Monitoring extra cellular fluid volume during renal function measurement

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STELLINGEN
behorende bij het proefschrift:

Monitoring Extra Cellular Fluid Volume during Renal Function Measurement

1. The cold war may be over, the salt war endures *(MH Alderman ;Am J Hypertens 1997)*
2. Bij elke 'tracer gemeten' GFR bepaling zou ECFV bepaald moeten worden *(dit proefschrift)*
3. Nierfunctie zou uitgedrukt moet worden in tijd die de nier nodig heeft om zijn werkvolume (lees: ECFV) van afvalstoffen te ontdoen: normalisatie van GFR ten opzichte van ECFV geeft deze maat *(dit proefschrift)*
4. Hoe hoger het kreatinine aanbod aan de nier, des te hoger de tubulaire excretie van kreatinine *(dit proefschrift)*
5. Het succes van diëten in de 1e week kan toegeschreven worden aan daling van ECFV bij verlaging van de zoutinname *(dit proefschrift)*
6. The fatter, the wetter *(dit proefschrift)*
7. Zoutgevoeligheid van bloeddruk wordt gekenmerkt door een verhoogde weefselactiviteit van het Renine-Angiotensine Systeem *(dit proefschrift)*
8. Bij studies naar anemie moet rekening gehouden worden met ECFV *(dit proefschrift)*
9. Matigheid is een deugd, omdat gulzigheid en dronkenschap slecht voor je gezondheid zijn *(Epicurus;Griekenland; 342 – 270 v.Chr)*
10. Door medisch handelen verdwijnt teleologie uit de humane fysiologie
11. Er zit meer geloof in wetenschap dan wetenschap in geloof
12. Believe nothing, no matter where you read it, or who said it, no matter if I have said it, unless it agrees with your own reason and your own common sense *(Siddhartha Gautama; aka Boeddha; Nepal; ca. 450-370 v.Chr)*
13. Tijdens de huidige kredietcrisis houdt het socialisme het kapitalisme in stand
14. Referenda zijn ondemocratisch
15. Voetbal is opium voor het volk
16. De mens is de maat van alle dingen *(Protagoras; Griekenland; 490-420 v.Chr)*

ECFV = Extra Cellular Fluid Volume (Extracellulair Volume)

Folkert Willem Visser
Groningen, 8 december 2008

RIJKSUNIVERSITEIT GRONINGEN

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CONTENTS

List of abbreviations

ADH	anti diuretic hormone
Aldo	plasma aldosterone
aldosteron ant	aldosteron antagonists
AngII	angiotensin II
ARB	angiotensin receptor blocker
Aza	Azathioprine
BMI	body mass index
BNP	brain natriuretic peptide
BSA	body surface area
BW	body weight
CKD	chronic kidney disease
CNI	Calcineurin Inhibitor
CHF	chronic heart failure
COV	coefficient of variation
CrCl	creatinine clearance
CRP	C-reactive protein
CsA	Cyclosporine A
DBP	diastolic blood pressure
ECFV	extra cellular fluid volume
eGFR	estimated GFR
EPO	erythropoietin
ERPF	effective renal plasma flow
FE_{creat}	fractional excretion of creatinine
FE_{Na+}	fractional excretion of sodium
FeSat	transferin saturation
FF	filtration fraction
FL_{Na+}	filtered load of sodium
GFR	glomerular filtration rate
HOMA	homeostatic model assessment
HS	high sodium diet
IOT	^{125}I-iothalamate
LS	low sodium diet
LVEF	left ventricular ejection fraction
MAP	mean arterial pressure
MMF	mycophenolate mofetil
NHANES	National Health And Nutrition Examination Survey
NYHA	New York Heart Association
ns	not significant
NT-proBNP	N-terminal proBNP
O/P ratio	Observed/Predicted ratio
PRA	Plasma renin activity
Pred	Prednisolon
RAAS	renin angiotensin aldosterone system
RBF	renal blood flow
RF	renal failure
rHuEPO	recombinant human erythropoietin
sbp	systolic blood pressure
SD	standard deviation
SR	sodium resistant blood pressure
SS	sodium sensitivity of blood pressure
TIBC	total iron binding capacity
TR_{Na+}	tubular reabsorption of sodium
Tx	transplantation
UAE	urinary albumin excretion
Vd	distribution volume

INTRODUCTION AND AIMS

Introduction

High salt intake as a pathogenetic factor

The association between a high salt intake and the risk for cardiovascular and renal disease has been a topic of long standing interest. Traditionally, the effect of high sodium intake on blood pressure is assumed to account for the association between salt intake and cardiovascular and renal disease. Epidemiological studies within and between different populations have provided extensive evidence for an association between mean sodium intake, blood pressure level, and the prevalence of hypertension[1-3]. Short term[4] and long term intervention studies (>1 year)[5-8] show that dietary sodium restriction results in a modest, but dose-dependent blood pressure lowering[9]. However, individual responses to sodium restriction are diverse. Approximately 50% of hypertensives are considered to be sodium-sensitive[10-13], meaning that blood pressure significantly drops when shifting from a normal to a low sodium diet. In normotensive subjects the proportion of sodium-sensitive subjects is some 30%[11]. Thus, although a relation between sodium and blood pressure is clearly present, the individual variability is large. This could implicate that not all subjects are equally sensitive to the pathogenetic effects of a high sodium intake (which is probably true[11,14]), but also, that blood pressure may not be the best or the only relevant parameter to assess the pathogenetic effects of a high sodium intake.

In line with the latter assumption, sodium intake has been shown to exert blood pressure independent effects as well[15]. First, blood pressure independent relations were found between higher sodium intake and parameters of cardiovascular end organ damage, such as a larger left ventricular mass[16-20], and a higher arterial resistance[21-24]. Moreover, animal data as well as human experimental studies provided evidence for blood pressure independent effects of a high sodium diet on

the vascular wall, such as endothelial dysfunction[25], and increased ACE activity[26,27]. Interestingly, blood pressure independent associations were also observed between high sodium intake and parameters of renal damage such as proteinuria[28], albuminuria[29,30] and glomerular hyperfiltration[31,32], in particular in association with overweight[33].

The relation between sodium intake and mortality has long been a matter of dispute. One of the factors fuelling the disparity is the difficulty in obtaining an accurate measure of sodium intake. During steady state 24 hour urinary sodium excretion provides a solid assessment of sodium intake, but the difficulties in obtaining large scale accurate 24 hour urine collections are considerable[34]. Accordingly, food-questionnaires have been used as an alternative in many studies, but unfortunately their accuracy in estimating true sodium intake is poor[35,36]. Yet, evidence has gradually accumulated over the years that support an adverse effect of sodium intake on mortality. First, in the INTERSALT study, an association was found between average sodium excretion per participating country and stroke mortality rate (figure 1)[37].

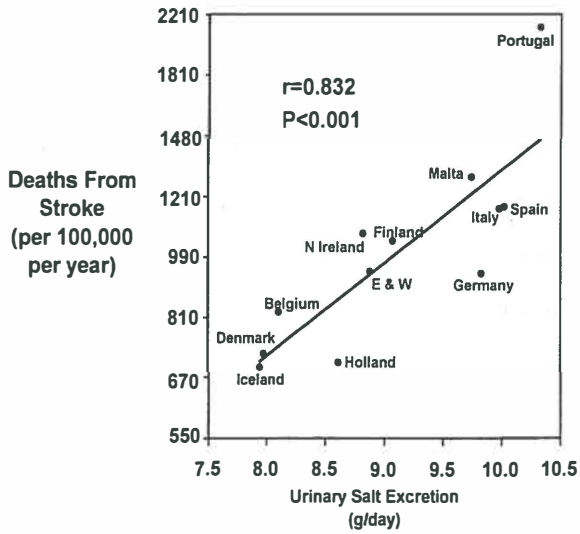

Figure 1 Relation between urinary Na^+ excretion and death from stroke in 12 European populations from the INTERSALT study (adapted from Perry et al[37].

In a prospective study of 2463 subjects in Finland, baseline urinary sodium excretion was directly correlated to mortality and risk of coronary events[38], the risk being increased by some 50% by a 6 gram increase in daily salt intake. Remarkably, in the latter two studies the association between sodium intake and increased morbidity and mortality was independent of blood pressure level. Furthermore, a recent study provided landmark data from the follow-up of the TOPH I and II trial[39], showing a reduced risk of cardiovascular events after 10-15 years of follow-up in the original intervention groups as compared to the control groups, of pre-hypertensive subjects. In the intervention group sodium intake had been reduced by some 50 mmol/day during the study period of 18-48 months. As the follow-up data were obtained by phone and post, unfortunately no data on blood pressure and sodium excretion during follow-up were available.

However, reports from the NHANES (National Health And Nutrition Examination Survey) illustrate that analyses of the effects of sodium intake for long term prognosis can be complicated and thus raise considerable controversy. In this large population-based cohort study, with a follow-up of 27-31 years, sodium intake at baseline was assessed by questionnaires. First, Alderman et al[40] showed an association between high sodium intake and survival benefit. Later, He et al[41] criticized the statistical analyses in the study from Alderman et al, and concluded from the same survey that a high sodium intake was associated with an increased cardiovascular morbidity and mortality. Interestingly, the analyses by He et al showed that high dietary sodium intake was associated with an increased risk only in overweight, but not in lean subjects, indicating an interaction between overweight and effects of sodium intake.

Sodium intake and the regulation of body fluid volume

Thus, whereas current evidence supports adverse effects of high sodium intake, controversies remain. So far, the large majority of studies on the adverse effects of high sodium focused on its effect on blood pressure. As it is becoming increasingly clear that sodium has also blood pressure independent effects, however, it might be fruitful to consider the physiological adaptations of the body to differences in sodium intake first.

Sodium, or Na^+, is the main positive electrolyte of the extra cellular fluid compartment. The concentration of the constituents of the different body fluid compartments, the intra cellular and the extra cellular fluid compartment, is kept within more or less stable limits by intricate regulation systems, thus ensuring stable conditions for the biochemical processes that constitute the human physiology. The maintenance of this stable condition is called *homeostasis* of the *milieu intérieur*, and is an important prerequisite for the normal function of cells and organs in the body. The extra cellular fluid compartment, that provides the environment for the cells, accounts for approximately one-third of the human body volume whereas the cells account for approximately two-third. As sodium, with its corresponding negative electrolyte Cl^- is the main electrolyte of the extra cellular fluid, it is also the main determinant of the extra cellular fluid volume (ECFV) and accordingly, regulation of sodium status is the key mechanism of volume homeostasis[42,43].

Sodium intake can vary extensively by differences in sodium content of the diet ranging from, for instance, 10 mmol/day in various indigenous populations, to over 800 mmol/day in Northern Japan[44]. Over this wide range the serum concentrations of Na^+ are maintained stable despite varying intake, while higher and lower sodium intake are accompanied by corresponding changes in ECFV.

This is accomplished by the combined effects of osmoregulation and volume regulation. A rise in sodium intake elicits a subtle rise in plasma [Na^+] that leads to release of the antidiuretic hormone (ADH) from the hypophysis in response to a rise in osmolality. ADH decreases water excretion and promotes thirst, to the extent that plasma [Na^+] and osmolality return to their original values, at the expense of a rise in ECFV. When the higher salt intake persists the ensuing rise in ECFV elicits a rise in natriuretic peptides from the heart, and moreover, suppresses the renin-angiotensin-aldosterone system (RAAS), which allows to gradually increase the rate of renal sodium excretion until it matches intake again. By then, a new steady state is achieved, characterized by a stable [Na^+], and an increase in total body sodium and a parallel increase in ECFV as illustrated in figure 2. Of note, in most healthy subjects this rise in ECFV, and consequently cardiac output, does not result in a rise in blood pressure, due to a concomitant decrease in peripheral vascular resistance.[43,45]

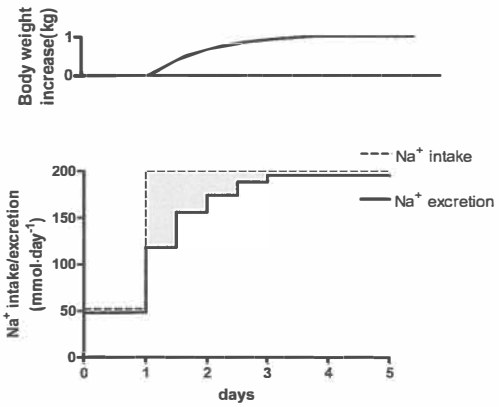

Figure 2 Shift in dietary sodium intake. Lower part: theoretical changes in Na^+ excretion after a dietary shift from 50 to 200 mmol Na^+·$24h^{-1}$ Upper part: corresponding change in body weight due to the change in dietary Na^+ intake and Na^+ balance. Figure adapted from Koomans et al[45].

In the Netherlands, in the general population[29] mean sodium intake is approximately 137 mmol·$24h^{-1}$ with an upper quintile of 220 mmol·$24h^{-1}$ and a lower quintile of 80 mmol·$24h^{-1}$. This amounts to an average difference in ECFV of approximately 1 liter[45,46]. It would be logical to assume that the corresponding differences in volume load for the heart bear pathophysiological relevance.

Accordingly, measurement of ECFV and its responses to differences in sodium intake might shed more light on both mechanisms of sodium-associated morbidity, and individual differences in susceptibility to the adverse effects of high sodium intake. However, large scale measurements of ECFV are not available so far.

Methods of extra cellular fluid volume assessment

The importance of assessment of ECFV is well-recognized in clinical practice, in particular in acute disease conditions. Yet, despite the fact that assessment of circulatory status is important in many clinical conditions, the measurement of ECFV has not become part of routine or even sophisticated clinical assessment, and has only a modest place in clinical research[47]. This relates to the fact that the available methods are either inaccurate, or considered too invasive for clinical routine. The distribution volume (Vd) of bromide provides the gold standard for ECFV measurement[48,49]. Its kinetics is well established and correction factors are proposed for correction of the Donnan equilibrium and for a small proportion of the bromide entering erythrocytes[50]. Its assessment however requires injection and timed follow-up, which is cumbersome in clinical practice. Bio-impedance has been proposed as a non-invasive method for measuring ECFV. However, its precision, reproducibility and accuracy are low. Especially the large inter-observer variability makes the method of less value in daily practice and also limits its value in scientific protocols[51,52].

An alternative way to measure ECFV may be by specific tracers used for measurement of glomerular filtration rate (GFR). Measurement of the renal clearance of specific tracers such as inulin, iothalamate, iohexol, and ^{51}Cr-EDTA, provides the gold standard for renal function measurement, and is used in top-clinical care as well as clinical research settings[53]. These methods are

predominantly based on the one-compartment technique, and therefore require tracers that distribute over the extra cellular space, since this is the fluid compartment which is cleared by the kidneys[54-56]. Some studies were reported that used the distribution volumes of inulin[57], ^{51}Cr-EDTA[54], ^{99m}Tc-DTPA[58] and iohexol[55], respectively, as measures for ECFV. Whereas these methods provide good estimates of ECFV, they are usually not applied to that purpose. As these tracers require injection and timed follow-up, they have no specific advantage over bromide. Yet, in patients in whom renal function is measured anyway they could provide an assessment of ECFV. As volume status, and in particular volume overload is an important pathophysiological factor in renal patients, this might not only be of scientific but also of clinical interest. Moreover, it has been suggested that normalization of renal function to ECFV could have specific advantages over normalization to body surface area (BSA), in particular in obese subjects[54,59-63].

In our centre measurement of GFR as the clearance of constantly infused ^{125}I-iothalamate (IOT)[64,65] is routinely used for renal function for top-clinical care, such as kidney donor screening and follow-up of transplant recipients, as well as clinical research. Animal studies have shown a uniform distribution of IOT over the ECFV [66-68], suggesting that the Vd of this tracer is well-suited for the assessment of ECFV, be it or not simultaneously with GFR. However, the feasibility of this tracer for assessment of ECFV in human has not been demonstrated, and requires specific validation. If this could be accomplished the renal function measurements would provide, at no extra effort or cost, a simultaneous assessment of ECFV, thus providing data on ECFV on a scale that is unique world-wide, mostly obtained in renal patients, i.e. a population where disturbances of volume regulation plays an important pathogenetic role.

Aims of the thesis

The aim of this thesis is, first, to develop and validate the method of measuring ECFV simultaneously with renal function measurement from the Vd of IOT, and second, to explore its implications in relation to cardiorenal risk parameters in several populations.

In ***chapter 1*** we validate the measurement of ECFV simultaneously with GFR using the constant infusion method of IOT. As mentioned above, IOT is a radio-labeled tracer used in scientific and top-clinical settings to accurately measure GFR. Whereas theoretically its Vd could be used as a measure for ECFV, studies calibrating and validating an ECFV assessment in the same procedure as the GFR measurement are lacking. This is accomplished in the studies described in chapter 1. Measurement of ECFV by the IOT method is compared with the gold standard, i.e. the Vd of bromide, and its reproducibility and sensitivity to detect changes in ECFV is assessed. Moreover, the simultaneous assessment of GFR and ECFV provides the possibility to express renal function indexed to ECFV in stead of indexed to BSA. The implications of indexing GFR to ECFV are also explored in this chapter.

Chapter 2 addresses the effects of weight excess on extra cellular volume regulation, renal function, and renal function assessment by creatinine clearance (CrCl). Weight excess, ranging from mild overweight to morbid obesity has become increasingly important as a cardiovascular and renal risk factor. Its effects on cardiorenal risk are mostly attributed to the concomitant presence of hypertension, insulin resistance and diabetes, but studies from our group demonstrated independent effects of weight excess as well. In many studies concerning the interaction between renal function and overweight, renal function

is estimated by creatinine-based methods. Whereas creatinine-based methods have been extensively explored in subjects with impaired renal function, their performance in subjects with normal, or even elevated renal function, has not been well-documented. The latter is relevant as the early changes of renal function in weight excess comprise a rise in GFR rather than a decrease. In ***chapter 2a*** therefore we study the performance of endogenous CrCl to estimate GFR in subjects without renal function impairment, and analyse for sources of systematic error, in particular in relation to body mass index (BMI). ***Chapter 2b*** investigates the effects of weight excess on the response of ECFV and renal sodium handling to a rise in sodium intake in healthy subjects. As noted above, in epidemiological studies, the association between higher sodium intake and cardiovascular and renal risk is particularly prominent in subjects with weight excess. We therefore hypothesize that BMI determines the responses of renal sodium handling, and consequently ECFV to a rise in sodium intake, with a larger rise in ECFV in subjects with higher BMI. To avoid effects secondary to co-morbid conditions such as hypertension and insulin resistance, this study is conducted in healthy volunteers, thus allowing to identify primary mechanisms in overweight-associated morbidity.

Chapter 3 addresses the mechanisms underlying sodium sensitivity of blood pressure in healthy subjects. Sodium sensitivity of blood pressure has been associated with an increased risk for mortality and cardiovascular morbidity, not only in hypertension, but also when blood pressure is in the normotensive range, supporting the clinical relevance of sodium sensitivity in the absence of hypertension. In hypertensive subjects sodium sensitivity of blood pressure has been attributed to an increased intra-renal activity of the RAAS, the main regulating system of sodium and fluid homeostasis. Since sodium sensitivity in

hypertensives might be secondary to subclinical hypertensive renal damage, the mechanisms of sodium sensitivity in normotensive subjects may be different. Therefore, we investigate whether sodium sensitivity of blood pressure is related to inappropriately increased activity of the intra-renal RAAS, and its possible effects on regulation of ECFV in healthy subjects.

In ***chapter 4*** we study the effect of unilateral nephrectomy on renal sodium handling and ECFV. The pathogenesis of hypertension in conditions of renal disease has been postulated to be due to impaired sodium excretion and consequent expansion of ECFV. Kidney donation provides a setting well-suited to test the impact of reduction of renal mass on volume status in humans without possible confounding effects of renal disease. Therefore, we study whether donating a kidney results in a rise in ECFV in healthy kidney donors.

In ***chapter 5*** we consider the possible contribution of altered ECFV regulation in the pathogenesis of cardiorenal anaemia in two different populations. Cardiorenal disorders are not only characterized by altered volume regulation, but also by the frequent occurrence of anaemia. ECFV expansion might contribute to anaemia by haemodilution, but the impact of this possible interrelationship has not been well-explored. Therefore, in ***chapter 5a***, we study the relation between haemoglobin and ECFV in a condition characterized by fluid retention, namely chronic heart failure (CHF) patients. Second, in ***chapter 5b***, we perform a corresponding analysis on the relation between haemoglobin levels and ECFV in stable renal transplant recipients.

Chapter 6, finally, gives an overview of the results from the studies, and discusses their implications for clinical practice and for further research

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CHAPTER 1

FEASIBILITY AND IMPACT OF THE MEASUREMENT OF EXTRA CELLULAR FLUID VOLUME SIMULTANEOUS WITH GLOMERULAR FILTRATION RATE BY ^{125}I-IOTHALAMATE

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Abstract

The feasibility, validity and possible applications of the assessment of extra cellular fluid volume (ECFV) simultaneous with glomerular filtration rate (GFR) were assessed in a serie of validation studies using the constant infusion method of ^{125}I-iothalamate (IOT).

In 48 subjects with a broad range of GFR, distribution volume (Vd) of IOT corresponded well with Vd-bromide (respectively 16.71 ± 3.0 and 16.73 ± 3.2 l, ns), with a strong correlation ($r=0.933$, $p<0.01$) and without systematic deviations. Reproducibility assessment in 25 healthy male subjects showed coefficients of variation of 8.6% of duplicate measurement of Vd_{IOT} during strictly standardized (50 mmol Na^+/day) sodium intake. An increase in dietary sodium intake (200 mmol Na^+/day) induced a corresponding rise in Vd_{IOT} of 1.11 ± 1.5 l ($p<0.01$). In 158 healthy prospective kidney donors we analyzed the impact of indexing of GFR to ECFV. Age, gender, height, and body surface area (BSA) were determinants of GFR. Whereas GFR, GFR/BSA and GFR/height were gender-dependent, GFR/ECFV was gender-independent and not related to height or BSA. This supports the potential of normalizing GFR by ECFV.

We conclude that ECFV can be simultaneously assessed with GFR by the constant infusion method using IOT. After appropriate validation, also other GFR tracers could be used for such a simultaneous estimation, providing a valuable resource of data on ECFV in renal studies and moreover, allowing GFR to be indexed to the body fluid compartment it clears: the ECFV.

Introduction

The gold standard for measuring glomerular filtration rate (GFR) is by specific tracers, such as inulin, Cr-EDTA, iothalamate and iohexol[1]. Since the distribution volume (Vd) of these tracers for GFR ideally equal extra cellular fluid volume (ECFV)[2-9], measuring GFR with such tracers could potentially be used for simultaneous assessment of ECFV.

Simultaneously measuring ECFV and GFR has several advantages. First, it will allow better insight into the (patho)physiological and clinical role of ECFV in renal disease and its complications, such as hypertension and left ventricular hypertrophy. Second, it has been proposed that the best way to normalize GFR between different individuals would be by ECFV rather than by body surface area (BSA)[6,10,11]. Whereas normalizing GFR for ECFV would be attractive from a theoretical perspective, it has not gained acceptance in practice as it is considered too cumbersome[12]. Validation of GFR measurement protocols for simultaneous assessment of ECFV would greatly increase the feasibility of normalizing GFR for ECFV.

Various GFR tracers were used for measuring ECFV[2-9], but their validation, reproducibility, and calibration against a gold standard for ECFV are not well-documented. In our centre accurate GFR measurement is performed as the clearance of ^{125}I-iothalamate (IOT) by the constant infusion method, simultaneously with effective renal plasma flow (ERPF)[13]. This renal function measurement is used in top-clinical care and for clinical research, and allows estimating GFR with a day-to-day variability of only 2.5%[14]. The aim of the current study was to validate this renal function protocol for assessing ECFV, and to use the combined assessment for normalizing GFR to ECFV. To this purpose, we studied first, the agreement of

Vd_{IOT} with Vd of bromide, the gold standard for ECFV measurements, over a wide range of renal function. Second, we assessed the reproducibility of ECFV measurements by assessing Vd_{IOT} under conditions of standardized sodium intake in healthy volunteers. Third, in these volunteers we tested the sensitivity of Vd_{IOT} to detect a change in ECFV. Finally, we analysed the impact of indexing GFR to ECFV in the above volunteers and in a cohort of 158 potential kidney donors.

Methods

All experiments were performed in adherence to the Declaration of Helsinki.

Measurement of renal function

Renal function measurements were performed using the constant infusion method with IOT and ^{131}I-Hippuran as described before[13-15]. After drawing a timepoint-0 blood sample, a priming solution containing 20 ml infusion solution (0.04 MBq of IOT and 0.03 MBq of ^{131}I-Hippuran) plus an extra of 0.6 MBq of IOT is given at 08.00 hours, followed by a constant infusion ranging from 6 $ml \cdot h^{-1}$ in subjects with impaired renal function to 12 $ml \cdot h^{-1}$ (based on previously known serum creatinine). Plasma concentrations of both tracers are allowed to stabilize during 1.5 hour equilibration, which is followed by two two-hour periods for simultaneous clearances of IOT and ^{131}I-Hippuran. The latter are calculated as $(U \cdot V)/P_{iot}$ and $(I \cdot V)/P_{hipp}$, respectively. $U \cdot V$ represents urinary excretion of the tracer; $I \cdot V$ represents the infusion rate of the tracer, which equals clearance from plasma during steady state. P represents tracer values in plasma at the end of each clearance period. The plasma clearance $(I \cdot V)/P_{hipp}$ equals its urinary clearance as there is no extrarenal clearance of this tracer. Thus, when plasma levels are in steady state ERPF equals $I \cdot V/P_{hipp}$. GFR is calculated as the urinary clearance of

IOT, corrected for voiding errors: $(U \cdot V/P)_{corr}$. As urinary clearance of ^{131}I-Hippuran equals plasma clearance in case of perfect urine collection, we routinely use the ratio of plasma-to-urinary clearance of ^{131}I-Hippuran to correct urinary clearance of IOT for voiding errors and dead space. By this method, coefficient of variation (COV) for GFR is 2.5% and for ERPF 5%[13].

Calculation of ECFV as Vd_{IOT}

Vd_{IOT} is calculated as: *amount of IOT in the patient divided by* P_{IOT} during steady state. The amount of IOT in the patient is calculated as: $[IOT_{infused} - IOT_{excreted}]$. The latter is calculated as: *[bolus + Σ(I·V)] - [urinary + extrarenal excretion of IOT].* Urinary excretion of IOT is measured as $\Sigma(U \cdot V)$, corrected for voiding errors as described above. Extrarenal excretion of IOT is calculated as described below. Taken together, Vd_{IOT} is calculated as: $\{[Bolus + \Sigma(I \cdot V)] - [\Sigma(U \cdot V) + extrarenal\ excretion]\} / P$

Calculation and validation of extrarenal clearance of IOT

IOT is not exclusively cleared by the kidney, as some biliary excretion occurs as well. The latter is negligible when renal function is normal but is considerable in patients with impaired renal function[13,14,16]. To calculate Vd_{IOT} as proposed above, extrarenal clearance has to be accounted for. In our set-up extrarenal clearance can be calculated from the difference between plasma and urinary clearance. Extrarenal excretion of IOT (% of input) is calculated during steady state as: $[(I \cdot V) - (U \cdot V)] / (I \cdot V) \cdot 100$ %. Subsequently total extrarenal excretion is calculated as: $\%ER\ excretion \cdot [bolus + \Sigma(I \cdot V)]$.

To validate this algorithm for calculation of extrarenal clearance of IOT we measured urinary recovery of IOT in 24h urine collected during the 24 hours following IOT infusion in 31 subjects (Mean GFR: 68 ± 32 ml·min^{-1}; range 20-152

ml·min^{-1}). Urinary recovery of IOT (% of input) was strongly and negatively correlated with calculated extrarenal clearance (r=-0.80, p<0.001). Mean total urinary recovery was 87 ± 8%, mirrored by a calculated extrarenal clearance of 14 ± 12% of total input, supporting the validity of our calculation of extrarenal clearance of IOT.

Calibration of Vd_{IOT} against $Vd_{bromide}$

24 males and 24 females (age 49 ± 13 yr), routinely visiting our Medical Centre for renal function measurements were included in this experiment. To include subjects with a wide GFR-range we included potential kidney donors (n=13), renal transplant recipients (n=32) and subjects with chronic kidney disease (CKD) (n=3). Renal function and Vd_{IOT} was assessed as described above.

On the same day as assessing Vd_{IOT}, subjects received oral NaBr in an approximate dose of 50 mg·kg^{-1}. Blood samples were, simultaneously to blood samples for IOT, drawn after 4.5 and 5.5 hours and $Vd_{bromide}$ was calculated as[17]: $Br\ dose\ /\ [Br]_{serum} \cdot 0.90 \cdot 0.95 \cdot 0.94$

In the latter formula, 0.90 is the fraction of bromide that is assumed to be distributed in non-extra cellular sites (principally erythrocytes), 0.95 is the Donnan equilibrium factor and 0.94 is the assumed amount of water in serum.

The ECFVs assessed by Vd_{IOT} and $Vd_{bromide}$ were compared by investigating agreement between the two measures as recommended by Bland&Altman[18] with calculation of 95% limits of agreement as mean ± 2 · SD of the difference between Vd_{IOT} and $Vd_{bromide}$.

