

**Water and salt:
from renal mechanisms
to clinical disorders**

ISBN 978-90-9021476-4

Hoorn, Ewout Jasper.

Water and salt: from renal mechanisms to clinical disorders.

Thesis, Erasmus University Rotterdam, The Netherlands.

© E.J. Hoorn 2007

All rights reserved. No part of this thesis may be reproduced or transmitted,
in any form or by any means, without prior written permission by the author.

Cover : Maaïke Koning

Lay-out : Ewout Hoorn

Printing : Haveka B.V., Alblasterdam

**Water and salt:
from renal mechanisms to clinical disorders**

**Water en zout:
van mechanismen in de nier tot klinische ziektebeelden**

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus
Prof.dr. S.W.J. Lamberts
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 28 februari 2007 om 13.45 uur

door

Ewout Jasper Hoorn

geboren te Meppel

Promotiecommissie

Promotor: : Prof.dr. W. Weimar

Overige leden : Prof.dr. A.H. Danser
Prof.dr. J. Lindemans
Prof.dr. J.H.P. Wilson

Copromotor : Dr. R. Zietse

The work presented in this thesis was performed at the Department of Pediatric Critical Care Medicine, The Hospital for Sick Children, University of Toronto, Toronto, Canada; in the Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, United States of America; and at the Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands.

Financial support by the Dutch Kidney Foundation and the Dutch Diabetes Research Foundation is gratefully acknowledged.

Financial support was also kindly provided by Amgen B.V., Astellas Pharma B.V. (producent van o.a. Prograft®), AstraZeneca B.V., Baxter B.V., Boehringer Ingelheim B.V., Bristol-Myers Squibb B.V., B. Braun Medical B.V., Cablon Medical B.V., Ferring Nederland, Fresenius Medical Care Nederland B.V., Genzyme Nederland, Janssen-Cilag B.V., LEO Pharma B.V., Menarini Farma Nederland, Merck Sharp & Dohme B.V., Novartis Pharma B.V., Novo Nordisk Farma B.V., Nycomed, Pfizer B.V., Roche Nederland B.V. en Servier Nederland Farma B.V.

*“Profundity must smile” – Thomas Mann
(Lotte in Weimar, 1939)*

Contents

<i>Chapter 1</i>	General introduction and aims of the thesis	11
------------------	---	----

Part A: Renal mechanisms of water and sodium balance regulation

<i>Chapter 2</i>	The application of DIGE-based proteomics to renal physiology. <i>Nephron Physiol</i> 2006	39
------------------	---	----

<i>Chapter 3</i>	Combined proteomics and pathways analysis of collecting duct reveals a protein regulatory network activated in vasopressin escape. <i>J Am Soc Nephrol</i> 2005	69
------------------	---	----

<i>Chapter 4</i>	Prospects for urinary proteomics: exosomes as a source of urinary biomarkers. <i>Nephrology (Carlton)</i> 2005	101
------------------	--	-----

<i>Chapter 5</i>	Hypokalemia in a mouse model of Gitelman's syndrome. <i>Am J Physiol Renal Physiol</i> 2006	125
------------------	---	-----

<i>Chapter 6</i>	Osmomediators natriuresis in humans: evidence for salinity sensing in the thick ascending limb. <i>Submitted</i>	147
------------------	--	-----

Part B: Clinical disorders of water and sodium balance

<i>Chapter 7</i>	Diagnostic approach to a patient with hyponatraemia: traditional versus physiology-based options. <i>QJM</i> 2005	165
------------------	---	-----

<i>Chapter 8</i>	Development of severe hyponatraemia in hospitalized patients: treatment-related risk factors and inadequate management. <i>Nephrol Dial Transplant 2006</i>	195
<i>Chapter 9</i>	Novel risk factors for hospital-acquired hyponatraemia: a matched case control study. <i>Clin Endocrinol (Oxf) 2007</i>	221
<i>Chapter 10</i>	Acute and concomitant deterioration of hyponatremia and renal dysfunction associated with heart and liver failure. <i>Clin Nephrol 2006</i>	245
<i>Chapter 11</i>	Hypernatremia in critically ill patients: too little water and too much salt. <i>Submitted</i>	265
<i>Chapter 12</i>	Preventing a fall in the effective plasma osmolality to minimize the likelihood of cerebral edema during therapy of children with diabetic ketoacidosis. <i>J Pediatr 2007</i>	291
<i>Chapter 13</i>	Acute hyponatremia related to intravenous fluid administration in hospitalized children: an observational study. <i>Pediatrics 2004</i>	313
<i>Chapter 14</i>	How to select optimal maintenance intravenous fluid therapy. <i>QJM 2003</i>	333
<i>Chapter 15</i>	Summary and conclusions (English)	363
	Summary and conclusions (Dutch)	372
	Future directions	384
	List of publications	392
	Acknowledgments and curriculum vitae	394

Abbreviations

2-DE	- two-dimensional electrophoresis
ACE	- angiotensin converting enzyme
ADH	- antidiuretic hormone (also: vasopressin)
ANP	- atrial natriuretic hormone
APACHE	- Acute Physiology and Chronic Health Evaluation
AQP	- aquaporin
ARB	- angiotensin receptor blocker
AVP	- arginine vasopressin (also: vasopressin)
BNP	- B-type natriuretic peptide
CaSR	- calcium sensing receptor
CDA	- clinical diagnostic algorithm
CDI	- central diabetes insipidus
CE	- cerebral edema
CNS	- central nervous system
CO ₂	- carbon dioxide
CRP	- C-reactive protein
DIGE	- Difference Gel Electrophoresis
DKA	- diabetic ketoacidosis
EC ₅₀	- 50% effective concentration
ECF	- extracellular fluid
EFW	- electrolyte free water
ENaC	- epithelial sodium channel
FE _{Ca}	- fractional calcium excretion
FE _{Mg}	- fractional magnesium excretion
FE _{Na}	- fractional sodium excretion
FHH	- familial hypocalciuric hypercalcemia
GCS	- Glasgow Coma Scale

GFR	- glomerular filtration rate
H ₂ O	- water
HS	- hypertonic saline
ICF	- intracellular fluid
ICU	- intensive care unit
IMCD	- inner medullary collecting duct
IPA	- Ingenuity Pathways Analysis ®
IS	- isotonic saline
IV	- intravenous
K ⁺	- potassium
LC	- liquid chromatography
MALDI	- matrix assisted laser desorption ionisation
MR	- mineralocorticoid receptor
MS	- mass spectrometry
MVB	- multivesicular body
Na ⁺	- sodium
NCC	- sodium chloride co-transporter
NDI	- nephrogenic diabetes insipidus
NKCC2	- sodium potassium chloride co- transporter type 2
NSAID	- non-steroidal anti-inflammatory drugs
ODS	- osmotic demyelination syndrome
PAGE	- polyacrylamide gel electrophoresis
P _{ADH}	- plasma antidiuretic hormone concentration
P _{Ca}	- plasma calcium concentration
P _{Eff osm}	- effective plasma osmolality
P _{Glucose}	- plasma glucose concentration
P _{Mg}	- plasma magnesium concentration

P_{Na}	- plasma sodium concentration (also: serum sodium concentration)
P_{Osm}	- plasma osmolality
PMF	- peptide mass fingerprinting
PTH	- parathyroid hormone
RAS	- renin angiotensin system
RD	- renal dysfunction
SIADH	- syndrome of inappropriate antidiuretic hormone secretion
S_{Creat}	- serum creatinine concentration
S_K	- serum potassium concentration
S_{Na}	- serum sodium concentration (also: plasma sodium concentration)
SNS	- sympathetic nervous system
SSRI	- selective serotonin reuptake inhibitors
S_{Urea}	- serum urea concentration
TAL	- thick ascending limb
TOF	- time of flight
U_{Creat}	- urine creatinine concentration
U_K	- urine potassium concentration
U_{Na}	- urine sodium concentration
U_{Osm}	- urine osmolality
V2R	- vasopressin 2 receptor

Chapter 1

**General introduction
and aims of the thesis**

General introduction

The regulation of water and sodium balance is crucial to the survival of mammals. Therefore, several homeostatic mechanisms have evolved to maintain water and sodium balance within narrow limits. The kidneys play a central role in water and sodium balance regulation, and do so by interacting with various endocrine, nervous, and hemodynamic systems. Although several interconnections exist, water balance regulation and sodium balance regulation can be seen as two separate systems. Central to the regulation of water balance is the sensing of the plasma osmolality by osmoreceptors, and it is therefore often referred to as “osmoregulation”. Similarly, the regulation of sodium balance depends on the sensing of the effective circulating volume, and is therefore referred to as “volume regulation”. Before discussing these two systems and their components, a brief description is given of how the kidney tubules and collecting ducts are designed to execute their task of water and sodium balance regulation.

I. Transport mechanisms in the renal tubule and collecting duct

The kidneys consist of nephrons, collecting ducts, and a unique microvasculature. The human kidney contains approximately one million nephrons, and each nephron is divided into a glomerulus and a tubule. The glomerulus is a tuft of capillaries that functions as a filter and forms an ultrafiltrate from plasma. In the tubule and the collecting duct, reabsorption and secretion of fluid and solutes occurs based on the needs of the body, after which pre-urine is formed and collected in the bladder. To be

able to perform these transport functions, the renal tubule and collecting duct cells are polarized, meaning that the membrane facing the tubular fluid has different properties from the membrane facing the blood [1]. All tubular transport mechanisms depend on the activity of the basolateral $\text{Na}^+\text{-K}^+\text{-ATPase}$. This primary active transport mechanism facilitates other forms of transport in renal cells by maintaining a low intracellular sodium concentration and thereby establishing a favorable electrochemical gradient. The other forms of transport include carrier-mediated diffusion, diffusion through a membrane channel (or pore), and, finally, passive diffusion.

The renal tubule can be divided into different segments, each with specialized transport characteristics, and includes the proximal tubule, the loop of Henle, and the distal convoluted tubule [2]. Figure 1 shows the nephron and collecting duct system and also gives an overview of the major transporters expressed along the renal tubule and collecting duct. Figure 2 shows the cell models of the proximal tubule, the thick ascending limb (which is part of the loop of Henle), the distal convoluted tubule, and the collecting duct.

The proximal tubule reabsorbs the bulk of the filtered water and sodium, and also plays an important role in the reabsorption of glucose, uric acid, phosphate, and amino acids.

Nephron segments including apical water, sodium, and potassium channels

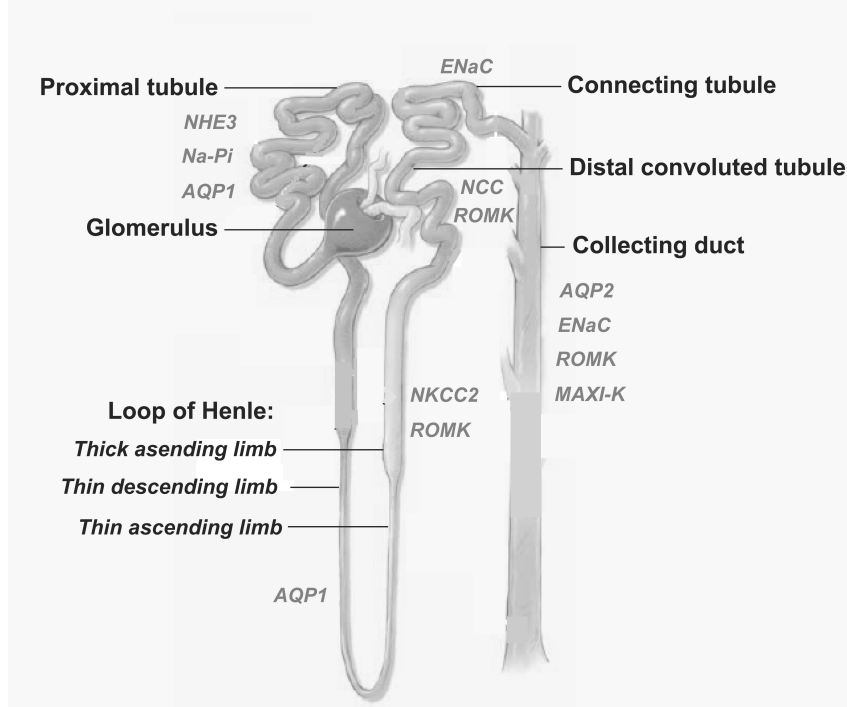


Figure 1: The nephron segments are depicted (black) including their apical water, sodium, and potassium channels (grey). For abbreviations, see text.

The loop of Henle consists of the thin descending limb (water permeable due to aquaporin-1 expression), thin ascending limb (water impermeable), and thick ascending limb (water impermeable). The thick ascending limb plays an important role in active sodium reabsorption and expresses the furosemide-sensitive $\text{Na}^+\text{-K}^+\text{-Cl-cotransporter 2}$ (NKCC2) and the renal outer medullary potassium channel (ROMK) [3]. Transport in the thick ascending limb is regulated by a number of circulating

hormones, including vasopressin (also: antidiuretic hormone), parathyroid hormone, and calcitonin. In addition, the thick ascending limb expresses the calcium sensing receptor, which also regulates transport by NKCC2 and ROMK. The thick ascending limb also contains the macula densa, which is part of the juxtaglomerular apparatus. The juxtaglomerular apparatus further comprises the extraglomerular mesangium, and the terminal portion of the afferent arteriole. The macula densa is made up of specialized cells that are believed to be capable of sensing the chloride concentration in tubular fluid. This mechanism, which is called tubuloglomerular feedback, allows the adjustment of the tone of the glomerular arterioles and thereby the glomerular filtration rate. The juxtaglomerular apparatus determines the amount of renin that is released into the systemic circulation, thereby regulating the activity of the renin-angiotensin-aldosterone system [4].

The distal convoluted tubule also plays an important role in active sodium reabsorption and expresses the thiazide-sensitive $\text{Na}^+\text{-Cl}^-$ co-transporter (NCC), and is regulated by the mineralocorticoid aldosterone [5].

The collecting duct system includes the connecting tubule and the collecting ducts [2]. This part of the nephron plays an important role in water balance regulation. To do so, the collecting duct is sensitive to vasopressin, which can activate the vasopressin 2 receptor (V2R). Activation of V2R stimulates an intracellular signaling cascade resulting in the insertion of the water-channel aquaporin-2 (AQP2) into the luminal membrane [6]. The collecting duct is also sensitive to aldosterone, which stimulates active sodium reabsorption and potassium secretion.

These effects are mediated by activation of the mineralocorticoid receptor (MR), which regulates the amiloride-sensitive epithelial sodium channel (ENaC) [7]. The MR-ENaC and V2R-AQP2 pathways are both expressed by the renal collecting duct principal cells (Figure 2). The other cell type in the collecting duct is the intercalated cell, which plays an important role in acid-base balance. As the last functional unit of the kidneys, the collecting duct plays an important role in the fine-tuning of fluid and electrolyte and acid-base balance.

Legend Figure 2: Cell models of tubular transport

Cell models of tubular transport in the proximal tubule, thick ascending limb, distal convoluted tubule, and collecting duct are shown. Intracellular pathways are shown schematically including the major regulatory proteins of the renal transporters. Finally, drugs that inhibit specific renal transporters (mostly diuretics) are shown.

Abbreviations: AC, adenylate cyclase; AQP, aquaporin; CaSR, calcium sensing receptor; ENaC, epithelial sodium channel; MR, mineralocorticoid receptor; NBC, sodium bicarbonate co-transporter; NHE3, sodium hydrogen exchanger type 3; NKCC2, sodium potassium chloride co-transporter type 2; PKA, protein kinase A; ROMK, renal outer medullary potassium channel; Sgk, serum and glucocorticoid regulated kinase; V2R, vasopressin 2 receptor; WNK, with no lysine.

The discovery of the renal tubular transport mechanisms described above was greatly facilitated by the development of several techniques, including electrophysiological methods (Ussing and colleagues), and the isolated perfused tubule technique (Burg and colleagues). These early renal physiologists described the transport characteristics of the different nephron segments with great detail, without knowing the responsible transport proteins. In the molecular era, the discovery of the first water-channel aquaporin-1 by Peter Agre in 1992 answered the long-standing question how water moves through plasma membranes, and was rewarded with the Nobel Prize in chemistry in 2003 [8]. The concept of specialized transport proteins led to the further discovery and cloning of the major water, sodium, and urea transporters (Boron, Hebert, Hediger, Lingrel, Rossier, Sasaki, Shull, and colleagues). The regulation of these renal transporters in health and disease has been an active field of research during the last decade, and was potentiated by the development of knock-out animal models for virtually all of the renal transporters (Fenton, Knepper, Nielsen, Shull, Takahashi, Verkman, and colleagues).

II. Osmoregulation and volume regulation

Renal tubular transport is regulated by various endocrine, nervous, and hemodynamic factors to ultimately establish whole-body homeostasis. This integrated response can be called the “body fluid homeostatic mechanism”, and is designed to maintain the plasma osmolality and the composition of the extracellular fluid volume, despite wide variations in water and solute intake (Table 1) [9]. Because most fluid and electrolyte disorders can be explained by one or more disturbances in this

system, the general principles of the body fluid homeostatic mechanism are discussed below.

Table 1: Osmoregulation versus volume regulation

	Osmoregulation	Volume regulation
What is being regulated	Water balance	Sodium balance
What is being sensed	Plasma osmolality	Effective circulating volume
Sensors	Hypothalamic osmoreceptors	• Carotic sinus • Afferent glomerular arteriole • Atria
Effectors	Antidiuretic hormone	• Sympathetic nervous system • Renin-angiotensin system • Natriuretic peptides • Pressure natriuresis • Antidiuretic hormone
What is affected	• Water excretion (via ADH) • Water intake (via thirst)	Sodium excretion

IIA. Osmoregulation

Water balance regulation depends on the release of vasopressin by the pituitary and its end-organ effect on the renal collecting duct system. This system is so efficient that under physiological circumstances the plasma osmolality does not vary by more than 1-2%, despite wide fluctuations in water intake. Vasopressin is a cyclic octapeptide of 1099 Dalton with a tail of three amino acid residues. It is synthesized and secreted by neurons of the supraoptic and paraventricular magnocellular nuclei in the hypothalamus, which project axon terminals to the neurohypophysis. Vasopressin release rises as a function of plasma osmolality. The following cascade is responsible for this relationship. A change in the plasma osmolality results in a change in cell volume. This change in cell volume is sensed by the osmoreceptors in the hypothalamus and increases the activity of mechanosensitive cation channels, which then affects membrane voltage and ultimately the action potential discharge [10]. The accelerated action potential discharge stimulates vasopressin secretion. The osmotic threshold for vasopressin release is 280-290 mOsm/kg, while the osmotic threshold for thirst occurs at approximately 290-295 mOsm/kg. Once released, circulating vasopressin can activate three types of G-protein coupled receptors, including V1a (vascular and hepatic), V1b (anterior pituitary), and V2 (renal collecting duct). The activation of the V2R (a 41 kDa protein of 371 residues with seven transmembrane domains) by vasopressin is the most important step in water balance regulation, because it increases renal water permeability by inserting AQP2 water-channels in the apical membrane of the collecting duct principal cell. Binding of vasopressin to the V2R activates the Gs adenyllyl

cyclase system, stimulating cAMP and protein kinase A, which results in phosphorylation of preformed AQP2.

IIB. Volume regulation

Total body water constitutes approximately 60% of the total body weight and exists in two compartments: the intracellular fluid compartment (ICF) and the extracellular fluid compartment (ECF) [11]. The intravascular fluid compartment makes up one-fourth of the ECF. Similar to water balance regulation, ECF regulation is also organized by sensor and effector elements (Table 1). There are volume sensors at several sites in the vasculature, including low-pressure baroreceptors in the cardiac atria, left ventricle, and pulmonary vasculature bed, and high-pressure baroreceptors in the carotid artery and aortic arch. These baroreceptors respond to volume expansion or contraction, and evoke a central response, that includes the activation of the sympathetic nervous system and the release of hormones such as atrial natriuretic peptide, vasopressin, adrenocorticotrophic hormone, norepinephrine, and endothelin [12]. The kidneys possess their own sensor-effector system with an intrarenal baroreceptor in the form of the juxtaglomerular apparatus. In the kidneys, volume contraction results in a decrease in renal perfusion (activating neuronal nitric oxide synthase and cyclo-oxygenase-2), a decrease in solute delivery to the macula densa, and an activation of renal sympathetic nerves. These three effects stimulate renin release and thus activate the renin-angiotensin-aldosterone system. Besides systemic effects, angiotensin II and aldosterone also regulate renal sodium excretion. Angiotensin II especially affects sodium reabsorption by the early proximal tubule, whereas aldosterone

primarily increases active sodium reabsorption in the distal nephron by activating NCC and ENaC [5,7].

III. Fluid and electrolyte disorders

Dysregulation of water and/or sodium balance can result in fluid and electrolyte disorders. Table 2 shows a selection of inherited and acquired diseases that can lead to fluid and electrolyte disorders, including their causes and mechanisms. While only the most prominent electrolyte disorders are shown in Table 2, many of these diseases are also characterized by changes in blood pressure, and calcium, magnesium, and acid-base balance. Inherited fluid and electrolyte disorders are relatively rare, but they have been extremely important for our understanding of transport physiology and pathophysiology. In contrast, acquired fluid and electrolyte disorders are much more common, and may cause substantial morbidity or even mortality. For example, hyponatremia is present in approximately 30% of hospitalized patients [13].

IIIA. Inherited fluid and electrolyte disorders

Inherited fluid and electrolyte disorders are usually caused by an activating or inactivating mutation of a gene encoding for a renal transport protein (Table 2). Starting with the most recently discovered disorder, Feldman *et al.* described nephrogenic SIADH, a syndrome in which missense mutations result in the constitutive activation of the V2R, thereby increasing renal water reabsorption and causing hyponatremia [14]. Nephrogenic diabetes insipidus is usually X-linked recessive and is often caused by mutations in the gene coding for V2R [15], although mutations in AQP2 have also been described [16]. These

mutations usually result in an intracellular entrapment of the V2R, which then fails to reach the basolateral plasma membrane. Nephrogenic diabetes insipidus is characterized by hypernatremia, thirst, and polyuria, potentially resulting in mental retardation and delayed development. Familial central diabetes insipidus is an autosomal dominant disorder in which a point mutation in the gene controlling the synthesis of the ADH precursor preprovasopressin-neurophysin II has occurred. This results in death of ADH-producing cells and causes similar symptoms as nephrogenic DI. In addition, a bright spot is visible on magnetic resonance imaging (perhaps due to the accumulated precursor) that is not seen in other forms of diabetes insipidus [17]. In Bartter, Gitelman, and Liddle syndromes, a genetic defect affects one of the major renal sodium transporters. The Bartter syndromes are autosomal recessive, and are caused by an *inactivating* mutation of the genes encoding NKCC2 (type I), ROMK (type II), ClC-Kb (type III), or barttin (type IV), or an *activating* mutation of the gene encoding the calcium sensing receptor (type V, or autosomal dominant hypocalcemia) [18]. All Bartter syndromes are characterized by hypokalemia, hypochloremic metabolic alkalosis, and renal sodium wasting. Gitelman syndrome is an autosomal recessive condition caused by a loss of function mutation of the NCC, and characterized by hypokalemia, metabolic alkalosis, hypocalciuria, and mild sodium wasting [19,20]. Liddle's syndrome is an autosomal dominant syndrome of hypertension, hypokalemia, and metabolic alkalosis. Liddle's syndrome is caused by overactivity of the sodium channel ENaC, because point mutations in the β - and γ -ENaC subunits prevent the binding of regulatory proteins [21,22].

Table 2: Selection of inherited and acquired diseases presenting with fluid and electrolyte disorders

Disease	Electrolyte disorder	Cause	Mechanism
<i>Inherited fluid and electrolyte disorders</i>			
• Nephrogenic SIADH	Hyponatremia	Activating Mutation	V2R ↑
• Nephrogenic DI	Hypernatremia	Inactivating Mutation	V2R-AQP2 ↓
• Central DI	Hypernatremia	Mutation	ADH ↓
• Bartter's syndrome	Hypokalemia	Inactivating mutation	NKCC2 ↓ CaSR↑, Clc ↓ or ROMK ↓
• Gitelman's syndrome	Hypokalemia	Inactivating mutation	NCC ↓
• Liddle's syndrome	Hypokalemia	Activating mutation	ENaC ↑
<i>Acquired fluid and electrolyte disorders</i>			
• SIADH	Hyponatremia	Drug or disease induced	ADH ↑
• Nephrogenic DI	Hypernatremia	Drug or disease induced	V2R-AQP2 ↓
• Central DI	Hypernatremia	Brain lesion	ADH ↓
• Cerebral salt wasting	Hyponatremia	Brain lesion	BNP ↑
• Heart or liver failure	Hyponatremia	Low effective circulating volume	ADH ↑
• Complications of diuretic use	Hyponatremia, hypokalemia	Drug induced	NKCC2 ↓ NCC↓, ENaC ↓
• Addison's disease	Hyponatremia, hyperkalemia	Auto-immune, infection, cancer	ADH ↑ Aldosterone ↓

IIIB. Acquired fluid and electrolyte disorders

In general, hyponatremia and hypernatremia (“dysnatremia”) are the result of a disturbance in water rather than sodium balance. Consequently, dysnatremia is usually associated with disturbances in the release or actions of vasopressin. For example, a study by Andersen *et al.* showed that increased secretion of vasopressin was present in 97% of the patients with hyponatremia [23]. However, it is important to remark that altered vasopressin metabolism alone is not sufficient to cause dysnatremia. Rather, it is the *combination* between altered vasopressin metabolism and the intake or administration of electrolyte free water that causes dysnatremia [11].

An important feature of the pathogenesis of hyponatremia is that not only osmotic factors, but also non-osmotic factors can cause vasopressin release. Non-osmotic factors causing vasopressin release include diseases (e.g., bronchogenic carcinoma, pneumonia, hydrocephalus), drugs (e.g., carbamazepine, selective serotonin reuptake inhibitors, vincristine), and symptoms such as pain and nausea [24]. Non-osmotic vasopressin release will cause electrolyte free water retention and, consequently, hyponatremia. This hyponatremic disorder is called the syndrome of inappropriate antidiuretic hormone secretion (SIADH), which was described in 1957 by Schwartz and Bartter [25].

The pathophysiology of the other hyponatremic disorders usually depends on a combination of non-osmotic and osmotic vasopressin release caused by ECF-contraction. Examples include diuretic-induced hyponatremia, Addison’s disease, and cerebral salt wasting (Table 2). The common feature of these

disorders is that there is renal sodium loss, caused by diuretic-induced inhibition of sodium reabsorption, aldosterone deficiency, or the release of natriuretic peptides, respectively. This will cause ECF-contraction and, if sufficiently severe, osmotic vasopressin release (Table 1). However, these disorders may also cause non-osmotic vasopressin release, and the responsible factors in the three examples include a direct effect of thiazide diuretics [26], glucocorticoid deficiency [27], and a brain lesion [28].

Hyponatremia can be accompanied by disorders of potassium balance. Concomitant hyponatremia and hypokalemia can occur in diuretic use, because of increased delivery of sodium and water to the potassium secretory site in the collecting ducts, and because of increased secretion of aldosterone due to volume depletion. Concomitant hyponatremia and hyperkalemia can occur in Addison's disease, because aldosterone regulates both sodium reabsorption and potassium secretion.

Finally, two important clinical disorders that are associated with the presence of hyponatremia, are heart and liver failure. A low cardiac output in heart failure and an increased systemic vasodilatation in liver failure will both result in a low effective circulating blood volume. A low effective circulating blood volume will activate the neurohumoral response, causing a rise in vasopressin secretion and consequently hyponatremia. The development of hyponatremia in cardiac and hepatic failure is predictive of a poor prognosis [29,30].

Hypernatremia is usually caused by increased insensible or enteral losses, or by a urinary concentrating defect such as diabetes insipidus [31]. In hypernatremic syndromes due to diabetes insipidus, the disturbance in vasopressin metabolism is exactly opposite to that in hyponatremic syndromes. That is, the release of vasopressin (central diabetes insipidus) or the actions of vasopressin (nephrogenic diabetes insipidus) are inhibited instead of augmented. Causes of central diabetes insipidus usually include a brain lesion (e.g., a neurotrauma, pituitary adenoma, or craniopharyngeoma), but can also be caused by medication (e.g., phenytoin). Causes of nephrogenic diabetes insipidus include diseases (e.g., myeloma kidney), drugs (e.g., lithium, amphotericin B, and ifosfamide), and electrolyte disorders (hypokalemia and hypercalcemia) [32]. Clinically, diabetes insipidus is characterized by polyuria with a urine osmolality lower than 200 mOsm/kg. Hypernatremia only develops during diabetes insipidus if the associated polyuria is not adequately compensated by oral fluid intake or intravenous fluid therapy.

IV. Symptomatology and treatment of hyponatremia and hypernatremia

The rate of development of hyponatremia and the presence of symptoms are used to classify hyponatremia in acute hyponatremia (development < 48 hours, usually symptomatic) and chronic hyponatremia (development > 48 hours, usually not symptomatic) [11]. Symptoms of acute hyponatremia are usually neuro-psychiatric and can include nausea, vomiting, headache, lethargy, reversible ataxia, psychosis, seizures, and coma. A recent study suggests that chronic hyponatremia may also

produce symptoms, including falls, unsteadiness, and attention deficits [33,34].

The symptomatology of acute hyponatremia can be explained by the development of cerebral edema and a rise in intracranial pressure. Cerebral edema develops because there is a fall in plasma osmolality, which causes a shift of water to the intracellular fluid compartment. For the same reasons, cerebral edema may also occur during the treatment of diabetic ketoacidosis, especially when a large fall in the plasma glucose concentration occurs in the context of a small rise in the plasma sodium concentration [35]. Although osmotic water shifts occur in all cells, the space in the skull limits the increase in brain volume (“rigid box”). This may also explain why children are more prone to the complications of cerebral edema, because they have more brain cells, and thus relatively less space for brain volume expansion [36]. Once brain cells swell, several defense mechanisms come into play to restore the brain cell volume [37]. Acute defense mechanisms (hours) include the active loss of cellular electrolytes (mainly potassium) to reduce the intracellular osmolality. More chronic defense mechanisms (days) rely on the cellular excretion of organic osmolytes, including myo-inositol, phosphocreatine, glutamine, and taurine.

Therapeutically, the distinction between acute hyponatremia and chronic hyponatremia is crucial because it determines the correction rate. If hyponatremia is acute and cerebral edema is progressive, brain stem herniation may occur, resulting in respiratory depression and death [38].

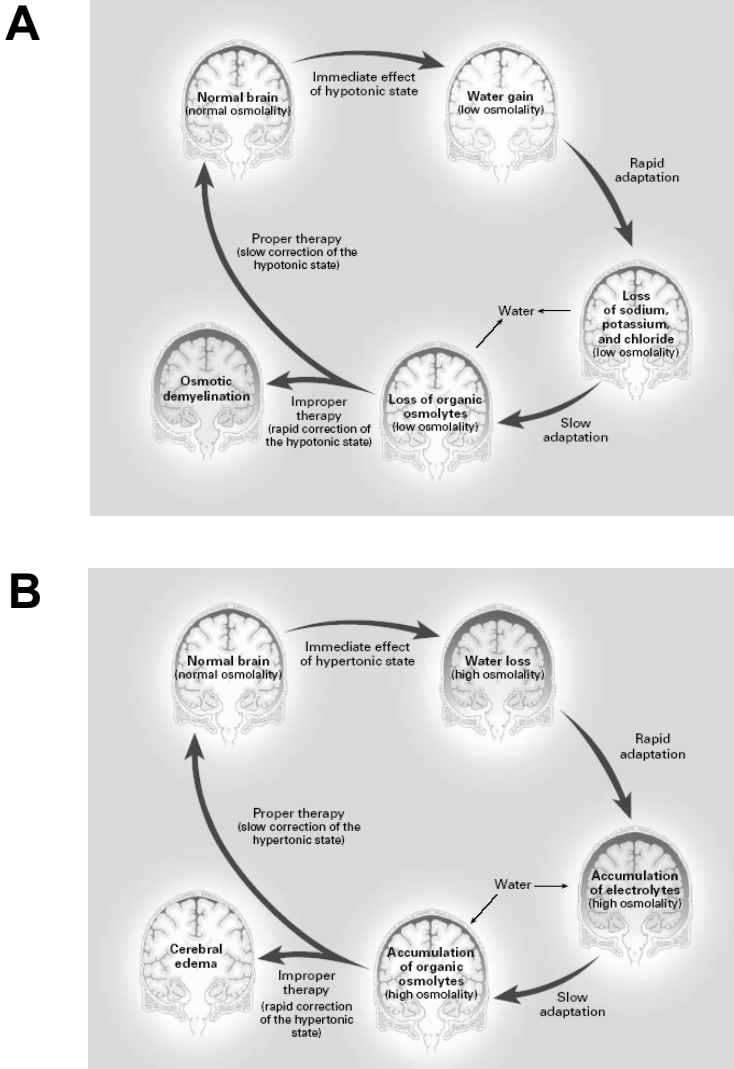


Figure 3: Dysnatremia and the brain

Brain volume changes during hyponatremia (Panel A) and hypernatremia (Panel B), including clinical dangers of improper therapy. With kind permission from NEJM.

Therefore, acute hyponatremia should be treated aggressively by establishing a rise in the plasma sodium concentration of 12 mmol/l/day, or higher. Conversely, if the brain has adapted to chronic hyponatremia, the adage is to “go slow” by not exceeding a rise in plasma sodium concentration of 8 mmol/day. The risk of too rapid correction of chronic hyponatremia is the osmotic demyelination syndrome (Figure 3), which has a wide spectrum of presentations, ranging from the absence of symptoms to a devastating disorder characterized by confusion, agitation, and, eventually, flaccid or spastic quadriparesis [39,40]. Despite these guidelines, the correction of hyponatremia remains a controversial subject, in part because there are few clinical indices to define whether hyponatremia is acute or chronic. A recent study suggests that low plasma urea levels, and increased fractional excretion of urea and uric acid may be helpful in diagnosing acute hyponatremia [41].

Brain volume changes during hypernatremia and its correction can be viewed as the opposite from those during the correction of hyponatremia (Figure 3). During acute hypernatremia brain cells shrink, which may produce symptoms of lethargy, weakness, and irritability, and can progress to twitching, seizures, and coma. The plasma sodium concentration may be restored relatively quickly during acute hypernatremia. In contrast, if the brain has adapted to chronic hypertonicity, cerebral edema may develop during too rapid correction of hypernatremia.

References

1. Shirley DG, Capasso G, Unwin RJ. Renal physiology. In: Johnson RJ, Feehally J, editors. *Comprehensive clinical nephrology*. 2nd ed. Edinburgh: Mosby; 2003. p. 13-26.
2. Kriz W, Bankir L. A standard nomenclature for structures of the kidney. The Renal Commission of the International Union of Physiological Sciences (IUPS). *Kidney Int* 1988; 33: 1-7.
3. Molony DA, Reeves WB, Andreoli TE. Na⁺:K⁺:2Cl⁻ cotransport and the thick ascending limb. *Kidney Int* 1989; 36: 418-26.
4. Briggs JP, Schnermann J. The tubuloglomerular feedback mechanism: functional and biochemical aspects. *Annu Rev Physiol* 1987; 49: 251-73.
5. Kim GH, Masilamani S, Turner R, Mitchell C, Wade JB, Knepper MA. The thiazide-sensitive Na-Cl cotransporter is an aldosterone-induced protein. *Proc Natl Acad Sci U S A* 1998; 95: 14552-7.
6. Nielsen S, Frokiaer J, Marples D, Kwon TH, Agre P, Knepper MA. Aquaporins in the kidney: from molecules to medicine. *Physiol Rev* 2002; 82: 205-44.
7. Masilamani S, Kim GH, Mitchell C, Wade JB, Knepper MA. Aldosterone-mediated regulation of EnaC alpha, beta, and gamma subunit proteins in rat kidney. *J Clin Invest* 1999; 104: R19-23.
8. Knepper MA, Nielsen S. Peter Agre, 2003 Nobel Prize winner in chemistry. *J Am Soc Nephrol* 2004; 15: 1093-5.
9. Schrier RW. Body fluid volume regulation in health and disease: a unifying hypothesis. *Ann Intern Med* 1990; 113: 155-9.
10. Oliet SH, Bourque CW. Mechanosensitive channels transduce osmosensitivity in supraoptic neurons. *Nature* 1993; 364: 341-3.
11. Halperin ML, Goldstein MB. *Fluid, Electrolyte, and Acid-Base Physiology*. 3rd ed. Philadelphia: W.B. Saunders Company; 1999.
12. Sklar AH, Schrier RW. Central nervous system mediators of vasopressin release. *Physiol Rev* 1983; 63: 1243-80.
13. Hoorn EJ, Lindemans J, Zietse R. Development of severe hyponatraemia in hospitalized patients: treatment-related risk factors and inadequate management. *Nephrol Dial Transplant* 2006; 21: 70-6.
14. Feldman BJ, Rosenthal SM, Vargas GA, Fenwick RG, Huang EA, Matsuda-Abedini M, et al. Nephrogenic syndrome of inappropriate antidiuresis. *N Engl J Med* 2005; 352: 1884-90.

15. Rosenthal W, Seibold A, Antaramian A, Lonergan M, Arthus MF, Hendy GN, et al. Molecular identification of the gene responsible for congenital nephrogenic diabetes insipidus. *Nature* 1992; 359: 233-5.
16. Deen PM, Verdijk MA, Knoers NV, Wieringa B, Monnens LA, van Os CH, et al. Requirement of human renal water channel aquaporin-2 for vasopressin-dependent concentration of urine. *Science* 1994; 264: 92-95.
17. Ito M, Jameson JL. Molecular basis of autosomal dominant neurohypophyseal diabetes insipidus. Cellular toxicity caused by the accumulation of mutant vasopressin precursors within the endoplasmic reticulum. *J Clin Invest* 1997; 99: 1897-905.
18. Hebert SC. Bartter syndrome. *Curr Opin Nephrol Hypertens* 2003; 12: 527-32.
19. Simon DB, Nelson-Williams C, Bia MJ, Ellison D, Karet FE, Molina AM, et al. Gitelman's variant of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter. *Nat Genet* 1996; 12: 24-30.
20. Gitelman HJ, Graham JB, Welt LG. A new familial disorder characterized by hypokalemia and hypomagnesemia. *Trans Assoc Am Physicians* 1966; 79: 221-35.
21. Schild L, Canessa CM, Shimkets RA, Gautschi I, Lifton RP, Rossier BC. A mutation in the epithelial sodium channel causing Liddle disease increases channel activity in the *Xenopus laevis* oocyte expression system. *Proc Natl Acad Sci U S A* 1995; 92: 5699-703.
22. Hansson JH, Nelson-Williams C, Suzuki H, Schild L, Shimkets R, Lu Y, et al. Hypertension caused by a truncated epithelial sodium channel gamma subunit: genetic heterogeneity of Liddle syndrome. *Nat Genet* 1995; 11: 76-82.
23. Anderson RJ, Chung HM, Kluge R, Schrier RW. Hyponatremia: a prospective analysis of its epidemiology and the pathogenetic role of vasopressin. *Ann Intern Med* 1985; 102: 164-8.
24. Moses AM, Miller M. Drug-induced dilutional hyponatremia. *N Engl J Med* 1974; 291: 1234-9.
25. Schwartz WB, Bennett W, Curelop S, Bartter FC. A syndrome of renal sodium loss and hyponatremia probably resulting from inappropriate secretion of antidiuretic hormone. *Am J Med* 1957; 23: 529-42.

26. Hamburger S, Koprivica B, Ellerbeck E, Covinsky JO. Thiazide-induced syndrome of inappropriate secretion of antidiuretic hormone. Time course of resolution. *JAMA* 1981; 246: 1235-6.
27. Kamoi K, Tamura T, Tanaka K, Ishibashi M, Yamaji T. Hyponatremia and osmoregulation of thirst and vasopressin secretion in patients with adrenal insufficiency. *J Clin Endocrinol Metab* 1993; 77: 1584-8.
28. Singh S, Bohn D, Carlotti AP, Cusimano M, Rutka JT, Halperin ML. Cerebral salt wasting: truths, fallacies, theories, and challenges. *Crit Care Med* 2002; 30: 2575-9.
29. Jalan R, Elton RA, Redhead DN, Finlayson ND, Hayes PC. Analysis of prognostic variables in the prediction of mortality, shunt failure, variceal rebleeding and encephalopathy following the transjugular intrahepatic portosystemic stent-shunt for variceal haemorrhage. *J Hepatol* 1995; 23: 123-8.
30. Lee DS, Austin PC, Rouleau JL, Liu PP, Naimark D, Tu JV. Predicting mortality among patients hospitalized for heart failure: derivation and validation of a clinical model. *JAMA* 2003; 290: 2581-7.
31. Palevsky PM, Bhargath R, Greenberg A. Hyponatremia in hospitalized patients. *Ann Intern Med* 1996; 124: 197-203.
32. Garofeanu CG, Weir M, Rosas-Arellano MP, Henson G, Garg AX, Clark WF. Causes of reversible nephrogenic diabetes insipidus: a systematic review. *Am J Kidney Dis* 2005; 45: 626-37.
33. Decaux G. Is asymptomatic hyponatremia really asymptomatic? *Am J Med* 2006; 119: S79-82.
34. Renneboog B, Musch W, Vandemergel X, Manto MU, Decaux G. Mild chronic hyponatremia is associated with falls, unsteadiness, and attention deficits. *Am J Med* 2006; 119: 71 e1-8.
35. Carlotti AP, Bohn D, Halperin ML. Importance of timing of risk factors for cerebral oedema during therapy for diabetic ketoacidosis. *Arch Dis Child* 2003; 88: 170-3.
36. Arieff AI, Ayus JC, Fraser CL. Hyponatraemia and death or permanent brain damage in healthy children. *BMJ* 1992; 304: 1218-22.
37. Sterns RH, Silver SM. Brain volume regulation in response to hypo-osmolality and its correction. *Am J Med* 2006; 119: S12-6.
38. Arieff AI. Hyponatremia, convulsions, respiratory arrest, and permanent brain damage after elective surgery in healthy women. *N Engl J Med* 1986; 314: 1529-35.

39. Sterns RH, Riggs JE, Schochet SS, Jr. Osmotic demyelination syndrome following correction of hyponatremia. *N Engl J Med* 1986; 314: 1535-42.
40. Sterns RH, Cappuccio JD, Silver SM, Cohen EP. Neurologic sequelae after treatment of severe hyponatremia: a multicenter perspective. *J Am Soc Nephrol* 1994; 4: 1522-30.
41. Hsu YJ, Chiu JS, Lu KC, Chau T, Lin SH. Biochemical and etiological characteristics of acute hyponatremia in the emergency department. *J Emerg Med* 2005; 29: 369-74.

Aims and outline of the thesis

The aims of this thesis are:

- 1. To study the renal mechanisms of water and sodium balance regulation in order to elucidate the pathophysiology of fluid and electrolyte disorders.*
- 2. To study the clinical aspects of fluid and electrolyte disorders in order to describe and improve preventive, diagnostic, and therapeutic approaches.*

Because of these two objectives, the thesis has been divided in two parts.

Part A is called “Renal mechanisms of water and sodium balance regulation” and mainly addresses the first objective of the thesis. The central subject of *Chapters 1 – 3* is “proteomics”, which is a relatively novel technique to study proteins on a large scale by combining protein separation techniques and mass spectrometry. *Chapter 2* is a general introduction to the application of the proteomics technique Difference Gel Electrophoresis (DIGE), and how it can be used to study the renal mechanisms of water and sodium balance. In *Chapter 3*, DIGE-based proteomics is applied to study the “vasopressin escape” phenomenon in a rat model. The vasopressin escape phenomenon is an important physiological process that limits the severity of the syndrome of inappropriate antidiuretic hormone secretion (SIADH) and other hyponatremic disorders.

Chapter 4 describes the potential of urinary proteomics, in which so-called “urinary exosomes” may be applied as biomarkers for the identification, differentiation and treatment of renal disease. In *Chapter 5* the pathogenesis of hypokalemia in Gitelman’s syndrome is investigated, using a $\text{Na}^+\text{-Cl}^-$ co-transporter (NCC) knock-out mouse model. In *Chapter 6*, the mechanism of osmomediated natriuresis is investigated in an infusion study with healthy human volunteers and patients with an inactivating mutation of the calcium sensing receptor (familial hypocalciuric hypercalcemia).

Part B is called “Clinical disorders of water and sodium balance” and mainly addresses the second objective of the thesis. In *Chapters 7 – 10* clinical studies on hyponatremia are presented, including studies addressing the diagnostic approach (*Chapter 7*), risk factors of hospital-acquired hyponatremia (*Chapters 8 and 9*), and the relationship between hyponatremia and renal dysfunction (*Chapter 10*). *Chapters 11 and 12* both deal with different aspects of hypernatremia, namely hypernatremia in critically ill patients (*Chapter 11*), and the role of hypernatremia during the treatment of diabetic ketoacidosis in children (*Chapter 12*), respectively. *Chapter 13* also focuses on fluid and electrolyte disorders in children, and presents a case control study analyzing the determinants of acute hyponatremia in hospitalized patients. Because of the important role of intravenous fluid therapy in fluid and electrolyte disorders, a discussion paper on how to select optimal intravenous fluid therapy concludes this thesis in *Chapter 14*.

Part A

Renal mechanisms of water
and sodium balance
regulation

Chapter 2

**The application of DIGE-based
proteomics to renal physiology**

**Ewout J. Hoorn, Jason D. Hoffert
and Mark A. Knepper**

Nephron Physiol 2006; 104: 61–72

Abstract

Proteomics is seeing increasing use as a means of identifying new mechanistic hypotheses in physiology. Proteomics based on two-dimensional electrophoresis (2-DE) has recently been optimized with the development of Difference Gel Electrophoresis (DIGE). In DIGE-based proteomics, the experimental and control samples are derivatized with different fluorophores and are run in the same gel, thereby minimizing technical variation. DIGE is currently one of the few techniques to perform quantitative proteomics, generating a statistical output to differences in protein abundances. In this review, we discuss the principles of DIGE-based proteomics, including sample preparation, 2-DE, statistical analysis of 2D-gels, and mass spectrometry. Strengths and weaknesses of DIGE are discussed, including possible solutions to overcome certain limitations, such as the identification of low abundance and integral membrane proteins. In addition, we provide a brief synopsis of our recent experiments in which DIGE-based proteomics was applied to study vasopressin signaling in the renal collecting duct. Finally, we illustrate how quantification based on the DIGE approach combined with bioinformatics may facilitate the study of systems biology of the kidney.

Introduction

The human genome project and related genome sequencing projects for other species have led to rejuvenation of the field of systems biology, which includes many genome wide discovery methodologies. One important set of discovery methods are the ‘proteomics’ approaches, i.e., large-scale study of proteins [1]. These approaches are particularly important in physiology because proteins are the prime mediators of biological function. According to Aebersold and Mann [2], the objective of proteomics is to ‘concurrently identify, quantify, and analyze a large number of proteins in a functional context’. Because of these characteristics, proteomics is seeing increasing use as a means of identifying new mechanistic hypotheses in physiology [3].

The last few years, the emphasis of proteomics research has shifted from identification to quantification [4,5]. Quantitative proteomics can be defined as the study of the relative differences in protein abundance between multiple samples [6]. In spite of promising alternative or complementary proteomics techniques that are under development (e.g., isotope coded affinity tags and multidimensional protein identification technology), two-dimensional electrophoresis (2-DE) is currently the only proteomics technique that can be routinely applied for parallel quantitative expression profiling of large sets of complex protein mixtures [7]. More recently, quantification in 2-DE has been optimized with the development of Difference Gel Electrophoresis (DIGE) [8]. In DIGE-based proteomics, the experimental and control samples are derivatized with different fluorophores and are run in the same gel, thereby minimizing technical variation, which may be as high as 20-30% [6].

DIGE-based proteomics is being pursued by several groups for the study of renal physiology and pathophysiology, including acute renal failure [9], diabetic nephropathy [10], acid-base disturbances [11], and fluid and electrolyte disorders [3,12]. Recently, we applied DIGE-based proteomics to study vasopressin signaling in the inner medullary collecting duct (IMCD) [3,12,13]. The focus of this review will be to discuss the principles of DIGE-based proteomics, including its strengths and weaknesses, and to provide a brief synopsis of our results, illustrating how quantification based on the DIGE approach may facilitate the study of systems biology of the kidney. We emphasize that, depending on the research question at hand, several proteomics techniques other than DIGE are available, including gel-free systems using liquid chromatography (LC) and chip-based systems using surface enhanced laser desorption/ionization, of which expert reviews have been published elsewhere [2,14,15].

Principles of DIGE-based proteomics

Before discussing the principles of DIGE-based proteomics, it is useful to consider the principles of any proteomics experiment. In this regard, the report on a recent proteomics meeting summarized important themes [16]. These were: (1) the need for simplification by selecting a subproteome; (2) the use of biological fluids versus tissue; (3) consideration of biological and experimental variation; (4) the creation of databases for integrated systems biology.

Indeed, we believe that these technical and methodological considerations are crucial to the success of proteomics research, especially quantitative proteomics. The use of biological fluids provides an important strategy for clinical proteomics, in particular for biomarker discovery (see our recent review on urinary proteomics in [17]). However, we argue that for studying cellular pathways, tissue is likely to remain an important substrate for proteomics experiments. Below we will discuss in more detail important technical and methodological aspects of DIGE-based proteomics, including sample preparation, 2-DE, statistical analysis of 2-D gels, and mass spectrometry. An integrated representation of these individual steps is shown in Figure 1. In addition, the strengths and limitations of DIGE-based proteomics are outlined in Table 1.

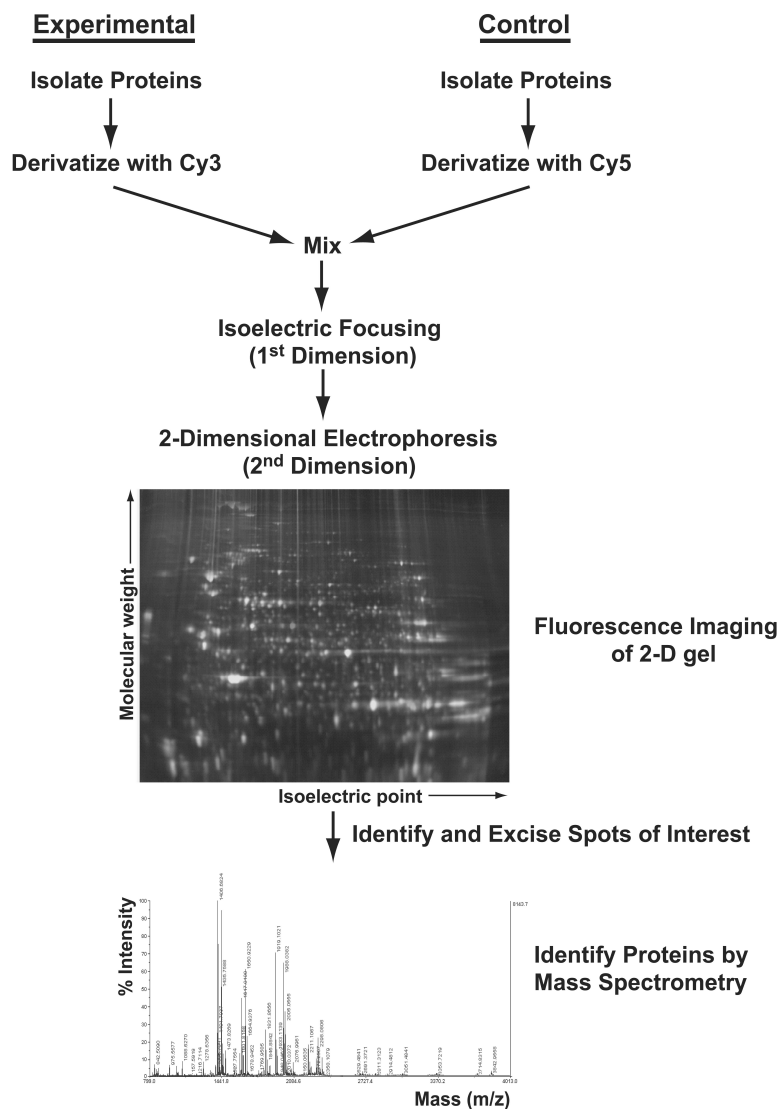


Figure 1: DIGE

Tissue samples are labeled with different fluorescent dyes (Cy3 and Cy5), and samples are mixed. Isoelectric focusing and 2-DE is carried out, and fluorescence images of the gels are obtained. Computer analysis identifies gel spots that increased or decreased in experimental vs. control. These spots can be cut out of the gel and trypsinized, and the responding proteins can be identified by mass spectrometry.

Table 1: Strengths and limitations of DIGE-based proteomics

1. Strengths DIGE-based proteomics

- Currently the only routine platform for quantitative proteomics
- Differential expression pattern with statistical output
- Low experimental variation due to mixing of experimental and control sample and inclusion of an internal standard
- Visualization of protein isoforms including splice variants and post-translational modifications
- Information on molecular weight and iso-electric point

2. Limitations DIGE-based proteomics

- Less efficient for hydrophobic proteins (e.g., integral membrane proteins)
- Low identification rate of low abundance proteins
- Potential false positives using traditional statistics
- Difficult quantification and identification of proteins with overlapping spots

3. Possible solutions to overcome limitations

- Use of other detergents during iso-electric focusing
 - Modified 2-DE (using 16-BAC and SDS)
 - Modified statistical procedures
 - Combination of targeted proteomics and DIGE-based proteomics
 - Combination of DIGE-based proteomics with bioinformatics pathways analysis
-

Sample preparation

Characteristic of sample preparation in DIGE-based proteomics is the covalent labelling of proteins with fluorescent dyes, which are matched so as to cause little or no shift in isoelectric point and an identical shift in molecular weight. For the labeling, two options called ‘minimal’ and ‘saturation’ labeling are available

[18]. In minimal labeling, cyanine-derived fluorophores called ‘CyDyes’ react with primary amino groups, typically the amino group of lysine side chains [18]. Three CyDyes are commonly used, namely Cy2, Cy3 and Cy5, which allow labelling of two samples of proteins plus an internal standard [19]. Minimal labeling is extremely sensitive (the detection limit is between 150 and 500 pg). Saturation labeling, in which thiol-reactive maleimide groups react with cysteines, has been shown to be even more sensitive [20], but is technically more challenging, and therefore usually only used when sample abundance is a limiting factor [18].

Besides this DIGE-specific step, other considerations are important for sample preparation in 2-DE. First of all, optimal conditions for protein separation in 2-DE should be created. Proteins need to be disaggregated, denatured, solubilized and reduced to enable optimal separation in 2-DE, while interfering substances need to be removed or inactivated [7]. Interfering substances include proteases, salts, and nucleic acids, which may cause protein loss or artefacts on 2-D gels. Depending on the sample under investigation, this may require the use of protease inhibitors, desalting methods (spin dialysis, precipitation with trichloroacetic acid, or commercially available 2-D clean-up kits), and removal of DNA and RNA (for example by nuclease treatment or shearing). Secondly, although 2-DE is a fractionation method itself, it may be necessary to use other pre-fractionation methods to select a subproteome of interest to enhance the abundance of proteins that are most relevant to the physiological process under study. Here, traditional biochemical separation methods are available to the investigator, including subcellular fractionation, sequential extraction, and laser

microdissection (for review, see [21]). Alternatively, the objective may be to enrich certain proteins, for example phosphoproteins prior to the application of proteomics methods [22]. Finally, although accurate measurement of protein concentration in the sample appears obvious, even small errors in protein concentration may have a large impact in quantitative proteomics. It may therefore be advantageous to confirm that equal amounts of protein will be used for the 2-DE prior to running the 2-D gel, for example by running a 1-D gel and staining with Coomassie Brilliant Blue.

Two-dimensional electrophoresis

During 2-DE, proteins are separated based on their isoelectric points (first dimension) and their molecular weights (second dimension) (Figure 1). In the first dimension, which is commonly called isoelectric focusing, protein separation is achieved in the context of a continuous pH gradient in which proteins migrate to the point in the gel where they have no net charge ('isoelectric point') [23]. The efficacy of isoelectric focusing has greatly benefited from the introduction of immobilized pH gradients, replacing the former carrier ampholytes, which were characterized by low reproducibility, and loss of alkaline proteins [24]. After the first dimension, a number of equilibration steps are performed to establish optimal physicochemical protein properties for the second dimension. During these equilibration steps, proteins are treated with sodium dodecyl sulfate (SDS; for protein adsorption), a reducing agent such as dithiothreitol (to reduce disulfide bonds), and an alkylating agent such as iodoacetamide (to stabilize newly formed –SH bonds) [23]. Subsequently, in the second dimension, electrophoresis is performed usually in a

polyacrylamide gel (hence the term 2D-PAGE), which acts as a sieving device. All proteins move toward the positive electrode because they are clad with negatively charged SDS molecules. The number of SDS molecule is roughly proportional to the size of the protein, thereby allowing separation based on the size of the proteins. Once stained, 2-D gels constitute a map of intact proteins whose characteristics depend not only on the proteins that are present but also on the relative abundance of various proteins and their pre- and post-translational modifications (Table 1) [7].

Statistical analysis of 2-D Gels

Typically, several gels will be run for a given experiment comparing several pairs of control and experimental samples. After 2-DE, a relative protein expression profile can be obtained by comparing the fluorescent signals of the experimental (labeled with one dye) and control gels (labeled with a second dye with different spectral properties). Several software applications are commercially available for normalization, quantification, and statistical analysis of the fluorescent analytical gel images. DeCyder (GE Healthcare) is a commonly used software application in DIGE-based proteomics. The ability to analyze differential expression represents the most important strength of DIGE-based proteomics, because it provides a degree of confidence that the differential expression cannot be explained by chance alone, or by an artefact in one of the individual samples of the group (Table 1).

However, there are also important caveats in the statistical analysis of DIGE-results using DeCyder. These caveats were recently discussed by Fodor *et al.* [25], who attempted to

overcome these limitations by applying alternative statistical methods derived from the microarray analysis literature. This approach decreased the number of potentially differentially expressed proteins, but increased the confidence in these detections [25]. Specifically, three caveats and potential solutions were discussed. First of all, the traditional Student's t-test used by DeCyder may not be the most optimal method for analyzing differential expression of many proteins in parallel. A proposed alternative are so-called 'moderated t-statistics', which are based on a hierarchical, hybrid classical/Bayes models, and have been shown to follow a t-distribution under certain assumptions [25,26]. Secondly, when analyzing the results of a Student's t-test, probability values (p-values) of less than 0.05 are traditionally used to define statistical significance. However, the number of proteins that is routinely analyzed in DIGE-based proteomics is approximately 2,000. This means that using a p-value of 0.05 or less will yield 50 potential false positives for each 1,000 resolved proteins [18]. Therefore, more stringent criteria for statistical significance may need to be applied to reduce the false-positive rate (Table 1). One popular, but stringent correction method is the Bonferroni correction, which multiplies the unadjusted p-values by the total number of tests performed [27]. Another less stringent and still practical method uses the calculation of a false discovery rate and consists of a sequential p-value procedure that controls the expected value of the false discovery rate [28]. The third and final caveat was that an evaluation of the DeCyder fold change analysis showed that the distribution of the standardized log abundances showed systematic biases within the gels and had different ranges across the gels [25]. This was also described by Kreil *et al.* [29] who demonstrated that dye-specific biases exist especially for spots

of low intensity, and who recommended using a so-called offset/scale normalization. In general, if responses are found using DIGE, regardless of the statistical method used, it appears wise to confirm the observed responses via independent methodologies such as immunoblotting.

Ultimately, statistical analysis of the DIGE results will generate a list of proteins of interest, usually proteins that show a statistically significant change between the experimental and the control setting, as well as a few reference proteins.

Prior to excising the proteins from the gels for identification by mass spectrometry, a so-called ‘picking gel’ is generated in which a large amount of unlabeled protein is added to the sample (up to 1 mg) and post-electrophoretic fluorescent staining is performed using either Coomassie Blue or Sypro Ruby. This is done because the labeled portion of the protein may migrate at a higher apparent molecular mass than the majority of the unlabeled protein, because the mass and hydrophobicity of the CyDyes influence protein migration during 2-DE [18]. The extra unlabeled protein added to the ‘picking gel’ sample increases the likelihood of identification by mass spectrometry (*vide infra*). The process of excising the spots of interest from the gel, trypsinizing them, and transferring them to the sample plate for mass spectrometry analysis is often automated through a robotic spot handling workstation.

Mass spectrometry

A simplified definition of a mass spectrometer is an instrument that measures the molecular weight of an ion with high accuracy. In general, this precision is so great as to permit identification of proteins from measurement of masses of peptides derived from trypsin digestion, recognizing that trypsin cuts proteins at specific sites, namely after lysines and arginines. A mass spectrometer consists of an ion source, a mass analyzer that measures the mass-to-charge ratio (m/z) of charged chemical species, and a detector that registers the number of ions at each m/z value (Figure 2) [2]. The mass spectrometer most commonly used in DIGE-based proteomics is a MALDI-TOF. MALDI stands for ‘matrix-assisted laser desorption/ionization’ and refers to the ionization source of the mass spectrometer. MALDI uses laser pulses to ionize and volatilize proteins and peptides that have been embedded in a dry, crystalline, organic matrix (Figure 2). TOF stands for ‘time-of-flight’ and refers to the principle used by the analyzer to separate ions. In TOF mass spectrometers, the TOF is related to the mass of the ions, because there is a fixed flight distance to the detector, and ions with different masses will travel at different speeds. Peptide ions with the same m/z will exhibit equal times of flight. The acquired spectra by MALDI-TOF mass spectrometry allow protein identification through peptide mass fingerprinting (PMF).

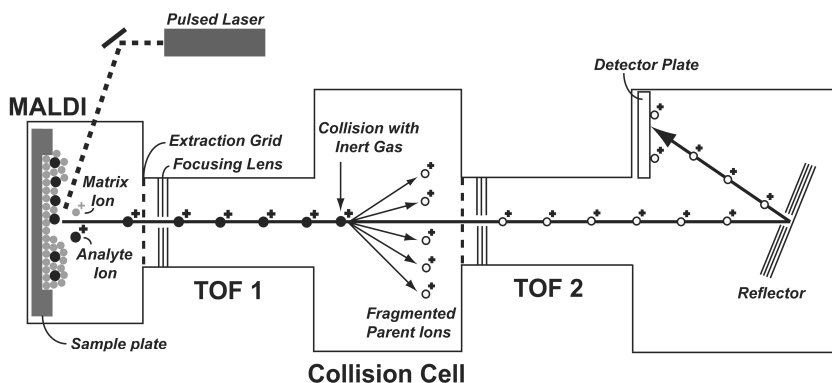


Figure 2: Mass spectrometry using MALDI-TOF/TOF

The process of mass spectrometry using MALDI-TOF/TOF is shown. The left portion of the figure shows the ionization source (MALDI, matrix assisted laser desorption/ionization), containing a pulsed laser that ionizes and volatilizes the sample. Both analyte and matrix ions are formed, but only analyte ions move into the instrument after passing an extraction grid and focusing lens, entering the first time-of-flight (TOF) tube. In the collision cell the analyte ions collide with an inert gas, causing fragmentation and the production of parent ions that continue to move to the second TOF tube. The reflector, finally, compensates for slight differences in kinetic energy and sends the ions to a detection plate, which amplifies and counts arriving ions, allowing calculation of mass-to-charge ratio's and subsequently protein identification.

PMF identifies a protein by measuring the molecular masses of all major trypsin products and matching these molecular masses with databases of theoretical sizes of trypsinized fragments from known protein sequences [1]. The disadvantages of PMF are that the sequence of the protein must be known and that mass changes of a specific protein (e.g., due to post-translational

modification) may hinder protein identification [30]. So-called ‘tandem mass spectrometry’ or MS/MS can avoid some of these disadvantages. Tandem MS uses collision-induced dissociation to fragment individual peptide ions (Figure 2) [2]. The collision cell contains an inert gas, which collides with the selection ion, causing fragmentation. The second mass-analyzer then measures the molecular masses of these fragments, creating a so-called ‘product ion spectrum’ [1]. This allows more detailed analysis of the selected ion, including a generation of its (partial) sequence. The acquired spectra by PMF and/or by MS/MS allow protein identification through database matching using online databases such as Mascot (www.matrixscience.com). Eventually, validated proteomics data can be cataloged in protein databases.

Examples of DIGE-based proteomics: vasopressin signaling in the inner medullary collecting duct

To provide more tangible examples of how DIGE-based proteomics can be applied to renal physiology, we give a synopsis of the studies we recently performed using this technique [3,12,13]. One of the objectives of our laboratory is to discover the fundamental mechanisms involved in regulation of the transport of water and ions, and to analyze how these regulatory processes are altered in various clinically important fluid and electrolyte disorders. One specific cellular pathway that is the subject of our research is how activation of the type 2 vasopressin receptor (V2R) ultimately results in recruitment of the water channel aquaporin-2 (AQP2), and how this pathway is regulated in health and disease [31] (Figure 3). In these experiments, we frequently focus on the inner medullary collecting duct (IMCD) because this nephron segment contains

principal cells that express both the V2R and AQP2 proteins, and therefore plays an important role in the regulation of water and salt balance. Unlike other parts of the collecting duct system, IMCD cells can be isolated in large numbers and in relatively high purity [32].

In one of the first studies using DIGE-based proteomics, we compared IMCD versus non-IMCD samples to identify proteins that are predominantly expressed in IMCD [13]. Inner medullae were dissected out of whole kidneys and IMCD tubules were isolated by collagenase digestion and low speed centrifugation [32]. Successful separation was confirmed because the collecting duct marker AQP2 was enriched 10-fold, compared to non-IMCD, which contains loops of Henle, vasa recta and interstitial cells. This approach illustrates how a specific subproteome can be selected prior to the application of DIGE-based proteomics, as emphasized earlier in this review. When IMCD versus non-IMCD samples were subsequently analyzed with DIGE-based proteomics, 50 proteins were identified that were highly expressed in IMCD, which included mostly enzymes, structural proteins, and signaling proteins. In contrast, 35 proteins were found predominantly in the non-IMCD cell types. Besides these physiologically relevant results, this first study was also aimed at validating and optimizing the DIGE technique. For this purpose, we assessed the intrinsic experimental variation, and demonstrated a low level of background variability and also the reproducibility of differential CyDye labeling. Finally, the protein identifications were tested and confirmed by more traditional biochemical methods, including immunoblotting and immunohistochemistry.

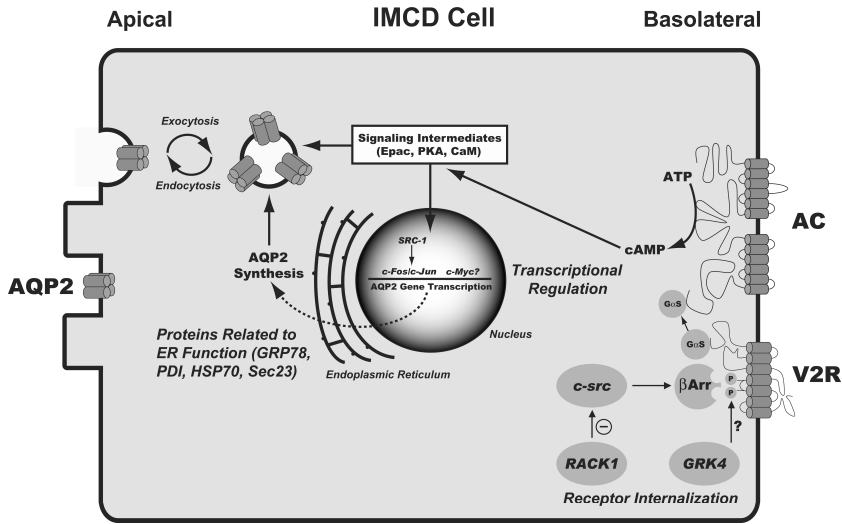


Figure 3: New hypotheses regarding vasopressin signaling in the inner medullary collecting duct

*Regulation of aquaporin-2 (AQP2) trafficking and expression in inner medullary collecting duct (IMCD) cells. Vasopressin acts on type 2 vasopressin receptors (V2R) in the basolateral plasma membrane, thereby activating an intracellular signaling cascade resulting in activation of adenylate cyclase (AC) and production of cAMP from ATP. cAMP then activates one or more signaling intermediates that may include Epac, protein kinase A (PKA) and/or calmodulin (CaM). These signaling intermediates regulate AQP2 in intracellular vesicles and also transcription factors such as c-Jun/c-Fos. Binding of these factors is thought to increase gene transcription of AQP2 resulting in synthesis of AQP2 protein, which in turn enters the regulated trafficking system. Novel processes and proteins identified by DIGE-based proteomics that are potentially involved in this pathway are shown in *italics*. These include: receptor internalization (lower right; *GRK4*, *c-src*, *RACK1*), transcriptional regulation (middle right; *c-Fos*, *c-Jun*, *c-Myc*, *SRC-1*), and proteins related to ER function (middle left; *GRP78*, *PDI*, *HSP70*, *Sec 23*). We emphasize that most of these processes and proteins have not yet been tested functionally and are therefore hypothetical. Figure in part adapted from [32], with kind permission.*

Experiments were taken to the next level when the effect of the V2R-selective vasopressin analog dDAVP on protein expression in IMCD was studied. To identify vasopressin-responsive proteins, experiments were performed in Brattleboro rats, which lack endogenous vasopressin because of a mutation in the neurophysin-vasopressin gene. These animals therefore exhibit central diabetes insipidus [33]. Experimental animals received dDAVP via osmotic minipump, while control animals received vehicle via osmotic minipump. Using DIGE-based proteomics, 43 proteins were found to be regulated in response to vasopressin infusion, including 18 that were increased in abundance, 22 that were decreased, and 3 that were shifted in the gel, presumably because of post-translational modification. Again, these results were selectively confirmed with immunoblotting and immunohistochemistry. The identified proteins pointed to several intracellular processes regulated by vasopressin, including determinants of nitric oxide levels in the cell (nitric oxide synthase 2, arginase 2, and NADPH oxidase or NOX4), the production of cAMP (adenylyl cyclase VI), receptor internalization (GPCR kinase 4), endoplasmic reticulum function (GRP 78, protein disulfide isomerases), and calcium-mediated processes (annexins II and V) (Figure 3).

In a third study, DIGE-based proteomics was applied to a clinically relevant disease model of hyponatremia and vasopressin escape [3]. Hyponatremia is the most common electrolyte disorder in hospitalized patients [34], and frequently results from non-osmotic secretion of vasopressin due to drugs or an underlying disease, resulting in the syndrome of inappropriate antidiuretic hormone secretion. However, the degree of hyponatremia is limited by a physiological process

called vasopressin escape, during which patients and experimental animals undergo a brisk water diuresis despite high levels of circulating vasopressin [35,36]. Our objective was to apply DIGE-based proteomics to identify IMCD proteins that trigger and maintain vasopressin escape. For this purpose, we used an animal model of vasopressin escape, in which animals continually received dDAVP via osmotic minipump, while experimental animals also received a water load. A set of 22 mostly high-abundance proteins was identified, many of which were confirmed by immunoblotting. These proteins were subsequently subjected to a bioinformatics pathways analysis, which indicated that 8 of the 22 proteins were part of a larger protein regulatory network consisting of 33 proteins, including low-abundance regulatory proteins and transcription factors. These identified proteins pointed to several candidate regulators of vasopressin escape, including proteins that could regulate the AQP2 gene (c-myc, c-fos, c-jun, SRC-1), proteins that may be involved in receptor internalization (c-src, RACK1), and proteins involved in protein folding in the endoplasmic reticulum (GRP78, protein disulfide isomerase, heat-shock protein 70) [3] (Figure 3). Furthermore, this approach represented a feasible strategy to combine the strengths of DIGE-based proteomics (quantitative analysis) and overcome its weakness (failure to identify low-abundance proteins), which will be discussed in more detail below.

Limitations of DIGE-based proteomics and possible solutions

The two major limitations of DIGE-based proteomics are the difficulty in identification of low-abundance proteins and hydrophobic proteins such as integral membrane proteins (Table 1).

Although virtually the complete proteome of the sample under investigation should be present in a 2D-gel, low-abundance proteins have been difficult to identify by mass spectrometry. Even optimized systems frequently have mass spectrometry identification rates of 50% or less. The reasons for this could lie anywhere from excision of the spot from the gel to the characteristics of the mass spectrometer used. For example, spot picking may have occurred in the periphery of the protein spot in the gel, the subsequent washing steps may have diluted the sample, trypsinization may have been incomplete, the spotting on the MALDI target plate may have been off-center, or the mass spectrometer used may have had insufficient sensitivity. This problem is less apparent using LC-based proteomics techniques, because the LC-step is usually directly coupled to the mass spectrometer (LC-MS). Some strategies to improve the identification of low abundance proteins include using higher protein concentrations, applying enrichment strategies [37], coating the MALDI target plate with hydrophobic compounds [38], and using a mass-spectrometer with higher sensitivity (Table 1).

