Aerobic exercise training lowers platelet reactivity and improves platelet sensitivity to prostacyclin in pre- and postmenopausal women

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Running head: Platelet function in pre- and postmenopausal women

Essentials

- Basal platelet reactivity was higher in recent postmenopausal women than late premenopausal women
- 3-months of high-intensity aerobic exercise spinning training in pre- and postmenopausal women:
 - Increased their platelet sensitivity to the inhibitory effect of arterially infused prostacyclin
 - Reduced basal platelet reactivity in the premenopausal women only

Summary

Background

The risk of atherothrombotic events in women increases after menopause. Regular physical activity is known to protect against cardiovascular disease and has been shown to reduce platelet reactivity in younger women, but it is unknown how regular exercise affects platelet function after menopause.

Objectives

To examine the effects of regular aerobic exercise in late pre- and recent postmenopausal women by testing platelet reactivity and sensitivity to the platelet inhibitors prostacyclin and nitric oxide.

Methods

25 sedentary, but healthy, late premenopausal (49 ± 0.4 years old) and 24 matched recently postmenopausal women (53 ± 0.6 years old), participated in a 3-month high-intensity supervised aerobic cycle-training intervention (1 hour, 3 times/week). Platelet reactivity was analyzed *in vitro* in platelet rich plasma obtained from venous blood as agonist-induced platelet aggregation in a 96-well light-transmission aggregometry assay. In a subgroup of 13 pre- and 14 postmenopausal women, platelet reactivity was tested *ex vivo* after femoral

arterial infusion of a prostacyclin analogue, acetylcholine, a cyclooxygenase inhibitor and after acute one-leg knee extensor exercise.

Results

Platelet reactivity was higher in the postmenopausal women at baseline. Exercise training reduced basal platelet reactivity in the premenopausal women only. After the training intervention, platelet aggregation was more inhibited by the prostacyclin infusion and the acute exercise in both pre- and postmenopausal women.

Conclusions

These results highlight previously unknown cardioprotective aspects of regular aerobic exercise in pre- and postmenopausal women, improving their regulation of platelet reactivity through an increased platelet sensitivity to prostacyclin, which may counterbalance the increased atherothrombotic risk associated with menopause.

Keywords:

Menopause, Nitric Oxide, Physical Activity, Platelet Aggregation, Prostacyclin

Background

Menopause, with the consequent loss of estrogen, is associated with an accelerated rate of endothelial dysfunction and postmenopausal women have an increased risk of atherothrombotic events compared to premenopausal women [1-3]. Estrogen stimulates endothelial production of the platelet inhibitors nitric oxide [4] and prostacyclin [5] and has been shown to have an inhibitory effect on platelet aggregation *in vitro* [6, 7]. Thus, loss of estrogen following menopause may result in increased platelet reactivity, but it is still debated if postmenopausal women have more reactive platelets than premenopausal women [8, 9].

Regular physical activity induces many of the same cardioprotective effects as estrogen on the cardiovascular system [10, 11] including modulating platelet function [12, 13]. The effects of regular exercise on platelet function have been studied in young pre-menopausal women where basal platelet reactivity [12] and platelet activation markers [13] were reduced by 2 months of regular aerobic exercise training. It is, however, unknown how regular physical activity influences platelet function after menopause.

The aim of the current study was to evaluate platelet reactivity in healthy pre- versus recently postmenopausal women of similar age, before and after a 3-month high-intensity aerobic exercise training intervention.

We hypothesized that postmenopausal women do have more reactive platelets compared to pre-menopausal women. In addition, we hypothesized that exercise training may compensate for the loss of estrogen during menopause by normalizing platelet reactivity and enhancing platelet sensitivity towards endothelium-derived prostacyclin and nitric oxide.

Materials and Methods

This cross-sectional, prospective study was part of an inter-disciplinary research initiative called the Copenhagen Women Study, looking into the effects of exercise training on women in the menopausal transition. More detailed information about the study design, recruitment of participants and training intervention are outlined in Mandrup et al [14]. The protocol for

this study was approved by the ethics committee of Copenhagen (H-1-2012-150). All subjects were informed of the risks and discomforts associated with the study and were recruited after providing their written informed consent to participate in the study in accordance with the Declaration of Helsinki. The study was registered at ClinicalTrials.gov (NCT02135575).

