RESEARCH ARTICLE

Higher Trace Elements and Lower Fatty Acids Levels in Erythrocytes as Predictors of Preeclampsia

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Abstract

BACKGROUND: Preeclampsia is one of the common causes of maternal death in Indonesia. Many studies only focus on the diagnosis and pharmacological treatment of preeclampsia. However, predictors of preeclampsia need to be observed to add more focus on the etiology and prevention of preeclampsia. The imbalances of trace elements and fatty acids play an important role in preeclampsia. Therefore, this study was conducted to evaluate the status of trace elements and fatty acids in preeclampsia patients as predictors of preeclampsia.

METHODS: A cross-sectional study was conducted in 3 hospitals, and involving 40 pregnant women classified into severe preeclampsia and normotensive groups. Trace elements and fatty acids were measured in serum and erythrocytes using Inductively Coupled Plasma and Gas Chromatography-Mass Spectrometry. Serum and erythrocytes fatty acid levels' cut-off value, sensitivity, and specificity were analyzed using Receiver Operating Characteristic (ROC) curve and Area Under the Curve (AUC) value.

RESULTS: Serum selenium, manganese, and iron levels were significantly different in the preeclampsia group than in the controls (p<0.05). Of all the heavy metals, higher concentrations of cadmium, arsenic, lead, and mercury were found in preeclampsia groups compared to control. Linoleic acid showed the highest predictive value to increase severe preeclampsia with AUC of 0.8. The ratio of high omega-6/omega-3 increases the risk of preeclampsia.

CONCLUSION: Selenium, manganese, iron, cadmium, arsenic, lead, and mercury levels are higher in the serum of preeclampsia patients. Almost all erythrocyte fatty acids were significantly higher in the control group compared to preeclampsia. Measurement of trace elements and fatty acids is needed as a predictor of preeclampsia. Erythrocyte fatty acids measurement is considered better than serum.

KEYWORDS: trace elements, fatty acids, preeclampsia

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Introduction

Preeclampsia is defined as a new-onset hypertension during pregnancy, commonly occurring after 20 weeks of gestation. It is accompanied by proteinuria or any proof of new organ damage.(1) Preeclampsia has profound implications for maternal health, and has become the second-highest etiology of maternal death in Indonesia, with annual cases around 5.3%.(2) Neonatal outcomes were also impacted, such as low birth weight, low Appearance-Pulse- Grimace-Activity-Respiration (APGAR) score, asphyxia, and preterm birth.(3,4) The simplified pathological mechanism of preeclampsia is the disruption of the development of

placental blood vessels.(3) Preeclampsia is associated with oxidative stress and inflammatory processes because of the changes in the placenta.(5) The imbalance in micronutrients, trace elements, and fatty acids has been shown to increase preeclampsia because of the effects on oxidative stress and the inflammatory process. Many studies have shown that essential trace minerals and fatty acids affect preeclampsia. For example, manganese might affect preeclampsia with its antioxidant effect to reduce preeclampsia in mice. In contrast, cadmium causes oxidative stress that leads to preeclampsia due to endothelial dysfunction in mice. However, large studies in humans regarding this issue are still limited.(6)

Several studies have also been conducted to assess micronutrient status in pregnant women, including a study that assessed the effect of iron and zinc supplementation, which found that iron levels decreased in the first and second trimesters while zinc levels decreased in the second and third trimesters.(1,6,7) Trace elements and fatty acids in serum and red blood cells/erythrocytes were measured to find the best way to measure each component. Some studies showed red blood cells have a rather long lifespan. Therefore, the fatty acids profile is considered a better long-term marker of fatty acids intake within a middle-term time period than serum.(8) Meanwhile, there is still limited study that compares trace elements in serum and red blood cells. Therefore, this study was performed to determine the status of micronutrient trace elements and fatty acids in serum and red blood cells in pregnant women with severe preeclampsia. These findings may contribute to developing more targeted interventions for the early detection and prevention of preeclampsia.

Methods

Study Design and Subjects Recruitment

This was a cross-sectional study conducted in 3 hospitals, which were Cipto Mangunkusumo National Referral Hospital, Koja Regional Hospital, and Tangerang Region Hospital, from January to May 2021. The protocol of study was approved by the Ethical Committee for Research in Human from the Faculty of Medicine, Universitas Indonesia (No. KET- 236/UN2.F1/ ETIK/PPM.00.02/2021).

Subjects in this study were classified into subjects with normal pregnancy (n=20) and preeclampsia subjects with severe features (n=20). The sample size of 20 subjects in each group was selected based on feasibility and preliminary estimation calculation. The inclusion criteria for preeclampsia subjects were patients with a gestational age of 20 weeks to term suffering from severe preeclampsia. Preeclampsia with severe features was determined based on the American College of Obstetricians and Gynecologists (ACOG) criteria.(1) Preeclampsia with severe features characterized by systolic blood pressure of 160 mmHg or more, or diastolic blood pressure of 110 mmHg or more, platelet count less than 100.000/L, elevated liver enzymes mor than twice of upper limit normal concentrations, severe persistent right upper quadrant or epigastric pain, serum creatinine concentrations more than 1.1 mg/dL or doubling of the latest baseline, pulmonary edema, visual disturbances, and new-onset headache persistent to medication.(1) The exclusion criteria in this study were subjects with multiple pregnancies, congenital anomalies, diabetes mellitus, infection, autoimmune diseases, cardiovascular diseases, and blood disorders. Meanwhile, the pregnant normotensive women were recruited as controls.

