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Associations of resting and peak fat oxidation with sex hormone profile and blood glucose control in middle-aged women

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Abstract *Background and aims:* Menopause may reduce fat oxidation. We investigated whether sex hormone profile explains resting fat oxidation (RFO) or peak fat oxidation (PFO) during incremental cycling in middle-aged women. Secondarily, we studied associations of RFO and PFO with glucose regulation.
Method and results: We measured RFO and PFO of 42 women (age 52–58 years) with indirect calorimetry. Seven participants were pre- or perimenopausal, 26 were postmenopausal, and nine were postmenopausal hormone therapy users. Serum estradiol (E2), follicle-stimulating hormone, progesterone, and testosterone levels were quantified with immunoassays. Insulin sensitivity (Matsuda index) and glucose tolerance (area under the curve) were determined by glucose tolerance testing. Body composition was assessed with dual-energy X-ray absorptiometry; physical activity with self-report and accelerometry; and diet, with food diaries. Menopausal status or sex hormone levels were not associated with the fat oxidation outcomes. RFO determinants were fat mass ($\beta = 0.44$, $P = 0.006$) and preceding energy intake ($\beta = -0.40$, $P = 0.019$). Cardiorespiratory fitness ($\beta = 0.59$, $P = 0.002$), lean mass ($\beta = 0.49$, $P = 0.002$) and physical activity (self-reported $\beta = 0.37$, $P = 0.020$; accelerometer-measured $\beta = 0.35$, $P = 0.024$) explained PFO. RFO and PFO were not related to insulin sensitivity. Higher RFO was associated with poorer glucose tolerance ($\beta = 0.52$, $P = 0.002$).
Conclusion: Among studied middle-aged women, sex hormone profile did not explain RFO or PFO, and higher fat oxidation capacity did not indicate better glucose control.

Abbreviations: AUC, area under the curve; COVID-19, coronavirus disease; E2, estradiol; FSH, follicle-stimulating hormone; HT, hormone therapy; NEFA, non-esterified fatty acids; OGTT, oral glucose tolerance test; PFO, peak fat oxidation; POST, postmenopause; PRE/PERI, pre- or perimenopause; RER, respiratory exchange ratio; RFO, resting fat oxidation.

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1. Introduction

Menopause follows the loss of ovarian follicular activity and leads to circulating 17β-estradiol (E2) decline with concomitant follicle-stimulating hormone (FSH) increase [1]. The E2 deficiency has been thought to reduce fat oxidation capacity [2,3]. Premenopausal women depend more on fat oxidation than men during exercise [4–7] but not necessarily at rest [8]. Whether the sex-related difference is lost [9,10] or not [11] after menopause is uncertain. However, E2 supplementation increases fat oxidation in men [12,13], and estrogen receptor α regulates myocellular fatty acid metabolism [14], supporting the role of E2 as a fat oxidation capacity determinant.

Only a few human studies have directly investigated whether fat oxidation responds to menopause. Lovejoy et al. [15] found that 24-h fat oxidation decreased in women who transitioned from premenopause to postmenopause during a 4-year follow-up. However, they could not show that the decrease differed from women who were still premenopausal at the time of the end measurements. In contrast, Albildgaard et al. [16] reported premenopausal women to use fatty acids more than postmenopausal participants while cycling for 45 min at 50% of the $\dot{V}O_{2MAX}$ intensity and the FSH levels to negatively correlate with fat oxidation rate. Notably, the relative exercise intensity at which individuals reach their peak fat oxidation (PFO) rates varies [4–7]. Using incremental exercise testing may therefore improve the assessment of fat oxidation capacity compared with single-stage testing [17]. To our knowledge, this approach has not been used to study associations between sex hormone profile and fat oxidation in middle-aged women.

The E2 levels can be raised with menopausal hormone therapy (HT). Nevertheless, fat oxidation did not differ between HT users and non-users during a 30-min treadmill test performed at 80% of $\dot{V}O_{2MAX}$ intensity [18]. The effects of HT on resting fat oxidation (RFO) have been suggested to depend on the administration route. Dos Reis et al. [19] reported lower RFO in women taking oral conjugated estrogens than in transdermal E2 users, perhaps because of the liver first-pass effect. However, randomized controlled crossover trials could not confirm this observation when conjugated estrogens were compared with transdermal E2 [20] or placebo [21] treatments. Moreover, whether administered orally or transdermally, E2-therapy did not affect RFO in hypogonadal girls with Turner syndrome [22].

Poor fat oxidation capacity may induce lipid intermediate accumulation and impaired insulin signaling in oxidative tissues [23]. Menopause is associated with disturbances in blood glucose regulation [24]. Therefore, investigating the relationship between fat oxidation and glucose control is especially relevant in middle-aged women. Both RFO [25] and PFO [26] have been associated with insulin sensitivity in other populations. However, the findings are not universal [7,27,28], and the studies have relied on fasting-based outcomes.

Aging is a confounder in menopause-related studies. Therefore, our primary aim was to investigate whether sex hormone profile is related to fat oxidation in women of similar ages but varying menopausal states. Our premise was that circulating E2 would be positively associated and FSH would be negatively associated with fat oxidation, especially during exercise. We also presumed that women with higher E2 levels, due to endogenous production or HT use, would exhibit higher fat oxidation than postmenopausal women. Our secondary aim was to investigate whether RFO and PFO are associated with blood glucose control during glucose challenge. We expected higher PFO to be associated with lower insulin release.

2. Methods

A detailed description of the study methodology is in **the Supplement**. The study was approved by the ethics committee of the Central Finland Health Care District (KSSHP Dnro 9U/2018) and complied with the Declaration of Helsinki. The participants provided informed consent.

