Study Protocol and Statistical Analysis Plan (SAP)

HYDRATION TO OPTIMIZE METABOLISM PILOT STUDY

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STUDY PROTOCOL

H2O-METABOLISM PILOT STUDY

Study Protocol, drafted by the Sponsor, LUND UNIVERSITY, through the Principal Investigator, Prof. Olle MELANDER, hereinafter referred to as "Sponsor" or "we".

PURPOSE AND AIMS

The number of patients with type 2 diabetes mellitus increases and represents a major public health threat as a potent risk factor for cardiovascular morbidity and mortality (CVD). Both diabetes and the pre-diabetic state also show clustering of other cardiovascular risk factors (e.g. hypertension and abdominal obesity) called the metabolic syndrome. Interestingly, many trials aimed at reducing risk of CVD in patients with established type 2 diabetes by treating hyperglycemia have failed to do so (*N Engl J Med* 2008,358:2545-59; *N Engl J Med* 2013,369:1317-26). One likely reason for this is that the diabetes-related macrovascular disease is manifest already at diagnosis of diabetes and difficult to reverse after years of exposure to hyperglycemia and other diabetes-related CVD risk factors. *Thus, the most efficient way of reducing both the galloping epidemic of diabetes and its CVD complications is to prevent the disease.* As the diagnosis of diabetes is based on elevation of the fasting plasma glucose concentration, *any preventive actions, which result in lowering of plasma glucose levels in the population, will reduce the incidence of type 2 diabetes.* Although dietary changes and physical exercise are the cornerstones in the prevention of obesity and diabetes, these preventive interventions have not been able to satisfactorily reduce obesity and diabetes rates and new preventive easy-to-implement life style interventions need to be discovered. As described below, high plasma concentration of vasopressin (VP) (i.e. antidiuretic hormone) is a novel and independent risk factor for type 2 diabetes, the metabolic syndrome, CVD and premature death (*Heart* 2016,102:127-32; *Am Heart J* 2015,169:549-556; *Int J Obes* 2013,37:598-603; *J Clin Endocrinol Metab* 2011,96:E1065-72; *Circulation* 2010,121:2102-8).

As VP can be suppressed by increasing water intake, we hypothesize that water supplementation in individuals with high VP can lower plasma glucose and prevent diabetes.

The aim of this study is to perform a pilot study to test if six weeks of moderate water supplementation of 1.5L extra water per day in low-drinkers with high plasma levels of VP can significantly alter hydration markers in general and reduce plasma VP (measured as the stable VP marker copeptin) in particular. The study was also designed to investigate study logistics, safety, compliance and drop-out rate before the start of a long term (1 year) RCT. Furthermore, as fasting plasma glucose will be the primary endpoint of the long term RCT, we also aim at investigating whether the 6-week water intervention can significantly reduce fasting plasma glucose concentration.

SURVEY OF THE FIELD

The main physiological role of VP is to maintain constant plasma osmolality. When water intake is low, increased pituitary VP secretion prevents hyper-osmolality by enhancing renal water reabsorption through VP stimulation of the V2-receptor in the renal collecting ducts, whereas at higher levels of water intake, VP secretion is supressed and circulating VP is low. Another effect of VP is that it increases release of adrenocorticotrophic hormone (ACTH) though binding to the VP-1B receptor in the pituitary gland and thereby leads to elevated adrenal cortisol secretion and a Cushing´s syndrome-like phenotype, i.e. diabetes, insulin

