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Sex differences in flow cytometry–based platelet reactivity in stable outpatients suspected of myocardial ischemia

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Abstract

Background: Antiplatelet therapy is the mainstay of secondary prevention of cardiovascular events. Studies suggest that women do not obtain equal therapeutic benefit from antiplatelet therapy compared with men. The link between sex differences in platelet biology and response to antiplatelet therapies is unclear. We therefore investigated the role of sex differences in platelet reactivity in a cohort of outpatients with chest pain, in response to treatment with antiplatelet agents.

Methods: Platelet reactivity was measured in 382 randomly selected patients participating in the Myocardial Ischemia Detection by Circulating Biomarkers (MYOMARKER) study, an observational cohort study of outpatients suspected of myocardial ischemia. In all patients, blood was collected during diagnostic workup, and platelet reactivity was assessed with a flow cytometry–based platelet activation test that quantifies both platelet degranulation (P-selectin expression) and platelet aggregation (fibrinogen binding to integrin αIIbβ3) in whole blood.

Results: Platelet reactivity was higher in women compared with men when activated with protease activating receptor 1–activating peptide SFLLRN (PAR1-AP) and adenosine 5′-phosphate (ADP), independent of age, basal activation status, estimated glomerular filtration rate < 60, platelet count, statin use, the use of P2Y12 inhibitors, or the use of aspirin. P2Y12 inhibitor use strongly reduced fibrinogen binding after stimulation with PAR1-AP, but only slightly reduced platelet P-selectin expression. Calculation of the relative inhibition in P2Y12 users indicated 62% inhibition of the response toward ADP. Stratified analysis showed that women (n = 14) using P2Y12

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inhibitors showed less inhibition of fibrinogen binding after PAR1-AP stimulation than men (n = 38) using P2Y12 inhibitors.

Conclusions: These findings call for further study of differential effects of P2Y12 inhibitors in women with suspected myocardial ischemia.

KEYWORDS
antiplatelet therapy, flow cytometry, platelet, platelet activation, sex differences

Essentials

- Sex-based differences may exist in therapeutic benefit from antiplatelet therapy.
- The link between sex differences in platelet biology and response to antiplatelet therapies is unclear.
- Platelet degranulation and fibrinogen binding were measured in patients undergoing myocardial perfusion imaging.
- Protease activating receptor 1–activating peptide SFLLRN and Adenosine 5′-phosphate dependent inhibition is lower in women on P2Y12 inhibitors using fluorescence activated cell sorting based assay.

1 | INTRODUCTION

Coronary artery disease (CAD) is characterized by obstructive or nonobstructive atherosclerotic plaque accumulation in the epicardial arteries. This process can be modified by lifestyle adjustments, pharmacologic therapies, and ultimately invasive treatments such as percutaneous intervention (PCI), coronary artery bypass grafting. The aims of pharmacologic management are to reduce angina symptoms and exercise-induced ischemia and to prevent cardiovascular events. Pharmacologic treatment consists of angina/ischemia relief (short-acting nitrates, β-blockers, and/or calcium channel blockers) combined with drugs for cardiovascular event prevention (statins and antiplatelet agents such as low-dose aspirin and clopidogrel).

Thrombosis is driven by platelet activation and aggregation and causes symptomatic coronary disease. Antiplatelet agents such as P2Y12 inhibitors are therefore the mainstay of antithrombotic therapy after a myocardial infarction or PCI.[1] Clopidogrel is a P2Y12 receptor inhibitor that blocks the ADP-induced signaling pathway for platelet activation. Interindividual responses to P2Y12 inhibitors have been described to vary from excessive platelet inhibition leading to bleeding complications, to insufficient inhibition of platelet reactivity leading to thrombotic events.[2,3] The latter is known as high-on-treatment platelet reactivity and is associated with an increased risk of secondary cardiovascular events.[4]

In the past decade, rates of cardiovascular deaths and recurrent atherothrombotic events in women have increased, while these rates have stabilized in men.[5,6] It is known that women have a higher bleeding risk and differentially benefit from antithrombotic therapy in terms of secondary prevention of ischemic events, compared with men.[5] Differences in platelet function between men and women have been reported, varying from differences in platelet counts, surface expression of receptors, or functional reactivity to agonist stimulation,[7] but the exact link between sex differences in platelet biology and response to antithrombotic therapies is unclear, which complicates defining the therapeutic window of antiplatelet therapy in women.[6] Better understanding of these differences and their clinical implications could lead to better optimized therapies for prevention of secondary cardiovascular events in women, without the risk of bleeding.

