ORIGINAL RESEARCH

Clopidogrel Improves Skin Microcirculatory Endothelial Function in Persons With Heightened Platelet Aggregation

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Background—Platelet activation can lead to enhanced oxidative stress, inflammatory response, and endothelial dysfunction. To quantify the effects of platelet inhibition on endothelial function, we assessed platelet activity of healthy persons before and after clopidogrel administration and evaluated its effects on endothelial function. We hypothesized that clopidogrel, by attenuating platelet activity, would result in enhanced endothelial function.

Methods and Results—Microcirculatory endothelial function was quantified by laser Doppler flowmetry (LDF) mediated by thermal hyperemia (TH) and postocclusive reactive hyperemia, respectively, in 287 and 241 relatively healthy and homogenous Old Order Amish persons. LDF and platelet aggregation measures were obtained at baseline and after 7 days of clopidogrel administration. Our primary outcome was percentage change in post- versus preclopidogrel LDF measures. Preclopidogrel TH-LDF and platelet aggregation were higher in women than in men ($P<0.001$). Clopidogrel administration was associated with ≈2-fold higher percentage change in TH-LDF in participants with high versus low baseline platelet aggregation (39.4±10.1% versus 17.4±5.6%, $P=0.03$). Clopidogrel also increased absolute TH-LDF measures in persons with high platelet aggregation (1757±766 to 2154±1055, $P=0.03$), with a more prominent effect in women (1909±846 to 2518±1048, $P=0.001$). There was no evidence that clopidogrel influenced postocclusive reactive hyperemia LDF measures.

Conclusions—The administration of clopidogrel in healthy persons with high baseline platelet aggregation results in improved TH-induced microcirculatory endothelial function. These data suggest that clopidogrel may have a beneficial effect on microcirculatory endothelial function, presumably through antiplatelet activity, and may confer additional vascular benefits.

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Key Words: clopidogrel • endothelial function • platelet aggregation • women

Platelet activation can contribute to cardiovascular disease through its role in thrombosis formation. This is mediated in part by the activation of the P2Y12 receptor (adenosine diphosphate [ADP] receptor) and subsequent overexpression of glycoprotein IIb/IIIa, a platelet aggregator receptor.[1] In addition to its role in thrombosis, platelet activation leads to an increase of circulating CD40-L, resulting in release of inflammatory and oxidative stress mediators.[2–5] This causes endothelial dysfunction and attenuated nitric oxide (NO) bioavailability, a sentinel event in the development and progression of both focal and systemic vascular disease and atherosclerotic-related morbidity.[6–11]

Clopidogrel, an ADP (P2Y12) receptor antagonist, is activated by hepatic cytochrome P450 (CYP) isoenzymes in a biologically active thiol metabolite, significantly reducing ADP-dependent platelet aggregation and thrombosis.[12–14] Along with the antithrombotic benefits, interest has been emerging in studying the effect of clopidogrel on endothelial function in persons with[15–18] and without coronary artery disease.[19] Furthermore, studies in rat models have shown that clopidogrel can improve endothelium-mediated vascular response, presumably via NO pathways.[20–22] In this study, we hypothesized that clopidogrel would result in enhanced microcirculatory endothelial function by primarily attenuating

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platelet activity. Bearing in mind the significant variability in baseline platelet aggregation and sex-specific variability in hypercoagulability state and endothelial function, we secondarily hypothesized that sex and baseline platelet aggregation level might affect the endothelial response to clopidogrel.

Considering that several methods exist for measuring microvascular endothelial function, we set out to measure the effect of clopidogrel on microcirculatory endothelial function and vascular response using 2 complementary measures: laser Doppler flowmetry (LDF) that was mediated by thermal hyperemia (TH) as our primary measure and by postocclusive reactive hyperemia (PORH) as a secondary measure. TH-LDF has been shown to be largely mediated by the endothelial release of NO and considered a valid proxy for endothelial function.[23,24] PORH-LDF captures a complex microvascular response to induced transient ischemia in which the endothelium plays a pivotal role, primarily independent of NO.[25] The study was performed in a relatively healthy and homogenous population to evaluate whether clopidogrel, by inhibiting platelet activity, can have a beneficial effect on microcirculatory endothelial function in clinically healthy persons.

Materials and Methods

Study Population

The Pharmacogenomics of Anti-Platelet Intervention (PAPI) study recruited relatively healthy Old Order Amish persons from Lancaster County, Pennsylvania, to evaluate the genetic determinants of platelet aggregation before and after administration of the antiplatelet drug clopidogrel. Detailed characteristics of the PAPI study and its participants were published previously.[26] Briefly, all participants were aged ≥20 years and were free of any known disease. Participants were excluded if any of the following criteria were met: history of cardiovascular disease, severe hypertension (blood pressure >180/105 mm Hg or antihypertensive medication), diabetes mellitus, anemia, cancer, thyroid malfunction, renal insufficiency, abnormal liver function tests, gastrointestinal bleeding, thrombocytosis or thrombocytopenia, current pregnancy or lactation, or continuation of any prescribed or over-the-counter medication that would interfere with the impact of clopidogrel. At least 1 week prior to the initial study visit, participants discontinued the use of all medications, vitamins, and supplements. This study was approved by the University of Maryland School of Medicine institutional review board. In this investigation, we evaluated a subgroup of 287 PAPI study participants who gave informed consent, and their LDF-based measures of endothelial function were recorded at baseline and after the clopidogrel intervention, which consisted of a 300-mg loading dose followed by 6 days of daily dosing at 75 mg. Baseline demographic and cardiovascular-related clinical variables were obtained under standardized protocols and were published previously.[26,27]

