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Hedonic and homeostatic appetite control in obesity and type 2 diabetes in the context of meal and exercise timing - The TIMEX study

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Abbrevations

BMI	Body mass index
CoEQ	Control of Eating Questionnaire
CoNSORT	Consolidated standards of reporting trials
ECG	Electrocardiography
IPAQ	International Physical Activity Questionnaire
MCQT	Munich Chronotype Questionnaire
MEQ	Morningness-Eveningness Questionnaire
MID	Minimal important difference
PSQI	Pittsburgh Sleep Quality Index
SBFPT	Steno Biometric Food Preference Task
SDCC	Steno Diabetes Center Copenhagen
SES	Socioeconomic status
SPIRIT	Standard protocol items: Recommidations for international trials statement
T2D	Type 2 diabetes
VAS	Visual analogue scales
VO_2peak	Peak oxygen uptake

Abstract

Introduction

The aim of this study is to investigate effects of acute exercise on appetite control and whether this differs between morning and evening in individuals with overweight/obesity with or without type 2 diabetes.

Methods and Analysis

The hedonic and homeostatic appetite control in obesity and type 2 diabetes in the context of meal and exercise timing (TIMEX) study is a randomised, controlled, cross-over trial. Fifty-eight women and men with overweight or obesity (BMI ≥25 kg/m^2) with or without type 2 diabetes will be recruited. All participants will complete a screening and baseline visit followed by four test visits: two morning visits and two evening visits. The participants will arrive in the fasted state during the visits. During one morning visit and one evening visit, the participants will engage in a 45-minute bout of acute exercise on an ergometer bicycle. The remaining two visits (one morning and one evening visit) will include 45 minutes of rest. Fifteen minutes after the rest or exercise period, the participants will be presented with an ad libitum meal. Blood samples will be collected and subjective appetite will be assessed using visual analogue scales in the fasted state before exercise/rest, immediately post-exercise/rest and at 15, 30, 45 and 60 minutes post-exercise/rest. Food reward and food preferences will be assessed using the validated diurnal version of Steno Biometric Food Preference Task in the fasted state before exercise/rest and 15 minutes after the ad libitum meal (45 minutes post-exercise/rest). The primary outcome is the difference in ad libitum energy intake after exercise compared to rest. Secondary outcomes include eating rate; 24-hour energy intake; appetite-related metabolites and hormones, and circulating biomarkers assessed from proteomics, metabolomics and lipidomics analyses; food choice, food attention and reaction time, explicit and implicit liking and wanting for different food categories, subjective appetite; ratings of perceived exertion during exercise. All outcomes will be compared between morning vs. evening and between participants with and without type 2 diabetes.

Ethics and dissemination

The study has been approved by the Ethics Committee of the Capital Region of Denmark (H-22019913) and the Capital Region of Denmark's Research Register (Privacy). The study will be conducted in accordance with the Declaration of Helsinki. All results will be published in national

and international peer-reviewed journals and will be disseminated at national and international conferences.

Trial registration

The trial is registered at ClinicalTrials.gov, identifier: NCT05768958

Strength and limitations

Strengths:

- Novel insights into the interplay between homeostatic (energy status-based) and hedonic (pleasure-based) appetite control in people with overweight, obesity and type 2 diabetes
- Two-by-two factorial design allows comparison of the effects of exercise vs. rest and the effects of morning vs. evening exercise in the same trial
- Cross-over study with a large sample size

Limitations:

- Short-term assessment of appetite/ad libitum energy intake
- Heterogenous study population (participants with and without type 2 diabetes) which increases the generalizability of the results, but also induces larger variation to the data
- No healthy control group (referred to as individuals with BMI between 18.5-24.9 kg/m^2) which prevents the study from investigating if the outcomes relates to just obesity

Introduction

The prevalence of overweight and obesity has increased significantly during the past decades (1). Similarly, the prevalence of type 2 diabetes (T2D) is continuingly increasing (2), while societal structures still fuel the rise in these public health concerns. The food environment of modern society has changed dramatically with highly palatable and energy-dense foods being ever more easily available. In such an obesogenic environment, food intake will to a larger extent be driven by hedonic and rewarding aspects of appetite control rather than homeostatic metabolic needs (3).

Food reward is a driver of eating behaviour and is defined as the momentary value of a food to the individual at the time of ingestion (4). 'Liking' and 'wanting' are key components of food reward, which occur both with (explicit) and without (implicit) conscious awareness (5,6). Intake of highly palatable and energy-dense foods is related to excess energy intake, high body mass index (BMI) and weight gain (7–9). Studies suggest that overweight and obesity are associated with a higher preference for foods high in fat and sugar and a higher reward response to food intake compared to lean individuals (10). Furthermore, results from brain imaging studies have suggested that the activity in central reward circuits in response to visual food stimuli and food intake in individuals with T2D are elevated compared to healthy controls (11,12). Insulin-reward pathways may be involved in this altered response as insulin resistance in the periphery is associated with insulin resistance in brain regions involved in the regulation of appetite and food reward (13). However, it remains to be established whether T2D per se alters food reward (14).

Circadian or diurnal rhythms may influence appetite control and ultimately energy balance. For example, in a group of British young adults without obesity, perceived hunger and reward for high-fat foods in response to a fixed test meal was greater in the evening compared to morning (15). This may promote overconsumption in the evening, but ad libitum energy intake was not assessed. Also, late-evening food intake is highly prevalent among individuals with T2D (16). Indeed, studies indicate that the timing of food intake can influence body weight (17). In healthy individuals, glucose tolerance to identical meals is greater earlier compared to later in the day (18). An important consideration in people with T2D is that insulin sensitivity has been shown to improve throughout the day and worsen throughout the night into the morning (19). Whether this shift affects appetite processes is unknown.

In addition to effects on body weight and composition (20), it is known that physical activity influences homeostatic (21) and hedonic (22) mechanisms of appetite. Exercise-induced anorexia has

been observed with acute bouts of exercise performed at ≥60% of VO_2 peak together with a short-term increase in circulating satiety-related hormones (23). Exercise-induced anorexia seem to return to control values within hours following exercise (23). Exercise has also been shown to act as a circadian time cue and exercise-induced signals could affect molecular clock genes (24). The diurnal timing of exercise also seems to have an impact on body weight, with greater losses in body weight seen with morning relative to evening exercise (25,26). A recent study conducted in mice suggests that exercise in the morning possibly relies more on glucose metabolism, whereas exercise in the evening relies more on fat metabolism – however, it remains unclear whether these mechanisms also applies in humans (27). Elucidating these mechanisms in humans remains to be explored. A few small studies in people at risk of or with T2D, have investigated effects of exercise timing on glucose control and suggested that exercise during evening is preferable compared to morning exercise (28–30), where as others did not observe differences (31). Understanding "when" to eat and exercise is of great importance as most clinical guidelines and interventions in prevention and treatment of obesity and related diseases focus on "what" and "how much" to eat or "how" and "how much" to exercise. Furthermore, a better understanding of appetite control and implicit and explicit processes of food reward in the context of meal and exercise timing in people with different weight and diabetes status is needed.

Objectives

The specific objectives of the TIMEX study are to investigate whether energy intake during an ad libitum meal differs following a 45-minute acute bout of exercise compared to rest; investigate whether the energy intake of the ad libitum meal differs between morning and evening; investigate if appetite ratings, food reward, metabolic markers following the ad libitum meal differ between morning and evening, and between individuals with and without T2D; and investigate circulating biomarkers that can be used to identify if ad libitum food intake is primarily driven by hedonic or homeostatic appetite.

Hypotheses

Hypotheses for the primary endpoint (ad libitum energy intake) are:

1. An acute bout of exercise reduces ad libitum energy intake compared with a rest condition
2. Time of day does not affect exercise-induced anorexia (i.e., reduction in energy intake after exercise)

Hypotheses for the secondary endpoints are:

3. Appetite sensations and reward for high-fat sweet foods will be greater in the evening relative to the morning and this effect will be more pronounced in individuals with T2D compared to individuals without T2D.
4. Appetite sensations and reward for high-fat sweet foods will be greater in response to exercise in the evening relative to the morning and this effect will be more pronounced in individuals with T2D compared to individuals without T2D.
5. Appetite and food reward will be associated with circulating biomarkers and these associations will be affected by T2D status.

Methods and Analysis

Study Design

This study is a randomised, controlled, cross-over trial (*Figure 1*). Fifty-eight individuals with overweight/obesity with or without T2D will be randomised after completion of a screening and baseline visit. Each participant will complete four test visits (two morning visits and two evening visits) on four separate days with a ≥3-day washout between visits. We aim for completion of all visits within a 3-month period for each participant to prevent potential changes in e.g., body weight, dietary intake, and physical activity. Primary and secondary outcomes are assessed during the baseline visit and test visits.

The study will be conducted at Steno Diabetes Center Copenhagen (SDCC) and will be reported according to the Consolidated Standards of Reporting Trials (CONSORT) (32). The study protocol follows the Standard Protocol Items: Recommendations for Interventional Trials statement (SPIRIT) (33). The study is registered at ClinicalTrials.gov (identifier: NCT05768958).

Participants

Women and men, 18-75 years, with overweight or obesity (BMI ≥25 kg/m^2) with or without T2D, who are eligible according to the inclusion and exclusion criteria (*Box 1*) will be included. We aim for an equal distribution of participants with and without T2D.

Eligibility criteria

Inclusion and exclusion criteria are listed in *Box 1*.

Outcomes

Primary outcome

The primary outcome is the difference in ad libitum energy intake after 45 minutes of acute exercise bout compared to rest.

