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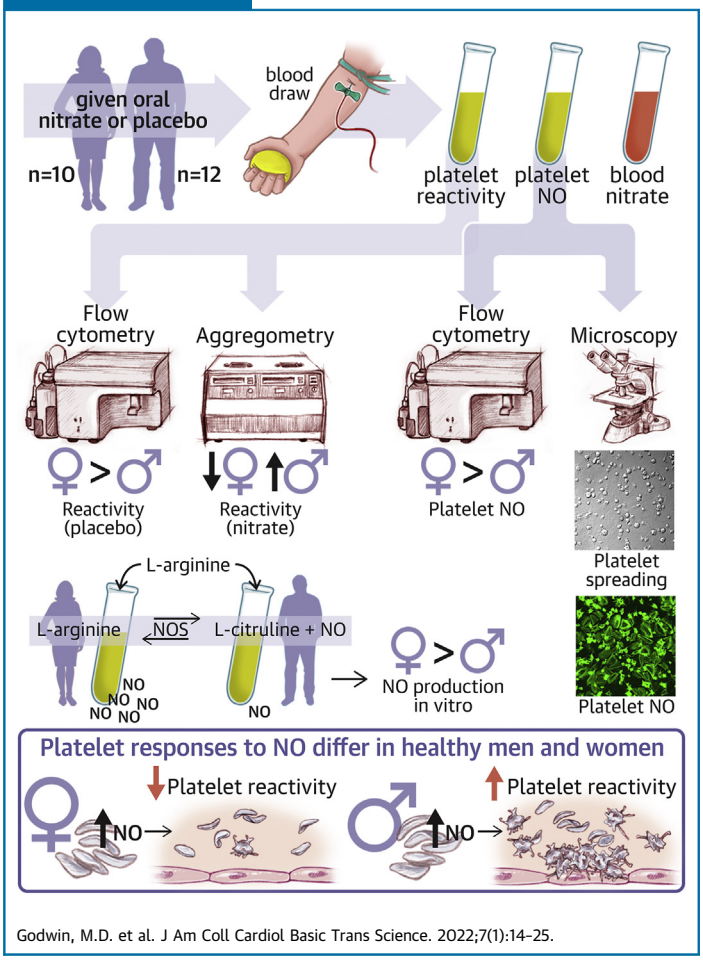
CLINICAL RESEARCH

Sex-Dependent Effect of Platelet Nitric Oxide

Production and Platelet Reactivity in Healthy Individuals

Matthew D. Godwin, BS,[a] Anu Aggarwal, PhD,[a] Zachary Hilt, PhD,[b] Shalini Shah, BS,[c] Joshua Gorski, MD,[c] Scott J. Cameron, MD, PhD[a,b,c,d,e]

VISUAL ABSTRACT



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HIGHLIGHTS

- **Platelet reactivity is greater in healthy women compared with men.**
- **Following an oral nitrate load, platelet nitric oxide production increased disproportionately more in healthy women than healthy men with attenuated platelet reactivity in women and enhanced platelet reactivity in men.**

From the [a]Cleveland Clinic Lerner College of Medicine, Cleveland Clinic Foundation, Cleveland, Ohio, USA; [b]Department of Medicine, Aab Cardiovascular Research Center, University of Rochester School of Medicine, Rochester, New York, USA; [c]Department of Medicine, Division of Cardiology, University of Rochester School of Medicine, Rochester, New York, USA; [d]Heart, Vascular, and Thoracic Institute, Department of Cardiovascular Medicine, Section of Vascular Medicine, Cleveland Clinic Foundation, Cleveland, Ohio, USA; and the [e]Taussig Institute, Department Hematology, Cleveland Clinic Foundation, Cleveland, Ohio, USA.

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SUMMARY

A nitrate-rich diet has many cardiovascular benefits, but the mechanism behind this is unclear. We hypothesized that the ingestion of nitrate augments nitrate to nitrite reduction, leading to nitric oxide (NO) production, which may suppress platelet reactivity. In a randomized, double-blinded, placebo-controlled study involving healthy individuals, ingestion of nitrate augmented saliva and plasma nitrite/nitrate concentration and enhanced platelet NO production disproportionately in women compared with men. The response of elevated platelet NO in men was increased platelet reactivity and the response of markedly elevated platelet NO in women slightly inhibited platelet reactivity. (J Am Coll Cardiol Basic Trans Science 2022;7:14–25)

ABBREVIATIONS AND ACRONYMS

ADP = adenosine diphosphate

DAF-FM = 4-amino-5-methylamino-2′, 7′-difluorofluorescein diacetate

LTA = light transmission aggregometry

NO = nitric oxide

PAR1 = protease-activated receptor 1

PKG = protein kinase G

VASP = vasodilator-stimulated phosphoprotein

Platelet reactivity and the response of antiplatelet agents may differ in health and disease. Various factors, including sex, diet, and clinical context, influence the phenotype and function of the platelet (1,2). We previously demonstrated that the platelet phenotype in peripheral artery disease and myocardial infarction is dysregulated so that platelets behave in an unpredictable manner and may show resistance to antiplatelet medications (3-5). Considering sex as a biological variable was not a significant feature of early preclinical antiplatelet medication studies. Platelet reactivity in women may not be the same as men, yet this is not taken into account when prescribing antiplatelet medications (2,5,6).

A plant-based diet may be recommended for patients with established vascular disease (7). The benefits of increased nitrate ingestion from a plant-based diet may include augmented NO production. NO was shown to inhibit platelet alpha granule and Weibel Palade body exocytosis in endothelial cells and in platelets through covalent modifications of N-ethylmaleimide-sensitive factor (8,9). Platelets have a dual role of regulating hemostasis as well as thrombosis. Platelet activation, therefore, is tightly regulated through controlled release of platelet-activating molecules including adenosine diphosphate (ADP), and thromboxane A_2, and platelet inhibitory molecules including prostaglandin E_2, prostaglandin I_2 (PGI_2), and nitric oxide (NO) (10,11).

We hypothesized that healthy men and women would have diminished platelet reactivity following oral nitrate ingestion if platelet NO production increases (12). As proof of principle that augmenting platelet NO production has beneficial effects, we conducted an investigation in which healthy men and women were administrated an oral nitrate load or placebo on 1 of 2 visits. We then assessed the effect of oral nitrate on blood nitrate concentration, platelet NO production, and platelet reactivity.

