2015•2016
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
*master in de biomedische wetenschappen*

# Masterproef

## Analysis of glycosaminoglycans as a sodium buffer network in patients with congestive heart failure


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Prof. dr. Jean NOBEN

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universiteit hasselt | Maastricht University

# Samenvatting

**Achtergrond:** Hartfalen is een serieuze conditie waarbij het lichaam onvoldoende wordt voorzien van zuurstofrijk bloed. Door de neurohormonale upregulatie wordt meer zout en water in het lichaam gehouden. Het gevolg hiervan is een vochtophoping in de longen of benen van patiënten. Glycosaminoglycanen (GAGs), die zich in het interstitium bevinden, hebben onlangs een grote aandacht gekregen in het reguleren van zouten in het lichaam. GAGs zijn lineaire, onvertakte polysachariden die talrijke negatieve sulfaat- en carboxylgroepen dragen. Natrium ionen kunnen hier aan binden waardoor in eerste instantie geen waterretentie wordt waargenomen. Bij overschrijding van een bepaalde dremplelwaarde verandert de structuur van GAGs waardoor natrium ionen weer vrij komen en osmotisch actief worden. Vochtophoping vindt plaats en de patiënt ontwikkelt oedeem. Opmerkelijk hierbij is dat de klinische presentatie van mensen met hartfalen varieert van geen tot een volledig ontwikkeld oedeem en dit onafhankelijk van hemodynamische factoren. Daarom wordt verondersteld dat GAGs, en vooral het aantal negatieve ladingen dat aanwezig zijn op GAGs, geassocieerd zijn met de klinische presentatie van patiënten met hartfalen.

**Materialen & methoden:** Volgens een protocol dat eerst opgesteld en geoptimaliseerd moest worden, werden GAGs bereid uit pronase E behandelde huidstalen van de kuit van patiënten met ofwel acuut gedecompenseerde (n = 8), ofwel chronisch stabiele hartfalen(n = 11) , alsook van de gezonde controles (n = 12). De concentratie (µg/mg drooggewicht) van de negatieve ladingen op GAGs werd bepaald met twee assays. Een carbazole assay werd uitgevoerd die de concentratie van uronzuren meet. Dit zijn de enkelvoudig geladen bouwstenen van GAGs. Een tweede assay met dimethylmethyleenblauw kleurstof werd uitgevoerd om het aantal sulfaatgroepen op GAGs te bepalen. Aanvullend werd ook hyaluronzuur, één van de GAG families, gemeten met behulp van ELISA. Als laatst werd er ook een profilering van GAGs opgesteld met HPLC-massaspectrometrie op chondroitinase-ABC behandelde en 2-aminoacridone gederivatiseerde stalen.

**Resultaten & discussie:** Een significant hogere concentratie gesulfateerde GAGs werd gevonden in acuut gedecompenseerde en chronisch stabiele patiënten in vergelijking met gezonde controles. Ook is de totale concentratie uronzuren, maar niet van hyaluronzuur zelf, significant verhoogd, en dit weliswaar alleen in gedecompenseerde patiënten.
Deze resultaten kunnen in de toekomst gebruikt worden om patiënten op te volgen en zo de risico op decompensatie te verlagen. Er zou veel vroeger in de behandeling ingegrepen kunnen worden met een betere prognose voor de individu en een lagere kostprijs voor de staat.
Mono-, di- en trigesulfateerde GAG disachariden konden aangetoond worden met HPLC-MS in teststalen van de huid. Een verdere optimalisering dient evenwel te gebeuren om ook een profilering te kunnen uitvoeren van de huidbiopten van de verschillende studiegroepen vanwege geringe hoeveelheden beschikbaar weefsel.

**Conclusie:** Periodieke symptomen van zout- en vochtophoping duiden op een verhoogd risico op sterven en re-hospitalisatie in hartfalen. Er is al veel gekend in verband met vrije zout regulatie. Onderzoek van het interstitium, als een nieuwe compartiment voor zoutregulatie, levert interessante nieuwe informatie op omtrent de pathofysiologie van hartfalen. Meer onderzoek is echter nodig om nieuwe technieken te kunnen ontwikkelen die onder meer gericht zijn op de verhoging van de bufferende capaciteit van het interstitium om het cardiovasculaire risico in patiënten met hartfalen te kunnen verlagen.

# Abstract

**Introduction**: Congestive heart failure is characterized by a fluid buildup in lungs and/or legs. It is a result of increased retention of sodium and water in the body, caused by upregulated neurohumoral systems. Recently, glycosaminoglycans (GAGs), the major component of the interstitium, gained major interest in sodium handling. GAGs are linear, unbranched polysaccharides, which are very polyanionic due to the presence of carboxyl and sulfate groups. Cations, such as sodium, can bind electrovalently, which are no longer osmotically active and therefore no fluid retention is observed in the individuals. This buffering function of GAGs, however, is not unlimited and after a certain sodium concentration GAG structure will alter and fluid accumulation will occur with eventual edema as result. However, the clinical presentation in heart failure varies from no edema to overt peripheral fluid buildup, which is independent of hemodynamic factors. We hypothesize that GAGs, and especially the amount of negative charges present on GAGs, are associated with the clinical presentation in heart failure patients.

**Materials & methods**: According to an optimized protocol that had to be set first, GAGs were prepared from pronase E treated skin biopsies of the lower leg of patients with either acute (n = 8) and chronic HF (n = 11), as well as control individuals (n = 12). The concentration (µg/mg dry weight) of negative charges present on GAGs was assessed by two assay. First, a carbazole assay was performed to detect the amount of uronic acids, which are the negatively charged building blocks of GAG chains. Secondly, a dimethylmethylene blue assay was done which only detected the sulfated groups present on GAGs. Additionally, hyaluronic acid, one of the GAG families, was measured with ELISA to detect possible differences between the groups. A profiling of GAGs in the samples was done by HPLC - mass spectrometry on chondroitinase-ABC treated and 2-aminoacridone derivatized samples.

**Results & discussion:** Significantly higher concentrations in the amount of sulfated GAGs were found in acute decompensated and chronic stable heart failure patients compared to healthy controls. Also the amount of uronic acids, but not of hyaluronic acids, was significantly higher, although only in decompensated patients.
These results are of important value, as they could be used in the future to follow-up patients and thus lower the risk of decompensation. Therapies could be adjusted earlier in the disease progression, having a major impact on the individuals itself and on the economic burden related to heart failure.
The presence of mono-, di-, as well as trisulfated GAG disaccharides was shown by HPLC – MS in ‘test’ skin samples. Nevertheless, to perform a GAG profiling in skin samples of the 3 study groups, additional optimization needs to be done since limited tissue was available.

**Conclusion:** Recurrent signs of sodium and fluid overload in heart failure are markers of mortality and re-hospitalizations. Much is already known about free sodium handling. Exploring the interstitium as a new compartment in sodium regulation provides new interesting information about the pathophysiology of heart failure. As research advances, new therapies could be developed to improve the buffering capacity of the interstitium and decrease the chance of decompensation in patients with heart failure.

# List of abbreviations

| | |
|---|---|
| **AD** | Acute decompensated |
| **AMAC** | 2-aminoacridone |
| **CS** | Chronic stable |
| **CV** | Coefficient of variation |
| **DAB** | 3,3-diamonobenzidine |
| **DMMB** | Dimethylmethylene blue |
| **DMSO** | Dimethyl sulfoxide |
| **DW** | Dry weight |
| **D0A0** | Chondroitin disaccharide standard (unsulfated) |
| **D0A6** | Chondroitin sulfate disaccharide standard (monosulfated) |
| **D0A4** | Dermatan sulfate (full GAG chain) |
| **GAG** | Glycosaminoglycan |
| **HA** | Hyaluronic acid |
| **HF** | Heart failure |
| **HPLC – MS** | High-performance liquid chromatography coupled to mass spectrometry |
| **SAX** | Strong anion exchange |
| **SD** | Standard deviation |
| **TFA** | Trifluoroacetic acid |
| **tsGAG** | Total sulfated GAG |
| **tUA** | Total uronic acid |
| **TWC** | Tissue water content |

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# Introduction

## Heart failure.

Heart failure is the state in which the heart is not able to supply the body with oxygen rich blood. More than 1 million people a year are hospitalized with heart failure and together with a high frequency of re-hospitalizations, it has a huge economic burden on the society. It is the leading cause of death worldwide (1). Despite, current medical progress, no absolute cure exists. Nowadays, many treatments focus on preventing further deterioration of the heart failure, lessen the symptoms and improving the daily life of a patient. In certain subgroups of heart failure patients, more advanced therapies are necessary such as device therapy (cardiac resynchronization therapy).

Heart failure is often characterized with a fluid buildup, most typical in the lungs (pulmonary edema) or ankles or feet, due to gravity forces. The term used to describe this condition is congestive heart failure. Congestion is the result of a defective cardiorenal interaction. Due to hemodynamic alterations (low cardiac output as well as high cardiac filling pressures) the kidneys activate several mechanisms to retain salt and fluid in the body. One of these mechanisms is the renin-angiotensin-aldosterone system (RAAS). This hormone regulated system increases the secretion of aldosterone which in turn is responsible for the increased reabsorption of sodium and water into the blood. Beside the increase in extracellular fluid, it also increases blood pressure.
Another mechanism that is activated in response to heart failure offset is the sympathetic nervous system (2). It increases the stroke volume and peripheral vasoconstriction to maintain mean arterial perfusion pressure (3). Furthermore, prolonged sympathetic activation adversely affects excitation-contraction coupling and enhances apoptotic pathway, playing an important role in the progression of chronic heart failure (4, 5).
Initially, these mechanisms are activated to compensate for the depressed myocardial function and to preserve cardiovascular homeostasis. On a long term, however, they are responsible for cardiac decompensation and heart failure progression and beside the heart, have delirious effects on other organs. Renin-angiotensin blockers (angiotensin converting enzyme inhibitors and spironolactone) together with beta-blockers are the cornerstone of current HF management. However, most patients need daily diuretics to prevent sodium and fluid retention.

## Sodium in body fluid homeostasis.

Much discussion has been going on about sodium intake for decades. A number of bodies, such as National Institutes of Health, Institute of Medicine and American Heart Association recommend a daily sodium intake of less than 2300 mg per day (6).Their starting point is that a high sodium intake is associated with increased hypertension and cardiovascular diseases due to increased water retention in the body. Nevertheless, others report that a reduction towards recommended sodium intake (< 2000 mg/day) would have a detrimental effect on the body (7, 8).
It is well recognized that sodium is essential for humans and animals to be able to live. Besides its role in the body fluid homeostasis, it is also responsible for muscular contraction and transmission of nerve signals. It is thus not surprising that these ions are tightly regulated by our body. Osmoreceptors in the hypothalamus sense a slight increase of salts in the body, stimulating thirst and arginine vasopressin (AVP) release to increase

the fluid. Subsequently, baroreceptors within the large arteries sense the increased volume and activate urinary sodium and water excretion to return the balance.
Although the classic idea of increased body fluid in response to increased sodium concentration is generally accepted, it may not always be true to explain fluid overload and increased cardiac filling pressure in patients with heart failure. It was found that even in healthy individuals, substantial fluctuations in total body sodium occur without the accompanied increase in total body fluid (9, 10).
Recently, is has been demonstrated that a large amount of sodium ions in the body is bound to glycosaminoglycan (GAG) networks in the interstitium. These networks can function as a buffer for sodium ions which are then not detectable by the receptors in the body. A better understanding of these interstitial networks is required to understand the total body sodium handling and possibly novel therapeutic targets that had been previously overlooked.

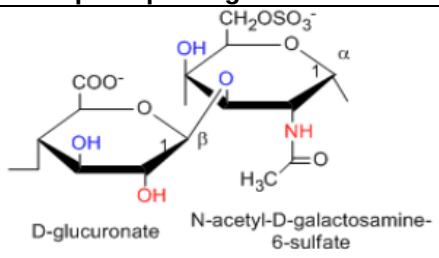
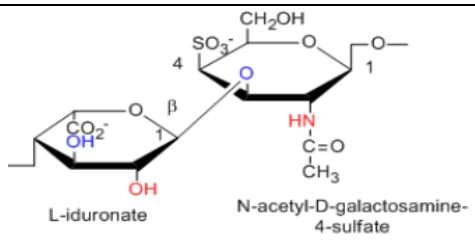
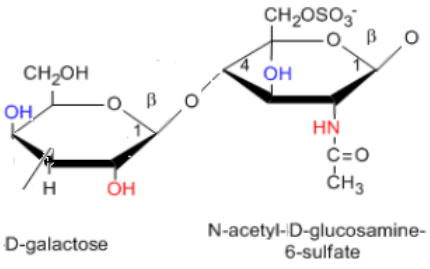
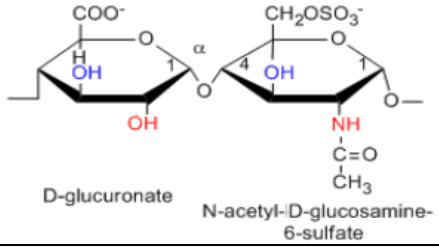
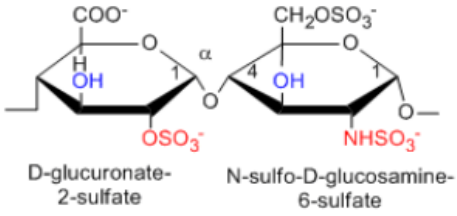
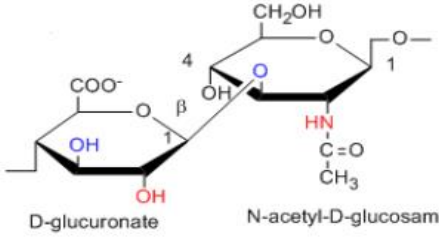
## Glycosaminoglycan diversity.

Glycosaminoglycans, formerly known as mucopolysaccharides, are long, unbranched polysaccharides composed of repeating disaccharide unit of an amino sugar (N-acetylglucosamine or N-acetylgalactosamine) and a uronic acid (glucuronic or iduronic acid) or galactose (11). Initially, they were thought to have a space filler function between the cells, necessary for the structure and compliance of the extracellular matrix (ECM) (12). Advances in glycobiology had led to the conclusion that GAGs reside not only in the ECM, but are also present in the cell membrane and in the vesicles inside the cells. Attached to various proteins, GAGs can act as key biological response modifiers by acting as signaling molecules, regulators of enzyme activity, mediators of cell communication and are able to initiate and control events associated with inflammation (13-15). It has been reported that the concentration of GAGs is increased in diseases like osteoarthritis, rheumatoid arthritis and psoriasis. This elevated concentration of GAGs may adversely influence the course of the disease, while others report a beneficial effect such as decreased inflammatory response (16). Furthermore, genetic defects in GAG biosynthesis lead to several signaling pathway dysfunctions with serious developmental consequences (17). Therefore, it is not surprising that researchers are interested in these molecules which probably have yet more undiscovered functions based on their diversity in structure, length and complexity.

In order to understand the importance of glycosaminoglycans as multi-functional entities, it is not only essential to know its building blocks, but also its posttranslational modifications and enormous variety of binding partners. There are four distinct GAG families: heparan sulfate (HS)/heparin, keratan sulfate (KS), chondroitin (CS)/dermatan sulfate (DS) and hyaluronan (HA). Except for HA, GAGs can bind covalently via a serine residue to a protein core, delineating them as proteoglycans (PG) (16). HA on the other hand can form aggregates with proteoglycans (18).
CS contains 2 alternating monosaccharides: D-glucuronic acid (GlcA) and N-acetyl-D-galactosamine (GalNac). Some GlcA residues are epimerized into L-iduronic acid (IdoA) and the resulting GAG is called DS ( or chondroitin sulfate B). Table 1 delineates the different GAG families and their structure. As shown in the figure, GAGs differ according to the type of hexuronic acid and hexosamine, as well as the geometry of the glycosidic linkage between these units (α or β). The amino sugar may also be sulfated on the carbons 6 and/or 4 or on the nitrogen while the sugar backbone can be sulfated at various positions. These makes that a simple octasaccharide could have over 1 000 000 different sulfation sequences (19). Together with the fact that GAGs chains bind to different protein

cores contributes to the diverse biological role of PGs (20). These interactions play important roles in normal physiology and are also involved in pathological processes.

**Table 1. Structure and distribution of different glycosaminoglycan families.** MW = molecular weight in g/mol. S = sulfate atom.

| Glycosaminoglycan | Localisation | Example repeating unit | MW |
|---|---|---|---|
| Chondroitin sulfate | Cartilage, tendon, ligaments, **skin**, aorta |  | 379.1 + 80 for each S |
| Dermatan sulfate (chondrotin sulfate B) | **Skin**, blood vessels, heart valves, lung |  | 379.1 + 80 for each S |
| Keratan sulfate | Cartilage, cornea |  | 379.1 + 80 for each S |
| Heparan sulfate | Basement membranes, cell surfaces |  | 379.1 + 80 for each S |
| Heparin | Mast cells (anticoagulant), arteries of the lungs, liver and skin |  | N-Ac = 379.1 N-S = 417.1 + 80 for each S |
| Hyaluronic acid | Joints, cartilage, umbilical cord, **skin**, vitreous humor of the eye |  | 379.1 |

## Glycosaminoglycans regulate sodium balance.

As mentioned before, patients with heart failure have increased concentration of salts present in their body (50). Recent studies have investigated the effect of increased and prolonged concentration of salts with a focus on glycosaminoglycans which seem to play an important role in sodium regulation (21, 22). Because of its many sulfate groups and carboxyl groups present on a GAG molecule, it is very anionic. The polyanionic nature allows them to bind different cations, preferentially sodium ions. After an acute increase in sodium ions in the body, such as a rich salt meal or acute heart failure, more GAGs are synthetized (23). Many several transcription factors are then activated and the GAG sulfation pattern increases to compensate for the increase in sodium ions. Due to high oncotic pressure in the interstitium, fluid starts to accumulate. Because of low compliance of interstitial GAG network and thus limited elastic potential, it 'presses' the fluid out into the lymphatic vessels and back to the circulation. Furthermore, the increased concentration of sodium ions indirectly activates vascular endothelial growth factor C (VEGF-C) and endothelial nitric oxide synthase expression, stimulating lymphangiogenesis to promote better lymph clearance (24). This state with a normal hydrostatic pressure and a high interstitial oncotic pressure is called the compensated state (fig. 1b). Those individuals have increased concentration of sodium ions in their body, but this increase is buffered by the interstitial GAG network. No increase in the concentration of sodium ions is observed in the blood. Hence, the pituitary osmoreceptors and renal regulatory function prevent the secretion of vasopressin (ADH) and activation of renin-angiotensin-aldosterone system respectively, causing no fluid retention in the body despite a higher sodium intake. An individual does not have any symptoms yet. Hence the name, compensated heart failure.

