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Novel aspects of exercise training to promote human metabolic health

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Novel aspects of exercise training to promote human metabolic health

DISSERTATION

To obtain the degree of Doctor at Maastricht University,
on the authority of the Rector Magnificus,
Prof. dr. Rianne M. Letschert
in accordance with the decision of the Board of Deans,
to be defended in public
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Table of content

CHAPTER 1	General introduction and outline	5
CHAPTER 2	Diurnal regulation of peripheral glucose metabolism: Potential effects of exercise timing	15
CHAPTER 3	Exercise training elicits superior metabolic effects when performed in the afternoon compared to morning in metabolically compromised humans	39
CHAPTER 4	Skeletal muscle mitochondrial inertia is associated with carnitine acetyltransferase protein activity and physical function in humans	59
CHAPTER 5	Invasive and non-invasive markers of skeletal muscle mitochondrial function in young healthy males	85
CHAPTER 6	High intensity interval training improves whole-body insulin sensitivity, skeletal muscle oxidative capacity and modifies intrahepatic lipid composition in overweight/obese individuals	109
CHAPTER 7	General discussion	139
CHAPTER 8	Summary	149
CHAPTER 9	Impact paragraph	155
CHAPTER 10	Acknowledgments	161
CHAPTER 11	About the author	167
CHAPTER 12	List of publications	171

CHAPTER 1

General introduction and outline

The obesity pandemic: its causes and consequences

The worldwide prevalence of obesity has increased exponentially in the last decades, reaching the alarming prevalence of 38% of adults globally in 2009-2010 and 42.4% in 2019 (1). Obesity is well recognized as the foremost contributing factor to the development of cardiometabolic complications, including type 2 diabetes mellitus (T2DM) and nonalcoholic fatty liver disease (NAFLD) (2), hence causing a major threat on healthcare expenses.

At the most fundamental level, obesity is the consequence of a positive energy balance stemming from a chronic energy consumption that exceeds the energy expenditure, resulting in the excessive storage of energy as fat into white adipose tissue (3). In consequence, white adipose tissue expands, causing a spill-over of free fatty acids into the systemic blood stream that triggers the uptake and storage of fatty acids in non-adipose tissues (e.g., cardiac, liver and skeletal muscle), the so-called ectopic fat accumulation (4). Ectopic fat accumulation is strongly associated with whole-body insulin resistance, a condition that is part of the T2DM pathogenesis (5).

Obesity-related insulin resistance: A metabolic concern at multiorgan scale

Insulin resistance refers to the impaired response by peripheral organs to the hormone insulin, causing a compensatory rise of insulin release by the pancreatic β-cells. This elevated secretion of insulin results in hyperinsulinemia and aims to preserve normal glucose homeostasis. Insulin-stimulated muscle glucose uptake is crucial to maintain normoglycemia, as nearly 80% of the insulin-mediated postprandial glucose disposal takes place in muscle tissue (6). Plasma glucose concentrations are also regulated by the action of insulin in the liver, mainly by the insulin-mediated inhibition of endogenous glucose production (7). Thus, hepatic insulin resistance implies a blunted insulin-mediated suppression of endogenous glucose production which contributes to hyperglycemia in postabsorptive and postprandial conditions. Furthermore, insulin resistance in white adipose tissue entails a deteriorated insulin-mediated inhibition of lipolysis, resulting in elevated circulatory levels of free fatty acids in bloodstream (8). White-adipose tissue insulin resistance contributes to obesity-related ectopic fat accumulation and promotes the competition for substrate oxidation in the postprandial condition. Long-term insulin resistance leads to pancreatic β-cell failure, and a subsequent decline in insulin production causing chronic hyperglycemia and T2DM (9).

Therefore, one can state that to improve plasma glucose homeostasis and to prevent T2DM, strategies to boost insulin sensitivity in multiple organs collectively are needed.

The metabolic and functional relevance of mitochondria

Mitochondria are organelles that fuel cellular energy needs via aerobic metabolism. To do so, mitochondria oxidize nutrients (glucose, fatty acids and some amino acids) in successive steps to generate chemical energy as ATP. Given that healthy mitochondria readily adapt to fluctuations in energy need and substrate availability, aberrations in mitochondria may therefore severely impair normal energy and substrate metabolism. Compromised mitochondrial function, as the consequence of reduced mitochondrial number and capacity, has been reported in obese, insulin resistant and metabolically compromised individuals (10, 11). A diminished mitochondrial function may contribute to the blunted switch between fatty acids and glucose oxidation at the transitions from fasted to fed conditions, hampering postprandial glucose clearance (12). Aberrations in mitochondrial function may also aggravate the mismatch between ATP demands and ATP production at the beginning of exercise, causing exercise intolerance and premature muscle fatigue (13). Indeed, the prevalence of premature muscle fatigue is higher in obese, metabolically compromised subjects as compared to healthy individuals (14). This evidence endorses the potential of targeting mitochondrial function to improve insulin sensitivity and functional capacities in metabolically compromised individuals.

The effects of exercise on insulin sensitivity and mitochondrial function.

Pioneering work by Lawrence et al. back in 1920 (15), showed that a single bout of exercise already promotes whole-body insulin sensitivity in humans, as healthy volunteers experienced blunted hyperglycemic events while recovering from exercise when carbohydrate were ingested. Since then, it has been shown consistently that the acute insulin sensitizing effects of exercise persists up to 48 hours (16-18) and more importantly, that regular exercise training triggers adaptations in muscle tissue that amplify the insulin-stimulated glucose disposal (19). Of note, regular exercise training also improves liver and white adipose tissue insulin sensitivity (20) and reduces fat accumulation in liver (21), pancreas (22) and heart (23). These benefits of regular exercise training are typically paralleled by enhanced skeletal muscle mitochondrial function, as expressed by elevated intrinsic mitochondrial respiration (24), maximal *in vivo* mitochondrial oxidative capacity (20) and augmented skeletal muscle mitochondrial content (25). These mitochondrial adaptations upon exercise are beneficial for metabolic health. Firstly, an improved skeletal muscle mitochondrial function facilitates the lipid oxidation at fasting and upon low-moderate intensity exercise as well as the insulin-stimulated glucose oxidation in the post-prandial state (26). These benefits might ameliorate

ectopic fat accumulation in muscle, next to contribute to improve whole-body glucose homeostasis. Secondly, the enhanced skeletal muscle mitochondrial responsiveness promotes optimal performance during daily life activities, resulting in a robust defense to sudden perturbations in energy homeostasis, and consequently, augmented resistance to fatigue.

Classically, exercise volume and intensity are regarded as the main factors determining the benefits of exercise on whole-body glucose homeostasis and skeletal muscle mitochondrial function. Hence, these benefits support the physiological rational of the conventional exercise recommendations that compromise performing at least 150 minutes of moderate intensity aerobic-type combined with resistant-type exercise per week (27). However, the effect of these conventional exercise programs is not equal in magnitude for all individuals, which gives rise to the still unanswered question how to optimize the effects of exercise in glucose metabolism and mitochondrial function.

NOVEL STRATEGIES TO OPTIMIZE THE BENEFITS OF EXERCISE

Emerging evidence indicates that other factors such as the timing of exercise sessions (28), food ingestion post exercise (29), and the long-term adherence to the exercise programs (30) might also determine the effects of exercise on whole-body glucose homeostasis and skeletal muscle mitochondrial function. Thus, novel strategies that explore these factors are urgently needed. This has led to studies aiming to identify the most optimal time of the day to exercise, next to identify time-efficient training methodologies and its interaction with food ingestion post exercise, when aiming to improve insulin sensitivity and skeletal muscle mitochondrial function. In this thesis, three novel approaches were investigated which are briefly introduced below.

The molecular clock and exercise timing – a missing link

Many physiological processes in the human body, such as hormone synthesis and glucose homeostasis, exhibit day-night cycles orchestrated by the central molecular clock which is located in the suprachiasmatic nucleus of the hypothalamus (31). Interestingly, peripheral organs such as liver, adipose tissue and skeletal muscle, contain their own molecular clocks (32). While the central molecular clock is mainly entrained by light (33), the peripheral molecular clocks are sensitive to behavioral cues such as feeding and exercise (34, 35).
Of note, skeletal muscle insulin sensitivity is higher in the morning as compared to evening hours (36, 37). In addition, experimentally induced circadian misalignment causes skeletal

muscle insulin resistance in normoglycemic individuals (38), which can be mitigated by performing acute bouts of high intensity interval exercise (39). Such insights that the biological clock, glucose homeostasis and exercise metabolism are tightly intertwined gave rise to the hypothesis that the timing of exercise can be used to optimize its insulin sensitizing and metabolic benefits. In this regard, recent evidence showed that 6 sessions of high intensity interval training during 2 weeks were more efficacious to acutely improve 24h glucose levels in type 2 diabetic patients when performed in the afternoon as compared to the morning training routine (28). Although these findings indicate that exercise timing can affect its efficacy to improve glucose homeostasis, it is still unknown whether the more sustained health effects of regular exercise training on insulin sensitivity and other metabolic outcome parameters are influenced by the timing of the regular exercise training.

High intensity interval training

Most adults with poor metabolic health fail to meet the recommendations of performing at least 150 minutes of physical activity per week. A “lack of time” is the most commonly cited barrier for this (40). In this regard, there has been a flourishing appreciation for the capability of high intensity interval training (HIIT) to improve metabolic health, as this methodology comprises, at least, 50% less time commitment as compared to the conventional exercise recommendations (41). Interestingly, it has been shown that HIIT improves the 24h glycaemic profile, enhances the maximal aerobic capacity and augments the expression of mitochondrial-related genes in muscle tissue from obese, insulin resistant individuals and type 2 diabetic patients (42). However, whether such HIIT-induced benefits on plasma glucose homeostasis are secondary to an improved whole-body insulin-mediated plasma glucose disposal has not been investigated, at least not in obese, metabolically compromised subjects. Similarly, if the augmented mitochondrial-related gene expression upon HIIT (42) translates into a greater mitochondrial oxidative capacity in this population is unknown so far. For high intensity interval training, timing of the exercise session might also be of relevance as the short-term nature of this training methodology favors a more readily implementation in the morning or evening hours.

The interplay between exercise and nutrients in the modern lifestyle

The high frequency of food ingestion in a typical day of our lifestyle indicates that exercise sessions will often be performed with elevated plasma glucose levels post-exercise. In fact, some evidence showed that carbohydrate ingestion shortly after a single exercise session blunts

its insulin sensitizing effects and reduces the expression of genes related with mitochondrial function in healthy, normoglycemic adults (43, 44). Nevertheless, it is still unknown whether the systematic rise of plasma glucose levels post-exercise affects the benefits of exercise on insulin sensitivity and mitochondrial function in obese, metabolically compromised subjects.

SLUGGISH MUSCLE MITOCHONDRIA AND EXERCISE INTOLERANCE

Of note, obesity-related metabolic disorders and sedentary aging are often accompanied by exercise intolerance (14). Exercise intolerance manifests as premature muscle fatigue when exposed to sudden increments in energy demand (13). To reap the most health benefits by exercise, it is crucial therefore to understand the underlying cause of exercise intolerance, next to elucidate potential targets of intervention.

At the transition from rest to exercise, phosphocreatine (PCr) degradation serves to meet the sudden increase in ATP demand, while it will take some time for the activation of glycolysis and mitochondrial ATP synthesis is to occur (45). At low to moderate intensity, mainly mitochondrial metabolism will be involved. In fact, PCr degradation reaches a new steady state when mitochondrial ATP synthesis fully copes with the new energy demand at the onset of exercise (45). Thus, the duration for PCr to reach a new steady state reflects the capability to activate mitochondria ATP synthesis, a phenomenon known as mitochondrial inertia (46). Given the finite PCr storage in skeletal muscle, a delayed activation of mitochondrial ATP synthesis would prompt an eventual PCr depletion and coincide or induce premature muscle fatigue. Hence, one can speculate that mitochondrial inertia contributes to exercise intolerance and might emerge as a target of intervention. Mechanistically, the activation of mitochondrial ATP synthesis at the onset of exercise relies on the intramyocellular acetyl-coa availability (46). The intramyocellular acetyl-coA availability is controlled through the reversible formation and degradation of acetylcarnitine by the mitochondrial enzyme carnitine acetyltransferase (CrAT). Previous evidence showed that CrAT protein activity and acetylcarnitine content in muscle tissue were significantly lower in metabolically compromised individuals as compared to endurance-trained volunteers (47). If a reduced CrAT protein activity and low acetylcarnitine content influence skeletal muscle mitochondrial inertia has not been investigated, neither the functional consequences of skeletal muscle mitochondrial inertia.

Thesis aim and outline

This thesis aims to investigate the effects of exercise timing and high intensity interval training on whole-body insulin sensitivity and skeletal muscle mitochondrial function. This thesis also aims to explore if the effects of high intensity interval training on whole-body insulin sensitivity and skeletal muscle mitochondrial function are affected by the systematic elevation of plasma glucose levels post exercise, next to identify potential targets of intervention to improve exercise tolerance.

In **chapter 2** of this thesis, the link between the skeletal muscle molecular clock and the benefits of exercise on glucose metabolism are reviewed. In this chapter, it is discussed how the nutritional status pre-post exercise, as well as the tissue-specific energy depots might influence the effects of exercise timing on glucose metabolism, with special emphasis on insulin resistant and type 2 diabetic individuals. This hypothesis is subsequently tested in **chapter 3**, in which state-of-the-art methodologies are used to investigate the effects of exercise timing on skeletal muscle insulin sensitivity and other clinical parameters in metabolically compromised individuals. In **chapter 4**, the responsiveness of skeletal muscle ATP synthesis at the onset of exercise and its potential underlying mechanisms are investigated in metabolically compromised and elderly individuals by using an invasive and a non-invasive approach. Furthermore, the association between the responsiveness of skeletal muscle ATP synthesis at the onset of exercise and various functional read-outs of exercise intolerance are determined. In **chapter 5**, the association and the validity of in vitro and in vivo biomarkers for mitochondrial oxidative capacity in healthy individuals ranging in maximal aerobic capacity is assessed. **In chapter 6**, the effects of 12 weeks of high intensity interval training on whole-body insulin sensitivity and skeletal muscle mitochondrial function in obese/overweight individuals is investigated. Furthermore, whether the effects of HIIT are affected by the regular co-ingestion of glucose and casein-hydrolysate after each session is also tested in **chapter 6**. Finally, the findings of the present thesis are integrated and discussed in a broader perspective in **chapter 7**. Moreover, suggestions for future research in this field are proposed.

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CHAPTER 2

Diurnal regulation of peripheral glucose metabolism: Potential effects of exercise timing.

Rodrigo Mancilla, Anna Krook, Patrick Schrauwen, Matthijs K.C Hesselink

Obesity (2020) 28, S38 – S45

Abstract

Diurnal oscillations in energy metabolism are linked to the activity of biological clocks and contribute to whole-body glucose homeostasis. Post-prandially, skeletal muscle takes up approximately 80% of circulatory glucose and hence is a key organ in maintenance of glucose homeostasis. Dysregulation of core clock components in skeletal muscle disrupts whole-body glucose homeostasis. Next to light-dark cycles, also non-photic cues such as nutrient intake and physical activity are potent cues to (re)set (dys)regulated clocks. Physical exercise is one of the most potent ways to improve myocellular insulin sensitivity. Given the role of the biological clock in glucose homeostasis and the power of exercise to improve insulin sensitivity, one can hypothesize that there might be an optimal time for exercise to maximally improve insulin sensitivity and glucose homeostasis. In this review, we aim to summarize the available information related to the interaction of diurnal rhythm, glucose homeostasis and physical exercise as a non-photic cue to correct dysregulation of human glucose metabolism.

Introduction

Whole-body glucose homeostasis is under the control of multiple hormones, including insulin, glucagon, cortisol, and adrenergic hormones. Insulin resistance of liver, skeletal muscle and white adipose tissue is an early hallmark of type-2 diabetes mellitus pathogenesis. Interestingly, whole-body glycemic regulation and insulin sensitivity exhibit diurnal variations (1, 2). These diurnal variations are mainly driven by circadian changes in circulatory glucoregulatory hormones, whole-body substrate oxidation and tissue-specific insulin responsiveness (3, 4). While circadian changes in hormonal patterns have been appreciated for some time, recent work has identified the molecular machinery present in mammalian cells that is able to control a roughly 24-hour pattern of mRNA expression. Thus, the importance of chronobiology and tissue-specific biological clocks in the regulation of metabolic health in humans has become an area of increasing focus during the past decade.

Virtually all cells of the human body have an internal timekeeping system (5) composed by the master molecular clock located in the hypothalamic suprachiasmatic nucleus of the brain. This clock is primarily entrained by light. In the reminder of this paper, we will refer to the master molecular clock as the "master clock" to clearly make the distinction from the peripheral biological clock referred to as "peripheral clock", with reference to the relevant tissue/organs, such as adipose tissue, liver, and skeletal muscle, that also possess biological clocks (6). The cell-autonomous autoregulatory mechanism of the master clock relies on transcriptional and post-translational negative feedback loops. The transcriptional factors Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle ARNT-Like (BMAL)-1 (or Aryl hydrocarbon receptor nuclear translocator like [ARNTL]) constitute the positive regulation of these feedback loops, which activate their target genes cryptochrome-2 (CRY), period (PER) and nuclear receptor subfamily 1 group D member 1 (Rev-erbA-α), which in turn, are negative regulators of clock feedback loops that eventually supress transcriptional activity (7). Recent results from animal models have indicated that the peripheral clock in skeletal muscle orchestrates a temporal organization of metabolic gene expression, thereby affecting glucose homeostasis (8). Similar observations have been reported in humans; laboratory-induced circadian misalignment interventions affect the diurnal pattern of peripheral clock gene expression in skeletal muscle (9). Moreover, in night-shift workers, master and peripheral clock disruption has been reported to coincide with an elevated risk for type 2 diabetes development (10).

Increased physical activity is a clinically proven, first-line strategy to improve glucose homeostasis. Most of the benefits noted in skeletal muscle that are associated with increased physical activity emerge from an upregulation of multiple components in the myocellular insulin signaling cascade after exercise training that jointly promote postprandial glucose clearance (11). In this regard, animal studies have revealed that the peripheral muscle clock is involved in the regulation of insulin-stimulated glucose disposal and exercise-induced changes in whole-body glucose homeostasis (12). Exercise is an external nonphotic (not related to light) modulator of the expression and rhythmicity of the peripheral muscle clock (13). This has led to the suggestion that peripheral clock dysfunction and its related metabolic effects may be corrected by resetting peripheral clocks in metabolically compromised participants. Considering the interplay between the peripheral clock in skeletal muscle and exercise as a nonphotic cue tool to readjust the clock, an emerging area of research aims to maximize the beneficial effects of exercise for glucose homeostasis by optimizing timing of the performed exercise (14).

In this review, we summarize current information related to the interaction of diurnal rhythm, glucose homeostasis, and physical exercise as a nonphotic cue to correct dysregulation of glucose metabolism.

Diurnal rhythmicity in substrate metabolism

Postprandial glucose disposal possesses a circadian pattern in healthy normoglycemic individuals as well as in subjects with impaired glucose tolerance. Plasma glucose clearance rates are higher in the morning compared with the evening in response to similar glucose loads (3, 15). Elegant studies using tissue-specific catheterization and continuous glucose infusion techniques have revealed that glycaemic dysregulation during evening time originates from a progressive impairment of skeletal muscle insulin responsiveness (4). Moreover, pancreatic insulin secretion exhibits rhythmicity in humans, with a lower rate of insulin secretion at evening hours (16). Thus, temporal fluctuation of skeletal muscle insulin sensitivity and a time-dependent insulin secretion rate both contribute to a diurnal pattern in whole-body glucose metabolism in humans. Additionally, diurnal fluctuations in key metabolic hormones like cortisol and melatonin (17), as well as diurnal elevations of non-esterified free fatty acids (NEFA) concentration in blood plasma (18), will affect whole-body glucose homeostasis. White adipose tissue also possesses a peripheral clock, contributing to oscillations in lipase activity (19, 20), triacylglycerol turnover (21) and

plasma NEFA levels. Tracer studies using continuous intravenous infusion of [$^{2}H_{2}$]-Palmitate reveal that postprandial storage of fatty acids in adipose tissue (NEFA buffering capacity) is higher during evening versus morning hours in lean participants (22). Interestingly, the postprandial NEFA buffering capacity of white adipose tissue is compromised in participants with obesity who do exhibit different diurnal variations upon consecutive meals throughout the day, variation patterns of which are similar to those noted in lean participants (22). Lower buffering capacity of adipose tissue in the evening contributes to a timing-dependent NEFA spillover to other peripheral tissues. This, combined with timing-dependent disinhibition of adipose tissue lipolysis (23), promotes intracellular substrate competition as well as lipid storage in ectopic tissues such as muscle. Augmented myocellular lipid storage often is associated with compromised insulin signalling. Thus, compromised adipose tissue NEFA buffering capacity and disinhibition of adipose tissue lipolysis may contribute to rhythmicity in insulin sensitivity.

Insulin-stimulated intramyocellular glucose disposal comprises oxidative and non-oxidative glucose disposal. While nonoxidative glucose disposal predominantly reflects glucose storage as muscle glycogen or conversion to lactate (24), oxidative glucose disposal is partly determined by mitochondrial oxidative capacity. We have shown rhythmicity in skeletal muscle mitochondrial oxidative capacity in humans, with peak oxidative capacity in the evening (23:00 hours). The peak in oxidative capacity coincided with a peak resting energy expenditure predominantly relying on carbohydrate oxidation (25). Whether this temporal variation of human skeletal muscle oxidative capacity and preferential glucose oxidation at evening times represents an intrinsic circadian-related oscillation or is merely a reflection of behavioural cues such as meal times or physical activity needs to be determined. Furthermore, if peak skeletal muscle mitochondrial function and predominant glucose oxidation in the evening are triggered to counteract impaired evening glycemic regulation to maintain glucose homeostasis warrants further investigation.

Non-invasive measurement of muscle and hepatic glycogen content by ^{13}C magnetic resonance spectroscopy indicated that glycogen content in liver and muscle was higher in the evening versus morning in healthy individuals (26). Although feeding-induced hyperglycemia *per se* upregulates both intracellular glucose disposal pathways (oxidation and storage), animal studies have highlighted a crucial role of tissue-specific of muscle- (12) or liver-specific peripheral clocks (27) in the circadian regulation of glucose metabolism. Muscle-specific ablation of BMAL-1 not only

disrupted glucose metabolism in muscle but also profoundly compromised whole-body glucose homeostasis (12). Liver-specific ablation of CLOCK dampened circadian variation in hepatic glycogen storage (27). Jointly, these observations coincide with fasting hyperglycemia and impaired glucose tolerance (12, 27) and stress the importance of peripheral clocks in the maintenance of glucose homeostasis.

Thus, the clock could play a role in orchestrating the anticipation of multiple insulin-responsive peripheral tissues to timely promote glucose disposal and avoid undue peaks in blood glucose. Eating behavior throughout wakefulness could contribute to maintain diurnal responses of peripheral clocks in different insulin target organs, which may serve to fine tune circadian regulation of plasma glucose clearance over the day. Nevertheless, more tightly controlled studies exploring the prime involvement of peripheral clocks and relevant downstream targets in maintaining glucose homeostasis in humans are required to elucidate whether the clock can be endorsed as a therapeutic target to improve glucose homeostasis.

The peripheral clock in skeletal muscle

Expression of clock genes in human skeletal muscle exhibits a profound day-night rhythmicity with alternating peak-expression of the positive (BMAL1 and CLOCK) and negative (PER and CRY) clock components (25). In addition, multiple clock-controlled genes related with substrate oxidation (pyruvate dehydrogenase complex), tricarboxylic acid cycle/respiratory electron transport chain (NADH dehydrogenase complexes) and ATP synthase (ATP5F1, ATP5G3, ATP5A1 and ATP5L) possess periodic expression, concomitant with an oscillatory mitochondrial respiratory capacity and mitochondrial dynamic in healthy individuals over a 24h period (25). Whether the temporal changes in expression of the peripheral muscle clock and its target genes in human skeletal muscle reflect an intrinsic circadian rhythmicity or originate from the interaction of the master clock with external cues such as meal times and daily physical activity is hard to disentangle. Furthermore, the physiological relevance of a peripheral clock in human skeletal muscle requires sequential muscle biopsies and hence is not straightforward to accomplish. Nevertheless, current findings indicate the peripheral muscle clock might sense and anticipate diurnal feeding-fasting and activity cycles, likely orchestrating diurnal changes gene expression and substrate selection in a circadian fashion (28). In addition, clock-governed events such as peak mitochondrial oxidative capacity match with a peak in carbohydrate oxidation (25) and a peak in

human physical performance (29) (Figure 1). This discloses new avenues of research pointing towards the timing of interventions to prime skeletal muscle metabolic capacity and optimize buffering responses to subsequent daily challenges such as feeding-fasting and physical activity-rest transitions.

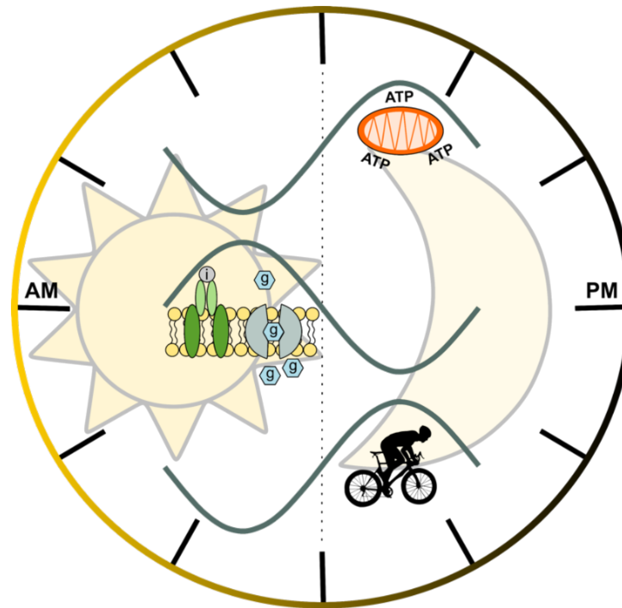

Figure 1: The master clock drives circadian variations in human metabolism.
The intracellular timekeeping system composed by the clock orchestrate temporal organization of organismal homeostasis. In humans, skeletal muscle mitochondrial oxidation capacity, whole-body insulin sensitivity, and maximal aerobic performance (upper, middle, and lower rhythms, respectively) all exhibit diurnal variations. Mitochondrial oxidative capacity is highest at evening time (23:00 hours) and coincides with peak resting energy expenditure predominantly fueled by glucose oxidation (25). Whole-body insulin sensitivity possesses diurnal fluctuations related to changes in glucoregulatory hormones and oscillations in glycogen content and glycolytic gene expression (52) with diurnal fluctuations in insulin sensitivity as consequence. Furthermore, maximal aerobic capacity displays diurnal variations, with a peak in maximal exercise performance at evening hours. As mitochondrial oxidative capacity affects maximal exercise performance and is paralleled by a shift in substrate selection (9), it can be speculated that distinct times throughout the day the muscle can more deadly meet its metabolic demands than during other periods.

Consistent with the results from human muscle biopsies samples, previous data has shown circadian rhythmicity of peripheral clock genes and other energy cellular sensor enzymes in primary human muscle cells after *in vitro* synchronization (30, 31). Remarkably, a dampened gene expression of the clock gene Rev-erbA-α and NAD-dependent deacetylase SIRT1 in cultured myotubes from metabolically compromised donors was reported (31). The blunted oscillation was in sharp contrast to profound fluctuations observed in the same genes in myotubes cultured from endurance trained individuals (31). Particularly, the nuclear receptor Rev-erbA-α and SIRT1 can modulate the oxidative phenotype in skeletal muscle. Importantly, both Rev-erb-α and SIRT1 are highly sensitive to feeding-fasting transitions and strongly associate with glucose homeostasis (32). Emerging evidence from animal studies has indicated that functional expression of peripheral clocks in muscle controls insulin-stimulated skeletal muscle glucose uptake, not only via transcriptional regulation of insulin signalling-related proteins (TBC1 domain family member 1 and Glucose Transporter [GLUT]4), but also via conserving a diurnal expression of glycolytic rate-limiting enzymes (8). Robust cellular metabolic networks that are under the control of the peripheral clock have recently been discovered. These interconnected cellular pathways anticipate time-dependent nutrient availability along with transient alterations in fuel oxidation upon feeding/exercise in mice skeletal muscle (33). In more detail, peripheral clock genes in muscle are involved in the regulation of the class I histone deacetylase Histone Deacetylation 3 (HDAC3) to adjust glycolysis and plasma glucose clearance along with muscle performance during exercise (33). Collectively, one can hypothesize that disruptions of the peripheral clock in skeletal muscle underlies the pathophysiology of insulin resistance and related compromised metabolic health (10, 34). In this regard, our group previously showed that short-term laboratory-induced day-night shift resulted in circadian misalignment of both the master and peripheral clocks, which was accompanied by lowered muscle insulin sensitivity in healthy volunteers (9). The compromised insulin sensitivity was mainly explained for by a deteriorated nonoxidative glucose disposal pathway, while oxidative glucose disposal remained unaltered. Clearly, more well-controlled human interventions studies are needed to examine if restoring master and peripheral clocks can be used as interventions aiming to promote insulin sensitivity and glucose homeostasis.

Exercise and the peripheral clock

The intimate interplay between the peripheral muscle clock and substrate metabolism indicates that intramyocellular clock functioning might dictate different periodic responses to exercise and modulates exercise-induced changes in gene expression. This interplay gives rises two different questions. Firstly, how much and in what context (for example diet) can exercise affect the peripheral muscle clock? Secondly, one may ask if the muscle clock dictates the effect of a given exercise bout (and thereby is a determinant of the outcome of an exercise training intervention). The latter notion is triggered by previous reports showing that muscle contractile bioenergetics and fiber-type-specific gene expression upon exercise are regulated by peripheral clocks in mice muscle cells (12, 35). In humans, exercise-induced hormonal responses are highly affected by the time of the day; this supports the notion that timing of exercise may also affect the hormonal responses and thereby energy metabolism and substrate selection postexercise (36). Although the impact of timing of exercise on exercise-induced improvements in glucose homeostasis remains to be determined, it is appealing to hypothesize that the therapeutic potential to optimize exercise timing can be used to maximize health outcomes in a clinical context. In this regard, one can argue for timing exercise when mitochondrial oxidative capacity peaks, as at that time, the muscle is primed to meet the energy requirements of contraction. Consequently, premature muscle fatigue may be diminished, and long-term adherence might be promoted. Considering that mitochondrial oxidative capacity in skeletal muscle plays a crucial role determining exercise performance, timing of exercise training might also be relevant to optimize (health) performance benefits. Alternatively, it could be hypothesised that exercising at times when mitochondrial oxidative capacity is lowest provides a relatively stronger trigger for adaptation, thus creating more room for improvement than applying the same type of exercise when mitochondrial capacity peaks.

Improvements in glucose metabolism in multiple peripheral tissues upon exercise have partly been attributed to endocrine properties of the skeletal muscle via release of contraction-induced myokines into systemic circulation (37). In fact, the synthesis of myokines interleukin 6, Monocyte chemoattractant protein-1, and interleukin 8 with a putative glucoregulatory role are under control of the peripheral muscle clock, as was shown in cultured human myotubes (38). This indicates that humoral effects of exercise via skeletal muscle might be affected by the status of the peripheral

muscle core and indeed display oscillatory changes over a 24-hour period. If the ability to secrete myokines upon muscle contraction indeed exhibits diurnal variation warrants further investigation. By mutating the CLOCK gene in somatic cells (39), it has become clear that disrupting the master clock has metabolic consequences as well as affects exercise performance (39). In a more tissue-specific manner, it has also been shown that deletion of CLOCK and BMAL-1 in pancreatic islet cells resulted in hypoinsulinemia and aberrations in glucose homeostasis (40). Muscle-specific loss of BMAL-1 resulted in compromised glucose uptake in muscle with disrupted systemic glucose homeostasis as consequence (12). Earlier studies using global knockout models of CLOCK (41) or BMAL-1 (42) revealed that life-long ablation of CLOCK was paralleled by hyperphagia and a sustained positive energy balance (41), while the global and life-long absence of BMAL-1 affected timing of locomotor behaviour and reduced spontaneous activity (42). It could hence be argued that some of the effects of clock disruption observed may be secondary to developmental defects and/or to the lifelong absence of a functional clock. However, it is important to note that mice with inducible muscle-specific ablation of BMAL-1 showed compromised insulin-stimulated glucose uptake in muscle, partly originating from downregulation of key insulin-signaling genes and lower levels of the main glucose transporter GLUT4 (8). Interestingly, compelling evidence from animal and human studies has illustrated that exercise is a potent nonphotic cue, able to reset the peripheral clock in muscle by modulating the expression of master-clock proteins (43). Day versus night exercise was also shown to differentially phase-shift peaks in the expression of PER2 in explants of muscle and lungs but not in suprachiasmatic nucleus explants, indicating that peripheral clock gene expression is responsive to exercise and the timing of exercise and suggesting that exercise can contribute to synchronization of peripheral and master clocks (13).

