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ORIGINAL RESEARCH

Influence of Sex on Platelet Reactivity in Response to Aspirin

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BACKGROUND: There are sex differences in the efficacy and safety of aspirin for the prevention of myocardial infarction and stroke. Whether this is explained by underlying differences in platelet reactivity and aspirin response remains poorly understood.

METHODS AND RESULTS: Healthy volunteers (n=378 208 women) and patients with coronary artery disease or coronary artery disease risk factors (n=217 112 women) took aspirin for 4 weeks. Light transmittance aggregometry using platelet-rich plasma was used to measure platelet reactivity in response to epinephrine, collagen, and ADP at baseline, 3 hours after the first aspirin dose, and after 4 weeks of daily aspirin therapy. A subset of patients underwent pharmacokinetic and pharmacodynamic assessment with levels of salicylate and cyclooxygenase-1–derived prostaglandin metabolites and light transmittance aggregometry in response to arachidonic acid and after ex vivo exposure to aspirin. At baseline, women had increased platelet aggregation in response to ADP and collagen. Innate platelet response to aspirin, assessed with ex vivo aspirin exposure of baseline platelets, did not differ by sex. Three hours after the first oral aspirin dose, platelet aggregation was inhibited in women to a greater degree in response to epinephrine and to a lesser degree with collagen. After 4 weeks of daily therapy, despite higher salicylate concentrations and greater cyclooxygenase-1 inhibition, women exhibited an attenuation of platelet inhibition in response to epinephrine and ADP.

CONCLUSIONS: We observed agonist-dependent sex differences in platelet responses to aspirin. Despite higher cyclooxygenase-1 inhibition, daily aspirin exposure resulted in a paradoxical attenuation of platelet inhibition in response to epinephrine and ADP over time in women but not in men.

Key Words: aspirin ■ platelets ■ sex differences

Historically, aspirin has been the drug of choice for primary and secondary prevention of coronary artery disease (CAD), myocardial infarction (MI), and stroke. However, in the past several years, the evidence for its efficacy for specific clinical indications has become much more complicated. Three recent large aspirin primary prevention trials[1–3] in patients from several different disease and demographic cohorts showed questionable benefit for prevention of major adverse cardiac events, which was counterbalanced by an increase in bleeding events. This resulted in a 2019 update to the American College of Cardiology/American Heart Association guidelines, which recommended against the routine use of aspirin for primary prevention of CAD.[4] Aspirin continues to have a major role in secondary prevention of major adverse cardiovascular and cerebrovascular events; however, many patients who are prescribed aspirin still experience atherothrombotic and/or bleeding events.[5]

There remains much to be understood about the nuances of aspirin response in specific subgroups of patients. In 2 of the 3 trials mentioned above, subgroup analysis revealed sex differences in outcomes: placebo was associated with a lower risk of mortality in men (but not women) in the ASPREE (Aspirin in Reducing Events in the Elderly) trial of elderly adults,

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CLINICAL PERSPECTIVE

What Is New?

- In an experimental cohort of patients who took aspirin for 4 weeks, cyclooxygenase-1–independent pathways involving agonist-specific platelet response to ADP, epinephrine, and collagen differed between men and women.
- These differences manifest themselves over time and were not present with ex vivo aspirin administration.

What Are the Clinical Implications?

- Future trials of antiplatelet therapy should take into account sex differences in mechanisms of action, absorption, and pharmacokinetics/pharmacodynamics in order to advance towards more personalized medical therapy for patients with cardiovascular disease.

Nonstandard Abbreviations and Acronyms

AA	arachidonic acid
ASCEND	A Study of Cardiovascular Events in Diabetes
ASPREE	Aspirin in Reducing Events in the Elderly
AUC	area under the curve
CAD	coronary artery disease
COX-1	cyclooxygenase-1
DCRU	Duke Clinical Research Unit
DM	diabetes mellitus
MI	myocardial infarction
PFA	platelet function analyzer
PRP	platelet-rich plasma

while in ASCEND (A Study of Cardiovascular Events in Diabetes) of patients with diabetes mellitus (DM), aspirin was associated with a lower rate of revascularization and serious vascular events in men (but not women). Prior clinical trial data indicate that aspirin is effective for the prevention of MI in men and for stroke in women but not vice versa,[6,7] while recent secondary analysis suggests that differences in clinical outcomes of men and women treated with aspirin may be overstated.[8] Evidence for sex differences in risk of bleeding is mixed, with a large meta-analysis showing no difference,[9] but 2 more recent geographic registry studies show higher rates of bleeding in men taking aspirin.[10,11] These trials suggest a reduced antithrombotic effect of aspirin in women compared with men, which motivates a deeper study of sex as a biological factor in the response to aspirin.

Ex vivo platelet aggregation assays can measure the effects of antiplatelet therapy.[12] Light transmittance aggregometry is commonly used to quantify changes in platelet aggregation in response to agonist stimulation. Multiple agonists are used to interrogate the multiple pathways by which antiplatelet agents achieve their effects. These agonists reflect elements of either the cyclooxygenase-1 (COX-1)–specific pathway (arachidonic acid [AA]) or COX-1–independent pathways, eg, ADP, epinephrine, or collagen. After exposure to aspirin, platelet response to AA (ie, the direct COX-1 pathway) is nearly completely suppressed in both men and women. However, COX-1–independent pathways likely play an additional and important role in the vascular biology of atherothrombotic disease.[13]

There is substantial preclinical evidence that there are baseline sex differences in platelet reactivity.[14–19] In addition to the baseline differences, there also appears to be differences in response to aspirin: a series of seminal studies demonstrated that women had higher platelet reactivity in response to ADP, epinephrine, and collagen, both at baseline and after treatment with aspirin 81 mg for 2 weeks[20] via COX-1–independent pathways, which persisted even with the use of high-dose aspirin.[21,22] However, several questions were not addressed in these prior studies: (1) whether there are differences in the acute (single-dose) versus chronic (days to weeks) response to aspirin exposure; and (2) the extent to which pharmacokinetic or pharmacodynamic differences could account for sex differences in aspirin response.

To address these knowledge gaps, we pursued the hypothesis that platelet reactivity in response to aspirin exposure differs between men and women. By studying serial measures of platelet aggregation from participants of an experimental protocol of aspirin exposure, we assessed: (1) the extent to which platelet aggregation in response to specific platelet agonists differs by sex; and (2) the effects of daily aspirin therapy on platelet aggregation by sex, and the extent to which any differences manifest over time.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Participants

This study analyzed samples from 4 cohorts of patients enrolled in a predefined, single experimental aspirin study protocol (Figure 1). Two cohorts, which

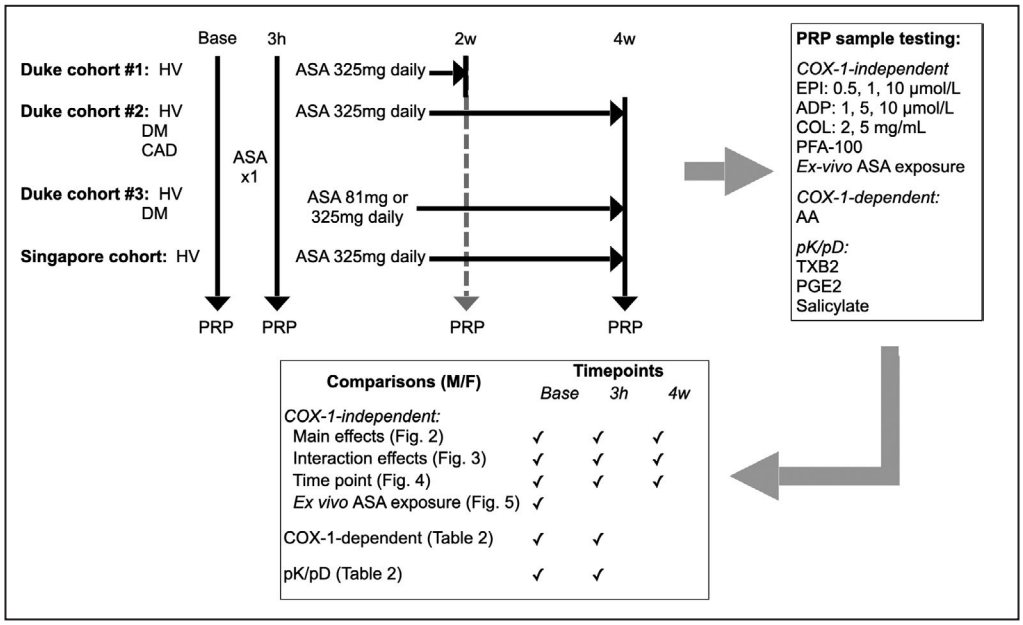

Figure 1. Experimental protocol, sample testing, and comparisons.
Patients in 4 experimental cohorts were exposed to aspirin (ASA) and samples were taken at baseline, 3 hours after ASA exposure, and after 2 or 4 weeks of daily ASA therapy. Platelet-rich plasma (PRP) samples were collected and multiple tests of platelet function were performed as shown. Men and women were compared using statistical models to determine the main effect of ASA treatment and the interaction effect of sex on ASA. Additional comparisons between experimental time points and for other platelet function data were made as shown. AA indicates arachidonic acid; CAD, coronary artery disease, COL, collagen; COX-1, cyclooxygenase-1; DM, diabetes mellitus; EPI, epinephrine; HV, healthy volunteer; M/W, men/women; PFA, platelet function analyzer; PGE2, prostaglandin E2; pK/pD, pharmacokinetic/pharmacodynamic; and TXB2, thromboxane B2.

have been previously described,[23] were recruited at the Duke Clinical Research Unit (DCRU, Durham, NC); a third cohort was also recruited at the DCRU (Data S1); and the fourth cohort was recruited at the SingHealth Investigational Medicine Unit in Singapore (Data S1). We enrolled participants of both sexes and multiple ethnic groups, and the study cohorts included patients with specific disease profiles: healthy volunteers, patients with known CAD, and patients with DM both with and without CAD. The profiles of each cohort were as follows:

1. Duke cohort #1 included healthy volunteers (25 men and 24 women);
2. Duke cohort #2 included healthy volunteers and patients with DM but without CAD, and patients with CAD (90 men and 151 women);
3. Duke cohort #3 included healthy volunteers and patients with DM both with and without CAD (110 men and 130 women);
4. Singapore cohort included healthy volunteers (50 men and 15 women).

