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Nathan P de Jong, Corey A Rynders, David A Goldstrohm, Zhaoxing Pan, Andrew H Lange, et al.. Effect of frequent interruptions of sedentary time on nutrient metabolism in sedentary overweight male and female adults. *Journal of Applied Physiology*, 2019, 126 (4), pp.984-992. 10.1152/jap-physiol.00632.2018 . hal-02108332

HAL Id: hal-02108332

<https://hal.science/hal-02108332>

Submitted on 26 May 2021

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

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31
32 **Keywords:** dietary fatty acid oxidation, carbohydrate oxidation, whole-room calorimetry,
33 microbouts of activity, physical inactivity

34
35 **Running title:** Breaking-up sedentary time and nutrient metabolism

36
37 **NCT#** NCT02258438

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ABSTRACT (248/250 WORDS)

Objective: This study compared 24-h nutrient oxidation responses between a sedentary condition (SED) and a condition where short 5-min bouts of moderate-intensity physical activity were performed hourly for 9 consecutive hours over 4-d (MICRO). To determine whether any shifts in fuel use were due solely to increases in energy expenditure we also studied a condition consisting of a single isoenergetic 45-min bout of moderate-intensity exercise (ONE). **Methods:** Twenty sedentary overweight or obese adults (10M/10F; 32.4±6.3 years; BMI, 30.6±2.9kg/m²) completed all three conditions (MICRO, SED, and ONE) in a randomized order. Each condition consisted of a 3 d free-living run-in followed by a 24-h stay in a whole-room calorimeter to measure total energy expenditure (TEE) and substrate utilization. Dietary fat oxidation was also assessed during the chamber stay by administering a [1-¹³C] oleic acid tracer at breakfast. Energy intake was matched across conditions. **Results:** Both MICRO and ONE increased TEE relative to SED resulting in a negative energy balance. HOMA-IR improved in both activity conditions. MICRO increased 24-h carbohydrate oxidation compared to both ONE and SED (p<0.01 for both). ONE was associated with higher 24-h total fat oxidation compared to SED, and higher 24-h dietary fat oxidation compared to both SED and MICRO. Differences in substrate oxidation remained significant after adjusting for energy balance. **Conclusion:** In overweight and obese men and women, breaking up sitting time increased reliance upon carbohydrate as fuel over 24-h while a single energy-matched continuous bout of exercise preferentially relies upon fat over 24-h.

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75 **News and Noteworthy**

- 76 • Insulin sensitivity as assessed by HOMA-IR was improved after 4-days of physical activity
77 independent of frequency and duration of activity bouts.
- 78 • Temporal patterns of activity across the day differentially affect substrate oxidation.
- 79 • Frequent interruptions of sedentary time with short bouts of walking primarily increases 24-h
80 carbohydrate oxidation whereas an energy-matched single continuous bout of moderate
81 intensity walking primarily increased 24-h fat oxidation.

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87 Introduction

88 Sedentary behavior is associated with several adverse health outcomes including obesity,
 89 cardiometabolic diseases, diabetes, certain types of cancer and premature mortality (23, 26).
 90 These associations have been observed across sex, age, ethnicity, and even among individuals
 91 who meet the current intensity-based physical activity guidelines (i.e., 150-min/week of
 92 moderate intensity or 75-min/week of vigorous exercise) (26). Isotemporal substitution modeling
 93 suggests that replacing 1h of sedentary time with either light or moderate-to-vigorous intensity
 94 physical activity (MVPA) in inactive adults was associated with lower mortality and risk factors of
 95 metabolic disease, with MVPA associated with the most potent health-enhancing time-
 96 dependent behavior (7, 24). Independent of potential confounders and time spent in other
 97 activities, reallocation of 30-min/day of sedentary time with an equal amount of MVPA is
 98 associated with lower blood triglycerides, glucose and insulin, and higher insulin sensitivity (7).
 99 Additionally, observational studies suggest that interrupting sedentary time with frequent bouts of
 100 physical activity is associated with lower plasma glucose and insulin, waist circumference,
 101 inflammatory marker C-reactive protein even in individuals who regularly exercise (15, 17).
 102 Adults whose sedentary time was mostly uninterrupted had less healthy cardiometabolic
 103 profiles, based on increased blood glucose and triglycerides, compared to those who had more
 104 frequent breaks in sedentary time (15-17), even when controlling for total sedentary time,
 105 MVPA, age, sex and ethnicity (15, 17).

