HOKKAIDO UNIVERSITY

Title	Platelet reactivity in twin pregnancies
Author(s)	Umazume, Takeshi; Yamada, Takahiro; Morikawa, Mamoru; Ishikawa, Satoshi; Furuta, Itsuko; Koyama, Takahiro; Matsuno, Kazuhiko; Minakami, Hisanori
Citation	Thrombosis Research, 138, 43-48 https://doi.org/10.1016/j.thromres.2015.12.019
Issue Date	2016-02
Doc URL	http://hdl.handle.net/2115/64445
Rights	© 2016. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/
Rights(URL)	http://creativecommons.org/licenses/by-nc-nd/4.0/
Type	article (author version)
File Information	manuscript.pdf

Platelet reactivity in twin pregnancies

Takeshi Umazume[a], Takahiro Yamada[a],* Mamoru Morikawa[a], Satoshi Ishikawa[a], Itsuko Furuta[a], Takahiro Koyama[a], Kazuhiko Matsuno[b], Hisanori Minakami[a]

a Department of Obstetrics, Hokkaido University Graduate School of Medicine, Sapporo, Japan
b Division of Laboratory and Transfusion Medicine, Hokkaido University Hospital, Sapporo, Japan

*Corresponding author:
Takahiro Yamada, MD, PhD,
Department of Obstetrics, Hokkaido University Graduate School of Medicine, Kita-ku
N14 W6, Sapporo 060-8638, Japan
TEL +81-11-706-6051 FAX +81-11-706-7981
E-mail address: taka0197@med.hokudai.ac.jp

Abstract

Background: Gestational thrombocytopenia is more likely to occur in twin than singleton pregnancies. However, it is unclear whether platelets are more reactive in twin than singleton pregnancies.

Methods: Changes in spontaneous platelet aggregation and concomitant fall in platelet count were examined over 90 minutes after blood sampling in 171 and 52 citrated whole blood (CWB) samples from 59 and 17 women with singleton and twin pregnancies, respectively. Soluble P-selectin (sP-selectin) levels in the plasma were also determined.

Results: CWB 60 min after blood sampling during 2nd trimester exhibited significantly larger numbers of platelet aggregates (1297 ± 1600 vs. $497\pm432/\mu l$, $P=0.040$) concomitant with significantly greater net decrease in platelet count (152 ± 55 vs. $115\pm45\times10^9/\mu l$, $P=0.036$) in twin than singleton pregnancies, respectively. This was followed by significantly lower 3rd trimester platelet count (181 ± 43 vs. $229\pm62\times10^9/l$, $P=0.009$) with significantly greater mean platelet volume (8.0 ± 1.2 vs. 7.1 ± 1.1 fl, $P=0.021$) in twin than singleton pregnancies, respectively. The 3rd trimester sP-selectin per platelet was significantly higher in twin than singleton pregnancies.

Conclusions: Platelets were more reactive in the 2nd trimester of twin than singleton pregnancies. This enhanced platelet reactivity may explain the decreased platelet count in the 3rd trimester of twin pregnancy.

Key words: gestational thrombocytopenia, HELLP syndrome, platelet aggregation assay, platelet reactivity, soluble P-selectin

List of abbreviations
CWB: citrated whole blood
HELLP syndrome: syndrome of hemolysis, elevated liver enzymes, and low platelet counts
IPF: immature platelet fraction
MPV: mean platelet volume
PA: platelet aggregate

Highlights
1. Gestational thrombocytopenia is likely to occur in twin pregnancy.
2. Spontaneous platelet aggregation (SPA) can occur in stirred citrated whole blood.
3. SPA of 2nd trimester blood was more active in twin than in singleton pregnancies.
4. SPA was associated with decrease in platelet counts.
5. Enhanced platelet reactivity may explain thrombocytopenia in twin pregnancy.

1. Introduction

Pregnancy is associated with a hypercoagulable state and increased risk of venous thromboembolism [1, 2]. As women with essential thrombocythemia have increased likelihood of experiencing adverse pregnancy outcomes, such as abortion and fetal demise [3, 4], the degree of platelet reactivity may be associated with pregnancy outcome. Theoretically, the circulating platelet count may decrease in the presence of disproportionately enhanced platelet reactivity for the platelet production in the bone marrow. Indeed, some pregnant women exhibit a gradual decline in platelet count during pregnancy (gestational thrombocytopenia) [5 – 7] and women with gestational thrombocytopenia are prone to the syndrome of hemolysis, elevated liver enzymes, and low platelet counts (HELLP syndrome) [6, 8, 9]. Gestational thrombocytopenia and HELLP syndrome occur significantly more often in women with twin than singleton pregnancies [5 – 7, 10].

In addition, the coagulation–fibrinolysis system is activated to a greater extent in twin than in singleton pregnancies. D-dimer levels are consistently higher [11] and blood fibrinogen level in the late stage of pregnancy is lower in twin than in singleton pregnancies, suggesting exaggerated fibrinogen consumption in twin pregnancy [12]. All of these observations suggest that platelet reactivity is enhanced to a greater extent in twin than in singleton pregnancies.

Traditional platelet function tests have limited clinical application as they require blood sample processing, are time consuming, labor intensive, and require skilled laboratory staff to perform and interpret the assay. However, when citrated whole blood (CWB) is stirred or mixed in some other way, spontaneous platelet aggregation and concomitant decrease in single platelet count occur as a result of platelet activation [13 – 16]. A hematology analyzer (CELL-DYN Sapphire Hematology System®; Abbott Diagnostics, Abbott Park, IL) with a newly developed software package can specifically count the numbers of platelet aggregates (PA) and single platelets simultaneously in CWB [17, 18]. The number of PA detected by this analyzer is suggested to reflect the degree of platelet reactivity [18].

Upon activation of platelets or endothelial cells, P-selectin is quickly expressed on the surface membrane [19]. Circulating degranulated platelets rapidly shed surface P-selectin [20], producing the circulating plasma protein soluble P-selectin (sP-selectin) [21]. Although P-selectin is present in both platelets and endothelial cells, several authors have concluded that platelets are the major source of sP-selectin in plasma [22, 23].

The present study was performed to determine whether the degree of spontaneous platelet aggregation in the CWB differs between women with singleton and twin pregnancies and association between spontaneous platelet aggregation and plasma sP-selectin levels.

2. Material and methods

This study was performed in accordance with the provisions of the Declaration of Helsinki and was conducted after receiving approval from the Institutional Review Board of Hokkaido University Hospital. Written informed consent was obtained from all participants prior to the study.

2.1. Participants

A total of 76 healthy women consisting of 59 and 17 women with singleton and twin pregnancies, respectively, participated in this study (Table 1). None of the 76 participants showed positivity for anti-HIV-1/2 antibody, and all 76 women had unremarkable medical histories, were normotensive during pregnancy and postpartum, and experienced uneventful current pregnancies. Therefore, all participants were considered healthy and not to have pathological hypercoagulability. Participants provided blood samples several times at various stages of pregnancy, including the 1st, 2nd, and 3rd trimesters, and postpartum days 2 – 6 (designated as P1) and 24 – 39 (P2) (Table 1). Among the 59 women with singleton pregnancies vs. 17 women with twin pregnancies, only one blood sample was available for 13 (22%) vs. 1 (5.9%), two blood samples were available for 11 (19%) vs. 1 (5.9%), three blood samples were available for 14 (24%) vs. 11 (65%), four blood samples were available for 11 (19%) vs. 4 (24%), and five blood samples were available for 10 (17%) vs. 0 (0.0%), respectively.

