DISSERTATION

SHORT-TERM METABOLIC EFFECTS OF BREAKING UP SEDENTARY BEHAVIORS

Submitted by

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ABSTRACT

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"Sit Less, Move More" has become a widespread public health message due in part to the recognition that sedentary behaviors (i.e., sitting) are associated with all-cause mortality and increased risk for obesity, diabetes, cardiovascular diseases and some cancers, even when accounting for time spent in moderate-to-vigorous physical activity. Recent epidemiological and experimental evidence from acute and short-term studies indicate that reducing and breaking up sedentary behaviors (i.e., sitting) may be a useful strategy for glucose control. Acute experimental trials (5-12 hr exposure) demonstrate that breaking up sedentary time with short-frequent bouts of physical activity is associated with lower postprandial glucose and insulin concentrations while a time-matched single-continuous bout is associated with lower postprandial triglyceride concentrations in response to standardized meals. This suggests differential substrate oxidation may be responsible for the changes in postprandial metabolites. However, what is unknown is (1) whether breaking up sedentary behaviors with short-frequent bouts of physical activity is a strategy that can be implemented in the daily life of sedentary, physically inactive adults; (2) whether the acute metabolic benefits previously observed are sustained or diluted beyond the acute exposure period (> 5-12 hr); (3) whether the effects are due to the active breaks *per se* or to increases in total energy expenditure and/or total active time and (2) the characterization of potential underlying physiological, cellular, and molecular mechanisms. The primary objective of this dissertation is to investigate the feasibility of implementing short-frequent bouts of physical activity to break up sedentary behaviors into daily life over the short-term (4-day) in those who are habitually inactive and the effect on nutrient metabolism when energy expenditure and balance are matched. We hypothesized that breaking up sedentary behaviors with short-frequent

bouts of physical activity is a feasible lifestyle intervention to increase physical activity which will be associated with attenuated glycemia by an increase in postprandial carbohydrate oxidation. In a randomized cross-over study, we compared the short-term effects (4-day) of breaking up sedentary behaviors with short-frequent bouts of moderate intensity physical activity (MICRO: 5-min walk bout every hour for 9 consecutive hours per day) to a time-matched single-continuous bout of moderate intensity physical activity (ONE: 45-min continuous walking bout per day), and a sedentary control (SED: habitual sedentary behaviors and physical inactivity each day) in inactive male and female adults with overweight or obesity. To reach our overall objective, three independent specific aims were pursued: 1) to determine the feasibility of implementing MICRO compared to ONE on daily time spent sitting and physically active over the short-term; 2) to determine the effect of MICRO compared to ONE on nutrient metabolism and insulin sensitivity; 3) to characterize the short-term effect of MICRO compared to ONE on permeabilized skeletal muscle fiber respiration and gene expression of proteins involved in the regulation of metabolic pathways. Results from this dissertation demonstrate that 1) MICRO is a feasible intervention to promote physical activity both on workdays and non-working days in those who are at high risk for metabolic disease; (2) At the same energy expenditure and balance, MICRO resulted in a greater reliance on carbohydrate as fuel during the waking period when the bouts were performed and over 24 hr. In contrast, a single isoenergetic continuous bout of moderate intensity walking increased 24 hr total and dietary fat oxidation. Both physical activity interventions lowered postprandial insulin and improved fasting indexes of insulin sensitivity compared to SED; 3) While no changes were observed after MICRO in mitochondrial oxidative capacity, MICRO is a sufficient stimulus to promote adaptations in skeletal muscle augmenting pathways associated with substrate oxidation. Independent of detectable differences in insulin sensitivity, total active time, and energy expenditure, breaking up sedentary behaviors with short-frequent bouts of physical activity spread throughout the day is a viable lifestyle intervention for glucose control compared to the same amount of physical performed as a single continuous bout with the rest of the day

spent sedentary. This evidence can be used to refine future physical activity guidelines to prevent and treat metabolic diseases, not in terms of intensity of exercise per day per week but in terms of avoidance of sedentary activities through short bouts of physical activity.

ACKNOWLEDGEMENTS

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1. Dr. Audrey Bergouignan's mentorship, guidance, and most importantly her friendship. You dedication to my training and trust in me allowed me to develop into a better scientist as well as a better person. You have sponsored my education and helped me develop independence and self-confidence. Thank you for listening, and teaching me throughout these years, especially during all the challenges and uncertainties.
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3. Allie, Betty Ann, Peggy, and Paul without my family none of this would be possible.
4. Mom, Dad, Jason, and Julie, thank you for your support and being able to describe to others what Human Bioenergetics is (*A fancy word for metabolism*).

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LIST OF DEFINITIONS

Activities of daily living: non-exercise activities, activities required for everyday living, including eating, bathing, toileting, dressing, getting into or out of a bed or chair, and basic mobility. Activities related to independent living, including preparing meals, managing money, shopping for groceries or personal items, and performing housework.

Aerobic physical activity: forms of activity that are intense enough and performed long enough to maintain or improve an individual's cardiorespiratory fitness. Aerobic activities commonly require the use of large muscle groups. Examples of aerobic activities include walking, basketball, soccer, wheelchair rolling, or dancing.

Body mass index: a weight-to-height ratio, calculated by dividing one's weight in kilograms by the square of one's height in meters.

Duration: the length of time for each session or bout.

Exercise: a component of physical activity; refers to activity that is planned, structured and repetitive for the purpose of improving or maintaining one or more components of physical fitness.

Frequency: the number of sessions or bouts of physical activity performed per day or per week.

Intensity: the rate of energy expended during the physical activity session or bout, usually in METs.

Interruptions to sedentary time: transition from sitting to standing or moving so that prolonged periods of sitting time are regularly interrupted.

Leisure time physical activity: discretionary activity performed when one is not working, transporting oneself to a different location, or doing household chores. Sports or exercise, going for a walk, and playing games (hopscotch, basketball), are examples of leisure-time physical activity.

Light intensity: activity requiring between 1.6 and 3.0 METs, such as walking at a slow pace (2 mph or less) or cooking, sweeping, light cleaning. Many non-exercise activities or daily life activities are light intensity.

Lying: refers to being in a horizontal position on a supporting surface.

Maximal oxygen uptake (VO2max): the body's capacity to transport and use oxygen during a maximal exertion involving dynamic contraction of large muscle groups, such as during running or cycling. It is also known as maximal aerobic power. Peak oxygen consumption ($VO2_{peak}$) is the highest rate of oxygen consumption observed during an exhaustive exercise test.

Metabolic Equivalent of Task (MET): A unit that represents the metabolic cost of physical activity. One MET is the rate of energy expenditure while sitting at rest, which, for most people approximates an oxygen uptake of 3.5 ml per kg per min. The energy expenditure of other activities is expressed in multiples of METs. For example, for the average adult, sitting and reading requires about 1.3 METs, strolling or walking slowly requires about 2.0 METs, and running at 5 miles per hour requires about 8.3 METS.

Metabolic health: having ideal levels of blood sugar, triglycerides, high-density lipoprotein (HDL) cholesterol, blood pressure, and waist circumference, without using medications. These factors directly relate to a person's risk for heart disease, diabetes, and stroke.

Metabolic syndrome: the name for a group of risk factors that raises your risk for heart disease and other health problems, such as diabetes and stroke. These risk factors are: abdominal obesity, high plasma triglyceride concentration, A low HDL cholesterol level, high blood pressure, and high fasting plasma glucose concentration.

Moderate intensity: activity requiring 3.0 to less than 6.0 METs, such as walking briskly (3 to 4 mph), mopping or vacuuming, or raking a yard.

Non-exercise physical activity: All physical activity that is not exercise.

Observational study: a study in which outcomes are measured but no attempt is made to change the outcome. The two most commonly used designs for observational studies are case-control studies and prospective cohort studies.

Occupational physical activity: activity performed at work, such as stocking shelves in a store, delivering packages in an office, preparing or serving food in restaurant, or carrying tools in a garage are examples of occupational physical activity.

Overweight: having a body mass index between 25 and 29 kg/m^2

Obese: having a body mass index between equal or above 30.0 kg/m^2.

Physical activity: any bodily movement produced by skeletal muscles that results in energy expenditure.

Physical activity guidelines for aerobic physical activity: adults should do at least 150 minutes to 300 minutes a week of moderate-intensity, or 75 minutes to 150 minutes a week of vigorous-intensity aerobic physical activity, or an equivalent combination of moderate- and vigorous-intensity aerobic activity.

Physical inactivity: an insufficient physical activity level to meet present physical activity guidelines for aerobic physical activity.

Prospective cohort study: a type of epidemiologic study in which the practices of the enrolled subjects are determined, and the subjects are followed (or observed) for the development of selected outcomes. It differs from randomized controlled trials in that the exposure is not assigned by the researchers.

Randomized controlled trial: a type of study design in which participants are randomly grouped based on an investigator-assigned exposure of interest, such as physical activity. For example, among a group of eligible participants, investigators may randomly assign them to exercise at three levels: no activity, moderate-intensity activity, and vigorous-intensity activity. The participants are then followed over time to assess the outcome of interest, such as change in abdominal fat.

Reclining: a body position between sitting and lying.

Resistance training: a method of muscle-strengthening activity or conditioning that involves the progressive use of resistance to increase one's ability to exert or resist force.

Sedentary behavior: any waking behavior characterized by an energy expenditure 1.0 to 1.5 metabolic equivalents (MET), while in a sitting, reclining, or lying posture.

Sedentary behavior pattern: the manner in which sedentary behavior is accumulated throughout the day or week while awake (e.g., the timing, duration and frequency of sedentary bouts and breaks).

Self- reported measures: the type of exposure assessment that has been most typically used in epidemiological studies on physical activity and health outcomes, often using 1- week recall via a self- completion survey or interview.

Sitting: a position in which one's weight is supported by one's buttocks rather than one's feet, and in which one's back is upright.

Standing: a position in which one has or is maintaining an upright position while supported by one's feet.

Total physical activity: sum of time spent in physical activity of light, moderate and vigorous intensity.

Total sedentary time: time spent in sedentary behavior that can be inferred from minimal measured movement based on an accelerometer reading; for example, the total time accumulated below a defined threshold.

Vigorous intensity: activity requiring 6.0 or greater METs, such as walking very fast (4.5 to 5 mph), running, mowing grass with a hand-push mower, or participating in an aerobics class.

Volume: the total amount of physical work performed in either a single session or over the course of an extended physical activity program.

LIST OF ACCOMPLISHMENTS

This dissertation work has been the topic for the following publications, oral and poster communications, funding applications, honors and awards, and workshops:

Peer Reviewed Publications:

- De Jong NP, Rudolph M, Sharp R, Houck J, Jones K, Jackman M, MacLean P, Bessesen DH, Bergouignan A. "*Short-term adaptations in skeletal muscle mitochondrial oxidative capacity and metabolic pathways to breaking up sedentary behaviors with short-frequent bouts of physical in adults with obesity*." Nutrients, 14 (3), 454.
- Le Roux E*, De Jong NP*, Blanc S, Simon C, Bessesen DH, Bergouignan A. (2021) "*Physiology of physical inactivity, sedentary behaviors and non-exercise activity: Insights from space bedrest model.*" Journal of Physiology, 600: 1037-1051 (* Co first authors) – *Invited review.*
- De Jong, NP, Rynders CA, Goldstrohm D, Pan Z, Lange A, Mendez C, Melanson EL, Bessesen DH, Bergouignan A. (2019) "*Effect of frequent interruptions of sedentary time on nutrient metabolism in sedentary overweight male and female adults.*" Journal of Applied Physiology, 126 (4), 984-992.
- De Jong NP, Debache I, Pan Z, Garnotel M, Lyden K, Sueur C, Simon C, Bessesen DH, Bergouignan A. (2018) "*Breaking up sedentary time in overweight/obese adults on work days and non-work days: results from a feasibility study.*" International Journal of Environmental Research and Public Health, 15 (11), 2566.
- Rynders CA, Blanc S, De Jong NP, Bessesen DH, Bergouignan A. (2018) "*Sedentary behavior is a key determinant of metabolic inflexibility.*" Journal of Physiology, 596 (8), 1319-1330. *Invited review*.

Publications In Preparation:

- De Jong NP, Lange A, Mendez C, Shreck L, Pan Z, Rynders, CA, Broussard, JL, Bessesen DH, Bergouignan A. "*Effects of one month of breaking up sedentary behaviors on glucose control and insulin sensitivity in physically inactive adults with overweight or obesity*."
- De Jong NP, Lange A, Mendez C, Goldstrohm D, Pan Z, Melanson EL, Bessesen DH, Bergouignan A. "*Effects of one month of breaking up sedentary behaviors with short frequent physical activity bouts on 24hr nutrient metabolism in physically inactive adults with overweight or obesity*."

Oral Presentations:

- De Jong NP, Rudolph MC, Jackman M, Sharp R, Jones K, Houck J, Pan Z, Reusch J,

MacLean P, Bessesen DH, Bergouignan A. "*Short-Term Skeletal Muscle Cellular and Molecular Responses to Breaking Up Sitting Time.*" The Obesity Society National Meeting, November 2021, virtual conference.

- De Jong NP, Bessesen DH, Bergouignan A. "*Effects of Breaking-up Sedentary Behaviors on Glycemic Control and Nutrient Metabolism."* Division of Endocrinology, Metabolism, and Diabetes Research Conference, October 2019, Aurora, CO.
- Schreck L, De Jong NP, Lange A, Mendez C, Glazer T, Goldsthrom DA, Melanson EL, Rynders CA, Broussard JL, Bessesen DH, Bergouignan A. Effect of breaking-up sedentary activity on metabolic flexibility and glycemia in free-living overweight/obese adults. American College of Sports Medicine, June 2019, Orlando, FL, USA.
- Laurens C, Schreck L, De Jong NP, Lange A, Mendez C, Glazer T, Goldsthrom D, Melanson EL, Rynders CA, Broussard JL, Bessesen DH, Bergouignan A. Effect of breaking-up sedentary activity on metabolic flexibility and glycemia in free-living overweight/obese adults. Société Française du Diabète, March 2019 Marseille, France.
- Bergouignan A, De Jong NP, Rynders CA, Melanson EL, Bessesen DH. Effect of breaking up sedentary time on substrate oxidation, metabolic flexibility and appetite in overweight sedentary adults. Benjamin Franklin-Lafayette Seminars, June 2017, Fréjus, France.
- De Jong NP, Bessesen DH, Bergouignan A. "*The Effect of Frequent Interruptions of Sedentary Time on Energy, Mood and Fatigue.*" Colorado School of Public Health, MAP ERC Research Day, March 2017, Denver, CO. Award - Outstanding Platform Presentation.
- Bergouignan A, De Jong NP, Rynders C, Melanson EL, Bessesen D. Effect of frequent interruptions on energy expenditure and substrate oxidation in overweight adults. 2016 American College of Sport Medicine, World Congress on the Basic Science of Energy Balance, Thematic Poster session: Energy balance – Innovative strategy, June 2016, Boston, MA.

Poster Presentations:

- De Jong NP, Schreck L, Lange A, Mendez C, Debache I, Garnotel M, Simon C, Bessesen DH, Bergouignan A. "*Effect of short bouts of activity spread throughout the day on the components of energy balance.*" The Obesity Society National Meeting, November 2019, Las Vegas, NV, USA.
- De Jong NP, Lange A, Mendez C, Debache I, Garnotel M, Zahariev, Simon C, Bessesen DH, Bergouignan A. "*Effect of one-month breaking up sedentary behaviors on metabolic profiles.*" The Obesity Society National Meeting, November 2019, Las Vegas, NV, USA.
- De Jong NP, Rynders C, Bessesen DH, Bergouignan A. "*Short-term effect of frequent short bouts of brisk walking on time spent sedentary and self-perceived levels of fatigue and vigor in free living overweight adults.*" American College Sports Medicine National Conference, Nov 2017, Denver, CO, USA.

- Rynders CA, Bergouignan A, De Jong NP, Melanson EL, Korht WM, Bessesen DH. Effect of acute exercise without energy replacement on fat oxidation and hormone profiles during sleep. American College of Sports Medicine, June 2017, Denver, Colorado, USA.

Funding Awards

NIH F31 Predoctoral Individual National Research Service Award

09/2020 – 03/2022: $48,043 - De Jong (PI)

Effects of breaking up sedentary behaviors on energy and substrate metabolism.

The training objectives outline a career development plan to supplement my doctoral training focused on the metabolic effects of physical activity to facilitate clinical research training in the use of stable isotope tracer methodology to track substrate metabolism, doubly labelled water method to derive total daily energy expenditure, use of accelerometry-based monitors to assess the daily patterns of sedentary behaviors and physical activity, and continuous glucose monitoring to assess daily glycemia.

Mentoring Team: Dr. Audrey Bergouignan PhD (primary), Dr. Ed Melanson PhD (co-primary), Dr. Jane Reusch MD, Dr. Dan Bessesen MD.

TL1 Program: Team Training across the Translational Sciences Spectrum

07/2018 – 06/2019: $41,484 - De Jong (PI)

Metabolic effects of breaking up sedentary time

The goal of this grant was to increase my exposure in wet lab work. I learned how to measure cellular and molecular changes in muscle-derived progenitor cells in response to a physical activity lifestyle intervention on physical activity and sedentary behaviors. This training facilitated the vertical integration of metabolism measured at the whole-body level down to the cellular and molecular mechanisms at the skeletal muscle level.

Mentoring team: Dr. Kristen Boyle PhD (primary) and Dr. Audrey Bergouignan PhD.

Funding applied not awarded

NIH NRSA F31 – A0	August 2019
NIH NRSA F31 – A1	April 2019
Chateaubriand Fellowship Program	January 2019
NIH NRSA F31 – A0	December 2018
BESST International Visiting Scientist	March 2018

Team Oriented Training across the Translational Sciences Spectrum (TL1) July 2017

Honors & Awards

Outstanding Graduate Student Colorado Nutrition Obesity Research Center 2018
University of Colorado – Anschutz Medical Campus, Aurora, CO

Outstanding Platform Presentation, 9th Annual Research Day Symposium 2017
Mountain and Area Plains Education & Research Consortium, Colorado School of Public Health, Aurora, CO

Workshops

Isotope Tracers in Metabolic Research Workshop November 2021
NIDDK (5-days).

Doubly Labelled Water Workshop March 2021
CO-NORC, University of Colorado (4-days)

Training in Nutrition and Obesity Research Methods September 2019
Pennington Louisiana NORC, Louisiana State University (2-days).

Indirect Calorimetry Workshop October 2019
CO-NORC, University of Colorado (3-days).

Technical Skills

- Participant recruitment, screening and enrollment
- Conductance of human clinical trial visits.
- Delivery of physical activity interventions.
- Objective measurement of physical activity and sedentary behaviors (ActivePal$_{micro}$ and Actigraph GT3X+).
- Nutrient metabolism (whole-room and hood indirect calorimetry, stable isotope tracer technique).
- Skeletal muscle mitochondrial respiration (permeabilized muscle fiber respirometry).
- Primary myoblast cell culture and differentiation.
- *In vitro* cellular metabolism (C14 glucose and fatty acid metabolism - star trap method).
- Molecular adaptations (RNA sequencing and pathway analysis, and capillary western blotting - JESS).

Professional Development

5 Choices for Extraordinary Productivity, Anschutz Health and Spring 2021
Wellness Center, University of Colorado (4-days).

Leadership for Innovative Team Science, TL1 Program, Colorado Clinical & Spring 2019

Translational Science Institute, University of Colorado (4-days).

CO-Mentoring Workshop, Colorado Clinical & Translational Science Institute, University of Colorado (completed with Dr. Audrey Bergouignan, 6-days). Fall 2018

Science of Team Science, TL1 Program, Colorado Clinical & Translational Science Institute, University of Colorado (2-days). Spring 2018

Teaching

HES 600 Research Design in Health/Exercise Science (Dr. Matthew Hickey – primary teaching mentor).

10/28/2020: Online class lecture: *How to be responsive in grant resubmissions.*

9/30/2020: Online class lecture: *Observational study designs & randomized trials.*

Class project: Reviewed and provided feedback on 15 x NIH F31 grant proposals

INTRODUCTION

1. THE TRANSITION OF PHYSICAL ACTIVITY

1.1. Historical perspective on the changes in physical activity patterns and sedentary behaviors observed in our modern societies

Most of current human biology and behaviors resulted from natural selection in *Homo Sapiens* about 200,000 years ago before advances in agriculture and technology [1]. Before agriculture, humans were hunting and gathering to access food, theoretically producing high levels of physical activity [2]. With the development of agriculture (~10,000 years ago), the former lifestyle cycle of short bouts of intense activities coupled with long resting periods (i.e., hunting and gathering) was replaced by continuous and sustained moderate intensity activities necessary to perform non-technological agriculture tasks [3]. It wasn't until the start of the Industrial Revolution (beginning at the end of the 18th century in the UK) that physical activity patterns dramatically shifted [4]. Major technological, socio-economic and cultural changes led to the replacement of a labor-based economy with one based on industry and machine manufacturing. Professional environments requiring high energy expenditure such as mines, forests or farms have been reduced in favor of sedentary environments with very low levels of physical activity. In the 1970's, 60-70% of workers had manual labor jobs but since the 1990s, 60-70% of workers have jobs in an office environment [5]. Not only observed in the professional environment but changes in physical activity have occurred in all contexts of daily life: domestic (dishwashers, washing machines, etc.), transport (car, bus, train) and leisure (video games, internet, television). All of which has greatly reduced the need for physical activity in everyday life.

1.2. Insufficient physical activity is a global public health concern

Globally more than a quarter of adults (1.4 billion) were not getting enough physical activity in 2016 [6]. Physical inactivity (physical activity level below the physical activity guidelines) is considered a key health risk factor for many chronic diseases [7]. The global burden of premature

mortality and non-communicable disease attributed to physical inactivity is 4% for type 2 diabetes, 4% for coronary heart disease, 2.2% for breast cancer and 2.5% for colon cancer [8]. The lack of physical activity has been estimated to cost INT$53.8 billion worldwide (INT$: international $, all costs converted to international to enable between country comparisons) [9]. Specifically in the United States, only 1 in 4 adults get the recommended levels of physical activity and physical inactivity is estimated to cost $117 billion USD in annual health care expense [10, 11]. The high cost of physical inactivity to the healthcare system may be driven by physical inactivity being associated with a higher risk for 35 chronic disease conditions including insulin resistance (a common precursor to type 2 diabetes), Alzheimer's disease and other diseases, or high cardiovascular risk factors leading to coronary artery disease [12]. In 2012, The Lancet published a first special issue on physical inactivity in which it was stated: "Due to its prevalence, wide geographical distribution and health effects, physical inactivity must be recognized as a pandemic with significant health, environmental, societal and economic consequences" [13]. The special issue on physical inactivity from The Lancet in 2016 urges governments to consider policies and recommendations that encourage physical activity, reduce sitting time, and provide funding and resources to implement these policies [14]. In consideration of the public health burden of physical inactivity the World Health Organization (WHO) and US based Healthy People 2030 have set a goal to reduce physical inactivity by 15% by 2030. However, without direct research on the determinants of physical inactivity (social, economic, and environmental) this goal may not be realized. While direct evidence on underlying mechanism is still scarce, physical inactivity seems to accelerate declines in important physiological factors that determine health status, including aerobic capacity, skeletal muscle mass and strength, and cognition [12].

2. PATHOPHYSIOLOGY OF PHYSICAL INACTIVITY

The pathophysiology of physical inactivity has been studies in a variety of different human models [15]. Briefly summarized, (1) detraining or training cessation models stop exercise training

is highly active people, (2) enforced bed rest exposes individuals to constant bed rest 24 hr/day which restricts muscle activity and postural changes, (3) enforced best rest combined with exercise; 24 hr/day bed rest except when combined with exercise that is performed while still in bed rest, (4) Limb immobilization/casting are characterized by periods in which a limb is immobilized using casting to mick the disuse that occurs following an injury, and (5) Imposed physical inactivity models whereby participants transition from high or normal to low daily ambulatory activity and/or increased sitting time. The bed rest model, more traditionally used by international space agencies to mimic the effects of microgravity on the body on Earth, is considered one of the strongest models for studying the direct physiological effects of physical inactivity, albeit an extreme model. However, while most of the findings described below came from bed rest models, these findings were also confirmed in imposed physical inactivity models [16, 17]. Bed rest model exposes healthy active adults free from any predisposition for chronic diseases and expose them to constant bed rest to study the pathophysiology of physical inactivity [18, 19]. Bed rest induced physical inactivity (Figure 1) produces a loss of body movement (hypokinesia) and loss of strength and power (hypodynamia) which leads to changes in all physiological systems [20]. Bed rest decreases muscle mass and function and produces a shift from slow oxidative to fast glycolytic muscle fibers [21-23] including reduced mitochondrial volume and oxidative capacity [24], and an impaired expression of genes involved in oxidative metabolism and mitochondrial function [25-27] which are associated with a reduction in lipid oxidation in favor of carbohydrate oxidation [26, 28]. In association with lower capacity and activity of key proteins involved in muscle glucose transport, phosphorylation and storage [29], bed rest lowers muscle insulin sensitivity [30]. Attenuated muscle insulin sensitivity leads to the development of glucose intolerance (hyperinsulinemia to main normal glucose disposal) which may be preceded by the development of a metabolically inflexible state (defined as an inability of the body to adjust substrate use to changes in substrate availability) [31]. These alterations are relevant in the postprandial state since the reduction in lipid oxidation in favor of carbohydrate oxidation leads to

decreased clearance of dietary lipids, which contributes to hyperlipidemia. Even with adipose tissue lipolysis [30, 32], elevated plasma lipids enhances fat accumulation in the visceral adipose depot [33] and ectopic fat storage in muscle, liver and bone [26, 34-36] which exacerbates insulin resistance. This cascade of metabolic alterations in healthy people exposed to bed rest are commonly observed in individuals with obesity, T2D or metabolic syndrome. Therefore, findings from bed rest studies support a key role of physical inactivity in the onset and development of metabolic disease.

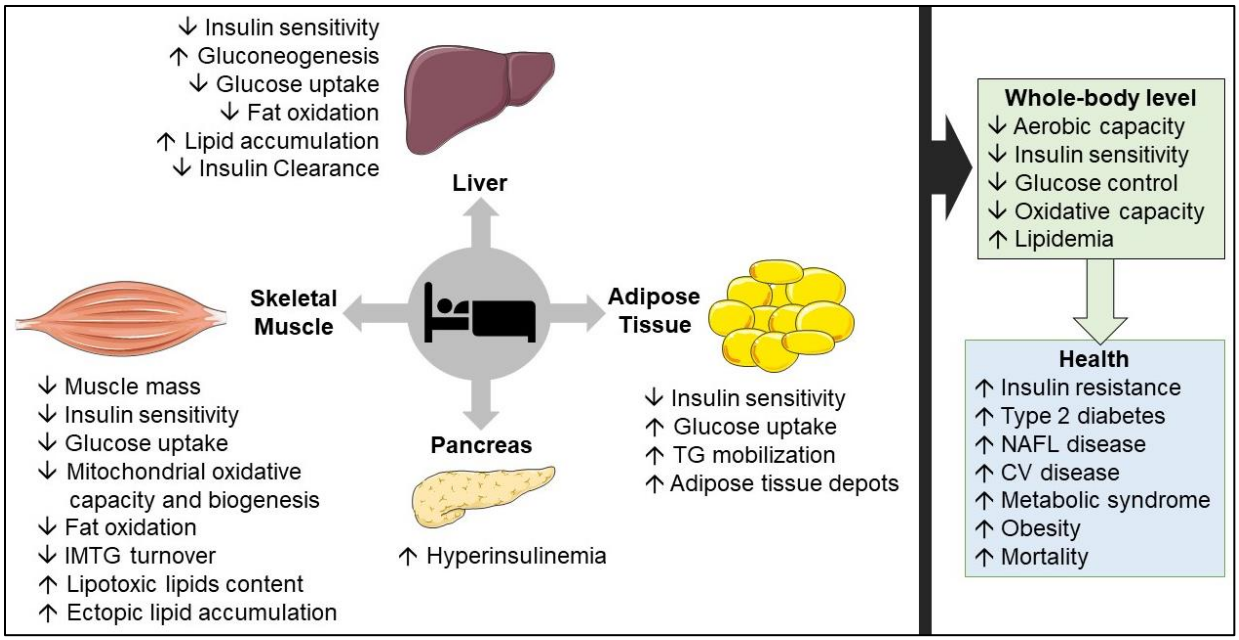

Figure 1: Pathophysiology of physical inactivity from bed rest model and health outcomes. CV: cardiovascular disease; IMTG: intramuscular triacylglycerols; TG: triacylglycerols. Adapted from Le Roux, E., and De Jong, N.P., et al. J Physiol, 2021 [18].

3. SEDENTARY BEHAVIORS, A NEW HEALTH BEHAVIOR

3.1. Definition

Through research on strategies to combat physical inactivity and along with the development of device-based physical activity monitors that can objectively measure all the components of physical activity in daily life (i.e., time spent sitting/lying, standing, stepping, and time spent in sedentary, light, and moderate to vigorous physical activity intensities), scientists have identified

another health risk behavior: sedentary behaviors. Sedentary behaviors are “any waking behavior characterized by an energy expenditure ≤1.5 metabolic equivalents while in a sitting, reclining, or lying posture” [37]. A PubMed keyword search for ‘sedentary behaviors’ showed 52 papers were published in 2000 and that number increased to 2218 published in 2020, highlighting the burgeoning field of sedentary behaviors research. Now, research suggests that being sedentary is distinct from being physically inactivity because of the detrimental associations between sedentary behaviors and health risk [38].

3.2. Prevalence

Sedentary behaviors are a ubiquitous behavior that has increased in every context of daily life over the past decades. While the prevalence of physical inactivity has not been reduced over the past 10 years, time spent in sedentary behaviors has increased [39]. Estimates from population health and large cohort studies using device-based measurements of sedentary behaviors and physical activity show that the average sedentary time in adults may be in the range of 7.7 to 11.5 hr/day, which corresponds up to 60% of adult wake time [40-42]. Most daily sedentary time is accumulated during occupational and leisure time with leisure time accounting for 47% (~4.3 hr/day) of total sedentary time [43]. In the US from 1950 to 2000, time spent watching TV increased by 61.4% and some individuals can spend 8 hr/day watching TV [44]. Sedentary activities such as watching television and videos or engaged in Internet and computer use accounted for 82% of sedentary leisure time [43]. In the modern work environment, office-based employees spend 66% of their total occupational time sedentary and 25% of total sedentary time in sedentary bouts longer than 55-min [45]. This shift from occupational tasks that require physical activity to more sedentary oriented occupational tasks is associated with a lower daily energy expenditure (> 100 kcals/day) that is associated with the increase in mean US body weight for men and women over the past 50 years [46]. This pattern of spending the majority of wakeful hours sedentary has been amplified by the COVID19 pandemic [47]. Risk mitigation strategies for the COVID19 pandemic included social distancing and “stay at home” orders which brought

large reductions in physical activity and considerable increases in sedentary time, particularly among previously physically active individuals [48, 49]. Many opportunities to be physically active were suspended, including fitness centers and public parks, school-based physical education and athletic programs, and outpatient cardiac rehabilitation. The increase in daily sedentary time is meaningful because each hour spent sitting increased the risk of obesity by 23%, type 2 diabetes by 22%, and early mortality by 5.9% [50-52].

