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Agreement between blood draw techniques for assessing platelet activation by flow cytometry

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Abstract

It is widely believed that assays of platelet activation are susceptible to pre-analytical variables related to blood draw technique. We assessed platelet activation by whole blood flow cytometry and investigated the effects of: 1) drawing blood into vacuum tubes or manually-aspirated syringes, and 2) discarding the first drawn blood sample (discard tube). Platelet P-selectin expression and platelet-monocyte complexes were measured by flow cytometry under both basal conditions and following stimulation with 0.1, 1 or 10μM ADP. Bland-Altman plots demonstrated agreement between results for vacuum tube and syringe-aspirated samples with an *a priori*-defined clinically relevant agreement limit of 5%. Agreement of results was also observed between discard tube and second draw samples for both vacuum-driven and manually aspirated blood. We conclude that a vacuum tube or a manually-aspirated syringe can be used when assessing platelet activation by flow cytometry and that there is no need for a discard tube.

Keywords: blood collection; preanalytical variability; flow cytometry; platelets; agreement statistics

Introduction

Unresponsiveness or hyper-reactivity of platelets can lead to disease, and assays of platelet function are commonly used diagnostically and experimentally. Since platelets are prone to become artificially activated *ex vivo*, rigorous attention is given to pre-analytical variables [1], particularly the technique used for blood collection [2]. Guidelines for the diagnostic evaluation of platelet disorders recommend either vacuum- or syringe-driven aspiration of blood into primary collection tubes [3]. However, many authorities believe that vacuum systems subject blood to higher levels of shear stress, resulting in artificial platelet activation [4]. Furthermore, the Clinical Laboratory Standards Institute (CLSI) recommends discarding the first drawn tube when blood is taken through a butterfly needle [5]. The rationale is that dead space in the tubing leads to underfilling of the primary collection tube leading to an incorrect anticoagulant/blood ratio [6,7].

Flow cytometry allows rapid *ex vivo* measurement of platelet activation through the assessment of surface receptors such as P-selectin, platelet-monocyte complexes, or fibrinogen binding [8]. Upon platelet stimulation, P-selectin from α-granules is rapidly translocated to the platelet membrane where it can be detected using labelled antibodies. Surface P-selectin mediates the rolling of platelets on endothelial cells, facilitates interactions with monocytes, and enables the initial platelet-platelet contacts needed for platelet aggregation [9]. P-selectin expression is often used as the 'gold standard' for assessing platelet activation, yet it can be rapidly shed from the platelet membrane so functional assays such as measurement of platelet-monocyte complexes are also performed [10].

The effect of pre-analytical variables on platelet activation assessed by flow cytometry has only been partially evaluated [11]. We, therefore, investigated the effect of blood draw technique on basal and stimulated P-selectin expression and platelet-monocyte complexes measured by whole blood flow cytometry. We applied statistical methods for assessing agreement between different techniques.

Methods

Patients and specimens

The study population comprised ten healthy volunteers (four males and six females; mean age 26), recruited from University staff who had not taken platelet-altering medication for a minimum of 14 days. The study was approved by the Ethics Committee of Hull York Medical School. Two consecutive blood samples (Vac-1 and Vac-2) were drawn from the from the median cubital vein in the right arm using a 21g butterfly needle (Becton Dickinson, UK) into 2.7 ml Vacutainers® containing 0.109 mol/l buffered sodium citrate (Becton Dickinson, UK). Immediately, two consecutive 2.7 ml samples (Syr-1 and Syr-2) were drawn from the opposing arm by manual aspiration into 5.0 ml syringes preloaded with 0.109 mol/l buffered sodium citrate. Other variables including tourniquet tightness and rest time were standardised. Samples were processed within 20 minutes of venepuncture and flow cytometry performed within 3 hours.