Reproducibility and sensitivity to detect changes in EFCV over time

25 healthy normotensive men were studied. Renal function and ECFV were measured four times, on day 7 and day 14 of two 14-day study periods, with a wash-out of at least 3 weeks in between. Each study period consisted of two 7-day periods with a different dietary sodium content, i.e. low sodium (50 mmol Na^+/day) and high sodium diet (200 mmol Na^+/day), in randomized order. Potassium intake was standardized at 80 mmol/day. Otherwise, the subjects continued their usual food habits. For assessment of dietary compliance, 24h urine was collected at day 6 during each 7-day period. During all periods, subjects were ambulant and continued normal activities. During study-days subjects reported at the research-unit at 08.00 hours, after having abstained from food and alcohol overnight. Height and body weight were measured, after which renal function and Vd_{IOT} was assessed.

Reproducibility was assessed by examining repeated measurements under the same sodium intake for bias and calculating the COV. Bias was investigated by examining the mean difference between repeated estimates. The COV was calculated as the within-subject variation as a percentage of the sample mean.

The averaged value of the duplicate measurements was further used to assess the sodium induced change in ECFV. Additionally, creatinine based renal function was calculated according to the simplified MDRD formula[19] and 24 hour creatinine clearance (CrCl). Creatinine in blood and urine were determined by Kodak Ektachem dry chemistry (Eastman Kodak, Rochester, NY, USA).

Between subjects normalization of GFR by ECFV

We assessed the impact of normalization of GFR by ECFV in 158 healthy subjects screened as potential kidney donors (table 2). In this population we assessed the impact of normalization by ECFV on the difference in renal function between men and women. BSA was calculated according to DuBois[20]. Serum creatinine based renal function was calculated according to the simplified MDRD formula[19]. To analyze the separate contributors to differences in GFR, we performed linear regression, with respectively GFR, GFR/1.73m^{2}BSA, GFR/height and GFR/ECFV as independent variables and age and gender as dependent variables. BSA or height was added as independent variable when appropriate. GFR, GFR/1.73m^{2}BSA and GFR/height are expressed in ml·min^{-1}, ml·min^{-1}·1.73m^{-2}BSA, and ml·min^{-1}·m^{-1}, respectively. GFR/ECFV is expressed as %·h^{-1}, corresponding to the % of the ECFV that is cleared per hour. This unit follows from dividing ml·min^{-1} (GFR) by ml (ECFV) · 60 (transforming min^{-1} in h^{-1}) and · 100 (expression in %).

Data analyses

Data are expressed as mean ± SD in text and tables and plotted as mean ± SEM in figures. Student's T-Test or paired Student's T-test were used for comparing means, correlations were assessed as Pearson's Correlation coefficient. All analyses were performed by the Statistical Package SPSS 14.0.

Results

Calibration of Vd_{IOT} against $Vd_{bromide}$

In the experiments comparing Vd_{IOT} and $Vd_{bromide}$, the GFR of the 48 included subjects ranged from 20 to 147 ml·min^{-1} (mean value 79 ± 41 ml·min^{-1}). After equilibration-time serum levels of IOT were 279 ± 67, 279 ± 76, 276 ± 83, 280 ± 90 and 284 ± 97 counts·ml^{-1}·min^{-1} (ANOVA, p>0.9), respectively at 1.5, 2.5, 3.5, 4.5 and 5.5 hours after start of the protocol. Thus, plasma levels of IOT were in steady state, a prerequisite for calculation of ECFV during the constant infusion method.

In figure 1 the results of the simultaneous assessments of Vd_{IOT} and $Vd_{bromide}$ are shown. The ECFV obtained as $Vd_{bromide}$ was 16.73 ± 3.15 l, and the ECFV obtained as Vd_{IOT} was 16.71 ± 2.96 l (ns). Vd_{IOT} and $Vd_{bromide}$ were strongly correlated (left panel, r=0.933, p<0.001). In the Bland-Altman plot (right panel) no systematic error was apparent, with 95% limits of agreement ranging from -2.3 to 2.2 l. Similar results were obtained in the 30 subjects in which GFR was below 75 ml·min^{-1}, i.e. subjects with a substantial extrarenal clearance of IOT, demonstrating the adequacy of our correction for extrarenal clearance of IOT.

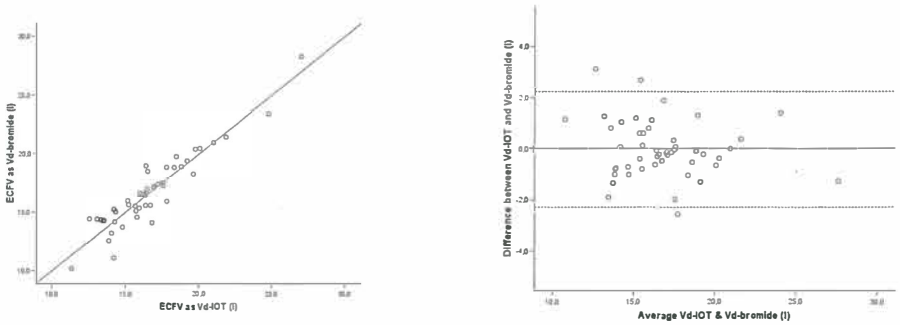

Figure 1 ***left panel: scatterplot of extra cellular fluid volume (ECFV) simultaneously obtained as distribution volume (Vd) of bromide (y-axis) and Vd of ^{125}I-iothalamate (IOT) (x-axis) with line of identity. Right panel: Bland Altman plot for $Vd_{bromide}$ and Vd_{IOT} with 95% limits of agreement***

Reproducibility and sensitivity to detect changes in ECFV

Data from the reproducibility experiment are given in table 1, showing the duplicate measurements on low (50 mmol Na^+/day) and high sodium (200 mmol Na^+/day). Dietary compliance was good during all four periods, as shown by 24h urinary sodium excretion. ECFV, body weight (BW) and GFR were virtually identical during the duplicate measurements on low and high sodium, respectively. Reproducibility of the ECFV measurement was assessed separately for the two low sodium periods and the two high sodium periods. During low sodium the mean difference in ECFV (bias) was 1.3%, with a COV of 8.6%. During high sodium, bias was 1.1% with a COV of 13.1%.

	Low sodium diet		High sodium diet	
	Period 1	**Period 2**	**Period 1**	**Period 2**
Na^+ excretion (mmol·24h^{-1})	35 ± 22	39 ± 16	250 ± 68	251 ± 65
Body Weight (kg)	80.1 ± 11.0	80.2 ± 11.0	81.8 ± 11.2	81.7 ± 11.3
GFR (ml·min^{-1})	127 ± 20	129 ± 18	138 ± 21	137 ± 20
ECFV as Vd_{IOT} (l)	19.7 ± 2.7	20.0 ± 2.4	20.8 ± 2.8	21.1 ± 3.2
MAP (mmHg)	85 ± 6	86 ± 8	87 ± 6	87 ± 7

Table 1 Measurements in healthy young men (n=25) on four separate occasions Glomerular filtration rate (GFR); extra cellular fluid volume (ECFV); mean arterial pressure (MAP)

In both periods, as anticipated, the shift from low to high sodium intake resulted in a rise in ECFV (Period 1: +1.09 ± 2.2 l ($p=0.02$); Period 2: +1.13 ± 2.0 l ($p<0.01$)) without statistical differences between the two periods. The mean rise in ECFV induced by high sodium was 1.11 ± 1.49 l ($p<0.01$), corresponding with a mean rise in BW of 1.60 ± 0.85 kg ($p<0.01$), as indicated in figure 2. The individual changes in BW were significantly and positively correlated to the individual changes in ECFV ($r=0.552$, $p<0.01$).

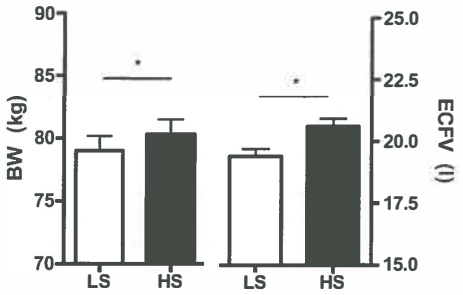

Figure 2 *Mean ± SEM for body weight (BW) and extra cellular fluid volume ECFV, respectively in subjects (n=25) in balance on low sodium (LS) and high sodium (HS) *p<0.001*

GFR increased significantly from low to high sodium (128 ± 18 versus 137 ± 18 ml·min^{-1}; p<0.01, figure 3). The sodium-induced rise in renal function was also significant for GFR/BSA (109 ± 13 versus 116 ± 12 ml·min^{-1}·1.73m^{-2}, p<0.01), as well as for GFR/BSA estimated by the MDRD formula (97 ± 18 versus 105 ± 19 ml·min^{-1}·1.73m^{-2}, p<0.01) and for 24h CrCl/BSA: 94 ± 20 versus 106 ± 21 ml·min^{-1}·1.73m^{-2}, p<0.01. However, renal function expressed as GFR/ECFV remained unchanged during the shift from low to high sodium: 39.0 ± 3.6%·h^{-1} and 39.6 ± 4.2%·h^{-1}(ns) as the rise in GFR was matched by a corresponding rise in ECFV.

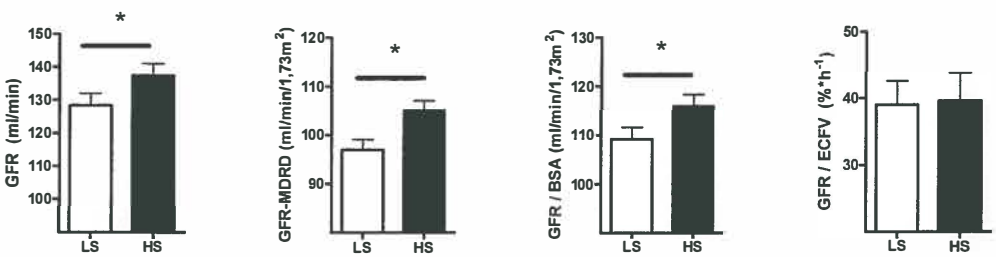

Figure 3 *Within-individual values: population of healthy young men (n=25), shown for subjects on low sodium diet (LS) and high sodium diet (HS), respectively. Mean glomerular filtration rate (GFR) ± SEM respectively for raw data, MDRD, indexed for body surface area (BSA) and indexed for extra cellular fluid volume (ECFV). *p<0.01- paired analyses.*

Between subject normalization of GFR by ECFV

To address the impact of expressing renal function as GFR/ECFV, we analysed data from 158 potential kidney donors. Their characteristics are given in table 2.

	Potential kidney donors	
	Male (n=73)	Female (n=85)
Age (years)	51 ± 11	52 ± 10
Body weight (kg)	91 ± 13	73 ± 11*
Height (cm)	183 ± 7	169 ± 6*
BSA (m^2)	2.07 ± 0.22	1.80 ± 0.20*
GFR ($ml \cdot min^{-1}$)	132 ± 30	105 ± 20*
ECFV as Vd_{IOT} (l)	22.9 ± 4.6	17.4 ± 2.5*

*Table 2 Characteristics of potential donor population (n=158). Body surface area (BSA); glomerular filtration rate (GFR); extra cellular fluid volume (ECFV); distribution volume of iothalamate (Vd_{iot}). *p<0.01 male versus female.*

We analysed for the determinants of GFR, GFR/BSA, GFR/height and GFR/ECFV by multivariate modelling, and found the models shown in table 3. As shown in the left column, age, BSA and gender are all independent determinants of GFR. Second, in line with the first model, age and gender are both independent determinants of GFR/BSA, and of GFR/height. However, when GFR is indexed to ECFV, only age is an independent determinant for GFR. Thus, in healthy subjects, indexing to ECFV appears to nullify gender, BSA- and height-related differences in GFR.

	GFR		GFR/BSA		GFR/h		GFR/ECFV	
	β	p	β	p	β	p	β	p
Age	-0.412	<0.01	-0.485	<0.01	-0.465	<0.01	-0.536	<0.01
Gender	-0.280	<0.01	-0.181	<0.01	-0.328	<0.01	0.058	ns
BSA	0.329	<0.01	-		-		-0.146	ns
Height *	0.342	<0.01	-		-		-0.092	ns

*Table 3 Linear regression models with respectively glomerular filtration rate (GFR) (r of model 0.702, p<0.001), GFR/body surface area (BSA) (r=0.518, p<0.01), GFR/height (r=0.580, p<0.01) and GFR/extra cellular fluid volume (ECFV) (r=0.568, p<0.01) as dependent variable. *Height is replacing BSA in the models; all other β values are given for the models including BSA.*

The impact of the various ways of indexing on the differences in renal function between men and women is illustrated in figure 4. Uncorrected GFR was higher in men (130 ± 28 versus 102 ± 25 ml·min^{-1}, p<0.01). BW and height, and consequently BSA, were higher in men (table 2), but indexing to BSA did not nullify differences in GFR between men and women (GFR/BSA 105 ± 20 versus 96 ± 22 ml·min^{-1}·1.73m^{-2}, p<0.01), and neither did indexing to height (GFR/height 72 ± 15 for men versus 62 ± 11 ml·min·m^{-1} for women, p<0.01). The same was true for GFR estimated by the MDRD: 85 ± 18 and 76 ± 14 ml·min^{-1}·1.73m^{-2}, p<0.01. However, GFR indexed to ECFV was similar for men and women, being 35 ± 7%·h^{-1} and 37 ± 6%·h^{-1}(ns).

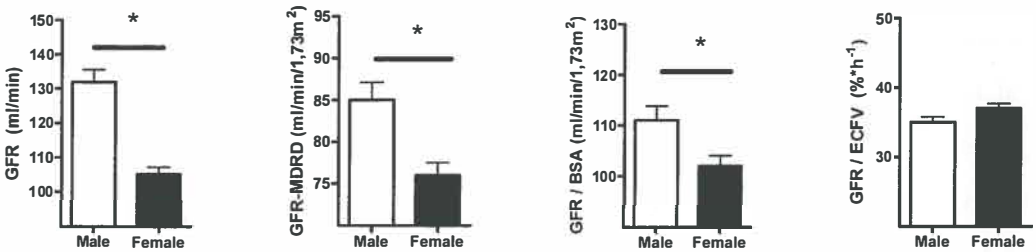

*Figure 4 Between-individual values: population of potential kidney donors shown by a break-up by men (n=73) and women (n=85). Mean glomerular filtration rate (GFR) ±SEM respectively for raw data, MDRD, indexed for body surface area (BSA) and indexed for extra cellular fluid volume (ECFV). *p<0.01*

Discussion

Our study demonstrates the validity and reproducibility of assessing ECFV simultaneously with GFR by the constant infusion method with IOT. This provides not only a useful tool for pathophysiological studies on ECFV in renal conditions, but also allows the indexing of GFR to ECFV without need for additional procedures or parameters. As the Vd of other GFR-tracers also equal ECFV, renal function protocols with other tracers could similarly be validated for simultaneous assessment of ECFV. This will increase the diagnostic yield of renal function measurements by specific tracers.

This is the first study to validate Vd_{IOT} as a measure for ECFV in human subjects. Vd_{IOT} was in good agreement with $Vd_{bromide}$ as a gold standard for ECFV over a wide range of renal function. Moreover, the day-to-day variation of Vd_{IOT} was low, at least during standardized sodium intakes in healthy subjects, i.e. conditions where the biological variation in ECFV is low. Finally, a change in ECFV induced by a shift in sodium intake could be adequately detected by the assessment of Vd_{IOT}.

Other GFR tracers, such as inulin, ^{99}mTc-DTPA, ^{51}Cr-EDTA and iohexol[2-9] have also been used for simultaneous assessment of GFR and ECFV. ECFV assessed as Vd of ^{51}Cr-EDTA had a day-to-day variation of 11.4%[7]; comparable to the reproducibility of Vd_{IOT} in our study. Most studies report that ECFV can be adequately assessed by the GFR tracer studied, without however calibrating Vd of the tracer against a gold standard for ECFV [4,6-8]. $Vd_{bromide}$ is considered the gold standard for ECFV assessment since its tissue content and serum distribution are well documented[21]. Only one small study, in 10 healthy subjects, calibrated tracer Vd against $Vd_{bromide}$.

In this study[2] $Vd_{iohexol}$ correlated well with $Vd_{bromide}$ but $Vd_{iohexol}$ was on average 0.7 l lower than $Vd_{bromide}$. For use in renal populations, however, the calibration in subjects with renal function impairment is needed, as renal function impairment can affect several factors relevant to the validity of the ECFV assessment, such as steady state kinetics and possible extrarenal clearance. Our study is by far the largest to provide calibration against $Vd_{bromide}$ and moreover is the only to include subjects with renal function impairment, thus allowing conclusions on the use of combined measurements of GFR and ECFV in renal populations.

The clearance of specific tracers such as iothalamate, iohexol or Cr-EDTA provides the gold standard for GFR measurement. As such measurements are relatively laborious and expensive, their use is mainly limited to specialized nephrological settings, such as screening of potential kidney donors, and research applications. Our data shows that the yield of GFR measurements can be increased by additionally providing an estimate of ECFV. As abnormalities in volume status are important in renal disease and its complications, this might prove a valuable tool for future studies.

Body dimensions differ between individuals. GFR is usually indexed to account for differences in body dimension, generally to BSA[22]. However, the use of BSA for indexing has been criticized by several authors[23-25]. Indexing to height or ECFV has been recommended, but neither gained broad acceptance despite studies supporting their superiority over indexing to BSA[11,26,27]. In our study, GFR was gender dependent. This is in line with data by Turner, showing a difference in GFR/1.73m^2 BSA between men and women, which could be traced back to a fallacy of indexing for BSA[27]. The difference in renal function between men and women, as found for GFR/1.73m^2 BSA was annihilated when GFR was indexed to ECFV. Indexing for height, as recommended by others[26,28], did not annihilate the

difference between males and females in our study either, providing an argument against indexing to height.

It has been pointed out recently that no gold standard is available to determine which indexing factor is best[12] and that the best indexing factor for GFR would be the one which provides the best clinical validity. Final proof whether ECFV is indeed the best indexing factor for GFR, should therefore be provided by long term follow-up studies in which superiority of GFR/ECFV as predictor of renal outcome is studied.

What is the physiological meaning of GFR/ECFV? ECFV is the body compartment which is cleared from toxins and waste products by the kidney. Expressing GFR as proportion of ECFV thus expresses clearance as a percentage of the volume compartment it clears. For instance, in our healthy volunteers GFR/ECFV was approximately 40%/hour, implicating that 40% of the ECFV is cleared per hour, and -the other way around-, that the kidneys need 2.5 hours to clear the complete ECFV.

ECFV is regulated within relatively narrow boundaries, but it is not fixed. It adapts to altered sodium intake, as also confirmed here. It could be argued that this hampers its suitability as indexing parameter. On the other hand, considering GFR in relation to the prevalent ECFV may allow better interpretation of changes in GFR, by distinguishing between changes in GFR secondary to altered volume status and changes in GFR dissociated from changes in ECFV. In the current study we induced a modest change in ECFV by a shift in sodium intake to investigate whether the estimate by V_{dIOT} was sufficiently sensitive to detect the change in ECFV. Indeed, V_{dIOT} was significantly higher during high sodium intake. The rise in ECFV matched the anticipated rise in GFR and accordingly, GFR indexed to

ECFV was similar during low and liberal sodium intake. It would be of interest therefore, to have the information on simultaneous values of ECFV and GFR also in disease conditions where GFR, ECFV or both are disturbed. In diabetes for instance, elevated GFR and volume expansion occur in incipient diabetic nephropathy[29,30]. Although in a small cohort hyperfiltration seemed to be independent of ECFV expansion[30], studies in large cohorts are lacking. Routinely implementing ECFV assessment in tracer-based GFR assessment potentially yields large study-cohorts which have an enormous explanatory potential for studying not only GFR, but also ECFV.

Several limitations should be mentioned. First, P_{IOT} is used for the calculation of both GFR and ECFV, so the two are not arithmetically independent. As a consequence, a correlation between GFR and ECFV cannot simply be interpreted as a biological association. It should be emphasized however, that this does not invalidate the use of GFR/ECFV, as in this ratio P_{IOT} falls out of the equation. For this reason, as also pointed out by others, assessment of GFR/ECFV is less sensitive to procedural errors than assessment of GFR alone[5,11]. Second, this is a single centre study, investigating one tracer and one measurement protocol only. Thus, for other settings separate validation and calibration is warranted. Yet, from a theoretical perspective and supported by several studies, single infusion methods using plasma disappearance curves are well-suited for ECFV assessment as well[4]. Finally, we studied mainly healthy subjects, and only a limited number of subjects with renal function impairment, none of them with clinically overt volume overload or oedema. Therefore, extrapolation of our findings to populations with gross abnormalities of ECFV and/or abnormal distribution of body fluid compartments needs more extensive validation.

In clinical practice creatinine-based approaches, be it renal function equations or CrCl, are the usual way to estimate renal function[31,32]. Creatinine-based renal function estimates are also generally indexed to body dimensions, usually BSA. However, the Vd of creatinine cannot directly be established, so our strategy is unfortunately not applicable to creatinine based renal function measurements.

In conclusion, our study demonstrates the feasibility and validity of measuring ECFV simultaneously with assessing GFR by ^{125}I-iothalamate clearance, without need for adapting the GFR protocol. This not only enables indexing of GFR to ECFV but also provides information on ECFV in studies on renal function. ECFV is a major physiological parameter and disturbances are common in renal patients. By our approach, that also could be implemented for other GFR tracers, information on ECFV can be conveniently obtained as an additional parameter in subjects in whom GFR is measured. This will increase the yield of measurements of true GFR.

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CHAPTER 2A

BODY MASS INDEX IS A MAIN DETERMINANT OF FRACTIONAL CREATININE EXCRETION: IMPLICATIONS FOR THE PREDICTIVE PERFORMANCE OF CREATININE CLEARANCE IN HEALTHY SUBJECTS

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Abstract

Accurate renal function measurement in subjects with normal or higher renal function is important for epidemiological screening and in the work-up for kidney donation. Creatinine based equations perform poorly in this range of renal function. Its 24h clearance (CrCl) may provide an alternative as tubular creatinine secretion is assumed absent when renal function is normal. Data on the validity of CrCl as estimate for glomerular filtration rate (GFR) in the normal or higher range are sparce. Therefore, we studied the predictive performance of 24h CrCl in 100 potential kidney donors, and moreover, studied tubular handling of creatinine (FE_{creat}) by simultaneous assessment of true GFR (iothalamate clearance) and CrCl.

Mean GFR was 117 ± 24 and 24 h CrCl 111 ± 31 $ml \cdot min^{-1}$. CrCl assessed simultaneously with GFR was 116 ± 27 $ml \cdot min^{-1}$. Mean bias of 24h CrCl was 5.6 $ml \cdot min^{-1}$; precision (r^2) 0.582, and 30%-accuracy 87%. Bias of 24h CrCl significantly correlated to body mass index (BMI) ($r=0.23$, $p<0.03$). FE_{creat} was 110 ± 11%. BMI ($r=0.388$, $p<0.01$) was independently associated with higher FE_{creat}.

In conclusion, the predictive performance of 24h CrCl as an estimate of GFR in healthy subjects was fair with however, systematic overestimation of GFR, in particular in overweight subjects. Tubular secretion of creatinine is present in healthy subjects, in particular in association with overweight. CrCl is useful to estimate GFR in the normal or higher range of renal function, but the impact of BMI on the systematic error should be taken into account.

Introduction

Extensive and long standing experience is available on the measurement of renal function in subjects with renal disease and renal function impairment[1,2]. More recently, measurement of renal function in subjects without renal disease has become a focus of interest for several purposes, such as early detection of renal disease in the general population[3], and screening of prospective kidney donors[4]. In addition, an elevated glomerular filtration rate (GFR) has recently been identified as an early manifestation of a metabolic risk profile[5;6,7], re-emphasizing the need for well-validated renal function estimates for populations where renal function is in the normal, and higher range.

The gold standard for GFR assessment is by the clearance of specific tracers, such as inulin and iothalamate[2], but these are not routinely available. Creatinine-based renal function equations are therefore recommended in several guidelines (i.e. KDOQI[8]), as these are simple and cheap, and thus well-suited to the outpatient setting[9,10]. However, these equations were empirically developed in populations with renal function impairment, and their performance is modest to poor in populations without renal function impairment[11-14]. Creatinine clearance (CrCl) calculated from 24h urine collection is not recommended currently as an alternative, for two main reasons. First, the inaccuracy due to inaccurate 24h urine collection (i.e. the non-systematic error), and second, the considerable overestimation of GFR by CrCl in subjects with moderate to severe renal function impairment, due to tubular secretion of creatinine (i.e. the systematic error)[15,16]. As tubular secretion is assumed to be absent in subjects with normal or high GFR, 24h CrCl could be a suitable alternative for renal function measurement in populations with normal or even elevated renal function, provided that adequate 24h urine collection is ensured by a dedicated setting with proper instructions for urine

collection. However, data on the predictive performance of 24h CrCl for true GFR in non-renal populations, are sparse.

The purpose of the current study is therefore, first, to determine the predictive performance of 24h CrCl in subjects without renal disease, and, second, to identify determinants of the systematic error, as the latter could potentially be eliminated by proper correction factors. To these purposes we measured 24h CrCl and true GFR (^{125}I-iothalamate (IOT) clearance) in a population of prospective kidney donors, and analysed for the determinants of bias. Moreover, we assessed 2h CrCl simultaneously with IOT clearance, in order to assess possible tubular creatinine handling in this population.

Methods

Study population

100 subjects screened as kidney donor between March 2006 and September 2007 are included in the study. Subject characteristics are shown in table 1.

Renal haemodynamic measurement

GFR was measured by constant infusion of IOT, with correction for voiding errors by simultaneous measurement of the clearance of ^{131}I-Hippuran as described by Donker et al. and Apperloo et al[17,18]. Briefly, an intravenous cannula was inserted for tracer infusion at 08.00 hours. The infusion fluid consisted of 4 MBq ^{131}I-Hippuran and 3 MBq IOT per 100 ml saline. First, a priming solution of 0.4 ml·kg^{-1} BW was administered plus an extra 0.6 MBq IOT to ensure steady state of the plasma tracers within the time frame of the measurement. Thereafter, a continuous infusion was started. After a stabilization period of 1.5 hour, two 2-hour clearance

periods followed. GFR was measured as the urinary clearance of IOT (U·V/P) and corrected for voiding errors by multiplying $U \cdot V/P_{iot}$ by the ratio of plasma clearance of ^{131}I-Hippuran to urinary clearance of ^{131}I-Hippuran. This correction method is based on the fact that, during steady state, the plasma clearance of ^{131}I-Hippuran equals its urinary clearance when urine collection is perfect. Thus, the voiding error can be calculated from the ratio of urinary clearance and plasma clearance of ^{131}I-Hippuran. This GFR measurement has a day-to-day coefficient of variation (COV) of 2.2%.

Creatinine Clearance

Creatinine was measured with the Roche enzymatic creatinine assay. Serum creatinine samples were obtained during the measurement of iothalamate-GFR. All subjects collected a single 24h urine sample the day preceding iothalamate-GFR assessment, being out of hospital. 24h CrCl was calculated as U·V/P, in where U represents the concentration of creatinine in urine, V represents the volume of the 24h urine sample and P represents the concentration of creatinine in serum.

CrCl was also simultaneously measured to 'true GFR'. During the measurement of iothalamate-GFR, creatinine was measured in a 2 hour urine portion also used for iothalamate measurement. CrCl in this portion was subsequently calculated according U·V/P and corrected for voiding errors by the ratio of plasma clearance of ^{131}I-Hippuran to urinary clearance of ^{131}I-Hippuran. From the same sample also fractional excretion of creatinine (FE_{creat}) was calculated as $(U \cdot V/P)_{creat} / (U \cdot V/P)_{iot}$.

The MDRD equation for estimated GFR (eGFR) was calculated as: $186 \cdot ([creat]/88.4)^{-1.154} \cdot age^{-0.203} \cdot 0.742$ *(if female)*[19]. As the MDRD equation was developed based on creatinine measurements in the Cleveland Clinic laboratory, the absolute value of

eGFR depends on proper calibration of the creatinine assay, i.e. calibration of the creatinine values measured in our laboratory against those of the Cleveland Clinic Laboratory. This provided the following calibration equation: (creatinine Cleveland)= 1.07 · (Groningen enzymatic) + 2.92. The data on eGFR are presented in the current paper after adjustment for this calibration factor. Data used for the calculation of CrCl were not adjusted for the calibration factor as calculation of CrCl also involves urinary creatinine, for which no calibration was performed.

Data analysis

Predictive performance of 24h CrCl, simultaneously assessed CrCl, MDRD and calibrated MDRD were analyzed according to the method proposed by Bostom et al[20], which expresses predictive performance of a measurement as bias, precision and accuracy. Bias is the mean prediction error and calculated as (CrCl-GFR). Precision is a value for the degree of spread and expressed as Pearson's correlation quotient (R^2). Accuracy is expressed as % of observations within respectively 10% and 30% of true GFR. For the comparison of 24h CrCl and GFR, we used the method described by Bland and Altman[33], plotting the average value of 24h CrCl and GFR against the difference (bias: 24h CrCl – GFR).

Statistical analyses were performed using SPSS software version 14.0 (SPSS Inc., Chicago, IL, USA). Data are given as mean ± standard deviation. Pearson's correlation coefficients were calculated to account for univariate correlations.

