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RESEARCH PAPER

Metabolic flexibility in postmenopausal women: Hormone replacement therapy is associated with higher mitochondrial content, respiratory capacity, and lower total fat mass

A. S. Kleis-Olsen[1] | J. E. Farlov[1] | E. A. Petersen[1] | M. Schmücker[1] | M. Flensted-Jensen[1] | I. Blom[1] | A. Ingersen[1] | M. Hansen[2] | J. W. Helge[1] | F. Dela[1,3,4] | S. Larsen[1,5,6]

[1]Xlab, Center for Healthy Aging, Department of Biomedical Sciences, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark

[2]Department of Public Health, Section of Sport Science, Aarhus University, Aarhus N, Denmark

[3]Department of Geriatrics, Bispebjerg-Frederiksberg University Hospital, Copenhagen, Denmark

[4]Department of Human Physiology and Biochemistry, Riga Stradiņš University, Riga, Latvia

[5]Clinical Research Centre, Medical University of Bialystok, Bialystok, Poland

[6]Institute of Sports Medicine Copenhagen, Department of Orthopedic Surgery, Copenhagen University Hospital – Bispebjerg and Frederiksberg, Copenhagen, Denmark

Correspondence
A. S. Kleis-Olsen, Xlab, Center for Healthy Aging, Department of Biomedical Sciences, Faculty of Health Sciences, University of Copenhagen, Blegdamsvej 3B, DK-2200, Copenhagen N, Denmark.
Email: ann-sofie.ako@sund.ku.dk

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Abstract

Aim: To investigate effects of hormone replacement therapy in postmenopausal women on factors associated with metabolic flexibility related to whole-body parameters including fat oxidation, resting energy expenditure, body composition and plasma concentrations of fatty acids, glucose, insulin, cortisol, and lipids, and for the mitochondrial level, including mitochondrial content, respiratory capacity, efficiency, and hydrogen peroxide emission.

Methods: 22 postmenopausal women were included. 11 were undergoing estradiol and progestin treatment (HT), and 11 were matched non-treated controls (CONT). Peak oxygen consumption, maximal fat oxidation, glycated hemoglobin, body composition, and resting energy expenditure were measured. Blood samples were collected at rest and during 45 min of ergometer exercise (65% VO_2peak). Muscle biopsies were obtained at rest and immediately post-exercise. Mitochondrial respiratory capacity, efficiency, and hydrogen peroxide emission in permeabilized fibers and isolated mitochondria were measured, and citrate synthase (CS) and 3-hydroxyacyl-CoA dehydrogenase (HAD) activity were assessed.

Results: HT showed higher absolute mitochondrial respiratory capacity and post-exercise hydrogen peroxide emission in permeabilized fibers and higher CS and HAD activities. All respiration normalized to CS activity showed no significant group differences in permeabilized fibers or isolated mitochondria. There were no differences in resting energy expenditure, maximal, and resting fat oxidation or plasma markers. HT had significantly lower visceral and total fat mass compared to CONT.

Conclusion: Use of hormone therapy is associated with higher mitochondrial content and respiratory capacity and a lower visceral and total fat mass. Resting energy expenditure and fat oxidation did not differ between HT and CONT.

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KEYWORDS
hormone replacement therapy, metabolic flexibility, mitochondrial function

1 | INTRODUCTION

The natural onset of menopause in women is characterized by the loss of follicles in the ovaries.[1] This transition impacts the secretion of female sex hormones causing changes in gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH) and leads to a systemic decline in plasma concentrations of estrogens and progesterone.[2,3] The alterations in female sex hormone concentrations have been associated with an increased risk of neurodegenerative diseases,[4] osteoporosis,[5] and increased cardiovascular and metabolic risk factors.[6,7] These risks encompass dyslipidemia, visceral adiposity, and insulin resistance,[8,9] which coincides with an elevated susceptibility to metabolic inflexibility and metabolic disorders, such as type 2 diabetes.[2,10] Furthermore, the menopausal transition leads to vasomotor symptoms including hot flashes, night sweats, and sleep disturbances decreasing the quality of life in up to 80% of women.[11] Therefore, some women choose to use hormone replacement therapy (HRT), mostly containing both estrogen and one type of synthetic progesterone to alleviate the symptoms.[2] Given the widespread distribution of estrogen and progesterone receptors in organs and tissues,[12,13] both hormonal deficiency and HRT in postmenopausal women may affect factors involved in metabolic flexibility.

Metabolic flexibility refers to the ability to efficiently respond or adapt to changes in metabolic demand to maintain energy homeostasis by matching fuel availability and energy demands. It relies on crosstalk between various tissues to control the availability, transportation, and utilization of substrates, constituting a close interaction between cellular and systemic metabolic processes and is therefore influenced by various factors at both cellular and whole-body level.[14] One crucial factor of metabolic flexibility is the mitochondrial capacity to handle and oxidize substrates at rest and during exercise. Typically, a high mitochondrial content, high mitochondrial respiratory capacity (MRC), and a high antioxidant capacity are linked to healthier metabolic phenotypes, whereas the opposite has been associated with metabolic disorders.[15,16] Previous studies have examined the acute effects of estrogen and progesterone on mitochondrial content, MRC, and antioxidant capacity, however only in animal models[17–19] and in cell cultures.[20] These studies found that estrogen, in the form of 17β-estradiol (estradiol), increased mitochondrial content[19,21] and MRC.[17] But, HRT may augment the emission of reactive oxygen species (ROS), which has been implicated in the development of several metabolic disorders,[22] though the interplay between female sex hormones and ROS remains poorly understood. As such, estrogen has been shown to decrease the emission of hydrogen peroxide (H_2O_2),[20] while progesterone appears to have the opposite effects as it has been associated with decreased MRC and increased H_2O_2 emission.[20]

Whether changes in mitochondrial characteristics and substrate oxidation capacity in postmenopausal women correspond with whole-body measures, such as resting metabolic rate and maximal fat oxidation (MFO), remains unknown. Resting metabolic rate decreases with age.[23] However, estrogens have been implicated as important regulators of both energy intake[24,25] and energy expenditure, which has been found to be higher in postmenopausal women undergoing HRT.[26] Additionally, D'eon et al. (2005) found that estrogen administration decreased adiposity in ovariectomized rodents compared to placebo models independently of reduced energy intake.[27] Regarding MFO, a high MFO measured from pulmonary gas exchange has been considered an indicator of metabolic flexibility in lean individuals.[28,29] Since obese individuals exhibit high MFO rates as well,[30] the interplay between molecular and whole-body measurements is needed to fully evaluate metabolic flexibility. In general, premenopausal women exhibit higher rates of MFO compared to age-matched men, but this difference becomes indiscernible after menopause.[31] This observation is reinforced by findings showing that premenopausal women have a higher MFO than postmenopausal women irrespective of age.[32,33] Furthermore, the acute administration of estradiol to men increase MFO.[34] Hence, it appears that the acute administration of female sex hormones may affect both cellular and systemic factors related to metabolic flexibility.

