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Time-restricted eating in people at high diabetes risk does not affect mitochondrial bioenergetics in peripheral blood mononuclear cells and platelets

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Overweight and obesity are linked to mitochondrial alterations, impaired glucose tolerance and a high risk of type 2 diabetes. Time-restricted eating (TRE) may aid in facilitating weight loss to prevent diabetes. Here, we investigated if TRE in individuals with overweight and prediabetes or obesity affects mitochondrial bioenergetics of peripheral blood mononuclear cells (PBMCs) and platelets using the Seahorse extracellular flux technology. In a 3-month randomized controlled trial, PBMCs/platelets were analyzed from 52 participants before and after a TRE intervention with a 10-h eating window or habitual living. PBMC and platelet respiratory function was evaluated through sequential addition of substrates, uncouplers, and inhibitors in living cells. After 3 months, there were no statistically significant differences in mitochondrial respiration within or between the TRE and control groups. Association analyses between PBMC/platelet respiration and clinical parameters including body mass index and fat mass showed no significant effects. In conclusion, 3 months of 10-h TRE does not alter the mitochondrial bioenergetics of PBMCs and platelets in individuals with high risk of type 2 diabetes.

Keywords Peripheral blood mononuclear cells (PBMCs), Bioenergetics, Seahorse extracellular flux, Mitochondria, Oxidative phosphorylation, Oxygen consumption rate (OCR), Time-restricted eating (TRE), Type 2 diabetes (T2D), Pre-diabetes, Overweight, Obesity, Platelets

Overweight and obesity are associated with insulin resistance leading to impaired glucose tolerance and development of type 2 diabetes (T2D). In the healthy state, metabolic processes in the human body are synchronized and made more efficient by the intrinsic circadian clock, which predicts the body's fluctuations in metabolic activity due to feeding/fasting and energy expenditure/rest[1,2]. However, a modern lifestyle with unlimited food availability around the clock, and irregular sleeping patterns interfere with this evolutionary energy balance system and are important contributary factors to overweight, insulin resistance and progression to T2D[3–6]. Time-restricted eating (TRE) has gained attention due to its potential to reduce body weight, improve insulin sensitivity, and reduce blood pressure in individuals with overweight, obesity, and/or T2D[7–9]. However, in a recent study (the RESET trial) we reported that 3 months of 10-h per day TRE in overweight or obese individuals at high risk of T2D only led to minor and not clinically relevant effects of TRE over habitual living on body weight[10] inferring that TRE may not always be effective to cause weight loss as this depends on the clinical phenotype and the exact TRE design. Furthermore, the extended fasting period with TRE and alignment of food intake to the circadian rhythmicity in metabolic functions e.g., glucose tolerance, may result in changes in energy metabolism and improvements in metabolic health outcomes beyond the effects related to an energy deficit/weight loss[11].

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In almost all eukaryotic cells, mitochondria are the central hub for cellular metabolism of glucose and fatty acids. However, in T2D and obesity the mitochondrial function in various tissues is altered, with changes incellular mitochondrial content, decreased metabolic flexibility and reduced mitochondrial oxidative capacity[12]. In rodents,, fasting has been shown to increase the expression of the mitochondrial biogenesis gene Pparg coactivator 1α (*PGC1α*) in hepatocytes[13], and misalignment of feeding to the active dark phase was shown to disturb the daily rhythm ofmitochondrial respiration in skeletal muscle[14] underlining a link between time of food intake and mitochondrial health. Therefore, TRE and impaired glucose and lipid metabolism linked to insulin resistance may converge at the level of the mitochondria.

Peripheral blood mononuclear cells (PBMCs) and platelets are exposed to changes in nutrients and stress signals in the circulation, and their mitochondrial bioenergetics are altered in various diseases, including diabetes[15–18]. These easily accessible cells are being used in nutrition and obesity studies as they may reflect alterations in internal tissue metabolism[19]. For instance, PBMC metabolic gene expression profiles seem to reflect gene expression changes in liver and adipose tissue in response to dietary conditions[20]. On the other hand, PBMCs do not reflect mitochondrial function in skeletal muscle in response to training intervention[21]. Despite this, information about mitochondrial function in PBMCs/platelets in response to dietary changes such as TRE could be relevant for understanding how intermittent fasting affects cellular respiration. Therefore, we, as part of our recent TRE study in individuals at high risk of T2D[10], evaluated if TRE affects mitochondrial bioenergetics of PBMCs and platelets by measuring the oxygen consumption rate (OCR) using extracellular flux technology.

Results

Study participants and PBMC/platelet composition

The protocol and primary outcome of our study investigating the effects of 3 months of 10-h TRE on body weight in 100 participants with either overweight (BMI ≥ 25 kg/m^2) and concurrent prediabetes (HbA1c 39–47 mmol/mol) or obesity (BMI ≥ 30 kg/m^2) have been published[10,22]. In brief, participants were randomly allocated to TRE or a control (habitual living) group for 3 months in a 1:1 ratio. Participants in the TRE group were instructed to consume all food and beverages within a self-selected consistent 10-h window placed between 6 a.m. and 8 p.m. each day. There were no restrictions regarding food quality or quantity. Participants in the control group continued their habitual lifestyles. Fifty-two of the participants were included in the present study and their baseline characteristics are presented in Table 1. Of these, 48 (23 in the TRE group and 25 in the control group) completed the trial. Adherence to the TRE intervention is presented in Supplementary Table 1.

PBMCs and platelets were isolated as described in the Methods section from participants at study start (visit 1) and after the 3-month TRE intervention or control habitual lifestyle period (visit 3). All cell preparations and whole blood were tested for cell composition. As expected, lymphocytes and monocytes were enriched in the PBMC fraction with neutrophils being depleted compared to whole blood (Supplementary Table 2). Although platelets were strongly depleted (>97%) in the cell fraction compared to whole blood, the relative number of platelets were still higher than lymphocytes and monocytes together.

