Diplomarbeit

Assessing Lymphatic Flow Changes in Patients with Lymphatic Disease: Effects of Therapy

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Sebastian Walbrodt

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Otto Loewi Forschungszentrum

Lehrstuhl für Physiologie

unter der Anleitung von

Assoz.-Prof. Priv.-Doz. Dr.med. Nandu Goswami

und

Prof. Dr.rer.nat. Andreas Rössler

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Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, Mai 9st, 2018 Sebastian Walbrodt eh.

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Last but not least, I would like to thank my parents and my spouse, who supported me during my entire studies.

Abstract

Introduction: Lymphedema is a disease of the lymphatic system that is disfiguring and still incurable today. While the causes are manifold, pathophysiologically there is always an imbalance between lymph formation and lymph drainage leading to a backlog of lymph which becomes visible as edema. The macromolecule hyaluronate is an integral part of the extracellular space and is present in almost all tissues, but especially in the skin. Several studies have shown that hyaluronic acid is transported in part via the lymph and subsequently reaches the bloodstream before it is finally degraded in the liver and kidneys and could therefore be used as a surrogate for lymphatic flow changes.

Aims and objectives: This diploma thesis aims to examine the role of Hyaluronan acid as a potential surrogate for lymph flow in persons with lymphedema. Moreover, this thesis tries to identify possible correlations with plasma density and weight.

Methodology: As part of the "Lymph" study of the Department of Physiology at the Medical University of Graz, in cooperation with the LKH Wolfsberg, we took blood samples on fixed days from eight patients with primary or secondary lymphedema, as they underwent a three-weeks treatment of complex decongestive therapy in the hospital.

Blood samples were taken at the first, second, seventh, 15th and 20th day before and after the treatment. The amount of hyaluronic acid was measured with a highly sensitive immunofluorescence test.

Results: Plasma levels of hyaluronic acid fluctuated strongly over the course of the therapy and between the subjects. Compared to pre-treatment levels, post-treatment levels were often lower, which was found to be statistically significant in the Wilcoxon test ($r = 0.35$, $P < 0.033$).

Discussion: It appears that complex decongestive therapy does not seem to affect pre- and post-therapy lymphatic flow, as assessed by hyaluronic acid levels. As the route of the hyaluronic acid molecules from connective tissue via the lymphatic system to the blood circulation is influenced by many factors, further research is required.

Zusammenfassung

Einleitung: Das Lymphödem ist eine bis heute nicht heilbare und entstellende Erkrankung des lymphatischen Systems. Während die Ursachen vielfältig sind, liegt pathophysiologisch immer ein Ungleichgewicht zwischen Lymphbildung und Lymphabfluss vor, in dessen weiterer Folge es zu einem Rückstau von Lymphe kommt welcher als Ödem sichtbar wird.

Das Makromolekül Hyaluron stellt einen integralen Bestandteil des Extrazellularraums dar und ist in nahezu allen Geweben, vor allem aber in der Haut vorhanden. Eine Reihe von Studien zeigten, dass Hyaluronsäure zu einem Teil über die Lymphe abtransportiert wird und in weiterer Folge die Blutbahn erreicht, bevor es endgültig in Leber und Nieren abgebaut wird und könnte somit als Verlaufsmarker für Änderungen im Lymphfluss einsetzbar sein.

Zielsetzung: Das Ziel dieser Diplomarbeit ist es, die Rolle von Hyaluronsäure als potentiellen Marker des Lymphflusses bei LymphödempatientInnen zu untersuchen und außerdem mögliche Korrelationen mit Plasmadichte und Gewicht zu identifizieren.

Methodik: Im Rahmen der Lymphstudie der Abteilung Physiologie der medizinischen Universität Graz haben wir in Zusammenarbeit mit dem LKH Wolfsberg von acht PatientInnen mit primärem oder sekundärem Lymphödem an mehreren Tagen Blutproben entnommen, welche im Rahmen einer dreiwöchigen komplexen physikalischen Entstauungstherapie stationär behandelt wurden.

Blutproben wurden am ersten, zweiten, siebten, 15. und 20. Tag jeweils vor und nach der Behandlung genommen. Der Hyaluronsäuregehalt wurde mit einem hochsensitiven Immunofluoreszenztest bestimmt.

Ergebnisse: Die Werte für die Hyaluronsäure schwankten im Verlauf und zwischen den ProbandInnen stark. Im Vergleich zur Messung vor der Behandlung waren die Messwerte nach der Behandlung häufig niedriger, welches sich im Wilcoxon-Test als statistisch signifikant zeigte (r = 0,35, $P < 0.033$).

Diskussion: Betrachtet man die Konzentration der Hyaluronsäure vor und nach der Therapie, so scheint die komplexe physikalische Entstauungstherapie keinen Einfluss auf den Lymphfluss zu haben. Es ist weitere Forschungsarbeit nötig, da der Weg der Hyaluronsäure vom Bindegewebe über das Lymphsystem bis hin zum Blutkreislauf von vielen Faktoren beeinflusst wird.

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List of Abbreviations

LMC	lymphatic muscle cell
ISF	interstitial fluid
HA	Hyaluronan acid
GlcA	D-glucuronic acid
GlcNAc	N-acetyl-D-glucosamine
HAS	Hyaluronan synthase
UDP	uridine diphosphate
HMW-HA	high molecular weight hyaluronan
LMW-HA	low molecular weight hyaluronan
mRNA	messenger ribonucleic acid
LE	lymphedema
QoL	quality of life
BIS	bioimpedance spectroscopy
ML	manual lymph drainage
APC	advanced pneumatic compression
LLLT	Low-Level Laser Therapy
LVA	lymph venous anastomosis
VLNT	Lymph Node Transfer
CDT	complex decongestive therapy

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1. Introduction

1.1 The Circulatory System

The circulatory system comprises the cardiovascular system and the lymphatic system. The cardiovascular system maintains the blood stream and transports nutrients, like oxygen and glucoses, as well as hormones to every cell in the human body. It is separated in a small, pulmonary circulation to oxygenate the red blood cells and a great, systemic circulation. Oxygenated blood is pumped through arteries from the left ventricle of the heart via the aorta to every organ. Simultaneously, the lumen of the vessels becomes smaller, while the overall artery diameter enlarges. The efflux of nutrients into the cell and uptake of CO2 and by-products proceeds in the capillary network that merges firstly into venules which continue into greater veins. The venous blood gathers in the vena cava inferior and vena cava superior, which both empty in the right atrium of the heart.

The lymphatic system evolved parallel with the blood circulation, but although it is integrated closely to the blood circulation, it differs radically in its composition, hydrodynamics and cellular content [1]. Apart from the cardiovascular system that works as a closed circle, the lymphatic system is a conducting network of vessels and nodes with an unidirectional flow starting in the capillary zone and ending as the right lymphatic duct and thoracic duct (left lymphatic duct) in one of the subclavian veins. While the right lymphatic duct carries the lymph of only one quarter of the body, namely the upper right side, the thoracic duct drains the upper left side and both lower extremities.

Lymph flow is the result of leaking plasma proteins and tissue fluid driven by pressure and concentration gradients directed towards the initial lymphatics and thus, the lymphatics have a crucial role in maintaining fluid homeostasis [2].

Lymphatic vessels are essential in transporting lymph, a fluid composed of interstitial fluid, macromolecules and triglycerides (during digestion in the intestinal lymphatics), but they also are a key highway via which immune cells are trafficked and shape and coordinate immune responses and therefore, the lymphatic vasculature is an integral component of the immune system [3].

Beside the lymphatic vessels, the primary lymphoid organs (Thymus and the bone marrow) and the secondary lymphoid organs (spleen, tonsils, lymph nodes) constitute another part of the lymphatic system.

1.2 Lymphatic System

1.2.1 Embryonic Development of the Lymph vasculature

The embryonic development of the lymphatic system starts early in the embryonic phase as a step-wise process with VEGRF-3+ and LYVE-1+ epithelial cells budding off from veins and forming primitive lymph sacs, which are the origin of further differentiation and maturing of the lymphatic system by forming lymphatic vessels and transmigrating the surrounding tissue (*fig. 1*) [4].

The formation of the lymph sacs is stimulated by unidentified inductive signals which lead to the induction of the transcription factor Prox1 in a subset of endothelial cells of the cardinal veins that become committed to the lymphatic lineage [5].

They also express additional lymphatic markers, which are less specific for lymphatic vessels like Podoplanin and Neuropilin.

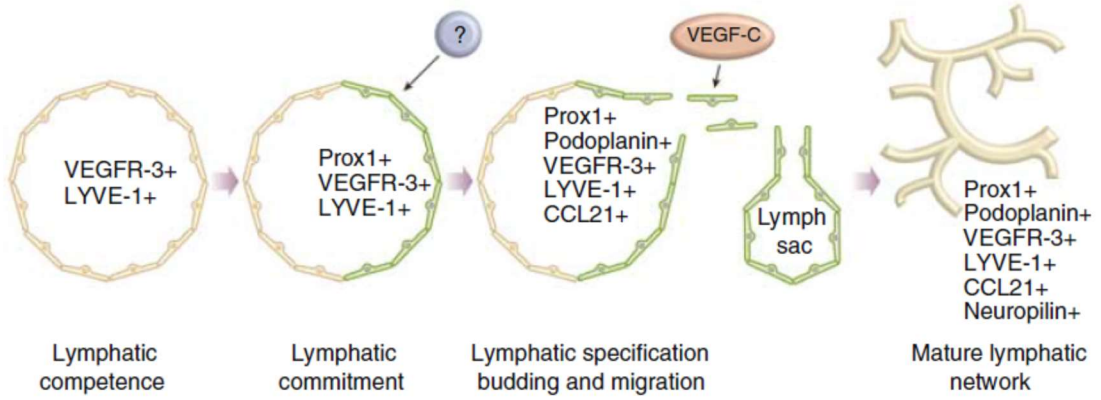

Fig. 1: The development of the lymphatic vasculature by an unknown signal inducing the formation of initial lymph sacs from cardinal veins and its maturation. Figure taken from [5].

1.2.2 Lymphatic Vessels

Lymphatic vessels and nodes form a fine network that carries tissue fluid, fat, colloids and cells including metastatic [6] cells from the interstitium of almost all organs via the thoracic duct and the right lymphatic duct and ends in the great veins of the body. Lymphatic vessels can be divided into three different types: initial lymphatics or lymphatic capillaries, pre-collectors and collecting lymphatics.

Originating in the tissue parenchyma, initial lymphatics form a fine network of non-contracting, blind-ending vessels with a diameter of 60-70 µm composed of a single layer of lymphatic endothelial cells with discontinuous, button-like junctions suggesting a fluid influx in regions between these buttons [7]. Lacking smooth muscle media and intraluminal valves unlike the contractile pre-collectors and collectors, the endothelium of initial lymphatics itself serves as a primary valve to obtain unidirectional lymph flow into the vessel [8]. Attached to interstitial collagen fibers by

anchoring filaments and surrounded by a discontinuous basement membrane, they form a distinct, “oak-leaf” shaped structure with their overlapping cellular extensions, which are only connected to each other at their bottom by Zonulae occludentes and adherences while the top can move freely [9]. Increased pressure in the interstitium exerts a higher tension in the anchoring filaments leading to small openings between the extensions [9].
In addition, a transcellular route by aquaporin-1 channels may contribute as another pathway as well [10].
The initial lymphatics coalesce in pre-collectors with a diameter of 150 µm, which exhibit both transporting and resorbing functions due to their inconsistent wall structure [9].
Therefore, pre-collectors resemble a combination of initial lymphatics with intraluminal, unidirectional valves and an accessory membrane of collagenous fibers and few smooth muscle cells. The intraluminal valves are bicuspid flaps composed of endothelial cells and connective tissue and referred to as system of “secondary valves” after the oak-leaf shaped endothelium of the initial lymphatics [11].

Unlike the pre-collectors, collectors have a diameter of 100-600 µm and morphologically resemble the three-layered wall of veins [9] and are lined with vascular smooth muscle cells that rhythmically contract to drive lymph flow [12]. The amount of smooths muscle cells increases from distal to proximal along the lymphatic route. Moreover, they contain further valves to maintain the unidirectional flow of lymph.
Functionally, the segment between two valves is termed lymphangion and its length differs from 2-3 mm (pre-collectors) to 6-10 cm (lower thoracic part of the thoracic duct) with a sac-like enhancement at its distal part narrowing to the proximal end [9].
Anatomically, collectors resemble a chain of lymphangions in series where each lymphangion is capable of contracting and pumping fluid into the subsequent one and backflow is prevented by intraluminal valves.
According to Kretz et al., the collectors can be subdivided in three sections based on their topography in the lymphatic system:

- The subcutaneous, epifascial system, draining the skin and the subcutis
- The subfascial system, collecting the lymph from the muscles, joints, tendon sheaths and nerves. The subcutaneous and subfascial system are connected by perforating vessels that mainly carry the lymph from the depth to the surface.
- The lymph vessels in the organs, which are adapted to the organ structure and therefore exhibit organ-specific differences [9].