Subjects

The study included basal platelet reactivity testing of platelets (see experimental day 1) from 25 premenopausal and 24 postmenopausal women. A more in-depth analysis of platelet reactivity in response to various arterial infusions and acute exercise (see experimental day 2) was performed in a subgroup of 13 premenopausal women and 14 postmenopausal women. The women were recruited from the Copenhagen area via newspaper advertisements where the inclusion criteria were: premenopausal women aged 45-53 still having normal and regular menstrual cycles and postmenopausal women aged 50-57 with amenorrhea for at least the past 2 years, BMI > 18.5 and <30, sedentary (no regular physical activity the last 2 years). Exclusion criteria included smoking, excessive alcohol intake, regular medication including hormone replacement therapy or chronic disease. Menopausal status was verified by laboratory measurements of hypothalamic hormones.

Exercise training intervention

Experimental days were performed before and after a 3-month supervised exercise training intervention where the subjects performed exercise on a cycling ergometer for 1 hour, 3 times/week. Heart rate was monitored in all subjects during every spinning training session (TEAM2 WearLink+, Polar). The aim of each supervised spinning training session was to provide interval training with intensities ranging from moderate to high intensity, with heart rates above 80% of maximal heart rate in all women for the majority of the training sessions [14]. Characterization of fitness level, by determination of pulmonary maximal oxygen uptake (VO_2 max, Oxycon Pro, Intramedic) was assessed by an incremental bicycle ergometer

exercise test (10-min warm-up at 50W, followed by an incremental increase by 25W every minute until exhaustion) before and after the training intervention.

Preparation of optimul platelet aggregometry 96-well plates

This protocol was adapted from Chan et al. [15, 16]. Briefly, platelet agonists adenosine diphosphate (ADP, final concentrations: 0.02–40 μ M, Sigma A2754), epinephrine (0.001–10 μ M Sigma E4375), Thrombin receptor activating peptide amide-6 (TRAP-6: 0.03–40 μ M, SFLLRN Bachem H2936), the thromboxane (TXA₂) analogue U46619 (0.02–40 μ M, Cayman Chemical Company 16450) and collagen (0.004 - 4 μ g/ml, Taceda 1130630) were added to individual wells of gelatin-coated, flat-bottom, 96-well half-area plates that were lyophilized, vacuum-packed, stored at room temperature and used within 4 weeks.

Experimental days

The subjects were advised not to exercise and to refrain from caffeine and alcohol within 24 hours before the experimental days and to avoid non-steroidal anti-inflammatory drugs for at least 2 weeks prior to the experimental days. All the experimental days in the premenopausal women were strictly timed to occur within the mid-follicular phase of their ovarian cycle (7-15 days after their last period) in order to avoid possible confounding effects on platelet function by the different phases of the menstrual cycle as previously reported [17].

Flow diagram over the prospective design of platelet reactivity testing in the Copenhagen Women Study.

Experimental day 1 - Determination of basal platelet reactivity and nitric oxide sensitivity

See Mandrup et al [14] for a detailed methodological description of this experimental day. All participants had fasted overnight and had been resting in the supine position for over 30 min when blood was drawn from their antecubital vein. The primary outcome of this experimental day was to test if the postmenopausal women had higher basal reactivity than the premenopausal women and if this was affected by exercise training. Basal platelet reactivity was defined as agonist-induced *ex vivo* platelet aggregation in platelet rich plasma (PRP) obtained from blood drawn at resting (basal) conditions. A secondary outcome of this experimental day was to examine platelet nitric oxide sensitivity in the women. Hence, PRP from a subgroup of n=12 pre- and n=12 postmenopausal women was pre-incubated *in vitro* for 1 min with the nitric oxide donor diethylamine NONOate (DEA/NONOate, D5431 Sigma final concentrations 1-10000nM) before stimulation with 4µg/ml of collagen in the optimul aggregation assay.

Experimental day 2 - Determination of platelet reactivity after arterial infusions and acute exercise

A full description of this experimental day can be found in Nyberg et al [18, 19]. A subgroup of 13 pre- and 14 postmenopausal women participated in this experimental day. In short, catheters were inserted by a medical doctor under local anesthesia into the femoral artery (for infusions) and into the femoral vein of the experimental leg (for blood sampling). After 20 min of rest, the infusion solutions were infused via a pump through the arterial catheter in the subject resting in a supine position. The infusion rates were based on the leg volume of the subject (calculated based on measurements of circumferences and partial lengths of the leg). The infusions were always performed in the same order and were separated by 30 min to avoid carry over effects. The ketorolac infusion was purposely performed as the last infusion, due to its long plasma half-life (4-6 hours). According to the manufacturer of epoprostenol, the half-life in human plasma (based on inhibition of platelet aggregation) is 10.6 min in females and it has been reported that platelet reactivity returns towards baseline 10 min after

discontinuation of an arterial acetylcholine infusion [20]. Hence the 30 min wash-out periods between the infusions used in our study protocol were sufficient to avoid carry-over effects between the infusions. This was confirmed by a full return to baseline of blood flow and platelet reactivity after the 30 min wash-out period.