Written informed consent was obtained from each patient, and the studies were approved by the Duke University Health System institutional review board and the SingHealth Centralized institutional review board.

Inclusion and Exclusion Criteria

The inclusion and exclusion criteria were nearly identical for all 4 cohorts. Study participants were between the ages of 30 and 75 years; were either healthy or had the requisite preexisting medical comorbidity profile; did not have a history of a bleeding disorder, gastrointestinal bleeding, intracranial bleeding, or known prior gastric ulcer without documented resolution; did not have known severe hepatic impairment; had not undergone surgery within the past 6 months; had not undergone prior gastric bypass surgery that could interfere with study drug absorption; and did not have aspirin allergy or known intolerance to aspirin. Healthy volunteers could not be taking any prescription medications other than oral contraceptives; could not exhibit regular use of tobacco or nicotine products or any known active use of illicit substances; could not have a known pregnancy or be currently breastfeeding; and could not have previously participated or have had a first-degree relative who participated in prior aspirin

studies. Patients in the diabetic and CAD cohorts were defined as having hypertension and/or hyperlipidemia based on the use of prescription antihypertensive and lipid-lowering medications, respectively. Patients who were already taking aspirin underwent a 4-week washout period, with the approval of their primary care physician before enrollment in the study.

Treatment Groups

All patients were studied under similar experimental protocols developed centrally to ensure compliance with prescribed doses including witnessed first and last doses, pill counts, and medication diaries. Aspirin doses were swallowed. Each study involved administration of open-label aspirin supplied by the study investigators as follows:

1. In Duke cohort #1, patients were given aspirin 325 mg daily for 2 weeks;
2. In Duke cohort #3, patients were given either aspirin 81 mg or 325 mg daily for 4 weeks, then were given the other aspirin dose for 4 weeks; and
3. In Duke cohort #2 and the Singapore cohort, patients were given aspirin 325 mg daily for 4 weeks.

Sample Collection and Safety Monitoring

Patients in each cohort had samples of platelet-rich plasma (PRP) collected at each study visit. At visits in which aspirin was initiated, patients had a baseline blood draw and then a second blood draw 3 hours after administration of the study drug. Demographic data including age, race, sex, vital signs, comorbid diseases, concomitant medications, timing of drug administration and blood draws, and determination of adherence to the study drug (as measured by self-reported missed doses as well as pill counts performed by study personnel) were collected at baseline and were updated at each visit. Monitoring of adverse events including bleeding, gastrointestinal discomfort, or respiratory difficulty was performed.

Sample Analysis

The protocol for measurement of platelet reactivity has been previously described.[23] Briefly, PRP samples were analyzed with light transmittance aggregometry using the method initially described by Born[24] to measure ex vivo platelet reactivity in response to epinephrine (0.5 μmol/L, 1 μmol/L, and 10 μmol/L), collagen (2 mg/mL and 5 mg/mL), and ADP (1 μmol/L, 5 μmol/L, and 10 μmol/L). Platelet agonists from the same source (Chronolog Corporation) were used at all sites. Area under the curve (AUC) was used as the primary measure of aggregation because the AUC captures several features of the aggregometry curve, including slope, maximal aggregation, and final aggregation. Higher AUC values reflect higher maximal and residual aggregation, slope, and velocities. To standardize AUC measurements across individuals and visits, we fixed the test duration at 12 minutes for epinephrine and 6 minutes for collagen, AA, and ADP. Additional testing was performed on whole blood samples in the Duke cohorts with the platelet function analyzer (PFA)-100 (Siemens Healthineers), which uses collagen and epinephrine as agonists. Finally, samples from Duke cohort #3 were additionally tested using light transmittance aggregometry after the addition of aspirin to the samples ex vivo at a concentration of 53 μmol/L, higher than the level achieved in vivo with 325 mg of oral aspirin,[25,26] for 10 minutes at room temperature.

Salicylate Concentrations

We adapted an enzyme immunoassay qualitative salicylate screening assay (Salicylates ELISA kit #133615; NeoGen Corporation) to measure salicylate levels from banked serum samples collected 3 hours after a witnessed aspirin dose in Duke cohort #3. The assay was run in accordance with NeoGen package insert instructions. Briefly, a salicylic acid (Sigma Aldrich cat #247588; Merck KGaA) standard solution at 1 mg/mL in methanol was used to generate serial dilutions with enzyme immunoassay buffer to cover a 0.0 μg/mL to 100.0 μg/mL range. A 4-parameter standard curve for best fit was used to estimate salicylate concentrations.

Oxylipin Measurements

Oxylipin profiling of PRP samples from Duke cohort #3 was used to measure prostaglandin metabolites before and after aspirin exposure using a custom assay built at the Duke Proteomics and Mass Spectrometry Shared Resource, which includes light chromatography-mass spectrometry structure-specific transitions for over 100 so-called oxylipin molecules (Waters Corporation). The performance metrics for this assay allow quantification for most analytes down to the 100 pg/mL range in 200 μL of plasma. Low, mid, and high plasma quality control standards at levels of 100 pg/mL, 1000 pg/mL, and 10 000 pg/mL for 40 compounds spanning all compound classes were prepared and utilized across all plates. Global and pooled study quality control samples were used to account for any batch effects. Included in this assay are 19 stable-isotope internal standards across all compound classes and 40 authentic standard curves. For oxylipin analysis, 200 μL of PRP were diluted with a solid-phase

extraction buffer containing butylated hydroxytoluene as an antioxidant as well as internal standards, and oxylipin molecules isolated by solid-phase extraction. Samples were dried in the presence of glycerol and resuspended in 50 µL acetonitrile/methanol and analyzed by electrospray ionization light chromatography-mass spectrometry with the same instrumentation as above. Molecules without a standard curve were quantified against the standard curve of their closest-eluting biosimilar compound. Once the raw data were acquired, peak integration and quantification against calibration curves was performed using Targetlynx software (Waters Corporation).

Statistical Analysis

Statistical Approach

Because each study cohort was designed to identify patients based on specific characteristics (eg, race and comorbidities), these factors were strongly (ie, perfectly) confounded with the cohort. As a result, a meta-analysis approach was used as this was felt to be more appropriate for making inferences about sex differences while controlling for important confounding factors. To account for multiple hypothesis testing, we used the Benjamini-Hochberg procedure with a lenient false discovery rate of 10% given the exploratory nature of our study. Bonferroni correction was not possible as the comparisons were not independent of each other.

Baseline Characteristics

Summary demographic data were collected at the time of enrollment and compiled within each study. Median and interquartile range of age, mean, and SD of body mass index, and percentages of patients of different races, in different disease cohorts (healthy, CAD, or DM), and with comorbidities (hypertension and hyperlipidemia) were calculated within each study.

Platelet Reactivity

Platelet reactivity was modeled as a function of sex and the interaction of treatment and sex. This linear mixed-effects model was fit using the maximum likelihood within each cohort. Parameter estimates and standard errors were combined across cohorts using the inverse-variance weighting method for hypothesis testing. Modeling was performed for each platelet agonist and concentration (including PFA-100). Additional subgroup analyses were performed to compare platelet reactivity at baseline versus in ex vivo aspirin-treated samples; at baseline versus at 3 hours after the first dose of aspirin; and at 3 hours after the first dose of aspirin versus after 4 weeks of therapy. The models controlled for age, hypertension, hyperlipidemia, CAD, DM, peripheral artery disease, race (fixed effects), and patient (random effect) for each agonist.

Oxylipin and Salicylate Data

Metabolites for which the smallest within-treatment group out-of-range rate was <40% and for which the replicate sample-pool coefficient of variation was <30% were retained for further analysis. Out-of-range values were imputed based on lower and upper detection limits. Metabolite-level batch scale factors were estimated and applied to achieve equal replicate sample-pool means between batches. Metabolites thromboxane B2 and prostaglandin E2 were selected from this panel to assess the activity of platelet cyclooxygenase activity. Similarly, salicylate concentration was log-transformed and batch-standardized such that the mean of each experimental batch was the same across all batches. Linear mixed effect models were used to fit the model drug levels as a function of treatment, sex, the interaction of treatment and sex, and body mass index, while controlling from within-patient repeated measures. Linear hypothesis testing was used to estimate the sex- or treatment-stratified effects and *P* values.