Results

Characteristics and measured variables of the population are given in table 1, showing that our population was middle-aged with a slight preponderance of women, and, as anticipated, normal renal function. Remarkably, FE_{creat} as calculated from the simultaneous clearances of IOT and creatinine was 110 ± 11%, suggesting that some 10% of CrCl was accounted for by tubular secretion.

Sex (male: female)	45:55
Age (years)	52 ± 11
Body weight (kg)	81.4 ± 15.1
Height (cm)	175 ± 9
BMI ($kg \cdot m^{-2}$)	26.4 ± 4.0
Systolic blood pressure (mmHg)	127 ± 15
Diastolic blood pressure (mmHg)	75 ± 10
MAP (mmHg)	92 ± 11
GFR ($ml \cdot min^{-1}$)	117 ± 24
GFR/BSA ($ml \cdot min^{-1} \cdot 1.73m^{-2}$)	102 ± 17
24h CrCl ($ml \cdot min^{-1}$)	123 ± 37
Simultaneously assessed CrCl ($ml \cdot min^{-1}$)	128 ± 37
FE_{creat} (%)	110 ± 11
eGFR ($ml \cdot min^{-1} \cdot 1.73m^{-2}$)	78.8 ± 13.2

Table 1 ***Baseline characteristics.***
Body mass index (BMI); mean arterial pressure (MAP); glomerular filtration rate (GFR); body surface area (BSA); creatinine clearance (CrCl); fractional excretion of creatinine (FE_{creat}), estimated GFR (eGFR).

In table 2 the predictive performance of 24h CrCl for the assessment of GFR is given, showing the mean prediction error (bias), the degree of spread (Pearson's R^2) and accuracy (% of observations within respectively 10% and 30% of true GFR). The predictive performance of simultaneously assessed CrCl is also given, which allows to evaluate predictive performance devoid of urine collection errors. Both 24h CrCl and simultaneously assessed CrCl significantly overestimated true GFR ($p=0.02$ and $p<0.01$, respectively) Whereas 10% accuracy was poor for both 24h

CrCl and for simultaneously assessed CrCl, the 30% accuracy was high, in particular for the simultaneously assessed CrCl. As anticipated, eGFR significantly underestimated true GFR, with a mean bias of -23.5 ± 13.7 ml·min·1.73m^{-2}. R^2 was 0.384, and 10%- and 30% accuracy were 21% and 74%, respectively.

	Bias (ml·min^{-1})	Precision (R^2)	10%-accuracy (%)	30%-accuracy (%)
24h CrCl	5.6 ± 22.4	0.582	48%	87%
Simultaneous CrCl	12.3 ± 13.9	0.826	58%	95%

Table 2 Predictive performance of 24h creatinine clearance (CrCl) and simultaneously assessed CrCl.

Individual values for GFR and 24h CrCl are plotted in figure 1 (left panel), showing a reasonably good correlation (r=0.763, p<0.01). A Bland-Altman plot of the correspondence between 24 hr CrCl and true GFR is given in figure 1 (right panel), showing a significant positive correlation (r=0.441, p<0.01), indicating a systematic error with overestimation of GFR by 24h CrCl in the higher range of GFR.

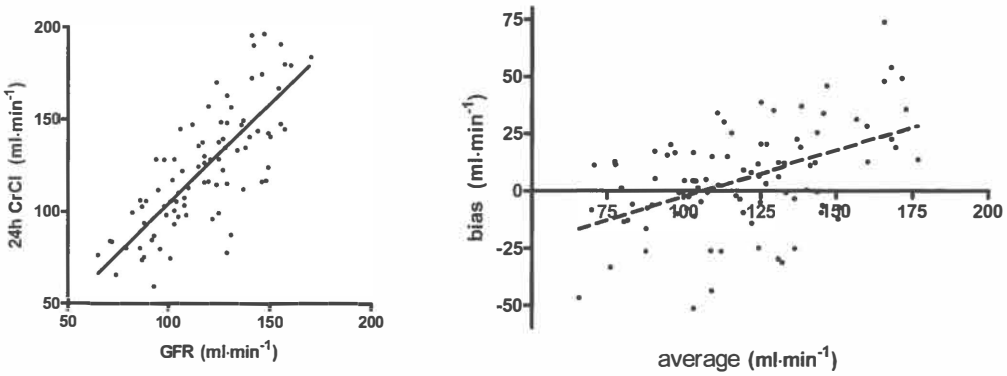

Figure 1 Left panel: scatterplot showing the univariate correlation between glomerular filtration rate (GFR; iothalamate clearance) and 24h creatinine clearance (CrCl); R^2=0.568, p<0.01). Right panel: Bland-Altman plot for GFR and 24h CrCl. The dotted line shows a significant association between level of renal function and bias (r=0.441; p<0.01).

To identify determinants of the systematic error in table 3 univariate correlations between bias of 24h CrCl (left column) and simultaneously measured CrCl (middle column), respectively, and patient characteristics are given.

	Bias 24h CrCl		*Bias simultaneous assessed CrCl*		*FE_{creat}*	
	r	p	r	p	r	P
Age	-0.098	0.36	-0.087	0.39	0.002	0.98
Gender	-0.148	0.16	**-0.241**	**0.02**	-0.161	0.11
Body weight	**0.311**	**<0.01**	**0.417**	**<0.01**	**0.305**	**<0.01**
Height	0.194	0.07	0.095	0.35	-0.011	0.91
MAP	0.100	0.35	0.012	0.91	-0.020	0.85
BMI	**0.234**	**0.03**	**0.459**	**<0.01**	**0.388**	**<0.01**
BSA	**0.284**	**<0.01**	**0.344**	**<0.01**	**0.225**	**0.03**
24h Creatinine excretion	**Nd ***		**0.302**	**<0.01**	0.180	0.08

Table 3 ***Univariate correlations (r) between baseline characteristics and respectively: bias in 24h Creatine clearance (24h CrCl); bias in simultaneously assessed CrCl and fractional excretion of creatinine (FE_{creat}). Mean arterial pressure (MAP); body mass index (BMI); body surface area (BSA).* data not given as 24h CrCl has a mathematical association with 24h creatinine excretion.***

The bias in 24h CrCl significantly and positively correlated with body weight (BW), body surface area (BSA) and body mass index (BMI; figure 2, right panel). When analysed by a break-up of BMI in weight categories, bias in 24h CrCl increased from 0.3 ± 17 ml·min^{-1} in lean subjects (BMI <25 kg·m^{-2}) to 8.2 ± 25 ml·min^{-1} in overweight (BMI 25-30) and 10.1 ± 24 ml·min^{-1} in obese subjects (BMI >30). A higher bias in simultaneously assessed CrCl (table 3, middle column) significantly and positively correlated to male gender, higher BW, BMI, BSA and 24h creatinine excretion. The univariate correlations between FE_{creat} and patient characteristics are given in the right column of table 3. FE_{creat} positively correlated with BW, BMI (figure 2, left panel) and BSA. In lean subjects FE_{creat} was 106 ± 12 %, in overweight and obese subjects it was 112 ± 11 % and 115 ± 8 %, respectively.

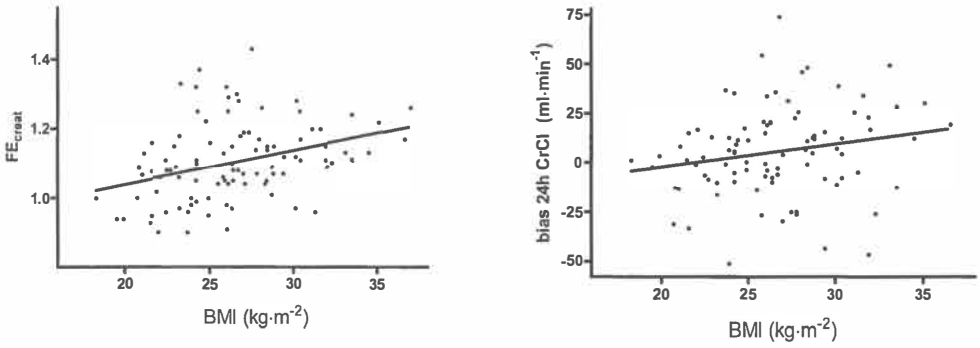

Figure 2 ***Scatterplot with univariate correlations for the association between body mass index (BMI) and fractional excretion of creatinine (FE_{creat}; left panel, r=0.388, p<0.01) and bias in 24h creatinine clearance (24h CrCl; right panel; r=0.234, p=0.03).***

To identify the independent contribution of the various univariate determinants, multivariate linear regression models were constructed. For simultaneously assessed CrCl the bias was best predicted by a model including gender (higher bias in men; β=0.205, p=0.04) and BMI (β=0.351, p<0.01), whereas 24h creatinine excretion dropped out of the model. In models including BW (R=0.380, p=0.001) or BSA (r=0.321, p<0.01) gender dropped out of the model; however, these models had a lower R. In the regression model with FE_{creat} as dependent variable, gender and 24h creatinine excretion dropped out of the model. A significant model could be built with only BMI (β=0.388, p<0.01) as independent variable. Models with only BW or BSA had lower R.

A higher BMI itself was correlated to a higher serum creatinine (r=0.283, p<0.01) and a higher 24h creatinine excretion (r=0.439, p<0.01). Moreover, males had a higher BMI (27.3 ± 3.8 $kg \cdot m^{-2}$) than females (25.8 ± 3.9 $kg \cdot m^{-2}$, p=0.04).

Discussion

In this study in healthy kidney donors the predictive performance of 24h CrCl for assessment of renal function was fair. Thus, in dedicated settings 24h CrCl can be useful to measure renal function in populations with renal function in the normal or higher range when gold standard methods are not available. However, a systematic error was identified with overestimation of GFR by CrCl that was particularly apparent in subjects with weight excess, amounting from approximately zero in lean subjects to approximately 10 ml·min^{-1} in obese subjects. FE_{creat} indicated net tubular secretion of creatinine in these healthy subjects. Remarkably, a higher BMI was an independent determinant of a higher FE_{creat}. Thus, the impact of BMI on the bias of CrCl and its impact on tubular handling of creatinine need to be accounted for in the interpretation of creatinine-based renal function assessment.

Our first goal was to assess the predictive performance of 24h CrCl in a population with renal function in the normal or higher range. Albeit not perfect, the predictive performance of CrCl was satisfactory, and better than for eGFR. The predictive performance of eGFR in our population was in line with reports in the literature on populations with normal or only slightly impaired renal function[11-13]. Our data indicate that, at least in a dedicated setting where subjects are highly motivated to accurately collect 24h urine, 24h CrCl is better suited than eGFR for estimation of renal function in subjects with renal function in the normal and higher range.

Remarkably, CrCl systematically overestimated GFR, suggesting net tubular secretion of creatinine. Overestimation of GFR due to tubular secretion of creatinine is well-established in subjects with moderate to severe renal function impairment[15,21] but, to the best of our knowledge, it has not been established in

subjects with normal renal function. To establish the extent of tubular secretion of creatinine we measured FE_{creat} during simultaneous assessment of CrCl and iothalamate-GFR, to circumvent confounding effects by urine collection errors or by the diurnal rhythm of GFR. The mean FE_{creat} of 110% strongly supports the presence of net tubular secretion of creatinine in these healthy subjects. Higher BW, BSA and BMI were all associated with a higher FE_{creat}, suggesting that larger body dimensions and larger net creatinine supply are the common denominator of a higher FE_{creat}. However, the association between FE_{creat} and 24h creatinine excretion was of borderline significance only, and thus does not unequivocally support this assumption.

Data from the literature nevertheless support an association between creatinine supply (i.e. creatinine levels in the peritubular capillaries) and tubular secretion of creatinine. First, the presence of net secretion of creatinine in subjects with renal function impairment and accordingly elevated creatinine levels, is well-established[15,21]. In healthy subjects recent studies demonstrated that infusion of exogenous creatinine leads to increases in FE_{creat} up to 200% immediately after infusion[22,23]. Thus, apparently, also in healthy subjects tubular secretion rate of creatinine increases along with the supply of creatinine. As BW, BSA and BMI are probably all associated with larger muscle mass, larger food intake and therefore larger creatinine supply, we consider it likely that creatinine supply is at least partially involved in tubular secretion of creatinine in our population. However, the supply hypothesis does not explain why BMI was the main determinant of FE_{creat}, rather than BSA or 24h creatinine excretion. Differences in signal-to-noise ratio for the different parameters could be involved, but alternatively, specific effects of weight excess on tubular function could be postulated, that deserve further investigation.

For clinical application the systematic error in 24h CrCl warrants proper attention. The average overestimation was smaller than for the simultaneously assessed CrCl, probably due to the diurnal rhythm of renal function with lower values during the night that are incorporated in 24h CrCl[24]. Nevertheless, a higher BMI was also significantly associated to a higher bias in 24h CrCl. Whereas the overestimation was absent or negligible in lean subjects, it amounted to 8 and 10 $ml \cdot min^{-1}$ in overweight and obese subjects, respectively. This systematic error will have to be taken into account when CrCl is used to evaluate renal function in subjects with weight excess. Increasing evidence supports an association between early metabolic abnormalities and elevated CrCl[25,26], assessed from creatinine-based renal function estimates. Whereas the association between weight excess and an increase in true GFR is well-established[27,28], our current data suggest that elevation in measured CrCl contains not only a component of elevated filtration, but also of elevated tubular secretion of creatinine. Whether accounting for this differential mechanism of increased creatinine clearance can improve its prognostic impact, would be highly interesting to pursue in epidemiological studies.

Our findings suggest that the validity of expressing tubular function as clearance of a substance divided by clearance of creatinine is biased in a BMI dependent way. For instance, FE_{Na+} would be underestimated by some 8 to 10 % in overweight and obese subjects, leading to biased conclusions on the extent of altered sodium handling in subjects with weight excess.

For better estimation of renal function from CrCl correction for BMI could be considered, as this can reduce or annihilate the BMI-related error. This would require data from larger populations. Furthermore, cimetidine can block tubular handling of creatinine[29], and may thus be useful as a tool to substantiate our

inferences on tubular secretion of creatinine. Finally, it should be noted that Cystatin C, rather than creatinine has been advocated as a suitable marker for renal function in the normal or upper range[30,31]. Further studies would be needed to determine the performance of CrCl with proper correction for tubular handling, as compared to Cystatin C.

Several limitations of our study should be considered. First, we used a single outpatient collection of 24h urine. Inaccurate urine collection is considered the most important threat to the validity of 24h urine. The use of 2 or 3 24h urine samples to improve the validity of 24h CrCl has been proposed[32], so possible the predictive performance of 24h CrCl could be improved. Second, we had no information on food intake, which may have been involved in some of the associations we observed. Finally, it should be mentioned that the absolute level of the bias strongly depends on the actual calibration of the creatinine assay. However, the absolute values of creatinine cannot explain the association of creatinine handling with BMI.

In conclusion, in this population of healthy kidney donors the predictive performance of 24h CrCl was satisfactory, demonstrating its feasibility for renal function assessment in subjects with renal function in the normal or upper range. Remarkably, our data support the presence of tubular secretion of creatinine in these healthy subjects, that was particularly apparent in overweight and obesity. The impact of BMI on tubular handling of creatinine will have to be accounted for in studies addressing the renal phenotype in overweight and obesity.

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CHAPTER 2B

HIGHER BODY MASS INDEX IS ASSOCIATED WITH A LARGER RISE IN EXTRA CELLULAR FLUID VOLUME IN RESPONSE TO HIGH SODIUM INTAKE IN HEALTHY MEN

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Abstract

A high sodium intake (HS) is associated to increased cardiovascular and renal risk, especially in overweight subjects. We hypothesized that abnormal sodium and fluid handling is involved, independent of hypertension or insulin resistance. Therefore, we studied the relation between body mass index (BMI) and sodium induced changes in extra cellular fluid volume (ECFV; distribution volume of ^{125}I-iothalamate) in 78 healthy men, not selected for BMI.

78 subjects with a median BMI of 22.5 (range: 19.2-33.9 $kg \cdot m^{-2}$) were studied after one week on a low sodium diet (LS, 50 mmol Na^+/day) and after one week on HS (200 mmol Na^+/day). The change from LS to HS resulted in an increase in ECFV of 1.2 ± 1.8 l. Individual changes in ECFV were correlated to BMI ($r=0.361$, $p<0.01$). Furthermore, in response to HS, a higher BMI was associated to a higher rise in filtered load of sodium ($FL_{Na+} = [Na^+] \cdot GFR$, $r=0.281$, $p<0.05$).

Thus, a shift to HS leads to a larger rise in ECFV in healthy subjects with higher BMI, associated with an elevated FL_{Na+} during HS. Although no hypertension occurred in these healthy subjects, our data provide a potential explanation for the interaction of sodium intake and BMI on cardiovascular and renal risk. Exaggerated fluid retention may be an early pathogenic factor in the cardio-renal complications of overweight.

Introduction

Several lines of evidence suggest an interaction between weight excess and high sodium intake on cardiovascular and renal risk profile. First, epidemiological studies have shown an association between high dietary sodium intake and an increased cardiovascular morbidity and mortality[1-3], that appears to be absent in lean subjects[4,5]. In line, in the PREVEND study an association between sodium intake and the cardiovascular and renal risk marker micro-albuminuria was found that was strong in overweight, and particularly obese subjects, but absent in lean subjects[6]. Moreover, weight excess is well-known to be associated with the sodium sensitivity of blood pressure[7,8]. Finally, we recently reported that high sodium elicits a renal hyperfiltration profile in overweight, but not lean young men[9]. Together, these data suggest that weight excess modulates the adverse effects of excess sodium intake on cardiorenal risk profile.

The mechanism underlying this interaction has not been well established, but effects of weight excess on renal sodium handling and volume homeostasis are likely. In obese subjects with the metabolic syndrome tubular sodium reabsorption is increased[10]. Moreover, hypertensive obese subjects have a higher extra cellular fluid volume (ECFV) than non-obese subjects without hypertension[11]. Metabolic syndrome and insulin resistance may well be involved in the association between weight excess and volume homeostasis[12], but data from our group demonstrate that renal effects of weight excess also occur independent of the metabolic syndrome and/or hypertension[9,13]. Of note, the effects of body mass index (BMI) on renal risk profile are not limited to overt or morbid obesity, but extend to well into the overweight range, i.e. a BMI between 25-30 kg/m^2, and perhaps even lower, thus extending to a considerable proportion of the population. To test the hypothesis that BMI is a determinant of volume homeostasis in healthy subjects we

studied renal sodium handling and ECFV in 78 normal subjects in balance on low and high dietary sodium intake, respectively, and analysed for a possible interaction between BMI and sodium homeostasis.

Methods

This study is a post-hoc analysis from a larger study published earlier on the impact of BMI on the renal haemodynamic adaptation to high sodium intake[9]. Recently, we showed that renal function assessment with the specific tracer ^{125}I-iothalamate (IOT), could also be used for estimation of ECFV[14]. The assessment of ECFV needs a single additional urine sampling that is not needed for glomerular filtration rate (GFR) assessment, to assess urinary excretion of IOT during the run-in period. This additional sampling was introduced only after the first 18 subjects had been studied. Accordingly, ECFV could be estimated in 78 out of the original 96 subjects; only these 78 healthy males were included in the current analyses. The subset of 78 subjects was not different to the total population of 96 subjects in age, blood pressure, body weight (BW), length, BMI or ECFV (all $p>0.5$). The study was approved by the local medical ethics committee, in accord with the Declaration of Helsinki Principles, and all participants gave written informed consent.

Study protocol

Subjects were studied at the end of two different 7-day periods, during which they used a low sodium diet (LS; 50 mmol Na^+/day) and a high sodium diet (HS; 200 mmol Na^+/day), respectively. Potassium intake was standardized at 80 mmol/day. Otherwise, the subjects continued their usual food habits. For assessment of dietary compliance and sodium balance, 24 h urine was collected at

day 4 and day 6 during each period. During both periods, the subjects were ambulant and continued their normal activities.

At day 7 of both study periods, the subjects reported at the research unit at 08.00 hours, after having abstained from food and alcohol overnight. Height and BW were measured at the start of this day. During the study day, subjects remained in a semi-supine position except during voiding. One intravenous cannula was inserted in each forearm. One was used for infusion of tracers and the other for infusion of fluids and blood sample withdrawal. Blood was collected for fasting glucose and insulin determination. At 11.00 hours, blood was withdrawn for determination of active plasma renin concentration and aldosterone. Sodium intake during the day was adjusted according to the actual diet in the concerning diet period. To ensure sufficient urine output, 250 ml of 5% glucose solution was administered in the right antecubital vein and subjects were provided with 250 ml of oral fluids every hour. After a 2 hour run-in period, GFR and ERPF were measured as the clearances of constantly infused IOT and ^{131}I-Hippuran, respectively. In this set-up, GFR is measured as the urinary clearance of IOT, and corrected for voiding errors by the ratio of plasma to urinary clearance of ^{131}I-Hippuran[15,16]. ECFV is measured as the distribution volume of IOT during steady state, as described in more detail previously[14]. Briefly, the distribution volume of IOT is calculated from the plasma level of IOT divided by the total amount of IOT in the body, which equals the amount infused minus the amount excreted. It is calculated as $\Sigma(I \cdot V)+Bolus-\Sigma(U \cdot V)/P$, and expressed as ECFV/BSA, i.e. l·1.73m^{-2}BSA. GFR, ERPF and ECFV measured as outlined above, has a day-to-day variation of 2.5, 5 and 9.2 %, respectively[14,15].

Blood pressure was assessed with an automatic device (Dinamap®) at 15 min intervals. Mean arterial pressure (MAP) was calculated as diastolic pressure plus

one-third of the pulse pressure. Data on sodium handling were calculated as the mean of the two one-hour clearance periods, simultaneously with the GFR measurements. Fractional excretion of sodium was calculated as (U·V)/P of sodium divided by GFR and expressed as %. Filtered load FL_{Na+} was calculated as $[Na^+]$·GFR and tubular reabsorption of sodium (TR_{Na+}) as FL_{Na+} minus urinary excretion; both were expressed in mmol·min^{-1}.

Calculation of BSA and BMI

Body Surface Area (BSA) and BMI were calculated from data obtained after a one-week low sodium diet. BMI was calculated as BW (in kg) divided by the square of height (m). BSA was calculated according to[17]: 0.007184·height (cm)$^{0.725}$·BW (kg)$^{0.425}$. ECFV is given indexed to 1.73·BSA, to make comparison between subjects possible.

Chemical analysis of urine and blood samples

Urinary concentrations of sodium and potassium and blood concentrations of sodium and lipids (fasting) were measured by standard auto-analyser technique (MEGA, Merck, Darmstadt, Germany). Insulin was determined on an AxSym with a threshold of 1.0 μU·ml^{-1} and intra-assay and inter-assay coefficients of variation of 2.6 and 4.3%, respectively (Abbott BV, Amstelveen, The Netherlands). Plasma glucose was determined by glucose-oxidase method (YSI 2300 Stat plus, Yellow Springs, OH, USA). Active plasma renin concentration was determined in terms of angiotensin I generation using a radioimmunoassay[18]. Aldosterone was measured with a commercially available radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA, USA). As a measure for insulin resistance, the HOMA index was calculated as[19] [insulin (fasting plasma level)·glucose (fasting plasma level)] / 22.5.

Data analysis

Data were analyzed using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SD in text and tables and as mean ± SEM in figure 1. Simple Pearson's parametric correlation was used for continuous analysis. Furthermore, the paired sample T-test was used for paired analyses (LS versus HS), the independent samples T-test for other parametric data and a Wilcoxon's signed rank test for other non-parametric data. Data on ECFV were analyzed both as crude values and after normalization for BSA. Since no essential differences between the two analyses were found, ECFV is only given normalized to BSA.

Results

The study population consisted of 78 healthy normotensive Caucasian men (age 24 ± 6 yrs) not selected for BMI. All subjects had normal blood pressure, with a systolic blood pressure <140 mm Hg and diastolic blood pressure <90 mm Hg, both after low sodium (LS) and high sodium diet (HS). All medical histories were without significant disease, and results of physical examination were unremarkable. In none of the subjects signs of diabetes or the metabolic syndrome were present. Height was 185 ± 7 cm; BW during low sodium 79.0 ± 10.4 kg. Median BMI was 22.5, ranging from 19.0 to 33.7 kg/m^2.

Subject characteristics by BMI: effect of sodium intake

In table 1 subject characteristics are shown for measurements after one week LS and one week HS diet, and by a break up by median BMI. No differences in blood pressure, fasting glucose, insulin, HOMA index, serum cholesterol, HDL cholesterol, LDL cholesterol or triglycerides were found between the subgroups with highest and lowest BMI. Blood pressure rose significantly when shifting from LS to HS (MAP: 86 ± 7 vs 88 ± 7 mmHg respectively, $p<0.01$); however, without differences between the BMI groups.

Adherence to the sodium diet was good and equal between the BMI groups. [Na^+] rose significantly when shifting from LS to HS (138 ± 3 vs 139 ± 3 mmol·l^{-1} respectively, $p=0.01$), without differences between the BMI groups. During LS intake all parameters of renal function and sodium handling, as well as ECFV·1.73m^{-2} were similar between the groups. However, during HS, significant differences in renal sodium handling and volume status emerged between the groups. ECFV·1.73m^{-2} was significantly higher in the higher BMI group, i.e. 16.7 ± 1.4 vs 18.1 ± 1.6 l·1.73m^{-2} ($p<0.001$) as also illustrated in figure 1. Renal sodium

handling was different between the groups during high sodium only, with a higher FL_{Na+} in the higher BMI group, mainly related to the significantly higher GFR. TR_{Na+} was higher as well; FE_{Na+} was lower in the higher BMI group, but this difference did not reach statistical significance (p=0.1).

	Low Sodium intake		High Sodium intake	
	BMI < median	**BMI > median**	**BMI < median**	**BMI > median**
MAP (mmHg)	85 ± 6	88 ± 8	87 ± 7	89 ± 6
Body Weight (kg)	73.4 ± 6.2	84.5 ± 10.8*	74.6 ± 6.4	86.0 ± 10.9*
Height (cm)	186 ± 6	185 ± 7	-	-
BMI ($kg \cdot m^{-2}$)	21.1 ± 0.9	24.6 ± 2.5*	21.5 ± 0.9	25.0 ± 2.5*
Glucose ($mmol \cdot l^{-1}$)	4.7 ± 0.7	4.6 ± 0.7	4.6 ± 0.6	4.5 ± 0.5
Insulin ($mU \cdot l^{-1}$)	11.1 ± 6.7	10.2 ± 4.9	9.4 ± 4.3	9.9 ± 5.3
HOMA	2.4 ± 1.7	2.1 ± 1.1	1.9 ± 1.0	2.0 ± 1.2
Total cholesterol ($mmol \cdot l^{-1}$)	4.3 ± 0.8	4.2 ± 0.6	4.1 ± 0.8	4.2 ± 0.7
Triglycerides ($mmol \cdot l^{-1}$)	1.2 ± 0.6	1.2 ± 0.6	1.1 ± 0.6	1.1 ± 0.5
HDL-cholesterol ($mmol \cdot l^{-1}$)	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3
LDL-cholesterol ($mmol \cdot l^{-1}$)	2.6 ± 0.6	2.5 ± 0.6	2.4 ± 0.6	2.6 ± 0.6
Na^+ excretion ($mmol \cdot 24h^{-1}$)	38 ±25	37 ± 21	241 ± 68	243 ±61
Serum [Na^+] ($mmol \cdot l^{-1}$)	138 ± 3	139 ± 3	139 ± 3	140 ± 3
ECFV ($l \cdot 1.73m^{-2}$)	16.3 ± 1.8	16.7 ± 1.4	16.7 ± 1.4	18.1 ± 1.6*
FL_{Na+} ($mmol \cdot min^{-1}$)	17.4 ± 2.2	18.4 ± 2.6	18.5 ± 2.3	20.2 ± 2.7*
TR_{Na+} ($mmol \cdot min^{-1}$)	17.3 ± 2.2	18.3 ± 2.6	18.1 ± 2.3	19.9 ± 2.8*
FE_{Na+} (%)	0.51 ± 0.4	0.45 ± 0.4	1.75 ± 0.6	1.52 ± 0.7
GFR ($ml \cdot min^{-1}$)	125 ± 16	132 ± 18	131 ± 15	145 ± 18*
PRA ($nmol \cdot l^{-1} \cdot h^{-1}$)	6.4 ± 3.2	6.5 ± 3.6	2.3 ± 1.2	2.3 ± 1.2
Aldo ($nmol \cdot l^{-1}$)	135 ± 71	160 ± 104	46 ± 26	42 ± 28

*Table 1 Characteristics after one week low sodium and one-week high sodium diet according to a break-up according to median body mass index (BMI); MAP, mean arterial pressure(MAP) ; homeostatic model assessment (HOMA); extra cellular fluid volume (ECFV); glomerular filtration rate (GFR); filtered load of sodium (FL_{Na+}); tubular reabsorption of sodium (TR_{Na+}; fractional excretion of sodium (FE_{Na+}); plasma renin activity (PRA); aldosterone (Aldo) . * p<0.01 low vs high BMI-group.*

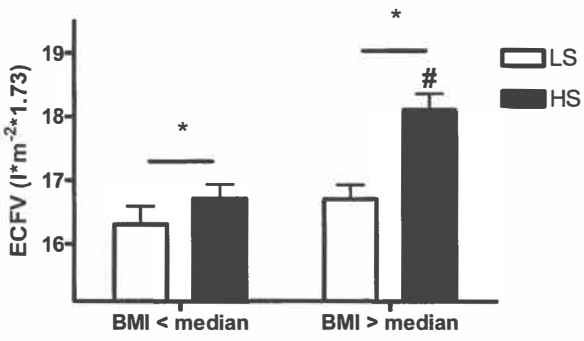

*Figure 1 Values for extra cellular fluid volume (ECFV), for respectively low (LS) and high dietary sodium (HS) intake shown for a break-up according to median body mass index (BMI). * p<0.01 low versus high sodium intake. # p<0.01 BMI below versus above median.*

In table 2 the sodium induced changes in renal sodium handling and ECFV are summarized. The shift from low to high sodium elicited a modest rise in ECFV ($p<0.05$) in the lower BMI group with a significantly larger rise in ECFV ($p<0.01$ low vs high BMI group) in the higher BMI group. FL_{Na+} increased more in the higher BMI group, reflecting a larger rise in GFR (+12.3 ± 9.8 vs +6.7 ± 12.0 ml·min^{-1} in the lower BMI group, $p=0.02$) and larger rise in filtration fraction (FF) (+0.97 ± 2 vs -0.16 ± 2%, $p=0.03$) in that group. TR_{Na+}, expressed in mmol·min^{-1} also increased more in the highest BMI group. Thus, the sodium-induced increase in TR_{Na+} was, in parallel to the increase in FL_{Na+} higher in the highest BMI group. As a measure for relative blunting of tubular reabsorption, FE_{Na+} rose less in the highest BMI group, but this finding did not reach statistical significance ($p=0.12$).