TRE does not affect PBMC/platelet mitochondrial bioenergetics

Freshly isolated PBMCs/platelets from the study participants at visit 1 (baseline) and visit 3 (end-of-study) were analyzed using the Seahorse extracellular flux technology which allows tracking of OCR in real-time. Injections of 0.5 µM oligomycin or 3 µM FCCP and then 2 µM rotenone plus 2 µM antimycin A during the OCR recordings allowed measurement of ATP-linked respiration, maximal substrate oxidation capacity, and non-mitochondrial respiration (see Method section for details and Supplementary Table 3). The contribution of fatty acid β-oxidation to the mitochondrial energy turnover was evaluated by pretreating cells with 5 µM etomoxir – an inhibitor of long-chain fatty acid transport into the mitochondria—prior to injecting oligomycin, FCCP, and rotenone/antimycin (A) In total, we obtained 17,023 individual OCR traces from the analyzed samples. All obtained OCR data were quality assessed (see Method section for details) resulting in omission of 3,910 individual traces. The obtained OCR traces for each group are shown in Fig. 1A,B All estimated differences in mitochondrial respiration parameters after 3 months of TRE intervention are shown in Fig. 1C; Table 2. There were no statistically significant differences in OCR between the TRE and control groups. In both groups, etomoxir treatment reduced most analyzed parameters of mitochondrial respiration by up to approx. 40%. However, there were no differences between the etomoxir-TRE and etomoxir-control groups for any mitochondrial respiration parameter analyzed (Table 3; Fig. 1C). For a subset (26 in total) of the PBMC/platelet preparations, we had a surplus of cells after seeding for the OCR experiments. We isolated RNA from these cells to examine the expression of key mitochondrial genes by quantitative PCR (qPCR). Analysis of hexokinase 1 (*HK1*), carnitine palmitoyltransferase 1A (*CPT1A*), and ubiquinol-cytochrome C reductase hinge protein like (*UQCRHL*) showed that *CPT1A* expression was relatively decreased and *UQCRHL* expression relatively increased in the TRE group compared the control group (Supplementary Fig. 1).

No associations between clinical characteristics and PBMC/platelet mitochondrial bioenergetics

Investigation of possible associations between mitochondrial ATP-linked respiration or mitochondrial respiratory spare capacity and clinical participant characteristics such as age, sex, BMI, body fat mass, blood glucose, or blood lipids showed no statistically significant associations (Table 4).

Discussion

In this study, we investigated if 3 months of TRE in individuals with high risk of T2D is associated with altered mitochondrial function in isolated PBMCs and platelets. We found that TRE was not associated with changes

Characteristic	Overall, $n = 52$[a]	Control, $n = 27$[a]	TRE, $n = 25$[a]
Sex			
Men	20 (38%)	10 (37%)	10 (40%)
Women	32 (62%)	17 (63%)	15 (60%)
Age (years)	60 (53, 67)	62 (56, 66)	56 (52, 67)
Smoking, n (%)			
No	52 (100%)	27 (100%)	25 (100%)
Lipid lowering medication, n (%)	3 (6%)	2 (7%)	1 (4%)
Body weight, kg	95 (85, 111)	98 (84, 111)	90 (89, 107)
Body weight, kg (women)	92.1 (19.3)	93.2 (22.7)	90.8 (15.3)
Body weight, kg (men)	109.9 (16.3)	111.9 (16.4)	107.9 (16.8)
BMI, kg/m^2	33.6 (6.0)	33.9 (7.3)	33.2 (4.3)
Fat mass, kg	37.1 (32.4, 42.3)	36.5 (33.1, 43.6)	38.0 (32.6, 42.2)
Fat free mass, kg (women)	52.0 (7.9)	52.4 (8.1)	51.5 (7.9)
Fat free mass, kg (men)	72.9 (8.0)	73.7 (7.8)	72.2 (8.6)
Fat percentage, % (women)	44 (4)	43 (5)	44 (3)
Fat percentage, % (men)	34 (4)	34 (5)	33 (4)
Daily eating duration median, hours	13.3 (12.4, 14.0)	13.2 (12.4, 13.9)	13.4 (12.6, 14.3)
Energy intake, kcal/day (women)	1,888 (1,566, 2,244)	1,567 (1,451, 2,121)	2,030 (1,824, 2,327)
Energy intake, kcal/day (men)	2,302 (2,103, 2,696)	2,358 (2,152, 2,701)	2,246 (2,007, 2,574)
HbA1c (mmol/mol)	39 (4)	39 (4)	38 (4)
Fasting glucose, mmol/l	5.8 (0.6)	5.8 (0.6)	5.7 (0.6)
Fasting insulin, pmol/l	71 (58, 100)	70 (63, 100)	76 (57, 96)
Total fasting cholesterol, mmol/l (women)	5.7 (0.9)	5.6 (0.8)	5.8 (1.0)
Total fasting cholesterol, mmol/l (men)	5.1 (0.8)	5.5 (0.6)	4.7 (0.8)
Fasting HDL, mmol/l (women)	1.48 (0.30)	1.55 (0.34)	1.39 (0.21)
Fasting HDL, mmol/l (men)	1.19 (0.22)	1.24 (0.15)	1.14 (0.26)
Fasting LDL, mmol/l (women)	3.5 (0.9)	3.4 (0.8)	3.6 (1.0)
Fasting LDL, mmol/l (men)	3.2 (0.7)	3.6 (0.5)	2.9 (0.7)
Fasting VLDL, mmol/l	0.70 (0.26)	0.70 (0.25)	0.69 (0.27)
Fasting triglyceride, mmol/l	1.52 (0.56)	1.52 (0.53)	1.52 (0.60)
Mitochondrial basal respiration-control (OCR)	68 (56, 88)	68 (52, 88)	68 (57, 88)
Mitochondrial basal respiration-etomoxir (OCR)	52 (39, 70)	52 (38, 73)	52 (40, 70)
Mitochondrial spare respiration-control (OCR)	215 (159, 282)	237 (156, 285)	209 (168, 277)
Mitochondrial spare respiration- etomoxir (OCR)	132 (77, 178)	138 (75, 176)	131 (85, 180)
Mitochondrial proton leak-control (OCR)	10.7 (7.5, 15.0)	11.9 (6.3, 14.3)	10.5 (8.1, 15.6)
Mitochondrial proton leak-etomoxir (OCR)	12.6 (9.8, 14.9)	12.9 (8.5, 15.0)	12.1 (10.8, 14.6)
Mitochondrial ATP-linked respiration-control (OCR)	52 (45, 66)	49 (43, 66)	54 (50, 66)
Mitochondrial ATP-linked respiration-etomoxir (OCR)	34 (27, 42)	34 (25, 42)	34 (30, 42)
Mitochondrial maximum respiration-control (OCR)	288 (212, 365)	309 (205, 367)	280 (216, 363)
Mitochondrial maximum respiration-etomoxir (OCR)	182 (124, 249)	182 (113, 245)	180 (127, 247)
Non-mitochondrial respiration-control (OCR)	17 (13, 24)	17 (12, 24)	19 (14, 24)
Non-mitochondrial respiration-etomoxir (OCR)	15 (10, 25)	18 (10, 23)	13 (9, 27)

