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ORIGINAL ARTICLE

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Platelet impedance adhesiometry: A novel technique for the measurement of platelet adhesion and spreading

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Abstract

Introduction: Thrombogenesis plays an important role in today's morbidity and mortality. Antithrombotics are among the most frequently prescribed drugs. Thorough knowledge of platelet function is needed for optimal clinical care. Platelet adhesion is a separate subprocess of platelet thrombus formation; still, no well-standardized technique for the isolated measurement of platelet adhesion exists. Impedimetry is one of the most reliable, state-of-art techniques to analyze cell adhesion, proliferation, viability, and cytotoxicity. We propose impedimetry as a feasible novel method for the isolated measurement of 2 significant platelet functions: adhesion and spreading.

Methods: Laboratory reference platelet agonists (epinephrine, ADP, and collagen) were applied to characterize platelet functions by impedimetry using the xCELLigence SP system. Platelet samples were obtained from 20 healthy patients under no drug therapy. Standard laboratory parameters and clinical patient history were also analyzed.

Results: Epinephrine and ADP increased platelet adhesion in a concentration-dependent manner, while collagen tended to have a negative effect. Serum sodium and calcium levels and age had a negative correlation with platelet adhesion induced by epinephrine and ADP, while increased immunoreactivity connected with allergic diseases was associated with increased platelet adhesion induced by epinephrine and ADP. ADP increased platelet spreading in a concentration-dependent manner.

Conclusion: Impedimetry proved to be a useful and sensitive method for the qualitative and quantitated measurement of platelet adhesion, even differentiating between subgroups of a healthy population. This novel technique is offered as an important method in the further investigation of platelet function.

KEYWORDS

ADP, collagen, epinephrine, impedimetry, platelet, platelet adhesion, platelet spreading

1 | INTRODUCTION

Platelet thrombus formation and platelet dysfunction are behind major cardiovascular and hematological diseases with the significant epidemiological role. Myocardial infarction or stroke contributes to present mortality and morbidity rates to great extent, while genetic platelet function defects being more rare conditions can pose a considerable diagnostic challenge. Antiplatelet and anticoagulant drugs are frequently prescribed drugs in daily clinical practice. Since decades, great effort has been made for investigation of the pathophysiology of platelet thrombus formation, which has been more relevant recently according to the improving number of implantable mechanical circulatory devices.

A great number laboratory techniques have been developed aiming to measure different aspects of platelet adhesion and aggregation. While the gold standard of the measurement of platelet aggregation and in the investigation of antithrombotic therapies today remains light transmission aggregometry,[1] further standardization is needed even in a clinical setting, because measurement protocols vary greatly between laboratories.[2,3] Techniques for the isolated measurement of platelet adhesion have been developed (Cone and Plate(let) Analyzer, Platelet Adhesion Assay, Retention Test Homburg)[4-6]; however, these tests are end-point assays, and as such, there is no possibility for real-time monitoring of platelet adhesion and examining of the dynamics of the process. Furthermore, they lack proper standardization so none of them is generally used in either clinical or research practice.

Impedimetry has been described in 1984 by Giaver and Keese as a noninvasive technique for the measurement of cell adhesion.[7] The basic principle is the fact that cells act as insulators in an electric circuit. The adhesion surface is an electrode microarray placed on the bottom of the wells of a specialized array (eg 96-well E-plate®).[8] Cell adhering to the electrodes generates a change in the measured electric impedance. This change directly correlates to area covered by cells, in consequence to the number of the adhering cells and the level of adhesion (degree of expansion of the adhering cells). Owing to the direct correlation between the measured impedance signal and the surface area covered by adhering platelets, it is also clear that platelet aggregates or clots—due to their smaller area than individual adhering platelets—do not generate an increased impedance signal. This ensures the absence of artifacts leading to false positive increased impedance measurements and allows to measure adhesion separately. The same principle is true with platelets simply "falling" to the electrodes: Because of their smaller surface than adhering platelets, they do not generate an increased impedance signal in contrast to adhering platelets. Another huge advantage of this technique is that impedimetry is a real-time measurement which gives us the opportunity to characterize the dynamics of adhesion. A variety of applications using impedimetry has been developed such as measurement of cell proliferation,[9] viability,[10] cellular permeability,[11] and cytotoxicity[12,13] as well as a number of systems using impedance-based approach have been also developed. While impedimetry has been used in research for decades in a number of ways, no clinical application has been developed yet.

While platelet adhesion is a separate and highly important process in platelet function, no common and well-applicable technique has been developed for platelet adhesion measurement yet. The available data on platelet adhesion—either its part in disease progression or as drug effect—are limited compared to that on platelet aggregation. We think that the lack of ideal platelet adhesion measurement method is at least partly compensated by monitoring electrochemical or electrophysical properties of the platelets. The aim of the current work was to propose impedimetry as a feasible method for the isolated measurement of platelet adhesion. In these experiments, we measured platelet adhesion by impedimetry on different platelet counts. Then, we examined platelet adhesion in 2 different setups: (i) We measured platelet adhesion in the presence of platelet activators; (ii) spreading of already adhered platelets was measured, and the effect of platelet activators on platelet spreading was investigated.

2 | MATERIALS AND METHODS

2.1 | Patients

Twenty patients were enrolled in this study. All of the patients were selected from the collection of Budakalasz Health Examination Survey organized by the Semmelweis University Heart and Vascular Center. All patients gave informed consent, and the Clinical Ethics Review Board of the Semmelweis University Budapest approved the protocols. The investigation conforms with the principles outlined in the Declaration of Helsinki.

Platelet samples were collected from each patient. All of the subjects were between the age of 20-40 years under no current medical drug treatment. Medical history was collected with questionnaire conducted by a physician.

2.2 | Blood collection and platelet isolation

Full blood was drawn by clean venipuncture with minimal venostasis using 18-G needle into 5-mL plastic 9NC coagulation sodium citrate 3.2% Vacuette® blood collection tubes. The first few milliliters of blood drawn was discarded.