106 A number of acute and short-term studies (≤ 3 days) have shown that frequent interruptions of
 107 prolonged sedentary activities with short bouts of walking decrease postprandial plasma
 108 glucose and insulin concentrations compared to a prolonged sedentary condition (2, 5, 9-11, 18,
 109 21, 27, 34). For example, Dunstan et al. (10) compared plasma glucose and insulin responses
 110 to uninterrupted sitting and 2-min bouts of activity every 20-min for 5-h in overweight adults.
 111 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 (5, 12, 18, 27, 34). 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 and Study Design

Participants

Eligible participants were aged 19-45 years, with a body mass index (BMI) between 27 and 33 kg/m², 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) (6) 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: $[(23.9 \times \text{FFM in kg}) + 372]$, where fat-free mass (FFM) was measured by dual energy X-ray absorptiometry (DXA, Hologic Delphi-W, Bedford, MA) (20, 35). 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 (8).

Inpatient study day

Figure 2 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. 1-¹³C 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 port-hole 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 O₂ consumption and CO₂ 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 (25). EE and substrate oxidation (13) 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 (30).

Dietary fatty acid oxidation

Participants collected an hourly breath sample for $^{13}\text{CO}_2$ by blowing through a tube into two 15 ml vacutainer tubes. Breath CO_2 was sampled directly from the vacutainer with a syringe, and $^{13}\text{CO}_2/^{12}\text{CO}_2$ 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 (4) and after correction for CO_2 entrapment in the bicarbonate pool and TCA cycle (1).

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 (11). 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 \pm 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 1. Twenty-five sedentary overweight adults ($n=12\text{M}/13\text{F}$; 31.6 ± 6.5 years; $\text{BMI}=30.5\pm 2.7$ kg/m^2) were recruited to participate in the study. Data are shown for twenty participants ($10\text{M}/10\text{F}$; 32.4 ± 6.3 years; BMI , 30.6 ± 2.9 kg/m^2) who completed all the procedures. Participant's characteristics are displayed in Table 1.

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

Daily patterns of EE and respiratory quotient (RQ) are presented in Supplemental Figure 1. Twenty-four hour energy intake, expenditure and balance are shown in Figure 3A. 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 ± 0.17 MJ/d, $p < 0.01$) and MICRO (-1.64 ± 0.17 MJ/d, $p < 0.01$) compared to SED. Twenty-four hour non-protein respiratory quotient (NPRQ) was lower in ONE compared to both SED and MICRO conditions (0.881 ± 0.006 versus 0.900 ± 0.006 and 0.900 ± 0.009 respectively, $p < 0.05$ for both; Figure 3D), 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 3C). 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 3C 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 3B).

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: $R = -0.84$, $p < 0.0001$).

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 4C) during the waking time when physical activity was performed. Waking NPRQ was lower in ONE compared to SED and MICRO (0.894 ± 0.006 versus 0.911 ± 0.005 and 0.914 ± 0.009 respectively, $p < 0.05$ for both). During sleep, NPRQ was lower in both ONE and MICRO compared to SED (0.847 ± 0.009 and 0.852 ± 0.008 versus 0.875 ± 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 4E).

Plasma metabolites and index of insulin sensitivity

Twenty-four-hour profiles of glucose, TG, insulin, and FFA are shown in 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 5). After 4-d of both active conditions HOMA-IR was decreased compared to SED (SED = 1.67 ± 0.87 , ONE = 1.20 ± 0.52 , MICRO = 1.42 ± 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 = $-16599 \pm 10871 \mu\text{IU/mL} \cdot 9\text{h}^{-1}$; $p < 0.0001$) and MICRO (MICRO = $-11570 \pm 8866 \mu\text{IU/mL} \cdot 9\text{h}^{-1}$; $p < 0.01$) compared to SED (SED = $-5283 \pm 10667 \mu\text{IU/mL} \cdot 9\text{h}^{-1}$) (Figure 5). No differences were noted over this same time period in plasma glucose, TG and FFA.

