Clinical Chemistry 59:8
1195–1204 (2013)

Microfluidic Assay of Platelet Deposition on Collagen by Perfusion of Whole Blood from Healthy Individuals Taking Aspirin

Ruizhi Li,[1] Susanne Fries,[2] Xuanwen Li,[2] Tilo Grosser,[2] and Scott L. Diamond[1*]

BACKGROUND: Microfluidic devices can create hemodynamic conditions for platelet assays. We validated an 8-channel device in a study of interdonor response to acetylsalicylic acid (ASA, aspirin) with whole blood from 28 healthy individuals.

METHODS: Platelet deposition was assessed before treatment or 24 h after ingestion of 325 mg ASA. Whole blood (plus 100 μmol/L H-D-Phe-Pro-Arg-chloromethylketone to inhibit thrombin) was further treated ex vivo with ASA (0–500 μmol/L) and perfused over fibrillar collagen for 300 s at a venous wall shear rate (200 s^{-1}).

RESULTS: Ex vivo ASA addition to blood drawn before aspirin ingestion caused a reduction in platelet deposition [half-maximal inhibitory concentration (IC_{50}) approximately 10–20 μmol/L], especially between 150 and 300 s of perfusion, when secondary aggregation mediated by thromboxane was expected. Twenty-seven of 28 individuals displayed smaller deposits (45% mean reduction; range 10%–90%; $P < 0.001$) from blood obtained 24 h after ASA ingestion (no ASA added ex vivo). In replicate tests, an R value to score secondary aggregation [deposition rate from 150 to 300 s normalized by rate from 60 to 150 s] showed $R < 1$ in only 2 of 28 individuals without ASA ingestion, with $R > 1$ in only 3 of 28 individuals after 500 μmol/L ASA addition ex vivo. At 24 h after ASA ingestion, 21 of 28 individuals displayed poor secondary aggregation ($R < 1$) without ex vivo ASA addition, whereas the 7 individuals with residual secondary aggregation ($R > 1$) displayed insensitivity to ex vivo ASA addition. Platelet deposition was not correlated with platelet count. Ex vivo ASA addition caused similar inhibition at venous and arterial wall shear rates.

CONCLUSIONS: Microfluidic devices quantified platelet deposition after ingestion or ex vivo addition of aspirin.

© 2013 American Association for Clinical Chemistry

Antiplatelet therapies are commonly used in acute treatment of coronary diseases and long-term prevention of cardiovascular events *(1)*. Inhibition of platelet cyclooxygenase 1 (COX-1)[3] by aspirin [acetylsalicylic acid (ASA)] and the consequent attenuation of thromboxane A_2 (TXA_2) production causes a decrease in secondary platelet aggregation and reduces excessive thrombus formation. ASA does not severely reduce the primary platelet response to the damaged vessel wall needed for homeostasis. Currently, about 50 million patients in the US take ASA at typical doses of 81 or 325 mg per day to reduce cardiovascular risks *(2)*.

Aspirin reduces the activation of platelets by irreversibly acetylating serine 529 of COX-1 and therefore reducing TXA_2 production *(3)*. The inhibition of COX-1 is irreversible and permanent for the lifetime of a platelet, because platelets lack the synthetic machinery to synthesize new protein *(4)*. The bleeding risks associated with aspirin led to the development of anti-inflammatory drugs selective for COX-2. Several COX-2–selective inhibitors cause fewer gastrointestinal side effects than traditional nonsteroidal anti-inflammatory drugs *(5)*, but they cause cardiovascular risks via inhibition of COX-2–dependent endothelial prostacyclin. All approved COX-2 inhibitors have been withdrawn or their prescription has been restricted *(6)*.

However, patients show a marked variability in laboratory responses to aspirin. Depending on the platelet function test, aspirin resistance has been reported in 5%–60% of patients *(7–9)*. Failure to actu-

[1] Institute for Medicine and Engineering, Department of Chemical and Biomolecular Engineering, University of Pennsylvania, Philadelphia, PA; [2] Institute for Translational Medicine and Therapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA.
* Address correspondence to this author at: Institute for Medicine and Engineering, Department of Chemical and Biomolecular Engineering, 1024 Vagelos Research Laboratories, University of Pennsylvania, Philadelphia, PA 19104. Fax 215-573-6815; e-mail sld@seas.upenn.edu.
Received October 21, 2012; accepted March 26, 2013.
Previously published online at DOI: 10.1373/clinchem.2012.198101
[3] Nonstandard abbreviations: COX, cyclooxygenase; ASA, acetylsalicylic acid; TXA_2, thromboxane A_2; PPACK, H-D-Phe-Pro-Arg-chloromethylketone; IC_{50}, half-maximal inhibitory concentration.

ally take the pill is a well-documented cause of apparent "aspirin resistance" *(10–12)*. Mutations in the thromboxane receptor would also cause aspirin insensitivity, but such mutations result in a modest bleeding phenotype that would be a contraindication for aspirin therapy *(13, 14)*. Patients may fail to benefit from aspirin therapy owing to increased platelet turnover, increased sensitivity to ADP and collagen *(4)*, or increased COX-1 synthesis. However, an association between aspirin resistance detected by a laboratory test in patients and a higher vascular event rate in a group of resistant individuals has yet to be convincingly shown *(15, 16)*.

Determination of the antiplatelet effectiveness of pharmacological agents often occurs in closed systems, with or without flow. Measurement of platelet calcium mobilization upon thromboxane stimulation is an example of a closed system with no flow *(17)*. Platelet aggregometry is a closed system with a poorly defined flow field. The efficacy of pharmacological agents in closed systems may not always predict antiplatelet benefits under flow conditions. For example, apyrase is a potent inhibitor in the tube but can potentiate thrombosis under flow due to the dynamics of local concentrations of adenosine triphosphate and adenosine diphosphate (ADP) in a concentration boundary layer *(18)*. During platelet deposition under flow conditions, the platelet deposit can reach platelet densities that are 50- to 200-fold greater than that of platelet-rich plasma. Because of this dense platelet deposit, the potency of pharmacological agents may depend on the local concentration of platelet release products that occur under flow conditions *(14)*. Microfluidic patterning techniques allow for spatially confined injury models distinct from current technologies, such as perfusion of whole blood through coated glass capillary flow chambers or parallel plate flow chambers. Furthermore, microfluidic devices recreate the progression of signaling and thrombotic pathways as they progress from a surface trigger, i.e., collagen and/or thrombin produced at the wall *(14, 19)*, to secondary aggregation processes driven by ADP and TXA_2.

