Dissertation

Effects of Physical Therapy on Fluid Mobilization, Hemodynamic Responses and Vascular Function in Lower Limb Lymphedema Patients

submitted by

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STATUTORY DECLARATION

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Guidelines of the Medical University of Graz on Good Scientific Practice “.

Graz, January 2021 e.h. Bianca Brix

DISCLOSURE

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LIST OF ABBREVIATIONS

A-to-V	Arteriolar-to-venous ratio
ADMA	Asymmetric dimethylarginine
BCRL	Breast cancer related lymphedema
BIS	Bioelectrical impedance spectroscopy
BMI	Body mass index
CDT	Complete decongestive therapy
CO	Cardiac Output
CRAE	Central retinal arteriolar equivalent
CRVE	Central retinal venular equivalent
DBP	Diastolic blood pressure
ECF	Extracellular fluid
FMD	Flow mediated dilatation
HCT	Hematocrit
HB	Hemoglobin
HDL	High-density lipoprotein
HR	Heart rate
ICF	Intracellular fluid
LVA	Lymphovenous anastomosis
MBP	Mean blood pressure
MetS	Metabolic Syndrome
MLD	Manual lymphatic drainage
MSOT	Multispectral optoacoustic tomography
NO	Nitric oxide
PD	Plasma density
pHA	Plasma hyaluronic acid
PV	Plasma volume
PVC	Plasma volume change
PWVcf	Carotid-femoral pulse wave velocity
QoL	Quality of life
RCT	Reverse cholesterol transport

SBP	Systolic blood pressure
SD	Standard deviation
SV	Stroke volume
TBW	Total body water
TPR	Total peripheral resistance
VEGF-C	Vascular endothelial growth factor-C
VEGFR-2	Vascular endothelial growth factor receptor-2
VLNT	Vascularized lymph node transfer

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KURZFASSUNG

Ziele: Das Lymphödem ist charakterisiert durch eine massive Gewebeschwellung aufgrund einer Dysfunktion des lymphatischen Systems. Die komplexe physikalische Entstauungstherapie (KPE) ist eine dreiwöchige physikalische Therapie, die aus manueller Lymphdrainage (MLD) sowie Kompressionsbandagen besteht. Sie zielt darauf ab die Lymphflüssigkeit zu mobilisieren und Volumen zu reduzieren. Obwohl bekannt ist, dass Lymphflüssigkeit in den Blutkreislauf zurückgeführt wird, ist nicht erforscht, wie der Körper auf die während der physikalischen Therapie mobilisierte überschüssige Flüssigkeit reagiert. Daher zielte diese Dissertation darauf ab, die Wirkung von KPE auf Flüssigkeitsverschiebungen, hämodynamische Reaktionen sowie die vaskuläre Funktion systematisch über drei Wochen und aufgrund der MLD zu untersuchen.

Methoden: Die Messungen wurden am 1., 2., 7., 14. und 21. Tage der KPE, vor - und nach - der MLD bei 13 Beinlymphödem-Patienten im Stadium II durchgeführt. Perometrie und bioelektrische Impedanzspektroskopie wurden zur Untersuchung von Beinvolumen, segmentaler- und Ganzkörper-Flüssigkeitszusammensetzung (Gesamtkörperwasser (limbTBW, %TBW), extrazelluläre (limbECF, %ECF) und intrazelluläre (limbICF, %ICF) Flüssigkeit, ECF/ICF und limbECF/limbICF) verwendet. Der Plasma-Hyaluronsäurespiegel (pHA), die Plasma-Volumenänderungen (PVC) und die Konzentrationen verschiedener Plasmakomponenten wurden im Blut bestimmt. Die hämodynamischen Reaktionen wurden während eines Sitzen-zu-Stehen-Tests, bestehend aus Baseline (im Sitzen), Stehen und einer Erholungsphase, kontinuierlich gemessen. Zur Bestimmung der endothelialen/vaskulären (Dys-)Funktion wurden Plasma Asymmetrisches Dimethylarginin (ADMA) Konzentrationen, die karotisch-femorale Pulswellengeschwindigkeit (PWVcf), die brachiale flussvermittelte Dilatation (FMD) und die retinale Mikrovaskulatur untersucht.

Ergebnisse: Das Beinvolumen, limbECF, limbICF, limbECF/limbICF, %TBW und %ECF nahmen während der dreiwöchigen KPE signifikant ab. Die größten Reduktionen traten innerhalb der ersten Woche der KPE auf. Die MLD führte zu einem signifikanten Anstieg von limbICF, %TBW und %ICF, während ECF/ICF aufgrund der MLD zurückging ($p=0.003$). Plasmavolumen, Albumin und Albumin/Globulin-Ratio stiegen nach der MLD signifikant an. Die MLD führte weiterhin zu einem Anstieg der weißen Blutkörperchen (Neutrophile) sowie zu einer Abnahme der eosinophilen Blutzellen. Die pHA-Werte veränderten sich durch die

Lymphödembehandlung nicht. Die dreiwöchige KPE-Behandlung führte zu einer Reduktion des diastolischen Ruheblutdrucks (p=0.048). Die Ergebnisse zeigten eine signifikant niedrigere Herzfrequenz während der orthostatischen Belastung nach der MLD an Tag 14 und Tag 21 der KPE. Obwohl Lymphödem-Patienten zu Beginn der Studie keine erhöhten Werte aufwiesen, reduzierte sich das ADMA aufgrund der MLD signifikant.

Schlussfolgerungen: Die physikalische Therapie führt bei Lymphödem-Patienten zu Flüssigkeitsverschiebungen, wobei die größten Effekte innerhalb der ersten Woche der Therapie auftreten. Flüssigkeitsverschiebungen durch die physikalische Therapie spiegelten sich auch in einem erhöhten Plasmavolumen, einer erhöhten Proteinkonzentration und einer veränderten Zellzahl wider. Der Hyaluronsäurespiegel im Plasma ist möglicherweise kein geeigneter Biomarker für den Lymphfluss bei Lymphödempatienten, die sich einer Therapie unterziehen. Lymphödempatienten scheinen kein höheres Risiko für orthostatische Unverträglichkeit und Stürze zu haben. Obwohl Lymphödempatienten zu Beginn der Therapie keine Anzeichen einer Endothelfunktion zeigen, scheint die manuelle Lymphdrainage einen positiven Effekt zu haben, da sie zu einer Senkung des AMDA-Spiegels führte.

Schlagwörter: Lymphatische Funktionsstörungen; komplexe physikalische Entstauungstherapie; manuelle Lymphdrainage; Flüssigkeitsmobilisierung; kardiovaskuläre Veränderungen; Blutdruckregulation; Endothelfunktion;

ABSTRACT

Purpose: Lymphedema is characterized by massive tissue swelling due to lymphatic system dysfunction. Complete decongestive therapy (CDT), a three-week physical therapy, consists of manual lymphatic drainage (MLD) and tissue compression. It aims at mobilizing lymphatic fluid and at volume reduction. Although lymphatic fluid is known to be returned into the blood stream, it is not yet known how the body copes with the excess fluid mobilized during physical therapy. Therefore, this thesis aimed at investigating the effect of CDT on fluid shifts, hemodynamic responses as well as vascular function systematically over three weeks of CDT and due to MLD.

Methodology: Measurements were conducted on day 1, 2, 7, 14 and 21 of CDT, before - and after - MLD in 13 patients with primary and secondary stage II lower lymphedema. Perometry and bioelectrical impedance spectroscopy were used to investigate limb volume, segmental and whole-body fluid composition (total body water (limbTBW, %TBW), extracellular (limbECF, %ECF) and intracellular (limbICF, %ICF fluid), as well as ECF/ICF & limbECF/limbICF). Blood samples were collected to assess plasma hyaluronic acid levels (pHA), plasma volume changes (PVC) and plasma component concentrations. Hemodynamic responses were evaluated continuously during a sit-to-stand test, consisting of seated baseline, standing and a recovery phase. Plasma asymmetric dimethylarginine (ADMA) levels, carotid-femoral pulse wave velocity (PWVcf), brachial flow mediated dilatation (FMD) and retinal microvasculature analysis were assessed to determine endothelial/vascular (dys-)function.

Results: Leg volume, limbECF, limbICF, limbECF/limbICF, %TBW and %ECF significantly decreased over three weeks of CDT. The greatest reductions occurred within the first week of CDT. MLD led to significant increases in limbICF, %TBW and %ICF, whereas ECF/ICF reduced due to MLD. Plasma volume, albumin and albumin to globulin ratio increased significantly post-MLD. MLD further led to the increase in white blood cell and neutrophils as well as a decrease in eosinophils. pHA levels did not change due to lymphedema therapy. Three weeks of CDT led to a significant reduction in resting diastolic blood pressure. Results further showed significantly lower heart rate during orthostatic loading post-MLD on day 14 and day 21 of CDT. Although lymphedema patients did not show elevated levels at baseline, ADMA reduced significantly due to MLD.

Conclusion: Complete decongestive therapy leads to fluid shifts in lymphedema patients. The major fluid shift effects occur within the first week of therapy. Fluid shifts due to lymphedema therapy were reflected in plasma volume and protein concentration increases as well as cell counts changes. Plasma hyaluronic acid levels might not be a suitable biomarker for lymphatic flow in lymphedema patients undergoing therapy. Lymphedema patients do not seem to be at a higher risk for orthostatic intolerance and falls. Although, lymphedema patients do not seem to show signs of endothelial function at baseline, manual lymphatic drainage seems to have a beneficial effect, as it led to reductions in AMDA levels.

Key words: Lymphatic disorders; complete decongestive therapy; manual lymphatic drainage; fluid mobilization; cardiovascular changes; blood pressure regulation; endothelial function;

1 INTRODUCTION

1.1 Problem statement

Distinct from the cardiovascular system, the adjunct lymphatic vasculature consists of another network of vessels. This vasculature plays an important, but not yet well-recognized and still poorly understood, role in normal physiology (1, 2). Principal functions of the lymphatic vessels include tissue pressure regulation, immune functions, absorption of dietary fat in the intestines, and the maintenance of fluid and colloid homeostasis through a network of open-ended vessels (3, 4). The lymphatic capillaries start blind-ended and are responsible for taking up a protein-rich lymphatic fluid, the exudate from the blood vessels. Blood plasma is continuously filtered from blood capillaries into the interstitial space via the dynamics of blood pressure and osmotic pressure (5). The permeable lymphatic endothelial cell junctions of the lymphatic capillaries enable the movement of interstitial fluid as well as macromolecules towards the lymphatic vessels. Lymphatic fluid is returned into the blood stream via an increasingly larger hierarchy of lymphatic vessels, reaching the blood circulation ultimately via the thoracic duct (4). There are different routes from the lymphatics to the blood circulation as well as different concentrations of lymph fluid described (3). Lymphatic fluid is transported from the initial lymphatic structure via an increasing hierarchy of lymphatic vessels over collecting lymphatics to bigger lymphatic trunks (6). During this process, they pass through lymph nodes. Lymphatic fluid can be partly reabsorbed in the lymph nodes, which results in a higher protein concentration post-nodal (4). Dependent on where the lymphatic fluid re-enters the blood vessels its composition can differ. Lymphatic fluid protein concentration is around 4% when taken up in the capillary bed. Concentration is estimated to be higher, reaching levels similar to blood plasma (6%), at the level of the thoracic duct (3). According to estimations, about eight liters lymphatic fluid is built every day, whereas after reabsorption processes at the lymph nodes, the efferent flow rate is about four liters per day (7). Gannon and Carati discuss another additional route of fluid entering lymphatic vessels. In an animal model Aquaporin-1 water channel expression was demonstrated in lymph nodes. This suggests that a transcellular water transport might be contributing to lymph node protein concentration (8, 9).

In any case of dysfunction within the lymphatic vasculature, accumulation of excess fluid within the tissue occurs. This can lead to a range of pathological conditions such as the

appearance of a regional swelling, especially in peripheral extremities, known as lymphedema (10, 11). Lymphedema is known as a progressive, chronic and disabling disease affecting lymphatic structures. The disease is characterized by fluid accumulation within the tissue, caused by lymphatic system insufficiency and/or dysfunctional lymphatic transport. Worldwide, it affects up to 300 Million patients (12). The disease arises when more plasma is filtered into the interstitial space than the lymphatic vessels can drain (13). Retention of fluid can reach up to 5-15 liters (14). Although there are several approaches in finding a cure for this disease, as e.g. surgery (15) or medication (10), no definite cure is currently available. Therapy of choice is a physical therapy known under the general term complete decongestive (physio-) therapy (CDT) according to Földi (16). It is comprised of manual lymphatic massage/drainage, compression bandaging as well as physical exercises, and aims at reducing the volume of the affected body part, therefore, reducing severity of lymphedema (14). Moreover, goal of therapy is to retain the disease in a rather lower stage, to prevent progression and to enhance quality of life (17). A study including six patients has shown that MLD increases lymphatic outflow, measured before-, during- and after-MLD using lymphoscintigraphy (18). Several studies show the effect of lymphedema therapy on the reduction of limb volume as well as increased quality of life as markers for successful therapy (17, 19-22). However, these studies determined the effect of different therapy modalities only before - and after - several days/weeks of therapy (17, 19-22).

Lymphatic dysfunction has been linked to risk factors leading to the development of cardiovascular diseases as e.g. obesity, hypertension and atherosclerosis. Studies used different mouse models and human samples to investigate a relationship between lymphatic vessel dysfunction and obesity (23). Lymphatic fluid leakage is associated with increased inflammatory cytokine release from adipose tissue and macrophages (24). It is further a primary contributor in metabolic syndrome, which is knowingly increasing the risk of developing cardiovascular diseases (25). Therefore, dysfunctions in the lymphatic system could play a relevant role in the development of cardiovascular diseases (26). Jones and Min (2011) suggested following pathway: The increased fluid volume, caused by dysfunctional lymphatic vessels (edema) leads to leaking chylomicrons. This affects the neighboring adipose tissue reservoirs. Increased inflammation and increased lymphangiogenesis due to the increased recruitment of macrophages to the fat tissue leads to further detriment of the lymphatic vessels. This process is eventually contributing to cardiovascular diseases. Another animal model study,

reported by Titze et al. (2003), shows that lymphatic vessels and macrophages contribute to blood pressure homeostasis by interstitial fluid control (27). They suggest roles of vascular endothelial growth factor-C (VEGF-C), promotor of lymphatic vessel growth, and vascular endothelial growth factor receptor-3 (VEGFR-3) on blood pressure regulation. Blocking VEGFR-3, for instance, lead to an increase in blood pressure in a high salt diet animal mouse model (27). Similar to this finding, higher concentration of VEGF-C in plasma of patients with refractory hypertension was found compared to normotensive control subjects (28). Chronic inflammation is often seen in lymphedema patients, resulting from lymphatic flow failure (29). In several models of inflammation, lymphatic function is impaired and may be affected in different inflammatory diseases, causing a worsening of the edema, inflammation and obesity. The chronic inflammatory disease atherosclerosis, is known to affect the wall of arteries. Plaques that are built up in the lumen of arteria lead to the narrowing of vessels (30). Immune cell recruitment and cholesterol accumulation are essential processes in this disease, which silently develops over decades (31). Only recently it was reported that lymphatic vasculature is actively involved in lipoprotein transport from the interstitial space into blood vessels (31-33). Studies indicate the involvement of lymphatic vessels in lipoprotein metabolism and plasma cholesterol concentrations (34-36). It is believed that endothelial dysfunction is an important factor in the pathogenesis of hypertension (37), atherosclerosis and other cardiovascular disease (38-40). Early detection of endothelial dysfunction may provide an incentive for therapeutic options designed to reverse this status.

Extensive literature research revealed that there here is no study available, which systematically investigates the effect of lymphedema therapy on fluid shifts and its effect on the cardiovascular system, especially hemodynamic responses and endothelial/vascular (dys-)function, in a time course manner over three weeks of physical therapy. Therefore, the overall aim of this thesis is to systematically assess the effect of lymphedema therapy on fluid mobilization, hemodynamic parameters at rest and in response to orthostatic loading as well as vascular function in lower leg lymphedema patient. Further, this thesis aims at advancing knowledge on the poorly understood area of lymphatic system function as well as mechanisms impacted by system dysfunction as observed in lymphedema and how these are modulated by physical therapy.

1.2 The vascular system

The term “vascular system” usually addresses to the cardiovascular system. However, what is often missed is that the vascular system indeed consists of two parts, the cardiovascular system including blood vessels and the lymphatic system including the lymphatic vasculature (31).

The cardiovascular system is comprised of the heart, the blood vessels as well as components transported in those vessels. Functions of the cardiovascular system includes the transport of the blood and its components, such as oxygen, nutrients, hormones and blood cells to all organs and tissues throughout the body. It can be distinguished between the smaller, pulmonary circulation where the red blood cells take up the oxygen and the systemic circulation. Oxygen rich blood is brought to the organs in the body where nutrients are brought to the cells. CO_2 as well as by-products are taken up in the capillary structure by venules, which further develop into greater veins. Venous blood is taken back to the heart through the vena cava inferior and vena cava superior (4).

The long time underestimated lymphatic vasculature is a second set of vessels that evolved and exists in parallel to the blood vasculature. It plays a crucial role in normal physiology. However, it is not yet fully understood. Apart from the lymphatic vessels, primary (e.g. the thymus or bone marrow), as well as secondary lymphoid organs (such as lymph nodes) form the lymphatic system. Compared to the cardiovascular system, it is a unidirectional system commencing in the capillary bed and finally ending at the thoracic duct (left lymphatic duct) and right lymphatic duct, respectively, into the subclavian veins. The blind-ended lymphatic vessels take up the exudate from the interstitial space, the so-called lymphatic fluid, at the capillary beds (41). Lymphatic flow is a product of the leakage of plasma proteins and tissue fluid, driven by pressure and concentration gradients into the original lymphatics. Thus, they play a vital role in maintaining fluid homeostasis (42). In addition, lymphatic structures are important for the transportation of lymph, a fluid made up of interstitial fluid, macromolecules and triglycerides. Moreover, they display a main highway through which immune cells are trafficked and immune responses are organized. This makes the lymphatics a crucial component of the immune system (43).

1.3 The lymphatic system

1.3.1 Organization of the lymphatic system

The lymphatic system includes a variety of structures and organs in different body parts. Lymphoid organs include for example the spleen, thymus and tonsils. Main focus here is laid on the lymphatic vasculature, which is - compared to the blood vasculature - a unidirectional transport system, starting at the peripheral located capillary beds of the cardiovascular system running throughout the whole body (4). The lymphatic vascular system is organized in a tree-like structure and can be distinguished into different sections dependent on the location (distal vs. central)(4): (i) initial lymphatic vessels; (ii) collector lymphatic vessels (pre- and post-nodal); (iii) lymph nodes; (iv) lymphatic trunks.

The *initial lymphatics (lymphatic capillaries)* are blind-ended vessels that are located in close proximity (only a few hundreds of micrometers) to the cardiovascular microvasculature (44), where the lymphatic fluid is formed. A single layer of endothelial cells and an interrupted basal lamina composes them. At the tip of each vessel, endothelial cells are overlapping and display intercellular junctions. These “button-like” connections let them appear in an “oak-leaf shape”. They can act as unidirectional valves, enabling interstitial fluid and proteins to drain into the lymphatic vasculature (3, 4). Schmid-Schonbein et al. (1990) defined those valves as primary valves in order to separate them from valves within the lumen in the bigger collector lymphatic vessels (44). Additionally, anchoring filaments can be found in the initial lymphatic vessels, which stabilize the blind-ended structures. Studies performed in mouse models and humans have reported that the diameter of the initial lymphatics differ a lot depending on the species as well as tissue and/or organ. In human skin, a diameter of 50-70µm was found (45).

The initial lymphatic vessels drain into larger structures, the *collecting lymphatic vessels*. Compared to initial lymphatics, the collector lymphatics present endothelial cells and smooth muscle layers, connective tissue and nerves innervating those vessels (44, 46). The number of smooth muscle cells are increasing with the increasing lymphatic vessel hierarchy (8). Collecting lymphatic vessels are organized in lymphangions, structural segments with unidirectional valves made of endothelial cells and connective tissue at each end. Their main role is to prevent backflow of lymphatic fluid. This is particularly important while standing in an upright position, as the lymph flux must be driven against gravity (4). Other important

structures of the lymphatic vasculature are lymph nodes, where immunological compounds are presented to immune cells. Lymphatic vessels approaching lymph nodes are defined as pre-nodal afferent lymphatic vessels. Several pre-nodal afferent vessels can enter whereas usually only one post-nodal efferent lymphatic vessel can be seen. Anatomically, pre- and post-nodal lymphatic vessels are identical and belong to the collecting lymphatics (3).

Located between initial and collecting lymphatics are the *precollectors*. They display valves but lack muscle cells and therefore, do not physically contract. Precollector lymphatic vessels are composed of a single endothelial layer and valves that prevent the backflow of lymphatic fluid. Those are needed, as they do now show to have a layer of smooth muscle.

Finally, the post-nodal collecting lymphatics lead towards lager *lymphatic trunks*. Similar to the smaller collecting lymphatics, they show a layer of smooth muscles which enables vascular contraction (47). Ultimately, the lymphatic vessels end at the right lymphatic and thoracic duct, which connects the lymphatic vasculature to the venous system at the subclavian veins. The lymphatic vessel diameter in this area average at about 2.2 mm inside diameter (36, 48). Whilst lymph from the upper right quarter of the body (right arm and right side of the head) flows into the right lymphatic duct, continuing into the right subclavian vein, the thoracic duct drains lymphatic fluid from the remaining body (legs, left arm and left part of the head) into the left subclavian vein (44).

1.3.2 Formation, transport and concentration of lymphatic fluid

In the cardiovascular system, arteries transport oxygenated blood and other plasma compounds to the peripheral/distal organs and tissues, whereas the counter-part is taken over by the veins in transporting blood back to the heart. There is a balance between how much is delivered and the amount being removed (4, 8). Within the microvasculature, the blood vessels continuously leak plasma, serving its role in the supply of fluid and nutrients to the tissues. This leakage of plasma into the interstitial space is driven by hydrostatic and osmotic pressure imbalance, described by the Starling equation, which was reversed by Levick et al. (2010) (5). Estimations suggest that eight liters of plasma are filtered by the microvasculature each day, generating interstitial fluid (5). As there is limited space for excessive fluid, the accumulation of such generates a pressure and is the driving force for lymph formation. As the initial lymphatic structures are in close proximity to the microvasculature, they are responsible for

taking up the excessive fluid and therefore, serve as the point of entry for the transportation of this fluid through the lymphatic vasculature and the return back into the venous system (4).

The above describes blind-ended vessels of the initial lymphatics take up fluid. When interstitial pressure rises, due to accumulation of fluid, over the pressure level of the lymphatic lumen, the valves open and fluid can enter. The one-directional valves inhibit backflow of the lymphatic fluid (49, 50). Anchoring filaments assist in maintaining the permeability of the vessels. Additionally, another mechanism has been discussed over decades. Already in the 1970s a trans-endothelial transport mechanism via vesicles has been postulated (51, 52). In 2009, a study done by Dixon et al. strongly suggested that such a transport mechanism contributes to lymph formation (53). Similar to this, several studies show that various chemical and physical stimuli are able to cause alteration of lymphatic endothelial cell shapes as well as barrier function (54-56). Additionally, several animal studies demonstrated that also forces such as heart beat or respiration can facilitate lymph formation (8).

Two primary forces are essential for fluid flowing through the collecting lymphatics. Extrinsic forces via e.g. muscle movement, heart contraction or breathing, as well as intrinsic forces. The structure of the collecting lymphatic vessels, namely the series of lymphangions together with muscle contraction, enables the vasculature to work as a pump. Each lymphangion individually is able to pump the lymph forward to the next section. Sequential peristaltic as well as segmented contractions of lymphangions, together with the lymphatic valves preventing backflow, achieve forward flow of the lymph (6, 57). This active, intrinsic pump mechanism plays a crucial role in the regular flow within the lymphatic system (58). The intrinsic contraction rate is about 10 per minute (59). To optimize its function, the lymphatic vasculature can respond to different stimuli such as neuronal and humoral stimuli, pressure or shear stress (47). Similar to blood vessels, flow-mediated nitric oxide production can be seen in lymphatic vessels as well (60). According to Olszewski et al. (1977), lymph flow can change up to 15-fold while standing up (58). Atrial and brain natriuretic peptides have shown the ability to modify the permeability of collecting lymphatics. Scallan et al. (2013) suggest a potential exchange mechanism between lymphatics and the tissue (61). The lymph fluid that is taken up by initially lymphatics consists of immune cells, proteins, electrolytes, lipids, lipoproteins and bacteria as well as their potential harmful compounds (4).

Traveling from the initial lymphatics to the trunks, lymph fluid crosses at least one lymph node. In the whole body, about 500-600 lymph nodes can be found. Within the lymph

node, bacteria and potentially harmful particles are eliminated by an adequate immune answer. Therefore, blood vessels can be found inside the nodes, through which fluid, proteins, and cells may be transported in either direction (4). Additional to the biological/immunological filtration, lymph is also being mechanically filtered within the lymph nodes. This allows protein-free fluid to pass through the blood-lymph barrier. Afferent lymph can therefore be concentrated by reabsorption of water (62), which results in a higher protein concentration in post-nodal lymphatic fluid (63). From the about eight liters of lymphatic fluid that is built every day, reabsorption processes at the lymph nodes reduce the efferent flow rate to about four liters (7). Additionally, Gannon and Carati (2003) discuss another route of fluid entering lymphatic vessels. They used a rat animal model to demonstrate the expression of aquaporin-1 water channels in lymph nodes. Their results indicate that a transcellular water transport might be contributing to lymph node protein concentration (9).

1.3.3 Assessing lymphatic flow

Lymphoscintigraphy is used as a diagnostic tool to image lymphatic vessels. It includes the injection of radioactive colloid particles. Accumulation of those within the lymphatic vasculature and lymph nodes can then be determined. It is currently used as the gold standard method to determine whether tissue swelling occurs due to lymphatic dysfunction (1). However, more suitable to show lymphatic flow is the use of near-infrared fluorescence dyes, in particular indocyanine green. This allows a quantitative analysis of lymph flow (29). Another option is magnetic resonance lymphangiography, which is highly suitable for visualizing lymphatic vessels, but scintigraphy is superior in detecting lymph nodes (64, 65). Near infrared fluorescence imaging uses fluorescent dyes, which are absorbed by the lymphatic vasculature, to visualize lymphatic vessels. This technique is routinely used in lymphatic-venous anastomosis surgery to find suitable vessels (1). Recently, a new promising assessment tool of lymphatic vessels has been proposed: Multispectral optoacoustic tomography (MSOT) is proposed to enable real-time tissue imaging allowing exact tissue identification using a hand-held probe. However, this methods needs be further evaluated in future clinical studies (66).

