**Abbreviations**

a2H 2H abundance

BIA Bio-impedance assessment

BMI Body Mass Index

BW Body water

2H2O Deuterium Oxide

ECF Extracellular Fluid

ICF Intracellular Fluid

ISF Interstitial Fluid

 NP Nocturnal Polyuria

TBW Total body water

**Abstract**

**Aim**

To evaluate the relevance of the use of deuterium oxide (2H2O) and Bio-Impedance Assessment (BIA) to assess the size and function of the interstitium and its further use in urological research.

**Methods**

Nineteenvolunteers were recruited in this prospective trail combining ingestion of 2H2O enriched water and BIA. Blood samples were obtained every 10 minutes after ingestion of 2H2O enriched water. Urine was collected before and after the experiment. BIA was done every 5 minutes. Body position (standing vs lying) was changed to study the effect on the fluid distribution. The 2H/H ratio was measured in all samples.

**Results**

A first order kinetic, representing the uptake of 2H2O from the gastronomic tract to the blood was assumed.  Sex seemed to have an influence on this exchange rate, with a significantly slower exchange for women compared to men (p = 0.041, men: 0.052 min -1; women 0.038 min-1).

Impedance measured in legs (men: p = 0.012, women: p = 0.008) and trunk (both p <0.001) decreased significantly while moving from lying to standing. These changes probably reflect the orthostatic redistribution of fluid with an increase of fluid in both trunk and legs.

**Conclusion**

Both methods were tested and found to be useful for further urological research. Significant sex differences were seen in uptake of 2H2O from the gastro-intestinal pool. An impact of posture changes on the electrical impedance measured in the legs and trunk was observed.

**Key words:** extracellular fluid – nocturia – deuterium oxide – electric impedance – body water

**Introduction**

The total body water (TBW) in humans can be subdivided in different water pools. The major pools are the extracellular fluid (ECF) and the intracellular fluid (ICF), where the ECF is further subdivided into plasma and interstitial fluid (ISF) [1]. The interstitium can be defined as the space between the capillary wall of veins or arteries and the surrounding cells. This interstitium is one of the main fluid compartments of the human body [2,3] and is a structurally complex space composed by collagen fiber bundles, proteoglycan filaments and free water [4]. Although, its composition and anatomy have been widely studied, the flow, exchange and storage mechanisms of the ISF are only poorly understood [5].

In urological practice a lot of questions remain on the impact of the interstitium in the pathophysiology of urological diseases. A typical example is the pathogenesis of edema and its connection with nocturnal polyuria (NP). In patients with spinal cord lesions for example, a high incidence of NP linked with a high incidence of edema is found. However, the exact mechanism explaining both observations is unknown [6,7]. Torimoto et al. found a higher incidence in edema during the evening in patients with NP compared to patients without NP [8]. Probably, the interstitium plays a crucial role in the pathogenesis, but this hypotheses has never been studied.

A direct way to quantify TBW is by using the principle of an isotopic pool dilution by a biological marker such as deuterated water (2H2O) [9,10]. Deuterium (2H) is a, stable isotope of hydrogen that distinguishes itself by an extra neutron in the nucleus compared to the more common isotope (99.985%) protium (1H). The natural abundance of 2H in our environment is 0.015 %, which causes a baseline concentration of this isotope in the human body [11]. In humans, 2H2O is mostly used in metabolic studies, to determine TBW, to control food intake, for determination of the hydration or nutrition status or as a marker in deuterium NMR spectrometry [11,12].

The TBW can also be measured by the use of Bio-Impedance Analysis (BIA), which is a non-invasive assessment, commonly used to observe volume status and body composition of dialysis patients [13,14]. BIA is based on measuring impedance (Ω), calculated as the sum of resistance and reactance, after conducting a low-amplitude, high frequency alternating electrical current trough the body. Resistance is linked with the diameter, length and composition of the material, whereas reactance is the capacity of a medium to maintain, store and interact with the electrical current. Typically, resistance and thus impedance are low in fluid containing tissues such as blood, urine and muscle cells, and high in fat and bone tissue [15]. In the human body, reactance can be interpret as the electrical current across the cellular membranes. However, at a low electrical frequency (1 kHz), the electrical current is not capable of penetrating this cellular membrane, resulting in the conduction trough the ECF alone [16].