resistance, hypertension, abdominal obesity and high risk of CVD morbidity and mortality (*J Physiol* 2007;584:235-44; *JCI* 2004;113:302-9; *Clin Endocrinol* 2004;60:192-200). We showed in 2010 that fasting plasma concentration of VP (measured by copeptin, a stable fragment of the VP precursor hormone) strongly predicts new onset type 2 diabetes independently of all other diabetes risk factors (*Circulation* 2010,121:2102-8), a finding that has later been replicated by other groups in large independent cohorts (*Diabetologia* 2012,55:1963-70; *JCEM* 2015,100:3332-9). Healthy subjects in the top quartile of copeptin (corresponding to plasma concentration of copeptin of >6.1 pmol/L in women and >10.7 pmol/L in men) had a 3-4 fold multivariate adjusted risk of developing diabetes as compared to subjects in the lowest quartile of copeptin (*Circulation* 2010,121:2102-8). Just like Cushing´s syndrome patients, subjects with high VP concentration are not only susceptible to diabetes. We showed that they are more likely to suffer from all components of the metabolic syndrome; i.e. abdominal obesity, insulin resistance, hypertension (*Int J Obes* 2013,37:598-603; *J Clin Endocrinol Metab* 2011,96:E1065-72; *Circulation* 2010,121:2102-8) and that high VP is an independent risk factor for CVD and premature mortality (*Heart* 2016,102:127-32; *Am Heart J* 2015,169:549-556). As a low water intake is the most likely cause of elevated VP levels, we tested in animals if high water intake leads to suppression of VP and amelioration of dysmetabolic phenotypes. Interestingly, whereas rats with high VP had deteriorated glucose tolerance, high water intake suppressed VP, ameliorated insulin resistance and markedly prevented hepatic steatosis, one of the hallmarks of the metabolic syndrome (*Diabetologia* 2015,58:1081-90). These data support a causal relationship between high VP, diabetes and metabolic syndrome, and suggest that water supplementation in humans with high VP may reduce the risk of diabetes and cardiometabolic disease.

PROJECT DESIGN

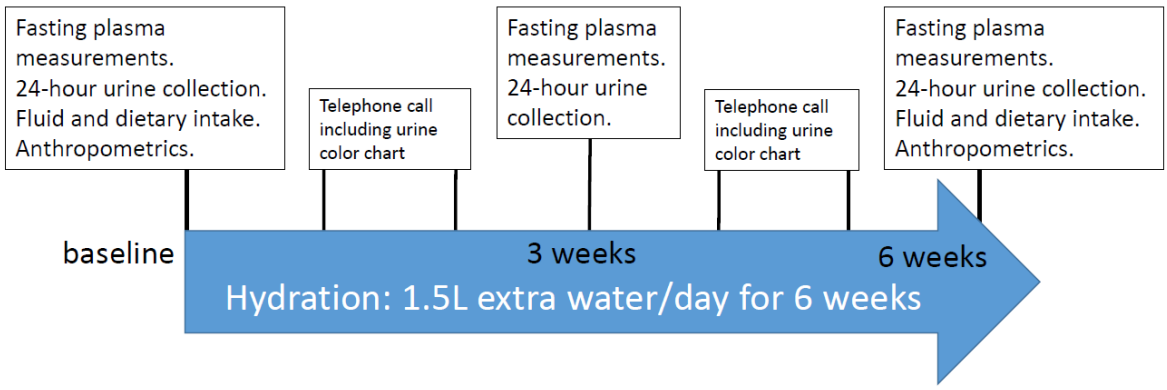
Population: We previously showed that subjects with high VP (i.e. copeptin of >6.1 pmol/L in women and >10.7 pmol/L in men), which corresponds to 25% of the population aged 20-75y, has 3-4 times elevated risk for diabetes development, i.e. a magnitude of risk that is similar to that of obesity, and a doubled risk of CVD mortality. In addition, the high VP target group of this study has a urine osmolality of >600 mosm/kg water, i.e. concentrated urine concordant with a low water intake.