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 (2, 5, 9-11, 18, 21, 27, 34). 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 (22, 32). Additionally, the current study did not include participants with impaired glucose tolerance or type 2 diabetes as has been done in previous studies (10, 11, 21). 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) (5). However, glycemic variability measured by continuous glucose monitoring was reduced in the two frequent break conditions, indicating improved glucose control (5). 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 (27). 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 (2). 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 (4). 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. (19) 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 was 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 (28). 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 (3). 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 (14, 33), 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 (29). For example, we showed that higher rates of nocturnal fat oxidation are associated with lower weight gain over five years in adults (31). 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 generalisability 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

443 dietary carbohydrate oxidation that may have potentialized the use of carbohydrate as fuel
444 during the MICRO condition. Additionally, while dietary fat oxidation was measured, we cannot
445 comment on the source of carbohydrate that was oxidized during the study day. Future studies
446 using both fat and carbohydrate stable isotope tracers will be needed.

449 **Conclusions and future directions**

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

459 **Acknowledgements**

460 The authors thank study volunteers for their time and participation as well as the medical,
461 nutrition and administrative staff of the University of Colorado CTRC.

463 **Conflict of interests**

464 The authors declare no conflict on interests.

467 **Author contributions**

AB and DHB designed the study, collected, analyzed and interpreted data, and drafted the manuscript. NPDJ collected, analyzed and interpreted data, and drafted the manuscript. ELM, CAR analyzed and interpreted data and drafted the manuscript. DG, AL, CM collected and analyzed data. ZP performed statistical analysis and drafted the manuscript. All authors gave final approval of the manuscript to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Disclosure

The contents are solely the responsibility of the authors and do not represent the views of the U.S. Department of Veterans Affairs, the United States Government, the Center for Disease Control, National Institute of Occupational Health and Safety and the Mountain and Area Plains Education and Research Center.

Funding sources

This work was supported by the National Institutes of Health grant numbers K99DK100465 (AB) and UL1 TR001082 (AB), K01DK113063 (CAR) and P30DK048520. This publication was also supported by Grant Number T42OH009229-10 (AB) from CDC NIOSH Mountain and Plains Education and Research Center.

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Figure legends

Figure 1: Trial CONSORT diagram

Figure 2: 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.

Figure 3: Absolute and relative twenty-four hour nutrient oxidation, balance and dietary fat oxidation

(A) 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 1-¹³C oleic acid stable isotope tracer) for each study condition: sedentary (SED), one bout (ONE) and microbouts (MICRO). Data are presented as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4: 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 \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5: 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 \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Supplemental Figures

Supplemental Figure 1: <https://doi.org/10.5281/zenodo.2399298>

Supplemental Figure 2: <https://doi.org/10.5281/zenodo.2399364>

Tables

Table 1

	Males	Females	Total
n	10	10	20
Age (years)	31.5 \pm 7.4	33.2 \pm 5.1	32.4 \pm 6.3
BMI (kg/m²)	28.9 \pm 3.0	31.9 \pm 2.0	30.6 \pm 2.9
Body Mass (kg)	88.0 \pm 13.6 **	83.5 \pm 7.2	85.7 \pm 10.8
FFM (kg)	63.2 \pm 10.0 ***	48.3 \pm 3.6	55.8 \pm 10.5
FM (kg)	24.6 \pm 4.4 ***	35.2 \pm 4.4	29.9 \pm 6.9
Fat mass (%)	28.1 \pm 2.5 ***	42.0 \pm 2.3	35.0 \pm 7.5
Fasting Glucose (mg/dL)	88.0 \pm 4.8	87.6 \pm 4.3	87.8 \pm 4.4
Fasting Triglyceride (mg/dL)	154.6 \pm 107.7 *	83.5 \pm 14.7	117.2 \pm 81.2
Fasting Insulin (μU/mL)	5.4 \pm 2.7	5.15 \pm 1.3	5.2 \pm 2.1
HOMA-IR (SED day fasting)	1.5 \pm 0.8	1.4 \pm 0.4	1.4 \pm 0.6
IPAQ self-reported sitting time (hrs/day)	9.6 \pm 2.9	11.3 \pm 3.5	10.5 \pm 3.3