2.2. Blood sample collection

Blood sampling was not performed at any particular time of the day, and without any particular time in relation to meal intake. A total of 9 – 10 ml of venous blood was drawn from the antecubital vein using a tourniquet and a 23-gauge needle connected to three successive vacuum tubes in the following order: 5 – 6 ml of blood in the first tube not used in this study, 2 ml of blood in the second tube containing 4.5 mg of EDTA, and 1.8 ml of blood in the third tube containing 0.2 ml of 3.2% sodium citrate solution. Two tubes containing whole blood anticoagulated with EDTA and citrate, designated as EDTA blood and CWB, respectively, were used in this study.

2.3. Measurement of platelet count, number of platelet aggregates (PA), and sP-selectin

Two tubes containing EDTA blood and CWB were applied to the CELL-DYN Sapphire Hematology System® (Abbott Diagnostics) 30 minutes after blood sampling at room temperature. These tubes were agitated in this system that took approximately 3 minutes to measure platelet count, number of PA, size of immature platelet fraction (IPF), and mean platelet volume (MPV) simultaneously. This procedure was repeated three times for each CWB to determine changes over time in number of PA and platelet count at 30, 60, and 90 minutes after blood sampling. For the platelet count in CWB, the corrected platelet count for the dilution with 0.2 ml of sodium citrate solution was used. Platelet count determined in EDTA blood was used as the baseline platelet count, MPV, and IPF of each blood sample. The antenatal EDTA blood (n=154, including 126 and 28 from women with singleton and twin pregnancies, respectively) after determination of these variables was centrifuged at 2000 g for 5 min at room temperature within 60 min after sampling and the plasma was stored at −20 °C until assay of sP-selectin using Human sP-selectin/CD62P Immunoassy® (R&D Systems Inc., Minneapolis, MN).

Data are presented as means ± SD. Statistical analyses were performed using the JMP® Pro11 statistical software package (SAS, Cary, NC). Differences in the means were tested using Wilcoxon's rank sum test between each group, and changes in variables within a group were compared using the Tukey–Kramer method. Pearson's product-moment correlation coefficient was used to measure linear correlations between two variables. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

3. Results

Neither maternal age nor fraction of nulliparous women differed significantly between the groups with singleton and twin pregnancies (Table 1). The sums of infant weight and placental weight were significantly greater in twin than in singleton pregnancies.

3.1. *Baseline characteristics of platelet in EDTA blood between singleton vs. twin pregnancy*

Baseline platelet count (in EDTA blood) increased significantly approximately 1 month postpartum in both singleton and twin pregnancies compared to the respective values in the 1st trimester (Fig. 1, A). Its level in the 3rd trimester of twin pregnancy was significantly reduced compared to that in singleton pregnancy. The MPV in the 3rd trimester was significantly greater in twin than in singleton pregnancies (Fig. 1, B). The IPF increased significantly within 1 week postpartum in both singleton and twin pregnancies compared to the respective values in the 1st trimester (Fig. 1, C).

3.2. *Spontaneous platelet aggregation in CWB of singleton vs. twin pregnancy*

The CWB exhibited an increase in number of PA over time after blood sampling in both singleton and twin pregnancies (Fig. 2). The degree of increase in PA count was lowest in the 1st trimester and greatest approximately 1 month postpartum in singleton as well as twin pregnancies. In singleton pregnancy, its degree appeared to increase with advancing gestation; the area under curve (AUC) was significantly greater in the 3rd trimester as well as at P2 (approximately 1 month postpartum) in comparison with that in the 1st trimester (Fig. 2, left). The number of PA counts in CWB of 2nd trimester at 60 minutes after sampling was significantly greater in twin than in singleton pregnancy (Fig. 3, left), resulting in a significantly greater net decrease in the platelet count of CWB at 30, 60, and 90 minutes after sampling in twin than in singleton pregnancy (Fig. 3, right).

3.3. *Relationship between PA count and net decrease in platelet count*

As expected, significant correlations between PA count and net decrease in platelet count were seen in singleton as well as twin pregnancies (Fig. 4, left). Varied size of PA containing varied number of single platelet explained the poor r- values around 0.55 on linear regression analysis (Fig. 4, right).

3.4. *Changes in sP-selectin and sP-selectin per platelet during pregnancy*

In singleton pregnancy, the plasma sP-selectin level was significantly higher for 3rd trimester than for 1st and 2nd trimesters, while no significant change was seen in twin pregnancy (Fig. 5, upper panel). There were no significant differences in sP-selectin levels between singleton and twin pregnancies. The 3rd trimester sP-selectin per platelet was significantly higher in twin than in singleton pregnancies (Fig. 5, lower panel).

3.5. *Association of plasma sP-selectin levels with PA counts 60 min after blood sampling and net decrease in platelet counts during 60 min after blood sampling*

Associations of sP-selectin levels with PA counts 60 min after blood sampling and net decrease in platelet counts during 60 min after blood sampling differed greatly between singleton and twin pregnancies (Fig. 6); in twin pregnancy, both PA counts 60 min after blood sampling and net decrease in platelet counts during 60 min after blood sampling became smaller significantly with increasing sP-selectin levels (Fig. 6, right panels), while

in singleton pregnancies, those increased significantly or tended to increase with increasing sP-selectin levels (Fig. 6, left panels).

4. Discussion

This study demonstrated significantly enhanced platelet reactivity in the 2nd trimester followed by significantly reduced platelet count with significantly increased MPV in the 3rd trimester of twin pregnancy compared to the respective parameters in singleton pregnancy. In addition, it was demonstrated that the 3rd trimester sP-selectin per platelet was significantly higher in twin than singleton pregnancies. To our knowledge, there have been no previous reports regarding platelet reactivity in twin pregnancy.

In this study, PA count was significantly positively correlated with the decrease in platelet count suggesting that "PA count by the CELL-DYN Sapphire Hematology System®" reflected "likelihood of spontaneous platelet aggregation." Early studies indicated a gradual decline in platelet count accompanied by an increase in PA of CWB [15 – 17]. Therefore, the significantly greater PA counts accompanied by the greater fall in platelet count in CWB of the 2nd trimester in twin than in singleton pregnancy implied that 2nd trimester platelets were more reactive in twin than in singleton pregnancies. This may explain why gestational thrombocytopenia is more likely to occur in twin than in singleton pregnancy. The 10th percentile value (137×10^9/l) of platelet count before delivery for twin pregnancies [6] corresponds to the 3rd – 4th percentile value for singleton pregnancies [5, 24, 25]. Gestational thrombocytopenia of less than 100×10^9/l was seen in 17% of twin pregnancies [26], while its rate was less than 1.0% of singleton pregnancies [27]. In this study, a significantly reduced platelet count was seen in the 3rd trimester of twin pregnancy compared to that of singleton pregnancy (181 ± 43 vs. $229 \pm 62 \times 10^9$/l, $P = 0.009$). As gestational thrombocytopenia is suggested to precede HELLP syndrome, which is a life-threatening pregnancy-specific complication [6, 8, 9], explaining why the HELLP syndrome is more likely to occur in twin than in singleton pregnancies, it may be clinically important to know that the platelet count is more likely to decrease in twin than in singleton pregnancy. The risks of HELLP syndrome were reported to be 0.2%, 0.9%, and 2.1% of singleton, twin, and triplet pregnancies, respectively [10].

