Statistical Analysis Plan

Influence of skin temperature gradient on the diurnal variation of metabolic hormones in climates with different ambient air temperatures in the summer

TEMP Study

Authors: Corina Konstantinou, Alexandra Akrotiriatou, Solomon Ioannou, Konstantinos C. Makris

Principal Investigator: Konstantinos C. Makris

Organization: Cyprus International Institute for Environmental and Public Health, Cyprus University of Technology

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1 Introduction

This statistical analysis plan will define and describe the analysis of the TEMP study data. The Cyprus International Institute for Environmental and Public Health of the Cyprus University of Technology is planning a randomised cross over trial of health indicators in relation to spatially varying climatic conditions ranging from the city to the mountainous environment. The purpose of the project is to understand the effect of fluctuations in external climatic conditions (e.g. air temperature) on the human body temperature and metabolic biomarkers (e.g., leptin, adiponectin, ghrelin) or stress hormones (cortisol, melatonin). The hypothesis of the project is that the number, duration and frequency of human exposures to wide gradient (> 8°C) of air temperature changes, as a way of dealing with high temperatures, may be related to metabolic alterations. An intervention potentially reducing the health risk associated with extended exposure to high temperatures in the summer for Cypriots may be the temporary (for a few days) stay in the mountains of Troodos (>800 m altitude). Most of the Troodos communities have consistently lower average ambient air temperatures of ~10°C than those in the cities, so we hypothesize not observing the metabolic hormone alterations anticipated in the urban environment.

2 Study Design

2.1 General aspects

A 2 x 2 cross-over pilot study of healthy adults is proposed to prospectively describe the effect of ambient temperature gradient on diurnal hormonal secretion patterns. A total of 50-60 subjects will be followed for a period of 7 days firstly in an urban environment or in a village of the mountainous rural environment of Troodos and later, for the same period, in the alternate setting, during the summer period in Cyprus. The two settings will allow for the comparison of the circadian patterns of hormones secreted by the adipose tissue among different gradient temperature changes. Continuous quantification of skin temperatures on one day in each setting will allow for characterization of how skin temperature is affected by ambient temperature. Collection of all urine voids on one day in each setting will allow for quantification of the hormone secretion by the adipose tissue. In Table 1 the study characteristics of the non-pharmacological intervention are presented and in Figure 1 the order and duration of various procedures of the study are displayed.

Table 1. Study characteristics

Study Design	Randomised cross-over trial
Study groups	Two study groups: **Group A**: People initially staying in their permanent house in the urban area and then moving to the rural area for at least 7 days (Troodos Mountains – holiday house). **Group B**: People initially staying in the rural mountainous area for at least 7 days (Troodos Mountains – holiday house) and then returning to their permanent house in the urban area.
Blinding	The blinding of the participants to group assignment is not possible with the present study design. The blinding of the researchers to the subjects' identity is achieved by the coding of all study materials (urine containers, temperature sensors, questionnaires and diaries). The study personnel who will obtain the outcome measurements will be not informed of the group assignment. The personnel who will deliver the

	intervention will not take any outcome measurements. All outcome assessors and data analysts will be kept masked to the allocation.
Randomisation	Random allocation will be made in blocks in order to keep the sizes of treatment groups similar. Block randomisation with block size=30 and subjects no=60, will be performed using the function RAND() in Excel, that provides a random generated list, by an investigator with no clinical involvement in the trial