METHODS

DEMOGRAPHICS. The randomized controlled trial was approved by the Institutional Review Board (IRB) at the University of Rochester. A separate protocol was approved by the IRB at the University of Rochester and also the IRB at the Cleveland clinic to study translational aspects of platelet biology in vitro. We evaluated in a randomized, double-blinded, placebo-controlled manner whether capsules containing nitrate (2 Berkeley Life Professional tabs, each containing 490 mg potassium nitrate) alter platelet NO production and receptor agonist sensitivity. Given that age and obesity were previously reported to affect platelet activation (13,14) 22 healthy Caucasian individuals were recruited (12 men, 10 women) who were without medical conditions, of similar age and body mass index, who were nonsmokers, not taking medications, and not taking supplements (**Figure 1**). Each subject presented twice; once for the administration of nitrate then placebo in a random order by an individual not involved in the study. One male subject had obesity class 1 and 1 female subject was postmenopausal. The washout period was a minimum of 2 weeks between treatments. This time period was chosen given that NO lasts for minutes in the body and this was a 1-time dose of nitrate. In

The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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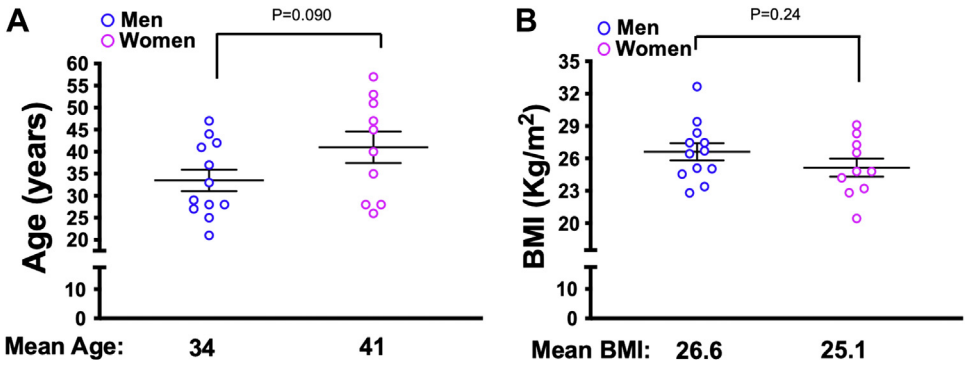
FIGURE 1 Demographics of Healthy Subjects



Healthy subjects were fasted and not taking medications or supplements. **(A)** Age was similar for men and women by Student's *t*-test. **(B)** Body mass index (BMI) was similar for men and women by Mann-Whitney *U* test. Only 1 female subject was postmenopausal and only 1 male subject had a BMI in the obese range. Data are represented as mean ± SEM for n = 12 men **(blue)** and n = 10 women **(pink)**.

addition, the entire population of platelets is replaced from bone marrow megakaryocytes by 7 days in humans.

NITRATE AND NITRITE ABSORPTION. Ninety minutes after oral administration of nitrate or placebo, saliva was analyzed for NO using a saliva test strip with colorimetric changes indicative of systemic NO production by an individual not involved in the study (Berkeley Life). One of the 22 subjects by choice did not complete saliva and plasma nitrate/nitrite assessment. EDTA plasma was stored for assessment of plasma nitrite/nitrate by the Griess reaction according to the manufacturer's instructions (Cayman Chemical) (15).

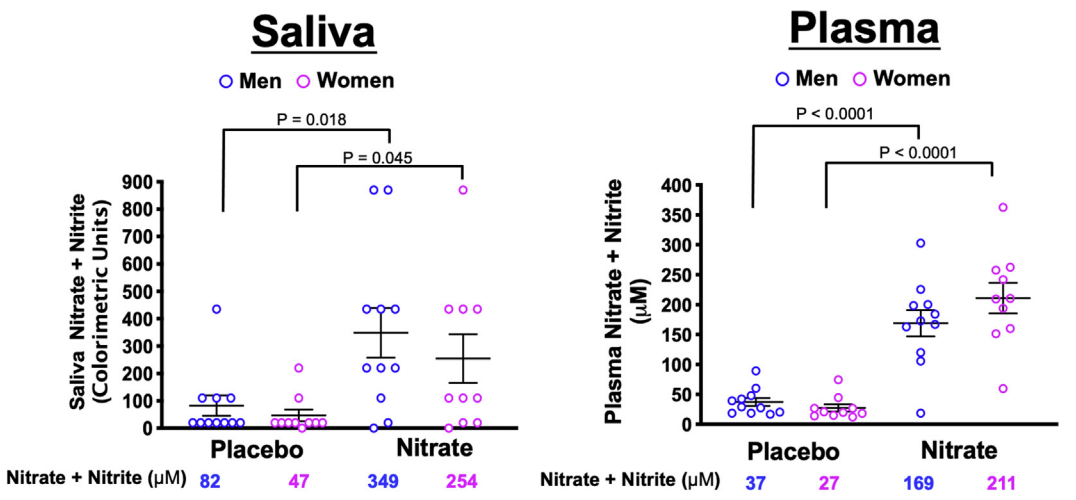
CONFOCAL MICROSCOPY AND STAINING. Ninety minutes after oral administration of nitrate or placebo, washed platelets were isolated from citrate plasma, spread on a fibrinogen matrix as we described previously (5), fixed with 2% formalin, permeabilized, and stained for P-selectin (BD Pharmingen) or NO with either a phycoerythrin-tagged P-selectin antibody or fluorophore 4-amino-5-methylamino-2′, 7′-difluorofluorescein diacetate (DAF-FM, Thermo Fisher), respectively. Stained platelets were visualized using confocal microscopy for localization of P-selectin and NO.

PLATELET FUNCTION. After administration of nitrate or placebo, washed platelets were isolated from citrate plasma. Washed platelets were stimulated with 4 concentrations of thrombin receptor-activating peptide 6 (TRAP6) (protease-activated receptor 1 [PAR1] agonist), U46619 (thromboxane receptor agonist), and ADP ($P2Y_{12}$ receptor agonist), and stained with a phycoerythrin-tagged P-selectin antibody (anti-CD62P antibody, BD Pharmingen). Platelet surface receptor density for the $P2Y_{12}$ receptor, the thromboxane receptor, and PAR1 was determined using fluorescent-tagged receptor antibodies, and quantified by flow cytometry. Platelet NO production was quantified by flow cytometry following DAF-FM (Thermo Fisher) incubation for 30 minutes at 37°C, followed by a wash step, and centrifugation with fresh PgI_2 to wash away excess dye while preserving platelet integrity. Light transmission aggregometry (LTA) (Chrono-log) was conducted using platelet-rich plasma.

