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DISSERTATION

**Long-term studies on sodium balance
and their relevance to human health**

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1. Table of contents	2
2. Summary	
2.1 German Abstract	3
2.2 English Abstract	4
2.3 Introduction	5
2.4 Methods	7
2.5 Results	11
2.6 Discussion	16
2.7 References	19
3. Participation in each report	20
4. Affidavit	22
5. Selected Publications:	
5.1 <i>Agreement Between 24-Hour Salt Ingestion and Sodium Excretion in a Controlled Environment</i>	23
5.2 <i>Long-term space flight simulation reveals infradian rhythmicity in human Na(+) balance</i>	31
5.3 <i>Skin sodium measured with ²³Na MRI at 7.0</i>	40
6. Curriculum vitae	51
7. Publications	53
8. Expression of thanks	55

2. Summary of the Dissertation based on publications

2.1 Deutsche Zusammenfassung

Natrium-Homöostase soll einen Gleichgewichtszustand aufrechterhalten werden. Eine rasche Anpassung der Natriumausscheidung auf vermehrte oder verminderte Natriumzufuhr hält dabei den Natriumgehalt im Körper konstant. Diese Theorie basiert auf relativ kurzen Experimenten, in deren Verlauf massive Veränderungen in der Kochsalzzufuhr vorgenommen wurden. Wir konnten im Rahmen eines simulierten Marsfluges die Natrium-Homöostase unter konstanten Bedingungen von drei Kochsalzmengen (6, 9 und 12 Gramm/Tag) über Wochen an 10 normalen männlichen Probanden untersuchen. Alle anderen Diätbestandteile wurden über diese Zeit konstant gehalten. Wir entdeckten bei konstanter Natriumzufuhr wöchentliche (circaseptane) Rhythmuszyklen in der Natriumausscheidung, die invers mit Aldosteronausscheidung und direkt mit Kortisolausscheidung kreuzkorrelierten. Der Gesamtkörpergehalt an Natrium schwankte mit noch langfristiger infradianer Rhythmik (Periodendauer >30 Tage). Diese Schwankungen des Gesamtkörperratriums traten ohne parallele Veränderungen von Gesamtkörperwasser, Körpergewicht oder Blutdruck auf. Wir testeten auch den prädiktiven Wert von 24-Stunden-Sammelurinproben zur Erfassung der täglichen Salzzufuhr. Aufgrund der infradianen Ausscheidungsrythmen erfasst nur jede zweite 24-Stunden-Sammelurinprobe die tatsächliche Natriumaufnahme innerhalb eines 3-g Prädiktionsintervalls korrekt. Unsere Befunde zur Natriumspeicherung beim Menschen stimmen mit unseren Tierversuchsergebnissen überein. Auch passen sie zu unseren Natrium-Magnetresonanztomographischen Untersuchungen, die wir in Berlin auf 7-Tesla haben erweitern können. Unsere Befunde könnten jetzige Überlegungen hinsichtlich Kochsalzzufuhr, Bluthochdruck und Endorganschaden beeinflussen und zu besseren therapeutischen Strategien führen.

2.2 Abstract

The steady-state theory of sodium homeostasis for maintenance of constant extracellular solute and fluid concentrations suggests that the body sodium (Na^+) content is maintained steady by rapid adjustment of renal excretion to variable intakes. This theory derives from studies with short-term exposure to dietary extremes. We recently performed the reverse experiment in that we fixed sodium intake for weeks at three levels of sodium intake (salt 6 g/day, 9 g/day, 12 g/day) and collected all urine made. We capitalized on a simulated flight to Mars, in which we could control intake and output of all nutrients for months. We found weekly (circaseptan) patterns in sodium excretion that were inversely cross-correlated with aldosterone and directly to cortisol. Total-body sodium was not dependent on sodium intake either but instead exhibited far longer (\geq monthly) infradian rhythms independent of extracellular water, body weight, or blood pressure. We also tested the utility of 24 hour urinary sample to predict the actual salt intake. Due to inherent changing patterns in Na^+ of retention and excretion and alterations in the body Na^+ content that are uncoupled from body fluid and blood pressure homeostasis, every second 24 hour urinary sample failed to predict salt intake within a 3 g range of real intake. Our findings are consistent with our ideas on tissue sodium storage and its regulation that we developed on the basis of animal research. We are implementing sodium magnetic resonance imaging to pursue open questions on sodium balance in patients. In Berlin, we could expand this technique to a 7-Tesla platform with greater resolution. The findings may recast thinking about how homeostasis of internal environment composition is achieved and become beneficial to diagnostic and therapeutic strategies, for instance in hypertension and target-organ damage.

2.3 Introduction

Sodium (as sodium chloride or salt) is essential for life. The cation is pivotal to the body's volume and water regulation, as well as numerous additional vital processes. However, since dietary sodium intake has been linked to high blood pressure and cardiovascular disease, almost all public-health recommendations addressing sodium intake call for a dietary reduction. Most authorities believe that sodium metabolism is well worked out and little further research is necessary on the issue. The outcomes of the studies reported in the current thesis underscore the fact that there is still much to be learned about sodium and its ultimate influence on health.

Sodium balance has been studied in humans for over 100 years but almost always short-term, generally a week to 10 days (1, 2). The findings indicate that ingested sodium is excreted in the urine when sweating is not excessive and diarrhea is not present. The assumption has been accepted that when sodium intake is increased to a higher level, urine sodium excretion increases accordingly until balance is re-attained, a process that takes several days. A concomitant increase in total-body sodium (TBNa) occurs. When sodium intake is decreased, the reverse process takes place. Sodium is an extracellular cation with osmotic activity and its amount in the body (total-body sodium) determines the extracellular fluid volume (two compartment model). Aside from relatively small amounts stored in bone, sodium accretion results in weight gain from extracellular volume retention and sodium depletion causes volume losses also accompanied by weight loss.