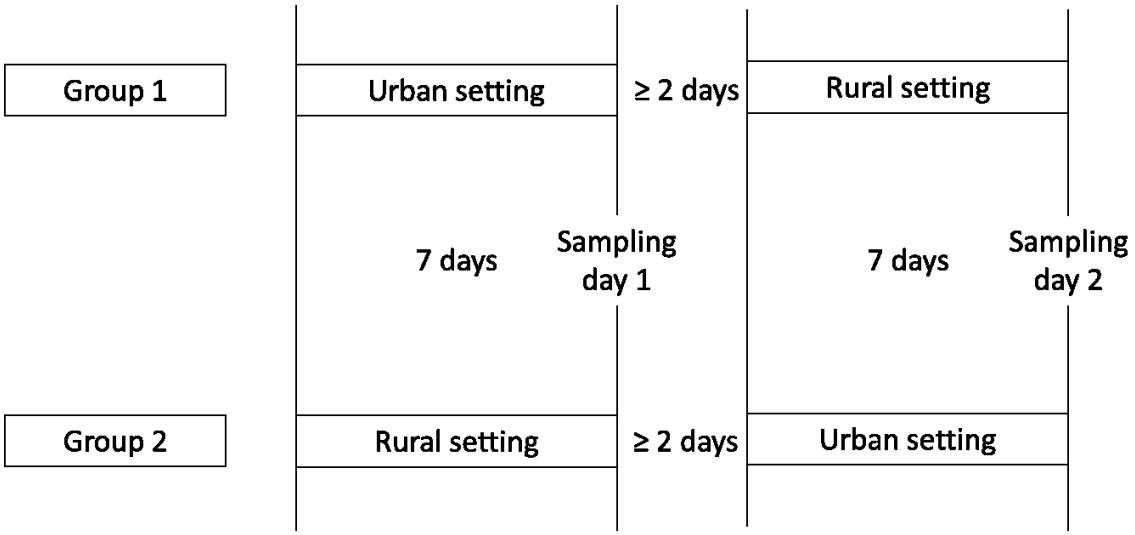

Figure 1. Study design

2.2 Study Objectives

Primary Objectives:

1. To investigate the effect of skin temperature gradient on the diurnal variation of adipokines and stress hormones in the two settings (urban and rural). (Null hypothesis: There is no association of skin temperature gradient with adipokine secretion.)

2. To investigate the circadian patterns of adipokines (leptin, adiponectin, and ghrelin hormones), cortisol, and melatonin in healthy individuals residing in two different climatological environments: urban and higher in latitude rural. (Null hypothesis: There is no difference in the circadian rhythm of the metabolic hormones between participants at the urban or rural environments.)

Secondary Objective:

1. To examine metabolite differences in the metabolomics-based profiles of healthy individuals with non-obese body weight in two study settings with distinctly different climatological characteristics.

2.3 Inclusion and Exclusion Criteria

Inclusion criteria	Exclusion criteria
Healthy adults between 20-60 years old Those that intend staying in Troodos area for the period of at least 7 consecutive days between July - September 2018.	Pregnant women and obese individuals (>30 kg m^{-2}) People with chronic conditions (hypertension, diabetes, metabolic syndrome, cancer). People receiving pharmaceutical treatment for impaired glucose levels or hypertension or antidepressants or thyroxin for thyroid disorders. Those that have traveled to another country within the past week of the study initiation.

Eligibility will be checked through telephone communications with questions that the researcher will make to those volunteers interested for the study. People who do not meet the criteria as stated in the protocol will be excluded from the study if they are younger or older than 20-60 years old, but they won't spend summer holidays there, they are planning to travel to another country during the study period, they are obese or suffer from a particular health problem or are pregnant women.

Based on Questionnaire A, eligibility will be confirmed for those that did not answer. Volunteers that are obese or suffer from a particular health problem or they take a particular medication or they travelled to another country within the past week of the study start date, will be excluded. In the questionnaire, the variables 'BMI', 'Travel' and 'Health' will be filled with >30kgm-2, tick on 'Yes' and specific country and tick on any of 'Diabetes TII / Kidney Disease / Liver Disease / Cardiovascular Disease / Respiratory System Disease / Specified Cancer / Specified Other'.

Diaries and Chronotype Questionnaires with irregular meal intakes and sleep/wake cycles will serve as another criterion to exclude volunteers from the study.

2.4 Recruitment process

Information about the study will be disseminated through flyers and through telephone communication via governmental and other non-profitable organizations (Troodos Development Company, Troodos Tourist Board, etc.) that will contact community leaders of the area and help us collect and create a contact list of "Potential Participants". This contacts' list will include names and phone numbers of citizens residing in one of the towns of Cyprus and who visit their house in Troodos Mountain during summer holidays. Using this list, potential participants will be informed about the study and screened for eligibility criteria via telephone.