Hyaluronic acid (HA), a biogenic macromolecule, is contributing to osmotic pressure as well as flow resistance. Therefore, it plays a crucial role in tissue hydration regulation and water as well as protein homeostasis. HA enters the blood circulation via the lymphatic vessels (42). The amount of HA found in the plasma depends on the balance between HA delivery and

removal from the plasma. Therefore, HA was reported to act as novel biomarker in assessing lymphatic outflow (67). Studies have shown that HA increases e.g. post-prandial (68) or during exercise (69) in healthy subjects. Roh et al. (2017) observed increased hyaluronic acid levels inside the lymphedematous tissues in an animal model (67). Analysis of human lymphedema tissue showed similar results (70). Recombinant hyaluronidase (67) or local heat therapy (71) showed promising results in mouse models to effectively break down high molecular weight hyaluronic acid, leading to a reduction of the accumulated fluid.

1.4 Lymphedema

Lymphedema is a chronic, progressive and disabling disease affecting the lymphatic vasculature, known to significantly reduce quality of life and therefore, being a massive psychological burden (72). The disease arises when it comes to an imbalance in lymphatic fluid transport homeostasis and dysfunctional lymphatic drainage. This leads to a localized form of swelling of the tissue, due to excessive retention of lymphatic fluid in the interstitial space. Usually, this occurs in the extremities, however, this disease can affect all parts of the body (73). Lymphedema development is described by Földi as "*a disequilibrium between lymphatic load and lymphatic transport capacity*" (74). Another concept of lymphedema is the definition as progressive irregular swelling of a limb and/or the adjacent quadrant due to the protein-rich fluid accumulation (75). In general, lymphedema is the result of an imbalance between lymphatic fluid development (lymphatic load) and its removal (lymphatic transport capacity) (76). Independent of the exact etiology, lymphedema is characterized by severe chronic swelling, atrophic skin changes, secondary infections and localized pain (77). Moreover, it significantly reduces quality of life of patients, not only due to mechanical impairment. The psychological aspect concerning the appearance of the affected limb are causing psychological burdens as well (77).

1.4.1 Epidemiology and etiology

Maclellan et al. (2015) describe that patients are often medical nomads. There is only a limited number of doctors specialized in this field (78). Often, there is a lack of exposure to this specialized field at medical school and during residency. Taken this together, lymphedema is often underestimated and misdiagnosed (78). Therefore, the patient numbers can only be estimated. Those numbers range from 140 Million up to 300 Million people worldwide suffering from lymphedema (72, 79-82). In a study including 255 patients, Maclellan et al. (2015) found that 25% of patients were officially diagnosed with "lymphedema" although they had another condition and the average time between the onset of the disease and the assignment to a lymphedema therapy program was 7.7 years (78). 71% of the patients in their study population were female. This is also supported by a study done by Neuhüttler and Brenner (2006). They found that lymphedema occurs much more often in females than in males (m:f=1:4.6). The patient numbers in this study was relatively low with 99 patients included

(83). However, the multi-center study LIMPRINT (2019) performed in France, Turkey, Italy and in the United Kingdom came to similar results with 6430 females out of 8140 included patients, which resembles around 79% (73). Although the exact pathophysiological mechanisms behind this disease are not yet fully elucidated and its heterogeneity in terms of root cause of the disease, it can be distinguished between primary and secondary lymphedema. Whereas primary lymphedema has a strong genetical background (inherited or spontaneous mutations), secondary lymphedema is usually acquired and develops, for example, upon obstruction of lymphatic vessels or deranged lymph nodes.

1.4.1.1 Primary lymphedema

Primary lymphedema is relatively rare showing a prevalence of about 1 from 100.000 individuals (79). This form of lymphedema can occur as a fault in lymphatic vessel development or spontaneous mutations, resulting in a structural and/or functional dysfunction that impairs drainage abilities (84). Primary forms of lymphedema have been associated with genetic mutations in 25-30% of primary lymphedema patients (36). About 50% of primary lymphedema cases involve the vascular endothelial growth factor-C (VEGF-C) and vascular endothelial growth factor receptor-3 (VEGFR-3) signaling axis (31). However, more than 20 genes (i.e.: CCBE1, FOXC2, GATA2, GJC2, PTPN14, SOX18 and VGFR-3) have been identified to have a link to lymphatic anomalies, leading to underdeveloped lymphatic vasculature or insufficient lymphatic outflow capacities. Lymphedema distichiasis, for examples, which is characterized by malformations of the valves within the collecting lymphatics is based on mutations in the FOXC2 gene (85, 86). Although more recent research shows there is a genetic background of primary lymphedema, the disease can develop later in life (80). Therefore, historically, primary forms of lymphedema have been classified depending on the age of onset of the disease. Following sub-groups were characterized: (a) congenital lymphedema: <1 year (b) lymphedema praecox, the most common form of primary lymphedema: 1-35 years (c) lymphedema tarda: <35 years (87). The precise incidence of primary lymphedema is not exactly known, but estimations assume it to be 5-10% higher in females compared to males (80). Additionally, lower extremities tend to be more susceptible to primary lymphedema. Although the exact mechanism behind this is still unclear, Ciudad et al. (2017) postulate it is highly possible that gravitation increases the problem and is part of the disease progression (15, 88, 89).

1.4.1.2 Secondary lymphedema

Secondary forms of lymphedema occur more often than primary forms of lymphedema. The prevalence of secondary lymphedema is estimated at about 1 in 1000 individuals. In average, patients are between 50 and 58 years old when the disease is firstly diagnosed (79). Secondary lymphedema develops after obstruction or damage of initially normal lymphatic vessels and is therefore an acquired disease. Possible reasons for this damage can be recurrent infections, obesity, trauma or surgery. Furthermore, it is also often seen a consequence of cancer therapy, such as radiotherapy or lymph node dissection (79, 87). Infection with the nematode *wuchereria bancrofti* is globally the number one cause of secondary lymphedema. According to the World Health Organization (WHO) fact sheet from March 2017, more than 120 Million individuals are infected and up to 40 Million people show to be disfigured and incapacitated due to this infection (90). It is mainly found in people living and/or traveling to sub-Saharan Africa and India (79, 90). Considering western countries, breast cancer related lymphedema (BCRL) is the most common cause of acquired lymphedema. About 15-20% of patients suffering from breast cancer are at risk of secondary lymphedema (84). A systematic review and meta-analysis of BCRL cases shows an overall incidence of 15.5% after cancer treatment (91). A report from 2010 stated that 16% of all patients treated with a combination of radiation therapy together with lymph node resection developed clinical signs of lymphedema (92), whereas the highest incidences at 31% were found in patients undergoing radiation therapy (91). Finally, other diseases as for example congestive heart failure or renal diseases can act as co-factors, resulting in secondary lymphedema (93).

1.4.2 Lymphatic dysfunction and cardiovascular diseases

Dysfunction within the lymphatic system can not only lead to lymphedema, but may also be involved in the development of cardiovascular diseases, due to its involvement in lipid absorption and transport as well as immune function. Recent research has more and more focused on investigating the relationship between lymphatic vessel dysfunction and cardiovascular risk factors such as dyslipidemia, inflammation, metabolic syndrome, obesity, hypertension, atherosclerosis and myocardial infarction (1, 2, 11, 25, 29-31, 36, 94).

1.4.2.1 Inflammation

Chronic inflammation is often seen in lymphedema patients, as a result from lymphatic flow failure (29). Protein-rich lymphatic fluid leads to an inflammatory response, when stagnant. This further facilitates adipocyte proliferation, fibrotic tissue changes and increased risk of infections, leading to more destruction of the lymphatic vessels (15). In several models of inflammation, lymphatic function is impaired and may be affected in different inflammatory diseases, causing a worsening of the edema, inflammation, and obesity. Lymphangiogenesis appears to act as a compensating mechanism for sustaining a physiological status in certain pathologies, but due to other inflammatory markers/cytokines, the lymphatic vasculature may become further compromised. Dependent on the tissue type and grade of damage, lymphangiogenesis induced by inflammation can have either beneficial or destructive effects (36, 95).

1.4.2.2 Obesity

Although the link between the lymphatic system and lipid transport is well-established, the role of the lymphatics in the metabolism of the adipose tissue as well as obesity has only been investigated recently. The connection between obesity and lymphatic vessels is bidirectional. Obesity is a recognized risk factor in the development of lymphedema (78). Savetsky et al. (2014) postulate that obesity enhances inflammation and therefore, impaired the function of the lymphatic vasculature (96). Further, impaired lymphatic function was observed in obese mice (97). Similar observations can be found in clinical data as well: Jamallo et al. (2013) determined a significant correlation between the body mass index (BMI) and cases of lymphedema in breast cancer (98). Further, a study by Ridner et al. (2011) showed that breast cancer survivors with a BMI of 30 or higher displayed a 3.6 times higher risk to develop secondary lymphedema compared to those showing a lower BMI (99). Oppositely, a new approach being researched is that lymphatic malfunction can serve as a factor in obesity development (94). Although the exact mechanism of how malfunction of the lymphatic system leads to adipose tissue accumulation has not been determined yet, animal studies using $Prox1^{+/-}$ mice showed that accumulation of fat in close proximity of the lymphatic vessels is associated with lymphatic dysfunction. Calorie intake was strictly controlled and can be excluded as reason for gaining weight. This malfunction in the lymphatics was observed before the animals

showed clinical signs of obesity. The degree of lymphatic impairment was strongly correlated with the body weight of the mice. The question that remains is how lymphatic dysfunction is associated with adipose tissue proliferation. It is believed that leaking lymphatics lead to hypertrophy of the adipocytes as lymphatic fluid, enriched with lipids, acts adipogenic (94). Studies have shown that lymphangiogenesis is enhanced during inflammatory processes. One possible explanation is that reaching a certain level of obesity impairs lymphatic function by induced inflammation (100, 101). Summarized, different mouse models and cases of human pathology suggest that there is relationship between dysfunctional lymphatic vessels and obesity: lymphatic fluid itself is able to cause preadipocytes maturation and adipose tissue growth. As obesity (associated with increased secretion of inflammatory cytokines) is a primary contributor in metabolic syndrome (which is known to lead to increased cardiovascular risk), a dysfunctional lymphatic system could play essential roles in the development of cardiovascular diseases (26, 94).

1.4.2.3 Metabolic syndrome

Evidence suggests that lymphedema and the accompanying pro-inflammatory state represent a higher risk for developing metabolic syndrome (MetS) and subsequently to cardiovascular diseases (25, 102). Metabolic syndrome is a term for a cluster of multiple risk factors and diseases. These include dyslipidemia, abdominal obesity, increased blood pressure and plasma glucose levels including insulin resistance. Atherosclerotic cardiovascular diseases are a direct consequence of MetS and its associated risk factors (25). The mesenteric lymphatics serve as an entry point for all dietary lipids (103-105). The so called chylomyron transports e.g. lipoproteins and cholesterol from the intestine to the blood circulation via the lymphatic vessels. Chakraborty et al. (2010) have shown that high fructose diet leads to an increase of adipose tissue in the area of the mesenteric lymphatic vessels, leading to a smaller vessel diameter and a reduced pumping activity (25). Differentiation to adipocytes and proliferation is promoted by both, lymph itself and the lipid-rich chylomicron part of lymph. Lymphatic vessel dysfunction can affect the transport of lipids, influence fat depositions and can develop to lymphedema along with the progression of MetS risk factors. However, future studies are still required to investigate the underlying mechanisms (25).

1.4.2.4 Blood pressure regulation and hypertension

Sodium homeostasis has been linked to blood pressure control (36). In animal experiments it was observed that lymphatic vessels and macrophages contribute to both, interstitial fluid balance as well as blood pressure control (106). Skin serves as storage for sodium, which is attached to proteoglycans without accompanying water retention (107). Available evidence suggests important roles of VEGF-C, which promotes lymphatic vessel growth, and VEGFR-2 in the regulation of blood pressure (27). Inhibition of both factors resulted in sodium accumulation and therefore, increased blood pressure (36). Similar to this, higher VEGF-C concentrations were found in plasma of patients with refractory hypertension compared to the normotensive control group (28). Two hypotheses of the underlying mechanism were discussed: (1) lymphatic network enables increased interstitial sodium clearance which excretion is regulated via the kidney and (2) endothelial nitric oxide (NO) production and vasodilation mediated via VEGFR-2 (36).

1.4.2.5 Atherosclerosis

The chronic, inflammatory disease atherosclerosis affects the walls of arteries. Plaques are built up in the lumen of arteria leading to the narrowing of the vessels. Immune cell recruitment and cholesterol accumulation are essential processes, which silently develops over decades (31). How cholesterol and inflammation work together in the progression of the disease is not yet elucidated. However, what has been observed is that regression of atherosclerosis is directly linked to cholesterol removal from the vascular endothelium (108). Simplified, the process responsible for cholesterol removal from macrophages is known as reverse cholesterol transport (RCT). RTC is involved the formation of high-density lipoprotein (HDL) (109). Only recently it was reported that the lymphatic vasculature is actively involved in HDL transport from the intestines to the blood stream (31-33). Lim et al. (2013) have investigated the effect of the administration of VEGF-C into $ApoE^{-/-}$ mice, which leads to the induction of lymphangiogenesis and further, to the reduction of cholesterol by an improved RCT (32). Moreover, reduced RCT to about 80% was seen when lymphatic vessels were harmed surgically (32). Another model lacking dermal lymphatics showed reduced RCT by up to 77% (33). This and further studies indicate the involvement of the lymphatic vasculature in

lipoprotein metabolism, cholesterol plasma levels and, additionally, that RCT is highly dependent on the lymphatic vasculature (34-36).

1.4.2.6 Myocardial lymphatics

Myocardial infarction can be the result of a preceded atherosclerotic disease (36). Inflammatory, angiogenic and lymphangiogenic responses are part of the healing process after myocardial infarction, together with tissue remodeling and scar development (110). A positive effect of VEGF-C has been investigated in animal models, leading to a reduction of edema (111) and fibrosis post-infarction, an increase in inflammatory cell clearance as well as lymphatic vessel function (112, 113). Additionally, improved diastolic function and 30% increase in ejection fraction was found. Intramyocardial VEGF-C treatment has also been used in phase II clinical studies including angina pectoris patients (114). Further, animal data also suggest the involvement of lymphatic vessels in fluid homeostasis of the cardiac interstitium. This is particularly important as small increases in fluid can dramatically affect heart function. Lymphatic vasculature prevents myocardial edema and cardiac dysfunction. Unsurprisingly, it is believed that impaired cardiac lymph drainage could potentially lead to myocardial incidents in humans (26). While human data are sparse, it is believed that lymphatic disruption following cardiac transplantation may be associated with post-operative mortality (115). Indeed, myocardial lymphatics showed a change of the phenotype in the endothelium post-transplantation (e.g. absolute density changes of endothelial markers in lymphatics). In patients who experienced at least one rejection a significantly lower VEGFR-3 density was observed (116).

1.4.3 Diagnosis of lymphedema

As referenced above, lymphedema is frequently underdiagnosed and undertreated. The variable way by which lymphedema is clinically diagnosed and defined is a confounding attribute in the diagnosis process (72, 117). Early detection of the disease is crucial for therapy approaches and outcome as well as for limiting/reducing disease progression. A recent study including 149 patients showed early detection along with a simple intervention can be highly effective in patients at risk for BCRL (118). However, early stages of lymphedema cannot always be easily differentiated from other causes leading to edema, as for example obesity,

venous diseases or lipedema (77). Schook et al. (2011) reported, that in 27% of pediatric patients, other anomalies such as post-traumatic swellings or lipedema are mistaken for lymphedema (119). Standard diagnostic tools do usually not enable diagnosis of lymphedema in a subclinical stage. Although several genes and biomarkers have been investigated (120, 121), up to know, most of the diagnoses are performed clinically through thorough anamnesis, including family history and a detailed physical examination considering palpation and inspection (77). Crucial for diagnosis is the patients' history (e.g. cancer treatment or trauma) as well as signs of typical symptoms (swelling, skin changes, recurrent infections). One of the most reported clinical signs in diagnosing the presence of lymphedema is the so-called Stemmer sign. If it is not possible to pinch a skinfold on the second toe, it is considered positive (122). In a study including 110 patients showing a positive Stemmer sign, lymphatic function was evaluated using lymphoscintigraphy. Results indicate that a positive Stemmer sign is indeed a reliable predictor for lymphedema (122). About 15% of the patients show skin changes such as hyperkeratosis or lymphorrhea (119). Other typical symptom, which is regularly described by lymphedema patients, is the feeling of heaviness in the affected limb. Together with acute swelling, this is a positive predictive value for the presence of lymphedema (121). Especially in early stages of lymphedema diagnosis can be challenging, as changes in volume or circumference are not clinically detectable. Diagnostic instruments that have shown good reliability and validity are tape measurement, water displacement, perometry and bioelectrical impedance spectroscopy (BIS). Out of those methods, BIS is the only method able to detect stage I lymphedema (123). Tape measurement can be highly observer-dependent. Therefore, perometry represents a more reliable tool with increased interobserver-reliability (124). Imaging techniques, namely lymphoscintigraphy, lymphangiography, or computer tomography are used to check for lymphatic flow impairment as underlying mechanism (77).

Lymphedema severity can be divided into four stages. The staging system according to Brunner (stage I-III) is most commonly used and was adapted by the latency stage (125), equivalent to stage 0. In this stage, the disease is clinically inconspicuous, despite a reduced transport capacity, which predisposes to developing edema. It includes patients that are considered to be at risk, e.g. breast cancer patients undergoing lymph node biopsies. Stage I is characterized by soft pitting edema, which is reversed, partially or completely upon limb elevation, whereas in stage II, patients suffer from swellings that are spontaneously irreversible and shows signs of tissue fibrosis. This means that elevation does not lead to a reduction of

swelling. Stage III is the severest form of lymphedema. It is characterized by tissue changes, such as fibrosis and adipocyte hypertrophy, and involves skin changes (14, 75).

1.4.4 Therapy approaches

Up to now, there is no definite cure available for lymphedema, except for obesity induced lymphedema (126, 127). Therefore, different strategies are currently researched. Recommendations state that lymphedema treatment should start as early as possible, as prolonged disease leads to fibrotic tissue changes and further destruction of the remaining functional lymphatic vessels (15). The major aims of different therapy approaches are to reduce the swelling in the affected body parts, by for instance promoting the development of alternative pathways from affected parts to body parts with intact lymphatic flow properties (128) and to improve the functional status (15) and therefore, prevent the progression of lymphedema (14).

Physical therapy

Physical therapy usually refers to exercises and treatments performed by specialized manual therapists. This includes lymph drainages, massages, all kinds of compression garment or intermitted pneumatic compression (129). Intermitted pneumatic compression devices are automated and simulate natural compression by sequential inflation of air. Although there are doubts about the efficacy of this intervention, recent meta-analysis has shown that it is effective in secondary lymphedema patients, applies alone or in combination with conservative therapies (10). These forms of therapies typically apply a positive pressure on the tissue. Gott et al. (2018) describe a different approach: negative pressure therapy, which represents a pulling/opening force and is already used in wound healing (130). However, clinical studies need to be performed in future to further evaluate this therapy (130). Kinesiology taping also displaces a form of negative pressure, however a meta-analysis report did not find evidence of efficacy, although quality of life (QoL) showed to be higher in patients treated with kinesiology (131). Other different forms of physical therapy being researched include for example fascia manipulation, heat treatment or extracorporeal shock wave therapy. Fascial manipulation is thought to be a promising tool in enhancing lymphatic flow in lymphedema patients by possibly breaking down hyaluronic acid molecules and by reducing lymphatic viscosity (132, 133). Mariana et al. (2011) investigated heat combined with mechanical lymph drainage, but could not find a synergic effect (134). Another pilot study showed a positive impact of extracorporeal

shock wave therapy in patients diagnosed with stage III secondary lymphedema, as it reduced arm circumference. However, this was only shown in a pilot study with seven patients enrolled (135). Far infrared radiation treatment showed promising results by significantly reducing limb circumference and QoL in a study with 32 lymphedema stage II and III patients (136).

Weight loss

Obesity is one of the known risk factors for lymphedema. It has been shown that weight loss interventions lead to a significant reduced limb volume in BCRL patients after a 12-week diet (80, 137). Although a mouse model has shown that obesity-induced lymphedema is reversible, a case study by Nitti et al. (2016) could not confirm this finding (126). Further, exercise and resistance training have been studied and the results show that both can decrease lymphedema severity by changing body composition (138).

Drug therapy

Diuretics support the excretion of body water in form of urine. Initially, it might have an effect in some of the patients, but as diuretics might lead to body fluid and electrolyte imbalance, administration is not purposeful and therefore, not recommended (128). Lymphedema has shown not to be affected by diuretic therapy (139). Benzopyrones may have a positive effect in hydrolyzing tissue proteins and activating the lymph transport route and therefore, promoting lymph absorption (128). Besides hepatopathic effects in long-term administration, trials did not confirm any effect of benzopyrones in reducing volume in the affected limb, pain or discomfort in lymphedema patients (140). Animal models showed a reversal effect of the disease in a mouse model with acquired, postsurgical lymphedema using Ketoprofen, a non-steroidal anti-inflammatory drug agent with anti-inflammatory mechanisms (141, 142). In an open-label and placebo-controlled pilot trial, Rockson et al. (2018) determined the potential benefit of anti-inflammatory therapy in lymphedema patients, mainly on skin thickness reduction, improved histopathology. It further led to a reduction in plasma granulocyte colony stimulating factor 1 expression (141). However, further studies are required to confirm these results. Gardenier et al. (2017) used a mouse model to explore the effect of Tacrolimus, an anti-T-cell immunosuppressive drug, as T-cells are presumed to be involved in lymphedema development by inhibiting lymphangiogenesis and promoting tissue fibrosis (143). Local application of the compound could prevent lymphedema formation and is also

effective if the disease is already established. However, this study was done in animal models and the results of clinical studies are awaited (143). Other clinical studies indicate that hyaluronic acid levels in lymphedematous regions are higher than in normal tissue. Around 1960, several researchers reported the approach of using hyaluronidase in the treatment of lymphedema and elephantiasis (144-146). In a study by Roh et al. (2017), animals with acquired lymphedema were treated with hyaluronidase, which was injected into lymphedematous tissue (67). The volume of the affected limb was reduced upon treatment. Their findings indicate that hyaluronidase promotes lymphangiogenesis and could potentially be a new treatment approach in lymphedema therapy by modifying HA fragment size. However, until now this was only tested in animal models and clinical studies need to be performed (67). Gene therapy delivery or external delivery of VEGF-C studies have shown to promote lymphangiogenesis and to decrease lymphedema in different animal models (147, 148). Several preliminary clinical studies suggest that mesenchymal stem cell, which differentiate to lymphatic endothelial-like cells, showed beneficial effects, hoping to provide new therapeutic options in future (81, 149-151). A one year follow up study, however, found no changes in arm volume and no improvement in lymphoscintigraphic evaluation after adipose-derived regenerative cells treatment (152). Although, different compounds are being actively research, drug therapy that can safely and effectively treat lymphedema is currently not available (76).

Surgical approaches

Traditionally, surgery was performed in patients where conservative therapy was not successful. Lately, due to advances in the field of microsurgery and moreover, due to the new insights into the lymphatic system itself, but also into the pathogenesis of the disease, new surgical procedures were introduced in animal models and clinical studies (15).

Generally, it can be distinguished between physiologic and excisional procedures. Physiologic procedures aim at improving lymphatic fluid flow and drainage by redirecting the lymphatic flow towards the venous system or by inducing new pathways by lymphangiogenesis. Lymphvenous Anastomosis (LVA) and vascularized lymph node transfer (VLNT) are the two most popular surgical approaches. LVA is mainly recommended at an early stage of lymphedema (stage I-II) and is used to redirect excess lymph into the venous system by connecting lymphatic vessels directly to venous vessels. Most patients experience an improvement in symptoms after LVA, however, wearing compression garments post-surgery

is still recommended. Interestingly, LVA seems to be more effective in the upper extremities than in lower limb lymphedema (15). VLNT is performed in advanced stages of lymphedema, where lymphatic vessels and/or lymph nodes are missing or dysfunctional. Different donor sites have been described: ileocecal, appendicular, jejunal, gastroepiploic or supraclavicular lymph nodes. The recipient site is chosen based on several factors such as the stage of lymphedema, former radiation, scar tissue or aesthetic appearance (153). Although the exact mechanism of action is not yet fully understood, two theories are presented: (1) transferred lymph node absorb lymphatic fluid accumulated nearby; and (2) lymph angiogenesis by releasing VEGF-C is induced by vascularized lymph nodes (15). Clinical studies show promising results for both, LVA and VLNT when it comes to a reduction of limb volume and episodes of cellulitis. However, these methods do not have a beneficial effect on reducing fibrosis and, moreover, data on long-term outcomes still need to be evaluated (81). Suction assisted lipectomy is an excisional procedure used for severe fibroadipose hypertrophy as can be seen in chronic lymphedema. This provides only minimal improvement of lymphedema, therefore, it is still required to apply compression bandages lifelong in order to prevent recurrence (81). Different studies confirmed the efficacy and also the long-term stability of lipectomy for reducing limb volume, although the outcome is highly dependent on the compliance with post-surgery recommendations (15). Radical reduction in lymphedema with preservation of perforators is a combination of physiological and excisional procedure and although some studies indicate good and long-term outcomes, it requires excellent microsurgical skills and has a higher risk of scar formation, infections and necrosis. It further requires longer operating times compared to the above-mentioned methods. It is used in advanced lymphedema (stage III) (15). The Charles' procedure involves the total removal of skin and subcutaneous tissue from the affected body part and is performed to reduce excessive volume and to control infection mainly in patients with a severe lymphedema in an advanced stage. However, long-term results are poor (15).