Secondary Exploratory Outcomes

All secondary outcomes include comparison between exercise and rest, morning and evening, and T2D status. Secondary outcomes include eating rate; 24-hour energy intake; appetite-related metabolites and hormones, and circulating biomarkers assessed from proteomics, metabolomics and lipidomics analyses; food choice, food attention and reaction time, explicit and implicit liking and wanting, subjective appetite; ratings of perceived exertion during exercise.

Recruitment

Screening/baseline visit

Participants are recruited via advertisements on www.Forskningnu.dk (social media). After reading the study information, they undergo a pre-screening phone interview to reduce screening failures. During the screening visit, participants are orally briefed on the study, provide written consent, and undergo a health exam, including medical history and eligibility assessment (*Box 1*). If eligible, participants proceed with height, weight, blood pressure, and ECG measurements. Those with approved ECGs complete a bicycle test to determine peak oxygen uptake (VO_2peak). After inclusion, participants are randomized into one of four test visit sequences, which are scheduled to reduce confounding variables.

Anthropometry and Blood Pressure

At the baseline visit, body weight will be measured to the nearest 0.1 kg with the participant wearing light clothes. Height will be measured to the nearest 0.1 cm. Height and weight will be measured using Seca 287 wireless ultrasonic measuring station (Seca gmbh & co. kg, Hamburg, Germany). Blood pressure and resting heart rate will be measured using a digital blood pressure gauge (Microlife BP A3L Comfort, Microlife AG Swiss Corporation, Widnau, Switzerland) and noted three times with 2-minutes intervals after a 10-minutes rest and the average of the two lowest values is reported.

Cardiorespiratory Fitness

VO_2peak as a measure of cardiorespiratory fitness will be measured by an incremental bicycle ergometer test (Corival CPET Medical Ergometer, Lode BV, Groningen, The Netherlands). Participants will complete a warm-up of 3 minutes at 25 watts followed by a linear increase in watts until exhaustion. Heart rate, ventilation rate, inspired oxygen and expired carbon dioxide levels are measured using a combined heart rate monitor and indirect calorimetry (Vyntus CPX, Vyaire Medical GmbH, Hoechberg, Germany). The VO_2peak test will be deemed valid if the respiratory exchange ratio is >1.15, a plateau in oxygen uptake is reached or heart rate is ±10 heartbeats from the estimated maximal heart rate (34).

Test Visits

All test visits will be conducted at SDCC. Prior to their visit, the participants are asked to standardize sleep (7-9 hours) if possible and dietary intake the day before all test visits. Participants will be asked to register their dietary intake 24 hours prior to and after each test visit. To obtain a somewhat equal baseline, the participants will further be asked to follow the same dietary pattern (to the best of their ability) during the 24 hours before each visit. On test days, participants will arrive at ~8:00 am after an ≥8-hour fast (morning visits) or at ~15:00 pm after a 3-hour fast (evening visits). Water is allowed until 2 hour before each visit. Smoking is allowed until 3 hours before each visit. Furthermore, no alcohol consumption or strenuous physical activity are allowed 48 hours prior to testing. Participants are instructed to avoid physically demanding transportation to the research facility. Participants are instructed to continue their regular medication regimen throughout the duration of the study. The schedule for the test visits is represented in *Figure 2*, and a schematic overview is presented in *Table 1*.

Blood Samples

Blood samples will be collected from an antecubital vein in the fasted state on the test visits and at 5 subsequent timepoints (0, 15, 30, 45, and 60 minutes after the completion of the exercise bout/rest). *Table 2* present the analyses that will be performed.

Body Composition

Body composition i.e., fat mass and fat free mass will be examined by electrical bioimpedance (seca mBCA 515, Seca gmbh & co. kg, Hamburg, Germany) at the first morning test visit with the participant wearing light clothes.

Dietary Intake

Participants will be instructed to weigh and register their food and drink intake 24 hours before and after each test visit. For the registration each participant receives a printed food diary containing written instruction for tracking, and tables for the tracking. All participants are offered to borrow a digital food scale. The hand-written, self-reported dietary intake will subsequently be logged into the online software Vitakost.dk (Vitakost, Kolding, Denmark) by the researchers.

Questionnaires

Participants will complete questionnaires on:

- Socioeconomic status (SES) (35)
- Eating behaviour (Control of Eating Questionnaire - CoEQ) (36)
- Physical activity (International Physical Activity Questionnaire - IPAQ) (37)
- Chronotype (Morningness/Eveningness Questionnaire; MEQ (38) and Munich Chronotype Questionnaire; MCTQ (39))
- Sleep (Pittsburgh Sleep Quality Index - PSQI) (40)
- Appetite (visual analogue scales - VAS) (41)
- Meal assessment (VAS) (41)

The participants will fill in the questionnaire CoEQ at the baseline visit, and the remaining (SES, IPAQ, MEQ, MCTQ and PSQI) at their first rest visit.

Subjective Appetite

Subjective appetite will be assessed using VAS (41), including ratings of hunger, fullness, satiety and prospective food consumption, thirst, desire to eat something sweet, salty, fatty, meat, and potential nausea. Palatability and liking of the ad libitum test meal will be rated. The ratings will be performed on a computer during all test visits.

Socioeconomic Position

Participants' socioeconomic position will be assessed using SES questionnaire (35) performed on a computer during the first rest visit. The SES questionnaire includes information on gender, educational level, work position, family orientation and annual household income.

Eating behaviour

Participants' subjective eating behavior will be assessed using CoEQ (36) performed on a computer during the baseline visit. The CoEQ includes questions on hunger, fullness, cravings and mood.

Physical activity

Participants' physical activity will be assessed using IPAQ (37) performed on a computer during the first rest visit. The IPAQ includes questions on frequency and intensity of physical activity on average over the past 7 days.

Chronotypic features

Data on participants' chronotypic features will be assessed using MEQ and MCQT (38,39). The questionnaire will be performed on a computer during the first rest visit. It will include questions on subjective fatigue, awakeness, energy level and hunger during mornings and nights.

Food preferences

Food preferences will be measured using the diurnally validated SBFPT (42,43), a computerized task adapted from the Leeds Food Preference Questionnaire (LFPQ) and tailored to fit a Danish food context (44). The task includes 16 images of common Danish foods, varying in fat content (high or low) and taste (sweet or savory). Before the study, an online survey validated the appropriateness of these images for morning and evening consumption among the target population (n=167). The SBFPT has two parts: a forced-choice task to measure food choice and implicit 'wanting,' and a rating task to assess explicit 'liking' and explicit 'wanting' on a scale from 0-100.

All responses are recorded in the digital software, iMotions 9.3 (iMotions A/S, Copenhagen K, Denmark) and used to compute mean scores for all abovementioned combined food categories. Prior to these two parts, participants are asked to familiarize themselves with a printed version of the 16 food imagesand explain what they see on the images. During the presentation of the 16 pictures, data on eye tracking and reaction time are collected. Eye tracking will be used to examine visual attention to food items through analyses of e.g., gaze duration bias and gaze direction bias. Eye tracking will be examined by tracking participants' eyes on the computer screen using the Tobii X2-60 device (Tobiipro, Stockholm, Sweden) integrated into the iMotions software.

Exercise Bout

The workload of the acute exercise bout will be individually calculated based on the peak workload ($Watt_{peak}$) achieved at the VO_2peak test. The total duration of the exercise bout is 45 minutes divided into intervals: 10 minutes warm-up (40% $Watt_{peak}$); 4 minutes high intensity (75% $Watt_{peak}$) and 3 minutes active rest (50% $Watt_{peak}$) intervals; 10 minutes cool-down (40% Wattpeak). In situations where a participant is not capable of completing the pre-calculated workload, the load will be manually downregulated to ensure a 45-minute exercise bout. Female participants are given 200 mL water and male participants are given 250 mL water during the 45-minute exercise bout, and asked to finish the water before completion. On the rest test days, participants will rest for the same duration as the exercise bout.

Ad Libitum Meal

Participants will be served an ad libitum meal 15 minutes after termination of the exercise bout/rest. The meal consists of savoury pie, from which each participant can choose between three different types: Bacon; Leeks; Pesto and tomato. The nutritional value of the different pies are presented by *Table 3*. The participants will be presented with 2 pies of 800 grams in total and instructed to eat until comfortably full/satiated. Participants will be presented with the same type of pie at all visits. Female and male participants receive 200 mL and 250 mL water, respectively, during the ad libitum meal, and are asked to finish the water before completing the meal. After completion of the meal, the remaining portion of pie will be measured, and the total weight of consumed pie will be calculated.

Statistical Methods

Sample Size

The study was designed to detect a minimally important difference (MID) of 500 kJ (45) with an alpha of 0.05 and beta of at least 0.8 for testing the hypotheses for the primary outcome in a hieararchal manner. Based on information from the literature (33,41,46–48) and observed data from similar ad libitum meal tests performed in one of our previous studies (49), we produced simulated data to use for the sample size calculation of the study. The primary scenario assessed was: Acute exercise reduces ad libitum intake by 1 MID both in the morning and in the evening. This scenario was used to test hypothesis 1 (superiority of exercise vs. rest for reducing ad libitum intake) and hypothesis 2 (equivalence of exercise in the morning vs. exercise in the evening). The simulated data

was analysed using a repeated measures regression model (mixed linear model, PROC MIXED, SAS 9.4, SAS Institutes Inc) specified as: ad libitum intake ~ sex + condition (morning/rest, morning/exercise, evening/rest, evening/exercise) + sequence (1-4), a repeat for condition on participant level, an unstructured covariance structure and alpha = 0.05. A total of 53 participants will be needed to complete the study to attain the desired statistical power. To account for potential loss-to-follow-up an additional 5 (10%) participants will be recruited.

