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Skeletal muscle mitochondrial inertia associates with carnitine acetyltransferase activity and physical function in humans

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BACKGROUND. At the onset of exercise, the speed at which PCr decreases towards a new steady state (PCr on-kinetics), reflects the readiness to activate mitochondrial ATP synthesis, which is secondary to Acetyl-CoA availability in skeletal muscle. We hypothesized that PCr on-kinetics are slower in metabolically compromised and older individuals, and associated with low carnitine acetyl-transferase (CrAT) protein activity and compromised physical function.

METHODS. We applied ^{31}P-Magnetic Resonance Spectroscopy (MRS) to assess PCr on-kinetics in two cohorts of human volunteers. Cohort 1: patients with type 2 diabetes, obese, lean trained and untrained individuals. Cohort 2: young and older individuals with normal physical activity and older trained. Previous results of CrAT protein activity and acetylcarnitine content in muscle tissue were used to explore the underlying mechanisms of PCr on-kinetics, along with various markers of physical function.

RESULTS. PCr on-kinetics were significantly slower in metabolically compromised and older individuals (indicating mitochondrial inertia) as compared to young and older trained volunteers, regardless of in vivo skeletal muscle oxidative capacity ($P<0.001$). Mitochondrial inertia correlated with reduced CrAT protein activity, low acetylcarnitine content and also with functional outcomes ($P<0.001$).

CONCLUSION. PCr on-kinetics are significantly slower in […]

Skeletal muscle mitochondrial inertia associates with carnitine acetyltransferase activity and physical function in humans

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Abstract

Background: At the onset of exercise, the speed at which PCr decreases towards a new steady state (PCr on-kinetics), reflects the readiness to activate mitochondrial ATP synthesis, which is secondary to Acetyl-CoA availability in skeletal muscle. We hypothesized that PCr on-kinetics are slower in metabolically compromised and older individuals, and associated with low carnitine acetyl-transferase (CrAT) protein activity and compromised physical function.

Methods: We applied ^{31}P-Magnetic Resonance Spectroscopy (MRS) to assess PCr on-kinetics in two cohorts of human volunteers. Cohort 1: patients with type 2 diabetes, obese, lean trained and untrained individuals. Cohort 2: young and older individuals with normal physical activity and older trained. Previous results of CrAT protein activity and acetylcarnitine content in muscle tissue were used to explore the underlying mechanisms of PCr on-kinetics, along with various markers of physical function.

Results: PCr on-kinetics were significantly slower in metabolically compromised and older individuals (indicating mitochondrial inertia) as compared to young and older trained volunteers, regardless of in vivo skeletal muscle oxidative capacity ($P<0.001$). Mitochondrial inertia correlated with reduced CrAT protein activity, low acetylcarnitine content and also with functional outcomes ($P<0.001$).

Conclusions: PCr on-kinetics are significantly slower in metabolically compromised and older individuals with normal physical activity compared to young and older trained, regardless of in vivo skeletal muscle oxidative capacity, indicating greater mitochondrial inertia. Thus, PCr on-kinetics are a currently unexplored signature of skeletal muscle mitochondrial metabolism, tightly linked to functional outcomes. Skeletal muscle mitochondrial inertia might emerge as a target of intervention to improve physical function.

Trial registration: clinicaltrials.gov: NCT01298375 and clinicaltrials.gov: NCT03666013

[Word count 250]

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Introduction

Regular physical activity is a major contributor to metabolic health. In light of an aging and predominantly sedentary population, the incidence of exercise intolerance has grown over the past decades (1). Of note, exercise intolerance typically manifests as premature muscle exhaustion upon objective measures of contractile performance (2) and it is associated with poor metabolic health (3). To take advantage of the health benefits of exercise, it is crucial to better understand the origin of exercise intolerance.

At the onset of exercise, phosphocreatine (PCr) rapidly buffers the sudden increase in energy demand. PCr decreases until a new steady state is reached during exercise when contractile activity is maintained by alternative sources of ATP synthesis, a fundamental process to prevent full PCr depletion and premature muscle fatigue (4). At low-moderate intensity exercise, ATP is mainly produced by mitochondrial oxidative phosphorylation. Given the importance of PCr as an ATP source at the onset of exercise and the role of mitochondrial ATP synthesis in reaching and maintaining the new steady state of PCr, the time that it takes to reach a steady state during exercise (here referred to as PCr on-kinetics) reflects the readiness to activate mitochondrial ATP synthesis upon a sudden increase in ATP demand at the beginning of exercise (5) (also referred to as mitochondrial inertia (6)). Thus, skeletal muscle mitochondrial inertia may well be a determinant of skeletal muscle physical performance and functional decline. In fact, the occurrence of functional decline (and associated exercise intolerance) has been shown to be higher in metabolically compromised and elderly sedentary individuals as compared to healthy and physically active peers (7). In line with this reasoning, we anticipate PCr on-kinetics to be slower in individuals with type 2 diabetes than in healthy individuals and similarly, we expect slower PCr on-kinetics in sedentary versus physically active elderly.

The activation of skeletal muscle mitochondrial metabolism at the onset of exercise is secondary to intramyocellular Acetyl-CoA availability (6). Acetyl-CoA availability largely depends on glycolysis and fatty acid oxidation, but a short-term Acetyl-CoA buffer is represented by the intracellular acetylcarnitine pool, which can be converted to Acetyl-CoA by the mitochondrial enzyme carnitine acetyltransferase (CrAT) (8). Interestingly, we previously reported that CrAT protein activity and acetylcarnitine content in human skeletal muscle were significantly lower in patients with type 2 diabetes and individuals with obesity as compared to endurance-trained people, supporting the notion that reduced CrAT protein activity and therefore a low capacity to form acetylcarnitine from Acetyl-CoA and carnitine might underlie metabolic inflexibility and impaired insulin sensitivity (9). Importantly, the CrAT enzyme also functions in the reverse direction to supply Acetyl-CoA from acetylcarnitine when energy demand suddenly increases. We here hypothesize that CrAT activity and acetylcarnitine content in skeletal muscle affect mitochondrial inertia and are determinants of PCr kinetics at the onset of exercise. This hypothesis was tested in four groups of human volunteers including: 1) patients with type 2 diabetes (T2DM), 2) normoglycemic individuals with obesity (OB), 3) young lean untrained (UT) and 4) young lean endurance trained (T). To examine the putative functional relevance of slow PCr on-kinetics, we included a second cohort of young and older human volunteers, with a wide range in skeletal muscle physical function as determined by functional markers of physical fitness in daily life (6-minute walk test and a chair-stand-test) and exercise efficiency.

Results

Participant characteristics

Participant characteristics from study cohort 1 are shown in table 1. Patients with T2DM and OB individuals were significantly older and had a higher body weight, BMI and fat mass (%) than T and UT counterparts ($P < 0.05$ for all comparisons). Patients with T2DM and OB individuals exhibited significantly lower whole-body insulin sensitivity as compared to T and UT volunteers (M value, $P < 0.001$). Maximal aerobic capacity (VO2max) was significantly higher in T as compared to UT, OB and T2DM individuals ($P < 0.001$ for all comparisons).

