

Diplomarbeit

Effects of lymphedema therapy on body salt homeostasis

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Graz, 05.06.2021

Affirmation

I solemnly vow, that this diploma thesis has been written and composed without assistance, independently and by myself only. It has neither been partly nor fully submitted as graded academic work. I herewith assure, that all references and sources – segments used directly or indirectly, exact wording as well as meaning – are stated and labeled as such.

Graz, 05.06.2021

Lukas Postlmayr eh.

Preface

This thesis represents a part of my studies. Because of this, I wanted to deal with a topic that on the one hand interests me very much and on the other hand can be useful for my upcoming time as a doctor.

Maybe due to my school journey through mechatronics, I became enthusiastic about the kidney as a filter organ but also as a hormonal regulatory organ. Therefore, I am very happy that the Chair of Physiology of the Otto Loewi Forschungszentrum für Gefäßbiologie, Immunologie und Entzündung, gave me an insight into one of their projects and thus enabled me to write my diploma thesis. I would especially like to thank Ao. Univ.-Prof. Mag. Dr. rer. nat. Andreas Rössler, who supported me during the whole time and could inspire me again and again with his great understanding and knowledge of physiology.

I would also like to thank Dr. scient. med. Bianca Brix MSc BSc, who helped me to familiarize myself with the subject matter, especially in the early days.

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Abbreviations

ECV – Extracellular Volume

Na – Sodium

PV – Plasma Volume

ICV – Intracellular Volume

K – Potassium

NaCl – Sodium Chloride

Cl – Chloride

ROMK – Renal outer medullary potassium channel

Ca – Calcium

Mg – Magnesium

MD – Macula Densa

ATP – Adenosin triphosphate

VA – Vas afferens

GFR – Glomerular filtration rate

ADH – Antidiuretic Hormone

ANP – Atrial natriuretic Peptide

ENaC – Epithelial Na-Channel

RAAS – Renin-Angiotensin-Aldosteron-System
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ACE – Angiotensin-Converting-Enzyme

BP – Blood Pressure

AT1R – Angiotensin type 1 receptor

AT2R – Angiotensin type 2 receptor

ATG – Angiotensinogen

AT1 – Angiotensin 1

AT2 – Angiotensin 2

MR – Mineralocorticoid receptor

HuR – Human antigen R

UTR – Untranslated region

ARR – Aldosterone-Renin-Ratio

PA – Primary Aldosteronism

ACTH – Adrenocorticotrophic hormone

NSAID – Non-steroidal anti-inflammatory drug

ADRR – Aldosterone-to-direct-Renin-concentration-ratio

ADH – Antidiuretic hormone

ANP – Atrial natriuretic peptide

LVS – Lymphatic vascular system

VEGF-C – Vascular endothelial growth factor C

VIII

LEC – Lymphatic endothelial cells

CLV – Collecting lymphatic vessel

MRI – Magnetic resonance imaging

MLD – Manual lymphatic drainage

HGF – Hepatocyte growth factor

GAGs – Glycosaminoglycans

PGs – Prostaglandins

HSD – High salt diet

LSD – Low salt diet

PRA – Plasma Renin Activity

PVC – Plasma volume change

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Zusammenfassung

Hintergrund

In den letzten Jahren wurde sehr viel im Bereich des Körpersalzhaushalts geforscht. Viele Studienergebnisse deuten darauf hin, dass es in der Haut von Menschen und anderen Säugetieren einen bisher unbekanntem Salzspeicher geben könnte. Gründe für diesen Speicher könnte eine Schutzfunktion gegenüber Mikroorganismen, Schutz vor Austrocknung, ein Regelsystem für wechselnde orale Salzzufuhr oder möglicherweise all diese Gründe zusammen sein. Bei Patienten*innen mit einem Lymphödem könnte es sein, dass man diese Mechanismen deutlicher und besser beurteilen kann, weshalb dieses Kollektiv genauer untersucht wurde.

Ziel

Ziel der Studie war es, Informationen über die Zusammensetzung der Lymphflüssigkeit zu erhalten und so mehr Wissen über die Einlagerung von Salz in der Haut zu gewinnen.

Methoden

Bei Patienten*innen mit Lymphödemen wurden vor und nach einer manuellen Lymphdrainage verschiedene Parameter der Wasser- und Salzregulation, wie Aldosteron, Renin und Natrium gemessen und analysiert.

Ergebnisse

Nach der manuellen Lymphdrainage stieg das Plasmavolumen im Median um 66.5 mL. Betrachtet man die Elektrolyte nach der manuellen Lymphdrainage, so lag der mediane Anstieg von Natrium bei 2.88 mmol/L und von Chlorid bei 2.54 mmol/L (Na: $p < 0.001$, Cl: $p < 0.001$). Es gab keine signifikanten Veränderungen der Plasma-Kalium-, -Calcium- und -Albumin-Konzentration. Während Natrium einen signifikanten Anstieg zeigte, sank die Plasma-Renin-Aktivität im Median um 0.04 ng/mL/h (Korrelation von Plasma-Renin-Aktivität - Natrium: $p = 0.029$, $r = -0.4$ (-0.66 – -0.05)).

Bei der Analyse des Renin-Angiotensin-Aldosteron-Systems wurden keine signifikanten Veränderungen oder Korrelationen zwischen der Plasma-Renin-Aktivität und der Aldosteron-Plasmakonzentration festgestellt.

Erkenntnisse und Ausblick

Die Ergebnisse dieser Arbeit veranlassen zu weiteren Untersuchungen des Salzspeichers Haut. Dadurch könnte es möglich sein, Krankheiten wie das Lymphödem, Diabetes Mellitus oder arterielle Hypertonie besser zu verstehen und somit besser behandeln bzw. im Idealfall vermeiden zu können.

Abstract

Background

Over the last years, different findings indicate an unknown salt storage in the skin of a human's body. Reasons may be, to create a barrier to defend the body of undesirable microorganisms, to protect the body from dehydration, being able to balance different amounts of oral salt intake, or all together. In case of a lymphedema, this system may unveil its function even better and so, patients suffering from this disease were investigated.

Objective

The aim of the study was, to obtain information about the composition of the lymphatic fluid and thus, to gain more knowledge about the skin salt storage.

Methods

Before and after a manual lymphatic drainage, performed on patients with a lymphedema, different parameters of water and salt regulation, such as Aldosteron, Renin or Sodium were investigated and analyzed.

Results

After manual lymphatic drainage, the median plasma volume increased by 66,5 mL. Looking at the electrolytes after the manual lymphatic drainage, the median increase in sodium was 2.88 mmol/L and in chloride 2.54 mmol/L (Na: $p < 0.001$, Cl: $p < 0.001$). There were no significant changes of plasma-kalium-, -calcium- and -albumin-concentration. While sodium showed a significant increase, the plasma renin activity decreased by 0.04 ng/mL/h of the median (correlation of plasma renin activity – sodium: $p = 0.029$, $r = -0.4$. (-0.66 – -0.05)).

Analyzing the Renin-Angiotensin-Aldosterone-System, no significant changes or correlations between plasma renin activity and aldosteron plasma concentration were seen.

Conclusion

The results of this thesis lead to much more investigation of the skin's salt storage and further of regulating variables of Aldosterone. It may be able to understand diseases like lymphatic edema, diabetes mellitus or arterial hypertension much better and so, modern medicine may be able to treat or prevent these diseases even better than at the moment.

1. Introduction

1.1 Kidney

The kidney is one of the most important organs in the human body. Because of its important function, to detoxify the blood and so the whole body, survival isn't possible for a long time without this organ. Although, there are many other features to regulate different parts of the body, which are important for a faultless performance. These are the regulation of: the body's fluid and electrolyte-homeostasis, the blood pressure, acid-base-balance, gluconeogenesis and the synthesis of hormones (1). Especially two of these functions are from main importance for this thesis, so they are described precisely.

1.1.1 Water, Sodium and Volume balance

A very big amount of the body's composition is water. About 50-70% of the body weight are water, and so it's obvious, that this compartment is highly regulated.

1.1.1.1 Extracellular Volume

One third of water is ascribed to the part of the body which is out of the cells. The biggest part of the extracellular volume (ECV) is the interstitial fluid with about 80% of ECV. This fluid is between and around all different kind of cells, contains different electrolytes (from were sodium (Na) is the most important one), specific proteins and will be relevant in case of a lymphedema. Due to the big amount of sodium in this fluid, it is obvious, that the regulation, to achieve sodium balance for maintenance of ECV and free water, is of great importance in the steady state (2). Another part of ECV is the Plasma Volume (PV). So, this Volume is built from the cellless part of the blood, making up about 20% of ECV and a permanent interaction with the interstitial compartment is given.

The last, very little part of ECV, is the so called third space. This fluid is secreted in serous cavities like the pleura, peritoneum, pericardium, liquor and a few others (1).

1.1.1.2 Intracellular Volume

The intracellular Volume (ICV) is the even bigger part of the body's water and contains about two third of it (1). Due to cells are the elements of which the body is built, it is also clear, that their condition is from main importance and so their volume is strictly regulated. Thus, they are surrounded by the interstitial fluid, a tight interaction of these two compartments is given.

1.1.1.3 Sodium and its renal duties

As the most represented solute in the ECV, and so PV, the sodium concentration in the primary urine is also very high, which causes the necessity of reabsorbing it in the tubular system. On the one hand, sodium itself has to be reabsorbed and on the other hand, sodium-reabsorption is used to get other solutes back from the primary urine.