Therefore, this study aimed to examine the effects of long-term use of combined HRT on metabolic flexibility in postmenopausal women. This involved linking whole-body parameters (MFO, resting fat oxidation, energy expenditure, and pertinent plasma markers) with cellular ex vivo measurements on mitochondrial function including mitochondrial content, MRC, and H_2O_2 emission measured in biopsies from m. vastus lateralis. It was hypothesized that women using HRT would show an enhanced mitochondrial function both at rest and after exercise, accompanied by a higher MFO compared

to non-treated controls. Additionally, it was hypothesized that plasma concentrations of lipids, insulin, and cortisol would be lower in HRT users, and HRT users would be characterized by higher resting energy expenditure and a healthier body composition compared to non-treated controls.

2 | RESULTS

2.1 | Characteristics and body composition

As shown in Table 1, the groups were matched by years since the last menstrual bleeding, fitness level (VO_2peak/kg), and BMI. Body composition was significantly different between the groups, as the HT group showed a significantly lower body fat percentage, fat mass, and visceral fat than the CONT group.

The blood profile is shown in Table 2. As expected, the HT group presented higher estradiol and lower FSH values than CONT, whereas endogenous progesterone levels did not differ between groups. Plasma concentrations of cortisol, insulin, and lipid profile markers (high-density, low-density lipoprotein, cholesterol, triglyceride) were not different between groups (Table 2). Glucose and free fatty acids (FFA) concentrations were measured at rest and during exercise after 30 and 45 min and did not differ significantly between groups (glucose: $p=0.06$. FFA: $p=0.45$), and no interaction effect was observed (glucose: $p=0.85$. FFA: $p=0.34$). However, exercise time increased plasma glucose ($p=0.0017$) and plasma FFA concentrations ($p<0.0001$) within groups significantly (Table 3). Oxygen consumption (VO_2) and respiratory exchange ratio (RER) were not different between groups ($p=0.50$ and $p=0.39$, respectively). However, exercise time increased VO_2 consumption from rest within groups (CONT: $p<0.001$. HT: $p<0.001$) significantly (Table 3).

2.2 | Mitochondrial function in permeabilized fibers

Absolute MRC (Figure 1) was affected significantly by treatment ($p=0.0085$). The HT group showed a significantly higher maximal MRC with complex I+II linked substrates (CONT: 70±13. HT: 83±14 pmol/s/mg. $p=0.03$) and a significantly higher ETS capacity than the CONT (CONT: 70±13. HT: 83±14 pmol/s/mg. $p=0.03$). Post-exercise, the HT group also showed significantly higher MRC with complex I linked respiration (CONT: 42±6. HT: 55±16 pmol/s/mg. $p=0.0014$), complex I+II linked respiration (CONT: 66±19. HT: 85±23 pmol/s/mg. $p=0.0001$), and ETS capacity (CONT: 74±18. HT: 99±24 pmol/s/mg. $p<0.0001$). Only in the CONT group a decrease was observed on ETS capacity after exercise compared to rest ($p=0.046$). No differences were found in leak respiration or mitochondrial efficiency measured as respiratory control ratio (RCR) between groups or in response to exercise (data not shown). When MRC in permeabilized fibers was normalized to mitochondrial content, no differences were observed between the groups.

TABLE 1 Subject characteristics.

	CONT	HT	*p*-Value
Subjects (*n*)	11	11	
Age (years)	56±4	55±5	0.47
Years past last bleeding	5±4	5±4	0.97
HbA1c (%)	5.4±0.3	5.4±0.3	0.95
Weight (kg)	66±5	63±8	0.17
BMI (kg/m^2)	24±2	22±2	0.16
VO_2peak/kg (mL/kg/min)	34±5	36±6	0.21
VO_2peak/kg FFM (mL/kg FFM/min)	48±8	50±6	0.64
Fat-free mass (kg)	45±4	46±5	0.49
Fat percentage (%)	34±7	27±6	0.02*
Fat mass (kg)	22±6	16±5	0.02*
Visceral fat (g)	527±348	227±115	0.02*
A/G ratio	0.77±0.3	0.74±0.2	0.78

Note: Subject characteristics for controls (CONT) and hormone-treated (HT) postmenopausal women.

Abbreviations: A/G ratio, android/gynoid ratio; BMI, body mass index; FFM, fat-free mass; HbA1C, glycated hemoglobin; VO_2peak, peak oxygen uptake.

* $p<0.05$.

TABLE 2 Plasma hormone concentrations and lipid profile.

	CONT	HT	*p*-Value
Estradiol (nmol/L)	<0.02	0.23 ± 0.1	<0.0001*
Progesterone (nmol/L)	0.19 ± 0.06	0.24 ± 0.07	0.2091
FSH (mIU/mL)	73.2 ± 18.9	39.5 ± 18.7	0.0013*
LH (mIU/mL)	35.5 ± 9.8	29.9 ± 17.0	0.4011
Cortisol (nmol/L)	379 ± 40	371 ± 69	0.7751
Insulin (pmol/L)	36 ± 14	29 ± 10	0.1977
HDL (mmol/L)	1.6 ± 0.3	1.7 ± 0.4	0.6033
LDL (mmol/L)	2.8 ± 0.7	2.6 ± 0.7	0.5392
Cholesterol (mmol/L)	4.7 ± 0.9	4.6 ± 0.5	0.8262
TG (mmol/L)	0.7 ± 0.3	0.9 ± 0.5	0.1520

Note: Subject characteristics for controls (CONT) and hormone-treated (HT) postmenopausal women.

Abbreviations: FSH, follicle-stimulating hormone; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LH, luteinizing hormone; TG, triglyceride.

* $p < 0.05$.

TABLE 3 Plasma glucose and FFA concentrations at rest and during exercise.

	CONT			HT		
	Rest	Ex30	Ex45	Rest	Ex30	Ex45
Glucose (mmol/L)	4.6 ± 0.8	5.3 ± 0.4*	5.4 ± 0.4*	4.9 ± 0.3	5.6 ± 0.5*	5.8 ± 0.9*
FFA (μmol/L)	412 ± 137	856 ± 291*	884 ± 242*	586 ± 202	905 ± 497*	884 ± 424*
VO_2 (mL/min)	199 ± 26	1401 ± 209‡	1435 ± 237‡	206 ± 24	1410 ± 236‡	1461 ± 215‡
RER	0.83 ± 0.02	0.86 ± 0.01	0.85 ± 0.03	0.83 ± 0.02	0.87 ± 0.03	0.85 ± 0.02

Note: Plasma glucose and FFA concentrations at rest and during exercise after 30 (Ex30) and 45 (Ex45) min for controls (CONT) and hormone-treated (HT) postmenopausal women.

Abbreviations: FFA, free fatty acids; RER, respiratory exchange ratio; VO_2, oxygen consumption.

* $p < 0.05$ significantly different from rest.

‡ $p < 0.001$ significantly different from rest.

Post-exercise, absolute H_2O_2 emission at maximal leak respiration (state 2) was higher in the HT compared to the CONT group (CONT: 0.8 ± 0.4. HT: 1.0 ± 0.3 pmol/s/mg. $p = 0.04$) (Figure 2A). When H_2O_2 emission was normalized to oxygen flux (Figure 2B), no differences were observed between groups ($p = 0.95$) or in response to exercise ($p = 0.75$).