Table 1. Baseline characteristics of the RESET participants included in the PBMC seahorse extracellular flux study. Values are presented as number (%), median (IQR) or mean (SD). [a]n (%); Median (IQR); Mean (SD), abbreviations: OCR (Oxygen Consumption Rate). The unit for OCR is pmol/min.

in any of the analyzed parameters of mitochondrial bioenergetics. Further, we found that around one third of aerobic energy production in PBMCs/platelets is derived from β-oxidation of fatty acids but is unaffected by TRE.

Our study was performed on isolated PBMCs and platelets from participants of the RESET trial[10]. Although other TRE studies in humans and animals have shown that TRE leads to modest weight loss[7,8], we did not observe a statistically significant reduction in body weight in response to TRE when compared to the control group. However, within the TRE group, there was a small but significant weight loss. The TRE intervention included dietary advice, but no restrictions on intake of macronutrients were imposed. It can be speculated that a more restrictive TRE intervention with shorter eating window would have had larger effects on body weight reduction and metabolic endpoints as a consequence hereof. The lack of effect of TRE on mitochondrial respiratory function, does align with the limited effect of TRE on clinical variables. Additionally, it indicates

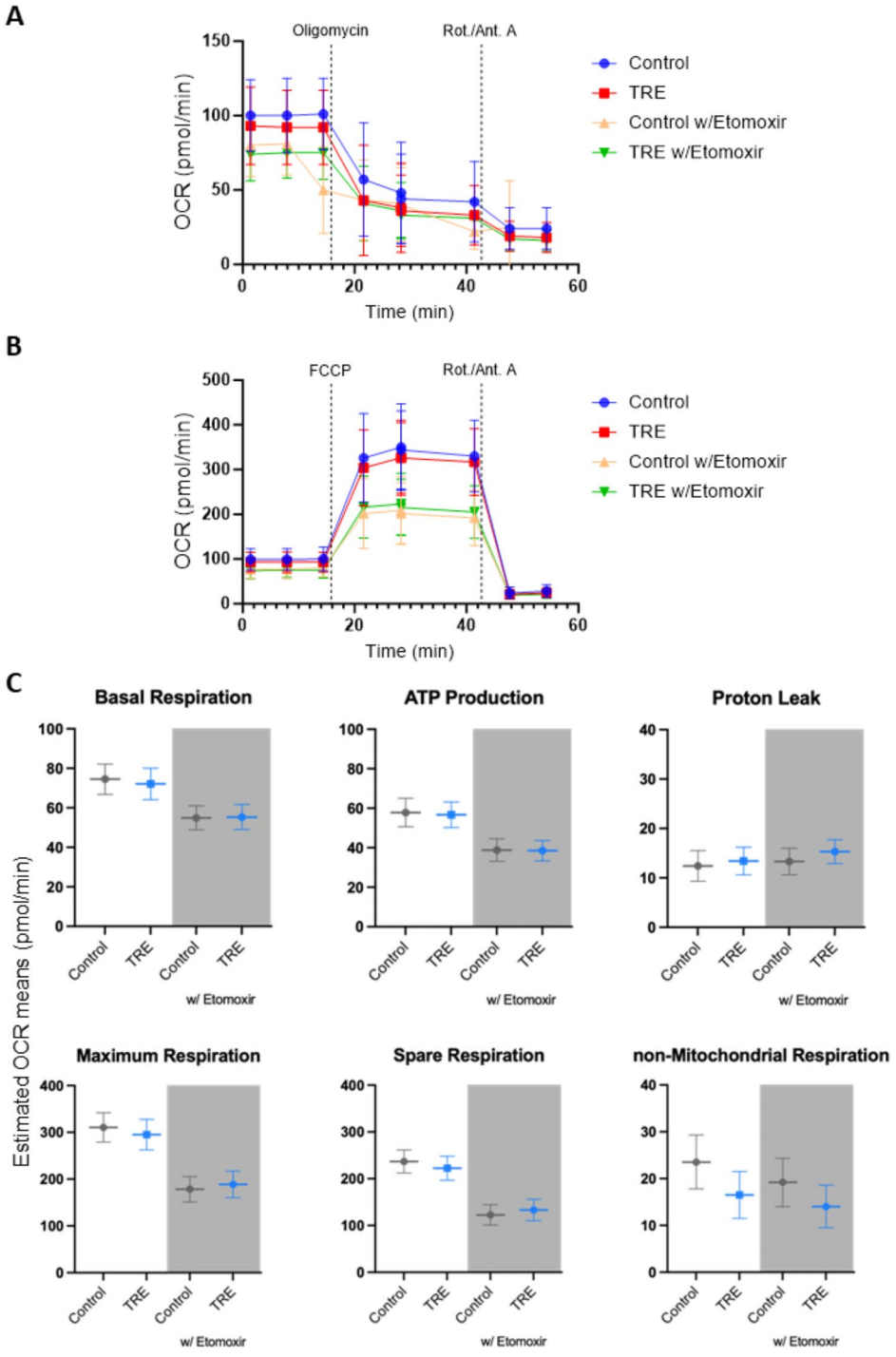

Fig. 1. TRE intervention does not change PBMC/platelet mitochondrial respiration. (**A** and **B**) OCR traces at visit 3 (end-of-study) from experiments with addition of either oligomycin or FCCP and then rotenone/antimycin A, incubated with or without etomoxir. Values are means ± SD of $n = 25$ (control group) and $n = 23$ (TRE group). (**C**) Estimated means of mitochondrial respiration parameters in PBMCs/platelets at visit 3 for control and TRE intervention group in the presence or absence of etomoxir. Values are means with 95% CI. OCR, oxygen consumption rate; TRE, time-restricted eating.