2.1. Study population

We recruited participants from the ERMA (The Estrogenic Regulation of Muscle Apoptosis) cohort [29] during its fourth-year follow-up, the EsmiRs (Estrogen and microRNAs as Modulators of Women's Metabolism) study. In total, 304 women participated in the basic EsmiRs measurements at the Health and Sports Laboratory of the University of Jyväskylä between January 2019 and March 2020. The measurements ended prematurely because of the coronavirus disease (COVID-19) pandemic.

For this EsmiRs Metabolism substudy, we recruited women who were either premenopausal or perimenopausal (PRE/PERI), postmenopausal (POST), or postmenopausal HT users (HT). The exclusion criteria were: 1) body mass index <18 or >30 kg/m^2, 2) oophorectomy or hysterectomy, 3) disease or medication use affecting metabolism, 4) hormonal contraception, and 5) regular smoking. A study nurse checked the participants' eligibility and group allocation during the basic EsmiRs measurements. We expected the PRE/PERI and HT groups to have higher systemic E2 levels and, therefore, higher PFO than the POST group. Using the data of Abildgaard et al. [16], we performed an *a priori* power calculation showing that 12 participants per group would be needed to detect a between-group difference in lean mass adjusted PFO with an alpha level of 0.05 and 80% power.

This substudy included two laboratory visits (Supplementary Fig. 1). At the first visit, the focus was on resting metabolism and glucose tolerance, and at the second visit, on exercise metabolism. The median (interquartile range) duration between the visits was 2 weeks (1–3 weeks).

2.2. Participants' hormonal status and final group assignments

Our sample was 13 PRE/PERI, 20 POST, and nine HT participants when the recruitment prematurely ended. In

the final menopausal status determination, the participants' self-reported menstrual data were supplemented with FSH measurements to ensure correct status assignment defined by the Stages of Reproductive Aging Workshop +10 guidelines [30]. During the process, we needed to reclassify six PRE/PERI women to be postmenopausal owing to their high FSH levels. Of the remaining seven PRE/PERI women, two were premenopausal, three were early perimenopausal, and two were late perimenopausal. Four POST women had extreme mean E2 levels ranging from 0.27 to 0.54 nmol/l, more than four times over the third quartile of 0.09 nmol/l. As we aimed to study the influence of E2 deficiency, we excluded these participants from the group comparisons. Of the HT users, seven used oral and two transdermal HT containing E2 and progestogen (see the Supplement for details). We considered combining women using different HT administration routes acceptable as oral E2 did not seem to limit RFO in a previous randomised controlled trial [22] or our sample (Supplementary Fig. 2).

We did not fully reach our recruitment goal owing to the COVID-19 pandemic. Therefore, we also performed an explorative analysis to test whether pooling the PRE/PERI ($n = 7$) and HT groups ($n = 9$) into a larger HIGH E2 group ($n = 16$) for comparison against the E2 deficient postmenopausal women (LOW E2 group, $n = 22$) would change our inferences from the primary analyses.

2.3. Diet and physical activity

We instructed the participants to avoid lifestyle changes during the study, abstain from exercise and alcohol for 48 h and caffeine for 12 h, and eat the last meal between 8:00 and 10:00 p.m. before the measurements. The participants drank 100 ml and 150 ml of water after waking up and after the body composition measurements, respectively. They minimised activities in the morning on the day of the measurements and arrived at the laboratory by car. The participants declared that they had followed the instructions.

Leisure-time physical activity was assessed with a structured questionnaire [31] and with a seven-day ActiGraph accelerometer (Pensacola, Florida, USA) wear between the basic EsmiRs and first substudy measurements [32,33]. The participants kept food diaries for 2 days and matched their diet 24 h before the substudy measurements. The intraclass correlation coefficients for energy intake and food quotient, the metabolisable respiratory quotient of the diet reflecting its macronutrient distribution, were 0.71 and 0.92, respectively ($P < 0.001$ for both).

2.4. Body composition

At the basic EsmiRs measurements, body composition was assessed using dual-energy X-ray absorptiometry (DXA Prodigy, GE Lunar Corp., Madison, WI, USA). Body composition was also assessed with InBody720 (Biospace, Seoul, Korea) at each visit.

2.5. Resting metabolism

Indirect calorimetry data were collected with a Vmax Encore 92 metabolic cart (Sensormedics, Yorba Linda, CA, USA). First, the participants rested for 30 min. Gas exchange was then measured for 30 min with the canopy method. The data collected during the first 10 min were discarded. Steady-state periods were determined as segments at which the coefficients of variation between the subsequent minutes and during the segments were $\leq$10% for $\dot{V}O_2$ and $\dot{V}CO_2$, and $\leq$5% for respiratory exchange ratio (RER). The median (interquartile range) steady-state duration was 20 min (13–20 min). RFO was calculated with the Frayn [34] equation, assuming that the urinary nitrogen excretion was negligible. The measurement of RFO was unreliable in three participants (PRE/PERI, $n = 2$; POST, $n = 1$) on the basis of high RER ($\geq$0.91) values [35].

2.6. Exercise metabolism

The exercise testing was performed with an Ergoselect 200 bicycle ergometer (Ergoline GmbH, Germany) with a cycling cadence of 70 $\pm$ 5 rpm. The protocol included the PFO and $\dot{V}O_{2PEAK}$ phases. Venous blood samples were drawn before and after the test.

