Three Weeks of Interrupting Sitting Lowers Fasting Glucose and Glycemic Variability, but not Glucose Tolerance, in Free-Living Women and Men with Obesity

Running title: Breaking sitting marginally lowers glycemic variability

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ABSTRACT

OBJECTIVE To determine whether interrupting prolonged sitting improves glycemic control and the metabolic profile of free-living adults with obesity.

METHODS Sixteen sedentary individuals (10 women/6 men; median [IQR] age 50 [44-53] years, BMI 32 [32-35.8] kg/m^2) were fitted with continuous glucose and activity monitors for 4 weeks. After a 1-week baseline period, participants were randomized into habitual lifestyle (Control) or Frequent Activity Breaks from Sitting (FABS) intervention groups. Each day, between 0800-1800 h, FABS received smartwatch notifications to break sitting with 3 min of low-to-moderate-intensity physical activity every 30 min. Glycemic control was assessed by OGTT and continuous glucose monitoring. Blood samples and *vastus lateralis* biopsies were taken for assessment of clinical chemistry and the skeletal muscle lipidome, respectively.

RESULTS Compared to baseline, FABS increased median steps by 744 (IQR [483-951]) and walking time by 10.4 (IQR [2.2-24.6]) min per day. Other indices of activity/sedentary behavior were unchanged. Glucose tolerance and average 24-h glucose curves were also unaffected. However, mean (±SD) fasting glucose levels (-0.34 [±0.37] mmol/L) and daily glucose variation (%CV; -2 [±2.2]%) reduced in FABS, suggesting a modest benefit for glycemic control that was most robust at higher volumes of daily activity. Clinical chemistry and the skeletal muscle lipidome were largely unperturbed, although 2 long-chain triglycerides increased 1.25-fold in FABS, post-intervention. All parameters remained stable in Control.

CONCLUSIONS Under free-living conditions, FABS lowered fasting glucose and glucose variability. Larger volumes of activity breaks from sitting may be required to promote greater health benefits.

Keywords: Obesity, insulin resistance, glycemia, lipids, prolonged sitting, activity breaks.

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New and Noteworthy

- Under free-living conditions, breaking sitting modestly increased activity behavior
- Breaking sitting was insufficient to modulate glucose tolerance or the skeletal muscle lipidome
- Activity breaks reduced fasting blood glucose levels and daily glucose variation compared to baseline, with a tendency to also decrease fasting LDLc
- This intervention may represent the minimal dose for breaking sedentary behavior, with larger volumes of activity possibly required to promote greater health benefits

Introduction

Technological advances have enabled lifestyles to become ever more sedentary. More than one third of Europeans are now physically inactive (24), spending ≈40% of leisure time watching television (23), and this accumulation of sedentary behavior is associated with impaired glucose tolerance and metabolic health (30). Every waking hour spent in sedentary postures (i.e. sitting or lying) increases risk for metabolic syndrome and type 2 diabetes (57), partly due to the detrimental effects of inactivity on whole-body insulin sensitivity (1, 25, 37, 43, 53). Reducing steps to ≈33% of habitual levels (i.e. ≈4,300 steps/day) impairs glycemic control after just 3 days (43) and 2 weeks at ≈12% of normal activity (i.e. ≈1,300 steps/day) lowers lean body mass, aerobic fitness ($\dot{\mathbf{V}}O_{2max}$), and skeletal muscle insulin sensitivity (37). In adults, sedentary lifestyles are also implicated in the development of obesity (28, 33). Individuals with severe obesity have decreased capacity for skeletal muscle fatty acid oxidation (34, 35) and often present with elevated concentrations of intramuscular lipids (21, 34), which may contribute to peripheral insulin resistance (11) and the proportionally greater risk of type 2 diabetes with increasing body mass index (BMI, kg/m^2) (8). However, in rodents, the inactivity-induced alterations in skeletal muscle lipid metabolism are reversed by low-intensity treadmill walking (6). Thus, even light physical activity may offer some protection against excessive lipid accumulation and associated detriments in skeletal muscle.

Modifiable lifestyle factors, including exercise, can combat the progression of impaired glucose tolerance towards type 2 diabetes (36, 39, 45). A single bout of exercise enhances whole-body insulin sensitivity for up to 48 h (42). Moreover, regular aerobic, resistance, or concurrent training improves glucose homeostasis and blood lipid profiles (52). Yet, compliance to current physical activity guidelines remains low (24). Hence, there is growing interest in establishing more accessible evidence-based intervention programs to reduce patterns of sedentary behavior, in order to stem the development of metabolic diseases.

Cross-sectional data suggests that individuals in the highest quartile for number of breaks in sedentary time per week (i.e. ≥673 breaks) have less central adiposity and better glucose tolerance than those in the lowest quartile (i.e. ≤506 breaks) (26). Controlled research trials provide further evidence that interrupting prolonged sitting with multiple breaks of light-to-moderate-intensity physical activity lowers postprandial glycemia and triglycerides (9, 40), and increases whole-body fat oxidation (56). Hence, breaking sedentary behavior may offer a pragmatic, easy to interpret public health intervention for improved insulin sensitivity and metabolic wellbeing. However, laboratory-based trials often report benefits when comparing activity breaks to conditions of uninterrupted sitting that are not necessarily indicative of free-living behaviors (3, 4, 13, 29, 32, 38, 47, 55), and results from short-term (i.e. ≤4 days) more ecologically valid trials are equivocal (7, 15-17, 50). As such, longer studies investigating the translational efficacy of breaking sedentary time in habitually active cohorts are needed.

Here, we investigated the effects of 3 weeks of frequent activity breaks from prolonged sitting on glycemia, clinical chemistry, and the skeletal muscle lipidome of women and men with obesity, under free-living conditions. We hypothesized that breaking sitting would improve glucose control, insulin sensitivity, and markers of metabolic health, concomitant with changes in skeletal muscle lipid content.

Materials and methods

Ethical approval

This parallel randomized control trial was approved by the regional ethics committee of Stockholm (2016/1768-32) and conducted in accordance with the Declaration of Helsinki. All participants gave their written and oral informed consent prior to enrolment. The study is registered as a clinical trial with the United States National Library of Medicine, at the National Institutes of Health (ClinicalTrials.gov identifier: NCT03083587).

Participants

Twenty adults with obesity were recruited for participation. Inclusion criteria were a self-perceived sedentary lifestyle, a sedentary occupation or unemployment, an age between 18-60 years, and a BMI of 30-45 kg/m^2. Exclusion criteria were regular exercise or physical activity, a prior diagnosis of diabetes or severe cardiovascular disease, and the use of anti-coagulant medications. A power calculation for sample size was not performed; however, this number was deemed adequate according to prior trials investigating the metabolic effects of breaking sitting in comparable demographics (3, 10, 13). Of the 20 prospective participants, 16 completed the trial: 2 withdrew before the study commenced, 1 was excluded due to regular physical activity that was not reported at initial screening, and 1 dropped-out during the baseline period.

Two individuals in the Control group and 1 individual in the Frequent Activity Breaks from Sitting (FABS) group were unemployed with a sedentary lifestyle, while the remainder of participants worked sedentary jobs (predominantly desk-based, n=11; bus driver, n=1; and musician, n=1). In the Control group, 1 individual was taking hypertensive medication (angiotensin II receptor blocker) and in the FABS group, 5 individuals were taking some form of medication(s) (selective serotonin reuptake inhibitors, n=4; Levothyroxine, n=1; calcium antagonist, n=1; Acetaminophen, n=1; non-steroidal anti-inflammatory, n=1; melatonin, n=1; vitamin D, n=1; iron, n=1). Medication remained constant throughout the study.

128 ***Study Design***

129 A schematic overview of the study design is shown in Figure 1. At visit 1, participants
130 reported to Danderyd Hospital, Stockholm, the morning after an overnight fast and having
131 refrained from any uncustomary physical activity for 48 h. Anthropometric measures and
132 blood samples (for baseline assessment of clinical chemistry) were taken. *Vastus lateralis*
133 biopsies were obtained using a Weil-Blakesley conchotome under local anesthesia
134 (mepivacaine hydrochloride, 10 mg/mL) and immediately cleared of visible adipose, vascular,
135 or connective tissues, before snap-freezing in liquid nitrogen and storing at -80°C until
136 subsequent analysis. A 2-h oral glucose tolerance test (OGTT, 75 g of glucose) was then
137 performed, with blood samples taken every 30 min. Participants were next fitted with
138 continuous glucose (CGM; FreeStyle Libre, Abbott Laboratories, Chicago, IL) and activPAL
139 (PAL Technologies, Glasgow, UK) monitors, and allocated by block randomization into no-
140 intervention (Control) or FABS groups.

141 During week 1 (Baseline), both groups continued with habitual living patterns to
142 establish baseline glucose and activity levels. Participants were then asked to maintain similar
143 dietary behaviors for the remainder of the trial. From weeks 2-4 (Intervention), the FABS
144 group received notification every 30 min, between 0800-1800 h, from a smartphone app
145 ('Rise and Recharge', Baker Heart and Diabetes Institute) connected to a smartwatch,
146 reminding them to break sitting. Upon notification, participants were to perform 3 min of low-
147 to-moderate-intensity physical activity (e.g. walking, stair-climbing, bodyweight squats etc.),
148 with a minimum threshold of ≥15 steps registering in the app as a successfully completed
149 activity break. During this period, the Control group continued with their habitual levels of
150 daily activity. At the end of week 4, participants returned to the clinic for visit 2, which was a
151 repeat of visit 1, under the same conditions. Participant recruitment started on the 02/01/2017
152 and data collection finished on the 07/31/2019.