Here we investigated an 8-channel microfluidic device in assessing ASA phenotype by use of fluorescently labeled platelets with ASA added to whole blood ex vivo, either before or 24 h after ASA intake by healthy individuals. We show a novel method to measure residual COX-1 function and evaluation of the COX-1–mediated TXA_2 pathway in a microfluidic platelet function test under venous flow conditions.

Materials and Methods

BLOOD COLLECTION, LABELING, AND DRUG ADMINISTRATION

We collected blood at 0 h and 26 h by venipuncture from 28 healthy individuals (11 men and 17 women, 18–55 years old), who self-reported not smoking and being free from oral medication and who abstained from caffeine, alcohol, and high-fat food for 24 h before and throughout the study. All volunteers received a 325-mg loading dose of aspirin by mouth at 2 h. Individuals returned after a 2-week washout period for a third blood draw (Fig. 1A). For arterial flow studies, we collected blood by venipuncture from 4 healthy men who were free of oral medication for 10 days and self-reported free from disease or bleeding disorders. All blood samples were drawn into H-D-Phe-Pro-Arg-chloromethylketone (PPACK) (100 μmol/L final concentration, Hematologic Technologies). PPACK is a potent inhibitor of thrombin that irreversibly and specifically inactivates thrombin at its active site. All volunteers provided informed consent in accordance with institutional review board approval and the Declaration of Helsinki. The blood was treated with phycoerythrin-conjugated mouse anti-human CD61 ($\alpha_{IIb}\beta_3$) antibody (BD Biosciences) in a ratio of 1:50. PPACK-treated whole blood was perfused through the microfluidic device within 1 h of phlebotomy. For ex vivo additions to whole blood, we dissolved ASA (Sigma Aldrich) in DMSO at 500 mmol/L. We then made dilutions of ASA to the desired final concentration in HEPES-buffered saline (HBS) (20 mmol/L HEPES, 160 mmol/L NaCl, pH 7.5) within 1 h of the test. Final ASA concentrations used were 0, 0.05, 0.5, 1, 5, 10, 50, and 500 μmol/L (final 0.1% DMSO in all samples). Blood was incubated for 30 min in ASA before initiation of the assay.

FABRICATION OF MICROFLUIDIC DEVICES, PLATELET DEPOSITION, AND REAL-TIME IMAGING

We fabricated microfluidic devices in poly(dimethylsioxane) (Sylgard 184, Ellsworth Adhesives) according to previously described techniques *(17)*. The device was fed by 8 distinct wells, with perfusion by withdrawal into a syringe pump (Harvard Apparatus) from a single outlet (Fig. 1B). The channels were spaced in close proximity to allow all channels to be imaged simultaneously with a 2× lens of an inverted microscope (IX81, Olympus America) equipped with a charge-coupled camera (Hamamatsu). A custom stage insert held 3 microfluidic devices, allowing 24 simultaneous clotting events to be imaged in 15-s intervals. We used a device with a single channel (5 cm × 250 μm × 50 μm) for patterning collagen. Equine fibrillar collagen type 1 (Chronopar, Chronolog) was diluted to 250 μg/mL in isotonic glucose solution (Chronopar, Chronolog). Before perfusion, channels were blocked with 0.5% BSA in HBS for 30 min. Samples were perfused at a venous wall shear rate of 200 s^{-1} (2 μL/min per channel) for 5 min. For arterial studies, we used a wall shear rate of 1000 s^{-1} (10 μL/min per channel) for 5 min. The respective shear