Hence, we aimed to determine whether there are sex differences in platelet reactivity in response to treatment with antiplatelet agents in a cohort of outpatients suspected for myocardial ischemia. Since high-on-treatment platelet reactivity is a prognostic risk factor for cardiovascular events in P2Y12 inhibitor users,[8] these results will help to understand whether sex differences in (residual) platelet reactivity will have implications for the efficacy of antiplatelet therapy in women.

2 | METHODS

2.1 | Study population

The Myocardial Ischemia Detection by Circulating Biomarkers (MYOMARKER) study (Dutch Trial Register Identifier: NTR5210) is an observational cohort study of patients undergoing myocardial perfusion imaging by Rubidium-82 positron emission tomography/computed tomography (^{82}Rb PET/CT) in the outpatient clinic as diagnostic workup for suspected myocardial ischemia. Patients were prospectively and consecutively enrolled between August 2014 and September 2016 from the Meander Medical Center in Amersfoort, the Netherlands. Patients younger than 18 years were excluded. The study was performed in accordance with the Declaration of Helsinki and was approved by the regional ethics committee and the institutional review board of Meander Medical Center in Amersfoort. Written informed consent was obtained from all patients.

2.2 | Reagents

Allophycocyanin (APC)-conjugated antiplatelet glycoprotein Ibα (GPIbα) antibody and R-phycoerythrin (RPE)-conjugated anti–P-selectin antibody were purchased from BD Pharmingen (Franklin Lakes, NJ, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-fibrinogen antibody from Dako (Glostrup). Adenosine 5′-phosphate (ADP) was obtained from Sigma Aldrich (Zwijndrecht), protease activating receptor 1 (PAR1)-activating peptide SFLLRN (PAR1-AP) from Bachem (Weil am Rhein) and U46619 from Cayman Chemical (Ann Arbor, MI, USA). Cross-linked collagen-related peptide (CRP-xL) was a generous gift from Professor Richard Farndale (Cambridge University). P2Y12 antagonist AR-C69931MX (AR-C; cangrelor) was kindly provided by AstraZeneca (Loughborough).

2.3 | Flow cytometric analysis of platelet activation markers in whole blood

Platelet reactivity was measured as described[9] in 382 randomly selected patients participating in the MYOMARKER study. Prior to ^{82}Rb PET/CT, peripheral venous blood was collected from the intravenous cannula routinely inserted in preparation for PET/CT into 3.2% sodium citrate Vacutainer tubes (BD Biosciences, Franklin Lakes, NJ, USA). All blood samples were processed within 1-6 hours after blood collection. Whole blood was diluted 1:10 (v/v) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline (10 mM HEPES, 150 mM NaCl, 1 mM MgSO4, 5 mM KCl, pH 7.4), containing APC-conjugated anti-GPIbα (1:25) either in combination with RPE-conjugated anti–P-selectin antibody (1:25) or FITC-conjugated antifibrinogen antibody (1:100) in the absence or presence of a single concentration of agonists (30 μM ADP, 1 μg/mL CRP-xL, 100 μM U46619, or 100 μM PAR1-AP alone or in combination with 1 μM AR-C). After 15 minutes at room temperature, samples were fixed (0.148% formaldehyde, 137 mM NaCl, 2.7 mM KCl, 1.12 mM NaH2PO4, 1.15 mM KH2PO4, 10.2mM Na2HPO4, 4mM ethylenediaminetetraacetic acid, pH 6.8) for 20 minutes at room temperature. Test strips were stored at 4°C until analysis (within 48 hours) on a fluorescence-activated cell sorter (FACS Canto II, BD Biosciences, San Jose, CA). Single platelets were identified on the basis of forward and sideward scatter properties and APC-conjugated anti-GPIbα antibody binding. Fibrinogen binding was determined as a measure of integrin αIIbβ3 activation and P-selectin expression as a measure of granule release. Data were analyzed with FACSDiva software 6.1.2 (BD Biosciences) and expressed as median fluorescence intensity (MFI) after normalization to correct for batch-to-batch variations.