Laboratory tests

40µl of whole blood was incubated at room temperature for 20 minutes in 40µl phosphate-buffered saline (PBS) containing anti-CD14-APC (4µL), anti-CD42b-AF488 (4µL) (BioLegend) and varying concentrations of adenosine diphosphate (0.1, 1 and 10µM ADP) (4µL). 800µL of FACS™ Lysing solution (BD Biosciences, UK) was added before performing two colour flow cytometry (BD FACSCalibur™). Monocytes were identified by their forward and side scatter properties and CD14 expression. The percentage of CD14+ monocytes forming platelet-monocyte complexes (CD14+ CD42b+) was calculated.

20µl of whole blood was incubated at room temperature for 20 minutes in 20µl PBS containing anti-CD62P-PE (6µl) (BioLegend) and varying concentrations of ADP (0.1, 1 and 10µM) (2µL). A separate sample was incubated in anti-CD42b-PerCP-Cy5.5 (BioLegend) (2µL) to confirm that the gated population was platelets. Samples were fixed with 400µL of 1% paraformaldehyde before flow cytometric analysis. Platelets were identified by their forward and side scatter properties and CD42b expression. 10,000 platelet events were acquired. We recorded the mean fluorescence intensity (MFI) and the percentage of platelets expressing CD62P with the positive region marker set at the 99th percentile of the isotype antibody fluorescence.

Statistical analysis

We used XLSTAT (Addinsoft, Paris, France), MedCalc version 18.2.1 (Ostend, Belgium) and GraphPad Prism version 7 (GraphPad Software, CA, USA) for statistical analyses. Measurements are reported as mean and 95% confidence internal (95% CI). Comparisons for Syr-1 vs Syr-2, Vac-1 vs Vac-2, Syr-1 vs Vac-1 and Syr-2 vs Vac-2 were performed using Pearson's correlation alongside Passing and Bablock regression. The Shapiro Wilk test confirmed normality of the differences between each of the pairs. Paired students t-tests assessed the significance of differences between samples and Bonferroni correction was employed for multiple testing. Bland-Altman plots were generated to assess agreement between the pairs for both platelet-monocyte complexes and platelet P-selectin expression [12]. The *a priori* acceptable agreement between methods was set at 5%. Further explanation of the statistical approach can be found in the supplementary information.

Results

Stimulation with ADP caused concentration-dependent increases in platelet-monocyte complexes and platelet P-selectin expression (Fig. S1). When comparing the different blood draw methods, Pearson's correlation coefficients (PCCs) ranged from 0.984-0.993 for platelet-monocyte complexes and 0.977-0.988 for platelet P-selectin expression. Passing and Bablock regression slopes were close to unity for Syr-1-Syr-2 (1.00, 0.989), Vac-1-Vac-2 (1.02, 0.979), Syr-1-Vac-1 (0.996, 0.953), Syr-2-Vac-2 (1.01, 0.963) for platelet-monocyte complexes and P-selectin expression respectively (Table S1). Differences between P-selectin expression (percent positive) and percentage platelet-monocyte complexes between samples were not statistically significant at any ADP concentration (Table I). Furthermore, no statistical significance was seen between P-selectin MFI at any ADP concentration (Table S2). When assessing agreement between methods using Bland-Altman plots, good agreement was demonstrated by the 95% limits of agreement (LOA) and the *a priori* 5% agreement limit for platelet-monocyte complexes and platelet P-selectin expression for all comparisons (Fig 1). Higher variability was seen between methods at higher levels of ADP stimulation (proportional bias) but was bidirectional and within acceptable limits (Fig 1).

Discussion

Evidence suggests that the pre-analytical stage is most vulnerable to laboratory error [13]. Although guidelines exist for the standardisation of pre-analytical variables when conducting platelet assays, recommendations are based on light-transmission aggregometry and the platelet function analyser-100 with no data arising from flow cytometric studies [3].

Bland-Altman plots allow systematic differences to be detected between two measurement techniques [12]. Studies investigating the influence of pre-analytical variables on platelet assays suffer from inappropriate statistical analysis [6,14]. Moreover, failure to define *a priori* clinically meaningful differences hampers interpretation of Bland-Altman plots [15,16]. In the present study, we display Bland-Altman plots displaying *a priori* clinically meaningful differences and data-defined LOA.