In line with these animal data, one-legged acute resistance exercise was also shown to trigger a rhythmic phase shift of the peripheral clock genes in muscle along with changes in glucose metabolism and mitochondrial function (43). These data indicate interaction of exercise timing and metabolism in humans and highlight the role exercise might have in the restoration of a transiently disturbed clock (e.g., upon travelling time zones and during shift work). In line with this, master-clock and clock-controlled gene expression in cultured human primary cells reflects the metabolic condition of the donors and fundamentally preserves a robust intrinsic rhythmicity in muscle cells upon long-term training of the donors (31, 38). Furthermore, the peripheral clock

in cultured human myotubes displayed a strong association with *in vivo* insulin sensitivity (31, 38). It was also observed that the amplitude of oscillations of the clock gene Rev-Erb Alpha was blunted in patients with type 2 diabetes compared with trained lean young individuals (31). This may suggest that regular exercise training endows the intracellular milieu with a tightly regulated time-keeping system that enables dampening fluctuations in substrates availability in a 24h cycle, thereby promoting insulin sensitivity. In summary, these findings indicate it is worth exploring if the interplay between exercise and circadian rhythmicity can be used to sustain and promote glucose homeostasis. To this end, we will need to integrate the knowledge from the chronobiology field with respect to the timing of exercise with the prescription of exercise regimes meant to improve, for instance, glucose homeostasis predominantly in metabolically deprived individuals. Future studies should examine if, indeed, appropriate timing of exercise helps to maximize exercise responsiveness (Figure 2).

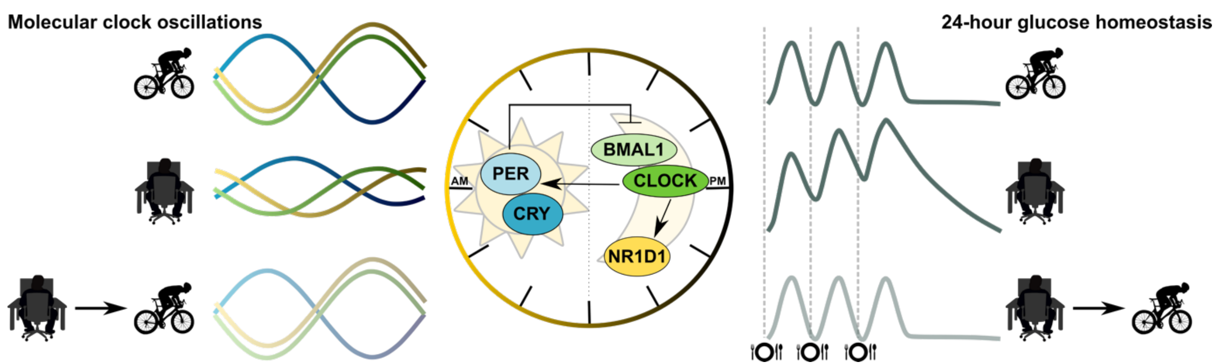

Figure 2: Exercise is a non-photic cue able to reset peripheral clocks.

Expression of clock genes in human skeletal muscle exhibits a profound day-night rhythmicity with alternating peak expression of the positive (BMAL1 and CLOCK) and negative (PER and CRY) clock components. The amplitude of these oscillations is more profound in physically fit, lean, young individuals (upper left-hand part) and is blunted in patients with type 2 diabetes (middle left-hand part). We hypothesize that exercise, as an external nonphotic (not related to light) modulator of the expression and rhythmicity of peripheral (muscle) clocks, can restore the amplitude in clock genes (lower left-hand part). At the right-hand side of the image, postprandial glucose peaks in physically fit, lean, young individuals (upper right-hand part) and in patients with type 2 diabetes (middle right-hand part), and the hypothetical exercise-mediated restoration of postprandial glucose peaks is shown schematically (lower right-hand part).

Classically, the insulin-sensitizing effects of exercise are explained via 5'-Adenosine Mono Phosphate-activated protein kinase (AMPK)-mediated GLUT4 translocation to the subsarcolemmal membrane, thereby promoting insulin-stimulated myocellular glucose uptake (44). Interestingly, phosphorylation of specific catabolic (α) and regulatory (β and γ) AMPK-containing subunits exhibit circadian variation in mammalian muscle cells (45), and mice lacking the gamma 3 subunit of AMPK do not display diurnal changes in the respiratory exchange ratio (RER) between the light and dark phase that are commonly observed in wild-type mice (46). In addition, some fundamental clock-network elements possess AMPK target activation sites (45, 47), providing a molecular mechanism for exercise-stimulated insulin sensitivity to interact with the clock genes. Bearing in mind that phosphorylation of specific AMPK subunit complexes upon exercise is highly dependent on intensity/duration and exercise type (48, 49), dosing precise exercise methodologies at certain times of the day might be crucial to maximize glycaemic outcome in metabolically compromised subjects.

Exercise timing

Timing of exercise can be related to clock time, timing relative to meals, timing relative to light exposure, and timing relative to substrate stores. Obviously, these tightly intertwined factors hamper making the distinction between the contributors of the individual components. In this section, we aim to summarize the effects of timing of exercise without the aim to disentangle the individual contributing components.

Studies in humans consistently revealed that interaction of exercise-induced AMPK phosphorylation with subsequent TBC1D4 (AS160)-GLUT4 activation upon insulin stimulation mitigates 24h hyperglycemia (50, 51). Time of day-specific responses to an acute exercise bout have been noted at both the transcriptomic and the metabolomics level in mice (52). Almost 25% of skeletal muscle transcripts responded to exercise in the early active phase. In the early rest phase, however, only around 5% of the transcripts responded to an exercise stimulus (52). In individuals with prediabetes, timing of exercise relative to meal time differentially affects blood glucose control; short term (6 x 1 minute) intense (90% maximal heart rate) 30 minutes of exercise prior to each meal was found to attenuate postmeal glucose peaks more profoundly than one 30-minute exercise session of moderate intensity (30% maximal heart rate) before dinner (53). It has, however, also been shown that low-intensity exercise after a meal lowers postprandial glucose

excursions (54), indicating that timing of the exercise relative to nutrient intake is not the sole determinant of the timing effects of exercise. In fact, nutritional status before as well as after exercise dictates substrate availability and hormonal concentration in plasma, consequently affecting the resynchronization of whole-body glucose homeostasis. For instance, performing short cycling bouts while being fasted triggers elevated synthesis of stress-related hormones in individuals with type 2 diabetes and does not result in improved glucose homeostasis directly post-exercise (55). Notably, such findings do not match the previously reported diminished postprandial glucose excursions when participants with type 2 diabetes perform similar high-intensity intermittent cycling bouts at fed condition (56).

In patients with type 2 diabetes, afternoon high-intensity interval training more effectively improved 48-hour blood glucose profile than morning training, which in fact had deleterious effects on blood glucose values (57). These findings are in line with mice data showing that glycolytic activation is specific to exercise at the early active phase rather than the early rest phase (52), suggesting that time of day affects the beneficial health effects of exercise. In a recent study (58), normoglycemic individuals with overweight and patients with type 2 diabetes followed a 12-week training program of 30 minutes of moderate-intensity walking combined with fours resistance-based exercises performed in the morning or afternoon. While the training program improved all glycemic markers, no differences were observed between morning and afternoon exercise (58). This may indicate that timing effects of exercise might be (partly) related to exercise intensity, but clearly more research is needed on this topic. Twelve weeks of multimodal exercise training improved glycemic control and postprandial glycemic responses in individuals with overweight with or without type 2 diabetes. However, no distinct glycemic benefits or alterations in circadian rhythm were associated with morning versus evening exercise when performed three times per week on this cohort.

Another crucial factor likely influencing temporal fluctuations on exercise-induced glycemic control is skeletal muscle glycogen content. It has been previously reported that skeletal muscle glycogen content *per se* stimulates glucose uptake by a negative feedback loop (high muscle glycogen content, low glucose uptake) (59, 60). Moreover, glycogen acts as a signalling molecule (61), altering, for instance, exercise-induced myokines synthesis (62-64) and mitochondrial-related gene expression (65). Subsequently, one can hypothesize that the beneficial effects of

exercise on blood glucose homeostasis are more pronounced after an overnight fast (when muscle glycogen depots may be lowered) relative to exercise in the postprandial state. On the other hand, it is important to highlight that exercise-induced skeletal muscle glycogen turnover is governed by absolute glycogen content (66, 67), which is expected to be higher in the evening. If exercise in the evening amplifies exercise-induced blood glucose homeostasis in the morning via higher rates of muscle glycogen degradation is currently unknown.

Exercise-induced catecholamine synthesis is the main humoral factor promoting white adipose tissue and visceral lipolysis (68). Catecholamines possess circadian variation with a peak during the day under resting conditions and two peaks in the morning and the evening upon exercise (69) indicating involvement of the master clock in catecholamine reactivity to exercise. Thus, interplay between the master clock and exercise triggers timing-dependent metabolic responses (Figure 3). On the one hand, it could be argued that performing exercise when the adrenergic responsiveness is greatest might maximize the exercise-induced lipolytic response and could lead to a higher free fatty acids overflow, thereby stimulating ectopic fat turnover. Consequently, peripheral fat accumulation might be reduced and aberrations in lipid-induced insulin signaling alleviated. On the other hand, however, the stimulatory effects of catecholamines on hepatic glucose output and inhibitory effects on peripheral glucose uptake may translate into elevated blood glucose levels (55, 70). Thus, it is currently unknown if alignment of exercise with the adrenergic circadian peak will be beneficial for glucose homeostasis.

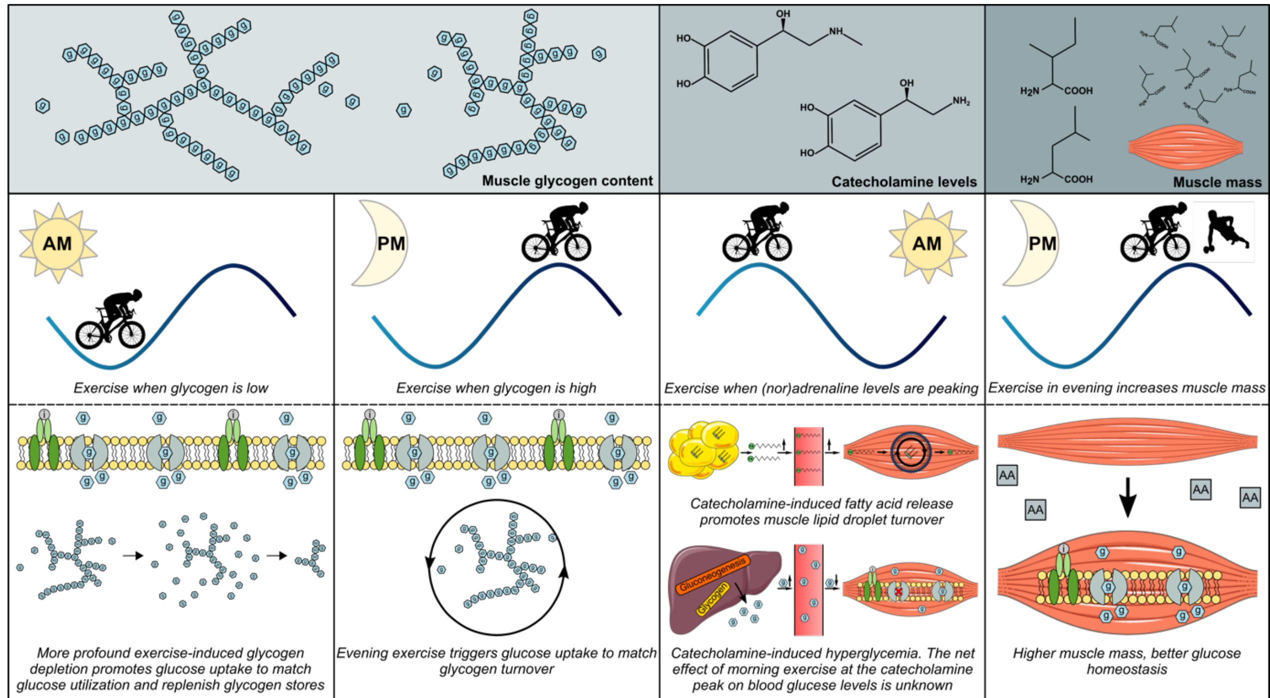

Figure 3: Exercise timing and hypothetical effects on glucose homeostasis.

A: In morning exercise, low glycogen content promotes glucose uptake to match exercise-mediated glucose utilization and to replenish glycogen stores. Evening exercise triggers glucose uptake to match glycogen turnover.

B: In morning exercise, catecholamine levels are peaking. This induces free fatty acids release from white adipose tissue and may promote uptake and utilization of plasma free fatty acids by skeletal muscle, thus preventing ectopic fat storage and putatively ameliorating insulin resistance. Also, catecholamine-induced hyperglycemia caused by increased hepatic glucose output and reduced peripheral insulin sensitivity can be alleviated by exercise. At the same time, it should be noted that exercise induces a second peak of catecholamines later in the day. The net effect on blood glucose homeostasis of exercise timed at the catecholamine peak remains to be elucidated.

C: Muscle mass is a major determinant of insulin-stimulated glucose disposal and glucose homeostasis. For optimal maintenance of muscle mass and to promote muscle mass accretion, data indicate that evening pre-meal resistance exercise promotes muscle protein synthesis by increasing diet-derived amino acid incorporation in muscle. If this effect is due to the mode of exercise, is due to the timing of the evening relative to the clock time, or is relative to the meal is currently unknown.

Preservation of muscle mass throughout lifespan is crucial for the maintenance or improvement of glucose homeostasis. In this regard, it is important to note that there is a synergistic interaction between resistance-type exercise training and protein (or protein hydrolysates) ingestion to stimulate muscle mass gain in healthy individuals (71) and/or in individuals at risk or diagnosed with type 2 diabetes (72). Resistance exercise training stimulates muscle protein synthesis via mammalian Target of Rapamycin-p70s6K signaling (73), which interacts with the peripheral clock to regulate cell growth in a circadian fashion (74). A study in male elderly patients showed that the overnight response of muscle protein synthesis to pre-sleep protein ingestion was stimulated by evening exercise (75). Interestingly, the stimulatory effect of exercise on muscle mass accretion was found to be more prominent in the evening than in the morning (76). In contrast, a recent meta-analysis concluded that the gain in muscle mass upon resistance exercise training does not differ when exercise is performed in the morning versus the evening. It should be noted, though, that the putative interaction between food ingestion and exercise timing was not taken into account in this meta-analysis (77), possibly explaining the difference with the study by Kuusmaa et al. (76), in which the participants were instructed to adhere to the national dietary guidelines. These findings suggest an interaction between exercise-induced skeletal muscle adaptations and the peripheral clock in humans.

As dietary-derived amino acids constitute the main substrate for a positive protein balance post-exercise (78, 79), it could be suggested that timed ingestion of amino acids can result in optimized amino acid supply to peripheral organs, thus stimulating muscle mass accretion and maintenance (80). Different regimes for diet and exercise timing have been explored with the aim to identify the optimal timing and mode of exercise to promote or maintain muscle mass (81-83). Resistance exercise performed in the evening followed by dietary amino acids ingested before sleep promotes *de novo* myofibrillar protein synthesis and results in a positive net protein balance during overnight sleep in young as well as in older subjects (75, 84).

Conclusion and future perspectives

Glucose homeostasis, and the regulation thereof, exhibits diurnal variation. Specifically, reduced skeletal muscle insulin responsiveness toward the evening coincides with dysregulation of plasma glucose. Alignment of master and peripheral clocks is most likely essential to maintain glucose homeostasis. Exercise training is a first line prevention/treatment strategy to circumvent glycemic

dysregulation in humans. Acute metabolic exercise-derived responses and transcriptional adaptations display periodic oscillations over the day. Thus, timing of the exercise session relative to clock time and meals is likely to affect (and possibly maximize) the consequences for glucose homeostasis. Dosing exercise at different times of the day translates into a natural interplay with the nutritional status of patients in a 24-hour cycle. Hence, we would like to stress the need to integrate the new knowledge from the chronobiology with the aim to understand how the synergistic interplay between exercise and nutritional cues can modulate human (patho)physiology. Importantly, exercise training and nutrition are two potent nonphotic cues that. can affect the master as well as peripheral clocks. Conversely, the master and peripheral clocks also affect exercise-mediated responses. Thus, for optimal alignment of the cellular microenvironment to oscillatory metabolic challenges throughout the day, more information on how the biological clock and physical exercise bidirectionally interact is needed.

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CHAPTER 3

Exercise training elicits superior metabolic effects when performed in the afternoon compared to morning in metabolically compromised humans.

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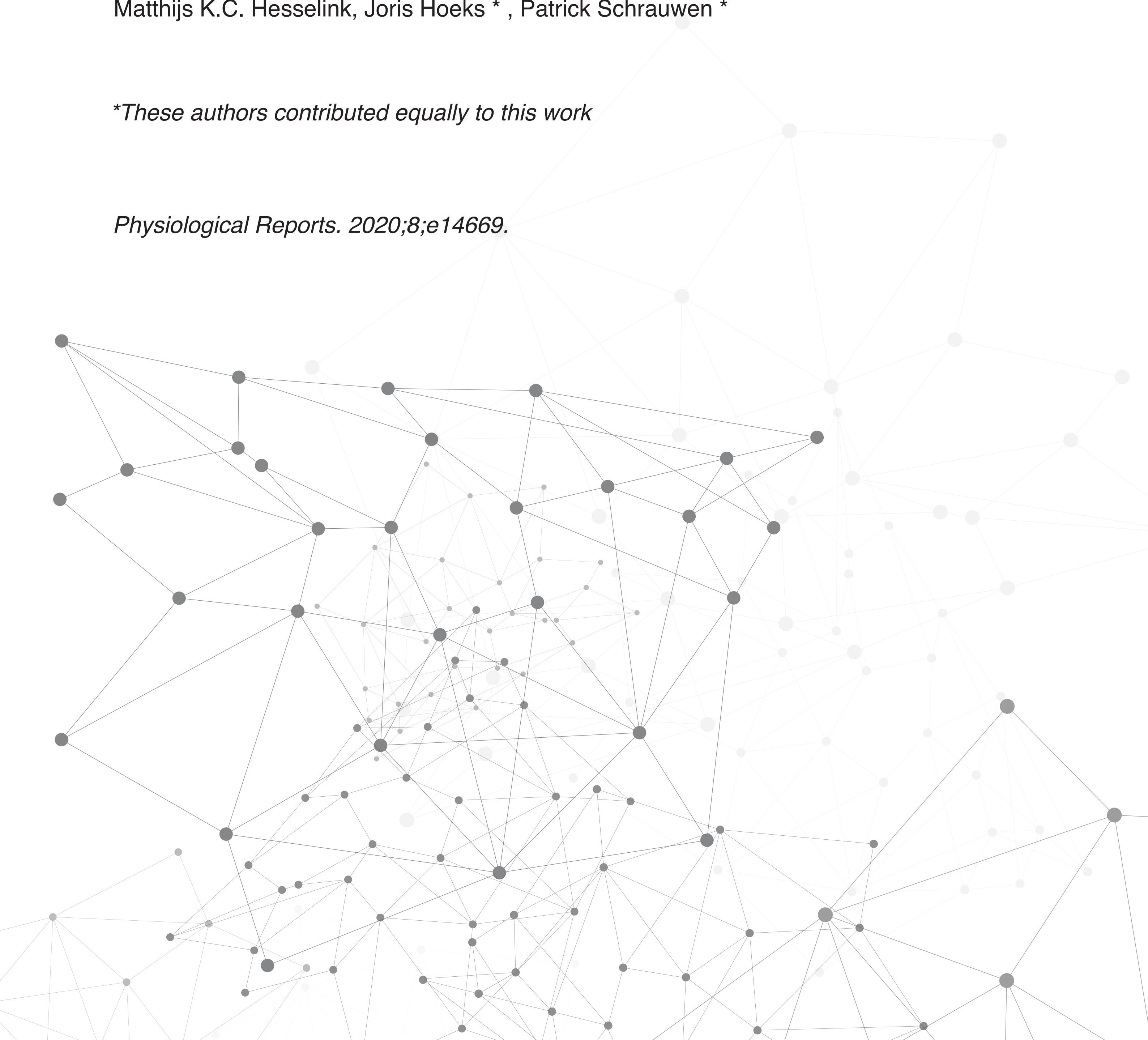

Abstract

The circadian clock and metabolism are tightly intertwined. Hence, specific timing of interventions that target metabolic changes may affect their efficacy. Here we retrospectively compared the metabolic health effects of morning versus afternoon exercise training in metabolically compromised subjects enrolled in a 12-week exercise training program. Thirty-two adult males (58 ± 7 yrs) at risk for or diagnosed with type 2 diabetes performed 12 weeks of supervised exercise training either in the morning (8.00-10.00 AM, N=12) or in the afternoon (3.00-6.00 PM, N=20). Compared to participants who trained in the morning, participants who trained in the afternoon experienced superior beneficial effects of exercise training on peripheral insulin sensitivity (+5.2 ± 6.4 vs -0.5 ± 5.4 μmol/min/kgFFM, p = 0.03), insulin-mediated suppression of adipose tissue lipolysis (-4.5 ± 13.7% vs +5.9 ± 11%, p=0.04), fasting plasma glucose levels (-0.3 ± 1.0 vs +0.5 ± 0.8 mmol/l, p = 0.02), exercise performance (+0.40 ± 0.2 vs +0.2 ± 0.1 W/kg, p=0.05) and fat mass (-1.2 ± 1.3 vs -0.2 ± 1.0 kg, p=0.03). In addition, exercise training in afternoon also tended to elicit superior effects on basal hepatic glucose output (p=0.057). Our findings suggest that metabolically compromised subjects may reap more pronounced metabolic benefits from exercise training when this training is performed in the afternoon versus morning.

Introduction

To cope with the dark and light cycles on earth and the alternating periods of feeding and fasting, and activity and recovery, the human body contains a biological clock that triggers anticipatory responses by setting a circadian rhythmicity. This circadian rhythmicity is regulated by a central clock, which is located in the hypothalamic suprachiasmatic nucleus (SCN), but also by multiple peripheral molecular clocks, located in distal organs such as skeletal muscle, liver and adipose tissue (1). While the central clock is entrained by daily light-dark cycles, the peripheral molecular clocks are robustly entrained by non-photic environmental signals (zeitgebers) such as exercise and feeding (2). Consequently, the central and peripheral clocks can desynchronize when cues derived from these behavioural zeitgebers are not aligned to the environmental light/dark cycle, eventually contributing to the development of insulin resistance (3, 4).

In that context, skeletal muscle insulin sensitivity is characterized by diurnal variations in healthy humans (5, 6), exhibiting higher insulin-stimulated plasma glucose clearance in the morning compared to the evening (7). Interestingly, we (8) and others (9, 10) have shown that a rapid day-night shift, as a model for shiftwork or jetlag, leads to a disturbed glucose homeostasis and skeletal muscle insulin resistance in humans, illustrating the indisputable role that central and peripheral molecular clocks play in regulating systemic energy homeostasis. As such, integrating different external cues at optimal times can potentially be used to improve metabolic health.

Exercise training is the first line strategy to counteract skeletal muscle insulin resistance and ameliorate elevated plasma glucose levels (11). Indeed, we (12, 13) and others (14, 15) have previously elucidated that exercise training programs of 2 months or longer result in training adaptations that improve skeletal muscle insulin resistance and mitochondrial function in obese volunteers and type 2 diabetes (T2D) patients. The recent insights in the role of the circadian clock in the etiology of T2DM has raised the suggestion that timing of exercise may affect the training-mediated effects on glucose homeostasis. Somewhat supportive for this notion, Savikj et al. reported that consecutive bouts of high intensity interval exercise during 2 weeks acutely induces more beneficial 24h glycaemic profiles in T2DM subjects when performed in the afternoon as compared to a morning training regime (16). However, so far it has not been revealed in humans if the classical, more sustained metabolic health effects of prolonged exercise training, including

beneficial training-adaptive effects on insulin sensitivity, exercise performance and body composition, are influenced by the timing of the exercise training sessions.

To test the hypothesis that timing of exercise affects long-term metabolic health training adaptations in metabolically compromised individuals, we retrospectively analyzed data from a study investigating the effect of exercise training on a large range of metabolic health outcomes (13). In this previous study, volunteers performed regular, supervised exercise training for 12 weeks either in the morning or afternoon but the effect of the timing of exercise was not previously analyzed (13). Here, we report that exercise performed in the afternoon lead to greater improvements in skeletal muscle and adipose tissue insulin sensitivity in conjunction with other beneficial metabolic health effects such as larger declines in fat mass and greater improvements in exercise performance when compared to morning training.

Material and methods

Ethical approval

All subjects gave their written informed consent. The protocol was reviewed and approved by the institutional medical ethics committee and the study was carried out at Maastricht University Medical Center, The Netherlands, following the declaration of Helsinki principles (ClinicalTrials.gov ID: NCT01317576).

Subjects

Thirty-five males with a BMI >26 kg/m^2 participated in a 12-week exercise training program. The baseline, metabolic characteristics of participants were previously reported in (17), whereas the effects of exercise training in obese and NAFL volunteers were reported in (13). The study was not designed to investigate the effect of timing of exercise and the reason for subjects performing exercise in the morning or evening was depending on the scheduling possibilities and personal preferences. For the current analysis, 32 volunteers were included that performed ALL of their exercise training sessions in either the morning (AM, N=12) or the afternoon (PM, N=20). Overall, 11 subjects were classified as overweight or obese, 9 subjects had a non-alcoholic fatty liver

(defined as liver fat ≥ 5% measured by proton magnetic resonance spectroscopy [^{1}H-MRS] and 12 subjects were classified as well-controlled type 2 diabetics patients on oral medication; those groups were equally distributed over the AM and PM group according to scheduling possibilities and personal preference where possible.

Unstable body weight (body weight variance of >3 kg in the last 3 months before enrolling the study), impaired renal and cardiac function, use of anti-thrombotic medication and beta-blockers, elevated resting blood pressure (>160/100 mmHg) and previously undergoing insulin therapy, were exclusion criteria for this study. Detailed inclusion criteria were reported previously by Brouwers et al. (13). The medication use did not change during the study, but antidiabetic medication was stopped 7 days before the hyperinsulinemic-euglycemic clamp.

Before, during and after 12 weeks of exercise training maximal work load (Wmax) and maximal aerobic capacity (VO_2max) were determined during a graded cycling test with ECG monitoring until exhaustion (18). Body composition was assessed by dual X-ray absorptiometry (Hologic Discovery A, Waltham, MA, USA). Participants were asked to maintain their regular dietary behavior throughout the study and to consume the same evening meal prior to the hyperinsulinemic-euglycemic clamp test before and after training.

Exercise training protocol

Subjects enrolled in a tightly controlled progressive exercise program for 12 weeks, combining aerobic-type and resistance-type exercises with 3 sessions per week. Twelve subjects performed all their exercise training sessions between 08:00 and 10:00 AM (AM group), whereas 20 subjects performed all training sessions between 15:00 and 18:00 PM (PM group). Compliance to the exercise training averaged 98%. All subjects performed twice a week aerobic exercise on a cycling ergometer for 30 minutes at 70% of a previously determined workload (Wmax), and once a week resistance exercise comprised of 3 series of 10 repetitions at 60% of the subject's previously determined maximal voluntary contraction (MVC). Resistance-type exercises targeted large muscle groups from the low and high limbs (leg extension, leg press, chess press, lat pull down, triceps and biceps curls, abdominal crunches and horizontal row). MVC was derived from 5 multiple repeated maximum testing. Total muscle strength was calculated as the sum of the derived MVC for all 8 muscle groups. Each training session involved a warming-up and cooling down phase by cycling 5 minutes on the ergometer at 45% Wmax at the beginning and at the end of the

training session respectively. Both muscle strength and Wmax were reassessed every 4 weeks to readjust training loads according to adopted exercise capacities. Training sessions were fully supervised and performed with 3-4 individuals at a time.

Hyperinsulinemic-euglycemic clamp

A 2-step hyperinsulinemic-euglycemic clamp (10 mU/m2/min and 40 mU/kg/min) was performed before and after the training period as originally described (19). Dietary habits were stable and physical activity was avoided during the 2 days prior to the clamp, which was performed 4 days before starting the regular training period and 48-72 hours after the last training session to prevent carry-over effect of the last exercise bout. Subjects were assisted to the laboratory after an overnight fast and clamp started with a primed continuous infusion of [6,6-2H2]-glucose (0.04 mg/kg/min) to determine the rates of endogenous glucose production (EGP), glucose appearance (Ra) and glucose disposal (Rd) as described previously (13, 17). After 3 hours of continuous glucose tracer infusion, a primed constant infusion of insulin (10 mU/m2/min) started for 4 hours, followed by high dose of insulin (40 mU/m2/min) infused continuously during 2 hours. During the last 30 minutes of the non-insulin stimulated period (t = 150-180) and under both insulin-stimulated steady states (t=390-420 and t= 510-540), blood samples were collected and substrate utilization was measured by indirect calorimetry (Omnical, Maastricht Instruments, The Netherlands).

Calculations

Steele's single pool non-steady state equations were used to calculate glucose rate of appearance (Ra) and glucose rate of disposal (Rd) (20). The distribution volume of glucose was assumed to be 0.160 l/kg. Insulin-stimulated glucose disposal was computed as the difference between Rd under insulin-stimulated conditions (40 mU/m^2/min) and under basal non-insulin-stimulated conditions (delta Rd). Glucose oxidation was computed with the assumption that protein oxidation was negligible, using Peronnet's equation (21). Non-oxidative glucose disposal (NOGD) was calculated as Rd (40 mU/m^2/min) – glucose oxidation (40 mU/m^2/min). Liver insulin sensitivity was computed as percent suppression of EGP during low dose (10 mU/m^2/min) insulin infusion. Adipose tissue insulin sensitivity was computed as percent suppression of plasma free fatty acids during low dose (10 mU/m^2/min) insulin infusion.

Blood sample analysis

Isotopic enrichment of plasma glucose was determined by electron ionization gas chromatography-mass spectroscopy (22). Arterialized blood samples were collected and immediately centrifuged at high speed. Plasma was frozen in liquid nitrogen and stored at -80°C until assayed. Plasma glucose and free fatty acids levels were determined with enzymatic assays automated on a Cobas Fata/Mira (FFA: Wako NEFA C test kit; Wako Chemicals, Neuss, Germany) (Plasma Glucose: Hexokinase method; La Roche, Basel, Switzerland).

Statistics

Data are presented as mean ± standard deviation (SD). Shapiro-Wilk normality test was carried out to evaluate normal distribution. An unpaired student's t-test was used to compare baseline subjects' characteristics for the morning and afternoon exerciser group. Post minus pre-exercise training changes in primary (whole body insulin sensitivity) and secondary outcome parameters (exercise performance and body composition) were compared between the morning and afternoon exercise group using unpaired student's t-test. A p-value ≤ 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 25.0 for Windows.

Results

Baseline characteristics

Table 1 shows the baseline subject characteristics of the volunteers that were included in the current analysis, classified by timing of exercise training either in the morning (AM) or afternoon training (PM). No significant differences in age, body weight, BMI and body composition were observed at baseline between the AM and PM groups (all $p>0.05$, Table 1). In addition, individuals with overweight or obesity, individuals with NAFL and individuals with T2DM were equally distributed over the two groups. Furthermore, baseline maximal aerobic capacity (VO_2max) and maximal power output (Wmax) were comparable between AM and PM groups (table 1). Also, fasting plasma glucose and free fatty acids levels were comparable between AM and PM groups at baseline ($p>0.05$, Table 1). No differences in any measures of insulin sensitivity were observed between AM and PM groups before exercise training (all $p>0.05$, Table 1).