1.5 Complete decongestive (physio-)therapy

Several surgical and non-surgical therapy approaches to treat lymphedema have been researched, however, the main therapy of choice remains to be a physical treatment known as complete decongestive (physio-)therapy (CDT). CDT is a rather preventive than curative treatment form (154). It is the gold-standard therapy according to the latest consensus document of the International Society of Lymphology (125). CDT is an empirically-driven, multicomponent therapy program aimed at decreasing limb volume and reducing the progression of lymphedema (117). CDT can be separated into two phases. Manual lymphatic drainage (MLD), multilayered compression bandages, exercise and meticulous skin care are part of the first phase. Phase two is mainly focused on self-care by wearing elastic sleeves and compression stocks as well as continue performing exercises (19). *Manual lymphatic drainage* is aimed at mobilizing and reducing fluid and overall volume. Distinct motions performed by lymph therapists during MLD involves flowing, rhythmic, stirring and occasionally scrubbing gestures with fairly low pressure (at about 30-40 mmHg) along with slow motions. The skin and subcutis are stretched during this procedure as well. MLD intervention starts in the area of healthy, adjoining body areas. The lymphatic vessels located there react with an increased motoric activity of the lymphangion, with a consequent increase in the formation of lymph and lymphatic outflow. MLD is then further applied to the obstructed trunk quadrants (ventral and dorsal) and to the impaired extremity (proximal, then distal). The *application of the compression bandages* is aimed at improving muscle and joint pump function. In addition, the associated respiratory movements that accompany the exercises are believed to assist in increasing lymphatic flow (155, 156). Additionally, *exercise therapy* (10), *psychological support* (72) as well as educational seminars on *skin care*, *skin restauration, nutrition* and *self-care* (157) are an important pillars of CDT. This physical therapy has been shown to mobilize accumulated fluid, and is believed to increase lymphatic outflow (74). Further, it is believed to enhance the excretion of up to one liter urine (after MLD) together with significant changes in the excretion of urinary neurohormones (158), to maintain the integrity of the skin and associated structures (117) and further, to improve quality of life of lymphedema patients (17). CDT has also shown to decrease the expression of pro-inflammatory gene sites (74). High load active exercise with compression therapy seems to be more effective than CDT alone (159). The main components of CDT are summarized in the call out box below:

- *Manual Lymphatic Drainage (MLD)*: Special hand movements (rhythmic, flowing or stirring) are performed by lymph therapists with a relatively low pressure of 30-40mmHg and a frequency mimicking the intrinsic frequency of the lymphangion. MLD is commenced in the area of healthy tissue and then expanded into adjacent areas towards the location of obstructed vessels (155, 156).
- *Compression bandages* are the only component for volume reduction and are further believed to improve muscle and joint pump function (155, 156)
- *Physical activity* such as ergometry are believed to support increasing lymphatic flow (10).
- *Skin care and skin restauration* (125)
- *Psychological support*, as lymphedema often is a huge psychological burden to patients, due to the disforming and disabling character of the disease (72)
- *Educational seminars* (157)

The main measured outcome parameters of CDT are the reduction of volume and circumference of the respective body part (19, 160) as well as increase of quality of life (22, 155). However, little is known about how much fluid is mobilized, where it is shifted to and how the body copes with the amount of fluid being mobilized. Bioelectrical impedance spectroscopy analysis can be used to determine the presence of lymphedema, however, it can also be useful in investigating extracellular and intracellular fluid shifts (161). A clinical study by Pereira de Godoy et al. (2013), investigated fluid mobilization before - and after - one week of intensive physical treatment using bioelectrical impedance in ten lower limb lymphedema patients (162). They found a significant reduction of total water, but an increase in intracellular water in the affected body part, whereas total water increased in the healthy body parts (upper limbs and trunk). It seems as if fluid is shifted from the affected body part to the healthy one (162). This was partly confirmed by a more recent investigation from the same researchers, which shows reduced intracellular and extracellular water post-therapy and both, intra - and extracellular - water increased in the trunk and upper extremities (163). Although CDT is the treatment of choice for all kinds of lymphedema etiologies (primary, secondary, upper and/or lower limbs), it seems to be more effective on lower limb lymphedema in terms of improving QoL (20). Additionally, dermal thickness was suggested as easy to assess marker for CDT effectiveness, however it only showed slight correlation with therapy outcome (164). Based on the main pillars of CDT, which are manual lymphatic drainage and compression, modified

therapies in terms of compression garment, duration, composition of therapy have been researched (21, 134, 165, 166).

Table 1 provides an overview of lymphedema and lymphedema therapy, highlighting the current knowledge as well as knowledge gaps and how the knowledge gaps could potentially be addressed.

Table 1: Overview of lower limb lymphedema and lymphedema therapy: current knowledge, knowledge gaps and how those can potentially be addressed. *Legend: ICF, intracellular fluid; ECF, extracellular fluid.*

Lymphedema		Complete Decongestive Therapy (CDT)
• Fluid accumulation • Chronic inflammatory state • Associated with risk factors leading to cardiovascular diseases	**Current knowledge**	• CDT leads to the mobilization of fluid from the lymphedematous tissue
• Effect of fluid accumulation in the lower limbs on cardio-postural control • Does lymphedema influence endothelial (dys-)function and vascular health?	**Knowledge gaps**	• Effect of CDT on mobilized fluid not known - Distribution of mobilized fluid unclear (ICF, ECF, blood circulation, lymphatic system) - Effects on plasma volume changes - Effects on plasma content (proteins, electrolytes) - Effects on hormonal responses - How does mobilized fluid affect hemodynamics and blood pressure? - How does fluid shift affect cardio-postural control → falls? • Effect of CDT on endothelial/vascular health?
• Assess hemodynamic responses (blood pressure, heart rate, cerebral blood flow) to orthostatic loading (via sit-to-stand test) • Vascular function assessments (Pulse wave velocity, flow mediated dilatation), including microvasculature assessment (retinal microvasculature analysis)	**How to address these knowledge gaps**	• CDT effects on fluid shifts can be assessed via perometry and bioelectrical impedance spectroscopy (distribution of fluids) • Plasma volume changes can be calculated via various formulae (e.g. based on plasma density, hematocrit, hemoglobin) • Plasma protein and electrolyte concentrations, osmolality, oncotic pressure and hormone assessments • Sit-to-stand test pre - and post - therapy to assess cardio-postural control • Vascular function assessments (pulse wave velocity, flow mediated dilatation), including microvasculature assessment (retinal imaging) over therapy

• Cardio-postural control could be impaired in lymphedema patients → orthostatic intolerance → increased risk of falls • Impaired endothelium/vascular function could increase cardiovascular risk	**Why addressing the knowledge gaps is important**	• Mobilized fluid could potentially lead to postural hypotension and falls • Manual lymphatic drainage could also affect cardio-postural control and blood pressure regulation leading to falls • Endothelial/vascular (dys-)function needs to be assessed over several weeks of therapy to assess effects of physical treatment
• Falls are associated with chronic hospitalization and increased costs • If detected early, signs of endothelial (dys-)function are reversible	**Long-term impacts**	• Minimize risk of falls during therapy, thus, reducing hospitalization duration, cost of care and improving quality of life • Cardiovascular risk is reduced if the treatment improves or prevents deterioration of endothelial function

2 AIMS AND OBJECTIVES

It has not been previously investigated how complete decongestive therapy (CDT) affects fluid shifts, the cardiovascular system as well as vascular health systematically over three weeks. As CDT leads to several liters of fluid being mobilized, this thesis is aiming to determine how the body copes with the excess fluid, which is returned back to the cardiovascular system. The overarching aim of this thesis is to **systematically assess the effect of** three weeks of **lymphedema therapy** on **fluid shifts, hemodynamic responses and endothelial/vascular (dys-)function** in lower leg lymphedema patients, and thereby **advancing knowledge on the poorly understood area of lymphatic system function** as well as **mechanisms impacted by systems' dysfunction** as observed in lymphedema. Finally, it aims at **investigating how these are modulated by physical therapy**. Therefore, following objectives were assessed systematically before-, during- and after-CDT, as well as pre - and post - MLD:

Objective #1: Determine fluid shifts before-, during- and after-CDT and due to MLD
Hypothesis #1: Overall leg volume as well as total- and extracellular fluid in the lower limbs will reduce over three weeks of CDT and post-MLD. The fluid mobilized from the lymphedematous leg will result in an increase in plasma volume, but will not lead to changes in plasma protein concentrations.

Rationale: Aim of CDT and its components such as MLD is to reduce volume and fluid within the affected body part. Several liters can be mobilized from each limb during three weeks of CDT. Previous studies have investigated such changes before- and after-CDT, however, there is no evidence how fluid is shifted due to MLD as well as during CDT. These fluid shifts, measured by perometry, bioelectric impedance spectroscopy (BIS) and plasma volume change calculations, were assessed systematically before- and after-MLD over several time points over the three weeks of CDT. Blood plasma components such as proteins and electrolytes were also assessed. As indicated above, protein concentration of the returned lymph fluid is estimated to be at the same level as blood plasma (3). Therefore, no changes in plasma protein concentrations were expected.

Objective #2: Determine lymphatic fluid outflow via plasma hyaluronic acid assessments

Hypothesis #2: Physical therapy in form of CDT and MLD will lead to increased plasma hyaluronic acid (HA) concentrations.

Rationale: As described above, lymphatic HA concentration is multiple times higher than plasma HA levels, which suggests that the lymphatic outflow back into the vasculature may be responsible for the majority of HA present in plasma. Therefore, hyaluronic acid has been proposed as a biomarker of lymphatic outflow (42, 69, 167, 168). However, those studies only included data from healthy participants. The physiological fluid dynamics surrounding lymphedema, as well as how CDT modulates lymphatic outflow are currently not well understood. Therefore, the effect of CDT and MLD on lymphatic outflow, via plasma hyaluronic acid concentration measurements, were assessed.

Objective #3: Assess hemodynamic changes in the system

Hypothesis #3: Lymphedema per se and lymphedema therapy will lead to altered hemodynamic responses at rest and during orthostatic loading (sit-to-stand test) over three weeks of CDT and due to MLD.

Rationale: Gravitational effects on the cardiovascular system during standing up (orthostasis) causes an initial drop in blood pressure (caused by blood pooling in the legs), which can lead to the loss of consciousness (syncope), if the body is not able to compensate for it. Orthostasis can display a massive issue in people with a history of dizziness when they stand up or in the elderly. In such cases, the cardiovascular system may not be able to regulate mean arterial pressure during orthostatic challenge, which can lead to a significant drop in cerebral blood flow, which may further result in fainting. The etiology of syncope is multifactorial and it can arise due to problems with cerebral autoregulation (169), hormonal factors (170, 171), autonomic dysfunction (172) or cardiac problems (173). In order to maintain a stable posture, the ability to detect posture changes and to elicit proper responses is necessary (174-177). Cardio-postural control may be affected by lymphedema treatment due to different volumes of fluid in the legs, pre- and post-therapy. However, there is no evidence of any study focusing on investigating orthostatic intolerance during CDT. As postural changes are accompanied by compensatory responses (178), changes in posture were carried out in form of a sit-to-stand test over several time points during CDT as well as pre- and post-MLD to assess changes in hemodynamic parameters at rest and hemodynamic responses to orthostatic loading.

Objective #4: Investigate the effects of physical therapy on vascular (dys-)function

Hypothesis #4: Lymphedema patients will show significant (subclinical) measures of endothelial/vascular dysfunction - which are related to micro-vascular dysfunction - at baseline. Further, CDT will improve these vascular function changes induced by lymphedema.

Rationale: Deranged lymphatic flow, as seen in lymphedema, can result in chronic inflammatory processes (36). Recent research has linked the lymphatic system and particularly lymphedema to inflammation, dyslipidemia, obesity, hypertension, metabolic syndrome or atherosclerosis (10, 24, 127, 179). All these factors have been implicated in the development of cardiovascular diseases (36). Endothelial dysfunction is the inability of vascular dilatation due to reduced levels of NO. It is a known risk factor in the pathogenesis of hypertension (37), atherosclerosis and other cardiovascular disease (38-40). It is considered as a key indicator of cardiovascular diseases. Therefore, vascular function, including measurements of endothelial parameters, before-, during- and following-CDT were studied. Endothelial (dys-)function and vascular health assessments in lymphedema patients were carried out via measurements of plasma asymmetric dimethylarginine (ADMA) concentrations, carotid-femoral pulse wave velocity (PWVcf) and flow mediated dilatation (FMD). Finally, microvascular changes were assessed via retinal microvasculature analysis.

3 MATERIALS AND METHODS

3.1 Pilot study

A pilot study was performed with the aim to investigate effects of three weeks of complete decongestive therapy on leg volume and lymphatic (out-)flow (assessed via plasma hyaluronic acid measurements). Plasma samples from 9 patients (3 male, 6 female, 55 years ± 13 years, 164.2 ± 6.1cm height, 85.3 ± 26.4 kg weight) with lower limb lymphedema stage II and III were obtained before - and after - three weeks of complete decongestive therapy (CDT)**.** Perometry was performed to assess leg volume. Plasma hyaluronic acid and volume regulating hormone responses were investigated as well**.** The main findings, as expected, were that lymphedema patients decrease leg volume due to CDT. Due to the high variances in-between patients, no significance was seen in plasma hyaluronic acid concentrations, which was used as a surrogate marker for lymphatic outflow. Furthermore, plasma total protein, plasma density, osmolality as well as sodium and chloride levels did not change before - compared to after - three weeks complete decongestive therapy. This pilot study indicated that there is a need to investigate these parameters not only before - and after - several weeks of lymphedema. Changes due to manual lymphatic drainage also need to be considered. Based on this pilot study, the study design was adapted as further detailed below (*Chapter 3.5*).

3.2 Patients

The study was conducted at the Center for Physical Medicine and Rehabilitation, Clinical Center for Lymphatic Disorders and General Hospital in Wolfsberg, Austria. Both, the Ethics Committee of Klagenfurt (EK: A 03/17) and the Ethics Committee of the Medical University Graz, Austria (EK: 29-090 ex 16/17) approved the study. The procedures throughout this study were performed in accordance with the Declaration of Helsinki (2013). Patients were recruited before undergoing three weeks of complete decongestive therapy. Strict inclusion and exclusion criteria were followed to enroll patients into this study. Included were patients with stage II primary or secondary lower limb lymphedema, in accordance with the latest consensus document of the International Society of Lymphology (2020) (125). Exclusion criteria were applied as follows: patients with signs of mental disorders, those with histories of cardiovascular diseases, syncope or alcoholism were excluded, as well as those on specific medications that potentially influence the measured parameters such as beta-blockers or diuretics. Finally, patients who received complete decongestive therapy within the last year prior to enrolment into this study as well as pregnant women were not allowed to participate. Each participant received detailed information about the study details and provided informed as well as written consent prior to participation. The signed informed consents are stored at the Division of Physiology, Otto Loewi Research Center, at the Medical University of Graz, Austria.

3.3 Sample size calculation

Fluid changes due to physical therapy, assessed via bioelectrical impedance spectroscopy was determined as the main outcome parameter. Based on previously published literature (162), a strong effect size could be expected ($f=0.5$). Considering $\alpha=0.05$ and a power=0.90, the a priori sample size calculation for repeated measures ANOVA resulted in an estimated total sample size of 13 patients.

3.4 Complete decongestive therapy protocol

Therapy protocols used in this research were equal to the treatment standards used at the Center for Physical Medicine and Rehabilitation at the Clinical Center for Lymphatic Disorders in Wolfsberg, Austria, which were based on the recommendations by Döller (156), as described in detail above (*Chapter 1.5*). Briefly, during three weeks of complete decongestive therapy, patients received manual lymphatic drainages every weekday for a duration of 30 minutes. Additionally, lymph therapists then applied compression bandages every day. These garments had to be worn by the patients during the whole day and at night. Furthermore, patients participated in physical exercise sessions such as ergometry and/or walking as well as measures for skin care and skin regeneration.

3.5 Study design

All measurements were conducted between 08:00 am and 12:30 pm in a moderately darkened and quiet room. The temperature remained constant between 22-25°C and the humidity was set between 50-55%. The detailed protocol of the experimental setup and all measurements performed are shown in Figure 1. As the pilot study highlighted the need to investigate the effects of lymphedema therapy systematically over three weeks as well as due to manual lymphatic drainage the following study design was developed: Fluid mobilization assessments (perometry, and bioelectrical impedance spectroscopy), blood collection, hemodynamic assessment (sit-to-stand test) and vascular measurements (carotid-femoral pulse wave velocity (PWVcf), brachial flow mediated dilatation (FMD) and retinal microvasculature analysis) were performed on five different days, systematically over three weeks of therapy in order to receive a time course of the effects due to treatment. Following time points were chosen: day 1, day 2, day 7, day 14 and day 21 of CDT. At each of these time points measurements were performed directly before - and after - 30 minutes of MLD in order to investigate its direct effect on fluid mobilization, cardiovascular responses and endothelial/vascular function.

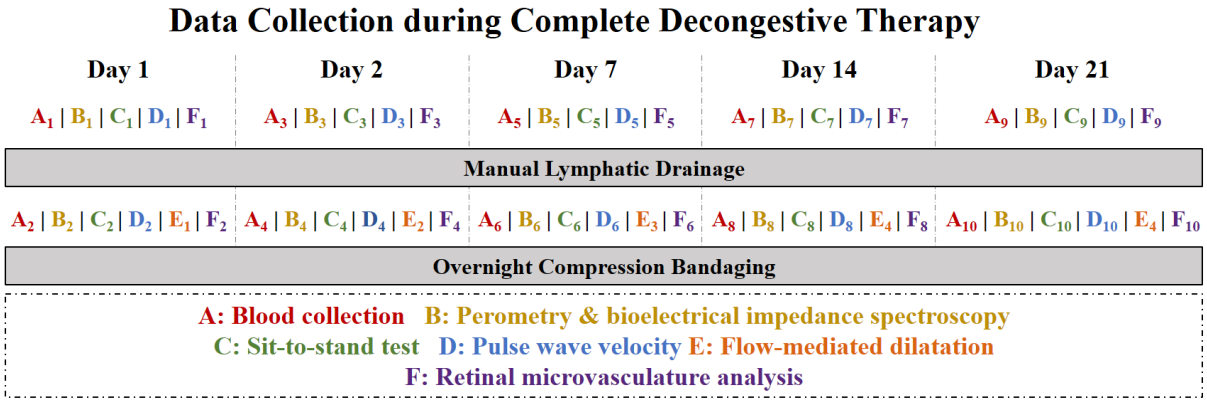

Figure 1: Detailed schematic of the data collection procedure. Over three weeks, measurements were performed on day 1, day 2, day 7, day 14 and day 21 of complete decongestive therapy (CDT). On each of these indicated time points (days), assessments were performed directly before - and after - manual lymphatic drainage. Following measurements were performed: A: Blood collection, B: Perometry and bioelectrical impedance spectroscopy (BIS), C: Sit-to-stand Test, D: Carotid-femoral pulse wave velocity, E: Brachial flow-mediated dilatation and F: Retinal microvasculature analysis.

3.6 Blood sampling and plasma analysis

At each of the above described time points, a total amount of 50mL venous blood was collected from the ante-cubital vein from seated patients. Following parameters were assessed at the routine blood analysis laboratory immediately after collection at the general hospital in Wolfsberg, Austria: Plasma protein concentrations (total protein, albumin and globulin), osmolality, oncotic pressure, electrolytes (sodium, chloride and potassium) and blood cell count. Additionally, EDTA blood samples were instantly centrifuged at 1500G for 15 minutes at 4°C. Supernatant plasma was obtained, aliquots were then stored at -80°C until used for further measurements, which were performed at the Medical University of Graz, Division of Physiology, Otto Loewi Research Center. Hematocrit (Hct) was assessed in duplicates (10 minutes at 10,000 rpm). Plasma density (PD) was assessed using a high-precision mass densitometry device (Model 602M, Paar KG, Graz, Austria) on 0.2 mL samples applying the mechanical oscillator technique. Density determinations were measured at 37.00 ± 0.02°C controlled by an ultra-thermostat (Hetofrig, Denmark). Corresponding PD and Hct values were used to calculate mass density (MD) of the fluid shifts. ADMA was measured using commercially available ELISA Kits.

3.7 Assessment of volume and fluid shifts

3.7.1 Perometry

Total volume of the lower limbs was assessed using a perometer type 550 T (Pero-System Messgeräte, Germany) along with the PeroPlus 2000 software. Both legs were analyzed at each of the ten indicated time points.

3.7.2 Bioelectrical impedance spectroscopy

Whole-body and segmental fluid composition was assessed with a SFB7 device (ImpediMed Ltd., Brisbane, Australia) together with the manufacturer's software (Bioimp 5.5.0.1., ImpediMed Ltd., Brisbane, Australia). Following protocol was strictly followed: Before the measurement, all patients were asked to empty their bladder. After disinfecting the respective skin parts, impedance electrodes were placed. Total body composition analysis as well as segmental measurements in the lower limbs were performed in supine position (180, 181). For whole-body composition assessment, electrodes were positioned on the left hand (midline of the ulnar styloid process on the wrist) and on the corresponding foot (between the medial and lateral malleolus bones, on the ankle). For segmental analysis, the leg electrode remained at the exact same position. The second electrode was positioned on the upper thigh, as high as possible. The exact position was marked for each individual patient and used at all measurement time points over the three weeks of therapy. Circumference at both electrode positions was measured and used to calculate segmental parameters (total body water, extracellular - and intracellular - fluid) of the legs (182, 183). Normalized to the individual patient's body weight, following parameter were calculated: percentages of total body water (%TWB), extracellular fluid (%ECF) and intracellular fluid (%ICF), as well as the ratio of whole-body extracellular - to intracellular - fluid (ECF/ICF). As the determination of the lower limbs individual weight was not possible, segmental fluid distribution - limb extracellular (limbECF) and limb intracellular fluid (limbICF) - were calculated from the segmental assessment. Calculations were based on the limb circumferences, limb length, extracellular impedance (Ri), intracellular impedance (R0), and gender specific resistances. Finally, the ratio between limb extracellular - and intracellular - fluid (limbECF/limbICF) was determined, expressed as intracellular impedance (Ri) to extracellular impedance (R0) ratio (180, 184).

3.7.3 Assessment of plasma volume expansion

Different equations were used to calculate relative plasma volume changes (PVC) based on (1) plasma density (PD) and hematocrit (Hct), (2) hematocrit only or (3) hematocrit and hemoglobin (Hb) changes. However, it is important to address that all these formulae are usually used to estimate plasma volume losses. Additionally, the formula according to Nadler (4) was used to estimate the absolute values of plasma volume changes.

(1) %ΔPV calculated by plasma density changes

Mass density (FD) of fluid shifts was calculated using the corresponding PD and values as described here in further detail (185):

$$FD = PD_B - \frac{Hct_B(1 - Hct_A)}{Hct_A - Hct_B}(PD_A - PD_B)$$

B is defined as the value before MLD and A is defined as the value post-MLD. Fluid shifts were expressed as relative changes in plasma volume compared to plasma volumes prior to MLD. With hemodilution (plasma volume expansion) the following equation applies:

$$\%\Delta PV = 100\frac{PD_B - PD_A}{PD_A - FD}PV_B$$

(2) %ΔPV calculated by hematocrit

ΔPV, expressed as percentages of the baseline values, was calculated from hematocrit variations according to Van Beaumont (186).

$$\%\Delta PV = \frac{100(Hct_B - Hct_A)}{Hct_A(100 - Hct_B)}$$

Whereas Hct_B is defined as the baseline value, Hct_A is defined as the value post-MLD.

(3) %ΔPV calculated by hematocrit and hemoglobin

Formulae have been developed to estimate the relative plasma volume changes (as compared to baseline). This equation uses the blood parameters hemoglobin (mg/dL) and hematocrit (%) and was originally intended for the usage in estimating acute changes in blood volumes during exercise, orthostatic stress or dehydration (187).

$$\%\Delta PV = 100\left\{\left(\frac{Hb_B}{Hb_A}\right)\frac{(100 - Hct_A)}{(100 - Hct_B)}\right\} - 1$$

In accordance with Dill & Costill the subscripts stand for “before” (B) and “after” (A).

(4) ΔPV calculated by anthropometric data and hematocrit

Additionally, absolute plasma volume increase was calculated based on Nadler's formula (188) used for estimating total blood and plasma volume which is using weight, height, sex, and hematocrit. This formula, however, could be criticized as being inaccurate for current estimates since patients with lymphedema have far just another body composition.

All parameters measured in the plasma that are dependent on plasma volume were corrected to plasma volume changes according to equation 2, as hematocrit as well as hemoglobin perfectly matched upon correction.

3.8 Assessing hemodynamic responses at rest and to orthostatic loading

A sit-to-stand test was performed to assess hemodynamic parameters at rest and hemodynamic responses to orthostatic loading. The Task Force Monitor® (TFM) (CNSystems, Graz, Austria) was used to continuously, non-invasively assess hemodynamic parameters. Therefore, electrocardiogram (ECG) electrodes were applied together with an upper arm as well as finger blood pressure cuff for continuous heart rate and blood pressure measurement. Electrode stripes for transthoracic bioimpedance cardiography were positioned in the neck and at the mid-clavicular line at the xiphoid process level (189). The patients were then asked to remain in a seated position (baseline) followed by 5 minutes of standing upright. During standing, the right arm was kept at heart level using a sling. Patients were asked to look straight towards a wall with their eyes opened, focused on a marked spot, and to breath normally. During

the sit-to-stand test, qualified personnel was present, being able to assist in any case of syncope signs during standing. Following parameters were continuously recorded: Heart rate (HR) was recorded via ECG. Plethysmography was used to measure beat-to-beat systolic blood pressure (SBP) and diastolic blood pressure (DBP), as well as mean blood pressure (mBP). Stroke volume (SV) was assessed using transthoracic bioimpedance cardiography. Continuously acquired data was then analyzed as described in Trozic et al. (2020). Briefly, following epochs were analyzed: (1) last 10 seconds of baseline, (2) 0-10 seconds, (3) 10-20 seconds, (4) 20-30 seconds, (5) 170-180 seconds (at 3 minutes) and (6) 150-260 seconds (at 5 minutes) of standing, as well as (7) first 10 seconds, (8) 10-20 seconds, (9) 20-30 seconds and (10) last 10 seconds of recovery phase (190). Figure 2 details the analyzed epochs.