Titze et al performed an earlier space-flight simulation balance study that cast doubt on these conventional ideas (3). In this study, he could not control sodium intake. However, he precisely knew daily sodium intake of each subject and collected 24 hour (24 h) urine samples daily to determine excretion. He found that sodium was retained in the body in some of the subjects over weeks in substantial amounts. However, this sodium retention was not accompanied *pari passu* by weight gain. The finding of sodium gain without weight gain is in contradiction to the widely accepted theory that changes in total-body sodium levels are accompanied by changes in extracellular volume. Titze et al suggested the existence of a sodium reservoir with the ability to store significant amounts of sodium in an osmotically inactive form (4). This reservoir could be located in bone, muscle, dense connective tissue, or cartilage.

In animal experiments Titze et al found that it was in fact the skin, that appears to be a major reservoir of sodium (5). Storage of sodium that carries a positive charge is associated with changes in negatively charged proteoglycans in the skin, which

resembles a kidney-like countercurrent system (4). We observed that immune cells outfitted with an osmosensor tightly regulate this third-space sodium storage depot (three compartment model) (5). These discrepancies with the “text-book” knowledge on sodium physiology made us pursue our experiments on the influence of salt in the living organism.

Because public policy apparently is based on such data, a critical re-evaluation of all what we know about sodium is needed.

The centerpiece of this thesis report concerns long-term sodium balance studies in humans in a project simulating a flight to Mars, termed Mars500. We varied salt intake between 6 and 12 g/d, all food intake ingredients were precisely determined in advance, and all urine made was collected in two Mars500 studies of 105 and 205 days duration, respectively. The intake of any-and-all other dietary constituents was maintained constant and only salt ingestion varied step-wise. We observed substantial variability in urinary sodium excretion (UNaV) and in total-body sodium despite constant salt intake and hypothesized that 24 h urinary samples are not reliable estimates of salt intake across the range tested. We found that a single, accurately collected, 24 h urine sample was not suitable for determining a 3 g difference in the subjects’ daily salt intake. Our findings suggest that estimating individual salt intake from urine collections could be less precise than supposed.

To overcome this obstacle, Titze et al developed a ^{23}Na magnetic resonance ($^{23}\text{NaMRI}$) technology at 3.0 Tesla, which allows to visualize and quantify sodium reservoirs in vivo in human tissues (7). We have adapted this method to 7.0 Tesla and transferred it to Berlin, where we have performed our first feasibility study on humans.

Our findings could find numerous clinical applications. We believe that the investigation of human sodium balance can benefit from state-of-the-art $^{23}\text{NaMRI}$ tools and should not be restricted solely to serum Na^+ measurements and 24 h urine collections.

2.4 Methods

Salt Balance Study Design

In the framework of Russian-German collaboration, aerospace scientists performed a project termed Mars500 in Moscow that simulated a flight to Mars. It consisted of two separate studies lasting 105 (pilot experiment) and 520 days (real- duration flight to Mars), or in other words Mars105 (105 Days study) and Mars520 (520 Days study).

These isolation studies were approved by several ethical boards of the Russian Federation and European Space Agency authorities. All studies were done as outlined in the Declaration of Helsinki.

12 young male healthy male volunteers provided written informed consent after due approval to spend 105 and 520 days in an enclosed habitat with a volume of 500 m³, the Mars500 simulator, consisting of hermetically sealed interconnecting modules (Figure 1).

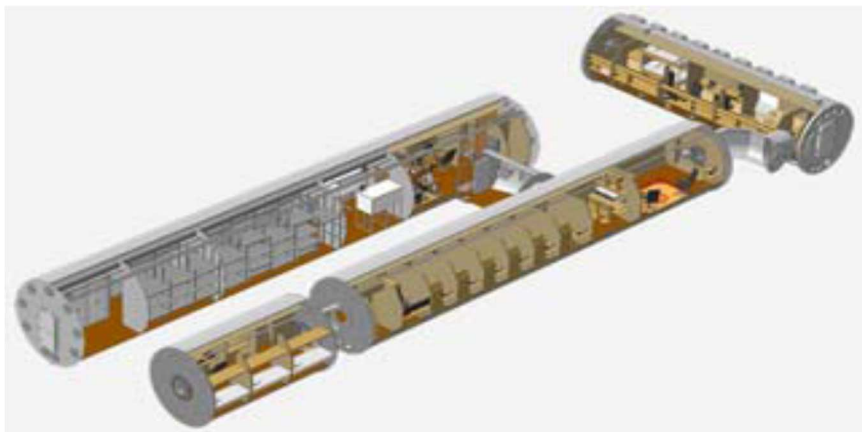


Figure 1. The Mars500 simulator.

(source: <http://mars500.imbp.ru/en/nek.html>). For general information see also <http://en.wikipedia.org/wiki/MARS-500>.

The crews lived and worked like cosmonauts on the international space station, locked up from the outside world for the entire course of the studies. They conducted scientific experiments and technical daily routines.

Environmental factors in the simulation facility were maintained constant. Microgravity and space radiation were not simulated. In our view, it was a perfect “metabolic ward for humans”, an experimental platform to conduct, for the first time ever, accurate long-term salt-and-water balance studies on humans.

Diets and Ultra-long Balance Approach

Nutritional intervention was performed during the complete Mars105 study and the first 205 days of Mars520 study. Various European food producers provided more than 200 different food items with pre-analyzed nutrient content for our nutritional intervention. Using the PRODI software, we calculated and individualized daily menu plans for each subject. The goal of the dietary intervention was to maintain all nutrients on a constant level throughout the studies; while only sodium ingestion was varied. The salt reduction was performed step-wise from 12 g salt per day over 9 g to 6 g salt per day in the 105 Days study, with a re-exposition of the subjects to 12 g salt per day after the 6 g salt / day depletion phase in 520 Days study (Figure 2).