Screening and inclusion process: Study subjects (n=30) will be recruited from the Malmö Offspring Study (n=1547) (http://www.med.lu.se/mos). Copeptin will be measured in -80 degree frozen plasma samples from this study. Assuming that the prevalence of having high plasma copeptin concentration of >6.1 pmol/L in women and >10.7 pmol/L in men will be approximately 25%, around 400 individuals will have high copeptin concentrations and will be eligible for the current study. These individuals will be contacted by letter and subsequently by telephone. Individuals interested in participation, who do not have any medication or disease implying immediate exclusion (see inclusion and exclusion criteria below), will be invited to participate in the current study.

Inclusion criteria will be provision of informed consent, age 20-75 years with high plasma concentration of VP (plasma concentration of copeptin of > 6.1 pmol/L in women and >10.7 pmol/L in men) and 24-hour urine osmolality ≥ 600 mosm/kg water.
Exclusion criteria will be 24-hour urine volume > 1.5 L, pregnancy or breastfeeding, plasma sodium <135 mM, use of diuretics, lithium or SSRI drugs, chronic kidney disease (eGFR<30mL/min), heart failure, inflammatory bowel disease, type 1 diabetes or type 2

diabetes treated with insulin and vulnerable subjects (subjects with legal guardian, with loss of personal liberty).

Figure 1. Study protocol



Intervention: All participants will be instructed to add 1.5 L of plain water daily on the top of habitual intake (intervention) during six weeks (Figure 1). At baseline, all participants also will receive general oral and written advice on diet and physical activity. Adherence to high water intake will be achieved by coaching at the clinic visit and by regular telephone contacts with the participants. We benefit from shared experience of how to successfully perform the water intervention by collaboration with Prof William Clark, London Health Science Centre, Canada (co-investigator in current project) who is PI of the Chronic Kidney Disease Water Intervention Trial (WIT) (same water vs control intervention as in current study but primary outcome = effect of water on decline of GFR) whose Data Safety Monitoring Report was completed June 2015 and showed excellent separation in water intake during follow-up (measured by 24-hour urine volume) and safety. In accordance with the protocol of the WIT, adherence to high water intake will be achieved by coaching at the clinic visits and by regular telephone contacts. Furthermore, we will provide urine color charts to use at home to facilitate adherence to target urine concentration.

Outcome: We will test if six weeks of water supplementation of 1.5L extra water per day in low-drinkers with high copeptin significantly alter hydration markers in general and reduce plasma copeptin in particular. Furthermore, as fasting plasma glucose will be the primary endpoint of the long term RCT, we will also investigate whether the 6-week water intervention could significantly reduce fasting plasma glucose concentration. Significance of differences between baseline and after intervention will be tested using paired t-test or Wilcoxons paired rank test depending on distribution. SPSS statistical software version 24 (SPSS Inc., Chicago, Ill., USA) will be used for all analyses. A 2-sided p value of <0.05 will be considered statistically significant.

Table 1. Schedule of visits and measures

				Follow-up [b]

	Visit 1 [a,c]	Visit 2 (Inclusion) [a,c]	Baseline (Week 0)	3 weeks	6 weeks
Informed Consent Process and Informed Consent Form Signature	+				
Exclusion criteria check-up including pregnancy	+				
SURVEY					
Demographics			+		
Diet (4-day report)			+		+
Health history			+		+
Health-related quality of life			+		+
Hydration coaching [c]			+	+	+
CLINICAL					
Height (cm)			+		+
Weight (Kg)			+		+
Waist circumference (cm)			+		+
Blood pressure (mm Hg)			+		+
Medications			+		+
BLOOD					
Blood sample	+		+	+	+
Fasting glucose (mmol/L)			+		+
Oral glucose tolerance test (OGTT) (mmol/L)			+		+
Fasting and 2h insulin (during OGTT) (mIE/L)			+		+
Fasting and 2h glucagon during OGTT) (pmol/L)			+		+
Fasting cortisol (nmol/L)			+		+
Fasting ACTH (pmol/L)			+		+
Fasting copeptin (pmol/L)			+		+
Fasting Lipids/lipoproteins [d] (mmol/L)/(g/L)			+		+
C-reactive protein (mg/L)			+		+
Creatinine (µmol/L)	+		+	+	+
eGFR (mL/min/1,73 m^2)	+		+		+
Sodium (mmol/L)	+		+	+	+
Potassium (mmol/L)			+	+	+
Urea (mmol/L)			+	+	+
Osmolality (mOsm/kg)			+	+	+
Erytrocyte Volume Fraction (%)			+		+
HbA1c (mmol/mol)			+		+
Plasma samples for long term storage			+		+
Whole blood for DNA isolation			+		
URINE					
24-hour urine sample		+		+	+
Urine volume (ml)		+		+	+
Creatinine (mmol/24h)		+		+	+