153 ***OGTT and insulin sensitivity analyses***

154 The primary outcomes for this trial were the assessment of glucose tolerance, by OGTT,
155 and insulin sensitivity, as defined by the homeostatic model assessment of insulin resistance
156 (HOMA2-IR), Matsuda (Insulin Sensitivity) Index, and hepatic insulin resistance index
157 (HIRI). Incremental areas under the curve (iAUC) for glucose and insulin were calculated
158 according to the trapezoidal rule for all peaks above fasting levels; HOMA2-IR was computed
159 from fasting glucose and insulin values using the HOMA2 Calculator (www.dtu.ox.ac.uk);
160 Matsuda Index was determined by the formula 10,000 / ($Glucose_{[0]}$ * $Insulin_{[0]}$ * $Glucose_{[mean]}$
161 * $Insulin_{[mean]}$)$^{1/2}$, where *0* and *mean* were fasting and mean values during the OGTT; and
162 HIRI as the product of glucose (mg/dL/min) and insulin (μU/mL/min) AUC during the first
163 30 min of the OGTT ([$Glucose_{(0\text{-}30)}$AUC x $Insulin_{(0\text{-}30)}$AUC] / 100) (48). In the FABS group,
164 1 participant was omitted from all analyses involving insulin because of sample hemolysis,
165 another was removed from glucose iAUC calculations due to a lack of intermittent blood
166 draws during the OGTT, and 1 was excluded from 2-h glucose and insulin comparisons also
167 due to sampling difficulty at this timepoint.

168 ***CGM and ActivPal data analysis***

169 A day of activPal data was considered valid if ≥10 h of wear time was registered during
170 waking hours, <95% of that time was spent in any one behavior (i.e. sedentary, standing, or
171 walking), and ≥500 steps were recorded (18). Thus, for all participants (Control, n=8; FABS,
172 n=8), activPal data was collected for 7 [2 to 7] baseline and 20.5 [15 to 21] intervention days
173 (median [range] of all subjects). Due to a malfunction of the CGM interstitial probe, no
174 baseline glucose data were obtained for 2 Control group participants and they were
175 subsequently excluded from CGM analyses. For the remaining participants (Control, n=6;
176 FABS, n=8), 7 [2 to 7] baseline days and 21 [12 to 21] intervention days were collected
177 (median [range] of all subjects).

For each participant, CGM and activPAL data were divided into hourly intervals. Average baseline and intervention 24-h curves were calculated as hour-of-day means (for glucose) or medians (for steps) of all collected baseline and intervention days. Total daily activity (i.e. number of steps, sit-to-stand transitions, and time spent walking or sitting) was calculated as the sum of all hourly values per day. To assess approximate adherence to our intervention, a *post hoc* analysis of participant activity was conducted. Given the low threshold of ≥15 steps every 30 min registering as a completed activity break, we considered successful adherence to be any increase in hourly steps and/or postural transitions above each participant's own, median, baseline levels between the hours of 08:00-18:00 (i.e. when smartwatch prompts to break sitting were received). Daily and, from that, weekly adherence was then calculated as a percentage of total quantified hours during which activity goal criteria was met.

Daily Continuous net glycemic action (CONGA) 1, 2, and 4 were calculated as a standard deviation of all $Glucose_{(t)} - Glucose_{(t-n)}$ differences within a day, where *t* was time-of-day and *n* was 1-, 2-, or 4-h for CONGA1, 2, and 4, respectively (41). Glucose standard deviation (SD) was calculated from all obtained values within a day, and coefficient of variation (%CV) as $Glucose_{(SD)} / Glucose_{(mean)} * 100$. Glycemic variability measurements (i.e. CONGA, SD, and %CV) were calculated for each subject per day using only days with ≥20 h of data collected. These calculations were made for 4 [1 to 6] baseline and 16 [7 to 21] intervention days (median [range] of all subjects).

FABS subgroup analysis of activity volume on glycemia

Participants in the FABS group were stratified into those with higher- *versus* those with lower- activity levels according to daily steps taken and postural transitions made during the intervention period. To normalize the contribution of steps and transitions towards the calculation of total activity volume, these indices were first scaled using the formula (X –

203 X_{mean}) / X_{SD}; where X was the total number of steps or transitions during a specific hour, and
204 X_{mean} and X_{SD} were the mean and standard deviation of hourly steps or transitions. FABS
205 participants were then ranked depending on the sum of their scaled activity and the most
206 active individuals were allocated to the 'high-activity' subgroup (n=4; 4 females), whereas the
207 least active individuals were assigned to the 'low-activity' subgroup (n=4; 1 female, 3 males),
208 prior to subsequent analyses.

209 ***Skeletal muscle lipidomics***

210 Frozen skeletal muscle biopsies were crushed to a homogenous powder using a cell-
211 crusher (Cellcrusher, Cork, Ireland) and aliquoted (16-48 mg) samples were shipped on dry
212 ice to the Swedish Metabolomics Centre (Swedish University of Agricultural Sciences, Umeå,
213 Sweden) for lipidomic analysis. Samples were lysed, lipids extracted, and solvent volumes
214 adjusted to biopsy weight (i.e. 600 µL for 16–26 mg, 900 µL for 29–37 mg, and 1200 µL for
215 40–48 mg). Equal volumes of lipid extracts were then loaded for ultra-high-performance
216 liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC/Q-TOF-MS).
217 Internal standards and a serial dilution curve were included to control for extraction efficiency
218 and injection volume. Lipid classes and species were annotated using ProFinder B.08 Agilent
219 MassHunter software (Agilent Technologies, USA). Raw lipid spectral counts were
220 normalized to the concentration of sample loaded for UHPLC/Q-TOF-MS (mg/µL).
221 Hierarchical clustering of Manhattan distances between samples was performed and one
222 sample, determined to be an outlier, was excluded from downstream analyses. The data were
223 then log transformed to obtain normal distribution.

224 ***Statistical analysis***

225 The Control group was used to confirm that differences observed in the FABS group were not
226 an artefact of time or randomization. Therefore, FABS *versus* Control group comparisons
227 were performed only at baseline, and intervention effects were determined within-group.

228 Statistical analyses were performed in Prism 8.4.3 (GraphPad, San Diego, CA, US) and R
229 3.6.3, in an unblinded manner, and data normality was assessed using the Shapiro-Wilk test.
230 Repeated measurements data were compared by mixed-design analysis of variance (i.e.
231 OGTT glucose and insulin curves, 24-h glucose and steps curves, and High- *versus* Low-
232 activity subgroup analyses) or Friedman's test (i.e. protocol adherence). Paired Student's
233 *t*/Wilcoxon signed-rank, or unpaired Student's *t*/Mann-Whitney U tests were applied for
234 within group (pre-to-post) or between group (baseline) comparisons, respectively. Student's *t*-
235 test was used to compare lipidomics data and significance threshold was set at $p<0.01$. For all
236 other analyses, $p<0.05$ was considered significant and trends <0.1 are reported. Effect sizes
237 were calculated for statistically changed clinical and glucose variability parameters in the
238 FABS group using Glass' delta (Δ) (i.e. $mean_1 - mean_2 / {}_{SD1}$, where $mean_1$ and ${}_{SD1}$ represented
239 baseline mean and SD, respectively). Data are presented as mean (±SD) or median [25% to
240 75% IQR], unless otherwise stated.

Results

FABS increased number of steps taken and time spent walking per day

Activity data was collected for 7 [2 to 7] baseline and 20.5 [15 to 21] intervention days (median [range] of all subjects, Figure 2A). *Post hoc* analysis of activity adherence was performed, considering successful adherence to be any increase in hourly steps and/or postural transitions above each participant's own, median, baseline levels between the hours of 08:00-18:00 (Figure 2B and 2C). The analysis suggested that participant adherence to the breaking sitting protocol was high in FABS during the first week of intervention (i.e. trial week 2) but dissipated towards baseline levels for 6/8 participants through weeks 3-4 (Figure 2D). Nevertheless, there was no statistical difference in adherence over time in either group (Friedman test, $p \geq 0.398$). Additionally, 1 participant in FABS did not increase their activity during the trial. The distribution of steps taken across the day differed between groups at baseline (Figure 2E, $p=0.036$ for time and group interaction). Number of steps taken varied across the day in both groups, but there was no effect of the intervention on 24-h stepping curves compared to baseline (Figures 2F, G). During intervention weeks, FABS altered activity levels compared to baseline, resulting in a median increase of 744 [483 to 951] steps per day (3,285 [2,058 to 4,014] baseline *versus* 3,926 [2,921 to 5,281] intervention, $p=0.008$) and a corresponding 10.4 [2.2 to 24.6] min more time spent walking (80.8 [52.8 to 103.2] baseline *versus* 96.8 [71.4 to 119.9] intervention, $p=0.008$) (Figures 2H, I). No changes in daily step count (4,211 [3,503 to 6,297] baseline *versus* 4,255 [3,412 to 6,208] intervention, $p=0.547$) or walking time (105.2 [75.2 to 146.5] baseline *versus* 110 [84.1 to 165.4] intervention, $p=0.461$) were observed in the Control group. Despite greater ambulation, other indices of sedentary behavior, such as the number of postural transitions made from sitting-to-standing (Control: 51 [±14] baseline *versus* 55 [±15] intervention, $p=0.136$; FABS: 52 [±12] baseline *versus* 56 [±12] intervention, $p=0.245$) and total time spent seated (Control: 491.6

[442.8 to 608.5] baseline *versus* 487 [424.9 to 600.8] min intervention, *p=0.945*; FABS 598 [533.5 to 665.8] baseline *versus* 599.8 [470.6 to 697.9] min intervention, *p=0.945*), were unchanged from baseline in either group (Figures 2J, K).