Table 1. Baseline subjects characteristics

	AM	PM
Sample size	12	20
T2D subjects	4	8
NAFL subjects	3	6
Healthy obese subjects	5	6
Age (year)	61 ± 5	57 ± 7
Body weight (kg)	94.7 ± 11.7	98.1 ± 10
BMI (kg/m^2)	30.3 ± 2.6	29.8 ± 2.3
Fat mass (kg)	27.4 ± 4.3	28.8 ± 5.6
Fat percentage (%)	28.6 ± 2.3	29 ± 3.2
Trunk fat mass (kg)	16.0 ± 2.5	16.2 ± 3.4
Fat free mass (kg)	65.4 ± 7.2	67.1 ± 5.1
$VO2_{max}$ (ml/kg/min)	26 ± 4.0	26.5 ± 4.5
W_{max} (W/kg)	1.9 ± 0.4	2.0 ± 0.3
Fasting glucose (mmol/l)	6.7 ± 2.1	6.8 ± 2.1
Fasting free fatty acids (μmol/l)	566 ± 171	615 ± 169
Clamp data		
Basal EGP (μmol/min/kgFFM)	7.4 ± 1.5	8.1 ± 3.5
Basal R_d (μmol/min/kgFFM)	7.8 ± 1.8	7.9 ± 3.6
Basal CHO_{ox} (μmol/min/kgFFM)	5.9 ± 3.9	5.3 ± 2.6
Insulin-induced suppression of plasma FFA (%)	-62.6 ± 9.5	-61.6 ± 16.0
Insulin-induced suppression of EGP (%)	-41.8 ± 26.3	-32.2 ± 28.9
Delta R_d (μmol/min/kgFFM)	16.9 ± 11.2	15.2 ± 9.0
Delta NOGD (μmol/min/kgFFM)	8.9 ± 7.2	8.2 ± 6.2

Data displayed as mean ± SD. BMI, body mass index; $VO2_{max}$, maximal aerobic capacity; W_{max}, maximal power output; EGP, endogenous glucose production; R_d, glucose disposal; CHO_{ox}, carbohydrate oxidation; FAT_{ox}, fat oxidation; FFA, free fatty acids; NOGD, non-oxidative glucose disposal. Insulin-induced suppression of EGP and plasma FFA were measured at 10 mU/m^2/min. Delta Rd was calculated as the difference between basal and insulin-stimulated glucose disposal at 40 mU/m^2/min infusion rate.

Afternoon exercise training improves peripheral insulin sensitivity and glucose homeostasis more profoundly than morning exercise training

Training-induced effects on insulin-stimulated peripheral glucose uptake, as expressed by the changes in plasma glucose disposal (delta R_d) from basal to insulin-stimulated conditions, were significantly affected by the timing of exercise (p= 0.03, figure 1A, table 2). Specifically, training-induced effects on delta R_d were greater in the PM group compared to the AM group (+5.2 ± 6.4 vs -0.5 ± 5.5 µmol/min/kgFFM, in PM vs AM). The superior effect of PM exercise training on insulin-stimulated glucose disposal was also reflected in a larger increase in training-induced change in glucose oxidation upon insulin effects (+0.8 ± 3.3 µmol/min/kgFFM in PM vs.-2.6 ± 2.4 µmol/min/kgFFM in AM, p= 0.04 respectively) (figure 1B, table 2), whereas insulin-stimulated non-oxidative glucose disposal (NOGD) was not significantly affected by timing of exercise (p=0.1, table 2). It should be noted though, that due to technical issues with tracer handling, the data on Rd (n=15 in PM) as well as glucose oxidation and NOGD (n=7 in AM and n=16 in AM and PM) are based on smaller group sizes.

The effect of exercise training on insulin-mediated suppression of plasma FFA (%FFA suppression), a marker of adipose tissue insulin sensitivity, was significantly larger in the PM vs AM group (-4.5 ± 13.7% vs +5.9 ± 11% in PM vs. AM, p=0.04, figure 1C, table 2), indicating that the effect of PM training on adipose tissue insulin sensitivity was more pronounced than AM training. However, changes in fasting plasma FFA levels upon exercise training did not reach statistical significance upon comparing PM and AM (-122.3 ± 198.6 µmol/l vs. -19.8 ± 139.8 in PM vs AM, p=0.13, table 2).

Training-induced changes in basal endogenous glucose production tended to be different between afternoon and morning exercisers (EGP: -0.01 ± 0.9 vs +1.08 ± 1.6 µmol/min/kgFFM in PM vs AM, p=0.057, table 2) but timing of exercise did not have significant effects on training-induced changes in insulin-mediated suppression of endogenous glucose production (%EGP suppression), a marker of liver insulin sensitivity (-10.6 ± 25.1 vs -2.3 ± 28.6 % in PM vs AM, p=0.4, table 2).

Changes in fasting plasma glucose levels upon training were significantly affected by timing of exercise (p=0.02, table 2), with a greater decline of fasting glucose levels in the PM group (-0.3 ± 1.0 mmol/l) vs. AM group (+0.5 ± 0.8 mmol/l, table 2).

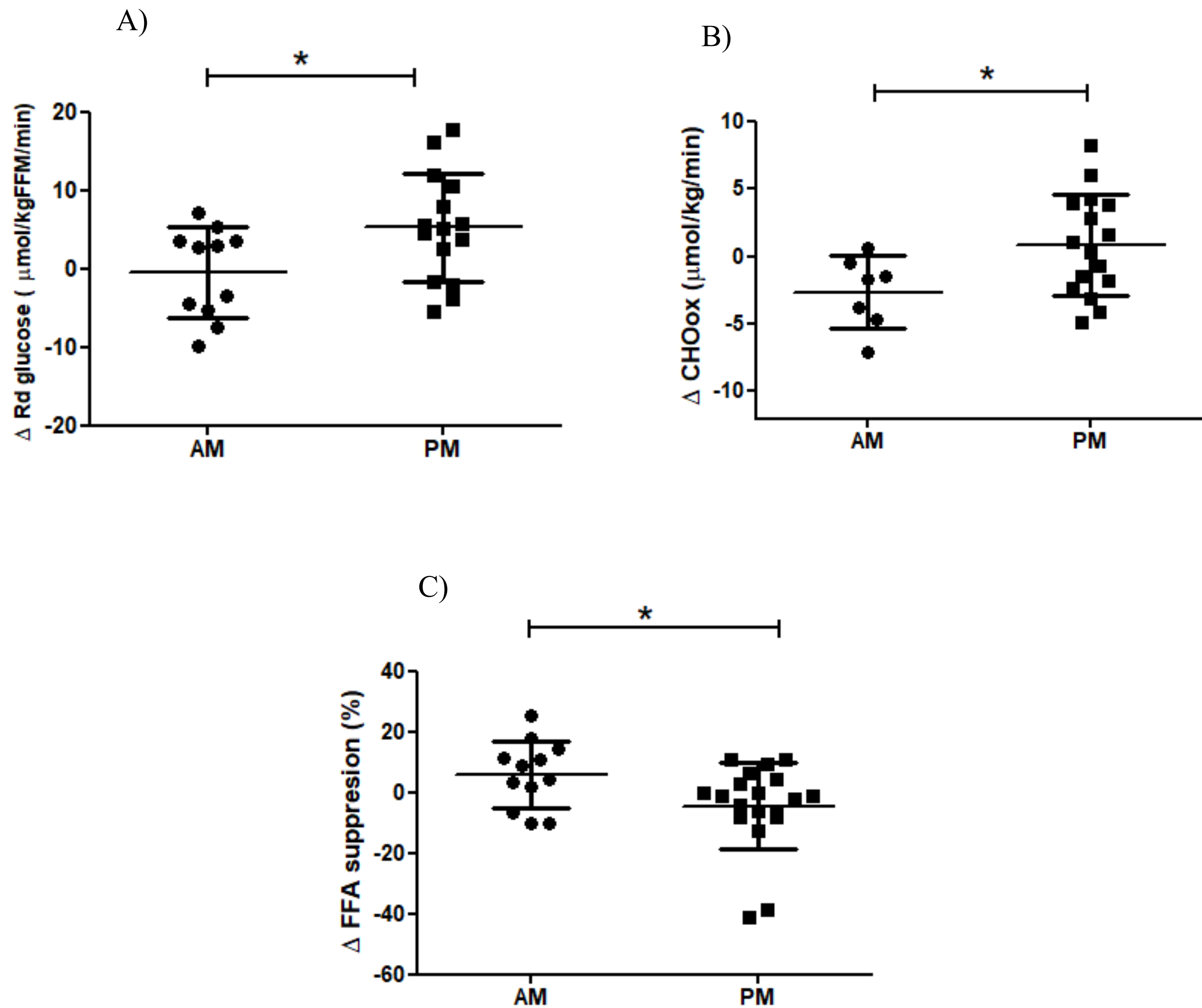

Figure 1. Changes on peripheral insulin sensitivity and glucose oxidation upon timing of exercise training either in the morning (AM) or in afternoon (PM).

A) Changes in peripheral insulin-stimulated glucose disposal (from basal to insulin-stimulated) were significantly greater in the PM group compared to AM group. * P <0.05. n=11 for AM group and n=15 for PM group. B) Changes in insulin-stimulated glucose oxidation (from basal to insulin-stimulated) were significantly greater in the PM group compared to AM group. * P <0.05. n=7 for AM group and n=16 for PM group. C) Changes in insulin-mediated suppression of plasma free fatty acids (%) improved significantly more in the PM group compared to AM group. * P<0.05. n=12 for AM group and n=19 for PM group. Data are expressed as means ± SD.

Exercise training performed in the afternoon has more profound effects on body composition than exercise training in the morning

Training-induced reductions in fat mass (-1.2 ± 1.3 vs -0.2 ± 1.0 kg in PM vs AM, p=0.03, figure 2A, table 2) and fat percentage (-1.0 ± 0.9 vs -0.3 ± 0.7 % in PM vs AM, p=0.03, table 2) were significantly larger in the PM group compared to AM group. Trunk fat mass tended to decrease more in the PM group compared to the AM group, although this effect did just not reach statistical significance (-0.7 ± 1.0 vs -0.07 ± 0.7 kg in PM vs AM, p=0.059, table 2).

Exercise training performed in the afternoon induces greater improvements on exercise performance compared to exercise training performed in the morning

Maximal power output (Wmax) tended to increase more in the PM vs AM group (+36.6 ± 22 vs +24.4 ± 11.3 Watt in PM vs AM, p=0.08) and this effect was statistically significant upon correction for body weight (0.40 ± 0.2 vs 0.24 ± 0.1 W/kg in PM vs AM, p=0.05, figure 2B, table 2). VO_2max tended to increase more in the PM group compared to the AM group, although this effect did not reach statistical significance (+3.0 ± 2.1 vs +1.8 ± 2.2 ml/kg/min in PM vs AM, p=0.1, table 2). Timing of exercise had no statistically significant effects on the training-induced changes in total muscle strength, body weight, BMI and fat free mass (FFM) (Table 2).

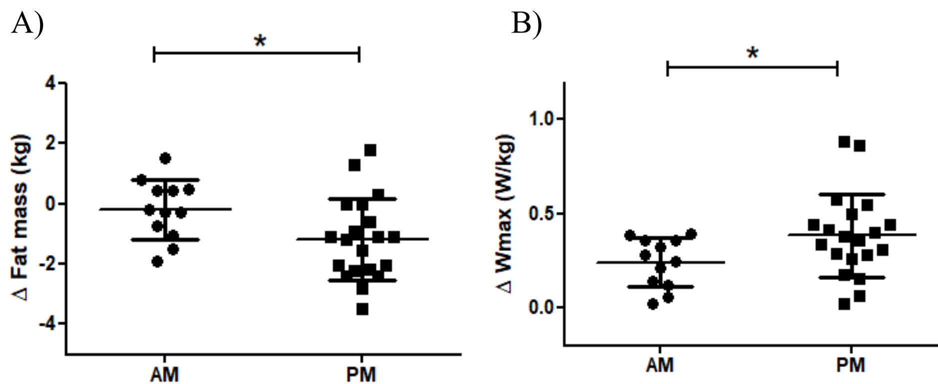

Figure 2. Changes on fat mass (kg) and Wmax (W/kg) upon timing of exercise training

A) Changes in Fat mass (kg) were significantly greater in the PM group compared to AM group. * P =0.03. n=12 for AM group and n=20 for PM group. B) Changes in Wmax (W/kg) were significantly greater in PM group compared to AM group. * P = 0.05. n=12 for AM group and n=20 for PM group. Data are expressed as means ± SD.

Table 2. Effects of morning and afternoon exercise training on insulin sensitivity and metabolic health

	AM		AM Δ	PM		PM Δ	P value
	Pre	Post		Pre	Post		
BMI (kg/m2)	30.3 ± 2.6	30.5 ± 2.5	0.2 ± 0.5	29.8 ± 2.3	29.6 ± 2.4	-0.2 ± 0.8	0.11
Body weight (kg)	94.7 ± 11.7	95.3 ± 11.6	0.7 ± 1.6	98.1 ± 10.0	97.5 ± 10.5	-0.6 ± 2.5	0.11
Fat mass (kg)	27.4 ± 4.3	27.2 ± 4.7	-0.2 ± 1.0	28.8 ± 5.6	27.6 ± 5.7	-1.2 ± 1.3	**0.03**
Fat free mass (kg)	65.4 ± 7.2	65.8 ± 7.7	0.4 ± 1.0	67.1 ± 5.1	67.4 ± 5.6	0.3 ± 1.5	0.82
Fat percentage (%)	28.6 ± 2.3	28.3 ± 2.4	-0.3 ± 0.7	29 ± 3.2	28 ± 3.1	-1.0 ± 0.9	**0.03**
Trunk fat mass (kg)	16.0 ± 2.5	16.0 ± 2.8	-0.07 ± 0.7	16.2 ± 3.4	15.4 ± 3.3	-0.7 ± 1.0	0.059
VO2max (ml/kg/min)	26.0 ± 4.0	27.7 ± 3.0	1.8 ± 2.2	26.5 ± 4.5	29.5 ± 5.0	3.0 ± 2.1	0.18
Wmax (W/kg)	1.9 ± 0.4	2.1 ± 0.3	0.2 ± 0.1	2.0 ± 0.3	2.4 ± 0.4	0.4 ± 0.2	**0.05**
Total muscle strength (kg)	79.4 ± 14	91.7 ± 12.3	12.3 ± 7.5	82.5 ± 15.8	98.4 ± 20.7	15.9±10.3	0.30
Fasting Free fatty acids (µmol/l)	567 ± 171	547 ± 174	-19.8 ± 139	615 ± 170	507 ± 177	-122 ± 198	0.13
Fasting glucose (mmol/l)	6.7 ± 2.1	7.2 ± 2.7	0.5 ± 0.8	6.8 ± 2.1	6.4 ± 2.0	-0.3 ± 1.0	**0.02**

Clamp data							
Basal EGP (µmol/min/kgFFM)	7.4 ± 1.5	8.5 ± 1.2	1.0 ± 1.6	8.1 ± 3.5	8.1 ± 3.7	-0.01 ± 0.9	0.057
Basal Rd (µmol/min/kgFFM)	7.8 ± 1.8	9.4 ± 2.5	1.0 ± 1.5	7.9 ± 3.6	7.7 ± 4.2	-0.3 ± 2.4	0.17
Basal CHOox (µmol/min/kgFFM)	5.9 ± 3.9	5.5 ± 3.2	0.2 ± 3.0	5.3 ± 2.6	5.6 ± 2.3	0.1 ± 2.1	0.89
Insulin-induced suppression EGP (%)	-41.8 ± 26.3	-42.8 ± 31.0	-2.3 ± 28.6	-32.2 ± 28.9	-40.8 ± 29.0	-10.6 ± 25.1	0.44
Insulin-induced suppression plasma FFA (%)	-62.6 ± 9.5	-56.7 ± 15.2	5.9 ± 11.0	-61.6 ± 16.0	-65.8 ± 12.4	-4.5 ± 13.7	**0.04**
Delta Rd (µmol/min/kgFFM)	16.9 ± 11.2	14.5 ± 13.8	-0.5 ± 5.5	15.2 ± 9.0	20.2 ± 13.6	5.2 ± 6.4	**0.03**
Delta CHOox (µmol/min/kgFFM)	6.7 ± 4.0	5.5 ± 3.8	-2.6 ± 2.4	6.3 ± 2.8	7.4 ± 4.0	0.8 ± 3.3	**0.04**
Delta NOGD (µmol/min/kgFFM)	8.9 ± 7.2	8.5 ± 12.0	-0.9 ± 5.3	8.2 ± 6.2	13.4 ± 10.5	4.2 ± 5.4	0.16
Delta FATox (µmol/min/kgFFM)	-1.8 ± 1.1	-1.6 ± 1.0	0.4 ± 0.5	-1.8 ± 0.7	-1.9 ± 1.0	-0.04 ± 0.7	0.24

Data are displayed as mean ± SD. BMI, body mass index; $VO2_{max}$, maximal aerobic capacity; W_{max}, maximal power output; EGP, endogenous glucose production; R_d, glucose disposal; CHO_{ox}, carbohydrate oxidation; FAT_{ox}, fat oxidation; FFA, free fatty acids; NOGD, non-oxidative glucose disposal. Insulin-induced suppression of EGP and plasma FFA were measured at 10 $mU/m^2/min$ infusion rate. Delta Rd was calculated as the difference between basal and insulin-stimulated glucose disposal at 40 $mU/m^2/min$ infusion rate. Bold font is used to indicate statistically significant differences between groups

Discussion

Exercise training is known to induce multiple beneficial effects on whole-body glucose homeostasis and metabolic health, although effects are not equal in magnitude for all individuals. Exercise volume and intensity are classically recognized as crucial determinants of the beneficial effect of exercise training. Triggered by novel insights in the role of the biological clock in metabolism, it has recently been postulated that also the time of day at which acute exercise is performed affects the metabolic adaptations to exercise, but evidence in humans is scarce. Here, we demonstrate that exercise training performed in the afternoon is more beneficial for improving peripheral insulin sensitivity (skeletal muscle and adipose tissue insulin sensitivity) in comparison with exercise training in the morning. The greater improvements in peripheral insulin-stimulated glucose disposal observed upon exercise training in the afternoon could primarily be attributed to increased insulin-stimulated glucose oxidation and was paralleled by greater improvements of fasting plasma glucose levels and exercise performance. In addition, a superior decline of fat mass was found upon exercise training in the afternoon compared to morning exercise training. Together, these data provide evidence in humans that timing of exercise may amplify the beneficial health effects of exercise training.

Insulin-stimulated skeletal muscle glucose uptake is crucial to maintain normal glucose homeostasis as approximately 80% of postprandial glucose clearance resides within skeletal muscle (23). By using animal models, recent studies revealed that insulin-stimulated skeletal muscle glucose clearance interacts with the peripheral muscle clock and such interplay can be affected by exercise timing (24, 25). Nevertheless, whether there is an optimal time to exercise and to reap greater improvements of insulin sensitivity in humans was unknown so far. Interestingly, we here show that exercising in the afternoon is more beneficial for skeletal muscle insulin sensitivity in comparison with exercise training in the morning. In this regard, our findings are in line with a report showing that 2 weeks of high intensity interval training (HIIT) in the afternoon acutely improved 24h glycaemic profile in subjects with T2D, while performing HIIT in the morning possessed detrimental effects on blood glucose levels throughout the day (16). However, it is important to note that in this cross-over design, Savikj et al. measured the effects of two-weeks HIIT training acutely; to the best of our knowledge, the effect of timing of exercise sessions on more classic longer-term training adaptations such as tissue-specific insulin sensitivity

had not been studied so far in humans. Interestingly, the superior benefits of afternoon exercise on insulin-stimulated skeletal muscle glucose uptake compared to morning training are observed in the absence of changes on body weight in any group, reflecting more optimal local-tissue adaptations in response to afternoon exercise timing. Whether these effects are due to an effect of exercise training on the skeletal muscle peripheral clock, on diurnal rhythms in hormonal secretion or involve other mechanisms needs to be further investigated.

In addition to skeletal muscle insulin sensitivity, hepatic glucose output and adipose tissue insulin sensitivity also contributes to the regulation of circulatory glucose levels. In this regard, it is relevant to highlight that afternoon exercise also triggered greater metabolic adaptations in other distal organs beyond those shown in skeletal muscle. Here, we reported that under hyperinsulinemic clamp conditions plasma FFA levels were significantly more suppressed by afternoon exercise training, possibly reflecting a greater antilipolytic activity of insulin in adipose tissue. Of interest, white adipose tissue FFA release and esterification feature diurnal oscillations controlled by the adipose tissue peripheral clock which responds to environmental cues such as meal ingestion and exercise timing (26). Uncontrolled adipose tissue lipolysis has been strongly associated to the development of skeletal muscle insulin resistance (27) and ectopic fat accumulation (28). It can be speculated that a lower insulin-mediated suppression of FFA release upon exercising in the morning could sustain FFA supply for oxidation, thus reducing glucose oxidation and diminishing insulin sensitivity compared to the effects of exercise training performed in the afternoon. Consequently, our results suggest that timing of exercise training affects adipose tissue FFA handling and that afternoon training is more beneficial for adipose tissue insulin sensitivity than morning training.

Interestingly, we observed that exercising in the afternoon is more beneficial for fasting plasma glucose levels in compared to exercise training in the morning. This differential response in fasting plasma glucose levels between the morning and afternoon training groups is consistent with the differences observed in the basal endogenous glucose production (EGP) upon exercise timing. Previous studies have indicated that the effects of exercise-training on basal EGP are profoundly affected by the pre-exercise nutritional status (29). Thus, a robust increase in hepatic glucose release, next to a rise in plasma glucose levels, was observed in subjects diagnosed with type 2 diabetes upon intense exercise in the fasted state as a consequence of aberrant increments of plasma

epinephrine and glucagon synthesis (29). On the other hand, it has been shown that hepatic glucose output is unaffected by exercising at fed conditions in subjects with T2D (30). In the current study, pre-exercise meal ingestion was not controlled, and exercise training was performed 2-3 hours after either breakfast or lunch. In addition, exercise may regulate appetite and thereby timing of exercise could affect post-exercise food intake. Clearly, future studies are needed to investigate if and how pre- and post-exercise food intake may affect the results of timing of exercise on glucose homeostasis.

Exercise training performed in the afternoon also induced a more profound increase in maximal power output than in the morning. Previous studies consistently reported that exercise performance, exercise efficiency and muscle strength display profound circadian variations (25, 31), all peaking in the afternoon. In this regard, our findings indicate that optimizing exercise timing can boost exercise-induced increases in physical performance. This gives rise to the notion that exercising at afternoon, when muscle is primed to meet the energy demand of contraction, might trigger greater benefits. Somewhat aligned with our findings, Küüsmaa et al. previously reported that 24 weeks of combined resistance-type and endurance type exercise training (2-5 times/week) executed in the afternoon induced a larger decrease in fat mass and lead to greater improvements in exercise performance (time to exhaustion) as compared to morning training in young healthy adults (32). Interestingly, here we elucidate that greater improvements of functional capacities upon afternoon exercise are observed in conjunction with superior metabolic health adaptations than morning exercise, which support the relevance of exercise timing into a clinical setting. Consistent with our findings regarding the more pronounced improvements in exercise performance in the afternoon training group, exercising at afternoon also induced larger decreases on fat mass compared to exercising in the morning, in absence of changes on body weight.

4.1 Limitations of the study

The present study addressed the clinical relevance of timing of regular exercise training by retrospectively analyzing existing research data and was consequently not designed to elucidate underlying mechanisms that might support our findings. Furthermore, the retrospective nature of the study poses some additional limitations. Thus, subjects were not randomized over the AM and PM group and more subjects were included in the PM group, which may have affected the outcome of the study. On the other hand, the retrospective analysis may also have the advantage that results

are not biased by prior knowledge on expected effects by volunteers and investigators. Also, due to small sample size, it was impossible to separate group-dependent metabolic adaptations upon exercise timing in healthy obese, NAFL or T2D individuals. Additionally, our investigation only included males and cannot be generalized to the entire population, although similar training-induced metabolic adaptations, regardless exercise timing, have been reported in adult males and females (33). To the best of our knowledge, gender differences in the effects of timing of exercise on health benefits have so far not been investigated in humans, and are eagerly awaited. Whether changes in food intake and dietary habits occurred during the supervised training period cannot be concluded from the present study, and cannot be excluded as part of the underlying mechanisms. However, considering that subjects were instructed to maintain their regular dietary habits throughout the training period and no significant changes in body weight were observed, our results support the notion that exercise-induced metabolic adaptations can be optimized by performing the exercise training at afternoon day-time. To our knowledge, it is the first report about the effects of timing of regular exercise training affecting insulin sensitivity and metabolic health in metabolically compromised subjects and T2D patients by using gold standard methods, which we consider a major strength of our study.

Conclusions

In conclusion, we here show that exercise training in the afternoon leads to more pronounced exercise-induced metabolic adaptations compared to training in the morning, in people who are metabolically compromised or have type 2 diabetes. Peripheral insulin sensitivity (skeletal muscle, adipose tissue and hepatic glucose output), in addition to fasting plasma glucose levels, exhibited greater improvements when exercise training was performed in the afternoon (3.00-6.00 PM) compared to the same exercise performed in the morning (8.00-10.00 AM). Furthermore, afternoon exercise triggered more profound benefits on improving exercise capacity and decreasing body fat content. Our data highlight that timing of an exercise training session is a crucial environmental cue when aiming to improve glucose homeostasis and elucidates that performing afternoon exercise training might be more optimal than exercising at morning hours. Considering the methodological limitations of the present study, future human interventions, especially larger prospective randomized controlled trials are required to confirm the present findings.

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CHAPTER 4

Skeletal muscle mitochondrial inertia is associated with carnitine acetyltransferase protein activity and physical function in humans.

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Abstract

Background: At the onset of exercise PCr decreases and at a constant exercise intensity, PCr restabilizes at a new steady state. The speed at which this new steady state is reached (PCr on-kinetics) reflects the readiness to activate mitochondrial ATP synthesis, which in turn is secondary to acetyl-coA availability. Here, we tested the hypothesis that PCr on-kinetics are slower in metabolically compromised and older individuals, and is associated with low carnitine acetyl-transferase (CrAT) protein activity and compromised physical function.

Methods: In this cross-sectional study, we applied ^{31}P-Magnetc resonance Spectroscopy (MRS) to assess PCr kinetics (quantified as half-time of the mono-exponential change of PCr) both at the onset and after exercise in 2 different cohorts of human volunteers: Cohort 1 consisted of 9 type 2 diabetes patients (T2DM), 8 obese sedentary participants (OB), 9 lean untrained individuals (UT) and 12 endurance-trained (T) volunteers. Cohort 2 consisted of 10 young (Y) and 15 older (O) individuals with normal physical activity as well as 17 older trained (OT) volunteers. Previous results of CrAT protein activity and acetylcarnitine content in muscle tissue from individuals of cohort 1 were used to explore the underlying mechanisms of PCr on-kinetics, along with various markers of physical function measured in individuals from cohort 2.

Results: In cohort 1, the halftime of PCr on-kinetic was significantly longer in T2DM patients (49.2 ± 4.3 s) and OB individuals (38.9 ± 2.6 s) as compared to T volunteers (19.6 ± 2.1 s; P < 0.001 both comparisons). Furthermore, the halftime of PCr on-kinetic was significantly longer in T2DM patients as compared to UT individuals (28.3 ± 3.2 s; P < 0.001). The halftime of PCr on-kinetic was strongly associated with skeletal muscle CrAT protein activity (r = -0.56; *P* = 0.001), *in vivo* skeletal muscle acetylcarnitine content (r = -0.47; *P* = 0.005) and ADP levels during exercise (r = 0.70; *P* < 0.001). In cohort 2, the halftime of PCr on-kinetic was significantly longer in O (33.1 ± 1.8 s) as compared to OT (24.7 ± 1.8 s; P = 0.005) and Y individuals (24.9 ± 2.2 s; *P* = 0.02). PCr on-kinetics were strongly associated with walking speed (r = -0.48; *P* = 0.002), chair sit-stand test performance (r = 0.54; *P* < 0.001), exercise gross (r = -0.56; *P* < 0.001) and net efficiency (r = -0.53; *P* < 0.001).

Conclusion/interpretation: PCr on-kinetics are significantly slower in metabolically compromised and older individuals with normal physical activity as compared to young,

healthy volunteers, regardless of *in vivo* skeletal muscle mitochondrial ATP synthetic capacity, indicating greater mitochondrial inertia. Thus, PCr on-kinetics are a currently unexplored signature of skeletal muscle mitochondrial metabolism. Furthermore, a reduced CrAT protein activity and higher ADP levels during exercise may indicate that lower acetyl-coA availability (due to lower CrAT protein function) or a lower sensitivity to ADP may underlie this reduced mitochondrial activation at the onset of exercise. PCr on-kinetics are tightly linked to functional outcomes, hence skeletal muscle mitochondrial inertia, might emerge as a relevant target of intervention to improve physical function.

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 obese individuals 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 a group of human volunteers that included type 2 diabetes patients, obese, normoglycemic individuals, as well as healthy young participants with normal physical activity and endurance-trained athletes. 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.

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, type 2 diabetic patients (T2DM), 8 older, obese sedentary volunteers (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. T2DM patients and obese individuals 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, if they had a medical history of cardiovascular disease. This study was approved by the medical Ethical Committee of the Maastricht University Medical Center. All individuals gave written informed consent and the study was conducted in accordance with the Declaration of Helsinki.

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) (10). 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 (11).

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 (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 (12) 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 (13). 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 (Figure 1).

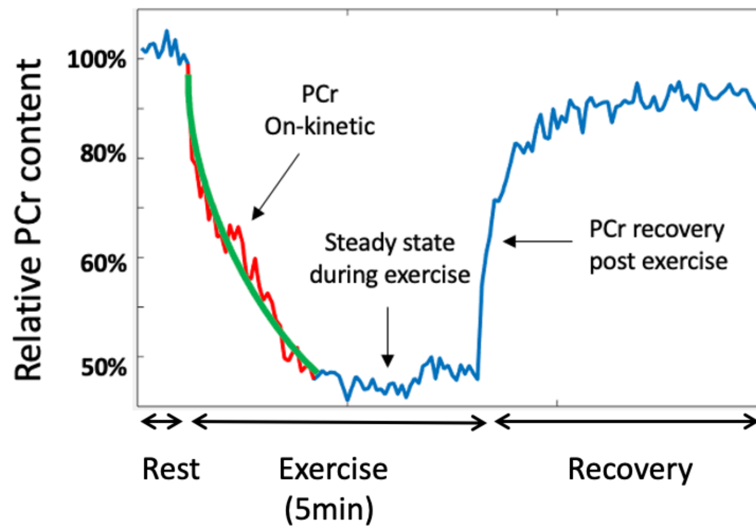

Figure 1. Time course of PCr in muscle during and after the knee-extension exercise assessed by ^{31}P-MRS. Red line indicates PCr on-kinetics towards a lower steady state during exercise, which was fitted with a mono-exponential function (green line). The half-time [s] of the fit was used as a parameter of PCr on-kinetics assumed to be a marker of skeletal muscle mitochondrial inertia.

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 (14) 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 (15). 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 (16):

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

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 (17). 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 $\chi2$ 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.

Results

Participant characteristics

Participant characteristics from study cohort 1 are shown in table 1. 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). T2DM patients 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).

Table 1. Participant characteristics

	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

Data are presented as mean ± SD. * p < 0.05 vs UT. † p < 0.05 vs T. ‡ p < 0.05 vs OB. BMI, body mass index; FPG, fasting plasma glucose level; VO2max, maximal oxygen consumption.

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

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 2A). 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 T2DM patients 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 T2DM patients as compared to OB, UT and T individuals ($P = 0.04$, $P < 0.01$, and $P < 0.01$ respectively). 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 T2DM patients 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 2B).

Reduced skeletal muscle CrAT protein activity and low acetylcarnitine content in type 2 diabetic and obese individuals are related to skeletal muscle mitochondrial inertia

We previously published that skeletal muscle CrAT protein activity was significantly lower in T2DM, OB and UT individuals as compared to T volunteers ($P < 0.01$) whereas skeletal muscle acetylcarnitine content was significantly lower in T2DM patients 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 2C and 2D). 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).