The aim of this pilot was to study how body water (BW) behaves between the different internal water pools of the human body, what the role and influence of the interstitium is and what the effect is of body position on the distribution and allocation of BW between different body parts (trunk, legs, arms). To study this, 2 techniques that have not been used before in urological research were tested on their usefulness:

* The first technique is a recent BIA device, which distinguishes itself from earlier devices by measuring impedance at different electrical currencies in different body parts (arm/limb/trunk). This technique made it possible to observe the influence of body position on the distribution of BW in these body compartments. Rationale for this research question is mainly the link between NP and edema that was found in earlier research and the fact that body position seemed to have an impact on the size of edema. For this reason, it can be suggested that body position and thus edema will have an impact on whether or not NP occurs.
* The second technique uses deuterium as a biological marker. With this technique the distribution, storage, allocation and excretion of BW was examined among the urological most relevant water pools (blood, urine and interstitium).

**Material & methods**

**Patient selection**

This prospective pilot-study was performed between May 2018 and November 2019. The study population consisted of healthy volunteers recruited trough posters and flyers spread across the hospital and university. All participants were aged between 20 and 30 years and had a Body Mass Index (BMI) between 20 kg/m2 and 24 kg/m2. Participants were excluded when they were students or employees related to the department of urology. In addition, participants with chronic diseases, implanted devices (pacemaker, IPG), on medication (except anti-conception) and pregnant women were excluded. In total 19 healthy volunteers (10 females and 9 males) aged between 21 and 24 years were recruited.

**Data collection**

The test protocol (fig 1) was conducted during the morning, after an overnight fast and a ban of alcohol for 24 hours. Firstly, body length (± 1 cm) and weight (± 0.1 kg) of each participant was measured. Subsequently, the volunteers’ TBW was estimated based on the Watson formula’s for both sexes [4].

Secondly, an urine sample was collected as reference sample. Thereafter a catheter was inserted in an antecubital vein to facilitate repeated blood sampling, and a reference blood sample was taken. At last, the electrodes of the BIA device were attached.

In the first part of the protocol the participants stood up for 5 minutes, laid down for 70 minutes, followed by 5 minutes in a seated position and ending with 30 minutes standing. BIA was done every 5 minutes. Fifteen minutes after the onset of the experiment, participants drunk 330 mL of deuterium enriched water while laying down. The deuterium enriched water was created by replacing a certain amount of water from a bottle of 330 ml mineral water with 2H2O (aD = 99.8%). The volume of 2H2O corresponded with 0.25% of the persons estimated TBW.

Blood samples were taken every 10 minutes, starting after the intake of the deuterated water. At the end of the test protocol a second urine sample was taken. Blood samples were given the time to clot and were centrifuged afterwards, (10 minutes, 1500 G). One ml serum was pipetted into another recipient. All samples were stored frozen at - 80°C until analysis of the deuterium abundance (a2H)The trial protocol was registered on clinicaltrails.gov (NCT04520477).

**Measurement of a2H**

To measure the 2H/H of deuterium oxide in the serum and urine samples, the water of these samples w extracted using a cryogenic distillation based on the method described in Bodé et al. [17], but was adapted for the specific sample matrixes. Shortly; The sample was transferred to a sample vial connected to a collection vial, via a glass pipe. Subsequently the sample was cooled in liquid nitrogen and the distillation setup was vacuumed to < 1 mbar. After assuring the air tightness of the setup, the sample vial was heated gradually in order to have a fractionation of 2H.To avoid boiling to 105°C, the collection vial was cooled in liquid nitrogen to collect the evaporating water. Finally, a2Hwas determined using cavity ringdown spectrometry equipped with a vaporizer and only micro pyrolysis module to remove the organic contaminants (WS-CRDS, L2120-I, Picarro, USA).

**Data processing**

TBW was measured and calculated using 3 different methods. Firstly, TBW was assessed by BIA in both standing and lying posture. Therefore, the measurements at 20 (lying) and 95 minutes (standing) after the onset (-15 minutes) of the protocol were used, as at this time points, volunteers were in a stable body position for 30 minutes.

Secondly, the pool size, based on urine and blood samples, was assessed using a mass balance of the dilution of the deuterium tracer by the pool as.

$$n\_{P} = n\_{LW}∙\frac{ a2HLW-a^{2}H\_{LP}}{a^{2}H\_{LP}-a^{2}H\_{T0}}$$

With nP and nLW being the amount (mol) of the water of the pool to be assessed and the labeled water (i.e. 330 mL ingested), respectively, anda2HLW,a2HLP, and a2HT0 being the a2H of the labeled water, the labeled pool and the pool before ingestion of the labeled water, respectively. For the a2H of the labeled water pool the last blood sample of the protocol was used. The 330 mL of water ingested during the isotopic labeling of the subjects was thus not considered as part of the BW pool.