This buffering function of GAGs is not unlimited. After a certain concentration of sodium ions, the threshold level for buffering is reached. Once this happens, the integrity of the whole interstitial network changes, leading to dysfunctional GAG molecules. The transudation of plasma fluid into the interstitium, that is normally opposed by the tensile forces of the GAG molecules is now lost, allowing fluid to accumulate (fig. 1c). Furthermore, the lymphatic drainage becomes less efficient, leading to leakage of lymph into the interstitium. Altogether, these effects lead to a local fluid accumulation in the extracellular matrix that can progress to a swelling and edema, a so called decompensated state. Except for edema development, this state may also be especially important in salt-sensitive hypertension and neurohumoral activation in HF (24).

The negative charges present on a GAG molecule to which sodium ions are attracted are thus very interesting targets to investigate and will be the main objective of this paper. It is thought that due to increased sodium concentration, a patient with chronic stable (compensated) heart failure will have more negative charges present on a GAG molecule. In contrast, a patient with acute decompensated HF with a dysfunctional GAG network will probably have significantly less negative charges, compared to a healthy individual. In short, it is hypothesized that the negative charge density related to GAGs in the interstitium is associated with the degree of interstitial edema in HF.

Three direct GAG quantification methods will be assessed, including high-performance liquid chromatography (HPLC), carbazole and dimethylmethylene blue (DMMB) assays. While the 2 former assays have been reported less reliable than a more precise HPLC quantification method due to small quantity and undersulfated GAGs that may be present

in some biological samples, they are still a lot cheaper and less labor intensive compared to HPLC and are thus important factors to be able to do a large screening study (17).

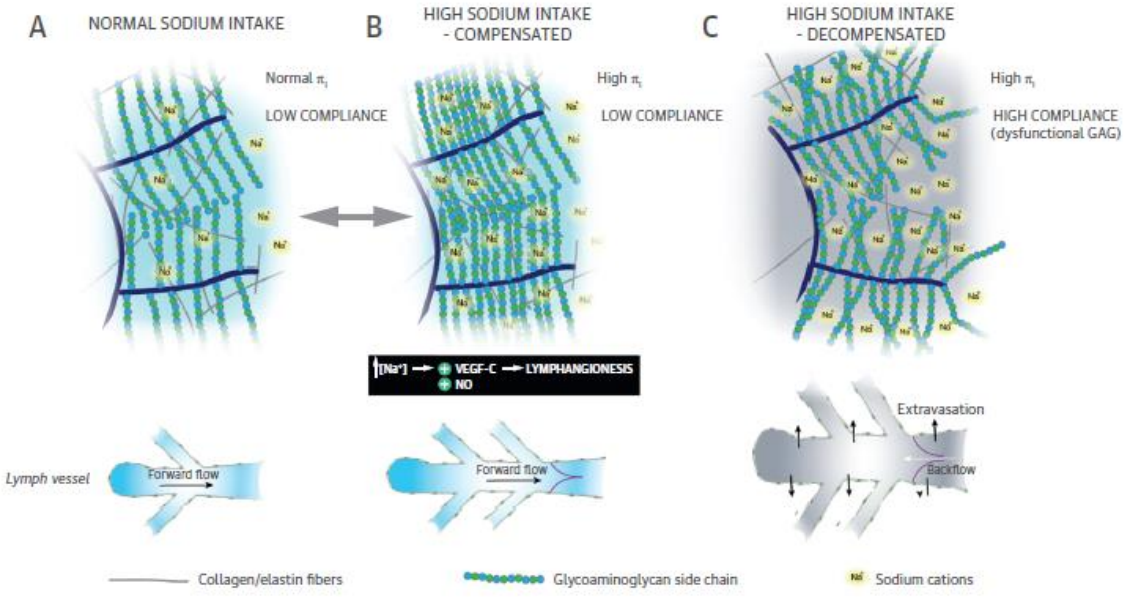



**Figure 1. The extracellular matrix or the interstitium.** (A) The compliance of the interstitium is determined by the presence of proteoglycans, together with elastin and collagen vessels. The fluid is drained by many lymphatic vessels. (B) After sodium intake, GAGs are synthetized that can accumulate greater number of sodium ions and lymphangiogenesis is promoted. Due to increased lymphatic capacity and low interstitial compliance, interstitial fluid is drained efficiently (compensated). (C) After certain threshold for buffering sodium ions is reached (heart failure patients), GAGs change their conformation and become dysfunctional, entering a high compliance state. In addition, lymphatic drainage becomes less efficient. Therefore, in heart failure, the combination of high interstitial oncotic pressure and high compliance makes the interstitial fluid to accumulate and the patient develops edema (decompensated) (24).

## GAG quantification.

<u>High-performance liquid chromatography.</u>

As said before, GAGs have an enormous variety of structural differences defining their activity. The negative charge density, and especially the sulfate groups, are of great interest. Modern analytical techniques such as HPLC are required to determine this structural information precisely.
HPLC is a widely used technique to separate, identify and quantify compounds in a mixture. A common approach to analyze GAGs in samples is to perform a depolymerization of GAG chains into disaccharide fragments with bacterial lyases (25). This results in a exposed reducing end at 4,5-unsaturated uronic acid residue which can be labeled with a reducing fluorophore 2-aminoacridone (AMAC) by means of reductive amination (fig. 2) (26). The hydrophobic nature of AMAC improves the chromatographic properties of reversed-phase HPLC and improves sensitivity. A profile of different sulfate patterns can then be set with a HPLC system and a fluorescent detector, based on different retention time of the disaccharide compounds. In addition, HPLC coupled to mass

spectrometry can provide certainness about an eluted peak on a chromatogram based on mass-to-charge ratio. The masses for different GAG disaccharides are listed in table 1.

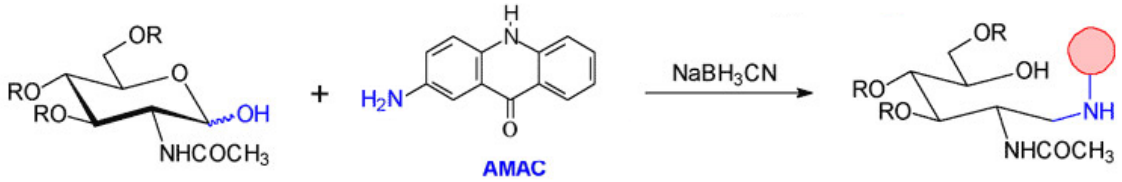



**Figure 2. The reductive amination of sugar with 2-aminoacridone.** Sodium cyanoborohydride is used to stabilize the binding.

Dimethylmethylene blue (DMMB) assay.

DMMB assay is a valuable spectrophotometric method to measure sulfated GAG content in biological fluids and tissue/cell extracts. The dye 1,-9 dimethylmethylene is a thiazine chromotrope agent that results in a change of the absorption spectrum due to induction of metachromasia when bound to sulfated GAGs. The absorption peak shifts from about 630 nm to about 530 nm in the presence of GAG polyanions. The degree of metachromasia is positively correlated with substrate-dye ratio (27, 28). The major problem here is the rapid precipitation of the substrate-dye complex, preventing accurate sGAG assessment.
Moreover, except for the duration of the reaction, it is also affected by parameters such as salt content, pH and the presence of interfering polyanions. Various modifications were made to enhance the stability, repeatability, sensitivity and accuracy of the assay discussed elsewhere (29, 30).
Other protocols to detect sGAGs initially allow the substrate-dye complex to precipitate, followed by dissolving the complex and measuring the absorption of the resuspended dye. A commercial kit tested in this paper (Blyscan Glycosaminoglycan Assay, Biocolot Ltd) is using this approach. Others measure the absorption of the remaining unbound dye after substrate-dye complex precipitation have occurred. The absorbance of the unbound dye is then inversely related to the sample sGAG content.
Important to note is that each different DMMB assay yields substantially different sGAGs concentrations in samples. Therefore, clinical information cannot be compared when variations of DMMB assays are used (31).

Carbazole assay.

Each GAG consists of a repeating unit of hexuronic acid and an amino sugar. To detect the negative charges from carboxyl groups on uronic acids, a simple assay, based on a reaction with chromophore carbazole, was developed. It involves treating the sample with a strong acid to hydrolyze the polysaccharides and dehydrate the sugars. The products are then treated with carbazole to create a stable chromophore in presence of sodium tetraborate (fig. 3).
The thee most important problems encountered in quantifying uronic acids since 1927, when the assay was developed, are the presence of colored reaction products from other components in mixture, the varying color yield of different uronic acids, and interference by certain salts (32, 33). Since then many modifications were made as for example inclusion of borate to reduce the varying color yields from different uronic acids. Some report overestimation of the concentration of GAGs (which is correlated with uronic acids)

(34). Nevertheless, it is a simple and rapid method, sufficiently sensitive for most laboratory situations and requires only routine laboratory equipment.

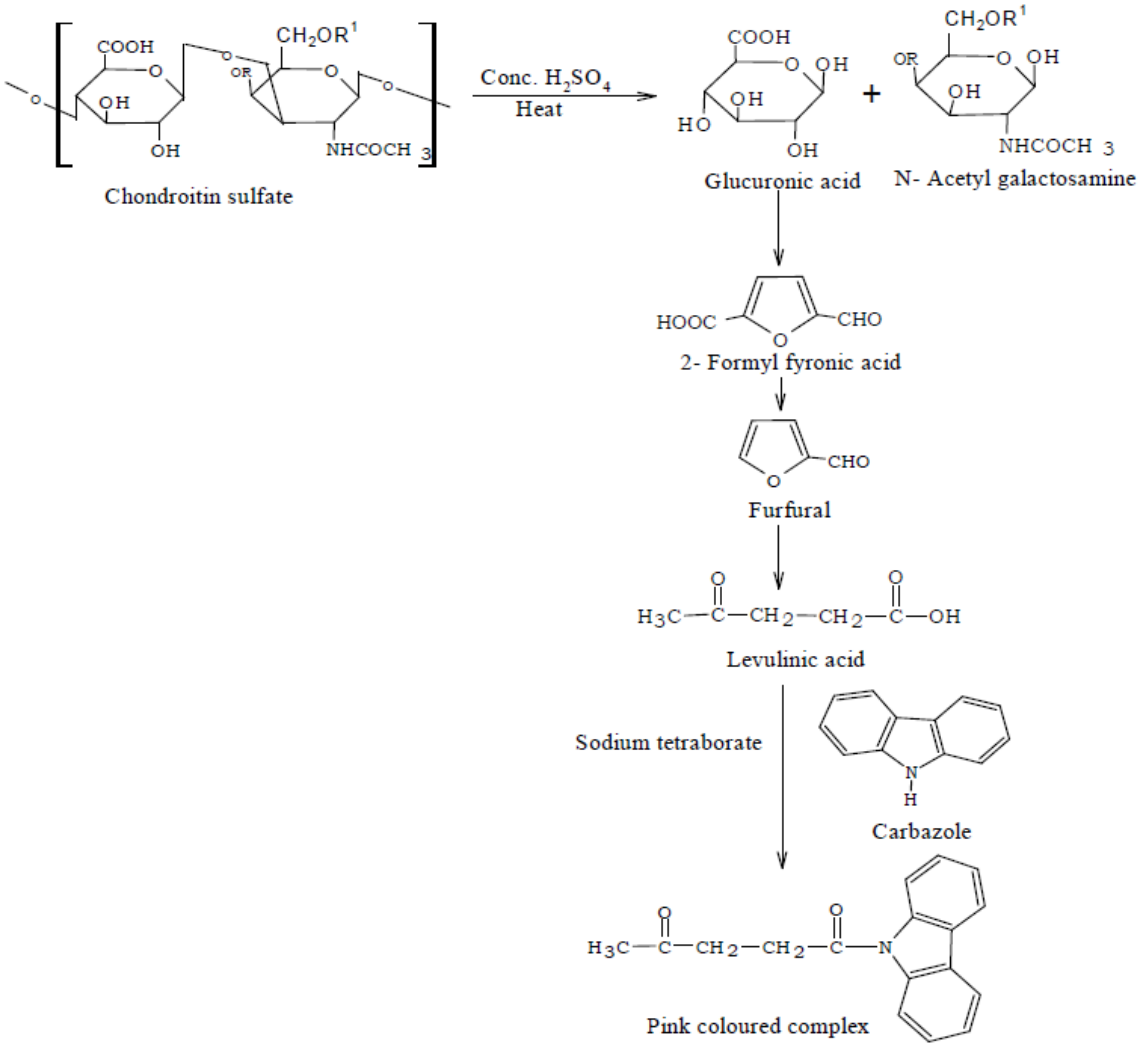



**Figure 3. Reaction scheme for chondroitin sulfate with carbazole.**

The possible differences in negative charge density between patients with HF and healthy individuals may help to identify heart failure patients at risk for acute decompensation and disease progression. This knowledge will allow to adjust heart failure therapy in an early stage of development, having a major impact on individual patient prognosis, morbidity and on economic problems related to heart failure. Furthermore, more insight will be created in the understanding of fluid homeostasis and thus in the pathophysiology of heart failure, allowing new therapies that target the interstitium to be developed.

# Materials & methods

## Human skin samples

Skin punch biopsies from 12 healthy, 11 stable heart failure (HF) and 8 acute decompensated HF patients were acquired from Hospital East Limburg (ZOL, Genk, Belgium). The grade of the edema at the time the biopsy was derived was recorded.
The patients could voluntarily participate in this study. If all in- and exclusion criteria were met (supplementary table 1) and informed consent signed, skin punch biopsies of the lower extremity (calf) were taken (supplementary attachment 2 for the procedure, supplementary fig. 2). The ethical permission was given by medical ethics committee of ZOL and Hasselt university.
Before analyzing patients samples, the analytical protocol was optimized using test samples taken from the skin of an abdomen, from persons who had undergone a fat removal surgery.

Fresh weight of each sample was measured with OHAUS, Analytical Plus 210 balance with a standard deviation of 0,1 mg in a pre-weighted microtube. The samples were then lyophilized (VirTis Freezemobile) until a constant dry weight was reached and the tissue water content was calculated. Vacuum centrifugation was also validated on test samples as a second way of drying the samples for comparison. Hereafter, dried samples were defatted at room temperature in chloroform/methanol 2:1, 1:1 and finally 1:2 (v/v) for 24 hours each. After 3 hours of lyophilizing, the microtubes were weighted and the net dry, defatted weight calculated. All samples were stored at -80°C.
During the processing of samples, the operator was blinded from which study group the biopsy was derived to eliminate unconscious bias that may occur.

### Tissue sample preparation

Initially, to extract GAG molecules from a skin sample, the samples were incubated in 300 µl pronase buffer (50 mM Tris/HCl, 1 mM $CaCl_2$, pH = 8) with 0,8 mg/ml pronase E overnight in a microtube shaker at 55°C. Later, it has become clear that this concentration may be too low to fully disintegrate the tissue and the concentration was raised to 2,4 mg/ml pronase E.
Samples were heated in boiling water for 15 minutes to inactivate pronase E, followed by centrifugation for 15 minutes at 12000 g. The supernatant was then divided for the 3 assays: 2 x 40 µl for the dimethylmethylene blue (DMMB) assay which was performed on the same day, 150 µl for carbazole assay which was overnight precipitated at -20°C with 90% ethanol, and 50 µl for hyaluronic acid (HA) ELISA which was stored at -80°C.

### DAB staining

Upon pronase E degradation of samples, a small pellicle remained in the pellet. Because pronase E may be resistant to keratin, a 3,3-diamonobenzidine (DAB) staining was performed on the pellicle with an anti-cytokeratin mice antibody to confirm this hypothesis. First, the pellicle was blocked in 0,5% $H_2O_2$ in MeOH for 30 minutes at room temperature. After several washings in 0,01M PBS; pH = 7,2 protein block was applied. After washing,

the pellicle was incubated with anti-cytokeratin mice antibody for 1 hour and washed intensively. Secondary anti-mouse peroxidase labelled antibody was applied for 30 minutes and washed. Then, DAB-peroxidase substrate (0,05% DAB; 0,015% $H_2O_2$; 0,01M PBS; pH = 7,2) was applied for several minutes and washed with distilled water. Hematoxylin was used as a counterstain and washed with tap water. Finally, the pellicle was mounted on a microscope slide with aqueous mounting agent (Aquatex, MerckMillipore, 108562), covered with a cover glass, and evaluated with light microscopy (Nikon, Eclipse 80i).

## GAG measurements

Total sulfated GAG measurement - The DMMB assay

A 'Blyscan Sulfated Glycosaminoglycan Assa Kit' (Biocolor Ltd., Belfast, UK) was tested according to the manufacturer's protocol (see supplementary attachment 4). Bovine tracheal chondroitin 4-sulfate, provided in the kit, was used as a standard molecule. Only abdomen test samples had been tested with this kit.

An alternative lower-cost assay, based on a thesis (35) was adopted as follows: sixteen milligrams of DMMB were dissolved in 5 ml of 95% ethanol and incubated for 30 minutes at room temperature. Next, 2 ml of formate buffer (40 mM formic acid; 80 mM NaOH in 500 µl Milli-Q water; pH = 3,5) were added and the volume was brought to 1L with Milli-Q water. The resultant DMMB dye buffer was stored protected from light at room temperature.

Forty microliters of pronase E digested skin sample in duplicate were diluted to 100 µl with pronase E buffer. Dermatan sulfate standards (1-10 µg) were prepared in a final volume of 100 µl (500 ng/µl stock solution diluted with pronase E buffer). To each sample or standard, 1350 µl DMMB buffer were added, vortexed thoroughly and incubated for 30 minutes at room temperature, protected from light. The tubes were then centrifuged at 12000 g for 15 minutes and 200 µl of the supernatant were applied in triplicate to a 96-well plate with a flat bottom. The absorbance was measured at 595 nm (FLUOstar OPTIMA, BMG Labtech). The wavelength was determined from the DMMB absorption spectrum with Bio-Rad SmartSpec Plus spectrophotometer. It was first allowed to warm up for at least 30 minutes. Blanking was done with the same DMMB buffer, but without the dimethylmethylene blue dye.

Total GAG measurement – The carbazole assay

The total uronic acid content in samples was measured by the carbazole reaction as described by Frazier (34) with some modifications. A volume of 150 µl of pronase E degraded sample was precipitated in 90% ethanol at -20°C overnight. After centrifugation at 12000 g and 4°C for 15 minutes, the pellet was resuspended in 90 µl Milli-Q water.
Forty µl of GAG sample in duplicate were added to pyrex disposable culture tubes (Corning Inc.) in a refrigerated chamber. Then, 200 µl of ice-cold solution of 25 mM sodium tetraborate in 98% sulfuric acid were added and vortexed. To make this solution, sodium tetraborate was first dissolved in hot water, followed by addition of ice-cold sulfuric acid. After heating at 100°C for 15 minutes and 10 minutes cooling at 4°C, 8 µl of 0,125 w/v% carbazole in absolute ethanol was added. The tubes were reheated at 100°C for 15

minutes and again 10 minutes cooled in the refrigerated chamber. Two hundred microliters were pipetted into the 96-well plate and the absorbance was read at 540 nm. The concentration of uronic acids was determined using an eleven points calibration curve of either dermatan sulfate standard (C3788, Sigma-Aldrich) for patient's skin samples, or glucuronic acid for measurements in test samples (0; 0,5; 1; 1,5; 2; 2,5; 5; 10; 15; 20 and 25 µg/40 µl).

