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Indexes of Fat Oxidation from Ramp vs. Graded Incremental Protocols in Postmenopausal Women

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Abstract: The maximal rate of fat oxidation (MFO, in g·min^{-1}) and the relative exercise intensity at which it occurs (FAT$_{max}$, as %$\dot{V}O_{2max}$) are indexes of metabolic flexibility. The time-consuming, graded exercise protocol required for MFO/FAT$_{max}$ determination hinders the extensive use of these indexes for individualized exercise prescription and monitoring. **Purpose:** validate ramp testing for MFO and FAT$_{max}$ measures in postmenopausal women. **Methods:** Seventeen healthy women (age: 54 ± 4 years, BMI 22 ± 3 kg·m^{-2}, and $\dot{V}O_{2max}$ 36.4 ± 5.3 mL·min^{-1}), who were 4 ± 3 years from menopause, performed on a cycle-ergometer, a ramp, and a graded incremental test. Based on $\dot{V}O_2$ and respiratory exchange ratio from the ramp and graded protocol (i.e., the 5th minute of each step), MFO and FAT$_{max}$ were determined. Data from the two protocols were compared using paired *t*-tests, linear regression, and Bland–Altman analysis. **Results:** The MFO measured with a ramp protocol was not different from (0.24 ± 0.09 vs. 0.20 ± 0.08 g·min^{-1}, $p = 0.10$), and moderately associated with, that of the graded protocol ($r^2 = 0.46$). FAT$_{max}$ occurred at similar exercise intensity for both protocols (47.8 ± 5.1 vs. 47.5 ± 4.3 %$\dot{V}O_{2max}$, $p = 0.91$, $r^2 = 0.52$). The comparison of MFO and FAT$_{max}$ across the protocols yields a non-significant bias but a relatively large limit of agreement (respectively, 0.05 g·min^{-1}, LOA = −0.08, and 0.19 g·min^{-1}; 0.3 %$\dot{V}O_{2max}$, LOA = −7.8, and 10.6 %$\dot{V}O_{2max}$). **Conclusions:** In postmenopausal women, ramp testing offers a valid alternative to the graded protocol for identifying MFO and FAT$_{max}$. The availability of a time- and cost-efficient approach, which can be incorporated into standard ramp incremental testing, can facilitate using these indexes of metabolic flexibility in research and medicine.

Keywords: FAT$_{max}$; fat oxidation; exercise testing; methodology; metabolic flexibility; adipose physiology

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1. Introduction

Metabolic flexibility is defined as the ability to maintain energy homeostasis in periods of caloric excess/restriction, periods of low/high energy demand, and changes in fuel availability; this is obtained through a short-term adjustment and/or a long-term adaptation of the metabolic rate and a shift in the nutrient oxidation balance between fats and carbohydrates [1]. Metabolically flexible individuals have a high oxidative capacity in skeletal muscle and, consequently, a high potential for fatty acid oxidation (FO) in response to lipid availability at rest; furthermore, they present a reduced reliance on

glucose oxidation, which enables them to preserve intramuscular glycogen stores during exercise; these characteristics are typically associated with a good level of cardiorespiratory fitness [2]. On the contrary, FO can be compromised by chronic inactivity and/or caloric excess [3]. Decreased reliance on FO and increased dependence on glucose oxidation may also precede insulin resistance in the offspring of patients with type 2 diabetes mellitus [4]. Moreover, metabolic inflexibility at rest and during exercise is associated with overweightness and obesity [3,5] and becomes increasingly prevalent in women as they transition to menopause [6,7]. Postmenopausal women exhibit lower fat oxidation and energy expenditure at rest [6] and during exercise [8], which may be partially responsible for the increased total and visceral fat mass [7,8]. Loss of muscle mass seems to be the most critical contributor to the observed changes in metabolism in postmenopausal women. Indeed, lower lean body mass is correlated with low whole-body fat oxidation and energy expenditure [9]. Moreover, epidemiological studies have shown that high follicle-stimulating hormone and low estrogen levels are associated with lower fat oxidation at rest and during exercise, changes in body composition, and central obesity [8]. Thus, decreasing estrogen levels, loss of muscle mass, and reduction in basal metabolic rate concur with a body's reduced ability to oxidize fat [6,8], a positive caloric balance, and a progressive increase in total and central body fat mass [10].

In addition, the higher prevalence of overweightness and obesity [11], sarcopenia [12], and metabolic syndrome/type 2 diabetes [13,14], which is associated with the higher longevity of women compared to men, coincide with an increase in the burden of disease, which calls for specific attention and intervention strategies.

Fat oxidation capacity can be characterized in vivo through the measurement of maximal fat oxidation (MFO) rate and the corresponding exercise intensity relative to maximal aerobic power (i.e., FAT_{max}) [15]. FO during exercise can be derived from the product of the fat oxidation in $g \cdot L^{-1}$ (as revealed by the respiratory exchange ratio (R)) and the oxygen consumption ($\dot{V}O_2$, in $L \cdot min^{-1}$, i.e., the absolute intensity) [16]. Typically, FO increases from rest up to a peak value and then markedly declines at the higher relative intensities, thus describing an inverted U-shape curve [15]. The peak of this curve identifies MFO and the corresponding exercise intensity relative to $\dot{V}O_{2max}$ (i.e., FAT_{max}). MFO and FAT_{max} are typically identified with the individual being in a fasted state and through graded testing protocols with step durations between 1 and 10 min, which entail reaching steady-state [16,17]. The workload increments for the graded steps and the estimation of FAT_{max} (i.e., $\%\dot{V}O_{2max}$) require a previous assessment of the maximal oxygen uptake ($\dot{V}O_{2max}$) through an additional incremental test to exhaustion, which is performed on a separate day [17]. However, numerous variations of graded protocols exist, and there is still no consensus on the gold standard [17].