Endothelial Function Measures

Laser Doppler flowmetry

LDF uses a laser light source and Doppler shift effect to noninvasively measure microvascular blood flow on a relative blood-perfusion-unit unitless scale, allowing for reliable measurement of change in microcirculatory perfusion in response to various interventions. All measurements were performed by a single trained technician according to an established protocol using a Perimed PF5020 unit in a temperature-controlled room.

Thermal hyperemia

Our primary microcirculatory endothelial function outcome was based on skin TH-LDF measures obtained from all study participants before and after clopidogrel administration. To obtain these measures, the LDF probe was placed on the volar aspect of the forearm and was heated to 44°C, causing dilatation of the skin vessels and increased flow. The LDF-based blood flow was recorded at baseline and then over 30 minutes as the probe heated the skin. We then calculated the percentage change in flow from baseline to the observed peak flow over the 30-minute time interval, which constituted our final TH-LDF measure. All results were abstracted by a single investigator who was blinded to the study participant status (F.S.).

Postocclusive hyperemia

PORH-LDF measurements were obtained on the same day as the TH-LDF measures in 247 participants who had TH-LDF measured and who consented to a second LDF-based measure during the same study visit. After immobilizing the left arm, the LDF probe was placed over the volar aspect of the forearm skin ≈5 to 10 cm proximal to the wrist to allow for the measure of baseline blood flow. Next, a sphygmomanometer cuff located on the left arm above the antecubital fossa was inflated 50 mm Hg above systolic blood pressure for 5 minutes to occlude arterial blood flow, resulting in ischemia-induced dilatation of resistance vessels.[28] The percentage increase in flow from baseline was used as a proxy of endothelial function and vascular reactivity.

Platelet aggregometry

Ex vivo platelet aggregometry was measured before and after clopidogrel intervention in platelet-rich plasma (200 000

platelets/μL), which was isolated from the whole blood, as described previously.[26] Following stimulation of platelet-rich plasma samples with 10 μg/mL of ADP or collagen, platelet aggregation was quantified by optical aggregometry (PAP8E aggregometer; Bio/Data Corp) using platelet-poor plasma as a reference, as described previously.[27] To categorize our participants as high versus low platelet aggregators, the quantile distribution plot of standardized platelet aggregation after stimulation with ADP or collagen was examined for each measure. Based on the preclopidogrel platelet aggregation distribution, participants in the upper quartile of either platelet aggregation measures were classified as having high composite platelet aggregation (n=67) (Figure A and B).

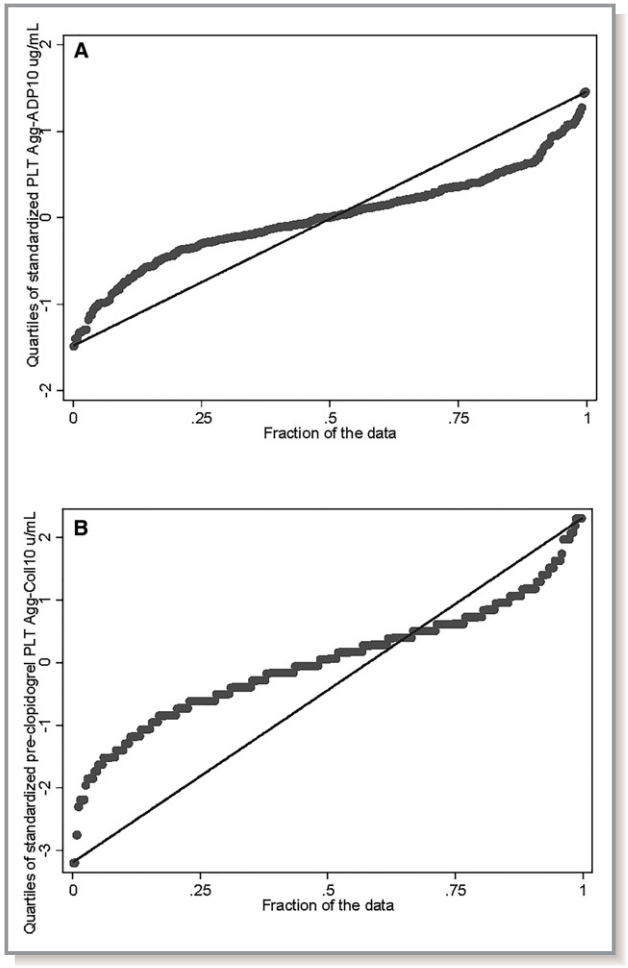

Figure. Distribution of ex vivo platelet aggregation stimulated by adding 10 μg/mL of adenosine diphosphate (ADP) or collagen. Participants with values for either platelet aggregation measure within the upper quartile of distribution were considered high aggregators. A, Ex vivo platelet aggregation stimulated by adding 10 μg/mL ADP. B, Ex vivo platelet aggregation stimulated by adding 10 μg/mL collagen.