Hyaluronic acid ELISA

A Hyaluronan Quantikine ELISA Kit (R&D systems, DHYAL0) was used to detect HA in pronase E treated skin samples. Thirty µl of sample, stored at - 80°C, was 1/200 diluted with 50 mM Tris/HCl, 1 mM $CaC2_2$, pH = 8. This solution was further 1/10 diluted with calibrator diluent, included in the kit. Except for that horizontal microplate shaker, set at 90 rpm, was used during incubation steps, the supplied protocol was followed closely.

## HPLC and MS

Standard solutions

Monosulfated (D0A6) or unsulfated (D0A0) chondroitin sulfate disaccharide standards were acquired from Iduron (CD003, CD001). A stock solution was prepared of 2,5 nmol/µl for D0A0 and 2 nmol/µl for D0A6 in Milli-Q water and stored at – 80°C. A calibration curve of 7 points for both standards was made with HPLC (0; 10; 25; 50; 100; 125; 250 pmol chondroitin standard).
Dermatan sulfate (D0A4) glycosaminoglycan powder was obtained from Sigma-Aldrich (C3788) and stored at – 4°C.

Chondroitinase-ABC activity assay

Chondroitinase-ABC from Proteus vulgaris (C3667-5UN) was acquired from Sigma-Aldrich. It was suspended in 0,01 w/v% BSA in water for a final concentration of 250 mU/ml and stored at -80°C in small aliquots in 1,5 ml tubes.
Chondroitinase-ABC activity was assessed in a temperature controlled spectrophotometer (Olis, DW2000). To a 1 ml quartz cuvette, 640 µl of chrondroitinase buffer (60 mM Tris/HCl; 40 mM NaAc; pH = 8) were added and prewarmed until 37°C. Then, 50 µl of 20 mg/ml dermatan sulfate in Milli-Q water were added immediately followed by 50 mU of chondroitinase-ABC. The absorbance of the stirred solution was monitored continuously at 232 nm, following the digestion of the GAG polymers into disaccharides. The resultant enzyme activity was calculated using the law of Lambert-Beer with extinction coefficient of 3800 L/mol.cm.

Sample preparation

Abdomen test skin samples (3-5 mg) were degraded with pronase E as stated above for GAG extraction. For larger test samples (35-45 mg) 4 mg/ml pronase E was used in a 500 µl volume per sample. Incubation was done overnight in a microtube shaker at 55°C. After heat inactivation and evaporation using vacuum centrifugation, either 50 mU (small

samples) or 250 mU (large samples) of chondroitinase-ABC were added in chondroitinase buffer (50 mM Tris/HCl; 60 mM NaAC; pH = 8) and incubated at 37°C overnight in a microtube shaker. After evaporation using vacuum centrifugation, the pellet was solubilized by vortexing in a variable amount of 0,1M 2-aminoacridone (AMAC) in glacial acetic acid:DMSO (3:17 v/v) solution, followed by the addition of a same volume of 1M $NaBH_3CN$ in ultrapure water. The final volume was between 20 to 100 µl. Five µl of each solution were sufficient to dissolve the chondroitin sulfate disaccharide standards D0A0 and D0A6 after evaporation. The samples and/or disaccharide standards were derivatized using AMAC at 45°C overnight in an oven. The next day, the AMAC solution with $NaBH_3CN$ was evaporated and resuspended in DMSO:Milli-Q (1:1 v/v) solution. Resultant solutions were analyzed with high-performance liquid chromatography (HPLC) coupled to a fluorescent detector.

Method

The AMAC-derivatized GAG disaccharides were analyzed by reversed-phase high-performance liquid chromatography using an Agilent 1200 instrument and a C18 X-Bridge Shield column (4.6 mm x 100 mm, 3.5 µm). The mobile phase comprised of 60 mM ammonium acetate, pH = 5,6 (solution A) and acetonitrile (solution B). GAG disaccharides were eluted using a linear gradient from 2% to 30% v/v solution B in 50 minutes. The mobile phase flow was set to 0,5 ml/min and samples (1-8 µl) were injected using an autosampler. The fluorescence excitation and emission wavelength was set to 428 nm and 525 nm, respectively.
For HPLC coupled to MS, the same settings were applied with the omitting of the fluorescent detector and coupling of the mass spectrometer (LCQ Classic, Thermo Scientific). Also, a different HPLC instrument was used (Merck Hitachi, L-6200). The samples were allowed to be identified by MS between minute 10 and 33. Before minute 10 and after 33, the mobile phase was guided to the waste.

Strong anion exchange (SAX)

A 100 µl pipette tip was used to construct a ‘stagetip’ column using 3 layers of solid phase extraction disk, empore anion-SR 47 mm (3M Company, 66888-U). It was inserted into a 1,5 ml microtube to collect fractions during purification. The column was first conditioned with 50 µl MeOH, followed by 50 µl $H_2O$, 50 µl 1M NaOH and 3 x 50 µl $H_2O$, centrifuging at 500 g in between. Then, variable amounts of GAG disaccharides derivatized with AMAC and solubilized in DMSO:$H_2O$ (1:1 v/v) were applied and centrifuged at 500 g. The column was then washed with 100 µl MeOH several times, followed by elution with 100 µl of 10% trifluoroacetic acid (TFA) in MeOH. All fractions were analyzed by HPLC. For 10% TFA eluted fraction, it was first evaporated and solubilized in DMSO:$H_2O$ (1:1 v/v).

## Statistical analysis

Statistical analyses for comparison of healthy individuals, acute decompensated and chronic stable heart failure patients were performed using non-parametric Kruskal-Wallis test (GraphPad Prism v. 7,00). Multiple comparisons between the 3 groups were done with a Dunn’s test. p-Values of less than 0,05 were considered statistically significant. Results were plotted as mean ± standard deviation (SD).

# Results

The mean weight ± standard deviation and the coefficient of variation for the skin samples of the 3 study groups are shown in table 2. The full table can be found in supplementary table 2. The weights of the 'test' skin samples, derived from the abdomen, are listed in supplementary table 3. No differences in the weights were observed between lyophilizing or vacuum centrifuging the test samples (supplementary figure 3).

Although the samples were taken by the same operator with the same technique (supplementary fig. 1), still considerable weight variation existed between healthy individuals and patients with HF (fig. 4A). After lyophilizing the samples, the differences became even more evident (fig. 4B). A possible layer of fat could be present in the samples, just beneath the dermis. After defatting was finished, the samples converged to an average weight of 4 mg for healthy individuals and about 2 mg for acute decompensated or chronic stable heart failure (HF) patients (fig 2C, table 2).

After pronase E degradation of samples, a pellicle always remained. An immunohistostaining in figure 5 revealed that pronase E resistant material was keratin which is normally protecting the epithelial cells from damage and stress. Due to the fact that all measurements are corrected for the sample dry weight, it was important to determine the weight of the pellicle and subtract from the determined dry weight. The residual sample weight varied between -0.3 and 0.5 mg, being at zero mg for most of them (supplementary table 4). Therefore, no subtraction correction was done.

**Table 2. The mean weight ± standard deviation (SD) of the skin samples derived from healthy individuals (n = 12), patients with acute decompensated (AD, n = 8) or chronic stable heart failure (CS, n = 11).** CV = coefficient of variation, TWC = tissue water content.

| State | Net fresh weight (mg) | Net dry weight (mg) | TWC (mg) | % TWC | Net dry defatted (mg) | Total fat (mg) | % FAT |
|---|---|---|---|---|---|---|---|
| **Healthy** | 18,1 ± 2,4 | 6,3 ± 2,0 | 11,8 ± 1,0 | 66,0 ± 6,3 | 4,0 ± 0,5 | 2,4 ± 1,6 | 34,2 ± 13,6 |
| CV% | 13,38 | 31,39 | 8,35 | 9,48 | 13,51 | 67,04 | 39,73 |
| **AD** | 13,0 ± 3,8 | 3,6 ± 1,5 | 9,4 ± 2,7 | 72,4 ± 7,2 | 1,8 ± 0,8 | 1,7 ± 0,9 | 46,1 ± 16,0 |
| CV% | 29,64 | 42,26 | 28,65 | 9,98 | 40,81 | 51,10 | 34,79 |
| **CS** | 14,2 ± 3,1 | 4,2 ± 1,4 | 10,0 ± 2,6 | 70,3 ± 7,1 | 2,3 ± 0,6 | 2,0 ± 1,5 | 42,2 ± 18,3 |
| CV% | 21,56 | 33,98 | 25,79 | 10,11 | 23,33 | 74,47 | 43,33 |

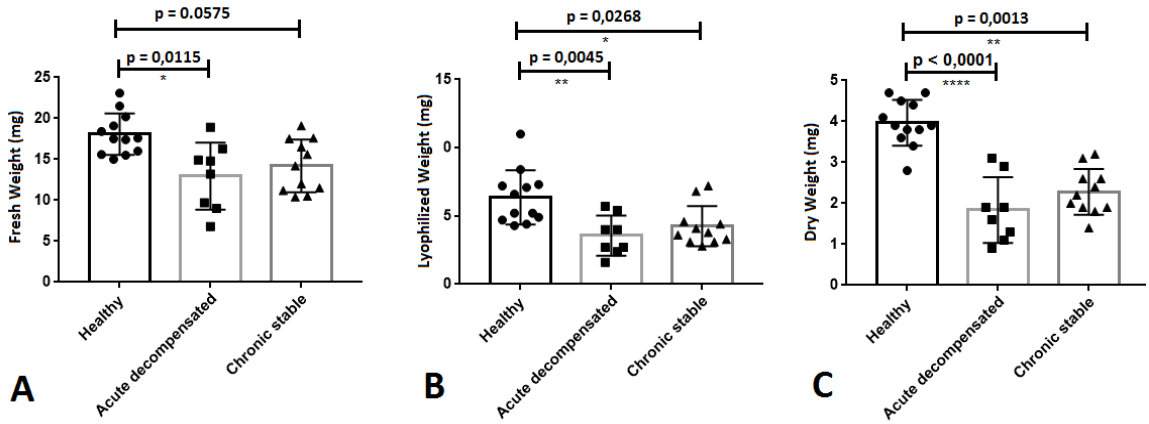



**Figure 4. The skin sample weights of healthy individuals (n = 12), patients with decompensated (n = 8) and chronic heart failure (n = 11).** The skin samples were weighted upon arrival (A = fresh weight), after freeze-drying, before defatting (B = lyophilizated), and after defatting, followed by freeze-drying (C = dry weight) and shown as mean ± SD

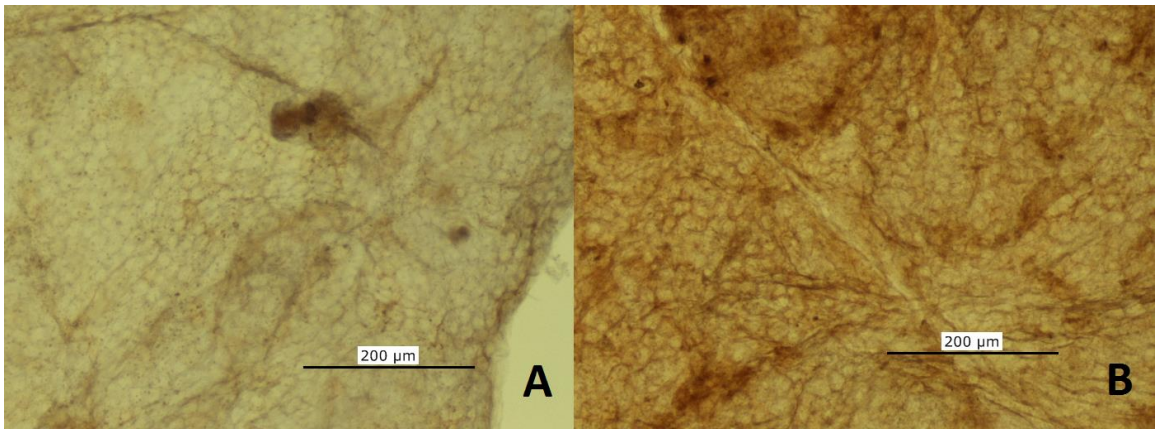
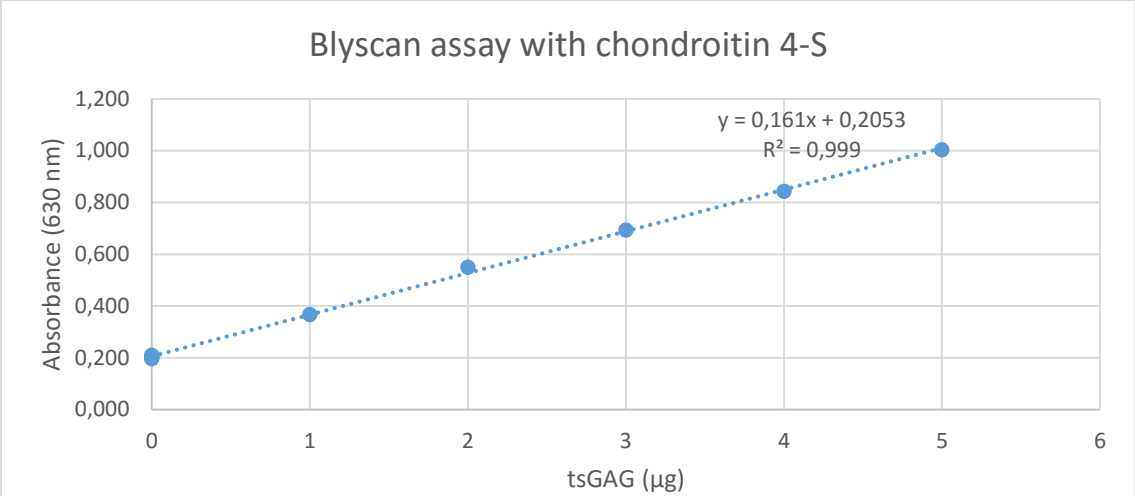



**Figure 5. Cytokeratin DAB staining on a pellicle after pronase E degradation of human skin sample.** Primary anti-cytokeratin antibody was omitted from A and serves as a control staining.

## Changes in the density of the negative charges on GAGs.

First of all, experiments had to be tested on test samples to determine the right concentration of pronase E, dilution factor, and to get familiar with the procedure of the assays itself. Figure 6 shows the results of a 'Blyscan sulfated GAG assay' with different concentrations of pronase E in a volume of 250 µl. Although, it was only tested on 3 samples, highest concentration of pronase E yielded the highest amounts of sGAG, compared to the lower concentrations of pronase E. With the decision to include hyaluronic acid ELISA, it was necessary to increase the pronase E buffer to 300 µl/sample.




| T46 0,8 mg/ml pronase E | T41 1,6 mg/ml pronase E | T40 2,4 mg/ml pronase E | T40 2,4 mg/ml pronase E |
|---|---|---|---|
| Dry weight = 5,8 mg<br>Total Volume = 250 µl | Dry weight = 5,3 mg<br>Total Volume = 250 µl | Dry weight = 5,8 mg<br>Total Volume = 250 µl | |
| **3,51 µg sGAG/mg DW** | **3,59 µg sGAG/mg DW** | **4,07 µg sGAG/mg DW** | **4,26 µg sGAG/mg DW** |

**Figure 6. A sulfated GAG Blyscan assay with different concentrations of pronase E on 3 different test skin samples.** A calibration curve with chondroitin 4-sulfate is shown. The highest concentration of pronase E (2.4 mg/ml) yielded the highest µg sGAG/mg dry weight (DW), shown in duplicate.

## Abdomen 'test' skin samples

Before proceeding to the patient's samples, it was necessary to optimize the protocol with test skin samples. They are derived from an abdomen of a person who underwent a fat removal surgery in the same way as the patient's leg samples. The results are supplementary to this article and therefore can be find in the supplementary section.

A lower cost alternative for the Blyscan sulfated GAG assay was performed with a 1-9 dimethylmetyhelene blue (DMMB) dye. The absorption wavelength had to be determined and was maximum at around 595 nm (fig. 7). The assay procedure was tested on test samples and the results are shown in supplementary figure 4.

It was not certain if the concentration of tUA and tsGAG in the skin of an abdomen would be the same as from a calf. Therefore, it was necessary to include one skin sample, isolated from a leg of an individual to be sure that all sample values fall into the calibration curve.
The results of the DMMB and carbazole assays are shown in table 3 and figure 8. Calibration curves for both DMMB and carbazole assays were linearly plotted ($R^2$ = 0,999 for DMMB and 0,9979 for carbazole) (fig. 8A and B).

DMMB and carbazole assays on the leg skin sample measured 7,86 µg sGAG/mg DW and 2,89 µg tUA/mg DW, respectively. Together with this sample, additional 4 abdomen 'test' samples were included in the test. The concentration of tsGAG varied between 9,51 and 11,85 µg/mg DW, while the concentration for tUA was between 3,23 and 3,99 µg/mg DW (table 3). These values were plotted against tissue dry weight (fig. 8C and D) and the correlation of tUA and tsGAG was determined (fig. 8E). A high correlation between the amount of tsGAG and tUA was observed($R^2$ = 0,9688).