Integral membrane proteins, particularly those with more than one transmembrane domain, have also been notoriously difficult to separate by 2-DE. The reason is that their solubilization

requires the use of strong detergents, such as SDS, which interfere with the isoelectric focusing step [1,39,40]. The introduction of thiourea as a chaotrope in addition to urea, and the introduction of dedicated zwitterionic detergents (such as CHAPS) has alleviated, but not solved this problem [1,39]. In some cases, other detergents, such as oligoxyethylene, sulfobetaine, dodecyl maltoside, and decaethylene glycol mono hexadecyl have been more successful in resolving membrane proteins using 2-DE [39,40]. Furthermore, a modified 2-DE technique has been described, in which two separations based on molecular weight are performed, the first with the cationic detergent benzyldimethyl-*n*-hexadecylammonium chloride and the second with SDS [41]. Because proteins show different migration properties in these two systems, a high resolution is maintained, while integral membrane proteins can be solubilized successfully. This method was described as early as 1989 by Macfarlane [41], later adapted by Hartinger *et al.* [42], and recently applied successfully by two other groups [43,44].

Besides these technical considerations, combining different methodological approaches could also contribute to overcoming the limitations of DIGE-based proteomics, while exploiting its strength in quantification. For example, in the case of the IMCD, the most important integral membrane proteins, which are channels and pumps, are well-characterized, whereas it is less known which intracellular signalling cascades regulate these proteins. Therefore, an approach called ‘targeted’ proteomics may be combined with DIGE-based proteomics [45]. In targeted proteomics, serial immunoblotting experiments are performed with ensembles of well-defined antibodies to investigate which membrane proteins are regulated after a given experimental

perturbation. Subsequently, DIGE-based proteomics may be used to identify which intracellular proteins are involved in the regulation of these proteins.

Another novel approach which we recently described, is to combine the proteomics analysis with a bioinformatic pathways analysis [3]. Proteins identified by DIGE were used as input data for this pathways analysis, which pointed to several additional proteins, including low-abundance regulatory proteins and transcription factors that could be involved in the process under investigation. These *in silico* identifications corresponded individually to ‘hypotheses’ that could be tested by immunoblotting asking whether each protein was up- or down-regulated as part of the process. Indeed, 10 of 10 proteins tested in this way displayed the hypothesized regulation, demonstrating a positive ‘goodness of fit’ with the proposed model [3].

Interpretation and implications of DIGE-based proteomics: towards systems biology of the kidney

The development and integration of proteomics and other ‘-omics’ techniques (genomics, transcriptomics, metabolomics) in combination with bioinformatics, has made it possible to study biology at systems level [46]. Aderem defined systems biology as ‘a comprehensive quantitative analysis of the manner in which all the components of a biological system interact functionally over time’ [47]. This definition again underscores the importance of a quantitative approach, and in this respect DIGE-based proteomics is expected to remain an important technique. Secondly, Aderem’s emphasis on the interaction of all components in a biological system implies that information

on proteins needs to be combined with data on genes, RNA, and their interactions and regulation. Furthermore, because proteins are regulated in several other ways than by abundance alone, new proteomics techniques studying post-translational modifications, for example phosphoproteomics, will become important [48]. In addition to these biological considerations, Liu [49] defined the major challenge of systems biology as ‘not biological but computational and organizational’. The latter illustrates the central role of bioinformatics in systems biology. Therefore, it may be insightful to give an example of how bioinformatics may be exploited to expand and contextualize biological knowledge. In Figure 4 (see Chapter 3, page 85) the protein network is shown that was generated by bioinformatics pathways analysis including the proteins regulated in the vasopressin escape phenomenon, as discussed above. The different gray levels in the figure illustrate how different techniques were combined to generate the network. Proteins that were identified by DIGE only are represented as light gray nodes, whereas proteins that were identified by DIGE and subsequently confirmed by immunoblotting are represented as black nodes. Proteins that were identified by the bioinformatics program only are represented as white nodes, whereas proteins that were identified by the bioinformatics program and then confirmed by immunoblotting are represented as dark gray nodes. For these experiments, we used the bioinformatics program Ingenuity (www.ingenuity.com, for other examples see [50,51]). Ingenuity uses the Ingenuity Pathways Knowledge Base, which is a very large, curated database consisting of millions of individually modeled relationships between proteins, culled from the biological literature. After uploading the regulated IMCD proteins, Ingenuity then identifies those gene

products that are known to directly interact with other gene products in the Ingenuity Pathways Analysis (IPA) Knowledge Base ('Focus Genes'). To build networks, Ingenuity queries the Ingenuity Pathways Knowledge Base for interactions between the focus genes and all other genes/gene products stored in the Ingenuity Pathways Knowledge Base. The interactions utilized for the network involve direct relationships that describe physical (binding) and functional (phosphorylation, transcription, proteolysis, etc.) interactions between proteins and genes. IPA computes a score for each network, which is derived from a p-value and indicates the likelihood of the Focus Genes in a network being found together due to random chance. Although the network in Figure 4 (see Chapter 3, page 85) consists of a limited number of proteins, this approach may represent an initial step towards studying the systems biology of the kidney. Inclusion of these proteins in databases, such as the one for renal collecting duct proteins, may facilitate and expedite future research initiatives [52].

It can be expected that an increasing number of similar networks will become available in the near future. At the same time, it will become increasingly important to standardize and validate these data. Unavoidably, incomplete, unstandardized, or false positive results will be included in these databases. Although the databases may be subjected to post-hoc validation analyses, quantitative proteomics with a statistically reliable output may enhance reliable database construction *a priori*. Further development of quantitative proteomics platforms, both DIGE- and LC-based, in concert with powerful bioinformatics techniques will likely become an important focus of systems biology in the near future. Ultimately, more formalized

modeling techniques need to be incorporated [53]. Such approaches as Bayesian network analysis [54] and differential equation based mathematical models [55] will be utilized, allowing interaction between mathematical modelling and experimental approaches [46]. Well-developed and tested models may then increase the understanding of how diseases perturb the normal network structures of a system [56]. Eventually, this may pave the way for more predictive and preventive medicine [56].

References

1. Knepper MA. Proteomics and the kidney. *J Am Soc Nephrol* 2002; 13: 1398-1408.
2. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature* 2003; 422: 198-207.
3. Hoorn EJ, Hoffert JD, Knepper MA. Combined proteomics and pathways analysis of collecting duct reveals a protein regulatory network activated in vasopressin escape. *J Am Soc Nephrol* 2005; 16: 2852-2863.
4. Steen H, Pandey A. Proteomics goes quantitative: measuring protein abundance. *Trends Biotechnol* 2002; 20: 361-364.
5. VanBogelen RA, Molloy MP. Exploring the proteome: reviving emphasis on quantitative protein profiling. *Proteomics* 2003; 3: 1833-1834.
6. Molloy MP, Brzezinski EE, Hang J, McDowell MT, VanBogelen RA. Overcoming technical variation and biological variation in quantitative proteomics. *Proteomics* 2003; 3: 1912-1929.
7. Gorg A, Weiss W, Dunn MJ. Current two-dimensional electrophoresis technology for proteomics. *Proteomics* 2004; 4: 3665-3685.
8. Unlu M, Morgan ME, Minden JS. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis* 1997; 18: 2071-2077.
9. de Graauw M, Tijdens I, Cramer R, Corless S, Timms JF, van de Water B. Heat shock protein 27 is the major differentially

- phosphorylated protein involved in renal epithelial cellular stress response and controls focal adhesion organization and apoptosis. *J Biol Chem* 2005; 280: 29885-29898.
10. Sharma K, Lee S, Han S, Francos B, McCue P, Wassell R, et al. Two-dimensional fluorescence difference gel electrophoresis analysis of the urine proteome in human diabetic nephropathy. *Proteomics* 2005; 5: 2648-2655.
 11. Curthoys NP, Taylor L, Hoffert JD, Knepper MA. Proteomic Analysis of the Adaptive Response of Rat Renal Proximal Tubules to Metabolic Acidosis. *Am J Physiol Renal Physiol* 2006 Aug 8 Epub.
 12. van Balkom BW, Hoffert JD, Chou CL, Knepper MA. Proteomic analysis of long-term vasopressin action in the inner medullary collecting duct of the Brattleboro rat. *Am J Physiol Renal Physiol* 2004; 286: F216-F224.
 13. Hoffert JD, van Balkom BW, Chou CL, Knepper MA. Application of difference gel electrophoresis to the identification of inner medullary collecting duct proteins. *Am J Physiol Renal Physiol* 2004; 286: F170-F179.
 14. Yates JR 3rd, Gilchrist A, Howell KE, Bergeron JJ. Proteomics of organelles and large cellular structures. *Nat Rev Mol Cell Biol* 2005; 6: 702-714.
 15. Petricoin EF, Liotta LA. SELDI-TOF-based serum proteomic pattern diagnostics for early detection of cancer. *Curr Opin Biotechnol* 2004; 15: 24-30.
 16. Pixton KL. Is it functional? Report on the first BSPR/EBI meeting on functional proteomics. *Proteomics* 2004; 4: 3762-3764.
 17. Hoorn EJ, Pisitkun T, Zietse R, Gross P, Frokiaer J, Wang NS, et al. Prospects for urinary proteomics: exosomes as a source of urinary biomarkers. *Nephrology (Carlton)* 2005; 10: 283-290.
 18. Lilley KS, Friedman DB. All about DIGE: quantification technology for differential-display 2D-gel proteomics. *Expert Rev Proteomics* 2004; 1: 401-409.
 19. Alban A, David SO, Bjorkesten L, Andersson C, Sloge E, Lewis S, et al. A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics* 2003; 3: 36-44.

20. Shaw J, Rowlinson R, Nickson J, Stone T, Sweet A, Williams K, et al. Evaluation of saturation labelling two-dimensional difference gel electrophoresis fluorescent dyes. *Proteomics* 2003; 3: 1181-1195.
21. Stasyk T, Huber LA. Zooming in: fractionation strategies in proteomics. *Proteomics* 2004; 4: 3704-3716.
22. Oda Y, Nagasu T, Chait BT. Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome. *Nat Biotechnol* 2001; 19: 379-382.
23. Klein E, Klein JB, Thongboonkerd V. Two-dimensional gel electrophoresis: a fundamental tool for expression proteomics studies. *Contrib Nephrol* 2004; 141: 25-39.
24. Gorg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, et al. The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 2000; 21: 1037-1053.
25. Fodor IK, Nelson DO, Alegria-Hartman M, Robbins K, Langlois RG, Turteltaub KW, et al. Statistical challenges in the analysis of two-dimensional difference gel electrophoresis experiments using DeCyderTM. *Bioinformatics* 2005; 21: 3733-3740.
26. Lonstedt I, Speed TP. Replicated micro-array data. *Stat Sin* 2002; 12: 31-46.
27. Bland JM, Altman DG. Multiple significance tests: the Bonferroni method. *BMJ* 1995;310:170.
28. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. *Behav Brain Res* 2001; 125: 279-284.
29. Kreil DP, Karp NA, Lilley KS. DNA microarray normalization methods can remove bias from differential protein expression analysis of 2D difference gel electrophoresis results. *Bioinformatics* 2004; 20: 2026-2034.
30. Arthur JM. Proteomics. *Curr Opin Nephrol Hypertens* 2003; 12: 423-430.
31. Nielsen S, Frokiaer J, Marples D, Kwon TH, Agre P, Knepper MA. Aquaporins in the kidney: from molecules to medicine. *Physiol Rev* 2002; 82: 205-244.
32. Stokes JB, Grupp C, Kinne RK. Purification of rat papillary collecting duct cells: functional and metabolic assessment. *Am J Physiol* 1987; 253: F251-F262.

33. Laycock JF. Review: the Brattleboro rat with hereditary hypothalamic diabetes insipidus as an ideal experimental model. *Lab Anim* 1976; 10: 261-270.
34. Adroge HJ, Madias NE . Hyponatremia. *N Engl J Med* 2000; 342: 1581-1589.
35. Jaenike JR, Waterhouse C. The renal response to sustained administration of vasopressin and water in man. *J Clin Endocrinol Metab* 1961; 21: 231-242.
36. Schwartz WB, Bennett W, Curelop S, Bartter FC. A syndrome of renal sodium loss and hyponatremia probably resulting from inappropriate secretion of antidiuretic hormone. 1957. *J Am Soc Nephrol* 2001; 12: 2860-2870.
37. Qin S, Ferdinand AS, Richie JP, O'Leary MP, Mok SC, Liu BC. Chromatofocusing fractionation and two-dimensional difference gel electrophoresis for low abundance serum proteins. *Proteomics* 2005; 5: 3183-3192.
38. Xiong S, Ding Q, Zhao Z, Chen W, Wang G, Liu S. A new method to improve sensitivity and resolution in matrix-assisted laser desorption/ionization time of flight mass spectrometry. *Proteomics* 2003; 3: 265-272.
39. Luche S, Santoni V, Rabilloud T. Evaluation of nonionic and zwitterionic detergents as membrane protein solubilizers in two-dimensional electrophoresis. *Proteomics* 2003; 3: 249-253.
40. Santoni V, Molloy M, Rabilloud T. Membrane proteins and proteomics : un amour impossible ? *Electrophoresis* 2000; 21: 1054-1070.
41. Macfarlane DE. Two dimensional benzyldimethyl-n-hexadecylammonium chloride-sodium dodecyl sulfate preparative polyacrylamide gel electrophoresis: a high capacity high resolution technique for the purification of proteins from complex mixtures. *Anal Biochem* 1989; 176: 457-463.
42. Hartinger J, Stenius K, Hogemann D, Jahn R. 16-BAC/SDS-PAGE: a two-dimensional gel electrophoresis system suitable for the separation of integral membrane proteins. *Anal Biochem* 1996; 240: 126-133.
43. Bunai K, Yamane K. Effectiveness and limitation of two-dimensional gel electrophoresis in bacterial membrane protein

- proteomics and perspectives. *J Chromatogr B Analyt Technol Biomed Life Sci* 2005; 815: 227-236.
44. Zahedi RP, Meisinger C, Sickmann A. Two-dimensional benzyltrimethyl-n-hexadecylammonium chloride/SDS-PAGE for membrane proteomics. *Proteomics* 2005; 5: 3581-3588.
 45. Knepper MA, Masilamani S. Targeted proteomics in the kidney using ensembles of antibodies. *Acta Physiol Scand* 2001; 173: 11-21.
 46. Westerhoff HV, Palsson BO. The evolution of molecular biology into systems biology. *Nat Biotechnol* 2004; 22: 1249-1252.
 47. Aderem A. Systems biology: its practice and challenges. *Cell* 2005; 121: 511-513.
 48. Kalume DE, Molina H, Pandey A. Tackling the phosphoproteome: tools and strategies. *Curr Opin Chem Biol* 2003; 7: 64-69.
 49. Liu ET. Systems biology, integrative biology, predictive biology. *Cell* 2005; 121: 505-506.
 50. Siripurapu V, Meth J, Kobayashi N, Hamaguchi M. DBC2 significantly influences cell-cycle, apoptosis, cytoskeleton and membrane-trafficking pathways. *J Mol Biol* 2005; 346: 83-89.
 51. Calvano SE, Xiao W, Richards DR, Feliciano RM, Baker HV, Cho RJ, et al. A network-based analysis of systemic inflammation in humans. *Nature* 2005; 437: 1032-1037.
 52. Legato J, Knepper MA, Star RA, Mejia R. Database for renal collecting duct regulatory and transporter proteins. *Physiol Genomics* 2003; 13: 179-181.
 53. Papin JA, Hunter T, Palsson BO, Subramaniam S. Reconstruction of cellular signalling networks and analysis of their properties. *Nat Rev Mol Cell Biol* 2005; 6: 99-111.
 54. Sachs K, Perez O, Pe'er D, Lauffenburger DA, Nolan GP. Causal protein-signaling networks derived from multiparameter single-cell data. *Science* 2005; 308: 523-529.
 55. Moraru II, Loew LM. Intracellular signaling: spatial and temporal control. *Physiology (Bethesda)* 2005; 20: 169-179.
 56. Hood L, Heath JR, Phelps ME, Lin B. Systems biology and new technologies enable predictive and preventative medicine. *Science* 2004; 306: 640-643.

Chapter 3

**Combined proteomics and pathways
analysis of collecting duct reveals a
protein regulatory network activated in
vasopressin escape**

**Ewout J. Hoorn, Jason D. Hoffert
and Mark A. Knepper**

J Am Soc Nephrol 2005; 16: 2852–63

Abstract

Low sensitivity is characteristic of many proteomics methods. Presented here is an approach that combines proteomics based on difference gel electrophoresis (DIGE) with bioinformatic pathways analysis to identify both abundant and relatively nonabundant proteins in inner medullary collecting duct (IMCD) altered in abundance during escape from vasopressin-induced antidiuresis. Rats received the vasopressin analog dDAVP by osmotic minipump plus either a daily water load (vasopressin escape) or only enough water to replace losses (control). Immunoblotting confirmed the hallmark of vasopressin escape, a decrease in aquaporin-2, and demonstrated a decrease in the abundance of the urea transporter UT-A3. DIGE identified 22 mostly high-abundance proteins regulated during vasopressin escape. These proteins were analyzed using pathways analysis software to reveal protein clusters incorporating the proteins identified by DIGE. A single dominant cluster emerged that included many relatively low-abundance proteins (abundances too low for DIGE identification), including several transcription factors. Immunoblotting confirmed a decrease in total and phosphorylated c-myc, a decrease in c-fos, and increases in c-jun and p53. Furthermore, immunoblotting confirmed hypothesized changes in other proteins in the proposed network: Increases in c-src, receptor for activated C kinase 1, calreticulin, and caspase 3 and decreases in steroid receptor co-activator 1, Grp78/BiP, and annexin A4. This combined approach proved capable of uncovering regulatory proteins that are altered in response to a specific physiologic perturbation without being detected directly by DIGE. The results demonstrate a dominant protein regulatory network in IMCD cells that is altered in association with vasopressin escape, providing a new framework for further studies of vasopressin signaling in IMCD.

Introduction

Escape from the antidiuretic action of vasopressin (“vasopressin escape”) is an important physiologic process that limits the severity of the syndrome of inappropriate antidiuresis (SIADH) and other hyponatremic disorders [1,2]. During vasopressin escape, humans and experimental animals undergo a brisk water diuresis, despite high circulating levels of vasopressin [2–4]. Vasopressin escape is of considerable clinical importance with regard to SIADH and other vasopressin-dependent dilutional hyponatremic states, because without escape, further water retention and hyponatremia could be fatal [4–6].

Studies in a rat model of vasopressin escape have demonstrated that the central feature of the vasopressin-independent increase in water excretion is a marked suppression of the expression of the water channel aquaporin-2 (AQP2) [2]. The fall in AQP2 protein abundance is due in part to decreased levels of AQP2 mRNA in collecting duct [2]. This response is associated with a decrease in the capacity of inner medullary collecting ducts (IMCD) to produce cAMP in response to vasopressin [7] in association with a fall in vasopressin-binding capacity of the V2R receptor [8]. In contrast, there is upregulation of AQP-3 [2] and the α -subunit of the epithelial Na channel (ENaC) [9], suggesting that the process that is responsible for suppression of AQP2 expression is selective. With regard to possible mediators of escape, roles have been suggested for nitric oxide and prostaglandins [10] and aldosterone [11]. However, little is known about which intracellular signaling processes orchestrate the escape and how the vasopressin escape phenomenon is triggered and maintained.

To address which proteins are co-regulated with AQP2 in IMCD during vasopressin escape, we used a relatively new differential proteomics method called difference gel electrophoresis (DIGE) coupled with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry to identify differentially expressed proteins. Recently, we demonstrated the feasibility of such an approach using DIGE-based proteomics to identify vasopressin-responsive proteins in the collecting duct of Brattleboro rats that were treated with the selective V2R agonist dDAVP [12].

To address further the signaling pathways that are activated during vasopressin escape, we analyzed the DIGE results using bioinformatic pathways analysis [13,14]. The pathways analysis software generates hypothetical protein networks, based on large databases of protein interactions culled from the biologic literature, including physical binding reactions, cis-trans interactions in transcriptional regulation, and enzyme–substrate relationships. Such networks can be used to predict signaling pathways that are activated during vasopressin escape. The rationale for using the pathways analysis approach was not only to facilitate the interpretation of the relationships between the identified proteins but also to identify relatively low-abundance proteins (abundances too low for DIGE identification) that may be involved in vasopressin escape [15]. The hypothetical changes in these low-abundance proteins then can be tested by immunoblotting. This integrated approach identified a single dominant network of proteins that includes several proteins that may play key regulatory roles in vasopressin escape

Methods

Animals and sample preparation

Osmotic minipumps (model 2001; Alzet, Palo Alto, CA) that deliver 5 ng/h dDAVP (Peninsula Laboratories, San Carlos, CA; ACUC Protocol 2-KE-3) were implanted subcutaneously in Male Sprague-Dawley rats (Taconic Farms, Germantown, NY). After 3 d, rats were divided into two groups: “escape” and “control.” Escape rats were given excess daily water (0.4 ml/g body wt) *via* a gelled-agar diet (71% water, 28% finely ground rat food, 1% agar; BACTO-AGAR; Difco Laboratories, Detroit, MI). This diet forced the rats to take the water load to consume the food ration. Control rats were given the same amount of food but with only enough water (0.075 ml/g body wt) to compensate for insensible losses plus 0.015 ml/g body wt per d urine. Rats did not receive *ad libitum* water. This represents a small modification from our previous studies in which control rats received no water mixed with the food but were allowed to receive *ad libitum* water [2,7,9]. The rats were maintained in metabolic cages in a temperature- and humidity-controlled room with a 12:12-h light-dark cycle, and urine was collected daily for measurement of volume and osmolality. Because the onset of escape is known to occur between 1 and 2 d after the start of water loading [2], three time points were analyzed (four control and four escape for each time point): After 1 and 2 d of water loading (early stages of escape) and after 4 d of water loading (late stages of escape). Thus, a total of 24 rats were separated into 12 control rats and 12 escape rats, and four *versus* four rats were selected arbitrarily for IMCD analysis at each of the three time points. The day 1 and day 4 time points were selected for DIGE analysis, whereas all three time points were analyzed by

immunoblotting. IMCD suspensions were prepared using the method of Stokes *et al.* [16] with some modifications [17] (Supplemental Materials available online at www.jasn.org).

Semiquantitative immunoblotting

Immunoblotting was carried out as described previously [18] (see Supplemental Materials). The IMCD pellet was solubilized in 5x Laemmli sample buffer (1 vol per 4 vol of sample) followed by heating to 60°C for 15 min before electrophoresis. Equal loading was confirmed by staining gels loaded for all three time points (24 samples) with Coomassie blue [18]. This loading gel was scanned with a linear fluorescence scanner (Odyssey; Li-Cor Biosciences, Lincoln, NE) at an excitation wavelength of 700 nm (Supplemental Figure 1 available online).

DIGE

DIGE analysis was carried out (for day 1 and day 4 time points, each four *versus* four samples) as described previously [12,19] (see Supplemental Materials). Briefly, before two-dimensional (2-D) gel electrophoresis, IMCD proteins were solubilized in 2-D sample buffer (7 M urea, 2 M thiourea, 30 mM Tris Cl, and 4% CHAPS [pH 8.5]). The samples were labeled on lysine side chains with Cy3- (control), Cy5- (escape), or Cy2- (mixture of control + escape samples, internal standard) fluorophores using *N*-hydroxysuccinimide chemistry (Amersham). Isoelectric focusing was performed using an IPGphor apparatus (Amersham, Piscataway, NJ). Isoelectric focusing strips were loaded onto Ettan DALT-6 electrophoresis unit (Amersham) and further separated on a 10% SDS-PAGE gel (5 W/gel).

Fluorescence analytical gel images were obtained (Typhoon scanner; 100 μm resolution; Amersham) using the following emission filters: Cy2 (520 BP 40), Cy3 (580 BP 30), and Cy5 (670 BP 30). Spot matching, quantification, and statistical analyses were performed using DeCyder software (Version 5.0; Amersham). The corresponding Cy3 (control) and Cy5 (escape) images were normalized to the pooled internal standard (Cy2) for that gel using a Least-Means-Squared-Gradient-Descent algorithm. One gel was chosen as the “master,” and all remaining analytical gels were matched and normalized to the Cy2 master spot map. The resulting protein abundance ratios, now represented as standardized log abundance values, were compared using an unpaired t test. The inverse log of these values is presented in Table 1 as protein abundance ratio. A protein abundance ratio > 1 corresponds to an increase in escape compared with control samples, whereas a ratio < 1 corresponds to a decrease in escape (significance criterion, $p \leq 0.05$). For picking, gels were fixed in 30% ethanol/7.5% acetic acid for 2 h followed by SYPRO Ruby (610 BP 30) staining overnight for total protein visualization.

A robotic workstation (Ettan; Amersham) was used to excise protein spots, perform in-gel tryptic digestion, extract peptides from the gel, and transfer the extracts onto a MALDI substrate. Spectra were acquired with an ABI 4700 MALDI-TOF/TOF mass spectrometer, and proteins were identified by database matching using Mascot.

Bioinformatic pathways analysis

Regulated proteins identified by DIGE were analyzed further by bioinformatic pathways analysis (Ingenuity Pathway Analysis [IPA]; Ingenuity Systems, Mountain View, CA; www.ingenuity.com). IPA constructs hypothetical protein interaction clusters on the basis of a regularly updated “Ingenuity Pathways Knowledge Base.” The Ingenuity Pathways Knowledge Base is a very large curated database that consists of millions of individual relationships between proteins, culled from the biologic literature. These relationships involve direct protein interactions, including physical binding interactions, enzyme substrate relationships, and cis-trans relationships in transcriptional control. The networks are displayed graphically as nodes (individual proteins) and edges (the biologic relationships between the nodes).

In practice, a data set that contains the GenBank identifiers of differentially expressed proteins identified in the DIGE experiment is uploaded into IPA. IPA then builds hypothetical networks from these proteins, and other non-DIGE-identified proteins from the database that are needed fill out a protein cluster. Network generation is optimized for inclusion of as many proteins from the inputted expression profile as possible and aims for highly connected networks.

IPA computes a score for each possible network according to the fit of that network to the inputted proteins. The score is calculated as the negative base-10 logarithm of the p-value that indicates the likelihood of the inputted proteins in a given network being found together as a result of random chance. Therefore, scores of 2 or higher have at least a 99% confidence

of not being generated by random chance alone. For previous studies using IPA, see Siripurapu *et al.* [13] and Raponi *et al.* [14].

Results

Verifying vasopressin escape

In this model of vasopressin escape, both control and experimental rats received a continuous dDAVP infusion starting on day -3, but only the experimental rats received a daily water load, mixed with the food, starting on day 0. As previously noted [2,7,9], rats began to “escape” from dDAVP-induced antidiuresis on the second day, *i.e.*, 24 to 48 h after initiation of water loading, as evidenced by a marked increase in urine excretion rate (Figure 1A). Urine osmolality (Figure 1B) was reduced significantly in escape on the second day of water loading. Plasma sodium levels (Figure 1C) showed an acute decrease between days 1 and 2 (from 137 ± 2 to 105 ± 3 mmol/L) and, subsequently, a partial recovery on day 4 (115 ± 3 mmol/L) in response to vasopressin escape. Plasma urea levels were significantly lower in escape animals both at early (day 1: 7.0 ± 0.7 *versus* 5.0 ± 0.4 mmol/L) and late (day 4: 7.0 ± 0.5 *versus* 5.0 ± 0.4 mmol/L) stages of vasopressin escape (Figure 1D).

Changes in abundances of transport proteins in IMCD

Figure 2 shows immunoblots for AQP2, α -EnaC, and collecting duct urea transporters in IMCD on day 4 of vasopressin escape and densitometry values for all three time points. Downregulation of AQP2 and upregulation of α -EnaC is consistent with previous studies [7,9].

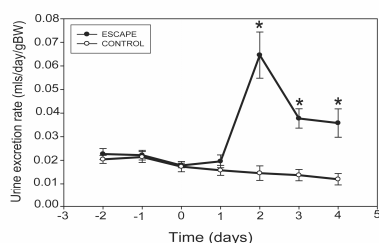
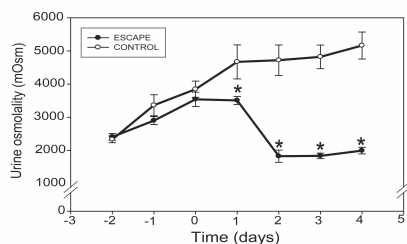
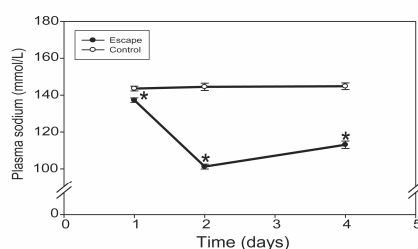
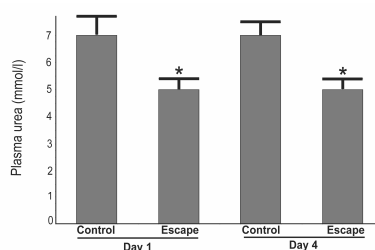
A Urinary excretion**B Urine osmolality****C Plasma sodium****D Plasma urea**

Figure 1: Urine excretion rate, urine osmolality, plasma sodium and plasma urea concentration during vasopressin escape

(A) Urine excretion rate over the course of the experiment with water loading commencing on day 0. Urine excretion rate was increased significantly from day 2 in the escape group relative to control (four versus four rats per time point). Negative time points represent the equilibration period.

(B) Urine osmolality over the course of the experiment. Osmolality was decreased significantly from day 1 to the end of the experiment in the escape group relative to control (four versus four rats per time point).

(C) Plasma sodium concentrations at days 1, 2, and 4. Plasma sodium was significantly lower in the escape group relative to control on all 3 d (four versus four rats per time point).

(D) Plasma urea concentrations at days 1 and 4. Plasma urea was significantly lower in the escape group relative to control on both days (four versus four rats per time point). * $p < 0.05$ for all.

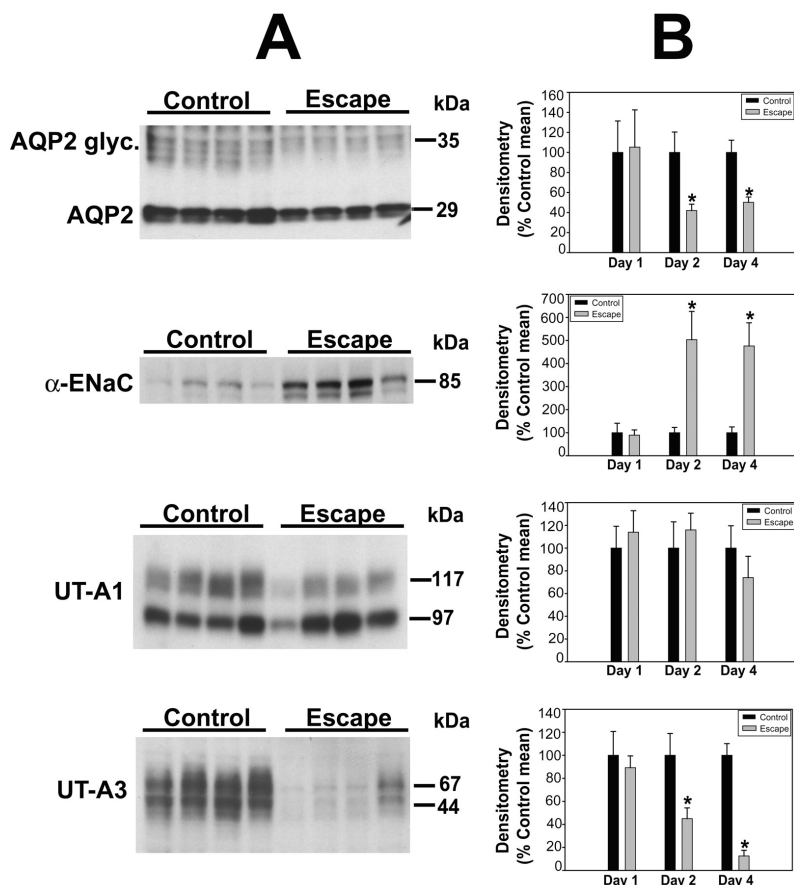


Figure 2: Immunoblots showing changes in abundances of aquaporin 2 (AQP2), α -subunit of epithelial Na channel (α -ENaC), and collecting duct urea transporters in inner medullary collecting ducts (IMCD) from rats that underwent vasopressin escape versus control rats. Immunoblots are of IMCD cells purified from rat renal medullas at late stage of vasopressin escape (day 4 time point). Each lane is loaded with a sample from a different rat ($n = 4$ rats per treatment). Thirty micrograms of total protein was loaded in each lane, and the resulting immunoblots were probed with anti-AQP2 antibody (L127), anti- α -ENaC (Q3560-2), anti-UT-A3 (Q2695-2), or anti-UT-A1 (L403). To the right of each immunoblot is a bar graph showing densitometry values for all three time points studied. $*p < 0.05$.

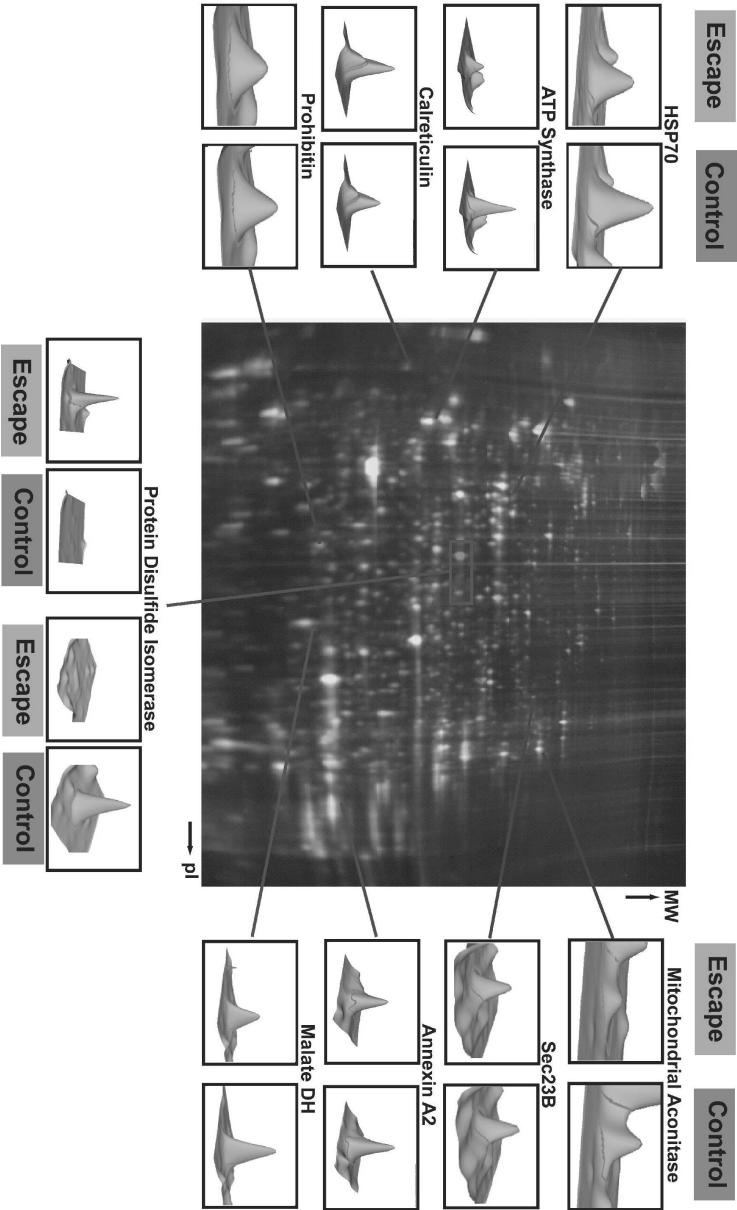
A novel finding was that UT-A3 showed a 50% decrease in band density on day 2 of vasopressin escape and was decreased further on day 4. Conversely, UT-A1 did not show a significant decrease.

Changes in rat IMCD proteome in vasopressin escape

Figure 3 shows an example of a DIGE gel with superimposition of Cy3 (control, red) and Cy5 (escape, green) images of the gel. Spots corresponding to proteins expressed at nearly equal levels in the two samples appear yellow, those upregulated in response to vasopressin escape appear green, and those downregulated in response to vasopressin escape appear red. Flanking the 2-D gel image are 3-D pixel density plots for nine selected proteins identified by MALDI-TOF/TOF mass spectrometry, including heat-shock protein 70 (HSP70), ATP synthase, calreticulin, prohibitin, mitochondrial aconitase, Sec23B, annexin A2, malate dehydrogenase, and protein disulfide isomerase (PDI). PDI had an apparent shift in isoelectric point, suggestive of a posttranslational modification. Only protein spots with statistically significant abundance ratios ($p \leq 0.05$ for four pairs of samples) were selected for MALDI-TOF/TOF identification. Moreover, only those identifications with expectation values (*i.e.*, an indicator of the degree of certainty of an identification) larger than 95% were accepted. A total of 22 protein spots were identified by MALDI-TOF/TOF mass spectrometry (Table 1). More proteins with altered abundance levels were identified at day 1 than at day 4.

Figure 3

Effects of Vasopressin Escape on IMCD Proteome



Legend Figure 3 (previous page):

Effects of vasopressin escape on IMCD proteome

Two-dimensional (2-D) gel showing changes in the IMCD proteome in vasopressin escape. Superimposed images from samples labeled with Cy3 (control, red pseudocolor), and Cy5 (escape, green pseudocolor) and 3-D representation of spot intensities. Spots that appear red or green represent proteins that are respectively down- or upregulated in vasopressin escape, whereas proteins that are equally abundant in both samples appear yellow. Full range of horizontal axis is from 3 pH units (left) to 10 pH units (right). Full range of vertical axis is 15 kD (bottom) to 120 kD (top). pI, isoelectric point; MW, molecular weight.

Those identified included proteins that were downregulated and upregulated and one protein that shifted position in the gel (Table 1; Figure 3) in response to vasopressin escape. Also listed in Table 1 are the theoretical molecular weights and theoretical isoelectric points. The molecular weights and isoelectric points for all of these proteins matched those derived from the spot position on the gel, providing additional verification of the identifications.

Table 1: IMCD proteins regulated at early (day 1) or later (day 4) stages of vasopressin escape

Ratio	Protein Identification	MW (kD)	pI	GenBank No.
<i>Decreased early in vasopressin escape (day 1)</i>				
0.39	ATP synthase β	50	5.0	gi 54792127
0.40	keratin complex 2, gene 8	51	5.7	gi 40786432
0.42	mitochondrial aconitase	86	7.9	gi 10637996
0.43	NADH dehydrogenase	81	5.5	gi 51858651
0.51	α B-crystallin	20	6.3	gi 16905067
0.58	GRP 75, stress 70 protein	75	5.9	gi 55584140
0.62	β -tubulin-1	51	4.9	gi 56754676
0.63	annexin A2	39	9.2	gi 584760
0.66	malate dehydrogenase 1	37	6.2	gi 15100179
0.71	F-actin capping protein β	31	5.7	gi 53734561
0.73	α -enolase	52	6.7	gi 56757324
0.74	pyruvate kinase	58	7.2	gi 125601
0.79	transketolase	68	7.2	gi 12018252
0.82	eukaryotic translation elongation factor 2	96	6.4	gi 8393296
0.82	α -1-antiproteinase	46	5.7	gi 1703024
0.83	Prohibitin	29	5.6	gi 34873234
0.88	Sec23B	87	6.5	gi 34858849
0.90	T-complex protein 1	58	6.6	gi 6981642
<i>Decreased later in vasopressin escape (day 4)</i>				
0.71	pyruvate kinase	58	7.2	gi 6981642
0.76	heat-shock protein 70	70	5.5	gi 450932
0.81	transketolase	68	7.2	gi 12018252
<i>Increased early in vasopressin escape (day 1)</i>				
1.28	calreticulin	48	4.3	gi 11693172
1.93	pyruvate dehydrogenase β	35	5.6	gi 1352624
<i>Spot shifted early in vasopressin escape (day 1)</i>				
0.19/25.15	protein disulfide-isomerase	57	5.9	gi 1352384

Pathways analysis of vasopressin escape

Figure 4 shows the largest protein cluster that was generated by the pathways analysis of the proteins listed in Table 1. This "vasopressin escape cluster" consists of a network of 33 proteins, including eight of the 22 proteins that were identified by DIGE-based proteomics and 25 additional proteins that were recognized as being related because of their reported interactions with the proteins identified by DIGE. The nodes represent individual proteins listed by gene name (see Table 2 for glossary), whereas the edges represent the interactions, which include direct physical binding, substrate–enzyme interactions, and/or cis-/trans relationship in transcriptional regulation. In Figure 4, nodes are displayed using different gray levels that represent how the protein was identified and studied. Proteins that were identified by DIGE only are represented as light gray nodes, whereas proteins that were identified by DIGE and studied further by immunoblotting are represented as black nodes. Proteins that were identified by IPA only are represented as white nodes, whereas proteins that were identified by IPA and studied further by immunoblotting are represented as dark gray nodes. Finally, the index protein AQP2 is shown. Because we were chiefly interested in identifying candidate proteins for follow-up by immunoblotting, we did not discriminate between the early and late time points for the pathways analysis. Annotation of the interactions in Figure 4 is provided as Supplemental Materials.

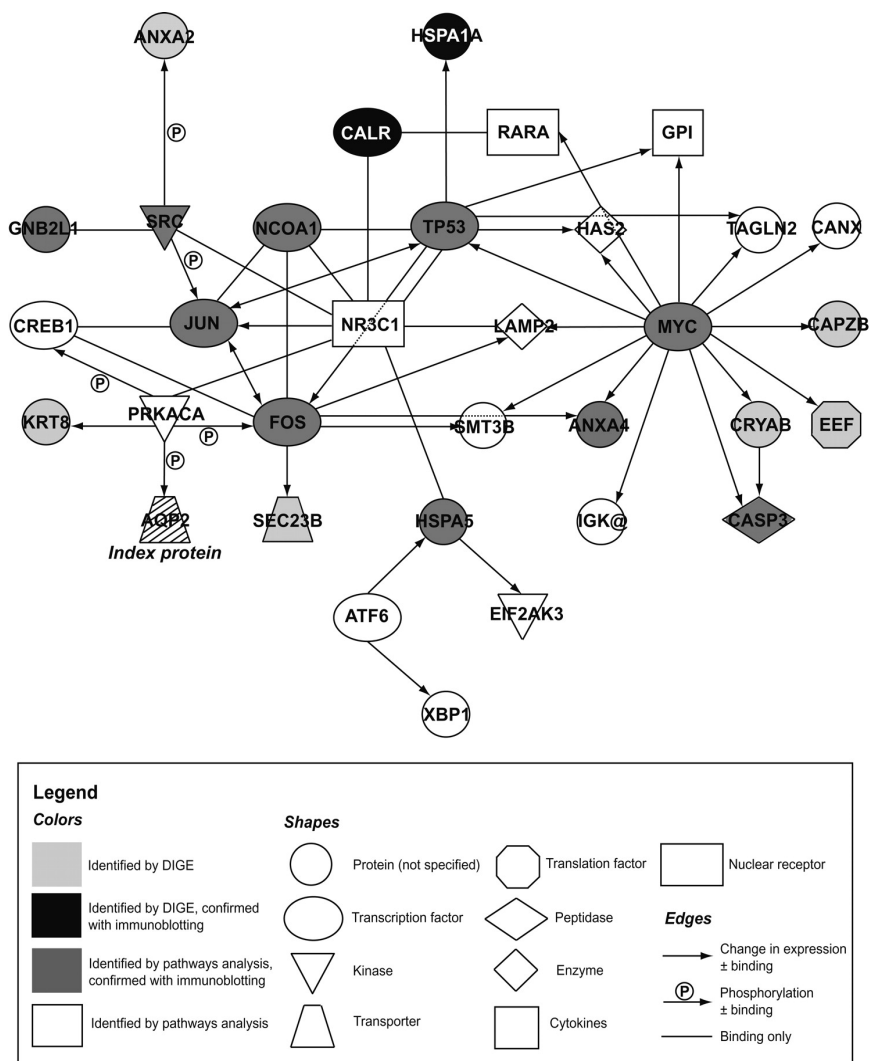


Figure 4: Protein regulatory network associated with vasopressin escape

Legend Figure 4 (previous page): *Protein regulatory network associated with vasopressin escape. Protein regulatory network was generated by bioinformatic pathways analysis through the use of the Ingenuity Pathways Analysis (IPA) software. Proteins that are listed in Table 1 were analyzed. Individual proteins are displayed as nodes, using different shades of gray to represent how the protein was identified and studied. In addition, different shapes are used to represent the functional class of the gene product (see figure insert). The edges describe the nature of the relationship between the nodes: An edge with arrowhead means that protein A acts on protein B, whereas an edge without an arrowhead represents binding only between two proteins. P indicates phosphorylation as a special case of the former. The gene names associated with the proteins are shown; see Table 2 for glossary. See Supplemental Materials for detailed descriptions of individual protein–protein interactions, including literature references. The overall score for the depicted network was 34, indicating that the probability of matching the indicated proteins by a purely random event was 10^{-34} .*

Confirmation by semiquantitative immunoblotting

Four of the 22 proteins that were identified by DIGE were selected for semiquantitative immunoblotting on the basis of the availability of high-quality antibodies. These were PDI, calreticulin, β -tubulin, and HSP70 (Figure 5). Although DIGE and immunoblotting gave percentage changes that differed somewhat for these four proteins, in general, immunoblotting confirmed the qualitative responses detected with the DIGE technique, as found previously [12,19]. Of the 25 proteins identified by pathways analysis as potentially involved in vasopressin escape (Figure 4), 10 were selected for semiquantitative immunoblotting on the basis of the central

positions in the protein cluster and of antibody availability. One of these is c-myc, a transcription factor that is regulated in part by phosphorylation. At early stages of escape, total c-myc abundance was decreased, whereas at later stages, the abundance of the phosphorylated form of c-myc was decreased (Figure 6).

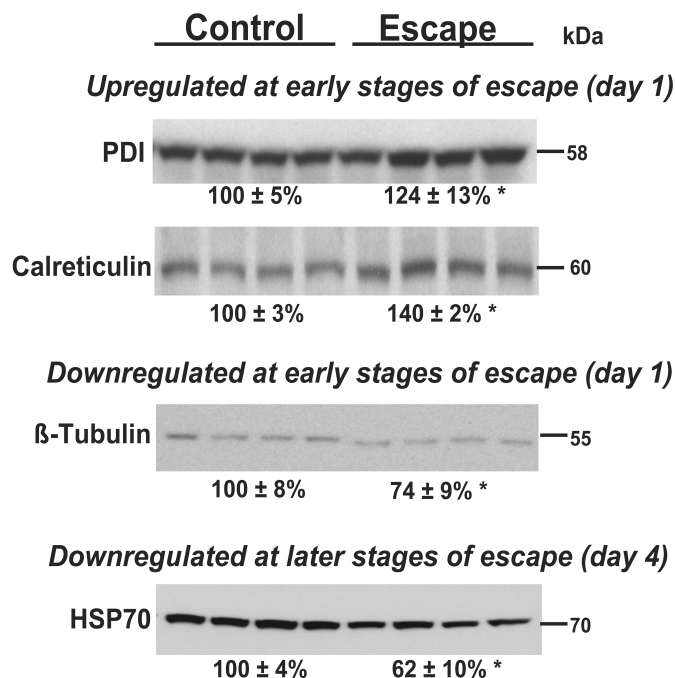


Figure 5: Immunoblots confirming selected protein abundance changes identified by DIGE-based proteomics

*IMCD suspensions, prepared at the day 1 and day 4 time points. Each lane is loaded with a sample from a different rat (n = 4 rats/treatment). Thirty micrograms of total protein were loaded in each lane, and the resulting immunoblots were probed with (for day 1) anti-protein disulfide isomerase (PDI), anti-calreticulin, anti-β-tubulin, and (for day 4) anti-heat shock protein 70 (HSP70). The apparent change in mobility of β-tubulin may represent an unidentified posttranslational modification. *p < 0.05.*

The abundances of four additional proteins that were identified by pathways analysis were decreased (annexin A4, GRP78/BiP, steroid receptor co-activator 1 [SRC-1], and c-fos; Figure 7A), whereas the abundances of five other proteins were increased (caspase 3, p53, receptor for activated C kinase 1 [RACK1], c-src, and c-jun; Figure 7B). The abundances of the proteins shown in Figures 5, 6, and 7 were changed only at the reported time point and were unchanged at the other time points (data not shown). To address whether the protein abundance changes are specific for the IMCD or occur in other tissues, we immunoblotted renal cortex and brain samples from the same experiments and probed with selected antibodies (Figure 8). Among the responding proteins in IMCD, only c-src showed a similar response in renal cortex, and only HSP70 showed a similar response in brain.

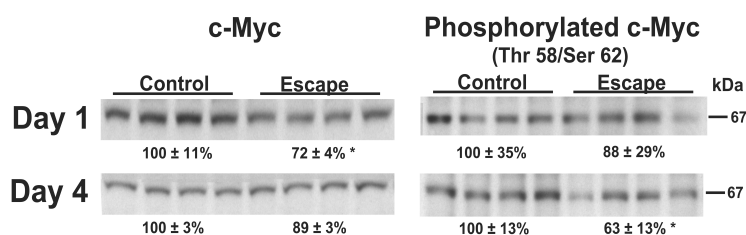


Figure 6: Immunoblots of IMCD proteins using antibodies to total and phosphorylated c-myc at early and late stages of vasopressin escape

*Each lane is loaded with a sample from a different rat ($n = 4$ rats/treatment). Thirty micrograms of total protein were loaded in each lane, and the resulting immunoblots were probed with anti-c-myc and anti-phosphorylated c-myc. * $p < 0.05$.*

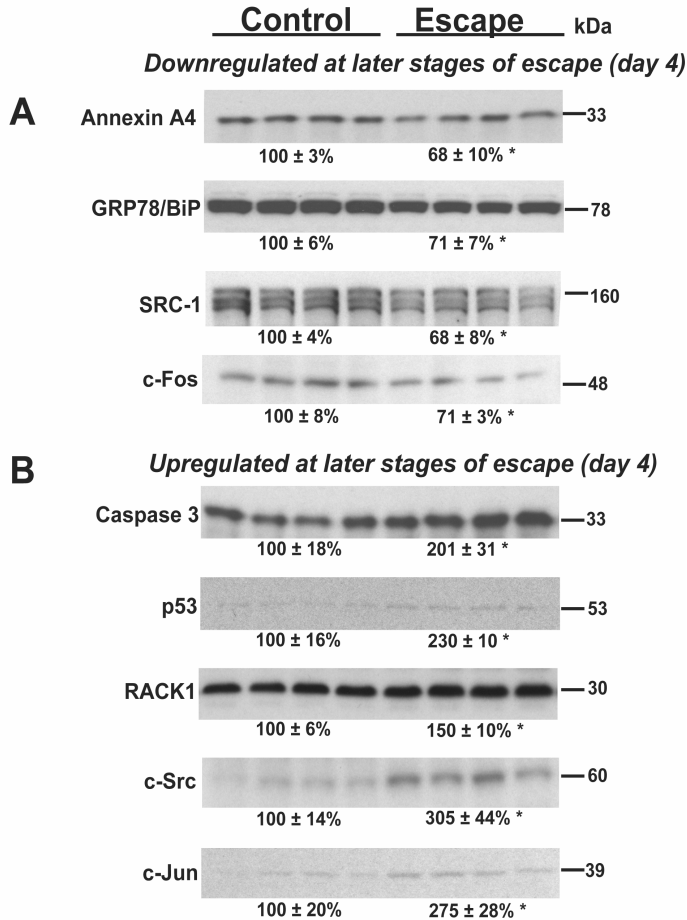


Figure 7: Immunoblots for selected proteins that were identified by pathways analysis

*IMCD proteins that were identified by pathways analysis were immunoblotted (30 µg of total protein per lane). Immunoblots show the regulation of proteins at late stages of vasopressin escape (day 4 time point); all proteins were unchanged at early stages of vasopressin escape (day 1; data not shown). Each lane is loaded with a sample from a different rat (n = 4 rats/treatment). Immunoblots were probed with antibodies to proteins indicated at left. *p < 0.05.*

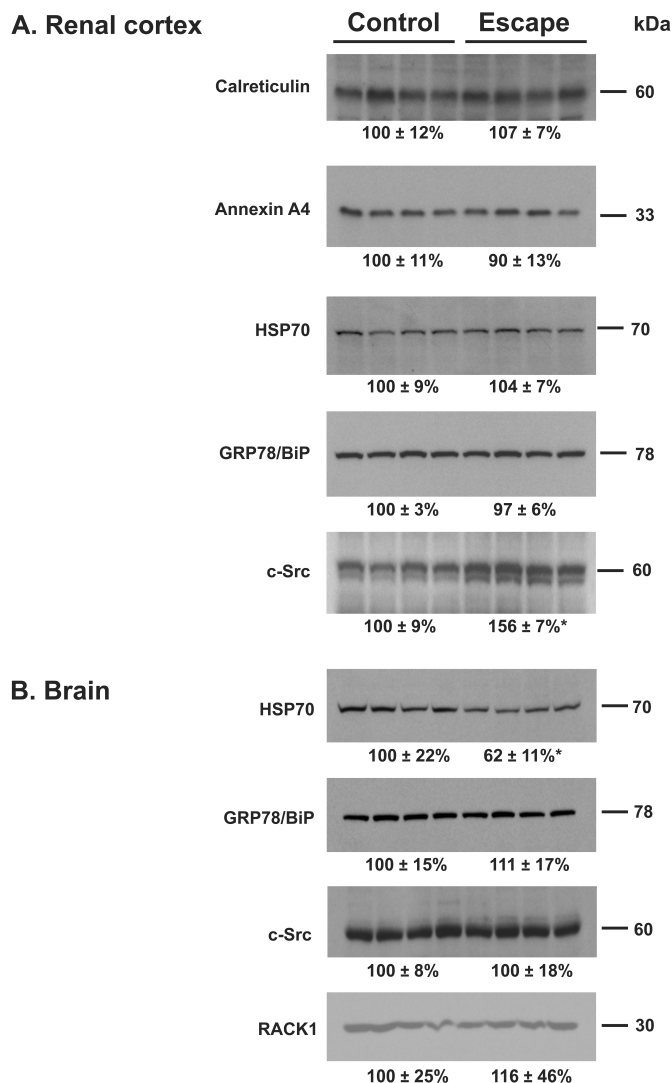


Figure 8: Immunoblots for selected proteins in renal cortex and brain tissue during vasopressin escape (day 4)

Each lane is loaded with a sample from a different rat ($n = 4$ rats/treatment), using renal cortex (A) and whole brain (B) homogenates. Thirty micrograms of total protein were loaded in each lane, and the resulting immunoblots were probed with antibodies to proteins indicated at left. * $p < 0.05$.

Discussion

Proteomics analysis is seeing increasing use as a means of identifying new mechanistic hypotheses in physiology [20]. An important disadvantage of all 2-D gel-based proteomics approaches is that low-abundance proteins, including many cell-signaling proteins, are unlikely to be identified [15,21]. Hence, alternative approaches are needed to discover transcription factors and other regulatory proteins that are involved in the responses to physiologic perturbations. In this study, we used a combination of DIGE-based proteomics and pathways analysis to identify a protein regulatory network whose state is altered in association with the vasopressin escape process and the associated downregulation of AQP2 expression. Proteins that were identified by DIGE were used as input data for bioinformatic pathways analysis, which pointed to several additional proteins that hypothetically could be involved in the escape process. These additional proteins corresponded individually to "hypotheses" that could be tested by immunoblotting asking whether each protein was up- or downregulated as part of the escape process. Indeed, 10 of 10 proteins that were tested in this way displayed the hypothesized regulation (Figures 6 and 7). The outcome of this integrated approach was a cluster of proteins, discussed below, that could participate in the onset and maintenance of escape or alternatively in secondary responses after vasopressin escape. Most of the demonstrated protein abundance changes occurred only in IMCD, not in renal cortex or brain, indicative of a selective response in the IMCD.

UT-A3 but not UT-A1 is downregulated during vasopressin escape

As a preliminary step in these experiments, we carried out immunoblotting to verify the downregulation of AQP2 seen in previous studies [2,9]. This study confirmed the decrease in AQP2 expression and showed that the α -subunit of ENaC is upregulated in IMCD in the same manner as in more proximal parts of the collecting duct [9]. In addition, we extended our studies to collecting duct urea transporter abundances and demonstrated that UT-A3 but not UT-A1 is markedly decreased in abundance during vasopressin escape (Figure 2). The decrease occurred in parallel with the reduction in AQP2. Because both UT-A1 (unchanged) and UT-A3 (downregulated) share the same transcription start site and upstream regulatory regions [22], it is unlikely that transcriptional regulation is the basis of the decrease in UT-A3 expression. Instead, recognizing that the 3' end of UT-A1 and UT-A3 transcripts differ [22], it seems possible that the differential regulation of these two proteins is based on the different 3' ends of the mRNA. Both mRNA stability regulation and translational regulation are based on specialized processes involving the 3' end of mRNA molecules [23], raising the hypothesis that either of these mechanisms may be involved in UT-A3 regulation.

The observed downregulation of UT-A3 could contribute to the increase in water excretion seen during the escape process by a mechanism similar to that demonstrated in knockout mice that lack both UT-A1 and UT-A3 [24]. The knockout mice developed a urinary concentrating defect because urea failed to accumulate in the inner medulla, resulting in a urea-dependent osmotic diuresis. Vasopressin escape is associated with modest

extracellular fluid volume (ECF) expansion and hypertension [10]. This ECF volume expansion could play a role in the decreased expression of collecting duct urea transporters, as suggested in a previous study that implicated aldosterone/salt-induced extracellular volume expansion in regulation of collecting duct urea transporter expression [25]. In our study, serum urea levels were markedly decreased as seen previously [11], a presumed consequence of UT-A3 downregulation.

Abundances of several transcription factors are altered during vasopressin escape process

Amid the protein regulatory network identified in this study are several transcription factors, whose cellular abundances are presumably too low to be detected *via* the DIGE technique. Several of these were demonstrated to undergo changes in abundance by immunoblotting. The earliest transcription factor to exhibit abundance changes was c-myc, whose abundance was found to be decreased just before the increase in water excretion (day 1). c-Myc is a basic helix-loop-helix leucine-zipper protein that binds as a heterodimer to so-called E-box cis-elements. The presence of three such E-box elements in the 5'-flanking region of the AQP2 gene [26] raises the possibility that c-myc abundance changes could contribute to the fall in AQP2 expression during the escape response. Although c-myc is best known as a tumor promotor oncogene, it also has physiologic functions in all cells that seem to be related to the state of differentiation and proliferation through general regulation of transcription and translation [27]. Its role in translational regulation was revealed in DNA array studies, which showed that changes in c-myc levels are associated with parallel changes in the expression of a host of ribosomal proteins [28]. The

demonstrated fall in c-myc expression therefore may be expected to be associated with a decrease in total cellular transcription and translation, at least at early stages of escape.

At later stages of vasopressin escape, we found a decrease in phosphorylated c-myc. The antibody recognizes c-myc phosphorylated at threonine-58 and serine-62. These modifications result in a decrease in half-life of the c-myc protein, thereby increasing the abundance of total c-myc. The phosphorylation is mediated largely by two kinases: c-jun N-terminal kinase [29] and glycogen synthase kinase-3 [30]. The decrease in phosphorylation may play a role in the restoration of total c-myc toward control levels on day 4 of the vasopressin escape protocol.

In addition to c-myc, several other transcription factors were identified whose abundances were altered at later stages of vasopressin escape, *viz.* c-fos (decreased), c-jun (increased), and p53 (increased). In addition, the abundance of a transcriptional co-factor, the SRC-1, was decreased.

c-Fos and c-jun together form the transcription factor called "activated protein 1" (AP-1), for which there is an enhancer site in the 5'-flanking region of the human AQP2 gene [31–33]. Previously, we demonstrated that c-fos and c-jun are upregulated in the rat renal inner medulla in response to long-term vasopressin infusion [34]. Previous studies have demonstrated that AP-1 and a cAMP response element are necessary for maximal transcriptional activation of the AQP2 gene in response to increased intracellular cAMP [31]. Conceivably, the demonstrated decrease in c-fos expression

contributes to the fall in AQP2 expression in vasopressin escape. c-Fos is itself regulated in part *via* a cAMP binding element in its 5'-flanking region [33]. SRC-1 is a transcriptional co-regulator that interacts with AP-1 and other transcription factors to mediate transcriptional regulation [35,36].

Abundances of several other regulatory proteins are altered during vasopressin escape

Aside from transcription factors, the protein regulatory network that was identified by pathways analysis included several other regulatory proteins that potentially could play a role in vasopressin escape. One of these was c-src, a nonreceptor tyrosine kinase whose abundance was increased three-fold during late stages of vasopressin escape (Figure 7). c-Src is a critical protein in the coupling between G-protein-coupled receptors and mitogen-activated protein kinase pathways [37]. c-Src as well as some of its substrates binds to β -arrestin 2 [38], a key protein in the internalization of the V2R. Note that c-src was also increased in renal cortical samples (Figure 8), suggesting that c-src upregulation may have been due to a systemic factor. Similarly, HSP70 was decreased in abundance not only in IMCD but also in brain, perhaps related to the fall in systemic tonicity [39]. Another protein whose abundance was upregulated was RACK1. Its function seems to be broader than its name suggests, because it constitutes a component of the ribosome and thus may play a role in translational regulation [40]. RACK1 is also antiapoptotic and a binding partner for c-src [41]. Finally, many of the identified regulated proteins also play a role in the endoplasmic reticulum (ER) stress response, including calreticulin, GRP78/BiP, PDI, and caspase 3. ER stress results

from situations in which the protein folding capacity of the ER is exceeded such as generalized acceleration of translation [42].

In conclusion, combined proteomics and pathways analysis served to identify a protein network that is associated with vasopressin escape and contains both high- and low-abundance proteins. The network included several transcription factors that may be involved in vasopressin escape as well as other relatively low-abundance regulatory proteins. These findings provide a new framework for the study of AQP2 regulation in the collecting duct, which is critical to the understanding of SIADH and other forms of hyponatremia.

Acknowledgments

This work was supported by the intramural budget of the National Heart, Lung, and Blood Institute (Z01-HL-01282-KE to M.A.K). E.J.H. was supported by the Dutch Kidney Foundation. We thank Dr. M. Michalak (University of Alberta, Canada) for kindly providing a calreticulin antibody, Angel Aponte for expert help with 2-D electrophoresis, Ellis Johns for assistance with immunoblotting, David Caden for expert help with blood chemistry, and Dr. R. Zietse for helpful discussions.

References

1. Adrogué HJ, Madias NE. Hyponatremia. *N Engl J Med* 2000; 342: 1581-1589.
2. Ecelbarger CA, Nielsen S, Olson BR, Murase T, Baker EA, Knepper MA, Verbalis JG. Role of renal aquaporins in escape from vasopressin-induced antidiuresis in rat. *J Clin Invest* 1997; 99: 1852-1863.
3. Jaenike JR, Waterhouse C. The renal response to sustained administration of vasopressin and water in man. *J Clin Endocrinol Metab* 1961; 21: 231-242.
4. Schwartz WB, Bennett W, Curelop S, Bartter FC. A syndrome of renal sodium loss and hyponatremia probably resulting from inappropriate secretion of antidiuretic hormone. 1957. *J Am Soc Nephrol* 2001; 12: 2860-2870.
5. Murase T, Ecelbarger CA, Baker EA, Tian Y, Knepper MA, Verbalis JG. Kidney aquaporin-2 expression during escape from antidiuresis is not related to plasma or tissue osmolality. *J Am Soc Nephrol* 1999; 10: 2067-2075.
6. Verbalis JG. Escape from antidiuresis: A good story. *Kidney Int* 2001; 60: 1608-1610.
7. Ecelbarger CA, Chou CL, Lee AJ, DiGiovanni SR, Verbalis JG, Knepper MA. Escape from vasopressin-induced antidiuresis: Role of vasopressin resistance of the collecting duct. *Am J Physiol* 1998; 274: F1161-F1166.
8. Tian Y, Sandberg K, Murase T, Baker EA, Speth RC, Verbalis JG. Vasopressin V2 receptor binding is down-regulated during renal escape from vasopressin-induced antidiuresis. *Endocrinology* 2000; 141: 307-314.
9. Ecelbarger CA, Knepper MA, Verbalis JG. Increased abundance of distal sodium transporters in rat kidney during vasopressin escape. *J Am Soc Nephrol* 2001; 12: 207-217.
10. Song J, Hu X, Khan O, Tian Y, Verbalis JG, Ecelbarger CA. Increased blood pressure, aldosterone activity, and regional differences in renal ENaC protein during vasopressin escape. *Am J Physiol Renal Physiol* 2004; 287: F1076-F1083.

11. Murase T, Tian Y, Fang XY, Verbalis JG. Synergistic effects of nitric oxide and prostaglandins on renal escape from vasopressin-induced antidiuresis. *Am J Physiol Regul Integr Comp Physiol* 2003; 284: R354–R362.
12. van Balkom BW, Hoffert JD, Chou CL, Knepper MA. Proteomic analysis of long-term vasopressin action in the inner medullary collecting duct of the Brattleboro rat. *Am J Physiol Renal Physiol* 2004; 286: F216–F224.
13. Siripurapu V, Meth J, Kobayashi N, Hamaguchi M. DBC2 significantly influences cell-cycle, apoptosis, cytoskeleton and membrane-trafficking pathways. *J Mol Biol* 2005; 346: 83–89.
14. Raponi M, Belly RT, Karp JE, Lancet JE, Atkins D, Wang Y. Microarray analysis reveals genetic pathways modulated by tipifarnib in acute myeloid leukemia. *BMC Cancer* 2004; 4: 56.
15. Knepper MA. Proteomics and the kidney. *J Am Soc Nephrol* 2002; 13: 1398–1408.
16. Stokes JB, Grupp C, Kinne RK. Purification of rat papillary collecting duct cells: functional and metabolic assessment. *Am J Physiol* 1987; 253: F251–F262.
17. Chou CL, DiGiovanni SR, Luther A, Lolait SJ, Knepper MA. Oxytocin as an antidiuretic hormone. II. Role of V2 vasopressin receptor. *Am J Physiol* 1995; 269: F78–F85.
18. Kim GH, Masilamani S, Turner R, Mitchell C, Wade JB, Knepper MA. The thiazide-sensitive Na-Cl cotransporter is an aldosterone-induced protein. *Proc Natl Acad Sci U S A* 1998; 95: 14552–14557.
19. Hoffert JD, van Balkom BW, Chou CL, Knepper MA. Application of difference gel electrophoresis to the identification of inner medullary collecting duct proteins. *Am J Physiol Renal Physiol* 2004; 286: F170–F179.
20. Thongboonkerd V, Barati MT, McLeish KR, Benarafa C, Remold-O'Donnell E, Zheng S, Rovin BH, Pierce WM, Epstein PN, Klein JB. Alterations in the renal elastin-elastase system in type 1 diabetic nephropathy identified by proteomic analysis. *J Am Soc Nephrol* 2004; 15: 650–662.
21. Thongboonkerd V. Proteomics in nephrology: Current status and future directions. *Am J Nephrol* 2004; 24: 360–378.

22. Fenton RA, Cottingham CA, Stewart GS, Howorth A, Hewitt JA, Smith CP: Structure and characterization of the mouse UT-A gene (Slc14a2). *Am J Physiol Renal Physiol* 2002; 282: F630–F638.
23. Audic Y, Hartley RS. Post-transcriptional regulation in cancer. *Biol Cell* 2004; 96: 479–498.
24. Fenton RA, Chou CL, Stewart GS, Smith CP, Knepper MA. Urinary concentrating defect in mice with selective deletion of phloretin-sensitive urea transporters in the renal collecting duct. *Proc Natl Acad Sci U S A* 2004; 101: 7469–7474.
25. Wang XY, Beutler K, Nielsen J, Nielsen S, Knepper MA, Masilamani S. Decreased abundance of collecting duct urea transporters UT-A1 and UT-A3 with ECF volume expansion. *Am J Physiol Renal Physiol* 2002; 282: F577–F584.
26. Ma T, Yang B, Kuo WL, Verkman AS. cDNA cloning and gene structure of a novel water channel expressed exclusively in human kidney: Evidence for a gene cluster of aquaporins at chromosome locus 12q13. *Genomics* 1996; 35: 543–550.
27. Schmidt EV. The role of c-myc in regulation of translation initiation. *Oncogene* 2004; 23: 3217–3221.
28. Louro ID, Bailey EC, Li X, South LS, McKie-Bell PR, Yoder BK, Huang CC, Johnson MR, Hill AE, Johnson RL, Ruppert JM. Comparative gene expression profile analysis of GLI and c-MYC in an epithelial model of malignant transformation. *Cancer Res* 2002; 62: 5867–5873.
29. Noguchi K, Kitanaka C, Yamana H, Kokubu A, Mochizuki T, Kuchino Y. Regulation of c-Myc through phosphorylation at Ser-62 and Ser-71 by c-Jun N-terminal kinase. *J Biol Chem* 1999; 274: 32580–32587.
30. Gregory MA, Qi Y, Hann SR. Phosphorylation by glycogen synthase kinase-3 controls c-myc proteolysis and subnuclear localization. *J Biol Chem* 2003; 278: 51606–51612.
31. Yasui M, Zelenin SM, Celsi G, Aperia A. Adenylate cyclase-coupled vasopressin receptor activates AQP2 promoter via a dual effect on CRE and AP1 elements. *Am J Physiol* 1997; 272: F443–F450.
32. Hozawa S, Holtzman EJ, Ausiello DA. cAMP motifs regulating transcription in the aquaporin 2 gene. *Am J Physiol* 1996; 270: C1695–C1702.

33. Uchida S, Sasaki S, Fushimi K, Marumo F. Isolation of human aquaporin-CD gene. *J Biol Chem* 1994; 269: 23451–23455.
34. Brooks HL, Ageloff S, Kwon TH, Brandt W, Terris JM, Seth A, Michea L, Nielsen S, Fenton R, Knepper MA. cDNA array identification of genes regulated in rat renal medulla in response to vasopressin infusion. *Am J Physiol Renal Physiol* 2003; 284: F218–F228.
35. Spencer TE, Jenster G, Burcin MM, Allis CD, Zhou J, Mizzen CA, McKenna NJ, Onate SA, Tsai SY, Tsai MJ, O'Malley BW. Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature* 1997; 389: 194–198.
36. Lee SK, Kim HJ, Na SY, Kim TS, Choi HS, Im SY, Lee JW. Steroid receptor coactivator-1 coactivates activating protein-1-mediated transactivations through interaction with the c-Jun and c-Fos subunits. *J Biol Chem* 1998; 273: 16651–16654.
37. Luttrell LM. 'Location, location, location:' Activation and targeting of MAP kinases by G protein-coupled receptors. *J Mol Endocrinol* 2003; 30: 117–126.
38. Miller WE, Maudsley S, Ahn S, Khan KD, Luttrell LM, Lefkowitz RJ. Beta-arrestin1 interacts with the catalytic domain of the tyrosine kinase c-SRC. Role of beta-arrestin1-dependent targeting of c-SRC in receptor endocytosis. *J Biol Chem* 2000; 275: 11312–11319.
39. Zhang Z, Ferraris JD, Brooks HL, Brisc I, Burg MB. Expression of osmotic stress-related genes in tissues of normal and hyposmotic rats. *Am J Physiol Renal Physiol* 2003; 285: F688–F693.
40. Sengupta J, Nilsson J, Gursky R, Spahn CM, Nissen P, Frank J. Identification of the versatile scaffold protein RACK1 on the eukaryotic ribosome by cryo-EM. *Nat Struct Mol Biol* 2004; 11: 957–962.
41. Chang BY, Harte RA, Cartwright CA: RACK1: A novel substrate for the Src protein-tyrosine kinase. *Oncogene* 21 : 7619–7629, 2002.
42. Schroder M, Kaufman RJ. ER stress and the unfolded protein response. *Mutat Res* 2005; 569: 29–63.

