Vasopressin, cardiorenal disease and hydration. The vasopressin system in relation to the risk of cardiorenal disease and how vasopressin levels are affected by salt and water interventions in humans.

Tasevska, Irina

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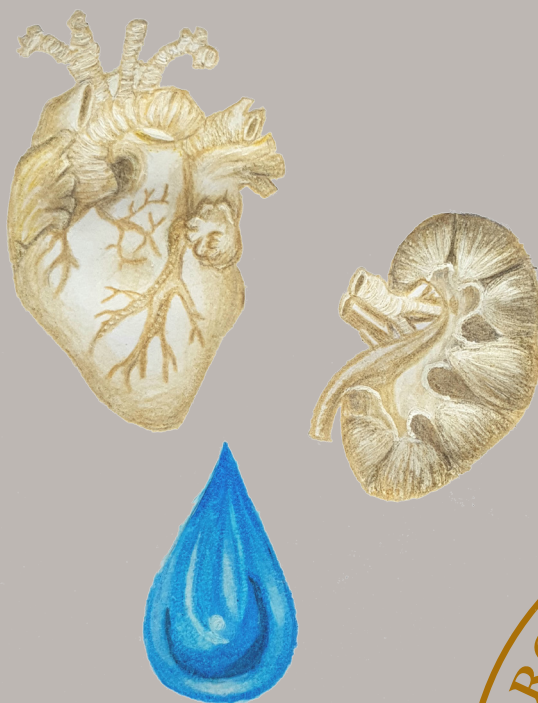
Vasopressin, cardiorenal disease and hydration

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Vasopressin, cardiorenal disease and hydration

Abstract

Aim: The aims of the first three studies were to investigate copeptin, a surrogate marker of vasopressin, in relation to salt sensitivity, coronary artery disease (CAD) as well as cardiovascular (CV) mortality and chronic kidney disease (CKD). In the fourth sudy, the aim was to investigate levels of copeptin and glucometabolic parameters in relation to increased water intake in humans.

Methods: In the first study, 39 healthy swedish individuals received meals containing 50 mmol NaCl and additionally capsules containing either 100 mmol NaCl or corresponding placebo capsules, in random order, during 4 weeks. Plasma copeptin was then compared do low respectively high dietary salt intake as well as the change in systolic blood pressure (salt sensitivity). In the second study, individuals recruited in The Malmö Preventive Project (MPP) (n=5386) were followed during a mean of 6.5 years and analyzed for incident CAD and CV mortality and related to levels of plasma copeptin. In the third study individuals recruited in The Malmö Diet and Cancer Cardiovascular Study (MDC-CS) (n=3186) were followed during 16.6 ± 1.5 years. Levels of plasma copeptin were then related to incident CKD, calculated by the MDRD formula, and yearly decline in eGFR. In the fourth study, 39 healthy individuals underwent, in random order, one week of high water intake (3L/d on top of habitual intake) and one week of normal (habitual) fluid intake (control) as well as an acute water load test. Levels of copeptin and glucometabolic parameters were then analyzed and compared during low respectively high water intake.

Results: Copeptin increased after a high compared to low dietary salt consumption in all subjects, $3,59 \pm 2,28$ versus $3,12 \pm 1,95$ ($P = 0,02$) but salt sensitivity i.e. blood pressure increase due to salt intake, was inversely correlated with salt-induced changes of copeptin, a finding only observed in females (r = -0.58; P=0.017). Copeptin was also inversely correlated with urinary volume, at both low ($r = -0,42$; $P = 0,001$) and high ($r = -0,60$; $P < 0,001$) salt consumption, as well as with the change in body weight ($r = −0,53$; $P < 0,001$). Among subjects free from CAD at baseline, the multivariate adjusted HR (95% confidence interval (CI)) per 1 SD increment of log-transformed copeptin for risk of CAD development was 1.20 (1.08 to 1.33) (p=0.001). Each SD increment of copeptin independently predicted CV mortality (1.36 (1.21 to 1.53); p<0.001). The results were significant both in diabetics (p=0.004)) and nondiabetics (p=0.02). Copeptin was independently associated with significantly greater annual decline of eGFR (ml/min/1.73 m^2) and each SD increment of copeptin independently predicted incident CKD with the MDRD formula calcutated as eGFR <60 (OR 1.19, 95% CI 1.04–1.36; p = 0.010). After acute intake of 1L of water, plasma copeptin was significantly reduced within 30 min with an average reduction of 39 % (95% CI 34-45) (p<0.001). One week of increased water intake led to a 15 % reduction (95CI 5-25) (p=0.003) in fasting copeptin and a subgroup responing well do hydration (low-drinkers) showed a significant water-induced reduction in fasting glucagon concentration. No significant water-induced difference was observed in glucose or insulin.

Conclusions: As suppression of copeptin on high versus low salt intake was associated with systolic salt sensitivity in women, our data suggest that high fluid intake and fluid retention may contribute to salt sensitivity. Copeptin predicts development of CAD and cardiovascular mortality both in diabetics and in nondiabetics and predicts decline in eGFR as well as greater risk of new-onset CKD. Finally, both acute and chronic water intake potently reduced plasma copeptin concentration in habitually low-water drinkers motivating long-term trials to assess the effects of water and on glucometabolic traits primarily in this sub-population.

Key words Copeptin, salt sensitivity, CAD, CV mortality, diabetes mellitus, CKD, hydration

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"Den skönaste och djupaste känsla vi kan erfara, är förnimmelsen av det hemlighetsfulla"

- Albert Einstein, 1879-1955

"The secret of getting ahead, is getting started"

- Mark Twain, 1835-1910

To my mother

♥

Content

List of publications

I. High salt intake increases copeptin but salt sensitivity is associated with fluid induced reduction of copeptin in women
Irina Tasevska, Sofia Enhörning, Philippe Burri and Olle Melander. International Journal of Hypertension. 2014.

II. Copeptin predicts coronary artery disease cardiovascular and total mortality
Irina Tasevska, Sofia Enhörning, Margaretha Persson, Peter M Nilsson,
Olle Melander. Heart. 2016.

III. Increased Levels of Copeptin, a Surrogate Marker of Arginine Vasopressin, Are Associated with an Increased Risk of Chronic Kidney Disease in a General Population
Irina Tasevska, Sofia Enhörning, Anders Christensson, Margaretha Persson, Peter M. Nilsson, Olle Melander. American Journal of Nephrology. 2016.

IV. Effects of an Acute Water Load and One-week Increased Hydration on Plasma Copeptin, Glycemia and Gluco-regulatory Hormones – a Water Intervention in Healthy Humans
Sofia Enhörning, Irina Tasevska, Ronan Roussel, Nadine Bouby, Margaretha Persson, Philippe Burri, Lise Bankir, Olle Melander

Abbreviations

ABP:	Ambulatory blood pressure measurement
AVP:	Arginine vasopressin
BMI:	Body mass index
CAD:	coronary artery disease
CKD:	chronic kidney disease
CONT-Wk:	Control week
CVD:	cardiovascular disease
DBP:	Diastolic blood pressure
eGFR:	Estimated glomerular filtration rate
HDL:	High density lipoprotein
HR:	Hazard ratio
HWI-Wk:	High water intake week
ICD:	International classification of diseases
LDL:	Low density lipoprotein
MDCS:	Malmö diet and cancer study
MDCS-CC:	Malmö diet and cancer study - Cardiovascular Cohort
MPP:	Malmö preventive project
OGTT:	Oral glucose tolerance test
OR:	Odds ratio
RCT:	Randomized controlled trial
SBP:	Systolic blood pressure
SD:	Standard deviation
V_{1a}:	Vasopressin receptor 1a
V_{1b}:	Vasopressin receptor 1b
V_2:	Vasopressin receptor 2
WHO:	World health organization

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Summary in Swedish

Vasopressin, hjärt- och njursjukdom samt hydrering

Salt- och vattenintervention hos människan

Från en strukturdel i vår hjärna, den så kallade hypofysen, frisätts ett hormon som heter vasopressin. Detta hormon frisätts framförallt när kroppen har för lite vätska i sig eller när koncentrationen av salter i blodet är hög. Anledningen till dess frisättning är att upprätthålla en normal koncentration (inte för utspädd och inte för underspädd) av salter och vätska i kroppen. Har ni tänkt på att ni urinerar mindre när ni dricker mindre? Det är tack vare detta hormon. Hormonet minskar då kroppens urinproduktion (genom receptorer i njurarna) för att behålla den vätska man har i kroppen och genom samma mekanism späder den ut koncentrationen av salter i blodet när salthalten är förhöjd. Utöver receptorer i njurarna påverkar vasopressin även receptorer i övriga delar i kroppen som hanterar vår omsättning av blodsocker, vilket gör hormonet intressant hos diabetiker. Vasopressin är svårt att mäta tillförlitligt i blodet genom ett vanligt blodprov eftersom att det bryts ner snabbt. Innan hormonet frisätts ut i blodet klyvs det på hälften och lämnar en lika stor mängd bredvid sig, ungefär som ett Kit Kat-choklad efter att det knäckts. Den ena delen (aktivt vasopressin) bryts ner snabbt, medan den andra delen är stabil och kvarstår längre i cirkulationen. Den stabila delen i detta fall kallas copeptin och är tekniskt mycket enklare att mäta genom ett blodprov eftersom den stannar i blodet en längre tid. Med andra ord, vi mäter copeptin som ett indirekt mått på vasopressin eftersom de två härstammar från samma modermolekyl och bildas i lika stort antal.

Vasopressin har således livsviktiga funktioner i vår kropp, men en överaktivering av systemet har i studier visat sig vara kopplat till risk för bland annat hjärt- och kärlsjukdomar samt diabetes. Vi har även i tidigare arbeten visat att förhöjda nivåer av copeptin är relaterat till en ökad risk för att utveckla diabetes. Då alltför många patienter upptäcks försent, när till exempel hjärtinfarken väl inträffat, är det av stor vikt och intresse att undersöka vilka förebyggande åtgärder som i tid kan vidtas. Det vill säga, undvika en potentiell sjukdom från alla första början. Om man tittar på diabetikerna idag, som man vet har en ökad risk för vissa sjukdomar, så följs dessa individer årligen för att i god tid kunna upptäcka till exempel en försämring av njurfunktionen. Men hur ser det ut i den övriga, till synes friska,

populationen som kanske också löper ökad risk för sjukdom? Kan man på något sätt fånga de individer som har den förhöjda risken och erbjuda dem en liknande screening och förebyggande behandling?

I min avhandling presenterar jag fyra arbeten där vi i det första undersöker om saltkänslighet, d.v.s. hur mycket vårt blodtryck stiger när vi äter mycket salt, är relaterat till våra nivåer av copeptin i blodet. Studien utfördes på 39 svenska individer (20 män och 19 kvinnor) med en medelålder på 53±11 år. Deltagarna fick under åtta veckor dagens alla måltider, innehållande 50 mmol koksalt, från sjukhuset. Som tillägg fick deltagarna kapslar innehållande antingen 100 mmol koksalt alternativt motsvarande placebokapslar under en period på fyra veckor. Därefter skedde ett byte till den kapsel deltagaren inte haft från början (koksalt alternativt placebo). Den totala tiden för studien var således åtta veckor. Detta resulterade i fyra veckor av högt saltintag (50+100 mmol) och fyra veckor av lågt saltintag (50+0). Vilken vecka som innehöll det höga respektive låga saltintaget visste inte individerna (studien var såkallad dubbelblindad och randomiserad).

Under tiden studien pågick gjordes även mätningar av 24h blodtryck, vikt samt 24h urinvolym, och blodprov togs för att mäta copeptin under fastande förhållanden. Mätningarna utfödes således både under högt (150 mmol/dag) samt lågt (50 mmol/dag) saltintag.

Vid analysen kunde vi se att copeptin ökade signifikant efter högt saltintag jämfört med låg saltkonsumption hos samtliga individer. Vi såg även att när copeptin steg minskade urinproduktionen (som ett indirekt mått på vattenitag) samt förändringen i vikt (som ett indirekt mått på vattenretention) och tvärtom. Saltkänslighet (blodtrycksstegring vid saltintag) var däremot kopplat till låga nivåer av copeptin men detta fynd var bara signifikant hos kvinnor. Vi kan från denna studie konkludera att ett högt saltintag ökar copeptin men att saltkänsligheten hos kvinnor är kopplad till en minskning av copeptin, troligtvis på grund av ett förhöjt vätskeintag.

I det andra arbetet undersökte vi närmre om förhöjda nivåer av copeptin är relaterade till ökad risk för att drabbas av hjärtinfarkt samt risken att dö av denna men även om förhöjda nivåer av copeptin ökar risken att dö av andra orsaker.

Hjärtinfarkt (förlust av hjärtmuskelceller på grund av syrebrist till hjärtat) orsakar en tredjedel av dödsfallen hos personer över 35 år. Trots att ett flertal metoder finns för att minska risken för hjärtinfarkt är det fortfarande en ledande orsak till död, varför det är viktigt att finna andra och nyare metoder för att förebygga sjukdomen.

I denna studie valde vi att följa 5836 helt friska personer med en medelålder på 69 år under 6.5 år och därefter analysera en eventuell utveckling av hjärtinfarkt och död. Dessa individer delades upp i 4 lika stora grupper där grupp 1 var de med

lägst värden av copeptin och grupp 4 de med högst. Därefter jämförde vi höga respektive låga nivåer av copeptin i blodet med risken att utveckla hjärtinfarkt och risken att dö av denna. De individer som tillhörde fjärde gruppen, de med högst nivåer av copeptin, löpte en dubblerad risk för att utveckla hjärtinfarkt jämfört med de med lägst nivåer. Grupp 4 hade även en 56% ökad risk att dö oavsett orsak jämfört med grupp 1, men hjärt-och kärlrisken drev detta fynd starkt, där de i grupp fyra hade 75% högre risk för att dö av hjärtrelaterad åkomma inom loppet av 6.5 år jämfört med individerna i grupp 1. Resultaten är signifikanta både hos individer med diabetes och utan även om dibetikerna hade en högre risk för ovan nämnda åkommor jämfört med de utan diabetes. Detta fynd skiljer sig från resultaten av vår tidigare studie på en medelålderspopulation där enbart de med diabets visade en riskökning. Resultaten visar även att risken är hög hos båda könen men högre hos kvinnorna. Varför är ännu oklart.

