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GLYCOSAMINOGLYCAN ANALYSIS OF BIOLOGICAL FLUIDS AND ORGANS IN AN ANIMAL MODEL OF MUCOPOLYSACCHARIDOSIS II (HUNTER SYNDROME): EFFECT OF TREATMENTS WITH ENZYME REPLACEMENT THERAPY AND GENISTEIN

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PREFAZIONE

La mucopolisaccaridosi II (MPS II) è una malattia genetica recessiva legata al cromosoma X, causata dal deficit dell'enzima lisosomiale iduronato-2-solfatasi (I2S). La mancanza di I2S fa sì che i glicosaminoglicani (GAG) come il condroitin solfato (CS), il dermatan solfato (DS) e l'eparan solfato (HS) si accumulino in tutti i tessuti del corpo, causando anomalie a diversi organi. Per MPS II, la terapia enzimatica sostitutiva (ERT) sembra essere il trattamento più efficace. Tuttavia, ERT non è in grado di attraversare la barriera emato-encefalica e mostra diversi limiti. L'isoflavone della soia genisteina è stato studiato come potenziale terapia per le MPS grazie alla sua presunta capacità di inibire la sintesi dei GAG e il loro accumulo. Inoltre, genisteina può attraversare la barriera ematoencefalica nei modelli murini, ma studi precedenti hanno mostrato esiti contrastanti.

Questo progetto è focalizzato sulla caratterizzazione qualitativa e quantitativa dei GAG in un modello murino di MPS II (I2S knock-out) non trattato, trattato con ERT o trattato con genisteina.

I GAG sono stati estratti da campioni di plasma, urina, fegato e cervello di 225 topi, secondo un protocollo standardizzato. La frazione grezza di GAG è stata digerita con enzimi specifici per isolare le unità disaccaridiche di acido ialuronico (HA), CS, DS e HS. I disaccaridi sono stati poi liofilizzati e contrassegnati con un fluoroforo. Infine, i disaccaridi derivatizzati sono stati quantificati mediante elettroforesi capillare interfacciata con LIF (Laser Induced Fluorescence).

Rispetto al Wild Type (WT), i campioni I2S KO hanno mostrato sin dalle prime settimane differenze significative per tutti i parametri analizzati. In questi soggetti, la concentrazione di CS-DS, HS e HA, così come la concentrazione totale di GAG, è aumentata in modo significativo rispetto al WT e rimane elevata durante tutte le settimane analizzate. Si evidenzia una elevata concentrazione di GAG, uno squilibrio del rapporto CS / HS verso HS, un aumento della densità di carica e un’alterazione del profilo di solfatazione. In particolare, CS-DS è principalmente solfatato nella posizione 2,4s, rispetto alla posizione

6s che è prevalente nei soggetti WT. Queste differenze sono particolarmente pronunciate nei campioni di fegato, suggerendo un maggiore accumulo di GAG negli organi solidi. Per quanto riguarda le terapie, possiamo notare come l'ERT possa ridurre molti dei parametri alterati, ma non possa ripristinare completamente la condizione di WT. Inoltre, ERT non ha determinato alterazioni nei campioni di cervello, confermando che l'enzima non può attraversare la barriera ematoencefalica. Il trattamento con Genisteina non ha mostrato alterazioni significative rispetto ai soggetti I2S KO non trattati.

In conclusione, confrontando i risultati ottenuti da topi WT e topi I2S KO, osserviamo valori più elevati per ciascun indice considerato. Possiamo anche evidenziare come l'aumento dei disaccaridi solfatati in posizione 2,4s indichi un accumulo di DS in questi organi, specifico della MPSII. Da questi risultati si può proporre l'analisi dell'indice di solfatazione 2,4s nel plasma, come strumento utile nella diagnosi di MPS II. Inoltre, come nei pazienti con MPS II, la terapia ERT può ridurre significativamente l'accumulo di GAG. Questi risultati rafforzano ulteriormente la coerenza di questo modello animale con la patologia MPS II. Tuttavia, ERT non è in grado di indurre la normalizzazione totale dei parametri. Al contrario, la terapia con genisteina non ha mostrato differenze significative nei soggetti I2S KO, in nessuno dei parametri considerati. Nella fase successiva, analizzeremo diversi organi. Inoltre, avremo più indicazioni sull'efficacia di diverse terapie, fornendo le basi per futuri studi clinici sull'uomo.

1. ABSTRACT

Mucopolysaccharidoses II is an X-linked recessive disorder caused by lysosomal enzyme iduronate-2-sulfatase (I2S) deficiency. The lack of I2S causes glycosaminoglycans (GAGs) such as chondroitin sulfate (CS), dermatan sulfate (DS) and heparan sulfate (HS) to accumulate in all body tissues, causing organ abnormalities. In severe cases, this leads to death during the teenage years. In MPS II, enzyme replacement therapy (ERT) appears to be the most effective treatment. However, ERT is unable to cross the blood-brain barrier and shows several limitations. The isoflavone genistein has been studied as a potential therapy for MPS because of its putative ability to inhibit GAG synthesis and subsequent accumulation. Furthermore, genistein can cross the blood-brain barrier in murine models, but previous studies showed variable outcomes.

This project is focused on GAGs qualitative and quantitative characterization in a murine MPS II model (I2S knock-out) non-treated, treated with ERT or treated with genistein, analyzed at different weeks after the beginning of therapy.

GAGs were extracted from plasma, urine, liver and brain samples of 225 mice, according to a standardized protocol. The crude GAGs fraction was digested with specific enzymes to isolate Hyaluronic Acid (HA), CS, DS and HS disaccharide units. Disaccharides were lyophilized and tagged with a fluorophore. Finally, derivatized disaccharides were separated and quantified by capillary electrophoresis interfaced with laser-induced fluorescence.

Compared to wild type (WT), the I2S KO samples showed from the first weeks significant differences for all the analyzed parameters. In these subjects, CS-DS, HS and HA concentration, as well as total GAGs concentration, increased significantly compared to WT subjects and remains high during all the analyzed weeks. We observe, higher GAGs concentration, CS / HS imbalance towards HS, increase in charge density, and alteration in the sulfation profile. In particular, CS-DS was mainly sulfated in the 2,4s position, compared to the 6s position which was prevalent in WT subjects. These differences are particularly pronounced in liver samples, suggesting a higher GAGs accumulation in solid organs. As

for the therapies, we can see how ERT can reduce many of the altered parameters, but it cannot restore the WT condition completely. Moreover, ERT did not show significant alterations in the brain samples, confirming that the enzyme cannot cross the blood-brain barrier. Genistein treatment, showed no significant alterations, either quantitatively or qualitatively, compared to non-treated I2S KO subjects.

Comparing the results obtained from WT mice and I2S KO mice we observe higher values of each index considered. We can also evidence how the increase in disaccharides sulfated in position 2,4s indicates an accumulation of DS in plasma, urine, liver and brain, specific of MPSII. From these results, we can propose the analysis of 2,4s sulfation index in plasma, as a useful tool in the diagnosis of this pathology. Moreover, as in MPSII patients, ERT therapy can significantly reduce GAGs accumulation. These findings further strengthen the coherence of this animal model to MPSII pathology. However, ERT is not able to induce total normalization of the parameters. On the contrary, genistein therapy did not show significant differences in I2S KO subjects, in any of the parameters considered. Therefore, this therapy would not seem to benefit the patient. In the next stage, we will also analyze different organs. Furthermore, we will have more indications of the effectiveness of different therapies in reducing GAGs accumulation, providing the basis for future clinical trials on humans.

2. INTRODUCTION

2.1 Glycosaminoglycans

Glycosaminoglycans (GAGs) are a family of linear polymers found in bacteria and animals. These heteropolysaccharide macromolecules originate from the repetition of fundamental disaccharide units represented by a hexosamine (N-acetylglucosamine GlcNAc or N-acetylgalactosamine GalNAc) and by a uronic acid (D-glucuronic or L-iduronic acid), except for keratan sulfate in which the uronic acid is replaced by a hexose (galactose) [1].

These GAGs, based on the nature of the hexosamine residues, are classified into two groups.

- Glucosaminoglycans (Hyaluronic Acid, Keratan sulfate, Heparan sulfate, Heparin)
- Galactosaminoglycans (Chondroitin sulfate, Dermatan sulfate).

The monosaccharide units are joined together by a glycosidic bond formed between the hydroxyl group of the anomeric carbon of a hexuronic acid and the hydroxyl group of a hexamine. The disaccharide units thus formed are linked together by another glycosidic bond. In this way, the long chains that distinguish the GAGs are obtained. The high variability of these macromolecules is also determined by the presence of sulfate groups on carbon atoms 4 and 6 and by the acetylation of the amino group of hexosamine, except for hyaluronic acid. The high content of carboxyl groups (-COO-) and esterified sulfate groups (-O-SO) gives GAGs a characteristic negative charge, and to reduce the repulsive forces between these negatively charged groups, GAGs assume an extended conformation forming helical rod structures.

GAGs have a molecular weight between 10 and 100 kDa and can be classified according to the saccharide residues that compose them, the geometry of the glycosidic bonds between them, and the presence/position of sulfate groups.

Five classes are distinguished:

- Hyaluronic acid (HA)

- Chondroitin sulfate (CS)
- Dermatan sulfate (DS)
- Heparin (Hep) and Heparan sulfate (HS)
- Keratan sulfate (KS)

Sulfated GAGs represent the largest group (among these CS, DS, KS, HS and Hep) but there are also non-sulfated GAGs such as hyaluronic acid. The saccharide backbone generally has sulfate groups in various positions [2].

The characteristic succession of sulfated and non-sulfated saccharide residues constitutes a recognition signal towards extracellular proteins which electrostatically bind the GAGs forming proteoglycans [1]. These represent the main components of the extracellular matrix and thanks to their ability to hydrate and retain water, these molecules confer a state of turgidity to the extracellular matrix which makes it resistant to compressive forces. Furthermore, the porous organization of these molecules allows the diffusion of water-soluble substances and cells migration.

Glycosaminoglycans also play an important role as cell surface receptors capable of mediating both signals from the extracellular environment and cell-cell recognition and binding [3]. It is evident how proteoglycans, and therefore GAGs, are fundamental in the correct functional and structural organization of cells and tissues.