For impedimetry measurements, platelet-rich plasma was prepared using a slightly modified version described by Rideg et al.[14] Platelets were isolated by centrifugation with 500×*g* for 7 minutes at room temperature. Thus, most of the red blood cells and white blood cells were separated in the sediment, and most of the platelets were present in the supernatant layer of plasma. Then, the supernatant was collected into sterile plastic test tubes. The platelet-rich plasma (average platelet density $1.26 \pm 0.17 \times 10^9$ platelet/mL) was diluted with 0.9% sodium-chloride infusion solution (normal saline, Salsol A, TEVA, Debrecen, Hungary) to 1:10 ratio for the adhesion and spreading measurements and used undiluted and diluted to different ratios (1:10, 1:100, 1:1000) for the platelet density measurements.

2.3 | Clinical laboratory measurements

Standard clinical laboratory measurements from the acquired blood samples were taken: complete blood count, blood chemistry, enzymes, and lipoprotein levels. Specialized test of the cardiovascular system, like hemoglobin A1c (HbA1c) and N-terminal pro-brain natriuretic peptide (NT pro-BNP) levels, was also measured. Among the clinical parameters, age, weight, height, waist size, hip size, systolic blood pressure (SBP), and left and right ankle-brachial index (ABI) were measured. From patient history, smoking, alcohol consumption, the presence of allergic disease (known food, pollen, etc. allergies), sporting activity, and physical activity were investigated.

2.4 | Impedance-based measurements

For impedance-based measurements, the xCELLigence SP® system (Roche Applied Science, Indianapolis, IN, USA) and E-plate 96® (Roche Applied Science, Indianapolis, IN, USA) were used. This technique uses impedimetry for adhesion measurement. Platelet adheres to gold electrode array placed at the bottom of well. Adhering platelets generate an increased impedance signal higher than the background impedance of the systems. This increased impedance signal is in direct correlation with the surface area covered by adhering platelets.

At first, wells were loaded with medium—normal saline — (80 μL/well), and then, baseline impedance level was measured for 1-3 hours till the curves reached equilibrium. After that, measurement of spreading or adhesion followed according to the applicable protocol (see below). The measured data were given as Cell Index (CI), and a dimensionless value was defined by the following formula:

$$CI = (Z_t - Z_b)/F \quad (1)$$

(Zt, measured impedance at any time point; Zb, baseline impedance; F, in-built factor depending on the used frequency of the alternating current).

During the whole experiment a 37°C, 5% carbon dioxide incubation was maintained. The measurements were executed in real-time mode (sampling frequency was 12/min) and were not paused during the administration of the platelet samples or agonists. For all samples, 3 parallels were measured (for all measurements SE of mean CI ≤ 0.359). The effect of the following agonists was investigated: epinephrine (Gedeon Richter PLC, Budapest, Hungary) in 5 μmol/L and 10 μmol/L concentrations, ADP (Sigma-Aldrich, St. Louis, MO, USA) in 5 μmol/L and 10 μmol/L concentrations, and collagen (Hormon-Chemie, Munich, Germany) in 2 μg/mL concentration.

2.5 | Measurement of adhesion

After equilibrium, agonists were added to wells (20 μL). After 20-60 minutes (adaptation period to horizontal phase), platelet samples were introduced into the wells (100 μL) and the adhesion activity of platelets was measured for 24 hours.

2.6 | Measurement of spreading

After the measurement of baseline as previously described, platelet samples were added to the wells (100 μL) and incubated till the adhesion of platelets reached a balanced state characterized by the curves reaching a plateau phase (5-8 hours). After that, agonists were introduced into the wells (20 μL) and change in spreading of the adhering platelets was measured for 24 hours.

2.7 | Statistical analysis

The statistical analysis was conducted using the in-built statistical program of the RTCA 2.0 Software (Roche Applied Science, Indianapolis, IN, USA) of the xCELLigence SP® system and Origin Pro 9. For quantitation, the slope parameter was used. The slope parameter is the rise of a straight line fitted to the measured data points in a chosen time period calculated by RTCA 2.0 Software. This measurement method generates "dimensionless" data: The slope parameter equals the tangent of the angle between the straight line and the "*x*" axis. Paired-sample *t* test was used for determining the statistical significance of the observed difference caused by platelet agonists. For further analysis, delta slope (DS) value was computed: Slope values were normalized to control by calculating the difference between the slopes of examined and control curves. The DS value, the difference between 2 dimensionless "slope" units, is also a dimensionless unit. Therefore, no units of measurement are indicated when data are shown in the Results section. Pearson correlation coefficient (*r*-value) between DS values and continuous variables (laboratory parameters, age) was calculated. A two-sample *t* test was used for discrete variables (age group, smoking, alcohol consumption, the presence of allergic disease, physical activity). For marking of confidence levels in the Figures, the *x*: $P < .05$; *y*: $P < .01$; *z*: $P < .001$ marks were used.