The increase in PA counts in CWB was lowest during the 1st trimester and greatest approximately 1 month postpartum in both singleton and twin pregnancies, consistent with the results of recent studies [18, 28]. Collagen-induced platelet aggregation is significantly reduced in the 1st trimester of pregnancy compared to non-pregnant women [28]. Platelet reactivity in CWB monitored by the increase in number of PA and the fall in platelet count is reduced in early pregnancy compared with non-pregnant healthy controls [18]. As, number of PA and the fall in platelet count 1 month postpartum in this study were comparable to those seen in non-pregnant control women in our previous study [18], we speculated that the platelet reactivity 1 month postpartum in this study represented the non-pregnant platelet reactivity level. Thus, platelet reactivity is reduced soon after the establishment of pregnancy via as yet unknown mechanisms in both singleton and twin pregnancies. Therefore, our observation of a greater enhancement of platelet reactivity in the 2nd trimester of twin pregnancy compared to singleton pregnancy suggested that a specific but as yet unknown factor(s) may have lifted early a restriction that worked to reduce platelet reactivity in twin pregnancy. Our expression, "the enhanced platelet reactivity in the 2nd trimester of twin pregnancy" may be misleading. Instead, "early lifting of restriction that works to reduce platelet reactivity in twin pregnancy" may be more appropriate to describe our observations.

In this study, the 3rd trimester sP-selectin level was significantly higher compared to those of 1st and 2nd trimester in singleton pregnancy, partly consistent with results of previous study by Holmes et al. [29] in which 2nd and 3rd trimester, but not 1st trimester sP-selectin levels were significantly higher than that in non-pregnant control women. In addition, the 3rd trimester sP-selectin per platelet was significantly higher in twin than singleton pregnancies in this study (Fig. 5). These results suggested that endothelial cell activation was occurring in late stage of twin pregnancies based on findings by Fijnheer et al.[22] in which sP-selectin per platelet is suggested to be a measure of endothelial cell activation, although the exact source of sP-selection was not determined in this study. None of participants with twin pregnancies had not clinical signs of preeclampsia in this study. However, twin pregnancy is a risk factor for preeclampsia [30] and endothelial cell dysfunction can precede the onset of preeclampsia [31], consistent with findings in this study. In such a condition of twin pregnancies having higher sP-selectin per platelet, this study suggested that spontaneous platelet aggregation was rather unlikely to occur (Fig. 6, right panels).

The placenta is an interface between the fetus of semi-allograft and the mother and was larger in twin than in singleton pregnancies (1.0 ± 0.2 vs. 0.6 ± 0.2 kg, respectively). Therefore, it was speculated that the volume of blood perfusion into the placenta would be greater in twin pregnancy, to allow the nourishment of two fetuses. The placenta is a well-known site of infarction [32]. In addition, platelet counts begin to increase soon after birth [6, 33], as was also confirmed in this study. It was speculated that perfusion of a greater blood volume into a larger placenta was associated with the early lifting of restriction that worked to reduce platelet reactivity in twin pregnancy.

5. Conclusion

In conclusion, this study was performed to test the hypothesis that platelet reactivity was enhanced to a greater extent in twin than in singleton pregnancy, and the results confirmed this hypothesis. This may explain why gestational thrombocytopenia is more likely to occur in twin than in singleton pregnancy. Based on the greater placental volume in twin than in singleton pregnancy, we speculated that the placenta may be the main site responsible for enhanced platelet reactivity.

Conflict of interest
The authors have no conflicts of interest to declare.

References

[1] Cerneca F, Ricci G, Simone R, Malisano M, Alberico S, Guaschino S. Coagulation and fibrinolysis changes in normal pregnancy. Increased levels of procoagulants and reduced levels of inhibitors during pregnancy induce a hypercoagulable state, combined with a reactive fibrinolysis. Eur J Obstet Gynecol Reprod Biol 1997;73:31 – 6.

[2] Ros Salonen H, Lichtenstein P, Bellocco R, Petersson G, Cnattingius S. Increased risks of circulatory diseases in late pregnancy and puerperium. Epidemiology 2001;12:456 – 60.

[3] Levy RA, Dos Santos FC, de Jesús GR, de Jesús NR. Antiphospholipid antibodies and antiphospholipid syndrome during pregnancy: diagnostic concepts. Front Immunol 2015 May 7;6:205. doi: 10.3389/fimmu.2015.00205.

[4] Umazume T, Yamada T, Akaishi R, Araki N, Nishida R, Morikawa M, et al. Essential thrombocythemia as a risk factor for stillbirth. Thromb Res 2014;133:158 – 61.

[5] Minakami H, Kuwata T, Sato I. Gestational thrombocytopenia: Is it new? Am J Obstet Gynecol 1996;175:1676 – 7.

[6] Minakami H, Watanabe T, Izumi A, Matsubara S, Koike T, Sayama M, et al. Association of a decrease in antithrombin III activity with a perinatal elevation in aspartate aminotransferase in women with twin pregnancies: Relevance to the HELLP syndrome. J Hepatol 1999;30:603–11.

[7] Tsunoda T, Ohkuchi A, Izumi A, Watanabe T, Matsubara S, Sato I, et al. Antithrombin III activity and platelet count are more likely to decrease in twin pregnancies than in singleton pregnancies. Acta Obstet Gynecol Scand 2002;81:840 – 5.

[8] Minakami H, Kohmura Y, Izumi A, Watanabe T, Matsubara S, Sato I. Relation between gestational thrombocytopenia and the HELLP syndrome. Gynecol Obstet Invest 1998;46:41 – 5.

[9] Minakami H, Sato I. Gestational thrombocytopenia. Lancet. 2000;356(9238):1354 – 5.

[10] Day MC, Barton JR, O'Brien JM, Istwan NB, Sibai BM. The effect of fetal number on the development of hypertensive conditions of pregnancy. Obstet Gynecol 2005;106:927 – 31.

[11] Yamada T, Kawaguchi S, Araki N, Takeda M, Nishida R, Yamada T, et al. Difference in the D-dimer rise between women with singleton and multifetal pregnancies. Thromb Res 2013;131:493 – 6.

[12] Yamada T, Morikawa M, Yamada T, Akaishi R, Kojima T, Minakami H. Fibrinogen levels in the late stage of twin pregnancy. Thromb Res 2015;135:318 – 21.

[13] Armstrong R, May JA, Lösche W, Heptinstall S. Factors that contribute to spontaneous platelet aggregation and streptokinase-induced aggregation in whole blood. Thromb Haemost 1995;73:297 – 303.

[14] Konstantopoulos K, Grotta JC, Sills C, Wu KK, Hellums JD. Shear-induced platelet aggregation in normal subjects and stroke patients. Thromb Haemost 1995;74:1329 – 32.

[15] Fox SC, Burgess-Wilson M, Heptinstall S, Mitchell JR. Platelet aggregation in whole blood determined using the Ultra-Flo 100 Platelet Counter. Thromb Haemost 1982;48:327 – 9.

[16] Saniabadi AR, Lowe GD, Barbenel JC, Forbes CD. A comparison of

spontaneous platelet aggregation in whole blood with platelet rich plasma: additional evidence for the role of ADP. Thromb Haemost 1984;51:115 – 8.

[17] Bowen KL, Procopio N, Wystepek E, Glazier J, Mattson JC. Platelet clumps, nucleated red cells, and leukocyte counts: a comparison between the Abbott CELL-DYN® 4000 and Coulter® STKS. Lab Hematol 1998;4:7 – 16.

