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DOI

[10.1016/S0002-9378\(97\)70212-6](https://doi.org/10.1016/S0002-9378(97)70212-6)

Publication date

1997

Published in

American journal of obstetrics and gynecology

[Link to publication](#)

Citation for published version (APA):

Konijnenberg, A., van der Post, J. A. M., Mol, B. W. J., Schaap, M. C. L., Lazarov, R., Bleker, O. P., Boer, K., & Sturk, A. (1997). Can flow cytometric detection of platelet activation early in pregnancy predict the occurrence of preeclampsia ? A prospective study. *American journal of obstetrics and gynecology*, 177, 434-442. [https://doi.org/10.1016/S0002-9378\(97\)70212-6](https://doi.org/10.1016/S0002-9378(97)70212-6)

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Can flow cytometric detection of platelet activation early in pregnancy predict the occurrence of preeclampsia?

A prospective study

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OBJECTIVES: An increased platelet activation status is present in patients with preeclampsia. Our purpose was (1) to establish by means of flow cytometry whether platelets circulate in an activated state during the first and second trimesters of pregnancy and (2) to establish whether early platelet activation predicts the onset of preeclampsia.

STUDY DESIGN: Consecutively, 244 pregnant women were included in a prospective study design. Platelets in whole blood samples from the pregnant women in the first trimester, the second trimester, and after delivery were labeled with the following antibodies associated with platelet activation: anti-CD62P (P-selectin, α -granule secretion), anti-CD63 (GP53, lysosomal secretion), anti-CD31 (GP1Ia), platelet endothelial cell adhesion molecule-1). The surface antigen exposure was determined by double-label flow cytometry with anti-CD42b (GPIb, a platelet-specific monoclonal glycoprotein) to select platelets and platelet-derived materials. Preeclampsia was defined as a diastolic blood pressure ≥ 90 mm Hg and proteinuria ≥ 0.3 gm in a 24-hour urine sample (International Society for Study of Hypertension in Pregnancy criteria).

RESULTS: Seventeen of 244 patients had preeclampsia (6.9%). Only first-trimester CD63 expression had an area under the curve >0.5 by receiver-operator characteristic curve analysis and was selected as a possible predictor of preeclampsia. We found a sensitivity of 47% and a specificity of 76% with use of a percentage of activated platelets above 2% as a positive test. Likelihood ratios were 1.94 for positive likelihood and 0.69 for negative likelihood. Univariate logistic regression analysis results were odds ratio 2.8 (95% confidence interval 1.0 to 7.6). Multivariate logistic regression analysis results were odds ratio 2.9 (95% confidence interval 0.92 to 8.9). However, the odds ratio of first antenatal diastolic blood pressure was two to four times higher than the odds ratio of first-trimester CD63 expression. The combination of first-trimester CD63 and first antenatal diastolic blood pressure increases the positive likelihood ratio from 1.94 to 9.4, with a sensitivity of 41%, a specificity of 96%, and a negative likelihood ratio of 0.62.

CONCLUSIONS: Increased first-trimester CD63 expression is an independent risk factor for development of preeclampsia. CD63 expression might be useful to identify a subgroup of patients with a high risk for development of preeclampsia, especially in combination with first-trimester antenatal diastolic blood pressure. This method of patient selection may enable more efficient intervention studies in patients at risk than do the selection methods used so far. (*Am J Obstet Gynecol* 1997;177:434-42.)

Key words: Preeclampsia, prediction, platelet activation, flow cytometry

Preeclampsia is a serious complication of pregnancy, with a high morbidity and mortality for mother and child.¹ The incidence of preeclampsia is approximately

5% of all pregnancies. Histologic characteristics of the placental bed are changed in preeclamptic patients, showing impairment of trophoblast invasion and acute atherosclerosis in spiral arteries.² Therefore preeclampsia is considered as a placental disease with involvement of various maternal organs. Local or systemic immunologic processes, endothelial cell damage, and platelet activation are supposed to play an important role in this disease, with involvement of multiple organs.^{3,4} In preeclampsia platelets are activated as demonstrated by elevated plasma concentrations of β -thromboglobulin, increased mean platelet volume, and increased platelet

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Received for publication October 29, 1996; revised March 7, 1997; accepted March 31, 1997.