3.3. Adverse health consequences of sedentary behaviors

Several epidemiological studies have reported associations between sedentary time and health outcomes including all-cause mortality, cardiovascular disease incidence and mortality, and risk of type 2 diabetes, metabolic syndrome and certain types of cancer [53]. Prospective epidemiological findings from device-based measurements show a positive association between total time spent sedentary and risk for metabolic disease [54]. High total sedentary time in a dose-response manner was associated with the higher risk for cardiovascular disease and type 2 diabetes in older women, even after controlling for health status, physical function, and moderate–vigorous physical activity [55]. In a cross-sectional analysis with 4,757 US based adults (respondents from 2003-04 and 2005-06 National Health and Nutrition Examination Survey) and objectively measured physical activity and sedentary behaviors, total sedentary time was detrimentally associated with waist circumference, body mass index, fasting triglycerides and C-reactive protein, and insulin sensitivity [56]. These associations were observed in both sexes and all ages and ethnicities, and were independent of adiposity. Also, these detrimental associations between higher total sedentary time and risk factors for cardiometabolic disease were present after controlling for physical activity level which suggests that sedentary behavior is a stand-alone factor in the relationship between physical activity and health. The physical activity guidelines promote a health enhancing behaviors that accounts for 3% of wake time each day (Figure 2) and the other 97% of wake time are a combination of non-exercise physical activity and sedentary

behaviors [41]. Physically active people, including those who go beyond the current physical activity guidelines, can be as sedentary as their inactive counterparts [57].

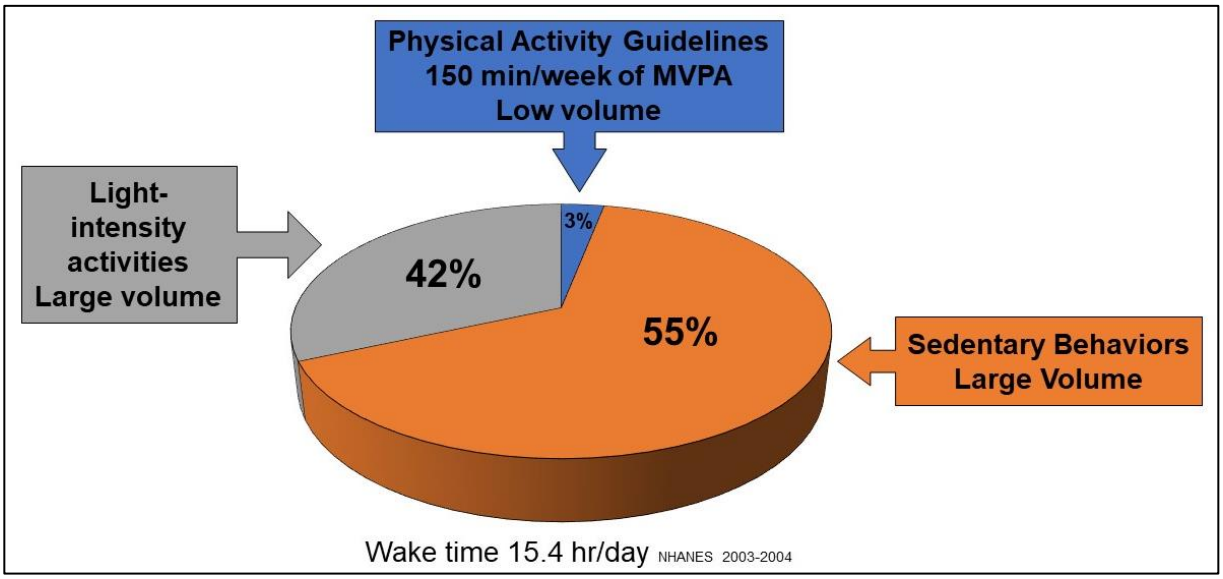

Figure 2: Traditional approach to the promotion of physical activity. While the physical activity guidelines stipulate 150-min of moderate to vigorous physical activity per week this volume of physical activity only represents 3% of wake time per day. This is a small proportion compared to the other 97% of wake time. The other 97% of wake time includes light intensity physical activity or non-exercise activities that correspond to any movement such as daily ambulation and low effort housework. * Adapted from Matthews, C.E., et al. Am J Epidemiol 2008 [41].

3.4. Physiology of sedentary behaviors

Clinical outcomes associated with large volumes of sedentary behaviors include hyperglycemia [58], dyslipidemia [59], and hypertension [60] which are all risk factors for the metabolic syndrome. Sedentary behaviors are further associated with elevated postprandial glucose, insulin, and triglyceride concentrations in response to standardized meals [61-67]. An early response to sedentary behaviors includes changes in markers of insulin action measured by oral-glucose tolerance test and whole-body glucose uptake [68, 69]. The acute effect of sitting on insulin action was measured in 14 young, nonobese, fit, men and women [68]. This randomized cross-over intervention compared three 24 hr interventions: an energy balanced high energy expenditure no sitting active condition (NO-SIT) with two low energy expenditure sitting

conditions, sitting trial without energy compensation (positive energy balance, SIT-POS) and sitting with energy compensation (energy balance, SIT-EB). Compared to NO-SIT, whole-body rate of glucose disappearance normalized to mean plasma insulin was reduced by 39% in SIT-POS and by 18% in SIT-EB. Independent of energy balance, sitting *per se* alters insulin action. In this same study, there was no change in hepatic and adipose tissue insulin action suggesting the changes in whole body insulin action in response to acute sitting were mediated by skeletal muscle. Low metabolic demand and energy turnover in muscle (Figure 3), particularly in the leg weight bearing muscles, may be a mechanism for the elevated concentrations of postprandial metabolites because during acute sedentary behaviors heart rate, blood flow and vascular shear stress remain at basal levels [70, 71].

Prolonged sedentary behaviors are associated with acute increases in blood pressure [72]. The magnitude of the effect of prolonged sedentary behaviors in increasing blood pressure seems to be greater in individuals with current cardiovascular disease risk factors including obesity and type 2 diabetes. While the mechanisms that may explain the higher blood pressure with prolonged sedentary behaviors remain hypothetical, current experimental evidence are starting to provide initial evidence on the physiological effects of prolonged sitting on cardiovascular function and risk markers [70, 73-79]. A seated position creates bends in major blood vessels of the lower body which may produce turbulent blood flow patterns that have been linked to atherosclerosis [80, 81]. Additionally, a lack of muscle contraction or activity associated with sedentary behaviors does not promote blood flow or vascular shear stress which are physiological stressors that may underlie adaptations in the endothelium from physical activity [70, 71]. Higher sympathetic nervous system activity may contribute to increases in blood pressure during prolonged sedentary behaviors. Higher plasma noradrenaline levels associated with sedentary behaviors [82] might result in vasoconstriction thereby increasing total peripheral resistance [83].

Along with associations with higher postprandial nutrient load and blood pressure, sedentary behaviors might further increase the risk of metabolic and cardiovascular disease through the development of sedentary behavior-induced “exercise resistance,” which is the attenuation of the typical responses observed after acute exercise [84]. While acute exercise attenuates plasma concentrations of glucose, insulin and triglycerides, 4-days of prolonged sedentary behaviors abolishes this effect of acute exercise [84, 85]. Additionally, when acute exercise is followed by prolonged sedentary behaviors, the blood pressure lowering effect of acute exercise is lower [86]. Considering this, sedentary behaviors may contribute to the risk of metabolic and cardiovascular disease through direct physiological consequences described above and also through the inhibition of the acute benefits of exercise. Future mechanistic studies will need to establish the physiology of sedentary behaviors to better understand the negative health effects and to better define the dose–response relationship between the components of physical activity and sedentary behaviors and key health outcomes.

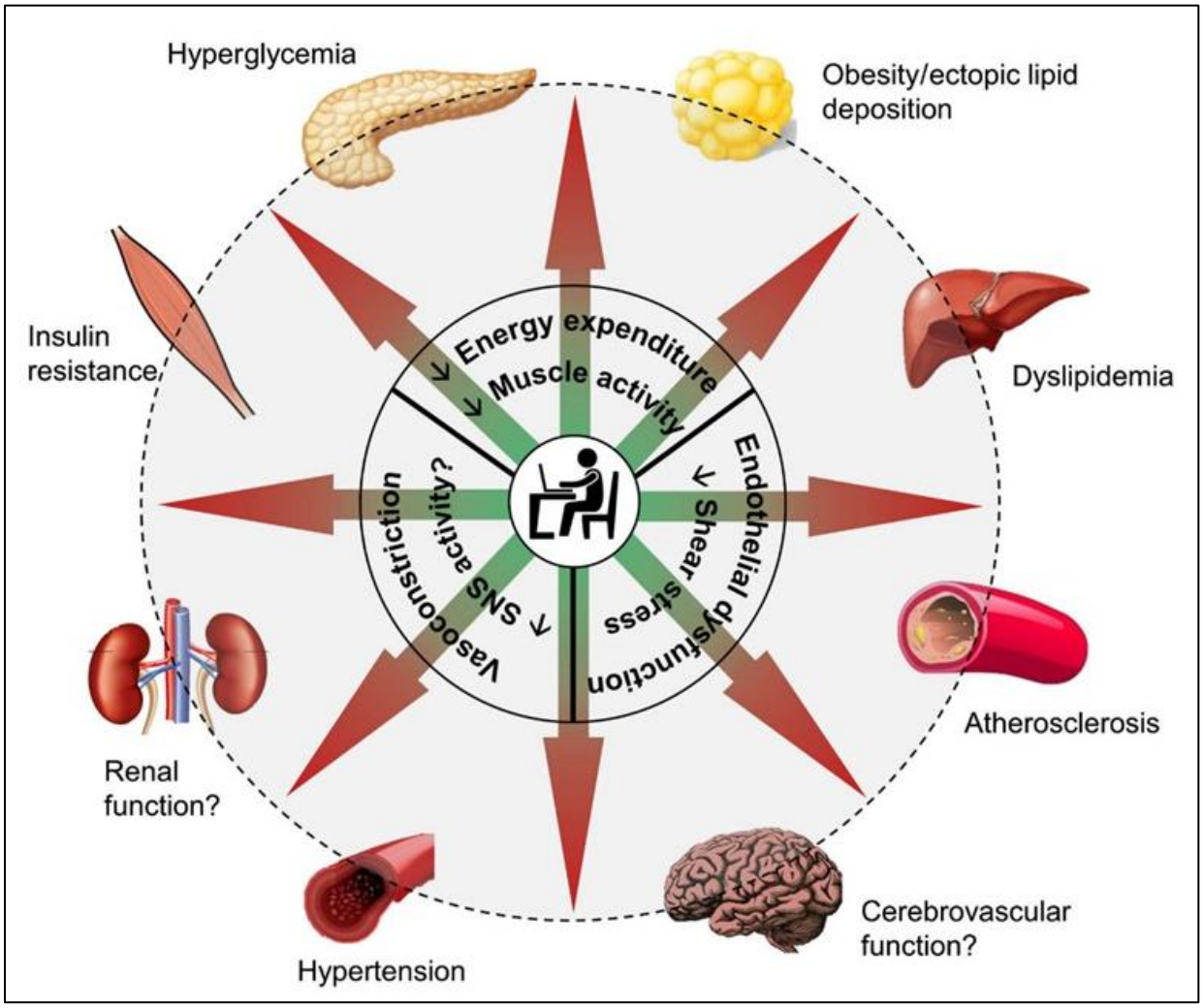

Figure 3: Linking risk factors for chronic disease with sedentary behaviors. Low metabolic demand and energy turnover in skeletal muscle may promote adaptations in peripheral organs that play a key role in metabolism, sympathetic nervous system, and blood flow. These adaptations are hypothesized from physical inactivity models of sedentary behaviors because direct randomized clinical trials are lacking. *Dempsey, P.C., et al. J Phys Act & Health, 2020 [87].

4. THE DOSE-RESPONSE RELATIONSHIPS BETWEEN PHYSICAL ACTIVITY, SEDENTARY BEHAVIORS, AND HEALTH OUTCOMES

How much sitting is too much? How much activity is needed to offset the adverse health effects of sedentary behaviors? Are the health effects the same to engage in light intensity physical activity or moderate-to-vigorous physical activity? A new area of research suggests that in highly sedentary individuals, physical activity performed as a single-continuous bout may not

be sufficient to offset the adverse health effects associated with sedentary behaviors. In a meta-analysis that included 1 million individuals, 60-75 min/day of moderate to vigorous physical activity is needed to prevent the risk of premature mortality that is associated with 9 hr/day or more of sedentary time [88]. While this level of physical activity seems reasonable to achieve, we know that adults do not choose to maintain this level of physical activity considering the low adherence rates to the physical activity guidelines. The same meta-analysis showed in individuals with the highest level of physical activity (>35.5 MET-hr/week) the mortality risk associated with sedentary time was not fully eliminated, suggesting that factors of sedentary behaviors can influence mortality risk [88]. Accumulating observational evidence suggests that prolonged, uninterrupted bouts of sedentary time compared to breaking-up sedentary time may be differentially associated with several cardiometabolic risk biomarkers and premature mortality [54, 55, 89, 90]. A recent study highlighted how even in the context of large volumes of moderate to vigorous physical activity, sedentary behaviors are detrimentally associated with metabolic health [91]. In this study, physical activity and sedentary behaviors were measured objectively with physical activity monitors and the authors determined that time spent in sedentary behaviors and physical activity were associated with metabolic syndrome in 54 older adults. Remarkably, this sample of older adults achieved high levels of moderate to vigorous physical activity (2.6 hr/day) which is 2 hr more than the current recommendations [92]. The results showed that greater sedentary time was associated with a higher metabolic risk score, independent of age and sex. The authors concluded that even among highly active older adults, sedentary behaviors are associated with increased metabolic risk. Therefore, extremely high levels of moderate to vigorous physical activity do not necessarily fully eliminate the negative health risks associated with sedentary behaviors.

In contrast to the growing body of epidemiological evidence currently available, experimental evidence supporting the adverse health effects of sedentary behaviors independent of time spent physically active is limited which is mainly due to challenges in isolating the effects of sedentary behaviors from those of physical activity. In a recent review [18], we addressed this question by

compiling data from bed rest studies with and without exercise to provide new insights into the relationship between sedentary behaviors, physical activity and cardiometabolic health. Beyond investigating the mechanistic underpinnings of the physiological adaptations to the space environment, International Space Agencies develop and test new countermeasures to prevent these adverse adaptations. Exercise is the cornerstone of the countermeasure programs. The bed rest model provides a unique paradigm to study the pathophysiology of physical inactivity and sedentary behaviors. During bed rest studies, physically active healthy participants free from any predisposition for chronic diseases stay in bed continuously 24 hr/day, 7 days/week. In this context, these individuals are both physically inactive and highly sedentary. During exercise countermeasure studies, exercise training is performed while in bed (Figure 4). They are both sedentary and physically active, and represent an extreme but unique model of 'sedentary exercisers'. Both resistance with and without aerobic exercise have been tested. Bed rest studies that used a combined resistance and aerobic exercise training protocol showed that skeletal muscle mass and function, and cardiorespiratory function are protected against very large volumes of sedentary behaviors induced by bed rest [18]. However, resistance and aerobic exercise totaling 300 min/week that increased total energy expenditure with aerobic training at 40-80% of VO_{2max} in healthy bed rested adults did not fully prevent the manifestation of metabolic dysfunction (i.e. whole-body insulin resistance, glucose intolerance, alterations of lipid metabolism, and systemic inflammation) [26, 93]. Along with exposing adults to long, unbroken time in bed only to exercise in continuous bouts while still lying-in bed, these bed rest plus exercise studies also eliminate non-exercise physical activity (i.e., daily physical activity that is not structured exercise). Daily life physical activity may modify the physiological adaptations associated with exercise training.

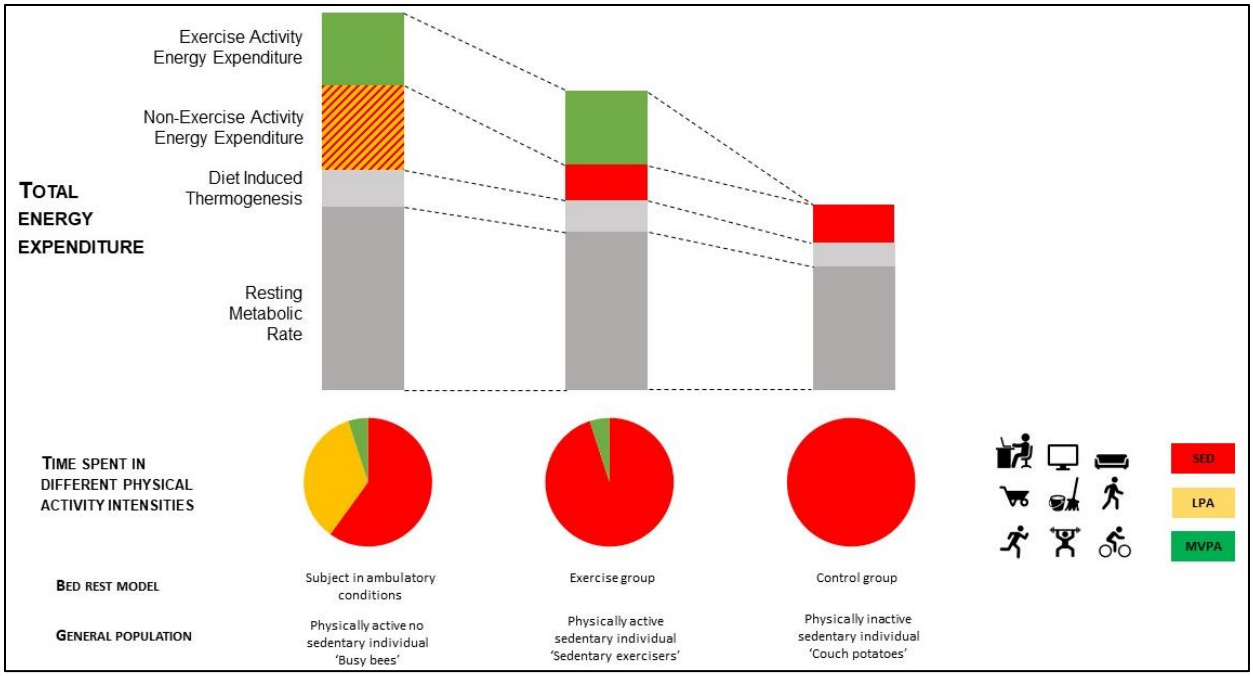

Figure 4: Schematic representation of the components of total energy expenditure during bedrest, conducted with or without exercise training. SED: sedentary activities; LPA: light-intensity physical activity; MVPA: moderate-to-vigorous physical activity. *Adapted from Le Roux & De Jong et al., 2021 [18].

Activities of daily living are inversely associated with sedentary behaviors [94]. In addition, large volumes of daily life physical activity, here considered as any body movements associated with activities of daily living, have been shown to confer health benefits. Knowing that lack of time is a major barrier to the practice of exercise and/or moderate to vigorous physical activity, reintroducing non-exercise activity into daily life could be an effective strategy to reduce sedentary time and prevent detrimental effects on metabolic health. In an elegant series of studies, Duvivier and colleagues compared the metabolic effects of replacing sedentary behaviors with light-intensity physical activity walking and standing to those of 1 hr/day of moderate to vigorous physical activity. Both interventions lasted four days and were matched for energy expenditure. Replacing sedentary behaviors with high volumes of light-intensity physical activity without any increase in moderate to vigorous physical activity, decreased postprandial insulin, fasting triglycerides and non-HDL cholesterol in healthy adults [95]. In adults with type 2 diabetes, increasing time spent standing and walking improved glucose control and insulin sensitivity, and

further reduced diastolic blood pressure, blood triglycerides and non-HDL cholesterol while increasing HDL cholesterol [96, 97]. The moderate to vigorous physical activity intervention tended to improve these metabolic parameters, but the effects were less pronounced. These studies show that when energy expenditure is matched, replacing sedentary behaviors with high volumes of light-intensity walking and standing is more beneficial than performing moderate to vigorous physical activity as a single continuous bout (i.e., structured exercise), at least for glucose control, insulin sensitivity and circulating lipids. In an 8-week exercise training study, non-exercise physical activity correlated with changes in aerobic capacity in while age, body mass index, while daily amount of physical activity training at any intensity level of exercise was not related [98]. These findings suggest that moderate to vigorous physical activity (i.e., exercise) and light-intensity standing, and walking might elicit differential cardiometabolic effects. Future studies will need to further compare the effects of light-intensity versus moderate to vigorous intensity physical activity on cardiometabolic health outcomes, including maximal aerobic capacity, muscle strength, substrate metabolism, glucose control, and insulin sensitivity in different populations and to investigate the mechanistic underpinnings of these physiological adaptations.

Taken together, sedentary behaviors and physical activity seem to be independent predictors of metabolic risk and the health consequences of sedentary behaviors are not fully eliminated even at extremely high levels of physical activity. This suggests that spending too much time in sedentary behaviors is different from not doing enough exercise. Two factors associated with this kind of sedentary behavior (eliminating non-exercise physical activity and exposure to prolonged uninterrupted bouts) may be significant factors in modifying the relationship between sedentary behaviors, exercise, and health.

5. THE BENEFICIAL EFFECTS OF BREAKING UP SEDENTARY BEHAVIORS WITH PHYSICAL ACTIVITY

Population health studies report that breaking up periods of sedentary behaviors is beneficially associated with cardiometabolic health risk factors [56, 90, 99, 100]. Adults who interrupt

sedentary time frequently had healthier waist circumference, body mass index, fasting glucose, triglyceride and C-reactive protein concentrations, and 2 hr postprandial glucose concentration compared to adults who interrupt sedentary time less frequently [100]. More importantly, these associations were observed even when accounting for total sedentary time and time spent in moderate to vigorous physical activity. Sedentary behaviors can be interrupted with many behaviors such as standing, walking, or other daily ambulatory activities (making the bed, preparing food, washing dishes, etc.) but whether all these activities reduce cardiometabolic risk factors equally is still being investigated. It is known that there is a negative relationship between aerobic capacity and morality risk, and while aerobic capacity has not been studied in response to breaking up sedentary behaviors with standing and walking, other key metabolic variables have been examined that shed light on these relationships. In a study with 154 adults, physical activity monitors that were worn on the trunk and upper leg for a week found that total standing time was positively associated with HDL concentrations and inversely associated with triglyceride concentrations [101]. Some experimental trials have shown that standing is beneficial for glycemic control [102-104], while other have not [73, 105]. The studies showing improved glycemic control with standing bouts tended to be in overweight to obese adults, and in those with impaired glucose regulation. The inconsistent results in experimental trials may be due to a lack of sensitivity to measure changes in glycemia from standing bouts as compared to walking bouts. The difference between standing and walking maybe be due to the energy expended. Standing has been shown to average 1.7 METs and walking has been shown to average 2.3 METs with increasing walking speed determining increased MET-value [106]. In support of breaking up sedentary behaviors with walking breaks instead of standing only breaks, isotemporal modeling approaches suggest that replacing bouts of sedentary time with light- or moderate-intensity physical activity is associated with reduced cardiometabolic risk factors, and all-cause and cardiovascular mortality risk with a higher risk reduction for adults who are less active [107-110]. Therefore, breaking up

bouts of sedentary behaviors with walking may be a potent physiological stimulus for health risk reduction.

Experimental evidence is beginning to accumulate on the biological associations between breaking up sedentary behaviors with short-frequent bouts of physical activity and a reduced risk of metabolic and cardiovascular disease. These studies showed that acutely breaking up sedentary behaviors with short-frequent bouts (<5 min/bout) of physical activity compared to a sedentary control condition is associated with lower postprandial plasma glucose, insulin, and triglyceride concentrations in adults who are lean, with obesity or with type 2 diabetes [61-64, 111-114]. Additionally, individuals at a higher risk for cardiovascular and metabolic disease (i.e., those who are physically inactive, sedentary, impaired glucose tolerance or with overt type 2 diabetes) have greater reductions in postprandial plasma glucose, insulin, and triglyceride concentrations with active breaks [72]. While findings from experimental trials consistently show lower postprandial glucose and insulin concentrations in response to standardized meals during activity breaks from sedentary time compared to uninterrupted sedentary behaviors, findings on fasting [69, 95] and postprandial plasma lipid responses [64, 67, 73, 103-105, 114-116] have been less consistent. A meta-analysis of experimental trials (N=37) that included 1-day and multiday (2-day) trials examining the effects of interrupting prolonged sitting with activity breaks on changes in postprandial triglyceride concentrations found the small effect of lower postprandial triglyceride concentrations was driven primarily by multiday trials suggesting a delayed response of physical activity on postprandial triglycerides [117-119]. Additionally, standardized meal composition (high fat vs. high glucose or test drink vs whole food), number of meals served, or the population studied (healthy vs. obese vs. type 2 diabetes) may account for inconsistencies in the results. Consequently, due to short experimental study designs, single (<24 hr) or multi day (<2 days) study designs, the metabolic effects of breaking up sedentary behaviors with short-frequent bouts

of physical activity compared to uninterrupted sedentary time beyond the acute exposure period is mostly unknown.

Very few experimental trials have controlled for energy expenditure between the experimental trials. When energy expenditure is matched, high volumes of sedentary behaviors replaced with non-exercise activity is more beneficial than performing structured exercise (moderate to vigorous physical activity as a single-continuous bout) for glucose control, insulin sensitivity, and circulating lipids [95-97]. This suggests that reducing sedentary time with activities of daily living are associated with beneficial changes in metabolic health outcomes. Specifically comparing the acute (9 hr) postprandial metabolite response to interrupting sedentary time with time-matched physical activity but differentiated by the frequency of physical activity bouts (short-frequent vs single-continuous), regular activity breaks reduced postprandial glycemia more than a single continuous bout of physical activity [64]. In this same study, postprandial triglyceride concentrations were attenuated only when physical activity was performed as a single continuous bout and not during regular activity breaks. The differential effects on changes in postprandial nutrients according to frequency of physical activity suggests there may be a differential metabolic response. However, it remains to be determined what physiological mechanisms may be responsible for the differential response in postprandial metabolites between time-matched physical activity trials.

A differential postprandial metabolite response observed between time-matched short-frequent bouts vs single-continuous bout in breaking up sedentary time suggests that changes in the rate of appearance or rate of disappearance for these postprandial nutrients depends on frequency of physical activity. Since dietary intake was matched in the acute investigation of changes in postprandial nutrients in response to prolonged sitting and time-matched physical activity [64], a possible mechanism for this difference between the two physical activity interventions could be a preferential oxidation of substrates. Short-frequent bouts of physical

activity may attenuate postprandial glucose concentrations because of a higher carbohydrate oxidation whereas a single continuous bout of physical activity may attenuate postprandial triglyceride concentrations through higher lipid oxidation. While this hypothesis has not been tested, some findings can shed light on the relevant physiological differences between sedentary behaviors and short-frequent bouts spread across the day. Experimental evidence from skeletal muscle biopsy samples has shown that interrupting prolonged sitting with regular activity bouts for 1 or 3 days increased the expression of proteins involved in both insulin-mediated and contraction-mediated glucose uptake pathways compared with continuous sitting [120]. Both pathways stimulate glucose transporter 4 (GLUT4) translocation to the plasma membrane, facilitating glucose uptake and thereby reducing blood glucose levels. Furthermore, physically active interruptions during prolonged sitting lead to the higher expression of skeletal muscle genes related to the regulation of carbohydrate metabolism compared with uninterrupted sitting [121]. Taken together, acute and short-term experimental evidence indicates the association between lower postprandial glucose and insulin concentrations and short-frequent bouts of physical activity may be the result of higher skeletal muscle glucose uptake via insulin-mediated and contraction-mediated pathways. However, no studies have examined the acute or short-term adaptations in skeletal muscle between time-matched short-frequent bouts of physical activity and a single-continuous bout of physical activity.

SCIENTIFIC PREMISE & SPECIFIC AIMS

Scientific Premise

Lower total sedentary time and more frequent breaks of sedentary time is associated with lower risk for metabolic disease. Acute experimental trials demonstrate that breaking up sedentary time with short-frequent bouts of physical activity is associated lower postprandial glucose, insulin, triglyceride concentrations in response to standardized meals compared to uninterrupted sedentary control. The magnitude of reduction in postprandial nutrients was greater in individuals with overweight to obesity compared to healthy. When physical activity time is matched between acute experimental trials that interrupt sedentary time, breaking up sedentary time with short-frequent bouts of physical activity is associated with lower postprandial glucose and insulin concentrations while a single-continuous bout is associated with lower postprandial triglyceride concentrations. Frequent short physical activity bouts spread across the day compared to uninterrupted sedentary behaviors is associated with a higher expression of contraction- and insulin-dependent nutrient uptake and pathways associated with the regulation of carbohydrate and fat metabolism. The scientific premise supports the argument that frequently interrupting sedentary behaviors with short-frequent bouts of physical activity is beneficial for the regulation of glycemia through a greater uptake and/or use of carbohydrate by the skeletal muscle. However, important gaps in knowledge exist:

1. Whether breaking up sedentary behaviors with short-frequent bouts of physical activity is a strategy that can be implemented in the daily life of sedentary, physically inactive adults.
2. Whether the acute metabolic benefits previously observed are sustained or diluted beyond the acute exposure period (> 5-12hrs).
3. Whether the metabolic effects are due to the breaks *per se* or the increase in physical activity and/or energy expenditure.

4. The characterization of the underlying physiological, cellular, and molecular mechanisms.

Central Objective and Hypothesis

The central objective of this dissertation is to investigate the short-term (4-day) effect of breaking up sedentary behaviors with short-frequent bouts of moderate intensity physical activity spread throughout the day in overweight to obese sedentary, physically inactive adults on nutrient metabolism and potential underlying mechanisms. The short-frequent bouts intervention was compared to a time-matched single-continuous bout of moderate intensity physical activity to separate the effects of the active breaks from the increases in physical activity and/or energy expenditure.

We hypothesized that breaking up sedentary behaviors with short-frequent bouts of physical activity may be better at improving glycemia than a time-matched single-continuous bout of physical activity. We proposed the following working model (Figure 5) to explain the greater benefits of breaking up sedentary behaviors on glucose control. Because intramuscular glycogen is immediately available as an energy source in muscle and circulating glucose is readily available in the postprandial period, short-frequent bouts of activity acutely increase carbohydrate oxidation relative to fat oxidation. Throughout the day circulating glucose is taken up by muscle to replenish intramuscular glycogen stores or to be consumed as an energy substrate, thereby attenuating glycemia. The repetition of these bouts over 4-days may optimize carbohydrate oxidation through higher mitochondrial oxidative capacity and regulation of carbohydrate metabolism through muscle contraction-transcription signaling. When performing a single-continuous bout of walking muscle glycogen is partially depleted, thus allowing lipid substrates to be oxidized for energy expenditure while muscle glycogen is replenished.

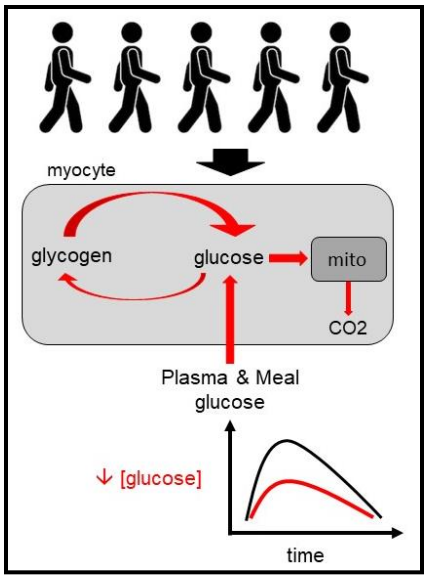

Figure 5: Working Model

Specific Aims and Hypothesis

In a randomized cross-over study, we compared the short-term effects (4-day) of breaking up sedentary behaviors with short-frequent bouts of moderate intensity physical activity (MICRO: 5-min walk bout every hour for 9 consecutive hours per day) to a time-matched single-continuous bout of moderate intensity physical activity (ONE: 45-min continuous walking bout per day), and a sedentary control (SED: habitual sedentary behaviors and physical inactivity each day) in inactive male and female adults with overweight or obesity. To reach our overall objective, three independent specific aims were pursued (Figure 6):

<u>Specific Aim 1</u>: To determine the feasibility of implementing MICRO compared to ONE in free-living adults with overweight or obesity on daily time spent sitting and physically active over the short-term.