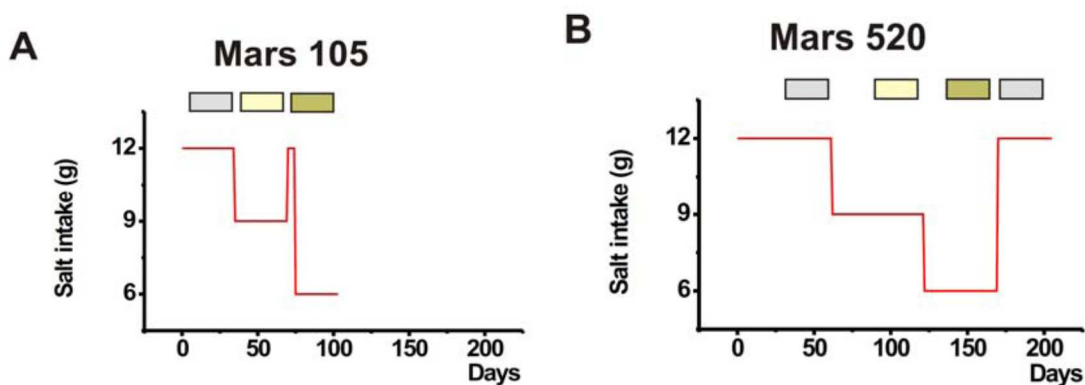


Figure 2. Design of the Mars105 (Panel A) and Mars520 (Panel B) salt balance studies. We decreased average daily salt intake stepwise from 12 g (grey) over 9 g (light brown) to 6 g (olive) with a re-exposition to a 12 g salt intake in Mars520. A short 12 g phase in the end of the 9 g phase in Mars105 was done on request of other researchers and is irrelevant to this study

We maintained each salt intake level constant for at least 29 days. The subjects collected all their urine made for a 24 h period on a day-to-day basis.

Each crewmember was handed a booklet with his daily individualized menu plans that served as protocols to document the actual food intake. During the studies subjects had free access to water.

Drop-out Criteria

We defined inadequate caloric intake and lack of accuracy in sodium balance as dropout criteria. Accuracy was evaluated by individual average daily urinary sodium excretion (UNaV) as percentage of actual daily Na⁺ intake. We excluded subjects from analysis when their weekly average UNaV was repeatedly less than 80% of sodium intake or when the subjects did not adhere to our daily menu plans. Only this strict focus on

experimental accuracy allowed us to implement a long-term balance approach. Because two subjects did not comply with these criteria, we had to exclude them from further analysis.

Specimen Collection

The subjects measured their body weight each morning after voiding the bladder. 24 h urine volumes were determined gravimetrically and four aliquots of 10 mL were transferred into test tubes to be frozen for later analysis. Morning blood pressure and heart rate were determined daily as triple measurements immediately after arousal. For measurements, subjects were in a quiet seated position and used (Medisana MTX (51080) or MTD ((51145);Neuss, Germany), upper arm blood pressure measuring devices with a cuff on the upper arm in 2 min intervals between measurements. On a monthly basis blood drawing was performed after arousal before breakfast, aliquots were frozen for later analysis.

Biochemical Methods

Sodium and potassium concentrations in each 24 h urine sample were measured by flame photometry (Eppendorff, Hamburg, Germany).

Urinary hormone excretion was measured using liquid chromatography and mass spectrometry (API 4000TM, Applied Biosystems, MDS Sciex, Foster City, USA).

Urinary creatinine was measured by isotope dilution analysis (Sigma; St. Louis, MO).

Analytical methods

Daily Na⁺ balances were calculated from the difference between daily Na⁺ intake (Na⁺ in) and urinary Na⁺ excretion (UNaV):

$$\text{Na}^+ \text{ balance Day } n \text{ (mmol / d)} = \text{Na}^+ \text{in Day } x \text{ n (mmol / d)} - \text{UNaV Day } x \text{ n (mmol / d)}$$

We then calculated the subjects' change in body Na⁺ content (Δ TBNa) from their daily Na⁺ balances:

$$\Delta \text{TBNa Day } n \text{ (mmol / d)} = \text{Na}^+ \text{ balance Day } n-1 \text{ (mmol / d)} + \text{Na}^+ \text{ balance Day } n \text{ (mmol / d)}$$

To correct ΔTBNa for extrarenal Na^+ loss (in average 12.7 mmol/d), the calculated ΔTBNa time series were detrended after non-linear best-fit analysis.

Sodium Magnetic Resonance Study ($^{23}\text{NaMRI}$)

In conventional magnetic resonance (MRI) scanners, hydrogen atoms are excited. However, any atom with an odd atomic number can be excited by magnetic resonance. Sodium has an atomic number 11, so the atom is susceptible to magnetic resonance. This physical feature has been used by our team to develop a $^{23}\text{NaCoil}$, in order to non-invasively measure sodium in human tissues. The coil has been adapted to the 7-Tesla MRI scanner in Berlin, in such a way that both proton and sodium images were possible to obtain. Proton images served for anatomical orientation and reproducible positioning of the human calf independent of the number of measurements.

In this study we focused on human skin to capture the sodium-storage depot. We performed an in vivo feasibility study on healthy male adult volunteers. Skin sodium content was determined using external agarose standards covering a physiological range of sodium concentrations. To ensure the intra-subject reproducibility, each volunteer was examined three to five times with each session including a 5-min walk in between the sessions. All images were processed with the ImageJ software package (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

For statistical analysis of infradian rhythmicity in human Na^+ balance we performed:

- Comparison of means either by paired t-test, or by multivariate analysis to test for the interaction of multiple effectors on various parameters (IBM SPSS Statistics Version 20, SSPS Inc.; Chicago, Illinois, USA).
- Time series analysis to test for rhythmical changing patterns by power spectral density estimation using a periodogram with a rectangular window function (Matlab (MathWorks Inc.; Natick, Massachusetts, USA)). We also used IBM SPSS Statistics Version 20 to detect time-shifted interrelations between rhythmical components within the time series by crosscorrelation analysis.