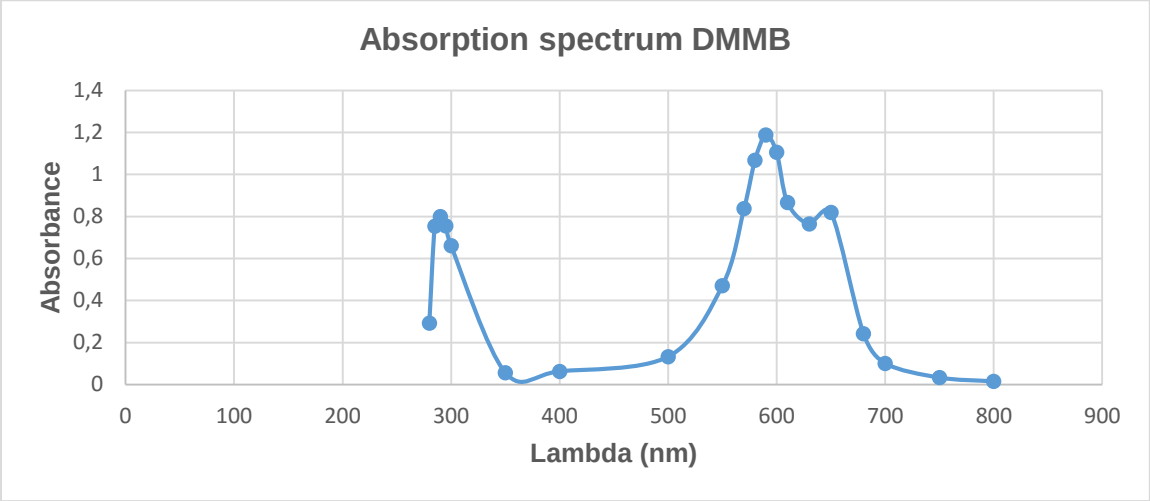



**Figure 7. An absorption spectrum of DMMB measured with SmartSpec plus spectrophotometer.**

**Table 3. The results of DMMB and carbazole assays in µg per mg tissue dry weight.** Glucuronic acid was used to set up a calibration curve for carbazole assay. Ab = Abdomen skin test sample, DW = tissue Dry Weight.

| | Leg Sample | Ab. 1 | Ab. 2 | Ab. 3 | Ab. 4 |
|---|---|---|---|---|---|
| **Dry Weight (mg)** | 4,2 | 3,2 | 3,3 | 3,9 | 3,5 |
| **µg tsGAG/mg DW** | 7,86 | 11,85 | 10,22 | 9,51 | 10,22 |
| **µg tUA/mg DW** | 2,89 | 3,99 | 3,6 | 3,23 | 3,61 |

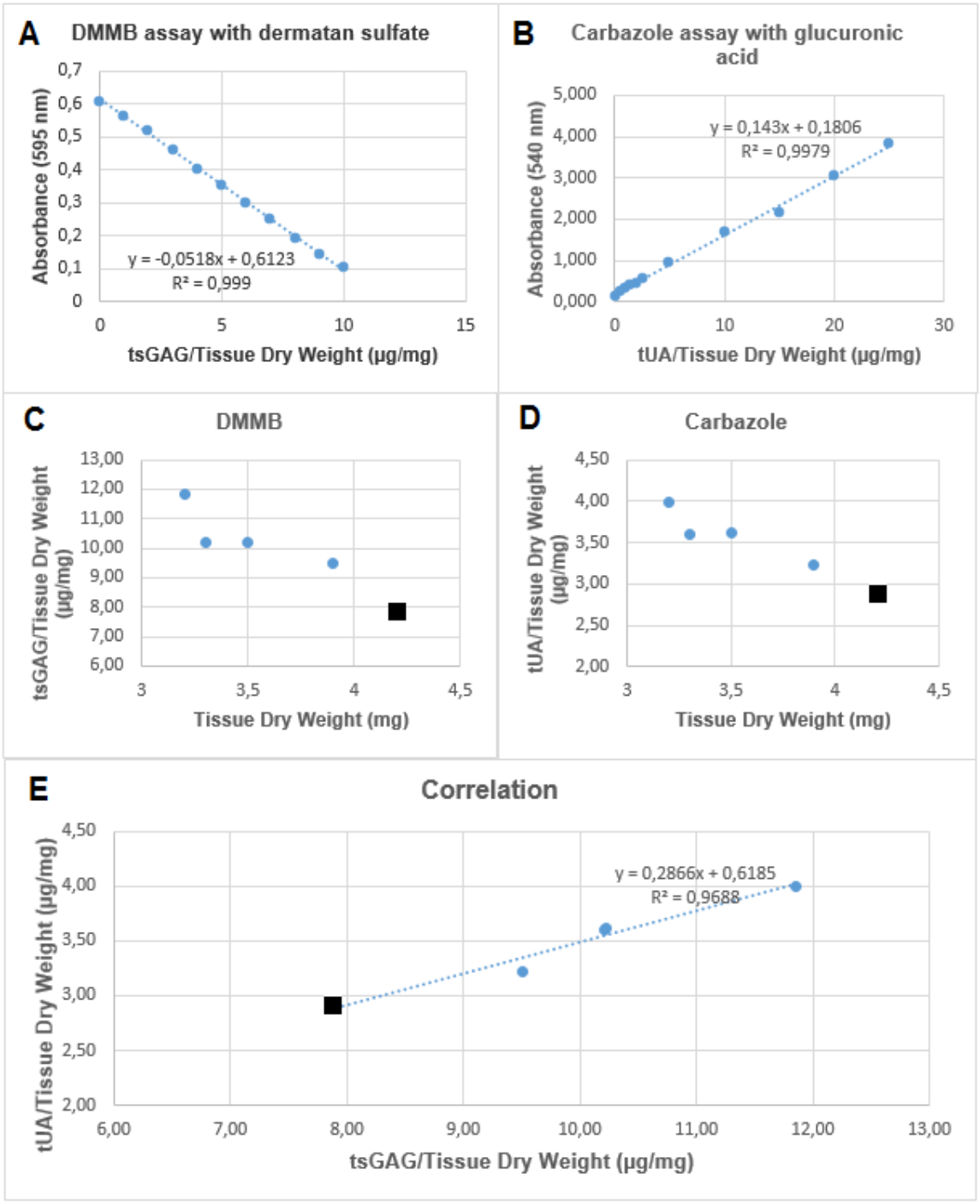



**Figure 8. Results of DMMB and carbazole assays on a leg skin sample, shown as black square, and 4 abdomen 'test' samples.** Calibration curves with coefficient of variation are reported for DMMB (A) and carbazole (B) assays. tsGAG and tUA per mg tissue dry weight (DW) were plotted against dry weight (C, D). The correlation between tsGAG and tUA is shown in E.

## Heart failure skin samples

After each skin biopsy was lyophilized and defatted, they were enzymatically treated with pronase E. Carbazole assay detected averaged about 10 µg tUA/mg dry weight for healthy individuals with a small standard deviation (table 4). The same applies for the amount of tsGAG/mg dry weight. Significant increase for tsGAG and UA was observed in acute decompensated HF patients compared to healthy controls (fig. 9A and B). Although the deviation is largest among all the groups, the p-value showed a significance level of 0,0003. Chronic stable HF patients showed only a significant increase in the amount of sGAG compared to healthy individuals. No significant differences for either tsGAG or tUA were observed between the 2 HF patient groups.
Hyaluronic acid contents seem not to change between the 3 groups investigated. Again, a large deviation is observed for acute decompensated patients compared to other groups (fig. 9C, table 4).
Water content in skin per mg dry weight was also measured. It showed a significant increase for both groups of HF patients (fig. 9D).

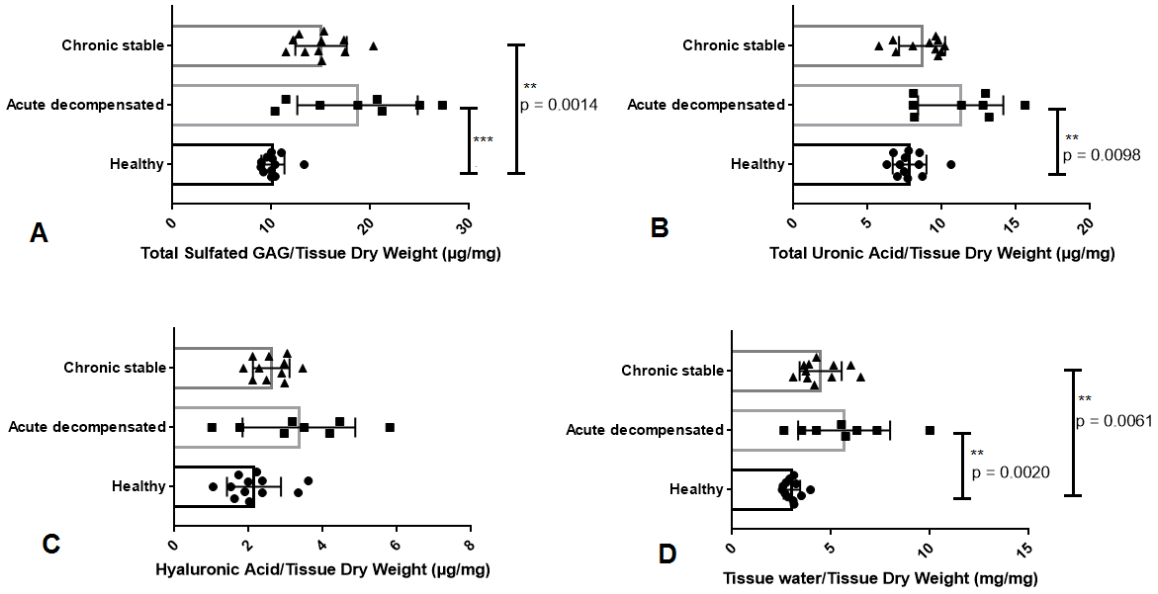



**Figure 9. Difference in HA, tsGAG, tUA and tissue water content per mg dry weight in skin samples.** Skin tissue was obtained from healthy individuals (n = 12), acute decompensated (n = 8) and chronically stable (n = 11) HF patients. tsGAG (A), tUA (B), HA (C) and tissue water (D) were measured and shown as mean ± SD. *** p = 0.0003.

**Table 4. Tissue water content, total sulfated GAG, total uronic acid and hyaluronic acid per mg dry weight (DW) were determined in skin samples of healthy individuals (n = 12), acute decompensated (AD, n = 8) and chronic stable HF patients (CS, n = 11).** Mean ± standard deviation (SD) are shown. Grade of edema: 0 = none; 1 = minimal; 2 = pitting edema; 3 = pronounced edema.

| State | Grade of edema | DW (mg) | µg sGAG/mg DW | µg uronic acid/mg DW | µg HA/mg DW | TWC/mg DW |
|---|---|---|---|---|---|---|
| Healthy | 0 | 2,8 | 13,32 | 13,38 | 3,61 | 3,96 |
| Healthy | 0 | 3,4 | 11,02 | 10,72 | 2,37 | 3,12 |
| Healthy | 0 | 3,6 | 10 | 9,85 | 2,22 | 3,5 |
| Healthy | 0 | 3,8 | 10,1 | 8,76 | 1,99 | 3,24 |
| Healthy | 0 | 3,8 | 10,39 | 9,78 | 1,62 | 3,13 |
| Healthy | 0 | 3,9 | 10,01 | 10,47 | 2,37 | 2,9 |
| Healthy | 0 | 3,9 | 9,55 | 9,5 | 2,02 | 2,82 |
| Healthy | 0 | 4,1 | 8,99 | 9,33 | 1,52 | 2,73 |
| Healthy | 0 | 4,4 | 10,01 | 8,76 | 1,9 | 2,57 |
| Healthy | 0 | 4,5 | 9,21 | 7,99 | 1,73 | 2,67 |
| Healthy | 0 | 4,7 | 10,37 | 10,34 | 3,34 | 3,09 |
| Healthy | 0 | 4,7 | 8,94 | 8,82 | 1,04 | 2,53 |
| **Mean ± SD** | / | 4,0 ± 0,5 | 10,2 ± 1,1 | 9,8 ± 1,3 | 2,1 ± 0,7 | 3,0 ± 0,4 |
| **State** | **Grade of edema** | **DW (mg)** | **µg sGAG/mg DW** | **µg uronic acid/mg DW** | **µg HA/mg DW** | **TWC/mg DW** |
| AD | 0 | 0,9 | 27,31 | 21,73 | 4,19 | 7,33 |
| AD | 2 | 1,1 | 25 | 19,94 | 3,5 | 10 |
| AD | 3 | 1,3 | 18,76 | 14,07 | 1,76 | 6,31 |
| AD | 2 | 1,6 | 21,2 | 17,58 | 4,46 | 2,62 |
| AD | 1 | 1,9 | 20,71 | 19,67 | 5,81 | 5,53 |
| AD | 2 | 1,9 | 14,92 | 15,38 | 3,18 | 5,74 |
| AD | 3 | 2,9 | 10,38 | 10,75 | 1,01 | 3,52 |
| AD | 3 | 3,1 | 11,47 | 10,55 | 2,96 | 4,26 |
| **Mean ± SD** | / | 1,8 ± 0,7 | 18,7 ± 5,7 | 16,2 ± 4,0 | 3,4 ± 1,4 | 5,7 ± 2,2 |
| **State** | **Grade of edema** | **DW (mg)** | **µg sGAG/mg DW** | **µg uronic acid/mg DW** | **µg HA/mg DW** | **TWC/mg DW** |
| CS | 0 | 1,4 | 17,34 | 15,24 | 2,89 | 6,5 |
| CS | 0 | 1,8 | 20,32 | 14,02 | 3,46 | 6 |
| CS | 0 | 1,9 | 17,45 | 13,64 | 2,28 | 4,16 |
| CS | 0 | 1,9 | 13,41 | 14,02 | 2,11 | 4,26 |
| CS | 1 | 2 | 14,77 | 10,55 | 2,11 | 3,8 |
| CS | 1 | 2,2 | 12,19 | 11,55 | 2,48 | 3,09 |
| CS | 2 | 2,4 | 15,1 | 12,78 | 2,97 | 3,88 |
| CS | 0 | 2,6 | 15,05 | 13,15 | 2,96 | 3,73 |
| CS | 0 | 2,6 | 15,32 | 12,13 | 3,04 | 5,04 |
| CS | 0 | 3,1 | 11,48 | 8,24 | 1,86 | 5,13 |
| CS | 2 | 3,2 | 12,8 | 9,3 | 2,55 | 3,63 |
| **Mean ± SD** | / | 2,3 ± 0,5 | 15,0 ± 2,5 | 12,2 ± 2,1 | 2,6 ± 0,5 | 4,5 ± 1,0 |

Tissue water content was evaluated to determine their correlation with GAG content in skin. A linear trend was observed between tissue water content and tsGAG, tUA, or HA for all 3 groups (fig. 10). When $R^2$ value was above 0.35, the analysis was accepted as a good linear correlation. $R^2$ values between 0.2 and 0.35 indicate a relatively weak linear correlation, while values below $R^2$ suggest no linear correlation at all. Having these criteria in mind, only tsGAG showed a significant positive correlation with tissue water content (fig. 10A). For tUA , a weak positive correlation was observed (fig. 10B). HA showed no linear correlation (fig. 10C).

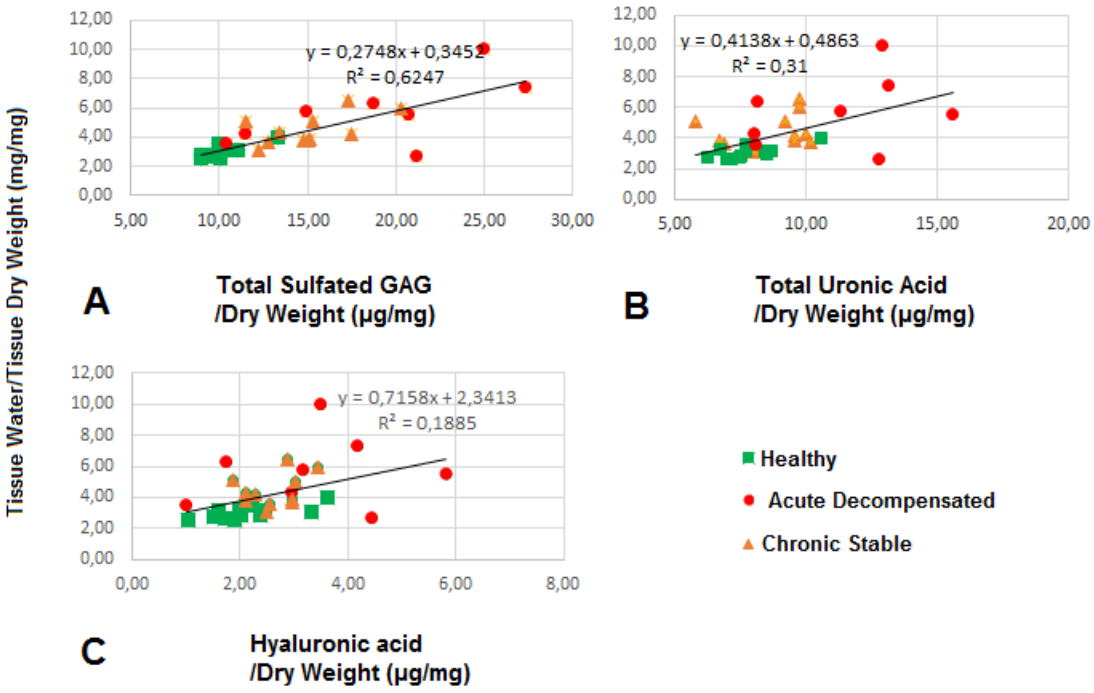



**Figure 10. Correlations between tissue water and GAG amount in skin samples for all 3 groups.** Water content in skin samples of healthy individuals (n = 12), acute decompensated (n = 8), and chronic stable HF patients (n = 11) was compared to tsGAG (A), tUA (B), or HA (C).

The results of hyaluronic acid, total sulfated GAGs, total uronic acid and tissue water content per mg dry weight were plotted against dry weights of the samples (fig. 11). It is notable that they all express a negative exponential trend. This was very obvious for tsGAG (fig. 11A). Because samples with low weight had the highest concentration of tsGAG, tUA or HA, while samples with highest weights converged to less or more one value, it was thought that pronase E did not entirely degrade the samples. Therefore, to test the validity of the results, the samples were once more treated with an aliquot of enzyme whereafter the DMMB assay was performed (supplementary table 5). No differences with the blanc measurements were found. Furthermore, this exponential trend was already visible before the samples were incubated with pronase E, as seen from the tissue water content/mg dry weight (fig. 11D). It is thus worthy to note that the measured differences in the results for tsGAG, tUA and HA are due to their biological host and their state at the moment the biopsy was derived and not due to technical/lab errors. Nevertheless, increasing the concentration of pronase E with increased dry weight of the sample might have been better, instead of adding the same volume of pronase E to each

sample. The problem stated above would then not even occur, but it would be hard to correct measured values for the blanc measurement, as several of them would be required.