The synthesis of glycosaminoglycans takes place by specific enzymes in the Golgi apparatus, or in the cell membrane for hyaluronic acid. In general, they are assembled by the sequential action of glycosyltransferases that transfer a monosaccharide from a nucleotide-linked derivative to an acceptor. This acceptor can be the reducing end of another sugar or a polypeptide [4].

2.1.1 Hyaluronic Acid

Hyaluronic acid has a molecular weight between 4 and 8000 kDa. Unlike most GAGs, it does not present sulfation nor bind covalently to proteins in the extracellular matrix.

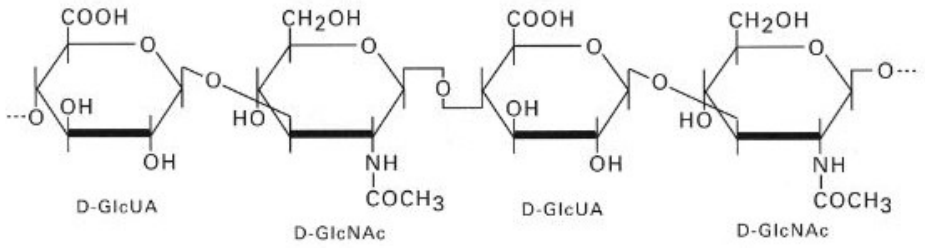


Figure 2.1: Hyaluronic Acid structure [5].

This molecule is present in the synovial fluid, in the vitreous humor of the eye, in the epithelial tissues, in the nervous tissue and in the extracellular matrix of the lax connective tissues where it acts as an excellent lubricant. A peculiar feature of HA is the ability to bind huge quantities of water.

From a structural point of view, HA is formed by the repetition of a fundamental unit given by a molecule of D-glucuronic acid linked via a β bond (1-4) to a residue of N-acetyl-D-glucosamine; these are then linked together through α bonds (1-4).

Unlike the other GAGs which are synthesized in the Golgi apparatus, HA is synthesized by transmembrane proteins called hyaluronic acid synthetase (HAS) [6]. Synthesis in the plasmatic membrane has the advantage of facilitating and speeding up hyaluronic acid extrusion in the extracellular environment. GAGs synthesis is strictly regulated at the transcriptional level: cytokines and growth factors can influence the expression of HAS which must then be phosphorylated to be activated. The substrates availability is also critical for HA synthesis and physiological homeostasis.

Another important mechanism is HA catabolism which always takes place inside the cell. HA is re-internalized into the cell by appropriate membrane receptors, CD44 [7] or LYVE-1 (lymphatic vessel endothelial receptor-1) [8]. HA degradation occurs in the lysosomal by specific hyaluronidase enzymes (HYALs): β-D-glucuronidase and β-N-acetyl-D-hexosaminidase [9].

The physiological turnover of this molecule is approximately 24 hours but can be influenced by the presence of reactive oxygen species. Hyaluronic acid is essential for correct tissue

homeostasis: it plays an important role in the ageing process, especially at the skin level [10].

2.1.2 Chondroitin Sulfate

Chondroitin sulfate has a molecular weight between 5-50 kDa, it is widely distributed in various connective tissues, especially cartilage, bones and heart valves. Thanks to its ability to bind large quantities of water, CS can uniformly distribute the forces exerted from the outside; its role is therefore important in connective tissues subjected to high pressure, such as the nucleus pulposus of intervertebral discs and joints cartilage. Moreover, CS can act as a filter, due to the high degree of hydration, salts and other low molecular weight compounds can diffuse through the proteoglycan gel, while proteins cannot. Furthermore, these GAGs are building blocks of basement membranes. They also participate in the cartilage calcification process, as they inhibit calcium sulfate crystallization.

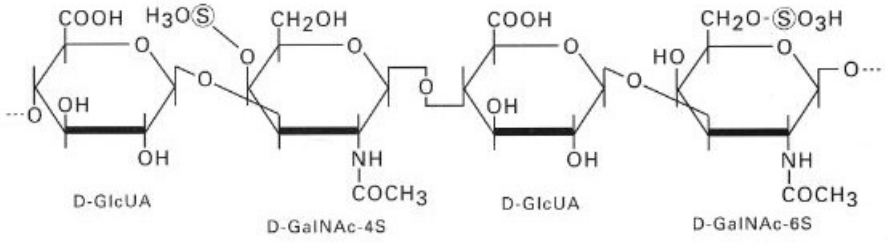


Figure 2.2: Chondroitin Sulfate structure [5].

From a structural point of view, CS consists of 10 to 200 repetitions of a disaccharide unit formed by a molecule of uronic acid (D-glucuronic or L-iduronic) and one of N-acetyl-D-galactosamine linked together by β (1-3) and β (1-4). The specific patterns of sulfation and the disaccharide units modifications allow the distinguishing of various types of CS. Nevertheless, the sulfation model allows specific interactions between CS and cytokines, growth factors and adhesion molecules regulating various biological functions [4].

The synthesis of chondroitin sulfate, as of dermatan sulfate, is very complex and takes place in the Golgi complex, where several specific enzymes collaborate in the saccharide sequence formation, which must then undergo at least two types of modification: one consists in the glucuronic acid transformation in its epimer, iduronic acid, the other in the

sulfation in various positions of the hydroxyl groups [11] [12]. These GAGs can present a sulfate group in position C-4 of galactosamine residue (chondroitin-4-sulfate or chondroitin sulfate A) or in position C-6 (chondroitin -6-sulfate or chondroitin sulfate C), which greatly complicate the structure.

2.1.3 Keratan Sulfate

Keratan sulfate is the most heterogeneous GAG, with a molecular weight between 4-19 kDa. It is found in cartilage, where it is aggregated with chondroitin sulfate, in the intervertebral discs, bones and cornea. Based on the tissues of origin, we can distinguish two types: Keratan Sulfate I or corneal (KS I) and Keratan Sulfate II or skeletal (KSII). Both have the same disaccharide structure, but KS I binds through an N-glycosidic bond to an L-asparagine residue while KS II forms an O-glycosyl bond with L-serine or L-threonine [13]. There is a third less represented form of KS (KS III) found mainly in the central nervous system proteoglycans [14].

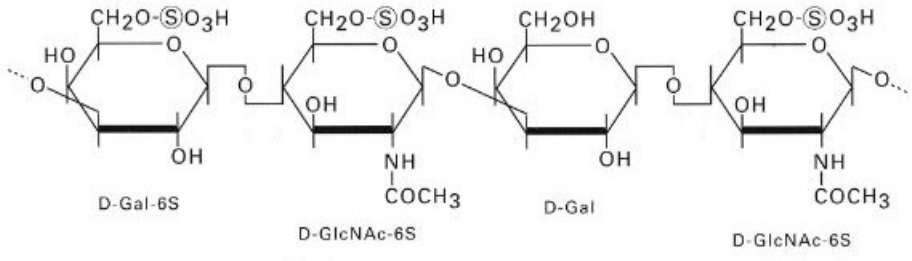


Figure 2.3: Keratan Sulfate structure [5].

Structurally, KS is formed by a disaccharide unit repeated several times and linked through β bonds (1-3), which is given by a galactose residue and one of N-acetyl-D-galactosamine which interact with β bonds (1-4). Given the presence of a galactose residue instead of uronic acid, KS differs from all other GAGs.

2.1.4 Dermatan Sulfate

Dermatan Sulfate, (also called Chondroitin Sulfate B), is an epimer of Keratan Sulfate since L-iduronic acid is found in the disaccharide unit instead of Uronic acid. This occurs by two enzymes: DS-epi1 and DS-epi2 which catalyze the carboxyl group repositioning on C5 [15].

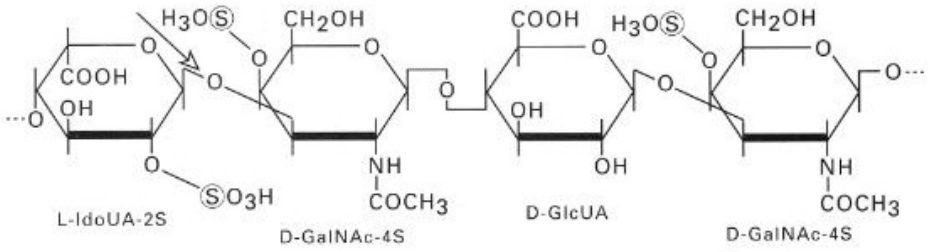


Figure 2.4: Dermatan Sulfate structure [5].

Therefore, the disaccharide is formed by L-iduronic acid linked α (1-3), or β (1-3) in the presence of d-iduronic acid, to N-acetylglucosamine. The galactosamine residue is sulfated at the C-4 position. The disaccharides are linked together by a β bond (1-4).

The molecular weight of DS is similar to CS, but generally, the chain of the former is longer. DS was found in the skin, vessel walls, and heart valves. GAGs containing L-iduronic acid, in addition to DS, Heparan Sulfate and Heparin, form insoluble complexes with serum low-density lipoproteins (LDL); this could favor the formation of atherosclerotic plaques [11]. Dermatan sulfate performs many physiological and pathological biological functions: it is involved in coagulation, cardiovascular diseases, carcinogenesis and the fibrotic process.

2.1.5 Heparin

Heparin has a much more complex structure than the other GAGs previously described. This GAG contains both Iduronic acid and Glucuronic acid and the glucosamine can be bonded to a sulfate or acetylated group. Compared to other GAGs, heparin is much more sulfated, containing up to three sulfate groups per disaccharide, which gives it considerable structural variability. The repeating units of Heparin are made up of L-iduronic acid which binds to D-glucosamine through α (1-4) bonds. The disaccharides are joined together by a BETA bond (1-4) [16]. The amino group in glucosamine can be either sulfated or acetylated. Heparin is the main component of mast cells' intracellular granules, present in the lung, liver and skin arteries.