Chapter 4

Prospects for urinary proteomics: exosomes as a source of urinary biomarkers

Ewout J. Hoorn, Trairak Pisitkun

Robert Zietse, Peter Gross, Jørgen Frøkiær

Nam Sun Wang, Patricia A. Gonzales

Robert A. Star and Mark A. Knepper

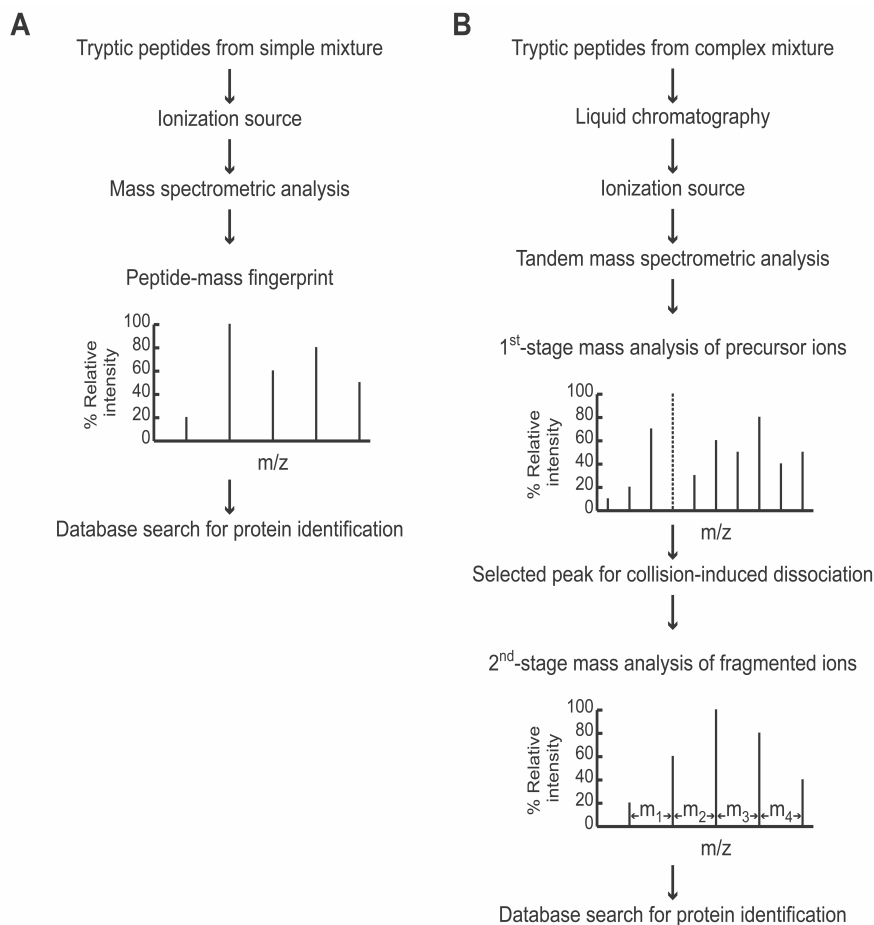
Nephrology (Carlton) 2005; 10: 283–90

Abstract

Recent progress in biotechnology offers the promise of better medical care at lower costs. Among the techniques that show the greatest promise is mass spectrometry of proteins, which can identify proteins present in body fluids and tissue specimens at a large scale. Because urine can be collected in large amounts in a non-invasive fashion, the potential exists to use mass spectrometry to discover urinary biomarkers that are early predictors of renal disease, or useful in making therapeutic choices. Recently, we demonstrated that both membrane proteins and cytosolic proteins from renal epithelia are highly enriched in low density urinary structures identified as exosomes. Exosomes were found to contain many disease-associated proteins including aquaporin-2, polycystin-1, podocin, nonmuscle myosin II, angiotensin converting enzyme, NKCC2, NCC, and ENaC. Potentially, other disease biomarkers could be discovered by mass spectrometry-based proteomic studies in well-defined patient populations. Here, we describe the advantages of using urinary exosomes as a starting material for biomarker discovery. In addition, the purpose of this review is to present an overall strategy for biomarker discovery in urine using exosomes and for developing cost-effective clinical assays for these biomarkers, which can potentially be used for early detection of disease, as a means of differential diagnosis, or as a means of guiding therapy. Finally, we emphasize potential barriers that need to be overcome before urinary proteomics can be applied clinically.

Introduction

A mass spectrometer is an instrument that measures the mass-to-charge ratio (m/z) of charged chemical species. Key breakthroughs were made in the early 1990's to create methods for ionizing peptides and delivering the resulting peptide ions to the near vacuum existing in a mass spectrometer [1,2]. These breakthroughs accounted for Nobel Prizes in Chemistry in 2002 to Fenn and Tanaka [1,2]. In general, a given protein can be identified by digesting it first with trypsin (or another protease) and then measuring the molecular masses of the resulting peptides with a mass spectrometer (a “peptide-mass fingerprint”) (Figure 1A). Each protein has a unique peptide-mass fingerprint, allowing its identification by comparison with the theoretical mass fingerprints of every known human or animal protein. Because of the success of the human genome project, human protein sequence databases are nearly complete. The most typical approach to peptide-mass fingerprinting uses two-dimensional electrophoresis to isolate individual proteins and MALDI-TOF mass spectrometry to identify them [3].

**Figure 1**

A. Peptide-mass fingerprint is used to identify proteins from a simple mixture (containing few proteins).

B. Tandem mass spectrometry coupling with upstream liquid chromatography (LC-MS/MS) is used to identify proteins from a complex mixture.

Abbreviations: m/z = mass-to-charge ratio, $m_{n(1-4)}$ = mass difference between two consecutive peaks representing residual mass of a particular amino acid.

The power of mass spectrometry has been made even greater by the addition of tandem mass spectrometers and by coupling tandem mass spectrometers to liquid chromatography to effect an initial separation of tryptic peptides (Figure 1B). Using such an LC-MS/MS approach, the specific proteins contained in a complex mixture can be identified without the need for initial purification of individual proteins. This LC-MS/MS approach and peptide-mass fingerprint approach have already been applied to identification of proteins in urine by a number of laboratories [4-6].

The purpose of this review is to present an overall strategy for biomarker discovery in exosomes isolated from urine and for developing cost-effective clinical assays for these biomarkers. We present a workflow paradigm for discovery of biomarkers, for validation of sensitivity and specificity of biomarker excretion in discrimination of target disease processes, and for creating cost-effective assays that can be made available for clinical use. We emphasize the major barriers to success with urinary proteomics including some seemingly mundane, but crucial questions such as how to collect urinary samples, how to store them, how to ship them, how to process them to enrich for low abundant markers, and how to quantify excretion rates of individual proteins. Finally, we will define criteria we believe the clinical syndrome under investigation should meet for urinary proteomics to be useful and we will provide examples to illustrate our point.

Sources of urinary proteins

A major challenge of urinary proteomics is to develop a rational means of reducing the complexity of the urinary proteome in order to enhance the detectability of relatively low abundance proteins that may have special pathophysiological significance. Theoretically, urinary proteins may originate from:

1. Glomerular filtration of plasma proteins
2. Renal tubular secretion of soluble proteins;
3. Whole cell shedding (sloughed cells);
4. Apical plasma membrane shedding (non-specific or apoptotic processes);
5. Glycosylphosphatidyl inositol (GPI) anchored protein detachment, e.g. Tamm-Horsfall Protein;
6. Exosome secretion;

Depending on the clinical syndrome, it might be prudent to select one or more of these protein-types as a starting material for biomarker discovery. Because we are interested in structural and/or functional defects in renal epithelia, in this paper we emphasize urinary exosomes as a starting material for biomarker discovery. Exosomes are small vesicles derived indirectly from the apical endosomal system and which contain many proteins already known to be associated with renal diseases [6]. The water channel aquaporin-2 (AQP2), an apical plasma membrane protein, is one biomarker present in exosomes that can be readily measured in urine [7] and which has been exploited in studies of various water balance disorders [8].

Secretion of urinary exosomes

After the discovery of AQP2 in the urine, various studies have been performed to determine the mechanism of its excretion. Immunoblot studies of different urinary fractions obtained from sequential centrifugation [7,9,10] demonstrated that urinary AQP2 and apical Na⁺ transporter proteins are present chiefly in small, low-density membrane vesicles obtained by high-speed ultracentrifugation. In addition, immuno-electron microscopy has confirmed the presence of AQP2 in small, low-density membrane vesicles [7,9]. This evidence suggested that whole cell or apical plasma membrane shedding was unlikely to be the main mechanism of apical plasma membrane protein excretion. However, further evidence was needed to explain the origin of small, low-density membrane vesicles. Wen and colleagues [9] revealed that urinary AQP2 is predominantly excreted via an apical pathway in the collecting duct because AQP2, but not AQP3 (a basolateral membrane marker), was excreted in urine at a significant level.

Recent studies have demonstrated that these small vesicles are so-called *exosomes* [6]. Urinary exosomes are the internal vesicles of multivesicular bodies (MVBs) that are delivered to the urinary space by fusion of the outer membrane of MVBs with the apical plasma membrane of renal tubule epithelial cells. This mechanism is shown and described in more detail in Figure 2.

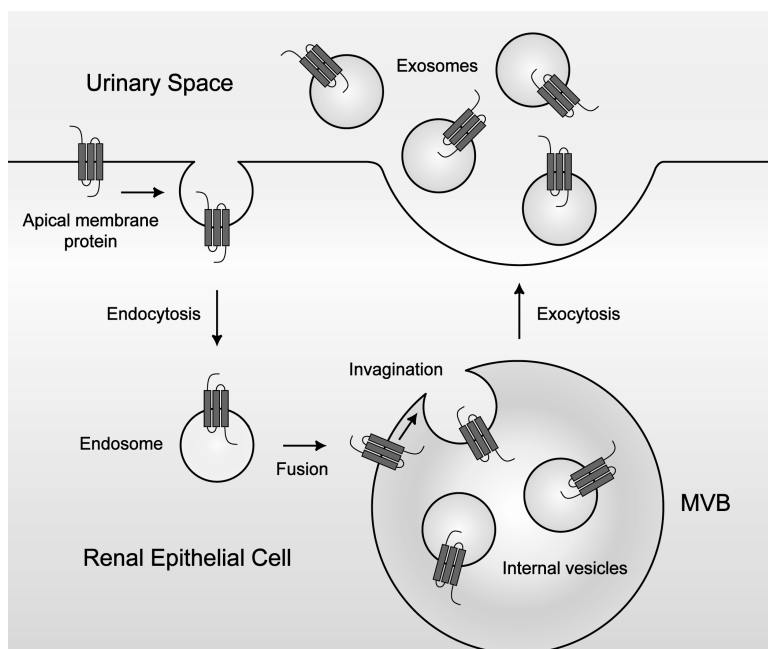


Figure 2: Urinary exosome formation

In the first step of the process apical membrane proteins undergo endocytosis. Secondly, the endosome is targeted to the multivesicular body (MVB). The signal that marks plasma membrane proteins for incorporation into MVBs is mono-ubiquitination. In the third step, the endosome fuses with the MVB. Consequently, the apical plasma membrane proteins are segregated in the MVB outer membranes and internalized by membrane invagination. Finally, the outer membrane of the MVB fuses with the apical plasma membrane releasing its internal vesicles, called “exosomes”, in the urinary space. Exosomes contain both membrane and cytosolic proteins.

Exosomes have been shown to derive from every epithelial cell type facing the urinary space from the podocyte to the transitional epithelium of the bladder. Exosomes have been previously demonstrated to be secreted by other cell types, including B cells, T cells, dendritic cells, reticulocytes,

mastocytes, enterocytes, platelets, erythroleukemia cells and other tumor cells.

The proteomic analysis of urinary exosomes using LC-MS/MS coupled with upstream 1-dimensional SDS-PAGE fractionation identified a roster of proteins directly involved in exosome biogenesis such as Class E vacuolar sorting proteins which have been found to mediate MVB formation in yeast [6]. Overall, the proteomic analysis of urinary exosomes identified 295 proteins, including at least 19 proteins already known to be associated with various renal diseases (Table 1).

Advantages of urinary exosomes as a starting material for biomarker discovery

The use of urinary exosomes for biomarker discovery has a number of important advantages, three of which will be addressed below.

1. Exosome isolation reduces the complexity of the urine proteome

The urine proteome contains a number of highly abundant proteins filtered from plasma as well as produced by renal tubular cells, especially Tamm-Horsfall protein. The presence of these abundant proteins complicates the detection of relatively low abundance proteins that may have special pathophysiological significance. This common dilemma is analogous to the proteomic analysis of blood plasma, which has been limited by the presence of highly abundant proteins such as albumin and immunoglobulins. Exosome isolation helps to minimize highly abundant proteins in urine and also enriches a subproteome that includes both membrane proteins and

cytosolic proteins trapped inside the exosomes. In the "barriers" section we will describe the urinary exosome isolation method in more detail, including strategies to remove high abundant proteins.

2. Exosomes contain important information from broad epithelial origins

Urinary exosomes contain cytosolic proteins entrained when the exosomes are formed by invagination of the outer membranes of multivesicular bodies (Figure 2), plus the proteome of the apical plasma membrane endocytic pathway, which plays a crucial role in the regulation of renal epithelial cell function. Our proteomic analysis of urinary exosomes identified proteins from all renal epithelia including the glomerular podocytes, the proximal tubule, the thick ascending limb of Henle, the distal convoluted tubule, the collecting duct, and the transitional epithelium of urinary bladder. Thus, based on our findings, urinary exosomes can be utilized to investigate the physiological or pathophysiological processes in virtually every epithelial cell type facing the urinary space.

3. Exosomes contain many disease associated proteins

The proteomic analysis of urinary exosomes identified at least 19 proteins that are known to be involved in specific kidney diseases or in blood pressure regulation (Table 1). Additional studies are likely to reveal many other disease related proteins in urinary exosomes. In the "barriers" section we will discuss how these potential biomarkers may lead to clinical studies of urinary proteomics.

Table 1: Proteins identified in urinary vesicles that are associated with kidney diseases or hypertension.

Kidney Diseases or Hypertension	Identified Proteins
Autosomal dominant and autosomal recessive nephrogenic diabetes insipidus	Aquaporin-2
Antenatal Bartter syndrome type 1	Sodium potassium chloride cotransporter-2
Gitelman's syndrome	Thiazide-sensitive Na-Cl cotransporter
Autosomal recessive pseudohypoaldosteronism type 1	Epithelial sodium channel α , β , γ
Liddle syndrome	Epithelial sodium channel β , γ
Familial renal hypomagnesemia	FXYD domain-containing ion transport regulator-2
Autosomal recessive syndrome of osteopetrosis with renal tubular acidosis	Carbonic anhydrase II
Proximal renal tubular acidosis	Carbonic anhydrase IV
Autosomal dominant polycystic kidney disease type 1	Polycystin-1
Medullary cystic kidney disease 2 and familial juvenile hyperuricemic nephropathy	Uromodulin
Autosomal recessive steroid-resistant nephrotic syndrome	Podocin
Fechtner syndrome and Epstein syndrome	Nonmuscle myosin heavy chain IIA
2,8-Dihydroxyadenine urolithiasis	Adenine phosphoribosyltransferase
Hypertension	Angiotensin I converting enzyme isoform-1 Aminopeptidase A Aminopeptidase N Aminopeptidase P Neprilysin Hydroxyprostaglandin dehydrogenase 15-(NAD) Dimethylarginine dimethylaminohydrolase-1

Workflow paradigm for discovery and validation of urinary biomarkers

Exosome isolation may provide an efficient first step in biomarker discovery in urine. Figure 3 shows the major steps that will be required for the development of urinary biomarker assays for routine clinical application. The first element is ‘discovery’ of potential biomarkers. For this, the power of LC-MS/MS is well established and application to the analysis of urinary exosomes may be predicted to identify one or more proteins excreted at a greater or lesser rate in the affected population than in an appropriate control group. Extremely well-defined patient populations are needed for these studies to avoid heterogeneity. An alternative to LC-MS/MS is to use two-dimensional electrophoresis [3], although this technique may be somewhat less efficient in the detection of membrane proteins and low abundance proteins. Certain biomarkers may be identified that are completely absent in the control subjects but strongly positive in the patients. More likely, however, quantitative changes in biomarker excretion rate will have to rely on discrimination of patients from controls or on discrimination between different subpopulations of patients. In principle, identification of differentially expressed proteins and quantification of proteins can be done by use of the isotope-coded affinity tag (ICAT) method [11] or by related methods.

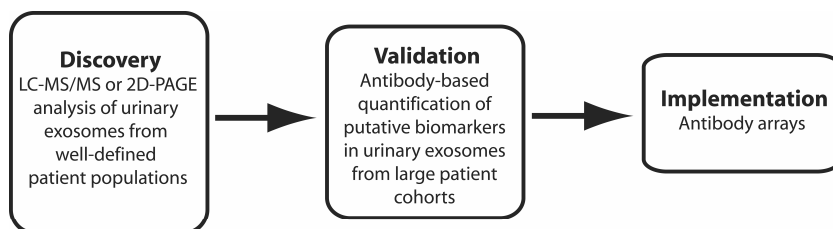


Figure 3: *This flowchart schematically represents the three major steps required for the development of urinary biomarker assays for routine clinical application. The flowchart consists of the ‘discovery’ of potential biomarkers by LC-MS/MS or 2D-PAGE, the subsequent validation of these biomarkers in larger clinical studies, and, finally, the clinical implementation through development of antibody arrays. Abbreviations: LC-MS/MS, Mass spectrometry coupled with upstream liquid chromatography; 2D-PAGE, Two-dimensional polyacrylamide gel electrophoresis.*

Once a set of potential biomarkers is discovered, it is necessary to carry out validation studies in larger groups of patients to test the hypothesis that measurements of biomarker excretion can discriminate patients from controls, or can discriminate among different subgroups of patients (Figure 3). In general, the strength of mass spectrometry is in protein identification and not in quantification, so it is likely that the validation phase can be completed more efficiently using antibody-based assays of protein abundance, rather than by quantitative mass spectrometry. The objective of these studies will be to get estimates of the sensitivity and specificity of the biomarker measurements in discriminating clinical groupings, prior to development of a product that can be applied to everyday clinical medicine. It is possible that a single biomarker will

allow adequate sensitivity and specificity for some renal disease processes, and a single enzyme-linked immunosorbent assay (ELISA) might prove cost effective. However, it is more likely that a combination of several biomarker measurements will prove necessary for disease discrimination. In this case, antibody arrays may provide an appropriate means of completing measurements for all relevant biomarkers at the lowest cost [12]. Antibody arrays are ensembles of antibodies specific for the relevant biomarkers, laid down as individual spots on a solid substrate such that urinary proteins will bind to them in proportion to their abundance in the original samples. Labeling of these antibodies with one fluorescent dye and a reference sample with another fluorescent dye allows quantification relative to the reference by two-channel fluorescence quantification [13]. Biomarkers relevant to a number of disease markers could be included in a single antibody array to allow a single array to be used for broad screening. For a more in-depth review on the workflow paradigm, we recommend a recent review by Hewitt *et al.* [14].

Barriers

Before urinary proteomics can proceed from bench to bedside, there are a number of important barriers that need to be overcome. These barriers are discussed below.

Collection, processing and storage of urinary samples

For quantitative comparisons, it is necessary to measure the rate of excretion of putative biomarkers. Thus a 24-hour urine collection would be desirable. However, this is an inconvenient procedure that is characterized by low patient compliance and thus unreliable results [15]. An alternative is to collect a spot urine sample and normalize the biomarker concentration by the creatinine concentration. For this, the first morning urine may be used, although this has the potential drawback of bladder and bacterial contamination due to a long residence-time in the bladder [16]. Thus, from the perspective of practicality and sterility, ideal initial conditions for urine collection may be the second morning urine. Studies on proteinuria have shown that the method of collecting random urine samples is valid and that the rate of protein excretion correlates well with that found in 24-hour urine [15].

Once the urine is collected, another potential problem is proteolysis [17], which can be dealt with through the use of protease inhibitors in the collection vessel [17]. However, some agents that are commonly used in the laboratory such as leupeptin and phenyl methyl sulfonyl fluoride (PMSF) are expensive. Hence, it will be important to determine the lowest cost set of protease inhibitors that are compatible with the protection of the collected human samples from degradation.

Alternatively, it is possible that protease inhibitors may not be needed for studies involving spot urine collections that are processed or frozen immediately.

Subsequently, before the urinary exosomes are isolated, procedures can be carried out to remove tubular casts, cells and abundant proteins that may obscure lower abundant proteins. Examples of the latter include the Tamm-Horsfall protein [6,18] and albumin when there is proteinuria [19]. Casts and intact cells can be removed by low speed centrifugation, albumin can be removed by albumin removal devices that use affinity ligands and, finally, Tamm-Horsfall protein can be removed with the use of reducing agents, like dithiothreitol [6]. Although the removal of Tamm-Horsfall protein is necessary for the analysis of exosomes, it was recently shown that this protein itself may have pathophysiological significance [20]. Hence, it could be possible that a simultaneous analysis of exosomes and other non-exosome proteins like Tamm-Horsfall may provide the most complete pathophysiological information. At least four different isolation protocols for urinary proteomics have been described [21]. Due to the variability in protein chemical and physical properties, different methods will isolate different subpopulations of urinary proteins [21], each of which may be appropriate for different clinical questions. We have used a protocol that employs ultracentrifugation to precipitate exosomes from urine (see above) [6]. This approach is potentially most valuable for the detection of defects in renal tubular function. It has been noted that this isolation protocol will enhance the detection of hydrophobic proteins in the apical endosomal pathway, whereas other isolation protocols, like the acetone precipitation technique, will increase the identification

of hydrophilic proteins that are derived from sources other than exosomes [21].

Another point that merits emphasis is that ultracentrifugation will be an impractical method to use for large-scale studies and for the ultimate clinical application of urinary proteomics because of the time and cost involved. Possible alternative methods for isolation of exosomes include filtration, adsorption, evaporation, dialysis, and/or immuno-isolation.

Storage of samples may be required either directly after the initial urine collection or after the actual exosome isolation. In addition to storage of samples, shipping of samples may also be required, especially if different laboratories collaborate in urinary proteomics projects. Prior to the undertaking of biomarker discovery studies in urine it would be important to develop standardized methods for storing and shipping of samples. Freezing of samples can potentially cause loss of exosomes due either to aggregation of vesicles or adsorption of exosomes to the surface of the collection vessel. Both problems could hypothetically be dealt with by adjusting sample pH or by adding a surfactant prior to freezing to minimize attraction between the vesicles. With regard to the degradation of frozen proteins over time, evidence from previous studies [22,23] suggests that there is considerable inter-protein variation in the degree of degradation. Furthermore, these studies concluded that if urinary proteins can be measured within four weeks after sampling, storage at 4 °C appears to give the best results. It may be desirable to add antibacterial agents like sodium azide or thymol. For longer time-periods, a storage temperature of -70 °C or -80 °C may be optimal. In both studies, storage at -20 °C

appeared least favorable both due to a higher degradation rate and the formation of a calcium-phosphate precipitate that was observed at this specific temperature [22,23]. The adjustment of pH prior to freezing did not appear to affect protein stability [23].

Quantification of urinary protein excretion and determination of its normal variability

A crucial step in urinary proteomics is the translation from biomarker discovery in a research setting to the diagnostic application in a clinical setting. This translation requires that a differentially expressed protein or group of proteins provides sufficient sensitivity, specificity and positive predictive value to be applied as a diagnostic test. As was discussed above in the working paradigm, this translation step should occur in the validation phase (Figure 3), where antibody-based assays of protein abundance rather than quantitative mass spectrometry is likely to be the most cost-effective technique. However, before differences in protein abundance can be interpreted, two questions should be addressed. The first question is how to quantify urinary protein excretion rates and the second question is how to establish the normal variability in excretion of a given urinary protein. Theoretically, the best choice for quantification of protein excretion is to carry out a timed urine collection and express the excretion rate in conventional rate units such as nanomols per hour. However, as discussed previously, patient compliance is a frequent problem with timed urine collections and most investigators have adopted approaches that can be applied to spot urines. A frequently used approach is to normalize by the excretion rate of creatinine, inulin or other filterable but non-reabsorbable markers, thereby eliminating the

time term in the variable [7]. If creatinine is used, it must be recognized that creatinine excretion rate is not invariant [24].

In the discovery phase, validation phase and clinical application phase, sample measurements in patients must be related to normal reference samples to draw conclusions. Thus, it is important to define a method for obtaining normal reference samples. To approach this problem, it would be useful to determine the intrinsic variability in the urinary proteome of normal human subjects. Potentially, the possible effects of age, gender, circadian rhythm, high and low salt diets and water-loading and restriction need to be investigated. The sensitivity, specificity and thus the clinical value of a candidate biomarker can only be defined relative to a carefully chosen control.

Criteria for and examples of urinary proteomics studies

Regardless of the specific diseases in which urinary proteomics may be applied, a number of general criteria that a disorder should meet for urinary proteomics to be useful should be defined. We argue that in this time of rising health care costs, biomarker discovery studies should be pursued only for patient populations for which measurements of the identified biomarkers have a realistic possibility of providing a significant improvement in patient care. We further argue that priority should be given to diseases for which interventions are likely to lead both to increased quality of life and reduced patient care costs. Finally, the diagnostic tools that are currently available for a specific disorder should be taken into consideration. Biomarker discovery may be pursued especially if the current

tools are nonexistent or inadequate due to cost, complexity, risk or accuracy.

In this paradigm, improved clinical decision making through the use of urinary proteomics may include the early detection of disease, the differential diagnosis of diseases with similar presentation but different therapeutic implications and guidance in the selection, evaluation or adjustment of a therapeutic regimen. A good but ambitious example in nephrology would be a situation where urinary proteomics could direct the physician to better diagnostic and therapeutic strategies that would either delay or even prevent end-stage renal failure.

Candidate biomarkers for the near future include the disease-associated proteins that were identified in urinary exosomes (Table 1), many of which may have diagnostic potential. One candidate biomarker is the Na^+/H^+ exchanger isoform 3 which in patients with acute renal failure was shown to be a marker of renal tubule damage, and may therefore help to differentiate prerenal azotemia, acute tubular necrosis, and intrinsic acute renal failure other than acute tubular necrosis [25]. Another important biomarker may be polycystin-1, the protein product of a gene responsible for autosomal dominant polycystic kidney disease, the most common genetic disease leading to renal failure [26]. Polycystin-1 is of low abundance in kidney tissue, but is readily detectable in urinary exosomes [6]. Lee and colleagues [27] demonstrated that immunoblot analysis of urinary exosomes can differentiate two different types of mutations for the thiazide sensitive Na-Cl co-transporter of the distal convoluted tubule, and thus may become a very simple and useful diagnostic tool to subclassify Gitelman's syndrome.

Furthermore, urinary proteomics has recently been applied in predicting diabetic nephropathy [28], in the early detection of acute renal failure [14,29] and acute allograft rejection [30], and in the assessment of the progression of renal disease in the renal Fanconi syndrome [5].

Besides the application of urinary proteomics to patient populations, another potential of clinical proteomics is what has been coined patient-tailored or personalized molecular medicine [31]. In a model for personalized cancer care, clinical proteomics was proposed for the early detection of cancer via serum proteomics patterns and subsequently in therapeutic fine-tuning via proteomic pathway profiling in diagnostic, post-therapy and post-relapse biopsies [31]. In analogy with this model, one could hypothesize a similar future role for urinary proteomics in hypertension, the treatment of which is currently largely empirical. With the kidney being the major regulator of blood pressure [32], one could argue that subtypes of essential hypertension may have unique fingerprints in the urinary proteome. If so, these fingerprints may be used to predict the optimal anti-hypertensive drug regimens for each subtype and may eventually help to personalize therapy and classify this heterogeneous disorder.

It should be borne in mind that the examples cited above represent first-level urinary proteomics and that the clinical value of these approaches remains to be determined in comparative studies. Furthermore, as was pointed out recently [33], the focus for the near future should be to further increase our understanding of the regulation of the urinary proteome and to design clinical urinary proteomics studies with small and

extremely well-characterized patient cohorts to identify new biomarkers for clinically important renal disease processes.

References

1. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. Electrospray ionization for mass spectrometry of large biomolecules. *Science* 1989; 246: 64–71.
2. Tanaka K, Waki H, Ido Y, Akita S, Yoshida Y, Yoshida T. Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom* 1988; 2: 151–3.
3. Klein E, Klein JB, Thongboonkerd V. Two-dimensional gel electrophoresis: a fundamental tool for expression proteomics studies. *Contrib Nephrol* 2004; 141: 25–39.
4. Thongboonkerd V, Klein JB, Arthur JM. Proteomic identification of a large complement of rat urinary proteins. *Nephron Exp Nephrol* 2003; 95: 69–78.
5. Cutillas PR, Norden AG, Cramer R, Burlingame AL, Unwin RJ. Detection and analysis of urinary peptides by on-line liquid chromatography and mass spectrometry: Application to patients with renal Fanconi syndrome. *Clin Sci (Lond)* 2003; 104: 483–90.
6. Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci USA* 2004; 101: 13368–73.
7. Kanno K, Sasaki S, Hirata Y et al. Urinary excretion of aquaporin-2 in patients with diabetes insipidus. *N Engl J Med* 1995; 332: 1540–45.
8. Funayama H, Nakamura T, Saito T et al. Urinary excretion of aquaporin-2 water channel exaggerated dependent upon vasopressin in congestive heart failure. *Kidney Int* 2004; 66: 1387–92.
9. Wen H, Frokiaer J, Kwon TH, Nielsen S. Urinary excretion of aquaporin-2 in rat is mediated by a vasopressin-dependent apical pathway. *J Am Soc Nephrol* 1999; 10: 1416–29.
10. McKee JA, Kumar S, Ecelbarger CA, Fernandez-Llama P, Terris J, Knepper MA. Detection of Na(+) transporter proteins in urine. *J Am Soc Nephrol* 2000; 11: 2128–32.

11. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 1999; 17: 994–9.
12. MacBeath G. Protein microarrays and proteomics. *Nat Genet* 2002; 32: 526–32.
13. Knepper MA. Proteomics and the kidney. *J Am Soc Nephrol* 2002; 13: 1398–408.
14. Hewitt SM, Dear J, Star RA. Discovery of protein biomarkers for renal diseases. *J Am Soc Nephrol* 2004; 15: 1677–89.
15. Bottini PV, Ribeiro Alves MA, Garlipp CR. Electrophoretic pattern of concentrated urine: comparison between 24-hour collection and random samples. *Am J Kidney Dis* 2002; 39: E2.
16. Russo LM, Bakris GL, Comper WD. Renal handling of albumin: a critical review of basic concepts and perspective. *Am J Kidney Dis* 2002; 39: 899–919.
17. Shinada M, Akdeniz A, Panagiotopoulos S, Jerums G, Bach LA. Proteolysis of insulin-like growth factor-binding protein-3 is increased in urine from patients with diabetic nephropathy. *J Clin Endocrinol Metab* 2000; 85: 1163–9.
18. Wiggins RC, Glatfelter A, Kshirsagar B, Brukman J. Procoagulant activity in normal human urine associated with subcellular particles. *Kidney Int* 1986; 29: 591–7.
19. Kennedy S. Proteomic profiling from human samples: the body fluid alternative. *Toxicol Lett* 2001; 120: 379–84.
20. Bachmann S, Mutig K, Bates J et al. Renal effects of Tamm-Horsfall protein (uromodulin) deficiency in mice. *Am J Physiol Renal Physiol* 2005; 288: F559–67.
21. Thongboonkerd V, McLeish KR, Arthur JM, Klein JB. Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation. *Kidney Int* 2002; 62: 1461–9.
22. Klasen IS, Reichert LJ, de Kat Angelino CM, Wetzels JF. Quantitative determination of low and high molecular weight proteins in human urine: influence of temperature and storage time. *Clin Chem* 1999; 45: 430–32.
23. Miki K, Sudo A. Effect of urine pH, storage time, and temperature on stability of catecholamines, cortisol, and creatinine. *Clin Chem* 1998; 44: 1759–62.

24. Edwards OM, Bayliss RI, Millen S. Urinary creatinine excretion as an index of the completeness of 24-hour urine collections. *Lancet* 1969; 29: 1165–6.
25. du Cheyron D, Daubin C, Poggioli J et al. Urinary measurement of Na⁺/H⁺ exchanger isoform 3 (NHE3) protein as new marker of tubule injury in critically ill patients with ARF. *Am J Kidney Dis* 2003; 42: 497–506.
26. Wilson PD. Polycystic kidney disease. *N Engl J Med* 2004; 350: 151–64.
27. Lee JW, Joo KW, Chang HR et al. Urine immunoblotting of sodium chloride cotransporter in patients with Gitelman's syndrome: its implications in differential diagnosis. *J Am Soc Nephrol* 2004; 15: 312A–313A..
28. Mischak H, Kaiser T, Walden M et al. Proteomic analysis for the assessment of diabetic renal damage in humans. *Clin Sci (Lond)* 2004; 107: 485–95.
29. Han WK, Bailly V, Abichandani R, Thadhani R, Bonventre JV. Kidney injury molecule-1 (KIM-1): a novel biomarker for human renal proximal tubule injury. *Kidney Int* 2002; 62: 237–44.
30. Schaub S, Rush D, Wilkins J et al. Proteomic-based detection of urine proteins associated with acute renal allograft rejection. *J Am Soc Nephrol* 2004; 15: 219–27.
31. Liotta LA, Kohn EC, Petricoin EF. Clinical proteomics: personalized molecular medicine. *JAMA* 2001; 286: 2211–14.
32. Guyton AC, Manning RD Jr, Hall JE, Norman RA Jr, Young DB, Pan YJ. The pathogenic role of the kidney. *J Cardiovasc Pharmacol* 1984; 6: S151–61.
33. Gross P. Renal transport proteins in excreted urine: gold mine or gold dust? *Kidney Int* 2004; 66: 1707–8.

Chapter 5

Hypokalemia in a mouse model of Gitelman's syndrome

Ryan G. Morris, Ewout J. Hoorn
and Mark A. Knepper

Am J Physiol Renal Physiol 2006; 290: F1416–20

Abstract

Hypokalemia is a prominent feature of Gitelman syndrome and a common side effect of thiazide use in the treatment of hypertension. It is widely recognized that genetic or pharmacological inhibition of the renal thiazide-sensitive sodium chloride cotransporter (NCC) initiates the potentially severe renal potassium loss observed in these settings. Surprisingly, hypokalemia has not been detected in NCC (-/-) mice (Schultheis *et al.* J. Biol. Chem 1998; 273: 29150-29155.) maintained on normal rodent diets. We now show that modest reduction of dietary potassium induced a marked reduction in plasma potassium and elevated renal potassium excretion in NCC (-/-) mice, which was associated with a pronounced polydipsia and polyuria of central origin. These findings are consistent with the development of potassium depletion in NCC (-/-) mice and were not seen in wild-type mice maintained on the same low potassium diet. In addition, plasma aldosterone levels were significantly elevated in NCC (-/-) mice even in the presence of a low potassium diet. Collectively, these findings suggest an early central component to the polyuria of Gitelman syndrome and show that both elevated aldosterone and dietary potassium content contribute to the development of hypokalemia in Gitelman syndrome. Therefore, NCC (-/-) mice are more sensitive to reductions in dietary potassium than wild-type mice and become hypokalemic thus more faithfully representing the Gitelman phenotype seen in humans.

Introduction

Hypokalemia is a cardinal feature of Gitelman's syndrome, a genetic renal disorder also characterized by alkalosis, hypomagnesemia, hypocalciuria and mild salt wasting [6]. Loss of function mutations in the thiazide-sensitive, sodium-chloride cotransporter (NCC) have been identified as underlying causes of Gitelman's syndrome [23]. Notably, administration of thiazide diuretics, agents widely used in the treatment of hypertension, generally results in the same physiological profile observed in Gitelman's subjects, including potentially severe hypokalemia. Therefore, inhibition of NCC either through pharmacological means or as a result of genetic inactivation can result in clinically significant renal potassium loss.

A mouse model of Gitelman's syndrome, NCC (-/-) mice, faithfully recapitulates many of the physiological findings observed in Gitelman's patients including hypomagnesemia, hypocalciuria [13,22], and alkalosis [13]. Surprisingly, hypokalemia has not been detected in this model. Here we sought to determine if NCC (-/-) mice were more sensitive to reductions in dietary potassium intake than wild-type mice.

Hypokalemia is often induced in rodent models by severely restricting dietary potassium for a period of two weeks. In addition to reducing plasma potassium, prolonged potassium deprivation also results in polyuria, polydipsia, renal hypertrophy and impaired renal concentrating ability [18]. Two weeks of potassium deprivation will induce the aforementioned changes in wild-type rodents with an intact NCC; therefore, we

chose to shorten the duration and degree of potassium restriction in an effort to highlight any sensitivity of the NCC (-/-) mice.

Methods

Animals

A colony of NCC (-/-) mice on a C57/Bl6 background was established at the NIH from breeding pairs provided by Dr. Gary Shull, (University of Cincinnati; Cincinnati, Ohio). Control mice, wild-type C57/Bl6 whose age and sex were matched to experimental NCC (-/-) mice were purchased from Taconic and allowed to equilibrate for a minimum of three days in the NIH animal housing facility before experimentation. All studies were approved by the NHLBI ACUC.

Serum chemistries

Mice were anesthetized with isofluorane and blood collected by retro-orbital puncture using a glass Pasteur pipette. Whole blood was transferred to a serum separator (StatSpin Inc, Norwood, MA) and plasma was isolated by centrifugation. Electrolyte content of 150 µl of serum was analyzed at the Clinical Center Laboratory of Medicine.

Metabolic cage studies

Mice were housed in metabolic cages for durations ranging from 7 to 10 days. Mice were initially fed a standard rodent pellet food with ad lib water. Mice were then fed gel food diets containing 4.5g/25g BW of sodium and potassium free rodent meal supplemented with appropriate amounts of sodium (0.5 mEq/day) and potassium chloride, 3mls of water, and agar were used to control potassium intake. All mice, regardless of

genotype, consumed essentially the entire daily portion of gel food. Our potassium replete diet contained 0.48 mEq of potassium chloride/day whereas the reduced potassium diet contained 0.048 mEq/day. In addition to the water present in the gel food, mice also had free access to water with the exception of the water restriction protocol. In the water restriction protocol, water intake was limited to 2.0mls of water/25g body weight in the gel food. Water intake was measured daily and mice were weighed at least every other day. Urine was collected under oil and urine volume and osmolality were determined gravimetrically and with a vapor pressure osmometer respectively. Electrolyte content of the urine was analyzed at the NHLBI Laboratory of Animal Medicine and Surgery.

Semi-quantitative immunoblotting

Mice were euthanized by cervical dislocation and right kidneys process as described previously [3]. Equal loading was confirmed by staining gels as described previously [12]. This gel was subsequently scanned with a linear fluorescence scanner (Odyssey, Li-Cor Biosciences) at an excitation wavelength of 700 nm. Affinity-purified primary antibodies against NKCC2, and AQP2 have been characterized previously.

Aldosterone measurements

Trunk blood was collected after decapitation and plasma collected via a serum separator. Plasma aldosterone was measured by radioimmunoassay (DPC; Los Angeles, California) per the manufacturer's instruction.

Statistics

ANOVAs were performed on multiple group comparisons followed by Bonferonni post-hoc tests. Significance was taken at $p < 0.05$. Student's T-tests were utilized on comparisons between two groups.

Results

Serum potassium

The plasma potassium concentration of NCC (-/-) mice on a low potassium diet was significantly decreased ($p < 0.01$) by roughly 1 mM compared to all other groups (Figure 1). As previously documented [22], NCC (-/-) mice displayed hypomagnesemia, and this was exacerbated by a low potassium diet (Figure 1). In contrast, plasma sodium and calcium concentrations were not significantly different between groups (data not shown).

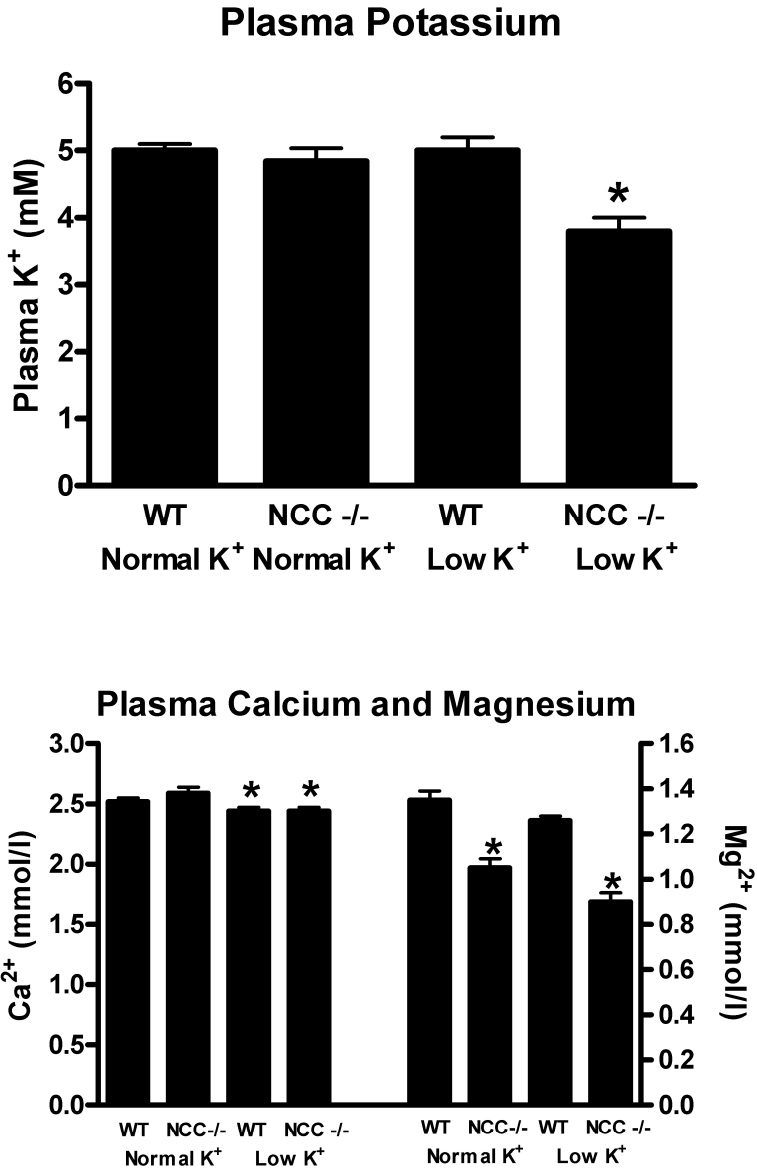


Figure 1: Plasma potassium, calcium and magnesium concentrations of wild-type and NCC (–/–) mice after 7 days on normal and low-potassium diets. * $p < 0.05$, by ANOVA.

Urinary potassium excretion

Rates of urinary potassium excretion were measured in NCC (-/-) and wild-type mice. After initiation of a low potassium diet, NCC (-/-) mice had significantly higher rates of renal potassium excretion compared to wild-type mice (Figure 2). Over time, the difference in urinary potassium excretion between NCC (-/-) and wild-type mice became indistinguishable, establishing a new steady state at a lower absolute rate of potassium excretion.

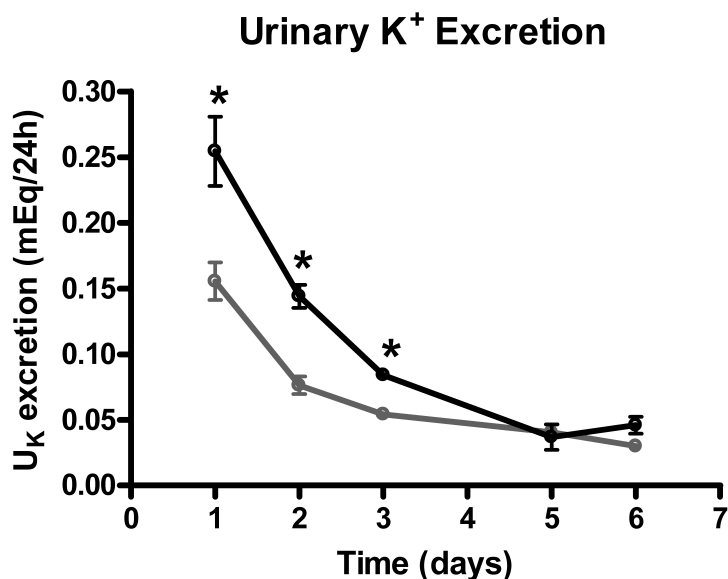


Figure 2: Urinary excretion of potassium in wild-type and NCC (-/-) mice maintained on low-potassium diets. * $p < 0.05$, by Student's *t*-test

Time course of water intake, urine volume and urine osmolality

Polydipsia and polyuria are well known consequences of hypokalemia [18]. A representative time course of water intake (panel A), urine output (panel B), and urine osmolality (panel C) of wild-type and NCC (-/-) mice are presented in Figure 3. In the basal condition, days 1-3, there was no significant difference in water intake, urine output, or urine osmolality between wild-type or NCC (-/-) mice. After two days on a reduced potassium diet (day 5 fig 3), there was a significant increase in the water intake of the NCC (-/-) compared to all other groups (Figure 3 panel A). This increase in water intake in the NCC (-/-) mice became increasingly pronounced over the duration of the experiment and was not observed in the other groups of animals. Significant increases in urine volume (panel B) and significant reductions in urine osmolality (panel C) of the NCC (-/-) low potassium group paralleled the increases in water intake. Among four metabolic cage studies performed, the onset of polyuria and polydipsia in the NCC (-/-) mice varied from two to four days after introduction of the low potassium diet but always persisted through the duration of the experiment.

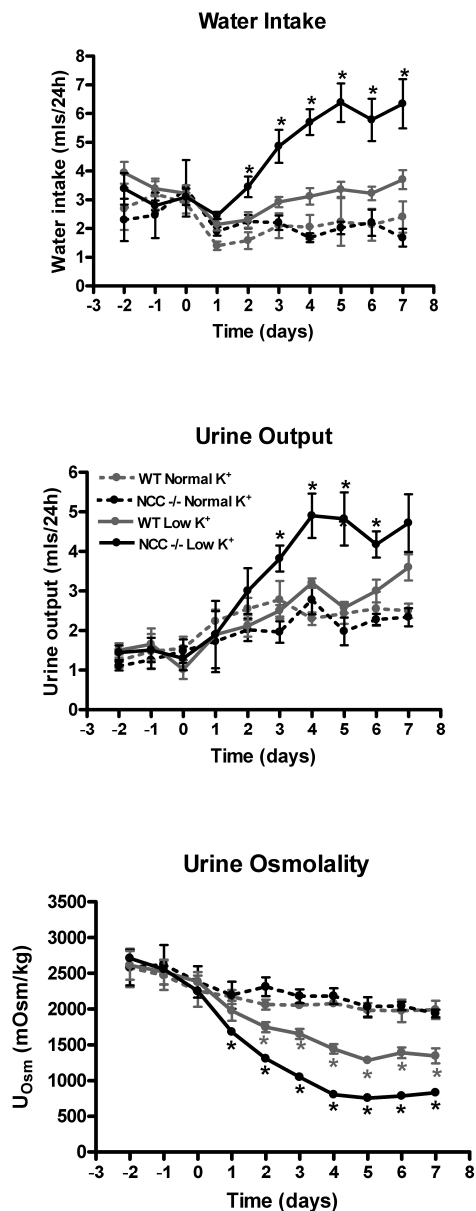


Figure 3: Time course of average water intake, urine output, and urine osmolality of wild-type and NCC (-/-) mice under basal conditions (days 1–3) and on switching to gel food diets containing normal and low potassium (days 4–10). * $p < 0.05$ by ANOVA.

Urinary concentrating ability

A urinary concentrating test was performed on all four groups of mice to investigate if the pronounced polyuria and polydipsia observed in the NCC (-/-) mice on a reduced potassium diet were associated with a reduced urine concentrating ability. Water intake, urine osmolality and urine volumes before and after 24 hours of water restriction for all four experimental groups are presented in Figure 4. All groups of animals significantly increased urine osmolality and decreased urine volume in response to water restriction. Urine osmolality and urine volume of wild-type and NCC (-/-) groups on low potassium diets were not significantly different after 24 hrs of water restriction.

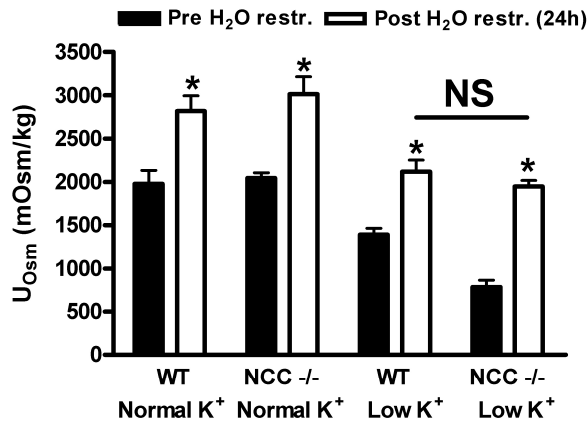


Figure 4: Urinary concentrating ability of wild-type and NCC (-/-) mice maintained on normal and low-potassium diets. Urine osmolality before and after 24 h of water restriction for all experimental groups are presented. * $p < 0.05$, by ANOVA. NS, no significant difference.

Kidney weight

Kidney weights and kidney weights normalized for body weight were analyzed for evidence of renal hypertrophy. Two of four individual experiments demonstrated a significant increase in kidney weight in NCC (-/-) mice maintained on a low potassium diet compared to knockout mice on a normal potassium diet (i.e. 0.116 ± 0.004 g vs. 0.149 ± 0.008 g, $N = 10$, $p < 0.002$). However, cumulative results from the 4 experiments (30-40 mice from each experimental group) did not demonstrate significant differences in kidney weight or kidney weight normalized to body weight despite the consistent polydipsia and polyuria (data not shown).

Western blotting analysis

Alterations in the expression pattern of transport proteins involved in renal concentrating ability were analyzed by Western blotting of whole kidney homogenates prepared from mice with ad libitum access to water. The protein abundance of the water channel aquaporin 2 (AQP2) was significantly reduced in both wild-type and NCC (-/-) mice on low potassium diets. In contrast, the abundance of NKCC2 was not significantly different among the groups (Figure 5).

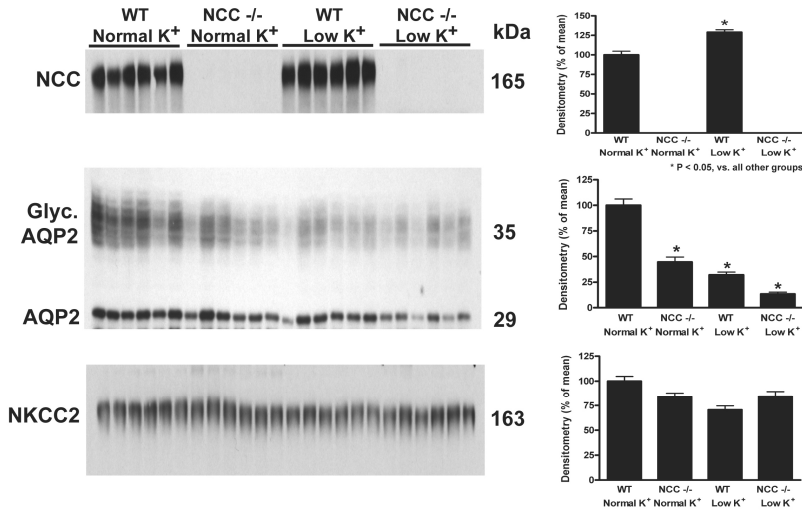


Figure 5: Western blot analysis of whole kidney homogenates demonstrating the effects of manipulating dietary potassium on the sodium-potassium-chloride cotransporter 2 (NKCC2) and aquaporin-2 (AQP-2) expression in wild-type and NCC (-/-) mice. * $p < 0.05$, by ANOVA.

Plasma aldosterone

Plasma aldosterone measurements were performed on the four experimental groups (Figure 6). Plasma aldosterone was significantly higher ($p < 0.001$) in NCC (-/-) mice compared to wild-type mice regardless of diet. As expected, low potassium diet suppressed plasma aldosterone levels although statistical significance was achieved only for the NCC (-/-) mice ($p < 0.01$). Even on a low potassium diet, aldosterone levels in NCC (-/-) mice were markedly elevated at 2.2 ± 0.5 nM (compared with the Kd of the mineralocorticoid receptor for aldosterone: 1.3 nM).

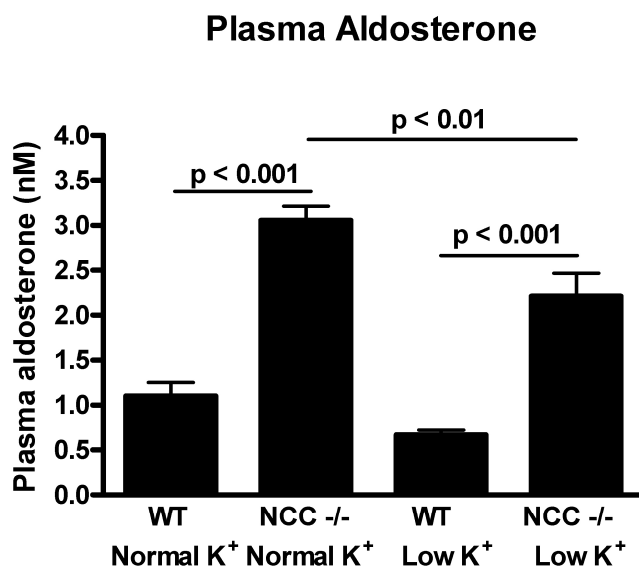


Figure 6: Plasma aldosterone levels of wild-type and NCC (-/-) mice maintained on normal or low-potassium diets for 7 days.

Discussion

Here we have examined the effects of manipulating dietary potassium intake in a mouse model of Gitelman's syndrome. In response to a diminished potassium intake, NCC (-/-) mice demonstrated a pronounced polydipsia and polyuria concurrent with elevated renal potassium excretion and significantly reduced serum potassium and magnesium concentrations; findings consistent with the development of potassium depletion. These findings were not observed in wild-type mice on a reduced potassium diet or wild-type and NCC (-/-) mice on a diet replete with potassium. Thus, the NCC (-/-) mice are more sensitive to dietary potassium restriction than wild-type mice.

In contrast to the seven day potassium depletion studies presented here, many experimental protocols for potassium depletion are two weeks in duration [1,2,9,15] and encompass both central and renal responses. For example, Berl *et al.* [1], have demonstrated polydipsia preceeds, and is independent of, the ultimate appearance of urinary concentrating defects in potassium depletion. Our results demonstrated the early onset of polydipsia and polyuria in the NCC (-/-) mice coincident with significant renal potassium loss. In NCC (-/-) mice, the polydipsia and polyuria persisted throughout the duration of the experiment despite wild-type and NCC (-/-) mice ultimately achieving similar rates of renal potassium excretion. The potassium loss accrued in the NCC (-/-) mice before establishing the new steady-state level of potassium excretion resulted in significantly lower plasma potassium in the NCC (-/-) mice which persisted throughout the duration of the experiments. The persistent polydipsia and polyuria is thought to be driven, at least in part, by the effects of hypokalemia on neural regulation of the thirst drive. However, many compensatory changes with various time courses are undoubtedly occurring in response to potassium depletion, including possible changes in the vasopressin axis, and angiotensin II levels which may directly or indirectly contribute to the polydipsia. Nevertheless, the absence of a frank renal concentrating defect at the specific time point examined in this model is consistent with an early-onset primary polydipsia. Such a process undoubtedly contributes to the polyuria seen in Gitelman patients.

Renal hypertrophy and a urinary concentrating defect are normally considered hallmarks of potassium depletion induced by restricting potassium intake. However, despite the obvious

polydipsia, polyuria and significantly reduced plasma potassium levels, at this time point there was no evidence of a concentrating defect or consistent findings of renal hypertrophy in the NCC (-/-) mice maintained on a low potassium diet. We propose that the lack of a urinary concentrating defect and inconsistent findings of hypertrophy are a result of the relatively short duration of low potassium diet which is further aggravated by the variable onset of polydipsia and polyuria.

The absence of a renal concentrating defect and inconsistent signs of renal hypertrophy would at first appear to preclude a renal phenotype at this specific time point in the model. However, there were significant decreases in renal AQP2 transporter abundance in both wild-type and NCC (-/-) mice in response to a low potassium diet. The diminished expression of AQP2 would be expected to contribute to the subsequent development of a renal concentrating defect known to be present after two weeks of potassium depletion [1,7,17]. Furthermore, the decreases in AQP2 abundance seen in wild-type and NCC (-/-) mice on low potassium diets are independent of water intake since water intake is significantly elevated only in the NCC (-/-) low potassium group, while AQP2 levels are decreased in both. Again, these findings are consistent with the observations that the ultimate development of a concentrating defect in potassium depletion is independent of water intake [1] and imply the kidneys are responding to dietary potassium through an unknown mechanism.

Given the exacerbated hypomagnesemia observed in these studies, the potential role of magnesium depletion in this model should be considered. Magnesium depletion is known to cause

tissue potassium depletion [24] but does not result in a urinary concentrating defect [16]. However, in models of primary magnesium depletion, there is no evidence of polyuria or polydipsia [16]. Furthermore, the hypomagnesemia described in this study is enhanced only in response to reductions in dietary potassium intake. Therefore, the exacerbated hypomagnesemia would appear to be secondary to alterations in potassium metabolism and not directly involved the polydipsia and polyuria.

There are many potential renal mechanisms contributing to the potassium deficiency in NCC (-/-) mice on a low potassium diet. First, even on a low potassium diet, circulating aldosterone in the NCC (-/-) mice is markedly elevated (presumably due to the salt wasting) and this would serve as a potent stimulus for potassium secretion by the aldosterone-sensitive segments of the renal tubule [4,19]. The hypocalciuria previously reported in the NCC (-/-) mice may also play an indirect role in promoting potassium secretion. Urinary calcium has been shown to inhibit active potassium secretion [20]; therefore, hypocalciuria could relieve this inhibition and promote potassium secretion. Furthermore, calcium also inhibits the activity of ENaC [5,8]. Again, a reduction of this inhibitory influence would allow enhanced electrogenic sodium reabsorption resulting in a depolarization of the apical membrane of the collecting duct principal cells, thereby increasing the driving force for potassium secretion [21]. Finally, increased luminal flow, as might be encountered in this model, has been shown to stimulate potassium secretion in in-vivo-perfused cortical collecting ducts [14]. Further investigation will be required to identify the precise molecular mechanisms in this model.

The pathogenesis of hypokalemia in Gitelman's syndrome remains incompletely understood [10]. Most explanations favor one or more of the aforementioned mechanisms. We have established that in a mouse model of Gitelman's syndrome a modest reduction in dietary potassium intake can induce the hypokalemic phenotype, allowing more detailed investigation of mechanisms contributing to the development of hypokalemia. Previous studies have shown that there are significant fluctuations in dietary potassium intake in humans, and that these are associated with blood pressure changes and cardiovascular risk [11]. Conceivably, hypokalemia in Gitelman's syndrome may be associated with a low dietary potassium intake, and may explain why some patients develop hypokalemia and polyuric spells, whereas others patients remain normokalemic.

In conclusion, NCC (-/-) mice are sensitive to reductions in dietary potassium and begin to demonstrate signs of potassium depletion before wild-type mice. Our findings are consistent with previous studies demonstrating polyuria and polydipsia occurring early in the development of potassium depletion, with alterations in renal concentrating ability and morphology occurring at later time points. Thus the absence of hypokalemia in NCC (-/-) mice on a normal diet appears to be due to compensation by sufficient amounts of dietary potassium.

Acknowledgments

We thank Dr. Gary Shull for the NCC (–/–) mice, David Caden at the NHLBI Laboratory of Animal Medicine and Surgery, and members of LKEM for helpful discussions.

References

1. Berl T, Linas SL, Aisenbrey GA, Anderson RJ. On the mechanism of polyuria in potassium depletion. The role of polydipsia. *J Clin Invest* 1997; 60: 620–625.
2. Cheval L, Duong Van Huyen JP, Bruneval P, Verbavatz JM, Elalouf JM, Doucet A. Plasticity of mouse renal collecting duct in response to potassium depletion. *Physiol Genomics* 2004; 19: 61–73.
3. Fenton RA, Chou CL, Stewart GS, Smith CP, Knepper MA. Urinary concentrating defect in mice with selective deletion of phloretin-sensitive urea transporters in the renal collecting duct. *Proc Natl Acad Sci USA* 2004; 101: 7469–7474.
4. Field MJ, Stanton BA, Giebisch GH. Differential acute effects of aldosterone, dexamethasone, and hyperkalemia on distal tubular potassium secretion in the rat kidney. *J Clin Invest* 1984; 74: 1792–1802.
5. Garty H, Lindemann B. Feedback inhibition of sodium uptake in K^+ -depolarized toad urinary bladders. *Biochim Biophys Acta* 1984; 771: 89–98.
6. Gitelman HJ, Graham JB, Welt LG. A new familial disorder characterized by hypokalemia and hypomagnesemia. *Trans Assoc Am Physicians* 1966; 79: 221–235.
7. Hollander W Jr, Winters RW, Willams TF, Bradley J, Oliver J, Welt LG. Defect in the renal tubular reabsorption of water associated with potassium depletion in rats. *Am J Physiol* 1957; 189: 557–563.
8. Ishikawa T, Marunaka Y, Rotin D. Electrophysiological characterization of the rat epithelial Na^+ channel (rENaC) expressed in MDCK cells. Effects of Na^+ and Ca^{2+} . *J Gen Physiol* 1998; 111: 825–846.
9. Jung JY, Madsen KM, Han KH, Yang CW, Knepper MA, Sands JM, Kim J. Expression of urea transporters in potassium-depleted mouse kidney. *Am J Physiol Renal Physiol* 2003; 285: F1210–F1224.

10. Kamel KS, Harvey E, Douek K, Parmar MS, Halperin ML. Studies on the pathogenesis of hypokalemia in Gitelman's syndrome: role of bicarbonaturia and hypomagnesemia. *Am J Nephrol* 1998; 18: 42–49.
11. Khaw KT, Barrett-Connor E. Dietary potassium and stroke-associated mortality. A 12-year prospective population study. *N Engl J Med* 1987; 316: 235–240.
12. Kim GH, Masilamani S, Turner R, Mitchell C, Wade JB, Knepper MA. The thiazide-sensitive Na-Cl cotransporter is an aldosterone-induced protein. *Proc Natl Acad Sci USA* 1998; 95: 14552–14557.
13. Loffing J, Vallon V, Loffing-Cueni D, Aregger F, Richter K, Pietri L, Bloch-Faure M, Hoenderop JG, Shull GE, Meneton P, Kaissling B. Altered renal distal tubule structure and renal Na⁺ and Ca²⁺ handling in a mouse model for Gitelman's syndrome. *J Am Soc Nephrol* 2004; 15: 2276–2288.
14. Malnic G, Berliner RW, Giebisch G. Flow dependence of K⁺ secretion in cortical distal tubules of the rat. *Am J Physiol Renal Fluid Electrolyte Physiol* 1989; 256: F932–F941.
15. Malnic G, Klose RM, Giebisch G. Micropuncture study of renal potassium excretion in the rat. *Am J Physiol* 1964; 206: 674–686.
16. Manitius A, Epstein FH. Some observations on the influence of a magnesium-deficient diet on rats, with special reference to renal concentrating ability. *J Clin Invest* 1963; 42: 208–215.
17. Manitius A, Levitin H, Beck D, Epstein FH. On the mechanism of impairment of renal concentrating ability in potassium deficiency. *J Clin Invest* 1960; 39: 684–692.
18. Mujais SK, Katz AI. Potassium deficiency. In: *The Kidney Physiology and Pathophysiology* (3rd ed.), edited by Seldin DW and Giebisch G. Philadelphia, PA: Lippincott Williams and Wilkins, 2000, p. 1615–1646.
19. Okusa MD, Velazquez H, Ellison DH, Wright FS. Luminal calcium regulates potassium transport by the renal distal tubule. *Am J Physiol Renal Fluid Electrolyte Physiol* 1990; 258: F423–F428.
20. O'Neil RG, Helman SI. Transport characteristics of renal collecting tubules: influences of DOCA and diet. *Am J Physiol Renal Fluid Electrolyte Physiol* 1977; 233: F544–F558.
21. Schafer JA, Troutman SL, Schlatter E. Vasopressin and mineralocorticoid increase apical membrane driving force for K⁺

- secretion in rat CCD. *Am J Physiol Renal Fluid Electrolyte Physiol* 1990; 258: F199–F210.
22. Schultheis PJ, Lorenz JN, Meneton P, Nieman ML, Riddle TM, Flagella M, Duffy JJ, Doetschman T, Miller ML, Shull GE. Phenotype resembling Gitelman's syndrome in mice lacking the apical $\text{Na}^+\text{-Cl}^-$ cotransporter of the distal convoluted tubule. *J Biol Chem* 1998; 273: 29150–29155.
23. Simon DB, Nelson-Williams C, Bia MJ, Ellison D, Karet FE, Molina AM, Vaara I, Iwata F, Cushner HM, Koolen M, Gainza FJ, Gitelman HJ, Lifton RP. Gitelman's variant of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter. *Nat Genet* 1996; 12: 24–30.
24. Whang R, Welt LG. Observations in experimental magnesium depletion. *Clin Res* 1962; 10: 68.

Chapter 6

**Osmomedi­ated natriuresis in humans:
evidence for salinity sensing
in the thick ascending limb**

Ewout J. Hoorn, M. Carola Zillikens
Huibert A.P. Pols, Frans Boomsma
and Robert Zietse

Submitted

Abstract

Objective: The mechanism of osmomediated natriuresis is unknown. We hypothesize that osmomediated natriuresis is mediated by the calcium sensing receptor (CaSR) in the thick ascending limb (TAL) of the loop of Henle.

Methods: Seven healthy volunteers received the same sodium load (3.85 mmol/kg) first as hypertonic saline (HS-group), and then as isotonic saline (IS-group). Two patients with familial hypocalciuric hypercalcemia (inactivating CaSR-mutation) also received hypertonic saline (HS-FHH-group).

Results: Compared to the IS-group, the HS-group developed mild hypernatremia (146 ± 1 vs. 141 ± 1 mmol/l, $p < 0.001$) and a larger natriuresis (501 ± 77 vs. 286 ± 34 μ mol/min, $p = 0.002$), confirming osmomediated natriuresis. The fractional calcium ($105 \pm 73\%$ vs. $-34 \pm 20\%$, $p = 0.02$) and magnesium ($21 \pm 28\%$ vs. $-25 \pm 10\%$, $p = 0.2$) excretions increased in the HS-group, but decreased in the IS-group. In the HS-FHH-group, despite a similar rise in plasma sodium concentration, urinary sodium excretion was attenuated (344 ± 70 μ mol/min), and the fractional calcium ($-25 \pm 4\%$) and magnesium ($-13 \pm 1\%$) excretions decreased. The HS-groups had an increase in antidiuretic hormone, while all groups exhibited a similar decrease in aldosterone and renin, and a similar increase in parathyroid hormone, atrial and B-type natriuretic peptides.

Conclusions: Osmomediated natriuresis is associated with increased renal calcium and magnesium excretion, suggesting the site of action to be the TAL. In FHH-patients who received hypertonic saline, urinary sodium, calcium, and magnesium excretion were attenuated. Thus, hypertonic saline might activate the CaSR (“salinity sensing”), thereby inhibiting the sodium-potassium-chloride cotransporter 2, and inducing a natriuresis.

Introduction

The effects of hyperosmolarity on water balance regulation are well known and include an activation of osmoreceptors, the release of antidiuretic hormone (ADH), and the insertion of water channels in the renal collecting duct to increase water permeability. Interestingly, hyperosmolarity also affects sodium balance regulation, because when the same sodium load is given as isotonic saline and as hypertonic saline, the latter will produce a greater natriuresis [1]. Surprisingly, the mechanism of this phenomenon of “osmomediated natriuresis” remains elusive. A previous infusion study for the first time demonstrated this phenomenon in healthy volunteers, who showed similar changes in the concentrations of renin, angiotensin II, aldosterone, atrial natriuretic peptide, the glomerular filtration rate, and hemodynamic parameters during hypertonic and isotonic infusion [2]. As expected, only ADH increased in subjects receiving hypertonic saline [2]. In the present study, we test the hypothesis that osmomediated natriuresis is mediated by the calcium sensing receptor (CaSR) in the thick ascending limb (TAL) of the loop of Henle. Recent evidence from cell and animal studies indicates that hyperosmolarity can decrease the 50% effective concentration (EC_{50}) of the CaSR for calcium [3,4], but no human studies have been performed. To address our hypothesis, we performed infusion studies in seven healthy volunteers and in two patients with an inactivating mutation of the CaSR (familial hypocalciuric hypercalcemia, FHH) [5].

Methods

Infusion studies

The experimental protocol was adapted from [2], and approved by the Ethics Committee of the Erasmus Medical Center (MEC-2005-326). Experiments were performed in 7 healthy volunteers (4 females, 3 males, 19 – 28 years, 50.4 – 72.6 kg, not taking medication) and 2 patients with FHH (father 57 years, 61.0 kg; daughter 23 years, 50.4 kg; both not taking medication). FHH was confirmed by sequence analysis, which identified a heterozygous nucleotide mutation in exon 4 (CGA>TGA=R392Stp). The subjects remained seated in an armchair throughout the experiment and were allowed to stand up only for micturition. Each experiment was divided into four 90-min periods (baseline, infusion, 2 post-infusion periods). After the baseline period, a sodium load of 3.85 mmol/kg body wt was infused over the following 90 min by an infusion pump. The load was infused as either 0.9% saline (“IS-group”, 25 ml/kg body wt) or as 5% saline (“HS-group”, 4.5 ml/kg body wt) on separate days. The FHH subjects only received an infusion of hypertonic saline (“HS-FHH-group”). Blood and urine were collected at the end of each 90-min period; blood was also collected at baseline and half-way the infusion. Withdrawn and excreted fluids were immediately replaced by tap water plus 45 ml (0.5 ml/min) as replacement for insensible water loss.

Measurements

Hematocrit (Hct) was determined by measuring impedance (XE 2100, Sysmex). Plasma concentrations of sodium (P_{Na}), calcium (P_{Ca}), magnesium (P_{Mg}), potassium, phosphate, and albumin, were measured by a multianalyzer (Hitachi 917, Roche). Plasma osmolality (P_{Osm}) was measured by freezing-point depression (Osmomet, Menarini). Concentrations of kreatinine were measured by conventional spectrophotometry based on the Jaffé reaction. Blood for hormone analyses was collected in prechilled polyethylene tubes containing EDTA. The samples were centrifuged at 4 °C immediately after sampling, and plasma was stored at -80 °C and analyzed within 6 months. Plasma ADH (P_{ADH}) and aldosterone were measured with commercially available radioimmunoassay kits (DiaSorin, Stillwater, MN, USA, and Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, USA, respectively). Enzymatically active renin was determined by radioimmunoassay as described previously [6]. Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) were measured with immunoradiometric assay kits (Shionoria, Osaka, Japan). Catecholamines were determined by high performance liquid chromatography with fluorimetric detection, as described previously [7]. Parathyroid hormone (PTH) was determined by a solid-phase sandwich chemoluminescence immunoassay (IMMULITE intact-PTH, DPC, The Netherlands). The percent change in plasma volume (PV) was calculated from the formula: $PV_t / PV_0 = 1 / ((1 - Hct_0) - 1) / 1 / ((1 - Hct_t) - 1)$. Finally, arterial systolic, diastolic, and mean arterial pressures were recorded semiautomatically by oscillometry 4 times during each 90-minute period.

Statistics

Results are presented as mean $\pm$ SEM. Data of the HS and IS-groups were subjected to a repeated measures general linear model, which analyzes the within and between group effects and generates one p-value for differences in the overall trends. A p-value of ≤ 0.05 was considered significant. No statistics were performed for the HS-FHH-group.

Results

Osmomeditated natriuresis, antidiuretic hormone, and sodium regulating hormones

As a result of the infusion, the HS-group developed mild hypernatremia (P_{Na} 146 ± 1 mmol/l) and hyperosmolarity (P_{Osm} 292 ± 3 mOsm/kg), which caused a rise in P_{ADH} (2.02 ± 0.85 pg/ml), reaching peak values at the end of the infusion (Figure 1). These courses were significantly different from those in the IS-group ($p < 0.05$ for all), in which P_{Na} , P_{Osm} and P_{ADH} remained relatively constant. In the HS-FHH-group, the initial P_{Na} , P_{Osm} , and P_{ADH} were lower, but rose with the same magnitude as in the HS-group.

All groups increased urinary sodium excretion during infusion. During the first post-infusion period (t 180 – 270 min), the increase in urinary sodium excretion persisted only in the HS-group ($40 \pm 24\%$), whereas it decreased in the IS-group ($-19 \pm 9\%$, HS vs. IS-group, $p = 0.002$), and more or less stabilized in the HS-FHH-group ($7 \pm 10\%$).

No significant differences were identified in the courses of plasma volume (Figure 1), hemodynamic parameters, and the plasma concentrations of aldosterone (Figure 1), renin, ANP, BNP, and catecholamines. The plasma renin activity decreased similar to plasma aldosterone (HS-group $-55 \pm 5\%$, IS-group $-48 \pm 7\%$, HS-FHH-group $-39 \pm 7\%$). During the infusion, plasma ANP (HS-group $41 \pm 22\%$, IS-group $52 \pm 18\%$, HS-FHH-group $105 \pm 84\%$) and BNP (HS-group $36 \pm 3\%$, IS-group $27 \pm 7\%$, HS-FHH-group $28 \pm 41\%$) increased, and then slightly decreased. The plasma norepinephrine concentrations rose in response to the infusions (HS-group $4 \pm 5\%$, IS-group $17 \pm 20\%$, HS-FHH-group $21 \pm 22\%$). Minimal changes ($< 10\%$) in plasma epinephrine concentrations were observed. Finally, heart rate, systolic, diastolic and mean arterial blood pressures remained stable during the experiments (data not shown).