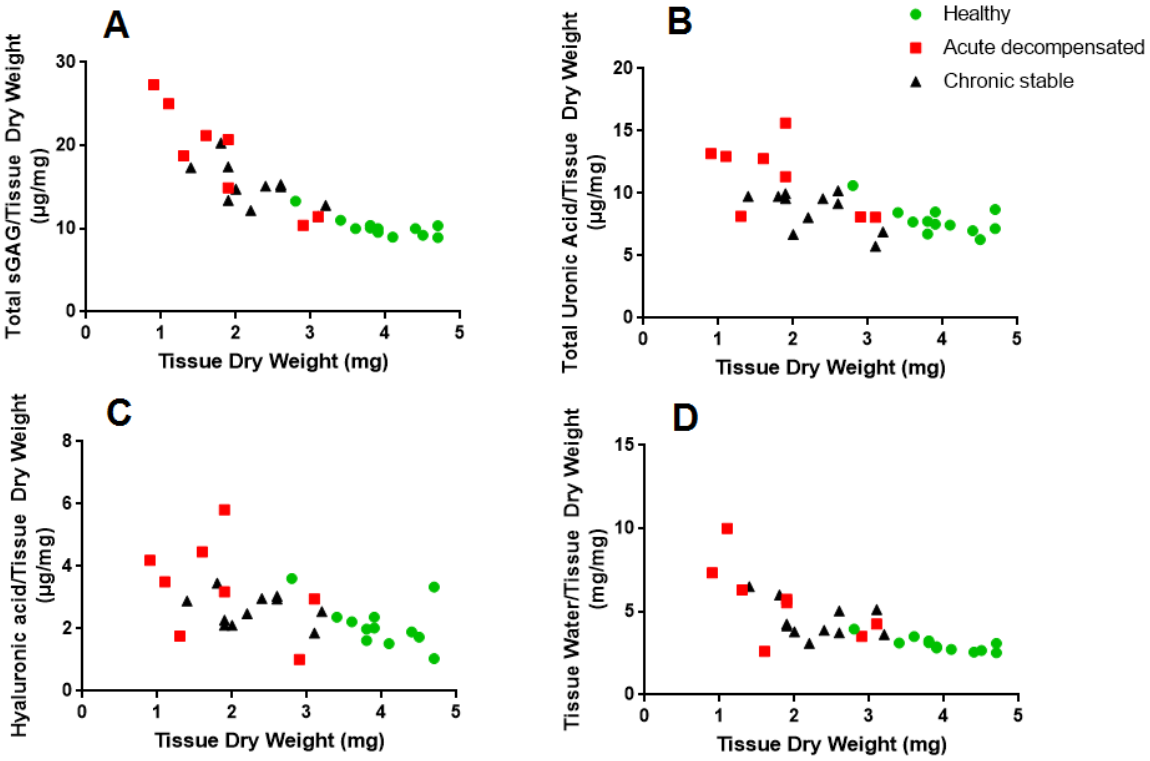



**Figure 11. Amount tissue water and GAGs in skin samples in function of its dry weight.** Skin tissues were obtained from healthy individuals (n = 12), acute decompensated (n = 8) and chronically stable (n = 11) HF patients**.** tsGAG (A), tUA (B), HA (C) and tissue water were analyzed.

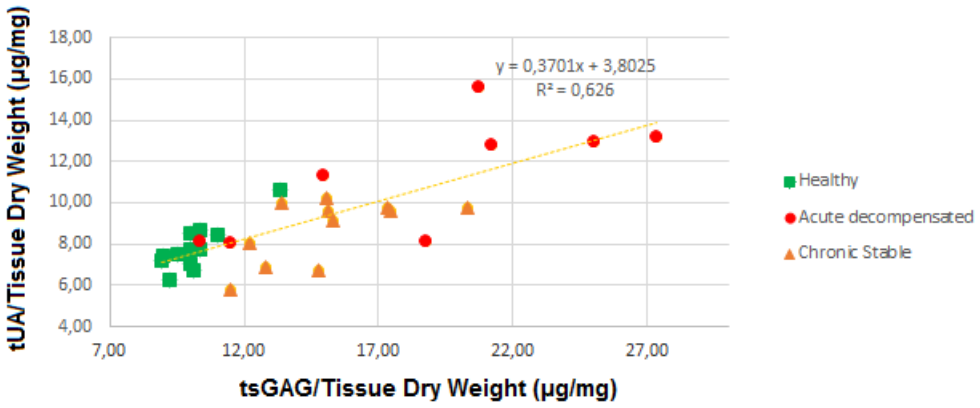



**Figure 12. Correlation between tsGAG and tUA in skin samples of healthy individuals (n = 12), acute decompensated (n = 8) and chronic stable (n = 11) HF patients.**

A plot of tsGAG/mg dry weight against tUA/mg dry weight resulted in a linear trend with $R^2 = 0.626$ (fig. 12). This is a strong correlation. Hence, samples with low sGAG content also have low tUA concentration..

TsGAG, tUA and HA were evaluated in function of the grade of edema (fig. 13). An individual could have been graded zero for no edema, 1 for discrete pitting edema, 2 for pitting edema below the knee, 3 for clinical pronounced edema with important pitting and 4 for anasarca edema above the knee. There were no patients present in this study with the most extreme form of edema. Most acute decompensated patients had a pronounced or pitting edema with the highest amount of tsGAG, tUA and HA content. For chronic stable HF patients, no remarkable differences were observed between having an edema or not. The tsGAG concentration remains significantly higher even in patients without edema, as stated before.
Remarkable is the acute decompensated patient with no edema (grade 0). This patient has a very low concentration of HA and tsGAG compared to the others. Therefore we omitted this patient out of analysis to compare only acute decompensated patients with edema (fig. 14). By comparing it with figure 9, tsGAG, tUA and tissue water between acute decompensated patients and healthy individuals become even more significant. Very important is the fact that HA content becomes significantly different between decompensated patients and healthy controls, which was not previously seen (compare fig. 14C and fig. 9C).

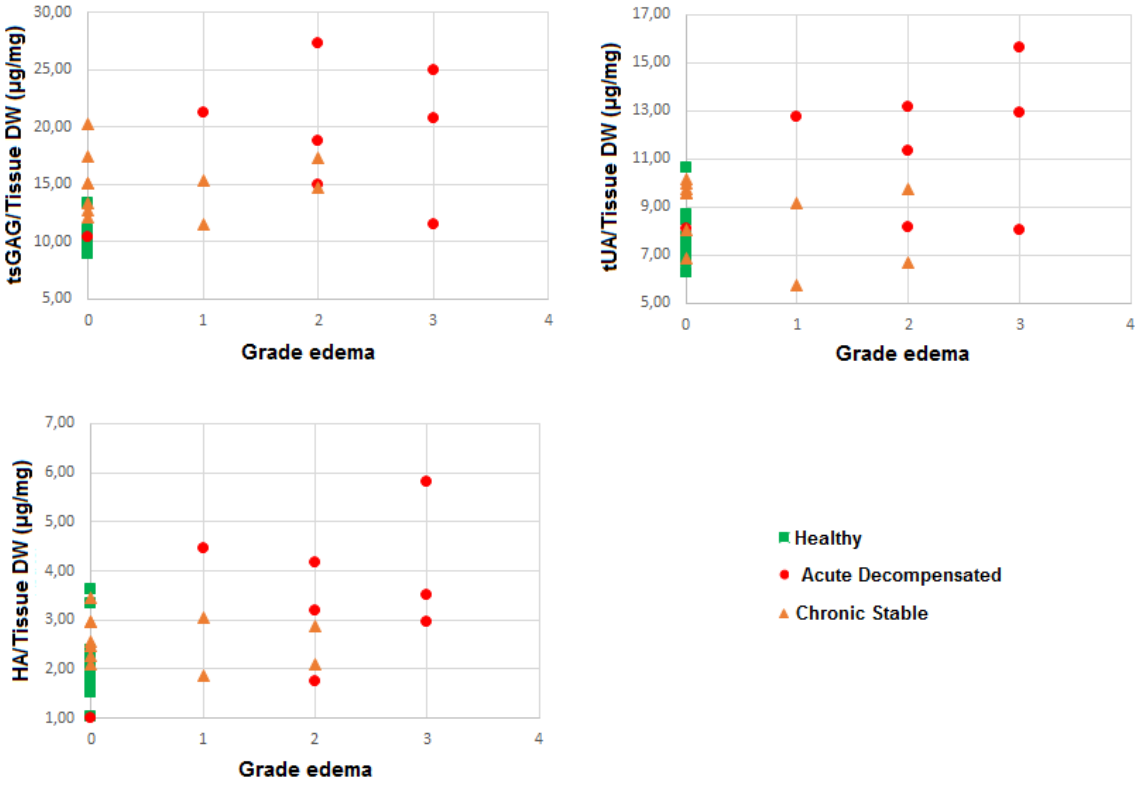



**Figure 13. The concentration of tsGAG, tUA and HA in µg per mg dry weight in function of the grade of edema.** Healthy individuals (n = 12), acute decompensated HF patients (n = 8), chronic stable HF patients (n = 11). Grade of edema: 0 = none, 1 = minimal, 2 = pitting edema, 3 = pronounced edema.

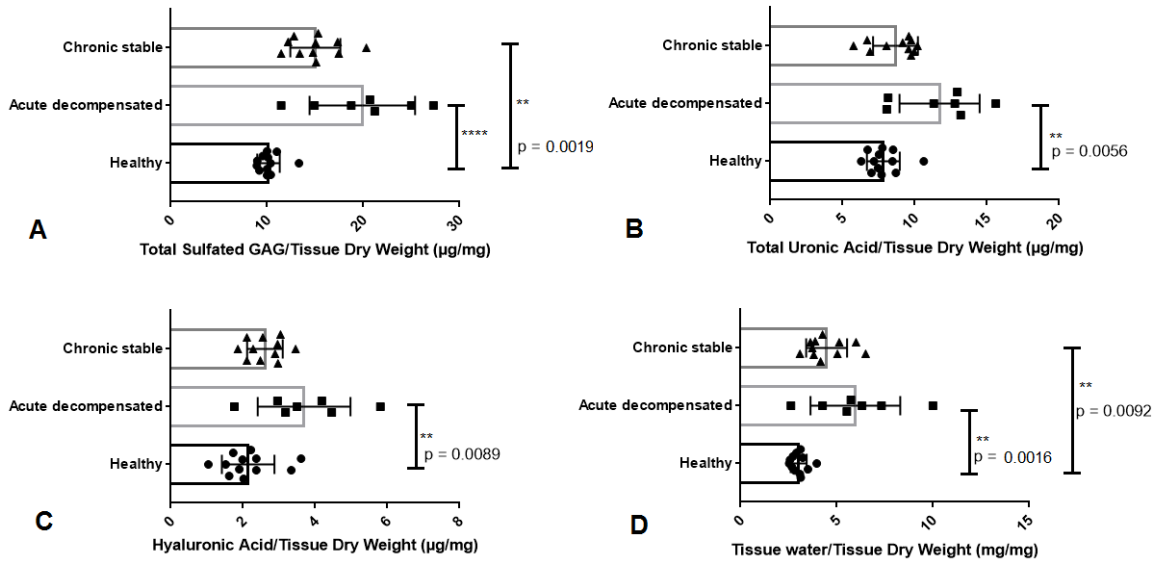



**Figure 14. Difference in HA, tsGAG, tUA and tissue water content per mg dry weight in skin samples.** Skin tissue was obtained from healthy individuals (n = 12), acute decompensated (n = 7) and chronically stable (n = 11) HF patients. Note that 1 AD patient without edema was left out of this analysis. tsGAG (A), tUA (B), HA (C) and tissue water (D) were measured and shown as mean ± SD. **** $p < 0.0001$.

## High-performance liquid chromatography and mass spectrometry.

No HPLC assay could have performed on patient's samples. This is due to too low sample volume that was available. Still, this technique is widely used to detect smallest concentrations of a component in a complex solution. Therefore, it was decided to set up a compositional mapping of GAGs with human abdomen test skin samples as described in material and methods.

Both chondroitin sulfate standards (D0A0 and D0A6) were analyzed by HPLC (fig. 15). The result is shown for 250 pmol for both standards. Three pronounced peaks are seen at different retention times. Sulfated GAG disaccharide standard was eluted first, followed by unsulfated, and eventually a saturated peak for unbound AMAC is observed. Different smaller peaks are unspecific and are AMAC related.
To quantify GAGs from samples with unknown concentration, calibration curves of both chondroitin sulfate standards were made. The peak area (luminescence * s ) measured by HPLC was plotted on the y-axis against known concentration of standards (fig. 16). Two different calibration curves were obtained. A calibration curve for di- or trisulfated GAG disaccharides would probably have yet another slope. This means that in order to precisely quantify GAGs in samples, different disaccharide standards are needed.

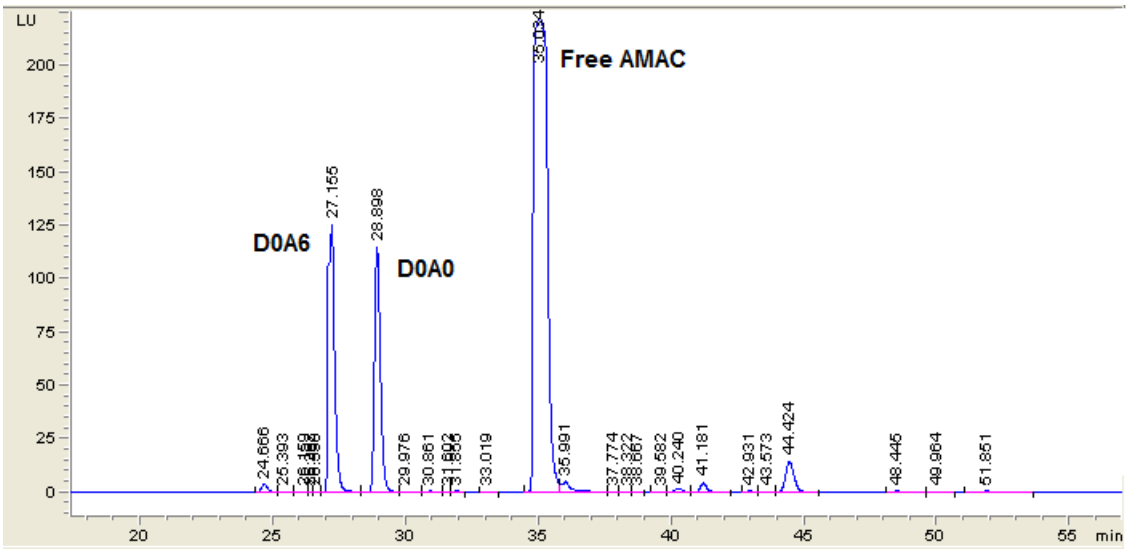



**Figure 15. A chromatogram as determined by HPLC after the separation of AMAC derivatized chondroitin sulfate disaccharide standards.** The initial concentration for both standards was 250 pmol. D0A0 = unsulfated chondroitin disaccharide standard, D0A6 = monosulfated chondroitin disaccharide standard.

Next, a HPLC analysis on chondroitinase-ABC digested abdomen test sample was performed (fig. 17). Many small peaks are observed. As all GAG disaccharides should elute before the retention time of the free AMAC, all the other peaks after this retention time are not specific. By comparing the retention times of unspecified peaks in figure 15 with those of figure 17, many of them are also detected in the abdomen sample, but in higher concentrations. This is due to higher amounts of AMAC and $NaBH_3CN$ that were used during derivatization, demonstrating that these peaks are AMAC related.
Furthermore, the peaks with the same retention times as those of chondroitin sulfate disaccharide standards can also be found in the abdomen test sample. This does not mean however, that these peaks are un- or monosulfated GAG disaccharides. Many other, unknown peaks are observed with similar area. Therefore, it was decided to do a mass spectrometric analysis that could distinguish peaks based on their mass-to-charge (m/z) ratio. Additionally, because relatively low height of peaks was observed in the test sample, ten times bigger skin sample was used for HPLC-MS analysis.

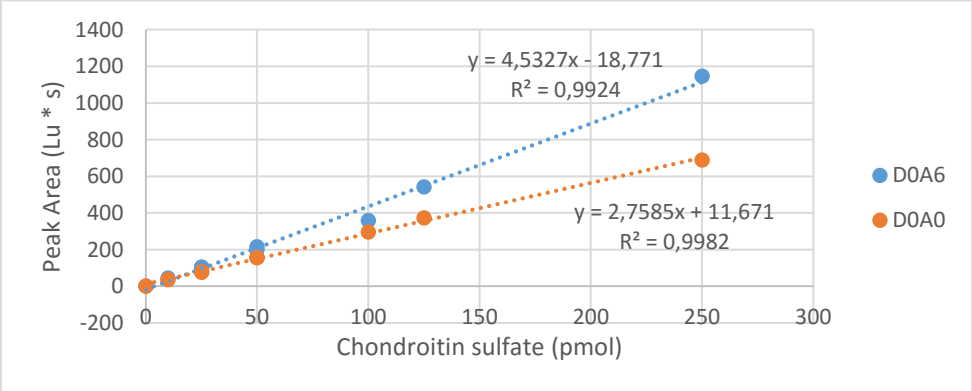



**Figure 16. Calibration curves for D0A0 and D0A6 chondroitin sulfate disaccharide standards after derivatization with AMAC and detection with HPLC by means of peak area.** D0A0 = unsulfated chondroitin disaccharide standard, D0A6 = monosulfated chondroitin disaccharide standard.

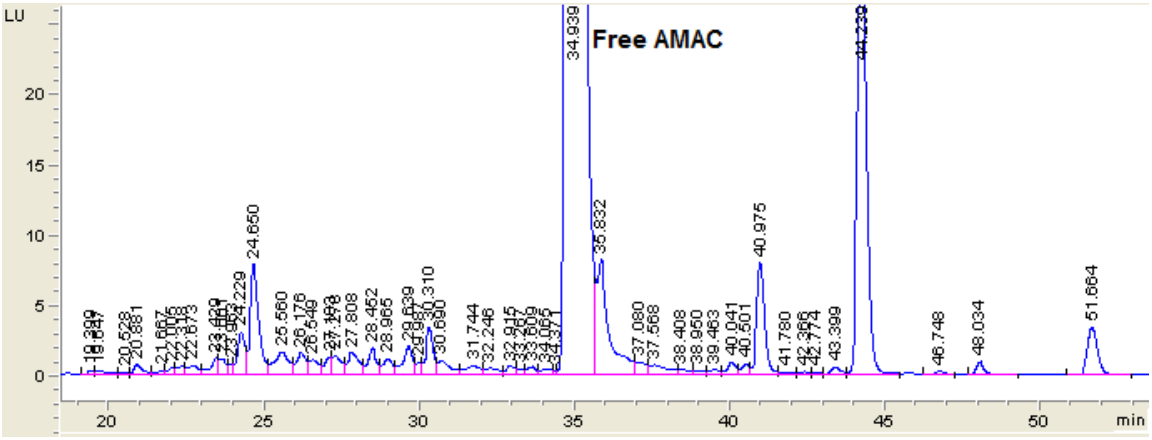



**Figure 17. A chromatogram as determined by HPLC after the separation of AMAC derivatized GAG disaccharides from an abdomen test sample.** Many unspecified peaks are present, which make the analysis difficult.

Figure 18 shows the results of HPLC-MS of chondoitinase-ABC digested large skin sample (42 mg), derivatized with AMAC. It was measured with a positive mode, as nothing was detected in negative one. Unsulfated, mono-, di-, and trisulfated GAG disaccharides were searched for with respective m/z of 574,1 + 80 g/mol for each sulfate. The m/z of 574,1 corresponds with the mass of unsulfated GAG disaccharide (379,1 g/mol; found in table 1) + the molecular weight of AMAC (194 g/mol) + 1 proton. The mass spectrometer was allowed to analyze the mobile phase between minute 10 and 33. Measurements before and after these time intervals were guided to the waste to minimize the pollution of MS.
Trisulfated GAG disaccharide with m/z of 814 was detected as 1 pronounced peak at a retention time of 22,38 on the chromatogram (fig. 18D). By looking at chromatograms for di-, mono- and unsulfated GAG disaccharides with the respective m/z ratio of 734; 654,1 and 574,1 in this order, one additional peak shows up later in the retention time. It is clear that different GAG disaccharides with different sulfate patterns were found in this sample. Less obvious is the fact that each chromatogram for a certain m/z ratio also showed the previously detected peaks at higher m/z. Chromatogram for unsulfated GAG disaccharides detects therefore all 4 peaks (tri-, di-, mono- and unsulfated) (fig. 18A). Probably, during the ionization process within the MS, sulfate ions become unstable and are fragmented. Therefore, all sulfate patterns are detected on a given retention time.
The peak at minute 10 for the detection of unsulfated GAG disaccharides is unspecific due to starting of the measurement.