This time-consuming protocol may limit the investigation of these indexes in research, sports, and clinical settings. In this context, the possibility of determining MFO and FAT_{max}, based on a shorter, non-steady-state, ramp incremental test, would have a great practical advantage. A ramp incremental test allows the time-efficient determination of cardiorespiratory fitness (i.e., $\dot{V}O_{2max}$) along with other valuable indexes, such as the ventilatory thresholds, efficiency of locomotion (i.e., O_2 gain), the $HR/\dot{V}O_2$ relationship, and the mean response time, which makes an individualized exercise prescription possible [18–22]. Accurately determining MFO and FAT_{max} in a single short visit would enable us to efficiently evaluate the body's potential for fat oxidation and facilitate the practical use of these parameters in research, sports, and clinical settings.

Two previous studies in young males tested the hypothesis that MFO and FAT_{max} in running [23] and cycling exercises [24] could be accurately determined based on a ramp incremental test, compared with the most commonly used graded protocol. Both studies

concluded that the ramp incremental could be a valid alternative for the determination of FAT_{max}; however, the comparison in the measure of MFO from ramp vs. graded protocols led to conflicting results, i.e., similar MFO [24] or 2-fold higher values for ramp vs. graded [23].

While the above results appear to support the possibility of obtaining at least an accurate FAT_{max} from incremental ramp testing, the extension of the above conclusions to different populations needs a specific validation: possible age, sex [25], and fitness-related [26] differences in the selection of substrates, in addition to the $\dot{V}O_2$/Power output relationship during incremental vs. constant-load exercise [27], might affect the correspondence between indexes derived from both protocols.

Therefore, given the importance of characterizing the potential role of fat oxidation as an index of metabolic flexibility in the female population, especially after the menopause transition, we aimed to extend and clarify the results of previous studies by testing post-menopausal women. To this end, we compared the performance of a time-efficient ramp to traditional graded, incremental testing for determining MFO and FAT_{max}.

We hypothesized that the MFO and FAT_{max} determined by the ramp test would be similar to the steady-state value obtained with the graded test.

2. Materials and Methods

2.1. Participants

Seventeen recreationally active postmenopausal women were recruited by advertisement within the local community and agreed to participate in this study. Inclusion criteria were female sex, age between 45 and 65 years, and menopause (i.e., absence of menstrual cycles for a minimum of 12 months); exclusion criteria were smoking and any condition that could influence the physiological responses during testing. Participants were not taking any medications that might alter their cardiorespiratory and metabolic responses to exercise. The participants were fully informed of any risk and discomfort associated with the experiments before giving their written consent to participate. Moreover, all participants had some previous physical activity experience, and ~half of them were engaged in organized aerobic physical activity programs at least once per week. All procedures were approved by the institutional committee for approval of human research (no. 16-2019).

2.2. Protocol

After medical clearance, participants visited the laboratory three times within one week. All tests were performed in an environmentally controlled laboratory (22–25 °C, 55–65% relative humidity). Participants were instructed to avoid caffeine consumption and physical activity for at least 8 h and 24 h, respectively, before each testing session. To minimize diurnal variation in whole-body fat and glycogen oxidation, all visits in each participant were conducted at the same time of day following a standardized meal consumed 2 h before each visit: 500 cc of water and 2 $g \cdot kg^{-1}$ of low glycaemic index carbohydrates [28].

On the first visit, anthropometric measures were taken by a single skilled investigator while participants were in a fasted state, wearing underwear and no shoes. The intra-evaluator measurement error (TEM) was calculated to evaluate the precision of the anthropometric measurements. Body mass (digital scale, Seca877, Seca, Leicester, UK) and height (vertical stadiometer, Seca, Leicester, UK) were determined to the nearest 0.1 kg and 0.5 cm ($\leq$1.0 %TEM), and the body mass index calculated. Skinfolds thickness was measured in triplicate at the pectoral, scapular, tri-ceps, iliac, abdominal, and thigh site (abdominal: 5.84 %TEM); percent body fat was estimated based on the sum of skinfolds [29].

Moreover, during the same visit, the participants' physical activity levels were assessed using the short form of the International Physical Activity Questionnaire [30]. The questionnaire was administered in a self-report format, with participants providing information about the frequency, duration, and intensity of physical activities over the past seven days as well as their sedentary behavior [30]. Total physical activity was calculated and expressed as metabolic equivalents (met·wk^{-1}), allowing for the categorization of participants' physical activity as low, moderate, or high as described elsewhere [30].

During the second and third visit, participants performed, on an electromagnetically braked cycle ergometer (Sport Excalibur, Lode, Groningen, The Netherlands), respectively: (i) A ramp incremental test to exhaustion; (ii) A graded incremental protocol.

Lastly, in a subsample of 10 individuals, a second ramp incremental test was performed on a different visit for test-retest reliability.

The ergometer position was chosen during the first visit and recorded for successive appointments.