	Sodium induced changes		Correlation (r) with BMI
	BMI< median	BMI > median	
Δ ECFV (l·m^{-2}·1.73^{-1})	0.5 ± 1.6	1.5 ± 1.5*	0.361**
Δ FL_{Na+} (mmol·min^{-1})	1.0 ± 1.6	1.9 ± 1.3*	0.329**
Δ TR_{Na+} (mmol·min^{-1})	0.8 ± 1.6	1.7 ± 1.2*	0.341**
Δ FE_{Na+} (%)	1.25 ± 0.6	1.12 ± 0.6	-0.210 (ns)

*Table 2 Sodium induced changes shown according to a break-up according to median body mass index (BMI) with in the right column the correlation coefficient (r) for the univariate correlation with BMI., extra cellular fluid volume (ECFV); filtered load of sodium (FL_{Na+}); tubular reabsorption of sodium (TR_{Na+}); fractional excretion of sodium (FE_{Na+}). *p<0.05 low vs high BMI group. **p<.05 for continuous univariate correlation with BMI.*

BMI as a determinant of ECFV during high sodium intake

A higher BMI significantly correlated to a larger rise in ECFV ($r=0.361$, $p<0.01$) as shown in figure 2 and table 2, right column. The correlation was still significant after exclusion of the 2 subjects with a BMI >30 $kg \cdot m^{-2}$ ($n=76$; $r=0.328$, $p<0.01$). These univariate data were confirmed by multivariate analysis. In the model, with an r^2 of 0.131, BMI was the only significant predictor of the change in ECFV ($\beta=0.361$, $p<0.01$) as dependent variable; forced entry of blood pressure, age and renal haemodynamics did not improve the model. Furthermore, as shown in table 2, BMI correlated with a larger sodium induced change in FL_{Na+} ($r=0.281$, $p<0.05$) and in TR_{Na+} ($r=0.293$, $p<0.05$), but not to the change in $[Na^+]$ or MAP. Correlations with the changes in FE_{Na+} did not reach statistical significance. During LS diet, FL_{Na+}, TR_{Na+} and FE_{Na+} did not correlate to BMI. During HS however, a higher FL_{Na+} (0.356, $p<0.01$), a higher TR_{Na+} ($r=0.373$, $p<0.01$) and a lower FE_{Na+} ($r=-0.263$, $p<0.05$) was significantly correlated to a higher BMI.

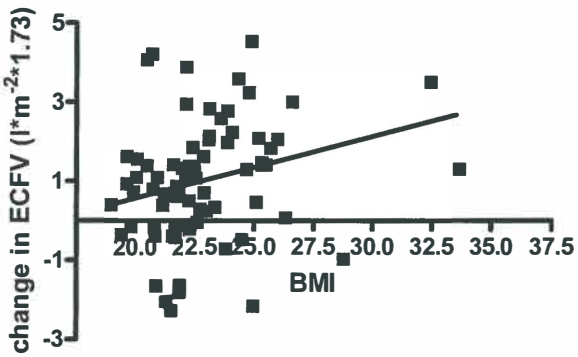

Figure 2 Scatterplot for the shift in extra cellular fluid volume (ECFV) from low sodium to high sodium intake versus body mass index (BMI). r=0.361, p<0.01

Results on correlations between BMI and sodium induced renal haemodynamic changes presented in earlier report[9], were reproduced in the subset used for the current study, namely a correlation between BMI and a sodium induced rise in GFR ($r=0.233$, $p<0.05$) and in FF ($r=0.274$, $p<0.05$); BMI was not related to the sodium induced rise in ERPF.

Discussion

The current study is the first to demonstrate that BMI determines the response of ECFV to a rise in sodium intake in healthy young adults. The rise in ECFV in response to a high sodium diet was larger in subjects with a higher BMI, even in the absence of overt obesity, or hypertension. As a consequence, during high sodium intake ECFV/BSA was significantly higher in overweight subjects than in lean subjects, whereas during low sodium diet it was not different. Our data suggest that effects of BMI on volume regulation may be involved in the combined effects of weight excess and sodium intake in long term cardiovascular risk in epidemiological studies.

Our study was performed in healthy young men. To be able to dissect the effects of a higher BMI as such from those of its complications, hypertension and diabetes were exclusion criteria. Moreover, none of the subjects in our study met the criteria of the metabolic syndrome, and HOMA was normal in all subjects, suggesting that insulin resistance was not involved. The effects on sodium homeostasis thus appear to be related to the higher BMI per se as rather than to any of its complications. Our population was not selected for weight excess, median BMI was 22.5 $kg \cdot m^{-2}$, and only two subjects were obese. Thus, the weight excess in our population was not particularly prominent and it is remarkable that clear-cut effects on volume status could nevertheless be observed. Yet, this is consistent with data demonstrating that the association between young adult BMI, metabolic risk factors, and long term risk also extends to the range of BMI below 25[9,13,20,21].

Altered sodium handling and volume excess have been reported previously in overt obesity and the metabolic syndrome[10,12,22-24]. In the Olivetti study obesity and the metabolic syndrome were associated with increased tubular sodium

reabsorption as well as hypertension[10,12], and Chagnac reported altered tubular sodium handling in morbidly obese subjects[24]. Our study is the first to demonstrate BMI-dependent altered volume homeostasis in the absence of overt obesity, i.e. in the overweight range, in healthy young adults. Apparently, overt obesity or presence of the metabolic syndrome are not a prerequisites for BMI dependent alterations in sodium status. This is consistent with the assumption that abnormal renal sodium may be a causal factor in overweight-associated morbidity rather than a consequence.

In our study the effects of BMI on volume status were not associated with an effect on blood pressure. Apparently in these normotensive subjects blood pressure was not volume-dependent - at least not over the range of volume change investigated here - and peripheral vasodilatation accounted for a stable blood pressure despite a higher ECFV. The absence of an association with higher blood pressure allows to conclude that the altered sodium handling was not secondary to the presence of hypertension in subjects with higher BMI. This is relevant to note, as most observations on altered sodium handling in obesity were made in hypertensive conditions, be it in animal studies or in human[10,12,22,24-26].

What could be the clinical relevance of an effect on sodium status without a blood pressure effect? Several lines of evidence support adverse effects of high sodium intake that are independent of blood pressure. For instance, the association between high sodium intake and left ventricular hypertrophy is on partly dependent on blood pressure[27]. Moreover, several epidemiological studies have shown an association between sodium intake and cardiovascular morbidity and mortality that is independent of blood pressure[1,2]. Remarkable, this also accounts for the combined effects of BMI and sodium intake on long term outcome[4,5]. Excess

expansion of ECFV, and its consequent volume load for the heart would be a plausible candidate mechanism underlying blood pressure independent effects of high sodium intake. As ECFV is not usually measured however, data to directly support this assumption are lacking.

We previously reported on the effect of BMI on the renal haemodynamic response to high sodium intake[9]. Our current report addresses the concomitant effects on ECFV in a large subset of this population. The effects on renal haemodynamics in this subset were fully in line with those of the whole population, namely a more pronounced rise in GFR in the subjects with the higher BMI. In fact, the impact of BMI on the responses of GFR and ECFV to high sodium was strikingly similar, suggesting that the more pronounced rise in GFR in the overweight subjects might be due to the more pronounced rise in ECFV. In this concept, overweight-associated hampered suppression of tubular reabsorption would be the primary phenomenon, and the exaggerated rise in ECFV and GFR its consequence, allowing the achievement of sodium balance by a larger increase in filtered load.

This study has several limitations. First, we used BMI as a measure for adipose tissue, although it is only an indirect assessment. Second, we approximated renal sodium handling by measuring the fractional excretion of sodium, without however data allowing to dissect between proximal and distal tubular sodium handling. Moreover, it should be mentioned that ECFV is directly related to body dimensions. Thus, differences between individuals should be interpreted with caution as these are less robust than those on the within-individual sodium-induced changes. Finally it should be mentioned that we investigated ECFV after only one week of altered sodium intake. Whereas this was sufficiently long to

achieve sodium balance again, it is unknown whether the differences observed here persist during long term follow-up.

From our data it could be hypothesized that a low sodium intake could have the potential to prevent part of the cardiovascular and/or morbidity associated with weight excess, but long term data would be needed to substantiate this assumption.

We conclude that in young healthy men a higher BMI is associated with a larger increase in ECFV during high sodium intake. These data suggest that altered sodium and fluid handling may be an early phenomenon in the pathophysiological consequences of weight excess, and that dietary sodium restriction may have preventive potential in overweight subjects.

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CHAPTER 3

RENAL RESPONSE TO ANGIOTENSIN II IS BLUNTED IN SODIUM SENSITIVE NORMOTENSIVE MEN

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Abstract

In hypertension, sodium sensitivity of blood pressure (SS) is associated with renal haemodynamic abnormalities, related to increased activity of the renal renin angiotensin aldosterone system (RAAS). The renal mechanisms of SS in normotensives are unknown. Therefore we studied whether SS is related to renal haemodynamics and renal responsiveness to angiotensin II (AngII) in young healthy adults.

Blood pressure and renal function and extra cellular fluid volume (ECFV; distribution volume (Vd) of ^{125}I-iothalamate (IOT)) were measured in 34 healthy men after one week low (LS; 50 mmol $Na^{+}\cdot 24h^{-1}$); one week high sodium diet (HS; 200 mmol $Na^{+}\cdot 24h^{-1}$); and one week HS-ACEi (enalapril 20 mg/day). The responses of effective renal plasma flow (ERPF; ^{131}I-Hippuran clearance) to graded infusion of AngII were assessed during each condition.

The sodium-induced change in mean arterial pressure (MAP) ranged from -7 to + 14 mmHg. SS (a sodium-induced increase in MAP >3 mmHg) was present in 13 subjects. ERPF was lower in SS subjects during LS and during HS-ACEi. ECFV was higher during HS intake. The AngII-induced decrease in ERPF was blunted in SS on LS (-25 ± 6 vs -29 ± 7% in sodium resistant subjects, $p<0.05$) and on HS (-30 ± 5 vs -35 ± 6%, $p<0.05$). The blunting was corrected by ACEi (-36 ± 6 vs -37 ± 7%, ns).

SS normotensive subjects have a blunted renal response to exogenous AngII. This is ameliorated by ACEi, supporting a role for inappropriately high intra-renal RAAS activity. As these findings cannot be attributed to subclinical renal hypertensive damage, high intra-renal RAAS activity and altered renal haemodynamics may be primary phenomena underlying SS.

Introduction

The response of blood pressure to a change in sodium intake is characterized by a wide interindividual variability. In hypertensive patients sodium sensitivity of blood pressure (SS) is associated with the presence of cardiovascular risk factors[1-3], an elevated cardiovascular and renal risk[2,4] and an elevated mortality[5]. Thus, SS appears to herald susceptibility to cardiovascular and/or renal damage. Therefore, elucidation of its underlying mechanisms is important.

The pathogenesis of SS is complex and multiple pathways appear to be involved, such as hampered action of natriuretic peptides, elevated endogenous ouabain like substances, reduced excretion of renal kallikrein, renal inflammation, oxidative stress and blunted arterial baroreflex sensitivity[6]. A consistent line of research points towards a role of the renin angiotensin aldosterone system (RAAS)[7]. Suppression of RAAS-activity, leading to vasodilatation in the renal and systemic vascular bed, and facilitating sodium excretion, plays a main role in the adaptation to a higher sodium intake[8]. A role for inappropriately high intra-renal RAAS-activity in SS, during high sodium in particular, is supported by studies in SS hypertensives. These studies showed SS hypertension to be associated with decreased renal plasma flow[1,2,9] and a blunted renal vasodilator response to high sodium intake[8,9]. Moreover, the renal haemodynamic response to exogenous angiotensin II (AngII) is blunted. The latter is corrected by RAAS blockade[8,9]. Finally, there is a more pronounced renal haemodynamic response to RAAS-blockade during high sodium intake[3,8]. Together these findings suggest a role for increased intra-renal RAAS-activity, in particular during high sodium intake.

Most human data on renal mechanisms of sodium sensitivity were obtained in middle-aged subjects with established hypertension and/or signs of target organ

damage[1-3,8,9]. Elegant animal data support a role for acquired subclinical renal interstitial damage in the pathogenesis of SS, as induction of interstitial changes consistently induced SS[10]. Thus, the RAAS-dependent renal mechanisms of SS as documented in SS hypertensives, might be the result of secondary subclinical renal damage due to the pre-existent state of hypertension.

In normotensive subjects SS can also be present, albeit at a lower prevalence than in hypertensive populations[11]. Interestingly, in normotensive subjects SS predicts mortality as well[5], supporting its pathophysiological relevance. The aim of the present study therefore, was to investigate renal mechanisms of SS, independent of possible subclinical hypertensive renal damage. In particular, we investigated whether the above-mentioned signs of inappropriate intra-renal RAAS activity, as documented in hypertensives, could also be detected in normotensive sodium sensitive young men.

Methods

Study population

34 healthy, non tobacco or medication using Caucasian men (26.5 ± 9.4 years) were recruited for the study. Their medical history revealed no significant diseases. Physical examination was unremarkable. All subjects were normotensive with a systolic blood pressure lower than 140 mmHg and diastolic blood pressure lower than 85 mmHg in the sitting position. Family history of hypertension was defined as at least one parent or sibling with hypertension. This study was approved by the medical ethical committee of the University Medical Centre Groningen and all participants gave written informed consent.

Study design

This cross-over protocol consisted of three randomized one-week periods, a 7-day period on a high sodium diet (HS; aim: 200 mmol Na^+/day); a 7-day period on a low sodium diet (LS; aim: 50 mmol Na^+/day) and a 7-day period on a high sodium diet combined with the use of Enalapril 20mg taken every morning (HS-ACEi), which was placebo-controlled and double-blind. A 7-day-period of sodium restriction is sufficient for stabilization of circulatory hormones[12], for induction of RAAS activation and for conducting sodium balance[13]. Sodium intake during the study was adjusted according to the prescribed diet, which was iso-caloric with a similar balance between protein, carbohydrate and fat during the study. On day 4 and day 6 of each dietary period, subjects collected 24-hour urine to assess dietary compliance and the achievement of a stable sodium balance. When sodium balance was not achieved the testing day was postponed for 3 or 7 days. On day 7 the subjects reported to the research unit at 08.00 hours after an overnight fast.

An intravenous cannula was inserted into the forearm for drawing blood samples; infusion of 250 ml·hour^{-1} of glucose 5% kept the cannula open. This infusion and 250 ml of oral water intake allowed hourly voluntary voiding. Into the contra-lateral arm another cannula was inserted for tracer and AngII infusion. Glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) were measured as previously described from the clearance of constantly infused ^{125}I-iothalamate (IOT) and ^{131}I-Hippuran, respectively[14]. The clearances were calculated using the formula U·V/P and I·V/P, respectively. U·V represents the urinary excretion of the tracer, I·V represents the infusion rate of the tracer and P represents the tracer plasma value during steady state, which was established after a run-in of 2 hours. The coefficients of variation (COV) for GFR and ERPF are 2.2% and 5.0% respectively. In this setting, we have found the reproducibility of dietary sodium induced shift in renal haemodynamics to be satisfactory (standard deviation of

ΔGFR: 9.9%; SD of ΔERPF: 10.1%)[15]. Extra cellular fluid volume (ECFV) was estimated from the distribution volume (Vd) of IOT and calculated as $[(I \cdot V + B \cdot V)-(U \cdot V)]/P_{iot}$ during steady state. $B \cdot V$ represents the bolus infusion of the tracer[16].

During the experiments the subjects remained in semi-supine position, except for standing up briefly for voiding. Blood pressure was measured, while the subjects were in semi-supine position at 15-minute intervals using a non-invasive device (Dinamap®; GE Medical systems, Milwaukee, USA). Baseline values for blood pressure were obtained from 10.00 to 12.00 hours. Between 12.00 and 15.00 hours AngII (Clinalfa, Merck Biosciences AG, Läufelfingen, Switzerland) was administered intravenously in constant infusion rates of 0.3; 1 and 3 $ng \cdot kg^{-1} \cdot h^{-1}$, each during one hour. The measurement of ERPF by clearance methods has a certain lag-time during acute changes. Our method is based on the assumption of steady state, which is by definition disturbed by acute interventions. We used one-hour clearances, which amount to four times the estimated half life of ^{131}I-Hippuran in normal renal function. Accordingly, the plasma levels at the end of the clearance period approximate the true steady state levels. To account for the lag-time in steady state, we only used the ERPF data from the last clearance period for the assessment of the quantitative relationship with sodium sensitivity. So, the nominal values of the ERPF response may be somewhat underestimated, but this is not likely to modify the between-individual relationship with blood pressure. During the AngII infusions blood pressure was measured at 5-minute intervals.

Laboratory measurements

Urinary sodium and serum cholesterol (fasting) were measured by a standard autoanalyzer technique (SMA-C, Technicon®). Insulin (fasting) was determined on an AxSym with a threshold of 1.0 μU/ml (Abbott BV, Amstelveen, The

Netherlands). Plasma glucose (fasting) was determined by glucose-oxidase method (YSI 2300 Stat plus, Yellow Springs, OH, USA). Urinary albumin excretion (UAE) was determined by nephelometry with a threshold of 2.3 $mg \cdot l^{-1}$ (Dade Behring Diagnostic, Marburg, Germany). Blood for measurement of humoral parameters was rapidly drawn in EDTA tubes, immediately centrifuged at 4 ºC and stored at – 20 °C. Aldosterone was measured with a commercially available radioimmunoassay kit (Diagnostic Products Corporation, Los Angeles, CA, USA). Plasma renin activity (PRA) was measured as described previously with a radioimmunoassay that detects the amount of angiotensin I produced per hour in the presence of excess exogenous angiotensinogen[17], with an incubation time of 1 hour.

Data analysis

Data are presented as mean ± standard deviation (SD) in text and table and as mean ± SEM in figures when normally distributed and as median (25^{th}–75^{th} percentile) when appropriate. The mean of all systolic and diastolic blood pressure recordings were calculated for baseline and for every infusion step of AngII. Subsequently, mean arterial pressure (MAP) was calculated as the mean systolic pressure plus two times the mean diastolic pressure, divided by three.

The sodium induced change in MAP was normalized for a targeted difference in sodium excretion between LS and HS of 150 mmol $Na^+ \cdot 24h^{-1}$. SS was calculated according to the following formula:

$$\frac{\text{Baseline MAP HS} - \text{Baseline MAP LS}}{\text{24h } Na^+ \text{ excretion HS} - \text{24h } Na^+ \text{ excretion LS}} \times 150$$

Data were analyzed in two ways; first we compared sodium sensitive subjects with sodium resistant subjects. To this purpose SS was defined as a sodium induced rise in MAP of more than 3 mmHg per 150 mmol Na^+, as used before[18], and which was shown to be reproducible (Cohen's κ=0.87)[19]. In our setting[15] repeated assessment of SS in healthy young males was reproducible with a Cohen's κ of 0.75 and a correlation of r=0.641 ($p<0.01$) for the individual subjects (unpublished data). Mann-Whitney U-tests were used for comparisons between the subgroups, Wilcoxon-sign-rank test for paired analyses. With the current study size, the power to detect a difference between subgroups in baseline ERPF of 50 $ml \cdot min^{-1} \cdot 1.73m^{-2}$ (SD 60 $ml \cdot min^{-1} \cdot 1.73m^{-2}$; α=0.05, two-tailed) is 63%. The power to detect a difference of 5% in response to AngII infusion (SD 5%, α=0.05, two-tailed) is 78%.

Second, since using a cut-off value is arbitrary, we also performed a continuous analysis, assessing correlation with individual responses of blood pressure to increased sodium intake with Spearman Rho correlation coefficient. Differences in baseline values between LS, HS and HS-ACEi were tested with the paired Student's T-tests for normally divided data, and Mann-Whitney U-tests for skewed distributed data.

Results

Baseline data

All subjects were normoalbuminuric (UAE <30mg·24h^{-1}) and their blood pressure remained in the normotensive range, during both LS and HS. 7 subjects had a positive family history of hypertension (22%, 2 subjects unknown).

	Low sodium	High Sodium	High sodium ACE inhibition
$(U \cdot V)_{Na+}$ (mmol/day)	37 ± 23	218 ± 57*	224 ± 72
Body weight (kg)	79.4 ± 9.4	80.7 ± 9.5*	80.5 ± 9.5
ECFV (l)	19.0 ± 2.9	20.4 ± 3.0*	20.3 ± 3.0
BMI (kg·m^{-2})	22.4 (21.3;24.7)	22.8 (21.8;24.8)*	22.7 (21.7;24.8)
SBP (mmHg)	117 ± 10	122 ± 12*	116 ± 10**
DBP (mmHg)	69 ± 8	70 ± 7	65 ± 13**
MAP (mmHg)	85 ± 7	88 ± 8*	82 ± 8**
ERPF (ml·min^{-1}·1.73m^{-2})	456 ± 61	488 ± 66*	511 ± 70**
GFR (ml·min^{-1}·1.73m^{-2})	105 ± 13	113 ± 14*	114 ± 13
PRA (ng angI·ml^{-1}·h^{-1})	6.2 (4.5;8.1)	2.8 (1.6;3.5)*	8.8 (3.6;14.0)**
Aldo (ng·l^{-1})	130 (81;180)	44 (24;57)*	31 (20;43)**
UAE (mg·24h^{-1})	6.6 (4;8)	4.5 (3;6)	5.4 (4;6)
glucose (mmol·l^{-1})	4.7 ± 0.7	4.6 ± 0 .7	4.5 ± 0.6
Insulin (mU·l^{-1})	8 (6;14)	8 (6;12)	10 (7;14)
Cholesterol (mmol·l^{-1})	4.5 ± 0.7	4.3 ± 0.7	4.4 ± 0.8

Table 1 ***Baseline characteristics***

24 h urinary sodium excretion ($(U \cdot V)_{Na+}$); extra cellular fluid volume (ECFV); body mass index (BMI); systolic blood pressure (SBP); diastolic blood pressure (DBP); mean arterial pressure (MAP); effective renal plasma flow (ERPF); glomerular filtration rate (GFR); plasma renin activity (PRA); plasma aldosterone(Aldo); urinary albumin excretion (UAE). Data expressed as mean (25^{th}-75^{th} percentile) or as mean ± SD for normal divided data. * p<0.05 compared to low sodium; **p<0.05 compared to high sodium

As indicated in table 1, urinary sodium excretion indicated satisfactory dietary compliance, accompanied by an adequate suppression of PRA and aldosterone by HS. HS induced a rise in body weight (BW) of 1.3 ± 1.0 kg, which was reflected by a rise in ECFV of 1.4 ± 2.2 l (p<0.05). As anticipated both GFR and ERPF increased

significantly in response to HS. MAP was slightly but significantly higher on HS compared to LS. ACE inhibition led to a significant decrease in blood pressure, a rise in ERPF and PRA and a drop in aldosterone. UAE, glucose, insulin and cholesterol were not influenced by sodium diet or use of ACEi.

Responses to angiotensin II

The responses of blood pressure to increasing doses AngII during LS, HS and HS-ACEi are shown in Figure 1 (upper panel) as group means. It shows that the lowest dose, 0,3 ng·kg^{-1}·min^{-1}, was a non-pressor dose, with subsequent significant dose-dependent increases during 1 and 3 ng·kg^{-1}·min^{-1}. The blood pressure responses to AngII were not significantly different between LS and HS or HS and HS-ACEi.

The responses of ERPF to AngII in the three periods are shown in Figure 1 (lower panel) as group means. ERPF significantly and progressively decreased in response to increasing doses of AngII during all periods. The magnitude of response was significantly more pronounced on HS compared to LS ($p<0.05$) and on HS-ACEi compared to HS ($p<0.05$). The range of the individual responses assessed during the 3 ng·kg^{-1}·min^{-1} dose was -40% to -16% (LS); -46 to -20% (HS) and -51 to -26% (HS-ACEi).

Baseline determinants of sodium sensitivity of blood pressure

The inter-individual differences in the blood pressure response from LS to HS ranged from -7 to +14 mmHg, with a rise in MAP over 3 mmHg·150mmol^{-1} Na^+ in 13 subjects (38%). The sodium sensitive subjects (SS; n=13) and the sodium resistant subjects (SR; n=21) were similar in age (respectively 28 ± 13 and 25 ± 6, p=0.5) and in body mass index (BMI) (respectively 22.5 (20.9;25.8) and 22.5 (21.7;24.0) kg·m^{-2}, p=0.9). 4 SS subjects (33%, 1 missing value) and 3 SR subjects (15%, 1 missing value) had a positive family history of hypertension (p=0.4).

Figure 1

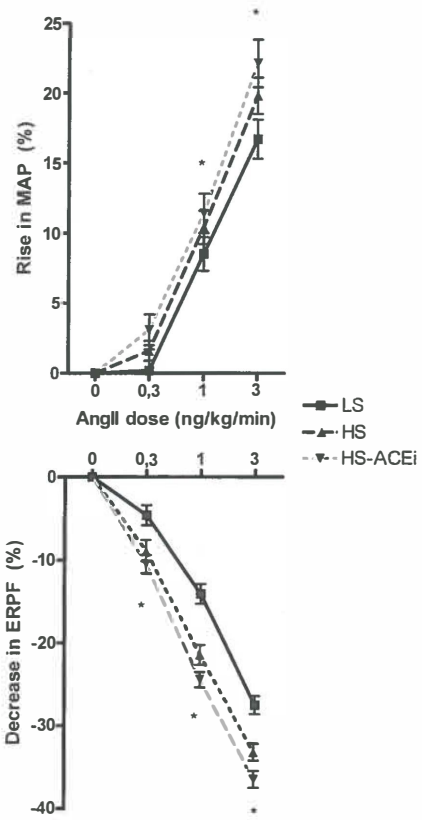

*Figure 1 The dose response curves for mean arterial pressure (MAP) (pressor response - upper panel) and effective renal plasma flow (ERPF) (renal response-lower panel) in response to angiotensin II (AngII) while subjects were on a low sodium diet (LS); a high sodium diet (HS) and HS combined with ACE inhibition (HS-ACEi). *p<0.05 compared to baseline values for LS, HS and HS-ACEi.*

As indicated in table 2 dietary compliance was similar in the two subgroups. Furthermore baseline blood pressure on LS was not significantly different between SS and SR subjects, but SS subjects had higher baseline MAP on HS (p=0.02). This difference between the groups disappeared on HS-ACEi. ERPF was lower in SS subjects during all conditions, although during HS the difference did not reach statistical significance (p=0.13). The shift from LS to HS induced an increase in ERPF of 30 ± 51 ml·min^{-1} in SR subjects (p=0.01) and 37 ± 51 ml·min^{-1} in SS subjects (p=0.02; SR vs SS subjects: p=0.7). Baseline GFR, FF, PRA, aldosterone, UAE, glucose, insulin and cholesterol were not different between SS and SR subjects, and the changes in these parameters induced by HS, and HS-ACEi were not statistically different between SS and SR either.