3 | RESULT

3.1 | Effect of platelet density

Before examining the effect of agonists on platelet spreading and adhesion, the effect of different platelet densities on the measured impedance was investigated. In Figure 1, the time interval −40-0 mins shows the end of the baseline equilibrium phase indicating that the impedance levels reached a balanced condition. Data point immediately before the administration of platelets into the wells was chosen as a baseline. After adding the platelet suspension to the wells, an immediate increase in the measured

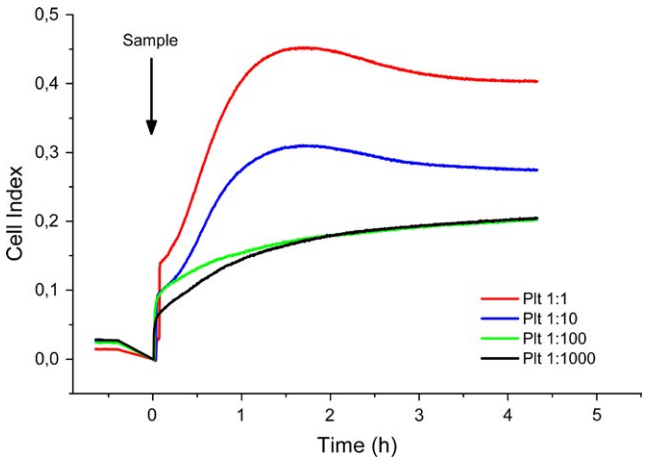

FIGURE 1 Effect of different platelet counts on platelet adhesion. Impedimetry (cell index-time) curves of different dilutions of platelet-rich plasma with normal saline (Plt 1:1, Plt 1:10, Plt 1:100, Plt 1:1000) [Colour figure can be viewed at wileyonlinelibrary.com]

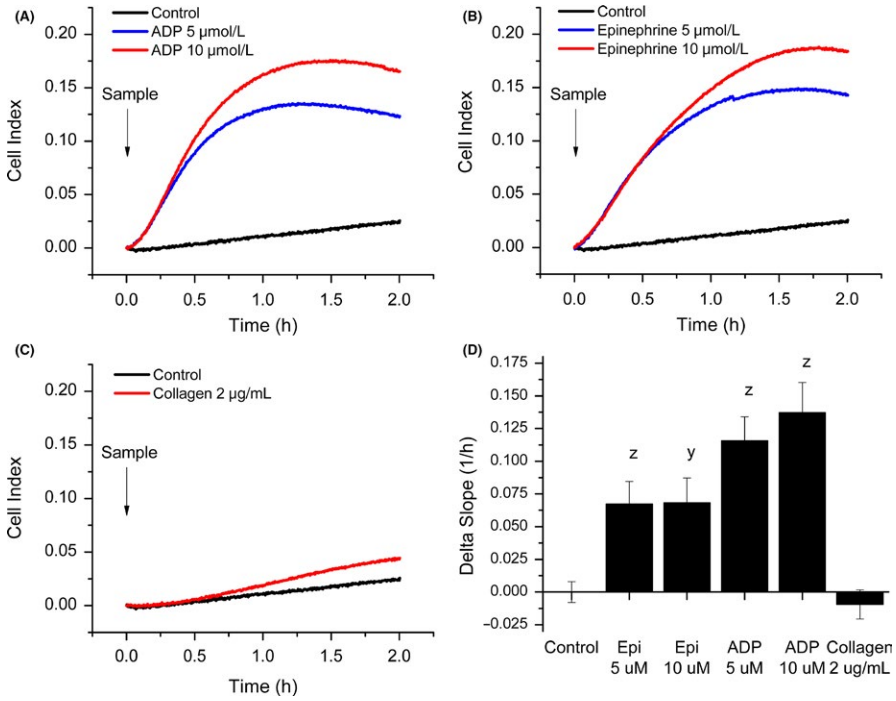

FIGURE 2 Effect of agonists on platelet adhesion. Representative impedimetry curves (cell index-time) of platelet samples in the presence of different types and concentrations of platelet agonists compared to control (normal saline): A, epinephrine 5 µmol/L and 10 µmol/L; B, ADP 5 µmol/L and 10 µmol/L; C, collagen 2 µg/mL. Impedimetry curves show the cell index change after addition of platelet samples. Comparison of the effect of different types and concentrations of platelet agonists on platelet adhesion (n = 10): D, Slope analysis of platelet adhesion impedimetry curves [for further explanation on process of statistical analysis, see Materials and Methods]; *x*: $P < .05$; *y*: $P < .01$; *z*: $P < .001$; Epi: epinephrine [Colour figure can be viewed at wileyonlinelibrary.com]

impedance (depending on the platelet count) was observed. After that, curves of the platelet sample by 1:1 and 1:10 dilution showed a slower but strong rise in the measured cell index representing strong platelet adhesion activity. Curves of the lower platelet counts (1:100 and 1:1000) had a much slower rise representing a smaller increase in impedance by the much fewer platelets. Because the objective of the following experiments was to examine the effect of activators on platelets, 1:10 dilution was chosen for the following examinations. To exclude the possible artifact caused by different platelet counts, in the following experiments the measured slope values of curves were normalized to that of the control curve.

3.2 | Adhesion measurements

In the first series of experiments (n = 10), we measured platelet adhesion in the presence of different platelet activators vs control. Epinephrine increased platelet adhesion activity on both 5 µmol/L and 10 µmol/L concentrations with DS values 0.067 ± 0.017 ($P < .001$) and 0.068 ± 0.019 ($P = .003$), respectively (Figure 2A, D). ADP increased platelet adhesion activity to an even greater extent on both 5 µmol/L and 10 µmol/L concentrations with DS values 0.116 ± 0.018 ($P < .001$) and 0.137 ± 0.023 ($P < .001$), respectively (Figure 2B, D). Collagen had no significant effect on platelet adhesion although we can observe slight negative tendency with DS value −0.009 ± 0.011 ($P = .315$); (Figure 2C, D).

Among complete blood count, MCHC (mean cell hemoglobin concentration) had significant positive correlation with platelet adhesion in the presence of 10 µmol/L epinephrine ($P = .036$) and showed significant positive correlation with platelet adhesion in the presence of both 5 µmol/L and 10 µmol/L ADP ($P = .016$ and .035, respectively). RDW had significant negative correlation with platelet adhesion in the presence of 5 µmol/L epinephrine ($P = .025$). Serum sodium level had significant negative correlation with platelet adhesion in the presence of epinephrine (5 and 10 µmol/L—$P = .010$ and .001, respectively) and ADP (5 and 10 µmol/L—$P = .038$ and .015, respectively) in both measured concentrations, while serum calcium had significant negative correlation with platelet adhesion in the presence of 10 µmol/L epinephrine ($P = .019$) as well as 5 µmol/L and 10 µmol/L ADP ($P = .023$ and .018, respectively). Serum glucose showed the same negative correlations (10 µmol/L epinephrine—$P = .005$; 5 and 10 µmol/L ADP—$P = .002$ and .003, respectively). CK and CKMB also had significant positive correlation with platelet adhesion in the presence of 5 µmol/L ADP ($P = .043$ and .038, respectively) (Table 1).

