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EXERCISE PHYSIOLOGY

Extreme duration exercise affects old and younger men differently

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Abstract

Aim & Methods: Extreme endurance exercise provides a valuable research model for understanding the adaptive metabolic response of older and younger individuals to intense physical activity. Here, we compare a wide range of metabolic and physiologic parameters in two cohorts of seven trained men, age 30 ± 5 years or age 65 ± 6 years, before and after the participants travelled ≈3000 km by bicycle over 15 days.

Results: Over the 15-day exercise intervention, participants lost 2–3 kg fat mass with no significant change in body weight. $\dot{V}O_2$max did not change in younger cyclists, but decreased ($p = 0.06$) in the older cohort. The resting plasma FFA concentration decreased markedly in both groups, and plasma glucose increased

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in the younger group. In the older cohort, plasma LDL-cholesterol and plasma triglyceride decreased. In skeletal muscle, fat transporters CD36 and FABPm remained unchanged. The glucose handling proteins GLUT4 and SNAP23 increased in both groups. Mitochondrial ROS production decreased in both groups, and ADP sensitivity increased in skeletal muscle in the older but not in the younger cohort.

Conclusion: In summary, these data suggest that older but not younger individuals experience a negative adaptive response affecting cardiovascular function in response to extreme endurance exercise, while a positive response to the same exercise intervention is observed in peripheral tissues in younger and older men. The results also suggest that the adaptive thresholds differ in younger and old men, and this difference primarily affects central cardiovascular functions in older men after extreme endurance exercise.

KEYWORDS

aerobic fitness, aging, cycling, endurance exercise, energy metabolism, fat oxidation, muscle biopsy

1 | INTRODUCTION

Acute and chronic exercise has pleiotropic beneficial effects on human metabolism and physiology. The beneficial effects of exercise include favorable changes in body composition, improved cardiorespiratory fitness, and improved metabolic health.[1] Furthermore, the muscle mitochondrial network volume, density, and function is improved and there is an improved overall inter-organ function and resilience.[1] Fully consistent with these observations, nearly all epidemiological studies demonstrate an inverse correlation between daily physical activity and all-cause mortality, metabolic syndrome, and type 2 diabetes mellitus.[2–8] However, although most physical activity promotes health in most individuals, there may be an upper limit to the health benefits of extreme physical activity.[9]

According to classical training physiology, the principle of supercompensation can be exploited to achieve maximal adaptation to exercise training. However, this approach requires knowledge of the volume, timing, type and intensity of the exercise to evaluate total load and the subsequent adaptation (ie to maintain adaptive homeostasis).[10] In general, the adaptive response range decreases with increasing age,[11] as older individuals experience decreased cardiorespiratory fitness (CRF) driven by decreased maximal heart rate, decreased fat free mass, and increased fat mass.[12–14] The physiological response to extreme exercise is also affected by environmental conditions, as demonstrated by studies conducted in connection with expeditions to Greenland and to Antarctica.[15–18] These studies report that excessive exercise at high altitude with exposure to cold temperature can be detrimental to performance and health. As these conditions influence the physiological response, these studies were not optimal for studying the physiological response to extreme exercise per se.

In an earlier study, we investigated the metabolic and physiologic impact of 14 days of consecutive cycling over a total distance of approximately 2700 km in a cohort of six older men (61 years). The study participants experienced a decrease in cardiorespiratory fitness, a decrease in maximal fat oxidation rate (MFO) and a decrease in insulin sensitivity (HOMA-IR) after the exercise intervention, suggesting that the intervention negatively impacted these markers of physiological and metabolic health.[19,20] We speculated that these apparently detrimental adaptations to extreme endurance exercise could have been age-specific and some support for this contention is provided by Easthope and colleagues who demonstrated somewhat larger muscle damage in 46 ± 6 years versus 31 ± 7 years old master and younger athletes, respectively after a 55 km trail running competition.[21] To test this possibility, we conducted the present study, which examines the physiologic and metabolic impact of repeated prolonged moderate intensity exercise (7–10 h/day for 15 consecutive days at ≈63% HR_{max}) in two cohorts of seven men, age 30 ± 5 years or age 65 ± 6 years. As such, the current study provides the opportunity to: (1) evaluate metabolic adaptation to

extreme endurance exercise using comprehensive testing before and after an exercise intervention; and (2) to determine whether age influences the capacity for adaptive response to extreme endurance exercise.

2 | RESULTS AND DISCUSSION

In this unique experimental study on the effects of extreme endurance exercise on two age-stratified cohorts of men, we show that younger and older study participants display similar adaptive responses in skeletal muscle, including increased skeletal muscle mass with stable mitochondrial respiratory capacity as well as similar metabolic peripheral responses, including improved glucose homeostasis and decreased fat oxidation. In contrast, participants in the older study cohort experienced decreases in cardiorespiratory fitness, maximal heart rate and strength, and increases in plasma GDF-15, while younger study participants did not show similar trends. These key findings, described below in greater detail, provide evidence that extreme endurance exercise differentially impacts peripheral and central organs, and that older and younger men experienced distinct adaptive thresholds to the extreme endurance exercise intervention in the context of the present study. The results suggest that age-related effects may exert its primary physiologic/metabolic effect through changes in central cardiovascular capacity and thresholds.

2.1 | Intervention and energy balance

For all study participants, resting heart rate was measured every morning for 2 weeks prior to the intervention and was initially similar in the two study cohorts (avg. young: 53 ± 7; old: 54 ± 5 beats/min). During the intervention, resting heart rate increased in the older cohort (during: avg. young: 51 ± 8; old: 60 ± 7 beats/min; post hoc: old before vs. during, $p = 0.02$), but did not change significantly in the younger cohort (young before vs. during, $p = 0.54$). Although in absolute terms, average heart rate (beats/min) was lower in older than in younger participants during the exercise intervention (Figure 1A), relative to the maximal heart rate, there was no difference between the two cohorts (main effect: age $p = 0.39$, Figure 1B). As the total volume (km) and the average relative intensity of the intervention was similar for the two cohorts, the total strain (volume × intensity) of the intervention was the same for both groups.

The body mass of the study participants was stable throughout the intervention, with a slight tendency toward a decrease in body mass, most pronounced in the younger cohort (Figure 1C, main effect: intervention $p = 0.06$). Before the intervention, fat free mass was higher ($p = 0.04$) and percent body fat was lower ($p = 0.03$) in the younger cohort than in the older cohort (Table 1).

It is well-established that fat free mass decreases and fat mass increases with increasing age[1,2]; however, a study of master athletes found that chronic exercise preserves fat free mass and that fat free mass loss commonly observed with age may reflect disuse rather than aging per se.[22] Despite smaller stature of the older men (Table 1), the present study demonstrates a similar degree of plasticity of lean and adipose tissues over the course of the exercise intervention in older and younger men. Notably, fat mass in younger and older cyclists decreased (−2.02 ± 2.17 and −3.04 ± 3.19 kg, respectively, Figure 1D) and fat free mass increased (+0.82 ± 1.63 and +2.96 ± 3.97 kg, respectively, Figure 1D). This age-independent increase in fat free mass was fuelled by a large energy intake of ~27 and 25 MJ/day in younger and older cyclists, respectively (Figure 1E), which is two-fold higher than total energy expenditure during the week prior to the start of the intervention (Figure 1G). Previous studies reported a comparably high daily energy intake by younger cyclists during extreme endurance events, such as the Tour de France.[23] During the intervention, the macronutrient composition of the energy intake changed such that carbohydrate energy% increased ($p < 0.0001$) and fat energy% decreased ($p < 0.0001$) in both cohorts, while protein energy% increased in younger cyclists from stage 2 + 3 to stage 5 + 6, and the opposite was seen in the older cyclists, such that protein energy% was higher in younger vs. old (interaction, $p < 0.01$) (Figure 1F). Details of the energy intake are given in Table S2.

Despite large daily energy intake, both younger and older cyclists experienced a negative energy balance over the course of the intervention, as reported previously for older elite athletes performing extreme exercise[24]; yet here, the negative energy balance was more pronounced in younger than in older study participants (younger: −6.4, old: −1.7 MJ/day). We speculate that there is an upper limit for daily energy intake around 25 MJ,[25–27] which is a barrier to maintaining energy homeostasis during extreme endurance activity. This appears to be relevant and critical for younger and older men alike.

While it seems paradoxical that fat free mass should increase despite a negative energy balance over the course of the exercise intervention, this observation should be considered in the context of a concomitant decrease in adipose tissue mass,[28–30] taking note of the distinct energy densities of lean and adipose tissue. However, we cannot exclude that the dietary food intake was underestimated and/or that an increase in total blood volume or water retention in some extracelluar compartments may explain this increase in fat free mass.