FABS had no effect on glucose tolerance

Participants in both groups were insulin resistant, as indicated by a HOMA2-IR >1.21 and a Matsuda Index <5 (49); however, the FABS group tended to have better HIRI (Control 161.2 [±41.3] *versus* FABS 55.9 [±41.3], *p<0.0001*) and HOMA2-%S (Control 52.9 [±17.0] *versus* FABS 93.4 ± [54.7], *p=0.067*) scores at baseline (Table 1). The intervention did not improve glucose tolerance (Figures 3A-F) or approximates of insulin resistance/sensitivity (Table 1) in Control or FABS groups. Accordingly, incremental areas under the curve (iAUC) for glucose (Control: 284.8 [±187.7] pre *versus* 298.8 [±215.1] mmol/L/2 h post, *p=0.645*; FABS: 228.6 [±143.3] pre *versus* 242.3 [±165.5] mmol/L/2 h post, *p=0.831*) and insulin (Control: 7339 [±1975] pre *versus* 7462 [±1990] mIU/L/2 h post, *p=0.895*; FABS: 8148 [±2938] pre *versus* 9227 [±5028] mmol/L/2 h post, *p=0.346*) excursions during an OGTT were also unchanged (Figures 3C, F). Post-trial fasting plasma glucose concentrations were reduced only in FABS (-0.34 [±0.37] mmol/L, *p=0.037*, Δ=0.33) (Table 1), although this group also had a tendency for serum insulin concentrations to be slightly higher at the 2-h timepoint of the post-trial OGTT compared to pre-trial levels (+20.2 [±21.8] mIU/L, *p=0.073*) (Table 1). Additionally, in the FABS group there was a non-significant reduction of fasting plasma low-density lipoprotein cholesterol (LDLc; -0.30 [-0.48 to -0.15] mmol/L, *p=0.078*), which was not present in the Control group (Table 1). Participants remained weight stable over the 4-week trial and all other clinical chemistry was unchanged relative to pre-trial values (Table 1).

FABS did not alter average interstitial glucose levels but marginally lowered glycemic variability

Continuous interstitial glucose readings were collected for 7 [2 to 7] baseline days and 21 [12 to 21] intervention days (Figure 4A; median [range] of n=14 participants; n=6 Control and n=8 FABS). Average interstitial fluid glucose readings, as measured by CGM, were not different between groups during the baseline week (Figure 4B). Glucose levels varied across the day; however, there was no effect of the intervention on 24-h glucose curves in either group (Figures 4C, D). Intra-day variations in glucose recordings were lower in 6 out of 8 FABS participants for CONGA1 (0.9 [±0.2] *versus* 0.8 [±0.1] mmol/L, *p=0.279*) and CONGA2 (1.0 [±0.2] *versus* 0.9 [±0.2] mmol/L, *p=0.409*) relative to baseline (Figures 4E, F), but no pattern of change was evident for CONGA4 (1.1 [±0.2] *versus* 1.0 [±0.2] mmol/L, *p=0.212*) (Figure 4G). Additionally, the daily standard deviation of glucose was reduced in 5 out of 8 participants in the FABS group during the intervention period (0.8 [±0.1] *versus* 0.7 [±0.1] mmol/L, *p=0.129*) (Figure 4H) and this subtle decrease reached statistical significance when normalized to each individual's average daily glucose level (i.e. %CV) (16.3 [±2.1] *versus* 14.3 [±2.7] %, *p=0.039*, Δ=0.94) (Figure 4I).

Greater volume of FABS more consistently and potently lowered glucose variability

FABS participants were divided into high- (n=4 females) and low- (n=4, 1 female and 3 males) activity subgroups based on the combined total steps and postural transitions performed per day during the intervention period (Figure 5A). Those participants who performed the most activity during the intervention had higher glucose levels during the post-trial OGTT, compared to those who performed less activity, as indicated by a main effect of subgroup (*p=0.044*) (Figure 5B). However, there were no between- or within-subgroup differences in glucose iAUC (Figure 5C) and insulin response was also unaffected by activity volume during the trial period (Figures 5D, E). The only female participant in the Low-activity subgroup was excluded from OGTT analyses, due to insufficient datapoints; as such, the impact of activity cannot be separated from any potential effect of sex in the assessment of

glucose tolerance. Individuals with higher activity levels tended to more consistently and potently lower their glucose variability (Figures 5F-J), as suggested by subgroup and time interactions for all parameters of dynamic glucose control (CONGA1, *p=0.018*; CONGA2, *p=0.028*; CONGA4, *p=0.092*; SD, *p=0.018*; %CV, *p=0.059*), driven by baseline-to-intervention differences in the High-activity subgroup (CONGA1, -0.21 [±0.10] mmol/L, *p=0.006,* Δ=0.85; CONGA2, -0.24 [±0.12] mmol/L, *p=0.013*, Δ=0.88; CONGA4, -0.25 [±0.17] mmol/L, *p=0.040*, Δ=0.97; SD, -0.20 [±0.10] mmol/L, *p=0.014*, Δ=1.24; %CV, -3.4 [±2.1]%, *p=0.016*, Δ=1.80) that were not present in the Low-activity subgroup.

FABS did not strongly perturb the skeletal muscle lipidome

Next, we performed a lipidomic analysis of *vastus lateralis* skeletal muscle biopsies obtained before and after the intervention. Triglycerides (TG) were the most abundant lipid class detected in this analysis, with 108 identified species, almost double the number of detected phosphatidylcholines (PC, 56 identified subspecies), which were the second most prominent class (Figure 6A). There was no difference in the skeletal muscle lipid profile between groups at baseline (Figure 6B, left panel); however, 2 long-chain saturated triglycerides were increased in the FABS group (both 1.25-fold, *p<0.01*), post-trial, whereas the lipid content of the Control group was unaltered (Figures 6B [center and right panels], C). Despite the observed changes in FABS, the overall tendency was for intramuscular triglycerides to decrease, as indicated by a median reduction of -0.59 log2 fold-change (Figure 6D). Furthermore, the intervention did not strongly affect the skeletal muscle lipidome, with lipid classes in both groups remaining largely unperturbed *versus* pre-trial levels (Figure 6D).

Discussion

In a free-living environment, reminders to break prolonged sitting resulted in a modest increase of stepping behavior across the day. Although insufficient to enhance glucose tolerance or impact the skeletal muscle lipidome, this change did lower fasting blood glucose and intra-day glucose variability, and tended to decrease LDLc levels, which could be clinically relevant.

The absence of improved glucose tolerance in our study somewhat contrasts with earlier investigations (9, 40). However, there are several differences in our free-living trial design that may account for this. Much of the literature reporting benefit from interrupting sedentary behavior comes from laboratory-based interventions (40) that increase the amount of time spent sitting in the control condition compared to habitual levels (46, 55). A single day of enforced sitting alters energy balance and attenuates whole-body insulin sensitivity (53). As such, previous trials may have inadvertently compared the effects of interrupting sitting to a control with reduced glucose tolerance, decreasing the ecological translatability of results.

Many of the controlled laboratory trials assessed effects on glucose and insulin levels when activity breaks were performed during the (≥4-h) postprandial period, after mixed macronutrient meals or drinks (3, 4, 13, 29, 32, 38, 47, 55, 56). Conversely, the post-trial glucose tolerance test in our study occurred the morning after the last day of intervention (i.e. in the absence of activity breaks) and contained only glucose, with glycemic responses measured over just a 2-h postprandial period. Thus, the timing and conditions of our glucose tolerance test might have contributed to the observed discrepancy with previous studies. The glucose- and insulin-lowering effects of 17 x 2-min intervals of light walking per day (i.e. every 20 min for 7 h; 34 min total) are not cumulative over 3 days (38); consequently, any positive effects from lower volumes of light-intensity breaks may be lost the following day,

such as when the glucose tolerance test was undertaken in our study. Therefore, daily repetition may be necessary to sustain any glycemic benefit.

Volume, intensity, and frequency of activity might modify the metabolic response to FABS. Activity breaks consisting of 2 min of low-intensity walking every 20 min (28 total min) attenuate glucose (3, 47) and insulin excursions, and improve Matsuda Index (47), whereas interrupting sitting with an equal frequency and volume of standing has no effect. Yet, standing can improve glucose tolerance when longer durations of total standing time are used (5, 29, 55). In the few free-living trials published to date, baseline step counts (i.e. ≈3000-4500 per day) were comparable to our study (15-17). Correspondingly, the interventions in these studies used much larger volumes of physical activity (i.e. ≥2.5 h standing plus ≥17,500 steps per day) to improve insulin sensitivity and blood lipid profiles (14). Our intervention had a low-threshold activity requirement (i.e. ≥15 steps, every 30 min) and only increased daily steps by 744. Together, this implies greater intensities, frequencies, and/or volumes of physical activity breaks from sitting may be required to have a lasting effect on insulin sensitivity, with volume perhaps being the most important of these variables for overall health (51).