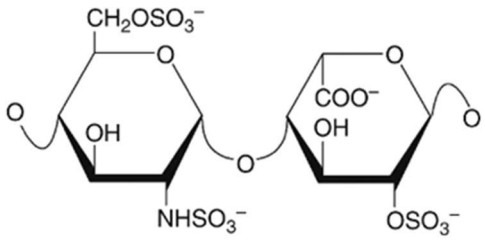


Figure 2.5: Heparin structure [5].

Heparin has been used for years in medical practice as an anticoagulant [17]. It is produced physiologically by mast cells associated with connective tissues, which store Heparin inside cytoplasmic granules, released in case of cell activation through degranulation. Heparin activates antithrombin III and inhibits the coagulation cascade. Only a small percentage of heparin is found in proteoglycans present in the extracellular matrices or bound to the cell surface, where they mediate extracellular signal transduction and play structural roles.

2.1.6 Heparan Sulfate

Heparan sulfate is closely related to heparin and contains the same monosaccharide constituents, bonded to each other as heparin [16]. Heparan sulfate consists of a residue of D-glucuronic acid and one of glucosamine, linked by α and β bonds (1-4). However, it differs from heparin because it contains fewer sulfate groups and fewer L-iduronic acid molecules.

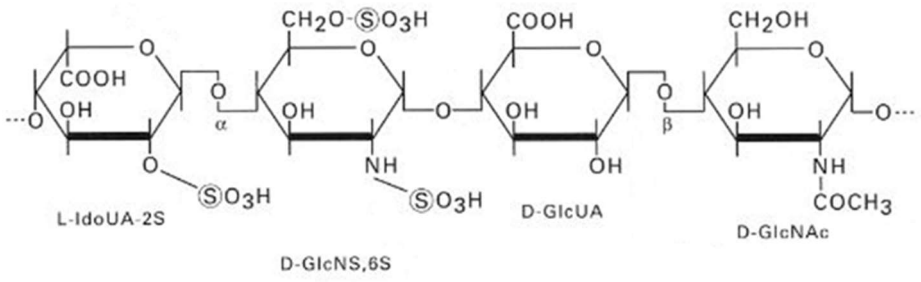


Figure 2.6: Heparan Sulfate structure [5].

Heparan sulfate is found in the basal membranes and interacts with various types of extracellular proteins involved in different biological processes: components of the extracellular matrix, enzymes, coagulation factors, cytokines, and growth factors. Recently

this GAG has been associated with the progression of neurodegenerative diseases regarding its ability to bind extracellular proteins, such as Tau and α-synuclein, and mediate their internalization in central nervous system cells [18].

2.2 Glycosaminoglycans Catabolism

Glycosaminoglycans catabolism occurs mainly in the lysosome. The process is highly organized and is mediated by the sequential action of hydrolases which cooperate in the degradation of the initial polymers to form saccharide and monosaccharide fragments. Those can be reused in the biosynthetic pathways. Hydrolases are substrate-specific and their catalytic activity depends on various parameters such as the stereoisomerism of the sugar, the type of glycosidic bond and the pH of the lysosomal compartment. Exoglycosidases work by releasing a monosaccharide from the non-reducing end of the polymer chain, while endoglycosidases cut specific sequences within the forming oligosaccharides. Other GAG-specific enzymes, such as sulfatase, hexosaminidase, and glucuronidase, intervene in the process.

The correct functionality of all these enzymes is essential to physiological balances: enzymatic deficiencies or alterations can lead to severe pathological conditions [19].

2.2.1 Hyaluronic Acid Catabolism

Hyaluronic acid is one of the most abundant GAGs in the human body. The degradation process of this molecule takes place at the intracellular level, through internalization mediated by the CD44 receptor; HA can be absorbed in blood and lymphatic vessels and then transported to the kidneys, liver and spleen where it will be degraded.

Catabolism involves the action of two hyaluronidases (Hyal-1 and Hyal-2), a β-glucuronidase and a β-N-acetylhexosaminidase. The first enzyme to intervene is Hyal-2 which, being anchored on the extracellular side of the plasmatic membrane, binds HA molecules present in the extracellular matrix and cuts the β bonds (1-4), leading to the formation of smaller oligosaccharides (about 20 kDa). The latter are internalized and

directed to the lysosomes; where Hyal-1 further degrades the molecules to form tetras and disaccharides. β-glucuronidase catalyzes the hydrolysis of the β bond (1-3) while β-N-acetylhexosaminidase hydrolyzes the remaining β bonds (1-4) leading to the release of GlcNAc and GlcUA [19].

2.2.2 Heparin and Heparan Sulfate Catabolism

For Heparan Sulfate, catabolism begins with an endoglucuronidase that cuts HS into smaller fragments, followed by an ordered degradation sequence. When a terminal iduronic acid sulfate is present, this must be desulfated by uronic acid-2-sulfatase to allow the action of α-iduronidase. Instead, when the sulfation is on glucuronic acid, the GlcA-2-sulfatase cuts the sulfate groups and allows the subsequent action of β-glucuronidase. At this point, glucosamine sulfate is the next sugar to be removed. First, N-sulfatase removes all the sulfate groups, then the newly formed glucosamine is acetylated to generate the α-N-acetylglucosaminidase substrate. When O-sulfated glucosamine is present, the sulfate groups are removed by a GlcNAc-6-sulfatase to allow the saccharide chain hydrolysis [19].

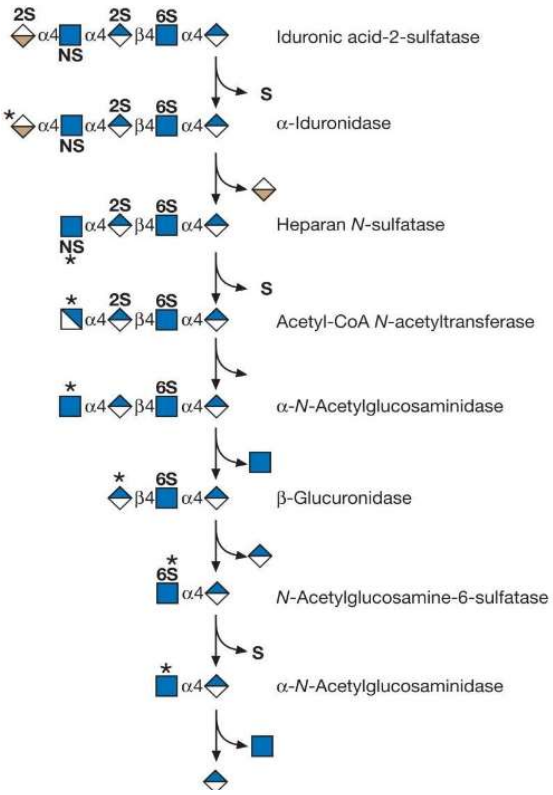


Figure 2.7: Heparan Sulfate catabolism [19].

2.2.3 Dermatan Sulfate and Chondroitin Sulfate Catabolism

Since DS and CS are two closely related GAGs, their catabolic process also occurs similarly through the intervention of lysosomal endoglycosidases, sulfatases and exoglycosidases. Regarding dermatan sulfate: terminal iduronic acid sulfate must undergo desulfation to be cleaved by α-iduronidase. At this point, the GalNAc-4-SO4 residue can be removed in two ways. In the first case, the sulfatase precedes the intervention of β-N-acetylhexosaminidase A or B, while in the second case, β-N-acetylhexosaminidase A detaches the glucosidic residue which will be subsequently desulfated. Finally, a β-glucuronidase is responsible for hydrolyzing glucuronic acid residues.

Instead, Chondroitin sulfate is catabolized by the action of GalNAc-6-SO4 sulfatase and GalNAc-4-SO4 sulfatase, of β-N-acetylhexosaminidase, A or B, and β-glucuronidase. Hyaluronidases may also participate in CS degradation process, but specific exoglycosidases for this GAG have not been observed [19].

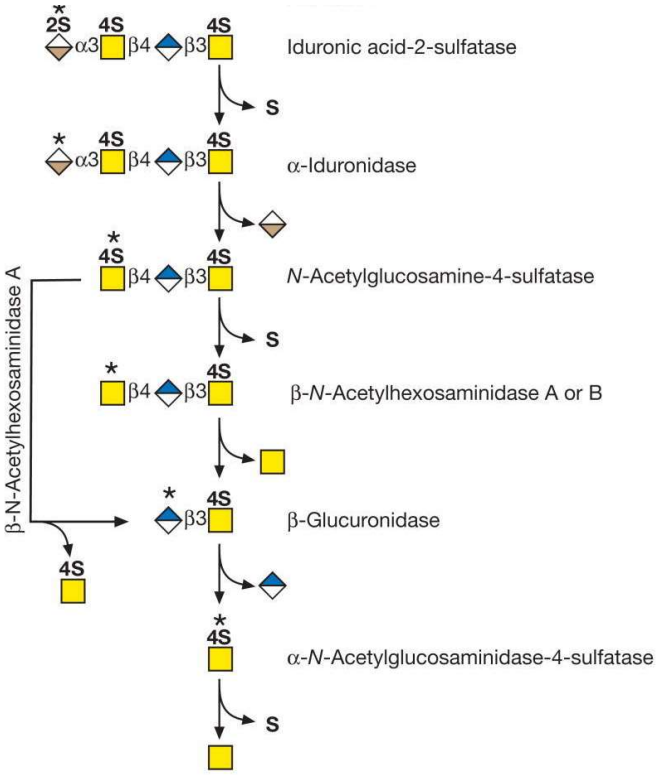


Figure 2.8: Dermatan Sulfate and Chondroitin Sulfate catabolism [19].

2.2.4 Keratan Sulfate Catabolism

Keratan sulfate degradation is carried out by the sequential action of exoglycosidases and sulfatases which act at the non-reducing terminal level. The first stage involves the desulfation of galactose-6-SO4 followed by the action of β-galactosidase to detach the galactose residue. GlcNAc-6-SO4, as in the case of CS, can be first desulfated and then hydrolyzed or vice versa [19].

2.3 Glycosaminoglycans Analysis

Various analytical approaches are reported in the literature, aimed at the qualitative-quantitative evaluation of GAGs in biological fluids and tissues for diagnostic and research purposes. The more classic electrophoretic methods are flanked by High-Performance Liquid Chromatography (HPLC) and Mass Spectrometry (MS).