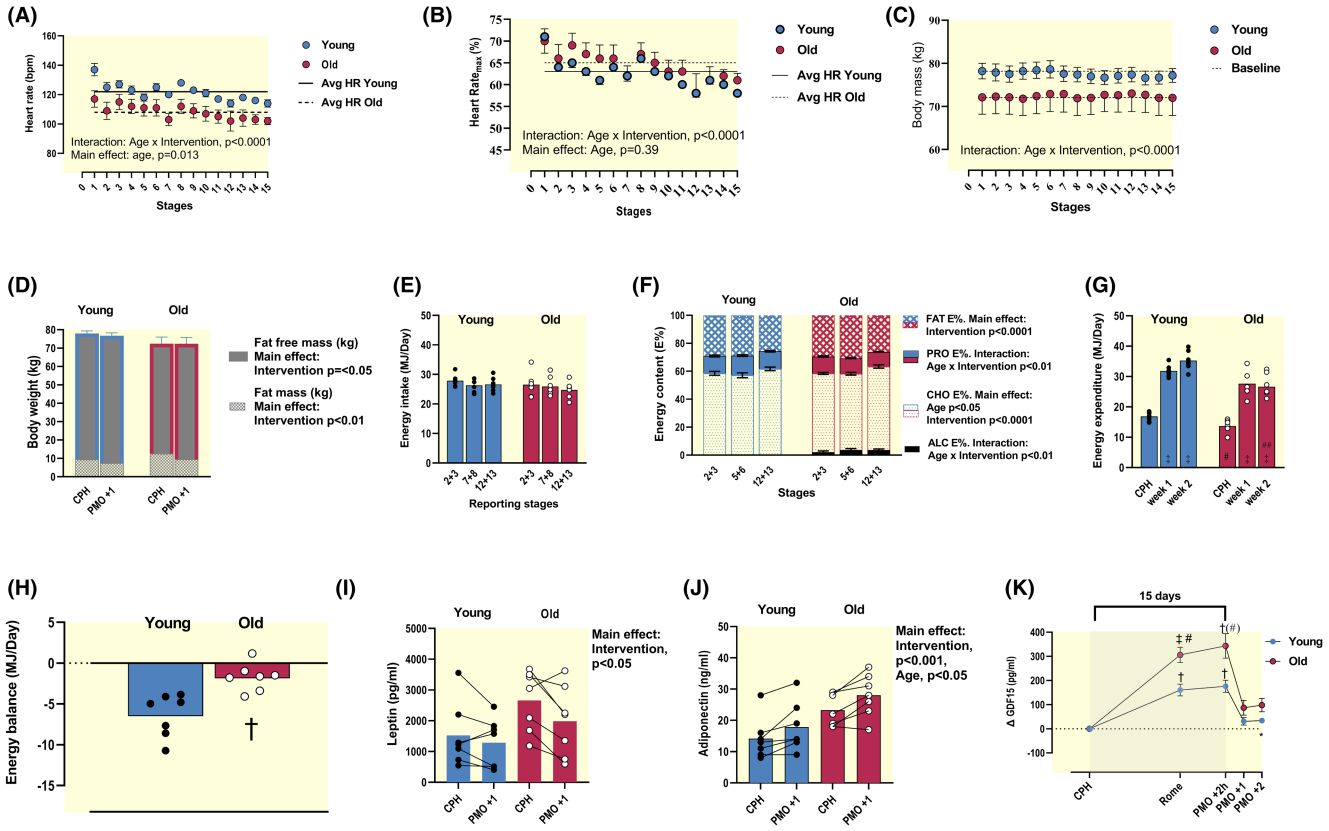

FIGURE 1 Body composition and energy intake and expenditure. (A) Absolute heart rate at each stage during the intervention and average intensity during the entire intervention (unpaired *t*, $p < 0.0001$). (B) Heart rate at each stage during the intervention relative to HR_{max} and average intensity during the entire intervention (unpaired *t*, $p = 0.13$). (C) Body mass each morning compared with baseline (dashed lines). (D) Body composition at baseline (CPH) and 1 day after the intervention (PMO + 1). (E) Average energy intake (MJ/day) from two reporting days (Bars) and individual data. (F) Macronutrient composition (E %) of the diet (also Table S2). (G). Average energy expenditure (Bars) 7 days prior to the intervention (CPH) and during the first 7 days (week 1) and last 7 days (week 2) of the intervention with individual data points (circles). (H) Energy balance (MJ/day) during the intervention (EE-EI) (unpaired *t*, $p < 0.01$). (I). Average and individual plasma leptin concentrations (pg/ml) at baseline (CPH) and 1 day after the intervention (PMO + 1) (Intervention, $p < 0.05$). (J) Average and individual plasma adiponectin concentrations (ng/ml) at baseline (CPH) and 1 day after the intervention (PMO + 1. (K) Change in circulating GDF15 from baseline (CPH) over ROME (stage 10) and at 2 h (PMO + 2 h), 1 (PMO + 1) and 2 (PMO + 2) days after the intervention. Post hoc multiple comparison analysis (Sidaks'): *different from CPH within group, $p < 0.05$, †$p < 0.01$, ‡$p < 0.001$. $^{\#}$Difference between groups within intervention point, $p < 0.05$, $^{\#\#}p < 0.01$

The plasma leptin concentration trended toward being lower in the younger than in the older cohort (main effects: age, $p = 0.10$; intervention, $p = 0.02$), and it decreased with the intervention (Figure 1I). Plasma adiponectin concentration was lower in the younger than in the older cohort (main effects: age, $p = 0.02$; intervention, $p < 0.001$) and it increased ($p < 0.001$) with the intervention in both cohorts (Figure 1J). Interestingly, in an earlier study, we found plasma leptin concentration to be unchanged in older men after 14 days of sustained exercise and a 2 kg loss of adipose tissue mass. Yet, an expeditionary study reported a decrease in plasma leptin and increase in adiponectin in line with the present findings.[31]

Recent studies have stimulated great interest in GDF15 (growth differentiation factor 15) for its pleiotropic metabolic and potential anti-obesity and appetite-regulating effects.[32] Circulating GDF15 appears to be influenced by exercise-induced metabolic stress more than by nutrition-induced metabolic stress associated with fasting or overeating.[33] In the present study, plasma GDF15 was lower ($p = 0.017$) in the younger cohort than in the older cohort before the intervention (271 ± 81 and 532 ± 156 pg/ml, respectively). It increased during the intervention and remained significantly elevated in the older cohort than the younger cohort after the intervention (Figure 1K). These results suggest that circulating GDF15 increases during extreme sustained exercise, with a greater proportional increase in older men. Furthermore, GDF15 remained elevated in the older cohort after 2 days of recovery (Figure 1K). Recently, GDF15

TABLE 1 Baseline (CPH) anthropometric characteristics

Variable	Young ($n = 7$)	Old ($n = 7$)	Main effect: Age (p-value)
Age (years)	30 ± 5.0	65 ± 6	–
Height (cm)	182 ± 5	175 ± 9	–
Body mass (kg)	78.0 ± 4.9	72.4 ± 10.4	0.28
BMI (kg/m^2)	23.6 ± 0.8	23.4 ± 1.2	0.94
Fat Free Mass (FFM) (kg)	68.5 ± 3.7	59.9 ± 9.4	0.04
FFM (% of body mass)	88.0 ± 3.8	82.6 ± 4.1	0.03
Fat mass (kg)	9.4 ± 3.3	12.6 ± 3.2	0.11
Body fat (%)	12.0 ± 3.8	17.4 ± 4.1*	0.03
VO_2max (ml/min)[a]	4767 ± 336	3467 ± 565†	<0.001
VO_2max (ml/min/kg)[a]	61.5 ± 2.2	46.8 ± 4.1†	<0.001

Note: Participant anthropometric characteristics. FFM was calculated by the doubly labeled water technique, and VO_2max from indirect calorimetry. Data are presented as mean ± SD.

Abbreviations: BMI, body mass index, FFM, fat free mass, VO_2max, maximal oxygen uptake.

[a]$n = 6$ in the old group, $n = 7$ in the young group.

*$p < 0.05$

†$p < 0.001$.

has also been proposed as a marker of overtraining, indicated by the bivariate correlation between circulating GDF15 and a decrease in maximal heart rate after a 3-week exercise intervention.[34] In the present study, we made a similar observation, as the linear regression between change in circulating GDF15 (CPH – PMO + 2) and change in maximal heart rate (CPH – PMO + 2) was robust ($r^2 = 0.64$, $p = 0.001$, data not shown). Together, these data show that despite an energy deficit (avg. 4 MJ/day) facilitated by a large energy expenditure, older and younger men alike were capable of increasing fat free mass and supporting energy demands during extreme endurance exercise through utilization of adipose tissue fatty acids. The changes in body composition reflect concomitant changes in the concentrations of circulating leptin, adiponectin, and the energy regulatory agent GDF15.

2.2 | Cardiorespiratory fitness and strength

For the $\dot{V}O_2$max, there was a trend toward a decrease in absolute terms ($p = 0.09$) in the older cohort, but it did not ($p = 0.21$) decrease in the younger cohort (from 3467 ± 565 to 3245 ± 822 and from 4767 ± 336 to 4898 ± 362 ml/min, respectively). However, expressed relative to fat free mass (ml/min/kg FFM), a decrease was observed in the older cohort (post hoc: $p = 0.02$), but no change was observed in the younger cohort (post hoc: $p = 0.86$) (Figure 2A). There was an overall interaction ($p = 0.02$) between groups and a response to the intervention. For individual participants, the response in $\dot{V}O_2$max was variable, with numerical increases in 6 of the 7 younger participants and a numerical decline in 4 of the 6 older participants (Figure 2A). We previously demonstrated that $\dot{V}O_2$max decreased in older men after 14 days of consecutive endurance exercise.[20]

Blood hemoglobin decreased with the intervention in the younger and the older cohorts group (main effect: intervention $p < 0.0001$) (Figure 2B). Furthermore, hemoglobin concentration remained depressed 2 days after the end of the intervention in the older cohort (CPH vs. PMO + 2, post hoc: $p = 0.001$), while hemoglobin concentrations in the younger cohort returned to baseline levels at the same point in time (Figure 2B). The changes (CPH →PMO + 2) in $\dot{V}O_2$max and hemoglobin concentrations were significantly correlated ($r^2 = 0.59$; $p = 0.002$) (Figure 2C) and we conclude that the decrease in $\dot{V}O_2$max in the older cohort may have been driven in part by lower hemoglobin. The underlying mechanism for this response is not yet known, but it could reflect an increase in erythrocyte destruction rate, a decrease in the rate of red blood cell production or a lower concentration of circulating erythropoietin. However, plasma sodium (data not shown) did not differ at PMO + 2 from baseline or between the two cohorts, implying that over hydration or water retention did not play a role.