Clopidogrel active metabolite quantification

Clopidogrel active metabolite was quantified in the blood collected in an EDTA tube containing 2 mmol/L (E)-2-bromo-3′-methoxyacetophenone (MPB; Sigma Aldrich, St Louis, Missouri, USA) within 1 hour after the last administration of clopidogrel using ultra–high-performance liquid chromatography–tandem mass spectrometry with an active metabolite calibration range of 0.1 to 150 ng/mL, as described previously.[27]

Genotyping

Our group and others have previously shown that a common loss-of-function *CYP2C19*2* variant (rs4244285) was associated with differential clopidogrel active metabolite formation and variability in platelet aggregation response to clopidogrel. To account for potential genotype effect, we used our previously obtained rs4244285 genotype results within the parent PAPI study as a covariate. The mean genotype call rate for the PAPI study population was 98.7%, and we observed >98% concordance in a subset of blind duplicates.[26,27]

Statistical Analysis

Our primary analysis was to compare percentage change in LDF measures of endothelial function before and after clopidogrel administration. Percentage change in postclopidogrel TH-LDF and PORH-LDF from preclopidogrel measures was calculated as follows: percentage change $TH=(TH_{postclopidogrel}-TH_{preclopidogrel})/(TH_{preclopidogrel})\times 100\%$. Similarly, percentage change $PORH=(PORH_{postclopidogrel}-PORH_{preclopidogrel})/(PORH_{preclopidogrel})\times 100\%$. In addition, secondary analyses of pre- and postclopidogrel absolute changes in the endothelial function measures were performed. To account for relatedness in the Old Order Amish participants, the Mixed Models Analysis for Populations and Pedigrees (MMAP) program was used (http://edn.som.umaryland.edu/mmap/index.php). MMAP utilizes a regression-based approach that models variation of the trait of interest as a function of measured covariate (eg, age) and a polygenic component that accounts for phenotypic correlation due to relatedness.[29,30] In the current analysis, to reduce the effects of age and family relatedness, endothelial function and platelet aggregation were separately analyzed using a mixed model adjusting for age and family relatedness using MMAP, and the residuals were then used for subsequent analyses. Moreover, to eliminate any unit-related effect, standardized endothelial function and platelet aggregation measures were created by subtracting the mean and dividing by the observed standard deviation for outcomes and platelet aggregation as the main risk factor of interest and other conventional covariates.

Table 1. Characteristics of the Study Participants

	Total, N=287	Women, n=154	Men, n=133	*P* Value*
Age, y	45.3±12.9	46.5±13.0	44.0±12.6	0.11
Sex, %				
Men	46.3	—	—	—
Women	53.7			
Menopausal state, %				
Yes	—	26.1	—	—
BMI, kg/m^2	26.9±4.6	27.7±5.2	25.9±3.7	0.006
<25, %	37.3	43.9	51.6	0.001
25 to <30, %	29.0	51.8	48.2	
≥30, %	23.7	72.1	27.9	
Current smoker, %	10.5	0	22.6	—
SBP, mm Hg	116.6±12.5	116.4±13.1	116.8±11.8	0.78
DBP, mm Hg	69.7±7.2	69.1±7.5	70.5±6.8	0.08
CRP, mg/dL, median (range)	0.9 (0.2–39.0)	1.1 (0.2–39.0)	0.7 (0.2–16.7)	0.09
Total cholesterol, mg/dL	209.1±42.8	208.8±38.8	209.4±46.0	0.72
LDL-C, mg/dL	133.4±39.3	130.1±41.6	137.3±36.4	0.04
HDL-C, mg/dL	61.2±15.3	64.2±15.4	57.6±14.5	0.0003
TG, mg/dL	72.9±41.8	75.6±44.5	69.8±38.3	0.50
PLTAgg-ADP10				
Before clopidogrel	69.5±11.3	71.8±10.4	66.9±11.7	0.003
After clopidogrel	30.6±11.2	31.4±11.3	29.6±11.1	0.16
PLTAgg-Coll10				
Before clopidogrel	86.2±7.7	86.3±8 .0	86.0±7.4	0.70
After clopidogrel	77.3±11.0	78.0±10.7	76.5±11.4	0.29
High composite platelet aggregation, n (%)				
Yes	67 (23.3)	39 (58.2)	28 (41.8)	0.39
No	220	115 (52.3)	105 (47.7)	
*CYP2C19**2 genotype				
0 allele	68.2 (n=195)	54.9 (n=107)	45.1 (n=88)	0.75
1 allele	28.7 (n=82)	50.0 (n=41)	50.0 (n=41)	
2 allele	3.1 (n=10)	55.5 (n=5)	44.5 (n=5)	
Clopidogrel active metabolite	19.2±9.6	19.5±9.2	19.8±10.1	0.90
TH before clopidogrel	1874±956	2158±1018	1546±756	0.00001
TH after clopidogrel	1907±956	2227±991	1537±764	0.00001
PORH before clopidogrel	441.6±191.6	460.5±199.0	424.5±183.9	0.12
PORH after clopidogrel	437.6±216.1	474.7±231.1	404.4±196.8	0.009

