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**First trimester elevations of hematocrit, lipid peroxidation and nitrates in women with twin pregnancies who develop preeclampsia**

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## **Abstract**

Twin pregnancies are considered a risk factor for preeclampsia, an obstetric complication with high maternal and infant morbi-mortality. We hypothesize that alterations in maternal hematocrit, plasma lipid peroxidation and nitrates in the first trimester of pregnancy are associated with preeclampsia development in twin pregnancies. Blood samples were extracted from 102 healthy women with twin pregnancies at tenth week of gestation to assess hematological parameters and plasma levels of malondialdehyde and nitrates. Logistic regression model showed an association between red blood cells (OR=38.8; p-value=0.009), hematocrit (OR=1.6; p-value=0.017), malondialdehyde (OR=1.5; p-value=0.002), and nitrates (OR=1.1; p-value=0.045) and preeclampsia development. **These parameters are potential biomarkers for early preeclampsia detection in twin pregnancies. Future research is needed to assess their value in predictive algorithms.**

**Keywords:** Biomarkers; Malondialdehyde; Nitric Oxide; Preeclampsia; Twin pregnancy

## 1. Introduction

The International Society for the Study of Hypertension in Pregnancy defines preeclampsia as de-novo hypertension (systolic blood pressure  $>140$  mm Hg or diastolic blood pressure  $>90$  mm Hg on two occasions) present after 20 weeks of gestation combined with proteinuria ( $>300$  mg/day), other maternal organ dysfunction, such as renal insufficiency, liver involvement, neurological or hematological complications [1]. Preeclampsia represents between 2-10% of the obstetric complications with high rate of morbidity and mortality for mother and fetus [2,3]. Twin pregnancies have 2-4 times increased risk to develop preeclampsia compared to singleton pregnancies [4]. Preeclampsia is a multifactorial disease, with several possible alterations implicated, including abnormal placentation and hypoperfusion, cardiovascular maladaptation, or immunological and angiogenic misbalance [5,6]. Some risk factors have been identified, including maternal habits (such as smoking) or previous history of preeclampsia. Low dose aspirin given to high-risk patients identified in the first trimester is useful as primary prevention of preeclampsia [6,7]. However, there are 2 problems. Screening tests in the first trimester need implementation, and lack of accurate early detection leads to false negatives, which are unnecessarily treated.

The majority of research on predictive biomarkers has focused on angiogenic factors. However, a focus on oxidative stress has been put forward, based on several lines of evidence [6]. Misbalance between reactive oxidative species (ROS) and antioxidants is present in preeclampsia [7], and elevations of lipid peroxidation products, such as malondialdehyde (MDA) are found in plasma at the end of pregnancies complicated with preeclampsia [8]. Other possible biomarkers are those related to nitric oxide (NO) metabolism, a potent vasodilator with key roles in the hemodynamic adaptation to pregnancy and placental function. Preeclampsia has been associated with alterations in NO production or its downstream mechanisms of action [9,10]. However, there are some conflicting results. In women with preeclampsia, plasma nitrates (which represent NO levels), have been shown to be increased in some studies [11,12], while others evidence a decrease [13,14]. Alterations in hematocrit can also be considered as potential biomarkers, based on the fact that they are increased in women with preeclampsia [15].

The majority of studies regarding preeclampsia biomarkers have been performed in women already diagnosed, and it would be desirable to identify women at risk before the disease is developed and to reduce the number of false positives, to avoid unnecessary treatment. The aim of our study was to contribute to the knowledge of biomarkers for early diagnosis focused

on twin pregnancies, which are a population of high risk. Based on the evidence in the literature, we hypothesize that alterations in hematological parameters, plasma lipid peroxidation or nitrates in the first trimester of pregnancy are associated with preeclampsia development in twin pregnancies.

## **2. Methods**

### *2.1. Study design*

The prospective study included 102 twin pregnant women, attended at the Obstetrics and Gynecology Service from La Paz University Hospital (HULP, Madrid, Spain). Enrolling was performed at ninth week of gestation **from January 2014 to December 2015**. The inclusion criteria were healthy women with twin pregnancy. Exclusion criteria were single pregnancy, and maternal chronic disease including overweight, previous hypertension, diabetes mellitus, kidney, immune and respiratory diseases. The study was performed in accordance with the Declaration of Helsinki regarding studies in human and it was approved by HULP Ethical Committee (Ref. PI-1490). An informed consent was signed by each participant and confidentiality of the data was guaranteed. Blood samples were collected at tenth week of gestation. Subjects were followed-up at the HULP until delivery, recording the following data: maternal age, gestational age, and preeclampsia (defined as blood pressure above 140/90 mmHg with proteinuria after 20 weeks of gestation).

### *2.2. Maternal biochemical variables at first trimester of pregnancy*

Blood sample was extracted from the participants by using venipuncture in Vacutainer® tubes, in fasting state, from 8:00 to 9:00 a.m., following the protocols established by the medical staff. The plasma was obtained by centrifugation (2100 rpm, 15 min at 4°C), within a maximum of 2h after extraction. The biochemical and hematological parameters were assayed by Laboratory Medicine Service from HULP according to hospital guidelines. The data were collected from the obstetrical record.