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0002-9378/97 \$5.00 + 0 6/1/82245*

turnover.⁴ Like others, we found with use of flow cytometry an increased platelet activation status in preeclamptic patients compared with normotensive pregnant women.^{5,6}

During platelet activation several changes occur at the platelet surface. Currently flow cytometry is the most sensitive technique to detect increased surface exposure of activation antigens on the platelet surface.⁷ Whole blood flow cytometric techniques can minimize the artifactual platelet activation often seen with other techniques such as platelet aggregation and measurement of β -thromboglobulin in plasma.⁷

There is evidence that platelets already circulate in an activated state before the clinical onset of preeclampsia. Zemel et al.,⁸ Zemel and Zemel,⁹ and van der Post et al.¹⁰ reported increased cytoplasmic Ca^{++} concentrations after *in vitro* stimulation of the platelets with arginine vasopressin, although these results were disputed. Janes et al.¹¹ found an increased CD63 expression on the surface of platelets in the circulation of pregnant women at 29 weeks of gestation before clinical symptoms of preeclampsia were present. This CD63 expression was detected by means of flow cytometry. Increased CD63 expression on the platelet surface represents platelet lysosomal release, one of the phenomena occurring during platelet activation.¹² We recently described an increased expression of CD63 and in addition an increased expression of P-selectin (α -granule secretion) and platelet endothelial cell adhesion molecule-1 (PECAM-1) on the surface of platelets of preeclamptic patients compared with those of pregnant women with an uncomplicated pregnancy.⁶ Therefore we anticipated that CD63 expression might be increased before the full development of preeclampsia and probably the expression of P-selectin and PECAM-1 as well. In the current study we used the antibodies anti-CD63, anti-P-selectin, and anti-CD31 (anti-PECAM-1).¹²⁻¹⁴ P-selectin and PECAM-1 are cell adhesion molecules, which regulate interactions of endothelial cells with activated leukocytes and platelets and thus may also be of importance in the pathophysiologic mechanisms of preeclampsia.¹⁴⁻¹⁷

Because preeclampsia is considered a placental disease, it might be hypothesized that platelets become activated early in pregnancy, during the early phases of trophoblast invasion, which occurs from 13 to 20 weeks' gestation.¹⁸ Therefore we tested the pregnant women at approximately 13 and 20 weeks of gestation. The aim of this study was to establish the extent of platelet activation during the first and second trimesters by means of flow cytometry and to determine whether this can be an indicator to predict the later onset of preeclampsia.

Methods

Patients. Between January 1994 and October 1995 consecutive pregnant women with an increased risk for

development of preeclampsia who attended the outpatient clinic of our hospital were included. Risk factors for preeclampsia were healthy primigravid women; pregnant women with multiple pregnancies; women with known preexisting diseases such as hypertension, diabetes mellitus, and vasculitis; and women with a previous pregnancy complicated by preeclampsia or intrauterine growth restriction (<2.3 rd percentile) with or without hypertension not caused by other factors such as congenital malformations or infectious fetal disease. The study was approved by the Ethics Committee of the Academic Medical Center, University of Amsterdam. After patients had given informed consent, blood samples were taken by venipuncture to determine the platelet activation status at 13 and 20 weeks' gestation and 6 weeks after delivery. The first 54 women of all patients included in the current study who had a pregnancy not complicated by a preeclampsia were also tested after delivery. Thus we investigated two issues: (1) a possible correlation between the expression of platelet surface antigens in nonpregnant women versus this expression in the first and second trimesters of their normal pregnancies and (2) a possible difference in the platelet activation parameters between women with a normal pregnancy and women with preeclampsia during pregnancy but each group tested in the nonpregnant phase.

Gestational age was determined by the date of the last menstrual period in combination with ultrasonographic examination at 13 weeks' amenorrhea. Our aim was to include a group of 250 pregnant women with an estimated risk of 6% for the development of preeclampsia (i.e., we expected a group of 15 patients to have preeclampsia during the course of the study).

Blood pressure was measured with a standard sphygmomanometer. During routine obstetric care proteinuria was established with repeated dipstick testing (Albym-test, Boehringer-Mannheim). In case of preeclampsia proteinuria was quantitated in a 24-hour urine sample. The pregnancy course was registered by the midwife or the gynecologist, who were unaware of the results of the laboratory measurements. Preeclampsia was defined according to the criteria of the International Society for Study of Hypertension in Pregnancy, as a diastolic blood pressure of ≥ 90 mm Hg in combination with a proteinuria of ≥ 0.3 gm in a 24-hour urine sample.¹⁹ A pregnant woman with a diastolic blood pressure ≥ 90 mm Hg at first antenatal visit was considered to have chronic hypertension. These chronic hypertensive patients had no proteinuria at the first visit. When those pregnant women had a proteinuria of ≥ 0.3 gm in a 24-hour urine sample they were considered to be (superimposed) preeclamptic patients. An independent investigator unaware of the results of the laboratory measurements evaluated medical records for the occurrence of preeclampsia according to the defined criteria.