Data are shown as mean±SD except as noted. All *P* value are shown after adjustment for age and family relatedness. BMI indicates body mass index; CRP, C-reactive protein; CYP2C19, cytochrome P450 2C19 system; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; PLTAgg-ADP10, platelet aggregation on adding 10 μg/mL adenosine diphosphate; PLTAgg-Coll10, platelet aggregation on adding 10 μg/mL collagen; PORH, postocclusive reactive hyperemia; SBP, systolic blood pressure; TG, triglyceride; TH, thermal hyperemia.
*Sex-based *P*-value.

We assessed the mean differences of preclopidogrel platelet aggregations in persons with high and low platelet aggregation states. In addition, the mean of preclopidogrel TH-LDF and PORH-LDF by categorical cardiovascular-related factors were compared using the Student *t* test. Then, univariate and multivariate regression analyses were

Table 2. Platelet Aggregation Response to Clopidogrel in Initial Composite High and Low Platelet Aggregation State

	High Composite PLTAgg-ADP10 or -Coll10	Low Composite PLTAgg-ADP10 or -Coll10
Preclopidogrel PLTAgg-ADP10	79.0±10.4	66.6±9.8
Postclopidogrel PLTAgg-ADP10	34.6±12.7	29.4±10.5
	P<0.00001	*P*=0.0007
Preclopidogrel PLTAgg-Coll10	95.1±5.5	83.0±6.1
Postclopidogrel PLTAgg-Coll10	81.1±10.6	76.2±11.0
	P<0.00001	*P*=0.002

PLTAgg-ADP10 indicates platelet aggregation on adding 10 μg/mL adenosine diphosphate; PLTAgg-Coll10, platelet aggregation on adding 10 μg/mL collagen.

performed to assess the association among platelet aggregation state (ie, high versus low), conventional cardiovascular risk factors, and percentage change in TH-LDF or PORH-LDF. Furthermore, to calculate the absolute change in postclopidogrel endothelial function measures from function before clopidogrel, a paired *t* test was used in all study participants and based on stratification by sex. We then compared absolute change in TH-LDF and PORH-LDF in all participants with high platelet aggregation, with secondary stratification by sex. STATA14 (StataCorp) was used for statistical analysis of the residual data. A *P* value <0.05 was considered statistically significance.

For pre- and postclopidogrel microcirculation, endothelial function heritability (h^2) was estimated while accounting for age and sex using the MMAP program.

Results

Baseline Characteristics and Sex Comparisons

The characteristics of the study participants are shown in Table 1. The mean age of the cohort was 45±13 years, and 54% of the participants were women, with no significant age discrepancy between the sexes (*P*=0.11). The clinically healthy study population was slightly overweight (mean body mass index [in kg/m^2] of 26.9±4.6), with elevated serum levels of low-density lipoprotein cholesterol (133.4±39.3 mg/dL) and high-density lipoprotein cholesterol (61.2±15.3 mg/dL). In Old Order Amish culture, women tend to not use tobacco products, resulting in 0% of women reporting smoking, whereas 22.6% of men reporting current smoking. Women had higher body mass index than men (27.7±5.2 versus 25.9±3.7, *P*=0.006). In addition, women had lower low-density lipoprotein cholesterol (130.1±41.6 versus 137.3±36.4 mg/dL, *P*=0.04) and higher high-density lipoprotein cholesterol serum concentrations (64.2±15.4 versus 57.6±14.5 mg/dL, *P*=0.0003). Of the participants, 23.3% (n=67) were classified as high composite platelet aggregators (based on ADP and/or collagen stimulation), of which >58.2% (n=39) were women (Table 1). In both initial high and low composite platelet aggregations, there was significant change in platelet aggregation after administration of clopidogrel, with more prominent change in initial high composite platelet aggregation (Table 2).

Ex vivo preclopidogrel ADP-stimulated platelet aggregation was significantly higher in women than in men (*P*=0.0002) (Table 1). The *CYP2C19**2 genotype and related serum concentration of the clopidogrel active metabolite were equally distributed between men and women (Table 1). Median C-reactive protein level was higher in participants with increased composite platelet aggregation compared with low composite platelet aggregation (1.3 versus 0.8 mg/dL, *P*=0.01).