Plasma MDA levels were measured by a spectrophotometry in a microplate reader, as previously described by Ramiro-Cortijo et al. [16]. Absorbance was measured at 532 nm and compared with a standard curve of 1,1,3,3-tetrathoxypropane.

To assess plasma nitrates, Griess reaction, modified to microplate reader was used, as previously described, measuring absorbance at 540 nm [17].

### *2.3. Statistical analysis*

The analysis was performed using R software (version 3.6.0, 2018, R Core Team, Vienna; Austria) within R Studio interface. Wilcoxon test was used to compare the difference rank-sum between groups. Univariate logistic regression was used to associate preeclampsia with biochemical variables, and the odd ratios (OR) with 95% confidence intervals were reported. Receiver operating characteristics (ROC) curves were used to evaluate the capacity of the biochemical variables to predict preeclampsia. The cut-off values, sensitivity and specificity were determined. The prediction efficacy was evaluated considering the area under the curve (AUC) with 95% confidence interval. Statistical significance was established at  $p\text{-value} < 0.05$ .

### 3. Results

In this cohort, 7.8% (8/102) of the women developed preeclampsia. **At the time of delivery**, maternity age and gestational age were not different between women who developed preeclampsia and no-preeclampsia pregnancy. **Smoking habits were not different between preeclamptic (25.0%) and non-preeclamptic women (29,5%;  $p=0.75$ ). Similarly, primiparity rates were not different between and preeclamptic women (37.5%) and non-preeclamptic women (36,5%;  $p=0.95$ ). Gestational diabetes (defined as) was present in 12,5% of preeclamptic and in 9,4% of non-preeclamptic women ( $p=0.78$ ). Fetal growth restriction was present in 37% of preeclamptic and in 10% of non-preeclamptic women ( $p < 0.05$ ). Low birth weight ( $< 1000\text{gr}$ ) was present in 75,0% of preeclamptic and in 46,2% of non-preeclamptic women ( $p=0.15$ ). Preterm birth ( $< 37$  weeks of gestation), was present in 50% of preeclamptic and in 39,3% of non-preeclamptic women ( $p=0.50$ ).**

At 10 weeks of pregnancy, red blood cells (RBC), hemoglobin, hematocrit, MDA and nitrates were significantly higher in women who developed preeclampsia than women without preeclampsia (Table 1). Logistic regression showed an association between preeclampsia and RBC (OR=38.8 [3.3;  $\infty$ ];  $p\text{-value}=0.009$ ), hematocrit (OR=1.6 [1.1; 2.6];  $p\text{-value}=0.017$ ), MDA (OR=1.5 [1.2; 2.0];  $p\text{-value}=0.002$ ), and nitrates (OR=1.1 [1.0; 1.2];  $p\text{-value}=0.045$ ). However, hemoglobin did not associate with preeclampsia (OR=1.1 [0.9; 1.2];  $p\text{-value}=0.33$ ), and this parameter was not included in the ROC analysis. RBC, hematocrit, MDA, and nitrates had significant differences in the AUC, with sensitivity over 80% and specificity over 65%, as shown in table 2. This model support 67% of variability in preeclampsia development.

### 4. Discussion

This study evidences that hematocrit, lipid peroxidation and NO levels in the first trimester of pregnancy **are potential** biomarkers to be included in predictive algorithms for preeclampsia detection in twin pregnancies.

An early elevation in RBC, hemoglobin and hematocrit were detected in women with twin pregnancy who later on developed preeclampsia. Alterations of RBC, hemoglobin, platelets or lymphocytes have been found in women with singleton pregnancies already diagnosed with preeclampsia [15,18]. Taken together these data and our findings in twin pregnancies, an early elevation of maternal hematocrit as potential early biomarker of preeclampsia. It would be interesting to analyze in a large population if hematological parameters, regularly detected in the first trimester of pregnancy, could be considered in predictive algorithms. **Since these parameters are routinely assessed in the first trimester of pregnancy, it would be interesting to evaluate retrospectively in a large cohort of women, including singleton pregnancies, their value as predictive tool, in order to translate this finding to the clinical setting.**

Our data also demonstrate a significantly increase of maternal plasma MDA in women who developed preeclampsia. It has been suggested that in preeclampsia, oxidative agents may be overproduced due to ischemic-reperfusion injury in the placenta [7]. Lipids are the first targets of oxidative damage in there is excessive ROS production [19]. Our data suggest that lipid peroxidation may be increased in pregnant women initiating the pathological process, which, later on, end up in preeclampsia. **Therefore, the possibility that stable products of lipid oxidation could be early biomarkers, deserves confirmation in a larger cohort. We assessed lipid peroxidation levels by MDA. We are aware of the limitations of this technique, and other stable products, such as HNE and F2-isoprostanes, could be assessed. However, none of them are devoid of artifacts (Tsikas D. Anal Biochem. 2017 May 1;524:13-30. doi: 10.1016/j.ab.2016.10.021).**