2.4 | Statistical analysis

Statistical analyses were performed with SPSS version 17.0 (SPSS, Chicago, IL). P values <.05 were considered statistically significant. Continuous variables were expressed as mean ± standard deviation (SD) and categorical variables as frequencies (%). Differences in continuous variables were compared with independent *t*-test. Dichotomous variables were compared with Fisher's exact test. Relative inhibition in P2Y12 users was calculated with the following formula:. Values were expressed as group means ± SD. Multivariable linear regression analysis was performed to adjust the difference in platelet reactivity per agonist between men and women for potential influential covariables, including age, the use of aspirin, the use of statins, kidney function (estimated glomerular filtration rate [eGFR]) <60, platelet count, and baseline activation status of platelets.

3 | RESULTS

3.1 | Baseline characteristics

The study population concerned 382 randomly selected patients participating in the MYOMARKER study. Thirty-nine percent (n = 150) of them were women. Baseline characteristics are provided in Table 1. Overall, the prevalence of classical risk factors such as diabetes (21%), hypertension (64%), hypercholesterolemia (58%), history of smoking (52%), obesity (median body mass index, 27.2 [interquartile range (IQR), 25-30]), and glomerular filtration rate <60 (27%) was high. Antiplatelet therapy included aspirin as monotherapy in 45% of the patients, P2Y12 inhibitor monotherapy in 2% of the patients, and dual antiplatelet therapy in 11% of the patients. Eighty-five percent of the P2Y12 inhibitor users received clopidogrel. History of CAD was present in 45% of patients but was less frequently observed in women compared with men (32% vs 53%; $P < .001$). Furthermore, women were older than men (69 vs 67 years; $P = .033$) and although still in the normal range, platelet counts were higher in women (median 262 × 10^9 cells/L [IQR, 217-295] vs. 222 × 10^9 cells/L [IQR, 191-256]; $P < .001$).

3.2 | Therapeutic P2Y12 Inhibitor use and platelet reactivity

Platelet activation is accompanied by activation of integrin αIIbβ3, the fibrinogen receptor, and the secretion of the content of the α and dense granules into the extracellular environment of the platelets. We first studied the effect of therapeutic P2Y12 inhibition on platelet reactivity by comparing platelet responses between P2Y12 inhibitor users and nonusers (Table 2). Since subanalysis on aspirin users and nonusers showed no differences in platelet activation (Table S1), patients with only aspirin use were also part of the P2Y12 inhibitor nonuser group. P2Y12 inhibitor use strongly reduced fibrinogen binding after stimulation with ADP, CRP-xL and PAR1-AP, as well as, to a lesser extent, P-selectin expression. To determine the efficacy of P2Y12 inhibitor use, we compared PAR1-AP–induced platelet reactivity with or without ex vivo incubation of platelets with cangrelor (AR-C69931MX; AR-C) in P2Y12 inhibitor users and nonusers. AR-C was previously reported to

TABLE 1 Baseline Characteristics

	Overall	Men	Women	*P* value
N	382	232 (61)	150 (39)	
Age, y, mean (SD)	67.9 (9.9)	67.0 (9.6)	69.2 (10.1)	.03
BMI, median (IQR)	27.2 (24.7-30.3)	27.4 (24.9-30.0)	26.7 (24.1-30.4)	.27
Risk factor, n (%)				
Diabetes	80 (21)	49 (21)	31 (21)	1.00
Hypertension	244 (64)	147 (63)	97 (65)	0.83
Hypercholesterolemia	223 (58)	134 (58)	89 (59)	0.83
Smoking				
Current	64 (17)	38 (16)	26 (17)	<0.01
Former (>30 d)	198 (52)	138 (60)	60 (40)	
Never	119 (31)	55 (24)	64 (43)	
Family history of CAD	110 (29)	66 (29)	44 (29)	0.91
Patient history, n (%)				
History of CAD	172 (45)	124 (53)	48 (32)	<0.01
History of MI	100 (26)	71 (31)	29 (19)	0.02
History of PCI	125 (33)	90 (39)	35 (23)	<0.01
History of CABG	68 (15)	47 (20)	21 (8)	<0.01
GFR < 60 mL/min	104 (27)	55 (24)	49 (32)	0.10
Medication, n (%)				
Aspirin	213 (56)	139 (60)	74 (49)	0.05
P2Y12 inhibitor	52 (14)	38 (16)	14 (9)	0.07
Clopidogrel	44 (85)	33 (87)	11 (79)	
Ticagrelor/Prasugrel	8 (15)	5 (13)	3 (2)	
Anticoagulant	71 (19)	46 (20)	25 (17)	0.50
Thrombin inhibitor	16 (4.2)	11 (5)	5 (3)	
Vitamin K antagonist	55 (14.4)	35 (15)	20 (13)	
Statin	232 (61)	153 (66)	79 (53)	0.01
Other lipid-lowering agent	31 (8)	19 (8)	12 (8)	1.00
Laboratory results, median (IQR)				
Platelet count 10^9/L	231 (200-276)	222 (191-256)	262 (217-295)	<0.01
Hemoglobin (mmol/L)	8.7 (8.2-9.4)	9.1 (8.5-9.5)	8.3 [7.9-8.8]	<0.01