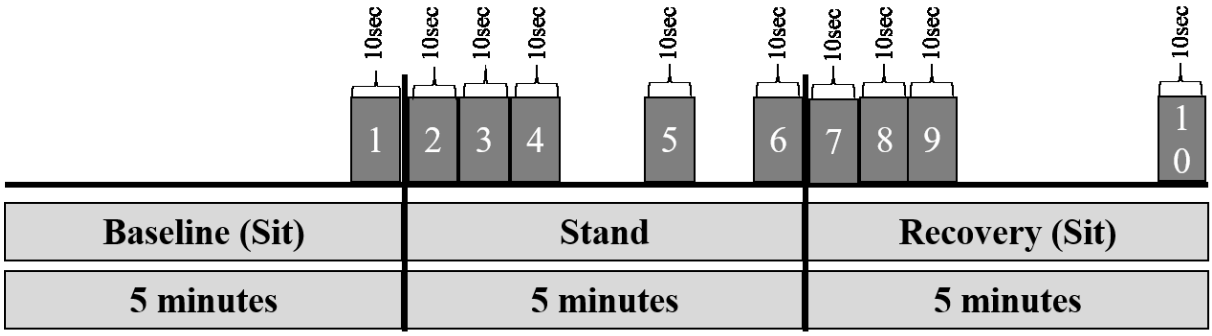

Figure 2: Epochs during sit-to-stand test, consisting of three phases: Baseline in seated position, standing and recovery (sitting). Following epochs were used for analysis (190): (1) last 10 seconds of baseline (2) 0-10 seconds of standing (3) 10-20 seconds of standing (4) 20-30 seconds of standing (5) 290-300 seconds of standing, (6) last 10 seconds of standing, (7) 0-10 seconds of recovery (8) 10-20 seconds of recovery (9) 20-30 seconds of recovery and (10) last 10 seconds of recovery.

3.9 Endothelial/vascular (dys-)function assessment

3.9.1 Pulse wave velocity

Vicorder® device (SMT medical, Germany) was used to assess carotid-femoral pulse wave velocity (PWVcf) in supine position, which is a measure for arterial stiffness (191). Therefore, one cuff was fixed around the neck, above the carotid artery and the second cuff was positioned around the right thigh as high as possible at the level of the femoral artery. Prior to cuff inflation, the distance between both cuffs (from the suprasternal notch to the middle of the femoral cuff) was measured. Carotid-femoral pulse wave velocity (PWVcf) data is presented in meters per second (m/s).

3.9.2 Flow-mediated dilatation

Flow-mediated dilatation (FMD) was assessed in supine position, strictly according to the physiological and methodological guidelines described by Thijssen et al. (192, 193). The patient's right arm was resting at 80° to allow a comfortable and relaxed position for assessing the brachial artery via ultrasound. A regular blood pressure cuff was positioned around the forearm, about 5-7cm from the medial epicondyle. After 10 minutes of baseline, the cuff was inflated to 200 mmHg for exactly 5 minutes. The artery was recorded until 150 seconds post-occlusion. Reactive hyperemia was verified by using pulse-wave Doppler scanning.

3.9.3 Retinal microvasculature imaging

A hand-held fundus camera (Smartscope Pro, Optomed, Finland) was used to assess microvascular changes of the eye. Retinal images of both eyes were obtained at all time points indicated in Figure 1. iFlexis software (VITO, Belgium) was used to analyze the optic disc-centered images. The analysis included the six biggest arterioles and venules located between 0.5- and 1-disc diameters from the optic disc margin (for details see (194-197)). Results are presented as central arteriolar equivalent (CRAE), central retinal venular equivalent (CRVE) and arteriolar-to-venous ratio (A-to-V).

3.10 Statistical analysis

Two-way analysis of variances ANOVA for repeated measures was performed in order to determine overall effects over three weeks of lymphedema therapy and manual lymphatic drainage averaged over three weeks. To avoid data loss in the repeated measures design and to maximize data output single time points were also analyzed separately using a pairwise comparison (t-test) of (i) daily averaged values with each indicated time point compared to the first day of therapy and (ii) pre- vs. post-MLD values for each indicated time point (Supplemental tables). Due to the limited number of complete data sets, hemodynamic responses were analyzed by two-way ANOVA for repeated measures with the following factors for each assessment day separately: epochs and pre-/post-manual lymphatic drainage. Correlation was tested using Pearson's correlation applying Fisher's z-transformation to calculate a mean correlation over all time points. *P*-values <0.05 were considered as statistically significant. For statistical analysis, SPSS Statistics (Version 23, IBM, USA) was used. GraphPad Prism (Version 8.4.1, GraphPad Software Inc., USA) was used to generate all figures. Hemodynamic data were analyzed using MATLAB-Software (Version 2016b, The MathWorks Inc., USA). Data are presented as mean ± standard deviation (SD). Mean values ± standard error of the mean (SEM) were used for the figures.

4 RESULTS

4.1 Patient details

A total number of 13 patients (3 males and 10 females, 57 ± 8.0 years old, 167.2 ± 8.3 cm height, 91.0 ± 23.4 kg weight, BMI: 32.4 ± 6.9 kg/m^2) with stage II primary (n=6) - or secondary (n=7) - lymphedema participated in this study. All patients were diagnosed with grade II lymphedema according to the staging system of the International Society of Lymphology (defined as pitting edema and not reversible upon limb elevation (77)) for at least 6 months before undergoing CDT at Wolfsberg Clinical Center for Lymphatic Disorders, Wolfsberg State Hospital, KABEG, Austria. Of those patients, 10 were diagnosed with bilateral lower limb lymphedema, the others (n=3) were diagnosed with unilateral forms of lymphedema. From the 13 patients, 8 patients were willing to provide blood samples. All patients were able to complete the study protocol. Patient details are displayed in Table 2. Over three weeks of therapy, patients lost in average 2.6 kg of body weight, from 93.7 ± 23.6 kg before CDT to 91.1 ± 23.1 kg post-CDT.

Table 2: Overview of patient demographics including age, sex, height and type of lymphedema.

ID	Blood sample	Sex	Age (years)	Height (cm)	Type of lymphedema
1	no	f	55	167	Secondary bilateral lymphedema
2	yes	m	63	180	Secondary bilateral lymphedema
3	no	f	53	178	Secondary unilateral lymphedema
4	yes	f	52	165	Secondary bilateral lymphedema
5	yes	f	46	158	Secondary bilateral lymphedema
6	no	f	55	159	Primary bilateral lymphedema
7	yes	m	56	180	Secondary bilateral lymphedema
8	no	f	71	159	Primary bilateral lymphedema
9	no	f	64	165	Secondary unilateral lymphedema
10	yes	m	59	176	Primary unilateral lymphedema
11	no	f	40	157	Primary bilateral lymphedema
12	yes	f	66	168	Primary bilateral lymphedema
13	no	f	55	161	Secondary bilateral lymphedema
Mean			**57**	**167.2**	
SD			**8**	**8.3**	

Table 3: Overview of weight (kg) and BMI (kg/m^2) changes over three weeks of complete decongestive therapy (CDT).

ID	Weight pre-CDT (kg)	Weight post-CDT (kg)	BMI pre-CDT (kg/m^2)	BMI post-CDT (kg/m^2)
1	61.8	60.8	22.2	21.9
2	117.1	114.4	36.1	35.2
3	116.7	113.8	36.9	36.0
4	95.0	91.7	34.9	33.8
5	63.4	60.8	25.2	24.4
6	101.4	97.0	40.0	38.4
7	90.5	85.6	28.1	26.5
8	102.9	100.9	40.7	40.0
9	103.2	100.5	37.8	37.1
10	137.1	133.3	44.2	42.9
11	73.7	72.2	30.0	29.2
12	60.1	58.7	21.3	29.9
13	95.5	94.6	37.0	36.6
Mean	**93.7**	**91.1**	**33.4**	**33.2**
SD	**23.6**	**23.0**	**7.1**	**6.1**

4.2 Fluid shifts due to physical therapy

4.2.1 Segmental fluid mobilization

Leg volume, assessed via perometry, decreased over three weeks of therapy from 12.881 ± 2.017 liters to 12.448 ± 1.976 liters ($F_{(2,37)}$=31; p<0.001). This equals a volume loss of 0.392 liters or 3.1% over three weeks of therapy (Figure 3). The greatest reduction of 1.5% (p<0.001) was shown between day 1 (12.840 ± 1.899 liters) and day 2 (12.640 ± 1.820 liters) of physical therapy (Table 3). Leg volume did not change due to MLD ($F_{(1,15)}$=0.42; p=0.527).

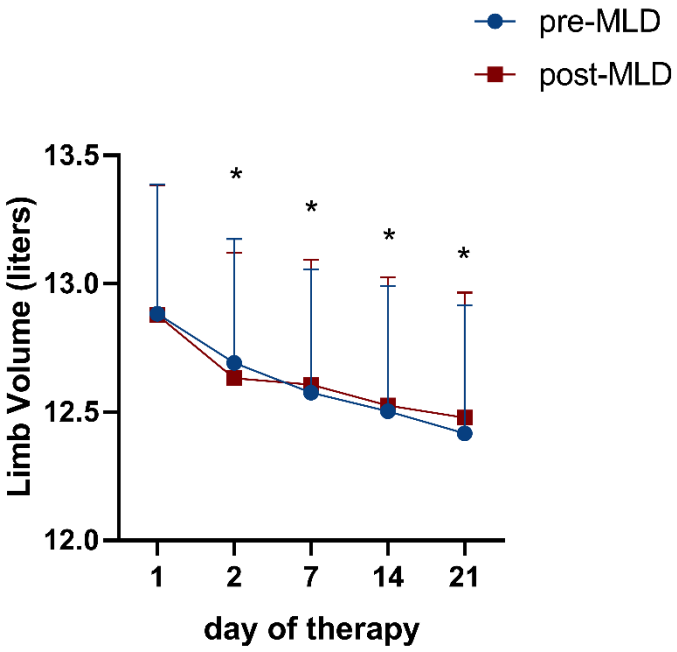

Figure 3: Time course over three weeks of complete decongestive therapy (CDT) of lower limb volume (liters) assessed via perometry. The blue dots represent values before manual lymphatic drainage (MLD), whereas the red boxes reflect values post-MLD. Asterisks (*) indicate significant (p<0.05) changes. [Data from *Brix et al. (2020)* (198)]

Three weeks of CDT further led to the reduction of extracellular limb volume (limbECF) ($F_{(4,76)}$=2.6; p=0.041). The greatest reduction could be observed between day 1 (3.068 ± 0.640 liters) and day 2 (2.795 ± 0.715 liters, $t_{(25)}$=3.03; p=0.006) as well as day 7 (2.905 ± 0.662 liters, $t_{(25)}$=2.75; p=0.011) of physical therapy (Figure 4B and Table 3).

Three weeks of CDT led to a decrease in intracellular fluid in the lower limbs (limbICF) ($F_{(4,76)}$=4.11; p=0.005), whereas limbICF increased due to MLD ($F_{(1,19)}$=6.85; p=0.017). A significant difference in limbICF was shown between day 2 (1.920 ± 0.453 liters) and day 14 (1.811 ± 0.430 liters) of therapy ($t_{(19)}$=3.43; p=0.003). MLD led to an average increase of limbICF from 1.837 ± 0.037 liters to 1.879 ± 0.056 liters (Table 2), which is an increase of 2.3% (Figure 4C and Table 3).

Total limb fluid (limbTBW) tended to decrease over three weeks from 4.987 ± 0.910 liters to 4.824 ± 0.926 liters ($F_{(3,51)}$=2.77; p=0.057). Further, limbTBW did not change due to MLD ($F_{(1,19)}$=3.05; p=0.097) (Figure 4A and Table 3).

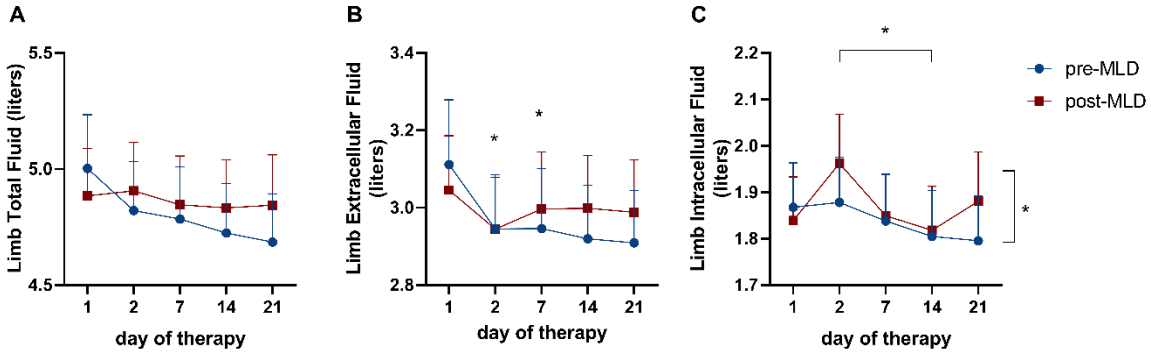

Figure 4: Overview of the time course effects of (A) total fluid, (B) extracellular fluid and (C) intracellular fluid changes in the lower limbs over three weeks of complete decongestive therapy (CDT) and due to manual lymphatic drainage (MLD). The blue dots reflect values pre-MLD, whereas the red squares represent values post-MLD. Significant changes are marked with asterisks (*). [Data from *Brix et al. (2020)* (198)]

Three weeks of CDT further led to the significant reduction of the ratio of limbECF normalized to limbICF (limbECF/limbICF) ($F_{(4,76)}$=4.49; p=0.003). LimcECF/limbICF did not change due to MLD ($F_{(1,19)}$=0.28; p=0.601). Post-test revealed that the major reduction occurred between day 1 and day 2 of CDT (p=0.003) (Figure 5 and Table 3).

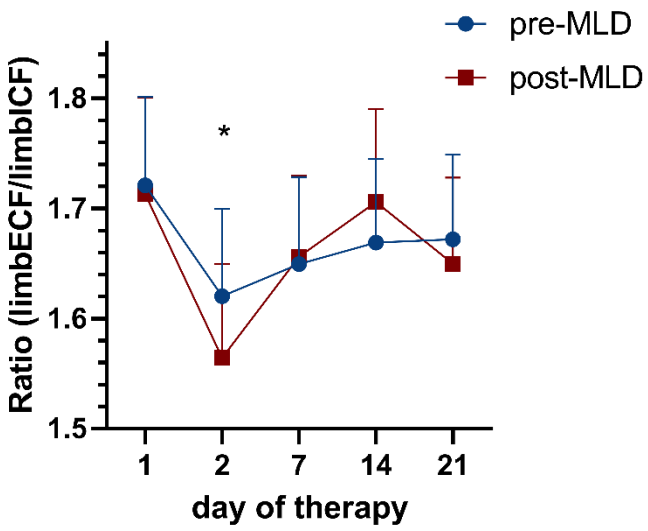

Figure 5: Time course of limb extracellular fluid, which was normalized to limb intracellular fluid (ratio) over three weeks of complete decongestive therapy (CDT) and due to manual lymphatic drainage (MLD). The blue dots display pre-MLD values, the red squares represent values after MLD. Asterisks (*) indicate significant differences (p<0.05). [Data from *Brix et al. (2020)* (198)]

Table 4: Overview of segmental fluid shifts over three weeks of complete congestive therapy (CDT), before - and after - manual lymphatic drainage (MLD). Data are shown as mean ± SD, including estimated marginal means.

		Complete Decongestive Therapy					
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**	*Estimated marginal means*
Limb Volume (liters) n=16	pre-MLD	12.884 ± 2.016	12.632 ± 1.933	12.576 ± 1.924	12.503 ± 1.954	12.417 ± 1.100	*1.2614 ± 1.962*
	post-MLD	12.878 ± 2.021	12.632 ± 1.954	12.607 ± 1.943	12.525 ± 2.002	12.478 ± 1.954	*1.2624 ± 1.970*
	Estimated marginal means	*12.881 ± 2.017*	*12.662 ± 1.943*	*12.591 ± 1.932*	*12.514 ± 1.977*	*12.448 ± 1.976*	
Limb total fluid (liters) n=20	pre-MLD	5.003 ± 1.038	4.821 ± 0.941	4.784 ± 1.010	4.725 ± 0.954	4.685 ± 0.933	*4.804 ± 0.962*
	post-MLD	4.885 ± 0.913	4.906 ± 0.934	4.846 ± 0.939	4.833 ± 0.926	4.845 ± 0.971	*4.863 ± 0.899*
	Estimated marginal means	*4.944 ± 0.930*	*4.864 ± 0.935*	*4.815 ± 0.970*	*4.779 ± 0.935*	*4.765 ± 0.944*	
Limb extracellular fluid (liters) n=20	pre-MLD	3.112 ± 0.750	2.945 ± 0.626	2.946 ± 0.697	2.920 ± 0.621	2.909 ± 0.605	*2.966 ± 0.648*
	post-MLD	3.046 ± 0.628	2.945 ± 0.603	2.997 ± 0.663	2.999 ± 0.613	2.988 ± 0.603	*2.995 ± 0.590*
	Estimated marginal means	*3.079 ± 0.657*	*2.945 ± 0.608*	*2.971 ± 0.680*	*2.959 ± 0.612*	*2.949 ± 0.599*	
Limb intracellular fluid (liters) n=20	pre-MLD	1.868 ± 0.429	1.878 ± 0.437	1.838 ± 0.452	1.805 ± 0.442	1.796 ± 0.434	*1.837 ± 0.429*
	post-MLD	1.839 ± 0.422	1.962 ± 0.475	1.849 ± 0.398	1.818 ± 0.424	1.881 ± 0.474	*1.870 ± 0.425*
	Estimated marginal means	*1.853 ± 0.416*	*1.920 ± 0.425*	*1.843 ± 0.429*	*1.811 ± 0.447*	*1.838 ± 0.599*	
Limb ECF/ICF (ratio) n=20	pre-MLD	1.72 ± 0.36	1.62 ± 0.36	1.65 ± 0.35	1.67 ± 0.34	1.67 ± 0.34	*1.66 ± 0.34*
	post-MLD	1.71 ± 0.39	1.56 ± 0.38	1.65 ± 0.33	1.71 ± 0.38	1.65 ± 0.35	*1.66 ± 0.35*
	Estimated marginal means	*1.71 ± 0.37*	*1.59 ± 0.36*	*1.65 ± 0.34*	*1.69 ± 0.36*	*1.66 ± 0.34*	

4.2.2 Correlation between limb volume and segmental fluid assessment

Perometry, which was used to assess limb volume, and BIS, used for the measurement of limb fluid (limbTBW) moderately correlated over all measurement time points (r=0.635).

4.2.3 Whole-body fluid shifts assessed via bioelectrical impedance spectroscopy

Percentage of total body water (%TBW) reduced over three weeks of physical therapy ($F_{(4,28)}$=7.04; p=0.001). Further, it showed to increase post-MLD ($F_{(1,7)}$=11.84; p=0.011). The greatest reduction in %TBW occurred between day 1 and day 2 of CDT ($t_{(13)}$=3.09; p=0.009), with a reduction from 50% to 46.5% total body water. Overall reduction of %TBW over three weeks of CDT was from 50% to 47%. The average increase due to MLD was from 46% pre-MLD to 48% post-MLD (Figure 6A and Table 4).

Relative changes in extracellular fluid (%ECF) occurred over three weeks of CDT ($F_{(4,40)}$=4.15; p=0.007), with the greatest decrease between the first and second day of lymphedema therapy ($t_{(13)}$=2.48; p=0.029). Overall reduction was 0.8% points (from 20.7% to 19.9%) in whole-body extracellular fluid. %ECF did not change due to MLD ($F_{(1,10)}$=2.17; p=0.172) (Figure 6B and Table 4).

Percentage of intracellular fluid (%ICF) increased due to MLD ($F_{(1,10)}$=15; p=0.003) by 1 percentage point. No changes in %ICF occurred over three weeks of CDT ($F_{(1,12)}$=4.04; p=0.063) (Figure 6C and Table 4).

MLD led to a significant change in the ratio of ECF/ICF over three weeks of CDT ($F_{(1,10)}$=8.97; p=0.013). CDT did not affect the ECF/ICF ratio over three weeks of therapy ($F_{(1,14)}$=0.56; p=0.521) (Figure 6D and Table 4).

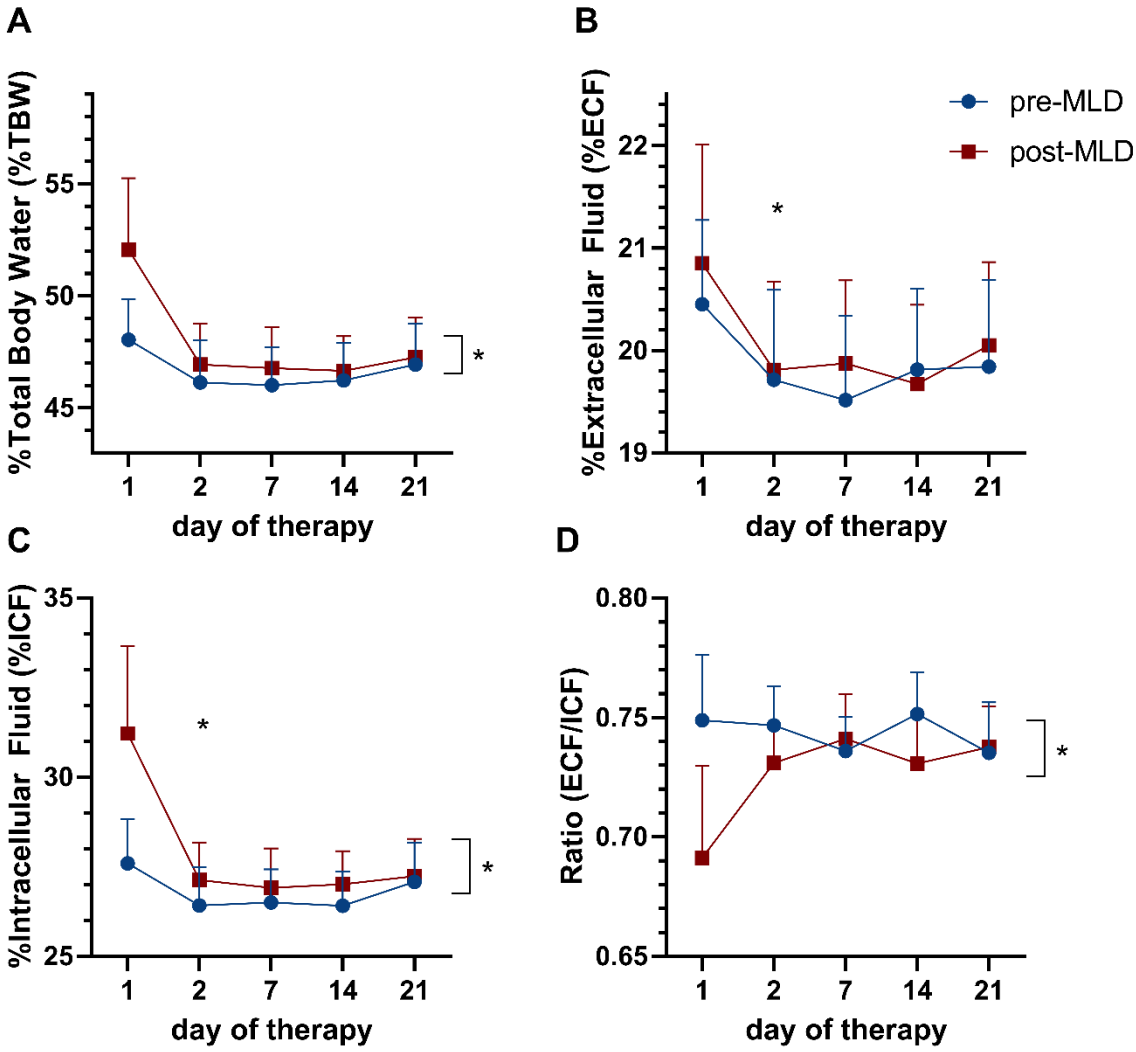

Figure 6: Time course of whole-body fluid shifts. (A) total body water (%TBW), (B) extracellular fluid (%ECF), (C) intracellular fluid (%ICF), relative to the individual patient's body weight, and (D) ECF/ICF ratio over three weeks of complete decongestive therapy (CDT) and due to manual lymphatic drainage (MLD). Blue circles represent values before MLD, whereas the red squared indicate post-MLD values. Asterisks (*) indicate significant changes ($p<0.05$). [Data from *Brix et al. (2020)* (198)]

Table 5: Overview of whole-body fluid shifts over three weeks of complete congestive therapy (CDT), before - and after - manual lymphatic drainage (MLD). Data are presented as mean ± SD, including estimated marginal means.

		Complete Decongestive Therapy					
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**	*Estimated marginal means*
%Total body fluid n=11	pre-MLD	48.0 ± 5.8	46.1 ± 6.3	46.0 ± 5.6	46.2 ± 5.5	46.9 ± 6.0	*46.7 ± 5.8*
	post-MLD	52.1 ± 10.6	46.9 ± 6.1	46.8 ± 6.1	46.7 ± 5.1	47.3 ± 5.9	*47.9 ± 6.2*
	Estimated marginal means	*50.1 ± 7.9*	*46.5 ± 6.2*	*46.4 ± 5.8*	*46.4 ± 5.3*	*47.1 ± 5.9*	
%Extracellular fluid n=11	pre-MLD	20.5 ± 2.7	19.7 ± 2.9	19.5 ± 2.7	19.8 ± 2.6	19.8 ± 2.8	*19.9 ± 2.7*
	post-MLD	20.8 ± 3.8	19.8 ± 2.9	19.9 ± 2.7	19.7 ± 2.5	20.0 ± 2.7	*20.0 ± 2.8*
	Estimated marginal means	*20.7 ± 3.2*	*19.8 ± 2.9*	*19.7 ± 2.7*	*19.7 ± 2.5*	*19.9 ± 2.7*	
%Intracellular fluid n=11	pre-MLD	27.6 ± 4.1	26.4 ± 3.6	25.5 ± 3.1	26.4 ± 3.2	27.1 ± 3.6	*26.8 ± 3.3*
	post-MLD	31.2 ± 8.1	27.1 ± 3.5	25.9 ± 3.7	27.0 ± 3.1	27.2 ± 3.4	*27.9 ± 3.9*
	Estimated marginal means	*29.4 ± 5.9*	*26.8 ± 3.5*	*26.7 ± 3.3*	*26.7 ± 3.1*	*27.2 ± 3.5*	
limbECF/limbICF (ratio) n=11	pre-MLD	0.75 ± 0.10	0.75 ± 0.05	0.74 ± 0.05	0.75 ± 0.06	0.74 ± 0.07	*0.74 ± 0.06*
	post-MLD	0.69 ± 0.13	0.73 ± 0.05	0.74 ± 0.06	0.73 ± 0.07	0.74 ± 0.06	*0.73 ± 0.07*
	Estimated marginal means	*0.72 ± 0.11*	*0.74 ± 0.05*	*0.74 ± 0.05*	*0.74 ± 0.06*	*0.74 ± 0.06*	

4.2.4 Plasma volume changes (PVC)

All four formulae for the calculation of plasma volume changes showed similar results. Plasma volume increased by approximately 1.5% ± 0.8 post-MLD. However, plasma volume did not change over three weeks of physical therapy ($F_{(2,24)}$=0.88; p=0.489) (Figure 7).