2.3.1 Electrophoretic Methods

Methods of choice for GAGs analysis are electrophoretic approaches, including electrophoresis in agarose gel, polyacrylamide gel, nitrocellulose or cellulose acetate, capillary electrophoresis, etc.

Two-dimensional agarose gel electrophoresis is probably one of the most used methods for GAGs qualitative evaluation in heterogeneous samples such as tissues and biological fluids. It is a simple method that allows the separation of different molecules based on their charge and molecular weight. Through bands densitometric analysis it is possible to obtain GAGs quantification [20].

Another relatively simple method required cellulose acetate strips on which different GAGs can be separated; depending on the type of buffer used, various types of analysis can be performed, from single GAGs separation to sulfation degree detection (57).

PAGE (Polyacrylamide Gel Electrophoresis) is a very useful tool in GAGs analytical investigations. Used in many experiments, it allows for the characterization of GAGs structures and properties [20].

Fluorophore Assisted Carbohydrate Electrophoresis (FACE) is also a very common method for GAGs analysis, it allows the electrophoretic separation of fluoro-labelled unsaturated disaccharides. GAGs need previous chemical or enzymatic digestion to obtain disaccharides, that will be linked to a fluorescent tag, such as 2-aminoacridone (AMAC). At the end of the run, a transilluminator allows gel reading [21].

2.3.2 Capillary Electrophoresis

Capillary Electrophoresis (CE) offers many advantages over other analytical methods, such as high separation efficiency, automated and reproducible analysis possibilities, moderate analysis time, low sample and buffer consumption, and coupling with ESI-MS for online structural evaluation [22].

The system consists of a 25 cm long fused silica capillary, with a 50 µm internal diameter, a high voltage power supply and two electrodes, a detector, and a device for injecting samples into the capillary.

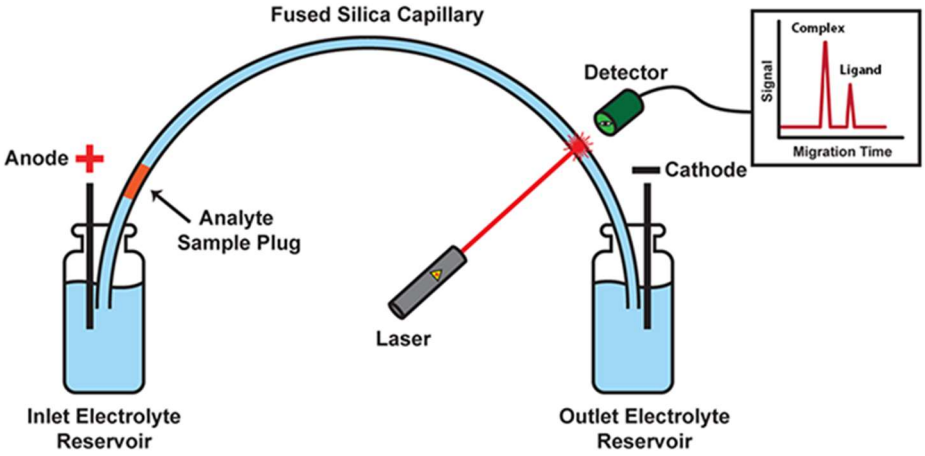


Figure 2.9: Main components of capillary electrophoresis.

All analytes travel the same distance from the injection point (positive electrode - anode) to the opposite end (negative electrode - cathode) where the detector is positioned: the

analytes are distinguished based on the time taken to travel this distance, called the migration time. The resulting graph is called an electropherogram which allows the identification of chemical compounds by analyzing the migration times, and sample quantification by calculating the peak's height and areas in the graph.

By applying a high-voltage electric field to the capillary (up to 25-30kV) the analytes migrate at different speeds, depending on their size and their charge, towards the oppositely charged electrode through an effect called electrophoretic mobility (EM). In addition to EM, electroosmotic mobility (EOF) is also recorded. Working at neutral or basic pH, the silanol groups of the silica deprotonate and interact ionically with the positive charges of the running buffer, forming an electric double layer close to the surface of the capillary: by applying an electric field, this double layer behaves like a piston which causes a net movement towards the cathode [23].

Therefore, the species separation in the capillary is carried out by the resultant force of the vector sum between the EOF and the EM: in the saccharides deriving from the enzymatic digestion of the GAGs, which are negatively charged, the EOF and the EM oppose each other and allows sugars migration towards the cathode. Capillary electrophoresis allows the separation of analytes towards the negative pole regardless of their electrical charge but with different migration times: positively charged species are detected first, then neutral species and finally negatively charged.

In this study, the detection method was laser-induced fluorescence (LIF): a laser, set at an appropriate wavelength, is used to excite a fluorescent compound, which is detected thanks to the following light emission. The laser possesses a high-intensity monochromatic light, which allows powerful and selective excitation of the derivatized saccharide residues [24].

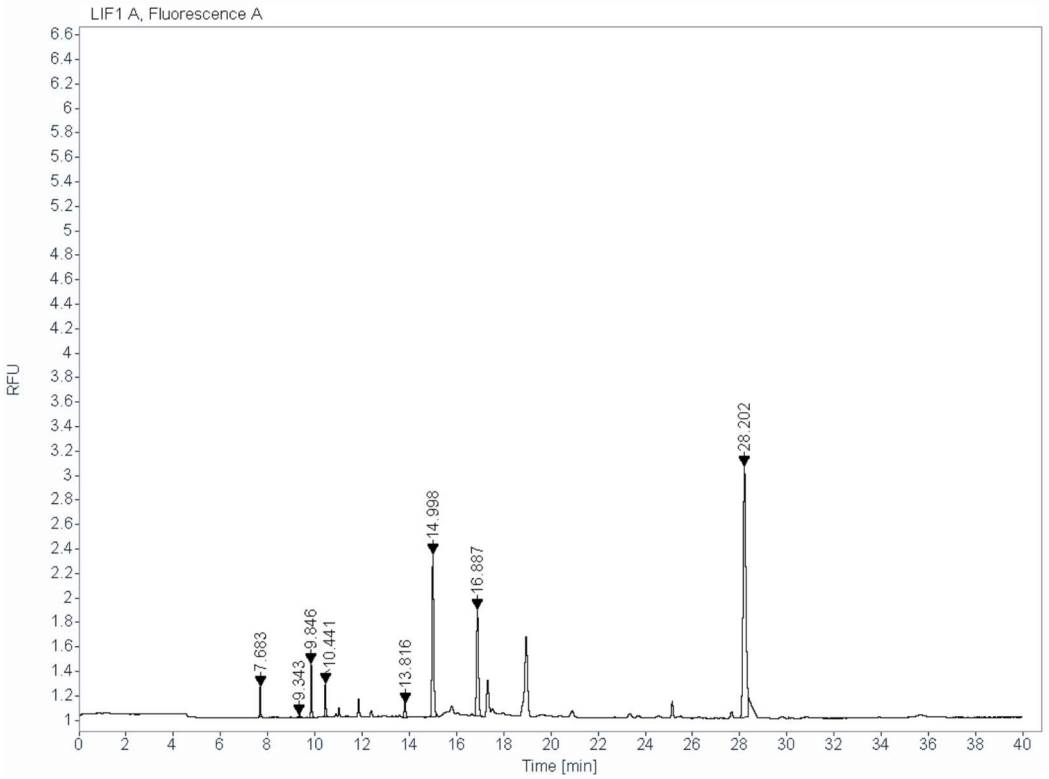


Figure 2.10: Electropherogram of Heparinase treated sample.

2.3.3 HPLC

High-performance liquid chromatography (HPLC) is a chromatographic method used to separate, identify and quantify the molecules contained in a mixture. It exploits the affinity balance between a stationary phase, placed inside the column, and a mobile or eluent phase, which flows through it. The instrumentation essentially consists of a pumping system which allows the creation of a solvent flow, a valve for the sample injection, a packed column in which the separation takes place and finally a detector. The system is connected to a computer necessary for chromatogram creation.

The heart of the method is represented by the column containing the stationary phase. The choice of this component is therefore fundamental for the type of analysis to be performed. The analytical sample particles tend to have more or less strong interactions with the stationary phase. The flow of eluent passing through the column will therefore detach these particles at different times depending on their chemical properties and the strength with which they interact with the stationary phase [25] [26].

2.3.4 Mass Spectrometry

Mass spectrometry is an analytical method widely applied in various biological fields, including glycan study. The methodology is based on the molecule ionization by a source. The ions thus obtained are sent to an analyzer which separates them according to their charge and mass (m/z) characteristics. The separated particles are finally picked up by a detector, obtaining a mass spectrum, the final output of this technique. The great versatility of this technology lies in the variety of sources and analyzers available which amplify its applicability. Another advantage is represented by the possibility of coupling mass spectrometry to a separating phase in HPLC. On the other hand, their use is limited by the high costs as well as the complexity of results interpretation which requires expert and qualified personnel.

In the field of glycobiology and the study of mucopolysaccharidosis, the mass spectrometer is often associated with HPLC or CE. In this way, it is possible to investigate biological fluids, obtaining both GAGs quantification and structural characterization [27] [28].

2.4 Mucopolysaccharidoses

Mucopolysaccharidoses (MPS) represent a group of rare diseases (1:25,000 live births [29]) and are part of a larger group of lysosomal storage diseases. In these pathologies, the non-degraded GAGs tend to accumulate in cells and tissues causing serious alterations and therefore a typical symptomatologic picture. MPS is caused by the deficiency of the enzymes that catalyze glycosaminoglycan degradation. Depending on the type of enzyme deficiency, the degradation of DS, CS, HS, KS and HA can be blocked or hindered, individually or in combination.