1. Epoprostenol infusion protocol

The synthetic prostacyclin analogue epoprostenol (Flolan, 25, 50, and 100ng min⁻¹L leg volume⁻¹, GlaxoSmithKline), was infused into the femoral artery for 2.5 min per dose and venous blood samples were collected at baseline and after 2.0 min of every infusion dose. Agonist-induced %platelet aggregation (platelet reactivity) testing was performed in PRP from these blood samples in order to test one of the primary outcomes of this study, namely if platelet sensitivity to prostacyclin is affected by menopausal status and/or by exercise training.

2. One-leg knee-extensor acute exercise test

The subject was transferred to the one-leg knee extensor (chair) ergometer and performed 5-min one-leg knee extensor exercise at 10Watt (W) and 20W respectively. Venous blood samples for platelet function testing were drawn at baseline and after the highest intensity bout (20W) in order to test the effects of acute (localized) exercise at moderate intensity on platelet reactivity (secondary outcome).

3. Acetylcholine infusion protocol

Acetylcholine (Miochol-E, 10, 25, and 100 µg min⁻¹ L leg volume⁻¹, Bausch & Lomb Inc.), was infused into the femoral artery for 2.5 min per dose and blood samples were collected at baseline and after 2.0 min of every infusion dose. Venous blood samples for platelet function testing were collected at baseline and at the end of the highest infusion rate (100µg min⁻¹L leg volume⁻¹) in order to test another primary outcome of the study: investigating the effects of acetylcholine-stimulated endothelial release of nitric oxide and prostacyclin on platelet reactivity in both groups of women, before and after the training intervention.

4. Ketorolac plus acetylcholine infusion protocol

Ketorolac (Toradol; $200\mu\text{g min}^{-1}\text{L leg volume}^{-1}$; Roche) was infused for 5 min before the acetylcholine protocol was repeated and kept constant until termination of the acetylcholine infusion. Venous blood samples for platelet function testing were collected after 4.5 min of ketorolac infusion and at the end of the highest acetylcholine infusion rate ($100\mu\text{g min}^{-1}\text{L leg volume}^{-1}$) in order to examine the contribution of platelet TXA_2 and endothelial prostacyclin and nitric oxide to platelet responses in each condition (secondary outcome).

Optimul assay of platelet aggregation

In both experimental day 1 and 2, blood was collected into vacutainers (sodium citrate, 3.2% Greiner-bio-one 454332) and immediately centrifuged for 10min (at 180g, 20°C), in order to separate PRP from the red blood cells. The remaining blood was further centrifuged for 2-min (15000g , 20°C) to obtain platelet poor plasma (PPP). $40\mu\text{l}$ PRP or PPP was pipetted into the appropriate test and control wells of the agonist coated optimul plate and placed on a shaker for 5 min (1200 rpm , 20°C , Eppendorf thermomixer EPP406) and read using a plate reader (Emax Precision Microplate Reader, Molecular Devices) and the light-absorbance by the PRP and PPP suspensions were measured at 595nm. The test was typically completed within 30 min of blood collection and timings and pipetting order were kept the same before and after the training intervention to avoid bias.

Blood analysis

Whole blood samples were analysed for estrogen (P-estradiol), follicle-stimulating hormone (FSH, P-follitropin) and luteinizing hormone (LH, P-lutropin) at the Department of Clinical Biochemistry at Rigshospitalet, using a competitive and a sandwich electrochemiluminescence immunoassay (all on a Modular E-Module) respectively. Plasma concentrations of the prostacyclin breakdown product 6-keto prostaglandin $\text{F}_{1\alpha}$ (6-keto $\text{PGF}_{1\alpha}$) was measured using an immunoassay kit (EIA; 515211, Cayman Chemical Co).

Statistical analysis

The study size was based on power calculations of the primary outcome measure (platelet function and vascular function), using the standardized difference method [21] where the α -level was set to 0.05 and the power level to 0.8, and was based on detecting a 20% difference in basal platelet reactivity after the training intervention based on previous reports [12] and a 25% inhibitory effect size by the acetylcholine and epoprostenol infusions [20]. Data are reported as mean $\pm$ standard error of mean (SEM). Differences in subject characteristics (Table 1) were detected using a two-tailed t-test for paired or unpaired data. Statistical Analysis were performed using GraphPad Prism5 and was analyzed using One-Way or Two-Way ANOVA as appropriate, with Bonferroni multiple comparison post-test. The Kolmogorov–Smirnov test and the Shapiro–Wilk test of normality confirmed that the data was normally distributed. Missing data points led to the exclusion of subjects from the matched ANOVA analysis, final n-numbers are indicated in each figure legend.