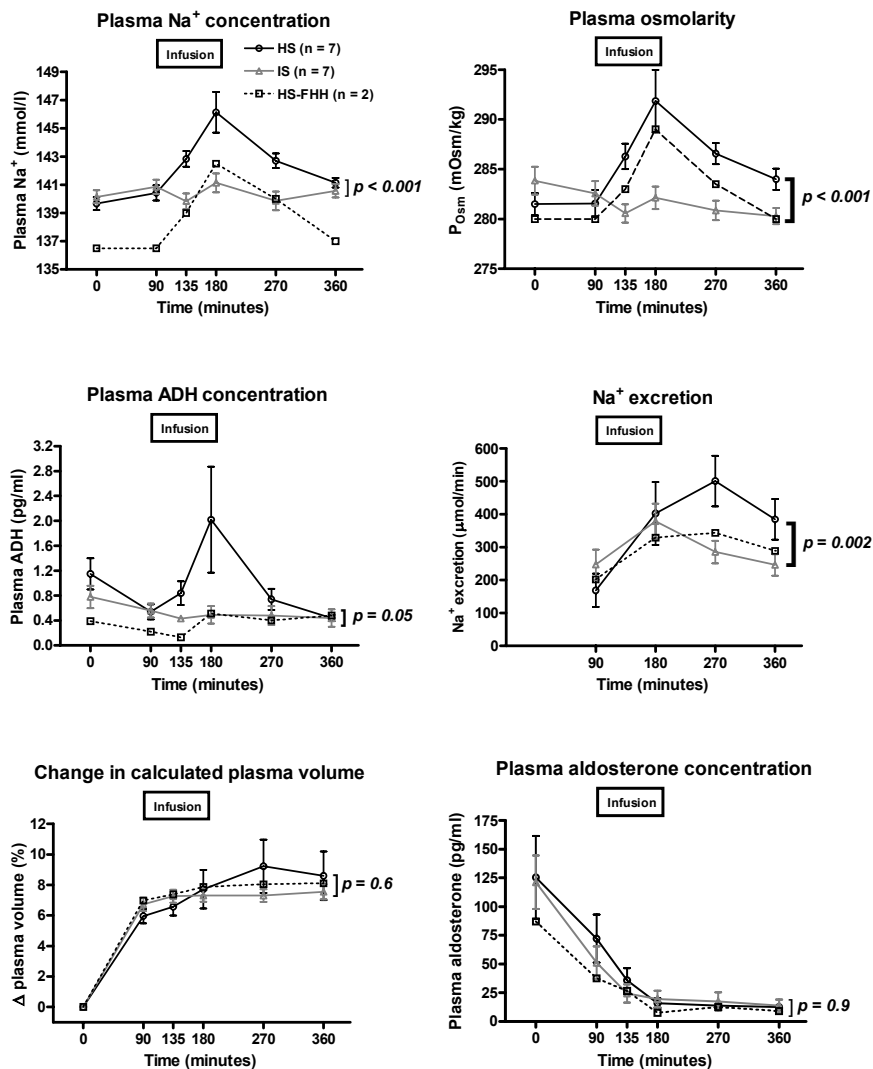


Figure 1: Effects of hypertonic and isotonic saline on indices of osmolarity, sodium excretion, and extracellular fluid volume

Legend Figure 1: *Seven healthy volunteers received the same sodium load as hypertonic saline (HS, solid black line) and as isotonic saline (IS, solid grey line), while two patients with familial hypocalciuric hypercalcemia received this sodium load only as hypertonic saline (HS-FHH, dashed black line). The effects of these infusions (given between $t = 90$ and 180 minutes) on renal sodium excretion and the courses of the calculated plasma volume, plasma osmolarity, and plasma sodium (Na^+), antidiuretic hormone (ADH), and aldosterone concentrations are shown. Statistical analysis was performed only for the HS and IS-groups using a repeated measures general linear model, which generated one p-value for the differences in the overall trend.*

Changes in calcium and magnesium metabolism

During the first half of the infusion, P_{Ca} fell in all groups, probably due to a combined effect of the doubling in plasma PTH and the fall in plasma albumin concentrations (4 g/l in all groups). During infusion, the fractional calcium excretion increased in the HS-group ($105 \pm 73\%$), whereas it decreased in the IS-group ($-34 \pm 20\%$) and the HS-FHH-group ($-25 \pm 4\%$). The P_{Mg} also fell during the infusion in all groups, but less so in the HS-group (decrease of 0.02 mmol/l vs. 0.07 mmol/l in the two other groups). Statistically, the course of P_{Mg} ($p = 0.04$), but not of the fractional magnesium excretion ($p = 0.2$) was different between the HS and IS-groups. All three groups showed a similar increase in the fractional phosphate and potassium excretions after the infusions (data not shown).

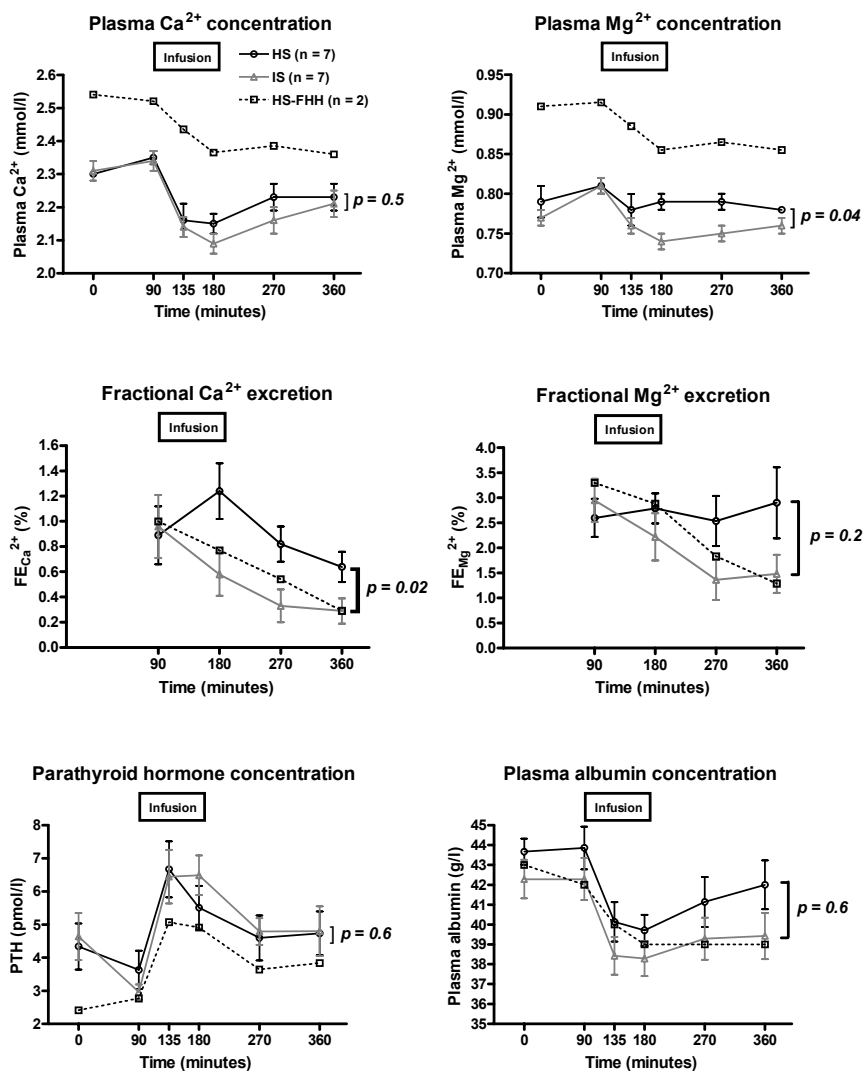


Figure 2: Effects of hypertonic and isotonic saline on calcium and magnesium metabolism

Legend Figure 2: *Seven healthy volunteers received the same sodium load as hypertonic saline (HS, solid black line) and as isotonic saline (IS, solid grey line), while two patients with familial hypocalciuric hypercalcemia received this sodium load only as hypertonic saline (HS-FHH, dashed black line). The effects of these infusions (given between $t = 90$ and 180 minutes) on the plasma calcium (Ca^{2+}) and magnesium (Mg^{2+}) concentrations, their fractional excretions, and the plasma parathyroid hormone (PTH) and albumin concentrations are shown. Statistical analysis was performed only for the HS and IS-groups using a repeated measures general linear model, which generated one p -value for the differences in the overall trend.*

Discussion

In the present study, we sought to clarify the mechanism of osmomediated natriuresis in humans, hypothesizing that the CaSR was involved in the process. To do so, an established infusion protocol [2] was used in which the same sodium load was either given as hypertonic saline (HS) or as isotonic saline (IS) to healthy volunteers, and to two FHH-patients with an inactivating mutation of the CaSR. Osmomediated natriuresis was confirmed in the HS-group, because hyperosmolarity was followed by a significantly higher urinary sodium excretion (Figure 1). The similar courses of the calculated plasma volume, the plasma renin activity, and the plasma concentrations of aldosterone, ANP, BNP, and catecholamines suggest that none of these sodium regulating factors were responsible for the increased natriuresis in the HS-group, although the sympathetic nervous system was not directly analyzed. ADH is unlikely to have caused a natriuresis, because it will increase cAMP production, favoring sodium reabsorption [2,8,9].

Thus, other explanations for the mechanism of osmomedi-ated natriuresis should be sought. We provide two pieces of evidence which suggest that osmomedi-ated natriuresis could be mediated by the CaSR in the TAL. First, the ability of the HS-FHH group to induce an osmomedi-ated natriuresis was attenuated, despite a similar degree of hyperosmolarity (Figure 1). Notably, the FHH-subjects also had a lower initial P_{Na} (as shown previously in [10]) and a smaller increase in P_{ADH} , suggesting a generally altered osmoregulation in these patients. Second, this study is the first to show that osmomedi-ated natriuresis is associated with altered renal calcium and magnesium handling (Figure 2). This suggests the renal site for osmomedi-ated natriuresis to be the TAL, because sodium, calcium, and magnesium excretion are regulated in this nephron segment [11]. Other nephron segments are less likely to be involved in the process, because no differences between groups were observed in the excretion of phosphate (proximal tubule) and potassium (collecting ducts), or the course of aldosterone (which regulates sodium excretion in the distal convoluted tubule and collecting ducts). Previously, el-Haij Fuleihan *et al.*, in an infusion study with a PTH clamp, showed that changes in P_{Ca} influenced renal calcium, magnesium, and sodium handling [12]. They also postulated that the CaSR in the TAL mediated this effect [12].

Hyperosmolarity might cause a natriuresis by decreasing the EC_{50} of the CaSR, rendering it to be more active at the same P_{Ca} [3,4]. Activation of the CaSR induces at least two intracellular signaling pathways that inhibit the sodium-potassium-chloride co-transporter type 2 (NKCC2), and could thereby cause a natriuresis [9]. An interesting illustration of this mechanism are patients with an activating mutation of the CaSR (autosomal dominant hypocalcemia), who also exhibit increased natriuresis (hence sometimes called type V Bartter's syndrome) [5,9].

Interestingly, the rise in the fractional calcium excretion in the HS-group occurred despite an increase in plasma PTH, possibly because activation of the CaSR in the TAL exceeded the effect of PTH on the TAL. The decrease in P_{Ca} (in all groups) could have been caused by extracellular fluid volume expansion induced by the infusions. In addition, approximately 50% of the decrease in P_{Ca} was accounted for by a fall in the plasma albumin concentration (applying a correction factor of 0.2 mmol/l for every 10 g/l decrease in plasma albumine concentration). The "true" decrease in P_{Ca} and/or extracellular fluid volume expansion may have caused the plasma PTH to rise [13]. However, if hypertonic saline would indeed decrease the EC_{50} of the CaSR, one would expect this effect both in the kidney and the parathyroid gland. In this reasoning, plasma PTH should fall in response to hypertonic saline, which it did not, at least not initially (Figure 2). Possible explanations include a kidney specific effect (e.g., to prevent kidney stone formation during hyperosmolarity [14]), or a higher local sodium chloride concentration in the basolateral TAL.

In conclusion, we showed that osmomodulated natriuresis in humans is associated with increased renal calcium and magnesium excretion, suggesting the site of action to be the TAL. In the TAL, hypertonic saline might indirectly activate the CaSR, inhibit the NKCC2, and cause a natriuresis. Evidence for this mechanism of “salinity sensing” was found by studying two patients with an inactivating mutation of the CaSR in whom urinary sodium, calcium, and magnesium excretion was attenuated. We acknowledge that our results are preliminary, and that further research is necessary. Nevertheless, our observations provide interesting new clues on the mechanism of osmomodulated natriuresis and suggest novel roles for the CaSR in human physiology.

References

1. Zucker IH, Kaley G. Natriuresis induced by intracarotid infusion of hypertonic NaCl. *Am J Physiol* 1976; 230: 427-33.
2. Andersen LJ, Andersen JL, Pump B, Bie P. Natriuresis induced by mild hypernatremia in humans. *Am J Physiol Regul Integr Comp Physiol* 2002; 282: R1754-61.
3. Quinn SJ, Kifor O, Trivedi S, Diaz R, Vassilev P, Brown E. Sodium and ionic strength sensing by the calcium receptor. *J Biol Chem* 1998; 273: 19579-86.
4. Nearing J, Betka M, Quinn S, Hentschel H, Elger M, Baum M, et al. Polyvalent cation receptor proteins (CaRs) are salinity sensors in fish. *Proc Natl Acad Sci U S A* 2002; 99: 9231-6.
5. Brown EM, Pollak M, Seidman CE, Seidman JG, Chou YH, Riccardi D, et al. Calcium-ion-sensing cell-surface receptors. *N Engl J Med* 1995; 333: 234-40.
6. Derkx FH, Tan-Tjong L, Wenting GJ, Boomsma F, Man in 't Veld AJ, Schalekamp MA. Asynchronous changes in prorenin and renin secretion after captopril in patients with renal artery stenosis. *Hypertension* 1983; 5: 244-56.

7. van der Hoorn FA, Boomsma F, Man in 't Veld AJ, Schalekamp MA. Determination of catecholamines in human plasma by high-performance liquid chromatography: comparison between a new method with fluorescence detection and an established method with electrochemical detection. *J Chromatogr* 1989; 487: 17-28.
8. Andersen LJ, Andersen JL, Schutten HJ, Warberg J, Bie P. Antidiuretic effect of subnormal levels of arginine vasopressin in normal humans. *Am J Physiol* 1990; 259: R53-60.
9. Hebert SC. Calcium and salinity sensing by the thick ascending limb: a journey from mammals to fish and back again. *Kidney Int Suppl* 2004; S28-33.
10. Kristiansen JH, Brochner-Mortensen J, Pedersen KO. Renal tubular function in familial hypocalciuric hypercalcemia. *Contrib Nephrol* 1987; 56: 210-4.
11. Greger R. Ion transport mechanisms in thick ascending limb of Henle's loop of mammalian nephron. *Physiol Rev* 1985; 65: 760-97.
12. el-Hajj Fuleihan G, Seifter J, Scott J, Brown EM. Calcium-regulated renal calcium handling in healthy men: relationship to sodium handling. *J Clin Endocrinol Metab* 1998; 83: 2366-72.
13. Doris PA. Plasma parathyroid hormone during acute volume expansion in the rat. *Life Sci* 1986; 38: 2097-102.
14. Sands JM, Naruse M, Baum M, Jo I, Hebert SC, Brown EM, et al. Apical extracellular calcium/polyvalent cation-sensing receptor regulates vasopressin-elicited water permeability in rat kidney inner medullary collecting duct. *J Clin Invest* 1997; 99: 1399-405.

Part B

Clinical disorders of
water and sodium
balance

Chapter 7

**Diagnostic approach to a patient with
hyponatraemia: traditional versus
physiology-based options**

**Ewout J. Hoorn, Mitchell L. Halperin
and Robert Zietse**

QJM 2005; 98: 529–40

Abstract

The usual diagnostic approach to a patient with hyponatraemia is based on the clinical assessment of the extracellular fluid (ECF) volume, and laboratory parameters such as plasma osmolality, urine osmolality and/or urine sodium concentration. Several clinical diagnostic algorithms (CDA) applying these diagnostic parameters are available to the clinician. However, the accuracy and utility of these CDAs has never been tested. Therefore, we performed a survey in which 46 physicians were asked to apply all existing, unique CDAs for hyponatraemia to four selected cases of hyponatraemia. The results of this survey showed that, on average, the CDAs enabled only 10% of physicians to reach a correct diagnosis. Several weaknesses were identified in the CDAs, including a failure to consider acute hyponatraemia, the belief that a modest degree of ECF contraction can be detected by physical examination supported by routine laboratory data, and a tendency to diagnose the syndrome of inappropriate secretion of antidiuretic hormone prior to excluding other causes of hyponatraemia. We conclude that the typical architecture of CDAs for hyponatraemia represents a hierarchical order of isolated clinical and/or laboratory parameters, and that they do not take into account the pathophysiological context, the mechanism by which hyponatraemia developed and the clinical dangers of hyponatraemia. These restrictions are important for physicians confronted with hyponatraemic patients and may require them to choose different approaches. We therefore conclude this review with the presentation of a more physiology-based approach to hyponatraemia, which seeks to overcome some of the limitations of the existing CDAs.

Introduction

There are two different, but not mutually exclusive, ways to arrive at a clinical diagnosis in a patient with hyponatraemia and to design appropriate therapy [1]. The first, which we shall call the traditional approach, uses a combination of clinical and laboratory parameters, and often relies on the use of clinical diagnostic algorithms (CDA). The second, which we shall call the physiology-based approach, emphasizes the underlying mechanisms that might have led to the development of hyponatraemia. It applies simple principles of physiology at the bedside, and relies on deductive reasoning and a quantitative analysis [1,2]. Our objective is to compare these two approaches by conducting a survey where physicians were asked to apply ten different CDAs for hyponatraemia in four selected cases. The outcome was compared to a physiology-based approach.

Methods

Literature review

Published CDAs were identified from a literature search using 'hyponatraemia' (Medical Subject Heading) limited to review articles published in the English language between January 1998 and August 2004. The search yielded 218 articles, of which 11 were review articles that included a CDA with an approach to the patient with hyponatraemia [3-13]. We also collected eight CDAs from textbooks of general internal medicine, nephrology and endocrinology [14-21]. Eliminating identical and overlapping CDAs, these 19 CDAs were reduced to 10 (Table 1). The parameters to evaluate included a clinical assessment of the extracellular fluid (ECF) volume (8/10), fluid challenge tests (1/10), and whether hyponatraemia was acute (1/10). In the

laboratory data, plasma osmolality (P_{Osm}) (4/10), plasma urate (1/10), renal function (1/10), urine sodium concentration (U_{Na}) (7/10), urine osmolality (U_{Osm}) (5/10), and the fractional excretion of urea and urate (1/10) were assessed (Table 1) [3-21].

Survey

Four challenging cases where hyponatraemia was a central diagnostic issue were selected [22-25]. To determine the value of each CDA in the differential diagnosis of hyponatraemia, 60 surveys containing the four cases and the ten CDAs were sent to physicians from five different countries (Canada, the Netherlands, South-Africa, Taiwan, USA); 46 surveys (from 27 residents, 6 fellows, and 13 staff physicians in internal medicine specialties) were available for complete analysis (77%). The reason that we chose the survey as our method of analysis is that it provides a reasonable way to evaluate the current diagnostic approaches to hyponatraemia.

Physicians were asked to provide a (differential) diagnosis in the first three cases using each CDA. Respondents could also indicate that they could not reach a diagnosis with the available information. Diagnoses were subsequently classified as correct, not correct, or not possible (Table 2). Because Case 4 lacked most of the information required for the CDAs, we chose to present a list of possible diagnoses for this case in multiple-choice format (Table 2). We were also interested in additional information such as the likelihood that hyponatraemia could be life-threatening, and which therapy the physicians would select. Finally, a case-specific question was asked (Table 2).

Table 1: Existing algorithms for hyponatraemia and their accuracy in three illustrative test cases

Algorithm	Algorithm hierarchy, parameters and cut-off values	References	Correct diagnosis in 3 test cases
1	ECF-volume	5, 9	6%
2	ECF-volume - U_{Na} (20-40)	6, 14, 20, 21	7%
3	ECF-volume U_{Na} (20)	3, 19	8%
4	Acuity of hyponatraemia ECF-volume	13	6%
5	ECF-volume U_{Na} (20) U_{osm} (500)	4, 16	12%
6	P_{osm} U_{osm} ECF-volume	8, 11, 12	9%
7	P_{osm} U_{osm} U_{Na}	17	11%
8	P_{osm} (280-295) ECF-volume U_{Na} (10 and 20)	15, 18	7%
9	P_{osm} (280) U_{osm} (100) U_{Na} (20-40) Fluid challenge tests FE_{urea} , FE_{urate} , P_{urate}	10	9%
10	U_{osm} (100) Renal function ECF-volume U_{Na} (20)	7	9%

Cases

Case 1

An 88-year-old man had complained of nausea and vomiting for 4 weeks [22]. A cutaneous B-cell lymphoma on his right cheek had been diagnosed 2 months ago. He was not taking medications, and had lost 2 kg in weight over the past several months. Physical examination was not consistent with a contracted ECF volume (normal pulse rate and blood-pressure, no orthostatic changes). Plasma sodium (P_{Na} 125 mmol/l), potassium (P_K 4.5 mmol/l), glucose (72 mg/dl; 4.0 mmol/l), creatinine (P_{creat} 0.9 mg/dl; 77 μ mol/l) and thyroid stimulating hormone (2.3 mIU/l) were measured on admission. His U_{Na} was 100 mmol/l. Plasma pH, plasma cortisol, and U_{osm} were not measured.

Case 2

A 19-year-old woman had myasthenia gravis [23]. Her main complaints were progressive weakness and fatigue over the past 6 months. Her appetite was poor and she had a 3 kg weight loss. In addition to a P_{Na} of 118 mmol/l, she had four other important abnormalities: hyperkalaemia (8.1 mmol/l), hypoglycaemia (45 mg/dl; 2.5 mmol/l), a low ECF volume (blood pressure 60/40 mmHg, heart rate 126 bpm in the absence of blood loss) and a low glomerular filtration rate (GFR) (P_{creat} 5.3 mg/dl; 461 μ mol/l). During the initial 12 h, she received 4.6 l isotonic saline and excreted 4.5 l urine with a U_{osm} of 438 mOsm/kg H_2O and a $U_{Na} + U_K$ of 80 mmol/l.

Case 3

A thin, 34-year-old woman ran several miles per day in a hot environment [24]. Because she sweated profusely, and because she thought it a healthy habit, she drank a large unmeasured volume of water per day. She was an ovolactovegetarian and had a restricted salt intake. She did not smoke, consume alcohol, or use illicit drugs or herbs. Her only symptom was polyuria (4–5 l/day). There were no findings on physical examination to indicate that her ECF volume was contracted. The principal lab findings were chronic hyponatraemia (131 mmol/l) with P_{osm} 268 mOsm/kg H₂O, U_{osm} 81 mOsm/kg H₂O, and U_{Na} 10 mmol/l. She did not have a high P_{creat} (0.9 mg/dl; 80 μ mol/l) or plasma urea (5.9 mg/dl; 2.1 mmol/l), or a low P_{K} (4.0 mmol/l). Follow-up studies did not reveal thyroid or adrenal insufficiency, a metabolic disease (e.g., porphyria), or lesions to explain why antidiuretic hormone (ADH) might be released.

Case 4

An 18-year-old female presented to the Emergency Department because she became unwell at a party [reference 25, case adapted for this review]. Shortly after arrival, she had a grand mal seizure. In blood drawn immediately after the seizure, her P_{Na} was 130 mmol/l; all other measured values except for her plasma chloride were within the normal range. Body temperature was 40°C; other vital and haemodynamic signs were unremarkable. During the first hour of hospitalization, she did not void and therefore there were no urine data. History from accompanying friends revealed that the patient had taken MDMA (methylenedioxymethamphetamine, ‘Ecstasy’), that she had consumed a considerable volume of water, but no alcohol, and that she had been dancing on a crowded and hot dance floor.

Table 2: Results from survey, algorithm diagnoses, and respondents' analysis of cases

Case	Algorithm diagnoses	Threat to life?	Therapy	Case-specific question
1	• SIADH 48% • Adrenal insufficiency 11% • Not possible 30% • Other 11%	• Yes 11% • No 89%	• Hypertonic solution 4% • Isotonic solution 26% • Hypotonic solution 2% • Water restriction 65% • Other 3%	<i>How important is ECF-volume in differentiating hyponatraemia?</i> • Most important 2% • Important 30% • Not very important 61% • Not important 4% • No answer 3%
2	• Adrenal insufficiency 19% • Primary renal disease 7% • Not possible 66% • Other 8%	• Yes 74% • No 26%	• Hypertonic solution 13% • Isotonic solution 57% • Hypotonic solution 9% • Water restriction 2% • Other 19%	<i>Which rate of correction?</i> • 12 mmol/l/24-h 22% • 8 mmol/l/24-h 61% • 5 mmol/l/24-h 2% • 0-4 mmol/l/24-h 15%
3	• Psychogenic polydipsia 53% • SIADH 12% • Not possible 21% • Other 14%	• Yes 0% • No 100%	• Hypertonic solution 0% • Isotonic solution 2% • Hypotonic solution 0% • Water restriction 80% • Other 18%	<i>Is hyponatraemia due to low Na intake possible?</i> • Yes 24% • No 70% • No answer 6%
4	<i>Multiple choice</i> • SIADH 11% • Water ingestion 13% • Na loss in sweat 28% • Not possible 17% • Other 31%	• Yes 59% • No 41%	• Hypertonic solution 24% • Isotonic solution 54% • Hypotonic solution 2% • Water restriction 9% • Other 9% • No answer 2%	<i>Is there a reason to believe that the P_{Na} is under- or overestimated?</i> • Yes 48% • No 52%

Results

Analysis of cases with the traditional approach

Table 1 depicts the ten CDAs, as well as how often each algorithm led to the correct diagnosis. Table 2 depicts the two most frequently chosen diagnoses in Cases 1, 2 and 3 and how often a diagnosis was considered not possible to make using the available data.

When compared to the final diagnoses (Table 3), the results demonstrate that for Cases 1, 2 and 3 a correct diagnosis was made in 11%, 19% and 0%, whereas an incorrect diagnosis was made by 59% (48 + 11), 15% (7 + 8) and 79% (53 + 12 + 14) of the respondents. In addition, 30%, 66% and 21% of the respondents felt that insufficient data were provided to establish a diagnosis in these three cases, respectively (Table 2). Table 2 also shows the respondents' opinion about whether hyponatraemia appeared to be life-threatening, which therapy they would have chosen, and which causes of hyponatraemia in Case 4 were thought to be likely.

Analysis and comparison with the physiology-based approach

Table 3 summarizes the four cases using a physiology-based approach. It includes the clinical diagnosis, diagnostic pitfalls that were recognized, the physiological principles that were applied and, finally, potential threats to survival.

Table 3: Analysis of cases with a physiology-based approach

Case	Presentation	Diagnosis	Diagnostic pitfalls	Applied physiological principles	Potential threats to survival
1	Hyponatraemia in the absence of a detectably contracted ECF-volume	“Normokalaemic” Addison’s disease	• Apparent normovolaemia • Normokalaemic Addison’s disease	• ECF-contraction may not be detectable [28-34] • Calculation of transtubular K gradient [2,70-72]	<i>Untreated:</i> haemodynamic collapse <i>Treatment:</i> osmotic demyelination
2	Excessive renal excretion of Na	Addison’s disease with an additional stimulus for Na excretion	• Acute presentation of chronic hyponatraemia • Second stimulus for high Na excretion	• Calculation of tonicity balance [1,2,43] • Calculation of fractional excretion of sodium [2,72]	<i>Untreated:</i> haemodynamic collapse <i>Treatment:</i> osmotic demyelination
3	Hyponatraemia with the ability to have a water diuresis	Hyponatraemia due to low delivery of solutes to the collecting duct	• Polyuria in the context of hyponatraemia • Hyponatraemia with minimal release of ADH	• Normal kidneys are able to excrete 12 liters of electrolyte free water [2] • Calculation of free water clearance [24]	<i>Increase in water balance:</i> cerebral oedema <i>Treatment:</i> osmotic demyelination
4	Acute hyponatraemia in a patient who took ‘Ecstasy’	Ecstasy-induced hyponatraemia	• Acute versus chronic hyponatraemia • Discrepancy between symptoms and P_{Na} • Rise in P_{Na} due to seizure	• Different pathophysiology acute and chronic hyponatraemia [59] • P_{Na} may be confounded after a seizure [55]	<i>Untreated:</i> cerebral oedema

Discussion

Each case will be analysed in more detail using a physiology-based approach, and this information will be compared to the outcome reached using the traditional approach. We shall organize this section by defining the problem in each case after presenting its outcome (for Cases 1 and 2), and by subsequently asking two questions that are clinically relevant and address the specific challenges of each case.

Case 1: Hyponatraemia without evidence of a contracted ECF volume

Continuation of the case: Because of the lymphoma, normal P_K , absence of signs of ECF volume contraction, and a U_{Na} of 100 mmol/l, 48% of the respondents selected the syndrome of inappropriate ADH secretion (SIADH) as the diagnosis (Table 2). The preferred treatment was water-restriction by 65% of the respondents and the authors of the paper [22]. With this therapy, hyponatraemia persisted and, unfortunately, the patient died in hospital 2 weeks after admission with haemodynamic collapse. Post-mortem examination revealed adrenal failure (plasma aldosterone 0 pmol/l) [22].

Definition of the problem: Addison's disease was not suspected and/or excluded because signs of a low ECF volume were not found and hyperkalaemia was absent. Although the symptoms nausea and vomiting may have been suggestive of corticotropic insufficiency [26], they are rather non-specific and can also be symptoms of hyponatraemia [27]. Most of the respondents (89%) stated that his hyponatraemia was not potentially life-threatening (Table 2). In conclusion, the two questions that arise

from this case are, ‘How reliable is the clinical assessment of the ECF volume?’, and, ‘Why was the P_K in the normal range?’

(i) How reliable is the clinical assessment of the ECF volume?

All but two CDAs included ECF volume as an important parameter to determine the cause of hyponatraemia, and in 4/10 (40%) it was the *first* parameter to assess (Table 1). In cases in which a low ECF volume cannot be established by clinical examination, this may lead to the exclusion of causes of hyponatraemia that typically present with a contracted ECF volume such as Addison's disease. In contrast to the importance the 8 CDAs gave to an evaluation of the ECF volume, the majority of the physicians (65%; 61 + 4) believed that determining ECF by physical examination is *not very reliable* and should only be supportive in the differentiation of hyponatraemia (Table 2). This opinion is supported by the literature. Several studies have analysed the validity of confirmatory tests for ECF contraction, performed by physical diagnosis [28-32], haemodynamic parameters (blood pressure, central venous pressure, blood volume, plasma volume, cardiac output) [33], laboratory analysis (ADH, aldosterone, catecholamines, renin, fractional excretions of Na or urea and total urates) [33], and/or urine electrolyte data [34], and all unequivocally showed a low sensitivity and specificity. Therefore, although clinical decision-making is often based on the assumption of ‘dehydration’, the degree of confidence in this impression is not supported by a strong database.

(ii) How can the absence of hyperkalaemia in Addison's disease be explained? Hyperkalaemia is not observed in 1/3 of patients with Addison's disease [35]; hence its absence does not exclude

this diagnosis. Perhaps normokalaemia reflects a poor intake of K, a deficiency of cortisol with sufficient aldosterone activity remaining to avoid hyperkalaemia, and/or the rate of excretion of K may be unusually high because of a high distal delivery of Na when Na reabsorption in an upstream nephron site was inhibited (aldosterone augments Na reabsorption in the distal convoluted tubule) [36,37].

Case 2: Excessive renal excretion of sodium

Continuation of the case: There was a presumptive diagnosis of Addison's disease on the basis of her auto-immune disease. As a result of the infusion of isotonic saline, there was a decrease in her P_K to 5.1 mmol/l and an increase in her P_{Na} to 129 mmol/l. Unfortunately, this increase in the P_{Na} led to the osmotic demyelination syndrome (ODS) (confirmed by magnetic resonance imaging) and the patient remains in a vegetative state with frequent attacks of myoclonus.

Definition of the problem: At first glance, Addison's disease appears to provide a sufficient explanation for the observed hyponatraemia and ECF volume contraction. However, this assumption conflicts with her Na + K excretion rate, which was close to 360 mmol in 12 h (80 mmol/l x 4.5 l) after therapy began. This can be extrapolated to 720 mmol/day (500 μ mol/min), a value that is almost 5-fold higher than the usual Na + K excretion rate [38,39]. Hence it is prudent to ask, 'Can low aldosterone levels be the sole explanation for hyponatraemia?' And, with regard to the development of ODS, 'How might ODS have been prevented?'

(i) *Can hypoaldosteronism explain her deficit of Na?* This question can be answered when this patient's renal function is evaluated. Because the GFR should be reduced when the blood pressure is low, the filtered load of Na will be much lower than normal. Moreover, Na reabsorption in nephron segments where aldosterone does not act should be stimulated by the low ECF volume. In quantitative terms, because her P_{Creat} was elevated 6-fold, her GFR should be $\sim 1/6$ of normal (20 vs. 120 ml/min). Therefore, her filtered load of Na should be 2360 $\mu\text{mol/min}$ (118 $\mu\text{mol/ml} \times 20 \text{ ml/min}$) with this GFR. Accordingly, the fractional excretion of Na (FE_{Na}) is $\sim 25\%$ (500/2360 $\mu\text{mol/min}$), a value that is much greater than the 'expected' 5% of the filtered load of Na. This high FE_{Na} suggests that a second 'renal lesion' contributed to the excessive excretion of Na [23]. The above analysis illustrates that in a case where hyponatraemia is associated with a low ECF volume and impaired renal function, it is useful to calculate the FE_{Na} . One CDA introduced the option of evaluating renal function in their strategy [7], but *none* suggested calculating the FE_{Na} (Table 1).

(ii) *How might ODS have been prevented?* This patient was treated with isotonic saline and developed ODS—her P_{Na} increased by 11 mmol/l in 24 h. Although a rate of ~ 12 mmol/l/day is said to be acceptable [40], it is not a target to be achieved, rather, it is an *upper limit not to be exceeded* [2]. Moreover, because this patient had chronic hyponatraemia and was catabolic, the risk of ODS was increased [41]. Therefore, in cases where there is an 'acute discovery of chronic hyponatraemia' the target for the daily rise in the P_{Na} should be much lower, $\sim 0\text{--}4$ mmol/l on the first day [2,23,42]. The reasons that her P_{Na} rose so quickly when isotonic saline was infused

becomes apparent when a tonicity balance is calculated [43]. There was a trivial positive water balance of $4.6 - 4.5 = 0.1$ l. In contrast, there was a large positive Na balance of 330 mmol ($4.5 \text{ l} \times 150 - 4.6 \text{ l} \times 80$). Prevention of this rapid rise in P_{Na} could have been achieved by matching the tonicity and volume of the infusate to that of the urine (in this case infusing close to half-isotonic saline). This emphasizes the importance of what can be called an intravenous (IV) fluid regimen that remains ‘isotonic to patient’ in *balance* terms. Of the respondents, the majority (78%; $61 + 2 + 15$) stated that although they would apply a correction rate of ~ 8 mmol/l/day or less, their choices of the type of IV fluid was isotonic (57%) or even hypertonic saline (13%), possibly as a result of the belief that her hyponatraemia was life-threatening (74%, Table 2). This therapy caused too rapid a rise in her P_{Na} and, consequently, the ODS.

Case 3: Hyponatraemia with the ability to have a water diuresis

Definition of the problem: In this case, the first impression is that hyponatraemia is due to the ingestion of large quantities of water. Indeed, most CDAs led to the diagnosis of primary polydipsia (53%, Table 2). However, if this were the case and if kidney function were normal, one would excrete the maximum volume of dilute urine, which is ~ 15 l/day [2]. Furthermore, because the U_{osm} is much lower than the P_{osm} , there appears to be very little ADH action. These considerations lead to the following questions: ‘How can hyponatraemia occur in the context of minimal ADH release?’, and, ‘Which groups of patients are susceptible to this type of hyponatraemia?’

(i) *How can hyponatraemia occur in the context of minimal release of ADH?* Water retention in this setting of minimal ADH release can occur when there is a low delivery of filtrate to the distal nephron [44]. To have a low distal volume delivery, there should be a low GFR and/or enhanced reabsorption of filtrate in the proximal convoluted tubule, responses that typically accompany a low intake of sodium chloride [45]. Second, there may be a small degree of water permeability in the distal nephron that could be the result of trace levels of ADH - levels that are not detected by conventional assays [46] and/or by ADH-insensitive water permeability (called basal water permeability [47]). In this patient, there was a very low distal volume delivery as reflected by the low rate of excretion of osmoles. Because urinary osmolality is a composite of both electrolytes (primarily sodium, potassium, and their accompanying anions) and excreted solutes, electrolyte-free water excretion is also dependent on the total rate of solute excretion [24]. Therefore, if solute excretion becomes very low, electrolyte-free water will be retained and hyponatraemia may ensue. In this patient, the low rate of solute excretion can be explained by the combination of a low-protein diet (low urea) and a low NaCl intake and/or a large non-renal or former renal NaCl loss. Because isolated groups that eat a diet with little NaCl do not suffer from hyponatraemia [48], a low P_{Na} will *not* develop solely because of low salt intake, contrary to the comments of 24% of the respondents (Table 2).

(ii) *Which groups of patients are likely to develop this type of hyponatraemia?* There are three settings where hyponatraemia is associated with a low rate of excretion of electrolyte-free water without having detectable levels of ADH in plasma—called

‘trickle-down hyponatraemia’ by Oh *et al* [49]. First, this can occur in elderly patients who consume tea (electrolyte-free water) and toast (low protein) diets, especially if treated with a thiazide diuretic and a low salt diet for hypertension. Second, it can be observed in patients who wish to control their body weight by diet and exercise, especially if they have a large intake of water. Although exercise causes a large sweat loss, if the diet is particularly low in NaCl, the net effect can result in a very low renal excretion of Na and Cl. The third setting for trickle-down hyponatraemia is beer potomania [50,51]. Because dietary carbohydrate, fat and ethanol all have carbon dioxide and water as end-products that are excreted in a 1:1 stoichiometry via the lungs [52], they will not usually produce many urinary osmoles because beer is low in protein and NaCl. In this patient, hyponatraemia was not considered to pose an immediate danger (0%, Table 2). However, this patient could be susceptible to brain swelling if water intake continued, but water loss in sweat (did not run that day) or urine (non-osmotic reason for ADH release) was prevented. In addition, brain damage (ODS) from rapid correction of hyponatraemia could occur, especially if she was malnourished and/or K-depleted [41].

Case 4: Acute hyponatraemia in a patient who took ‘Ecstasy’

Definition of the problem: In this case, there appears to be a discrepancy between the measured P_{Na} and the severity of the symptoms. Hence the questions are: ‘Might there be a confounding issue?’, and, ‘Should the emphasis in management be further diagnostic tests or immediate therapy?’

(i) *Might there be a confounding issue?* Symptoms related to acute hyponatraemia (<48 h) most commonly occur if the P_{Na} is < 125 mmol/l [53,54]. Nevertheless, the presenting symptom in this case was a seizure, which can raise the P_{Na} acutely by 10–15 mmol/l [55]. The mechanism involves the generation of new osmoles that are retained in skeletal muscle cells (which account for 50% of total body water) and cause water to shift from the ECF to the intracellular compartment [55]. A similar situation may be seen when severe rhabdomyolysis causes hypernatraemia [2]. The P_{Na} should therefore be re-evaluated after the seizure to reveal the steady-state P_{Na} . Almost half of the respondents recognized that the P_{Na} may represent a non-steady-state value (48%, Table 2). In conclusion, this case illustrates the importance of analysing possible confounding factors in the differential diagnosis of hyponatraemia. The most common examples include ‘pseudohyponatraemia’ [56], and situations where an ‘effective’ osmole in the ECF prevents water from moving into the intracellular compartment (e.g., hyperglycaemia, therapy with mannitol, surgery with lavage fluids) [2,57,58].

(ii) *Should the next focus be diagnostic tests or therapy?* In the patient with acute symptomatic hyponatraemia, therapeutic considerations dominate over diagnostic ones [59]. Once acute, symptomatic hyponatraemia is suspected, it is imperative to infuse hypertonic saline, because irreversible changes in brain function can occur in a very short time [60]. A minority of the respondents chose a hypertonic solution (24%, Table 2), although 59% did believe that the hyponatraemia was life-threatening (Table 2). Remarkably, only one recently published algorithm included the distinction of acute vs. chronic

hyponatraemia in the diagnostic approach (Table 1) [13], despite ample literature on this subject [61-63]. This may delay recognizing the dangers of acute hyponatraemia, which should always be the primary focus. Finally, with regard to the pathophysiology of Ecstasy-induced hyponatraemia, different possible mechanisms have been described, including ADH release [64], water intoxication [65], and reduced intestinal motility [25].

Concluding remarks

For a classification to be useful, it must permit the clinician to reach the correct diagnosis and implement the appropriate therapy. It must also rely on criteria that are valid and available in a timely fashion. Unfortunately, as demonstrated by the selected (and biased) cases, the existing CDAs did not live up to these standards (Table 1). In part, the low accuracy can be explained because many physicians felt it was impossible to establish a diagnosis when they applied the available data to the CDAs (Table 2). However, the requirement that certain values should be available before being able to proceed in the CDA, is in itself a weak point, and may unnecessarily delay diagnosis and treatment. In our analysis, the most serious error was the failure to rule out acute hyponatraemia as the first step. A second weak point was the mistaken belief that a clinician could detect a mild to modest degree of ECF volume contraction by physical examination supported by routine laboratory data. This was most evident in Case 1, because it led to an incorrect diagnosis (SIADH) and improper treatment (water restriction) that could have been responsible for the fatal outcome, due to eventual haemodynamic collapse [22]. Although more laboratory tests

may have been desirable, this case appears to represent a more common pitfall where the clinical scenario resembles SIADH, which subsequently is not reconsidered and may lead to an adverse outcome [22,26,66,67]. Of note, SIADH should be a diagnosis of exclusion, and should only be considered if adrenal, thyroid and pituitary insufficiency are absent [26,66,67]. Another problem that merits attention is that the traditional approach often relies on generalizations rather than reliable data. Examples cited were the need to find hyperkalaemia to diagnose Addison's disease [55] and to assume a stable P_{Na} under circumstances where water-shifts are likely (e.g., seizures, rhabdomyolysis) [2,35].

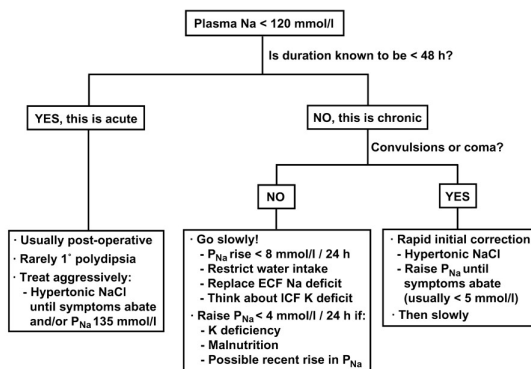
In summary, the typical architecture of the current CDAs represents a hierarchical order of isolated clinical and/or laboratory parameters. They do not take into account the pathophysiological context, the mechanism by which hyponatraemia developed and the clinical dangers of hyponatraemia. These restrictions are important for physicians confronted with hyponatraemic patients and may require them to choose different approaches.

Based on the above, we provide a different type of algorithm that is designed to recognize the dangers of hyponatraemia, and to consider the pathophysiology of hyponatraemia before analysing diagnostic parameters (Figure 1). This 'physiology-based algorithm' is separated in three steps. First, a distinction between acute and chronic hyponatraemia is made, and therapy is designed accordingly. If the patient is classified as chronic, but does have symptoms of hyponatraemia, a rapid initial restoration is recommended, because 'acute on chronic'

hyponatraemia is likely. In contrast, slow correction rates are advised when hyponatraemia is chronic and not symptomatic, especially when there is concomitant hypokalaemia and/or malnutrition, because in those situations the risk of ODS is high [23,41].

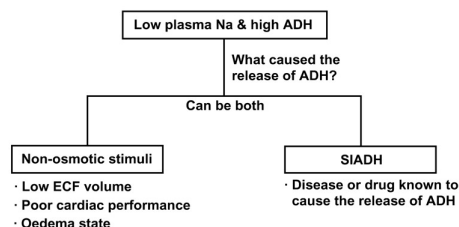
In the second step, the pathophysiology of hyponatraemia is reviewed, connecting it to the clinical situation at hand. In addition, the unique pathophysiology of hyponatraemia with low circulating levels of ADH is presented. The final step of our algorithm was intentionally organized in a tabular format so that all causes of hyponatraemia can still be taken into consideration without excluding others (Step 3, Figure 1). The philosophy behind this is that relying on one single value may be misleading, as expected values may vary depending on the situation. This has been illustrated for urinary values [36,68] and for diagnostic tests [26]. Here, a physiological analysis using simple formulae can be used simultaneously and synergistically with the traditional analysis (Figure 1 and Table 3) [2,24,69-74]. We emphasize that our algorithm is not intended to completely replace the other existing CDAs, but rather, to provide a physiology-based alternative, which seeks to overcome some of the identified limitations of the existing CDA's.

STEP 1: Determine if hyponatraemia is acute or chronic and design therapy accordingly



STEP 2: Consider the pathophysiology of hyponatraemia

A. Low plasma Na and high ADH



B. Low plasma Na and low ADH

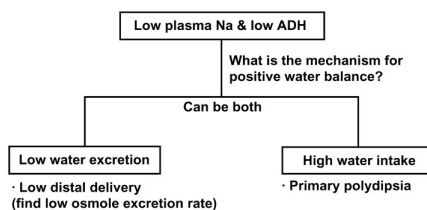


Figure 1: Physiology-based approach to hyponatraemia.

In Step 2, 'High ADH' and 'Low ADH' refer to pathophysiological considerations in the patient, and do not necessarily imply that the determination of ADH levels is required clinically; an estimate about ADH levels may also be inferred from (for example) the urine osmolality.

STEP 3: Confirm final diagnosis by integrated diagnostic approach

Mechanism	History	Possible ECF contraction	Possible findings in plasma	Typical U_{Na}	Physiological analysis	Final diagnosis
Low delivery of filtrate to distal nephron	Beer-potomania; low-solute diet.	No	- Low P_{urea} - Non-detectable ADH levels	Low	- $P_{osm} > U_{osm}$ - Calculate water clearance* [5]	Trickle-down hyponatraemia
Excessive water intake or administration	Presence of psychosis; hypotonic IV-fluids	No		Low	- $P_{osm} > U_{osm}$ - Intake > 15 L / 24 hours	Psychogenic polydipsia; hypotonic IV-fluids
Renal Na loss and physiological ADH secretion	Diuretic-use: often recent and often thiazides	Yes	- Low P_K - Metabolic alkalosis	High	- High U_K - High $U_{HCO_3^-}$	Diuretic-induced hyponatraemia
	Vomiting	Yes	- Low P_K - Metabolic alkalosis	High	- Low U_{Cl} - High $U_{HCO_3^-}$	Hyponatraemia due to vomiting
	Adrenal pathology: auto-immunity, tuberculosis, cancer, haemorrhage	Yes	- High P_K - Low cortisol, aldosterone - Metabolic acidosis	High	- Transtubular K gradient < 2 [†] [69-71]	Addison's disease; adrenal insufficiency
	Intracerebral lesion; polyuria	Yes		High	- Time-course U_{Cl} [73]	Cerebral salt wasting
Non-renal Na loss	Diarrhea; burns; ileus; pancreatitis	Yes	- Metabolic acidosis (in diarrhea)	Low		Hyponatraemia due to non-renal Na loss
Low cardiac output or low albumin	History of heart, liver or renal disease	No; oedema is possible	- Low P_{Alb} - Metabolic acidosis (in renal failure)	Low	- FE_{Na} (in renal failure)* [72]	Hyponatraemia associated with heart, liver or renal failure
Non-physiological release of ADH	Disease or drug known to cause release ADH	No	- Normal P_K - Low-normal P_{urea} and P_{urate}	High	- FE_{urate} [74] - Exclude (ad)renal, pituitary, thyroid insufficiency	Syndrome of inappropriate ADH secretion

* Free water clearance = urine output $\times (1 - U_{osm} / P_{osm})$

† Transtubular potassium gradient = $U_K / (U_{osm} / P_{osm}) / P_K$

‡ Fractional excretion of sodium = $(U_{Na} \times P_{creat}) / (P_{Na} \times U_{creat})$

Acknowledgments

We would like to thank Dr. R.H. Sterns, Dr. M.R. Davids and Dr. S.H. Lin for their cooperation with collecting the surveys.

References

1. Halperin ML, Bohn D. Clinical approach to disorders of salt and water balance. Emphasis on integrative physiology. *Crit Care Clin* 2001; 18: 249–72.
2. Halperin ML, Goldstein MB. Fluid, electrolyte and acid-base physiology: a problem-based approach, 3rd edn. Philadelphia, WB Saunders, 1999.
3. Kumar S, Berl T. Sodium. *Lancet* 1998; 352: 220–8.
4. Smith DM, McKenna K, Thompson CJ. Hyponatraemia. *Clin Endocrinol (Oxf)* 2000; 52: 667–78.
5. Adroge HJ, Madias NE. Hyponatremia. *N Engl J Med* 2000; 342: 1581–9.
6. Fall PJ. Hyponatremia and hypernatremia. *Postgrad Med* 2000; 107: 75–82.
7. Kugler JP, Hustead T. Hyponatremia and hypernatremia in the elderly. *Am Fam Physician* 2000; 6: 3623–30.
8. Palmer BF. Hyponatremia in a neurosurgical patient: syndrome of inappropriate ADH secretion versus cerebral salt wasting. *Nephrol Dial Transplant* 2000; 15: 262–8.
9. Schmitz PH, de Meijer PH, Meinders AE. Hyponatraemia due to hypothyroidism: a pure renal mechanism. *Neth J Med* 2001; 58: 143–9.
10. Milionis HJ, Liamis GL, Elisaf MS. The hyponatraemic patient: a systematic approach to laboratory diagnosis. *CMAJ* 2002; 166: 1056–62.
11. Palmer BF, Gates JR, Lader M. Causes and management of hyponatremia. *Ann Pharmacother* 2003; 37: 1694–702.
12. Palmer BF. Hyponatremia in patients with central nervous system disease: SIADH versus CSW. *Trends Endocrinol Metab* 2003; 14: 182–7.
13. Yeates KE, Singer M, Morton AR. Salt and water: a simple approach to hyponatraemia. *CMAJ* 2004; 170: 365–9.

14. Brenner BM, ed. *The Kidney*, 7th edn. Philadelphia, WB Saunders, 2004.
15. Braunwald E, Kasper DL, Fauci AS, Hauser SL, Longo DL, Jameson JL, eds. *Harrison's principles of internal medicine*, 15th edn. New York, McGraw-Hill, 2001.
16. Longmore M, Wilkinson I, Torok E. *Oxford handbook of clinical medicine*, 5th edn. New York, Oxford University Press, 2002.
17. Rose BD, Post TW. *Clinical physiology of acid-base and electrolyte disorders*, 5th edn. New York, McGraw-Hill, 2003.
18. Tierney LM, McPhee SJ, Papadakis MA, eds. *Current medical diagnosis & treatment*, 43rd edn. New York, McGraw-Hill, 2003.
19. Johnson R, Feehally J, eds. *Comprehensive clinical nephrology*, 2nd edn. New York, Mosby, 2003.
20. Warrell DA, Cox TM, Firth JD, eds. *Oxford textbook of medicine*, 4th edn. New York, Oxford University Press, 2003.
21. DeGroot LJ, Jameson JL, eds. *Endocrinology*, 4th edn. Philadelphia, WB Saunders, 2001.
22. Van Steijn JH, Gans RO. Clinical thinking and decision making in daily practice. An elderly man with hyponatraemia. *Ned Tijdschr Geneeskd* 2002; 146: 404–10.
23. Lin SH, Hsu YJ, Chiu JS, Chu SJ, Davids MR, Halperin ML. Osmotic demyelination syndrome: a potentially avoidable disaster. *QJM* 2003; 96: 935–47.
24. Thaler SM, Teitelbaum I, Berl T. 'Beer potomania' in non-beer drinkers: effect of low dietary solute intake. *Am J Kidney Dis* 1998; 31: 1028–31.
25. Cherney DZ, Davids MR, Halperin ML. Acute hyponatraemia and 'ecstasy': insights from a quantitative and integrative analysis. *QJM* 2002; 95: 475–83.
26. Smith JC, Siddique H, Corrall RJ. Misinterpretation of serum cortisol in a patient with hyponatraemia. *BMJ* 2004; 328: 215–16.
27. Verbalis JG. Adaptation to acute and chronic hyponatremia: implications for symptomatology, diagnosis, and therapy. *Semin Nephrol* 1998; 18: 3–19.
28. McCance RA. Medical problems in mineral metabolism III. Experimental human salt deficiency. *Lancet* 1936; 230: 823–30.

29. Spital A. Clinical assessment of extracellular fluid volume in hyponatremia. *Am J Med* 1988; 84: 562.
30. Chung HM, Kluge R, Schrier RW, Anderson RJ. Clinical assessment of extracellular fluid volume in hyponatremia. *Am J Med* 1987; 83: 905–8.
31. Gorelick MH, Shaw KN, Murphy KO. Validity and reliability of clinical signs in the diagnosis of dehydration in children. *Pediatrics* 1997; 99: E6.
32. McGee S, Abernethy WB, Simel DL. The rational clinical examination. Is this patient hypovolemic? *JAMA* 1999; 281: 1022–9.
33. Oh MS, Carroll HJ. Cerebral salt-wasting syndrome. We need better proof of its existence. *Nephron* 1999; 82: 110–14.
34. Kamel KS, Ethier JH, Richardson RM, Bear RA, Halperin ML. Urine electrolytes and osmolality: when and how to use them. *Am J Nephrol* 1990; 10: 89–102.
35. Nerup J. Addison's disease-clinical studies. A report of 108 cases. *Acta Endocrinol* 1974; 76: 127–41.
36. Gagnon RF, Halperin ML. Possible mechanisms to explain the absence of hyperkalemia in Addison's disease. *Nephrol Dial Transplant* 2001; 16: 1280–4.
37. Kim GH, Masilamani S, Turner R, Mitchell C, Wade JB, Knepper MA. The thiazide-sensitive Na-Cl cotransporter is an aldosterone-induced protein. *Proc Natl Acad Sci USA* 1998; 24: 14552–7.
38. Taubes G. The (political) science of salt. *Science* 1998; 281: 898–901, 903–7.
39. Simpson FO. Sodium intake, body sodium, and sodium excretion. *Lancet* 1988; 2: 25–9.
40. Arieff AI. Management of hyponatraemia. *BMJ* 1993; 307: 305–8.
41. Laureno R, Karp BI. Myelinolysis after correction of hyponatraemia. *Ann Intern Med* 1997; 126: 57–62.
42. Sterns RH, Cappuccio JD, Silver SM, Cohen EP. Neurologic sequelae after treatment of severe hyponatraemia: a multicenter perspective. *J Am Soc Nephrol* 1994; 4: 1522–30.
43. Mallie JP, Bichet DG, Halperin ML. Effective water clearance and tonicity balance: the excretion of water revisited. *Clin Invest Med* 1997; 20: 16–24.
44. Oh MS, Carroll HJ, Roy A, Denault N, Ledoux S, Remillard G, et al. Chronic hyponatremia in the absence of ADH: possible role of decreased delivery of filtrate. *J Am Soc Nephrol* 1997; 8: 108A.

45. Hollenberg NK. Set point for sodium homeostasis: surfeit, deficit, and their implications. *Kidney Int* 1980; 17: 423–9.
46. Kern PA, Robbins RJ, Bichet D, Berl T, Verbalis JG. Syndrome of inappropriate antidiuresis in the absence of arginine vasopressin. *J Clin Endocrinol Metab* 1986; 62: 148–52.
47. Lankford SP, Chou CL, Terada Y, Wall SM, Wade JB, Knepper MA. Regulation of collecting duct water permeability independent of cAMP-mediated AVP response. *Am J Physiol* 1991; 261: F554–66.
48. Oliver WJ, Cohen EL, Neel JV. Blood pressure, sodium intake, and sodium related hormones in the Yanomamo Indians, a ‘no-salt’ culture. *Circulation* 1975; 52: 146–51.
49. Oh MS, Halperin ML. The mechanism of urine concentration in the inner medulla. *Nephron* 1997; 75: 384–93.
50. Hilden T, Svendsen TL. Electrolyte disturbances in beer drinkers. A specific ‘hypo-osmolality syndrome’. *Lancet* 1975; 2: 245–6.
51. Fenves AZ, Thomas S, Knochel JP. Beer potomania: two cases and review of the literature. *Clin Nephrol* 1996; 45: 61–4.
52. Halperin ML, Rolleston FS. *Clinical Detective Stories: A problem-based approach to clinical cases in energy and acid-base metabolism*, 1st edn. London, Portland Press, 1993.
53. Arieff AI. Hyponatremia, convulsions, respiratory arrest, and permanent brain damage after elective surgery in healthy women. *N Engl J Med* 1986; 314: 1529–35.
54. Arieff AI, Ayus JC, Fraser CL. Hyponatraemia and death or permanent brain damage in healthy children. *BMJ* 1992; 304: 1218–22.
55. Welt LG, Orloff J, Kydd DM, Oltman JE. An example of cellular hyperosmolality. *J Clin Invest* 1950; 29: 935–9.
56. Turchin A, Seifter JL, Seely EW. Clinical problem-solving. Mind the gap. *N Engl J Med* 2003; 349: 1465–9.
57. Sunderrajan S, Bauer JH, Vopat RL, Wanner-Barjenbruch P, Hayes A. Posttransurethral prostatic resection hyponatraemic syndrome: case report and review of the literature. *Am J Kidney Dis* 1984; 4: 80–4.
58. Davids MR, Lin SH, Edoute Y, Cheema-Dhadli S, Halperin ML. Hyponatraemia and hyperglycaemia during laparoscopic surgery. *QJM* 2002; 95: 321–30.

59. Soupart A, Decaux G. Therapeutic recommendations for management of severe hyponatremia: current concepts on pathogenesis and prevention of neurologic complications. *Clin Nephrol* 1996; 46: 149–69.
60. Spital A. Hyponatremic encephalopathy. *Am J Med* 1997;103:251–2.
61. Gross P, Reimann D, Henschkowski J, Damian M. Treatment of severe hyponatremia: conventional and novel aspects. *J Am Soc Nephrol* 2001; 17: S10–14.
62. Gross P, Reimann D, Neidel J, Doke C, Prosperi F, Decaux G, Verbalis J, Schrier RW. The treatment of severe hyponatremia. *Kidney Int* 1998; 64: S6–11.
63. Lauriat SM, Berl T. The hyponatraemic patient: practical focus on therapy. *J Am Soc Nephrol* 1997; 8: 1599–607.
64. Holden R, Jackson MA. Near-fatal hyponatraemic coma due to vasopressin over-secretion after ‘ecstasy’ (3,4-MDMA). *Lancet* 1996; 347: 1052.
65. Wilkins B. Cerebral oedema after MDMA (‘ecstasy’) and unrestricted water intake. Hyponatraemia must be treated with low water input. *BMJ* 1996; 313: 689–90.
66. O’Rahilly S. Secretion of antidiuretic hormone in hyponatraemia: not always ‘inappropriate’. *BMJ* 1985; 290: 1803–4.
67. Waise A, Young RJ. Pitfalls in the management of acute adrenocortical insufficiency: discussion paper. *J R Soc Med* 1989; 82: 741–2.
68. Penney MD, Walters G. Are osmolality measurements clinically useful? *Ann Clin Biochem* 1987; 24: 566–71.
69. Chacko M, Fordtran JS, Emmett M. Effect of mineralocorticoid activity on transtubular potassium gradient, urinary [K]/[Na] ratio, and fractional excretion of potassium. *Am J Kidney Dis* 1998; 32: 47–51.
70. Zettle RM, West ML, Josse RG, Richardson RM, Marsden PA, Halperin ML. Renal potassium handling during states of low aldosterone bio-activity: a method to differentiate renal and non-renal causes. *Am J Nephrol* 1987; 7: 360–6.
71. West ML, Marsden PA, Richardson RM, Zettle RM, Halperin ML. New clinical approach to evaluate disorders of potassium excretion. *Miner Electrolyte Metab* 1986; 12: 234–8.
72. Coulthard MG, Haycock GB. Distinguishing between salt poisoning and hypernatraemic dehydration in children. *BMJ* 2003; 326: 157–60.

73. Carlotti AP, Bohn D, Rutka JT, Singh S, Berry WA, Sharman A, Cusimano M, Halperin ML. A method to estimate urinary electrolyte excretion in patients at risk for developing cerebral salt wasting. *J Neurosurg* 2001; 95: 420–4.
74. Maesaka JK, Batuman V, Yudd M, Salem M, Sved AF, Venkatesan J. Hyponatraemia and hypouricemia: differentiation from SIADH. *Clin Nephrol* 1990; 33: 174–8.

Chapter 8

**Development of severe hyponatraemia
in hospitalized patients:
treatment-related risk factors and
inadequate management**

**Ewout J. Hoorn, Jan Lindemans
and Robert Zietse**

Nephrol Dial Transplant 2006; 21: 70–6

Abstract

Background: Although hyponatraemia (plasma sodium [P_{Na}] < 136 mmol/l) frequently develops in hospital, risk factors for hospital-acquired hyponatraemia remain unclear.

Methods: Patients who presented with severe hyponatraemia ($P_{Na} \leq 125$ mmol/l) were compared with patients with hospital-acquired severe hyponatraemia in a 3 month hospital-wide cohort study.

Results: Thirty-eight patients had severe hyponatraemia on admission ($P_{Na} 121 \pm 4$ mmol/l), whereas 36 patients had hospital-acquired severe hyponatraemia ($P_{Na} 133 \pm 5 \rightarrow 122 \pm 4$ mmol/l). In hospital-acquired hyponatraemia, treatment started significantly later (1.0 ± 2.6 vs 9.8 ± 10.6 days, $p < 0.001$) and the duration of hospitalization was longer (18.2 ± 11.5 vs 30.7 ± 23.4 days, $p = 0.01$). The correction of P_{Na} in hospital-acquired hyponatraemia was slower after both 24 h (6 ± 4 vs 4 ± 4 mmol/l, $p = 0.009$) and 48 h (10 ± 6 mmol/l vs 6 ± 5 mmol/l, $p = 0.001$) of treatment. Nineteen patients (26%) from both groups were not treated for hyponatraemia and this was associated with a higher mortality rate (seven out of 19 vs seven out of 55, $p = 0.04$). Factors that contributed to hospital-acquired hyponatraemia included: thiazide diuretics (none out of 38 vs eight out of 36, $p = 0.002$), drugs stimulating antidiuretic hormone (two out of 38 vs eight out of 36, $p = 0.04$), surgery (none out of 38 vs 10 out of 36, $p < 0.001$) and hypotonic intravenous fluids (one out of 38 vs eight out of 36, $p = 0.01$). Symptomatic hyponatraemia was present in 27 patients (36%), and 14 patients died (19%).

Conclusions: The development of severe hyponatraemia in hospitalized patients was associated with treatment-related factors and inadequate management. Early recognition of risk factors and expedited therapy may make hospital-acquired severe hyponatraemia more preventable.

Introduction

Hyponatraemia is the most common electrolyte disorder in hospitalized patients, and is generally defined as a plasma sodium concentration (P_{Na}) of < 136 mmol/l [1–3]. Although different definitions of severe hyponatraemia have been used, ranging from 110 to 125 mmol/l [3–7], several studies have established an association between severe hyponatraemia and increased morbidity and mortality rates [3,8–10]. This adverse outcome may be the result of the underlying disease and/or direct complications of hyponatraemia, including cerebral oedema in acute hyponatraemia [8,9] and the osmotic demyelination syndrome (ODS) after overly rapid correction of chronic hyponatraemia [10].

Hyponatraemia is frequently acquired or aggravated in hospital [3]. Although several predisposing factors have been identified in selected patient populations [1,8,11], no studies have directly investigated which factors contribute to hospital-acquired hyponatraemia in a general hospital population. Therefore, we conducted the present hospital-wide cohort study in which we compared patients who were admitted with severe hyponatraemia with patients with hospital-acquired severe hyponatraemia, analysing factors related not only to the underlying disorder but also to the treatment and management during hospitalization.

Methods

Study group

In a study period of 3 months (August–October 2003), all P_{Na} levels measured in adult hospitalized patients in the Erasmus Medical Center, an 831-bed urban university hospital in Rotterdam, The Netherlands, were reviewed. Hyponatraemia was defined as $P_{Na} < 136$ mmol/l and severe hyponatraemia as $P_{Na} \leq 125$ mmol/l. Patients with severe hyponatraemia were studied in further detail. Patients entered the study either when they were admitted with a $P_{Na} \leq 125$ mmol/l (admission hyponatraemia) or when they were already hospitalized and the level decreased in hospital to $P_{Na} \leq 125$ mmol/l (hospital-acquired hyponatraemia). Patients were identified from determined P_{Na} values of hospitalized patients, which were sent electronically twice daily from the clinical chemistry department to one of the investigators (E.J.H.). P_{Na} values were determined with ion-selective electrodes (Hitachi 917, Roche, according to the manufacturer's instructions) and in all of these samples plasma osmolality and plasma glucose concentration were determined simultaneously. Because pseudo-hyponatraemia may still be a problem despite the use of ion-selective electrodes [12], in all patients the first step consisted of the exclusion of pseudo-hyponatraemia, through analysis of plasma osmolality, total protein, triglyceride and cholesterol concentrations.

Evaluation

After inclusion, we analysed which factors may have contributed to the development of hyponatraemia (underlying disorders, medication and in-hospital procedures) and which symptoms of hyponatraemia the patient had developed.

Subsequently, the patient cohort was followed-up prospectively until discharge or death, to evaluate if further symptoms developed, if and how P_{Na} was restored and if patients developed ODS [7,10]. Clinical data were collected by way of chart review and interview, observation and (neurological) examination of patients. The definition of symptomatic hyponatraemia was based on a clinical assessment of symptomatology, including the presence of sensorium changes, seizures, and/or respiratory depression, applying previously established criteria [8,9,13]. Sensorium changes comprised acute confusional states, stupor, delirium and/or coma in the absence of dementia, psychiatric illness and substance abuse. The syndrome of inappropriate antidiuretic hormone secretion (SIADH) was defined by the criteria described by Verbalis [14]. Drugs that can increase the non-osmotic release of antidiuretic hormone (ADH) or potentiate its renal action (ADH-stimulating drugs) were recorded, with the exception of commonly prescribed drugs that rarely produce hyponatraemia, including acetaminophen and non-steroidal anti-inflammatory drugs [15]. Patients were screened for ODS based on clinical grounds (i.e. the development of confusion, agitation, or flaccid or spastic paralysis during or after correction of hyponatraemia), but no magnetic resonance imaging scans (the golden standard for ODS) were performed [7,10].

In order to study the clinical management of hyponatraemia, all medical charts and discharge letters were reviewed to evaluate whether severe hyponatraemia was documented, which diagnoses for hyponatraemia were established, if therapy was instituted and if so which therapy. Treatment of hyponatraemia was defined as the institution of any of the described therapeutic modalities for hyponatraemia [1,2,15] and/or the discontinuation

of a potential causative factor (e.g., diuretics). During the study period, the treating physicians remained responsible for the care of the patients reported in this study and the investigators did not intervene at any point. Informed consent was received from all patients.

Statistical analysis

Data were analysed using SPSS (version 12.0, Chicago, IL). Nominal data were analysed using Fisher's exact test and ordinal data using the Mann–Whitney rank sum test. A *P*-value of 0.05 was considered significant. All data are expressed as mean $\pm$ SD

Results

Incidence and department distribution of hyponatraemia

During the 3 month study period, P_{Na} was determined in 2907 out of 5437 (54%) hospitalized patients. In those patients, 30% (880 out of 2907) had at least one episode of hyponatraemia ($P_{Na} < 136$ mmol/l) (Table 1). P_{Na} was determined significantly less often in the departments of surgery (39%), gynaecology (13%) and ear, nose and throat (38%) (all $p < 0.001$, compared with all other departments). The incidence of hyponatraemia was significantly higher in the departments of internal medicine (36%), surgery (32%) and intensive care (38%) (all $P < 0.05$, compared with all other departments). Severe hyponatraemia ($P_{Na} \leq 125$ mmol/l) was present in 76 patients (3%), and 74 of those patients were studied in more detail, excluding two patients with mannitol-induced hyponatraemia. In this study group, there were no patients with pseudo-hyponatraemia (average plasma osmolality 254 ± 10 mOsm/kg) or

hyperglycaemia-induced hyponatraemia (average plasma glucose 6.0 ± 1.5 mmol/l, range 3.4 – 9.5 mmol/l).

Table 1: Incidences and department distribution of hyponatraemia and severe hyponatraemia

Departments	P _{Na} determined No./total no. (%)	P _{Na} < 136 mmol/l No. (%)	P _{Na} ≤ 125 mmol/l No. (%)
Medicine	872/1278 (68)	316 (36) ^B	37 (4)
Surgery	596/1545 (39) ^A	190 (32) ^B	13 (2)
Cardiology	541/769 (70)	144 (27)	10 (2)
Pulmonology	105/181 (58)	35 (33)	3 (3)
Intensive care	203/203 (100)	78 (38) ^B	4 (2)
Neurology	149/181 (82)	36 (24)	5 (3)
Gynaecology	54/418 (13) ^A	19 (35)	1 (2)
Urology	132/233 (57)	28 (21)	1 (1)
Ear-nose-throat	142/375 (38) ^A	28 (20)	2 (1)
Psychiatry	113/119 (95)	6 (5)	0 (0)
All	2907/5437 (54)	880 (30)	76 (3)

^A $p < 0.001$, compared to all other departments

^B $p < 0.05$, compared to all other departments

Outcome, course and management of severe hyponatraemia

Thirty-eight patients (51%) were admitted with severe hyponatraemia, whereas 36 patients (49%) had hospital-acquired severe hyponatraemia. The demographic and outcome characteristics of these patients are shown in Table 2. Twenty-seven patients (36%) had symptoms that were attributed to hyponatraemia and, because these patients also lacked alternative explanations for these symptoms, they were judged to have symptomatic hyponatraemia. These symptoms always developed when P_{Na} was ≤ 125 mmol/l. No patients were directly admitted for analysis of severe hyponatraemia, although in some patients symptoms attributable to severe hyponatraemia (e.g., vertigo with falls, lethargy and nausea) were the main reason for admission. Fourteen patients (19%) died (seven with symptomatic hyponatraemia), and these patients were more often female (11 out of 14 vs 28 out of 60, $p = 0.02$).

The primary causes of death were respiratory arrest (seven patients), cardiac arrest (two patients), multi-organ failure (two patients) or unknown (three patients), and especially in patients with respiratory arrest and unknown causes of death severe hyponatraemia may have contributed to this outcome [8,13]. Eleven patients (15%) were admitted to the intensive care unit, and for five patients this was directly related to the complications of symptomatic hyponatraemia (three patients with respiratory insufficiency and two patients with seizures).

Table 2: Demographic and outcome characteristics of 74 patients with admission and hospital-acquired severe hyponatraemia

	Admission Group 1 (n = 38)	Acquired Group 2 (n = 36)	P- value
Age, years	58 ± 15	64 ± 18	0.1
Female, n (%)	21 (54)	18 (46)	0.8
Symptomatic hyponatraemia, n (%)	15 (40)	12 (33)	0.6
- Sensorium changes	14 (37)	10 (28)	0.5
- Seizures	3 (8)	1 (3)	0.6
- Respiratory depression	0 (0)	5 (14)	0.02
Mortality, n (%)	9 (24)	5 (14)	0.4
- During/after correction of hyponatraemia	8 (21)	3 (8)	0.1
- P _{Na} at time of death, mmol/l	136 ± 9	131 ± 9	0.2
- Time from lowest P _{Na} until death, days	12.8 ± 12.8	5.3 ± 7.2	0.07
Osmotic demyelination during therapy, n (%)	0 (0)	0 (0)	1.0

The course and management of patients with admission and hospital-acquired hyponatraemia are shown in Table 3. By definition, all patients with hospital-acquired hyponatraemia had a fall in P_{Na}, which averaged 11 ± 6 mmol/l, and was more rapid compared with the few patients with admission hyponatraemia, who also had a fall in P_{Na} (eight patients). The P_{Na} decrease rates in symptomatic patients and patients who died were 0.9 ± 1.0 and 0.7 ± 0.9 mmol/l/day, respectively.