Maximal heart rate did not change in the younger cohort (CPH: 195 ± 11 vs PMO + 2:196 ± 12 beats/min), but it decreased markedly after the intervention in the older cohort (CPH: 168 ± 12 vs PMO + 2:156 ± 18 beats/min $p < 0.0001$) (Figure 2D). Thus, an attenuated ability to achieve HR_{max} in older participants after excessive sustained exercise, as observed previously,[35] and in the current study (Figure 2D), may contribute to the observed lower post-intervention $\dot{V}O_2$max in older participants. Resting HR was also elevated in the older cohort after the intervention, such that several

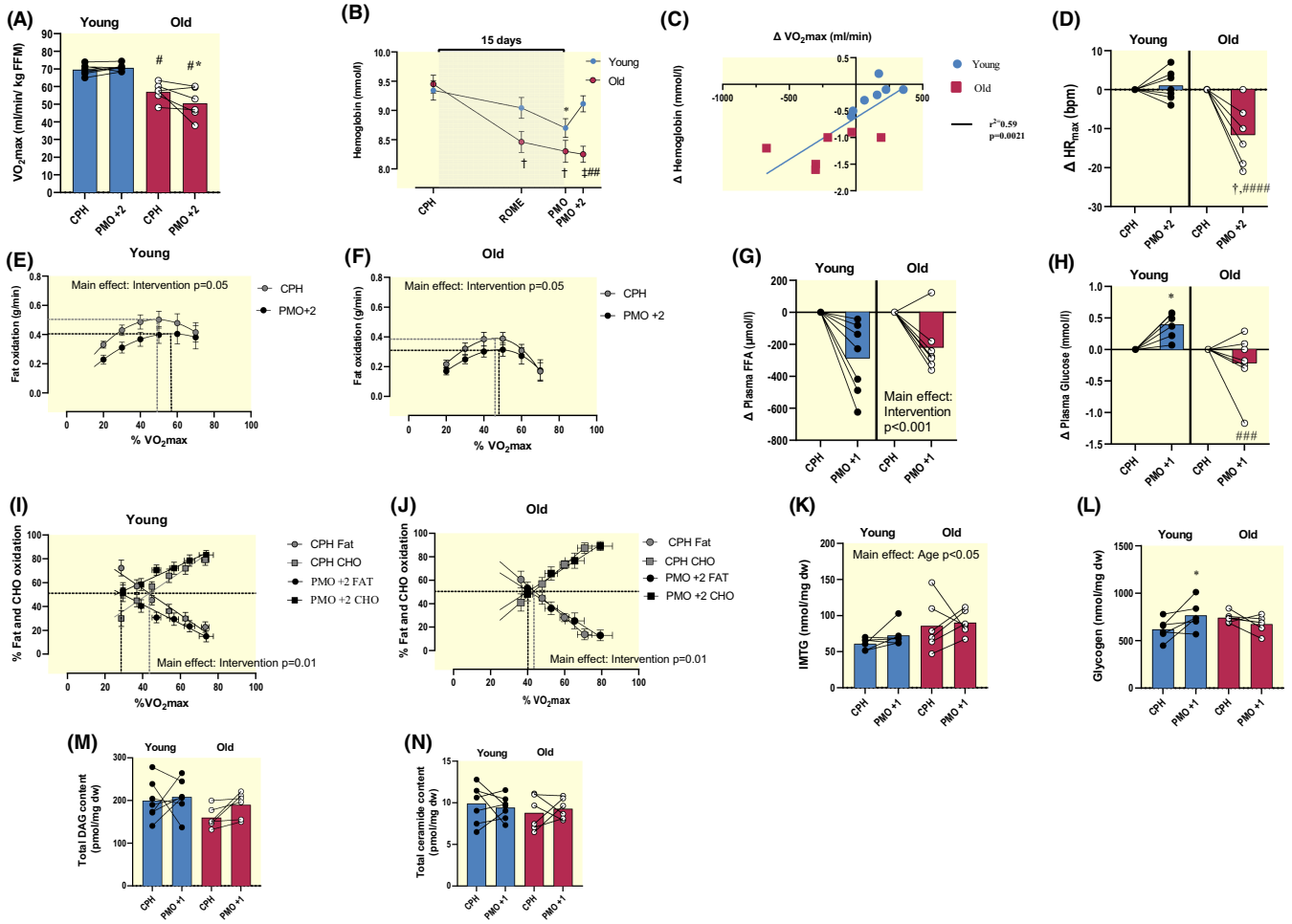

FIGURE 2 Cardiorespiratory fitness and substrate oxidation during exercise (A) VO_2max relative to fat free mass in young (blue bars indicate average and individual data points in black and circles) and old ($n = 6$, red bars indicate average and individual data points in white circles). (B) Blood hemoglobin concentrations at baseline (CPH), in ROME (Stage 10), 2 h after the intervention (PMO + 2 h), 1 day (PMO + 1) and 2 days (PMO + 2) after the intervention. (C) Linear regression analysis between the change in VO_2max and change in circulating hemoglobin from baseline (CPH) to 2 days after the intervention (PMO + 2) (young subjects are blue circles and old subjects are red squared, $n = 6$). (D) Change in HRmax during the VO_2max test from CPH to PMO + 2 (Old, $n = 6$). (E) Fat oxidation rate (g/min) at 20%, 30%, 40%, 50%, 60%, and 70% of VO_2max in the young group in CPH and at PMO + 2. Dashed vertical lines represent FatMax and horizontal lines represent MFO. (F) Fat oxidation rate (g/min) at 20%, 30%, 40%, 50%, 60%, and 70% of VO_2max in the old group ($n = 6$) in CPH and at PMO + 2. Dashed vertical lines represent FATmax and horizontal lines represent MFO. (G) Change in fasting plasma FFA concentrations from CPH to PMO + 1 in young and old. (H) Change in fasting plasma glucose concentrations from CPH to PMO + 1 in young and old. (I) Relative contribution of fat (circles) and CHO (squares) in CPH (gray) and at PMO + 2 (black) at the relative intensity (% VO_2max) during the graded exercise test in the young group. Horizontal dashed line indicates 50% contribution from each substrate, and vertical dashed lines represent the cross-over points. (J) Relative contribution of fat (circles) and CHO (squares) in CPH (gray) and at PMO + 2 (black) at the relative intensity (% VO_2max) during the graded exercise test in the old group ($n = 6$). Horizontal dashed line indicates 50% contribution from fat and CHO, and vertical dashed lines represent the cross-over points. (K) IMTG (mmol/mg dw) content in m. vastus lateralis in CPH and at PMO + 1 in the young and old group. (L) Glycogen (mmol/mg dw) content in m. vastus lateralis in CPH and at PMO + 1 in the young and old group. (M) Total DAG content (pmol/mg dw) in m. vastus lateralis in CPH and at PMO + 1 in the young and old group (Specified DAG analysis data in Table S5). (N) Total ceramide content (pmol/mg dw) in m. vastus lateralis in CPH and at PMO + 1 in the young and old group (Specified ceramide analysis data in Table S4). Post hoc multiple comparison analysis (Sidaks'): *different from CPH within group, $p < 0.05$, $^{\dagger}p < 0.01$, $^{\ddagger}p < 0.001$. $^{\#}$Difference between groups within intervention point, $p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$

of these factors in combination may have reduced the maximal cardiac output in the older cohort.

Interestingly, handgrip strength, which is independent of cardiac output, was similar at baseline for the younger and older cohorts (48.0 ± 1.8 and 43.8 ± 3.3 kg, respectively), and it decreased (post hoc: $p < 0.01$) in the older (36.9 ± 3.1 kg) but not in the younger cohort (50.8 ± 2.3 kg) after the intervention. This may indicate

central neural fatigue among older but not younger study participants.[36] However, we cannot exclude that there are other age-related factors that could have influenced this, such as the longer cycling time and/or differences in position on the cycle in the older group.

These findings clearly show maladaptation in central cardiovascular and regulatory functions in the older but not in the younger cohort group. This could reflect the fact that blood hemoglobin returned to baseline levels more quickly in younger than in older participants, as well as the greater ability of younger participants to achieve HR_{max} after sustained endurance exercise.

2.3 | Substrate oxidation

Maximal fat oxidation rate (MFO) decreased with the intervention in the younger and older cohorts (from 0.53 ± 0.14 to 0.43 ± 0.15 and from 0.41 ± 0.11 to 0.35 ± 0.10 g/min, respectively; main effect: intervention, $p = 0.05$) (Figure 2E,F). MFO also decreased with the intervention when expressed relative to FFM (main effect: Intervention $p = 0.04$, Data not shown). The percentage of $\dot{V}O_2$max corresponding to MFO (FATmax) did not change with the intervention (main effect: Intervention $p = 0.16$) for younger (48 ± 6 to 51 ± 7% $\dot{V}O_2$max) or older cohorts (44 ± 5 to 46 ± 8% $\dot{V}O_2$max) (Figure 2E,F). The cross-over point (the $\dot{V}O_2$max where 50 percent of substrate is carbohydrate) was lower after the intervention (main effect: intervention $p = 0.01$) (Figure 2I,J).

The decrease in MFO in both groups should be considered in light of the marked decrease (main effect, $p < 0.001$) in resting plasma FFA concentration in both groups and increase in plasma glucose ($p = 0.02$) in the younger but not in the older cohort (Figure 2G,H).

Interestingly, according to an extensive review, overtraining syndrome is characterized by a decrease in respiratory exchange ratio (ie increased fat oxidation).[35] This is in marked contrast with the results of the present study. In particular, fat oxidation during exercise reported as MFO (g/min, Figure 2E,F) or as the cross-over point (% of VO_2max, Figure 2I,J) decreased after the intervention in young and old study participants. It is not clear whether this reflects improved glucose-oxidative capacity, attenuated fatty acid oxidative-capacity or both.