Results

Subject Characteristics

The premenopausal women were ~4 years younger compared to the postmenopausal women (Table 1). All the premenopausal women had regular menstrual bleeding and the menopausal status of all participants was verified by their hormonal levels (Table 1). There was no difference in fitness level ($VO_2\text{max}$, mL/min per kg) between the groups of women before the training intervention (Table 1) and exercise training increased $VO_2\text{max}$ similarly in the two groups (by $8.9 \pm 1.5\%$ in the pre- and by $8.8 \pm 1.6\%$ in the postmenopausal women). Platelet count was within normal range ($150\text{--}450 \times 10^9/\text{L}$), not different between the groups, and was not affected by exercise training (Table 1). Mean platelet volume was also similar between the pre- and postmenopausal women, before and after the training intervention (approx. 9 fL, $n=16$).

Basal platelet reactivity

The steepest part of the basal platelet reactivity aggregation response curves to TRAP-6 and U46619 were shifted to the left in the postmenopausal women, (Figure 1 C+D) and a smaller but similar shift to the left was found in the aggregation response curves to epinephrine and ADP (Figure 1B+E). These left-hand shifted curves indicate that platelets from the postmenopausal women required less stimuli to aggregate and therefore had a higher reactivity to these agonists compared to platelets from the premenopausal women.

Exercise training had an inhibitory effect on collagen-induced platelet aggregation in the premenopausal women only, indicating that exercise training lowered basal platelet reactivity to this agonist (Figure 1A). No other alterations in basal platelet reactivity were observed following the training intervention.

Inhibitory effects on platelet reactivity by the in vivo infusion of prostacyclin

Femoral arterial infusion of the prostacyclin analogue epoprostenol resulted in potent, dose-dependent, inhibitory effects (right-hand shifts and reduction in maximal aggregation) on the collagen, TRAP-6 and ADP-induced platelet aggregation curves (Figure 2 and online-only data supplement, S1). The platelet sensitivity to the inhibitory effects of the epoprostenol infusion was similar between the groups before the training intervention (Figure 2C). The epoprostenol infusion had a larger anti-aggregatory effect after the training intervention in both the pre- and the postmenopausal women (Figure 2A+B), which indicates that exercise training increased platelet sensitivity to the inhibitory effects of prostacyclin. This effect was greater in the postmenopausal women when compared to the premenopausal women (Figure 2D).

Venous plasma levels of the prostacyclin breakdown product 6-keto PGF_{1α} were not different between the groups of women at baseline and increased ~20-fold after the highest epoprostenol dose (Figure 4A+B). These levels did not change after the training intervention.

Platelet reactivity after acute one-leg knee extensor exercise

The acute one-leg knee extensor exercise (at 20W moderate intensity) had an inhibitory effect on platelet aggregation that was enhanced after the training intervention in both the pre- and the postmenopausal women (Figure 3 and online-only data supplement, S2). This inhibitory effect was more pronounced in the premenopausal women (Figure 3A+C and online-only data supplement, S2). The 6-keto PGF_{1α} plasma levels were reduced after the exercise in both groups of women to a similar extent, both before and after the training intervention, and the levels did not differ between the groups of women (Figure 4C+D). Additionally, the acute exercise did not affect platelet count, before or after the training intervention in either group (online-only data supplement, Table S1).

Platelet reactivity after arterial acetylcholine and ketorolac infusions

The arterial acetylcholine infusion had an inhibitory effect on platelet aggregation in both groups of women (Figure 5A+B). After the exercise intervention, the inhibitory effect of the acetylcholine infusion had increased in the premenopausal women only (Figure 5A and online-only data supplement, S3).

Arterial infusion of the non-selective cyclooxygenase inhibitor ketorolac led to a right-hand shift of the aggregation curves and a reduction in maximal aggregation to a similar degree as acetylcholine alone (online-only data supplement, S4). The co-infusion of acetylcholine and ketorolac further reduced platelet aggregation (Figure 5C). The inhibitory effect of this co-infusion was enhanced in the premenopausal women after the training intervention (online-only data supplement, S4).

The acetylcholine infusion led to a ~20-fold increase in venous plasma 6-keto PGF_{1α} levels [18] which were similar in both groups and unaffected by the training intervention.

In vitro testing of platelet nitric oxide sensitivity

The two highest concentrations of DEA/NONOate inhibited the collagen-induced platelet aggregation to a similar degree in both groups of women. There was no difference in platelet sensitivity to inhibition by DEA/NONOate after the training intervention (Figure 6).

Discussion

The primary outcomes of the current study were: (i) sedentary, recently postmenopausal women had more reactive platelets compared to matched late premenopausal women of similar age; (ii) a period of high-intensity aerobic exercise training reduced basal platelet reactivity in the premenopausal, but not the postmenopausal women; (iii) the training intervention enhanced platelet sensitivity to the inhibitory, anti-aggregatory effects of the arterial prostacyclin infusion and acute one-leg knee extensor exercise in both groups of women.