- Both MICRO and ONE will be associated with higher time spent in moderate to vigorous physical activity compared to SED as measured by 3D-activity monitors (ActiGraph and ActivPal).

- MICRO will result in lower total time spent sedentary and lower time spent in prolonged sedentary bouts (>60-min/bout) compared to ONE and SED as measured by inclinometry (ActivePal).
- MICRO will be associated with higher subjective feeling of vigor and lower feeling of fatigue compared to ONE and SED as measured by validated questionnaires.

Exploratory Aim 1: To compare the effect of MICRO versus ONE, both compared to SED, on daily time spent sitting and physically active on work and non-workdays.

<u>Specific Aim 2</u>: To determine the effect of MICRO compared to ONE in adults with overweight to obesity on nutrient metabolism and insulin sensitivity.

- MICRO will lead to higher 24 hr total and postprandial carbohydrate oxidation as measured by whole-room calorimetry, and lower postprandial glucose and insulin concentrations in response to standardized meals compared to both ONE and SED.
- ONE will lead to higher 24 hr total and postprandial fat oxidation as measured by whole-room calorimetry, and lower postprandial triglyceride concentrations in response to standardized meals compared to both MICRO and SED.
- Both MICRO and ONE will improve indexes of insulin sensitivity compared to SED (HOMA-IR and insulin/glucose).

<u>Specific Aim 3</u>: To characterize the short-term effect of MICRO compared to ONE on skeletal muscle substrate oxidation and gene expression associated with energy metabolism in adults with overweight to obesity.

- Permeabilized skeletal muscle fibers collected after 4 days of MICRO will exhibit higher mitochondrial respiration in the presence of carbohydrate-associated substrates as measured by high resolution respirometry (Oroboros) along with enhanced expression of

genes and pathways (mRNA sequencing and Ingenuity Pathway Analysis) associated with the regulation of carbohydrate metabolism when compared to the sedentary condition.

- Permeabilized skeletal muscle fibers collected after 4 days of ONE will exhibit higher lipid-associated mitochondrial respiration in the presence of lipid-associated substrates (high resolution respirometry) along with higher expression of genes and pathways associated (mRNA sequencing and Ingenuity Pathway Analysis) with the regulation of lipid metabolism when compared to the sedentary condition.

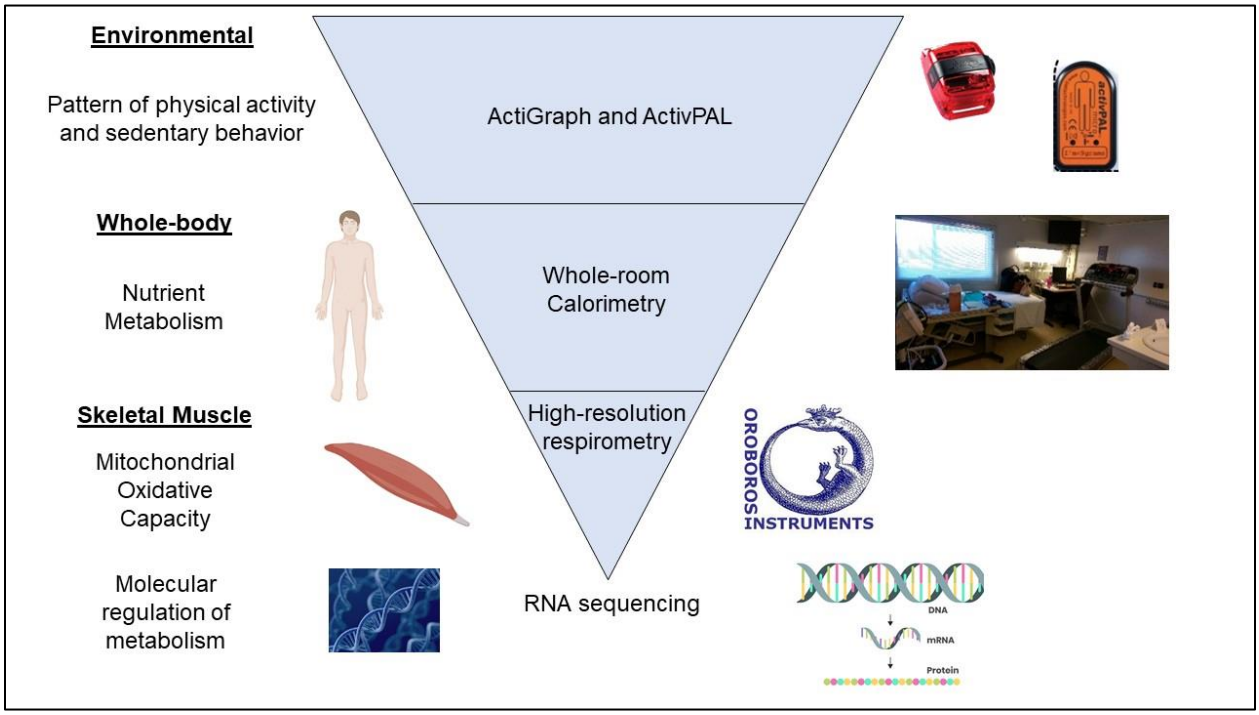

Figure 6: Primary measurements at different physiological levels

OVERALL STUDY DESIGN

The primary objective of this section is to present the overall study design. In each of following chapters the methods and sample size are presented specific to that chapter.

Clinical Trial: This dissertation was conducted in the context of a clinical trial funded by the National Institute of Health (K99DK100465, 2014-2016) to Principal Investigator Dr. Audrey Bergouignan, PhD, Director of Research at the French National Center for Scientific Research (CNRS) and Assistant Professor in the Division of Endocrinology, Metabolism, and Diabetes, School of Medicine at the University of Colorado Anschutz Medical Campus. My role in this study started before my entry into the Human Bioenergetics PhD program as Colorado State University. During this time, I served as the primary professional research assistant and recruited and retained all research participants, scheduled and completed all study related visits, and database management. After my entry in the PhD program, I contributed to the sample and data analysis, preparation of activity reports, ancillary studies and manuscripts development, submission, and navigation of the peer-review process.

ClinicalTrials.gov identifier: NCT02258438

Participants: Eligible participants were aged 19-45 years, with a body mass index between 27 and 33 kg/m^2, weight stable for ≥ 3 months, and self-reported > 6 hr/day sitting. Women were pre-menopausal but could use oral contraceptives. Exclusion criteria included clinically diagnosed diabetes, taking glucose- and/or lipid-lowering medication, dyslipidemia, smoking, or being physically active (>150-min/week moderate-intensity exercise). Participants were recruited between October 2014 and October 2016 from advertisements, public announcements, and flyers in the Denver and Aurora areas, Colorado, USA. This study was approved by the Colorado Multiple Institutional Review Board and was in accordance with the Declaration of Helsinki.

Demographics for all enrolled participants (including participants who have been withdrawn):

	Native American	Asian	Black	White	Hispanic	Other	Total
Female	0	0	2	9	2	0	13
Male	0	2	0	8	4	1	15
Unknown	0	0	0	0	0	0	0
Total	0	2	2	17	6	1	28

Colorado Multiple Institutional Review Board identifier: 14-0429

Sample Size: The primary outcomes were 24 hr non-protein respiratory, total substrate oxidation and postprandial plasma glucose and insulin. Secondary outcomes included skeletal muscle mitochondrial oxidative capacity, mRNA sequencing and pathway analysis, self-perceived fatigue and vigor and perception of the interventions. Our main hypothesis was that MICRO will lead to higher 24 hr total and postprandial carbohydrate oxidation (whole-room calorimetry), and lower postprandial glucose and insulin concentrations in response to standardized meals compared to ONE and SED, while ONE will lead to higher 24 hr total and postprandial fat oxidation (whole-room calorimetry), and lower postprandial triglyceride concentrations in response to standardized meals compared to MICRO and SED. In a study by Dunstan et al. [63], 19 overweight adults interrupted prolonged sitting bouts with moderate-intensity activity (2-min every 20-min) reduced 5 hr postprandial glucose and insulin iAUC by 29% and 23%, respectively, compared to uninterrupted sitting. Following Littell [122] and assuming that the effect sizes between groups are similar to the Dunstan et al. study [63], we estimated that there will be over 80% power to detect the direct treatment effect with a sample size of 24 participants. Specifically, there will be 0.77 power to compare SED and MICRO and 0.89 power to detect the difference between SED and ONE. We recently showed in overweight adults that a sample size of 12 allows for detecting a significant difference in 24 hr non-protein respiratory quotient, fat oxidation and carbohydrate oxidation between a day spent in sedentary conditions and a day with one bout of 45-min of moderate-intensity exercise when energy expended during the exercise is not replaced [123]. A

sample size of 24 will therefore be sufficient to detect differences in 24 hr non-protein respiratory quotient and substrate use. Accounting for a 25% drop out rate, we anticipate enrolling 30 participants.

Study Design: This was a three-arm cross-over randomized trial (Figure 7). The study interventions were separated by a 28 day wash out period. Participants were randomized to one of three possible trial-intervention orders using balanced blocks prepared for male and female participants. Each eligible participant completed three separate 4-day interventions that consisted of three days in free-living conditions followed by 24 hr in a whole-room calorimeter and a muscle biopsy was harvested in fasting conditions at 7:30 AM the morning after the whole-room calorimeter. The three Interventions were:

- Sedentary (SED): During the 3-day free-living period, participants were asked to maintain usual levels of daily activity and sedentary behaviors, and were asked to refrain from structured exercise. On day 4, participants remained sedentary in the whole-room calorimeter.
- Sedentary + 1 continuous bout of activity (ONE): During the 3-day free-living period, participants were instructed to perform 45-min of moderate-intensity walking once per day and maintained usual levels of daily activity and sedentary behaviors the remainder of the day. On day 4, participants remained sedentary in the whole-room calorimeter except to perform one bout of 45-min moderate-intensity treadmill walking at 10:00 AM and remained sedentary the remainder of the day.
- Sedentary + microbouts of activity (MICRO): During the 3-day free-living period, participants performed 5-min of moderate intensity walking bouts each hour for 9 consecutive hours and maintained usual levels of daily activity and sedentary behaviors the remainder of the day. On day 4, participants performed 5-min moderate-intensity treadmill walking every hour for 9 consecutive hours from 10:00 AM to 6:00 PM and remained sedentary the remainder of the day.

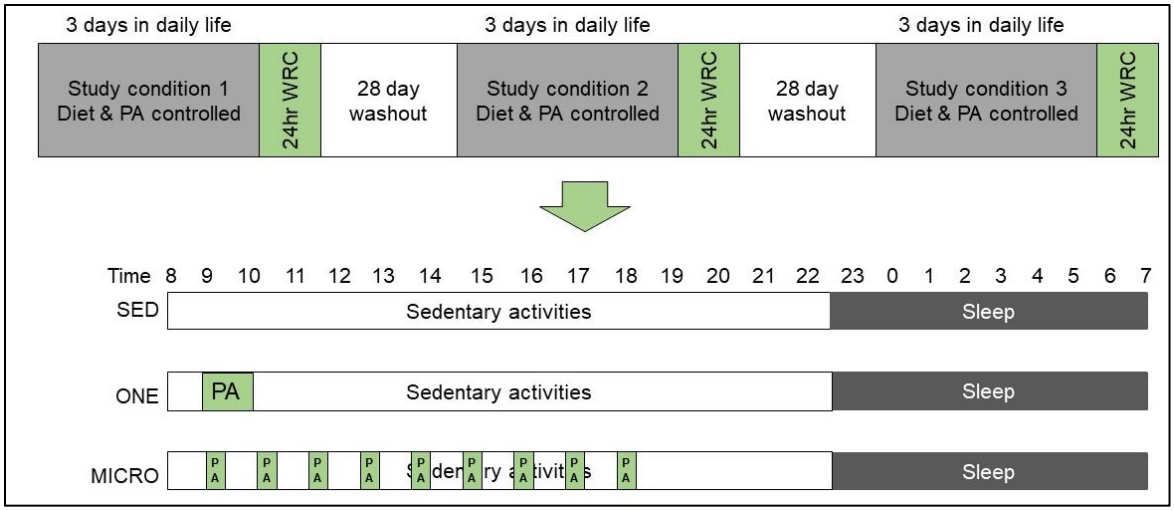

Figure 7: Study design. WRC: whole-room calorimetry, SED: sedentary, ONE: one bout study condition, MICRO: microbouts study condition, PA: physical activity.

STUDY DESIGN CONSIDERATIONS

- **Why have a 28 day wash out period between interventions?** Each study intervention was separated by 28 days for females to ensure that all experimental conditions were completed in the same phase of the menstrual cycle in women, i.e. follicular phase. Indeed, past studies on the impact of menstrual cycle suggest differential metabolic physiology related to each phase of the menstrual cycle [124]. To reduce inter-individual variation between each study condition men's study visits were also separated by 28 days.
- **Why 5-min for short-frequent bouts of physical activity?** A main goal was to move this novel strategy out of the lab and test the feasibility in people's daily lives. In their study, Dunstan et al (2012) had participants complete 2-min walking bouts every 20 minutes and were able to observe reduction in postprandial glucose and insulin concentrations over a 5 hr experimental period. We thought this frequency of physical activity bouts was not feasible outside the laboratory. Therefore, we thought five minutes of walking every hour to be a practical strategy that can be implemented into daily life.

- **Why include a 45-min single-continuous walking bout as an experimental trial?** The majority of previously published experimental trials examining the metabolic health effects of breaking up sedentary behaviors with short-frequents bouts of physical activity have only been compared to an uninterrupted sedentary condition. We included a time-matched single-continuous bouts intervention to help separate the effects from an increase in physical activity and/or energy expenditure and the breaks in sedentary time.
- **Why include a 3-day run-in period?** We provided a standardized diet during the 3 day run-in period to minimize any potential diet-induced variability in the metabolic profile and primary outcomes. This was particularly important for the measurement of nutrient use.
- **Why balance sample size equally between males and females?** While this study was not powered to detect differences based on sex, we balanced the samples size equally between males and females to explore potential sex differences. Trends observed would provide a rationale to plane future investigations. Also, the majority of previously published studies have not balanced experimental samples by sex. Therefore, we wanted to begin to address this knowledge gap in the literature.
- **Why not maintain energy balance during the measurement of substrate oxidation during the whole-room calorimeter study day?** We did not increase energy intake to match energy expenditure because evidence has shown that participants do not spontaneously increase their food intake in response to exercise or if they do, they do not match total energy expenditure and are still in energy deficit [125-127].

BREAKING UP SEDENTARY TIME IN OVERWEIGHT/OBESE ADULTS ON WORKDAYS AND NON-WORKDAYS: RESULTS FROM A FEASIBILITY STUDY

Adapted from:

"Breaking up Sedentary Time in Overweight/Obese Adults on Work Days and Non-Work Days: Results from a Feasibility Study."

***De Jong NP**, Debache I, Pan Z, Garnotel M, Lyden K, Sueur C, Simon C, Bessesen DH, and Bergouignan A.*

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Synopsis

Many experimental trials have shown that regular brief interruptions to prolonged sitting time can improve cardiometabolic risk markers (reduced postprandial metabolites in response to standard meal) in healthy, overweight to obese, and type 2 diabetic adults [HDR 68]. However, exposure to breaking-up sedentary behaviors with short-frequent bouts of physical activity has mostly been acute (≤ 2 days) and conducted in the controlled laboratory setting. While these laboratory studies are informative, applying this strategy in the real world over multiple days is key for translating evidence into practice. In a randomized crossover trial (6 hr/condition), we have shown that healthy normal weight adults felt higher feelings of energy, vigor and mood, and lower feelings of fatigue throughout the day in normal weight adults while interrupting sitting time with short-frequent bouts (5 min/hr for 6 hr consecutive, moderate intensity treadmill walking) of physical activity [128]. Additionally, there were no reported effects on cognitive function supporting the notion that microbouts of physical activity could be implemented into daily life in work and non-work environments without affecting energy, mood, or work productivity.

In the following paper, we investigated the feasibility of a 3-day intervention that interrupted sedentary time with short-frequent bouts of physical activity in sedentary adults with overweight to obesity during work and non-workdays outside the controlled laboratory environment. This three-arm randomized cross-over study included a time matched single-continuous bout of physical activity, and a sedentary control condition to test whether potential differences in outcomes were due to the frequent interruptions of sedentary time or total active time. The main outcomes were objectively measured time spent sitting/lying, standing, stepping and daily steps, and time spent in different physical activity intensities. Secondary outcomes included indexes of insulin sensitivity, objectively measured activity energy expenditure, physical activity level and subjective perceptions of the interventions.

We were able to show that short-frequent bouts of physical activity spread across the day are a feasible intervention to promote physical activity in those who are at high risk for metabolic disease (adults with overweight to obese, physically inactive and sedentary). This low-cost intervention can be applied in both work and non-work contexts of daily life. Both physical activity interventions similarly improved indexes of insulin sensitivity and were associated with an activity level prescribed by the physical activity guidelines, at least over the short-term. Future studies need to test if this daily pattern of physical activity can be maintained over a longer term (several weeks) and what physiological mechanisms may be responsible for improved insulin sensitivity indexes.

Introduction

Sedentary behavior, i.e. sitting time, have been associated with adverse health outcomes including body mass index, cardio-metabolic outcomes, mental health and premature mortality [51, 129-136], and has emerged as an important public health concern [53]. In addition to total daily sitting time, prolonged unbroken sitting time has been negatively associated with cardiometabolic health biomarkers [56, 100].

Over the past few decades, advances in technology and computer-based tasks have increased time spent sitting at the workplace [137]. It has been found that office-based employees spend 66% of their total work time sitting with 25% of total sitting time in bouts longer than 55 minutes [45]. These changes in the workplace have been associated with reduced daily occupational energy expenditure. Since the 1960s, in the USA and the UK, population levels of occupational physical activity have declined by more than 30% [46]. Facing this developing public health challenge, the World Health Organization has recently published new guidelines for employers to promote healthier occupational environments [138]. Among the four major components of the guidelines, limiting prolonged sitting and increasing physical activity is one of them. While guidelines exist, they still need to be translated into practical strategies that can be implemented on a large scale. In this context, there has been increasing interest in understanding the efficacy of a broad range of interventions targeting sedentary behavior in the workplace.

A growing number of studies have examined environmental changes in the occupational setting to reduce sitting time such as active workstations and include sit-to-stand desks, treadmill desks and seated active workstations utilizing portable pedal machines [139-141]. These interventions have shown mixed results. While individual sit-to-stand desk interventions have not been shown to decrease sedentary time [142], interventions with multi-level components targeting the individual but also social and built environment showed that stand-up desk options reduce sitting and increase standing time [143]. However, no effect on stepping time was observed. A

personalized consultation with weekly emails aimed to reduce prolonged sitting time did not decrease total daily sedentary time but reduced the occurrence of sedentary bouts of more than 30 min [144, 145]. Another study using hourly computer screen prompts and text messages to break up sitting decreased total time spent sitting and increased the number of daily steps, but failed at increasing the number of sit-to-stand transitions [146]. Another goal of these interventions is to increase energy expenditure. The implementation of treadmill desks and seated active workstations can reduce daily sitting time, increase time spent in physical activity [147, 148] and almost triple the energy expenditure of that measured while sitting. For example, walking at 1.8 km/h can induce an expenditure above 0.41 MJ/h, which could beneficially impact energy balance if sustained for several hours per day [149, 150]. However, long-term adherence to these interventions (12 months) are poor [147, 148], treadmill desks are costly and present a safety hazard. Therefore, a cost effective, easy to implement intervention that can reduce total time spent sitting, prevent prolonged sitting bouts as well as increase time spent active and energy expenditure is still needed. Implementing frequent short bursts of walking could fulfill these requirements.

Such interventions have already been tested in the laboratory setting. Past studies showed beneficial effects of frequent interruptions of sitting time with short bouts of activity varying in mode, frequency, duration and intensity on metabolic, cognitive and hemodynamic outcomes [61-64, 73, 102, 111-114, 151-153]. Regardless of adiposity, sex and age frequent interruptions of sedentary actives with walking breaks have been associated with attenuated postprandial plasma glucose and insulin concentrations in obese and type 2 diabetic adults [61-64, 73, 102, 111-114]. We have shown that interrupting sedentary behavior with short bursts of treadmill walking increases self-perceived feelings of energy, vigor and mood and decreases feelings of fatigue throughout the day in normal weight adults [153]. The effect of such an intervention on the profile of physical activity and energy expenditure in free-living conditions is unknown.

While the workplace has been identified as a priority setting for addressing this chronic disease factor, it may be important to target sedentary behavior in other contexts such as on non-working days. Non-working days also comprise a large portion of a working adult's week and have also been associated with a large amount of time attributed to sedentary activities [154]. Because workers who spend more time in sedentary pursuits during work hours do not compensate by being more active in non-working periods (19), there is a need to test interventions that aim at reducing time spent sedentary both during work days and non-work days outside of the controlled laboratory environment.

Based on the data generated by the past intervention studies conducted in laboratory setting and the real-world, we hypothesized that an intervention aimed at breaking up sedentary time with short bouts of activity could prevent time spent sitting, increase daily physical activity and energy expenditure, and positively impact metabolic health and well-being in office workers. Our purpose was to test the feasibility to implement such an intervention over a short period of time (3 days) in the daily life of overweight sedentary male and female adults during work days and non-work days, and thus test this hypothesis. To test whether the effects on time spent sitting, time spent physically active and energy expenditure were due to the frequent interruptions of sedentary time with short bouts of activity or to the total time spent active, we used a three-arm cross-over randomized design. Frequent interruptions of sedentary time with short bouts of physical activity were compared to a duration-matched single continuous bout of physical activity, and a sedentary control condition. Further, we compared the effect of the interventions on self-perceived vigor and fatigue and an index of insulin sensitivity. We finally assessed how difficult it was for participants to implement these interventions in their daily life on work days and non-work days.

Methods

Participants

This study was approved by the Colorado Multiple Institutional Review Board (COMIRB) and was in accordance with the Declaration of Helsinki (COMIRB# 14-0429). Eligible participants were between 19-45 years old with an occupation that requires sitting time, had a body mass index (BMI) between 27-33 kg/m^2, were weight stable for at least 3 months, insulin sensitive (fasting plasma insulin concentration below 25 µIU/mL), and self-reporting > 6hrs/day of occupational sitting. All women enrolled in the study were pre-menopausal and could use birth control medications. Exclusion criteria included clinically diagnosed diabetes, taking glucose- and/or lipid-lowering medications, dyslipidemia, smoking, or meeting the American College of Sports Medicine (ACSM) physical activity recommendations (>150 min/week MVPA). Participants were recruited between October 2014 and October 2016 from newspaper advertisements, public announcements, and flyers in the Denver and Aurora areas in Colorado, USA. Participants were randomized to one of three possible trial-condition orders using balanced blocks separately prepared for male and female participants. The study statistician (Z.P.) prepared the computer-generated randomization lists and sealed envelopes for randomization [155].

Study Design

Eligible volunteers completed three separate 3-day trial phases under free-living conditions. The study phases were separated by a 28-day wash out period and women were all studied in the follicular phase of their menstrual cycle. All the study related visits were conducted at the Clinical and Translational Research Center of University of Colorado (CTRC). The three trial conditions were administered in random order:

Sedentary (SED)*:* Free-living subjects maintained their usual levels of daily activity during the three days of measurement and were asked to refrain from structured exercise.

Sedentary + 1 continuous bout of activity (ONE)*:* During the 3-days of measurement, subjects were asked to perform 45-min of moderate-intensity walking once per day and maintain their usual sedentary lifestyle the rest of the day.

Sedentary + microbouts of activity (MICRO)*:* During the 3-days of measurement, participants were asked to perform a 5-min bout of moderate-intensity walking bout each hour for 9 consecutive hours throughout the day and maintain their usual sedentary lifestyle the rest of the time.

For both interventions, the intensity of the activity was defined during the screening visit. On each day of measurement, participants were asked to complete a diary log and record the time the participant went to sleep and woke up from sleep, the time the bouts of physical activity were performed and if it was a work day or not.

Screening Visit

Subjects were screened, consented, and underwent a review of medical history and physical examination and a blood draw to verify fasting plasma insulin concentrations for eligibility. Resting Metabolic Rate (RMR) was measured by indirect calorimetry for 30 minutes in the fasted state, under resting conditions and at thermoneutrality. Body composition including fat-free mass (FFM) and fat mass (FM) was measured by dual energy X-ray absorptiometry (DXA, Hologic Delphi-W, Bedford, MA). The short version of the International Physical Activity Questionnaire (IPAQ) was completed to assess habitual physical activity and time spent sitting [156]. Subjects then performed an incremental exercise test on a treadmill (increments of 0.3 miles/hr every 2-min) to determine a walking pace that was then prescribed for ONE and MICRO conditions. For each exercise level, subjects rated their perceived effort on a Borg scale from 0 (very light) to 20 (maximal exertion). The aim was to identify the walking speed that subjects associated with a perceived exertion level of 13 (somewhat hard). Subjects were instructed to walk at this pace for each bout of activity during the intervention.

Measurement of time spent sitting/lying, standing, stepping and daily steps

Time spent sitting/lying, standing, stepping and daily steps were quantified using an ActivPAL™ triaxial accelerometer/inclinometer (PAL Technologies Ltd, Glasgow, Scotland) during the three days of measurement in each condition. Participants were instructed to always wear the monitor. The device was worn midline on the anterior aspect of the thigh and wrapped with a nitrile sleeve, allowing for 24hr measurement. The monitor produces a signal related to thigh inclination and is a valid and reliable measurement tool for determining posture and motion during activities of daily living [157]. When the monitor is oriented horizontally, it classifies the activity as sitting/lying. Vertical positioning of the monitor is classified as standing. Step cadence and number of steps were recorded by the monitor when a participant was walking.

The ActivPAL™ has been validated for use in adults to distinguish between sitting/lying, standing, and stepping activities [157-160]. Data event files from the ActivPAL™ were used to quantify sitting/lying, standing, and stepping time. In these files, the ActivPAL™ records each time an activity changes and the time that the activity changed. Sitting/lying, standing, and stepping time were calculated by summing the duration of each event and the number of breaks from sitting time were quantified as a transition from sitting/lying to either standing or stepping. Sitting bouts lasting longer than 30-min and 60-min were also used to test the effect of the conditions on the sitting bout length. A customized R program (www.r-project.org) was used to convert the event data file to a second-by-second data file to estimate additional metrics of sedentary behaviors and time spent sitting/lying, standing, stepping. The following metrics of sedentary behaviors were computed over 24hr: total sedentary time (total time spent in sitting/lying events), total breaks in sedentary time (number of times a sitting/lying event was followed by a standing or stepping event), and time (minutes/day) in sedentary bouts ≥30 and ≥60-minutes. The same outcomes were also reported as percentage of waking time. Because sleep time was removed, we assumed that sitting/lying time mainly corresponded to sitting time during waking hours. The R package (PAactivPAL) is available for researchers to generate these metrics [161].

Measurement of physical activity intensity, activity energy expenditure and physical activity level

Activity energy expenditure (AEE) and time spent in different activity intensities were determined using the ActiGraph GT3X tri-axial accelerometer (ActiGraph, Pensacola, Fla., USA). Participants were instructed to wear the accelerometer during wake time by attaching it to their right hip directly above their right knee using an elastic belt that was provided. A sampling rate of 30-Hz was used. After each of the 3-day study conditions, data were downloaded using software provided by the manufacturer (ActiLife 6.13 Pensacola, USA) and AEE per minute (J/kg/min) was estimated using the Freedson vector magnitude combination model [162, 163]. Total energy expenditure (MJ/d) was calculated as (AEE + RMR) / 0.9, where RMR was resting metabolic rate (MJ/d). Physical activity level (PAL) was calculated as the ratio between TEE over measured RMR. Cut-points of <1.5 and <3 METs and >3METs (metabolic equivalents) were used for very light intensity activity, light intensity activity and moderate-to-very vigorous activity, respectively. Minute-data during waking hours were summed to obtain data per day. Although sedentary behavior has been defined as activities with an energy expenditure below 1.5 METs while in a sitting, reclining, or lying posture [37], activities with METs <1.5 were referred to as very light intensity activity in our study. By only measuring energy expenditure without recognition of the concomitant posture, we are including activities such as standing that are not sedentary activities. By choosing the term “very light intensity activity” we are more conservative and avoiding any misinterpretation.

Perception of the challenges associated with the conditions, self-perceived vigor, and fatigue

At the end of each intervention or control day participants filled out online 100mm visual analog scales (VAS) designed to capture their perception of the study condition [164]. The VAS addressed the following question *“Please indicate on the scale how challenging you found the day.”* The anchors for this question were “*Extremely Easy”* and *“Extremely Challenging.”*

Immediately after the first survey, participants then completed an online modified version of the Perception of Mood survey (POMs) to assess changes in feelings of vigor and fatigue [165]. Only the POMs-Fatigue (POMs-F; $n = 7$ items) and the POMs-Vigor (POMs-V; $n = 8$ items) subscales were used for analysis.

Plasma metabolic outcomes

The morning after each 3-day trial, the participants reported to the CTRC for a fasting blood collection which was analyzed for glucose and insulin. Whole blood was added to a preservative (3.6 mg EDTA plus 2.4 mg glutathione in distilled water). Insulin concentrations were measured using a standard double antibody radioimmunoassay (EMD Millipore, St. Charles, Missouri). Serum glucose concentrations were determined using the hexokinase method (Wako Diagnostics, Mountain View, CA). These analyses were performed on the Beckman Coulter AU480 Chemistry Analyzer (Brea, CA).

Statistical analysis

Based on the diary log information, data were recorded on 43, 47 and 43 work days while in SED, ONE and MICRO conditions, respectively. Consequently, 23, 19 and 23 study days were non-work days when participants were in SED, ONE and MICRO conditions, respectively. The analysis of the working status effect (work day versus non-work day) was *a posteriori* analysis. This is why the number of work days and non-work days are unbalanced across the three conditions and the work status.

If there was more than one measure assessed at different days per condition and work status, the mean value of the repeated measures served as outcome in the model. Linear mixed models were used to test differences in the two activity monitor outcomes, self-perceived challenge, vigor and fatigue, with sequence, period, condition (SED, MICRO and ONE), work status (work day vs non-work day) and condition-by-work status interaction as fixed effects and subjects as random effect with a compound symmetry covariance. Contrasts were used, under this model, to test for the between work status difference under each condition, the between condition differences

separately on workdays and non-work days and the between work status difference with respect to the between condition difference. No correction for multiple comparisons was applied. Fasting plasma insulin and glucose concentrations measured on the morning of day 4 were also analyzed using linear mixed model but work status was not considered. Indeed, within the three days prior to the blood draw, days could have been randomly spent at work or not, it was therefore impossible to know if the interaction between the condition and the work status had any influence on index of insulin sensitivity. Data are expressed as mean ± SD, unless otherwise stated. All statistical analyses were performed with SAS 9.4 (SAS Institute, Cary, NC).

Results

Subjects' characteristics and compliance with the interventions

Subjects' characteristics are displayed on Table 1. On average over the 3-days of intervention, participants performed 97.6 ± 0.0% and 98.4 ± 0.1% of the prescribed physical activity bouts in MICRO and ONE, respectively. High levels of compliance with both interventions were attained despite reporting that performing the physical activity interventions was more challenging than spending a day being sedentary (Intervention effect: p=0.007; Figure 8). While participants reported that MICRO was challenging to perform on work days (p=0.004 vs. SED), ONE was perceived to be more challenging to comply with on non-work days compared to both SED (p=0.05) and MICRO (p=0.04).

Table 1. Study participant's anthropological characteristics & habitual sitting.