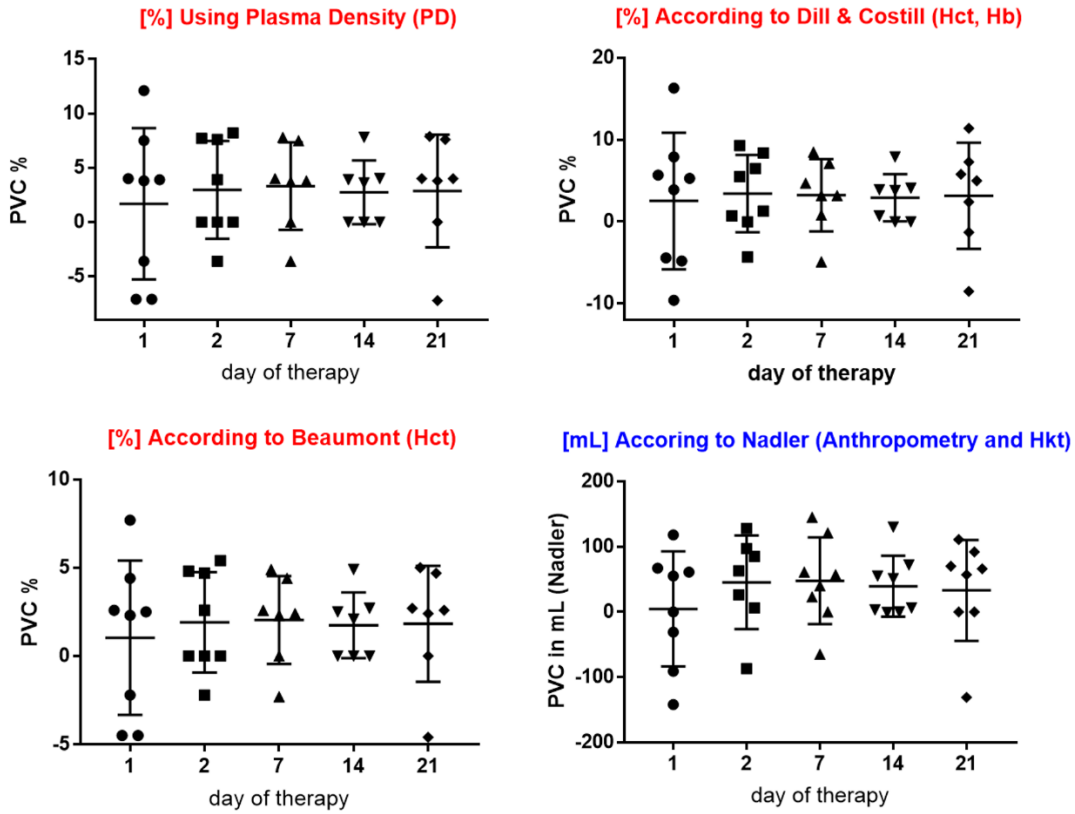

Figure 7: Overview of plasma volume changes (PVC) based on different equations used. Plasma density (PD) and hematocrit were used to calculate the mass density of the fluid shifts. Formula according to Dill & Costill (187), utilizing hemoglobin (mg/dL) and hematocrit (%). Values calculated from hematocrit variations according to Van Beaumont (20). Formula according to Nadler to estimate absolute plasma volume increases in milliliters taking into account weight, height, sex, and hematocrit (188). [Adapted from *Brix et al. (2020)* (198)]

4.2.5 Plasma protein increases due to physical therapy

Albumin, as well as albumin to globulin ratio (A/G ratio), increased from 4.33 ± 0.31 to 4.48 ± 0.38 mg/L and 1.61 ± 0.24 to 1.67 ± 0.23 mg/L due to MLD ($F_{(1,5)}$=23.71; p=0.005 and $F_{(1,5)}$=6.63; p=0.049, respectively), as detailed in Table 5. This reflects an increase of 3.3% and 4.1%, respectively (Figure 8B and 8D). Total plasma protein levels tended to increase post-MLD ($F_{(1,6)}$=5.04; p=0.066), whereas it did not change over three weeks of complete decongestive therapy ($F_{(4,24)}$=0.96; p=0.445) (Figure 8A). Globulin levels did not change due to MLD ($F_{(1,5)}$=0.47; p=0.522) and not over three weeks of CDT ($F_{(4,20)}$=0.23; p=0.916) (Figure 8C).

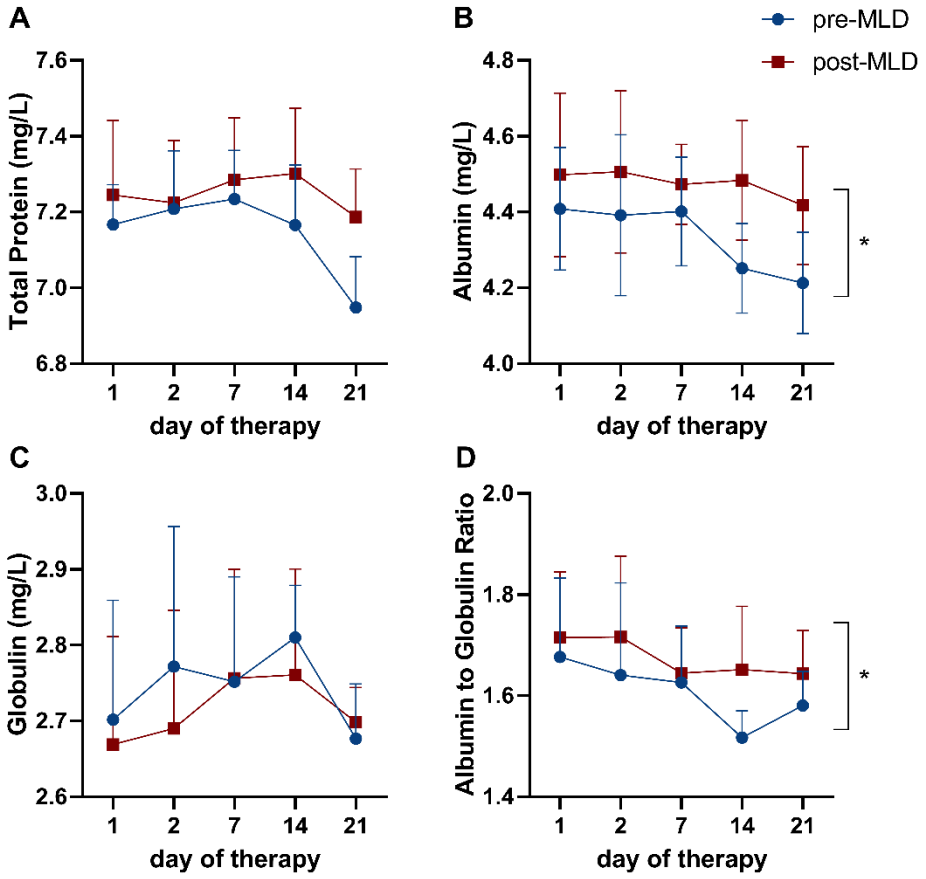

Figure 8: Time course of changes in (A) total protein (mg/L), (B) albumin (mg/L), (C) globulin (mg/L) and (D) ratio between albumin and globulin (A/G) over three weeks of complete decongestive therapy (CDT) and due to manual lymphatic drainage (MLD). The blue dots represent values before MLD, the red squares represent concentrations after MLD. Asterisks (*) display significant increases in albumin (p=0.005) and A/G ratio (p=0.049) pre- compared to post-MLD. [Data from *Brix et al. (2020)* (198)]

Table 6: Overview of plasma proteins changes over three weeks of physical therapy (complete decongestive therapy), before - and after - manual lymphatic drainage (MLD). Values are shown as mean ± SD, including estimated marginal means.

		Complete Decongestive Therapy					
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**	*Estimated marginal means*
Total protein (mg/L) n=7	pre-MLD	7.12 ± 0.28	7.21 ± 0.41	7.23 ± 0.34	7.17 ± 0.42	6.95 ± 0.35	*7.14 ± 0.31*
	post-MLD	7.25 ± 0.52	7.22 ± 0.43	7.28 ± 0.43	7.30 ± 0.45	7.19 ± 0.34	*7.25 ± 0.37*
	Estimated marginal means	*7.21 ± 0.34*	*7.22 ± 0.40*	*7.26 ± 0.37*	*7.23 ± 0.43*	*7.07 ± 0.33*	
Albumin (mg/L) n=6	pre-MLD	4.41 ± 0.39	4.39 ± 0.52	4.40 ± 0.35	4.25 ± 0.29	4.21 ± 0.33	*4.33 ± 0.31*
	post-MLD	4.50 ± 0.53	4.51 ± 0.53	4.47 ± 0.26	4.48 ± 0.39	4.42 ± 0.38	*4.48 ± 0.38*
	Estimated marginal means	*4.45 ± 0.45*	*4.45 ± 0.52*	*4.44 ± 0.56*	*4.37 ± 0.33*	*4.32 ± 0.34*	
Globulin (mg/L) n=6	pre-MLD	2.70 ± 0.39	2.77 ± 0.45	2.75 ± 0.34	2.81 ± 0.17	2.68 ± 0.18	*2.74 ± 0.24*
	post-MLD	2.67 ± 0.35	2.69 ± 0.38	2.76 ± 0.35	2.76 ± 0.34	2.70 ± 0.11	*2.72 ± 0.22*
	Estimated marginal means	*2.69 ± 0.34*	*2.73 ± 0.40*	*2.75 ± 0.13*	*2.79 ± 0.24*	*2.69 ± 0.13*	
Albumin to Globulin Ratio n=6	pre-MLD	1.68 ± 0.38	1.64 ± 0.45	1.63 ± 0.27	1.52 ± 0.13	1.58 ± 0.16	*1.61 ± 0.24*
	post-MLD	1.72 ± 0.32	1.72 ± 0.39	1.64 ± 0.22	1.65 ± 0.31	1.64 ± 0.21	*1.67 ± 0.23*
	Estimated marginal means	*1.70 ± 0.34*	*1.68 ± 0.41*	*1.64 ± 0.23*	*1.58 ± 0.20*	*1.61 ± 0.17*	

4.2.6 Plasma osmolality and oncotic pressure

CDT did not lead to changes in plasma osmolality as well as oncotic pressure over three weeks ($F_{(4,12)}$=1.01; p=0.440 and $F_{(1,6)}$=1.02; p=0.366, respectively). Further, both parameters did not change due to MLD ($F_{(1,3)}$=2.14; p=0.239 and $F_{(1,5)}$=0.08; p=0.789, respectively). Detailed concentrations can be found in Table 6.

Table 7: Osmolality and oncotic pressure over three weeks of physical therapy (complete decongestive therapy), before - and after - manual lymphatic drainage (MLD). Values are shown as mean ± SD, including estimated marginal means.

		Complete Decongestive Therapy					
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**	*Estimated marginal means*
Osmolality (mOSM/kg) n=4	pre-MLD	296 ± 11.1	293 ± 10.8	295 ± 10.1	293 ± 8.3	2950 ± 8.2	*294 ± 9.5*
	post-MLD	293 ± 10.6	301 ± 17.5	299 ± 18.3	296 ± 15.1	303.9 ± 12.3	*299 ± 13.5*
	Estimated marginal means	*294 ± 9.1*	*297 ± 13.6*	*297 ± 14.0*	*294.8 ± 11.4*	*299.4 ± 10.2*	
Oncotic pressure (mmHg) n=6	pre-MLD	31 ± 2.8	30 ± 4.1	30 ± 2.3	30 ± 1.0	29 ± 1.9	*30 ± 1.8*
	post-MLD	29 ± 4.0	29 ± 4.1	30 ± 4.0	31 ± 3.9	29 ± 2.9	*30 ± 2.8*
	Estimated marginal means	*30 ± 3.0*	*30 ± 4.0*	*30 ± 2.8*	*31 ± 2.2*	*28.7 ± 1.7*	

4.2.7 Plasma electrolyte concentrations

An interaction was found between therapy days and MLD for plasma electrolyte levels (*Sodium:* $F_{(4,16)}$=4.35; p=0.014; *Chloride:* $F_{(4,16)}$=4.35; p=0.014; *Potassium:* $F_{(4,12)}$=4.62; p=0.011). MLD led to a reduction of electrolyte levels on the first day of therapy (Figure 9 and Table 7). However, on all measurement days, electrolyte concentrations tended to increase after MLD. Both, CDT as well as MLD did not lead to significant changes in sodium ($F_{(4,16)}$=0.86;

p=0.509 and $F_{(1,4)}$=1.00; p=0.374), chloride ($F_{(1,16)}$=1.10; p=0.389 and $F_{(1,4)}$=0.35; p=0.585) and potassium ($F_{(4,12)}$=0.92; p=0.474 and $F_{(1,3)}$=0.06; p=0.824) concentrations.

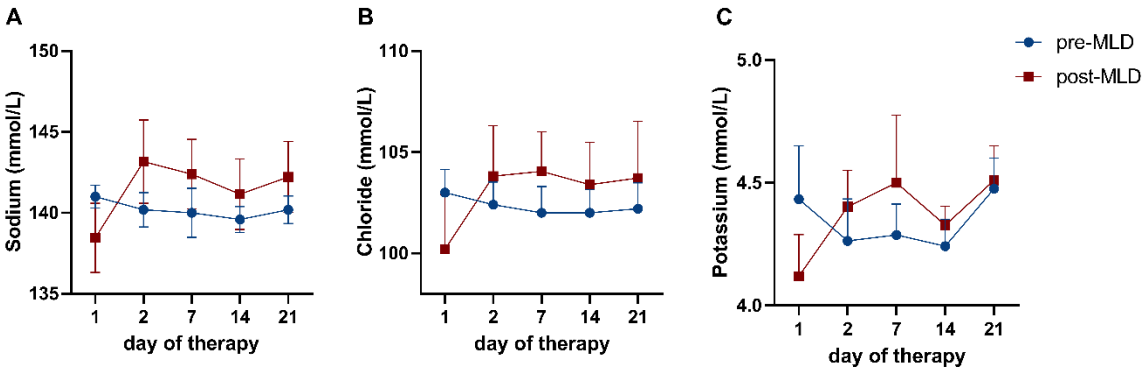

Figure 9: Time course of plasma electrolyte concentrations over three weeks of complete decongestive therapy, before (blue circles) - and after (red boxes) - manual lymphatic drainage (MLD): (A) sodium (mmol/L), (B) chloride (mmol/L) and (C) potassium (mmol/L). [Data from *Brix et al. (2020)* (198)]

Table 8: Changes in plasma electrolyte concentrations over three weeks of complete decongestive therapy, before - and after - manual lymphatic drainage (MLD). Values are shown as mean ± SD, including estimated marginal means.

		Complete Decongestive Therapy					
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**	*Estimated marginal means*
Sodium (mmol/L) n=5	pre-MLD	141 ± 1.6	140 ± 2.4	140 ± 3.4	140 ± 1.8	140 ± 1.9	*140 ± 1.9*
	post-MLD	138 ± 4.8	143 ± 5.8	142 ± 4.8	141 ± 4.9	142 ± 4.9	*141 ± 4.3*
	Estimated marginal means	*140 ± 3.1*	*142 ± 4.0*	*141 ± 4.0*	*140 ± 3.1*	*141 ± 3.4*	
Cloride (mmol/L) n=5	pre-MLD	103 ± 2.5	102 ± 2.5	102 ± 3.0	102 ± 2.5	102 ± 2.9	*102 ± 2.5*
	post-MLD	100 ± 6.1	104 ± 5.6	104 ± 4.4	103 ± 4.7	104 ± 6.3	*103 ± 5.1*
	Estimated marginal means	*102 ± 4.1*	*103 ± 4.0*	*103 ± 3.6*	*103 ± 3.6*	*103 ± 4.5*	
Potassium (mmol/L) n=5	pre-MLD	4.4 ± 0.5	4.3 ± 0.4	4.3 ± 0.3	4.2 ± 0.2	4.5 ± 0.3	*4.3 ± 0.3*
	post-MLD	4.1 ± 0.4	4.4 ± 0.3	4.5 ± 0.6	4.3 ± 0.2	4.5 ± 0.3	*4.4 ± 0.3*
	Estimated marginal means	*4.3 ± 0.4*	*4.3 ± 0.3*	*4.4 ± 0.4*	*4.3 ± 0.1*	*4.5 ± 0.2*	

4.2.8 Changes in blood cell count due to manual lymphatic drainage

White blood cells increased due to MLD ($F_{(1,5)}$=7.92; p=0.037), from 5.33 ± 1.60 x10^3 cells/µL before - to 6.49 ± 1.66 x10^3 cells/µL after - MLD, which is an increase of 22% (Figure 10A and Table 8). No changes appear over three weeks of CDT ($F_{(4,20)}$=0.98; p=0.438) (Figure 10A and Table 8). Neutrophils increased post-MLD ($F_{(1,5)}$=7.38; p=0.042) from 3.06 ± 0.90 x10^3 cells/µL pre-MLD to 4.26 ± 1.35 x10^3 cells/µL, representing an increase of 39% (Figure 10B and Table 8). In contrast to this, eosinophil cell count changed due to MLD ($F_{(1,5)}$=8.48; p=0.033), showing an average decrease after lymphatic massage from 184 ± 71.6 cells/µL pre-MLD to 129 ± 42.5 cells/µL post-CDT, which equals a reduction of 30% (Figure 10C and Table 8). No changes occurred in these blood cells over three weeks of physical therapy (*Eosinophils*: $F_{(4,10)}$=1.14; p=0.367; *Neutrophils*: $F_{(4,20)}$=0.85; p=0.512).

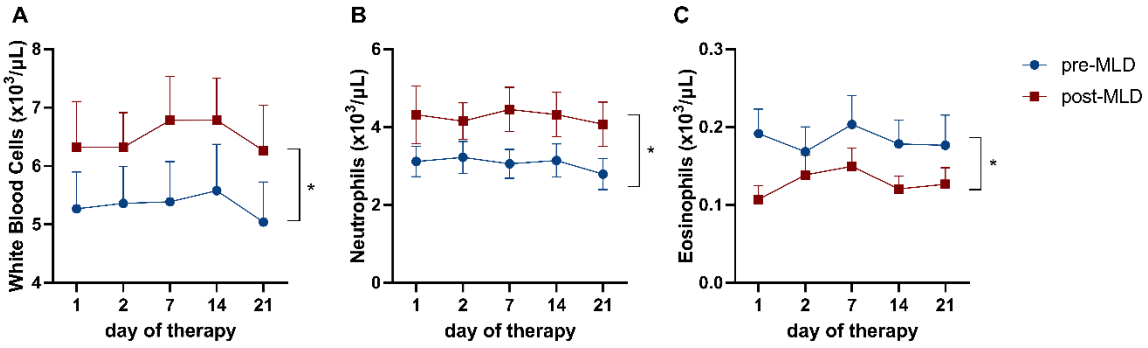

Figure 10: Time course of blood cell count changes over three weeks of complete decongestive therapy (CDT) and due to manual lymphatic drainage (MLD). (A) white blood cells (x10^3 cells/µL), (B) neutrophils (x10^3 cells/µL) and (C) eosinophils (x10^3 cells/µL). Cell counts pre-MLD are shown as blue dots, cell counts post-MLD are displayed as red boxes. Significant changes due to MLD are marked with asterisks (*).

Table 9: Overview of changes in white blood cell, neutrophil and eosinophil cell count over three weeks of complete decongestive therapy, before - and after - manual lymphatic drainage (MLD). Values are shown as mean ± SD, including estimated marginal means.

		Complete Decongestive Therapy					
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**	*Estimated marginal means*
White blood cells ($x10^3$ cells/µL) n=6	pre-MLD	5.27 ± 1.55	5.36 ± 1.55	5.39 ± 1.68	5.58 ± 1.95	5.04 ± 1.68	*5.33 ± 1.60*
	post-MLD	6.32 ± 1.92	6.32 ± 1.45	6.78 ± 1.85	6.78 ± 1.76	6.26 ± 1.90	*6.49 ± 1.66*
	Estimated marginal means	*5.80 ± 1.46*	*5.84 ± 1.38*	*6.08 ± 1.70*	*6.18 ± 1.74*	*5.65 ± 1.77*	
Neutrophils ($x10^3$ cells/µL) n=6	pre-MLD	3.12 ± 0.96	3.22 ± 1.00	3.06 ± 0.91	3.14 ± 1.04	2.79 ± 0.97	*3.06 ± 0.90*
	post-MLD	4.32 ± 1.82	4.15 ± 1.15	4.45 ± 1.40	4.32 ± 1.40	4.073 ± 1.40	*4.26 ± 1.35*
	Estimated marginal means	*3.72 ± 1.15*	*3.69 ± 0.94*	*3.76 ± 1.06*	*3.73 ± 0.98*	*3.43 ± 1.15*	
Eosinophils (cells/µL) n=6	pre-MLD	192 ± 77.6	168 ± 78.3	204 ± 91.1	178 ± 74.7	177 ± 95.2	*184 ± 71.6*
	post-MLD	107 ± 43.6	139 ± 62.4	150 ± 57.0	121 ± 40.0	127 ± 51.9	*129 ± 42.5*
	Estimated marginal means	*150 ± 59.6*	*154 ± 63.4*	*176 ± 65.1*	*150 ± 47.4*	*152 ± 65.6*	

4.3 Lymphatic fluid outflow assessed via plasma hyaluronic acid

Three weeks of complete decongestive therapy did not lead to changes in plasma hyaluronic acid concentrations ($F_{(2,11)}$=0.58; p=0.683). Further, pHA levels did not change due to MLD as well ($F_{(1,6)}$=0.97; p=0.364) (Table 9).

Table 10: Plasma hyaluronic acid concentrations over three weeks of complete decongestive therapy, before - and after - manual lymphatic drainage (MLD). Data are presented as mean ± SD, including estimated marginal means.

		Complete Decongestive Therapy					
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**	*Estimated marginal means*
Plasma hyaluronic acid (ng/mL) n=7	pre-MLD	112.81 ± 126.12	79.24 ± 53.62	74.22 ± 58.69	119.46 ± 165.35	75.63 ± 83.38	*92.27 ± 93.49*
	post-MLD	102.64 ± 100.03	81.06 ± 77.23	94.08 ± 121.08	58.96 ± 61.90	67.45 ± 56.33	*80.84 ± 69.60*
	Estimated marginal means	*107.73 ± 111.45*	*80.15 ± 60.71*	*84.15 ± 89.14*	*89.21 ± 113.26*	*71.54 ± 68.89*	

4.4 Hemodynamics responses to lymphedema therapy

4.4.1 Hemodynamic parameters at rest

Resting (baseline, epoch 1) diastolic blood pressure (dBP) significantly decreased over three weeks of complete decongestive therapy ($F_{(4,12)}$=3.31; p=0.048) from 90.9 ± 10.6 mmHg to 82.4 ± 4.0 mmHg (Figure 11C, Table 10). Resting values in heart rate (HR) ($F_{(4,16)}$=1.17; p=0.362) , systolic blood pressure (sBP) ($F_{(4,12)}$=2.65; p=0.085) and mean blood pressure (mBP) ($F_{(4,12)}$=2.89; p=0.069) did not change due to three weeks of complete decongestive therapy (Figure 11A, B & D and Table 10).

No changes occurred due to manual lymphatic drainage in heart rate ($F_{(1,4)}$=1.17; p=0.699), systolic blood pressure ($F_{(1,3)}$=0.00; p=0.998), diastolic blood pressure ($F_{(1,3)}$=2.28, p=0.632) and mean blood pressure ($F_{(1,3)}$=0.07; p=0.815) (Figure 11A-D and Table 10).

Stroke volume, total peripheral resistance and cardiac output did not change due to CDT and/or MLD (Supplemental Table S15 and S16).