Other important molecule for normal vascular function is NO. We found an increase in the stable product nitrate, which suggests an overproduction, in agreement with a study in women with already established preeclampsia [20, 21]. Our results indicate a systemic upregulation of NO pathway, which may be a maternal response to the initiation of a vascular alteration in the placenta. Our model support 67% of variability in preeclampsia development. The heterogeneous nature of preeclampsia, makes necessary the combination of clinical, biophysical and biochemical parameters into multivariate algorithms to predict the disease, [22]. Other models studied in single pregnancies had reported 43-63% of variability in the first, 65-99% in the second, and around 44% in the third trimester. However, fewer models included

additional biomarkers, such as pregnancy-associated plasma protein A or placental growth factor [23], or emerging risk factors such as lipid peroxidation or NO pathways.

Our data suggest that hematological parameter, lipid oxidation products and altered NO metabolism, may be considered. Regarding the possibility to translate our findings to the clinical setting, hematological parameters are routinely assessed in the first trimester of pregnancy. Therefore, it would be interesting to assess in a cohort of women with already diagnosed preeclampsia, if RBC or hematocrit are of prognostic value. Regarding MDA and nitrates need to be assessed by complex methods, such as HPLC-MS, which are not common in the clinical setting.

The present study evidenced that women who developed preeclampsia tended to be older and had lower gestational age compared to non-preeclampsia women. The relationship between preeclampsia and gestational age [24] or maternal age [25] has been previously found single pregnancies. In fact, not only the maternal age but also the paternal age could have an effect on pregnancy-induced hypertension diseases [25]. Therefore, together with biochemical markers, social and clinical variables should be considered in predictive algorithms.

## **5. Conclusions**

Our data demonstrate the relationship between first trimester biochemical and hematological alterations, and preeclampsia development in twin pregnancies. Confirmation of our results in a larger cohort, could help to conclude on the value of these biomarkers in predictive algorithms to identify women at risk. The present work could improve preeclampsia guidelines, establishing prophylaxis and close follow-up protocols.



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**Declaration of conflicting interests.** The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Ethical approval.** This study was in accordance with the Declaration of Helsinki (modified in 2013) regarding studies in human and it was approved by HULP Ethical Committee (Ref. PI-1490).

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**Table 1.** Maternal characteristics and biochemical parameters according to the development of preeclampsia in twin pregnancies.

Parameters	No-Preeclampsia	Preeclampsia	p-value
Maternal age (years)	35.0 (32.0; 38.0)	37.5 (33.5; 40.0)	0.31
Gestational age (weeks)	37.0 (35.0; 38.0)	36.2 (33.8; 37.0)	0.11
Red blood cells ( $10^6$ /mL)	4.2 (3.9; 4.4)	4.6 (4.5; 4.8)	0.002
Hemoglobin (g/dL)	13.3 (12.3; 18.9)	14.1 (13.7; 24.5)	0.049
Hematocrit (%)	38.3 (36.3; 40.0)	41.2 (40.7; 41.8)	0.004
Leukocytes ( $10^6$ /mL)	8.3 (7.4; 9.8)	7.7 (7.1; 10.0)	0.70
Platelets ( $10^3$ /mL)	250 (226; 290)	270 (226; 293)	0.79
Cholesterol (mg/dL)	178 (159; 198)	193 (159; 200)	0.70
Triglycerides (mg/dL)	94.0 (75.0; 125.0)	124.0 (97.5; 141.3)	0.07
Total bilirubin (mg/dL)	0.43 (0.36; 0.56)	0.48 (0.38; 0.56)	0.95
Protein (mg/dL)	6.8 (6.5; 7.0)	6.9 (6.8; 6.9)	0.67
Uric acid (mg/dL)	3.1 (2.6; 3.4)	3.4 (2.9; 4.2)	0.21
Glucose (mg/dL)	82.0 (77.0; 88.0)	84.0 (81.5; 104.8)	0.35
Malondialdehyde ( $\mu$ mol/L)	5.5 (3.6; 7.5)	11.0 (9.9; 16.9)	0.001
Nitrates ( $\mu$ M)	2.9 (1.6; 4.7)	7.5 (3.7; 11.7)	0.034

Data were expressed as median and interquartile range (Q1; Q3), p-value was reported by Wilcoxon test.

**Table 2.** Receiver operating curve for biochemical parameters associated with preeclampsia in twin pregnancies.

	<b>Cut-off</b>	<b>Sensitivity</b>	<b>Specificity</b>	<b>AUC [95% CI]</b>	<b>p-value</b>
Red blood cells ( $10^6$ /mL)	3.98	1.00	0.69	0.87 [0.73; 0.95]	0.002
Hematocrit (%)	37.0	1.00	0.69	0.85 [0.74; 0.93]	0.005
Malondialdehyde ( $\mu$ mol/L)	3.85	1.00	0.71	0.85 [0.61; 0.95]	0.002
Nitrates ( $\mu$ M)	1.85	0.88	0.70	0.70 [0.49; 0.89]	0.031

Area Under the Curve (AUC) and 95% of confidence interval (CI).