2.3 | Muscle mitochondrial content and enzyme activity

CS activity was measured in the muscle tissue as a marker of mitochondrial content. Muscle CS activity was significantly higher in the HT compared to the CONT group (CONT: 128 ± 24 μmol/g/min. HT: 161 ± 45 μmol/g/min. $p = 0.04$) (Figure 3A).

The activity of 3-hydroxyacyl-CoA dehydrogenase (HAD) was significantly higher in the HT compared to the CONT group (CONT: 77.6 ± 23 μmol/g/min. HT: 106.6 ± 37 μmol/g/min. $p = 0.01$) (Figure 3B).

2.4 | Mitochondrial function in isolated mitochondria normalized to CS activity

There was no difference in CS activity in isolated mitochondria between groups (CONT: 3.45 ± 2.13 μmol/g/min. HT: 3.13 ± 0.8 μmol/g/min. $p = 0.35$). Additionally, MRC normalized to CS activity was not affected by treatment in neither the carbohydrate (CHO) ($p = 0.11$) or fatty acid-supported protocol ($p = 0.22$) (Figure 4A,B). Leak respiration and mitochondrial efficiency measured as RCR (not shown) and P/O ratio (Figure 4C,D) were not different between groups.

H_2O_2 emission normalized to CS activity in isolated mitochondria was unaffected by treatment and exercise in both the CHO-supported ($p = 0.7$ and $p = 0.5$, respectively) and fatty acid-supported ($p = 0.6$ and $p = 0.6$, respectively) protocol (Figure 5). Additionally, no differences were observed when H_2O_2 emission was normalized to oxygen flux in any of the protocols (CHO-supported: $p = 0.4$. Fatty acid-supported: $p = 0.4$).

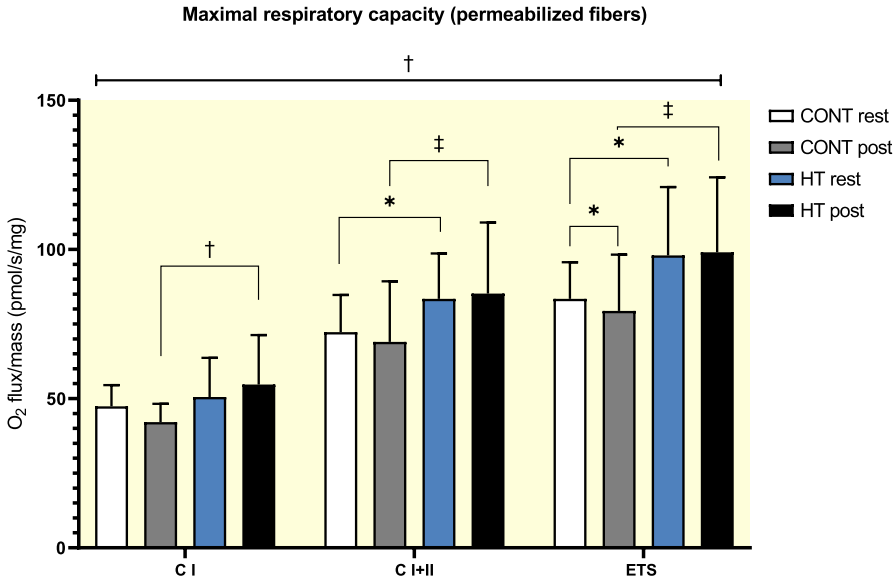

FIGURE 1 Maximal respiratory capacity in permeabilized fibers for controls (CONT) and hormone-treated (HT) postmenopausal women. C I, complex I (malate, pyruvate, glutamate, ADP); C I+II, complex I+II (malate, pyruvate, glutamate, ADP, succinate); ETS, electron transport system (FCCP). *$p < 0.05$; †$p < 0.01$; ‡$p < 0.001$.

2.5 | Resting energy expenditure and fat oxidation

Resting energy expenditure, resting fat oxidation, and MFO during exercise are shown in Table 4. No differences were observed between groups in these variables.

MFO was not correlated to MRC in isolated mitochondria or permeabilized fibers (all R values <0.3, all p-values >0.4), indicating no link between whole-body substrate oxidation and local mitochondrial capacity for substrate oxidation in the muscle tissue. Similarly, HAD activity was not correlated to MFO (all R values <0.5, all p-values >0.2). A significant negative correlation between MFO and visceral fat (Figure 6) was observed ($R = -0.43$, $p = 0.04$), and was driven by the CONT group ($R = -0.65$. $p = 0.02$. HT: $R = 0.41$. $p = 0.2$). MFO did not correlate with fat mass ($R = -0.2$, $p = 0.4$).

No differences were observed in RER, substrate use (%), VO_2, and heart rate between the groups during 45 min of cycling exercise. However, both groups showed a main effect of exercise time on RER ($p = 0.0004$) which increased in both groups during exercise (data not shown).

3 | DISCUSSION

The primary findings of this study showed that HRT in postmenopausal women was associated with (1) higher absolute MRC and H_2O_2 emission (at maximal leak) in permeabilized fibers likely driven by higher mitochondrial content measured as CS activity; (2) no differences in MRC and H_2O_2 emission in isolated mitochondria or in mitochondrial efficiency in both isolated mitochondria and permeabilized fibers; (3) lower fat mass, body fat percentage, and visceral fat mass, but no group differences in plasma concentrations of lipids, glucose, FFA, cortisol and insulin; (4) no effect on resting fat oxidation, MFO and resting energy expenditure compared to non-treated postmenopausal women.

3.1 | Mitochondrial function

The current findings of a higher absolute MRC in the HT group are in line with previous findings in animal and cell culture studies, which have shown that MRC can be enhanced in the presence of estradiol alone, as well as in combination with progesterone.[17,20,35] Mechanistically, this is explained in mice by findings showing that estradiol reduces mitochondrial membrane viscosity and thereby improves the complexes' mobility and activity, thus increasing the rate of electron transfer.[17] However, most previous studies,[18,36–38] but not all,[17,20] suggest that estradiol enhances MRC by increasing mitochondrial biogenesis via activation of the estrogen-receptor-α (ERα) pathway. In line with these studies, the HT group in the present study showed a significantly higher CS activity in skeletal muscle tissue. Since no differences were observed in MRC normalized to CS activity in neither permeabilized fibers nor isolated mitochondria, this suggests that the higher absolute MRC was mainly explained by a higher mitochondrial content in the HT users. The HRT in the present study included the use of both estradiol and synthetic progestin. In relation to that, previous findings suggest that it is estradiol and not progesterone/progestin that enhances mitochondrial biogenesis.[18,36] This was exemplified in cultured MCF-7 cells, where estradiol increased transcription and protein expression of nuclear respiratory factor 1 (NRF-1), resulting in increased mitochondrial biogenesis and elevated protein levels of two