Skeletal muscle mitochondrial inertia at the onset of exercise in individuals with type 2 diabetes and obesity

PCr half-time at the onset of exercise was significantly different across the groups (T2DM: 49.2 ± 4.3 s, OB: 38.9 ± 2.6 s, UT: 28.3 ± 3.2 s, and T: 19.6 ± 2.1 s; $P < 0.001$, Figure 1A). Post-hoc analysis revealed that PCr on-kinetics were significantly slower in T2DM and OB individuals as compared to T volunteers ($P < 0.001$ for both comparisons). Furthermore, PCr on-kinetics were significantly slower in patients with T2DM as compared to UT individuals ($P < 0.001$).

We previously reported (9) that in vivo skeletal muscle mitochondrial capacity, as determined by the rate constant of PCr recovery post-exercise, was significantly different across the groups ($P < 0.001$), with a significantly slower PCr recovery in patients with T2DM as compared to OB, UT and T individuals ($P = 0.04$, $P < 0.01$, and $P < 0.01$ respectively). Also, PCr recovery post-exercise was significantly slower in T2DM patients as compared to OB and UT ($P < 0.005$). In order to investigate whether a slow PCr on-kinetics

simply reflects lower mitochondrial function, we adjusted the PCr on-kinetics for PCr recovery post-exercise. Of note, the significant differences in PCr on-kinetics observed in the current study between patients with T2DM and T and UT individuals remained even upon correction for PCr recovery post-exercise (P = 0.006 and P = 0.010). The significant difference in PCr on-kinetics between OB and T individuals also remained upon correction for PCr recovery post-exercise (P = 0.010). Furthermore, PCr on-kinetics (mitochondrial inertia) and PCr recovery rate post exercise were strongly associated (n = 37, r = 0.67; P < 0.001, Figure 1B).

Reduced skeletal muscle CrAT protein activity and low acetylcarnitine content in individuals with type 2 diabetes and obesity are related to skeletal muscle mitochondrial inertia

We previously published that skeletal muscle CrAT protein activity was significantly lower in patients with T2DM, OB and UT individuals as compared to T volunteers (P < 0.01) whereas skeletal muscle acetylcarnitine content was significantly lower in patients with T2DM as compared to T individuals (P = 0.017) (9). In the current study we tested if skeletal muscle CrAT protein activity and acetylcarnitine content associated with PCr on-kinetics. Interestingly, we observed strong negative correlations between both skeletal muscle CrAT protein activity (n = 31, r = -0.56, P = 0.001) and in vivo skeletal muscle acetylcarnitine content (n = 35, r = -0.47, P = 0.005) with PCr on-kinetics half-time (Figure 1C and 1D). Interestingly, the association between in vivo skeletal muscle acetylcarnitine content and PCr on-kinetics half-time remained significant after adjusting for the PCr recovery post-exercise (r = -0.40, P = 0.034). In contrast, the association between CrAT protein activity and PCr on-kinetics half-time was not significant after adjusting for PCr recovery post exercise (r = -0.17, P = 0.37).

Skeletal muscle mitochondrial inertia is associated with elevated ADP levels in muscle of metabolically compromised individuals.

Prior to exercise, ADP levels (μM) at rest were similar across the groups. At the onset of exercise, ADP levels increased rapidly in all individuals (Figure 2A). When PCr reached a new steady state during exercise, ADP levels were significantly different across the groups (patients with T2DM: 55.4 ± 4.7 μM, OB individuals: 52.3 ± 3.0 μM, UT individuals: 46.3 ± 6.6 μM, T individuals: 37.8 ± 2.2 μM; $P = 0.018$, Figure 2B), with significantly higher levels in patients with T2DM as compared to T volunteers ($P = 0.025$). When PCr reached a new steady state during exercise, ADP levels were significantly associated with PCr on-kinetics (n = 35, r = 0.70, $P < 0.001$; Figure 2C). Interestingly, this association remained significant after adjusting for the PCr recovery post-exercise (r = 0.61, $P < 0.001$). Intracellular pH was not significantly different across the groups, neither at rest (patients with T2DM: 7.1 ± 0.02, OB individuals: 7.1 ± 0.03, UT individuals: 7.1 ± 0.01, T individuals: 7.1 ± 0.02; $P = 0.90$) nor at the end of exercise (patients with T2DM: 7.1 ± 0.02, OB individuals: 7.1 ± 0.04, UT individuals: 7.02 ± 0.03 and T individuals: 7.00 ± 0.02; $P = 0.08$).

Subsequently, we sought to determine whether PCr on-kinetics, hence skeletal muscle mitochondrial inertia, are related to age, training status and functional capacities in a second cohort consisting of young (Y) and older (O) individuals with normal physical activity as well as trained older adults (OT). The characteristics of study cohort 2 are shown in table 2.

Skeletal muscle mitochondrial inertia at the onset of exercise in older individuals is rescued by exercise training

In line with our findings from individuals of cohort 1, PCr on-kinetics were significantly different across groups (Y: 24.9 ± 2.2 s, OT: 24.7 ± 1.8 s, O: 33.1 ± 1.8 s; $P = 0.002$, Figure 3A) with significantly longer values for the halftime in O individuals as compared to OT ($P = 0.005$) and Y individuals ($P = 0.02$). Of note, PCr on-kinetics were not significantly different between OT and Y groups ($P > 0.05$). PCr recovery half-time post-exercise was not significantly different across groups (Y: 18.5 ± 0.7 s, OT: 19.3 ± 1.1 s, O: 21.5 ± 0.8 s: s; $P = 0.133$, Figure 3B). Similar to our findings in cohort 1, PCr on-kinetics (mitochondrial inertia) and PCr recovery half-time post exercise (in vivo mitochondrial function) were significantly associated (n = 42, r = 0.31; $P = 0.04$, Figure 3C). Interestingly though, the significant difference in PCr on-kinetics between O and OT groups remained even upon adjustment for PCr recovery post exercise ($P = 0.023$), and the differences between O and Y individuals tended to remain significant ($P = 0.08$).

Skeletal muscle mitochondrial inertia at the onset of exercise is associated with functional outcomes and exercise efficiency

During the 6-minute walk test (6-MWT), both the distance covered and walking speed were not significantly different across the groups (Y: 630 ± 15 m and 1.75 ± 0.104 m/s, OT: 639 ± 17 m and 1.77 ± 0.04 m/s, O: 583 ± 24 m and 1.62 ± 0.06 m/s; $P = 0.12$ for both comparisons, Figure 3D). Also, the chair stand test did not reveal significant differences between the groups (Y: 9.0 ± 2.5 s, OT: 9.0 ± 1.9 s, O: 10.2 ± 1.6 s; $P = 0.14$, Figure 3E).