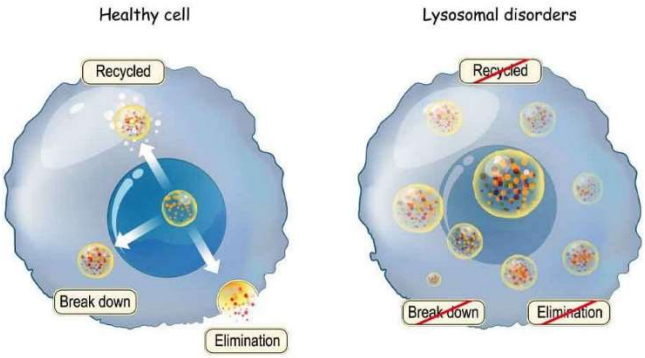


Figure 2.11: Cell with normal lysosomal catabolism vs cell with lysosomal accumulation due to enzyme deficiency [30].

MPS are genetic diseases with autosomal recessive transmission except for MPS II which has a recessive transmission linked to the X chromosome. The clinical manifestations that characterize MPS are essentially related to the amount and type of substance accumulated. The consequence is GAGs intracellular accumulation which results in cellular, tissue, organ dysfunction and GAGs abnormal urinary excretion. Since lysosomes are contained in all body cells, except for red blood cells, the metabolic defect occurs simultaneously in various organs and systems. MPS infants are asymptomatic, although the accumulation begins in the fetal period [31]. Like all storage diseases, MPS are also progressive, with variable onset in the first years of life and sharing some clinical characteristics such as coarse facial features, macrocrania, hypopostaturality, skeletal anomalies, hepatosplenomegaly, mental retardation, corneal opacity and deafness, clinical findings do not appear in all forms. The central nervous system can suffer serious consequences, such as cognitive or behavioral disorders, furthermore, severe mental retardation characterizes some forms of MPS while in others CNS may not be involved. Severe mental retardation is present in MPS I (Hurler syndrome), in the severe form of MPS II (Hunter syndrome), and in all subtypes of MPS III (Sanfilippo syndrome). In the most serious cases, they are extremely disabling pathologies and can lead to early death. The mucopolysaccharidosis characteristic is the presence of partially degraded GAGs fragments in urine, blood and cerebrospinal fluid [32]. Eleven known deficient enzymes give rise to seven distinct forms of MPS as indicated in Table 1.

MPS	ENZYME DEFICIENCY	GAG
MPS I	α-L-iduronidase	DS HS
MPS II	Iduronate-2-sulfatase	DS HS
MPS III A-D	A: heparan *N*-sulfatase	HS
	B: α-*N*-acetylglucosaminidase	
	C: acetyl-CoA- α-glucosaminide acetyltransferase	
	D: *N*-acetylglucosamine 6-sulphatase	
MPS IV A, B	A: galactose 6-sulphatase	KS, CS
	B: β-galactosidase	KS
MPS VI	Arylsulphatase B	DS, CS
MPS VII	β-Glucuronidase	DS, HS, CS
MPS IX	Hyaluronidase	HA

Table 1: MPS Classification.

2.4.1 Mucopolysaccharidosis Type I

Mucopolysaccharidosis type I is caused by a defect in the enzyme α-L-iduronidase. The altered gene is located on the short arm of chromosome 4, is approximately 19 kb in size, and contains 14 exons [33].

Three variants are distinguished according to severity: Hurler syndrome which is the most severe form, Scheie syndrome which is the mildest and Hurler-Scheie syndrome which presents an intermediate phenotype. Hurler/Scheie's S. and Scheie's S. are also called "attenuated" forms.



Figure 2.12: MPS I patients with severe or attenuated phenotype [34].

Mucopolysaccharidosis I/H (Hurler Syndrome) manifests itself from the first year of life with characteristic clinical manifestations such as a flattened nose, prominent forehead, short stature and severe mental retardation. Corneal opacity, chronic hearing loss, dysostosis multiplex, and organomegaly are typical. The patients are also subject to cardiovascular and respiratory problems which often lead to the subject's death within the first decade of life.

Mucopolysaccharidosis I/S (Scheie's syndrome) is a very rare, late-onset disease; in fact, it is rarely diagnosed in childhood. Clinical signs are mild joint limitations, mild hepatomegaly, corneal opacity, and heart valve involvement, but there is no mental retardation or stature deficit. The prognosis is good, with most patients reaching advanced adulthood.

Mucopolysaccharidosis IH/S (S. of Hurler/Scheie) is characterized by an intermediate phenotype between the two forms described above; the disease manifests itself in childhood and evolves slowly, allowing survival between 20 and 30 years. Patients may experience mild mental retardation, dysostosis multiplex, corneal opacity, or micrognathism. Cardiovascular and respiratory problems can also arise [34].

From a laboratory point of view, the urinary excretion of HS and DS is observed due to α-L-iduronidase enzyme deficiency, dosed on cultured leukocytes or fibroblasts. Molecular analysis of the gene located on chromosome 4 (4p16.3) allows diagnosis confirmation [35].

2.4.2 Mucopolysaccharidosis Type II

MPS II (Hunter Syndrome) is the only form of MPS that has an X-linked inheritance, so males are affected more than females. The deficient enzyme is iduronate-2-sulfatase (I2S), a key enzyme in the catabolism of DS and HS; the incidence of Hunter syndrome is estimated to be about 1 in 165,000 male births [29].

Clinically, a severe form is distinguished from a mild one, the symptoms of the severe form generally appear around the first year of life and reach full expression at 3-4 years of age. The most severe form presents progressive mental retardation and motor disability; progression is slow and survival is estimated at 15-20 years of life. The mild form is compatible with adult life and mental retardation may be mild or absent. In general, subjects may have joint problems, mental retardation, muscle weakness, breathing problems, hearing loss, and behavioral problems.



Figure 2.13: Two patients with MPS II. Patient A manifest a high degree of severity. Patient B shows an attenuated phenotype of the disease [36].

From a laboratory point of view, the urinary excretion of HS and DS is observed due to the deficiency of the enzyme iduronate-2-sulfatase, measured on cultured leukocytes or fibroblasts. Molecular analysis of the gene located on the X chromosome long arm (Xq27-

28) allows diagnosis confirmation [37] [38]. The gene responsible for Hunter syndrome is 24 kb and contains 9 exons [39] [40].

For the Hunter syndrome study, knock-out mouse models were generated by targeting exon 4 and part of exon 5 of the gene, and it has been characterized [41].

2.4.3 Mucopolysaccharidosis Type III

MPS III (Sanfilippo Syndrome) is the most frequent form of MPS and is due to four distinct enzymatic defects (forms A-D), all involved in the degradation of HS.



Figure 2.14: Patient with MPS III. Facial abnormalities, typical of the pathology [42].

From the clinical point of view, it is mainly characterized by the rapid and progressive mental retardation associated with mild facial dimorphisms. Psychomotor development is mostly normal; subsequently, there is a slowdown, an arrest and finally a more or less rapid regression associated with considerable hyperactivity which makes patients management very difficult [43]. The final stage is characterized by profound dementia often associated with convulsions, athetosis and spastic quadriplegia. Death occurs in the second decade of life. This pathology is classified according to enzyme deficiency:

- MPS IIIA, the most severe form, is due to a deficiency of the enzyme heparan N-sulfoglucosamine sulfohydrolase (chromosome 17q25.3) [44].
- MPS IIIB is caused by α-N-acetylglucosaminidase (chromosome 17q21) enzyme deficiency [45].

- MPS IIIC is linked to the deficiency of another lysosomal enzyme, α-glucosaminide N-acetyltransferase (chromosome 8p11.1) [46].
- MPS IIID is due to the enzymatic deficiency of N-acetylglucosamine-6-sulfatase (chromosome 12q14) [47].

Laboratory tests show urinary excretion of HS.

2.4.4 Mucopolysaccharidosis Type IV

MPS IV (Morquio Syndrome) is characterized by skeletal dysplasia and urinary excretion of keratan sulfate, it can be caused by two different enzyme defects: galactosamine-6-sulfatase (MPS IV A chromosome 16q24.3) and β-galactosidase (MPS IV B chromosome 3p21.33) [48]. The first clinical manifestations are observed between 18 and 24 months of life and include growth retardation and initial signs of bone dysplasia [49].



Figure 2.15: Patients with MPS IV [50].

The clinical picture is complete around 4-6 years. Clinically, the two forms can manifest with a wide range of symptoms: skeletal malformations, dwarfism, corneal opacity and vision disturbances. Patients do not have cognitive problems or mental retardation; the prevalent symptoms are those affecting the skeletal system. The stature development is seriously compromised with a definitive height that stands between 115 and 120 cm. The two forms are almost similar, except for a generally less severe phenotype in form B, in form A it is

possible to evaluate an accumulation of both CS and KS. The laboratory diagnosis is based on the evaluation of KS excretion in urine and the analysis of the enzyme deficiency in leukocytes or fibroblasts.

2.4.5 Mucopolysaccharidosis Type VI

MPS VI (Maroteaux-Lamy Syndrome) is caused by aryl sulfatase B deficiency, resembles Hurler syndrome in clinical characteristics, but does not show mental retardation. Heart failure due to valvular involvement is a frequent finding, the symptoms show short stature, heart problems, joint stiffness and facial dysmorphism [51].

The urinary dosage of mucopolysaccharides shows an increased excretion of CS and DS and the diagnosis is based on the enzymatic activity determination and the molecular investigation of the gene encoding aryl sulfatase B, mapped on chromosome 5 (5q11-5q13).

2.4.6 Mucopolysaccharidosis Type VII

MPS VII (Sly Syndrome) is due to the deficiency of β-glucuronidase enzyme, which is involved in the catabolism of three glycosaminoglycans: DS, CS and HS. The gene is located on chromosome 7 (7q11.21). Very few cases have been described in the literature and the clinical picture is variable; there are forms with onset in the prenatal period which often cause stillbirth, forms with onset in the neonatal period associated with dysmorphisms, hernias, hepatosplenomegaly, dysostosis, hypotonia and neurological deficits with severe cognitive impairment and short stature and finally forms with onset in adolescent or adult age who are discovered following presentation with thoracic kyphosis [52].

2.4.7 Mucopolysaccharidosis Type IX

MPS IX (Natowicz syndrome) is caused by hyaluronidase enzyme deficiency, whose gene is located on chromosome 3 (3p21). It is an extremely rare condition, known patients present nodular masses of soft tissue located at the joints level, motor difficulties and short stature. The laboratory diagnosis is based on the analysis of the enzymatic deficiency carried out on fibroblasts or leukocytes [53].