Materials. Anti-CD63 was obtained from Immunotech, Marseilles, France. The antibodies anti-GPIb (CLB-MB45), anti-P-selectin (CLB-thromb/6) and anti-PECAM-1 (CLB-ES12F11) were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. Anti-GPIb was biotinylated by the method of Hnatowitch et al.²⁰ and the fluorescein isothiocyanate labeling was performed by the method of The and Feltkamp.²¹ Phycoerythrin-conjugated streptavidin was obtained from Dakopatts, Glostrup, Denmark. Only monoclonal antibodies were used.

Preparation of blood samples. Blood was obtained from an antecubital vein through a butterfly needle (1.0 mm) with either no or a light tourniquet. The first 4 ml of blood was discarded. Then a 2.7 ml sample of blood was collected in 0.3 ml of 3.2% sodium citrate. Within 3 minutes 5 μ l aliquots of the citrated blood were added to tubes containing 35 μ l of HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) buffer (137 mmol/L sodium chloride, 2.7 mmol/L potassium chloride, 1.0 mmol/L magnesium chloride, 5.6 mmol/L glucose, 20 mmol/L HEPES, 1 mg/ml albumin, 3.3 mmol/L sodium phosphate, pH 7.4), 5 μ l anti-GPIb (1.5 μ g/ml final concentration) and 5 μ l of a fluorescein isothiocyanate-labeled second antibody. Final concentrations were 10 μ g/ml anti-P-selectin, 5 μ g/ml anti-CD63, and 1 μ g/ml anti-PECAM-1. In preliminary in vitro experiments these concentrations yielded optimal detection of the platelet antigens. Of circulating cells, GPIb is solely confined to platelets, so anti-GPIb monoclonal antibodies can be used to select platelets and platelet-derived material in the flow cytometric analyses.

After careful mixing and 15 minutes of incubation at ambient temperature in the dark, 5 μ l of 10-fold diluted phycoerythrin-conjugated streptavidin was added. Incubation continued for another 15 minutes, and finally the samples were diluted by the addition of 2.0 ml of HEPES buffer.

With each study of a preeclamptic patient or a normal pregnant woman, blood was simultaneously collected from a healthy nonpregnant control subject and processed identically. Those samples served to set the threshold for the platelet activation in the flow cytometric analysis at approximately 1% to 3%.

Flow cytometric analysis. Double-label flow cytometry was performed as described by Shattil et al.⁷ Within 2 hours after collection the samples were analyzed in a FACScan flow cytometer with PC-lys software (Becton-Dickinson, San Jose, Calif.). Forward light scatter and sideward light scatter were set at logarithmic gain. Platelet material was identified by analyzing the biotinylated GPIb content of particles at 585 nm. The platelets (70% to 75% of all platelet-derived particles) were distinguished from microparticles and platelet-leukocyte com-

plexes by measuring their size on gating in the forward scatter versus side scatter mode.

The surface expression of activation markers on a population of 5000 platelets was determined by the fluorescence intensity of the fluorescein isothiocyanate-labeled antibodies at 515 nm. The data were analyzed as percentages of platelet activation because we found in a cross-sectional study that in preeclampsia a subpopulation of platelets is activated and not the whole platelet population.⁶ Analysis of the percent platelet activation above a certain threshold provides information about activation of a subpopulation, whereas the other method of analysis, the analysis of mean fluorescence intensities, is indicative of activation of the total platelet population. For the data analysis to determine the percent of activated platelets, the threshold for platelet activation was set at approximately 1% to 3% fluorescence-positive platelets in healthy nonpregnant control samples. Subsequently, the percentages of platelets from preeclamptic patients or normotensive pregnant women with fluorescein isothiocyanate fluorescence above these thresholds were determined. The percentage of positive platelets in the nonpregnant control sample was then subtracted to obtain the final result as the percentage of activated platelets. We decided to use this method of analysis instead of a comparison with a nonspecific control antibody to exclude accidental in vitro platelet activation as evidenced in the healthy control sample tested simultaneously.