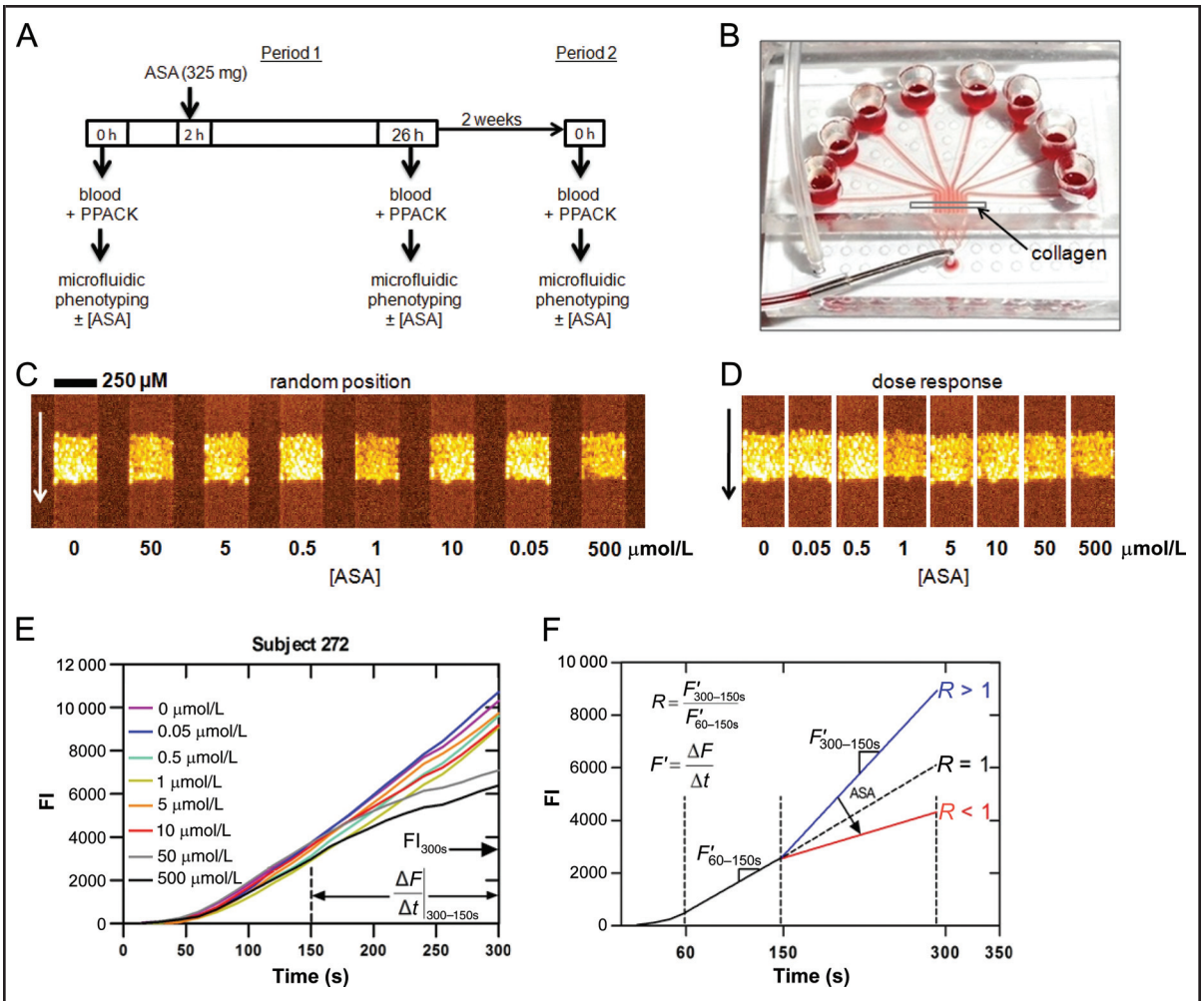

Fig. 1. Protocol and 8-channel microfluidic device used for dose–response experiments.

(A), Clinical study protocol. Blood samples were taken at $t = 0$ h and $t = 26$ h with ASA administration to each individual at $t = 2$ h, followed by an additional blood draw 2 weeks later. For each blood sample, PPACK and increasing amounts of ASA were added before microfluidic testing (3 replicate tests per blood sample). (B), Photograph of the 8-channel microfluidic device with location of collagen strip indicated digitally. (C and D), Micrographs of fluorescently labeled platelet accumulation at collagen and platelet interaction zone with various ASA concentrations, either in randomized positions (C) or ordered by ASA dose (D). (E), Fluorescence values over time for each ASA concentration. (F), Schematic summary of secondary platelet aggregation and definition of R value on the basis of deposition rates (F').

rates for venous and arterial studies were established previously in other assays with the same microfluidic device *(18, 19)*.

CALCULATION OF HALF-MAXIMAL INHIBITORY CONCENTRATION AND ASA SENSITIVITY

We fitted background-corrected fluorescence values with a 4-parameter dose–response model:

$$\mathrm{FI} = A + \frac{A - B}{1 + 10^{[(\mathrm{Log\ IC_{50}} - C) \cdot D]}},$$

where C represents the ASA concentration; FI, the background corrected fluorescence of the corresponding region; A and B, the minimum and maximum intensities, respectively; and D, the Hill coefficient. The data were fitted by use of a log(inhibitor) vs response routine in GraphPad Prism 5.00 (GraphPad Software). We also analyzed platelet deposition on collagen using the change in the background-corrected fluorescence values between time intervals 60 and 150 s for collagen deposition

Table 1 Donor attributes.

Subject ID	Age, years	Body mass index, kg/m^2	Sex	Platelet count, ×1000 platelets/μL		Hematocrit, %
				Period 1	Period 2	
217	25	27.08	M	190	187	43
246	30	24.28	F	183	171	36
272	22	21.65	F	280	275	39
259	43	20.14	M	220	247	39
262	37	24.18	M	274	233	41
225	31	24.47	F	216	217	36
250	44	24.77	F	227	216	38
241	26	19.47	F	204	227	40
283	37	28.28	F	206	176	38
268	31	27.59	M	293	262	44
203	33	29.71	M	216	226	44
248	33	20.84	F	300	288	38
239	52	24.58	F	225	301	37
216	27	24.98	F	182	195	36
226	51	23.51	F	226	222	34
235	21	20.73	F	189	216	36
238	26	17.54	F	150	183	37
240	48	31.79	M	182	149	40
255	25	20.02	F	183	181	44
212	29	23.92	M	272	191	44
231	35	27.33	M	209	209	45
243	23	19.46	F	235	230	40
278	22	20.37	F	261	258	36
293	37	20.59	F	283	285	39
253	44	26.18	M	224	245	46
256	29	19.12	M	176	176	40
277	25	20.34	F	196	182	36
282	44	28.52	M	243	234	45
Mean (SD)	33 (9)	23.62 (3.70)		223 (40)	220 (39)	40 (3.5)

and 150 and 300 s for TXA_2-dependent secondary aggregation ($F' = \Delta F/\Delta t$ between 60 and 150 s or 150 and 300 s). We then took the ratio of these 2 slopes (R value) as an internally normalized value for each individual, with $R > 1$ when secondary aggregation was prominent and $R < 1$ when secondary aggregation was attenuated relative to the primary response to collagen (primary deposition) (see Supplemental Table S1 and Fig. S2, which accompany the online version of this article at http://www.clinchem.org/content/vol59/issue8).

Results

A protocol was established (Fig. 1A) for the recruitment of healthy donors (n = 28) (Table 1) to obtain 3 venous blood samples. Relevant characteristics of the individuals are reported in Table 1. The first blood sample was collected at $t = 0$ h, followed by ASA ingestion at $t = 2$ h, and collection of a second sample at $t = 26$ h, 24 h after in vivo ingestion of 325 mg ASA by all individuals (period 1). A third venous sample was collected after a 2-week washout period at $t = 0$ h (period 2). With each of the 3 blood samples obtained from each donor during periods 1 and 2, ASA was added ex vivo at increasing doses from 0 to 500 μmol/L ASA before running the 300-s microfluidic test (8 doses run in triplicate per blood draw at wall shear rate of 200 s^{-1}), for a total of 2304 individual clotting events. All results shown are means of triplicate measurements.