2.5 Mucopolysaccharidoses Diagnosis

As far as mucopolysaccharidoses are concerned, the variety of phenotypes and symptoms usually leads to diagnosis delay. This leads to substrate accumulation and irreversible tissue damage; the only option is to limit symptom progression. For this reason, is essential to anticipate the diagnosis times to the subject's first stages of life [54].

The diagnostic investigation involves an accurate assessment of the patient's clinical condition followed by confirmatory laboratory tests. The presence of substrates in the urine is verified by dosage and investigation by two-dimensional electrophoresis and DMB staining [55]. This type of approach does not allow for discrimination between MPS forms, for this reason, the next step is the enzymatic activity test, to identify the deficit and therefore the pathology. The lysosomal hydrolytic enzymes quantification is performed on cultured leukocytes or fibroblasts.

Biochemical investigations are followed by molecular investigations to evaluate the mutated gene presence and the family history evaluation, defining healthy carriers with the mutated allele.

Very interesting is the DBS (dried blood spots) research for the early diagnosis of rare genetic diseases. Many of these studies involve lysosomal storage diseases, such as mucopolysaccharidosis. In the first place, the use of DBS facilitates the pre-analytical process. Sample collection is less invasive which is why it is welcomed with greater adherence by the subjects.



Figure 2.16: Dried Blood Spots card.

Transportation and storage are also simpler when compared to venous blood or urine specimens. Another important DBS advantage is the possibility to carry out both enzymatic

and molecular biology investigations. Given all these characteristics, analytical approaches that allow the use of dried blood samples are excellent candidates for screening tests development, to be performed on newborns. Applied to pathologies such as MPS, they would allow for a better and more timely therapeutic approach [56].

As for MPS II diagnosis, the "gold standard" is the demonstration of I2S enzyme deficiency in leukocytes, fibroblasts and plasma. Once the test is positive, it is crucial to monitor the enzymatic activity of at least one of the other sulfatase to avoid the presence of multiple deficient sulfatase pathologies. Genetic testing may be useful in male patients who display an atypical phenotype, or for those whose enzyme testing is negative. Genetic testing can be useful when no cases of MPS II are known in the family history, to identify female carriers. In prenatal diagnosis, the enzymatic activity of I2S can also be tested in cells cultured from amniotic fluid, in cells obtained from a chorionic villus biopsy or fetal blood [37] [57].

2.6 Mucopolysaccharidoses Therapy

In the majority of mucopolysaccharidoses forms, the prognosis is very poor both in terms of mortality and morbidity, so there has always been a considerable commitment to identify a therapy capable of modifying the natural history of these diseases. Historically, the treatment of mucopolysaccharidoses relied on palliative care to treat the symptoms. Currently, for some forms of MPS, therapies are available. These approaches aim to reduce GAGs accumulation in cells and tissues by the administration of the deficient enzyme, directly, through enzyme replacement therapy (ERT) or, indirectly, through the transplantation of hematopoietic stem cells (HSCT) or cells genetically modified to express the missing enzyme (gene therapy).

2.6.1 Enzymatic Replacement Therapy

The treatment of choice for subjects affected by MPS type I, II, IVA and VI is represented by Enzyme replacement therapy (ERT). In MPS II, ERT (Elaprase®) involves the weekly administration by intravenous infusion (dose of 0.5mg/kg of body weight) of the recombinant I2S enzyme produced in a human cell line. The enzyme maintains the same pattern of post-translational modifications, including the characteristic glycosylation, responsible for the lysosome internalization, according to the endocytic pathway mediated by the mannose-6-P (M6P) receptor.

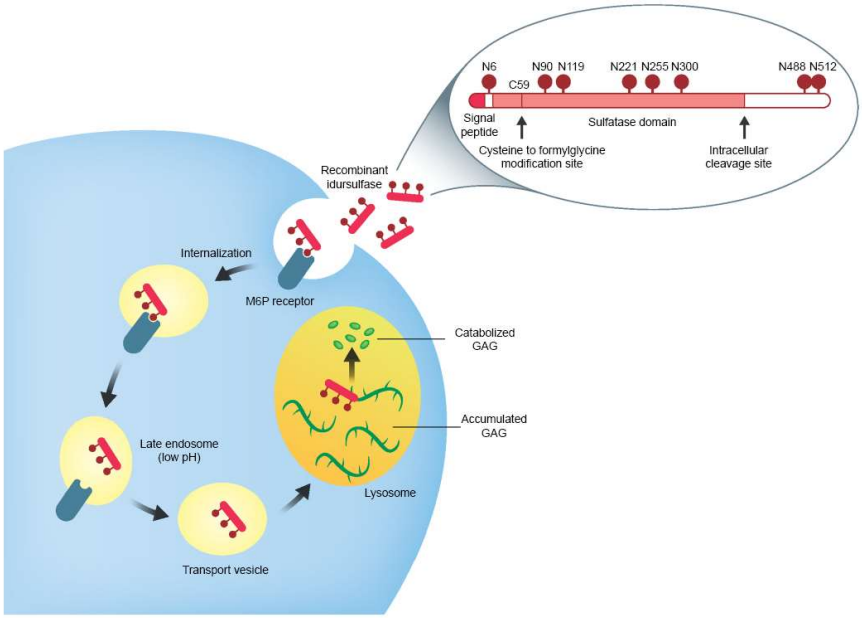


Figure 2.17: Recombinant enzyme cellular uptake mechanism [58].

The treatment efficacy and safety are confirmed, especially concerning visceral organs such as liver, spleen and kidneys, in which improvements such as size reduction, are already appreciable after a few months of therapy. The enzyme has a short half-life in circulation, precisely due to the intense endocytosis mediated by the M6P receptors. Only a small fraction reaches other areas of interest such as bones and cartilage, where the effects, even after prolonged periods of treatment, are limited. The main defect of this therapy consists, however, of the inability to cross the blood-brain barrier, having no beneficial effect on the central nervous system [59]. Although positive results have been reported following ERT in

neonates [60] or using doses far higher than those conventionally used in clinical treatment [61]. With the current dosages and formulation, enzyme diffusion across the BBB is almost non-existent [62]. For this reason, the intrathecal injection of the recombinant enzyme (IT-ERT), i.e., the direct administration into the cerebrospinal fluid, is still being studied. This would seem to slow down, but not resolve, the progression of neurological decline [63]. Furthermore, new approaches are under study with fusion proteins to circumvent the obstacle of the blood-brain barrier, exploiting the receptor-mediated mechanism that allows transferrin to enter the CNS. The I2S was combined with a portion of human anti-transferrin receptor antibody, following intravenous injections the HS levels in the cerebrospinal fluid and brain, significantly decreased in animal models and humans, parallel to a systemic efficacy not inferior to the canonical ERT [64]. However, ERT is extremely expensive, approximately 400 000 euros year−1 for a 30 kg patient.

2.6.2 Hematopoietic Stem Cell Transplantation

Among current non-pharmacological therapies, hematopoietic stem cell transplantation (HSCT) is available for MPS I, II and VI, while studies have not found improvements for MPS III and IV [65]. In subjects undergoing HSCT, a reduction in the onset of symptoms was observed. Improvements have been reported for respiratory, cardiovascular, sight, hearing and joint mobility systems. The nervous system also benefits from this type of treatment which prevents mental retardation [66]. The efficacy of the therapy depends on the timeliness of the treatment and the age of the subject.

While there are major hurdles to overcome, such as finding a matched donor and reducing procedure-related mortality rates, HSCT can provide a long-lasting source of enzymes that rapidly reduce GAGs accumulation and alter the course of the pathology, especially preserving the neurological involvement in patients with Hurler syndrome, treated before the second year of age.

2.6.3 Genetic Therapy

In recent years, gene therapy has attracted particular attention due to the possibility of guaranteeing a stable resource of the enzyme with possible localization in all organs, including the brain. Adenoviral or lentiviral vectors are exploited depending on the transduction method used, respectively in vivo (intravenous, intracranial or direct intrathecal injection of the vector); or ex vivo, autologous HSCT in which the patient's stem cells are transfected with the vector and then transplanted. Studies have shown efficacy in mice affected by MPS I, II and III regarding GAGs accumulation in the CNS, while trials currently in progress reveal that the combined injection of vector and specific zinc-fingers for the gene insertion at the correct locus leads to a reduction in urinary GAGs levels without serious side effects. This method greatly reduces the risk of rejection and problems in finding a matching donor [63].

2.6.4 Substrate Reduction Therapy: Genistein

Substrate reduction therapy (SRT) has recently been shown to be a promising strategy for the treatment of CNS-related symptoms of MPS. SRT exploits small molecules that potentially cross the blood-brain barrier and act in CNS, altering GAGs synthesis and therefore re-establishing a balance between anabolism and catabolism in cells. Among the molecules under study, genistein (GENI), a natural soy isoflavone (4',5,7-Trihydroxyisoflavone) is of particular interest.

Genistein has recently been identified as a potential new treatment for MPS [67] [68]. It belongs to a group of plant-derived compounds (phytotherapeutics) used to treat disorders such as osteoporosis [69], cardiovascular disease [70] and menopause [71]. Further therapeutic potential has been shown for cancer [72] and other chronic diseases.

Studies in MPS IIIB and MPS II mice demonstrated that genistein led to complete behavioral correction in long-term treatments for MPS IIIB, and CNS improvements in short-term treatments for MPS II.

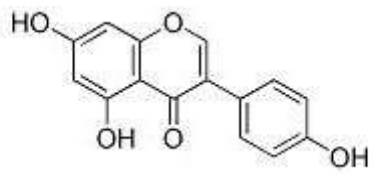


Figure 2.18: Isoflavone genistein structure [73].

As for genistein action mechanism, the maximum level of GAGs synthesis is reached when there is a link between the growth factor EGF (epidermal growth factor) and its tyrosine kinase receptor which, once activated, induces the start of a kinase cascade that ends with the activation of particular transcription factors. Genistein acts as an inhibitor of the EGF receptor, consequently, it limits GAGs biosynthesis by altering the gene expression of the enzymes involved in GAG synthesis [74]. Genistein could therefore be considered as a new form of SRT, based on inhibition of GAG synthesis and thus be applied in therapeutic protocols for MPS.