	Group	Estimated mean at V1 (95% CI)	Estimated mean at V3 (95% CI)	Within group changes (95% CI)	*P*-value	Difference from control (95% CI)	*P*-value
Basal respiration	Control	71 (63.5: 78.5)	74.5 (66.8: 82.1)	3.4 (− 6.4: 13.3)	0.488		
	TRE	71 (63.5: 78.5)	72.1 (64.1: 80)	1.1 (− 9.1: 11.2)	0.836	2.4 (− 8.7: 13.5)	0.668
ATP-linked respiration	Control	55.6 (48.6: 62.6)	57.8 (50.6: 65)	2.2 (− 7.1: 11.6)	0.633		
	TRE	55.6 (48.6: 62.6)	56.7 (50.2: 63.1)	1.1 (− 7.9: 10)	0.814	1.2 (− 8.7: 11)	0.810
Proton leak	Control	13.7 (9.7: 17.7)	12.4 (9.3: 15.5)	− 1.3 (− 6.2: 3.5)	0.583		
	TRE	13.7 (9.7: 17.7)	13.4 (10.6: 16.2)	− 0.3 (− 5.1: 4.4)	0.886	− 1 (− 5.2: 3.2)	0.634
Maximal respiration	Control	302.2 (255.2: 349.1)	310.6 (279.3: 341.9)	8.4 (− 42.7: 59.6)	0.743		
	TRE	302.2 (255.2: 349.1)	295.2 (262.6: 327.8)	− 6.9 (− 59.1: 45.2)	0.792	15.4 (− 29.9: 60.6)	0.496
Spare capacity	Control	231.2 (190.4: 272.1)	236.8 (212.1: 261.4)	5.5 (− 37.7: 48.8)	0.799		
	TRE	231.2 (190.4: 272.1)	222.5 (196.9: 248.1)	− 8.7 (− 52.7: 35.3)	0.695	14.2 (− 21.3: 49.7)	0.423
Non-mitochondrial respiration	Control	21 (16.9: 25.1)	23.5 (17.8: 29.3)	2.5 (− 4.2: 9.3)	0.454		
	TRE	21 (16.9: 25.1)	16.5 (11.5: 21.5)	− 4.5 (− 10.7: 1.7)	0.150	7 (− 0.7: 14.8)	0.074

Table 2. Estimated differences in PBMC bioenergetics between control and TRE groups. Analyses were adjusted for baseline values at visit 1 for all participants. *V1* visit 1 (baseline), *V3* visit 3 (end-of-study), *CI* confidence interval. Values are in pmol/min (oxygen consumption rate).

	Group	Estimated mean at V1 (95% CI)	Estimated mean at V3 (95% CI)	Within group changes (95% CI)	*P*-value	Difference from control (95% CI)	*P*-value
Basal respiration	Control	54.8 (49.4: 60.2)	54.9 (48.9: 61)	0.1 (− 7.4: 7.6)	0.977		
	TRE	54.8 (49.4: 60.2)	55.3 (49: 61.7)	0.5 (− 7.2: 8.3)	0.889	− 0.4 (− 9.3: 8.4)	0.922
ATP-linked respiration	Control	36.7 (32.3: 41.2)	38.8 (33.1: 44.5)	2 (− 4.5: 8.6)	0.534		
	TRE	36.7 (32.3: 41.2)	38.5 (33.4: 43.6)	1.8 (− 4.5: 8.1)	0.577	0.3 (− 7.5: 8)	0.940
Proton leak	Control	14.7 (10.8: 18.6)	13.3 (10.6: 16)	− 1.4 (− 6: 3.2)	0.548		
	TRE	14.7 (10.8: 18.6)	15.3 (12.9: 17.7)	0.6 (− 3.9: 5.1)	0.792	− 2 (− 5.7: 1.7)	0.278
Maximal respiration	Control	192.9 (165: 220.8)	178.4 (151.2: 205.5)	− 14.6 (− 50: 20.9)	0.415		
	TRE	192.9 (165: 220.8)	188.8 (160.4: 217.2)	− 4.1 (− 40.7: 32.5)	0.823	− 10.4 (− 50: 29.1)	0.597
Spare capacity	Control	138.2 (114.9: 161.4)	122.6 (100.8: 144.4)	− 15.6 (− 44.4: 13.3)	0.286		
	TRE	138.2 (114.9: 161.4)	133.1 (110.1: 156.1)	− 5.1 (− 35: 24.8)	0.736	− 10.5 (− 42.3: 21.4)	0.510
Non-mitochondrial respiration	Control	17.5 (14: 20.9)	19.2 (14: 24.3)	1.7 (− 4.1: 7.4)	0.560		
	TRE	17.5 (14: 20.9)	14 (9.5: 18.6)	− 3.5 (− 8.8: 1.9)	0.200	5.1 (− 1.8: 12)	0.141

Table 3. Estimated differences in PBMC bioenergetics between control and TRE groups in the presence of etomoxir. Analyses were adjusted for baseline values at visit 1 for all participants. *V1* visit 1 (baseline), *V3* visit 3 (end-of-treatment), *CI* confidence interval. Values are in pmol/min (oxygen consumption rate).

that measuring mitochondrial health in such a mild intervention setup does not convey further insight than the measurement of clinical variables.

In animal models, time-restricted feeding in alignment with the active dark phase is associated with increased β-oxidation, leading to a reduction in liver free fatty acids and subsequent reduction in inflammation[23]. Fasting has also been shown to improve fat oxidation in humans[24], and both animal- and human cell models indicate that mitochondrial adaptations, such as nutrient-induced fission, are important for the response[25–2724]. Based on these notions, we tested if TRE would lead to a preferential utilization of fatty acids as substrate for oxidative phosphorylation. However, as all bioenergetic parameters in the TRE and control groups were affected similarly by pretreatment of the PBMCs/platelets with etomoxir, this is likely not the case in our study. A plausible reason for this could be that the fasting plasma lipid concentrations (triglycerides, LDL, and HDL) were not significantly different between the TRE and control groups. Therefore, the availability of fatty acids for β-oxidation and energy production in PBMCs/platelets was similar in both the TRE and control groups. Etomoxir reduced the energy production in our experiments by approximately one-third, confirming that β-oxidation of fatty acids is an important energy source, as previously reported in PBMCs[28]. Interestingly, we observed that the relative expression of *CPT1A*, the rate-limiting enzyme in fatty acid β-oxidation, at end-of-treatment vs. baseline was slightly decreased in the TRE group compared to control supporting a possible link between TRE and fatty acid β-oxidation. It's important to note, however, that dietary changes such as fasting could alter mitochondrial respiratory parameters in a manner dependent on nutrient and substrate availability which will not be seen in the Seahorse assay where substrate availability is saturated. We also observed a small increase in the relative expression of *UQCRHL* in the TRE group compared to control. However, these modest changes at the gene level were not sufficient to cause changes in mitochondrial activity. The relative expression of *HK1*, the rate limiting