WESTERN BLOTTING. Platelet lysate from citrate plasma was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and proteins were transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked for an hour in 3% bovine serum albumin and incubated overnight for vasodilator-stimulated phosphoprotein (VASP), VASP phosphorylated on Ser-239, Protein Kinase G (PKG), endothelial nitric oxide synthase (eNOS), and Tubulin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control. All antibodies were purchased from Cell Signaling Technology.

STATISTICAL TESTS. Statistical tests were conducted using GraphPad Prism 7 (GraphPad Software, Inc) after Shapiro-Wilks testing to assess for normalcy. Nonparametric group comparisons were made by the Mann-Whitney *U* test and, for 3 or more group comparisons, the Kruskal-Wallis test followed by Dunn's Post test. For Gaussian-distributed data between 2

FIGURE 2 **Absorption of Oral Nitrate in Healthy Individuals**



Healthy subjects were randomized to placebo or nitrate capsule in a double-blinded manner before blood analysis 90 minutes later. **(Left)** Saliva nitrate/nitrite concentration was assessed using a test strip for n = 11 men **(blue)** and n = 10 women **(pink)** by the Mann-Whitney *U* test. **(Right)** Plasma nitrate/nitrite concentration for n = 11 men **(blue)** and n = 10 women **(pink)** was assessed by enzymatic assay (Griess reaction) and significance determined by Mann-Whitney *U* test. Data are represented as mean ± SEM.

comparative groups, the *t*-test was used. The parametric and nonparametric statistical tests used are shown in each figure legend. Significance was accepted as a *P* value <0.05.

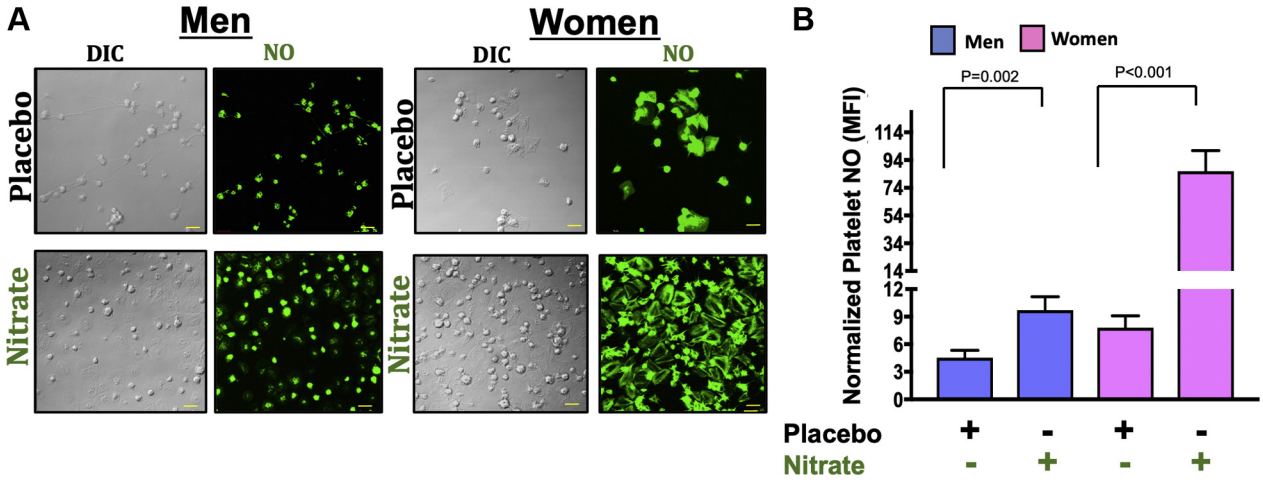
RESULTS

ADVERSE AND UNEXPECTED EVENTS. There were no serious adverse events. One healthy female subject experienced transient facial flushing and diaphoresis. One healthy male subject described transient belching. At the conclusion of the study, it was determined that both subjects were randomized to nitrate capsules.

NITRATE BIOAVAILABILITY AND CONVERSION TO NO. Ninety minutes following oral nitrate ingestion, saliva nitrite/nitrate concentration increased by 4.3-fold ($P = 0.018$ vs. placebo) in men and 5.4-fold ($P = 0.045$ vs placebo) in women, whereas plasma nitrite/nitrate concentration increased 4.6-fold in men ($P < 0.0001$ vs placebo) and 7.8-fold ($P < 0.0001$ vs placebo) in women (Figure 2). Interestingly, the subject with the highest plasma nitrate and nitrite concentration measured at 363 μM following an oral nitrate load was several standard deviations above the mean and the only subject on a plant-based diet. This finding suggests the possibility of enhanced efficiency in absorption or metabolism of nitrate in individuals accustomed to a plant-based diet (16). Plasma nitrate to nitrite conversion was coincident with a dramatic increase in platelet NO production in healthy women compared with men when visually inspected by confocal microscopy (Figure 3A) and approximately 2-fold and approximately 10-fold increase in platelet NO production in the platelets of women compared with men, respectively, when assessed quantitatively by flow cytometry (Figure 3B).

PLATELET RESPONSES FOLLOWING AN ORAL NITRATE LOAD. Surface P-selectin exteriorization from intracellular alpha granules to the platelet surface membrane is well-known to reflect activation of individual platelets (3,17). Following an oral nitrate load, platelet surface P-selectin expression was less in women compared with men when visually inspected by confocal microscopy (Figure 4A) and quantitatively reflected by a 2-fold decrease of platelet surface P-selectin by flow cytometry only in women after an oral nitrate load (Figure 4B). Platelet reactivity following agonist stimulation of surface receptors was dramatically higher in younger women compared with younger men through the thromboxane receptor, the $P2Y_{12}$ receptor, and PAR1, which agrees with a recent observation in an older population (5). After nitrate ingestion, platelets from healthy women were slightly inhibited, whereas male platelets, conversely, showed enhanced platelet reactivity following agonist stimulation (Figure 5). Although platelet surface P-selectin expression by flow

FIGURE 3 Platelet NO Detection After an Oral Nitrate Load



(A) Healthy subjects were randomized to placebo or nitrate capsule in a double-blinded manner. After 90 minutes, platelets were isolated, stained with a fluorescent probe, 4-amino-5-methylamino-2′, 7′-difluorofluorescein diacetate (DAF-FM), visualized by confocal microscopy on a fibrinogen matrix (representative images, **left**). **(B)** In separate experiments, platelet DAF-FM fluorescence was quantified in quadruplicate by fluorescence-activated cell sorting as mean ± SEM, n = 12 men **(blue)** and n = 10 women **(pink)**. Level of significance is noted, Mann-Whitney *U* test. **Yellow bar** = 5 μm. DIC = differential interference contrast; MFI = mean fluorescence intensity; NO = nitric oxide.

