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Pravastatin suppresses inflammatory cytokines and endothelial activation in patients at risk of developing preeclampsia: INOVASIA study

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ORIGINAL ARTICLE

Pravastatin suppresses inflammatory cytokines and endothelial activation in patients at risk of developing preeclampsia: INOVASIA study

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ABSTRACT

Introduction: The Indonesian INOVASIA study is an ongoing multicentre randomized, open controlled trial of pravastatin for the prevention of preeclampsia in patients deemed to be high risk. Here we evaluate the effects of pravastatin on circulating inflammatory and endothelial markers, i.e. Vascular Endothelial Growth Factor (VEGF), Interleukin-6 (IL-6), Endothelin-1 (ET-1), and Nitric Oxide (NO).

Methods: Pregnant women deemed to be at a high risk of developing preeclampsia women were recruited based on the Fetal Medicine Foundation preeclampsia screening test or a history of preterm preeclampsia, or clinical risk factors in combination with an abnormal uterine artery Doppler flow pattern at 11–20 week's gestation. This is a nested cohort study within the larger trial (INOVASIA); 38 patients were consecutively recruited and assigned to the pravastatin group and the control group. Participants in the pravastatin group received pravastatin (2 × 20 mg p.o) in addition to a standard regimen of aspirin (80 mg p.o) and calcium (1 g p.o), from 14 to 20 weeks until delivery. Blood samples to measure the various biomarkers were obtained in consecutive patients before starting the research medication and just before delivery (pre and post-test examination).

Result: The number of samples on the 2 time points for the various biomarkers was: VEGF: 38, IL-6: 30, ET-1: 38, and NO: 35. IL-6 levels decreased significantly in the pravastatin group (mean ± SD): (191.87 ± 82.99 vs. 151.85 + 48.46, $p = .013$), while levels in the control group did not change significantly (median (interquartile range)) (144.17 (53.91) vs. 140.82 (16.18), $p = .177$). ET-1 levels decreased significantly in the pravastatin group (3.64 ± 0.85 vs. 3.01 ± 0.74, $p = .006$) while the control group had more or less stable levels (3.57 ± 1.12 vs. 3.78 ± 0.73 $p = .594$). NO was the only serum marker that showed significant changes in both groups. NO levels increased in pravastatin group (11.30 (17.43) vs. 41.90 (53.18), $p = .044$) and decreased in control group (38.70 (34.80) vs. 10.03 (26.96), $p = .002$). VEGF levels appeared to follow opposite trends in the 2 groups (NS) (Pravastatin: 3.22 (0.62) vs. 3.28 (0.75), $p = .402$. Control: 3.38 (0.83) vs. 3.06 (0.74), $p = .287$).

Conclusion: Administration of 40 mg pravastatin resulted in an improvement in NO levels, and a decrease in IL-6 and endothelin (ET-1) levels. The direction of the effect of pravastatin on these biomarkers appears to underpin the potential for a beneficial effect of pravastatin in the prevention of preeclampsia.

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Pravastatin; vascular endothelial growth factor (VEGF); interleukin-6 (IL-6); nitric oxide (NO); endothelin-1 (ET-1)

Introduction

Preeclampsia is the second cause of maternal mortality and morbidity worldwide. Preeclampsia occurs in 2–8% pregnancy, and contribute to 70,000 maternal death and 500,000 neonatal death annually worldwide [1]. In many developing countries, preeclampsia is the leading cause of maternal death (15–20%), perinatal death, preterm birth, and intrauterine growth restriction (IUGR) [2–4]. Indonesia, the 4th most populous country in the world, still faces a significant problem of maternal mortality caused by preeclampsia. Indeed, the relative contribution of preeclampsia to overall

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maternal mortality may even be on the increase: 21.5% (2010) to 27.1% (2013) [5]. In Dr. Soetomo Hospital (the primary tertiary referral center for East Java) over the 2016–2017 period, 778 cases of preeclampsia were admitted, including 78 cases of eclampsia, tragically resulting in 26 cases of maternal death; a tremendous case fatality rate (CFR) of 3.3% (unpublished data).