RESULTS

Baseline Characteristics

The baseline characteristics of patients in the 4 cohorts are shown in Table 1. There were expected demographic differences between groups based on the differing recruiting strategies as well as the respective populations of North Carolina and Singapore. The Singapore cohort had fewer women than the 3 Duke cohorts, and while the 3 Duke cohorts were composed of primarily black and white patients, the Singapore study was made up entirely of patients of Chinese descent. Patients were adherent to the aspirin regimen: 6.2% of patients missed a single dose during the study, and no patients reported missing >1 dose.

Variation in Baseline Platelet Reactivity by Sex at Baseline and on Aspirin

Baseline differences in platelet reactivity are displayed in Figure 2 and Table S1. Women had higher platelet aggregation levels in response to ADP at the lower concentrations ($P=2.5\times10^{-5}$ and $P=0.035$ for 1 µmol/L and 5 µmol/L, respectively) but no significant differences were seen at the highest

Table 1. Baseline Characteristics of Study Patients

	Duke Cohort #1		Duke Cohort #2		Duke Cohort #3		Singapore Cohort		Overall	
	Men	Women	Men	Women	Men	Women	Men	Women	Men	Women
No.	25	24	90	151	110	130	50	15	275	320
Age (IQR)	24.2 (2)	29.7 (18)	48.9 (16)	48.3 (22)	56.5 (24.8)	50.3 (17)	35.8 (12.3)	38.2 (20.5)	47.3 (23)	47.2 (21)
BMI (SD)	25.1 (2.9)	26.3 (5.7)	32.3 (7.4)	30.9 (7.9)	29.0 (5.6)	30.8 (7.3)	24.5 (5.4)	22.2 (3.6)	28.4 (7.6)	29.9 (7.6)
Race, %										
White	68.0	58.3	45.6	43.0	76.4	46.9	0	0	51.6	43.8
Black	16.0	20.8	46.7	46.4	20.9	50.0	0	0	25.1	43.8
Asian	12.0	16.7	1.1	3.3	2.3	1.8	100	100	20.4	8.4
Other	4.0	4.2	7.8	5.3	0.9	0.8	0	0	3.0	4.0
Cohort, %										
Healthy	100	100	62.2	72.8	35.5	45.4	100	100	61.8	65.0
CAD	0	0	0	0	34.5	7.7	0	0	13.8	3.1
DM	0	0	37.8	27.2	30.0	46.9	0	0	24.4	31.0
Comorbidities, %*										
Hypertension	0	0	12.2	16.6	49.1	40.8	0	0	23.6	24.7
Hyperlipidemia	0	4.0	14.4	13.9	43.6	33.8	0	0	22.5	20.3

Twenty-eight patients included in the individual cohort totals had missing sex data and were not included in the sex differences analyses. BMI indicates body mass index; and IQR, interquartile range.

*Patients were divided into healthy, coronary artery disease (CAD), and diabetes mellitus (DM) subcohorts; they were excluded from the CAD cohort if they had DM and vice versa. Patients were not excluded from cohorts based on the presence of hypertension or hyperlipidemia.

concentration (10 µmol/L). For collagen, women had higher platelet aggregation at 5 mg/mL (P=0.018) but not at 2 mg/mL. There were no significant sex differences in platelet aggregation in response to epinephrine or PFA closure time. Use of an alternative statistical analysis using a censored linear regression (tobit) model for PFA closure time (which has a maximum value of 300) did not alter this result.

On average, women had significantly higher levels of platelet aggregation at all concentrations of ADP (P=9.1×10^{-4}, P=0.036, and P=0.020 for 1 µmol/L, 5 µmol/L, and 10 µmol/L, respectively) (Figure 2) on aspirin. There were no significant sex differences in platelet aggregation in response to epinephrine or collagen or PFA closure time. Using the same tobit model mentioned above for PFA closure time for patients taking aspirin, women had significantly shorter PFA closure times (ie, increased platelet aggregation; P=0.0049).

Therefore, both at baseline and after aspirin exposure, women had higher aggregation compared with men in response to ADP. Platelet function was similar, however, between men and women at baseline and on aspirin using epinephrine and collagen in both isolated platelets and in whole blood.

Platelet Reactivity Changes in Response to ASA Exposure

To determine whether change in platelet reactivity in response to aspirin exposure differed by sex, we tested the interaction of aspirin and sex on platelet reactivity measures (Figure 3 and Table S2). Aspirin inhibited platelet aggregation in both sexes, for every platelet agonist at every concentration. Women had significantly enhanced platelet inhibition (ie, a larger relative reduction in platelet aggregation) compared with men for low concentrations of epinephrine (0.05 µmol/L and 1 µmol/L, P=2.8×10^{-3} and P=0.031, respectively) but not at 10 µmol/L. For collagen, women had significantly decreased platelet inhibition (ie, a smaller relative reduction in platelet aggregation) for both concentrations (2 mg/mL and 5 mg/mL, P=0.023 and P=6.8×10^{-4}, respectively) compared with men. No significant differences were seen for ADP at any of the tested concentrations.

Therefore, the change in platelet reactivity in response to aspirin exposure reveals sex differences for epinephrine (enhanced platelet inhibition for women) and collagen (diminished platelet inhibition for women). Consistent with the higher baseline and on-treatment platelet aggregation, the change in ADP measures was equivalent between sexes.

Innate Platelet Response to Aspirin

To assess whether men and women differ in their innate baseline ability to respond to aspirin, we used 2 complementary approaches: (1) the response to a single witnessed aspirin dose and (2) the response to the ex vivo addition of aspirin to baseline platelet samples.

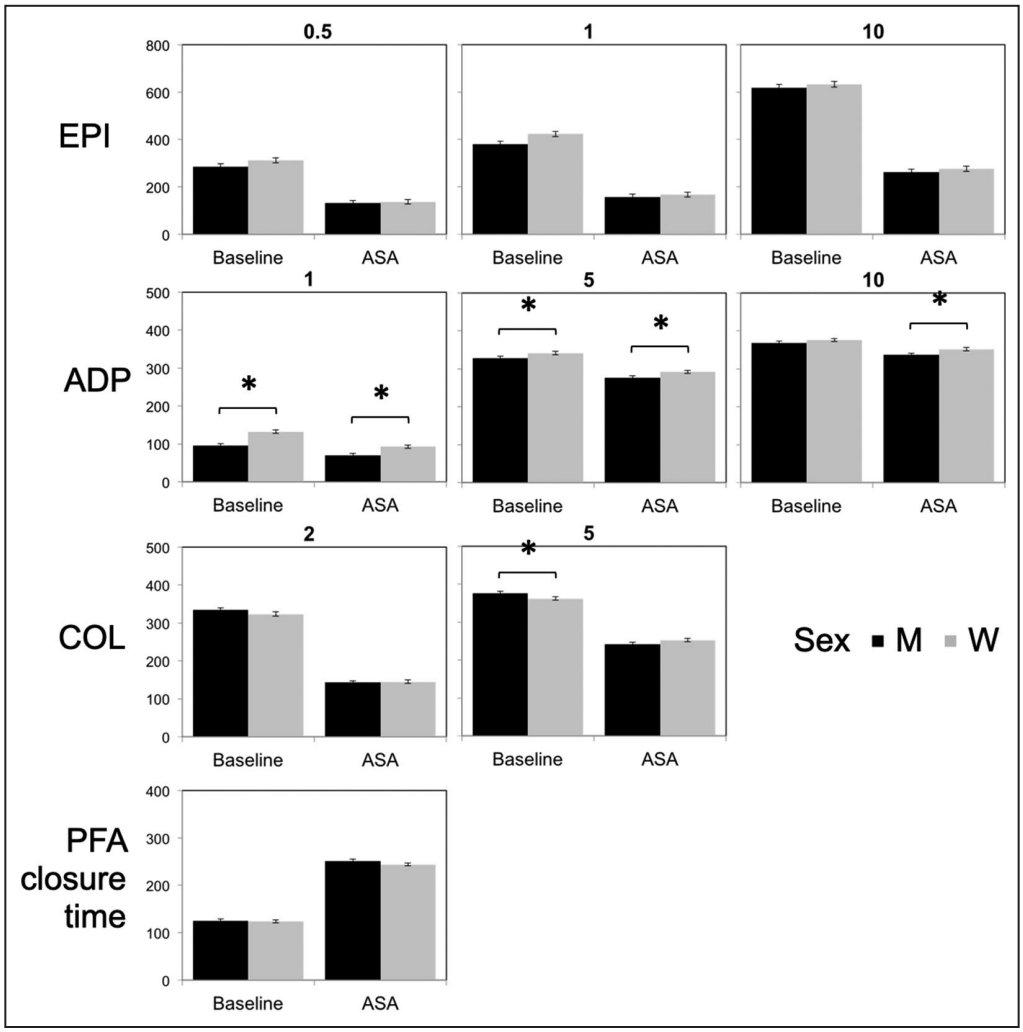

Figure 2. Sex differences in platelet aggregation both at baseline and after treatment with aspirin (ASA).
Values for epinephrine (EPI), ADP, and collagen (COL) are expressed as standardized mean area under the curve of light transmittance aggregometry with standard error bars. Agonist concentrations are denoted above each frame and are expressed in μmol/L for EPI and ADP and as mg/mL for COL. Significant differences (P<0.05) are noted by an asterisk. The data used to generate this figure are reported in Table S1. M indicates men; PFA, platelet function analyzer; and W, women.