Guidelines advocate that a discard tube of blood is needed when a butterfly device is used [5]. We demonstrate agreement in basal and stimulated platelet P-selectin expression and platelet-monocyte complexes between the first and second collection tubes. We conclude it is unnecessary to discard the first tube of blood drawn.

Agreement was also established between results of blood aspiration via syringe or vacuum tube, which is consistent with guidance that blood may be drawn by either method [3].

The present study represents the first attempt to apply agreement statistics to pre-analytical determinants of platelet activation assessed by flow cytometry. We have shown that the initial blood drawn, or subsequent samples drawn by butterfly needle into a vacuum tube or manual syringe, did not influence platelet activation assessed by flow cytometry.

Acknowledgements

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Author Contributions

Performed the experiments: EW. Conceived and designed the experiments: EW, MC. Analysed the data: EW, SH. Wrote the paper: EW, SH

Declaration of interest

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Figure and Table Legend

Table I.

Difference in percentage of platelet-monocyte complexes and percentage of platelets positive for P-selectin under basal and stimulated conditions, in whole blood collected into either BD Vacutainers containing 0.109mol/l buffered sodium citrate or manual syringes preloaded with 0.109mol/l buffered sodium citrate. *Mean difference for paired observations. Syr: syringe driven aspiration; Vac: vacutainer driven aspiration; ADP: adenosine diphosphate. Values are shown as mean and 95% CI. Significance at P<.00125 (after Bonferroni correction).

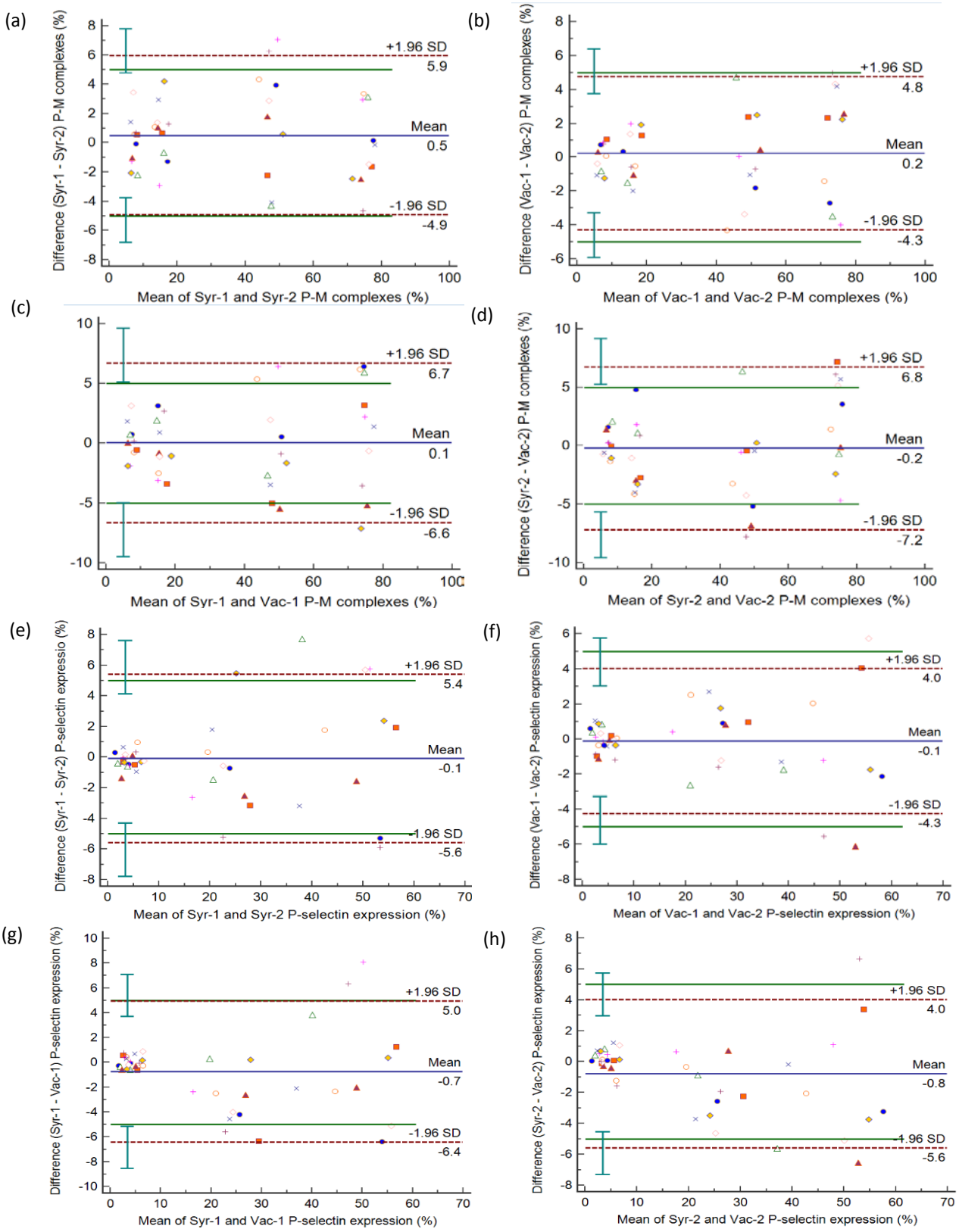
Fig 1.