We previously observed a decrease in MFO in older men after an extreme exercise intervention.[20] Yet, our present finding shows that this response is not age-specific. While a similar metabolic adaptation has been described previously in the expeditionary literature, those studies were conducted under hypobaric and hypoxic environmental conditions, making it difficult to compare the two sets of observations.[15,16] Here, the decrease in fat oxidation capacity and shift toward a more carbohydrate-based oxidation profile was supported by a major decrease in plasma FFA, independent of participant age, which was also observed in our previous study (Figure 2G).[20]

During exercise catecholamines stimulate lipolysis and increases plasma FFA availability, but the effect of repeated very prolonged exercise on the lipolytic response has not been investigated. We hypothesize that a marked decrease in circulating FFA may reflect chronic increase in circulating catecholamines during the intervention. This could desensitize the beta-adrenergic lipolytic response, which may be present 2 days after the end of the intervention in both groups. Consistent with this being a lipolytic maladaptation, fatty acid transport capacity (FAT/CD36, FATPm, Figure 3B) HAD-activity and mitochondrial respiration (Figure 5A,B) remained unchanged. IMTG content was higher after the intervention (Figure 2K). Consistent with the unchanged fat transport and oxidation capacity also the lipid droplet membrane-associated proteins (PLN2, PLN3 and PLN5, Figure 3C) were unchanged, yet the higher IMTG content is supported by a trend toward higher DGAT content (Figure 3C). Higher glucose oxidation (Figure 2E,F,I,J) is consistent with a ~60% increase in GLUT4 content in both cohorts (Figure 3A) and a numerical increase in HKII content (the latter lacking statistical significance due to large variation).

The muscle glycogen content increased ($p = 0.04$) in the younger cohort and remained unchanged in the older cohort (Figure 2L). Furthermore, IMTG was higher in the older than in the younger cohort ($p < 0.05$; Figure 2K). For the total DAG content there was a trend toward being higher (main effect: age $p = 0.07$) in the older than in the younger cohort, with no effect of the intervention on either cohort (Figure 2M). The DAG subspecies were not significantly influenced by age or the exercise intervention (Table S5). The total ceramide and ceramide subspecies content of muscle was similar in both cohorts before and after the intervention (Table S4). Similarly, the sphinganine, sphingosine, and sphingosine-1-phosphate content of muscle was not influenced by age or the intervention (Table S4).

Glycogen content and fasting plasma glucose increased from baseline in the younger group, but remained unchanged in the older group (Figure 2H,L). It is well-known that the macronutrient content of the diet influences substrate oxidation during exercise.[37–39] In the present study, the absolute (g/day) content of dietary fat and protein decreased during the intervention, while dietary carbohydrate remained at a high constant amount, namely, an average of 929 ± 31 and 848 ± 46 g/day for younger and older participants, respectively (Table S2), corresponding to ~12 g/kg BW/day (Table S2). It is very likely that this massive intake of dietary carbohydrate had a significant

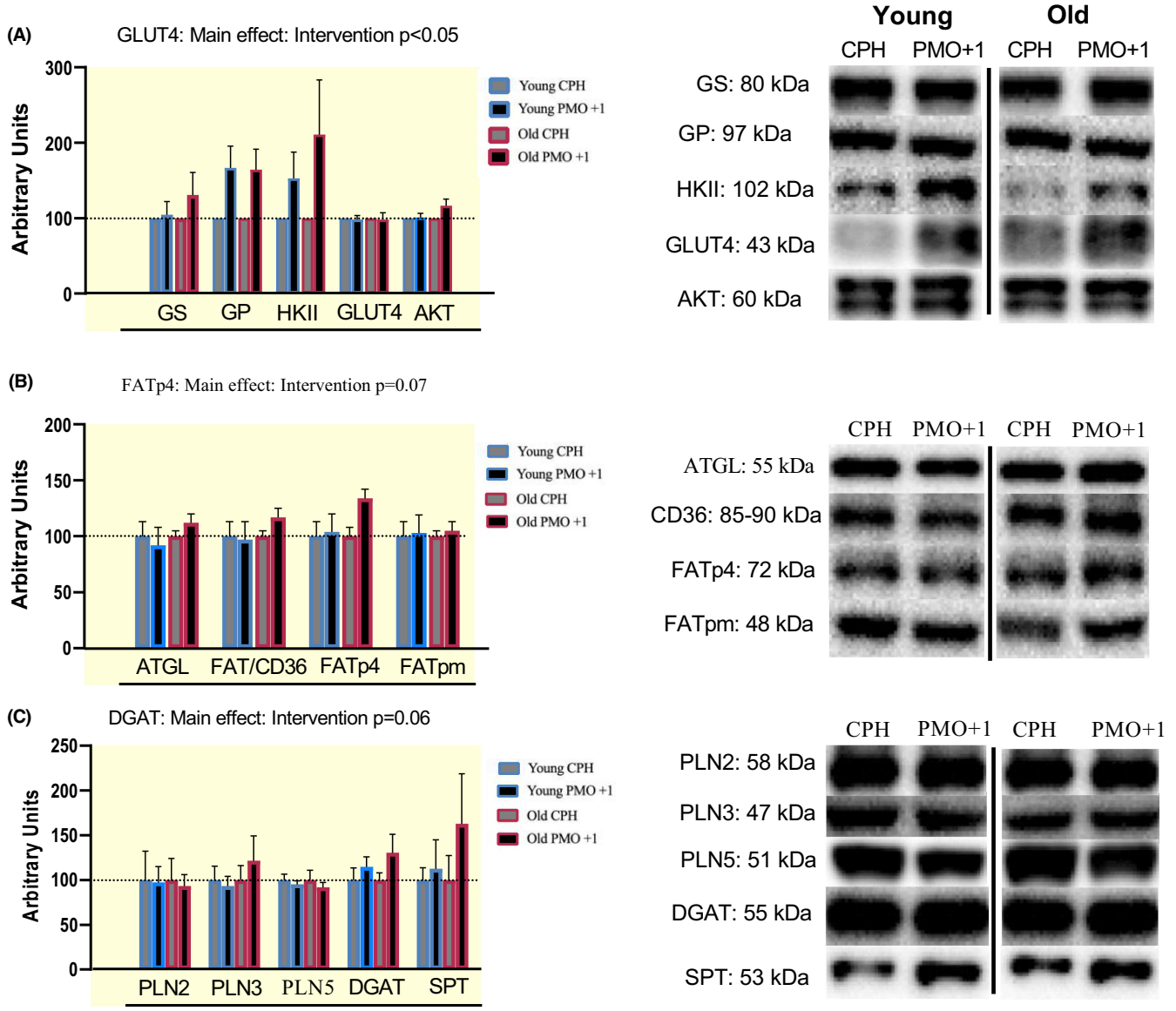

FIGURE 3 Western-blot analyzed proteins indexed to 100 and with representative blots. (A) Glucose-associated proteins (arbitrary unites/indexed) in m. vastus lateralis of the young blue (in CPH = gray fill in bar, PMO + 1 = black fill in bar) and the old red (in CPH = grey fill in bar, PMO + 1 = black fill in bar) group, GS, glucose synthase, GP, glucose phosphatase, HKII, hexokinase II, GLUT4, glucose transporter type 4, AKT (PKB), protein kinase B. (B) Fat-associated proteins (arbitrary unites/indexed) in m. vastus lateralis of the young blue (in CPH = gray fill in bar, PMO + 1 = black fill in bar) and the old red (in CPH = gray fill in bar, PMO + 1 = black fill in bar) group. ATGL, adipose triglyceride lipase, FAT/CD36, fatty acid translocase, FATp4, long-chain fatty acid transport protein 4. FATpm, plasma membrane fatty acid binding protein. (C) Intramuscular fat-associated proteins (arbitrary unites/indexed) in m. vastus lateralis of the young blue (in CPH = gray fill in bar, PMO + 1 = black fill in bar) and the old red (in CPH = gray fill in bar, PMO + 1 = black fill in bar) group. PLN2, perilipin 2, PLN3, perilipin 3, PLN 5, perilipin 5, DGAT, diglyceride acyltransferase, SPT, serine palmitoyltransferase

impact on substrate oxidation in study participants during the intervention.

Nevertheless, an increase in glucose oxidation after endurance exercise was not expected, according to the classical notion that exercise training leads to a relatively higher oxidation of fats during exercise.[40,41] On the other hand, very few studies have examined the impact of an extreme exercise intervention on substrate oxidation during exercise, and to our knowledge, the effect of the age of participants on this endpoint has not been described in published literature to date. Thus, the present study provides the first evidence of an age-independent extreme exercise-induced decrease in fat oxidation and increase in glucose oxidation. However, it is important to acknowledge that the massive carbohydrate intake and the negative energy balance that accompany the extreme exercise also contribute to shift the substrate oxidation.

Proteins involved in muscle glucose metabolism, including GS, GP, HKII, GLUT4 and AKT, were expressed at similar levels in both study groups at baseline (Figure 3A). Expression of GLUT4 increased significantly in both the younger and the older cohort (+67 ± 29% and +64 ± 27%, respectively; main effect: intervention $p = 0.02$) (Figure 3A). HAD activity was similar in both study groups and did not change with the intervention (young: from 147 ± 18 to 147 ± 24 μmol/g/min; old: from 121 ± 19 to 118 ± 20 μmol/g/min, CPH and PMO + 1, respectively).

Similarly, proteins involved in muscle lipid metabolism, including ATGL, FATp4, FABPpm, PLN2, PLN3, PLN5, DGAT, SPT (Figure 3B,C) were expressed at similar levels in both study groups before and after the intervention, with the exception that DGAT and FATP4 increased during the intervention (main effect, $p < 0.07$) (Figure 3C).

2.4 | Glucose homeostasis and health parameters

Glucose tolerance did not change with the intervention, and AUC for glucose was similar for both cohorts (main effect: age $p = 0.15$; Figure 4A,B,E,F). However, insulin concentration during the OGTT was significantly lower after the intervention in both study cohorts (main effect: intervention: $p < 0.05$; Figure 4C,D,G,H). The C-peptide response to the OGTT resembled the insulin response, albeit with no significant change ($p = 0.08$; Figure 4I). The glucagon response was higher after the intervention ($p = 0.09$; Figure 4J). The incretin response GLP-1 (AUC) and GIP (AUC) were unchanged and decreased, respectively, in response to the OGTT (GIP main effect, intervention $p < 0.05$) (Figure 4K,L).