Upon performing a submaximal cycling test, gross and net exercise efficiencies were significantly different across the groups (Y: 19.7 ± 0.7 % and 23.2 ± 0.90 %, OT: 18.3 ± 0.4 % and 21.4 ± 0.5 %, O: 15.9 ± 0.4 % and 19.0 ± 0.4 %; P < 0.001 for gross and net efficiency respectively, Figure 3F and 3G). Gross and net exercise efficiencies were significantly lower in O individuals as compared to both Y (P < 0.001 for both comparisons) and OT individuals (P = 0.003 and P = 0.01). Gross and net exercise efficiencies were not significantly different between Y and OT volunteers (gross efficiency P = 0.23 and net efficiency P = 0.11). The Y and OT individuals performed the sub-maximal cycling test at a similar but significantly higher absolute workload (Y: 109.1 ± 8.4 W, OT: 97. ± 6.8 W; P = 0.64) as compared to O (74 ± 6.2 W; P < 0.05 for both comparisons). Resting energy expenditure was not significantly different across the groups (P = 0.22, Table 2).

Next, we aimed to test if the PCr on-kinetics were related to these physical function parameters. Indeed, we found that slower PCr on-kinetics (mitochondrial inertia) were strongly associated with lower walking speed (n = 39, r = -0.48; P = 0.002, Figure 4A), chair stand test performance (n = 40, r = 0.54; P < 0.001, Figure 4B), gross exercise efficiency (n = 42, r = -0.56; P < 0.001, Figure 4C) and net exercise efficiency (n = 42, r = -0.53; P < 0.001, Figure 4D). Considering that Y, OT and O groups differ in terms of age and sex, as well as to investigate whether a slow PCr on-kinetics simply reflects lower mitochondrial function, we recomputed the associations between PCr on-kinetic and the different functional parameters upon adjusting for age, sex and PCr recovery half-time post exercise (in vivo mitochondrial function). Interestingly, the significant associations of PCr on-kinetic with these functional parameters remained: walking speed (r = -0.49; P = 0.002), chair stand test performance (r = 0.58; P < 0.001), gross exercise efficiency (r = -0.40; P = 0.018) and net exercise efficiency (r = -0.34; P = 0.046).

Discussion

Intramyocellular PCr buffers ATP as the sudden increase in energy demand at the onset of exercise would otherwise lead to ATP depletion. The delay in activating mitochondrial ATP synthesis in response to such sudden increase in ATP demand is known as skeletal muscle mitochondrial inertia. Slower activation of mitochondrial ATP synthesis will result in a more pronounced PCr depletion at the onset of exercise. Assessment of skeletal muscle mitochondrial inertia at the onset of exercise has been hampered by the invasive nature of repeated muscle biopsies. In this cross-sectional study, we applied ^{31}P-MRS methodology to non-invasively quantify the in vivo PCr kinetics at the onset of exercise (PCr on-kinetics) in two different cohorts. It was observed that PCr on-kinetics were significantly slower in older, metabolically compromised volunteers as compared to young, endurance-trained individuals as well as in older, normally physically active individuals as compared to young individuals and older exercise-trained participants. Moreover, we observed that PCr on-kinetics strongly correlated with multiple markers linked to physical function such as walking speed, chair sit-to-stand transitions and mechanical efficiency of exercise. Finally, the observed differences in PCr on-kinetics, and its association with functional outcomes, was strongly associated with CrAT protein activity, acetylcarnitine content and ADP concentration during exercise in muscle tissue. Collectively, our results support the hypothesis that skeletal muscle mitochondrial inertia is greater in metabolically compromised and elderly individuals and is closely related to physical function. Furthermore, our findings suggest that a diminished ability of CrAT protein to supply Acetyl-CoA groups for oxidation may underlie skeletal muscle mitochondrial inertia. Alternatively, insensitivity to ADP to stimulate oxidative metabolism may play a role.

Previous investigations from cross-sectional and interventional studies have used similar approaches applying ^{31}P-MRS techniques to investigate in vivo PCr on-kinetics in young, healthy volunteers (5, 10, 11) and found that the halftime of PCr on-kinetics is related to the physical fitness of individuals. In fact, the halftime values reported are consistent with our results from endurance-trained and healthy untrained volunteers as well as our data obtained in young, physically active individuals. However, to the best of our knowledge, no previous studies have specifically examined the PCr kinetics at the onset of exercise in metabolically compromised and elderly individuals. PCr utilization upon exercise largely depends on the rate of energy supply by mitochondrial oxidative phosphorylation (OXPHOS) (4). Thus, an impaired ability of mitochondria to respond and produce ATP upon sudden increases in energy demand might prompt a more prolonged reliance on PCr. This is also referred to as mitochondrial inertia. In line with this notion, we show here that PCr on-kinetics are significantly slower in metabolically compromised volunteers and in elderly individuals with normal physical activity as compared to their young and elderly trained counterparts. To investigate whether mitochondrial inertia is simply a reflection of mitochondrial capacity, we also adjusted the results for PCr recovery half-time, which is considered a measure of maximal mitochondrial ATP synthetic capacity. Interestingly, the significant differences on PCr on-kinetics remained even after adjusting by in vivo PCr recovery post-exercise. Our results suggest that PCr kinetics at the onset of exercise are a unique characteristic of -not yet clearly defined- skeletal muscle mitochondrial function which reflects the readiness of skeletal muscle mitochondria to produce ATP.

To further explore the functional relevance of this yet unexplored signature of skeletal muscle mitochondrial function, we performed a series of correlative analysis between PCr kinetics at the onset of exercise and various parameters of physical function. PCr kinetics

at the onset of exercise proved to be strongly correlated with walking speed and the speed of sit-to-stand transitions, regardless of in vivo skeletal muscle mitochondrial capacity (as determined by PCr recovery post-exercise). As we investigated the response to sudden increase on ATP demand which is typical in the initiation of exercise, this can be especially important when initiating movements and furthermore support the contention that skeletal muscle mitochondrial activation is a salient contributor to performance in circumstances that mimic daily life situations. Hence, PCr kinetics at the onset of exercise might be an important factor of exercise intolerance and therefore, be a potential target of intervention. In this regard, a previous study reported that 5 weeks of regular endurance-type exercise training resulted in an improvement of the PCr kinetics at the onset of exercise in concert with improving exercise tolerance in young healthy individuals (5).Whether exercise training also improves PCr kinetics at the onset of exercise (mitochondrial activation), along with improved muscle functional capacity, in individuals who are prone to premature muscle fatigue warrants future investigation. Furthermore, PCr on-kinetics strongly correlated with exercise efficiency, defined as the ratio between mechanical work and energy expenditure. Interestingly, we observed that PCr on-kinetics correlate with exercise efficiency even upon correction for skeletal muscle mitochondrial capacity (PCr recovery post exercise). This statistical adjustment suggests a relationship between mitochondrial inertia and functional outcomes, independent of mitochondrial maximal ATP-synthesis rate and hence reveals mitochondrial inertia as a unique and new signature of mitochondrial function. However, considering the close link between skeletal muscle mitochondrial function and exercise efficiency (12), it is difficult to disentangle the interdependence of PCr on-kinetics and skeletal muscle mitochondrial function. Therefore, the mechanistic link between PCr on-kinetics and exercise efficiency requires further study.