Friso et al. analyzed a set of I2S-knock-out mice treated alternately with 5 mg/kg or 25 mg/kg of genistein per day, for 10 or 20 weeks and surprisingly the efficacy of the therapy correlated inversely with the duration of the same, indicating that sustained EGFR saturation induces its long-term up-regulation, as endogenous ligands exert pressure on the transcription rate of the receptor [75].

A possible problem related to genistein long-term administration in children could relate to its phytoestrogenic activity, potentially influencing puberty and fertility [67]. However, studies of genistein toxicity in vivo showed no adverse effects in dogs up to 500 mg·kg−1·day−1 [76]. Administration in humans of doses of genistein up to 600 mg·day−1 (8 mg·kg−1·day−1) for about 3 months resulted in minor side effects related to its oestrogenic activity [77], while cytogenetic analysis in peripheral blood lymphocytes, obtained from subjects under treatment for prostate cancer, showed no increase in DNA strand breaks [78]. Genistein is much less expensive than ERT, and it can be taken orally. In addition, this molecule can cross the BBB in rats and mice [79] and GAG reduction in vitro mediated by genistein was recently described [67] [74].

Therefore, genistein could help in solving ERT limitations, as well as providing hope for those forms of MPS for which ERT is not yet available. Because of these potential benefits to MPS, genistein merits a full evaluation in terms of efficacy.

3 OBJECTIVES

In MPS study, the importance of early diagnosis has emerged. As well as constituting the basis for the formulation of genetic advice to the family, early diagnosis is the fundamental prerequisite for allowing an early therapeutic approach before irreversible damage has occurred in those diseases for which effective therapy is currently available. In this regard, new frontiers are being crossed in current and future therapies study; therefore, the need emerges for a careful evaluation of the treatment to optimize its use, evaluate its risks and monitor its effectiveness.

The present work, through the modification of already known laboratory methods (analysis of HA, CS, DS and HS total concentration, disaccharide composition, chemical properties and overall structure dosage) allows the characterization of glycosaminoglycans in urine, plasma, brain and liver of a mouse model of MPS II I2S-KO non-treated, treated with ERT or treated with genistein, analyzed at different weeks after the beginning of therapy.

The analysis of GAGs in this model allows the molecular characterization of a mouse model for MPS II analysis, to determine the behavior of this model compared to wild type mice and facilitate the identification of new biomarkers for the early diagnosis of MPSII also in humans.

Furthermore, the follow-up analysis of this mouse model treated with different therapeutic approaches can give us important information on the efficacy and modulation of the therapy during its course.

Finally, the results obtained from GAGs analysis in a new therapeutic strategy such as genistein could give further information for the application of substrate reduction therapy, providing the basis for future clinical trials on humans.

4 MATERIALS AND METHODS

4.1 Mouse Model

The MPS II mouse model was obtained by the JAX® Mice & Services (JAX stock #024,744; The Jackson Laboratory, Bar Harbor, Maine, USA), originally generated by gene disruption of the murine *i2s* gene [41] [80]. I2S knock-out mice (I2S-KO) were expanded by the University of Padua animal facility in light- and temperature-controlled conditions, with water and food, provided ad *libitum*. This study was conducted in strict accordance with the European Directive 2010/63/EU. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Padua and authorized by the Italian Ministry of Health (project n.410/2015, approved on May 21, 2015). In the experiments here reported, hemizygous I2S-KO male mice were treated, starting at 12 weeks of age, by weekly administration of Elaprase® (Shire Lexington, MA, USA, now Takeda Pharmaceutical Co.), at a dosage of 1 mg/ kg. Administrations were carried out through tail vein injection, for 0, 2, 4, 6, 12, 18, or 24 weeks. As controls, I2S-KO and WT mice were injected with 0.9% NaCl following the same schedule. Before treatment and 1 week after the last injection, plasma samples were obtained by submandibular vein bleeding, and urine samples were collected using metabolic cages for 24 h.

Regarding genistein treatment, hemizygous I2S-KO male mice were fed, starting at 12 weeks of age with modified pellet (Mucedola, Milan, Italy) with the addition of genistein (Soyfem, Biofarm, Pozna&#324, Poland) in the dosage of 5 mg/kg/day. This dosage equals the human daily intake used in the published pilot study [68] in patients with MPS III. As a control, 3 WT mice were fed similarly, while I2S-KO and WT mice were fed unmodified pellets. Before treatment and 1 week after the last time point, plasma samples were obtained by submandibular vein bleeding, and urine samples were collected using metabolic cages for 24 h.

Upon reaching each time point 6-7 mice were sacrificed. Liver and Brain tissues were harvested after administration of PBS pH 7.4 to eliminate organs' blood perfusion. Liver brain and urine were frozen until analysis. Serum was obtained by centrifugation at 10,000 g for 10 minutes, then frozen until use. All samples were sent to the University of Modena and Reggio Emilia where biochemical GAGs analysis were performed on plasma, urine, liver and brain.

4.2 GAGs Isolation and Purification

GAGs were extracted from plasma, urine and tissues according to a standardized protocol [81], [82].

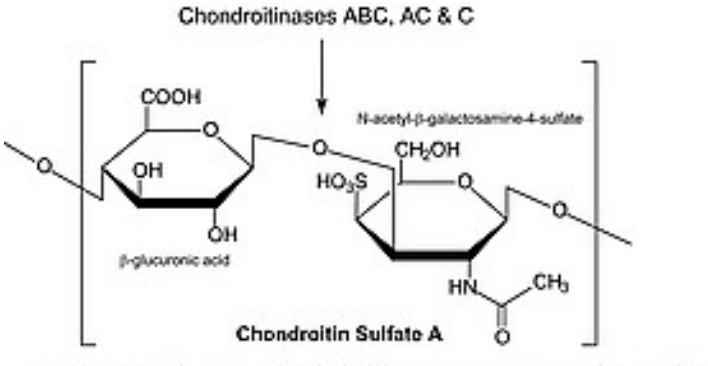
In the protocol first steps, samples were treated differently depending on the original tissue:

- Plasma: 100 µl of sample was freeze-dried and treated with Actinase E (Streptomyces grisou-type XIV protease, E.C 3.4.21.80, Sigma-Aldrich, Germany) at 37°C overnight. The sample was then boiled for 10 minutes and centrifuged to collect the supernatant.
- Urine: 500 µl of sample was precipitated by adding 5 ml of ethanol overnight at -20°C, to favor GAGs precipitation. After being centrifuged, the supernatant was discarded, the pellet preserved, and then dried. Afterwards, the dried pellet was dissolved in 1 ml of distilled water. Urine creatinine analysis was also performed.
- Tissues: 5 ml of acetone were added to 300 mg of sample to favor organ dehydration and lipid fraction dissolution. The tissue was then homogenized and centrifuged. The collected pellet was desiccated and treated with Actinase E. Afterwards, the sample was boiled for 10 minutes and centrifuged to collect the supernatant. At this point, the sample was treated with sodium borohydride 0.1 g/mL and NaOH 2M overnight at 4°C. This method allows the breaking of the bonds between glycosaminoglycans and amino acid residues, covalently linked through serine hydroxyl groups. In a β-elimination reaction, the bond between xylose of the bridging

sugar sequence (xylose-galactose-galactose) and serine is cleaved. The reaction was then neutralized with 2M HCl and 5 mL of absolute ethanol were added to precipitate the polysaccharides overnight at – 20°C. The sample was centrifuged and the pellet obtained was dried and dissolved with 1 ml of distilled water.

All the obtained samples were then loaded onto a 0.5-2cm anion exchange chromatographic column, prepared by adding 500 µl of QAE-SEPHADEX A-25 resin (Sigma-Aldrich, Germany) re-equilibrated in water. The GAGs retained by the column resin were recovered by competition by adding 1 ml of 2.5M NaCl solution to the chromatographic column. The eluate was then filtered on YM3 Amicon Ultra-0.5ml filters (Merck Millipore, USA) and the retentate, containing the GAGS chains, was freeze-dried.

Quantitation and structural characterization of glycosaminoglycans (HA and HS) and galactosaminoglycans (CS and DS) were performed by constitutive disaccharides. Lyophilized aliquots of isolated GAGs were reconstituted with suitable buffers and treated with chondroitinases or heparinases. Two aliquots of extracted GAGs were reconstituted with ammonium acetate 0.1 M pH 7.9 and treated with 20 milliUnits (mU) of chondroitinase ABC (from Proteus Vulgaris [EC 4.2.2.4], Sigma-Aldrich, Germany) for the determination of HA, CS, and DS. Chondroitinase ABC enzyme catalyzes the eliminatory degradation of polysaccharides containing (1-4)-β-D-hexosaminyl and (1-3)-β-D-glucuronosyl or (1-3)-α-L-iduronosyl linkages to disaccharides containing 4-deoxy-β-D-gluc-4-enurosyl groups. Then it acts on chondroitin 4-sulfate, chondroitin 6-sulfate and dermatan sulfate, and mildly on hyaluronic acid.