**Statistical analysis**

Statistical analysis was performed using SPSS v.25. Descriptive statistics are reported as median (interquartile range). A Wilcoxon signed-ranked test was used to observe differences of BIA in different postures. A Mann Whitney U test was used to observe sex differences within the group. A p-value of < 0.05 was considered statistically significant. A Bland-Altmann plot was designed to observe proportional bias between TBW calculated based on the a2H of the blood samples and the TBW based on BIA measurements in a lying position. The study was approved by the institutions review board (EC 2017/1636). Written informed consent was obtained from all participants.

**Results**

**2H abundance**

1. *Serum samples*

In men the maximum a2Hwas reached 50 minutes after ingestion (0.31% (0.28 % - 0.32%)), while in women the maximum was reached more slowly after 60 minutes (0.25% (0.23% - 0.27%)) (Fig 2 A-B)). During the first period (from uptake of the isotopic label till reaching the maximal a2Hof the blood) a first order kinetic, representing the exchange of water from the gastronomic tract to the blood, was assumed. An exchange rate could be determined using a correlation analyses on following equation:

$$a^{2}H\_{t, S}= a^{2}H\_{T0,S}+a^{2}H\_{max,S}(1-e^{\left(-k∙t\right)})$$

With a2Ht,S, a2HT0,S, a2Hmax,S, being the a2Hof the serum at specific time, at start point T0 and at the point of maximal isotopic enrichment respectively. While obvious individual differences could be noted, sex had a clear effect on the exchange rate, with a significantly slower exchange rate for female volunteers (Mann Whitney U test, p = 0.041, men: 0.052 min -1; women: 0.038 min -1).

1. *Urine samples*

After termination of the test protocol, the median a2Hin urine was similar for both sexes s (female: 0.26 % (0.22 % – 0.28 %), male: 0.26 % (0.21 % - 0.31 %)). Nevertheless, it was not possible to do multiple observations and to determine kinetic parameters due to practical and ethical limitations of the experiment (short duration, limited bladder filling).

**BIA measurements**

* + - 1. *At 50 kHz*

Impedance measured at 50 kHz (Fig 3 A-B) showed a significant difference in the trunk between the lying and the standing position, as it increased significantly between - 15 (standing) and -10 minutes (lying) and decreased significantly with the next posture chance between 55 (lying) and 65 minutes (standing) (p < 0.001 for both sexes s). These changes reflect the redistribution of fluid with an increase of fluid in the trunk while switching positions from lying to standing.

The impedance measured in the arms did not differ significantly between the standing and the supine position. Posture changes did influence the impedance in legs for both sexes, as impedance decreased when standing up after 65 minutes of the test protocol (p = 0.012 in men and p = 0.008 in women). As for the trunk, an increased fluid volume in the legs can be mentioned as the reason for the significant lower impedance.

* + - 1. *At 1 kHz*

Impedance measurements at 1 kHz (Fig 3 C-D) show the conduction through the ECF alone, as the cell membrane could not be passed at this low frequency. Equal to measurements at 50khz, posture changes did not impact the impedance of the arms at 1 kHz. For the trunk, a posture change from lying to standing up (65 minutes) is reflected with a significant lower impedance. However after this initial decrease, no changes in trunk impedance could be measures between consecutive measurements in the standing position. In the legs, a significant lower impedance is observed while moving from lying to standing (men: p = 0.011, and women p = 0.007), suggestion a fast increase of fluid in the ECF in de lower limbs. In contrary to the trunk, a further significant decrease in leg impedance could be observed during repeated measurements in the standing position (men, p = 0.018, women p = 0.011).

**Prediction of the TBW**

Table 1 reports TBW calculations using different methods. However, some precautions should be taken for this deuterium serum measurements, as an equilibrium was not yet attained at the end of the experiment. For this reason, calculations based on deuterium measurements are mentioned as BW instead of TBW. A Bland Altmann compared the TBW measurements based on the a2H serum samples and the TBW by BIA in a lying position. Although the TBW based on a2H of the serum samples was not optimal due to the limited time frame of the study, the linear regression analysis did not describe proportional bias between both methods (p = 0.3).