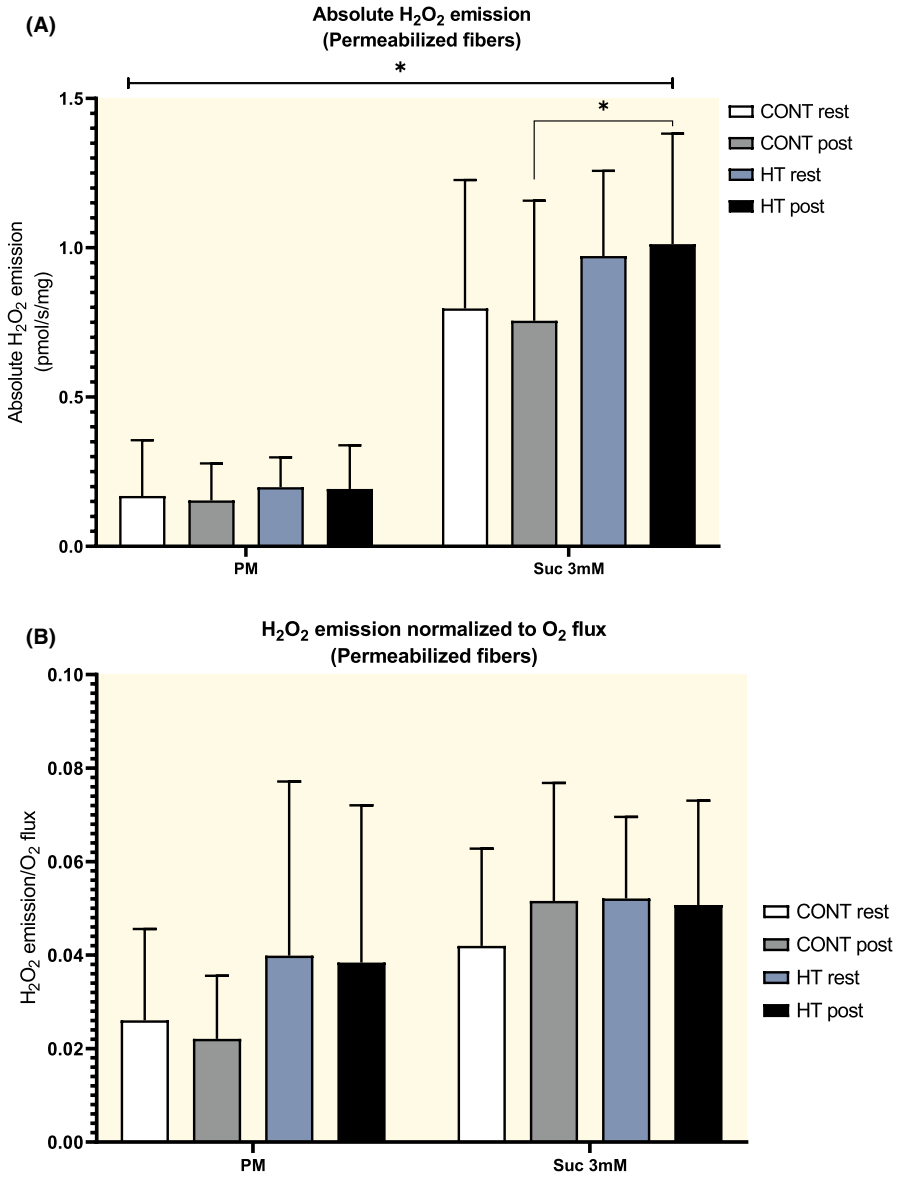

FIGURE 2 (A) Absolute and (B) H_2O_2 emission normalized to O_2 flux in permeabilized fibers of controls (CONT) and hormone-treated (HT) postmenopausal women. PM, pyruvate, malate; Suc, Succinate.

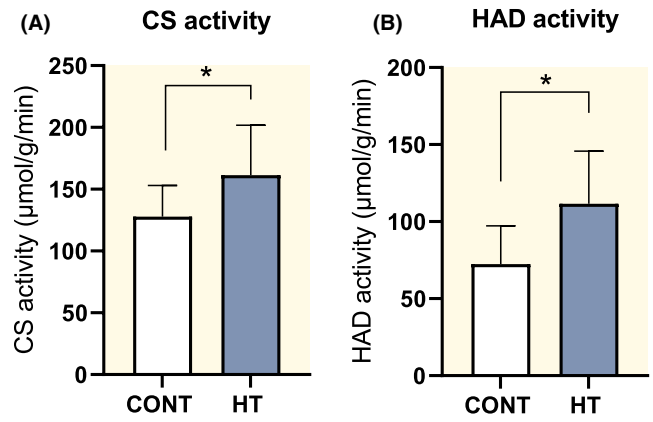

FIGURE 3 (A) CS activity in permeabilized fibers and (B) HAD activity in permeabilized fibers. CONT, controls; HT, hormone-treated. *$p < 0.05$.

complex IV units.[21] Other studies have reported similar effects of estradiol in skeletal muscle from rats[39] as well as other tissues.[40] Furthermore, estradiol seems to activate other co-activators and transcription factors such as adenosine monophosphate-activated protein kinase, proliferator-activated receptor-gamma coactivator alpha, peroxisome proliferator-activated receptors, and mitochondrial transcription factor A, all of which are linked to mitochondrial biogenesis or the upregulation of mitochondrial proteins.[41–44] Therefore, it can be speculated that similar mechanisms operate in human skeletal muscle within the HT group and explain the higher CS activity.

Typically, elevated CS activity, thus mitochondrial content, correlates with increased VO_2peak.[45] Notably, in our investigation, despite identifying a higher mitochondrial content in the HT group, no differences in VO_2peak