	Males	Females	All
n	10	12	22
Age (year)	31.5 ± 7.4	32.0 ± 6.1	31.8 ± 6.6
BMI (kg/m^2)	28.8 ± 2.9	31.7 ± 1.8	30.5 ± 2.7
FM (kg)	24.6 ± 4.3***	36.0 ± 4.7	30.9 ± 7.3
FFM (kg)	63.1 ± 9.9***	49.9 ± 5.0	56.0 ± 10.1
FM (%)	28.1 ± 2.4***	41.8 ± 2.4	35.6 ± 7.4
Self-reported sitting time (h/d)	9.0 ± 3.2	10.6 ± 1.1	9.5 ± 4.1

*Data are presented as mean ± SD. * p<0.05, ** p<0.01, *** p<0.0001 vs. Female. n, number of subjects; BMI, body mass index; FFM, fat-free mass; FM, fat mass; Self-reported sitting time was estimated from the IPAQ, international physical activity questionnaire.*

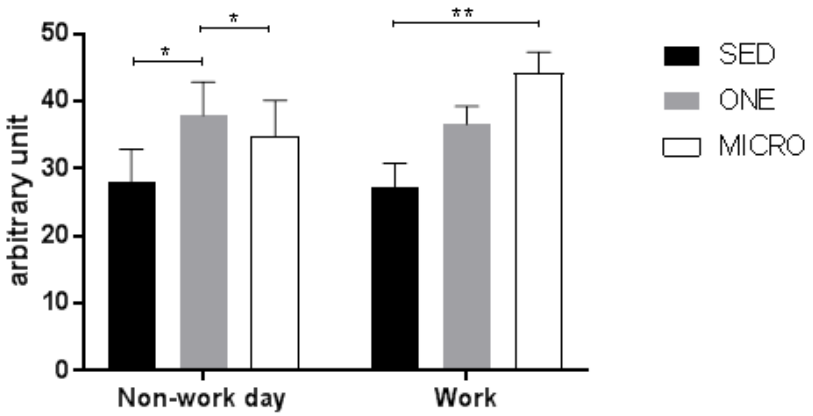


Figure 8. Visual analog scale representing the perception of the challenges associated with the conditions. At the end of each intervention or control day participants filled out online 100mm visual analog scales (VAS) designed to capture their perception of the study condition. The VAS addressed the following question "Please indicate on the scale how challenging you found the day." The anchors for this question were "Extremely Easy" and "Extremely Challenging." SED, indicates the sedentary condition; ONE, indicates the one-bout intervention; MICRO, indicates the microbouts intervention. * p<0.05, ** p<0.01, *** p<0.0001 vs. sedentary control.

Effect of the physical activity interventions on time spent sitting/lying, standing and stepping

Time spent sitting/lying, standing, and stepping over 24hr is reported in Table 2. One ActivPAL™ was lost and two were defective, we are therefore reporting data obtained in 19 subjects. Both MICRO (11. 4 ± 4.7 vs. 9.2 ± 3.4%, p=0.009) and ONE (13.9 ± 3.5% vs. 9.2 ± 3.4%, p<0.0001) increased the percentage of waking time spent stepping compared to SED on work days but not on non-work days. This resulted in 0.4 ± 0.1 hour more spent stepping in ONE than in MICRO (p=0.01). As a result, the number of daily steps increased from 7,125 ± 2,554 to 12,257 ± 3,145 in ONE (p<0.0001) and 10,036 ± 4,262 in MICRO (p=0.0002) on work days; participants took more steps when performing ONE than MICRO (p=0.005). Both ONE (+2,967 ± 456, p=0.005) and MICRO (+2,841 ± 552, p=0.02) led to a greater number of daily steps compared to SED on non-working days. However, time spent sitting and standing, the average duration of the sedentary bouts and the number of transitions from the sitting to standing position (index of breaking up prolonged sitting) were not significantly different across conditions and days (p>0.05 for all). Surprisingly, the sitting bouts of more than 30 minutes tended to occur more often in MICRO than in both SED (p=0.057) and ONE (p=0.051) when in leisure contexts.

Table 2. Time spent sitting/lying, standing and stepping over 24hr and as percent of wake time.

	SED		ONE		MICRO	
	Non-work day	Work	Non-work day	Work	Non-work day	Work
Sitting/lying (hr/d)	9.8 ± 2.0	10.6 ± 2.3	9.6 ± 1.9	10.2 ± 2.4	9.6 ± 2.5	10.5 ± 2.2
Standing (hr/d)	3.5 ± 1.8	3.4 ± 1.8	3.0 ± 1.8	3.4 ± 1.5	3.6 ± 2.1	3.2 ± 1.9
Stepping (hr/d)	1.4 ± 0.5	1.4 ± 0.5	1.7 ± 0.4	2.1 ± 0.5***	1.7 ± 0.4	1.7 ± 0.7**δ
Sitting (% waking time)	66.6 ± 14.2	68.4 ± 13.5	67.2 ± 12.7	64.5 ± 10.4	64.0 ± 15.3	67.8 ± 14.1
Standing (% waking time)	23.9 ± 12.3	22.3 ± 11.6	20.7 ± 11.9	21.4 ±8.8	24.0 ± 14.5	20.7 ± 12.6
Stepping (% waking time)	9.4 ± 3.6	9.2 ±3.4	11.9 ± 2.4	13.9 ± 3.5***	11.9 ± 2.9	11.4 ± 4.7**δ
Sit-to-stand transitions (#)	48.8 ± 15.1	47.2 ± 17.7	42.5 ± 13.6	50.1 ± 22.3	46.1 ± 12.4	50.7 ± 21.3
Sitting bouts > 30-min (#)	5.6 ± 1.7	6.2 ± 2.2	5.5 ± 1.7	6.1 ± 1.7	6.7 ± 2.7*δ	7.4 ± 2.7
Sitting bouts > 60-min (#)	3.1 ± 1.4	3.1 ± 1.5	2.6 ± 1.1	3.1 ± 1.6	2.3 ± 1.6	2.8 ± 2.0
Step count (#)	6,409 ± 2,843	7,125 ± 2,554	9,376 ± 2,387**	12,257 ± 3,149***	9,250 ± 2,291*	10,036 ± 4,262**δδ

Data are presented as the mean ± SD. * p<0.05, ** p<0.01, *** p<0.0001 compared to SED control within the same location. δ p<0.05, δ δ p<0.01, δ δ δ p<0.0001 different from ONE within same location. Sitting/lying (hr/d), number of hours per day spent siting; Standing (hr/d), number of hours per day spent standing; Stepping (hr/d), number of hours per day spent standing; Sitting (% waking time), percent of waking hours spent sitting; Standing (% waking time), percent of waking hours spent sitting; Stepping (% waking time), percent of waking hours spent stepping; Sitting bouts > 30-min, number of sitting bouts lasting at least 30 minutes; Sitting bouts > 60-min, number of sitting bouts lasting at least 60 minutes; Sit-to-stand transitions, number of times a participant rose from a seated position; Step Count (#), is the number of steps taken per day.

Effect of the physical activity interventions on time spent in very-light, light, moderate and vigorous intensity physical activity

Time spent in very-light, light, moderate-and-very vigorous intensity physical activity during waking hours is shown in Figure 9. One ActiGraph GT3X was lost; data are reported for 21 subjects. On work days, waking time spent in very light intensity activities tended to be lower in ONE compared to SED (12.5 ± 1.3 vs 13.5 ± 1.1 h/d, p=0.055), but not different between MICRO and SED or ONE. Light intensity activities were not different across conditions (p>0.05 for all). On non-work days, MICRO significantly reduced time spent in light intensity activities compared to SED (1.5 ± 0.5 vs. 1.9 ± 0.8 h/d, p=0.040), but was associated with more time spent in very light intensity activities than ONE (13.7 ± 0.5 vs. 11.5 ± 0.5 h/d, p=0.002). Both MICRO (work day: +23.4 ± 6.6 min, non-work day: +21.6 ± 8.4 min) and ONE (work day: +40.2 ± 6.6, non-work day: +36.0 ± 9.0 min) significantly increased time spent in MVPA compared to SED on both non-work and work days (p<0.01 for all). On work days, MVPA was even greater in ONE than in MICRO (p=0.02).

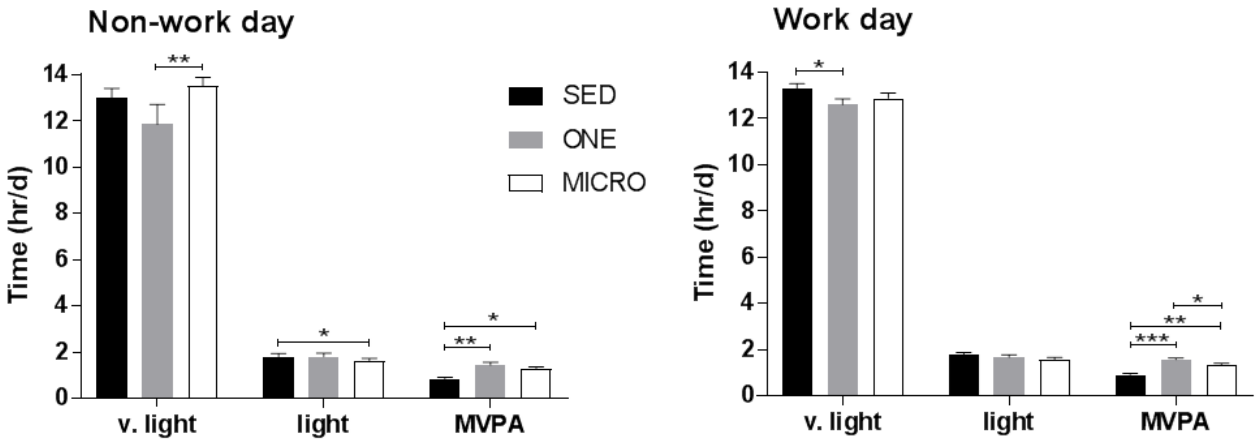
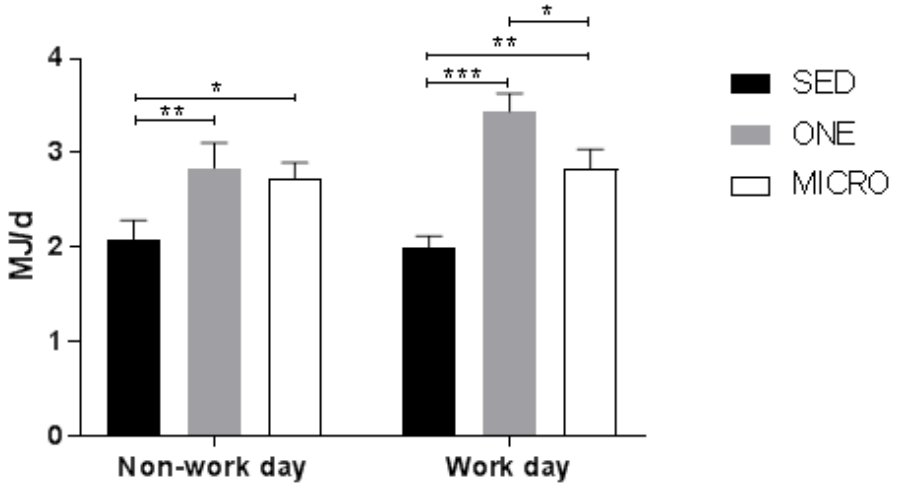


Figure 9: Waking time per day performing very light, light and moderate-to-vigorous intensity physical activity. Accelerometry data collected from ActiGraph GT3X tri-axial accelerometer are displayed by location (work or non-work day) and by physical activity intensity. V. light, very light intensity physical activity; MVPA, moderate-to-very vigorous intensity physical activity; SED, sedentary condition; ONE, one-bout intervention; MICRO, microbouts intervention. * p<0.05, ** p<0.01, *** p<0.0001 vs. sedentary control condition.

Effect of the physical activity interventions on 24hr activity energy expenditure and physical activity level

Changes in MVPA induced by the physical activity interventions translated into parallel changes in AEE (Figure 10). Both MICRO and ONE significantly increased AEE compared to



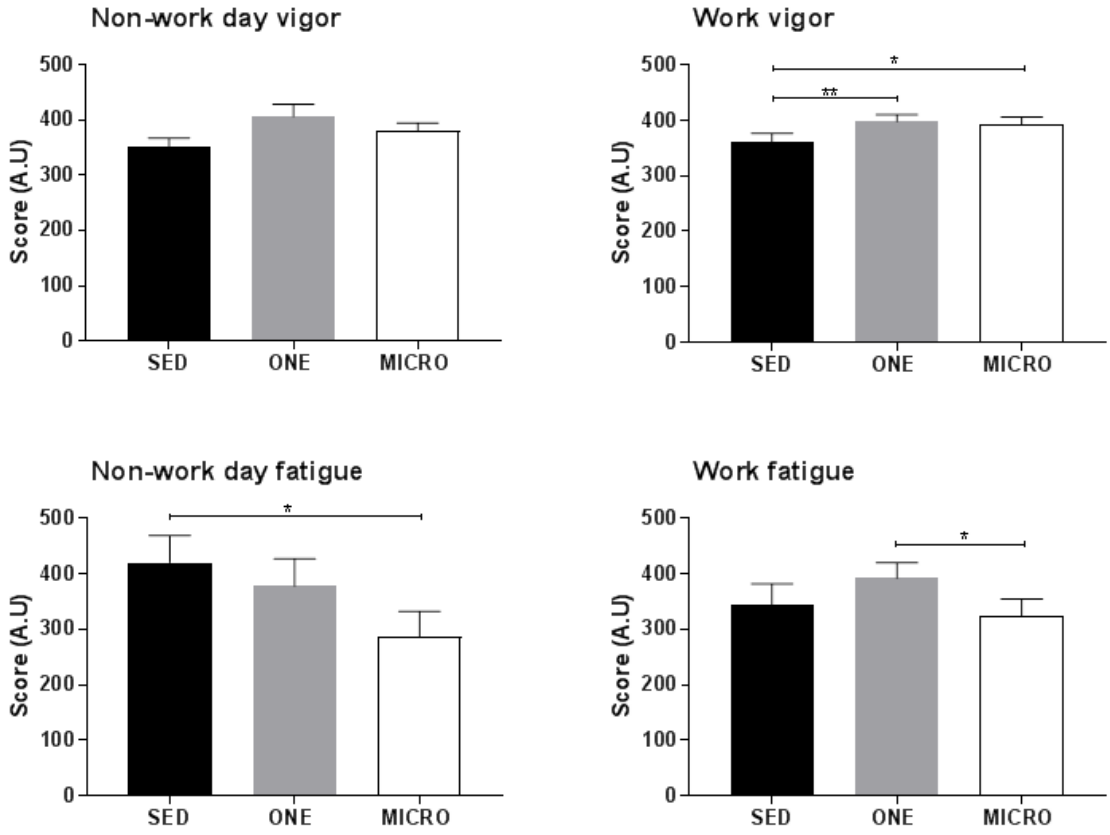
SED on both work and non-work days ($p<0.05$ for all). Physical activity level (PAL) was significantly lower in SED compared to ONE on non-work days (SED: 1.46 ± 0.04, ONE: 1.62 ± 0.04, $p=0.004$) and compared to both ONE and MICRO on work days (SED:1.43 ± 0.03, ONE: 1.65 ± 0.03, $p<0.001$, MICRO:1.55 ± 0.03, $p=0.003$). PAL was further higher in ONE than in MICRO on work days ($p=0.008$).

Figure 10: Activity energy expenditure. The activity energy expenditure (MJ/d) estimated from ActiGraph GT3X tri-axial accelerometer is displayed by location (work or non-work day). SED, sedentary condition; ONE, one-bout intervention; MICRO, microbouts intervention. * $p<0.05$, ** $p<0.01$, *** $p<0.0001$ vs. sedentary control condition.

Effect of the physical activity interventions on self-perceived vigor and fatigue

No significant differences in self-perceived vigor were noted across conditions on non-work days ($p>0.05$ for all, Figure 11). On working days, participants reported a greater level of self-perceived vigor at the end of the day in both MICRO (386.7 ± 27.9, $p=0.01$) and ONE (403.4 ± 28.1, $p=0.002$) compared to SED (314.1 ± 28.0). They further reported feeling less fatigue on

work days after a day performing MICRO than after a day performing ONE (-119.7 ± 52.5, p=0.03). On non-work days, they tended to feel less fatigue on MICRO compared to both SED (-128.9 ±



65.6, p=0.054) and ONE (-124.5 ± 67.3, p=0.069).

Figure 11: Self-perceived fatigue and vigor. At the end of each study day participants rated their self-perceived feeling of fatigue and vigor (arbitrary unit). SED, sedentary condition; ONE, one-bout intervention; MICRO, microbouts intervention. * p<0.05, ** p<0.01, *** p<0.0001 vs. sedentary control condition.

Effect of the physical activity interventions on index of insulin sensitivity

On the morning of day 4, fasting insulin and glucose concentrations were measured (Table 3). MICRO and ONE significantly decreased fasting insulin concentration by 37.3% (p=0.03) and 43.6% (p=0.02) respectively compared to SED. Fasting glucose concentrations remained unchanged. As a result, insulin:glucose ratio, an index of insulin sensitivity, was reduced by both

MICRO (p=0.03) and ONE (p=0.02) compared to SED, suggesting an improvement in insulin sensitivity. No differences were observed between the two active conditions.

Table 3: Fasting plasma glucose and insulin concentrations.

	SED	ONE	MICRO
Fasting glucose (mg/dL)	90.1 ± 7.3	88.4 ± 7.7	88.7 ± 10.6
Fasting insulin (uI/mL)	10.8 ± 8.9	6.1 ± 3.0*	6.7 ± 6.1*
I/G	0.121 ± 0.101	0.069 ± 0.341*	0.075 ± 0.063*

*Data are presented as the mean ± SD. * p<0.05, ** p<0.01, *** p<0.0001 compared to SED control. I/G, insulin/glucose ratio.*

Discussion

In this randomized feasibility study, we showed that sedentary, physically inactive, overweight/obese individuals were able to implement physical activity interventions consisting either of frequent bouts of activity or one continuous bout, the latter being more commonly promulgated by public health promotion initiatives and healthcare providers. Overall, these two physical activity interventions had similar effects. Both interventions increased daily steps, MVPA, AEE and PAL on both working and non-working days compared to the sedentary control. These increases were more pronounced with a daily single bout of physical activity as compared to microbouts. The greater physical activity and energy expenditure were further associated with higher self-perceived feelings of vigor at the end of the day and improved fasting insulin sensitivity. Microbouts of activity were also associated with lower feelings of fatigue at the end of the day both on work days and non-work days. Neither of the interventions decreased time spent sitting or standing, the number of breaks from the sitting position and the average duration of a sitting bout.

Because office employees are vulnerable to the adverse health effects of prolonged sitting, an increasing number of interventions have targeted the work environment [166]. Strategies that promote body movements, such as passive pedaling or treadmill desks have been shown to increase physical activity and energy expenditure and to some extent reduce time spent sitting [140-142, 148-150]. However, they are relatively expensive, can be a safety hazard and may be impractical to implement on a large scale. Therefore, we proposed that an intervention involving frequent short bouts of brisk walking could be an inexpensive, safe, easy to implement physical activity promotion intervention. Contrary to our hypothesis, microbouts of activity spread out across the day did not reduce the number or duration of sitting bouts and did not increase the number of transitions from the sitting position to standing or stepping. This may be because asking individuals to break-up prolonged sitting nine times a day, every hour for nine consecutive hours to perform 5-min of walking is not a sufficient stimulus. In support of this interpretation, a recent study used hourly computer screen prompts or text messages to break up sitting. Sitting time was broken up with 7 minutes of walking to accumulate 30-60 minutes of walking per day. Additionally, there was an additional 6,000 step count goal. This intervention was 7 days measured in overweight/obese and resulted in a decrease in total sitting time by 1.85 h/d on average [146]. Despite the frequency of activity being more frequent (every 30-60min), this study also failed to show an increase in the number of sedentary breaks (sit-to-stand transitions) [146]. In our study, the number of sitting bouts longer than 30-min was even greater when participants were asked to perform microbouts of activity compared to single bouts on non-work days. This suggests that people tend to stay seated until they have to stand up and be active. Therefore, future studies may need to test specific interventions that primarily target breaks from sitting in addition to sitting time, daily steps, or bouts of physical activity.

The American College of Sports Medicine, the American Heart Association and the American Diabetes Association recommend that adults perform at least 150-300 min/wk (21.4 - 42.8 min/day) of MVPA to maintain and promote cardiovascular health and insulin sensitivity [167].

Implementing frequent short bouts of 5-min brisk walking across the day in our study led to a significant 22.5-min/day increase in MVPA on average. In addition, the microbouts intervention produced an increase in AEE of 0.54 MJ/d (129 kcal/d) on non-working days and 0.78 MJ/d (187 kcal/d) on work days. It has been proposed that a very small energy gap – the difference between energy intake and energy expenditure –plays a role in weight gain [168]. A difference of 100 kcal/day at the population level could theoretically prevent weight gain in 90% of the U.S. adult population. Consequently, the increase in AEE along with the suppressive effect on appetite previously reported with microbouts of activity (at least in normal-weight individuals) [150, 153] may help mitigate weight gain. Implementing microbouts of activity at work could be a viable strategy, among other strategies, to slow down weight gain. In addition, large prospective cohort studies of diverse populations have shown that an AEE of approximately 4.18 MJ/wk (1,000 kcal/wk) is associated with lower rates of cardiovascular disease and premature mortality [167]. It would therefore be important to study the effect of this intervention over the long-term and verify whether a 1,000 kcal/wk energy expenditure could be reached. Finally, our feasibility study showed that three days of microbouts of activity performed in daily life improves insulin sensitivity, which adds to the increasing body of data collected in the laboratory settings on the beneficial effect of frequent interruptions of prolonged sitting on insulin action [61-64, 73, 102, 111-114, 120]. This is the first study to show that an intervention using small bouts of activity promotes overweight sedentary adults to comply with the current physical activity guidelines, at least in the short term. As a result, this strategy may have positive effects on body weight control and cardiometabolic health. However, we need to acknowledge that the single bout intervention we tested in the same subjects induced greater increases in MVPA (40 min/work day) and AEE 1.41 MJ/work day (+337 kcal/work day). The subjects thus attained a PAL of 1.65 that is characteristic of people who are moderately active. Future studies are needed to test the long-term effects of the microbouts of activity versus single bout of activity on the daily pattern of physical activity and energy balance regulation (appetite, energy intake, energy expenditure).

The long-term goal will be to test this type of intervention in the public on a large scale. The modern occupational environments promote increased sedentary time [169], and has therefore been identified as an ideal environment to target sedentary behaviors. This is even more important because adults who spend more time being sedentary at work do not compensate by being more active during non-working periods [170]. Interestingly, we showed that the beneficial effects of the microbouts intervention on physical activity and self-perceived fatigue were observed on both work and non-work days. This means that if implemented in occupational contexts this intervention, if sustained on weekends, could also increase physical activity on non-work days. A limitation is that instead of shifting time from very light to MVPA intensity activities as observed with the single bout of activity, the microbouts of activity increased MVPA in detriment of light intensity activity on non-work days. Another potential issue for future implementation of such intervention is the fact that participants reported the microbouts of activity to be more challenging to perform at work. But in our study, participants were the only employees performing these activities at their workplace. If the environment was designed to support breaking up sitting, participants may find this approach less challenging. It is well known that socio-ecological approaches acting on both the microenvironment (individual) and macro environment (socio-professional environment, office layout, alternative workstations, active vs sitting meetings, etc.) are key when aiming to implement new interventions changing behavior for a sustained period of time. Developing strategies to self-motivate individuals in adopting this new behavior is also crucial [171]. The fact that our overweight/obese participants perceived less fatigue at the end of a workday performing the microbouts than a single continuous bout of activity, as we previously reported it in normal weight individuals, could be used to encourage employers to incorporate microbouts of activity into the daily routines of their office employees [153]. Additionally, strategies aiming to reduce time spent sitting have not been shown to affect productivity or cognitive functions [151-153]. Most likely, a combination of the two interventions to target both occupational

and non-work time may be the best approach. It could also provide individuals with different tools to choose from according to their mood that day at the office or outside the office.

Several limitations need to be acknowledged. The main limitation is that the study was conducted over 3-days and so conclusions about whether the weekly level of recommended MVPA could be reached and sustained for longer time periods cannot be drawn. The comparison between work days and non-work days was not *a priori* powered and led to an unbalanced number of days spent in the two different settings. Because participant's knew their physical activity was being tracked by two physical activity monitors there could have been an effect of increased activity [172]. Indeed Clemes et al. showed that wearing activity monitors for three days induces a spike in physical activity levels that regresses back to the mean after 7-days [172]. However, other studies have shown no evidence of reactivity to physical activity monitors [173, 174]. In addition, the cross-over design may have limited the reactivity effect to the monitors. Another strength was that the pattern of physical activities was assessed using two complementary activity monitors, one specifically designed to detect changes in sitting and the other one designed to determine time spent in activities of different intensities and the associated energy expenditure. Finally, this feasibility study testing a novel lifestyle intervention to prevent sedentary behavior was conducted in overweight/obese, sedentary, physically inactive adults, which represent a high-risk group for metabolic diseases.

Conclusion

In this feasibility study, we showed in overweight/obese physically inactive sedentary adults that regardless of the terms of the intervention, promoting physical activity led to an increase in physical activity and energy expenditure, and improved insulin sensitivity and vigor. However, none reduced total daily sitting time or the length of sitting bouts. This suggests that more efforts are needed in the workplace to increase physical activity along with a concomitant reduction in the number and duration of sitting bouts. It may be that frequent prompts to rise from sitting in

combination with encouragements for either microbouts or single bouts of activity may represent the best overall strategy. This will need to be tested as part of a multicomponent intervention at the organizational, environmental, and individual levels. Therefore, the overall public health message should communicate that any increase in physical activity can be beneficial when performed consistently over time.

EFFECT OF FREQUENT INTERRUPTIONS OF SEDENTARY TIME ON NUTRIENT METABOLISM IN SEDENTARY OVERWEIGHT MALE AND FEMALE ADULTS

Adapted from:

"Effect of frequent interruptions of sedentary time on nutrient metabolism in sedentary overweight male and female adults."

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Synopsis

Implementing short-frequent bouts of physical activity into daily life over the short-term has been shown to be a feasible lifestyle intervention in adults who are overweight to obese, sedentary, and physically inactive. This is important because epidemiologic evidence supports the scientific premise that interrupting sedentary behaviors with short-frequent bouts of physical activity is associated with lower risk factors for metabolic risk [100]. Acutely (≤ 48 hr) breaking up sedentary behaviors with short-frequent bouts of physical activity has been shown to significantly lower postprandial metabolite (plasma glucose, insulin, and triglyceride) concentrations in response to standardized meals compared to a sedentary control. However, it remains unclear (1) whether the lower postprandial metabolites associated with short-frequent bouts of physical activity is due to the breaks in sitting time *per se* or mainly driven by the increase in physical activity and/or energy expenditure, (2) whether the acute metabolic benefits are sustained or diluted beyond the acute exposure period, and (4) what are the underlying physiological mechanisms that may be responsible for the lower postprandial metabolites.

In the following paper, we report the short-term (4-day) metabolic effects of breaking up sedentary behaviors with short-frequent bouts of physical activity in sedentary, physically inactive adults with overweight to obesity. This three-arm randomized cross-over study included a time and energy matched single-continuous bout of physical activity and a sedentary control condition. The primary outcomes were 24 hr total substrate oxidation and changes in postprandial glucose, insulin, and triglyceride concentrations.

At the same energy expenditure and balance, we showed that breaking up sedentary time with short-frequent bouts of moderate intensity walking breaks lead to a greater reliance on carbohydrate as fuel during the waking period when the bouts were performed and over 24 hr compared to a sedentary control condition. In contrast, a single isoenergetic continuous bout of moderate intensity walking increased 24 hr fat oxidation. Importantly, both physical activity interventions had lower postprandial insulin and fasting HOMA-IR. These findings indicate that

when total energy expenditure is equal between the two active conditions, breaking up sedentary time with short-frequent bouts of physical activity impacts daily patterns in fuel utilization differently than daily single-continuous bout, with potentially a more positive effect on carbohydrate metabolism.

Introduction

Sedentary behavior is associated with several adverse health outcomes including obesity, cardiometabolic diseases, diabetes, certain types of cancer and premature mortality [175, 176]. These associations have been observed across sex, age, ethnicity, and even among individuals who meet the current intensity-based physical activity guidelines (i.e., 150-min/week of moderate intensity or 75-min/week of vigorous exercise) [175]. Isotemporal substitution modeling suggests that replacing 1h of sedentary time with either light or moderate-to-vigorous intensity physical activity (MVPA) in inactive adults was associated with lower mortality and risk factors of metabolic disease, with MVPA associated with the most potent health-enhancing time-dependent behavior [42, 177]. Independent of potential confounders and time spent in other activities, reallocation of 30-min/day of sedentary time with an equal amount of MVPA is associated with lower blood triglycerides, glucose and insulin, and higher insulin sensitivity [177]. Additionally, observational studies suggest that interrupting sedentary time with frequent bouts of physical activity is associated with lower plasma glucose and insulin, waist circumference, inflammatory marker C-reactive protein even in individuals who regularly exercise [56, 100]. Adults whose sedentary time was mostly uninterrupted had less healthy cardiometabolic profiles, based on increased blood glucose and triglycerides, compared to those who had more frequent breaks in sedentary time [56, 100, 178], even when controlling for total sedentary time, MVPA, age, sex and ethnicity [56, 100].

A number of acute and short-term studies (≤ 3 days) have shown that frequent interruptions of prolonged sedentary activities with short bouts of walking decrease postprandial plasma glucose and insulin concentrations compared to a prolonged sedentary condition [61-64, 73, 111-114]. For example, Dunstan et al. (10) compared plasma glucose and insulin responses to uninterrupted sitting and 2-min bouts of activity every 20-min for 5-h in overweight adults. Sitting was either interrupted by light (3.2 km/h) or moderate intensity (5.8-6.4 km/h) treadmill walking.

Relative to uninterrupted sitting, glucose, and insulin iAUC in response to standardized meal were both significantly reduced after the activity-break conditions. A possible hypothesis for the reduction in plasma glucose concentrations is that carbohydrate oxidation is increased to support the increased energy expenditure. To test this hypothesis, we used room calorimetry and stable isotope tracers to compare the short-term effects (4-d) of activity microbouts (5-min of moderate-intensity physical activity performed hourly for 9 consecutive hours) to a sedentary condition on 24-h nutrient oxidation in physically inactive adults with overweight and obesity. Because a shift in carbohydrate oxidation with activity microbouts might be solely due to increased energy expenditure we also studied an energy matched single bout of moderate-intensity physical activity performed in the morning. Only few studies have controlled for energy expenditure by comparing the effect of frequent interruptions of sedentary behavior with bouts of physical activity to an isocaloric continuous bout of physical activity, which remains the most common form of recommended physical activity [64, 95, 111-113]. A finding that short frequent bouts of activity promote carbohydrate oxidation in sedentary persons may inform on an approach to personalize exercise prescription for individuals who have difficulty complying with traditional physical activity recommendations and reducing the exposure to increased plasma glucose observed in highly sedentary populations.

Methods

Participants

Eligible participants were aged 19-45 years, with a body mass index (BMI) between 27 and 33 kg/m^2, weight stable for >3 months, had fasting plasma insulin concentrations below 25 µIU/mL, and self-reported > 6h/day sitting. Women were pre-menopausal but could use oral contraceptives. Exclusion criteria included clinically diagnosed diabetes, taking glucose- and/or lipid-lowering medication, dyslipidemia, smoking, or being physically active (>150-min/week

moderate-intensity exercise). Participants were recruited between October 2014 and October 2016 from newspapers advertisements, public announcement, and flyers in the Denver and Aurora areas, Colorado, USA (see CONSORT diagram Figure 1).