While the preferred management of preeclampsia is delivery of the baby and placenta, prevention is, of course, the more desirable strategy to reduce maternal and perinatal mortality and morbidity. The primary prevention strategy recommended for preeclampsia right now is by giving low dose aspirin [6]. In a recent meta-analysis study by Roberge et al., it was highlighted that low dose aspirin needs to be started early in pregnancy (<16 weeks), because initiation of low dose aspirin after 16 weeks was shown to have a minimal impact on the risk of PE and severe PE [7]. Low dose aspirin also did not decrease the risk of preeclampsia >37 weeks gestational age and preeclampsia <37 weeks in multiparous women. A *post hoc* analysis of the ASPRE trial demonstrated that low dose aspirin does not prevent preeclampsia in chronic hypertensive women [8–10]. New strategies to prevent preeclampsia need to be developed for these reasons.

Ahmed et al. were the first to propose statins as a potential new approach in the prevention/management of preeclampsia. The first pilot study (STAMP trial: pravastatin to ameliorate early-onset preeclampsia) in the UK, showed no evidence of prolonging pregnancy in early-onset preeclampsia treated with pravastatin [11]. However, Costantine et al., in his pilot RCT showed a promising result of pravastatin used to prevent preeclampsia in high-risk women (although the sample size was small) (Costantine et al., 2016) [12]. The rationale to use statin as a drug for preeclampsia is based on animal studies showing that pravastatin (3-hydroxy-3-methylglutaryl coenzyme-A-reductase inhibitor) has a possible protective role in the uteroplacental interface and on vascular endothelial cells [13]. Pravastatin has pleiotropic effects that may be protective against preeclampsia such as endothelial protection, antioxidant properties, anti-inflammatory effects, anti-thrombotic effects, and possibly the most relevant pro-angiogenic effects [14]. The research on pravastatin has so far focused on its effect on angiogenic-antiangiogenic factors (sFlt and PlGF). The current study aimed to evaluate the effects of pravastatin on various additional inflammatory and endothelial markers (VEGF, ET-1, IL-6, and NO) in patients deemed to be a high risk of developing preeclampsia.

Methods

Recruitment patients, inclusion and exclusion criteria

The study was performed in Dr. Soetomo General Academic Hospital and Universitas Airlangga Hospital between July 2017 and December 2018. This study was part of the multicenter trial INOVASIA (Indonesia Pravastatin to Prevent Preeclampsia study, Clinical trial gov ID: NCT03648970). The study was approved by the Ethical Committee in Health Research Dr. Soetomo General Academic Hospital Surabaya (427/Panke.KKE/VII/2017) and Ethical and Law Committee of Universitas Airlangga Hospital (122/KEH/2017). The inclusion criteria for this study were: gestational age 10−20 weeks, with a history of previous preterm preeclampsia or patients with a combination of risk factors deemed to be at risk of at least 30% risk of developing preeclampsia). Consecutive patients recruited in this study included 11 patients based on a history of preterm preeclampsia and 27 patients based on a combination of clinical risk factors plus an abnormal uterine artery Doppler flow velocity waveform ($n = 38$). These risk factors included at least 2 of the following criteria: clinical risk factors (obesity, family history of preeclampsia, all type of diabetes, chronic hypertension, multiple pregnancy, nulliparity, age >40 years old, IVF, mean arterial pressure (MAP) >90 mmHg) plus an abnormal uterine artery Doppler. Obesity was defined as body mass index (BMI) value ≥30 kg/m^2, and it was measured during pregnancy [15]. The patients who had a mother or (a) sister(s) with a previous history of preeclampsia were defined as having a positive family history of preeclampsia. Chronic hypertension was defined as a high blood pressure before pregnancy or <20 weeks gestation [16].