[18] Umazume T, Yamada T, Morikawa, Ishikawa S, Furuta I, Koyama T, et al. Platelet aggregation in citrated whole blood of the first trimester of pregnancy. Clinica Chimica Acta 2015;448:60 – 4.

[19] Tedder TF, Steeber DA, Chen A, Engel P. The selectins: vascular adhesion molecules. FASEB J 1995;9:886 – 73.

[20] Michelson AD, Barnard MR, Hechtman HB, MacGregor H, Connolly RJ, Loscalzo J, et al. In vivo tracking of platelets: circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. Proc Natl Acad Sci USA 1996;93:11,877 – 11,882.

[21] Dunlop LC, Skinner MP, Bendall LJ, Favaloro EJ, Castaldi PA, Gorman JJ, et al. Characterization of GMP- 140 (P-selectin) as a circulating plasma protein. J Exp Med 1992;175:1147 – 50.

[22] Fijnheer R, Frijns CJM, Korteweg J, Rommes H, Peters JH, Sixma JJ, et al. The origin of P-selectin as a circulating plasma protein. Thromb Haemost 1997;77:1081 – 5.

[23] Semenov AV, Romanov YA, Loktionova SA, Tikhomirov OY, Khachikian MV, Vasil'ev SA, et al. Production of soluble P-selectin by platelets and endothelial cells. Biochemistry 1999;64:1326 – 35.

[24] Freedman J, Musclow E, Garvey B, Abbott D. Unexplained periparturient thrombocytopenia. Am J Hematol 1986;21:397 – 407.

[25] Burrows RF, Kelton JG. Fetal thrombocytopenia and its relation to maternal thrombocytopenia. N Engl J Med 1993;329:1463 – 6.

[26] Morikawa M, Yamada T, Kataoka S, Cho K, Yamada H, Suzuki S, et al. Changes in anti- thrombin activity and platelet counts in the late stage of twin and triplet pregnancies. Semin Thromb Hemost 2005;31:290 – 6.

[27] Morikawa M, Yamada T, Yamada T, Koyama T, Akaishi R, Ishikawa S, et al. Antithrombin activity, platelet count, hemoglobin concentration and hematocrit value determined immediately before vaginal delivery among healthy women. Hokkaido Igaku Zasshi 2012;87:141 – 6.

[28] Burke N, Flood K, Murray A, Cotter B, Dempsey M, Fay L, et.al. Platelet reactivity changes significantly throughout all trimesters of pregnancy compared with the nonpregnant state: a prospective study. BJOG 2013;120:1599 – 604.

[29] Holmes VA, Wallace JM, Gilmore WS, McFaul P, Alexander HD. Soluble P-selectin levels during normal pregnancy: a longitudinal study. BJOG. 2002;109:997 – 1002.

[30] Bdolah Y, Lam C, Rajakumar A, Shivalingappa V, Mutter W, Sachs BP, et al.Twin pregnancy and the risk of preeclampsia: bigger placenta or relative ischemia? Am J Obstet Gynecol 2008;198:428.e1 – 6.

[31] Khan F, Belch JJF, MacLeod M, Mires G. Changes in endothelial function precede the clinical disease in women in whom preeclampsia develops. Hypertension 2005;46:1123 – 8.

[32] Vinnars MT, Vollmer B, Nasiell J, Papadogiannakis N, Westgren M. Association between cerebral palsy and microscopically verified placental infarction in extremely preterm infants. Acta Obstet Gynecol Scand 2015;94:976 – 82.

[33] Morikawa M, Yamada T, Turuga N, Cho K, Yamada H, Sakuragi N, et al.

Coagulation-fibrinolysis is more enhanced in twin than in singleton pregnancies J Perinat Med 2006;34:392 – 7.

Figure Captions

Fig. 1 Baseline characteristics of platelets according to pregnancy stage
IPF, immature platelet fraction; MPV, mean platelet volume; 1st, 2nd, and 3rd; trimesters of pregnancy; P1 and P2; postpartum day 2 – 6 and postpartum day 24 – 39, respectively. *, $P < 0.05$ vs. level of 1st trimester within a group. †, $P < 0.05$ between two groups. The numbers of women examined at each pregnancy stage are shown in Table 1. The platelet counts were 181 ± 43 vs. $229 \pm 62 \times 10^9$/l ($P = 0.009$) and the MPV was 8.0 ± 1.2 vs. 7.1 ± 1.1 fl ($P = 0.021$) for EDTA blood samples from women in 3rd trimester twin vs. singleton pregnancy, respectively. The IPF increased significantly within 1 week postpartum in singleton as well as twin pregnancy compared to the 1st trimester (from $3.6 \pm 2.2\%$ to $5.8 \pm 2.5\%$, $P = 0.003$ for singleton pregnancy and from $2.2 \pm 0.7\%$ to $6.9 \pm 2.7\%$, $P = 0.014$ for twin pregnancy).

Fig. 2 Changes in number of platelet aggregates (PA) according to time after blood sampling and pregnancy stage
*, The areas under the curve for the 3rd trimester and for P2 (approximately 1 month postpartum) were significantly greater compared to the 1st trimester ($P = 0.024$ and $P = 0.002$, respectively). The numbers of women examined at each pregnancy stage are shown in Table 1.

Fig. 3 Differences in PA counts and net decrease in platelet count according to pregnancy stage between singleton vs. twin pregnancy

†, $P < 0.05$ between singleton vs. twin pregnancy. The numbers of women examined at each pregnancy stage are shown in Table 1. The left figures were abstracted from Fig. 2. In CWB, the increase in PA count occurred concomitantly with a net decrease in platelet count (fall in platelet count from the baseline platelet count determined in EDTA blood). In the 2nd trimester, the PA counts were 1297 ± 1600 vs. 497 ± 432/µl ($P = 0.040$) in the CWB 60 minutes after blood sampling from women with twin vs. singleton pregnancy, respectively. In the 2nd trimester, the net decreases in platelet count were 124 ± 66 vs. $83 \pm 43 \times 10^9$/µl ($P = 0.044$), 152 ± 55 vs. $115 \pm 45 \times 10^9$/µl ($P = 0.036$), and 165 ± 52 vs. $125 \pm 42 \times 10^9$/µl ($P = 0.034$) for CWB at 30, 60, and 90 minutes after blood sampling from women with twin vs. singleton pregnancy, respectively.

Fig. 4 Relationship between PA count and net decrease in platelet count

Statistically significant correlations between PA count and net decrease in platelet count were seen in both singleton and twin pregnancies (left). PA of various sizes were seen microscopically in CWB of both singleton and twin pregnancies. The right figure shows a large PA in which a large number of platelets were aggregated (large black arrow), a smaller PA in which a smaller number of platelets were aggregated (white arrow), and a single platelet (small black arrow) among many erythrocytes. Actual scatter plots by CELL-DYN Sapphire Hematology System® were presented previously [18].