1.2.3 Lymph nodes

In the human body approximately 600-700 lymph nodes exist, which belong to the group of secondary lymphoid organs constituted of lymphoreticular tissue [9]. Lymph nodes are present in the entire body and frequently organized in groups in distinct regions (within or near the trunk and the head). They are kidney or oval shaped organs with an almost continuous capsule of collagenous fibers, few elastic fibers and smooth muscle cells with trabecular arranged extensions that divide cortex and medulla of the node into smaller compartments termed lymph lobules [13].

Furthermore, the lymph node contains a meshwork of reticular fibers which can be subdivided into four structurally distinct regions correlating with the location of distinct immune cell subsets where immunological processes take place [14]. Below the capsule is the subcapsular sinus, in which the afferent lymphatic vessels drain. The cortex underneath the subcapsular sinus consists of an outer zone with B cells and a deep zone named paracortex where T cells interact with dendritic cells.

The medulla is the inner portion of the lymph node and contains sinuses, blood vessels and medullary cords with further cell types of the lymphatic line. Furthermore, the medulla is in contact with the hilum, where one or - less frequently - two efferent lymph vessels leave the node and which is also the passageway for the supplying arterial and venous vessels.

Alongside their competence in filtering and surveilling lymph for quick immunological response, lymph nodes participate in the catabolism of hyaluronan through their capacity for the absorption and metabolic degradation of hyaluronan [15], but also their ability to produce and release hyaluronan of predominant low molecular weight into the outflowing lymph [16].

1.2.4 Innervation and modulation

It is supposed that the lymphatic vasculature has a sympathic and parasympathic innervation due to several findings in different species, although the role of the sympathicus with its adrenergic nerves is researched best, little is known about the fundamental mechanisms and how they contribute to the propulsion of lymph fluid [9].

It has been hypothesized, lymph vessels work similarly to blood vessels, where innervation and vasoactive substances play a key role in the vessel diameter and therefore contribute to the distribution of blood volume [17, 18].

It has also been shown that a various number of agents released from the lymphatic tissue such as prostaglandin, acetylcholine, neuropeptide Y, vasoactive intestinal peptide (VIP), substance P (SP) and bradykinin can influence lymph flow by modulating muscle tension, contractibility and chronotropy [19, 20, 21].

1.2.5 Lymph propulsion

Lymph propulsion is the result of a combination of extrinsic and intrinsic forces pumping the lymph against the hydrostatic pressure gradient.

Engset et al. revealed that, at rest, 1/3 of lymph transport in the lower extremities is passively achieved by the compression of nearby skeletal muscles (extrinsic), while 2/3 depend on active pumping within the lymphatic vessels by a layer of smooth lymphatic muscle cells (LMCs) in the walls of the pre-collectors and collectors (intrinsic) [22].

According to *Scallan et al.*, this lymphatic pump functionally resembles the cardiac pump in many aspects as preload, afterload, contractility and contraction frequency are determinants of its function [23]:

Similar to the heart, preload describes the end-diastolic pressure within the lymph vessel and analogous to the Frank Starling relationship, an increased filling pressure results in a raised pump output.

Afterload comprises all counter forces lymph pump must overcome to provide sufficient lymph flow, e.g. the central venous pressure, but also outflow obstructions and gravitational shifts [23].

On one hand, contractility of the LMC is afterload-dependent (contractility rises in response to elevated afterload) [24], on the other hand it is regulated by a various number of agents (*see also 1.2.4*).

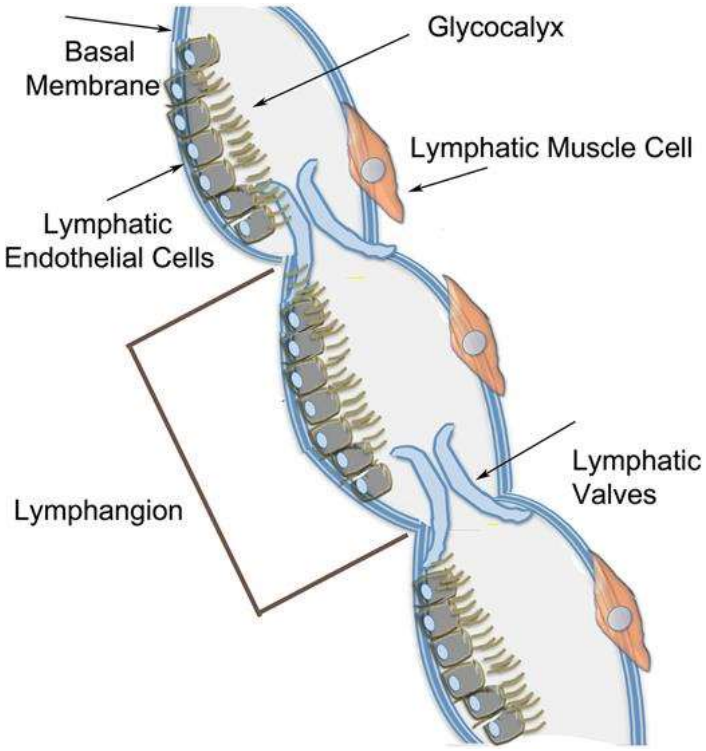

Fig. 2: Schematic of a lymphatic collector. Figure taken from [28]

Contraction frequency, which is another necessary parameter for the lymphangion to adopt to flow changes, initially rises with rapid pressure increase, but declines and stabilizes over the time [25].

Lymph movement runs in synchronized waves through the lymphangion and backflow is prevented by the intraluminal valves at the beginning and ending of each lymphangion (*fig. 2*).

A pump cycle starts with both valves closed while intraluminal pressure rises under the myogenic constriction of the LMC layer until it exceeds the output pressure resulting in the opening of the output valve and ejection of lymph [26]. Under the relaxation of the LMC the intraluminal pressure decreases, and the output valve closes thus allowing the lymphangion to uptake the lymph from the preceding lymphangion.

The LMC layer has the ability to contract spontaneously at an inherent frequency or by electric coupling between LMC layers resulting in a synchronized wave of contractions along the lymphatic vessel [27].

1.2.6 What is lymph?

Fundamentally, lymph is the successor of interstitial fluid (ISF), which occupies the space between cells and enters the lymphatic vasculature by drainage. Approximately 12L of the total body water are in the interstitial space, while only a smaller portion of approx. 3L water contribute to the blood volume [2]. According to the Starling principle, interstitial fluid itself is formed as an ultrafiltrate of the capillary microvasculation of blood plasma, which is determined by the net balance between hydrostatic and osmotic pressures across the microvascular endothelium (*fig. 3*) [28].

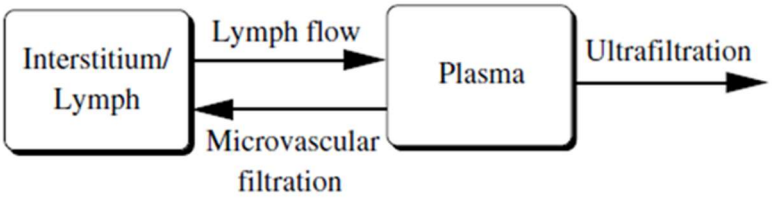

Fig. 3: Fluid shifts between Plasma and Lymph. Figure taken from [2].

Deduced from the principle of irreversible thermodynamics, Starlings principle can be expressed as an equation:

$$\frac{J_v}{A} = L_P\{\Delta P - \sum \sigma_n \Delta \Pi_n\} \quad (1)$$

where J_v is the volume filtration rate per unit endothelial area A, L_P is hydraulic conductivity of the membrane and ΔP the difference in hydrostatic pressure between the capillary blood (P_c) and ISF (P_i). $\sum\sigma_n\Delta\Pi_n$ represents the sum of the differences in osmotic pressure exerted across the vessel walls by all the solutes in plasma and ISF quantified by their leakiness with Staverman's osmotic reflection coefficient σ [29].

In this model, the steady-state fluid exchange is regarded as filtration in the arterial and reabsorption in the venous branch of the capillary bed. New insights in the last 25 years revealed, that fluid reabsorption in the venous capillaries is rather exceptional due to the usually higher pressure in venules [30, 31], which depends on temperature and heart level [32, 33]. In fact, the glycocalyx of the endothelium is thought to work as semipermeable membrane and the oncotic gradient is between the oncotic pressure of the subglycocalyceal space and the intravascular oncotic pressure [29].

Moreover, absorption appears to be a transient phenomenon as *Michel and Phillips* observed in capillaries of the frog mesentery [34].

These findings indicate a constant, low level of fluid exchange driven by the hydrostatic pressure

towards the ISF and further into the lymphatics during the steady-state (*fig. 4*).

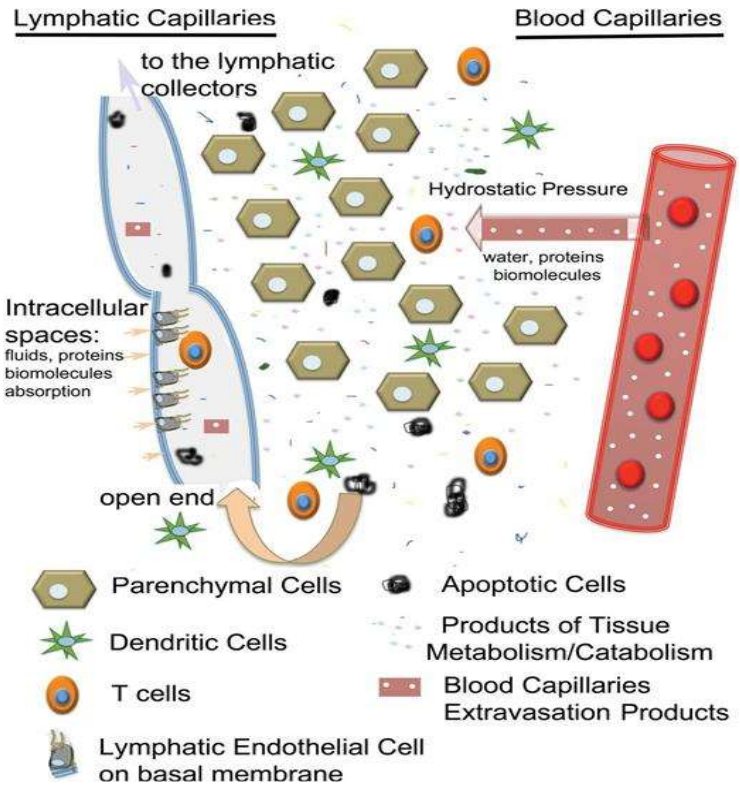

Fig. 4: Schematic of lymph formation. Figure taken from [28]

As a consequence of being the successor of ISF and thus of blood plasma, lymph fluid was generally thought to have a similar composition. However, it has been shown that the occurrence of proteins in lymph is likely to be uniquely defined by the anatomical region from which it was derived depending on the local metabolism of the parenchyma [35].

In addition, the absorption and transport of dietary lipids during digestion in the intestine is predominantly restricted to the lymph.

1.3 Assessment of lymph flow

1.3.1 Lymphoscintigraphy

Lymphedema is defined as a condition of restricted lymphatic drainage; therefore, methods of clinical feasibility are required, which assess the lymphatic flow rate. Quantitative Lymphoscintigraphy is referred to as the best method currently available in quantitively assessing lymph flow due to its high sensitivity of functional information [36].

A radioactive, gamma emitting tracer, usually Technetium (TC)-99 m combined with a carrier (e.g. human serum albumin) is injected in the skin facilitating to backtrace the efferent flow of the radiopharmaceutical agent through lymph vessels and nodes by a gamma camera system, which allows to gain anatomical and functional information [37].

Alternatively, the removal of the tracer can be measured with a scintillation counter, which does only provide functional information.

The tracer is a radiolabeled macromolecule, usually plasma proteins with a hydrodynamic radius between 3.6–5.6 nm or larger colloid particles between 40–100 nm in diameter, which do not escape by capillary re-uptake due to their size and concentration and the pressure gradient towards the interstitium [36].

1.3.2 Hyaluronan

1.3.2.1 Chemical properties

The Hyaluronic acid, first described by Meyer and Palmer in the 1930s as the mucus of the vitreous body of a bovine eye [38], is a linear, acid polysaccharide and belongs to the group of glycosaminoglycans (GAG), which additionally comprises Heparan sulfate, Heparin, Chondroitin 4-(6-) sulfate, Dermatan sulfate and Keratan sulfate. GAGs are single chain polymers of disaccharide units containing N-acetylhexosamine and hexose and the second sugar habitually is a hexuronic acid except for keratan sulphate, which contains galactose instead.