	Covariate	Beta (95% CI)	*P*-value	Beta (95% CI) (+ etomoxir)	*P*-value
ATP-linked respiration	Sex	2 (− 4.6: 8.7)	0.541	5.3 (− 3.3: 13.9)	0.222
	Age (years)	0.2 (− 0.2: 0.5)	0.363	0.2 (− 0.3: 0.7)	0.373
	Fasting glucose, mmol/L	1.5 (− 4: 7.1)	0.582	1.4 (− 6.2: 9)	0.717
	BMI, kg/m^2	0.3 (− 0.2: 0.8)	0.240	0.5 (− 0.1: 1.1)	0.117
	Fat mass, kg	0.2 (0: 0.5)	0.081	0.3 (0: 0.7)	0.052
	Fat percentage, %	0.5 (− 0.2: 1.2)	0.134	0.8 (− 0.1: 1.7)	0.075
	Total fasting cholesterol, mmol/L	− 0.9 (− 4.3: 2.5)	0.593	− 2.8 (− 7: 1.4)	0.185
	Fasting LDL, mmol/L	− 0.9 (− 4.8: 3)	0.645	− 3.3 (− 8.3: 1.6)	0.179
	Fasting HDL, mmol/L	11.9 (− 1.2: 25.1)	0.073	13.8 (− 3.9: 31.5)	0.123
	Fasting triglycerides, mmol/L	− 3.2 (− 7.6: 1.2)	0.152	− 3.4 (− 8.9: 2.1)	0.214
Spare capacity	Sex	14.9 (− 15.1: 44.8)	0.324	8.6 (− 28.6: 45.7)	0.645
	Age (years)	1.3 (− 0.5: 3.1)	0.146	1.2 (− 1: 3.5)	0.270
	Fasting glucose, mmol/L	− 8.8 (− 34.4: 16.8)	0.495	− 5.5 (− 38.2: 27.3)	0.739
	BMI, kg/m2	− 0.2 (− 2.6: 2.1)	0.848	0.6 (− 2.3: 3.5)	0.674
	Fat mass, kg	0.1 (− 1.1: 1.3)	0.862	0.1 (− 1.4: 1.7)	0.868
	Fat percentage, %	0.9 (− 2.2: 4.1)	0.552	0.1 (− 3.8: 4)	0.967
	Total fasting cholesterol, mmol/L	2.1 (− 13.5: 17.6)	0.790	− 4.6 (− 23.7: 14.6)	0.634
	Fasting LDL, mmol/L	3.5 (− 14.5: 21.5)	0.702	− 4.5 (− 26.9: 17.8)	0.686
	Fasting HDL, mmol/L	48.9 (− 9.5: 107.2)	0.099	25.2 (− 53.9: 104.4)	0.525
	Fasting triglyceride, mmol/L	− 8 (− 28.4: 12.4)	0.436	− 5.6 (− 29.6: 18.4)	0.641

Table 4. Associations between clinical participant characteristics and PBMC ATP-linked respiration or spare respiration. *BMI* body mass index, *LDL* low density lipoprotein, *HDL* high density lipoprotein. Values are in pmol/min (oxygen consumption rate).

enzyme in glycolysis, was not different between groups. Of note, as we only obtained RNA from half of the study participants, the gene expression data should be interpreted with caution.

We did not find any associations between clinical characteristics such as age and BMI of the participants and the measured parameters for PBMC/platelet mitochondrial bioenergetics. Other studies showed an association of aging and mitochondrial function[29–31]. These studies, however, used skeletal muscle instead of circulating cells. In addition, as age-dependent changes in respiratory capacity were observed in older populations than ours, it is possible that our study population would not yet show signs of mitochondrial dysfunction. We considered dividing the study participants into smaller subgroups before analyzing the mitochondrial data, but this would give too small sample sizes for meaningful statistical evaluations.

Other smaller studies have performed PBMC bioenergetic measurements at single sample collection points[15,32]. To our knowledge, however, our study is the first to perform longitudinal measures of PBMC/platelet bioenergetics from women and men with overweight and prediabetes or obesity. An elegant study in individuals with and without T2D included a similar number of participants per group as our study and demonstrated increased mitochondrial oxidative phosphorylation in freshly isolated PBMCs from individuals with T2D compared to controls[28]. Our study investigated if the mitochondrial respiratory function in people at high risk of T2D could be improved by a TRE intervention. The inclusion of healthy controls could have helped to establish if mitochondrial function in the trial population was impaired at the study start. We recently reported increased proton leak and glycolysis in PBMCs from patients with type 1 diabetes compared to healthy controls[33]. Comparing the obtained OCR data of the current study with that of the healthy control group from our previous study, suggests that all mitochondrial parameters are similar except for maximal and spare respiration, which are higher in the current study. However, this will need further exploration in future studies to fully clarify.

We observed that the OCR measurements of the fresh PBMCs/platelets from the same participants between visit 1 and 3, i.e., before and after the 3-month intervention period, had a rather large degree of variation between the repeated measures in both the TRE and control groups, as exemplified by the Spearman correlation analysis (Supplementary Fig. 2). The reason(s) for this remains unclear but is likely a result of natural fluctuations in experimental procedures including cell harvesting and seeding performed with months in between. Others have shown that factors such as donor age and time of year can influence PBMC respiration[34]. Unfortunately, cryopreservation of PBMCs is not suited for extracellular flux analysis as this significantly decreases mitochondrial respiration[35,36] so cryopreserving the harvested cells from all study participants and visits and then analyzing them simultaneously at the end of the study is not feasible.