Mechanistically, PCr kinetics at the onset of exercise, hence skeletal muscle mitochondrial inertia, is governed by the intramyocellular Acetyl-CoA availability (13). Thus, a momentary deficit of Acetyl-CoA groups would restrict the rate of ATP production via oxidative metabolism, thereby causing a prolonged reliance on substrate level phosphorylation (14). Here, we show for the first time in humans that CrAT protein activity in muscle tissue, the enzyme that essentially buffers the intramyocellular Acetyl-CoA content via acetylcarnitine formation and breakdown, is strongly associated with PCr kinetics at the onset of exercise. Furthermore, skeletal muscle acetylcarnitine content was also reduced at rest in metabolically compromised individuals and significantly associated with slow PCr kinetics at the onset of exercise. In line with the current results, we previously reported by using a loss-of-function mouse model that CrAT protein activity prevents the Acetyl-CoA deficit upon exercise via transferring Acetyl-CoA groups from the intracellular acetylcarnitine pool for mitochondrial oxidation (15). Elegantly, we revealed that CrAT-mediated Acetyl-CoA buffering prevents a further reliance on PCr hydrolysis and skeletal muscle glycogen breakdown upon exercise, concluding that CrAT protein function mitigates skeletal muscle mitochondrial inertia and promotes exercise tolerance (15). Other studies reported that elevated Acetyl-CoA/acetylcarnitine content in muscle tissue prior to exercise results in a lower PCr degradation at the onset of exercise and improved exercise tolerance, independent of increases on muscle blood flow (14, 16). Our findings are consistent with the premise that the stockpiled Acetyl-CoA groups buffered as acetylcarnitine via CrAT protein function are instrumental for skeletal muscle mitochondrial activation, as this might reflect more readily available substrate to fuel the TCA cycle at the onset of exercise. Indeed, we show strong correlations between CrAT protein activity and PCr on-kinetics.

An alternative mechanistic explanation for the observed differences in PCr kinetics across groups at the onset of exercise may be an inherently lower sensitivity of the OXPHOS system to ADP levels to regenerate ATP by active muscles in metabolically challenged groups. Considering the lower intrinsic ex vivo ADP sensitivity previously reported in metabolically compromised (17) and elderly individuals (18), one might expect that higher ADP levels are needed to stimulate the OXPHOS system in these individuals.

We calculated ADP concentrations, assuming creatine kinase reaction to be at equilibrium (19) and in line with the notion of ADP sensitivity being of importance, we found higher ADP levels during exercise in patients with type 2 diabetes and in obese individuals as compared to endurance-trained individuals. These results suggest that a higher metabolic stress is needed to activate oxidative ATP formation in metabolically compromised individuals, while prompting the reliance on substrate level phosphorylation for a longer time. The underlying mechanism explaining such differences in sensitivity to metabolic stress remains inconclusive and next to ADP, also other metabolites have been suggested to underlie activation of oxidative metabolism (20). If a stronger reliance on PCr is a contributor to reduced physical function, increasing the PCr pool (e.g by creatine supplementation) (21, 22) or the acetylcarnitine content (e.g by carnitine supplementation) (23) in muscle tissue, or even a combined administration, may be beneficial nutritional interventions. Interestingly, a short-term creatine supplementation (5 g/day, for 11 days) increased the intramyocellular PCr pool at rest and during exercise and enhanced ATP re-synthesis in young healthy individuals (21). Furthermore, we previously showed that long-term carnitine supplementation (2 g/day, for 36 days) increased the intramyocellular acetylcarnitine levels at rest and the acetylcarnitine formation capacity upon exercise in pre-diabetic volunteers (23). Nevertheless, it is

unknown whether this affects PCr kinetics at the onset of exercise and eventually, would improve exercise performance.

The use of a non-invasive approach to explore skeletal muscle metabolism during exercise in two different cohorts of human volunteers is the main strength of the present study. The high time resolution of dynamic ^{31}P-MRS allows us to investigate the response of PCr at the onset of exercise in muscle tissue in detail, thereby to assess the phenomenon of skeletal muscle mitochondrial inertia in metabolically compromised and elderly individuals. In addition to explore the potential underlying mechanisms of PCr kinetics at the onset of exercise, hence skeletal muscle mitochondrial inertia, we also investigated the functional relevance of this phenomenon by measuring PCr kinetics at the onset of exercise along with classical read-outs for functional markers of physical function (6-minute walk test and a chair sit-to-stand-test) and exercise efficiency. A limitation of the present study is its cross-sectional nature, therefore, no conclusions can be drawn on whether PCr kinetics at the onset of exercise improves upon exercise training or any other lifestyle intervention. Moreover, the design of the study makes it hard to disentangle putative age and sex effects: typically, patients with type 2 diabetes are of older age and higher BMI than trained individuals. Thus, the effects observed in cohort 1 may originate from differences in age, BMI and metabolic status. The effects observed in cohort 2, suggest that mitochondrial inertia may relate to age and training status without a sec effect: the correlation between PCr on kinetics and markers of physical function remain upon adjustment for sex.

In conclusion, we show here that PCr kinetics at the onset of exercise are significantly slower in older, metabolically compromised individuals as compared to young, endurance-trained volunteers as well as in older individuals with normal physical activity as compared to young, healthy and older exercise-trained counterparts, regardless of in

vivo skeletal muscle ATP synthetic capacity. Moreover, we report that PCr kinetics at the onset of exercise are a -yet unexplored- signature of skeletal muscle mitochondrial metabolism tightly linked to physical function, which coexist with reduced CrAT activity, low acetylcarnitine levels and elevated ADP concentration in muscle tissue during exercise. These results indicate that PCr kinetics at the onset of exercise, hence skeletal muscle mitochondrial inertia, might emerge as a target for intervention to blunt exercise intolerance.

Methods

Participants

Here, we used data from 2 previous studies which were registered at clinicaltrials.gov with identifiers NCT01298375 (study cohort 1) and NCT03666013 (study cohort 2).

Study cohort 1

As reported previously (9), thirty-eight male volunteers, including 9 older patients with type 2 diabetes (T2DM), 8 older individuals with obesity (OB), 9 young, lean, untrained volunteers (UT, VO2max < 45 ml/kg/min) and 12 young, lean, endurance-trained individuals (T, VO2max > 55 ml/kg/min) participated as cohort 1 in the present study. Patients with T2DM and individuals with obesity as well as endurance-trained and lean sedentary volunteers were matched for age and BMI.