At the proximal tubule, which has overall a very high transport capacity, the Na-K-ATPase is located at the basolateral membrane of tubular cells. Its function is, as in most other cells in the body, to get sodium out of the cell and potassium (K) into it. As a result, the concentration gradient of Na between the tubular lumen and the cell is very high, forcing Na to migrate back into the cell. During this migration, Na takes many other solutes with it (like glucose, amino acids, lactate and phosphate) and acts as symporter. For other solutes like hydrogen, there is a Na-Antiport, which pumps Na into the cell and the solute out of it.

At the loop of Henle, an important part of concentrating the urine happens. Sodium chloride (NaCl, Cl) and water are diffusing into the renal medulla at the descending part of the loop. As a result, the medulla gets hypertonic. The following ascending part is impermeable for water but contains the Na-K-2Cl-Symporter, pumping K and Cl by means of the Na-concentration gradient into the cells. Na leaves the cell via the Na-K-ATPase, K gets back into the lumen through special K-channels, the renal outer medullary potassium channels (ROMK), and also back into the blood with a basolateral KCl-Symporter. Due to ion distribution (K in the lumen, and Cl leaving basolateral), a lumen-positive electrical gradient occurs, which pushes cations like

Na, Calcium (Ca) and Magnesium (Mg) out of the lumen. The result of the passage through the loop of Henle is a hypotonic urine.

Arrived at the distal bundle, a NaCl-Symporter at the apical membrane carries even more NaCl back into the cell and the basolateral Na-K-ATPase pushes the Na further on into the blood. The remaining Cl is carried with a KCl-Symporter into the blood also. Another important mechanism in renal regulation of Na is the tubuloglomerular feedback-mechanism. The distal bundle has at one part of its course a very near relationship to the Macula Densa (MD). MD acts as a sensor for NaCl-concentration in the urine and reacts to a too high NaCl-concentration with a release of Adenosine triphosphate (ATP). ATP gets broke down to Adenosine, which effects the vas afferens (VA) as constrictor. If VA contracts, the glomerular filtration rate (GFR) decreases, and with it, filtration of NaCl.

The last station where modification of urine is performed, is the collecting duct. The principal cells have epithelial Na-Channels (ENaC) and ROMKs (per each Na-ion which goes into the cells, one K-ion goes into the lumen). Further, Na is pumped again with Na-K-ATPase into the blood, K is going in circles – from cell, to blood, from blood to cell. But because of the apical Na-K-exchange, the more Na is reabsorbed, the more K leaves to the lumen and is eliminated (1).

1.1.2 Regulation

1.1.2.1 Renin-Angiotensin-Aldosteron-System

The kidney has to guarantee enough pressure all the time for filtration and so has its own autoregulation, the Renin-Angiotensin-Aldosteron-System (RAAS, Figure 1). “A key feature of this enzymatic cascade is the tightly regulated secretion of renin by juxtaglomerular cells. They are able to sense and respond precisely to changes in blood pressure or extracellular fluid volume. But not only juxtaglomerular cells, also a subset of mesangial cells, arteriolar smooth muscle cells, interstitial pericytes and tubular cells produce renin and serve in a local or systemic capacity to ensure blood pressure and fluid–electrolyte homeostasis”(3). Renin is a Protease, which is able to catalyze the metabolism from angiotensinogen to angiotensin I. To prevent a premature onset of this effect, prorenin, a precursor is synthesized and stored in

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granules. Inside of these granules, prorenin transforms to renin and is released through endocytosis in a controlled manner, at the proper moment (1). “Three major physiological mechanisms control renin synthesis and release: the concentration of sodium chloride (NaCl) in the MD, whereby a decrease in NaCl concentration stimulates renin synthesis and release; perfusion pressure as sensed by renal baroreceptors, whereby a decrease in perfusion pressure stimulates renin release; and β -adrenergic receptor activity, whereby activation of β -adrenergic receptors in juxtaglomerular cells by circulating or locally released catecholamines stimulates renin release”(3).

In this way, renin gets into the blood circulation, where it meets angiotensinogen and so, Angiotensin I results. Angiotensinogen is synthesized in the liver (4) and also in the fatty tissue (1) and its plasma availability is a way higher than those of renin. This suggests, if renin is released, it definitely has enough substrate to act. If Angiotensin I arrives at the lung, Angiotensin-Converting-Enzyme (ACE) catalyzes the metabolism to Angiotensin II, which finally has the ability to, speak verbosely, increase blood pressure (BP). “Angiotensin II acts at 2 receptors, the angiotensin type-1 and type-2 receptors (AT1R and AT2R)” (3,4). “Angiotensin II's actions at the AT1R leads to increased sodium retention, vasoconstriction (including preferential constriction of the efferent arteriole of the kidney), stimulation of thirst and desire for salt, enhanced sympathetic nervous system activity, and aldosterone release from the adrenal gland's zona glomerulosa. Actions of AT2R stimulation are counter regulatory to those of the AT1R, where type-2 receptor stimulation leads to anti-inflammatory, anti-fibrotic, and vasodilatory effects, but does play a less role in the adult” (4). Finally, a negative Feedback mechanism leads to a suspended renin-release, whereby, it is not clear if the increased BP and other effects of RAAS results in a downregulation of itself, or effects of angiotensin II on type 1 angiotensin II-receptors are responsible for this (3).

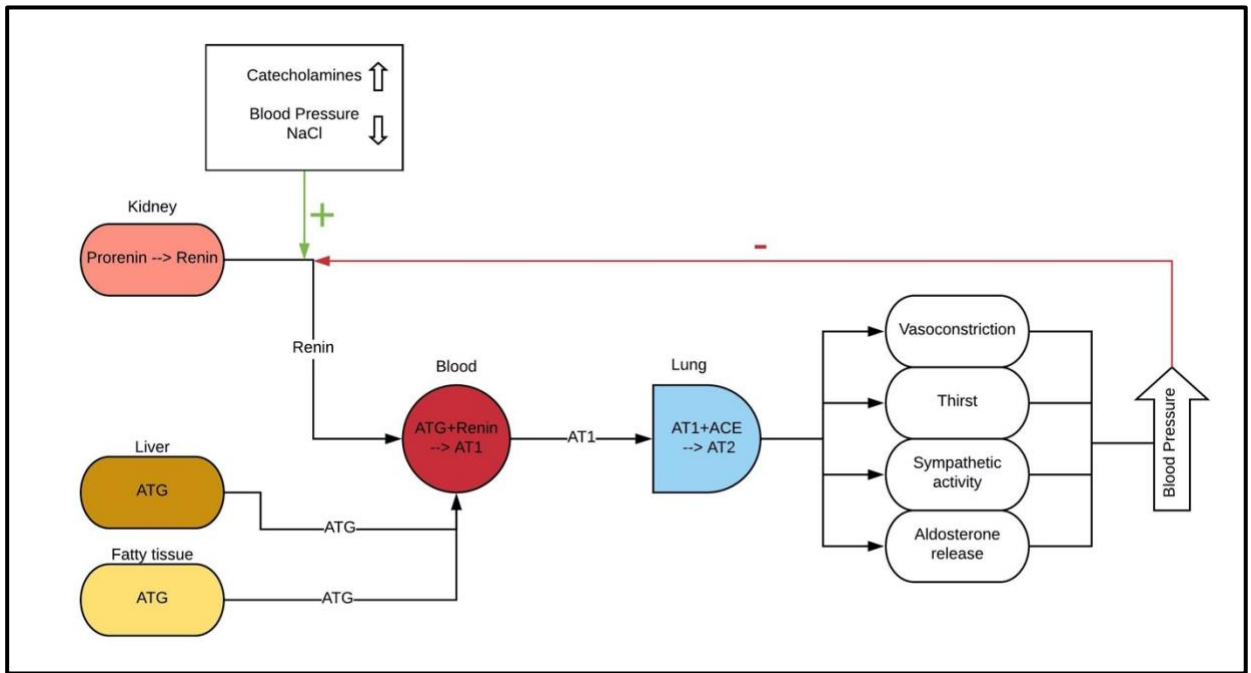


Figure 1 – RAAS; ATG – Angiotensinogen, AT1 – Angiotensin I, AT2 – Angiotensin II, ACE – Angiotensin converting enzyme

1.1.2.2 Aldosteron

As described above, one of the effects of angiotensin II is the release of aldosterone. Aldosterone is a hormone, which is synthesized mainly at adrenal gland's zona glomerulosa. Another important stimulus is hyperkalemia. Making aware of its functional mechanism, both release factors make sense. Not only in the kidneys, but also in the intestine, sweat and salivary glands (4) more ENaCs, ROMKs and Na-K-ATPases are integrated into the cell membranes, causing an increase of Na and water retention and so an increase of K excretion (1). Furthermore, aldosterone is also built in nonepithelial tissues, like the retina, brain, myocardium, vascular smooth muscle cells, fibroblasts and adipocytes. But in this context, it causes inflammatory processes, with the result of hypertrophy and fibrosis. These findings are corroborated by cases of heart failure, which shows an improved cardiovascular morbidity and mortality, after treatment with spironolactone or eplerenone (Spironolactone/Eplerenone are aldosterone antagonists) in combination with

RAAS-inhibitors and beta-blockers (4,5). Moreover, aldosterone and angiotensin II may be responsible for an increased fibrosis and a decreased fibrolysis in the heart and the kidney, triggered by cytokines like tumor necrosis factor alpha, plasminogen-activator-inhibitor, nuclear factor kappa-B, and transforming growth factor beta. Underpinned again from a cardioprotective impact by inhibiting mineralocorticoid-receptors (MR) at macrophages (4). Another finding by patients with metabolic syndrome exhibits increased aldosterone plasma levels, which are decreasing clearly, if these patients lose weight. This correlation raises the question of responsibility. Hypothesis are on the one hand, the production of aldosterone by adipocytes, or on the other hand, stimulation of aldosterone synthesis by adipokinin, insulin, tumor necrosis factor related protein, fatty acids or products of oxidation (5). The circumstance, that aldosterone is part of a control loop, stimulated in times of hypotonicity, leads us to the interesting mechanism of posttranscriptional signaling. An RNA-binding protein, the human antigen R (HuR), stays in the nucleus during times of isotonicity and causes the synthesis of a special MR-type, the delta 6 MR. Delta 6 MR lacks the whole exon 6 and so it isn't as functional as a full-length MR. In case of hypotonicity, HuR leaves the nucleus and is transferred to the cytoplasm, where it interacts with MR's 3'-untranslated region (UTR) and so stabilizes the MR transcript. In addition, this feature enhances the effect of aldosterone at the kidney (6).