To statistically check for the difference between true (=recorded) sodium intake and urinary sodium excretion, we applied:

— Bland–Altman plots. We considered a ± 25 mmol sodium (corresponding to ± 1.5 g salt) deviation from the mean difference between the recorded sodium intake and renal sodium excretion as true positive urine sample (correct prediction of salt intake). UNaV samples, which were outside this range, were considered as true negative (misclassification of salt intake).

To test statistically the effect of salt intake on UNaV measures we used:

— Multilevel modeling with linear mixed models. We tested a random intercept versus a random intercept–slope model and selected the best-fit model. A P value < 0.05 was considered statistically significant. Data analysis was performed with IBM/SPSS software.

2.5 Results

The Mars500 human balance studies

In earlier human balance studies, subjects were exposed to varying (commonly extreme) sodium intakes and the effects on balance, total-body sodium, and daily excretion were investigated (8). As soon as steady state appeared to be achieved, the studies were terminated. In the Mars500 studies, we aimed to test the reverse experimental approach, namely the response of the body to constant salt intake at three different levels. Thus, the Mars500 balance studies were not only designed to examine sodium balance in response to abrupt increases in dietary salt (traditional step change approach), but also to investigate sodium metabolism in response to ultra-long-term constant (ultra-long-term constancy) salt intake (9). Such experiments cannot be performed under daily life conditions, because precisely controlled food intake and specimen collection by each test subject over months would be necessary.

The subjects' daily menus consisted of 27 279 individual servings, of which 83.0% were completely consumed, 16.5% completely rejected, and 0.5% incompletely consumed. As the subjects accurately indicated days and meals which they had completely rejected, we adjusted our data set and analyzed whether average recorded Na^+ intake matched average urinary output at each dietary salt level. Urinary recovery of dietary salt was 92% of recorded intake over the entire period of the studies, indicating long-term steady-state sodium balance. Moreover, as another proof of excellent adherence to the sodium balance study, we found out that from the 14.8 kg of dietary salt

that were loaded into the Mars500 simulator within the processed foods for both studies, the 10 subjects recovered 13.7 kg of salt (92%) in their urine.

Urinary creatinine excretion was constant at all salt intakes (not shown). Less than 0.1% of our 1646 urine samples showed creatinine excretion below 0.8 g/d, or above 2.4 g/d. We therefore used all samples for analysis.

The design of the Mars500 sodium balance studies allowed us to address the question of what happens to sodium balance when sodium intake is constant. A dramatic observation in the Mars500 studies was the fact that although salt intake was maintained constant for days on end at each level of intake, the 24 h daily urinary sodium excretion

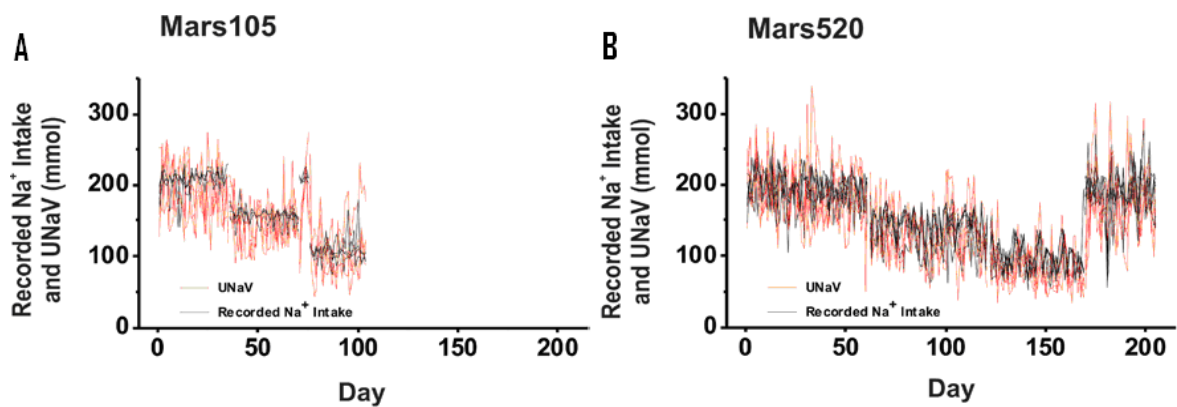


Figure 3. Recorded sodium intake (mmol / d; black) and 24 h urinary sodium excretion (mmol / d; red) of all subjects during the 105 Days (Panel A) and the 205 Days (Panel B) studies.

fluctuated markedly around the mean sodium intake (Figure 3). In the 105 days study sodium intake was clamped more rigidly than in the 205 days study, which resulted in a more pronounced urinary Na^+ oscillation pattern around the constant sodium intake than in the Mars520 study. The daily fluctuations in UNaV were profound, even at the lowest level of salt intake so that any given UNaV did not reflect salt intake.

To find out regulatory mechanisms of such variability, we measured urinary aldosterone and urinary cortisol excretion. We found that at constant salt intake, daily

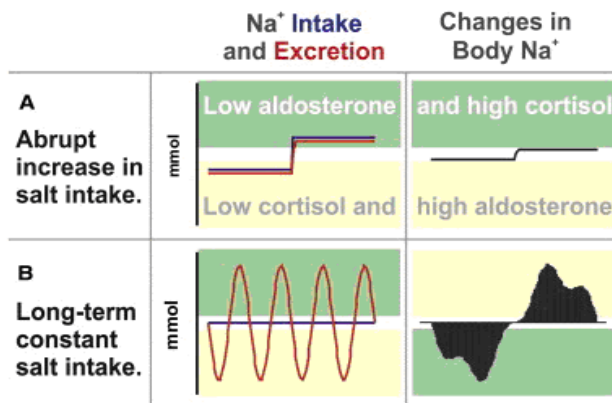


Figure 4. Na⁺ intake and excretion, and changes in total-body sodium in response to abrupt increase in salt intake or long-term constant salt intake. Panel A: at low (6 g/day salt) sodium intake (represented by the yellow field), daily aldosterone excretion was relatively high, while daily cortisol and cortisone excretions were low. When sodium intake was raised abruptly (from 6 g/day salt to 9 g/day or 12 g/day), urinary aldosterone excretion decreased, while cortisol excretion increased. Panel B: when salt intake is constant, a daily fluctuation in U_{Na} occurred that cross-correlated inversely with aldosterone excretion and directly with cortisol excretion. We also observed that changes in total-body sodium had their own even far longer infradian rhythms that were independent of salt intake and also blood pressure (not shown).