After a single oral aspirin dose, platelet aggregation decreased as expected in both men and women for every agonist and every concentration (Figure 4A and Table S3). For epinephrine at a concentration of 1 μmol/L, women had significantly enhanced platelet inhibition (P=0.014) compared with men and a similar magnitude of enhanced platelet inhibition at the higher concentrations (P=0.16 and P=0.17 for 0.5 μmol/L and 10 μmol/L, respectively), although this did not meet statistical significance. There were no significant differences seen for any of the other agonists and concentrations tested.

To normalize any differences in absorption, distribution, and metabolism of aspirin, we used the ex vivo addition of a fixed concentration of aspirin. Ex vivo aspirin inhibited platelet aggregation for each of the agonists and concentrations tested (ADP 2 μmol/L, collagen 2 mg/mL, and epinephrine 0.5 μmol/L) similarly between men and women (Figure 5 and Table S4).

These results suggest that platelet inhibition resulting from a witnessed dose of non-enteric-coated, oral aspirin is similar between men and women using collagen and ADP, yet, with epinephrine, is enhanced in women. In contrast, when aspirin-naïve platelets were directly exposed to a fixed concentration of aspirin ex vivo, there was no inherent difference by sex in platelet inhibition by aspirin.

Temporal Changes in Platelet Response After Aspirin Administration

To study how platelet responses in men and women change over time, we compared platelet reactivity

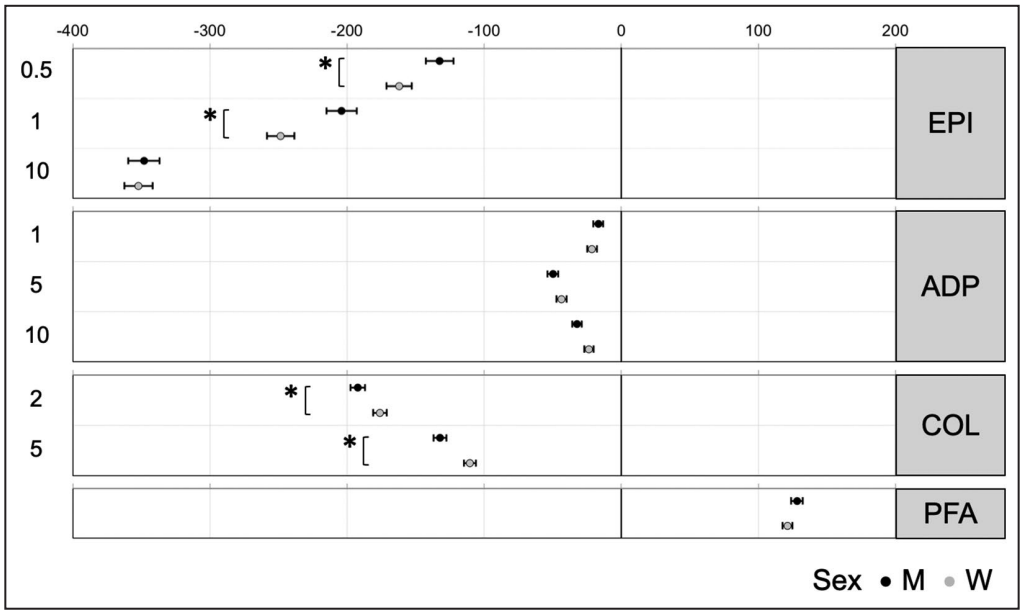

Figure 3. Treatment effect of aspirin stratified by sex, standardized in comparison to baseline platelet aggregation for each agonist, which is set at 0.
Significant sex differences are seen for the change in platelet aggregation in response to platelet agonists epinephrine (EPI) (at 0.5 and 1 μmol/L) and collagen (COL) (at 2 and 5 mg/mL) after treatment with aspirin. Agonist concentrations are denoted on the left and are expressed in μmol/L for EPI and ADP and as mg/mL for COL. Standard error bars are displayed, and significant differences are noted by an asterisk. M indicates men; PFA, platelet function analyzer; and W, women.

3 hours after the first aspirin dose (when COX-1 is suppressed with 325 mg aspirin) with that after 4 weeks of daily aspirin dosing. Comparing the platelet samples taken 3 hours after the first dose of aspirin with samples taken after patients had been taking aspirin daily for 4 weeks, there were no significant differences in platelet aggregation in men for any agonist or concentration (Figure 4B and Table S3).

In women, however, there was a significant increase in platelet aggregation over the time interval for epinephrine at every concentration (P=3.6×10^{-4}, P=7.6×10^{-5}, and P=0.016 for 0.5 μmol/L, 1 μmol/L, and 10 μmol/L, respectively, for the light transmittance aggregometry AUC) and for ADP at the higher concentrations (P=0.026 and P=9.1×10^{-4} for 5 μmol/L and 10 μmol/L, respectively); however, there were no significant differences seen for collagen or for ADP 1 μmol/L.

These results suggest that over the course of several weeks of therapy, the effects of aspirin on platelets as assessed by epinephrine and ADP are attenuated over time in women but remain stable in men.

Pharmacokinetic/Pharmacodynamic Differences by Sex

To assess whether differences in platelet responses to aspirin over time could be explained by differences in aspirin pharmacodynamics, we assessed COX-1 activity using AA-induced platelet aggregation and metabolites that are sensitive to platelet COX-1 activity—thromboxane B2 and prostaglandin E2—in a subset of healthy patients (Table 2). Aspirin, at doses of 81 mg and 325 mg, significantly decreased AA-induced platelet aggregation. There was no difference in AA-induced platelet aggregation with 81 mg of aspirin, but a higher proportion of women achieved effective inhibition of platelet aggregation (ie, <20% of baseline platelet aggregation) at a dose of 325 mg (P=2×10^{-4}). Thromboxane B2 and prostaglandin E2 levels were decreased to similar levels with 81 mg and 325 mg of aspirin with a trend toward higher thromboxane B2 levels in men, which did not reach statistical significance. To assess whether exposure to aspirin was different in men versus women, we measured salicylate concentrations (the stable metabolite of aspirin) 3 hours after their final, witnessed aspirin dose and found that women had higher concentrations of salicylate compared with men after low- and high-dose aspirin (P=0.017 and P=1.3×10^{-3} for 81 mg and 325 mg, respectively).

DISCUSSION

Multiple potential factors, including sex, influence the clinical response to antiplatelet agents. A large body of evidence has shown significant sex differences in