HALF-MAXIMAL INHIBITORY CONCENTRATION FOR ASA DURING THROMBOSIS UNDER FLOW

Ex vivo addition of ASA to whole blood was tested in the device in a dose–response manner to calculate half-maximal inhibitory concentration (IC_{50}) values for collagen-induced platelet deposition (Fig. 1B). In each experiment, 24 simultaneously forming thrombi were imaged in real time. Fluorescently labeled platelets aggregated only at the site of collagen exposure, with no deposition upstream or downstream of the patterned collagen (Fig. 1, C and D). Platelet adhesion due to collagen occurred within 60 s, and secondary platelet accumulation/aggregation continued after 150 s of perfusion (Fig. 1E; see online Supplemental Fig. S1). Inhibition of secondary platelet adhesion by ASA was measured via the *R* value (Fig. 1F). Determination of surface fluorescence at 300 s (FI_{300s}) allowed the determination of an IC_{50} of 22 μmol/L ASA (Fig. 2A) for an ex vivo ASA incubation time of 30 min for subject 272. The collagen-induced platelet activation was temporally separate and preceded the ASA-sensitive deposition regime. A dose-dependent decrease in platelet deposition rate (F' from 150 to 300 s) was observed with increasing ASA concentration, whereas the initial deposition rate (F' between 60 and 150 s) was ASA insensitive (Fig. 2B). Finally, internal normalization (Fig. 2C) of late-stage $F'_{150–300s}$ divided by early-stage $F'_{60–150s}$ provides an *R* value of 1.0 near the ASA IC_{50} of 10 μmol/L. This *R* value showed more dynamic range and sensitivity related to secondary platelet aggregation due to TXA_2 release, causing a minor left shift in the IC_{50} value to 10 μmol/L. The ASA dose response was also obtained by ex vivo addition of ASA to whole blood 24 h after ingestion of a single 325-mg dose. Representative subject 255 lacked response to ex vivo addition of ASA, demonstrating strong inhibition of platelet COX-1 activity by the 325 mg dose (Fig. 2D).

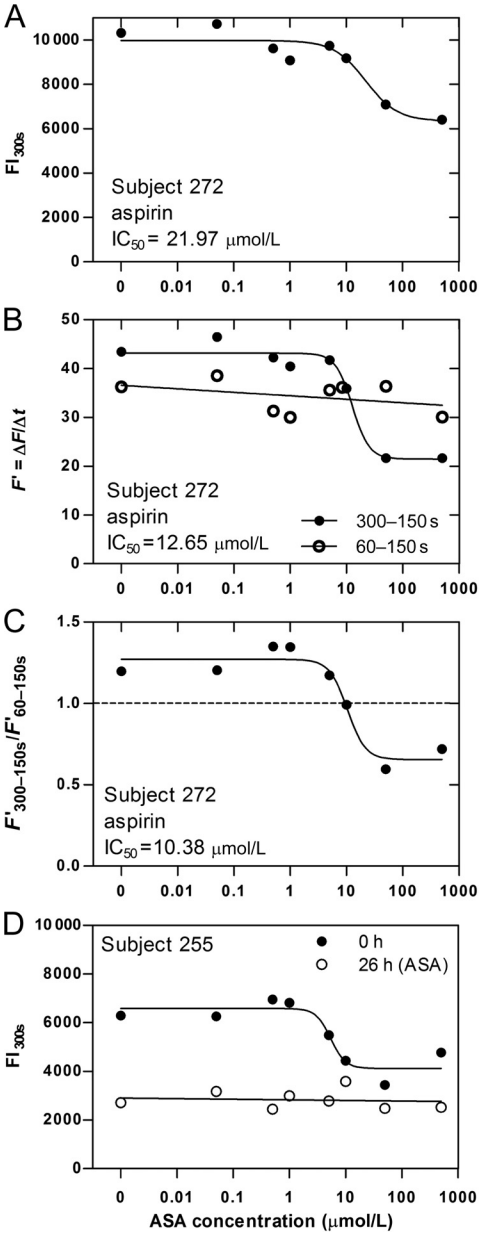

Fig. 2. Platelet deposition from PPACK-treated whole blood with increasing ex vivo addition of ASA at 200 s^{-1} wall shear rate over fibrillar collagen for period 1 (t = 0 h, no ASA ingestion).

(A), Representative IC_{50} curve for ASA dose–response. (B), To separate COX-1–mediated TXA_2 production from the collagen response, $F' = \Delta F/\Delta t$ was calculated for the initial time interval of 60–150 s and secondary aggregation during the time interval of 150–300 s. Initial deposition rate from 60 to 150 s was ASA insensitive. (C), Normalization of individual response to ASA using the *R* value. (D), Ex vivo addition of ASA at 26 h (○) had no efficacy after donors took ASA (325 mg) at 2 h.

MICROFLUIDIC ASA PHENOTYPING AND EX VIVO ADDITION OF ASPIRIN AFTER ORAL ADMINISTRATION

Microfluidic phenotyping of whole blood 24 h after ingestion of a single 325-mg dose showed that 27 of 28 individuals had some reduction in total platelet accumulation relative to deposition measured in blood before ASA ingestion (Fig. 3A). Only subject 253 displayed a slight increase in platelet deposit, which was not statistically significant. Marked interindividual variation in the amount of baseline platelet deposition was observed, with FI_{300s} values ranging from <4000 to >12 000. However, the microfluidic assay allowed detection of the decrease in total platelet accumulation following in vivo ASA ingestion. Platelet deposition from blood obtained 24 h after ASA ingestion (no additional ex vivo ASA added) was inhibited relative to the platelet deposition measured for blood before ASA