TH-mediated LDF

Of the 308 participants with preclopidogrel TH-LDF measurements, postclopidogrel measures could not be obtained in 10 persons. In addition, 11 TH-LDF measures were excluded secondary to body motion artifacts resulting in unreliable measures. Preclopidogrel TH-LDF was higher in women than men (2158±1018 versus 1546±755, *P*=0.00001) (Table 1). Of all examined cofactors, sex was the strongest determinant of preclopidogrel TH-LDF endothelial function (*P*=0.001) (Table 3). Women with higher composite platelet aggregation had lower preclopidogrel TH-LDF measures compared with those with lower platelet aggregation (1909±846 versus 2242±1061, respectively; *P*=0.04). Preclopidogrel TH-LDF was not significantly different for pre- and postmenopausal women (2201±1062 versus 2042±919, *P*=0.49). In univariate regression analysis, high composite platelet aggregation (β=0.33, *P*=0.01) was significantly associated with percentage change in TH-LDF, and in our multivariate analysis, platelet aggregation (β=0.29, *P*=0.03) was the sole risk factor that was significantly associated with percentage change in TH-LDF. There were no significant associations between other conventional cardiovascular disease risk factors and preclopidogrel TH-LDF measure (Table 4).

Postclopidogrel TH-LDF was significantly higher in women than in men (2227±991 versus 1537±764, *P*=0.0001). There was a significant percentage change in postclopidogrel TH-LDF (17.4±82.9 versus 39.4±82.4, *P*=0.03) in participants with low versus high composite platelet aggregation, respectively. There was no overall significant absolute changes in postclopidogrel TH-LDF from preclopidogrel TH-LDF in the

Table 3. Mean of Preclopidogrel TH-LDF and PORH-LDF by Categorical Cardiovascular-Related Factors

	Preclopidogrel TH-LDF, Mean±SD, *P* Value	Preclopidogrel PORH-LDF, Mean±SD, *P* Value
High composite platelet aggregation		
No	1909±1006	440.25±192.4
Yes	1757±765, 0.25	445.6±190.7, 0.90
Age, y		
<55	1875±955	422.2±181.3
≥55	1868±973, 0.65	395.1±137.3, 0.42
Sex		
Men	1546±759	424.5±183.8
Women	2158±1018, 0.001	460.5±199.0, 0.60
Menopausal state		
No	2202±104	467.3±210.7
Yes	2042±133, 0.40	447.4±171.8, 0.63
BMI, kg/m^2		
<30	1825±942	426.3±179.4
≥30	2033±991, 0.11	383.5±151.2, 0.86
Current smoker		
No	1562±783	447.3±190.2
Yes*	1488±680, 0.64	395.7±200.5, 0.17
SBP, mm Hg		
<130	1900±983	430.2±170.8
≥130	1834±917, 0.56	398.3±179.5, 0.50
DBP, mm Hg		
<80	1896±972	418.4±174.3
≥80	1619±725, 0.18	408.1±183.1, 0.82
CRP, mg/dL		
<1.0	1870±908	434.4±173.8
≥1.0	1901±1023, 0.80	387.7±168.1, 0.18
LDL-C, mg/dL		
<160	1844±921	425.7±178.8
≥160	1986±1079, 0.79	456.5±202.6, 0.09
HDL-C, mg/dL		
≥40 for men or ≥50 for women	2299±1051	494.0±216.4
<40 for men or <50 for women	1821±932, 0.01	437.8±189.7, 0.25
TG, mg/dL		
<200	1872±961	440.2±192.3
≥200	2090±229, 0.50	519.2±143.1, 0.41
*CYP2C19**2 genotype†		
0 allele	1853±973	422.7±173.5
1 allele	1851±863	409.9±185.3
2 allele	2564.8±931, 0.08	389.1±133.9, 0.81

BMI indicates body mass index; CRP, C-reactive protein; CYP2C19, cytochrome p450 2C19 system; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDF, laser Doppler flowmetry; LDL-C, low-density lipoprotein cholesterol; PORH, postocclusive reactive hyperemia; SBP, systolic blood pressure; TG, triglyceride; TH, thermal hyperemia.
*Smoking in men.
†*P* value based on Kruskal–Wallis.

nonstratified analysis of composite platelet aggregation (1907±956 versus 1874±956, *P*=0.6).

Although there was no overall significant absolute change in postclopidogrel TH-LDF from preclopidogrel TH-LDF in women (2227±991 versus 2158±1018, *P*=0.55) or men (1537±764 versus 1546±755, *P*=0.46) (Table 1), there was a significant absolute change in postclopidogrel TH-LDF from preclopidogrel TH-LDF in participants with high composite platelet aggregation (2154±1055 versus 1757±766, *P*=0.03) (Table 6). Moreover, premenopausal women with high composite aggregability showed significant absolute change in postclopidogrel from preclopidogrel TH-LDF (2520±986 versus 1888±840, *P*=0.002, n=36). In women with high composite aggregability, percentage change in TH-LDF was marginally higher in non-menopausal versus menopausal women (33.07±9.3 [n=25] versus 14.9±15.1 [n=11], *P*=0.1). No association was observed between TH and other cardiovascular disease risk factors (Table 4).