sodium excretion exhibited aldosterone-dependent, weekly (circaseptan) rhythms, resulting in periodic sodium storage. Changes in TBNa ($\pm 200\text{--}400$ mmol) exhibited longer infradian rhythm periods (about monthly and longer period lengths) without parallel changes in body weight (not shown) and extracellular water (not shown) and were inversely related to urinary aldosterone excretion and directly to urinary cortisol, suggesting rhythmic hormonal control (9). Our findings define rhythmic sodium excretory and retention patterns independent of blood pressure (not shown), which occur independent of salt intake (Figure 4).

Our finding that Na⁺ intake and urinary Na⁺ excretion are not coupled within 24 hours and that and body Na⁺ in the body is not necessarily a function of Na⁺ intake encouraged us to re-analyze the data from the Mars500 simulation studies for another purpose.

The 24 h urinary Na⁺ excretion is fundamental in clinical medicine and research. We rely on 24 h U_{NaV} as a gold standard for assessing daily Na⁺ intake. Our trust underlies a belief that Na⁺ content in the urine is related to any given intake, which requires a steady-state between Na⁺ intake and Na⁺ excretion every day.

Our previous findings in the Mars500 subjects and our previous animal data cast doubt

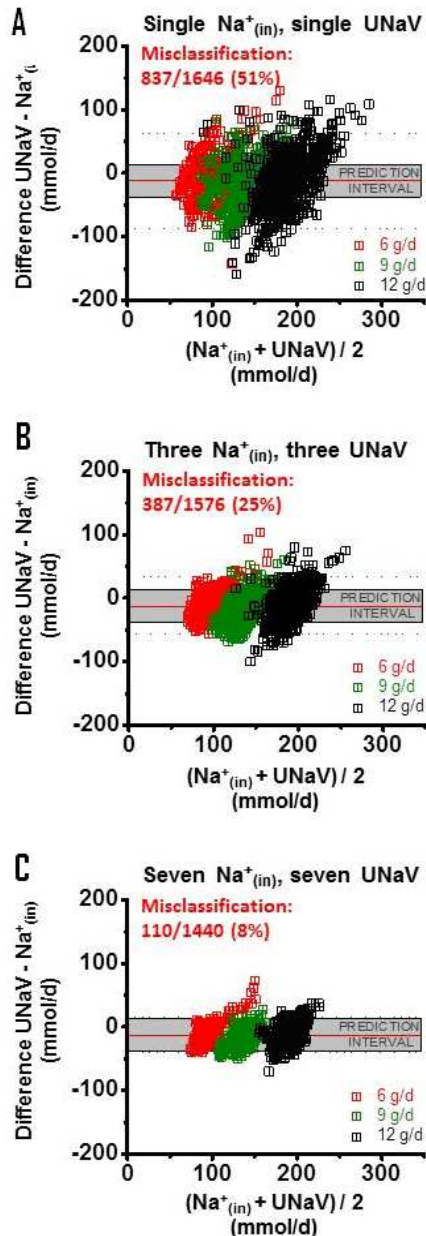


Figure 5. Analysis of agreement between sodium intake and excretion in Bland-Altman-Plots. Panel A: Bland-Altman plot to test the agreement between single recorded 24 h Na^+ intakes and single 24 h UNaV. The prediction interval to accurately predict Na^+ intake by UNaV is defined as ± 25 mmol/d of the mean difference between sodium intake and UNaV. Panel B: Analysis of agreement between three consecutively recorded Na^+ intakes and three UNaV collections. Multiple collections reduce the variability and thereby improve the predictive value of UNaV. Panel C: Seven consecutive collections reduce the number of misclassifications of UNaV to less than 10%. Red solid line indicates regression line; the dotted line: upper and lower confidence level.

as to whether or not body Na^+ content is really maintained constant within such narrow limits (3, 10). We analyzed the predictive value of a single 24 h urine sample to accurately estimate real salt intake (Figure 5). We compared true sodium intake with measured 24 h sodium excretion in the urine. Because current computerized models often calculate the projected effect of a 3-gram reduction in salt intake on cardiovascular outcome, we tested the accuracy of 24 h UNaV to correctly estimate real salt intake within a 3-gram (50 mmol) range.

Accuracy of each individual UNaV for correct assessment of daily salt intake was performed by definition of true positives of salt intake. We considered a ± 25 mmol (corresponding to ± 1.5 gram salt) deviation from the mean difference between the recorded sodium intake and renal sodium excretion as true positive urine sample (correct prediction of salt intake). UNaV samples, which

were outside this range, were considered as true negative (misclassification of salt intake).

Because of the biological variability in UNaV, only every other daily urine sample correctly classified a 3-g difference in salt intake (51%). By increasing the observations to 3 consecutive 24 h collections and sodium intakes, classification accuracy improved to 75%. Collecting seven consecutive 24 h urines and sodium intake samples improved classification accuracy to 92% (Figure 5).

We conclude that single 24 h urine collections at intakes ranging from 6 to 12 g salt per day were not suitable to detect a 3-g difference in any individual salt intake. Repeated measurements of 24 h UNaV improve precision. This knowledge could be relevant to patient care and the conduct of intervention trials.