Sample Preparation

Blood venous samples from non-fasting subjects were collected using 15 mL Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ, USA). Blood samples were transferred into a Serum Separator Tube (SST). The samples were slowly shaken 5-10 times and were left upright for 30 minutes at 25°C until the blood clots. The samples were transported to the laboratory at 2-8°C to prevent sample degradation.

Erythrocytes Isolation

A heparin royal blue trace element vacutainer was used for trace element measurement. Blood was taken as much as 6 mL and shaken slowly 5-10 times until homogeneous. Then, it was immediately divided into 2 polypropylene tubes; the first tube was separated from the plasma for micronutrient (trace elements) examination and the second tube remained in whole blood heparin for the manufacture of erythrocyte lysate at 2-8°C.

The erythrocytes samples used to analyze trace elements and fatty acids were previously isolated by gradient centrifugation and washing techniques. Isolation of erythrocytes was started by pipetting 2 mL of the blood sample into a polypropylene tube of 2 mL of Phosphate Buffered Saline (PBS) and homogenized. Then, 3 mL of Ficoll-Paque Plus solution was added, homogenized, and centrifuged at 400 g for 30 minutes at 19°C until a layer formed. After forming the erythrocyte layer and supernatant, part of the supernatant layer was removed, and 2 mL of PBS was added. Then, it was washed 3 times until clean by

centrifugation at a speed of 100 g for 10 minutes. After the last washing, PBS was added to the final volume of 2 mL, and then the erythrocyte sample was ready to be used to measure trace elements and fatty acids.

Trace Elements Measurement

A total of 1 mL of erythrocytes sample was lysed with 1 mL of cold water. Then, the sample was diluted with a dilution factor of 100 times with an alkaline solution containing an internal standard of Indium. The concentration was known for the measurement of metals vanadium (V), chromium (Cr), manganese (Mn), ferrum (Fe), cobalt (Co), nickel (Ni), cuprum (Cu), zinc (Zn), and selenium (Se). The samples were diluted 20 times with an alkaline solution containing an internal standard of Indium with known concentrations for measurement of metals arsenic (As), cadmium (Cd), mercury (Hg), titanium (Ti), and lead (Pb). After that, it was injected into the inductively coupled plasma-mass spectrometry (ICP-MS) 7700x system with the MassHunter software (Agilent, Santa Clara, CA, USA).

Trace elements and heavy metals were measured and calculated using standard internal calculations. The trace elements and heavy metals concentrations were expressed in units of ug/L and/or ug/dL. These results were corrected for the erythrocytes count with the final units ag (atogram)/erythrocytes and fg (femtogram)/erythrocyte specifically for Fe and Zn. The sample dilution factor was 100× for V, Cr, Mn, Fe, Co, Ni, Cu, Zn, and Se, and 20× for As, Cd, Hg, Ti, and Pb. The results had been multiplied by the dilution factor.

Fatty Acids Measurement

A total of 1 mL of erythrocyte sample was lysed with 1 mL of cold water. Then, protein precipitation was performed with 300 L of methanol. After that, 600 L of chloroform was added, which contains an internal standard whose concentration was known for the liquid-liquid extraction process. This process was carried out on samples homogenized using a vortex and centrifuged at a speed of 2500 g for 5 minutes until two layers, the supernatant and the chloroform, were formed. Then the supernatant was separated and dried using nitrogen for 30 minutes. After that, it was reconstituted with 190 L of n-hexane and 10 L of Tetramethyl Ammonium Hydroxide (TMAH), homogenized, and incubated for 2 hours to separate into two layers. Then, the hexane phase in the top layer was taken, transferred into a vial, injected into the gas Chromatography-Mass Spectrometry (GC-MS) system, and quantified with the MassHunter software (Agilent).

The measurement of fatty acids was carried out quantitatively and calculated against the calibration standard using standard internal calculations. Fatty acid concentrations are expressed in units of umol/L. These results have been corrected for the erythrocyte count with the final unit Amol (atomol)/erythrocyte (1 atomol = $10^{-}18$ moles). The concentration factor of the sample was 10 times higher. The results were divided by concentration factor. The measurements were developed and validated by the Mass Spectrometry Laboratory of PT. Prodia Widyahusada (Jakarta, Indonesia) and only used for research scale.

Statistical Analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS) version 26.0 (IBM Corporation, Armonk, NY, USA). The numeric data were checked for normal distribution using the Shapiro Wilk Test, then presented as mean±SD or median (IQR) based on the data distribution. Statistical analysis of this study was carried out according to the proposed hypothesis by using unpaired numerical comparative analysis in 2 groups.

To analyze the appropriate cut-off value, sensitivity and specificity of the variable erythrocyte fatty acid levels are examined using the Receiver Operating Characteristic (ROC) curve. Then, the Area Under the Curve (AUC) value was obtained based on the ROC curve. The AUC score used as a parameter was 0.6. The AUC could range between 0·5 and 1, with higher values indicating better discrimination between high-risk and low-risk patients. AUC score <0.6 was considered low, and were not taken into account.(9,10) Therefore, AUC >0.6 was chosen as the cut-off value.

Bivariate and multivariate analyses to see the relationship between erythrocytes fatty acid variables in patients with severe preeclampsia were performed using logistic regression adjusted for maternal age, gestational age, and body mass index. Based on our demographic data, these three parameters (maternal age, gestational age, and body mass index) were related to preeclampsia. All analysis results with $p<0.05$ are considered significant.