Bland-Altman plots of agreement for percentage platelet-monocyte complexes (a-d) and percentage P-selectin expression (e-h) between (a, e) Syr-1 and Syr-2, (b, f) Vac-1 and Vac-2, (c, g) Syr-1 and Vac-1, (d, h) Syr-2 and Vac-2. Horizontal blue lines and dotted red lines represent the mean difference between the two collection methods and the 95% limits of agreement (LOA) respectively. Horizontal green lines represent the *a priori* maximum allowed difference of ±5% between methods. Vertical error bars represent 95% Cis for the LOA. Syr: syringe driven aspiration; Vac: vacutainer driven aspiration.

Table I

Percentage Platelet-Monocyte Complexes																
ADP Concentration (μM)	Syr-1	Syr-2	Vac-1	Vac-2	Syr-1 vs Syr-2			Vac-1 vs Vac-2			Syr-1 vs Vac-1			Syr-2 vs Vac-2		
					% difference			% difference			% difference			% difference		
	Values	Values	Values	Values	**Mean difference* (%)**	**95% confidence interval**	***P* value**	**Mean difference (%)**	**95% confidence interval**	***P* value**	**Mean difference (%)**	**95% confidence interval**	***P* value**	**Mean difference (%)**	**95% confidence interval**	***P* value**
Basal	7.27 (6.47, 8.07)	7.26 (6.42, 8.10)	7.10 (6.2,7.99)	7.06 (6.37, 7.76)	**0.010**	**(-1.22, 1.24)**	**0.986**	**0.037**	**(-0.585, 0.659)**	**0.896**	**0.173**	**(-0.948, 1.293)**	**0.735**	**0.200**	**(-0.642, 1.04)**	**0.604**
0.1	15.7 (14.6, 16.9)	14.9 (13.7, 16.2)	16.0 (14.6, 17.4)	15.9 (14.8, 17.0)	**0.767**	**(-0.688, 2.22)**	**0.263**	**0.133**	**(-0.912, 1.18)**	**0.780**	**-0.310**	**(-2.014, 1.394)**	**0.690**	**-0.944**	**(-3.08, 1.19)**	**0.343**
1	48.4 (46.4, 50.3)	46.74 (44.7, 48.7)	48.8 (47.4, 50.2)	48.9 (46.7, 51.2)	**1.62**	**(-1.28, 4.53)**	**0.238**	**-0.108**	**(-2.09, 1.88)**	**0.905**	**-0.464**	**(-3.383, 2.455)**	**0.727**	**-2.19**	**(-5.17, 0.785)**	**0.130**
10	75.2 (73.4, 77.1)	75.6 (74.0, 77.1)	74.3 (72.4, 76.3)	73.4 (71. 8, 75.1)	**-0.325**	**(-2.28, 1.63)**	**0.716**	**0.904**	**(-1.57, 3.38)**	**0.430**	**0.903**	**(-2.586, 4.392)**	**0.573**	**2.13**	**(-0.751, 5.02)**	**0.129**
Combined					**0.519**	**(-0.372, 1.41)**	**0.246**	**0.242**	**(-0.499, 0.982)**	**0.513**	**0.076**	**(-1.009, 1.160)**	**0.889**	**-0.202**	**(-1.34, 0.939)**	**0.723**