2.3. Ramp Incremental Protocol

For all individuals, the ramp incremental protocol consisted of a 6 min cycling at 80 W, followed by 4 min baseline cycling at 30 W, and thereafter, a 10–15 watt·min^{-1} linear increase in power output until volitional exhaustion [31]. Increments in power output were individually tailored based on the anticipated fitness level of the individual [32] so that the incremental phase of the test would last between 8 and 12 min. Participants were asked to pick a self-selected cadence in the 70–90 rpm range and maintain it throughout all tests. Breath-by-breath pulmonary gas exchange, ventilation, and heart rate were continuously measured using a metabolic cart (Quark B2, Cosmed, Rome, Italy). Capillary blood samples (20 µL) were drawn from the ear lobe before and at the 1st, 3rd, and 5th min after exhaustion. The samples were immediately analyzed using an electro-enzymatic technique (Radiometer ABL90 FLEX, Radiometer Medical ApS, Brønshøj, Denmark), and the highest value was considered the peak of blood lactate accumulation for the incremental test.

2.4. Graded Incremental Protocol

The graded incremental protocol consists of a 3 min baseline cycling at 30 watts, followed by 5-step increments lasting 5 min. The absolute power output for each of the five steps was chosen to elicit a $\dot{V}O_2$ equal to 40, 50, 60, 70, and 80% of the previously identified $\dot{V}O_{2max}$. To this end, the individual $\dot{V}O_2$/power output relationship derived from the incremental exercise was corrected for the $\dot{V}O_2$ mean response time and slow component (see data analysis) [18]. Breath-by-breath pulmonary gas exchange, ventilation, and heart rate were continuously measured using the same method described for the ramp incremental.

2.5. Data Analysis

For both protocols, gas exchange variables and heart rate were sampled breath-by-breath; aberrant data points (outside 3 standard deviations from the local mean) were removed, and thereafter, data were linearly interpolated at 1 s and then mediated at 5 s intervals. For the incremental test, the gas exchange threshold and respiratory compensation point were determined with the standard technique by visual inspection of gas exchange variables by three blinded expert reviewers [22]. The steady-state $\dot{V}O_2$, measured during the 80 W bouts prior to the ramp incremental test, was used to correct the $\dot{V}O_2$/power output relationship for the mean response time [20]. To correct the ramp-identified power output above the gas exchange threshold, an additional correction to account for the $\dot{V}O_2$ slow component was applied [18]. The power outputs associated with the target $\dot{V}O_2$ (i.e., 40, 50, 60, 70, and 80 %$\dot{V}O_{2max}$) were obtained using the mathematical model developed by

Caen et al. [18]. $\dot{V}O_{2max}$, $HR_{max,}$ and peak power output were determined, respectively, as the average $\dot{V}O_2$ of the last 30-s of exercise and the highest HR and power output achieved upon exhaustion [33]. FO ($g \cdot min^{-1}$) was calculated for both testing protocols based on $\dot{V}O_2$ and respiratory exchange ratio (R) data and the following formula [34]:

$$FO\ (g \cdot min^{-1}) = 1.67 \times (1 - R) \times \dot{V}O_2\ (L \cdot min^{-1})$$

A 5 s average was computed for ramp incremental, while a one-minute average at the 5th minute of each step was used for the graded incremental protocol. Individual data from both protocols were then interpolated every 5% of $\dot{V}O_{2max}$ from 40 to 80 percent to allow data averaging and comparisons between intensities and testing protocols.

For each participant, the interpolated FO data of the two protocols were plotted as a function of relative exercise intensity ($\%\dot{V}O_{2max}$) (Figure 1). Then, a 2nd order polynomial curve ($y = ax^2 + bx + c$) was fitted through the data for each protocol [17]. Maximal FO (MFO) and the corresponding relative exercise intensity (FAT_{max}) were determined with the following formulas [17]:

$$MFO = y_{max} = -\Delta/4a \quad (1)$$

where $\Delta = (b^2 - 4ac)$

$$FAT_{max} = x_{@MFO} = -b/2a \quad (2)$$

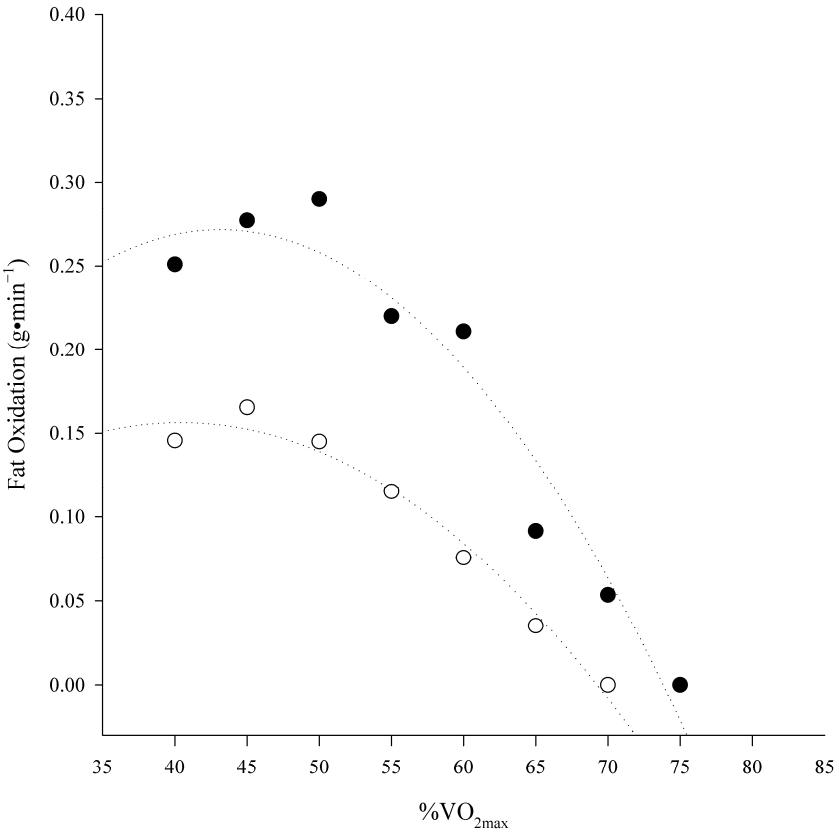

Figure 1. Fat oxidation during graded incremental protocol as a function of exercise intensity relative to the maximal oxygen uptake ($\%\dot{V}O_{2max}$) in two representative participants along with the 2nd order curve fitting.

Lastly, the individual values of $\dot{V}O_2$, HR, and power output at FAT_{max} were identified for each protocol using the linear relationship with $\%\dot{V}O_2$.

2.6. Statistical Analysis

All data are presented as mean ± standard deviation (SD). A two-way repeated-measures ANOVA was performed to compare $\dot{V}O_2$, HR, R, power output, and fat oxidation between testing protocols over different relative intensities (intensity × protocol). Post hoc analyses were performed using the Bonferroni test. To test the between-day fat oxidation variability, the within-subject coefficient of variation was determined by comparing the last-minute warm-up (i.e., 30 watts) period performed before each protocol. An adjusted r^2 was calculated to validate the regression models (i.e., 2nd order polynomial curve) fitted through FO and $\%VO_{2max}$ for each protocol.

After assumptions verification (i.e., normality, homogeneity of variance), a paired *t*-test was applied to compare FAT_{max} and the values of MFO, $\dot{V}O_2$, HR, and power output at the intensity corresponding to FAT_{max} between the two exercise protocols. To test the relationship, concordance, and agreement between the measures of FAT_{max} and MFO, which were between the two protocols, a linear regression, the concordance correlation coefficient (CCC), and Bland–Altman analysis were performed. Differences from "0" for the detected Bland–Altman bias between the two protocols were tested by a one-sample z-test.

Lastly, in the subsample of 10 individuals who performed a second ramp incremental test on a separate day, initial and final $\dot{V}O_{2max}$, MFO, and FAT_{max} were compared by paired *t*-test, linear regression, CCC, and Bland–Altman analysis. Power analysis was conducted a priori (G*Power 3.1) based on the expected SD of fat oxidation reported during constant load exercise in previous articles as the primary variable [35]. Thus, to identify significant differences, with an α error of 0.20 and a statistical power $(1 - \beta)$ of 0.80, an n value of 17 subjects was estimated. All statistical analyses were performed using SigmaPlot (version 14.0). Statistical significance was accepted when $p < \alpha$, α was set in advance at the 0.05 level, and *Cohen's d* determined.

3. Results

The subjects' characteristics are reported in Table 1. The average time from menopause was 4 ± 3 yrs (from 1 to 8), while the average body mass index and % body fat indicated a normal weight population (22 ± 3 $kg \cdot m^{-2}$ and 25 ± 4 %). The high average $\dot{V}O_{2max} \cdot kg^{-1}$ and the International Physical Activity Questionnaire results indicated a moderately active lifestyle (36.4 ± 5.3 $mL \cdot min^{-1} \cdot kg^{-1}$ and 2250 ± 150 $met \cdot wk^{-1}$).

Table 1. Overview of anagraphic, anthropometrics, and fitness variables.

	Age	**Time from Menopause**	**Height**	**Weight**	**BMI**	**%BF**	**FFM**	$\dot{V}O_{2max}$	**PA**
	(yrs)	**(yrs)**	**(cm)**	**(kg)**	**($kg \cdot m^{-2}$)**	**(%)**	**(Kg)**	**($mL \cdot min^{-1} \cdot kg^{-1}$)**	**($met \cdot wk^{-1}$)**
Mean ± SD	54 ± 4	4 ± 3	164 ± 5	59 ± 8	22 ± 3	25 ± 4%	43 ± 4	36.4 ± 5.3	2250 ± 150

Age, time from menopause, height, weight, % body fat (BF), fat-free mass (FFM), maximum oxygen consumption ($\dot{V}O_{2max}$), and habitual level of physical activity (PA). Values expressed as Mean ± SD.

Participants' mean ± SD of $\dot{V}O_{2max}$, peak power output, and HR_{max}, measured at the end of the ramp incremental, were 2.12 ± 0.26 $L \cdot min^{-1}$, 172 ± 22 watts, and 171 ± 9 bpm, respectively. Furthermore, the values of $\%HR_{max}$, R_{max}, and maximal lactate concentration reached upon exhaustion indicate that a maximal effort was reached (101 ± 5%, 1.19 ± 0.07, and 8.6 ± 1.1 $mmol \cdot L^{-1}$, respectively). Participants' gas exchange ratio and respiratory compensation point were detected at $\dot{V}O_2$ of 1.22 ± 0.18 $L \cdot min^{-1}$ (58 ± 9 $\%\dot{V}O_{2max}$) and 1.72 ± 0.18 $L \cdot min^{-1}$ (81 ± 5 $\%\dot{V}O_{2max}$) respectively.