Results

Characteristics of Subjects

There was a significant difference ($p<0.008$) in the education level. In the comparison of blood pressure, there was a significant difference in blood pressure between the two groups with systolic ($p<0.000$) and diastolic ($p<0.000$). Likewise, pre-pregnancy body mass index was significantly

different (p<0.042) between preeclampsia patients compared to controls, with the majority of preeclampsia patients being obese (Table 1).

Trace Elements in Serum and Erythrocytes

Based on the distribution of trace elements in serum and red blood cells, it was shown that between preeclampsia and the control group, Se,Fe, Cd, and Pb significantly differ in serum and erythrocytes (p<0.05). Meanwhile, Mn, As, and Hg only differ in serum. Conversely, Cu and Co significantly differed only in erythrocytes (Table 2). Analysis with ICP-MS were performed, an analysis result example of the serum sample of preeclampsia subject for the measurement of Ni, V, Cr, Mn, Fe, Co, Cu, Zn, Se, and Mo was presented in Table 3; while a result example from erythrocytes for the measurement of Cd, Tl, As, Hg, and Pb from the same preeclampsia subjects was presented in Table 4.

Fatty Acids in Serum and Erythrocytes

A comparison of fatty acids between serum and erythrocytes was shown in Table 5. It was found that only alpha lipoic acid (ALA) and arachidonic acid (AA) have significant differences in both serum and red blood cells (p<0.05). In contrast, almost all fatty acids consisting of ALA, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), omega-3, linoleic acid (LA), gamma-linolenic acid (GLA), dihomo-gamma-linolenic acid (DGLA), AA, omega-6, and oleic acid were significant only in erythrocytes measurement. Analysis with GC-MS was performed, Figure 1 shown an analysis results calibration in form of curve and compound graphics which show comparation between preeclampsia subject and a normal pregnancy/control subject.

Receiver Operating Characteristic Curve

Based on Figure 2 and Table 6, it was known that the AUC value of the ROC curve ranges from 0.62 to 0.8, which indicates that these cut-off values were considered to have low to moderate effects on the stratification of preeclampsia patients based on trace elements and fatty acids levels. The cut-off values ranged in sensitivity between 45-95% and specificity from 45-85%. The highest score in the prediction of severe preeclampsia had an AUC of 0.8 (95% CI: 0.658–0.942) in LA with a cut-off point of 54.25 Amol/erythrocyte and a DGLA of 0.8 (95% CI: 0.664–0.936%) with a cut-off point 3.88 Amol/erythrocyte. The lowest value was omega-6/omega-3 with an AUC value of 0.62 (95% CI: 44–80%) and a cut-off value of 12.35 Amol/erythrocyte, with a sensitivity of 45% and specificity of 85%.

Bivariate and Multivariate Analysis

In bivariate analysis, there were significant differences (p<0.05) between severe preeclampsia and control in almost all variables at concentrations of AA/EPA, omega-3 index, ALA, EPA, DHA, omega-3, LA, DGLA, AA, omega-6, and

Table 1. Characteristics of subjects.

Variables	Preeclampsia (n = 20)	Control (n = 20)	*p*-value
Age (year), mean±SD	34±5.9	31±5.4	0.671[a]
Gestational age (week), mean±SD	30.6±4.3	31.5±4.1	0.676[a]
Education, n (%)			0.008*[,b]
Senior high school	19 (95%)	12 (60%)	
Undergraduate	1 (5%)	8 (40%)	
Job, n (%)			0.292[b]
Housewife	19 (95%)	17 (85%)	
Employee	1 (5%)	3 (15%)	
Parity, n (%)			0.519[b]
Nulliparity	7 (35%)	9 (45%)	
Multipara	13 (65%)	11 (55%)	
Systolic BP (mmHg), mean±SD	168.1±19.2	114.2±12.9	0.000*[,a]
Diastolic BP (mmHg), median (IQR)	108 (80–130)	74 (67–96)	0.000*[,a]
Body Mass Index	28.7±6.4	24.9±4.9	0.042*[,a]
Underweight (<18.5)	0 (0%)	1 (5%)	
Normal (18.5–22.9)	5 (25%)	9 (45%)	
Overweight (23–24.9)	2 (10%)	1 (5%)	
Obese (≥25)	13 (65%)	9 (45%)	

*Significant with p<0.05. [a]Tested with Independent t-test; [b]Tested with Chi-square.

Table 2. Distribution comparison of serum and erythrocytes trace elements.