1.1.2.3 Aldosterone-Renin-Ratio

The Aldosterone-Renin-Ratio (ARR) is commonly used in diagnosis of primary aldosteronism (PA). Patients suffering from PA, have an increased ARR and interestingly, patients with lymphedema exhibit increased ARR also. To calculate the ARR, aldosterone concentration and either plasma renin concentration or plasma renin activity are measured and are set into relation. It is controversial discussed, if renin-concentration or renin activity is the better parameter to calculate the ARR (7). For a reliable result, it is simply important to choose one way and go with it.

PA is the most common reason for secondary hypertension. Pathophysiological, an

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elevated aldosterone-level increases blood pressure on the one hand, and on the other hand leads to fibrosis and inflammation. Especially due to the second point, patients with PA have an increased cardiovascular risk, compared to patients with essential hypertension at the same blood pressure-levels, so a better detection of PA and furthermore the proper treatment would prevent patients of premature cardiovascular events (5).

Under physiological conditions, a heightened renin level leads to an increase of aldosterone (function of RAAS), so both hormones are upregulated. In case of an PA, huge amounts of aldosterone are secreted. This elevated aldosterone inhibits the renin production due to the negative feedback loop. This mechanism increases the ARR in turn and so can be used for diagnosis much better, than looking for aldosterone or renin separately. PA is mainly caused either by an unilateral aldosterone producing adenoma or a bilateral hyperplasia of the adrenal gland. Further, carcinoma or an ectopic aldosterone producing tumor could be responsible for PA (7–9).

Avoiding false positive or false negative results isn't easy, because there are many disruptive factors, affecting aldosterone and renin and so the ARR. Ahead of these factors, the first problem is the definition of a threshold because most guidelines have their own opinion and the cut-off level depends on the measuring method also (9).

Posture increases aldosterone levels, because the drop of blood pressure activates RAAS and additionally the metabolic clearance of aldosterone is reduced, due to a lower hepatic blood flow. This leads to a 5 to 15 minute gap between physical activity and taking the blood sample, to avoid this bias. Time of day affects this circumstance also, because in the morning, posture has a bigger impact than in the afternoon. Further, if PA is established for a long time, renin is always very low, which leads aldosterone to be strongly dependent of the production of adrenocorticotrophic hormone (ACTH) and ACTH is highest at about 8 am and falls rapidly after (7,8). These facts lead to a recommendation for taking samples in the morning, being two hours out of bed, in a sitting position (9). Moreover, women's hormones estrogen and progesterone influences ARR notable, so, at certain periods in the women's

cycle, they are tending to be false positive (10).

Dietary salt intake affects ARR in two ways. As salt restriction increases both renin and aldosterone, which leads to more false negative testing, salt excess decreases renin strongly when affecting aldosterone not that much, so a false positive ARR occurs (7).

As described above, hyperkalemia stimulates aldosterone release, so by implication, during hypokalemia aldosterone levels would be low. This circumstance implicates false negative testing, so the potassium level has to be checked, what isn't easy due to the fact, that a big issue of blood sample collection is a cytolysis-induced hyperkalemia (9).

Another disruptive factor is renal impairment, accompanying with RAAS activation leading to high angiotensin II and aldosterone levels, but proportional lower renin elevation, because of a lower secretion by juxtaglomerular cells, resulting in an false positive bias (7,8,11).

Medication affects ARR in both ways and should be mentioned before sample collection. Examples of agents are β -blockers, clonidine, methyldopa, non-steroidal anti-inflammatory drugs (NSAIDs), ACE- and angiotensin-receptor-blockers and different diuretics. Wash-out periods should be done before testing (8).

New findings suggest the use of aldosterone-to-direct-renin-concentration-ratio (ADRR) instead of ARR, because of a "satisfying sensitivity and specificity". One advantage for example, is the independence of renin substrate availability (12).

Despite these extensive impairments, there is enough evidence, showing, that in most cases it is able to do a sufficient assertion. Only if ARR is close to the threshold, beneath or above, disruptive factors should be mentioned (9).

1.1.2.4 Antidiuretic Hormone

On the basics of physics, osmosis and diffusion have a huge impact on the compartments of water. While the interstitial fluid is more flexible in its extension, the cells' volume can only differ in a little range, to hold up their function. Because of their dependence on the osmolarity of their surrounding fluid, the interstitial fluid and subsequently the ECV has to be controlled very tight. Beside the physiological aspect, this is necessary, because water and sodium intake and output differs every single day, due to variation in food and fluid consumption, physical stress, sweat and so on. Daily, the kidneys can excrete 0.5–25 L of urine with osmolality that varies from 40 to 1400 mOsm/kg H₂O. This tight control is accomplished primarily through alterations in sodium and water reabsorption via mechanisms stimulated by volume and tonicity of plasma (2).

While the mechanisms described above, take its main part at the kidney, further control loops are located in other parts of the body, but also effecting the kidney to maintain body homeostasis. The concentration of osmotically active solutes is about 300 mosm/L, but more important is the osmolality, which is about 290 mosm/kgH₂O. The osmolality is measured from special neurons at the hypothalamus (Lamina terminalis, Organum vasculorum). A hypotonic environment at these neurons causes osmosis of water into them and their volume increases, whereas hypertonicity is extracting water of them. So, during hypertonicity in their environment, these neurons are getting hyperosmolar and so the permeability of mechanically controlled Cation-channels decreases. As a result, the neurons depolarize and activates Ncl. Supraopticus and Ncl. Paraventricularis, which releases antidiuretic hormone (ADH, Vasopressin). ADH causes an increased concentration of Aquaporin 2, a water channel, in the renal collecting ducts and so, more water is removed from excreted urine. Furthermore, the Na-K-2Cl-Symporter is activated at the ascending part of the loop of Henle. This system is this sensitive, only 1% change in osmolality is enough, to release ADH. Due to its mode of action, ADH has a big influence on free water, so the osmolality is decreasing, what is intended, but also causes a stop of ADH-release at about 280mosm/L (1).

1.1.2.5 Thirst

Another mechanism to decrease plasma tonicity, is thirst. Only 2–3% of change in plasma osmolality is a potent stimulus of it (1,2). To take a closer look, this mechanism is called the osmotic thirst, which is also regulated from the hypothalamus and has a big correlation with plasma levels of ADH (13). Another stimulus of thirst is hypovolemia, but not as potent as the osmotic way. To stimulate hypovolemic thirst, it needs about 10% loss of extracellular fluid (2).

1.1.2.6 Atrial Natriuretic Peptide

As a result of the mechanisms above, PV increases. As PV is controlled, an increase of it causes atrial natriuretic peptide (ANP) release from the myocardium, which effects vasodilatation in peripheral veins (14), followed by a shift of blood volume from central to peripheral. Due to the vasodilatation in renal veins, the osmotic gradient in the renal medulla is washed out, which causes an increased diuresis to decrease PV, but now with effects on water AND sodium, allowing an adjustment in osmolality (1,14).

1.2 Lymphatic vascular system

The lymphatic vascular system (LVS) is fundamental known in most of the world, but an often shabbily treated body function. Due to the properties of the cardiovascular system, especially the capillary system, there is a need of transporting the lacking plasma volume back to the blood circulation. In case of failure, lymphedema occurs and with it many other problems, affecting a patient's quality of life dramatically. The LVS plays also an important role in terms of immune system and in transporting absorbed nutrients, especially lipids, from the intestine.

1.2.1 Anatomy

The LVS arises at the very distal part of every capillary field and starts with a subepithelial lymphatic capillary network. This network is also called initial lymphatics and have a single-layered endothelial covering and only a very sparse basal membrane with big gaps between. Vascular endothelial growth factor C (VEGF-C) is the major differentiating factor for lymphatic endothelial cells, forcing pre-stages of venous endothelial cells to differentiate into lymphatic endothelial cells (LEC)(15). LECs are arranged in a manner to build a kind of a flap valve with the effect, that interstitial fluid is able to get inside the vessel, but once in, it is hard to get out of the lumen, so the fluid is kept inside (16). These capillaries are attached with special filaments to the interstitial tissue, what is very important for their function. In case of an increased interstitial volume, the whole tissue extends, and due to the filaments, the junctions between the endothelial cells open up, so more fluid is able to get into the LVS and so back into the cardiovascular system. The so called precollector vessels are the first structures, which contains little valves, to allow only a proximal directed flow of the fluid. Collecting lymphatic vessels (CLV) are the next bigger structure. They have continuous adherence junctions and so they are incapable for fluid uptake for the most part (17). CLVs have valves in a constant distance from 6 to 20 mm, to secure only one flow direction and the part between two valves is called "lymphangion". Moreover, they have a special type of smooth muscle cells, which contains a cross of cardiac, skeletal and

smooth muscle myosin (17,18), are arranged circular and are contracting periodically to ensure lymphatic flow (19).