Data are presented as the mean \pm 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; HOMA-IR, homeostatic model assessment of insulin resistance; IPAQ, international physical activity questionnaire

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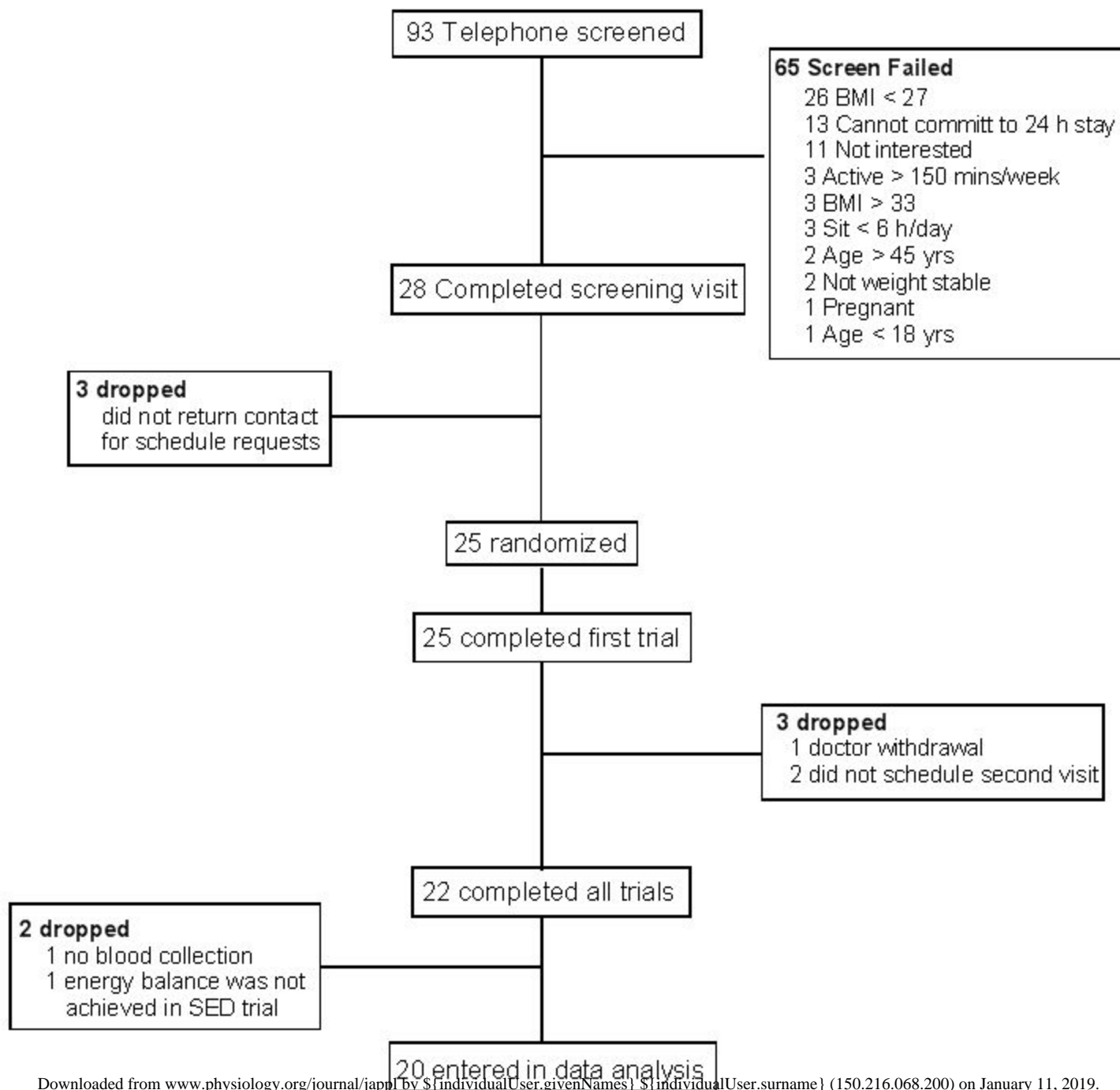
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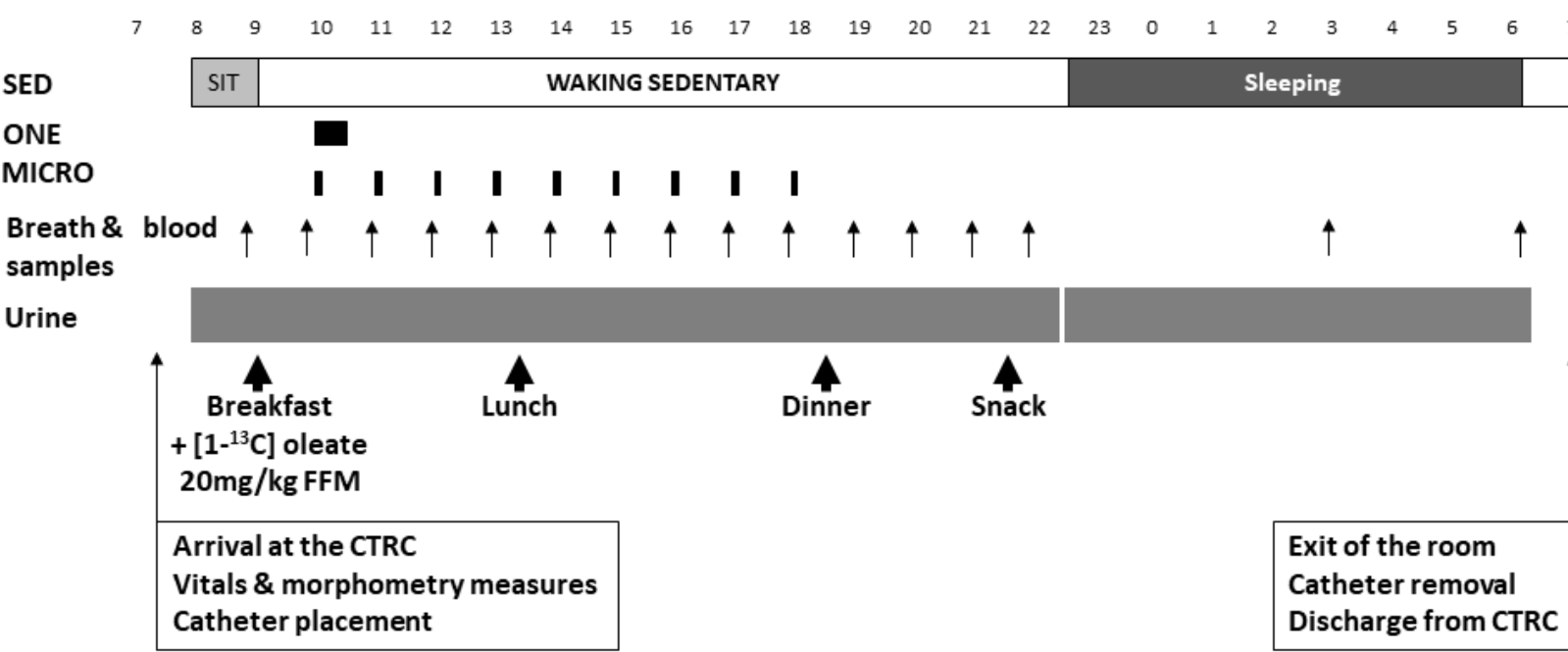
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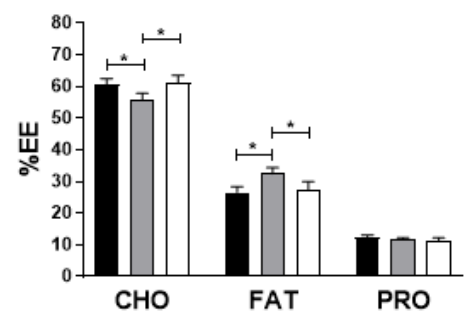