The PFO phase started at 20 W. The workload was increased by 20 W every 4 min until RER reached 1.0 or the seventh test stage. Two participants completed the PFO phase without reaching a RER of 1.0., but their fat oxidation had started to decline before the last stage. Gas exchange was measured breath by breath and recorded as rolling 30-s averages. Fat oxidation for the last minute of each stage was calculated with the Frayn equation [34]. PFO was determined as the highest calculated fat oxidation rate; and FAT_{MAX} ($\%\dot{V}O_{2PEAK}$), as the exercise intensity at which PFO was reached.

The $\dot{V}O_{2PEAK}$ phase directly followed the PFO phase. The phase started at 100 W, and the workload was increased by 1 W/3 s. Gas exchange was recorded as 10-s rolling averages. We determined the $\dot{V}O_{2PEAK}$ as the highest $\dot{V}O_2$ average during a 30-s period and W_{MAX} as the highest workload reached. The exercise test ended with a 5-min cooldown at 50 W. The gas exchange data were unreliable in two HT participants because of metabolic cart failure or mask-wearing difficulties. One PRE/PERI participant could not continue to the $\dot{V}O_{2PEAK}$ phase due to fatigue resulting from low energy diet.

2.7. Blood glucose control and biomarkers

An oral glucose tolerance test (OGTT) followed the resting metabolism measurement. Venepuncture samples were drawn before and 30, 60, 90, and 120 min after ingestion of a 75-g glucose solution (GlucosePro 250 ml, Comed Oy, Ylöjärvi, Finland). The plasma glucose levels were analyzed with Konelab 20 XT (Thermo Fisher Scientific, Vantaa, Finland); and the serum insulin levels, with IMMULITE 2000 (Siemens Medical Solution Diagnostics, Los Angeles, CA, USA). We determined insulin sensitivity with the

Matsuda index and glucose tolerance by calculating the total glucose area under the curve (AUC). We also calculated fasting-based homeostatic model assessment for insulin resistance (HOMA-IR) index and AUC for insulin.

For the quantification of other biomarkers, serum was stored at −80 °C. The levels of sex hormones (E2, FSH, progesterone, and testosterone) and sex hormone-binding globulin were analyzed with IMMULITE 2000. Non-esterified fatty acids (NEFA) levels were analyzed with Indiko (Thermo Fisher Scientific, Vantaa, Finland). Glycerol and 3-hydroxybutyrate levels were measured using nuclear magnetic resonance spectroscopy (Nightingale Health Ltd., Helsinki, Finland) [36].

2.8. Statistical analyses

We used the R statistical environment (version 4.0.5) for analyses. The code is available at https://osf.io/afmu7. We performed analyses using linear regression, analysis of variance, or analysis of covariance, confirmed the model assumptions before accepting the results, and used an alpha level of 0.05 for statistical significance (detailed description in the Supplement). The main models included lean mass as a covariate to control the analyses for the oxidative tissue mass. We refrained from using ratio scaling as it misrepresents the relationship between the numerator and denominator [37,38].

First, we identified the RFO and PFO determinants by using the fat oxidation measures as the outcomes and the variable of interest as the exposures. We also constructed multivariable explanatory models on the basis of previous research. Next, we tested the associations between sex hormone levels and fat oxidation measures and whether the sex hormones improved the explanatory models. We then compared RFO and PFO between the menopausal groups. We also performed an explorative analysis by comparing the HIGH E2 and LOW E2 groups.

Lastly, we studied the associations between fat oxidation and blood glucose regulation by using OGTT measures as the outcomes and RFO or PFO as the exposure. The OGTT measures were log-transformed to improve residual normality. We also performed sensitivity analyses by excluding participants with incomplete OGTT data or including NEFA levels as a covariate. NEFA levels were used as a proxy for participants' energy balance and metabolic state.

Table 1 Participant characteristics in the whole study sample and in the pre- or perimenopausal (PRE/PERI), postmenopausal (POST), and postmenopausal hormone therapy user (HT) groups. Four POST women were excluded from group comparisons for having high estradiol levels.