PORH-mediated LDF

Of the participants consented for the primary TH-LDF measure, 247 were available and agreed to participate in the supplementary PORH-LDF measurement arm of the study. In addition, 6 participants were excluded because of low-quality PORH-LDF readings related to body motion artifacts, resulting in a total of 241 participants with both pre- and postclopidogrel PORH-LDF measurements. There was no significant difference between women and men in preclopidogrel PORH-LDF (*P*=0.12) (Table 1). In addition, preclopidogrel PORH-LDF was not significantly different between premenopausal and menopausal women (*P*=0.60). Furthermore, in both univariate and multivariate analyses, no association was observed between conventional cardiovascular disease risk factors and preclopidogrel PORH-LDF measures, except for sex (Table 5).

Table 4. Univariate and Multivariate Regression Models for Association Between Various Cardiovascular Risk Factors and Percentage Change in Postclopidogrel TH-LDF

	Univariate Models, Percentage Change TH-LDF, β±SD, *P* Value	Multivariate Model, Percentage Change TH-LDF, β±SD, *P* Value
High composite platelet aggregation		
Yes	0.33±0.10, 0.01	0.29±0.02, 0.03
Age, y		
≥55	−0.002±0.001, 0.9	−0.02±0.05, 0.80
Sex		
Women	0.08±0.10, 0.50	0.0.08±0.10, 0.40
Menopausal state		
Yes	0.04±0.08, 0.90	—
BMI, kg/m^2		
≥30	−0.08±0.13, 0.60	−0.04±0.10, 0.40
Current smoker*		
Yes	−0.19±0.19, 0.30	−0.06±0.08, 0.40
SBP, mm Hg		
≥130	0.001±0.004, 0.80	−0.003±0.007, 0.66
DBP, mm Hg		
≥80	0.01±0.008, 0.14	0.02±0.01, 0.08
CRP, mg/dL		
≥1.0	−0.05±0.1, 0.67	−0.10±0.05, 0.45
LDL-C, mg/dL		
>130	−0.02 (0.010), 0.80	−0.01±0.10, 0.90
HDL-C, mg/dL		
<40 for men or <50 for women	0.18±0.10, 0.34	0.11±0.20, 0.60
TG, mg/dL		
≥200	0.01±0.06, 0.90	0.04±0.60, 0.90
*CYP2C19**2 genotype		
1 allele	—	0.03±0.01, 0.80
2 allele		−0.48±0.30, 0.15

Percentage change TH-LDF was calculated as follows: preclopidogrel TH-LDF−postclopidogrel TH-LDF/preclopidogrel TH-LDF)×100. BMI indicates body mass index; CRP, C-reactive protein; CYP2C19, cytochrome P450 2C19; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDF, laser Doppler flowmetry; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; TG, triglyceride; TH, thermal hyperemia.
*Smoking in men.

Table 5. Univariate and Multivariate Regression Models for Association Between Various Cardiovascular Risk Factors and Percentage Change in Postclopidogrel PORH-LDF

	Univariate Models, Percentage Change PORH-LDF, β±SD, *P* Value	Multivariate Model, Percentage Change PORH-LDF, β±SD, *P* Value
High composite platelet aggregation		
Yes	−0.20±0.10, 0.15	−0.19±0.16, 0.22
Age, y		
≥55	−0.15±0.10, 0.35	−0.05±0.08, 0.52
Sex		
Women	0.30±0.10, 0.01	0.48±0.16, 0.003
Menopausal state		
Yes	0.35±0.20, 0.10	—
BMI, kg/m^2		
≥30	−0.02±0.17, 0.90	−0.07±0.19, 0.70
Current Smoker*		
Yes	−0.40±0.20, 0.04	0.02±0.20, 0.90
SBP, mm Hg		
≥130	−0.08±0.14, 0.55	−0.03±0.17, 0.84
DBP, mm Hg		
≥80	−0.15±0.26, 0.50	−0.40±0.30, 0.21
CRP, mg/dL		
≥1.0	0.01±0.14, 0.90	0.07±0.17, 0.67
LDL-C, mg/dL		
>130	0.13±0.10, 0.28	0.30±0.15, 0.06
HDL-C, mg/dL		
<40 for men or <50 for women	−0.17±0.25, 0.50	−0.05±0.33, 0.88
TG, mg/dL		
≥200	−0.040±0.50, 0.54	−0.13±0.60, 0.80
*CYP2C19**2 genotype		
1 allele	0.05±0.15, 0.73	0.05±0.10, 0.73
2 allele	0.06±0.35, 0.87	0.06±0.30, 0.86

Percentage change PORH-LDF was calculated as follows: preclopidogrel PORH-LDF−postclopidogrel PORH-LDF/preclopidogrel PORH-LDF)×100. BMI indicates body mass index; CRP, C-reactive protein; CYP2C19, cytochrome P450 2C19; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; LDF, laser Doppler flowmetry; LDL-C, low-density lipoprotein cholesterol; PORH, postocclusive reactive hyperemia; SBP, systolic blood pressure; TG, triglyceride.
*Smoking in men.