Lymph nodes are integrated in the system of outflow and every drop of fluid passes not less than one lymph node (16). They have important functions in terms of filtration and concentration of the lymphatic fluid, and they play an important role for the immune system. A Lymph node is fed by a few afferent lymph vessels, containing most of the time fluid with less than a half of protein concentration, compared to plasma. At the efferent vessels, protein concentration is approximately equal to plasma, leading to the fact, that lymph nodes absorb water (17). Findings also exhibited, that lymph nodes are able to dilute extremely concentrated lymph fluid (16).

The more proximal the CLVs get, the bigger they get and finally they are called lymphatic ducts. The biggest one is the thoracic duct, which collects the lymphatics of the whole body, except the right upper limb, right thoracic half and right half of the head. These parts are collected by the ductus lymphaticus dexter. These ducts lead to the cardiovascular system at the left and right angulus venosus (20). At these confluences, lymphovenous valves and a platelet thrombus hinder blood to flow backwards into the lymphatic system (17).

Beside this fundamental structure, every limb has an epifascial lymphatic system to transport the lymph fluid of the skin and the layers underneath and a subfascial lymph system for the muscles and joints. Additionally, both are connected through many little lymphatic vessels perforating the fascia (20).

1.2.2 Physiology

In the capillary system, the blood loses water and many of its solutes, due to the hydrostatic pressure and the permeability of the endothelium at the arterial side of a capillary. This is desirable on the one side, to allow different compartments to interact with each other in terms of hydration and nutrition, but on the other hand this fluid couldn't stay at the interstitial space and has to be brought back to the cardiovascular system. The gradient of the pressure decreases on the way to the venous side and finally it turns around, so water and solutes are pushed back into

the blood. Furthermore, the colloid osmotic pressure inside the capillary gets higher and higher towards the venous side, because big proteins, especially albumin, couldn't pass through the endothelial barrier, leading to a turnaround of the pressure direction also (1). The amount of fluid which stays in the interstitial space gets through the lacking endothelial structure inside the initial lymphatic vessels and stays there. To carry lymph away, different pumping strategies are established. Periodical spontaneous contractions of the vessel's muscles pump it forward. Moreover, an increase of tension inside the vessel causes a myogenic response, which spreads at the surrounding lymphangions. Due to contraction, the diameter of the vessels gets smaller, increasing the inside pressure and so the valves open up, allowing lymph fluid to flow. These mechanisms of transporting are called the intrinsic pump, because they are performed of the LVS itself. Other mechanisms like muscle pump, joint pump or arterial pulsation are used also and are able to increase transport capacity many times over, called the extrinsic pump (16). There are about eight liters of lymph fluid produced per day and so the necessity of a system like the LVS is given. About half of the fluid is reabsorbed in regional lymph nodes and the remaining four liters passes the big lymphatic vessels and gets back through the thoracic duct (1,16).

Lymph of the intestine is also drained into the thoracic duct. This special lymph is called chyle and contains nearly all dietary lipids. Enterocytes absorb these lipids from the intestinal lumen, prepare them for further transport (building of chylomicrons) and finally carry them via exocytosis into the lymphatic vessel, which is established in every villi of the small intestine (18,21).

In terms of the immune system, the LVS plays an important role. Up to 90% of the immune cells, found in lymph, are T-lymphocytes, especially CD4+ effector memory T-cells and dendritic cells. Also, monocytes, granulocytes and B-cells are found (18). Nevertheless, in phases of health, not many cells enter the lymphatic system, but in case of an inflammation the number of cells strongly increases. The impact of cytokines changes the structure of the lymphatic endothelial cells and their junctions, resulting in an eased entry for immune cells into the lymphatic vessels. Amoeboid moving of the cells and a directing chemotaxis are used to guide them

into the LVS (17). Presenting antigens at the lymph nodes and inducing an immediate immunological reaction is a very important function. Happening in this way is much faster than only by migration of immune cells itself and so pathogens can be combated much earlier (16). Interestingly, a reduced lymph flow affects the migration of immune cells only a little, especially because the gradient of chemotaxis is higher during these times, because the messenger agents are washed away lesser by fluid flow (17).

1.2.3 Pathophysiology

If this system starts to fail, fluid is kept in the interstitial tissue. There are two possibilities of failure. First, the capacity of fluid which can be transported, may be surpass the capacity of the LVS, which is called a high-volume-insufficiency. Or, a disruptive factor effects the LVS, so it isn't able to do its work, even if a normal volume has to be cleared. This is called a low-volume-insufficiency (20). In case of failure, during the first period, changes are seen only at the lymphatics itself and not in the soft tissue, so no symptoms are noticed by patients. Single parts of the system like an incompetence of the valves or a destruction of contractile elements leads to a failure of the whole system. At the beginning, these crashes can be compensated and maybe repaired, and the patient never minds anything. But if the process progresses, spreading at the soft tissues is unstoppable and a vicious circle begins to run, a lymphedema is established. A progressive swelling in fat, skin and connective tissue is seen due to stasis of fluid. For one thing, interstitial fluid is a good breeding ground for bacterial and fungal microorganisms and additionally immune response in these defect areas is impaired. Not only of that, also because lymphocytes are not carried away as during normal function, but all together results in chronic inflammation. An inflammatory reaction always releases many cytokines, which are responsible for further fibrosis of the soft tissues in an ongoing chronic process, resulting in an even worse lymphatic function (22). Fatty tissue is also increasing in areas of lymphedema and there are findings exhibiting lymph itself stimulates adipocytes (23).

But not only the local lymphatic system in limbs is affected, also the thoracic duct

shows pathological changes. A study with 32 patients with secondary lymphedema (12 in upper limb, 20 in lower limb) after surgical cancer treatment, injuries or infections, exhibits morphological changes of the terminal thoracic duct, compared with 20 healthy volunteers. Changes were thickened walls, irregularly narrow lymph vessels, significant decreased diameter of thoracic duct and lymph deposits were found in the lumen of thoracic duct (24). These findings lead to the question, if there are maybe primary changes of the lymphatic system, which are compensated during normal life, but in case of a surgery, injury and so on, the system decompensates, and lymphedema occurs. Or, maybe local inflammation and cytokines leading to fibrosis in the limb are washed up with lymph fluid and on their way, they lead to further fibrosis of the bigger central structures like the thoracic duct.

Another study examined lymph flow by means of magnetic resonance imaging (MRI) with contrast agent. 27 patients with primary lymphedema were investigated. Affected limbs were compared to the healthy one. Contrast enhancement, peak time and peak enhancement were significantly decreased in affected limbs and a delay in outflow of inguinal lymph nodes was detected. Furthermore, postcontrast MRI shows abnormal lymph flow pathways within lymph nodes in affected limbs (25).

1.3 Lymphedema

As described above, lymphedema occurs if the LVS is overstained with volume or is structural impaired. Beside many different reasons leading to a lymphedema, the pathology can be divided into two main groups: primary and secondary lymphedema.

1.3.1 Primary lymphedema

1.3.1.1 Definition

„Primary lymphedema is a lymphatic system malformation characterized by swelling of an extremity that can be associated with other lymphatic effusions, due to an underlying developmental anomaly of the lymphatic system (abnormal lymphoangiogenesis). It can be hereditary or not and be congenital or late onset.” (26)

1.3.1.2 Prevalence

Primary lymphedema shows a prevalence of 1-3/10.000, with a female to male-ratio of 3,5 : 1 (22).

1.3.1.3 Classification

Due to medical improvement, different autosomal mutations are found in patients and allow a classification:

- Hereditary Type 1: (congenital) Type Nonne-Milroy, usually symmetrical
- Hereditary Type 2: Type Meige, occurring during puberty
- Idiopathic:
 - Before the age of 35 → Lymphedema præcox
 - After the age of 35 → Lymphedema tardum
- A few other types, which are combined with different syndromes (20)

Furthermore, primary lymphedema can be confined to only one limb, but affection of more limbs is also possible and also head, neck, genitals and breast can be affected. The lower limb is involved more frequently (16,27).

1.3.1.4 Symptoms

Of course, swelling of the limb is the central impairment and during the first phase of establishing the edema, pain and feeling of heaviness or tightness can occur. Due to chronic inflammatory processes as described above, changes of skin like hyperkeratosis, papillomatosis or lymphangiectasia are seen often and also dysplasia of nails occurs. These further clinical pictures implicate a kind of irreversible changes (27).

As it is a systemic disease, other parts of the LVS can be involved too, leading to chylothoraces, pericardial effusions or intestinal lymphangiectasia (27,28).

1.3.2 Secondary lymphedema

This kind of lymphedema is much more common (about 90% of lymphedema) and occurs, if the LVS is damaged in any way. Reasons could be surgery, trauma, inflammation (bacterial, viral, parasites, fungal, uninfected), treatment of cancer (surgery, radiation). In the western world, cancer treatment is the leading cause of secondary lymphedema, whereas worldwide filariasis is the main reason (20,22).

1.3.3 Treatment of lymphedema

Particularly because lymphedema is an incurable disease, continuous treatment is very important to keep the situation under control and so, hold up the patient's quality of life.

1.3.3.1 Conservative therapies

Conservative therapies play the biggest role in treatment. There are many different kinds of techniques to help the patients and all of them are highly variable, so for one patient technique A works, another patient needs a combination of technique C

and D and so on. Some of the therapies can be done by the patient itself and some has to be performed every day.

Compression therapy:

The idea of compression is to diminish ultrafiltration and so the expression of the lymphedema. It is used on the one hand early in disease, to slow down the formation of the lymphedema and also later on and especially after lymphatic drainage, to keep the effect of the therapy for a longer time. Different kinds of treatment are used, such as bandaging, kinesio tape, special garments and intermitted pneumatic compression. Time of compression is also highly variable and could reach from minutes to hours, may need the whole day or is performed during the night (29).