Despite no improvement in the post-trial OGTT response, the potential for larger volumes of activity breaks from sitting to confer a greater benefit on glycemic control is supported by our collective analyses of CGM, which suggest that FABS marginally lowers intra-day interstitial glucose variation (41) compared to baseline and that this response is most robust in participants with higher daily activity levels. Decreased glycemic variability may be partially accounted for by better peripheral blood perfusion. Inactivity promotes microvascular dysfunction (25), whereas light-intensity bodyweight exercise every 30 min increases flow-mediated femoral artery dilation (10, 54). Such improvements in glucose dispersion through- and subsequent uptake from- the skeletal muscle interstitium could

contribute to the greater 24-h oxidation of carbohydrates when 5-min bouts of moderate-intensity walking (i.e. Borg scale 13/20 RPE) are performed hourly, for 9 h (12). Nevertheless, the adherence data in our study question the feasibility of implementing frequent, longer-duration activity breaks from sitting under free-living conditions, especially during working hours; as successful compliance would be expected to increase indices of activity behavior above levels observed in FABS across all intervention weeks. For the benefit of public health, future effort should be dedicated to establishing breaking sitting protocols that integrate clinical efficacy with real-world practicality.

Here we report reductions in fasting blood glucose and a trend for decreased LDLc levels after 3 weeks of breaking sedentary behavior. The mechanisms by which fasting glucose concentrations are lowered might include improvements in blood flow to skeletal muscle (10, 54), as well as the attenuation of systemic inflammation. Inflammatory and endoplasmic reticulum stress pathways are upregulated in skeletal muscle after 9 days of bed rest inactivity (1); whereas 2-min bouts of low-intensity walking, every 20 min during a 5-h postprandial period, downregulate inflammatory pathways in subcutaneous adipose tissue (22). Furthermore, cross-sectional analysis of 4,757 individuals associates FABS with lower levels of C-reactive protein (CRP) (27). As CRP positively correlates with fasting glucose (2), subsequent trials may look to determine the long-term impact of interrupting sitting on inflammatory markers, as well as skeletal muscle hemodynamics. Intriguingly, the risk of developing coronary heart disease might be ≈12% lower for individuals with fasting glucose between 3.9-5.59 *versus* 5.6-6.99 mmol/L (20). Thus, if the modest effect on fasting glucose in our intervention group is maintained, it could have a clinically meaningful impact on metabolic health.

Retrospective analysis of shorter-term free-living studies provides evidence that replacing sitting with a combination of standing and walking lowers non-HDLc (14). Our data

suggest FABS may also cause specific changes in LDLc. Exercise increases the rate at which LDLc is cleared from circulation and this effect is abolished in LDL-receptor knockout mice (58), suggesting that physical activity increases the tissue density and/or activity of LDL-receptors. Upregulation of the LDL-receptor may take time to manifest into notable changes in LDLc and further trials of sufficient duration are required to replicate this finding, before establishing the dose-response and time course of effect.

Contrary to our hypothesis, we found little impact of FABS on the skeletal muscle lipidome, with only 2 lipid species changed and no alteration in the overall abundance of any lipid class observed. Interrupting sitting (29, 32) or reducing overall sedentary behavior (15) elevates non-esterified fatty acids and/or attenuates triglycerides in circulation, and 2 min of light-intensity walking every 20 min, for 8 h, increases whole-body fat oxidation *versus* uninterrupted sitting (56). These effects may be mediated, in part, by the contraction-induced upregulation of skeletal muscle lipoprotein lipase (LPL) activity (6, 31), further suggesting that breaking sitting may influence intramuscular lipid composition. We previously reported that reductions of specific intramuscular lipids correlate with approximates of systemic insulin sensitivity in men and women with obesity, after 3 weeks of low-calorie dieting (44). As such, interventions that break sedentary time with more robust disruptions to homeostasis may be required to perturb the skeletal muscle lipidome in the absence of weight loss.

We investigated the impact of breaking sitting using a free-living design. This approach increases the ecological validity and translational efficacy of our findings, but introduces logistical limitations, such as a lack of standardized nutrition. Energy content and glycemic index of meals can influence subsequent glucose and insulin excursions (4, 46). Because dietary intakes were not recorded, we cannot exclude the possibility that nutritional differences from baseline (e.g. calorie content and macro-/micro- nutrient composition per meal, or meal frequency) affected our findings, despite study groups being encouraged to

maintain typical dietary behaviors. Nevertheless, that participants were weight-stable indicates overall energy consumption during the trial was consistent with habitual calorie intakes. A lack of standardized meal timing also meant we were unable to perform targeted analysis of postprandial glycemia during intervention weeks. The use of CONGA*n* allows for the assessment of intra-day glucose variation without the need for defined meal or exercise times (41). An additional limitation of our intervention includes the absence of menopausal assessment or menstrual cycle normalization over baseline and intervention weeks. In premenopausal women, surrogate insulin resistance (i.e. HOMA1-IR) fluctuates mildly across the menstrual cycle, such that resistance is lowest at menses, increases around ovulation, and is highest during the luteal phase (59). Variations in HOMA1-IR reflect changes in serum insulin and are positively associated with circulating levels of estradiol and progesterone, but inversely related to follicle-stimulating hormone (59). Lastly, it is possible that we were underpowered to detect subtle differences resulting from FABS in some of the outcomes measured.

To our knowledge, this is the longest duration study to investigate the therapeutic impact of FABS and, as such, our findings have important translational implications. Insulin sensitivity and glucose tolerance remained unaltered, while fasting blood glucose and daily glucose variation were modestly reduced, with a trend for lowered LDLc. Our intervention may represent the minimum effective dose for breaking sedentary behavior, with larger volumes of total activity required to elicit greater health benefits. Future studies should establish the relationship between frequency, intensity, and volume of activity breaks, and how these variables interact with demographics across the spectrum of metabolic health. Importantly, the activity data reported in our study question the ecological practicality of regular 3-min bouts of activity. Since exercise offers some distinct cardiometabolic benefits (14), studying the potential of combining activity breaks from sitting with formal exercise

462 regimens could provide the most effective solution for public health improvement, consistent
463 with recent calls promoting the message of *sit less, move more, more often* (19).

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Declarations of Interest

The authors declare no relevant conflicts of interest.

Author contributions

J.A.B.S. researched data and wrote the manuscript; M.S. researched data and wrote the manuscript; P.S. researched data and reviewed/edited the manuscript; S.P. researched data and reviewed/edited the manuscript; J.A.H. contributed to the study design and reviewed/edited the manuscript; D.D. contributed to the study design and discussion, and reviewed/edited the manuscript; B.M.G. researched data and reviewed/edited the manuscript; A.K. contributed to the study design and discussion and reviewed/edited the manuscript; J.R.Z. contributed to the study design and discussion and wrote the manuscript; E.N. contributed to the study design and discussion, and wrote the manuscript.

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712 **Tables**

713 **Table 1. Participant Characteristics and Clinical Chemistry**

	Control (n = 8; 5 females, 3 males)		FABS (n = 8; 5 females, 3 males)	
	Pre	Post	Pre	Post
Anthropometry				
Age (years)	47 ± 5		49 ± 10	
Body mass (kg)	95.4 ± 14.2	95.4 ± 14.3	97.6 ± 13.8	97.6 ± 13.9
BMI (kg/m^2)	34.3 ± 3.9	34.4 ± 4.0	33.4 ± 3.6	33.3 ± 3.6
Waist circumference (cm)	107.3 ± 8.5	107.0 ± 8.9	109.9 ± 10.3	109.3 ± 11.4
Clinical chemistry				
HbA1c (%)	5.6 ± 2.5	5.6 ± 2.6	5.2 ± 2.7	5.2 ± 2.4
HbA1c (mmol/mol)	37.4 ± 4.3	37.3 ± 4.6	33.3 ± 6.2	32.8 ± 4.3
Fasting glucose (mmol/L)	5.7 ± 0.5	5.6 ± 0.5	5.7 ± 0.9	5.4 ± 0.7
[a]2-h glucose (mmol/L)	7.8 ± 2.9	7.2 ± 3.3	6.5 ± 2.2	7.8 ± 2.5
[a]Fasting insulin (mU/L)	16.4 ± 8.0	16.7 ± 6.7	10.8 ± 5.8	12.1 ± 7.2
[b]2-h insulin (mU/L)	79.9 ± 40.9	74.1 ± 35.2	74.1 ± 72.6	94.3 ± 65.0[#]
Matsuda Index	2.8 ± 0.9	2.8 ± 1.2	4.4 ± 2.9	4.5 ± 3.3
[a]HIRI	161.2 ± 41.3	163.7 ± 86.5	55.9 ± 21.0*	62.8 ± 34.4
[a]HOMA2-IR	2.2 ± 1.1	2.2 ± 0.9	1.4 ± 0.8	1.6 ± 0.9
[a]HOMA2-%β	117.9 ± 19.8	128.8 ± 46.2	100.7 ± 32.7	112.1 ± 41.8
[a]HOMA2-%S	52.9 ± 17.0	54.5 ± 27.9	93.4 ± 54.7[†]	100.0 ± 86.1
Triglycerides (mmol/L)	1.4 ± 0.8	1.3 ± 0.6	1.7 ± 0.7	1.9 ± 1.0
Cholesterol (mmol/L)	5.4 ± 1.3	5.4 ± 1.1	5.2 ± 1.0	5.1 ± 0.6
HDLc (mmol/L)	1.6 ± 0.7	1.3 ± 0.2	1.3 ± 0.4	1.3 ± 0.4
LDLc (mmol/L)	3.1 ± 1.3	3.5 ± 0.9	3.2 ± 0.7	3.0 ± 0.5[#]

714 Data are presented as mean ± SD. [a]n=7 in FABS group, [b]n=6 in FABS group. [#]*p≤0.078*,
715 FABS post *versus* FABS pre. **p<0.0001* and [†]*p=0.067*, FABS pre *versus* Control pre. Paired
716 and unpaired Student's *t*-test.