To investigate the within-assay coefficient of variation of the platelet activation analysis, blood was obtained from one healthy volunteer and used to prepare 10 mixtures for flow cytometric analysis with the HEPES buffer, first and second antibody, as described above. The between-assay coefficient of variation was established by testing a volunteer on 10 different occasions between April 1996 and January 1997. The within-assay coefficient of variation of the mean fluorescence intensities with anti-P-selectin, anti-CD63, and anti-PECAM-1 were 1.4%, 1.0%, and 0.7%, respectively. Between-assay coefficients of variation of the mean fluorescence intensities were 7.8%, 7.9%, and 12.5%, respectively. The within-assay coefficients of variation of the data analysis of the percentage of activated platelets with anti-P-selectin, anti-CD63, and anti-PECAM-1 were 15% (range of percentage of activated platelets 1.8% to 2.9%), 6.2% (range 2.2% to 2.5%), and 8.7% (range 1.7% to 2.3%), respectively. The between-assay coefficient of variation of the percentage of activated platelets has no meaning because the percentage of activated platelets on that day is always, arbitrarily, set at 1% to 3% with the sample from the healthy individual. The reproducibility of this biologic assay was considered appropriate.

Statistics. The capacity of the three platelet surface antigens CD63, P-selectin, and PECAM-1 to predict the

Table I. Patient characteristics

	<i>Not preeclamptic</i>	<i>Preeclamptic</i>	<i>Significance</i>
No. of individuals	227	17	
Age (yr)	29 (16-43)	32 (26-42)	$p = 0.83$
Amenorrhea at booking (days)	94 (45-143)	96 (64-124)	$p = 0.53$
Initial diastolic BP (mm Hg)	67 (45-95)	77 (55-105)	$p = 0.21$
Amenorrhea second visit	140 (102-166)	132 (114-150)	$p = 0.89$
Diastolic BP second visit	66 (45-110)	79 (60-110)	$p = 0.07$
Maximal diastolic BP	82 (60-125)	106 (90-125)	$p = 0.000$
Proteinuria (gm/24 hr)	Negative	3.6 (0.3-19.0)	$p = 0.000$
Birth weight (gm)	3182 (674-4200)	2515 (440-4200)	$p = 0.000$
Amenorrhea at delivery	276 (173-297)	246 (117-292)	$p = 0.000$
Days after delivery	66 (42-412)	47 (46-178)	$p = 0.49$
Diastolic BP after delivery	73 (50-90)	85 (70-115)	$p = 0.03$

Differences are established with Mann-Whitney *U* test. Values are expressed as mean and range. BP, Blood pressure

occurrence of preeclampsia was evaluated with receiver-operator characteristic curve analysis. We determined a cutoff value of 2%. For this cutoff value we constructed 2×2 tables. Subsequently sensitivity, specificity, and likelihood ratios were calculated. The likelihood ratio of a positive test result indicates the likelihood of a positive test result in a patient with the disease over the likelihood of a positive test result in a patient without the disease. The likelihood ratio of a negative test result indicates the likelihood of a negative test result in a patient with the disease over the likelihood of a negative test result in a patient without the disease. The positive likelihood ratio is therefore calculated as (Sensitivity/[1 - Specificity]) and the positive likelihood ratio as ([1 - Sensitivity]/Specificity). Subsequently, the association between patient characteristics and the occurrence of preeclampsia was evaluated with use of logistic regression analysis. Variables considered singly and in combination in the logistic regression model were test result of the platelet activation marker(s) that can predict preeclampsia, age >36 years, first antenatal diastolic blood pressure, first antenatal systolic blood pressure, a history of a previous pregnancy complicated by preeclampsia or the syndrome of hemolysis, elevated liver enzymes, and low platelets (HELLP). Differences in patient characteristics between the preeclamptic and the pregnant women with uncomplicated pregnancy were established with a Mann-Whitney *U* test.

Results

Patient characteristics. A total of 287 eligible consecutive women attending the outpatient clinic of our hospital were included. Thirty-three pregnant women did not give informed consent. During the study 10 women were excluded because of various reasons: preterm delivery mean amenorrhea 131 days (range 125 to 139 days, $n = 4$, only one of these patients had an increased expression of CD63, 7.9 % activated platelets), refusal after the first venipuncture ($n = 2$, none of these women had an increased CD63 expression), and because