I det tredje arbetet undersökte vi om risken för försämrad njurfunktion och utveckling av kronisk njursvikt var relaterad till förhöjda nivåer av copeptin. I 5252 individer från databasen Malmö Diet and Cancer Cardiovascular Cohort (MDCC-C) mättes copeptin och njurfunktionen vid studiens start. Dessa individer följdes under 16.6 ± 1.5 år och på grund av olika anledningar (tex flytt, död, ovilja att vidare delta), fanns det vid återundersökningen 3186 indivder kvar för analys. Vid återundersökningen relaterades copeptin till utveckling av kronisk njursvikt och försämrad njurfunktion och individerna delades upp i fem lika stora grupper beroende på nivåer av copeptin. De med högst värden tillhörde grupp fem och de med lägst grupp ett (referensgruppen). När vi sedan jämförde grupperna, kunde vi se att förhöjda nivåer av copeptin ledde till en årlig försämring av njurfunktionen. Undersökningen visade även att förhöjda nivåer av copeptin utgör en risk för utveckling av kronisk njursvikt på sikt, där de individer tillhörande grupp fem hade en 48% ökad risk att utveckla njursvikt (stadie 2 av 5) jämfört med referensgruppen och en risk på 57% att utveckla njursvikt stadie 3 av 5.

Hur kan man då minska risken av ovan nämnda sjukdomar om man har förhöjda värden av copeptin?

Om man i tid kan lämna ett blodprov och ta reda på om man har förhöjda värden av copeptin, och på så sätt får reda på att man löper en ökad risk för framtida hjärtinfarkt och/eller njursvikt, ökar också möjligheterna att förebygga framtida sjukdomar. När man då vet om man tillhör riskgruppen eller inte är det dags för den förebyggande interventionen. **Det viktigaste budskapet** från dessa studier är att förhöjda värden av copeptin utgör en markör för ovan nämnda risker och att detta bör vässa läkarnas uppmärksamhet gällande den förhöjda risk de friska individerna, och inte bara diabetikerna, löper. Som tidigare nämns screenas diabetikerna så tidigt som möjligt för att i tid upptäcka en eventuell sjukdomsutveckling. Genom att fånga upp de friska individer som också har en

förhöjd risk (dvs. de med höga värden av copeptin) kan vi optimera förebyggande åtgärder i tid och förhindra uppkomsten av hjärtinfarkt eller njursvikt från första början genom att till exempel erbjuda blodtrycksbehandling i tidigare skede eller mer aggressivt försöka sänka ett förhöjt blodtryck.

Om det då är som vi tror, att ökade nivåer av vasopressin (mätt med copeptin) leder till utveckling av dessa sjukdomar på sikt, vad kan vi då mer göra för att hjälpa dessa individer att minimera risken mer än att försöka fånga dem tidigt? Är själva risken behandlingsbar? Detta är något vi ämnar svara på i den fjärde studien. Om nu copeptin släpps ut vid förhöjd koncentration av salt i blodet och för lite vätska i kroppen, borde inte frisättningen hämmas om vi upprätthåller en normal koncentration av salter i blodet och en normal vätskebalans i kroppen? Detta är något som vi tror på och har därför valt att utföra en vattenintervetionsstudie hos människor.
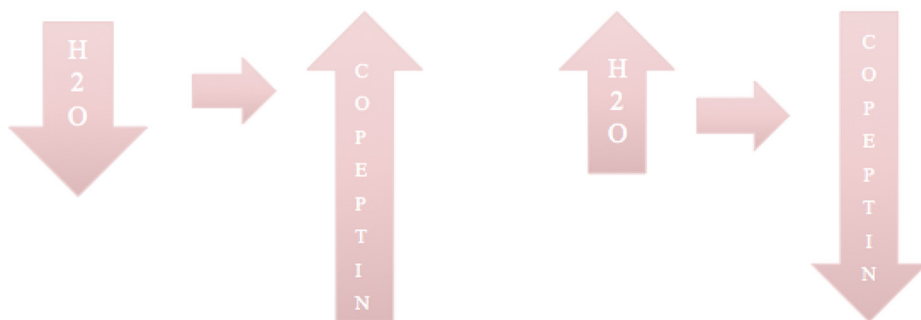
Figure 1: The relationship between water and copeptin

I den fjärde studien rekryterade vi 37 st individer och lät dem genomföra en vecka med ett högt vattenintag (vattenveckan) och en vecka med sitt vanliga vattenintag som jämförelse (kontrollveckan). Individerna var i botten helt friska och mellan 20 och 70 år gamla. Första dagen i undersökningen (dag 1) lämnade individerna blodprover (bland annat på copeptin) och mätte även längd, vikt samt blodtryck. Under denna dag fick de även genomgå ett akut vattentest. Här fick de under 20 minuter antingen dricka 1L vatten (om de hade sin vattenvecka) eller 10 ml vatten (om de hade sin kontrollvecka). Därefter togs upprepade copeptin mätningar under en period på 4h. Sedan påbörjade individerna antingen sin vattenvecka där de då drack 3L per dag eller sin kontrollvecka där de drack som de vanligtvis gör. Efter 5 dagar kom de igen för att lämna blodprover som en säkerhetsåtgärd, då framförallt för att mäta salterna i blodet och upptäcka en eventuell överdriven utspädning av blodplasman. Dag 9 kom de åter för att ta proverna (bland annat copeptin) på nytt och då även genomgå ett så kallat oralt glukostoleranstest. Detta

är ett test där individen dricker en glukoslösning (socker) och sedan tas prover på blodsocker för att se hur kroppen med hjälp av blodsocker-reglerande hormon (insulin och glukagon) sköter hanteringen av det givna glukoset.

När mätningarna var gjorda analyserades copeptin i förhållande till det höga respektive låga vattenintaget, men eventuella samband med glukos, glukagon och insulin analyserades också.

Om vi börjar med resultaten från det akuta testet (dag 1) kunde vi här se att ett akut intag av 1L vatten sänkte copeptinnivåerna signifikant inom 30 min, nivåerna var som lägst vid 90 min (39% sänkning i medel) och nivåerna kvarstannade låga under hela testperioden (4h). Under det längre testet, 1 vecka, ledde ett ökat vattenintag till 15% reduktion av copeptinnivåerna när man jämförde med kontrollveckan. Den största sänkningen sågs bland de individer som vanligtvis hade höga värden av copeptin och koncentrerat urin, båda tecken till att man kanske vanligen dricker förlite. Då dessa individer svarade med den största sänkningen av copeptin valde vi att kalla dem för "water-responders". Övriga individer, d.v.s. de utan den uttalade sänkningen, kallade vi för non water-responders. Går vi vidare till analysen av glukos, insulin och glucagon såg vi här att water-responders hade en vatteninducerad minskning av glukagon (ett hormon som höjer sockernivån i blodet). Varken water-responders eller non water-responders visade någon påverkan på glukos och insulin.

Studie fyra har fått oss att vilja undersöka om vi kan påverka nivåerna av copeptin med hjälp av ökat vatten-intag under en längre period. Vi har funderat över vilka individer vi i så fall ska inrikta oss på. Nästa mål kommer bli att rekrytera den del av befolkningen som är water-responders, d.v.s. de som vanligtvis dricker så lite vatten att deras urin är koncentrerad och deras copeptin-nivåer är förhöjda. Då denna population har förhöjda nivåer av copeptin är de även i risk för hjärt- och kärlsjukdom samt njursvikt. Tanken är sedan att öka deras dagliga vätskeintag under en längre tidsperiod för att på så vis sänka vasopressinsystemets aktivitet och/eller påverka kroppens hantering av blodsocker och då förhoppningsvis även sänka de risker som en förhöjd aktivitet av vasopressin har visats orsaka.
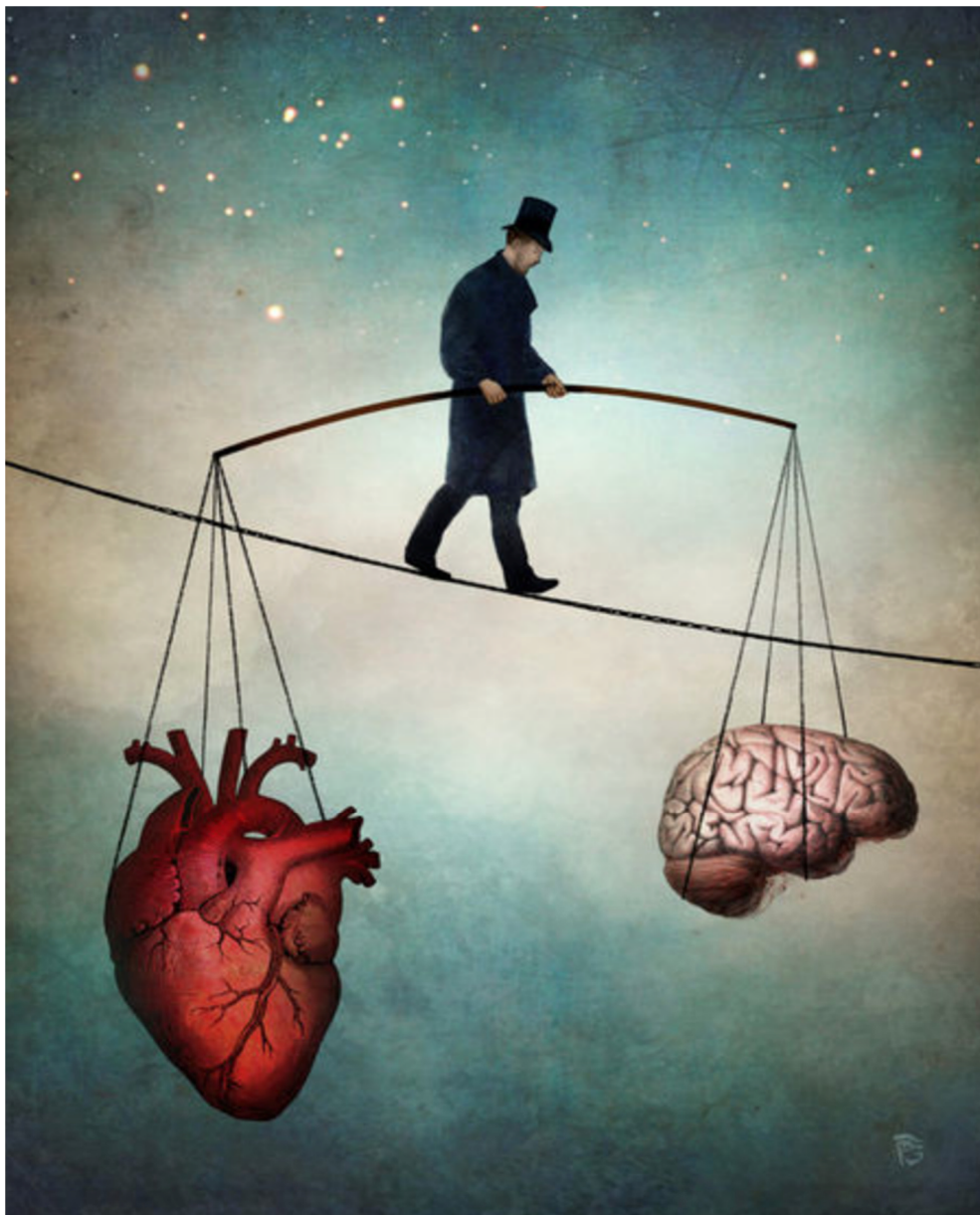
Se kapitel "future research".

Photo: Balance by unknown

Introduction

Cardiovascular disease and chronic kidney disease are large public health problems with the former one being a leading cause of death worldwide. Even though we know several successful preventive strategies the diseases are still associated with a high mortality rate. The health issues of these diseases make them interesting for even more thorough research in order to find other and new preventive interventions. A lot of focus is given to both secondary and primary prevention, especially in individuals with a known increase in risk, but it is harder to identify those individuals with an increased risk without any signs of illness. Our main interest highlights the field of primary prevention and finding individuals at risk in order to optimize preventive strategies.

Overactivation of the vasopressin (AVP) system has been linked to components of the metabolic syndrome with hypertension being one of them. Furthermore, the relationship between hypertension and increase in salt intake has been under a long debate. As increased osmolality is a powerful stimuli of AVP release, we wanted to investigate the relationship between vasopressin concentration and change in blood pressure during an increase in salt intake. Also, increased activity of vasopressin system and development of cardiovascular disease (mainly in individuals with diabetes mellitus)[1] as well as kidney damage (mainly in animals)[2] have been shown. In line with these findings, we wanted to investigate the vasopressin system in relation to the risk of developing cardiovascular disease and chronic kidney disease in a healthy human population. By studying the relationship between increased levels of vasopressin and potential harmful effects on different organs, a path towards intervention was opened, leading us to the last study. As the main stimuli of vasopressin release is high plasma osmolality, a human water intervention experiment was performed in order to study the effects of vasopressin in relation to increased water load. Increased water intake was used aiming to decrease the participants' plasma osmolality and thus their vasopressin concentration in plasma.

The Vasopressin system and Copeptin

AVP, also known as antidiuretic hormone, is a key hormone in the human body serving importat physiological functions such as homeostasis of fluid balance, vascular tonus and regulation of endocrine stress response. Measurement of AVP in plasma has been shown to be difficult due to a short half-life and a small size of the molecule. Holwerda first described during 1972 a glycopeptide C-terminal part of the AVP molecule[3] and Roger Acher later named it copeptin[4]. AVP and copeptin are derived from the same amino acid precursor (CT-proAVP) protein and produced in a ratio 1:1. Due to the facts that **copeptin** is a more stable molecule than AVP and procuced in equal amounts with AVP it is therefore measured to indirectly reflect the levels of AVP.

Vasopressin synthesis

Pro-AVP is the precursor peptide of AVP produced and released by two mechanisms. The pro-AVP constitutes of a signal peptide, the 9 aminoacid (aa) long AVP, neurophysin II, and the 39 aa long glycosylated peptide[5]. The latter two components probably assist the correct folding of AVP[6].