Among the measured clinical parameters (Table S1), age had significant negative correlation with platelet adhesion in the presence of 5 µmol/L epinephrine ($r = -.708$, $P = .022$), while height had significant positive correlation with platelet adhesion in the presence of 5 µmol/L epinephrine ($r = .773$, $P = .042$). Systolic blood pressure (SBP) had significant positive correlation with platelet adhesion in the presence of collagen ($r = .893$, $P = .007$).

When taking age as a discrete variable, platelet adhesion in the presence of 5 µmol/L epinephrine was significantly higher in those below 35 years than those over it (0.095 ± 0.015 vs 0.040 ± 0.011, $P = .018$). Platelet adhesion was significantly higher in individuals with known allergic disease compared to those without in the presence of 10 µmol/L epinephrine (0.11 ± 0.011 vs 0.024 ± 0.012, $P = .008$), 10 µmol/L ADP (0.224 ± 0.022 vs 0.08 ± 0.025, $P = .02$), and 5 µmol/L ADP (0.171 ± 0.027 vs 0.073 ± 0.018, $P = .035$). There was no significant difference in platelet adhesion in the presence of either investigated agent between smokers and nonsmokers,

TABLE 1 Measured laboratory values (mean ± standard error/SE) and correlation coefficients (*r*) between platelet adhesion or spreading and laboratory values

			Platelet adhesion						Platelet spreading					
				Correlation coefficient (*r*)						Correlation coefficient (*r*)				
	Laboratory parameters	Normal range	Mean ± SE	Epi 10 µmol/L	Epi 5 µmol/L	ADP 10 µmol/L	ADP 5 µmol/L	Coll 2 µg/mL	Mean ± SE	Epi 10 µmol/L	Epi 5 µmol/L	ADP 10 µmol/L	ADP 5 µmol/L	Coll 2 µg/mL
	WBC (10^3/mm^3)	4.0-10.0	8.26 ± 0.98	−.05	−.24	.07	.02	.60	7.42 ± 0.61	.20	.43	.26	.31	.50
	Neutrophil (%)	45.0-79.0	58.23 ± 3.96	−.42	−.56	−.27	−.28	.13	58.00 ± 1.45	.65	.42	.35	.30	.01
	Lymphocyte (%)	16.0-45.0	32.33 ± 3.51	.47	.65	.28	.29	−.18	31.35 ± 2.48	−.74	−.48	−.60	−.50	.14
⇨	Monocyte (%)	0-10.0	6.89 ± 0.65	−.05	−.21	.13	.15	−.12	7.15 ± 1.34	**.87**[x]	.44	.31	.19	.18
	Eosinophil (%)	0-4.0	2.24 ± 0.47	.13	.15	.02	.00	.47	3.22 ± 0.91	−.29	.02	.63	.59	−.64
	Basophil (%)	0-1.0	0.27 ± 0.08	−.05	.23	−.29	−.35	−.24	0.28 ± 0.05	−.17	−.46	.00	−.07	−.21
	RBC (10^3/mm^3)	3.5-5.0	4.80 ± 0.14	.01	−.24	.22	.26	.29	4.42 ± 0.25	.50	.01	.11	.06	.69
	Hemoglobin (g/dL)	12.0-16.0	14.37 ± 0.36	.18	.05	.28	.36	.18	13.25 ± 0.45	.43	−.06	.24	.18	.50
	Hematocrit (%)	35.0-47.0	41.87 ± 0.85	−.27	−.38	−.15	−.09	.16	39.20 ± 1.35	.60	.18	.32	.24	.50
⇨	MCV (µmol/L^3)	76-100	87.53 ± 1.34	−.46	−.12	−.69	−.68	−.39	89.30 ± 2.39	−.20	.27	.15	.14	**−.85**[x]
⇨	MCH (pg)	27.0-33.0	30.00 ± 0.39	.26	.59	−.01	.05	−.34	30.18 ± 0.81	−.42	.00	.08	.10	**−.87**[x]
➡	MCHC (g/dL)	31.0-35.0	34.30 ± 0.45	**.79**[x]	.71	**.79**[x]	**.85**[x]	.12	33.80 ± 0.29	−.73	**−.92**[x]	−.23	−.16	−.08
⇨	RDW (%)	11.0-15.0	13.06 ± 0.35	−.60	**−.82**[x]	−.38	−.40	.16	12.50 ± 0.21	.21	.47	.54	.53	.23
	Plt (10^3/mm^3)	150-400	238.57 ± 16.41	−.46	−.32	−.60	−.67	.17	247.67 ± 20.34	−.22	.31	.15	.19	.10
➡	Sodium (mmol/L)	135-145	137.29 ± 0.36	**−.96**[y]	**−.87**[x]	**−.85**[x]	**−.78**[x]	−.66	138.33 ± 0.49	.46	.70	.43	.38	.27
	Potassium (mmol/L)	3.5-5.1	4.30 ± 0.08	.05	.06	−.03	−.16	.42	4.07 ± 0.09	.15	−.10	.54	.45	−.57
➡	Calcium (mmol/L)	2.15-2.55	2.44 ± 0.07	**−.84**[x]	−.69	**−.84**[x]	**−.82**[x]	−.38	2.35 ± 0.09	.12	−.26	−.16	−.21	.60
	Magnesium (mmol/L)	0.66-1.07	0.89 ± 0.02	.43	.51	.41	.51	−.26	0.93 ± 0.03	.42	.77	.20	.15	−.42
	Creatinine (µmol/L)	44-80	72.71 ± 5.75	−.10	−.30	.18	.28	−.35	76.50 ± 5.96	.53	.59	.64	.47	−.42
	Urea (mmol/L)	1.0-8.3	4.76 ± 0.39	.53	.44	.46	.32	.63	4.40 ± 0.32	.45	.39	.19	.17	.69
➡	Glucose (mmol/L)	4.11-5.89	5.31 ± 0.10	**−.91**[y]	−.74	**−.93**[y]	**−.94**[y]	−.39	5.41 ± 0.13	−.60	−.63	−.06	.13	.14
	T. bilirubin (µmol/L)	1.0-21.0	5.86 ± 0.82	.02	.06	.11	.21	−.51	5.41 ± 1.18	−.29	−.81	−.41	−.39	.42
	D. Bilirubin (µmol/L)	0.5-3.4	1.60 ± 0.14	.26	.35	.27	.34	−.55	1.45 ± 0.32	−.19	−.77	−.41	−.41	.35
	Protein (g/L)	66.0-87.0	73.70 ± 1.29	.00	−.34	.24	.20	.58	74.77 ± 1.84	.05	.05	.47	.39	−.17
	Albumin	34.0-48.0	49.20 ± 0.85	.60	.69	.41	.42	.45	47.85 ± 0.93	−.12	−.25	.02	.07	.68
	HsCRP (mg/L)	0.1-5	3.13 ± 2.18	−.55	−.67	−.42	−.45	.01	4.25 ± 1.70	.48	.41	−.33	−.41	−.19
	Cholesterol (mmol/L)	0-5.2	5.31 ± 0.35	−.24	−.36	−.15	−.17	.47	5.32 ± 0.42	−.20	.10	.61	.68	.12