Study cohort 2

In the current study, we used data from a previous study from individuals in which PCr on-kinetics could be reliably determined; In total, forty-two participants including 10 young, with normal physical activity (Y, 20-30 years), 15 older, with normal physical activity (O, 65-80 years) and 17 older, trained (OT, 65-80 years) participants were included in the present study cohort 2.

Individuals were considered normally physically active if they completed no more than one structured exercise session per week, while individuals were considered as trained if they engaged in at least 3 structured exercise sessions of at least 1 hour per week for an uninterrupted period of at least the past year.

Participants were excluded from the study if they reported MRI contra-indications and if they had a medical history of cardiovascular disease.

Body composition and maximal aerobic capacity

Body composition in cohort 1 was assessed by dual X-ray absorptiometry (DEXA scan, Hologic Discovery A, Waltham, MA, U.S.A). For study cohort 2, body composition was assessed by air displacement plethysmography (BodPod®, COSMED, Inc., Rome, Italy) (24). Maximal aerobic capacity (VO2max) was determined in both study cohorts by a graded maximal cycling test until exhaustion via indirect calorimetry (Omnical, Maastricht, The Netherlands), as described previously (25).

In vivo skeletal muscle PCr on-kinetics and PCr recovery

All MR measurements were performed on a 3T clinical MRI scanner (Achieva 3T-X Phillips Healthcare, Best, The Netherlands) with a 6 cm surface coil. ^{31}P-MRS was employed to examine in vivo skeletal muscle metabolism in the m. vastus lateralis during and post-exercise as reported earlier with a time resolution of 4 seconds (9). To circumvent the influence of exercise intensity on PCr utilization and kinetics, we standardized an exercise protocol to individuals' maximal capacities aiming to reach a similar PCr depletion rate in all participants. A knee-extension protocol was performed on a custom-built MRI compatible ergometer with a pulley system for 5 min at 50-60% of the individuals' pre-determined maximal knee-extension capacity, aiming at PCr depletion of 30-50% in all subjects. The ^{31}P-resonances were quantified in MATLAB by peak fitting. The decrease of PCr over time from the onset of exercise to a lower steady-state was fitted with a mono-exponential function using a custom-written MATLAB script. The half-time [s] of the fit was used as a parameter of PCr on-kinetics assumed to be a marker of skeletal muscle mitochondrial inertia. Here, a longer half-time indicates a

longer reliance on PCr as a source of ATP and therefore a more pronounced skeletal mitochondrial inertia (Supplemental Figure 1). The time course of the PCr recovery rate post exercise (off-kinetics) was also fitted with a mono-exponential function and the halftime was used as a marker of oxidative capacity as reported earlier (9). In vivo mitochondrial function is therefore expressed as the PCr recovery half-time [s] post exercise, with a shorter half-time indicating a faster recovery and thus a better mitochondrial function.

In vivo acetylcarnitine content in skeletal muscle

Skeletal muscle acetylcarnitine content was quantified in volunteers from study cohort 1 by ^{1}H-MRS at rest before the ^{31}P-MRS protocol, as described earlier (9).

In vivo ADP levels in skeletal muscle during exercise

The spectra from volunteers of study cohort 1 were additionally fitted in jMRUI by a time domain-fitting routine using the AMARES algorithm (26) in order to calculate ADP levels assuming creatine kinase (CK) to be at equilibrium with constant (K_{CK} = 1.66 * 10^{9} M^{-1}) and upon the assumptions that PCr represents 85% of the total creatine concentration at rest and [ATP] equal to 8.2 mM (27). Resting ADP levels were calculated from the average peak areas (amplitude) of the first 5 dynamic scans (TR = 4 sec) recorded at rest. In addition, ADP levels at the onset of exercise were computed from the average peak areas (amplitude) of the last 3 dynamic spectra (TR = 4 sec) before the PCr levels reached a new and lower steady state (Supplemental Figure 1).

Physical function and exercise efficiency parameters

Functional outcomes were determined in volunteers from cohort 2, who performed the chair-stand exercise test as previously reported (28) and the 6 minutes walking test (6 MWT) using the treadmill of the CAREN-system (Computer Assisted Rehabilitation Environment Extended, CAREN; Motekforce Link, Amsterdam, the Netherlands).

In addition, volunteers from cohort 2 performed a single, submaximal cycling bout of 60 minutes on a cycle ergometer at 50% of their individual maximal power output with oxygen consumption (O2) and carbon dioxide (CO2) production being measured for 15 minutes at minute 15 and 45. Resting energy expenditure (REE) and energy expenditure during steady-state exercise (EEE) was calculated using the Weir equation (29). Gross efficiency (GE) was computed as the ratio of power output (watts converted in kJ/min) to exercise energy expenditure during the submaximal cycling test and expressed as percentage as follows:

$$GE\ (\%) = (Work_{(kJ/min)} / EEE_{(kJ/min)} * 100$$

Net efficiency (NE) was computed as the ratio of power output (watts converted to kJ/min) to exercise energy expenditure (EEE) minus resting energy expenditure (REE) and expressed as percentage as previously described (30):

$$NE\ (\%) = (Work_{(kJ/min)} / (EEE_{(kJ/min)} - REE_{(kJ/min)}) * 100$$

Whole-body insulin sensitivity and muscle biopsy

For the characterization of the metabolically compromised individuals included in the present study, we here report the outcomes of whole-body insulin sensitivity from individuals of study cohort 1 who underwent a 2 step, hyperinsulinemic-euglycemic

clamp (10 and 40 mU/kg/min) as originally described (31). Before starting the glucose and insulin infusion, a muscle biopsy was taken from the m. vastus lateralis under local anesthesia (2% lidocaine), directly frozen in melting isopentane and stored at -80°C until further analysis. CrAT protein activity was assessed using 0.01 mg of soluble protein lysate in 50 mM Tris, pH 7.4, 1 mM EDTA, 0.1 M DTNB, 1.0 mM Acetyl-CoA, and 5 mM l-carnitine at 25° C (9).

Statistics

Participant characteristics are expressed as mean ± SD, while all other results are expressed as mean ± SEM. Statistical analysis was performed using SPSS, version 21.0 (IBM Corp. Armonk, NY, USA). Shapiro-Wilk normality test was carried out to evaluate normal distribution. A one-way ANOVA with Bonferroni *post hoc* correction was used to test for statistical differences across groups for participants' characteristics and outcome parameters in both study cohorts. Further comparisons of the differences in our primary outcome (PCr on-kinetic) across groups in both study cohorts were conducted using 1-way ANCOVA, implementing the PCr recovery rate post-exercise as covariate. Sex distribution across the groups of study cohort 2 was determined by $\chi 2$ test. To test for significant linear association between variables by using individual data, we conducted bivariate Pearson's correlation and partial correlation analyses corrected for PCr recovery rate post-exercise, age and sex. In all tests, a P value < 0.05 was set to be statistically significant.