Findings from previous studies that have examined platelet reactivity in pre- and postmenopausal women have been inconsistent [8, 9], and a main confounding factor may have been the sizeable age differences (10 to 15-year age variance) between the pre- and post-menopausal women studied. The current study was designed to minimize the age difference between the pre- and postmenopausal women, leading to a mean age difference of only about 4 years. In agreement with our initial hypothesis, we found that platelets from postmenopausal women were more reactive to aggregate when challenged with several different platelet agonists, compared to platelets from premenopausal women. Our findings may, together with other risk factors such as elevated blood pressure, endothelial dysfunction and raised cholesterol, explain the increased incidence of atherothrombotic events in postmenopausal women.

The underlying mechanism behind the hyperactive platelets in the postmenopausal women could potentially be due to their diminished levels of estrogen following menopause. Both

megakaryocytes and circulating human platelets have been reported to contain the estrogen receptor β [22, 23] and 17β -estradiol have been shown to have direct inhibitory effects on platelet aggregation *in vitro* [6, 7]. Hence, circulating estrogen in the premenopausal women may have a direct inhibitory effect on platelet reactivity that is missing in the postmenopausal women. Additionally, estrogen may have a genomic effect on megakaryocytes to produce platelets with a less reactive phenotype, and consequently when the hormonal milieu is altered after menopause, megakaryocytes may be reprogrammed to produce platelets that are more reactive. Alternatively, since estrogen has been shown to stimulate endothelial production of nitric oxide [4] and prostacyclin [5] the hyperactive platelets in the postmenopausal women may also be due to endothelial dysfunction resulting in impaired endothelial regulation of platelet reactivity. Our group have previously published that the postmenopausal women in the current study did have a reduced vasodilatory response to the prostacyclin and acetylcholine infusion compared to the premenopausal women, indicating that their vascular endothelial function was indeed impaired [18]. Their vasodilatory response (vascular function) did however improve with the exercise training, whilst their basal platelet reactivity remained unaltered. This indicates that endothelial dysfunction was not the reason behind the hyperactive platelets in the postmenopausal women.

Our finding that basal platelet reactivity was reduced by the exercise training in the premenopausal women only, was in contrast to what we hypothesized, and indicates that this particular adaptation to aerobic exercise training is estrogen-dependent. A role for estrogen is further supported by previous findings on the effects of aerobic exercise training in young pre-menopausal women [12]. It is noteworthy that this inhibitory effect on basal platelet reactivity by the exercise training in the premenopausal women was collagen specific since platelet responses towards the other agonists were not affected. Accordingly, Wu et al reported that 17β -estradiol inhibited collagen-induced platelet aggregation more potently than other agonists [6]. Future studies should focus on investigating platelet-collagen interactions to see if for instance collagen receptors (i.e. glycoprotein VI) are affected by exercise training

and estrogen levels. In the current study, through a unique approach of *in vivo* arterial infusion of the prostacyclin analogue (epoprostenol), we found that platelets from both the pre- and postmenopausal women were significantly more inhibited by prostacyclin after the training intervention. Since plasma levels of the prostacyclin hydrolysis breakdown product (6-keto PGF_{1α}) during the infusion were the same before and after the intervention, this confirms the consistency of the protocol and that exercise training does not alter prostacyclin metabolism/clearance. Hence, the enhanced inhibitory effect of the prostacyclin infusion after the training intervention can be explained by the exercise training increasing the sensitivity of platelets to prostacyclin, which confirms our initial hypothesis. In accordance with these results, a study from Sinzinger et al. observed increased platelet sensitivity to prostacyclin (added *in vitro*) after a one year jogging intervention in younger men and women [24]. Dix et al. also reported a higher platelet prostacyclin sensitivity in marathon runners compared to non-runners and they reported that this was due to a greater activity of the membrane-bound adenylate cyclase enzyme in their platelets, which generates inhibitory cyclic AMP [25]. In the current study, using a unique human *in vivo* model, we have been able to replicate these findings both in a more physiological setting and in older pre- and postmenopausal women, indicating that this important platelet adaptation to exercise training is preserved with older age and after menopause.

Contrary to what we hypothesized, there was however no difference in nitric oxide sensitivity between the groups of women, before or after the training intervention, suggesting that neither the menopausal transition nor exercise training affects platelet nitric oxide sensitivity.