	Low Sodium			High Sodium			High Sodium – ACE inhibition		
	SR	SS	p	SR	SS	p	SR	SS	p
n	21	13		21	13		21	13	
$(U \cdot V)_{Na+}$ (mmol/day)	32 ± 21	46 ± 23	0.99	227 ± 58	204 ± 55	0.26	227 ± 74	220 ± 73	0.78
MAP (mmHg)	87 ± 5	83 ± 10	0.18	**85 ± 6**	**92 ± 8**	**0.02**	83 ± 7	83 ± 9	0.66
BW (kg)	77.9 ± 7.0	81.9± 12.3	0.24	79.0 ± 7.1	83.2± 12.4	0.22	79.4 ± 7.3	82.3± 12.3	0.40
ECFV ($l \cdot 1.8m^{-1}$)	17.9 ± 2.0	19.2 ± 3.2	0.14	**18.9 ± 2.0**	**21.0 ± 3.2**	**0.03**	19.4 ± 1.7	20.1 ± 3.5	0.50
ERPF ($ml \cdot min \cdot 1.73m^{-2}$)	**472 ± 56**	**429 ± 60**	**0.04**	502 ± 64	467 ± 67	0.13	**531 ± 65**	**480 ± 69**	**0.04**
GFR ($ml \cdot min \cdot 1.73m^{-2}$)	108 ± 12	101 ± 14	0.18	114 ± 12	112 ± 17	0.75	117 ± 10	110 ± 17	0.23
FF (%)	23.0 ± 2.5	23.8 ± 2.7	0.41	22.9 ± 2.3	24.1 ± 2.1	0.12	22.1 ± 1.8	23.1 ± 2.0	0.19
PRA ($nmol \cdot l \cdot h^{-1}$)	5.8 (5.1;8.2)	6.5 (3.0;8.5)	0.70	3.0 (1.9;3.7)	1.8 (1.2;3.2)	0.12	10.5 (4.2;15.5)	5.8 (3.0;13.2)	0.38
Aldo ($nmol \cdot l^{-1}$)	130 (81;216)	130 (81;171)	0.84	43 (24;57)	45 (27;60)	0.89	29 (20;40)	35 (20;49)	0.58
UAE ($mg \cdot 24h^{-1}$)	5.9 (3;7)	6.8 (6;15)	0.37	4.0 (3;6)	4.7 (3;7)	0.63	5.4 (4;6)	5.3 (4;10)	0.12
glucose ($mmol \cdot l^{-1}$)	4.5 ± 0.6	4.9 ± 0.9	0.29	4.5 ± 0.5	4.8 ± 0.9	0.29	4.4 ± 0.4	4.7 ± 0.8	0.32
Insulin ($mU \cdot l^{-1}$)	8 (6;14)	8 (6;13)	0.68	9 (6;12)	9 (6;11)	0.58	11 (7;14)	8 (7;14)	0.38
Cholesterol ($mmol \cdot l^{-1}$)	4.5 ± 0.8	4.5 ± 0.7	0.89	4.3 ± 0.7	4.3 ± 0.7	0.87	4.5 ± 0.9	4.3 ± 0.6	0.62

Table 2 ***Baseline characteristics sodium resistant (SR) vs sodium sensitive (SS) subjects. 24 h urinary sodium excretion ($(U \cdot V)_{Na+}$); mean arterial pressure (MAP); body weight (BW); extra cellular fluid volume (ECFV); effective renal plasma flow (ERPF); glomerular filtration rate (GFR); filtration fraction (FF); plasma renin activity (PRA); plasma aldosterone(Aldo); urinary albumin excretion (UAE).***

Data expressed as mean (25^{th}-75^{th} percentile) or as mean ± SD for normal divided data. P values given for the comparison between SR and SS.

In SS subjects ECFV was higher than in SR subjects during HS (table 2); during LS there was a tendency for a difference as well but this did not quite reach statistical significance. (p=0.10). As a consequence the HS induced change in ECFV was not

statistically different between the subgroups, as indicated in figure 2. After ACEi ECFV was not significantly different between the groups (p=0.32). As indicated in figure 2 (right panel) the ECFV response to ACEi seemed to be stronger in SS subjects (-0.88 ±2.4 l in SS vs +0.25 ±1.9 l in SR), but this difference did not reach statistical significance (p=0.17).

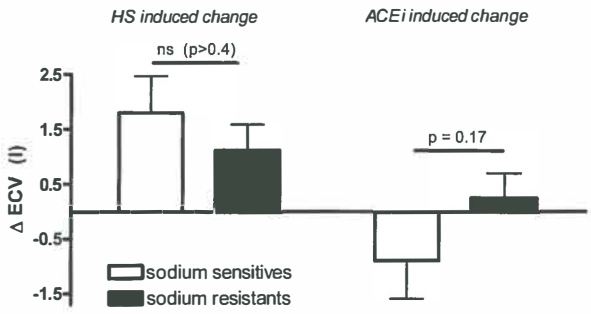

Figure 2 Changes in baseline extra cellular fluid volume ECFV between low sodium and high sodium diet period (left panel) and between high sodium and high-sodium combined with ACE-inhibitor period (right panel).

Renal angII response as determinant of sodium sensitivity of blood pressure

In Figure 3 the renal responses to 3 ng/kg/min AngII are shown for respectively LS, HS and HS-ACEi. In SS subjects the renal response to AngII was less pronounced than in SR during LS (-25 ± 6 vs -29 ± 6%; SS vs SR; p<0.05), as well during HS (-30 ± 5 vs -35 ± 6%; p<0.05), but not during HS-ACEi (-36 ± 6 vs -37 ± 6%). As a consequence the renal response to AngII was significantly enhanced by RAAS blockade (HS vs HS-ACEi) in SS but not in SR.

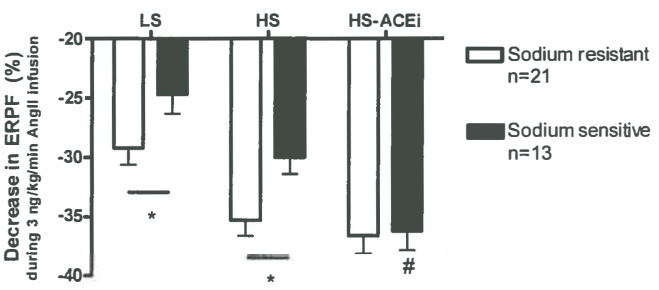

*Figure 3 Mean effective renal plasma flow (ERPF) responses to 3 ng·kg^{-1}·min^{-1} AngII while subjects were on a low sodium diet (LS), a high sodium diet (HS) or HS combined with ACE inhibition (HS-ACEi), shown for the subgroup of sodium sensitive subjects (n=13) and sodium resistant subjects (n=21). * p<0.05 between subgroups. # p<0.05 HS-ACEi vs HS.*

When analyzed as a continuous variable, SS negatively correlated with the renal responses to AngII during HS as shown in figure 4 (r=-0.400, p=0.019). This correlation was abolished by ACEi. During LS, there was a trend for association between sodium sensitivity of blood pressure and the response of ERPF to AngII (r=-0.298, p=0.087). No significant association between SS and the Ang II induced responses of urinary excretion of sodium, and circulating levels of PRA and Aldosterone was detected.

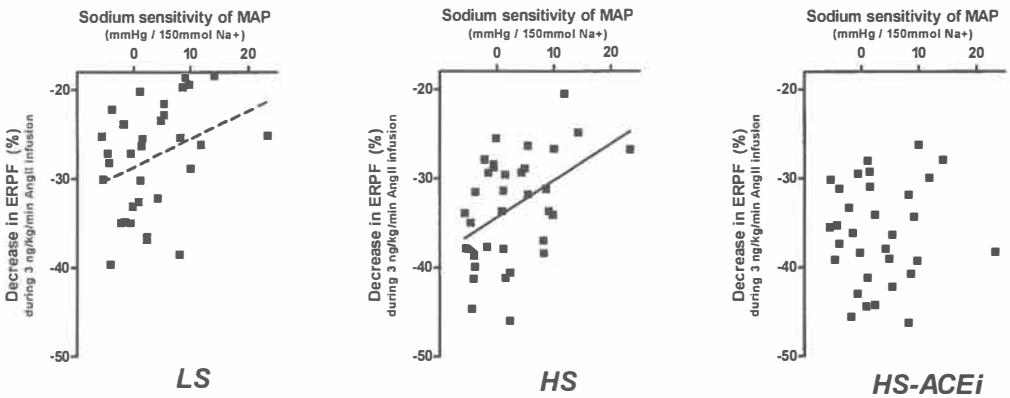

Figure 4 Scatterplot for sodium sensitivity of mean arterial pressure (MAP) and the renal response to 3 n·kg^{-1}·min^{-1} AngII (change in effective renal plasma flow (ERPF) (%), during respectively LS (r=-0.298, p=0.087), HS (r=-0.400, p=0.019) and HS-ACEi (r=0.09, p=0.6).

Discussion

This study shows that SS in healthy young men is associated with distinct haemodynamic characteristics. First, SS was generally associated with lower baseline ERPF, although during HS this association did not reach statistical significance. Second, in SS the renal vasoconstrictor response to exogenous AngII is blunted, which is ameliorated by ACEi, suggesting an inappropriately high intra-renal RAAS activity. Finally, our data suggest that SS is accompanied by relative ECFV expansion irrespective of sodium intake. As these observations were made in healthy normotensives, the abnormalities cannot be due to hypertensive organ damage, and might thus be primary factors in SS.

In our population ERPF was generally lower in SS subjects, which parallels findings in SS hypertensives[1,2,9], but to the best of our knowledge has not been shown in SS normotensives before. During HS the difference in ERPF between SS and SR subjects did not reach statistical significance, which may be due to a lack of power of our study. This less clear-cut difference under HS is at variance with SS hypertensives, in which a lower ERPF is especially observed during HS intake. Consequently, the response of ERPF to sodium loading was normal in our population, whereas this response is blunted in SS hypertensives[3,8,9]. As a poor renal vasodilator response to HS can be expected to hamper urinary sodium excretion by its effects on peritubular Starling forces, such an abnormal response has been postulated to play a pathogenic role in SS in hypertension[3]. Whereas this may be true, our data demonstrate that an abnormal vasodilator response to HS is not a prerequisite for SS as such.

Moreover, ACEi increased ERPF in SR as well as SS, but did not abolish the difference between the groups. This suggests that the lower ERPF in SS is not due

to a RAAS-mediated increase in renal vascular tone. However, whether ACEi results in full blockade of the intra-renal RAAS is questionable[20], and more extensive blockade of the RAAS, for instance by renin-inhibition, would be needed to conclusively exclude a role for the RAAS in the lower ERPF in SS normotensives. Other factors linked to SS[6,21] may give alternative explanations: lower nephron numbers, increased sympathetic tone or endothelial dysfunction could all lead to lower ERPF.

We used the renal vasoconstrictor response to AngII as an indirect estimate of intra-renal RAAS-activity. The mean renal vasoconstrictor response to AngII was increased by HS, which is well in line with earlier findings[9]. The increase in AngII response during HS is attributed to an upregulation of AngII type 1 receptors[22], reflecting suppression of intra-renal RAAS-activity and lower intra-renal AngII, i.e the appropriate response to increased sodium intake. In our population, as anticipated, ACEi further increased the mean vasoconstrictor response to AngII, thus reflecting pharmacological inhibition of the intra-renal RAAS-activity.

In SS subjects the renal response to AngII was blunted during HS, and this blunting was corrected by ACEi. Together these results suggest inappropriately high intra-renal RAAS-activity in SS subjects during HS. However, the results of the comparison between SS and SR can be strongly influenced by the cut-off used to define SS. Therefore we sought to confirm the results by analysing SS as a continuous variable as well, i.e. as the change in blood pressure elicited by HS. This analysis confirmed the results of the dichotomous analysis, showing a significant correlation between a higher SS and less pronounced renal AngII response during HS, which was no longer present during HS-ACEi, supporting the robustness of our finding. These data parallel findings in hypertensives, in whom a

blunted renal vasoconstrictor response to AngII during HS was present, that could be corrected by ACEi. Accordingly, it has been attributed to poor suppression of the intra-renal RAAS by HS[8,9].

To our knowledge, no data are available on the renal response to AngII during LS in relation to SS, neither in hypertensive nor in normotensive subjects. In the current study, the renal response to AngII was blunted in SS not only during HS, but also during LS. Thus, also during LS intra-renal RAAS activity may be higher in SS subjects. These SS subjects may therefore have a constitutively increased intra-renal RAAS activity as primary phenomenon, with genetic factors[23], lower nephron numbers[21] or both involved. However, when analysed as continuous variable, the association between SS and renal response to AngII was of borderline significance only. So the findings on LS are less robust than on HS, and need further confirmation.

A rise in ECFV is the basis of the adaptation to an increased sodium intake[6]; in our population this is shown by an average increase in ECFV of 1.4 l. Subgroup-analyses on ECFV in our population were unfortunately underpowered, but the results can serve to elicit the hypothesis that independent of sodium intake SS subjects are characterized by an increased ECFV, relative to SR subjects. We hypothesize that not adaptation to HS intake is impaired and causes fluid retention, but that volume homeostasis is set at a higher set-point in SS subjects. Interestingly, the difference in ECFV between SS and SR subjects was no longer apparent during ACEi, suggesting a role of increased activity of the RAAS in the higher set-point for volume regulation in SS subjects.

Our results were obtained in healthy young volunteers, without influence of factors previously linked to SS in hypertensives as race[24], obesity[25], hyperinsulinemia[26], hypercholesterolemia[27] and albuminuria[1]. Thus, these factors are apparently not a prerequisite, or a causal factor for sodium-sensitivity.

Our study has several limitations. First, our study population was relatively small, which relates to the demanding study protocol. Second, our population may have contained subjects that will develop hypertension at older age, but we have no means of identifying these. Third, our low sodium diet was not very strict, with a target intake of 50 mmol Na^+/day, whereas other studies on SS use 10-20 mmol Na^+/day, which hampers direct comparison with these studies. Fourth, the reproducibility of the renal AngII response has not been established. Furthermore, the duration of the diet periods was only one week. This is sufficient to re-establish sodium balance, but does not necessarily reflect the state of sodium balance on long term. Finally, we only studied males, as in women regulation of the RAAS and sodium responses are under influence of the menstrual cycle[28]. Since it has been proposed that woman are characterized by a different reactivity to sodium[29], translation of our results to women is not straight-forward.

Our study demonstrates that SS in healthy young men is associated with a lower ERPF during LS intake, with a normal renal response to high sodium intake, but with a blunted renal response to AngII, which is ameliorated by pharmacological ACE-inhibition. Our findings are compatible with the assumption of inappropriate activity of the intra-renal RAAS as a mechanism underlying sodium sensitivity of blood pressure. The data on ECFV suggest that not the adaptation of ECFV to a change in sodium intake, but rather the setpoint of ECFV may be altered in SS. As these observations were made in healthy young men, it is unlikely that these renal

characteristics reflect subclinical hypertensive renal damage, and they may therefore reflect a primary phenomenon. Whether these abnormalities could be involved in the increased risk for development of cardiovascular risk factors, and even more importantly, in the elevated mortality in sodium-sensitive subjects, should be the subject of further studies.

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CHAPTER 4

EFFECT OF REDUCTION OF RENAL MASS AND RENAL FUNCTION BY KIDNEY DONATION ON EXTRA CELLULAR FLUID VOLUME

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Abstract

In chronic kidney disease a decline in renal function is associated with fluid retention. Whether this relates to the renal function impairment as such, or to renal parenchymal abnormalities is unknown. To investigate the effect of reduction in renal mass per se, we measured extra cellular fluid volume (ECFV), as well as renal haemodynamics before and 2 months after donor nephrectomy.

194 consecutive living kidney donors (88 men; 106 women; 49 ± 11 yr) were included. No dietary restrictions were applied. Glomerular filtration rate (GFR) was measured as the clearance of ^{125}I-iothalamate (IOT) and ECFV as its distribution volume. GFR decreased by 37 ± 7%, effective renal plasma flow (ERPF) by 33 ± 7% (both $p<0.01$). Consequently, filtration fraction (FF) decreased from 27.3 ± 3.2 to 25.8 ± 2.9% respectively before and after donation ($p<0.01$). ECFV decreased from 19.7 ± 3.4 to 18.5 ± 3.0 l ($p<0.01$). Blood pressure, serum sodium levels and daily sodium excretion were similar before and after donation. In a subset of 37 subjects we assessed renal sodium handling. Filtered load of sodium per kidney increased by 33 ± 16% ($p<0.01$), with a corresponding rise in tubular sodium reabsorption of 32 ± 16%. Fractional excretion of sodium (FE_{na+}) increased by 18 [-18-83]% ($p=0.02$).

In conclusion, in healthy subjects ECFV decreases after unilateral nephrectomy despite reduction in renal mass and renal function. The lower FF after donation, with consequently altered peritubular starling forces, may reflect an adaptive response to facilitate renal sodium excretion with a lower nephron mass.

Introduction

Living kidney donation programs have become increasingly important due to shortage of kidneys from deceased donors. Donor safety is an important consideration to justify living kidney donation programs. Fortunately, so far long term donor and kidney survival after kidney donation is excellent[1,2], probably due to careful screening and selection of kidney donors. Caution is warranted however, as the current donor selection criteria tend to become more liberal as regards older donor age, and presence of hypertension, and long term follow up of the current donor population will have to substantiate the safety of current donation practice.

Donor nephrectomy leads to a substantial reduction in renal mass and renal function. In renal disease, as well as animal models of renal disease, reduction of renal mass and renal function are often associated with volume expansion and systemic and glomerular hypertension[3-7]. These are assumed to reflect adaptive responses to nephron loss, aimed at preservation of glomerular filtration rate (GFR), at the expense however of long term systemic and glomerular hypertensive damage[8]. However, whether this is related to the loss of renal mass as such, or to presence of parenchymal abnormalities has not been well-established. After donor nephrectomy clear-cut adaptive responses occurs in the remaining kidney, leading to substantial increases in single kidney GFR and effective renal plasma flow (ERPF), respectively[9,10]. Whether this adaptation is associated with expansion of the extra cellular fluid volume (ECFV) is unknown. In the current study, therefore, we measured ECFV and renal haemodynamics in a cohort of 194 living kidney donors, both before and 2 months after kidney donation.

Methods

Data from 194 consecutive living kidney donors that participated in the donor screening protocol with subsequent donation between 2000 and 2007 were included in the present analyses. None had a history of kidney disease, diabetes, and cardiovascular events. 13 subjects used anti-hypertensive drugs in a stable dose during the observation period. Physical examination did not reveal abnormal findings. Assessments were made 138 ± 170 days before and at 59 ± 13 days after the kidney donation procedure. No dietary restrictions were applied at any time during screening or follow-up.

Assessment of renal haemodynamics and extra cellular fluid volume

Renal haemodynamics, i.e. GFR, ERPF and filtration fraction (FF) and ECFV were measured as part of the screening and follow-up protocol during the constantly infusion protocol of ^{125}I-iothalamate (IOT) and ^{131}I-Hippuran, as described in detail previously[11,12]. Briefly, GFR was measured as the urinary clearance (U·V/P) of IOT. Simultaneous infusion of ^{131}I-Hippuran was used to correct the GFR values for inaccurate urine collection as described previously. The coefficient of variation (COV) for this GFR measurement is 2.2%. ERPF was measured as the plasma clearance (I·V/P) of ^{131}I-Hippuran. The COV for this ERPF measurement is 5%. FF was calculated as GFR/ERPF * 100%. ECFV was calculated as the distribution volume (Vd) of IOT. Previously, we found a COV for this ECFV assessment of 8.6% and good agreement between this method and the gold standard in ECFV assessment: bromide Vd[13].

The procedure was as follows. The donors were in a quiet room, in semi-supine position. After drawing a blank blood sample, a priming solution containing 0.04 ml·kg^{-1} body weight (BW) of the infusion fluid (4 MBq of ^{131}I-Hippuran and 3 MBq

of IOT per 100 ml saline) plus a bolus of 0.6 MBq of IOT was administered at 08.00 hours, after which the radio-isotopes were infused at a constant rate of 12 $ml \cdot h^{-1}$. After an equilibration period of 1.5 hour to allow for stable plasma concentrations, two clearance periods of 2 hours were conducted and the mean value of these two periods was used for analysis. Urine was collected by spontaneous voiding. Blood samples were drawn at start, middle and end of each clearance period.

Blood pressure

Blood pressure was measured with a semi-automated device (Dinamap® 1846, Critikon, Tampa, FL, USA). Mean arterial pressure (MAP) was calculated as the sum of systolic blood pressure and 2 times diastolic blood pressure divided by 3.

Renal Sodium Handling

Renal sodium handling was assessed in a subset of 37 subjects (18 men; 19 women, age 49 ± 10) from 24h urine sodium excretion and from a 2-hour urine collection obtained simultaneously with the assessment of GFR. Calculations were as follows:

- Filtered load of sodium (FL_{Na+}) = GFR · $[Na^+]$
- Fractional excretion of sodium (FE_{Na+}) = $(U \cdot V)_{Na+}$/ $[Na^+]$ divided by $(U \cdot V)_{creat}$ / [creat], in where V drops out of the equation, leaving:
  $FE_{Na+} = (U_{Na+} / [Na^+]) / U_{creat} / [creat]$
- Tubular reabsorption of sodium (TR_{Na+}) = $FL_{Na+} \cdot (1\text{-}FE_{Na+})$

Statistical analysis

Analyses were performed using SPSS software version 14.0 (SPSS Inc., Chicago, IL, USA). Data are given as mean ± standard deviation or median [25^{th}-75^{th} percentile]

when not normally distributed. Pearson's correlation coefficients were calculated to account for univariate correlations. Student's paired T-test was used to compare values pre- and post donation when data was normally divided, otherwise Wilcoxon non-parametric test was used. When post-donation are expressed as % of pre-donation values, these values are tested with an one-sample T-test against the value of 100%.

Exclusion in the analysis of the 13 subjects using anti-hypertensive treatment did not influence the results presented in the results section. Therefore, these are not presented separately.

Results

88 men (age 49 ± 12) and 106 women (age 49 ± 9) were included in this study. In table 1, characteristics before and after nephrectomy are given. MAP was equal before and after kidney donation. BW was 0.5 ± 2.6 kg (p=0.02) lower after nephrectomy. BMI before unilateral nephrectomy was 26.0 ± 4.1 $kg \cdot m^{-2}$ without differences between men and women.

ECFV decreased with -1.2 ± 2.2 l (p<0.01) after kidney donation. ECFV as a % of BSA decreased as well, by -1.1 ± 1.9 $l \cdot 1.73 \cdot m^{-2}$ (p<0.01). The decrease in ECFV was not related to gender, age, BMI, MAP or the use of anti-hypertensives. After the kidney donation GFR decreased to 63 ± 7% and ERPF to 67 ± 7% (both p<0.01) of their pre-donation values. Consequently, single-kidney GFR increased with 26 ± 13% and single kidney ERPF with 33 ± 13% (both p<0.01), leading to a slight but significant decrease in FF to 95 ± 9% (p<0.01) of its pre-donation value. Urinary albumin excretion was low and well within the normal range both before and after donation.

	Before donation	After donation	p-value
MAP (mmHg)	93 ± 11	93 ± 10	ns
Body Weight (kg)	79.0 ± 14.0	78.5 ± 13.8	<0.02
ECFV (l)	19.7 ± 3.4	18.5 ± 3.0	<0.01
GFR ($ml \cdot min \cdot 1.73m^{-2}$)	105 ± 15	66 ± 10	<0.01
ERPF ($ml \cdot min \cdot 1.73m^{-2}$)	390 ± 69	259 ± 44	<0.01
FF (%)	27.3 ± 3.2	25.8 ± 2.9	<0.01
Serum creatinine ($umol \cdot l^{-1}$)	85 ± 13	115 ± 22	<0.01
Urinary albumin ($mg \cdot l^{-1}$)	2.3 [1.0-3.8]	2.3 [1.1-3.8]	ns

Table 1 Donor characteristics before and after living kidney donation. Mean arterial pressure (MAP); extra cellular fluid volume (ECFV); glomerular filtration rate (GFR); effective renal plasma flow (ERPF; filtration fraction (FF.)

Renal sodium handling

Table 2 gives data on renal sodium handling as assessed in a subset of 37 subjects simultaneously with GFR. Serum sodium levels were not significantly different before and after kidney donation. In this subset the decrease in ECFV was similar to that in the total population, namely 1.2 ± 1.8 l ($p<0.01$). 24 hour urinary sodium excretion was equal before and after donation as shown in table 2. Also in the 2-hour portion sodium excretion was not significantly different before and after donation (0.23 ± 0.1 mmol·min^{-1} and 0.21 ± 0.01 mmol·min^{-1}, respectively). The 'total body' FL_{Na+} and TR_{Na+} both decreased after donation. However, the single kidney values increased by approximately one-third. FE_{Na+}, i.e. the % of the filtered load that is actually excreted, increased significantly by an absolute value of 0.23 [-0.28-0.62] % (p=0.02).

	Before donation	**After donation**	**p**	
[Na^+] (mmol·l^{-1})	142 ± 2	141 ± 2	ns	
ECFV (l)	20.0 ± 3.7	18.8 ± 3.1	<0.001	
$(U\cdot V)_{Na+}$(mmol·24h^{-1})	178 ± 70	171 ± 69	ns	**Δ per kidney (%)**
FL_{Na+} (mmol·min^{-1})	17.18 ± 3.3	11.28 ± 2.1	<0.001	→ +33 ± 16*
TR_{Na+}(mmol·min^{-1})	17.11 ± 3.2	11.15 ± 2.0	<0.001	→ +32 ± 16*
FE_{Na+} (%)	1.12 [0.8-1.54]	1.39 [1.06-1.66]	0.02	→ +18 [-18-83]*

***Table 2** Sodium handling in a subset of n=37. Extra cellular fluid volume (ECFV); sodium excretion ($(U\cdot V)_{Na+}$); filtered load of sodium (FL_{Na+}); tubular reabsorption of sodium (TR_{Na+}); fractional excretion of sodium (FE_{Na+}). The latter three are also given as donation induced relative change (Δ) per kidney (right column). *p<0.05: change is different from 0.*

Discussion

Donor nephrectomy was associated with a significant decrease in ECFV two months after donation, despite the substantial reduction in renal mass and in renal function. This decrease is remarkable, since in renal disease and in animal models of renal damage, reduction of renal mass has been reported to be associated with an increased ECFV[14]. The adaptive response of renal haemodynamics, with a lower FF after donation, may have accounted for facilitated excretion of sodium, due to altered peritubular Starling forces.

The average decrease in ECFV was some 5%, without a detectable effect on blood pressure. Other reports on kidney donors are not uniform as regards blood pressure, and some have reported an increased prevalence of hypertension[15-17]. Our data suggest that expansion of ECFV, at least on the relative short term studied here, is not a likely causal factor for the small increases in blood pressure reported in some studies. The other way around, in the current study the decrease in ECFV did not result in a lower blood pressure, so apparently blood pressure was not volume dependent in our population. Generally homeostatic mechanisms such as activation and suppression of the renin angiotensin aldosterone system (RAAS) ensure stable blood pressure during changes in volume status[14]. However, we have no data on parameters of the RAAS to substantiate this in the current population.

Dietary sodium intake is a main determinant of ECFV[13]. Dietary intake was not standardized in our population, so a decrease in dietary sodium intake could theoretically be involved in the decrease in ECFV after donation. However, in a subset of the population we documented 24h sodium excretion. In this subset, that was not different from the total population in any of the tested characteristics; 24h urinary sodium excretion after donation was similar before and after donation.

From these data it is unlikely that a reduction in habitual sodium intake accounted for the post-donation decrease in ECFV.

In our study, ECFV and renal haemodynamics were measured approximately 2 months after donor nephrectomy. This period of time is considered long enough for achieving stable renal haemodynamics, and sodium and water balance[9,18]. As sodium intake was unaltered, apparently a lower set-point for ECFV must be present. What could be its underlying mechanism? Uninephrectomy is associated with a substantial adaptation of glomerulo-tubular balance[9,10,18-20]. In the single kidney, filtered load of sodium is substantially increased, due to the adaptive rise in single-kidney GFR. Single kidney tubular sodium reabsorption increases to a smaller extent with, consequently a rise in fractional excretion of sodium. Several mechanisms could be involved in the resetting that can lead to the negative sodium balance.

First, the time course of glomerular and tubular adaptation is probably dissimilar, with a fast rise in single kidney GFR, and a slower rise in tubular reabsorption, as shown in rat studies where this was associated with net natriuresis early after uninephrectomy[18,19]. However, this mechanism cannot explain why a lower ECFV is maintained after the initial phase. Second, the observed reduction of the filtration fraction could be involved[21]. A lower FF leads to altered peritubular Starling forces, with a lower hydrostatic and a higher oncotic pressure, together facilitating sodium excretion[22]. Finally, after uninpehrectomy the single-kidney load for nutritional waste products is increasing, which could promote natriuresis by modulation of glomerulo-tubular balance, as suggested by experimental studies[23]. We have no data however, to substantiate this assumption for the current study.

What could be the implications of our findings? Expansion of ECFV is assumed to be a pathogenetic factor in cardiovascular and renal damage[24]. Thus, the decrease in ECFV observed here could be a favourable prognostic sign. Yet, a lower ECFV could also have adverse consequences, for instance when dehydration from other causes is superimposed. However, from our data it cannot be derived whether the reduction in ECFV is maintained during long term follow-up, and what its effects on long term cardiorenal risk profile might be. These issues should be subject of further research.

Our study has several limitations. First, we have only data at one time-point after kidney donation. Second, sodium intake was not standardized. Furthermore, data on renal sodium handling, especially tubular sodium handling were assessed indirectly, and experiments which could discriminate proximal and distal tubular functions would be of additive value.

In conclusion, ECFV decreases after donor nephrectomy in healthy living kidney donors. An altered setpoint of glomerulo-tubular balance might be involved in this decrease in ECFV, related to the lower filtration fraction observed after donation. A lower ECFV may contribute to long-term cardiovascular and renal health in kidney donors, but long term studies are warranted to confirm this hypothesis.