The $\dot{V}O_2$, HR, R, and power output were increased as a function of exercise intensity ($p < 0.001$ for all variables) with no differences among testing protocols ($p > 0.05$ for all variables) (Figure 2A–D). The mean coefficient of variations for the FO measured within subjects between days was 19.9 ± 5.9%. As a function of relative exercise intensity, FO displayed the well-known inverted U-shaped curve (Figure 2E). The mean adjusted r^2 values for the fitting regression were respectively for the ramp incremental protocol 0.93 ($p < 0.001$) and for the steady state protocol 0.92 ($p < 0.001$). The FO was increased as a function of exercise intensity ($p < 0.001$), yet there was no significant main effect due to the exercise protocol ($p = 0.17$) nor a significant interaction between intensity and protocol ($p = 0.99$).

The maximum fat oxidation rate was not significantly different between the ramp (0.24 ± 0.09 g·min^{-1}) and the graded (0.20 ± 0.08 g·min^{-1}) incremental protocol ($p = 0.10$, d = 0.53) (Table 2). The regression analysis and CCC showed, respectively, moderate correlation (r = 0.68, $p < 0.01$, SEE = 0.05 g·min^{-1}) and concordance (CCC = 0.60). Bland–Altman analysis showed no significant bias between measures (0.05 g·min^{-1}, z-score < 1.96) but relatively low precision (0.07 g·min^{-1}) (Figure 3). The relative intensity at MFO was not different between the ramp incremental protocol (47.5 ± 4.2 %$\dot{V}O_{2max}$) and the steady-state protocol (47.8 ± 5.1 %$\dot{V}O_{2max}$) ($p = 0.91$, d = 0.04) (Table 1). The regression analysis and CCC showed, respectively, moderate correlation (r = 0.69, $p < 0.01$, SEE 3.15%) and concordance (CCC = 0.69). Furthermore, Bland–Altman analysis showed no significant bias between measures (0.26 %$\dot{V}O_{2max;}$ z-score < 1.96) but relatively low precision (4.7 %$\dot{V}O_{2max}$) (Figure 3). Values of $\dot{V}O_2$, HR, and power output at FAT_{max} and relative statistics analysis are shown in Table 2.

Table 2. Variables at FAT_{max} intensity.

	MFO (g·min^{-1})	**FAT_{max} (%$\dot{V}O_{2max}$)**	**$\dot{V}O_2$ (L·min^{-1})**	**Power Output (watt)**	**HR (bpm)**
Ramp	0.24 ± 0.09	47.5 ± 4.3%	1.00 ± 0.13	42 ± 14	109 ± 13
Graded	0.20 ± 0.08	47.8 ± 5.1%	1.03 ± 0.14	47 ± 14	105 ± 11
t-test	0.10	0.91	0.29	0.14	0.11
r^2	0.46	0.52	0.80	0.73	0.76
SEE	0.05	3.9	81	8.1	6.1
CCC	0.60	0.69	0.69	0.20	0.80
Bias ± SD	0.05 ± 0.07	0.3 ± 4.7	−0.04 ± 0.08	4.8 ± 8.5	2.2 ± 6.5
LLOA	−0.08	−7.8	−0.19	−11.8	−10.5
ULOA	0.19	10.6	0.12	21.4	14.9

Values are expressed as mean ± SD. Maximal fat oxidation (MFO), the intensity at MFO relative to $\dot{V}O_{2max}$ (FAT_{max}), oxygen consumption ($\dot{V}O_2$), power output, and heart rate (HR) at the intensity corresponding to MFO are reported for the ramp and graded incremental protocol. Results from the *t*-test, regression analysis (as a coefficient of determinations, the standard error of estimate (SEE)), the concordance correlation coefficient (CCC), and Bland–Altman analysis (Bias ± SD and lower and upper limits of agreements) are shown.

The variables detected during the two incremental tests performed in the subset of 10 participants are shown in Table 3. Test-retest reliability revealed no significant differences between the first and second ramp incremental tests ($p > 0.05$ for all comparisons). The regression analysis and CCC showed good correlation and mild to good concordance between measures (Table 3. Bland–Altman analysis showed no significant bias between measures; however, relatively high coefficients of variation for MFO and FAT_{max} only (MFO, cv = 17%, FAT_{max} cv = 2% while $\dot{V}O_{2max}$ cv = 0.13%).