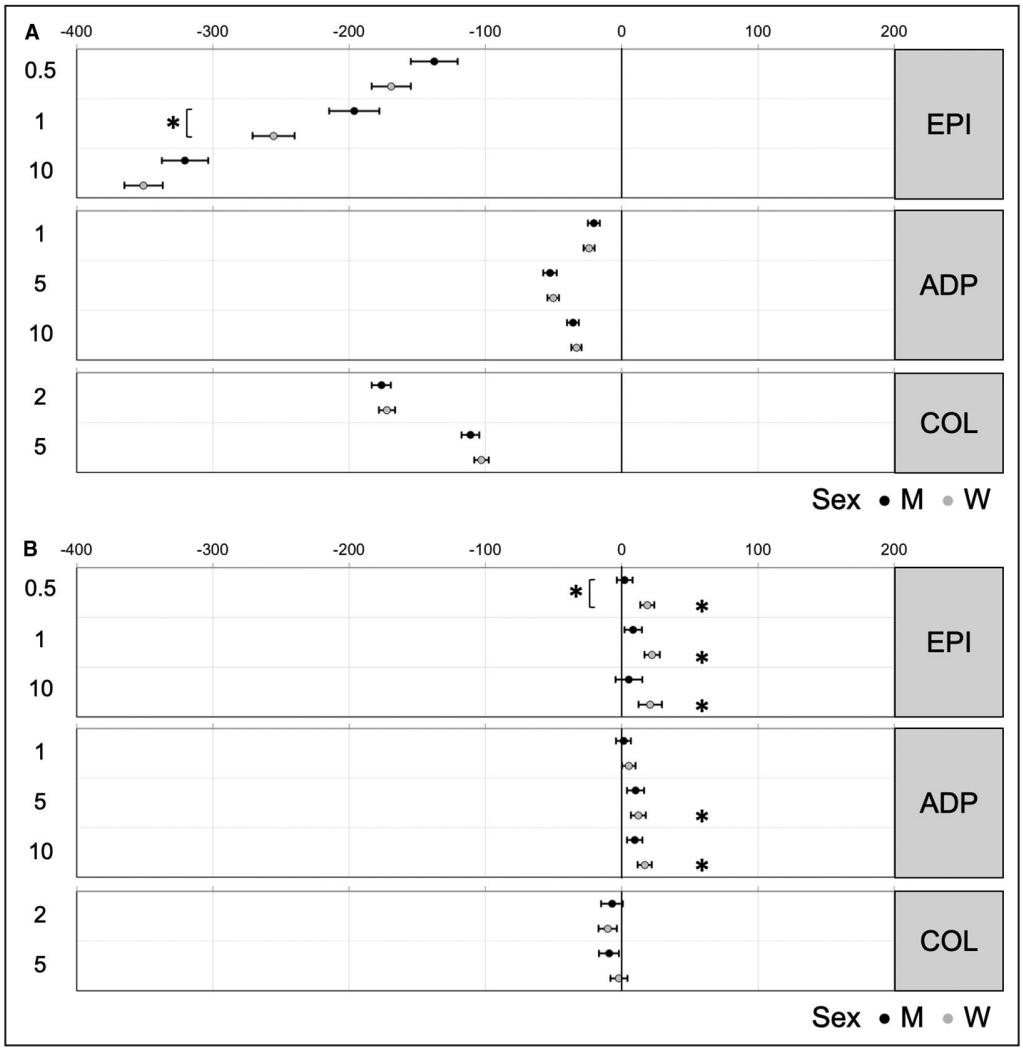

Figure 4. Changes in platelet aggregation during the course of treatment.
A, Platelet aggregation at 3 hours after first aspirin exposure was compared with baseline platelet aggregation, which is standardized and set at 0. Agonist concentrations are expressed in μmol/L for epinephrine (EPI) and ADP and as mg/mL for collagen (COL). Standard error bars are displayed. None of the 95% CIs for the individual values include 0, indicating a significant change in response over the time period. Significant differences between the sexes (P<0.05) are noted with a bracket and asterisk. **B**, Platelet aggregation after 4 weeks of aspirin treatment was compared with platelet aggregation 3 hours after the first aspirin dose, which is standardized and set at 0. Agonist concentrations are expressed in μmol/L for EPI and ADP and as mg/mL for COL. Standard error bars are displayed. Individual values for which the estimated 95% CI does not include 0 (indicating a significant change in response over the time period) are noted with an asterisk. Significant differences between the sexes (P<0.05) are noted with a bracket and asterisk. The data used to generate these figures are reported in Table S3. M indicates men; and W, women.

platelet biology and the response of platelets to antiplatelet agents,[27,28] with higher platelet reactivity in women via multiple COX-1–dependent and COX-1–independent pathways. Because other extrinsic factors such as concomitant medications, medication adherence and as-yet-unknown genomic factors may also affect antiplatelet responses, studying response to antiplatelet medications in an experimental setting is critical. In our study of platelet aggregation measured in purified platelets, we observed significant sex differences both at baseline and in patients treated with aspirin that were specific to the agonist and concentration used to analyze platelet aggregation. Women had higher ADP-induced platelet reactivity at baseline and after aspirin exposure. While the acute and innate platelet responses to ADP were similar between the sexes, there was an attenuation of platelet inhibition over time with daily aspirin dosing in women that was not seen in men. In contrast, with epinephrine, we observed a biphasic response with

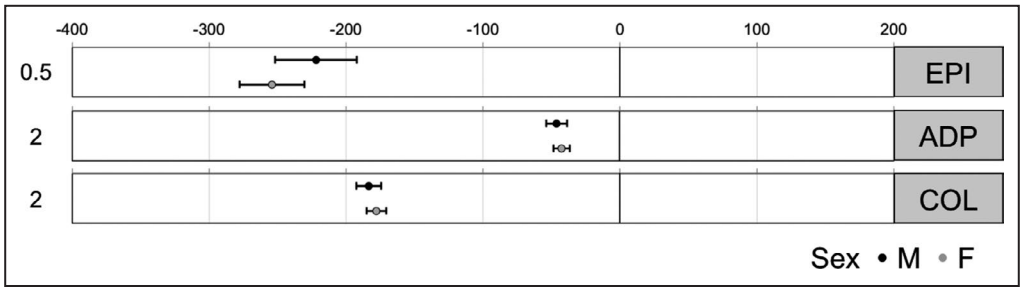

Figure 5. Platelet response to ex vivo aspirin exposure.
Ex vivo aspirin exposure results in a decrease in platelet aggregation, with no significant sex differences. Values are standardized in comparison to baseline platelet aggregation, which is set at 0. Agonist concentrations are denoted above each frame and are expressed in μmol/L for epinephrine (EPI) and ADP and as mg/mL for collagen (COL). Standard error bars are displayed. M indicates men; and W, women.

enhanced acute response to oral aspirin in women followed by a paradoxical rebound in platelet reactivity with daily aspirin dosing. We found no evidence of sex differences in COX-1 inhibition to explain the rebound in platelet reactivity in women versus men. These findings shed light on the temporal response to aspirin and show that the inhibitory effects of aspirin on non–COX-1 measures of platelet function, but not COX-1 inhibition, are attenuated in women over time.

The foundational work in this area was by Becker et al,[20] who showed that women were found to have significantly higher platelet reactivity both before and after treatment, with agonist-dependent sex trends in the change in platelet reactivity after treatment. Of note, in this study, there were differences between whole blood samples (no significant sex differences in the change in platelet reactivity in response to ADP and collagen) and in PRP samples (decreased platelet inhibition in response to ADP and collagen but increased inhibition in response to epinephrine at baseline and after aspirin exposure, which mirrors the relative changes found in our study). The discrepancies between whole blood and PRP analyses may be attributable to factors extrinsic to platelets in nonplatelet blood components.

Our findings build on these initial observations by providing a window into the acute response to oral versus ex vivo aspirin. Based on our finding that oral but not ex vivo aspirin exposure leads to enhanced platelet inhibition in women versus men, we can conclude that any sex differences in circulating, aspirin-naïve

Table 2. Pharmacokinetic and Pharmacodynamic Differences by Sex

	No.	Baseline	*P* Value	Aspirin 81 mg	*P* Value	Aspirin 325 mg	*P* Value
AA							
Women	*	351.9 (39.0)	0.27	92.8%	0.13	85.6%	2×10^{-4}
Men		338.2 (48.1)		86.3%		95.5%	
Thromboxane B2							
Women	138	9.88 (0.81)	0.062	2.33 (0.06)	0.60	2.33 (0.14)	0.060
Men	74	10.68 (0.72)		3.09 (0.83)		2.41 (0.15)	
Prostaglandin E2							
Women	138	5.88 (0.73)	0.21	3.29 (0.51)	0.95	3.29 (0.57)	0.93
Men	74	6.30 (0.53)		3.27 (0.61)		3.32 (0.50)	
Salicylate							
Women	65	1.48 (0.11)	0.19	2.76 (0.14)	0.017	3.98 (0.15)	1.3×10^{-3}
Men	50	1.38 (0.12)		2.54 (0.15)		3.68 (0.13)	

Arachidonic acid (AA)–induced platelet aggregation values at baseline are expressed as standardized mean area under the curve of light transmittance aggregometry (LTA); for aspirin 81 mg and aspirin 325 mg, values are expressed as percentage of patients whose levels were less than the accepted cutoff of <20% of baseline aggregation indicating effective antiplatelet effect for aspirin. Thromboxane B2 and prostaglandin E2 are reported as mean log-transformed pg/mL, and salicylate levels are reported as mean log-transformed μg/mL. Standard error values are in parentheses. *P* values are for chi-square tests of homogeneity of proportions for men and women (AA on-aspirin comparisons) and Welch unpaired *t* tests comparing means between women and men (all other comparisons).

*For the AA measurements, the number varies by aspirin dose: there were 206 baseline samples for women and 191 for men; 138 aspirin 81 mg samples for women and 73 for men; and 245 aspirin 325 mg samples for women and 215 for men.

platelets do not result in a difference in aspirin response. Instead, differences in the acute response likely involve pharmacokinetic factors (ie, absorption, metabolism, or elimination) that lead to higher aspirin exposure in women that are normalized through the ex vivo addition of a fixed concentration of aspirin. This suggests that in settings where acute platelet inhibition with aspirin is important (eg, acute coronary syndromes or percutaneous coronary intervention), intravenous aspirin may be required to ensure similar antiplatelet activity between sexes.

Further, our study provides novel insights into temporal changes in aspirin response. Because aspirin is used chronically to prevent cardiovascular disease, we analyzed platelet response in samples collected in both the acute and chronic phase of aspirin response. In contrast, prior studies in this area only compared samples taken at baseline and after 2 weeks of daily aspirin dosing. We show that platelets from women were more inhibited, primarily in response to epinephrine, at 3 hours after an initial dose of aspirin, but that after an additional 4 weeks of treatment, there was significantly less inhibition, ie, a biphasic response to aspirin. While response to ADP was similar after 3 hours, the same time-dependent attenuation in platelet reactivity was seen in women at 4 weeks. This attenuation of platelet inhibition over time with epinephrine and ADP suggests that there may be physiologic pathways at the megakaryocyte level that feedback and/or reset as the platelet pool turns over during chronic aspirin exposure. We hypothesize that these treatment-emergent pathways may differ between sexes. Previous work examining the effects of aspirin has described attenuation of the inhibition of platelet reactivity in response to ADP after repeated dosing; the treatment intervals in these studies were 8 weeks to 24 months. However, one study enrolled only men[29] and the other did not analyze sex differences in response.[30] Our study clarifies the changes in platelet response to aspirin treatment over time, identifies important sex differences in this response, and suggests that future studies of platelet response to antiplatelet agents should collect data at multiple time points to more fully analyze the temporal nature of response to antiplatelet therapy.

Our findings also provide mechanistic insight into sex differences in aspirin's utility for the prevention of stroke and MI. For example, the increased levels of AA- and epinephrine-induced platelet inhibition in women may be more closely connected to the increased protection aspirin confers against stroke in women. In contrast, higher aggregation at baseline and on treatment and attenuation over time in response to ADP may be more relevant for MI where women seem to derive less benefit from aspirin. The lack of significant sex differences in aspirin pharmacodynamics suggest that the different responses to ADP, collagen, and epinephrine are unlikely to be related to COX-1 inhibition, since, based on our analysis of COX-1–sensitive metabolites, this appears to be sufficiently inhibited regardless of sex. We can conclude that the attenuation of aspirin's effects on platelets over time with epinephrine and ADP in women is neither caused by insufficient aspirin exposure nor an inability of aspirin to inhibit COX-1 in women compared with men.