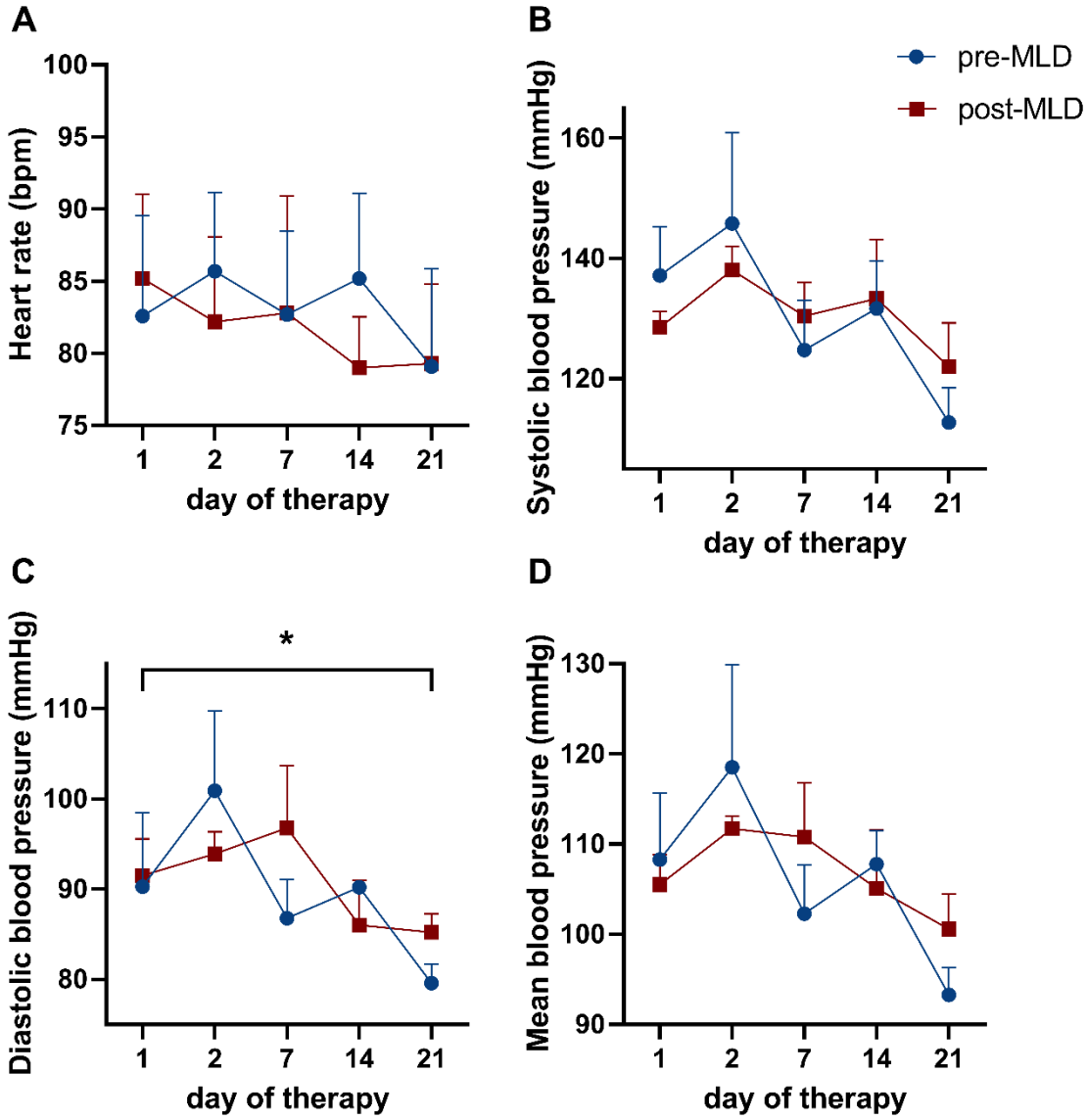

Figure 11: Time course of hemodynamic baseline values over three weeks of complete decongestive therapy (CDT) and due to manual lymphatic drainage (MLD). (A) Heart rate (bpm), (B) systolic blood pressure (mmHg), (C) diastolic blood pressure (mmHg) and (D) mean blood pressure (mmHg). The blue circles indicate values pre-MLD, whereas the red squares represent values post-MLD. Significant changes are marked with asterisks (*).

Table 11: Overview of changes in hemodynamic parameters at rest (baseline) over three weeks of complete decongestive therapy, before - and after - manual lymphatic drainage (MLD). Values are presented as mean ± SD, including estimated marginal means.

		Complete Decongestive Therapy					
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**	*Estimated marginal means*
Heart rate (bpm) n=5	pre-MLD	82.6 ± 15.6	85.7 ± 12.2	82.7 ± 12.9	85.2 ± 13.2	79.1 ± 15.2	*83.1 ± 13.5*
	post-MLD	85.2 ± 13.1	82.2 ± 13.2	82.8 ± 18.2	79.0 ± 7.9	79.3 ± 12.4	*81.7 ± 11.8*
	Estimated marginal means	*83.9 ± 13.8*	*84.0 ± 11.6*	*82.7 ± 15.5*	*82.1 ± 9.2*	*79.2 ± 12.4*	
Systolic blood pressure (mmHg) n=4	pre-MLD	137.2 ± 16.4	145.8 ± 30.3	124.8 ± 16.5	131.7 ± 15.9	112.7 ± 11.5	*130.4 ± 12.9*
	post-MLD	128.5 ± 5.4	138.1 ± 7.8	130.4 ± 11.1	133.3 ± 19.6	122.0 ± 14.5	*130.4 ± 6.8*
	Estimated marginal means	*132.8 ± 10.2*	*142.0 ± 18.4*	*127.6 ± 13.4*	*132.5 ± 13.1*	*117.3 ± 12.4*	
Diastolic blood pressure (mmHg) n=4	pre-MLD	90.3 ± 16.4	100.9 ± 17.7	86.8 ± 8.7	90.2 ± 1.2	79.6 ± 4.3	*89.6 ± 7.4*
	post-MLD	91.5 ± 8.2	93.9 ± 4.9	96.8 ± 13.9	86.0 ± 10.0	85.2 ± 4.1	*90.7 ± 5.6*
	Estimated marginal means	*90.9 ± 10.6*	*97.4 ± 7.5*	*91.8 ± 11.3*	*88.1 ± 5.2*	*82.4 ± 4.0*	
Mean blood pressure (mmHg) n=4	pre-MLD	108.3 ± 14.8	118.5 ± 22.8	102.3 ± 10.9	107.8 ± 7.4	93.3 ± 6.1	*106.1 ± 9.4*
	post-MLD	105.5 ± 6.7	111.7 ± 2.8	110.8 ±12.1	105.1 ± 13.0	100.6 ± 7.8	*106.8 ± 5.3*
	Estimated marginal means	*106.9 ± 9.5*	*115.1 ± 12.3*	*106.6 ± 11.2*	*106.4 ± 8.5*	*97.0 ± 6.6*	

4.4.2 Hemodynamic responses to orthostatic loading (via sit-to-stand test)

Manual lymphatic drainage led to a significant reduction in heart rate over all epochs on day 14 ($F_{(1,9)}$=6.07; p=0.036) and day 21 ($F_{(1,9)}$=5.24; p=0.048) of CDT (Figure 12). An overall effect in the epochs during orthostatic loading was seen in heart rate on day 1 ($F_{(9,72)}$=12.53; p=0.014) and day 7 ($F_{(9,81)}$=6.56; p<0.001), day 14 ($F_{(2,21)}$=5.14; p<0.001) and day 21 ($F_{(2,19)}$=6.67; p=0.006) of CDT. No changes in blood pressure responses (sBP, dBP and mBP) during a sit-to-stand test were observed before - compared to after - manual lymphatic drainage on each individual assessment day.

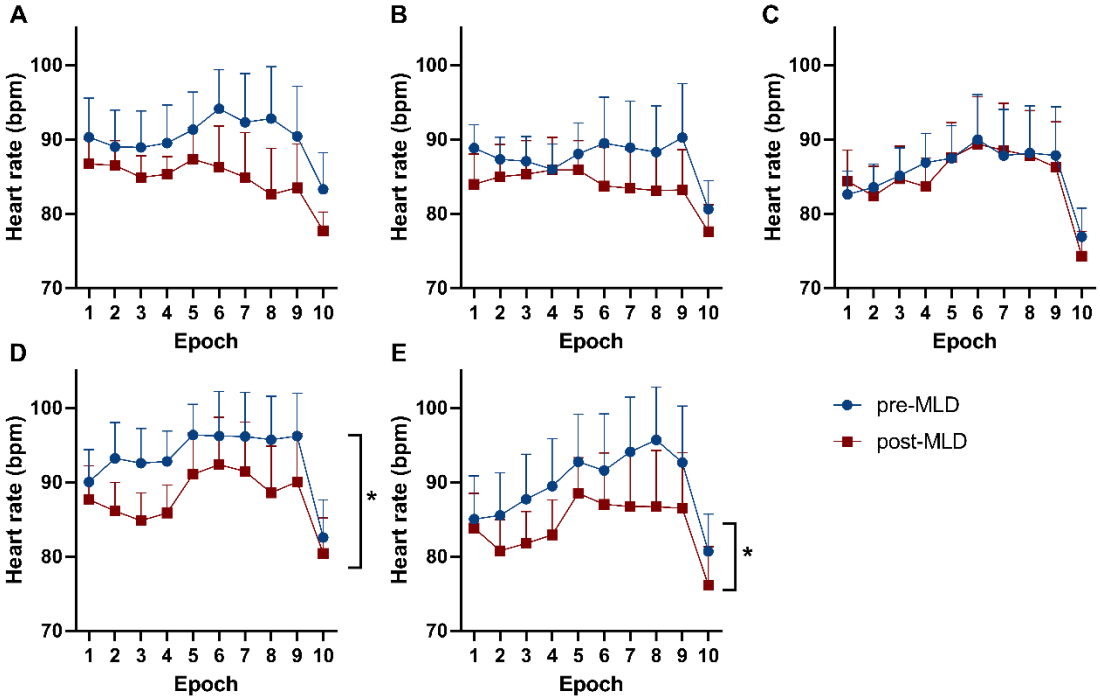

Figure 12: Overview of heart rate responses during orthostatic loading (10 Epochs during a sit-to-stand test) over three weeks of complete decongestive therapy (CDT) and due to manual lymphatic drainage (MLD). (A) Day 1, (B) day 2, (C) day 3, (D) day 4 and € day 5 of CDT. The blue circles represent values pre-MLD, whereas the red squares represent values post-MLD. Asterisks (*) indicate significant changes in heart rate pre- vs. post-MLD on day 14 (p=0.036) and day 21 (p=0.048) of CDT.

4.5 Endothelial function/vascular health

Plasma ADMA significantly reduced from 0.54 ± 0.03 µmol/L to 0.50 ± 0.04 µmol/L due to MLD ($F_{(1,6)}$=16.75; p=0.006), averaged over three weeks. Further, ADMA concentrations tended to decrease over three weeks of CDT ($F_{(4,24)}$=2.76; p=0.051) with a decrease from 0.54 ± 0.05 µmol/L to 0.49 ± 0.03 µmol/L (Figure 13, Table 11). Interaction between both factors, however, did not reach significance ($F_{(4,24)}$=0.24; p=0.999).

PWVcf and FMD showed a weak, but significant correlation (r=0.361, p=0.010). However, MLD did not lead to changes in PWVcf ($F_{(1,7)}$=0.05; p=0.830) and retinal microvasculature analysis (*CRAE:* $F_{(1,5)}$=2.20; p=0.198; *CRVE:* $F_{(1,5)}$=0.25; p=0.640 and *A-to-V:* $F_{(1,5)}$=1.15; p=0.332) (Table 11). Further, three weeks of complete decongestive therapy did not lead to changes in PWVcf ($F_{(4,28)}$=0.34; p=0.848), FMD ($F_{(4,32)}$=1.41; p=0.252) as well as retinal microvasculature parameters (CRAE: $F_{(4,20)}$=1.47; p=0.249; CRVE: $F_{(4,20)}$=2.55; p=0.071 and A-to-V: $F_{(4,20)}$=2.16; p=0.110) (Table 11).

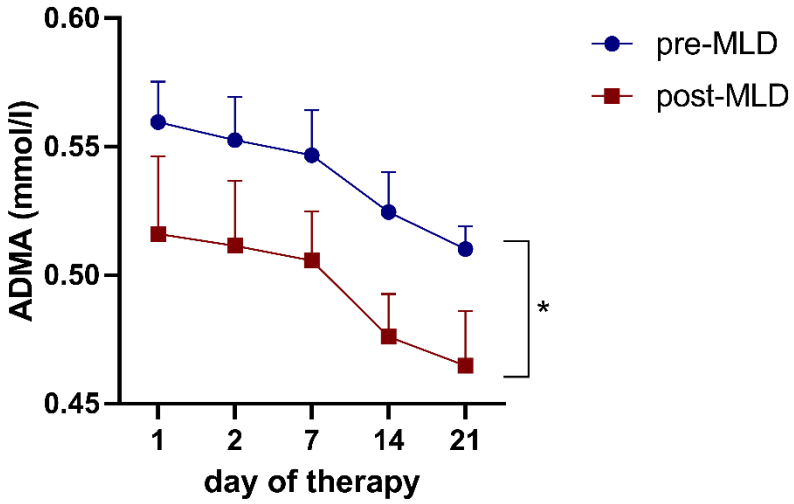

Figure 13: Time course of plasma asymmetric dimethylarginine (ADMA) (µmol/L) level changes over three weeks of complete decongestive therapy (CDT) and due to manual lymphatic drainage (MLD). ADMA levels pre-MLD are represented as blue dots, whereas ADMA levels after lymphatic drainage are shown as red boxes. The asterisk (*) represents significant difference between pre-MLD and post-MLD ADMA measurements (p=0.0064).

Table 12: Overview of endothelial - and vascular - function parameters before - and after - manual lymphatic drainage (MLD), over three weeks of complete decongestive therapy (CDT). Estimated marginal means are included in the table as well. Data are presented as means ± SD.

		Complete Decongestive Therapy					
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**	*Estimated marginal means*
ADMA (µmol/L) n=7	pre-MLD	0.56 ± 0.04	0.55 ± 0.04	0.55 ± 0.05	0.52 ± 0.04	0.51 ± 0.02	*0.54 ± 0.03*
	post-MLD	0.52 ± 0.08	0.51 ± 0.07	0.51 ± 0.05	0.48 ± 0.04	0.46 ± 0.06	*0.50 ± 0.04*
	Estimated marginal means	*0.54 ± 0.05*	*0.53 ± 0.05*	*0.53 ± 0.04*	*0.50 ± 0.04*	*0.49 ± 0.03*	
PWVcf (m/s) n=8	pre-MLD	9.8 ± 3.4	9.0 ± 1.2	9.0 ± 1.1	9.3 ± 3.2	8.9 ± 2.1	*9.2 ± 2.1*
	post-MLD	9 ± 1.4	9.4 ± 2.9	9.4 ± 1.9	9.3 ± 2.4	9.1 ± 2.3	*9.2 ± 2.0*
	Estimated marginal means	*9.3 ± 2.3*	*9.2 ± 2.0*	*9.2 ± 1.4*	*9.3 ± 2.7*	*9.0 ± 2.2*	
FMD (%) n=13	post-MLD	7.3 ± 2.8	8.2 ± 4.4	9.1 ± 2.8	9.9 ± 2.9	10 ± 3	
CRAE (µM) n=6	pre-MLD	130 ± 23	129 ± 21	135 ± 12	138 ± 16	125 ± 11	*131 ± 11*
	post-MLD	143 ± 12	131 ± 11	130 ± 17	145 ± 20	134 ± 16	*137 ± 8*
	Estimated marginal means	*136 ± 17*	*130 ± 14*	*132 ± 9*	*142 ± 13*	*130 ± 7*	
CVRE (µM) n=6	pre-MLD	210 ± 25	234 ± 44	215 ± 26	241 ± 21	219 ± 16	*223 ± 19*
	post-MLD	206 ± 27	230 ± 25	222 ± 15	216 ± 18	221 ± 31	*220 ± 15*
	Estimated marginal means	*208 ± 15*	*232 ± 22*	*218 ± 16*	*228 ± 16*	*220 ± 18*	
A-to-V Ratio n=6	pre-MLD	0.63 ± 0.08	0.58 ± 0.14	0.64 ± 0.04	0.57 ± 0.01	0.58 ± 0.01	*0.60 ± 0.05*
	post-MLD	0.71 ± 0.11	0.59 ± 0.08	0.59 ± 0.09	0.68 ± 0.06	0.62 ± 0.01	*0.64 ± 0.05*
	Estimated marginal means	*0.67 ± 0.05*	*0.58 ± 0.09*	*0.61 ± 0.04*	*0.62 ± 0.03*	*0.60 ± 0.06*	

Legend: ADMA, asymmetric dimethylarginine; A-to-V, arteriolar-to-venous ratio; CDT, complete decongestive therapy; CRAE, central arteriolar equivalent; CRVE, central retinal venular equivalent; MLD, manual lymphatic drainage; PWVcf, carotid-femoral pulse wave velocity.

5 DISCUSSION

The aim of this thesis was to investigate the effects of three weeks of complete decongestive therapy and manual lymphatic drainage on fluid shifts, hemodynamic responses and endothelial/vascular (dys-)function in lower limb lymphedema patients. The main findings are:

1) Limb volume decreased due to three weeks of lymphedema therapy. Segmental assessment of limb fluid showed that extracellular - and intracellular - fluid decreased over three weeks of therapy as well. Total leg fluid tended to decrease due to physical therapy. Manual lymphatic drainage led to an increase in intracellular limb fluid. Assessments of total leg volume (perometry) and total leg fluid (bioelectrical impedance spectroscopy) moderately correlated. Total body - and extracellular - fluid reduced over three weeks of complete decongestive therapy, whereas they increased due to manual lymphatic drainage. Limb extracellular -, whole-body total - and extracellular - fluid analysis suggest that the greatest changes in fluid shifts occurred between the first and second day of therapy, whereas afterwards no further changes were seen. The observed fluid shifts were also reflected in changes in plasma volume. Plasma volume increased by about 1.5% after manual lymphatic drainage. Additionally, albumin levels as well as albumin to globulin ratio increased after manual lymphatic drainage. Manual lymphatic drainage further led to an increased white blood cell and neutrophil cell count, and to a decrease in eosinophils.

2) Plasma hyaluronic acid concentrations did not change due to complete decongestive therapy and/or manual lymphatic drainage over three weeks.

3) Resting diastolic blood pressure decreased over three weeks of complete decongestive therapy, whereas no changes occurred in other baseline hemodynamic parameters due to lymphedema therapy. Hemodynamic responses showed significantly lower heart rate responses to orthostatic loading on day 14 and day 21 of complete decongestive therapy.

4) Plasma ADMA concentrations were at normal range at baseline, however, significantly reduced due to manual lymphatic drainage averaged over three weeks of therapy. Carotid-femoral pulse wave velocity and brachial flow-mediated dilatation significantly correlated. Further, no changes in carotid-femoral pulse wave velocity, brachial flow mediated dilatation, as well as in retinal microvasculature analysis (as indicator of microvascular health) were determined due to three weeks of complete decongestive therapy in lymphedema patients.

5.1 Fluid mobilization over three weeks of complete decongestive therapy

5.1.1 Changes in lower limb volume

Over three weeks of complete decongestive therapy, limb volume reduced by 392 mL or 3.1% ($p<0.001$). A case study performed by Cohen et al. (2011) reported a similar reduction (368 mL) after eight therapy sessions within three months (199). The results presented here show that a shorter treatment protocol (three weeks vs. three months) led to similar reductions in limb volume. An explanation for this could be the higher frequency of therapy sessions (daily manual lymphatic drainage vs. eight sessions within three months) used in this study. Kostanoğlu et al. (2019) reported that a reduction between 296 mL and 1038 mL in leg volume was determined after four weeks of physical therapy. The amount of volume reductions was dependent on the stage of lymphedema (200). In a more recent study, Cavezzi et al. (2020) demonstrated that limb volume reduced after six days of therapy using perometry (201). The observed reduction by Cavezzi et al. was similar to what was observed in this study after 21 days.

Manual lymphatic drainage, however, did not led to a change in leg volume ($p=0.527$). This finding is novel, as in previous studies, perometry has been used to assess leg volume as an outcome parameter for several weeks of physical therapy (21, 155, 200, 202). However, none of these studies assessed leg volume directly before - and after - a manual lymphatic drainage treatment. Only a recent study by Habnouni et al. (2020) reported that no changes in limb volume occurred after 30 minutes of manual lymphatic drainage. However, they could observe a reduction in leg circumference in children with lymphedema after manual lymphatic drainage (203). Their findings are in line with the results of what could be observed in this study. These results indicate that perometric assessment of leg volume might be too insensitive in detecting short-term fluid mobilization due to physical treatment in patients with lower limb lymphedema.

5.1.2 Lower limb fluid changes

Extracellular - as well as intracellular - fluid decreased in the lower limbs over three weeks of physical therapy. Further, total limb fluid tended to reduce after three weeks of therapy ($p=0.057$). Bioelectrical impedance spectroscopy has been used in different studies, which investigated extracellular - vs. intracellular - fluid shifts due to lymphedema therapy (123, 184, 204). For example, a significant reduction in total -, extracellular - and intracellular - limb fluid was reported after one week of intensive physical treatment in a study by Pereira de Godoy et al. (2013), performed in lower limb lymphedema patients (162). They further report a significant reduction in the lower limbs, whereas total water increased in the upper extremities and in the trunk (healthy body areas). Based on their findings, they concluded that fluid is mobilized from the affected extremities to healthy areas of the body (162). Similar results are reported in a more recent study (163). Those are in accordance with the here presented results as well, which show a significant reduction of limbECF within the first week of CDT (that is, baseline values as compared to those of the second and 14^{th} day of therapy).

Yamamoto et al. (2008) reported a volume decrease of 55% between the first - and second - day of therapy and further, a volume reduction of 11% between the second - and third - day of therapy. Afterwards, no further significant decrease was seen (205). In agreement with Yamamoto et al. (2008), Lasinski et al. (2012) postulate that the major effect of CDT occur within the first five days of therapy, but longer compression is needed to maintain this positive effect (19). This is also what was observed in this study, which confirms that the greatest effect of physical therapy occurs within the first week of treatment. Overall, the results of this thesis indicate that the body is able to adapt to perturbations rather rapidly (in this case within one week) and that no further changes happen afterwards. This is in accordance to what has previously been reported, for example, in studies investigating the effects of space flight on the human body or in bed rest studies (206-210).

As manual lymphatic drainage has been reported to enhance lymphatic fluid outflow, it was hypothesized that extracellular fluid of the limbs (limbECF) decreases after 30 minutes of manual lymphatic drainage, and further, that no changes will occur in limb intracellular fluid. However, the results presented here disprove this hypothesis, as manual lymphatic drainage in the limbs did, as opposed to what was expected, not lead to changes extracellular limb fluid. Whereas no changes in extracellular fluid was seen, an increase in intracellular limb fluid

occurred due to manual lymphatic drainage (p=0.017). This is in line with the results of a study performed by Maher et al. (2012). They did also not observe changes in limbECF after manual lymphatic drainage. However, their study was performed in arm lymphedema patients (211). Tambour et al. (2018) agree with this finding, as they demonstrated that no additional beneficial effect is added when manual lymphatic drainage is performed during complete decongestive therapy as compared to lymphedema therapy without manual lymphatic drainage in arm lymphedema patients (212).

Although the different individual components of complete decongestive therapy were not investigated separately, the results presented here indicate that complete decongestive therapy does lead to fluid shifts, whereas manual lymphatic drainage alone is not sufficient enough to influence segmental fluid shifts (assessed directly before - and - after lymphatic drainage). This is also in agreement with what has been previously reported. Previous findings indicate that manual lymphatic drainage does not add further benefits to lymphedema therapy (212-214), especially in moderate to severe lymphedema cases (215).

5.1.3 Correlation between perometry and bioelectrical impedance spectroscopy

Perometry (assessing leg volume) and bioelectrical impedance spectroscopy analysis (assessing leg fluid) moderately correlated over all measurement time points (r=0.635). Jain et al. (2010) previously reported that bioelectrical impedance spectroscopy is able to produce reliable and valid data, compared to perometry, the gold standard tool for volume assessment (216). However, this was investigated in patients diagnosed with cancer-related arm lymphedema. Another study done by Bundred et al. (2015) also showed a moderate correlation between both methods used in the early detection of arm lymphedema in cancer patients (217). Despite extensive literature review, no previous study was found that has previously investigated how these two methods for the assessment of physical therapy outcome (202) correlate in lower limb lymphedema patients.

5.1.4 Whole-body fluid shifts

Another hypothesis that was tested was that overall total - and extracellular - fluid will decrease due to three weeks of complete decongestive therapy. Reductions in total body water (%TBW) and extracellular fluid (%ECF) occurred after three weeks of physical therapy, which

is in accordance with the proposed hypothesis. The largest fluid reduction was seen between the first and the second day of complete decongestive therapy (TBW: p=0.009 and ECF: p=0.029). This is similar to what was observed in the segmental analysis of the lower limbs. These results suggest that one week of treatment might be sufficient, as no further changes were observed afterwards, at least in terms of fluid shifts.

Additionally, it was observed that whole-body intracellular fluid (%ICF) changed over the three weeks of therapy. Pereira de Godoy et al. (2019) reported, that 7 days of a specific intensive therapy led to an increase in intracellular fluid. In contrast to what was reported by Pereira de Godoy et al. (2019), a reduction of intracellular fluid over three weeks of complete decongestive therapy as well as an increase of intracellular fluid after manual lymphatic drainage was observed in this current study. The differences in these findings could be explained by the methodology used: assessment of only the trunk and upper abdomen (163), versus overall body fluid changes, as reported here.

Further, manual lymphatic drainage led to the reduction of the ratio of extracellular - to intracellular - fluid (ECF/ICF) of the whole body, averaged over three weeks of complete decongestive therapy. This is in contrast with what has previously been reported. A recent review by Thompson et al. (2020), for instance, reported that manual lymphatic drainage might not add additional benefits to the treatment using complex decongestive therapy patients in higher stages of lymphedema (stage II-III) (215). Although this may be true for changes in limb fluid, the novel findings presented here show that manual lymphatic drainage alone is indeed able to affect whole-body fluids.

5.1.5 Plasma volume and plasma component concentration changes

Manual lymphatic drainage has been reported to lead to an enhanced lymphatic activity in healthy patients (218) and further, to improve transport of radiotracers in the lymphatic vessels in lymphedema patients (18). Therefore, the hypothesis was tested that the fluid shifts, which occur due to physical therapy would also be detectable in changes in plasma volume. This could potentially be used as an indirect indicator for lymphatic outflow. Indeed, lymphatic drainage led to an average increase in plasma volume of 1.5% ± 0.8. Those observed plasma volume increases occurred after manual lymphatic drainage and therefore, suggest that mobilized lymphatic fluid enters the blood stream. Typically, plasma volume expansions have been described during exercise, heat exposure or pregnancy (219). During exercise, for

instance, plasma volume increases of 9 to 15% have been previously reported (220). Compared to this, plasma volume changes due to manual lymphatic drainage in lymphedema patients reached lower levels (1.5%). Extensive literature research revealed that no previous study has investigated short-term plasma volume changes due to physical therapy.