Physiotherapy:

Specially trained physical therapists are performing manual lymphatic drainage (MLD) to mobilize fluid and bring it out of the interstitial space. They use light pressure and go from distal to proximal. Many different kinds of techniques are established, but there is rare evidence if these therapies help or not and which one is the right one and for which type of disease.

There are also techniques, which can be performed by the patient itself, what is handy for them, because they are able to do it at home and don't have to visit to the therapist (29).

1.3.3.2 Surgical therapies

If opportunities of conservative therapy are used up and the lymphedema is still in progression, a surgical intervention can be mentioned. A few techniques are investigated, and the lympho-lymphatic anastomosis shows very good experimental results with about 80% of success. Trials exhibit also, how important an early intervention is, because sclerosis and fibrosis effects the outcome negatively. The first half year after onset of disease seems to be the best time for a surgical intervention.

For patients with lymphedema after breast cancer, the autologous lymph vessel transplantation exhibits at least two third of volume reduction in 60% of the patients. Lymphatic vessels are taken from a leg and are transplanted into the axilla. Patients suffering from unilateral genital- or leg-lymphedema are treated with a lymph vessel transposition. Inguinal lymphatic vessels are prepared at the contralateral leg, at distal groin, and then are transferred to the affected leg (30).

1.3.3.3 Experimental therapies

New approaches of healing are tried and so many different ideas in different trials are found. Various studies suggest that lymph angiogenesis can be stimulated by cytokines like vascular endothelial growth factor C and -D, angiopoietin-1 or fibroblast growth factor. Hepatocyte growth factor (HGF) also affects the lymphatic vascular system, as shown in a study with mice, simulating breast cancer treatment. They showed a postoperative volume reduction at the affected limb and extraanatomical lymph flow after treatment with HGF (22).

Another trial with rats tried a drainage device, after removal of popliteal and inguinal lymph nodes and irradiation. A fenestrated tubing with a miniaturized pumping system was implanted subcutaneously and showed 51% +/- 18 volume reduction, compared with the lymphedematous situation before the implantation (31).

1.3.4 Complications

The most common complications are infections, especially fungal and bacterial origin, relieved of skin changes like papillomatosis, hyperkeratosis and lymphatic fistulas. Particularly an untreated lymphedema may develop malign cancer (Stewart-Treves-Syndrom) (20).

1.3.5 Prognosis

The lymphedema is a chronic disease which is unable to cure. An early beginning of treatment allows the best opportunities for the patient's life, as irreversible changes of skin may be prevented. Nevertheless, a close relationship to the medical team is needed, not only to prevent the patient of physical consequences, but also for his/her mental health (20).

1.4 Skin salt storage

The idea of a salt storage in the skin is, that our body has a buffer system to react on different amounts of sodium intake, beside the kidney's function to excrete acute sodium loads. Different studies exhibit, that skin and its interstitial matrix could be involved in this. Newest findings exhibited a steady state of Na intake and Na excretion over weeks or months, caused by spontaneous rhythmical release of aldosterone and cortisol independent of the current Na intake. These findings are against the previous assumption of an immediate clearance of surplus Na throughout the kidney and leads to the question of a Na storage. These facts were examined during a Mars-flight simulation of once 105 days, and once 205 days (32). During a long-term Na intake, in the first few days, water and Na increase in the same way, but at the end of the study, after 135 days, a difference was seen. The amount of Na was higher, than the amount of water, which would be needed to compensate the osmotical impact of Na. This leads to the assumption, that Na is stored in an osmotically inactive state (33). Underpinned by findings, exhibiting increased Glycosaminoglycans (GAGs) and decreased Prostaglandins (PGs) during high-salt-diet and the reverse effect during low-salt-diet, Na may be linked to GAGs and gets osmotically inactive through this mechanism (34). Another study investigates the impact of high-salt-diet (HSD) on skin-osmolality. First, they described a higher Na-accumulation than water accumulation, underpinning the hypothesis, that Na is stored osmotically inactive. During low-salt-diet (LSD) dermis osmolality was equal to plasma's, but HSD shows a 12mosmol/kg higher osmolality of dermis than in plasma. Contrary, interstitial fluid's osmolality was measured with a subcutaneous wick fluid procedure and was at the same levels as plasma. An osmolyte gradient between epidermis and dermis was determined, which was higher during HSD. Investigation of which osmolytes cause this gradient, exhibits urea as mainly involved, while Na and K weren't responsible for it. A difference between osmolality of plasma and lymph fluid wasn't found (35). Furthermore, during high-salt diet, a weekly rhythm of aldosterone and cortisol driven random Na-excretion occurs, independent of the current salt intake, so any kind of salt storage

have to exist. Also, a decrease in water uptake was seen in contrast to the elevated salt intake. An endogenous water production forced by glucocorticoids is suggested. Another consequence of these slightly increased glucocorticoid levels over a long period (if somebody consumes much Na over a lifetime – industrial nations), are the negative effects of aldosterone as described above. These hormonal influence could lead to chronic diseases like diabetes mellitus, different cardiovascular diseases and so on (34).

In case of a lymphedema and its treatment, the hypothesis is, that during MLD not only water is coming out of the affected limb, also dissolved substances, especially Na, are flushed back into the cardiovascular system. The observation of therapists, that patients have to run to the toilette immediately after MLD but simultaneously are very thirsty, underpins this hypothesis, since both are counter regulations of plasma hyperosmolality.

To take a closer look on this circumstance, seven patients suffering from lymphedema were accompanied during their stay at LKH Wolfsberg and at defined points, blood and urine samples were taken to investigate this hypothesis.

2. Material and Methods

This thesis analysis a part of the collected data of the study “Effects of Orthostatic Loading on Lymphatic Flow and Vascular Function Assessments in Health and Disease”, performed by the Otto Loewi Forschungszentrum of the medical university of Graz, at LKH Wolfsberg (Carinthia).

2.1 Background

The background of the study was, to understand fundamental relationships between the cardiovascular system and its regulation and the impact on lymphatic flow. Particularly inflammation, functions of the vascular endothelium, gravitational effects on the cardiovascular system during orthostasis and hormonal changes were investigated. Further, new findings, exhibiting the possibility of a skin sodium storage, leading to the main subject of this thesis.

2.2 Study design

The patients were at LKH Wolfsberg, a specialized clinic for lymphatic diseases, for three weeks. During this stay, they were treated in all different fields, which were food and dietary, moderate physical activities and different physical therapies, especially manual lymphatic drainage.

In this study, lymphatic and vascular stress responses to orthostatic challenge were studied in two cohorts (healthy subjects and patients with lymphedema). The impact of treating lymphedema (MLD) on lymphatic (assessed by hyaluronic acid estimations) and endothelial function (assessed by flow mediated dilatation in the brachial artery using ultrasonography, retinal imaging and blood sampling), cardiovascular autonomic function (via heart rate variability), cerebral blood flow, hormonal responses and blood coagulation were evaluated.

For this thesis, I got all values, concerning water and electrolyte regulation, from the department for analysis.

2.3 Study population

For this study, 16 patients were examined. Especially for this thesis, it was possible to use data of seven patients, from six of them a complete set of data was made, one patient broke up after the second data collection, so 32 sets of before and after-values were collected.

The patients were at the age of 46 to 70 years, two men, five women.

All the patients in this study gave verbal and written informed consent. Research was performed in accordance with the Declaration of Helsinki (1989) of the WMA.

2.3.1 Inclusion criteria

All Patients with clinical diagnosed lymphedema.

2.3.2 Exclusion criteria

Other forms of edema, which are lipedema, edema of cardiac backlog and other forms of secondary lymphedema.

2.4 Protocol

At day one, day two, day seven, day 14, and day 21 blood and urine samples were taken before and after MLD. MLD was performed by different therapists. Due to the very tight schedule of the patients and the primary motivation to analyze various parameters and their behavior, it was not possible to adapt the daily routine of Wolfsberg's procedures on perfect and strict sampling-conditions. The blood collection before MLD was done in another room, in another floor, so the patients have to walk between collection and MLD. After the MLD, the patients have to go the same way back, to do the blood sampling for the after-values. Additionally, between blood collection and MLD, a sit-to-stand test (five minutes sitting, six minutes standing, and again five minutes sitting) was performed to collect orthostatic data.

2.5 Measurements and lab methods

2.5.1 Electrolytes, Hormones of water and electrolyte regulation

These parameters are standard parameter and were measured by routine labor.

2.5.2 Aldosterone

Aldosterone was quantified with an ELISA-kit of Crystal Chem High Performance Assays. This kit uses an antigen-antibody-technique with a few steps of incubating and washing out, and finally the Aldosterone-parameter can be measured by colour intensity. (Catalog #80951)

2.5.3 Plasma renin activity

Plasma renin activity (PRA) was quantified with an ELISA-kit of Crystal Chem High Performance Assays. PRA is quantified via measuring the built of Angiotensin I after a defined period of time. (Catalog #80970)

2.5.4 Aldosterone-Renin-Ratio

To calculate the ARR, aldosterone-concentration was divided by plasma renin activity (9).

$ARR = \text{Aldosterone-concentration} / \text{Plasma Renin Activity}$

2.5.5 Correction of values

To analyze the data, it was necessary to do a correction of the values which were taken after the MLD. Due to the incoming fluid of MLD and accompanying plasma volume changes, parameters may be affected and get false low. These changes were corrected with Van Beaumont's formula. Dill's correction was also to choice, but is more fitting in situations of plasma volume loss and active body exercise (36–38).

2.5.6 Plasma volume

To calculate plasma volume and so plasma volume changes (PVC), first of all, the blood volume was calculated by Nadler's formula (39). This calculated blood value was multiplied with the measured hematocrit, and so the volume of blood cells was generated. Finally, the volume of the blood cells has to be subtracted of the blood volume to get the plasma volume.