In the first mechanism, pro-AVP is produced in **magnocellular neurons** in supraoptic and paraventricular parts of the hypothalamus. Then, it is released during axonal transport, through infundibulum, to **posterior** lobe of the **pituitary gland** where it is stored for later release in order to regulate homeostasis.
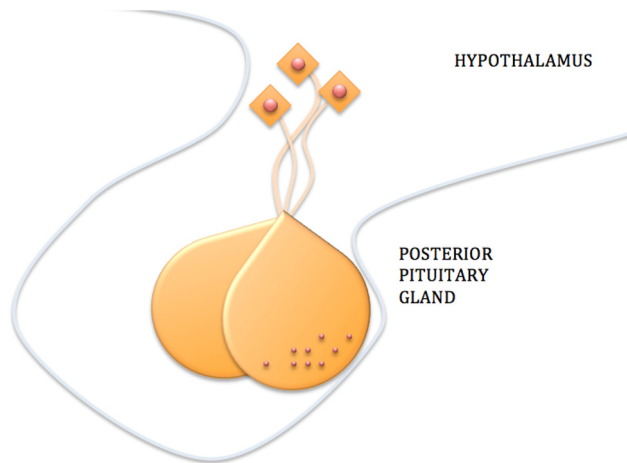
Figure 2: Synthesis of vasopressin in magnocellular cells.

In the second mechanism, pro-AVP is, together with other releasing hormones such as corticotropin-releasing hormone (CRH), produced in **parvocellular neurons** of the hypothalamus. It is released into the pituitary portal system to the **anterior pituitary gland**, to directly act on endocrine cells of the adenohypophysis, resulting in adrenocorticotropic hormone (ACTH) and cortisol release. This is a mechanism performed in synergy with CRH[7] [8]making AVP a part of the endocrine stress response.
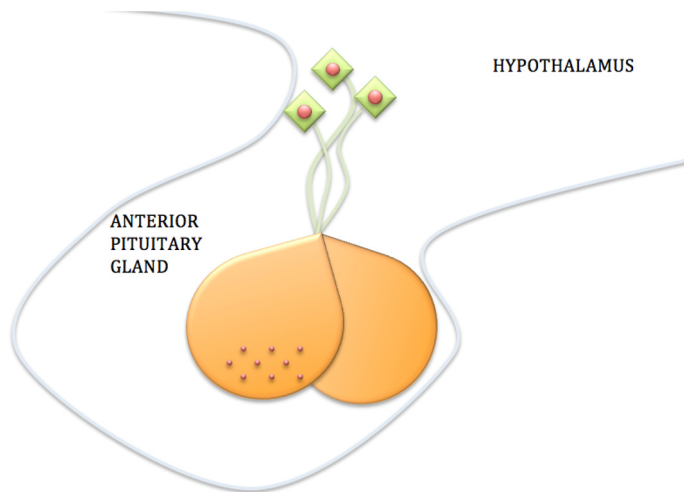
Figure 3: Synthesis of vasopressin in parvocellular cells.

Vasopressin recepors and effects

AVP is released mainly in response to hypovolemia and increased osmolality. In the body, the hormone acts on different recepors and contributes to several effects.

V_{1a}-receptor: Vasoconstriction

The Vasopressin$_{1a}$ receptor (V_{1a}R) is present in many tissues and is the most prevalent receptor in the body. It influences blood pressure, circulation and coagulation. Through a G-protein-induced action on vascular smooth muscle cells it causes vasoconstriction by increasing intracellular calcium. The V_{1a}R is also present in cardiac myocytes, but the action that can be attributed to the receptor in the myocytes is under debate. By indirectly stimulating Factor VII and von Willebrand factor release the receptor causes platelet activation.

Through action on the V_{1a}R, AVP is involved in gluconeogenesis as well as glucogenolysis in the liver[9-12]. In general, higher concentrations of AVP are needed in order to act on this receptor when compared with Vasopressin$_2$ receptor (V_2R) mediated effects in the kidney.

V_2 receptor: Water balance

The V_2R is located in cells of the renal collecting tubules. By increasing cyclic adenosine monophosphate (cAMP), via the Gs pathway, it serves two main functions of water homeostasis[13]. Firstly, it stimulates the production of mRNA that encodes aquaporin 2. Secondly, it inreases transportation of aquaporin 2 vesicles into the membrane of the collecting duct, resulting in water-absorpion from the urine [13] and thus directly influencing water uptake. More specifically, the V_2R is located in principal cells of the collecting ducts, and the insertion of aquaporin 2-rich vesicles occurs in the luminal membrane of the cells. In addition, in the cortical and outer medullary collecting duct, vasopressin stimulates sodium reabsorption by acting on luminal sodium channel ENaC which drives water and thus concentrates all other solutes in the lumen[14]. Also, in the terminal inner medullary collecting duct, vasopressin increases permeability to urea by activating ureatransporters UT-A1 and UT-A3 which allows concentrated urea to diffuse into the interstitium favoring water reabosrpition[15, 16]. Summarizing, these effects contribute to urine concentration

V_{1b} receptor: ACTH, insulin and glucagon secretion

The *Vasopressin $_{1b}$* receptor (V_{1b}R) is also known as V_3 receptor. It is present in cells of the adenohypophysis and involved in secretion of ACTH. Furthermore by acting on cells in the pancreas, V_{1b}R mediates insulin and glucagon secretion, thus suggesting a role in the glucose metabolism[17, 18].

Copeptin

The exact physiological role of copeptin is not fully known. Copeptin has been shown to interact with the calnexin/calreticulin system, which is a system involved in the monitoring of protein folding, and is thus suggested to be involved in structural formation of pro-AVP[19, 20]. Copeptin is measurable in urine, which it indicates that its elimination is, at least partly, renal. As stated before, copeptin is released in equal amounts with AVP, and due to its stable properties it is measured to indirectly reflect levels of AVP. For more specifics concering measurement, see chapter "clinical examination and assays".

AVP has vital functions by regulation the body's cardiovascular homeostasis. However, an overactivity of the AVP system has been shown to increase morbidity and mortality in certain diseases (see next chapters). By understanding the underlying mechanisms of action of the AVP system and studying the potential harmful effects of its overactivity, more knowledge can be gained in order to optimize preventive interventions.

Hypertension and salt sensitivity

The heart pumps the blood into the vascular tree where the vessels are the carriers of the blood to the rest of the body. The blood pressure is the force created of blood pushing against the walls of the vessels originating from the heart (arteries). Or in other words, blood pressure is a function of cardiac output and peripheral resistance (arterial blood pressure = cardiac output x peripheral resistance). The more pressure there is here, the more the heart has to work to pump the blood. A high blood pressure, named hypertension, is a condition in which the pressure in the blood vessels is raised persistently. In the majority of the patients the exact cause of hypertension is rather multifactorial and not fully known (primary or essential hypertension) whereas in some patients a secondary cause (secondary hypertension) can be identified. Our research focuses on the former one.

In Sweden, around 2 million individuals (27%) suffer from hypertension and in individuals aged above 65 years, the incidence rate is more than 50%. The incidence increases with age and the complications can be both cardial and renal. Hypertension is the most treatable risk factor for cardiovascular disease. Despite this, the control of the disease is far from adequate with only 50% of the patients reaching the treatment goals [97]. One of the potential reasons for this is that hypertensive individuals are usually free from symptoms and because of this the disease is not detected in time. Furthermore, the medications have side effects challenging the physician. Risk factors for developing hypertension include

modifiable ones such as overweight, smoking and physical inactivity as well as non-modifiable ones such as family history and gender.

As previously shown, apart from former mentioned risk factors, high salt intake is a known and well-debated risk factor for developing hypertension. In some parts of the world where salt intake is low, it has been shown that hypertension rarely develops nor increases with age [21]. When blood pressure increase is related to increase in salt intake it is termed salt sensitivity.

Studies have shown that hypertensive patients are more salt sensitive than normotensive individuals [22] and there is evidence of salt sensitivity being a heritable trait [23]. Normotensive individuals having a first degree relative with hypertension have been shown to be more salt sensitive than those with no family history of hypertension [24]. Furthermore, Melander et al have previously shown, in the same study subjects as those presented in paper I in this thesis, that a reduction of daily salt intake by 100 mmol (150 versus 50 mmol) leads to a substantial decrease in blood pressure[25]. In the same population it is found that genetic variation in NEDD4L seems to affect salt sensitivity in normotensive individuals, thus suggesting that genotyping of NEDD4L may be clinically useful in order to identify subjects who would benefit from dietary salt restriction in the prevention of hypertension[26]. Other genetic studies have shown a correlation between salt sensitivity and renal defect. Most known monogenic forms of hypertension are caused by an increase in renal sodium reabsorption[27] and have a mutated gene expression in the kidney which is central in the pathogenesis [27-35]. These findings suggest that genetic defects in the kidney might increase the susceptibility to salt sensitivity in the more common umbrella diagnosis of primary hypertension.

Apart from classical risk factors (smoking, physical inactivity and family history for example), primary hypertension is known to co-occur with other diseases such as type 2 diabetes, obesity and dyslipidemia, leading us to the glucometabolic field. In fact, insulin resistance is characteristic in patients with primary hypertension [36, 37]. The previously mentioned normotensive individuals who were shown to be more salt sensitive if they had a first degree relative with hypertension, have in addition also shown features of insulin resistance[38, 39]. The findings of insulin resistance and salt sensitivity highlight two common features of primary hypertension and possible hallmarks of inherited hypertension.

The vasopressin system in hypertension and salt sensitivity

We know that increased osmolality leads to increased levels of AVP. Further on, a hyperactivity of the AVP system has been linked to components of the metabolic system including both hypertension and diabetes incidence[1, 40, 41]. What has

not been studied is the third link in the process, namely the relation between copeptin and increased salt intake as well as salt sensitivity. We believe that AVP, measured as copeptin, can be manipulated by increased salt intake, making AVP a new possible contributing factor in the process. Based on this background, we hypothesized that the vasopressin system may be involved in development of hypertension and salt sensitivity.

Cardiovascular and chronic kidney disease

Cardiovascular disease

The cardiovascular diseases (CVDs) are a collection of disorders of the heart and blood vessels. They include coronary artery disease (CAD), cerebrovascular disease (stroke), peripheral artery disease, rheumatic heart disease, congenital heart disease and deep vein thrombosis as well as pulmonary embolism. According to WHO, cardiovascular disease is the leading cause of death world wide representing 31% of all (yearly) global deaths with the majority (7.4 million) being due to CAD and the rest (6.7 million) due to stroke. Our research focuses on CAD, which was defined as coronary revascularisation, fatal or non-fatal myocardial infarction, or death due to ischaemic heart disease (see the method section). Myocardial infarction usually results from a blockage in the vessels that prevents blood from flowing to the heart and thereby causing ischemia to the heart muscle. The most common reason for this blockage is a build-up of lipid deposits on the inner walls of the vessels, which eventually lead to atherosclerosis.

In the majority of CAD cases, the disease can be prevented by modifying behavioural risk factors such as tobacco use, obesity, unhealthy diet, physical inactivity and alcohol abuse. Other determinants of CAD that are non-modifiable include male gender, high age and family history of CAD. Individuals with co-morbidities such as hypertension, diabetes and dyslipidemia are also at high risk of developing CAD stressing the important need of early detection and proper prevention in those indidivuals. Regarding the diabetes-related CAD, the underlying mechanisms are still not fully known. Two potential explanations for this might be that macrovascular damage starts early, even before the onset of diabetes, and that therapy is initiated too late, alternatively that other factors than hyperglycemia are responsible for diabetes-related CAD. This highlights the need of identification of drug and lifestyle modifiable factors regarding causality both in diabetes and CAD in order to optimize preventive strategies and catching high-risk individuals.

We have previously shown that increased activity of the AVP system is linked to development of incident diabetes [1, 42, 43]. Furthermore, we have shown that in a middle aged, diabetic population, increased levels of copeptin are related to increased risk of developing incident CAD but not in the corresponding non-diabetic population [44]. In contrast to this previous finding, our second paper shows an increased risk of CAD in an elderly population both among those with and those without diabetes. We therefore believe that the vascular tree ages earlier in the diabetic population than in the non-diabetic one. In fact, premature vascular ageing has been seen in diabetics [45] and hyperglycaemia has been associated with provocation of endothelial dysfunction, vascular smooth muscle cell proliferation and inflammatory phenotype changes in macrophages [46-49]. Furthermore, chronic exposure to glucose has also been shown to enhance collagen cross-linking in the arterial wall [50] and to upregulate enzymes (metalloproteinase-2 and metalloproteinase-9) responsible for degradation of elastin.

Chronic kidney disease

Chronic kidney disease (CKD) is a loss of kidney function. The kidney function is measured by estimeted GFR, or eGFR, that describes the flow rate (ml/min) of filtered fluid through the kidney.

Creatinine

Creatinine is a substance widely used in equations of estimating GFR. Creatinine is a cleavage product of creatinine phosphate, a substance produced in constant rate in the muscles. Apart from its constant production, creatinine is also excreted from the urine in a constant rate making it suitable for measurement of filtration. Levels of creatinine increase during renal impairment (renal excretion decreases) and creatinine is therefore usually used as an indirect sign of renal dysfunction. However, some factors can affect the levels of creatinine making it partially unreliable as a maker. For example, in well-trained individuals with high muscle mass creatinine levels are generally higher and the opposite effect is seen in elderly individuals with low muscle mass. Lastly, women generally have lower levels of creatinine than men and Afro-american individuals have higher than Caucasians.

Estimated glomerular filtration rate (eGFR)

An effective way of measuring kidney function is to calculate eGFR by adjusting for cofounders in different formulas estimating the GFR. The most widely used formula is the Modification of Diet in Renal Disease (MDRD) Study equation and the Cockcroft-Gault formula (CG). The differences in these formulas are the adjusting factors where the MDRD adjusts for gender, age and if relevant afro-american race whereas the CG equation in addition to this adjusts for body weight.