the follow-up of their pregnancy outcomes was not known ($n = 4$, one of these women had an increased CD63 expression, 11.1 % activated platelets). A group of 244 pregnant women remained for analysis. This group consisted of 199 healthy primigravid women; 11 women were labeled as having essential hypertension on the basis of the medical history, 12 women had a twin pregnancy, 2 women had insulin-dependent diabetes mellitus, and 20 women had a previous pregnancy complicated by preeclampsia or HELLP syndrome. According to the criteria of the International Society for Study of Hypertension in Pregnancy, 17 women had preeclampsia out of this group of 244 pregnant women (6.9%). Of the 17 preeclamptic patients, 9 patients came from a total of 199 healthy primigravid women and 8 patients came from the group of 45 women with a preexisting disease or multiple pregnancy or a previous pregnancy complicated by preeclampsia or HELLP syndrome. The preeclamptic patients differed significantly from those without preeclampsia in maximal diastolic blood pressure during pregnancy but also in diastolic blood pressure after delivery, in proteinuria, in amenorrhea at delivery, and in birth weight of the newborns (Table I). Six of the 244 pregnant women had a diastolic blood pressure ≥ 90 mm Hg at the first antenatal visit. Of these 6 women 2 had superimposed preeclampsia.

We calculated correlation coefficients to determine whether the test result of first-trimester CD63 expression is correlated with the severity of disease. The factors considered were test result of first-trimester CD63 expression, birth weight, first antenatal and maximal diastolic blood pressure, and proteinuria. We did not find any significant correlation between these factors.

In 4 cases of the 17 preeclamptic patients, proteinuria could not be quantitated by a predelivery 24-hour urine sample because the women involved were delivered within 24 hours of attending the clinic. In these cases dipstick testing was performed in two random urine samples and always showed a test result of 2+ or more. A test result of 2+ or more corresponds to a proteinuria of

Fig. 1. Receiver-operator characteristic curves are represented for CD63 expression in first (*solid line*) and second (*dotted line*) trimesters.

≥ 1.0 gm/L.²² One patient of these 4 preeclamptic cases was admitted to our hospital with severe intracerebral hemorrhage. Her baby was rapidly delivered by cesarean section and was in good condition. Unfortunately, the mother did not survive. Proteinuria was not determined, but this patient showed all laboratory characteristics of the HELLP syndrome (ranges of reference values are between parenthesis): maximal diastolic blood pressure 90 mm Hg, hemoglobin 5.7 mmol/L (7.5 to 9.8 mmol/L), hematocrit 0.28 L/L (0.38 to 0.45 L/L), aspartate aminotransferase 462 U/L (<30 U/L), alanine aminotransferase 480 U/L (<26 U/L), and thrombocytes 39.10^9 /L (150 to 350/L).

Platelet activation analyses. Of the 17 preeclamptic patients, 13 were tested after the delivery. Of the other 4 patients, 1 patient had preeclampsia complicated by a cerebral hemorrhage and did not survive and 3 patients did not give consent for testing after delivery. The first 54 women in the study who had a pregnancy not complicated by preeclampsia were also tested after delivery. We did not observe a difference in the expression of CD63, P-selectin, and PECAM-1 between those 54 women with a normal pregnancy and the 13 preeclamptic patients tested at this study period. We could also not find any correlation between the expression of the platelet activa-

tion markers in the first or second trimester of pregnancy with the expression of these markers after the delivery in the 54 women with normal pregnancy.

In Fig. 1 receiver-operator characteristic curves are presented for the first (Fig. 1, A) and second trimesters (Fig. 1, B) of the three markers that we anticipated a priori to be increased because of the results found in the cross-sectional study⁶: CD63, P-selectin, and PECAM-1. Receiver-operator characteristic curve analysis demonstrated that only first-trimester anti-CD63 expression had an area under the curve of 0.63, which is clearly >0.5 and was selected as a potential predictor. All other receiver-operator characteristic curves in the first and second trimesters had an area under the curve <0.5 (Fig. 1). The area under the receiver-operator characteristic curve (95% confidence interval) was for first-trimester CD63 0.63 (0.47 to 0.78) and for P-selectin 0.47 (0.33 to 0.6) and for PECAM-1 0.52 (0.34 to 0.7) and for second-trimester CD63 0.44 (0.30 to 0.57), P-selectin 0.38 (0.26 to 0.49), and PECAM-1 0.39 (0.25 to 0.53). P-selectin and PECAM-1 were therefore not selected as useful predictors of preeclampsia. For first-trimester CD63 expression, a percentage of activated platelets $>2\%$ was determined to be the best cutoff point with the highest value of sensitivity and specificity (Table II). With this cutoff point

Table II. Clinical usefulness of first-trimester CD63 expression and first antenatal diastolic blood pressure

	Preeclampsia		Total
	+	-	
First-trimester CD63 expression*			
$\geq 2\%$	8	55	63
$< 2\%$	9	172	181
TOTAL	17	227	244
First antenatal diastolic blood pressure†			
≥ 75 mm Hg	12	57	69
< 75 mm Hg	5	170	175
TOTAL	17	227	244
Combination of first-trimester CD63 expression and first antenatal blood pressure ≥ 75 mm Hg‡			
Both tests positive	7	10	17
Remaining pregnant women	10	217	227
TOTAL	17	227	244

+, Positive; -, negative.