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CHAPTER 5A

ANAEMIA IN CHRONIC HEART FAILURE IS NOT ONLY RELATED TO RENAL DYSFUNCTION AND BLUNTED ERYTHROPOIETIN PRODUCTION, BUT TO FLUID RETENTION AS WELL

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Abstract

Anaemia is prevalent in the chronic heart failure (CHF) population, but its cause is often unknown. The present study aimed to investigate the relation between anaemia, renal dysfunction, erythropoietin (EPO) production and fluid retention in CHF patients.

We studied 97 patients with CHF, of which 15 had anaemia (Hb <13.0 g/dl in men) and Hb <12.0 g/dl in women), without haematinic deficiencies. Glomerular Filtration Rate (GFR) and extra cellular fluid volume (ECFV) were measured as the clearance and the distribution volume of constantly infused ^{125}I-iothalamate, respectively. Effective renal plasma flow (ERPF) was determined as the clearance of ^{131}I-Hippuran. Anaemic CHF patients displayed significantly reduced GFR (p=0.002), ERPF (p=0.005) and EPO production (p=0.001), and an elevated ECFV (p=0.015). Multivariate analysis demonstrated that lower GFR (p=0.003), lower ERPF (p=0.004), lower EPO production (p=0.006), and a higher ECFV (p=0.001), were significant independent predictors of lower haemoglobin levels.

Anaemia in CHF is not only independently associated with impaired renal function and blunted EPO production, but to fluid retention as well.

Introduction

Anaemia is present in a substantial part of the chronic heart failure (CHF) population, ranging from 15-55%, depending on the definition of anaemia and severity of disease[1]. Anaemia is independently associated with increased morbidity and impaired prognosis, although the cause of anaemia is often unknown[1-5]. CHF is associated with elevated levels of erythropoietin (EPO), suggesting impaired erythropoietic activity in the bone marrow[1,6,7]. Recently we demonstrated that anaemia in CHF could partly be explained by increased serum levels of AcSDKP, a negative regulator of haematopoietic stem cell proliferation[8]. In addition, we hypothesize that CHF will compromise renal perfusion resulting in impaired EPO production, thereby causing anaemia. Finally, it has been suggested that anaemia in CHF may be partly explained by fluid retention and consequent haemodilution[9]. However, the relative contribution of renal perfusion, EPO production, and fluid retention to the presence of anaemia in CHF has so far not been well described. We therefore evaluated the relation between anaemia, effective renal plasma flow (ERPF), EPO production and extra cellular fluid volume (ECFV) in CHF patients.

Methods

Patient population

Clinically stable CHF patients on outpatient follow-up at our department were asked to participate, as described in detail previously[10]. Approximately 121 patients were asked to participate. 110 patients were included into the original analysis and finished the study. Owing to missing haemoglobin levels, 13 patients were excluded from analysis, leaving 97 subjects for analysis. Briefly, inclusion criteria were age >18 years and left ventricular ejection fraction (LVEF) <45%. All patients used renin angiotensin aldosterone system (RAAS) blockers, and

medication had remained stable for at least one month. Exclusion criteria included stroke, myocardial infarction or cardiac revascularization procedures within the last 3 months or scheduled for these procedures, unstable angina, primary renal disease, prior organ transplant, or chronic use of renal function compromising medication.

Cardio-renal haemodynamic parameters

LVEF was determined by nuclear ventriculography or echocardiography using Simpsons rule. Mean arterial pressure (MAP) was calculated from systolic and diastolic blood pressure measurements obtained immediately before ^{125}I-iotholamate (IOT) and ^{131}I-Hippuran clearance measurements from 10 consecutive measurements in supine position using an automated system. N-terminal proBNP (NT-proBNP) was determined by electrochemiluminescence immunoassay on the Roche Elecsys (Roche diagnostics, The Netherlands). Glomerular filtration rate (GFR) and ERPF were measured by constant infusion of radiolabelled tracers IOT and ^{131}I-Hippuran[11]. Briefly, after drawing a blank blood sample, a priming solution containing 0.4 ml·kg^{-1} body weight (BW) of the infusion solution (0.04 MBq of IOT and 0.03 MBq of ^{131}I-Hippuran) plus an extra amount of 0.6 MBq of IOT was given at 08.00 hours, followed by infusion at 12 ml·h^{-1}, adapted to 9 ml·h^{-1} in subjects with renal function impairment as estimated from previously obtained serum creatinine values. This ensures steady-state plasma levels of ^{131}I-Hippuran and IOT after a run-in period of 2 hours, as verified by hourly blood samples. Subsequently, clearances of IOT and ^{131}I-Hippuran and the distribution volume (Vd) of IOT were measured during steady state. The GFR and ERPF were calculated as $(U \cdot V)/P_{(iothalamate)}$ and $(I \cdot V)/P_{(hippuran)}$, respectively, and $(U \cdot V)/P_{(iothalamate)}$ was corrected for voiding errors by the ratio of the urinary to plasma clearance of ^{131}I-Hippuran. U·V represents the urinary excretion of the tracer, I·V represents the

infusion rate of the tracer; P represents the values in plasma calculated from the samples bracketing each clearance period. The body surface area (BSA) was calculated as $0.007184 \cdot weight^{0.425} \cdot length^{0.725}$, and GFR and ERPF were expressed per $1.73m^2$ of BSA. Renal blood flow (RBF) was calculated as ERPF/1-haematocrit. The filtration fraction (FF) was calculated as the ratio of GFR and ERPF and expressed as percentage. ECFV was estimated from the Vd of IOT[12,13] and calculated as $[(I \cdot V + B \cdot V) - U \cdot V] / P_{(iothalamate)}$ during steady state. B·V represents the bolus infusion of the tracer. ECFV was expressed as l/kg BW.

Haemoglobin levels, haematinic parameters and EPO levels

Haemoglobin, iron, ferritin, transferin, vitamin B11 and B12 levels were determined at the local laboratory facilities. EPO levels were determined by IMMULITE EPO assay (DPC, Los Angeles, California, USA). To define the relation between EPO levels and a given Hb we included 15 reference subjects referred to our department with complaints of chest pain or palpitations. The reference subjects had a mean age of 50 ± 4.5 and had normal LVEF (LVEF >60%), normal renal function, no signs of inflammation, or symptoms of CHF[14]. An exponential regression equation of serum EPO vs. Hb ($mmol \cdot l^{-1}$) was calculated, resulting in the following equation: log EPO = 3.015 - (0.130·Hb). Predicted log EPO and observed/ predicted (O/P) log EPO ratio (log serum EPO/predicted log EPO) were calculated with this equation. Mean O/P ratio in reference subjects was 0.90 ± 0.029 (95% CI 0.64 - 1.12). Total iron binding capacity (TIBC) was calculated by multiplying serum transferrin with 20. Transferin saturation (FeSat) was calculated as serum (serum iron/TIBC)·100%. Iron deficiency was defined as ferritin levels <30 $\mu g \cdot l^{-1}$ or FeSat <15%. According to local laboratory reference ranges, deficiency in vitamin B11 and B12 was defined as levels below 142 $pmol \cdot l^{-1}$, and 50 $pmol \cdot l^{-1}$ respectively.

High sensitive C-reactive protein (CRP) was determined by nepholometry. The threshold for detection was 0.156 $mg \cdot l^{-1}$; When CRP levels were below the detection limit, they were assigned the value 0.156 $mg \cdot l^{-1}$ for statistical purposes.

Statistics

Data are given as mean ± standard deviation (SD) when normally distributed, as median and interquartile range when skewed distributed and as frequencies and percentages for categorical variables. Differences between groups were compared with Student's T-test, Mann Whitney-U test or Fisher's exact test when appropriate. A p-value <0.05 was considered statistically significant, and all reported probability values are two-sided. Correlation between Hb, EPO or O/P ratio and various other variables was performed using Pearson's correlation coefficients. Non-normally distributed continuous variables were log-transformed. The variables age, sex, pharmacological treatments, New York Heart Association (NYHA) functional class, LVEF, ERPF, GFR, FF, ECFV, NT-proBNP, CRP, and MAP were assessed for univariate linear association with Hb or log EPO. Variables that showed a significant ($p<0.15$) univariate association were included stepwise in a multivariable linear regression model on the basis of on the strength of the univariate associations. All the variables described earlier were added to the final model simultaneously to assure that addition of these variables did not significantly increase the predictive accuracy of the model. The final model was assessed for first line interaction.

Results

Patient characteristics

76% of subjects were male and age ranged from 27 to 81 years. NYHA functional classes I, II, III and IV comprised 14%, 44%, 31%, and 10% of patients, respectively.

Differences in characteristics between anaemic and non-anaemic CHF patients

In the total population, 19 patients (20%) were anaemic according to the WHO criteria (Hb <13.0 $g \cdot dl^{-1}$ in men and Hb <12.0 $g \cdot dl^{-1}$ in women). Iron deficiency was present in 4 out of 19 anaemic (21%) and 3 out of 78 non-anaemic (4%) CHF patients. Other haematinic deficiencies were not observed. The iron-deficient patients were excluded from further analysis, leaving 75 non-anaemic subjects and 15 subjects with unexplained anaemia. Differences in characteristics between anaemic and non-anaemic subjects are summarized in table 1.

Anaemic subjects were significantly older and in a higher NYHA class. Although LVEF was comparable, anaemic patients showed more severe haemodynamic impairment, reflected by reduced MAP (86.8 ± 13 vs. 76.8 ± 14 mmHg; p=0.007) and ERPF (286 ± 83 vs. 219 ± 74 $ml \cdot min^{-1} \cdot 1.73m^{-2}$; p=0.005) and RBF (502 ± 150 vs. 348 ± 121 $ml \cdot min^{-1} \cdot 1.73m^{-2}$; p<0.001) and elevated NT-proBNP levels (10 (260-1355) vs. 1004 (720-1904) $pg \cdot ml^{-1}$; p=0.029). Anaemic CHF patients more often used diuretics (65 vs. 87%; p=0.045), and despite this displayed a significantly elevated ECFV (0.25 ± 0.5 vs. 0.29 ± 0.4 $l \cdot /kg^{-1}$; p=0.015), implicating fluid overload.

The fluid retention was subclinical, as anaemic patients did not display oedema, nocturia, or dyspnoea more frequently (data not shown). Plasma sodium levels and fractional sodium excretion were similar, implicating that the elevated ECFV was not caused by excess sodium intake.

	Non-anaemic (n= 75)	Anaemic (n=15)	p-value
Age (years)	56.3 ± 12	65.6 ± 9	0.004*
Sex (n % male)	58 (85)	10 (71)	0.510
NYHA class	2.3 ± 0.8	2.8 ± 0.8	0.036*
BMI ($kg \cdot m^{-2}$)	27.8 ± 3.9	26.1 ± 3.0	0.140
Ischemic etiology (n %)	38 (50)	7 (47)	1
Cardiorenal haemodynamic parameters			
Heart rate	65 ± 2	64 ± 3	0.834
MAP (mmHg)	86.8 ± 1.5	76.8 ± 3.6	0.007*
LVEF (%)	28 ± 10	26 ± 6	0.525
NT-proBNP ($pg \cdot ml^{-1}$)	510 (260-1355)	1004 (720-1904)	0.029*
Creatinine ($mg \cdot dl^{-1}$)	1.2 (1-2)	1.2 (1.1-1.7)	0.150
Urea ($mg \cdot dl^{-1}$)	19 (16-22)	35 (23-40)	<0.001*
GFR ($ml \cdot min^{-1} \cdot 1.73m^{-2}$)	79 ± 25	56 ± 28	0.002*
ERPF($ml \cdot min^{-1} \cdot 1.73m^{-2}$)	286 ± 83	219 ± 74	0.005*
RBF ($ml \cdot min^{-1} \cdot 1.73m^{-2}$)	502 ± 150	348 ± 121	<0.001*
FF (%)	28 (26-30)	26 (20-29)	0.080
FE_{Na+} (%)	0.88 ± 0.33	0.97 ± 0.52	0.370
ECFV/BW ($l \cdot kg^{-1}$)	0.25 ± 0.1	0.29 ± 0.1	0.015*
Mild RF (GFR <60)	16 (21%)	9 (60%)	0.004*
Severe RF (GFR <30)	4 (5%)	3 (20%)	0.088
Erythropoietic and inflammatory parameters			
Hb ($mg \cdot dl^{-1}$)	15 ± 0.7	12.7 ± 0.4	<0.001*
Serum EPO ($U \cdot l^{-1}$)	15.7 (11-21)	18.5 (12-31)	0.357
O/P ratio	1.15 ± 0.20	0.93 ± 0.18	0.001*
CRP ($mg \cdot l^{-1}$)	2.15 (0.95-4.06)	2.38 (0.77-5.66)	0.733
Medication			
ACE inhibitors, n (%)	66 (88)	12 (80)	1
ARB, n (%)	9 (12)	3 (20)	0.414
Beta blockers, n (%)	62 (83)	13 (87)	1
Diuretic, n (%)	49 (65)	13 (87)	0.045*
Aldosteron ant., n (%)	51 (88)	7 (47)	0.144

Table 1 Characteristics of anaemic and non-anaemic CHF patients.
All continuous variables are presented as mean ± SD if normally distributed and as median value with 25^{th}-75^{th} percentile when skewed distributed. New York Heart Association (NYHA); body mass index (BMI); mean arterial pressure (MAP); left ventricular ejection fraction (LVEF); N-terminal proBNP (NT-proBNP); glomerular filtration rate (GFR); effective renal plasma flow (ERPF); renal blood flow (RBF); filtration fraction (FF); fractional excretion of sodium (FE_{Na+}); extra cellular fluid volume (ECFV); body weight (BW); renal failure (RF); erythropoietin (EPO); observed/predicted ratio (O/P ratio); C-reactive protein (CRP); angiotensin receptor blocker (ARB); aldosteron antagonists (aldosteron ant.).
**p<0.05*

Although creatinine levels were comparable between groups, urea levels were elevated (19 (16-22) vs. 35 (23-40) $mg \cdot dl^{-1}$; p=0.007) and GFR (79 ± 25 vs. 56 ± 28 $ml \cdot min^{-1} \cdot 1.73m^{-2}$; p=0.002) was significantly reduced in the anaemic patients. Moreover, anaemic patients had a higher incidence of moderate renal failure (RF) (GFR <60 $ml \cdot min^{-1} \cdot 1.73m^{-2}$) and a trend towards more severe RF (GFR <30 $ml \cdot min^{-1} \cdot 1.73m^{-2}$) (21 vs. 60%, p<0.005 and 5 vs. 20%, p=0.088), respectively.

By definition, Hb level was significantly lower in anaemic subjects. However, EPO levels were comparable between anaemic and non-anaemic CHF patients. Additionally O/P ratio was significantly reduced in anaemic subjects (1.15 ± 0.2 vs. 0.93 ± 0.2, p=0.001), indicating blunted EPO production. O/P ratio was significantly higher in non-anaemic CHF patients compared to reference subjects (p=0.026), whereas O/P ratio in anaemic CHF patients and controls were comparable. Thus, EPO production is elevated both in anaemic and non-anaemic CHF patients, but based on their Hb, it should have been higher in anaemic CHF patients. It therefore seems that the compensatory rise in response to anaemia is impaired.

Hb levels and EPO production in the CHF population

As previously described, CHF patients displayed a relatively moderate negative correlation between EPO and Hb levels (r=-0.281, p=0.007). A moderate significant correlation was also observed between EPO levels and both CRP (r=0.281, p=0.007) and NYHA class (r=0.210, p=0.05), and a trend with NT-proBNP (r=0.193, p=0.07). No significant correlation was observed between EPO levels and other markers for cardiorenal haemodynamic status, or renal function parameters. O/P ratio correlated with Hb (r=0.397, p=0.001), GFR (r=0.237, p=0.024) and FF (r=0.285, p=0.007).

Predictors of HB and serum EPO

Univariate and multivariate linear associations between Hb and EPO levels are displayed in tables 2 and 3, respectively.

Haemoglobin								
	Univariate			Multivariate				
	B	SE	p-value	B	SE	β	Part.cor	p-value
Sex	-1.41	0.30	<0.001	-1.189	0.258	-0.394	-0.449	<0.001
Age	-0.022	0.012	0.077					
GFR	0.02	0.005	<0.001	0.037	0.009	0.744	0.414	<0.001
ERPF	-0.004	0.002	0.013	-0.008	0.003	-0.534	-0.310	0.004
NYHA	-0.04	0.17	0.013					
EPO	-1.55	0.57	0.007	-1.422	0.430	-0.266	-0.399	0.001
ECFV	-6.36	2.95	0.034	-7.120	2.215	-0.259	-0.330	0.004
MAP	-0.03	0.01	0.002					
BNP	-0.399	0.144	0.007					

Table 2 Univariate and multivariate predictors of Hb levels.
Glomerular filtration rate (GFR); effective renal plasma flow (ERPF); New York Heart Association (NYHA); erythropoietin (EPO); extra cellular fluid volume (ECFV); mean arterial pressure (MAP). Adjusted R^2 = 0.436. β, standardized beta; part.cor., partial correlation

EPO							
	Univariate			Multivariate			
	B	SE	p-value	B	SE	β	p-value
Sex	0.03	0.233	0.6				
Age	0.027	0.6	0.233				
Hb	-0.052	0.019	0.007	-0.044	0.019	-0.233	0.025
NYHA	-0.06	0.031	0.061				
CRP	0.345	0.126	0.006	0.125	0.054	0.234	0.024

Table 3 Univariate and multivariate predictors of serum erythropoietin (EPO) levels. New York Heart Association (NYHA); C-reactive protein (CRP).
Adjusted R^2 = 0.111; standard error (SE); standardized beta (β;).

Sex, lower EPO (figure 1a), lower GFR (figure 1b), lower ERPF (figure 1c), and higher ECFV (figure 1d) were independently associated with lower Hb levels, accounting for 31-44% of the variance in Hb levels. The variables CRP, NYHA class, NT-proBNP, and Hb showed significant univariate association with plasma EPO. However, higher CRP and lower Hb levels were the only independent predictors of higher serum EPO levels. Inclusion of the full list of possible predictive variables did not result in a significant increase in the adjusted r^2, slope, or partial correlation coefficient of the variables in our model.

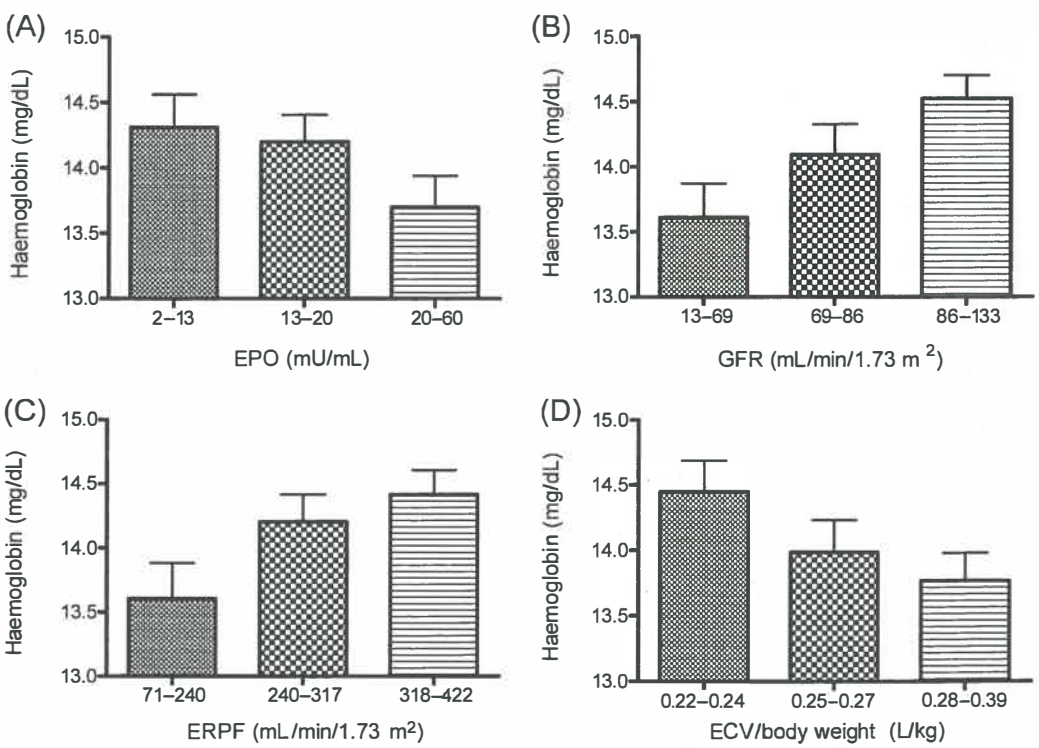

Figure 1 *Relation between haemoglobin levels and erythropoietin (EPO), glomerular filtration rate (GFR), effective renal plasma flow (ERPF) and extra cellular fluid volume (ECFV).*

Discussion

The present study demonstrated that anaemia in CHF patients was not only independently related to impaired renal haemodynamics and blunted EPO production, but to an increased ECFV as well. However, in contrast to our expectations, serum EPO levels were not directly related to renal perfusion.

The association between anaemia in CHF and impaired EPO production has been suggested previously[3,7]. Nevertheless, the presence of defective endogenous EPO production was not formally evaluated until recently. In a comprehensive retrospective analysis on the cause of anaemia in CHF patients, Opasich et al.[15] found that 50% of anaemic CHF patients showed evidence of impaired EPO production. Our data further substantiate these findings.

The relation between renal perfusion and EPO levels has been evaluated preciously in two populations comprising 13 and 14 CHF patients[16,17]. In these studies, EPO production inversely correlated with RBF, ERPF, and renal oxygen delivery, suggesting that impaired renal oxygenation caused the elevated EPO levels. However, in our far larger cohort, these findings could not be reproduced. Although there was no relation between ERPF and EPO, an univariate moderate correlation between EPO production and GFR was observed, which might implicate that blunted EPO production results from impaired renal function and structural renal damage. Furthermore, circulating inflammatory cytokines and ACE-inhibitors can directly inhibit EPO production in the kidney and might contribute to the blunted EPO production[18,19]. Additionally, impaired GFR could attenuate the excretion of circulating erythropoiesis-inhibiting factors (e.g. AcSDKP), leading to enhanced plasma levels, as has been demonstrated in a haemodialysis population[20].

The non-anaemic CHF patients displayed higher EPO levels and O/P ratios than reference subjects, as has been described previously. Elevated EPO levels were independently related to higher CRP levels, suggesting that elevated EPO production is directly related to an enhanced inflammatory state. Several pro-inflammatory cytokines have inhibitory effects on erythropoiesis, and are established as the cause of anaemia associated with chronic inflammatory disease[21]. CHF is associated with enhanced expression of a variety of pro-inflammatory cytokines, possibly contributing to the development of anaemia[6]. In addition, we recently demonstrated that anaemia in CHF could be partially explained by elevated levels of AcSDKP, a negative regulator of haematopoietic stem cells[8]. These circulating factors inhibit erythropoiesis and can eventually result in elevated EPO requirements. Indeed although EPO production was blunted, the circulating EPO levels in anaemic CHF patients were not reduced but slightly elevated compared to non-anaemic patients. The slightly elevated EPO levels were however insufficient for the prevailing Hb, reflected by significantly impaired O/P ratio. Hence, anaemia in CHF does not result from the inability to produce EPO, but an inability to further increase baseline EPO production.

As expected, anaemic patients displayed elevated ECFV, which was independently related to lower Hb levels. Impaired renal haemodynamics in CHF causes activation of RAAS and vasopressin systems, resulting in salt and fluid retention and consequently increased ECFV. Fluid retention in CHF can cause haemodilution, resulting in pseudo-anaemia, which carries even a worse prognosis than true anaemia[9]. In the present study, anaemic subjects more frequently received diuretics but nonetheless displayed elevated ECFV. Importantly, although fluid retention was related to anaemia, signs and symptoms of fluid retention were absent. Thus, haemodilution seems to precede the clinical presentation of

fluid retention. Therefore, starting or increasing the dose of diuretics should be considered before starting with EPO treatment. Since the aetiology of anaemia in CHF seems multifactorial, the preponderant cause should be identified on an individual basis, for instance, by determining the O/P ratio or ECFV in addition to regular diagnostic procedures.

Although the reduced renal function, the blunted EPO production and higher ECFV could be the cause of anaemia in CHF, they could also be a consequence. Lower Hb levels can result in peripheral tissue hypoxia, causing vasodilatation and consequently reducing blood pressure[22]. This will result in activation of the RAAS and further compromise of RBF by renal vasoconstriction and fluid retention. The compromised kidney seems unable to meet the increased demand, and anaemia ensues. The vicious cycle of CHF causing anaemia, and anaemia causing further deterioration of CHF has been described as the cardiorenal anaemia syndrome[23].
Thus, anaemia in CHF is directly related to an impaired haemodynamic state, compromising renal perfusion, attenuating EPO production, and increasing fluid retention. Therefore, improvement of cardiac function and cardio-renal haemodynamics would be the most rational approach for the treatment of anaemia in CHF. Additionally, administration of recombinant human EPO might also break the vicious cycle by replenishing the insufficient EPO levels[24]. It is however uncertain whether supplementation of EPO in anaemic CHF patients will decrease morbidity and mortality, as anaemia might merely be a marker for impaired cardiac function. This will emerge from scheduled randomized clinical trials. However, whether this will improve Hb and outcome in this population is uncertain.

Our study has limitations. Apart form the obvious cross-sectional design, the CHF population contained relatively few anaemic CHF patients and anaemic subjects had relatively mild anaemia. Therefore our data might not be representative for more severe forms of anaemia and should be regarded as hypothesis generating.

We conclude that anaemia in CHF is not only independently associated with impaired renal function and blunted EPO production, but to fluid retention as well.

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CHAPTER 5B

HIGHER EXTRA CELLULAR FLUID VOLUME IS A DETERMINANT OF LATE POST-TRANSPLANT ANAEMIA, INDEPENDENT OF GLOMERULAR FILTRATION RATE

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Abstract

Anaemia is common after renal transplantation, and bears prognostic impact. Its pathogenesis is multifactorial, but incompletely elucidated. Recent data in cardiac patients, demonstrating an independent role for a higher extra cellular fluid volume (ECFV) in anaemia in stable heart failure, elicited the hypothesis that a higher ECFV might be involved in late post-transplant anaemia.

103 stable renal transplant recipients, who underwent renal function measurements with constant infusion of ^{125}I-iothalamate (IOT) between March and October 2006, were included. Glomerular filtration rate (GFR) was measured as the clearance of IOT and ECFV as its distribution volume. In anaemic subjects GFR, serum iron and folic acid were significantly lower than in non-anaemic subjects (all $p<0.05$). ECFV, renin angiotensin aldosterone system inhibiting medication usage and proteinuria were higher in anaemic subjects (all $p<0.05$). Age, length, body mass index, mean arterial pressure, erythropoietin, smoking, immunosuppressive medication and diuretics use were not different at $p<0.05$ level. On multivariate analysis female sex ($p<0.005$), higher ECFV ($p<0.004$), lower GFR ($p<0.003$), more proteinuria ($p<0.05$) and lower folic acid ($p<0.05$) were independent predictors for lower Hb.

Thus, a higher ECFV is independently associated with a lower Hb in stable renal transplant recipients. Volume expansion may contribute to the adverse long term prognostic impact of a lower Hb level.

Introduction

Anaemia is common after renal transplantation with estimates ranging from 20% up to 76%[1], depending on the definition of anaemia and the time after transplantation. Late post transplant anaemia is defined as anaemia at more than six months after transplantion[2,3], i.e. when peri-operative events can be assumed to have abated. Possible causes of late post transplant anaemia include chronic renal failure with its associated erythropoietin (EPO) deficiency or EPO resistance[3], as well as possible bone marrow suppression due to immunosuppressants, iron/ folic acid/ vitamine B12 deficiency, inflammation, infections and use of renin angiotensin aldosterone system (RAAS) blocking medication[3,4] It has been pointed out that unidentified, non-transplant-related factors may also be involved[5,6]. Considering the prognostic impact of anaemia after renal transplantation[7-10], it would be important to unravel the contribution of the various possible determinants of anaemia after renal transplantation, as this could guide the most appropriate way of intervention.

In a recent study in patients with chronic heart failure (CHF) and heart failure-related renal function impairment we showed that an increased extra cellular fluid volume (ECFV) was an independent determinant of anaemia. Remarkably, this was independent of renal function[11] . Whether ECFV is also a determinant of anaemia in renal transplant recipients has not been evaluated so far. In our centre, renal function measurement by ^{125}I-iothalamate (IOT) is used for monitoring of renal function in transplant recipients[12,13]. This allows to estimate ECFV as the distribution volume (Vd) of IOT[11]. In the current study, therefore, we investigated whether ECFV is a determinant of haemoglobin levels in a cross-sectional analysis in stable transplant recipients, and, if so whether such an effect was independent of renal function and EPO levels.