The MDRD equation was developed from patients with a rather low eGFR (on average an eGFR of 40 ml/minute/1.73 m^2). This makes the fomula less suitable for individuals with an eGFR >60 ml/min/1.73 m^2 as there is a risk of underestimating the true GFR. Furthermore, the fact that the CG equation includes measurement of weight makes this formula to overestimate GFR in obese and vice verca, underestimating it in underweight subjects. Lastly, a third formula has started to be widely accepted; the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI 2009) formula. In contrast to the other two formulas mentioned, this formula adjusts for over and underestimation of CKD depending on high or low creatinine levels. This formula can be calculated by using creatinine alone or combining it with cystatin C.

Levles of CKD

The different levels of the loss of function are divided into 5 stages according to the "Kidney Disease Outcomes Quality Initiative" (KDOQI) criteria. Stage 1 indicates a normal kidney function, but structural abnormalities or genetic traits point towards kidney disease, and is measuread as an eGFR above or equal to 90 ml/min. This stage does not need any treatment but solely has to be observed, and blood pressure needs to be controlled. Stage 2 (eGFR 60-89 ml/min), indicates a mildly reduced kidney function, and as stage 1, it needs to be obseved. Stage 3 (eGFR 30-59 ml/min) includes a moderately reduced kidney function and needs specific management whereas stage 4 (eGFR 15-29 ml/min) indicates severly reduced kidney function and need planning for end stage renal failure. Lastly, an eGFR less than 15 occurs in stage 5 and equals to end stage, or very severe, kidney failure.

Table 1: Classification of chronic kidney disease

Stage	eGFR (ml/min/1.73 m2)
1 Kidney damage, preserved eGFR	≥ 90-120
2 Kidney damage, mildly impaired eGFR	60-89
3 Moderately impaired eGFR	30-59
4 Severely impaired eGFR	15-29
5 Kidney failure	< 15 (or in dialysis)

CKD has become a public health issue and in 2016 the global prevalence of CKD was 11-13% [51]. In addition, the health care problem is highest during the early stages of CKD (due to increased prevalence) occuring in 35% of individuals aged above 70 [52]. The early stages of CKD are asymptomatic and the diagnosis is usually set in the progrediated stages. This underline the importance of finding strategies to early identify individuals with increased risk in order to optimize prevention. There are many reasons for developing CKD and apart from the classic risk factors such as age, gender and smoking, decline in renal function is accelerated in hypertension, diabetes and obesity. Furthermore, CKD is an accelerator of CVD risk. It is found to be an independent risk factor for CVD events [53], and several studies have found an inverse and independent relationship between CVD risk and eGFR[54-57].

CKD and the vasopressin system

Causes of CKD development can be divided into three categories: prerenal, renal and postrenal. An example of prerenal CKD is hypovolemia, whereas glomerulosclerosis can be classified as a renal cause and for example obstruction in the ureter is classified as a postrenal cause. In addition, dehydration/hypovolemia is a powerful stimulus of vasopressin release making vasopressin a potential factor in the pathogenesis of CKD. When further looking at the renal causes of CKD that are possibly linked to AVP, animal studies have shown that in rodent models of diabetes, infusion of AVP induces hypertension, glomerular hyperfiltration, albuminuria and glumerulosclerosis suggesting a role of the AVP system in the pathogenesis of renal function decline[2]. Furthermore, elevated levels of AVP are found to be associated with greater decline in eGFR in individuals with type 2 diabetes [58] as well as with a doubling of plasma creatinine in patients with both type 2 diabetes and albuminuria[59]. A study performed on healthy kidney donors showed that eGFR decreased significantly post donation, but levels of copeptin remained unchanged, suggesting that AVP may in fact be causally related to the decline in eGFR rather than a marker of decline in eGFR[60]. Since an increased activity of the AVP system has been linked to both cardiac and renal findings, studying the AVP system in relation to

development of morbidity and mortality in cardiorenal disease has become of great interest for us. Since vasopressin is released during a dehydrated state, experimental studies of rats, trying to reduce levels of vasopressin by increasing water intake, have been performed. These studies showed that increased water intake suppresses vasopressin, reduces proteinuria and improves creatinine clearance (16-18). Regarding human studies, a randomised controlled pilot trial in Canadian patients having stage 3 chronic kidney disease, was performed. This study showed that adults with CKD stage 3 successfully can be randomised to drink 1L more per day than controls and that the increase in water intake significantly reduced levels of copeptin[61].

The city and population of Malmö

The city of Malmö is Sweden's third largest city, located in southwest part at the coast and belonging to the province of Skåne. By the 8km long Öresund bridge, with a combined rail- and motorway between Malmö and Copenhagen, the city connects with the rest of Europe. Not only does the city have beautiful parks and an outstanding football team, but the international food culture in the city of Malmö is at its fully bloom.

During 2016, the number of inhabitants in Malmö was 328 494. The ethnic background of the Malmö population has changed over the years. At early 70s, the population amounted to 243 591 out of which 19 308 (7.9%) had foregin ancestry. This is to be compared with the current population of 328 494 inhabitants (162 184 men and 166 310 women), out of which 145 641 (44%) are born oversea (32%) or have both parents with foregin background (12%)[98]. The individuals with foreign ancestry are currently representing 178 different nationalities. The mean age for the Malmö citizen is 38.5 years and the share of individuals continuing their studies at a higher educational level (meaning after high school) has increased from 41% (2008) to 48% (2015)[98]. In comparision, the percentage in Sweden in general is 42%.

Socioeconomic status among the population in Malmö has also been changing over the years. The share of low-income households has increased over the period 1999-2011 (18% respectively 27%) but the statistics differ significantly between different regions of Malmö. In a region named Rosengård, which is a region with poor socioeconomic status in Malmö, women aged between 55-59 years have a sick leave rate of 36%. This is to be compared with a sick leave rate of 6% among women of the same age in the region Limhamn-Bunkeflo, which is a region with richer socioeconomic status in Malmö The share of homeless individuals in Malmö has increased during the time period 2003-2013 from 531 to 973

individuals [98]. Lastly, the usage of tobacco has changed, showing a decreasing trend over the years. 13% of the women and 17% of the men in the city of Malmö smoke on a daily basis, when compared to the 60's where every second man and every fourth woman was a smoker [98].

These numbers show that the population in the city is constantly changing, resulting in several factors that can challange a researcher. In this thesis, the study participants in all of the papers are individuals from the city of Malmö representing a Caucasian population. In study II and III, the individuals were recruited during the early 70's (the Malmö Preventive Project Cohort) as well as during the early 90's (the Malmö Diet and Cancer Cohort). Here, 10% of the individuals were born in another country than Sweden with the majority born in Denmark, Finland and former Republic of Yugoslavia. Even though the population has changed, the collected data represents a big part of the individuals living in Malmö.

Photo: Turning Torso by Igor Tasevski

Study population

In paper I, a study population of 39 individuals, without history of hypertension, diabetes and renal disease, were examined. The subjects were initially recruited between January and December in 2005 (n=46) through local newspaper advertisements in the city of Malmö. Out of these individuals, a number of 7 could not complete the study because of fever (n=2) or refusal to take the study capsules regulary (n=5), leavning 39 subjects to study. The mean age of the participants were 53±11 years, BMI was 26.4±3.1 kg/m^2 and 20 were men.

Based on the Dietary Approaches to Stop Hypertension (DASH) study a powercalculation was made. The DASH study was performed in Caucasial, normotensive individuals. Assuming a SD of 7 mmHg of the change in systolic blood pressure induced by a 100 mmol/24 h reduction in NaCl intake, at least 25 study participants would be required to detect a mean systolic blood pressure reduction of 4 mmHg with 80% power at a significance level of 5% (STATA; STATA Corp., College Station, Texas, USA).

In paper II, the data collection constitued of study subjects from the **Malmö Preventive Project** (**MPP)**, a Swedish single-centre prospective population-based study. The collection of the study material begun in the early 70's as a screening survey in the middle-aged population of Malmö. The aim of the study was to find high-risk individuals for preventive interventions concerning cardiovascular diseases, alcohol abuse and breast cancer. Individuals from the city of Malmö were invited for clinical examination, questionnaire and blood sampling. At the end of the initial recruitment in 1992, the cohort consisted of 33 346 individuals (71 % of everyone invited), out of which 22 444 were men and 10 902 women. The baseline examination consisted of screening for traditional risk factors of all-cause mortality, cardiovascular disease and alcohol abuse. A questionnaire was given that mainly included life style and socioeconomic factors. All the participants also left blood tests and went through a physical examination including blood pressure measurement, height, weight and lung function tests and some of them also had mammography performed. The blood samples were stored in −80° for later analyses. Men were mostly screened during the first half of the period (1974-1982) and women in the latter half (1981-1992) implying different follow-up time for the different genders. On nearly 25 % of the screened

individuals, various interventions such as lifestyle modifications, drug therapy and referral to special outpatient clinics were carried out.

During the period 2002-2006 all subjects who were alive were invited for a re-examination, and a number of 18 240 individuals attended the follow-up. Cardiovascular risk factors were reassessed and plasma samples were drawn and frozen to −80° for later analyses.

The follow-up period between 2002 and 2006 is the baseline examination in our study. Out of the 16 835 individuals that had complete data on cardiovascular risk factors at follow-up, a random sample of 5 386 individuals was selected for copeptin analysis. The only exclusion criterium was previous participation in the Malmö Diet and Cancer Study (MDCS). Out of the 5 386 individuals, 513 have had a prevalent cardiovascular event leaving 4873 individuals for analyses of incident CAD.

In paper III, data from a large population based prospective cohort study – the **Malmö diet and cancer study MDCS** was used. The main goal of this study was to gain more knowledge on the impact of diet on cancer incidence and mortality, or more specifically to clarify whether a Western diet high in fat and total calories and low in vegetables, fruit and fibres, increases the risk of certain forms of cancer such as cancer of the breast, colon, rectum, pancreas, ovary, endometrium and prostate [62]. The MDCS baseline examination includes dietary assessment, a self-administered questionnaire, anthropometric measuring and collection of blood samples stored in a biological bank[62]. The data collection of the MDCS begun 1st of January 1991 and ended the 25th of September 1996. Men and women born 1926-1945 (ages 44-74 years) were recruited and resulted in 53 325 individuals as the first group. The cohort was then redefined every third month including individuals that had moved to Malmö and identifying diseased subjects as well as those who had moved from the city. In this way, the subjects could be removed from the sampling frame instead of classified as non-responders. During 1995, the study was extended to include younger women (born up to 1950) in order to study breast cancer among premenopausal women [63]. In all, a number of 74 138 individuals were enrolled. The study subjects were randomly invited by letters and if they did not respond to the first one, two more letters were sent. In addition to this, the recruitment also reached out by advertisements in local newspapers, public places and primary health care centers.

Individuals were excluded from the baseline of the study for different reasons. In seventeen subjects, it was not possible to identify any civil registration number, whereas some had been registered twice. A number of 3 017 individuals died or moved before receiving the invitation letter, 224 subjects died before completing the baseline examination, 21 817 did not reply to the invitation and 16 942 individuals were unwilling to participate[63]. The only exclusion criterias were

mental retardation and not knowing the Swedish language (since they would have difficulties to respond to the questionnaire), resulting in 1975 excluded individuals[62]. Sujbects completing the questionnaire, the anthropometric body composition measurements and the dietary assessment were regarded as complete participants and here a number of 2 048 did not fulfill these examinations and were therefore excluded. In all, a number of 28 098 participants, 11 063 men and 17 035 women, had complete data. This resulted in a participation rate of 40.8%, for men 38.3% and for women 42.6% [63]. The mean age of these study subjects was at baseline 56.4 ± 5.7 years.

During the time period from November 1991 until February 1994 every second individual was invited to participate in additional examinations to study the epidemiology of carotid artery disease with ultrasonography of carotid arteries, with a special interest in intima media thickness. This cohort is referred to as the **MDCS Cardiovascular Cohort (MDCS-CC)**[64]. A number of 6 103 subjects had complete data and out of these subjects, 5 400 provided fasting blood samples. From frozen fasting blood samples, copeptin was successfully measured in 5252 subjects. During the reexamination between 2007 and 2012 (mean follow-up time 16.6 ± 1.5 years), 3 186 of the re-investigated individuals had baseline fasting plasma copeptin concentration available and in these individuals eGFR was determined.

In paper IV, 55 healthy subjects, aged between 20 and 70 years, were recruited during 2011 via advertisement in the local newspaper "Metro", through the homepage of Medical University of Lund and through telephone contacts with individuals that previously participated in MPP and MDCS. Of these, 39 subjects (71%) completed the study and 37 subjects had complete copeptin measurements, which are the ones used in our study. The recruitment included both men and women. The blood samples were stored in $-80°C$ for later analyses. Undiagnosed individuals, if any, with diabetes, impaired oral glucose tolerance test or increased blood pressure could be identified and then offered treatment.

Aims

The aims of this thesis are to investigate following

- if copeptin is related to salt sensitivity
- if copeptin is related to the risk of coronary artery disease, cardiovascular mortality and total mortality
- if copeptin is related to decline in renal function and development of chronic kidney disease
- if copeptin can be suppressed by increase in water intake and if so, glucometabolic parameters can be affected as well.

Methods

In paper I, 46 healthy individuals were recruited via advertisements in local newspapers to participate, as described in the previous chapter. Out of these 46 individuals, 39 individuals completed the study. 20 of them were men and 19 were women. The study subjects were unmedicated and had no history of hypertension, diabetes mellitus or renal disease.

At baseline, the individuals were examined having their habitual diets, i.e. nonstandardized salt intake. The examinamtion constituted of blood tests, 24h blood pressure measurement and 24h urine collection.

Then, for a period of 8 weeks, following the baseline visit, the subjects were all given meals and drinks provided by our metabolic ward, produced and packed at Findus R&D AB (Bjuv, Sweden). The diet was designed by a dietician and the daily energy intake was adjusted according to body weight and gender (2000–2600 kcal/day). The individuals were prohibited from ingesting anything else during the study apart from the received food and tap water. In addition to the given food that contained 50 mmol of NaCl, all of the subjects received capsules either containing 100 mmol NaCl (6 grams of NaCl per day administered as 3×500 mg capsules 4 times daily) during 4 weeks or corresponding placebo capsules during 4 weeks, in a double-blinded random order. This resulted in 4 weeks of high salt intake i.e 150 mmol (9 grams) daily (50 mmol + 100 ml) and 4 weeks of low salt intake i.e 50 mmol (3 grams), (50 mmol + 0 ml) daily. The NaCl and placebo capsules were blinded, coded and packed by Apoteket AB (Swedish state pharmacy).