2.5 Intervention

Table 2 presents the details of the visits and the sample collection processes.

Table 2. Visits details and sample collection process in chronological order.

Visits/Sampling days	Description
Visit 1	The researcher visits the participant one day before the sampling day 1 (6 days after being in the urban/rural setting) and the following procedures are performed: • Administrates the consent form and receives it signed by the participant • Administrates the Questionnaire A and Chronotype Questionnaire and receives them completed by the participant • Provides coded vials for urine sampling in plastic bag and explains • Provides activity diary and explanations • Places temperature sensors on the participant (after activating them on place) and explains • Provides the instructions for urine collection and temperature sensors

	• Arranges appointment for the sample collection two days later (ideally in the morning)
Sampling day 1	The participant is wearing the sensors continuously from the previous day and collects the urine voids of the day (at least 4) except for the first morning void. The participant stores the urine vials in a plastic bag in the freezer/fridge. The participant notes in the activity diary, the activities of the day (including entrance/exit from buildings, sleep/wake cycle, physical activities and meals) and their corresponding time and the times that each urine void was collected.
Visit 2	The participant collects the first morning void of the day after the sampling day and continues wearing the temperature sensors and noting in the diary. The researcher visits the participant one day after the sampling day on the arranged time and the following procedures are performed: • Collection of the urine samples and sensors • Collection and check of the completed activity diary • Sensors data are transferred immediately on a laptop, to check that data were recorded • Arrangement for the next visit that will take place in the other setting (urban/rural) with at least 2 days of washout period and on the 6th day in the alternate setting.
Visit 3	The researcher visits the participant one day before the sampling day 2 (6 days after being in the urban/rural setting) and the following procedures are performed: • Provides coded vials for urine sampling in plastic bag and explains • Provides activity diary and explains • Places temperature sensors on the participant (after activating them on place) and explains • Provides the instructions for urine collection and temperature sensors

	• Arranges appointment for the sample collection two days later (ideally in the morning)
Sampling day 2	The participant is wearing the sensors continuously from the previous day and collects the urine voids of the day (at least 4) except for the first morning void. The participant stores the urine vials in a plastic bag in the freezer/fridge. The participant notes in the activity diary, the activities of the day (including entrance/exit from buildings, sleep/wake cycle, physical activities and meals) and their corresponding time and the times that each urine void was collected.
Visit 4	The participant collects the first morning void of the day after the sampling day and continues wearing the temperature sensors and noting in the diary. The researcher visits the participant one day after the sampling day on the arranged time and the following procedures are performed: • Collection of the urine samples and sensors • Collection and check of the completed activity diary • Sensors data are transferred immediately on a laptop, to check that data were recorded

2.6 Adherence

In order to achieve adherence the script for telephone communications and visits plan is presented in the SOP report. In this way, researchers will be trained to: a) inform participants for the study and answer any question and b) ask from the participant to follow specific instructions regarding to sampling days.

The assessment for researcher's adherence will be examined through completion of the 'Tools List'. The participant's adherence includes the diary of meal intakes, where participants should note the time for all important activities, such as sleep/wake time, urine sample taken, working hours, etc.

2.7 Withdrawals

Participants can withdraw from the study at any point without having to provide any explanation. Participants who discontinue completing the data collection prior to the end of the trial period will be withdrawn but their already collected data will remain available for analysis, unless they request otherwise. Reasons for withdrawal will be documented wherever possible.

2.8 Outcomes

The primary outcomes are the adipokines (leptin, adiponectin and ghrelin) and stress hormones (melatonin and cortisol) and their diurnal variations and circadian patterns in the two different climatological settings. The secondary outcome is the metabolic hormonal profiles of the participants.

2.9 Sample Size

To estimate the sample size, the sample size program by the MGH Biostatistics Center was used (Schoenfeld, 2010). We used the paired t test study design for calculating the power of this test, assuming that each subject has a pair of values (one for the urban setting and one for the rural mountainous setting). The variable used for the estimation of the sample size was one of the adipokines (serum leptin), since it is one the primary outcomes for the trial and there is no available information for urinary adipokines.