Table 3: Time-course in plasma sodium concentration and observed management in admission and hospital-acquired severe hyponatraemia

	Group 1 (n = 38)	Group 2 (n = 36)	P-value
Admission P_{Na} , mmol/l	121 ± 4	133 ± 5	-
Lowest P_{Na} , mmol/l	120 ± 4	122 ± 4	0.02
Duration $P_{Na} \leq 125$ mmol/l, days	2.8 ± 3.0	3.1 ± 2.4	0.3
P_{Na} at discharge or death, mmol/l	134 ± 6	132 ± 6	0.1
Rate of decrease, mmol/l/day	0.9 ± 3.2	1.5 ± 0.9	< 0.001
Rate of increase*, mmol/l/day	1.5 ± 1.6	3.7 ± 13.9	0.2
P_{Na} determinations / day (before lowest P_{Na})	2.4 ± 2.3	0.8 ± 1.0	0.001
P_{Na} determinations / day (after lowest P_{Na})	1.4 ± 1.0	2.2 ± 2.6	0.2
No therapy for hyponatraemia, n (%)	10 (26)	9 (25)	1.0
Time until start therapy after $P_{Na} < 136$ mmol/l, days	1.0 ± 2.6	9.8 ± 10.6	< 0.001
No documentation of hyponatraemia †, n (%)	16 (42)	25 (69)	0.02
Duration hospitalisation, days	18.2 ± 11.5	30.7 ± 23.4	0.01

* Calculated as the restoration from lowest P_{Na} until discharge or death.

† Defined as any reference to hyponatraemia in charts and/or discharge letters.

Fifty-five patients (74%) were treated for severe hyponatraemia. In hospital-acquired hyponatraemia, the time until therapy was started after the first P_{Na} value < 136 mmol/l was recorded was significantly longer (1.0 ± 2.6 vs 9.8 ± 10.6 days, $p < 0.001$).

An overview of how severe hyponatraemia was treated in each aetiological category is shown in Table 4. Of the 19 patients in whom treatment was lacking, seven died, resulting in a higher mortality rate in untreated patients (seven out of 19 vs seven out of 55, $p = 0.04$); this mortality rate did not differ between admission and hospital-acquired hyponatraemia. During the first 24 and 48 h of treatment, P_{Na} was corrected more rapidly in admission hyponatraemia compared with hospital-acquired hyponatraemia (Figure 1). This also appeared to be the case in patients who died, although this difference was not statistically significant, perhaps because of the small number of patients (eight vs three patients). However, patients with symptomatic hyponatraemia were corrected with similar correction rates in the two groups. Finally, untreated patients (not shown in Figure 1) did have a minor rise in P_{Na} (4 ± 4 mmol/l for both 24 and 48 h), possibly because the stimulus for ADH abated, or because 'escape' from the effects of ADH occurred [16].

Table 4: Observed treatment of severe hyponatraemia in major aetiological groups^a

Treatment^b / Cause hyponatraemia	None n (%)	Normal saline	Hyper-tonic saline	Water restriction	NaCl Tablets	Stop therapy^c
Liver failure (n = 11)	7 (64)	0 (0)	0 (0)	1 (9)	0 (0)	3 (27)
Heart failure (n = 11)	4 (36)	1 (9)	0 (0)	0 (0)	0 (0)	6 (5)
SIADH (n = 13)	2 (15)	5 (38)	1 (8)	4 (31)	1 (8)	0 (0)
Diuretics (n = 10)	2 (13)	5 (33)	0 (0)	0 (0)	2 (13)	6 (40)
Post-operative (n = 10)	3 (27)	3 (27)	2 (18)	1 (9)	0 (0)	2 (18)
Drug-induced (n = 6)	0 (0)	1 (17)	1 (17)	1 (0)	1 (17)	2 (33)
Miscellaneous (n = 13)	1 (8)	8 (62)	0 (0)	0 (0)	4 (31)	0 (0)
All patients (n = 74)	19 (24)	23 (29)	4 (5)	7 (9)	8 (10)	19 (24)

^a More underlying reasons for severe hyponatraemia may have been present (see also Table 5).

^b Some patients were treated in more than one way; percentages are calculated based on the total number of treatments, not patients.

^c Discontinuation of the factor that potentially caused hyponatraemia (e.g., diuretics).

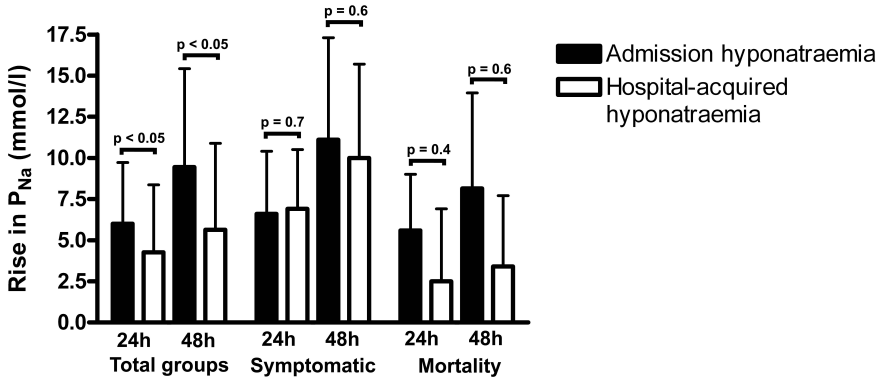


Figure 1: Correction rates of severe hyponatraemia in the first 24 and 48 h of therapy are shown in admission and hospital-acquired severe hyponatraemia (38 vs 36 patients). In addition, correction rates of two subgroups are shown, including patients who had symptomatic hyponatraemia prior to their correction (15 vs 12 patients), and patients who died during the correction of hyponatraemia (eight vs three patients).

Factors contributing to hospital-acquired hyponatraemia

To evaluate which factors that may have contributed to hyponatraemia were present on admission and which were introduced during hospitalization, those underlying disorders, drugs and in-hospital procedures that may affect water and sodium balance were compared (Table 5). This analysis identified two hospital-associated factors that were more often present in patients with hospital-acquired hyponatraemia, namely surgery (none out of 38 vs 10 out of 36, $p < 0.001$) and hypotonic intravenous (i.v.) fluids (one out of 38 vs eight out of 36, $p = 0.01$). Secondly, during hospitalization, but not on admission, the hospital-acquired group received significantly more thiazide diuretics (none out of 38 vs eight out of 36, $p = 0.002$) and ADH-stimulating drugs (two out of 38 vs eight out of

36, $p = 0.04$), either because these drugs were started in hospital, or because they were discontinued in patients with admission hyponatraemia. ADH-stimulating drugs included haloperidol (four patients), hypoglycaemic medication (three patients), chemotherapeutics (bortezomib and thalidomide), sodium valproate (two patients), carbamazepine, serotonin re-uptake inhibitors and 1-desamino-8-D-arginine vasopressine (dDAVP).

Underlying disorders that may have affected water and sodium homeostasis were approximately equally distributed in the two groups (Table 5). Most underlying disorders were already present on admission, although gastrointestinal losses and renal insufficiency often developed in hospital, but not significantly more so in hospital-acquired hyponatraemia.

The exception were four patients, three of whom developed severe hyponatraemia as a result of SIADH due to stroke, and another patient who developed severe hyponatraemia as a result of a myocardial infarction [17]. Additional diseases in which patients had the clinical and biochemical characteristics of SIADH included pneumonia (two patients), lung carcinoma (two patients), oesophageal carcinoma, hydrocephalus, neurotrauma, Guillain–Barré syndrome and encephalitis [14,15].

Table 5: Analysis of factors contributing to the development of severe hyponatraemia during hospitalisation

	Factors on admission		Factors in hospital	
	Group 1	Group 2	Group 1	Group 2
<i>Underlying disorder</i>				
- Liver failure, n (%)	7 (18)	4 (11)	7 (18)	4 (11)
- Heart failure	4 (11)	6 (17)	4 (11)	7 (20)
- Renal insufficiency	8 (21)	12 (33)	10 (26)	16 (44)
- Gastro-intestinal losses	9 (24)	4 (5)	10 (26)	8 (22)
- SIADH	8 (21)	2 (6)	8 (21)	5 (14)
<i>Medication</i>				
- Loop diuretics, n (%)	12 (32)	9 (25)	10 (26)	16 (44)
- Thiazide diuretics	5 (13)	7 (19)	0 (0)	8 (22) *
- K sparing diuretics	8 (21)	8 (22)	8 (21)	10 (28)
- RAS-acting agents	3 (8)	5 (14)	1 (3)	5 (14)
- ADH-stimulating drugs	6 (16)	2 (6)	2 (5)	8 (22) *
<i>Treatment in-hospital</i>				
- Surgery, n (%)	-	-	0 (0)	10 (28) *
- Hypotonic IV-fluids	-	-	1 (3)	8 (22) *
<i>Total number of factors</i>	2.1 ± 1.4	2.0 ± 1.2	1.7 ± 1.5	3.3 ± 1.6 *

* $p < 0.05$, comparing group 1 vs group 2

The 34 patients with symptomatic hyponatraemia and/or hyponatraemia-associated mortality often had advanced medical conditions, such as liver failure (five deaths, two symptomatic patients), heart failure (one death, one symptomatic patient) and SIADH due to stroke (two deaths, one symptomatic patient), and it is possible that hyponatraemia was predominantly a secondary feature in these patients. However, in patients with post-operative hyponatraemia (one death, four symptomatic patients), diuretic-induced hyponatraemia (two deaths, two symptomatic patients), drug-induced hyponatraemia (five symptomatic patients) and in some patients with SIADH (five symptomatic patients), it is likely that hyponatraemia contributed significantly, or caused the observed morbidity and mortality.

In particular, hospital-acquired hyponatraemia appeared multifactorial, because in this group the total number of possible causative factors during hospitalization was significantly higher than in patients with hyponatraemia on admission. Common combinations of causative factors included: diuretics and ADH-stimulating drugs (10 patients), heart failure and renal insufficiency (nine patients), surgery and hypotonic i.v. fluids (eight patients), liver failure and renal insufficiency (eight patients) and, finally, SIADH and ADH-stimulating drugs (four patients). Less common factors (not shown in Table 5) that may have contributed to hyponatraemia included Addison's disease (two patients) and hypothyroidism (two patients). There were no patients with apparent polydipsia, although most patients continued their habitual fluid intake, which may have contributed to hyponatraemia in some cases.

Discussion

In the present study, we sought to identify differences between patients who were admitted with severe hyponatraemia and those who developed severe hyponatraemia in hospital. The principal results demonstrate that hospital-acquired severe hyponatraemia was associated with treatment-related factors and inadequate or delayed management, thereby allowing a further decrease in P_{Na} , and exposing these patients to the dangers of severe hyponatraemia [8,9].

Overall, the following picture of severe hyponatraemia in hospitalized patients emerged. Half of the patients presented with severe hyponatraemia, whereas the other half developed severe hyponatraemia in hospital (Table 2). In both groups, severe hyponatraemia was associated with substantial symptomatology and mortality (Table 2). When severe hyponatraemia was already present on admission, the time until treatment was initiated was significantly shorter than when severe hyponatraemia was hospital acquired (Table 3). Thus, there appeared to be a low recognition of deteriorating hyponatraemia, an assumption that was substantiated further by the observations that hospital-acquired hyponatraemia was less often documented and P_{Na} less often determined prior to the lowest P_{Na} (Table 3). Although severe hyponatraemia was also corrected significantly more slowly in hospital-acquired hyponatraemia, patients with symptomatic hyponatraemia were corrected equally rapidly in both groups (Figure 1). In addition, no cases of ODS were observed during the correction phase (Table 2). Finally, there appeared to be no relationship between the rate of correction and mortality (Figure 1), although few

extreme correction rates were observed, and patients with hospital-acquired hyponatraemia appeared to die earlier (Table 2). However, untreated patients did have a higher mortality rate. Furthermore, a number of patients received inappropriate and potentially hazardous treatment for severe hyponatraemia, for example isotonic saline for SIADH (Table 4) [18]. In summary, in this study, permitting severe hyponatraemia and the associated symptomatology and mortality to develop in hospital was of greater clinical impact than how severe hyponatraemia was corrected.

A difficult question remains as to whether symptomatology and mortality in hyponatraemic patients are caused directly by severe hyponatraemia, the underlying disorder, or both [19]. Factoring in co-morbidity, we believe that symptomatic hyponatraemia and hyponatraemia-associated mortality could have been more preventable in patients with hospital-acquired hyponatraemia who lacked a severe underlying disorder associated with hyponatraemia, for example in post-operative, diuretic- or drug-induced severe hyponatraemia. We emphasize that although the decrease rates of P_{Na} appeared to be relatively slow for the development of symptomatic hyponatraemia [20], hyponatraemia may have been more acute in many patients because of infrequent P_{Na} measurements. Except for adverse patient outcomes, the duration of hospitalization in hospital-acquired hyponatraemia was also significantly longer (Table 3), thus increasing health care costs [19].

The awareness of severe hyponatraemia has been addressed previously by Arieff, who almost 20 years ago described that even in hyponatraemic patients with severe neurological

symptoms, hyponatraemia was often not recognized [8]. More recently, Movig *et al.* evaluated the discharge diagnosis 'hyponatraemia' in 2632 cases of hyponatraemia and identified a similar low recognition rate, with only 30% of patients in whom severe hyponatraemia was classified [21]. A possible explanation as to why severe hyponatraemia is not readily recognized may be that the underlying disorder diverts attention from hyponatraemia and/or may give the impression that the cause of hyponatraemia and the associated symptoms are solely a result of that underlying disorder.

Previously, Palevsky *et al.* in a study on hypernatraemia in hospitalized patients showed results that were very similar to ours, namely an association between inadequate or delayed treatment and hospital-acquired hypernatraemia [22]. They suggested that better management may be achieved by physician education and development of preventive hospital systems, and our results suggest that these recommendations can also be extrapolated to hyponatraemia [22]. Indeed, Paltiel *et al.* showed that the implementation of a hospital warning system improved the management of electrolyte disorders, in their case hypokalaemia [23].

The optimal treatment for severe hyponatraemia remains controversial, because the importance of slow correction to prevent ODS [7,10] must be weighed against a significantly improved survival rate associated with more rapid correction [19,24,25]. The latter was demonstrated by Ayus and Arieff who showed that patients with chronic hyponatraemia whose P_{Na} was restored by 22 mmol/l in 35 h with intravenous NaCl had a better neurological outcome than those whose P_{Na} was restored

by 3 mmol/l in 41 h with water restriction [24]. In a recent review, Martin showed that the recommended correction rates for hyponatraemia have steadily declined over the years to ~ 8 mmol/l/day [26]. The question is if this decreasing trend has overstepped its goals and if part of the poor outcome could have been prevented by more aggressive therapy [11,27], provided that risk factors for ODS are taken into account [28].

In the second part of this study, we assessed which factors were associated with hospital-acquired hyponatraemia. In principle, hyponatraemia can develop in hospital when there is a deterioration of the underlying disease, or when factors are being introduced in the hospital that can potentially disturb sodium and water balance. In this study, it appeared that most of the factors that contributed to hospital-acquired hyponatraemia were either maintained or introduced in hospital, and included thiazide diuretics, ADH-stimulating drugs, hypotonic i.v. fluids and surgery (Table 5). Nevertheless, because the average admission P_{Na} of patients with hospital-acquired hyponatraemia was already in the hyponatraemic range ($P_{Na} 133 \pm 5$ mmol/l), it is conceivable that underlying disorders or medication already led to moderate hyponatraemia at the time of admission in some patients, which was then aggravated further by factors introduced in the hospital.

The post-operative state and thiazides are well-established causes of hyponatraemia, but have chiefly been described in selected patient populations [8,29–31]. Hypotonic i.v. fluids as a cause of hospital-acquired hyponatraemia have mainly been described in hospitalized children, often with adverse outcome [32,33]. The ADH-stimulating capacity of some of the drugs

identified in this study has long been known [15,34,35], whereas we also identified novel chemotherapeutic [36], antiepileptic [37] and antidepressant [38] drugs that probably contributed to hyponatraemia. The mechanisms by which the above factors can cause hyponatraemia are through the non-osmotic release of ADH followed by reabsorption of electrolyte-free water that was orally ingested or provided in hypotonic i.v. fluids, and/or by the promotion of renal sodium loss [39]. In particular, the development of hospital-acquired hyponatraemia appeared to depend on a combination of these factors, because the total number of possible causes of hyponatraemia during hospitalization was significantly higher, once more illustrating the multi-factorial nature of this condition (Table 3) [4,40,41].

This is one of the largest reported series of patients with severe hyponatraemia. The majority of the previous cohort studies were retrospective chart reviews, and usually reviewed a similar number of patients, but with lower P_{Na} levels using a longer study period [3–7]. In the present study, by choosing a relatively short study period, and a P_{Na} level in which the likelihood of symptomatic hyponatraemia is increased [20], we sought to investigate the frequently encountered cases of hyponatraemia. Indeed, hyponatraemia remains a very common condition, with one in every three hospitalized patients having an episode of hyponatraemia, and one in every 30 hospitalized patients having an episode of severe hyponatraemia (Table 1). Two notable differences in this study compared with previous studies should be mentioned. First, the number of patients with SIADH was relatively low, although we argue that previous studies did not always use stringent criteria for SIADH, which is a diagnosis of exclusion [14]. Secondly, the low incidence of hyponatraemia in

psychiatric patients is remarkable because many psychotropic drugs, including haloperidol and serotonin reuptake inhibitors, frequently produce hyponatraemia [35,41]. A possible explanation is that in our institution psychotropic drugs are frequently withdrawn for diagnostic purposes.

Finally, a number of important limitations should be mentioned. The possible awareness of this study in our institution may have created bias toward the clinicians' approach to hyponatraemia. However, the expectation would be that the presence of the study would only lead to a higher index of suspicion for hyponatraemia. The sample size limited the robustness with which we could analyse the data, and hence limits the power of some of the observations. We believe that a future study which compares patients with hospital-acquired hyponatraemia with a hospitalized control group without hyponatraemia would be important to identify further risk factors associated with the development of hospital-acquired hyponatraemia.

In conclusion, this study identified treatment-related factors associated with hospital-acquired severe hyponatraemia, including factors that contributed to its development (thiazide diuretics, ADH-stimulating drugs, hypotonic i.v. fluids and surgery), and factors that influenced its course (low recognition, delayed therapy and slow correction). These factors should be prevented if possible or lead to frequent monitoring of P_{Na} and early intervention if P_{Na} decreases to < 136 mmol/l. Increased recognition and expedited therapy may be achieved by hospital warning systems, automated consultations or other surveillance systems.

Acknowledgments

The authors would like to thank Dr. Mitchell Halperin for helpful discussion and Gert Verheij for his technical support with the retrieval of laboratory values. This work was presented in a poster session at the American Society of Nephrology Renal Week 2004, St Louis, MO.

References

1. Adrogue HJ, Madias NE. Hyponatremia. *N Engl J Med* 2000; 342: 1581–1589.
2. Palmer BF, Gates JR, Lader M. Causes and management of hyponatremia. *Ann Pharmacother* 2003; 37: 1694–1702.
3. Anderson RJ, Chung HM, Kluge R, Schrier RW. Hyponatremia: a prospective analysis of its epidemiology and the pathogenetic role of vasopressin. *Ann Intern Med* 1985; 102: 164–168.
4. Saeed BO, Beaumont D, Handley GH, Weaver JU. Severe hyponatraemia: investigation and management in a district general hospital. *J Clin Pathol* 2002; 55: 893–896.
5. Sterns RH. Severe symptomatic hyponatremia: treatment and outcome. A study of 64 cases. *Ann Intern Med* 1987; 107: 656–664.
6. Nzerue CM, Baffoe-Bonnie H, You W, Falana B, Dai S. Predictors of outcome in hospitalized patients with severe hyponatremia. *J Natl Med Assoc* 2003; 95: 335–343.
7. Ellis SJ. Severe hyponatraemia: complications and treatment. *QJM* 1995; 88: 905–909.
8. Arieff AI. Hyponatremia, convulsions, respiratory arrest, and permanent brain damage after elective surgery in healthy women. *N Engl J Med* 1986; 314: 1529–1535.
9. Fraser CL, Arieff AI. Epidemiology, pathophysiology, and management of hyponatremic encephalopathy. *Am J Med* 1997; 102: 67–77.
10. Laureno R, Karp BI. Myelinolysis after correction of hyponatremia. *Ann Intern Med* 1997; 126: 57–62.
11. Anderson RJ. Hospital-associated hyponatremia. *Kidney Int* 1986; 29: 1237–1247.

12. Turchin A, Seifter JL, Seely EW. Clinical problem-solving. Mind the gap. *N Engl J Med* 2003; 349: 1465–1469.
13. Ayus JC, Arieff AI. Pulmonary complications of hyponatremic encephalopathy. Noncardiogenic pulmonary edema and hypercapnic respiratory failure. *Chest* 1995; 107: 517–521.
14. Verbalis JG. The syndrome of inappropriate antidiuretic hormone secretion and other hypoosmolar disorders. In: Schrier RW, ed. *Diseases of the Kidney and Urinary Tract*. LLW, Philadelphia, PA; 2001: 2518.
15. Parikh C, Kumar S, Berl T. Disorders of water metabolism. In: Johnson RJ, Feehally J, eds. *Comprehensive Clinical Nephrology*, 2nd edn. Mosby: Edinburgh, 2003.
16. Ecelbarger CA, Nielsen S, Olson BR et al. Role of renal aquaporins in escape from vasopressin-induced antidiuresis in rat. *J Clin Invest* 1997; 99: 1852–1863.
17. Evans JR, McIntosh JP, McIntosh HJ, Mitchell PE. Hyponatremia in patients admitted to a coronary care unit. *Clin Chem* 1990; 36: 322–325.
18. Reimann D, Gross P. Sudden onset of unconsciousness and hyponatraemia. Facts and myths about salt and water in SIAD. *Nephrol Dial Transplant* 1996; 11: 2101–2102.
19. Adroge HJ. Consequences of inadequate management of hyponatremia. *Am J Nephrol* 2005; 25: 240–249.
20. Lauriat SM, Berl T. The hyponatremic patient: practical focus on therapy. *J Am Soc Nephrol* 1997; 8: 1599–1607.
21. Movig KL, Leufkens HG, Lenderink AW, Egberts AC. Validity of hospital discharge International Classification of Diseases (ICD) codes for identifying patients with hyponatremia. *J Clin Epidemiol* 2003; 56: 530–535.
22. Palevsky PM, Bhagrath R, Greenberg A. Hypernatremia in hospitalized patients. *Ann Intern Med* 1996; 124: 197–203.
23. Paltiel O, Gordon L, Berg D, Israeli A. Effect of a computerized alert on the management of hypokalemia in hospitalized patients. *Arch Intern Med* 2003; 163: 200–204.
24. Ayus JC, Arieff AI. Chronic hyponatremic encephalopathy in postmenopausal women: association of therapies with morbidity and mortality. *JAMA* 1999; 281: 2299–2304.
25. Gross P, Reimann D, Neidel J et al. The treatment of severe hyponatremia. *Kidney Int Suppl* 1998; 64: S6–S11.

26. Martin RJ. Central pontine and extrapontine myelinolysis: the osmotic demyelination syndromes. *J Neurol Neurosurg Psychiatry* 2004; 75 [Suppl 3]: iii22–iii28.
27. Moritz ML, Ayus JC. Hospital-acquired hyponatremia: why are there still deaths? *Pediatrics* 2004; 113: 1395–1396.
28. Lohr JW. Osmotic demyelination syndrome following correction of hyponatremia: association with hypokalemia. *Am J Med* 1994; 96: 408–413.
29. Chow KM, Szeto CC, Wong TY, Leung CB, Li PK. Risk factors for thiazide-induced hyponatraemia. *QJM* 2003; 96: 911–917.
30. Sonnenblick M, Friedlander Y, Rosin AJ. Diuretic-induced severe hyponatremia. Review and analysis of 129 reported patients. *Chest* 1993; 103: 601–606.
31. Ayus JC, Wheeler JM, Arieff AI. Postoperative hyponatremic encephalopathy in menstruant women. *Ann Intern Med* 1992; 117: 891–897.
32. Halberthal M, Halperin ML, Bohn D. Lesson of the week: acute hyponatraemia in children admitted to hospital: retrospective analysis of factors contributing to its development and resolution. *BMJ* 2001; 322: 780–782.
33. Hoorn EJ, Geary D, Robb M, Halperin ML, Bohn D. Acute hyponatremia related to intravenous fluid administration in hospitalized children: an observational study. *Pediatrics* 2004; 113: 1279–1284.
34. Moses AM, Miller M. Drug-induced dilutional hyponatremia. *N Engl J Med* 1974; 291: 1234–1239.
35. Rider JM, Mauger TF, Jameson JP, Notman DD. Water handling in patients receiving haloperidol decanoate. *Ann Pharmacother* 1995; 29: 663–666.
36. O'Connor OA, Wright J, Moskowitz C et al. Phase II clinical experience with the novel proteasome inhibitor bortezomib in patients with indolent non-Hodgkin's lymphoma and mantle cell lymphoma. *J Clin Oncol* 2005; 23: 676–684.
37. Asconape JJ. Some common issues in the use of antiepileptic drugs. *Semin Neurol* 2002; 22: 27–39.
38. Movig KL, Leufkens HG, Lenderink AW et al. Association between antidepressant drug use and hyponatraemia: a case-control study. *Br J Clin Pharmacol* 2002; 53: 363–369.

39. Halperin ML, Goldstein MB. Fluid, Electrolyte, and Acid–Base Physiology. W. B. Saunders: Philadelphia, PA, 1999.
40. Gardner LB, Preston RA. University of Miami Division of Clinical Pharmacology Therapeutic Rounds: the water-intolerant patient and perioperative hyponatremia. *Am J Ther* 2000; 7: 23–30.
41. Rosner MH. Severe hyponatremia associated with the combined use of thiazide diuretics and selective serotonin reuptake inhibitors. *Am J Med Sci* 2004; 327: 109–111.

Chapter 9

**Novel risk factors for
hospital-acquired hyponatraemia:
a matched case control study**

**Carolien M. Beukhof*, Ewout J. Hoorn*
Jan Lindemans and Robert Zietse**

Clin Endocrinol (Oxf), in press

* Authors contributed equally

Abstract

Background: Hospital-acquired hyponatraemia is a common and potentially serious condition. Risk factors for hospital-acquired hyponatraemia have not been studied in a controlled fashion.

Methods: From 1,501 patients in whom serum sodium (S_{Na}) was determined, 50 cases with hospital-acquired hyponatraemia (in-hospital decrease in $S_{Na} \geq 7$ mmol/l to < 136 mmol/l) were identified. They were matched by age, gender, and department to 69 normonatraemic controls.

Results: In cases S_{Na} fell from 141 ± 2 mmol/l to 130 ± 4 mmol/l, while controls remained normonatraemic. During the development of hyponatraemia, C-reactive protein increased in controls (median from 23 to 146 mg/l), whereas it decreased in controls (median from 31 to 24 mg/l, $p = 0.008$). Additional factors associated with hospital-acquired hyponatraemia included diabetes mellitus (16/50 vs. 10/69, $p = 0.009$), and the use of insulin (12/50 vs. 4/69, $p = 0.007$), antibiotics (41/50 vs. 38/69, $p = 0.006$), and opioids (32/50 vs. 27/69, $p = 0.005$). Multivariate conditional logistic regression showed that the use of insulin (odds ratio 10.5, 95% confidence interval 1.5 to 72.4), antibiotics (4.5, 1.4 to 14.6), and opioids (2.9, 1.1 to 7.8) were also independently associated with hospital-acquired hyponatraemia. Mortality (6/50 vs. 1/69, $p = 0.04$) and intensive care admission (15/50 vs. 7/69, $p = 0.008$) were higher in cases.

Conclusions: An increase in C-reactive protein and the use of insulin, antibiotics and opioids are novel risk factors for hospital-acquired hyponatraemia. These factors represent interesting new clues regarding the pathophysiology of hospital-acquired hyponataemia, suggesting that the acute phase response, pain and/or direct drug effects could be involved in the release of vasopressin.

Introduction

Hyponatraemia, defined as a serum sodium concentration (S_{Na}) < 136 mmol/l, is a common and potentially serious electrolyte disorder in hospitalised patients [1-4]. Previous studies have shown that hyponatraemia frequently develops in hospital [5,6]. The development of hyponatraemia usually requires two elements: electrolyte-free water and increased antidiuretic hormone to prevent its excretion [5,7]. The past decades several factors that may contribute to hospital-acquired hyponatraemia have been identified, including diuretics [8], surgery [1], hypotonic intravenous fluids [9], colonoscopy [10], and several drugs and diseases that promote the non-osmotic release of antidiuretic hormone [5,6,7,11]. However, the majority of these studies was either performed in a non-controlled fashion [5,6,12], or conducted in selected subpopulations [1,2,10]. As a result, it remains unclear which factors contribute to hospital-acquired hyponatraemia in a general hospital population, thereby complicating efforts to prevent this condition [13]. Therefore, we conducted a case-control study, in which patients with hospital-acquired hyponatraemia were matched to control patients who remained normonatremic during hospitalisation.

Patients and methods

Patient population and laboratory measurements

In a study-period of two months (April – May 2005), we reviewed all S_{Na} -values ordered in the departments of internal medicine, haematology, surgery, neurology, neurosurgery, cardiology, transplantation, and gynaecology in the Erasmus Medical Center, an 813-bed urban university hospital in Rotterdam, The Netherlands. S_{Na} -values were determined with ion-selective electrodes (Hitachi 917, Roche, according to the manufacturer's instructions). Because pseudohyponatraemia may still be a problem despite the use of ion-selective electrodes [14], the clinical chemistry department automatically determined lipid and protein concentrations from the same sample. In addition, patients with hyperglycaemia-induced hyponatraemia were excluded from analysis.

Cases and controls

Cases consisted of patients with hospital-acquired hyponatraemia, which was defined as an admission S_{Na} between 136 and 145 mmol/l and a subsequent decrease in S_{Na} of at least seven mmol/l (calculated from the highest S_N -value) which resulted in at least one $S_{Na} < 136$ mmol/l. In the absence of formal criteria for hospital-acquired hyponatraemia, we selected these criteria to minimise the chance that the development of hyponatraemia could be explained by minor metabolic fluctuations and/or the standard deviation of the laboratory test (which may be as high as 1 - 2 mEq/l) [15]. Each case was age- and gender-matched to one, two or three control patients, who were admitted to the same department, and who remained normonatraemic during admission (defined as at least two

available S_{Na} -values between 136 and 145 mmol/l and no decrease or increase in S_{Na} exceeding seven mmol/l). In addition, the time in which cases developed hospital-acquired hyponatraemia was matched to a similar hospitalisation time for controls, allowing a variation of $\pm 25\%$. The time at which hospital-acquired hyponatraemia was present for the first time, was called 'inclusion'.

Data collection and analysis

All factors that may have contributed to hospital-acquired hyponatraemia, and that were present during the time from admission until inclusion, were recorded. These included: demographic data, biochemical parameters, underlying disorders, disease severity, medication, intravenous fluids, and in-hospital procedures. With regard to biochemistry, we focused on parameters that may reflect severity of disease, including C-reactive protein (CRP), albumin, and creatinine measured at the time of admission and inclusion $\pm$ two days. Disease severity was estimated using the Mortality Probability Model [16]. Although originally designed for the intensive care, this model might also give an estimate of disease severity for non-intensive care patients, and has the advantage of assessing variables that occur during hospitalisation [16]. Examples of variables that are used in the model include cancer, infection, creatinine, surgery, and age [16]. In a subanalysis, cases with hyponatraemia and a concomitant rise in serum creatinine were compared to cases whose serum creatinine remained stable or decreased. Both subgroups were also compared to their matched controls. In the absence of complete urinary values, serum creatinine may serve as a rough estimate for whether hyponatraemia was more likely

to be “dilutional” or “depletional”. All data were collected through the electronic hospital information system, by way of chart-review, and review of discharge letters. During the study-period, the treating physicians remained responsible for the care of the patients reported in this study, and the investigators did not intervene at any point. Consequently, the availability of some of the biochemical parameters was less than 100%. Because these parameters were not determined more frequently in cases, we accepted this bias and included the parameters in our univariate analysis only. Patients were followed until discharge, and intensive care admission (as an index of the severity of the patient’s condition) and/or death during hospitalisation were defined as outcome variables.

Statistical analysis

Data were analysed by SPSS (version 13.0, Chicago, IL). Biochemical parameters were first tested for normality of distribution using the Kolmogorov-Smirnov test, and log-transformed when not normally distributed. They were then compared between groups at the time of admission and inclusion using a repeated measures general linear model. Other factors that were present from admission until inclusion (i.e., co-morbidity, medication, and in-hospital procedures) were analysed using univariate conditional logistic regression. Significant variables from the univariate analyses were further analysed using multivariate regression with a backward conditional approach. Conditional logistic regression is the method of choice to analyse matched cases and controls. To perform conditional logistic regression, we used the Cox proportional hazards regression model because it allows a comparison between cases and controls in the same matched set

[17]. In SPSS, this was done by choosing Cox Regression and entering a constant for “time”, case or control for “status”, and their match numbers for “strata”. For the outcome analysis and the subanalysis, Fisher’s Exact test was used. A p-value of < 0.05 was considered significant.

Results

Study group characteristics and outcome

During the two months, 4,848 S_{Na} values were ordered in 1,501 patients, of whom 383 patients (26%) had at least one S_{Na} value < 136 mmol/l. Fifty patients met our criteria of hospital-acquired hyponatraemia and they were matched to 69 control patients. One patient with hyperglycaemia-induced hyponatraemia (serum glucose 37.3 mmol/l) was excluded from analysis; there were no patients with pseudohyponatraemia. Patients were admitted to the departments of internal medicine (14 cases, 21 controls), haematology (10 cases, 10 controls), surgery (9 cases, 16 controls), neurology and neurosurgery (6 cases, 8 controls), cardiology (5 cases, 7 controls), transplantation (4 cases, 4 controls), and gynaecology (2 cases, 3 controls).

Table 1 shows matching variables, disease severity, and outcomes. From admission until inclusion, mortality probability was not higher in cases (median 4.2 vs. 2.7, $p = 0.1$). From admission until discharge, intensive care admission (15/50 vs. 7/69, $p = 0.008$), and mortality (6/50 vs. 1/69, $p = 0.04$) were significantly higher in cases. The median lowest S_{Na} -value of patients who died was 132 mmol/l (range 120 – 136 mmol/l). Two patients died during hyponatraemia (causes of death cardiogenic shock and multi-organ failure), whereas the other

patients died after S_{Na} had been restored (causes of death multi-organ failure, haemorrhagic shock, septic shock, heart failure). One control patient died because of heart failure. No major symptoms of hyponatraemia (seizures or encephalopathy) were observed in patients who survived.

Table 1: Matching variables and outcome

	Cases (n = 50)	Controls (n = 69)	P- value*
Matching variables			
- Female, n (%)	23 (46)	31 (45)	-
- Age, years	57 ± 16	57 ± 18	-
- Time until inclusion, days	15 ± 12	11 ± 9	-
Disease severity			
- Mortality probability [†] , %	4.2 (0.4 – 83.4)	2.7 (0.3 – 90.6)	0.1
Outcomes			
- Intensive care admission, n (%)	15 (30)	7 (10)	0.008
- Mortality in hospital, n (%)	6 (12)	1 (1)	0.04
- S_{Na} at discharge or death, mmol/l	137 ± 5	140 ± 2	< 0.001

* Comparing cases versus controls using Fisher's Exact test.

[†] Assessed using the Mortality Probability Model [16].

Biochemistry

Figure 1 shows S_{Na} , serum CRP, and serum creatinine concentrations at the time of admission and inclusion for both groups. Cases were admitted normonatremic (S_{Na} 139 ± 2 mmol/l), on average slightly increased their S_{Na} to 141 ± 2 mmol/l (data not shown), and subsequently developed hyponatraemia (lowest S_{Na} 130 ± 4 mmol/l) (Figure 1A). Available CRP levels (Figure 1B) increased significantly in cases (median from 23 to 146 mg/l), whereas they decreased in controls (median from 31 to 24 mg/l). The difference between cases and controls, as analysed by a repeated measures general linear model, was significant ($p = 0.008$). In contrast, the changes in serum albumin (data not shown) and creatinine levels (Figure 1C) were not significant between groups (120 ± 163 μ mol/l to 128 ± 154 μ mol/l in cases vs. 121 ± 134 μ mol/l to 94 ± 56 μ mol/l in controls, $p = 0.06$).

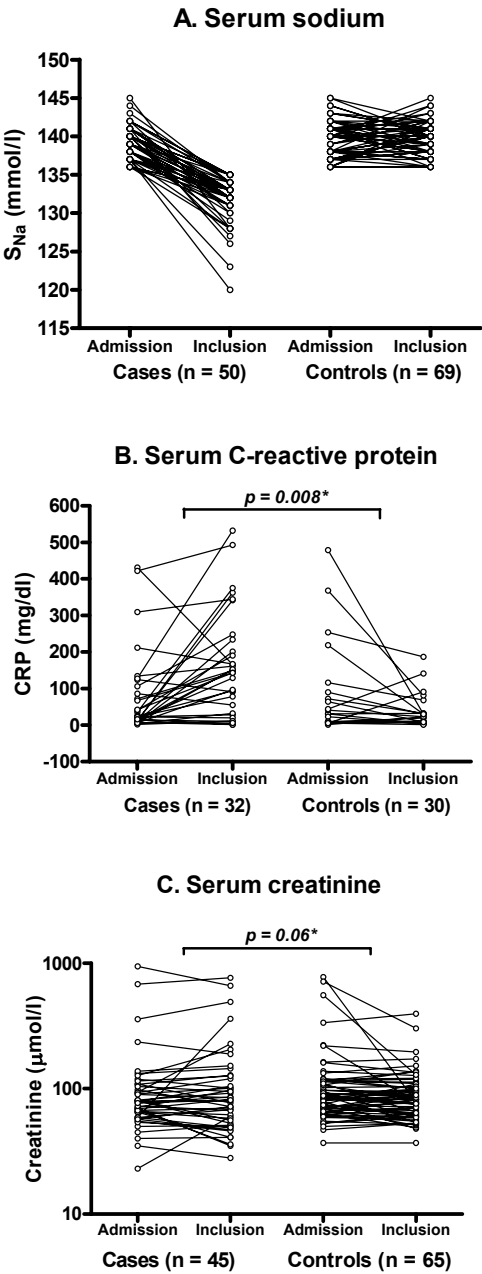


Figure 1: Course of serum sodium, C-reactive protein, and creatinine

Legend Figure 1: Course of serum sodium, C-reactive protein, and creatinine

Selected biochemical parameters in cases and controls shown as individual data points and measured at the time of admission or inclusion ± 2 days. C-reactive protein and creatinine were not normally distributed and therefore log transformed for analysis. Creatinine is log scaled for presentation.

** Results from general linear model comparing the change from admission until inclusion between cases and controls.*

Co-morbidity, medication, and in-hospital procedures

Tables 2 and 3 show co-morbidity and medication in cases and controls. Diabetes mellitus was more common in cases (16/50 vs. 10/69, $p = 0.009$). Furthermore, cases more often received insulin (12/50 vs. 4/69, $p = 0.007$), antibiotics (41/50 vs. 38/69, $p = 0.007$), and opioids (32/50 vs. 27/69, $p = 0.005$). The following antibiotics were used most frequently: beta-lactams (20 cases, 18 controls), antimycotics (14 cases, 7 controls), quinolones (11 cases, 8 controls), cephalosporins (10 cases, 6 controls), and vancomycine (7 cases, 0 controls). Common opioids included tramadol (18 cases, 14 controls), morphine (10 cases, 4 controls), and fentanyl (6 cases, 6 controls). Finally, no significant differences were found with regard to in-hospital procedures, including surgery (15/50 vs. 18/69, $p = 0.2$), heart catheterisation (4/50 vs. 5/69, $p = 0.7$), and colonoscopy (6/50 vs. 9/69, $p = 0.5$).

Table 2: Co-morbidity

Variable	Cases (n = 50)	Controls (n = 69)	P-value*
Heart failure, n (%)	6 (12)	6 (9)	0.5
Liver failure, n (%)	5 (10)	5 (7)	0.8
Infectious disease, n (%)	26 (52)	24 (35)	0.1
Pneumonia, n (%)	7 (14)	6 (9)	0.4
Acute neurological disease, n (%)	6 (12)	2 (3)	0.2
Diabetes mellitus, n (%)	16 (32)	10 (15)	0.009
Gastro-intestinal losses, n (%)	10 (20)	9 (13)	0.3
Cancer, n (%)	19 (38)	19 (28)	0.4

* Comparing cases versus controls using univariate conditional logistic regression.

Table 3: Medication

Medication	Cases (n = 50)	Controls (n = 69)	P-value*
Diuretics			
- Loop, n (%)	21 (42)	19 (28)	0.05
- Potassium sparing, n (%)	6 (12)	4 (6)	0.2
- Thiazides, n (%)	0 (0)	3 (4)	0.4
Analgetics			
- Opioids, n (%)	32 (64)	27 (39)	0.005
- NSAIDs, n (%)	14 (28)	25 (36)	0.5
- Acetaminophen, n (%)	27 (54)	34 (49)	0.4
Pscyoactive drugs			
- Benzodiazepine, n (%)	23 (46)	19 (28)	0.05
- SSRIs, n (%)	2 (4)	6 (9)	0.4
- Haloperidol, n (%)	4 (8)	4 (6)	0.7
Intravenous fluids			
- Isotonic, n (%)	19 (38)	24 (35)	0.7
- Hypotonic, n (%)	10 (20)	12 (17)	1.0
- Mannitol, n (%)	7 (14)	2 (3)	0.2
Antidiabetic drugs			
- Oral antidiabetics, n (%)	5 (10)	8 (12)	0.9
- Insulin, n (%)	12 (24)	4 (6)	0.007
Miscellaneous			
- Antibiotics, n (%)	41 (82)	38 (55)	0.007
- Chemotherapeutics, n (%)	9 (18)	8 (12)	0.6
- ACE-Is and ARBs, n (%)	17 (34)	21 (30)	0.8

* Comparing cases versus controls using univariate conditional logistic regression.

Abbreviations: NSAID, non-steroidal anti-inflammatory drugs; SSRI, selective serotonin reuptake inhibitors; ACE-Is, angiotensin converting enzyme inhibitors; ARBs, angiotensin receptor blockers.

Multivariate conditional logistic regression

After these univariate analyses, a multivariate conditional logistic regression analysis was performed (Table 4). In this model, we included the mortality probability (Table 1), and the significant variables from the univariate analyses ($p < 0.05$, Tables 2 and 3). Because CRP data were incomplete, this variable was not analyzed in the multivariate analysis. From this analysis, the use of insulin (odds ratio 10.5, 95% confidence interval 1.5 to 72.4), antibiotics (4.5, 1.4 to 14.6), and opioids (2.9, 1.1 to 7.8) were identified as independent factors associated with hospital-acquired hyponatraemia.

Table 4: Results of multivariate conditional logistic regression

Variable	Parameter estimate	Standard error	Wald Chi-square	P-value	OR (95% CI)
Insulin	1.3	0.6	5.6	0.02	9.9 (1.5-63.8)
Antibiotics	2.7	0.7	6.8	0.009	5.5 (1.5-19.7)
Opioids	2.3	1.0	5.9	0.02	3.7 (1.3-10.8)

Subanalysis on hyponatraemia and renal function

During the development of hyponatraemia, 23 cases had a rise in serum creatinine (subgroup 1, from 92 ± 65 $\mu\text{mol/l}$ to 131 ± 106 $\mu\text{mol/l}$) while in 20 cases serum creatinine remained stable or decreased (subgroup 2, from 83 ± 42 $\mu\text{mol/l}$ to 66 ± 35 $\mu\text{mol/l}$). Seven (pre) dialysis patients were excluded from analysis. Loop diuretic treatment was more common in subgroup 1 (14/23 vs. 6/20, $p = 0.04$), whereas cancer (14/20 vs. 5/23, $p = 0.001$) and

chemotherapy (8/20 vs. 1/23, $p = 0.004$) were more common in subgroup 2. When the subgroups were compared to their matched controls, antibiotics (19/23 vs. 16/34, $p = 0.02$) were more common in subgroup 1, while opioids (14/20 vs. 9/30, $p = 0.04$) and benzodiazepines (13/20 vs. 9/30) were more common in subgroup 2. Again, the course of the available CRP levels from admission until inclusion was significantly different between cases and controls in both subgroups, as analysed by two separate general linear models (Subgroup 1: 23 to 143 mg/l in cases vs. 37 to 22 mg/l in controls, $p = 0.05$; Subgroup 2: 22 to 152 mg/l in cases vs. 31 to 24 mg/l in controls, $p = 0.05$).

Discussion

In the present study, we sought to identify risk factors for hospital-acquired hyponatraemia using a matched case-control study design. The principal results demonstrate that increasing CRP levels, and the use of insulin, antibiotics and opioids are novel factors associated with hospital-acquired hyponatraemia. In addition, a multivariate analysis showed that insulin, antibiotics, and opioids were also independently related to hospital-acquired hyponatraemia.

In principle, hospital-acquired hyponatraemia may develop when the underlying disease deteriorates, and/or when factors are being introduced in the hospital that can potentially disturb water and sodium balance [18]. The association between an increase in CRP and the development of hyponatraemia could have reflected the course of the underlying disease. More specific, it could indicate the presence of the acute phase response, which can activate a neuroendocrine response,

including the release of antidiuretic hormone through interleukin-6 [19,20]. The acute phase response could also explain why antibiotics, as an indirect measure of infection, were used more often in patients with hospital-acquired hyponatraemia. A direct effect of antibiotics on antidiuretic hormone release or renal electrolyte handling is also possible. Previously, Ferreira Da Cunha *et al.* described that hyponatraemia developed three times more often in surgical patients with the acute phase response [21]. Of interest is also a case of hyponatraemia associated with infectious disease, in which elevated interleukin-6, CRP and antidiuretic hormone levels were the only positive findings [22]. Recently, Siegel reviewed the possible roles of cytokines in exercise associated and other forms of hyponatraemia [23]. A possible explanation why infection and surgery were not directly associated with hospital-acquired hyponatraemia in this study, is that the presence of either does not necessarily mean that the acute phase response was present. However, to confirm the presence of the acute phase response other indices such as serum albumin (which was measured infrequently in our patients) and leukocytes would be important.

A second novel factor associated with hospital-acquired hyponatraemia was the use of opioids, which were used 1.5 times more often in cases. Because opioids are commonly prescribed for pain relief, and pain is a very potent stimulus for antidiuretic hormone release [24], this may explain their relationship with hyponatraemia, although a direct effect of these drugs also remains a possibility [25].

The fact that increasing CRP, and the use of antibiotics and opioids were identified as risk factors for hospital-acquired hyponatraemia, also suggests that the condition of cases was generally worse than that of control subjects. Although a higher intensive care admission and mortality appear to support this impression, the calculated mortality probability during the development of hyponatraemia was not higher (Table 1). Nevertheless, the possibility cannot be excluded that the identified factors associated with hospital-acquired hyponatraemia, were predominantly surrogate markers for disease severity. One could therefore speculate that hyponatraemia could be a prognostic factor in a general hospital population [26], as has also been shown in several subpopulations, including hyponatraemic patients with heart failure [27], liver failure [28], and cancer [29]. Interestingly, however, insulin, antibiotics, and opioids remained independent predictors of hospital-acquired hyponatraemia, even when disease severity was included in the multivariate analysis (Table 4).

Finally, diabetes mellitus and the use of insulin were related to hospital-acquired hyponatraemia. Because patients with hyperglycaemia-induced hyponatraemia were excluded and the use of oral antidiabetics was similar between groups, other reasons for this association should be sought. It is well known that antidiuretic hormone is increased in diabetics [30], but the occurrence of hyponatraemia has only been described anecdotally [31]. There is some experimental evidence that insulin could have a direct effect on water homeostasis [32].

Previously, several other factors that potentially contribute to hospital-acquired hyponatraemia have been identified in non-controlled studies, including diuretics [8], surgery [1], hypotonic intravenous fluids [6,9], heart catheterisation [32], colonoscopy [10], antidepressants [34], and antipsychotics [35]. Remarkably, in this matched case-control study, these factors were not confirmed. Apparently these factors could be important in isolated cases, but they do not appear to play a major role on a day-to-day basis in a general hospital population. Alternatively, these factors may be involved in “hospital-aggravated” rather than hospital-acquired hyponatraemia. Indeed, we recently identified some of the aforementioned factors (diuretics, surgery, hypotonic intravenous fluids) in a population of patients who had mild hyponatraemia on admission and subsequently developed severe hyponatraemia in-hospital [6]. In this study, antibiotics, insulin, and opioids were not associated with admission hyponatraemia or hospital aggravated hyponatraemia (CRP was not analysed) [6], again emphasising their association with hospital-acquired hyponatraemia. Another implication of not identifying hypotonic intravenous fluids as a risk factor for hospital-acquired hyponatraemia, is that apparently the average oral fluid intake in hospitalised patients in combination with increased antidiuretic hormone was sufficient for the development of hospital-acquired hyponatraemia.

Although not a specific subject of this study, we performed a subanalysis on patients whose serum creatinine rose or fell during the development of hyponatraemia, thereby assessing whether hyponatraemia was more likely to be depletional or dilutional [36]. Loop diuretics appeared to be associated with depletional hyponatraemia, whereas cancer and chemotherapy

appeared to be associated with dilutional hyponatraemia. These observations are in agreement with the literature [1,3,4,7,8,11]. When the subgroups were compared to their controls, only antibiotics (depletional), and opioids and benzodiazepines (dilutional) were significantly different between cases and controls, although this may have been caused by a loss of statistical power. The increase in CRP levels remained significant in both subgroups.

Although this is one of the largest cohorts of hyponatraemic patients described in the literature, a number of limitations should be mentioned. The sample size and availability of the data limited the robustness with which we could analyse our data, and hence limits the power of some of the observations. Especially, because CRP values were incomplete, we were not able to assess if increasing CRP was independently associated with hospital-acquired hyponatraemia.

In conclusion, novel risk factors for hospital-acquired hyponatraemia that were identified in this study are an increase in CRP, and the use of insulin, antibiotics and opioids. These factors represent interesting new clues regarding the pathophysiology of hospital-acquired hyponatraemia, suggesting that the acute phase response, pain and/or direct drug effects could be involved in the release of vasopressin. Clearly, further research is needed to elucidate the pathophysiological background of some of the associations found in this study.

Acknowledgments

The authors are indebted to Dr. T. Stijnen and Dr. W.C. Hop for statistical advice and to Gert Verheij for assistance with retrieval of laboratory data.

References

1. Arieff AI. Hyponatremia, convulsions, respiratory arrest, and permanent brain damage after elective surgery in healthy women. *N Engl J Med* 1986; 314: 1529-1535.
2. Laureno R, Karp BI. Myelinolysis after correction of hyponatremia. *Ann Intern Med* 1997; 126: 57-62.
3. Adrogue HJ, Madias NE. Hyponatremia. *N Engl J Med* 2000; 342: 1581-1589.
4. Jamieson MJ. Hyponatraemia. *BMJ (Clin Res Ed)* 1985; 290: 1723-1728.
5. Anderson RJ, Chung HM, Kluge R, Schrier RW. Hyponatremia: a prospective analysis of its epidemiology and the pathogenetic role of vasopressin. *Ann Intern Med* 1985; 102: 164-168.
6. Hoorn EJ, Lindemans J, Zietse R. Development of severe hyponatraemia in hospitalized patients: treatment-related risk factors and inadequate management. *Nephrol Dial Transplant* 2006; 21: 70-76.
7. Halperin ML, Goldstein MB. Fluid, electrolyte, and acid-base physiology. 1999, 3rd ed. Philadelphia, PA: W.B. Saunders.
8. Spital A. Diuretic-induced hyponatremia. *Am J Nephrol* 1999; 19: 447-452.
9. Hoorn EJ, Geary D, Robb M, Halperin ML, Bohn D. Acute hyponatremia related to intravenous fluid administration in hospitalized children: an observational study. *Pediatrics* 2004; 113: 1279-1284.
10. Ayus JC, Levine R, Arieff AI. Fatal dysnatraemia caused by elective colonoscopy. *BMJ* 2003; 326: 382-384.
11. Moses AM, Miller M. Drug-induced dilutional hyponatremia. *N Engl J Med* 1974; 291: 1234-1239.
12. Sterns RH. Severe symptomatic hyponatremia: treatment and outcome. A study of 64 cases. *Ann Intern Med* 1987; 107: 656-664.

13. Moritz ML, Ayus JC. Hospital-acquired hyponatremia: why are there still deaths? *Pediatrics* 2004; 113: 1395-1396.
14. Turchin A, Seifter JL, Seely EW. Clinical problem-solving. Mind the gap. *N Engl J Med* 2003; 349: 1465-9.
15. Ross JW, Miller WG, Myers GL, Praestgaard J. The accuracy of laboratory measurements in clinical chemistry: a study of 11 routine chemistry analytes in the College of American Pathologists Chemistry Survey with fresh frozen serum, definitive methods, and reference methods. *Arch Pathol Lab Med* 1998; 122: 587-608.
16. Lemeshow S, Teres D, Avrunin JS, Gage RW. Refining intensive care unit outcome prediction by using changing probabilities of mortality. *Crit Care Med* 1988; 16: 470-477.
17. Petrie A, Sabin C. *Medical statistics at a glance*. 2nd ed 2005. Oxford: Blackwell Publishing.
18. Anderson RJ. Hospital-associated hyponatremia. *Kidney Int* 1986; 29: 1237-1247.
19. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med* 1999; 340: 448-454.
20. Papanicolaou DA, Wilder RL, Manolagas SC, Chrousos GP. The pathophysiologic roles of interleukin-6 in human disease. *Ann Intern Med* 1998; 128: 127-137.
21. Ferreira da Cunha D, Pontes Monteiro J, Modesto dos Santos V, Araujo Oliveira F, Freire de Carvalho da Cunha S. Hyponatremia in acute-phase response syndrome patients in general surgical wards. *Am J Nephrol* 2000; 20: 37-41.
22. Murakami T, Matoba H, Kuga Y, Ozawa S, Kubota K, Yoshida S. Hyponatremia in a patient with chronic inflammatory disease. *Internal Medicine* 1998; 37: 792-795.
23. Siegel AJ. Exercise-associated hyponatremia: role of cytokines. *Am J Med* 2006; 119: S74-S78.
24. Kendler KS, Weitzman RE, Fisher DA. The effect of pain on plasma arginine vasopressin concentrations in man. *Clin Endocrinol (Oxf)* 1978; 8: 89-94.
25. Bozkurt P, Kaya G, Yeker Y, Altintas F, Bakan M, Hacibekiroglu M, Kavunoglu G. Effects of systemic and epidural morphine on antidiuretic hormone levels in children. *Paediatr Anaesth* 2003; 13: 508-514.

26. Tierney WM, Martin DK, Greenlee MC, Zerbe RL, McDonald CJ. The prognosis of hyponatremia at hospital admission. *J Gen Intern Med* 1986; 1: 380-385.
27. Lee DS, Austin PC, Rouleau JL, Liu PP, Naimark D, Tu JV. Predicting mortality among patients hospitalized for heart failure: derivation and validation of a clinical model. *JAMA* 2003; 290: 2581-2587.
28. Jalan R, Elton RA, Redhead DN, Finlayson ND, Hayes PC. Analysis of prognostic variables in the prediction of mortality, shunt failure, variceal rebleeding and encephalopathy following the transjugular intrahepatic portosystemic stent-shunt for variceal haemorrhage. *J Hepatol* 1995; 23: 123-128.
29. Berghmans T, Paesmans M, Body JJ. A prospective study on hyponatraemia in medical cancer patients: epidemiology, aetiology and differential diagnosis. *Support Care Cancer* 2000; 8: 192-197.
30. Bankir L, Bardoux P, Ahloulay M. Vasopressin and diabetes mellitus. *Nephron* 2001; 87: 8-18.
31. Uchida D, Nishikawa T, Omura M, Takaya T, Myojo S, Motoyoshi M, Yoshida S. Possible involvement of hypersecretion of ADH in hyponatremia in a diabetic patient complicated with severe neuropathy. *Horm Res* 1993; 39: 247-252.
32. Bustamante M, Hasler U, Kotova O, Chibalin AV, Mordasini D, Rousselot M, Vandewalle A, Martin PY, Feraille E. Insulin potentiates AVP-induced AQP2 expression in cultured renal collecting duct principal cells. *Am J Physiol Renal Physiol* 2005; 288: F334-F344.
33. Aronson D, Dragu RE, Nakhoul F, Hir J, Miller A, Boulos M, Zinder O, Green J, Mittleman MA, Markiewicz W. Hyponatremia as a complication of cardiac catheterization: a prospective study. *Am J Kidney Dis* 2002; 40: 940-946.
34. Movig KL, Leufkens HG, Lenderink AW, van den Akker VG, Hodiamont PP, Goldschmidt HM, Egberts AC. Association between antidepressant drug use and hyponatraemia: a case-control study. *Br J Clin Pharmacol* 2002; 53: 363-369.
35. Rider JM, Mauger TF, Jameson JP, Notman DD. Water handling in patients receiving haloperidol decanoate. *Ann Pharmacother* 1995; 29: 663-666.

36. Hoorn EJ, Lindemans J, Zietse R. Acute and concomitant deterioration of hyponatremia and renal dysfunction associated with heart and liver failure. Clin Nephrol 2006; 65: 248-255.

Chapter 10

**Acute and concomitant deterioration of
hyponatremia and renal dysfunction
associated with heart and liver failure**

**Ewout J. Hoorn, Jan Lindemans
and Robert Zietse**

Clin Nephrol 2006; 65: 248–55

Abstract

Background: Although hyponatremia with concomitant renal dysfunction has been described anecdotally, little is known about how these two conditions are related, which type of renal dysfunction usually occurs, and which patients are at risk.

Methods: From 3029 measured serum sodium (S_{Na}) concentrations in 3 months, patients with at least one $S_{Na} \leq 125$ mmol/l were selected, and divided in patients with at least one $S_{Creat} \geq 125$ μ mol/l (study-group, $n = 20$), and patients whose S_{Creat} remained normal (control-group, $n = 50$).

Results: During the first 5 days of hospitalization, S_{Creat} almost doubled in the study-group from 125 ± 40 μ mol/l to 207 ± 72 μ mol/l, while S_{Na} decreased concurrently from 130 ± 2 mmol/l to 122 ± 3 mmol/l. The peak S_{Creat} and S_{Na} values coincided, and the average courses were highly correlated ($r = 0.88$, $p < 0.001$). The study-group more often had heart failure (10/20 vs. 1/50, $p < 0.001$) and liver failure (7/20 vs. 4/50, $p = 0.009$), and more often received loop diuretics (13/20 vs. 12/50, $p = 0.002$), spironolactone (11/20 vs. 7/50, $p = 0.001$), and/or angiotensin converting enzyme inhibitors (5/20 vs. 2/50, $p = 0.02$). Four patients were admitted to the intensive care (10 in control-group, $p = 1.0$), and 5 patients died (10 in control-group, $p = 0.7$).

Conclusions: Renal dysfunction is common in severe hyponatremia and usually develops in an acute-on-chronic fashion with concomitant deterioration of both conditions. This concourse is associated with heart failure, liver failure, and/or a renal drug regimen. Given the recent results of clinical trials, this patient group may be especially suitable for treatment with vasopressin antagonists.

Introduction

Severe hyponatremia and renal dysfunction (RD) are frequently acquired in the hospital and are associated with increased morbidity and mortality, either because of direct complications of these conditions, or because of the severity of the underlying disease [1,2]. Previous cohort studies of patients with hyponatremia have reported that RD is a common finding in this population [3-7]. The majority of these studies showed a prevalence of around 9%, although higher numbers of 17% [5] and 50% [7] have also been reported. However, none of these studies directly investigated the association between hyponatremia and RD, the type of RD in hyponatremia, and which patients are at risk for this combination.

In principle, because water and sodium balance and glomerular filtration rate (GFR) are regulated by similar homeostatic control systems, including the renin-angiotensin-system (RAS), the sympathetic nervous system (SNS), and antidiuretic hormone (ADH), it is conceivable that perturbations in one or more of these control systems may lead to concomitant hyponatremia and RD. Thus, the objective of the present study was to analyze the relationship between hyponatremia and RD in a cohort of hyponatremic patients.

Methods

Subjects

All serum sodium concentrations (S_{Na}) measured in adult hospitalized patients in a study-period of three months in 2003, in the Erasmus Medical Center, an 831-bed urban university hospital in Rotterdam, The Netherlands, were reviewed. Severe hyponatremia was defined as at least one $S_{Na} \leq 125$ mmol/l, and RD was defined as at least one serum creatinine concentration (S_{Creat}) ≥ 125 μ mol/l. The study-group consisted of patients with severe hyponatremia and RD, whereas the control-group consisted of patients with severe hyponatremia but without RD. Patients with isotonic or hypertonic hyponatremia (defined by measured serum osmolality values) and patients with end-stage-kidney disease were excluded from the analysis. S_{Na} values were determined with ion-selective electrodes, and S_{Creat} values with the modified Jaffé reaction.

Evaluation

The following data were included in our analysis: age, gender, weight, reason of admission, underlying disorders, medication including intravenous (IV) fluids, assessment of the extracellular fluid (ECF) volume, hemodynamic and serial biochemical parameters. Data were collected by way of clinical examination of patients, the laboratory and hospital information systems, and chart review.

Statistical analysis

Data were analyzed using SPSS (v. 12.0, Chicago, IL). For selected pairs of biochemical parameters, it was analyzed if their courses during hospitalization were correlated (using Pearson's r), using the daily averages of the study-group. Nominal data were analyzed using the Fisher's exact test and ordinal data using the Student's T-test. A p-value of 0.05 was considered significant. All data are expressed as mean $\pm$ SD.

Results

Study group

Of the 3029 hospitalized patients in whom S_{Na} was determined, 81 patients (2.7%) had at least one S_{Na} value ≤ 125 mmol/l. Twenty patients (25%) were included in the study-group (severe hyponatremia with RD), and 50 patients in the control group (severe hyponatremia without RD). Seven patients with isotonic or hypertonic hyponatremia (due to mannitol or hyperglycemia) and 4 patients with end-stage kidney disease (three of which were on hemodialysis) were excluded from analysis. The demographic and outcome characteristics of the study and control groups are shown in Table 1.

Table 1: Demographic and outcome characteristics

	Hyponatremia with RD (n = 20)	Hyponatremia without RD (n = 50)	P-value
Age, years	53 ± 16	64 ± 16	0.01
Female sex, n (%)	7 (35)	28 (56)	0.2
Weight, kg	73 ± 10	63 ± 10	< 0.001
ICU-admission, n (%)	4 (20)	10 (20)	1.0
Mortality, n (%)	5 (25)	10 (20)	0.7
Duration of admission, days	18 ± 14	26 ± 20	0.1

RD, renal dysfunction; ICU, intensive care unit

Course of hyponatremia and renal dysfunction

The paired courses of S_{Na} , S_{Creat} , the serum urea concentration (S_{Urea}), and the serum potassium concentration (S_K) during hospitalization are shown in Figure 1. In the 20 patients with hyponatremia and RD, the average S_{Creat} around admission was already elevated at $137 \pm 65 \mu\text{mol/l}$ ($1.55 \pm 0.74 \text{ mg/dl}$), then showed an initial moderate decrease during the first two days to $125 \pm 40 \mu\text{mol/l}$ ($1.41 \pm 0.45 \text{ mg/dl}$), after which S_{Creat} almost doubled in the following three days to $207 \pm 72 \mu\text{mol/l}$ ($2.34 \pm 0.81 \text{ mg/dl}$). Concurrently, S_{Na} decreased from $130 \pm 2 \text{ mmol/l}$ on admission to $122 \pm 3 \text{ mmol/l}$ five days later.

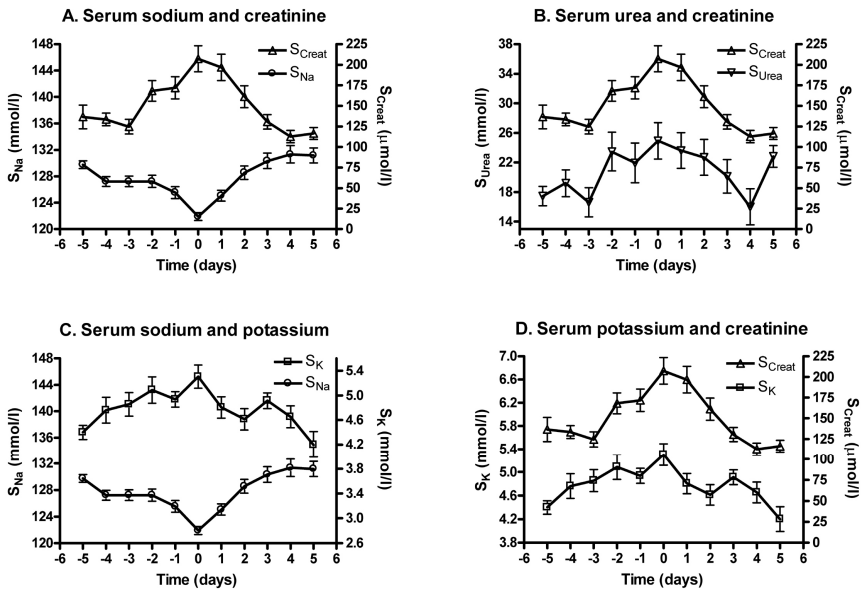


Figure 1: Paired courses of serum sodium (S_{Na}), creatinine (S_{Creat}), urea (S_{Urea}), and potassium (S_K) concentrations are shown to illustrate parallel trends. Each data-point represents the average of 20 values, with all peak values set at day 0. For clarity of the figure, error bars represent the standard error of the mean rather than the standard deviation. See text for correlations between the average courses.

The peak S_{Na} and S_{Creat} values coincided, and their average courses were highly correlated ($r = 0.88$, $p < 0.001$). In addition, S_{Urea} and S_K also rose in association with the rise in S_{Creat} and fall in S_{Na} , again with coinciding peak values (Figure 1). The average course of S_{Urea} was correlated with the average course of S_{Creat} ($r = 0.77$, $p = 0.006$), while the average course of S_K was correlated with the average courses of both S_{Na} ($r = 0.75$, $p = 0.008$) and S_{Creat} ($r = 0.64$, $p = 0.03$). The correlations between the average courses of S_{Urea} and those of S_{Na} and S_K were less strong ($r = 0.56$ and $r = 0.34$, respectively), and not statistically

significant ($p > 0.05$). Fractional excretions of sodium (FE_{Na}), calculated as $(U_{Na} * S_{Creat}) / (S_{Na} * U_{Creat})$, were available in 5 patients (U_{Creat} unavailable in other patients), and were all $> 1\%$ ($12 \pm 8\%$). For 15 patients, this was the first time RD was observed, while 5 patients already had a moderately impaired renal function for one to four weeks.

Biochemical and hemodynamic parameters

A comparison of selected biochemical and hemodynamic parameters in the study and control-groups is shown in Table 2. This analysis showed that the courses of S_{Na} were approximately equal, whereas patients with severe hyponatremia and RD had significantly higher uric acid concentrations (0.64 ± 0.26 vs. 0.23 ± 0.11 , $p < 0.001$), lower urinary sodium concentrations (28 ± 20 vs. 69 ± 22 mmol/l, $p < 0.001$), and lower systolic blood pressures (119 ± 22 vs. 133 ± 27 mm Hg, $p = 0.04$). Finally, although of limited sensitivity and specificity in hyponatremia [8], assessment of the ECF volume showed more ECF expansion in the study group.

Factors associated with hyponatremia and renal dysfunction

In order to identify factors that may have contributed to RD in patients with severe hyponatremia, the reasons of admission, and the medication that was used during the period in which S_{Na} fell and S_{Creat} rose, were compared to those in the control group (Table 3). Patients with severe hyponatremia and RD more often had heart failure and liver failure, and were more often receiving loop diuretics, spironolactone, and/or angiotensin converting enzyme (ACE) inhibitors.

Table 2: Selected biochemical and hemodynamic parameters *

	Hyponatremia with RD (n = 20)	Hyponatremia without RD (n = 50)	P-value
Serum			
- Sodium - admission, mmol/l	130 ± 2	127 ± 8	0.1
- Sodium - lowest	122 ± 3	121 ± 4	0.3
- Sodium - discharge/death	133 ± 5	133 ± 7	1.0
- Uric acid, $\mu\text{mol/l}$ †	0.64 ± 0.26	0.23 ± 0.11	< 0.001
- Albumin, g/l †	33 ± 9	33 ± 7	1.0
Urine (spot)			
- Sodium, mmol	28 ± 20	69 ± 22	< 0.001
- Potassium, mmol	49 ± 33	38 ± 33	0.2
- Osmolality, mOsm/kg	447 ± 219	465 ± 226	0.8
Hemodynamics			
- Systolic blood pressure, mm Hg	119 ± 22	133 ± 27	0.04
- Diastolic blood pressure, mm Hg	68 ± 14	73 ± 15	0.2
- Heart rate, bpm	92 ± 18	85 ± 18	0.1
Extracellular fluid volume			
- Signs of ECF expansion ‡, n (%)	13 (65)	13 (25)	0.005
- Signs of ECF contraction ‡	0 (0)	6 (12)	0.2

* Measured at the time of lowest serum sodium (S_{Na}) values except for admission and discharge/death S_{Na} .

† Reference values for uric acid 0.12 – 0.34 $\mu\text{mol/l}$ (females), 0.20 – 0.42 $\mu\text{mol/l}$ (males), and for albumin 35 – 55 g/l.

‡ ECF expansion defined as the presence of peripheral edema, pleural effusions and/or ascites. ECF contraction defined as the presence of orthostatic hypotension, low capillary refill, increased skin turgor, and/or dry mucous membranes.

Frequently, a combination of these factors was present, including 6 patients with heart failure who were also receiving loop diuretics, spironolactone and ACE-inhibitors, and 5 patients with liver failure who were also receiving loop-diuretics and spironolactone. Of the remaining 9 patients, 4 patients with heart failure or liver failure had different drug combinations, 3 patients had heart failure or liver failure only, and 2 patients had different underlying disorders (infectious disease). Frequently, treatment with the aforementioned drugs was initiated or intensified in the hospital prior to the time that hyponatremia and RD worsened. Loop diuretics were started in 7 patients and their dosage was increased in 5 patients, spironolactone was started in 2 patients, and ACE-inhibitors were started in 1 patient. Other factors that may have contributed to the deterioration of hyponatremia and/or RD included non-steroidal anti-inflammatory drugs (1 patient), other nephrotoxic drugs (7 patients), hypotonic IV-fluids (3 patients), and/or gastrointestinal losses (5 patients; 13 patients in the control-group, $p = 1.0$; not shown in Table 3). Common drug combinations in the control group were loop diuretics with thiazides or spironolactone (8 patients), and loop diuretics with other nephrotoxic drugs (2 patients).

The 10 patients with heart failure and RD were admitted for complications or further deterioration of heart failure (8 patients), or for heart transplantation screening (2 patients); all patients were in New York Heart Association classes III or IV. Echocardiography was available in 9 patients (8 study-group, 1 control), and showed severe systolic dysfunction (6 patients), moderate systolic dysfunction (2 patients), and severe diastolic dysfunction (1 patient).

Table 3: Reasons of admission and medication that patients received during deterioration of hyponatremia and/or renal dysfunction

	Hyponatremia with RD (n = 20)	Hyponatremia without RD (n = 50)	P-value
Reason of admission			
- Heart failure, n (%)	10 (50)	1 (2)	< 0.001
- Liver failure	7 (35)	4 (8)	0.009
- Infectious disease	2 (10)	4 (8)	1.0
- Malignancy	0 (0)	16 (32)	0.003
- Neurological disorder	1 (5)	11 (22)	0.2
- Analysis internal medicine	0 (0)	7 (14)	0.2
- Elective surgery	0 (0)	10 (20)	0.05
Medication			
- Loop diuretics, n (%)	13 (65)	12 (24)	0.002
- Thiazide diuretics	2 (10)	13 (26)	0.2
- Spironolactone	11 (55)	7 (14)	0.001
- ACE-inhibitors	5 (25)	2 (4)	0.02
- NSAIDs	1 (5)	5 (10)	0.7
- Other nephrotoxic drugs*	7 (35)	11 (22)	0.4
- Isotonic IV-fluids	0 (0)	2 (4)	1.0
- Hypotonic IV-fluids	3 (15)	7 (14)	1.0

* Including allopurinol, aminoglycosides, carbamazepine, cytotoxic agents, and immunosuppressive agents.

Abbreviations: ACE, angiotensin converting enzyme; NSAID, non-steroidal anti-inflammatory drugs; IV, intravenous.

Similarly, the 7 patients with liver failure and RD were admitted for complications or further deterioration of liver failure (5 patients), or for liver transplantation screening (2 patients); all patients had Child-Pugh classifications B or C.

The management of hyponatremia in the two groups is shown in Table 4. In patients with hyponatremia and RD, the recovery of S_{Na} and S_{Creat} appeared to be associated with isotonic IV-fluid therapy (9 patients), or discontinuation of diuretics (9 patients), whereas in two patients there appeared to be a spontaneous recovery.