Some measurements resulted in a negative PVC. This circumstance may be caused by the way, PV was calculated and the difficult sampling conditions. Since the hypothesis is, that MLD washes fluid from the limb back into the body, underpinned by clinical observation of urgency, it would have made no sense, to use a negative PVC in this analysis. Also, very small PVC's, of a few mL, but huge changes of electrolyte concentrations at the same time weren't used, because that would have meant, that an extremely high concentrated fluid was washed in. A cutoff of PVC <20 mL was defined to be crossed out.

2.5.7 Sodium-Protein correction

As plasma proteins may affect the measurement of Na, a corrected Plasma Na-value was used for all analysis (40). The correction-formula is:

$$\text{Na-C} = (\text{Na-M} / 0,93 * (100 - \text{TPP})) / 100$$

Na-C...corrected Na concentration (mmol/L)

Na-M...measured Na concentration (mmol/L)

TPP...Total plasma protein (g/dL)

2.6 Statistical evaluation

To analyze the data, first of all, a Shapiro-Wilk-test was performed, to check, if the analyzed data show a normal distribution. Data with a normal distribution was analyzed with a paired or unpaired t-test, depending on the sample. Not normal distributed data was analyzed with the Wilcoxon-test. Correlations were investigated with Pearson-Correlation.

A p-value of $< 0,05$ was defined to be statistically significant.

For analysis, Graphpad Prism 8 was used.

3. Results

At all, seven patients with lymphedema were tested five times. One of the patients broke up after the second measurement, so 32 sets of before- and after-datasets were made overall.

3.1 Incoming fluid

PV before and after MLD was estimated as described above ([Plasma volume](#)). Further, plasma electrolyte concentrations of Na, Cl, K and Ca were measured, and so, it was possible to calculate the electrolyte concentration of the incoming fluid with the following formula:

$$EC = (PV2 * C2 - PV1 * C1) / (PV2 - PV1)$$

EC...Electrolyte concentration of the incoming fluid (mmol/L)

PV1...Plasma volume before (L)

PV2...Plasma volume after (L)

C1...Plasma electrolyte concentration before (mmol/L)

C2...Plasma electrolyte concentration after (mmol/L)

After removing all samples which shows a negative PV-balance, and also those with a very small PV-gap but a very big change in plasma electrolyte level (Reasons see at [Plasma volume](#)), 20 usable samples of Na and 18 of Cl are remaining:

Table 1 - Fluid and Electrolyte changes

		PV in L	PVC in mL	Na-Delta	Na in mmol/L	Cl-Delta	Cl in mmol/L
1.	before	3,27					
	after	3,34	72,00	0,34	155,69	2,07	195,03
2.	before	2,28					
	after	2,40	118,00	9,27	323,28	8,49	268,73
3.	before	2,35					
	after	2,41	63,00	2,69	238,26	-0,53	neg.
4.	before	3,00					
	after	3,06	61,00	1,92	236,10	2,75	241,76
5.	before	2,99					
	after	3,09	97,00	6,85	358,34	3,64	221,94
6.	before	2,96					
	after	3,02	61,00	3,61	317,54	3,77	290,59
7.	before	3,02					
	after	3,07	51,00	3,88	372,86	2,85	276,17
8.	before	2,93					
	after	2,99	66,00	2,51	255,61	1,77	186,41
9.	before	2,16					
	after	2,22	55,00	1,52	199,08	0,46	118,51
10.	before	2,18					
	after	2,20	26,00	-4,36	neg.	-1,98	neg.
11.	before	2,21					
	after	2,23	23,00	-5,20	neg.	-4,98	neg.
12.	before	2,10					
	after	2,16	55,00	0,19	142,92	-1,64	neg.
13.	before	3,16					
	after	3,23	67,00	3,52	311,09	0,40	124,50
14.	before	3,10					
	after	3,23	128,00	7,38	327,85	4,76	222,00
15.	before	3,18					
	after	3,31	121,00	6,47	317,61	2,99	185,79
16.	before	3,16					
	after	3,29	130,00	7,25	325,15	5,09	232,95
17.	before	3,13					
	after	3,20	70,00	1,87	226,21	3,49	262,19
18.	before	2,75					
	after	2,81	57,00	3,07	290,91	2,33	216,53
19.	before	2,72					
	after	2,83	111,00	4,70	260,28	3,72	196,71
20.	before	2,15					
	after	2,24	85,00	2,64	211,41	1,78	149,85
21.	before	2,17					
	after	2,21	40,00	0,15	151,86	1,44	182,29
22.	before	2,20					
	after	2,30	92,00	4,43	252,24	4,07	203,54

As shown by table 1 and table 2, the Na concentration exhibits in most cases a clear increase after MLD, keeping in mind, that Na is a very tight regulated parameter.

The changes of plasma Na concentration compared to the amount of incoming fluid shows a very good and significant correlation. ($r=0.88$, $0.72-0.94$, $p<0.001$)

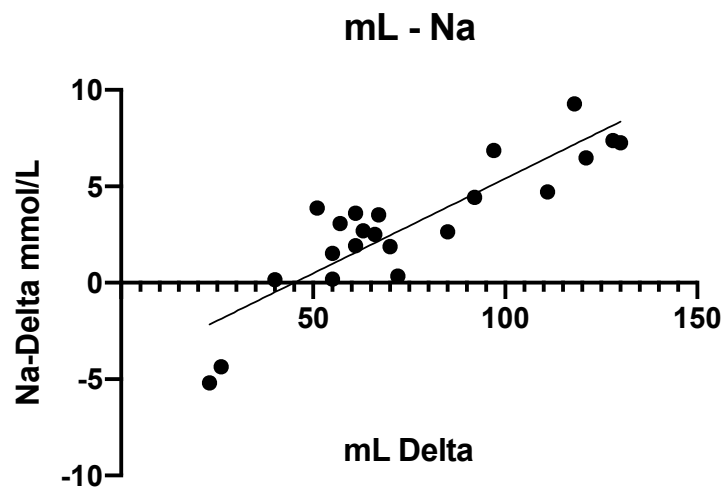


Figure 2 - Na-changes compared to the amount of incoming fluid

Another indication for an incoming hyperosmolar fluid is the significant elevation of Cl, $r=0.77$, $0.51-0.89$, $p<0.001$.

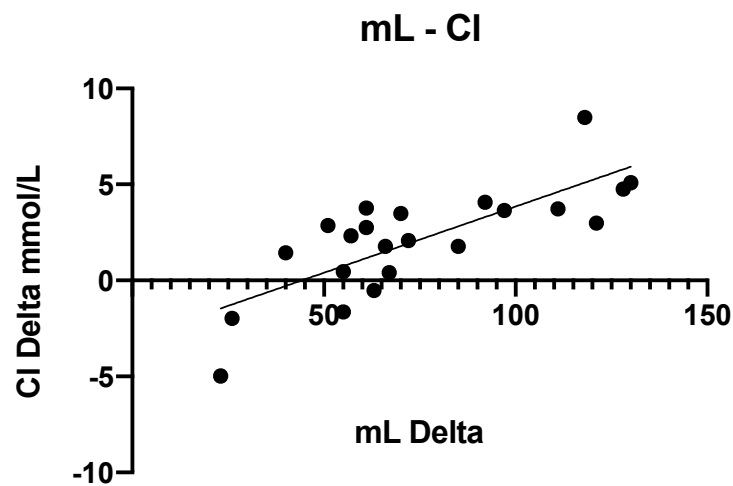


Figure 3 - Cl-changes compared to the amount of incoming fluid

Cl increases the same way as Na does, as shown by the following graph. Pearson correlation between Na- and Cl-changes (Figure 4): $r=0.92$, $0.83-0.96$, $p<0.001$.

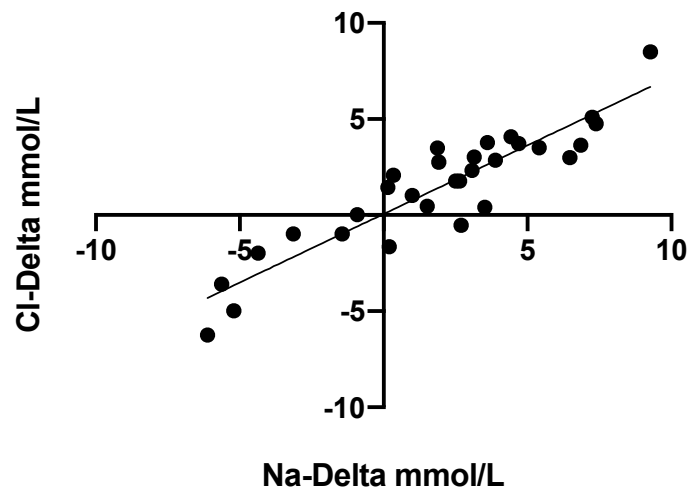


Figure 4 – Plasma Sodium- and Chloride-Delta changes

These findings lead to the assumption, that skin and the interstitium contains and maintains a very hypertonic and salty environment, and due to the MLD washes this high concentrated fluid in the cardiovascular system these changes may appear.

While a good correlation is seen between Na and Cl and the amount of incoming fluid and also with each and other, K ($r=0.12$, $-0.32-0.52$, $p=0.6$), Ca ($r=0.4$, $-0.06-0.71$, $p=0.083$) and Albumin ($r=0.05$, $-0.38-0.46$, $p=0.82$) don't show similar changes in a related form.

Albumin exhibits the weakest changes compared to PVC. Although, analyzing all samples with a positive PVC, there is a mean increase of Albumin of $0.134 \text{ g/dL} \pm 0.154$.

3.2 Hormone changes caused by sodium

Since these huge amounts of electrolytes are washed into the system, it's obvious that hormones, which regulate these electrolytes may change too. In this case, Na seems to be the most important ion, so hormone systems which are part of its regulation were investigated.