Study approval

This study was approved by the medical Ethical Committee of the Maastricht University Medical Center. All individuals gave written informed consent before enrollment and the study was conducted in accordance with the Declaration of Helsinki.

Author Contributions

R.M, J.H and P.S, V.S and M.H, conceived and designed the study. R.M, L.L, LG, T.K, D.M, J.H, P.S, V.S and M.H analyzed and interpreted the data. R.M, V.S and M.H wrote the manuscript. R.M and M.H revised and approved the final version of the manuscript. All authors reviewed and approved the final version of the manuscript. M.H is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure 1. Pronounced skeletal muscle mitochondrial inertia in metabolically compromised individuals. (A) Halftime of PCr on-kinetics; (B) Linear association between PCr on-kinetics and PCr recovery post exercise; (C) Linear association between PCr on-kinetics and CrAT protein activity; (D) Linear association between PCr on-kinetics and in vivo skeletal muscle acetylcarnitine content. [s]; seconds; T2DM: Patients with type 2 diabetes; OB: Individuals with obesity; UT: Lean Untrained; T: Trained. Circles in blue: T2DM (n=8); circles in red: OB (n=8); circles in green: UT (n=11); circles in grey: T (n=12). Data are shown as individual points and mean ± SEM. * $p < 0.05$ vs. Untrained individuals, † $p < 0.05$ vs. Trained individuals. PCr on-kinetics could not be measured in one T2DM patient due to unreliable steady state and therefore excluded for the analysis. Statistical test were ANOVA with Bonferroni *post hoc* correction and Pearson's correlation.

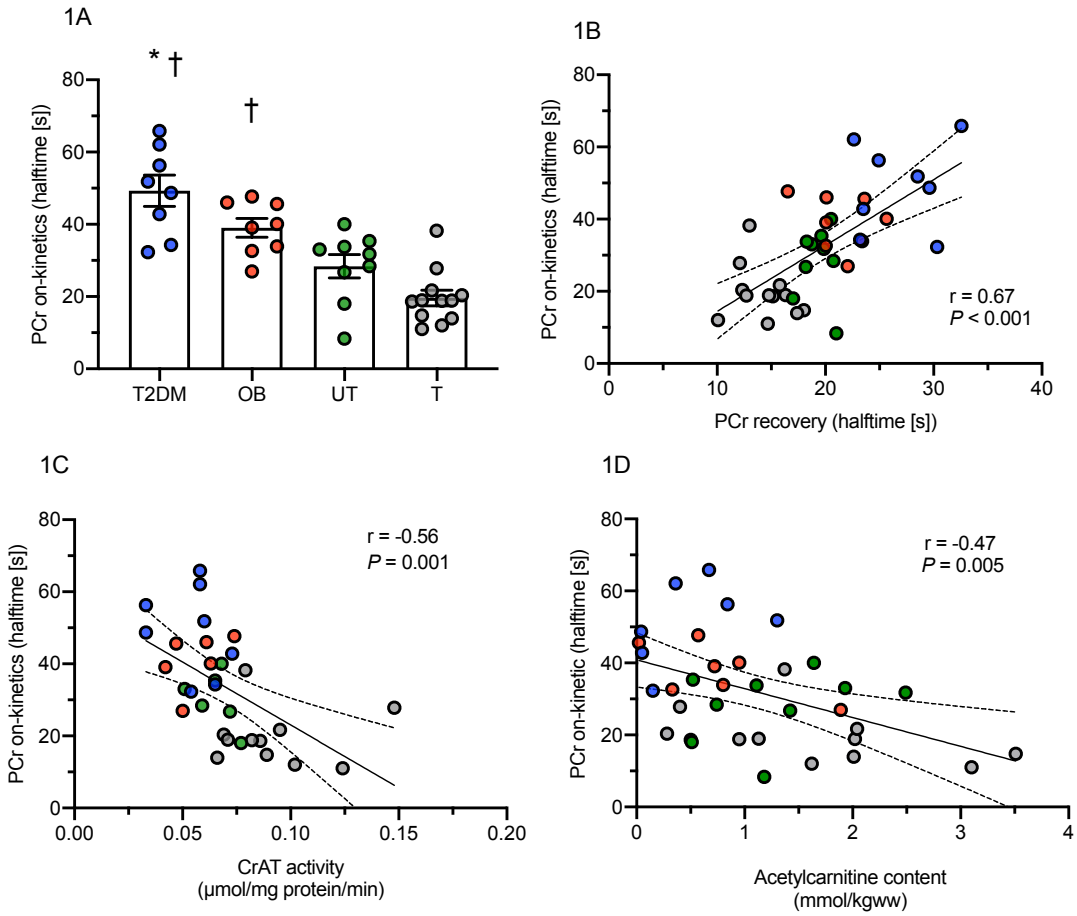

Figure 2. Skeletal muscle mitochondrial inertia coexists with elevated ADP accumulation in muscle tissue during exercise. (A) ADP levels in muscle tissue increases upon exercise onset; (B) ADP levels when PCr utilization reaches a new steady state at the onset of exercise; (C) Linear association between PCr on-kinetics and ADP levels when PCr reached a new steady state during exercise; [s]: seconds; T2DM: patients with type 2 diabetes; OB: individuals with obesity; UT: Lean Untrained; T: Trained. Circles in blue: T2DM (n=8); circles in red: OB (n=7); circles in green: UT (n=8); circles in grey: T (n=12). Data are shown as individual points and mean ± SEM. † $p < 0.05$ vs. Trained volunteers. Statistical test were ANOVA with Bonferroni *post hoc* correction and Pearson's correlation.