Table 4: Management of hyponatremia

Management	Hyponatremia with RD (n = 20)	Hyponatremia without RD (n = 50)	P-value
Normal saline, n (%)	9 (45)	18 (36)	0.6
Hypertonic saline	0 (0)	3 (6)	0.6
Water restriction	0 (0)	7 (14)	0.2
NaCl tablets	0 (0)	8 (16)	0.1
Stop diuretic	9 (45)	9 (18)	0.03
No therapy	2 (10)	14 (28)	0.1

Discussion

In the present study, we sought to investigate the relationship between severe hyponatremia and renal dysfunction (RD). The principle results demonstrate that approximately one in every four patients with severe hyponatremia also had RD, which usually developed in an acute-on-chronic fashion, during which there was concomitant deterioration of hyponatremia and renal function. As expected, during this concourse, S_K and S_{Urea} also rose and their average courses were highly correlated with those of S_{Na} and S_{Creat} (Figure 1). Because in most patients S_{Creat} rose between 1.5 and 2 times the baseline-value, according to the RIFLE-criteria (Risk of renal dysfunction, Injury to the kidney, Failure of kidney function, Loss of kidney function, End-stage kidney disease), RD should be classified as either “risk of RD”, or “injury to the kidney” [9]. Factors associated with the development of RD in severe hyponatremia usually consisted of a combination of underlying disorders (heart failure, liver failure) and renal drugs (loop-diuretics, spironolactone, ACE-inhibitors). Because this renal drug regimen was often initiated or intensified in-hospital, further worsening of hyponatremia and RD appeared to be related, at least in part, to what has been coined the delicate balance of modern polypharmacy [10]. Although hyponatremia and RD have individually been described as side-effects of loop diuretics [11,12], spironolactone [13], and ACE-inhibitors [14], this is one of the first reports describing a simultaneous deterioration of both conditions.

Diagnostically, it is difficult to assess whether RD should be ascribed to prerenal disease or acute tubular necrosis, especially because many patients were receiving diuretics [15]. However, the available FE_{Na} values, the high uric acid values, and the fact that RD responded to treatment, and usually did not improve spontaneously, appear to indicate that a prerenal cause for RD was likely. This may also explain the initial decrease in S_{Creat} (Figure 1), because periodic downward fluctuations due to variations in renal perfusion are suggestive of a prerenal cause [16]. For more conclusive answers, however, urinalysis or FE of urea could have been useful tests [17]. Another diagnostic consideration is if RD in patients with liver failure may have been due to the hepatorenal syndrome. Of interest is that one of the criteria of hepatorenal syndrome is in fact a decreased S_{Na} (< 130 mmol/l) [18]. However, again, what militates against the hepatorenal syndrome, is the recovery of renal function upon treatment, which makes a prerenal cause more likely [18].

A possible pathophysiological link between hyponatremia and RD is the neurohumoral response, which can be activated by decreased cardiac output (heart failure), systemic vasodilatation (liver failure, sepsis), and/or hypovolemia (diuretics, gastrointestinal losses) [19]. This neurohumoral response will lead to increased vasoconstrictor forces including activation of the RAS and SNS, and stimulation of ADH-release, all of which can affect water-sodium balance and renal function [19]. Dzau *et al.* demonstrated that in patients with heart failure and hyponatremia, plasma renin, angiotensin II, and aldosterone levels were significantly higher than those in heart-failure-patients without hyponatremia, and that this pronounced vasoconstrictor response appeared to be compensated by

vasodilator mechanisms through increased prostaglandin synthesis [20]. Similarly, in patients with liver failure, the degree of activation of the RAS, SNS, and ADH was found to be proportional to the severity of the hemodynamic insufficiency [21,22].

The fact that hyponatremia and renal function were deteriorating concurrently suggests that the delicate balance between vasoconstrictor and vasodilator forces was disrupted, likely because of a deterioration of the underlying disease and/or because of changes in the renal drug regimen. Increased vasoconstrictor forces could then result in RD if the autoregulatory capacity of the kidney is overwhelmed. In addition, increased ADH-release due to arterial underfilling and/or decreased delivery to the distal nephron due to a lower GFR may have been responsible for hyponatremia [23]. Indirect evidence for high circulating ADH levels comes from the high urinary osmolality values (Table 2). Animal models of heart failure and liver failure have indeed demonstrated that the pathogenesis of hyponatremia in these disorders is related to high levels of ADH and increased expression of the water channel aquaporin-2 [24]. In this setting, the infusion of hypotonic IV-fluids can further aggravate hyponatremia, and should therefore be avoided [25].

In the literature, we were able to find one other study that documented concomitant deterioration of hyponatremia and RD [26]. In this study, three patients with acute-on-chronic renal failure and hyponatremia associated with hypothyroidism were reported, and the course of S_{Creat} and S_{Na} strikingly resembled our observations [26]. Because hypothyroidism causes a

decrease in cardiac output, the mechanism of hyponatremia and renal failure may have been similar to the one present in our series.

From our results, it is clear that hyponatremic patients with RD are distinctly different from hyponatremic patients without RD, in whom post-operative hyponatremia and malignancy-related hyponatremia are more common (Table 3). Therefore, an important question is if hyponatremia in the presence of RD needs to be managed differently. While guidelines for diuretic therapy in heart failure and liver failure with concomitant RD recommend intensified loop diuretic therapy and/or the addition of a thiazide-diuretic [27], this may not be desirable in the context of hyponatremia. Although sodium retention is a characteristic of progressive heart failure and liver failure, for hyponatremia to develop, water retention must exceed sodium retention. Therapeutically, this implies that either the increased ADH-release needs to be reversed, or that the retained electrolyte-free-water needs to be excreted [28]. Theoretically, a reduction in ADH-levels could be achieved by re-expanding the contracted intravascular compartment with isotonic saline, although this may lead to further cardiac or hepatic decompensation. Loop diuretics in combination with small-volume hypertonic saline may be used to achieve a net excretion of electrolyte-free-water [29], although increased diuretic resistance may prevent the diuretic to reach the lumen-side of the nephron to exert its effects [30].

A potentially promising alternative to diuretics may therefore be treatment with the recently introduced vasopressin-receptor antagonists [31]. In fact, results from a recent trial, in which the

vasopressin-receptor antagonist tolvaptan was tested in patients with heart failure, not only showed a correction of hyponatremia, but also improved survival, especially in patients with RD [32]. These results may follow from the observations that hyponatremia and RD have both been identified as predictors of mortality in heart failure patients [33,34]. This association is less clear in patients with liver failure [35], although successful correction of hyponatremia with a vasopressin-receptor antagonist was also demonstrated in this patient population [36]. In our series a quarter of the patients with severe hyponatremia and RD died, but this was not significantly higher than in patients with severe hyponatremia without RD (Table 1). Nevertheless, the potential dangers of deteriorating hyponatremia and RD are evident and include neurological complications, uremia, hyperkalemia, and progression of renal failure [2,15].

In conclusion, RD is common in severe hyponatremia, and usually develops in an acute-on-chronic fashion during which there is concomitant deterioration of hyponatremia and renal function. Severe hyponatremia with RD usually occurs in patients with advanced heart or liver failure, who are receiving loop diuretics, spironolactone and/or ACE-inhibitors.

References

1. Nolan CR, Anderson RJ. Hospital-acquired acute renal failure. *J Am Soc Nephrol* 1998; 9: 710-8.
2. Adroge HJ, Madias NE. Hyponatremia. *N Engl J Med* 2000; 342: 1581-9.
3. Anderson RJ, Chung HM, Kluge R, Schrier RW. Hyponatremia: a prospective analysis of its epidemiology and the pathogenetic role of vasopressin. *Ann Intern Med* 1985; 102: 164-8.

4. Ellis SJ. Severe hyponatraemia: complications and treatment. *QJM* 1995; 88: 905-9.
5. Erasmus RT, Matsha TE. The frequency, aetiology and outcome of severe hyponatraemia in adult hospitalised patients. *Cent Afr J Med* 1998; 44: 154-8.
6. Nzerue CM, Baffoe-Bonnie H, You W, Falana B, Dai S. Predictors of outcome in hospitalized patients with severe hyponatremia. *J Natl Med Assoc* 2003; 95: 335-43.
7. Saeed BO, Beaumont D, Handley GH, Weaver JU. Severe hyponatraemia: investigation and management in a district general hospital. *J Clin Pathol* 2002; 55: 893-6.
8. Chung HM, Kluge R, Schrier RW, Anderson RJ. Clinical assessment of extracellular fluid volume in hyponatremia. *Am J Med* 1987; 83: 905-8.
9. Bellomo R, Ronco C, Kellum JA, Mehta RL, Palevsky P. Acute renal failure - definition, outcome measures, animal models, fluid therapy and information technology needs: the Second International Consensus Conference of the Acute Dialysis Quality Initiative (ADQI) Group. *Crit Care* 2004; 8: R204-12.
10. Thomas MC. Diuretics, ACE inhibitors and NSAIDs--the triple whammy. *Med J Aust* 2000; 172: 184-5.
11. Spital A. Diuretic-induced hyponatremia. *Am J Nephrol* 1999; 19: 447-52.
12. Sonnenblick M, Rosin AJ. Significance of the measurement of uric acid fractional clearance in diuretic induced hyponatraemia. *Postgrad Med J* 1986; 62: 449-52.
13. Tamirisa KP, Aaronson KD, Koelling TM. Spironolactone-induced renal insufficiency and hyperkalemia in patients with heart failure. *Am Heart J* 2004; 148: 971-8.
14. Oster JR, Materson BJ. Renal and electrolyte complications of congestive heart failure and effects of therapy with angiotensin-converting enzyme inhibitors. *Arch Intern Med* 1992; 152: 704-10.
15. Klahr S, Miller SB. Acute oliguria. *N Engl J Med* 1998; 338: 671-5.
16. Espinel CH, Gregory AW. Differential diagnosis of acute renal failure. *Clin Nephrol* 1980; 13: 73-7.

17. Carvounis CP, Nisar S, Guro-Razuman S. Significance of the fractional excretion of urea in the differential diagnosis of acute renal failure. *Kidney Int* 2002; 62: 2223-9.
18. Bataller R, Sort P, Gines P, Arroyo V. Hepatorenal syndrome: definition, pathophysiology, clinical features and management. *Kidney Int Suppl* 1998; 66: S47-53.
19. Schrier RW, Gurevich AK, Cadnapahornchai MA. Pathogenesis and management of sodium and water retention in cardiac failure and cirrhosis. *Semin Nephrol* 2001; 21: 157-72.
20. Dzau VJ, Packer M, Lilly LS, Swartz SL, Hollenberg NK, Williams GH. Prostaglandins in severe congestive heart failure. Relation to activation of the renin--angiotensin system and hyponatremia. *N Engl J Med* 1984; 310: 347-52.
21. Henriksen JH, Bendtsen F, Gerbes AL, Christensen NJ, Ring-Larsen H, Sorensen TI. Estimated central blood volume in cirrhosis: relationship to sympathetic nervous activity, beta-adrenergic blockade and atrial natriuretic factor. *Hepatology* 1992; 16: 1163-70.
22. Arroyo V, Bosch J, Gaya-Beltran J, Kravetz D, Estrada L, Rivera F, et al. Plasma renin activity and urinary sodium excretion as prognostic indicators in nonazotemic cirrhosis with ascites. *Ann Intern Med* 1981; 94: 198-201.
23. Halperin ML, Bichet DG, Oh MS. Integrative physiology of basal water permeability in the distal nephron: implications for the syndrome of inappropriate secretion of antidiuretic hormone. *Clin Nephrol* 2001; 56: 339-45.
24. Nielsen S, Frokiaer J, Marples D, Kwon TH, Agre P, Knepper MA. Aquaporins in the kidney: from molecules to medicine. *Physiol Rev* 2002; 82: 205-44.
25. Shafiee MA, Bohn D, Hoorn EJ, Halperin ML. How to select optimal maintenance intravenous fluid therapy. *QJM* 2003; 96: 601-10.
26. Woodrow G, Brownjohn AM, Turney JH. Acute-on-chronic renal failure and hyponatraemia associated with severe hypothyroidism. *Nephrol Dial Transplant* 1993; 8: 557-9.
27. Brater DC. Diuretic therapy. *N Engl J Med* 1998; 339: 387-95.

28. Porzio P, Halberthal M, Bohn D, Halperin ML. Treatment of acute hyponatremia: ensuring the excretion of a predictable amount of electrolyte-free water. *Crit Care Med* 2000; 28: 1905-10.
29. Licata G, Di Pasquale P, Parrinello G, Cardinale A, Scandurra A, Follone G, et al. Effects of high-dose furosemide and small-volume hypertonic saline solution infusion in comparison with a high dose of furosemide as bolus in refractory congestive heart failure: long-term effects. *Am Heart J* 2003; 145: 459-66.
30. Kramer BK, Schweda F, Riegger GA. Diuretic treatment and diuretic resistance in heart failure. *Am J Med* 1999; 106: 90-6.
31. Wong LL, Verbalis JG. Vasopressin V2 receptor antagonists. *Cardiovasc Res* 2001; 51: 391-402.
32. Gheorghiade M, Gattis WA, O'Connor CM, Adams KF, Jr., Elkayam U, Barbagelata A, et al. Effects of tolvaptan, a vasopressin antagonist, in patients hospitalized with worsening heart failure: a randomized controlled trial. *JAMA* 2004; 291: 1963-71.
33. Lee DS, Austin PC, Rouleau JL, Liu PP, Naimark D, Tu JV. Predicting mortality among patients hospitalized for heart failure: derivation and validation of a clinical model. *JAMA* 2003; 290: 2581-7.
34. Fonarow GC, Adams KF, Jr., Abraham WT, Yancy CW, Boscardin WJ. Risk stratification for in-hospital mortality in acutely decompensated heart failure: classification and regression tree analysis. *JAMA* 2005; 293: 572-80.
35. Porcel A, Diaz F, Rendon P, Macias M, Martin-Herrera L, Giron-Gonzalez JA. Dilutional hyponatremia in patients with cirrhosis and ascites. *Arch Intern Med* 2002; 162: 323-8.
36. Gerbes AL, Gulberg V, Gines P, Decaux G, Gross P, Gandjini H, et al. Therapy of hyponatremia in cirrhosis with a vasopressin receptor antagonist: a randomized double-blind multicenter trial. *Gastroenterology* 2003; 124: 933-9.

Chapter 11

**Hypernatremia in critically ill patients:
too little water and too much salt**

Ewout J. Hoorn, Michiel G.H. Betjes
Joachim Weigel and Robert Zietse

Submitted

Abstract

Objective: To study the risk factors and mechanisms of hypernatremia (serum sodium ≥ 150 mmol/l) in patients admitted to the intensive care unit (ICU), thereby challenging the assumption that hypernatremia usually develops because of a negative water balance.

Design: 1:2 matched case-control study and fluid balance studies.

Setting: Internal, neurological and surgical ICUs of a university hospital.

Patients: From 2642 ICU-patients, 130 patients had ICU-acquired hypernatremia. They were matched to 260 normonatremic controls.

Interventions: None.

Measurements and main results: In cases, serum sodium rose from 141 ± 3 mmol/l to 156 ± 6 mmol/l in 48 ± 4 hours. Risk factors for ICU-acquired hypernatremia were ($p < 0.05$ for all): hypokalemia (69/130 vs. 89/260), hypercalcemia (22/130 vs. 15/260), renal insufficiency (69/130 vs. 35/260), uremia (55/130 vs. 57/260), mannitol (13/130 vs. 3/260), hyperglycemia (56/130 vs. 81/260), and sodium bicarbonate (30/130 vs. 1/260). Mortality was higher in cases (62/130 vs. 27/260, $p < 0.001$). Multivariate analyses showed that these associations with hypernatremia were independent of the severity of disease. During the development of hypernatremia, 80 patients had a negative fluid balance (-31 ± 2 ml/kg/day), while 50 patients had a positive fluid balance (72 ± 3 ml/kg/day). In patients with a positive fluid balance, the tonicity of the administered fluids was higher (148 ± 2 vs. 133 ± 3 mEq/l, $p < 0.001$), while they received a similar small amount of electrolyte free water (3.5 ± 0.6 vs. 4.7 ± 0.9 ml/kg/day, $p = 1.0$).

Conclusions: Critically ill patients with hypernatremia more often have a reason to lose renal water due to a concentrating defect, osmotic diuresis, or renal insufficiency. This deficit is then corrected not only with too little water, but also with too much sodium. Therapy should therefore rely on adding electrolyte free water and/or creating a negative sodium balance. Adjustments in intravenous fluid regimens may prevent hypernatremia.

Introduction

Fluid and electrolyte disorders are common in critically ill patients, and often pose a challenge to the treating clinician [1]. Of the dysnatremias, hypernatremia has received far less attention than hyponatremia, with the result that limited evidence is available for rational management strategies.

Nevertheless, it is generally assumed that hypernatremia most commonly develops as a result of a negative water balance [2-4]. Previous studies identified a urinary concentrating defect, increased insensible and enteral losses, and inadequate fluid management as responsible factors for this negative water balance [5,6]. However, in principle, hypernatremia may also develop as a result of a positive sodium balance. This can occur when the tonicity of the administered fluids exceeds the urine's tonicity, but only case reports [7-9] and one cases series [10] have illustrated this mechanism.

In the present study, hypernatremia in critically ill patients was studied in two ways. First, a 1:2 matched case control study was performed to identify risk factors for ICU-acquired hypernatremia. Second, fluid balance studies were carried out in cases to see which fluids were administered and how often hypernatremia developed with a negative or a positive fluid balance. We demonstrate that one third of the cases had a positive fluid balance during the development of hypernatremia. This suggests that hypernatremia due to a positive sodium balance is more common than previously appreciated, potentially affecting the approach to the critically ill patient with hypernatremia.

Materials and methods

Patient population and laboratory measurements

The study protocol was approved by the Institutional Review Board (MEC-2005-190). All serum sodium values (S_{Na}) ordered in 2005 in patients admitted to the Erasmus Medical Center, an 813-bed urban university hospital in Rotterdam, The Netherlands, were reviewed. Hypernatremia was defined as at least one $S_{Na} \geq 150$ mmol/l. All four ICUs (surgical, medical, neurological and cardiac ICUs), with a total of 42 beds, were included in the initial analysis. S_{Na} -values were determined with ion-selective electrodes (Hitachi 917, Roche). Other biochemical disorders were defined as follows: hypokalemia as serum potassium ≤ 3.5 mmol/l, hypercalcemia as ionized calcium ≥ 1.29 mmol/l, renal insufficiency as serum creatinine ≥ 100 μ mol/l for females and ≥ 125 μ mol/l for males, uremia as serum urea ≥ 8 mmol/l, hyperglycemia as serum glucose ≥ 10 mmol/l, and rhabdomyolysis as creatine kinase ≥ 200 U/l.

Data collection

The following data were recorded: reason of admission, Acute Physiology And Chronic Health Evaluation II (APACHE II) score, Glasgow Coma Score (GCS), vital signs, biochemical parameters, medication, and fluid balances. Data were retrieved from the ICU data management system (PICIS Care Suite 7.1, Wakefield, MA) and reviewed manually. A review of charts and discharge letters was also performed.

Matched case control study

To analyze which factors contributed to the development of hypernatremia in ICU patients, a matched case control study was performed. Cases consisted of patients who were admitted to the medical, surgical, or neurological ICUs with $S_{Na} < 145$ mmol/l, and who subsequently developed hypernatremia in the ICU. The cardiac ICU was not included, because hypernatremia was relatively uncommon in this ICU and fluid balances were not regularly recorded. Each case was matched to two control patients, who were admitted to the same ICU and who remained normonatremic (S_{Na} between 136 and 145 mmol/l). Furthermore, ICU exposure was matched, meaning that the ICU admission time of controls needed to be at least as long as the time that hypernatremia developed in cases. If more than two controls were available, they were chosen randomly using a random number generator (www.random.org). Risk factors were recorded in cases in the period between the last normal S_{Na} and the highest S_{Na} , and in controls using an equivalent time period.

Fluid balance studies

Fluid balances were calculated during the development of hypernatremia (in ml/kg/day), using all input (intravenous [IV] fluids, oral intake, nutrition, blood products) and output (24-hour urine, insensible and enteral losses, blood loss, and wound drains) values. For insensible loss, an average of 14 ml/kg/day was used, adding 3.5 ml/kg/day per degree above 37 °C [11]. The amount of sodium (Na^+), potassium (K^+) and electrolyte-free water (EFW), and the tonicity of the fluids administered through the IV and/or oral routes were calculated, as described

previously [12]. Polyuria was defined as a urine output of ≥ 40 ml/kg/day [2].

Statistical analysis

Data were analyzed using SPSS (version 13.0, Chicago, IL). Cases were compared to controls using conditional logistic regression. To perform conditional logistic regression, we used the Cox proportional hazards regression model, because it allows a comparison between cases and controls in the same matched set. Two multivariate analyses (using a backward conditional approach) were performed to identify independent predictors for hypernatremia and for mortality. In the fluid balance studies, cases with a negative fluid balance were compared to cases with a positive fluid balance using non-parametric tests. Data are expressed as mean $\pm$ SD, except not normally distributed variables (reported as median and range and log-transformed before analysis) and fluid balance data (mean $\pm$ SEM). For all analyses, a p-value of ≤ 0.05 was considered significant.

Results

Epidemiology of hypernatremia

Although hyponatremia was more common than hypernatremia, the “ICU incidences” of hypernatremia ≥ 145 mmol/l (744/1272 vs. 1295/4687, $p < 0.001$) and hypernatremia ≥ 150 mmol/l (153/340 vs. 295/1225, $p < 0.001$) were both higher (Table 1). Also of interest, because 1245 ICU patients remained normonatremic, 642 patients (24%) must have had episodes of hyponatremia and hypernatremia.

Table 1: Epidemiology of dysnatremia in ICU and non-ICU patients

Types of dysnatremia	Total (n =12838)	ICU patients (n = 2642)	Non-ICU patients (n = 10196)	ICU incidence*
Hypernatremia, n (%) ($S_{Na} \geq 145$ mmol/l)	1272 (10)	744 (28)	528 (5)	58%
Hypernatremia, n (%) ($S_{Na} \geq 150$ mmol/l)	340 (3)	153 (6)	187 (2)	45%
Hyponatremia, n (%) ($S_{Na} \leq 136$ mmol/l)	4687 (37)	1295 (49)	3392 (33)	28%
Hyponatremia, n (%) ($S_{Na} \leq 130$ mmol/l)	1225 (10)	295 (11)	930 (9)	24%

* Calculated by dividing the number of ICU patients with hypernatremia or hyponatremia by the total number of patients.

Course of intensive care hypernatremia

Of the 153 patients with a $S_{Na} \geq 150$ mmol/l in Table 1, 140 patients were studied, excluding 13 patients from the cardiac ICU. Of these, 130 patients (93%) developed hypernatremia in the ICU, and their S_{Na} increased from 141 ± 3 to 156 ± 6 mmol/l in 48 ± 4 h. Ten patients (7%) presented hypernatremic ($S_{Na} 156 \pm 6$ mmol/l). Hypernatremia was observed for a median of 45 h (range 0.25 – 603 h). Thirty-three patients (24%) died hypernatremic. In the remaining 107 patients, S_{Na} returned to normal in a median of 83 h (range 5 – 639 h). Forty-five patients (32%) had hyponatremia prior to hypernatremia (lowest $S_{Na} 130 \pm 5$ mmol/l), whereas 42 patients (30%) developed

hyponatremia after their hypernatremic episode (lowest S_{Na} 130 ± 4 mmol/l).

Characteristics and outcome of cases and controls

Patients were admitted to the neurological ICU (61 cases, 122 controls), surgical ICU (43 cases, 86 controls), and internal ICU (26 cases, 52 controls). The reasons of admission to the ICU were: trauma (20 cases, 21 controls), cerebral hemorrhage (17 cases, 32 controls), respiratory insufficiency (16 cases, 32 controls), sepsis (12 cases, 6 controls), intoxication (1 case, 9 controls), post-operative care after gastro-intestinal surgery (24 cases, 54 controls), brain tumor surgery (2 cases, 31 controls), and abdominal aneurysm surgery (8 cases, 10 controls). Cases were more often admitted directly to the ICU with higher APACHE II scores and lower GCS (Table 2). Cases were more often admitted to ICU after emergency surgery, and more often ventilated during admission. With regard to outcome, cases had a longer ICU stay and a higher mortality rate.

Table 2: General characteristics and outcomes of cases and controls

Variable	Cases (n = 130)	Controls (n = 260)	P-value
<i>Demographics</i>			
Age, years	57 ± 18	54 ± 18	0.2
Female sex, n (%)	53 (41)	117 (45)	0.4
<i>Admission</i>			
Directly to ICU, n (%)	74 (57)	121 (47)	0.04
APACHE II score	22 (6-44)	16 (2-35)	< 0.001
Glasgow Coma Score	7 (3-15)	10 (3-15)	< 0.001
<i>Vital signs*</i>			
Heart rate, bpm	91 ± 16	82 ± 15	< 0.001
Systolic BP, mmHg	130 ± 22	130 ± 20	0.9
Diastolic BP, mmHg	66 ± 12	67 ± 12	0.5
Temperature, °C	36.8 ± 0.8	36.6 ± 0.6	0.04
<i>Interventions</i>			
Elective surgery, n (%)	24 (19)	86 (33)	0.003
Emergency surgery	36 (28)	19 (7)	< 0.001
Ventilation	120 (92)	151 (58)	< 0.001
<i>Outcomes</i>			
Duration ICU, days	11 (1-152)	3 (1-75)	< 0.001
Mortality, n (%)	62 (48)	27 (10)	< 0.001
Hypernatremia at death	32 (25)	-	-

* Average values during the development of hypernatremia.

Abbreviations: ICU, intensive care unit; APACHE II, Acute Physiology And Chronic Health Evaluation; BP, blood pressure.

Factors contributing to hypernatremia

Table 3 shows that cases more often had hypokalemia, hypercalcemia, renal insufficiency, uremia, hyperglycemia, and more often received mannitol and sodium bicarbonate during the development of hypernatremia. Except for hyperglycemia, these factors remained significantly more common in cases ($p < 0.05$) when the APACHE II score and GCS were included in the comparison. In cases, biochemical disorders were more severe for hypokalemia (lowest serum potassium 3.1 ± 0.4 mmol/l vs. 3.2 ± 0.2 mmol/l, $p = 0.01$), hypercalcemia (highest ionized calcium 1.45 ± 0.25 mmol/l vs. 1.34 ± 0.07 mmol/l, $p < 0.001$), uremia (highest serum urea 18.1 ± 9.9 mmol/l vs. 13.8 ± 6.4 mmol/l, $p = 0.002$), and hyperglycemia (highest serum glucose 14.5 ± 9.1 mmol/l vs. 11.9 ± 1.8 mmol/l, $p = 0.03$). An analysis of other biochemical disorders, showed that hypoalbuminemia was also more common (118/130 vs. 126/260, $p < 0.001$) and more severe (22 ± 6 vs. 26 ± 5 g/l, $p < 0.001$) in cases. Hypomagnesemia, metabolic and respiratory acid-base disturbances were not more common in cases (data not shown). In patients with hypokalemia, hypomagnesemia, acid-base disturbances and/or diuretic use were not more common (data not shown).

Table 3: Factors potentially contributing to hypernatremia

Mechanisms	Causes	Cases (n = 130)	Controls (n = 260)	P-Value
Diabetes insipidus	Diseases associated with CDI*, n (%)	9 (7)	12 (5)	0.3
	Drugs associated with NDI [†]	29 (22)	39 (15)	0.08
	Hypokalemia [‡]	69 (53)	89 (34)	< 0.001
	Hypercalcemia	22 (17)	14 (6)	0.001
Altered kidney function	Loop diuretics	29 (22)	65 (25)	0.4
	Renal insufficiency	69 (53)	35 (14)	< 0.001
Osmotic diuresis	Uremia	55 (42)	57 (22)	< 0.001
	Mannitol	13 (10)	3 (1)	0.001
	Hyperglycemia	56 (48)	81 (36)	0.04
Shift of water	Rhabdomyolysis [¶]	49 (47)	64 (51)	1.0
Sodium gain	Sodium bicarbonate	30 (23)	1 (0.4)	< 0.001
Non-renal water loss	Lactulose	14 (11)	18 (7)	0.2

* Includes: any neurotrauma, pituitary adenoma, and craniopharyngeoma [2].

[†] Includes: amphotericin B, dexamethasone, dopamine, ethanol, rifampin, and/or triamterene-hydrochlorothiazide [31].

[‡] For definition of biochemical disorders, see Methods.

[¶] Creatine kinase was available in 105 cases (81%) and 124 controls (48%).

Multivariate analysis

The first multivariate analysis showed that there was an independent association between hypernatremia and all but one of the significant contributing factors ($p < 0.05$ in Table 3), including hypokalemia (odds ratio 2.3, 95% confidence interval 1.1 to 4.7), hypercalcemia (3.5, 1.1 to 10.5), renal insufficiency (7.8, 3.4 to 17.9), uremia (2.2., 1.1 to 4.5) and the use of mannitol (20.0, 2.7 to 149.1), and sodium bicarbonate (6.4, 1.9 to 20.6). In the second multivariate analysis predictors for mortality were studied, including hypernatremia, the APACHE II score, GCS, and renal insufficiency in the model. Hypernatremia (3.7, 2.3 to 6.0) and renal insufficiency (2.1, 1.1 to 4.1) were found to be independent predictors of mortality in our population.

Fluid balance studies

During the development of hypernatremia in the ICU, 80 patients (62%) had a negative fluid balance (-31 ± 2 ml/kg/day), while 50 patients (38%) had a positive fluid balance (72 ± 3 ml/kg/day) (Figure 1).

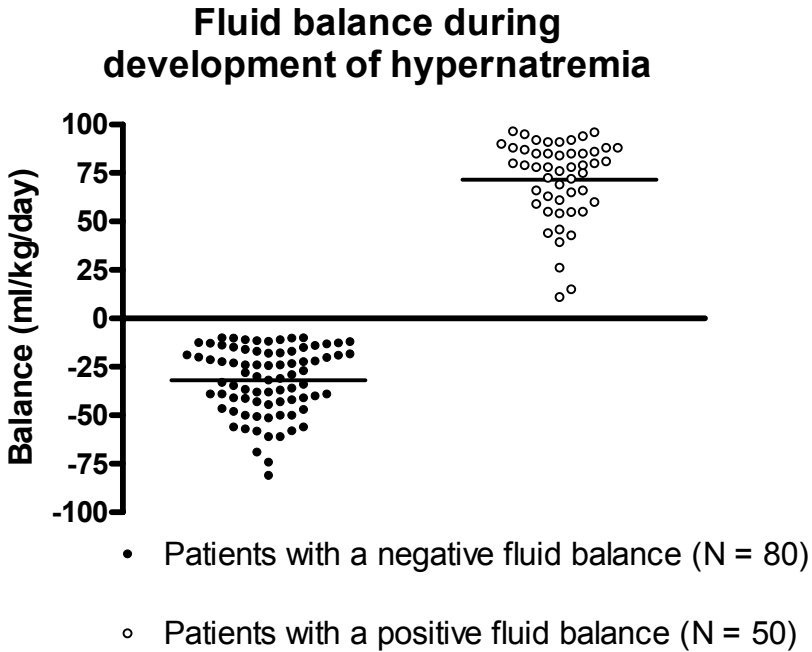


Figure 1: Fluid balance during the development of hypernatremia
Fluid balances were calculated during the development of hypernatremia using all input (intravenous fluids, oral intake, nutrition, blood products) and output (24-hour urine, insensible and enteral losses, blood loss, and wound drains) values.

The tonicity of the administered fluids (amount of $\text{Na}^+ + \text{K}^+$ per volume) was higher in patients with a positive fluid balance (148 ± 2 vs. 133 ± 3 mmol/l, $p < 0.001$), while both subgroups received a similar small amount of EFW (with 52 patients receiving no EFW) (Figure 2). A positive fluid balance developed, because more saline, blood, colloids, and Ringer's lactate were given to these patients (Figure 3A). No cases were treated with hypertonic saline. Excreted fluids were largely

comparable between the two subgroups, except for blood losses, which were higher in patients with a positive fluid balance (24 ± 11 vs. 17 ± 17 ml/kg/day, $p = 0.002$) (Figure 3B). On average, cases were polyuric (40 ± 5 ml/kg/day). Urinary output in cases with hypokalemia (45 ± 4 ml/kg/day), hypercalcemia (55 ± 7 ml/kg/day), mannitol use (57 ± 5 ml/kg/day), and hyperglycemia (40 ± 4 ml/kg/day) was also in the polyuric range. The majority of the risk factors listed in Table 3 were evenly distributed between patients with negative and positive fluid balances, except for renal insufficiency, which was more common in patients with a negative fluid balance (52/80 vs. 17/50, $p = 0.001$).

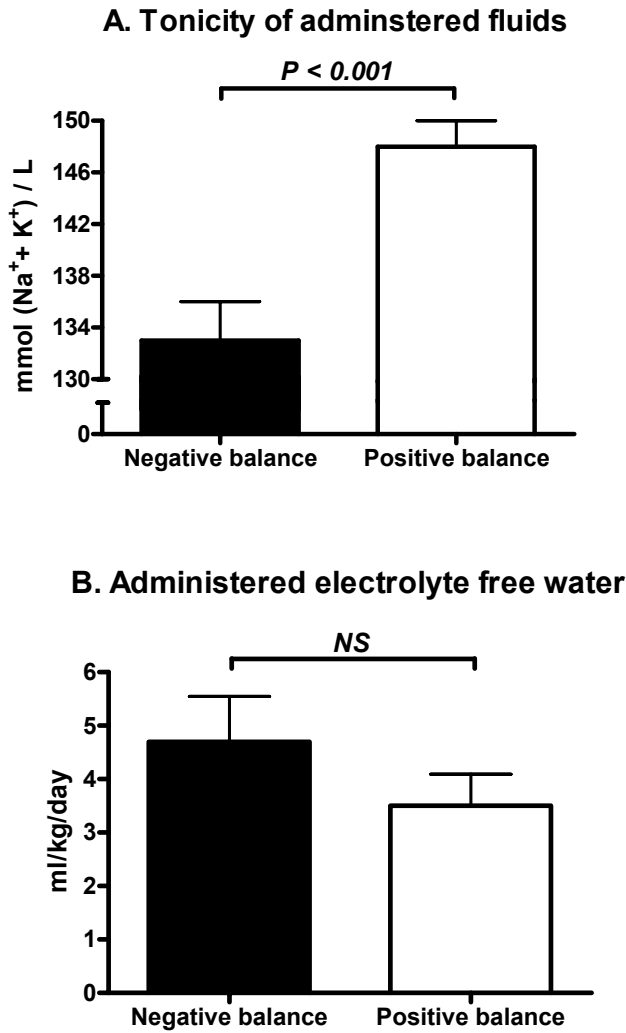


Figure 2: Tonicity and electrolyte free water content of the administered fluids

Data are a composite of intravenous fluids, oral intake, enteral nutrition, blood transfusion and bicarbonate administration. Tonicity was calculated by dividing the $\text{Na}^+ + \text{K}^+$ content by total volume. Electrolyte free water was calculated as described in the Methods. Statistical analyses were performed by non-parametric testing. Data are expressed as mean $\pm$ SEM.

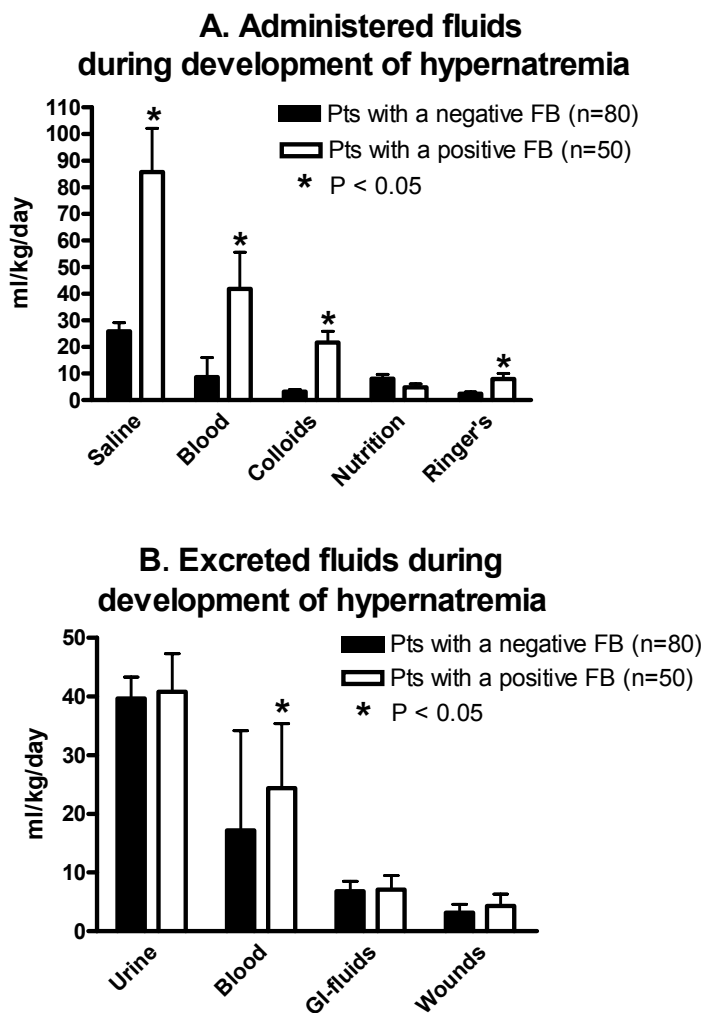


Figure 3: Type and amount of fluids administered and excreted during the development of hypernatremia

Panel A shows the amount of administered saline (isotonic only), blood (from transfusions), colloids, nutrition (mainly enteral), and Ringer's (lactate) in patients with a negative and a positive fluid balance (See Figure 1). Statistical analyses were performed by non-parametric testing. Data are expressed as mean $\pm$ SEM. **Abbreviations:** Pts, patients; FB, fluid balance; GI, gastro-intestinal.

Discussion

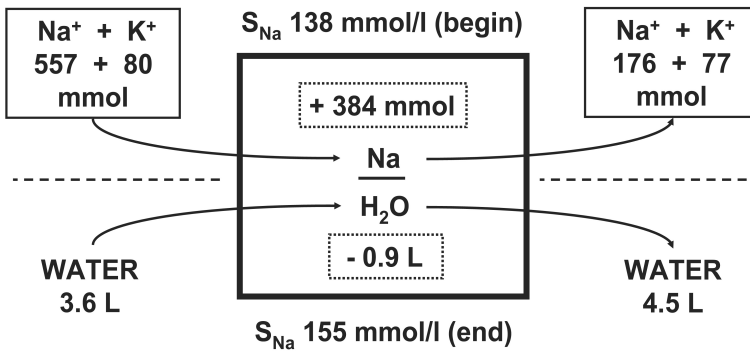
Here, we studied the risk factors and mechanisms of hypernatremia in critically ill patients using case control and fluid balance studies. The principal results demonstrate that hypernatremia is a typical ICU problem (Table 1) with a high mortality rate (Table 2), which developed because there was a reason to lose renal water (Table 3), and was then corrected with IV-fluids containing too little water and/or too much sodium (Figures 1 and 2).

It seems important to emphasize the combination of factors that likely contributed to hypernatremia. First, cases more often had a reason to lose renal water (Table 3), and the responsible mechanisms will be discussed below. This may have caused an initial rise in S_{Na} , which is known to produce a strong thirst stimulus [13]. Second, because cases were more often unconscious and required ventilation (Table 2), their ability to express thirst and access water was limited. Consequently, the defense of their water homeostasis depended completely on the treating clinicians. Therefore, the third and final step in the development of hypernatremia appeared to be inadequate IV-fluid administration, which did not prevent, or even aggravate hypernatremia. Previously, several studies [5-7,14], including ours [12], have shown the relationship between inadequate IV-fluid therapy and the development of dysnatremia, and some authors consider it a negative indicator of quality of care [5].

A novel finding was that hypernatremia with a positive fluid balance was much more common than previously appreciated [2-4]. Hypernatremia with a positive fluid balance can only

develop when sodium balance is more positive than water balance [2]. A positive sodium balance was likely caused by the continuous administration of near-isotonic fluids (Figure 2A), while patients at the same time had reasons to excrete a hypotonic urine, and also lost other hypotonic fluids, such as gastro-intestinal fluids (Figure 3B). A positive sodium balance likely also contributed to hypernatremia in patients with a negative fluid balance, because a negative water balance alone cannot completely account for the rise in S_{Na} . For example, for S_{Na} to rise from 141 mmol/l to 156 mmol/l in a 70-kg patient due to a negative water balance, total body water would need to be reduced from 42 L to $(42 \times 141) / 156 = 38$ L. This equals a deficit of 4 L or 57 ml/kg, which is almost twice as high as the number we found (Figure 1). The development of hypernatremia with a positive and a negative fluid balance is illustrated by the tonicity balances of two representative cases who had complete balance data (Figure 4).

Hyponatremia due to a negative water and positive sodium balance



Hyponatremia due to a more positive sodium than water balance

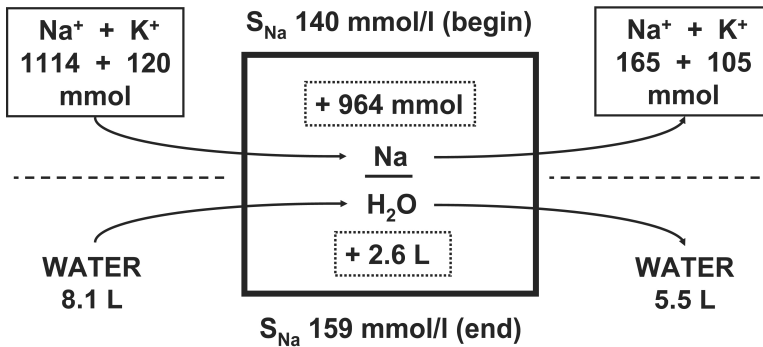


Figure 4: Tonicity balances illustrating two mechanisms of hyponatremia

Two mechanisms of hyponatremia are shown in two representative patients. The first patient developed hyponatremia in four days, and the second patient in one day. The large darker rectangles represent total body water with the serum sodium concentration measured at the beginning and end of the observation shown on top and bottom of this rectangle, respectively. The quantities of sodium (Na^+) plus potassium (K^+) infused and excreted are shown in the two flanking rectangles, and the volumes of water (H_2O) infused and excreted are depicted below.

The primary mechanisms by which cases lost renal water appeared to be nephrogenic diabetes insipidus and osmotic diuresis (Table 3). Hypokalemia can induce nephrogenic diabetes insipidus by a downregulation of aquaporin-2 [15], while hypercalcemia interferes with sodium and water reabsorption through activation of the calcium sensing receptors in the loop of Henle and collecting ducts [16,17]. We speculate that hypokalemia was more common in cases because of high aldosterone and/or catecholamine levels (especially in patients with a negative fluid balance) [18]. Causes for critical care hypercalcemia may have included primary hyperparathyroidism and/or overcorrection of hypocalcemia [19,20]. Uremia, hyperglycemia, and mannitol can all produce an osmotic diuresis, during which more water than sodium is lost [21]. Causes for uremia may have included a catabolic state, gastrointestinal hemorrhage, or high protein tube feeding. A recent study showed that hypernatremia was more common in patients treated with mannitol on the neurological ICU [22]. Patients who had risk factors for diabetes insipidus or an osmotic diuresis, were nearly always polyuric, suggesting that these mechanisms were indeed present. However, confirmative studies would have required urine osmolality or a trial of vasopressin.

Possible explanations for the association between renal insufficiency and hypernatremia include the presence of acute tubular necrosis (which can have a polyuric phase with a water diuresis) [23], and/or an incapacity to conserve water [24,25]. Although the available data showed that rhabdomyolysis was not more common in cases, it may have contributed to hypernatremia in some patients by causing a shift of water to the intracellular fluid compartment [2,26]. Other mechanisms that

could have contributed to hypernatremia include high glucocorticoid and/or catecholamine levels, which can inhibit the release or actions of vasopressin [27-29]. However, vasopressin is usually elevated during critical illness [30]. Other factors that have previously been associated with hypernatremia were not more common or uncommon in our series, including diseases associated with central diabetes insipidus [2], drugs associated with nephrogenic diabetes insipidus [31], burns, citrate dialysis, and the use of charcoal, phenytoin, lactulose, and hypertonic saline [2-4].

Clinically, an important question is how these results could assist in preventing and treating hypernatremia in critically ill patients. To prevent hypernatremia, close monitoring of factors that could result in the excretion of a hypotonic urine may be indicated. If polyuria develops, the amount and tonicity of the IV-fluids should be matched to the urinary output in order to maintain fluid and electrolyte balance. If a water or osmotic diuresis is present, isotonic fluids should be switched to more hypotonic solutions to prevent a positive sodium balance from developing. Although aggressive fluid resuscitation to defend the extracellular fluid volume may have been required initially in patients who developed a positive fluid balance (e.g., because of on-going blood loss), it seems advisable to reduce the infusion rate once hemodynamic stability is achieved.

The mainstay of the treatment of hypernatremia is to increase electrolyte free water administration [32]. However, if hypernatremia is not only a problem of too little water but also of too much salt, the goal of therapy should be to create a negative balance of $\text{Na}^+ + \text{K}^+$, for example with diuretics [33].

Therapy should also focus on a low effective circulating volume, which is often present in patients with hypernatremia and a positive fluid balance [10]. There does not appear to be a broad indication for vasopressin treatment in these patients, because water losses appeared to be mostly of renal and not of central origin (Table 3).

Although not a specific subject of this study, an interesting observation was that *hyponatremia* preceded or followed *hypernatremia* in approximately one third of the patients. This could be related to fluctuations in vasopressin release [34], but overcorrection of dysnatremia likely also played an important role.

Finally, a strenuous argument to prevent hypernatremia is its high mortality rate, which has also been shown by others [6,35,36]. Although it has been suggested that the underlying disease rather than hypernatremia itself is usually responsible for this high mortality rate [6], we found that hypernatremia in critically ill patients was an independent predictor for mortality, together with renal insufficiency. Part of the complexity may be that severe hypernatremia produces few specific neurological symptoms, which are even more difficult to assess in patients who often already have neurological disorders. Nevertheless, hypernatremia or its correction can result in brain damage from osmotic demyelination, cerebral edema and/or intracranial hemorrhage [1-4]. Although hypernatremia may be protective in some settings, for example in children with brain trauma [37], we feel that in this population it was clearly associated with adverse outcome, and should therefore be prevented.

One obvious limitation of our study is that the presence of a positive *sodium* balance was only illustrated indirectly by demonstrating a positive *fluid* balance, because osmolality and electrolytes were not routinely determined in 24-h urine. Although insensible losses were estimated, it seems unlikely that the positive fluid balances were due to higher than calculated insensible losses, because the majority of patients would still have had a positive fluid balance even if insensible losses would have been twice as high.

Conclusion

ICU-acquired hypernatremia is much more common than ICU-admission hypernatremia. The likely mechanisms of hypernatremia in critically ill patients constitute a reason to excrete a hypotonic urine, which is corrected with IV-fluids containing too little water and/or too much sodium. Hypernatremia due to a positive sodium balance appears more common than previously appreciated, implying that in those cases treatment should also rely on creating a negative sodium balance. Adjustments in intravenous fluid regimens may prevent hypernatremia.

Acknowledgments

The authors would like to thank N. Tahitu, T. Bontje and G. Lansbergen for their help with data retrieval, and Dr. M. ten Berg, Dr. P. Stolk, and Dr. W. Scholte op Reimer for statistical advice.

References

1. Moritz ML, Ayus JC. Dysnatremias in the critical care setting. *Contrib Nephrol* 2004; 144: 132-57.
2. Halperin ML, Goldstein MB. *Fluid, Electrolyte, and Acid-Base Physiology*. 3rd. ed. Philadelphia: W.B. Saunders Company; 1999.
3. Adroque HJ, Madias NE. Hypernatremia. *N Engl J Med* 2000; 342: 1493-9.
4. Rose BD, Post TW. *Clinical physiology of acid-base and electrolyte disorders*. 5th ed. New York, NY: McGraw-Hill; 2001.
5. Polderman KH, Schreuder WO, Strack van Schijndel RJ, Thijs LG. Hypernatremia in the intensive care unit: an indicator of quality of care? *Crit Care Med* 1999;27:1105-8.
6. Palevsky PM, Bhagrath R, Greenberg A. Hypernatremia in hospitalized patients. *Ann Intern Med* 1996; 124: 197-203.
7. Liamis G, Kalaitzidis R, Elisaf M. Hypernatremia during correction of hypercalcemia. *Nephron* 2000; 86: 358.
8. Davids MR, Edoute Y, Halperin ML. The approach to a patient with acute polyuria and hypernatremia: a need for the physiology of McCance at the bedside. *Neth J Med* 2001; 58: 103-10.
9. Kaufman AM, Kahn T. Congestive heart failure with hypernatremia. *Arch Intern Med* 1986; 146: 402-3.
10. Kahn T. Hypernatremia with edema. *Arch Intern Med* 1999; 159: 93-8.
11. Cox P. Insensible water loss and its assessment in adult patients: a review. *Acta Anaesthesiol Scand* 1987; 31: 771-6.
12. Hoorn EJ, Geary D, Robb M, Halperin ML, Bohn D. Acute hyponatremia related to intravenous fluid administration in hospitalized children: an observational study. *Pediatrics* 2004; 113: 1279-84.
13. Baylis PH, Thompson CJ. Osmoregulation of vasopressin secretion and thirst in health and disease. *Clin Endocrinol (Oxf)* 1988; 29: 549-76.
14. Millionis HJ, Liamis G, Elisaf MS. Hypernatremia in hospitalized patients: a sequel of inadvertent fluid administration. *Arch Intern Med* 2000; 160: 1541-2.

15. Marples D, Frokiaer J, Dorup J, Knepper MA, Nielsen S. Hypokalemia-induced downregulation of aquaporin-2 water channel expression in rat kidney medulla and cortex. *J Clin Invest* 1996; 97: 1960-8.
16. Sands JM, Naruse M, Baum M, Jo I, Hebert SC, Brown EM, et al. Apical extracellular calcium/polyvalent cation-sensing receptor regulates vasopressin-elicited water permeability in rat kidney inner medullary collecting duct. *J Clin Invest* 1997; 99: 1399-405.
17. Galla JH, Booker BB, Luke RG. Role of the loop segment in the urinary concentrating defect of hypercalcemia. *Kidney Int* 1986; 29: 977-82.
18. Weisberg LS, Szerlip HM, Cox M. Disorders of potassium homeostasis in critically ill patients. *Crit Care Clin* 1987; 3: 835-54.
19. Ariyan CE, Sosa JA. Assessment and management of patients with abnormal calcium. *Crit Care Med* 2004; 32: S146-54.
20. Lind L, Ljunghall S. Critical care hypercalcemia--a hyperparathyroid state. *Exp Clin Endocrinol* 1992; 100: 148-51.
21. Gennari FJ, Kassirer JP. Osmotic diuresis. *N Engl J Med* 1974; 291: 714-20.
22. Aiyagari V, Deibert E, Diringer MN. Hypernatremia in the neurologic intensive care unit: how high is too high? *J Crit Care* 2006; 21: 163-72.
23. Miller PD, Krebs RA, Neal BJ, McIntyre DO. Polyuric prerenal failure. *Arch Intern Med* 1980; 140: 907-9.
24. Teitelbaum I, McGuinness S. Vasopressin resistance in chronic renal failure. Evidence for the role of decreased V2 receptor mRNA. *J Clin Invest* 1995; 96: 378-85.
25. Bedford JJ, Leader JP, Walker RJ. Aquaporin expression in normal human kidney and in renal disease. *J Am Soc Nephrol* 2003; 14: 2581-7.
26. Abramovici MI, Singhal PC, Trachtman H. Hypernatremia and rhabdomyolysis. *J Med* 1992; 23: 17-28.
27. Rouch AJ, Kudo LH. Alpha 2-adrenergic-mediated inhibition of water and urea permeability in the rat IMCD. *Am J Physiol* 1996; 271: F150-7.

28. Sonnenblick M, Algur N. Hypernatremia in the acutely ill elderly patients: role of impaired arginine-vasopressin secretion. *Miner Electrolyte Metab* 1993; 19: 32-5.
29. Papanek PE, Sladek CD, Raff H. Corticosterone inhibition of osmotically stimulated vasopressin from hypothalamic-neurohypophyseal explants. *Am J Physiol* 1997; 272: R158-62.
30. Jochberger S, Mayr VD, Luckner G, Wenzel V, Ulmer H, Schmid S, et al. Serum vasopressin concentrations in critically ill patients. *Crit Care Med* 2006; 34: 293-9.
31. Garofeanu CG, Weir M, Rosas-Arellano MP, Henson G, Garg AX, Clark WF. Causes of reversible nephrogenic diabetes insipidus: a systematic review. *Am J Kidney Dis* 2005; 45: 626-37.
32. Sterns RH. Hypernatremia in the intensive care unit: instant quality--just add water. *Crit Care Med* 1999; 27: 1041-2.
33. Carlotti AP, Bohn D, Mallie JP, Halperin ML. Tonicity balance, and not electrolyte-free water calculations, more accurately guides therapy for acute changes in natremia. *Intensive Care Med* 2001; 27: 921-4.
34. Lindsay RS, Seckl JR, Padfield PL. The triple-phase response--problems of water balance after pituitary surgery. *Postgrad Med J* 1995; 71: 439-41.
35. Qureshi AI, Suri MF, Sung GY, Straw RN, Yahia AM, Saad M, et al. Prognostic significance of hypernatremia and hyponatremia among patients with aneurysmal subarachnoid hemorrhage. *Neurosurgery* 2002; 50: 749-55.
36. Daggett P, Deanfield J, Moss F, Reynolds D. Severe hypernatraemia in adults. *BMJ* 1979; 1: 1177-80.
37. Peterson B, Khanna S, Fisher B, Marshall L. Prolonged hypernatremia controls elevated intracranial pressure in head-injured pediatric patients. *Crit Care Med* 2000; 28: 1136-43.

Chapter 12

**Preventing a fall in the effective
osmolality to minimize the likelihood of
cerebral edema during therapy of
children with diabetic ketoacidosis**

Ewout J. Hoorn, Ana P.C.P. Carlotti

Leila A.A. Costa, Beth MacMahon

Gareth Bohn, Robert Zietse

Mitchell L. Halperin and Desmond Bohn

J Pediatr, in press

Abstract

Objective: To test if a fall in effective plasma osmolality ($P_{\text{Eff osm}}$; $2 \times \text{plasma sodium } [P_{\text{Na}}] + \text{plasma glucose concentrations}$) during therapy for diabetic ketoacidosis (DKA) is associated with an increased risk of cerebral edema (CE), and if the development of hypernatremia to prevent a fall in the $P_{\text{Eff osm}}$ is dangerous.

Study design: Retrospective comparison of a CE-group ($n = 12$) to non-CE groups with hypernatremia ($n = 44$) and without hypernatremia ($n = 13$).

Results: The development of CE (at $6.8 \pm 1.5\text{h}$) was associated with a fall in $P_{\text{Eff osm}}$ from 304 ± 5 to 290 ± 5 mOsm/kg ($p < 0.001$). Control patients did not show this fall in $P_{\text{Eff osm}}$ at 4h (1 ± 2 and 2 ± 2 vs. -9 ± 2 mOsm/kg, $p < 0.01$), because of a larger rise in P_{Na} and/or a smaller fall in plasma glucose. During this period, the CE-group received more near-isotonic fluids (69 ± 9 vs. 35 ± 2 and 27 ± 3 ml/kg, $p < 0.001$). The CE-group had a higher mortality ($3/12$ vs. $0/57$, $p = 0.003$), and more neurological sequelae ($5/12$ vs. $1/57$, $p < 0.001$).

Conclusions: CE during therapy for DKA was associated with a fall in $P_{\text{Eff osm}}$. An adequate rise in P_{Na} may be needed to prevent this fall in $P_{\text{Eff osm}}$.

Introduction

Cerebral edema (CE) is the most common cause of morbidity and mortality in diabetic ketoacidosis (DKA) in pediatric patients [1-3]. However, there is a lack of consensus on how to prevent its development [3-6]. Because CE usually occurs during the first 5-15 hours of treatment of DKA [7], it is possible that therapy contributes to its development [8]. Several associations with CE have been identified, including younger age [9], newly diagnosed diabetes [3,9], higher initial plasma urea concentration [1,7], higher initial plasma glucose (P_{Glucose}) [1,9], severity of the acidosis [1,7,10,11], and therapy with sodium bicarbonate [7].

In addition to the above, fluid and electrolyte disturbances and their management may add to the risk of developing CE during therapy of DKA [1,7,10-15]. One important observation in this regard was that a smaller increase in the plasma sodium concentration (P_{Na}) during therapy was associated with CE [7,10,15]. Because the P_{Na} is the most important determinant of the effective plasma osmolality ($P_{\text{Eff osm}}$), our objective was to test the hypothesis that a fall in $P_{\text{Eff osm}}$ (defined as $2 \times P_{\text{Na}} + P_{\text{Glucose}}$) during therapy for DKA is associated with the development of CE. Furthermore, because a rise in the P_{Na} is necessary to maintain $P_{\text{Eff osm}}$ when the P_{Glucose} falls, we also wanted to evaluate if hypernatremia in this context had untoward effects.

Patients and methods

Approval was obtained from the Institutional Research Ethics Boards to conduct a retrospective review of patients who were admitted with DKA to the Hospital for Sick Children (HSC) in Toronto, Canada, or to the University Hospital of São Paulo (USP), Ribeirão Preto, Brazil.

Definitions

To be included in the DKA population, patients required a $P_{\text{Glucose}} > 11$ mmol/l (200 mg/dl) *and* a venous pH < 7.30 , or a plasma bicarbonate concentration < 15 mmol/l [16,17]. CE was defined as a clinically assessed alteration in mental status (obtundation or disorientation) *in combination with* radiographically or pathologically confirmed CE, or specific treatment for CE (hyperosmolar therapy and/or controlled ventilation) that was followed by prompt clinical improvement [7]. $P_{\text{Eff osm}}$ was calculated as $2 \times P_{\text{Na}} + P_{\text{Glucose}}$ in mmol/l terms [18].

Study groups

Three groups of DKA patients were included in this study. The “CE group” consisted of all patients who developed CE during therapy for DKA, and who were admitted to the pediatric intensive care units (ICU) of HSC (between 1994 and 1999) or USP (between 1996 and 2005). These data were collected from two institutes by the same physician (APCP) to get a sufficiently large group. The characteristics of HSC and USP patients were similar (data not shown). The CE group was compared to two control groups, who did not develop CE during DKA and who were admitted to HSC in the same years as the cases. The first

group (“controls with hypernatremia”) consisted of ICU and non-ICU patients who had at least one $P_{Na} \geq 150$ mmol/l (hypernatremia) during the first 8 hours of hospitalization. The second group (“controls without hypernatremia”) consisted of ICU patients who did not have hypernatremia during DKA treatment.

Data collection

A retrospective chart review was performed using a computer database (for intensive care patients), and/or a ‘diabetes mellitus flow sheet’ (for other departments). The first 24 hours of treatment of DKA were reviewed, starting when therapy began. Data of patients who were originally admitted to other hospitals prior to transfer were also included in our analysis. Data collection included: biochemical and hemodynamic parameters, medication, all input values (IV-fluids and/or oral fluids) – including those received at the referral hospitals – available output values, and outcome. With regard to biochemical parameters, we focused on the $P_{Eff\ osm}$, P_{Na} , and $P_{Glucose}$, which were recorded at 4-h intervals; other biochemical parameters and hemodynamic parameters are reported at the time of admission only. The amount of sodium (Na^+) and potassium (K^+), volume and tonicity of the fluids (i.e. the amount of $Na^+ + K^+$ per liter) administered through the IV and/or oral routes were evaluated. All urinary values were analyzed and fluid balances calculated when data were available. Outcome measures included neurological sequelae and mortality during or shortly after treatment of DKA.

Statistical analysis

Group comparisons of normally distributed variables were performed by one-way analysis of variance (ANOVA) with Least Significant Difference post-hoc tests (reporting the p-values of the latter). Categorical data were analyzed by Fisher's Exact test (analyzing the control groups separately and combined). In order to compare changes in biochemical parameters over time, a repeated measures general linear model was used. A p-value of ≤ 0.05 was considered significant. Data are expressed as mean $\pm$ SEM.

Results

Patients

The CE group consisted of 12 patients with DKA (7 from HSC and 5 from USP), who developed CE 6.8 ± 1.5 hours after therapy for DKA began (range 0.5 – 20 h). Forty-four control patients with hypernatremia (peak P_{Na} 161 ± 1 mmol/l) and 13 control patients without hypernatremia were identified (all directly admitted to HSC). Six patients were excluded because of a central nervous system infection and/or substance abuse, while an additional 11 patients were excluded, because they were admitted for less than 24h and/or had incomplete data. CE was diagnosed clinically in all patients of the CE group, and confirmed by computed tomography (CT) scan in 9 patients, and by autopsy in 1 patient. In contrast, there was no clinical suspicion of CE in control patients; in three patients, CT-scans were performed and confirmed the absence of CE. Treatment for CE consisted of: mannitol and ventilation (6 patients), mannitol, hypertonic saline and ventilation (2 patients), hypertonic saline

and ventilation (1 patient), mannitol only (2 patients), and ventilation only (1 patient).

Table 1: Biochemistry and hemodynamics at time of admission

	CE group (n = 12)	Controls with hyponatremia (n = 44)	Controls without hyponatremia (n = 13)
<i>Plasma biochemistry</i>			
Osmolality, mOsm/kg*	304 ± 5	342 ± 4 ^A	301 ± 2
Sodium, mmol/l	133 ± 2	149 ± 1 ^A	136 ± 1
Glucose, mmol/l	38 ± 4	40 ± 3	20 ± 2 ^B
pH	7.09 ± 0.05	7.11 ± 0.02	7.09 ± 0.04
Bicarbonate, mmol/l	8.0 ± 1.6	7.7 ± 0.9	6.6 ± 0.8
Creatinine, µmol/l	109 ± 11	100 ± 10	59 ± 8
Urea, mmol/l	11.6 ± 1.2	10.0 ± 0.7	4.8 ± 0.7 ^A
Hematocrit, %	39 ± 4	31 ± 3	46 ± 2 ^B
<i>Hemodynamics</i>			
Heart rate, bpm	115 ± 7	137 ± 4	130 ± 5
SBP, mm Hg	107 ± 4	105 ± 2	120 ± 5 ^B
DBP, mm Hg	67 ± 3	63 ± 2	76 ± 5 ^B

* Calculated as $2 \cdot P_{Na} + P_{Glucose}$

^A Values higher (effective osmolality and sodium) or lower (urea) compared to the two other groups ($p < 0.05$ by ANOVA).

^B Values higher (hematocrit, systolic and diastolic blood pressure) or lower (glucose) compared to the control patients with hyponatremia ($p < 0.05$ by ANOVA).

SBP, systolic blood pressure; DBP, diastolic blood pressure.

Biochemistry data and hemodynamics

Biochemical and hemodynamic parameters in the two groups at the time of admission are shown in Table 1. $P_{\text{Eff osm}}$ and P_{Na} were significantly higher in control patients with hypernatremia. Other differences included a lower P_{Glucose} and plasma urea, and a higher hematocrit, systolic and diastolic blood pressure in the control patients without hypernatremia (Table 1).

Treatment and outcomes

The CE group received a bolus of insulin significantly more often than control patients (11/12 vs. 9/57, $p < 0.001$), while treatment with fluid boluses or sodium bicarbonate was comparable (Table 2). Outcome was poorest in the CE group, as evidenced by a higher number of deaths (3/12 vs. 0/57, $p = 0.003$), and more CE patients developed neurological sequelae (5/12 vs. 1/57, $p < 0.001$). Neurological sequelae consisted of hemiparesis (2 patients), other motor disturbances (3 patients), visual disturbances (3 patients), speech disturbances (3 patients), and cognitive problems (2 patients). One control patient developed severe hypernatremia (P_{Na} rose from 149 mmol/l to 189 mmol/l) with neurological sequelae (speech disturbances and cognitive problems).

Table 2: Treatment and outcome characteristics

	CE group (n = 12)	Controls with hyponatremia (n = 44)	Controls without hyponatremia (n = 13)
Demographics			
Age, years	6.2 ± 1.2	7.7 ± 0.9	10.8 ± 1.2
Weight, kg	21.2 ± 4.1 ^B	32.1 ± 3.3	42.5 ± 6.3
Female sex, n (%)	8 (67)	29 (66)	7 (54)
New onset diabetes	6 (50)	31 (71)	9 (69)
Treatment			
Fluid bolus *, n (%)	6 (50)	17 (39)	2 (15)
Insulin bolus †	11 (92) ^A	8 (18)	1 (8)
Sodium bicarbonate	8 (67)	15 (34)	4 (31)
Outcome			
Brain damage, n (%)	5 (42) ^A	1 (2)	0 (0)
Mortality	3 (25) ^A	0 (0)	0 (0)

* Defined as ≥ 10 ml/kg in 30-60 minutes

† Defined as ≥ 0.1 U/kg in single application

^A Values higher compared to both control groups ($p < 0.05$ by Fisher's exact for control groups separately, and combined).

^B Values lower compared to control patients without hyponatremia ($p < 0.05$ by ANOVA).

Course of effective plasma osmolality

Figure 1A shows the time courses for changes in the $P_{\text{Eff osm}}$, P_{Na} , and P_{Glucose} in the three groups during the first 24 hours in hospital. In the CE group, there was a significant decrease in $P_{\text{Eff osm}}$ in each patient as well as in group average (304 ± 5 mOsm/kg on admission vs. 290 ± 5 mOsm/kg at the time of CE, $p < 0.001$). The $P_{\text{Eff osm}}$ fell during the first 8 hours of therapy (after which hyperosmolar therapy was given to treat CE), whereas it remained constant or decreased minimally in the control groups. This difference was statistically significant ($p = 0.002$), as analyzed by a general linear model. The fall in $P_{\text{Eff osm}}$ in the CE group was most prominent in the first four hours after admission (-9 ± 2 mOsm/kg), and was significantly greater than in controls with hypernatremia (1 ± 2 mOsm/kg, $p = 0.003$) and without hypernatremia (2 ± 2 mOsm/kg, $p = 0.001$) (Figure 1B). In hypernatremic control patients, the small change in $P_{\text{Eff osm}}$ was the result of a larger rise in P_{Na} (7 ± 1 vs. 3 ± 1 mmol/l, $p = 0.03$), whereas in random control patients it was mainly the effect of a smaller fall in P_{Glucose} (-4 ± 2 vs. -14 ± 2 , $p = 0.01$) (Figure 1B).

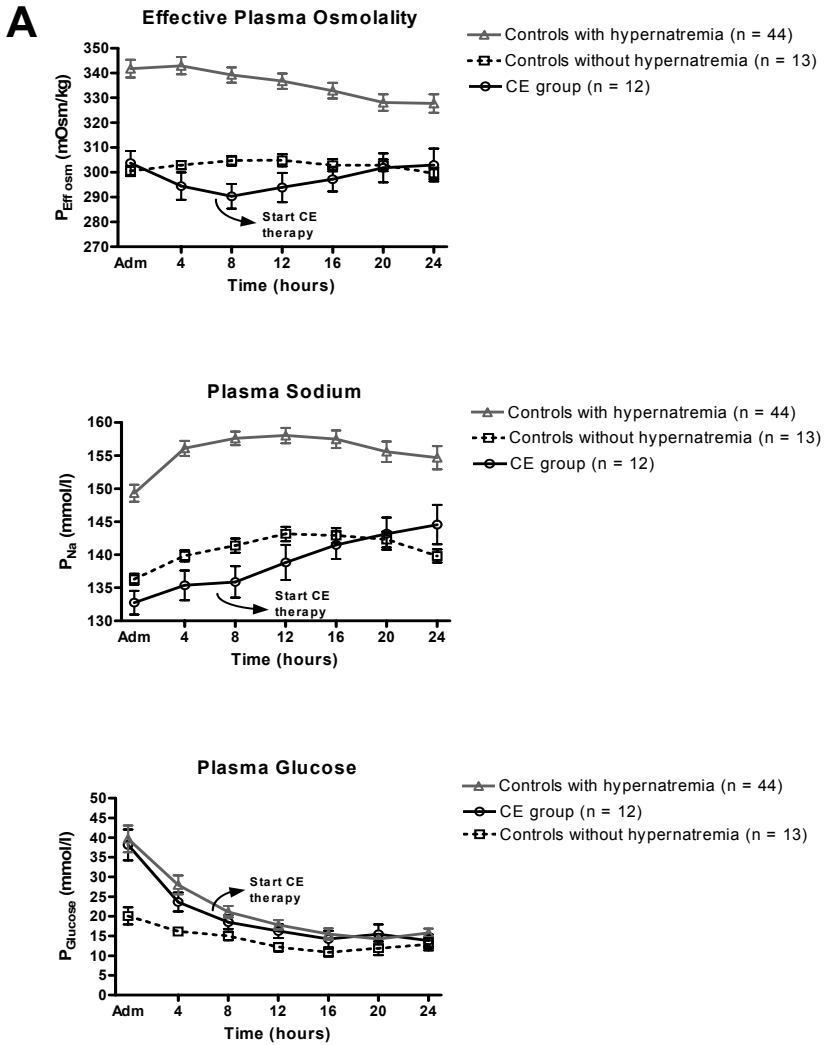
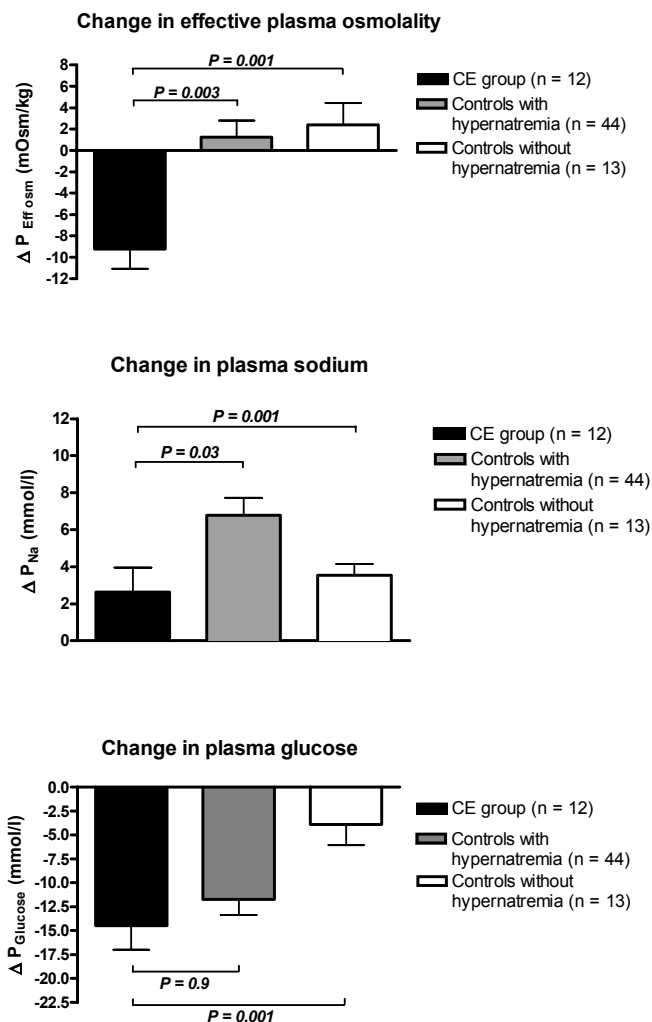


Figure 1: Time courses for effective plasma osmolality, plasma sodium, and plasma glucose concentrations

Panel A shows the courses in effective plasma osmolality, plasma sodium, and plasma glucose concentrations. Data are shown from the start of therapy until 24 hours of therapy, and divided in 4-hour intervals. Data were available for all patients at each time-point. The average time at which therapy for CE was started is indicated.

B**Figure 1 (continued)**

Panel B shows the changes in the same parameters during the first 4 hours of therapy. Group comparison were performed using ANOVA and post-hoc tests. P-values from the post-hoc tests are shown. To convert glucose concentrations from mmol/l to mg/dl, multiply by 18.

Abbreviations: CE, cerebral edema; Adm, admission.

Input and output values

The time interval from 4 to 8 hours was the time in which $P_{\text{Eff osm}}$ reached its nadir and this is when CE developed in most of these patients. Therefore, we were interested in evaluating input and output values from admission until a time-point within this interval (the 6-hour time-point was selected). At 6 hours of therapy, the CE group received more fluid (69 ± 9 vs. 35 ± 2 and 27 ± 3 ml/kg) and more $\text{Na}^+ + \text{K}^+$ (10 ± 2 vs. 6 ± 0.5 and 4 ± 0.5 mmol/kg) compared to the control groups with and without hyponatremia (Figure 2, $p < 0.05$ for all). The tonicity of these fluids was lower for the CE group compared to the controls with hyponatremia (142 ± 7 vs. 163 ± 4 , $p = 0.05$). A similar analysis was performed for urinary output and balance data, which were available in 6 of the 12 CE-patients, 39 of the 44 controls with hyponatremia and all of the controls without hyponatremia. The CE group had a more positive balance (52 ± 19 ml/kg vs. 8 ± 2 and 3 ± 4 ml/kg, $p < 0.001$ for both) and a higher urine output (64 ± 27 ml/kg vs. 23 ± 2 and 24 ± 5 ml/kg, $p < 0.001$ for both) compared to the control groups. The administration of hypertonic saline to treat CE was not included in these calculations because it was always administered after the 6-hour time-point.