	Full sample (N = 42)	PRE/PERI (n = 7)	POST (n = 22)	HT (n = 9)
Age, years	55.3 (1.6)	54.9 (1.5)	55.6 (1.6)	55.4 (1.4)
Sex hormones, mean of two measurements				
E2, nmol/l	0.11 (0.06–0.25)	0.18 (0.15–0.22)	0.06 (0.05–0.09)	0.27 (0.25–0.42)
FSH, IU/l	66.0 (37.4)	20.8 (14.2)	89.2 (31.5)	49.8 (25.7)
Progesterone, nmol/l	0.35 (0.23–0.57)	3.27 (1.23–7.55)	0.31 (0.17–0.39)	0.35 (0.26–0.39)
Testosterone, nmol/l	0.54 (0.30)	0.61 (0.29)	0.47 (0.29)	0.65 (0.33)
SHBG, nmol/l	75.2 (33.1)	72.3 (14.4)	69.0 (29.2)	93.4 (47.4)
Body composition				
Height, cm	166.0 (4.9)	166.7 (4.6)	165.8 (5.0)	166.6 (6.0)
Body mass, kg	67.8 (8.1)	69.9 (8.9)	67.2 (7.7)	68.9 (10.1)
BMI, kg/m^2	24.6 (2.5)	25.1 (2.8)	24.4 (2.4)	24.8 (3.2)
Lean mass, kg	41.2 (3.8)	42.4 (3.0)	40.9 (3.6)	40.2 (3.7)
Fat mass, kg	24.1 (6.1)	24.8 (6.4)	23.8 (6.1)	26.2 (6.6)
Percent body fat, %	36.5 (5.5)	36.4 (5.1)	36.3 (5.8)	38.9 (4.4)
Leisure-time physical activity				
Self-reported, MET-h/d	4.5 (2.3–7.5)	4.5 (3.4–6.8)	4.4 (2.3–7.5)	2.9 (1.5–4.5)
Accelerometry, MVPA min/d[a]	49.0 (26.0), n = 40	71.5 (28.8), n = 5	49.2 (23.6)	32.4 (15.8)
Glucose tolerance				
Fasting glucose, mmol/l	5.3 (5.1–5.5)	5.5 (5.2–5.7)	5.2 (5.0–5.5)	5.4 (5.2–5.5)
2-h glucose, mmol/l	5.6 (4.7–6.9), n = 41	6.1 (5.3–7.0)	5.1 (4.7–6.3), n = 21	7.4 (6.0–7.8)
Fasting insulin, IU/l	4.0 (2.4–5.7)	3.1 (1.5–4.4)	4.3 (2.2–7.0)	3.8 (3.2–8.0)
2-h insulin, IU/l	45.4 (38.5–57.9), n = 41	41.0 (36.6–52.2)	44.6 (35.0–51.6), n = 21	57.9 (49.0–72.5)
HOMA-IR	0.85 (0.49–1.36)	0.81 (0.37–1.06)	0.75 (0.46–1.44)	0.91 (0.62–1.94)
Glucose AUC, mmol/l/h	12.9 (11.3–14.5)	13.8 (12.0–15.0)	12.3 (10.8–13.5)	16.0 (13.8–16.4)
Insulin AUC, IU/l/h	75.7 (66.8–112.2)	75.8 (64.2–101.2)	72.0 (61.4–114.9)	106.7 (76.1–131.2)
Matsuda index	7.3 (5.4–9.4), n = 41	8.5 (6.9–10.7)	7.2 (5.4–11.1), n = 21	6.3 (3.7–8.8)
Cardiorespiratory fitness				
$\dot{V}O_{2PEAK}$, l/min	2.1 (0.3), n = 39	2.2 (0.4), n = 6	2.2 (0.3)	2.0 (0.3), n = 7
$\dot{V}O_{2PEAK}$, ml/kg/min	31.7 (5.1), n = 39	31.3 (4.0), n = 6	32.5 (5.3)	27.7 (3.5), n = 7
$\dot{V}O_{2PEAK}$, ml/kg LM/min	51.9 (6.9), n = 39	51.2 (5.4), n = 6	53.0 (7.7)	48.3 (6.4), n = 7
W_{MAX}, W	183 (26), n = 41	188 (23), n = 6	185 (24)	167 (29)

Data as means (standard deviation) or medians (interquartile range).
AUC, area under the curve; BMI, body mass index; E2, 17β-estradiol; FSH, follicle-stimulating hormone; HOMA-IR, homeostatic model assessment of insulin resistance; LM, lean mass; MET, metabolic equivalent of task; SHBG, sex hormone-binding globulin; $\dot{V}O_{2PEAK}$, peak oxygen uptake, W_{MAX}, maximal workload.
[a] moderate-to-vigorous intensity leisure-time physical activity minutes per day.

3. Results

The participants were 52- to 58-year-old women, free of chronic diseases and metabolism affecting medication verified by a medical examination performed at the first visit (Table 1). On the basis of OGTT, two HT users had elevated fasting glucose levels (>6.0 mmol/l), and five women (POST, $n = 2$; HT, $n = 3$) had impaired glucose tolerance (2-h glucose measurement value, 7.8–11.0 mmol/l).

Age ($P = 0.56$), percent body fat ($P = 0.46$), and self-reported leisure-time physical activity ($P = 0.74$) did not significantly differ across the menopausal groups (Supplementary Table 1). As expected, the PRE/PERI and HT groups had higher E2 levels and lower FSH levels than the POST group (Fig. 1). Testosterone ($P = 0.25$) and sex hormone-binding globulin levels ($P = 0.39$) did not differ between the groups; however, the PRE/PERI group had higher progesterone levels compared with the POST group ($P = 0.002$) and the HT group ($P = 0.026$) (Supplementary Table 1).

3.1. Resting and peak fat oxidation determinants and mediators

The indirect calorimetry, dietary, and metabolite data are shown in Table 2. Fat mass was positively, and prior energy