Percentage Platelets positive for P-selectin																
Basal	2.74 (2.23, 3.24)	2.88 (2.40, 3.37)	2.67 (2.23, 3.11)	2.67 (2.10, 3.24)	**-0.146**	**(-0.528, 0.236)**	**0.409**	**0.003**	**(-0.552, 0.558)**	**0.991**	**0.067**	**(-0.282, 0.416)**	**0.674**	**0.216**	**(-0.035, 0.467)**	**0.083**
0.1	5.20 (4.38, 6.03)	5.42 (4.69, 6.14)	5.20 (4.48, 5.93)	5.35 (4.48, 6.22)	**-0.212**	**(-0.608, 0.184)**	**0.256**	**-0.144**	**(-0.508, 0.220)**	**0.393**	**-0.001**	**(-0.349, 0.347)**	**0.995**	**0.067**	**(-0.592, 0.726)**	**0.823**
1	22.2 (19.5, 24.9)	23.0 (20.3, 25.7)	25.3 (22.2, 28.5)	24.9 (21.8, 27.9)	**-0.862**	**(-2.99, 1.27)**	**0.383**	**0.463**	**(-0.797, 1.72)**	**0.427**	**-3.162**	**(-4.753, -1.57)**	**0.002**	**-1.84**	**(-3.16, -0.515)**	**0.012**
10	49.0 (44.2, 53.9)	48.1 (42.7, 53. 6)	48.8 (43.4, 54.3)	49.6 (44.6, 54.6)	**0.933**	**(-2.47, 4.34)**	**0.551**	**-0.794**	**(-3.50, 1.91)**	**0.524**	**0.193**	**(-3.20, 3.58)**	**0.900**	**-1.53**	**(-4.57, 1.51)**	**0.283**
Combined					**-0.072**	**(-0.967, 0.824)**	**0.872**	**-0.118**	**(-0.791, 0.555)**	**0.725**	**-0.726**	**(-1.66, 0.205)**	**0.123**	**-0.772**	**(-1.56, 0.015)**	**0.052**

Fig 1


(a)
Difference (Syr-1 - Syr-2) P-M complexes (%)
+1.96 SD
5.9
Mean
0.5
-1.96 SD
-4.9
Mean of Syr-1 and Syr-2 P-M complexes (%)
(b)
Difference (Vac-1 - Vac-2) P-M complexes (%)
+1.96 SD
4.8
Mean
0.2
-1.96 SD
-4.3
Mean of Vac-1 and Vac-2 P-M complexes (%)
(c)
+1.96 SD
6.7
Mean
0.1
-1.96 SD
-6.6
Mean of Syr-1 and Vac-1 P-M complexes (%)
(d)
Difference (Syr-2 - Vac-2) P-M complexes (%)
+1.96 SD
6.8
Mean
-0.2
-1.96 SD
-7.2
Mean of Syr-2 and Vac-2 P-M complexes (%)
(e)
Difference (Syr-1 - Syr-2) P-selectin expressio (%)
+1.96 SD
5.4
Mean
-0.1
-1.96 SD
-5.6
Mean of Syr-1 and Syr-2 P-selectin expression (%)
(f)
Difference (Vac-1 - Vac-2) P-selectin expression (%)
+1.96 SD
4.0
Mean
-0.1
-1.96 SD
-4.3
Mean of Vac-1 and Vac-2 P-selectin expression (%)
(g)
Difference (Syr-1 - Vac-1) P-selectin expression (%)
+1.96 SD
5.0
Mean
-0.7
-1.96 SD
-6.4
Mean of Syr-1 and Vac-1 P-selectin expression (%)
(h)
Difference (Syr-2 - Vac-2) P-selectin expression (%)
+1.96 SD
4.0
Mean
-0.8
-1.96 SD
-5.6
Mean of Syr-2 and Vac-2 P-selectin expression (%)