Sodium (mmol/24h)		+		+	+
Potassium (mmol/24h)		+		+	+
Urea (mmol/24h)		+		+	+
Osmolality (mOsm/kg)		+		+	+
Albumin/creatinine ratio (g/mol)		+			+
Cortisol (nmol/24h)		+			+
Urine for long term storage		+			+
FAECES					
Sample for microbiome seq			+		+
Bristol stool score			+		+
Check for adverse events				+	+

[a]Pre-intervention.
[b]Time after baseline.
[c]In addition to coaching during telephone contacts.
[d]triglycerides, LDL, HDL, ApoA1, ApoB.

Protocol: All visits will take place at Skåne University Hospital. Recruitment and intervention will be ongoing from February to August 2017 (rolling enrollment, 6 weeks follow up). Coaching and enquire about adherence and tolerance to water treatment will take place both during clinical visits after baseline, and during telephone contacts (Table 1, Figure 1) to optimize compliance. Color charts will be used at all telephone contacts, and measures of urine volume and urine osmolality will be used at clinical visits, to motivate the participants to increase their water supplementation/compliance if lack of compliance is indicated, suspected or documented based on these parameters.

Clinic visits will be performed at baseline, after 3 weeks of intervention and after 6 weeks (i.e. at the end of the intervention). For the sequence of events during visits please see Table 1. Between visits, telephone contacts will be scheduled for coaching (Figure 1). At baseline, and at the end of the water intervention, plasma copeptin, electrolytes, C-reactive protein and fasting glucose will be measured, anthropometry will be assessed, and 24h urine will be collected. Furthermore, current medication and medical history will be recorded, and fluid/water and dietary intake will be assessed using "*Riksmaten 2010*", a validated web-based 4-day record tool developed by the Swedish National Food Administration and used in the latest national diet survey in Swedish adults (*J Nutr Sci* 2016,5:e39). In addition to Riksmaten recording, participants will fill in a food propensity questionnaire to assess foods that may not be consumed within the 4 days (like fish, berries or sugar-sweetened beverages). To help with the registration, participants will receive a food/drink dairy and a portion guide with pictures of 24 different food categories. In addition, there is an instruction video available via YouTube https://www.youtube.com/watch?v=DB3bzD0FJMg. Blood pressure will be measured after a 5-minute rest. At the 3-week visit, plasma electrolytes will be measured, and 24h urine will be collected for measurement of urine volume and urine osmolality. These measures will, together with urine color charts, be used to motivate the participants to keep up or, in case of indications of lack of compliance, increase their water intake.

Laboratory measurements: All plasma laboratory analyses (Table 1), except copeptin, will be performed using certified methods at the Skane University Hospital's central clinical lab. Plasma copeptin will be measured in our lab in fasting plasma samples stored at -80°C using a