In conclusion, our study suggests that 3 months of 10-h per day TRE in individuals with high risk of T2D does not alter PBMC/platelet mitochondrial bioenergetics. We encourage careful considerations to study design and experimental procedures when designing future longitudinal studies aimed at investigating cellular bioenergetic alterations in freshly isolated cells following an intervention period.

Methods

Participants and trial design

This study is a predefined sub-study of the RESET trial (15) a (RCT) registered at ClinicalTrials.gov, identifier: NCT03854656.

The RESET study investigated the effects of 3 months of 10-h TRE on body weight, metabolism, and behavior in 100 participants aged 30–70 years with either (1) overweight (BMI ≥ 25 kg/m^2) and concurrent prediabetes (HbA1c 39–47 mmol/mol) or (2) obesity (BMI ≥ 30 kg/m^2) with or without prediabetes (HbA1c ≤ 47 mmol/mol). Participants were randomly allocated to TRE or a control group for 3 months in a 1:1 ratio. Participants allocated to TRE were encouraged to consume all food and beverages within the same self-selected 10-h window placed between 6 a.m. and 8 p.m. each day. There were no restrictions regarding food quality or quantity. In the remaining 14 h, they were encouraged to only drink water. Participants in the control group continued their habitual lifestyles. All participants were advised to follow the official Danish dietary recommendations (https://en.foedevarestyrelsen.dk/food/nutrition-and-health/the-official-dietary-guidelines). The study design is described in detail in (15). Study visits were scheduled at baseline (visit 1) and after 3 months (visit 3). For conditions prior to testing e.g., fasting duration etc. see protocol paper (15).

The present study included a sub-group of 52 participants of which 48 completed to study. Participants were continuously recruited from March 2019 to March 2021. All participants of the RESET study with visits during this period were included, unless there were schedule incompatibilities. Blood samples taken were picked up at an agreed collection location by a person who had no knowledge of participants grouping information until final data analysis. The study was approved by the Danish Data Protection Agency and was conducted according to the Declaration of Helsinki and all procedures involving study participants were approved by the Ethics Committee of the Capital Region of Denmark (H-18059188). Written and verbal informed consent was obtained from all study participants before inclusion and testing. Baseline characteristics of the 52 participants in this study can be found in Table 1.

PBMC/platelet isolation

Twenty mL of fasting whole blood samples for cellular bioenergetic analyses were collected from each participant at visit 1 and 3. PBMCs and platelets were isolated from fresh peripheral blood through density gradient centrifugation, 30 min to 1 h after collection. Briefly, blood was diluted 1:1 in DPBS without Ca^{2+} and Mg^{2-} (Gibco) and layered on top of Lymphoprep (Stemcell technologies) in equal volumes in 50 mL Sepmate tubes (Stemcell technologies). After 10 min centrifugation at 1200 g, plasma and cell layer was collected onto a new 50 mL tube and centrifuged for 5 min at 300×g. The cell pellet was washed in DPBS without Ca^{2+} and Mg^{2-} and centrifuged one last time at 170×*g* for 8 min to get rid of most of the platelets. Cells were resuspended in a volume of DPBS without Ca^{2+} and Mg^{2-} equal to the initial blood volume for cell count.

PBMC/platelet and complete blood cell assessment

Using the manual mode in a Sysmex Xn550, a minimum of 100 µL of blood or purified cell suspension were analyzed for cell count for each participant visit. The number of red blood cells, white blood cells, and platelets were obtained. We also recorded the number of each white blood cell type, i.e., neutrophils, lymphocytes, and monocytes.

PBMC/platelet bioenergetics

A Seahorse XFe24 Analyzer (Agilent Technologies, Santa Clara, CA, USA) using the Mito Stress protocol was used to measure real-time parameters of mitochondrial oxidative phosphorylation. 5×10^5 of freshly isolated PBMCs/platelets were seeded per well of a Seahorse XFe24 plate coated with poly-D-lysine (Millipore A-003-E) in XF base medium without phenol red (Agilent 100335) supplemented with 5mM HEPES, 2mM L-glutamine, 1mM Sodium Pyruvate (Gibco 11360070) and 5mM Glucose (Sigma G8644). Cells were incubated for 1 h at 37 °C in a CO_2-free incubator prior to the start of the experiment and 5 µM etomoxir (Sigma, E1905), an inhibitor of the CPT1a Acetyl-CoA transporter, was added to half the plate 20 min prior to OCR measurements for evaluation of the contribution of fatty acid β-oxidation to mitochondrial energy production. Three basal OCR measurements per well were performed, followed by an injection of 0.5 µM oligomycin (Sigma O4876), an inhibitor of ATP synthase allowing measurement of leak respiration and post-calculation of the ATP-linked respiration of the cells, or injection of 3 µM FCCP (carbonylcyanide-p-(trifluoromethoxy)phenylhydrazone, Sigma C2920) which uncouples the ATP synthase and, hence, mitochondrial respiration allowing to determine the maximal substrate oxidation capacity. Both oligomycin and FCCP effects were recorded during three OCR measurements per well. Finally, a combined injection of 2 µM rotenone (Sigma, R8875) and 2 µM antimycin A (Sigma A8674), inhibitors of complex I and III, respectively, was done followed by two OCR measurements per well to enable correction of the data for non-mitochondrial respiration. For all conditions, between 3 and 5 technical replicates were done. All OCR recordings from all study participants were done within 5 h of sample collection. Seahorse data were exported from the Wave software to Excel. OCR traces were created using Graphpad Prism software.

Prior to data analysis, wells with negative OCR readings were excluded, as well as basal measure points with an OCR below 20 pmol/min, as this is the lower optimal OCR detection limit of the Seahorse extracellular flux analyzer as recommended by the manufacturer (Agilent). If a participant had less than 3 technical replicates left for each parameter measured after data clean-up, that was removed from the data analysis. One participant was omitted from the analysis due to experimental technical error.

Statistical analysis

The main endpoints of this study were ATP-linked respiration and spare mitochondrial respiration. The distribution of continuous variables included in Table 2 were assessed using QQ-plots; those with a Gaussian distribution are presented as mean and standard deviation (SD), otherwise medians and quartiles are presented.