**Discussion**

For many years research concerning the human water storage and excretion has been conducted. The results of this pilot study demonstrate the potential usefulness of deuterated water and BIA for further research concerning the link between NP and edema. Subsequently, the kinetics of exchange between the intravascular, slow and fast exchangeable pool and the impact of different body positions on the fluid contain of different body parts could be determined.

**Deuterium dilution experiment**

In literature, the first increase of a2H in serum samples has been reported 2.5 minutes after ingestion [18]. In the current study, a sample taken 10 minutes after ingestion already reflects the spread of 2H2O in the intravascular system. The uptake of 2H2O seems to be divided into a fast and a slow exchangeable pool. This is in line with results found by other authors [19,20]. This fast exchangeable pool represents c.a. 80% of the TBW (78%, ± 11% for men, and 86%, ± 6% for women). Besides the intra- individual differences in this pool exchange rates, men tended to have a statistically faster exchange rate compared to women (0.052 min-1 VS 0.038 min-1). Other studies mainly included male volunteers causing a lack of data about sex differences [19,20]. After this a2H intravascular peak, a decrease in the intravascular a2H is observed, probably reflecting the exchange between the intravascular compartment and other slower exchanging pools. Possibly, this slower exchangeable pool reflects the interaction with the ISF and can be calculated as 22% of the TBW for men and 14% of the TBW for women. However, due to the limited time frame of this protocol, this slowly interacting pool could not be fully characterized. Deuterium dilution analysis based on urine samples is rather new, and has not been done by many researcher. Given only two urinary samples were taken per person it is difficult to draw any conclusions.

**BIA**

The advanced features of this BIA device (measurement at different electrical currencies and in different body parts) proofed in this pilot trail to be useful to observe the effect of body positions on the fluid distribution of different limbs. The trunk and leg impedance did decrease significantly with posture changes (at both 1 and 50 kHz) from lying to standing, which most likely visualizes the orthostatic increase of fluid volume in both trunk and legs while standing up. This increase in volume of the trunk can be declared by an influx of blood from the head, whereas the inflow of blood from the trunk into the legs can explain the decreased leg impedance. Equal observations were made in literature [16]. At 1 kHz, this leg impedance tend to decrease significantly with repeated measurements, suggesting a further inflow of fluid into the leg ECF over time. This observation suggests that for the ECF of the legs two potentially orthostatic time curves could be proposed: an immediate (first 5 -10 minutes) decrease in impedance reflecting an influx into the vascular system of the legs, and subsequently a less rapid decrease potentially reflecting the spread of fluid inflow into both the intravascular and ISF of the legs.

However, in this study it was only possible to observe the interaction of ECF without the possibility to make a distinction between plasma and ISF. This BIA technique is appropriate to measure differences in ICF and ECF, but it lacks the strength to observe the ISF shift between the different body compartments.

**Estimation of TBW**

Although differences could be measured between the calculated TBW’s, all methods were appropriate to estimate TBW in young, healthy volunteers. However, for older or comorbid patients the Watson formula is not feasible, as it does not consider body composition (fat or muscle constitution), fluid storage problems or intake of (anti)diuretic medication. For those patients, methods using deuterium dilution or BIA probably provide a better estimation of the TBW. Despite literature suggests an overestimation of TBW calculated by a BIA device, only a trend could be visualize in the current study [11]. Based on the results of Bland Altman plot, both techniques (deuterium dilution and BIA measurements in lying) position are comparable methods for calculation the TBW without proportional bias. , , however in our opinion the deuterium assessment using the a2H in blood, seems the most suitable method to observe TBW in all patients, as it can be defined as the most exact medium for calculating TBW after achieving an equilibrium.

**Interstitium**

During this research, the volume, spread and change of the ISF could not be visualized or calculated using both methods. However, the interaction of the ingested 2H2O with the ISF can be suggested through interaction between the intravascular and a slower exchangeable pool which probably contains the interstitium. Additionally, BIA measurements in the legs at 1 kHz, suggest a potential interaction and fluid accumulation in the ISF after changing positions.

**Limitations**

However, this study has some limitations: Firstly, the concentration of deuterium due to evaporation in sweat and the loss of deuterium through saliva was not measured. Moreover, the first serum sample was taken 10 minutes after ingestion, which leads to the fact that the initial moment of appearance could not be reported. Multiple urine sampling to observe and to determinate the first appearance of deuterium oxide into the urine was not possible due to practical difficulties (short duration, limited bladder filling).