Fig. 5 Changes in sP-selectin and sP-selectin per platelet during pregnancy

*, $P < 0.05$ vs. levels of 1st and 2nd trimesters within a group. †, $P < 0.05$ between two groups. The plasma sP-selectin level (ng/mL) was 32.8 ± 9.4 vs. 40.4 ± 16.0 with $P = 0.195$ for 1st trimester, 37.9 ± 11.0 vs. 45.2 ± 20.8 with $P = 0.144$ for 2nd trimester, and $45.3 \pm$

12.9 vs. 51.0±17.5 with $P = 0.071$ for 3rd trimester of singleton vs. twin pregnancies, respectively. The sP-selection concentration per platelet (fg/platelet) was 0.122±0.037 vs 0.143±0.073 with $P = 0.775$ for 1st trimester, 0.150±0.046 vs. 0.159±0.081 with $P = 0.790$ for 2nd trimester and 0.187±0.064 vs. 0.235±0.089 with $P = 0.018$ for 3rd trimester of singleton vs. twin pregnancies, respectively.

Fig. 6 Association of plasma sP-selectin levels with PA counts 60 min after blood sampling and net decrease in platelet counts during 60 min after blood sampling

In 126 antenatal blood samples from singleton pregnancies, PA counts 60 min after blood sampling increased significantly with increasing sP-selectin levels and net decrease in platelet counts during 60 min after blood sampling tended to increase with increasing sP-selectin levels (left panels). However, in 28 antenatal blood samples from twin pregnancies, both PA counts 60 min after blood sampling and net decrease in platelet counts during 60 min after blood sampling became smaller significantly with increasing sP-selectin levels (right panels).
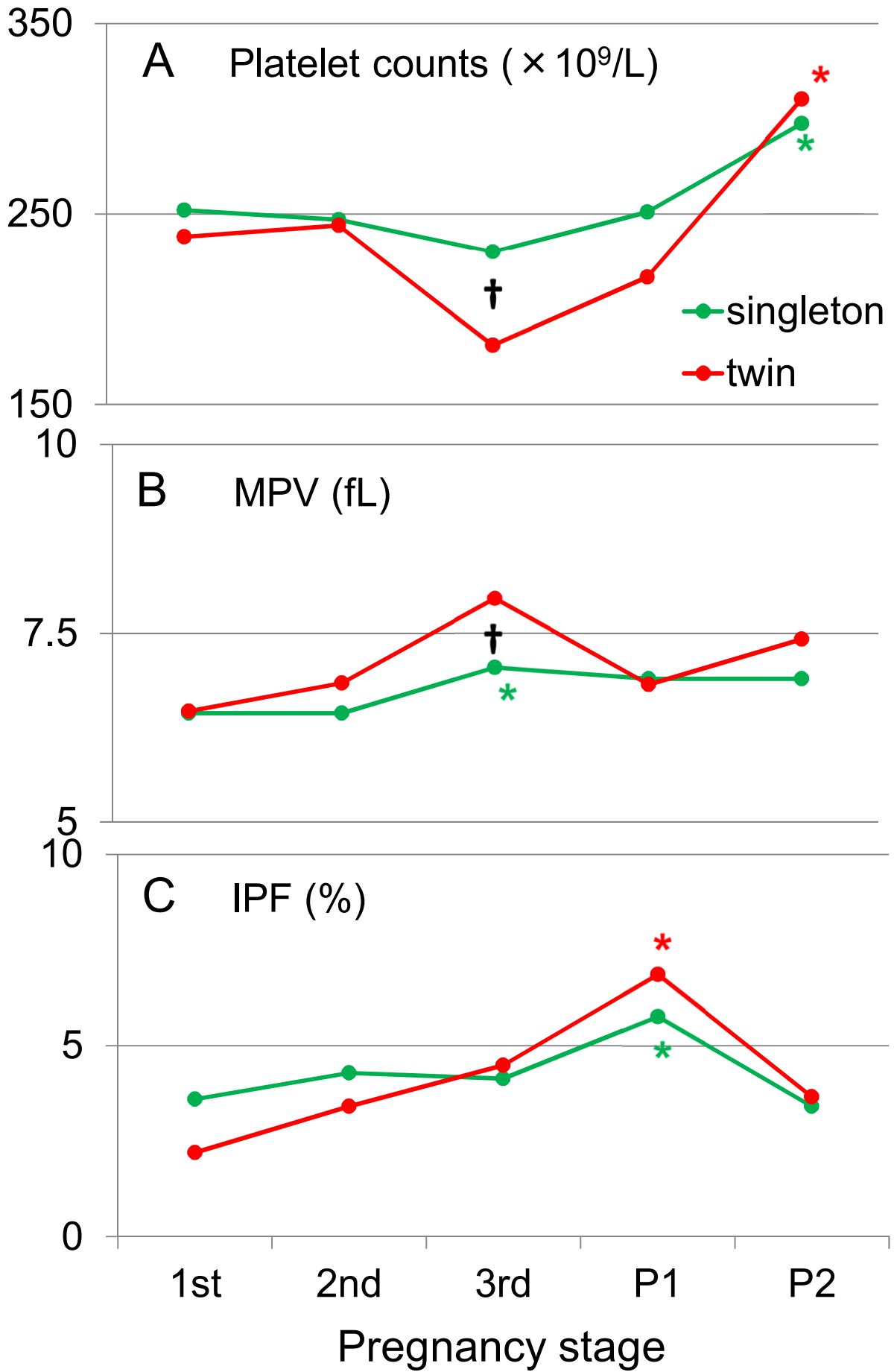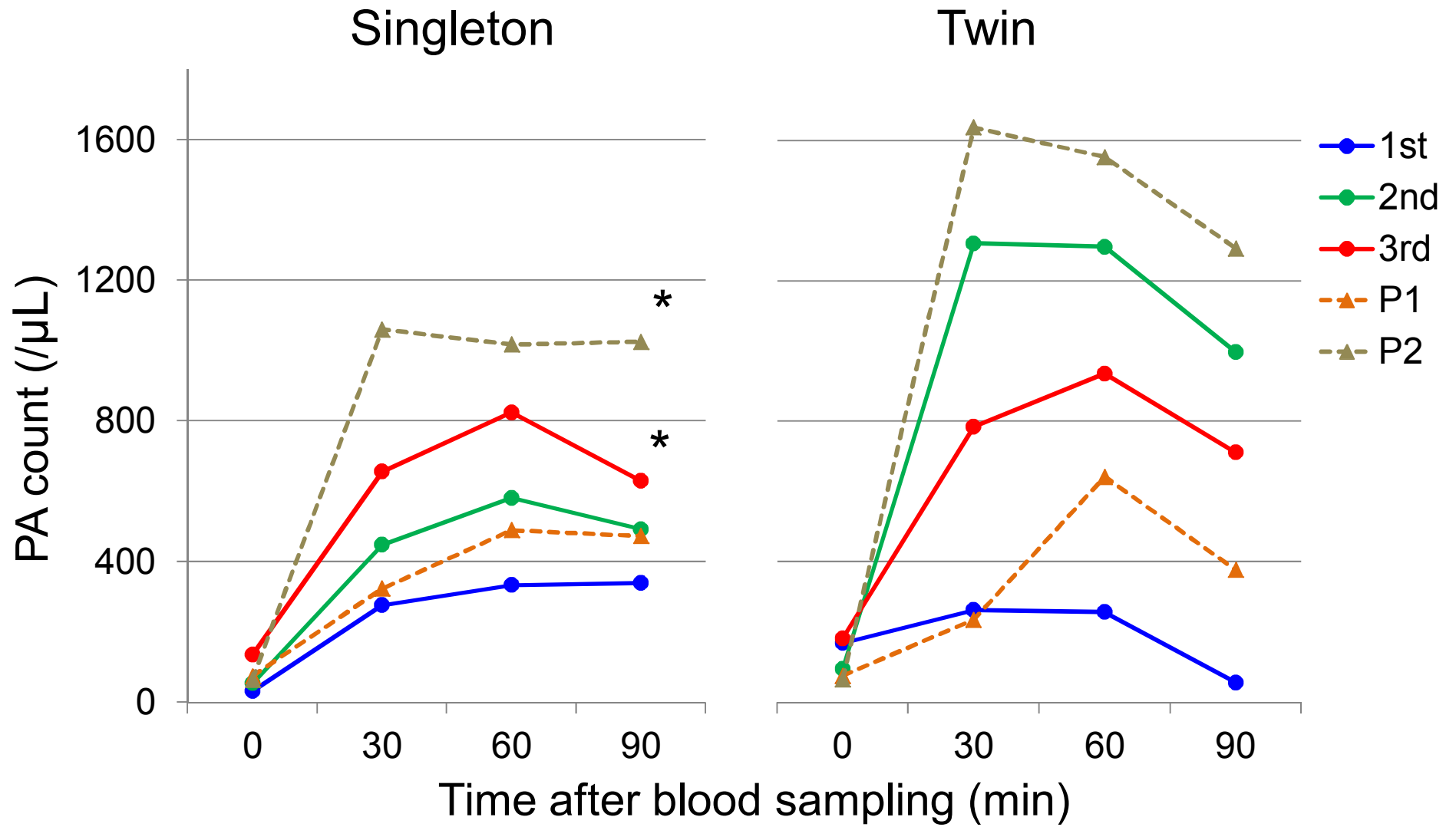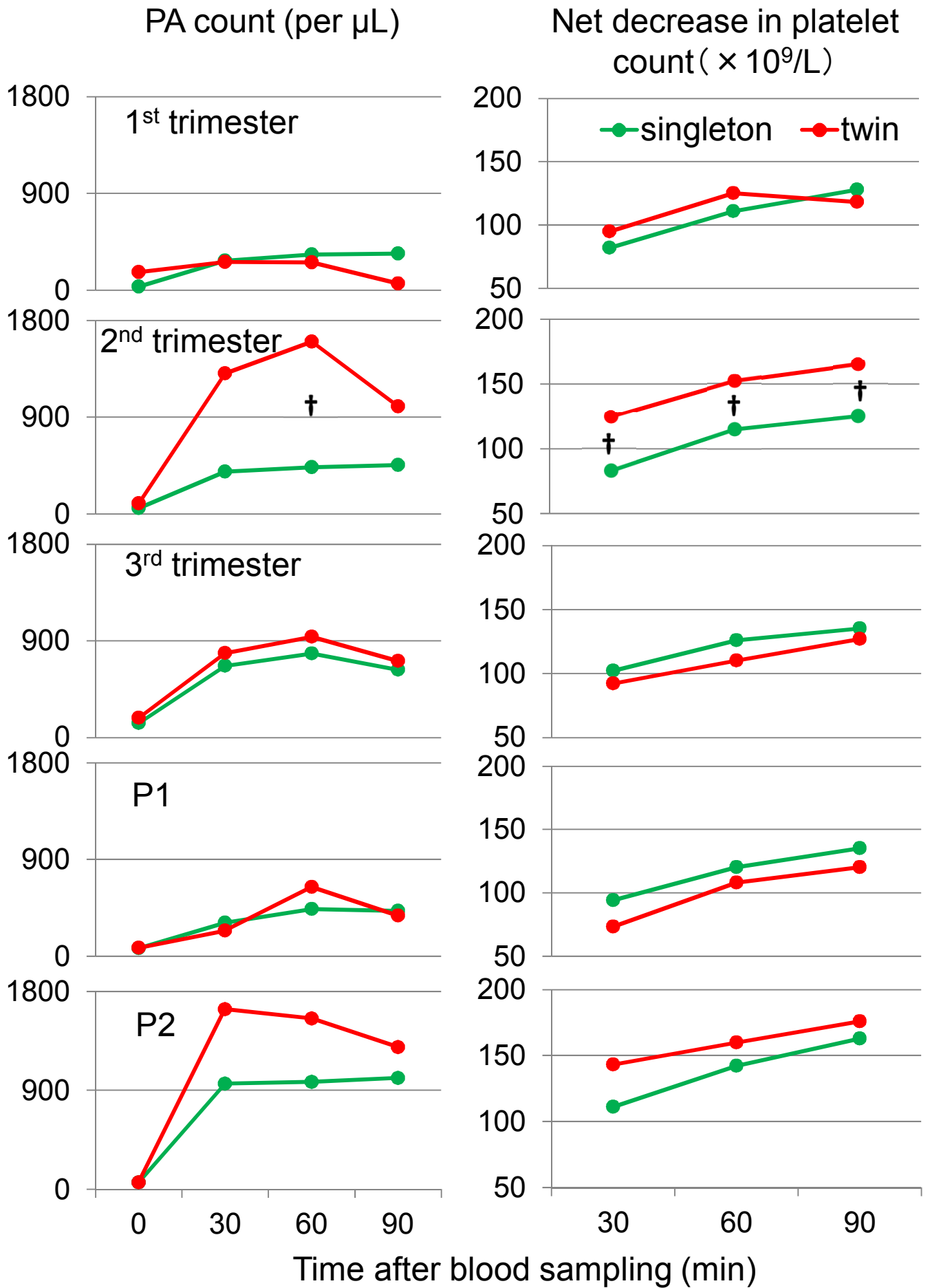
Fig 1