Unlike other GAGs, Hyaluronic acid is an unbranched molecule and does not exhibit any sulfate groups.

In 1986, Balazs and coworkers suggested the term Hyaluronan (HA) for this polysaccharide generally, irrespective of its degree of dissociation (remark of the author: as acid or its salt Hyaluronate) [39] to avoid the difficulty of cation identification and to take its polyanionic property in vivo into account [40].

The molecular appearance of HA is consistent with a linear polymer made of repeating disaccharides units of D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc) (structure:

…[GlcA (1-β-3) GlcNAc (1-β-4)]$_n$... (*fig. 5*)).

The molecule is stabilized by hydrogen bonds [41], consequently forming a double helical configuration resulting in an "overall expended coil structure" in solution [42]. This specific structure allows HA to bind approximately the 1000-fold of its own size as water by mechanically immobilizing the main part of the water within the coil [42].

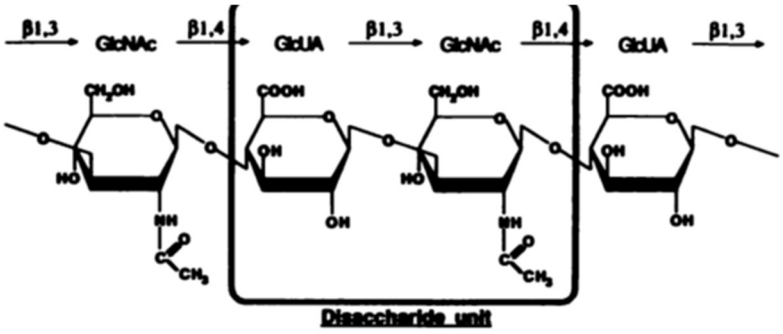

Fig. 5 Chemical structure of HA with alternating Disaccharide. Figure taken from [42]

Moreover, hydrogen bonds and chain-chain reactions give more stiffness and induce the development of more complex structures accounting for the special viscoelastic properties, which are relevant to the role of HA in biological environments [43].

HA tends to form an entangled network that resists the flow of a solvent [42], therefore it impedes fluid fluxes through the tissue compartments [44].

Furthermore, HA has a distinct and anomalous viscoelasticity, which is, depending on the applied pressure, more elastic with a high viscosity in the case of low shear stress, but when subjected to high shear forces it can drop 1000-fold [45].

Unlike other GAGs, which have a 100 - 1000 times lower molecular weight, HA molecules can occur with a very high molecular mass, ranging from about 10^5 to 10^7 Da due to their very long chains, but they can also occur as smaller fragments and oligosaccharides depending on the underlying conditions [46].

1.3.2.2 Synthesis and Distribution

HA is synthesized mainly in mesenchymal cells by a membrane-bound synthase on the inner surface of the plasma membrane, where a Hyaluronan synthase (HAS) alternatively adds UDP-GlcA and UDP-GlcNAc monomers to the reducing end of the emerging molecule [47]. During processing, the molecule is directly extruded into the extracellular space by mechanisms which remain unclear, since there is no final evidence for the existence of a pore in vertebrate cells and findings for ABC transporter mediated extrusion are inconsistent [48].

The Hyaluronan synthase comprises the three isoforms HAS1-3, each possessing two binding domains for UDP sugars. While HAS2 and HAS3 contribute most to the production of HA, HAS1 plays only a minor role, maybe due to its lower synthesis rate [49], but its activity can be upregulated during inflammatory processes [50]. In addition, it has been ascertained, that HAS1 and HAS2 synthesize HA of high molecular weight (HMW-HA) (up to 2,000 kDa), whereas HA by HAS3 is of

low molecular weight (LMW-HA) (100-1,000 kDa) [49]. The activity of HAS is modulated by numerous external stimuli, such as cytokines and growth factors, but is also related to mRNA expression, cell density [51] and the availability of UDP-sugar precursors [52].
Moreover, the expression of mRNA for HA synthases depends on the cell type and external stimuli have an impact on the HA chain length [51].

The outstanding role of HA as main contributor to the extracellular matrix is its ubiquitous distribution in all body tissues, although its concentration varies. It is high in the synovial fluid (1400-3600µg/g) and low in blood serum (0.01-0.1µg/g) [53].
Elevated levels of HA concentration in blood serum can be found in pathologic conditions, e.g. rheumatoid arthritis and liver cirrhosis [54].
The estimated amount of HA in an adult human is approximately 20g and the skin presumably contains about half of the total HA in the body [55]. Besides its occurrence in the extracellular matrix, HA is also present on the surface of some cells, where it features as a pericellular coat, which potentially mediates inflammatory responses [56], but is also involved in cell differentiation and morphogenesis [53].
More recent studies suggest, that HA also occurs intracellular, where it could play a role as a component of the nuclear matrix, the cell skeleton and during mitosis [57],

1.3.2.3 Turnover and Degradation

Currently, there are three pathways known for HA turnover and degradation, but the contribution of each has been discussed controversially:

1. HA is removed from the ECM by drainage into the lymphatics, where further degradation occurs in lymph nodes and subsequently in the liver [58] and kidneys after reaching the blood stream via the thoracic duct. The share of the total HA degradation in lymph nodes presumably varies between the nodes but can be as high as 90% [45].

   Approximately 25% of the plasma HA is cleared every minute, but HA of higher molecular weight (>50 kDa) cannot be excreted by the kidney and therefore is exclusively cleared by the liver cells [2] via a molecular weight-dependent receptor uptake of HA, which prefers higher molecular masses.
   The relevance of the lymphatic route has been outlined by several studies, who investigated lymph flow in the intestine, lung [59, 60, 61] and skin [62].
   Moreover, the concentration of HA in the lymphatics is 0.2–50 mg L^{-1} and 10-100 times higher than that in plasma [63].

2. Local catabolism, a concerted interaction between various enzymes and receptors, is another contributor of HA turnover. Moreover, the local degradation occurs both inside the cell and in the ECM. HA in the ECM is cleaved and internalized by the hyaluronidase Hyal2, which is anchored on the outer membrane surface and hydrolyzes the hexosaminidic β (1–4) linkages between the *N*-acetyl-d-glucosamine and d-glucuronic acid. Inside the cell, further degradation is facilitated by Hyal1 and p-exoglycosidases, which can also cleave HA into smaller oligosaccharides.

   Next to the hyaluronidase group, recently published studies identified hyaluronidase activity for two cell surface proteins suggesting a much more complex degradation process of HA [64].

   The share of local catabolism on total HA turnover is not absolute clear due to conflicting results in some studies. While *Laurent et al.* suggest that local catabolism plays only a minor role in the elimination of HA, and estimate the share of HA turnover in skin between 12 to 25% [65], *Bell and Armstrong* suggest that only a pool of moveable HA (mainly LMW-HA) is free and reaches the lymphatics, while another pool of HA (mainly HMW-HA) is deeply integrated in the ECM and therefore not easily moveable, emphasizing the importance of local catabolism by HAases or free radicals [66]
3. Free radicals in the ECM constitute the last option to degrade HA by cleaving the molecule chain in smaller fragments in the presence of cations [67, 68]. It has also been suggested that there is some form of coordination between free radicals and HAases.

From the foregoing it can be concluded that HA underlies a rapid turnover in the ECM by HAases and free radicals and that after its degradation into HA of lower molecular weight it is likely to be free and moveable. The increased movability and a constant fluid flow towards the lymphatics suggests an influx of HA into the lymphatics where further degradation takes place until LMWHA reaches bloodstream.

1.3.2.4 Estimating lymph flow derived from plasma Hyaluronan concentration

In order to use Hyaluronan as a surrogate to estimate lymph flow three conditions must be fulfilled:

1. A sufficient amount of Hyaluronan must be moveable, reach the lymphatics and may not bypass the lymphatic route, because of direct uptake by blood capillaries.

   As discussed in *1.2.6*, furthermore, microvascular filtration is usually a constant flow of plasma towards the ISF and further into the lymphatics, while capillary uptake is rather unlikely. In addition, the pool of free and unbound HA in the interstitium was estimated at

25% [62], while only a small amount of the total HA pool (approx. 10%) is drained into the lymphatics [69]. Moreover, an animal study with tracer labeled HA showed that the molecule, once injected into the skin, is able to reach the blood stream after 45min under normal conditions [62].

Lastly, the lymphatic route for soft tissue HA has been proven with labelled HA, which was injected into the skin and showed lymphatic drainage [62, 65] .

2. Hyaluronan may neither be degraded completely in the lymphatics, nor extruded by them.

   The degradation of HA in lymph nodes is well known, but since efferent lymphatics contain the same amount of HA as the thoracic duct, it is evident that HA will not be degraded totally. Biosynthesis and extrusion of LMWHA in lymph nodes was reported by *Brown et al.*, but it has not been quantified yet and might therefore be negligibly low [16].

3. Other conditions for increased HA levels in blood serum must be ruled out.

   Technically, the level of HA in blood plasma is rather constant and ranges between 0.01-0.1µg/g, but it has been ascertained that some pathophysiological conditions, such as burn injuries, sepsis and cirrhosis, but also physiological processes, e.g. digestion and physical activity [70], can increase the level of HA in blood plasma. The mechanisms remain rather unclear, but it has been suggested that lymphatic flow changes may be responsible. Those conditions must be considered, since they can distort the results.

It is clear from the aforementioned that HA levels in plasma should rise after daily therapy, due to an increased lymph flow, while baseline levels and levels at the end of therapy should not differ significantly, because accumulation is rather unlikely, considering the fast metabolization of HA in blood.

In addition, a slight decline of plasma density after daily therapy is expected, deriving from a plasma dilution due to an increased lymph flow.

Furthermore, it has been reported that lymphedematous tissue contains higher amounts of HA than normal tissue [71]. Assuming that weight loss is a direct result of edema fluid loss, percentage changes of HA could correlate with percentage weight changes.

1.4 Lymphedema

Lymphedema (LE) describes the accumulation of a protein-rich fluid in the interstitium as well as the fat deposition and inflammation caused fibrosis which lead to a swelling of the affected part of the body. The swelling occurs in the subcutis and appears to be circumferential and symmetrical, while structures beneath the muscle fascia are not affected [72]. The condition is characterized by a chronic progression with recurrent skin infections and is currently not curable. Patients become increasingly impaired in their daily life resulting in a significantly diminished quality of life(QoL). LEs occur most commonly in limbs, but neck, face, genitals or torso also can be affected.

Although LEs are rather common, little is known about their prevalence among the population [73], which is why estimations vary from 140 to 250 million people worldwide [74]. In the developed world, a lymphedema is mainly the result of iatrogenic cancer treatment, while in the third world filariasis infections are the main cause, as one in three out of 120 million infected patients develops a lymphedema [75].

A questionnaire based study by *Neuhüttler* and *Brenner* revealed that the condition is much more common in women than in men (m : f = 1 : 4,6), though the number of participants was relatively low [76].

Földi describes the development of lymphedemas as a disequilibrium between lymphatic load and lymphatic transport capacity. On one hand, high lymphatic load and normal transport capacity describe a state of high output failure without lymphatic stasis caused by cardiogenic or nephrogenic failure as well as venous diseases or traumata. On the other hand, low output failure lymphedemas arise from an abnormal transport capacity but normal lymphatic load and lead to lymphatic stasis.

A reduced lymphatic transport capacity is usually caused by interrupted or constricted lymph vessels hampering the flow of interstitial fluid to the lymphatic ducts and induces a local fluid retention in the interstitium as well as tissue swelling.

A combination of high and low output failure, the increased lymphatic load and reduced transport capacity is referred to as combined insufficiency [74].

1.4.1 Classification

1.4.1.1 Primary Lymphedema

Primary lymphedemas are low output failure lymphedemas with congenital anomalies of lacking, extended or dysfunctional lymph vessels due to genetic disorders [74].

To date, the underlying molecular mechanisms in the development of the primary lymphedema are still poorly understood and therefore subject of intensive research. Primary LEs occur twice as often in women than in men and affect predominantly the lower extremities [77]. In contrast, pediatric

primary LE affects boys and girls equally, typically occurring postnatally in male babies, but mostly during adolescence in young women [78].

They may be classified either by their occurrence as hereditary or sporadic or alternatively by the age of onset: congenital L. (present at birth), L. praecox (occurs between birth and 35) and L. tardum (develops after the age of 35) [74]. Congenital lymphedema and L. praecox represent most primary lymphedemas with over 90% [79].