The height of the detected peaks and the corresponding areas in both small and large skin samples seemed to be low (results not shown). Therefore, enzymatic activity of chondroitinase-ABC was tested on the efficiency of GAG depolymerization into disaccharide units (fig. 19). As mentioned before, this was necessary to expose the reducing end of a saccharide for 2-aminoacridone (AMAC) binding during derivatization process (fig. 2). Fifty mU of chondroitinase-ABC were tested and the efficiency calculated with the law of Lambert-Beer. It was found that the actual enzymatic activity, measured between minute 2 and 6, was 8,8 mU or 17,6% efficiency. An inquiry was sent to Sigma-

Aldrich with the described results (supplementary attachment 3). The company responded by gifting a new chrondrotinase-ABC enzyme with another lot-number. The test was performed once again with the resultant measured activity of 13,5 mU or 27% efficiency (results not shown).

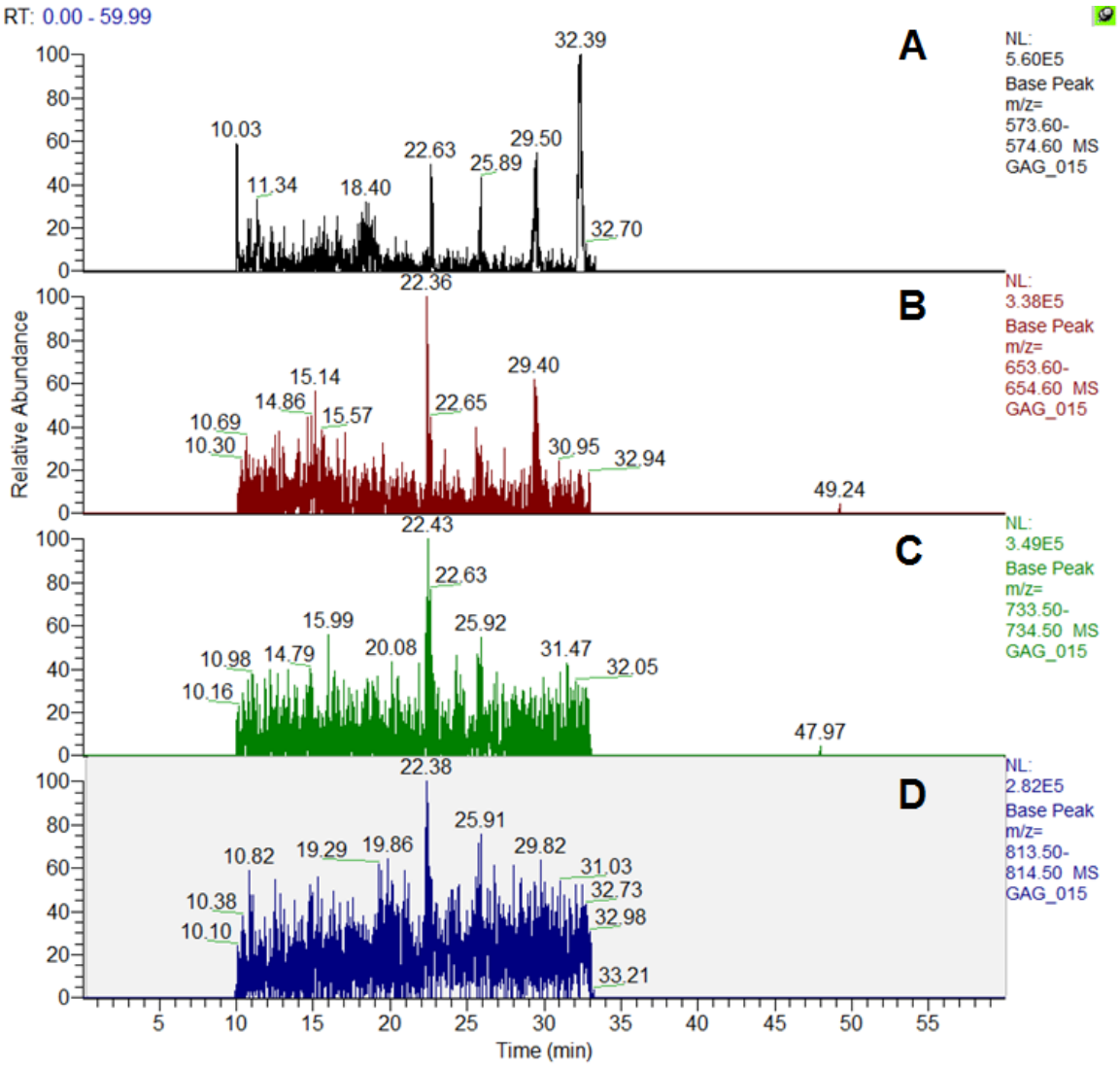



**Figure 18. Chromatogram of chondrotinase-ABC digested and AMAC derivatized large skin sample, separated by HPLC and detected with MS.** The mobile phase comprised of 60 mM ammonium acetate, pH = 5,6 and acetonitrile, which was gradually increased from 2% to 30% v/v from start till minute 50. Before minute 10 and after minute 33, the mobile phase was guided to the waste. Mass-to-charge ratios of 574,1 (+ 80/160/240) were analyzed.

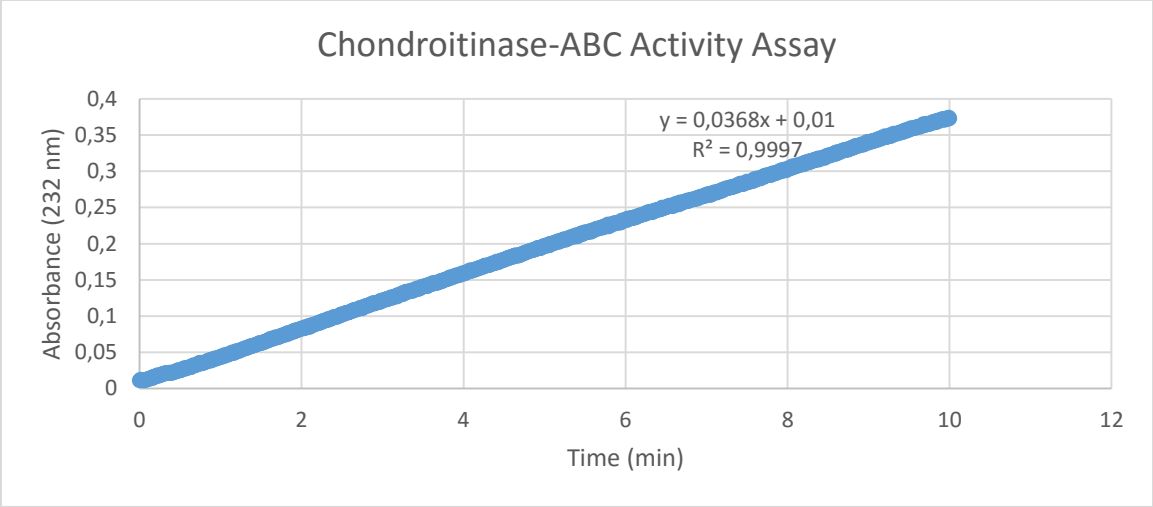



**Figure 19. A linear depolymerization of dermatan sulfate standard into disaccharides was achieved during chrondroitinase-ABC digestion, measured with temperature-controlled spectrophotometer at 37°C.** Fifty mU of the enzyme were tested, constantly monitored during 10 minutes. The actual enzyme activity was calculated between minute 2 (Abs = 0,083) and minute 6 (Abs = 0,233).

## Strong anion exchange

Many unspecified peaks were detected during the separation of either standards, or samples by HPLC. To distinguish between GAG disaccharides and AMAC-related peaks, SAX was performed. Furthermore, it was interesting to perform mass spectrometric analysis during the complete run from zero till 50 minutes. Since the instrument becomes polluted by the high presence of free AMAC or AMAC-related dirt, purification was necessary.

A stagetip column was constructed containing 3 layers of solid phase extraction disk membrane (fig. 20). After conditioning of the membrane, the negatively charged carboxyl groups on D0A0 or both sulfated and carboxyl groups on D0A6 were allowed to bind with quaternary ammonium groups in the membrane. After washing unbound molecules, the GAG disaccharides were eluted with strong acid. All fractions were analyzed by HPLC and the percentage purified GAG disaccharides calculated (table 5).

**Figure 20. A stagetip column containing 3 layers of solid phase extraction disk in a 100 µl pipette tip.** The tip was inserted into the microtube to collect fractions during purification.

**Table 5. Strong anion exchange fractions of chondroitin sulfate disaccharide standards were analyzed by HPLC.** Area was compared and the percentage of purified disaccharides standards calculated.

| | Area D0A6 (Lu*s) | Area D0A0 (Lu*s) | Area AMAC (Lu*s) |
|---|---|---|---|
| **Before SAX** | 1970,1 | 1344,6 | 6364 (saturated) |
| **Breakthrough** | / | / | 973,6 |
| **Washing 1** | / | / | 3799,2 |
| **Washing 2** | / | / | / |
| **Elution 1** | 1438,6 = 73% | 1259,6 = 93,7% | 1057,6 |
| **Elution 2** | 92,9 = 9,4% | 7,0 = 1,0% | 9,3 |
| **% Purified** | 82,4 | 94,7 | |

# Discussion

Heart failure is a condition caused by structural and/or functional cardiac abnormalities resulting in a reduced cardiac output and/or elevated intracardiac pressures (36). These abnormalities lead to neurohumoral activation which is initially an adaptive response to restore tissue perfusion. However, upregulated neurohumoral systems become detrimental and cause disease progression, mainly by the retention of sodium and water in the body. This causes fluid accumulation in different organs, clinically most recognized as peripheral or pulmonary edema. Nevertheless, patients with congestive heart failure are not always present with an overt edema. The reason for this is difficult to explain based on patient's hemodynamics or neurohumoral activation. Therefore, other factors must play an important role. Recently, it was hypothesized that the interstitium may play a decisive role. The interstitium consists of glycosaminoglycans, and the degree of negative charges related to these macromolecules may be associated with the degree of fluid buildup in this compartment in patients with heart failure.

The skin is one of the largest organs in the human body. Skin biopsies were acquired from legs of participating individuals. The skin is a protecting layer against external factors, made up of multiple layers of ectodermal tissue. It functions as an insulation layer, it regulates temperature and is responsible for sensation (37). Because it is an external body organ, taking a biopsy is relatively easy compared to the internal organs. The leg skin biopsy also represents very well the state of a patient related to heart failure and edema development. Due to gravity forces, the redundant fluid in the body as a result of heart failure eventually accumulates there. At last, skin has the largest available interstitium compared to interstitia of other organs. Therefore, it is not surprising why a calf skin biopsy was opted over other possibilities to measure interstitial (sulfated) GAG and water content.

We hypothesized that there would be important differences in the skin interstitium of healthy subjects and patients with heart failure related to negative charge density on GAGs. Moreover, we hypothesized that patients with chronic stable heart failure would have more of the interstitial negative charges as compared to healthy individuals, while acute decompensated heart failure patients with overt edema would have less.

To investigate our hypothesis, human skin samples from healthy subjects, stable heart failure patients and patients with acute decompensation were analyzed for GAG content, sulfated GAG content and water content. The main findings are that there is a significant difference in GAG content and sulfation-pattern between healthy controls and patients with heart failure and that there is a strong correlation between higher sulfation bounds of GAGs and tissue water content.

## Differences in GAG content and tissue sulfation.

The values for tsGAG, tUA and hyaluronic acid per mg dry tissue correspond with the values found by Oh et al. in skin specimens of buttock for healthy individuals (38). Although it was expected to find an increase in tUA and tsGAG for chronically stable HF patients and a decrease for acute decompensated HF patients compared to healthy individuals, it is shown in this study that chronic stable as well as acute decompensated patients have significantly higher amounts of sGAG and tUA as compared to healthy controls (fig. 9).

In the compensated state, it was hypothesized that the patients will have a larger negative charge density compared to healthy individuals. This is due to upregulation of the synthesis of GAGs in response to high sodium concentration by the body. More GAGs synthetized results in more binding sites to sequester sodium ions and thus keeping osmotic value in range. As only GAG sulfation is significantly upregulated in this state, it must be concluded that especially sulfate ions are responsible for binding and buffering of sodium ions in the compensated state (fig. 9A).
At the time of decompensation, not only GAG sulfation is increased, but also the total amount of GAGs, as seen from the total uronic acid concentration (fig. 9B). This could be explained by a transition of compensated towards decompensated state. As said before, during compensated state, more GAGs (and especially more sulfate groups) are synthetized in response to increased sodium concentration. At this stage, no overt edema is observed as fluid accumulation into the interstitum is opposed by the tensile forces of GAGs. This ongoing state of GAG synthesis reaches its maximum at the state of decompensation. When the GAG tensile forces suddenly lessen due to altered conformation, fluid accumulation and subsequent edema development are allowed to happen. This does not mean that at the time of decompensation, GAGs become desulfated or have less uronic acid. The half-life of dermatan sulfate and chondroitin sulfate in elderly rats is 2 and 4 days respectively (39). In humans, it might be just even more. That's why, it may be possible that by the time the patients arrive in a hospital after decompensation (most often at the same day), they still have considerable amounts of tUA and tsGAG present in their skin interstitium when the biopsy is taken. Another factor contributing to this theory is the fact that acute decompensated patients show a large standard deviation for both tsGAG and tUA.
Remarkably, hyaluronic acid did not show any significant differences between the 3 groups. It is a unique molecule among GAGs in that it is not sulfated. Due to the fact that especially sulfate pattern plays an important role in compensated state, no differences at this stage are seen compared to healthy individuals. In acute decompensated state however, a high standard deviation is observed. It may suggest that a possible upregulation of HA still happens in decompensated patients. But due to a short half-life of less than a day in skin, this theoretical increase may be lost (40).

## Tissue water content is correlated with tsGAG.

Since no differences in weight were observed in the test skin samples between drying by lyophilization and vacuum centrifugation, it was chosen to lyophilize the upcoming samples as they require to be frozen during the procedure.
There were significant differences in the dry weights between skin samples of healthy individuals and samples of heart failure patients after freeze-drying (fig. 5B). Nevertheless, only evaluating the water that had been sublimated during freeze-drying gives a faulty impression. It seems that the volume lost during lyophilization is not significantly different between the 3 groups (supplementary fig. 5). But, it is important to note that already before the lyophilization of samples had started, the fresh skin sample weights were significantly different (fig. 5A). A ratio of the volume water that was lyophilized and the net dry weight corrects for these initial weight differences (fig. 9D). This ratio however, is only a rough estimate of the real value. At the moment of taking a punch biopsy, a certain volume of water already leaked out at the place of the incision due to abundant fluid that is present in patients with heart failure. As water also takes in a certain volume during the incision process, this may account for the observed initial weight differences between healthy individuals and patients with heart failure.

Tissue water content per mg dry weight had a strong linear trend with tsGAG, a weak correlation was observed with tUA and no correlation, but a risking trendline, with HA for the 3 groups together (fig. 12). Most acute decompensated heart failure patients had a pronounced or pitting edema and thus a high initial volume of tissue water. According to correlation analysis, it means that acute decompensated HF patients should have highest amounts of tsGAG, tUA and HA content, healthy individuals the lowest content, while chronic stable patients in between. This outcome is in accordance with the results described above.

The above stated results up till now are described for the individual groups (healthy/acute decompensated/chronic stable), which are compared to each other. The 2 groups with heart failure can actually be splitted up in 4 groups, based on their clinical presentation of the edema at the time the biopsy was taken (fig. 13). Healthy individuals obviously do not have edema and therefore cannot be splitted up. As only 8 acute decompensated and 11 chronic stable HF patients are available, which are further splitted up in 4 groups, no statistical significance could be tested.
It is very remarkable that one acute decompensated patient with no edema has one of the lowest concentrations for HA, tsGAG and tUA compared to all individuals. A possible explanation is that not all individuals gradually progress from compensated towards decompensated stage. Very interesting is the fact that when we omit this acute decompensated patient without edema out of the analysis, HA content becomes significantly different compared to healthy individuals. Also tsGAG and tUA content become even more significant (fig. 14).

## High-performance liquid chromatography

HPLC with a fluorescent detection was described as a reliable method to detect even the smallest amounts of a compound in a mixture. Nevertheless, it has not been performed on patient's samples. There are three reasons for this. First, a carbazole method can be as reliable as a HPLC assay in detecting and quantifying uronic acids in samples (41). Second, only 150 µl out of 300 µl sample was required to do the carbazole assay, leaving enough volume to do the DMMB and HA assays. HPLC however, required to use the whole sample volume. Third, even with 300 µl of sample, it had poor results in detecting GAGs, probably due to malfunctioning of the chondroitinase ABC enzyme. Hence, the carbazole assay was the preferred method of choice to detect GAG uronic acid.

Concerning the profiling of GAGs, it requires much further exploration. Although we succeeded in determining mono, di- and trisulfated GAGs in bigger skin samples with mass spectrometry, it needs further optimizations. Furthermore, the method that had been used to separate GAGs on HPLC was meant to use with the negative mode of MS. Here, a positive mode was used, as nothing was detected with a negative one. Additionally, poor enzyme activity of chrondroitinase-ABC and the need of more GAG disaccharide standards, which have a high cost, hampered future exploration.
Although 82,4 and 94,7% was recovered for D0A6 and D0A0 respectively by using SAX, the experiment was not carried out on the skin samples. There future experimentations are needed.

## Study limitations

The most important limitation of this study is that no salt measurements were done in the skin biopsies. Salt could be an important marker of decompensation and disease progression. Together with GAG sulfation, a stronger correlation could be found between clinical presentation of patients with heart failure and healthy individuals. Even between acute decompensated and chronic stable patients mutually, possible differences could be found.

Another limitation is the low number of acute decompensated heart failure patients. To get more reliable results, more patients are required. As 12 healthy controls and 11 chronic stable patients participated in this study, at least 10 acute decompensated patients were required (and not 8 as in this case). Patients with acute decompensation however, are the most difficult patients to recruit. They already suffer from decompensation, are hospitalized, and in addition need to give their permission to invasively take a skin biopsy. Therefore it is obvious that not everyone wanted to give their agreement to participate in this study.