(Continues)

TABLE 1 (Continued)

			Platelet adhesion						Platelet spreading					
				Correlation coefficient (*r*)						Correlation coefficient (*r*)				
	Laboratory parameters	Normal range	Mean ± SE	Epi 10 µmol/L	Epi 5 µmol/L	ADP 10 µmol/L	ADP 5 µmol/L	Coll 2 µg/mL	Mean ± SE	Epi 10 µmol/L	Epi 5 µmol/L	ADP 10 µmol/L	ADP 5 µmol/L	Coll 2 µg/mL
	Tg (mmol/L)	0-2.2	1.74 ± 0.44	.34	.02	.59	.61	.36	2.58 ± 0.73	.29	.57	.74	.63	−.45
⇨	HDL (mmol/L)	1.15-1.68	1.45 ± 0.14	−.18	.00	−.40	−.48	.39	1.44 ± 0.17	−.59	**−.94**^{y}	−.56	−.47	.15
	LDL (mmol/L)	0.10-3.34	3.20 ± 0.26	−.27	−.41	−.13	−.15	.38	3.09 ± 0.43	−.02	.23	.47	.55	.35
	Uric acid (mmol/L)	143-339	288.14 ± 24.45	−.17	−.41	.12	.14	−.21	268.33 ± 39.22	.46	.54	.31	.22	.32
⇨	GOT (U/L)	0-32	20.29 ± 3.26	−.14	−.40	.18	.26	−.16	20.00 ± 2.58	−.07	−.54	−.51	−.46	**.90**^{x}
⇨	GPT (U/L)	0-33	18.14 ± 3.33	−.19	−.47	.13	.18	−.14	19.33 ± 3.76	.52	.11	−.10	−.12	**.87**^{x}
⇨	GGT (U/L)	5-36	17.14 ± 2.84	.05	−.09	.17	.20	.27	17.67 ± 3.43	**.83**^{x}	.65	−.02	−.12	.61
	ALP (U/L)	35-104	68.43 ± 5.69	.00	−.14	.12	.21	.08	63.50 ± 7.98	.44	.79	.62	.53	−.11
⇨	CK (U/L)	3-167	97.00 ± 14.47	.74	.69	.73	**.77**^{x}	.04	199.83 ± 63.06	.31	.38	−.28	−.30	.70
⇨	CKMB (U/L)	0-25.0	12.21 ± 0.42	.59	.32	.75	**.78**^{x}	.44	13.65 ± 0.90	−.33	−.60	−.54	−.52	.53
	LDH (U/L)	240-480	267.71 ± 18.64	.53	.32	.63	.62	.52	281.33 ± 15.56	−.32	−.27	−.46	−.40	.62
	HBa1c (mmol/mol)	20-42	35.63 ± 0.99	.18	−.13	.40	.36	.56	32.45 ± 0.85	.76	.19	−.12	−.21	.68
	HBa1c (%)	4.8-5.9	5.39 ± 0.10	.19	−.11	.39	.35	.59	5.12 ± 0.08	.72	.16	−.08	−.17	.70
	NT-pro-BNP (pg/mL)	5-125	32.54 ± 8.92	−.06	.07	−.10	−.06	−.36	55.43 ± 7.47	−.13	.31	.30	.22	−.81

ADP, adenosine-diphosphate; Coll, collagen; Epi, epinephrine; Plt, platelet count; RBC, red blood count; WBC, white blood count.

x: $P < .05$, y: $P < .01$; empty arrows: significant correlation was found between the laboratory value and platelet adhesion or spreading in 1 case; solid arrows: significant correlation was found between the laboratory value and platelet adhesion or spreading in multiple cases. Values in bold represent statistically significant data compared to the identical controls.