Variables	Serum		Erythrocytes	
	Preeclampsia (n=20)	Control (n=20)	Preeclampsia (n=20)	Control (n=20)
Zn	56.05±8.95[a]	53.45±8.22[a]	1.37 (0.46–2.86)[b]	1.31 (0.94–2.10)[b]
Se	89.80±15.13[a]	73.50±15.41*[,a]	19.39 (8.23–38.92)[b]	21.48 (17.05–28.08)*[,b]
Mn	0.79 (0.3–1.9)[b]	0.62 (0.3–1.1)*[,b]	7.97±2.78[a]	8.08±1.71[a]
Cu	2304.20±451.70[a]	2151.15±357.20[a]	60.24 (20.40–108.43)[b]	57.07 (34.53–82.43)*[,b]
Fe	119.55 (28.0–348.0)[b]	74.23 (16.0–232.9)*[,b]	65.01 (22.90–137.12)[b]	72.14 (45.21–92.17)*[,b]
Co	0.30 (0.1–0.8)[b]	0.46 (0.1–1.2)[b]	0.22±0.16[a]	0.12±0.07*[,a]
Cd	1.03 (0.0–3.4)[b]	0.45 (0.1–0.8)*[,b]	0.13±0.07[a]	0.05±0.04*[,a]
As	3.26 (1.0–9.7)[b]	1.82 (0.8–3.5)*[,b]	0.51±0.25[a]	0.40±0.08[a]
Pb	4.38 (1.7–8.9)[b]	2.42 (1.2–8.9)*[,b]	9.55±4.70[a]	6.00±2.20*[,a]
Hg	5.39 (1.6–9.2)[b]	6.03 (2.4–16.0)*[,b]	0.89 (0.34–1.40)[b]	0.93 (0.34–1.38)[b]

*Significances compare to each control group (p<0.05). [a]Tested with unpaired T-test (Data presented in Mean±SD); [b]Tested with Mann Whitney test (Data presented in Median (IQR)).

oleic. The results of multivariate analysis after adjusting for the variables of maternal age, gestational age, and body mass index found that there was an increased risk of severe preeclampsia with high AA/EPA (OR: 11.02; 95%CI: 1.74–69.75) and low omega-3 index (OR: 6.25; 95%CI: 1.13–34.55). From the group of omega-3 fatty acids among ALA, EPA, and DHA, the highest increased risk of preeclampsia was found at low EPA (OR: 14.53; 95%CI: 2.21–95.41). A high concentration of oleic acid has the risk 72.46 times of developing severe preeclampsia than the control (Table 7).

Discussion

The data characteristics of this study's populations showed significant differences in body mass index between preeclampsia patients and controls. Sixty-five percent of patients with severe features were obese. Obesity is linked with the risk of preeclampsia, although it may also be influenced by increased fluid retention in patients with severe features.(11,12) However, another study also showed

Table 3. Analysis example using ICP-MS for serum trace elements.

Compound	Mass	Conc	Units	Count	Quant by	Det	Ratio	ISTD
Ni	60	1.946	ug/L	1,217	Area	Pulse		
V	51	0.568	ug/L	91	Area	Pulse		
Cr	52	0.152	ug/L	255	Area	Pulse	1.856E-01	In
Mn	55	0.748	ug/L	122	Area	Pulse	8.946E-02	In
Fe	56	87.387	ug/dL	100,206	Area	Pulse	7.293E+01	In
Co	59	0.276	ug/L	80	Area	Pulse	7.293E+01	In
Cu	63	2.373.728	ug/L	503,461	Area	Pulse	3.401E+02	In
Zn	66	63.286	ug/dL	20,714	Area	Pulse	1.508E+01	In
Se	78	120.291	ug/L	167	Area	Pulse	1.215E-01	In
Mo	95	1.093	ug/L	154	Area	Pulse	1.117E-01	In

Table 4. Analysis example using ICP-MS for erythrocytes trace elements.

Compound	Mass	Conc	Units	Count	Quant by	Det	Ratio	ISTD
Cd	111	0.553	ug/L	68	Area	Pulse	5.381E-03	In
Tl	205	0.335	ug/L	177	Area	Pulse	5.381E-03	In
As	75	3.020	ug/L	61	Area	Pulse	4.126E-02	In
Hg	202	5.541	ug/L	223	Area	Pulse		
Pb	208	2.181	ug/dL	5,075	Area	Pulse	3.419E+00	In

Table 5. Comparison distribution of serum fatty acids erythrocytes fatty acids.

Variables	Serum		Erythrocytes	
	Preeclampsia (n=20)	Control (n=20)	Preeclampsia (n=20)	Control (n=20)
Omega-6/omega-3	14.26±5.15[a]	12.64±3.47[a]	12.09±0.00[b]	8.72±0.00[b]
AA/EPA	61.50 (16–124)[b]	44.15 (15–103)*[,b]	112.10 (22.18–381.72)[b]	70.04 (24.70–223.30)[b]
Omega-3 index	2.35 (1–5)[b]	2.70 (2–5)[b]	0.028±0.02[a]	0.041±0.02[a]
ALA	41.05 (18–75)[b]	45.15 (21–111)*[,b]	0.18±0.14[a]	0.36±0.22*[,a]
EPA	16.00 (5–35)[b]	16.00 (8–33)[b]	0.36±0.38[a]	0.77±0.51*[,a]
DHA	376.45 (203–626)[b]	379.75 (272–622)[b]	9.64±8.93[a]	16.35±8.81*[,a]
Omega-3	433.00 (269–699)[b]	440.85 (330–709)[b]	10.17±9.29[a]	17.49±9.31*[,a]
LA	4708.40±1318.75[a]	4504.00±1146.58[a]	46.35±19.87[a]	68.91±19.67*[,a]
GLA	11.15±3.62[a]	10.75±4.51[a]	0.09±0.05[a]	0.14±0.05*[,a]
DGLA	298.40±81.13[a]	271.70±72.84[a]	4.49±2.53[a]	7.78±3.13*[,a]
AA	708.05 (457–1301)[b]	583.60 (390-928)*[,b]	23.78±15.54[a]	39.35±13.40*[,a]
Omega-6	5726.10±1321.65[a]	5370.05±1194.92[a]	74.72±36.05[a]	116.18±33.6*[,a]
Oleic	5132.35±1669.02[a]	4219.90±1433.10[a]	54.22±9.44[a]	62.72±9.37*[,a]