Recently, the age-of-onset classification has come under scrutiny after several authors have been criticizing it for over-simplifying and neglecting different phenotypes as well as the underlying genetic aberrations of the primary lymphedema and therefore Connell et al.'s algorithm for diagnosing primary lymphedema, which includes age of onset, affected sites and associated features, has been recommended [77, 80]. This algorithm categorizes primary lymphedemas in **syndromic** (e.g. Turner, Noonan, Prader-Willi) and **systemic conditions** (including two subcategories: multisegmental lymphatic dysplasia with systemic involvement (MLDSI) and generalized lymphatic dysplasia (GLD)), **disturbed growth** (e.g. Proteus Syndrom, WILD Syndrom), **congenital onset primary lymphedema** (e.g. Milroy Disease, Milroy-like lymphedema, MCLMR syndrome) and **late onset primary lymphedema** (e.g. Lymphoedema distichiasis syndrome, Meige disease, Emberger syndrome) [80].

1.4.1.2 Secondary Lymphedema

Secondary lymphedemas develop as low output failures after pathological processes like traumata, tumor growth, parasitic infections, iatrogenic interventions or recurring lymphangitides constrict or destroy the vessels. Complications of cancer treatment after breast cancer – referred to as the most common tumor affecting the lymph flow by removal or radiation of lymph nodes - represent the most frequent underlying cause for developing secondary lymphedemas in industrialized countries, whereas parasitic infections (e.g. filariasis) are the most frequent cause in the third world [81].

1.4.2 Risk Factors for Lymphedema

Several risk factors have been discovered over the last decades for developing lymphedema. Genetic mutations are one key player in developing lymphedema and amongst the best researched are genetic mutations encoding proteins related to the VEGFR3 pathway occurring in 36% of inherited lymphedema, but only in 8% of the sporadic forms [82].

Moreover, genetic mutations may also contribute to the development of secondary lymphedemas. It has been demonstrated that HGF/MET gene mutations lead to an altered susceptibility for developing lymphedemas [83]. Furthermore, preliminary findings have revealed that single nucleotide

polymorphisms (SNPs) in Potassium Channel genes are associated with developing lymphedema after breast cancer surgery [84].

In several studies, obesity has turned out to decrease lymphatic function, which may be due to the reduced clearance of macromolecules from the skin [85]. *Földi* affiliates the exacerbation of a preexisting lymphedema under obesity to the higher thoracic pressure hampering lymph flow in the thoracic duct [74]. Exceeding a BMI threshold of over 54 [86], obesity itself can induce secondary lymphedema, which may cause irreversible lymphatic dysfunction [87]. However, the beneficial effect of dietary weight reduction on lymphedema-related volume excess remains controversial [88].

Iatrogenic interventions affecting the lymphatics such as axillary sampling and to a minor extent sentinel node biopsy [89], are further risk factors of the secondary lymphedema. Radiotherapy alone contributes little to the development of LEs [77], but combined with surgery it significantly increases the incidence [90].

If the capacity of the lymphatics is subnormal due to malformed or damaged vessels, infections will increase the lymphatic load and thus can predispose the development of lymphedema or worsen it [74].

1.4.3 Stages

The most commonly used staging system of lymphedemas was established by Brunner, originally comprising the stages I - III, a preceding stage of latency has been added later.

Stage 0 or Ia, latency stage:

At this stage, lymphedemas are clinically inapparent despite a subnormal transport capacity of the lymphatic vasculature. It has been demonstrated, that acute lymphedemas after injury of the lymphatics may regress spontaneously after a few days and may stay inapparent for months or years before the edema recurs [74].

Stage I:

At this stage, the edema is still reversible and has a characteristic soft, dough-like consistency. By depressing the swollen area with a finger, an indentation remains for a while, which is referred to as pitting edema. *Földi* describes the swelling as an accumulation of a protein-rich fluid in the interstitium with few fibrotic changes of the tissue. Furthermore, the swelling subsides completely or partially if the limb is elevated.

Stage II:

The condition has become spontaneously irreversible and elevating does not reduce the swelling. Spontaneously means that without therapy, elevating the affected limb does not induce reducing of the swelling. The tissue is hard and pressure does not leave an indentation. In addition, the tissue has

undergone a strong fibrosclerotic transformation.

Skin infections are common, because of local immune deficiency [74, 91].

Stage III:

This stage encompasses the lymphostatic elephantiasis, which represents the terminal stage of lymphostasis. It evolves facultatively, usually after exacerbating erysipelas [74]. The swelling of the affected limb has reached an enormous size resulting in a complete deformation; while the appearance of the cutis can be normal or exhibit trophic skin changes, such as fissures, acanthosis, fat deposition, fibrosis and warty overgrowths [91]. A pitting sign is absent or slightly pronounced, where fat tissue dominates the lymphedema [92].

Besides this staging system, other staging systems exist of whom some classify the edema based on circumference measurements or volume changes (e.g. by using water displacement).

1.4.4 Clinical presentation

The assessment of lymphedema starts with a precise anamnesis and physical examination considering palpation and inspection. The anamnesis must include inquiry of the patient's travels to areas endemic for filariasis, any axillary or inguinal injury, any cancer treatments, any episodes of cellulitis, any familial cases of LE as well as consideration of the edema's progression [93]. Moreover, prior to diagnosing primary lymphedema, especially the late-onset phenotypes, other causes like cancer must be ruled out.

In early stages, diagnosing lymphedema can be difficult due to non-detectable changes in volume or circumference.

Furthermore, the lymphedema must be distinguished from other "local" diseases like chronic venous stasis or lipedema and global conditions like congestive heart failure, renal failure, hypalbuminemia and drug-induced edemas.

Schook et al. revealed, that in pediatric population other conditions, such as other vascular anomalies and non-vascular diseases e.g. hemi-hypertrophy, posttraumatic swellings, lipedemas or rheumatologic diseases are mistaken for lymphedema in 27% of all cases [94].

During the physical examination, the anatomical location of the edema plays a key role. Lymphedema usually involves the distal extremity, is the hand or food spared out, lymphedema is less likely [93]. Moreover, 99% of all lymphedemas are localized to an extremity, while the other localizations are rare [93].

Pitting edemas occur usually in early stages and may be absent in late stages due to fat deposition in the subcutis.

The probably most important clinical sign in diagnosing lymphedema is the Stemmer sign, it is

positive if pinching and lifting a skinfold at the base of the second toe/middle finger is not possible. Remarkably, it is more sensitive than specific; if the sign is positive it is likely that the patient has lymphedema [93].
Skin appearance is another aspect of the examination. Although a primary lymphedema typically presents itself with normal skin, changes such as hyperkeratosis, bleeding from vesicles and lymphorrhea can occur in 15% of the patients [78]. Scars in the axillary or inguinal region can be a hint for damages of the lymphatics.

Although LEs are considered to be non-painful, musculoskeletal discomfort can be distressing due to the enlargement of the extremity [93]. Other symptoms correlating with lymphedema are fatigue, paresthesia, mobility disturbances and sensations of heaviness of the affected limb [95]. In particular, sensations of heaviness over the last year and a current swelling have a positive predictive value for the presence of a lymphedema [96].

1.4.5 Differential Diagnosis

Swelling of a limb may have many causes which must be considered as a differential diagnosis for lymphedema. To discuss all causes would go beyond the scope of this work and therefore all possible differential diagnosis will only be listed for the sake of completeness (see *table 1*).

Vascular anomalies	Genetic diseases (sporadic/in-hereted)	Tumor	Miscellaneous
Capillary Malformation (causing circumferential and longitudinal overgrowth)	CLOVES Syndrom	Lipofibromatosis	Hemihypertrophy
Infantile Hemangioma	Klippel-Trénaunay Syndrome		Lipedema
Kaposiform Hemangioendo-thelioma	Parker Weber Syndrom		Obesity
Lymphatic Malformation			Systemic Diseases
Non-eponymous combined Vascular Malformation			Trauma
Venous Malformation			Venous Insufficiency

Table 1: Causes of limb swelling

However, lipedema is one of the more frequent differential diagnosis with some similarities to lymphedema that has not yet been properly researched and will be discussed briefly as follows:
Lipedema is a condition characterized by the enlargement of both legs because of fat accumulation in the subcutis. The causes are still unknown, but the occurrence of lipedema, especially in women at

some point during puberty, indicates a genetic susceptibility in combination with hormonal shifts of estrogen and progesterone as triggers.
Another characteristic of this condition is the disturbance of fat deposition resulting in a weight gain in lipedematous areas and weight loss in non-lipedematous areas.
The clinical presentation encompasses symmetrical, disproportionately large and column-like legs or arms and possible affection of the hips and buttocks, while sparing feet or hands, allowing the discrimination from lymphedema with its asymmetrical enlargement of an extremity and affected arm/foot. In addition, the sign of Stemmer is always negative.

1.4.6 Lymphedema Assessment

1.4.6.1 Bioelectrical Impedance Techniques

The principles of bioelectrical impedance techniques are differences in the electrical conducting of body compartments. Applying an alternating current with 0,8 mA at typically 50 kHz results in a voltage drop and maximal phase shift, which are measurable. The resistance of the alternating current, impedance (Z), comprises two partial oppositions: reactance (Xc) and resistance (R): Reactance describes the capacitance of cell membranes, whereas resistance is the electrical opposition of tissue fluid [97].
From the group of bioelectrical impedance techniques, bioimpedance spectroscopy (BIS) is recommended in assessing lymphedema [97]. BIS facilitates measurements at low frequencies (5kHz), where the capacitive resistance of cell membranes functions as a barrier for the current flow, therefore it can only take the route through the extracellular fluids [97].
It has been shown, that BIS is a reliable method to identify lymphedema and monitoring the efficacy of the therapy [98].

1.4.6.2 Tissue dielectric constant measurement

Tissue dielectric constant (TDC) measurements radiate a 300 Mhz microwave via a coaxial probe into the tissue of interest and measure the reflections to calculate a reflection coefficient, which allows to estimate specific tissue properties [99].
The reflection coefficient is further needed to calculate the dielectric constant (TDC), which can be used to estimate the tissue water percentage. It has been shown, that in lymphedematous arms TDC values are significantly higher than in non-affected arms, but also that at-risk arms with pre-clinical lymphedema could potentially identified by their TDC value [100].

1.4.6.3 Imaging modalities

The common conventional imaging modalities used in clinical practice comprise magnet resonance

imaging (MRI), computer tomography (CT) and ultrasonography.

Although lymphedema is a clinical diagnosis, imaging modalities are useful to confirm the diagnosis and further give an insight into the anatomical and functional status of the lymphatics to identify the underlying etiology. MRI is the preferred modality with fat-sensitive T1 sequence and fluid-sensitive sequences or fat-suppressed T2-weighted sequences being most helpful in the visualizing of lymphedema [101]. Additionally, Gadolinium-enhanced T1 sequences are necessary in diagnosing secondary lymphedema or inflammatory processes in primary lymphedemas [101].

MRI findings correlating with primary lymphedema are circumferential distribution of edema within the epifascial compartment with reticular honeycomb pattern, as well as circumferential thickening of the subcutaneous soft tissue, while in chronic processes MR images further feature subcutaneous accumulations of fluid and fat [101].

Beside conventional MRI, magnet resonance lymphography (MRL), similar to MR angiography, is a rather new technique providing a better spatial and temporal resolution than lymphoscintigraphy with slightly worse specificity in detecting lymph node changes, but better sensitivity for abnormal lymph vessels [102].

A water-soluble MR contrast agent combined with Lidocaine 1% is injected into the subcutis of the dorsal aspect of each foot or hand in the region of the four interdigital web spaces [103].

The examination includes s heavily T_2-weighted 3D sequence to define the severity and extent of the edema and a high-resolution dynamic 3D gradient echo imaging after contrast agent injection, which is repeated every 5 minutes for several times to visualize the lymphatic vessels [104].

Findings in primary Lymphedema include three types: (1) **only nodes** are affected; (2) **only vessels** are affected with hypoplasia, aplasia or hyperplasia [lacking or hypoplastic in number and diameter vs. varicose-like vessels]; (3**) both lymph vessels and nodes** are affected consistent with dilated, enhanced and radiating vessels as well as abnormal lymph flow pathways within lymph nodes [105, 106].

In secondary lymphedema MRL demonstrates tortuous and dilated collecting lymphatics in lymphedematous limbs, whereas lymph nodes are shown to be altered due to the underlying cause e.g. postoperatively, nodes are smaller, decreased and irregular with poor enhancement [105].

The advantages of MRL due to its anatomical fidelity with better depiction of lymph vessels than the lymphoscintigraphy as gold-standard could contribute to plan microsurgical operations (e.g. lymphaticovenous anastomosis) or to surveil conservative treatments.

CT can be useful to assess lymphedema in patients where MRI is not feasible due to technical or safety issues, because of its rapidity and less necessary compliance [101].

Marotel et al. found in a CT study, evaluating 150 cases of primary and secondary lymphedema skin

thickening, increased subcutaneous tissue surface, thickening of the perimuscular aponeurosis and different types of fat infiltration [107].
Finally, CT can be used to surveil therapeutic response to compression therapy [108].