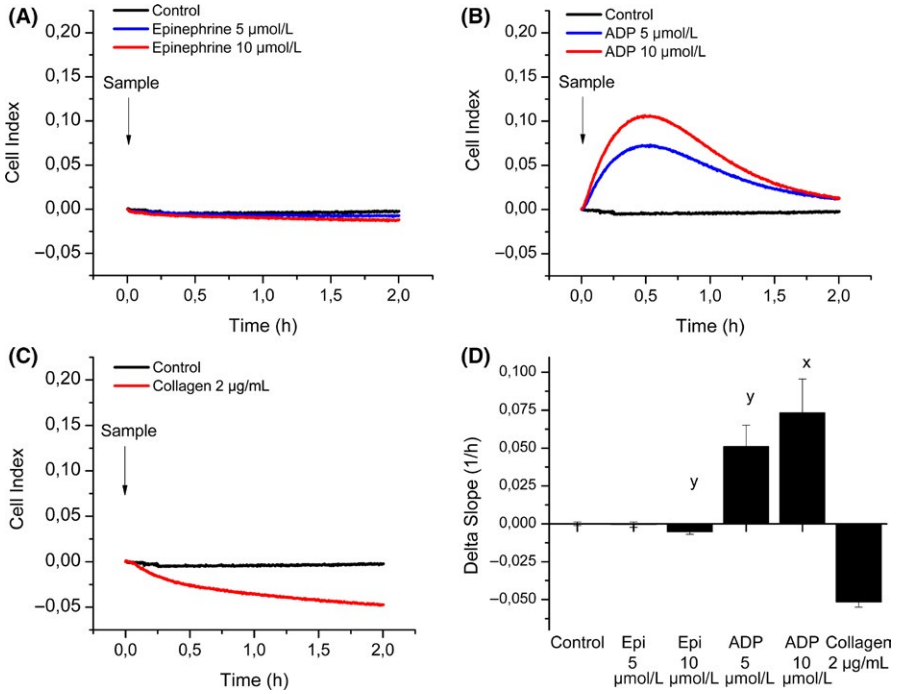

FIGURE 3 Effect of agonists on platelet spreading. Representative impedimetry curves (cell index-time) of platelet samples after the addition of different types and concentrations of platelet agonists compared to control (normal saline): A, epinephrine 5 µmol/L and 10 µmol/L; B, ADP 5 µmol/L and 10 µmol/L; C, collagen 2 µg/mL. Impedimetry curves show the cell index change after addition of agonist. Comparison of the effect of different types and concentrations of platelet agonists on platelet spreading (n = 10): D, Slope analysis of platelet spreading impedimetry curves [for further explanation on process of statistical analysis, see Materials and Methods]; *x*: $P < .05$; *y*: $P < .01$; *z*: $P < .001$; Epi: epinephrine [Colour figure can be viewed at wileyonlinelibrary.com]

individuals with different level of alcohol consumption and individuals with or without regular physical activity (Table S2).

3.3 | Spreading measurements

In the second series of experiments (n = 10), we measured the effects of platelet activators vs control on platelets that had adhered to the electrode surface previously. Epinephrine decreased platelet spreading in 10 µmol/L concentration with DS value values −0.005 ± 0.002 ($P = .001$), while the lower concentration (5 µmol/L) of epinephrine showed no effect with DS value −0.0005 ± 0.002 ($P = .680$) (Figure 3A, D). On the other hand, ADP increased platelet spreading in 5 µmol/L and 10 µmol/L concentrations with DS values 0.051 ± 0.014 ($P = .008$) and 0.073 ± 0.022 ($P = .011$), respectively (Figure 3B, D). Collagen also showed decreased platelet spreading with DS value −0.052 ± 0.004 ($P < .001$) in 2 µg/mL concentration (Figure 3C, D).

From clinical laboratory parameters, monocyte ratio had a significant positive correlation with platelet spreading induced with 10 µmol/L epinephrine ($P = .025$). MCV and MCH had a significant negative correlation with collagen-induced platelet spreading ($P = .032$ and 0.024, respectively), while MCHC had a significant negative correlation with 5 µmol/L epinephrine-induced platelet spreading ($P = .010$). HDL also had a significant negative correlation with 5 µmol/L epinephrine-induced platelet spreading ($P = .005$). Of the measured liver enzyme levels, GOT and GPT had a significant positive correlation with platelet spreading in the presence of collagen ($P = .015$ and .024, respectively) and GGT showed a positive correlation with platelet spreading in the presence of 10 µmol/L epinephrine ($P = .039$) (Table 1).

None of the measured clinical parameters showed significant correlation with platelet spreading. There was no significant difference of platelet spreading between different age groups, smokers and nonsmokers, individuals with different levels of alcohol consumption, individuals with or without regular physical activity or individuals with or without allergic disease. (Tables S1 and S2).

4 | DISCUSSION

Platelet function testing is one of the focus points in current cardiovascular medicine. Although several platelet function tests are available, a clinically applicable, reliable, and well-standardized test with high sensitivity and specificity for investigation of platelet function and determining the usefulness of the monitoring of antiplatelet therapies is still an unmet need. Furthermore, most of these tests are focusing on the measurement of platelet aggregation, while there is no established method for the isolated measurement of platelet adhesion.[15]

The xCELLigence system is a real-time technique for measurement of cell adhesion using an impedimetric approach. Its reliability and high sensitivity have been demonstrated with a variety of cell types and applications.[9-13] Platelets are cell fragments with considerably smaller dimensions than whole cells; therefore, a highly sensitive method is needed for detection of changes in adhesion and spreading. The xCELLigence system met these expectations, and the impedance signal of adhering platelets, although with smaller Cell Index values than it is usual for whole cells, was easily detectable and measurable. Even more, xCELLigence was able to differentiate between various platelet counts per sample (Figure 1). Although in this experiment, the effect of dilution of the plasma itself cannot be excluded, different platelet densities no doubt played a role in the different result.

4.1 | Impedimetric platelet adhesion and spreading measurements

Effect of routinely used reference platelet agonists, epinephrine, ADP, and collagen[2] on platelets was measured by impedimetry. Two weak platelet agonists, epinephrine, and ADP increased platelet adhesion in a concentration-dependent manner. ADP showed the same concentration-dependent positive effect on platelet spreading, but epinephrine did not on platelets already adhering to a surface. This underlines the high sensitivity of impedimetry and xCELLigence: It was able to differentiate between 2 closely connected processes of platelet cell biology.