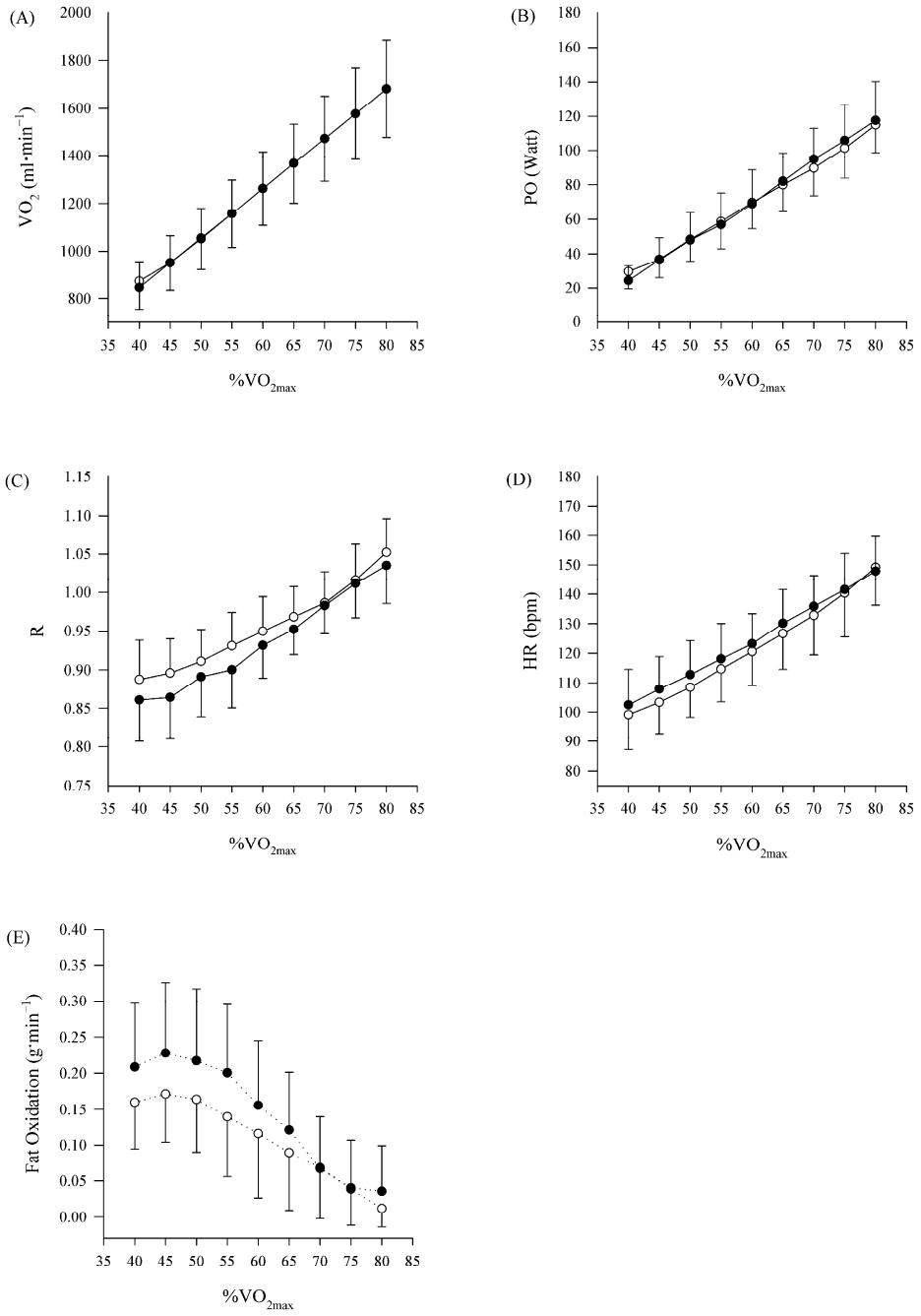

Figure 2. Data were collected during ramp (●) and graded (o) incremental protocol as a function of exercise intensity relative to maximal oxygen uptake (%$\dot{V}O_{2max}$): (**A**) Oxygen Uptake ($\dot{V}O_2$); (**B**) Power Output (PO); (**C**) Respiratory Exchange Ratio (R); (**D**) Heart Rate (HR); (**E**) Fat Oxidation. Values are expressed as mean ± SD.

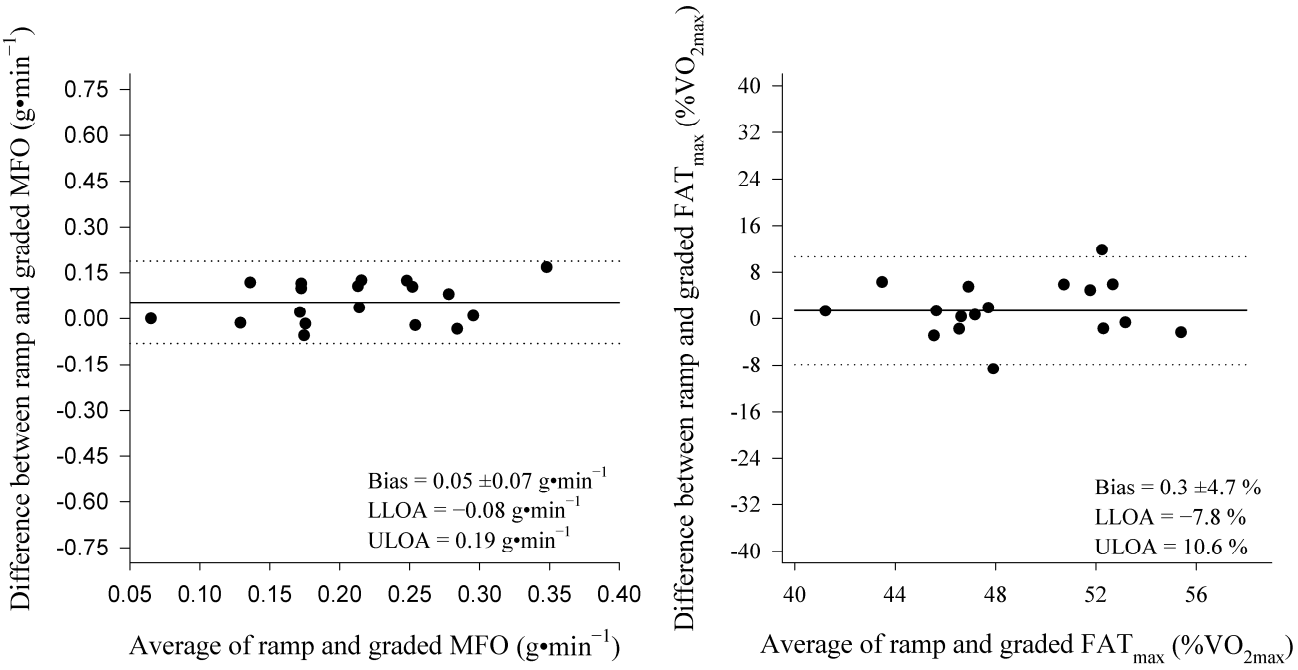

Figure 3. Bland–Altman analysis between the ramp and graded MFO (**left panel**) and FAT_{max} (**right panel**). Bias ± SD and lower and upper limits of agreement are shown.