After 8 weeks, the subjects came for a follow-up visit and were examined with a similar protocol as the one used during baseline, i.e blood tests (including copeptin), 24h ABP and 24h urine collection. Salt sensitivity was defined as the difference between systolic 24h ABP after high and low salt intake.
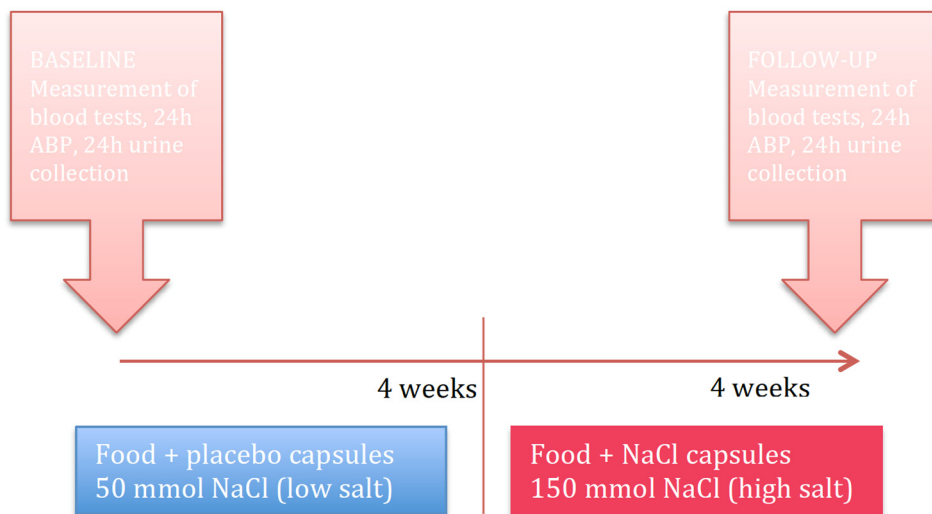
Salt sensitivity = ΔSBP high salt - ΔSBP low salt

Figure 4: Method description paper I

In paper II, we used study subjects from the MPP cohort as previously described. The only exclusion criterion was prior participation in the other large population-based prospective cohort study from Malmö, that is, the MDCS. We chose a random sample of 5386 individuals to measure copeptin in and analyse copeptin in relation to cardiovascular and total mortality, as two of our endpoints. Our third endpoint focuses on copeptin and the risk of developing incident coronary artery disease (CAD). During baseline (before 2002-2006), 513 subjects had already developed CAD and were therefore excluded, leaving 4873 indiciduals for analysis of incident CAD.

At the clinical examination during baseline, participants underwent a medical history, physical examination and laboratory measurements that were stored in minus −80° for later analyses. The study subjects were then followed for all-cause mortality, cardiovascular mortality and a first incident CAD event until 31 December 2010 resulting in a follow-up time of 6.5 years. During the follow-up time, the events were identified by linking a 10-digit personal identification number of each Swedish citizen with three registers: 1. The Swedish Hospital Discharge Register, 2. The Swedish Cause of Death Register and 3. The Swedish Coronary Angiography and Angioplasty Registry (SCAAR). The registers have been previously described and validated for classification of outcomes [65, 66]. CAD was defined as coronary revascularisation, fatal or non-fatal myocardial infarction, or death due to ischaemic heart disease. Myocardial infarction was defined on the basis of International Classification of Diseases, ninth revision (ICD-9) code 410 or International Classification of Diseases, tenth revision (ICD-

10) code I21. Death attributable to ischaemic heart disease was defined as ICD-9 codes 412 and 414 or ICD-10 codes I22, I23 or I25. Coronary artery bypass surgery was identified from national Swedish classification systems of surgical procedures and defined as procedure codes 3065, 3066, 3068, 3080, 3092, 3105, 3127 or 3158 (the Op6 system) or procedure code FN (the KKÅ97 system). Percutaneous intervention was identified from the SCAAR. Cardiovascular mortality was defined as primary cause of death classified as ICD-9 diagnoses 390–459 and ICD-10 diagnoses I00–199.

In paper III, data from the MDCS cohort was used. As described in previous chapter, individuals born between 1923-1945 were recruited during 1991-1996 resulting in a number of 28098 participants at baseline. Out of these, 6103 individuals were also a part of the MDCS Cardiovascular Cohort (MDCS-CC) and here 5400 were randomly selected for copeptin analyses where 5252 had complete data. Copeptin was measured at baseline in plasma samples that had been stored in -80°C. The study subjects were then followed until 2007-2012 (16.6 ± 1.5 years) leaving 3186 individuals at the follow-up analyses. At the re-examination, new blood testing and physical examination were performed.

Calculationg eGFR was performed using different, but yet well established, formulas. Both the Modification of Diet in Renal Disease (MDRD) formula and the CKD Epidemiology Collaboration (CKD-EPI) formula were used.

MDRD formula (using creatinine)

eGFR = 186 x (Creatinine / 88.4)$^{-1.154}$ x (Age)$^{-0.203}$ x (0.742 if female) x (1.210 if black)

CKD-EPI formula (using creatinine and cystatin C)

eGFR = 133 x min($S_{creatinine}$/0.8, 1)$^{-0.499}$ x max ($S_{creatinine}$/0.8, 1)$^{-1.328}$ x 0.996^{Age} x 0.932 (if female)

eGFR = 133 x min($S_{cystatin}$/0.8, 1)$^{-0.499}$ x max (S_{cys}/0.8, 1)$^{-1.328}$ x 0.996^{Age} x 0.932 (if female)

For the continous analyses, eGFR was calculated as decline in eGFR (ΔeGFR) over time by substracting follow up eGFR from eGFR during baseline and dividing by the follow-up time in years (i.e., annual decline of eGFR in ml/min/1.73 m^2).

ΔeGFR = <u>eGFR baseline – eGFR follow up</u>
        follow up time in years

Copeptin was then related to decline in renal function and development of incident chronic kidney disease. Incident CKD was defined by the use of three different cutoff levels of eGFR.

- **eGFR <60** ml/min/1.73 m^2 (CKD_60), subjects having prevalent CKD_60 at baseline were excluded.

- **eGFR <45** (CKD_45) ml/min/1.73 m^2 subjects with prevalent CKD_45 at baseline were excluded

- **eGFR <30** (CKD_30) ml/min/1.73 m^2, subjects with prevalent CKD_30 at baseline were excluded

Our primary analysis was made using the MDRD formula (CKD_60$_{MDRD}$, CKD_45$_{MDRD}$ and CKD_30$_{MDRD}$, respectively). In addition, the CKD-EPI formula of eGFR (CKD_60$_{CKD-EPI}$, CKD_45$_{CKD-EPI}$ and CKD_30$_{CKD-EPI}$, respectively) was also used.
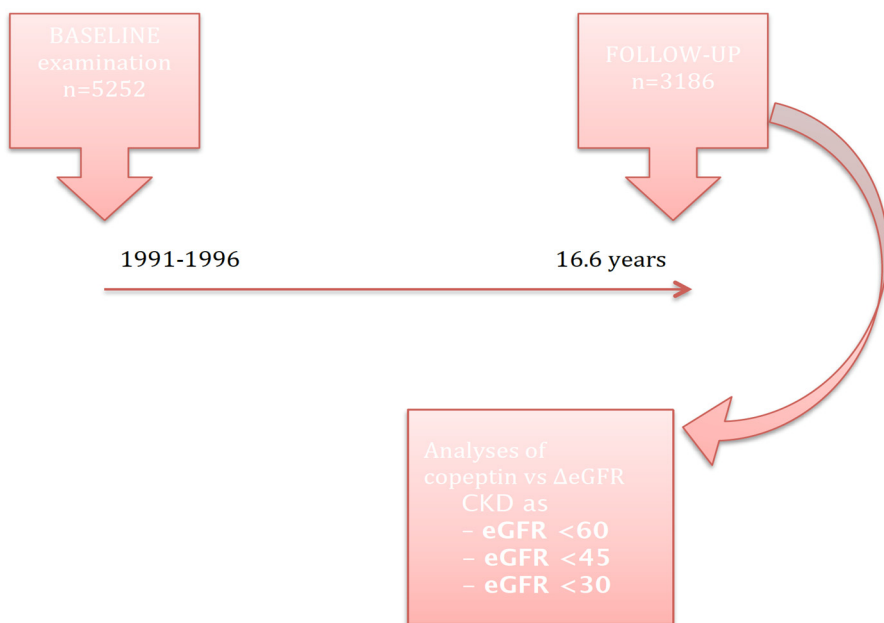
Figure 5: Method description paper III

In paper IV, 55 healthy subjects aged between 20-70 years were analysed. As described previously, the individuals were recruited via advertisement in local newspapers as well as telephone contact with individuals that had previously participated in the MPP and MDCS. The number of of subjects completing the study was 39 (71%), and 37 of those had complete data on plasma copeptin.

The intervention project was divided into two parts, one being a high intake water week (HWI-Wk) and one being a control week (CONT-Wk). During HWI-Wk, the study subjects were exposed to increased water intake during one week, and

42

during the CONT-Wk there was no change from usual fluid intake. The interventions were performed in a randomized order. In the beginning of the water week the study subjects were also exposed to an acute water load as described below.

During the HWI-Wk, each participant was instructed to ingest 3L water per day in addition to his/her own fluid intake. Here, the participants were provided with two bottles (1.5L+1.5L) of still water per day (10 mg/L sodium). During the CONT-Wk, the participants ingested their habitual water intake. In addition to this during day 1, the participants were instructed to, during a maximum time period of 20 minutes, acutely ingest either 1L of still bottled water (during the HWI-Wk) or only 10 ml of water during the CONT-Wk. Day 1 of HWI-Wk then continued with the additional 2L (in total 3L) water intake. The acute effects of water on copeptin were mapped by blood sampling for copeptin measurement every 30 minutes during a period of 4h after the acute intake of water.

Each subject participated in both HWI-Wk and CONT-Wk, thus serving as its own control. The intervention weeks were separated by three weeks of each subject's usual fluid intake as a wash-out period.

The absolute differences, or Δvalues, were calculated as the difference of variables between the end of CONT-Wk and the end of HWI-Wk. The Δvalues were analysed for fasting plasma copeptin, glucose, insulin and glucagon and osmolality. As these variables were measured fasting both on day 8 and 9, a mean value of those values was used. In addition, Δvalues of 120 min measurements during an OGTT between day 9 of CONT-Wk and day 9 of HWI-Wk, were calculated for glucose, insulin and glucagon. Finally, Δ values between habitual (CONT-Wk) and post intervention (HWI-Wk) urine osmolality and urine volume were calculated, based on the 24h urine collections returned on day 9.

HWI-WK

- Blood tests + height and weight
- 1.5+1.5L water given
- Acute 1L water ingestion
- 24h urine collection start
- 24 ABP

CONT-WK

- Blood tests + height and weight
- Acute 10 ml water ingestion
- 24h urine collection start
- 24 ABP

1 week

HWI-WK

- Blood tests + weight
- OGTT
- 24h urine collection end
- 24h ABP

CONT-WK

- Blood tests + weight
- OGTT
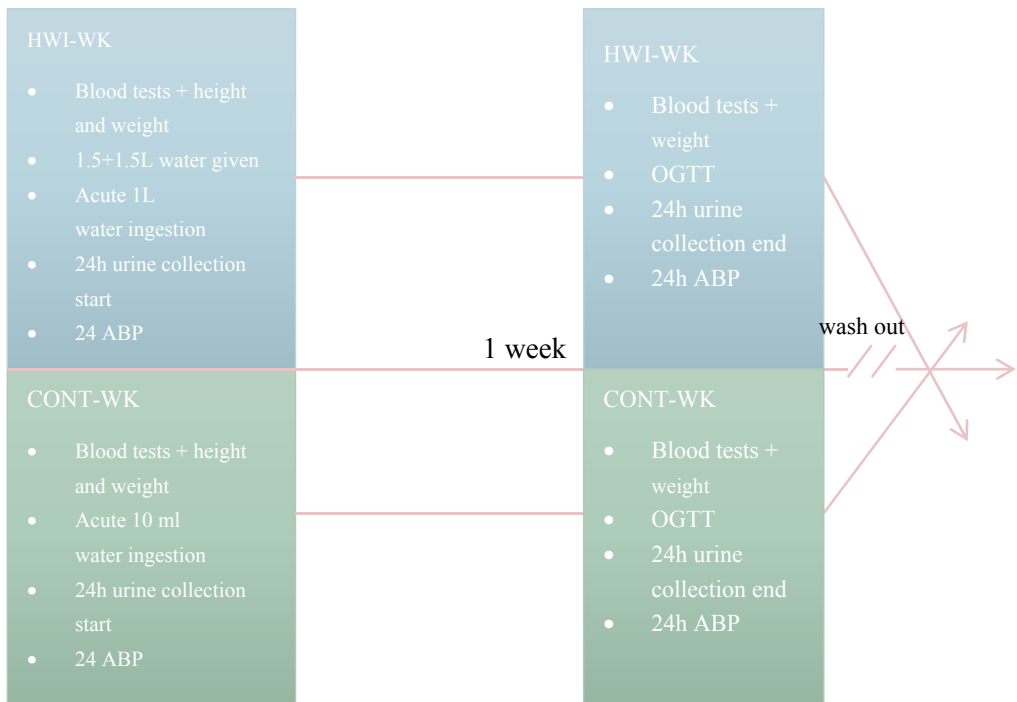- 24h urine collection end
- 24h ABP

wash out

Figure 6: Method description paper IV

Ethical considerations

For all the studies, the study protocols were approved by the regional Ethics Committee of Lund University and all the participants were provided written informed consent.