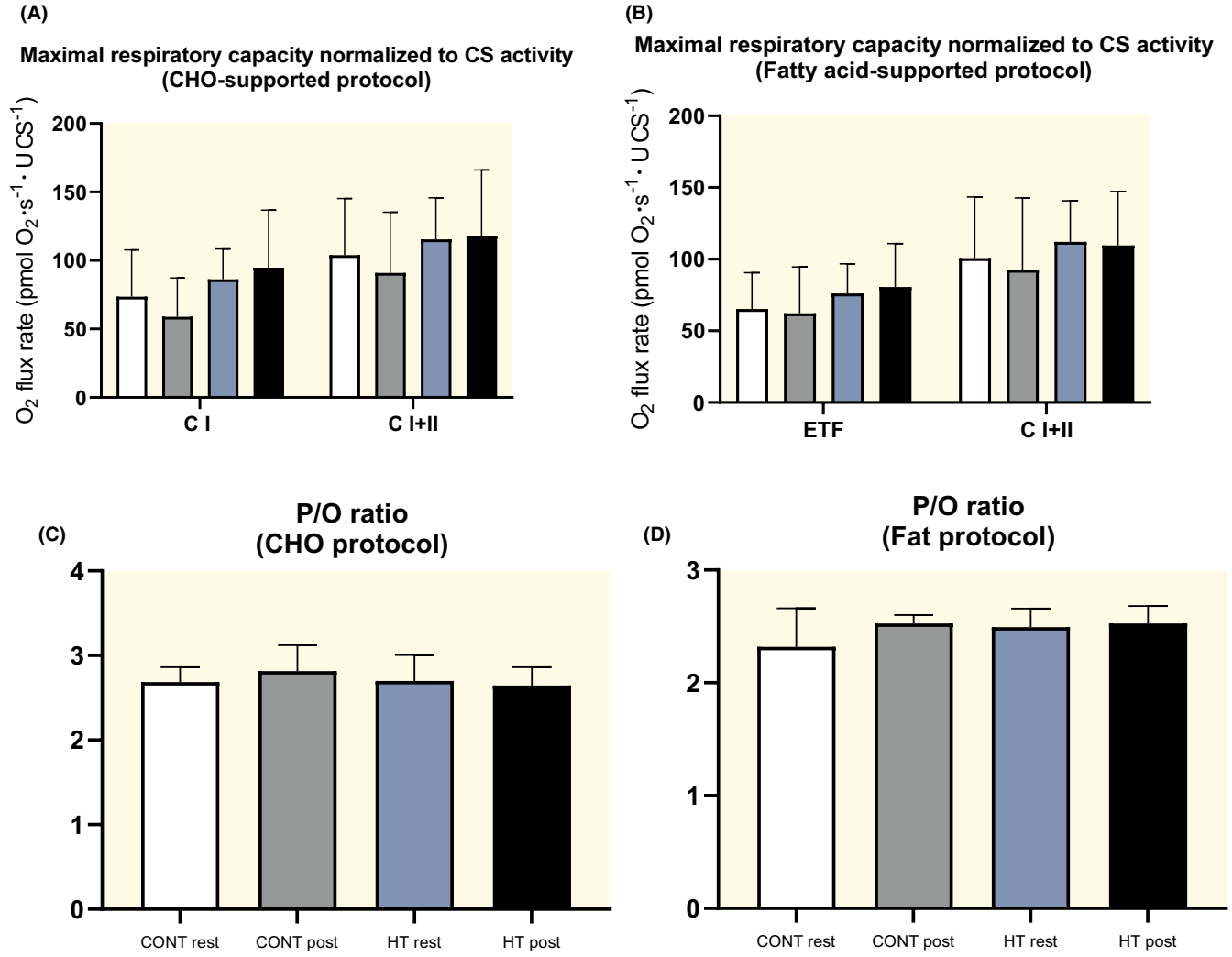

FIGURE 4 Maximal respiratory capacity in isolated mitochondria normalized to CS activity. (A) CHO-supported protocol (malate, pyruvate). (B) Fatty acid-supported protocol (malate, PalmC). (C) P/O ratio in CHO-supported protocol. (D) P/O ratio in the fatty acid-supported protocol. C I, complex I; C I+II, complex I+II; Post, post 45 min exercise.

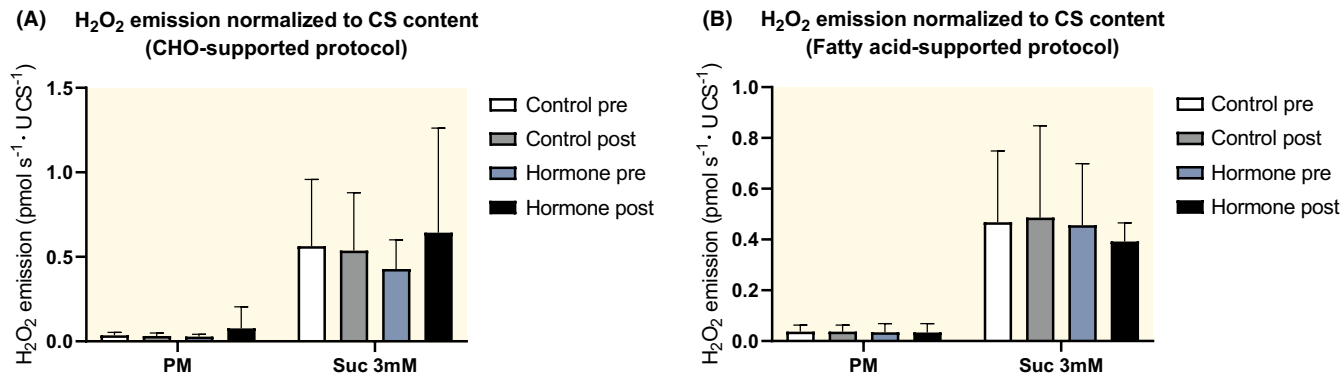

FIGURE 5 (A) H_2O_2 emission normalized to CS activity in the CHO-supported protocol. (B) H_2O_2 emission normalized to CS activity in the fatty acid-supported protocol. PM, pyruvate, malate; Suc, Succinate.

per FFM were observed between groups. It is pertinent to acknowledge that VO_2peak is predominantly dictated by central parameters, whereas peripheral adaptations, inclusive of augmented mitochondrial function or content, play a crucial role in mitigating peripheral fatigue.[46] Consequently, speculation arises concerning the potential

	CONT	HT	*p*-Value
Resting energy expenditure (kcal/day)	1363 ± 187	1423 ± 174	0.45
Fat oxidation rest (g/min)	0.08 ± 0.02	0.07 ± 0.02	0.39
Maximal fat oxidation (g/min)	0.29 ± 0.06	0.33 ± 0.07	0.10
Maximal fat oxidation (mg/kg FFM/min)	6.47 ± 1.6	7.25 ± 1.5	0.28

TABLE 4 Energy expenditure and fat oxidation.

Note: Energy expenditure, resting and maximal fat oxidation for controls (CONT) and hormone-treated (HT) postmenopausal women.

Abbreviations: FFM, fat-free mass; kcal, kilocalories.

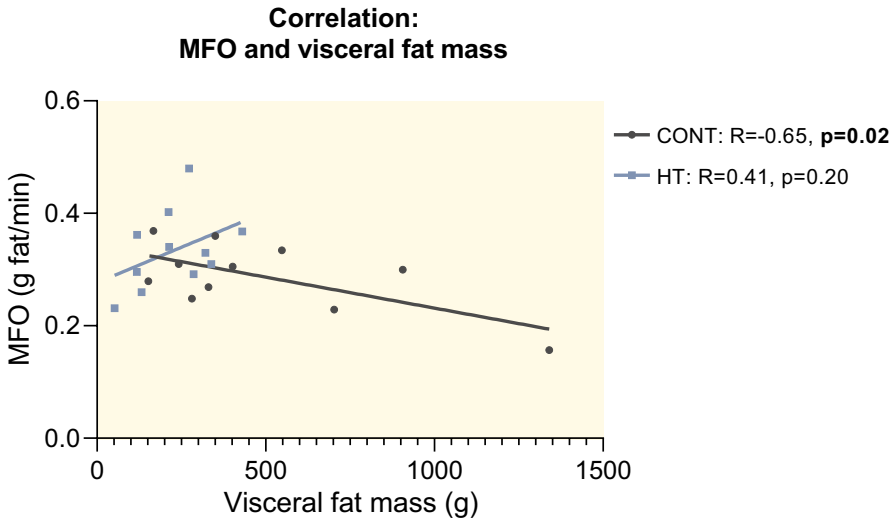

FIGURE 6 Correlation between MFO and visceral fat mass in CONT (controls) and HT (hormone-treated) postmenopausal women.

of the HT group to endure prolonged exercise or exhibit lower plasma lactate levels than the CONT group at equivalent absolute intensity. However, this was not assessed in the current study. Nevertheless, when CS activity was measured in isolated mitochondria, no significant difference between the CONT and HT groups was observed in the present study. The contrasting findings may be attributed to the isolation process of the mitochondria, which maximally yields 50% of the mitochondria from the tissue.[47] Furthermore, it is worth noting that although CS activity is a widely used marker for mitochondrial content,[48] it may be influenced by acute exercise.[49] Therefore, all flux rates in the present study were normalized to CS activity at rest, given the limited expectation of alterations in mitochondrial content within 45 min of exercise. This should therefore be considered when interpreting data.