Figure Legends

Figure 1. Schematic summary of the Frequent Activity Breaks from Sitting (FABS) intervention. Participants (n=16) were randomized to Control (n=8) or FABS (n=8) groups. Activity and glucose continuous monitoring data were collected for 1 week of baseline and 3 weeks of intervention. Blood and skeletal muscle samples were taken, anthropometric measurements made, and an oral glucose tolerance test (OGTT) performed at visits 1 and 2 (V1, V2).

Figure 2. FABS increased number of steps taken and time spent walking per day. (A) Distribution of continuous activity data collected during baseline (days 1–7) and intervention (days 8–28) periods for Control (n=8) and FABS (n=8) groups. Shades of blue represent number of hours of continuous data collected and grey represents complete lack of data. (A, B) Individual participant's median (A) steps and (B) transitions per hour from 08:00 to 18:00 during baseline (i.e. week 1). (C) Weekly adherence to intervention protocol (%). Weeks 2-4 are intervention weeks, black lines indicate median group adherence for each week, and connecting lines represent patterns of adherence for each participant. (E) Pattern of daily stepping activity during baseline for Control (blue, n=8) and FABS (orange, n=8) groups. Data are mean (±SD) of median steps taken per hour. Mixed-design analysis of variance (Time, Intervention), #overall time affect and ‡time and group interaction ($p<0.05$). (F, G) Pattern of daily stepping activity during intervention weeks compared to baseline. Data are mean (±SD) of median steps taken per hour. Paired mixed-design analysis of variance (Time, Intervention), #overall time affect ($p<0.05$). (H-K) Median number of daily (H) steps taken, minutes spent (I) walking and (J) sitting, and (K) transitions made from sitting to standing postures during intervention weeks compared to baseline. Wilcoxon signed-rank (within-group) and Mann-Whitney U (baseline between-group) test, *$p<0.05$. Control group (blue, n=8), FABS group (red, n=8).

Figure 3. FABS had no effect on glucose tolerance. Pre-to-post trial 2-h OGTT curves and incremental areas under the curves (iAUC) for (A–C) glucose and (D–F) insulin. Data are mean (±SD) for Control (blue, n=8) and FABS (red, n=7) groups. Paired mixed-design analysis of variance (Time, Intervention), #overall time affect ($p<0.05$).

Figure 4. FABS did not alter average interstitial glucose levels but marginally lowered glycemic variability. (A) Distribution of collected continuous interstitial glucose data during baseline (days 1–7) and intervention (days 8–28) periods for Control (n=8) and FABS (n=8) groups. Shades of blue represent number of hours of continuous data collected and grey represents complete lack of data. (B) 24-h hourly glucose means during baseline for Control (blue, n=6) and FABS (orange, n=8) groups. Data are mean (±SD). Mixed-design analysis of variance (Time, Intervention), #overall time affect ($p<0.05$). (C, D) 24-h hourly glucose means during intervention weeks compared to baseline. Data are mean (±SD) for Control (blue, n=6) and FABS (red, n=8) groups. Paired mixed-design analysis of variance (Time, Intervention), #overall time affect ($p<0.05$). (E-I) Indices of dynamic glucose control. Mean daily continuous overall net glycemic action (CONGA) for (E) 1-, (F) 2-, and (G) 4-h intervals, and mean daily glycemic (H) standard deviation (SD) and (I) coefficient of variation (%CV) during intervention weeks compared to baseline. Wilcoxon signed-rank (within-group) and Mann-Whitney U (baseline between-group) test, *$p<0.05$. Control group (blue, n=6), FABS group (red, n=8).

Figure 5. Greater volume of FABS more consistently and potently lowered glucose variability. (A) FABS group participants were separated into Low (orange) and High (green) activity levels, based on the median steps and postural transitions per day during intervention weeks. (B-E) Pre- (baseline) and post- (intervention) trial individual 2-h oral glucose tolerance test (OGTT) curves and incremental areas under the curves (iAUC) for (B, C) glucose and (D, E) insulin. Mixed-design analysis of variance (Time, Subgroup); Baseline

#overall time effect ($p<0.05$); Intervention #overall time effect ($p\leq0.08$), †overall subgroup effect ($p<0.05$). Low subgroup (orange, n=3 males), High subgroup (green, n=4 females). (F-J) Indices of dynamic glucose control color-coded according to participant activity level in FABS. Continuous overall net glycemic action (CONGA) for (B) 1-, (C) 2-, and (D) 4-h intervals, and mean daily glycemic (E) standard deviation (SD) and (F) coefficient of variation (%CV) during intervention weeks compared to baseline. Mixed-design analysis of variance (Time, Subgroup), #overall time effect ($p\leq0.052$), ‡time and subgroup interaction ($p\leq0.092$). Low subgroup (orange; n=4, 1 female and 3 males), High subgroup (green; n=4 females).

Figure 6. FABS did not strongly perturb the skeletal muscle lipidome. (A) Lipid classes and total number of lipid species within these classes identified in the skeletal muscle lipidomics analysis. TG = triglycerides, PC = phosphocholines, PE = phosphoethanolamines, CAR = fatty acyl carnitines, SM = sphingomyelins, PI = phosphoinositols, PG = phosphoglycerols, PS = phosphoserines, DG = diacylglycerols, Cer = ceramides. (B) Volcano plots of baseline differences in lipid species between groups (left) and pre-to-post trial changes of detected lipid species in Control (center) and FABS (right) groups. Dashed lines indicate significance threshold, paired (within-group) and unpaired (baseline between-group) Student's *t*-test, *$p<0.01$. (C) Individual participant data of the 2 triglycerides that exceeded the significance threshold in (B). (D) Median pre-to-post trial fold changes within each lipid class in the Control (left) and FABS (right) groups. Line denotes the median, box represents IQR, and error bars show minimum and maximum change for Control (n=8) and FABS (n=7) groups, respectively.