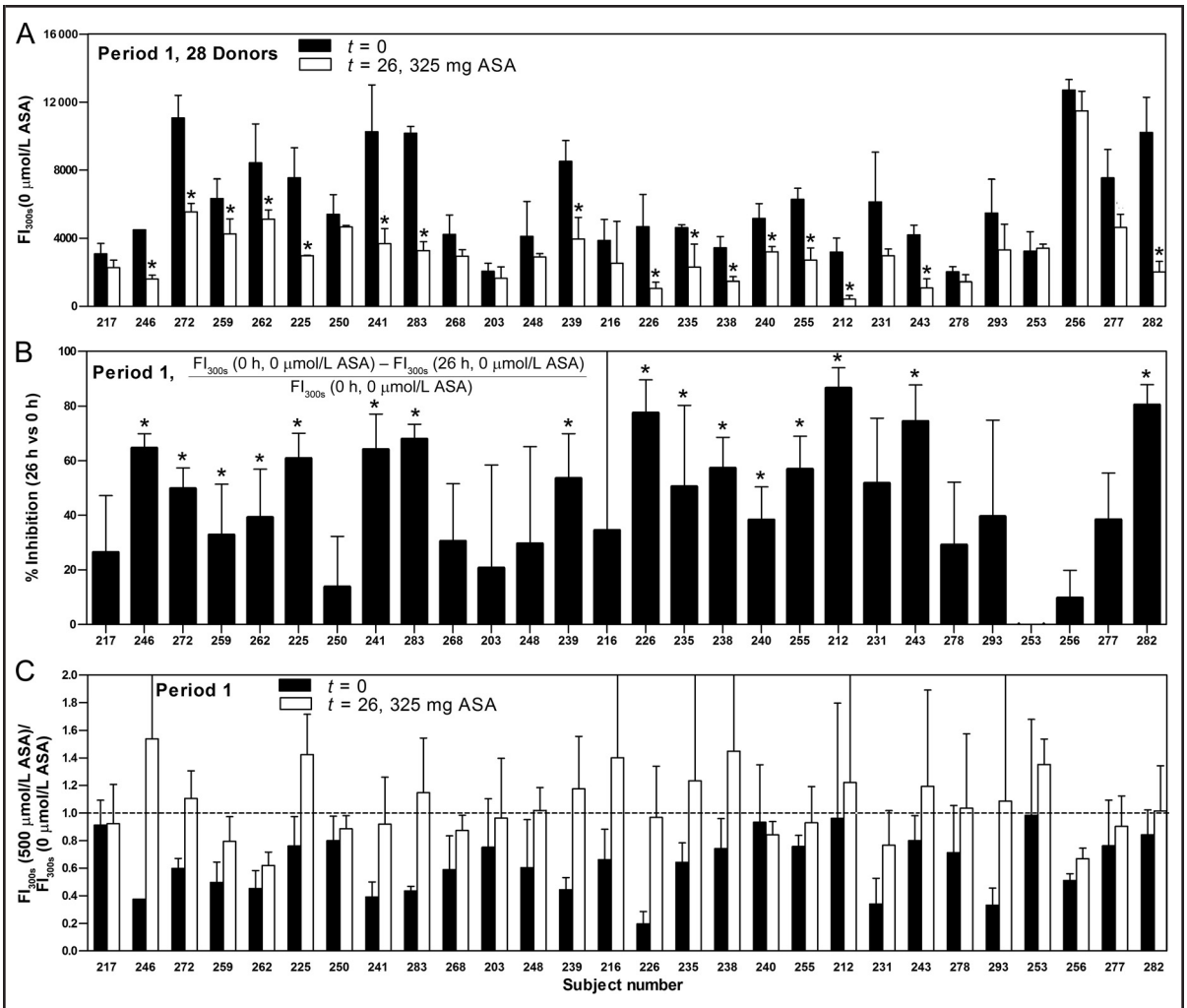

Fig. 3. Microfluidic ASA phenotyping of donors.

(A), Ingestion of ASA at $t = 2$ h resulted in smaller platelet deposits compared to the response obtained from blood before ASA ingestion at $t = 0$ h (28 donors). $^*P < 0.05$, relative to each donor at $t = 0$ h. (B), Percent inhibition of platelet deposition as a measure of in vivo ASA efficacy at 26 h, as assessed by 8-channel microfluidic assay. $^*P < 0.05$, relative to each donor at $t = 0$ h. (C), Comparison of high-dose ex vivo addition of 500 μmol/L ASA measured for whole blood obtained before ($t = 0$) or 26 h after oral ASA administration (at 2 h). Data are mean (SD) (3 replicates).

ingestion (Fig. 3B). The degree of inhibition of platelet deposition for these 28 individuals at 24 h after ASA dosage ranged from 10% to 90% [mean (SD) 45% (23%); $P < 0.001$, n = 28) (Fig. 3B). When comparing each individual at 26 h to their response at 0 h, those with ≥25% inhibition (16 of 28 individuals) displayed statistically significant inhibition ($P < 0.05$) (Fig. 3B). In comparing platelet deposition (FI_{300s}) in response to ex vivo 500 μmol/L ASA addition relative to no ex vivo ASA addition, the ex vivo addition of high-dose ASA was very potent in blood obtained before ASA ingestion: 24 of 28 individuals responded to ex vivo ASA addition (Fig. 3C). Only 4 of 28 individuals (subjects 217, 250, 240, and 253) lacked a significant inhibition with ex vivo ASA addition to blood obtained before in vivo ASA ingestion. In contrast, only 7 of 28 individuals displayed sensitivity to 500 μmol/L ASA addition ex vivo 24 h after ASA ingestion (Fig. 3C).

MICROFLUIDIC ASSAY FOR MEASURING PLATELET DEPOSITION IN THE PRESENCE OF ASPIRIN

For the 28 healthy donors with platelet counts within reference intervals, no correlation between the extent of platelet deposition on collagen and platelet count