Although collagen is a strong platelet agonist, using xCELLigence a tendentially negative effect of collagen on platelet adhesion and spreading was measured. For this interesting finding, 2 different explanations are presented here. In our experiments, samples diluted with saline were used. It is possible that von Willebrand factor (vWf) levels became too low for the effect of collagen to take place[16] leading to decreased impedance levels. On the other hand, platelets do have direct collagen receptors (GP VI and integrin $\alpha 2\beta 1$) on their surface that does not require vWf for collagen binding with GP VI playing a major role in platelet activation upon collagen binding. Collagen was applied in these experiments in similar concentration as used in light transmission aggregometry.[17] It is possible that—despite the intentionally lowered platelet count in samples—collagen actually triggered platelet aggregation. The total surface of platelet aggregates is much smaller than the combined surface of individual adhering platelets which accounts for the decrease in impedance. This finding suggests that weak platelet agonists (epinephrine and ADP) are the reference platelet agonists of choice in impedimetric platelet adhesion testing because smaller stimuli are required than in platelet aggregometry.

Most impedimetry curves showed an initial increase and a subsequent decrease in the measured impedance. As discussed above, the initial increase shows the phase of adhesion or spreading. The decrease could indicate a degree of reversibility in the platelet adhesion or spreading; however, degradation of platelets is also a reasonable possibility.

4.2 | Influence of clinical factors on platelet adhesion and spreading

To further analyze the suitability of impedimetry for measuring platelet adhesion and spreading, we investigated in vivo parameters that could potentially affect platelet adhesion. Increased platelet activity and reactivity in acute and chronic allergic or other hyperimmune states are well documented.[18-21] Impedimetric measurement detected similar, increased platelet adhesion in individuals with a history of allergic diseases. The association between blood glucose levels and platelet aggregability has already been described: Ahuja et al found decreased platelet aggregation in postprandial samples.[22,23] We found a same negative correlation between platelet adhesion and blood glucose levels. Although we have to note that these were not fasting blood glucose levels, also, no correlation between platelet adhesion and serum HbA1c levels—all in the normal range—was observed. Increased platelet reactivity and decreased response to antiplatelet agents among the elderly is well-documented.[24-27] In our data, age showed a negative correlation with platelet adhesion in the presence of low concentration epinephrine. Our subjects were between the age of 20 and 40 years, a population with lower cardiovascular risk, the subjects of the aforementioned studies belonging to the mostly elderly population. The fact that platelet impedance adhesiometry was able to detect subtle differences in platelet adhesion between subgroups of a healthy population emphasizes the significance of the techniques in detecting potential prognostic factors in asymptomatic patients.

4.3 | Correlation between laboratory parameters and impedimetric measurements

To investigate further the clinical usefulness of platelet impedance adhesiometry, we examined the relationship between platelet adhesion and spreading measured by impedimetry and standard clinical laboratory parameters. Partly because of the low number and nonroutine use of platelet adhesion measurement techniques, there are very few data available on this topic, so further investigation is required. Platelet adhesion induced by epinephrine and ADP had a negative correlation with serum sodium and calcium levels. While calcium is component of almost every hemostatic process, the increase in ADP and epinephrine-induced platelet aggregation and activation in citrated plasma samples is documented.[28] Our results, while cannot be a direct effect, correlate with these results and indicate the sensitivity of the impedimetric method. Although multiple correlations were found between platelet adhesion and MCHC, no correlation was found between hemoglobin concentration and hematocrit, which suggests that this cannot be a direct correlation. However, it is possible that these 2 parameters are indirectly associated with a state of increased thrombogenicity. This is supported by a study published in 2016, where MCHC was found to be an independent prognostic factor of acute myocardial infarction.[29] Change in spreading of platelets already adhering to surface is a much weaker reaction than adhesion indicated by our results with changes on the much smaller scale than adhesion. Examining correlation between change in platelet spreading and measured laboratory or clinical variables shows that there is a less significant correlation; also, tendencies are less clear. Parameters have less variability in the healthy population, and further standardization is needed in targeted patient groups.

5 | CONCLUSION

Platelet adhesion and spreading proved to be a qualitatively and quantitatively well-measurable by a high-throughput impedimetry system. The easy application of the sample, short runs of assays, as

well as the in-built software offer the opportunity of an easy to use, semi-automated measurement in research and clinics, too. The effect of platelet agonists used as laboratory reference on platelet adhesion and spreading in a healthy population was demonstrated by this novel technique. Impedimetry was able to differentiate between the 2 separate physiological processes. These results correlated well with the measured laboratory and clinical parameters, which may have an influence on platelet adhesion and spreading. Although in the future, more reference studies of specific patient groups are required to explore the changes in platelet adhesion and spreading characteristic to different clinical scenarios, even the slight differences in a healthy patient group could be demonstrated with impedimetry. All in all, the observations and data presented above support our confidence that impedimetry is a dedicated and useful method for more accurate investigation of platelet functions in a clinical setting, providing diagnostic assistance and guiding therapy in the close future.

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DISCLOSURES/DUALITY OF INTEREST

None declared.