Postclopidogrel PORH-LDF was significantly higher in women than in men (474.7±231.1 versus 404.4±196.8, *P*=0.009) (Table 1). There was no association between composite platelet aggregation or conventional cardiovascular risk factors and percentage change in PORH-LDF (Table 4). There was no significant absolute change in postclopidogrel PORH-LDF from preclopidogrel PORH-LDF in women or men (Table 1), despite higher baseline composite platelet aggregation in women (*P*=0.13) (Table 6). Pretreatment platelet volume was measured but was not significantly associated with percentage change in TH-LDF or PORH-LDF (data not shown).

Table 6. Paired Change From Post- to Preclopidogrel TH- and PORH-Mediated Laser Doppler Flowmetry in the High Composite Platelet Aggregation Group

	Total	Women	Men
Preclopidogrel TH	1757±766	1909±846	1445±589*
Postclopidogrel TH	2153.8±1054.5	2518±1048	1646±845*
	n=67, P=0.03†	n=39, P=0.001†	n=28, P=0.70†
Preclopidogrel PORH	445.6±190.7	493.1±192.0	396.3±179.4
Postclopidogrel PORH	404.0±195.8	412.7±200.6	394.9±193.8
	n=59, P=0.30†	n=30, P=0.13†	n=29, P=0.90†

Data are shown as mean±SD. PORH indicates postocclusive reactive hyperemia; TH, thermal hyperemia.
*P<0.05 for TH difference between men and women, the analysis is of the residual data after adjustment for age and family relatedness.
†P-value for difference between pre- and postclopidogrel TH.

Heritability of Pre- and Postclopidogrel TH-LDF and PORH-LDF Measures

TH-LDF showed significant heritability when measured before clopidogrel administration (h^2 0.36±0.2, P=0.05), accounting for age and sex. Heritability was slightly higher after the administration of clopidogrel (h^2 0.46±0.2, P=0.02). Additional adjustment for *CYP2C19**2 attenuated postclopidogrel TH-LDF heritability (h^2 0.32±0.16, P=0.05). The heritability estimate for PORH-LDF was not significant.

Discussion

A complex interplay exists between platelet function and the endothelium in which platelet activation can affect endothelial function and endothelial dysfunction may, in turn, influence platelet aggregation. Given this multifaceted reciprocal relationship, we evaluated the response of skin microcirculation endothelial function quantified by TH-LDF and PORH-LDF to clopidogrel administration in healthy persons. In addition, we assessed the effect of clopidogrel on microcirculatory endothelial function in persons with high versus low baseline composite platelet aggregation. We also assessed the association of microcirculatory endothelial function measures and conventional cardiovascular disease risk factors in persons without established coronary artery disease. Finally, we calculated the estimated heritability of LDF measures of endothelial function before and after exposure to clopidogrel.

We found that clopidogrel administration improves TH-LDF–based measures of microcirculatory endothelial function in healthy persons with high composite platelet aggregability and that the endothelial response was more robust among women. We did not find a relationship between clopidogrel use and our PORH-LDF measures.

The discrepancy between PORH-LDF and TH-LDF results is consistent with the fact that they measure different biological components of endothelial function and vascular reactivity, which was the initial rationale for using both measures. PORH-LDF captures a complex microvascular response to induced transient ischemia in which the endothelium plays a pivotal role, primarily independent of NO and more dependent on other metabolic vasodilators, the myogenic response, and sensory nerves.[25] In contrast, TH-induced vasodilatation in skin microcirculation is contingent mainly on endothelial cell–dependent NO bioavailability.[25,28,31] Previous in vivo studies found that clopidogrel can enhance the bioavailability of NO release,[15,16,21] and studies in animal models showed that clopidogrel may not confer its beneficial effect on endothelial function merely through P2Y12 receptors but possibly by interfering with the interaction between platelets and leukocytes, resulting in attenuated inflammation and NO bioavailability.[20–22] These in vivo and animal-based studies implicating a beneficial role of clopidogrel in NO bioavailability are consistent with our results showing improvement in TH-LDF, which is mediated primarily by NO.[23] Clinical studies have demonstrated that using antiplatelet therapy before percutaneous coronary intervention attenuates platelet activation and endothelial dysfunction, supporting favorable outcomes in the patients with myocardial infarction.[18,32] In addition, studies have shown that antiplatelet therapy improves endothelial function by diminishing inflammation and oxidative stress biomarkers, thereby enhancing NO bioavailability.[15–17,33] In the current study, postclopidogrel TH-LDF was increased in the participants with higher baseline platelet aggregation, and that finding is consistent with the hypothesis that the effect of clopidogrel results from modulation of high platelet activity.