Statistical Analysis Plan

Outcomes measured on the test days will as a general rule be analysed using a mixed model identical to the one described in the section "Sample Size". Model assumptions will be assessed using graphical methods. If needed outcomes will be log-transformed for analysis and results back-transformed for presentation. Outcomes that do not fit the model will be analysed using a generalized mixed model or by comparing the observed data using nonparamtric null-hypothsis tests. Modelled outcomes will be presented as estimated levels (95% confidence intervals (95%CI)) on test days and comparisons between test days will be presented as estimated differences (95% confidence intervals (95%CI), p-values). All outcomes will additionally be reported as summary of the raw data using appropriate summary statistics. Values are presented as mean ± SD if normally distributed, as median [IQR] if non-normally distributed and as n (%) if categorical.

P-values < 0.05 will be regarded as statistically significant. The false positive rate related to the hypothesis for the primary outcome will be controlled by using a hierarchal testing procedure. Secondary/descriptive outcomes will not adjusted for multiplicity, apart from omics outcomes (proteomics, metabolomics and lipodomics) where a false discovery rate cut-off (<0.1) will be applied.

Laboratory Analysis

Glucose, insulin, total cholesterol, triglycerides, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, alanine aminotransferase (ALAT), aspartate transaminase (ASAT), natrium, kalium, c-reactive protein (CPR) and glycated haemoglobin (HbA1C) analyses will be performed immediately at the Department of Clinical Biochemistry, Herlev Hospital, Denmark (adjacent to SDCC).

One blood sample (3 mL) will be treated with dipeptidyl peptidase 4-inhibitor "ValPyr" at the time of collection. Blood samples not immediately analysed will be centrifuged at 4°C and 3000 rpm for 10 minutes. Plasma will then be transferred to cryotubes, and stored at -20°C while the test visit is ongoing, and then immediately transferred to a -80°C freezer for storage after the visit. All other analyses will be performed after study completion in thawed samples in collaboration with the specific laboratory of excellence.

Omics Analysis

The proteomics analysis, Liquid Chromatographic-Mass Spectrometry (LC-MS), will be performed at Novo Nordisk A/S.

Metabolomics analysis (Gas Chromatographic-Mass Spectrometry (GC-MS)), and lipidomics analysis (ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-QTOF/MS)) will be performed at SDCC.

Ethics and Dissemination

Ethics

The knowledge obtained from this study will contribute to a better understanding of appetite control in response to acute exercise and timing of exercise in people with overweight/obesity, and people with overweight/obesity and T2D. The study will be conducted in accordance with the ethical principles in the Declaration of Helsinki, with national laws and regulations for clinical research. The study has been registered at ClinicalTrials.gov (NCT05768958). Participants will provide informed consent orally and in writing. All study results (positive, negative, and inconclusive) will be published in international peer-reviewed scientific journals and presented at national and international conferences.

Reporting Patient and Public Involvement in Research

Individuals in the target group were involved in the intial stages of the TIMEX study. Their involvement covered two parts of the study:

1) To ensure a diurnally-validated version of the SBFPT that would allow us to examine food reward both in the morning and evening using the same food images, we conducted an online questionnaire containing 28 food items (see *Test Visits; Steno Biometric Food Preference Task*) (43). The details are elaborated elsewhere (43). Based on the answers, we selected 16 items to be included into the SBFPT. This involvement from participants in our target group ensured a diurnally-validated version of the food images and thus a higher quality of the SBPFT.
2) Individuals within our target group actively participated in selecting an appropriate ad libitum meal for the test visits. The process included 3 phases: The first phase involved SDCC employees engaged in the TIMEX study and members of our research group. Together we compiled a list of meals suitable for consumption in the morning and evening, with high calorie density and high fat content. The second phase involved colleagues and members of our target group. Insights from individuals with T2D helped us clarify a meal balancing low carbohydrate content with high caloric density. The third phase involved meal testing in our laboratory to ensure consistent preparation and ease of disassembly. Experience from the laboratory resulted in a test meal choice of savoury pie. We opted to provide participants with a selection of three different pies to account for individual taste preferences, allergies, and dietary restrictions (e.g., religious considerations).

   Incorporating the target group into these considerations proved highly beneficial. The insights provided by participants with T2D were particularly valuable. Participants with T2D exhibited a reluctance toward consuming foods high in carbohydrates compared to those without T2D. Without their involvement, there was a risk of selecting a meal that would not align with the preferences of people with T2D, potentially hindering recruitment for the study.

Discussion

Despite current clinical guidelines and interventions to prevent and treat obesity, the global prevalence of obesity continues to increase. Additionally, there is a growing number of individuals living with T2D. Guidelines focusing on "what" and "how much" to eat or exercise are unmanageable for many people and are too time consuming, explaining the need for more feasible strategies. An increasing focus on hedonic mechanisms, diurnal rhytms and food reward systems indicates differences between lean individuals and individuals with overweight/obesity and/or T2D. This novel research raises important questions; Does acute exercise affect appetite in people with overweight, obesity and T2D and is the effect influenced by the timing of exercise? If physical activity can

influence hedonic aspects of appetite control, does exercise timing affect this? Does obesity with or without T2D alter food reward? Building upon these inquiries, the TIMEX study aims to investigate whether or to what extend acute exercise and timing of exercise can affect appetite control in individuals with obesity with and without T2D.

The cross-over study design enables comparison of appetite ratings, metabolic response, and subsequent food intake following an acute exercise bout compared to rest condition where each participant serves as their own control. Furthermore, how this might differ between morning and evening, and between individuals with obesity with or without T2D will also be examined. Blood samples taken during each test visit will reveal how appetite-related metabolites, hormones, and other biomarkers are influenced by the timing of exercice, and how these effects differ between weight and diabetes status.

Authors' contributions

KB, GF, KF, and JSQ conceived the idea and initiated the study. JSQ is principal investigator. NPL, HEP, MBB, MKG, GF, KB, KF, LGG, and JSQ contributed to the study design, statistics and methodology. LJ, NPL, HEP, and MMJ performed the clinial examinations and LJ, NPL, MMJ, LGG, and JSQ drafted the manuscript. All authors have critically reviewed the manuscript and approved the final version.

Funding Statement and Economical Compensation

The study is initiated by the investigator and co-investigators at SDCC. The total budget of the study is 2,970,400 DKK and will cover expenses for laboratory analyses, salaries, some equipment, and operating expenses. The study is funded by a NovoSTAR grant from Novo Nordisk A/S (2,970,400 DKK) granted investigator, Jonas Salling Quist. The grant is paid to and handled by SDCC. If needed or if possible, additional funding will be sought from relevant partners and foundations. The Ethics Committee of the Capital Region of Denmark and the study participants will be informed of grant source and amount, if additional funding is obtained.

Study participants receive DKK 400 for each study visit completed (excluding the screening visit). The remuneration is compensation for the inconvenience associated with participation in the study. Travel expenses are covered if individuals live more than 10 km away with a maximum of DKK 500 per visit. This applies to both use of public transportation and private transportation by car (car expenses of 2.16 DKK per kilometer). Transportation by car will be registered as transport subsidy. There is no treatment gain for the participants in the study. Participants who withdraw or are excluded from the study will receive renumeration corresponding to the number of study visits completed. Participants will not receive other benefits of economic value.

Competing interests

Lea Jalking, Natja Poder Launbo, Marie Møller Jensen, Martin Bæk Blond, Louise Groth Grunnet and Jonas Salling Quist are employed by SDCC. SDCC is a hospital providing health services for the public health care system. SDCC is funded by the Novo Nordisk Foundation through unrestricted grants. The Novo Nordisk Foundation has no economic interests in the study. The Novo Nordisk Foundation will not have influence on 1) the study design; 2) the collection, analysis, and interpretation of data; 3) the writing of the study report or any publication; and 4) the decision to

submit the paper for publication. The investigators employed at SDCC will not benefit economically from conducting the study.

Co-investigators Marina Kjærgaard Gerstenberg and Rune Ehrenreich Kuhre are employed at Novo Nordisk A/S and will be responsible for exploratory investigations using the data collected in the TIMEX study. Due to the collaborative nature of the study, Novo Nordisk A/S has been involved in designing the study, and will be involved in analyzing and interpreting data. Novo Nordisk A/S will align with SDCC if and when data and/or biosamples from the study are used for publications. HEP and KF are employed by Novo Nordisk A/S. iMotions A/S is a collaborator on the project and give advice for the use and analysis of biometric methods in the study design phase. iMotions A/S have no influence on 1) the study design; 2) the collection, analysis, and interpretation of data; 3) the writing of the study report or any publication; and 4) the decision to submit the paper for publication. LGG and KF own shares in Novo Nordisk A/S. The sponsor and principal investigor, JSQ, have no economic interest in the results of the study.