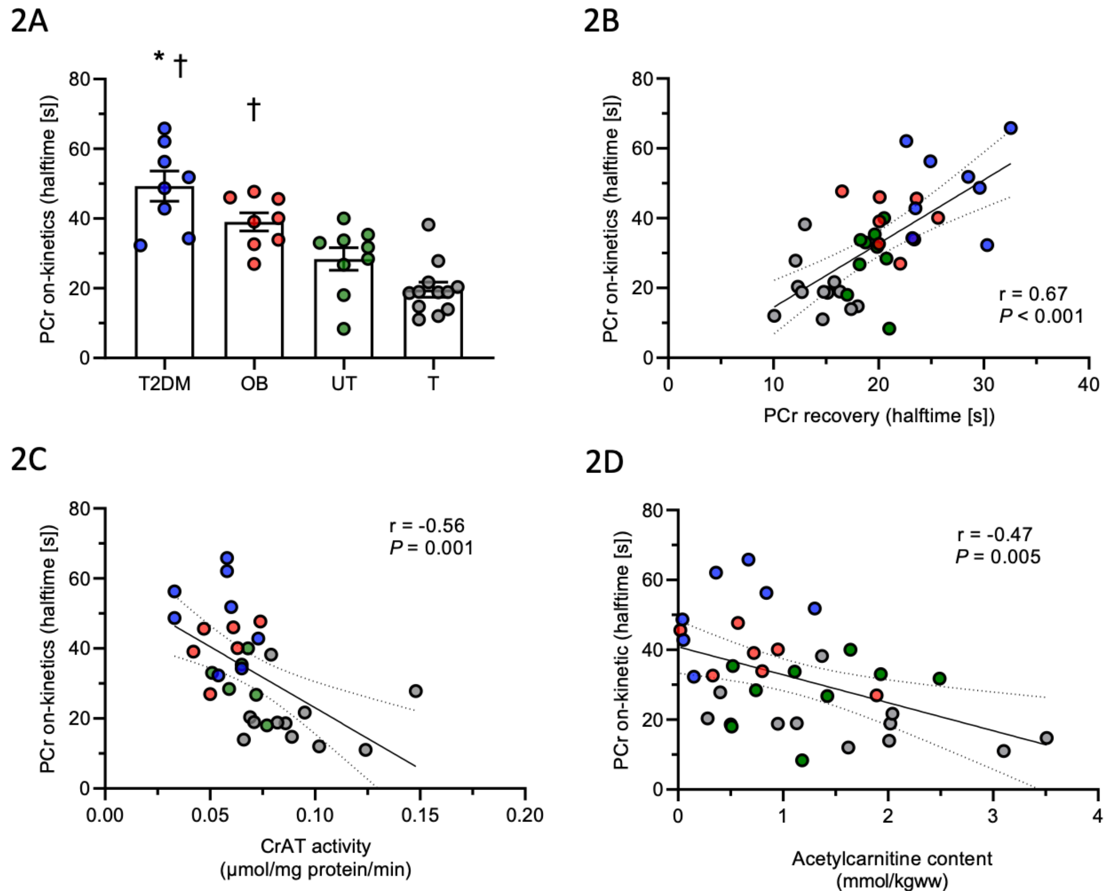

Figure 2. Pronounced skeletal muscle mitochondrial inertia in metabolically compromised individuals. (A) Halftime of PCr on-kinetics; (B) Linear association between PCr on-kinetics and PCr recovery post exercise; (C) Linear association between PCr on-kinetics and CrAT protein activity; (D) Linear association between PCr on-kinetics and *in vivo* skeletal muscle acetylcarnitine content. [s]; seconds; T2DM: Type 2 diabetic patients; OB: Obese individuals; UT: Lean Untrained; T: Trained. Circles in blue: T2DM; circles in red: OB; circles in green: UT; circles in grey: T. Data are shown as 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.

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

Prior to exercise, ADP levels (mmol) at rest were similar across the groups. At the onset of exercise, ADP levels increased rapidly in all individuals (Figure 3A). When PCr reached a new steady state during exercise, ADP levels were significantly different across the groups (T2DM patients: 55.4 ± 4.7 mmol, OB individuals: 52.3 ± 3.0 mmol, UT individuals: 46.3 ± 6.6 mmol, T individuals: 37.8 ± 2.2 mmol; $P = 0.018$, Figure 3B), with significantly higher levels in T2DM patients 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 3C). 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 (T2DM patients: 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 (T2DM patients: 7.1 ± 0.02, OB individuals: 7.1 ± 0.04, UT individuals: 7.02 ± 0.03 and T individuals: 7.00 ± 0.02; $P = 0.08$).

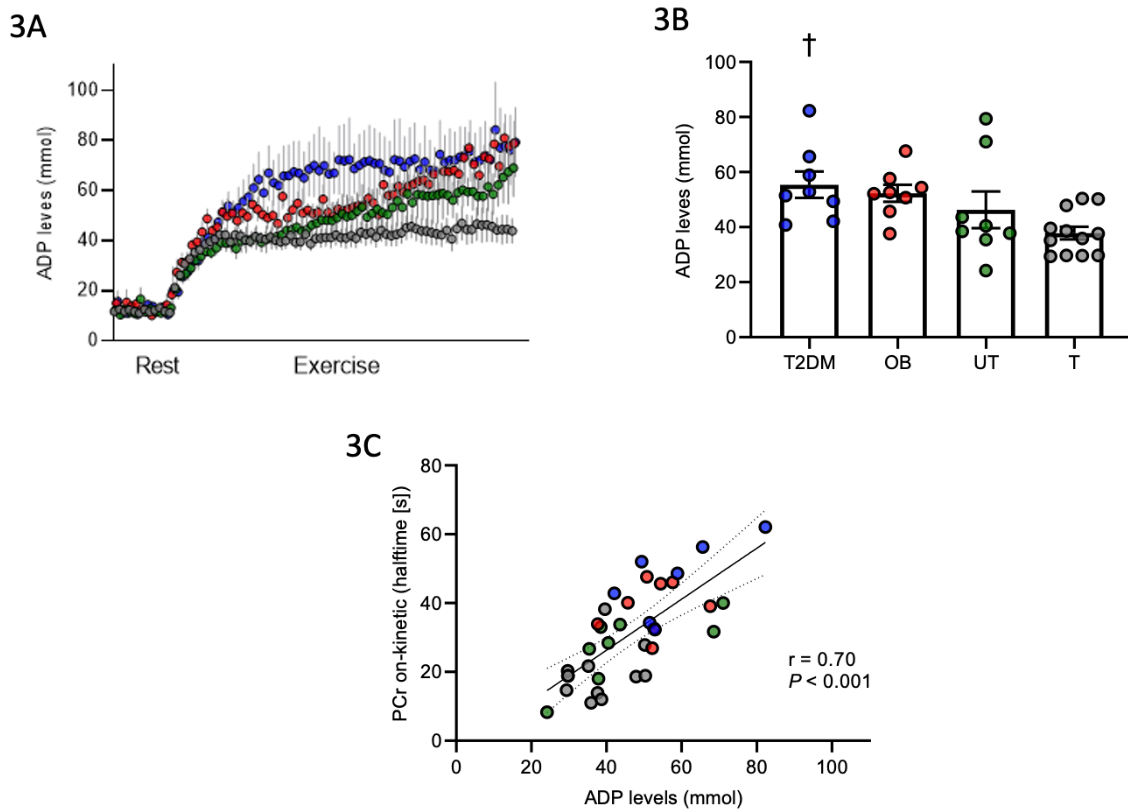

Figure 3. Skeletal muscle mitochondrial inertia coexists with elevated ADP accumulation in muscle tissue during exercise. (A) ADP levels in muscle tissue increases upon exercise onset; (B) ADP levels when PCr utilization reaches a new steady state at the onset of exercise; (C) Linear association between PCr on-kinetics and ADP levels when PCr reached a new steady state during exercise; [s]: seconds; T2DM: Type 2 diabetic patients; OB: Obese individuals; UT: Lean Untrained; T: Trained. Circles in blue: T2DM; circles in red: OB; circles in green: UT; circles in grey: T. Data are shown as mean ± SEM. † $p < 0.05$ vs. Trained volunteers. ADP levels could not be measured in one T2DM patient due to unreliable steady state. This is the same participant excluded for the PCr on-kinetics analysis.

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.

Table 2. Participant characteristics

	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

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.

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 4A) 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 4B). 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 4C). 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 ($P = 0.08$).

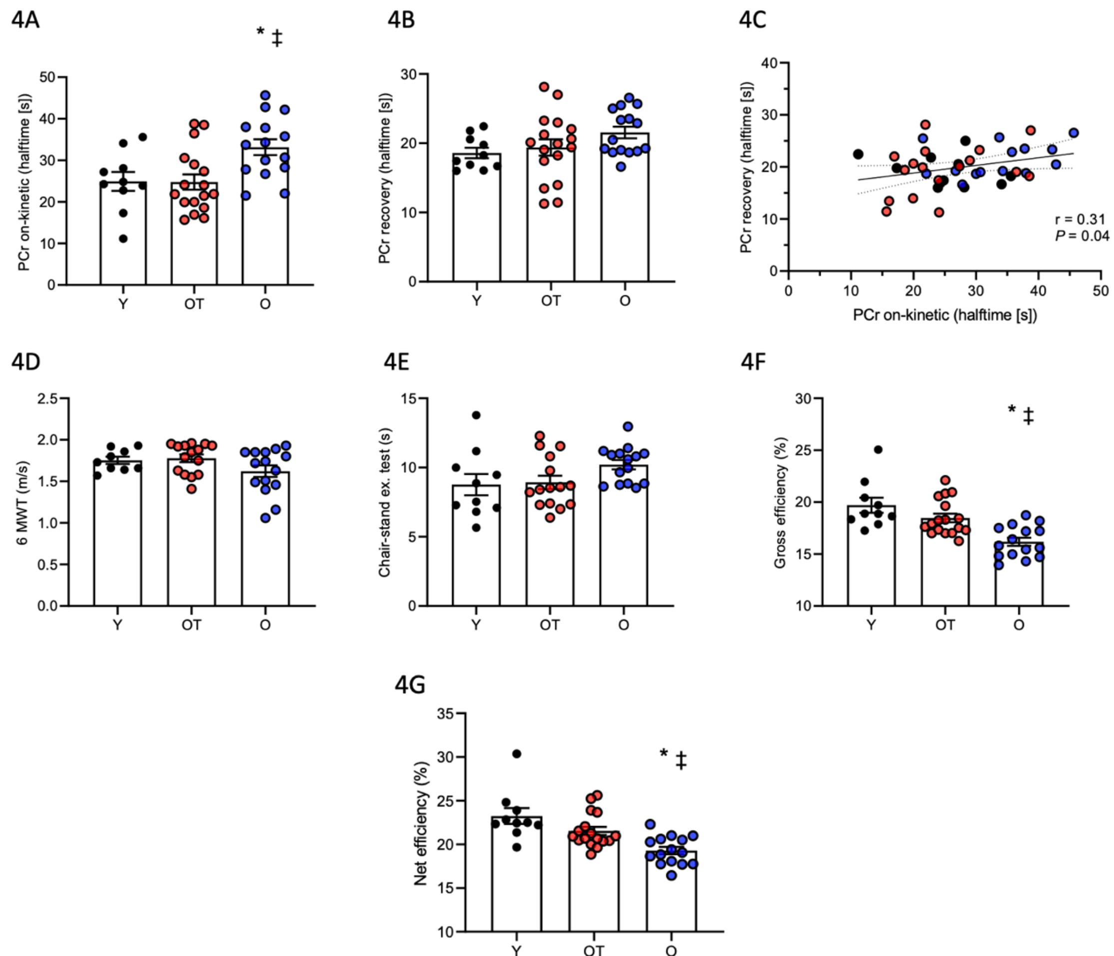

Figure 4. Skeletal muscle mitochondrial inertia and reduced functional outcomes in elderly sedentary individuals. (A) Halftime of PCr on-kinetics; (B) Halftime of PCr recovery post-exercise; (C) Linear association between PCr on-kinetics and PCr recovery post-exercise; (D) Walking speed upon performing 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; circles in red: OT; circles in blue: O. Data are shown as 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.

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 4D). 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 4E).

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 4F and 4G). 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 5A), chair stand test performance (n = 40, r = 0.54; $P < 0.001$, Figure 5B), gross exercise efficiency (n = 42, r = -0.56; $P < 0.001$, Figure 5C) and net exercise efficiency (n = 42, r = -0.53; $P < 0.001$, Figure 5D). 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; $P = 0.046$).

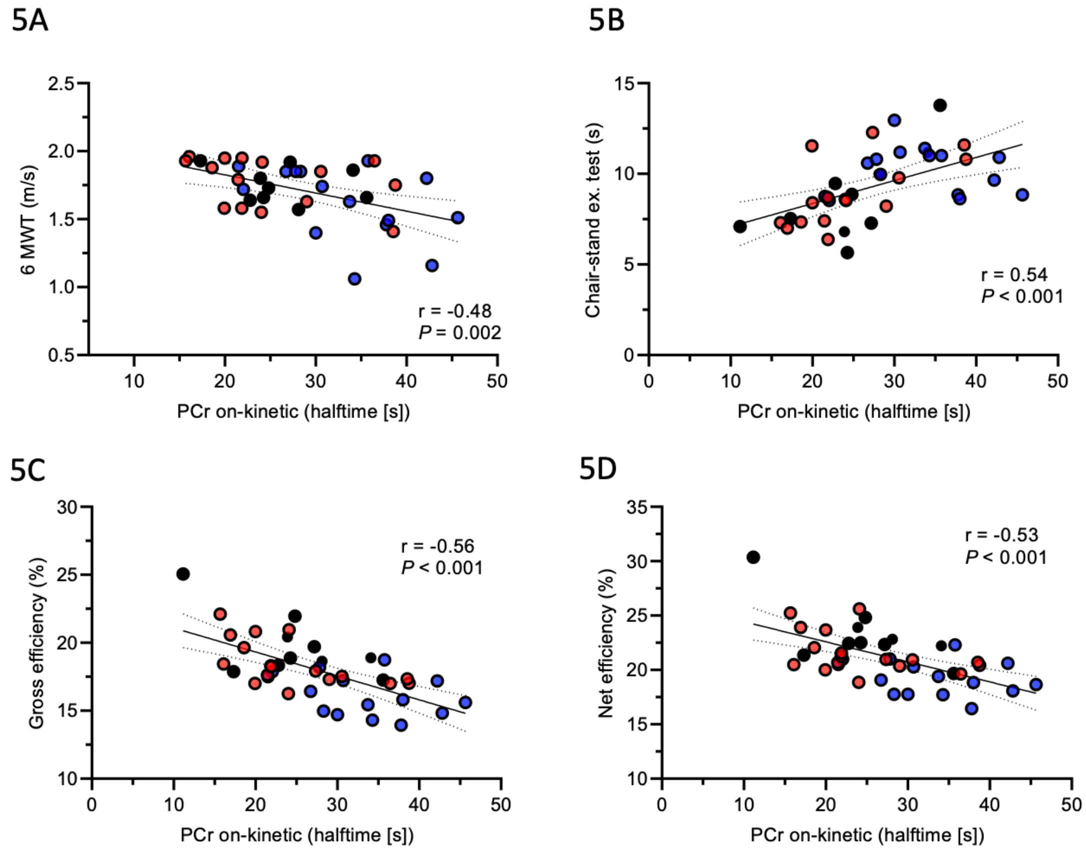

Figure 5. Skeletal muscle mitochondrial inertia is associated with physical function and exercise efficiency in humans. Linear association between PCr on-kinetics and (A) walking speed, (B) chair seating-standing exercise performance, (C) gross and (D) net exercise efficiency. [s]: seconds; Y: young; OT: Older trained; O: Older with normal physical activity. Circles in black: Y; circles in red: OT; circles in blue: O.

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, 18, 19) 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 that there is a relationship between mitochondrial inertia and functional outcomes which is independent of maximal ATP-synthetic capacity of mitochondria. However, considering the close link between skeletal muscle mitochondrial function and exercise efficiency (20), 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 (21). 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 (22). 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 our results, Seiler et al. 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 (23). Elegantly, they 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 (23). 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 (22, 24). 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 (25) and elderly individuals (26), one might expect that higher ADP levels are needed to stimulate the OXPHOS system in these individuals. In line with this notion, we found higher ADP levels during exercise in type 2 diabetic patients and 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.

If a stronger reliance on PCr is a contributor to reduced physical function, increasing the PCr pool (e.g by creatine supplementation) (27, 28) or the acetylcarnitine content (e.g by carnitine supplementation) (29) 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 (27). 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 (29). 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.

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.

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CHAPTER 5

Invasive and non-invasive markers of skeletal muscle mitochondrial function in young healthy males.

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Abstract

Background

Mitochondria are organelles that fuel cellular energy requirements by ATP formation via aerobic metabolism. Given the wide variety of methods to assess skeletal muscle mitochondrial capacity, we tested how well different invasive and non-invasive markers of skeletal muscle mitochondrial capacity reflect mitochondrial respiration in permeabilized muscle fibers.

Methods

Nineteen young males (mean age: 24 ± 4 yrs, VO2max: 31.4 – 55.7 ml/kg/min) were recruited and a muscle biopsy was collected to determine mitochondrial respiration from permeabilized muscle fibers and to quantify markers of mitochondrial capacity/content such as citrate synthase (CS) activity, mitochondrial DNA copy number, TOMM20, VDAC and protein content for complex I-V of the oxidative phosphorylation (OXPHOS) system. Additionally, all participants underwent several non-invasive assessments of mitochondrial capacity. Thus, PCr recovery post exercise was assessed by phosphorous magnetic resonance spectroscopy whereas maximal aerobic capacity and exercise efficiency were determined by cycling exercise.

Results

From the invasive markers, Complex V protein content showed the strongest concordance (Rc = 0.72) with maximally uncoupled mitochondrial respiration. Complex V protein content and CS activity showed the strongest concordance (Rc between 0.50 to 0.72) with ADP-stimulated coupled mitochondrial respiration, fueled by various substrates. From the non-invasive markers, exercise efficiency showed the strongest concordance with maximally uncoupled mitochondrial respiration (Rc = 0.67). For ADP-stimulated coupled mitochondrial respiration, gross exercise efficiency, VO2max and PCr recovery exhibited concordance values between 0.50 and 0.77.

Conclusion

From the invasive markers, Complex V protein content and CS activity are surrogates that best reflect skeletal muscle mitochondrial respiratory capacity. From the non-invasive markers, exercise efficiency and PCr recovery post exercise most closely reflect skeletal muscle mitochondrial respiratory capacity.

Introduction

Mitochondria are organelles that fuel cellular energy requirements by ATP formation via aerobic metabolism and hence largely determine the oxidative capacity of a cell. Alterations in mitochondrial function may therefore negatively impact energy metabolism and have been associated with a wide variety of metabolic diseases such as obesity-related insulin resistance (1) and type 2 diabetes (2). Skeletal muscle is the largest and most metabolically active organ in humans with a crucial role in enabling muscle contraction and therefore motion. Due to the limited storage capacity for ATP, human skeletal muscle is highly enriched with mitochondria to sustain contractile activity.

In humans, skeletal muscle mitochondrial function can be determined by a wide variety of invasive measurements, ranging from the assessment of the activity and/or content of (regulatory) proteins and enzymes of mitochondrial metabolism up to the quantification of oxygen consumption and ATP production rates in muscle specimens and/or isolated mitochondria. Furthermore, non-invasive *in vivo* approaches using magnetic resonance spectroscopy (MRS) have also been applied to determine skeletal muscle oxidative capacity. Thus, *in vivo* skeletal muscle oxidative capacity can be estimated by measuring the recovery rate of phosphocreatine (PCr) after exercise, using phosphorus magnetic resonance spectroscopy (^{31}P-MRS) (3).

While there are numerous methods available to measure skeletal muscle mitochondrial function and oxidative capacity, these various readouts are not always in full agreement with each other. This indicates that other factors may interfere with (some of) the outcome measures and/or that the different markers for mitochondrial function may, partly, reflect different characteristics of mitochondrial metabolism. For instance, it was previously shown that *in vivo* skeletal muscle oxidative capacity as measured by ^{31}P-MRS was significantly associated with skeletal muscle citrate synthase (CS) activity in young, healthy human volunteers with varying level of physical activity, but not with cytochrome c oxidase (COX); both widely used markers of skeletal muscle mitochondrial function (4). Similarly, mitochondrial respiratory capacity from permeabilized muscle fibers was associated with specific markers of skeletal muscle mitochondrial content in young, healthy individuals with a widely different training status (5), such as cardiolipin and CS, but not with mitochondrial DNA copy number (mtDNA) (5).

In the current study, we aimed to examine which commonly used invasive (VDAC, mtDNA, TOMM20, CS, OxPhos) and non-invasive (PCr recovery, VO2max, exercise efficiency) markers of skeletal muscle mitochondrial capacity correlate best with the mitochondrial

respiratory capacity in permeabilized human muscle fibers. The latter was deemed the most relevant outcome for skeletal muscle oxidative capacity in biopsies, since it encompasses factors such as mitochondrial content, enzymatic activity and protein content. Therefore, it is interesting to investigate how this parameter is related to other markers of oxidative capacity determined in biopsies and to *in vivo* measures of oxidative capacity. These associations were examined in young, healthy individuals encompassing a wide range of maximal aerobic capacity based on VO2max. Next, to investigate whether there is a linear relationship between variables, we also studied the agreement between parameters by evaluating their concordance.

Methods and materials

Participants

Participants included in this study were derived from 2 different studies, both performed at Maastricht University and approved by the Ethics Committee of the Maastricht University Medical Center+. Studies were registered at http://clinicaltrials.gov with identifiers NCT03697928 and NCT03666013. For the current study, nineteen young, healthy, male volunteers (aged 18 – 40 years), were included, thirteen individuals from the study with identifier NCT03697928 and 6 individuals from the study with identifier NCT03666013. The study was conducted in accordance with the principles of the declaration of Helsinki and all participants provided their written informed consent. Prior to inclusion and after an overnight fast, all participants underwent a medical screening that included a venous blood sample, a resting electrocardiogram (ECG) and a medical history questionnaire. Exclusion criteria were contraindications for MRI examination, uncontrolled hypertension, smoking and excessive alcohol consumption or drug abuse.

Study design

Participants reported to the university under fasting conditions (5-10 hours) on 2 different days with at least 72h of rest in between test days. On the first day, subjects performed an incremental cycling test until exhaustion to determine maximal aerobic capacity (VO2max) (6). Body composition as well as total body mass were measured using air displacement plethysmography (BODPOD®, Cosmed, Rome, Italy) (7). On the second day, a muscle biopsy was taken while resting. Subsequently, phosphorus magnetic resonance spectroscopy (^{31}P-MRS) was performed to measure *in vivo* skeletal muscle oxidative capacity in *m. vastus*

lateralis as previously described (8) and participants performed a submaximal cycling test at 50% of their maximal power output (as assessed during the VO2max test) to determine gross mechanical exercise efficiency. Participants were instructed to maintain their habitual diet and to refrain from any strenuous physical activity during the three days directly preceding the test days.

Submaximal cycling test and exercise efficiency

During the submaximal exercise test, oxygen consumption (O2) and carbon dioxide (CO2) production were measured by indirect calorimetry for at least 10 minutes. Participants were instructed to maintain a cadence between 60-80 rpm throughout the test. To calculate the energy expenditure upon exercise, the Weir equation (9) was used from the measurements of O2 consumption and CO2 production. Gross energy efficiency (GEE) was calculated as the ratio of power output (watts converted in kJ/min) over exercise energy expenditure (EEE) (kJ/min) and expressed as percentage, as previously reported (10):

GEE (%) = (Work (kJ/min) / EEE (kJ/min)) * 100

Skeletal muscle biopsy

A muscle biopsy was obtained from the *m. vastus lateralis* according to the Bergström method (11) using a side-cutting needle under local anesthesia (1.0% lidocaine without epinephrine). A portion of the muscle biopsy was immediately placed in ice-cold preservation medium (BIOPS, OROBOROS Instruments, Innsbruck, Austria) and used for the assessment of mitochondrial respiratory capacity in permeabilized muscle fibers. The remaining portion of the muscle biopsy was immediately frozen in melting isopentane and stored at -80°C until further analysis.

***Ex vivo* skeletal muscle mitochondrial respiration**

Permeabilized muscle fibers were prepared from the muscle tissue collected in the BIOPS preservation medium, as described previously (12). Subsequently, the permeabilized muscle fibers (~2.5 mg wet weight) were analyzed for *ex vivo* mitochondrial respiration assessment using high-resolution respirometry (Oxygraph, OROBOROS Instruments, Innsbruck, Austria) (13). To prevent oxygen deprivation during the measurement, the respiration chambers were hyper-oxygenated up to ~400 μmol/L O_2. Next, a multi-substrate protocol was used in which

different substrates were added consecutively at saturating concentrations. State 2 respiration was measured upon the addition of malate (4 mmol/L) plus octanoyl-carnitine (50 μmol/L). Subsequently, an excess of 2 mmol/L of ADP was added to determine coupled (state 3) respiration supported by a fatty acid substrate. Coupled (state 3) respiration was then maximized by the subsequent addition of the complex I-linked substrate glutamate (10 mmol/L) and the complex II-linked substrate succinate (10 mmol/L). Finally, the chemical uncoupler carbonylcyanide-4-(trifluromethoxy)-phenylhydrazone (FCCP) was titrated to assess the maximal capacity of the electron transport chain (state u respiration). The integrity of the outer mitochondrial membrane was assessed by the addition of cytochrome C (10 μmol/L) upon maximally coupled respiration. In case cytochrome C increases oxygen consumption >10%, the measurement was excluded from statistical analysis. All measurements were performed in quadruplicate and data are expressed per mg wet weight.

Western blot analysis

Western blot analyses were performed in Bioplex-lysates of human muscle tissue as previously described (14). Equal amounts of proteins were loaded on gradient Bolt 4-12% gels (Novex, Thermo Fisher Scientific, Bleiswijk, The Netherlands). Proteins were transferred to nitrocellulose with the Trans-Blot Turbo transfer system (Bio-Rad Laboratories). The following antibodies and dilutions were used in this study: a cocktail of mouse monoclonal antibodies directed against human OXPHOS (dilution 1:5.000; ab110411, Abcam, Cambridge, UK), as well as antibodies directed against TOMM20 (dilution 1:10.000; aba186734; Abcam), porin/VDAC (dilution 1:1.000; sc-390996; 1:5000, Santa Cruz biotechnology). The specific proteins were detected using secondary antibodies conjugated with IRDye680 or IRDye800 and were quantified with the CLx Odyssey Near Infrared Imager (Li-COR, Westburg, Leusden, The Netherlands).

Quantification of mitochondrial DNA content and citrate synthase activity

Mitochondrial DNA (mtDNA) copy number was determined using quantitative real-time PCR, based on the TaqMan probe method, as described previously (15). mtDNA copy number was calculated from the ratio of NADH dehydrogenase subunit 1 (ND1) to lipoprotein lipase (LPL) (mtDNA/nuclear DNA), as described previously (16). For citrate synthase (CS) activity, ~10 mg of the muscle tissue sample was cut with a cryostat (-20°C), dissolved in 150 μl cold SET buffer (containing 250 mmol/l sucrose, 2 mmol/l EDTA and 10 mmol/l Tris-HCl, adjusted pH

at 7.4) and homogenized. The supernatant was used for the determination of citrate synthase activity according to Shepherd and Garland (17). CS activity was expressed as units per gram of protein.

Magnetic Resonance Spectroscopy

All magnetic resonance spectroscopy (MRS) experiments were performed on a 3T MRI scanner (Achieva 3T-X; Phillips Healtcare, Best, The Netherlands). Food intake was standardized by offering participants a light lunch at noon and asked them to refrain from food until completion of the test day. At 17.00h, participants were positioned in the MRI scanner and ^{31}Phosphorus Magnetic Resonance Spectroscopy (^{31}P-MRS) was performed to measure *in vivo* skeletal muscle oxidative capacity in m. vastus lateralis as previously described (8), using a 6 cm surface coil. A series of 150 unlocalized ^{31}P-spectra were acquired using the following parameters: single acquisition (NSA = 1); repetition time (TR) = 4000ms; spectral bandwidth = 3000 Hz; number of points = 1024. Of the series of 150 spectra, 10 spectra were acquired at rest, 70 spectra acquired during one-legged knee extension and flexion exercise, and 70 spectra during recovery. The exercise was performed inside the scanner using a custom-built device with an adjustable weight. The exercise intensity was chosen to correspond to 50-60% of the one-legged exercise capacity (determined on a separate day). Spectra were analyzed with a custom-built MATLAB script (MATLAB 2018a, Mathworks Inc, Natick, Massachusetts, USA). Inorganic phosphate (Pi), PCr and ATP peaks were fitted, and pH was determined. The PCr recovery was fitted with a mono-exponential function and the rate constant (κ in s^{-1}) was determined as previously reported (8). The rate constant κ of PCr resynthesis is almost entirely dependent on ATP produced by oxidative phosphorylation, hence can be used as a parameter of *in vivo* oxidative capacity (18).

Statistical analyses

Participant characteristics are reported as mean ± standard deviation. Data are presented for the 19 individuals unless otherwise indicated in the table/figure legends. 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. Two-sided Pearson's correlation was calculated between the *ex vivo* mitochondrial respiratory capacity and the invasive and non-invasive markers of mitochondrial oxidative capacity. For the Person's correlations that revealed significance, we subsequently examined the concordance using the Lin's concordance

test, which takes into account the variation of the individual data from the line of identity. Lin's scale is between 0 and 1, where 0 indicates no concordance and 1 indicates perfect concordance. This analysis was performed using relative data, as individual values were related to the mean value in all measures. A P value < 0.05 was set to be statistically significant.

Results

Participant characteristics

The participants' characteristics are shown in table 1. It is important to note that the participants included in the current study presented a wide range of maximal aerobic capacity, from relatively untrained up to well-trained individuals. The other characteristics further classify the participants as young and generally healthy, although also body weight, BMI and body composition displayed a rather wide range.

Table 1. Participant characteristics

Characteristics (n = 19)	**Mean ± SD**	**range**
Age (years)	24.7 ± 4.5	20.0 – 40.0
Body weight (kg)	72.7 ± 9.7	56.9 – 91.0
BMI (kg/m^2)	23.7 ± 2.4	19.3 – 28.0
FM (kg)	16.0 ± 5.8	6.5 – 28.0
Fat Percentage (%)	22.0 ± 7.5	10.4 – 40.4
FFM (kg)	56.6 ± 9.3	41.3 – 72.9
Fasting plasma glucose (mmol/l)	5.0 ± 0.3	4.5 – 5.5
VO2max (ml/kg/min)	43.0 ± 6.8	31.4 – 55.7
Wmax (watts)	243 ± 66	148– 366

Data are presented as mean ± standard deviation. BMI: body mass index; FM: fat mass; FFM: fat-free mass; VO2max: maximal aerobic capacity; Wmax: maximal power output.

Mitochondrial oxygen consumption in permeabilized skeletal muscle fibers

In the present study, we assessed the respiratory capacity in permeabilized muscle fibers and used this as our reference outcome for skeletal muscle mitochondrial capacity. The minimal and maximal values (range) of ADP-stimulated (state 3) mitochondrial respiration, upon the addition of different substrates, as well as the maximally uncoupled mitochondrial respiration (state u) are shown in table 2.

Linear correlations between invasive markers of skeletal muscle mitochondrial capacity and ex vivo mitochondrial respiration in permeabilized skeletal muscle fibers

Besides analyzing mitochondrial respiratory capacity in freshly prepared, permeabilized skeletal muscle fibers, part of the same muscle biopsy was frozen for subsequent determination of several markers of mitochondrial content and/or capacity. Of these markers, citrate synthase (CS) activity, TOMM20 protein content, VDAC protein content as well as the protein content of structural components of most complexes of the oxidative phosphorylation system (OXPHOS) were significantly associated with the ADP-stimulated (state 3) mitochondrial respiration upon the addition of different substrates (Table 2). Correlation coefficients (r) of these significant associations varied between 0.45 to 0.73. Mitochondrial DNA copy number (mtDNA) was not significantly associated with any measure of ADP-stimulated (state 3) mitochondrial respiration upon the addition of different substrates. All markers, except for VDAC and complex II protein content, were significantly associated with the maximal FCCP-induced uncoupled respiration (state u) in permeabilized muscle fibers (Table 2). Correlation coefficients (r) of the significant associations between the various markers and maximally uncoupled respiration ranged between 0.52 to 0.74.

Table 2. Associations between invasive markers of mitochondrial content/capacity and *ex vivo* mitochondrial respiration in permeabilized muscle fibers

		State 3						State u	
		MO3		MOG3		MOGS3			
	Range	r	*P*	r	*P*	r	*P*	r	*P*
MO3 ($pmol*mg^{-1}*s^{-1}$)	22 – 50								
MOG3 ($pmol*mg^{-1}*s^{-1}$)	44 – 79								
MOGS3 ($pmol*mg^{-1}*s^{-1}$)	69 – 114								
State 3 u ($pmol*mg^{-1}*s^{-1}$)	89 – 165								
mtDNA copy number (ND1/LPL)	3.63×10^3 – 7.97×10^3	0.44	0.08	0.32	0.23	0.32	0.23	**0.52**	**0.03**
CS activity (µmol/min/g)	60 – 137	**0.73**	**0.001**	**0.66**	**0.004**	**0.61**	**0.010**	**0.72**	**0.001**
TOMM20 protein content (AU)	0.60 – 1.4	0.41	0.08	**0.51**	**0.02**	**0.57**	**0.01**	**0.62**	**0.005**
VDAC protein content (AU)	0.65 – 1.4	0.002	0.98	**0.53**	**0.02**	0.43	0.06	0.34	0.14
Complex I protein content (AU)	0.14 – 2.5	**0.45**	**0.05**	**0.53**	**0.01**	**0.60**	**0.006**	**0.68**	**0.002**
Complex II protein content (AU)	0.36 – 1.9	0.14	0.57	0.41	0.08	**0.53**	**0.02**	0.36	0.13
Complex III protein content (AU)	0.66 – 1.5	**0.50**	**0.02**	0.34	0.14	**0.56**	**0.01**	**0.63**	**0.004**
Complex IV protein content (AU)	0.40 – 1.5	**0.46**	**0.04**	**0.48**	**0.03**	**0.57**	**0.01**	**0.56**	**0.01**
Complex V protein content (AU)	0.62 – 1.3	**0.66**	**0.002**	**0.62**	**0.005**	**0.57**	**0.01**	**0.74**	**<0.001**

ADP-stimulated respiration fueled by malate + octanoyl-carnitine (MO3), by malate + octanoyl-carnitine + glutamate (MOG3) and by malate + octanoyl-carnitine + glutamate + succinate (MOGS3); State 3u: maximal FCCP-induced uncoupled mitochondrial respiration; r; Pearson correlation coefficient; *P*; significance value; CS: citrate synthase; AU: arbitrary units. Data for CS activity and mtDNA are missing from 2 participants due to the limited size of their muscle biopsy.