The effects of acute exercise on platelet function appears to be intensity dependent since moderate exercise has been shown to suppress platelet reactivity whereas acute strenuous exercise has been shown to temporarily increase platelet reactivity [26, 27]. The acute one-leg dynamic knee-extensor exercise-test used in this study was conducted at moderate intensity and the benefit of using this model is that it allows for assessment of a localized effect of a single active muscle group [28] with blood sampling obtained from the femoral

vein draining the exercising leg. By use of this model, we report for the first time that a bout of one-leg knee-extensor exercise had an inhibitory effect on platelet aggregation and that this effect was significantly enhanced after the training intervention period in both the pre- and the postmenopausal women. One possible explanation for the greater inhibitory effect of the acute exercise after training could be an enhanced nitric oxide bioavailability as endothelial nitric oxide synthase (eNOS) protein levels were higher in thigh skeletal muscle samples from both the pre- and postmenopausal women, after the training intervention [19]. In addition, the greater platelet sensitivity to prostacyclin observed in the infusion experiments could have contributed to a larger inhibitory effect of exercise-induced prostacyclin release after the training period.

Study limitations

One potential limitation in this study is that the platelet reactivity assay used in this study requires that the blood samples are centrifuged to obtain PRP and thus there is a time delay (~10min) after blood collection. This could have affected the magnitude of the inhibitory effects that were detected on the platelet responses following the acute exercise and arterial infusion protocols. The investigator performing this assay was not blinded to the identity of the blood samples, but since this is a quantitative test of platelet aggregation, the potential for bias was minimal.

Another limitation is the lack of a non-training control group. We decided not to include one since our primary objective was to evaluate differences in training response in the pre- and postmenopausal women. Furthermore, we did not choose a cross-over design for the study since it would be difficult to control for carry-over effects from the physical activity intervention.

One more limitation with our study was that our study population were probably healthier than the general population (considering they had normal weight and were normotensive [14, 18]), and hence, the generalizability of the current results would need to be confirmed in less

healthy (for instance overweight and hypertensive) pre-and postmenopausal women in larger-scale studies such as the ongoing platelet function testing in the Framingham study.

Conclusion

In conclusion, the present study demonstrates that basal platelet reactivity is increased in women within only a few years after menopause, which may contribute to the rapid increased risk of cardiovascular events in postmenopausal women. Moreover, the novel finding that regular exercise training can increase platelet sensitivity to prostacyclin in both pre- and postmenopausal women highlights a previously unknown mechanism behind the cardioprotective effects of regular exercise.

Addendum

MH. Lundberg Slingsby: concept and design, acquisition of data, analysis and/or interpretation of data, critical writing and revising the intellectual content. M Nyberg: concept and design, acquisition of data and revising the intellectual content. J Egelund: acquisition of data and revising the intellectual content. C.M Mandrup: concept and design, acquisition of data and revising the intellectual content, R Frikke-Schmidt concept and design and revising the intellectual content. NS Kirkby: concept and design, analysis and/or interpretation of data and revising the intellectual content. Y Hellsten: concept and design, acquisition of data, analysis and/or interpretation of data, critical writing and revising the intellectual content.

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Disclosures

There are no conflicts of interest.