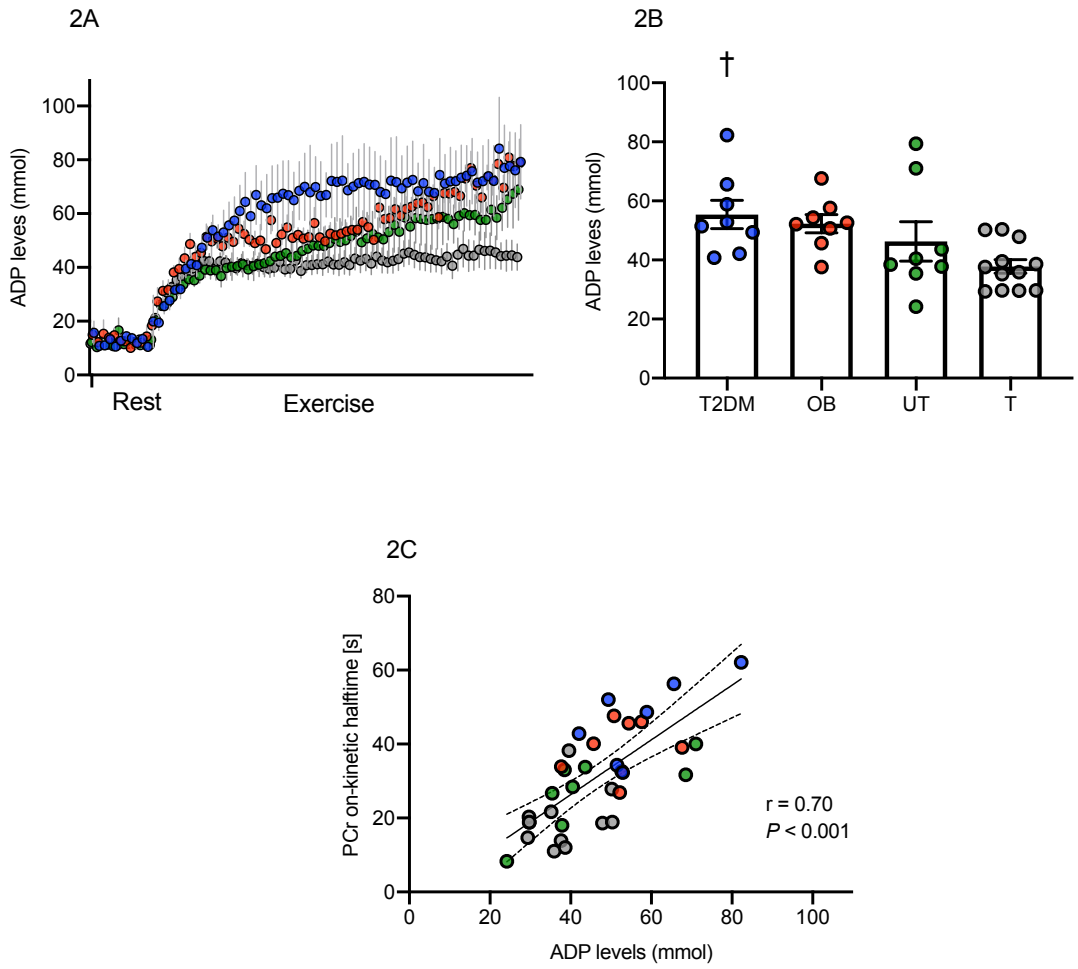

Figure 3. Skeletal muscle mitochondrial inertia and reduced functional outcomes in elderly sedentary individuals. (A) Halftime of PCr on-kinetics; (B) Halftime of PCr recovery post-exercise; (C) Linear association between PCr on-kinetics and PCr recovery post-exercise; (D) Walking speed upon performing the 6-minutes walking test (6MWT); (E) Seating and standing upon performing the chair-stand test; (F) Gross exercise efficiency and (G) Net exercise efficiency upon performing a submaximal cycling test. [s]: seconds; Y: young; OT: Older trained; O: Older with normal physical activity. Circles in black: Y (n=10); circles in red: OT (n=17); circles in blue: O (n=15). Data are shown as individual points and mean ± SD. ‡ $p < 0.05$ vs. Y individuals, * $P < 0.05$ vs. OT individuals. One participant from Y and 2 participants from OT did not perform the 6MWT due to scheduling issues. Two other participants from OT did not perform the chair-stand test due to scheduling issues. Statistical test were ANOVA with Bonferroni *post hoc* correction and Pearson's correlation.

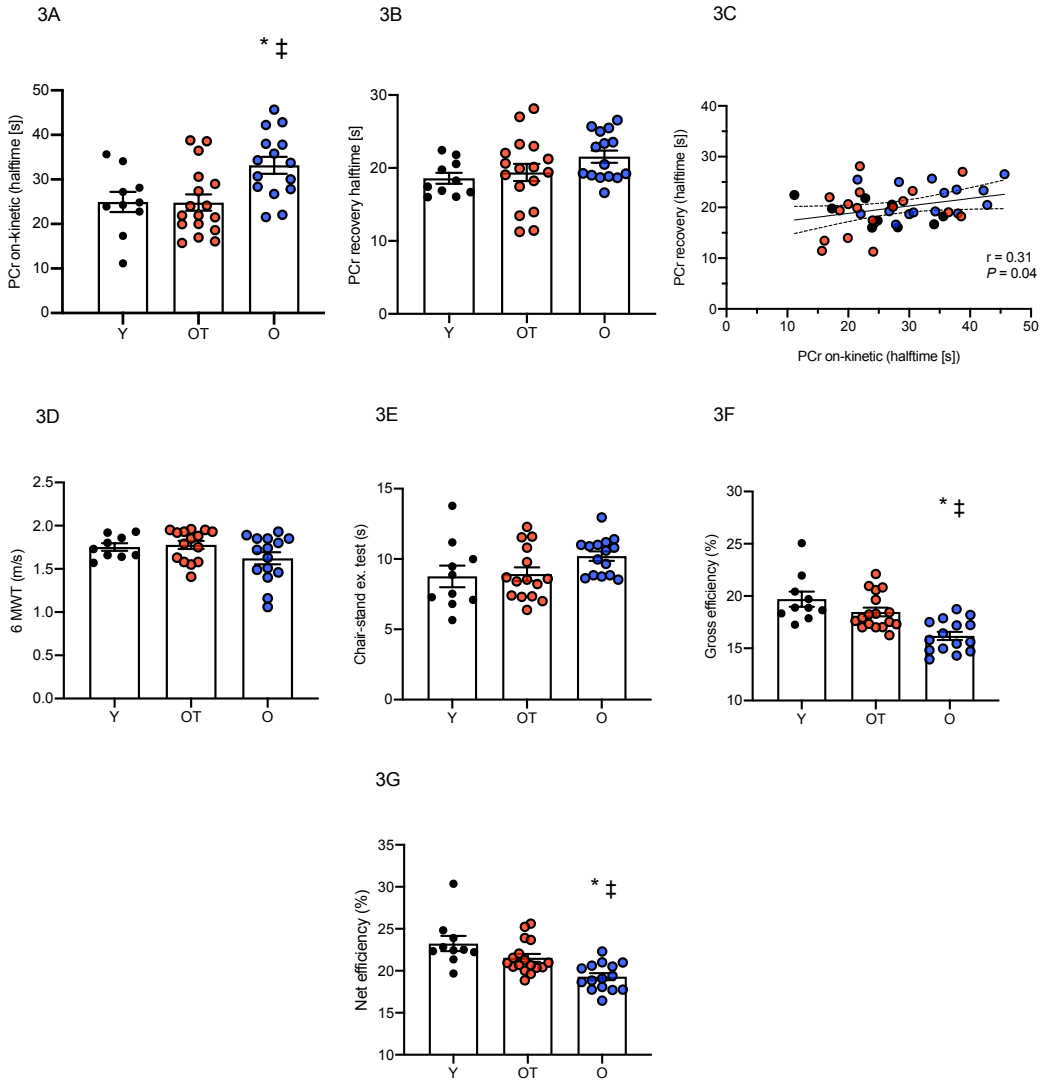

Figure 4. Skeletal muscle mitochondrial inertia is associated with physical function and exercise efficiency in humans. Linear association between PCr on-kinetics and (A) walking speed, (B) chair seating-standing exercise performance, (C) gross and (D) net exercise efficiency. [s]: seconds; Y: young; OT: Older trained; O: Older with normal physical activity. Circles in black: Y (n=10); circles in red: OT (n=17); circles in blue: O (n=15). Statistical test was Pearson's correlation.