*Significances compare to each control group (p<0.05). [a]Tested with unpaired T-test (Data presented in Mean±SD); [b]Tested with Mann Whitney test (Data presented in Median (IQR)).

no correlation in body mass index between preeclampsia and normal pregnancy.(13)

This study evaluates many trace elements in pregnant women that act as cofactors of antioxidant enzymes to reduce the effect of free radicals in the body. Free radicals cause many negative effects on the physiological functions of female reproduction, from oocyte maturation to luteal maintenance during pregnancy.(14,15)

Se levels in serum were higher in the preeclampsia group, but the levels in erythrocytes were lower in the preeclampsia group. It remains unclear whether the best method to measure Se. If plasma or serum Se describes its level in the short term, erythrocytes may be able to describe its concentration status in the long term.(16) In contrast with these findings, a previous case-control study had a results of lower plasma Se concentrations in preeclampsia patients compared to the normal pregnancy group. Other studies also support the importance of optimal Se supplementation to reduce the incidence of preeclampsia. The hypothesis is the reduced levels of Se can affect the activities of selenoproteins, which leads to the inability to protect the endothelium against oxidative damage. The study that detects Se levels in erythrocytes is limited.(17,18)

Mn was only 4.4% in plasma or serum, but it is significantly higher in preeclampsia patients' serum than in controls. Low levels of Mn can reduce manganese superoxide dismutase activity and lead to the accumulation of reactive oxygen species that can develop into preeclampsia.(19)

The serum Zn was not significant between both groups. Both of the serum Zn levels of severe preeclampsia and control groups were considered low. Research conducted in Jakarta supports these findings because it also showed low serum zinc levels in pregnant women from the first to the third trimester. This illustrates that Zn levels in normal pregnant women are still lacking as well, especially in pregnant women with complications such as preeclampsia. However, several investigators have noted that women with preeclampsia, compared with normotensive pregnant women, have lower Zn concentrations.(20)

In this study, serum Cu levels were also not significant, but both groups showed increased levels of Cu in serum. Conversely, Cu concentration in erythrocytes is significantly higher in severe preeclampsia. Cu plays an important role in scavenging free radicals and reduces oxidative stress. It is also an essential substrate or cofactor for adequate activation of antioxidant enzymes. Impaired Cu-Zn Superoxide Dismutase (Cu-Zn SOD) activity contributes to oxidative damage in the body. Consistent with this study in which Cu levels were higher in preeclampsia, there was a meta-analysis in which 8 of the 12 included studies reported significantly higher Cu levels in preeclampsia patients. But, another study reported that Cu levels were significantly lower in preeclampsia patients than in controls.(20)

This study shows higher levels of Fe in the serum of preeclampsia patients. These results align with a meta-analysis conducted in China and a study in Saudi Arabia,