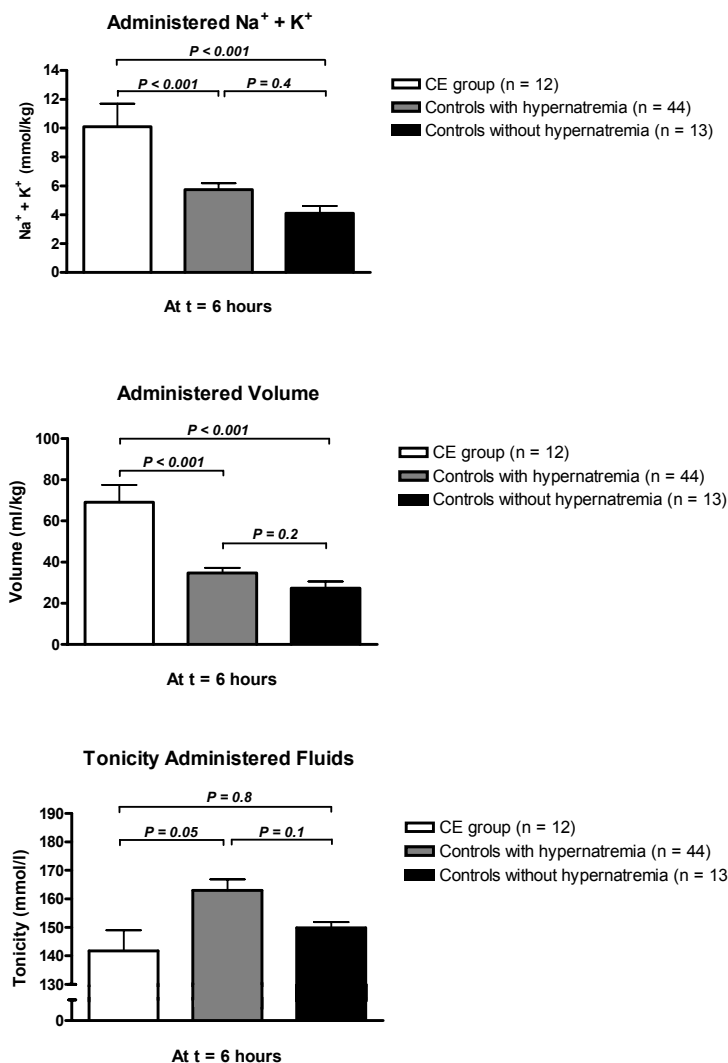


Figure 2: Sodium and potassium content, volume, and tonicity of the administered fluids

Data are a composite of intravenous fluids, oral intake and bicarbonate administration. Tonicity was calculated by dividing the $\text{Na}^+ + \text{K}^+$ content by total volume. Statistical analyses were performed by ANOVA and post-hoc tests. P-values from the post-hoc tests are shown. Data represent situation prior to treatment for CE.

Discussion

The objective of the present study was to investigate if a fall in $P_{\text{Eff osm}}$ was associated with CE during treatment of DKA, and if the development of hypernatremia to maintain $P_{\text{Eff osm}}$ may be dangerous. For this purpose, 12 DKA patients who developed CE were compared to control groups with and without hypernatremia. The principal finding was that patients who developed CE had a larger fall in $P_{\text{Eff osm}}$ early during therapy, which was not present in controls because they either had a larger rise in P_{Na} or a smaller fall in P_{Glucose} (Figure 1). The changes in $P_{\text{Eff osm}}$ became most prominent during the first eight hours after therapy for DKA had begun, which appeared to be a time window with a large risk for the development of CE.

When fluid and electrolyte management was analyzed, we found that the CE group received a larger volume of near-isotonic fluids during this period (Figure 2). They also had a higher urine output and a more positive fluid balance than control patients. Thus, this study adds to the evidence that, at least in some settings, fluid and electrolyte management during DKA might be causally linked to the development of CE [1,7,10-15]. In addition, our data confirm results from two previous studies, one by Bello *et al.* [9] who found that CE was related to a marked reduction in measured plasma osmolality, and the second by Durr *et al.* [10] who found that CE correlated with a small rise in P_{Na} and a large fall in $P_{\text{Eff osm}}$. However, because not only osmotic factors, but also vasogenic [19] and cytotoxic factors [20] have been implicated in brain edema formation, one should be hesitant to conclude that the large fall in $P_{\text{Eff osm}}$ alone caused CE. Finally, because the development of hypernatremia during

DKA in patients who had near-normal P_{Na} values on admission prevented a large fall in $P_{Eff\ osm}$ and was associated with better outcome, it might be a goal of therapy for this subset of patients.

The next important issue is to define how fluid and electrolyte management may have caused the large fall in $P_{Eff\ osm}$ in the CE group. Because there were so few measured data in the urine, we could not evaluate specific mechanisms, but three possibilities will be considered based on the available evidence.

First, the fall in $P_{Eff\ osm}$ may have been caused by infusing more IV-fluids with a lower $Na^+ + K^+$ concentration [21]. Since the tonicity of the infusate was somewhat hypotonic to the patients, the infusion of larger volumes may have resulted in a positive electrolyte-free water balance, which prevented an adequate rise in P_{Na} despite decreasing $P_{Glucose}$ levels.

Second, if the infused volume was sufficiently high to cause the extracellular fluid volume to become expanded, this may have triggered a “desalination” process [22]. To have a fall (or a smaller rise) in the P_{Na} in these patients, one must find a negative balance for $Na^+ + K^+$ and/or a positive balance for water. What is known about the composition of the urine in patients with DKA is that the concentration of Na^+ in the urine is ~40-50 mmol/l during the osmotic diuresis phase [23]. Nevertheless, once the $P_{Glucose}$ falls appreciably, glucosuria will diminish and the $Na^+ + K^+$ concentration in the urine can rise appreciably. It is at this latter time that desalination of infused saline could contribute to the smaller rise in the P_{Na} . Of relevance, patients who developed CE in our study received almost 2-fold more intravenous saline.

Third, although not obvious on clinical grounds, a factor that merits attention is the role of hypotonic fluid retention in the upper gastrointestinal tract. When fruit juice and/or sweetened pop are ingested, there may be a large volume of water with little Na^+ in the stomach and its rate of emptying could be rapid early during therapy as described in a previous case [24]. If the CE group drank predominantly water prior to coming to hospital and their stomach emptying was more rapid, this may have caused a smaller rise in the P_{Na} .

The fourth factor that was identified in this study as a potential contributor to CE was the higher incidence of an insulin bolus administration (Table 2). When a bolus of insulin is given, the sodium:hydrogen ion (H^+) exchanger becomes active [25]. Consequently, there will be a gain of Na^+ and a loss of H^+ in the intracellular fluid compartment. This results in an increased number of intracellular solutes, because exported H^+ would be largely bound to intracellular buffers. This rise in the number of solute molecules inside cells could expand the intracellular volume because water moves rapidly across cell membranes to achieve osmotic equilibrium. This could explain how insulin bolus administration could contribute to CE, especially when insulin was given at the onset of treatment, when the blood brain barrier might be more permeable [26-28].

Clinically, an important question is how these ideas could assist in optimizing fluid and electrolyte management in pediatric patients who present with DKA. The two major goals for IV-fluid therapy should be to deal with hemodynamic emergencies due to a low extracellular fluid volume (at the outset recognizing that true emergencies are not common), and, second, to avoid a

rapid fall in $P_{\text{Eff osm}}$ to minimize the risk of developing CE [16,17,29].

With regard to the first goal, based on the available biochemical and hemodynamic data on admission (Table 1), it is questionable that there was a hemodynamic indication to infuse such a large amount of $\text{Na}^+ + \text{K}^+$ (Figure 2). For example, the amount of infused $\text{Na}^+ + \text{K}^+$ in the CE group exceeded 9 mmol/kg, which is roughly equal to 30% of the extracellular fluid volume [28]. A more moderate fluid regimen may therefore be advisable if there is no compelling evidence to be aggressive [15,30-33]. We previously showed that the degree of extracellular fluid contraction during hyperglycemia is best assessed by serial plasma hematocrit or total protein levels [34], and these should therefore be determined more frequently during fluid resuscitation in DKA.

With regard to the second goal, the $P_{\text{Eff osm}}$ was not well maintained in the patients that developed CE. Regular assessments of the $P_{\text{Eff osm}}$ and paying more attention to the urine volume and its electrolyte concentrations in these potentially vulnerable groups might help to define whether the $P_{\text{Eff osm}}$ is likely to fall. In addition, a low P_{Na} on admission may serve as a warning sign. If $P_{\text{Eff osm}}$ falls, infusing an appropriate volume of hypertonic saline would be a therapeutic option to consider [35]. Because the $P_{\text{Eff osm}}$ was remarkably well maintained in control patients, the development of hypernatremia might be needed in those patients with a high P_{Glucose} and near-normal P_{Na} to prevent an excessive fall in the $P_{\text{Eff osm}}$ early in therapy. This is further supported by the good clinical outcome and few adverse events except in one unusual case, for which a lesser rise in P_{Na} and a

small fall in the $P_{\text{Eff osm}}$ may have been needed. We speculate that the majority of control patients developed hypernatremia because they either had a larger osmotic diuresis (with the consequent loss of more electrolyte-free water) and/or received isotonic fluids while excreting a hypotonic urine [18]. Another way to maintain $P_{\text{Eff osm}}$ is to establish a smaller fall in P_{Glucose} , as occurred in the controls without hypernatremia (Figure 1B), although it must be noted that their degree of hyperglycemia on admission was less severe (Table 1).

Because of the descriptive nature of this study, a number of limitations should be mentioned. First, this study was designed to test a single hypothesis in relation to CE, and therefore other factors were not weighed in a multivariate approach. Second, the way cases and controls were selected does not allow a representative estimate of the incidence of CE and hypernatremia during DKA.

In conclusion, this is the first study to suggest that the production of a modest degree of hypernatremia might be needed to prevent a fall in the $P_{\text{Eff osm}}$ and the development of CE in children with DKA. This may explain why a smaller rise in P_{Na} might be associated with CE [7,10,15]. If a fall in $P_{\text{Eff osm}}$ was prevented during therapy of DKA, and this required that hypernatremia be present, perhaps achieving this abnormal electrolyte concentration should be a goal of therapy when the P_{Na} is not appreciably low in a patient with a serious degree of hyperglycemia.

References

1. Lawrence SE, Cummings EA, Gaboury I, Daneman D. Population-based study of incidence and risk factors for cerebral edema in pediatric diabetic ketoacidosis. *J Pediatr* 2005; 146: 688-92.
2. Edge JA, Ford-Adams ME, Dunger DB. Causes of death in children with insulin dependent diabetes 1990-96. *Arch Dis Child* 1999; 81: 318-23.
3. Edge JA, Hawkins MM, Winter DL, Dunger DB. The risk and outcome of cerebral oedema developing during diabetic ketoacidosis. *Arch Dis Child* 2001; 85: 16-22.
4. Dunger DB, Edge JA. Predicting cerebral edema during diabetic ketoacidosis. *N Engl J Med* 2001; 344: 302-3.
5. Edge JA. Cerebral oedema during treatment of diabetic ketoacidosis: are we any nearer finding a cause? *Diabetes Metab Res Rev* 2000; 16: 316-24.
6. Levitsky LL. Symptomatic cerebral edema in diabetic ketoacidosis: the mechanism is clarified but still far from clear. *J Pediatr* 2004; 145: 149-50.
7. Glaser N, Barnett P, McCaslin I, Nelson D, Trainor J, Louie J, et al. Risk factors for cerebral edema in children with diabetic ketoacidosis. The Pediatric Emergency Medicine Collaborative Research Committee of the American Academy of Pediatrics. *N Engl J Med* 2001; 344: 264-9.
8. Brown TB. Cerebral oedema in childhood diabetic ketoacidosis: is treatment a factor? *Emerg Med J* 2004; 21: 141-4.
9. Bello FA, Sotos JF. Cerebral oedema in diabetic ketoacidosis in children. *Lancet* 1990; 336: 64.
10. Durr JA, Hoffman WH, Sklar AH, el Gammal T, Steinhart CM. Correlates of brain edema in uncontrolled IDDM. *Diabetes* 1992; 41: 627-32.
11. Mahoney CP, Vlcek BW, DelAguila M. Risk factors for developing brain herniation during diabetic ketoacidosis. *Pediatr Neurol* 1999; 21: 721-7.
12. Duck SC, Weldon VV, Pagliara AS, Haymond MW. Cerebral edema complicating therapy for diabetic ketoacidosis. *Diabetes* 1976; 25: 111-5.
13. Duck SC, Wyatt DT. Factors associated with brain herniation in the treatment of diabetic ketoacidosis. *J Pediatr* 1988; 113: 10-4.

14. Hale PM, Rezvani I, Braunstein AW, Lipman TH, Martinez N, Garibaldi L. Factors predicting cerebral edema in young children with diabetic ketoacidosis and new onset type I diabetes. *Acta Paediatr* 1997; 86: 626-31.
15. Harris GD, Fiordalisi I, Harris WL, Mosovich LL, Finberg L. Minimizing the risk of brain herniation during treatment of diabetic ketoacidemia: a retrospective and prospective study. *J Pediatr* 1990; 117: 22-31.
16. Dunger DB, Sperling MA, Acerini CL, Bohn DJ, Daneman D, Danne TP, et al. ESPE/LWPES consensus statement on diabetic ketoacidosis in children and adolescents. *Arch Dis Child* 2004; 89: 188-94.
17. Dunger DB, Sperling MA, Acerini CL, Bohn DJ, Daneman D, Danne TP, et al. European Society for Paediatric Endocrinology/Lawson Wilkins Pediatric Endocrine Society consensus statement on diabetic ketoacidosis in children and adolescents. *Pediatrics* 2004; 113: e133-40.
18. Halperin ML, Goldstein MB. Fluid, electrolyte, and acid-base physiology. 3rd ed. Philadelphia, PA: W.B. Saunders; 1999.
19. Glaser NS, Wootton-Gorges SL, Marcin JP, Buonocore MH, Dicarlo J, Neely EK, et al. Mechanism of cerebral edema in children with diabetic ketoacidosis. *J Pediatr* 2004; 145: 164-71.
20. Cameron FJ, Kean MJ, Wellard RM, Werther GA, Neil JJ, Inder TE. Insights into the acute cerebral metabolic changes associated with childhood diabetes. *Diabet Med* 2005; 22: 648-53.
21. Hoorn EJ, Geary D, Robb M, Halperin ML, Bohn D. Acute hyponatremia related to intravenous fluid administration in hospitalized children: an observational study. *Pediatrics* 2004; 113: 1279-84.
22. Steele A, Gowrishankar M, Abrahamson S, Mazer CD, Feldman RD, Halperin ML. Postoperative hyponatremia despite near-isotonic saline infusion: a phenomenon of desalination. *Ann Intern Med* 1997; 126: 20-5.
23. Halperin ML, Goguen JM, Scheich AM, Kamel KS. Clinical consequences of hyperglycemia and its correction. In: Giebisch G, editor. *Clinical disturbances of water metabolism*. New York: Raven Press Ltd; 1993. p. 249-272.
24. Davids MR, Edoute Y, Stock S, Halperin ML. Severe degree of hyperglycaemia: insights from integrative physiology. *QJM* 2002; 95: 113-24.

25. Van der Meulen JA, Klip A, Grinstein S. Possible mechanism for cerebral oedema in diabetic ketoacidosis. *Lancet* 1987; 2: 306-8.
26. Hoffman WH, Steinhart CM, el Gammal T, Steele S, Cuadrado AR, Morse PK. Cranial CT in children and adolescents with diabetic ketoacidosis. *AJNR Am J Neuroradiol* 1988; 9: 733-9.
27. Krane EJ, Rockoff MA, Wallman JK, Wolfsdorf JI. Subclinical brain swelling in children during treatment of diabetic ketoacidosis. *N Engl J Med* 1985; 312: 1147-51.
28. Carlotti AP, Bohn D, Halperin ML. Importance of timing of risk factors for cerebral oedema during therapy for diabetic ketoacidosis. *Arch Dis Child* 2003; 88: 170-3.
29. Inward CD, Chambers TL. Fluid management in diabetic ketoacidosis. *Arch Dis Child* 2002; 86: 443-4.
30. Harris GD, Fiordalisi I. Physiologic management of diabetic ketoacidemia. A 5-year prospective pediatric experience in 231 episodes. *Arch Pediatr Adolesc Med* 1994; 148: 1046-52.
31. Felner EI, White PC. Improving management of diabetic ketoacidosis in children. *Pediatrics* 2001; 108: 735-40.
32. Shafiee MA, Bohn D, Hoorn EJ, Halperin ML. How to select optimal maintenance intravenous fluid therapy. *QJM* 2003; 96: 601-10.
33. Bohn D, Daneman D. Diabetic ketoacidosis and cerebral edema. *Curr Opin Pediatr* 2002; 14: 287-91.
34. Napolova O, Urbach S, Davids MR, Halperin ML. Assessing the degree of extracellular fluid volume contraction in a patient with a severe degree of hyperglycaemia. *Nephrol Dial Transplant* 2003; 18: 2674-7.
35. Curtis JR, Bohn D, Daneman D. Use of hypertonic saline in the treatment of cerebral edema in diabetic ketoacidosis (DKA). *Pediatr Diabetes* 2001; 2: 191-4.

Chapter 13

**Acute hyponatremia related to
intravenous fluid administration
in hospitalized patients:
an observational study**

**Ewout J. Hoorn, Dennis Geary
Maryanne Robb, Mitchell L. Halperin
and Desmond Bohn**

Pediatrics 2004; 113: 1279–84

Abstract

Objective: To develop hyponatremia (plasma sodium concentration [P_{Na}] < 136 mmol/L), one needs a source of water input and antidiuretic hormone secretion release to diminish its excretion. The administration of hypotonic maintenance fluids is common practice in hospitalized children. The objective of this study was to identify risk factors for the development of hospital-acquired, acute hyponatremia in a tertiary care hospital using a retrospective analysis.

Methods: All children who presented to the emergency department in a 3-month period and had at least 1 P_{Na} measured ($n = 1586$) were evaluated. Those who were admitted were followed for the next 48 hours to identify patients with hospital-acquired hyponatremia. An age- and gender-matched case-control (1:3) analysis was performed with patients who did not become hyponatremic.

Results: Hyponatremia ($P_{Na} < 136$ mmol/L) was documented in 131 of 1586 patients with ≥ 1 P_{Na} measurements. Although 96 patients were hyponatremic on presentation, our study group consisted of 40 patients who developed hyponatremia in hospital. The case-control study showed that the patients in the hospital-acquired hyponatremia group received significantly more EFW and had a higher positive water balance. With respect to outcomes, 2 patients had major neurologic sequelae and 1 died.

Conclusion: The most important factor for hospital-acquired hyponatremia is the administration of hypotonic fluid. We suggest that hypotonic fluid not be given to children when they have a $P_{Na} < 138$ mmol/L.

Introduction

Hyponatremia is the most frequently encountered electrolyte disorder in hospitalized patients [1,2] and suggests that there is a surplus of water and/or a deficit of Na^+ in the extracellular fluid (ECF) compartment. Hence, there must be a source of water and actions of antidiuretic hormone secretion (ADH) to impair its excretion [3]. In children, the source of water is frequently the administration of hypotonic intravenous fluids. When the plasma sodium concentration (P_{Na}) falls acutely to < 130 mmol/L, brain cell swelling may develop and be sufficient to lead to a devastating neurologic outcome. The most frequent clinical setting for acute hyponatremia is after elective surgery [4-6]. In this situation, the stimuli for the release of ADH are usually nonosmotic (pain, anxiety, nausea, and the use of pharmacologic agents such as narcotics and inhalational anesthetics). The problem is compounded when hypotonic fluids are given while the excretion of hypotonic urine is impaired [7].

We recently reported on the development of acute hyponatremia in children who received hypotonic intravenous fluids [6]. We identified during a 10-year period 23 patients who had a rapid reduction in P_{Na} after surgery or in association with the administration of large amounts of hypotonic fluids. There was a 30% adverse outcome rate (death or neurologic injury). However, because that study was based on either a hospital discharge diagnosis of acute hyponatremia or patients who were referred to the critical care unit because of cerebral edema and brainstem herniation, it is unlikely to be an accurate reflection of the numbers at risk for an adverse neurologic event. We therefore conducted the present study to determine the

importance of intravenous fluid therapy and the underlying diseases in its development.

Methods

Approval was obtained from the Institutional Research Ethics Board to conduct a retrospective review of patients who were seen in or admitted through our hospital emergency department.

Study group

Hyponatremia was defined as a $P_{Na} < 136$ mmol/L. During the 3-month period from November 2000 to February 2001, there were 13 506 visits to the emergency department at the Hospital for Sick Children in Toronto. Those who had at least 1 P_{Na} value < 136 mmol/L were identified. We then focused on patients who had a fall in P_{Na} in hospital—the hospital-acquired hyponatremia group.

The following general clinical data were included in our analysis: age, gender, weight, diagnosis, and medications. We looked for possible central nervous system symptoms of acute hyponatremia (headache, nausea, vomiting, seizures, and changes in sensorium) as well as the volume of oral and intravenous fluid intake. Output values were also included when data were recorded. Data suggestive of a contracted ECF volume were included when documented in the chart (low blood pressure, rapid heart rate, and reduced capillary refill time). For each patient, the volume and type of fluid administered were compared with those recommended for maintenance fluid requirements in children based on the formula using body weight originally published by Holliday and Segar.⁸ Cases in which

deficits were replaced (eg, a contracted ECF volume) were incorporated into the final analysis.

Analysis of the basis for a fall in the plasma sodium concentration

Patients who developed hyponatremia in hospital were analyzed in greater detail because we could evaluate risk factors that contributed to its development. We calculated the amount of electrolyte-free water (EFW) input using the tonicity and volume of the administered fluid [9]. For example, the commonly used solution for maintenance fluids in our institution is 3.3% dextrose in 0.3% NaCl (51 mmol of Na⁺ per liter), which is one third of the amount present in an isotonic saline. Therefore, two thirds of the volume of this solution can be thought of as EFW [10]. In our calculations, we included potassium (K⁺) in defining tonicity [11]. These calculations were also performed for oral solutions.

The influence of EFW on the P_{Na} was analyzed using the initial measured P_{Na} and total body water (TBW) estimated as 60% of body weight, except for neonates in whom TBW was calculated as 70% of body weight. If, for example, the P_{Na} fell from 140 to 135 mmol/L as a result of a positive balance for EFW, then the TBW in a 10-kg person would have to increase from 6000 mL to 6220 mL (positive balance of 220 mL of EFW). Included in calculations for output were insensible losses, using an average of 14 mL/kg/day in the absence of fever [12]. Finally, we recorded likely reasons for high ADH levels from data in the history (disease, symptoms, drugs, and surgery) and physical examination (ECF contraction).

We also compared the patients with hospital-acquired hyponatremia with a control group of age-, gender-, and weight-matched patients who had ≥ 2 P_{Na} measurements in which the P_{Na} was > 136 mmol/L, using a 1:3 match. Cases with a reason for a shift of water from the intracellular fluid to the ECF compartment (e.g., hyperglycemia) or those who were given hypertonic mannitol were excluded from this analysis. We identified all patients who had ≥ 1 serum electrolyte measurements from the hospital laboratory database.

Analytical methods and calculations

A retrospective case-control study was performed using a t test and a χ^2 test. Correction for multiple variable testing included using the Bonferroni correction [13].

Results

Patients

A total of 432 patients had ≥ 2 P_{Na} measurements, 97 of which had a $P_{Na} < 136$ mmol/L. The remaining 335 patients were not hyponatremic and formed the basis of our control group. Sixty-two patients were hyponatremic on presentation, whereas 35 of 97 developed hyponatremia after presentation. In 12 of 62 of these patients, the P_{Na} remained < 136 mmol/L, whereas in 50 of 62, it increased to > 136 mmol/L but then fell again to < 136 mmol/L in 5 patients on a subsequent measurement. Thus, the total number of patients who developed hospital-acquired hyponatremia was 40 of 432. The P_{Na} in these 40 patients with hospital-acquired hyponatremia fell from a mean of 139 ± 3 mmol/L to 133 ± 2 mmol/L, a decline of 6 ± 1 mmol/L in 19 ± 10 hours.

Our next step was to relate the amount of EFW given (orally and/or intravenously) to that needed to cause their observed fall in P_{Na} ; there were 2 near-equal groups (Figure 1): 1 received sufficient (or more) EFW to explain their fall in P_{Na} (all points on or above the line of identity), and the other did not receive enough EFW to explain their fall in P_{Na} (points below the line of identity).

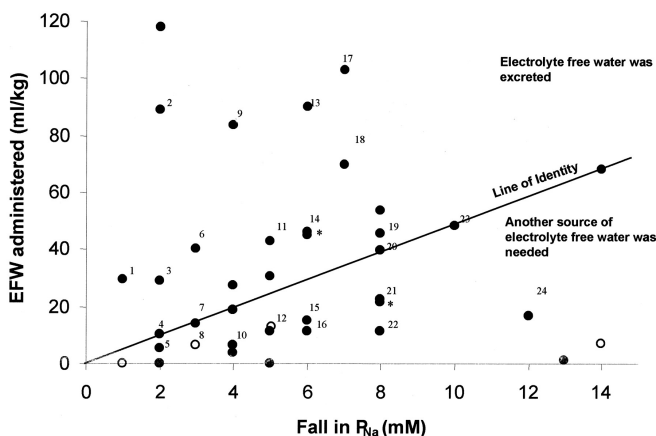


Figure 1: The relationship between EFW administration and the fall in P_{Na} . *Overlap of 2 points. Points represent individual patients with hospital-acquired hyponatremia ($n = 37$). The case numbers next to the dots refer to patients whose urine output is known and correspond with those in Figure 2. The solid line represents the amount of EFW that would need to be retained to cause the observed fall in P_{Na} . The patients whose points fell on or above the line of identity could have retained enough EFW to explain their fall in P_{Na} ; any extra water either was excreted in the urine or sweat or was turned into an isotonic saline as a result of a positive balance for Na^+ . In the patients whose points fell below the line of identity, the amount of EFW given can be only a partial explanation for their fall in P_{Na} . These patients required a concurrent excretion of hypertonic Na^+ ($+K^+$) and/or an occult source of EFW to achieve their observed fall in P_{Na} .

The source of this EFW load was predominantly the infusion of hypotonic fluids (66%), whereas in the remainder, the fall in P_{Na} could be attributed to the oral intake of EFW; these latter patients could have had an occult source of water intake, a reason to shift EFW out of cells (e.g., a catabolic state [14], the excretion of hypertonic urine [7,15]). The main reason for this ECF volume expansion may have been the bolus infusion of more isotonic saline than needed to reexpand the ECF volume.

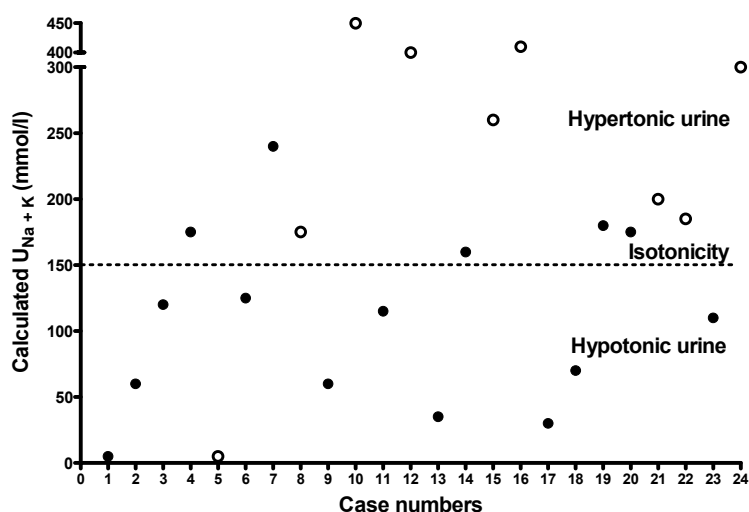


Figure 2: Calculated urine Na^+ concentration

Dots represent the urine Na^+ concentration for patients with known urine outputs. The case numbers correspond to the case numbers in Figure 1. Open dots refer to patients whose points fall under the line of identity in Figure 1. Patients whose points fell below the line representing isotonicity have urine Na^+ concentrations exceeding 150 mmol/L. In those patients, ADH levels should be high. In 2 patients, urine outputs were noted to be small, which explains their values close to 0 mmol/L (for validation of this method, see Carlotti et al. [46]).

We identified 16 patients with insufficient EFW to cause the observed degree of fall in their P_{Na} (Figure 1); 11 received a bolus of 0.9 NaCl (45 ± 42 mL/kg/hour; 15% expansion of ECF if all retained) on the basis of the presumption that they had ECF contraction. None of the patients on or above the line of identity received boluses of fluid.

Case-control study

The in-hospital group with a fall in their P_{Na} received 3-fold more EFW and had a greater positive fluid balance than the control group ($p < 0.001$ and $p = 0.02$, respectively; Table 1). Although this in-hospital group received less Na^+ per kilogram of body weight, this difference was not statistically significant. The amount of fluid infused was not only significantly higher in this in-hospital group but also well above that recommended by the standard formula for maintenance fluid administration [8] and well above what we now calculate for maintenance fluids [16]. Our analysis showed that there were no significant differences related to the underlying disease, that the symptoms of nausea and vomiting were significantly more prevalent in the in-hospital group, and that patients in the in-hospital group underwent surgery more frequently ($p < 0.05$). Finally, likely reasons for high levels of ADH in patients with hospital-acquired hyponatremia were found to be mainly of nonosmotic origin (symptoms, drugs, and disease; Table 1).

Table 1: Results 1:3 age- and gender matched case-control study (* $p < 0.05$)

Category	Variable	Cases (n = 37)	Controls (n = 111)
Demographics	Age, years	7 ± 6	7 ± 6
	Gender, n (%)	15 (41) F	45 (41) F
	Weight, kg	25 ± 18	28 ± 21
	Surgery, n (%)	6 (16)	6 (5)
Water and sodium	P _{Na} , mmol/l	139 ± 3 → 133 ± 2	140 ± 2
	Decrease in P _{Na} , mmol	6 ± 3 in 19 ± 10 h	-
	EFW, mL	2 ± 2	1 ± 1*
	Na ⁺ , mmol/kg/h	0 ± 1	1 ± 1
	Positive water balance, ml/kg/h	4 ± 5	2 ± 3*
IV-fluid regimen	Amount of fluid, ml/h	98 ± 77	47 ± 46*
	% that received more than recommended maintenance	73	23*
Disease categories	GI disorders, n (%)	11 (30)	19 (17)
	Neoplasia	8 (22)	14 (13)
	Respiratory infections	5 (14)	28 (25)
	Renal disease	1 (3)	6 (5)
Cause elevated ADH	Disease, n (%)	5 (14)	-
	Symptoms	23 (62)	-
	Drugs	9 (24)	-
	Hypovolemia	0 (0)	-
Symptoms	Nausea, n (%)	10 (27)	3 (8)*
	Headache	2 (5)	11 (10)
	Vomiting	25 (68)	46 (41)*
	Seizures	1 (3)	6 (5)
	Sensorium changes	7 (19)	13 (12)

Discussion

The principal results in this study confirm that it was not uncommon for hyponatremia to develop in the first 48 hours of admission to hospital, related in large part to intravenous fluid administration. The level of P_{Na} that we used for eligibility criterion is consistent with previously published definitions [17] and was the median level found in a large published series of children who presented to a hospital with acute medical illnesses [18]. Groups of children who previously have been reported to be at risk are those with meningitis, encephalitis, head injury, bronchiolitis, gastroenteritis, and chronic lung disease of prematurity and in association with chemotherapy [19-27]. This list was not all-inclusive because other nonosmotic stimuli were present in our population with hospital-acquired hyponatremia (Table 1). Hyponatremia is also a common event after elective surgery [2,28-30] and when acute (< 48 hours) can lead to catastrophic neurologic sequelae [5,6,31]. Children with chronic hyponatremia are not at risk for the development of cerebral edema [32,33].

There are 2 requirements for a fall in P_{Na} : the presence of ADH and a source of water input. Although it should not be surprising to find elevated ADH levels in acutely ill patients [18], this will not cause hyponatremia in the absence of water input. The major source for water input in our study was the infusion of a large amount of hypotonic fluid. Because close to half of the cases received a higher amount of intravenous fluid than recommended for maintenance on the basis of the formula of Holliday and Segar [8], the amount given was a contributing causative factor. The infusion of a large volume of saline was likely attributable to the belief that the ECF volume was

contracted. For the group that did not have a recorded input of sufficient water to explain their fall in P_{Na} (Figure 1, points below line of identity), one would suspect that they had an occult water intake (e.g., ice chips, water residing in the lumen of the gastrointestinal tract after admission, electrolyte-free water generation by the kidney secondary to the excretion of hypertonic urine; open dots in Figure 2). For this latter mechanism, one needs the combination of an infusion of isotonic saline and the excretion of hypertonic urine [7]. It is possible that this desalination process may have been triggered by the acute expansion of the ECF volume as a result of the administration of isotonic saline, because we could find recorded evidence that the ECF volume was contracted in only 10% of these patients. We emphasize that the clinical assessment of the degree of ECF volume contraction is a method of limited sensitivity and specificity [34-37].

Dangers of acute hyponatremia

Previous studies have shown that children with acute hyponatremia have an appreciable risk for neurologic damage [5,6,38,39]. With respect to the potential dangers of acute hyponatremia in our patient population, it is possible that the observed fall in P_{Na} led to serious severe neurologic outcomes in 2 of 40 patients. One of these (Figure 1; fall in P_{Na} of 14 mmol/L) had an underlying seizure disorder and had a convulsion during the hyponatremic period. This highlights the need to be more vigilant about the fall in P_{Na} when an underlying medical condition places the patient at risk. We also emphasize a diagnostic caveat: that a seizure may raise the P_{Na} transiently by an average of 13 mmol/L, masking the original degree of hyponatremia [40]. The second patient (fall in P_{Na} of 13 mmol/L

from 142 to 128 mmol/L in 1.5 hours) had a cardiac arrest. Although she was resuscitated initially, she ultimately died. Postmortem examination revealed brain cell swelling. The high incidence of nausea and vomiting (Table 1) may indicate more cases of symptomatic hyponatremia; however, because these symptoms are also known to be potent stimuli of ADH release, this deduction is not possible from this retrospective study.

Rationale of the choice of intravenous fluid: hypotonic versus isotonic

The almost universal practice of the use of hypotonic fluids in children is based on calculations that linked energy expenditure to water and electrolyte losses, published nearly 50 years ago. Applying this formula results in the administration of large amounts of EFW, which then has to be excreted by the kidney. We believe that linking energy expenditure to water losses in hospitalized patients significantly overestimates the need for maintenance fluid. In a recent commentary [16], we reevaluated the factors used to calculate water and electrolyte requirements in Holliday and Segar's original paper. Moreover, these calculations did not factor in the unpredictable effect of nonosmotic stimuli for ADH secretion in the acutely ill child, which can result in retention of water and hyponatremia [18]. Our conclusion was that the water requirements and the renal ability to excrete hypotonic urine were overestimated. Therefore, our general recommendation was that the P_{Na} be measured once the ECF volume is expanded $> 10\%$ (30 mL/kg); and if the P_{Na} is < 138 mmol/L, then do not infuse hypotonic fluids [16].

The option of selecting isotonic rather than hypotonic for maintenance fluid in children has been advocated by some

authors [6,41-43]. In a recent publication, Moritz and Ayus [43] drew attention to this idea, and our data support this position. This generally has not been accepted because of concerns about excessive administration of Na^+ and the development of hypernatremia. The comparative studies in children, although few, do not support this perceived danger. In a randomized trial of different fluid protocols in children with meningitis, Powell *et al.* [20] compared a fluid-restricted group who received hypotonic saline with a fluid-deficit replacement plus maintenance regimen using predominantly isotonic solutions. Children in the isotonic group received an average of 6 mmol Na^+ /kg/day and had normal P_{Na} levels, whereas those in the hypotonic group received an average of 2 mmol Na^+ /kg/day and became hyponatremic. Likewise, in the study by Gerigk *et al.* [18] of acutely ill children in which the median P_{Na} was 136 mmol/L at the time of admission to hospital, those who were given isotonic fluid had a more rapid fall in their ADH levels than those who received hypotonic fluids.

Children who undergo surgical procedures are particularly at risk from hyponatremia because of the association between anesthetic agents and opiates and nonosmotic ADH secretion. Moreover, the syndrome of inappropriate ADH secretion has been frequently reported in association with spinal surgery [30,44]. Burrows *et al.* [30] compared hypotonic with isotonic intravenous fluids in children who underwent surgery for scoliosis. Both groups had a fall in their P_{Na} in the postoperative period, but the reduction was greater in those who received the hypotonic solution.

Patients who have findings of hyponatremia, with impaired excretion of EFW as a result of actions of ADH in the absence of obvious stimuli for ADH release (an ECF volume contraction of at least 8%), or either adrenal insufficiency or hypothyroidism are said to have the syndrome of inappropriate ADH secretion [45]. In this syndrome, the urine usually contains an appreciable quantity of Na^+ . Therefore, we could have said that hyponatremia developed in our patients as a result of the syndrome of inappropriate ADH secretion. Notwithstanding, we have used a different way to describe the basis of hyponatremia in our population. Our description begins with the pathophysiology. Our patients had multiple nonosmotic stimuli for the release of ADH. The source of the EFW was hypotonic fluids given by the physician (hypotonic intravenous solutions), health care workers (e.g., ice chips), and/or the family of the patient (oral drinks containing water). In addition, EFW could be generated by the kidneys when the urine has a higher $\text{Na}^+ + \text{K}^+$ concentration than the net of all inputs [7]. Regardless of the terminology, the most important factor is the net input of EFW in this setting because ADH is likely to be present for the nonosmotic reasons described above. Moreover, although patients have this type of ADH release, they need not develop a significant degree of hyponatremia because as their P_{Na} falls, thirst is suppressed and there is no longer a physiologic stimulus causing a large input of water. In contrast, in hospital, the physician rather than the patient determines the water intake.

Study limitations

This study was retrospective and hence has the imperfections that characterize such studies. By evaluating every patient who arrived in our emergency department in a 3-month period, we

attempted to minimize this limitation. Our actual incidence of hyponatremia is probably an overestimation because the P_{Na} was measured in only ~10% of the total population, a group that had indications for this measurement. In addition to these limitations, there was the problem of not measuring urine electrolytes and plasma ADH levels. Also, some of the patients and especially those who received insufficient EFW to explain their fall in P_{Na} could have had an occult source of water. Occult sources of water include water in the gastrointestinal tract that was not absorbed before the first measurement of the P_{Na} , the use of ice chips, or a parent giving his or her child a drink without informing the nurse so that there is no record of that input in the hospital chart. We think that a prospective study to answer some of the obvious questions would be helpful.

Conclusions

The development of hyponatremia is unacceptably high in hospitalized children. This is attributable in large part to the administration of excessive amounts of water as hypotonic saline in situations in which ADH is secreted for nonosmotic reasons. The original guidelines for maintenance fluid may not be applicable in an era when the complexity and the severity of illness seen in hospitalized children who receive intravenous fluid therapy has radically changed (e.g., leukemia, complex congenital heart disease) and irregularities of ADH secretion are more likely to be commonplace. We believe that hospital-acquired hyponatremia unnecessarily puts children at risk for the development of adverse neurologic events and is largely preventable. We suggest that the current recommendations for intravenous fluid therapy in hospitalized children be revised. Hypotonic fluids should not be used routinely in the

intraoperative or postoperative period or when a patient has a P_{Na} in the low-normal or distinctly hyponatremic range (< 138 mmol/L). In addition, boluses of isotonic saline should be given only when there are clear hemodynamic indications for that infusion.

References

1. Anderson RJ. Hospital-associated hyponatremia. *Kidney Int* 1986; 29: 1237–1247.
2. Kennedy PG, Mitchell DM, Hoffbrand BI. Severe hyponatraemia in hospital inpatients. *BMJ* 1978; 2: 1251–1253.
3. Halperin ML, Goldstein MB. Sodium and Water. 3rd ed. Philadelphia. PA: WB Saunders; 1999
4. Arieff AI. Hyponatremia, convulsions, respiratory arrest, and permanent brain damage after elective surgery in healthy women. *N Engl J Med* 1986; 314: 1529–1535.
5. Arieff AI, Ayus JC, Fraser CL. Hyponatraemia and death or permanent brain damage in healthy children. *BMJ* 1992; 304: 1218–1222.
6. Halberthal M, Halperin ML, Bohn D. Acute hyponatraemia in children admitted to hospital: retrospective analysis of factors contributing to its development and resolution. *BMJ* 2001; 322: 780–782.
7. Steele A, Gowrishankar M, Abrahamson S, Mazer CD, Feldman RD, Halperin ML. Postoperative hyponatremia despite near-isotonic saline infusion: a phenomenon of desalination. *Ann Intern Med* 1997; 126: 20–25.
8. Holliday MA, Segar WE. The maintenance need for water in parenteral fluid therapy. *Pediatrics* 1957; 19: 823–832.
9. Rose BD. New approach to disturbances in the plasma sodium concentration. *Am J Med*. 1986; 81: 1033–1040.
10. Goldberg M. Hyponatremia. *Med Clin North Am* 1981;65:251–269.
11. Edelman IS, Leibman J. Anatomy of body water and electrolytes. *Am J Med* 1959; 27: 256–277.
12. Blumenfeld JD, Vaughan ED. Regulation of Water Balance. 7th ed. Philadelphia, PA: WB Saunders; 1998

13. Bland JM, Altman DG. Multiple significance tests: the Bonferroni method. *BMJ* 1995; 310: 170.
14. Gowrishankar M, Chen CB, Mallie JP, Halperin ML. What is the impact of potassium excretion on the intracellular fluid volume: importance of urine anions. *Kidney Int* 1996; 50: 1490–1495.
15. Gowrishankar M, Chen CB, Cheema-Dhadli S, Steele A, Halperin ML. Hyponatremia in the rat in the absence of positive water balance. *J Am Soc Nephrol* 1997; 8: 524–529.
16. Shafiee MA, Bohn D, Hoorn EJ, Halperin ML. How to select optimal maintenance intravenous fluid therapy. *QJM* 2003; 96: 601–610.
17. Adroge HJ, Madias NE. Hyponatremia. *N Engl J Med* 2000; 342: 1581–1589.
18. Gerigk M, Gnehm HE, Rascher W. Arginine vasopressin and renin in acutely ill children: implication for fluid therapy. *Acta Paediatr* 1996; 85: 550–553.
19. Shann F, Germer S. Hyponatraemia associated with pneumonia or bacterial meningitis. *Arch Dis Child* 1985; 60: 963–966.
20. Powell KR, Sugarman LI, Eskenazi AE, et al. Normalization of plasma arginine vasopressin concentrations when children with meningitis are given maintenance plus replacement fluid therapy. *J Pediatr* 1990; 117: 515–522.
21. Kojima T, Fukuda Y, Hirata Y, Matsuzaki S, Kobayashi Y. Changes in vasopressin, atrial natriuretic factor, and water homeostasis in the early stage of bronchopulmonary dysplasia. *Pediatr Res* 1990; 27: 260–263.
22. Hazinski TA, Blalock WA, Engelhardt B. Control of water balance in infants with bronchopulmonary dysplasia: role of endogenous vasopressin. *Pediatr Res* 1988; 23: 86–88.
23. Bhalla P, Eaton FE, Coulter JB, Amegavie FL, Sills JA, Abernethy LJ. Hyponatraemic seizures and excessive intake of hypotonic fluids in young children. *BMJ* 1999; 319: 1554–1557.
24. Moritz ML, Ayus JC. La Crosse encephalitis in children. *N Engl J Med* 2001; 345: 148–149.
25. Milionis HJ, Bourantas CL, Siamopoulos KC, Elisaf MS. Acid-base and electrolyte abnormalities in patients with acute leukemia. *Am J Hematol* 1999; 62: 201–207.

26. Padilla G, Leake JA, Castro R, Ervin MG, Ross MG, Leake RD. Vasopressin levels and pediatric head trauma. *Pediatrics* 1989; 83: 700–705.
27. Abe T, Takaue Y, Okamoto Y, et al. Syndrome of inappropriate antidiuretic hormone secretion (SIADH) in children undergoing high-dose chemotherapy and autologous peripheral blood stem cell transplantation. *Pediatr Hematol Oncol* 1995; 12: 363–369.
28. Chung HM, Kluge R, Schrier RW, Anderson RJ. Postoperative hyponatremia. A prospective study. *Arch Intern Med* 1986; 146: 333–336.
29. Judd BA, Haycock GB, Dalton N, Chantler C. Hyponatraemia in premature babies and following surgery in older children. *Acta Paediatr Scand* 1987; 76: 385–393.
30. Burrows FA, Shutack JG, Crone RK. Inappropriate secretion of antidiuretic hormone in a postsurgical pediatric population. *Crit Care Med*. 1983; 11: 527–531.
31. Teyssier G, Rayet I, David T, Serio A, Damon G, Freycon F. Hyponatremia and convulsions in the postoperative period in children [in French]. *Arch Fr Pediatr* 1988; 45: 489–491.
32. Porcel A, Diaz F, Rendon P, Macias M, Martin-Herrera L, Giron-Gonzalez JA. Dilutional hyponatremia in patients with cirrhosis and ascites. *Arch Intern Med* 2002; 162: 323–328.
33. Usberti M, Federico S, Meccariello S, et al. Role of plasma vasopressin in the impairment of water excretion in nephrotic syndrome. *Kidney Int* 1984; 25: 422–429.
34. McCance RA. Medical problems in mineral metabolism III: experimental human salt deficiency. *Lancet* 1936; 230: 823–830.
35. Chung HM, Kluge R, Schrier RW, Anderson RJ. Clinical assessment of extracellular fluid volume in hyponatremia. *Am J Med* 1987;83:905–908.
36. Gorelick MH, Shaw KN, Murphy KO. Validity and reliability of clinical signs in the diagnosis of dehydration in children. *Pediatrics* 1997;99:E6.
37. Mackenzie A, Barnes G, Shann F. Clinical signs of dehydration in children. *Lancet* 1989; 2: 605–607.
38. Varavithya W, Hellerstein S. Acute symptomatic hyponatremia. *J Pediatr* 1967; 71: 269–283.

39. Dunn K, Butt W. Extreme sodium derangement in a paediatric inpatient population. *J Paediatr Child Health* 1997; 33: 26–30.
40. Welt LG, Orloss J, Kydd DM, Oltman JE. An example of cellular hyperosmolarity. *J Clin Invest* 1950; 29: 935–939.
41. Moritz ML, Ayus JC. Disorders of water metabolism in children: hyponatremia and hypernatremia. *Pediatr Rev* 2002; 23: 371–380.
42. Judd BA, Haycock GB, Dalton RN, Chantler C. Antidiuretic hormone following surgery in children. *Acta Paediatr Scand* 1990; 79: 461–466.
43. Moritz ML, Ayus JC. Prevention of hospital-acquired hyponatremia: a case for using isotonic saline. *Pediatrics* 2003; 111: 227–230.
44. Lieh-Lai MW, Stanitski DF, Sarnaik AP, et al. Syndrome of inappropriate antidiuretic hormone secretion in children following spinal fusion. *Crit Care Med* 1999; 27: 622–627,
45. Schwartz WB, Bennett W, Curelop S. A syndrome of renal sodium loss and hyponatremia probably resulting from inappropriate secretion of antidiuretic hormone. *Am J Med* 1957; 23: 529–542.
46. Carlotti AP, Bohn D, Rutka JT, et al. A method to estimate urinary electrolyte excretion in patients at risk for developing cerebral salt wasting. *J Neurosurg* 2001; 95: 420–424.

Chapter 14

How to select optimal maintenance intravenous fluid therapy

Mohammad A.S. Shafiee, Desmond Bohn
Ewout J. Hoorn and Mitchell L. Halperin

QJM 2003; 96: 601–10

Abstract

Hyponatraemia is the commonest electrolyte abnormality in hospitalized patients. If the plasma sodium concentration (P_{Na}) declines to ~ 120 mmol/l in < 48 h, brain cell swelling might result in herniation, with devastating consequences. The volume and/or the composition of fluids used for intravenous therapy often contribute to the development of acute hyponatraemia. Our hypothesis is that the traditional calculation of the daily loss of insensible water overestimates this parameter, leading to an excessive daily recommended requirement for water. We offer suggestions to minimize the risk of iatrogenic hyponatraemia.

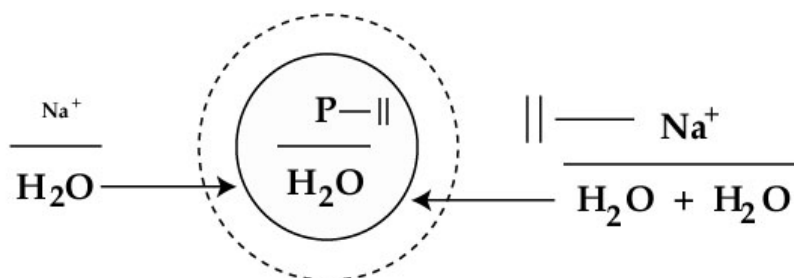
Introduction

Hyponatraemia is defined as a plasma Na^+ concentration (P_{Na}) $< 136 \text{ mmol/l}$ [1]. While this could result from a deficit of sodium (Na^+), it is most often due to a surplus of water (Figure 1, Table 1). To create a positive water balance [1,2], input of water must exceed output. A low water output is usually due to the release of vasopressin, often because of non-osmotic stimuli (Table 2) [3]. When this occurs, patients cannot produce the large water diuresis that prevents a fall in their P_{Na} [4]. In fact, vasopressin acts throughout the 24-h period in normal volunteers despite episodic intake of water (Figure 2). Hence normal humans rarely have a water diuresis unless their arterial P_{Na} falls below 136 mmol/l . In contrast, when hospitalized adults [4] and paediatric [5] patients have a P_{Na} that is $< 136 \text{ mmol/l}$, they do not have a dilute urine because of renal actions of vasopressin, released in response to non-osmotic stimuli.

Table 1: Physiological classification of hyponatraemia

<i>I. Deficit of Na^+ in the ECF compartment</i>
1. Non-renal loss of Na^+ (sweat, gastrointestinal tract) 2. Renal loss of Na^+: • Lack of a stimulator for Na^+ reabsorption (e.g., aldosterone) • Presence of an inhibitor (e.g., ECF volume expansion or a diuretic) • Inborn errors that interfere with the normal reabsorption of Na^+
<i>II. Surplus of electrolyte-free water in the ECF compartment</i>
1. Determine the source of electrolyte-free water: • Gastrointestinal tract • Intravenous fluids • Urine (need to excrete urine with a $\text{Na}^+ + \text{K}^+$ concentration $> P_{\text{Na}}$ or the infused fluids [4]) 2. Determine why there is too little excretion of electrolyte-free water: • Vasopressin is present (see Table 2) • Vasopressin can be absent • Low distal delivery of filtrate ('trickle-down hyponatraemia' [46]) • Renal failure

Note that hyperglycaemia and/or high mannitol-like solutes in plasma might be contributing to the degree of hyponatraemia.

Na⁺ LOSS**WATER GAIN****Figure 1: Water movement across cell membranes**

The solid circle represents the normal ICF volume and the dashed circle represents the expanded ICF volume when hyponatraemia is present. Water channels permit the cell membranes to be permeable to water. Osmotic forces are immense. Therefore, independent of whether hyponatraemia is due to a negative balance for Na⁺ as shown on the left, or a positive balance for water shown on the right, the ICF volume will rise to the same extent because the number of particles restricted to the ICF compartment (P) does not change appreciably in most cells, and Na⁺ ions are restricted to the ECF compartment for the most part.

Table 2: High vasopressin levels in patients with hyponatraemia

1. Causes that are readily reversible
• Low ‘effective’ circulating volume to cause the release of vasopressin • Anxiety, stress, pain, nausea • Drugs causing nausea (e.g., many of the currently used chemotherapeutic agents), the central release of vasopressin (e.g., morphine, barbiturates, ‘ecstasy’) or enhancement of the renal effects of vasopressin (e.g., certain oral hypoglycaemics, non-steroidal anti-inflammatory agents, anticonvulsants such as carbamazepine) • Endocrine causes (e.g., hypothyroidism, adrenal insufficiency) • Exogenous dDAVP, oxytocin
2. Causes that cannot be reversed rapidly
• Vasopressin-producing tumours • CNS or lung lesions (may cause ‘reset osmostat’) • Granulomas • Certain metabolic lesions (e.g., porphyria)

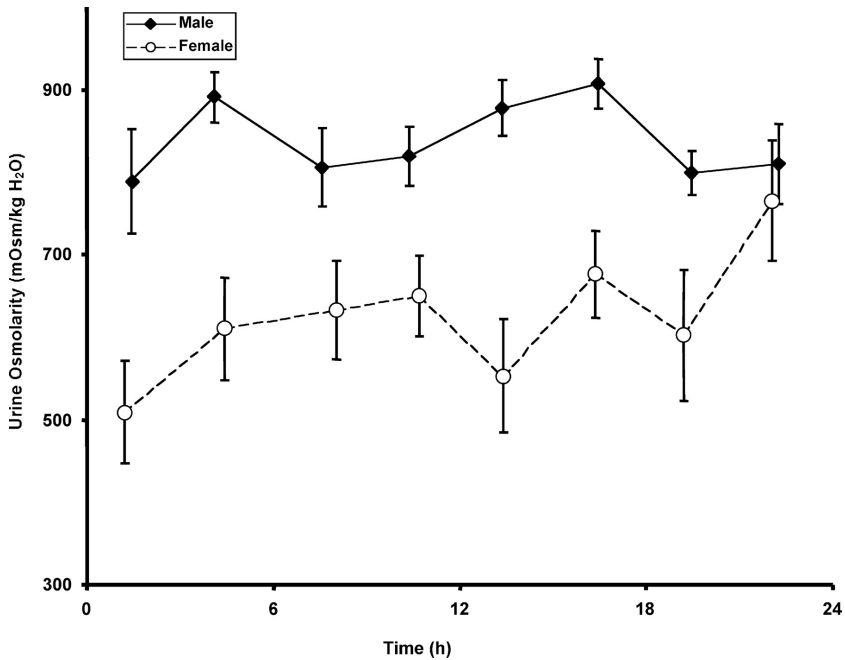


Figure 2: Diurnal variation in the urine osmolality

Data were gathered in 75 volunteers (aged 17–64 years). Voluntary voiding occurred at 2-h intervals for the 16 h while awake and when the subject woke up at night to void. There were no restrictions on water intake, diet, or activity. No subject took medications. The males ($n = 45$) are depicted by the solid diamonds connected by a solid line and the females ($n = 30$) by the open circles connected by a dashed line.

Outline of the problem

Intravenous fluid administration is common and more than 75% of currently recommended maintenance fluids are given in the form of electrolyte-free water (0.2% saline) [6,7]. Because patients who are ill often have non-osmotic stimuli for the release of vasopressin, it is not surprising that hyponatraemia is

the commonest electrolyte disorder in hospitalized patients, in part due to this electrolyte-free water input [8]. A large fall in the P_{Na} in < 48 h should cause cell swelling because the ICF volume is inversely related to the P_{Na} (Figure 1) [1]. Because brain cells occupy approximately 2/3 of the intracranial volume, brain-cell swelling is very likely to increase intracranial pressure and predispose to brain herniation, since the brain resides in a rigid bony structure and there is only a small volume of intracranial water that can be lost when there is a small rise in intracranial pressure (the volume in the cerebrospinal fluid) [9]. Children are at greater risk because their brains have a larger ICF volume (more cells) per total skull volume. Nevertheless, not all causes of acute hyponatraemia are associated with brain cell swelling. For example, when a patient undergoes a transurethral resection of the prostate, lavage fluid may enter the vascular compartment and be retained in the ECF compartment [10]. If this fluid contains osmoles such as sorbitol or mannitol that remain extracellularly, the ECF volume will be expanded by an iso-osmotic, Na^+ -free solution. Because most of these solutions are half-isotonic, there will be a modest degree of expansion of the ICF compartment. Accordingly, little of this added Na^+ -free water will enter cells and cause brain-cell swelling despite the large fall in P_{Na} [11]. Hence it is important to include in the clinical analysis the condition that caused the hyponatraemia, and not simply treat a laboratory P_{Na} value.

Our view is that a fall in the P_{Na} is preventable if intravenous fluid therapy is tailored to the needs of individual patients [5]. Hence the first question to ask is why do we currently infuse so much hypotonic saline.

Usual reasons for intravenous fluid administration

1. Defend normal blood pressure

A reduced ECF volume, if accompanied by haemodynamic collapse, has a greater initial priority than all but the most serious degrees of expansion of the ICF volume. Isotonic saline is the initial therapy, but hypertonic saline should be given if there is both a low ECF volume and an acutely low P_{Na} , as seen in people with excessive sweat loss accompanied by a large intake of water [12,13].

Patients who are anaesthetized usually have a fall in blood pressure in the absence of a contracted ECF volume because anaesthetic agents diminish venous tone [4]. An infusion of isotonic saline helps avoid this type of hypotension.

2. Return the ICF volume to normal

In a patient with acute hyponatraemia, the ICF volume in the brain rises and could become dangerously high with a more prominent decline in the P_{Na} . Shrinking the ICF volume becomes an urgent priority only when there are CNS symptoms or a P_{Na} in the mid-to-low 120 mmol/l range.

When there is a large water deficit in the ICF compartment (indicated by a $P_{Na} > 140$ mM), electrolyte-free water (often 5% dextrose in water or D₅W) should be infused. Care should be taken to avoid inducing a significant degree of hyperglycaemia [14].

3. Replacing ongoing renal losses

This therapy is directed at patients who do not have an antecedent condition that led to the retention of salt and water.

4. Giving maintenance fluids to match insensible losses

The traditional guidelines for maintenance fluid infusion focus on the need to replace insensible loss of water for heat dissipation—the latter was related to caloric expenditure based on data and deductions published almost 50 years ago [15], and was reiterated recently [16,17]. These authors stated: *‘Maintenance requirements for water in individuals is determined by their caloric expenditure. By means of the following formula (summarized in Figure 3) the caloric expenditure of hospitalized patients can be estimated with sufficient accuracy by weight alone. Significant deviations from this relation exist, including excessive obesity, the elderly, and patients with infections.’* Notwithstanding, alternative views have been expressed [5,18], because traditional recommendations require that *hypotonic* saline be infused. We emphasize that there is a danger in infusing a large volume of electrolyte-free water because it will accumulate when vasopressin acts, providing that there is not a very large volume of sweat. Moreover, it is not uncommon for patients to seek medical care and arrive in hospital with a low P_{Na} because they drank electrolyte-free water while vasopressin was released secondary to their illness (Table 2).

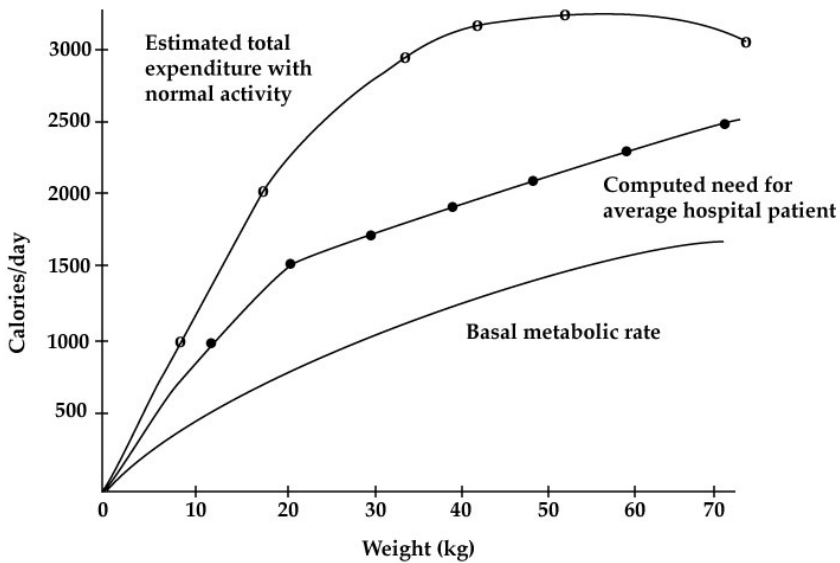


Figure 3: Comparison of energy expenditure in the basal and ideal state

This figure was redrawn, based on a publication by Holliday and Segar [15]. The upper and lower lines were plotted from the data of Butler and Talbot [47]. Weights at the 50th percentile level were selected for converting calories at various ages to calories related to weight. The computed line was derived from the following equations: ages 0–10 kg, 100 cal/kg; 10–20 kg, 1000 cal + 50 cal/kg for each kg over 10 kg; 20 kg and up, 1500 cal/kg + 20 cal/kg for each kg over 20 kg.

It would be a grave error to give them electrolyte-free water. In the next section, we shall provide evidence to suggest that the classical calculations of losses of insensible water lead to an overestimation of the need for hypotonic fluid administration. This error is compounded by a perceived need to excrete a larger urine volume with a more ‘comfortable’ urine osmolality [15].

5. The need for glucose as a fuel for the brain

A theoretical advantage of infusing 2 l of D₅W is that this infusion provides 100 g of glucose, which is almost equivalent to the 120 g of glucose oxidized daily by the brain [19]. The unstated assumption is that this infused glucose is not oxidized by other organs, which in turn is dependent on their ability to oxidize fatty acids [20], because fatty acid oxidation inhibits the oxidation of glucose [21]. Therefore, if the infusion of glucose raises the plasma glucose concentration sufficiently to stimulate the release of insulin [22], little of this infused glucose will be available for oxidation in the brain, because of the antilipolytic actions of insulin. On the other hand, if glucose were needed (i.e. to prevent the development of hypoglycaemia and/or to minimize protein catabolism), it need not be given as D₅W with its electrolyte-free water load—rather glucose could be added to infused saline.

Analysis of insensible water loss

Heat is produced continuously by intermediary metabolism [23]. This heat must be dissipated to avoid a life-threatening rise in body temperature. Part of this vital function is served by evaporation of surface water from the skin and the respiratory tract. While this rationale is correct, we question whether the recommendations derived from it are correct from a quantitative perspective [15].

Water production by metabolism and its loss via the lungs

We begin with this component of water balance to largely dismiss it as a factor that would change the P_{Na}. Our rationale is as follows—metabolic production of CO₂ and water occur in a 1:1 proportion during the oxidation of carbohydrates (C_n(H₂O)_n)

(equation 1) and fatty acids $(-\text{CH}_2)_n$ (equation 2). Moreover, these two end-products are eliminated together in alveolar air in a 1:1 proportion, providing that the arterial pCO_2 is close to 40 mmHg (Figure 4) [23]. The reason for this parallel excretion of water and CO_2 is that the partial pressures of water vapour and CO_2 are virtually equal in alveolar air (47 and 40 mmHg) and in inspired air (close to zero after air is warmed to 37°C [24]). Therefore, water loss via exhaling alveolar air is equal to its metabolic production, so these two pathways can be ignored unless the patient is hyperventilating and/or is on a ventilator and is inspiring humidified air warmed to body temperature [23]. Only water evaporation from the upper respiratory tract results in a negative water balance.

Water loss in sweat

This type of water loss is the major reason to administer electrolyte-free water to hospitalized patients. We emphasize that an exogenous source of water should be replaced only in patients who have a $\text{P}_{\text{Na}} > 140$ mmol/l, indicating a deficit of intracellular water. Sweat usually contains 15–30 mmol of Na^+ per litre [25,26].

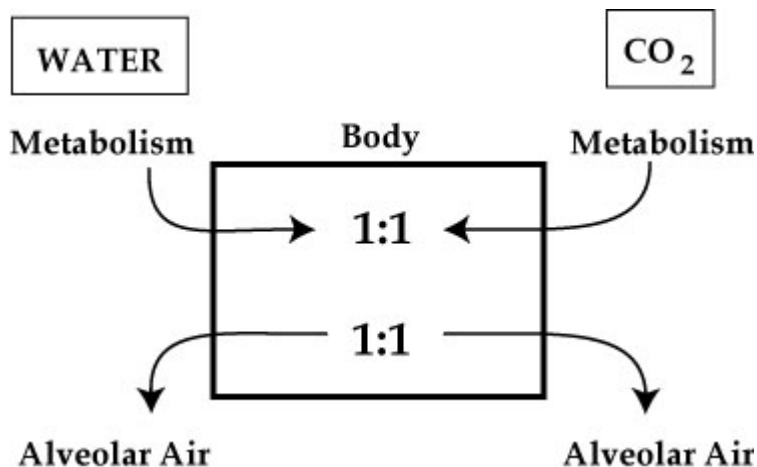


Figure 4: Fate of metabolic water

The body is depicted by the large rectangle. Events describing water are shown to the left while events describing CO₂ are shown to the right of this rectangle. Production of water and CO₂ (top arrows) as well as their loss (bottom arrows) are both in close to a 1:1 proportion. Hence there is no net change in water balance as a result of metabolism and alveolar ventilation.

Hence when 1 l sweat containing 25 mmol/l Na⁺ evaporates, there is a loss of 0.83 l electrolyte-free water (Figure 5). This link between water evaporation (heat dissipation) and caloric expenditure (heat production) is the cornerstone for the traditional prescription for maintenance fluid administration [15]. These two components are analyzed in the following paragraphs.

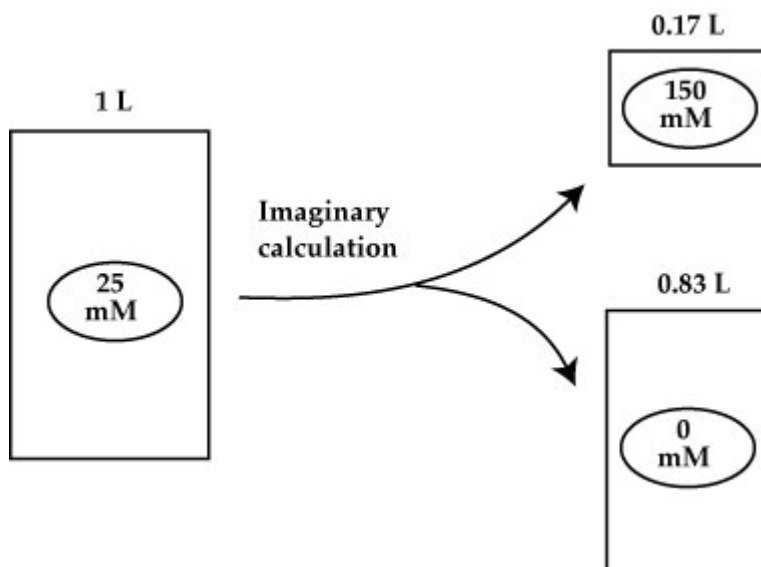


Figure 5: The electrolyte-free water content of sweat

The larger rectangle to the left of the arrows represents 1 l of sweat. Its Na⁺ concentration is 25 mmol/l, shown in the oval inside that rectangle. An imaginary calculation is performed where the 25 mmol of Na⁺ are restricted to a volume of 1/6 l to have a final concentration of 150 mmol/l. The remaining 5/6 l is electrolyte-free water.

(a) Heat dissipation

In a healthy, fed, 70 kg adult male in steady state, approximately 2500 kcal are ingested and expended each day. The volume of insensible water loss is close to 1 l/day, but this causes a loss of only slightly > 500 kcal [24]. Hence ~2000 kcal of heat are removed daily by conduction and convection. The exact quantitative contribution of these latter routes of heat loss depends on environmental factors such as temperature, air circulation, humidity, cutaneous blood flow, clothing, etc. Hence they cannot be quantified from general guidelines.

When additional heat loss is needed, there is an increased production of sweat together with its evaporation. Therefore an appreciable volume of sweat can be anticipated during exercise or a febrile state, unless heat loss by conduction and/or convection can be augmented. Conversely, little water loss in sweat might occur when caloric expenditure declines, if there is little change in heat loss by conduction and convection.

(b) Heat production

There are three classes of nutrients, carbohydrate, fat, and protein [23]. On a typical Western diet, oxidation of carbohydrates and fatty acids generate ~85% of the adenosine triphosphate (ATP) needed to perform biological work. The limiting factor for oxidation of these fuels is the availability of adenosine diphosphate (ADP) in cells [27]. Thus the rate of fuel oxidation is dependent on how quickly ADP is regenerated during biological work and/or if oxidative phosphorylation is uncoupled [28,29].

There are three categories of biological work: biosynthetic, mechanical, and electrical work. To digest food, enzymes must be synthesized. For absorption of nutrients, there is a need for ion transport [30]. During the conversion of dietary fuels to storage forms of energy, there is biosynthesis of proteins [31], glycogen, and triglycerides. Hence it is not surprising that caloric expenditure is much lower when there is little physical activity (e.g., bed rest) and little dietary intake. In quantitative terms, there is a 50% decline in daily caloric expenditure during prolonged fasting with inactivity [19]. If a hospitalized patient is afebrile, does not eat, and is physically inactive, much less heat

production can be anticipated, and thereby there is a diminished need for water infusion to match its loss by evaporation.

Caloric expenditure (heat production) exceeds that needed for metabolic work if there is uncoupling of oxidative phosphorylation. This form of heat production is catalysed by uncoupler proteins [28,29]. The major uncoupler proteins that have an impact on the endogenous heat production in human subjects are those in skeletal muscle—their estimated contribution to energy metabolism in the rat is about 25% of resting energy turnover [28]. Moreover, the uncoupler proteins in skeletal muscle rise 5–10-fold during caloric deprivation [32]. This topic is discussed in more detail in reference [33].

In summary, it is unlikely that the contribution of uncoupler protein-catalyzed energy metabolism will be constant in a patient, and it should be extremely difficult to predict their total caloric expenditure while receiving much of his/her caloric supply from endogenous sources. Therefore relating body needs for electrolyte-free water in an *individual* patient is dependent on unreliable criteria—*estimated* energy expenditure based on body weight [15].

Analysis of electrolyte-free water loss in the urine of hospitalized patients

The kidneys regulate the excretion of water and Na^+ by independent mechanisms.

1. Excretion of water

In most hospitalized patients, there will be very little if any excretion of electrolyte-free water, because vasopressin makes the later parts of the distal nephron permeable to water [3]. Exceptions to this rule occur in diabetes insipidus, during a glucose- or urea-induced osmotic diuresis [34], and when there is a major renal concentrating defect.

2. Generation of electrolyte-free water

The infusion of isotonic saline can lead to a positive electrolyte-free water balance when this salt load is excreted in hypertonic form. The most common example of this type of generation of electrolyte-free water is when a large volume of isotonic saline is infused to prevent an appreciable fall in blood pressure in anaesthetized patients [4]. Later, when the level of the anaesthetic agent declines, the rate of excretion of $\text{Na}^+ + \text{K}^+$ rises due to a higher central venous pressure and the urine becomes hypertonic due to actions of vasopressin (Figure 6) [37].

Quantitatively, close to half of the infusate can be *desalinated* because the urine Na^+ plus K^+ concentration can rise to ~ 300 mmol/l [4]. Therefore, a large urine volume is not a ‘good urine output’ because it leads to the net addition of electrolyte-free water to the body, and thereby the development of acute hyponatraemia [4]. In this setting, measures to lower the urine

Na^+ plus K^+ concentration include a loop diuretic or inducing an osmotic diuresis with urea or mannitol [35].

3. Excretion of concentrated urine and extra renal work

One reason proposed to infuse electrolyte-free water to hospitalized patients is to minimize the work of the kidney [15]. Energy is expended for active, but not passive reabsorption of Na^+ [38]. While it has been suggested that work is required to excrete concentrated urine [39], we doubt that extra renal work was required for this task. Our rationale is that normal kidneys reabsorb 99.5% of filtered Na^+ , whether or not the final urine is concentrated (see Appendix for more discussion). Water reabsorption in the kidney is passive, and is controlled by the induction of water permeability of luminal membrane of the distal nephron [40]—this requires the expenditure of very little energy, so it can be ignored in the overall quantitative analysis of renal work.

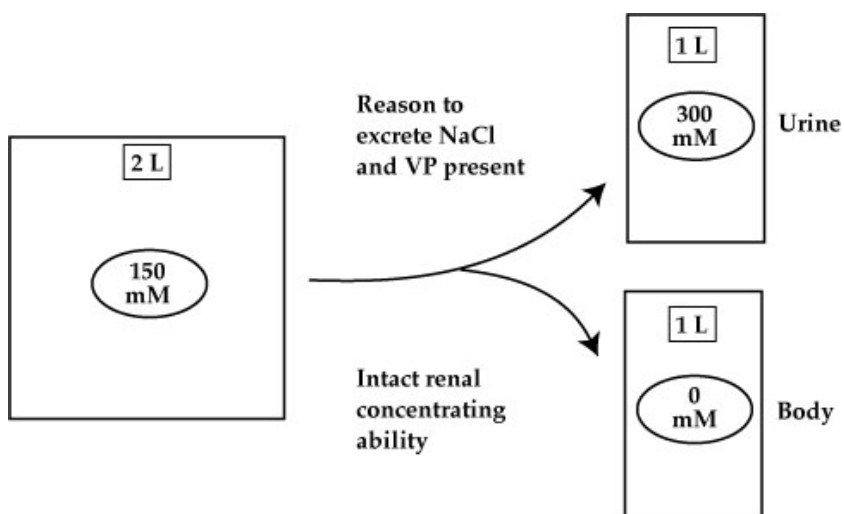


Figure 6: Desalination of administered isotonic saline

The larger rectangle to the left of the arrows represents the infusion of 2 l of isotonic saline; the concentration of Na^+ is shown in the oval inside that rectangle. A similar depiction is used for the excretion of Na^+ and water and they are shown to the right of the arrows. To have a concentration of Na^+ in the urine that is 300 mmol/l, vasopressin (VP) must act and there must be a reason to excrete NaCl. The remaining 1 l of electrolyte-free water is retained in the body [4].