The Doppler examination of the uterine artery was performed according to the protocol from the Maternal-Fetal Medicine Foundation [17–19]. Abnormal Doppler of uterine artery was defined based on the criteria: first-trimester screening: pulsatility index (PI) >95th centile; or second-trimester scanning: the presence of early diastolic notching or resistance index (RI) >0.58) [19–22].

Exclusion criteria were: contraindication to statin use (hypersensitivity to pravastatin, active liver disease, pre-pregnant renal disease), current use of statin, participation in any other clinical intervention trial investigation. Twenty-seven patients who came to our hospital were screened at first trimester (11–14 weeks) to identify the high-risk group. Eleven patients were booked at a later gestational age (14–20 weeks) (Table 1).

Table 1. Recruitment criteria.

1		History of preeclampsia Or	
2	Combination of 2 clinical risk factors	• Obesity • Family history of preeclampsia • Chronic hypertension • All type of diabetes • Multiple pregnancies • Nuliparity • Maternal ages >40 years old • IVF pregnancy • MAP >90 mmHg And	
	Abnormal uterine artery Doppler	First-trimester screening (11–14 weeks) Second-trimester screening (14–20 weeks)	PI >95th percentile • Early diastolic notching • RI >0.58

Blood sampling and treatment

Enrolled patients were randomly assigned into pravastatin or control group using SPSS software The pravastatin group received additional therapy of pravastatin (Novell Pharmaceutical Laboratories) (2 × 20 mg) in addition to the standard regimen of low dose aspirin (1 × 80 mg, peroral and calcium (1000 mg, per oral) (control group). Pravastatin administration was started directly at 14–20 weeks following recruitment and continued up to the time of delivery. Low dose aspirin and calcium were given started from 12 weeks of pregnancy and stopped at 36 weeks gestational age or when the patients developed preeclampsia.

In the first 40 consecutive patients, pretreatment samples 5–10 ml maternal blood were collected just before the first administration of pravastatin, and just before delivery. A blood sample was immediately sent to the laboratory (PRODIA Surabaya) to be further processed. A blood tube was left at room temperature for 60 min, and then centrifuged 1000 RPM for 15 min. Serum than were stored in a −80 °C freezer within a storage box, until the mother completed her involvement in the trial. After the mother completed her involvement in the trial, all samples were processed and analyzed. Forty kits were available for this particular study; differences in sample numbers were caused by sampling handling error within the hospital, and sample loss during transport.

Biomolecular analysis

The serum biomarkers were measured using specific reagent-kits: VEGF (RayBio® Human VEGF-A ELISA KIT. Ray Biotech Inc, 3607 Parkway Lane, Suite 200, Peachtree Corners, GA 30092, USA), IL-6 (RayBio® Human IL-6 ELISA KIT, RayBiotech Inc), ET-1 (IBL Human Endothelin-1 ELISA KIT. IBL International GMBH, Flughafenstrasse 52a, D-22335 Hamburg, Germany), and NO (QuantiChrom™ Nitric Oxide Assay Kit D2NO-100. BioAssay System, 3191 Corporate Place, Hayward, CA 94545, USA). Intra-assay and Inter-assay Coefficient of Variance using this kit in each biomarkers were: VEGF (<10% & <12%), IL-6 (<10% & <12%), ET-1 (4.6–8.5 & 3.1–8.1%), and NO (2.4% & 3.7%). VEGF, IL-6, and ET-1 were quantitatively measured using *in vitro* enzyme-linked immunosorbent assay (ELISA) sandwich methods in serum following the manufacturer's instructions. The serum NO levels were measured by applying the improve Griess reduction method.

Statistical methods

The data were first analyzed using descriptive statistical methods to measure the mean, standard deviation, median, and interquartile range. The normality of the data then analyzed using the Shapiro-Wilk test (since the number of samples less than 50). *p*-Value <.05 (Shapiro–Wilk test) indicate the abnormal distribution of the data. Maternal characteristic data were compared using Independent Sample *T*-Test (normal distribution) and Mann–Whitney Test (abnormal distribution data). The serum biomarkers pre and post-treatment data in each group then compared with the Paired Sample *T*-Test for the normally distributed data and Wilcoxon test (Non-Parametric test) for the abnormally distributed data. $p < 05$ indicated significant value. The data were presented as a mean + standard deviation (SD) for the normally distributed data and mean (interquartile range) for the abnormally distributed data. IBM© SPSS© Statistics version 25 was used for this statistical analysis.