3.2.1 RAAS

Since the Renin-Angiotensin-Aldosterone-System is the most important regulator of blood pressure, there may be reactions, caused by fluid and electrolyte load.

Table 2 - Sodium and RAAS changes

		Plasma Na mmol/L	Na-Delta	Aldosteron ng/dL	Aldosteron-Delta	PRA ng/mL/h	PRA-Delta	ARR	ARR-Delta
1.	before	143,21		8,13		0,54		15,12	
	after	148,62	5,41	6,11	-2,01	0,40	-0,14	15,29	0,16
2.	before	140,27		4,76		0,27		17,76	
	after	143,43	3,15	8,33	3,57	0,39	0,12	21,32	3,56
3.	before	136,93		5,85		0,35		16,74	
	after			3,12	-2,73	0,38	0,03	8,22	-8,52
4.	before	139,80		7,59		0,38		19,92	
	after	140,14	0,34	2,80	-4,78	0,35	-0,03	7,91	-12,01
5.	before	142,54		2,85		0,31		9,26	
	after	141,10	-1,44	1,70	-1,15	0,49	0,18	3,49	-5,77
6.	before	134,64		3,86		0,28		13,80	
	after	143,91	9,27	0,99	-2,87	0,32	0,04	3,10	-10,70
7.	before	135,39		3,13		0,26		11,95	
	after	138,07	2,69	1,56	-1,57	0,26	-0,01	6,09	-5,86
8.	before	139,91		0,70		0,56		1,26	
	after	141,83	1,92	0,98	0,28	0,58	0,02	1,70	0,43
9.	before	140,23		0,53		1,02		0,52	
	after	147,07	6,85	0,65	0,13	0,57	-0,45	1,15	0,64
10.	before	139,16		0,61		0,50		1,22	
	after	142,77	3,61	0,54	-0,07	0,58	0,08	0,93	-0,29
11.	before	139,66		0,43		0,96		0,45	
	after	143,54	3,88	0,49	0,06	0,66	-0,31	0,75	0,30
12.	before	141,74		0,33		1,33		0,25	
	after	144,25	2,51	1,31	0,98	0,91	-0,42	1,43	1,18
13.	before	137,91		0,82		0,99		0,83	
	after	139,43	1,52	0,64	-0,18	1,43	0,44	0,45	-0,38
14.	before	136,75		0,86		0,77		1,12	
	after	132,39	-4,36	0,52	-0,34	1,23	0,46	0,42	-0,70
15.	before	137,10		0,70		0,67		1,05	
	after	131,91	-5,20	0,57	-0,13	1,02	0,35	0,56	-0,49
16.	before	135,50		0,75		0,85		0,88	
	after	135,69	0,19	0,47	-0,28	1,04	0,19	0,46	-0,42
17.	before	135,52		0,81		0,62		1,31	
	after			0,60	-0,21	0,99	0,37	0,61	-0,70
18.	before	141,40		0,54		0,29		1,85	
	after	144,92	3,52	1,77	1,23	0,17	-0,12	10,44	8,58
19.	before	141,88		2,07		0,37		5,55	
	after	149,26	7,38	2,78	0,70	0,18	-0,20	15,77	10,22
20.	before	140,99		1,71		0,40		4,28	
	after	147,46	6,47	1,62	-0,09	0,27	-0,13	5,97	1,69
21.	before	141,54		1,09		0,17		6,24	
	after	148,79	7,25	1,28	0,19	0,29	0,12	4,41	-1,83
22.	before	140,94		1,10		0,36		3,04	
	after	142,81	1,87	0,97	-0,13	0,36	0,00	2,71	-0,34
23.	before	141,12		6,73		2,06		3,26	
	after	135,49	-5,63	3,81	-2,92	4,60	2,54	0,83	-2,43
24.	before	139,60		3,16		2,24		1,41	
	after	140,59	0,99	1,26	-1,90	0,97	-1,27	1,29	-0,12
25.	before	139,89		4,17		1,86		2,25	
	after	142,96	3,07	7,45	3,28	2,73	0,88	2,73	0,48
26.	before	141,33		8,78		2,47		3,55	
	after	138,19	-3,14	4,21	-4,57	2,62	0,15	1,61	-1,95
27.	before	140,60		5,12		1,79		2,86	
	after	145,30	4,70	3,56	-1,57	2,94	1,15	1,21	-1,65
28.	before	143,32		0,89		0,34		2,59	
	after	137,20	-6,12	0,53	-0,36	0,62	0,28	0,85	-1,74
29.	before	141,89		0,83		0,49		1,70	
	after	144,53	2,64	0,50	-0,33	0,39	-0,10	1,27	-0,43
30.	before	143,52		1,06		1,17		0,91	
	after	143,67	0,15	0,32	-0,74	0,91	-0,26	0,35	-0,56
31.	before	138,04		1,08		0,32		3,39	
	after	137,12	-0,92	0,22	-0,87	0,40	0,08	0,54	-2,85
32.	before	141,57		0,99		0,52		1,92	
	after	146,01	4,43	0,41	-0,58	0,51	-0,01	0,81	-1,11

As the initial part of the system, Plasma Renin Activity, has to increase, if there are any notable changes. Shown in Figure 5, PRA is changing not very effectively, if correlated with PVC ($r=-0.13$, $-0.50-0.28$, $p=0.536$). PRA correlated with changes of Plasma-Na, exhibits an even better and in particular significant correlation ($r=-0.4$, $-0.66- -0.05$, $p= 0.029$) (Figure 6).

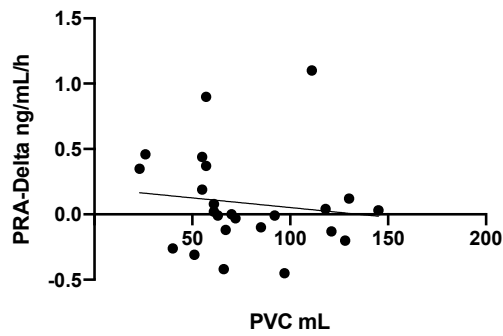


Figure 5 – PRA changes, depending on PVC

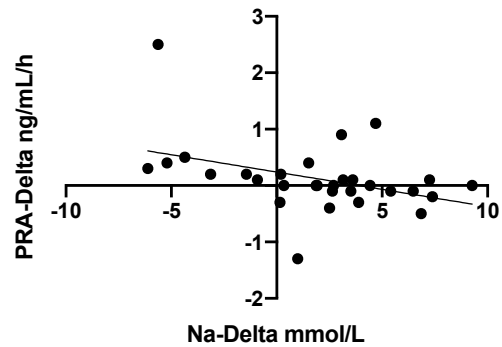


Figure 6 - PRA changes, depending on Sodium change

The Aldosteron reaction is ambivalent. Looking on Figure 7, the overall reaction, which is seen, is, that the more fluid is coming in, the less Aldosteron is released ($r=-0.26$, $-0.59-0.16$, $p=0.221$). But, taking a look on the reaction of Aldosteron, depending on Na-change, the opposite happens. The more Na is washed in, the more Aldosteron is released ($r=0.23$, $-0.14-0.54$, $p=0.219$). Both trends and distributions are not significant, and the single values are widely spread too (subject of discussion in [Protocol](#)).

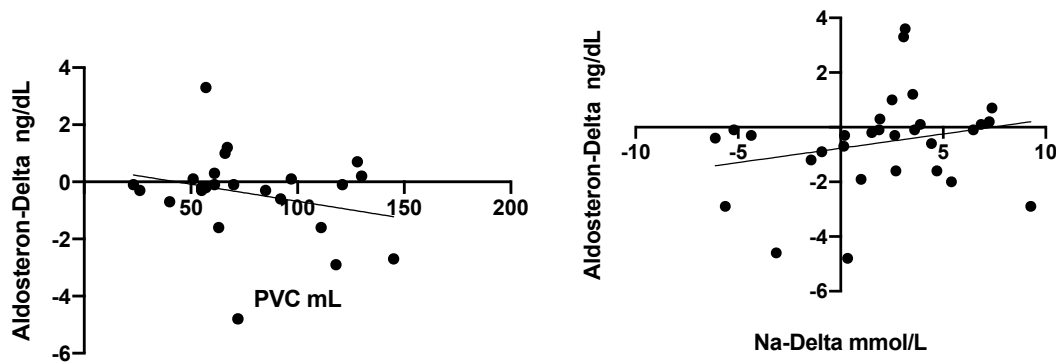


Figure 7 - Aldosteron-Delta, depending on PVC Figure 8 - Aldosteron-Delta, depending on Sodium change

A correlation between PRA and Aldosteron isn't given, as the following graph shows. ($r=0.055$, $-0.34-0.44$, $p=0.79$)

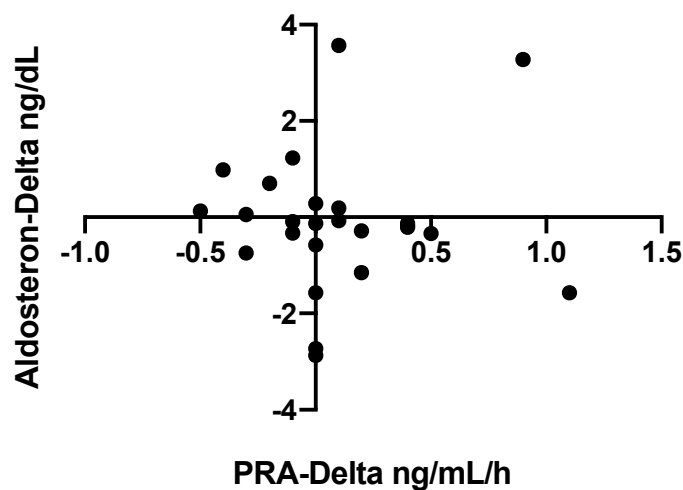
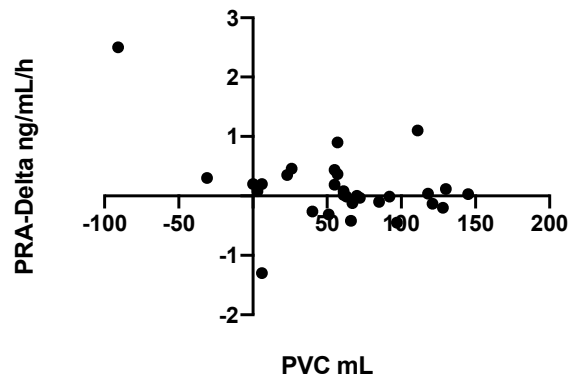


Figure 9 – Correlation between PRA- and Aldosteron-changes

Calculating the ARR, the values spread also widely, correlated with PVC ($r = -0.11$, $-0.5-0.3$, $p=0.588$). A correlation with the change of Plasma Na concentration exhibits a correlation-coefficient of 0.17 , $-0.20-0.49$, $p=0.372$.