*Prevalence 6.9%, sensitivity 47%, specificity 76%, likelihood ratio of positive test result 1.94, likelihood ratio of negative test result 0.69.

†Sensitivity 75%, specificity 75%, likelihood ratio of positive test result 2.8, likelihood ratio of negative test result 0.39.

‡Sensitivity 41%, specificity 96%, likelihood ratio of positive test result 9.4, likelihood ratio of negative test result 0.62.

we found a sensitivity of 47%, a specificity of 76%, a likelihood ratio for a positive test result of 1.94, and a likelihood ratio for a negative test result of 0.69.

Univariate analysis demonstrated that first-trimester CD63 expression is a possible predictor of preeclampsia (odds ratio 2.8, 95% confidence interval 1.03 to 7.6) (Table III). Also, multivariate logistic regression analysis demonstrated that increased first-trimester CD63 expression still had an increased odds ratio of 2.9 (95% confidence interval 0.92 to 8.9), which means that it can be considered as an independent predictor for the occurrence of preeclampsia (Table III). Some interaction existed between other known risk factors for the development of preeclampsia, such as a history of a previous pregnancy complicated by preeclampsia, an age older than 36 years, or a first antenatal diastolic blood pressure of ≥ 75 mm Hg (Table III).

Comment

We hypothesized that platelet activation can be detected in early pregnancy in those patients in whom preeclampsia will develop later in pregnancy. Because preeclampsia is considered to be a placental disease with impaired trophoblast invasion, which might cause specific pathologic changes in the spiral arteries and their branches of the maternal circulation, this should result

Table III. Logistic regression analysis

	Univariate analysis: Odds ratio and 95% CI	Multivariate analysis: Odds ratio and 95% CI
CD63 13 wk	2.8 (1.0-7.6)	2.9 (0.92-8.9)
Age > 36 yr	2.7 (0.95-7.8)	1.7 (0.34-8.0)
RR_{dia} 75-80 mm Hg	3.9 (0.88-17)	5.4 (1.13-25)
RR_{dia} 80-85 mm Hg	8.1 (2.2-30)	5.7 (1.38-24)
RR_{dia} > 85 mm Hg	12 (2.9-53)	6.8 (1.23-38)
RR_{sys} > 130 mm Hg	5.5 (1.6-20)	2.6 (0.50-13.3)
History of preeclampsia	3.0 (0.79-12)	2.6 (0.49-14)

Univariate and multivariate logistic regression analysis, odds ratios and 95% confidence intervals are represented. CD63 13 weeks indicates first-trimester CD63 test result. History of preeclampsia means a previous pregnancy complicated by a preeclampsia or HELLP syndrome. *CI*, Confidence interval; RR_{dia} , booking diastolic blood pressure; RR_{sys} , booking systolic blood pressure.

in platelet activation.² Therefore we took blood samples and examined the platelet activation status at an average of 13 and 20 weeks' gestation age, the periods coinciding with the invasion of the trophoblast.¹⁸ Moreover, during normal pregnancy the invasion of the trophoblast is presumed to be controlled by a maternal immune response that is altered in patients with preeclampsia.^{15, 18, 23} During immunologic responses activated platelets interact with activated endothelial cells and leukocytes.¹⁵ Indeed, in this study we found evidence of increased activation of platelets at 13 weeks' gestation in patients destined to have preeclampsia compared with women with uneventful pregnancies. This was confined to CD63 expression. This observation indicates that an increased platelet activation status is present on average 21 weeks (range 3 to 27 weeks) before the development of preeclampsia, which opens new perspectives for early identification of a population at risk and also for early clinical intervention with, for instance, aspirin. However, some reservations should be made.