Clinical examination and laboratory assays

Blood pressure measurement

In papers I and IV, blood pressure measurements were obtained by an ABPM 90207 device (Spacelabs Medical Inc., Redmond, WA, USA). The device was applied on the left arm for 24h ambulatory blood pressure (ABP) measurement and depending on the arm circumference of the subject, two cuff sizes were used (24–32 cm and 32–42 cm, respectively). The study subjects were instructed to maintain the left arm relaxed along the body during each measurement. In studies two and three, blood pressure was measured using an oscillometric device twice after 10 min of rest in the supine position.

Laboratory measurements

In papers I and IV, the study subjects were instructed to sample urine in given containers for a 24h urine collection. The collection followed procedures developed at the Department of Endocrinology, Skåne University Hospital, and consisted of a comprehensible written instruction aimed at ensuring accurate and complete collection of urine. Laboratory tests included sodium measured in serum as well as in urine, by standard biochemical methods at the Department of Clinical Chemistry, Malmo University Hospital. Furthermore, in the fourth study, additional measurements of plasma urea, creatinine, potassium, glucose, glucagon, insulin and urine osmolarily were performed.

In the second and third study, the laboratory measurements included fasting serum total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, creatinine and cystatin C that were made according to standard procedures at the Department of Clinical Chemistry, University Hospital Malmö. Low-density lipoprotein (LDL) cholesterol was calculated according to Friedewald's formula. Plasma creatinine was analyzed with the Jaffé method and traceable to the International Standardization with isotope dilution mass spectrometry. The same

technique was used for measuring plasma creatinine at baseline and at the follow-up. Plasma cystatin C was measured using a particle-enhanced immunonephelometric assay (N Latex Cystatin; Dade Behring, Deerfield, Illinois) at baseline and follow-up. The values of cystatin C were not standardized because they were analyzed before the introduction of the world calibrator in 2010. The reference value for the method was 0.53-0.95 mg/l.

In paper IV, an oral glucose tolerance test (OGTT) was performed. Subjects ingested 75g of glucose over a maximum period of 3min, starting sometime between 7:30 and 9:00 AM (after an over-night fast, i.e. no meals or drinks after 10PM the evening before), followed by blood sampling for glucose and insulin measurement at 30, 60 and 120min and glucagon measurement at 120 min.

Finally, copeptin was measured in fasted EDTA plasma using a commercially available assay in the chemiluminescence/coated tube format (B.R.A.H.M.S AG, Hennigsdorf, Germany).

The participants' weight (kg) and height (cm) were measured by trained nurses.

Diabetes mellitus was defined as fasting plasma glucose ≥ 7.0 mmol/L, a self-reported physician diagnosis of diabetes or use of antidiabetic medication.

Cigarette smoking was elicited by a self-administered questionnaire, with current cigarette smoking defined as any use within the past year.

Statistics

All data were analyzed with SPSS statistical software. The distribution of copeptin was skewed to the right and therefore transformed using the natural logarithm.

In paper I, version 21, SPSS Inc., Chicago, IL, USA was used. Significance of differences of paired variables (i.e., changes induced by different levels of salt intake) was tested by paired t-test or Wilcoxon's paired rank test, where appropriate, whereas significance of differences between groups was tested with t-test. Pearson's test of correlations (r) was used to calculate correlations.

In paper II, copeptin was related to risk of development of CAD (first myocardial infarction or coronary revascularisation), cardiovascular and total mortality using multivariate adjusted Cox proportional hazards models. All models were adjusted for age, gender, systolic blood pressure, antihypertensive therapy, smoking, diabetes, LDL and HDL cholesterol. The proportional hazards assumption was met using weighted residuals. Two interaction terms (LN-transformed copeptin x diabetes status as well as LN-transformed copeptin x gender) were introduced on top of all other covariates to test for multiplicative interactions on risk CAD and the other study outcomes.

In paper III, copeptin was related to the decline in eGFR using multivariate adjusted linear regression models and to the risk of developing incident CKD by use of multivariate adjusted logistic regression. All models were adjusted for baseline level of eGFR, age, gender, systolic blood pressure, antihypertensive therapy, smoking, diabetes, LDL cholesterol, HDL cholesterol and follow-up time. Continuous variables are shown as means ± SD if normally distributed and as medians and interquartile range if skewed. Categorical variables are shown as numbers and percentages. SPSS statistical software version 22.0 (SPSS Inc., Chicago, Ill., USA) was used for all calculations.

Finally, in paper IV, using paired t-test or Wilcoxons sign rank test, depending on normality, the significance of differences between end of HWI-Wk and end of CONT-Wk, as well as differences in copeptin at different times after acute water loading compared to time 0min (pre-water load) was tested. Subjects were a posteriori divided into "water-responders" and "non water-responders" according to the amplitude of the copeptin decline (Δcopeptin) calculated as the difference in copeptin concentration at the end of CONT-Wk and the end of HWI-Wk. Water-

responders were defined as subjects in the top tertile of the copeptin decline. Significance of differences between these two subgroups was tested using independent sample t-test or Mann-Whitney U-test depending on normality. We used linear regression analysis of crude and serum albumin-corrected residuals between water-induced changes (end of HWI-Wk vs end of CONT-Wk) of copeptin versus changes of glucose, insulin and glucagon during the same time period. Here, SPSS statistical software version 23 (SPSS Inc., Chicago, Ill., USA) was used for all analyses.

A two-sided p value of <0.05 was considered statistically significant.

Results

In paper I, the change of copeptin during low respectively high salt intake is shown in table 2 (paper I). A positive correlation between increase in salt intake and levels of copeptin was seen in all subjects when analyzed together but only in females when analyzed separately. The opposite correlation was shown when analyzing the change in body weight (table 3, paper I) where increased body weight was associated with decreased levels of copeptin in all individuals (table 4, paper 1). Analyses of 24h urinary production did not show any significant correlation with copeptin concentration except when analyzing men separately, where a negative correlation with levels of copeptin was found.

The change of 24h ABP, i.e. systolic salt sensitivity, correlated inversely with copeptin when going from low to high salt intake, but the correlation was only seen in females (Table 5, paper I). Analyses of salt sensitivity and the change in body weight and 24h urinary production did not show any significant correlations.

Due to the gender differences, where significant results were obtained in females but not in males, we divided the women in two categories - premenopausal and postmenopausal. The intention was to analyze whether estrogens contribute to the sensitivity of copeptin [67]. A significant increase in levels of copeptin by increasing dietary salt intake was seen in premenopausal women (defined as age below 51)[68] whereas postmenopausal women did not show any significant correlations (table 6, paper 1). When comparing the salt-induced change of copeptin with salt sensitivity in premenopausal and postmenopausal women, respectively, no significant correlations were found. There was also no significant relationship when analyzing the correlation between salt sensitivity and saltinduced change of body weight as well as urinary volume neither in pre- nor in postmenopausal women (Table 7, paper I).

The 24 h urinary excretion of sodium indicated a good compliance to the high and low dietary salt intake (Table 1, paper I).

In paper II, at the baseline of the study, the mean age was 69 years, 69.8% were men, the prevalence of diabetes was 11.7% and the mean follow-up time was 5.6±1.4 years (table 1, paper II). In the population free from CAD at baseline, the analyses showed a 20 % increased risk of CAD per 1 SD increment of logtransformed copeptin (table 2, paper II). Dividing the study population into

quartiles of copeptin, the top quartile showed a 44 % increased risk of developing CAD compared with the reference quartile (table 2, figure 1, paper II). A borderline significant interaction was found between copeptin and diabetes on the risk of CAD (p=0.08). The association between elevated levels of copeptin and increased risk of CAD was significant in both the diabetic part of the population and in the non-diabetic part. However, the point estimate of the HR for CAD per 1 SD increment of log-transformed copeptin was nominally higher in diabetes subjects (1.49 (1.14 to 1.95); p=0.004) compared to the non-diabetic part of the population (1.15 (1.02 to 1.50); p=0.02).

A similar calculation dividing the population based on gender showed a significant interaction between copeptin and gender for outcome of CAD (1.35 (1.07 to 1.70); p=0.013). The females had an increased risk compared to the men; HR per 1 SD increase of copeptin in women was (1.51 (1.23 to 1.86); p<0.001) and in men (1.11 (0.98 to 1.26); p=0.089) (table 2, paper II).

Furthermore, a highly significant association between copeptin and increased risk of total mortality that was independent of cardiovascular risk factors (table 3, paper II) was found. When dividing the study population in quartiles, the relative risk of total mortality was >50% higher in the top when comparing to the bottom quartile of copeptin, with a significant trend over quartiles (p<0.001) (table 3 and figure 2, paper II). No significant correlation between total mortality and diabetes status was found (p=0.985), this in contrast to the CAD analysis that showed a borderline significant interaction. The HR for total mortality (95% CI) per 1 SD increment of copeptin was 1.22(1.03 to 1.45); p=0.023 in diabetic subjects and 1.32 (1.22 to 1.43); p<0.001 in non-diabetic subjects. Analyzing the links between copeptin and gender for outcome of total mortality, no significant interaction was found (1.11 (0.94 to 1.31); p=0.230). However, an analysis for total mortality stratified by gender showed significant results in both men and women with HR per 1 SD increase of copeptin in women of (1.43 (1.22 to 1.67); p<0.001) and (1.28 (1.18 to 1.39); p<0.001) of men (table 3, paper II).

Finally, we found that the association between copeptin and risk of total mortality was mainly driven by the cardiovascular mortality rate (table 4 and figure 3, paper II). Subjects belonging to the top quartile of copeptin had a 1.75-fold increase in risk of dying from a cardiovascular event when compared to the reference quartile (table 4, paper II). No significant interaction was found between copeptin and diabetes on the risk of cardiovascular mortality (p=0.154). However, when analyzing diabetic and non-diabetic subjects separately, the HR (95% CI) per 1 SD increment of copeptin was 1.39 (1.06 to 1.81); p=0.017 in diabetic subjects and 1.32 (1.15 to 1.51); p<0.001 in non-diabetic subjects. Furthermore, the gender stratified analyses of copeptin and cardiovascular mortality showed no significant interaction between copeptin and gender (p=0.256). When stratifying our

population by gender, we found copeptin to be significantly associated with cardiovascular mortality in both gender with HR per 1 SD increase of copeptin in women of (1.54 (1.17 to 2.03); p=0.002) and in men of (1.32 (1.16 to 1.50); p<0.001) (table 4, paper II).

In this study additional adjustments for BMI, DBP, PP, use of ACEi/ARBs, spironolactone, heart failure, and glucose were calculated and this did not change the results (data not shown). Lastly, there were significant (p<0.001) associations between copeptin and all risk factors that were adjusted for in all Cox regressions except for smoking even though the r values were generally small (r=0.079–0.197).

Copeptin positively correlated to age, systolic blood pressure, antihypertensive therapy and diabetes mellitus and negatively to HDL and LDL. In the population free from prevalent CAD (n=4873), 370 incident CAD events occurred during follow-up, and in the entire population (n=5386), 757 individuals died of whom 284 were cardiovascular deaths.

In paper III, at the baseline of the study, the mean age was 56.4 ± 5.7 and 39,8% were men. The mean SBP was 138 ± 18.1, the mean GFR_MDRD_at baseline was 74.7±14.6 and the mean GFR_CKD_EPI at baseline was 90.1±13.0 (table 1, paper III). We found that each SD increment of copeptin was significantly associated with a greater yearly decline of eGFR both according to the MDRD formula and CKD-EPI formula, (OR 0.057, 95% CI 0.022-0.093; p = 0.001) and (OR 0.050, 95% CI 0.022-0.077; p < 0.001) respectively (table 2, paper III). Analyses that was not adjusted for baseline eGFR gave similar results, MDRD formula (OR 0.046, 95% CI 0.007-0.085; p = 0.020) and CKD-EPI formula (OR 0.030, 95% CI 0.000-0.060; p < 0.05.

Each SD increment of copeptin independently predicted incident CKD_60_{MDRD} (OR 1.19, 95% CI 1.04-1.36; p = 0.010), CKD_45_{MDRD} (OR 1.33, 95% CI 1.04-1.71; p = 0.026) and CKD_30_{MDRD}(OR 3.69, 95% CI 1.41-9.66; p = 0.008), as shown in table 3, paper III. Dividing the study subjects into quintiles of copeptin, the top quintile showed a significantly increased risk of developing CKD_60_{MDRD} (OR 1.48, 95% CI 1.03-2.12; p = 0.032) when compared to the reference quintile (Q1), and there was a significant trend over quintiles (p = 0.009). The top quintile did not show any significant risk of developing CKD_45_{MDRD} but there was there was a significant trend across quintiles. The quintile analysis of copeptin in relation to $CKD30_{MDRD}$ could not be assessed due to lack of incident CKD_30_{MDRD} cases in the reference quintile (Q5).

Using the CKD-EPI formula to calculate eGFR in the continous analysis, copeptin did not significantly predict incident $CKD\_60_{CKD-EPI}$ (OR 1.09, 95% CI 0.99-1.19; p = 0.084). However, each SD increment of copeptin independently and

significantly predicted incident CKD_45$_{CKD-EPI}$ (OR 1.18, 95% CI 1.01-1.38; p = 0.043) as well as CKD_30$_{CKD-EPI}$ (OR 1.88, 95% CI 1.13-3.12; p = 0.015; table 4, paper 3). Dividing the study subjects inte quintiles of copeptin, there was an increased risk of developing CKD_60$_{CKD-EPI}$ in Q5 compared to the reference quintile as well as of developing CKD_45$_{CKD-EPI}$ in Q5 compared to the reference quintile (table 4; figure 1, paper III), and a significant trend over quintiles (p = 0.009) and (p = 0.028) respectively. Furthermore, the analysis of CKD_30$_{CKD-EPI}$ showed a significant trend over quintiles (p = 0.035), and subjects in the highest quintile group (Q5) had a borderline significantly increased risk of developing CKD_30$_{CKD-EPI}$ when compared to the reference quintile (table 4, supplementary figure 1, paper III).

As a last step in this study, a sensitivity analysis (CKD-EPI) was made near the studied threshold. Study subjects with eGFR >65 ml/min/1.73 m^2 at baseline were incorporated when studying eGFR <60, <45 and <30 ml/min/1.73 m^2 as end points (supplementary table 2, paper III).