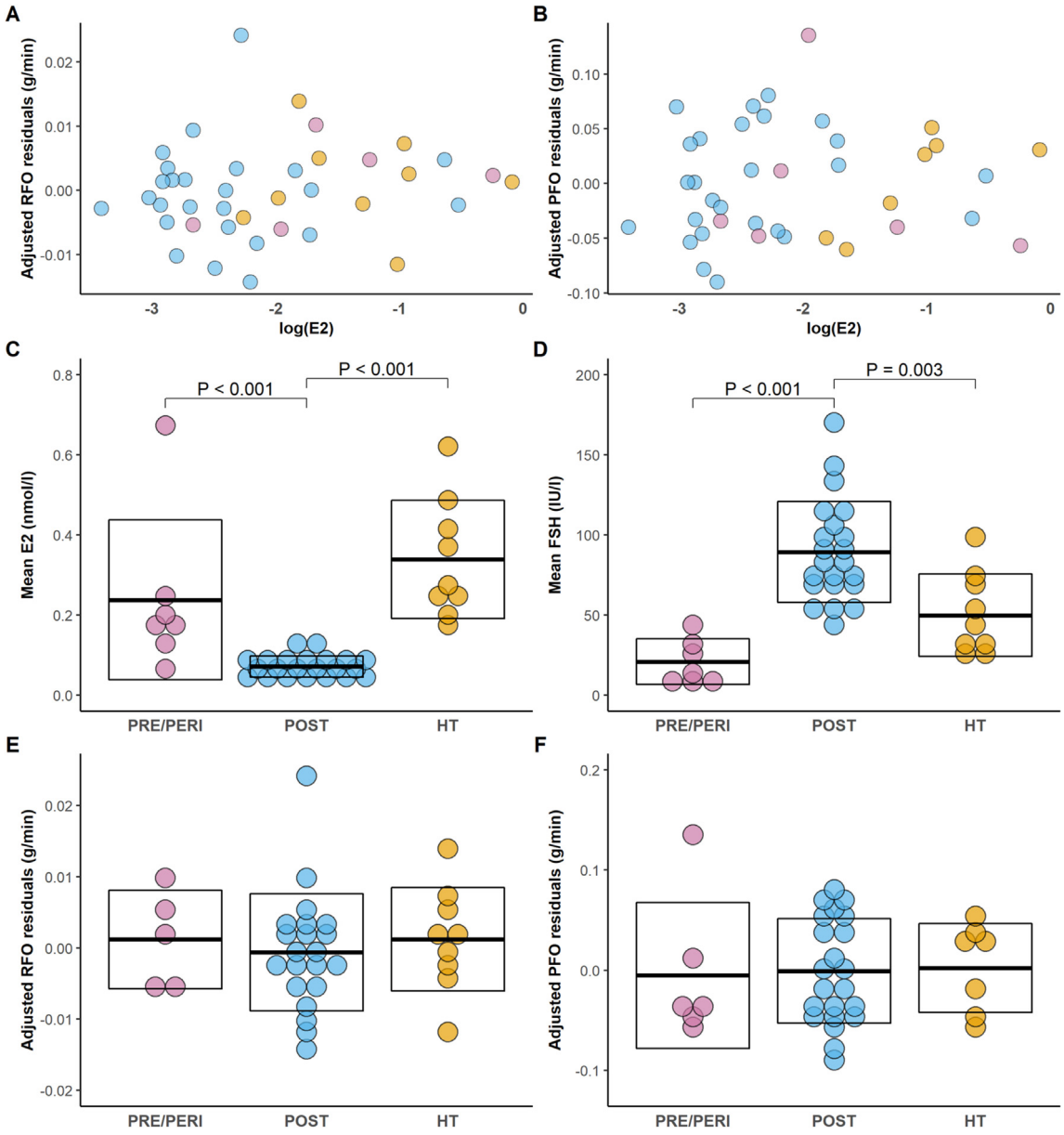

Figure 1 The associations of log-transformed E2 levels with adjusted resting fat oxidation residuals (RFO; Fig. 1A) and peak fat oxidation residuals (PFO; Fig. 1B), and comparison of female sex hormone levels (Fig. 1C and D) and adjusted RFO and PFO residuals (Fig. 1E and F) between the menopausal groups. The adjusted residuals represent the difference between the measured fat oxidation and the fat oxidation predicted by the linear regression when adjusted for fat oxidation main determinants (RFO: lean mass, fat mass, and energy intake; PFO: lean mass, cardiorespiratory fitness, and self-reported leisure-time physical activity).

intake was negatively associated with RFO (Table 3). The explanatory model including lean mass ($\beta = 0.26$; $P = 0.075$), fat mass ($\beta = 0.47$; $P = 0.002$) and energy intake ($\beta = -0.43$; $P = 0.005$) explained 40% of the RFO variance (adjusted $R^2 = 0.35$, $P < 0.001$). NEFA and 3-hydroxybutyrate levels were positively associated with RFO (Table 3).

RFO and PFO, measured on separate days, were not significantly associated with each other ($\beta = 0.10$, $P = 0.53$). VO_{2PEAK}, W_{MAX}, FATMAX, and self-reported and accelerometer-measured leisure-time physical activity were positively associated with PFO (Table 3). In the explanatory model, lean mass ($\beta = 0.16$; $P = 0.33$), W_{MAX} ($\beta = 0.46$; $P = 0.032$) and self-reported leisure-time physical activity ($\beta = 0.21$; $P = 0.23$) explained 43% of the PFO variance (adjusted $R^2 = 0.39$; $P < 0.001$). Pre-exercise NEFA and pre- and post-exercise glycerol levels were positively associated with PFO when post-exercise analyses were adjusted for metabolite pre-exercise concentration (Table 3).

3.2. Sex hormone profile associations with resting and peak fat oxidation

Neither E2 (Fig. 1) nor the other sex hormone levels (Supplementary Fig. 3) were significantly associated with RFO or PFO. Moreover, they did not contribute to RFO or PFO explanation when included in the explanatory models with the main fat oxidation determinants (Table 4).

We could not show that women with different menopausal statuses differed in absolute (F [2,32] = 1.46, $P = 0.25$, $\eta^2p = 0.08$) or lean mass adjusted (F [2,31] = 1.68, $P = 0.25$, $\eta^2p = 0.10$) RFO. The lack of group differences appeared even more clear when we included the main RFO determinants as covariates (F [2,29] = 0.25, $P = 0.78$, $\eta^2p = 0.02$) (Fig. 1). Neither did the menopausal groups differ in PFO (F [2,33] = 0.32, $P = 0.73$, $\eta^2p = 0.02$), whether adjusted for lean mass (F [2,32] = 0.18, $P = 0.84$, $\eta^2p = 0.01$), or the main PFO determinants (F [2,29] = 0.01, $P = 0.99$, $\eta^2p = 0.00$) (Fig. 1). The explorative analyses pararelled the results from primary analyses and did not reveal significant RFO or PFO differences between the HIGH E2 and LOW E2 groups (Supplementary Table 2).