In contrast to what was hypothesized, manual lymphatic drainage led to elevated albumin levels. This is also reflected albumin to globulin ratio changes. Plasma protein concentrations were at normal range at all time-points for each individual patient (total protein: 6-8 mg/dl, albumin: 3.5-5.2 mg/dl (221)). Therefore, it is not expected that those levels would influence the disease condition directly. However, it has previously been reported that plasma protein concentrations are affected by plasma volume changes (185, 222). Nevertheless, this has been explored in physiological perturbations associated with the loss of plasma volume (e.g. post-presyncope or during postural changes) and not in cases of increased plasma volume, as observed after lymphatic drainage. Albumin concentrations were corrected to the plasma volume changes that occurred due to physical therapy. As hematocrit did not change due to manual lymphatic massage, it can be excluded that the changes in albumin occurred due to the fluid shifts only. Therefore, these results indicate that the fluid, which is entering into the blood stream (that is, the lymphatic fluid mobilized due to physical therapy) has a higher albumin content than plasma. This has not been reported previously.

The increased concentrations of plasma albumin after manual lymphatic drainage further led to the assumption that oncotic pressure might be influenced by such changes as well. However, oncotic pressure did not change due to lymphedema therapy.

Furthermore, plasma electrolyte levels (sodium, chloride and potassium) were not affected by lymphedema therapy. A possible explanation could be that the plasma volume increase was relatively small (approximately 1.5%). In accordance to this, osmolality did not change as well. Interestingly, on the first day of therapy, osmolality and electrolyte levels (sodium, chloride and potassium concentrations) show a tendency to decrease after manual lymphatic drainage, whereas the same parameters tended to increase after lymphatic drainage at all other measurement time points (which were on day 2, day 7, day 14 and day 21 of therapy). Therefore, statistical analysis of variances did not result in significant changes comparing electrolyte levels pre - vs. post - manual lymphatic drainage, averaged over three weeks of therapy. This could be based on the major changes in fluid shifts occurring between the first and second day of complete decongestive therapy.

Finally, white blood cells (WBC) were affected, not over three weeks of therapy, but due to manual lymphatic drainage. This has not been previously reported. Whereas neutrophil cell counts increased, eosinophils decreased after lymphatic drainage. Red blood cell count and hematocrit did not change, indicating that the observed changes in white blood cells do not occur due to plasma volume changes or correction errors of the observed plasma volume changes. Lymphedema has been associated with chronic inflammatory responses (24). Pivetta et al. (2016), for examples, showed that neutrophils infiltrate at the area of acquired lymphedema in a mouse model (223). In agreement with this, Rockson et al. (2018) also report an increase in neutrophil cell counts in induced lymphedema animal models (141). The underlying mechanism of the elevated neutrophil cell counts observed in this study is not known. It could be speculated that manual lymphatic drainage may lead to an increased mobilization of neutrophils (from the tissue or the mobilized lymphatic fluid), which can be found in an increased number in the plasma after lymphatic drainage. However, this novel finding needs to be investigated in further detail in future studies.

In summary, the assessment of plasma volume changes using the Van Beaumont equation seems to be a suitable tool to investigate minor changes in plasma volume, as they could be observed due to fluid mobilization during physical therapy in lymphedema patients. Future studies are needed to evaluate plasma volume and plasma component assessment as a promising and cost-effective method for the measurement of lymphatic (out-)flow and further, lymphatic fluid concentration in different types of lymphedema patients (e.g. different grades, primary vs. secondary lymphedema).

5.2 Lymphatic (out-)flow assessed via plasma hyaluronic acid levels

No significant changes in plasma hyaluronic acid concentrations, a surrogate for lymphatic fluid (out-)flow (42, 167, 224), were observed over three weeks of complete decongestive therapy and also not due to manual lymphatic drainage. It has previously been demonstrated that hyaluronic acid levels in blood plasma increase post-prandial (225) or during exercise (69) in studies with healthy participants. A possible explanation for the lack of significance in lymphedema patients undergoing complete decongestive therapy could be the high standard deviation seen in hyaluronic acid values between patients even at baseline (pre-therapy). The results are in contrast to the postulated hypothesis that three weeks of compression therapy together with exercises would lead to increases in lymphatic outflow, which would be reflected in elevated plasma hyaluronic acid concentrations. Furthermore, the obtained results raise the question whether plasma hyaluronic acid concentrations can indeed act as a reasonable indicator of lymphatic fluid outflow in lymphedema patients. In those patients, the hyaluronic acid molecule might be retained within the lymphedematous tissue due to its large molecular size. It is possible that physical therapy mobilizes fluid from the affected limbs, but without accompanied hyaluronic acid molecules in lymphedema patients. As hyaluronic acid is known to be a very large molecule, it may be resistant to breakdown and wash out by local physical pressure application such as during manual lymphatic drainage. Indeed, recently investigated treatment options show that recombinant hyaluronidase (67) or heat therapy applied locally (71) might be successful in breaking down high molecular weight hyaluronic acid. However, this has only been investigated in mouse models so far and therefore, needs to be evaluated in future clinical studies.

Several studies have previously reported that exercise is an important co-factor in enhancing lymphatic flow in lymphedema patients (13, 58, 226). This could not be confirmed in this study, which is suggesting that lymphatic outflow is not increased after physical therapy. The discrepancy to other studies, which report increased lymphatic flow due to physical therapy and exercise (13, 58, 226), could be due to different lymphedema patient characteristics that were included or different methodologies of lymphatic flow assessments used in other studies.

5.3 Hemodynamic changes at rest and in response to orthostatic loading

A significant decrease in resting diastolic blood pressure from 90.9 ± 10.6 mmHg to 82.4 ± 4.0 mmHg was observed due to three weeks of complete decongestive therapy ($p=0.048$). This is a novel finding, as no previously published study was found that reported changes in blood pressure due to complete decongestive therapy. However, as exercise is one component of lymphedema therapy, together with compression bandages and manual lymphatic drainage, it cannot be determined if the reduction in diastolic blood pressure is a results of lymphedema therapy per se or occurred due to exercise that was performed as part of complete decongestive therapy. Studies have shown that 30-60 minutes of exercise per week already lead to significant reduction of diastolic blood pressure in essential hypertensives (227). Daily steps by pedometer as well as the daily exercise time was not recorded in this study. However, as CDT was performed in a clinical environment with strict time-schedules and therapy as well as exercise sessions, the amount of daily/weekly exercise was the same throughout the three weeks for all patients: approximately 60 minutes per day.

No changes occurred due to manual lymphatic drainage in hemodynamic parameters at rest. Previously reported studies investigating cardiovascular changes due to manual lymphatic drainage report inconclusive results (228-230). Esmer et al. (2019) investigated acute cardiovascular responses in heart rate and blood pressure after manual lymphatic drainage in different body parts (228). They reported a reduced systolic - and diastolic - blood pressure after manual lymphatic drainage in the lower limbs (228). This is in contrast to the results shown here. Manual lymphatic drainage did not lead to any changes in heart rate and blood pressure in the patients included into this study. Similar results were reported by Ramos et al. (2015), which showed no changes in blood pressure during - and after - one session of manual lymphatic drainage (229). In another study, by Leduc et al. (2011), echocardiography was used to determine cardiac parameter changes due to manual lymphatic drainage. They reported a significant decrease in heart rate, whereas blood pressure did not change (230). A possible explanation for the different findings could be the participants included into the different studies. Whereas some included healthy participants (228, 229), others included patients with heart disease (230). In this thesis, lower limb lymphedema patients were investigated, which has not been previously reported.

It was further hypothesized that in lymphedema patients will show signs of orthostatic hypotension due to the different fluid volumes in the lower limbs, pre - vs. post - therapy, which was shown by an increase of plasma volume of about 1.5%. All patients were able to complete the sit-to-stand test. No signs of orthostatic hypotension (defined as a reduction in systolic blood pressure of 20 mmHg or a decrease diastolic blood pressure of 10 mmHg during standing (190, 231)) were seen in lymphedema patients at baseline and also not over three weeks of lymphedema therapy. These results presented here indicate that lymphedema patients are not at a higher risk for orthostatic intolerance and therefore, at a higher risk of falls.

Further, manual lymphatic drainage led to a significant reduction in heart rate over all epochs on day 14 ($p=0.036$) and day 21 ($p=0.048$) of CDT. Averaged over all epochs, the reductions in heart rate were 4 bpm on day 14 and 5 bpm on day 21. Therefore, manual lymphatic drainage seems to potentially have beneficial effects on hemodynamic responses to orthostatic loading in lymphedema patients undergoing lymphedema therapy. Finally, blood pressure responses, did not change due to complete decongestive therapy and/or manual lymphatic drainage.

5.4 Endothelial/vascular (dys-)function

One hypothesis of this thesis was that lymphedema patients will show subclinical signs of endothelial dysfunction already at baseline, before starting with CDT (Figure 1, time point A1). The results show that plasma ADMA concentrations were within a normal range in lymphedema patients. Plasma ADMA concentrations in patients - before undergoing lymphedema therapy - showed to be 0.56 ± 0.04 µmol/L, which is within the range as can be seen in healthy persons (0.25 - 0.92 µmol/L) (232). Therefore, the results suggest that patients with lymphedema do not appear to display endothelial dysfunction. This are novel results, as no other study previously specifically investigated endothelial (dys-)function/vascular health in lymphedema patients. As endothelial dysfunction is correlated with a higher risk of developing cardiovascular diseases (233, 234), these results indicate that lymphedema patients in general might not be at a higher cardiovascular risk.

Another observation was related to the effects of lymphedema therapy. The results reported in this thesis show a significant reduction of plasma ADMA from 0.54 ± 0.02 µmol/L to 0.49 ± 0.02 µmol/L (p=0.0064) after 30 minutes of manual lymphatic drainage. Limited studies are available, which examine short-term effects of various interventions and/or perturbations on plasma ADMA concentrations. A study by Zinellu et al. (2017), for example, reports a significant reduction of ADMA by 8% after a single exercise intervention (235). Similar reductions were observed after lymphatic drainage, although a much more strenuous intervention was used in the study performed by Zinellu and colleagues (9% vs 8% decrease). Although the baseline values were not elevated in lymphedema patients, results indicate a potential beneficial effect of manual lymphatic drainage on endothelial function. This is a novel finding, as extensive literature research did not reveal any study reporting the effects of physical therapy on endothelial (dys-)function markers. Nevertheless, this needs to be further investigated in future clinical studies, including more lymphedema patients in different stages of the disease as well as different durations of lymphedema, as this can also influence endothelial (dys-)function and possibly predispose those patients to an elevated risk of cardiovascular diseases.

Additionally, the effect of decongestive therapy over three weeks on endothelial health was investigated. The results showed that ADMA tended to reduce over three weeks of physical therapy (p=0.0506). As no other study previously published has specifically investigated

ADMA over three weeks of decongestive complete therapy, other studies in which ADMA was measured over varying interventions were studied. For example, Rudofsky et al. (2011) observed a reduction of ADMA levels during a weight loss therapy program of 12 weeks (236). Another study reported reductions in ADMA concentrations after two months of physical exercise training in type 1 diabetes patients (237).

Pulse wave velocity and flow mediated dilatation did not change due to manual lymphatic drainage and not over three weeks of physical therapy. As these methods are used to assess vascular changes in rather large arteries (*a. carotis, a. femoralis* and *a. brachilis*) (194), it is possible that assessment of these arteries is not sensitive enough detect endothelial function changes in lymphedema patients. Or, it could be that such changes in endothelial/vascular function might only appear in the venous system or in later stages of the disease.

ADMA did not have any relationship with pulse wave velocity ($r=-0.044$, $p=0.739$) and flow-mediated dilatation ($r=-0.112$, $p=0.550$). This is surprising, as previous reports have shown an association between these measurements of endothelial health and vascular function assessment (238-240). Protopsaltis et al. (2012) observed a correlation between ADMA and pulse wave velocity in prediabetic patients (240). An inverse correlation between ADMA and flow-mediated dilatation was previously reported in healthy individuals (241), prediabetic women (242) and patients with rheumatoid arthritis (239). While such associations could not be confirmed in the measurements in this study, the results show that there is a correlation between pulse wave velocity and flow-mediated dilatation ($p=0.010$). This in agreement to previous reports that have shown such correlations (194, 243, 244).

Despite retinal measurement being suggested as indicator of microvascular health (196, 245), no changes in the ophthalmic vessels in lymphedema patients at baseline and not following lymphatic drainage or as a result of three weeks of physical therapy were seen. In addition, retinal microvasculature parameters did not show any correlation to ADMA. Although a previous study reported spastic and stagnant microcirculation in leg lymphedema patients (measured via laser Doppler flowmetry in the legs), as well as improvement of such following physical therapy (246), such microvascular changes in the retina did not occur in the patients included in this study. It is possible that the microvascular changes in the retina occur much later (or are absent) in those patients, as most of them had localized lymphedema in the legs. Future research should investigate whether vascular function changes occur in different vascular beds or differ depending on the regions in lymphedema patients.

5.5 Limitations

A possible limitation of this thesis could be that patients with primary and secondary lymphedema were included. Although the underlying mechanisms both forms of lymphedema are contrasting (247), the treatment approach is identical (129). This was also the case in other studies investigating fluid shifts, which also included primary and secondary lymphedema patients (162, 200). For example, Noh et al. (2015) reported no significant differences in leg volume (as indicator for therapy outcome) after physical therapy between patients diagnosed with primary and those diagnosed with secondary lymphedema (20). Additionally, detailed statistical analysis did not show any differences when both groups were analyzed individually.

As the majority of patients included into this study were diagnosed with bilateral lower limb lymphedema (Table 2), the data sets of all lower limbs were pooled. Therefore, the ratios between affected and non-affected legs were not calculated due to the fact that the number of non-affected lower limbs available for statistical analysis was limited to n=3. This could be a possible limitation. However, this should not be considered as a major limitation, as detail analysis revealed that the results were not different after excluding the non-affected legs.

A further possible limitation could be the limited number of patients that participated in this study (n=13). However, a priori sample size calculation resulted in an estimated total sample size of 13 patients. Additionally, the strength on this clinical study is that each patient served as his/her own control. Therefore, each patient was followed over three weeks of complete decongestive therapy and the identical measurements were performed in the same patient over several time points. Different durations and stages of the disease, different etiologies of lymphedema (primary vs. secondary), in different extremities (upper and/or lower body) or by varying therapies (physical treatments, surgery and/or medications) could be investigated in larger cohorts in future studies.

Due to the limited number of patients included into the study, statistical analysis of different subgroups according to the BMI (e.g. separated according to BMI<25 and BMI>35) was not possible. However, none of the patients included into this study were diagnosed with obesity-induced lymphedema. Therefore, the improvements of lymphedema symptoms would not be expected to be explained by exercise and/or weight loss only (248).

Another limiting factor of this study could be that more female patients (n=10) were enrolled to the study as compared to the number of male patients (n=3). However, this should

not be a major limitation, as it is known that far more females are affected by this chronic, progressive and debilitating disease (73).

6 CONCLUSIONS

In conclusion, it was observed that segmental and whole-body fluid shifts occurred over three weeks of complete decongestive therapy as well as due to manual lymphatic drainage. Within the first of the three weeks of therapy, the greatest effect in terms of fluid shifts was observed. Both assessments that were used to investigate volume/fluid loss showed a moderate correlation. However, perometry (used for assessing limb volume) did not seem to be sensitive enough for the detection of minor fluid shifts as for example seen due to manual lymphatic drainage. The fluid shifts seen due to lymphedema therapy (manual lymphatic drainage) were also reflected in changes in plasma volume as well as plasma proteins and blood cell counts. This has not been reported previously. Therefore, this assessment could potentially serve as an indirect indicator of lymphatic outflow and composition of lymphatic fluid that is mobilized from the lymphatic vasculature towards the blood vessels during physical therapy.

Hyaluronic acid, used as surrogate marker for lymphatic outflow, did not change over three weeks and due to manual lymphatic drainage. Assessing plasma hyaluronic acid concentrations might not be a good indicator of lymphatic outflow in lymphedema patients, compared to healthy persons.

Lymphedema patients seem not to display an increased risk for orthostatic intolerance and falls, as they did not display any signs of orthostatic hypotension at baseline and over complete decongestive therapy. Blood pressure regulation did not change due to complete decongestive therapy and/or manual lymphatic drainage. Manual lymphatic drainage, however, could have potential beneficial effects on hemodynamic responses to orthostatic loading in lymphedema patients undergoing lymphedema therapy.

Lymphedema patients do not show signs of endothelial dysfunction, which indicates that those patients may not be at a higher risk of developing cardiovascular diseases. Finally, a novel finding was that manual lymphatic drainage seems to have a beneficial effect on endothelial/vascular health, which has not been reported previously.

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SUPPLEMENTAL TABLES

Table S1: Overview of daily averaged values of segmental fluid shift parameters (mean ± SD). Displayed is the mean value (pre + post manual lymphatic drainage (MLD)) of each measurement day over the three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) of day 1 with each indicated time point separately. Significant differences are shown in bold.

	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Limb Volume (liters)	12.840 ± 1.899	12.642 ± 1.828	12.577 ± 1.816	12.514 ± 1.977	12.448 ± 1.976
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*4.98*	*5.57*	*6.08*	*9.92*
	p	***<0.001***	***<0.001***	***<0.001***	***<0.001***
	n	*18*	*18*	*16*	*16*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Limb total fluid (liters)	4.797 ± 0.961	4.517 ± 1.101	4.726 ± 0.938	4.779 ± 0.936	4.824 ± 0.926
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*2.15*	*2.25*	*2.29*	*2.37*
	p	*0.041*	*0.034*	*0.033*	*0.027*
	n	*28*	*26*	*22*	*22*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Limb extracellular fluid (liters)	2.997 ± 0.668	2.743 ± 0.714	2.905 ± 0.664	2.959 ± 0,614	2.986 ± 0.599
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*3.02*	*2.75*	*2.37*	*2.29*
	p	***0.006***	***0.011***	*0.028*	*0.032*
	n	*28*	*26*	*22*	*22*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Limb intracellular fluid (liters)	1.792 ± 0.404	1.776 ± 0.478	1.821 ± 0.401	1.811 ± 0.430	1.858 ± 0.432
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*0.31*	*0.33*	*1.27*	*0.04*
	p	*0.760*	*0.745*	*0.220*	*0.969*
	n	*28*	*26*	*20*	*22*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Limb ECF/ICF (ratio)	1.72 ± 0.33	1.58 ± 0.33	1.63 ± 0.33	1.68 ± 0.35	1.66 ± 0.34
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*6.22*	*3.92*	*1.82*	*2.31*
	p	***<0.001***	***0.001***	*0.082*	*0.310*
	n	*28*	*26*	*22*	*22*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/4=0.0125).

Table S2: Overview of segmental fluid shift parameters (mean ± SD) before - and after - manual lymphatic drainage (MLD), over three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) pre- vs. post-MLD for each indicated time point separately. Significant p-values are in highlighted in bold.

		Complete Decongestive Therapy (CDT)				
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Limb Volume (liters)	pre-MLD	12.838 ± 1.898	12.095 ± 2.057	12.023 ± 1996	11.926 ± 2.165	11.889 ± 2.170
	post-MLD	12.841 ± 1.902	12.073 ± 2.061	12.052 ± 2014	11.945 ± 2.202	11.917 ± 2.170
	n	*18*	*25*	*26*	*22*	*22*
	t	*-0.10*	*0.90*	*-1.40*	*-0.73*	*-1.11*
	p	*0.922*	*0.378*	*0.173*	*0.476*	*0.278*
Limb total fluid (liters)	pre-MLD	4.865 ± 1.009	4.301 ± 1.529	4.678 ± 0.973	4.725 ± 0.954	4.747 ± 0.915
	post-MLD	4.637 ± 1.037	4.734 ± 0.975	4.775 ± 0.910	4.833 ± 0.926	4.901 ± 0.952
	n	*30*	*28*	*26*	*20*	*22*
	t	*1.64*	*-1.74*	*-2.74*	*-2.77*	*-3.04*
	p	*0.112*	*0.092*	*0.011*	*0.012*	***0.006***
Limb extracellular fluid (liters)	pre-MLD	2.999 ± 0.726	2.635 ± 0.968	2.868 ± 0.684	2.920 ± 0.621	2.949 ± 0.603
	post-MLD	2.892 ± 0.715	2.851 ± 0.626	2.943 ± 0.647	2.999 ± 0.613	3.024 ± 0.613
	n	*30*	*28*	*26*	*20*	*22*
	t	*1.16*	*-1.45*	*-3.83*	*-2.75*	*-2.69*
	p	*0.256*	*0.158*	***<0.001***	*0.013*	*0.014*
Limb intracellular fluid (liters)	pre-MLD	1.850 ± 0.438	1.668 ± 0.624	1.810 ± 0.422	1.805 ± 0.442	1.816 ± 0.419
	post-MLD	1.745 ± 0.425	1.884 ± 0.464	1.832 ± 0.385	1.818 ± 0.424	1.899 ± 0.455
	n	*30*	*28*	*26*	*20*	*22*
	t	*1.72*	*-2.11*	*-1.12*	*-0.61*	*-2.75*
	p	*0.096*	*0.045*	*0.275*	*0.548*	*0.012*
Limb ECF/ICF (ratio)	pre-MLD	1.67 ± 0.36	1.62 ± 0.33	1.62 ± 0.35	1.67 ± 0.34	1.67 ± 0.34
	post-MLD	1.70 ± 0.35	1.57 ± 0.35	1.64 ± 0.32	1.71 ± 0.38	1.65 ± 0.34
	n	*30*	*26*	*26*	*20*	*22*
	t	*-0.70*	*1.86*	*-1.06*	*-1.58*	*1,07*
	p	*0.489*	*0.075*	*0.298*	*0.131*	*0.298*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/5=0.01).

Table S3: Overview of daily averaged values of whole-body fluids (mean ± SD). Displayed is the mean value (pre + post manual lymphatic drainage (MLD)) of each measurement day over the three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) of day 1 with each indicated time point separately. Significant differences are shown in bold.

	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
%Total body fluid	51.7 ± 9.0	47.1 ± 5.8	46.9 ± 5.5	47.0 ± 5.0	47.5 ± 5.6
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*3.10*	*3.25*	*3.06*	*2.62*
	p	***0.009***	***0.007***	***0.010***	*0.022*
	n	*13*	*13*	*13*	*13*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
% Extra-cellular fluid	21.7 ± 4.1	20.1 ± 2.8	20.0 ± 2.6	20.1 ± 2.5	20.2 ± 2.6
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*2.48*	*2.54*	*2.41*	*2.30*
	p	*0.029*	*0.026*	*0.033*	*0.040*
	n	*13*	*13*	*13*	*13*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
%Intra-cellular fluid	30.0 ± 5.9	27.0 ± 3.3	26.9 ± 3.1	26.9 ± 2.9	27.2 ± 3.3
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*2.70*	*2.78*	*2.68*	*2.24*
	p	*0.019*	*0.017*	*0.020*	*0.045*
	n	*13*	*13*	*13*	*13*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
ECF/ICF Ratio	0.74 ± 0.11	0.75 ± 0.05	0.74 ± 0.05	0.75 ± 0.06	0.74 ± 0.06
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*-0.523*	*-0.383*	*-0.620*	*-0,406*
	p	*0.610*	*0.708*	*0.547*	*0.692*
	n	*13*	*13*	*13*	*13*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/4=0.0125).

Table S4: Pair Overview of whole-body fluid shift parameters (mean ± SD) before - and after - manual lymphatic drainage (MLD), over three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) pre- vs. post-MLD for each indicated time point separately. Significant p-values are in highlighted in bold.

		Complete Decongestive Therapy (CDT)				
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
%Total body fluid	pre-MLD	48.7 ± 6.7	46.3 ± 6.0	46.5 ± 5.3	46.8 ± 5.3	47.3 ± 5.9
	post-MLD	54.8 ± 13.7	47.1 ± 5.8	47.3 ± 5.7	47.2 ± 4.9	47.7 ± 5.8
	n	*13*	*12*	*13*	*13*	*12*
	t	*-2.05*	*-2.40*	*-2.12*	*-0.81*	*-0.91*
	p	*0.063*	*0.035*	*0.055*	*0.435*	*0.382*
%Extra-cellular fluid	pre-MLD	20.9 ± 2.7	19.9 ± 2.8	19.8 ± 2.6	20.1 ± 2.5	20.0 ± 2.8
	post-MLD	22.5 ± 6.2	20.0 ± 2.8	20.2 ± 2.6	20.0 ± 2.5	20.3 ± 2.7
	n	*13*	*12*	*13*	*13*	*12*
	t	*-1.21*	*-1.36*	*-6.32*	*0.33*	*-1.51*
	p	*0.249*	*0.202*	***<0.001***	*0.747*	*0.158*
%Intra-cellular fluid	pre-MLD	27.8 ± 3.8	26.4 ± 3.4	26.7 ± 2.9	26.6 ± 3.0	27.2 ± 3.5
	post-MLD	32.3 ± 8.8	27.1 ± 3.3	27.1 ± 3.4	27.2 ± 2.9	27.4 ± 3.4
	n	*13*	*12*	*13*	*13*	*12*
	t	*-2.49*	*-2.58*	*-1.12*	*-2.08*	*-0.55*
	p	*0.028*	*0.026*	*0.286*	*0.060*	*0.592*
ECF/ICF	pre-MLD	0.76 ± 0.09	0.75 ± 0.06	0.74 ± 0.05	0.76 ± 0.06	0.74 ± 0.07
	post-MLD	0.71 ± 0.13	0.74 ± 0.06	0.75 ± 0.06	0.74 ± 0.07	0.74 ± 0.05
	n	*13*	*12*	*13*	*13*	*12*
	t	*2.51*	*2.71*	*-0.33*	*1.72*	*-0.35*
	p	*0.028*	*0.020*	*0.745*	*0.112*	*0.731*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/5=0.01).

Table S5: Overview of daily averaged values of plasma protein concentrations (mean ± SD). Displayed is the mean value (pre + post manual lymphatic drainage (MLD)) of each measurement day over the three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) of day 1 with each indicated time point separately. Significant differences are shown in bold.