The fact that plasma glucagon increased during the intervention is somewhat surprising. According to the intra-islet insulin hypothesis,[42] release of glucagon is inhibited by glucose-stimulated release of insulin, which is consistent with the higher plasma glucagon in type 2 diabetics,[43] likely due to a reduced incretin effect.[44] Here, we also found that GIP was lower during the OGTT after the intervention, which is in line with previous findings.[45] A decrease in GIP would be expected to suppress secretion of insulin, as seen here, and it is possible that higher glucagon is secondary to lower insulin in the context of the present study. However, it is possible that the increased glucagon may be driven by an increased plasma amino acid concentration consistent with the rather high protein intake and thus acting to regulate hepatic amino acid metabolism and ureagenesis.[46]

In both young and old, total cholesterol, LDL-C and TG concentrations in the blood were in the lower part of the normal range before the intervention (Figure 4M). Even so, total cholesterol (post hoc $p < 0.001$), LDL (post hoc $p < 0.001$) and TG (post hoc $p = 0.01$) decreased significantly in the older cohort in response to the intervention, while HDL (post hoc $p < 0.001$) increased in the younger cohort (Figure 4M).

The plasma hsCRP was lower (main effect: age, $p = 0.03$) and CK higher (main effect: age $p = 0.04$) in the younger than in the older cohort, but these values were unaffected by the intervention (Table S1). Plasma ASAT and ALAT concentrations were similar in the younger and older cohorts, but both were higher after the intervention (main effect: intervention, $p = 0.01$ and $p = 0.02$, respectively) (Table S1).

The current evidence on the effect of age and training on bioactive lipids in muscle of healthy individuals is somewhat equivocal, as both higher[47] and lower[48] content with age and positive or no effect of training have been reported.[49] In this study, the basal total DAG content of muscle was higher in the older study cohort, while ceramide content was similar in younger and older study participants (Figure 2M,N). The abundance of bioactive lipids, ceramide, and DAGs and their subspecies in muscle has been linked to insulin resistance by virtue of the impact of these biomolecules on insulin signaling,[50] although in man this conclusion remains controversial.[51,52] In the present study, the exercise intervention had no significant effect on total bioactive lipids, ceramide, and DAGs and their subspecies in muscle of younger or older study participants (Tables S4 and S5). Consistent with this, expression of serine palmitoyl transferase and the abundance of intermediates in ceramide synthesis and breakdown were similar between groups and unchanged by the intervention. Furthermore, metabolically, these results indicate that the decrease in insulin response to glucose load, of borderline statistical significance, is not linked to changes in bioactive lipids; instead, it is more likely related to higher expression of GLUT4 in muscle.

2.5 | Mitochondrial respiration

Citrate synthase (CS) activity is a useful biomarker for mitochondrial content.[53] In this study, the intervention did not affect CS activity in the younger or older cohort, but CS activity was higher in younger study participants (young: CPH: 260 ± 32; PMO + 1:255 ± 23 μmol/g/min, Old: CPH: 214 ± 33; PMO + 1:214 ± 20 μmol/g/min). Mitochondrial respiratory capacity (coupled and uncoupled respiration) was comparable in the study cohorts, and an interaction was seen with the intervention (Figure 5A). Intrinsic mitochondrial respiratory capacity (mitochondrial respiratory capacity normalized to mitochondrial content/CS

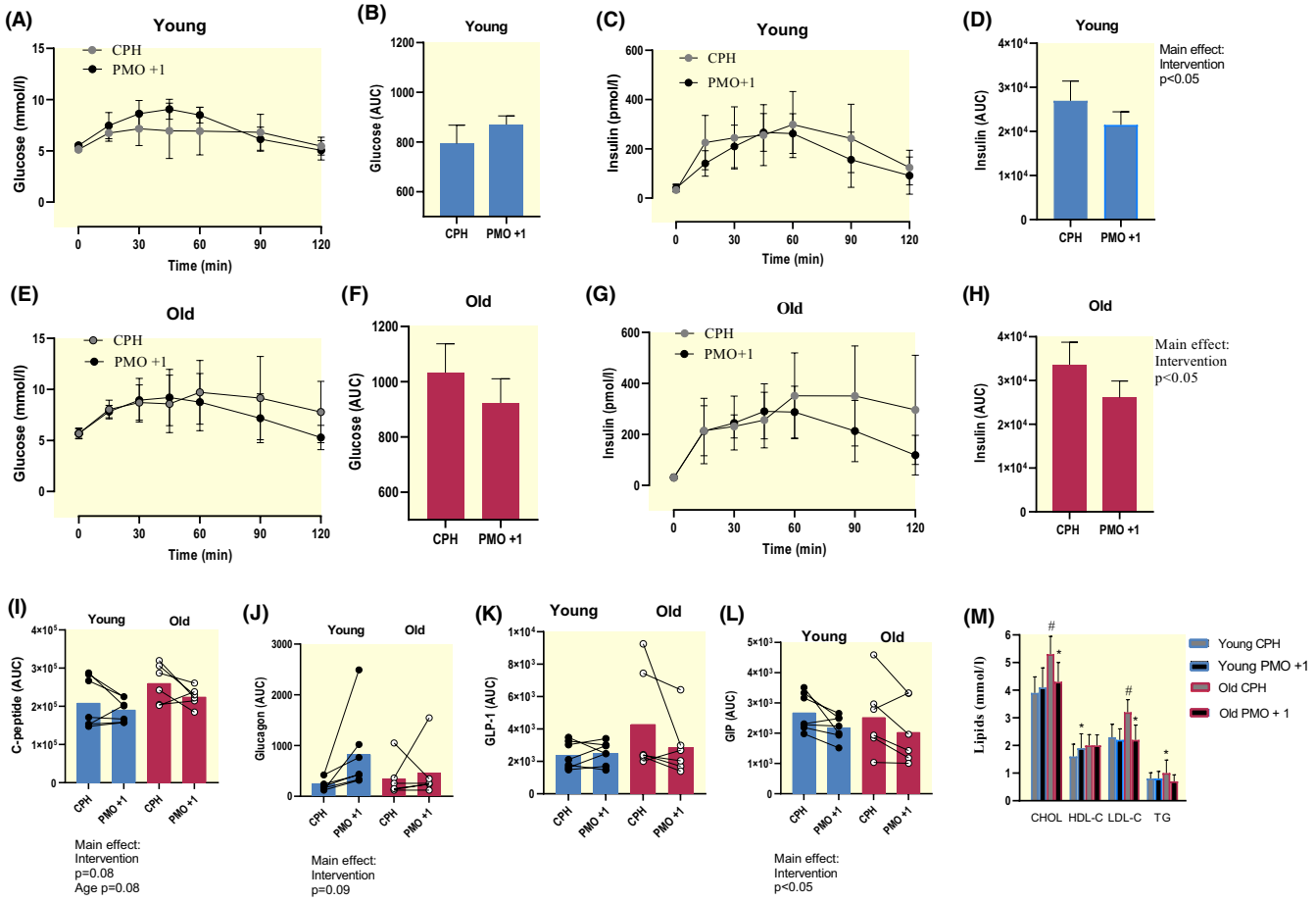

FIGURE 4 Oral glucose tolerance and blood lipids. (A) OGTT: Plasma glucose concentrations at 0, 15, 30, 45, 60, 90, and 120 min after the ingestion of 75 g of glucose dissolved in 400 ml water, in the young group in CPH (gray) and 1 day after the intervention (PMO + 1, black). (B). Area under the curve for plasma glucose concentrations during the OGTT in CPH and PMO + 1 in the young group. (C) OGTT: Plasma insulin concentrations at 0, 15, 30, 45, 60, 90, and 120 min in the young group in CPH (gray) and 1 day after the intervention (PMO + 1, black). (D). Area under the curve for plasma insulin concentrations during the OGTT in CPH and PMO + 1 in the young group. (E) OGTT: Plasma glucose concentrations at 0, 15, 30, 45, 60, 90, and 120 min after the ingestion of 75 g of glucose dissolved in 400 ml water, in the old group in CPH (gray) and 1 day after the intervention (PMO + 1, black). (F). Area under the curve for plasma glucose concentrations during the OGTT in CPH and PMO + 1 in the old group. (G) OGTT: Plasma insulin concentrations at 0, 15, 30, 45, 60, 90, and 120 min, in the old group in CPH (gray) and 1 day after the intervention (PMO + 1, black). (H). Area under the curve for plasma insulin concentrations during the OGTT in CPH and PMO + 1 in the old group. (I) Area under the curve for plasma C-peptide concentrations (at 0, 15, 30, 45, 60, 90, and 120 min) the OGTT in CPH and PMO + 1 in the young (blue bars) and the old (red bars) group. Individual values for the young black and old white circles. (J) Area under the curve for plasma glucagon concentrations (at 0, 30, 60, and 120 min) during the OGTT in CPH and PMO + 1 in the young (blue bars) and the old (red bars) group. Individual values are in circles for the young black and old white. (K, L) Area under the curve for plasma incretins (GLP-1 and GIP) concentrations (at 0, 30, 60, and 120 min) during the OGTT in CPH and PMO + 1 in the young (blue bars) and the old (red bars) group. (M) Plasma lipid total cholesterol, HDL- and LDL-cholesterol and triglyceride concentrations in CPH (gray fill) and 1 day after the intervention (PMO+1, black fill) in young (blue border) and old (red border). Post hoc multiple comparison analysis (Sidaks'): *different from CPH within group, $p < 0.05$. #Difference between groups within intervention point, $p < 0.05$

activity) was higher in the older than in the younger cohort before the intervention, and a borderline interaction ($p = 0.09$) was seen with the intervention (Figure 5B).