3.3. Associations of resting and peak fat oxidation with blood glucose regulation

Neither RFO nor PFO was significantly associated with Matsuda on HOMA-IR indexes (Table 5, Supplementary Fig. 4). However, RFO was positively associated with glucose and insulin AUCs. The associations between RFO and glucose AUC ($\beta = 0.50$; $P = 0.007$) and insulin AUC ($\beta = 0.39$; $P = 0.042$) remained significant when adjusted

Table 2 Fat oxidation, preceding diet, and lipolysis-related metabolites in the whole study sample and in the pre- or perimenopausal (PRE/PERI), postmenopausal (POST), and postmenopausal hormone therapy user (HT) groups. Four POST women were excluded from group comparisons for having high estradiol levels.

	Full sample	PRE/PERI	POST	HT
Resting metabolism	$n = 39$	$n = 5$	$n = 21$	$n = 9$
RER	0.83 (0.03)	0.83 (0.03)	0.84 (0.03)	0.81 (0.02)
RFO, g/min	0.050 (0.010)	0.053 (0.009)	0.048 (0.010)	0.054 (0.009)
RFO, mg/kg LM/min	1.22 (0.23)	1.25 (0.19)	1.18 (0.25)	1.34 (0.18)
Diet two days before testing				
Energy intake, kcal/d	1795 (394)	1884 (324)	1853 (458)	1595 (197)
Energy intake, kcal/kg/d	27 (6)	26 (5)	28 (6)	23 (2)
Food quotient	0.86 (0.03)	0.83 (0.05)	0.86 (0.02)	0.87 (0.01)
Metabolites				
Non-esterified fatty acids, μmol/l	351 (255–493)	216 (213–256)	379 (255–489)	482 (325–727)
Glycerol, μmol/l	123 (92–150), $n = 36$	89 (82–95), $n = 4$	123 (94–153), $n = 19$	129 (125–166)
3-hydroxybutyrate, μmol/l	47 (23–83)	93 (38–168)	35 (19–54)	73 (58–117)
Exercise metabolism	$n = 40$	$n = 7$	$n = 22$	$n = 7$
PFO, g/min	0.22 (0.07)	0.23 (0.10)	0.22 (0.06)	0.21 (0.05)
PFO, mg/kg LM/min	5.3 (1.4)	5.4 (2.1)	5.3 (1.3)	5.0 (1.2)
FAT_{MAX}, $\%VO_{2PEAK}$	34 (9)	37 (15), $n = 6$	33 (8)	33 (5)
Diet two days before testing				
Energy intake, kcal/d	1887 (360)	1984 (388)	1898 (356)	1624 (224)
Energy intake, kcal/kg/d	28 (6)	29 (5)	28 (6)	23 (2)
Food quotient	0.86 (0.03)	0.85 (0.04)	0.86 (0.03)	0.88 (0.03)
Metabolites pre-exercise				
Non-esterified fatty acids, μmol/l	408 (319–566)	336 (277–553)	400 (266–531)	444 (382–724)
Glycerol, μmol/l	99 (84–136), $n = 34$	93 (86–112), $n = 6$	103 (89–142), $n = 19$	103 (84–164), $n = 6$
3-hydroxybuturate, μmol/l	61 (24–102), $n = 39$	46 (28–213)	42 (22–78), $n = 21$	74 (37–138)
Metabolites post-exercise				
Non-esterified fatty acids, μmol/l	503 (428–817), $n = 38$	601 (463–659)	486 (429–861), $n = 21$	688 (427–927), $n = 6$
Glycerol, μmol/l	269 (219–315), $n = 34$	240 (201–265), $n = 6$	278 (221–323), $n = 21$	304 (259–338), $n = 6$
3-hydroxybuturate, μmol/l	102 (83–128), $n = 38$	107 (94–167)	94 (82–117), $n = 21$	125 (83–152), $n = 6$

Data as means (standard deviation) or medians (interquartile range).
LM, lean mass; PFO, peak fat oxidation; RER, respiratory exchange ratio; RFO, resting fat oxidation.

for NEFA levels. Excluding participants with incomplete OGTT data did not influence the association of RFO and glucose AUC ($\beta = 0.51$; $P = 0.007$). The association with insulin AUC did not remain significant ($\beta = 0.31$; $P = 0.12$).

4. Discussion

This study investigated the associations between sex hormone profile and fat oxidation in middle-aged women. We were unable to show that menopausal status or sex hormone levels explain RFO or PFO in the study sample. We also examined the associations of RFO and PFO with blood glucose regulation during glucose challenge. Higher fat oxidation at rest or during exercise did not relate to insulin sensitivity; however, higher RFO preceded poorer glucose tolerance.

4.1. Sex hormone profile and resting fat oxidation

Fasting substrate use depends on the energy balance and diet composition [39,40]. In agreement, the RFO determinants in this study were variables describing tissue mass and energy intake. NEFA and 3-hydroxybutyrate levels were also positively associated with RFO, further linking RFO to lipolysis rate and energy balance. Therefore unsurprisingly, we did not observe associations between sex hormone levels and RFO. Previous studies also questioned the relationship between E2 levels and RFO in premenopausal [41] and postmenopausal women [42]. Resting substrate use was also unaltered by conjugated estrogens when the diet was controlled before measurements [43].

Ultimately, menopause will unlikely affect RFO directly. In this study, RFO was indeed very similar between the menopausal or HIGH and LOW E2 groups, especially after confounder adjustment. The laws of conservation of mass and energy also make it challenging to accept that the menopause-associated increase in fat mass [44,45] results from decreased fat oxidation ability [39].