Concordance between invasive markers of skeletal muscle mitochondrial capacity and *ex vivo* mitochondrial respiration in permeabilized skeletal muscle fibers

Next, if a significant linear relationship between parameters was found, we also aimed to test how well the two parameters agreed, in other words, whether the variability in the mitochondrial respiratory capacity in permeabilized muscle fibers is similarly reflected in the various invasive skeletal muscle markers for mitochondrial content/capacity. For this purpose, we computed Lin's concordance coefficient (Rc) for those skeletal muscle markers that were significantly associated with either maximal FCCP-induced uncoupled mitochondrial respiration (state u) or the coupled ADP-stimulated mitochondrial (state 3). The results of this analysis are displayed in table 3 and in figure 1, where the various skeletal muscle markers are listed according to their Rc rank in relation to the maximal uncoupled mitochondrial respiration (state u).

Complex V protein content showed the strongest concordance (Rc = 0.72, figure 1A) with maximally uncoupled respiration, followed by CS activity (Rc = 0.70, figure 1B), complex III protein content (Rc = 0.60), TOMM20 protein content (Rc = 0.60), mtDNA content (Rc = 0.49), complex IV protein content (Rc = 0.43) and complex I protein content (Rc = 0.36) (Table 3). With respect to maximal ADP-stimulated respiration, CS activity displayed the strongest concordance (Table 3) upon the different substrate combinations (Rc between 0.50 - 0.72, figure 1C-E), whereas complex V protein content exhibited the second strongest concordance (Rc between 0.50 - 0.64, figure 1F-H). TOMM20 protein content, VDAC protein content and protein content for complex I-IV of the OXPHOS system exhibited lower concordance values with ADP-stimulated respiration upon the various substrate combinations, ranging between 0.23 – 0.48 (table 3).

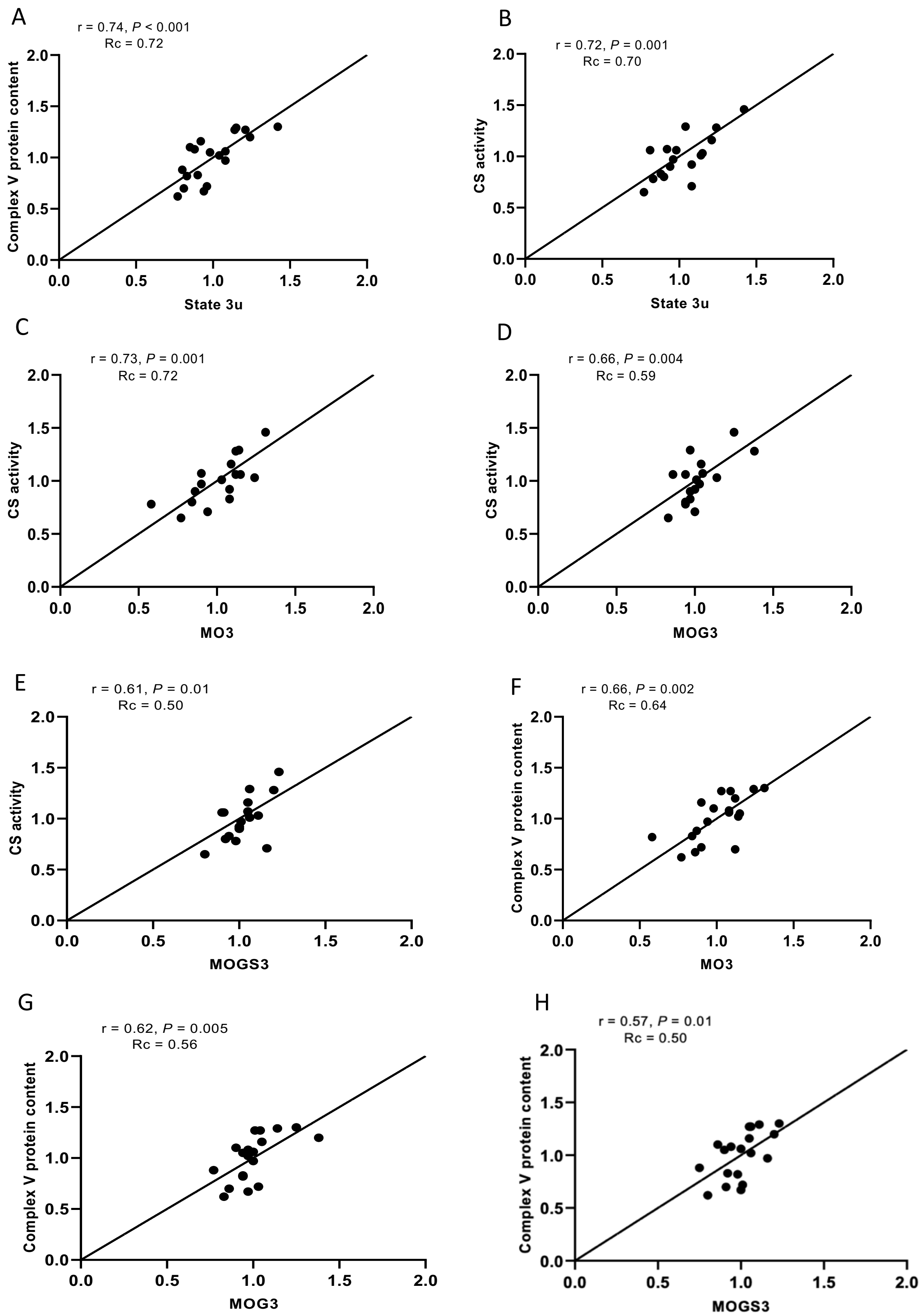

Figure 1. Concordance (or agreement) between mitochondrial respiration in permeabilized muscle fibers and invasive markers of mitochondrial content/capacity. Examples with strongest concordance (Rc) are shown. The black lines represent the line of identity (slope = 1, off-set =0) between variables.

Table 3. Linear correlation coefficients and concordance between invasive markers of mitochondrial content/capacity and *ex vivo* mitochondrial respiration in permeabilized muscle fibers

	State 3									State 3u		
	MO3			MOG3			MOGS3					
	r	*P*	Rc	r	*P*	Rc	r	*P*	Rc	r	*P*	Rc
Complex V protein content (AU)	0.66	0.002	0.64	0.62	0.005	0.56	0.57	0.01	0.50	0.74	<0.001	0.72
CS activity (µmol/min/g)	0.73	0.001	0.72	0.66	0.004	0.59	0.61	0.01	0.50	0.72	0.001	0.70
Complex III protein content (AU)	0.50	0.02	0.48				0.56	0.01	0.47	0.63	0.004	0.60
TOMM20 protein content (AU)				0.51	0.02	0.46	0.57	0.01	0.47	0.62	0.005	0.60
mtDNA copy number (ND1/LPL)										0.52	0.03	0.49
Complex IV protein content (AU)	0.46	0.04	0.38	0.48	0.03	0.34	0.57	0.01	0.38	0.56	0.01	0.43
Complex I protein content (AU)	0.45	0.05	0.23	0.53	0.01	0.25	0.60	0.006	0.25	0.68	0.002	0.36
Complex II protein content (AU)							0.53	0.02	0.32			
VDAC protein content (AU)				0.53	0.02	0.45						

ADP-stimulated respiration fueled by malate + octanoyl-carnitine (MO3), by malate + octanoyl-carnitine + glutamate (MOG3) and by malate + octanoyl-carnitine + glutamate + succinate (MOGS3); State 3u: maximal FCCP-induced uncoupled mitochondrial respiration; r; Pearson correlation coefficient; *P*; significance value; Rc: Lin's concordance coefficient; CS: citrate synthase; AU: arbitrary units.

Linear correlations between non-invasive measures of oxidative capacity and *ex vivo* mitochondrial respiration in permeabilized skeletal muscle fibers

Because mitochondrial respiratory capacity in permeabilized human muscle fibers is the gold standard, invasive measure of mitochondrial function, we were also interested to investigate how this parameter related to non-invasive readouts for skeletal muscle and whole-body oxidative capacity. We found that the maximal *in vivo* skeletal muscle oxidative capacity, expressed by the phosphocreatine (PCr) recovery rate constant after exercise, was significantly associated with MO3 ($r = 0.81$, $P < 0.001$), MOG3 ($r = 0.62$, $P = 0.008$) and the maximally uncoupled respiration ($r = 0.61$, $P = 0.001$) (Table 4). Furthermore, also maximal aerobic capacity (i.e., VO2max) and gross exercise efficiency were significantly associated with ADP-stimulated and maximally uncoupled mitochondrial respiration (r coefficients between 0.51 and 0.69, all P values < 0.05; Table 4).

Table 4. Associations between non-invasive measures of oxidative capacity and mitochondrial respiration in permeabilized muscle fibers

		State 3						State 3u	
		MO3		MOG3		MOGS3			
	Range	r	P	r	P	r	P	r	P
PCr recovery constant rate [s^{-1}]	0.025 – 0.045	**0.81**	**<0.001**	**0.62**	**0.008**	0.43	0.08	**0.61**	**0.001**
VO2max (ml/kg/min)	31.4 – 55.7	**0.54**	**0.01**	**0.51**	**0.02**	**0.61**	**0.005**	**0.54**	**0.01**
Gross exercise efficiency (%)	14.6 – 25.4	**0.54**	**0.01**	**0.62**	**0.005**	**0.62**	**0.004**	**0.69**	**0.001**

ADP-stimulated respiration fueled by malate + octanoyl-carnitine (MO3), by malate + octanoyl-carnitine + glutamate (MOG3) and by malate + octanoyl-carnitine + glutamate + succinate (MOGS3); State 3u: maximal FCCP-induced uncoupled mitochondrial respiration; r; Pearson correlation coefficient; *P*; significance value CS: citrate synthase; AU: arbitrary units. PCr recovery data is missing in one participant due to implication of the SARS-CoV-19 outbreak and one other subject has been excluded from the PCr recovery data analysis due to a pH decline below 6.9.

Concordance between non-invasive measures of oxidative capacity and *ex vivo* mitochondrial respiration in permeabilized skeletal muscle fibers

Similar to the invasive markers for skeletal muscle mitochondrial capacity, we next aimed to determine the agreement of the non-invasive readouts of skeletal muscle and whole-body oxidative capacity by calculating the Lin's concordance coefficient (Rc) for those measures that significantly associated with *ex vivo* mitochondrial respiration. The results of this analysis are displayed in table 5, where the various measures are again ranked according to their Rc value for maximally uncoupled mitochondrial respiration.

Gross exercise efficiency showed the strongest concordance with maximally uncoupled mitochondrial respiratory capacity (Rc = 0.67, figure 2A), followed by PCr recovery rate constant (Rc = 0.59, figure 2B) and maximal aerobic capacity (VO2max) (Rc = 0.53). For maximally coupled, ADP-stimulated respiration upon complex I- and complex I+II-linked substrates, gross exercise efficiency also displayed the strongest concordance (Rc = 0.62 and 0.61, respectively, figure 2C-D, table 5) followed by PCr recovery rate constant (Rc = 0.62 for MOG3) and VO2max (Rc = 0.50 for MOG3 and Rc = 0.60 for MOGS3, table 5). PCr recovery rate constant exhibited the strongest concordance with ADP-stimulated respiration fueled by a lipid substrate (Rc = 0.77, figure 2E), followed by maximal aerobic capacity (Rc = 0.54) and exercise efficiency (Rc = 0.53).

Finally, we also determined the association and concordance among the non-invasive measures of oxidative capacity (supplemental table 1). PCr recovery post exercise, exercise gross efficiency and VO2max exhibited significant associations (r coefficients between 0.53 and 0.64) with a moderate to substantial concordance (Rc ranged between 0.51 to 0.63).

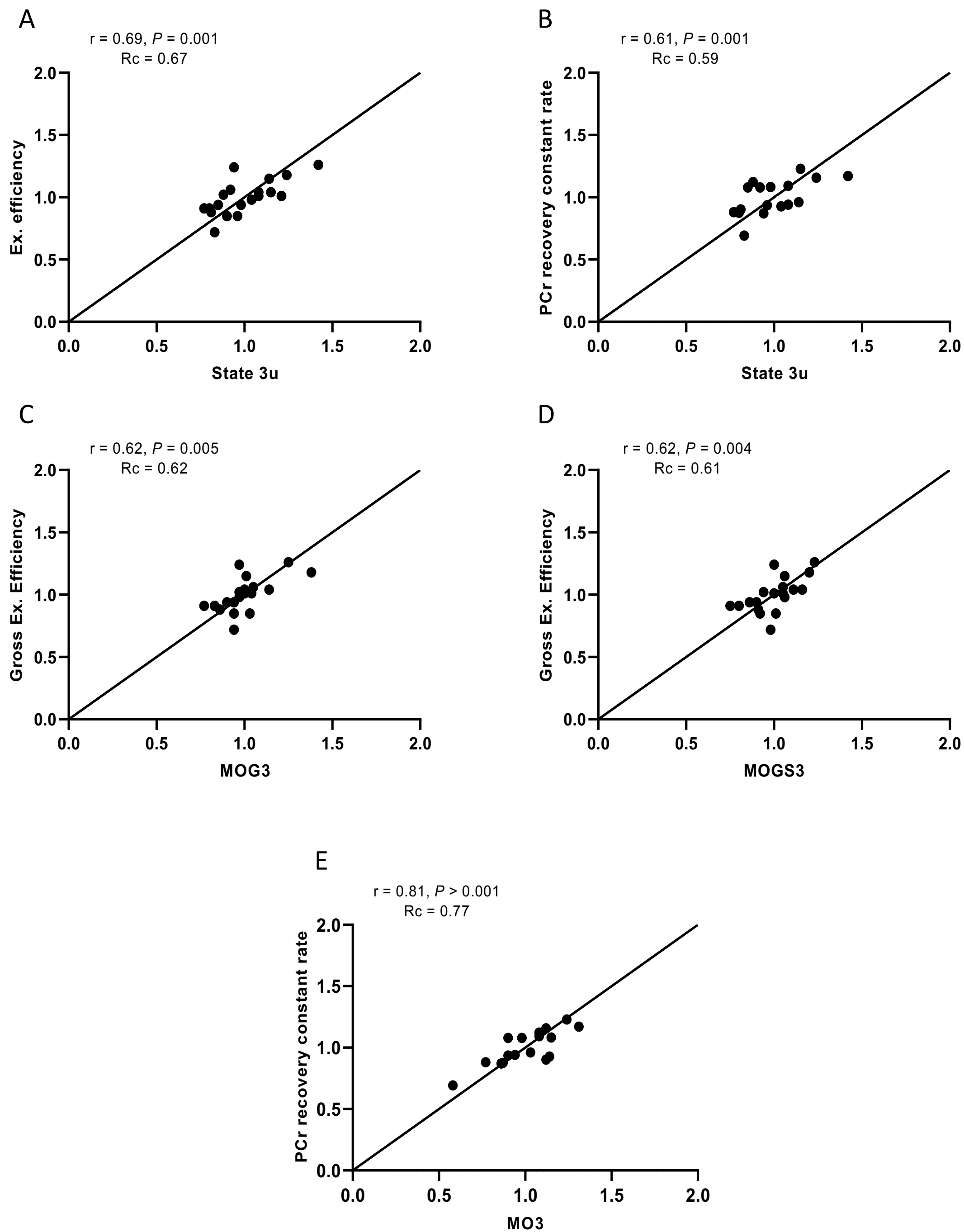

Figure 2. Concordance between mitochondrial respiration in permeabilized muscle fibers and the non-invasive readouts of exercise efficiency (A-D) and PCr recovery rate constant (E) that exhibited the strongest concordance (Rc). The black lines represent the line of identity (slope = 1, off-set =0) between variables.

Table 5. Linear correlation coefficients and concordance between non-invasive measures of oxidative capacity and *ex vivo* mitochondrial respiration in permeabilized muscle fibers

	State 3									State 3u		
	MO3			MOG3			MOGS3					
	r	*P*	Rc	r	*P*	Rc	r	*P*	Rc	r	*P*	Rc
Gross exercise efficiency (%)	0.54	0.01	0.53	0.62	0.005	0.62	0.62	0.004	0.61	0.69	0.001	0.67
PCr recovery constant rate [s^{-1}]	0.81	<0.001	0.77	0.62	0.008	0.62				0.61	0.001	0.59
VO2max (ml/kg/min)	0.54	0.01	0.54	0.51	0.02	0.50	0.61	0.005	0.60	0.54	0.01	0.53

ADP-stimulated respiration fueled by malate + octanoyl-carnitine (MO3), by malate + octanoyl-carnitine + glutamate (MOG3) and by malate + octanoyl-carnitine + glutamate + succinate (MOGS3); State 3u: maximal FCCP-induced uncoupled mitochondrial respiration; r; Pearson correlation coefficient; *P*; significance value; Rc: Lin's concordance coefficient; CS: citrate synthase; AU: arbitrary units.

Discussion

We here aimed to examine how well different invasive and noninvasive markers of mitochondrial content/capacity reflect mitochondrial respiratory capacity in permeabilized muscle fibers in young, healthy individuals characterized by a wide range of maximal aerobic capacity. The main findings of the present study were that several markers of skeletal muscle mitochondrial content/capacity determined in muscle biopsy specimens were significantly associated with mitochondrial respiratory capacity in permeabilized skeletal muscle fibers and also showed a fair to substantial concordance. The invasive markers with the strongest association and strongest concordance with mitochondrial respiratory capacity were protein content for complex V of the OXPHOS system and citrate synthase (CS) activity. In addition, we showed that various non-invasive readouts for skeletal muscle and whole-body oxidative capacity were significantly associated with mitochondrial respiratory capacity and exhibited a moderate to substantial concordance. The non-invasive readout with the strongest association with mitochondrial respiratory capacity along with the strongest concordance was gross exercise efficiency, followed by PCr recovery post-exercise and maximal aerobic capacity.

Invasive markers of skeletal muscle mitochondrial content/capacity

Classically, the overall volume of the mitochondrial pool is thought to reflect its functional capacity (19). In line with this reasoning, our findings showed that different markers of mitochondrial content mirror skeletal muscle mitochondrial respiratory capacity to a certain extent, with CS activity and protein content of complex V showing the highest level of concordance. Given the relatively small sample size of the present study in combination with the modest difference between these markers, it cannot be concluded from the current study which one is a better reflection of mitochondrial respiratory capacity.

Interestingly, our findings also showed that some invasive markers appear to be more closely related to state 3 respiration, whereas others associate closer with the capacity of the electron transport chain (as reflected by maximally uncoupled respiration). This suggests that different invasive markers of mitochondrial capacity may reflect distinct aspects of mitochondrial metabolism.

Our results are in agreement with a previous study that compared the content and activity of different markers of mitochondrial content and also tested their concordance with the maximal coupled mitochondrial respiration in permeabilized muscle (5). However, that study also indicated that assessment of the enzymatic activity for complex II and complex IV of the OXPHOS system was superior to CS activity, mtDNA copy number and the enzymatic activity

and protein content for complex I, III and V of the OXPHOS system to reflect maximal coupled mitochondrial respiration in healthy young individuals (5). We also showed that TOMM20, VDAC and mtDNA are relatively poor proxies in estimating mitochondrial respiratory capacity. In light of the fact that these markers are widely used in the field to explore skeletal muscle mitochondrial adaptations upon exercise training (20-22), their poor reflection of mitochondrial respiratory capacity is an important and somewhat unanticipated finding.

The poor concordance of TOMM20, VDAC and mtDNA with mitochondrial respiratory capacity may in part be explained functionally, as these markers of mitochondrial content are not directly involved in the electron transport chain and/or phosphorylation system.

For studies that aim to investigate skeletal muscle mitochondrial respiratory capacity, but do not have the opportunity to measure mitochondrial oxygen consumption or ATP production rates via high resolution respirometry methodology, it is essential to make a good choice in regard to the stronger (CS activity and protein content for complex V of the OXPHOS system) and weaker (mtDNA and VDAC protein content) markers of mitochondrial oxidative capacity.

Non-invasive measures of oxidative capacity and ex vivo mitochondrial respiration in permeabilized muscle fibers

We found that non-invasive readouts for skeletal muscle and whole-body oxidative capacity, such as PCr recovery post exercise, maximal whole body aerobic capacity as well as exercise efficiency were significantly associated and exhibited a moderate to substantial concordance with skeletal muscle mitochondrial respiration. Consistent with our results, prior investigations have documented significant associations between PCr recovery post exercise (23, 24), maximal whole-body aerobic capacity (25) and exercise efficiency (26, 27) with mitochondrial respiration from permeabilized muscle fibers. Thus, it was previously shown that the maximal skeletal muscle oxidative capacity, as estimated by PCr recovery post-exercise via ^{31}P-MRS, exhibits a strong linear relationship with skeletal muscle mitochondrial respiration and displays a robust test-retest reliability in a similar cohort of human volunteer as in the present study (24). Furthermore, PCr recovery constant rate was shown to be significantly associated with citrate synthase activity (28).

In addition, improvements in exercise efficiency (26) and maximal aerobic capacity (29) have been shown to parallel increases in skeletal muscle mitochondrial respiration after regular physical activity and exercise training. However, the agreement between exercise efficiency and maximal aerobic capacity with mitochondrial respiration has not been reported before.

Our data extend such previous findings and surprisingly indicate that especially exercise efficiency shows a high degree of agreement with the skeletal muscle mitochondrial respiratory capacity. Furthermore, skeletal muscle oxidative capacity as determined by PCr recovery post-exercise was in good agreement and therefore support the widespread use of PCr recovery post-exercise as a valid muscle-specific marker of mitochondrial respiration.

These findings suggest that similar limitations exist in mitochondrial respiration in permeabilized muscle fibers and *in vivo* function in the current, generally healthy population. In addition, these findings may benefit studies that aim to investigate mitochondrial function but are without the opportunity to collect muscle biopsies.

In the present study, we included young healthy volunteers characterized by a physiological range of maximal aerobic capacity that can be targeted by exercise training interventions. Yet, there was not a perfect concordance between the various invasive and non-invasive markers of mitochondrial capacity with mitochondrial respiration in permeabilized muscle fibers. This indicates that the positive effects of exercise training on skeletal muscle mitochondrial function are not guaranteed to be detected, depending on the selected marker to be analyzed. Certainly, the selection of a poor marker of mitochondrial function can hamper conclusions to be drawn.

Obviously, it should be acknowledged that various differences between the *ex vivo* and *in vivo* assessments contribute to the fact that there is no perfect agreement between these markers, i.e., these markers may (at least in part) reflect different aspects of mitochondrial metabolism. Thus, when assessing the respiratory capacity of permeabilized muscle fibers, the oxygraph chamber is hyperoxygenated and supplied with excess concentrations of substrates, hence oxygen and substrate (transport) are not limiting. Moreover, the permeabilization procedure of the sarcolemma further expedites the diffusion of oxygen and substrates into the mitochondria. In vivo, blood flow to the active limbs affects oxygen availability within the contracting muscle tissue, hence affecting the ATP synthesis capacity (30). Conversely, the concentration of substrates and reducing equivalents as well as ADP during exercise *in vivo* are dictated by the energy stress imposed by contraction and often regulated by intracellular substrate transport. Even the level of concordance among the various non-invasive measures of oxidative capacity was surprisingly modest. Because these non-invasive measures share multiple factors that integrally determine oxidative capacity (e.g., muscle blood flow, mitochondrial content and mitochondrial enzyme activity, oxygen and substrate transport), we had anticipated a higher level of concordance among these outcomes. These data indicate that factors that underly muscle-specific oxidative capacity still differ to those that govern whole-body oxidative

capacity. Similarly, these data suggest that factors that underly maximal aerobic capacity still differ to those that determine exercise efficiency.

In conclusion, the present study shows that protein content for complex V of the OXPHOS system and CS activity are invasive markers of mitochondrial function that best reflect skeletal muscle mitochondrial respiratory capacity as assessed in permeabilized muscle fibers. Other invasive markers of skeletal muscle mitochondrial density such as mtDNA, TOMM20, VDAC and protein content for complex I-IV of the OXPHOS system, represent less accurate surrogates for testing mitochondrial respiration from permeabilized muscle fibers. Moreover, the present study shows that PCr recovery post-exercise, maximal aerobic capacity, and exercise efficiency display high agreement with mitochondrial respiration from permeabilized muscle fibers. Exercise efficiency was the noninvasive marker that reflects mitochondrial respiratory capacity best, followed by PCr recovery post-exercise and maximal aerobic capacity (VO2max). These results are of relevance for studies that aim to investigate skeletal muscle mitochondrial function but do not possess the high resolution respirometry methodology and/or are unable to obtain muscle biopsies.

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Supplementary table 1. Linear correlation coefficients and concordance between non-invasive measures of oxidative capacity

	PCr recovery constant rate [s]			Gross exercise efficiency (%)			VO2max (ml/kg/min)		
	r	*P*	Rc	r	*P*	Rc	r	*P*	Rc
PCr recovery constant rate [s]				0.55	0.02	0.54	0.53	0.03	0.51
Gross exercise efficiency (%)	0.55	0.02	0.54				0.64	0.003	0.63
VO2max (ml/kg/min)	0.53	0.03	0.51	0.64	0.003	0.63			

r; Pearson correlation coefficient; *P*; significance value; Rc: Lin's concordance coefficient; CS: citrate synthase; [s]; seconds

CHAPTER 6

High intensity interval training improves whole-body insulin sensitivity, skeletal muscle oxidative capacity and modifies intrahepatic lipid composition in overweight/obese individuals

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Abstract

Background

High intensity interval training (HIIT) is considered a time-efficient alternative for conventional exercise training and may hence be a promising exercise modality to counteract obesity-related metabolic impairments. Here, we investigated if HIIT prompts beneficial effects on insulin sensitivity, muscle oxidative capacity and intrahepatic lipid (IHL) content in overweight/obese individuals. Carbohydrate-rich and insulinogenic sports drinks are frequently consumed after training and may impact the outcome of the training. Hence, we also explored if co-ingestion of a standardized glucose and casein hydrolysate post-exercise affects the anticipated HIIT-mediated metabolic improvements.

Methods

Twenty-three, overweight and obese adults (10 males and 13 females, mean age: 64 ± 7.5 yr, BMI: 31.8 ± 3.3 kg/m^2) completed 12 weeks of progressive HIIT on a stationary bike while consuming either water during/post exercise (HIIT+WTR) or with co-ingestion of a glucose and casein hydrolysate immediately post-exercise (HIIT+CHO/PRO). Before and after the HIIT program, whole-body insulin sensitivity was assessed by a 2-step hyperinsulinemic-euglycemic clamp while muscle oxidative capacity and IHL content and composition were determined by magnetic resonance spectroscopy.

Results

HIIT significantly improved maximal aerobic capacity (*P* < 0.001) as well as skeletal muscle oxidative capacity (*P* < 0.001) to a similar extent in both groups. Thus, VO_2max improved from 41.0 ± 1.7 to 44.8 ± 1.8 ml/FFM/min in the HIIT+WAT group and from 42.8 ± 1.9 to 47.5 ± 2.0 ml/FFM/min in HIIT+CHO/PRO, whereas the PCr recovery rate constant (parameter of muscle oxidative capacity) improved in the HIIT+WAT group from 0.030 ± 0.002 to 0.041 ± 0.003 s^{-1} and in the HIIT+CHO/PRO group from 0.031 ± 0.002 to 0.038 ± 0.003 s^{-1}. HIIT tended to decrease IHL content (*P* = 0.06), significantly increased the PUFA fraction (*P* = 0.048) and tended to decrease the MUFA fraction (*P* = 0.08) in the liver, whereas the SFA fraction did not change. No differences were observed between groups with respect to IHL content or composition. Whole-body insulin sensitivity, as reflected by the M-value, improved significantly upon HIIT, to a similar extent in both groups (HIIT+WAT: Δ 8.2 ± 3.7 and

HIIT+CHO/PRO: Δ 6.9 ± 4.1 μmol/FFM/min, $P = 0.01$). No changes in body weight and body composition were observed.

Conclusion

HIIT is an effective training method to enhance insulin sensitivity, muscle oxidative capacity, and modify intrahepatic lipid composition in overweight/obese individuals. HIIT also tends to decrease intrahepatic lipid content in overweight/obese individuals. These benefits can be attained regardless of co-ingesting glucose and proteins after exercise and occurred in the absence of changes in body weight and body composition.

Introduction

Obesity-related skeletal muscle insulin resistance is an early signature of type 2 diabetes mellitus (T2DM) pathogenesis which coexists with diminished *in vivo* and *ex vivo* mitochondrial capacity (1, 2), systemic hyperinsulinemia, and ectopic fat accumulation in muscle, liver, pancreas and heart (3). Regular exercise training - as part of promoting a healthy lifestyle - is, in combination with caloric restriction, the first-line strategy for the prevention and treatment of skeletal muscle insulin resistance. In fact, conventional exercise training methods, consisting of moderate intensity aerobic-type exercise, sometimes combined with resistance-type exercise, have been shown to enhance skeletal muscle insulin sensitivity in obese, metabolically compromised individuals (4) as well as in patients with T2DM (5). The insulin sensitizing effects of conventional exercise training are thought to be (partly) mediated by the exercise-induced skeletal muscle glycogen turnover (6), which in turn is largely affected by exercise intensity (7). Moreover, conventional exercise training is paralleled by improved skeletal muscle mitochondrial function (8) and reduced intrahepatic lipid content (9), both of which likely contribute to improved glucose homeostasis as well. Adherence to a conventional exercise regimen, however, is notoriously low in people at risk for developing T2DM compromising the efficacy of exercise programs in preventing metabolic disease. In this context, there is a growing appreciation for the potential of high intensity interval training (HIIT), as a time efficient and generally well-tolerated (10) alternative exercise methodology, to enhance metabolic health in obese, metabolically compromised individuals. Furthermore, since HIIT specifically targets skeletal muscle glycogen turnover due to its high intensity nature, this exercise modality may optimally convey beneficial effects on whole-body and skeletal muscle insulin resistance.

Indeed, a HIIT protocol comprising just ten 1-minute bouts of cycling at 90% of maximal heart rate, interspersed by 1 minute of recovery, was shown to reduce 24-hour glucose levels in obese and T2DM patients after performing only 6 sessions over a 2-week period (11). Furthermore, it has also been shown that HIIT promotes GLUT-4 protein content (11, 12) and expression of mitochondria-related genes (11, 13) in skeletal muscle from obese, metabolically compromised individuals. However, whether such HIIT-induced adaptations coincide with improved whole-body insulin-mediated glucose disposal and augmented skeletal muscle oxidative capacity has not yet been investigated in overweight/obese individuals at risk for developing diabetes. Therefore, the main aim of the present study was to test if 12 weeks of HIIT, performed 3 times per week, improves whole-body insulin sensitivity and *in vivo* skeletal muscle oxidative

capacity in overweight/obese individuals. In addition, we also assessed the effects of HIIT on intrahepatic lipid content and composition.
Another aspect that we investigated was the effect of the co-ingestion of carbohydrate and protein on the HIIT-induced physiological and metabolic adaptations. The consumption of soft or sports drinks has increased exponentially in the past decade (14) and ingestion of insulinogenic, carbohydrate rich drinks after an exercise session is not uncommon. Insulinogenic, carbohydrate rich drinks are likely to interfere with post-exercise skeletal muscle glycogen metabolism (15) and may hence blunt the insulin sensitizing effects of exercise (16). Therefore, the second aim of this study was to test if ingestion of a casein-hydrolysate with added maltodextrin after each training session blunts the effect of HIIT on insulin sensitivity and *in vivo* skeletal muscle oxidative capacity in overweight/obese individuals.