Study Design

Following a screening visit, each eligible subject completed three separate 4-day trial conditions that consisted of three days in free-living conditions followed by 24-h in a whole-room calorimeter. The three trial conditions were administered in random order:

- *Sedentary* (SED)*:* During the 3-day free-living period, subjects were asked to maintain usual levels of daily activity, and were asked to refrain from structured exercise. On day 4, subjects remained sedentary in the whole-room calorimeter.
- *Sedentary + 1 continuous bout of activity* (ONE)*:* During the 3-day free-living period, subjects were instructed to perform 45-min of moderate-intensity walking once per day in the morning and maintained usual levels of daily activity the rest of the day. On day 4, participants remained sedentary in the whole-room calorimeter except to perform one bout of 45-min moderate-intensity treadmill walking at 10:00AM.
- *Sedentary + microbouts of activity* (MICRO)*:* During the 3-day run-in period, subjects performed 5-min of moderate intensity walking bouts each hour for 9 consecutive hours in daily life and maintained usual levels of daily activity the rest of the time. On day 4, participants performed 5-min moderate-intensity treadmill walking every hour for 9 consecutive hours from 1000h to 1800h and remained sedentary the rest of the day.

The study conditions were separated by a 28-day wash out period and women were studied in the follicular phase of the menstrual cycle. All the visits were conducted at the Clinical and Translational Research Center of University of Colorado Hospital (CTRC). This study was approved by the Colorado Multiple Institutional Review Board (COMIRB) and was in accordance with the Declaration of Helsinki.

Screening visit

At screening, written consent was obtained and participants were screened for exclusion criteria. This included a medical history and physical examination. The short version of the International Physical Activity Questionnaire (IPAQ) [156] was completed to assess eligibility based on inclusion criteria for habitual physical activity and time spent sedentary. Subjects then performed a test on a motorized treadmill to determine the walking pace that was prescribed for the ONE and MICRO conditions. The walking test started at a pace of 2.4 mph and the pace increased by increments of 0.3 mph every 2-min. At each level, subjects rated their perceived effort on a Borg scale from 0 (very light) to 20 (maximal exertion). The aim was to identify the speed that participants associated with a level of effort reaching 13 (somewhat hard). The walking test was stopped when the participant rated the speed of the treadmill with an RPE of 16 (hard to very hard). The treadmill speed associated with 13 RPE was the pace that was prescribed on day 4 for ONE and MICRO. For the three days of the run-in period, subjects were instructed to walk at a pace similar to what was established with the walking test, i.e. the participants perceived moderate-intensity walking pace, an RPE of 13.

Randomization

Participants were randomized to one of three possible trial-condition orders using balanced blocks prepared for male and female participants. The study statistician (Z.P.) prepared the computer-generated randomization lists and sealed envelopes for randomization. Once informed consent was obtained, a study member opened the sealed randomization envelope revealing the trial-condition order.

Run-in diet and physical activity

A 3-day standard diet was provided by the CTRC Metabolic Kitchen during the run-in to each inpatient study visit. The macronutrient composition of the diet was 30% fat, 55% carbohydrate (CHO) and 15% protein of total energy intake. Daily energy needs were calculated based on an estimate of resting metabolic rate (RMR) derived from the average of 1) direct measurement by hood indirect calorimetry and 2) an estimate using the following equation: [(23.9 x FFM in kg) +

372], where fat-free mass (FFM) was measured by dual energy X-ray absorptiometry (DXA, Hologic Delphi-W, Bedford, MA) [179, 180]. The estimated RMR was then multiplied by an activity factor (1.4-1.7) based on the time spent physically active self-reported in the IPAQ. Participants were instructed to eat all food and bring back leftovers to the Metabolic Kitchen. They were also asked to abstain from consuming alcohol and to consume the same amount of caffeine (number of cups) for 24-h before each whole-room calorimeter stay. Daily energy needs in the chamber were estimated using the same equations as described above but applying an activity factor of 1.3. Compliance with the activity prescriptions (SED, ONE or MICRO) during the 3-day run in period was objectively assessed with the use of an inclinometer (ActivPAL, Glasgow, UK) and accelerometer (Actigraph GT3X+, Fort Walton Beach, FL). Results on physical activity during the run-in diet were reported elsewhere [181].

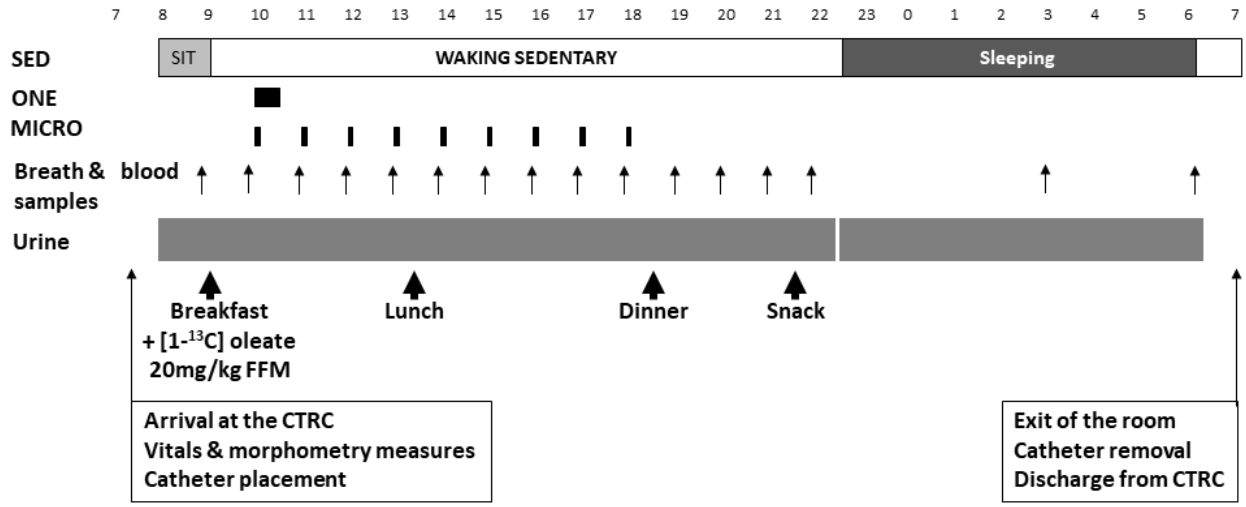

Figure 12: Protocol of the study day in the whole-room calorimeter. SED, sedentary condition in which participants remained sedentary (no exercise); ONE, one bout condition in which participants remained sedentary for the day except for completing one bout of moderate intensity treadmill walking for 45-minutes; MICRO, microbouts condition in which participants remained sedentary for the day except for completing 9 bouts of 5-min moderate intensity treadmill walking once an hour for 9 consecutive hours. ▬ indicates 45-minute bout of walking. ▮ indicates 5-minute walking bout. ↑ indicates blood collection. ▲ indicates meals.

Inpatient study day

Figure 12 depicts the inpatient study protocol. Participants reported to the CTRC at 0730h, voided and were weighed. An IV catheter was placed into the antecubital vein for blood sampling.

Subjects then entered the whole-room calorimeter for a 23-h stay. Subjects remained seated for the first hour to achieve a steady state within the individual and the whole-room calorimeter. A fasting blood sample was obtained at 0855h. Breakfast, lunch, dinner and snack were given at 0900h, 1300h, 1830h and 2130h and contained 30%, 35%, 25% and 10% of daily energy needs, respectively. The macronutrient composition of each meal was 65% CHO, 20% fat and 15% protein of total energy intake; a moderately-high carbohydrate diet was used to optimize our ability to observe differences in fuel utilization between the three conditions. 1-^{13}C oleic acid (20mg/kg of FFM, 99% enrichment, Cambridge Isotopic Laboratories, MA) was mixed and administered with the liquid breakfast meal. Breath and blood samples were then collected every hour for 14-h from 0900h-2200h, and at 0300h. Final blood and breath samples were collected at 0700h the following day. In MICRO, blood samples were collected prior each activity bout x 9 bouts. Blood samples were obtained by having subjects extend their arm through a porthole imbedded in the wall of the whole-room calorimeter. Lights-out and a sleep opportunity was scheduled from 2230h to 0630h. Urine was collected in one jug for the "waking time" from the start of the study day to 2230h (bedtime), and in a second jug during "sleep time" from 2230h (bedtime) to 0700h the following morning. Study participants exited the whole-room calorimeter at 0700h.

Energy expenditure and substrate oxidation

Twenty-three-hour respiratory gas exchange data were extrapolated to 24-h values. TEE and substrate oxidation were determined using O_2 consumption and CO_2 production determined from the flow rates and differences in gas concentrations between air entering and air exiting the calorimeter and nitrogen excretion in the urine, as previously described [182]. EE and substrate oxidation [183] were determined over 24-h, waking time and sleeping time. Twenty-four-hour energy balance was calculated as the difference between 24-h energy intake and 24-h TEE. Activity energy expenditure (AEE) was calculated as TEE – 10% of TEE - SMR, where 10% TEE is the thermic effect of food and SMR (sleep metabolic rate) was the sleep metabolic rate measured from 0100h to 0300h [184].

Dietary fatty acid oxidation

Participants collected an hourly breath sample for $^{13}CO_2$ by blowing through a tube into two 15 ml vacutainer tubes. Breath CO_2 was sampled directly from the vacutainer with a syringe, and $^{13}CO_2/^{12}CO_2$ was measured with isotopic ratio mass spectrometer (IRMS, Delta V, Thermo Electron, Bremen, Germany). The average baseline enrichment value was subtracted from the subsequent values for each subject, and each time point was expressed as the increase in enrichment relative to the subject's own baseline. By using time matched CO_2 production rates from the whole-room calorimeter, 1-^{13}C oleic acid oxidation was calculated as the instantaneous percentage recovery of ^{13}C in expired CO_2 per hour for 14-h and after 24-h cumulative oxidation rates were also calculated over 24-h as previously described [17] and after correction for CO_2 entrapment in the bicarbonate pool and TCA cycle [185].

Plasma analysis

Whole blood was added to a preservative (3.6 mg EDTA plus 2.4 mg glutathione in distilled water). Plasma and serum were separated after spinning and stored at -80°C until analyzed. EDTA plasma samples were assayed for triglycerides (TG), glucose and insulin. Insulin concentrations were measured using a standard double antibody radioimmunoassay (EMD Millipore, St. Charles, Missouri). Serum glucose concentrations were determined using the hexokinase method, TG were measured using the enzymatic assay and non-esterified fatty acids (NEFA) by using commercial enzymatic assay (Wako Diagnostics, Mountain View, CA); all samples were run on the Beckman Coulter AU480 Chemistry Analyzer (Brea, CA).

Data and statistical analysis

The sample size was calculated using data from a previous study [63]. In that study, the authors observed in 19 overweight men that interruptions of prolonged sitting with moderate-intensity activity (2-min every 20-min) reduced 5-h glucose and insulin incremental area under the curve (iAUC) by 29% and 23%, respectively, compared to uninterrupted sitting. Assuming the effect sizes between conditions would be similar to this study, we estimated that 20 paired

observations were needed to achieve an 80% power to detect a direct treatment effect, while adopting a two-tailed testing and alpha < 0.05.

iAUCs were calculated with the trapezoidal rule for plasma metabolites and insulin. Linear mixed models (LMM) were used to test differences in total substrate use, TG, FFA, glucose and insulin iAUCs and dietary fat oxidation with intervention as repeated effect, sequence, period and intervention as fixed effects and subjects as random effect with a compound symmetry covariance. Energy balance was taken into account as a covariate when necessary. Least significant difference (LSD) post-hoc tests were used to examine between condition differences. Carryover effects were expected to be minimal because of the minimum 28-days washout period between consecutive interventions. Pearson correlation coefficients were calculated to examine the relationships among the outcomes, i.e. TEE, AEE, energy balance, substrate use, dietary fatty acid oxidation, and plasma metabolites and insulin iAUCs. Data are expressed as mean (SD), unless otherwise stated. All statistical analyses were performed with SPSS (v22.0, IBM, SPSS Statistics Inc., Chicago, IL).

Results

Participant characteristics

The Consolidated Standards of Reporting Trials (CONSORT) diagram is shown as Figure 13. Twenty-five sedentary overweight adults (n=12M/13F; 31.6 years (SD 6.5); BMI=30.5 kg/m^2 (SD 2.7) were recruited to participate in the study. Data are shown for twenty participants (10M/10F; 32.4 years (SD 6.3); BMI, 30.6 kg/m^2 (SD 2.9) who completed all the procedures. Participant's characteristics are displayed in Table 4.

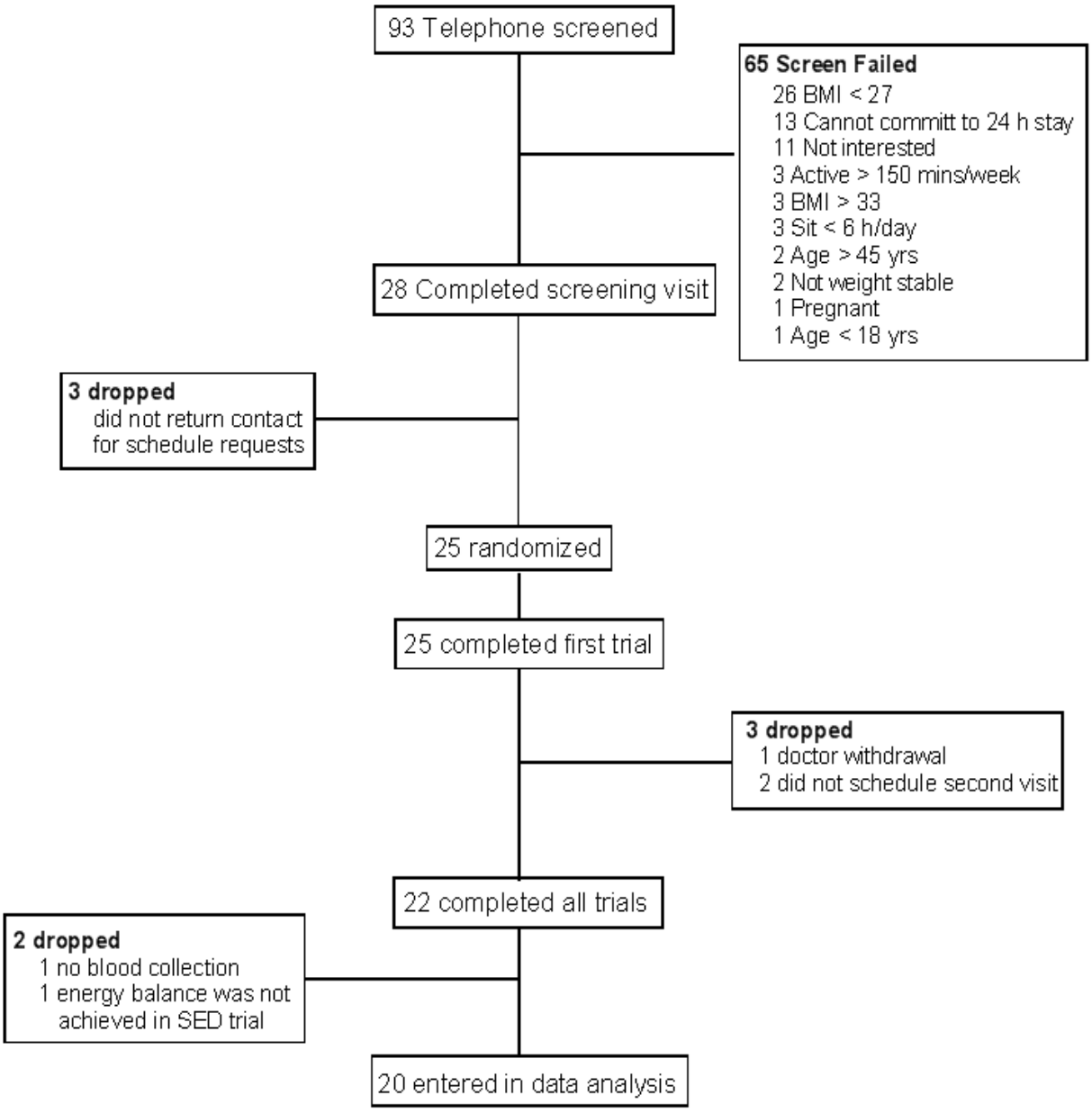

Figure 13: Trial CONSORT diagram

Table 4. Participant Characteristics

	Males	Females	Total
N	10	10	20
Age (years)	31.5 (SD 7.4)	33.2 (SD 5.1)	32.4 (SD 6.3)
BMI (kg/m^2)	28.9 (SD 3.0)	31.9 (SD 2.0)	30.6 (SD 2.9)
Body Mass (kg)	88.0 (SD 13.6)**	83.5 (SD 7.2)	85.7 (SD 10.8)
FFM (kg)	63.2 (SD 10.0)***	48.3 (SD 3.6)	55.8 (SD 10.5)
FM (kg)	24.6 (SD 4.4)***	35.2 (SD 4.4)	29.9 (SD 6.9)
Fat mass (%)	28.1 (SD 2.5)***	42.0 (SD 2.3)	35.0 (SD 7.5)
Fasting Glucose (mg/dL)	88.0 (SD 4.8)	87.6 (SD 4.3)	87.8 (SD 4.4)
Fasting Triglyceride (mg/dL)	154.6 (SD 107.7)*	83.5 (SD 14.7)	117.2 (SD 81.2)
Fasting Insulin (µIU/mL)	5.4 (SD 2.7)	5.15 (SD 1.3)	5.2 (SD 2.1)
HOMA-IR (SED day fasting)	1.5 (SD 0.8)	1.4 (SD 0.4)	1.4 (SD 0.6)
IPAQ self-reported sitting time (hrs/day)	9.6 (SD 2.9)	11.3 (SD 3.5)	10.5 (SD 3.3)

*Data are presented as the mean (SD). N, number of subjects; BMI, body mass index; FFM, fat-free mass; FM, fat mass; HOMA-IR, homeostatic model assessment of insulin resistance; IPAQ, international physical activity questionnaire. * p<0.05, ** p<0.01, *** p<0.0001 vs. Female*

Twenty-four-hour energy intake, expenditure, balance and nutrient oxidation

Daily patterns of EE and respiratory quotient (RQ) are presented in the Annex Supplemental Figure 1. Twenty-four-hour energy intake, expenditure and balance are shown in Figure 14A. By design, both TEE and AEE were matched between the two active conditions and significantly higher than SED (p<0.0001 for both). Because total energy intake was the same across all three study conditions, participants were in negative energy balance in both ONE (-1.50 MJ/d (SD 0.17), p<0.01) and MICRO (-1.64 MJ/d (SD 0.17), p<0.01) compared to SED. Twenty-four hour non-protein respiratory quotient (NPRQ) was lower in ONE compared to both SED and MICRO conditions (0.881 (SD 0.006) versus 0.900 (SD 0.006) and 0.900 (SD 0.009) respectively, p<0.05

for both; Figure 14D), even when accounting for differences in energy balance. MICRO was associated with higher 24-h carbohydrate oxidation compared to both ONE ($p<0.01$) and SED ($p<0.001$; Figure 14C). In contrast ONE was associated with higher 24-h total fat oxidation compared to SED ($p<0.001$) and higher 24-h dietary fat oxidation compared to both SED and MICRO ($p<0.05$ for both; Figure 14C and E). When taking energy balance into account, both 24-h total and dietary fat oxidation were higher and 24-h carbohydrate oxidation lower in ONE compared to MICRO ($p<0.05$ for all). When expressing nutrient oxidation as a percentage of TEE, we observed that ONE had greater reliance on fat as fuel and less on carbohydrate compared to SED. Nutrient oxidation as percentage of TEE was not altered in MICRO (Figure 14B).

In ONE, changes in both 24-h total and dietary fat oxidation were positively correlated with the increase in 24-h EE ($R^2=0.66$, $p<0.001$; $R^2= 0.58$, $p<0.01$, respectively) but these associations were not observed in MICRO even though the increase in 24-h EE was similar. In both active conditions, changes in 24-h fat oxidation were negatively correlated with changes in 24-h carbohydrate oxidation (ONE: $R=-0.79$, $p<0.0001$; MICRO: $R=-0.84$, $p<0.0001$).

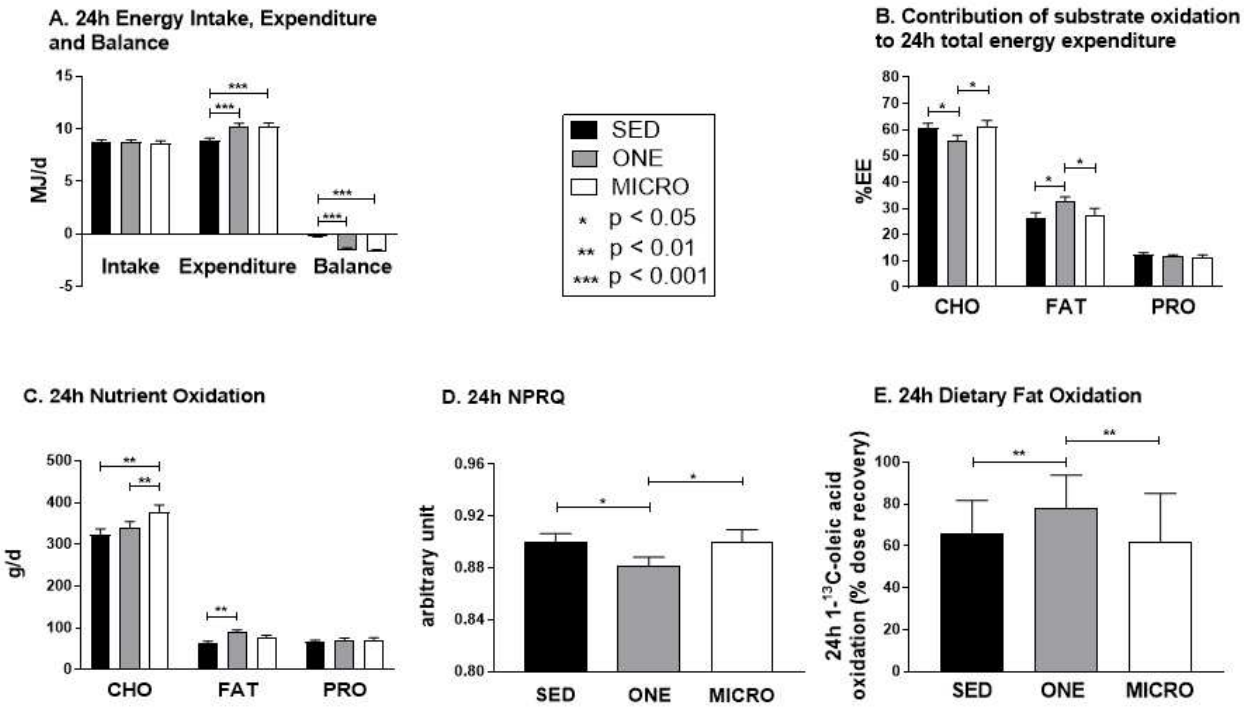

Figure 14: Absolute and relative twenty-four-hour nutrient oxidation, balance and dietary fat oxidation. Energy intake (MJ/d), energy expenditure (MJ/d) and energy balance (MJ/d) during the study day in the whole room calorimeter. (B) Relative contribution (%) of carbohydrate (CHO), fat (FAT) and protein (PRO) oxidation to total 24-h energy expenditure. (C) Absolute nutrient oxidation (grams/day) for carbohydrate (CHO), fat (FAT) and protein (PRO). (D) 24-h non-protein respiratory quotient for each study condition: sedentary (SED), one bout (ONE) and microbouts (MICRO). (E) 24-h dietary fat oxidation (percent dose recovery from 1-^{13}C oleic acid stable isotope tracer) for each study condition: sedentary (SED), one bout (ONE) and microbouts (MICRO). Data are presented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Substrate oxidation during waking and sleeping periods

As expected, EE was significantly higher in ONE and MICRO compared to SED ($p<0.0001$ for both; Figure 15C) during the waking time when physical activity was performed. Waking NPRQ was lower in ONE compared to SED and MICRO (0.894 (SD 0.006) versus 0.911 (SD 0.005) and 0.914 (SD 0.009) respectively, $p<0.05$ for both). During sleep, NPRQ was lower in both ONE and MICRO compared to SED (0.847 (SD 0.009) and 0.852 (SD 0.008) versus 0.875 (SD 0.011), respectively, $p<0.05$ for both). In both active conditions, carbohydrate oxidation decreased during the night. Compared to SED this was associated with greater fat oxidation in ONE, but increased protein oxidation in MICRO ($p<0.05$ for all; Figure 15E).

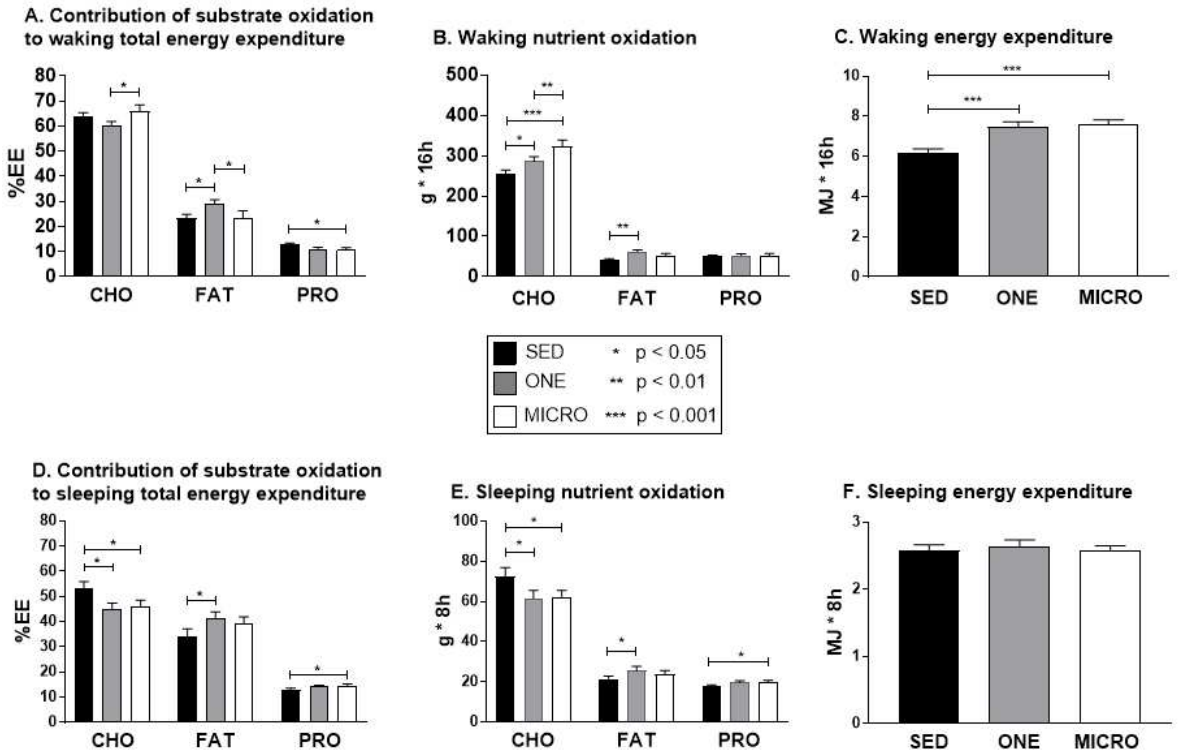

Figure 15: Waking and sleeping substrate oxidation. Absolute and relative substrate oxidation during waking (0800h-2230h) and sleeping (2230h-0630h) time. (A) Relative contribution (%) of carbohydrate (CHO), fat (FAT) and protein oxidations to waking energy expenditure. (B) Waking absolute nutrient oxidation (g) of carbohydrate (CHO), fat (FAT) and protein oxidations for waking energy expenditure. (C) Waking energy expenditure. (D) Relative contribution (%) of carbohydrate (CHO), fat (FAT) and protein oxidations to sleeping energy expenditure. (E) Sleeping absolute nutrient oxidation (g) of carbohydrate (CHO), fat (FAT) and protein oxidations for sleeping energy expenditure. (F) Sleeping energy expenditure. %EE, percent contribution to energy expenditure; g, grams; MJ, mega Joules. Data are presented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Plasma metabolites and index of insulin sensitivity

Twenty-four-hour profiles of glucose, TG, insulin, and FFA are shown in the Annex Supplemental Figure 2. There were no differences between conditions in fasting concentrations or 24-h iAUC values for plasma glucose, insulin, FFA and TG (Figure 16). After 4-d of both active conditions HOMA-IR was decreased compared to SED (SED=1.67 (SD 0.87), ONE= 1.20 (SD 0.52), MICRO=1.42 (SD 0.70); $p<0.05$ for both), indicating an improvement in fasting insulin sensitivity. In addition, insulin iAUC measured from the first to the last microbout of activity (1000h – 1800h) was reduced in ONE (ONE= -16599 µIU/mL.$9h^{-1}$ (SD 10871); $p<0.0001$) and MICRO

(MICRO= -11570 µIU/mL.9h^{-1} (SD 8866); p<0.01) compared to SED (SED= -5283 µIU/mL.9h^{-1} (SD 10667) (Figure 16). No differences were noted over this same time period in plasma glucose, TG and FFA.

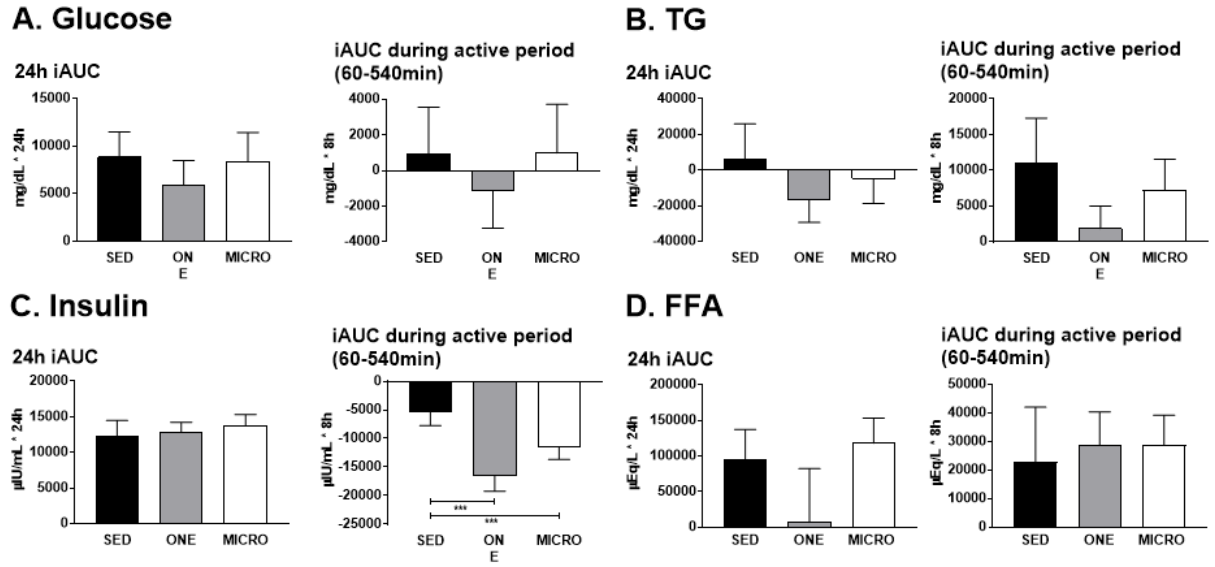

Figure 16: Twenty-four hour and active period iAUCs for plasma metabolites. iAUCs for plasma glucose, TG, insulin and FFAs measured during the inpatient study day. (A) 24-h and active period plasma glucose iAUCs. (B) 24-h and active period plasma TG iAUCs. (C) 24-h and active period plasma insulin iAUCs. (D) 24-h and active period plasma FFA iAUCs. Active period iAUC measured from the first to the last microbout of activity (1000h – 1800h). TG, triglyceride; FFA, free fatty acids; SED, sedentary exposure; ONE, one bout (45-min moderate intensity at 1000h) walking exposure; MICRO, microbouts exposure (5-min walking per hour for 9 consecutive hours at moderate intensity from 1000h-1800h). Data are presented as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001.