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Inclusion criteria

- Adults with overweight or obesity (BMI >25 kg/m2) with and without T2D
- Age 18 – 75 years
- HbA1c ≥48 mmol/mol for people with T2D

Exclusion criteria

- Not able to eat ad libitum meal because of e.g., allergy or intolerance
- Not able to perform the exercise bout because of e.g., musculoskeletal conditions
- Daily smoking
- For women: Pregnancy / planned pregnancy (within the study period) / lactating
- Self-reported history of an eating disorder in the past 3 years
- Self-reported weight change (>5 kg) within three months prior to inclusion
- Treatment with antidepressants
- Treatment with fast acting insulin, combination insulin products and sulfonylureas
- Alcohol/drug abuse or in treatment with disulfiram (antabus) at time of inclusion
- Uncontrolled medical issues including but not limited to cardiovascular pulmonary, rheumatologic, hematologic, oncologic, infectious, gastrointestinal, or psychiatric disease; diabetes or other endocrine disease; immunosuppression
- Current treatment with medication which significantly affect appetite or energy balance (e.g., GLP-1 receptor agonists)
- Bariatric surgery
- Unable to understand the informed consent and the study procedures
- Concomitant participation in intervention studies
- Incapable of understanding Danish

Box 1. Inclusion and exclusion criteria for participation in the TIMEX study. Abbreviations: T2D, Type 2 Diabetes; BMI, Body mass index

Table 1. Schematic overview of study visits				
Visit	Screening	Baseline visit	Rest test visit	Exercise-test visit
Participant-related information				
Informed consent	X			
Assessment of in- and exclusion criteria	X			
Anthropometrics		X		
Medical history (including concomitant medications)		X		
Clinical assessment				
Blood pressure		X		
ECG		X		
Bioimpedance			X (morning)	
VO_2peak		X		
Efficacy outcomes				
Fasting blood samples + HbA1c			X	X
Post exercise/rest blood samples			X	X
Post ad libitum meal blood samples			X	X
VAS *(appetite)*			X	X
VAS *(meal assessment)*			X	X
SBFPT			X	X
Acute exercise bout				X
RPE *(Borg scale)*				X
Ad libitum meal			X	X
Questionnaires				
Socioeconomic status (SES)			X (morning)	
Control of Eating Questionnaire (CoEQ)	X			
International Physical Activity Questionnaire (IPAQ)			X (morning)	

Morningness/Eveningness Questionnaire (MEQ)			X (morning)	
Munich Chronotype Questionnaire (MCTQ)			X (morning)	
Pittsburgh Sleep Quality Index (PSQI)			X (morning)	
Safety outcomes				
Adverse events		X	X	X
Abbrevations: ECG (Electrocardiography), DXA (Dual-energy x-ray absorptiometry), RPE (Ratings of perceived exertion), VO2peak (Peak oxygen consumption), SBFPT (Steno Biometric Food Preference Task), VAS (Visual Analog Scale)				

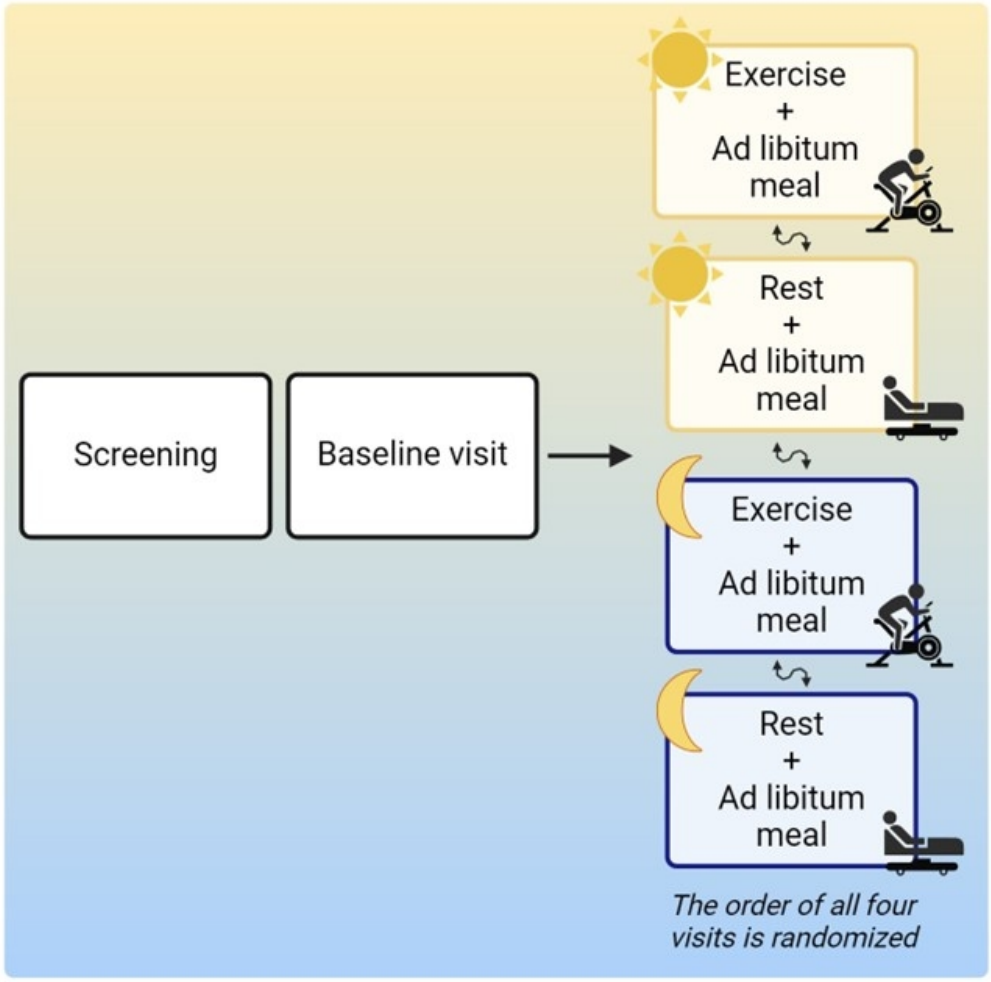
Table 2. Schematic overview of blood sample measures

	Fasted state	Post exercise/rest
HbA1c	X	
CRP	X	
ALAT	X	
ASAT	X	
Natrium	X	
Kalium	X	
Glucose	X	X
Insulin	X	X
C-peptide	X	X
Triglycerides	X	X
LDL cholesterol	X	X
HDL cholesterol	X	X
Appetite-related hormones	X	X
Proteomics	X	
Metabolomics	X	X
Lipidomics	X	X

Abbreviations: Glycated haemoglobin (HbA1c), C-reactive protein (CRP), Alanine aminotransferase (ALAT), Aspartate transaminase (ASAT), Low density lipoprotein (LDL), High density lipoprotein (HDL), Gastric inhibitory polypeptide (GIP), Glucagon-like-peptide (GLP-1), Peptide Tyrosine Tyrosine (PYY), Pancreatic polypeptide (PP), Fibroblast growth hormone 21 (FGF21), Growth differentiation factor 15 (GDF15)

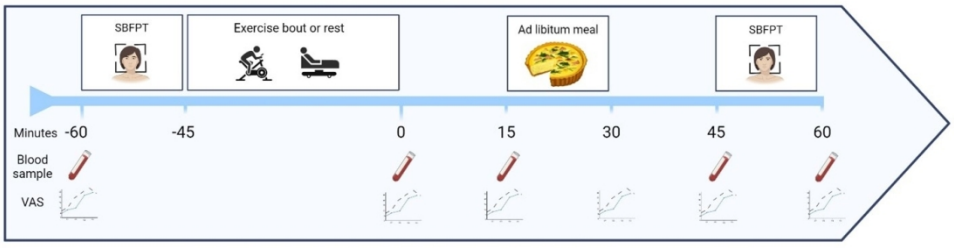
Table 3. Nutritional value of ad libitum meal options

Pie-variant	**Kcal/KJ per 100 grams**	**Nutrition in grams (E%)**
Bacon	270 kcal/1134 KJ	Fat: 17 g (57%) Carbohydrates: 19 g (28%) Protein: 10 g (15%)
Leeks	210 kcal/882 KJ	Fat: 11 g (48%) Carbohydrates: 20 g (39%) Protein: 7 g (13%)
Pesto and tomato	234 kcal/983 KJ	Fat: 12 g (46%) Carbohydrates: 22 g (38%) Protein: 9 g (16%)



TIMEX study design. Baseline visits are completed after screening on the same day. Morning visits are initiated at 08:00-08:30 and evening visits are initiated at 15:00-15:30. All visits are completed within 2.5 hours, and with a minimum of a 3-day washout.

169x167mm (96 x 96 DPI)



Schedule for the test visits. Abbreviations: SBFPT, Steno Biometric Food Preference Task. VAS, visual analogue scales

169x45mm (220 x 220 DPI)

BMJ Open

Effects of exercise and exercise timing on energy intake and appetite control in Danish individuals with overweight or obesity with and without type 2 diabetes: a protocol for a randomized controlled crossover trial

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Title: Effects of exercise and exercise timing on energy intake and appetite control in Danish individuals with overweight or obesity with and without type 2 diabetes: a protocol for a randomized controlled crossover trial

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Abbrevations

BMI	Body mass index
CoEQ	Control of Eating Questionnaire
CoNSORT	Consolidated standards of reporting trials
ECG	Electrocardiography
IPAQ	International Physical Activity Questionnaire
MCQT	Munich Chronotype Questionnaire
MEQ	Morningness-Eveningness Questionnaire
MID	Minimal important difference
PSQI	Pittsburgh Sleep Quality Index
SBFPT	Steno Biometric Food Preference Task
SDCC	Steno Diabetes Center Copenhagen
SES	Socioeconomic status
SPIRIT	Standard protocol items: Recommendations for international trials statement
T2D	Type 2 diabetes
VAS	Visual analogue scales
VO_2peak	Peak oxygen uptake

Abstract

Introduction

The aim of this study is to investigate effects of acute exercise on appetite control and whether this differs between morning and late afternoon in individuals with overweight/obesity with or without type 2 diabetes.