Sodium Magnetic Resonance Study ($^{23}\text{NaMRI}$) at 7.0 Tesla

Clinical investigation of body Na^+ has traditionally been indirect, relying on estimates of extracellular sodium content on the basis of serum Na^+ concentration. Sodium concentration in serum is maintained within narrow limits. Normal serum sodium levels lie between approximately 135 and 145 mmol / L. It is generally believed that after consumption of high amounts of salt, sodium, as an osmotic agent, consequently draws water into the vascular system, which leads to an increase in blood pressure. The mechanism of pressure natriuresis “repairs” the imbalance by forcing excretion of excess sodium out of the body. Traditionally, it has been believed that sodium concentration in the interstitial tissue is equal to that in plasma.

The lack of a simple, non-invasive technology to assess sodium in the human body did not give us an opportunity to detect hidden Na^+ reservoirs in the volunteers of the Mars500 studies, which could be responsible for the rhythmical retention and release of this cation.

Our animal experiments clearly suggest that interstitial sodium is not a direct reflection of plasma sodium concentration (10), that different compartments of the body can serve as a reservoir of water-free sodium storage (4), the main of which appears to be the skin and muscle (10), which are practically the largest organs in the body.

Titze et al therefore designed a magnetic resonance imaging (^{23}Na MRI) method at 3.0 Tesla, that “visualizes” Na^+ rather than H^+ as in conventional MRI (7). The resolution of 3.0 Tesla MRI is not perfect enough to precisely detect Na^+ in the skin, which is only 2-3 mm thick. For this reason, we have adapted this method to 7.0 Tesla MRI and transferred this method to Berlin. We have developed a sodium radio-frequency (RF) coil suitable for a 7.0 Tesla device to measure sodium in the human skin.

For our feasibility study, we recruited 17 healthy male adult volunteers, aged 20-79 years and after due approval of the local ethical committee and obtaining of written informed consents, we performed our in vivo skin sodium measurements on humans. The volunteer study revealed inter-volunteer differences in skin Na^+ content. For example, the

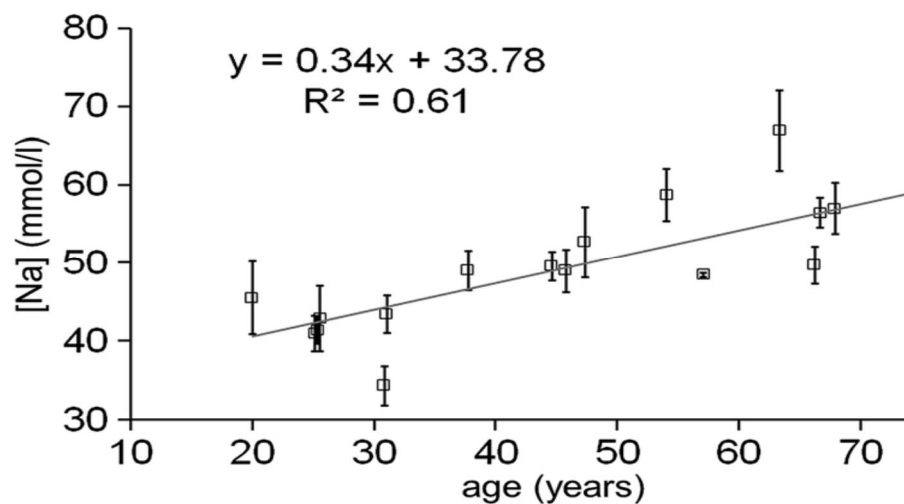


Figure 6. Human skin Na^+ content versus age obtained from ^{23}Na MRI at 7.0 Tesla. The preliminary in vivo ^{23}Na MRI data (n = 17, men) suggest an increase in skin Na^+ content with age of 0.34 ± 0.07 mmol / (L year).

skin Na^+ content of a 25-year-old man was found to be 41 ± 2 mmol/L. In comparison, a 67-year-old man showed a skin Na^+ content which was approximately 1.4-fold higher (57 ± 3 mmol/L). Our ^{23}Na MRI in vivo data

suggested an age-dependent increase in the skin Na^+ content as illustrated in Figure 6.

2.6 Discussion

In our Mars500 salt balance studies we intended to test whether salt intake and daily UNaV are related, whether total-body Na^+ is necessarily a function of salt intake and whether 24 h urinary salt content is an adequate measure of salt intake. We applied an experimental approach, in which we tested the response of the body to long-term constant salt intake. Thus, the Mars500 balance studies were not only designed to examine Na^+ balance in response to abrupt increases in dietary salt (traditional step change approach) but also to investigate Na^+ metabolism in response to ultra-long-term constant salt intake

(ultra-long-term constancy). Strictly controlled enclosed habitat (thermoconstancy, little variable atmospheric pressure and humidity), identical daily routines with restricted information from the outside world, compliance to the experimental protocols allowed us to avoid many confounders, which exist in real life. Such experiments cannot be performed under daily life conditions, because precisely controlled food intake monitoring and daily specimen collection by each test subject over months would be necessary.

We found that UNaV in humans was characterized by weekly (circaseptan) rhythmic change patterns, which were not related to salt intake but instead were paralleled by inversely changing patterns in urinary aldosterone and directly changing patterns in urinary cortisol. Rhythmic retention and excretion of Na⁺ resulted in additional long-term variability of total-body Na⁺ that was not related to body weight, blood pressure changes, or salt intake, suggesting that Na⁺ was rhythmically stored and released from the body without parallel changes in water content. Total-body sodium storage displayed even longer, monthly (circaseptan) rhythms. Our studies raise questions, whether endogenous free-running weekly and monthly circadian sodium cycles exist in humans and whether or not it is true that body Na⁺ content is steadily maintained within very narrow limits via rapid urinary excretion of dietary salt. Our findings suggest that current physiological concepts on Na⁺ metabolism may underestimate the time frame and variability by which such sodium steady state can be really achieved.

The wide degree of variability in UNaV at constant salt intake levels that we observed in our studies also questioned the widely accepted notion that a single 24 h UNaV sample is a valid measure of daily salt intake in humans.