Previous studies have suggested that platelet activation leads to the recruitment of inflammatory cells, releasing cytokines even in healthy women.[34,35] Activated platelets release CD40L, resulting in a local inflammatory response.[2] In addition, activated platelets release platelet factor 4, which upregulates the expression of E-selectin on endothelial cells, resulting in further recruitment and adhesion of inflammatory cells to the endothelial cells.[1] Zhang et al showed that clopidogrel improved macrovascular endothelial function measured by flow-mediated dilation in a healthy Chinese population.[19] This study was performed in only 12 participants and focused mainly on the macrovascular effect of clopidogrel and *CYP2C19**2 polymorphism and did not stratify or report effect modification based on platelet function or sex. A previous study looking at the effect of

clopidogrel and/or clopidogrel with aspirin on PORH-LDF brachial artery reactivity test did not find any effect[36]; however, this study had only 79 older patients (mean age of 63 years) with comorbidities and only 6 female patients. Moreover, the authors did not explore the influence of high platelet aggregability and did not measure microcirculatory response. In our larger study, we found a response in TH-LDF measures based on baseline hyperaggregability, with a stronger effect on women. In contrast with the study by Ostad et al, another study in relatively older participants showed long-term beneficial effect of clopidogrel on endothelial function in patients with coronary artery disease;[15] however, this study used acetylcholine-mediated brachial vasodilation and not PORH-LDF or TH-LDF–based response. In another small study of 20 participants with coronary artery disease, postclopidogrel endothelial function was measured based on a significantly different pulse wave amplitude system (no measure of blood flow), and it showed a favorable response.[37] Last, in a study of 91 participants with established coronary artery disease and effect of clopidogrel, high platelet aggregability was associated with decreased endothelial function; however, the study did not have pre- and postclopidogrel measures.[38] Limited reports note that P2Y12 receptors reside on the vascular cells[39–41] but show no evidence that P2Y12 receptors are located on endothelial cells. Moreover, in animal models, it has been shown that clopidogrel primarily mediates its effect on vascular endothelium by NO pathways, presumably independent of P2Y12 receptors.[20–22] These findings, with ours, suggest that platelet aggregation is associated with decreased endothelial function and that clopidogrel can confer a beneficial effect on endothelial function, probably by a NO-dependent pathway.

We did not find an association between our LDF measures and conventional clinical cardiovascular risk factors except for sex. We believe that the primary reason for this lack of association pertains to the fact that our population was healthy, as opposed to most studies, which included and/or selected patients with chronic diseases. Differences in blood pressure in a primarily normotensive population, for example, might not be associated with sufficient vascular disease to affect endothelial function significantly enough to be detectable in a study population with a modest sample size such as ours. In a distinct larger study of brachial flow-mediated dilation in >500 healthy participants, we similarly found no association between flow-mediated dilation and any established cardiovascular risk factors (Shabnam Salimi, MD, MSc, and Afshin Parsa, MD, MPH, unpublished data, 2016).

In the current study, we also found that the endothelial function-related benefit of clopidogrel in healthy persons with high platelet aggregability was more robust in women. Some studies reported increasing acute coronary syndrome in young women.[42–44] Doughty et al reported that participants with younger age constituted >10% of their patients with acute myocardial infarction, and >25% of those participants were women.[42] Our sex-specific findings in younger women could also suggest a potential interaction between endothelial function and platelet aggregation or perhaps sex-specific hormones. Moreover, we found, for the first time, significant heritability of baseline and postclopidogrel TH-LDF endothelial function, suggesting a significant genetic component to microcirculatory-based endothelial function.

Strengths and Limitations

Our study is the largest study to date aimed at testing the effect of clopidogrel on measures of microcirculatory endothelial function in a healthy population. The use of 2 complementary LDF-based methods enabled us to more comprehensively test for dermal microcirculatory endothelial function and vascular reactivity. The use of a highly homogenous founder population allowed us to minimize potential confounders, and *CYP2C19**2 genotype testing and our measures of platelet aggregability all allowed for careful analyses. Nevertheless, stratification by sex and baseline composite platelet aggregation resulted in smaller subgroups, limiting overall power. The lack of a secondary control group with non–clopidogrel-mediated platelet inhibition did not allow us to rule out a non–platelet-mediated effect of clopidogrel on endothelial function. Given that the positive effect of clopidogrel was noted only in those with high platelet aggregability, a non–platelet-mediated pathway seems less likely. Finally, pre- and postclopidogrel serum proinflammatory cytokines measurements and oxidative stress markers could have shed more light on the findings.

Conclusion

Our findings demonstrated that clopidogrel can improve TH-LDF, a proxy of NO bioavailability, which may have vascular-related benefits in addition to its well-established antithrombotic effects. The TH-LDF response to clopidogrel appears to be most pronounced in premenopausal women with high baseline composite platelet aggregation. This sex-based difference suggests a possible interaction among sex-specific hormones, platelet function, and TH-LDF. In addition, we found a genetic component to TH-LDF–based response, providing further impetus to study the genetic determinant of microcirculatory endothelial function. In summary, these findings implicate potential additional clinical benefits of clopidogrel on microcirculatory NO-mediated endothelial cell response in persons with high platelet aggregability.

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Disclosures

None.

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