commercially available chemiluminescence sandwich immunoassay copeptin ProAVP kit with coated tubes (Thermo Scientific BRAHMS Copeptin proAVP KRYPTOR).
All urine laboratory analyses except for 24h urine osmolality will be performed using certified methods at the Skane University Hospital's central clinical lab, whereas 24h urine osmolality will be measured at the clinical site using an i-Osmometer basic (Löser, Germany).
The gut microbiota composition has been suggested to mediate systemic metabolic changes. As stool consistency, in turn highly affected by water intake, has been shown to be one of the main factors determining the microbiota composition (*Science* 2016;352:560-4), we will measure the gut microbiota in order to test if such changes may mediate part of any beneficial effects of water supplementation on metabolism. Furthermore, as recent work shows that genetic factors play a role in determining basal level of VP (measured by copeptin) (J Clin Endocrinol Metab 2016;101:2432-9), it can be hypothesized that the magnitude of the copeptin lowering effect by water – and the potential effects on metabolism from lower copeptin concentration – varies according to such genetic factors. Therefore, we will also isolate DNA from buffy coats in order to determine such genetic variation.

Handling of blood samples: The amount of blood drawn is 3 ml at visit 1, ~80 ml at the baseline visit, ~20 ml at the 3 week visit, and finally ~80 ml at the 6 week visit. This includes blood for direct analyses of routine chemistry (Table 1) as well as biobank samples. Copeptin will not be measured continuously during the trial but in one batch after all study subjects have completed the 6 week protocol.

The buffy-coat of one of the centrifuged plasma tubes will be collected and stored frozen for later DNA extraction. We are planning to genotype genes coding for AVP and its receptors, loci associated with urine and plasma osmolality and genes associated with altered copeptin concentration.

The routine (directly analyzed) blood/urine samples are transported in air temperature from the clinical site to the University Hospital's central clinical lab by site staff. The lab is located in the area of Skane University Hospital Malmö, only a 3 minute-walk from the clinical site, which is located at Inga Marie Nilssons gata 50, Skåne University Hospital, Malmö.

Blood samples for long-term storage (biobank) are centrifuged immediately after blood draw. Then they are stored in the fridge for approximately 1-3 hours before being aliquoted and transferred (transported on ice) to the -80 degree freezer in another close-by building, approximately a 5 minute-walk from the clinical site. These samples are also transported by site staff. Assays on the biobank samples will be performed after all study subjects have completed the protocol in the same batch of reagents (includes analysis of copeptin, see above).

Procedure for OGTT: After an over-night fast (no meals or drinks after 10PM the evening before), subjects will ingest 75 g of glucose over a maximum period of 3 minutes, starting sometime between 730 and 9 AM, followed by blood sampling for glucose measurement at 30, 60 and 120 min.

Handling of urine samples: 24-hour urine samples will be collected at Visit 2 before baseline and again at the 3-weeks and 6-weeks visit (Table 1). 24-hour urine collections follow procedures developed at the Department of Endocrinology, Skåne University Hospital, and consists of a comprehensible written instruction aimed at ensuring accurate and complete collection of urine. The procedure has been used by us in many large research studies (see for

example applicant references 5 and 10). The participants start the 24-hour urine collection one day before a planned visit, and bring their container on the day of visit. For example, if their visit is on a Wednesday they start collecting urine on a Tuesday. The first urine on Tuesday morning is not collected. The subject notes the time of collection start on a form for urine collection. From this point and 24 hours forward they collect ALL urine. They are instructed to try and collect the first morning urine on Wednesday at approximately the same time as they started the collection on Tuesday morning. Prior to the urine collection they are provided with one big container and 3-4 small ones. They are asked to keep the big container in the fridge at all time, if possible. Subjects are instructed to bring the small containers with them when they leave home for work and such. These containers should also be kept in fridge if possible, but if this is not possible the participants are allowed to keep them in room temperature during the day and then pour the content into the large container when they get home.

Procedure for stool collection: Participants will be instructed via a video how to collect their faeces sample at home. The participants will be asked to store the samples in a freezer until delivery to the clinic. At the clinic, the 4 aliquots will be stored in a -80 degree freezer.