It is notable that the differences in platelet reactivity seen in our study were absent when samples were tested with high concentrations of platelet agonists. This may indicate that the platelet pathways underlying sex differences in platelet response to aspirin may only be detectable using low platelet agonist concentrations. Indeed, Yee et al[31] have previously shown that using submaximal doses of platelet agonists can identify an unusual hyperreactive platelet response, which interestingly was more common in women. Our findings suggest that future work in this area include low concentrations of platelet agonists.

Despite the strengths of our study, we acknowledge some limitations. We combined multiple cohorts recruited over several years and locations with the goal of improving statistical power. Although we used centrally developed protocols, each cohort was analyzed individually and results were combined using meta-analysis; our approach may have increased heterogeneity in the results. Additionally, in our primary analysis we did not account for menstrual cycles, whether women were taking hormonal contraception, or differences in body mass index, which may influence the pharmacokinetics of aspirin. While most patients take antiplatelet agents chronically, we evaluated patients who were treated with aspirin for only 4 weeks; as such, we do not know to what extent the temporal differences identified in our study would persist or expand over time. Last, more than half of the aspirin exposures were at a dose of 325 mg/d, with the rest of the exposures at 81 mg/d. Therefore, the extent to which our findings extend to low-dose aspirin are not known.

CONCLUSIONS

We have identified agonist-specific sex differences in platelet response to aspirin, which were not present with ex vivo aspirin administration. These findings have important clinical implications for the route of administration of aspirin (oral versus intravenous) in situations in which potent and rapid platelet inhibition is desired (eg, acute MI). In addition, we describe sex-specific attenuation of aspirin's inhibition of epinephrine- and ADP-induced platelet aggregation after 4 weeks of therapy in women, which may contribute to differences in outcomes between men and women treated with antiplatelet therapy for the prevention of cardiovascular

and cerebrovascular disease. Further study of these differences in clinical trials of antiplatelet therapy and continued attention to sex differences in mechanisms of action, absorption, and pharmacokinetics/pharmacodynamics will be critical to advancing our understanding of cardiovascular disease treatment and achieving the goal of personalized medical therapy, especially for women.[32]

ARTICLE INFORMATION

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Disclosures

None.

Supplementary Materials

Data S1
Tables S1–S4
Reference 32

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SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

Study protocols for Duke cohort #3 and Singapore cohort

Duke cohort #3

The Duke Clinical Research Unit (DCRU, Durham, NC, USA) performed an aspirin and ticagrelor challenge study to examine gene expression profiles in healthy volunteers and patients with type 2 diabetes. Patients were recruited from January 2014 to June 2016.

Study protocol

Study cohorts. This antiplatelet exposure study used two separate cohorts: 1) healthy adult volunteers 2) patients with Type 2 diabetes.

Prior aspirin use and aspirin washout. For patients at risk for cardiovascular disease who met inclusion/exclusion criteria and who consented to the study, a letter was sent to the patient's physician requesting his/her approval or disapproval for the patient to hold aspirin for four weeks. If the physician approved, the patient was contacted and taken off of aspirin for four weeks prior to the first study visit. In addition and by design, all antiplatelet agents were held for an additional four weeks between the third and fourth study visits.

Randomization. Patients in each cohort were randomized using a cross-over design comparing low- and high-dose aspirin as well as a non-aspirin platelet inhibitor ticagrelor. Randomization was performed after informed consent and baseline

clinical/medical data was collected. Patients were randomly assigned to one of the following drug sequences:

1. Aspirin 81 mg daily for four weeks, then aspirin 325 mg daily for four weeks, then a four-week aspirin washout, then ticagrelor 90 mg twice daily; or
2. Aspirin 325 mg daily for four weeks, then aspirin 81 mg daily for four weeks, then a four-week aspirin washout, then ticagrelor 90 mg twice daily.

Study visits. Patients were instructed to come to the DCRU before or on the same day as the first visit to meet with the study coordinator for screening, to review the consent form and to ask any questions regarding participation. After consent was obtained, patients were scheduled for the study visits. Patients were asked to fast for at least six hours prior to each visit and to refrain from tobacco, alcohol, nicotine product use and intensive exercise for at least six hours prior to each clinic visit. In addition patients were asked to refrain from non-steroidal anti-inflammatory agents (NSAIDs) and if a sexually active female of child-bearing potential, to use appropriate contraception during the entire study period once consent is obtained and for two weeks afterwards. If these guidelines were not followed then patients were rescheduled. A urine pregnancy test was collected at the first study visit, prior to beginning aspirin use, if a female patient had sex without contraception or if she thought she may be pregnant. In addition, a serum pregnancy test was performed after the consent form was signed in all female patients of childbearing potential and also on those whose menopausal state or surgical hysterectomy could not be confirmed.

Demographic data was collected at the screening visit and included date of birth/age, sex, medications (prescription, over-the-counter, and as needed), self-reported

race (Caucasian, African-American, Asian/Pacific Islander, Indian, Native American, Latino, Other), ethnicity, medical history (both groups), and regular tobacco use (packs/week). Risk factors and comorbidities were identified including: hypertension and/or hyperlipidemia (defined as requiring prescription medication); coronary artery disease (diagnosed by a positive stress test or significant > 50% stenosis on coronary angiography, prior percutaneous coronary intervention, or prior coronary artery bypass surgery); peripheral artery disease (diagnosed by ankle brachial index < 0.9, > 50% stenosis on peripheral angiography or noninvasive imaging including CT, MRI, or ultrasound), cerebrovascular disease (including cerebral infarction or transient ischemic attack), and deep venous thrombosis or pulmonary embolus.

At each visit, patients were asked about the following: any adverse drug events that may have occurred since the previous visit; any changes to medication list since consent or last visit; recent prescription medication use within the prior seven days; last menstrual period (in women of child bearing potential); contraceptive use (if sexually active female patient of childbearing potential; last use of tobacco, nicotine products, or alcohol (if within six hours of the visit, the visit was rescheduled); last aspirin or NSAID use (if within 14 days of the visit, the visit was rescheduled), and last intensive exercise (if within six hours of the visit, the visit was rescheduled). Blood pressure and heart rate were measured. It was confirmed that each patient had been fasting for six hours prior to the study visit (patients in the healthy volunteer cohort could drink water and patients with diabetes could drink fruit juice). If patients had symptoms of fever, flu, and/or upper respiratory infection in the past seven days or had recently received a live nasal influenza vaccine, the visit was rescheduled for seven days after resolution of symptoms or

vaccination. Patients were instructed to keep a medication log and the number of pills dispensed, number of pills returned, and number of missed doses based on the medication log was recorded.

At the first study visit, the first phlebotomy sample was drawn for baseline platelet function and biological sample collection, and then the first dose of aspirin (81 or 325 mg) was administered. Patients remained fasting for three hours and then gave a second blood sample for platelet function analysis three hours after the time of first aspirin ingestion. Patients were then provided 34 tablets of aspirin (81 or 325 mg), a medication log, and a list of medications to avoid during the study. Patients were instructed to take one aspirin tablet daily with a full glass of water and to record the date/time of each dose. (If a patient missed a dose of aspirin, they were allowed to take it later in the day.)

On the morning of the second and third study visit, patients were instructed not to take their scheduled dose of aspirin and instead to come to the DCRU to receive their final aspirin dose with a full glass of water. They remained fasting for three hours after aspirin ingestion. At the three-hour timepoint, a blood sample was drawn for platelet function testing and biological sample collection.

After the conclusion of the third study visit, all patients were instructed to discontinue the use of all aspirin or NSAIDs for the next four weeks to allow sufficient time for the genomic content of platelets to return to baseline and for the entire platelet pool to regenerate ~2-3 times.

At the fourth study visit, patients arrived at the DCRU to have their baseline platelet function and biological sample collection. They were then given a loading dose (180 mg) of ticagrelor with a full glass of water. Patients remained fasting for three hours

and then gave a second blood sample for platelet function analysis three hours after the time of first ticagrelor ingestion. Patients were provided120 ticagrelor tablets (90 mg), a medication diary, and a list of medications to avoid during the study. Patients were instructed to take one ticagrelor tablet twice daily with a full glass of water and record the date/time of each dose. (If a patient missed a dose of ticagrelor, they were allowed to take it later in the day.)

On the morning of the fifth study visit, patients were instructed not to take their scheduled dose of ticagrelor and instead to come to the DCRU to receive their final ticagrelor dose with a full glass of water. They remained fasting for three hours after ticagrelor ingestion. At the three-hour timepoint, a blood sample was drawn for platelet function testing and biological sample collection.

Medication adherence. During the study (i.e. from the time of consent), patients were instructed to refrain from using NSAIDs or over the counter medications that contain NSAID medications (see list for examples). Instead, for analgesic or antipyretic purposes, patients could use products containing acetaminophen as needed. Subjects were asked to review the active ingredient lists of over the counter medications taken during the study period and to compare to the allowed list.