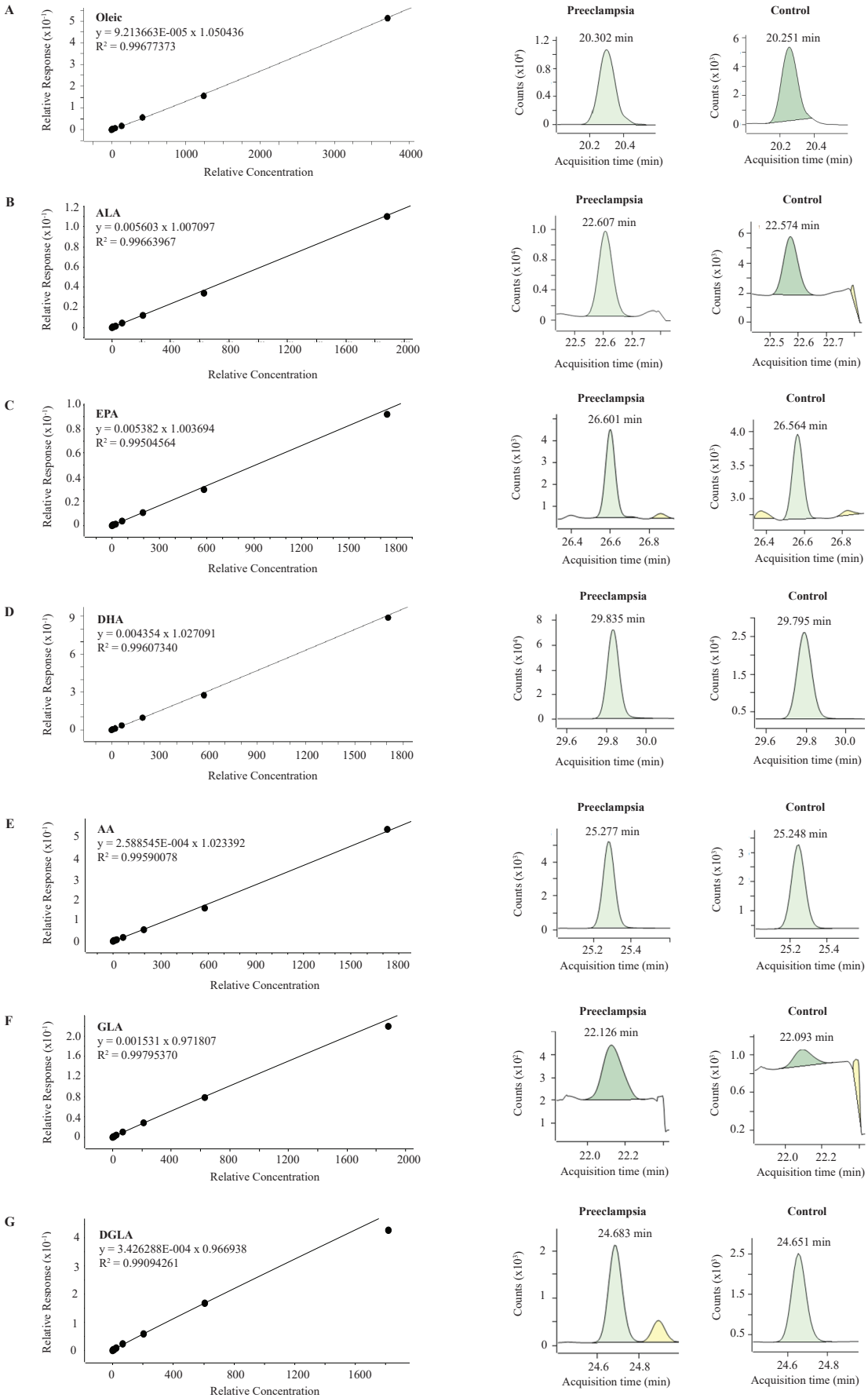

Figure 1. Examples of fatty acids measurement in preeclampsia and normal pregnancy/control subjects measured by GC-MS, in form of calibration curve and compound grapics. A: Oleic. B: ALA. C: EPA. D: DHA. E: AA. F: GLA. G: DGLA.

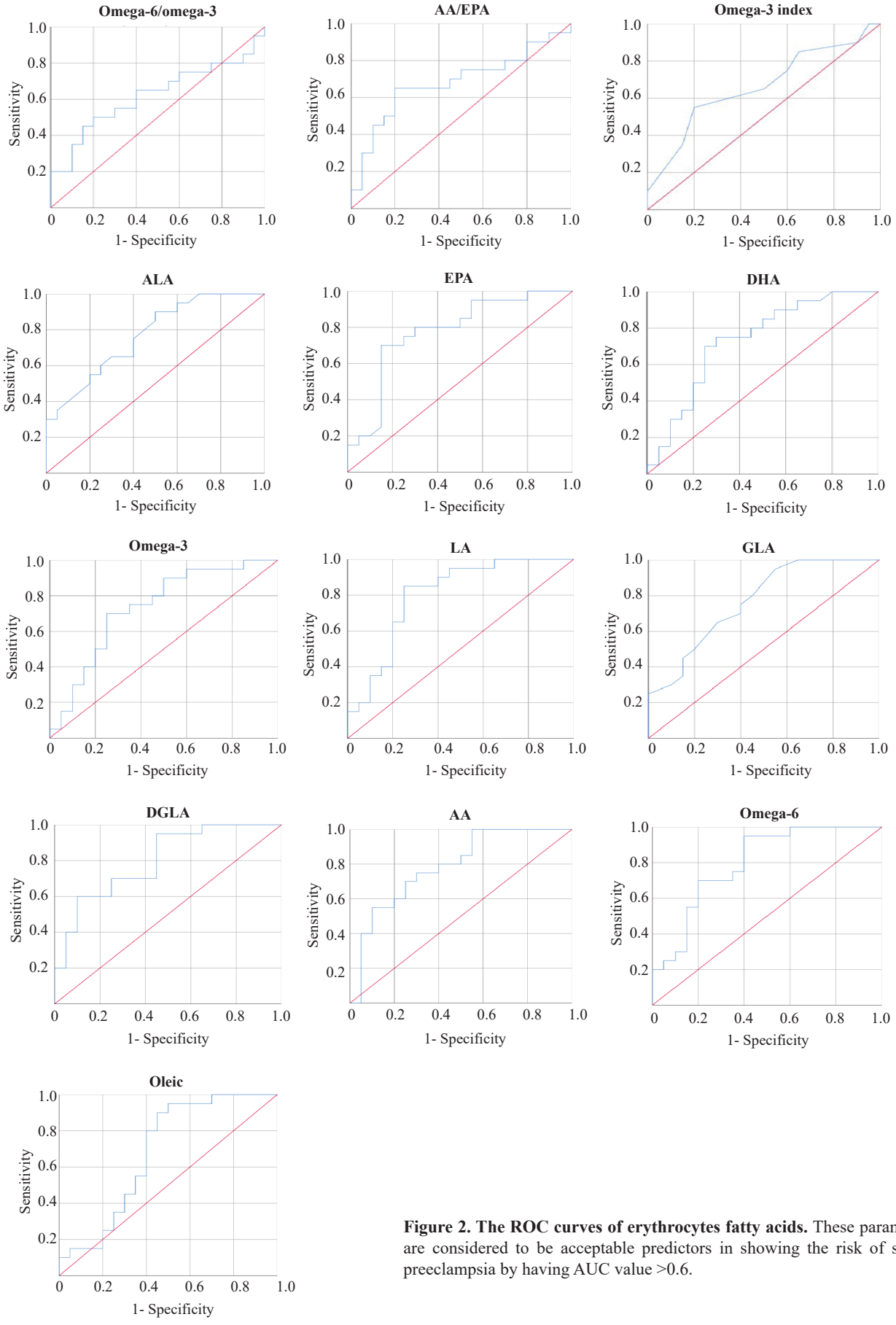

Figure 2. The ROC curves of erythrocytes fatty acids. These parameters are considered to be acceptable predictors in showing the risk of severe preeclampsia by having AUC value >0.6.