Interestingly, a recent study of mitochondrial function in young men and women who engaged in excessive HIIT-training reported that CS activity increased stepwise with increasing training load.[54] The same study demonstrated that excessive HIIT-exercise correlated with a decrease in mitochondrial respiratory capacity (assessed as complex I, complex II, or complex I and II), whereas light and moderate HIIT-exercise did not.[54] However, in that study,[54] biopsies were taken 14 h after the last HIIT-exercise bout, which may explain the discrepancy with the present study where the biopsies were obtained app. 40 h after arrival. It has also been shown that changes in CS-activity are related to changes in $\dot{V}O_2$max in younger individuals,

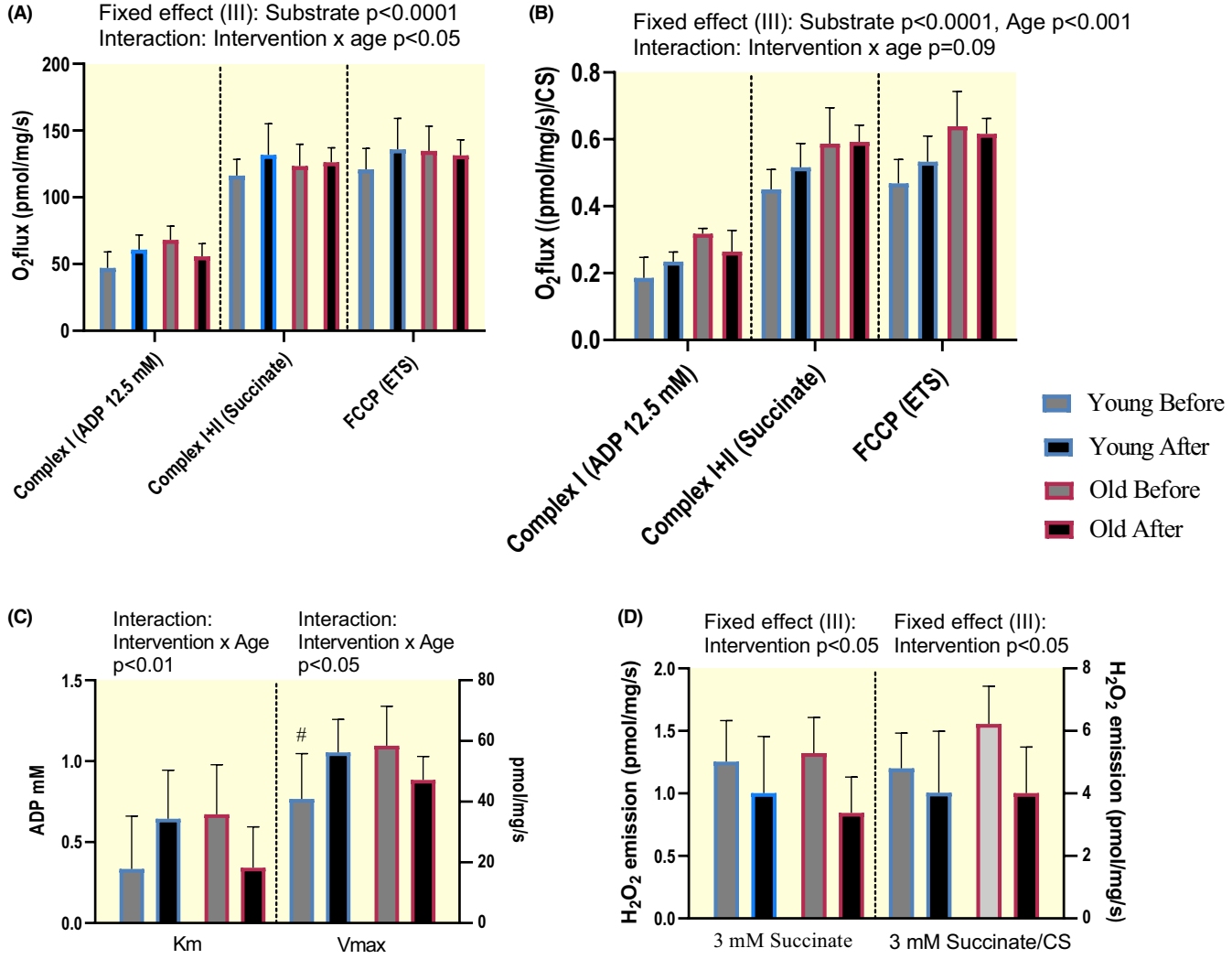

FIGURE 5 Mitochondrial respiration, ADP sensitivity, and ROS-production. (A) Mitochondrial respiration (pmol/mg/s) in m. vastus lateralis of the young with the addition of 12.5 mM ADP, succinate, and FCCP. (B) Intrinsic mitochondrial function ([pmol/mg/s]/CS) with the addition of 12.5 mM ADP, succinate, and FCCP. (C) ADP sensitivity in m. vastus lateralis of the young reported as Km (ADP mM, left y-axis) and Vmax (pmol/mg/s, right y-axis). (D) Reactive oxygen species (ROS) production (hydrogen peroxide, H_2O_2, pmol/mg/s) in m. vastus lateralis with the addition of 3 mM succinate and 3 mM succinate normalized to CS activity (right y axis). In all sub figures in the young (in CPH = blue border gray fill bar, PMO + 1 = blue border black fill bar) and the old (in CPH = red border gray fill bar, PMO + 1 = red border black fill bar) group. #Difference between groups within intervention point, $p < 0.05$

although this may not occur to the same extent in older individuals.[55]

In the present study, the activity of β-hydroxyacyl-CoA dehydrogenase (HAD), a key enzyme in beta-oxidation, was lower in the older cohort, but was unaffected by the intervention in either cohort. This suggests a lower mitochondrial volume/density in older study participants, which is supported by the CS activity findings. Nevertheless, as mentioned above, mitochondrial respiratory capacity through Complex I or Complex I + II was similar in younger and older cohorts, when normalized to tissue weight; in contrast, mitochondrial respiratory capacity normalized to CS activity (e.g., intrinsic mitochondrial respiratory capacity), measured through Complex I or Complex I + II, was higher in the older cohort before the intervention. Interestingly, it has previously been reported that bed rest causes a similar adaptation.[56]

In the older cohort, the sensitivity to ADP trended toward an increase ($p = 0.06$) (Km decreased) while the V_{max} for ADP trended toward a decrease ($p = 0.10$) after the intervention, whereas the opposite pattern was observed in the younger cohort (Figure 5C, Intervention × Age interaction, $p = 0.01$). It has previously been reported that an exercise intervention decreased ADP sensitivity in middle-aged subjects,[57] and this was confirmed in a cross-sectional study that compared ADP sensitivity in well-trained and untrained subjects.[58]

H_2O_2 emission was comparable in both cohorts before the intervention, and a significant decrease was seen after the intervention (main effect, $p < 0.05$), whether normalized to weight of tissue or to mitochondrial content (CS activity) (Figure 5D). This is supported by data from our laboratory, showing that 6 weeks of high-intensity training reduced emission of H_2O_2 in untrained obese subjects.[59]

2.6 | Skeletal muscle fiber types and capillarization

Interestingly, minimal differences in muscle fiber type composition and capillarization were detected between the younger and older study participants (Table S3), but a trend toward smaller type IIA fibers ($p = 0.06$) and significantly lower Cap/type IIA fiber was observed. We speculate that the high level of training and aerobic fitness in the older cohort counteracted the decrement in fiber area and capillarization across all fibers, but particularly in type II fibers, that is normally observed with increasing age.[60] The intervention had no effects on muscle fiber type composition and capillarization in either group, which was expected, given that the intervention was relatively short in duration and that the participants were very trained cyclists. Furthermore, given the fairly large daily protein intake (>2 g/kg body mass per day), where normal recommendations for intensive endurance training would be 1.6–1.8 g/kg body mass per day and the primarily concentric muscle use during cycling,[61] the intervention would not be expected to alter muscle fiber type/content. While small changes in fiber types have been documented in arm and leg muscle in response to extreme repeated prolonged exercise in cold temperatures for 4½ weeks,[62] the conditions of the intervention in the present study would not be expected to produce similar effects.

2.7 | Limitations

We undertook the field-based research study described here in order to examine the effects of a "real-life" extreme exercise intervention on a group of age-stratified study participants over an extended time frame. Although the study presented unique and difficult challenges, it would be difficult if not impossible to simulate or replicate the study or the intervention in a controlled laboratory environment. Furthermore, the best available methodology was applied, given the conditions at hand, to produce a robust set of high-quality data. Because of the rather extreme nature of the intervention, the number of study participants was less than desirable and this may explain that for some parameters only a trend could be observed.

We acknowledge that the study has several limitations. First, participants in the older cohort cycled at a slower pace than participants in the younger cohort, and therefore they exercised for a longer absolute period of time. On the other hand, participants in the younger cohort travelled at higher velocity, resulting in exponentially higher external workload per unit time. Accordingly, we choose to match the absolute distance travelled and the relative intensity (ie % HRmax) for all study participants.

Secondly, for logistic reasons, the younger cohort departed Copenhagen 3 days after the older cohort. Therefore, the two cohorts experienced slightly different ambient conditions throughout the intervention (*ie* temperature, wind, precipitation on a given stage were slightly different for the two cohorts). Nevertheless, it is highly unlikely that minor differences in ambient conditions during the intervention had a significant impact on study endpoints, outcomes, or conclusions. In the present study, the subjects food consumption was free both before and during the intervention, and this obviously adds an extra layer of complexity to the interpretation of the results.

Thirdly, we do think we achieved recruiting reasonably homogenous groups, but clearly exceptional in terms of being able to cycle 7–10 h per day for 15 consecutive days.

The measured heart rate during the intervention was used to quantify relative internal workload (i.e., % HRmax), energy expenditure, and body composition. This methodology is based on the assumption that total body water content is stable. While this is a fair and accurate assumption in general, exceptional circumstances can occur. For example, one older study subject experienced peripheral edema during stages 13 and 14 of the intervention, which largely resolved during stage 15 (the last stage of the intervention). Therefore, we cannot exclude a change in the total body water content or displacement of water content from one tissue to another, circumstances that could have affected some calculations of energy expenditure and body composition in the later stages of the study.