**Further research**

The biggest limitation of this pilot is the limited duration of the experiment and the lack of numerous time framed urine samples. At the end of this experiment, the pools were not yet in an equilibrium fase. To further test these hypotheses, an extended study protocol with a longer duration (± 5 hours) and numerous urine collections are necessary. Ideally, this study can be done in patients using CISC or in patients with a chronic transurethral catheter, to obtain a continuously urine collection and in patients with fluid storage or excretion problems, such as edema. These patient groups will most certainly provide more insight into the functioning of the interstitium, as they have a deviant fluid handling and a potential disabled function of the interstitium.

**Conclusion**

In this pilot study, both methods to observe fluid distributions in patients were tested and found to be appropriate for their further use in urological research concerning the impact of edema on NP.

BIA showed a clear effect of body composition on the fluid distribution of different body parts. The slow decrease in impedance when changing from a lying to a standing posture suggests a potential fluid accumulation in both the plasma and ISF of the legs. TBW can be assessed using BIA measurements and deuterium dilution without proportional bias.

Moreover, a fast exchangeable pool reflecting the absorption of 2H2O from the gastrointestinal tract into the intravascular pool could be calculated. Clear sex differences in the exchange rate of this fast exchangeable pool were observed. After achieving the maximal intravasculara2H , exchange with a slower exchangeable pool, most probably the interstitium, was visualized.

Further research with a long-lasting protocol in patients with edema, will potentially provide more insights into the functioning of the interstitium and the possible link with edema and diuresis.

**Figures**

**Table 1** **Overview of TBW and Body Water (BW) calculation based on different methods for 19 volunteers**: Based on Watson formula, calculated based on the maximal 2H abundance in urine, serum and obtained through BIA in standing and lying position. However, some precautions should be taken for this deuterium serum measurements, as an equilibrium was not yet attained at the end of the experiment. For this reason, calculations based on deuterium measurements are mentioned as BW instead of TB

**Figure 1** **Overview of the test protocol** with exact time of the ingestion of deuterium enriched water and the different sampling times of blood and urine.

In the first part of the protocol the participants stood up for 5 minutes, laid down for 70 minutes, followed by 5 minutes in a seated position and ending with 30 minutes standing. BIA was done every 5 minutes. Fifteen minutes after the onset of the experiment, participants drunk 330 mL of deuterium enriched water while laying down. The deuterium enriched water was created by replacing a certain amount of water from a bottle of 330 ml mineral water with 2H2O. The volume of 2H2O corresponded with 0.25% of the persons estimated TBW based on the Watson formula. Blood samples were taken every 10 minutes, starting after the intake of the deuterated water. At the end of the test protocol a second urine sample was taken.

**Figure 2 (A-B) a2H(%) in serum measured during different moments of the test protocol in both sexes** (A for women and B for men).At point -15 minutes, a reference serum sample was taken, subsequently patients drunk deuterium enriched water at time point 0 minutes and a serum sample was taken every 10 minutes. In men, the maximum a2H was reached 50 minutes after ingestion (0.31% (0.28 % - 0.32%)), while in women the maximum was reached more slowly after 60 minutes (0.25% (0.23% - 0.27%))

**Figure 3 (A – D): Impedance measured at both 50 kHz and 1 kHz in different body segments and both sexes.** Conduction at 50 KHz showing impedance at a normal frequency, conducted through ICF and ECF. Impedance at 1 kHz is reflecting conduction through the ECF only, as the current at this low frequency is too low to pass the cell membrane. All volunteers are standing up on point -15 minutes, lying down from point -10 minutes through point 60 minutes, were seated on a chair at measurement 65 minutes and were standing from minute 70 till the end of the experiment.

**A)** Trunk impedance at 50 kHz showing a significant difference with posture change from lying to standing (Wilcoxon, both sexes p < 0.001)

**B)** Leg Impedance at 50 kHz, showing a significant difference with posture change from lying to standing in both sexes (Wilcoxon, p = 0.012 in men and p = 0.008 in women).

**C)** Trunk impedance at 1 kHz showing a significant decrease with posture change from lying to standing (Wilcoxon, men p = 0.012, women p = 0.005)

**D)** Leg impedance at 1 kHz showing a significant decrease with posture change from lying to standing (Wilcoxon, men p = 0.01, women, p = 0.007)

**Figure 4: Bland Altman plot** observing no proportional bias ( p = 0.03) between TBW based on a2H of serum samples and TBW based on BIA measurements in lying position. The lines on the plot observe the mean (1.085, SD 7.17) and 95% CI (-12.99 – 13.22) of the difference of both variables.

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