Methods and Analysis

The hedonic and homeostatic appetite control in obesity and type 2 diabetes in the context of meal and exercise timing (TIMEX) study is a randomised, controlled, cross-over trial. Fifty-eight women and men (aged 18 – 75 years) with overweight or obesity (BMI ≥25 kg/m^2) with or without type 2 diabetes will be recruited. All participants will complete a screening and baseline visit followed by four test visits: two morning visits and two late afternoon visits. The participants will arrive in the fasted state during the visits. During one morning visit and one late afternoon visit, the participants will engage in a 45-minute bout of acute high-intensity interval exercise on an ergometer bicycle. The remaining two visits (one morning and one late afternoon visit) will include 45 minutes of rest. Fifteen minutes after the rest or exercise period, the participants will be presented with an ad libitum meal. Blood samples will be collected and subjective appetite will be assessed using visual analogue scales in the fasted state before exercise/rest, immediately post-exercise/rest and at 15, 30, 45 and 60 minutes post-exercise/rest. Food reward and food preferences will be assessed using the validated diurnal version of the Steno Biometric Food Preference Task in the fasted state before exercise/rest and 15 minutes after the ad libitum meal (45 minutes post-exercise/rest). The primary outcome is the difference in ad libitum energy intake after exercise compared to rest. Secondary outcomes include eating rate; 24-hour energy intake; appetite-related metabolites and hormones, and circulating biomarkers assessed from proteomics, metabolomics, and lipidomics analyses; food choice, food attention and reaction time, explicit and implicit liking and wanting for different food categories, subjective appetite; ratings of perceived exertion during exercise. All outcomes will be compared between morning and late afternoon and between participants with and without type 2 diabetes.

Ethics and dissemination

The study has been approved by the Ethics Committee of the Capital Region of Denmark (H-22019913) and the Capital Region of Denmark's Research Register (Privacy). The study will be conducted in accordance with the Declaration of Helsinki. All results will be published in national

and international peer-reviewed journals and will be disseminated at national and international conferences.

Trial registration

The trial is registered at ClinicalTrials.gov, identifier: NCT05768958

Strength and limitations

Strengths:

- Comprehensive assessment of appetite control including various, multidisciplinary methods: ad libitum food intake, subjective appetite ratings, appetite-regulating hormones, assessment of food preferences, and 24-hour diet records
- Two-by-two factorial design allows comparison of the effects of exercise vs. rest and the effects of morning vs. late afternoon exercise in the same trial
- Cross-over study with a large sample size in people with overweight or obesity with and without type 2 diabetes

Limitations:

- Heterogenous study population (participants with and without type 2 diabetes) which increases the generalizability of the results, but also induces larger variation to the data
- No healthy control group (referred to as individuals with BMI between 18.5-24.9 kg/m^2) which prevents the study from investigating if the outcomes relate to overweight and obesity per se

Introduction

The role of exercise in appetite control and weight management, as well as in the treatment of metabolic disorders such as type 2 diabetes (T2D), has been a prominent focus in numerous research studies. The phenomenon of exercise-induced anorexia was investigated and described by King, Burley & Blundell in 1994[1], who demonstrated that subjective appetite ratings were lower immediately following an acute bout of exercise compared with pre-exercise levels. Although energy intake after the exercise was not suppressed, relative to the energy expended, a long duration (≈50 min), high intensity exercise bout led to a lower relative energy intake[1]. Consistent findings have since shown that post-exercise energy intake, relative to the energy expenditure during exercise, is often reduced compared to resting condition. This indicates that exercise can create an energy deficit that is not fully compensated for by short-term increases in energy intake. However, the effects of exercise on absolute energy intake remain inconclusive, with reports of increased, decreased and no difference in energy intake compared to rest[2–4]. These discrepancies may be attributed to methodological variations, including differences in exercise intensity, the timing of the post-exercise meal, and the characteristics of the study population.

Circadian or diurnal rhythms may influence appetite control and consequently, energy balance. Exercise has been identified as a circadian time cue, with exercise-induced signals shown to affect molecular clock genes[5]. The diurnal timing of exercise also seems to impact body weight, with greater losses in body weight observed with morning compared to evening exercise[6,7]. However, research on the effects of exercise timing on appetite and energy intake has yielded mixed results. Some studies found no significant differences in energy intake at any meal or overall during 26 hours post-exercise or macronutrient preferences between morning and evening exercise[8]. However, others reported that morning exercise may lead to greater perceived satiety 15 min post-exercise in women with overweight, although 24-hour post-exercise energy intake was similar[9]. Exercise timing can also affect food preferences, with greater wanting for low-fat sweet foods after morning compared with evening exercise and greater wanting for high-fat sweet foods after evening compared with morning exercise[10]. The impact of exercise timing may be influenced by individual chronotype, with early chronotypes experiencing greater hunger suppression after morning exercise and late chronotypes after evening exercise[10]; however, there is a need for a better understanding of the interaction between chronotype and exercise timing for appetite control and weight management[11].

Furthermore, whether exercise affects appetite control differently in people living only with overweight/obesity or with overweight/obesity and type 2 diabetes is currently unknown. In healthy individuals, glucose tolerance to identical meals is greater earlier compared to later in the day[12]. However, in people with T2D, insulin sensitivity has been shown to improve throughout the day and worsen throughout the night into the morning[13]. Whether this diurnal variation affects appetite control remains unclear. A few small studies in people at risk of or with T2D, have investigated effects of exercise timing on glycaemic control, with some suggesting that afternoon/evening exercise may lead to better glucose regulation compared to morning exercise[14–16]. However, other studies have not observed significant differences[17,18]. Additionally, people living with T2D are often prone to late-night eating[19], which could serve as a potential target for exercise interventions. If evening exercise could suppress appetite and reduce energy intake during the hours following the exercise bout, it may provide a viable strategy to mitigate late-night eating behavior in this population.

Understanding "when" to eat and exercise is of great importance as most clinical guidelines and interventions in prevention and treatment of obesity and related diseases focus on "what" and "how much" to eat or "how" and "how much" to exercise. This study will investigate how exercise and exercise timing affects appetite control and subsequent energy intake after an acute, high intensity exercise bout in people with overweight/obesity and with and without T2D.

Objectives

The specific objectives of the TIMEX study are to investigate whether energy intake during an ad libitum meal differs following a 45-minute acute bout of high-intensity interval exercise compared to rest; investigate whether the energy intake of the ad libitum meal differs between morning and late afternoon; investigate if appetite ratings, food reward, metabolic markers following the ad libitum meal differ between morning and late afternoon, and between individuals with and without T2D; and investigate circulating biomarkers that can be used to identify if ad libitum food intake is primarily driven by hedonic or homeostatic appetite.

Hypotheses

Hypotheses for the primary endpoint (ad libitum energy intake) are:

1. An acute bout of exercise reduces ad libitum energy intake compared with a rest condition
2. Time of day does not affect exercise-induced anorexia (i.e., reduction in energy intake after exercise)

Hypotheses for the secondary endpoints are:

3. Appetite sensations and reward for high-fat sweet foods will be greater in the late afternoon relative to the morning and this effect will be more pronounced in individuals with T2D compared to individuals without T2D.
4. Appetite sensations and reward for high-fat sweet foods will be greater in response to exercise in the late afternoon relative to the morning and this effect will be more pronounced in individuals with T2D compared to individuals without T2D.
5. Appetite and food reward will be associated with circulating biomarkers and these associations will be affected by T2D status.

Methods and Analysis

Study Design

This study is a randomised, controlled, cross-over trial (*Figure 1*). Fifty-eight individuals with overweight/obesity with or without T2D will be randomised after completion of a screening and baseline visit. Each participant will complete four test visits (two morning visits and two late afternoon visits) on four separate days with a ≥3-day washout between visits. We aim for completion of all visits within a 3-month period for each participant to prevent potential changes in e.g., body weight, dietary intake, and physical activity. Primary and secondary outcomes are assessed during the baseline visit and test visits.

The study will be conducted at Steno Diabetes Center Copenhagen (SDCC) and will be reported according to the Consolidated Standards of Reporting Trials (CONSORT)[20]. The study protocol follows the Standard Protocol Items: Recommendations for Interventional Trials statement (SPIRIT)[21]. The study is registered at ClinicalTrials.gov (identifier: NCT05768958).

Participants

Women and men, 18-75 years, with overweight or obesity (BMI ≥25 kg/m^2) with or without T2D, who are eligible according to the inclusion and exclusion criteria (*Box 1*) will be included. We aim for an equal distribution of participants with and without T2D.

Eligibility criteria

Inclusion and exclusion criteria are listed in *Box 1*.

Outcomes

Primary outcome

The primary outcome is the difference in energy intake during an ad libitum meal after 45 minutes of acute exercise bout compared to rest. The ad libitum meal will be served 15 min post-exercise.

Secondary Exploratory Outcomes

All secondary outcomes include comparison between exercise and rest, morning and late afternoon, and T2D status. Secondary outcomes include eating rate; 24-hour energy intake (comprised of the energy intake from the ad libitum meal and self-reported energy intake 24-hours post-visit); appetite-related metabolites and hormones, and circulating biomarkers assessed from proteomics, metabolomics and lipidomics analyses; food choice, food attention and reaction time, explicit and implicit liking and wanting, subjective appetite; ratings of perceived exertion during exercise.

Recruitment

Screening/baseline visit

Participants are recruited via advertisements on www.Forskningnu.dk (social media). After reading the study information, they undergo a pre-screening phone interview to reduce screening failures. During the screening visit, participants are orally briefed on the study, provide written consent (Supplemental material), and undergo a health exam, including medical history and eligibility assessment (*Box 1*). If eligible, participants proceed with height, weight, blood pressure, and ECG measurements. Those with approved ECGs complete a bicycle test to determine peak oxygen uptake (VO_2peak). After inclusion, participants are randomized into one of four test visit sequences, which are scheduled to reduce confounding variables.