The logistic regression analysis demonstrated that an initial diastolic blood pressure > 75 mm Hg is far more predictive for the development of preeclampsia than the test result of first-trimester CD63 expression. The odds ratio for initial diastolic blood pressure > 75 mm Hg is almost twice higher than the odds ratio of 2.9 for first-trimester CD63 expression (Table III). This is in contrast with the study of Sibai et al.²⁴ investigating clinical risk factors in healthy nulliparous women. Systolic blood pressure at 13 weeks appeared to be highly predictive and diastolic blood pressure was the least predictive.²⁴ In our study logistic regression analysis demonstrated that the initial systolic blood pressure is not an independent predictive factor for the development of preeclampsia (Table III). How can we explain this? First, this might be due to a different study group. In the article of Sibai et al.²⁴ only healthy primigravid

women were included. Second, we measured blood pressure with a standard sphygmomanometer. Possibly in the study of Sibai et al. a different device for assessment of blood pressure levels was used, which might alter the relationship between diastolic and systolic blood pressure, unlike our measurements.

Still, the independence of increased CD63 expression on platelets as a predictor for the development of preeclampsia among factors such as initial blood pressure confirms the involvement of platelets in the pathogenesis of preeclampsia.³ We speculated that a combination of a platelet activation test with initial blood pressure might be a tool to identify pregnant women at risk for development of preeclampsia and that intervention with, for instance, aspirin might be more beneficial in women selected this way. Therefore we analyzed the combination of first-trimester CD63 expression and initial diastolic blood pressure ≥ 75 mm Hg. This combination increases the likelihood ratio of a positive test result from 1.94 to 9.4 (Table II). The sensitivity is 41%, the specificity is 96%, and the likelihood ratio of a negative test result is 0.62. By applying this combined test, we might have found 7 of all 17 preeclamptic patients plus 10 pregnant women who did not have preeclampsia. In the whole patient group of 244 patients, 17 women were found with both measurements positive. How would this compare to the aspirin intervention studies? In the study by Schiff et al.²⁵ a randomized placebo-controlled study to investigate the capacity of aspirin to prevent preeclampsia, a patient group with the same patient characteristics compared with our study was used. In the placebo arm of that study, with use of the rollover test to select patients at risk, they were able to identify 22.6% of all preeclamptic patients. Applying our method of risk selection, on the basis of platelet behavior in combination with blood pressure, possibly almost twice as many patients would have been identified. Moreover, the most important effect of aspirin probably lies in blocking the thromboxane A_2 synthesis in activated platelets. Increased CD63 expression directly indicates platelet activation. It is therefore, in combination with blood pressure, more likely to identify those patients who will benefit from aspirin treatment than tests based solely on vascular reactivity, such as a rollover test. This needs confirmation in future prospective studies.

The increased CD63 expression indicates platelet activation. It may seem surprising that the expression of P-selectin and PECAM-1 appeared not to be predictive for preeclampsia in the first trimester of pregnancy, whereas CD63 is, if all three parameters are measures of platelet activation. However, PECAM-1 is localized at various sites in the platelet, not only the α -granule that also contains P-selectin. Its appearance on the surface membrane on platelet activation is still under investigation. In *in vitro* studies we observed that the expression

of PECAM-1 actually becomes decreased on platelet activation, possibly because of its relocation from the platelet surface into the open canalicular membrane system (unpublished results). A discrepancy between PECAM-1 and CD63 is therefore not surprising. On the other hand, two markers of the platelet secretory reaction (i.e. P-selectin from the α -granule and CD63 from the lysosomal granule) might be expected to give similar results. However, P-selectin can be shed from the platelets after its appearance on the platelet surface and measured as soluble component in the plasma. Such shedding has so far not been reported for CD63 but cannot be excluded. Also, the composition of the platelet surface is continuously undergoing adjustments as a result of a continuous transport of many components from the surface into the interior of the platelet and vice versa. The expression of CD63, PECAM-1, and P-selectin can therefore not be compared directly and each marker should be checked for its clinical usefulness.

It might also be argued that the increased CD63 expression is found by chance. This seems unlikely for three reasons. First, the multiple logistic regression analysis demonstrated that first-trimester CD63 expression is an independent factor for the prediction of preeclampsia (Table III). Second, Janes et al.¹¹ reported an increased expression of CD63 at a gestational age of 29 weeks before the development of the classic clinical symptoms of preeclampsia. And third, we found an increased expression of CD63 in combination with an increased expression of the cell adhesion molecules P-selectin and PECAM-1 in third-trimester preeclamptic patients.⁶

Remarkably, we did not find activated platelets at a gestational age of 20 weeks in the women in whom preeclampsia will develop, which is difficult to explain. We can only hypothesize that the second wave of trophoblast invasion is less immunogenic than the first wave, causing no detectable platelet activation. Another possibility might be that, because of biologic variability in trophoblast invasion between individuals, we tested some of the women at the wrong moment. Sampling at more different time points might have provided more precise information about early platelet activation. This was not feasible in this study.