Results

Maternal characteristics

Twenty-seven patients were recruited in the 1st trimester, six patients (22.2%) based on a history of preeclampsia, and 21 patients based on clinical risk

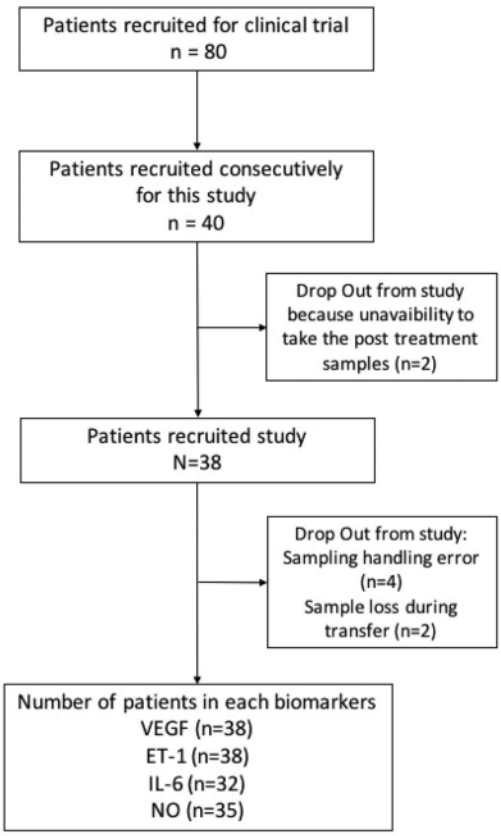

Figure 1. Patients recruitment algorithm.

factors and abnormal uterine artery doppler. Eleven patients were booked in the second trimester (14–20 weeks), with only 5 (45.4%) patients based on the history of preterm preeclampsia. The total sample recruited in this study included 38 patients, with a loss of 6 IL-6 and 3 NO samples caused by sampling handling error and sample loss during transfer (Figure 1).

There were no significant differences in maternal characteristics (age, body mass index (BMI), parity, gestational age at recruitment, gestational age at delivery) between the pravastatin and control group ($p > .05$) (Table 2). Except for the initial systolic blood pressure which was found to be slightly higher in pravastatin group (122.65 ± 11.16 vs. 116.37 ± 15.05; $p = .037$). No adverse side effects were observed in the pravastatin group.

Serum biomarkers levels in pravastatin and control group

Table 3 provides an overview of the pre-pravastatin and the pre-delivery levels of the various serum biomarkers. IL-6 levels showed a significant decrease in the pravastatin group (191.87 ± 82.99 vs. $151.85 + 48$. $p = .013$), compared to IL-6 level changes in control group (144.17 (53.91) vs. 140.82 (16.18). $p = .177$) (Figure 2). Also, the ET-1 levels in the pravastatin group decreased significantly (3.64 ± 0.85 vs. 3.01 ± 0.74. $p = .006$) while levels remained more or less static in the control group (3.57 ± 1.12 vs. 3.78 ± 0.73. $p = .594$) (Figure 3). NO level was the only serum marker that showed significant opposite changes between both groups. NO levels in the pravastatin group showed a significant increase after starting pravastatin (11.30 (17.43) vs. 41.90 (53.18). $p = .025$), while levels decreased significantly in the control group (38.70 (34.80) vs. 10.03 (26.96). $p = .025$) (Figure 4). VEGF level in both group did not show any statistical differences ($p = .463$; $p = .355$), VEGF in pravastatin group tended to increase (3.22 (0.62) vs. 3.28 (0.75)), while levels tend to decrease in control group after treatment (3.38 (0.83) vs. 3.06 (0.74)) (Figure 5).