3.2 | Mitochondrial efficiency

No differences between HT and CONT were found in mitochondrial efficiency measured as RCR and P/O ratio in permeabilized fibers and isolated mitochondria. The effect of HRT on mitochondrial efficiency has not previously been studied in humans, but animal studies have reported divergent findings on the influence of estradiol, and none on progesterone/progestin, on mitochondrial efficiency.[50–52] One study found that estradiol enhanced mitochondrial efficiency by suppressing uncoupling protein 3 (UCP3) in myoblasts in mice.[51] In contrast, others found no differences in ERα-knockout mice measured as P/O ratio, suggesting no direct influence of estradiol on mitochondrial efficiency.[50] However, the contrasting findings in the studies might be explained by the different methods used to assess mitochondrial efficiency. In support of the latter observations,[50,51] our findings are the first to suggest no difference in mitochondrial efficiency between postmenopausal women who are users or non-users of HRT.

3.3 | H_2O_2 emission

The absolute H_2O_2 emission was higher post-exercise in the HT group in the permeabilized fibers. This finding is in contrast with the majority of prior research in cell cultures and rodent models, which have indicated antioxidant effects of estradiol, however only at rest. The antioxidant effects of estradiol involved upregulation of mRNA levels of antioxidants such as glutathione peroxidases (GPX), ultimately leading to a reduction in H_2O_2 emission.[17,20,53,54] However, progesterone seems to increase state 4 H_2O_2 emission,[20] which could explain the current findings on

higher absolute H_2O_2 emission at maximal leak in the HT group, if progestins exert similar effects in humans. Still, previous studies indicate that the effect of progesterone is overruled by the antioxidant effects of estradiol.[17,20] Conversely, estradiol has also been described to exert pro-oxidant effects due to the structure of the hormone. When estradiol is oxidized into estrone or estriol, superoxide ($O_2^{\bullet-}$) is produced, thus increasing ROS formation.[55,56] This has been documented in liver, breast cancer cells, and the uterus,[56,57] but is not examined in skeletal muscle cells. However, as ROS is known to be an important signaling molecule essential for cellular function,[58] a higher absolute H_2O_2 emission could possibly reflect a modified signaling mechanism in the HT group. It has previously been shown that exercise likely induces the ROS production outside the mitochondria through the activation of nicotinamide-adenine-dinucleotide-phosphate (NADPH) oxidases and xanthine oxidases.[59,60] Since the higher absolute H_2O_2 emission was only observed post-exercise and in permeabilized fibers, this could support a better muscle signaling in the HT users in response to exercise compared to CONT. It is important to mention that our study did not examine antioxidant markers, which leaves the possibility that the activity of antioxidants might have increased, corresponding to the elevated absolute H_2O_2 emission post-exercise. Nevertheless, no differences were observed in H_2O_2 emission normalized to CS activity for both permeabilized fibers and isolated mitochondria. This suggests that the higher absolute H_2O_2 emission in the HT group in permeabilized fibers could be attributed to differences in mitochondrial content rather than any potential pro- or antioxidant effects of estradiol.

3.4 | Metabolic flexibility

HAD is a key enzyme of the beta-oxidation pathway that breaks down fatty acids to acetyl-coA before entering the tricarboxylic cycle. Notably, the HT group exhibited significantly higher HAD activity, consistent with the higher mitochondrial content and absolute MRC in muscle. Enhancements in both mitochondrial biogenesis and the activities of enzymes related to substrate oxidation, such as HAD, have been associated with improved metabolic flexibility.[14] Therefore, it seems plausible that HRT may augment metabolic flexibility in postmenopausal women when evaluated at the mitochondrial level. However, this was not reflected on a whole-body level in the measure of MFO which did not differ between the groups. Additionally, MFO did not correlate with HAD activity nor mitochondrial content, indicating no direct link between oxidative capacity at the mitochondrial level and whole-body level. Since previous studies have found that MFO and resting fat oxidation were lower in post- compared to premenopausal women with higher plasma estradiol concentrations[32,33] the comparable MFO rates in the groups of the present study might be due to other confounding factors.

MFO is affected by various factors such as age, gender, training status and VO_2peak, fasting period, fat mass, and plasma FFA,[30,31,61,62] which can influence the potential effects of female sex hormones on MFO. To account for some of these parameters, age and training status were matched between the groups, and all measurements were made after an overnight fast in the present study. In relation to this, plasma FFA concentrations and RER values were not different between the groups. However, we cannot exclude that the significantly higher fat mass, body fat percentage and visceral fat mass in the CONT group have equalized the direct effect of HRT on MFO. A previous study has found that obese unfit women exhibit a higher MFO compared to lean unfit women related to a higher fat mass.[30] Based on these findings, it was hypothesized that the higher total fat mass in the CONT group could potentially elevate their MFO and thereby compromise for any potential hormone-induced effects in the HT group. However, no correlation between fat mass and MFO in the CONT group was observed to support this ($R=-0.47$, $p=0.13$). Contrarily to total fat mass, a high visceral fat mass has been associated with lower MFO rates in pre-, peri-, and postmenopausal women.[33] Similar to these observations, we observed that visceral fat mass was significantly negatively correlated to MFO in the CONT, but not the HT group (CONT: $R=0.65$, $p=0.02$. HT: $R=0.41$, $p=0.2$). However, the significance of the correlation in the CONT group was largely driven by one subject. In support of the negative relationship between visceral fat mass and MFO in non-treated postmenopausal women, another study reported that a high android/gynoid ratio was associated with lower MFO rates,[62] which may reflect the accumulation of visceral fat in the android region. Yet, this was not significantly evident in the present study (CONT: $R=-0.52$, $p=0.09$. HT: $R=-0.1$, $p=0.76$).

Our findings suggest that while whole-body MFO seems to be preserved in the CONT group, there might be a compromised capacity for central and visceral fat oxidation, indicating that fat oxidation capacity might depend on fat location, when not influenced by female sex hormones. As these negative associations were not seen in the HT group, this potentially indicates that HRT contributes to maintaining the capacity for fat oxidation, regardless of the location of the fat. This may partly explain the favoring of a healthier body composition observed in the HT group. In support of this, a longitudinal study reported a higher increase in fat mass, mainly in the android region, in non-treated women compared to HRT users.[63]

3.5 | Limitations

In this study, HRT was used in varying doses between the individuals. Furthermore, the timing of HRT administration differed between the subjects in perspective to the test time points during the experimental day, which potentially affected the hormone plasma concentrations. However, as the women had been in HRT for >6 months, more or less stable levels of the synthetic hormones have been reached in plasma[64] and therefore only small daily fluctuations within each participant can be expected.