The concentration of tsGAG and tUA found by DMMB and carbazole assay respectively, varied amongst different sets of test samples (compare supplemental figure X, table 4..). This may be due to different external factors which can vary from day to day. DMMB assay for example, can be performed by several methods. The method used in this paper is called the indirect method, but there is also a direct spectrophotometric method (better known as Farndale method) and a microplate DMMB assay (35). All 3 methods result in a different sGAG value determined. Furthermore, several variants of the DMMB assay exist within the 3 assays, what provides an even bigger variation on the results (30). Also salts can interfere with the results of both assays. Especially for the detection of uronic acids with carbazole assay, salts can generate false positive results (34). The acidic pH of 3,5 for DMMB assay however, minimizes the effects of salts and also other interfering contaminants such as DNA for this assay. Nevertheless, the discussed results for the measured content of total and sulfated GAGs have an important clinical value, as long as these measured values are not compared to values obtained by other methods.

# Conclusion & synthesis

Nowadays, clinical presentation in patients with heart failure varies from overt peripheral edema to no edema at all. The classic idea of increased concentration of salts in the body and upregulated neurohumoral system cannot always explain this phenomenon. Therefore, interstitium, as a possible new compartment for sodium regulation, was investigated in this study. The focus herein lied on the density of the negative charges of glycosaminoglycans, which can bind and buffer sodium cations. We performed several assays which quantified the amount of either sulfate groups present on GAGs, or uronic acids which exhibit a carboxyl group and are building blocks for GAG full chains. Our findings are that there is a significant difference in GAG content and sulfation pattern between heart failure patients and healthy controls and that these measurements are correlated with tissue water content and thus clinically observed edema.

The initial hypothesis was that the amount of these negative charge present on GAGs would differ between healthy individuals and individuals with heart failure. More concrete, we thought that chronic stable patients would have a high amount, while decompensated patients low amount of negative charges compared to healthy individuals.
Especially post-translational sulfation pattern on GAGs was increased in both acute decompensated and chronic stable HF patients. The concentration of uronic acids becomes significantly higher with the progression of heart failure towards decompensation, compared to healthy controls. Two possible reasons can account for this. First, sulfate – sodium binding is stronger and might be preferred over the carboxyl groups for buffering sodium ions. Secondly, sulfation is a post-translational modification which happens faster than the full synthesis of GAG chains. In either way, at the time of decompensation both tsGAG content and the total amount of GAGs, which are correlated with total uronic acid, are upregulated. This is in contrast with our initial thought that decompensated patients would have significantly lower negative charges compared to healthy controls. As mentioned before, at a certain sodium threshold, interstitial GAG network becomes dysfunctional, releasing buffered sodium ions which then become osmotically active thereby allowing individuals to develop edema. Dysfunctional does not mean that GAGs are suddenly cleaned up by various enzymes. The half-life for hyaluronic acid is less than 1 day and for chondroitin and dermatan sulfate approximately 2-4 days. Together with the fact that the biopsy was taken at the day of decompensation may be the reason for the high content of measured GAGs in decompensated state.

These results may have a big impact on patients with heart failure. Since there is a high rate of mortality and re-hospitalizations in heart failure, new targets to improve outcome are needed. First, we showed indirectly that interstitium is indeed being used as a sodium buffer network with available techniques for its evaluation. The problem is that it requires a skin biopsy to be taken in an invasive way. Future techniques may enable to identify interstital GAG networks without the need of an invasive biopsy. In this way, patients could be followed up with repetitive measurements to decrease the chance of decompensation. Secondly, interstitium could become a target for new heart failure therapies. Strategies to lower interstitial sodium and fluid content as well as therapies able to stabilize the GAG buffer network could have tremendous impact on the individual itself as well as on the economic costs related to heart failure. As persistent signs of sodium and fluid overload in heart failure are nowadays being used as important predictors for mortality and re-hospitalization, interstitial sodium concentration might also be an important indicator to guide therapy and could even be used as a new cardiovascular risk factor.

## Acknowledgements.

First, I would like to thank prof. dr. med. Mullens who started this interesting research as well as dr. Petra Nijst for her help from within the hospital east Limburg (ZOL, Genk) for the delivery of samples for this research.
Of course, thank you to all members of Biomedical Research Institute in Diepenbeek, in particular prof. dr. Jean-Paul Noben who helped me day by day in establishing new experiments and ideas. Erik Royackers also deserves a kind word to be said about for his help in the laboratory and with the equipment. At last, thank you to Igna Rutten, Annelies Bronckaers and Roland Valcke for their time spent helping me with the experiments.

Prof. dr. med. W. Mullens and dr. P. Nijst are researchers for the Limburg Clinical Research Program (LCRP) UHasselt – ZOL – Jessa, supported by the foundation Limburg Sterk Merk (LSM), Hasselt University, Hospital East Limburg (ZOL) and Jessa Hospital.

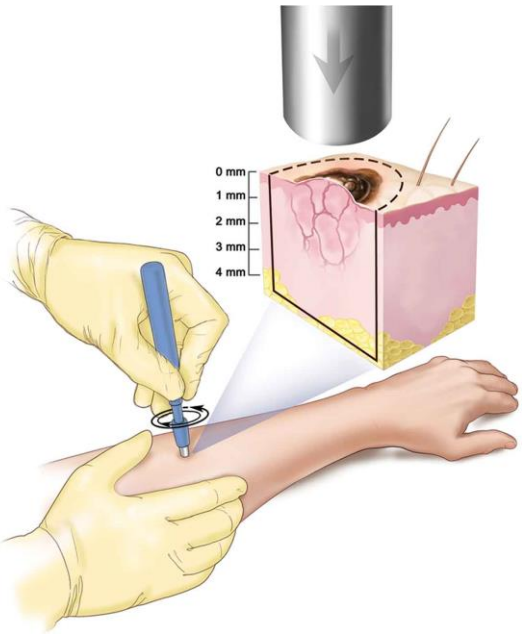
# Supplementary section.

**Supplementary table 1. The in- and exclusion criteria to participate in this study**. Ten healthy, ten stable heart failure (HF) patients and ten acute decompensated heart failure patients (ADHF) will be recruited. *NYHA = New York Heart Association: Class I (cardiac disease with no symptoms) – class IV (severe cardiac limitations even at rest). * LVEF = Left ventricular ejection fraction. ** = ACE inhibitors, beta-blockers, aldosterone antagonists and lifestyle modifications.*

| | **Healthy** | **Stable HF patients** | **ADHF** |
|---|---|---|---|
| **Inclusion criteria** | >18 years | >18 years | >18 years |
| | | History of chronic (>6 months) HF with NYHA I-II symptoms. Stable symptoms | Currently diagnosed with acute decompensated heart failure |
| | | LVEF* ≤ 40% | LVEF ≤ 40% |
| | | Absence of congestive signs | Current signs and symptoms of volume overload |
| | | Receiving guideline-recommended medical therapy** | |
| **Exclusion criteria** | History of chronic cardiac or renal disease | Unable to provide informed consent or comply with study protocol | Unable to provide informed consent or comply with study protocol |
| | Other causes of lower extremity edema | Other causes of lower extremity edema | Other causes of lower extremity edema |
| | Intake of loop diuretic, aldactone, other diuretics, ACE-inhibitor | Severe advanced heart failure (NYHA ≥ III) | |
| | | On renal replacement therapy | |

**Attachment 2. Skin punch biopsy of the lower extremity (calf).**

Skin punch biopsy is considered the primary technique for obtaining diagnostic full-thickness skin specimens. The technique involves the use of a circular blade that is rotated down through the epidermis and dermis, and into the subcutaneous fat, yielding a 3- to 4-mm cylindrical core of tissue sample. This results in a 3-mm diameter elliptical-shaped wound, which may be closed by a single suture. Properly administered local anesthesia makes this a painless procedure (emla hydrophilic cream, 5% lidocaine).




**Supplementary figure 1. An example of a skin punch biopsy from an arm.**


**Supplementary figure 2. The skin samples taken by a punch biopsy.** Several punch biopsies were taken from the skin and the dimensions measured (A). Examples of test samples taken by a skin punch biopsy (B).

**Supplementary table 2. The weights of the skin samples from healthy individuals (n = 12), patients with decompensated (n = 8) and chronic stable heart failure (n = 11).** TWC = tissue water content; AD = acute decompensated heart failure; CC = chronic stable heart failure.

| State | Net fresh weight (mg) | Net dry weight (mg) | TWC (mg) | % TWC | Net dry defatted (mg) | Total fat (mg) | % FAT |
|---|---|---|---|---|---|---|---|
| Healthy | 15,6 | 4,5 | 11,1 | 71,2 | 2,8 | 1,9 | 40,4 |
| Healthy | 15 | 4,4 | 10,6 | 70,7 | 3,4 | 1 | 22,7 |
| Healthy | 17,6 | 5 | 12,6 | 71,6 | 3,6 | 1,6 | 30,8 |
| Healthy | 17,4 | 5,1 | 12,3 | 70,7 | 3,8 | 1,4 | 26,9 |
| Healthy | 20,2 | 8,3 | 11,9 | 58,9 | 3,8 | 4,6 | 54,8 |
| Healthy | 17,5 | 6,5 | 11 | 62,9 | 3,9 | 2,7 | 40,9 |
| Healthy | 15,5 | 4,2 | 11,3 | 72,9 | 3,9 | 0,4 | 9,3 |
| Healthy | 16 | 4,8 | 11,2 | 70,0 | 4,1 | 0,8 | 16,3 |
| Healthy | 18,4 | 7,1 | 11,3 | 61,4 | 4,4 | 2,9 | 39,7 |
| Healthy | 19,1 | 7,1 | 12 | 62,8 | 4,5 | 2,6 | 36,6 |
| Healthy | 23,1 | 11,2 | 11,9 | 51,5 | 4,7 | 6,3 | 57,3 |
| Healthy | 21,5 | 7 | 14,5 | 67,4 | 4,7 | 2,5 | 34,7 |
| | | | | | | | |
| AD | 9 | 2,4 | 6,6 | 73,3 | 0,9 | 1,5 | 62,5 |
| AD | 14,8 | 3,8 | 11 | 74,3 | 1,1 | 2,9 | 72,5 |
| AD | 9,7 | 1,5 | 8,2 | 84,5 | 1,3 | 0,3 | 18,8 |
| AD | 6,8 | 2,6 | 4,2 | 61,8 | 1,6 | 1,1 | 40,7 |
| AD | 14,9 | 4 | 10,9 | 73,2 | 1,9 | 2,1 | 52,5 |
| AD | 13,2 | 2,7 | 10,5 | 79,5 | 1,9 | 0,8 | 29,6 |
| AD | 16,3 | 6,1 | 10,2 | 62,6 | 2,9 | 2,5 | 46,3 |
| AD | 18,9 | 5,7 | 13,2 | 69,8 | 3,1 | 2,6 | 45,6 |
| | | | | | | | |
| CS | 12 | 2,9 | 9,1 | 75,8 | 1,4 | 1,7 | 54,8 |
| CS | 17,6 | 6,8 | 10,8 | 61,4 | 1,8 | 5 | 73,5 |
| CS | 11,5 | 3,6 | 7,9 | 68,7 | 1,9 | 1,7 | 47,2 |
| CS | 11,2 | 3,1 | 8,1 | 72,3 | 1,9 | 1,2 | 38,7 |
| CS | 10,4 | 2,8 | 7,6 | 73,1 | 2 | 0,8 | 28,6 |
| CS | 10,5 | 3,7 | 6,8 | 64,8 | 2,2 | 1,6 | 42,1 |
| CS | 16,5 | 7,2 | 9,3 | 56,4 | 2,4 | 4,8 | 66,7 |
| CS | 14,2 | 4,5 | 9,7 | 68,3 | 2,6 | 2 | 43,5 |
| CS | 17,5 | 4,4 | 13,1 | 74,9 | 2,6 | 1,8 | 40,9 |
| CS | 19,1 | 3,2 | 15,9 | 83,2 | 3,1 | 0,2 | 6,1 |
| CS | 15,6 | 4 | 11,6 | 74,4 | 3,2 | 0,9 | 22 |

**Supplementary table 3**. **The weights of the first set of 'test' skin samples, derived from the abdomen.** TWC = tissue water content.

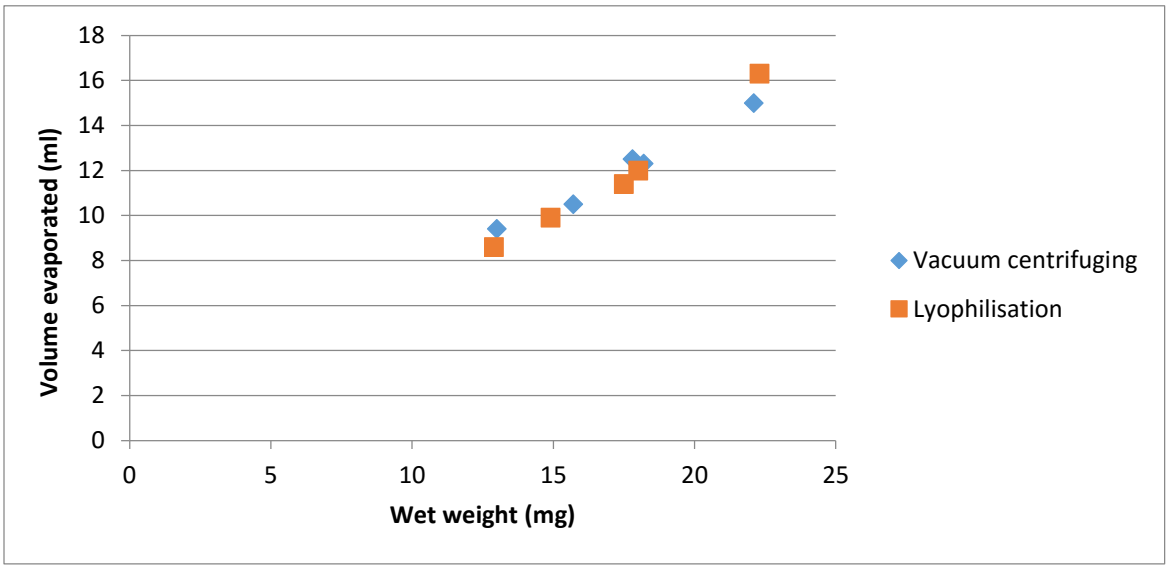
| Sample | Net fresh weight (mg) | Net dry weight (mg) | TWC (mg) | % TWC | Net dry defatted (mg) | Total fat (mg) | % FAT |
|---|---|---|---|---|---|---|---|
| T31 | 11 | 2,8 | 8,2 | 74,6 | 2,5 | 0,3 | 10,7 |
| T48 | 15,5 | 3,9 | 11,6 | 74,8 | 3,9 | 0 | 0,0 |
| T24 | 18,5 | 5,2 | 13,3 | 71,9 | 4 | 1,2 | 23,1 |
| T32 | 16,3 | 4,2 | 12,1 | 74,2 | 4 | 0,2 | 4,8 |
| T39 | 17 | 5 | 12 | 70,6 | 4,1 | 0,9 | 18,0 |
| T26 | 19,4 | 4,9 | 14,5 | 74,7 | 4,2 | 0,7 | 14,3 |
| T27 | 18,9 | 5,1 | 13,8 | 73 | 4,2 | 0,9 | 17,7 |
| T50 | 17,3 | 5,4 | 11,9 | 68,8 | 4,2 | 1,2 | 22,2 |
| T23 | 17,5 | 4,6 | 12,9 | 73,7 | 4,4 | 0,2 | 4,4 |
| T25 | 19,4 | 5,4 | 14 | 72,2 | 4,4 | 1 | 18,5 |
| T29 | 17,8 | 5,2 | 12,6 | 70,8 | 4,4 | 0,8 | 15,4 |
| T43 | 16,9 | 4,6 | 12,3 | 72,8 | 4,4 | 0,2 | 4,4 |
| T45 | 18,2 | 5,3 | 12,9 | 70,9 | 4,4 | 0,9 | 17,0 |
| T47 | 17,4 | 5,5 | 11,9 | 68,4 | 4,4 | 1,1 | 20,0 |
| T44 | 18 | 5,3 | 12,7 | 70,6 | 4,4 | 0,9 | 17,0 |
| T36 | 21 | 7,3 | 13,7 | 65,2 | 4,6 | 2,7 | 37,0 |
| T30 | 21 | 7 | 14 | 66,7 | 4,6 | 2,4 | 34,3 |
| T38 | 18,6 | 5 | 13,6 | 73,1 | 4,6 | 0,4 | 8,0 |
| T34 | 22 | 6,9 | 15,1 | 68,6 | 4,9 | 2 | 29,0 |
| T35 | 19,3 | 5,2 | 14,1 | 73,1 | 4,9 | 0,3 | 5,8 |
| T37 | 21,4 | 6,7 | 14,7 | 68,7 | 4,9 | 1,8 | 26,9 |
| T42 | 19,5 | 5,8 | 13,7 | 70,3 | 4,9 | 0,9 | 15,5 |
| T41 | 21 | 6,7 | 14,3 | 68,1 | 5,3 | 1,4 | 20,9 |
| T28 | 23,9 | 7,6 | 16,3 | 68,2 | 5,4 | 2,2 | 29,0 |
| T22 | 33,1 | 14,6 | 18,5 | 55,9 | 5,5 | 9,1 | 62,3 |
| T49 | 23,2 | 7,9 | 15,3 | 66 | 5,5 | 2,4 | 30,4 |
| T33 | 19,7 | 6,3 | 13,4 | 68 | 5,6 | 0,7 | 11,1 |
| T46 | 25,1 | 8,4 | 16,7 | 66,5 | 5,8 | 2,6 | 31,0 |
| T40 | 26,9 | 9,1 | 17,8 | 66,2 | 5,8 | 3,3 | 36,3 |
| T21 | 31,2 | 12,6 | 18,6 | 59,6 | 5,9 | 6,7 | 53,2 |

**Supplementary table 3 (continuation)**. **The weights of the second set of 'test' skin samples, derived from the abdomen.** TWC = tissue water content.