The input parameters were: significance level, within patient standard deviation, power and minimal detectable difference in means. Based on the literature, the within subject standard deviation of plasma leptin was assumed to be 3.53ppb for females and 0.01ppb for males (Saad et al., 1998), and we hypothesized a minimal detectable difference in leptin levels between the urban and rural mountainous settings to be atleast 1.9 ppb, based on other intervention studies (Hibi et al., 2017; Fontes-Villalba et al., 2016). Assuming a power of 80% and a two-sided 5% significance level, the sample size for this two-treatment crossover study was estimated to be 57 subjects.

3 Statistical Analysis

3.1 Timing of Analysis

The final analysis will be performed after all urine samples and temperature data are collected from all participants and ‘Data and Recruitment Form’ is completed.

Also, the final analysis will be performed on data transferred to the file 'Full Data'. This file will be the merge of the files 'Questionnaires', 'Urine Analysis Data' and 'Temperature Data':

- **Questionnaires**: answers from questionnaire A (baseline characteristics), Chronotype questionnaire and diaries.
- **Urine Analysis Data**: data on adipokines (ghrelin, adiponectin and leptin), cortisol, melatonin and other metabolites concentrations.
- **Temperature Data**: data on ambient air and skin temperature.

3.2 Populations

Depending on the analysis, different characteristics for inclusion are defined in the population. For example:

In the Full Analysis, population is defined as:

- All subjects who were randomised.
- All subjects who completed both sampling days providing complete follow-up, independent of the duration of the sampling days.
- All subjects who completed both sampling days providing complete follow-up, independent of the stability of sleep/wake cycles and meal intakes.
- All subjects who completed both sampling days providing complete follow-up, independent of the number of received urine vials.

In the Final Analysis, population is defined as:

- All subjects who completed both sampling days providing complete follow-up, except those who dropped out.
- All subjects who adhere to the major criteria in the protocol (e.g. all subjects who stayed healthy or did not travel to another country within the study or completed both sampling days and completed the whole study duration).
- All subjects who did not substantially deviate with regards to sleep/wake cycles and meal intakes
- All subjects except those that have a health problem (diabetes and hypertension) and did not know by the beginning of the study.
- All subjects except those women that were pregnant and did not know by the beginning of the study.

3.3 Levels of confidence and p values

Statistical tests and confidence intervals will be two-sided. 95% confidence intervals will be presented wherever possible. The statistical significance level set will be at the 5% level.

3.4 Unadjusted and adjusted analyses

The urinary variables will be used with adjustment for creatinine levels to account for urine dilution. If outcomes are not normally-distributed and the analyses require the normality assumption, then outcomes will be log-transformed (natural logarithm).

3.5 Multiple testing

Multiple testing will be accounted for, using the Benjamini-Hochberg (false discovery rate, FDR) method considering all the regression parameters of the primary and post-hoc analyses. Q-value <0.05 indicates statistical significance of an association after controlling the false discovery rate at 5%.

3.6 Missing Data and data below LOD

The underlying assumption for missing data in non-statistical terms include participants voluntarily or due to another factor (pregnancy, diabetes, hypertension) dropping out of the study or due to incomplete data in the diaries – questionnaires. Those kinds of data are considered censored and missing not at random and they will be denoted as 'NA' or as an "empty box" in any files with data.

For the urinary data below the limit of detection (LOD), imputation will be performed based on existing suggestions for handling non-detect urinary measured concentrations data (Helsel, 2005). For outcomes that contain <20% values below the detection limit, values <LOD will be imputed as LOD/2 and for outcomes that contain ≥20% values below detection, values <LOD will be imputed with regression on order statistics (ROS). With the ROS method, non-detect data are replaced based on a probability plot of detects. Outcomes with >70% values below detection, will be used as binary variables in the primary, post-hoc and sensitivity analyses.

3.7 Derived and Study Variables

Table 3 includes all variables that will be collected, the type of variable and the file that will be included, whereas in Table 3 there is a description of the frequency and timing of relevant variable observations and assessments.