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REFERENCES

1. Aradi D, Komocsi A, Price MJ, et al. Efficacy and safety of intensified antiplatelet therapy on the basis of platelet reactivity testing in patients after percutaneous coronary intervention: systematic review and meta-analysis. *Int J Cardiol*. 2013;167:2140-2148.
2. Mylotte D, Foley D, Kenny D. Platelet function testing: methods of assessment and clinical utility. *Cardiovasc Hematol Agents Med Chem*. 2011;9:14-24.
3. Aradi D, Magyarlaki T, Tokes-Fuzesi M, Rideg O, Vorobcsuk A, Komocsi A. Comparison of conventional aggregometry with VASP for monitoring P2Y12-specific platelet inhibition. *Platelets*. 2010;21:563-570.
4. Varon D, Dardik R, Shenkman B, et al. A new method for quantitative analysis of whole blood platelet interaction with extracellular matrix under flow conditions. *Thromb Res*. 1997;85:283-294.
5. Nowak G, Wiesenburg A, Schumann A, Bucha E. Platelet adhesion assay–a new quantitative whole blood test to measure platelet function. *Semin Thromb Hemost*. 2005;31:470-475.
6. Krischek B, Morgenstern E, Mestres P, et al. Adhesion, spreading, and aggregation of platelets in flowing blood and the reliability of the retention test Homburg. *Semin Thromb Hemost*. 2005;31:449-457.
7. Giaever I, Keese CR. Monitoring fibroblast behavior in tissue culture with an applied electric field. *Proc Natl Acad Sci USA*. 1984;81:3761-3764.
8. Atienza JM, Zhu J, Wang X, Xu X, Abassi Y. Dynamic monitoring of cell adhesion and spreading on microelectronic sensor arrays. *J Biomol Screen*. 2005;10:795-805.
9. Pick H, Terrettaz S, Baud O, Laribi O, Brisken C, Vogel H. Monitoring proliferative activities of hormone-like odorants in human breast cancer cells by gene transcription profiling and electrical impedance spectroscopy. *Biosens Bioelectron*. 2013;50:431-436.
10. Lei KF, Wu MH, Hsu CW, Chen YD. Real-time and non-invasive impedimetric monitoring of cell proliferation and chemosensitivity in a perfusion 3D cell culture microfluidic chip. *Biosens Bioelectron*. 2014;51:16-21.
11. Sun SQ, Sursal T, Adibnia Y, et al. Mitochondrial DAMPs increase endothelial permeability through neutrophil dependent and independent pathways. *PLoS ONE*. 2013;8:e59989.
12. Kiss L, Walter FR, Bocsik A, et al. Kinetic analysis of the toxicity of pharmaceutical excipients Cremophor EL and RH40 on endothelial and epithelial cells. *J Pharm Sci*. 2013;102:1173-1181.
13. Opp D, Wafula B, Lim J, Huang E, Lo JC, Lo CM. Use of electric cell-substrate impedance sensing to assess in vitro cytotoxicity. *Biosens Bioelectron*. 2009;24:2625-2629.
14. Rideg O, Komocsi A, Magyarlaki T, et al. Impact of genetic variants on post-clopidogrel platelet reactivity in patients after elective percutaneous coronary intervention. *Pharmacogenomics*. 2011;12:1269-1280.
15. Gorog DA, Fuster V. Platelet function tests in clinical cardiology: unfulfilled expectations. *J Am Coll Cardiol*. 2013;61:2115-2129.
16. Sadler JE. Low von Willebrand factor: sometimes a risk factor and sometimes a disease. *Hematology Am Soc Hematol Educ Program*. 2009;2009:106-112.
17. Paul R, Banerjee AK, Guha S, et al. Study of platelet aggregation in acute coronary syndrome with special reference to metabolic syndrome. *Int J Appl Basic Med Res*. 2013;3:117-121.
18. Berlacher MD, Vieth JA, Heflin BC, et al. FcgammaRIIa ligation induces platelet hypersensitivity to thrombotic stimuli. *Am J Pathol*. 2013;182:244-254.
19. Kasperska-Zajac A, Rogala B. Platelet activation during allergic inflammation. *Inflammation*. 2007;30:161-166.
20. Kasperska-Zajac A, Brzoza Z, Rogala B. Platelet function in cutaneous diseases. *Platelets*. 2008;19:317-321.
21. Nastalek M, Potaczek DP, Wojas-Pelc A, Undas A. Plasma platelet activation markers in patients with atopic dermatitis and concomitant allergic diseases. *J Dermatol Sci*. 2011;64:79-82.
22. Ahuja KD, Adams MJ, Robertson IK, Ball MJ. Acute effect of a high-carbohydrate low-fat meal on platelet aggregation. *Platelets*. 2009;20:606-609.
23. Ahuja KD, Thomas GA, Adams MJ, Ball MJ. Postprandial platelet aggregation: effects of different meals and glycemic index. *Eur J Clin Nutr*. 2012;66:722-726.
24. Marcucci R, Cenci C, Cioni G, Lombardi A, Giusti B, Gensini GF. Antiplatelets in acute coronary syndrome: personal perspectives. *Expert Rev Cardiovasc Ther*. 2012;10:1487-1496.
25. Silvain J, Cayla G, Hulot JS, et al. High on-thienopyridine platelet reactivity in elderly coronary patients: the SENIOR-PLATELET study. *Eur Heart J*. 2012;33:1241-1249.
26. Gremmel T, Steiner S, Seidinger D, Koppensteiner R, Panzer S, Kopp CW. Adenosine diphosphate-inducible platelet reactivity shows a pronounced age dependency in the initial phase of antiplatelet therapy with clopidogrel. *J Thromb Haemost*. 2010;8:37-42.

27. Cuisset T, Quilici J, Grosdidier C, et al. Comparison of platelet reactivity and clopidogrel response in patients </= 75 Years Versus > 75 years undergoing percutaneous coronary intervention for non-ST-segment elevation acute coronary syndrome. *Am J Cardiol*. 2011;108:1411-1416.
28. Lages B, Weiss HJ. Dependence of human platelet functional responses on divalent cations: aggregation and secretion in heparin- and hirudin-anticoagulated platelet-rich plasma and the effects of chelating agents. *Thromb Haemost*. 1981;45:173-179.
29. Huang YL, Hu ZD. Lower mean corpuscular hemoglobin concentration is associated with poorer outcomes in intensive care unit admitted patients with acute myocardial infarction. *Ann Transl Med*. 2016;4:190.

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