Supplementary Information

Methods

Statistical Approach

To assess agreement a stepwise approach was undertaken. Although it is assumed that two techniques for measuring the same output will be closely related, Pearson's correlation coefficient (PCC) was calculated to clarify the presence of a linear relationship between Syr-1-Syr-2, Vac-1-Vac-2, Syr-1-Vac-1, Syr-2-Vac-2. PCC is highly sensitive to the range of values and lacks information about systematic difference therefore PCC does not assess agreement as a high degree of correlation is possible when agreement is poor [1]. Passing and Bablock regression analysis was then undertaken on the above pairs. This is a non-parametric linear regression procedure which is non-sensitive to outliers and fits the parameters of a and b in the linear equation $y = a + bx$. This reveals constant (regression line intercept a) and proportional (regression line slope b) difference with confidence intervals of 95% (95% Cis). Therefore, if the 95% Cis for a include zero one can conclude that there is no constant difference between methods. Additionally, if the 95% Cis for b include the value one, then it can be concluded that there is no proportion difference between methods. Overall this allows the for the assumption that $x = y$ and agreement between methods to be presumed. The primary fallacy with the Passing and Bablock regression model is that it derives the agreement of two methods from the data and neglects whether this is within clinically relevant parameters [2]. Therefore, Bland-Altman analysis was undertaken (explained in the main text) to assess whether the agreement between the above pairs was within an a priori 5% agreement limit. With this analysis, a paired students t-test was also computed testing the

null hypothesis H0 that the mean of the differences between the results does not differ from 0, against the alternative Ha that it does. Finally, Bonferroni correction was applied to adjust the significant p value for paired students t-tests to account for multiple comparisons.

Referances for Supplementary Information

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Figure Legend for Supplementary Tables

Table SI.

Results of Passing and Bablock regression analysis for Syr-1 vs Syr-2, Vac-1 vs Vac-2, Syr-1 vs Vac-1 and Syr-2 vs Vac-2 for percentage platelet-monocyte complexes and percentage platelet P-selectin expression. Syr: syringe driven aspiration; Vac: BD vacutainer™ driven aspiration

Table S2.

Platelet P-selectin mean fluorescent intensity (MFI) under basal and stimulated conditions, in whole blood collected into either BD Vacutainers containing 0.109mol/l buffered sodium citrate or manual syringes preloaded with 0.109mol/l buffered sodium citrate. Syr: syringe driven aspiration; Vac: vacutainer driven aspiration; ADP: adenosine diphosphate. Values are shown as mean and 95% CI. Significance at P<.00125 (after Bonferroni correction).

Figure Legend for Supplementary Figure

Fig S1.

Platelet activation status as expressed by platelet-monocyte complexes (a) or platelet P-selectin expression (b) under basal conditions and when stimulated with 0.1µM ADP, 1µM ADP, 10µM ADP in both manual syringe and vacuum-aspirated blood. Syr: syringe driven aspiration; Vac: BD vacutainer™ driven aspiration; ADP: adenosine diphosphate. Data are expressed as mean and 95% confidence interval.