Lastly, we tried to anticipate possible interactions between different post-intervention assessments and tests. However, we did not anticipate but did observe that the OGTT performed on the first test day in Palermo may have influenced the overnight-fasted plasma glucose and FFA concentrations on the second test day in Palermo. We speculate this is the reason for the discrepancies between plasma glucose and FFA measured on test days 1 and 2 in Palermo (data not shown). However, other explanations cannot be excluded.

Despite these recognized and other possibly unrecognized limitations, we regard this as a study that provides

unique insight into the physiologic and metabolic responses to extreme endurance exercise in younger and older men.

3 | CONCLUSIONS

In summary, we provide evidence for negative [central] cardiovascular but primarily positive peripheral adaptive responses to extreme endurance exercise in older men, but no evidence of a negative cardiovascular adaptive response in younger men. Therefore, we propose the novel idea that the adaptive thresholds associated with extreme exercise vary by tissue/organ and participant age. Lastly, while in the context of the present study, extreme endurance exercise is associated with a maladaptive cardiovascular response in older men, additional studies are needed to ascertain the significance of the latter observation.

4 | MATERIALS AND METHODS

Seven younger (30 ± 5 years) and seven older (65 ± 6 years) well-trained male cyclists of Caucasian origin participated in this study. The older participants voluntarily planned to do this cycling trip and contacted us and were keen on participating in relevant research coupled to their planned trip. The younger group was recruited from local groups of very trained healthy endurance athletes. On a weekly base, prior to the study endurance training was performed for 9.1 ± 3.7 h/week in the younger and 5.2 ± 1.3 h/week in the older group and all had done this for at least 5 years and most much longer. The two cohorts were matched according to Body Mass Index (BMI: 23.6 ± 0.8 and 23.4 ± 1.2 kg/m^2, young and old, respectively).

The participants were informed about the safety and risks of participating in the study, and written consent was obtained. The study was approved by the Greater Copenhagen Region science ethical committee (H-16049145). The study included two consecutive days of experimental testing in Copenhagen (CPH) 7–14 days prior to start of the 15-day intervention. The older cohort of cyclists departed Copenhagen on May 15th 2017 and the younger cohort departed Copenhagen on May 18th 2017. In Rome, blood samples were obtained overnight fasted in the morning on day 10. Blood samples were obtained 2 h after arrival in Palermo (PMO). The participants refrained from any exercise for 36 h after arrival in PMO, followed by 2 consecutive days of testing (PMO + 1 and PMO + 2). The data sets collected before and after the intervention were identical, and were performed using the same laboratory equipment, which was shipped from Copenhagen to Palermo, Italy. A schematic overview of the intervention and experimental test days is provided in Figure 6.

4.1 | Intervention

The intervention consisted of approximately 3000 km of cycling from Copenhagen, Denmark, to Palermo, Italy over 15 days. Participants used their own bikes and equipment throughout the intervention, but were assisted with spare bike parts ie tubes, tires, gear parts etc The participants followed a similar and pre-defined route on each of the 15 stages. Each group was assisted by a team of researchers and students. GPS files were uploaded daily. Heart rate and body weight were recorded daily and on specific days urine samples were obtained and stored. Food and drink intake were monitored on stages 2 + 3, 6 + 7, and 12 + 13 (Figure 6).

4.2 | Exercise volume and intensity

The total travelled distance and the time spent were uploaded daily from a combined GPS and heart rate monitor (Garmin Vivoactive, Garmin, Olathe, Kansas, USA). The total exercise time and distance travelled only included active time on the bike. The relative heart rate during the intervention was calculated as the % of the maximal heart rate obtained during the initial $\dot{V}O_2$max test.

All participants followed the same route and slept at the same hotels. The total distance travelled by the younger cohort was 3087 km (206 ± 36 km/day) and for the older cohort was 2928 km (195 ± 31 km/day), and this difference was due to road closures, road works, and detours and one occasion, where all seven older participants were transported by car for the last 15 km of the stage, due to darkness. Furthermore, in two cases, an individual in the older cohort did not complete the full distance of the daily stage due to not feeling well (stomach pain). Initially, there were eight participants in the younger cohort, but one of the participants left the study early due to injury (cyclist's palsy). The total active cycling time of the intervention was 108.1 h (7.2 h/day) for the younger cohort and 126.6 h (8.4 h/day) for the older cohort. The average cycling speed was 28.5 and 23.1 km/h for the younger and old cohorts, respectively.

4.3 | Body weight and composition

Participants were weighed (Uniscale-200-50, VETEK, Sweden) in the morning before breakfast in similar and minimal clothing. Body composition was estimated from

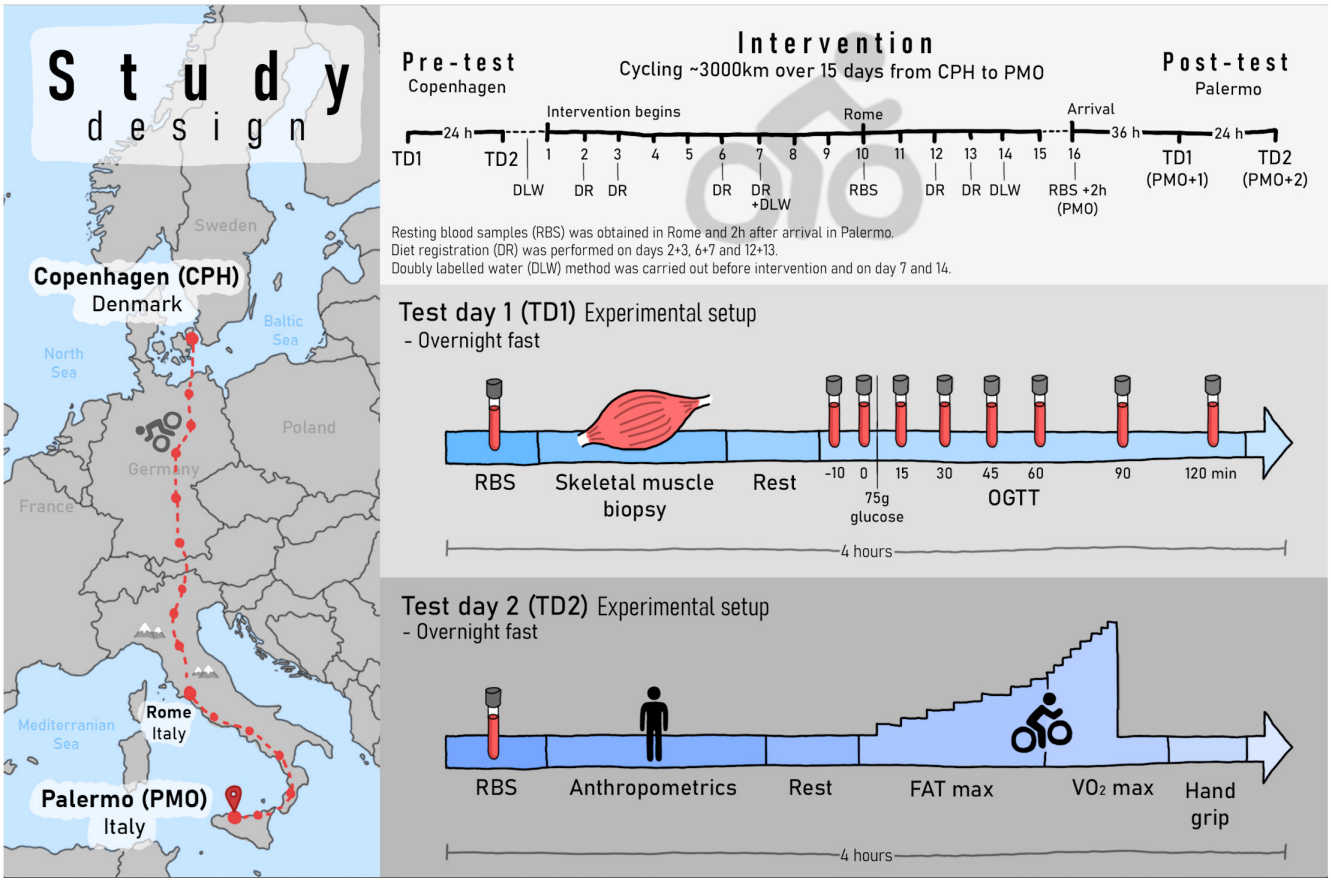

FIGURE 6 Study design. The two testing days were conducted in similar order in Copenhagen (CPH) and Palermo (PMO) CPH and PMO + 1 (rest day) followed by CPH and PMO + 2 (rest days). In Palermo a blood sample was obtained 2 h after the last exercise bout (PMO + 2 h)

the total volume of body water (TBW) calculated from ^{2}H isotope dilution according to the Maastricht protocol.[63] From TBW, the body composition was estimated 7 days before the intervention started and after stage 15 of the intervention. Fat-free-mass (FFM) was calculated from TBW ÷ 0.73.[24] Fat mass was calculated as total body mass minus fat-free-mass. Body composition was also measured before the intervention by dual-energy X-ray absorptiometry (DXA; Lunar iDXA Series; GE Medical Systems). When body composition measured by DXA and the ^{2}H-isotope dilution technique was compared, a *strong linear relationship was observed revealing an* $R^2 = 0.95$ *for LBM and* $R^2 = 0.87$ *for Fatmass (%).*

4.4 | Metabolism and physical performance tests

An incremental exercise test was performed before departure (CPH) and 2 days after arrival in Palermo (PMO + 2) (Figure 6). The subjects were asked to refrain from performing physical activity the last 24 h before the first test days in Copenhagen and Palermo. On the test days, the subjects arrived from very early morning to mid morning keeping the same order of testing in Copenhagen and Palermo. The testing schedule on day 1 and day 2 is outlined in Figure 6. During the initial testing, one subject in the old cohort experienced discomfort during the graded exercise test. Baseline resting substrate oxidation was measured over 5 min with the subjects sitting and resting on the cycle ergometer. Subsequently, the subjects performed an incremental exercise protocol commencing at 95 watts (W) followed by 35 W increases every 3 min. The $\dot{V}O_2$ and $\dot{V}CO_2$ during last 90 seconds was used to compute a third-order polynomial regression curve fit, to determine the maximal fat oxidation rate (MFO) and the relative workload at which this occurs (FATmax). The exercise protocol was adapted from Achten et al ($GE_{35/3}$ protocol).[64] When subjects reached a respiratory exchange ratio (RER) >1.0 increments of 35 W were added every minute until voluntary exhaustion. $\dot{V}O_2$max values were accepted when a plateau or an attenuated increase in $\dot{V}O_2$ was reached (increases of <2 ml/min/kg) despite increasing workloads and when an RER >1.15 was observed. Pulmonary $\dot{V}O_2$ and $\dot{V}CO_2$ were measured breath-by-breath with an automated online system (COSMED

– Quark CPET, Rome, Italy). The gas analyzers were calibrated with a 5.00% CO_2–16.00% O_2 gas mixture before each test.