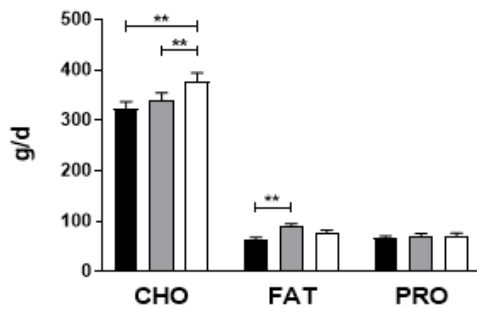
A. 24h Energy Intake, Expenditure and Balance



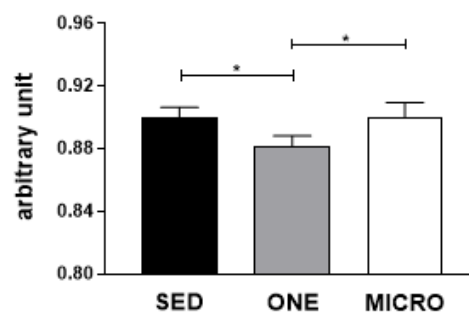
B. Contribution of substrate oxidation to 24h total energy expenditure



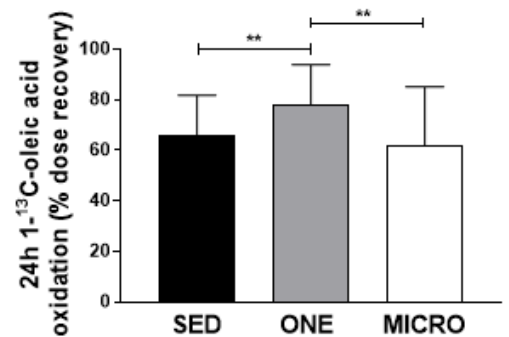
C. 24h Nutrient Oxidation



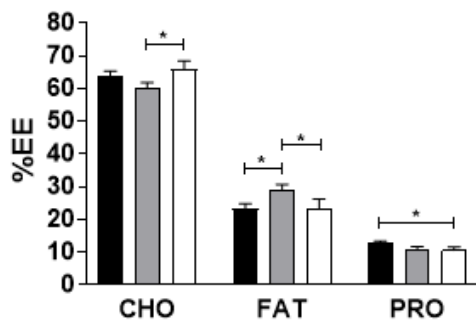
D. 24h NPRQ



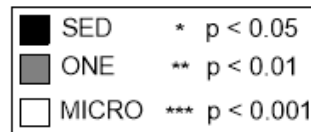
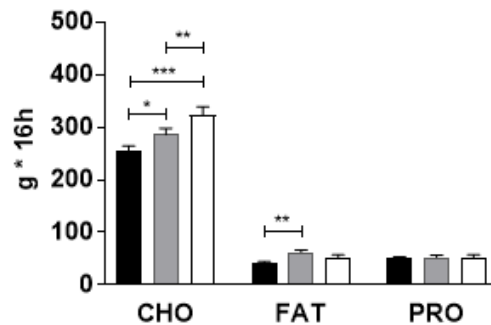
E. 24h Dietary Fat Oxidation



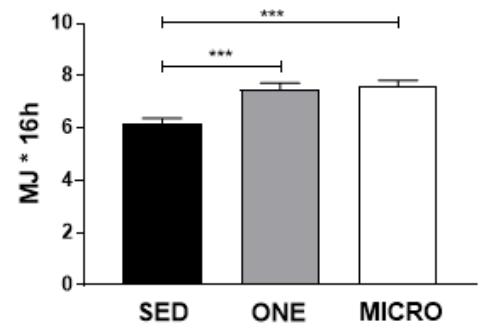
A. Contribution of substrate oxidation to waking total energy expenditure