4.2. Sex hormone profile and peak fat oxidation

The sex hormone levels or menopausal and E2 group statuses were not associated with PFO. Therefore, we could not reproduce the findings of Abildgaard et al. [16] by using incremental testing. Besides the testing approaches, a key difference between the studies is that our study did not have a pure premenopausal group. Our results agree with those reported by Johnson et al. [18], who observed similar fat oxidation rates in HT users and non-users, although, their selected testing intensity (80% of VO_{2MAX}) likely influenced their results [46].

Factors other than female sex hormone levels seem to determine PFO in women of all ages. The menstrual cycle phase or circulating E2 levels were not associated with PFO in reproductive women [47]. Moreover, controlling for the menstrual cycle phase did not improve the day-to-day reliability of PFO assessment [48]. In this study, PFO was associated with its known determinants: cardiorespiratory

Table 3 Associations between potential fat oxidation determinants, mediators, and resting fat oxidation or peak fat oxidation during exercise using linear regression models adjusted for lean mass.

	Resting fat oxidation				Peak fat oxidation			
	β	95% CI	*P*-value	*n*	β	95% CI	*P*-value	*n*
Univariable model								
Lean mass, kg	0.24	−0.08 to 0.56	0.132	39	0.49	0.20–0.79	**0.002**	40
Multivariable models with lean mass as a covariate								
Fat mass, kg	0.44	0.14–0.75	**0.006**	39	−0.03	−0.33 to 0.27	0.855	40
Leisure-time physical activity								
Self-reported, MET-h/d	−0.17	−0.50 to 0.16	0.300	39	0.37	0.06–0.68	**0.020**	40
Accelerometery, min/d	0.11	−0.24 to 0.45	0.530	39	0.35	0.05–0.65	**0.024**	38
Diet two days before the assessment								
Energy intake, kcal/d	−0.40	−0.73 to −0.07	**0.019**	39	0.05	−0.28 to 0.37	0.760	40
Food quotient	−0.09	−0.41 to 0.24	0.600	39	0.07	−0.22 to 0.37	0.613	40
Exercise test outcomes								
VO_{2PEAK}, l/min	−0.13	−0.53 to 0.28	0.526	36	0.47	0.14–0.79	**0.006**	39
W_{MAX}, W	−0.03	−0.44 to 0.37	0.864	38	0.59	0.23–0.94	**0.002**	39
FAT_{MAX}, $\%VO_{2PEAK}$	−0.16	−0.53 to 0.20	0.364	36	0.33	0.03–0.62	**0.033**	39
Lipolysis-related metabolites at rest or pre-exercise								
NEFA, μmol/l	0.46	0.16–0.75	**0.003**	39	0.33	0.05–0.61	**0.024**	40
Glycerol	0.26	−0.07 to 0.59	0.122	36	0.31	0.04–0.57	**0.024**	34
3-hydroxybuturate, μmol/l	0.52	0.24–0.81	**0.001**	39	0.19	−0.10 to 0.47	0.189	39
Lipolysis-related metabolites post-exercise, also adjusted for metabolite pre-exercise concentration								
NEFA, μmol/l					0.19	−0.12 to 0.50	0.222	38
Glycerol					0.42	0.16–0.68	**0.003**	34
3-hydroxybutyrate, μmol/l					0.37	−0.05 to 0.80	0.084	36

P-values < 0.05 are in bold.
β, standardized regression estimate; CI, confidence interval; MET, metabolic equivalent of task; NEFA, non-esterified fatty acids; Std, standardized; VO_{2PEAK}, peak oxygen uptake; W_{MAX}, maximal workload.

fitness, lean mass, and leisure-time physical activity [4–7]. Genetic pleiotropy contributes to the associations, and higher PFO does not result solely from an active lifestyle [28,49]. Still, 12-week endurance-focused training increased fat oxidation during exercise but not at rest in postmenopausal women [50], showing that fat oxidation capacity can be improved in E2 deficiency.

Overall, we could not show that the sex hormone profile influences PFO in middle-aged women. Our finding possesses uncertainty, and more work is needed to clarify whether menopause modifies fat oxidation during exercise and, if so, whether the effect has clinical significance. Cross-sectional designs may not be sensitive enough to accomplish this task. Ideally, longitudinal fat oxidation changes over the menopausal transition or after HT initiation should be measured. Duplicate measurements would benefit PFO assessment [7,48].

4.3. Fat oxidation and blood glucose regulation

The role of RFO as a metabolic health marker is controversial [51]. Some [25] but not all [52] studies have reported that RFO is inversely associated with fasting insulin levels. The relationship is believable as insulin levels decline and fat oxidation increases according to fasting duration [53]. However, these findings are likely caused by differences in metabolic status rather than metabolic health. In this study, higher RFO preceded poorer glucose tolerance when OGTT followed the RFO assessment, as in our previous study in 30-year-old men [28]. Because NEFA and 3-hydroxybutyrate levels were positively associated with RFO, we thought the observed association resulted from fat oxidation-induced inhibition of glucose use [54]. However, we could not explain the association by NEFA adjustment.

The rationale for PFO as a metabolic health marker is more robust compared with RFO because it reflects fiber type distribution [55], concentrations of lipid handling proteins [55–57], and mitochondrial volume density [58] in skeletal muscles. PFO has been shown to be associated with fasting insulin sensitivity surrogates [26,52], although the findings are not unanimous [7,27]. PFO was not associated with insulin sensitivity or glucose tolerance in our present or earlier study [28]. However, PFO was associated with lower insulin AUC in the previous study in men, but we could not replicate the finding in the present study in women. Besides participant characteristics, a difference between our studies was OGTT timing. In the study in men, OGTT was performed the day after the PFO assessment, and in this study in women, 1–3 weeks before PFO testing. As PFO [48] and OGTT outcomes [59] exhibit significant day-to-day variability, their association may be time-dependent. Song et al. [60] recently challenged the concept of metabolic flexibility by showing that insulin resistance does not result from mitochondrial substrate preference, and our results follow their finding.