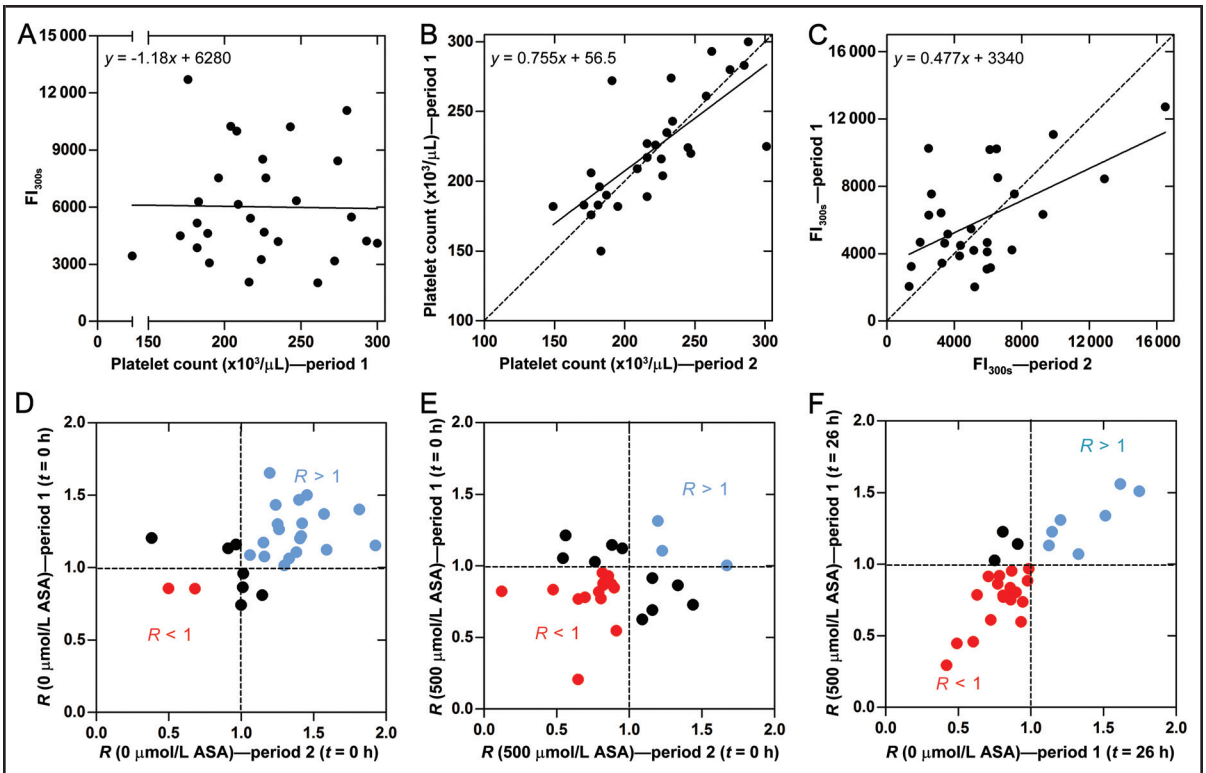

Fig. 4. Microfluidic assay characterization.

(A), Final thrombus size without ASA at $t = 300$ s vs donor platelet count in period 1 (no ASA ingestion). (B), Donor platelet count in period 1 vs donor platelet count in period 2 after 2-week ASA washout period. (C), Final thrombus size without ASA at $t = 300$ s in period 1 vs final thrombus size without ASA at $t = 300$ s in period 2. (D), *R* value for secondary platelet aggregation (no ASA ingestion; no ex vivo added ASA) in both periods. (E), *R* value for high-dose ex vivo ASA addition inhibition of secondary platelet aggregation in periods 1 and 2 (no ASA ingestion). (F), *R* value for high-dose ex vivo ASA vs zero ex vivo dose ASA in period 1 in blood exposed in vivo to ASA (ASA ingestion at 2 h).

was observed (Fig. 4A). Individual platelet counts were highly correlated between period 1 and period 2, indicating the ability of the body to control total platelet count (Fig. 4B). Total platelet deposition (FI_{300s}) showed a weak but positive correlation between period 1 and period 2 (Fig. 4C).

Taking the *R* value as a normalized metric of secondary aggregation for each individual, 22 of 28 individuals had $R > 1$ in period 1 and 23 of 28 individuals had $R > 1$ in period 2 (Fig. 4D). Interestingly, only 2 of 28 individuals had $R < 1$ in both trial periods (Fig. 4D), suggesting that almost all donors have a secondary aggregation response (i.e., $R > 1$) that was detectable in a microfluidic assay when aspirin was absent. All 28 cohorts as a group had $R = 1.21$ (0.34) and 1.16 (0.22) in period 1, 0 h, and period 2, 0 h, respectively, from 168 determinations of this metric, displaying sensitivity and specificity to score secondary aggregation upon no ex vivo ASA treatment in this microfluidic assay ($P <$ 0.05, n = 28). In contrast, with 500 μmol/L ASA added ex vivo, only 3 of 28 donors displayed $R > 1$ in both period 1 and 2 (Fig. 4E), demonstrating that almost all individuals had platelets that were sensitive to inhibition by ASA. With respect to the in vivo activity of ASA, 21 of 28 individuals had $R < 1$ at 24 h after ingestion of a 325-mg dose (no ASA added ex vivo) (Fig. 4F). In these individuals, the addition of 500 μmol/L ASA ex vivo did not further inhibit platelet function, since the platelets had already been exposed to ASA in vivo. Interestingly, 7 of 28 individuals who had $R > 1$ following ASA ingestion also displayed insensitivity following ex vivo ASA addition (Fig. 4F).

An ROC analysis (see online Supplemental Figs. S3 and S4) indicated that a cutoff of $R = 1$ resulted in a true-positive rate of 71% (sensitivity) and a false-positive rate of 36% (or 64% specificity) when comparing donors before and after ASA ingestion. Similarly, when comparing donors before and after ex vivo ASA

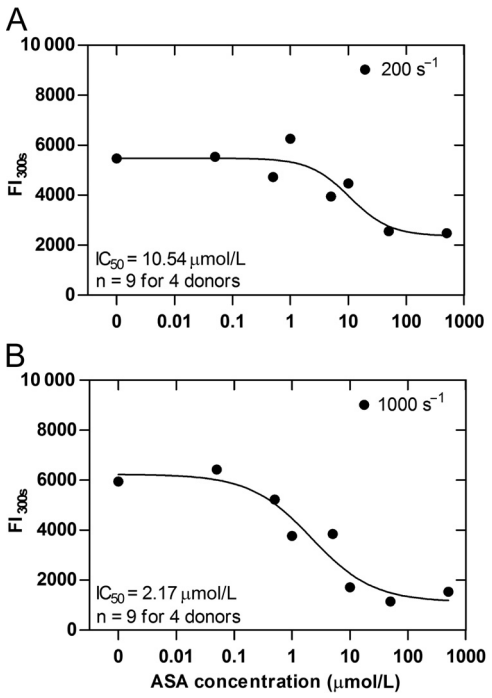

Fig. 5. Response to ex vivo ASA dosing at venous and arterial wall shear rate.