Design a prescription for intravenous fluid administration

It is important to recognize that intravenous fluid prescriptions should be *individualized*. The priorities for the infusion of fluids are summarized in Table 3. Focusing on maintenance fluid administration, our view is that caloric expenditure should *not* be used to quantify its need [15].

1. What are the indications and contraindications to infuse hypotonic fluids?

Do not infuse hypotonic solutions if the P_{Na} is < 138 mmol/l unless the patient is having a rapid water diuresis and you want to limit the rise in P_{Na} . Rather, hypotonic fluids should be given to match the daily loss of electrolyte-free water in sweat in a patient who has a $P_{Na} > 138$ mmol/l. This volume is < 1 l/day unless the patient is febrile. There are two circumstances to infuse electrolyte-free water rapidly: first, when the P_{Na} is significantly > 145 mmol/l and if the patient is symptomatic. It should be noted that the P_{Na} can rise transiently by ~ 15 mmol/l during a seizure [41]. Second, replace renal loss of electrolyte-free water in a large water or osmotic diuresis, or if there is a non-renal loss via the gastrointestinal tract or the skin. The goal will be to create a positive balance for electrolyte-free water. To lower the P_{Na} by 1 mmol/l, the positive balance of electrolyte-free water should be $([1 / P_{Na}] \times \text{total body water, usually } 50\text{--}70\% \text{ weight depending on skeletal muscle and adipose tissue mass})$. For example, in a 50 kg person who has 28 l total body water, a positive balance of 0.2 l of electrolyte-free water would lower the P_{Na} from 140 to 139 mmol/l.

2. Do certain patients have unique factors that place them at greater risk of developing a more severe decline in their P_{Na} when given a particular volume of electrolyte-free water?

The answer is yes, and two factors should be considered. The first is the age of the patient, because brain cell number decreases with age, placing children and young adults at greater risk. The second factor concerns skeletal muscle mass relative to body weight, because 50% of body water is in skeletal muscle in normal subjects. Therefore in patients who have marked muscle atrophy due to disuse, nutritional problems, and/or a disease that involves skeletal muscle, much less electrolyte-free water needs to be retained to cause a serious decline in P_{Na} and, as a result, a greater degree of swelling of brain cells if the hyponatraemia is acute.

Concluding remarks

Patients who receive intravenous fluids often have a fall in their P_{Na} in hospital. This provides a rationale to re-evaluate current recommendations for intravenous therapy. Once adequate blood pressure is established, deficits and ongoing losses should be replaced. General guidelines that follow from the underlying physiology discussed in this paper are as follows. Do not give hypotonic solutions if the patient has a surplus of water in cells (a $P_{Na} < 138$ mmol/l in the absence of hyperglycaemia).

Table 3: Indications for the prescription of intravenous fluids

<i>I. Highest priority</i>
a) Defend haemodynamics 1. Re-expand a severely contracted ECF volume 2. Prevent a fall in blood pressure when venous tone is low (e.g., anaesthesia) b) Return the ICF volume towards normal 1. Acute hyponatraemia that is symptomatic <i>Infuse hypertonic saline to raise the P_{Na} by 5 mmol/l in 1–2 h</i> 2. Chronic hyponatraemia with a seizure <i>Infuse hypertonic saline to raise the P_{Na} by 5 mmol/l, but maximum is 8 mmol/l/day; a lower target should be set if the patient is malnourished or K^+-depleted</i> 3. Chronic asymptomatic hyponatraemia <i>Raise the P_{Na} by up to 8 mmol/l/day, slower rate if the P_K is low in a malnourished patient</i>
<i>II. Moderate priority</i>
1. Re-expand a modestly contracted ECF volume <i>Replace ongoing losses</i> <i>Avoid oliguria</i> <i>Match estimated electrolyte-free water loss in sweat and in the GI tract</i>

Do not administer isotonic saline in the absence of an indication to do so, because electrolyte-free water may be generated by desalination (Figure 6) [4]. In this context, a ‘good urine output’ can be a danger sign. The P_{Na} should be examined before infusing > 1–2 l hypotonic intravenous solutions. Be aware of occult source of oral water administration (e.g., ice chips to treat a dry mouth). Be even more cautious when planning intravenous

therapy for young patients and those with a small muscle mass. Do not advise subjects to drink a large quantity of water if they are taking drugs such as ‘ecstasy’ that are known to cause the release of vasopressin independent of the P_{Na} (reviewed in reference 42).

Appendix: a more detailed analysis of renal work

Renal work is almost exclusively due to active reabsorption of Na^+ in individual nephron segments, providing that Na^+ that was reabsorbed by a transcellular route [43], including the Na-K-ATPase—3 Na^+ pumped per ATP hydrolysed [44].

The proximal convoluted tubule

Not every Na^+ that enters proximal convoluted tubule cells is exported via the Na-K-ATPase. For example, when Na^+ is reabsorbed via H^+ secretion to reabsorb filtered HCO_3^- , one-third of the Na^+ that exits these cells is passively linked to $3HCO_3^-$ ($Na(HCO_3)_3^{2-}$). The resting membrane potential, a by-product of the Na-K-ATPase flux, drives this passive reabsorption of Na^+ . In quantitative terms, this passive process accounts for ~10% of proximal convoluted tubule Na^+ reabsorption.

Loop of Henle

The active reabsorption of Na^+ is in the thick ascending limb of the loop of Henle and involves the Na-K-2 Cl cotransporter. This Na-K-2 Cl cotransporter flux reabsorbs one Na^+ and two Cl^- ions. The second Na^+ is reabsorbed passively through the tight junctions between cells, driven by a lumen-positive voltage, because these junctions are permeable to Na^+ . Thus this half of Na^+ reabsorption in the thick ascending limb of the loop of

Henle bypasses the Na-K-ATPase and does *not* directly require the hydrolysis of ATP.

The reabsorption of Na^+ in the medullary thick ascending limb of the loop of Henle is necessary for both concentrating *and* diluting the urine. A higher medullary interstitial osmolality is important when the urine is maximally dilute, because it leads to the reabsorption of almost 2/3 of the 60 l of filtrate delivered daily from the proximal convoluted tubule in the descending thin limb of the loop of Henle [45]. This places an upper limit on water diuresis.

Distal nephron segments

Because all the reabsorption of Na^+ in the distal nephron traverses cells and because there is no passive transport of Na^+ across basolateral membranes that leads to a bypass of the Na-K-ATPase, more energy would need to be expended by the kidney to reabsorb the same amount of Na^+ daily by the kidney if less Na^+ were reabsorbed in its thick ascending limb.

References

1. Sterns RH, Silver SM, Spital A. Hyponatremia. In: Seldin DW, Giebisch G, eds. *The Kidney: Physiology & Pathophysiology*. Philadelphia PA, Lippincott Williams & Wilkins, 2000: 1217–38.
2. Halperin ML, Goldstein MB. *Fluid, Electrolyte and Acid-Base Physiology*, 3rd edn. Philadelphia PA, WB Saunders Company, 1998.
3. Robertson GL. Vasopressin. In: Seldin DW, Giebisch G, ed. *The Kidney: Physiology & Pathophysiology*. Philadelphia PA, Lippincott Williams & Wilkins, 2000: 1133–52.
4. Steele A, Gowrishankar M, Abrahamson S, Mazer D, Feldman R, Halperin ML. Postoperative hyponatremia despite isotonic saline infusion: A phenomenon of ‘desalination’. *Ann Intern Med* 1997; 126: 20–5.
5. Halberthal M, Halperin ML, Bohn D. Acute hyponatremia in hospitalized children: A quantitative analysis of factors contributing to the development and resolution of acute hyponatremia in hospitalized children. *BMJ* 2001; 322: 780–2.
6. Goldberg M. Hyponatremia. *Med Clin North Am* 1981;65:251–69.
7. Rose BD. New approach to disturbances in the plasma sodium concentration. *Am J Med* 1986; 81: 1033–40.
8. Anderson RJ. Hospital associated hyponatremia. *Kidney Int* 1986; 29: 1237–47.
9. Arieff AI. Hyponatremia, convulsions, respiratory arrest, and permanent brain damage after elective surgery in healthy women. *N Engl J Med* 1986; 314: 1529–35.
10. Hahn RG. The transurethral resection syndrome. *Acta Anaesthesiol Scand* 1991; 35: 557–67.
11. Gowrishankar M, Lin SH, Mallie JP, Oh MS, Halperin ML. Acute hyponatremia in the perioperative period: Insights into its pathophysiology and recommendations for management. *Clin Nephrol* 1998; 50: 352–60.
12. Noakes TD. Running, the kidneys and drinking too much—the hyponatraemia of exercise. *S Afr Med J* 2001; 91: 843–4
13. Smith H, Dhath G, Melia W, Dickinson J. Cystic fibrosis presenting as hyponatremic heat exhaustion. *BMJ* 1995; 310: 579–80.

14. Halperin ML, Skorecki KL. Interpretation of the urine electrolytes and osmolality in the regulation of body fluid tonicity. *Am J Nephrol* 1986; 6: 241–5.
15. Holliday M, Segar W. The maintenance need for water in parenteral fluid therapy. *Pediatrics* 1957; 19: 823–32.
16. Chesney RW. The maintenance need for water in parenteral fluid therapy. *Pediatrics* 1998; 102: 399–400.
17. Holliday MA, Segar WE. Reducing errors in fluid therapy management. *Pediatrics* 2003; 111: 424–5.
18. Moritz ML, Ayus JC. Prevention of hospital-acquired hyponatremia: A case for using isotonic saline. *Pediatrics* 2003; 111: 227–30.
19. Cahill GFJ. Starvation in man. *N Engl J Med* 1970; 282: 668–75.
20. Randle PJ. Fuel selection in animals. *Biochem Soc Trans* 1986; 14: 799–806.
21. Denton RM, McCormack JG, Rutter GA. Ca^{2+} and the regulation of intramitochondrial dehydrogenases. *Benzon Symposium* 1991; 30: 309–19.
22. Shepherd PR, Kahn BB. Glucose transporters and insulin action. *N Engl J Med* 1999; 341: 248–57.
23. Halperin ML, Rolleston FS. *Clinical Detective Stories: A Problem-Based Approach to Clinical Cases in Energy and Acid-Base Metabolism*, 1st edn. London, Portland Press, 1993.
24. Schmidt-Nielsen K. *Animal physiology: Adaptation and Environment*, 5th edn. Cambridge, Cambridge University Press, 1997.
25. Sato K, Kang WH, Saga K, Sato KT. Biology of sweat glands and their disorders. *J Am Acad Dermatol* 1989; 20: 537–63.
26. Quinton P. Physiology of sweat secretion. *Kidney Int* 1986; 32: S-102–8.
27. Flatt JP. On the maximal possible rate of ketogenesis. *Diabetes* 1972; 21: 50–3.
28. Brand MD. Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Exp Gerontol* 2000; 35: 811–20.
29. Lowell BB, Spiegelman BM. Towards a molecular understanding of adaptive thermogenesis. *Nature* 2000; 404: 652–60.
30. Field MJ. Intestinal ion transport and the pathophysiology of diarrhea. *J Clin Invest* 2003; 111: 931–43.

31. Cheema-Dhadli S, Halperin ML. Relative rates of appearance of nitrogen and sulphur: implications for postprandial synthesis of proteins. *Can J Physiol Pharm* 1993; 71: 120–7.
32. Boss O, Samec S, Dulloo A, Seydoux J, Muzzin P, Giacobino J-P. Tissue-dependent upregulation of rat uncoupling protein 2-expression in response to fasting or cold. *FEBS Lett* 1997; 412: 111–14.
33. Davids MR, Eduote Y, Jungas RL, Cheema-Dhadli S, Halperin ML. Facilitating an understanding of integrative physiology: Emphasis on the composition of body fluid compartments. *Can J Physiol Pharmacol* 2002; 80: 835–50.
34. Spira A, Gowrishankar M, Halperin ML. Factors contributing to the degree of polyuria in a patient with diabetes mellitus in poor control. *Am J Kidney Dis* 1997; 30: 829–35.
35. Davids MR, Edoute Y, Halperin ML. The approach to a patient with acute polyuria and hypernatremia: A need for the physiology of McCance at the bedside. *Netherlands J Med* 2001; 58: 103–10.
36. Davids M, Edout Y, Mallie J-P, Bichet DG, Halperin ML. Body compartment volumes and composition after giving a vasopressin antagonist: changes are revealed by a tonicity balance. *Nephrol Dial Transplant* 2002; 17: 300–3.
37. Carlotti AP, Bohn D, Mallie JP, Halperin ML. Tonicity balance and not electrolyte-free water calculations more accurately guide therapy for acute changes in natremia. *Intensive Care Med* 2001; 27: 921–4.
38. Oh MS, Carroll HJ. External balance of electrolytes and acids and alkali. In: Seldin DW, Giebisch G, eds. *The Kidney: Physiology & Pathophysiology*. Philadelphia PA, Lippincott Williams & Wilkins, 2000: 33–60.
39. Bankir L. Vasopressin and water conservation: The good and the evil. *Am J Kidney Dis* 1997; 30: xlv–xlvii.
40. Nielsen S, Frokiaer J, Marples D, Kwon TH, Agre P, Knepper MA. Aquaporins in the kidney: from molecules to medicine. *Physiol Rev* 2002; 82: 205–44.
41. Welt LG, Orloss J, Kydd DM, Oltman JE. An example of cellular hyperosmolarity. *J Clin Invest* 1950; 29: 935–9.
42. Cherney DZI, Davids MR, Halperin ML. Acute hyponatraemia and ‘ecstasy’: insights from a quantitative and integrative analysis. *QJM* 2002; 95: 475–83.

43. Mandel L, Balaban R. Stoichiometry and coupling of active transport to oxidative metabolism in epithelial tissues. *Am J Physiol* 1981; 240: F357–71.
44. Russell JM. Sodium-potassium-chloride cotransport. *Physiol Rev* 2000; 80: 211–76.
45. Oh MS, Halperin ML. The mechanisms of urine concentration in the inner medulla. *Nephron* 1997; 75: 384–93.
46. Halperin ML, Bichet DG, Oh MS. Integrative physiology of basal water permeability in the distal nephron: implications for the syndrome of inappropriate secretion of antidiuretic hormone. *Clin Nephrol* 2001; 56: 339–45.
47. Butler AM, Talbot NB. Parenteral-fluid therapy. 1. Estimation and provision of daily maintenance requirements. *N Engl J Med* 1944; 231: 585–90.

Chapter 15

**Summary, conclusions
and future directions**

Summary and conclusions

Chapter 1 is a brief introduction to the renal mechanisms and clinical disorders of water and sodium balance. The aims of the thesis are presented. The thesis is divided into two parts. Part A (*Chapters 2 – 6*) presents studies investigating the renal mechanisms of water and sodium balance regulation, while Part B (*Chapters 7 – 14*) presents studies investigating clinical disorders of water and sodium balance.

Chapter 2, the first chapter of Part A, is an introduction to the proteomics technique Difference Gel Electrophoresis (DIGE). Proteomics is a promising new technique with the ability to study cellular signaling pathways and identify clinical disease biomarkers. DIGE is currently one of the few techniques to perform quantitative proteomics, generating a statistical output to differences in protein abundances. The chapter discusses the principles of DIGE-based proteomics, including sample preparation, two-dimensional electrophoresis (2-DE), statistical analysis of 2D-gels, and mass spectrometry. Strengths and weaknesses of DIGE are discussed, including possible solutions to overcome certain limitations, such as the identification of low abundance and transmembrane proteins. By giving a synopsis of our recent proteomics studies, we illustrate how DIGE may be used to study the renal mechanisms of water and sodium balance regulation. Finally, we illustrate how quantification based on the DIGE approach combined with bioinformatics may facilitate the study of systems biology of the kidney.

In *Chapter 3*, DIGE-based proteomics is applied to a rat model of the “vasopressin escape” phenomenon. Escape from the antidiuretic action of vasopressin is an important physiologic process that limits the severity of the syndrome of inappropriate antidiuresis (SIADH) and other hyponatremic disorders. However, little is known about which intracellular signaling processes orchestrate the escape and how the phenomenon is triggered and maintained. Immunoblotting confirmed the hallmark of vasopressin escape, a decrease in aquaporin-2 (AQP2), and demonstrated a novel finding, namely a decrease in the abundance of the urea transporter UT-A3. DIGE identified 22 mostly high-abundance proteins regulated during vasopressin escape, which were subsequently analyzed using bioinformatics pathways analysis. One single dominant cluster emerged that pointed to several candidate regulators of vasopressin escape, including proteins that could regulate the aquaporin-2 gene (c-myc, c-fos, c-jun, SRC-1), proteins that may be involved in receptor internalization (c-src, RACK1), and proteins involved in protein folding in the endoplasmic reticulum (GRP78, protein disulfide isomerase, heat-shock protein 70). The regulation of these proteins in vasopressin escape was selectively confirmed by immunoblotting. These findings provide a new framework for the study of AQP2 regulation in the collecting duct, which is critical to the understanding of SIADH and other forms of hyponatremia.

In *Chapter 4*, prospects for urinary proteomics are discussed by presenting an overall strategy for biomarker discovery using urinary exosomes. Urinary exosomes are the internal vesicles of multivesicular bodies that are delivered to the urinary space by fusion of the outer membrane of multivesicular bodies with the

apical plasma membrane of renal tubule epithelial cells. A previous proteomic analysis of urinary exosomes identified 295 proteins, including many disease-associated proteins such as aquaporin-2, polycystin-1, podocin, nonmuscle myosin II, angiotensin converting enzyme, the $\text{Na}^+\text{-K}^+\text{-Cl}^+$ co-transporter 2 (NKCC2), the $\text{Na}^+\text{-Cl}^+$ co-transporter (NCC), and the epithelial sodium channel (ENaC). Although rigorous clinical testing is needed, urinary exosomes can be potentially used as biomarkers for early detection of disease, as a means of differential diagnosis, or as a means of guiding therapy.

In *Chapter 5*, the pathogenesis of hypokalemia in Gitelman's syndrome was investigated. Gitelman's syndrome is a genetic renal disorder caused by a loss of function mutation in the $\text{Na}^+\text{-Cl}^-$ cotransporter (NCC). Clinically, Gitelman's syndrome is characterized by hypokalemia, metabolic alkalosis, hypomagnesemia, hypocalciuria, and mild salt wasting. Surprisingly, hypokalemia was not detected in NCC knock-out (-/-) mice maintained on normal rodent diets, whereas the other features of Gitelman's syndrome were present. We showed that modest reduction of dietary potassium induced a marked reduction in plasma potassium and elevated renal potassium excretion in NCC (-/-) mice, which was associated with a pronounced polydipsia and polyuria of central origin. The possible mechanisms for elevated renal potassium excretion include increased circulating aldosterone levels, a reduction of the inhibitory influence of calcium on the activity of ENaC, or increased luminal flow. These results also provide insight to other forms of hypokalemia due to renal potassium loss, and emphasize the interactions that exist between the regulation of water, sodium, and potassium balance.

In *Chapter 6*, the mechanism of osmomediated natriuresis was investigated. Osmomediated natriuresis refers to the phenomenon that experimental animals and humans, when given the same sodium load, demonstrate a greater natriuresis when this sodium load is given in hypertonic form. We tested the hypothesis that osmomediated natriuresis is mediated by the calcium sensing receptor in the thick ascending limb of the loop of Henle. Using an established infusion protocol, we showed that osmomediated natriuresis in humans is associated with increased renal calcium and magnesium excretion, suggesting the site of action to be the thick ascending limb. In the thick ascending limb, hypertonic saline might indirectly activate the calcium sensing receptor by decreasing the 50% effective concentration of the calcium sensing receptor for calcium. Activation of the calcium sensing receptor can inhibit NKCC2, and cause a natriuresis. Evidence for this mechanism of “salinity sensing” was found by studying two patients with an inactivating mutation of the CaSR (familial hypocalciuric hypercalcemia). When these patients were given hypertonic saline, their urinary sodium, calcium, and magnesium excretion was attenuated, compared to the healthy volunteers. Our observations provide interesting new clues on the mechanism of osmomediated natriuresis and suggest novel roles for the calcium sensing receptor in human physiology.

In *Chapter 7*, the first chapter of Part B, we test the accuracy and utility of the traditional clinical diagnostic algorithms for hyponatremia, which are based on the clinical assessment of the extracellular fluid (ECF) volume, and laboratory parameters such as serum osmolality, urine osmolality and/or urine sodium concentration. Several weaknesses were identified, including a

failure to consider acute hyponatremia, the belief that a modest degree of ECF contraction can be detected by physical examination supported by routine laboratory data, and a tendency to diagnose SIADH prior to excluding other causes of hyponatremia. We conclude that the typical architecture of the clinical diagnostic algorithms for hyponatremia represents a hierarchical order of isolated clinical and/or laboratory parameters, and that they do not take into account the pathophysiological context, the mechanism by which hyponatremia developed and the clinical dangers of hyponatremia. These restrictions are important for physicians confronted with hyponatremic patients and may require them to choose different approaches. We therefore present a novel, more physiology-based approach to hyponatremia, which seeks to overcome some of the limitations of the existing algorithms.

In *Chapters 8 and 9*, risk factors for hospital-acquired hyponatremia were analyzed. Hospital-acquired hyponatremia is a common and potentially serious condition. Our objective was to identify disease or treatment related factors that predispose to the development of hyponatremia in hospital. In *Chapter 8*, patients who developed severe hyponatremia (serum sodium ≤ 125 mmol/l) in hospital were compared to patients who were admitted with severe hyponatremia. Hospital-acquired severe hyponatremia was mainly associated with treatment-related factors, including the use of thiazide diuretics, drugs stimulating antidiuretic hormone, surgery, and hypotonic intravenous fluids. The correction of hospital-acquired hyponatremia was slower than that of admission hyponatremia. The mortality rate was higher in patients who were not at all treated for hyponatremia. Therefore, the conclusion of the study was that early recognition

of risk factors and expedited therapy could make hospital-acquired severe hyponatremia more preventable. In *Chapter 9*, a matched case control study was conducted to further study hospital-acquired hyponatremia. On admission to the hospital, both cases and controls had a normal serum sodium concentration, while only the cases developed hyponatremia in hospital. During the development of hyponatremia, C-reactive protein increased in cases, whereas it decreased in controls. Furthermore, diabetes mellitus, and the use of insulin, antibiotics, and opioids were significantly more common in cases. Therefore, these factors may represent novel risk factors for hospital-acquired hyponatremia. Pathophysiologically, these factors may contribute to increased vasopressin release, which could be mediated by the acute phase response, pain and/or direct drug effects.

In *Chapter 10*, we focused on the relationship between hyponatremia and renal dysfunction. Little is known about how these two conditions are related, which type of renal dysfunction usually occurs, and which patients are at risk. The principal results demonstrate that renal dysfunction is common in severe hyponatremia and usually develops in an acute-on-chronic fashion with concomitant deterioration of both conditions. This concurrence is associated with heart failure, liver failure, and/or a renal drug regimen (loop diuretics, spironolactone, angiotensin converting enzyme inhibitors). A possible pathophysiological link between hyponatremia and renal dysfunction is the neurohumoral response. The neurohumoral response leads to increased vasoconstrictor forces including activation of the renin-angiotensin system and the sympathetic nervous system, and stimulation of antidiuretic hormone release, all of which can

simultaneously affect renal function and water-sodium balance. Given the recent results of clinical trials, this patient group may be especially suitable for treatment with vasopressin antagonists.

In *Chapter 11* the risk factors and mechanisms of hypernatremia (serum sodium ≥ 150 mmol/l) in patients admitted to the intensive care unit (ICU) were analyzed, thereby challenging the assumption that hypernatremia usually develops because of a negative water balance. The following risk factors for ICU-acquired hypernatremia were identified: hypokalemia, hypercalcemia, hyperglycemia, uremia, renal insufficiency, and the administration of mannitol or sodium bicarbonate. A multivariate analysis showed that these factors contributed to hypernatremia independent of the severity of the underlying disease. These factors point towards three mechanisms by which cases could have lost renal water, including nephrogenic diabetes insipidus (hypokalemia, hypercalcemia), osmotic diuresis (hyperglycemia, mannitol, uremia), and renal insufficiency. Another interesting finding was that hypernatremia due to a positive sodium balance was more common than previously appreciated. Apparently, hypernatremia in critically ill patients is not only a problem of too little water, but also of too much salt. Therapy should therefore rely on adding electrolyte free water and/or creating a negative sodium balance. Adjustments in intravenous fluid regimens may prevent hypernatremia.

Chapters 12, 13 and 14 study the role of intravenous fluid therapy in fluid and electrolyte disorders in children. In *Chapter 12*, it was shown that a fall in the effective serum osmolality during therapy for diabetic ketoacidosis was associated with an increased risk of cerebral edema. The effective osmolality fell because there was an insufficient rise in the serum sodium concentration, or because there was a large fall in the serum glucose concentration. Indeed, children who developed cerebral edema received larger volumes of intravenous fluids with a lower sodium concentration, and more insulin. The development of hypernatremia seemed to prevent cerebral edema, because it was associated with a stable course of the effective osmolality. The conclusion of *Chapter 12* was that an adequate rise in serum sodium concentration during the treatment of diabetic ketoacidosis in children is needed to prevent a fall in the effective serum osmolality. In *Chapter 13*, the basis of acute hyponatremia in hospitalized children was studied, demonstrating that acute hyponatremia was associated with the administration of hypotonic intravenous fluids and a positive fluid balance. Hospital-acquired hyponatremia unnecessarily puts children at risk for the development of adverse neurologic events and is largely preventable. The original guidelines for maintenance fluid may therefore not be applicable in an era when the complexity and the severity of illness seen in hospitalized children who receive intravenous fluid therapy has radically changed (e.g., leukemia, complex congenital heart disease) and irregularities of antidiuretic hormone secretion are more likely to be commonplace. Therefore, *Chapter 14* concludes this thesis with a discussion on how to select optimal maintenance fluid therapy, emphasizing strategies to prevent iatrogenic hyponatremia.

Samenvatting en conclusies

Het handhaven van de water en zout balans is cruciaal voor de overleving van mens en dier. De nieren spelen een centrale rol in het reguleren van deze water en zout balans. Afhankelijk van de behoefte van het lichaam, zorgen de nieren ervoor dat water en zout, maar ook andere stoffen zoals kalium, magnesium, calcium en fosfaat, uitgescheiden of juist vastgehouden worden. Hiertoe ontvangen de nieren verschillende signalen uit het lichaam, die vooral door hormonen in het bloed doorgegeven worden. Twee belangrijke hormonen zijn het antidiuretisch hormoon, dat vooral de waterbalans reguleert, en aldosteron, dat vooral de zoutbalans reguleert. In de zogenaamde tubuli van de nieren bevinden zich receptoren voor deze hormonen. Als het hormoon de receptor bereikt heeft, worden speciale water en zout kanalen geactiveerd die ervoor zorgen dat water en zout terugkeren naar de bloedsomloop. Deze speciale water en zout kanalen werden ongeveer tien jaar geleden ontdekt en betekende een revolutie in het denken over nierfysiologie. Zoals gezegd is het handhaven van de water en zout balans belangrijk voor het functioneren van alledag. Hierdoor zijn we bijvoorbeeld in staat om 's nachts niet te hoeven drinken, te sporten en ons aan te passen aan een warm klimaat. Het belang van de water en zout balans wordt echter vooral duidelijk op het moment dat deze balans verstoord raakt. Mensen kunnen namelijk ziek worden, en in het ergste geval zelfs overlijden, als ze te veel water vasthouden (hyponatriëmie), of juist te veel water uitscheiden (hypernatriëmie). Daarnaast speelt de water en zout balans een belangrijke rol in andere ziektebeelden zoals een hoge bloeddruk (hypertensie), hartfalen en leverfalen.

Dit proefschrift heeft twee doelstellingen: om de mechanismen in de nier te bestuderen die betrokken zijn bij de water en zout regulatie (deel A) en om de klinische aspecten van stoornissen in de water en zout balans (of: electrolytstoornissen) te onderzoeken (deel B). Hieronder zullen de belangrijkste resultaten en conclusies van dit proefschrift per hoofdstuk besproken worden.

Hoofdstuk 1 is een korte introductie over de mechanismen in de nier die betrokken zijn bij de regulatie van de water en zout balans en de klinische ziektebeelden die kunnen optreden bij een verstoring van die balans.

Hoofdstuk 2, het eerste hoofdstuk van Deel A, geeft een overzicht van de proteomicstechniek Difference Gel Electrophoresis (DIGE). Proteomics is een veelbelovende, nieuwe techniek, waarmee met behulp van grootschalig eiwitonderzoek cellulaire cascades en biomarkers van klinische ziekten geïdentificeerd kunnen worden. Zo kan proteomics worden ingezet om te onderzoeken welke eiwitten betrokken zijn bij het reguleren van water en zout kanalen (zie *Hoofdstuk 3*). Klinisch zou proteomics van bijvoorbeeld urine gebruikt kunnen worden voor het opsporen, differentiëren en vervolgen van nefrologische en andere aandoeningen (zie *Hoofdstuk 4*). Van de proteomics technieken is DIGE op dit moment één van de weinige technieken waarmee proteomics op een kwantitatieve manier uitgevoerd kan worden. Dit betekent dat verschillen in eiwitconcentraties met DIGE statistisch geanalyseerd kunnen worden. Het hoofdstuk behandelt de principes van DIGE-proteomics, inclusief het prepareren van monsters voor proteomics, zogenaamde twee-dimensionele

electroforese, de statistische analyse van twee-dimensionele gels en massa spectrometrie. De voor- en nadelen van DIGE worden besproken, evenals mogelijke oplossingen om met DIGE toch eiwitten die in lage concentraties aanwezig zijn en transmembraan eiwitten te identificeren. Tenslotte illustreren we hoe met de combinatie van DIGE-proteomics en bio-informatica de studie van de nier op het niveau van de systeembioogie uitgevoerd kan worden.

In *Hoofdstuk 3* wordt DIGE gebruikt om het zogenaamde “vasopressine escape” fenomeen te bestuderen. Vasopressine escape is een belangrijk fysiologisch proces dat de ernst van SIADH (een syndroom waarin er een teveel aan antidiuretisch hormoon is) en andere vormen van hyponatriëmie beperkt. Er is weinig bekend over welke processen in de cel betrokken zijn bij het activeren en handhaven van het vasopressine escape fenomeen. Daarom onderzochten wij met de technieken DIGE, immunoblotting (een immunologische techniek waarmee eiwitconcentraties bepaald kunnen worden) en bio-informatica welke eiwitten betrokken waren bij het vasopressine escape fenomeen in de verzamelbuis van de nier. Met immunoblot-technieken werd eerst de aanwezigheid van het vasopressine escape fenomeen bevestigd. Dit werd gedaan door aan te tonen dat de concentratie van het waterkanaal aquaporine-2 afgenomen was. Met immunoblot-technieken werd eveneens een nieuw resultaat geboekt, namelijk dat ook de concentratie van het ureumkanaal UT-A3 gedaald was ten tijde van vasopressine escape. Vervolgens identificeerde DIGE-proteomics 22 eiwitten die een rol leken te spelen in het vasopressine escape fenomeen. De resultaten van DIGE werden met immunoblotting herhaald en bevestigd. Deze eiwitten werden vervolgens met bio-

informatica technieken geanalyseerd om te bestuderen op welke manier ze aan elkaar gerelateerd waren. Met deze analyse werd één cluster van eiwitten gevonden die het vasopressine escape fenomeen lijkt te reguleren. Drie categorieën eiwitten werden geïdentificeerd: eiwitten die het gen dat codeert voor aquaporine-2 kunnen reguleren (c-myc, c-fos, c-jun, SRC-1), eiwitten die betrokken zijn bij het internaliseren van bepaalde receptoren (c-src, RACK1), en eiwitten die betrokken zijn bij het vouwen van andere eiwitten in het endoplasmatisch reticulum (GRP78, protein disulfide isomerase, heatshock protein 70). Deze resultaten verschaffen een nieuw kader voor het bestuderen van de regulatie van het waterkanaal aquaporine-2 in de verzamelbuis van de nier. Dit is belangrijk om de pathofysiologie van SIADH en andere vormen van hyponatriëmie beter te leren begrijpen.

In *Hoofdstuk 4* wordt besproken hoe proteomics van zogenaamde urine exosomen gebruikt kan worden om biomarkers voor ziekten van de nier te identificeren. Urine exosomen zijn vesikels die gevormd worden door het epitheel van de niertubuli. Een eerdere proteomics analyse van deze urine exosomen toonde de aanwezigheid van 295 eiwitten aan. Veel van deze eiwitten zijn betrokken bij bepaalde nierziektes, zoals aquaporine-2, polycystine-1, podocine, nonmuscle myosine II, angiotensin convertende enzyme, de natrium-kalium-chloride co-transporter 2, de natrium-chloride-co-transporter, en het epitheliale natriumkanal. Hoewel nog uitgebreide klinische studies noodzakelijk zijn, kunnen urine exosomen in de toekomst mogelijk ingezet worden bij de vroegdiagnostiek, differentiaal diagnose en behandeling van nierziekten.

In *Hoofdstuk 5* werd de ontstaanswijze van hypokaliëmie (een laag kaliumgehalte in het bloed) in het syndroom van Gitelman onderzocht. Het syndroom van Gitelman is een genetische nierziekte waarbij er een inactiverende mutatie is van de natrium-chloride-co-transporter (NCC). Het syndroom van Gitelman wordt gekenmerkt door hypokaliëmie, metabole alkalose (relatief basisch bloed), hypomagnesiëmie (een laag magnesiumgehalte in het bloed), hypocalciurie (een lage calcium uitscheiding), en mild renaal natrium verlies. In een muizenmodel van het syndroom van Gitelman (NCC knock-out muizen) werd eerder géén hypokaliëmie gezien, terwijl alle andere kenmerken van het syndroom wel aanwezig waren. Wij toonden aan dat het ontstaan van hypokaliëmie afhankelijk is van de hoeveelheid kalium in het dieet van deze muizen. Bij een vermindering van het kaliumgehalte in het dieet ontwikkelden de muizen hypokaliëmie met een verhoogde kaliumuitscheiding door de nier. Tegelijkertijd ontwikkelden de muizen een indrukwekkende polydipsie en polyurie van centrale oorsprong (d.w.z. ontstaan in het centrale zenuwstelsel). Deze bevindingen geven inzicht in de manier waarop hypokaliëmie ontstaat in het syndroom van Gitelman. Mogelijke mechanismen zijn: verhoogde aldosteronconcentraties, een vermindering van de remmende werking van calcium op de activiteit van het epitheliale natriumkanal ENaC in de verzamelbuis en/of een verhoogde stroomsnelheid in het distale nefron. Deze bevindingen geven ook inzicht in het ontstaan van andere vormen van hypokaliëmie en de interacties tussen de regulatie van de water, natrium en kalium balans.

In *Hoofdstuk 6* werd het mechanisme van osmogemedieerde natriurese onderzocht. Osmogemedieerde natriurese is het fenomeen dat proefdieren en mensen méér natrium uitscheiden als dezelfde hoeveelheid natrium in geconcentreerde vorm (hyperosmolair) gegeven wordt. Het mechanisme van osmogemedieerde natriurese is onbekend. In de studie werd de hypothese getoetst dat osmogemedieerde natriurese gemedieerd wordt door de zogenaamde calcium-sensing receptor (CaSR) in het dikke, opstijgende been van de lis van Henle. Door middel van infusiestudies in gezonde vrijwilligers toonden wij aan dat de verhoogde uitscheiding van natrium eveneens gepaard gaat met een verhoogde uitscheiding van calcium en magnesium. Dit suggereert inderdaad dat het mechanisme van osmogemedieerde natriurese via het dikke, opstijgende been van de lis van Henle verloopt. Het zou daarom kunnen dat hypertoon zout de CaSR activeert door de gevoeligheid van de receptor voor calcium te vergroten. Het activeren van de CaSR kan vervolgens de natrium-kalium-chloride-co-transporter type 2 remmen, zodat er minder natrium gereabsorbeerd wordt en zo een natriurese ontstaat. Aanvullend bewijs voor dit mechanisme werd gevonden door ook twee patiënten te bestuderen die een defect in de CaSR hebben. Het ziektebeeld dat deze patiënten hebben heet familiale hypocalciurische hypercalciëmie. Het toedienen van hypertoon zout aan deze twee patiënten resulteerde in minder uitscheiding van natrium, calcium en magnesium dan in de gezonde vrijwilligers. Deze observaties verschaffen nieuwe inzichten in het mechanisme van osmogemedieerde natriurese en suggereren een nieuwe rol voor de CaSR in de humane fysiologie. Het mechanisme van osmogemedieerde natriurese lijkt te bestaan om hypernatriëmie maar bijvoorbeeld ook het ontstaan van nierstenen te voorkomen.

In *Hoofdstuk 7*, het eerste hoofdstuk van Deel B, werd de nauwkeurigheid en bruikbaarheid getoetst van de bestaande algoritmen voor hyponatriëmie. Deze algoritmen kunnen de arts helpen om de oorzaak van hyponatriëmie op te sporen. Ze worden daarom frequent gebruikt in de klinische praktijk. Echter, wij vonden meerdere onvolkomenheden in de algoritmen. Zo maakten de algoritmen geen onderscheid tussen acute en chronische hyponatriëmie. Ook gingen de algoritmen ervan uit dat de volumestatus van de patiënt bepaald kan worden door het lichamelijk onderzoek in combinatie met laboratoriumonderzoek. Tenslotte werd de diagnose SIADH te snel gesteld, zonder eerst andere oorzaken van hyponatriëmie uit te sluiten. Onze conclusie was daarom dat de huidige algoritmen voor hyponatriëmie te weinig rekening houden met de pathofysiologische context, de manier waarop hyponatriëmie ontstaat, en de klinische gevaren van hyponatriëmie. Deze beperkingen zijn belangrijk voor artsen die regelmatig met patiënten met hyponatriëmie geconfronteerd worden. Daarom kan een andere benadering noodzakelijk zijn. Wij presenteren daarom een nieuwe benadering voor de diagnostiek van hyponatriëmie, die meer gebaseerd is op fysiologische principes en waarin getracht wordt de geconstateerde beperkingen van de huidige algoritmen te verhelpen.

In *Hoofdstukken 8 en 9* worden de risicofactoren voor hyponatriëmie die in het ziekenhuis ontstaat geanalyseerd. Ziekenhuisgeassocieerde hyponatriëmie is een veel voorkomende en potentieel ernstige aandoening.

In *Hoofdstuk 8* werden patiënten met een ernstige ziekenhuisgeassocieerde hyponatriëmie (een natriumconcentratie van ≤ 125 mmol/l) vergeleken met patiënten die bij opname al een ernstige hyponatriëmie hadden. Het bleek dat de ontwikkeling van ziekenhuisgeassocieerde hyponatriëmie gerelateerd was aan de therapie van de patiënt. De geïdentificeerde risicofactoren voor ziekenhuisgeassocieerde hyponatriëmie waren namelijk: thiazide diuretica, geneesmiddelen die de afgifte van het antidiuretisch hormoon stimuleren, chirurgie, en hypotone infuusvloeistoffen. Daarnaast bleek de correctiesnelheid van ziekenhuisgeassocieerde hyponatriëmie langzamer dan die van hyponatriëmie die al bij opname aanwezig was. Bij patiënten die helemaal niet voor hyponatriëmie behandeld werden, was de mortaliteit hoger. De conclusie van de studie was dat de risicofactoren van ziekenhuisgeassocieerde hyponatriëmie onderkend en, indien mogelijk, vermeden moeten worden. Als ziekenhuisgeassocieerde hyponatriëmie aanwezig is, moet deze adequaat behandeld worden.

In *Hoofdstuk 9* werden twee groepen patiënten vergeleken in een gematched case-controle onderzoek. Bij opname in het ziekenhuis hadden zowel de cases als de controles een normale natriumconcentratie. Vervolgens ontwikkelden de cases ziekenhuisgeassocieerde hyponatriëmie (natriumconcentratie < 136 mmol/l, daling ≥ 7 mmol/l), terwijl de natriumconcentratie in de controles normaal bleef (tussen de 136 en 145 mmol/l, fluctuatie < 7 mmol/l). Tijdens het ontstaan van hyponatriëmie, steeg het zogenaamde C-reactive protein in de cases, terwijl het daalde in de controles. Daarnaast kwamen diabetes mellitus (suikerziekte) en het gebruik van insuline, antibiotica en

opioïden (zware pijnstillers) vaker voor bij de cases. Deze factoren lijken daarom nieuwe risicofactoren te zijn voor ziekenhuisgeassocieerde hyponatriëmie. Een pathofysiologische verklaring voor deze resultaten zou kunnen zijn dat de acute fase respons (C-reactive protein, infectie), pijn (opioïden) en/of een direct effect van geneesmiddelen (antibiotica, opioïden, insuline) bijdragen aan een niet-fysiologische afgifte van het antidiuretisch hormoon. Ook hier geldt dat bij de aanwezigheid van één van deze factoren, de natriumconcentratie nauwkeurig gecontroleerd moet worden en hyponatriëmie tijdig behandeld dient te worden.

In *Hoofdstuk 10* werd de relatie onderzocht tussen het gelijktijdig optreden van hyponatriëmie en nierinsufficiëntie. Er is weinig bekend over hoe deze twee aandoeningen aan elkaar gerelateerd zijn, welk type nierinsufficiëntie meestal optreedt, en welk soort patiënten risico loopt op deze combinatie. De resultaten laten zien dat er vaak een gelijktijdige en acute verslechtering optreedt van zowel de hyponatriëmie als de nierinsufficiëntie. Dit gelijktijdige beloop blijkt met name voor te komen in patiënten met hartfalen of leverfalen die geneesmiddelen gebruiken die van invloed zijn op zowel de water en zout balans als op de nierfunctie (lisdiuretica, spironolacton, en remmers van het angiotensine convertend enzyme). De pathofysiologische verklaring voor het gelijktijdig optreden van hyponatriëmie en nierinsufficiëntie is de neurohumorale respons. De neurohumorale respons leidt zowel tot meer vasoconstrictie (waardoor het renine angiotensine systeem en het sympathische zenuwstelsel geactiveerd worden) als tot afgifte van het antidiuretisch hormoon. Op deze manier kan gelijktijdig de water en zout balans en de nierfunctie nadelig

beïnvloed worden. Gezien de resultaten van recente klinische trials, lijkt behandeling met vasopressine receptor antagonisten geschikt voor patiënten met gelijktijdige hyponatriëmie en nierinsufficiëntie.

In *Hoofdstuk 11* werd onderzocht wat de risicofactoren zijn en wat het mechanisme is van hypernatriëmie die ontstaat in patiënten die opgenomen worden op de intensive care. Hypernatriëmie werd gedefinieerd als een natriumconcentratie van ≥ 150 mmol/l. In deze studie werd ook onderzocht of de veronderstelling waar is, dat hypernatriëmie zich meestal ontwikkelt ten gevolge van een negatieve waterbalans. De volgende risicofactoren voor het ontwikkelen van hypernatriëmie op de intensive care werden gevonden: hypokaliëmie, hypercalciëmie, hyperglycemie, uremie, nierinsufficiëntie en het toedienen van mannitol en natrium bicarbonaat. Een multivariaat analyse liet zien dat deze factoren onafhankelijk van elkaar en onafhankelijk van de ernst van de onderliggende ziekte bijdragen aan hypernatriëmie. Deze factoren suggereren dat hypernatriëmie op drie manieren kan ontstaan: via nefrogene diabetes insipidus (hypokaliëmie, hypercalciëmie), een osmotische diurese (hyperglycemie, mannitol, uremie), en nierinsufficiëntie. Deze factoren leiden vaak tot het uitscheiden van hypotone (verdunde) urine. Hypernatriëmie ontstaat vervolgens omdat het lichaam meer water dan zout verliest en omdat er in het infuus vaak een hogere natriumconcentratie aanwezig is dan in de urine. Een ander resultaat van deze studie was dat hypernatriëmie ten gevolge van een positieve zoutbalans vaker voorkwam dan voorheen werd aangenomen (één derde van de gevallen). De

conclusie van de studie was daarom ook dat hypernatriëmie niet alleen een probleem van te weinig water, maar ook van te veel zout is. De behandeling van hypernatriëmie op de intensive care zou daarom zowel op het toedienen van water als op het creëren van een negatieve zoutbalans gericht moeten zijn. Aanpassingen in het infuusbeleid zouden hypernatriëmie op de intensive care meer kunnen voorkomen.

De *Hoofdstukken 12 en 13* bestuderen de rol van infuusvloeistoffen bij het ontstaan van stoornissen in de water en zout balans in kinderen. Kinderen zijn extra gevoelig voor de complicaties van electrolytstoornissen.

In *Hoofdstuk 12* werd de relatie onderzocht tussen de effectieve osmolaliteit (de som van de natrium, kalium en glucose concentraties in het bloed) en het ontstaan van hersenoedeem tijdens de behandeling van diabetische ketoacidose (ontregelde suikerziekte). Hersenoedeem kan blijvende hersenschade veroorzaken of zelfs dodelijk zijn. Wij toonden aan dat de effectieve osmolaliteit daalde in de kinderen die hersenoedeem ontwikkelden, terwijl deze stabiel bleef of steeg in de kinderen die geen hersenoedeem ontwikkelden. Hypernatriëmie voorkwam de ontwikkeling van hersenoedeem. De reden dat de effectieve osmolaliteit daalde was dat de natriumconcentratie in het bloed tijdens de behandeling van diabetische ketoacidose onvoldoende steeg óf dat de glucose concentratie te veel daalde. De oorzaak hiervan was dat de kinderen die hersenoedeem ontwikkelden meer infuusvloeistof met een lagere natriumconcentratie én meer insuline ontvingen. De conclusie van *Hoofdstuk 12* was dat er een adequate stijging van de natriumconcentratie nodig is tijdens de behandeling van

diabetische ketoacidose in kinderen. Dit voorkomt een daling in de effectieve osmolaliteit en daarmee het ontstaan van hersenoedeem. Hiertoe moet het infuus- en insulinebeleid aangepast worden.

In *Hoofdstuk 13* werd de oorzaak van acute ziekenhuisgeassocieerde hyponatriëmie in kinderen bestudeerd. Het bleek dat dit gerelateerd was aan het toedienen van hypotone infuusvloeistoffen, zoals nog regelmatig gebeurd in de kindergeneeskunde. Hypotone infuusvloeistoffen bevatten relatief weinig natrium en relatief veel water. Deze vorm van hyponatriëmie brengt kinderen daarom onnodig in gevaar voor de neurologische complicaties van hyponatriëmie en kan dus voorkomen worden. De oorspronkelijke richtlijnen voor het infuusbeleid in kinderen lijken niet meer toepasbaar in een tijd waarin de complexiteit en de ernst van de ziekten waarmee kinderen worden opgenomen in het ziekenhuis radicaal veranderd is. Hierdoor zal het vaker zo zijn dat het antidiuretisch hormoon in hogere concentraties aanwezig is, waardoor deze kinderen meer water zullen vasthouden en daarom meer kans hebben om hyponatriëmie te ontwikkelen.

Omdat het infuusbeleid electrolytstoornissen kan veroorzaken of kan verergeren, besluit *Hoofdstuk 14* dit proefschrift, waarin algemene richtlijnen voor het infuusbeleid in volwassenen en kinderen gegeven worden, waarbij de nadruk ligt op het voorkomen van iatrogene hyponatriëmie.

Water and salt: future directions

I. From the outside to the inside: WNK kinases

The discovery of the major renal transport proteins along the nephron has revolutionized the understanding of renal physiology. The last decade, research has focused on the regulation of renal transport proteins in health and disease [1]. More recently, the emphasis has shifted from renal transporters to their intracellular regulatory proteins. An illustration of this “outside to inside” trend is the discovery of the WNK kinases. WNK stands for “with no K”, where K stands for the amino-acid lysine. The WNK family of serine-threonine kinases is unique in having a substitution of cysteine for lysine in subdomain II of the catalytic core of the kinase domain [2]. The connection between WNK kinases and kidney ion transport was made through positional cloning studies of patients with familial hyperkalemic hypertension (also known as pseudo-hypoaldosteronism type II or Gordon’s syndrome) [3]. Familial hyperkalemic hypertension has been described as the “mirror image” of Gitelman’s syndrome and was also shown to be a disorder of the distal convoluted tubule (DCT) [3]. Consequently, WNK kinases appeared to be involved in renal sodium and potassium handling by the DCT. The rapidly evolving cell model for the DCT currently shows prominent roles for (the transcripts of) WNK1, WNK3, and WNK4 in the regulation of NCC, ROMK, and ENaC [2,3]. Physiologically, the WNK kinases may function as a “molecular switch” to enable aldosterone to reabsorb sodium during hypovolemia, and secrete potassium during hyperkalemia (Figure 1) [3]. In this respects, it may be the “molecular answer” to the aldosterone

paradox, which was originally described by Halperin and colleagues, addressing the question how aldosterone could at the same time be a sodium reabsorbing and potassium secreting hormone [4].

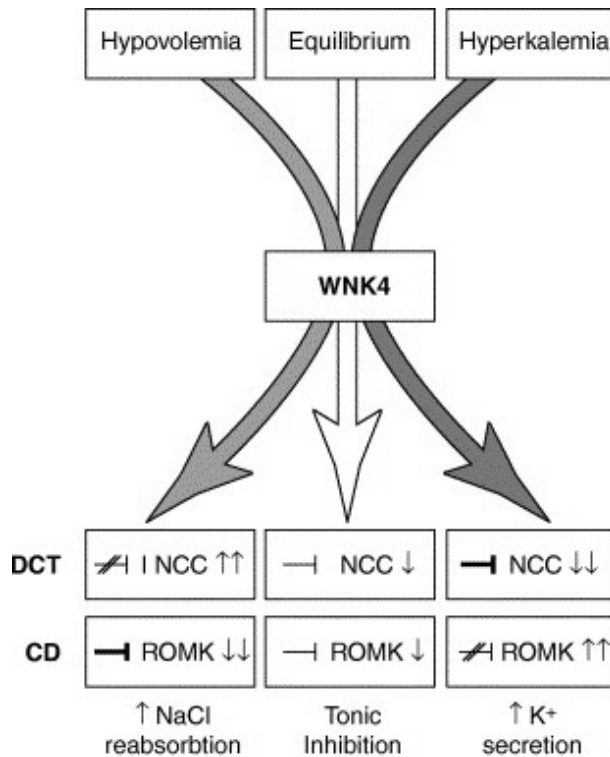


Figure 1: *WNK4 controls the balance between renal reabsorption of NaCl and secretion of KCl in the aldosterone-sensitive distal nephron by differentially regulating the thiazide-sensitive NCC and the ROMK. With kind permission from [2].*

II. From the inside to the outside: urinary proteomics

Urinary proteomics is a promising method to identify urinary biomarkers for renal disease (Reference 5, see also *Chapter 4*). In general, urinary proteins may originate from the glomerular filtrate, renal tubular secretion, cell shedding, proteolytic cleavage of glycoproteins (e.g., Tamm-Horsfall), or exosomes. Urinary exosomes may be of special interest, because exosome formation appears to be an active and potentially regulated process by which renal transporters and intracellular proteins are excreted into the urine [5,6]. In addition, proteomic profiling of urinary exosomes demonstrated them to contain several disease associated proteins [5,6]. The first urinary proteomics study was carried out by Kanno *et al.*, who demonstrated that the water-channel aquaporin-2 can be measured in human urine, and that its concentration can differentiate between diabetes insipidus of central and renal origin [7]. Currently, early urinary proteomics studies are being conducted, studying various diseases, including genetic renal electrolyte disorders, minimal change disease, membranous nephropathy, focal segmental glomerular sclerosis, diabetic nephropathy, acute renal allograft rejection, urolithiasis, acute renal failure, renal and bladder cancer, and autosomal dominant polycystic kidney disease [5,8]. Especially in diseases where early identification, differential diagnosis or therapeutic monitoring is important, urinary proteomics may be applicable as a non-invasive and cost-effective technology. In this regard, one could hypothesize a future role for urinary proteomics in hypertension, the treatment of which is currently largely empirical. If unique fingerprints in the urinary proteome exist for different types of (essential) hypertension, this may

eventually help to personalize therapy and classify this heterogeneous disorder. The application of urinary proteomics to hypertension was recently illustrated in a study by Olivieri *et al.* [9]. In patients with primary aldosteronism and hypertension, they demonstrated a specific pattern of the urinary excretion of prostasin, which is a membrane-bound serine protease regulating the epithelial sodium channel (ENaC) in the renal collecting duct [9]. Thus, urinary prostasin was postulated to be a marker for ENaC activation in humans, making it an attractive candidate marker for primary aldosteronism and other forms of hypertension.

III. From investigation to intervention: “aquaretics”

The study of the vasopressin axis is an interesting example of how “investigation” has ultimately led to “intervention”. Because several clinical disorders are associated with inappropriate vasopressin release and action, the challenge was to specifically inhibit the vasopressin axis. As early as the 1970s, Manning and Sawyer started developing linear peptide vasopressin 2 receptor (V2R) antagonists by modifying the potent antidiuretic peptide desmopressin [10]. Although these agents exhibited V2R antagonism in animals, they still had agonistic properties in humans. Because the peptide antagonists also had a poor bioavailability and a short half-life, other investigators started developing nonpeptide antagonists by using functional screening strategies [10]. To date, this quest has yielded four vasopressin antagonists, including SR-121463, lixivaptan, tolvaptan, and conivaptan, the latter of which also has V1a antagonistic properties [11].

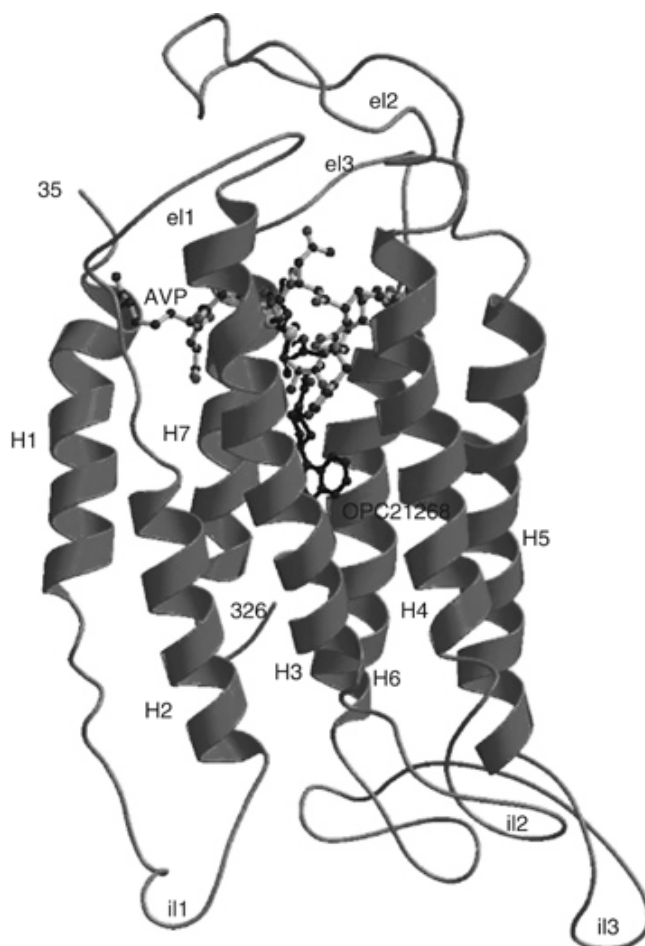


Figure 2: V2 receptor shown as a ribbon diagram.

For the receptor, loops labeled 'e' are extracellular and loops labeled 'i' are intracellular. Models of arginine vasopressin (AVP) and a V2 receptor antagonist (OPC21268) are shown at the sites where they would dock were the other not present. Binding sites are distinct with partial overlap. The antagonist, which lodges deeper in the receptor than AVP, prevents AVP docking but does not interact with the receptor's active site in the H1 helix. With kind permission from [10].

Phase 3 clinical trials in patients with dilutional hyponatremia have shown that all of these agents induce a water diuresis (as evidenced by an increase in free water clearance and a decrease in urine osmolality) and, consequently, correct hyponatremia [11]. Few side effects were observed, including no effect on renal sodium, chloride, and potassium excretion, no thromboembolic events (despite a steep increase in vasopressin levels), and no apparent symptoms of osmotic demyelination. Although these preliminary results hold promise, especially for the treatment of hyponatremia in SIADH, heart and liver failure, important questions remain to be answered. For example, the differences between V2-specific and mixed V1a/V2 receptor antagonists, and the general long-term effects of these agents should be evaluated.

IV. Back to the future: integrative physiology

The molecular developments described above are important to increase the understanding of fluid and electrolyte physiology, and, ultimately, to improve patient care. However, by definition, molecular biology focuses on minute components of the overall physiological system, bearing the danger of a reductionistic view. Therefore, “integrative physiology”, in which physiological principles are applied to clinical challenges, remains a useful strategy to the practice of modern medicine [12]. This is especially true for fluid and electrolyte disorders, which often require a physiology-based approach with a focus on the underlying mechanisms. Examples of integrative physiology concepts include the “effective” plasma osmolality, which may be used to analyze osmotic shifts, the tonicity balance, which may be used to evaluate the pathogenesis and

therapeutic goals in dysnatremia, and the transtubular potassium gradient, which is a physiological measure of mineralocorticoid action on the collecting tubule [12,13,14]. Several investigators have contributed to the application of integrative physiology to clinical medicine, including Halperin [13], McCance [15], and Rose [14].

References

1. Nielsen S, Frokiaer J, Marples D, Kwon TH, Agre P, Knepper MA. Aquaporins in the kidney: from molecules to medicine. *Physiol Rev* 2002; 82: 205-44.
2. Kahle KT, Wilson FH, Lifton RP. Regulation of diverse ion transport pathways by WNK4 kinase: a novel molecular switch. *Trends Endocrinol Metab* 2005; 16: 98-103.
3. Subramanya AR, Yang CL, McCormick JA, Ellison DH. WNK kinases regulate sodium chloride and potassium transport by the aldosterone-sensitive distal nephron. *Kidney Int* 2006; 70: 630-4.
4. Kamel KS, Oh MS, Halperin ML. Bartter's, Gitelman's, and Gordon's syndromes. From physiology to molecular biology and back, yet still some unanswered questions. *Nephron* 2002; 92 Suppl 1: 18-27.
5. Hoorn EJ, Pisitkun T, Zietse R, Gross P, Frokiaer J, Wang NS, Gonzales PA, Star RA, Knepper MA. Prospects for urinary proteomics: exosomes as a source of urinary biomarkers. *Nephrology (Carlton)* 2005; 10: 283-90.
6. Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A*. 2004; 101: 13368-73.
7. Kanno K, Sasaki S, Hirata Y, Ishikawa S, Fushimi K, Nakanishi S, Bichet DG, Marumo F. Urinary excretion of aquaporin-2 in patients with diabetes insipidus. *N Engl J Med*. 1995; 332: 1540-5.
8. O'Riordan E, Goligorsky MS. Emerging studies of the urinary proteome: the end of the beginning? *Curr Opin Nephrol Hypertens* 2005; 14: 579-85.
9. Olivieri O, Castagna A, Guarini P, Chiecchi L, Sabaini G, Pizzolo F, Corrocher R, Righetti PG. Urinary prostatic acid phosphatase: a candidate marker of

- epithelial sodium channel activation in humans. *Hypertension* 2005; 46: 683-8.
10. Greenberg A, Verbalis JG. Vasopressin receptor antagonists. *Kidney Int* 2006; 69: 2124-30.
 11. Palm C, Pistrosch F, Herbrig K, Gross P. Vasopressin antagonists as aquaretic agents for the treatment of hyponatremia. *Am J Med* 2006; 119: S87-92.
 12. Halperin ML, Bohn D. Clinical approach to disorders of salt and water balance. Emphasis on integrative physiology. *Crit Care Clin* 2002; 18: 249-72.
 13. Halperin ML, Goldstein MB. *Fluid, Electrolyte, and Acid-Base Physiology*. 3rd. ed. Philadelphia: W.B. Saunders Company; 1999.
 14. McCance RA. Medical problems in mineral metabolism. III. Experimental human salt deficiency. *Lancet* 1936; 230: 823–830.
 15. Rose BD, Post TW. *Clinical physiology of acid-base and electrolyte disorders*. 5th ed. New York, NY: McGraw-Hill; 2001.

List of publications

1. Hoorn EJ, Betjes MG, Zietse R, Halperin ML. Clinical thinking and decision making in daily practice. An old man with hyponatremia (Letter). *Ned Tijdschr Geneeskd* 2002; 22; 146: 1206.
2. Shafiee MA, Bohn D, Hoorn EJ, Halperin ML. How to select optimal maintenance intravenous fluid therapy. *QJM* 2003; 96: 601-10.
3. Hoorn EJ, Geary D, Robb M, Halperin ML, Bohn D. Acute hyponatremia related to intravenous fluid administration in hospitalized children: an observational study. *Pediatrics* 2004; 113: 1279-84.
4. Hoorn EJ, Halperin ML, Zietse R. Diagnostic approach to a patient with hyponatraemia: traditional versus physiology-based options. *QJM* 2005; 98: 529-40.
5. Hoorn EJ, Pisitkun T, Zietse R, Gross P, Frokiaer J, Wang NS, Gonzales PA, Star RA, Knepper MA. Prospects for urinary proteomics: exosomes as a source of urinary biomarkers. *Nephrology (Carlton)* 2005; 10: 283-90.
6. De Vogel S, Hoorn EJ, Papademetriou V. Losartan in cardiovascular disease. *Future Cardiology* 2005; 1: 433-46.
7. Hoorn EJ, Hoffert JD, Knepper MA. Combined proteomics and pathways analysis of collecting duct reveals a protein regulatory network activated in vasopressin escape. *J Am Soc Nephrol* 2005; 16: 2852-63.
8. Hoorn EJ, de Vogel S, Zietse R. Insulin resistance in an 18-year-old patient with Down syndrome presenting with hyperglycaemic coma, hypernatraemia and rhabdomyolysis. *J Intern Med* 2005; 258: 285-8.

9. Hoorn EJ, Lindemans J, Zietse R. Development of severe hyponatraemia in hospitalized patients: treatment-related risk factors and inadequate management. *Nephrol Dial Transplant* 2006; 21: 70-6.
10. Morris RG, Hoorn EJ, Knepper MA. Hypokalemia in a mouse model of Gitelman's syndrome. *Am J Physiol Renal Physiol*. 2006; 290: F1416-20.
11. Hoorn EJ, Lindemans J, Zietse R. Acute and concomitant deterioration of hyponatremia and renal dysfunction associated with heart and liver failure. *Clin Nephrol* 2006; 65: 248-55.
12. Hoorn EJ, Hoffert JD, Knepper MA. The application of DIGE-based proteomics to renal physiology. *Nephron Physiol* 2006; 104: p61-72.
13. Beukhof CM, Hoorn EJ, Lindemans J, Zietse R. Novel risk factors for hospital-acquired hyponatraemia: a matched case-control study. *Clin Endocrinol (Oxf)*, in press.
14. Hoorn EJ, Carlotti AP, Costa LA, MacMahon B, Bohn G, Zietse R Halperin ML, Bohn D. Preventing a fall in the effective plasma osmolality to minimize the likelihood of cerebral edema during therapy of children with diabetic ketoacidosis. *J Pediatr*, in press.
15. Hoorn EJ, Zietse R. Combined renal tubular acidosis and diabetes insipidus in hematological disease. *Nat Clin Pract Nephrol*, in press.
16. Hoorn EJ, Pisitkun T, Knepper MA. Proteomic approaches for the study of cell signaling in the renal collecting duct. *Contrib Nephrol*, in press.

Dankwoord

Bladerend door de proefdrukken lijkt het proefschrift op een reisdocument. Figuurlijk, omdat de verschillende studies vertrek- en aankomstpunten zijn in een wetenschappelijke ontwikkeling: van desillusie tot eureka en van ontsnapte muizen tot geaccepteerde artikelen. Letterlijk, omdat de studies in Toronto, Washington D.C. en Rotterdam werden uitgevoerd met kleurrijke persoonlijkheden van verschillende nationaliteiten. Dit alles maakte het een ontzettend vormende, boeiende en mooie reis. Graag wil ik daarom iedereen die direct of indirect aan dit proefschrift heeft bijgedragen bedanken.

Mijn promotor: prof.dr. W. Weimar. Beste Willem, hoewel dendritische cellen aquaporines tot expressie brengen, heeft ons onderzoek verder weinig raakvlakken. Dat stond een positieve reactie van jouw kant op dit onderzoek en dit proefschrift nooit in de weg en dat waardeer ik zeer. Dank dat je mijn promotor wilt zijn.

Mijn copromotor: dr. R. Zietse. Beste Bob, het Rotterdamse water en zout onderzoek kreeg vorm onder jouw bezielende begeleiding. Ik zal onze woensdagmiddagbesprekingen niet snel vergeten, hardop filosoferend over duizelige exosomen, de fysiologie van de vampire bat en de geheimen van de distal convoluted tubule. Maar ook de extra-renale avonturen zijn blijvende herinneringen: jouw Jack Nicholson act in het keurige 1789, de schietstoelen in de Amtrak trein en de letterlijke waterzooi in Brussel. Dank voor alles, to be continued en yakshemash!

Prof. Halperin, dear Mitch, it is a great honour to have you in my PhD committee. Your approach of integrative physiology to clinical medicine is truly unique, and this thesis would have looked completely different without your input. I hope to have captured something of your critical appraisal. Thank you, and, cheers!

Prof. Bohn, dear Des, this adventure started in your department. Your enthusiasm for medicine and science made it a kick-start – thank you for your terrific support. Also thanks to the other (alumni) PICU department members, especially Dr. Chris Parshuram (for the X-factor), Dr. Sian Jagger (both socially and shoulderly), Dr. Peter Cox (for altruistic co-dislocation), and Helena Frndova (the quotes!). Dr. Ana Carlotti, thank you for our collaboration on the DKA study. In deze context wil ik ook graag drs. E. van der Voort bedanken. Beste Edwin, uiteindelijk begon ook alles via jou (“reisbureau Sophia”) en ik heb je enthousiaste begeleiding zeer gewaardeerd. Natuurlijk kan ik dan ook niet Caroline Pijpers vergeten: bedankt voor jouw functie als “matchmaker”.

Dr. Knepper, dear Mark, it was a great pleasure to work in your famous lab. You introduced me to the intriguing aspects of molecular renal physiology and you were an inspiring tutor along the way. Thanks also to the other (alumni) LKEM-members, especially Dr. Ryan Morris (“small but mighty”), Dr. Jason Hoffert (Team DIGE), Dr. Trairak Pisitkun (the proteomics prince), Dr. Robert Fenton (for pre-eminence and 24h of Cork), Dr. Shyama Masilamani, and Angel Aponte.

Prof. Knoers, water en zout minnend Nederland kan niet om Nijmegen heen – hartelijk dank dat u zitting wilde nemen in de promotiecommissie.

Prof. Devuyst, thank you for being on my PhD committee. Your Aquaporins conference was superb and I hope we will be able to collaborate on mutual interests in the near future.

De overige leden van de promotiecommissie, de hoogleraren Danser, Lindemans en Wilson, dank ik voor hun zitting in de promotiecommissie en de snelle beoordeling van het proefschrift.

Graag wil ik de mensen van een aantal afdelingen in het Erasmus Medisch Centrum bedanken. Allereerst de afdelingen nefrologie en dialyse, in het bijzonder Willij Zuidema en Adela den Breejen. Mijn speciale dank gaat uit naar dr. E.H. Ie, beste Han-Yo, dank voor al jouw “hoe te promoveren” tips en voor alle interessante gesprekken, met als onbetwist hoogtepunt ons bezoek aan Meg & Jack. Alle andere HRU-leden (jullie weten waarvoor de “H” staat), NIO’s en nefrologen: dank voor de prettige samenwerking en sfeer. Vervolgens het klinisch chemisch laboratorium, in het bijzonder prof.dr. J. Lindemans (tevens voor deelname in de promotiecommissie) en Gert Verheij. De afdeling directie informatie: met name Trudy Bontje, Natascha Tahitu en Guido van Lansbergen. Voor de samenwerking bij de infusiestudie wil ik drs. Carola Zillikens, Bianca en Marjolijn (immer vrolijk ondanks haperende venflons en piepende infuuspompen) en dr. Frans Boomsma bedanken. Uiteraard ook dank aan alle patiënten en vrijwilligers die meewerkten aan de dorstig makende infusiestudie!

Alle studenten met wie ik heb samengewerkt wil ik bedanken voor hun inspanningen: Carolien Beukhof, Daphne Hotho, Meindert Crop, Lotte Cremers en Merel Hamer (alias K2), Roeland Zoutendijk en Jurriaan Hamstra. Ik hoop een beetje van het enthousiasme voor electrolytstoornissen aan jullie te hebben overgebracht.

Graag wil ik de Nierstichting hartelijk danken voor hun ondersteuning bij meerdere wetenschappelijke projecten uit dit proefschrift.

Alle arts-assistenten en internisten uit het Medisch Centrum Rijnmond Zuid wil ik bedanken voor de prettige samenwerking en jullie tolerantie ten aanzien van mijn standaardvraag tijdens de overdracht: “Hoe zit dat eigenlijk met die hyponatriëmie?”

Fotografe Maaïke Koning wil ik bedanken voor de voorkant van het proefschrift – onze tweede medische co-productie!

De redacteuren van het universiteitsblad Erasmus Magazine, drs. A. Hofstede en ir. G. van der Ende, wil ik bedanken. Beste Ad en Gert, jullie hebben mijn pen bijgeslepen van mooischrijverij naar journalistiek, en dat kwam zeer van pas bij dit proefschrift.

Beste Gitara Edward, Maarten ten Berg, Wouter de Jong en Pieter Stolk: wie had gedacht toen we een biertje op vrijdagmiddag in de Madison Pub in Toronto dronken dat we nu allemaal aan het promoveren zouden zijn! Het is een eer om de eerste te zijn en ik kijk met veel belangstelling uit naar jullie proefschriften.

Frans de Weert, Matthijs de Noord en Harald van Beek (“Meppeler Boys”) wil ik danken voor onze bijzondere vriendschap. Beste Frans en Matthijs, dank ook dat jullie mijn paranimf willen zijn: wederom in stijl, maar dit keer zonder klompen.

Mijn ouders en broer wil ik bedanken voor de betrokkenheid en ondersteuning op weg naar dit proefschrift en uiteraard het plezierige contact door de jaren heen, dat zeker heeft bijgedragen aan een kritische geest en een onmisbaar relativiseringsvermogen.

Lieve Sanne, sinds vogel vlieg ik! Net toen ik de hypothese dat jij de allerleukste bent als bewezen beschouwde, moest ik zo nodig in Amerika nog een paar hypothesen toetsen. Maar jij kwam ook en inmiddels kan ik niet meer zonder jouw vrolijke kijk op het leven. Eindconclusie: jouw bijzondere aantrekkingskracht wint het van de aantrekkingskracht van het natrium ion.

Curriculum vitae

Ewout Jasper Hoorn werd op 16 oktober 1978 geboren in Meppel. Hier voltooide hij in 1997 het gymnasium aan de rijksscholengemeenschap “Stad en Esch”. Aan de Erasmus Universiteit Rotterdam haalde hij in 2001 het doctoraal examen geneeskunde en in 2003 het artsexamen. De eerste aanzet tot dit proefschrift vormde een afstudeeronderzoek naar hyponatriëmie aan de University of Toronto in Toronto, Canada, onder leiding van prof. D. Bohn en prof.dr. M.L. Halperin, die beiden betrokken bleven bij vervolgstudies in dit proefschrift. Via prof.dr. Halperin ontmoette hij dr. R. Zietse, met wie het Rotterdamse “water en zout” onderzoek opgestart werd en die copromotor van dit proefschrift is. Van 2004 tot 2005 werkte hij een jaar in het Laboratory of Kidney and Electrolyte Metabolism aan de National Institutes of Health in Bethesda, Verenigde Staten. Onder supervisie van dr. M.A. Knepper werd hier basaal wetenschappelijk onderzoek verricht naar de renale mechanismen van water en zout regulatie, onder andere gebruikmakend van proteomics technieken. Van 2005 tot 2006 verrichtte hij basaal en klinisch onderzoek in het Erasmus Medisch Centrum. Sinds 2006 is hij in opleiding tot internist in het Medisch Centrum Rijnmond Zuid (opleider dr. A. Berghout) en het Erasmus Medisch Centrum (opleider dr. J.L.C.M. van Saase).