Table 3. List of all variables collected through the study

File	Description	Variables	Type
Quest A	Baseline Characteristics	BMI	Continuous
		Age	Continuous
		Education	Categorical (4 Levels)
		Work	Continuous
		Working Experience	Continuous
		Smoking	Categorical (3 Levels)
		Cigarettes/day	Continuous
		Years smoking	Continuous
		Age ex-Smoker	Continuous
		Alcohol	Categorical (4 Levels)
		Alcohol Units	Continuous
		Exercise	Categorical (2 Levels)
		Exercise Hours	Continuous
		A/C Rural	Categorical (4 Levels)
		A/C Urban	Categorical (4 Levels)
		Travel	Categorical (2 Levels)

		Airplane Frequency	Categorical (3 Levels)
		Screen Hours	Continuous
		Health	Categorical
Chronotype Quest	Sleep/Wake Cycles		
Diary	Meal Intakes	Time / Meal Time / Drink Time	Continuous
		Diary / Meal Description / Drink Description	Categorical
Urine Analysis	Outcome	Adipokines (leptin, adiponectin, ghrelin)	Continuous
	Exposures	Cortisol Melatonin Metabolites (metabolomics)	Continuous
Temperature Analysis	Exposures	Skin temperature Ambient air temperature	Categorical/continuous

3.8 Baseline characteristics

The baseline characteristics (recorded through questionnaire A (Table 3)) will be summarized overall and by study group. Continuous variables will be checked for normality with QQ plots and histograms. Categorical variables will be described with frequencies and percentages, normally-distributed continuous variables with means and standard deviations, and non-normal continuous variables with medians and interquartile ranges (25th-75th percentiles).

Baseline data will be tested for difference between both study groups by

- Chi-squared test for categorical variables

- T-test for normally distributed continuous variables
- Wilcoxon test for non-normally distributed continuous variables

3.9 Primary analysis of outcomes

3.9.1 Cosinor method

An exploratory analysis will be formed by plotting the data as a function of time for each sampling day. This will allow for visual observation of possible trends and for comparisons between sampling days. Residuals will also be plotted as a function of time that will provide valuable information on how to proceed for further analysis. A histogram will also be used to assess normality of the results.

Since prior information suggests a presence of rhythm with period of 24-hours, data will be stacked in classes in an attempt to reduce noise and reveal the rhythm's waveform. Class means will be used to test for a statistically significant time effect.

Once a rhythm has been validated, the single cosinor method will be used to model the rhythm by fitting a cosine curve, a procedure similar to the least square methods (Cornelissen, 2014). For each subject, a single-component cosinor will be created for each sampling day, and for each group, a population-mean cosinor will be created for each sampling day. These methods also involve certain assumptions that need to be satisfied hence; regression diagnostics will be implemented at this stage to assess the model's adequacy. Further statistical testing will be implemented to test the equality of parameters such as the MESORs, amplitudes and acrophases (Cornelissen, 2014) of the two groups in the two settings.

3.9.2 Exposure: Temperature gradient

We define ambient temperature gradient as any change in ambient air temperature of ± 2◦C. We define skin temperature gradient as a minimum change of ±0.1◦C in skin temperatures. We will examine how exposures in temperature gradient affect the outcome in 3 different ways:
i) Frequency: The number of temperature gradient changes within a day.
ii) Magnitude: The increase/decrease in temperature exposure metric from its previous in time background value of the gradients.
iii) Duration: The time each temperature gradient lasts.

3.9.3 Primary Analysis

Linear mixed effect models of the adipokines, cortisol and melatonin levels will be used as a function of the treatment (rural vs urban), accounting for both between and within subject variability by having random intercepts for the participants with unstructured covariance matrix. Non-linear mixed effects models of the adipokines, cortisol and melatonin levels, accounting for their diurnal variation as a function of the treatment, will be also constructed.

3.10 Secondary Analysis of outcomes

An untargeted metabolomics analysis will be conducted to investigate the differential expression of metabolic profiles in the volunteers residing in two climatologically-varying settings.

4 References

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