Representative IC_{50} curves for ASA at venous (A) and arterial (B) shear rates (4 donors). A total of 9 microfluidic assays were run at each shear rate for the 4 donors.

addition, the $R = 1$ cutoff resulted in a true-positive rate of 75% (sensitivity) and a false-positive rate of 33% (or 67% specificity).

EFFECT OF ASA AT ARTERIAL FLOW CONDITIONS

ASA is used to reduce the incidence of arterial thrombosis. In the microfluidic assay, we have observed that arterial shear rates of 1000–2000 s^{-1} cause unstable thrombi with pronounced embolism, especially when thrombin or fibrin is absent *(19)*. This is especially relevant for experiments conducted at constant flow rate (as opposed to constant pressure drop), in which clot growth can cause high shear stresses to occur as the clot partially occludes the flow path. To investigate the pharmacological potency of ASA at arterial shear rates, IC_{50} curves were calculated with the microfluidic device from platelet accumulation with collagen under the influence of ASA at 1000 s^{-1}. Comparison of the same 4 donors who did not participate in the study protocol previously mentioned at both venous (Fig. 5A) and arterial (Fig. 5B) shear rates indicated the efficacy of ASA under both conditions. The slightly lower value of the IC_{50} at 1000 s^{-1} compared to that observed at venous shear rate, although not significantly different, may be due to the reduced concentrations of TXA_2 in the boundary layer at higher shear rates *(14)*. Further work will be necessary to determine if the IC_{50} actually depends on wall shear rate.

Discussion

We demonstrated that microfluidic devices allow the generation of an aspirin dose–response curve under flow conditions based on final platelet fluorescence (FI_{300s} in Fig. 2A), platelet deposition rate between 150 and 300 s ($F'_{300-150s}$ in Fig. 2B), or the R value, which is a normalized metric of secondary aggregation (Fig. 2C). For each of these metrics, the IC_{50} of aspirin was between 10 and 20 μmol/L, which is quite consistent with the known $K_d = 4.5$ μmol/L of ASA against COX-1 *(20)*. The effect of COX-1 inhibition in donors observed in this assay after 150 s is consistent with previous findings in the same device and a multiscale neural network and Monte Carlo simulation of platelet deposition under flow *(14)*. Furthermore, this onset of action of ASA is consistent with aggregometry results showing action of COX-1 inhibitors at the later stages after primary aggregation *(21)*. The assay partially detected the in vivo efficacy of ASA ingestion on platelet COX-1 when whole blood was tested ex vivo (Fig. 3A). Lack of statistical significance of in vivo modulation of platelet COX-1 activity due to ASA ingestion in 12 of the 28 individuals tested could be attributed to varying interdonor functionality of thromboxane, $P2Y_1$, and $P2Y_{12}$ receptors. Interdonor changes in $P2Y_1$ and $P2Y_{12}$ receptor response modulate ADP flux from the growing thrombus under flow conditions, causing variations in total platelet accumulation and the R value. Healthy individuals who ingested 325 mg ASA displayed a mean of 45% smaller platelet deposits when the blood was tested 24 h after dosing (Fig. 3B). Whereas healthy donors have platelets that display thromboxane-dependent secondary aggregation in the flow assay (only 2 of 28 donors had $R < 1$) (Fig. 4D), healthy donors have platelets that respond to ASA administered ex vivo (only 3 of 28 donors had $R > 1$ in duplicate tests of 500 μmol/L ASA added ex vivo) (Fig. 4E).

The microfluidic assay brings together contributions of platelet collagen signaling, granule release, ADP signaling, thromboxane synthesis, and adhesion strength under flow conditions. Thus, the pharmacological effects of COX-1 inhibition were investigated under far more complex conditions than an assay that measures thromboxane synthesis. Although the effects of aspirin ingestion or ex vivo aspirin addition are clearly detectable in our assay, the capabilities of this

microfluidic assay to detect "aspirin resistance" for patients with cardiovascular diseases remains unknown, as this study was limited to healthy volunteers.

Several platelet function tests currently exist; however, these technologies to assess aspirin sensitivity in whole-blood methodologies have limitations, especially with respect to running a dose–response assay requiring multiple tests. Dose–response assays are a fundamental pharmacological tool to quantify drug potency or remaining enzyme activity. PFA-100 and VerifyNow are tests used to assess inhibition of platelet function by aspirin using whole blood. PFA-100 deploys a cartridge to create flow and measure puncture closure time with an activating coating of collagen/ADP or collagen/epinephrine *(22)*. The assay is highly dependent on plasma von Willebrand factor, high shear stress, and epinephrine, thus presenting difficulties in isolating the inhibitory effects of aspirin *(23)*. VerifyNow Aspirin is a point-of-care assay using whole blood in standard cartridges containing a preparation of human fibrinogen-coated beads and arachidonic acid as agonists *(22)*. VerifyNow Aspirin is an assay based on light transmittance but lacks a hemodynamic flow field. Most recently, development of a perfusion chamber allows for study of ASA-dependent changes in platelet deposition *(24)*. Our methodology is distinct from that of Stephens et al. *(24)*, where whole blood was perfused through an entire glass capillary coated with human type III fibrillar collagen at 1000 or 1500 s^{-1}. A collagen-coated capillary creates a distance of many centimeters of collagen for platelets to activate, adhere, embolize, and recapture on the surface. Platelet deposition is a function of distance from the entrance in this assay, due to boundary-layer depletion of platelets over the unusually long length of collagen. Because only 1 capillary is run at a time, dose–response testing is particularly cumbersome. In contrast, our 8-channel microfluidic perfusion of whole blood over fibrillar collagen exposes platelets to a spatially confined 250-micron long "injury" site where all the data are collected (and there is no dependence on distance down the channel). The 8-channel microfluidic device facilitated the ex vivo testing of antiplatelet drugs in a dose-dependent manner in a large cohort of healthy donors.