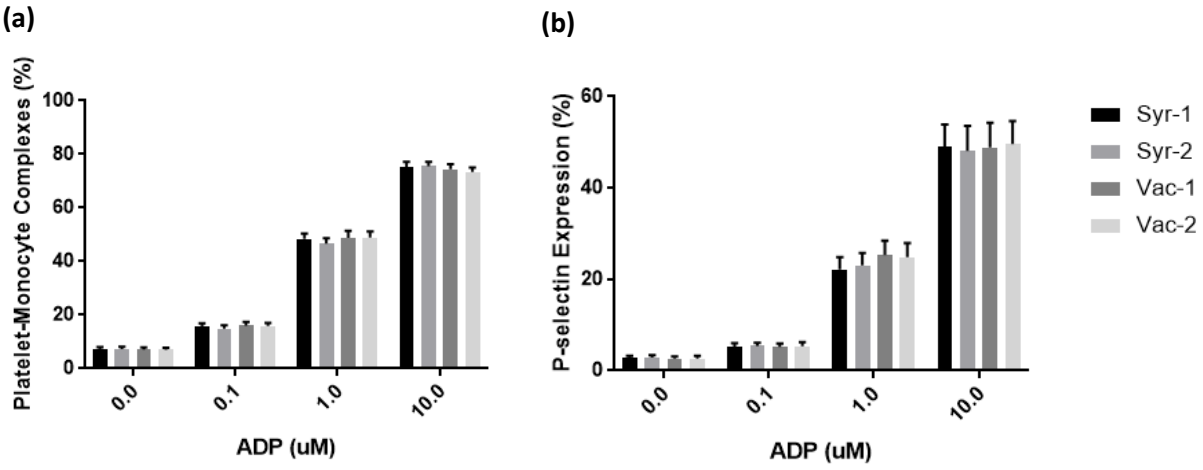
Table SI.

		Intercept	95% CI	Slope	95% CI
% Platelet-Monocyte Complexes	Syr-1 vs Syr-2	0.614	-0.384 to 1.328	1.000	0.967 to 1.047
	Vac-1 vs Vac-2	-0.302	-1.389 to 0.558	1.020	0.978 to 1.053
	Syr-1 vs Vac-1	-0.133	-1.403 to 1.152	0.996	0.940 to 1.047
	Syr-2 vs Vac-2	-0.795	-2.014 to 0.901	1.010	0.961 to 1.073
% Platelet P-selectin expression	Syr-1 vs Syr-2	-0.243	-0.650 to 0.203	0.989	0.926 to 1.054
	Vac-1 vs Vac-2	0.068	-0.250 to 0.457	0.979	0.951 to 1.036
	Syr-1 vs Vac-1	0.021	-0.345 to 0.514	0.953	0.895 to 1.013
	Syr-2 vs Vac-2	0.412	0.080 to 0.793	0.933	0.898 to 0.979

Table S2

MFI Platelet P-selectin expression								
ADP Concentration (μM)	Syr-1	Syr-2	Vac-1	Vac-2	Syr-1 vs Syr-2	Vac-1 vs Vac-2	Syr-1 vs Vac-1	Syr-2 vs Vac-2
	Values	Values	Values	Values	*P* value	*P* value	*P* value	*P* value
Basal	30.6 (29.0, 32.1)	30.1 (28.4, 31.8)	30.0 (27.8, 32.3)	30.1 (28.4, 31.7)	0.610	0.969	0.498	0.948
0.1	39.8 (38.1, 41.4)	38.8 (37.4, 40.2)	41.2 (38.3, 44.2)	39.9 (38.6, 41.2)	0.292	0.379	0.289	0.249
1	74.6 (72.4, 76.9)	73.6 (71.4, 75.9)	77.0 (75.3, 78.6)	73.9 (71.9, 75.9)	0.541	0.116	0.186	0.065
10	145 (145, 150)	145 (143, 147)	145 (145, 150)	146 (145, 149)	0.097	0.433	0.919	0.135

Fig S1


(a)
Platelet-Monocyte Complexes (%)
100
80
60
40
20
0
0.0
0.1
1.0
10.0
ADP (uM)
(b)
P-selectin Expression (%)
60
40
20
0
0.0
0.1
1.0
10.0
ADP (uM)
Syr-1
Syr-2
Vac-1
Vac-2