Table 3. Variables for the first and second ramp incremental test in the subgroup.

	MFO	**FAT_{max}**	**$\dot{V}O_{2max}$**
	(g·min^{-1})	**(%$\dot{V}O_{2max}$)**	**(L·min^{-1})**
First Ramp	0.21 ± 0.09	46.9 ± 3.1%	2.10 ± 0.31
Second Ramp	0.25 ± 0.08	46.7 ± 4.3%	2.09 ± 0.33
t-test	0.33	0.87	0.96
r^2	0.53	0.77	0.82
SEE	0.04	2.1	48
CCC	0.65	0.83	0.95
Bias ± SD	0.04 ± 0.05	0.21 ± 7.8	0.21 ± 0.18
LLOA	−0.07	−4.1	−0.17
ULOA	0.11	6.9	0.12

Values are expressed as mean ± SD. Maximal fat oxidation (MFO), the intensity at MFO relative to maximal oxygen uptake (FAT_{max}), and maximal oxygen consumption ($\dot{V}O_{2max}$) are reported for the first and second ramp incremental protocols.

4. Discussion

This study tested the performance of a ramp compared to the traditional graded incremental test for determining the maximal fat oxidation rate (MFO) and the corresponding exercise intensity relative to $\dot{V}O_{2max}$ (FAT_{max}) in postmenopausal women.

The present study confirmed that that ramp incremental test allows the detection of both FAT_{max} and MFO. Indeed, MFO during ramp incremental tests were similar and occurred at comparable relative exercise intensity to the traditional, more time-consuming graded protocol. The present results suggest that a graded protocol may not be strictly needed to evaluate fat metabolism in postmenopausal women.

The individual and anthropometric characteristics (body mass index and %body fat) of the participants enrolled in the study were in line with what was expected from the existing literature for this population [36]. However, the average relative high value of $\dot{V}O_{2max}$ of 36.4 ± 5.3 mL·min^{-1}·kg^{-1} places them between the 75th and 80th percentile of the age-specific ACSM (American College of Sport Medicine) fitness distribution [37].

Four previous studies, with an overall sample size of 58 individuals, specifically investigated the indexes of fat oxidation in healthy, postmenopausal women [8,38–40]. The present study confirmed values of MFO and FAT_{max} similar to those reported in previous studies (respectively for the graded incremental protocol: 0.20 ± 0.08 g·min^{-1} and 47 ± 5% $\dot{V}O_{2max}$) in a comparable population. The growing research interest in women's metabolic profile and flexibility after menopause is driven by the increasing prevalence of metabolic disorders in this population [9,41]. Changes in female sex hormone concentrations after menopause have been associated with increased metabolic and cardiovascular risk factors as well as neurodegenerative diseases and osteoporosis [41–44]. These risks include insulin resistance, visceral adiposity, and dyslipidemia, which is associated with metabolic inflexibility and disorders such as type 2 diabetes [41,45,46]. MFO is a key marker of metabolic flexibility in individuals with metabolic disorders as it represents the body's capacity to utilize fat as a predominant fuel source during physical activity [15]. For these reasons, recent articles are investigating how exercise interventions can enhance fat oxidation to mitigate the adverse metabolic effects associated with menopause [42,47,48].

The present study provided the first data on the direct comparison between ramp incremental and graded protocol in postmenopausal women: virtually identical inverted U-shape profiles of fat oxidation as a function of exercise intensity relative to $\dot{V}O_{2max}$ (Figure 2E) were found, and MFO displayed similar values and occurred at comparable relative exercise intensity in both protocols.

The first validated protocol to detect MFO and FAT_{max} was developed by Achten et al. [15] based on a graded incremental test with a 3-min duration stage. Afterward, several protocols were proposed considering participants' sex, age, fitness level, or body weight [17]. Stage duration (e.g., from 1 to 10 min) and workload increment (e.g., from 10 to 50 watts) are the main modified characteristics [17]. A longer stage duration, by allowing the reaching of the steady state in $\dot{V}O_2$ and $\dot{V}CO_2$ even in sedentary individuals, allows higher accuracy at the expense of a considerably longer test duration. Likewise, applying relatively high workload increments can reduce the test duration with reduced accuracy [17]. Moreover, the following should be noted: (i) The set-up workload increments for the graded protocol; (ii) The estimation of FAT_{max} (i.e., $\%\dot{V}O_{2max}$) require a previous assessment of the $\dot{V}O_{2max}$ through an additional incremental test to exhaustion, which was performed on a separate day without being in a fasted state condition.

Conversely, a less time-consuming ramp testing allows the identification within a single visit of fat metabolism, metabolic flexibility, and the traditional cardiorespiratory fitness indexes (e.g., $\dot{V}O_{2max}$), making this protocol more useful for routine assessments [24]. In fact, according to the present results, ramp incremental protocol can combine accurate measurements of fat metabolism and other physiological variables such as $\dot{V}O_{2max}$, gas exchange ratio, respiratory compensation point, the O_2 gain, the $HR/\dot{V}O_2$ relationship, and the mean response time that are bases of an individualized exercise prescription.