| Sample | Net fresh weight (mg) | Net dry weight (mg) | TWC (mg) | % TWC | Net dry defatted (mg) | Total fat (mg) | % FAT |
|---|---|---|---|---|---|---|---|
| T113 | 13,5 | 3,9 | 9,6 | 71,1 | 2,3 | 1,6 | 41,0 |
| T106 | 14,7 | 5 | 9,7 | 66 | 3 | 2 | 40,0 |
| T119 | 14,6 | 4,9 | 9,7 | 66,4 | 3 | 1,9 | 38,8 |
| T122 | 14,5 | 4,6 | 9,9 | 68,3 | 3 | 1,6 | 34,8 |
| T103 | 14,6 | 4,1 | 10,5 | 71,9 | 3,1 | 1 | 24,4 |
| T112 | 15,3 | 4,7 | 10,6 | 69,3 | 3,2 | 1,5 | 31,9 |
| T114 | 19,6 | 6,2 | 13,4 | 68,4 | 3,2 | 3 | 48,4 |
| T121 | 15,4 | 4,8 | 10,6 | 68,8 | 3,2 | 1,6 | 33,3 |
| T126 | 14,2 | 4,7 | 9,5 | 66,9 | 3,3 | 1,4 | 29,8 |
| T117 | 17,8 | 5,7 | 12,1 | 68 | 3,3 | 2,4 | 42,1 |
| T118 | 18 | 5,8 | 12,2 | 67,8 | 3,3 | 2,5 | 43,1 |
| T111 | 14,3 | 4,3 | 10 | 69,9 | 3,4 | 0,9 | 20,9 |
| T116 | 16,9 | 4,9 | 12 | 71 | 3,4 | 1,5 | 30,6 |
| T124 | 16,2 | 6,3 | 9,9 | 61,1 | 3,4 | 2,9 | 46,0 |
| T108 | 16,2 | 4,8 | 11,4 | 70,4 | 3,4 | 1,4 | 29,2 |
| T107 | 17,3 | 6 | 11,3 | 65,3 | 3,5 | 2,5 | 41,7 |
| T129 | 16,6 | 5,5 | 11,1 | 66,9 | 3,5 | 2 | 36,4 |
| T100 | 16,5 | 5 | 11,5 | 69,7 | 3,6 | 1,4 | 28,0 |
| T125 | 19,1 | 6,4 | 12,7 | 66,5 | 3,6 | 2,8 | 43,8 |
| T105 | 16,8 | 5 | 11,8 | 70,2 | 3,7 | 1,3 | 26,0 |
| T120 | 21,3 | 8,5 | 12,8 | 60,1 | 3,9 | 4,6 | 54,1 |
| T128 | 16,9 | 5,3 | 11,6 | 68,6 | 3,9 | 1,4 | 26,4 |
| T115 | 21 | 7,9 | 13,1 | 62,4 | 4 | 3,9 | 49,4 |
| T130 | 15,8 | 5,1 | 10,7 | 67,7 | 4 | 1,1 | 21,6 |
| T110 | 17,4 | 5,2 | 12,2 | 70,1 | 4,1 | 1,1 | 21,2 |
| T104 | 20,7 | 6 | 14,7 | 71 | 4,2 | 1,8 | 30,0 |
| T109 | 18 | 5,1 | 12,9 | 71,7 | 4,2 | 0,9 | 17,7 |
| T123 | 16,7 | 5,8 | 10,9 | 65,3 | 4,2 | 1,6 | 27,6 |
| T102 | 20,8 | 6 | 14,8 | 71,2 | 4,4 | 1,6 | 26,7 |
| T101 | 19,6 | 5,2 | 14,4 | 73,5 | 4,4 | 0,8 | 15,4 |
| T127 | 19,7 | 6,4 | 13,3 | 67,5 | 4,9 | 1,5 | 23,4 |

**Supplementary table 3 (continuation)**. **The weights of the third set 'test' skin samples, derived from the abdomen.** TWC = tissue water content.

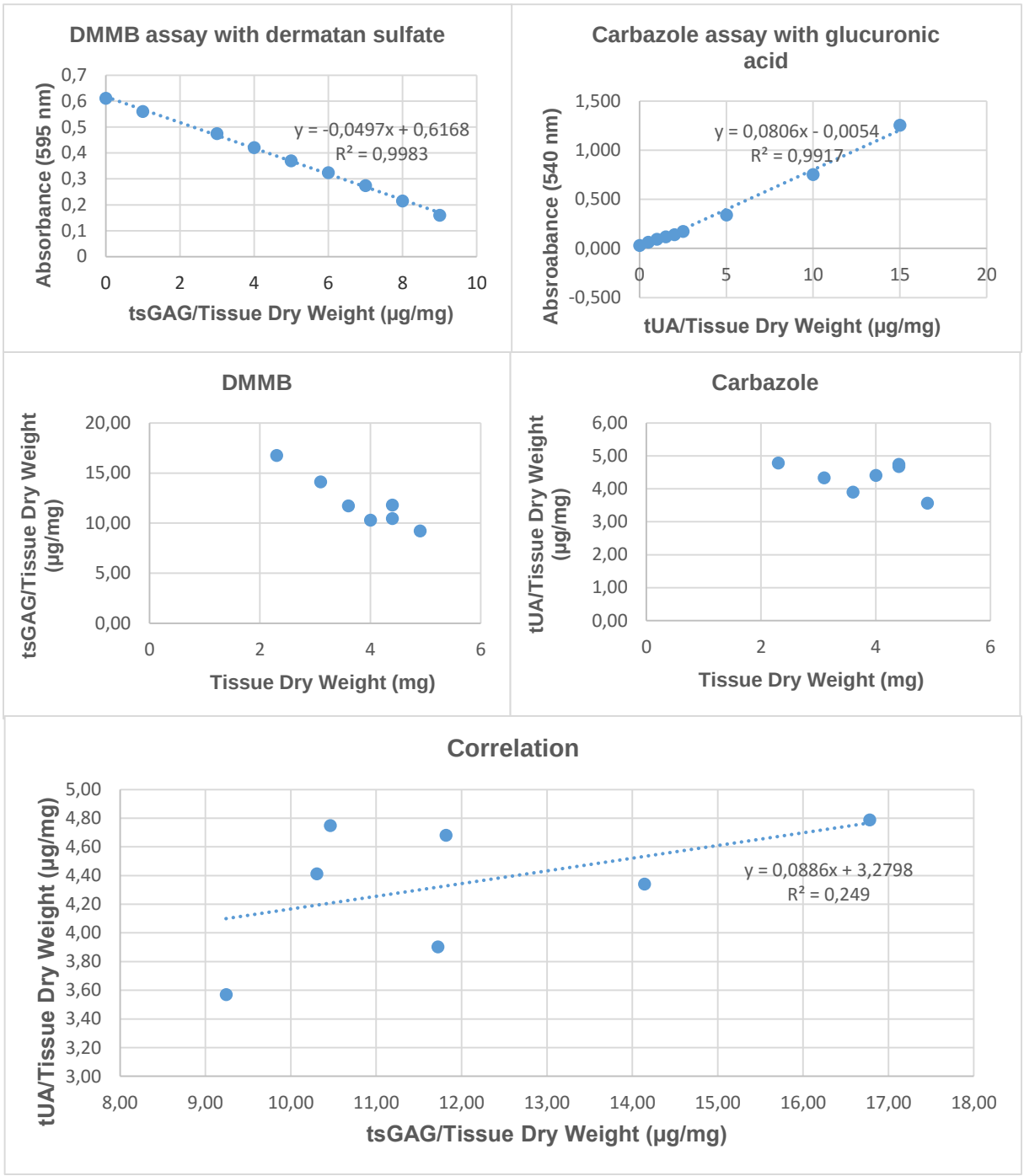
| Sample | Net fresh weight (mg) | Net dry weight (mg) | TWC (mg) | % TWC | Net dry defatted (mg) | Total fat (mg) | % FAT |
|---|---|---|---|---|---|---|---|
| T151 | 19,3 | 9,4 | 9,9 | 51,3 | 2,5 | 6,9 | 73,4 |
| T153 | 15,8 | 3,6 | 12,2 | 77,2 | 3,2 | 0,4 | 11,1 |
| T179 | 14,8 | 3,9 | 10,9 | 73,7 | 3,2 | 0,7 | 18,0 |
| T169 | 24,2 | 13,8 | 10,4 | 43 | 3,3 | 10,5 | 76,1 |
| T156 | 17,5 | 5,5 | 12 | 68,6 | 3,5 | 2 | 36,4 |
| T158 | 19,5 | 8,4 | 11,1 | 56,9 | 3,5 | 4,9 | 58,3 |
| T160 | 15,8 | 5,4 | 10,4 | 65,8 | 3,5 | 1,9 | 35,2 |
| T173 | 17 | 8,2 | 8,8 | 51,8 | 3,5 | 4,7 | 57,3 |
| T177 | 18 | 5,6 | 12,4 | 68,9 | 3,8 | 1,8 | 32,1 |
| T154 | 23,4 | 10 | 13,4 | 57,3 | 3,9 | 6,1 | 61,0 |
| T171 | 17,4 | 6,1 | 11,3 | 64,9 | 3,9 | 2,2 | 36,1 |
| T155 | 16,5 | 3,9 | 12,6 | 76,4 | 4 | -0,1 | -2,6 |
| T164 | 16,6 | 4,9 | 11,7 | 70,5 | 4 | 0,9 | 18,4 |
| T176 | 20,1 | 8,5 | 11,6 | 57,7 | 4 | 4,5 | 52,9 |
| T159 | 21,2 | 8,2 | 13 | 61,3 | 4,1 | 4,1 | 50,0 |
| T175 | 15,2 | 3,9 | 11,3 | 74,3 | 4,1 | -0,2 | -5,1 |
| T152 | 16,1 | 4 | 12,1 | 75,2 | 4,2 | -0,2 | -5,0 |
| T167 | 16,7 | 5,2 | 11,5 | 68,9 | 4,2 | 1 | 19,2 |
| T157 | 15,7 | 4,4 | 11,3 | 72 | 4,2 | 0,2 | 4,6 |
| T150 | 25,6 | 8,3 | 17,3 | 67,6 | 4,3 | 4 | 48,2 |
| T163 | 19,3 | 6 | 13,3 | 68,9 | 4,3 | 1,7 | 28,3 |
| T178 | 20,4 | 6,6 | 13,8 | 67,7 | 4,5 | 2,1 | 31,8 |
| T162 | 21,1 | 7,7 | 13,4 | 63,5 | 4,7 | 3 | 39,0 |
| T165 | 16,9 | 5,3 | 11,6 | 68,6 | 4,8 | 0,5 | 9,4 |
| T168 | 18,2 | 5,7 | 12,5 | 68,7 | 4,8 | 0,9 | 15,8 |
| T166 | 18,2 | 5,3 | 12,9 | 70,9 | 4,9 | 0,4 | 7,6 |
| T161 | 20,9 | 10,3 | 10,6 | 50,7 | 5 | 5,3 | 51,5 |
| T172 | 19,9 | 6,8 | 13,1 | 65,8 | 5 | 1,8 | 26,5 |
| T174 | 21,4 | 6,9 | 14,5 | 67,8 | 5 | 1,9 | 27,5 |
| T170 | 22,4 | 7,9 | 14,5 | 64,7 | 5,2 | 2,7 | 34,2 |
| T127 | 19,7 | 6,4 | 13,3 | 67,5 | 4,9 | 1,5 | 23,4 |




**Supplementary figure 3. Comparison of the 2 techniques to dry the skin biopsies.** Five test samples per technique were used. No significant differences are observed.

**Supplementary table 4. The net weights of the residues after enzymatic degradation of patient's skin samples with pronase E and evaporating using vacuum centrifugation.** The microtube was weighted before and after sample degradation and the net rest weight calculated. AD = acute decompensated heart failure; CC = chronic stable heart failure.

| State | Microtube empty weight (mg) | Microtube empty weight after pronase E (mg) | Net rest weight after pronase E (mg) |
|---|---|---|---|
| Healthy | 1010,8 | 1011,1 | 0,3 |
| Healthy | 1018,7 | 1019 | 0,3 |
| Healthy | 1008,8 | 1008,8 | 0 |
| Healthy | 1013,1 | 1013,3 | 0,2 |
| Healthy | 1004,2 | 1004,1 | -0,1 |
| Healthy | 1014,6 | 1014,8 | 0,2 |
| Healthy | 1007,7 | 1007,8 | 0,1 |
| Healthy | 1016,3 | 1016,5 | 0,2 |
| Healthy | 998,9 | 999,2 | 0,3 |
| Healthy | 1004,1 | 1004,4 | 0,3 |
| Healthy | 1007,6 | 1008,1 | 0,5 |
| Healthy | 999,6 | 999,6 | 0 |
| | | | |
| AD | 1005,8 | 1005,5 | -0,3 |
| AD | 1009,3 | 1009,6 | 0,3 |
| AD | 1004,7 | 1004,8 | 0,1 |
| AD | 1008,3 | 1008,5 | 0,2 |
| AD | 999,6 | 999,6 | 0 |
| AD | 996,5 | 996,5 | 0 |
| AD | 1008,5 | 1008,5 | 0 |
| AD | 1009 | 1009 | 0 |
| | | | |
| CS | 990,8 | 991,2 | 0,4 |
| CS | 1005,9 | 1005,9 | 0 |
| CS | 1011,1 | 1011,2 | 0,1 |
| CS | 1006,2 | 1006,1 | -0,1 |
| CS | 1003,7 | 1003,9 | 0,2 |
| CS | 997 | 996,9 | -0,1 |
| CS | 997,7 | 997,8 | 0,1 |
| CS | 1013,7 | 1013,8 | 0,1 |
| CS | 991,4 | 991,4 | 0 |
| CS | 1006,6 | 1006,6 | 0 |
| CS | 996,3 | 996,1 | -0,2 |




**Supplementary figure 4. The results of the DMMB and the carbazole assay on the 7 test skin samples, derived from the abdomen.**

**Supplementary table 5. The results of a DMMB assay, which was performed second time on the residues left after the first pronase E degradation of patient's skin samples.** The results indicate that the samples were fully degraded after the first pronase E treatment.

| State | DW (mg) | µg sGAG/mg DW |
|---|---|---|
| Healthy | 2,8 | -0,15 |
| Healthy | 3,4 | 0,43 |
| Healthy | 3,6 | 2,48 |
| Healthy | 3,8 | 0,44 |
| Healthy | 3,8 | 0,15 |
| Healthy | 3,9 | -0,37 |
| Healthy | 3,9 | 0,46 |
| Healthy | 4,1 | -0,29 |
| Healthy | 4,4 | -0,31 |
| Healthy | 4,5 | 0,44 |
| Healthy | 4,7 | 0,11 |
| Healthy | 4,7 | -0,57 |
| | | |
| AD | 0,9 | -0,66 |
| AD | 1,1 | 0,87 |
| AD | 1,3 | 1,05 |
| AD | 1,6 | 0,33 |
| AD | 1,9 | -0,49 |
| AD | 1,9 | 3,29 |
| AD | 2,9 | -0,18 |
| AD | 3,1 | -2,53 |
| | | |
| CS | 1,4 | -0,30 |
| CS | 1,8 | 1,81 |
| CS | 1,9 | 0,01 |
| CS | 1,9 | -2,47 |
| CS | 2 | -1,73 |
| CS | 2,2 | -1,51 |
| CS | 2,4 | 0,02 |
| CS | 2,6 | -0,29 |
| CS | 2,6 | 0,59 |
| CS | 3,1 | -0,87 |
| CS | 3,2 | -0,75 |

**Supplementary attachment 3. An e-mail to Sigma-Aldrich about poor efficiency of chondroitinase-ABC and their response.**

Dear sir/madame,

Not long ago I have bought Chondroitinase ABC from Sigma (C3667, 5 UN). We have done several tests with failure. We eventually came to a conclusion that chondroitinase may work unsufficiently.
Let me start with shortly introducing the steps:

1) Chondroitinase ABC powder (0.48 mg) was solved in 25 ml of 0.01% BSA solution. As we were interested in degrading glycosaminoglycans such as chondrotine and dermatan sulfate, we have used the specification sheet where it was stated that the powder should contain 6.24 units of the enzyme. Thus, the final concentration would then be 250 mU/ml (6,24/25). We have used 200 µl for 50 mU for degrading the sample as our samples are light weighted (3-5 mg) and as stated in other protocols. Also a buffer containing 40 mM Tris/HCl, pH = 8; 60 mM sodium acetate as the final concentration was used. The skin samples were first degraded with pronase E, releasing the glycosaminoglycans, and pronase was heat-inactivated before adding the chondroitinase ABC.

2) After many, low signal results, we decided to perform an activity assay using a temperature controlled spectrophotometer, set at 37°C.

Firstly, to 640 µl 50 mM Tris/HCl, 60 mM sodium acetate, pH = 8; 50 µl of 1 mg/ml dermatan sulfate standard (bought from Sigma for this test, C3788 25 mg) was added. Alter stirring, 50 mU (200 µl) of chondroitinase ABC was added and compared to the blanc without enzyme. The reaction was measured in time at 37°C and at 232 nm (released reduced disaccharides) against the blanc. These results, you can find in the attachment.

Using the law of Lambert-Beer, it was found that the chondroitinase-ABC had about one sixth of its expected activity. Instead of 50 mU, we only found 8 mU.

Could you please explain these unsatisfying results?
Thank you in advance,

Greetings,
Mikhail

**Scientist's Response Via Email (Astrid)**

Dear Mikhail,

Thank you for your e-mail.
Regarding your inquiry about Chondroitinase ABC from Proteus vulgaris, product C3667.
We are sorry to hear that you are experiencing products with our product in your application.
You have very transparently described your steps and troubleshooting so we believe that your complaint is justified.
I have found in our system that this is, unfortunately enough, a known complaint for this product.
As far as I can see this problem has not yet been further investigated, so I will discuss this internally and will escalate this to our quality department.

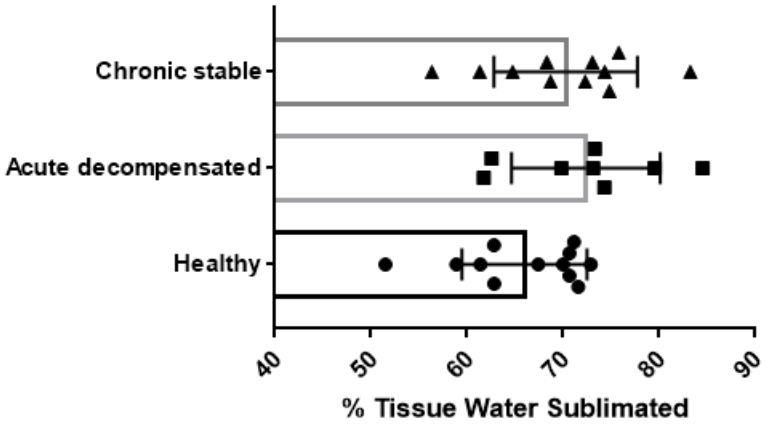
I have registered a complaint for this order with number 300303545.
At this point we can offer you one of the three following options, to compensate you for the inconvenience:

1) Alternative batch; at the moment we have for C3667-5UN LOT 065M4170V on stock,
2) Alternative product; the only option in this case is C2905. Please note that for this product the activity is lower!
3) Credit note. Because you already pointed out that you urgently need the enzyme, this may be the least preferred option.

I look forward to your reply, so I can further process your complaint.
Apologies for all the inconvenience.

With kind regards,

Astrid Provoost
Specialist Life Science
Merck
European Technical Service - Benelux




**Supplementary figure 5. Percentage of tissue water that was sublimated after freeze-drying of lyophilizing of skin samples from healthy individuals (n = 12), acute decompensated (n = 8) and chronic stable heart failure patients (n = 11).** No significant differences are observed.

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