Methods

Study population

Data from 138 consecutive stable renal transplant recipients, who visited our outpatient clinic between March and October 2006 for assessment of glomerular filtration rate (GFR) by IOT clearance, and of whom all clinical data were available, were considered for inclusion. Inclusion criteria were a minimal time after transplantation of one year, a functioning graft and age >18. 103 recipients were included. Clinical data included gender, age, height, length, time since transplantation, immunosuppressive regimen, use of diuretics and RAAS blockers and previous history of smoking. The immunosuppressive regimen consisted of triple therapy with cyclosporine A (CsA) or tacrolimus, azathioprine and steroids (15 patients); if treatment started after May 1997 the regime consisted of CsA or tacrolimus, mycophenolate mofetil (MMF) and steroids (53 patients). 19 patients were on a dual immunosuppressive regimen after stepwise withdrawal of CsA or tacrolimus and another 16 patients were on a dual regimen consisting of a calcineurin inhibitor with steroids.

Renal function measurements

During their visit, recipients underwent renal function measurement with IOT and ^{131}I-Hippuran by the constant infusion method[12,13], to measure GFR and effective renal plasma flow (ERPF), respectively, as part of their routine follow-up. In short, participants arrived at 08.00 hours. A baseline blood sample was drawn, which was used for laboratory measurements. Thereafter, a priming solution was given with 0.4 ml per kg body weight (BW) of the infusion solution (0.04 MBq of IOT and 0.03 MBq of ^{131}I-Hippuran) plus an extra amount of IOT (0.6 MBq), followed by constant infusion at 9 ml·h^{-1}. After one-and-a-half hour, steady state of IOT and ^{131}I-Hippuran was achieved. In steady state, over a 4 hour period, hourly blood

samples were taken and urine was collected every two hours. GFR was calculated as the renal clearance of IOT by the formula U·V/P wherein U·V represents the amount of IOT excreted in the urine and P represents the plasma concentration of IOT. ERPF was calculated as the plasma clearance rate, by the formula I·V/P, where I·V represents the infusion rate and P represents the plasma concentration of ^{131}I-Hippuran. Correction of GFR measurement for voiding errors was done using the ratio of urinary to plasma clearance of ^{131}I-Hippuran: $(U \cdot V/P)_{Iothalamate} \cdot (I \cdot V/P)_{Hippuran} / (U \cdot V/P)_{Hippuran}$.

Body surface area (BSA) was calculated according to Boyd's formula ($0.0178 \cdot Height^{0.5} \cdot Weight^{0.484}$)[14] and GFR and ERPF expressed as ml·min^{-1} per 1.73m^2 of BSA. ECFV was calculated as the Vd of IOT[11,15] in steady state as $(B \cdot V + I \cdot V - U \cdot V)/P_{Iothalamate}$, wherein B·V represents the bolus given at 08.00, I·V represents the infusion rate and P represents the plasma concentration of IOT. ECFV was expressed as a percentage of BW.

Laboratory measurements

Haematological parameters were determined from blood samples drawn at baseline. Haemoglobin, haematocrit, iron, ferritin, transferrin, folic acid, vitamin B12, N-terminal proBNP (NT-proBNP) and EPO levels were determined at the local laboratory facilities, as well as sodium and creatinine levels. EPO levels were measured on the Immulite 2000 assay (DPC, Los Angeles, CA, USA), NT-proBNP levels on the Roche Modular E170 (F. Hoffmann-La Roche Ltd., Basel, Switzerland). Anaemia was defined based on local laboratory values. Anaemia in males was defined as Hb <8.7 mmol·l^{-1} (14.0 g·dl^{-1}) and anaemia in females as Hb <7.5 mmol·l^{-1} (12.1 g·dl^{-1}). To define the relation between haemoglobin and EPO levels, an Observed/Predicted (O/P) ratio for EPO levels was calculated using the

formula as used by Westenbrink et al.[11], log Predicted EPO = 3.015-(0.130·Hb). Fractional excretion of sodium (FE_{Na+}) was calculated as $((U \cdot V)_{Na+} \cdot P_{creat}) / (P_{Na+} \cdot GFR) \cdot 100$.

Statistics

To identify the determinants of anaemia in our study population, data were analyzed in a dual way. First, the study population was divided into two groups, based on presence or absence of anaemia. Second, multivariate analysis was performed with Hb as the dependent variable.

Data are given as mean ± standard deviation (SD) when normally distributed and as median and 25^{th}-75^{th} quartile when distribution was skewed. Frequencies and percentages are given for categorical variables. Differences between groups were compared with T-test, or χ^2 test for categorical variables. A p-value <0.05 (two-sided) was considered to be significant. Non-normally distributed values were log transformed as appropriate.

For multivariate analysis, all differences found between the anaemic and the non-anaemic group with $p<0.1$ were included as independent variable into a backward stepwise multivariate linear regression model with Hb as a continuous dependent variable.

Results

Patient characteristics

Patient characteristics are given in table 1, for the whole population and with a break up by presence or absence of anaemia. Of all 103 renal transplant recipients included, 57 were male. Mean age was 53 ± 13 years with a minimum of 19 years. Mean GFR was 54 ± 19 $ml \cdot min^{-1} \cdot 1.73m^{-2}$. Proteinuria >0.3 g/day was present in 35 recipients (34%). All patients used immunosuppressive medication, 86 using calcineurin inhibitors in combination with prednisolon, potentially combined with azathioprine or MMF. None of the recipients used iron suppletion therapy or recombinant human erythropoietin (rHuEPO).

Differences between anaemic and non-anaemic patients

Fifty patients (49%) were classified as anaemic. Among the anaemic patients men were overrepresented (66% male versus 45% in the non-anaemic group, p<0,05). Anaemic patients had, by definition, significantly lower Hb levels (7.4 ± 0.7 $mmol \cdot l^{-1}$ vs. 9.1 ± 0.8 $mmol \cdot l^{-1}$; p<0.001), a lower GFR (48 ± 17 vs. 60 ± 19 $ml \cdot min^{-1} \cdot 1.73m^{-2}$; p=0.002), higher ECFV (20.6 ± 3.6l vs. 18.4 ± 3.5 l (p<0.002), corresponding to 25.6 ± 4.6% vs. 22.9 ± 3.8% of BW; p<0.003), more proteinuria (0.59 $g \cdot 24h^{-1}$ vs. 0.30 $g \cdot 24h^{-1}$; p<0.05), lower serum iron levels (13.3 vs. 15.7 $\mu mol \cdot l^{-1}$; p<0.03) and lower serum folic acid levels (9.8 vs. 12.0 $nmol \cdot l^{-1}$; p<0.004). Immunosuppressive medication, use of diuretics and 24-hour Na_+ excretion was not different between the groups. However, use of RAAS blocking medication was higher in the anaemic patients. EPO levels were not significantly different between the groups, yet the O/P ratio was significantly lower in the anaemic group (0.56 vs. 1.20; p<0.001). Analysing the data for men and women separately did not alter the differences between anaemic and non-anaemic recipients (data not shown).

	Total (n=103)	Anaemic (n= 50)	Non-anaemic (n = 53)	p-value
Sex *(male/female)*	57/46	*33/17*	*24/29*	0.04
Age (years)	53 ± 13	53 ± 14	53 ± 13	ns
Length (m)	1.73 ± 0.09	1.75 ± 0.09	1.71 ± 0.09	ns
Weight (kg)	81.7 ± 15.5	82.3 ± 16.3	81.1 ± 14.7	ns
MAP (mmHg)	98.8 ± 11.6	100.5 ± 12.3	100.0 ± 12.1	ns
Time after Tx (months)	59 *(12-121)*	*67 (12-119)*	*82 (12-123)*	ns
Haemoglobin ($mmol \cdot l^{-1}$)	8.3 ± 1.1	7.4 ± 0.7	9.1 ± 0.8	<0.001
Haematocrit (%)	41 ± 5	37 ± 3	44 ± 4	<0.001
EPO ($U \cdot l^{-1}$)	15.9 ± 9.7	17.3 ± 11.3	14.6 ± 7.9	ns
O/P ratio EPO	0.89 ± 0.70	0.56 ± 0.31	1.20 ± 0.82	<0.001
GFR ($ml \cdot min^{-1} \cdot 1.73m^{-2}$)	54 ± 19	48 ± 17	60 ± 19	0.002
ERPF ($ml \cdot min^{-1} \cdot 1.73m^{-2}$)	197 ± 64	209 ± 62	185 ± 65	ns
ECFV (l)	19.5 ± 3.7	20.6 ± 3.6	18.4 ± 3.5	0.002
ECFV (% of BW)	24.2 ± 4.4	25.6 ± 4.6	22.9 ± 3.8	0.002
24-h Na Excretion ($mmol \cdot 24h^{-1}$)	161 ± 60	169 ± 71	153 ± 47	ns
Proteinuria ($g \cdot 24h^{-1}$)	0.25 *(0.19-0.40)*	0.26 *(0.14-0.54)*	0.24 *(0.20-0.32)*	0.05
Smoking n *(%)*	19 *(18)*	8 *(16)*	11 *(21)*	ns
Serum Iron ($\mu mol \cdot l^{-1}$)	14.2 ± 4.7	13.3 ± 5.1	15.7 ± 4.8	0.02
Folic Acid ($nmol \cdot l^{-1}$)	11.0 *(5-81)*	*9.8 (8.4-11.0)*	12.0 *(9.5-15.3)*	0.003
Vitamin B12 ($pmol \cdot l^{-1}$)	*297 (197-335)*	*270 (214-324)*	*254 (202-343)*	ns
Ferritin ($\mu g \cdot l^{-1}$)	*192 (36-199)*	*97 (33-260)*	*123 (56-296)*	ns
Transferrin ($g \cdot l^{-1}$)	2.56 ± 0.53	2.52 ± 0.47	2.60 ± 0.50	ns
NT-ProBNP ($ng \cdot l^{-1}$)	*282 (116-702)*	*292 (141-993)*	*237 (90-648)*	ns
RAAS inhibiting medication, n *(%)*	50 *(48)*	33 *(66)*	17 *(32)*	<0.001
Diuretics, n *(%)*	45 *(44)*	24 *(48)*	21 *(40)*	ns
CNI + Aza + Pred, n *(%)*	15 *(14)*	10 *(20)*	5 *(9)*	ns
CNI + MMF + Pred, n *(%)*	53 *(54)*	22 *(44)*	31 *(59)*	ns
CNI + Pred, n *(%)*	16 *(16)*	8 *(16)*	8 *(15)*	ns
Aza + Pred, n *(%)*	9 *(9)*	4 *(8)*	5 *(9)*	ns
MMF + Pred, n *(%)*	10 *(10)*	6 *(12)*	4 *(8)*	ns

Table 1 Characteristics of 50 anaemic and 53 non-anaemic renal transplant patients.
All continuous variables are presented as mean ± SD if normally distributed and as median value with 25th-75th percentile when skewed distributed. Mean arterial pressure (MAP); erythropoietin (EPO); Observed/Predicted (O/P) ratio; glomerular filtration rate (GFR); effective renal plasma flow (ERPF); extra cellular fluid volume (ECFV); N-terminal proBNP (NT-proBNP); renin angiotensin aldosterone system (RAAS); Calcineurin Inhibitor (CNI); Azathioprine (Aza); Prednisolon (Pred); mycophenolate mofetil (MMF).

Determinants of Hb

The univariate correlation between ECFV and Hb, analysed as a continuous variable, is given in figure 1 for the population as a whole. It shows a significant negative correlation between ECFV and Hb (r=-0.34; p<0.001). When analysed by a break-up for GFR above (triangles) or below (circles) the median (45 ml·min^{-1}·1.73m^{-2}), the graphs shows that the negative association between ECFV and Hb was present both in subjects with lower GFR (r=-0.43; p<0.002) and subjects with higher GFR (r=-0.26; p<0.05), with a lower Hb for any given level of ECFV in the subgroup with lower GFR.

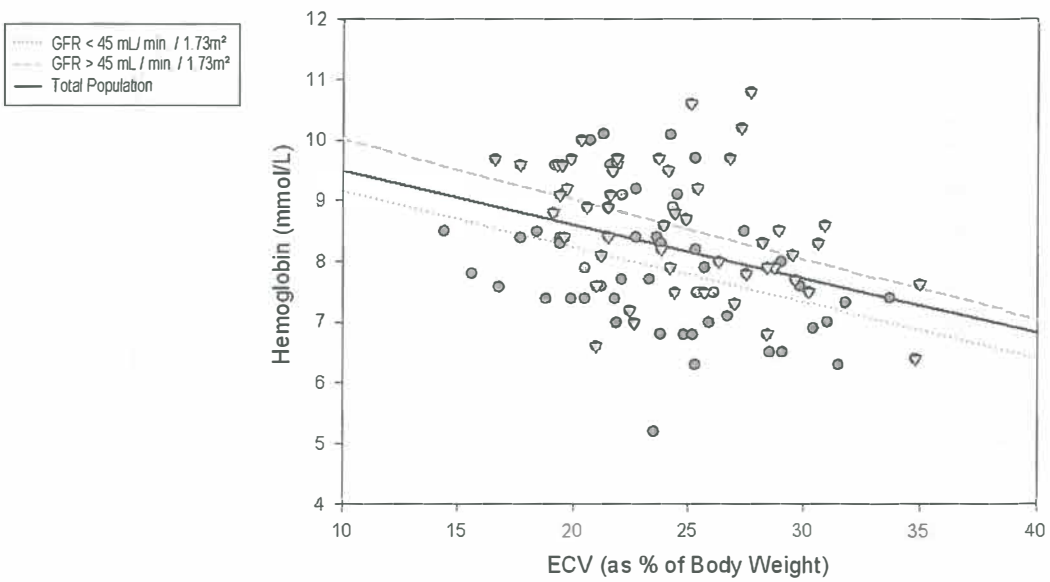

Figure 1 *Correlations between extra cellular fluid volume (ECFV) and haemoglobin levels for subjects with glomerular filtration rate (GFR) <45 ml·min^{-1}·1.73m^{-2} (r = -0.43; p<0.002); subjects with GFR > 45 ml·min^{-1}·1.73m^{-2} (r = -0.26; p<0.05) and total study population (r = -0.34; p<0.001)*

The independent associations of GFR and ECFV with Hb were confirmed on multivariate analysis, as were the independent contributions of female sex, lower EPO levels, use of RAAS blockade, and proteinuria, with an r^2 of the model of 0.451 ($p<0.001$; table 2). The colinearity index, an index of colinearity, remained below 15, indicating that GFR and ECFV were not co-linear. Furthermore, no interaction between GFR and ECFV could be detected. Serum iron levels and ERPF fell out of the model. Forced inclusion of possible additional variables, such as immunosuppressive regimen, smoking, sodium excretion and body mass index (BMI) did not improve the model or increase the adjusted r^2.

	β	SE	p-value
Sex	-0.330	0.178	<0.001
EPO	-0.177	0.010	<0.03
ECFV (% of BW)	-0.418	0.027	<0.001
GFR/1,73m²	0.474	0.006	<0.001
RAAS Inhibitors	-0.244	0.179	<0.005
Proteinuria	-0.171	0.271	<0.05

Table 2 Multivariate analysis of the predictors of Hb in renal transplant recipient. Erythropoietin (EPO); extra cellular fluid volume (ECFV); glomerular filtration rate (GFR); renin angiotensin aldosterone system (RAAS). Adjusted $r^2 = 0.451$.
Excluded variables via backward regression model analysis: ERPF/1,73m², Serum Iron, Ferritin, Transferrin, Folic Acid and Vitamin B12, Diuretics use and Cyclosporin dose.

Discussion

This study demonstrates that a lower haemoglobin level in stable renal transplant recipients is not only determined by a worse renal function and blunted EPO production, but also by higher ECFV.

Anaemia is common in renal transplant recipients and its importance has recently been underlined by its prognostic impact for mortality and graft loss[6,10]. Its pathogenesis is multifactorial[3], and it has been pointed out that reduced kidney function is not the only explanation for anaemia in renal transplant recipients[5]. In our study we identified several differences between anaemic and non-anaemic subjects, with a preponderance of men, and with lower GFR, O/P ratio of EPO, and serum iron and folic acid in anaemic subjects, as well as higher ECFV and proteinuria, and use of RAAS blockade in the anaemic subjects. As the validity of this comparison could be affected by the cut-off used for definition of anaemia[6], we also analysed for the determinants of Hb as a continuous variable. Thus, we could confirm the independent contributions of GFR, ECFV, EPO, RAAS blocking medication and proteinuria, in addition to the anticipated effect of sex.

The effect of GFR was anticipated and in line with other studies on anaemia in renal transplantation and in native kidney disease. A blunted response of EPO to anaemia, due to malfunction of the EPO producing cells[16] is probably a main mechanism underlying this association[17,18]. In our study EPO level in anaemic subjects was not significantly different from the EPO level in non-anaemic subjects, despite the lower haemoglobin level. This is in line with a blunted EPO response to anaemia, which was confirmed by the lower O/P ratio for EPO level[19,20] in the anaemic subjects. It would be of interest to know whether EPO O/P ratio would be independently associated with Hb level, but we refrained from multivariate

analysis on this issue as EPO O/P ratio and Hb level are arithmetically associated, invalidating such an analysis.

A higher ECFV was independently associated with a lower Hb, independent of GFR. No co-linearity of ECFV and GFR could be detected, supporting the independent contribution of a higher ECFV to a lower Hb. This was in line with our prior observation in patients with heart failure[11]. In heart failure haemodilution has been demonstrated to be involved in the association between ECFV and anaemia[21]. Our data are the first to demonstrate an association between a higher ECFV and a lower Hb in renal transplant recipients, and moreover, in only mild anaemia. It should be noted that this association was found in the absence of clinical signs of fluid retention in these stable transplant recipients, and that, apparently, the difference in ECFV of approximately 2 liters can go unnoticed by clinical assessment. An association between ECFV and anaemia was reported previously in patients with severe chronic anaemia (haematocrit 9-16%) in whom ECFV was increased by approximately 4 liters. It was associated with peripheral vasodilation, lower blood pressure and reduced renal blood flow (RBF) and activation of the sympathetic nervous system and the RAAS. The volume retention was attributed to the anaemia as such, as a compensatory response to maintain tissue oxygenation[22,23]. It should be pointed out that the anaemia in our study was much milder with a mean haematocrit of 37% in the anaemic subjects, in whom ECFV was some 2 liters higher than in the non-anaemic subjects. Our study design unfortunately does not allow to dissect cause and consequence, so it cannot be derived from our data whether the anaemia contributed to the observed ECFV expansion. The other way round, ECFV expansion could have induced haemodilution and consequently a lower haemoglobin level.

The use of RAAS blocking medication is known to affect haemoglobin levels[24,25]. ACE inhibitors may even be used to treat polycythemia vera.[26] Various mechanisms have been suggested, including prevention of the stimulatory effect of angiotensin II on the synthesis of EPO[27]; increased renal plasma flow, reducing the hypoxic stimulus for EPO formation[4,28]. A direct effect on red blood stem cells has also been suggested[29].

Proteinuria was also a determinant of a lower Hb. An adverse effect of proteinuria on Hb has been reported in nephrotic range proteinuria in native kidneys, attributed to urinary loss of EPO and transferrin[30], but to the best of our knowledge this is the first time that proteinuria is identified as a determinant of Hb in renal transplant recipients. Proteinuria in our study was not in the nephrotic range and its effect on Hb was independent of ECFV. Theoretically, proteinuria might also have contributed to a lower Hb by inducing volume retention. As its predictive value was independent of ECFV an effect on ECFV cannot explain the association between proteinuria and anaemia in this population. We cannot exclude however that proteinuria may contribute to anaemia by inducing volume retention

Our study has several limitations. First, it was a cross-sectional analysis and thus includes a variety of patients at various times after transplantation, and on unrestricted, non-standardized sodium intake. Furthermore, most patients had relatively mild anaemia, which may have influenced our results. No data on cardiac function in these patients were available. The effect of deteriorating cardiac function, a symptom often seen in renal transplant recipients[23] which may influence volume status[21], on anaemia in this population therefore could not be established. Finally, we have no information on plasma volume or red cell mass, so we were unable to infer that our findings are due to haemodilution.

Anaemia is a predictor of cardiovascular morbidity and mortality in renal patients[31-33;34] and in recent studies anaemia had prognostic impact in renal transplant recipients as well, with an adverse effect on graft survival and mortality[7-10,35]. It has been pointed out that the mechanisms of anaemia in transplant recipients may partly correspond to those in native kidney disease, but transplant-specific mechanisms, such as effects of immune suppression may be involved as well[3]. Unravelling the underlying mechanisms may be relevant for intervention strategies. In spite of the association between anaemia and cardiovascular morbidity and mortality in chronic kidney disease (CKD) patients the effect of correction of Hb by rHuEPO on cardiovascular endpoints has so far been disappointing. Correction of anaemia with rHuEPO did not decrease left ventricular hypertrophy[36] and moreover, in two recent large studies[37,38] normalization of haemoglobin levels did not reduce cardiovascular end points in CKD patients. The discrepancy between observational data showing a predictive effect of anaemia for cardiovascular mortality, and the lack of therapeutic benefit of anaemia correction in controlled trials has been called the "anaemia paradox"[39] in CKD. So far, data in transplant recipients are insufficient to support or reject such a paradox for transplant recipients[8,35]. Our data suggest that explicit consideration of volume status might be relevant in the work-up and management of anaemia in transplant recipients. Whereas most nephrologists would generally agree that proper control of volume status is relevant in the management of renal patients, be it in native or transplanted kidneys, in clinical practice intervention aimed at control of volume status is limited to the treatment of hypertension, and of clinically overt fluid overload, such as peripheral and pulmonary oedema. In this respect, it would be important to develop a reliable, convenient indicator of ECFV for use in clinical practice.

We conclude that ECFV is independently associated with anaemia in renal transplant recipients, and that this is independent of renal function. This extends prior observations in heart failure, and in primary severe anaemia to the renal transplant population and to relatively mild anaemia. Further studies should elucidate the mechanisms underlying this association, and its consequences for the management of anaemia and volume status in transplant recipients.

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CHAPTER 6

SUMMARY AND GENERAL DISCUSSION

Summary

A high sodium intake is associated with increased cardiovascular and renal morbidity, and higher mortality. This association is particularly prominent in overweight or obese subjects. Although elevation of blood pressure is generally considered an important intermediate mechanism, high sodium intake also exerts adverse effects independent of blood pressure. A rise in sodium intake initially elicits a positive sodium balance. Consequently, when the higher sodium intake is maintained, extra cellular fluid volume (ECFV) is increased. As a higher ECFV leads to a higher volume load for the heart, ECFV might be an intermediate factor in the adverse effects of excess sodium intake. ECFV can reliably be measured as the distribution volume (Vd) of bromide, considered as a gold standard. However, this method requires bromide injection and timed blood sampling, which is considered impractical in clinical practice, and unfeasible for large scale epidemiological studies. Since no reliable alternatives are available, large scale data on ECFV are not available. Consequently, the role of ECFV in the adverse effects of excess sodium intake in largely unexplored.

Interestingly, the Vd of tracers used as gold standards for renal function assessment, such as inulin, iohexol and iothalamate, equals ECFV. Although these methods are laborious and expensive, nevertheless their use is relatively common in top-clinical nephrology settings as well as in clinical research. Validation of such renal function assessments for simultaneous assessment of ECFV would provide data on ECFV on a scale that is unique world-wide, without extra effort or costs, mostly obtained in renal patients, i.e. a population where disturbance of volume status is an important pathogenetic factor.

The studies in this thesis were devoted, first, to development and validation of ECFV measurement from the distribution volume of ^{125}I-iothalamate (IOT), simultaneously with the renal function measurement by its urinary clearance that is performed routinely in our center. Additionally, after validation and calibration of the ECFV assessment, we used this method to study ECFV in relation to several risk factors for cardiovascular and renal disease, namely weight excess, sodium sensitivity of blood pressure, loss of renal mass, and cardiorenal anaemia.

In Chapter 1 we present studies on the validation and calibration of the Vd of IOT as a measure for ECFV. As noted above, IOT is the tracer routinely used for precise assessment of glomerular filtration rate (GFR) in our centre. ECFV assessed as the Vd of IOT was in good accordance with the Vd of bromide, i.e. the gold standard method for ECFV, and showed a fair reproducibility with day-to-day variations of 8.6% and 13.1%, respectively, during low and high sodium diet. Moreover, the method could reproducibly detect the change in ECFV elicited by a shift from low to high sodium intake over a clinically and physiologically relevant range, i.e. from a daily intake of 3 to 12 grams of sodium. Thus, simultaneous measurement of renal function and ECFV is possible, feasible and reliable, and this combined measurement could be used to evaluate ECFV in several relevant conditions.

Simultaneous assessment of renal function and ECFV provides the possibility to express renal function indexed to ECFV, rather than to body surface area (BSA), which is current practice. Indexing of renal function to body dimensions is required to meaningfully compare renal function in different individuals, and BSA is usually used to this purpose. However, the validity of indexing to BSA has been questioned, as body composition can vary considerably between individuals despite a similar BSA. This for instance applies to obesity, and to the differences in body composition between men and women, as for a given BSA women have a

larger fat mass. In the second part of chapter 1 we examined whether indexing GFR to ECFV would provide benefits over indexing to BSA. In our population, that consisted of healthy subjects screened for kidney donation, the difference in renal function between men and women, as observed for GFR/BSA, disappeared when GFR was indexed to ECFV. Renal function indexed to ECFV was neither associated to BSA, length or body weight. This suggests that ECFV is the most appropriate indexing parameter.

In chapter 2 we focused on weight excess, a risk factor for cardio-renal damage, in association with sodium status. In subjects with weight excess an elevated renal function has been observed, that is considered an early metabolic risk marker. Moreover, elevated GFR may be a pathogenetic factor in the long term renal damage associated with weight excess. To analyze these interrelationships, appropriate evaluation of renal function and its relation to BMI is required. In chapter 2A we found that a higher BMI is associated with a systematic error in creatinine clearance, one of the main methods for measurement of renal function in clinical practice, in healthy subjects. Whereas creatinine clearance provided a satisfactory assessment of renal function in our population, remarkably, overweight was associated with overestimation of true renal function by creatinine clearance. This was due to net tubular creatinine secretion in subjects with higher BMI. Whereas elevated true GFR has indeed been documented in obesity, our data show that an elevated creatinine clearance in obesity is partly explained by altered tubular creatinine handling. Our data suggest that a higher creatinine supply to the kidney, by either larger muscle mass, dietary intake or both, leads to net tubular creatinine secretion. So far, it is generally assumed that tubular creatinine secretion occurs only, or particularly in subjects with moderate to severe renal function impairment. Our data demonstrate a systematic error in healthy subjects as well,

indicating that creatinine clearance should be interpreted with care, in particular in subjects with weight excess.

In chapter 2B we studied the impact of BMI on renal sodium handling and volume homeostasis in young healthy volunteers. A higher BMI was associated with a larger increase in ECFV when shifting from a low to a high sodium diet. As a consequence, during high sodium intake overweight subjects had a higher ECFV than lean subjects, whereas during low sodium diet ECFV was similar. These data suggest that BMI-associated differences in volume homeostasis could be involved in the interaction between high sodium intake, weight excess and long term cardiovascular morbidity and mortality. During high sodium intake, tubular sodium reabsorption as well as filtered load of sodium were higher in the overweight subjects than in the lean subjects, indicating that BMI modulated glomerulo-tubular balance during high sodium intake, possibly by its association with a higher filtration fraction.

In studies on the adverse effects of high sodium intake on blood pressure it has long been noted that individual differences in the response to altered sodium intake, the so-called sodium-sensitivity, are large in normotensive as well as hypertensive subjects. Interestingly, sodium-sensitivity as such is associated with long term mortality, independent of blood pressure. Its underlying mechanisms are therefore of interest. We studied the possible role of intra-renal activity of the renin angiotensin aldosterone system (RAAS) with sodium sensitivity in healthy subjects. Our data provided support for an increased activity of the intra-renal RAAS during both low sodium and high sodium intake in sodium sensitive subjects as compared to sodium-resistant subjects. During ACE-inhibition these differences were annihilated. Interestingly, ECFV tended to be higher in sodium

sensitive subjects during either sodium intake. As our study was not quite powered to draw conclusions on between-group differences in ECFV, these data require confirmation in larger studies.

The amount of functional renal tissue is assumed to be important in volume homeostasis, and reduction of renal mass, by renal disease or in animal models, has been shown to be associated with volume expansion. However, whether this relates to the reduction in renal mass and function, or to the presence of renal parenchymal abnormalities, has not been dissected. In chapter 4, therefore, we studied the effect of uninephrectomy in healthy kidney donors on ECFV and renal sodium handling. Remarkably, in these healthy subjects uninephrectomy resulted in a decrease in ECFV three months after donation, despite a substantial reduction in total GFR. Thus, reduction of renal mass and renal function is not invariably associated with ECFV expansion. In our healthy kidney donors a clear-cut adaptive response was present after donation, with a substantial increase in single-kidney GFR in the remaining kidney. Consequently, after donation filtered load of sodium was higher, as were tubular reabsorption rate of sodium, and fractional sodium excretion. We assume that an altered setpoint of glomerular and tubular sodium handling resulted in the observed decrease of ECFV. This might be due to different adaptative responses of filtration and reabsorption, to the observed reduction of filtration fraction, to the increased filtered load of bioactive substances such as amino-acids, or a combination of these.