Associations between treatments and the mitochondrial bioenergetic-related outcomes were assessed using a baseline-corrected repeated measures regression model (Proc Mixed, SAS) with Treatment, Etomoxir, Visit (factorial)*Treatment as fixed effects/interactions. The model was specified with a restricted maximum likelihood estimation method, the Kenward-Roger degrees of freedom method, and a repeat (visit) on participant level (unstructured covariance structure). Model fit was assessed using graphical methods. Association analyses between ATP-linked respiration/spare mitochondrial capacity and other participant characteristics (predictor) were done using a similar regression model with the following fixed effects: Treatment, Predictor, Visit (factorial)*Treatment. This analysis was performed separately for measurements with and without etomoxir.

A p-value < 0.05 was considered statistically significant. Results were not adjusted for multiplicity. Statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC, USA) and R version 3.6.1.

Data availability

Data are available upon reasonable request to the corresponding author.

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References

1. Poggiogalle, E., Jamshed, H. & Peterson, C. M. Circadian regulation of glucose, lipid, and energy metabolism in humans. *Metabolism* **84**, 11–27. https://doi.org/10.1016/j.metabol.2017.11.017 (2018).
2. van Moorsel, D. et al. Demonstration of a day-night rhythm in human skeletal muscle oxidative capacity. *Mol. Metab.* **5**, 635–645. https://doi.org/10.1016/j.molmet.2016.06.012 (2016).
3. Leproult, R., Holmback, U. & Van Cauter, E. Circadian misalignment augments markers of insulin resistance and inflammation, independently of sleep loss. *Diabetes* **63**, 1860–1869. https://doi.org/10.2337/db13-1546 (2014).
4. Morris, C. J. et al. Endogenous circadian system and circadian misalignment impact glucose tolerance via separate mechanisms in humans. *Proc. Natl. Acad. Sci. U S A.* **112**, E2225–2234. https://doi.org/10.1073/pnas.1418955112 (2015).
5. Scheer, F. A., Hilton, M. F., Mantzoros, C. S. & Shea, S. A. Adverse metabolic and cardiovascular consequences of circadian misalignment. *Proc. Natl. Acad. Sci. U S A* **106**, 4453–4458. https://doi.org/10.1073/pnas.0808180106 (2009).
6. Sondrup, N. et al. Effects of sleep manipulation on markers of insulin sensitivity: A systematic review and meta-analysis of randomized controlled trials. *Sleep. Med. Rev.* **62**, 101594. https://doi.org/10.1016/j.smrv.2022.101594 (2022).
7. Anton, S. D. et al. The effects of time restricted feeding on overweight, older adults: A pilot study. *Nutrients* **11**(7), 1500. https://doi.org/10.3390/nu11071500 (2019).
8. Gabel, K. et al. Effects of 8-h time restricted feeding on body weight and metabolic disease risk factors in obese adults: A pilot study. *Nutr. Healthy Aging* **4**, 345–353. https://doi.org/10.3233/NHA-170036 (2018).
9. Hutchison, A. T. et al. Time-Restricted feeding improves glucose tolerance in men at risk for type 2 diabetes: A randomized crossover trial. *Obes. (Silver Spring)* **27**, 724–732. https://doi.org/10.1002/oby.22449 (2019).
10. Quist, J. S. et al. Effects of 3 months of 10-h per-day time-restricted eating and 3 months of follow-up on bodyweight and cardiometabolic health in Danish individuals at high risk of type 2 diabetes: The RESET single-centre, parallel, superiority, open-label, randomised controlled trial. *Lancet Healthy Longev.* **5**, e314–e325. https://doi.org/10.1016/S2666-7568(24)00028-X (2024).
11. Manoogian, E. N. C., Chow, L. S., Taub, P. R., Laferrere, B. & Panda, S. Time-restricted eating for the prevention and management of metabolic diseases. *Endocr. Rev.* **43**, 405–436. https://doi.org/10.1210/endrev/bnab027 (2022).
12. Koliaki, C. & Roden, M. Alterations of mitochondrial function and insulin sensitivity in human obesity and diabetes mellitus. *Annu. Rev. Nutr.* **36**, 337–367. https://doi.org/10.1146/annurev-nutr-071715-050656 (2016).
13. Rodgers, J. T. et al. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature* **434**, 113–118. https://doi.org/10.1038/nature03354 (2005).
14. de Goede, P. et al. Time-restricted feeding during the inactive phase abolishes the daily rhythm in mitochondrial respiration in rat skeletal muscle. *FASEB J.* **36**, e22133. https://doi.org/10.1096/fj.202100707R (2022).
15. Czajka, A. et al. Altered mitochondrial function, mitochondrial DNA and reduced metabolic flexibility in patients with diabetic nephropathy. *EBioMedicine* **2**, 499–512. https://doi.org/10.1016/j.ebiom.2015.04.002 (2015).
16. Mosconi, L. et al. Reduced mitochondria cytochrome oxidase activity in adult children of mothers with Alzheimer's disease. *J. Alzheimers Dis.* **27**, 483–490. https://doi.org/10.3233/JAD-2011-110866 (2011).
17. Perl, A., Hanczko, R. & Doherty, E. Assessment of mitochondrial dysfunction in lymphocytes of patients with systemic lupus erythematosus. *Methods Mol. Biol.* **900**, 61–89. https://doi.org/10.1007/978-1-60761-720-4_4 (2012).
18. Widlansky, M. E. et al. Altered mitochondrial membrane potential, mass, and morphology in the mononuclear cells of humans with type 2 diabetes. *Transl Res.* **156**, 15–25. https://doi.org/10.1016/j.trsl.2010.04.001 (2010).
19. Reynes, B., Priego, T., Cifre, M., Oliver, P. & Palou, A. Peripheral blood cells, a transcriptomic tool in nutrigenomic and obesity studies: Current state of the Art. *Compr. Rev. Food Sci. Food Saf.* **17**, 1006–1020. https://doi.org/10.1111/1541-4337.12363 (2018).
20. Costa, A. et al. Use of human PBMC to analyse the impact of obesity on lipid metabolism and metabolic status: A proof-of-concept pilot study. *Sci. Rep.* **11**, 18329. https://doi.org/10.1038/s41598-021-96981-6 (2021).
21. Hedges, C. P. et al. Peripheral blood mononuclear cells do not reflect skeletal muscle mitochondrial function or adaptation to high-intensity interval training in healthy young men. *J. Appl. Physiol. (1985).* **126**, 454–461. https://doi.org/10.1152/japplphysiol.00777.2018 (2019).
22. Quist, J. S. et al. Protocol for a single-centre, parallel-group, randomised, controlled, superiority trial on the effects of time-restricted eating on body weight, behaviour and metabolism in individuals at high risk of type 2 diabetes: The restricted eating time (RESET) study. *BMJ Open.* **10**, e037166. https://doi.org/10.1136/bmjopen-2020-037166 (2020).
23. Hatori, M. et al. Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. *Cell. Metab.* **15**, 848–860. https://doi.org/10.1016/j.cmet.2012.04.019 (2012).
24. Chiu, C. H. et al. Five days of time-restricted feeding increases fat oxidation rate but not affect postprandial lipemia: A crossover trial. *Sci. Rep.* **12**, 9295. https://doi.org/10.1038/s41598-022-13387-8 (2022).
25. Castro-Sepulveda, M. et al. The fasting-feeding metabolic transition regulates mitochondrial dynamics. *FASEB J.* **35**, e21891. https://doi.org/10.1096/fj.202100929R (2021).
26. Martinez-Lopez, N. et al. mTORC2-NDRG1-CDC42 axis couples fasting to mitochondrial fission. *Nat. Cell. Biol.* **25**, 989–1003. https://doi.org/10.1038/s41556-023-01163-3 (2023).