The study staff were available to discuss new medications that may interfere with the study. Although new medications or prohibited medications did not necessarily result in exclusion from the trial, each instance was reviewed by the study physician. Subjects were instructed that the over the counter products on the list were allowed if they are taken as needed and not on a daily basis.

Inclusion and exclusion criteria

Healthy cohort. In the healthy cohort, inclusion criteria included age between 30 and 70, non-smoker, and a requirement that female patients should not exceed 55% o the cohort.

Exclusion criteria included history of bleeding disorder (gastrointestinal bleeding, intracranial bleeding or known prior gastric ulcer without documented resolution; current regular use of antiplatelet agents (aspirin, cilostazol, prasugrel, clopidogrel, dipyridamole, ticagrelor, or ticlopidine), nonsteroidal anti-inflammatory agents (NSAIDs), oral corticosteroids (i.e. prednisone), and/or anticoagulants (warfarin, dabigatran, apixaban, rivaroxaban, enoxaparin); known, severe hepatic impairment; surgery within the last 6 months, at the discretion of the PI; prior gastric bypass surgery (or equivalent) that interferes with absorption at the discretion of the PI; aspirin allergy or known intolerance to aspirin or ticagrelor; comorbid conditions including hypertension (requiring prescription medication), hyperlipidemia (requiring medications), type 1 or 2 diabetes, coronary artery disease (diagnosed by a positive stress test or significant stenosis at coronary angiography, or any of the following: myocardial infarction, percutaneous coronary intervention, coronary artery bypass surgery), peripheral artery disease (diagnosed by ankle brachial index, angiography, or noninvasive imaging; CT, MRI, or ultrasound), cerebrovascular disease (cerebral infarction, cerebral hemorrhage, subarachnoid hemorrhage, and/or transient ischemic attack), deep venous thrombosis or pulmonary embolus, known HIV, hepatitis B or C virus infection, chronic liver disease, and chronic kidney disease; use of daily prescription medications other than oral

contraceptives; regular use (defined as any use within the past 7 days) of tobacco (cigarette, cigar, or chewing tobacco) and/or nicotine products (gum, patch, or inhaler); current breastfeeding or known pregnancy; personal or prior family member participation in prior aspirin studies at the Duke Clinical Research Unit; and known active substance use, at the discretion of the study investigator.

Diabetic cohort. In the diabetic cohort, inclusion criteria included age over 45 for men and over 50 for women, type 2 diabetes requiring prescription medications, approval by the patient's physician to allow aspirin washout prior to participation if aspirin was already prescribed or agreement by the patient to submit to a four-week aspirin washout period if patient self-administers aspirin, and a requirement that female patients should not exceed 55% of the cohort.

Exclusion criteria included a history of bleeding disorder (gastrointestinal bleeding, known prior gastric/duodenal ulcer without documented resolution, or intracranial bleeding); current regular use of antiplatelet agents other than aspirin (cilostazol, clopidogrel, prasugrel, ticagrelor, dipyridamole, or ticlopidine), NSAIDs, oral corticosteroids (i.e. prednisone), or anticoagulants (warfarin, dabigatran, apixaban, rivaroxaban, or enoxaparin); current regular use of CYP3A inhibitors (including atazanavir, clarithromycin, indinavir, itraconazole, ketoconazole, nefazodone, nelfinavir, ritonavir, saquinavir, telithromycin and voriconazole) or inducers (including rifampin, dexamethasone, phenytoin, carbamazepine, and phenobarbital); known, severe hepatic impairment; surgery within the last 6 months; aspirin allergy or known intolerance to aspirin or to ticagrelor; current breastfeeding; conditions where aspirin washout is likely to expose the patient to excessive risk of thrombosis including atrial fibrillation and

known history of arterial thrombosis (myocardial infarction, stroke, or transient ischemic attack); other comorbid conditions including chronic liver disease, chronic kidney disease, HIV or hepatitis B or C virus infection, type 1 diabetes, DVT or pulmonary embolus; known pregnancy in women of child bearing potential; personal or prior family member participation in prior aspirin studies at the Duke Clinical Research Unit; and known active substance use, at the discretion of the study investigator.

Sample collection

Phlebotomy protocol for all visits. At each study visit, patients were first asked to rest in a supine position for 10 minutes. Approximately 90 mL of blood was collected in multiple tubes to allow for serum, plasma, and platelet analysis as well as genetic analysis (DNA, RNA).

Side effect and safety monitoring

Patients in the study were able to report side effects and adverse events at any time during the study and were queried for the development of side effects and adverse events at every study visit.

Patients taking aspirin were asked to report side effects and adverse events including gastrointestinal (stomach upset, heartburn, nausea, abdominal pain), allergic (rash, hives, itching, difficulty breathing, wheezing, chest tightness, swelling of the mouth, face, lips or tongue), tinnitus, hearing loss, bruising, and bleeding.

Patients taking ticagrelor were asked to report side effects and adverse events including shortness of breath, bleeding (hematoma, epistaxis, gastrointestinal bleeding, subcutaneous or dermal bleeding), allergy (rash, itching).

Patients on aspirin were instructed to not have more than one drink (for females) or two drinks (for males) of alcohol per day.

Patients who developed gastrointestinal side effects were allowed to take gastric acid suppressant medications (prescription or OTC) during the study if necessary.

Female patients who were found to be pregnant at the screening or any of the study visits terminated the study immediately.

All serious adverse events, both unexpected and related to the study medications, were reported to the study investigator. If patients could not continue the study due to an adverse event or uncontrolled side effects, they were able to reschedule visits or exit the study.

Singapore cohort

The SingHealth Investigational Medicine Unit (Singapore) performed an aspirin and ticagrelor challenge study to examine gene expression profiles in healthy volunteers. Patients were recruited from December 2013 to November 2014.

Study protocol

At the initial screening visit, healthy volunteers were screened for eligibility and consented for participation in the study. Demographic data was collected, medication list was reviewed, a complete blood count and serum pregnancy test (for all women of childbearing age) were collected, and patients were instructed to refrain from any non-permitted medication use and to return for visit 1 in a fasting state for at least 6 hours.

Measurement of vital signs (blood pressure, heart rate, height, and weight), review of medical history, exercise habits, and recent use of tobacco, alcohol, and NSAIDs, and review of contraceptive requirements was performed at each study visit. At the first study visit, patients first gave blood samples for genetic and platelet function tests. They were then randomized 1:1 to take a single dose of either open-label aspirin 325 mg or ticagrelor 180mg (two 90 mg tablets). Three hours after the first supervised dose, they again gave blood samples. Patients were then provided 35 aspirin 325 mg tablets or 70 ticagrelor 90 mg tablets (depending on randomization arm), a medication log, and a list of medications to avoid during the study. Patients were instructed to take either one aspirin tablet every morning or one ticagrelor tablet twice daily in the morning and evening, with a full glass of water each time, until visit 2.

At the second study visit, patients were instructed to fast for at least 6 hours prior to arrival. The last dose of study drug was taken on arrival to the research facility, and three hours after arrival, patients underwent repeat blood sampling.

After visit 2, patients underwent a 28-day washout period. At the third study visit, patients again gave blood for genetic and platelet function testing. In this crossover study, patients had previously been randomized to open-label aspirin were crossed over to open-label ticagrelor and vice versa. Subjects were given either ticagrelor 180mg (two 90 mg tablets) or aspirin 325 mg. They were provided with either 70 ticagrelor 90 mg tablets or 35 aspirin 325 mg tablets, a medication log, and a list of medications to avoid during the study. Patients were instructed to take either one ticagrelor tablet twice daily or one aspirin tablet every morning, with a full glass of water each time, until visit 4.

At the fourth study visit, patients were instructed to fast for at least 6 hours prior to arrival. The last dose of study drug was taken on arrival to the research facility, and three hours after arrival, patients underwent repeat blood sampling.

All subjects were reminded to refrain from using prescription or over-the-counter NSAIDs. For analgesic or antipyretic purposes, subjects could take acetaminophen-containing products. Medication logs were recorded by the patients and reviewed at the final study visit.

Inclusion and exclusion criteria

This study included healthy volunteers greater than 21 years old. Patients were selected from a Chinese population; all four grandparents must have been of Chinese ancestry. The following exclusion criteria applied to all cohorts: history of bleeding

disorder, gastrointestinal bleeding, or known prior gastric ulcer without documented resolution; history of anemia; hemoglobin less than 10 g/dL or platelets < $100x10^9$/L at the screening visit; any active infection including HIV, hepatitis B virus, or hepatitis C virus; current regular use of antiplatelet agents (aspirin, aspirin/dipyridamole, cilostazol, ticlopidine, prasugrel, ticagrelor), inflammatory agents (NSAIDs), oral corticosteroids, anticoagulants (warfarin or enoxaparin); surgery within the last six months; aspirin allergy or known intolerance to 325 mg of aspirin; ticagrelor allergy or known intolerance to 90 or 180 mg of ticagrelor; comorbid conditions including hypertension requiring medication, hyperlipidemia requiring medication, type 1 or 2 diabetes, coronary artery disease (positive stress test, significant stenosis identified by coronary angiography, prior myocardial infarction, percutaneous coronary intervention, or coronary artery bypass surgery), peripheral artery disease (diagnosed by ankle-branchial index, angiography, or noninvasive imaging including CT, MRI, or ultrasound); cerebrovascular disease (including history of cerebral infarction, cerebral hemorrhage, subarachnoid hemorrhage, or transient ischemic attack), history of deep vein thrombosis or pulmonary embolus; use of any prescription medications other than oral contraceptives; regular use (defined as one or more uses per day) of tobacco (cigarette, cigar), and/or nicotine products (gum, patch, inhaler, etc.) or alcohol; current breastfeeding or known pregnancy; family member participating in the study; history of malignancy (at the discretion of the study physician); presence of a condition which, in the opinion of the study physician, could present a concern for patient safety or difficulty with data interpretation; and females of childbearing age who declined to practice adequate contraceptive measures.