The two previous studies in young individuals that compared measures of MFO and FAT_{max} from a ramp vs. graded, incremental protocol agreed that FAT_{max} could be accurately estimated with both protocols and in both cycling and running tests [23,24]. However, MFO, during running exercise, was reported to be overestimated by the ramp incremental protocol [23]. The differences in the physiological response to running vs. cycling and the fitness level between the two studies may be responsible for this discrepancy. In particular, recent studies showed that different types of exercise influence the MFO value mainly according to the muscle recruitment patterns (proportion of type II muscle fiber) and the muscle mass involved in various types of locomotion [24,49].

The present study indicated that both FAT_{max} and MFO can be identified through standard cyclo-ergometer ramp incremental testing in postmenopausal women. A small

(0.04 g·min^{-1}, +20%), non-significant overestimation of MFO has been found, as determined from the ramp incremental compared to the graded test protocol. This discrepancy in MFO can be explained by the difference in the speed of adjustment (i.e., the kinetics) of the $\dot{V}O_2$ and $\dot{V}CO_2$ upon metabolic transitions. Both signals typically display an exponential rise in the moderate and heavy exercise domain, reaching a steady state within 3–5 min [50]. However, due to the 20 times higher diffusion coefficient of CO_2 compared to O_2, $\dot{V}CO_2$ typically displays a faster kinetic. Therefore, the resulting R displays a transitory peak in the first 2–3 min of exercise, eventually adjusting to a lower steady-state value within 5 min. Moreover, the speed of adjustment of $\dot{V}O_2$ and $\dot{V}CO_2$ is affected by the fitness level of the subjects, with slower kinetics characterizing less fit individuals [32]. Since the estimate of fat oxidation through indirect calorimetry depends on the product of g·L^{-1} (derived from R) and L·min^{-1} (derived from $\dot{V}O_2$), the duration of the exercise stage will affect the estimate; theoretically, this would be more so in less fit subjects (characterized by a slower speed of adjustment of $\dot{V}O_2$). Therefore, based on the fact that less aerobically fit individuals have slower $\dot{V}O_2$ and $\dot{V}CO_2$ kinetics at exercise onset, we expected in postmenopausal women a larger discrepancy in the FO derived from ramp vs. grade incremental test (where R-value at 1 min into a given workload is lower than the steady state value at the 5th min). However, compared to Takagi's work on young, healthy males, our postmenopausal women displayed a smaller difference between MFO derived from the two protocols [23]. This unexpected finding may be attributed to a possible larger blood substrate availability in relation to the different pre-test nutritional states (i.e., fasting vs. standard meal in our study compared to Takagi's works). However, it could also be related to a lower range of potential variation for fat oxidation in postmenopausal women than in young individuals [51]. Indeed, healthy younger individuals can increase their resting fat oxidation by about 2.5–4 fold; our postmenopausal women have a reserve of less than 1.8 of the resting value.

Therefore, the results need to be confirmed in a more heterogeneous population regarding sex, age, and aerobic fitness level, and further investigations are needed to clarify the best approach to measure and analyze MFO and FAT_{max} during exercise.

Exercise intensity target is often prescribed using an external load such as watt, speed, or pace [27,52]. Interestingly, while Takagi S. et al. [23] did not mention the absolute external load at FAT_{max}, Michalik K. et al. [24] reported a significant difference between the two protocols (~18 Watts). To some extent, this discrepancy could be explained by the difference in the $\dot{V}O_2$/Power output relationship from a non-steady-state vs. steady-state incremental test [18,27]. In fact, during ramp protocol, due to the continuously changing metabolic demands, the measured $\dot{V}O_2$ (and the derived FO) lags the true metabolic needs (i.e., the actual steady-state $\dot{V}O_2$ and FO) for any given power output. The magnitude of the "gap" between the ramp and steady-state exercise depends on the individual $\dot{V}O_2$ kinetics and different methodological factors (e.g., ramp slope, pedal rate). Failing to account for this "gap" may interfere with the translation of a given metabolic intensity (e.g., FAT_{max}) into an external load equivalent [18,27]. In the present study, the correction strategy adopted to adjust the power output derived from the ramp test could explain the observed similar power output at FAT_{max} among protocols compared with Michalik's work.

The main limitation of the present study lies in the intrinsic variability of the FO measurement; while it is plausible that overnight fasting may increase test-retest reliability through increased homogeneity of substrate availability as well as facilitate the comparison with previous work, we opted instead for a standardized meal to resemble the ecological conditions of a standard cardiorespiratory testing session while also containing an important source of variations. Indeed, the main aim of this study was to evaluate the accuracy of the ramp vs. graded incremental approach in determining fat metabolism performed

in the same preanalytical and ecological conditions. Notably, the observed within-subject variability between subsequent tests on FO, $FAT_{max,}$ and MFO was similar to that described in the existing literature (10–25%) and compatible with our choice of using a standard meal rather than a "fasting night" [35,53].

5. Conclusions

In summary, in postmenopausal women, ramp testing offers a valid alternative to a graded protocol for identifying MFO and FAT_{max} and could facilitate the practical use of these parameters in research and clinical settings. This has important practical implications for a longitudinal and large-scale evaluation of fat metabolism in postmenopausal women, which also offer a means for individualized exercise prescription.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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