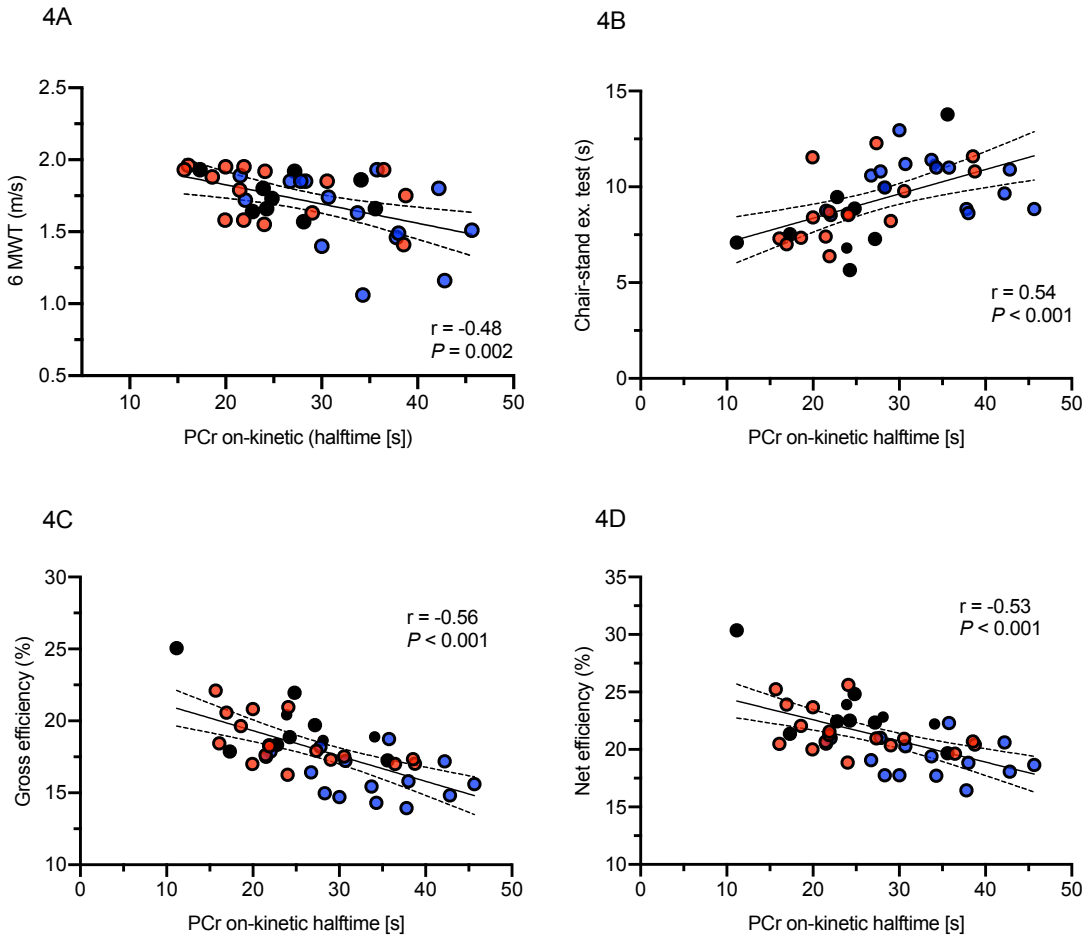

Table 1. Participant characteristics: Data are presented as mean ± SD. T2DM: patients with type 2 diabetes, OB: individuals with obesity, UT: Lean untrained individuals, T: Lean trained individuals. BMI, body mass index; FPG, fasting plasma glucose level; VO2max, maximal oxygen consumption; M value, mean glucose infusion rate during euglycemic hyperinsulinemic clamp reflects whole-body insulin sensitivity * $p < 0.05$ vs UT. † $p < 0.05$ vs T. ‡ $p < 0.05$ vs OB.

	T2DM volunteers (T2DM)	**Obese Individuals (OB)**	**Lean Untrained (UT)**	**Endurance Trained (T)**
N	9	8	9	12
Age (yrs)	64 ± 7 *†	59 ± 7*†	22 ± 4	25 ± 4
Body weight (Kg)	93.3 ± 8.9*†	97.2 ± 11.4*†	71.7 ± 6.7	71.6 ± 7.0
BMI (kg/m^2)	30.5 ± 1.4*†	31.2 ± 1.6*†	21.9 ± 2.1	21.2 ± 1.6
Fat mass (%)	27.9 ± 6.1*†	36.4 ± 6.8*†	17.8 ± 4.7	13.0 ± 2.1
FPG (mmol/L)	8.1 ± 2.0‡*†	5.4 ± 0.3	5.1 ± 0.2	5.1 ± 0.3
M value (µmol/kg/min)	23.6 ± 8.1*†	32.4 ± 14.0*†	62.0 ± 16.2	76.8 ± 16.7
VO2max (ml/kg/min)	24.8 ± 4.7*†	27.7 ± 4.5*†	41.0 ± 1.2†	59.6 ± 3.8

Table 2. Participant characteristics: Data are presented as mean ± SD. * $p < 0.05$ vs. OT. † $p < 0.05$ vs. O. Sex distribution across groups was determined by χ^2 test ($p = 0.941$). BMI, body mass index; FPG, fasting plasma glucose level; VO2max, maximal oxygen consumption; REE, resting energy expenditure; PA, physical activity.

	Young individuals (Y)	**Older trained (OT)**	**Older normal PA (O)**
N	10	17	15
Males/Females	5/5	9/8	8/7
Age (yrs)	23 ± 1*†	69 ± 2	70 ± 2
Body weight (kg)	70.0 ± 11.5	67.9 ± 8.9	72.8 ± 12
BMI (kg/m^2)	23.2 ± 3.3	23.5 ± 1.9	25.6 ± 3.4
Fat Mass (%)	26.0 ± 9.1	26.5 ± 7.8	33.4 ± 9.7
FPG (mmol/L)	5.08 ± 0.1*	5.4 ± 0.5	5.3 ± 0.4
VO2max (ml/kg/min)	40.7 ± 8.4†	36.0 ± 7.2†	26.8 ± 6.3
Wmax (Watts)	210 ± 53†	194.0 ± 61†	144.1 ± 47
REE (Kj/min)	4.7 ± 0.6	4.3 ± 0.7	4.3 ± 0.7