Figure 1

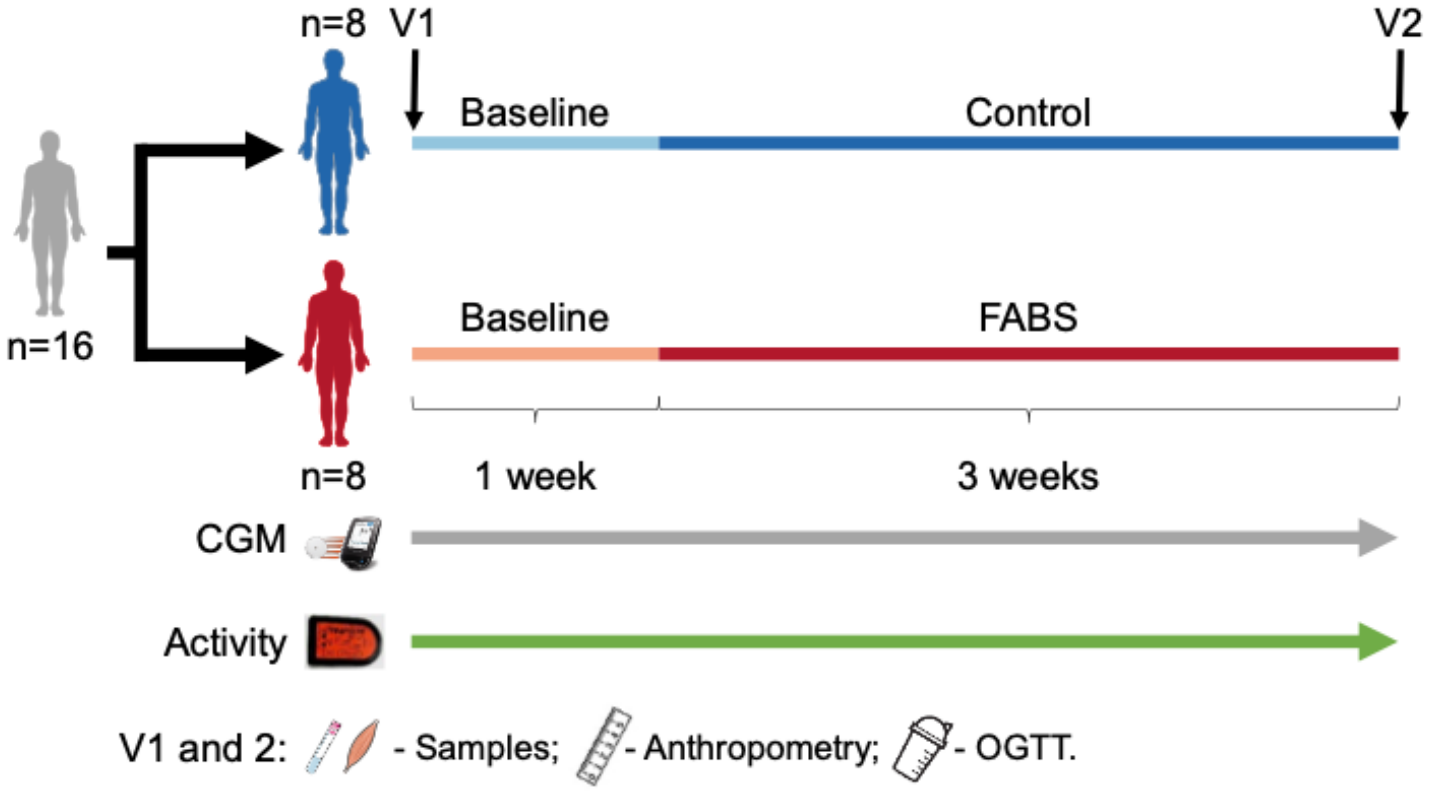


Figure 2

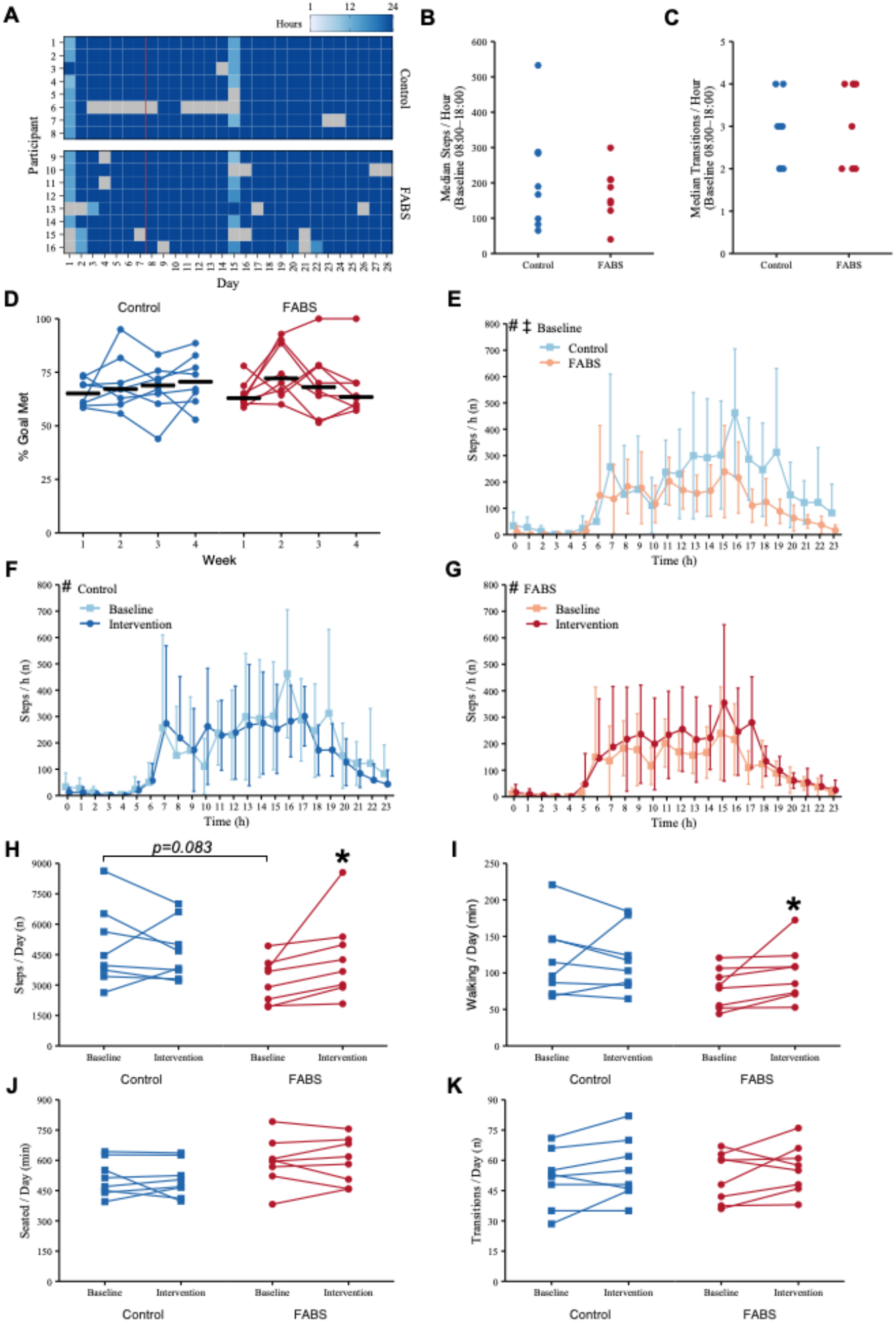


Figure 3

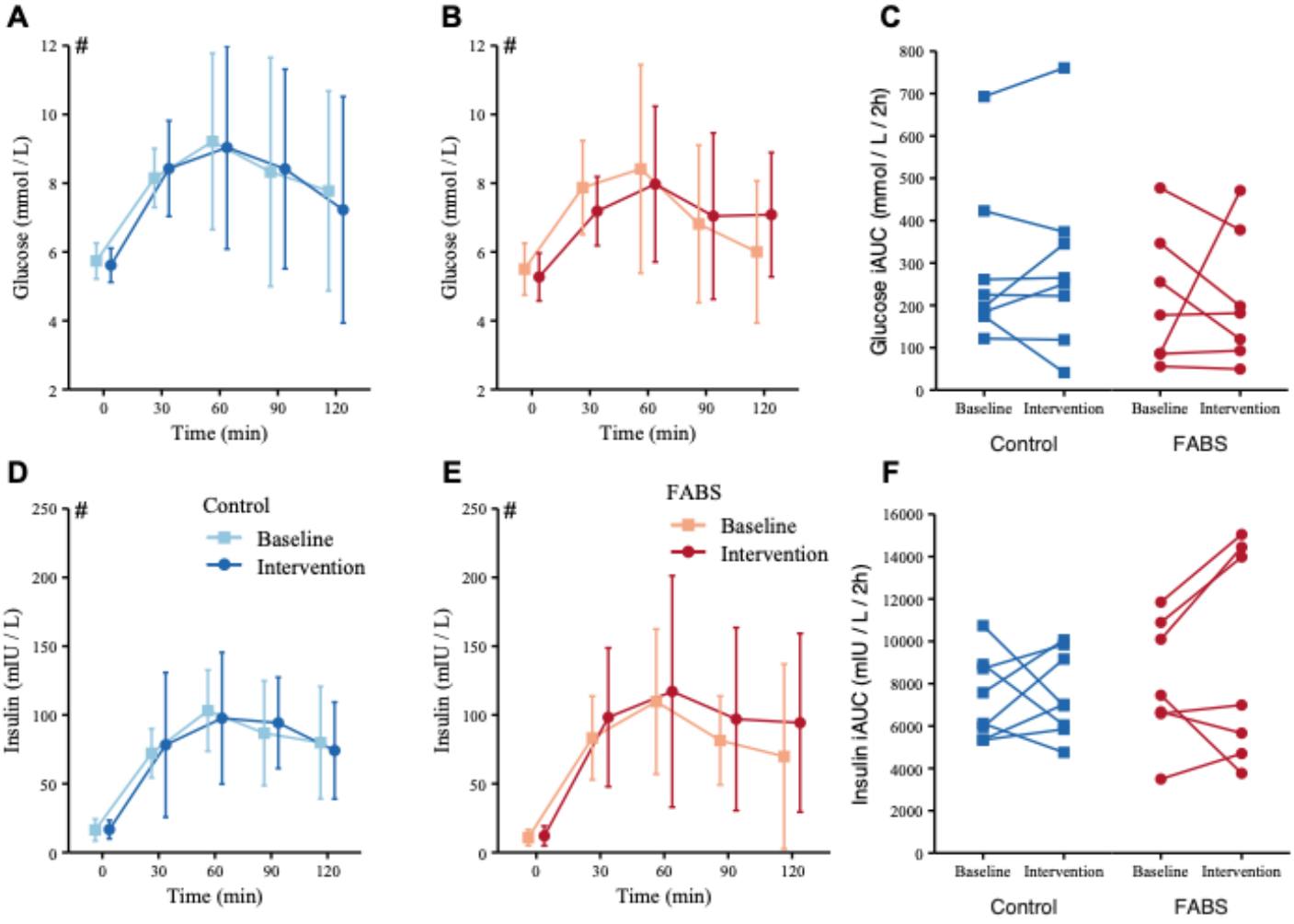