Table 6. Predictive value of fatty acids parameters in showing the risk of severe preeclampsia.

Variable	AUC	95% CI	Optimal Cut-off Value	Sensitivity (%)	Specificity (%)
Omega-6/omega-3	0.62	0.440–0.800	12.35	45	85
AA/EPA	0.68	0.508–0.852	80.97	65	80
Omega–3 index	0.66	0.492–0.883	0.02	55	80
ALA	0.77	0.625–0.912	0.16	90	50
EPA	0.78	0.627–0.926	0.54	70	85
DHA	0.73	0.572–0.890	10.58	70	75
Omega-3	0.73	0.574–0.891	11.37	70	75
LA	0.8	0.658–0.942	54.25	85	75
GLA	0.77	0.621–0.912	0.07	95	45
AA	0.79	0.645–0.930	33.74	70	75
DGLA	0.80	0.664–0.936	3.88	95	55
Omega-6	0.79	0.658–0.937	75.46	95	60
Oleic	0.68	0.509–0.858	52.42	95	50

which found higher serum Fe levels in pregnant women with preeclampsia compared to normal controls.(21,22) In preeclampsia patients, there is a histological feature of severe vascular damage in the placenta that explains the process of cell injury and Fe release. It leads to lipid peroxidation and increased oxidative stress in the placenta and blood vessels. This mechanism explains the findings of endothelial cell damage in preeclampsia patients.(23) The differences in the results of preeclampsia patients' Fe levels in serum and erythrocytes may be due to impaired metabolism and hemostasis of Fe. Studies on erythrocytes Fe levels in preeclampsia are still very limited and almost non-existent.(24)

In this study, the trace elements of the heavy metal group, such as Cd, As, Pb, and Hg, were also examined. There was a significant difference ($p<0.05$) with higher levels of heavy metals in the preeclampsia serum compared to normotensive patients. The accumulation in the body of these heavy metals causes various toxic effects on tissues and organs. Heavy metals interfere with cellular activities, from growth to apoptosis. It also induces toxicity, increasing oxidative stress, weakening antioxidant defenses, and inactivating enzymes.(25)

In this study, the measurement of heavy metals in erythrocytes was also found to be significantly higher in Co, Cd, and Hg in preeclampsia with severe features. Heavy metals such as Cr, Cd, and Hg can accumulate in the placental tissue and interfere with placental function. Exposure to those heavy metals will trigger the secretion of pro-inflammatory factors and apoptotic signals in the placenta and trophoblast cells through oxidative stress.(25)

The measurement of fatty acids in this study also carried out on serum and erythrocytes of preeclampsia as well as the normotensive pregnant women. In the serum of preeclampsia patients, higher concentrations of AA/EPA and lower ALA were found. Meanwhile, the concentrations of ALA, EPA, DHA, omega-3, LA), gamma linoleic acid (GLA), AA, dihomogamma linolenic acid (DGLA), omega-6, and oleic acid on erythrocytes measurement were significantly lower in preeclampsia compared to the control group. It is known that fatty acids, especially the long-chain unsaturated fatty acids, play a role in many processes and reduce the risk of preeclampsia. Likewise, fatty acids in the erythrocyte membrane are needed to maintain membrane stability and play a role in maintaining membrane fluidity.(26)

In contrast to trace elements, where studies are mostly carried out on serum, fatty acids have mainly been carried out on erythrocytes. However, daily clinical measurements of fatty acids are still being done on serum. A study comparing erythrocytes and plasma fatty acids showed that DHA concentration as a biomarker was found to be better in erythrocytes than plasma. Several studies have been conducted to compare the correlation of erythrocytes and plasma in the same population. However, sometimes it is still difficult to conclude the mechanism due to many factors such as dietary variations, laboratory measurement errors, different dietary measurement methods, and biological variability. However, as described above, fatty acid erythrocytes are known to reflect long-term fatty acid intake better due to lower sensitivity to recent dietary intakes and slower turnover rates.(26)

Table 7. Fatty acid (omega profile) parameters showing the risk of severe preeclampsia.