Discussion

This study revealed that pravastatin induced change in the levels of the various biomarkers supporting the potential beneficial effect in the prevention of preeclampsia. Pravastatin use led to a significant increase in NO levels and a decrease in ET-1 levels suggesting a normalization or optimization of endothelial functioning. IL-6 levels decreased in the pravastatin group, indicating a decrease in overall maternal inflammation status. VEGF levels did not show any differences in both groups.

Preeclampsia is known as a syndrome with multiple complex and interacting pathogenetic pathways. The increasing imbalance between increasing anti-angiogenic factors over decreasing pro-angiogenic factors has been generally accepted as one of the main pathways in the pathophysiology of preeclampsia [23,24]. Endothelin-1 (ET-1) is considered to be a robust marker of marked pathological endothelial cell activation level ('cry of the dying endothelial cell'). ET-1 is a polypeptide with very potent vasoconstrictor activity. Besides its role as a general vasoconstrictor, ET-1 also induced oxidative stress and typically triggered a marked inflammatory response [25,26]. IL-6 is one of the multifunctional pro-inflammatory cytokines, which has a significant role in the acute phase reaction in the inflammation process. Several studies have found increased levels of IL-6 in preeclampsia patients, correlating with the severity of the disease [27,28].

Table 2. General maternal characteristics in each study group.

Variable	Pravastatin group ($n = 38$)	Control group ($n = 38$)	p
Maternal age (years old)	31.98 ± 5.72	29.75 ± 5.93	.092[a]
BMI (kg/m^2)	28.32 ± 5.71	26.15 ± 5.82	.097[a]
Parity			
Primi	9 (22.5%)	16 (40%)	.091[b]
Multi	31 (77.5%)	24 (60%)	
Gestational age at recruit (weeks)	16.60 ± 2.25	16.08 ± 2.24	.304[c]
Gestational age at delivery (weeks)	37.47 ± 1.48	36.05 ± 3.84	.315[c]

Significance value: $p < 0.05$. [a]Independent *T*-test; [b]Chi-square test; [c]Mann–Whitney test.

Table 3. Comparison serum biomarkers level before and after treatment.

Serum level	Before treatment	After treatment	p Value
IL-6			
Pravastatin group (ng/l)	191.87 ± 82.99	151.85 ± 48.46	.013*[a]
Control group (ng/l)	144.17 (53.91)	140.82 (16.18)	.177[b]
ET-1			
Pravastatin group (pg/ml)	3.64 ± 0.85	3.01 ± 0.74	.006*[a]
Control group (pg/ml)	3.57 ± 1.12	3.78 ± 0.73	.594[a]
NO			
Pravastatin group (mc/l)	11.30 (17.43)	41.90 (53.18)	.025*[b]
Control group (mc/l)	38.70 (34.80)	10.03 (26.96)	.025*[b]
VEGF			
Pravastatin group (pg/ml)	3.22 (0.62)	3.28 (0.75)	.463[b]
Control group (pg/ml)	3.38 (0.83)	3.06 (0.74)	.355[b]

The data were presented as a mean+SD for the normal distribution, and median (interquartile range) for the abnormal distribution. [a]Paired Sampel *T*-test; [b]Wilcoxon test; *indicate significant p value <0.05.

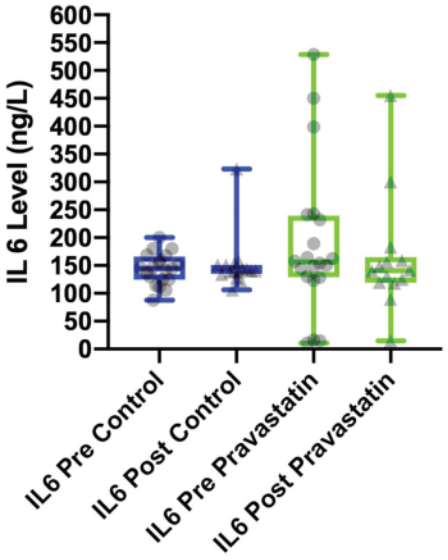

Figure 2. Box and whisker plot of the serum IL-6 pre and post treatment in control vs. pravastatin group.