Regarding findings on mitochondrial function, ex vivo mitochondrial analyses may not fully represent in vivo conditions, and the handling of biopsies may influence the results. All the post-exercise biopsies were obtained within 5 ± 1.5 min after completion of the exercise bout. However, the preparation of both isolated mitochondria and permeabilized fibers requires 60–80 min. Therefore the post-exercise results may not completely reflect the acute effect of exercise. However, the present study was able to detect post-exercise differences in absolute MRC, which indicate that some alterations can be discovered after exercise despite relatively long preparation time.

Moreover, as this is a cross-sectional study it has limitations in establishing causality. Therefore, a future longitudinal randomized controlled study is needed to fully clarify the effects of HRT on metabolic flexibility in postmenopausal women.

4 | METHODS

The study was approved by the Ethical Committee for the Capital Region of Denmark (H-21049123) and conducted in accordance with the Declaration of Helsinki. Participants were informed verbally and written about the study and potential risks before written consent was obtained.

4.1 | Participants

This cross-sectional study included 22 postmenopausal women. Inclusion criteria were as follows: female between 45 and 60 years of age, amenorrheic for >12 months, and no previous cardiovascular events. The exclusion criteria included any other medication than HRT, hypertension, and elevated long-term blood glucose levels (Glycated hemoglobin (HbA1C) >48 mmol/mol or 6.5%).

All participants completed a questionnaire with information on health and physical activity level (International Physical Activity Questionnaire, IPAQ) before any measurements were done.

One group ($N = 11$) consisted of postmenopausal women in oral HRT for 3.6 ± 2 years (HT), and another group ($N = 11$) consisted of VO_2peak- and BMI-matched non-treated controls (CONT). The groups were further matched by years after last menstrual bleeding. The HT group all received a combination of estradiol and progestin (norethisterone acetate). As the HRT was prescribed by the participants' personal clinicians the concentration in estradiol and norethisterone acetate differed in the participants from 0.5–2 mg and 0.1–1 mg, respectively. The drugs included were Activelle (Novo Nordisk), Noresmea (Sandoz), and Kliogest (Novo Nordisk). All non-treated participants were later confirmed postmenopausal with FSH values >30 IU/L.[65] The detection of norethisterone acetate was inaccessible as no commercial appropriate kit was available, and therefore, norethisterone acetate levels are not presented.

4.2 | Study overview

Participants were scheduled for two separate laboratory visits at least 48 h apart. On both days, participants arrived after an overnight fast and had restrained from strenuous physical activity 48 h prior to both visits. Participants were instructed to log their dietary intake the day prior to the screening and to repeat it the day prior to the experimental day to standardize dietary factors, minimize individual variability, and facilitate the isolation of specific experimental effects.

The screening included measurements of HbA1c (DCA Vantage Analyzer), anthropometry (height, weight, hip/waist ratio), blood pressure, electrocardiogram (ECG), and body composition by Dual-energy X-ray absorptiometry (DXA) (Lunar iDXA, G&E Medical Systems Lunar, Wisconsin, USA, Encore Version 14.10.022). The participants completed two exercise tests on a bicycle ergometer (COSMED, E100 ergoline, Germany), a MFO test and a peak oxygen consumption (VO_2peak) test separated by 5 min of rest. Pulmonary gas exchange of VO_2 and CO_2 was collected by a breath-by-breath analyzer system (Quark CPET, COSMED, Italy). The fat oxidation test was started by 5 min at rest, followed by a 5 min warm up at 50 W. The test then proceeded with an increase of 20 W every third min until an RER of 1.0 was reached. MFO was calculated as described previously.[31] The initial workload of the VO_2peak test was 50 W and increased 20 W every min until exhaustion. To verify if subjects had reached their VO_2peak, two of the following criteria had to be attained: RER >1.15, plateau in VO_2 or heart rate despite an increase in workload, or inability to maintain a cadence above 60 rpm. At least two of three criteria were met in all tests.

On the experimental day, resting energy expenditure was measured by a ventilated hood system (COSMED, Q-NRG,

Italy) after participants had rested for 20 min in a supine position. A vein catheter was inserted into the antecubital vein and blood was collected twice at rest and twice during exercise (after 30 and 45 min). The exercise consisted of 45 min on a cycle ergometer at 65% ± 2% VO_2peak (CONT: 65% ± 2%, HT: 64% ± 3% VO_2peak, $p = 0.8$). To obtain similar relative exercise intensity, the load was controlled and if necessary, adjusted within the first 12 min of exercise. A biopsy from m. vastus lateralis was obtained with a Bergström needle with manual suction[66] at rest and immediately (5 ± 1.5 min) after completion of exercise. Biopsies were separated in three and placed in either isolation buffer (Sucrose 100 mM, KCl 100 mM, Tris–HCl 50 mM, KH_2PO_4 1 mM, EGTA 0.1 mM, BSA 0.2%, at pH 7.4) for analysis of isolated mitochondria, BIOPS (100 mM CaK_2EGTA, 100 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2 \cdot 6H_2O$, 20 mM Taurine, 15 mM Na_2Phospho-creatine, 20 mM Imidazole, 0.5 mM DTT, 50 mM MES) for analysis of permeabilized fibers or snap frozen in liquid nitrogen for later analysis.

4.2.1 | Permeabilization of muscle fibers

The permeabilization process was completed as previously described.[67] After permeabilization with saponin approximately 2 mg (range: 1.41–2.43 mg) wet weights of tissue were added to each oxygraph chamber containing MIR05 (sucrose (110 mM), potassium lactobionate (60 mM), EGTA (0.5 mM), $MgCl_2 \cdot 6H_2O$ (3 mM), taurine (20 mM), KH_2PO_4 (10 mM), HEPES (20 mM), BSA (1 g/L), pH 7.1) at 37°C for high-resolution respirometry or fluorometry. All absolute measures were normalized to mg wet weight of tissue in the chamber. The analyses were started immediately after permeabilization.

4.2.2 | Isolation of mitochondria

Mitochondria were isolated from the biopsy via different centrifugation steps as explained elsewhere[68] and diluted in suspension buffer (Sucrose 75 mM, Mannitol 225 mM, Tris base 10 mM, EDTA 0.1 mM, at pH 7.4). The isolated mitochondria suspension (10 µL) was added to each chamber containing MIR05 at 37°C for high-resolution respirometry or fluorometry. All analyses of isolated mitochondria were started within 45 min after isolation was finished.

4.2.3 | High-resolution respirometry

All measurements were performed in duplicate in 5 different oxygraphs (Oxygraph-2k, Oroboros, Austria). Measurements on permeabilized fibers were performed in hyperoxygenated chambers (450 nmol O_2/mL), and the following protocol was used: Pyruvate (5 mM), malate (2 mM), and glutamate (10 mM) were added to determine state 2 respiration with complex I linked substrates. Then cytochrome c (0.01 mM) was added to control for outer membrane integrity (as no changes were observed after titration of cytochrome c, the integrity of the outer mitochondrial membrane was maintained (data not shown)). To determine state 3 with complex I-linked substrates, ADP was added in stepwise increments (5.0 mM). Succinate (10 mM) was added to determine state 3 respiration with complex I + II linked substrates, followed by a stepwise titration (0.5 µM) of FCCP to reach maximal uncoupling.