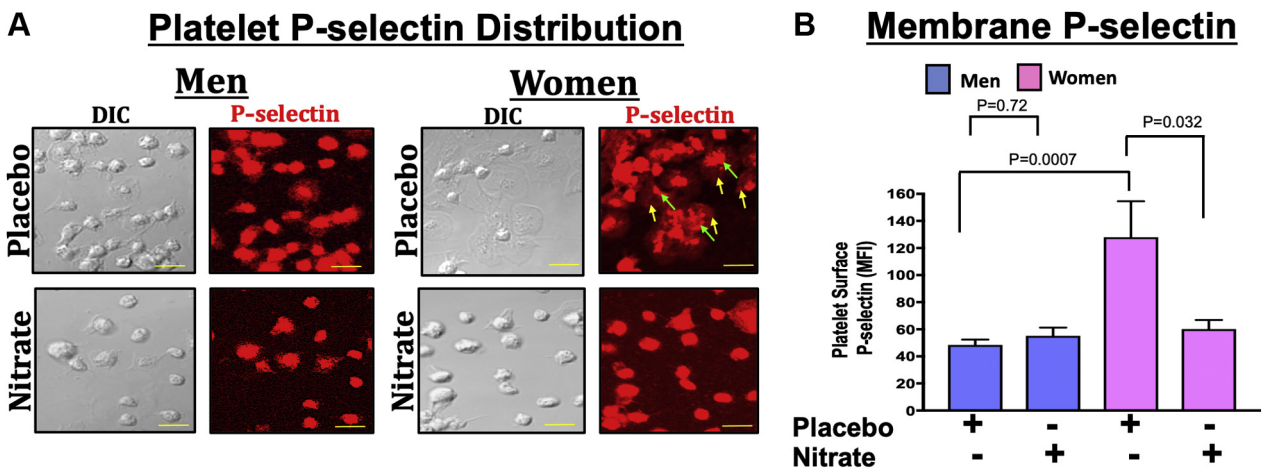
cytometry is generally accepted as a marker of individual platelet reactivity through degranulation, we also assessed the effect of nitrate ingestion on light transmission aggregometry (LTA), which measures platelet-to-platelet interaction before thrombus formation. Comparing healthy subjects not taking any medications or supplements with subjects who take nitrate capsule supplements daily, we also observed differences in platelet activation by LTA between men and women (Figures 6A to 6D). Curiously, the effect of enhanced platelet reactivity in women was most pronounced when using the PAR1 agonist TRAP6.

PLATELET RESPONSES FOLLOWING DIRECT NITRATE EXPOSURE. To evaluate the possibility that the mechanism for sex differences in platelet nitrate exposure requires chemical conversion to nitrite in the gut, we incubated platelets from healthy men and women with nitrate, and found platelet NO production is enhanced only in men. To evaluate for sex differences in endogenous platelet NO production as well as to confirm the specificity of DAF-FM as a fluorophore for NO, we incubated washed platelets from men and women with the NO synthase (NOS) substrate L-Arginine in the presence of either vehicle or an NOS inhibitor L-N^G-Nitro arginine methyl ester (L-NAME) (Figure 7A). Platelet NO production was enhanced with NOS substrate supplementation only in women by more than 4-fold, an effect that was fully reversible with L-NAME, confirming both the specificity of DAF-FM as well as fundamental sex differences is the response to supplements that augment platelet NO through increased NOS activity (Figure 7B).

Nitrate ingestion did not affect platelet count, white blood cell count, or red blood cell count (Supplemental Figure 1). A previous investigation on the effect of erythrocytes on NO (which did not document the sex of platelet donors) revealed erythrocyte reduction of nitrate to NO and a change in platelet reactivity (18). Because hemoglobin can scavenge NO (19,20), we measured hemoglobin concentration of men and women following placebo administration and found it to be 14.6 ± 0.41 and 13.3 ± 0.65, respectively (P = 0.100). For nitrate-treated subjects, the hemoglobin concentration was 14.6 ± 0.82 and 14.4 ± 0.53, respectively (P = 0.84). This provides assurance that the markedly higher platelet NO production in women compared with men is not from a sex-dependent difference in blood hemoglobin concentration.

Platelet $P2Y_{12}$ surface receptor density was the only receptor with marked differences between healthy men and women, and greater in women (Supplemental Figure 2). The discordance between platelet reactivity and surface receptor expression suggests that differences in postreceptor signal

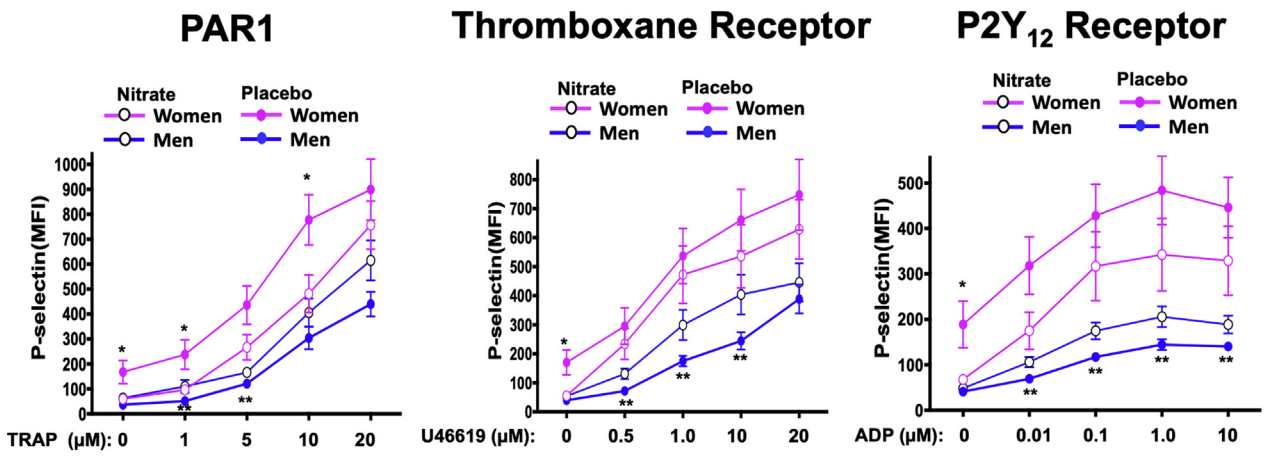
FIGURE 4 Platelet P-Selectin Distribution After an Oral Nitrate Load