Variable	Preeclampsia [n (%)]	Control [n (%)]	*p*-value	Unadjusted OR (CI 95%)	Adjusted OR (CI 95%)
Omega-6/ omega-3 (amol/erythrocyte)					
High risk (≥12.33)	8 (40)	3 (15)	0.157	3.78 (0.83–17.25)	2.66 (0.47–14.94)
Low risk (<12.33)	12 (60)	17 (85)		1.0	1.0
AA/EPA (amol/erythrocyte)					
High risk (≥80.97)	13 (65)	4 (20)	0.011	7.43 (1.78–31.04)	11.02 (1.74–69.75)
Low risk (<80.97)	7 (35)	16 (80)		1.0	1.0
Omega-3 index (amol/erythrocyte)					
High risk (≥0.02)	11 (55)	4 (20)	0.050	4.89 (1.2–19.94)	6.25 (1.13–34.55)
Low risk (<0.02)	9 (45)	16 (80)		1.0	1.0
ALA (amol/erythrocyte)					
High risk (≥0.16)	10 (50)	2 (10)	0.016	9.00 (1.64–49.45)	12.54 (1.62–97.06)
Low risk (<0.16)	10 (50)	18 (90)		1.0	1.0
EPA (amol/erythrocyte)					
High risk (≥0.54)	16 (80)	6 (30)	0.004	9.33 (2.18–39.96)	14.53 (2.21–95.41)
Low risk (<0.54)	4 (20)	14 (70)		1.0	1.0
DHA (amol/erythrocyte)					
High risk (≥10.58)	15 (75)	6 (30)	0.011	7.00 (1.74–28.17)	12.07 (1.86–78.5)
Low risk (<10.58)	5 (25)	14 (70)		1.0	1.0
Omega-3 (amol/erythrocyte)					
High risk (≥10.58)	14 (70)	6 (30)	0.027	5.44 (1.41–21.05)	9.88 (1.51–64.56)
Low risk (<10.58)	6 (30)	14 (70)		1.0	1.0
LA (amol/erythrocyte)					
High risk (≥54.25)	14 (70)	3 (15)	0.001	13.22 (2.79–62.67)	17.11 (2.76–106.68)
Low risk (<54.25)	6 (30)	17 (85)		1.0	1.0
DGLA (amol/erythrocyte)					
High risk (≥3.88)	11 (55)	1 (5)	0.002	23.22 (2.58–208.61)	43.62 (2.96–643.47)
Low risk (<3.88)	9 (45)	19 (95)		1.0	1.0
AA (amol/erythrocyte)					
High risk (≥33.74)	15 (75)	7 (35)	0.026	5.57 (1.42–21.86)	7.37 (1.37–39.7)
Low risk (<33.74)	5 (25)	13 (65)		1.0	1.0
Omega-6 (amol/erythrocyte)					
High risk (≥ 75.46)	8 (40)	19 (95)	0.001	28.50 (3.16– 257.44)	94.21 (4.79–1852.03)
Low risk (<75.46)	12 (60)	1 (5)		1.0	1.0
Oleic (amol/erythrocyte)					
High risk (≥52.42)	10 (50)	1 (5)	0.005	19.00 (2.12–170.38)	72.46 (3.76–1394.92)
Low risk (< 52.42)	10 (50)	19 (95)		1.0	1.0

Based on the result of fatty acid measurement in erythrocytes, which were significantly different in almost all omega-3, -6, and -9 variables, further analysis was carried out using the ROC curve. It aims to obtain the cut-of-point value on the fatty acid measurement in erythrocytes as predictors of the risk of preeclampsia based on the fatty acid levels. High total serum of polyunsaturated fatty acids (PUFA) concentration and low DHA percentage in preeclampsia with severe features. The long-chain PUFA (LCPUFA) parameter was also shown to increase the risk of severe preeclampsia, especially ALA 53 mol/L with an OR of 5.44 and 95% CI of 1.16-25.42.(27) In this study, the risk of preeclampsia was found higher in patients with EPA below 0.54 (OR: 14.53; 95%CI: 2.21-95.41). These omega-3 fatty acids have several important roles, from early placentation until delivery. The low levels of omega-3 will cause disturbances in the early development of the placenta and lead to poor pregnancy outcomes.(28)

The risk of preeclampsia increases when the total concentration of omega-6 is above 75.46 Amol/erythrocytes, followed by the increased DGLA, LA, and AA fatty acids. However, this study has a wide confidence interval of

DGLA, omega-6 total, and LA analysis. Therefore, further analysis should be carried out. AA, with a cut of point 33.74, will increase the risk of becoming preeclampsia 7.37 times if there is an increase in its concentration (95% CI: 1.37-39.7). Oleic acid, a group of omega-9 fatty acids, with concentrations greater than 52.42 Amol/erythrocytes, increased the risk of severe preeclampsia. However, this study has a very wide confidence interval. Therefore, further analysis must be done.

Unfortunately, ruptured membranes and intrauterine growth restriction fetuses cases were not included in this study. This study also did not assess the subjects' physical activity levels, smoking status, and daily intake which could influence the trace elements and fatty acids levels. The fasting blood test may also yield a more accurate result. Further analysis is needed to give more accurate benefits of trace elements and fatty acids as predictors for preeclampsia.

Conclusion

The results of this study showed that there are equally significant differences in both serum and erythrocytes of Se, Fe, Cd, and Pb levels. Meanwhile, the levels of Mn, As, and HG are found to be significantly different in serum only. In contrast, significantly different in erythrocytes only were found in Cu and Co levels. Of all the heavy metals, Cd, As, Pb, and Hg, are significantly different in serum. Comparison of fatty acids are shown to be significantly differences in serum and erythrocytes for ALA and AA. While almost all fatty acids, such as ALA, EPA, DHA, omega-3, LA, GLA, DGLA, AA, omega-6, and oleic are significant on erythrocytes measurement only. These results suggest that measurement of trace elements and fatty acids are better measured using Erythrocytes compared to serum samples for preeclampsia subjects.

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Authors Contribution

All authors designed the study. RWRP conducted the study. DP, NW, and RI monitored the process. RW, RI, and YP performed the analysis and interpretation of data. RW, DP, and NW wrote the manuscript draft. RI, YP, YBS, DP and NW revised the paper and had primary responsibility for the final content. All authors agreed to the published version of the manuscript.

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