Figure 4

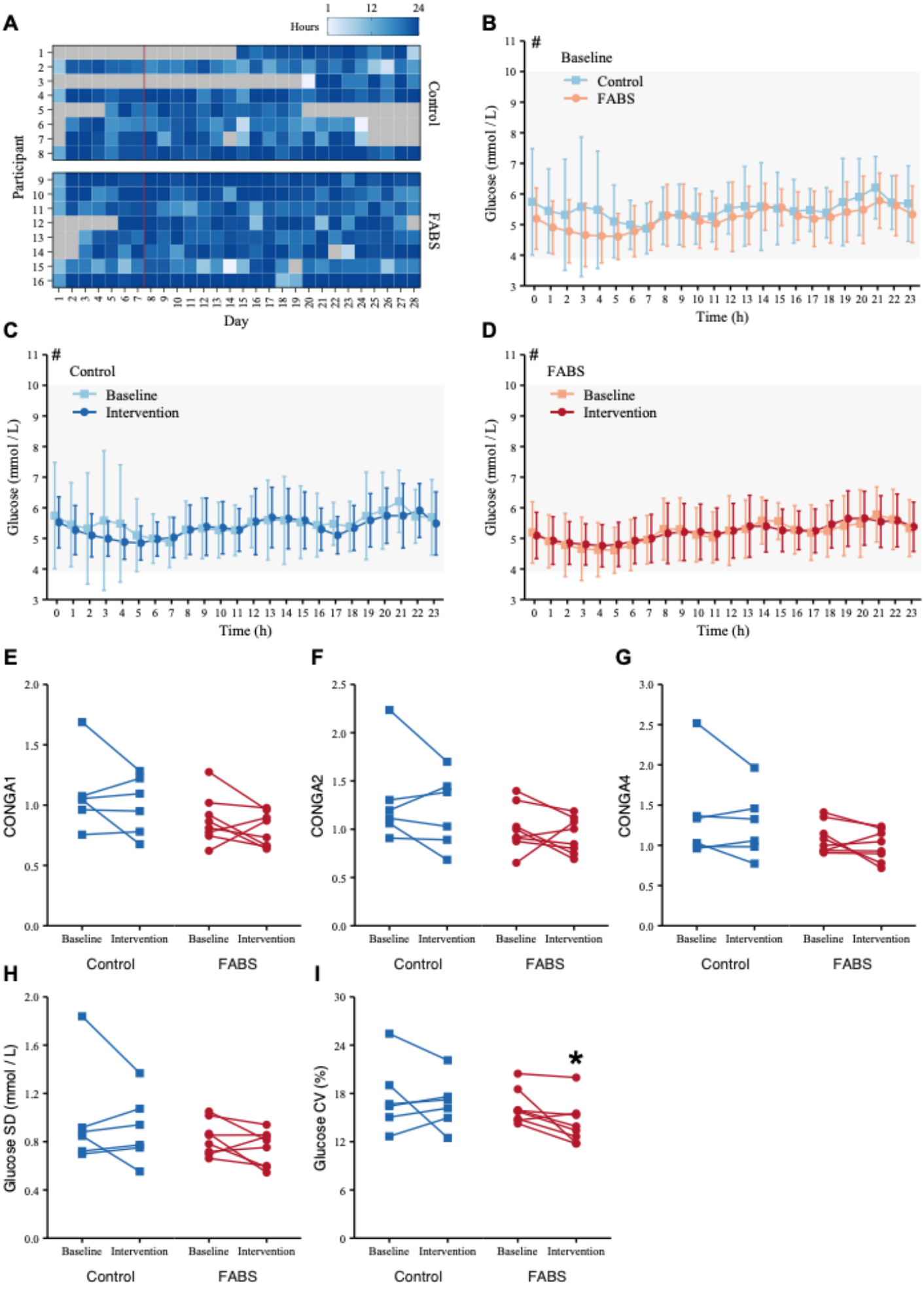


Figure 5

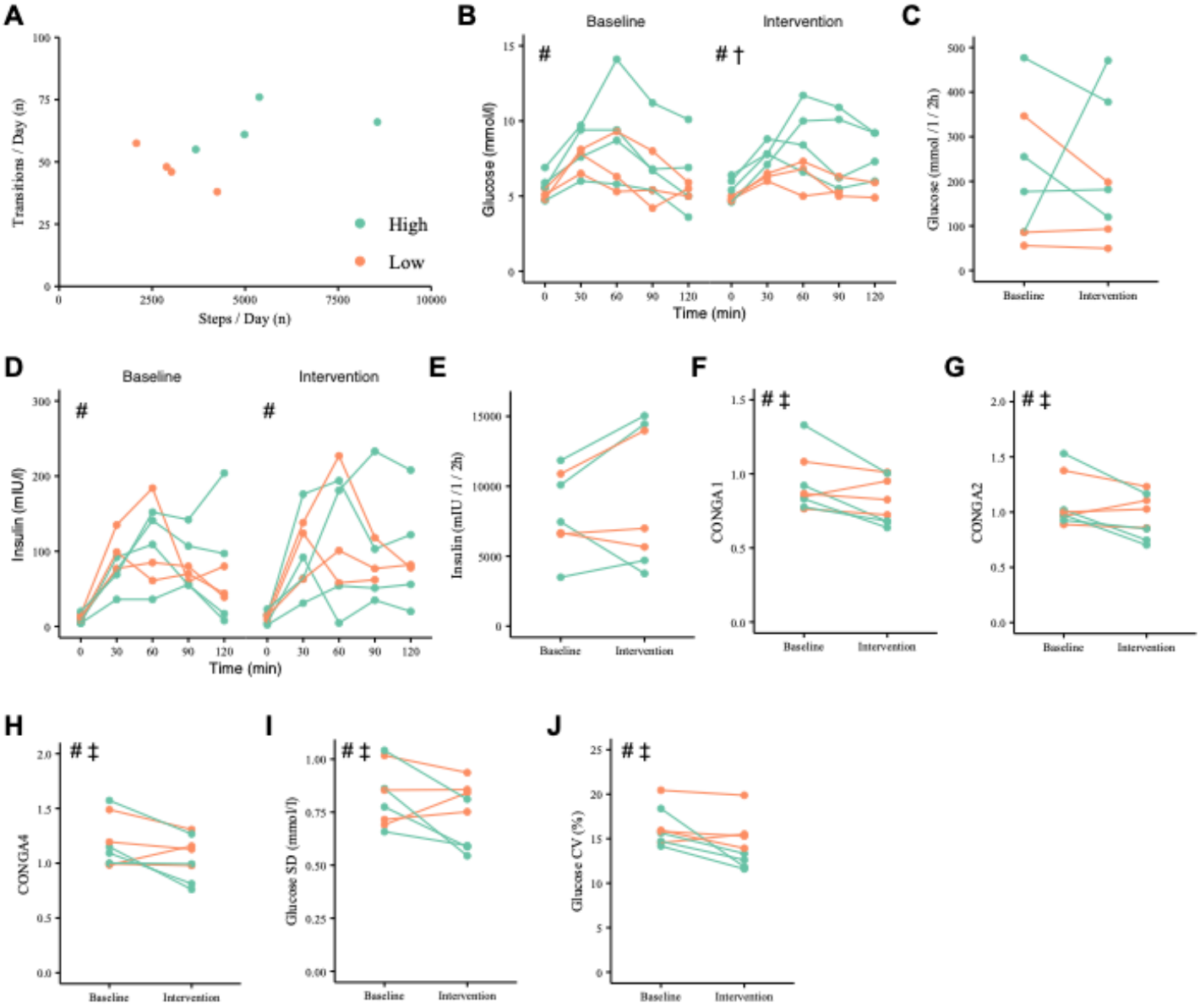
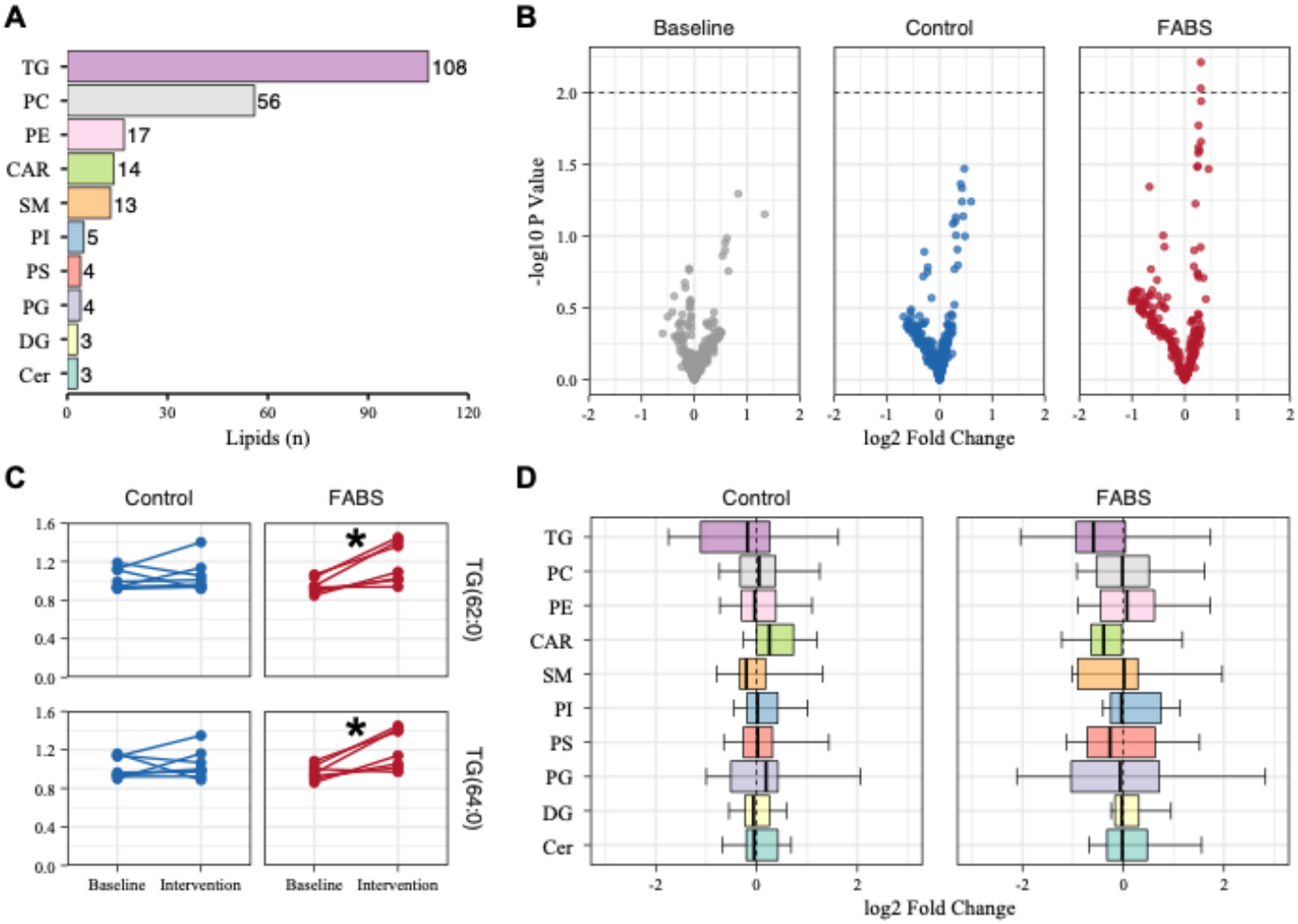