The collection of 24 h urine specimens is currently the “gold standard” of estimating sodium (salt) intake. We tested the utility of this gold standard within the range of 6 and 12 g / day salt intake. The amount of 6 g salt / day is the World Health recommendation for the general population, 9 g salt / day is the level that an international study named INTERSALT found that most human populations ingest (11), and 12 g / day is the level that experts claim the society, including the Germans, actually eat. In our Mars500 studies every second single 24 h UNaV failed to detect a 3-g difference in sodium intake. Multiple collections improved precision. Luft et al (12) made similar observations to those reported here in humans living under daily-life conditions. Their subjects varied their salt intake daily around a given mean intake, as is likely the case in real life, whereas in this study salt intake was more rigorously fixed. They found that 9 collections were optimal to predict salt intake, and that nocturnal (first-morning-voided urines) collections were of no value.

Our Mars500 studies have clear-cut clinical implications. If the “gold standard” fails to provide credible information, a more reliable method of estimating salt intake and salt content in the body is necessary. Our challenge was to develop a tool to actually “see” the sodium in the body. $^{23}\text{NaMRI}$ has apparently filled that gap. The method was first established for 3.0 Tesla MRI scanners and has shown that the sodium-storage depot can be affected by treatment in patients with primary aldosteronism (13), the depot is affected by age (14), by hemodialysis (15), and the presence of hypertension (16). Recent work in Berlin here at the Experimental Clinical Research Center (ECRC) suggests that at 7.0 Tesla, even greater resolution and acquisition of more precise data is possible (14).

A continuous movement from human subjects (bedside) to bench (animal-related) and back to human subjects characterizes our work. This process has been in place for over a century but has recently been termed “translational” research.

Whether or not our data speak to the public health issue about recommended salt intake is unclear.

However, we believe that good hypotheses should be rigorously tested. Our long-term Mars500 balance study and our $^{23}\text{NaMRI}$ evidence may provide new evidence on sodium balance and its relevance to human health.

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3. Candidate's participation in these studies

Natalia Rakova played the following role in the publications listed below:

●→ Publikation 1: **Rakova N**, Jüttner K, Dahlmann A, Schröder A, Linz P, Kopp C, Rauh M, Goller U, Beck L, Agureev A, Vassilieva G, Lenkova L, Johannes B, Wabel P, Moissl U, Vienken J, Gerzer R, Eckardt KU, Müller DN, Kirsch K, Morukov B, Luft FC, Titze J. Long-term space flight simulation reveals infradian rhythmicity in human Na(+) balance. Cell Metab. 2013 Jan 8;17(1):125-31.

Natalia Rakova participated in the design of the study, in obtaining ethical approval from Russian regulatory authorities, saw to formal contractual issues. She was in charge of acquisition of the necessary food items in Germany, organizing their transport to Moscow (to the site of the Mars520 project), including customs clearance and compliance with formal issues. She transferred all scientific equipment to Russia, purchased consumables for the studies and loaded the simulation facility (Mars500 ground-based aircraft) with materials necessary for both long-term balance studies prior to their start. Apart from logistical tasks, Natalia Rakova trained the subjects on the protocols of the studies, including managing the equipment, data and biological material collection. A challenging part of her pre-study assignment was to establish a trust-based contact with the subjects, spark their interest and motivation in the balance studies, as it was one of the crucial points to ensure compliancy. She spent the entire time on-site in Moscow as part of the "ground control team", contacted the subjects on a regular basis and saw to their needs. She transferred more than 6 000 frozen urinary samples and over 50 frozen blood aliquotes to Germany, processed the samples, and performed the electrolyte measurements. She participated in data analysis and manuscript preparation.

●→ Publikation 2: Lerchl K, **Rakova N**, Dahlmann A, Rauh M, Goller U, Basner M, Dinges

DF, Beck L, Agureev A, Larina I, Baranov V, Morukov B, Eckardt KU, Vassilieva G, Wabel P, Vienken J, Kirsch K, Johannes B, Krannich A, Luft FC, Titze J. Agreement Between 24-Hour Salt Ingestion and Sodium Excretion in a Controlled Environment. Hypertension 2015 Oct;66(4):850-7.

She participated in data analysis and manuscript preparation.

●→ Publikation 3: Linz P, Santoro D, Renz W, Rieger J, Ruehle A, Ruff J, Deimling M, **Rakova N**, Muller DN, Luft FC, Titze J, Niendorf T. Skin sodium measured with ²³Na MRI at 7.0 T. NMR Biomed. 2015 Jan; 28(1):54-62.

Natalia Rakova participated in adapting this technology to the 7-Tesla MRI scanner in Berlin. She helped recruit and examine the subjects in the ECRC. She guided the subjects through their scans in the MRI unit and was responsible for their welfare as physician. Natalia Rakova analyzed MRI images and participated in manuscript preparation

Signature, date, and stamp of thesis advisor and supervisor.

Signature of PhD candidate and date

4. Affidavit

I, Natalia Rakova, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic „Long-term studies on sodium balance and their relevance to human health“ I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date

Signature

5. Selected Publications

5.1 Publication 1

(IF 2013 - 16,747)

Rakova N, Jüttner K, Dahlmann A, Schröder A, Linz P, Kopp C, Rauh M, Goller U, Beck L, Agureev A, Vassilieva G, Lenkova L, Johannes B, Wabel P, Moissl U, Vienken J, Gerzer R, Eckardt KU, Müller DN, Kirsch K, Morukov B, Luft FC, Titze J. Long-term space flight simulation reveals infradian rhythmicity in human Na(+) balance. *Cell Metab.* 2013 Jan 8;17(1):125-31.

<http://dx.doi.org/10.1016/j.cmet.2012.11.013>

5.2 Publication 2

(IF 2014 / 2015 - 6.480)

Lerchl K, **Rakova N**, Dahlmann A, Rauh M, Goller U, Basner M, Dinges DF, Beck L, Agureev A, Larina I, Baranov V, Morukov B, Eckardt KU, Vassilieva G, Wabel P, Vienken J, Kirsch K, Johannes B, Krannich A, Luft FC, Titze J. Agreement Between 24-Hour Salt Ingestion and Sodium Excretion in a Controlled Environment. *Hypertension* 2015 Oct;66(4):850-7.