During the 16-year follow-up time a number of 1172 (43.9%) subjects developed hypertension (diagnosed and treated) and 471 (15.7%) developed diabetes.

The number of excluded subjects, depending on which definition of prevalent CKD that was used, was 391 subjects if defined by CKD_60$_{MDRD}$, 19 if defined by CKD_45$_{MDRD}$, 1 if defined by CKD_30$_{MDRD}$, 39 if defined by CKD_60$_{CKD-EPI}$, 4 if defined by CKD_45$_{CKD-EPI}$ and 0 if defined by CKD_30$_{CKD-EPI}$.

In paper IV, the 37 study participants had a median age of 53 y (inter-quartile range 37-68) years. Nine were men and mean BMI was of 25.2 kg/m^2 (SD 4.4). We found that a rapid oral water load of 1L (acute test) resulted in a significant decrease in levels of copeptin within 30 min, reaching its maximum decrease at 90 min and a suppression that was sustained for at least 4h, meaning the entire duration of the test (figure 1, paper IV). The average recuction of copeptin was 39% (95CI 34-45%; p<0.001). In comparison, one week of increased water intake resulted in a 15% (95CI 5-25%; P=0.003) reduction of copeptin when compared to the control week (table 1, paper IV). In study subjects with indices of habitually lower water intake (mild dehydration) during the CONT-Wk, meaning subjects with higher habitual copeptin (r=0.63, p<0.001), higher habitual urine osmolality (r=0.62, p<0.001) and lower habitual urine volume (r=-0.52, p=0.001), a more pronounced reduction at the end of HWI-Wk when compared with the CONT-Wk was seen. The water responders, i.e. those belonging to the top tertile of water-induced copeptin reduction (n=12), had a greater decrease in copeptin of 41% (95CI 34–49; P<0.001) when compared to the non water-responders (n=25) that showed a non-significant reduction, 2.7% (95CI -8.3–14; P=0.61), table 2 paper IV. As above mentioned, there were three characteristics separating water-responders from non water-responders: habitually higher copeptin, higher urine

54

osmolality and lower urine volume. All of these characteristics are indices of having a less hydrated state (table 2, figures 3a and 3b, paper IV).

Continuing to the results of hydration and glucometabolic parameters there was over all no significant differences in glucose, insulin or glucagon at the end of HWI-Wk when compared to end of CONT-Wk (table 3, paper IV). Neither was there any significant correlation between change in copeptin (Δcopeptin) and Δglucose at 0 min (r=-0.08, p=0.6), Δglucose at 120 min (r=0.02, p=0.9), Δinsulin at 0 min (r=-0.15, p=0.39) or Δ insulin at 120 min (r=-0.16, p=0.36) of an OGTT. Adjusting for water-induced plasma albumin (as a proxy for water-induced plasma dilution) did not change the results.

However, we found a significant association between Δcopeptin and Δglucagon both at 0 min (r=0.37, p=0.03) and 120 min (r=0.39, p=0.02) of an OGTT when going from habitual water intake to high water intake. An analysis adjusted for plasma albumin concentration was also performed to make sure that the water-induced reductions of glucagon were not a result of volume expansion. After the adjustment, the correlations still remained significant (p=0.01 at 0 min and p=0.02 at 120 min of the OGTT).

Calculating the water-induced change of glucagon in a fasting state and at 120 min, the results differed significantly between water-responders and non water-responders (figures 3a and 3b, paper IV). In the water-responders, an increased water intake was followed by a significant reduction of glucagon during the fasting state (p=0.04) and a borderline significant glucagon reduction at 120 min of an OGTT (p=0.07). There was no such reduction found among the non water-responders (figures 2a and 2b, paper IV).

The habitual glucometabolic parameters did not significantly differ between the water-responders and non water-responders (supplemental table 1, paper IV).

Discussion

The main finding in paper I was that copeptin increased after high salt intake, but a high salt-induced change of copeptin was inversely correlated with degree of salt sensitivity. This phenomenon was only seen in females.

It has previously been shown that, in healthy subjects, copeptin is associated with components of the metabolic syndrome [40, 41] including hypertension and that copeptin independently predicts development of diabetes mellitus [1]. Since the question regarding a causal relationship of copeptin and previously mentioned endpoints remains unclear, it is of interest to investigate environmental stimuli that might alter levels of copeptin. Then, it can further be studied whether these environmental stimuli can ameliorate components of the metabolic syndrome by reduction or worsen them by stimulation. Since vasopressin release is stimulated by increased osmolality and hypovolemia, we found it interesting to investigate if increased salt intake, and thus increase in osmolality, could alter levels of copeptin, making salt the environmental stimuli of study interest.

We found that four weeks of controlled high salt intake, as compared to four weeks of controlled low salt intake, resulted in an increase of plasma copeptin levels. Based on this, one may suggest that salt-induced increase of osmolality is a more potent stimulus for AVP release than salt-induced increase of blood volume which would be expected to inhibit AVP release. If there is a causal relationship between AVP (measured by copeptin) and components of the metabolic syndrome, a high salt intake would have negative effects on these components.

Furthermore, as expected we found that decreased levels of copeptin were associated with an increase in urinary volume as well as change in weight, and vise verca when going from low salt intake to high. Since we did not have data on fluid intake, urinary production was used as an indirect measure of fluid intake and weight gain as an indirect measure of fluid retention. The inverse relationship is thought to be due to gradual supression of AVP release by an increase in water intake, assuming that the low levels of copeptin are a result of high water intake.

The role of salt-induced water intake and water retention in salt sensitivity is rather controversial, and we therefore tested if the change in copeptin with an increase in salt intake was associated with salt sensitivity. We found a significant association

among women only, suggesting that in women high water intake, water retention or both, may play a crucial role for salt sensitivity.

Summarizing, high levels of salt intake leads to increased levels of copeptin and in women only, salt sensitivity is associated with a decrease in copeptin presumably through an increase in water intake and/or water retention. We therefore believe that an increase in salt intake needs to be accompanied by an increase in fluid intake or fluid retention in order to reslut in elevated blood pressure in women. Since these results were neither found to be significant in men nor in postmenopausal women, it is suggested that there are different underlying mechanisms of salt sensitivity in the different groups.

In paper II, the main finding was that copeptin significantly and independently predicted development of incident CAD as well as total and cardiovascular mortality. In contrast to our previous finding in a middle-aged population in which the increased risk was only seen in diabetics, this study showed that both the diabetics and non-diabetics in the elderly (mean age 70 years) population had an increased risk, even though the diabetic part of the population had the highest risk. In the interaction analysis for the outcome of CAD, a trend towards an interaction between copeptin and diabetes was found, and the point estimate of the effect size for CAD was higher among diabetic individuals when compared with non-diabetic individuals. On the other hand, the association between copeptin, total and cardiovascular mortality appeared to be equally strong in both diabetics and non-diabetics. As previously shown, copeptin predicts new onset diabetes [1, 43] and is associated with several cardiometabolic risk factors [40, 41, 69]. Even though one could expect to find significant association with cardiovascular endpoints in both the diabetic and non-diabetic populations this has not been previously shown. One possible explanation for this is that increased levels of circulating vasopressin may specifically contribute to diabetes-related cardiovascular risk. Our findings regarding the outcome of CAD partially support this theory due to the trend found towards an interaction between copeptin and diabetes, at least suggesting a stronger effect among the diabetics. Furthermore, in this study, copeptin significantly predicted CAD, cardiovascular and total mortality both in subjects with diabetes and those without. This finding brings the term vascular ageing into discussion as a possible explanation for significant risk development in non-diabetics first once they become older, as compared to the diabetics who develop vascular ageing earlier. The hypothesis here is that the vascular age of the middle-aged diabetics resemble that of the older non-diabetics.

If AVP, manifesting with increased levels of plasma copeptin, is causally related to cardiovascular disease, this may accelerate atherosclerotic events once the vascular wall has become vulnerable to arterial stiffness and plaque formation either due to diabetes and/or ageing. It is not known if AVP, measured by

58

copeptin, only reflects underlying causes that in turn are responsible for cardiometabolic outcome, or if it is actually causally related to the outcome. Low water intake and dehydration are known potent stimulants of AVP release during normal condition and based on that, an increase in water intake is a potential candidate of preventive intervention in high-risk individuals such as middle-aged diabetics and elderly individuals. In addition, a low water intake has been associated with development of CVD risk factors, for example hyperglycemia [70] and abdominal obesity[41].

The current study thus encourages further studies to investigate if, or not, the adverse cardiometabolic risk profile is associated with high levels of copeptin, if the relationship is causal or not and whether it can be affected by increased water intake. It should however be mentioned that in subjects with subclinical conditions such as reduced left ventricular function as well as individuals with medical treatment that may affect AVP release or electrolyte disturbances, increased water intake may be harmful and should be evaluated carefully in this population. Also, subclinical reduction of left ventricular function may also explain part of the association between copeptin and our end points.

Furthermore, significant interaction between copeptin and female gender on incident CAD was shown, whereas no such gender interaction was observed regarding the outcomes of total and cardiovascular mortality. The results in men were not significant but there was a similar trend. Therefore, a possible gender difference in the relationship between copeptin and CAD merits further investigation in other studies.

The main finding in paper III was that in a healthy urban population, copeptin independently predicted decline in eGFR over time and development of new-onset CKD. The risk of CKD_60_{MDRD} was doubled when comparing the top quintile of copeptin with the reference quintile. Also, a French community-based study found high copeptin levels to be predictive of new onset CKD [71] making our study a replicate and extension of those findings in a Northern European population.

It is unknown if the found associations between high levels of copeptin, indirectly reflecting leves of AVP, and development of incident CKD, are causal. However, in the literature, evidence obtained from animal studies and human intervention studies of patients suggest that high AVP levels may in fact be causally related to impaired renal function. Infusion of V2R agonists in rats have been linked to proteinuria and increased mortality [2]. In line line with this, V2R antagonists in rat model of type 1 diabetes, have been shown to have beneficial effects on renal function [72]. In addition, in Brattleboro rats lacking AVP expression, and being partially nephrectomized, CKD is less likely to develop [2]. In the same rat model,

a three-fold increase in water intake ameliorated proteinuria and glomerulosclerosis [73]. This indicates that water-induced suppression of AVP may be of renal protective importance. In humans there are no known interventions studies of the general population. However, a study in patients with polycystic kidney disease,[74] showed that treatment with tolvaptan, being a V2R antagonist, resulted in a slower progress of cyst growth, reduced decline in eGFR and reduced change in total kidney volume when compared with the controls. In the analysis adjusted for baseline eGFR, the findings were not significantly changed and this argues against copeptin only being a filtration marker. Also, a study in normal rats and healthy humans showed that AVP increases urinary albumin excretion (a pathological state seen both in diabetes mellitus and hypertension)[75]. In this study, acute infusion of the V2R agonist dDAVP tripled urinary albumin excretion without changing creatinine and beta2-microgobulin excretion. The same study showed similar results in patients with central diabetes insipidus and in patients having aquaporin 2 mutation (heredetary nephrogenic diabetes insipidus). According to these results, the albuminuric effects seemed to result from increased glomerular leakage and required functional V2R, bringing additional support for involvement of AVP. Based on these findings, we can speculate that, presumably acting through V2R, AVP plays a role in the development of CKD making water intake or pharmacological AVP blockade interesting candidates for primary prevention of a decline in eGFR and development of CKD. In addition, high urine volume in adults with normal kidney function has been linked to slower decline in eGFR[76] and increase water intake has been linked to significant decrease in levels of copeptin in patients with CKD stage 3 [61].

Previously, increased levels of copeptin have been associated with faster decline in eGFR in a high-risk population, in this case patients with type 2 diabetes, for CKD development[58]. Together with recent data from a general French population [71] the current study extends the prior findings in these patients to be valid in a general population as well and include prediction of incident CKD. Copeptin may therefore identify high-risk individuals without well-known risk factors such as diabetes. When looking at the diabetic population, screening for microalbuminuria is initiated early being obligatory in the screening process because of the known risk development of CKD. Our current study highlights the potential value of screening those in the general population, without diabetes but with increased levels of copeptin, in interest of targeted primary prevention of CKD. We have previously shown that copeptin is related both to incidence of diabetes and hypertension and based on this we find it likely that part of the association between copeptin and CKD is mediated by new onset hypertension [40] and diabetes [41]. We thus believe that a more intense screening program with frequent measurements of renal function, and possibly more aggressive treatment

of hypertension (similar to the one applied in the diabetic population), may be motivated in individuals with elevated levels of copeptin, in order to prevent CKD.

The results of incident CKD differed depending on the cutoff level and wether the MDRD or the CKD-EPI formula was used. When looking at the constitution of the different formulas used, the MDRD was developed using data from patients with an average measured eGFR of 40 ml/min/1.73 m^2 making this formula less suitable for individuals with a eGFR >60 ml/min/1.73 m^2 as it results in an underestimate of the true GFR. However, in those who developed CKD, this formula increases the accuracy even though the population was healthy. In contrast, the other fomula used, CKD-EPI, was derived from a population that resembles the general population with a mean GFR of 68ml/min/1.73 m^2. Despite these differences between the equations, both of the formulas showed a significant decline of continuous eGFR in relation to baseline levels of copeptin as well as in the quintile analyses where similar trends were found.

We believe that part of the crude relationship between copeptin and eGFR is explained by the fact that copeptin is renally excreted. Further we emphasize that the known correlation between copeptin and eGFR is partially explained by copeptin being excreted by the kidneys. In order to extract the proportion of variance in copeptin explained by differences in kidney function at baseline we adjusted the analyses for baseline eGFR and still found that the decline in eGFR was independent of baseline eGFR.

The main finding of paper IV was that a significant reduction of copeptin after an increased water intake during one week, as compared with habitual water intake, was seen in 37 healthy study subjects. During the acute water ingestion test, a 40% reduction of copeptin was observed and the effect was sustained at least over 4h. However, the copeptin lowering effect varied substantially between the subjects. The one third of the population that had the greatest copeptin reduction (water responders) were characterized by indices of relatively low habitual water intake. Looking at our second endpoint, the glucometabolic parameters, the increased water intake during one week did not alter glycemia, insulin or glucagon concentrations when compared to habitual concentrations, i.e. concentrations at the end of control week. However, in water-responders increased water intake led to a significant reduction of fasting glucagon concentration when compared to non water-responders.