27. Song, J. E. et al. Mitochondrial fission governed by Drp1 regulates exogenous fatty acid usage and storage in Hela cells. *Metabolites* **11**(5), 322. https://doi.org/10.3390/metabo11050322 (2021).
28. Hartman, M. L. et al. Relation of mitochondrial oxygen consumption in peripheral blood mononuclear cells to vascular function in type 2 diabetes mellitus. *Vasc. Med.* **19**, 67–74. https://doi.org/10.1177/1358863X14521315 (2014).
29. Conley, K. E., Jubrias, S. A. & Esselman, P. C. Oxidative capacity and ageing in human muscle. *J. Physiol.* **526 Pt 1**, 203–210. https://doi.org/10.1111/j.1469-7793.2000.t01-1-00203.x (2000).
30. Joseph, A. M. et al. The impact of aging on mitochondrial function and biogenesis pathways in skeletal muscle of sedentary high- and low-functioning elderly individuals. *Aging Cell.* **11**, 801–809. https://doi.org/10.1111/j.1474-9726.2012.00844.x (2012).
31. Short, K. R. et al. Decline in skeletal muscle mitochondrial function with aging in humans. *Proc. Natl. Acad. Sci. U S A.* **102**, 5618–5623. https://doi.org/10.1073/pnas.0501559102 (2005).
32. Tyrrell, D. J. et al. Blood-cell bioenergetics are associated with physical function and inflammation in overweight/obese older adults. *Exp. Gerontol.* **70**, 84–91. https://doi.org/10.1016/j.exger.2015.07.015 (2015).
33. Lopes de Melo, J. M. et al. Increased mitochondrial proton leak and glycolysis in peripheral blood mononuclear cells in type-1-diabetes. *Heliyon* **8**, e12304 (2022). https://doi.org/10.1016/j.heliyon.2022.e12304
34. Tessema, B., Riemer, J., Sack, U. & Konig, B. Cellular stress assay in peripheral blood mononuclear cells: factors influencing its results. *Int. J. Mol. Sci.* **23**(21), 13118. https://doi.org/10.3390/ijms232113118 (2022).
35. Jones, N. et al. Bioenergetic analysis of human peripheral blood mononuclear cells. *Clin. Exp. Immunol.* **182**, 69–80. https://doi.org/10.1111/cei.12662 (2015).
36. Keane, K. N., Calton, E. K., Cruzat, V. F., Soares, M. J. & Newsholme, P. The impact of cryopreservation on human peripheral blood leucocyte bioenergetics. *Clin. Sci. (Lond).* **128**, 723–733. https://doi.org/10.1042/CS20140725 (2015).

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Author contributions

J.S.Q., and K.F. conceived the clinical study idea and designed the study. K.F. is sponsor and principal investigator of the study. J.M.L.M., J.S.Q., V.H.J., K.F., and J.S. designed the PBMC/platelet bioenergetics part of the study. J.S.Q., H.P., M.M.C. and K.K.B.C. were involved in the trial conduct. J.M.L.M. and V.H.J. collected the PBMCs and performed the bioenergetic analyses. J.M.L.M. and M.B.B. conducted the formal data analysis. M.B.B. performed statistical analyses. J.M.L.M. wrote the first draft of the manuscript commented by J.S. All authors critically reviewed and approved the final manuscript. 222Authors declare no direct competing interests. J.S.Q. has received funding from Novo Nordisk A/S for other studies. H.P. is currently employed at Novo Nordisk A/S and is co-investigator on the project which was part of her Industrial PhD project in collaboration with iMotions A/S, where Pedersen was employed. K.F. is currently employed at Novo Nordisk A/S, has received research grants from Novo Nordisk A/S, and holds shares in Novo Nordisk A/S. J.M.L.M. and K.K.B.C. are currently employed at Novo Nordisk A/S and hold shares in Novo Nordisk A/S. M.M.J is currently funded by a grant from Novo Nordisk A/S but not at the time of the present study. J.S. and V.H.J. hold shares in Novo Nordisk A/S.

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Declarations

Competing interests

The authors declare no direct competing interests. J.S.Q. has received funding from Novo Nordisk A/S for other studies. H.P. is currently employed at Novo Nordisk A/S and is co-investigator on the project which was part of her Industrial PhD project in collaboration with iMotions A/S, where Pedersen was employed. K.F. is currently employed at Novo Nordisk A/S, has received research grants from Novo Nordisk A/S, and holds shares in Novo Nordisk A/S. J.M.L.M. and K.K.B.C. are currently employed at Novo Nordisk A/S and hold shares in Novo Nordisk A/S. M.M.J is currently funded by a grant from Novo Nordisk A/S but not at the time of the present study. J.S. and V.H.J. hold shares in Novo Nordisk A/S.

Additional information

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