US is another modality providing a noninvasive tool to assess lymphedema. It can reveal thickening of the skin and interstitial fluid accumulation and furtherly the degree of fibrosis [101]. It also has been showed, that the amount of interstitial fluid accumulation and the presence of fibrosis seen in US correlates with the manifestation of the edema: few fluid accumulation and tissue fibrosis present with no pitting, while intense fluid accumulation with absent or rare signs of fibrosclerosis present with soft edema [109].

1.4.7 Lymphedema Treatment

There is a broad consensus that lymphedema treatment is always mandatory to prevent progression of this chronical disease. Early treatment often results in rapid clinical improvement and may prevent further progression to the chronic phase of the disease [110].
The treatment comprises operative and non-operative management options.
According to the AWMF guidelines, the complex decongestive therapy (CDT) represents the gold-standard in non-operative methods of lymphedema treatment and should be considered first before choosing an operative procedure in adult patients.
However, taking into account the LE stage no clear recommendations do exist neither when to begin an operative therapy nor the preferential operation technique.

1.4.7.1 Non-operative Management

Basically, complex decongestive therapy includes a reductive phase and a maintenance phase [111] and comprises several components, which will be described briefly as follows:

Manual lymph drainage (ML)
The purpose of ML is a stretching of the walls of lymphatic vessels by four distinct hand maneuvers stimulating LMCs and resulting in an increased pulsation of the lymphangion with a higher uptake of interstitial fluid in the lymph collectors and consecutively a reduction of tissue fluid.

Compression therapy
The principle of compression therapy is that an applied pressure by garments or bandages leads to an increased interstitial pressure and therefore reduced capillary filtration in the affected limb.

Bandaging can also reduce fibrosis in the skin [112]. The use of compression is the basic therapy throughout the lymphedema population. Bandages are good for initial controlling the LE, while garments are used in long-term treatment. Major key elements for the success of this therapy are individualization and compliance. Garments must be replaced regularly depending on the patient's activities, they also must be good fitted to the body by standardized measurements and compression must be adapted continuously [113].

Pressure dosage by the garments is categorized in 4 classes according to the European Standardization Committee [114]:

	RAL-GZG	Compression at the ankle[1]
CCL I	Mild	18-21 mmHg
CCL II	moderate	23-32 mmHg
CCL III	strong	34-46 mmHg
CCL IV	very strong	49- mmHg

Table 2. [1] The values indicate the compression exerted by the hosiery at a hypothetical cylindrical ankle.

Moreover, compression therapy alone leads to a stepwise reduction of excess volume with the most significant changes within 3 months alone [115], while a combination with other operative and non-operative treatments may intensify the effect [115, 116].

Skin care

Compression garments stress the skin immensely and therefore skin care is mandatory. Moreover, skin damages, e.g. injuries or sun burns increase lymphatic load and must be avoided.

Meticulous personal hygiene with pH-adjusted soaps and a skin care regimen based on the usage of cremes with an increased part of fat and antibacterial agents are recommended.

Exercises

Specific exercises are beneficial for LE patients [117]. They activate the muscle pump and its muscle contractions result in an increased interstitial pressure facilitating lymph flow. It is recommended, that compression garments should be worn during activity [117].

Patient information and training

CDT is always time-consuming and onerous for the patient, because the disease is not curable and therapy should be adhered lifelong. Thus, the patient should be motivated to stick to the therapy.

Other non-invasive therapies

Recent non-invasive methods are advanced pneumatic compression (APC) and Low-Level Laser Therapy (LLLT).

APC therapy can play a supportive role in CDT either in early or late phases [117] by mimicking the pump effect of muscular contraction on the lymphatic system [118].
LLLT is also a supportive therapy and usually combined with CDT. It can support the formation of new lymph vessels, improves the lymphatic motricity and prevents fibrosis [119].

1.4.7.2 Operative Management

The operative management includes reductive techniques, such as liposuction and various kinds of direct excision methods with the Charles procedure as the extreme, and more physiologic techniques, such as lymphatic venous anastomosis, lymphatico-lymphatic by-pass, and lymph node transfer.
Despite recent improvements in technology to assist in identifying lymphatic vessels, an operative management should be the second choice and considered only if the patient's distress lasts or skin changes secondary to LE rise although the successful establishment of a recommended treatment regimen with a good patient adherence for at least 6 months [120].

Reductive techniques

Reductive techniques are effective in volume reduction, but do not fix the decreased lymph flow. Liposuction directly removes adipose and fibrotic tissue, while direct excisions have a more aggressive approach with the resection of subcutaneous tissue and sometimes skin allowing primary closure. As a very first technique of these, the Charles procedure, published in 1912, represents the most disfiguring and most aggressive one and is nowadays only considered in high stages of LE.
Moreover, direct excision techniques can require skin grafting and have an elevated risk for lengthy wound healing and blood transfusion, while liposuction has almost no complications [121].

Physiologic techniques

Lymphatic Venous Anastomosis (LVA) is a microsurgery technique used at all LE stages, where lymphatic vessels are anastomosed with nearby veins. A variation of this technique is the interposition of an autologous vein graft between two lymphatic vessels (lymphatic-venous-lymphatic, LVL anastomoses), where a direct shunt between lymphatic and venous system is not feasible due to coexisting venous diseases [122]. Intraoperatively, fluorescence aides the identification of lymphatic vessels and an operating microscope is used to assist in anastomosing the vessels [123].
Carl et. al revealed in a systematic review, that LVA is a rather safe and effective technique with a good volume reduction and improved QoL [121].

Vascularized Lymph Node Transfer (VLNT) is another surgical option in lymphedema therapy. In this procedure one or more lymph nodes from a non-edematous region of the body are transferred to

the affected limb. The harvested nodes can be accompanied by a dermal flap or solely implanted at the recipient site [124]. In order to avoid necrosis and gain proper functionality, the implant also needs a vascularization in form of arterial and venous anastomoses.

It has been shown that VLNT leads to an increased lymph flow accompanied by volume reduction and elevated QoL [121], although its exact mechanisms are poorly understood.

It has been hypothesized that the transferred node simply acts as a pump which drains tissue fluid adjacent to the flap into the venous anastomoses, while the arterial influx provides a strong hydrostatic pressure [125].

Despite the method's good outcome several complications can occur. For instance, harvesting lymph nodes can cause LE at the donor site, which is - along with cellulitis, lymphocele, donor site pain and seroma - the most common complication [121].

2. Aims and Objectives

The rate of lymph flow is important for the maintenance of the blood volume. However, its direct measurement by cannulation of lymph vessels and indirect measurement by tracer appearance rate are demanding and results of the latter method are not always accurate.

The macromolecule and extracellular space filler hyaluronan is part of the regulation of tissue hydration and maintenance of water and protein homeostasis due to its distinct properties. In addition, it has been shown, that the concentration of the HA in lymph is several times higher than in plasma, suggesting that the lymphatic route may account for the majority of HA found in plasma [2].

Hypothesizing that HA is a surrogate for lymph flow, the aim of this diploma thesis is to investigate, how HA plasma concentrations change in patients with lymphedema who undergo a three-week treatment of complex decongestive therapy.

As laid out in the introduction it seems very likely that a rise of HA level in blood after daily therapy is caused by an increased lymph flow. Thus, measuring HA concentration in plasma could be a simple and useful lab test and might be beneficial in assessing and monitoring lymphedema therapies.

Finally, these findings could redound to a better understanding of the lymph flow and its contributions to maintain the blood volume and blood pressure but may also give a deeper insight in the pathophysiological processes accounting for the development of lymphedema, which are still unknown in detail.

In order to achieve this aim, blood samples will be collected before and after daily therapy and plasma HA concentrations will be measured.

Percentage changes of HA concentrations will be compared with percentage plasma density and weight changes to find correlations.

3. Methods

Eight subjects (age 46 – 70, Ø 59.5 years, five females, three males) with primary or secondary leg lymphedema, stage II or higher, recruited at the "Lymphklinik" department of the LKH Wolfsberg, have been randomly selected for blood sampling. All subjects were part of the "LYMPH" study conducted by the department for physiology, LKH Graz, which examined the effects of CDT on various parameters. All subjects underwent a treatment of CDT for three weeks. Blood samples were taken before and after daily manual therapy at the first, second, seventh, 15. and 20. day of the treatment. Exclusion criteria were: mental disorders, inflammatory diseases, heart and kidney diseases, pregnancy, history of syncope and alcoholism.

All participants were informed about the purpose and were familiarized with the protocol and procedures of the study before agreeing to participate.

The study complies with the principles of the declaration of Helsinki and approval was given by the ethics commission of the Medical University of Graz (*see Appendix A*).

Therapy Protocol

All participants underwent a three-week treatment of CDT with manual lymph drainages every day for 30 minutes except on weekends. Compression bandages were then applied by lymph therapists every day and the bandages were worn the whole day and overnight. Additionally, the patients took part in exercise therapy. Therapy standards that were used in this study are according to Döller. These are briefly summarized as follows:

Mobilizing and eliminating edema fluid by special hand movements is the main intention of ML.

Lymph therapists perform four hand maneuvers, which encompass circulating, rhythmic, pumping and scrubbing motions with relatively low pressure (30-40 mmHg). In addition, maneuvers are performed slowly to adapt to the spontaneous frequency of the lymphangion (10/min).

The stretching of the skin and the subcutis, which starts in healthy areas next to the LE, is followed by increased lympho-angiomotoric activity of the local lymph vessels leading to increased lymph formation and lymph flow. Subsequently, the treatment is extended to the blocked trunk quadrants (ventral and dorsal) and furtherly to the affected extremity (proximal before distal).

In addition to ML, patients participated in physical exercises with applied compression bandages. The exercises were at the same day, but usually not directly after the ML. It is supposed that physical exercises improve the function of the muscle and joint pump as well as the breathing mechanics, which all together contribute to the increase of the lymph venous outflow. Moreover, sport has beneficial effects on health and psyche.

Test protocol

The test protocol was conducted before and after CDT and encompassed next to blood sampling scaling, measuring of the LE by tape, bio impedance measurements, retina scans and a sit-to-stand test for 15 minutes. Flow-mediated vasodilation and carotid intima thickness measurements were conducted additionally after CDT.

Blood Sampling

Venous blood was collected before patients started the complex decongestive therapy and after completing the therapy. 20 mL were taken from each patient at both collecting time points. Blood samples were centrifuged at 1500 G for 15 minutes at °6 C. Plasma was collected and stored in aliquots at -70°C until being used for further measurements.

Laboratory methods

HA was measured with an ultrasensitive time-resolved fluorescence immunoassay using the streptavidin–biotin system [126]. Hematocrit was measured at the laboratory of the LKH Wolfsberg with standard laboratory equipment. Plasma density measurements were conducted with the density meter DMA 58 (Anton Paar GmbH, Graz, Austria).

Calculations

Changes in plasma volume (ΔPV) were expected due to physical activity and perspiration during CDT and therefore were calculated using van Beaumont's formula [127] as follows:

$$\Delta PV(\%) = \frac{100}{100 - HCT_{pre}} \times \frac{100 \times (HCT_{pre} - HCT_{post})}{HCT_{post}}$$

where HCT is in %.

Hyaluronic acid concentrations after CDT were corrected due to plasma volume shifts as follows:

$$[HA]_c = [HA]_u \times (1 + \frac{\Delta PV(\%)}{100})$$

where c and u sub-indices indicate corrected and uncorrected concentration, respectively.

Statistical analysis

All data variables were analyzed for normality by Shapiro-Wilk Test and log-transformed to achieve normality where necessary. Likewise, the difference between log-transformed before and after HA values had been calculated and examined for normal distribution.

A paired T-test has been conducted to assess differences in the mean values of all HA values before and after CDT.

In addition, a paired Wilcoxon signed-rank test was used to compare non-transformed HA data.

In order to explore the overall effect of therapy on HA turnover, the first HA level at day one and the first level at day five have been compared by a paired sample sign test and a paired Wilcoxon signed-rank test, respectively.

Pearson's correlation coefficients were calculated to examine the strength of the effect on association. Furthermore, to find concordances, ANOVA and regression analyses were conducted between percentage changes of HA concentration and weight (between test days) and percentage changes of HA concentration and plasma density (before and after daily CDT), respectively.

The statistical analyses were performed using SPSS, version 23 (IBM Corp., Armonk, New York, USA). The results were considered statistically significant at $P < 0.05$. Data were expressed as mean $\pm$ one standard deviation.

4. Results

4.1 Weight characteristics

Characteristics and diagnosis of the subjects are shown in table 3. All subjects were diagnosed with LE stage II. One subject (*no. 2*) disembarked on the study after the second test day, apart from that all other participants completed the entire test routine for all 5 days. Weight measurements for subject *no. 1* started at the end of the second day due to an absent scale.