This study demonstrated that pravastatin leads to a significant decrease in ET-1 levels. The ET-1 results of this clinical study are in line with Vitro study by Brownfoot et al., demonstrating that pravastatin significantly decreased expression of ET-1 mRNA in HUVECs (Human Umbilical Vein Endothelial Cells) treated with TNF-A or trophoblast conditioned media

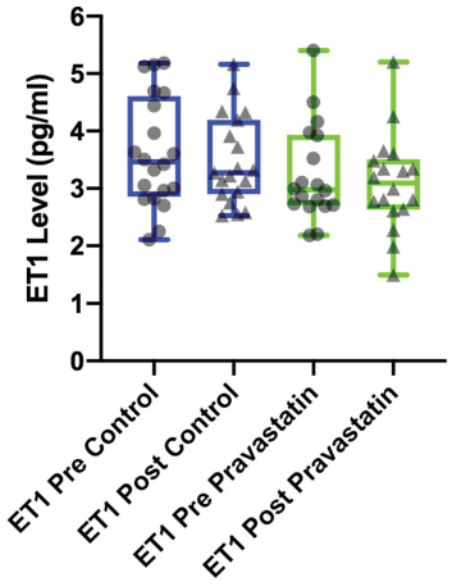

Figure 3. Box and whisker plot of the serum ET-1 pre and post treatment in control vs. pravastatin group.

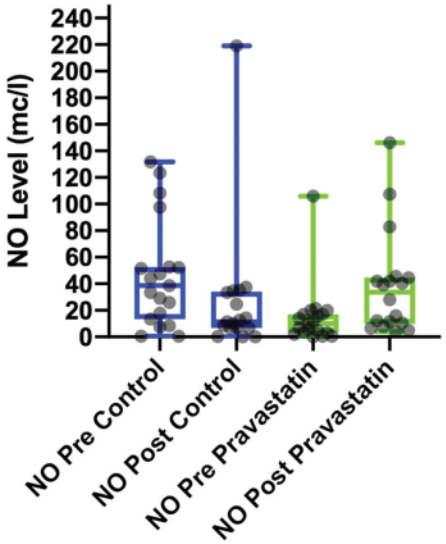

Figure 4. Box and whisker plot of the serum NO pre and post treatment in control vs. pravasatatin group.

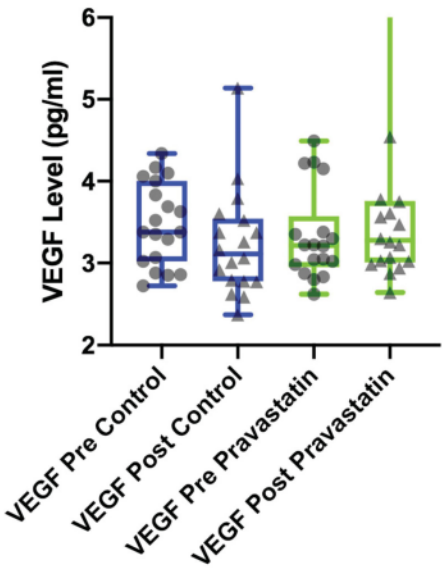

Figure 5. Box and whisker plot of the serum VEGF pre and post treatment in control vs. pravastatin group.

[29]. Pravastatin, through its HO/CO protective pathways, prevents endothelial dysfunction by inhibiting sFlt-1 and sEng release from the effects of hypoxia and reperfusion on the syncytiotrophoblast, and may stimulate the production of VEGF, and PlGF [30,31]. This will result in a lower production of ET-1 from endothelial cells and increased NO production via reduced oxidative stress [32].