<http://dx.doi.org/10.1161/HYPERTENSIONAHA.115.05851>

5.3 Publication 3

(IF 2014 / 2015 - 3.044)

Linz P, Santoro D, Renz W, Rieger J, Rühle A, Ruff J, Deimling M, **Rakova N**, Müller DN, Luft FC, Titze J, Niendorf T. Skin sodium measured with ²³Na MRI at 7.0 T. NMR Biomed. 2015 Jan;28(1):54-62.

<http://dx.doi.org/10.1002/nbm.3224>

6. Curriculum vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

7. Publications

1. Jens Titze, **Natalia Rakova**, Christoph Kopp, Anke Dahlmann, Jonathan Jantsch, Friedrich C. Luft. Balancing wobbles in the body sodium. 2015 Sep 25. pii: gfv343. [Epub ahead of print]
2. Lerchl K*, **Rakova N***, Dahlmann A, Rauh M, Goller U, Basner M, Dinges DF, Beck L, Agureev , Larina I, Baranov V, Morukova, Eckardt KU, Vassilieva G, Wabel P, Vienken J, Kirsch K, Johannes B, Krannich A, Luft FC, Titze J. Agreement Between 24-Hour Salt Ingestion and Sodium Excretion in a Controlled Environment. *Hypertension* 2015 Oct;66(4):850-7.
3. Jantsch J, Schatz V, Friedrich D, Schröder A, Kopp C, Siegert I, Maronna A, Wendelborn D, Linz P, Binger KJ, Gebhardt M, Heinig M, Neubert P, Fischer F, Teufel S, David JP, Neufert C, Cavallaro A, **Rakova N**, Küper C, Beck FX, Neuhofer W, Muller DN, Schuler G, Uder M, Bogdan C, Luft FC, Titze J. Cutaneous na(+) storage strengthens the antimicrobial barrier function of the skin and boosts macrophage-driven host defense. *Cell Metab.* 2015 Mar 3;21(3):493-501.
4. Linz P, Santoro D, Renz W, Rieger J, Ruehle A, Ruff J, Deimling M, **Rakova N**, Muller DN, Luft FC, Titze J, Niendorf T. Skin sodium measured with ²³Na MRI at 7.0 T. *NMR Biomed.* 2015 Jan;28(1):54-62.
5. Titze J, Dahlmann A, Lerchl K, Kopp C, **Rakova N**, Schröder A, Luft FC. Spooky sodium balance. *Kidney Int.* 2014;85(4):759-67.
6. **Rakova N**, Muller DN, Staff AC, Luft FC, Dechend R. Novel ideas about salt, blood pressure, and pregnancy. *J Reprod Immunol.* 2014;101-102:135-9.
7. Wiig H, Schröder A, Neuhofer W, Jantsch J, Kopp C, Karlsen TV, Boschmann M, Goss J, Bry M, **Rakova N**, Dahlmann A, Brenner S, Tenstad O, Nurmi H, Mervaala E, Wagner H, Beck FX, Müller DN, Kerjaschki D, Luft FC, Harrison DG, Alitalo K, Titze J. Immune cells control skin lymphatic electrolyte homeostasis and blood pressure. *J Clin Invest.* 2013;123(7):2803-15.

8. **Rakova N***, Juttner K*, Dahlmann A, Schröder A, Linz P, Kopp C, Rauh M, Goller U, Beck L, Agureev A, Vassilieva G, Lenkova L, Johannes B, Wabel P, Moissi U, Vienken J, Gerzer R, Eckardt KU, Müller DN, Kirsch K, Morukov B, Luft FC, Titze J. Long-term space flight simulation reveals infradian rhythmicity in human Na(+) balance. *Cell Metab.* 2013;17:125-31.
9. Novikova N, De Boever P, Poddubko S, Deshevaya E, Polikarpov N, **Rakova N**, Coninx I, Mergeay M. Survey of environmental biocontamination on board the International Space Station. *Res Microbiol.* 2006;157(1):5-12.

* These authors contributed equally to this work.

First-Coauthor on Cell Metab 2013 and Hypertension 2015.

8. Expression of thanks

I am particularly grateful to Prof. Jens Titze (University of Erlangen and Vanderbilt University). Prof. Titze recruited me for the Mars500 studies and supported me in every possible way. He also made it possible for me to come to Germany, first to Erlangen and then to Berlin. He instructed me in flame photometry, atomic absorption spectrometry, silver nitrate titration method, ashing method, as well as in statistical analysis of original data and data presentation. Clearly, he is the driving force behind these studies.

I thank Prof. Friedrich C. Luft, who has collaborated with Prof. Titze for 15 years. These studies also carry his unmistakable influence, since he has studied sodium metabolism for over 40 years. He established my presence and projects at the ECRC. He was instrumental in establishing my electrolyte laboratory at the ECRC. He has also tirelessly served as my thesis advisor and counseled me in the preparation for the current report.

I thank Prof. Dominik N. Müller, who is also intimately involved in these projects. Prof. Müller particularly helped me to conduct our first clinical feasibility study using $^{23}\text{NaMRI}$ at 7.0 Tesla. Prof. Müller surely continues his support of my interests here in Berlin.

I thank Dr. Peter Linz. He is an MRI physicist and designed the coil instrumental in our $^{23}\text{NaMRI}$ studies here in Berlin. From him I learned about the MRI principles of work and how to use this technology in clinical studies, including data interpretation.

I thank Dr. Michael Boschmann and Dr. Knut Mai, who were not directly involved in these particular studies. However, we have an intense collaboration outside of this report, which will also result in fruitful clinical science. Both have been extremely kind and helpful.