Note: P values are calculated for differences between male and female patients.

BMI, body mass index; CABG, coronary artery bypass grafting; CAD, coronary artery disease (defined as patients who received coronary revascularization [either percutaneous or surgical] or documented MI by a cardiologist; GFR, glomerular filtration rate; IQR, interquartile range; MI, myocardial infarction; PCI, percutaneous coronary intervention; SD, standard deviation.

completely inhibit ADP-induced platelet aggregation, fibrinogen binding, and P-selectin expression and has a strong inhibiting effect on PAR1-AP–induced fibrinogen binding.[2] Similar to these observations, incubation of platelets with AR-C resulted in almost complete inhibition of PAR1-AP–induced fibrinogen binding, but only slightly decreased PAR1-AP–induced P-selectin expression, showing that PAR1-AP–induced fibrinogen binding depends on secondary activation by ADP released from activated platelets (Table S2). Furthermore, the inhibitory effect of AR-C on fibrinogen binding upon stimulation with PAR1-AP was substantially stronger than the effect of therapeutic P2Y12 inhibitor use on PAR1-AP–induced fibrinogen binding in our cohort, which indicates residual platelet activity toward ADP in patients on therapeutic P2Y12 inhibitors. Calculation of the relative inhibition in P2Y12 users indicated 62% (±33) inhibition of the response toward ADP.

3.3 | P2Y12 inhibitor use and sex-specific differences in platelet reactivity

We first compared platelet activation in response to agonist stimulation between women and men in our total cohort. Baseline platelet

TABLE 2 Platelet reactivity stratified by P2Y12 inhibitor use and sex

	Total			Men			Women		
	No P2Y12 inhibitor use	P2Y12 inhibitor use	*P*	No P2Y12 inhibitor use	P2Y12 inhibitor use	*P*	No P2Y12 inhibitor use	P2Y12 inhibitor use	*P*
	n = 330	n = 52	value	n = 194	n = 38	value	n = 136	n = 14	value
Fibrinogen binding, MFI, AU, mean (SD)									
Basal activation status	74 (24)	69 (20)	.14	73 (23)	67 (20)	.18	76 (25)	73 (20)	.70
PAR1 - AP	3014 (1420)	1582 (1268)	.00	2815 (1403)	1414 (1109)	<.01	3297 (1402)	2036 (1582)	<0.01
PAR1 - AP + AR-C	533 (248)	527 (282)	.89	512 (245)	507 (295)	.91	562 (249)	583 (243)	.76
ADP	3192 (1558)	1245 (1330)	<.01	3126 (1534)	1056 (1092)	<.01	3288 (1591)	1759 (1777)	<0.01
CRP-xL	5117 (1864)	3738 (2119)	<.01	5137 (1983)	3393 (1855)	<.01	5088 (1687)	4674 (2555)	.41
U46619	2544 (2327)	2407 (2299)	.69	2609 (2498)	2500 (2500)	.80	2451 (2064)	2156 (1886)	.61
P-selectin expression, MFI, AU, mean (SD)									
Basal activation status	97 (47)	80 (38)	.02	96 (42)	75 (26)	.004	98 (54)	94 (59)	.80
PAR1 - AP	15 894 (5388)	14 185 (5024)	.03	15 407 (5221)	13 551 (4447)	.04	16 588 (5563)	15 905 (6196)	.67
PAR1 - AP + AR-C	13 526 (4696)	12 720 (4408)	.25	13 084 (4550)	12 250 (4236)	.30	14 155 (4843)	13 997 (4770)	.91
ADP	5327 (2140)	2132 (2405)	<0.01	5091 (2055)	1697 (1335)	<.001	5663 (2220)	3315 (3945)	<0.01
CRP-xL	14 657 (5121)	13 142 (5360)	.05	14 399 (5166)	12 470 (4356)	.03	15 025 (5053)	14 965 (7336)	.97
U46619	7481 (5729)	6832 (5246)	.44	7481 (5992)	7068 (5470)	.70	7481 (5352)	6189 (4710)	.39

Note: Results are displayed as mean ± SD.