Healthy subjects were randomized to placebo or nitrate capsule in a double-blinded manner. **(A)** After 90 minutes, platelets were isolated, permeabilized, and stained with a phycoerythrin-tagged P-selectin antibody, and visualized by confocal microscopy on a fibrinogen matrix (representative images, left). Punctate P-selectin inside platelet alpha granules is indicated by **green arrowheads**, and diffuse platelet membrane P-selectin staining is noted by **yellow arrowheads**. **(B)** In separate experiments, platelet surface P-selectin was quantified in quadruplicate by fluorescence-activated cell sorting as mean ± SEM. Men **(blue)**, n = 12, and women **(pink)**, n = 10. Level of significance is noted, Mann-Whitney *U* test. Abbreviations as in Figure 3.

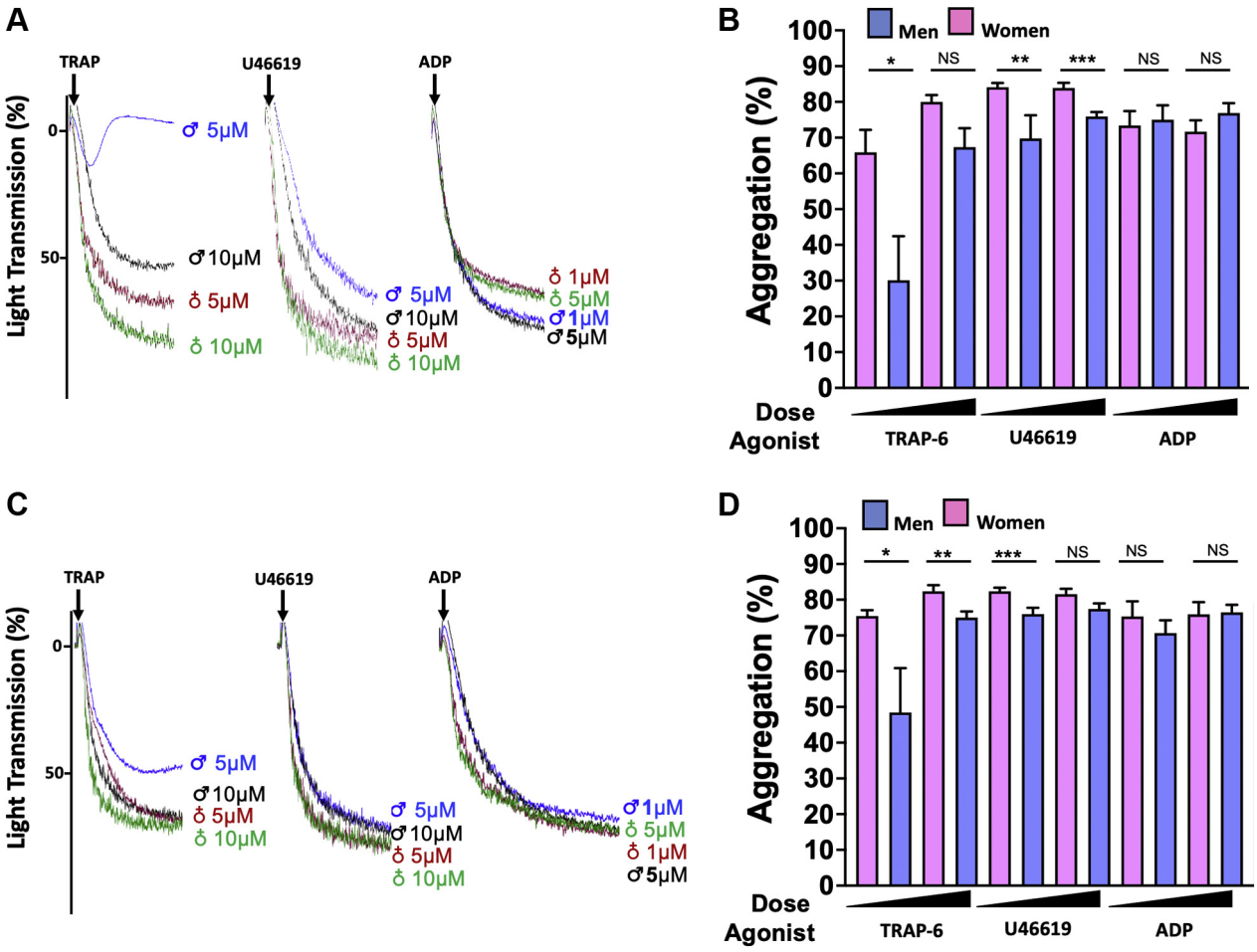
transduction pathways may exist between men and women in response to NO. Given that eNOS expression and an intact PKG module was previously reported in platelets (21), we confirmed this observation and determined eNOS expression was greater in healthy women than men (Figure 8A). Because NO stimulates platelet guanylate cyclase to hydrolyze guanosine triphosphate (GTP) to cyclic

FIGURE 5 Platelet Alpha Granule Exocytosis After Oral Nitrate Load Decreases in Females and Increases in Males



Healthy subjects were randomized to placebo or nitrate capsule in a double-blinded manner. After 90 minutes, platelets were isolated and stimulated with TRAP (PAR1 agonist), U46619 (thromboxane receptor agonist), and ADP ($P2Y_{12}$ receptor agonist); then stained with a phycoerythrin tagged P-selectin antibody and quantified in quadruplicate by fluorescence-activated cell sorting as mean ± SEM, n = 12 men **(blue)** and n = 10 women **(pink)**. *$P < 0.05$ between female placebo groups vs female nitrate groups and **$P < 0.05$ for male placebo groups vs male nitrate groups at the concentration shown. Group differences analyzed by the Kruskal-Wallis test followed by Dunn's posttest correction. ADP = adenosine diphosphate; MFI = mean fluorescence intensity; PAR1 = protease-activated receptor 1; TRAP = thrombin receptor-activating peptide.