Measurements on isolated mitochondria were performed at atmospheric oxygen levels (200 nmol O_2/mL) and 2 different protocols were used; a CHO-supported and a fatty acid-supported protocol. They progressed as follows: Addition of isolated mitochondria solution (10 µL) followed by the addition of malate (2 mM) and pyruvate (5 mM) in the CHO-supported protocol and malate (2 mM) and palmitoylcarnitine (PalmC) (10 µM) in the fatty acid-supported protocol to determine state 2 leak respiration. To determine P/O ratio both protocols proceeded with the addition of a small non-saturating amount of ADP (200 µM), which caused the respiratory flux to increase. As the flux returned to baseline, the chambers were opened to restore the oxygen levels inside the chambers before the addition of ADP (5 mM) and $MgCl_2$ (3 mM). In the fatty acid-supported protocol pyruvate (5 mM) was then added. Finally, glutamate (10 mM) and succinate (10 mM) were added in both of the protocols to determine state 3 respiration with complex I + II linked substrates.

Mitochondrial efficiency was determined as P/O ratio by dividing the amount of ADP added in the non-saturating step with the difference in oxygen levels before and after the flux rate returned to baseline. The calculation relies on the assumption that all added ADP is phosphorylated,[69] and is further explained in Flensted-Jensen et al., 2023[70]:

$$\mathrm{P/O\ ratio} = \mathrm{ATP}\frac{200\ \mu\mathrm{M}}{\mathrm{oxygen1} - \mathrm{oxygen2}} * 2$$

The P/O ratios are presented as the mean value of two chambers.

RCR was also assessed to evaluate mitochondrial efficiency and determined in both isolated mitochondria and permeabilized fibers. It was calculated by the ratio between maximal ADP-supported respiration (state 3 with complex I + II linked substrates) and state 2 leak respiration:

$$\mathrm{RCR} = \frac{\mathrm{State\ 3}}{\mathrm{LEAK}}$$

Leak respiration refers to mitochondrial respiration in the absence of ATP synthesis, as only substrates that stimulate specific complexes in the electron transport system are added and no ADP is available.[71]

4.2.4 | High-resolution fluorometry

In the fluorometry protocol for the permeabilized fibers, blebbistatin (5 μM) was added first, to prevent muscle fiber contraction.[72] It proceeded with the addition of superoxide dismutase (1.7 μM), Amplex Red (4 μM), and horseradish peroxidase (HRP) (4 μM) to catalyze the dismutation of $O_2^{\bullet -}$ to $H_2O_2 + O_2$ and to produce the fluorescent molecule, resorufin. Pyruvate (5 μM) and malate (10 μM) were added to determine state 2 H_2O_2 emission with complex I linked substrates. Then a three-step succinate titration was performed (1–2–3 mM). To calibrate the flourometer, a 0.1 μM H_2O_2 injection was performed after the addition of HRP. Hereafter, three additional 0.1 μM H_2O_2 injections were performed (after malate/pyruvate, after succinate (3 mM), and after ADP/$MgCl_2$), allowing for a detection of any changes in assay sensitivity.

The protocols for isolated mitochondria were similar; however, no blebbistatin was added. The protocols for isolated mitochondria were performed as a CHO-supported (pyruvate (5 μM) + malate (10 μM)) protocol and a fatty acid-supported (PalmC (2 μM) + malate (10 μM)) protocol.

Enzyme activities

CS and HAD activity were determined in freeze-dried dissected muscle tissue as previously described[73] and determined per gram wet weight. CS activity is used as a marker of mitochondrial content[48] in the present study.

Blood samples

Blood was obtained into sampling tubes with anticoagulants (Dipotassium (K_2) ethylene diamine tetraacetic acid (EDTA)/Aprotinin; tripotassium (K3) EDTA/Aprotinin; lithium heparin; sodium fluoride (NaF)/potassium oxalate (K-oxalate)), and centrifuged (ROTINA 380R, Axeb lab solutions) for 10 min at 4000 *g* and 4°C to separate the plasma. The plasma was pipetted into Eppendorf tubes and frozen at −80°C until analysis. COBAS e601 was used to analyze plasma samples using electrochemiluminescence immunoassay (ECLIA).

4.3 | Statistical analysis

All data were tested for normality by the Shapiro–Wilk test, QQ-plots, and residual plots before analysis. The data were normally distributed and are presented as mean values ± standard deviation (SD). A mixed linear effects model accounting for repeated measures was performed in Rstudio (Version Mountain Hydrangea 2023.06.0) on all mitochondrial data and plasma concentrations of glucose and FFA during exercise, to test the potential effects and interaction between the dependent variable, respirometry/fluorometry or glucose/FFA, and the independent variables, group and time. Unpaired t-tests were used to compare mean and SDs between the groups for resting plasma concentrations, anthropometric measures, and exercise tests. GraphPad Prism (Version 9.01, GraphPad Software, Inc., La Jolla, California, USA) was used for illustrative figures. In all analyses, an alpha level of $p < 0.05$ was considered statistically significant.

5 | CONCLUSION

We predicted that long-term use of HRT would reflect metabolic flexibility by firstly, an enhanced mitochondrial MRC and H_2O_2 emission at rest and post-exercise, accompanied by higher MFO at a whole-body level. Secondly; lower plasma concentrations of lipids, cortisol, and insulin and the favoring of a healthier body composition when compared to non-users of HRT. We found that the use of HRT, in part, was associated with metabolic flexibility, as the HT users showed larger capacity for substrate oxidation at the mitochondrial level, due to higher mitochondrial content and HAD activity. However, resting fat oxidation, MFO, resting energy expenditure, and plasma markers did not differ between HRT users and non-users of HRT. HRT was associated with lower total fat mass, visceral fat mass, and overall body fat percentage compared to non-users of HRT.

AUTHOR CONTRIBUTIONS

A. S. Kleis-Olsen: Investigation, project administration, writing – original draft. J. E. Farlov: Investigation, writing – original draft. E. A. Petersen: Investigation, writing – review & editing. M. Schmücker: Investigation, writing – review & editing. M. Flensted-Jensen: Investigation, writing – review & editing. I. Blom: Investigation, writing – review & editing. A. Ingersen: Investigation, writing – review & editing. M. Hansen: Investigation, writing – review & editing. J. W. Helge: Conceptualization, resources, funding acquisition, writing – review & editing. F. Dela: Resources, funding acquisition, writing – review & editing. S. Larsen: Conceptualization, investigation, supervision, resources, funding acquisition, project administration, writing – review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data supporting the findings in the study are available from the corresponding author (A. S. Kleis-Olsen) or last author (S. Larsen) upon reasonable request.

ORCID

A. S. Kleis-Olsen https://orcid.org/0009-0004-4987-8698
E. A. Petersen https://orcid.org/0009-0006-0902-7009
M. Schmücker https://orcid.org/0009-0007-7133-5683
M. Flensted-Jensen https://orcid.org/0000-0002-5288-9783
I. Blom https://orcid.org/0000-0003-1602-3031
A. Ingersen https://orcid.org/0000-0001-6112-3563
M. Hansen https://orcid.org/0000-0002-9383-8202
J. W. Helge https://orcid.org/0000-0001-9724-5423
F. Dela https://orcid.org/0000-0001-9970-9535
S. Larsen https://orcid.org/0000-0002-5170-4337

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