Research design and methods

Participants

Twenty-nine, overweight and obese adults (14 males and 15 postmenopausal females, BMI ≥ 27 – 38 kg/m^2, between 45-75 years old) were recruited from the general population in the vicinity of Maastricht through advertisements. Secondary inclusion criteria were stable body weight (no body mass fluctuations > 3 kg in the past three months) and sedentary behavior (self-reported < 150 minutes/ week regular physical activity). Exclusion criteria were T2DM (HbA1c > 6.5%), uncontrolled hypertension, alcohol consumption > 30 g/day, use of beta-blockers and medication known to interfere with glucose homeostasis/metabolism. Of these twenty-nine recruited participants, twenty-three individuals completed the study (10 males and 13 females). One participant (from the HIIT+WTR group) dropped out before starting the training intervention, whereas the other two participants (from the HIIT+WTR group) dropped out in the last week of the training intervention, all three due to personal reasons. One participant was diagnosed with serious adverse event during the training intervention, not related to the study. Therefore, this participant was considered as a drop-out and not included in the data analysis. Furthermore, two individuals (from the HIIT+CHO/PRO group) had to stop the training period (half-way of the training intervention) due to the SARS-CoV-2 (COVID-19) outbreak in The Netherlands in early 2020. The study protocol was approved by the institutional Medical Ethical Committee and conducted in accordance with the declaration

of Helsinki. All subjects provided their written informed consent after the study protocol was explained and before the first measurement was performed.

Study overview

All volunteers underwent a supervised and personalized progressive high intensity interval training (HIIT) program three times per week, during twelve weeks at Maastricht University. Prior to inclusion, volunteers underwent a medical screening visit after 10-12 hours fasting that included the collection of a fasted blood sample, a medical history questionnaire, determination of blood pressure, body weight, height and an electrocardiogram (ECG) at rest. After the screening procedure and when eligible, baseline measurements were performed on three different days. On test day 1, whole-body insulin sensitivity was assessed via a 2-step hyperinsulinemic-euglycemic clamp. On test day 2, intrahepatic lipid (IHL) content and composition were determined via proton magnetic resonance spectroscopy (^{1}H-MRS), followed by the evaluation of body composition (BodPod) and maximal aerobic capacity (VO_2 max following incremental exercise on a stationary bike until voluntary exhaustion). On test day 3, skeletal muscle oxidative capacity was estimated by measuring the recovery rate of phosphocreatine (PCr) post-exercise by phosphorus MRS (^{31}P-MRS). Subsequently, participants were randomly allocated to two different groups and balanced for age, sex, BMI and maximal aerobic capacity by use of randomization by minimization. In a parallel design, both groups performed the same HIIT-based training protocol, during which one group consumed water *ad libitum* during and after each training session (HIIT+WTR, n = 12), whereas the other group (HIIT+CHO/PRO, n=11) - next to *ad libitum* access to water - co-ingested 15 grams of Maltodextrin (Fantomalt, Nutricia, The Netherlands, commercially available) mixed with 10 grams of casein hydrolysate (PeptoPro, Body&Fit, The Netherlands, commercially available) dissolved in 250 ml of tap water, within a time window of 30 minutes after each training session. At the end of the training period, all measurements were repeated within 48-72 hours after the last training session with measurements being performed at the same clock time. Participants were instructed to maintain their regular eating and daily physical activity behavior throughout the intervention period. The experimental design is schematically shown in figure 1.

Whole-body insulin sensitivity, as reflected by the M-value, was the primary outcome parameter. Secondary outcomes included metabolic flexibility, *in vivo* skeletal muscle oxidative capacity and intrahepatic lipid content as well as intrahepatic lipid composition.

Exercise training protocol

The HIIT protocol consisted of 10x 1 minute cycling bouts at 80-90% of maximal power output (Wmax) interspersed with 2 minutes of recovery, 3 sessions per week during 12 weeks. HIIT sessions were carried out on a cycle ergometer set in a constant load mode at a cadence between 60-80 rpm. Throughout every exercise session, participants wore a heart rate monitor (Polar A300 + H7 HR sensor), with heart rate being recorded at the beginning and at the end of each cycling bout. During the 2 minutes of recovery, participants rested or pedaled slowly at the resistance of 50 watts. Each training session included a 5-minute warming up at 50 watts at a pedal cadence between 60-80 rpm. Including the warming up period, the total time commitment of the HIIT sessions was 35 minutes. Every 4 weeks, Wmax was reassessed upon a progressive maximal cycling test and cycling loads during the consecutive training sessions were adjusted accordingly.

HIIT sessions were performed either in the morning (08.00 – 10.00h) or afternoon (15.00 – 17.00h) depending on the participants availability and personal preferences, with maximally 3 participants at a time and tightly supervised. All exercise sessions were supervised by one of the researchers.

Hyperinsulinemic-euglycemic clamp (HIEC)

All participants underwent a 2-step (10 and 40 $mU/m^2/min$ of insulin) hyperinsulinemic-euglycemic clamp 4-5 days before starting the HIIT period and within 48-72 hours after the last training session. On the evening prior to the clamp, participants consumed a standardized high carbohydrate meal and were instructed to avoid alcohol consumption and vigorous physical activity during the 2 days prior to the clamp. Participants reported to the research unit after a 10-12 hour overnight fast. After 180 minutes, a low dose insulin infusion was started (10 $mU/m^2/min$) for 3 hours, subsequently followed by high dose insulin infusion (40 $mU/m^2/min$) for 2.5 hours. During each insulin infusion phase, plasma glucose concentrations were maintained by concomitant infusion of glucose enriched with [6,6-$^2H_2$]-glucose to maintain a steady-state plasma glucose level of approximately 5.0 mmol/L. Subsequently, whole-body insulin sensitivity was determined by calculating the M-value (μmol/FFM/min).

During the last 30 minutes of basal phase (t = 150-180 min), low insulin infusion (t = 330-360 min) and high insulin infusion (t = 480-510 min), blood samples were collected and substrate oxidation was measured by indirect calorimetry (Omnical, Maastricht Instruments,

Maastricht). Here, only data regarding substrate oxidation at basal state and high insulin infusion phase are reported.

Metabolic flexibility

Carbohydrate and lipid oxidation in the basal state and under insulin stimulated conditions were calculated according to Peronnet et al. (17). Metabolic flexibility was expressed as the change in the respiratory exchange ratio (Δ delta RER) from the fasted state (t = 150-180 min) to high insulin-stimulated condition (t = 480-510 min).

Magnetic resonance spectroscopy

Intrahepatic lipid content and composition

All magnetic resonance spectroscopy (MRS) measurements were performed on a 3-Tesla whole body MRI scanner (Achieva T-X, Philips, Healthcare, Best, The Netherlands). Proton magnetic resonance spectroscopy (^{1}H-MRS) was applied to quantify intrahepatic lipid (IHL) content and composition at 07.00 AM after 10-12 hours overnight fasting. For IHL content and composition, a 32- channel sense cardiac/torso coil (Philips Healthcare, Best, The Netherlands) was used. As previously reported (18), all spectra were acquired by using a STEAM sequence (19) with a repetition time (TR) 4500 ms and an echo time (TE) of 20 ms with a mixing time (TM) of 16 ms, spectral bandwidth 2000 Hz and data points 2048. We used a voxel size of 30 x 30 x 30 mm and VAPOR water suppression (20) was applied and 128 acquisitions were averaged after individual phasing and frequency alignment. A water reference scan was obtained without water suppression and averaging 32 spectra. Resonances were quantified with a time-domain fitting algorithm in a custom-written MATLAB script and lipid composition was calculated as reported earlier (18). IHL content is reported as T2 corrected ratios (21 ms for water and 58.68 ms for the CH2 lipid resonance) of the signal intensities of the CH_2 resonance relative to the sum of CH_2 and unsuppressed water resonance. Ratios were converted to weight percentages, according to Szczepaniak et al (21) and Longo et al. (22).

Skeletal muscle oxidative capacity

To assess *in vivo* skeletal muscle oxidative capacity, ^{31}P-MRS was applied to quantify the post-exercise phosphocreatine (PCr) recovery rate constant (1). Measurements were performed at 18.00h after 5 hours fasting since lunch time, on a 3T clinical MRI scanner (Achieva Tx, Phillips, Healthcare, Best, The Netherlands), using a 6 cm (double tuned ^{1}H/^{31}P) surface coil

fixed to the vastus lateralis muscle. Participants performed a knee-extension exercise protocol within the scanner on a custom-built magnetic resonance compatible ergometer with a pulley system for 5 min at 50-60% of the volunteers' pre-determined maximal knee extension capacity. A series of 150 unlocalized ^{31}P-spectra were acquired in total, with 10 spectra acquired at rest, 70 during exercise and 70 spectra after exercise, with the following parameters: NSA = 1 (single acquisitions); repetition time (TR) = 4000ms; spectral bandwidth = 3000 Hz; number of points = 1024. Acquired spectra were analyzed with a custom-written MATLAB script (The Mathworks Inc. Natick, MA, USA). The time course of the PCr recovery post-exercise was fitted with a mono-exponential function and the rate constant (k) [s^{-1}] was used as a marker of *in vivo* muscle mitochondrial capacity as previously reported (1) with a higher rate constant [s^{-1}] reflecting a better *in vivo* mitochondrial capacity. The pH was determined in every spectrum from the chemical shift difference between inorganic phosphate and PCr. When pH dropped below 6.9 the exercise protocol was repeated with a slightly lower intensity. If the pH still dropped below 6.9 after the exercise protocol was repeated, the participants were excluded from the current analysis.
Prior to assessing *in vivo* skeletal muscle mitochondrial capacity, subjects consumed the same light lunch at the same clock time in both periods before reporting to the University.

Body composition
Body composition (fat mass and fat-free mass) was determined at 08.00 AM after 10-12 hours fasting by using air displacement plethysmography (BodPod®, COSMED, Inc,. Rome, Italy) (23).

Maximal aerobic capacity
Maximal aerobic capacity (VO_2max) and maximal power output (Wmax) were assessed upon a graded cycling test until exhaustion, in the fasted state and after the body composition assessment. In summary, after a warm-up period of 5 minutes cycling at 50 watts, the power output was increased with 25 Watts every 2.5 minutes until subjects were no longer able to maintain a cadence between 60-80 rpm. Oxygen (O_2) consumption and carbon dioxide (CO_2) production were measured continuously throughout the cycling test using indirect calorimetry (Omnical, IDEE, Maastricht, The Netherlands) (24).

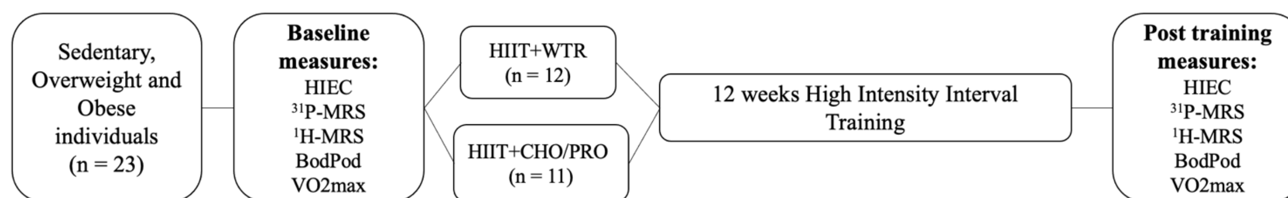

Figure 1. Overview of study design.

HIEC: 2 step hyperinsulinemic-euglycemic clamp. ^{31}P-MRS: 31-phosphorus magnetic resonance spectroscopy. ^{1}H-MRS: 1-proton magnetic resonance spectroscopy. VO_2max: maximal aerobic capacity. WTR: water. CHO: carbohydrate. PRO: casein hydrolysate protein.

Statistics

All values are reported as mean ± SEM, unless stated otherwise. Shapiro-Wilk test was used to assess normal distribution. Homogeneity of the variance was measured by the Levene's test. Statistical analysis was performed two sided using SPSS, version 26 (IBM Corp. Armonk, NY, USA) and statistically significant differences were set at $P < 0.05$. The distribution of sex across groups was determined by $\chi 2$ test. A two-way mixed ANOVA model was applied using HIIT+WTR vs. HIIT+CHO/PRO as between subjects factor and training as a within subjects factor. Unpaired Student's t-tests were used to analyze baseline differences between HIIT+WTR and HIIT+CHO/PRO with respect to subjects' characteristics, *in vivo* skeletal muscle mitochondrial capacity, intrahepatic lipid content/composition, whole-body insulin sensitivity and metabolic flexibility. Pearson's correlation analyses were performed to test linear association between HIIT-induced changes on whole-body insulin sensitivity and HIIT-induced changes on maximal aerobic capacity, *in vivo* skeletal muscle mitochondrial capacity and intrahepatic lipid content as well as composition.

Results

Baseline characteristics

No significant differences in gender distribution, age, body weight, BMI, systolic and diastolic blood pressure or body composition were observed at baseline between HIIT+WTR and HIIT+CHO/PRO groups (P > 0.05 for all comparisons, Table 1). Also, baseline maximal aerobic capacity (VO_2max) and maximal power output (Wmax) were comparable between HIIT+WTR and HIIT+CHO/PRO groups (P > 0.05 for both comparisons). In addition, no differences in fasting plasma glucose, insulin and HbA1c levels were observed between HIIT+WTR and HIIT+CHO/PRO groups prior to the training program (P > 0.05 for all comparisons). Compliance to the training sessions was high and comparable between groups (Table 1).

Table 1.- Baseline participants' characteristics

	HIIT + WTR	**HIIT + CHO/PRO**	***P* value**
Sample size (f/m)	7/5	6/5	0.85
Age (years)	61.7 ± 7.9	65.6 ± 6.7	0.22
Body weight (kg)	92.6 ± 15.3	86.3 ± 12.7	0.29
BMI (kg/m^2)	32.4 ± 3.2	31.0 ± 3.3	0.34
SBP (mmHg)	136 ± 15	134 ± 15	0.89
DBP (mmHg)	84 ± 7	81 ± 6	0.21
Fat mass (kg)	39.4 ± 7.6	36.8 ± 11.6	0.54
Fat percentage (%)	43.0 ± 7.7	42.2 ± 9.9	0.83
Fat-free mass (kg)	53.2 ± 13.8	49.5 ± 8.9	0.45
VO_2max (ml/FFM/min)	41.0 ± 6.8	42.8 ± 5.3	0.50
Wmax (watt)	155.9 ± 52.8	158.0 ± 53.2	0.92
Fasting glucose (mmol/L)	5.5 ± 0.3	5.3 ± 0.3	0.25
Fasting insulin (pmol/L)	64.7 ± 36.8	61.1 ± 22.8	0.79
HbA1c (%)	5.4 ± 0.2	5.3 ± 0.5	0.43
Training compliance (%)	99.5 ± 1.6	97.2 ± 4.1	0.08

Data are expressed as mean ± SD. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FFM: fat-free mass; Wmax: maximal power output. F: female; M: male.

High intensity interval training improves maximal aerobic capacity and exercise performance without effects on body weight and body composition.

As anticipated, maximal aerobic capacity (VO_2max) significantly increased upon HIIT (training effect $P < 0.001$, Figure 2A, Table 2). The absolute increase in VO_2max upon HIIT was similar between HIIT+WTR and HIIT+CHO/PRO groups (+3.8 ml/FFM/min and +4.7 ml/FFM/min, respectively; training X group $P = 0.62$, Table 2). Similarly, maximal power output (Wmax) significantly improved upon HIIT (training effect $P < 0.001$, Figure 2B, Table 2). The absolute increase in Wmax upon HIIT was similar between groups (HIIT+WTR: +21.1 Watts and HIIT+CHO/PRO: +14 Watts; training X group $P = 0.946$, Table 2). HIIT did not induce any changes in body weight, fat mass (FM), fat percentage, fat free mass (FFM) and fasting plasma glucose values in any group (training effect $P > 0.05$ and training X group $P > 0.05$ for all comparisons, Table 2).

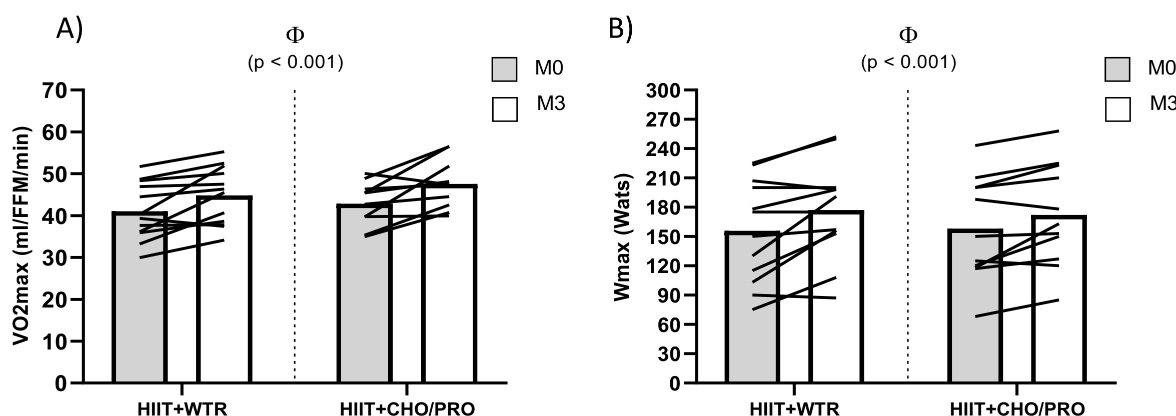

Figure 2. The effects of HIIT on A) maximal aerobic capacity (VO_2max) and B) exercise performance (Wmax). Grey bars represent mean values at baseline. White bars represent mean values after training. Black lines represent values of individual volunteers. Φ denotes the training effects. VO_2max data from 1 participant in the HIIT+CHO/PRO group was excluded of the analysis due to problems with the indirect calorimetry system during test. No significant group and training X group interaction effects were found in any variable. HIIT+WTR: High intensity interval training group which consumed water ad libitum during and after exercise (n = 12). HIIT+CHO/PRO: High intensity interval training group which co-ingested glucose and protein post exercise (n = 11).

High intensity interval training enhances *in vivo* skeletal muscle oxidative capacity in overweight/obese individuals

In vivo skeletal muscle oxidative capacity, as expressed by the PCr recovery rate constant after exercise, was comparable between groups at baseline ($P > 0.05$, Table 2). In line with our hypothesis, PCr recovery rate constant significantly improved upon HIIT (training effect $P < 0.001$, Figure 3, Table 2). This increase in *in vivo* skeletal muscle oxidative capacity was similar in both groups (HIIT+WTR: +37% and HIIT+CHO/PRO: +23%; training X group $P = 0.421$, Table 2), although more individuals showed an increasing response in the HIIT+WTR group. There were no differences in the pH values post-PCr depleting exercise neither at baseline nor after HIIT (HIIT+WTR: 6.99 ± 0.02 and 6.98 ± 0.02 at baseline and post training and HIIT+CHO/PRO: 7.01 ± 0.02 and 7.00 ± 0.02 at baseline and post training, respectively, $P > 0.05$ for all comparisons).

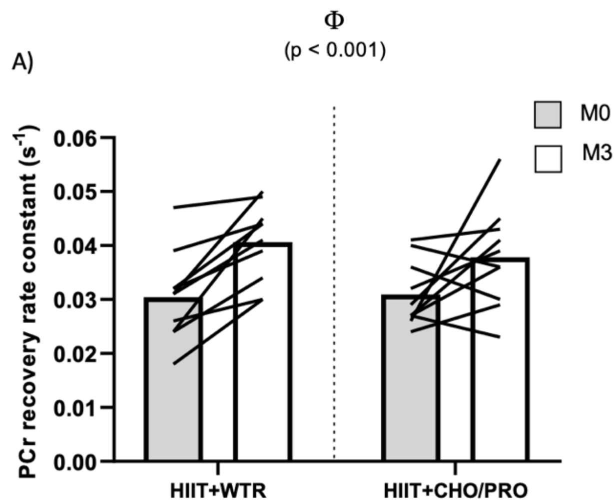

Figure 3. The effect of HIIT on *in vivo* skeletal muscle oxidative capacity as determined by the PCr recovery rate constant [s^{-1}]. Grey bars represent mean values at baseline. White bars represent mean values after training. Black lines represent values of individual volunteers. Φ denotes the training effects. Data from 2 participants in the HIIT+WTR group were excluded from the analysis due to a pH decline below 6.9. Data could not be acquired in 1 participant from the HIIT+CHO/PRO group after training due to implications of the SARS-CoV-19 outbreak. No significant group and training X group interaction effects were found. Abbreviations: HIIT+WTR: High intensity interval training group which consumed water ad libitum during and after exercise (n = 10). HIIT+CHO/PRO: High intensity interval training group which co-ingested glucose and protein post exercise (n = 10). PCr: Phosphocreatine.

High intensity interval training tends to decrease intrahepatic lipid content and modifies intrahepatic lipid composition in overweight/obese individuals.

IHL content was similar between groups at baseline ($P > 0.05$, Table 2) and tended to decrease upon HIIT (training effect $P = 0.065$). This effect was not significantly different between the HIIT+WTR group (-18%) as compared to the HIIT+CHO/PRO group (-4%; training X group $P = 0.374$, Figure 4A, Table 2).

In terms of IHL composition, fractions of PUFA, MUFA and SFA were comparable between groups at baseline ($P > 0.05$ for all comparisons, Table 2). Interestingly, HIIT significantly increased the intrahepatic fraction of PUFA (training effect $P = 0.048$, Figure 4B, Table 2). The increase in fraction of PUFA upon HIIT was similar between groups (HIIT+WTR: from 18.0% to 20.7% and HIIT+CHO/PRO: from 14.5% to 18.8%; training X group $P = 0.616$, Table 2). Furthermore, HIIT tended to decrease the intrahepatic fraction of MUFA (training effect $P = 0.088$) and did so to a similar extent in both groups (HIIT+WTR: from 43.0% to 39.7% and HIIT+CHO/PRO: from 43.3% to 40.3%; training X group $P = 0.933$, Figure 4C, Table 2). The intrahepatic fraction of SFA remained unaffected by HIIT, irrespective of group (training effect P = 0.874, training X group $P = 0.951$, Figure 4D, Table 2).

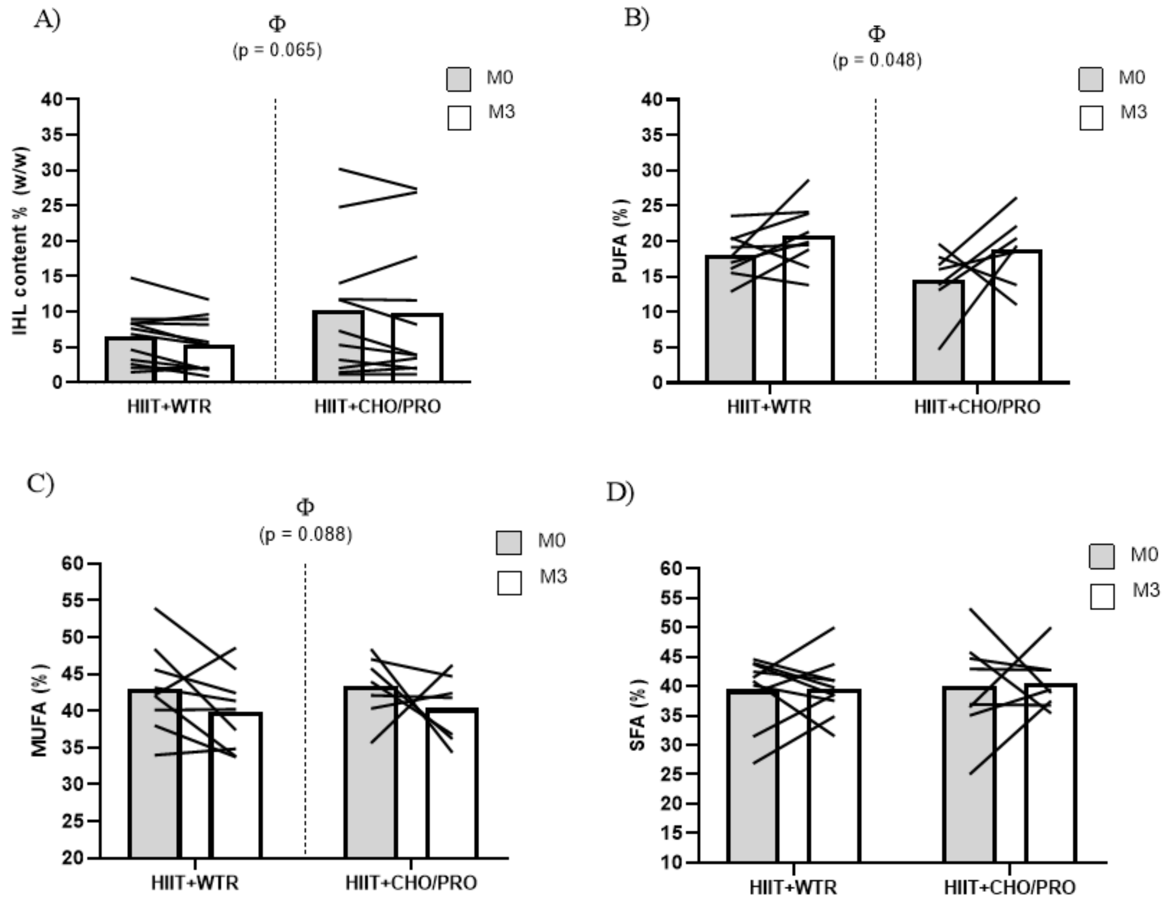

Figure 4. The effects of HIIT on IHL content and composition as measured by means of ^{1}H-MRS. A) HIIT tended to decrease IHL content. B) HIIT significantly increased intrahepatic PUFA fraction. C) HIIT tended to decrease intrahepatic MUFA fraction ($P = 0.088$). D) HIIT did not change intrahepatic SFA fraction. All responses were similar in the two groups ($P > 0.05$ for all comparisons). Grey bars represent mean values at baseline. White bars represent mean values after training. Black lines represent values of individual volunteers. Φ denotes the training effects. SFA data from 2 participants in HIIT+WTR group and from 3 participants in the HIIT+CHO/PRO group were excluded due to insufficient spectral quality to quantify the SFA fraction. PUFA and MUFA data from the same individuals were excluded of the analysis. In addition, PUFA and MUFA data from 1 participant in the HIIT+WTR group and 1 from participant from the HIIT+CHO/PRO group were also excluded from the analysis due to insufficient spectral quality to differentiate between PUFA and MUFA species. No significant group and training X group interaction effects were found in any variable. Abbreviations: HIIT+WTR: High intensity interval training group which consumed water ad libitum during and after exercise (n = 12). HIIT+CHO/PRO: High intensity interval training group which co-ingested glucose and protein post exercise (n = 11).

High intensity interval training improves whole-body insulin sensitivity and metabolic flexibility in overweight/obese individuals

Whole-body insulin sensitivity, as expressed by the M-value, was comparable between groups at baseline ($P > 0.05$, Table 2). In line with our hypothesis, HIIT significantly improved the M-value (training effect $P = 0.013$, Figure 5A, Table 2). This increase in whole-body insulin sensitivity upon HIIT was similar in the two groups (HIIT+WTR: +15% and HIIT+CHO/PRO: +13%; training X group $P = 0.831$, Table 2).

Metabolic flexibility, expressed as the change in the respiratory exchange ratio (Δ delta RER) from basal (fasted) to high insulin-stimulated conditions, was comparable between groups at baseline (Table 2). HIIT significantly improved metabolic flexibility (training effect $P = 0.032$, Figure 5B, Table 2), and did so similarly in both groups (HIIT+WTR: +24% and HIIT+CHO/PRO: +15%; training X group $P = 0.644$). Accordingly, the insulin-stimulated increase in glucose oxidation (training effect $P = 0.019$, Figure 5C, Table 2) and the insulin-induced suppression of fat oxidation (training effect $P = 0.028$, Table 2) were more pronounced after HIIT. These HIIT-induced adaptations in insulin-stimulated glucose (HIIT+WTR: +19% and HIIT+CHO/PRO: +23%) and fat (HIIT+WTR: -20% and HIIT+CHO/PRO: -17%) oxidation were not differentially affected by group.

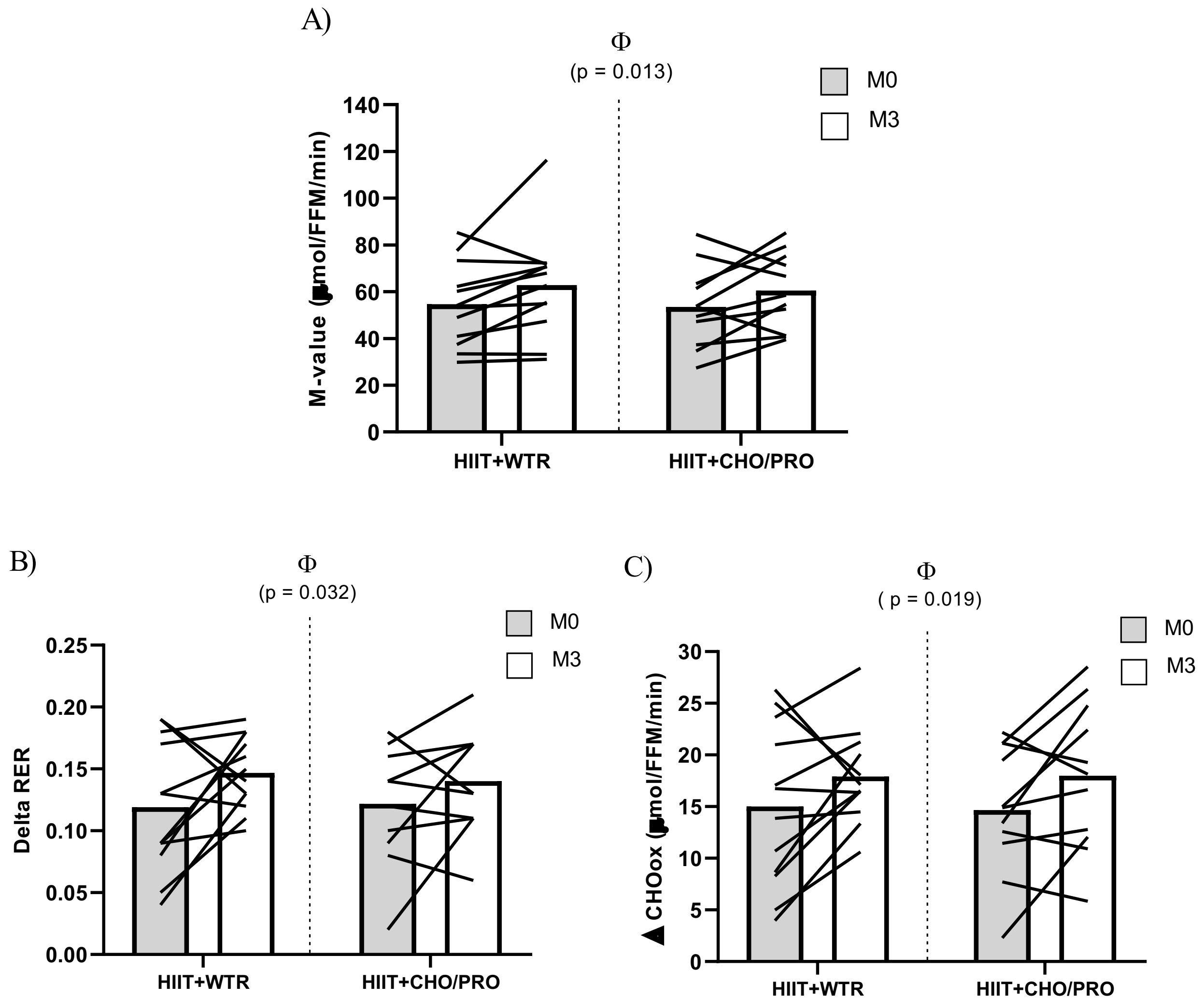

Figure 5. Effects of HIIT on: A) Whole-body insulin sensitivity as computed based on M-value, B) Metabolic flexibility measured as the change on RER from the basal (fasted) to the high insulin-stimulated condition and C) Insulin-stimulated increase on glucose oxidation from basal to high insulin-stimulated condition. Grey bars represent mean values at baseline. White bars represent mean values after training. Black lines represent values of individual volunteers. Φ denotes the training effects. No significant group and training X group interaction effects were found in any variable. Abbreviations: HIIT+WTR: High intensity interval training group which consumed water ad libitum during and after exercise (n = 12). HIIT+CHO/PRO: High intensity interval training group which co-ingested glucose and protein post exercise (n = 11). M-value: Mean glucose infusion rate. RER: respiratory exchange ratio. Δ CHOox: changes in carbohydrate oxidation upon insulin stimulation (from basal to high insulin infusion).