References

1. Kannel WB, Hjortland MC, McNamara PM, Gordon T. Menopause and risk of cardiovascular disease: the Framingham study. *Ann Intern Med.* 1976;85(4):447-52.
2. Taddei S, Virdis A, Ghiadoni L, Mattei P, Sudano I, Bernini G, Pinto S, Salvetti A. Menopause is associated with endothelial dysfunction in women. *Hypertension.* 1996;28(4):576-82.
3. Barrett-Connor E. Clinical review 162: cardiovascular endocrinology 3: an epidemiologist looks at hormones and heart disease in women. *J Clin Endocrinol Metab.* 2003;88(9):4031-42.
4. Darkow DJ, Lu L, White RE. Estrogen relaxation of coronary artery smooth muscle is mediated by nitric oxide and cGMP. *Am J Physiol.* 1997;272(6 Pt 2):H2765-73.
5. Wakasugi M, Noguchi T, Kazama YI, Kanemaru Y, Onaya T. The effects of sex hormones on the synthesis of prostacyclin (PGI₂) by vascular tissues. *Prostaglandins.* 1989;37(4):401-10.
6. Wu GJ, Lee JJ, Chou DS, Jayakumar T, Hsiao G, Chen WF, Sheu JR. Inhibitory signaling of 17beta-estradiol in platelet activation: the pivotal role of cyclic AMP-mediated nitric oxide synthase activation. *Eur J Pharmacol.* 2010;649(1-3):140-9.
7. Bar J, Lahav J, Hod M, Ben-Rafael Z, Weinberger I, Brosens J. Regulation of platelet aggregation and adenosine triphosphate release in vitro by 17beta-estradiol and medroxyprogesterone acetate in postmenopausal women. *Thromb Haemost.* 2000;84(4):695-700.
8. Singla A, Bliden KP, Jeong YH, Abadilla K, Antonino MJ, Muse WC, Mathew DP, Bailon O, Tantry US, Gurbel PA. Platelet reactivity and thrombogenicity in postmenopausal women. *Menopause.* 2013;20(1):57-63.
9. Roshan TM, Normah J, Rehman A, Naing L. Effect of menopause on platelet activation markers determined by flow cytometry. *Am J Hematol.* 2005;80(4):257-61.
10. Pedersen BK, Saltin B. Exercise as medicine - evidence for prescribing exercise as therapy in 26 different chronic diseases. *Scand J Med Sci Sports.* 2015;25 Suppl 3:1-72.
11. Nyberg M, Seidelin K, Andersen TR, Overby NN, Hellsten Y, Bangsbo J. Biomarkers of vascular function in premenopausal and recent postmenopausal women of similar age: effect of exercise training. *Am J Physiol Regul Integr Comp Physiol.* 2014;306(7):R510-7.
12. Wang JS, Jen CJ, Chen HI. Effects of chronic exercise and deconditioning on platelet function in women. *J Appl Physiol (1985).* 1997;83(6):2080-5.
13. Heber S, Assinger A, Pokan R, Volf I. Correlation between Cardiorespiratory Fitness and Platelet Function in Healthy Women. *Med Sci Sports Exerc.* 2016;48(6):1101-10.
14. Mandrup CM, Egelund J, Nyberg M, Lundberg Slingsby MH, Andersen C, Logstrup S, Bangsbo J, Suetta C, Stallknecht B, Hellsten Y. Effects of high-intensity training on cardiovascular risk factors in premenopausal and postmenopausal women. *Am J Obstet Gynecol.* 2016.
15. Chan MV, Armstrong PC, Papalia F, Kirkby NS, Warner TD. Optical multichannel (optimul) platelet aggregometry in 96-well plates as an additional method of platelet reactivity testing. *Platelets.* 2011;22(7):485-94.
16. Chan MV, Warner TD. Standardised optical multichannel (optimul) platelet aggregometry using high-speed shaking and fixed time point readings. *Platelets.* 2012;23(5):404-8.
17. Wang JS, Jen CJ, Lee HL, Chen HI. Effects of short-term exercise on female platelet function during different phases of the menstrual cycle. *Arterioscler Thromb Vasc Biol.* 1997;17(9):1682-6.
18. Nyberg M, Egelund J, Mandrup CM, Nielsen MB, Mogensen AS, Stallknecht B, Bangsbo J, Hellsten Y. Early Postmenopausal Phase Is Associated With Reduced Prostacyclin-Induced Vasodilation That Is Reversed by Exercise Training: The Copenhagen Women Study. *Hypertension.* 2016;68(4):1011-20.
19. Nyberg M, Egelund J, Mandrup CM, Andersen CB, Hansen K, Hergel IF, Valbak-Andersen N, Frikke-Schmidt R, Stallknecht B, Bangsbo J, Hellsten Y. Leg vascular and skeletal muscle mitochondrial adaptations to aerobic high-intensity exercise training are enhanced in the early postmenopausal phase. *J Physiol.* 2017;595(9):2969-83.

20. Diodati JG, Dakak N, Gilligan DM, Quyyumi AA. Effect of atherosclerosis on endothelium-dependent inhibition of platelet activation in humans. *Circulation*. 1998;98(1):17-24.
21. Whitley E, Ball J. Statistics review 4: sample size calculations. *Crit Care*. 2002;6(4):335-41.
22. Khetawat G, Faraday N, Nealen ML, Vijayan KV, Bolton E, Noga SJ, Bray PF. Human megakaryocytes and platelets contain the estrogen receptor beta and androgen receptor (AR): testosterone regulates AR expression. *Blood*. 2000;95(7):2289-96.
23. Miller VM, Jayachandran M, Hashimoto K, Heit JA, Owen WG. Estrogen, inflammation, and platelet phenotype. *Gend Med*. 2008;5 Suppl A:S91-S102.
24. Sinzinger H, Fitscha P. Jogging causes a significant increase in platelet sensitivity to prostacyclin. *Int J Sports Med*. 1986;7(6):338-41.
25. Dix CJ, Hassall DG, Bruckdorfer KR. The increased sensitivity of platelets to prostacyclin in marathon runners. *Thromb Haemost*. 1984;51(3):385-7.
26. Wang JS, Cheng LJ. Effect of strenuous, acute exercise on alpha2-adrenergic agonist-potentiated platelet activation. *Arterioscler Thromb Vasc Biol*. 1999;19(6):1559-65.
27. Heber S, Volf I. Effects of Physical (In)activity on Platelet Function. *Biomed Res Int*. 2015;2015:165078.
28. Andersen P, Saltin B. Maximal perfusion of skeletal muscle in man. *J Physiol*. 1985;366:233-49.