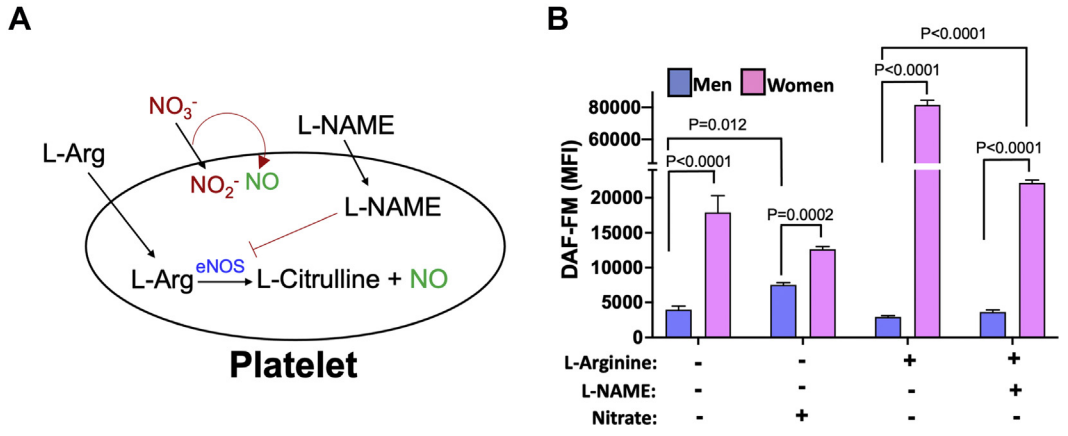
FIGURE 6 Platelet Aggregometry in Healthy Men and Women



(A) Platelet-rich plasma (PRP) was isolated from the blood of healthy women and men then stimulated with agonists for PAR1 (TRAP6), the thromboxane receptor (U46619), and the $P2Y_{12}$ receptor (ADP) at the concentrations indicated. Representative curves for each condition are shown by light transmission aggregometry. **(B)** Summary data of light transmission aggregometry on isolated rich plasma (PRP) from the blood of healthy women and men before stimulation with low- (5 μM) or high-dose (10 μM) TRAP6, with low- (5 μM) or high-dose (10 μM) U46619, with low- (1 μM) or high-dose (5 μM) ADP, and with low- (1 μg/mL) or high-dose (5 μg/mL). Data are quantified in as mean ± SEM, n = 3 men **(blue)** and n = 3 women **(pink)**. Group differences analyzed as indicated in parentheses depending on data distribution. **P* = 0.04 (Mann-Whitney *U*), ***P* = 0.0007 (*t*-test), ****P* = 0.0008 (Mann-Whitney *U*). **(C)** PRP was isolated from the blood of healthy women and men taking supplemental nitrate capsules daily before stimulation with and low (5 μM) or high (10 μM) TRAP6, with low (5 μM) or high (10 μM) U44619, and low (5 μM) or high (10 μM) ADP. Representative curves for each condition are shown by light transmission aggregometry. **(D)** Summary data of light transmission aggregometry on isolated rich plasma (PRP) from the blood of healthy women and men taking supplemental nitrate capsules daily before stimulation with low (5μM) or high (10 μM) TRAP6, with low (5 μM) or high (10 μM) U46619, and low (1 μM) or high (5 μM) ADP. Data are quantified in as mean ± SEM, n = 3 men **(blue)** and n = 3 women **(pink)**. **P* = 0.04 (*t*-test), ***P* = 0.008 (*t*-test), ****P* = 0.009 (*t*-test). NS = not significant; other abbreviations as in Figure 5.

guanosine diphosphate (cGMP) (22), we assessed downstream activated (phosphorylated) vasodilator-stimulated phosphoprotein that inhibits platelet reactivity (23). Although platelet PKG was similarly increased in men and women after an oral nitrate load (not shown), phospho-VASP (pVASP) on serine residue 239 was increased only in women (Figure 8B). A pictorial representation of the effect of platelet NO in men and women is shown in Figure 9. The key mechanistic differences with respect to NO production in platelets are that women require enteric absorption and processing of nitrate to generate nitrite and liberate NO in platelets, whereas platelets from men harbor a mechanism whereby

FIGURE 7 **Platelet NO Production Is More Pronounced in Women**



(A) Blood was drawn, and washed platelets were isolated from healthy men and women and incubated with a L-Arginine (1 mM, 30 minutes), which is a substrate for endothelial nitric oxide synthase (eNOS) known to be expressed in platelets. NO is released following conversion to L-Citrulline in the presence and absence of a competitive eNOS inhibitor L-NG-Nitro arginine methyl ester (L-NAME) (300 μM, 30 minutes). Nitrate (NO_3^-) capsules (1 mg/mL, 30 minutes) were also exposed to healthy platelets ex vivo and the effect on NO production in the platelet was assessed. **(B)** Platelet DAF-FM fluorescence, which is an indicator of NO concentration, was quantified in quadruplicate by fluorescence-activated cell sorting as mean ± SEM, n = 4 men **(blue)** and n = 4 women **(pink)**, and significance between indicated groups by the Kruskal-Wallis test followed by Dunn's posttest correction, as noted. DAF-FM = 4-amino-5-methylamino-2′, 7′-difluorofluorescein diacetate; L-Arg = L-Arginine; MFI = mean fluorescence intensity; NO = nitric oxide.

nitrate can be used as a substrate for NO production. Conversely, NOS can readily use L-Arginine as a substrate to markedly augment NO production in platelets from healthy women but there is little effect in platelets from healthy men.