Fig 2

Fig 3

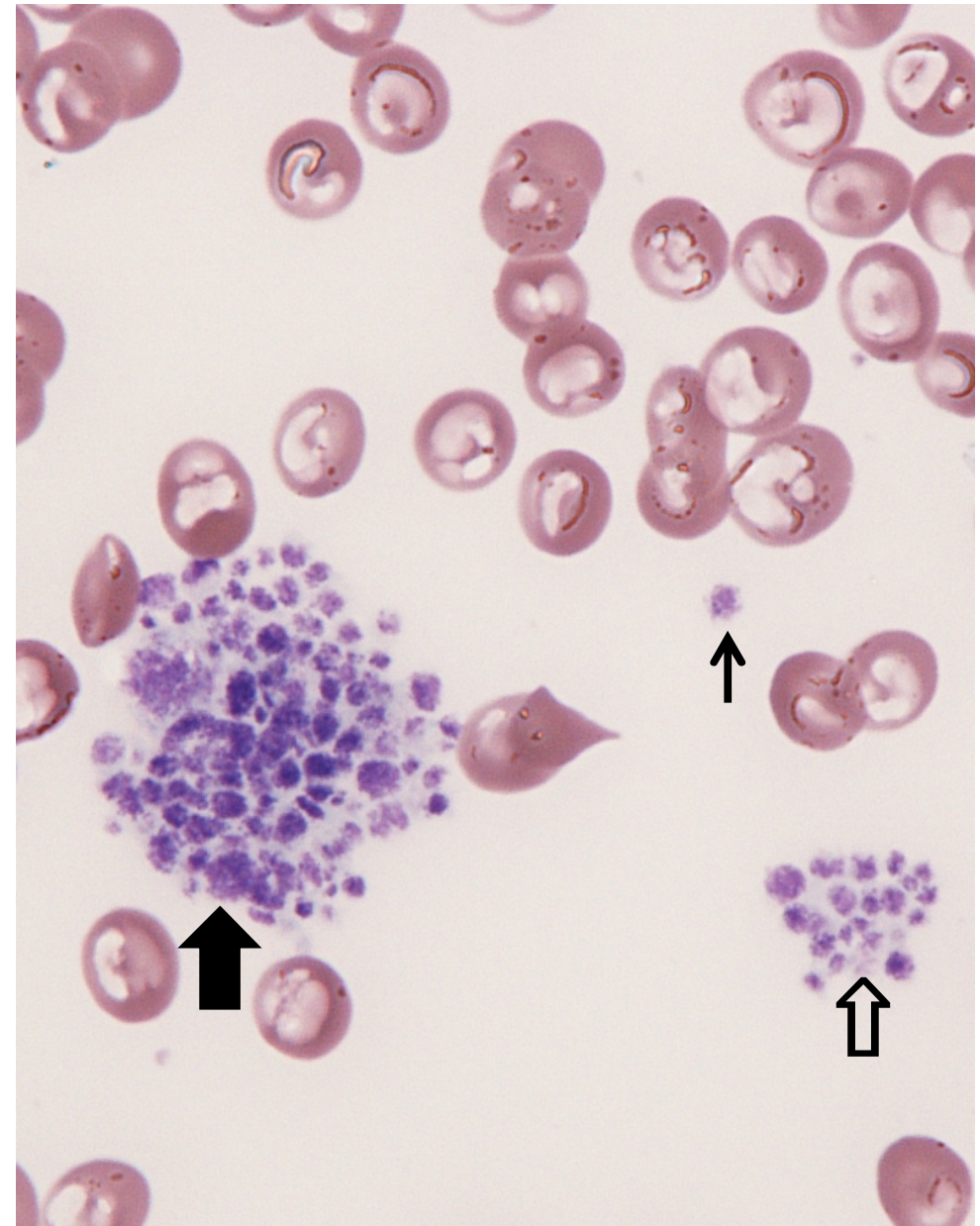**PA count (per μL)** / **Net decrease in platelet count (×10⁹/L)**