Anthropometry and Blood Pressure

At the baseline visit, body weight will be measured to the nearest 0.1 kg with the participant wearing light clothes. Height will be measured to the nearest 0.1 cm. Height and weight will be measured using Seca 287 wireless ultrasonic measuring station (Seca gmbh & co. kg, Hamburg, Germany). Blood pressure and resting heart rate will be measured using a digital blood pressure gauge (Microlife BP A3L Comfort, Microlife AG Swiss Corporation, Widnau, Switzerland) and noted three times with 2-minutes intervals after a 10-minutes rest and the average of the two lowest values is reported.

Cardiorespiratory Fitness

VO_2peak as a measure of cardiorespiratory fitness will be measured by an incremental bicycle ergometer test (Corival CPET Medical Ergometer, Lode BV, Groningen, The Netherlands). Participants will complete a warm-up of 3 minutes at 25 watts followed by a linear increase in watts until exhaustion. Heart rate, ventilation rate, inspired oxygen and expired carbon dioxide levels are measured using a combined heart rate monitor and indirect calorimetry (Vyntus CPX, Vyaire Medical GmbH, Hoechberg, Germany). The VO_2peak test will be deemed valid if the respiratory exchange ratio is >1.15, a plateau in oxygen uptake is reached and heart rate is ±10 heartbeats from the estimated maximal heart rate[22].

Test Visits

All test visits will be conducted at SDCC. Prior to their visit, the participants are asked to standardize sleep (7-9 hours) if possible and dietary intake the day before all test visits. Participants will be asked to register their dietary intake 24 hours prior to and after each test visit. To obtain a somewhat equal baseline, the participants will further be asked to follow the same dietary pattern (to the best of their ability) during the 24 hours before each visit. On test days, participants will arrive at ~8:00 am after an ≥8-hour fast (morning visits) or at ~3:00 pm after a 3-hour fast (late afternoon visits). Water is allowed until 2 hours before each visit. Smoking is allowed until 3 hours before each visit. Furthermore, no alcohol consumption or strenuous physical activity are allowed 48 hours prior to testing. Participants are instructed to avoid physically demanding transportation to the research facility. Participants are instructed to continue their regular medication regimen throughout the duration of the study. The schedule for the test visits is represented in *Figure 2*, and a schematic overview is presented in *Table 1*.

Blood Samples

Blood samples will be collected from an antecubital vein in the fasted state on the test visits and at 4 subsequent timepoints (0, 30, 45, and 60 minutes after the completion of the exercise bout/rest). *Table 2* present the analyses that will be performed.

Body Composition

Body composition i.e., fat mass and fat free mass will be examined by electrical bioimpedance (seca mBCA 515, Seca gmbh & co. kg, Hamburg, Germany) at the first morning test visit with the participant wearing light clothes.

Dietary Intake

Participants will be instructed to weigh and register their food and drink intake 24 hours before and after each test visit. For the registration each participant receives a printed food diary containing written instruction for tracking, and tables for the tracking. All participants are offered to borrow a digital food scale. The hand-written, self-reported dietary intake will subsequently be logged into the online software Vitakost.dk (Vitakost, Kolding, Denmark) by the researchers.

Questionnaires

Participants will complete questionnaires on:

- Socioeconomic status (SES)[23]
- Eating behaviour (Control of Eating Questionnaire - CoEQ)[24]
- Physical activity (International Physical Activity Questionnaire - IPAQ)[25]
- Chronotype (Morningness/Eveningness Questionnaire; MEQ[26] and Munich Chronotype Questionnaire; MCTQ[27])
- Sleep (Pittsburgh Sleep Quality Index - PSQI)[28]
- Appetite and meal assessment (visual analogue scales - VAS)[29]

The participants will fill in the questionnaire CoEQ at the baseline visit, and the remaining (SES, IPAQ, MEQ, MCTQ and PSQI) at their first rest visit.

Subjective Appetite

Subjective appetite will be assessed using VAS[29], including ratings of hunger, fullness, satiety and prospective food consumption, thirst, desire to eat something sweet, salty, fatty, meat, and potential nausea. We will collect the VAS measurements in the fasted state on the test visits and at 5 subsequent timepoints (0, 15, 30, 45, and 60 minutes after the completion of the exercise bout/rest).

Palatability and liking of the ad libitum test meal will be rated. The ratings will be performed on a computer during all test visits.

Socioeconomic Status

Participants' socioeconomic status will be assessed using SES questionnaire [23] performed on a computer during the first rest visit. The SES questionnaire includes information on gender, educational level, work position, family orientation and annual household income.

Eating behaviour traits

Participants' eating behaviour traits will be assessed using CoEQ [24] performed on a computer during the baseline visit. The CoEQ includes questions on hunger, fullness, cravings and mood over the past 7 days.

Physical activity

Participants' physical activity will be assessed using IPAQ [25] performed on a computer during the first rest visit. The IPAQ includes questions on frequency and intensity of physical activity on average over the past 7 days.

Chronotypical features

Data on participants' chronotypical features will be assessed using MEQ and MCQT [26,27]. The questionnaire will be performed on a computer during the first rest visit. It will include questions on subjective fatigue, awakeness, energy level and hunger during mornings and nights.

Food preferences

Food preferences will be measured using the diurnally validated SBFPT[30,31], a computerized task adapted from the Leeds Food Preference Questionnaire (LFPQ) and tailored to fit a Danish food context[32]. The task includes 16 images of common Danish foods, varying in fat content (high or low) and taste (sweet or savory). Before the study, an online survey validated the appropriateness of these images for morning and late afternoon consumption among the target population (n=167). The SBFPT has two parts: a forced-choice task to measure food choice and implicit 'wanting,' and a rating task to assess explicit 'liking' and explicit 'wanting' on a scale from 0-100.

All responses are recorded in the digital software, iMotions 9.3 (iMotions A/S, Copenhagen K, Denmark) and used to compute mean scores for all abovementioned combined food categories. Prior to these two parts, participants are asked to familiarize themselves with a printed version of the 16 food images and explain what they see on the images. During the presentation of the 16 pictures, data on eye tracking and reaction time are collected. Eye tracking will be used to examine visual attention

to food items through analyses of e.g., gaze duration bias and gaze direction bias. Eye tracking will be examined by tracking participants' eyes on the computer screen using the Tobii X2-60 device (Tobiipro, Stockholm, Sweden) integrated into the iMotions software.

Exercise Bout

The workload of the acute exercise bout will be individually calculated based on the peak workload ($Watt_{peak}$) achieved at the VO_2peak test. The total duration of the exercise bout is 45 minutes divided into intervals: 10 minutes warm-up (40% $Watt_{peak}$); 4 x 4 minutes high intensity (75% $Watt_{peak}$) with 3 minutes active rest (50% $Watt_{peak}$) between intervals; 10 minutes cool-down (40% Wattpeak). Initially the high intensity intervals were set at 85% $Watt_{max}$, however, after 4 participants we changed the workload to 75% $Watt_{max}$ since participants were not able to complete the exercise at the 85% $Watt_{max}$. In situations where a participant is not capable of completing the pre-calculated workload, the load will be manually downregulated to ensure a 45-minute exercise bout. During the exercise bout, heart rate, workload, and revolutions per minute (RPM) be measured continuously. Participants will be asked to rate their perceived exertion (using the Borg Scale) at the end of the warm-up, the end of the last low-intensity and high-intensity interval, and at the end of the cool-down period. Female participants are given 200 mL water and male participants are given 250 mL water during the 45-minute exercise bout and asked to finish the water before completion. On the rest test days, participants will rest for the same duration as the exercise bout.

Ad Libitum Meal

Participants will be served an ad libitum meal 15 minutes after termination of the exercise bout/rest. The meal consists of savoury pie, from which each participant can choose between three different types: Bacon; Leeks; Pesto and tomato. The nutritional value of the different pies is presented in *Table 3*. The participants will be presented with 2 pies of 800 grams in total and instructed to eat until comfortably full/satiated. Participants will be presented with the same type of pie at all visits. The pies will be divided into randomly sized portions to minimize the likelihood of participants estimating their consumption based on previous visits. The portions will be served on a large plate for consistency in presentation. Female and male participants receive 200 mL and 250 mL water, respectively, during the ad libitum meal, and are asked to finish the water before completing the meal.

After completion of the meal, the remaining portion of pie will be measured, and the total weight of consumed pie will be calculated.

Statistical Methods

Sample Size

The study was designed to detect a minimally important difference (MID) of 500 kJ[33] with an alpha of 0.05 and beta of at least 0.8 for testing the hypotheses for the primary outcome in a hierarchal manner. Based on information from the literature[2,21,34–36] and observed data from similar ad libitum meal tests performed in one of our previous studies[37], we produced simulated data to use for the sample size calculation of the study. The primary scenario assessed was: Acute exercise reduces ad libitum intake by 1 MID both in the morning and in the late afternoon. This scenario was used to test hypothesis 1 (superiority of exercise vs. rest for reducing ad libitum intake) and hypothesis 2 (equivalence of exercise in the morning vs. exercise in the late afternoon). The simulated data was analysed using a repeated measures regression model (mixed linear model, PROC MIXED, SAS 9.4, SAS Institutes Inc) specified as: ad libitum intake ~ sex + condition (morning/rest, morning/exercise, late afternoon/rest, late afternoon/exercise) + sequence (1-4), a repeat for condition on participant level, an unstructured covariance structure and alpha = 0.05. A total of 53 participants will be needed to complete the study to attain the desired statistical power. To account for potential loss-to-follow-up an additional 5 (10%) participants will be recruited. Participants who withdraw after randomization, but before completing any test visits will be replaced.

Statistical Analysis Plan

All outcomes will be reported as summary of the raw data using appropriate summary statistics. Descriptive data are presented as mean ± SD if normally distributed, as median [IQR] if non-normally distributed and as n (%) if categorical.