	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Total protein (mg/L)	7.29 ± 0.42	7.24 ± 0.38	7.26 ± 0.37	7.23 ± 0.43	7.07 ± 0.33
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*0.51*	*-0.44*	*-0.44*	*1.68*
	p	*0.623*	*0.673*	*0.676*	*0.144*
	n	*8*	*7*	*7*	*7*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Albumin (mg/L)	4.45 ± 0.41	4.39 ± 0.47	4.43 ± 0.28	4.38 ± 0.30	4.29 ± 0.32
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*1.02*	*-0.13*	*0.42*	*1.42*
	p	*0.343*	*0.901*	*0.689*	*0.206*
	n	*8*	*7*	*7*	*7*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Globulin (mg/L)	2.84 ± 0.41	2.86 ± 0.43	2.83 ± 0.35	2.87 ± 0.31	2.77 ± 0.26
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*-0.21*	*-0.50*	*-1.24*	*0.09*
	p	*0.842*	*0.638*	*0.263*	*0.931*
	n	*8*	*7*	*7*	*7*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Albumin to globulin ratio	1.61 ± 0.34	1.59 ± 0.40	1.59 ± 0.24	1.55 ± 0.21	1.56 ± 0.21
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*0.44*	*0.56*	*1.24*	*0.62*
	p	*0.675*	*0.599*	*0.263*	*0.555*
	n	*8*	*7*	*7*	*7*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/4=0.0125).

Table S6: Pair Overview of plasma protein concentrations (mean ± SD) before - and after - manual lymphatic drainage (MLD), over three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) pre- vs. post-MLD for each indicated time point separately. Significant p-values are in highlighted in bold.

		Complete Decongestive Therapy (CDT)				
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Total protein (mg/L)	pre-MLD	7.26 ± 0.37	7.24 ± 0.38	7.23 ± 0.34	7.17 ± 0.42	6.95 ± 0.35
	post-MLD	7.32 ± 0.53	7.25 ± 0.41	7.28 ± 0.43	7.30 ± 0.45	7.19 ± 0.34
	n	*8*	*8*	*7*	*7*	*7*
	t	*-0.43*	*-0.22*	*-0.49*	*-1.878*	*-3.46*
	p	*0.680*	*0.831*	*0.640*	*0.109*	*0.014*
Albumin (mg/L)	pre-MLD	4.41 ± 0.36	4.34 ± 0.46	4.39 ± 0.32	4.25 ± 0.29	4.18 ± 0.32
	post-MLD	4.49 ± 0.46	4.44 ± 0.48	4.47 ± 0.24	4.48 ± 0.39	4.41 ± 0.35
	n	*8*	*8*	*7*	*6*	*7*
	t	*-1.20*	*-4.63*	*-1.72*	*-3.68*	*-3.08*
	p	*0.270*	***0.002***	*0.137*	*0.014*	*0.021*
Globulin (mg/L)	pre-MLD	2.85 ± 0.43	2.90 ± 0.46	2.85 ± 0.40	2.81 ± 0.17	2.77 ± 0.30
	post-MLD	2.83 ± 0.43	2.82 ± 0.42	2.82 ± 0.36	2.76 ± 0.34	2.78 ± 0.24
	n	*8*	*8*	*7*	*6*	*7*
	t	*0.20*	*1.07*	*0.28*	*0.48*	*-0.17*
	p	*0.850*	*0.320*	*0.787*	*0.649*	*0.870*
Albumin to globulin ratio	pre-MLD	1.59 ± 0.36	1.55 ± 0.42	1.57 ± 0.29	1.52 ± 0.13	1.52 ± 0.21
	post-MLD	1.62 ± 0.33	1.62 ± 0.39	1.61 ± 0.22	1.65 ± 0.31	1.60 ± 0.23
	n	*8*	*8*	*7*	*6*	*7*
	t	*-0.56*	*-1.38*	*-0.48*	*-1.34*	*-1.46*
	p	*0.590*	*0.211*	*0.651*	*0.239*	*0.195*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/5=0.01).

Table S7: Overview of daily averaged values of osmolality and oncotic pressure (mean ± SD). Displayed is the mean value (pre + post manual lymphatic drainage (MLD)) of each measurement day over the three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) of day 1 with each indicated time point separately. Significant differences are shown in bold.

	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Osmolality (mOSM/kg)	294 ± 6.6	299 ± 14.8	296 ± 10.9	294 ± 8.9	297 ± 8.6
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*-1.30*	*-1.17*	*-0.05*	*-1.50*
	p	*0.242*	*0.293*	*0.965*	*0.193*
	n	*7*	*6*	*6*	*6*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Oncotic pressure (mmHg)	30 ± 3.	30 ± 3.8	30 ± 2.8	31 ± 2.2	29 ± 1.8
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*1.22*	*0.93*	*-0.15*	*2.65*
	p	*0.269*	*0.387*	*0.883*	*0.038*
	n	*7*	*7*	*7*	*7*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/4=0.0125).

Table S8: Pair Overview of osmolality and oncotic pressure (mean ± SD) before - and after - manual lymphatic drainage (MLD), over three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) pre- vs. post-MLD for each indicated time point separately. Significant p-values are in highlighted in bold.

		Complete Decongestive Therapy (CDT)				
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Osmolality (mOSM/kg)	pre-MLD	293 ± 9.4	291 ± 8.8	295 ± 7.8	293 ± 8.0	296 ± 7.0
	post-MLD	294 ± 8.6	299 ± 14.4	301 ± 15.1	298 ± 12.2	299 ± 11.2
	n	*7*	*6*	*7*	*6*	*7*
	t	*-0.31*	*-2.30*	*-1.43*	*-1.54*	*-0.90*
	p	*0.769*	*0.069*	*0.204*	*0.184*	*0.403*
Oncotic pressure (mmHg)	pre-MLD	31 ± 2.8	30 ± 3.8	30 ± 2.3	30 ± 1.1	30 ± 1.8
	post-MLD	29 ± 4.0	30 ± 3.8	30 ± 3.9	31 ± 3.6	29 ± 2.6
	n	*6*	*7*	*6*	*7*	*7*
	t	*0.94*	*0.80*	*0.20*	*-0.91*	*0.16*
	p	*0.391*	*0.455*	*0.852*	*0.398*	*0.879*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/5=0.01).

Table S9: Overview of daily averaged values of plasma electrolyte concentrations (mean ± SD). Displayed is the mean value (pre + post manual lymphatic drainage (MLD)) of each measurement day over the three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) of day 1 with each indicated time point separately. Significant differences are shown in bold.

	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Sodium (mmol/L)	141 ± 3.3	140 ± 4.2	140 ± 4.2	140 ± 3.0	141 ± 3.7
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*0.16*	*0.47*	*0.62*	*0.05*
	p	*0.875*	*0.654*	*0.560*	*0.965*
	n	*8*	*7*	*7*	*7*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Cloride (mmol/L)	102 ± 3.6	102 ± 3.6	102 ± 4.0	101 ± 3.6	102 ± 4.2
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*-0.26*	*0.36*	*0.39*	*-0.61*
	p	*0.804*	*0.729*	*0.711*	*0.563*
	n	*8*	*7*	*7*	*7*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Potassium (mmol/L)	4.4 ± 0.4	4.3 ± 0.3	4.3 ± 0.4	4.3 ± 0.11	4.5 ± 0.20
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*0.96*	*-0.14*	*0.32*	*-2.20*
	p	*0.369*	*0.891*	*0.758*	*0.070*
	n	*8*	*7*	*7*	*7*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/4=0.0125).

Table S10: Pair Overview of plasma electrolyte concentrations (mean ± SD) before - and after - manual lymphatic drainage (MLD), over three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) pre- vs. post-MLD for each indicated time point separately. Significant p-values are in highlighted in bold.

		Complete Decongestive Therapy (CDT)				
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Sodium (mmol/L)	pre-MLD	140 ± 2.7	139 ± 2.6	139 ± 3.3	139 ± 2.1	140 ± 2.1
	post-MLD	141 ± 5.4	141 ± 6.0	141 ± 6.0	140 ± 4.4	142 ± 4.3
	n	*8*	*8*	*6*	*7*	*6*
	t	*-0.15*	*-1.37*	*-0.77*	*-0.92*	*-1.21*
	p	*0.887*	*0.212*	*0.476*	*0.392*	*0.280*
Cloride (mmol/L)	pre-MLD	101 ± 3.3	102 ± 2.3	102 ± 2.9	101 ± 2.7	103 ± 2.8
	post-MLD	102 ± 5.4	102 ± 4.9	102 ± 5.7	102 ± 4.7	104 ± 5.6
	n	*8*	*8*	*6*	*7*	*6*
	t	*0.10*	*-0.90*	*-0.68*	*-1.17*	*-0.73*
	p	*0.921*	*0.400*	*0.528*	*0.286*	*0.500*
Potassium (mmol/L)	pre-MLD	4.5 ± 0.4	4.3 ± 0.4	4.3 ± 0.3	4.2 ± 0.2	4.5 ± 0.3
	post-MLD	4.3 ± 0.4	4.3 ± 0.3	4.5 ± 0.6	4.3 ± 0.1	4.5 ± 0.3
	n	*8*	*8*	*6*	*7*	*6*
	t	*1.54*	*-0.37*	*-0.90*	*-0.96*	*-0.10*
	p	*0.167*	*0.730*	*0.410*	*0.374*	*0.922*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/5=0.01).

Table S11: Overview of daily averaged values of white blood cell, neutrophil and eosinophil cell counts (mean ± SD). Displayed is the mean value (pre + post manual lymphatic drainage (MLD)) of each measurement day over the three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) of day 1 with each indicated time point separately. Significant differences are shown in bold.

	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
White blood cells (x10^3 cells/µL)	6.29 ± 1.57	6.10 ± 1.30	6.46 ± 1.84	6.46 ± 1.75	5.99 ± 1.84
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*1.04*	*-1.51*	*-1.29*	*0.05*
	p	*0.334*	*0.182*	*0.246*	*0.964*
	n	*8*	*7*	*7*	*7*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Neutrophils (x10^3 cells/µL)	3.91 ± 1.09	3.74 ± 0.87	3.92 ± 1.06	3.80 ± 0.92	3.56 ± 1.10
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*1.20*	*-0.86*	*-0.31*	*0.96*
	p	*0.269*	*0.425*	*0.765*	*0.374*
	n	*8*	*7*	*7*	*7*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Eosinophils (cells/µL)	146 ± 55.1	153 ± 64.6	180 ± 64.6	152 ± 47.5	151 ± 65.4
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*-1.06*	*-2.48*	*0.07*	*0.17*
	p	*0.325*	*0.048*	*0.944*	*0.868*
	n	*8*	*7*	*7*	*7*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/4=0.0125).

Table S12: Overview of white blood cell, neutrophil and eosinophil cell counts (mean ± SD) before - and after - manual lymphatic drainage (MLD), over three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) pre- vs. post-MLD for each indicated time point separately. Significant p-values are in highlighted in bold.

		Complete Decongestive Therapy (CDT)				
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
White blood cells (x10^3 cells/µL)	pre-MLD	4.82 ± 0.29	4.82 ± 0.34	4.82 ± 0.36	4.84 ± 0.39	4.73 ± 0.31
	post-MLD	9.31 ± 2.47	4.80 ± 0.31	4.84 ± 0.35	4.85 ± 0.39	4.74 ± 0.38
	n	*8*	*7*	*7*	*7*	*7*
	t	*0.11*	*1.38*	*-1.45*	*-0.86*	*-0.16*
	p	*0.924*	*0.218*	*0.197*	*0.425*	*0.879*
Neutrophils (x10^3 cells/µL)	pre-MLD	3.38 ± 1.00	3.29 ± 0.93	3.18 ± 0.89	3.18 ± 0.95	2.80 ± 0.89
	post-MLD	4.44 ± 1.59	4.33 ± 1.15	4.66 ± 1.39	4.42 ± 1.31	4.32 ± 1.44
	n	*8*	*7*	*7*	*7*	*7*
	t	*-1.96*	*-2.73*	*-4.08*	*-2.40*	*-4.38*
	p	*0.090*	*0.034*	***0.007***	*0.053*	***0.005***
Eosinophils (cells/µL)	pre-MLD	185 ± 70.9	164 ± 72.3	210 ± 85.0	180 ± 68.3	176 ± 87.0
	post-MLD	106 ± 42.5	128 ± 64.0	150 ± 52.1	125 ± 38.2	126 ± 47.4
	n	*8*	*7*	*7*	*7*	*7*
	t	*5.71*	*3.30*	*2.81*	*2.57*	*2.60*
	p	***<0.001***	*0.016*	*0.031*	*0.043*	*0.041*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/5=0.01).

Table S13: Overview of daily averaged values of plasma hyaluronic acid concentrations (mean ± SD). Displayed is the mean value (pre + post manual lymphatic drainage (MLD)) of each measurement day over the three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) of day 1 with each indicated time point separately. Significant differences are shown in bold.

	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Plasma hyaluronic acid (ng/mL)	102.09 ± 104.41	76.97 ± 56.92	84.34 ± 89.23	89.21 ± 113.26	71.54 ± 68.89
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*1.25*	*0.96*	*0.61*	*43466*
	p	*0.253*	*0.372*	*0.566*	*0.277*
	n	*8*	*7*	*7*	*7*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/4=0.0125).

Table S14: Overview of plasma hyaluronic acid concentrations (mean ± SD) before - and after - manual lymphatic drainage (MLD), over three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) pre- vs. post-MLD for each indicated time point separately. Significant p-values are in highlighted in bold.

		Complete Decongestive Therapy (CDT)				
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Plasma hyaluronic acid (ng/mL)	pre-MLD	4.47 ± 0.38	4.28 ± 0.38	4.30 ± 0.25	4.22 ± 0.20	4.49 ± 0.25
	post-MLD	4.27 ± 0.41	4.32 ± 0.28	4.45 ± 0.56	4.32 ± 0.14	4.50 ± 0.28
	n	*8*	*8*	*6*	*7*	*6*
	t	*1.54*	*-0.37*	*-0.90*	*-0.96*	*-0.10*
	p	*0.167*	*0.726*	*0.410*	*0.374*	*0.922*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/5=0.01).

Table S15: Overview of daily averaged values of baseline hemodynamics (mean ± SD). Displayed is the mean value (pre + post manual lymphatic drainage (MLD)) of each measurement day over the three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) of day 1 with each indicated time point separately.

	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Heart rate (bpm)	90.8 ± 12.3	87.5 ± 10.6	85.5 ± 12.6	86.7 ± 11.6	88.7 ± 17.8
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*1.45*	*1.81*	*1.49*	*0.48*
	p	*0.179*	*0.101*	*0.167*	*0.642*
	n	*11*	*11*	*11*	*11*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Systolic blood pressure (mmHg)	126.5 ± 16.4	130.1 ± 17.2	119.8 ± 20.4	118.9 ± 17.9	109.9 ± 13.3
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*-0.67*	*0.79*	*1.76*	*2.29*
	p	*0.518*	*0.451*	*0.117*	*0.051*
	n	*10*	*10*	*9*	*9*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Diastolic blood pressure (mmHg)	87.3 ± 13.1	90.5 ± 9.4	83.7 ± 11.7	79.5 ± 14.5	77.5 ± 8.4
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*-0.93*	*0.86*	*2.35*	*2.02*
	p	*0.379*	*0.414*	*0.047*	*0.078*
	n	*10*	*10*	*9*	*9*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Mean blood pressure (mmHg)	102.8 ± 13.8	106.5 ± 12.3	98.6 ± 13.9	95.5 ± 16.2	90.6 ± 10.2
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*-0.93*	*0.75*	*1.95*	*2.14*
	p	*0.377*	*0.471*	*0.087*	*0.065*
	n	*10*	*10*	*9*	*9*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Stroke volume (mL)	57.0 ± 11.6	54.2 ± 9.2	53.5 ± 9.4	52.9 ± 9.0	54.8 ± 10.4
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*1.49*	*1.25*	*1.38*	*0.98*
	p	*0.170*	*0.243*	*0.200*	*0.355*
	n	*10*	*10*	*10*	*9*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Total peripheral resistance (dyn·s/cm^5)	1632.0 ± 335.7	1813.9 ± 297.8	1731.5 ± 400.9	1690.0 ± 527.8	1598.4 ± 387.0
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*-1.79*	*-0.83*	*-0.16*	*0.51*
	p	*0.108*	*0.430*	*0.877*	*0.626*
	n	*10*	*10*	*9*	*9*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Cardiac Output (liters/min)	5.02 ± 0.92	4.66 ± 0.67	4.52 ± 0.69	4.55 ± 0.86	4.63 ± 1.00
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*1.64*	*1.93*	*1.43*	*1.25*
	p	*0.136*	*0.085*	*0.188*	*0.246*
	n	*10*	*10*	*10*	*9*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/4=0.0125).

Table S16: Overview of baseline hemodynamics (mean ± SD) before - and after - manual lymphatic drainage (MLD), over three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) pre- vs. post-MLD for each indicated time point separately.

		Complete Decongestive Therapy (CDT)				
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
Heart rate (bpm)	pre-MLD	90.3 ± 15.8	88.8 ± 10.0	82.6 ± 10.0	89.1 ± 13.4	85.4 ± 17.9
	post-MLD	86.7 ± 10.0	84.0 ± 13.0	84.4 ± 13.3	87.0 ± 13.8	84.1 ± 14.0
	n	*9*	*10*	*10*	*11*	*11*
	t	*1.07*	*1.60*	*-0.85*	*0.55*	*0.40*
	p	*0.317*	*0.145*	*0.417*	*0.596*	*0.694*
Systolic blood pressure (mmHg)	pre-MLD	130.3 ± 23.3	131.9 ± 25.5	117.7 ± 18.6	117.6 ± 22.0	105.9 ± 11.0
	post-MLD	120.6 ± 19.8	127.3 ± 13.2	117.4 ± 19.4	118.5 ± 23.4	111.6 ± 15.0
	n	*9*	*10*	*8*	*11*	*11*
	t	*1.08*	*0.78*	*0.04*	*-0.17*	*-1.95*
	p	*0.310*	*0.453*	*0.968*	*0.870*	*0.080*
Diastolic blood pressure (mmHg)	pre-MLD	88.4 ± 16.2	93.2 ± 15.1	82.8 ± 11.4	79.4 ± 18.1	74.8 ± 5.4
	post-MLD	84.0 ± 16.7	87.4 ± 7.8	87.2 ± 14.8	79.2 ± 18.6	78.3 ± 11.4
	n	*9*	*10*	*8*	*11*	*11*
	t	*0.68*	*1.30*	*-1.01*	*0.07*	*-1.32*
	p	*0.514*	*0.227*	*0.347*	*0.949*	*0.216*
Mean blood pressure (mmHg)	pre-MLD	104.7 ± 17.5	108.7 ± 19.1	97.4 ± 13.9	95.0 ± 19.8	87.7 ± 7.1
	post-MLD	98.5 ± 17.3	103.5 ± 9.1	99.7 ± 16.2	94.7 ± 20.7	91.4 ± 13.3
	n	*9*	*10*	*8*	*11*	*11*
	t	*0.90*	*1.09*	*-0.39*	*0.07*	*-1.24*
	p	*0.397*	*0.303*	*0.709*	*0.949*	*0.244*
Stroke volume (mL)	pre-MLD	56.1 ± 14.8	58.6 ± 11.0	55.4 ± 8.0	57.4 ± 12.8	55.4 ± 10.0
	post-MLD	51.2 ± 6.5	53.8 ± 7.1	54.2 ± 11.1	53.4 ± 12.8	55.3 ± 10.4
	n	*8*	*9*	*10*	*9*	*10*
	t	*1.22*	*1.82*	*0.52*	*1.57*	*0.06*
	p	*0.263*	*0.106*	*0.614*	*0.155*	*0.952*
Total peripheral resistance (dyn·s/cm^5)	pre-MLD	1667.2 ± 260.7	1739.7 ± 472.4	1685.3 ± 345.4	1462.5 ± 561.2	1501.3 ± 327.1
	post-MLD	1756.4 ± 500.2	1895.8 ± 328.8	1805.3 ± 410.2	1695.5 ± 821.3	1623.6 ± 483.9
	n	*8*	*9*	*8*	*9*	*10*
	t	*-0.54*	*-0.94*	*-1.20*	*-1.67*	*-1.24*
	p	*0.606*	*0.374*	*0.268*	*0.133*	*0.248*
Cardiac Output (liter/min)	pre-MLD	5.04 ± 1.08	5.09 ± 0.84	4.53 ± 0.62	5.13 ± 0.77	4.68 ± 0.83
	post-MLD	4.50 ± 0.76	4.35 ± 0.74	4.51 ± 0.86	4.62 ± 1.01	4.72 ± 1.23
	n	*8*	*9*	*10*	*9*	*10*
	t	*1.90*	*2.90*	*0.11*	*2.57*	*-0.12*
	p	*0.100*	*0.020*	*0.911*	*0.033*	*0.907*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/5=0.01).

Table S17: Overview of daily averaged values of endothelial and vascular function parameters (mean ± SD). Displayed is the mean value (pre + post manual lymphatic drainage (MLD)) of each measurement day over the three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) of day 1 with each indicated time point separately. Significant differences are shown in bold. *Legend (alphabetical order): ADMA, asymmetric dimethylarginine; A-to-V, arteriolar-to-venous ratio; CDT, complete decongestive therapy; CRAE, central arteriolar equivalent; CRVE, central retinal venular equivalent; MLD, manual lymphatic drainage; PWVcf, carotid-femoral pulse wave velocity.*

	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
ADMA (µmol/L)	0.53 ± 0.05	0.53 ± 0.05	0.53 ± 0.04	0.50 ± 0.04	0.49 ± 0.04
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*0.52*	*0.90*	*1.79*	*2.16*
	p	*0.621*	*0.403*	*0.123*	*0.074*
	n	*8*	*7*	*7*	*7*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
PWVcf (m/s)	9.3 ± 2.0	9.3 ± 1.8	9.1 ± 1.3	9.3 ± 2.7	9.2 ± 2.0
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*0.25*	*0.55*	*0.29*	*0.51*
	p	*0.811*	*0.595*	*0.780*	*0.166*
	n	*19*	*9*	*8*	*10*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
CRAE (µm)	134 ± 17	134 ± 20	124 ± 14	148 ± 21	132 ± 8
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*-0.02*	*1.21*	*-1.28*	*0.04*
	p	*0.987*	*0.052*	*0.247*	*0.969*
	n	*12*	*11*	*7*	*7*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
CRVE (µm)	205 ± 26	227 ± 22	211 ± 24	226 ± 16	214 ± 24
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*-2.09*	*-0.14*	*-4.41*	*-1.28*
	p	*0.060*	*0.891*	***0.005***	*0.250*
	n	*12*	*11*	*7*	*7*
	Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
A-to-V Ratio	0.67 ± 0.12	0.61 ± 0.10	0.59 ± 0.05	0.66 ± 0.10	0.63 ± 0.10
		Day 1 vs day 2	**Day 1 vs day 7**	**Day 1 vs day 14**	**Day 1 vs day 21**
	t	*1.92*	*3.64*	*0.08*	*0.79*
	p	*0.081*	***0.005***	*0.938*	*0.458*
	n	*12*	*11*	*7*	*7*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/4=0.0125).

Table S18: Overview of endothelial and vascular function parameters (mean ± SD) before - and after - manual lymphatic drainage (MLD), over three weeks of complete decongestive therapy (CDT), including a pairwise comparison (t-test) pre- vs. post-MLD for each indicated time point separately. Significant p-values are in highlighted in bold. *Legend (alphabetical order): ADMA, asymmetric dimethylarginine; A-to-V, arteriolar-to-venous ratio; CDT, complete decongestive therapy; CRAE, central arteriolar equivalent; CRVE, central retinal venular equivalent; MLD, manual lymphatic drainage; PWVcf, carotid-femoral pulse wave velocity.*

		Complete Decongestive Therapy (CDT)				
		Day 1	**Day 2**	**Day 7**	**Day 14**	**Day 21**
ADMA (µmol/L)	pre-MLD	0.55 ± 0.04	0.55 ± 0.05	0.55 ± 0.05	0.52 ± 0.04	0.51 ± 0.02
	post-MLD	0.52 ± 0.07	0.51 ± 0.06	0.51 ± 0.05	0.48 ± 0.04	0.46 ± 0.06
	n	*8*	*8*	*7*	*7*	*7*
	t	*1.49*	*2.12*	*1.75*	*3.68*	*2.41*
	p	*0.180*	*0.071*	*0.130*	***0.010***	*0.052*
PWVcf (m/s)	pre-MLD	9.7 ± 3.1	9.1 ± 1.3	9.0 ± 1.0	9.3 ± 2.9	9.2 ± 1.9
	post-MLD	9.3 ± 2.5	9.5 ± 2.5	9.3 ± 1.8	9.6 ± 2.3	9.0 ± 2.0
	n	*13*	*10*	*9*	*10*	*12*
	t	*0.64*	*-0.60*	*-0.86*	*-0.70*	*0.40*
	p	*0.537*	*0.566*	*0.412*	*0.450*	*0.697*
CRAE (µm)	pre-MLD	127 ± 23	132 ± 26	127 ± 14	132 ± 20	123 ± 13
	post-MLD	139 ± 22	129 ± 9	120 ± 21	141 ± 19	147 ± 29
	n	*13*	*11*	*11*	*8*	*9*
	t	*-1.38*	*0.27*	*1.00*	*-1.03*	*-1.90*
	p	*0.193*	*0.792*	*0.342*	*0.338*	*0.094*
CRVE (µm)	pre-MLD	197 ± 33	228 ± 36	209 ± 27	231 ± 26	206 ± 25
	post-MLD	207 ± 29	221 ± 25	212 ± 32	214 ± 16	224 ± 30
	n	*13*	*11*	*11*	*8*	*9*
	t	*-0.95*	*0.54*	*-0.28*	*1.86*	*-1.31*
	p	*0.359*	*0.604*	*0.782*	*0.105*	*0.226*
A-to-V Ratio	pre-MLD	0.66 ± 0.11	0.60 ± 0.17	0.62 ± 0.05	0.57 ± 0.04	0.61 ± 0.09
	post-MLD	0.70 ± 0.19	0.60 ± 0.08	0.57 ± 0.07	0.66 ± 0.06	0.66 ± 0.12
	n	*13*	*11*	*11*	*8*	*9*
	t	*-0.81*	*-0.09*	*2.08*	*-3.96*	*-1.34*
	p	*0.435*	*0.927*	*0.064*	***0.005***	*0.218*

Note: A corrected significance level for pairwise comparisons was applied (p=0.05/5=0.01).