Figure 6



Tables

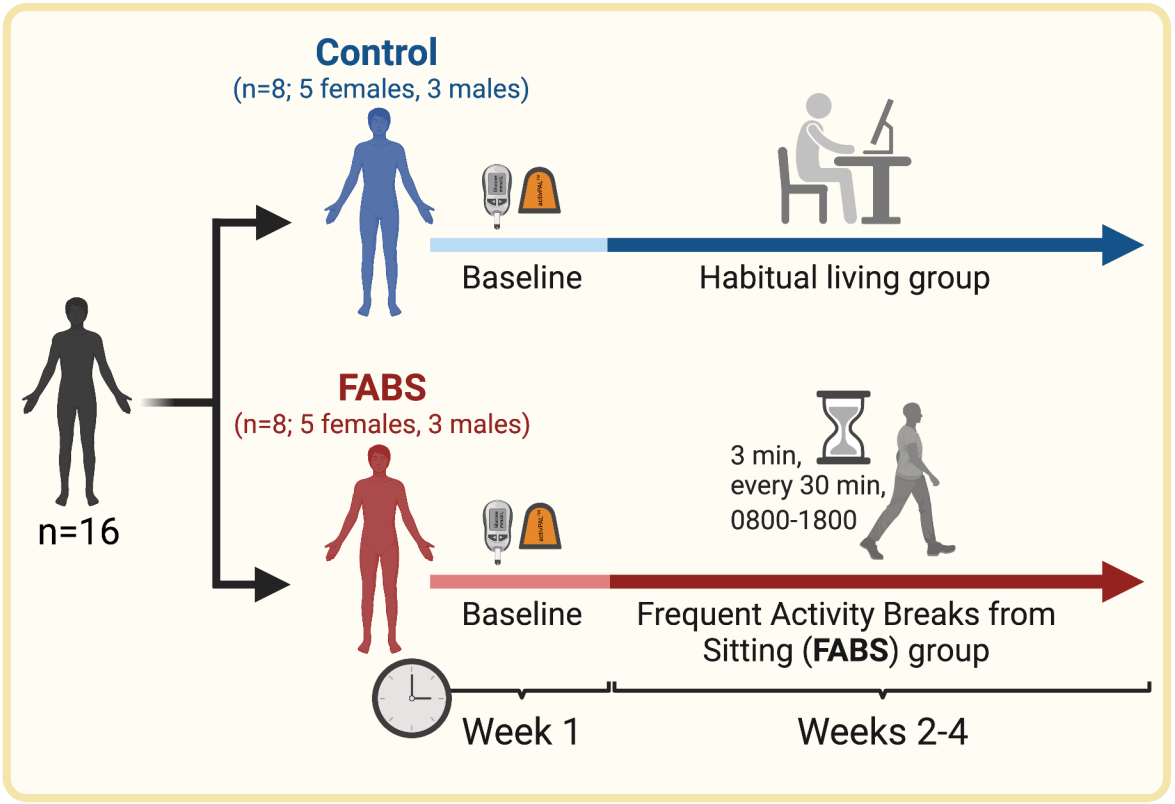
Table 1. Participant Characteristics and Clinical Chemistry

	Control (n = 8; 5 females, 3 males)		FABS (n = 8; 5 females, 3 males)	
	Pre	Post	Pre	Post
Anthropometry				
Age (years)	47 ± 5		49 ± 10	
Body mass (kg)	95.4 ± 14.2	95.4 ± 14.3	97.6 ± 13.8	97.6 ± 13.9
BMI (kg/m^2)	34.3 ± 3.9	34.4 ± 4.0	33.4 ± 3.6	33.3 ± 3.6
Waist circumference (cm)	107.3 ± 8.5	107.0 ± 8.9	109.9 ± 10.3	109.3 ± 11.4
Clinical chemistry				
HbA1c (%)	5.6 ± 2.5	5.6 ± 2.6	5.2 ± 2.7	5.2 ± 2.4
HbA1c (mmol/mol)	37.4 ± 4.3	37.3 ± 4.6	33.3 ± 6.2	32.8 ± 4.3
Fasting glucose (mmol/L)	5.7 ± 0.5	5.6 ± 0.5	5.7 ± 0.9	5.4 ± 0.7
[a]2-h glucose (mmol/L)	7.8 ± 2.9	7.2 ± 3.3	6.5 ± 2.2	7.8 ± 2.5
[a]Fasting insulin (mU/L)	16.4 ± 8.0	16.7 ± 6.7	10.8 ± 5.8	12.1 ± 7.2
[b]2-h insulin (mU/L)	79.9 ± 40.9	74.1 ± 35.2	74.1 ± 72.6	94.3 ± 65.0[#]
Matsuda Index	2.8 ± 0.9	2.8 ± 1.2	4.4 ± 2.9	4.5 ± 3.3
[a]HIRI	161.2 ± 41.3	163.7 ± 86.5	55.9 ± 21.0*	62.8 ± 34.4
[a]HOMA2-IR	2.2 ± 1.1	2.2 ± 0.9	1.4 ± 0.8	1.6 ± 0.9
[a]HOMA2-%β	117.9 ± 19.8	128.8 ± 46.2	100.7 ± 32.7	112.1 ± 41.8
[a]HOMA2-%S	52.9 ± 17.0	54.5 ± 27.9	93.4 ± 54.7[†]	100.0 ± 86.1
Triglycerides (mmol/L)	1.4 ± 0.8	1.3 ± 0.6	1.7 ± 0.7	1.9 ± 1.0
Cholesterol (mmol/L)	5.4 ± 1.3	5.4 ± 1.1	5.2 ± 1.0	5.1 ± 0.6
HDLc (mmol/L)	1.6 ± 0.7	1.3 ± 0.2	1.3 ± 0.4	1.3 ± 0.4
LDLc (mmol/L)	3.1 ± 1.3	3.5 ± 0.9	3.2 ± 0.7	3.0 ± 0.5[#]

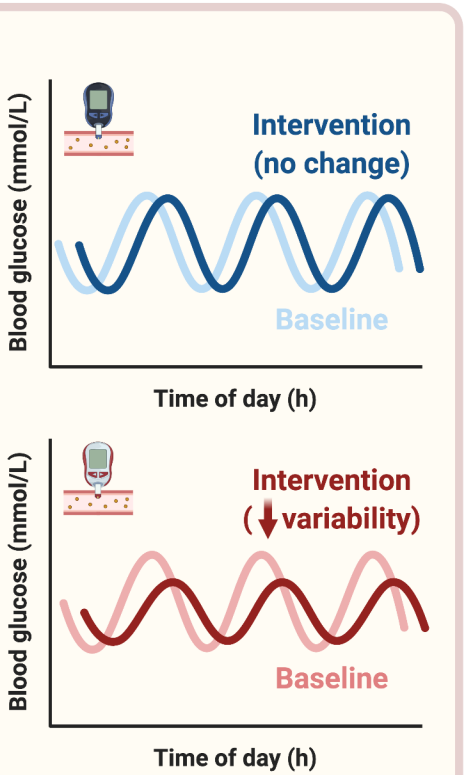
Data are presented as mean ± SD. [a]n=7 in FABS group, [b]n=6 in FABS group. [#]*p≤0.078*, FABS post *versus* FABS pre. **p<0.0001* and [†]*p=0.067*, FABS pre *versus* Control pre. Paired and unpaired Student's *t*-test.

Three Weeks of Interrupting Sitting Lowers Fasting Glucose and Glycemic Variability, but not Glucose Tolerance, in Free-Living Women and Men with Obesity

Methods



Results



Conclusion

Under free-living conditions, FABS marginally lowered fasting glucose and glucose variability. Larger volumes of activity breaks from sitting may be required to promote greater health benefits.