Subject Characteristics	Premenopausal women		Postmenopausal women	
	Experimental day 1 (n=25) <i>Subgroup Exp. Day 2 (n=13)</i>		Experimental day 1 (n=24) <i>Subgroup Exp. Day 2 (n=14)</i>	
Variable	Before training	After training	Before training	After training
Age (y)	49.1 ± 0.4 <i>50.3 ± 0.6</i>	-	53.7 ± 0.6 * <i>53.7 ± 0.8 *</i>	-
Time from last menstrual cycle (y)	-	-	3.1 ± 0.5 <i>3.0 ± 0.5</i>	-
P-estradiol (nmol/L)	0.723 ± 0.129 <i>0.5 ± 0.1</i>	-	0.034 ± 0.002 * <i>0.1 ± 0.01 *</i>	-
P-follicitropin (Follicle Stimulating Hormone, IU/L)	13 ± 2 <i>13 ± 4</i>	-	72 ± 8 * <i>93.3 ± 6.4 *</i>	-
P-lutropin (Luteinizing Hormone, IU/L)	15 ± 3 <i>18.7 ± 4</i>	-	24 ± 4 * <i>34.9 ± 4 *</i>	-
Height (m)	1.68 ± 0.01	-	1.68 ± 0.01	-
Weight (kg)	67.6 ± 1.4	67.1 ± 1.4	66.6 ± 1.6	65.8 ± 1.5
Body Mass Index (kg/m ²)	24.0 ± 0.5	23.8 ± 0.5	23.7 ± 0.4	23.4 ± 0.4 †
VO _{2max} (mL/min)	2124 ± 53	2328 ± 62 †	2007 ± 48	2177 ± 54 †
VO _{2max} (mL/min per kg)	31.5 ± 0.6	34.8 ± 0.9 †	30.4 ± 0.9	33.5 ± 1.1 †
Platelet count (x10 ⁹ /L)	262 ± 11	252 ± 12	274 ± 11	258 ± 9

Table 1. Subject characteristics. The data from the n=25 pre- and n=24 postmenopausal women included in experimental day 1 (testing basal platelet reactivity before and after training) are highlighted in bold. Data confirming menopausal status of the subgroup study of the n=13 pre- and n=14 postmenopausal women that participated in experimental day 2 (arterial infusions and acute exercise protocol) are highlighted in italics. * $P < 0.05$: between pre- and postmenopausal women. † $P < 0.05$: from before training.

Figure 2. Collagen- induced platelet aggregation in platelet rich plasma from the femoral vein at baseline and following femoral arterial infusion of 25, 50 and 100 $\text{ng min}^{-1} \text{L}^{-1}$ epoprostenol (prostacyclin analogue) in (A) premenopausal women ($n=12$) and (B) postmenopausal women ($n=12$) before and after a 3-month aerobic exercise training intervention. (C) %inhibition of 4 $\mu\text{g/ml}$ collagen-induced platelet aggregation in pre- vs. postmenopausal women before and (D) after the training intervention. $\square P < 0.05$: before versus after training and $* P < 0.05$: between groups.

Figure 3. Collagen-induced platelet aggregation concentration response curves and area under the curve (AUC) in platelet rich plasma from the femoral vein at baseline and after 10 minutes of acute one-leg knee-extensor exercise at 20 Watt (W) in (A+C) premenopausal (n=12) and (B+D) postmenopausal women (n=13). (#) P<0.05 compared to baseline before and after the training intervention and (α) P<0.05 compared to the 20W exercise before the training intervention.

Figure 4. Venous plasma levels of the prostacyclin breakdown product 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) at baseline and after arterial infusion of 100 ngmin⁻¹L⁻¹ epoprostenol (prostacyclin analogue) in (A) premenopausal (n=10) and (B) postmenopausal (n=10) women and after 10 minutes of acute one-leg knee extension exercise 20Watt (W) in (C) premenopausal (n=12) and (D) postmenopausal women (n=14). (#) $P < 0.05$ compared to baseline before and after the training intervention.

Figure 6. Collagen (4 $\mu\text{g/mL}$)-induced platelet aggregation inhibited by the nitric oxide (NO) donor, Diethylamine NONOate (DEA/NONOate) in (A) premenopausal ($n=8$) and (B) postmenopausal women ($n=12$) before and after the 3-month aerobic exercise training intervention. (C) Pre-vs. postmenopausal women before the training intervention and (D) after the training intervention.