DISCUSSION

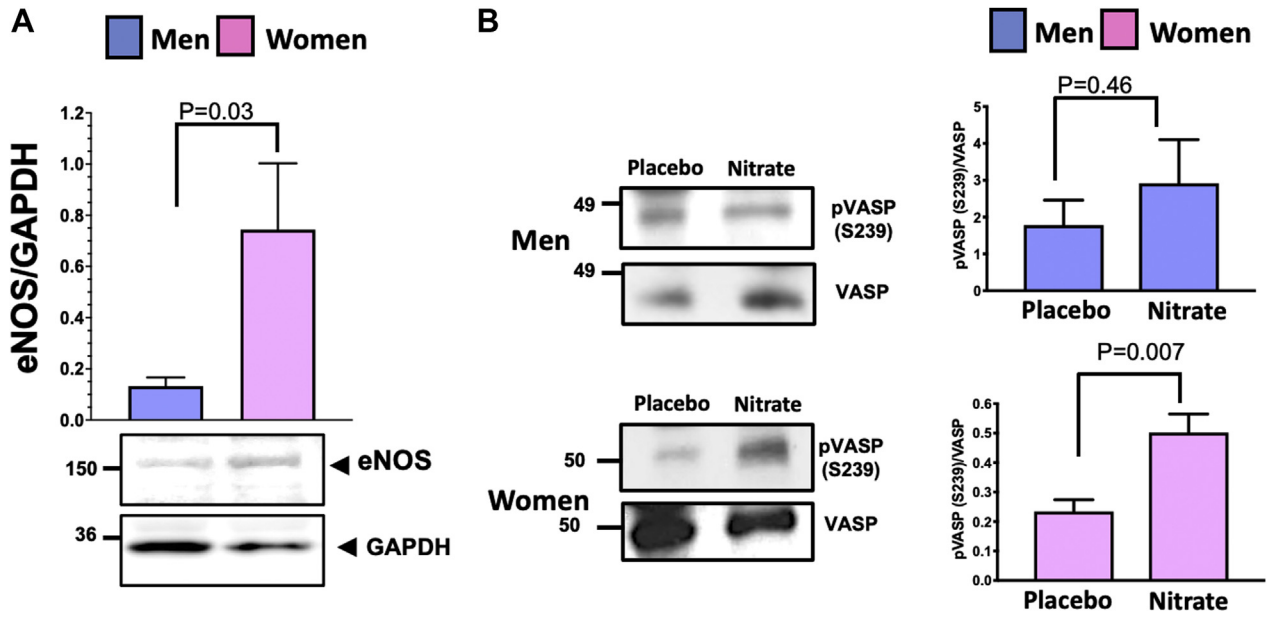
In this randomized and double-blinded investigation, we confirm that platelet reactivity is markedly greater in young healthy women compared with young healthy men and especially through platelet PAR1. We now show that oral nitrate, a molecule found in many vegetables, increases platelet reactivity in men and attenuates platelet reactivity in women coincident with augmented platelet NO production.

A critical distinction in the data between men and women is that enteric processing and absorption of nitrate is required for nitrate to augment platelet NO production in women, which does not appear to be a requirement for men. Loading platelets with the NOS substrate L-Arginine could readily liberate NO only in platelets from female donors, and incubation of nitrate could liberate NO only in platelets from male donors. These data suggest that nitrate requires processing in the gastrointestinal tract of female individuals to generate platelet NO, whereas male platelets may have a direct nitrate uptake transporter and biochemical capabilities of NO liberation outside of the gastrointestinal tract. The different responses in platelets obtained from blood after an oral nitrate load compared with nitrate given to washed platelets ex vivo may also be from nitrite reacting differently in the presence of erythrocytes found in whole blood as previously reported (18). A prior study showed that sialin (a product of the *SLC175* gene) is a membrane nitrate shuttle (24). Sialin is highly expressed in human platelets (25) and a sex-specific difference in expression could explain why nitrate given to washed platelets ex vivo increased platelet NO generation in men but not women.

We demonstrated 10-fold greater NO production in platelets from women compared with men after an oral nitrate load. Because plasma and saliva nitrate were similar in men and women, this also suggests the possibility of a nitrate shuttle that permits reduction to nitrite and NO release locally inside the platelet. Facultative anaerobes in the gut are well-known to reduce nitrate to nitrite enzymatically (26). Another explanation for our data is that the composition of gut microbiota required for NO reduction from nitrate may differ in men and women, as suggested (27).

Our data confirm previous reports that NO has an inhibitory effect on platelet activation, although we

FIGURE 8 Platelet Signaling in Healthy Men and Women After Nitrate



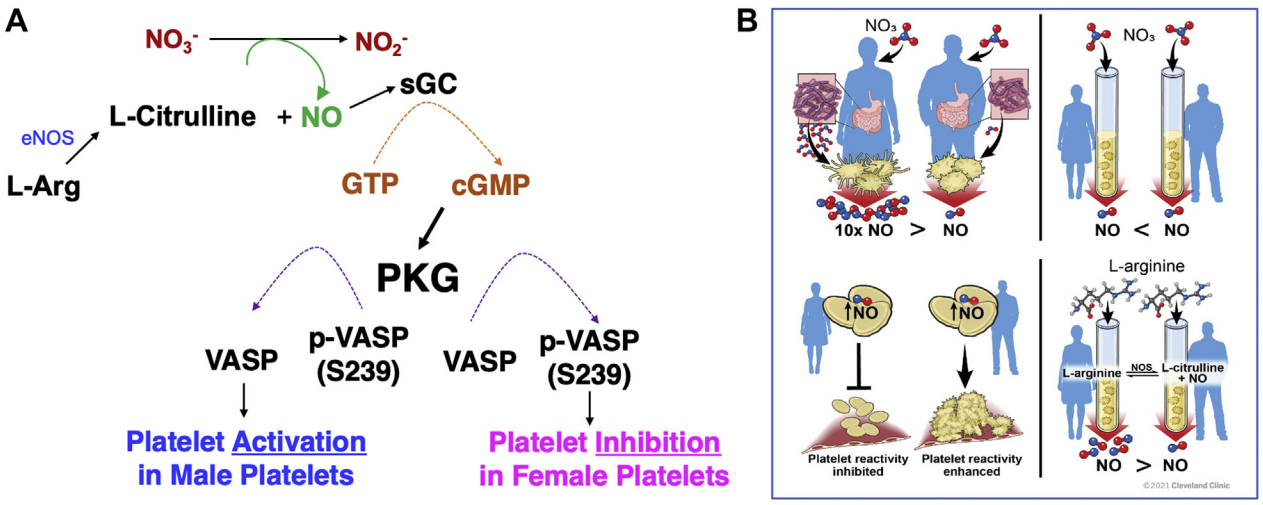
(A) Platelet eNOS expression in healthy men and women was assessed by immunoblotting. GAPDH is a loading control. Data are quantified as mean ± SEM, n = 5 men **(blue)** by Mann-Whitney *U* test and n = 4 women **(pink)** by Mann-Whitney *U* test. Level of significance is noted. **(B)** Healthy subjects were randomized to placebo or nitrate capsule in a double-blinded manner. After 90 minutes, platelets were isolated, and subjected to western blotting for VASP phosphorylated on Ser-239 (pVASP), and nonphosphorylated VASP is a loading control. Data are quantified as mean ± SEM, n = 4 men **(blue)** and n = 5 women **(pink)**. Level of significance is noted by Student's *t*-test. eNOS = endothelial nitric oxide synthase; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; VASP = vasodilator-stimulated phosphoprotein.