ID	Sex	Diagnosis	Age	Height	Weight at the beginning (Kg)	Weight at the end (Kg)	Total weight change (Kg)	av. weight change between measurements (Kg)
1	m	LE, both legs	63	180	117,1(day 2)	114,4	-2,7	-0,45
2	w	LE, left leg	63	167	63,3	63,9(day 2)	0,6	0,20
3	w	LE, both legs	53	178	116,8	113,8	-3	-0,33
4	w	LE, left leg, questionable lipedema right leg	46	158	62,9	61,3	-1,6	-0,18
5	m	LE, right leg	56	180	90,2	86	-4,2	-0,47
6	w	primary LE, both legs	70	159	102,7	101,2	-1,5	-0,17
7	m	LE, both legs	59	176	137,1	133,5	-3,6	-0,40
8	w	LE, left leg	66	168	59,9	59,1	-0,8	-0,09

Table 3: Subject characteristics

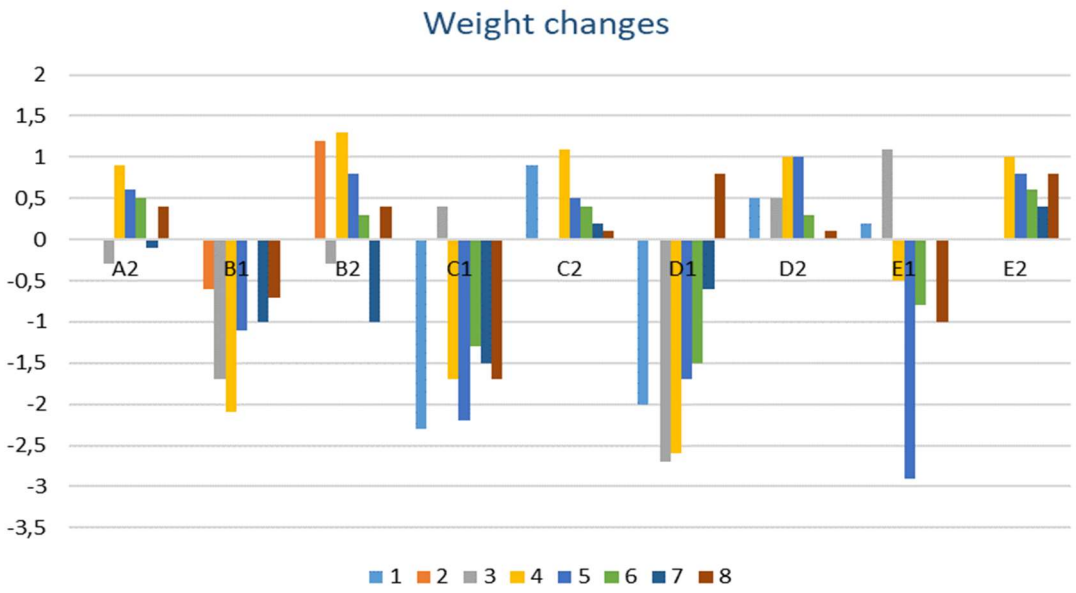

Figure 6: Weight changes (in kilogram) of all eight subjects between days and measurements. A-E correspond to the test days 1 - 5. '1' indicates the weight change between the last measurement of the previous day and the first measurement of the current day. '2' denotes weight changes during CDT of the same day.

A more detailed focus on the weight changes is given in figure 6. Weight losses occur usually between test days, while minor weight gains are recognizable at the measurements after CDT, most likely due to water and food consumption during breakfast. Moreover, weight loss appears to be strongest between the second and 7th (Ø 1,5 KG ± 0,8 KG) and 7th and 15th (Ø 1,5 KG ± 1,1 KG) test day. In general, all subjects experienced a weight loss over the course of the entire three weeks treatment,

except for *no. 2*.

The average weight loss between the first and the last test day was 2,1 KG ± 1,5 KG.

4.1 Blood characteristics

Hematocrit (HCT) level was an average 39% ± 2,4 for male and 44,7% ±2,1 for women, respectively. Maximum and minimum values for HCT were 49% (*no.1* at day 4) and 37% (*no. 3* at day 2 and 4, *no. 4* at day 5), therefore all subjects had normal HCT values regarding their sex.

Plasma density values of all participants were within the normal range. Figure 7 shows the average plasma density before and after CDT for each day.

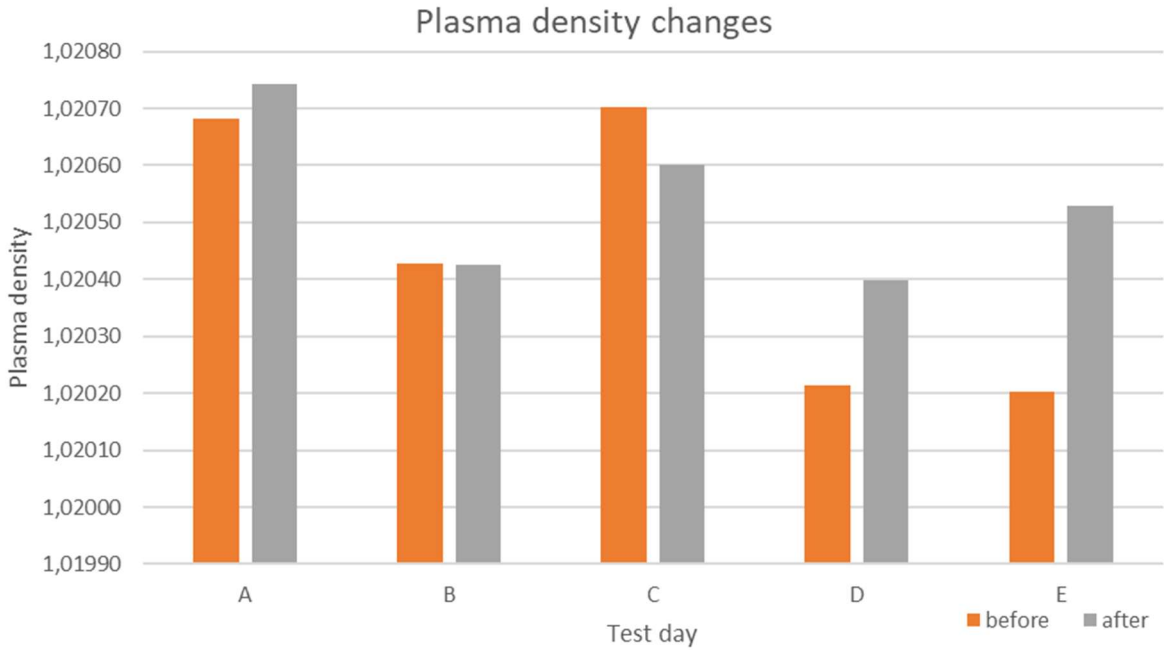

Figure 7: Average plasma density in g/cm^3 for each day. Capital letters A-E denote the test days 1 – 5.

Plasma volume changes (calculated in accordance with Van Beaumont's formula after CDT) were within the range of 13,3% (*no. 2* at day 1) and -7,8% (*no. 6* and *7* at day 1, *no 7* at day 5). Altogether, plasma volume slightly increased after CDT on average with a high standard deviation (*see table 4*).

Test Day	av. Plasma volume change (%)
1	1,9 ± 7,2
2	3,3 ± 4,6
3	3,7 ± 5,1
4	3,0 ± 3,0
5	3,2 ± 5,2

Table 4: Plasma volume changes according to Van Beaumont's formula

4.2 Hyaluronic acid characteristics

Detailed information about the measured HA concentrations for each subject are given in figure 11. HA concentrations have been found to vary widely among subjects. Altogether, *no. 1* had the highest HA concentration on average ranging between 83 ng/ml (day 2) and 17 ng/ml (day 5) while decreasing inconstantly over the time. Several other subjects (*no. 3,4,7* and *8*) had rather low thus normal HA concentrations in blood for the entire therapy. No 6 had the highest measured HA level of the entire group with 88 ng/ml (at day 4). Mean values of HA (normal and log-transformed) for each day are given in table 5. Boxplots of HA values are given in figure 8.

Test day	preCDT	postCDT	preCDT (log-transformed)	postCDT (log-transformed)
1	28,38 ± 28,53	20,24 ± 18,89	1,22 ± 0,44	1,15 ± 0,35
2	20,28 ± 14,3	20,98 ± 24,67	1,19 ± 0,33	1,12 ± 0,39
3	21,19 ± 19,13	20,72 ± 24,32	1,17 ± 0,36	1,06 ± 0,46
4	29,39 ± 33,37	14,53 ± 14,01	1,19 ± 0,47	0,95 ± 0,43
5	16,78 ± 16.01	13,90 ± 10,05	1,05 ± 0,38	1,05 ± 0,28

Table 5: Mean values of HA (normal and log-transformed) for each day before and after CDT. Normal HA values in ng/ml.

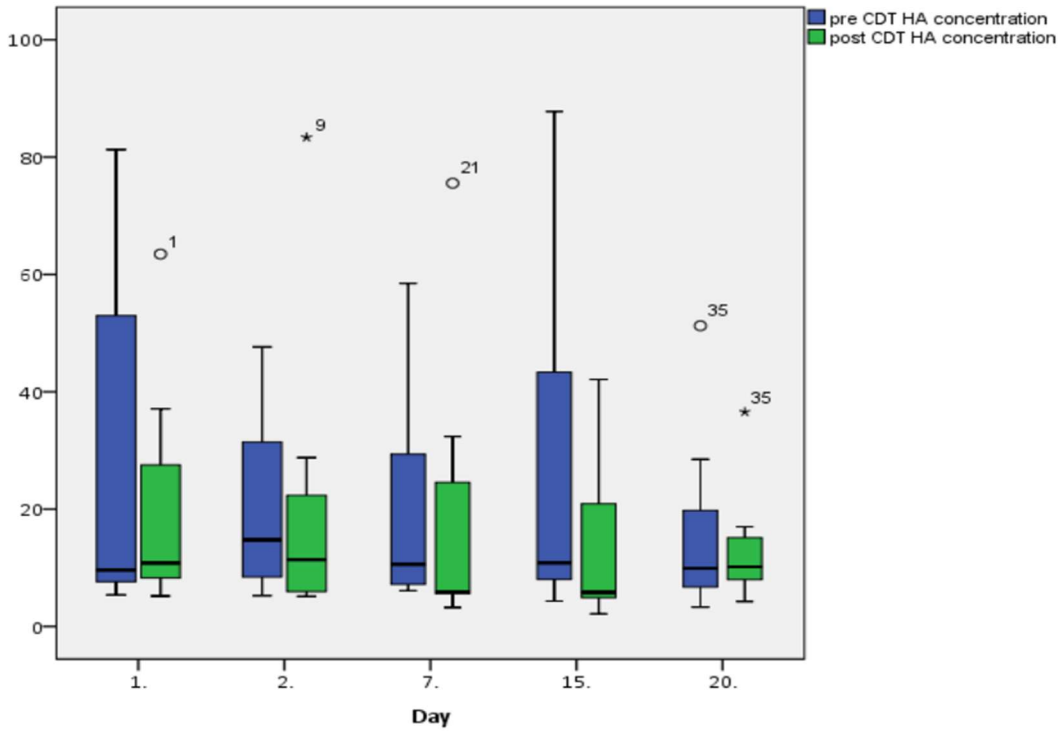

Figure 8: Boxplots of HA values before and after daily CDT for all test days clarifies the huge distribution of measured HA levels. Post-CDT Levels are slightly lower than pre-CDT levels.

The chronological sequence of HA concentrations for each subject is displayed in figures 9 and 10. Figure 9 shows the HA concentrations for the subjects with general 'low level' HA concentrations. HA concentrations of subjects *no. 3* and *7* increased after CDT and decreased between test days, while subjects *no. 4* and *8* had a decrease of HA after CDT and an increase between test days. In general, no one had a consistent trend downwards or upwards. Moreover, *no. 3* and *7* deviated from their typical pattern for at least one day (*n*o. *3*, day C, *no. 7*, days C, D).