Table 2. Effects of High Intensity Interval Training (HIIT) on metabolic health

	Pre-training (M0)		Post-training (M3)		*P* values		
	WTR	**CHO/PRO**	**WTR**	**CHO/PRO**	**Training**	**Group**	**Group x Training**
Body weight (kg)	92.6 ± 4.0	86.3 ± 4.2	92.0 ± 4.2	85.8 ± 4.4	0.21	0.31	0.88
Fat mass (kg)	39.4 ± 2.8	36.8 ± 2.9	38.5 ± 3.0	37.4 ± 3.1	0.83	0.67	0.29
Fat percentage (%)	43.0 ± 2.5	42.2 ± 2.6	42.4 ± 2.7	43.0 ± 2.8	0.79	0.98	0.27
Fat-free mass (kg)	53.2 ± 3.3	49.5 ± 3.5	53.4 ± 3.5	48.3 ± 3.6	0.40	0.38	0.22
VO_2max (ml/FFM/min)	41.0 ± 1.7	42.8 ± 1.9	44.8 ± 1.8	47.5 ± 2.0	**<0.001**	0.38	0.62
Wmax (watt)	155.9 ± 15.3	158.0 ± 15.9	177.0 ± 14.6	172.0 ± 15.3	**<0.001**	0.94	0.38
Fasting glucose (mmol/L)	5.5 ± 0.09	5.3 ± 0.10	5.4 ± 0.12	5.3 ± 0.13	0.43	0.41	0.57
PCr recovery constant rate(s^{-1})	0.030 ± 0.002	0.031 ± 0.002	0.041 ± 0.003	0.038 ± 0.003	**<0.001**	0.69	0.42
Liver fat content (w/w)	6.4 ± 2.0	10.2 ± 2.1	5.2 ± 2.1	9.8 ± 2.2	0.06	0.17	0.37
PUFA (%)	18.0 ± 1.3	14.5 ± 1.5	20.7 ± 1.5	18.8 ± 1.7	**0.048**	0.09	0.61
MUFA (%)	43.0 ± 1.7	43.3 ± 1.9	39.7 ± 1.6	40.3 ± 1.8	0.08	0.83	0.93
SFA (%)	39.4 ± 2.2	40.0 ± 2.5	39.6 ± 1.5	40.4 ± 1.7	0.87	0.75	0.95
M value µmol/min/kgFFM	54.6 ± 5.0	53.4 ± 5.2	62.8 ± 5.6	60.4 ± 5.9	**0.013**	0.80	0.83
Carbohydrate oxidation (µmol/min/kgFFM)							
Basal	8.1 ± 1.1	8.7 ± 1.1	8.0 ± 1.2	7.3 ± 1.3	0.51	0.95	0.52
High insulin	23.1 ± 2.2	23.3 ± 2.2	26.0 ± 1.8	25.3 ± 1.9	**0.007**	0.94	0.58
Δ Delta	15.0 ± 2.0	14.6 ± 2.1	17.9 ± 1.7	17.9 ± 1.7	**0.019**	0.95	0.86
Fat oxidation (µmol/min/kgFFM)							
Basal	6.2 ± 0.3	6.1 ± 0.3	6.1 ± 0.3	6.7 ± 0.4	0.27	0.65	0.25
High insulin	2.8 ± 0.3	2.6 ± 0.3	2.1 ± 0.2	2.6 ± 0.2	0.09	0.75	0.11
Δ Delta	-3.3 ± 0.4	-3.4 ± 0.4	-3.9 ± 0.3	-4.0 ± 0.3	**0.028**	0.87	0.88
Respiratory exchange ratio (RER)							
Basal	0.77 ± 0.009	0.77 ± 0.009	0.77 ± 0.010	0.76 ± 0.010	0.40	0.97	0.67
High insulin	0.89 ± 0.014	0.89 ± 0.015	0.91 ± 0.010	0.90 ± 0.010	**0.042**	0.95	0.25
Δ Delta	0.11 ± 0.015	0.12 ± 0.015	0.14 ± 0.010	0.14 ± 0.011	**0.032**	0.89	0.64

Table 2. Values are presented as mean ± SEM. Bold *P* values indicate significant training effects. WTR: HIIT+water group, CHO/PRO: HIIT+ carbohydrate/protein drink group.

The improved maximal aerobic capacity upon HIIT is associated with changes in whole-body insulin sensitivity, but not with changes in skeletal muscle mitochondrial capacity and intrahepatic lipid content.

HIIT-induced changes in maximal aerobic capacity were significantly associated with HIIT-induced changes in whole-body insulin sensitivity (n = 22, r = 0.45, *P* = 0.03; Figure 6A) but not with HIIT-induced changes in *in vivo* skeletal muscle mitochondrial capacity (n = 19, r = 0.08, *P* = 0.72) and intrahepatic lipid content (n = 19, r = -0.07, *P* = 0.75). Furthermore, the HIIT-induced changes in whole body insulin sensitivity were not associated with the HIIT-induced changes in intrahepatic lipid content (n = 23, r = -0.04, *P* = 0.84; Figure 6B).

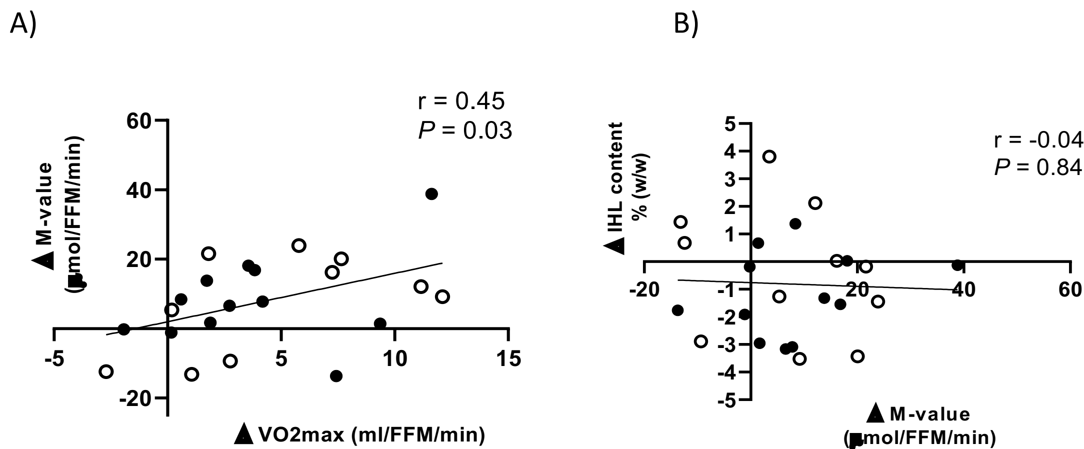

Figure 6. Linear associations between HIIT-induced changes in A) maximal aerobic capacity and whole-body insulin sensitivity and B) whole-body insulin sensitivity and intrahepatic lipid content. Open circles represent individual data from the HIIT+WTR group whereas closed circles represent individuals from the HIIT+CHO/PRO group.

Discussion

Previous studies investigating the effects of high intensity interval training (HIIT) in humans have shown the potential of HIIT as an exercise modality to improve oxidative capacity and metabolic health. Here, we investigated if 12 weeks of HIIT, performed 3 times per week, improves insulin sensitivity and *in vivo* skeletal muscle oxidative capacity in overweight and obese participants. We also investigated if the co-ingestion of glucose and casein-hydrolysate after each training session impacts the physiological and metabolic adaptations of HIIT. The current study showed that 12 weeks of HIIT significantly improves whole-body insulin sensitivity and robustly enhances *in vivo* skeletal muscle oxidative capacity. Furthermore, we demonstrated that 12 weeks of HIIT promotes metabolic flexibility. The improved metabolic flexibility mainly originates from a more profound increase of insulin-stimulated glucose oxidation and more pronounced insulin-induced suppression of fat oxidation after training, whereas basal glucose/fat oxidation remained unchanged. In addition, we showed that 12 weeks of HIIT tended to decrease intrahepatic lipid content. The tendency to lower hepatic lipid content was paralleled by changes in intrahepatic lipid composition. The intrahepatic PUFA fraction was elevated upon HIIT, while the MUFA fraction tended to decrease and the SFA fraction remained unaltered after HIIT. Interestingly, these metabolic benefits and adaptative responses attributed to our HIIT method were observed regardless of the systematic glucose and casein hydrolysate co-ingestion post-training, and occurred in the absence of changes in body weight and body composition. In aggregate, our data indicate that HIIT is a powerful strategy to improve metabolic health in overweight and obese individuals and hence represents a feasible and time-efficient training method, essentially involving only 30 minutes of net exercise per week.

The insulin sensitizing effect of regular exercise training in obese, metabolically compromised individuals is well-recognized. Conventional exercise training, such as continuous aerobic exercise, whether or not combined with resistance-type exercise at moderate intensity, improves insulin-stimulated whole-body glucose disposal in obese, insulin resistant and type 2 diabetic subjects (5, 8, 25). Long-term adherence to these training modalities is, however, low. Hence, more time-efficient, clinically effective training strategies to improve metabolic health are warranted. Preliminary studies explored the potential benefits of HIIT to improve plasma glucose homeostasis (11, 26, 27). Of note, only 6 sessions of HIIT (10 bouts of 1 minute cycling at 80-90% maximal heart rate, interspersed by 1 minute of recovery between bouts) performed

over the period of 2 weeks sufficed to mitigate 24-hour hyperglycemic peaks in type 2 diabetic patients (11). Similar observations were made in obese, insulin resistant individuals (28). In addition, other reports elucidated the benefits of HIIT to reduce fasting plasma glucose and insulin levels (29, 30) as well as post-prandial glucose values in insulin resistant volunteers (31). Even though such promising results have contributed to a wider acceptance of HIIT to improve plasma glucose homeostasis in subjects with poor metabolic health, the direct effects of HIIT on insulin resistance as measured by gold standard methods have not consistently been studied. Here, we show that regular HIIT improves insulin-stimulated whole-body plasma glucose disposal by ~15% as measured during hyperinsulinemic-euglycemic clamp conditions in overweight and obese individuals. These observations are in line with previous findings reporting similar insulin sensitizing effects of HIIT in type 2 diabetic patients (32, 33). The order of magnitude of the changes we observed in whole-body insulin sensitivity upon HIIT are similar to observations in obese individuals that have been previously reported by Ryan et al. (34). Interestingly, the findings by Ryan et al. (34) were attributed to the acute (remnant) effects of the final training session rather than a HIIT-mediated adaptative response whereas in our study the assessment of insulin stimulated whole-body glucose disposal was always performed within 48-72 hours after the final exercise session, reducing the likelihood of an acute effect. Next to these methodological considerations, it is relevant to note that in our study, only cycling exercise was employed and at a lower training volume (3 times per week, for 12 weeks) as compared to the intervention by Ryan et al. (34), which comprised cycling, rowing and treadmill/elliptical exercises, 4 times per week for 12 weeks.

Impaired metabolic flexibility is a common characteristic of obese, metabolically compromised individuals (35), mainly reflected by a diminished ability to adapt substrate oxidation upon changes in substrate availability. Here, we showed that HIIT significantly promotes metabolic flexibility as expressed by an augmented insulin-stimulated increase in glucose oxidation and a more pronounced insulin-mediated suppression of fat oxidation, while basal fat/glucose oxidation remained unaltered. Interestingly, the observed improvement in metabolic flexibility in the present study is in contrast with a previous study (8), reporting unaltered metabolic flexibility upon conventional aerobic-type exercise combined with resistant-type exercise in obese, normoglycemic individuals (8). Given that metabolic flexibility might be secondary to the changes in intracellular glucose availability for oxidation (36), the differential response of the two studies in terms of metabolic flexibility might be due to the dissimilar training effects on insulin sensitivity. In this context, we showed in the current study that the HIIT-induced

changes on metabolic flexibility were paralleled by HIIT-induced improvements in whole-body insulin sensitivity, whereas the lack of change in metabolic flexibility in obese normoglycemic individuals reported by Meex et al (8) coexisted with unaltered insulin sensitivity upon conventional exercise training. Of note, the HIIT-induced improvements on metabolic flexibility and whole-body insulin sensitivity in the present study were observed in absence of changes in body weight and body composition. Thus, our findings suggest that HIIT is a feasible method to ameliorate plasma glucose homeostasis and post-prandial glucose oxidation. These benefits are of relevance, especially at the transitions from fasting-to-fed conditions in obese, metabolically compromised individuals.

Twelve weeks of HIIT improved *in vivo* skeletal muscle oxidative capacity as measured by ^{31}P-MRS. Similar enhancements in *in vivo* skeletal muscle oxidative capacity have been previously reported upon HIIT in young, healthy individuals (37, 38). To the best of our knowledge, the effects of HIIT on *in vivo* skeletal muscle oxidative capacity in overweight and obese individuals was unknown so far. Of interest, our findings show that HIIT resembles previously reported improvements in *in vivo* skeletal muscle oxidative capacity upon conventional exercise interventions in obese adults (8). In the present study, HIIT induced a 36.5% improvement in *in vivo* skeletal muscle oxidative capacity in the HIIT+WTR group and a 22.5% improvement in the HIIT+CHO/PRO group. These improvements are of similar magnitude as reported previously by Meex et al (8), who reported a 28% improvement in *in vivo* skeletal muscle oxidative capacity in obese normoglycemic adults after 12 weeks of endurance-type exercise combined with resistance-type exercise training. The current findings extend previous evidence showing that HIIT improved *ex vivo* respiratory capacity in permeabilized muscle fibers in older adults (39, 40), and increased the expression of markers of mitochondrial function and mitochondrial content (i.e. CS activity and proteins from the oxidative phosphorylation system) (11, 39). Together, these results elucidate the potential of HIIT as an alternative training program to effectively improve skeletal muscle oxidative capacity in overweight and obese individuals.

Next to the HIIT-induced improvements in skeletal muscle oxidative capacity, 12 weeks of HIIT significantly improved whole-body oxidative capacity, measured as VO_2max. Interestingly, these beneficial changes in oxidative capacity did not correlate, indicating that other, yet to be determined and to be exercise targeted, factor(s) also contribute to the improved oxidative capacity. Maximal cardiac output and muscle oxygenation are likely candidates to

contribute to improvements in oxidative capacity (41) whose adaptive responses were, however, not measured in the present study.
The magnitude of the observed benefits on maximal aerobic capacity and exercise performance are in accordance with previous literature reporting increments of ~10-15% in healthy males and obese, metabolically compromised individuals by performing a similar HIIT method as it was applied here (12, 42). We found that HIIT-induced changes on maximal aerobic capacity were associated with HIIT-induced improvements in whole-body insulin sensitivity. Such findings are relevant in the context of promoting metabolic health via regular exercise training, especially considering that aerobic fitness is a strong, independent predictor of insulin sensitivity in adult humans (43).

In the present study, we found lower levels of intrahepatic lipid content after HIIT in 15 out of 26 individuals. While this effect failed to reach statistical significance, the remodeling of the lipid composition that occurred in parallel in the liver upon HIIT, indicates that hepatic fat can be targeted by HIIT. This is in line with findings by Oh et al. who demonstrated that 12 weeks of HIIT reduces intrahepatic lipid content in obese individuals with non-alcoholic fatty liver disease (NAFLD) (44). Interestingly, Oh et al. showed that HIIT confers similar intrahepatic lipid lowering effects as compared to aerobic-type exercise, but with a significantly lower time commitment (44). In pre-diabetic individuals, it has been shown that a reduction in intrahepatic lipid content can already be observed after just 2 weeks of HIIT (45). In contrast, a recent study reported no HIIT effects on intrahepatic lipid content in obese, normoglycemic adults after 12 weeks of training (34). However, differences in the timing of the measurement relative to the last training session may contribute to the discrepancy in results.
Besides the HIIT-induced changes on intrahepatic lipid content, HIIT also altered intrahepatic lipid composition in the present study. More specifically, our HIIT modality significantly elevated the PUFA fraction while the MUFA fraction tended to decrease. To our best knowledge, this is the first human intervention study examining the effects of HIIT on the intrahepatic PUFA, MUFA and SFA fraction separately *in vivo,* and extend previous findings that the intrahepatic PUFA fraction increased upon 7 consecutive sessions of aerobic-type exercise in obese subjects with nonalcoholic fatty liver disease (46).

Our final aim was to examine if the physiological and metabolic effects of HIIT are affected by the co-ingestion of glucose and casein-hydrolysate post-exercise. Here, we showed that the adaptations upon HIIT with respect to maximal aerobic capacity, whole-body insulin

sensitivity, skeletal muscle oxidative capacity and intrahepatic lipid content as well as intrahepatic lipid composition were observed irrespective of the systematic co-ingestion of glucose and casein hydrolysate post exercise. Given the putative hyperinsulinemic properties of this sports-type drink, our findings contrast previous evidence showing that the rapid elevation of plasma glucose and insulin levels post exercise blunts the insulin sensitizing effects of acute exercise in healthy individuals (47) and impedes skeletal muscle mitochondrial adaptations (48). Thus, our data support the beneficial effects of implementing HIIT in a modern lifestyle, irrespective of consuming carbohydrates and proteins post training.

Limitations

It should be noted that throughout this study, the SARS-CoV-2 (COVID-19) outbreak in early 2020 hampered the inclusion of participants in both HIIT+WTR and HIIT+CHO/PRO training groups, which clearly delayed inclusion of the participants and the more demanding sample analysis. The study was originally designed to focus on the effect of HIIT on insulin-stimulated oxidative and non-oxidative glucose disposal in skeletal muscle. To this end we co-infused the [6,6-$^{2}H_2$]-glucose tracer. Time constraints, however, prevented analysis of the tracer samples. Hence, we cannot yet draw conclusions on liver versus muscle insulin sensitivity and cannot make valid computations for oxidative and non-oxidative glucose disposal. In addition, we acknowledge some limitation regarding the specific detection of the PUFA fraction due to its low peak amplitude, especially in subjects with low intrahepatic lipid content. Such limitation hampered the subsequent detection of MUFA fraction, reducing our power to explore the HIIT effects on intrahepatic lipid composition in all participants.

Conclusion

In conclusion, the present study shows that 12 weeks of HIIT in overweight/obese individuals improves whole-body insulin sensitivity and robustly enhanced *in vivo* skeletal muscle oxidative capacity, maximal aerobic capacity and exercise performance. These changes went along with improved metabolic flexibility. HIIT also tended to decrease IHL content, and significantly modified IHL composition as expressed by the elevation of the PUFA fraction and the decline, albeit not significant, of the MUFA fraction, whereas SFA remained unaltered. These beneficial effects of HIIT were observed regardless of the co-ingestion of glucose and casein hydrolysate after each training session and in the absence of changes in body weight as well as body composition. Our findings highlight HIIT as a feasible training modality to

ameliorate metabolic health in overweight and obese individuals even when followed by nutrient ingestion.

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CHAPTER 7

General discussion

What factors determine the benefits of exercise in human metabolic health?

Although the benefits of regular exercise on metabolic health have been widely recognized, we only start to understand which factors, and to what extent, determine exercise effectiveness to promote metabolic health
In essence, the benefits of exercise training on metabolic health are mediated by episodic exercise-induced disruptions of whole-body energy homeostasis (1). Classically, in obese, metabolically compromised individuals and type 2 diabetic patients, low-to-moderate intensity (40-70% Wmax) endurance-type, whether or not combined with resistance-type exercise (40-70% of maximal strength), has been prescribed to lower plasma glucose levels (2), and to improve whole-body and skeletal muscle oxidative capacity (3). Interestingly, it has been shown that there is a dose-response relationship between the intensity of exercise and the magnitude of the improvement on insulin sensitivity and other metabolic outcome parameters (4). Mechanistically, this dose-response relationship has been mainly attributed to exercise-induced skeletal muscle glycogen breakdown (5). In support, other investigations have reported that high intensity exercise confers greater adaptations on whole-body insulin sensitivity (6), skeletal muscle mitochondrial function (7) and cardiorespiratory fitness (8) as compared to low-moderate intensity exercise in humans, even when energy expenditure was matched between exercise programs. Nevertheless, it is important to acknowledge the limited feasibility and the cardiovascular risks associated with intense, continuous exercise especially in individuals who are not used to exercise on a regular basis. In this regard, high intensity interval training (HIIT) is described as a time–efficient and well-tolerated methodology to improve metabolic health. HIIT typically encompasses short bouts of exercise performed at high intensity (between 30s to 4min performed at >80% Wmax) interspersed by short periods of complete or partial resting in between (9). In **chapter 6**, we showed that HIIT training is feasible for obese, metabolically compromised individuals, who are not used to regular exercise and that HIIT improves whole-body insulin sensitivity, skeletal muscle oxidative capacity and maximal aerobic capacity in these individuals. Therefore, our findings strengthen the view that regular high intensity exercise is an effective training methodology for improving metabolic health. These observations support the notice that HIIT can be used as an alternative exercise methodology even in obese individuals characterized by a sedentary lifestyle.

Next to exercise intensity, the timing of exercise may also play an important role in determining the effects of training on metabolic health. The fact that multiple exercise related parameters,

such as maximal voluntary contraction (10), substrate oxidation and energy economy during exercise (11) exhibit diurnal variations, raises the idea that there might be an optimal time of the day for exercise to maximize the health outcomes. Emerging evidence from animal models shows that exercise timing significantly affects tissue-specific insulin responsiveness (12). These effects were dependent on the expression and functioning of the biological clock in muscle tissue, revealing the molecular connection between exercise timing and glucose homeostasis. Although these findings still require validation in human studies, it is not surprising that timing of exercise might impact energy and substrate metabolism as we may have evolved to perform best at certain times of the day. In line with this reasoning, recent findings suggest that exercise timing might also differently modulate insulin sensitivity in humans, with afternoon exercise being more efficient to acutely improve the 24h glycaemic profile in type 2 diabetic patients as compared to morning exercise (13). In **chapter 3**, we performed a retrospective analysis of exercise training effects when performed in the morning vs. the late afternoon on insulin sensitivity and other metabolic health outcome parameters in obese, metabolically compromised individuals. Interestingly, we found that afternoon exercise training confers more pronounced benefits on tissue-specific insulin sensitivity (skeletal muscle, white adipose tissue and liver), fasting plasma glucose levels, maximal aerobic capacity and body composition as compared to morning exercise training. These findings identify exercise timing to be another factor that affects the beneficial effects of exercise on metabolic health. Thus, these data support the idea that synchronizing exercise interventions to the molecular clock might better exploit the exercise-induced health benefits to prevent and treat metabolic disorders. Future research is needed to investigate whether the effects of exercise timing on metabolic health are due to the impact of exercise on the central and/or peripheral molecular clock.

Timing of exercise goes beyond the molecular clock, because we eat at certain time of the day and it is quite likely that the nutritional status (fasted vs postprandial state) of individuals may also determine the physiological responses to exercise and therefore training adaptations. Interestingly, it has been shown that the acute ingestion of carbohydrates post-exercise blunts its insulin sensitizing effects in healthy normoglycemic individuals (14). Authors stated that such carbohydrate ingestion post-exercise might have facilitated an early skeletal muscle glycogen replenishment, hence reducing the insulin-stimulated plasma glucose disposal. However, whether exercising in the fasted state or after lunch/dinner differs in the exercise-induced benefits on metabolic health is yet unclear. In **chapter 6**, we found that the regular co-ingestion of carbohydrate and casein-hydrolysate after each HIIT session during 12 weeks did

not affect the training-induced benefits on whole-body insulin sensitivity, skeletal muscle mitochondrial function, maximal aerobic capacity and intrahepatic lipid metabolism in obese, metabolically compromised individuals. Hence, our findings do not support the idea that physiological adaptations, induced by regular exercise are affected by the acute elevations of plasma glucose and insulin levels upon meal and/or drink ingestion after training sessions. Therefore, we did not find any basis for advocating exercise to be performed preferentially either before a meal or without food ingestion shortly afterwards.

How to assess skeletal muscle mitochondrial inertia at the onset of exercise?

Skeletal muscle mitochondrial adaptations are at the core of the exercise-induced benefits on metabolic health. Mitochondrial function is usually defined as the maximal capacity to synthesize ATP. Another important characteristic of mitochondria may be their responsiveness to abrupt changes on energy demand, for instance at the transition from rest to exercise. In fact, the responsiveness of skeletal muscle mitochondria at the onset of exercise is functionally relevant in humans as the slow mitochondrial ATP synthesis would prompt premature muscle fatigue, thereby exercise intolerance. Nevertheless, the responsiveness of skeletal muscle mitochondria to increase ATP synthesis and match ATP demand at the onset of exercise, is currently not well studied. One of the reasons is the lack of readily accessible (non-invasive) tools to investigate mitochondrial inertia. One approach to investigate mitochondrial inertia is to study the dynamic behavior of PCr content in muscle upon the initiation of exercise. Up to now, the study of PCr content upon exercise initiation, was performed by taking multiple muscle biopsies in a short time window (15). However, the invasiveness and limited time-resolution of successive muscle biopsies is an obvious limitation of this approach, affecting the dynamic assessment of mitochondrial responsiveness. Another approach to study mitochondrial responsiveness at the onset of exercise is the assessment of the whole-body oxygen consumption on-kinetic, which is a non-invasive method, but not muscle specific (16). Thus, noninvasive alternatives are needed to measure skeletal muscle mitochondrial responsiveness in humans, to obtain novel insights about the role of mitochondrial activation in premature muscle fatigue and exercise intolerance. In **chapter 4** of this thesis, we applied a novel ^{31}P-MRS methodology to quantify the *in vivo* skeletal muscle PCr kinetics at the onset of moderate intensity exercise in two different cohorts of human volunteers. We found that skeletal muscle PCr on-kinetic is significantly slower in metabolically compromised volunteers as compared to endurance-trained individuals, as well as in elderly individuals with normal

physical activity as compared to their elderly and young trained counterparts. These findings suggest that the responsiveness of mitochondria is a novel signature of mitochondrial function which is part of the (patho)physiology in obese, type 2 diabetic patients, and elderly individuals, and it can be modulated through regular exercise training. So far, this aspect of mitochondrial function is understudied and in order to address the important issue of exercise intolerance, it should be investigated in more detail. To determine if mitochondrial inertia can be manipulated and whether it is a potential target of intervention for improving exercise intolerance warrants future investigations.

How should muscle metabolism be measured in a more physiologically relevant way?

In this thesis, we studied physiological adaptations of muscle tissue to regular exercise. The classical ways of investigating metabolic parameters in the most standardized circumstances is in the morning after an overnight fast. Although such classical approach gives important insights, novel understandings on the dynamic nature of metabolism also poses new demands on the techniques to be applied.

In fact, skeletal muscle metabolism shows a striking dynamic over a 24h cycle in healthy individuals and the energy production and substrate utilization varies considerably (17). Interestingly, the day-night rhythm was shown to be absent in skeletal muscle metabolism of insulin resistant individuals (18), who were also characterized by impaired oxidative ATP synthetic capacity (19). These malleable properties of muscle tissue call for dynamic measurements of muscle metabolism at different time points over the day and when exposed to elevated energy demands.

The dynamic assessment of muscle metabolism in a 24h cycle would be beneficial for two main reasons. Firstly, the inter-subject differences in biological clock time may trouble the conclusions about muscle metabolism. Secondly, it facilitates the investigation of the time sensitivity of interventions. This last is important in the context of the hypothesis reviewed in **chapter 2**, that the alignment of interventions (e.g., exercise, feeding time) with muscle metabolism in a 24h cycle might amplify the associated benefits on metabolic health. If multiple time points should be measured, non-invasive techniques are much better suitable than invasive testing. In this thesis, we applied such non-invasive MRS techniques for the quantification of metabolites in muscle and in the liver, which can be repeated many times without hazardous effects. This advantage also holds true for other dynamic assessments of

human metabolism that are minimally invasive, such as blood sampling or continuous glucose monitoring (CGM).

Furthermore, muscle metabolism rapidly changes when challenged, as for example during exercise. To investigate such dynamic responses in muscle metabolism, higher time resolution is needed than for 24 h measurements. The dynamic assessment with a high time resolution during exercise offers the opportunity to evaluate muscle metabolism in a physiological context where multiple factors (e.g., muscle blood flow) work in concert aiming to preserve energy homeostasis. Therefore, the dynamic assessments of muscle metabolism during exercise promotes the detection of potential targets of intervention, for instance in individuals who are prone to experience premature muscle fatigue. In this regard, there is a growing appreciation for the potential of MRS as the state-of-the-art methodology to study muscle metabolism in humans. Also, other non-invasive (or minimally invasive) techniques, such as indirect calorimetry and a wide range of novel sensors (e.g., continuous glucose monitors, 24h accelerometers, 24h blood pressure monitors, oxygen tension and body temperature sensors) will facilitate the investigation of human muscle metabolism in a more dynamic fashion.

Concluding remarks and future perspectives

This thesis provides evidence that exercise timing plays a major role in determining the benefits of exercise training on metabolic health and shows that exercise training performed in the afternoon confers greater benefits than morning exercise in obese, metabolically compromised individuals. Therefore, an interesting future step would be to investigate if the benefits of exercise on metabolic health can be optimized if timed to the central or peripheral muscle clock. In this regard, future human trials should consider that metabolically compromised adults exhibit a blunted circadian rhythmicity of clock protein expression in muscle tissue. Interestingly, this coincides with a disrupted transitions from predominantly fat oxidation in fasting conditions to predominantly glucose oxidation upon feeding over a 24h cycle (18). Whether regular exercise training restores the molecular clock machinery, and thereby improves 24h fluctuations on substrate oxidation has not been investigated yet. Furthermore, whether synchronizing exercise sessions to the molecular clock amplifies metabolic outcome parameters warrants also future investigation.

We showed in **chapter 6** that 12 weeks of high intensity interval training improves whole-body insulin sensitivity, skeletal muscle mitochondrial function and maximal aerobic capacity in obese, metabolically compromised individuals. Therefore, this type of training might be considered as a time-efficient training alternative to the conventional exercise programs, which comprises low-to-moderate intensity aerobic-type combined with resistant-type exercise.
In essence, insulin sensitivity comprises oxidative (stimulation of glucose oxidation by insulin) and non-oxidative glucose disposal (NOGD), with the latter mainly reflecting glucose storage as glycogen. In absolute terms, NOGD contributes to a greater extent than the insulin-stimulated glucose oxidation disposal to skeletal muscle insulin resistance, as expressed by an impaired glycogen resynthesis capacity (20). Given that HIIT strongly triggers skeletal muscle glycogen turnover, it would be interesting to explore whether HIIT improves the insulin-stimulated NOGD, potentially underlying the restoration of insulin sensitivity in insulin resistant individuals.

Metabolically compromised as well as elderly individuals are prone to exercise intolerance. In essence, exercise intolerance stems from a mismatch between the imposed energy demand by contractile activity and the energy production by oxidative metabolism. In **chapter 4**, we applied a non-invasive ^{31}P-MRS technique to assess *in vivo* muscle metabolism at the onset of exercise and observed that metabolically compromised and elderly subjects with normal physical activity exhibit a refractory responsiveness of muscle mitochondria to produce energy. Interestingly, the slow responsiveness of mitochondria was strongly related to a reduced acetyl-coa availability in the form of acetylcarnitine and augmented ADP levels, with the latter implying an impaired ADP sensitivity in muscle. Of note, the slow responsiveness was negatively associated with different functional parameters that mimic daily life situations, suggesting that the responsiveness of mitochondria can be a target of intervention to improve functional capacities. In order to improve the responsiveness of skeletal muscle mitochondrial at the onset of exercise, interventional studies that elevate either the skeletal muscle acetylcarnitine formation (e.g.,carnitine) or augment the ADP affinity of mitochondria (e.g., nitrate) should be initiated. Whether increasing the responsiveness of muscle mitochondria at the onset of exercise indeed leads to improved functional outcomes in metabolically compromised and elderly subjects also needs to be investigated.