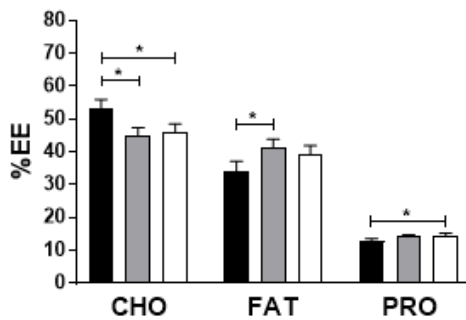
B. Waking nutrient oxidation



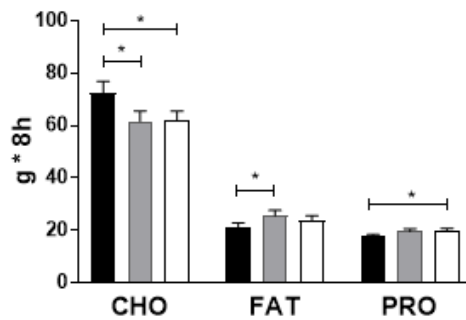
C. Waking energy expenditure



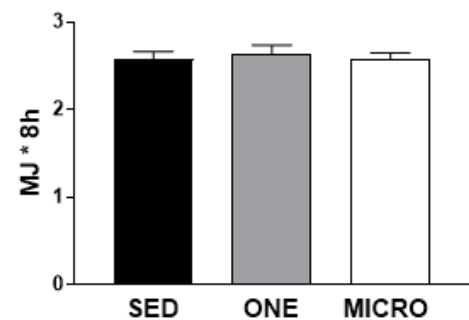
D. Contribution of substrate oxidation to sleeping total energy expenditure



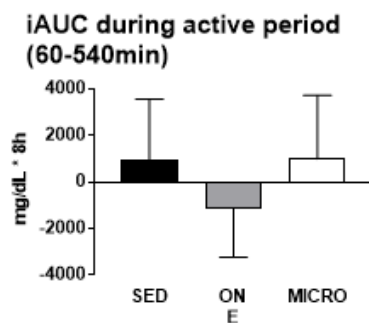
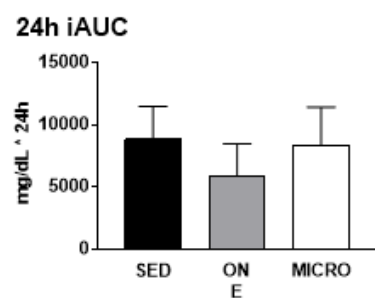
E. Sleeping nutrient oxidation



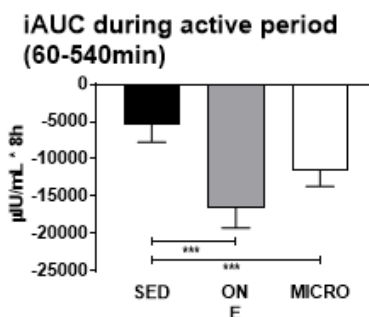
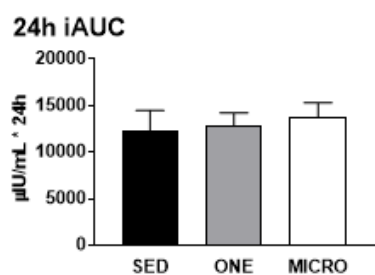
F. Sleeping energy expenditure



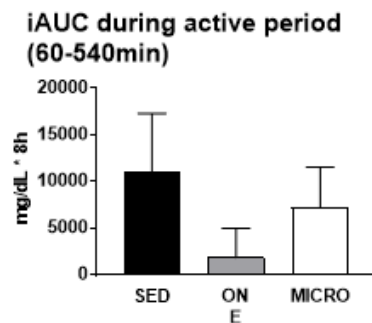
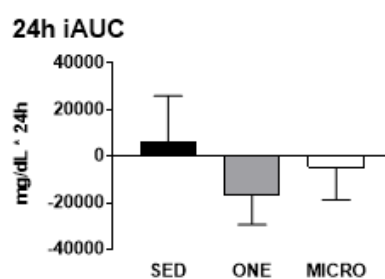
A. Glucose



C. Insulin



B. TG



D. FFA