were surprised that this effect was observed only in female individuals. A prior investigation, notably enrolling mostly men, revealed platelet tolerance to inorganic nitrate ingestion might be related to the NO-scavenging effect of the free radical O_2^- (28). In isolated platelets ex vivo and in in vitro studies, NO production may be protective by inhibiting platelet granule exocytosis and platelet activation and by inhibiting endothelial granule exocytosis (8). The results of our study in male platelets in vivo suggests the opposite, that low levels of NO production augment alpha granule exocytosis and platelet reactivity. This observation is consistent with the study by Li et al. (29) that demonstrated NO production promotes platelet granule exocytosis and platelet activation.

In a study that did not reveal the sexual identity of experimental mice, manipulating the blood nitrite/nitrate balance through dietary restriction or antibiotic use alters platelet reactivity (30), invoking the gut microbiome as a potential explanation for sex-specific differences in platelet NO production. Sex-dependent differences in platelet reactivity and responsiveness to antiplatelet medications were previously suggested (2,5,6,31). In a recent study of men and women at the time of myocardial infarction, we demonstrated that protease-activated receptor signaling switches in opposite directions compared with healthy conditions in a pathway that likely involves changes in the expression of G-protein-coupled receptor subunits, and downstream platelet calcium mobilization (5). In the present study of healthy men and women, we show phosphorylation of VASP is increased by almost 2.5-fold with nitrate ingestion and platelet NO production only in women. Because VASP phosphorylation is well-known to inhibit platelet function (23,32), and phosphorylation of VASP at serine 239 is reported as a mechanism of platelet inhibition through the NO-VASP-PKG module (33), we offer this pathway as a potential mechanism for the sex-dependent difference in platelet reactivity to NO observed.

STUDY LIMITATIONS. A limitation of our study lies in the inclusion of premenopausal women with blood draws throughout the menstrual cycle. One report suggests differences in plasma-soluble P-selectin and

FIGURE 9 Platelet NO Production Depends on Sex and the Medium of Nitrate Delivery



(A) NO activates platelet-soluble guanylate cyclase (sGC), hydrolyzing guanosine triphosphate (GTP) cyclic guanosine monophosphate (cGMP), which activates protein kinase G (PKG). PKG is activated and phosphorylates VASP on serine residue 239 only in female platelets and attenuates platelet reactivity. **(B)** Oral nitrate reduction to nitrite in vivo leads to platelet NO generation and is more pronounced in women. Oral nitrate (NO_3^-) reduction to nitrite (NO_2^-) in vitro leads to platelet NO generation, which is more pronounced in men, whereas L-Arg loading of platelets and conversion to NO by eNOS is more efficient in women. eNOS = endothelial nitric oxide synthase; L-Arg = L-Arginine; NO = nitric oxide; VASP = vasodilator-stimulated phosphoprotein.

platelet reactivity in the follicular compared with the luteal phase of the menstrual cycle (34). In addition, we assessed platelet NO production and platelet reactivity 90 minutes after a single oral load of nitrate. The response may be more pronounced under steady-state conditions if the study was repeated in individuals after multiple daily doses of nitrate. Diet contributes to the composition of an individual's microbiota (35) that is required for NO reduction from nitrate (27). Differences in individual microbiota, as a result of diet, may determine how an individual can produce and tolerate nitrites (36,37). Although surface P-selectin is a common and useful marker for looking at activation of individual platelets, our data do suggest that confirming platelet responses using additional techniques, and especially LTA, which models platelet-to platelet interactions in vivo, is important to fully support findings obtained in translational research studies involving platelets.

CONCLUSIONS

In summary, we show that platelet reactivity individually and during aggregation is greater in healthy women than men, especially through PAR1, and we describe a divergent effect of oral nitrate on platelet NO production and platelet reactivity that is sex-specific and potentially involving a complex interaction with the gut microbiome. Women are typically underrepresented in cardiovascular investigations. Plasma nitrate/nitrite and the effect on platelet NO should be prospectively evaluated in men and women with respect to cardiovascular outcomes in patients who subscribe to a plant-based diet.

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ADDRESS FOR CORRESPONDENCE: Dr Scott J. Cameron, Cleveland Clinic Foundation, Heart Vascular and Thoracic Institute, Department of Cardiovascular Medicine, Section of Vascular Medicine, J3-5, 9500 Euclid Avenue, Cleveland, Ohio 44195, USA. E-mail: cameros3@ccf.org.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: There are many beneficial cardiovascular effects of a plant-based diet. Green, leafy vegetables are enriched in nitrate which is converted to nitrite by intestinal flora causing NO liberation. NO has several protective properties, including anti-inflammatory and vasodilatory functions, as well as the inhibition of thrombosis. The beneficial effect of dietary constituents, however, may be sex-specific. In a small double-blinded, randomized controlled trial, nitrate ingestion led to equivalent nitrite concentration in the blood of healthy men and women, but markedly increased NO production in the platelets of women compared with men. Increased platelet NO production in women was coincident with inhibition of platelet reactivity but we conversely observed platelet activation in men.

TRANSLATIONAL OUTLOOK: Platelet reactivity in healthy women is far greater than healthy men. Low levels of platelet NO production activates platelets, whereas high levels of platelet NO production inhibits platelets. Because the conversion of ingested nitrate to nitrite in in men and women was similar, this suggests the possibility that nitrate is processed differently by gut flora or that NO is liberated locally in the platelet in a sex-dependent manner. Sex-specific differences in the response to dietary substances require more careful consideration and study.

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APPENDIX For supplemental figures, please see the online version of this paper.