Discussion

In this crossover study, we showed in sedentary overweight or obese adults that four days of frequent interruptions in sedentary time with short moderate intensity walking breaks every hour for nine hours leads to greater reliance upon carbohydrate as fuel compared to a sedentary control condition. In contrast and to our surprise, a single isoenergetic continuous bout of moderate-intensity walking led to greater total and dietary fat oxidation. These findings suggest that when energy expenditure is equal between the two active conditions, breaking up sedentary time

impacts daily patterns in fuel utilization differently than when exercise is performed as a single bout in the morning.

Breaking up sedentary behavior with short bouts of activity resulted in improved postprandial glucose and insulin metabolism in several previous acute and short intervention studies [61-64, 73, 111-114]. In the present study, we observed a significant reduction in plasma insulin iAUC when participants were performing microbouts of activity, but postprandial glucose concentration was not reduced over the 9h active period. There are several possible reasons for the discrepancy in postprandial glucose responses in response to short frequent breaks of prolonged sitting compared to sedentary control. Previous studies supplied liquid meal replacement shakes as the study day energy intake. While the use of liquid meal replacement shakes ensures accurate standardization of macronutrient intake it does not reflect the postprandial responses of foods that are regularly consumed by the target population. The current study administered food preference questionnaires and provided meals composed of whole foods including dietary fiber. Dietary fiber has been shown to attenuate postprandial plasma glucose responses [186, 187]. Additionally, the current study did not include participants with impaired glucose tolerance or type 2 diabetes as has been done in previous studies [62, 63, 114]. Along this line, Blankenship *et al.* (2014) studied overweight and obese participants similar to the present study but also did not observe differences in plasma glucose responses to a meal tolerance test administered at the end of a day after a protocol involving either frequent long or short activity breaks (conditions matched for EE) [112]. However, glycemic variability measured by continuous glucose monitoring was reduced in the two frequent break conditions, indicating improved glucose control [112]. Peddie et al (2013) showed a reduction in postprandial glucose iAUC in young healthy male adults compared to sedentary condition when interrupting sitting with walking breaks of 1-min and 40-s every 30-min (30-min total walking) over 9h [64]. Similarly, Bailey et al showed in overweight young adults a beneficial effect on postprandial glycemia when sitting was interrupted by 2-min bouts of light walking every

20-min (28-min total) for 6-h [73]. In the current study, independent of changes in substrate use, both modalities of physical activity improved insulin sensitivity after four days as indicated by the decrease in HOMA-IR in the fasted state the following morning and the decrease in postprandial insulin iAUC during the active period. Taken together, both exercise interventions improved indexes of insulin sensitivity but through a differential metabolic response to varying frequencies of activity bouts. These studies suggest that while less apparent in non-diabetic populations, frequent interruptions of sedentary time help control glucose metabolism via a better insulin sensitivity and greater use of carbohydrate as fuel in postprandial state and over 24-h thus lowering glycemia mean and variability. The magnitude of the effects may be related to the frequency of the interruptions. Interestingly, these effects seem to be independent of energy balance, and likely related to the frequent interruptions of sedentary time.

To distinguish the effect of energy expenditure from those resulting from the frequent interruptions of sedentary time, we included an isoenergetic single continuous bout of moderate intensity walking in the study design. At the same energy expenditure and energy deficit, frequent interruptions of sedentary activity with short bouts of moderate intensity physical activity primarily rely upon carbohydrate substrate to maintain energetic homeostasis over 24-h, while a single bout of moderate-intensity activity favors the oxidation of both 24-h total and dietary fatty acids, as previously reported [17]. One could assume that the greater use of fat oxidation observed with physical activity performed as a continuous long bout may result over the long run in a greater weight loss than what could be attained by performing multiple short bouts of activity. Jackicic et al. [188] showed that multiple short bouts and time-matched long continuous bouts of activity induced similar weight loss after 18 months of intervention in sedentary overweight women. It is however important to note that the short bouts were of 10-min duration which could be long enough to trigger fat oxidation compared to 5-min bout of activity. Future studies looking at the

long-term impact of microbouts of activity versus long bouts of activity on body weight regulation will be needed to further test this hypothesis.

The differential effects between these two active conditions were also observed over the waking and sleeping periods when examined separately. During the waking period, frequent interruptions in sedentary time was associated with greater carbohydrate oxidation while the performance of a single bout of walking led to an increase in both carbohydrate and lipid oxidation. As suggested by the tight correlation between carbohydrate and fat oxidation observed over 24-h for each active condition, nutrient oxidation is likely the result of competition between substrates entering the TCA cycle [189]. One may assume the potential following scenario. Glucose was preferentially used with the short bouts of activity because it was readily available as skeletal muscle glycogen, especially during the first minutes of the bout of activity. The regular muscle contractions spread across the day with the frequent interruptions of sedentary time may have further stimulated the translocation from the cytoplasm to the membrane of the glucose transporter GLUT4, as previously shown [120]. This triggered the uptake and oxidation of glucose by the cell to provide energy. Because the microbouts of activity were performed in the postprandial state only, glucose was constantly available and competing against fat. When performing 45-min of walking, glycogen storage was at least partly depleted thus allowing fat to be oxidized for energy expenditure and glycogen pools were re-filled. Because of the close relationship between energy and fat balances [190, 191], we observed that at the equal energy expenditure, fat oxidation was increased but only when physical activity was performed as one single bout.

During the sleeping periods, carbohydrate oxidation was reduced in the two active conditions compared to the sedentary condition. Interestingly, while this was in favor of increased fat oxidation after 45-min of moderate-intensity walking performed in the morning, it was associated with an increase in protein oxidation following a day performing microbouts of activity. However,

measurement of protein oxidation via urinary nitrogen excretion is not a direct measure of protein oxidation but an assessment of protein deamination. The greater disappearance of protein may rather reflect a use of protein for gluconeogenesis to replenish muscle glycogen than a use of protein as fuel for the body. Over 24-h, the microbouts of activity likely trigger the use of glycogen stores and its replenishment, thus enhancing glycogen turnover; future studies will be needed to test this hypothesis. These differences and changes in nocturnal nutrient metabolism are important given the growing body of data pointing towards a key role of sleep in the regulation of energy homeostasis and metabolism [192]. For example, we showed that higher rates of nocturnal fat oxidation are associated with lower weight gain over five years in adults [193]. Future research is needed to better understand the changes induced by the two different types of physical activity interventions on the changes observed in waking and sleeping nutrient metabolism.

A major strength of this study is that it was a randomized controlled trial testing energy-matched active conditions to isolate the respective effects of the frequency of interruptions of sedentary time from energy expenditure. Also, diet, alcohol and caffeine consumption were controlled, and nutrient metabolism was measured over 24-h in a whole-room calorimeter. There are some methodological factors that limit generalizability of the present findings. Comparing the active conditions to the sedentary conditions in stable energy balance would have been more rigorous, and most likely representative of what happened in daily life in chronic situations. We however mathematically and statistically accounted for differences in energy balance, and the two active conditions were in similar energy imbalance. Another limitation was the artificial elevation of dietary carbohydrate oxidation that may have potentialized the use of carbohydrate as fuel during the MICRO condition. Additionally, while dietary fat oxidation was measured, we cannot comment on the source of carbohydrate that was oxidized during the study day. Future studies using both fat and carbohydrate stable isotope tracers will be needed.

Conclusions and future directions

In conclusion, we showed that while four days of frequent interruptions in sitting time primarily relies upon carbohydrate as fuel, a single long bout of activity primarily influences lipid metabolism. This suggests that the beneficial effects of interrupting sedentary time on glucose control that was previously reported is likely related to a greater reliance upon carbohydrate as fuel. This effect does not appear to be related to energy expenditure and balance, but rather to increasing the frequency of muscle contractions spread across the day. Underlying mechanisms at play as well as the role of moderating factors such as weight status, insulin resistance and sex need to be examined in the future.

SHORT-TERM ADAPTATIONS IN SKELETAL MUSCLE MITOCHONDRIAL OXIDATIVE CAPACITY AND METABOLIC PATHWAYS TO BREAKING UP SEDENTARY BEHAVIORS IN OVERWEIGHT OR OBESE ADULTS

Adapted from:

"Short-Term Adaptations in Skeletal Muscle Mitochondrial Oxidative Capacity and Metabolic Pathways to Breaking Up Sedentary Behaviors in Overweight or Obese Adults."

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Synopsis

When total active time and energy expenditure were matched, breaking up sedentary behaviors with short-frequent bouts of physical activity was associated with a higher postprandial and 24 hr total carbohydrate oxidation while a daily single-continuous bout of physical activity was associated with higher postprandial and 24 hr lipid oxidation. Both physical activity interventions were associated with lower postprandial insulin concentrations and indexes of insulin sensitivity. Because skeletal muscle is responsible for the majority of substrate oxidation that accompanies physical activity and a main driver of insulin sensitivity, we wondered if responses in skeletal muscle to frequent, short bouts or a single-long bout of physical activity would mirror those observed at the whole-body level. Acute moderate intensity (5 hr) and short-term light intensity (3-day) exposure to short-frequent physical activity bouts acutely activated both non-oxidative and oxidative glucose uptake via contraction-mediated and insulin-dependent pathways compared to a sedentary control in overweight adults [120]. However, it remains unclear whether the acute adaptations previously observed are similar or different than those triggered by a single-continuous bout of physical activity over the short-term. Also, higher mitochondrial oxidative capacity is associated with increased insulin sensitivity [194, 195]. A potential mechanism in our study for lower postprandial insulin concentrations for both physical activity interventions could be a higher mitochondrial oxidative capacity relative to sedentary behaviors.

In the following study, the primary outcome was adaptations in mitochondrial oxidative capacity. We hypothesized that 4-days of breaking up sedentary behaviors with short-frequent bouts of physical activity would result in higher mitochondrial respiration in the presence of carbohydrate-associated substrates along with enhanced expression of genes and pathways associated with the regulation of carbohydrate metabolism, as measured by mRNA expression when compared to the sedentary condition. We also hypothesized that 4-days of daily single-continuous bouts of physical activity matched for total active time would exhibit higher lipid-linked

mitochondrial respiration and enhanced expression of genes and pathways associated with the regulation of lipid metabolism when compared to the sedentary condition. We observed a higher mitochondrial ability to burn fat as fuel and the activation of molecular and cellular signaling pathways was more pronounced in muscle samples harvested after 4-days of physical activity performed as a daily single-continuous bout than as multiple short bouts spread throughout the day. Contrary to our hypothesis, mitochondrial respiratory states and coupling did not differ across groups with carbohydrate-associated substrates. Functional adaptations in mitochondrial oxidative capacity following changes in gene expression may require more time to manifest.

Introduction

Prolonged sedentary behaviors (SB), defined as “any waking behavior characterized by an energy expenditure ≤ 1.5 metabolic equivalents while in a sitting, reclining, or lying posture” [37], are detrimental for health. It has been negatively associated with common risk factors for cardiometabolic disease such as waist circumference, body mass index (BMI, kg/m2), 2 h postprandial glycemia, and fasting plasma glucose, triglycerides (TG) and C-reactive protein. Conversely, frequently breaking up SB with short bouts of physical activity (PA) confers metabolic health benefits independent of total time spent in moderate to vigorous physical activity (MVPA) and total daily sitting time [56, 100]. It is now well established that acute (≤ 12 h) and short-term (≤ 4 days) exposures to active breaks reduce postprandial glucose and insulin concentrations compared to a sitting control in adults who are lean, with obesity and type 2 diabetes [61-65, 111-114].

To investigate the independent effects of the active breaks, the metabolic response of short-frequent PA bouts performed throughout the day have been compared to a time-matched single-continuous bout. Those studies have highlighted that for same total active time and/or energy expenditure, differential metabolic effects are elicited by these different PA modalities. Short-frequent bouts of PA decrease postprandial plasma glucose and insulin concentrations, while a single-continuous bout of PA reduces postprandial TG levels, both compared to prolonged sedentary periods [64, 113]. In a randomized cross-over trial, we further showed that breaking up SB with short-frequent bouts of moderate intensity walking increases 24 h carbohydrate (CHO) oxidation, while a single-continuous bout of moderate intensity walking increases 24 h fatty acid (FA) oxidation in physically inactive adults with overweight or obesity [196]. However, the mechanisms underlying these differential metabolic effects are poorly known [87].

So far, only two human clinical trials have investigated some of the underlying mechanisms in skeletal muscle, which is a critical site for glucose disposal and nutrient metabolism both at rest

and during PA [197]. Acute light- and moderate intensity walking breaks (2 min bouts every 20 min for 5 h) have been associated with differentially ex-pressed genes involved in CHO and lipid metabolism (pyruvate dehydrogenase kinase 4 [PDK4] and nicotinamide N-methyltransferase [NNMT], respectively) as well as in cellular development, growth, and proliferation (myogenic factor 6 [MYF6], epithelial mem-brane protein 1 [EMP1], and dynein, light chain, LC8-type 1, transcript variant 1 [DYNLL1]) in adults with overweight or obesity [121]. Performing frequent active breaks for 3 days (2 min bouts every 20 min for 6 h/day) was shown to activate both non-oxidative and oxidative glucose uptake via insulin-independent and dependent pathways [120], which may optimize insulin action and both glucose oxidation and storage [195]. It remains unclear whether these molecular adaptations align with, or are different from, those triggered by a time-matched single-continuous bout of PA, and whether they can explain the differential metabolic responses observed at the whole-body level [198].

In this ancillary study, we tested the hypothesis that 4 days of breaking up SB with short-frequent bouts of PA would enhance mitochondrial respiration in the presence of CHO-associated substrates and skeletal muscle would increase expression profiles favoring CHO metabolism. Because prolonged PA is known to be associated with increases in whole-body fat oxidation rates, especially in post-absorptive conditions [199], we further hypothesized that after 4 days of a daily single-continuous bout of PA matched for total active time would result in greater lipid-linked mitochondrial respiration and expression of genes associated with the regulation of lipid metabolism. Our findings provide muscle-specific evidence that molecular and functional adaptations depend on the terms of PA, i.e., frequency, duration and/or volume, and could contribute to whole-body energetics to improve metabolic health outcomes.

Methods

Experimental Protocol

The detailed description of the study design and main outcomes have been published previously [196]. Following the screening visit, three interventions were completed by each participant in a randomized balanced order. Each intervention period lasted 4-days. The first 3-days in free-living conditions and the 4th day was in a whole-room calorimeter. All interventions were separated by a 28-day wash out period. Study procedures were performed during the same phase of the menstrual cycle in all women. All the visits were conducted at the Clinical and Translational Research Center of University of Colorado Hospital. This study was approved by the Colorado Multiple Institutional Review Board and listed at ClinicalTrials.gov: NCT02258438 posted 10-07-2014.

Participants

Study volunteers were recruited from October 2014 to October 2016. Inclusion criteria included: age 19 – 45 yr, BMI 27 – 33 kg/m^2, weight stable for ≥ 3 months, and being sedentary defined as sitting ≥ 6 h/day. Women were premenopausal but could use oral contraceptives. Exclusion criteria included clinically diagnosed diabetes, consumption of glucose- and/or lipid-lowering medication, dyslipidemia, smoking, or being physically active (> self-reported 150 min/wk MVPA).

Screening Visit

Written informed consent was obtained at the screening visit. Following verification of eligibility criteria, participants completed a treadmill walk test to determine a self-perceived moderate intensity walking pace (mph). Participants were asked to walk at a similar moderate intensity pace in free-living conditions and completed the same treadmill walking pace in the whole-room calorimeter.

Study Interventions

Participants were randomized to one of three possible trial-condition sequences using balanced blocks, separately prepared for male and female participants. The study statistician (Z.P.) prepared the computer-generated randomization assignments and placed them in sealed envelopes which were opened the day before the first intervention to reveal the order of study interventions.

Sedentary behaviors (SED): During the 3-day free-living period, participants were asked to refrain from structured exercise and maintain habitual levels of daily PA and SB. On day 4, participants remained sedentary in the whole-room calorimeter.

Sedentary + single-continuous bout of walking (ONE): During the 3-day free-living period, participants were instructed to perform 45-min of moderate-intensity walking once per day and to maintain habitual levels of daily PA and SB the remainder of the day. On day 4, participants performed one 45-min bout of moderate intensity walking on a treadmill at 10:00 AM but otherwise remained sedentary in the whole-room calorimeter.

Sedentary + microbouts of walking (MICRO): During the 3-day free-living period, participants were instructed to perform a 5-min bout of moderate intensity walking each hour for 9 consecutive hours and to maintain habitual levels of daily PA and SB the remainder of the day. On day 4, participants performed a 5-min bout of moderate-intensity treadmill walking every hour for 9 consecutive hours from 10:00AM to 6:00PM and remained sedentary the rest of the day.

Compliance was verified with objectively measured time spent sitting and lying, stepping and daily steps with ActivPAL™ PA monitors (PAL Technologies Ltd., Glasgow, Scotland) and PA intensity was confirmed with ActiGraph GT3X tri-axial accelerometer (ActiGraph, Pensacola, FL, USA). Data on time spent sedentary and daily patterns of PA have been previously published [181].

Standardized Diet

Each participant consumed a 3-day standardized diet leading up to the whole-room calorimeter study visit. The macronutrient content of the control diet was 30% fat, 55% CHO, and 15% protein of total energy intake. On day 4, the macronutrient composition was 20% fat, 65% CHO and 15% protein. Daily energy needs were calculated based on resting metabolic rate and fat free mass measured by dual energy X-ray absorptiometry (DXA, Hologic Delphi-W, Bedford, MA), as previously reported [196]. By design, participants received the same amount of food across the three interventions and as a result were in similar state of negative energy balance in the two PA conditions [196].

Skeletal Muscle Biopsy

Following an overnight fast, a *vastus lateralis* skeletal muscle biopsy was collected under local anesthesia (2% wt/vol lidocaine HCl) using the Bergstrom biopsy needle technique on the morning of day 5. From the total biopsy sample, ~10-20 mg was placed immediately in ice cold BIOPS solution (10mM Ca–EGTA buffer, 0.1M free calcium, 20mM imidazole, 20mM taurine, 50mM potassium 2-[N-morpholino]-ethanesulfonic acid, 0.5mM dithiothreitol, 6.56mM MgCl2, 5.77mM ATP, and 15mM phosphocreatine [PCr], pH 7.1) and ~40-60 mg was snap frozen in liquid nitrogen and stored at -80^{o}C for future analysis.

Preparation of Permeabilized Muscle Fiber Bundles

Muscle fiber bundles were gently teased apart in a petri dish containing ice cold BIOPS solution with fine-nosed forceps and a dissecting microscope. Approximately 2-5 mg fiber bundles were then permeabilized in saponin (5 mg/ml) for 30 minutes at 4°C and then washed once for 10 minutes at 4°C with Mir05 respiration medium (0.5mM EGTA, 3mM MgCl2·6H2O, 60mM K-lactobionate, 20mM taurine, 10mM KH2PO4, 20mM HEPES, 110mM sucrose, and 1g/L BSA, pH 7.1), all on an orbital shaker [200]. The permeabilized muscle fiber bundles were blotted, weighed,

and placed in the high-resolution respirometry (HRR) chambers of an Oxygraph 2K (Oroboros Inc., Innsbruck, Austria) for analysis.

Skeletal Muscle Mitochondrial Respiration Protocols

HRR of permeabilized muscle fibers offers an integrative *ex vivo* measure of the dynamics of coupled metabolic pathways [201]. Quantification of oxygen consumption in permeabilized fiber bundles was conducted at 37°C in the oxygen concentration above 200 nmol O_2/ml over a ~2 h period. Substrate-uncoupler-inhibitor titration (SUIT) protocols for CHO and FA respiration was performed following stabilization of the O_2 trace. The addition of Cytochrome C was used to confirm the integrity of the outer mitochondrial membrane and the quality control threshold was set at ≤ 10% increase in respiration. Steady state O_2 flux for each respiratory state was determined and normalized to dry fiber bundle weight using DatLab 5 software (Oroboros Inc.).

In the CHO SUIT, LEAK state without adenylates respiration (i.e., substrate driven non-phosphorylating inner membrane proton leak) was determined by the addition of saturating concentrations of pyruvate (2 M) and malate (0.4 M). State 3 coupled respiration supported by electron flux through complex I was measured with the addition of ADP (0.5 M). Oxidative phosphorylation (OXPHOS) was achieved by adding saturating concentrations glutamate (2 M) and succinate (1 M). The addition of oligomycin (5 mM) inhibited ATP synthase and titrations of the uncoupler FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone 0.1 mM) were performed to assess maximal electron transport system (ETS c1+c2) respiration. Lastly, complex I inhibitor rotenone (1 mM) was then added to measure the rate of respiration through complex II alone (ETS c2). Finishing, malonic acid (2 M) and antimycin A (5 mM) were added to inhibit complex II and III, respectively. With total electron transport chain respiration inhibition, residual oxygen consumption was then quantified to be a correction factor for all mitochondrial respiratory rates.

In the FA SUIT, LEAK state without adenylates respiration was measured by the addition of octanoylcarnitine (0.1 M) and malate (0.4 M). This was followed by ADP (0.5 mM) for State 3 coupled respiration and then pyruvate (2 M), glutamate (2 M) and succinate (1 M) for OXPHOS. The remainder of the FA SUIT protocol was the same as the CHO SUIT.

For both substrate specific SUIT protocols, the respiratory control ratio (RCR) was calculated (State 3/LEAK) to determine the degree of coupled respiration and the flux control ratio (FCR, each respiratory state normalized to maximal respiratory rate, i.e. ETS c1+c2) to determine oxygen flux in different respiratory control states.

RNA sequencing

Samples were sequenced as previously described [202, 203]. Briefly, frozen muscle samples were mechanically homogenized (~40-60 mg) in 1mL of TRIzol, and RNA was isolated according to manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). RNA samples were submitted to the University of Colorado Denver's Genomics and Microarray Core where quality was assessed with the Agilent 2100 Bioanalyzer both before and after library preparation, which used Nugen's Universal Plus mRNA-seq kit according to manufacturer's protocols (NuGEN, Redwood City, CA). Sequencing was performed with the NovaSEQ6000 Ilumina sequencing platform as 150 nucleotide paired end reads, sequenced at 80 million paired reads per sample. Samples were run in two batches. Generated reads were trimmed, mapped to the human genome (GRCh38) using gSNAP, fragments per kilobase of transcript per million mapped reads (FPKM) were calculated using Cufflinks, and R for discovery of differential gene expression [204, 205]. Genes were removed if their FPKM expression was less than 5 in either group of the statistical comparisons or if the difference in means was > 5. Raw mRNA sequencing data has been archived under the GEO accession number GSE186715.

Pathway Analysis

Canonical pathway and pathway comparison was conducted as previously described using IPA (Ingenuity Pathway Analysis, Qiagen, Hilden, Germany) [206]. Briefly, pathway analysis was performed using statistically significant differentially expressed genes with p-values of <0.05. Comparison analyses if the differentially expressed gene (DEG) lists between PA modes (ONE v SED, MICRO v SED, and MICRO v ONE) were used to calculate biological pathways and functions that were in common among DEG lists. The output lists were ordered by pathway enrichment significance (Benjamini-Hochberg false discovery rate [FDR]) and pathways were colored by predicted activation Z-score (red = positive, blue = negative, white = N/A).

Data and Statistical Analysis

One-way analysis of variance (ANOVA) was used to test for differences in participant characteristics between three subgroups: participants from the parent study (N=20) [196], participants included for Oroboros experiments to measure mitochondrial respiration in permeabilized muscle fibers (N=19) and participants for RNAseq analysis for molecular analysis (N=8). All initial respiratory states (LEAK, State 3, OXPHOS, ETS c1+c2 and ETS c2) that qualified for inclusion in data analysis (≤ 10% change in respiration after the addition of cytochrome C) were assessed with the ROUT method to identify and remove outliers from the data set [207]. Linear mixed models tested for between-condition differences in permeabilized muscle fiber respiratory states (LEAK, State 3, OXPHOS, ETS c1+c2 and ETS c2), and ratios (RCR and FCR) with intervention as repeated effect, sequence, period, and intervention as fixed effects and participants as random effects with variance components as covariance structure. A $P \leq 0.1$ was considered significant for model interactions. The least significant difference post-hoc test was used to examine between-condition differences, a $P \leq 0.05$ was considered significant. Differentially expressed genes were analyzed with unpaired t-test between (SED vs. MICRO, SED vs. ONE, and ONE vs. MICRO) followed by false discovery rate (FDR) which was considered significant ≤ 0.05 by the Benjamini and Hochberg multiple testing correction.

Spearman correlational analysis stratified by intervention was performed on mitochondrial oxygen consumption dependent variables with previously published whole-body metabolic outcomes as independent variables [196]. Linear mixed effect model and between-group comparison of correlations were fit to the dependent variables for the three interventions simultaneously while accounting for the correlation of repeated observations from the same participants. $P \leq 0.1$ was considered significant for model interactions and $P \leq 0.05$ was considered significant for between-group differences in correlation. Data are provided as mean ± SD, unless specified otherwise.

Results

Participant Characteristics

As previously reported [196], twenty participants (n=10 males and 10 females) completed all study related procedures, mean age was 32.4 ± 6.3 yr and BMI 30.6 ± 2.9 kg/m^2. A subsample of participants had fasting muscle biopsies harvested for measurement of mitochondrial oxygen consumption in permeabilized muscle fibers (N=19, 9 males and 10 females) and a subsample for RNA sequencing and pathway analysis (N=8, 4 males and 4 females). No differences in the participant characteristics were noted between the two subsamples and the sample from the parent clinical trial (Table 5).

Table 5: Participant characteristics

	Muscle mitochondrial respiration (Oroboros)	Muscle molecular adaptations (RNA seq)
N (#)	19 (9m/10f)	8 (4m/4f)
Age (years)	32.2 ± 6.4	34.3 ± 8.4
BMI (kg/m^2)	30.3 ± 3.0	29.9 ± 2.4
Body Mass (kg)	85.2 ± 10.9	84.8 ± 11.6
FFM (kg)	55.2 ± 10.6	55.9 ± 11.0
FM (kg)	30.0 ± 7.1	28.9 ± 8.3
Fat mass (%)	35.3 ± 7.7	34.2 ± 8.8
Fasting Glucose (mg/dL)	87.8 ± 4.6	86.6 ± 4.8
Fasting Triglyceride (mg/dL)	102.7 ± 52.8	115.9 ± 67.5
HOMA-IR (SED day fasting)	1.4 ± 0.7	1.2 ± 0.6
IPAQ self-reported sitting time (h/day)	10.6 ± 3.3	8.5 ± 3.0

Data are presented as mean ± SD. N, number of participants; BMI, body mass index; kg, kilograms; FFM, fat-free mass; FM, fat mass; HOMA-IR, homeostatic model assessment of insulin resistance was calculated from the blood draw collected on the morning of day 4 during the habitual sedentary behaviors (SED) intervention; IPAQ, international physical activity questionnaire.

Mitochondrial capacity for fat oxidation is enhanced by a single continuous bout of PA, but not by multiple short bouts of PA

In the FA SUIT, no between-intervention differences in mitochondrial respiratory states were observed (Figure 17A), but RCR (i.e., degree of mitochondrial coupling) differed across interventions (intervention effect: P=0.008). In the presence of FA-associated substrates (Oct + M + ADP; Figure 17E), the RCR was higher after completing 4-days of ONE (4.8 ± 2.5) compared to both SED (2.3 ± 1.0, P=0.002) and MICRO (3.1 ± 1.1, P=0.024). The higher coupling in the presence of FA-associated substrates was likely driven by a decrease in LEAK respiration. LEAK FCR (i.e., contribution of mitochondrial respiratory state to overall flux; Figure 17C) differed across

the three interventions (intervention effect: P = 0.020); it was lower in ONE (0.035 ± 0.008) compared to SED (0.075 ± 0.010, P=0.006), but not to MICRO (0.052 ± 0.005, P=0.21). In the CHO SUIT, there were no between-intervention differences observed for mitochondrial respiratory states (Figure 17B), FCR (Figure 17D), and RCR (Figure 17F).

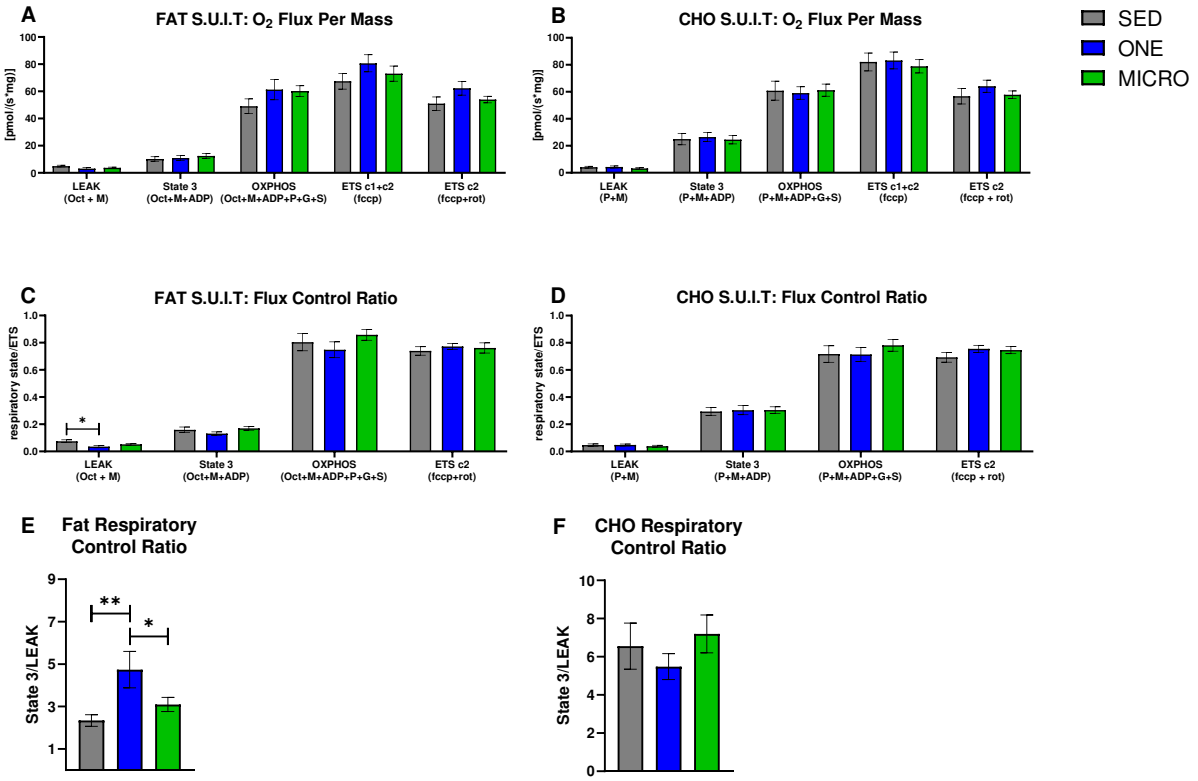

Figure 17: Carbohydrate- and fat-associated mitochondrial respiratory states, flux control and respiratory control in permeabilized skeletal muscles fibers. Substrate–uncoupler–inhibitor titration protocols associated with carbohydrate (CHO) and fat mitochondrial substrates were used for the evaluation of mitochondrial respiratory states, flux control ratio and respiratory control in permeabilized skeletal muscle fibers collected in the fasting state from adults with overweight-to-obesity adults (n = 19, 9m/10f) after completing three 4-day study interventions: SED – sedentary control, ONE – single-continuous bout of 45-min/day of moderate-intensity physical activity, MICRO – hourly 5-min bouts of moderate intensity physical activity performed for 9 consecutive hours per day. **(A)** Fat-associated mitochondrial respiratory states: LEAK: octanol (Oct) + malate (M); State 3: Oct + M + adenosine diphosphate (ADP); Oxidative Phosphorylation (OXPHOS): Oct + M + ADP + Pyruvate (P) + Glutamate (G) + succinate (S); Electron transport system (ETS) through complex 1 and 2 (ETS c1+c2): fccp (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone); ETS through complex 2 (ETS c2): fccp + rotenone (rot). **(B)** CHO-associated mitochondrial respiratory states: LEAK: P + M; State 3: P + M + ADP; OXPHOS: P + M + ADP + G + S; ETS c1 & c2: fccp; ETS c2: fccp + rot. The flux control ratio was calculated for each respiratory state by normalizing the respiratory state to ETS c1 &c2 which quantifies the contribution of each respiratory state to overall electron flux independent of mitochondrial content, mitochondrial preparations and experiment specific assay conditions. **(C)** Fat-associated FCR.