Handgrip strength was measured in the standing position with a handheld dynamometer (HandGrip; Takei Grip-D TKK5401, Japan). Participants performed three compressions with each hand with at least a 45 second break between compressions. The highest load (kg) with each hand was reported as handgrip strength.

4.5 | Energy balance

Energy expenditure (EE) was measured by the double-labeled water (DLW) technique applied according to the Maastricht protocol[63] and modified to the expected high level of energy expenditure by applying DLW each week instead of every second week.[24] In brief, we applied every dosage of DLW in the evening before sleep and shortly after a urine sample. Participants ingested an amount of DLW (calculated from body weight) that would ensure baseline amount of $^2H_2{}^{18}O$ and ^2H to increase by 100–150 and 200–250 parts per million, respectively. Urine samples were obtained in the evening on days −8 and −1 before the intervention and after stages 7 and 14 of the intervention. Morning urine samples were obtained on days −7 and before stages 1, 8, and 15. All urine samples were divided in two identical vials and immediately stored at 5°C. All morning urine samples were taken before any physical activity. Carbon dioxide (CO_2) production was calculated from difference in ^{18}O and ^2H elimination rates as part of the DLW technique. The CO_2-production was converted to EE by metabolic fuel quotient (assuming an average RER of 0.85) from dietary records and the use of body energy stores.[65] To control for potential geographical changes in concentrations of ^{18}O and ^2H in local water sources (tap water) as a confounding factor, four non-cyclist subjects travelling with the cohorts also provided urine samples.

Total energy intake (EI) was measured by diet registration over two consecutive days at stages 2 + 3, 6 + 7 and 12 + 13. All food and drinks during every meal were weighed on portable food scales by the assisting team of researchers. Before cycling at all stages of the intervention, participants were offered carbohydrate-rich sports drinks and energy bars, with a known amount of energy and macronutrient content. On the diet recording days, participants were instructed to store all packaging from sports bars, and any remaining sports drinks were weighed in order to calculate the total amount ingested. Dietary records were processed using appropriate software (Dankost 3000, Dansk Catering Service), and EI and macronutrient composition of the diet were calculated.

4.6 | Blood analyses

Venous blood samples for analysis of plasma concentration of glucose, free fatty acids (FFA), glycerol, insulin, C-peptide, glucagon, adiponectin, leptin, gastric inhibitory peptide (GIP), total glucagon-like-peptide 1 (GLP-1), C-reactive protein (hsCRP), aspartate-aminotransferase (ASAT), alanine-aminotransferase (ALAT), and creatine kinase (CK) were obtained before (CPH) the intervention and one day after arrival in Palermo (PMO + 1). In addition, concentrations of hemoglobin (Hgb) in blood and concentrations of growth/differentiation factor 15 (GDF15) in plasma was also measured during the intervention at stage 10 (ROME), 2 h after arrival (PMO), at PMO + 1 (only GDF15), and 2 days after arrival in Palermo (PMO + 2). Before (CPH) and after the intervention (PMO + 1), a 120 min oral glucose tolerance test (75 g of glucose dissolved in 400 ml of water) was performed in the overnight fasting state, and plasma concentrations of FFA and glycerol were measured at −10 min and 0 min, while glucose, insulin, C-peptide, and glucagon were measured at 0, 15, 30, 45, 60, 90, and 120 min. GIP and GLP-1 were measured at −10, 0, 30, 60, and 120 min. Blood samples were always immediately centrifuged at 4.000 g for 10 min at 4°C (Centrifuge Hettich Universal 30 RF; Hettich, Tuttlingen, Germany) and the plasma fraction was collected and stored at −80°C for later analysis.

Concentrations of glucose, FFA, glycerol, ASAT, ALAT, CK, hsCRP, insulin, and C-peptide were measured by spectrophotometry (Cobas 6000 c 501, Roche, Glostrup, Denmark). GIP and total GLP-1were measured by RIA and GDF15 by ELISA as previously described.[33,66,67] Hgb was measured on an ABL90 FLEX blood gas analyzer (Radiometer, Bronshoj, Denmark).

4.7 | Skeletal muscle analyses

Skeletal muscle biopsies from vastus lateralis were obtained before (CPH) and after (PMO + 1) the intervention with a Bergström needle after applying local anesthesia. Two (CPH and PMO + 1) muscles biopsies were obtained from each leg in a randomized order. The biopsy (app. 200 mg) was immediately divided into three separate samples for further analysis. One part was immediately placed in BIOPS buffer medium for mitochondrial respirometry and fluorometry analysis. Another part was embedded in TissueTek® (VWR Chemicals, Leuven, Belgium) and frozen in isopentane cooled in liquid nitrogen, and the last part was snap frozen in liquid nitrogen and stored at −80°C for later analysis. Muscle fiber composition was analyzed as previously described.[68] Protein expression of glycogen synthase (GS), glycogen phosphorylase (GP), hexokinase

II (HKII), glucose transporter protein 4 (GLUT4), and protein kinase B (Akt), adipose triglyceride lipase (ATGL), fatty acid translocase (FAT/CD36), long-chain fatty acid transport protein 4 (FATp4), plasma membrane fatty acid-binding protein (FATpm), perilipin 2 (PLN2), perilipin 3 (PLN3), perilipin 5 (PLN5), diglyceride acyltransferase (DGAT), and serine palmitoyltransferase (SPT) was performed by western blotting (for details, see Supporting Information). Analysis of enzyme activities of citrate synthase (CS) and 3-hydroxyacyl CoA dehydrogenase (HAD) was performed using approximately 2 mg of freeze-dried and dissected muscle tissue. Homogenization was conducted in 600 µl 0.3 M K2 HPO4, 0.05% bovine serum albumin (BSA) at a pH of 7.7 for 2 min using a Tissuelyser (Qiagen, Venlo, Limburg, the Netherlands). Six microliter of 10% Triton was added and the muscle samples were left on ice for 15 min and then stored at −80°C for later analyses by spectrophotometry (Cobas 6000 c 501, Roche, Glostrup, Denmark). IMTG was analyzed on freeze-dried dissected muscle tissue, which was homogenized in methanol and chloroform, incubated in tetraethylammonium hydroxide and HCl, and glycerol concentration was analyzed by spectrophotometry (Cobas 6000 c 501, Roche, Glostrup, Denmark). Muscle glycogen was analyzed on freeze-dried dissected muscle tissue, hydrolyzed with acid (HCl), and glycosyl units determined (Cobas 6000 c 501, Roche, Glostrup, Denmark). Diacylglycerols (DAG) and ceramides were analyzed on app. 5 mg of freeze-dried dissected muscle tissue. DAG were measured as previously described.[69] The internal standard used was deuterated DAG (Deuterated DAG Mixture I and Mixture II, Avanti Polar Lipids, Alabaster, AL, USA) mixtures, and the measurements were made on Sciex QTRAP 6500+ AB Sciex (Germany GmbH, Darmstadt, Germany). Ceramides were measured as previously described.[70] Detailed description of DAG and Ceramide analyses can be found in the Supporting Information.

4.8 | Mitochondrial respiratory capacity and H_2O_2 emission

Muscle fibers for mitochondrial respiratory capacity and H_2O_2 emission were prepared for analysis as previously described.[53,71] Briefly, the muscle fibers were mechanically separated in their respective preservation buffers (BIOPS for mitochondrial respiratory capacity and buffer X for H_2O_2 emission) on ice using sharp needles and chemically permeabilized with saponin (50 µg/ml) for 30 min on ice. This was followed by two washes (10 min) in mitochondrial respiration medium (MiR05 for mitochondrial respiratory capacity and buffer Z for H_2O_2 emission). The respiratory capacity protocol is described in detail in the supplemental material. The protocol used for H_2O_2 emission has been described previously.[71]

4.9 | Statistical analysis

All data in tables and graphs are shown as mean ± SD or individual data points, if not otherwise stated. When normal distribution failed, data were log-transformed. A simple unpaired Students t-test was applied to analyze baseline differences between groups. CPH vs. PMO-data were analyzed using a Two-way ANOVA analysis of variance, when a significant effect of the intervention or an interaction of group × intervention was present, we conducted a Holm–Sidak post-hoc analysis. When missing data were apparent, we used a linear mixed effect model (maximum likelihood and Geisser-greenhouse correction), with intervention and age (intervention × age) as fixed effects type III. Data analysis and graphical work were conducted using Sigmaplot (Systat Software, San Jose, CA, USA), GraphPad prism 8.0 (GraphPad Software, La Jolla, CA, USA), SPSS vers. 25 (IBM software), and Microsoft PowerPoint (Microsoft office 2018). We applied an α of 0.05.

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CONFLICT OF INTEREST

There are no conflict of interest for this paper.

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