Pravastatin significantly increased serum NO levels while high-risk women on just low-dose aspirin showed a decrease in NO levels. Garret et al., in her experimental study using pravastatin in a PE mice model, showed similar results. The addition of pravastatin to the PE mice model showed preservation of NO level similar to the normotensive control group. In contrast, in a group without pravastatin therapy, the NO level decreased significantly [33]. Statin has positive pleiotropic effects on the NO pathway by increasing its expression and activity, leading to increased NO bioavailability [34]. Statins increase NO synthesis via several mechanisms: prolong the stability of NO mRNA, reduce free radical oxidant formation, upregulating expression of NO mRNA, reduction of caveolin-1 levels in the plasma membrane [35–37]. Statin can also stimulate Akt activation (by inducing its translocation to the discrete site), which leads to phosphorylation of eNOS and directly activate NO. Statin also induces Heat Shock Protein-90 (HSP-90), which interacts with Akt to enhance the activity of NO [38]. Nevertheless, the pretreatment (baseline) value of the NO in both groups was different, with a significantly lower level in the pravastatin group. With these relatively small numbers, this might be related to the slightly more obese women in the pravastatin group and other uncontrolled variables such as dietary habits, medication intake, and possibly NO boosting supplement.

Pravastatin in this study appeared to attenuate the inflammatory process. Serum level IL-6 in pravastatin group were substantially decreased after treatment, while the level in the control group did not change. In another study using a PE mice model, the addition of Pravastatin reduced IL-6 levels equal to normal normotensive mice. While in PE mice model without pravastatin, IL-6 level was increased fivefold compared to control [33]. Preeclampsia has been long viewed as the result of excessive inflammatory reaction caused by a decompensated response to pregnancy or other chronic stimuli [39]. IL-6 levels are increased significantly in preeclampsia women [27,40] and may correlate with disease severity [41]. Statins have apparent anti-inflammation properties in the vascular wall by desensitizing the activation of pathways such as nuclear factor kappa B (NF-kB) and activator protein-1 (AP-1), and as such reducing pro-inflammatory gene expression (involving IL-6). In an in-vitro study (human mononuclear cells and vascular smooth muscle), Pravastatin, in particular, was found to have a higher inhibitory effect on IL-6 production compared to simvastatin and atorvastatin (60% vs. 53% vs. 50%) [42]. This in-vitro study also revealed that a combination of a statin with aspirin had an even higher effectivity in inhibiting the production of IL-6 from SMC and MNC [42].

VEGF was the only biomarker that did not show any significant difference between both groups. However, this should be interpreted carefully, since there are several known limitations on the measurement of VEGF levels worldwide. Until now, the international standard for the preparation required for measuring VEGF has not been established [43]. The calibrant has also not been standardized since a study on the differences of commercially available calibrant methods could produce significant inter-assay differences [44]. The type of VEGF measured may also vary, from circulating VEGF, total VEGF, free VEGF, and specific VEGF isotopes. In this study, we measured only the free VEGF because of its bio-availability. Some researchers are still in doubt that free VEGF could genuinely depict the VEGF production (or total VEGF) since it was affected by the degradation rates and or binding to carrier proteins. The last important issue is

the type of method used. In this study, we used "sandwich" methods, where the monoclonal antibodies detect antigen VEGF. The soluble receptors of VEGF (sFlt-1) could mask the epitope of VEGF so that it cannot be detected by the monoclonal antibodies [43].

The result of this study was promising, but further study with a larger sample needs to be performed to support and reassure this finding.

Conclusion

Based on our findings, we propose that Pravastatin has a potential beneficial role in preventing various pathological changes in women at a high risk of developing preeclampsia women, based on its pleiotropic effects and through its metabolite, HO-1 (*Hemeoxygenase*-1) and CO (*Carbon Monoxide*). Although these findings are exciting, the potential use of pravastatin in the prevention of hypertensive disorders of pregnancy should await the outcome of sizeable multicenter RCTs like the Indonesian INOVASIA study.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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