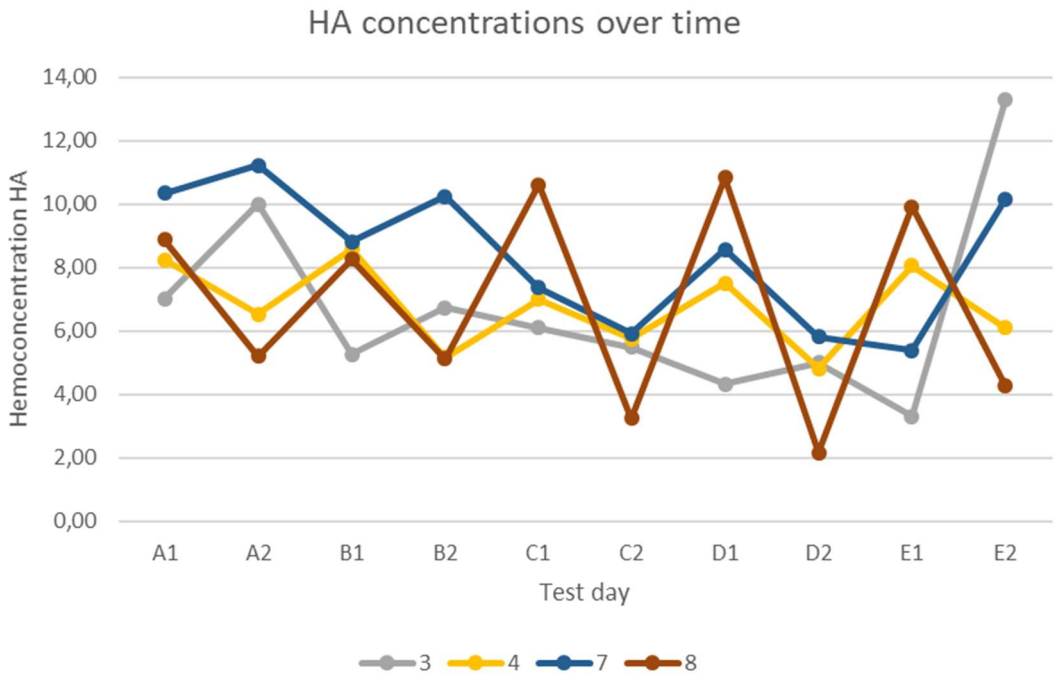

Figure 9: Chronological sequences for subjects *no. 3,4,7* and *8*. Capital letters A-E denote the days 1-5. Numbers 1 and 2 indicate before and after daily CDT, respectively. HA concentration is given in ng/ml.

Figure 10 shows the chronological sequences of subjects with general 'high level' HA concentrations, except for subject *no. 5*, which is included due to a rise of HA concentration from the first to the second day to over 15 ng/ml. All other subjects in this figure show a decrease of HA concentration by the end of the treatment compared to the start, but this decrease is never continuous. Contrary to the 'low level HA' subjects a typical pattern is not apparent.

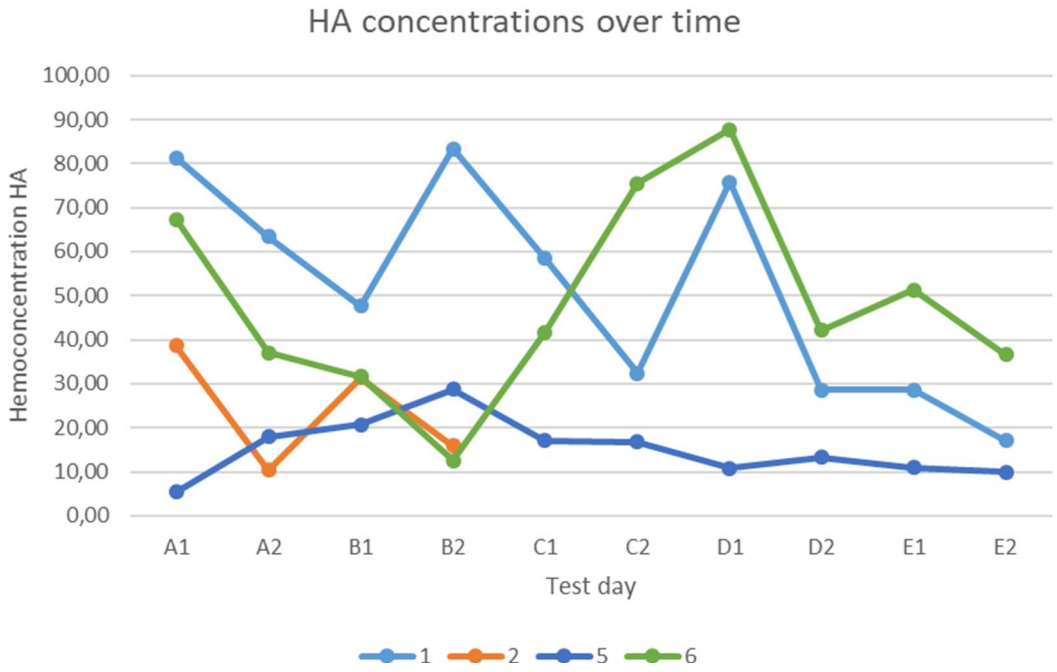

Figure 10: Chronological sequences for subjects *no. 1,2,5* and 6. HA concentration is given in ng/ml.

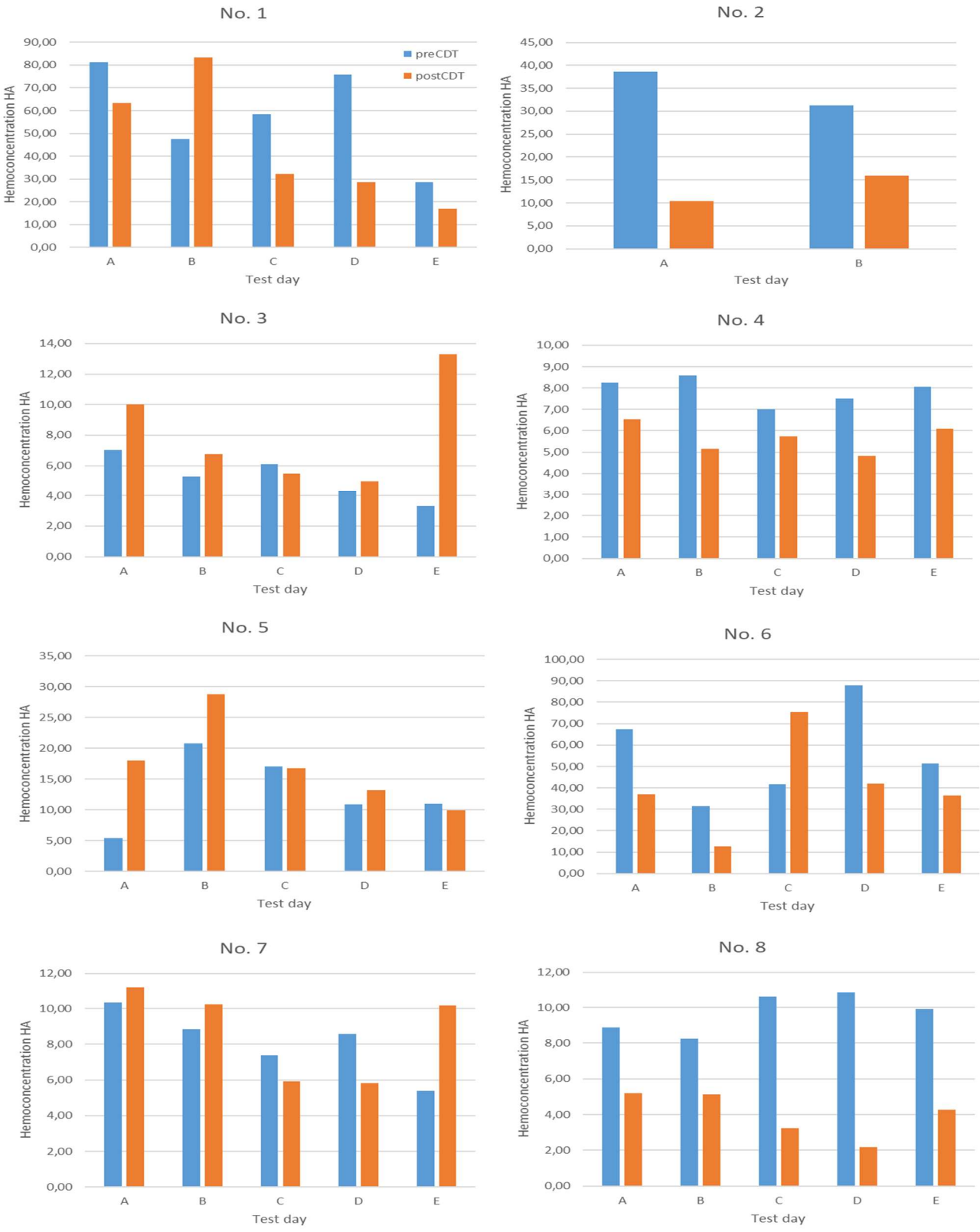

Figure 11: Shows the HA concentrations in ng/ml before and after CDT for each subject and day. postCDT HA concentrations have been corrected to plasma volume. Subjects no. 1,2,4,6 and 8 had usually higher HA levels in blood before CDT, while subjects no. 3, 5 and 7 had a lower amount of HA in blood before CDT. Furthermore, HA concentration have been found to vary widely among the subjects.

In order to explore effects on HA levels during CDT, a paired t-test with 'before' and 'after CDT' HA concentrations was conducted showing a moderate effect ($r = 0.33$, $P < 0.04$).
An additionally conducted paired Wilcoxon signed-rank test showed a similar result ($r = 0{,}35$, $P < 0.033$) with a high moderate effect.
Altogether, HA levels in plasma were frequently lower after CDT than before.

Paired Wilcoxon signed-rank test and paired sample sign test were conducted to explore differences between baseline values at the beginning of the treatment (first measurement /test day one) and at the end (first measurement / test day five) showing no significant effect of the entire three-weeks treatment on HA levels (paired sample sign test: $P < 0.453$; Wilcoxon signed-rank test: $P < 0.237$). Due to scant data normality was not achievable, thus a paired t-test could not be conducted.

Correlations between HA and weight changes between test days and HA and changes in plasma density before and after CDT, respectively, were investigated by ANOVA analysis. Results for HA/plasma density ($P < 0.207$) and HA/weight ($P < 0.713$) showed no significant correlations. Results were plotted in figures 12 and 13.

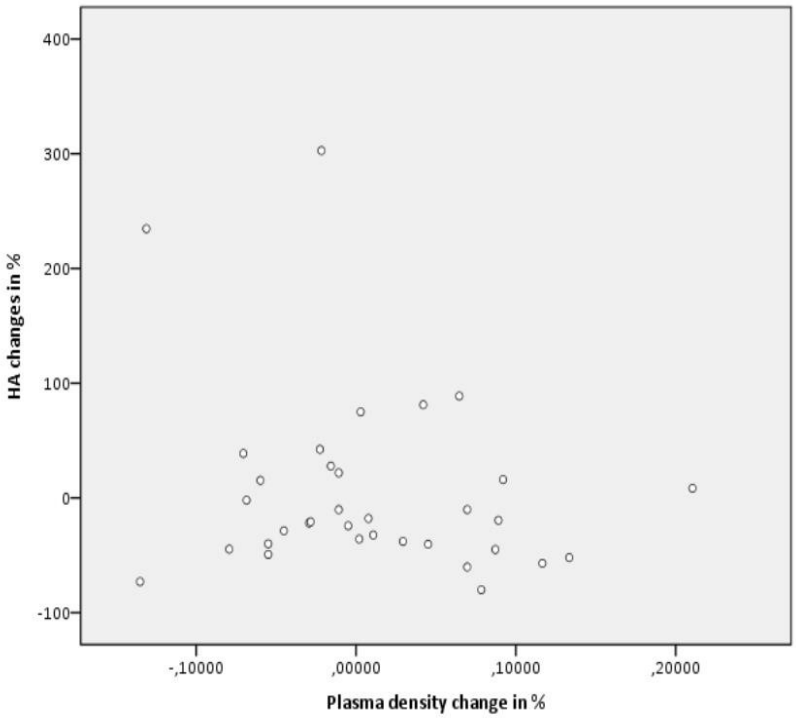

Figure 12: shows no correlation between percentage changes of HA and plasma density before and after daily CDT.

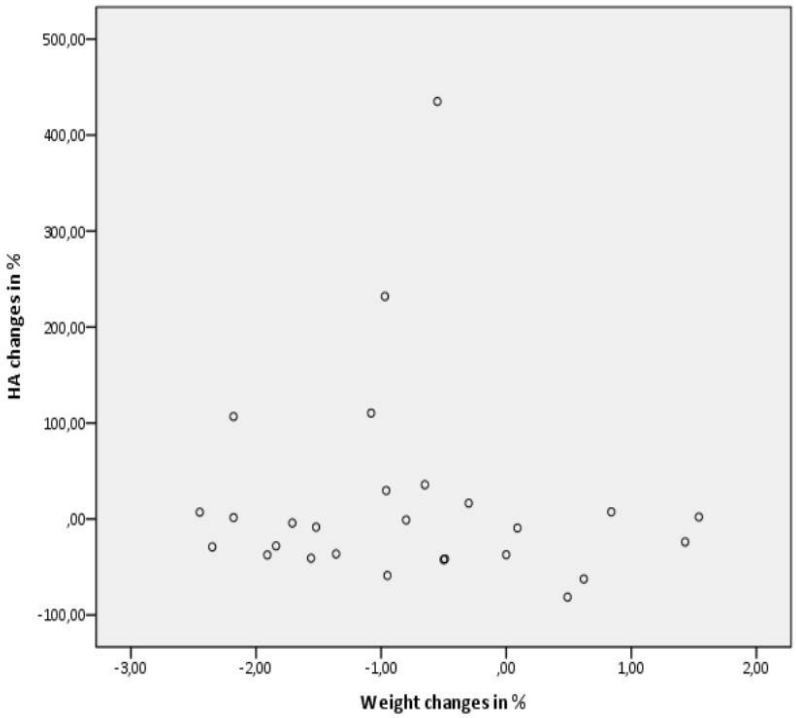

Figure 13: shows no correlation between percentage changes of HA and weight between test days.