4.4. Limitations

Our study has several limitations. First, we had to discontinue the participant recruitment early and did not reach our recruitment goal. Therefore, our study may have lacked the power to detect significant group differences between the menopausal groups. We pooled PRE/PERI and HT women into a HIGH E2 group for explorative analyses with larger group sizes to counteract this limitation. This strategy has important limitations as cyclical endogenous hormone production and daily exogenous HT likely affect metabolism differently. For example, besides the apparent difference in how the hormones enter circulation, the two also have discordant temporal kinetics. Second, HT use was not standardized and reflected real-life differences in HT

Table 4 Associations between serum sex hormone levels and resting fat oxidation (RFO) or peak fat oxidation (PFO) during exercise in the whole study sample, with the adjusted R^2 values representing the variance proportions the explanatory variables explain together and the P-values in the last column showing the statistical significance of the whole model adjusted for fat oxidation main determinants.

RFO (n = 39)	Lean mass adjusted				Adjusted for main determinants[a]				
	β	**95% CI**	***P*-value**	R^2**adj**	β	**95% CI**	***P*-value**	R^2**adj**	***P*-value**
Model without the sex hormone				0.03				0.35	0.0004
$_{log}$E2	0.22	−0.10 to 0.53	0.170	0.06	0.10	−0.17 to 0.37	0.439	0.34	0.0010
FSH	−0.21	−0.54 to 0.12	0.200	0.05	−0.09	−0.38 to 0.19	0.504	0.34	0.0011
$_{log}$P4, n = 34	0.20	−0.15 to 0.56	0.251	0.01	0.13	−0.17 to 0.43	0.381	0.32	0.0038
Testosterone	0.27	−0.06 to 0.59	0.105	0.08	0.12	−0.17 to 0.40	0.412	0.34	0.0010
SHBG	0.07	−0.26 to 0.40	0.667	0.01	−0.03	−0.34 to 0.27	0.821	0.33	0.0013
PFO (n = 40)	β	**95% CI**	***P*-value**	R^2**adj**	β	**95% CI**	***P*-value**	R^2**adj**	***P*-value**
Model without the sex hormone				0.21				0.39	0.0002
$_{log}$E2	0.01	−0.28 to 0.30	0.949	0.19	0.04	−0.22 to 0.31	0.736	0.37	0.0005
FSH	0.16	−0.13 to 0.44	0.278	0.22	0.14	−0.11 to 0.40	0.266	0.39	0.0003
$_{log}$P4	0.24	−0.03 to 0.51	0.085	0.25	0.19	−0.06 to 0.44	0.130	0.41	0.0002
Testosterone	0.18	−0.11 to 0.46	0.217	0.22	0.20	−0.05 to 0.45	0.116	0.41	0.0002
SHBG	0.01	−0.28 to 0.31	0.927	0.19	0.01	−0.25 to 0.27	0.940	0.37	0.0005

β, standardized regression estimate; CI, confidence interval; E2,17β-estradiol, FSH, follicle-stimulating hormone; P4, progesterone; SHBG, sex hormone-binding globulin.

[a] RFO: lean mass, fat mass, and energy intake; PFO: lean mass, $W_{MAX,}$ and self-reported physical activity.

Table 5 Lean mass adjusted associations between resting fat oxidation (RFO, n = 39) or peak fat oxidation (PFO, n = 40) and oral glucose tolerance test outcomes, with adjusted R^2 values representing the variance proportions that lean mass and the fat oxidation measure explain together and the P-values in the last column showing the statistical significance of the whole model.

	β	95% CI	*P*-value	R^2adj	*P*-value
HOMA-IR					
RFO	0.21	−0.15 to 0.56	0.244	0.04	0.496
PFO, $n = 39$	−0.37	−0.79 to 0.06	0.092	0.10	0.154
Matsuda index					
RFO, $n = 38$	−0.33	−0.68 to 0.02	0.063	0.10	0.165
PFO, $n = 38$	0.26	−0.19 to 0.72	0.249	0.04	0.507
Glucose AUC					
RFO	0.52	0.21–0.82	**0.002**	0.27	**0.004**
PFO	0.02	−0.34 to 0.38	0.913	0.01	0.786
Insulin AUC					
RFO	0.42	0.10–0.75	**0.012**	0.18	**0.027**
PFO	−0.06	−0.44 to 0.32	0.752	0.04	0.451

P-values < 0.05 are in bold.
AUC, total area under the curve; β, standardized regression estimate; CI, confidence interval; HOMA-IR, homeostatic model assessment of insulin resistance.

prescription. Third, even though we standardized the participant preparation between the two laboratory visits, the metabolic states of the participants may have varied to some extent. The interindividual variation in metabolic states may have also influenced the assessment of the associations between sex hormone levels and fat oxidation.

5. Conclusions

In this study in middle-aged women, sex hormone profile did not explain fat oxidation at rest or during exercise. Higher fat oxidation in either condition did not indicate better blood glucose control. RFO was mainly related to energy balance and PFO to cardiorespiratory fitness. Therefore, we encourage middle-aged women interested in improving their fat oxidation capacity to engage in regular leisure-time physical activity.

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Declaration of competing interest

The authors have nothing to disclose.

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Appendix A. Supplementary data

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