Outcomes measured on the test days will as a general rule be analysed using a linear mixed model (LMM). LMMs will be used to assess energy intake differences across conditions (rest vs. exercise), time of day (morning vs. late afternoon), and their interactions, as well as the randomized visit sequence, while accounting for within-subject variability with a random intercept on participant level. We will calculate levels for each condition and the contrasts between them (estimated levels and

differences with 95%CI will be calculated and presented). The interaction with T2D status on the outcomes will also be explored using T2D as an interaction term in the model. In the LMM with T2D interaction we will adjust for age, sex, and BMI to account for potential differences between groups.

Model assumptions will be assessed using graphical methods, including Q-Q plots, residual vs. predicted plots, and histograms of residuals. If needed outcomes will be log-transformed for analysis and results back-transformed for presentation. Outcomes that do not fit the model will be analysed using a generalized mixed model or by comparing the observed data using nonparametric null-hypothesis tests.

Modelled outcomes will be presented as estimated levels (95% confidence intervals (95%CI)) on test days and comparisons between test days will be presented as estimated differences (95%CI, p-values).

P-values < 0.05 will be regarded as statistically significant. The false positive rate related to the hypothesis for the primary outcome will be controlled by using a hierarchal testing procedure. Secondary/descriptive outcomes will not be adjusted for multiplicity, apart from omics outcomes (proteomics, metabolomics and lipidomics) where a false discovery rate cut-off (<0.1) will be applied.

Laboratory Analysis

Glucose, insulin, total cholesterol, triglycerides, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, alanine aminotransferase (ALAT), aspartate transaminase (ASAT), natrium, kalium, c-reactive protein (CPR) and glycated haemoglobin (HbA1C) analyses will be performed immediately at the Department of Clinical Biochemistry, Herlev Hospital, Denmark (adjacent to SDCC).

One blood sample (3 mL) will be treated with dipeptidyl peptidase 4-inhibitor "ValPyr" at the time of collection. Blood samples not immediately analysed will be centrifuged at 4°C and 3000 rpm for 10 minutes. Plasma will then be transferred to cryotubes and stored at -20°C while the test visit is ongoing, and then immediately transferred to a -80°C freezer for storage after the visit. All other analyses will be performed after study completion in thawed samples in collaboration with the specific laboratory of excellence. The hormones scheduled for analysis following study completion include: Ghrelin, Glucagon, Gastric inhibitory polypeptide (GIP), Glucagon-like-peptide (GLP-1),

Peptide Tyrosine Tyrosine (PYY), Pancreatic polypeptide (PP), Cholecystokinin (CCK), Fibroblast Growth Factor 21 (FGF21), Growth Differentiation Factor 15 (GDF15).

Omics Analysis

The proteomics analysis, Liquid Chromatographic-Mass Spectrometry (LC-MS), will be performed at Novo Nordisk A/S.

Metabolomics analysis (Gas Chromatographic-Mass Spectrometry (GC-MS)), and lipidomics analysis (ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-QTOF/MS)) will be performed at SDCC.

Ethics and Dissemination

Ethics

The knowledge obtained from this study will contribute to a better understanding of appetite control in response to acute exercise and timing of exercise in people with overweight/obesity, and people with overweight/obesity and T2D. The study has been approved by the Ethics Committee of the Capital Region of Denmark (H-22019913) and the Capital Region of Denmark's Research Register (Privacy). The study will be conducted in accordance with the Declaration of Helsinki. The study has been registered at ClinicalTrials.gov (NCT05768958). Participants will provide informed consent orally and in writing. All study results (positive, negative, and inconclusive) will be published in international peer-reviewed scientific journals and presented at national and international conferences.

Reporting Patient and Public Involvement in Research

Individuals in the target group were involved in the initial stages of the TIMEX study. Their involvement covered two parts of the study:

1) To ensure a diurnally-validated version of the SBFPT that would allow us to examine food reward both in the morning and late afternoon using the same food images, we conducted an online questionnaire containing 28 food items (see *Test Visits; Steno Biometric Food Preference Task*) [31]. The details are elaborated elsewhere [31]. Based on the answers, we selected

16 items to be included into the SBFPT. This involvement from participants in our target group ensured a diurnally-validated version of the food images and thus a higher quality of the SBPFT.

2) Individuals within our target group actively participated in selecting an appropriate ad libitum meal for the test visits. The process included 3 phases: The first phase involved SDCC employees engaged in the TIMEX study and members of our research group. Together we compiled a list of meals suitable for consumption in the morning and late afternoon, with high calorie density and high fat content. The second phase involved colleagues and members of our target group. Insights from individuals with T2D helped us clarify a meal balancing low carbohydrate content with high caloric density. The third phase involved meal testing in our laboratory to ensure consistent preparation and ease of disassembly. Experience from the laboratory resulted in a test meal choice of savoury pie. We opted to provide participants with a selection of three different pies to account for individual taste preferences, allergies, and dietary restrictions (e.g., religious considerations).

Incorporating the target group into these considerations proved highly beneficial. The insights provided by participants with T2D were particularly valuable. Participants with T2D exhibited a reluctance toward consuming foods high in carbohydrates compared to those without T2D. Without their involvement, there was a risk of selecting a meal that would not align with the preferences of people with T2D, potentially hindering recruitment for the study.

Discussion

Despite current clinical guidelines and interventions to prevent and treat obesity, the global prevalence of obesity continues to increase. Additionally, there is a growing number of individuals living with T2D. Guidelines focusing on "what" and "how much" to eat or exercise are unmanageable for many people and are too time consuming, explaining the need for more feasible strategies. An increasing focus on hedonic mechanisms, diurnal rhythms and food reward systems indicate differences between lean individuals and individuals with overweight/obesity and/or T2D. This novel research raises important questions; Does acute exercise affect appetite in people with overweight, obesity and T2D and is the effect influenced by the timing of exercise? If physical activity can influence hedonic aspects of appetite control, does exercise timing affect this? Does obesity with or without T2D alter food reward? Building upon these inquiries, the TIMEX study aims to investigate whether or to what extend acute exercise and timing of exercise can affect appetite control in individuals with obesity with and without T2D.

The cross-over study design enables comparison of appetite ratings, metabolic response, and subsequent food intake following an acute exercise bout compared to rest condition where each participant serves as their own control. Furthermore, how this might differ between morning and late afternoon, and between individuals with obesity with or without T2D will also be examined. Blood samples taken during each test visit will reveal how appetite-related metabolites, hormones, and other biomarkers are influenced by the timing of exercise, and how these effects differ between weight and diabetes status.

Authors' contributions

Conceptualization: MKG, GF, KB KF and JSQ; Methodology: NPL, HEP, MBB, GF, KB, KF, LGG and JSQ; Software: NPL, MMJ, HEP, MBB; Investigation: LJ, NPL, MMJ, HEP, MBB; Writing – original draft: LJ and NPL; Writing – review and editing: LJ, NPL, MMJ, HEP, MBB, MKG, GF, KB, KF, LGG and JSQ; Visualization: LJ and NPL; Supervision: GF, KB, KF, LGG, JSQ; Project administration: LJ, NPL, HEP, MMJ; Funding acquisition: MKG, GF, KB, KF, LGG and JSQ; Guarantor: JSQ.

Funding Statement and Economical Compensation

The study is initiated by the investigator and co-investigators at SDCC. The total budget of the study is 2,970,400 DKK and will cover expenses for laboratory analyses, salaries, some equipment, and operating expenses. The study is funded by a NovoSTAR grant from Novo Nordisk A/S (2,970,400 DKK) granted investigator, JSQ. The grant is paid to and handled by SDCC. If needed or if possible, additional funding will be sought from relevant partners and foundations. The Ethics Committee of the Capital Region of Denmark and the study participants will be informed of grant source and amount, if additional funding is obtained.

Study participants receive DKK 400 for each study visit completed (excluding the screening visit). The remuneration is compensation for the inconvenience associated with participation in the study. Travel expenses are covered if individuals live more than 10 km away with a maximum of DKK 500 per visit. This applies to both use of public transportation and private transportation by car (car expenses of 2.16 DKK per kilometer). Transportation by car will be registered as transport subsidy. There is no treatment gain for the participants in the study. Participants who withdraw or are excluded from the study will receive renumeration corresponding to the number of study visits completed. Participants will not receive other benefits of economic value.

Competing interests

LJ, NPL, MMJ, MBB, LGG and JSQ are employed by SDCC. SDCC is a hospital providing health services for the public health care system. SDCC is funded by the Novo Nordisk Foundation through unrestricted grants. The Novo Nordisk Foundation has no economic interests in the study. The Novo Nordisk Foundation will not have influence on 1) the study design; 2) the collection, analysis, and interpretation of data; 3) the writing of the study report or any publication; and 4) the decision to

submit the paper for publication. The investigators employed at SDCC will not benefit economically from conducting the study.

Co-investigator MKG is employed at Novo Nordisk A/S and will be responsible for exploratory investigations using the data collected in the TIMEX study. Due to the collaborative nature of the study, Novo Nordisk A/S has been involved in designing the study and will be involved in analyzing and interpreting data. Novo Nordisk A/S will align with SDCC if and when data and/or biosamples from the study are used for publications. HEP and KF are now employed by Novo Nordisk A/S. iMotions A/S is a collaborator on the project and give advice for the use and analysis of biometric methods in the study design phase. iMotions A/S have no influence on 1) the study design; 2) the collection, analysis, and interpretation of data; 3) the writing of the study report or any publication; and 4) the decision to submit the paper for publication. LGG and KF own shares in Novo Nordisk A/S. The sponsor and principal investigator, JSQ, have no economic interest in the results of the study. All other authors have no competing interests to declare.