ADP, adenosine diphosphate; AU, arbitrary units; CRP-xL, crosslinked collagen-related peptide; MFI, mean fluorescence intensity; PAR1-AP, protease-activated receptor 1–activating peptide; PAR1-AP + AR-C, protease-activated receptor 1–activating peptide + P2Y12 antagonist AR-C69931MX (cangrelor); SD, standard deviation.

activation status was similar in men and women. Platelet fibrinogen binding and P-selectin expression in response to stimulation with the agonists PAR1-AP and ADP were higher in women than in men (Table S3). To study sex-specific differences in the efficacy of therapeutic P2Y12 inhibitors, fibrinogen binding and P-selectin expression were compared in P2Y12 inhibitor users and nonusers stratified by sex (Table 2). In both men and women, P2Y12 inhibitor use caused a decrease in the PAR1-AP and ADP-stimulated fibrinogen binding capacity, and a decrease in fibrinogen-binding capacity after CRP-xL stimulation in men.

Next, differences in efficacy of P2Y12 inhibitors between men and women were assessed. The relative inhibition in women on P2Y12 inhibitors was 55% (±38) compared with 64% (±31) in men; this difference was not significant ($P = .417$). Although complete inhibition was not achieved in either male or female patients, P2Y12 inhibitors were more effective in men than in women. Differences in PAR1-AP–induced fibrinogen-binding capacity and ADP-induced P-selectin expression were independent of possible influencing covariables such as age, kidney function (eGFR) <60, use of aspirin, use of statins, use of P2Y12 inhibitors, platelet count, and baseline activation status of platelets (Table 3).

4 | DISCUSSION

Because the link between sex differences in platelet biology and response to antiplatelet therapies is unclear, we compared the efficacy of antiplatelet therapy in women and men with a flow cytometry–based platelet activation test in a general outpatient chest pain cohort. Overall, agonist-induced P-selectin expression and fibrinogen binding were higher in women compared with men. In both men and women, P2Y12 inhibitors were not able to completely block agonist-induced platelet reactivity, but, interestingly, the efficacy of P2Y12 inhibitor use was lower in women compared with men for both fibrinogen binding and P-selectin expression. Several explanations have been proposed for the sex-specific differences in platelet reactivity.

The presence of more vulnerable plaques and more severe structural and functional abnormalities in epicardial coronary arteries could be one of the causes of lower platelet reactivity in men.[10] Endothelial irregularities can overstimulate the platelets, thereby desensitizing platelet receptors and depleting the remaining platelet reactivity. In other words, highly reactive platelets will form aggregates and are rapidly removed from the circulation, leaving behind

TABLE 3 Average difference in platelet reactivity in women compared with men adjusted for possible influential covariables

	Average difference	95% CI
Fibrinogen binding, MFI, AU, mean (SD)		
PAR1-AP	441	24 to 858
PAR1-AP + AR-C	65	−16 to 147
ADP	436	−16 to 889
CRP-xL	399	−154 to 952
U46619	−374	−1078 to 330
P-selectin expression, MFI, AU, mean (SD)		
PAR1-AP	1006	−582 to 2594
PAR1-AP + AR-C	883	−523 to 2288
ADP	839	191 to 1488
CRP-xL	790	−691 to 2271
U46619	−796	−2607 to 1016

Note: Multivariable linear regression analysis, results are displayed as the average difference in platelet reactivity (in MFI arbitrary units) in women compared with men adjusted for aspirin use, statin use, P2Y12 inhibitor use, age, kidney function (eGFR) <60, platelet count, and baseline activation status with corresponding 95% CI.

ADP, adenosine diphosphate; AU, arbitrary units; CI, confidence interval; CRP-xL, crosslinked collagen-related peptide; MFI, mean fluorescence intensity; PAR1-AP, protease-activated receptor 1–activating peptide; PAR1-AP + AR-C, protease-activated receptor 1–activating peptide + P2Y12 antagonist AR-C69931MX (cangrelor); SD, standard deviation.

the less responsive and the preactivated platelets that are less susceptible to further stimulation.[8] In our study population, spontaneous coronary artery dissection was more often present in men than women. However, in our cohort, platelet count was normal in both men and women, and no significant differences were observed when comparing the baseline platelet activity in men and women.