Patients were allowed to take the following over-the-counter medications: paracetamol/acetaminophen, dextromethorphan, pseudoephedrine, guaifenesin, diphenhydramine, chlorpheniramine maleate, loratadine, and benzocaine. Patients were not allowed to take ibuprofen, naproxen sodium, ketoprofen, aspirin or acetylsalicylic acid.

Sample collection

At the screening visit, a single sample of blood was taken for complete blood count for safety purposes.

At each study visit, 40 mL of blood was collected for platelet function testing and 30 mL of blood was collected for genetic testing. Blood was collected after a 10-minute supine rest period.

Side effect and safety monitoring

Patients in the study were able to report side effects and adverse events at any time during the study and were queried for the development of side effects and adverse events at every study visit.

Patients taking aspirin were asked to report side effects and adverse events including gastrointestinal (stomach upset, heartburn, nausea, abdominal pain), allergic (rash, hives, itching, difficulty breathing, wheezing, chest tightness, swelling of the mouth, face, lips or tongue), tinnitus, hearing loss, bruising, and bleeding.

Patients taking ticagrelor were asked to report side effects and adverse events including shortness of breath, bleeding (hematoma, epistaxis, gastrointestinal bleeding, subcutaneous or dermal bleeding), allergy (rash, itching).

Patients on aspirin were instructed to not have more than one drink (for females) or two drinks (for males) of alcohol per day.

Patients who developed gastrointestinal side effects were allowed to take gastric acid suppressant medications (prescription or OTC) during the study if necessary.

Female patients who were found to be pregnant at the screening or any of the study visits terminated the study immediately.

All serious adverse events, both unexpected and related to the study medications, were reported to the study investigator. If patients could not continue the study due to an adverse event or uncontrolled side effects, they were able to reschedule visits or exit the study.

Platelet function testing

Platelet samples were analyzed using light transmittance aggregometry to measure serum platelet aggregation in response to:

1. Epinephrine at low, intermediate, and high concentrations;
2. Adenosine diphosphate at low, intermediate, and high concentrations;
3. Collagen at low, intermediate, and high concentrations;
4. Arachidonic acid.

Genetic studies

Analysis of gene expression was performed using Affymetrix GeneChip microarrays (Santa Clara, CA, USA). Expression of a unique set of 60 co-expressed genes which were represented on the Affymetrix microarray was measured to determine the aspirin response signature which has been previously described.[32] Genomic analysis of the aspirin response signature and the entire microarray dataset was performed at Duke-National University of Singapore in collaboration with investigators at Duke University (Durham, NC, USA).

IRB and informed consent

The above studies were approved by the local institutional review committee. Subjects gave written informed consent prior to enrollment in the study. The procedures described herein were in accordance with institutional guidelines.

Table S1. Sex differences in platelet aggregation both at baseline and after treatment with aspirin.

Agonist		Baseline			ASA		
		Males	**Females**	**P**	**Males**	**Females**	**P**
EPI	0.5 μmol/L	272.1 (14.8)	309.6 (14.2)	0.068	135.8 (4.9)	142.4 (4.7)	0.33
	1 μmol/L	378.8 (16.3)	418.0 (14.8)	0.075	162.8 (5.2)	167.0 (4.9)	0.56
	10 μmol/L	612.4 (14.1)	628.7 (12.7)	0.39	269.7 (7.5)	279.6 (7.4)	0.34
ADP	1 μmol/L	79.4 (5.9)	122.2 (6.8)	**2.5×10^{-6}**	73.5 (4.2)	93.1 (4.1)	**9.1×10^{-4}**
	5 μmol/L	324.0 (4.8)	337.5 (4.2)	**0.035**	279.1 (4.5)	291.2 (3.7)	**0.035**
	10 μmol/L	362.9 (3.9)	371.6 (3.5)	0.10	340.1 (4.1)	352.3 (3.3)	**0.020**
COL	2 mg/mL	331.2 (4.5)	323.7 (3.8)	0.20	142.7 (5.0)	147.4 (4.6)	0.49
	5 mg/mL	372.0 (3.8)	360.4 (3.1)	**0.018**	243.4 (5.0)	252.8 (4.2)	0.15
PFA-100		125.4 (2.2)	123.5 (2.0)	0.53*	251.9 (3.6)	243.6 (3.1)	0.084*

Values are expressed as standardized mean area under the curve of light transmittance aggregometry with standard errors in parentheses. Significant differences ($p < 0.05$) are noted in **bold**. ADP = adenosine diphosphate; ASA = aspirin; COL = collagen; EPI = epinephrine; PFA = platelet function analyzer.*As mentioned in the manuscript, use of an alternative statistical analysis using a censored linear regression (tobit) model for PFA-100 closure time changes the P values to 0.56 and 0.0049, respectively, for baseline and on-ASA comparisons.

Table S2. Treatment effect of aspirin stratified by sex, standardized in comparison to baseline platelet aggregation for each agonist which is set at 0.

Agonist		Treatment Effect		
		Males	**Females**	**P**
EPI	0.5 μmol/L	-132.5 (10.1)	-162.0 (9.2)	**0.031**
	1 μmol/L	-204.0 (11.0)	-248.5 (10.0)	**0.0028**
	10 μmol/L	-348.2 (11.4)	-352.2 (10.4)	0.80
ADP	1 μmol/L	-16.8 (3.6)	-21.4 (3.6)	0.36
	5 μmol/L	-49.9 (3.9)	-43.6 (3.7)	0.24
	10 μmol/L	-32.3 (3.5)	-23.6 (3.3)	0.070
COL	2 mg/mL	-192.2 (5.2)	-176.0 (4.9)	**0.023**
	5 mg/mL	-132.2 (4.7)	-110.4 (4.3)	**6.8×10^{-3}**
PFA-100		128.1 (4.3)	121.3 (3.6)	0.22

Agonist concentrations are expressed in μmol/L for EPI and ADP and as mg/ml for COL; values for PFA closure time are in seconds. Standard errors are noted in parentheses. Significant differences ($p < 0.05$) are noted in **bold**.

Table S3. Changes in platelet aggregation during the course of treatment.

Agonist		Baseline to 3h			3h to 4 weeks		
		Males	**Females**	**P**	**Males**	**Females**	**P**
EPI	0.5 μmol/L	**-137.6 (17.1)**	**-169.1 (14.5)**	0.16	2.1 (5.8)	**18.8 (5.3)**	**0.034**
	1 μmol/L	**-196.2 (18.5)**	**-255.5 (15.4)**	**0.014**	8.4 (6.4)	**22.3 (5.6)**	0.10
	10 μmol/L	**-320.5 (17.0)**	**-351.0 (14.1)**	0.17	5.3 (9.8)	**20.8 (8.6)**	0.23
ADP	1 μmol/L	**-20.3 (4.4)**	**-23.9 (4.0)**	0.55	1.3 (5.6)	5.2 (5.0)	0.60
	5 μmol/L	**-52.6 (4.9)**	**-50.2 (4.2)**	0.71	10.2 (6.2)	**12.2 (5.5)**	0.81
	10 μmol/L	**-35.9 (4.4)**	**-33.2 (3.8)**	0.64	9.6 (5.7)	**17.0 (5.1)**	0.34
COL	2 mg/mL	**-176.4 (7.1)**	**-172.2 (6.0)**	0.65	-7.1 (8.0)	-10.2 (6.8)	0.76
	5 mg/mL	**-111.1 (6.5)**	**-102.9 (5.4)**	0.33	-9.4 (7.3)	-2.1 (6.3)	0.45

A. Platelet aggregation at 3 hours after first aspirin exposure was compared to baseline platelet aggregation, which is standardized and set at 0.

B. Platelet aggregation after 4 weeks of aspirin treatment was compared to platelet aggregation 3 hours after the first aspirin dose, which is standardized and set at 0.

For both tables, agonist concentrations are expressed in μmol/L for EPI and ADP and as mg/ml for COL; standard errors are noted in parentheses. Individual values for which the estimated 95% confidence interval does not include 0 (indicating a significant change in response over the time period) are noted in **bold**. Significant differences between sexes ($p < 0.05$) are also noted in **bold**.

Table S4. Platelet response to *ex vivo* aspirin exposure.

Agonist		Response to *ex vivo* aspirin exposure	
		Males	**Females**
EPI	0.5 μmol/L	-222.0 (29.9)	-254.1 (23.7)
ADP	5 μmol/L	-46.3 (7.6)	-42.6 (6.0)
COL	2 mg/mL	-183.4 (9.1)	-177.9 (7.2)

Ex vivo aspirin exposure results in a decrease in platelet aggregation, with no significant sex differences. Values are standardized in comparison to baseline platelet aggregation which is set at 0. Agonist concentrations are expressed in μmol/L for EPI and ADP and as mg/ml for COL; standard errors are noted in parentheses.