Finally, looking on the values without doing the PVC-cutoff of <20 mL, the following results are exhibiting.

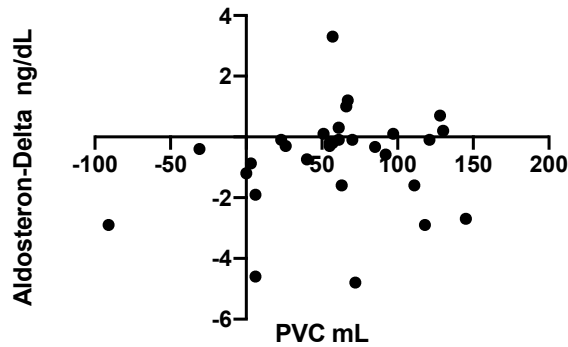


$r = -0.39$, $-0.66-0.036$, $p=0.033$

Figure 10 - PRA-Delta, depending on PVC

PRA shows a better correlation ($r=-0.39$), compared to $r=-0.13$ (with <20 mL cutoff). But this circumstance results only because of the one value in the upper left quadrant. Removing them as 'statistical outlier', results in a r -value of 0.014 , $p=0.94$.

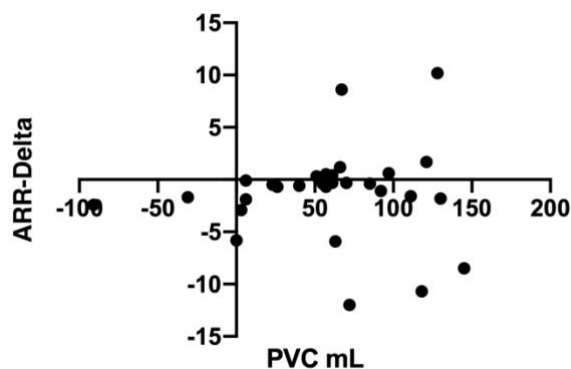
The r-value of Aldosteron-Delta, depending on PVC without the cutoff shows a similar poor correlation of 0.18 (Figure 11) but in the opposite way, compared to the 20mL-cutoff-r-value of -0.26.



$r = 0.18, -0.20-0.50, p=0.353$

Figure 11 - Aldosterone-Delta, depending on PVC

Completing this analysis, the r-value of ARR-Delta, depending on PVC without 20mL-Cutoff is 0.038, similar to the 20mL-cutoff-r-value of -0.11, but as Aldosterone, the opposite way.



$r = 0.038, -0.33-0.39, p=0.841$

Figure 12 - ARR-Delta, depending on PVC

Closing this RAAS-analysis, these outstanding insignificant hormonal changes, compared to PVC and Na load, have to be highlighted, keeping in mind, that the overall doctrine of RAAS describes a direct control loop of these parameters.

3.2.2 ANP

There are no significant changes of ANP correlated with PVC and Na change ($r=-0.02$, $-0.42-0.39$, $p=0.931$ and $r=-0.08$, $-0.28-0.43$, $p=0.670$).

3.2.3 AVP

There are no significant changes of AVP correlated with PVC and Na change ($r=0.38$, $-0.05-0.69$, $p=0.08$ and $r=0.16$, $-0.24-0.51$, $p=0.43$).

3.2.4 Pro-BNP

There are no significant changes of Pro-BNP correlated with PVC and Na change ($r=-0.17$, $-0.54-0.24$, $p=0.419$ and $r=0.075$, $-0.43-0.30$, $p=0.701$).

4. Discussion

4.1 Sampling

4.1.1 Protocol

One of the biggest points of criticism is the way sampling was done. The patients had to walk long distances inside the hospital, first for getting the initial value, then the massage. The rooms for these interventions were on different floors. Additionally, between the first sampling and massage, other tests, like a sit to stand test, were performed to get also information about orthostatic stress and its regulation. After the MLD, it would be ideal to take the second blood sample right at the lying position, but instead, the patients had to walk back downstairs in the other room. A sit-to-stand-test was performed again. The combination of all these movements and walking ways may had a big impact on Aldosterone and the whole RAAS-regulation (9). There is also no information about the patient's medication and definitely no wash-out period of concerning agents occurred (9). According to these points, the findings relating to RAAS have to be critical questioned and should be mentioned definitely in further studies.

4.1.2 Baseline

Another point of criticism is the missing baseline of all values. Changes of different electrolytes occur more or less considerable, but without knowing how the individual patient reacts on circumstances coming up by sampling and lying around half an hour during massage, no exact effect and further on statistical significance can be described. So, in following studies, the patients should run a "baseline-round", at which all parameters and needed movements are done, but without performing the MLD. It will be possible to assess the single effects of MLD even better, without having different biases coming up by movement and orthostatic stress.

4.1.3 Calculation of Plasma Volume change

A limitation of this calculation is a negative PV-balance, because of the underlying idea, MLD shouldn't shift volume from the body in the affected legs, but the opposite way. Also, very little changes in PV-balance may result in false-high and unrealistic electrolyte concentrations of the incoming fluid. In this case, it is obvious, that the estimated PV-balance isn't correct.

Since the clinical examination leads to the assumption, that fluid is going from the limb back into the body, a negative PV-balance at all is a fact, that should be questioned precisely. This leads to the way PVC was determined as a whole. Direct measuring of the incoming fluid isn't possible. Therefore an invasive device inside the vena iliaca communis of the affected side and also inside the ductus thoracicus would be needed, because nobody knows exactly where and how much of lymph fluid is coming back, especially during this kind of stress (16,17). So, the other way, calculating the PVC by means of changes in hematocrit is a logical, but vulnerable method. Vulnerable, due to a very long period of time between the before-blood-collection and the after-blood-collection, of about two hours. During this time, the body is able to react on these volume changes. On the one hand, to shift the fluid anywhere else into the interstitium and on the other hand, the kidneys do their job, as seen by a massive urgency after the MLD. So, because of these circumstances, the PVC might be underestimated, which leads to false-high electrolyte concentrations of the incoming fluid. The same bias applies for electrolyte regulation also, which would result in a false-low electrolyte concentration of the incoming fluid. Maybe both mechanisms cancel each other, but that would be pure speculation.

The fact, that a significant PRA-decrease correlated with Na changes is seen (Figure 6), leads to the circumstance, that something is happening at all. To evaluate if Na alone, a considerable amount of fluid or something else is the trigger, further investigations have to be done.

4.2 RAAS contradiction

The results from RAAS analysis are very interesting but also astonishing. While the reaction of PRA to an increase of Na was as expected, the reaction of Aldosteron to the decrease of PRA wasn't. A decrease of PRA should be followed by a decrease of Aldosteron, but instead, there were widely spread values without any significance. On the one hand, RAAS couldn't be approved as a control loop (Figure 9) and on the other hand, these findings raise the question, if there is another factor, affecting Aldosteron. First, Na was discussed as influence factor. The fact that Aldosteron causes an increase of Na and water retention, leads to the consideration, that an acute Na load causes a decrease of Aldosteron. But as seen in Figure 8, no correlation between these two values is seen. Similar findings were seen in further studies, in which also hormonal changes after various orthostatic stress was investigated (41,42). These findings need further investigations and especially more proper examination conditions, to get valid Aldosterone values (discussed at [Sampling](#)).

4.3 The therapist's impact

The changes of K were investigated due to the question, if MLD affects single cells negatively in their function, especially muscle cells and erythrocytes. If this would be the case, the increases of Na and Cl may result from these cell damages, although intracellular concentration of Na and Cl isn't very high. But as this analyze exhibits, Na and Cl increase much stronger as K, which leads to the assumption, that these Na- and Cl-ions has their origin somewhere else, keeping the intracellular electrolyte-ratios in mind.

It was not possible to identify the origin of the K-changes. The fact of changing K-behavior, sometimes an increase, sometimes a decrease, couldn't be traced back on single patients or on the manner of different therapists.

As K is a very important ion in terms of myocardial function, a baseline-blood-collection would provide clarification if these changes were normal fluctuations or a result of MLD.

5. Conclusion

In this theses, the human's electrolyte and water homeostasis during acute fluid load, caused by a manual lymphatic drainage, was investigated, to get more information about the composition of the lymphatic fluid. The different electrolyte fluctuations, especially Na and Cl, could indicate relevant concentrations in the massaged lymph fluid and give rise to further investigations in this field.

It was seen that this kind of investigation needs strict conditions to enable confident statements. A study with more patients and a more stringent protocol would allow a deeper insight into this topic.

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