Evidence is increasing that maternal disorders are predisposing to the development of preeclampsia.²⁶ To test whether the differences in platelet activation might be an inherent maternal characteristic, postpartum CD63 expression, as well as P-selectin and PECAM-1 expression, were compared between the preeclamptic patients and normal pregnant women. No differences were found between the two groups. We also could not demonstrate a correlation between the expression of the platelet activation markers during and after the delivery in the women with a normal pregnancy.

Correlation coefficients were determined to investigate whether the test result of first-trimester CD63 expression is correlated with severity of disease because in our previous study we did observe a correlation between platelet CD63 expression in preeclamptic patients and disease severity.⁶ We did not find any significant correlation between these factors. This may be due to the lower extent of platelet activation found in first-trimester pregnant women in whom preeclampsia will develop (median percent of activated platelets 0.67%, range -1.09% to 9.05%) compared with the extent of platelet activation in diseased third-trimester preeclamptic patients (median percent of activated platelets 8.0%, range -1.6% to 38.6%). Also, platelet activation in third-trimester preeclamptic patients is likely to be caused by generalized endothelial damage, which is unlikely to be the cause of platelet activation in the first trimester. Additionally, the severity of disease of the preeclamptic patients in the previous study was more severe compared with the severity of disease of the patients in the current study. The third-trimester preeclamptic patients had lower mean birth weights (1528 gm, range 230 to 3020 gm) and higher mean maximal diastolic blood pressures (113 mm Hg, range 100 to 130 mm Hg) compared with the 17 preeclamptic patients from this study. This may explain the lower extent of platelet activation found in the 17 preeclamptic patients in the current study.

Previously we reported that only a subpopulation of the platelets appears to be activated in preeclampsia.⁶ It may be wondered why not all the platelets are activated because they all circulate. This question cannot definitely be answered. Possibly all platelets happen to become somewhat activated at some stages during their lifespan in the circulation, even in nondiseased subjects, for instance, if the platelets come across an endothelial injury, become temporarily trapped at sites of turbulent flow at vessel bifurcations (frequently the sites of thrombi), or because of other stimuli. These activations may either be reversible or exceed a certain activation threshold that leads irreversibly to adhesion or aggregation at the activation site. Only the platelets that still circulate are being analyzed with this technique, and the most extensively activated platelets will be trapped at the activation site. It is then possibly not surprising that most of the circulating platelets are still, or again, in the nonactivated state as the result of an inherent capability to become either reversibly activated or irreversibly and thus lost from the circulation.

Of course, the study group was somewhat heterogeneous with regard to the risk factors for the development of preeclampsia. It would have been of interest to stratify the results on the basis of subgroups (e.g., prior hypertension, multiple gestation, or nulliparity), but unfortunately these subgroups are too small to perform such an analysis.

It may be wondered whether the CD63 measurement should now be recommended for routine clinical use. Costwise this seems feasible. The reagent cost involved in a double-label flow cytometric analysis with anti-GPIb and anti-CD63 is an estimated U.S. \$2.50 per sample, including the blood collection device, fluorescently labeled antibodies, buffers, and materials of the flow cytometer. If the time involved in the blood collection is excluded, then the actual processing time of one sample in the laboratory and the measurement at the flow cytometer would be about 30 minutes but could also include up to 10 patients in the same period. The data analysis would require some 5 minutes per sample. So, all in all, routine use seems attainable from a cost point of view. Still, we do not recommend this. At the current stage its use should be limited to research protocols, for instance, to attempt to identify the pregnant women at risk for the development of preeclampsia in prospective trials on the preventive treatment with low-dose aspirin, as mentioned previously. If such trials prove effective, routine use could be in place.

Finally, if application of this test in routine antenatal care is to be considered at some stage in the future, then the test needs to be modified to make it more useful for routine laboratory practice. In the current study the flow cytometric analysis had to be performed within 2 hours after blood collection. Currently, our laboratory is performing experiments to enable analysis up to 24 hours after blood collection by fixation of the platelets with paraformaldehyde.

In conclusion, we observed in the first trimester that an increased CD63 expression is a predictive factor for the development of preeclampsia. However, the odds ratio for this test is low compared with the other known risk factors (i.e., first antenatal diastolic blood pressure). A combination of these two factors seems promising to identify in the first trimester of pregnancy those pregnant women who are at risk for development of preeclampsia. This combined approach of patient selection may enable more efficient intervention studies in patients at risk than did the selection methods used before.

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