Data handling: The case report form (eCRF) of the present study consists of a database (Redcap).The questionnaire based data and 4-day web-based dietary and fluid intake data will be electronically stored in the Redcap database, using code linkage. The laboratory results from the hospital clinical chemistry are automatically imported into the Redcap when analyses are available. Anthropometric data, blood pressure and urine osmolality (which is analyzed on site and not sent to the lab) are entered manually into the eCRF.

Archiving of data: Data is archived for at least 10 years.

Ethical considerations: this study is conducted in compliance with the principles of the 'World Medical Association Declaration of Helsinki' (59th WMA General Assembly, Fortaleza, Brazil, October 2013), ICH guidelines for Good Clinical Practice as appropriate for nutritional products, and local legislation of the country in which the research is conducted, whichever affords the greater protection to the participants.

This protocol and supportive documentation provided to the subjects, such as information and informed consent sheets and Forms, were submitted to the applicable Ethics Committee in Lund by Olle Melander according to local regulations. Approval from the Ethics Committee was obtained on 15 Dec 2016.

The signature of the study informed consent form marks the inclusion of the subject in the study. This signature will be obtained after having fully briefed the subject about the study and answered all of his/her questions about the study.

STATISTICAL ANALYSIS PLAN

H2O-METABOLISM PILOT STUDY

1. Aims and objectives

The aim of this study is to perform a pilot study in 30 subjects, before the start of a long term RCT. Thus, we have not performed a power calculation. In the study we will test for the first time if six weeks of moderate water supplementation (1.5L extra water per day) in low-drinkers with high plasma levels of copeptin can significantly reduce plasma copeptin concentration. The primary outcome measure of the study is the change of fasting plasma copeptin between baseline and 6 weeks of water intervention. Furthermore, the study is designed to investigate study logistics, safety, compliance and drop-out rate before the start of a long term (1 year) RCT. Finally, as fasting plasma glucose will be the primary endpoint of the long term RCT, we also aim at investigating whether the 6-week water intervention can significantly reduce fasting plasma glucose concentration.

2. Sample size

A pilot study with 30 participants.

3. Outcomes

This section will present the outcomes investigated to answer the study aims and objectives. The analyses are described in section 5 Analyses.

3.1 Primary outcome

Fasting plasma copeptin (measured at baseline and at 6 weeks).

3.2 Secondary outcomes

24 hour urine osmolality (measured at baseline, at 3 weeks and at 6 weeks).
24 hour urine volume (measured at baseline, at 3 weeks and at 6 weeks).
Drinking water (measured at baseline and at 6 weeks).
Total water (measured at baseline and at 6 weeks).
Fasting plasma glucose concentration (measured at baseline, at 3 weeks and at 6 weeks).

Surveys
Health-related quality of life, fluid/water and dietary intake (using Riksmaten 2010), stool form (Bristol Stool Scale).

3.3 Safety outcomes

Adverse events
Adverse events are reported at each clinic visit.

Concomitant medications
Usage of medications during study period will be recorded.

4. Populations and subgroups to be analysed

4.1 Populations

Per Protocol (PP)

All included study subjects completing the whole study period (complete cases) will be seen as the primary population for the analysis. For a specific analysis, study subjects with missing data on any of the variables in the model will be excluded from the analysis.

5. Analyses

All outcomes will be presented using descriptive statistics; normally distributed data by the mean and standard deviation (SD) and skewed distributions by the median and interquartile range (IQR). Binary and categorical variables will be presented using counts and percentages. SPSS statistical software version 24 (SPSS Inc., Chicago, Ill., USA) will be used for all statistical analysis.

5.1 Primary outcome

The primary analysis will compare fasting plasma copeptin concentration between baseline and 6 weeks using a paired T-test or Wilcoxons paired rank test depending on distribution.

5.2 Secondary outcomes

The secondary analyses will compare 24 hour urine osmolality, 24 hour urine volume, drinking water, total water and fasting plasma glucose concentration between baseline and 6 weeks using a paired T-test or Wilcoxons paired rank test depending on distribution.