The biology of thrombosis includes the interaction of platelets with potent vessel wall-derived stimuli including collagen and thrombin. As a layer of platelets becomes activated on collagen, they recruit additional platelets via release of ADP and TXA_2. By targeting the secondary wave of platelet recruitment with $P2Y_{12}$ antagonists and/or COX-1 inhibitors, an antithrombotic effect is achieved, with reduced risk of bleeding in comparison to other antithrombotic therapies that target biologically active clotting factors, such as warfarin. Few assays recreate the sequential process of collagen adhesion followed by the release of autocrine factors. Because platelet recruitment, adhesion, and the convective diffusion of autocrine agents are dictated by prevailing hemodynamic conditions *(14)*, microfluidic assays partially recreate the disease environment in which antiplatelet agents must act. We demonstrate the use of the microfluidic assay to evaluate healthy individual response to aspirin.

Supplemental information about this study, including additional discussion topics and cases, is presented in the online Supplemental Data.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: T. Grosser, PLx Pharma.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: R. Li, University of Pennsylvania Institute for Translational Medicine and Therapeutics; X. Li, the American Heart Association Postdoctoral Fellowship AHA12POST11890008; T. Grosser, University of Pennsylvania Institute for Translational Medicine and Therapeutics; S.L. Diamond, University of Pennsylvania Institute for Translational Medicine and Therapeutics, the NIH National Center for Advancing Translational Sciences, grant UL1TR000003, R01 HL-103419.
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: The authors acknowledge Thomas V. Colace (University of Pennsylvania) for his advice and expertise in microfluidic flow assays.

References

1. Patrono C, Coller B, FitzGerald GA, Hirsh J, Roth G. Platelet-active drugs: the relationships among dose, effectiveness and side effects. Chest 2004; 126:234s–64s.
2. Campbell CL, Smyth S, Montalescot G, Steinhubl SR. Aspirin dose for the prevention of cardiovascular disease. JAMA 2007;297:2018–24.
3. Vane JR, Botting RM. The mechanism of action of aspirin. Thromb Res 2003;110:255–8.
4. Michelson AD, Frelinger LA, Furman. MI. Resistance to antiplatelet drugs. Eur Heart J Supp 2006;8(Suppl G):G53–8.
5. Fries S, Grosser, T. The cardiovascular pharmacology of COX-2 inhibition. Hematology Am Soc Hematol Educ Program 2005;445–51.
6. Grosser T, Yu, Y, FitzGerald, G. Emotion recollected in tranquility: lessons learned from the COX-2 saga. Annu Rev Med 2010;61:17–33.
7. Michelson AD. Platelet function testing in cardiovascular diseases. Circulation 2004;110:e489–93.
8. Michelson AD, Canntaneo M, Ekielboom JW, Gur-

bel PA, Kottke-Merchant K, Kunicki TJ, et al. Aspirin resistance: position paper of the Working Group on Aspirin Resistance. J Thromb Haemost 2005;3:1309–11.
9. Bhatt DL. Aspirin resistance: more than just a laboratory curiosity. J Am Coll Cardiol 2004;43: 1127–9.
10. Cattaneo M. Aspirin and clopidogrel: efficacy, safety and the issue of drug resistance. Arterioscler Thromb Vasc Biol 2004;24:1980–7.
11. Cuisset T, Frere C, Quilici J, Gaborit B, Bali L, Poyet R, Faille, D, et al. Aspirin noncompliance is the major cause of 'aspirin resistance' in patients undergoing coronary stenting. Am Heart J 2009; 157:889–93.
12. Schwatz KA, Schwatz DE, Barber K, Reeves M, De Franco AC. Non-compliance is the predominant cause of aspirin resistance in chronic coronary arterial disease patients. J Transl Med 2008;6:46.
13. Hirata T, Kakizuka A, Ushikubi F, Fuse I, Okuma M, Narumiya S. Arg 60 to Leu mutation of the human thromboxane A2 receptor in a dominantly inherited bleeding disorder. J Clin Invest 1994;94: 1662–7.
14. Flamm MH, Colace TV, Chatterjee MS, Jing H, Zhou S, Jaeger D, Brass LF, Sinno T, Diamond SL. Multiscale prediction of patient specific platelet function under flow. Blood 2012;120:190–8.
15. Frelinger A, Li Y, Linden M, Barnard M, Fox M, Christie, D, et al. Association of cyclooxygenase-1-dependent and -independent platelet function assays with adverse clinical outcomes in aspirin-treated patients presenting for cardiac catheterization. Circulation 2009;120:2586–96.
16. Reny J, Berdaque P, Poncet A, Barazer I, Nolli S, Fabbro-Peray P, et al. Antiplatelet drug response status does not predict recurrent ischemic events in stable cardiovascular patients: results of the Antiplatelet Drug Resistances and Ischemic Events study. Circulation 2012;125:3201–10.
17. Chatterjee MS, Purvis, JE, Brass LF, Diamond SL. Pairwise agonist scanning predicts cellular signaling responses to combinatorial stimuli. Nature Biotechnol 2010;28:727–32.
18. Maloney SF, Brass LF, Diamond SL. $P2Y_{12}$ or $P2Y_1$ inhibitors reduce platelet deposition in a microfluidic model of thrombosis while apyrase lacks efficacy under flow conditions. Integr Bio (Camb) 2010;2:183–92.
19. Colace TV, Muthard RW, Diamond SL. Thrombus growth and embolism on tissue factor-bearing collagen surface under flow: role of thrombin with and without fibrin. Arterioscler Thromb Vasc Biol 2012;32:1466–76.
20. Cryer B, Feldman M. Cyclooxygenase-1 and cyclooxygenase-2 selectivity of widely used anti-inflammatory drugs. Am J Med 1998;104:413–21.
21. Rinder CS, Student LA, Bonan JL, Rinder HM, Smith BR. Aspirin does not inhibit adenosine diphosphate-induced platelet alpha-granule release. Blood 1993;82:505–12.
22. Lordkipanidze M, Pharand C, Schampaert E, Turgeon J, Palisaitis DA, Diodati JG. A comparison of six major platelet function tests to determine the prevalence of aspirin resistance in patients with stable coronary artery disease. Eur Heart J 2007; 28:1702–8.
23. Hankey GJ, Eikelboom JW. Aspirin resistance. Lancet 2006;367:606–17.
24. Stephens G, He M, Wong C, Jurek M, Luedemann HC, Shapurian G. Development of a perfusion chamber assay to study in real time the kinetics of thrombosis and the antithrombotic characteristics of antiplatelet drugs. Thrombosis J 2012;10: 1–11.