(D) CHO-associated FCR. The respiratory control ratio (RCR) quantifies the degree of coupled respiration by State 3/LEAK. **(E)** Fat-associated RCR. **(F)** CHO-associated RCR. Data are mean ± SEM. Significant between-interventions * $P < 0.05$, ** $P < 0.01$.

Correlations between skeletal muscle mitochondrial oxidative capacity and whole-body metabolic outcomes

Mitochondrial coupling (i.e., RCR) in the presence of lipid-associated substrates was positively associated with fasting insulin concentrations in both PA interventions (slope ONE = 0.65, P = 0.0008 and slope MICRO = 0.24, P = 0.02; Table 6). The slopes of these associations were significantly different from SED (slope ONE vs. SED: P = 0.015 and slope MICRO vs SED: P = 0.05). Furthermore, ONE and MICRO differed (P = 0.03), the association was stronger in ONE than in MICRO. Similarly, mitochondrial coupling in the presence of lipid-associated substrates was positively associated with fasting insulin/glucose, an index of insulin sensitivity, in both PA interventions (slope ONE = 58.11, P = 0.001 and MICRO = 23.24, P = 0.012). The slope for ONE was significantly different from SED (slope ONE vs. SED: P = 0.002) and the slope for MICRO tended to differ from the one observed in SED (slope MICRO vs SED: P = 0.07). Sleeping CHO oxidation (i.e., proxy of fasting CHO oxidation) was negatively correlated with CHO-supported LEAK respiration (slope = -36.06, P = 0.03) but not with OXPHOS and ETS c1+c2 (P for interaction: OXPHOS P = 0.401 and ETS c1+c2 P = 0.194). Sleeping fat oxidation (i.e. proxy of fasting fat oxidation) was not correlated with fat-supported LEAK, OXPHOS, and ETS c1+c2 (P for interaction P = 0.919, P = 0.357, and P = 0.115, respectively).

Table 6: Relationship between mitochondrial oxidative capacity and whole-body metabolic measures

Dependent Variable	Independent Variable	Intervention	Slope Estimate	P-value	P for interaction
RCR fat	Fasting Insulin	SED	0.08	0.96	0.003 **
		ONE	0.65	0.008 ##	
		MICRO	0.24	0.02 #	
		SED - ONE	-0.64	0.015 #	
		SED - BREAK	-0.24	0.05 #	
		ONE - BREAK	0.40	0.03 #	
RCR fat	Insulin/Glucose	SED	4.27	0.58	0.005 **
		ONE	58.11	0.001 ##	
		MICRO	23.24	0.012 #	
		SED - ONE	-53.84	0.002 ##	
		SED - BREAK	-18.97	0.07	
		ONE - BREAK	34.87	0.03 #	
CHO LEAK	Sleeping CHO (g/min)	SED	-8.63	0.56	0.077 *
		ONE	27.24	0.17	
		MICRO	-36.06	0.03 #	
		SED - ONE	-35.87	0.11	
		SED - BREAK	27.45	0.31	
		ONE - BREAK	63.32	0.03 #	
CHO OXPHOS	Sleeping CHO (g/min)	SED	42.22	0.76	0.401
		ONE	-97.52	0.55	
		MICRO	-320.65	0.15	
		SED - ONE	139.74	0.53	
		SED - BREAK	362.86	0.18	
		ONE - BREAK	223.13	0.42	
CHO ETS c1+c2	Sleeping CHO (g/min)	SED	-36.61	0.78	0.194
		ONE	229.91	0.15	
		MICRO	-200.32	0.33	
		SED - ONE	-266.53	0.18	
		SED - BREAK	163.71	0.49	
		ONE - BREAK	430.24	0.09	
FAT LEAK	Sleeping FAT (g/min)	SED	-2.47	0.94	0.919

		ONE	-8.59	0.81	
		MICRO	16.99	0.75	
		SED - ONE	6.11	0.89	
		SED - BREAK	-19.47	0.75	
		ONE - BREAK	-25.59	0.68	
FAT OXPHOS	Sleeping FAT (g/min)	SED	-105.42	0.71	0.357
		ONE	-460.21	0.15	
		MICRO	344.18	0.46	
		SED - ONE	354.79	0.41	
		SED - BREAK	-449.60	0.41	
		ONE - BREAK	-804.40	0.16	
FAT ETS c1+c2	Sleeping FAT (g/min)	SED	-61.82	0.85	0.115
		ONE	-170.15	0.57	
		MICRO	936.09	0.04	
		SED - ONE	108.33	0.80	
		SED - BREAK	-997.91	0.08	
		ONE - BREAK	-1106.24	0.04	

Spearman correlational analysis between fasting insulin concentration and mitochondrial coupling in the presence of lipid-associated substrates stratified by intervention. A linear mixed-effect model was fit to the dependent variable for each trial simultaneously while accounting for the correlation of repeated observations from the same participant. Under the linear mixed-effect model, slopes of the dependent variable vs. independent variable were estimated and be-tween-group comparison of the slopes were conducted. The between-group difference in slope assesses the difference between the two groups with respect to the correlation. SED, sedentary control; ONE, single-continuous bout of 45 min/day of moderate-intensity physical activity; MICRO, hourly 5 min bouts of moderate intensity physical activity performed for 9 consecutive hours per day; g/min, gram per minute substrate oxidation. Significant model interaction: * $p \leq 0.05$, ** $p \leq 0.01$. Significant between-intervention: # $p \leq 0.05$, ## $p \leq 0.01$.

Differential gene expression leads to similar pathway enrichment for MICRO and ONE with more biological pathways enriched in ONE

Differentially expressed genes (DEG) were calculated (FDR ≤ 0.05) relative to the SED condition, and 1,126 DEG were identified in the ONE and 678 DEG were identified in the MICRO (Figure 18A). About 14% of DEG were shared between ONE and MICRO (Figure 18B). Ingenuity Pathway Analysis software was used to evaluate biological pathways and functions (Figure 19),

providing a global view of the gene expression differences for muscle adaptations to PA mode. Significantly different gene lists were analyzed using the Comparison Analysis tool in IPA, and the pathway enrichment scores (sum of -log10(FDR)) were calculated as an indication of the proportion of DEGs that populate a given pathway. The fold changes of individual DEGs within a pathway were used to calculate whether a given pathway was predicted to be activated (red), inhibited (blue), or no prediction (white) [206]. In general, this pathway analysis revealed similar DEG enrichment and predicted activation states for ONE and MICRO compared to SED. For example, the oxidative phosphorylation pathway was predicted to be activated (SED vs ONE: $P < 0.0001$; SED vs MICRO: $P < 0.0001$) and the sirtuin signaling pathway was predicted to be inhibited (SED vs ONE: $P < 0.0001$; SED vs MICRO: $P < 0.0001$). Critical cellular energy and metabolism sensors had significantly enriched pathways following ONE ($P < 0.01$, parenthetical values) but not MICRO ($P > 0.05$) when compared to SED. For instance, the mTOR, ERK/MAPK, AMPK, Nrf2-mediated oxidative stress response, insulin receptor, calcium signaling, necroptosis, and HIF1α pathways were enriched following ONE but not MICRO. Comparing both PA interventions (MICRO vs ONE) several pathways were enriched including sirtuin signaling, oxidative phosphorylation, mTOR, ERK/MAPK, AMPK, and NRF2-mediated oxidative stress response pathways ($P < 0.01$ for all, parenthetical values). Oxidative phosphorylation pathway was predicted to be less activated following MICRO (MICRO vs ONE activation *z*-score: -3.357, $P < 0.001$). Additionally, while these pathways were not enriched, higher expression of calcium signaling and necroptosis signaling pathways were observed for MICRO ($P < 0.05$).

When comparing the significantly different genes modulated in the Oxidative Phosphorylation pathways for ONE or MICRO (relative to SED), more genes were similarly ex-pressed in common between ONE and MICRO (N = 19/45), while some were unique only to MICRO (N = 10/45) or ONE (N = 16/45) (Figure 4A). Several genes regulated in common between both PA trials included downregulation of the mitochondria trafficking inesin-binding protein 1 (TRAK1) and

apoptosis activator Bcl2-associated agonist of cell death (BCL2), mitochondrial ATP synthase subunit epsilon 5E (ATP5E), cytochrome c oxidase genes (COX6, −7A2 and −7B), and NADH dehydrogenase subcomplex subunits (NDUFA2, −4, −8, 11, and −13, NDUFB5, −9, NDUFS3, and NDUFV2), mitochondrial fission 1 protein (FIS1), and mitochondrial peroxiredoxin-5 (PDX5).

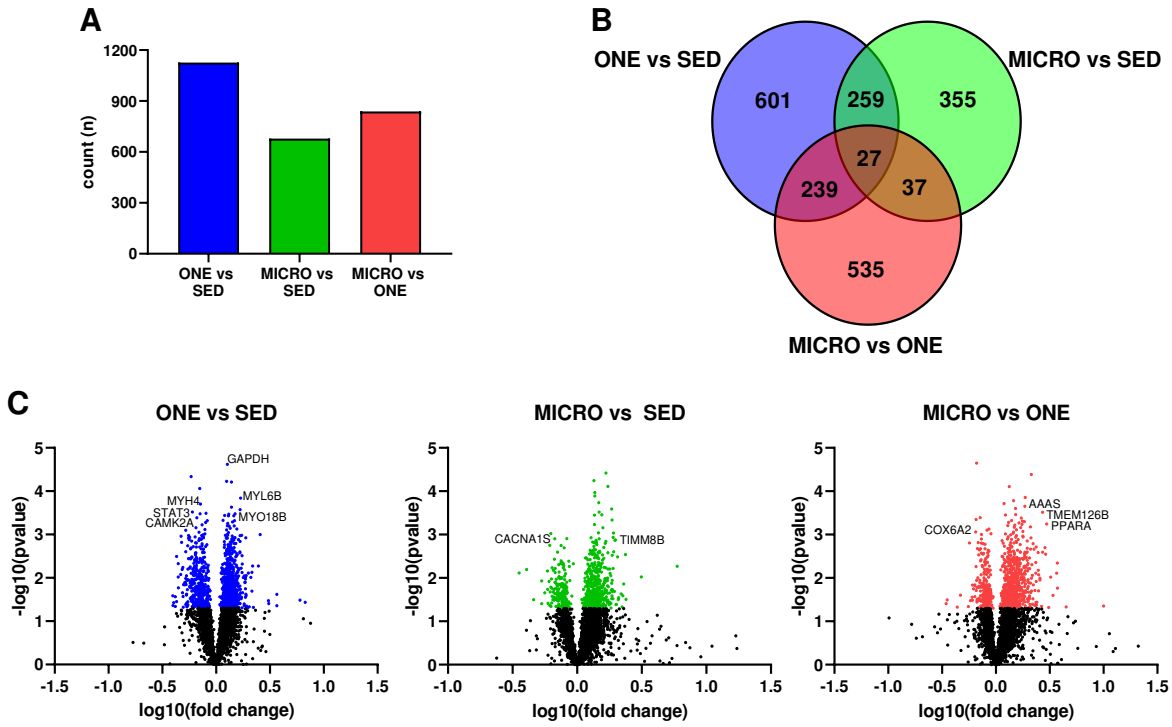

Figure 18: Comparison of muscle gene expression signatures in fasting skeletal muscle biopsies. (A) counts of differentially expressed genes by comparison, (B) Venn diagrams displaying the counts differentially expressed genes for each comparison, (C) Volcano plots of differentially expressed genes. Genes related to metabolic outcomes selected from top 30 indicated on plot. Skeletal muscle biopsies were collected in the fasting state from adults with overweight-to-obesity adults (n = 8, 4m/4f) after completing three 4-day study interventions: SED – sedentary control, ONE – single-continuous bout of 45-min/day of moderate-intensity physical activity, MICRO – hourly 5-min bouts of moderate intensity physical activity performed for 9 consecutive hours per day.

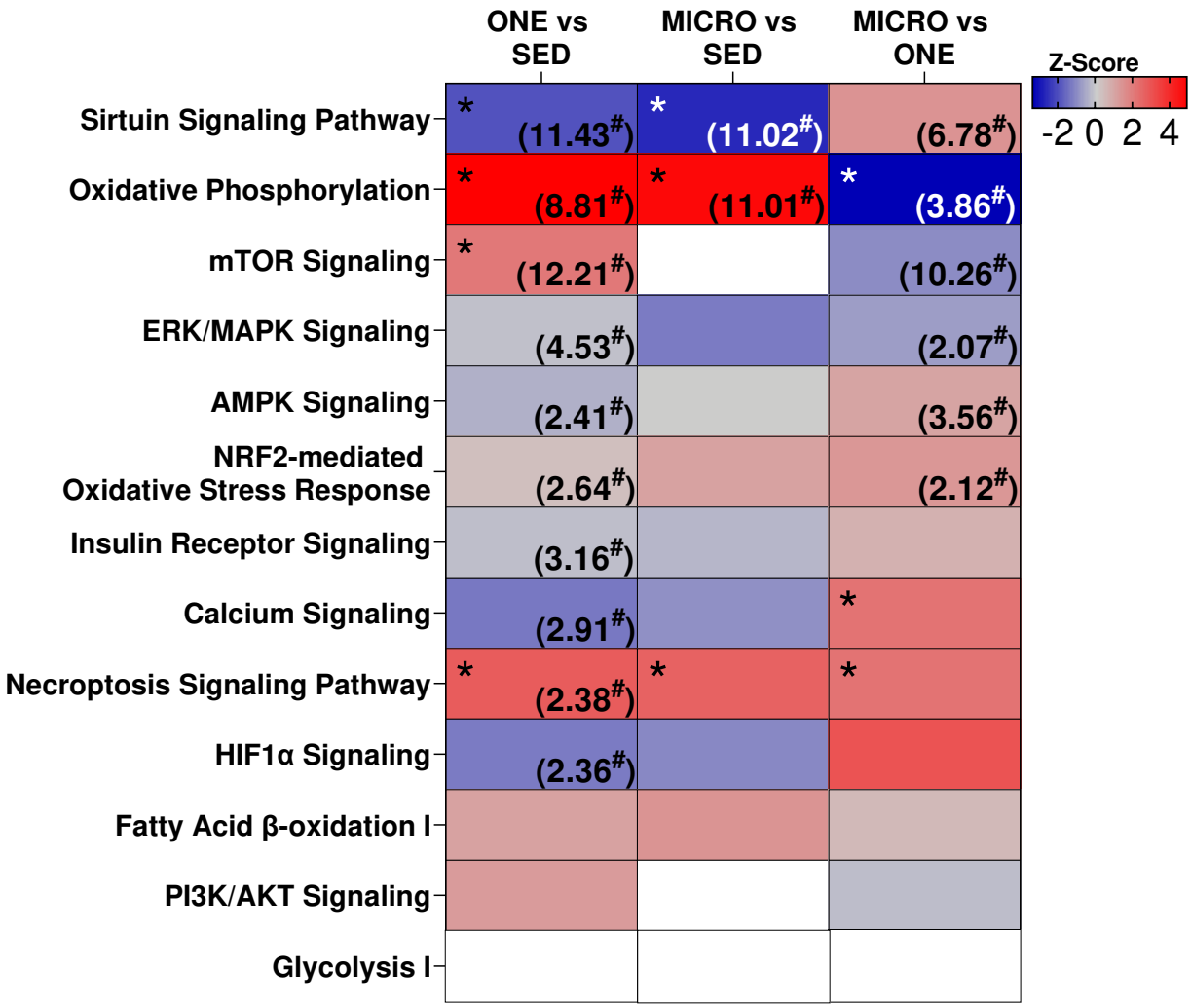

Figure 19: Comparison of metabolic and exercise-responsive pathways. Pathway enrichment was calculated -log10(FDR) for each comparison (value indicated in parentheses). For pathway ranking, total FDR score was calculated as the sum across the row for each FDR score. Pathway activation state colored by z-score (white = N/A, red = activated, blue = inhibited). A portion (n=8 – 4m/4f) of the skeletal muscle samples were analyzed by RNA sequencing to identify differentially expressed genes and pathway analysis was performed with Ingenuity Pathway Analysis (IPA, Qiagen Inc., Hilden Germany). Skeletal muscle biopsies were collected in the fasting state from adults with overweight-to-obesity adults (n = 8, 4m/4f) after completing three 4-day study interventions: SED – sedentary control, ONE – single-continuous bout of 45-min/day of moderate-intensity physical activity, MICRO – hourly 5-min bouts of moderate intensity physical activity performed for 9 consecutive hours per day. * significant z-score: $P < 0.05$. # significant pathway enrichment: $P < 0.05$.

The Sirtuin Signaling pathway, critical for sensing cellular energy status and metabolism [208], was also regulated following both ONE and MICRO. Significantly different genes that were regulated in common between ONE and MICRO included forkhead box protein O4 (FOXO4,

decreased), mitochondrial import inner membrane translocase subunits (TIMM8 increased, -23 increased and -50 increased, and PAM16 increased), and the mitochondrial cholesterol transport protein (TSPO increased) (Figure 20B). Significantly regulated genes in ONE included 5'-AMP-activated protein kinase catalytic subunit alpha-1 (PRKAA1, de-creased), superoxide dismutase 1 and 2 (increased SOD1 and decreased SOD2), lipogenic transcription factor sterol regulatory element-binding protein 1 (SREBF1, decreased), and inflammatory response signal transducer and activator of transcription 3 (STAT3, de-creased). Furthermore, ONE upregulated unique mitochondrial import inner membrane translocase subunits (TIMM13 and -17A), as well as mitochondrial import receptor subunits (TOMM6, -7, and 22) and several MAP kinase signal transduction genes (MAPK1, -3, and -12). In contrast to ONE, MICRO had unique regulation of mitochondrial isocitrate dehydrogenase 2 (IDH2, decreased), skeletal muscle autophagy regulators forkhead box protein O3 (FOXO3, decreased) and ubiquitin-like-conjugating enzyme (ATG3, increased), microtubule components (TUBA1A in-creased, TUBA1B increased, and TUBA8 increased), as well as complex V ATP synthase subunit gamma (ATP5C1 increased).

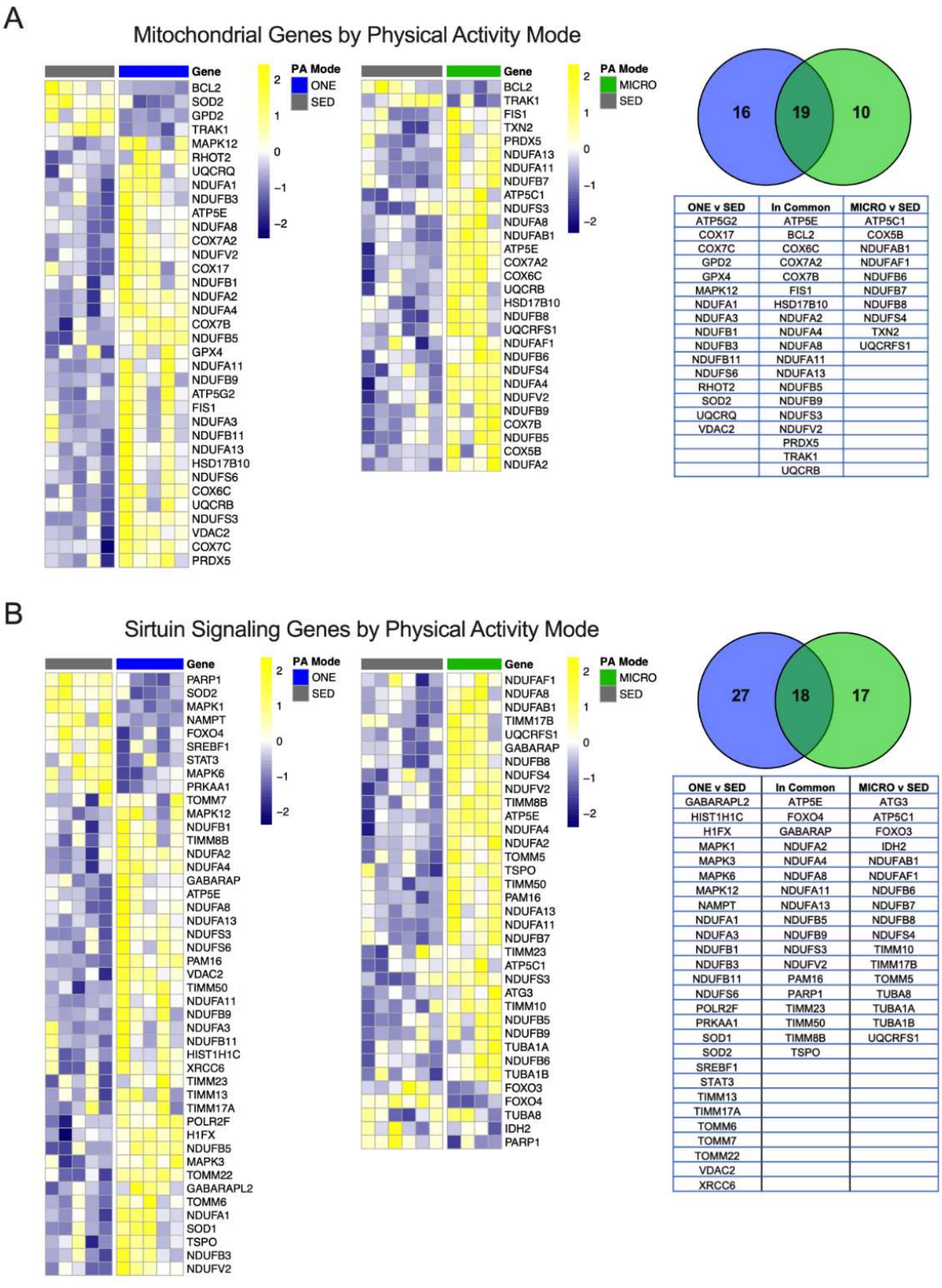

Figure 20. Physical activity mode induces mitochondrial and sirtuin signaling genes unique to the mode. Significantly different genes between either ONE vs SED or MICRO vs SED belonging to the mitochondria **(A)** or the Sirtuin Signaling pathway **(B)** were plotted by

heatmap. In the mitochondria, 19 genes were in common, while 18 genes were in common between PA modes. Several NADH dehydrogenase subcomplex subunits (NDUF), cytochrome c oxidase (COX), and ATP synthase subunits were regulated specifically by a particular mode of PA. FOXO family member 4 was downregulated by both PA modes and may be an important regulatory transcription factor responding to acute PA in human skeletal muscle. All genes presented are significant following multiple testing correction (FDR $p \leq 0.05$).

Discussion

In this randomized cross-over study, we compared the functional and molecular adaptations in skeletal muscle biopsies to short-frequent bouts of PA spread throughout the day to a time-matched single-continuous bout of PA, both compared to sedentary control conditions, in adults with overweight or obesity. We identified changes in the regulation of gene expression and mitochondrial functional capacity that are in common to both PA interventions and others that are unique to either ONE or MICRO. This indicates that biological pathways in skeletal muscle differentially respond to the terms of PA, i.e., volume, frequency, and/or duration of the bouts.

Similar enrichment and activation of two key biological pathways known to regulate substrate oxidation, the Oxidative Phosphorylation pathway (including electron transport chain components) and the Sirtuin Signaling pathway, were observed after ONE and MICRO, suggesting that adaptations within these pathways depend on total daily active time rather than bout frequency and/or duration. These changes were observed 14 h after the last microbout of PA and 21 h after the single-continuous bout of PA, indicating a sustained response beyond the acute period. A potential regulator of muscle adaptation to acute forms of PA is FOXO4, which was downregulated following both ONE and MICRO. FOXO4 and family members are transcription factors known to abolish the effects of insulin and IGF-1 [209], potentially supporting improved insulin sensitivity in skeletal muscle following PA. This is in line with the improved insulin sensitivity noted at the whole-body level after both ONE and MICRO [196] [7].

Contrary to our hypothesis, we observed no functional adaptations in mitochondrial oxidative capacity in the presence of CHO-associated substrates following both PA interventions. This suggests that in the short-term, short-frequent bouts of PA can trigger changes in the regulation of gene expression in pathways associated with the regulation of metabolism, although these changes do not translate into higher mitochondrial function. This further suggests that mechanisms other than adaptations in mitochondrial oxidative capacity may contribute to the increase in 24 h CHO oxidation we observed previously in MICRO [196]. Because short-frequent bouts of brisk walking were completed throughout the day, participants were in the postprandial state. Therefore, the greater oxidative rates of CHO may be the result of CHO availability to provide energy for movement. Of note, substrate-driven LEAK respiratory state in the presence of CHO-associated substrate was negatively correlated with sleeping CHO oxidation (an index of fasting CHO oxidation) and no associations were observed for OXPHOS and ETS mitochondrial respiratory states. A potential early mechanism for adaptations in mitochondrial function may be the decrease in substrate-driven LEAK respiration through a more robust electron transport chain in which more protons are used for ATP production. Potential mitochondrial adaptations to MICRO may therefore take more time to develop. Longer-term studies will be needed to test if short-frequent bouts of PA can ultimately enhance overall skeletal muscle mitochondrial function.

We found that ONE, but not MICRO, resulted in a higher RCR when permeabilized muscle fibers were provided lipid-associated substrates, suggesting an improved coupling efficiency. The observed higher RCR in the presence of lipid-associated substrates may be due to a decrease in LEAK state FCR, which indicates a reduced contribution of the LEAK respiratory state to overall electron flux and improved coupling efficiency because fewer protons are lost to non-coupled respiration. Besides one study testing the effect of an acute 60 min bout of PA in 21 healthy untrained male participants [210], the evidence for acute and short-term adaptions in mitochondrial oxidative capacity are to our knowledge limited. Therefore, it is interesting to note

that adaptations can be detected after 4 days of PA. In addition, this improved mitochondrial oxidative capacity for lipids, along with the molecular adaptations favoring substrate oxidation, may contribute to the higher 24 h FA oxidation we observed previously in response to a single-continuous bout intervention [196].

The daily single-continuous bout of intervention also involved more biological pathways that were enriched with DEGs than the short-frequent PA bouts intervention (Figure 3). These include the AMPK Signaling pathway, which is sensitive to changes in ATP turnover, the Calcium Signaling pathway, which is sensitive to oscillations in calcium concentrations from skeletal muscle contractile function, and the HIF1α Signaling pathway, which is sensitive to the intracellular partial pressure of oxygen [197]. Additionally, pathways associated with skeletal muscle nutrient uptake, growth, differentiation, survival, and the oxidative stress response were enriched after the daily single-continuous bout of PA intervention, including the Insulin Receptor Signaling, mTOR, ERK/MAPK Signaling, and NRF2-mediated Oxidative Stress Response pathways. This constellation of enriched pathways after the single-continuous PA bout intervention is known to be associated with PGC-1α [211-214], a master regulator of mitochondrial biogenesis [215], which may explain why we observed higher mitochondrial coupling after ONE. Of note, these results are confounded by an energy deficit, as energy expended during PA was not re-placed and future studies will need to confirm if similar results are observed under stable energy balance.

The enrichment of several PA-associated transcriptional response pathways may be due to the sustained nature of the single-continuous PA bout intervention more than total active time. The longer bout length may create a greater challenge to skeletal muscle homeostasis, therefore eliciting a greater adaptive stress response. This might be particularly true for the Oxidative Phosphorylation pathway because, when comparing both PA interventions together, this pathway was predicted to be less expressed in the short-frequent bouts intervention. However, the Calcium

Signaling pathway was predicted to be positively expressed following the exposure to frequent active breaks, suggesting that the frequency rather than the length of the PA bouts may impact this pathway. Sarcolemma de-polarization during muscle contractions increases cytosolic Ca++ thereby activating the Calcium Signaling pathway. The frequent nature of the PA bouts may trigger waves of cytosolic Ca++ release that lead to increased expression of the Calcium Signaling pathway, which has been shown to be associated with mitochondrial biogenesis [215].

A strength of the study was the randomized cross-over design of the interventions; each participant served as their own control and all females were studied in the same phase of their menstrual cycle. We were able to compare the respective effects of interrupting sedentary time from increased energy expenditure and/or PA by including a time-matched control. Compliance with each intervention period was verified with PA monitors. The results of this ancillary study should also be considered along with several limitations. Muscle biopsies were harvested at two different time intervals after the last bout of PA (21 h post the single-continuous PA bout in ONE and 14 h after the last bout of the short-frequent PA bouts in MICRO). The current experiments quantified mitochondrial respiratory capacity and not the actual respiration directly following completion of each 4-day PA trial. Additionally, the sample size for RNA sequencing was small and the RNA sequencing results were not confirmed with RT-quantitative PCR due to limitations of muscle tissue availability.

Conclusion

Breaking up SB with short-frequent bouts of PA spread throughout the day over the short-term (4 days) is a sufficient stimulus to promote changes in the regulation of skeletal muscle gene expression in pathways associated with substrate oxidation. These changes were similar to those elicited by a time-matched daily single-continuous bout of PA, suggesting that total daily active

time and/or energy expenditure is a primary trigger for those pathways. However, these molecular changes translated into an improved capacity for mitochondrial fat oxidation and were associated with higher expression in muscle con-traction transcription signaling pathways only when PA was performed as a single-continuous bout. In contrast, the Calcium Signaling pathway was only activated when PA was completed as multiple short-frequent bouts. Therefore, this study showed for the first time that biological pathways in skeletal muscle are differentially regulated by the terms of PA, i.e., total active time, bout length, and bout frequency. Future studies will need to determine whether these differential responses are sustained over time and translate into differential functional and whole-body changes.

DISCUSSION

SUMMARY

Despite the well-known health benefits of exercise, most adults do not meet the physical activity recommendations, and the prevalence of metabolic diseases continues to rise. Traditionally, physical activity is typically prescribed as a single-continuous bout of 30-60 min per day. The newly released physical guidelines no longer require physical activity to be in 10-min bouts and suggest reducing sedentary behaviors. This is important because epidemiologic and experimental evidence suggest that breaking up sedentary behaviors with short-frequent bouts of physical activity acutely lowered glycemia, even in individuals who exercise regularly [56, 63, 64, 100]. Acute experimental trials (5-12 hr exposure) demonstrate that breaking up sedentary time with short-frequent bouts of physical activity is associated with lower postprandial glucose and insulin concentrations while a time-matched single-continuous bout is associated with lower postprandial triglyceride concentrations in response to standardized meals [64]. This suggests differential substrate oxidation may be responsible for the changes in postprandial metabolites. However, critical gaps in knowledge remain including (1) whether breaking up sedentary behaviors with short-frequent bouts of physical activity is a strategy that can be implemented in the daily life of sedentary, physically inactive adults; (2) whether the acute metabolic benefits previously observed are sustained or diluted beyond the acute exposure period (> 5-12hrs); (3) whether the effects are due to the active breaks *per se* or to increases in total energy expenditure and/or total active time and (4) the characterization of potential underlying physiological, cellular, and molecular mechanisms.