Fig 4
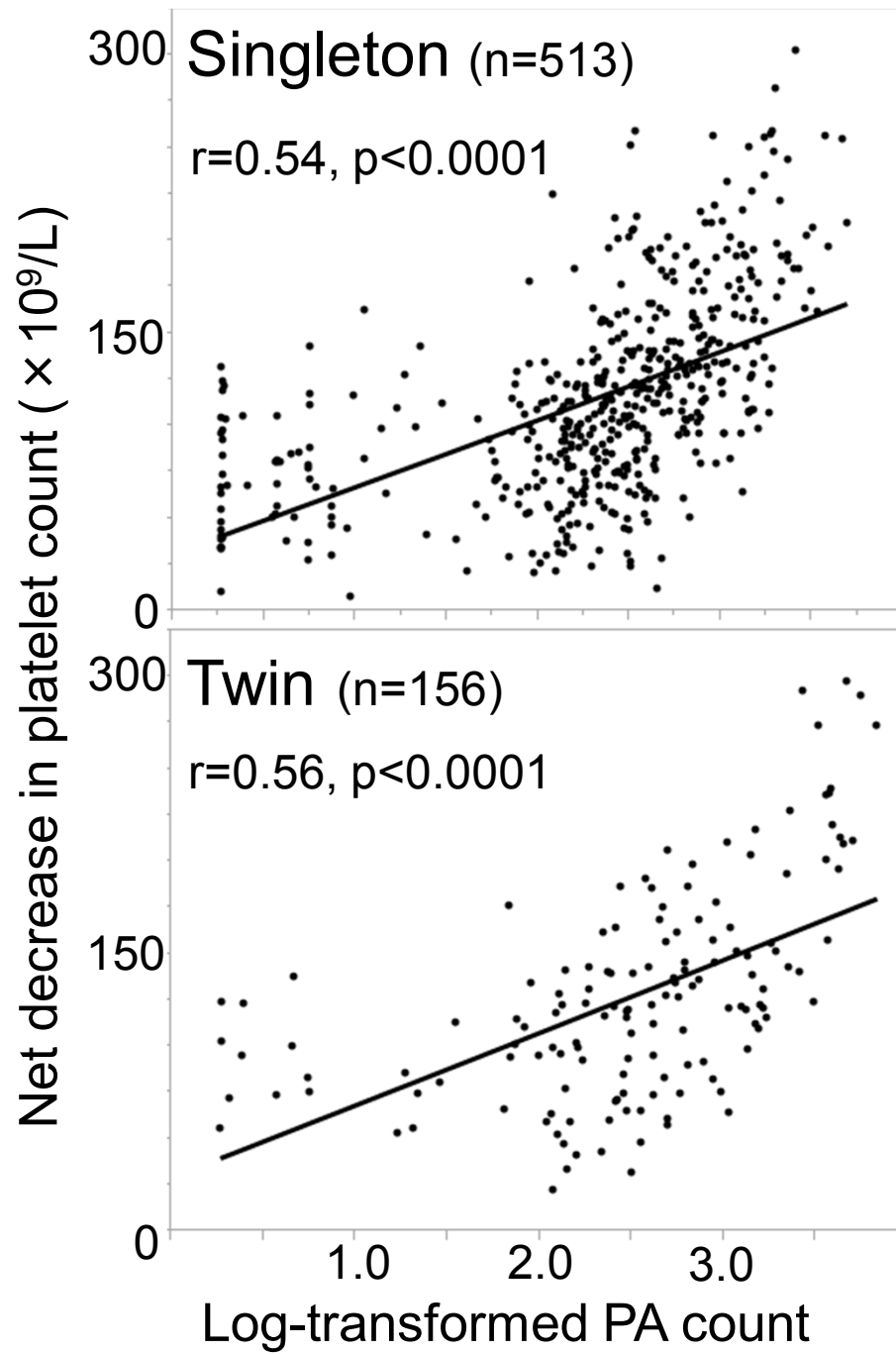
Fig 5
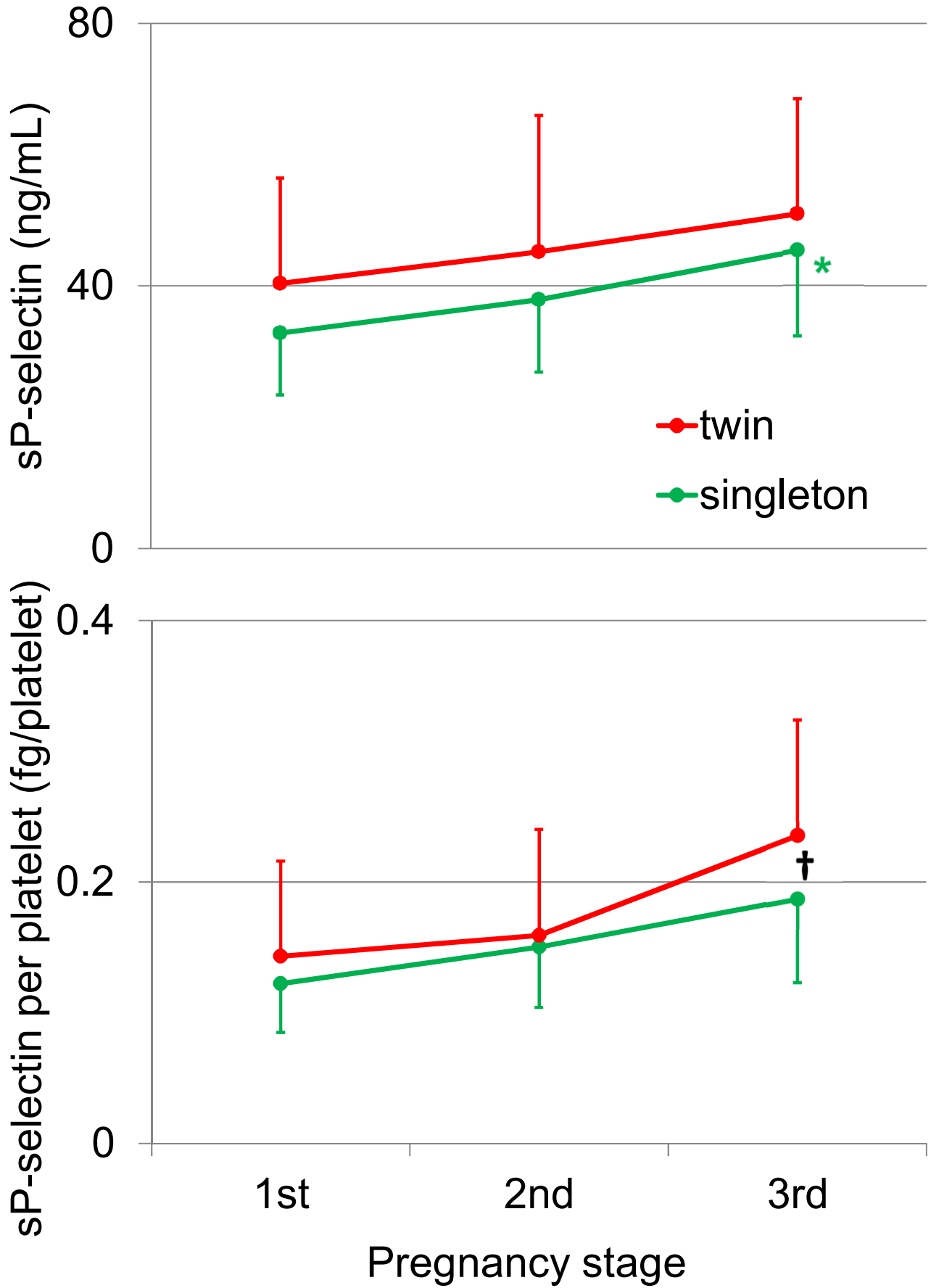
Fig 6
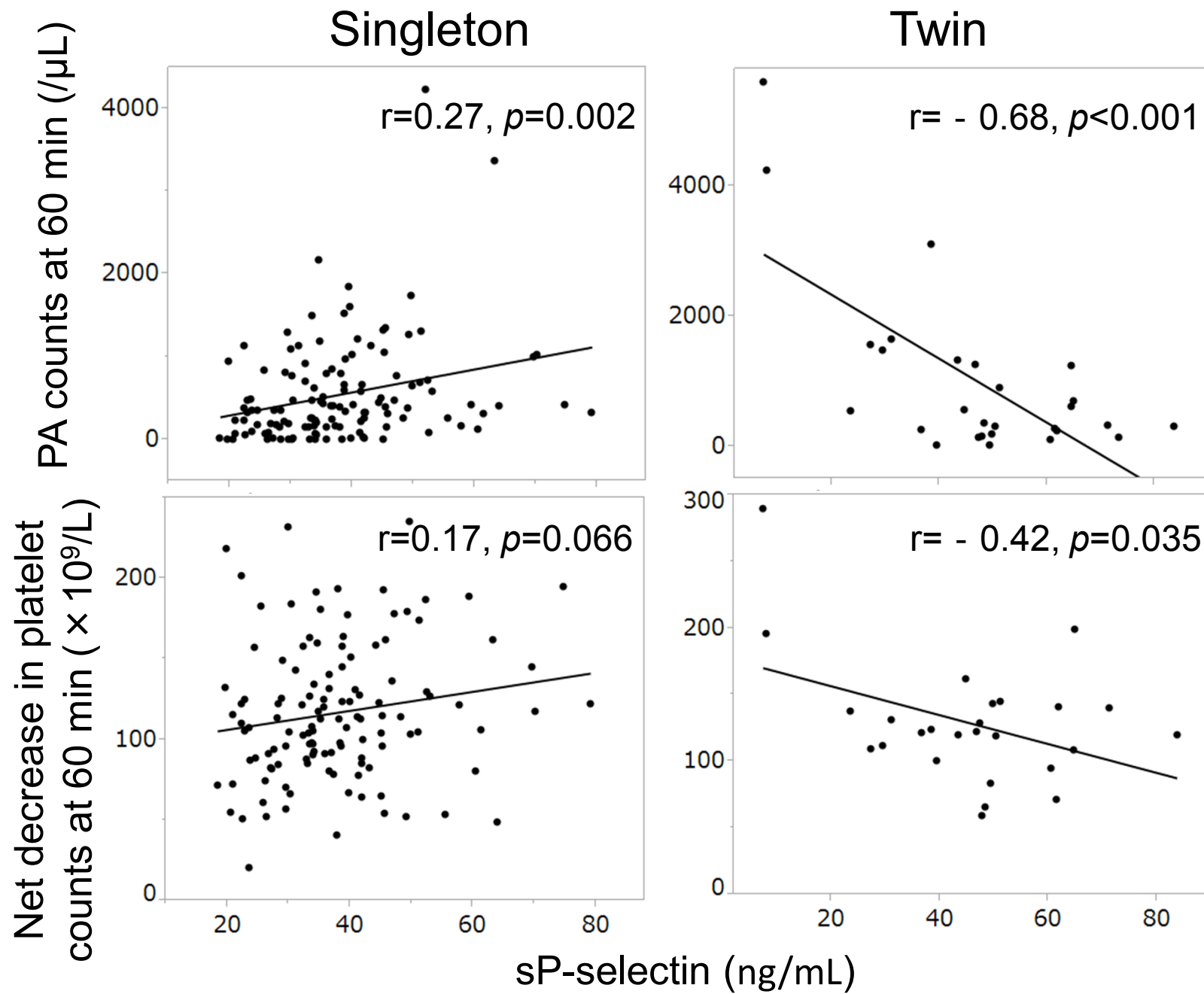
Table 1. Demographic characteristics between two groups

	Singleton	Twin
No of women	59	17
Age (year)	34.2±5.2	31.5±6.1
Nulliparous women	33 (56%)	13 (76%)
GW at delivery	38.6±1.3	36.1±3.3*
Placenta weight (kg)†	0.6±0.2	1.0±0.2*
Infant weight (kg)†	3.0±0.4	4.3±1.0*
Total no. of blood samples	171	52
No. of blood samples/person	2.9±1.4	3.1±0.7
GW at blood sampling		
First trimester	11.6±1.0 [49]	11.9±1.3 [4]
Second trimester	25.4±0.9 [42]	24.7±2.6 [10]
Third trimester	36.2±0.5 [35]	35.1±0.8*[14]
PPD 2−6 (P1)	3.1±0.8 [21]	3.8±1.1 [12]
PPD 24−39 (P2)	29.8±3.9 [24]	30.9±3.3 [12]

*, $P<0.05$ vs. women with singleton pregnancies; †, sum for twins; GW, gestational week; PPD, postpartum day. The numbers of women that provided blood samples are indicated in square brackets.