Several studies showed higher platelet reactivity and reduced effectiveness of P2Y12 inhibitors among elderly patients.[11-15] This could be explained by a decline in the cytochrome P450 (CYP450) enzymes in patients with advanced age or an impairment in platelet turnover as consequence of age.[12-14,16] Age has also been associated with increased plaque instability and vulnerability.[17] In our study, age differences between both sexes were minimal, and even after correction for age in the multivariable regression analysis, the differences in platelet reactivity remained present.

Whether the observed differences in platelet reactivity might also be explained by differences in levels of sex hormones is not entirely clear. Some studies report an increase in aggregation and clotting parameters in postmenopausal women due to lower estradiol levels.[7,18] Estradiol is suggested to inhibit platelet aggregation by stimulating the production of prostacyclin. Testosterone, on the other hand, has a prothrombotic effect by increasing the synthesis of and responsiveness to thromboxane A2.[7] Yet other studies find sex hormones as the sole reason for the differences in efficacy of antiplatelet agents unlikely[7] and did not find evidence that menopausal status has an effect on an increased platelet reactivity.[19]

Finally, it is known that CYP450 polymorphisms are associated with variable responses to clopidogrel. Since these polymorphisms are equally distributed among men and women, we expect that differences in outcome due to genotype dissimilarities are very unlikely, and therefore we did not investigate it in this study.[5]

In contrast to the proposed theories for existing differences in platelet reactivity between men and women, a recent meta-analysis of randomized trials of potent P2Y12 inhibitors, including prasugrel, ticagrelor, and intervenous cangrelor, in patients with coronary artery disease, showed comparable efficacy and safety of potent P2Y12 inhibitors in men and women.[20] However, compared to potent P2Y12 inhibitors, clopidogrel shows more variability in inhibiting platelets with a considerable individual heterogeneity.[3] Increased event rates among patients with a decreased platelet inhibition response to clopidogrel have been reported.[21] Consistent with our study, some studies found that women more often have a decreased platelet inhibition response to clopidogrel, but the clinical implications of these findings remain unclear.[21] A meta-analysis that examined cardiovascular efficacy of clopidogrel opposes the above-mentioned results and suggests no significant difference in efficacy between men and women.[22]

4.1 | Limitations

Comparisons made between subgroups are based on small numbers of patients (38 male and 14 female patients in P2Y12 inhibitor users) and therefore need to be interpreted with caution. Furthermore, this study was not powered to investigate the effect of the difference in platelet reactivity on clinical outcomes. This study was also underpowered to perform subanalyses on the effect of different types of antiplatelet agents. Therefore, clinical implications of our findings remain unclear. Furthermore, patients included in this study were referred for perfusion imaging, indicating a population with a higher cardiovascular risk profile. This could have led to selection bias.

5 | CONCLUSIONS

We showed for the first time in a stable outpatient chest pain cohort that platelet reactivity is higher in women compared with men, independent of age, basal activation status, or the use of P2Y12 inhibitors and/or the use of aspirin. Additionally, with our flow cytometry–based platelet activation test, we were able to demonstrate that women showed higher residual on P2Y12 treatment platelet reactivity compared with men. Based on this study alone, clinical implications are unclear. More randomized controlled trials that equally focus on women are needed to optimize gender-specific therapy and further improve clinical outcomes in women.

RELATIONSHIP DISCLOSURE

SJAK and RTU are stockholders in U-PACT BV, a spin-off company from UMC Utrecht. The other authors have no disclosures that would be a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Study design: FW, MD, IEMB, SK, RTU, DPVdeK, and LT. Data collection: FW, MD, IEMB, AMS, and AM. Data analysis and interpretation: FW, MD, IEMB, SJAK, RTU, GJdB, DEG, AM, DPVdK, and LT. Drafting article and figures: FW, MD, IEMB, SJAK, RTU, GJdB, DEG, AM, DPVdKleijn, and LT. Critical revision and final approval: FW, MD, IEMB, SJAK, RTU, GJdB, GP, AMS, DEG, AM, DPVdK, and LT.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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