We, among others, have previously shown that AVP, measured by copeptin, is an independent risk factor for diabetes, the metabolic syndrome, CAD, CVD, CKD and premature death in the population [1, 77, 78]. It is also well established that certain diseases such as heart failure, acute myocardial infarction, hemorrhage and sepsis result in elevation of copeptin [79-82], although in the general population the most likely cause of having elevated copeptin is a low water intake. Based on

the several reports showing an independent relationship between elevated levels of copeptin and risk of cardiometabolic diseases, the potential usage of increased hydration as a preventive strategy for these diseases has acquired increasing interest. In animal models, increased hydration has shown beneficial effects on glucose metabolism through AVP reduction and the opposite effect, that elevation of AVP deteriorate glucose tolerance, has also been observed [83], pointing at a likely causal relationship. Furthermore, a recent study provided additional support of causality when showing that a genetic variation in the human AVP gene was associated with both elevated copeptin and increased risk of hyperglycemia[84]. If the relationship is indeed causal, it would open the possibility of water intake-based metabolic intervention. However, even if this is the case, it is not known to what extent an increased water intake is instrumental in decreasing AVP secretion. Based on this, the first aim of paper IV was to test if, and to what extent, copeptin can be reduced in healthy humans by increased water intake both acutely and over one week. A study in younger subjects has previously demonstrated the acute effect of a large oral water load on copeptin [85]. Our study extends the findings and importantly shows from a therapeutic point of view that the copeptin reduction was sustained throughout the 4h of the test and, judging by the shape of the curve, the effect probably lasted even longer (figure 1 paper IV). This finding suggests that water does not have to be continuously ingested to achieve sustained reduction of copeptin, but that the same amount can be drunk during a short period of time (<20min) with a sustained effect over at least 4h.

In the analysis of the effect of increased water intake on copeptin over one week, a comparision between copeptin after HWI-Wk and CONT-Wk was made. Even though the study subjects were instructed to ingest the 3L water on top of their habitual water intake, the difference in 24h urine volume after the two periods suggests that the achieved difference in water intake was close to 2L per day (table 1, paper 4). It therefore seems likely that when adding 3L of water, the habitual intake decreases.

The average reduction of copeptin of 15% was largely driven by the water-responders, who showed an average copeptin reduction of 41% (table 2, paper IV). The water-responders are thus the individuals that would benefit the most from increased water intake, if used therapeutically in order to decrease vasopressin AVP levels. To identify these individuals, we compared measures of hydration during habitual water intake (end of CONT-Wk) between water-responders and non water-responders (table 2, paper IV). This analysis showed that water-responders were characterized by higher copeptin, higher urine osmolality and lower urine volume, all being indices of a relatively lower water intake. Based on this, intervention studies aiming at lowering AVP by increasing water intake should preferably focus on the one-third of the population with high copeptin and

low water intake in order to target those at risk of developing cardiorenal disease and catch the responders in order to intervene.

As previously mentioned, copeptin has been repeatedly shown to be a strong independent risk factor for diabetes development. Therefore, one of our aims was to study was to analyze the relationship between water induced reduction of copeptin and glucometabolic parameters, or more specifically glucose, insulin and glucagon concentrations. We did not observe any difference in these metabolic parameters at the end of the HWI-Wk when compared to the end of the CONT-Wk. One may speculate that one week of water intervention may be a too short period to observe metabolic alterations reflected by these measures. Another possibility is that the study was underpowered to detect an existing effect. On the other hand, as an additional explanation for the default effects, we may speculate that the metabolic effects from increased water intake are only seen in subjects whose copeptin is in fact prominently reduced by increased water intake, i.e., in water responders. In contrast to the above neutral findings, glucagon was indeed significantly reduced by hydration in water-responders during fasting state and borderline significantly reduced after the oral glucose challenge (figures 3a and 3b, paper IV). Even though this finding needs replication, it suggests that during only one week of increased hydration of subjects with habitual low water intake, a marked reduction of AVP is observed which is paralleled by a reduction of glucagon secretion.

Previous studies have shown that high glucagon secretion is an important risk factor for impaired glucose tolerance and type 2 diabetes [86]. Type 2 diabetes is furthermore associated with increased glucagon concentration throughout the day [87] and both type 2 diabetes and impaired glucose tolerance are associated with impaired suppression of glucagon secretion [88, 89]. It is also shown that increased levels of glucagon secretion manifest long before the onset of impaired glucose tolerance [89]. By activation of $V_{1b}R$ in α-cells of pancreatic islets [18] AVP stimulates glucagon secretion, an effect that is concordant with our finding that fasting glucagon concentration was reduced upon water-induced suppression of copeptin. Additionally, in rodent models, we have found that during conditions of high AVP, selective pharmacological blockade of V1bR with SR149415 resulted in reduction of plasma glucagon [90]. These findings, together with our current finding that water induces reduction of copeptin and glucagon in water-responders, encourage long-term studies of anti-diabetic effects of water supplementation in subjects with low water intake.

Futhermore, we previously showed that the risk of future diabetes development among normoglycemic subjects was 3.5-fold in the top quartile when compared to the bottom quartile of fasting plasma copeptin concentration, despite adjustment for known diabetes risk factors. The top quartile had levels of copeptin of >6.1

pmol/L in females and >10.7 pmol/L in males [1]. In the current study population, a 23% of the subjects had habitual fasting plasma copeptin concentration above this threshold, denoting high diabetes risk, and 89% out of these high diabetes risk subjects, were water-responders. Taking this finding into account, we suggest that approximately 25% of the population would represent an ideal target group for studying the effects of water supplementation on diabetes risk. The majority of these subjects, apart from having a high diabetes risk, are also water-responders with low habitual water intake.

Lastly it should also be mentioned that the AVP action of urea transporters combined with a low urine flow are factors known to increase the reabsorption of urea along the collecting ducts, leading to an increase in plasma urea [91, 92]. This likely explains the significantly lower urea concentration observed after the HWI-Wk than after the CONT-Wk (table 1, paper IV) meaning an improvement in fractional excretion of urea occuring when urine flow rate increases. By showing water loweing effects of copeptin mainly in the water-responders, who also are at increased risk of cardiorenal disease, and suppression of glucagon in subjects that are at risk for developing diabetes, the studied population represents an ideal target for further studies. By selecting the subjects at risk, further studies can focus on a long duration randomized controlled intervention trial testing the effects of hydration on cardiometabolic outcomes. In addition, a recent study on healthy younger individuals showed that levels of copeptin are positively associated with 24h urine osmolality, and in individuals consuming low-to-moderate fluid intake, a 6-week water intervention reduced circulating levels of copeptin[93]. The ideas for extending this knowledge are presented in the chapter "future research".

Summarizing the four papers included in this thesis, we have shown that in the first three copeptin is independently associated with salt sensitivity in women, development of CAD as well as CV and total mortality in both diabetics and non-diabetics and lastly that increased levels of copeptin are associated with a decline in eGFR and development of CKD. By showing the risk development of the different endpoints in relation to the AVP system, indirectly measured as copeptin, it is of primary interest to capture the high-risk individuals in order to provide optimal primary preventive interventions. This does not only account for offering early screening methods or more aggressive therapeutic goals of components of the metabolic syndrome but also makes a new field of non-medical treatment of great interest. By inhibiting powerful stimuli of AVP release with an increase in water intake we are getting more knowledge of how to and whom we should treat in order to apply primary prevention.

Conclusions

Paper I: Investigated if copeptin is related to salt senisitivity (i.e increase in systolic blood pressure).

We can here conclude that

- Increase in dietary salt consumption significantly correlates with the increase in levels of copeptin in all individuals when calculated together but only in females when calculated separately.
- Levels of copeptin inversely correlate with urinary volume (as an indirect measure of fluid intake)
- Levels of copeptin inversely correlate with change in body weight (as an indirect measure of fluid retention).
- Copeptin can be a useful marker for the amount of fluid consumption as well as fluid retention.
- Taking the gender difference into account, we also hypothesize that estrogens are contributing factors to the degree of salt sensitivity.

Paper II: Investigated if copeptin is related to development of indicent CAD, cardiovascular mortality and total mortality rates.

We can from this study conclude that
- Increased levels of copeptin are related to development of CAD, cardiovascular mortality and total mortality rates with subjects belonging to the top quartile of copeptin had a >70% increased risk of dying from cardiovascular disease when compared to the bottom quartile.
- The results show an increase in risk in both diabetics and non-diabetics.
- If the associations between copeptin and poor cardiovascular outcome are causal, interventions targeted at the vasopressin system appear as interesting candidates for primary prevention of CAD both in diabetics and in older, healthy individuals.

Paper III: Investigated if elevated levels of copeptin are related to decline in renal function and development of chronic kidney disease, in a middle-aged population.

Our analyses show that
- Increased levels of copeptin independently and significantly predict a decline in eGFR and development of chronic kidney disease in the population with a previous eGFR of more than 60 ml/min/1.73 m^2 at baseline.

- Our data highlights the potential role of copeptin as a biomarker in primary prevention of CKD and can thus be used to identify high-risk individuals in order to apply early preventive interventions.

Paper IV: Investigated if copeptin can be suppressed by increase in water intake and if so, glucometabolic parameters can be affected as well.

We can here conclude that
- High water intake acutely results in a significant reduction of copeptin. This is an effect that is sustained over at least four hours.

- During one week of intervention, the water reduction of copeptin was more pronounced in subjects with habitually high copeptin levels (and thus inreased risk of previously studied endpoints as well as diabetes) and signs of low water intake (i.e. water-responders).

- The water-induced recution of copeptin is about 40% but fasting glycemia and insulin are not changed by one week of high water intake.

- Water-responders show reduced concentrations of fasting glucagon.

Limitations

There are several limitations of our studies to acknowledge. Firstly, when looking at paper I, the major limitation of this study is the small number of subjects included, especially when looking at the gender stratified analyses. The study needs replication, not to overestimate the magnitude of the presented associations. However, the salt sensitivity testing was performed under controlled conditions including a double-blinded rancomized procedure and 24h ABP was used to measure blood pressure, resulting in a more exact blood pressure measurement.

Secondly, in paper II, our study population is likely to be healthier than the general population resulting in a healthy cohort effect. Our study population is thus not fully representative when comparing with the average population of the corresponding age, since our population is still alive from the initial MPP examination 1974–1992. When analyzing the results, we adjust for several factors but measure of renal function is not one of them because of absence of this data in our cohort. On the other hand, in our previous study [44], this adjustment did not affect the association between copeptin and cardiovascular endpoints. Furthermore, we state in the discussion part of paper II that the significant relations between copeptin and outcomes in diabetics and non-diabetics partially can be explained by vascular ageing. We need to highlight that this statement is only a hypothesis since vascular ageing has several definitions. Also, when comparing diabetics and non-diabetics we have to keep in mind that the diabetic population is small (12%) and we are therefore underpowered to rule out non-significant interactions in the calculated endpoints.

When comparing the limitations of paper II and paper III, there are some similarities. The healthy cohort effect is applicable in both of these papers. As the study populations had a participation rate of 71% in MPP (paper II) and only 40% in MDCS (paper III) they can be assumed to consist of healthier individuals than the general population. Also, we do lack data on confounding variables such as dietary data, salt and water intake as well as albuminuria that is required for the diagnosis of CKD stages one and two. As both paper II and paper II are observational studies, we do not know if the associations between copeptin and the examined endpoints reflect a causal relationship or not.

Lastly, in paper IV, we did not control the participants' food intake. As shown in the results, the osmolar excretion was significantly higher at the end of HWI-Wk when compared to the end of CONT-Wk. The explaination of this observation is thought to result from a greater food intake when the hydration is increased. Similar observations have been made in previous rat studies where the rats tend to eat more when they drink more[73, 94]. If the food intake did not increase, it is possible that the copeptin levels would be more suppressed. On the other hand, there was no difference in osmolar excretion rate between the water-responders and non water-responders as was the case at the end of the study period. Thus, the glucagon-lowering effect seen in water responders is unlikely to result from from differences in the amount of food ingested.

Future research

The studies of this thesis have shown that increased levels of AVP, measured as copeptin, are associated with salt sensitivity in females and increased risk of cardiorenal disease both in a diabetic population and an elderly, non-diabetic population. We have also shown that levels of copeptin can be reduced by increased water intake in water responders, making increased hydration an interesting candidate as an early preventive tool.

Now we need more knowledge of how much water is appropriate to ingest and which individuals that benefit from this the most. In paper IV we partially answer these questions, but to have a more solid ground we intend to do a long-term analysis of increased hydration in study subjects. Firstly, a pilot study of 6 weeks of water intervention will be performed and studied. Secondly, a parallel-group randomized controlled trial (RCT) with two arms will be performed during 12 months. Subjects will be randomized either to the water-intervention (0.5 L x 3 daily on the top of habitual intake) or to control group. Both groups will receive general life style advice (general oral and written advice on diet and physical activity) and water (bottled Evian water) will be provided to participants in the active treatment arm. Both the pilot study and the main study are designed the same way.

As previously mentioned, there are several animal studies pointing towards a causal relationship between the AVP system and disease development but no such relationship has been shown in humans. By studying human genetics, we intend to get closer to the answer of wheter there is a causal relationship or not. The data collection process in ongoing and currently we have collected genome-wide association study data from approximately 20 000 individuals with copeptin measured (the goal is 25 000 individuals).

Lastly, by specializing in the field of anesthesiology and intensive care, but yet keeping the AVP system close to heart, it would be interesting to start a project on AVP in this field of specialty. As for now, AVP is widely used, therapeutically, in acute settings. However, long-term effects of elevated levels of copeptin, indirectly reflecting levels of AVP, have shown disadvantageous effects. Ristagno et.al showed that increased levels of copeptin correlate with organ dysfunction in intensive care setting [95] and another study recently showed that elevated levels of copeptin at ICU admission are associated with increased 30-day mortality[96].

Data on copeptin and glucometabolic effects during acute settings and its long-term effects are lacking and this field is of special interest to study in the future.

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