In this dissertation, we compared in a cross-over randomized study the short-term metabolic effects of breaking up sedentary behaviors with short-frequent bouts of physical activity (MICRO: 5-min moderate-intensity walking bout every hour for 9 consecutive hours per day for 4-days, total

activity time 45-min/day) to those of a time- and energy-matched single-continuous bout of moderate intensity physical activity (ONE: 45-min continuous walking bout per day) and to a sedentary control condition (SED: maintain habitual sedentary behaviors) in overweight to obese, sedentary, physically inactive male and female adults. By matching the two active conditions for total active time, we aimed to separate the effects of an increase in physical activity and/or energy expenditure from those of the active breaks in sedentary time. Findings show that sedentary adults were able to implement MICRO into both work and non-workdays and felt less fatigue at the end of the day compared to days during which they performed a single continuous bout of brisk walking. Both physical activity interventions promoted in previously sedentary, physically inactive adults to reach the currently recommended levels of physical activity. At the same energy expenditure and balance, MICRO led to a greater reliance on postprandial and 24 hr total carbohydrate oxidation to provide energy to the body while ONE led to a greater reliance on lipid oxidation over the same time periods. Independent of substrate oxidation, both ONE and MICRO improved indexes of insulin sensitivity after 4 days. Although more biological pathways associated with muscle contraction-transcription signaling and a higher capacity for mitochondrial fat oxidation were observed in skeletal muscle after 4 days of ONE compared MICRO, both physical activity interventions enhanced expression of the Oxidative Phosphorylation and Sirtuin Signaling pathways. Altogether these findings showed that breaking-up sedentary behaviors with short, frequent bouts spread throughout the day is a promising strategy to interrupt prolonged sitting and increase daily physical activity and improves metabolic outcomes in people at risk for developing chronic metabolic diseases. It further suggests that by preferentially relying upon carbohydrate for fuel it may help with glucose control. Future studies will need to confirm this assumption and test the long-term metabolic health effects of the active breaks.

SHORT-FREQUENT BOUTS OF PHYSICAL ACTIVITY SPREAD THROUGHOUT THE DAY TO INCREASE PHYSICAL ACTIVITY AND DECREASE SEDENTARY BEHAVIORS

At least over the short-term, MICRO is a feasible strategy to promote physical activity in those who are at high risk for metabolic disease (overweight to obese, physically inactive, sedentary adults). Both time in moderate to vigorous physical activity and daily step count were similarly higher in both MICRO and ONE compared to SED. This low-cost intervention can be applied in both work and non-work contexts of daily life. At the end of the work day after MICRO, participants reported feeling less fatigue and more vigor and this may potentially benefit well-being and productivity at work. In an acute study (N = 30 sedentary healthy lean adults), breaking up sedentary behaviors over 6 hours (5-min walking bout ever hour) was associated with perceptions of more energy, improved mood, more vigor and less fatigue and did not negatively affect cognitive performance [153] (see Appendix for publication). Altogether these findings suggest that MICRO is a viable stratgy to implement in the work environment where office workers are particularly vulnerable to the adverse health effects of sedentary behaviors. Surprisingly, total sedentary time and number of prolonged sedentary bouts (sedentary bouts > 60-min) were not lower when completing MICRO nor were they in ONE in both work and nonwork contexts. Larger volumes of physical activity may be needed to significantly produce a shift from sedentary activities to physical activities. Previous research has however shown that encouraging breaking up sedentary behaviors throughout a work day and prescribing a daily step count goal resulted in lower total sedentary in adults with overweight to obesity [146]. Additionally, it seems that participants stay sedentary in between microbouts because on non-work days, time spent in light intensity physical activity was decreased in favor of more sedentary time. Potential spontaneous behavioral compensation may exist, and would need to be further studied to better tailor those physical activity prescriptions. Taken together, future public health messaging may need to specifically target both physical activity (i.e., daily steps) and sedentary behaviors (limit total sedentary behaviors and break up sedentary time) seperately to efficiently increase physical activity and reduce both total sedentary time and prolonghed sedentary bouts.

THE METABOLIC HEALTH EFFECTS OF TIME-MATCH PHYSICAL ACTIVITY TRIALS DIFFERENTIATED BY THE FREQUENCY OF ACTIVITY BOUTS

The greater use of carbohydrate during MICRO may explain the reduced postprandial glycemia repeatedly observed in response to acute and short-term exposure to physical activity breaks [62-64, 114] and the higher lipid oxidation may explain the reduced postprandial triglycerides in the same acute study [64]. The differential fuel oxidation profile between MICRO and ONE does not appear to be related to energy expenditure and balance but instead to the increase in the frequency of physical activity spread across the day. This supports the observations from population health studies showing a beneficial association between breaks in sedentary time and cardiometabolic healh risk factors such as waist circumference, body mass index, fasting blood glucose, cholesterol concentrations, and C-reactive protein concentrations, and 2 hr postprandial glucose concentrations [56, 100, 216]. The frequency of the physical activity bouts spread across the day may have enhanced carbohydrate oxidation when breaking up sedentary behaviors with short-frequent bouts of physical activity because glucose was constantly available in the postprandial state while microbouts were performed. Previously, an acute laboratory study exposed physically inactive healthy weight adults (N = 70) to prolonged sitting for 9 hr and two time-matched physical activity conditions to break up sitting time with a single-coninuous bout of 30-min treadmill walking and short-frequent bouts of treadmill walking (1:40 min active bout every 30-min). Respiratory gases were measured by face mask indirect calorimetry at regular hourly intervals throughout all conditions and showed that the average respiratory exchange ratio during regular physical activity breaks trial was higher (0.93 ± 0.02) compared to a time-matched single continous bout condition (0.90 ± .02, $P < 0.025$) and a sedentary control (0.90 ± .02, $P < 0.033$) [64]. While correlational analysis was not run in the previous study, the higher respiratory exchange ratio (indicating a higher relative contribution of carbohydrate over fat to substrate oxidation) may be responsible for the lower postprandial

glucose and insulin concentrations oberserved with regular activity breaks to break up sedentary time. In the current study, we were able to extend these results by showing a higher postprandial and 24 hr total carbohydrate oxidation (grams/day) by respiratory gases measured by whole-room indirect calormetry. While the relative contribution of carbohydrate to energy expenditure over 24 hr was not higher for MICRO compared to ONE and SED, the relative contribution of carbohydrate to waking energy expenditure was significantly higher in MICRO (65.9 ± 2.5%) compared to ONE (60.1 ± 1.8%, $P < 0.05$) which suggests the higher carbohydrate oxidation observed over 24 hrs was mainly driven by changes in wake time substrate oxation. Therefore, the higher carbohydrate oxidation observed with breaking up sedentary behaviors with short-frequent bouts of physical activity spread across the day may be lead to higher postprandial glucose clearance, and thus to postprandial glycemia reduction.

The dose-response relationship between frequency of active bouts and carbohydrate oxidation is unclear. Previously, respiratory gases were collected during the last 2 hours of an 8 hr cross-over experimental trial comparing uninterrupted sitting (SIT) to sitting interupted by treadmill walking matched for physical activity time but bouts at different frequencies: sitting interrupted by 2 min of walking every 20 minutes (INT20), sitting interrupted by 6 min of walking every hour (INT60), and sitting interrupted by 12 min of walking every second hour (INT120) in 14 sedentary obese men [217]. Compared to uninteruppted sitting all physical activity trials were associated with higher total carbohydrate oxidation with no change in fat oxidation compared to a sedentary control (difference from SIT carbohydrate oxidation: INT20: +189.9 ± 68.1 kcal, INT60: +215.1 ± 67.6 kcal, INT120: +212.2 ± 67.7 kcal, $P < 0.05$ for all). This suggests that while multiple bouts of activity promotes carbohydrate oxidation, the frequency of those bouts do not influence the oxidative rates. In this dissertation, we did not include an experimental trial that changed the frequency of the short-frequent bouts of physical activity but we did show breaking up sedentary behaviors compared to an isocaloric single-continuous bout was associated with

higher carbohydrate oxidation. Taken together, irrespective of the frequency of physical activity bouts, simply just breaking up sedentary behaviors with short-frequent bouts of physical activity is associated with higher carbohydrate oxidation to meet daily energy demands. Future studies should investigate how many daily breaks (or active bouts) are required to stimulate carbohydrate oxidation and thus help control glucose homeostasis. This will be important to be studied in people with normal glycemia but also in those with glucose intolerance.

Both physical activity interventions improved indexes of insulin sensitivity but through a differential metabolic response to varying frequencies of activity bouts and no change in postprandial glycemia. A possible explanation why we observed no change is postprandial glucose concentrations during the active period but did observe lower insulin concentrations in response to standardized meals is that our study sample did not include participants with impaired glucose tolerance or insulin resistance contrary to most previous studies [62, 63, 114]. The influence of breaking up sedentary behaviors with short-frequent bouts of physical activity spread across the day on glucose metabolism may depend on the degree of underlying metabolic impairement of the population being studied [58, 218, 219]. Experimental trials conducted in normal weight, normoglycemic individuals tend to report reductions in postprandial insulin concetrations when breaking up sedentary behaviors with short-frequent bouts of physical activity [64, 66]. Yet, reductions in postprandial glucose concetrations have been inconsistent [64, 73, 220]. Individuals who have some degree of metabolic impairement (overweight to obese, impaired glucose tolerance, type 2 diabetes) exhibit a greater magnitude of reduction in postprandial glucose concentrations [62, 63, 103, 114]. This hypothesis is suported by a pooled analysis from 3 randomized cross-over laboratory-based trials (n = 62) that examined the postprandial glucose- and insulin-lowering effects of sitting interrupted by regular brief activity breaks vs. prolonged sitting in overweight to obese adults who exhibited normal, impaired glucose metabolism or type 2 diabetes not treated by insulin [58]. The magnitude of reduction in postprandial glucose and

insulin responses between prolonged sitting and sitting interrupted by regular brief activity breaks was driven by baseline levels of fasting glucose, insulin and/or surrogate markers of β-cell function and insulin resistance. This suggests that those with elevated underlying levels of insulin resistance may gain greater benefits on glucose control from regularly interrupting prolonged sitting than their healthier counterparts. Therefore, breaking up sedentary behaviors with short-frequent bouts of physical activity may reduce the amount of insulin required to maintain normal glycemia in metabolically healthy individuals and for those with varying degress of insulin resistance, breaking up sedentary time with physical activity bouts may derive a greater benefit by improving glucose clearance as well as insulin sensitivity.

While MICRO induced similar expression of 2 pathways known to regulate substrate oxidation 14 hours after the last microbout, the short nature of our intervention may not have provided enough time for the observed adaptations in gene expression to fully develop into enhanced mitochondrial function. Pathways associated with carbohydrate metabolism were not expressed after MICRO and mitochondrial oxidative capacity in the presence of carbohydrate substrate was not altered. The increase in postprandial and 24 hr substrate oxidation associated with MICRO is likely related to carbohydrate availbility during the postprandial period rather than adaptations in mitochondrial oxidative capacity. Even though the sustained greater expression of the Oxidative Phosphorylation pathway (including the electron transport chain) did not lead to functional adaptations on mitochondrial oxidative capacity the LEAK respiratory state in the presence of CHO-associated substrate was negatively correlated with sleeping CHO oxidation, an index of fasting CHO oxidation, while no associations were observed for OXPHOS and ETS mitochondrial respiratory states. An early mechanism for adaptations in mitochondrial function may be the decrease in LEAK respiration through a more robust electron transport chain in which more protons are used for ATP production. We did not quantify protein expression, so we don't know if the upregulation of the Oxidative Phosphorylation pathway translated to more electron transport

chain proteins. Future studies should exmaine if a longer exposure to short-frequent bouts of physical activity has the potential to translate to higher mitochondrial function.

Concerning improved indexes of insulin sensitivity, MICRO did not express more genes known to be associated with insulin receptor signaling but ONE did, indicating the sustained nature of the single-continuous may drive this adaptation. However, the Calcium Signaling pathway was predicted to be positively expressed in MICRO suggesting the frequent bouts of physical activity rather than bout length may impact this pathway. The frequent nature of the physical activity bouts and muscle contraction may trigger waves of cytosolic Ca++ release that lead to increased expression of the calcium signaling pathway which is associated with mitochondrial biogenesis [221]. It seems muscle-contraction glucose uptake may be the primary mechanism for consistently lower postprandial insulin concentrations in response to standardized meals and while breaking up sedentary time with short-frequent bouts of physical activity, especially in those at the healthy end of the metabolic spectrum [64, 65, 219]. Acute (5 h) and short-term (3-day) exposure to short-frequent physical activity bouts activates both non-oxidative and oxidative glucose uptake via insulin-independent and dependent pathways [120], which may optimize insulin action and both glucose oxidation and storage (Figure 21) [222]. Acute exercise activates molecular signals that can bypass defects in skeletal muscle insulin signaling, resulting in an insulin-independent increase in glucose uptake [223]. This pathway may be the driver behind lower postprandial insulin concentrations because less insulin is required for skeletal muscle glucose uptake. Furthermore, a single bout of aerobic exercise increases the effect of insulin on skeletal muscle glucose uptake [223-227] which may be sustained for 48 hr after exercise [228-230]. Therefore, a combination of insulin-independent glucose uptake and physical activity induced muscle insulin senstivity maybe the mechanisms by which indexes of insulin sensvitity are improved.

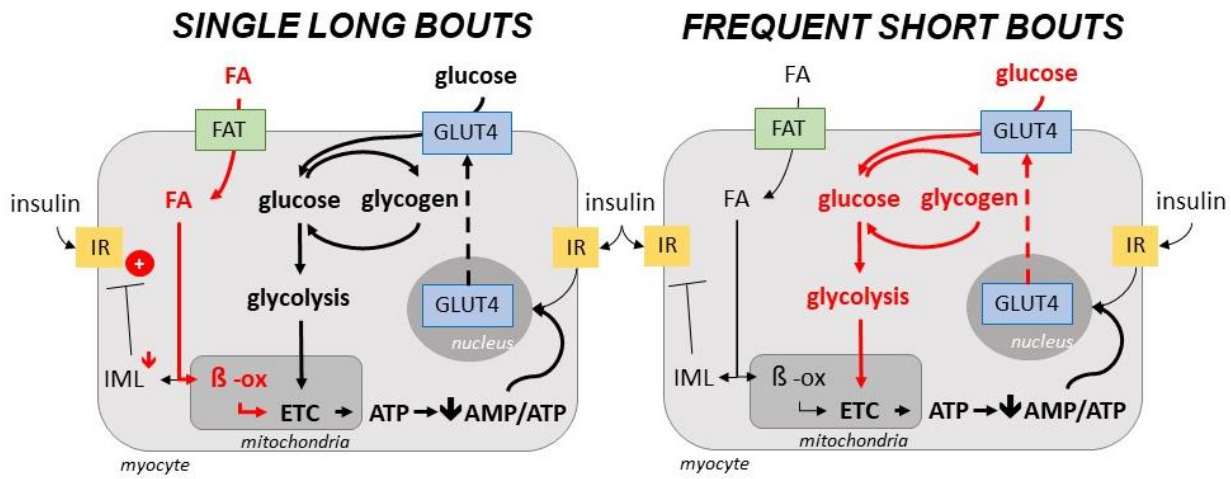

Figure 21: Pathways of skeletal muscle nutrient uptake. FA: fatty acid, FAT: fatty acid transporter, GLUT4: glucose transporter 4, IR: insulin receptor, β-ox: beta oxidation, IML: intramyocellular lipid, ETC: electron transport chain, ATP: adenosine triphosphate, AMP: adenosine monophosphate.

INTEGRATING THE EVIDENCE WITH THE WORKING MODEL

Over the short-term, even though participants rated breaking up sedentary behaviors with short-frequent bouts of physical activity to be challenging, this lifestyle intervention is a feasible option to increase time spent in moderate to vigorous physical activity and daily step count in work and non-work contexts in those who are habitually inactive. Breaking up sedentary behaviors with short-frequent bouts of physical activity is a viable strategy to control postprandial glucose concentrations through contraction-mediated glucose uptake and potentially improved insulin sensitivity in skeletal muscle. To fully elucidate the benefits of physical activity breaks on glucose control (Figure 5), exogenous and endogenous glucose fluxes should be examined in detail in future research. One hypothesis is the greater reliance on acute carbohydrate oxidation of the short-frequent bouts of physical activity may first come from intramuscular glycogen sources to meet energy demand. Accordingly, skeletal muscle glucose disposal increases to replenish glycogen stores after each microbout and postprandial glycemia is reduced because of increased dietary glucose uptake. Over time, breaking up sedentary behaviors with short-frequent bouts of

physical activity increases the capacity of muscle glucose uptake which may lead to a greater reliance on exogenous glucose uptake rather than endogenous muscle glycogen sources. Therefore, data from this dissertation suggest that performing multiple short bouts of activity throughout the day to frequently interrupt sedentary time may be an effective strategy to control glucose than the standard approach of a daily single-continuous bout of physical activity. Future investigations on this area of research will begin to establish how breaking up prolonged sitting, a cost-effective and clinically useful population wide approach, that improves glucose control (independent of physical activity) in adults with prediabetes, a population that is at high risk of developing type 2 diabetes.

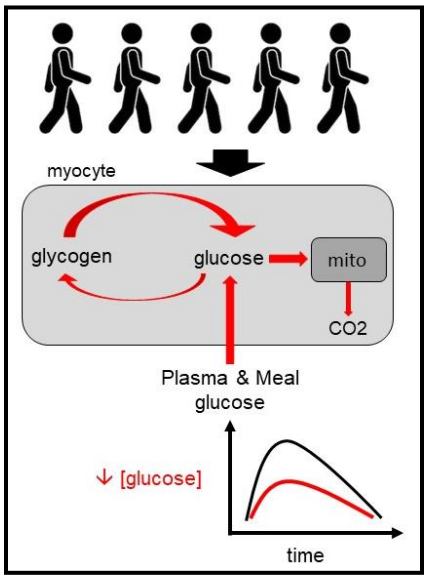

Figure 5: Working Model

A WHOLE-DAY APPROACH TO PROMOTE PHYSICAL ACTIVITY

Given the beneficial effects of increasing moderate to vigorous on cardiorespiratory fitness, contraction-mediated glucose uptake, insulin sensitivity and musculoskeletal health [231], promoting physical activity should remain a key target for type 2 diabetes prevention and management. Even though we know much more about the health benefits of moderate to vigorous

physical activity, at the population level increases in moderate to vigorous physical activity have been unrealized [232-235]. The first formally recognized physical activity guidelines created in 2008 was based on 50 years or more of research that focused on moderate to vigorous physical activity and exercise, but the evidence was not strong enough to consider how other components of physical activity (i.e., sedentary behaviors and light-intensity physical activity) influence health. Now, more is known about how moderate to vigorous physical activity may have limited scope in displacing total daily sedentary time and that high amount of sedentary time is detrimental for health [236, 237]. The new physical activity guidelines recognize the detrimental health effects of a high amount of sedentary and encourage any physical activity is better than none. A “whole day” approach to increasing physical activity rather than focusing on long bouts of moderate to vigorous physical activity may prove useful in its own right for enhancing glycemic control. This may also be an acceptable starting point for those who are sedentary, deconditioned, or unable or reluctant to add or transition into structured physical activity. Importantly, this approach aims at reducing and breaking up sitting time.

EMERGING AREAS OF RESEARCH

The state of current knowledge around the metabolic health effects of breaking up sedentary behaviors with short-frequent physical activity bouts spread across the day is centered on acute and short-term experimental trials on glycemic control, insulin sensitivity, and the regulation of blood flow [72, 117, 219, 238]. Preliminary evidence indicates breaking up sedentary behaviors with short-frequent physical activity may lower subjective feelings of hunger and appetite. In an acute experimental trial, we showed adults exposed to short-frequent activity breaks report subjective feelings of lower appetite at the end of the day in response to standardized meals and moderate-intensity physical activity breaks [153]. However, we did not measure blood biomarkers of appetite regulation. Holmstrup et al. showed increased feelings of satiety and reduced hunger with short-frequent bouts of physical activity to a greater extent than continuous physical in obese

individuals but reported no change in circulating appetite hormones [239]. Bailey et al found that regularly breaking up sedentary behaviors with light physical activity did not change circulating biomarkers of appetite regulation, Ghrelin and PYY [240]. Therefore, it seems short-frequent bouts of physical activity influences subjective feelings of appetite and hunger but not hormone regulators of appetite which may be due to quick transient changes in the circulation of these appetite regulating hormones. The preponderance of research examining the acute response of ghrelin and PYY to exercise has shown that moderate to high intensity continuous exercise (~60-70 VO_{2max}) is associated with a suppression of ghrelin and elevated PYY in lean healthy males [241]. However, these changes are short and normal circulating levels are restored soon after exercise [242, 243]. Future studies, similar to design in this dissertation could examine subjective feelings of appetite and hunger along with serial blood sample collection at the end of the short-term intervention to tease apart differences in subjective feelings of appetite and how this relates to changes in circulating biomarkers of appetite regulation.

Major gaps in knowledge exist with respect to sex specific glycemic response to breaking up sedentary behaviors, particularly in clinical populations such as individuals at risk of developing type 2 diabetes. A recent meta-analysis suggests that attenuations in glucose incremental area under the curve in response to acute, frequent, short active breaks to sitting is more pronounced in female compared to male adults [117]. Experimentally, type 2 diabetic females more than males showed a higher suppression of postprandial glycemia in response to standardized meal during exposure to light walking breaks to break up sedentary behaviors [62]. Therefore, preliminary evidence indicates that physical activity breaks to fragment sedentary time may be a better strategy at improving postprandial glycemia in women than in men. Comparing sex differences in response to short frequent bouts of physical activity will be instrumental when defining future prescriptions of physical activity for female and male adults targeting the prevention and/or treatment of type 2 diabetes and associated cardiovascular complications. This is particularly

relevant as the active breaks may become an alternative strategy to exercise for women at risk for or with type 2 diabetes who have greater exercise impairments than men with type 2 diabetes [244] and appear to suffer worse cardiovascular consequences than their male counterparts with type 2 diabetes [245].

LIMITATIONS

Energy expended during physical activity was not replaced by energy intake and so by design participants were in energy deficit which was the same for both physical activity groups. Comparing the active trials in energy balance would have been more rigorous and most likely representative of daily life over the long-term. We decided to not increase energy intake to match energy expenditure because evidence has shown that participants do not spontaneously increase their food intake in response to exercise or if they do, they do not match total energy expenditure and are still in energy deficit [126].

Indexes of insulin sensitivity (fasting HOMA-IR, ratio Insulin/Glucose, postprandial insulinemia) are not the strongest methods available to determine insulin sensitivity. It is important to assess changes in insulin sensitivity with more rigorous methods than these crude indexes. More rigorous methods include oral glucose tolerance test, intravenous glucose tolerance test, or the gold standard hyperinsulinemic euglycemic clamp. The short duration of the study timeline and cost of the above measurements were prohibitive. We know by experience that scheduling a whole-room calorimeter stay followed by measurement of insulin sensitivity with one of the above measures would have been a big participant burden because this would require almost 2 full days to complete, and most people can't take that time off to participate in research. However, the evidence from this dissertation provides preliminary evidence that peripheral insulin sensitivity is augmented by short-frequent bouts of physical activity and provides evidence to investigate further how short-frequent bouts of physical activity improves insulin sensitivity compared to a time matched single-continuous bout of physical activity.

CONCLUSIONS

Contemporary societies have engineered physical activity out of daily life through the industrial and technological revolutions that changed the way we work and live but also promoted the general adoption of sedentary behaviors that occur in every domain of daily life: transportation (planes, trains, and automobiles), domestic (washing machines and dishwashers), and leisure time activities and social events (video games, computers, going out to dinner). Although public-health recommendations to engage in moderate to vigorous physical activity have been widely promulgated by the government, most people do not achieve this level of physical activity. This may be due to inconvenience and competing priorities and/or to the fact that exercise does not reduce sedentary behaviors and can on the contrary even lead to a spontaneous compensatory increase in sedentary activities. In this dissertation, we showed that when physical activity is performed as frequent, short bouts spread throughout the day, thus frequently interrupting prolonged sitting, the benefits on glucose control are greater than when the same amount of physical is performed as a single continuous bout with the rest of the day being sedentary. These improvements seem to be independent of detectable differences in insulin sensitivity, total daily active time and energy expenditure. This evidence can be used to refine future guidelines to prevent and treat metabolic diseases, not in terms of intensity of exercise per day per week but in terms of avoidance of sedentary activities through short bouts of physical activity.

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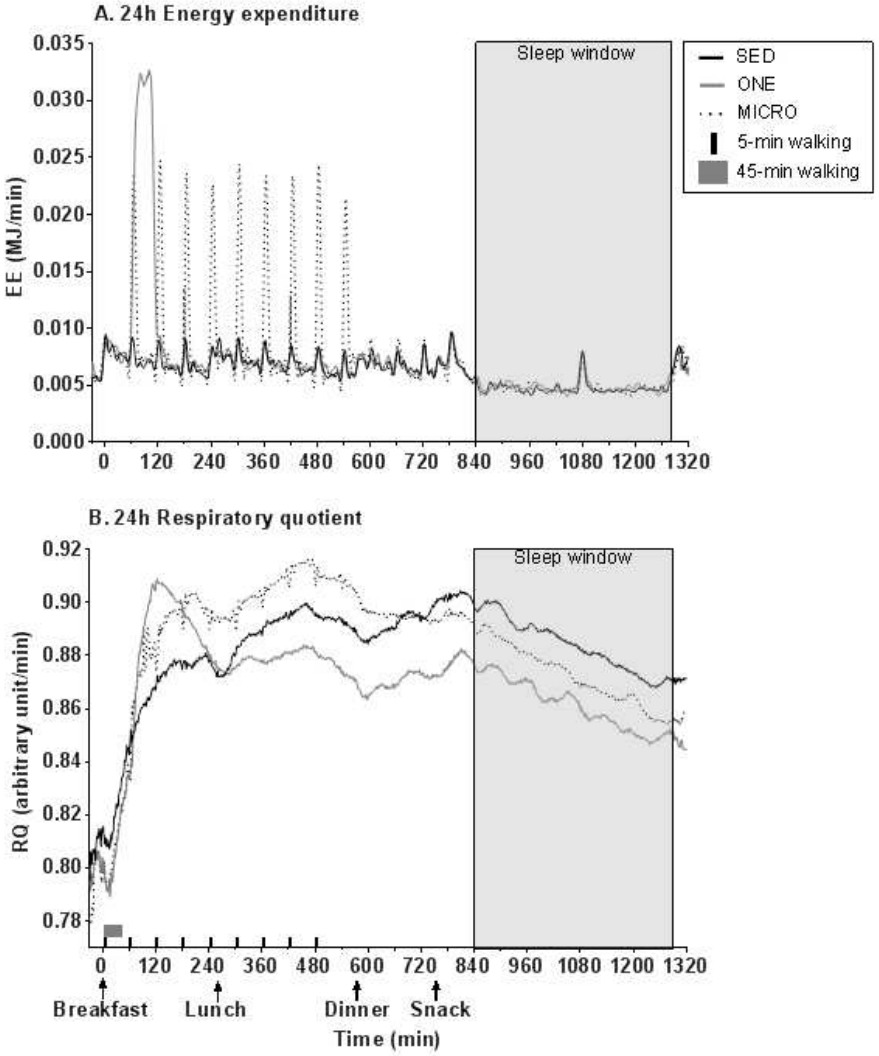
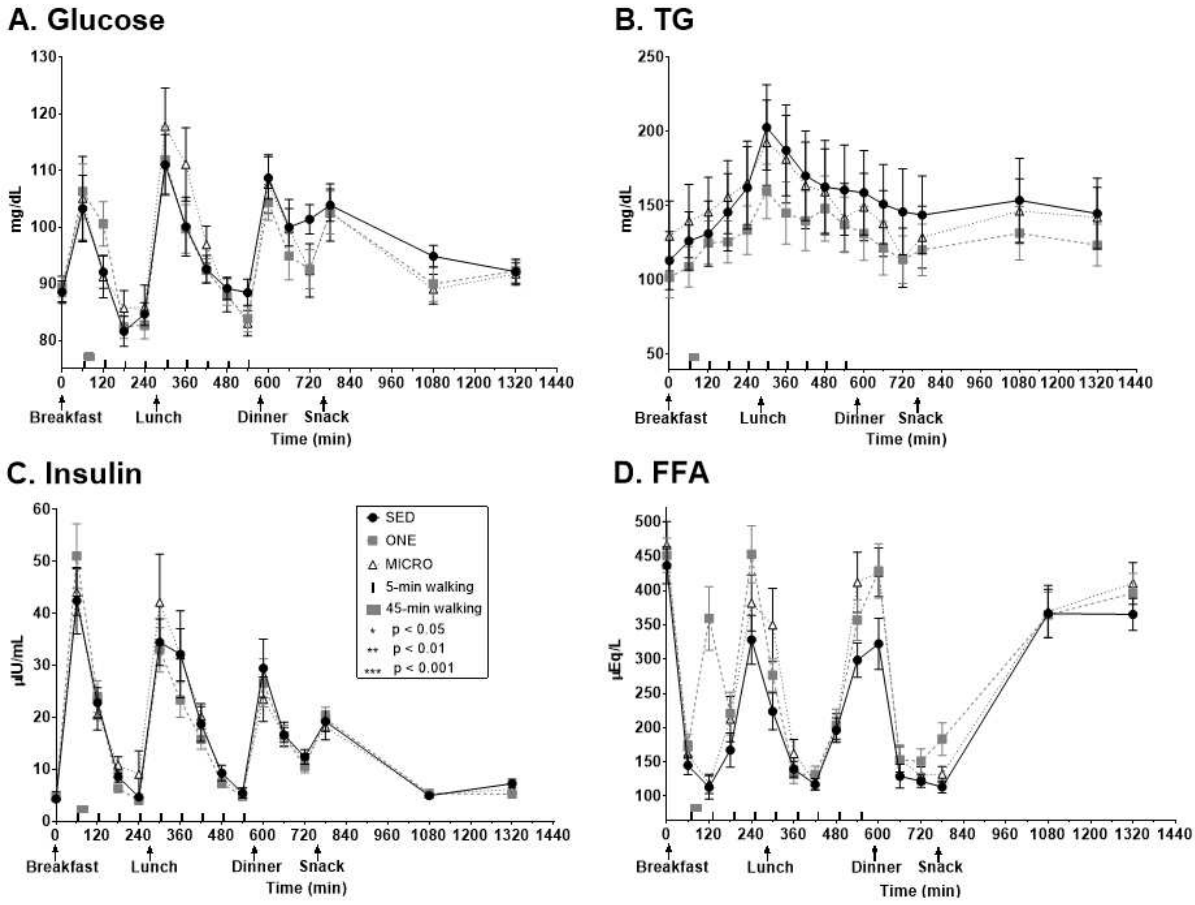
ANNEX

Supplemental Figure 1



Supplemental Figure 1: Twenty-four hour respiratory quotient and energy expenditure. The pattern of (A) 24-h energy expenditure throughout each study day sedentary (SED), one bout (ONE) and microbouts (MICRO); (B) 24-h respiratory quotient throughout each study day sedentary (SED), one bout (ONE) and microbouts (MICRO). Sleep opportunity was scheduled for 2230-0630. ▬ indicates 45-minute bout of walking. ▮ indicates 5-minute walking bout. ↑ indicates meals. Data are presented as mean per minute.

Supplemental Figure 2



Supplemental Figure 2: Twenty-four-hour plasma metabolite. The pattern of change in plasma metabolite concentrations during the study day. (A) Pattern of change for glucose through the study day. (B) Pattern of change for triglyceride through the study day. (C) Pattern of change for insulin through the study day. (D) Pattern of change for FFAs through the study day. TG, triglyceride; FFA, free fatty acid. Data are presented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. ▬ indicates 45-minute bout of walking. ▌ indicates 5-minute walking bout. ↑ indicates meals. Data are presented as mean per minute.