5. Discussion

It appears that complex decongestive therapy does not seem to affect pre- and post-therapy lymphatic flow, as assessed by hyaluronic acid levels. As the route of the hyaluronic acid molecules from connective tissue via the lymphatic system to the blood circulation is influenced by many factors, further research is required.

However, our results show a tendency towards slightly decreased HA levels after CDT rather than an increase, despite the latter being expected to happen because of accumulating HA in the blood originating from increased lymphatic flow that carries HA with it and from the edematous tissue, where HA had been "freed" during CDT.

This tendency was common in all subjects, while only subjects with abnormal high mean levels of HA (>19 ng/ml [63]) also showed an inconsistent decrease over time (*no. 1, 2* and *6*). Within the 'low level' group, this characteristic could only be seen in *no. 3* (with an unexpected rise over baseline HA at the second measurement of the last day).

This might suggest, that a decrease over time is only perceivable in subjects with considerable high HA levels due to rapid HA turnover in blood. However, high levels of HA in plasma must be considered as pathological and the underlying condition might be distinct from lymphedema. As a result, this would mitigate the validity of HA as a marker for lymph flow because of interferences in the HA production and turnover.

As for now, the behavior of HA cannot be explained as it is apparently influenced by multiple factors, suggesting it is not useful to utilize HA as a surrogate for lymph flow.

One reason for inconsistent HA levels after CDT may be accounted for by the time lag between therapy and blood sampling. Although times for blood sampling were scheduled directly after manual therapy and bandaging, short delays occurred due to organizational problems, but even with perfect timing, blood sampling might still be late due to the fast metabolism of HA within a few minutes.

Moreover, lymph propulsion might not be immediately affected by manual therapy and a recognizable rise of HA therefore might be timed after blood sampling or could not be detected, if the impact of therapy on lymph flow is low. This is very likely in subjects where the damage to the lymphatics is more severe. For instance, *Tan et al.* revealed in a near-infrared fluorescence imaging study that manual therapy appeared to be more effective in healthy and asymptomatic LE subjects regarding lymph velocity and lymph propulsion rate, though even symptomatic LE patients showed a clearly increased lymph velocity by 23% and a decreased lymph propulsion rate by 9% after manual therapy [128].

Another reason for inconsistent results may be due to the measuring method for HA, which does not take into account the molecular size of HA. It is evident, that the size of HA differs widely depending

on its occurrence and degradation takes place at multiple sites. While the polymer prevails in very large states in the tissue it becomes degraded into smaller residues either during its passage through the lymphatics or already in the tissue. Moreover, it has been reported that lymph nodes have the capacity to uptake and degrade considerable amounts of HA and its larger polymers are more rapidly removed [15].

Nevertheless, it is likely that only small fragments of HA have the capability to move freely and mostly unaffected by nodal degradation, whereas HMWHA is bound in the tissue until it is cleaved by hyaluronidases or free radicals into LMWHA. This is in line with studies of *Reed et al.* and *Armstrong and Bell* who investigated the turnover of HA in tissue and lymph and observed an increase of LMWHA together with an increased lymphatic flux, while the tissue did not show significant amounts of LMWHA [66].

However, since both LMWHA and HMWHA occur in plasma the ratio of these HA entities should be considered to identify the small HA fragments from the lymphatics. The unexpected decline of HA levels after CDT could be due to the higher affinity of the liver endothelial cells for the HMWHA [129] resulting in a decrease of both HMWHA and total HA content while LMWHA could be unaffected or above the baseline level. However, in this hypothesis the origin of HMWHA in the circulation would remain unclear.

In addition, induced lymphedemas in a mouse model study decreased in size when treated with hyaluronidase, suggesting that tissue fluid can escape when HMWHA content is cleaved into smaller fragments [130]. In fact, it has been reported, that the HA content in lymphedematous leg is approx. eight times higher than the HA content of the contralateral leg [71], which is very likely a result of its impaired lymphatic removal.

Due to its specific water-binding properties and its tendency to form an entangled network within the ECM, HA could function like a sponge and therefore play a crucial role in the pathogenesis of LE.

In histopathological sections, *Roh et al.* also showed an accumulation of LMWHA in edematous tissue after hyaluronidase treatment, suggesting that even smaller HA molecules cannot enter the lymphatics in lymphedematous conditions and the observed alleviation of LE has been a consequence of increased lymphangiogenesis promoted by interactions between LMWHA and the LYVE-1 receptor [130].

Finally, the inconsistent findings regarding HA levels may have been completely random and CDT could not affect HA at all. Although a decline in HA levels after CDT is more frequent than an increase, the number of participants was low and almost everyone showed aberrations even if they exhibited a HA pattern (*see fig. 10*).

Subjects of the “high level group” even exhibited no HA pattern at all (except *no. 2*).

Moreover, pre and post CDT HA levels usually showed only marginal difference, especially in the "low level group", where subjects had normal HA levels.

In order to investigate associated factors, correlation analyses were performed considering percentage changes in plasma density during daily CDT and percentage changes in weight between test days.

An impediment in performing this study was to gather information about the real lymph flow since lymphoscintigraphies were not conducted. Assuming that changes in plasma density could be caused by a dilution or contraction of plasma and facilitated - beside other factors - by lymphatic influx into the circulation, an increased lymph flow would mean a decline of plasma density due to dilution. Conversely, an increase of plasma density would represent a lowered lymph flow due to the hampered uptake of plasma constituents.

However, the analysis could not identify a correlation between percentage changes in plasma density and in HA levels.

In another approach the correlation of percentage changes in weight with percentage changes in HA between test days has been explored.

During CDT, weight changes could potentially correlate with changes in the size of the LE, because patients were not subjected to keep a calorie-reduced diet it could be assumed that weight loss would basically be a reduction of edematous fluid. This reduction would be promoted by an increased lymph flow and excess fluid from the edematous region would be eliminated by the kidneys. Unfortunately, weight measurements are rather imprecise and biased by hydration status and food consumption, which is why the significance is low. In addition, weight changes and HA did not exhibit any correlation.

In summary, these two parameters – weight change and plasma density change, respectively – have both a poor and rather theoretical significance for lymph flow and may possibly lead to false conclusions. A reliable and convincing conclusion about the correlation of HA levels with lymph flow cannot be drawn without a direct assessment of the flow by lymphoscintigraphy.

5.1 Limitations

Since this study was conducted in humans, standardization was not easily obtainable. A fact, which potentially lowers the validity of the results. As mentioned above, distortions occurred due to non-standardized blood sampling times, which is why a strict time schedule could be helpful with fixed intervals for measurements of HA, although this is hardly feasible in a clinical setting.

Furthermore, the number of subjects was small, to achieve a higher statistically significance higher numbers of participants are important.

Finally, the effect of CDT on HA should be researched in healthy individuals to draw comparisons

between those and LE patients.

5.2 Conclusion and further implications

In the present study we investigated the influence of CDT on Hyaluronan for the first time. Although it has been proposed in experimental animal studies that HA of low molecular weight is drained by the lymphatics and further reaches the blood stream, we did not see a forecasted rise of plasma HA. This could possibly be due to the fast turnover of HA in blood by liver cells or complete degradation in lymph nodes by hyaluronidases. However, a decrease of HA after daily CDT was strikingly frequent, therefore, more research should be conducted to investigate and quantify other influencing factors of the turnover of HA, since the turnover of HA is complex and not fully understood yet. At this stage, it is not possible to make certain assumptions about lymph flow by utilizing HA as a surrogate.

To prove if HA could be a surrogate for lymph flow, a new approach should also assess lymph flow by lymphoscintigraphy or by direct cannulation in animal studies. Until now, BIS data from the LYMPH study, which could have provided better information about the lymph flow, have not been analyzed and could therefore not be utilized to interpret HA levels. Moreover, the polymorphism of HA regarding its molecular size should be considered, since there is strong evidence that both LMWHA and HMWHA have different and sometimes converse biological functions.

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Appendix A

Ethikkommission

Medizinische Universität Graz

Auenbruggerplatz 2, A-8036 Graz
ethikkommission@medunigraz.at
Tel.: +43 / 316 / 385-13928, Fax: -14348

VOTUM

gültig bis 03.03.2018

EK-Nummer:	29-090 ex 16/17
Studientitel:	Effects of Orthostatic Loading on Lymphatic Flow and Vascular Fuction Assessments in Health and Disease
Prüfer:	Assoc. Prof. Dr. Nandu Goswami Medizinische Universität Graz
Sponsor:	Medizinische Universität Graz
Ansprechpartner:	Assoc. Prof. Nandu Goswami, 8010 Graz , Harrachgasse 21/V
CRO:	-
Antragsteller:	Medizinische Universität Graz
Ansprechpartner:	Assoc. Prof. Nandu Goswami, 8010 Graz, Harrachgasse 21/V

Die o.a. Studie wurde von der Ethikkommission erstmals im 'expedited Review' am 02.11.2016 behandelt. Die Ethikkommission ist zu folgendem Schluss gekommen:

Es besteht kein Einwand gegen die Durchführung der Studie in der vorliegenden Form.

Kommissionsmitglieder, die für diesen Tagesordnungspunkt als befangen anzusehen waren und daher gemäß Geschäftsordnung an der Entscheidungsfindung und Abstimmung nicht teilgenommen haben: keine

Zur Beurteilung vorliegende Dokumente:

Dokumente eingegangen am 27.10.2016, begutachtet im 'expedited Review' am 02.11.2016	
✓ Cover Letter Cover Letter 1	21.10.2016
✓ Antragsformular ECS	27.10.2016
Originalprotokoll Studienprotokoll_vers01_21102016 1	21.10.2016
Informed Consent Form Probandeninformation_vers01_21102016 1	21.10.2016
Dokumente eingegangen am 03.11.2016 (in der nächsten Begutachtung mitbegutachtet)	
✓ Antragsformular ECS unterschrieben	27.10.2016
Dokumente eingegangen am 23.01.2017 (in der nächsten Begutachtung mitbegutachtet)	
✓ Cover Letter Stellungnahme zur Bearbeitungsmitteilung	23.01.2017
✓ Informed Consent Form track changes 01	20.10.2016
Dokumente eingegangen am 01.03.2017, begutachtet im 'expedited Review' am 03.03.2017	
✓ Informed Consent Form 03	21.02.2017

Die Ethikkommission geht - rechtlich unverbindlich - davon aus, dass es sich um keine klinische Prüfung nach AMG bzw. MPG handelt.

Es handelt sich um eine Studie im Rahmen einer Diplomarbeit.

Das Votum der Ethikkommission berührt in keiner Weise die alleinige Verantwortung der Prüferin / des Prüfers / der Prüfer für die ordnungsgemäße Durchführung der Studie unter Einhaltung aller einschlägiger gesetzlicher Bestimmungen und Richtlinien.

Weiters machen wir darauf aufmerksam, dass der Kommission unverzüglich zu melden sind:

- Abweichungen vom Protokoll aus Sicherheitsgründen oder Protokolländerungen

- Änderungen, die das Risiko der Teilnehmer/-innen erhöhen oder die Durchführung der Studie wesentlich beeinflussen

- Mutmaßliche unerwartete schwerwiegende Nebenwirkungen - SUSARs (AMG-Studien ab 1.5.2004) oder schwerwiegende unerwünschte Ereignisse - SAEs (andere Studien)

- Jegliche Information über sonstige Umstände, die die Sicherheit der Teilnehmer/-innen oder die Durchführung der Studie beeinträchtigen können

zusätzliche Auflagen: Das Votum ist nur für das Zentrum Med. Universität Graz - Assoc.Prof.Dr. Nandu Goswami gültig.

Dieses Votum gilt für ein Jahr ab dem Datum der Ausstellung. Bei längerer Studiendauer ist rechtzeitig vor Ablauf der Gültigkeit des Votums ein Zwischenbericht vorzulegen (Berichtsformular), um eine etwaige Verlängerung zu erlangen.

Graz, 03. März 2017

Univ.Prof.DI Dr.Josef Haas
Vorsitzender

Univ.Prof.Dr.Hermann Toplak
Stv. Vorsitzender

Achtung: Bitte bei allen das Projekt betreffende Schreiben oder telefonischen Anfragen die EK-Nummer angeben!

Medizinische Universität Graz, Auenbruggerplatz 2, A-8036 Graz. www.medunigraz.at
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