Data Sharing PlanL

Data from the trial will not be made publicly available as individual data from participants are protected by EU's GDPR (General Data Protection Regulation) laws and Danish Data Protection laws. Any anonymized datasets combining all trial participant data can be made available upon reasonable request from the corresponding author.

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Inclusion criteria

- Adults with overweight or obesity (BMI >25 kg/m2) with and without T2D
- Age 18 – 75 years
- HbA1c ≥48 mmol/mol for people with T2D
- Waist to height ratio ≥0.5 or waist circumference ≥88/102 cm for women and men, respectively

Exclusion criteria

- Not able to eat ad libitum meal because of e.g., allergy or intolerance
- Not able to perform the exercise bout because of e.g., musculoskeletal conditions
- Daily smoking
- For women: Pregnancy / planned pregnancy (within the study period) / lactating
- Self-reported history of an eating disorder in the past 3 years
- Self-reported weight change (>5 kg) within three months prior to inclusion
- Treatment with antidepressants
- Treatment with fast acting insulin, combination insulin products and sulfonylureas
- Alcohol/drug abuse or in treatment with disulfiram (antabus) at time of inclusion
- Uncontrolled medical issues including but not limited to cardiovascular pulmonary, rheumatologic, hematologic, oncologic, infectious, gastrointestinal, or psychiatric disease; diabetes or other endocrine disease; immunosuppression
- Current treatment with medication which significantly affect appetite or energy balance (e.g., GLP-1 receptor agonists)
- Bariatric surgery
- Unable to understand the informed consent and the study procedures
- Concomitant participation in intervention studies
- Incapable of understanding Danish

Box 1. Inclusion and exclusion criteria for participation in the TIMEX study. Abbreviations: T2D, Type 2 Diabetes; BMI, Body mass index

Table 1. Schematic overview of study visits				
Visit	Screening	Baseline visit	Rest test visit	Exercise-test visit
Participant-related information				
Informed consent	X			
Assessment of in- and exclusion criteria	X			
Anthropometrics		X		
Medical history (including concomitant medications)		X		
Clinical assessment				
Blood pressure		X		
ECG		X		
Bioimpedance			X (morning)	
VO_2peak		X		
Efficacy outcomes				
Fasting blood samples + HbA1c			X	X
Post exercise/rest blood samples			X	X
Post ad libitum meal blood samples			X	X
VAS *(appetite)*			X	X
VAS *(meal assessment)*			X	X
SBFPT			X	X
Acute exercise bout				X
RPE *(Borg scale)*				X
Ad libitum meal			X	X
Questionnaires				
Socioeconomic status (SES)			X (morning)	
Control of Eating Questionnaire (CoEQ)	X			
International Physical Activity Questionnaire (IPAQ)			X (morning)	

Morningness/Eveningness Questionnaire (MEQ)			X (morning)	
Munich Chronotype Questionnaire (MCTQ)			X (morning)	
Pittsburgh Sleep Quality Index (PSQI)			X (morning)	
Safety outcomes				
Adverse events		X	X	X

Abbrevations: ECG (Electrocardiography), DXA (Dual-energy x-ray absorptiometry), RPE (Ratings of perceived exertion), VO2peak (Peak oxygen consumption), SBFPT (Steno Biometric Food Preference Task), VAS (Visual Analog Scale)

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Table 2. Schematic overview of blood sample measures

	Fasted state	Post exercise/rest
HbA1c	X	
CRP	X	
ALAT	X	
ASAT	X	
Natrium	X	
Kalium	X	
Glucose	X	X
Insulin	X	X
C-peptide	X	X
Triglycerides	X	X
LDL cholesterol	X	X
HDL cholesterol	X	X
Appetite-related hormones (Ghrelin, Glucagon, GIP, GLP-1, PYY, PP, CCK, FGF21, GDF15)	X	X
Proteomics	X	
Metabolomics	X	X
Lipidomics	X	X

Abbreviations: Glycated haemoglobin (HbA1c), C-reactive protein (CRP), Alanine aminotransferase (ALAT), Aspartate transaminase (ASAT), Low density lipoprotein (LDL), High density lipoprotein (HDL), Gastric inhibitory polypeptide (GIP), Glucagon-like-peptide (GLP-1), Peptide Tyrosine Tyrosine (PYY), Pancreatic polypeptide (PP), Cholecystokinin (CCK), Fibroblast Growth Factor 21 (FGF21), Growth Differentiation Factor 15 (GDF15)

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Table 3. Nutritional value of ad libitum meal options		
Pie-variant	**Kcal/KJ per 100 grams**	**Nutrition in grams (E%)**
Bacon	270 kcal/1134 KJ	Fat: 17 g (57%) Carbohydrates: 19 g (28%) Protein: 10 g (15%)
Leeks	210 kcal/882 KJ	Fat: 11 g (48%) Carbohydrates: 20 g (39%) Protein: 7 g (13%)
Pesto and tomato	234 kcal/983 KJ	Fat: 12 g (46%) Carbohydrates: 22 g (38%) Protein: 9 g (16%)

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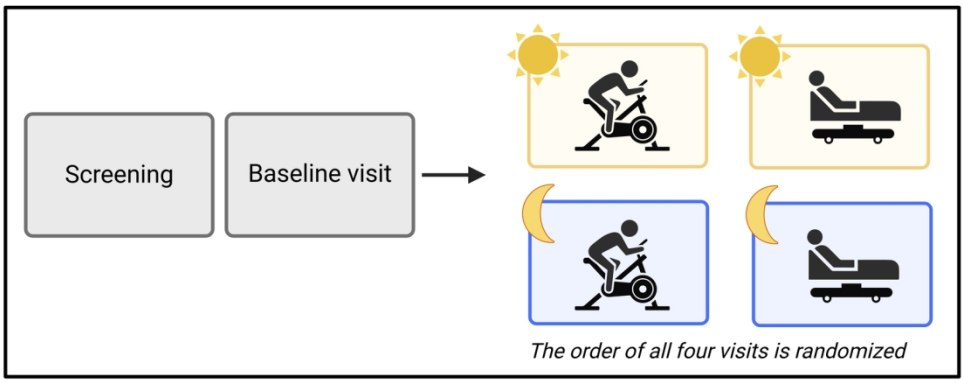
Figure Legends

Figure 1: TIMEX study design. Baseline visits are completed after screening on the same day. Morning visits are initiated at 08:00-08:30 and late afternoon visits are initiated at 15:00-15:30. All visits are completed within 2.5 hours, and with a minimum of a 3-day washout.

Figure 2: Schedule for the test visits. Abbreviations: SBFPT, Steno Biometric Food Preference Task. VAS, visual analogue scales



TIMEX study design. Baseline visits are completed after screening on the same day. Morning visits are initiated at 08:00-08:30 and late afternoon visits are initiated at 15:00-15:30. All visits are completed within 2.5 hours, and with a minimum of a 3-day washout.

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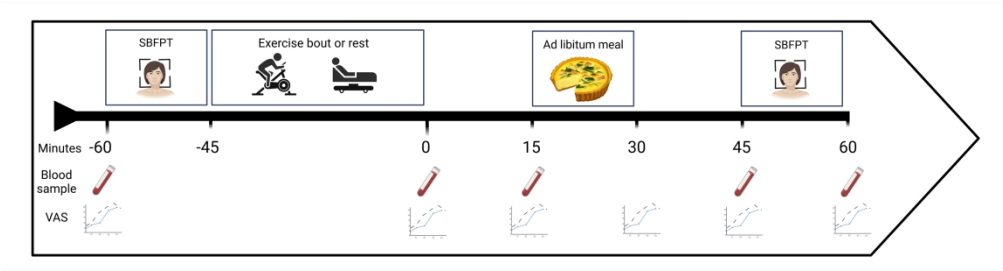

Schedule for the test visits. Abbreviations: SBFPT, Steno Biometric Food Preference Task. VAS, visual analogue scales

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THE SCIENTIFIC ETHICAL COMMITTEE SYSTEM

(S4)

Informed consent to participate in a health science research project.

Title of the research project: What role does morning or afternoon exercise play on food intake among individuals with overweight or with overweight and type 2 diabetes.

Declaration from the participant:

I have received written and oral information, and I know enough about the purpose, methods, benefits, and disadvantages to agree to participate.

I understand that participation is voluntary, and that I can withdraw my consent at any time without losing my current or future rights to treatment.

I consent to participate in the research project and to the collection of my biological material for storage in a research biobank. I have received a copy of this consent form and a copy of the written project information for my own use.

Participant's Name: _____

Date: _____ Signature: _____

If any new significant health information arises about you during the research project, you will be informed. If you do not wish to receive information about new significant health findings, please mark here: _____ (check)

Would you like to be informed about the project's results and any potential implications for you?

Yes _____ (check) No _____ (check)

Standard Consent Form prepared by The Scientific Ethical Committee System, August 2016.

Declaration by the person providing information:

I declare that the participant has received oral and written information about the research project.

In my opinion, sufficient information has been given to make a decision about participation in the trial.

Name of the person providing information: _____

Date: _____ Signature: _____

THE SCIENTIFIC ETHICAL COMMITTEE SYSTEM

Declaration from the participant regarding information about future research projects:

By signing, I agree that staff at Steno Diabetes Center Copenhagen and their collaborators may send me information about research projects that they deem relevant for me to participate in.

I understand that participation is always voluntary, and I can always decline to participate.

My information will not be shared with third parties.

I can withdraw this consent at any time without losing my current or future rights to treatment.

Participant's Name:

_____

Participant's Contact Information (email/phone)

_____

_____

Standard Consent Form prepared by The Scientific Ethical Committee System, August 2016.

Date:_____ Signature:_____

Project Identification: H-22019913

Version 1.0, 08.09.2022

Standard Consent Form prepared by The Scientific Ethical Committee System, August 2016.