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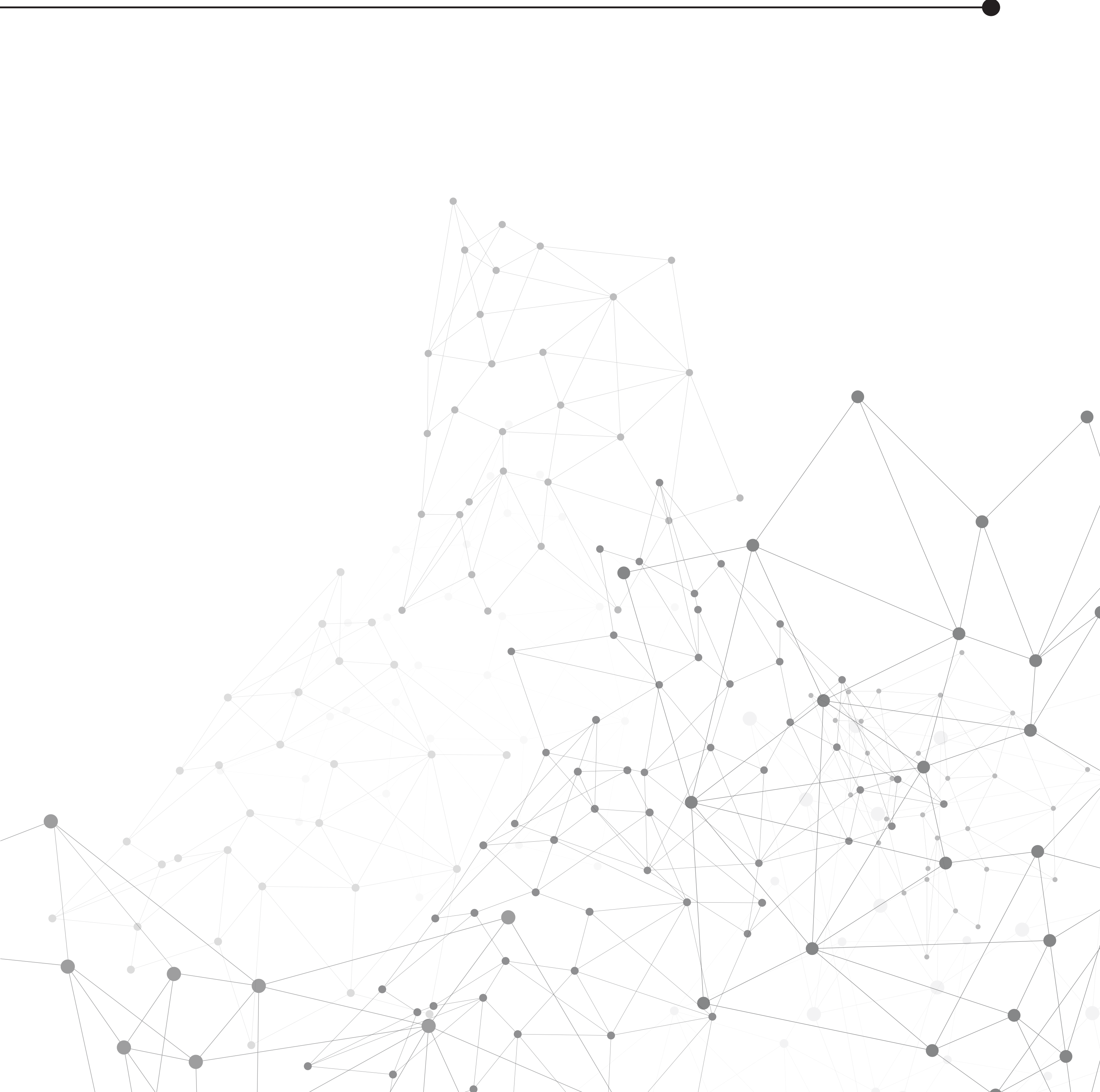
CHAPTER 8

Summary



Summary

The prevalence of obesity has increased exponentially worldwide (1). Obesity is a crucial contributing factor to the development of cardiovascular disorders, type 2 diabetes mellitus (T2DM) and non-alcoholic fatty liver disease (2). In fact, obese/overweight individuals are characterized by an impaired whole-body insulin sensitivity (3) and reduced skeletal muscle mitochondrial function (4), which in turn negatively affect plasma glucose homeostasis.
Regular exercise training is a well-recognized lifestyle intervention to prevent and counteract obesity-related metabolic disorders. Classically, exercise volume and intensity are regarded as the main factors determining the benefits of exercise in metabolic health. Hence, exercise volume and intensity provide the physiological rationale of the conventional recommendations of performing at least 150 minutes of moderate intensity aerobic-type combined with resistant-type exercise per week (5). However, the effects of these conventional exercise recommendations differ in magnitude among individuals, showing the need to come up with novel strategies to optimize the effects of exercise.

Many physiological processes in the human body, such as hormone synthesis and glucose homeostasis, exhibit day-night cycles, orchestrated by the central molecular clock which is located in the suprachiasmatic nucleus of the hypothalamus (6). Interestingly, peripheral organs such as liver, adipose tissue and skeletal muscle, contain their own molecular clocks (6). Of note, experimentally induced circadian misalignment causes skeletal muscle insulin resistance in normoglycemic individuals (7), which can be mitigated by performing acute bouts of high intensity interval exercise (8). Such insights that the biological clock, glucose homeostasis and exercise metabolism are tightly intertwined substantiate the hypothesis that the timing of exercise can be used to optimize its insulin sensitizing and metabolic benefits. The link between the skeletal muscle molecular clock and the benefits of exercise on glucose metabolism are reviewed in **chapter 2**. In this chapter, it is discussed how the nutritional status pre-post exercise, as well as the tissue-specific energy depots might influence the effects of exercise timing on glucose metabolism, with special emphasis on insulin resistant and type 2 diabetic individuals. This hypothesis was subsequently tested in **chapter 3**, in which state-of-the-art methodologies are used to investigate the effects of exercise timing on skeletal muscle insulin sensitivity and other clinical parameters in metabolically compromised individuals. We showed that afternoon exercise training confers superior benefits as compared to morning

exercise training in skeletal muscle insulin sensitivity, body composition and exercise performance in obese, metabolically compromised individuals.

Of note, obesity-related metabolic disorders and sedentary aging are often accompanied by exercise intolerance (9), likely explained by a refractory myocellular ATP synthesis via mitochondrial metabolism at the onset of exercise. The delayed responsiveness of skeletal muscle mitochondrial ATP synthesis is known as mitochondrial inertia (10). The responsiveness of skeletal muscle mitochondrial ATP synthesis at the onset of exercise relies on the intramyocellular acetyl-coa availability (10), which is controlled by the mitochondrial enzyme carnitine acetyltransferase (CrAT) (11). In **chapter 4**, the responsiveness of skeletal muscle mitochondrial ATP production at the onset of exercise, its potential underlying mechanisms, and its association with functional read-outs of exercise intolerance were determined in two different cohorts of human volunteers. The responsiveness of skeletal muscle ATP synthesis at the onset of exercise was slower in older, metabolically compromised individuals as compared to young, endurance-trained volunteers as well as in elderly, normally physically active individuals as compared to young, healthy and elderly exercise-trained counterparts. The responsiveness of skeletal muscle ATP synthesis at the onset of exercise was tightly linked to functional outcomes, which coexisted with reduced CrAT activity, low acetylcarnitine levels and elevated ADP concentration in muscle tissue during exercise. These results indicate that mitochondrial inertia at the onset of exercise might emerge as a target for intervention to improve exercise tolerance.

Given the wide variety of methods to assess skeletal muscle mitochondrial function, a direct comparisons and validation of the different markers of mitochondrial function with gold standard measures are needed. In **chapter 5** we determined to what extent different in vitro markers of mitochondrial content and in vivo functional readouts for skeletal muscle and whole-body oxidative capacity reflect muscle mitochondrial respiratory capacity as determined ex vivo, by high resolution respirometry.
Of the in vitro markers, protein content for complex V of the oxidative phosphorylation system and citrate synthase (CS) activity are the most valid surrogate markers of skeletal muscle mitochondrial respiratory capacity. From the in vivo readouts for skeletal muscle and whole-body oxidative capacity, PCr recovery post exercise, maximal aerobic capacity and exercise efficiency are valid reflection of skeletal muscle mitochondrial respiratory capacity. These results are of relevance for researchers to make a good choice from the multitude of techniques

that are available, when determining mitochondrial function. In addition, these findings might benefit researchers aiming to investigate skeletal muscle mitochondrial function but do not posse the gold standard technique to do so, and for studies that may not be able to incorporate muscle biopsies.

Most adults with poor metabolic health fail to meet the recommendations of performing at least 150 minutes of physical activity per week, with a "lack of time" being the most commonly cited barrier (12). In this regard, there has been a flourishing appreciation for the capability of high intensity interval training (HIIT) to improve metabolic health, as this methodology needs at least 50% less time commitment as compared to exercise performed according to the conventional exercise recommendations (13). In **chapter 6**, we investigated if 12 weeks of HIIT prompts beneficial effects on insulin sensitivity, muscle mitochondrial capacity and intrahepatic lipid (IHL) content in obese adults. Given the frequent consumption of carbohydrate rich and insulinogenic sports drinks after a training session, we also explored if co-ingestion of a standardized glucose and casein hydrolysate post-exercise affects the HIIT-mediated metabolic improvements. HIIT enhanced insulin sensitivity, skeletal muscle mitochondrial capacity, decreased intrahepatic lipid content and modified intrahepatic lipid composition in obese adults. These benefits were attained regardless of co-ingesting glucose and proteins after exercise and in the absence of changes in body weight and body composition. Taken together, HIIT is an effective training method to enhance metabolic health in obese adults.

Overall, the results from this thesis provide novel information about exercise timing as contributing factor to optimize the benefits of exercise in metabolic health. Also, the results of this thesis demonstrate that high intensity interval training is an effective training modality to improve whole-body insulin sensitivity, skeletal muscle mitochondrial capacity and to decrease and modify liver fat content and composition in obese adults. In addition, the results of this thesis indicate that skeletal muscle mitochondrial inertia is a novel signature of skeletal muscle mitochondrial function that correlates with markers for daily life activity. Thus, mitochondrial inertia is a putative target of intervention to improve physical function in metabolically compromised and elderly individuals. Furthermore, this thesis shows which markers of mitochondrial content and functional readouts of muscle oxidative capacity reflect muscle mitochondrial respiratory capacity *ex vivo*. Thus, the results of this thesis provide valuable

additions to the current literature about how to exploit the benefits of regular exercise training in human metabolic health.

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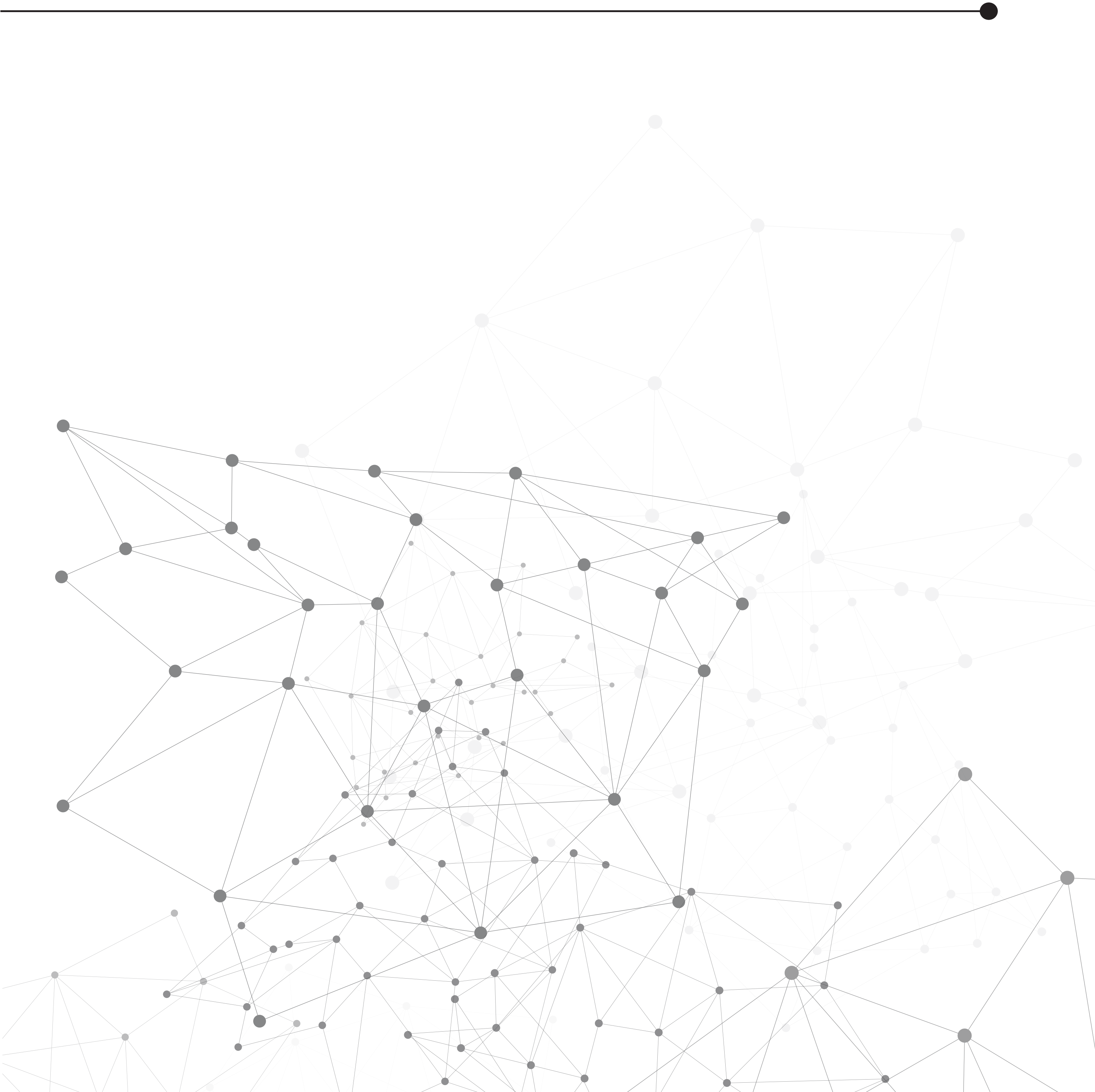
CHAPTER 9

Impact paragraph



What is the contribution of the results from this thesis to the scientific community and societal challenges?

To address the growing prevalence of obesity and obesity-related co-morbidities, effective strategies to treat obesity-related co-morbidities are urgently needed. In this regard, the results of **chapter 2, 3 and 6** illustrate the following:

1) Exercise training performed in the afternoon confers greater benefits than exercise training performed in the morning when it comes to improvements in skeletal muscle insulin sensitivity, body composition and exercise performance in obese, metabolically compromised individuals.

2) High intensity interval training (HIIT) is an effective and time-efficient exercise modality to improve metabolic health in obese adults.

Thus, these results contribute to the still unsolved questions about how to optimize the benefits of exercise training in human metabolic health. Also, these results indicate that HIIT is an alternative training regime to conventional combined aerobic/resistance exercise, which might circumvent the common report of lack of time by individuals to not adhere to exercise programs nowadays.

The results of **chapter 4** provide novel insights about potential targets of intervention to treat exercise intolerance in metabolically compromised and elderly individuals. These finding may inspire future research to explore specific nutritional supplements aiming to enhance exercise tolerance. The relevance of these results is that premature muscle fatigue prompts a poor exercise adherence and increases the risk of stumbling and falls and compromises subjects independence.

Since obesity and aging are major societal challenges in the last decade, associated with a variety of co-morbidities causing a substantial health care cost, the targeted treatment of exercise intolerance and premature muscle fatigue can enhance the quality of life of subjects and reduce the health care costs.

As last, the results of **chapter 4, 5 and 6** underscore the use the magnetic resonance spectroscopy (MRS) technique to investigate how muscle and liver adapt to exercise training. Interestingly, the MRS technique was applied to investigate dynamic responses of the muscle tissue to exercise *in vivo* with a high time resolution, which was (up to now) mostly investigated via taking multiple muscle biopsies in a short time window.

To whom are the research results interesting and relevant?

The results of this thesis are interesting and of relevance for different entities such as researchers, metabolically compromised and elderly individuals, health care professionals and clinical practice, as well as nutritional and pharmaceutical companies.

Researchers

The results of **chapter 2 and 3** demonstrate that exercise timing impacts the benefits of exercise in human metabolic health. Thus, researchers might take advantage of such findings to investigate if the benefits of exercise on metabolic health can be optimized or possibly even amplified if timed to the central or peripheral muscle clock. In this regard, food ingestion before and after exercise is an important aspect to consider as nutrients are known to interact with the molecular clock machinery and to influence exercise responses.

In addition, researchers interested to investigate skeletal muscle mitochondrial function can benefit from the results in **chapter 5** of the present thesis as here we validated the use of surrogate markers from human biopsies relative to the gold standard measures (high resolution respirometry). Thus, researchers who do not possess such gold standard technique can analyze specific markers from muscle samples to conclude about mitochondrial function. Moreover, we also validate non-invasive markers for skeletal muscle and whole-body oxidative capacity and investigate to what extent they reflect skeletal muscle mitochondrial function, from which in turn researchers can benefit who cannot obtain muscle biopsies from human volunteers.

Metabolically compromised and elderly individuals

The results of chapter **3, 4 and 6** are of interest for metabolically compromised and elderly individuals, especially to understand the potential of regular exercise training to promote metabolic health. These results can be helpful to stimulate a healthy lifestyle and to motivate individuals to engage in regular exercise programs. To attain those goals, it is crucial to disseminate the scientific results of the studies outlined in this thesis into layman's terms.

Health care professionals and clinical practice

The results of **chapter 6** of the present thesis can be helpful for health care professionals who support obese, metabolically compromised and elderly individuals while performing therapeutic physical activity. Nowadays, endurance-type combined with strength-type exercise are conventional exercise regimes recommended for obese and elderly individuals aiming to improve plasma glucose homeostasis. The results of chapter 6 show that high intensity interval

training (HIIT) is an effective methodology to improve plasma glucose homeostasis, which in turn highlights HIIT as an alternative exercise strategy to be implemented into the clinical practice. In addition, the results of this thesis demonstrate that timing of an exercise training session is a crucial environmental cue when aiming to improve glucose homeostasis and elucidates that performing afternoon exercise training might be more optimal than exercising at morning hours.

Nutritional and pharmaceutical companies

The results of **chapter 4** of the present thesis reveal the slow responsiveness of skeletal muscle mitochondria at the onset of exercise as a potential underlying cause of premature muscle fatigue in metabolically compromised and elderly individuals, which can be used as target of interventions. These findings can be of interest to nutritional companies as the supplementation with products able to elevate the acetyl-coa content in muscle (e.g., carnitine and ketone bodies mono-esters beverages) might accelerate the mitochondrial responsiveness at the onset of exercise, and eventually improve physical function. For these compounds to be optimally effective, it is however essential that they reach the muscle in their active form and supplement the endogenous stores. Thus, smart delivery and targeted release approaches may need to come into play here. Also, for this, involvement of food tech industry would clearly be an asset.
In addition, the present findings can draw attention to pharmaceutical companies to elaborate specific acetyl-coA precursors in muscle tissue or to develop controlled substances that promote the enzymatic activation of CrAT protein.

How can these target groups be involved in and informed about the research results, so that the acquired knowledge can be used in the future?

The results of the present chapters are or will be published as original scientific articles in international, peer reviewed and open access journals. Hence, the findings of this thesis will become fully available online and will be of free access for the different entities interested worldwide. Importantly, some of the results of this thesis have been disseminated to the scientific community on national (Annual Dutch Diabetes Research Meeting) and international scientific conferences (Cell Symposia: Exercise Metabolism, Muscle Clocks and Diabetes Symposia, Metabolism in Action – Copenhagen and at the European Association for the Study of Diabetes) via poster and oral presentations. In such way, the acquired knowledge from the

present thesis was and will be spread within the scientific community to promote novel research ideas as well as to generate collaborative work with (inter)national peers.

Next to presenting the results of this thesis to the scientific community, all individuals who voluntarily participated in the studies received an information brochure summarizing the study results. One way to disseminate the research results to the no scientific community in layman terms is the use of social media. Also, meetings can be organized with individuals interested in research to inform them about the investigation results as well as the importance of a healthy lifestyle.

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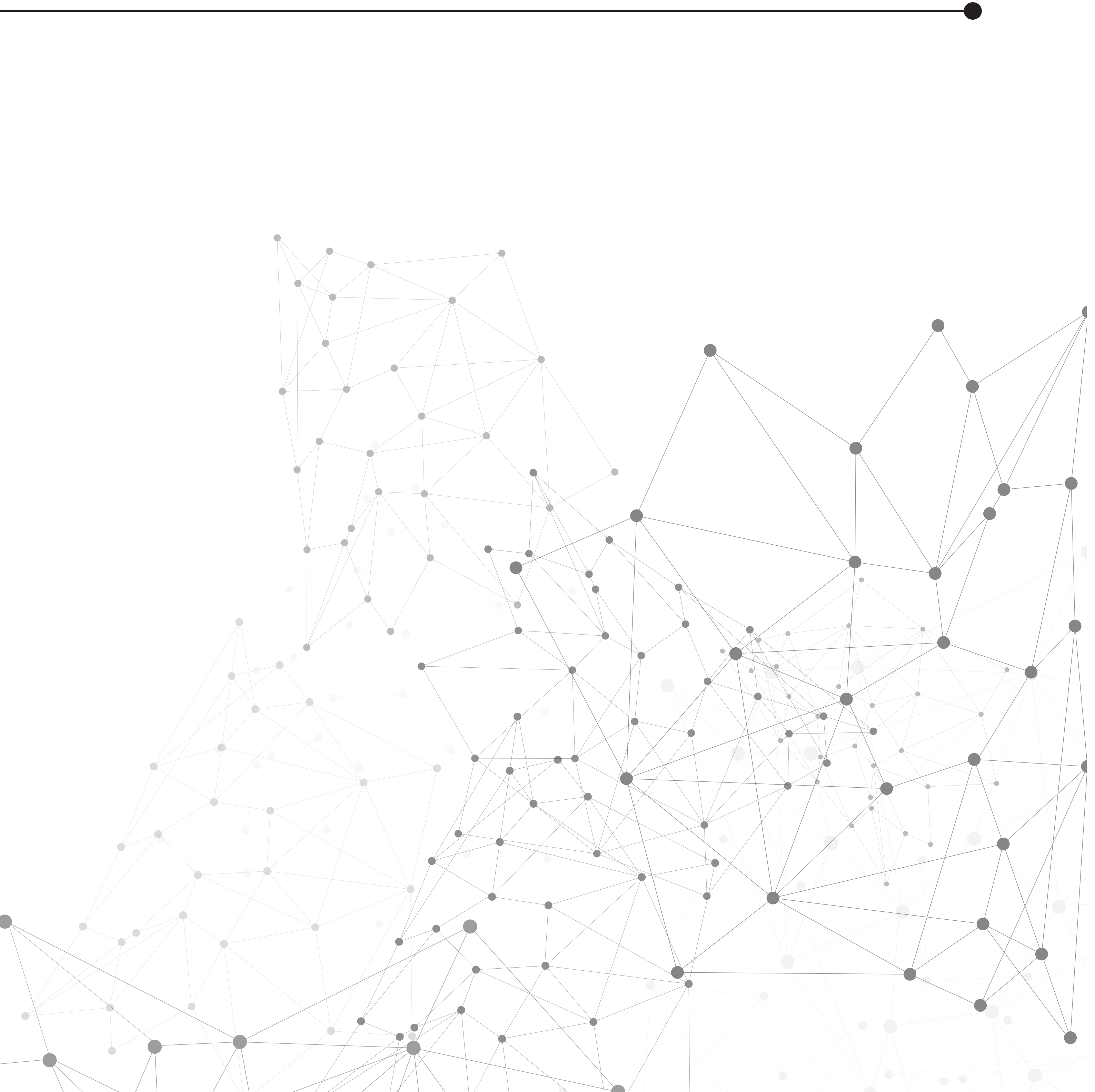

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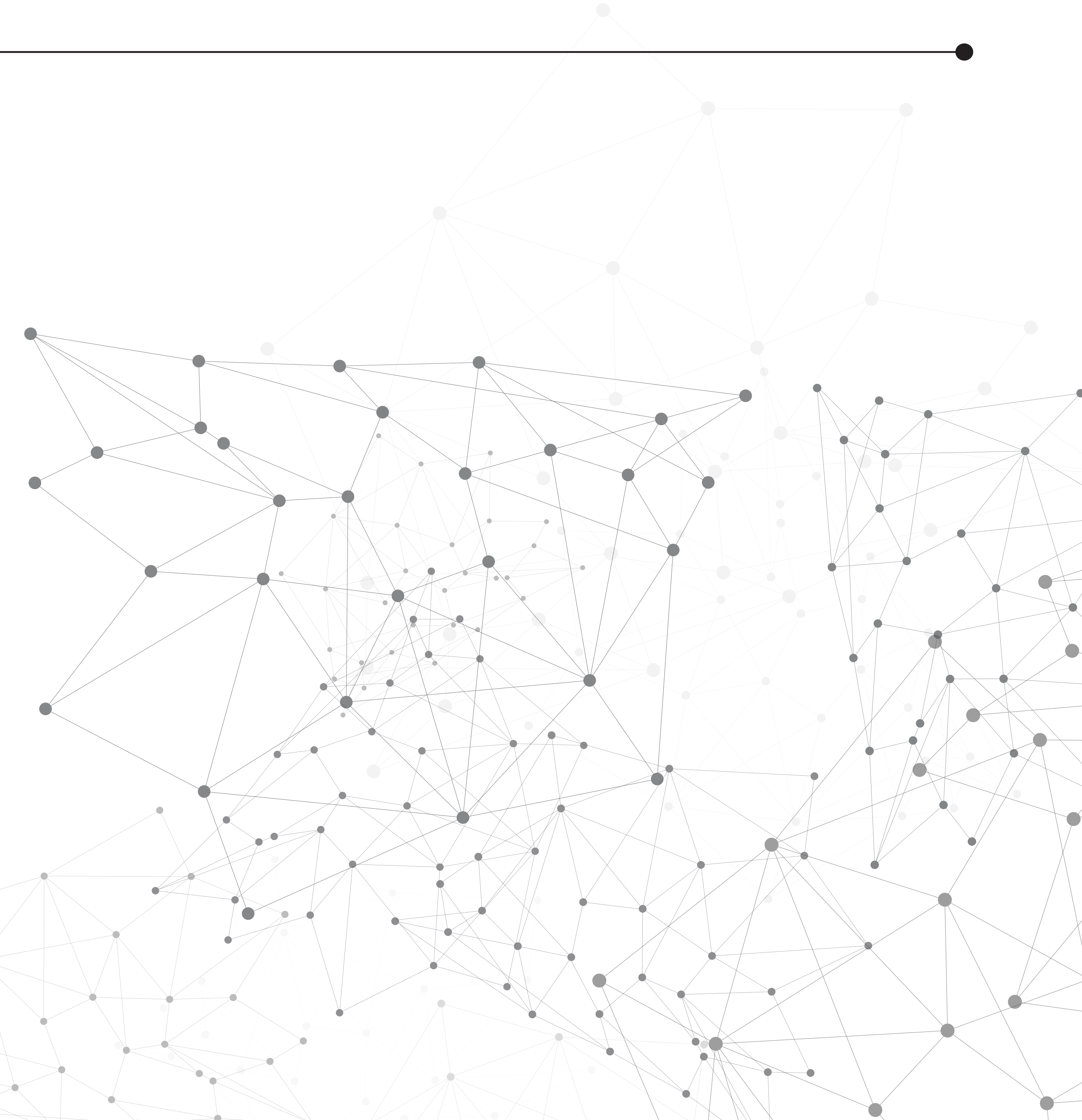
Quienes siempre creyeron en mi.

This achievement is dedicated to my family, Friends and to my mentor Erik Diaz;
Who always believed on me.

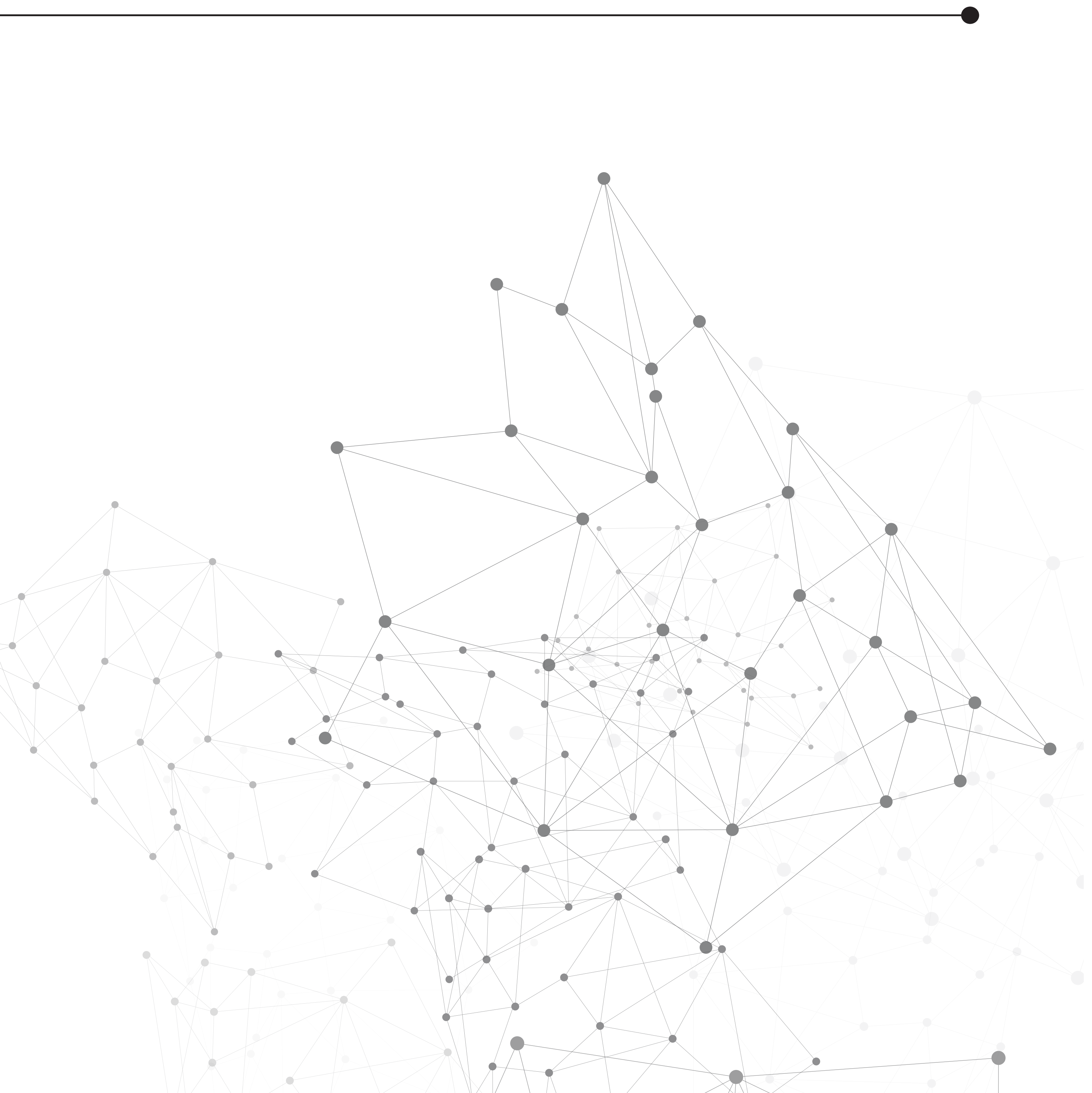
ABOUT THE AUTHOR



Rodrigo Mancilla was born in Coyhaique city, in southern Chile in 1989. In 2007, Rodrigo started his bachelor degree in Physical Education at the Universidad de la Frontera, Temuco - Chile. In 2011, he began his first master in physical activity and health at Universidad de la Frontera, under the supervision of Dr. Erik Diaz. Working at the center for investigating human metabolism and health (CIEMETS), Rodrigo published his thesis about the effects of high-intensity interval training on glycaemic control in pre-type 2 diabetic subjects. In 2014, Rodrigo received a national award from the Chilean commission of investigation, sciences and technology (CONICYT) to pursue a second master degree at Maastricht University, specifically the human movement sciences master program. During his internship, Rodrigo worked under the supervision of Prof. Matthijs Hesselink and Dr. Vera Schrauwen-Hinderling, studying the in-vivo diurnal metabolic variations of human liver tissue by using magnetic resonance spectroscopy (MRS) techniques.

In 2016, Rodrigo received a second award from CONICYT to pursue his PhD at the department of Nutrition and Movement science at Maastricht University under the supervision of Prof. Matthijs Hesselink and Dr. Vera Schrauwen-Hinderling. During his PhD, Rodrigo primarily focused on the effects of regular exercise training and nutrition in skeletal muscle tissue remodeling in relation to insulin sensitivity and mitochondrial function in insulin resistant subjects, taking into account a cellular, tissular and whole body approach. Upon finalization of his PhD, Rodrigo will continue exploring how to optimize the synergic interaction between exercise and nutrition to improve glucose homeostasis and skeletal muscle mitochondrial function in humans, as well as other novel environmental cues to promote human metabolic health.

LIST OF PUBLICATIONS



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3.- High intensity interval trainig improves whole-body insulin sensitivity, skeletal muscle oxidative capacity and modifies intrahepatic lipid composition in overweight/obese individuals. **Mancilla R*,** Bergman M*, Veeraiah P, de Wit V, Bruls Y, Hoeks J, Schrauwen-Hinderling V, Hesselink M.K.C.