Finally, in chapter 5 we analysed the association between anaemia and ECFV in two different disease conditions. First, in chapter 5A we describe a patient population with chronic heart failure, i.e. a condition characterized by primary fluid overload but with intrinsically normal kidneys. In this population anaemia

was not only related to impaired renal function and blunted EPO production, but remarkably a higher ECFV was an independent determinant of anaemia as well. Second, in chapter 5B we analysed a population of stable renal transplant recipients. In this population a higher ECFV was an independent determinant of anaemia as well. These studies thus demonstrate a consistent association between volume status and anaemia that should be considered appropriately when studying the pathophysiology of anaemia. Our studies suggest that it might be fruitful to explore the effects of correction of volume overload as part of a therapeutic intervention regimen in cardiorenal anaemia.

General Discussion

The importance of assessment of volume status is undisputed in clinical medicine for many acute and chronic disease conditions. Yet, measurement of ECFV has not made its way into clinical practice, and moreover, it has an only modest place in clinical research. This is, among others, due to the fact the available methods are either inaccurate or considered too invasive for clinical routine. In the studies described in this these we demonstrate the feasibility to estimate ECFV from the data generated during the accurate measurement of renal function as the clearance of IOT.

Obviously, we do not want to advocate large scale application of IOT measurements in any population where assessment of ECFV would be relevant. Rather, we wanted to enhance the yield of accurate renal function measurements that are done for various purposes, with an estimate of ECFV, thus creating a data resource that is not available so far anywhere in the world. Accurate renal function measurement is mostly done in specific populations with renal or cardiovascular

disease. Since disturbances of volume homeostasis are of considerable pathophysiological relevance in these populations, simultaneous assessment of ECFV might be highly useful, not only for research purposes, but potentially also for clinical patient management. Indeed, if measurement of ECFV would be implemented in all instances where GFR is measured with specific tracers, this would provide a tremendous resource of data that might of great value to better address the role of altered volume homeostasis in renal disease, and its cardiovascular complications. In this paragraph we will discuss the feasibility, objectives and possible merits of ECFV measurement in renal settings. Moreover, we will discuss the novel insights on the role of altered regulation of renal sodium handling and ECFV in relation to risk markers of cardiovascular disease, that we obtained by analysing ECFV data from renal function measurements in several populations.

Implementation of ECFV assessment in renal function measurement

Assessment of renal function by specific tracers is laborious and therefore expensive. However, the accuracy and precision are far superior over creatinine-based renal function assessments[1,2]. Therefore, in scientific and top-clinical nephrology settings, measurement of renal function by specific tracers is relatively common. In our centre, 800-1000 measurements are performed yearly, most in protocollized follow-up of transplant recipients, but also in patients with native kidney disease, or chronic heart failure, and finally for screening and follow-up of healthy kidney donors. Routinely measuring ECFV simultaneously with the renal function measurements would increase the yield of these measurements, and open-up a resource of data on ECFV world-wide.

Several specific tracers are available to accurate renal function measurement, such as inulin, iohexol, IOT and ^{51}CrEDTA[1]. Our studies focused on IOT as this tracer is used for renal function measurement in our centre. It is important to note that the kinetics and renal handling of other renal function tracers are not essentially different from IOT[3]. Accordingly, other renal function tracers could similarly be used to estimate ECFV. For some tracers clinical validation is available supporting their use for ECFV measurement, albeit not as extensive as pursued here for IOT[4-9]. A main feature of GFR assessments in many other centres is that renal function is calculated from the plasma-disappearance curve, whereas our constant infusion method relies on steady state plasma levels. Plasma-disappearance curve are also suitable for ECFV assessments, and do not require the algorithm that we used for extrarenal clearance.

Which insights could we gain from measurement of ECFV simultaneously with renal function? As noted above, renal function is usually indexed to BSA to be able to compare renal function between individuals. However, the validity of indexing GFR to BSA has been questioned by several authors, pointing to possible confounding by obesity and male-female differences[10,11]. An alternative would be indexing GFR to ECFV[12]. The latter is attractive since the ECFV is the body compartment from which the kidney clears the waste products[9]. The results from chapter one, showing that indexing to ECFV annihilates the alleged male-female differences in renal function, strongly suggest that indexing to ECFV has advantages over indexing to BSA. This is supported by findings from our other studies. In chapters 1, 2 and 3 the sodium induced changes in GFR paralleled those in ECFV. This observation suggests that in normal physiology sodium-induced changes in renal function are linked to those in ECFV. Therefore, indexing renal function for ECFV could not only prove to be important for between-individual

comparisons, but also for follow-up of individual subjects. Furthermore, in chapter 2, we found that the association between BMI and GFR paralleled the association between BMI and ECFV. These findings suggest that the drawbacks of indexing to BSA in obese subjects do not apply to indexing to ECFV. However, as has been pointed out recently[13], there is no gold standard as to the best way to index renal function, and the final proof for superiority of one practice over another will have to come from studies evaluating their predictive potential for long term renal function.

When GFR is expressed per liter of ECFV, its unit reflects is the virtual volume of ECFV which is cleared per unit of time. By indexing GFR ($ml \cdot min^{-1}$) to ECFV (l) the volume expression drops out of the equation, and thus GFR/ECFV reflects the proportion of the ECFV cleared per unit of time. The other way around ECFV/GFR reflects the time needed to clear the complete ECFV. This way of considering renal function may help to appreciate renal function relative to the metabolic and homeostatic requirements of the body[14]. Our data in chapter 4 nicely illustrates this approach. Before uninephrectomy GFR/ECFV, i.e. the proportion of the ECFV cleared per hour was 36 %. After donation, as anticipated, GFR fell considerably, but due to a rise in GFR in the remaining kidney, post-donation GFR was not 50% but on the average 63% of its pre-donation value. ECFV decreased slightly. Consequently the proportion of the ECFV cleared per hour decreased to $24\% \cdot h^{-1}$. The other way around ECFV/GFR increased from 2.8 to 4.3 hour. Thus, the remaining kidney needs approximately 53% more time to clear the complete ECFV; had there been no changes in single kidney GFR or ECFV, this time would have doubled. It is tempting to speculate that the combined changes of GFR and ECFV reflect a concerted adaptive response to uninephrectomy, but we have no means to substantiate this assumption from our current data.

ECFV assessment by specific tracers, including ours, requires injection and timed follow-up, which is impractical in clinical practice and in large scale epidemiological surveys. Our data strongly suggest that specific assessment of ECFV may provide important pathophysiological insights, for instance on blood-pressure independent mechanisms underlying the combined effects of weight excess and sodium intake on long term cardiovascular outcome. For assessment of ECFV in clinical practice and for epidemiological surveys simple, cheap, and reliable markers for volume status are needed. From the physiology of sodium handling, it would be of interest to explore the potential of natriuretic peptides to this purpose. NT-proBNP is a stable natriuretic peptide that is widely used for diagnosis and prognostic evaluation of heart failure[15]. Somewhat surprisingly, the contribution of volume overload, relative to the contribution of cardiac status as such, to the considerable elevation of NT-proBNP in cardiac patients has not been well-characterized. More subtle increases in NT-proBNP are associated with long term mortality in other populations, including the general population[16-18]. Preliminary data of our own group have shown that increased sodium intake leads to a rise in NT-proBNP in healthy subjects, in the order of magnitude as observed to be prognostic in general population-based cohorts. It is tempting to speculate that such subtle elevations on NT-proBNP reflect subclinical ECFV expansion. It would be highly interesting to explore this issue, in particular because volume expansion is accessible to intervention by dietary sodium restriction and/or diuretics.

ECFV in relation to cardiorenal risk markers

In chapter 2 and 3 we presented data on abnormalities in ECFV in relation to BMI and sodium-sensitivity of blood pressure. Weight excess was associated with a larger rise in ECFV due to high sodium intake, and consequently, a higher ECFV in

overweight subjects as compared to lean subjects during high, but not during low sodium intake. These differences in ECFV were not associated with differences in blood pressure. As this observation was made in healthy young subjects, apparently the alteration in volume homeostasis is an early feature of the metabolic risk profile associated with weight excess. Our data moreover demonstrate that the early changes in volume homeostasis in subjects with weight excess are not a consequence of possible sub-clinical hypertensive renal damage, or of insulin resistance but due to the weight excess as such. Our data strongly suggest that effects on volume homeostasis are involved in the combined effects of high sodium intake and weight excess on cardiovascular, and possibly, renal risk. Whereas this hypothesis requires further confirmation, it could have considerable clinical implications since, as noted above, volume excess is accessible to therapeutic intervention by dietary sodium restriction and/or diuretic therapy.

In sodium sensitive healthy subjects we found evidence for increased activity of the intra-renal RAAS. Also, their ECFV tended to be higher than in sodium-resistant subjects irrespective sodium intake, but this difference did not quite reach statistical significance. However, relative to the other study parameters, the study was not adequately powered to detect between-group differences in ECFV. Yet, a higher ECFV is clearly one of the effects that can be anticipated from the increased activity of the intra-renal RAAS that we observed in the sodium-sensitive subjects. Of note, a constitutively higher ECFV in sodium-sensitive subjects might also provide an explanation for the blood pressure-independent association between sodium-sensitivity and long term mortality[19]. So, the hypothesis that differences in ECFV are involved in differences in sodium-sensitivity in normotensive subjects is attractive, and needs exploration in further, larger studies.

Our data in healthy kidney donors allow to address the effect of reduction in renal mass on ECFV. Reduction of renal mass, and its corresponding reduction in renal function are generally assumed to be associated with adverse cardiovascular effects, not only due to the accumulation of uremic waste products, but also due to disturbed volume homeostasis and volume overload[20]. However, the available evidence was largely obtained in conditions where the reduction in renal mass was due to renal disease[21-23], or, in case of animal models, very severe and associated with early renal parenchymal damage[24,25]. So, it was unclear whether the association of reduction in renal mass and volume expansion was due to the reduction in renal mass as such, or to renal parenchymal damage, or a combination of the two. Our data in healthy kidney donors demonstrate that loss of renal mass and renal function does not invariably lead to a rise in ECFV, as actually ECFV decreased despite the substantial reduction in total GFR. This suggests that fluid overload in renal disease is related to the renal parenchymal abnormalities, and consequent alterations in renal sodium handling, rather than to nephron loss per se. Put otherwise: qualitative differences in the kidney are more important than quantitative differences. Apparently, in a healthy kidney the adaptive responses that aim to preserve GFR after contralateral nephrectomy, do not involve expansion of the ECFV. It would be logical to assume that a lower set-point for ECFV is a pathophyiologically favourable adaptation to the single kidney state, but for the moment we have no data to substantiate this assumption.

Finally, our data in chapter 5 demonstrate how volume status can be closely interrelated with other manifestations of cardiorenal disease, namely anaemia. As ECFV was elevated in anaemia in heart failure as well as in stable renal transplant recipients, the association between ECFV and anaemia appears to be a robust finding. The relationship between ECFV and anaemia however is complicated.

Anaemia itself leads to lowering of oxygenation and thereby fluid retention can occur in order to increases cardiac output, as observed in severe anaemia[26]. However, vice versa, primary abnormalities of fluid regulation may lead to haemodilution, and thus mimic anaemia[27]. Third, anaemia and fluid excess may also have common pathophysiological pathways, such as a decrease in healthy renal mass[28]. Finally, it should be noted that blockade of the RAAS, ubiquitous in heart failure and increasingly common in renal transplant recipients, may well have influenced haemoglobin level in our studies, as RAAS-blockade is well-established to be able to reduce haemoglobin levels, and even induce anaemia[29-31]. It would be of considerable clinical relevance to further explore the interrelationship between volume expansion and anaemia in cardiorenal disease, as this might help the integrated management of the diverse components of cardiorenal damage. It would be important to establish whether the elevated ECFV is indeed associated with haemodilution, and even more importantly, whether control of volume excess can contribute to the management of cardiorenal anaemia. Studies addressing this issue will start shortly in our department.

Taken together, our measurement of the ECFV simultaneous with renal function has provided a wealth of data on the regulation of ECFV in health and cardiorenal disease. However, as our data by definition are derived from renal function measurements that are performed only in specific populations, we do not provide a comprehensive overview of abnormalities in the regulation of ECFV. Yet, our approach allows to collect data on ECFV on a scale that is so far unique. Our current studies demonstrate the potential of the approach in analyses that were so far cross-sectional, but in future studies long term follow-up data should be obtained that will allow to determine the prognostic impact of changes in ECFV for cardiovascular and renal outcome, as well as the possible merits of indexing GFR

to ECFV over BSA. Measurement of ECFV might shed new light on long-standing issues of research in our group, such as the mechanisms underlying potentiation of the effects of RAAS-blockade by sodium restriction and/or diuretics[32,33]. Moreover, it will be useful in analysing the regulation and consequences of altered volume status in type I diabetes. In this condition volume overload is assumed to be involved in systemic and glomerular hypertension, and the pathogenesis of renal damage[34,35]. However, dietary sodium restriction is associated with adverse effects on renal haemodynamic profile[36]. The role of sodium restriction in the treatment of diabetes is therefore still unknown and a matter of debate[37].

We conclude that assessment of ECFV may be of great help to delineate the (patho) physiological effects of sodium. Sodium intake is a modifiable factor probably involved in a wide spectrum of pathology, ranging from cardiovascular risk in the general population to optimalization of treatment in specific situations. The role of ECFV as a factor mediating the (patho-) physiological effects of dietary sodium intake is so far unsatisfactory explored; the results presented in this thesis are a guide and start for further exploration of sodium status and ECFV in (patho) physiology.

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SAMENVATTING IN HET NEDERLANDS

met uitleg voor de leek

Samenvatting

Het eten van veel zout geeft een verhoogd risico op hart- en vaatziektes, en een hoger risico om te sterven. Deze gegevens komen naar voren uit grote studies die inwoners van verschillende landen (met verschillende eetgewoontes), maar ook verschillen tussen personen binnen een land bestudeerden. De relatie tussen het eten van veel zout en risico op hart- en vaatziektes is sterker in mensen met overgewicht. Het ontstaan van hoge bloeddruk kan belangrijk zijn als tussenstap, maar het verhoogde risico is ook aanwezig onafhankelijk van een verhoogde bloeddruk.

Wat gebeurt er als iemand (veel) zout tot zich neemt? Ten eerste is het belangrijk om te weten dat de zouthuishouding en de waterhuishouding nauw samengaan. Zoutinname zorgt voor een subtiele verhoging van de zoutconcentratie wat een verhoging geeft van het ADH (anti-diuretisch hormoon), in andere woorden: het hormoon wat de productie van urine (diurese) tegengaat. Inname van zout gaat dus gepaard met vasthouden van water. Om deze reden verandert de concentratie van zout in het lichaam nauwelijks. Het zout, of liever gezegd de elektrolyt Na^+, bevindt zich met name buiten de lichaamscellen (extra cellulair; dit is in de bloedbaan en in de ruimte tussen de cellen). Na zoutinname stijgt de zout- en waterhoeveelheid, de concentratie blijft echter nagenoeg gelijk; er vindt dus een stijging plaats van het Extra Cellulair (vloeistof) Volume.

Een verhoging van het ExtraCellulaire Volume (ECV) zorgt ervoor dat er eiwitten uit het hart (natriuretische peptides = natrium-uitscheidende eiwitten) uitgescheiden worden en dat het Renine-Angiotensine Systeem (enzymsysteem welke het zout vasthouden door de nier stimuleert) geremd wordt. Uiteindelijk past het lichaam zich op deze manier aan zodat de zoutinname en de zoutuitscheiding (door de nieren) weer aan elkaar gelijk zijn. Dit hele proces kost

echter tijd (2-3 dagen) en daarom is het uiteindelijke resultaat dat iemand die veel zout tot zich neemt, een verhoging krijgt van het ECV (=hoeveelheid water in de extracellulaire ruimte).

Als we teruggaan naar de relatie tussen zoutinname en risico op hart- en vaatziektes, is de gedachte dat stijging van ECV hierin een belangrijke rol kan spelen. Immers, meer volume in de extracellulaire ruimte geeft meer druk op hart- en bloedvaten en dit zou op de lange termijn wel eens schadelijk kunnen zijn. Het meten van ECV is daarom belangrijk als je meer wilt weten over de relatie tussen zoutinname en hart- en vaatziektes. Echter, er is een probleem en dat is dat het meten van ECV niet eenvoudig is. Er zijn wel enkele methoden, maar deze zijn allemaal erg bewerkelijk, moeilijk uit te voeren en vaak slecht onderzocht. De 'gouden standaard' voor het meten van ECV is het meten van het Broom verdelingsvolume. In dit proefschrift echter doe ik een voorstel om ECV te meten tijdens een meting die al dagelijks wordt uitgevoerd in het UMCG: een nauwkeurige meting van de nierfunctie.

Het nauwkeurig meten van de nierfunctie is ook niet eenvoudig. In het UMCG gebruiken we een radioactieve stof (^{125}I-iothalamaat). Dit stofje wordt met een constante snelheid in de bloedbaan gespoten, net zolang totdat de uitscheiding via de nieren even snel gaat als het inspuiten via een infuus: op dat moment is er een evenwicht (steady state). Dit stofje iothalamaat heeft een eigenschap die lijkt op Na^+, dit stofje blijft namelijk ook met name buiten de cellen en verdeelt zich vrij gelijkmatig over de extra-cellulaire ruimte. Als je dus weet hoeveel iothalamaat er in het lichaam gespoten is (van nature komt dit stofje niet voor in je lichaam) en je weet wat de concentratie is in de extracellulaire ruimte (= ook de bloedbaan), dan kun je uitrekenen hoe groot de Extracellulaire ruimte is.

Met de gedachte uit de vorige alinea hebben we in **Hoofdstuk 1** het ECV, berekend aan de hand van het verdelingsvolume van iothalamaat, vergeleken met ECV gemeten met de gouden standaard: het verdelingsvolume van Broom. Beide waardes kwamen goed met elkaar overeen; dit leek zo te zijn voor verschillende nierfuncties, waarbij gezegd moet worden dat er wel een correctie nodig is bij slechte nierfunctie, omdat dan iothalamaat niet alleen door de nieren wordt uitgescheiden, maar er ook een gedeelte via waarschijnlijk de darmen en galvorming verdwijnt.

In het overige deel van hoofdstuk 1, gaan we verder in op normalisatie van de nierfunctie. Het is namelijk zo dat de nierfunctie van een klein persoon lager kan zijn dan van een groot persoon (vergelijk een klein dun omaatje met een grote bodybuilder), maar dit hoeft niet te betekenen dat de nierfunctie ook slechter is. Een klein persoon heeft nou eenmaal een minder hoge nierfunctie nodig. Met deze gedachte wordt de nierfunctie altijd gecorrigeerd voor lichaamsoppervlak (Body Surface Area- BSA); welke niet gemeten wordt, maar berekend aan de hand van lengte en gewicht. Deze correctie aan de hand van lichaamsoppervlak krijgt veel kritiek, omdat enerzijds de bepaling met de formule onnauwkeurig is, maar ook omdat verschillen tussen dikke en dunne personen en bijvoorbeeld mannen en vrouwen niet goed tot uitdrukking komt. Een alternatief kan zijn: nierfunctie normaliseren voor ECV. Ten eerste is het zo dat een klein persoon een kleiner ECV zal hebben dan een groot persoon (onder normale omstandigheden), ten tweede is het ook logisch om de nierfunctie te normaliseren voor ECV als we kijken wat nierfunctie nou eigenlijk betekent. De nieren zuiveren het lichaam van afvalstoffen. De nieren gebruiken het Extra-cellulaire lichaamscomponent hiervoor. Het is dus niet zozeer dat de nierfunctie hoger zou moeten zijn als het lichaamsoppervlak hoger is, maar de nierfunctie zou hoger moeten zijn als het

ECV hoger is. Omdat nierfunctie en ECV fysiologisch met elkaar verbonden zijn, zou het logisch zijn om de nierfunctie te normaliseren voor ECV. In Hoofdstuk 1 doen we dit ook bij mensen die zich hebben opgegeven om een nier af te staan (gezonde mensen dus). Het blijkt dat de nierfunctie (lichaamsoppervlak gecorrigeerd) tussen mannen en vrouwen verschillend is. Als we echter corrigeren aan de hand van ECV is de nierfunctie niet meer verschillend. Er is geen reden dat de door ons onderzochte vrouwen een slechtere nierfunctie zouden hebben dan mannen, daarom vinden wij dat onze resultaten een aanwijzing geven dat normalisatie voor ECV beter is dan normalisatie voor lichaamsoppervlak.

In Hoofdstuk 2 gaan we verder in op de relatie tussen nierfunctie, ECV en overgewicht. In **hoofdstuk 2A** gaan we eerst in op een andere methode van het meten van nierfunctie: het verzamelen van 24-uurs urine en vervolgens het meten van de Kreatinine-klaring. Kreatinine is een afvalstof uit de spieren en wordt door de nieren uit het lichaam verwijderd. Hoewel het minder nauwkeurig wordt geacht dan metingen met radio-actieve stoffen, wordt deze meting zeer vaak uitgevoerd. Als we er achter komen, waarom deze meting minder nauwkeurig is, kunnen we hiervoor corrigeren en kan de meting wellicht nauwkeuriger worden. In hoofdstuk 2 A meten we de hoeveelheid Kreatinine die niet alleen gefiltreerd wordt door de nier, maar daarbovenop door de tubulus worden uitgescheiden (de tubulus is het buisje na de glomerulus (=filter van de nier), waar veel water, zout en andere stoffen juist terug de bloedbaan in gaan). Het is algemeen bekend dat deze tubulaire uitscheiding van Kreatinine met name voorkomt bij mensen met een slechte (filtrerende) functie van de nieren, maar wij laten zien dat het ook voorkomt bij gezonde mensen. Bij deze gezonde mensen lijkt deze extra uitscheiding van Kreatinine een relatie te hebben met mate van

overgewicht (bepaald door het berekenen van Body Mass Index (BMI)), oftewel hoe hoger de BMI, hoe meer de Kreatinine uitscheiding en hoe meer de Kreatinine-klaring de nierfunctie overschat. Het zou kunnen zijn dat bij mensen met overgewicht de tubulus actiever is (door stofjes uit vetcellen), het zou ook kunnen zijn dat mensen met overgewicht meer spieren hebben (en dus meer Kreatinine als afvalstof) en meer Kreatinine via hun voeding binnenkrijgen en dat daardoor de nieren juist bij deze mensen meer Kreatinine uitscheiden.

In **Hoofdstuk 2B** laten we zien (weer bij jonge gezonde mannen) dat het ECV van iemand met overgewicht meer stijgt dan het ECV van iemand zonder overgewicht als die persoon van een zoutbeperkt naar een zoutverrijkt dieet gaat. In de inleiding van dit hoofdstuk vertelden we dat de relatie tussen zoutinname en risico op hart- en vaatziektes sterker is bij mensen met overgewicht. Dat mensen met overgewicht na verhoging van de zoutinname meer stijgen in hun ECV zou hiervoor een verklaring kunnen zijn. De druk in hart- en bloedvaten stijgt daardoor misschien bij deze mensen wel meer. Het is ook zo dat de filtratie-druk (gemeten als filtratie-fractie) in de nieren hiermee samenhangt. Of dit een oorzaak of gevolg is, of stoffen die vrijkomen uit vetweefsel hiermee samenhangen, of dat de zout-hantering door de nieren op een andere manier afhankelijk is van overgewicht is nog onbekend en zal verder onderzocht moeten worden.

In **Hoofdstuk 3** gaan we in op zoutgevoeligheid van bloeddruk. Zoutgevoeligheid van bloeddruk is de stijging in bloeddruk na het veranderen van een zoutbeperkt naar een zoutverrijkt dieet. Lange termijn studies laten zien dat deze zoutgevoeligheid ook een risicofactor is op het ontwikkelen van hart- en vaatziektes. In dit hoofdstuk laten we zien dat er bij gezonde jonge mannen een

relatie is tussen zoutgevoeligheid van de bloeddruk en reactie van de nierdoorstroming (gemeten als ERPF) op infusie van angiotensine II. De reactie van de nierdoorstroming op geïnfundeerd angiotensine II (en het vervolgens teniet te doen door ACE-remmers) is een maat voor de activiteit van het renine-angiotensine aldosteron systeem (RAAS) in de nier. De activiteit van dit RAAS systeem in het nierweefsel heeft een matige overeenkomst met de activiteit in de bloedbaan; meten van de nieractiviteit is echter zeer lastig, maar kan op deze indirecte manier. Het RAAS heeft een belangrijke rol in de water- en zouthuishouding. Een grotere zoutgevoeligheid van bloeddruk lijkt echter niet samen te gaan met een grotere stijging van het ECV bij de overgang van zoutbeperkt naar zoutverrijkt dieet. Wel lijken de zoutgevoelige mensen 'overall' (zowel tijdens zoutbeperkt als zoutverrijkt dieet) een hoger ECV te hebben; waarbij dit verschil wegvalt als het RAAS geremd wordt (door ACE-remmers). Oftewel, zoutgevoeligheid van bloeddruk lijkt samen te gaan met een verhoogde activiteit van het RAAS in de nier en een verhoogd ECV. Twee kenmerken die een rol spelen in de relatie tussen zoutgevoeligheid en verhoogd risico op hart- en vaatziektes?

In **hoofdstuk 4** onderzoeken we wat er met het ECV gebeurt als gezonde mensen een nier afstaan. Na afstaan van een nier daalt de nierfunctie naar ongeveer 60%, oftewel de overgebleven nier gaat 20% harder werken. Het ECV daalt, wat in strijd is met de gedachte dat een lagere nierfunctie (minder werkend nierweefsel) gepaard gaat met een verhoging in het ECV. Wat de reden is voor de daling in ECV weten we niet. Het zou kunnen zijn dat het lichaam direct na verwijdering van een nier zich moet aanpassen aan de nieuwe situatie en dat verhogen van het terughalen van natrium via de tubulus hierin de stap is die het langst duurt, wat samen kan gaan in verlies van natrium en daarmee met een verlaging van het

ECV. Het zou ook kunnen zijn dat het setpunt tussen filtratie en terughalen via de tubulus veranderd is, met als resultaat verlaging van ECV.

In **hoofdstuk 5** leggen we een relatie tussen bloedarmoede (anemie, een verlaagd Hemoglobine gehalte) en een verhoogd ECV. Dit doen we in twee verschillende patiëntengroepen. Ten eerste (hoofdstuk 5A) bij patiënten met hartfalen en ten tweede (hoofdstuk 5B) bij patiënten die een niertransplantaat hebben ontvangen. Opvallend is dat de gevonden relaties onafhankelijk zijn van de nierfunctie, de daarmee samenhangende EPO productie en dat er geen tekenen bij lichamelijk onderzoek waren van overvulling. Als er onderzoek gedaan wordt naar bloedarmoede moet er dus ook rekening gehouden worden met het ECV. Een mogelijke relatie tussen het RAAS (en dus zout en volumehuishouding) en bloedarmoede is in dit licht een interessant punt om verder te onderzoeken.

Concluderend doen we in dit proefschrift een voorstel om zoveel mogelijk tijdens nierfunctie-metingen ook het ECV te meten. Dit maakt studies met betrekking tot zoutinname, de zout- en waterhuishouding en het ontstaan van hart- en vaatziektes mogelijk. In dit proefschrift maken we een begin met zulke studies, maar er zijn nog veel openliggende vragen en er zijn daardoor nog veel studies te verrichten.

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Toch wil ik ook enkele mensen, danwel groeperingen specifiek benoemen. Ik begin met de personen die zich vaak belangeloos hebben overgeleverd aan de door mij uitgevoerde experimenten: de proefpersonen. Zonder proefpersonen kunnen er nog zoveel briljante onderzoeken worden uitgedacht, maar dan komt de medische wetenschap geen stap verder. Jullie zijn de werkelijke motor van de medische vooruitgang, daarom: super bedankt jongens. Tevens veel dank aan patiënten uit het UMCG en uit het Twenteborg te Almelo die mee wilden werken: zonder jullie was het werk uit dit proefschrift niet mogelijk geweest.

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Enkele personen die ik wel specifiek wil noemen zijn: Menno, bedankt hoe jij me ooit hebt opgevangen en me hebt laten wennen en hebt ingewerkt bij de nefrologie. Titia, Femke en Mieneke: jullie wil ik bedanken als roommates, als collaborateurs voor verschillende studies, als klankbord voor frustraties, als bron van inspiratie en ga zo maar door. Toppunt was misschien wel het tripje naar Madrid, waarvan sommige anderen zich af hebben gevraagd hoe ik dat heb overleefd, maar met zulke topmeiden was dat geen probleem. Inge completeerde de topmeiden in Madrid: ook jij was zo'n topper. Arjan, Ferdau en Steef: jullie hebben misschien wel de meeste data gegenereerd die in dit proefschrift beschreven staat. Jullie zijn begonnen als student bij de nefrologie, waar ik ook een rol mocht spelen in jullie

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CURRICULUM VITAE & LIST OF PUBLICATIONS

Curriculum Vitae

Folkert Willem Visser was born on June 19, 1980 in Dokkum, The Netherlands. After he finished pre-university education (VWO) at the Menso Alting College in Hoogeveen, he started studying Biology, but switched after one year to study Medicine. During his internships Folkert already started doing research and in januari 2004, he was accepted by the Junior Scientific Masterclass to start a MD/PhD track at the department of Nephrology of the University Hospital in Groningen. In September 2005 Folkert graduated in Medicine to become a Medical Doctor. Until December 2007 Folkert was doing full-time research, which results in a defence of his thesis at December 8, 2008. From januari 2008 Folkert is working as Medical Doctor in traineeship to become an internist in the Deventer Hospital in Deventer.

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