Alternating copoly (β-glucuronic acid-(1-3))-N-acetyl-β-galactosamine-4-sulfate

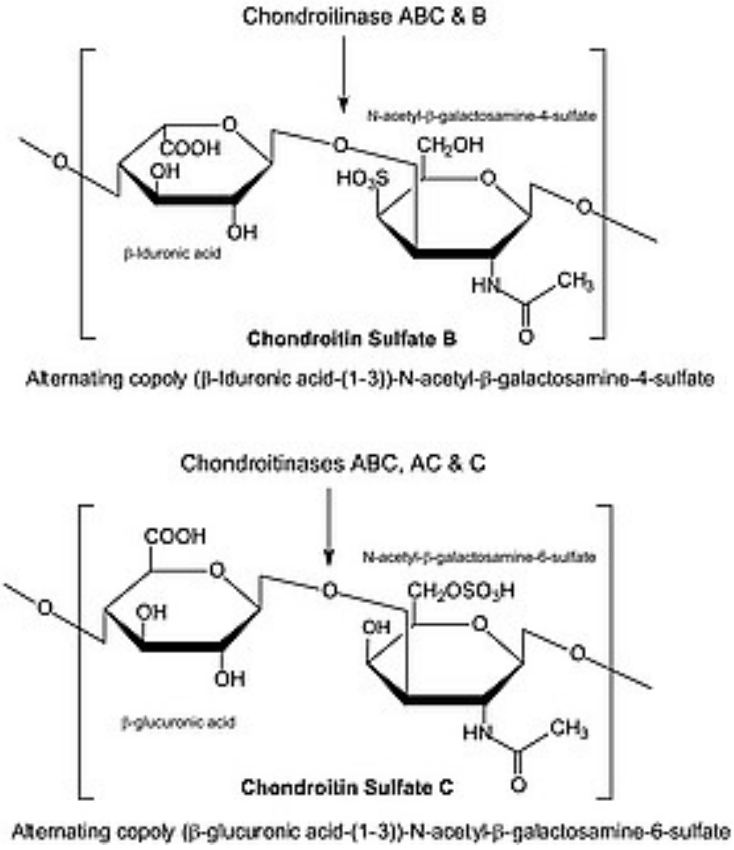


Figure 4.1: Mechanism of chondroitinases ABC, AC and B. The enzymes work by degrading the glycosidic bond between uronic acid and hexosamine [83].

Another aliquot was reconstituted with 0.1 M sodium acetate and 0.1 M calcium acetate pH 7.0 and treated with a mixture of 1 mU of heparinase I from Flavobacterium heparinum [EC 4.2.2.7] and 0.1 mU of heparinases II (from Flavobacterium heparinum [EC number not assigned]) and III (from Flavobacterium heparinum [EC 4.2.2.8]) (Grampian Enzymes, Orkney, UK) at 35 °C overnight, for the release of HS disaccharides.

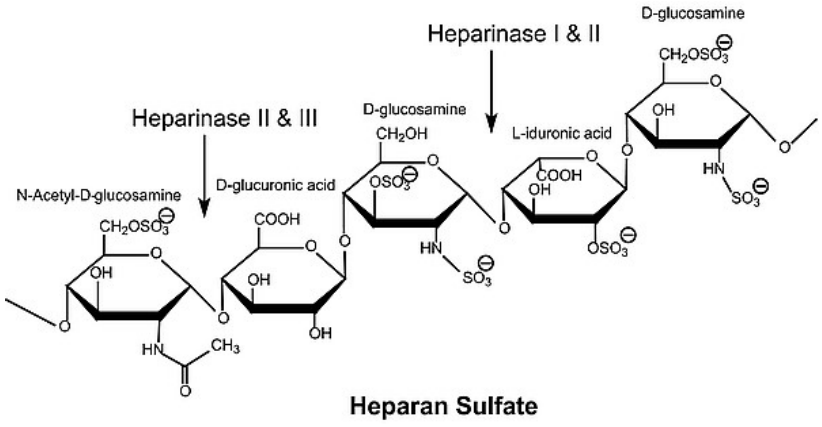


Figure 4.2: The action of heparinases against a molecule of HS. Enzymes I and II hydrolyze the bond between hexosamine and iduronic acid, II and III the bond between hexamine and glucuronic acid [84].

4.3 Capillary Electrophoresis

After lyophilization, generated unsaturated disaccharides were fluorotagged with 2-aminoacridone (AMAC) (Sigma-Aldrich, Germany) [85] and separated by CE-LIF, as previously reported [82] [86]. The lyophilized mixtures of Δ-disaccharides were reconstituted with a 0.1 M AMAC solution in glacial acetic acid-DMSO and a freshly prepared 1 M sodium cyanoborohydride in water. After centrifugation at 11,000 g for 3 min, derivatization was performed by incubation at 45 °C for 4 h. Finally, 50% v/v DMSO was added to the samples and aliquots were taken for CE-LIF analysis. Disaccharides fluorotagged with AMAC were analyzed using an HPCE system (Agilent Technologies, Wilmington, DE, USA) equipped with a ZETALIF (Picometrics, France) detector (λex = 488 nm). Resolution and analysis were performed on uncoated fused-silica capillary columns at 25 °C using 50 mM phosphate buffer (pH 3.8) with a voltage of 30 kV under reversed polarity. The capillary was flushed with HPLC-grade water, 0.1 M NaOH, water, and operating buffer between each run. The operating buffer was filtered through a 0.22 μm membrane filter. Samples were introduced using the pressure mode (50 mbar × 5 s) at the cathode.

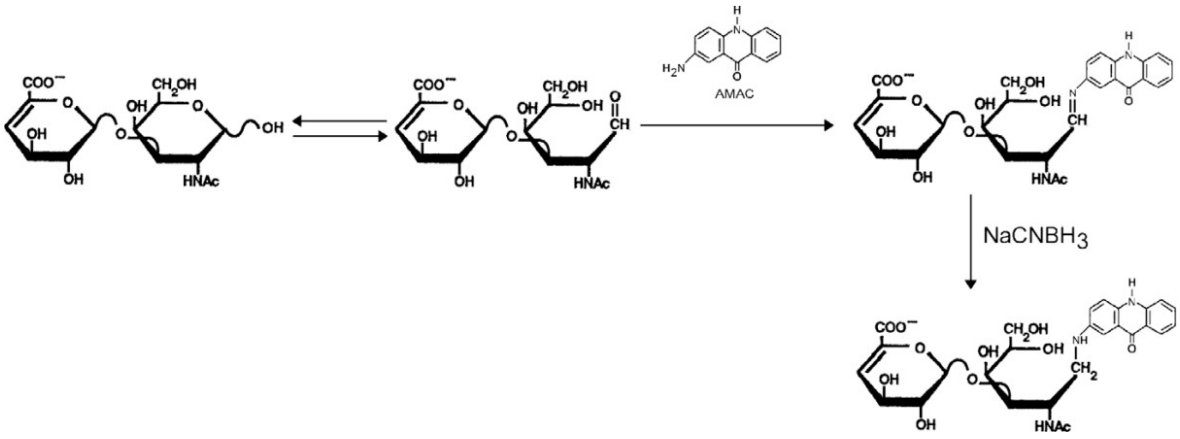


Figure 4.3: AMAC derivatization [85].

4.4 Disaccharides Analysis

The single HA, HS, CS and DS, contents were calculated against real standards (Sigma-Aldrich, Germany) and enzymatically treated as reported above. The absolute content of different GAGs in plasma was expressed as μg/ml, in urine, the values were normalized to creatinine (CR) and reported as μg/mg CR. For tissues, the values were reported as μg/mg of tissue. From these concentrations, the ratio (CS + DS)/HS was calculated. Moreover, the relative percentages of unsaturated disaccharides of HS (eight disaccharides) and galactosaminoglycans (eight disaccharides) were measured (Table 2) [87].

Glycosaminoglycan	Disaccharide
CS + DS	0s: ΔUA-GalNAc
	2s: ΔUA2S-GalNAc
	6s: ΔUA-GalNAc6S
	4s: ΔUA-GalNAc4S
	2,6dis: ΔUA2S-GalNAc6S
	4,6dis: ΔUA-GalNAc4S6S
	2,4dis: ΔUA2S-GalNAc4S
	2,4,6tris: ΔUA2S-GalNAc4S6S
HS	0s: ΔUA-GlcNAc
	Ns: ΔUA-GlcNS
	6s: ΔUA-GlcNAc6S
	2s: ΔUA2S-GlcNAc
	N,6dis: ΔUA-GlcNS6S
	N,2dis: ΔUA2S-GlcNS
	2,6dis: ΔUA2S-GlcNAc6S
	N,2,6tris: ΔUA2S-GlcNS6S

Table 2: Unsaturated disaccharides of chondroitin- (CS), dermatan- (DS), and heparan-sulfate (HS).

Starting from these data, the following parameters were calculated: the ratio between the % of disaccharide 4s and the % of non-sulfated disaccharide (4s/0s) and the ratio between the content of disaccharide sulfated in position 4 of GalNAc and the content of disaccharide sulfated in position 6 of GalNAc (4s/6s). The charge density of sulfated GAGs

(galactosaminoglycans and HS) was also calculated considering the presence and the percentages of sulfated groups for each kind of disaccharide.

Finally, specific structural features of HS were determined as the percentage of N-acetyl, N-sulfated, 2-sulfated, and 6-sulfated groups. On the whole, along with the absolute content of the various GAGs, further structural features (CS + DS and HS charge densities; CS + DS/HS ratio; 4s/0s and 4s/6s CS + DS ratios; N-acetyl, N-sulfated, 2-sulfated, and 6-sulfated HS groups) were measured.

4.5 Statistical Analysis

Statistically significant differences between I2S-KO vs WT and I2S-KO vs treated I2S-KO mice groups were determined by applying the nonparametric Mann–Whitney U test using GraphPad Prism 9.3.1 (La Jolla, CA, USA). Significance was set at $p < 0.05$. Data are presented as mean ± standard deviation.

5 RESULTS

5.1 Quantitation of Total GAGs by CE-LIF

In this study we performed a qualitative and quantitative analysis of GAGs extracted from different tissues samples and biological fluids (plasma, urine, liver and brain), in a mouse model of MPS II at different weeks of treatment, starting at 12 weeks of age (0, 2, 4, 6, 12, 18 and 24 weeks of treatment). The same analyzes were performed on WT mice, I2S KO mice, ERT-treated I2S KO mice, and GENI-treated I2S KO mice. Furthermore, we compared the results with those obtained from GENI-treated WT mice samples to evaluate whether GENI was able to modify the WT phenotype by itself.

First, we noticed that GAG analysis of all biological fluids and tissues showed significant differences already from the first weeks of treatment. However, we did not find significant differences between the different weeks of treatment for the I2S KO mice, nor for the samples treated with different therapies. For this reason, and given the small number of samples, we decided to analyze samples by treatment class and not by week, as to increase results' statistical significance.

From quantitative analysis, we can see how total GAGs (figure 5.1) content increased in all I2S KO subjects compared to WT. In particular, total GAGs increase by 35% in plasma, 908% in urine, 7920% in liver and 951% in brain.

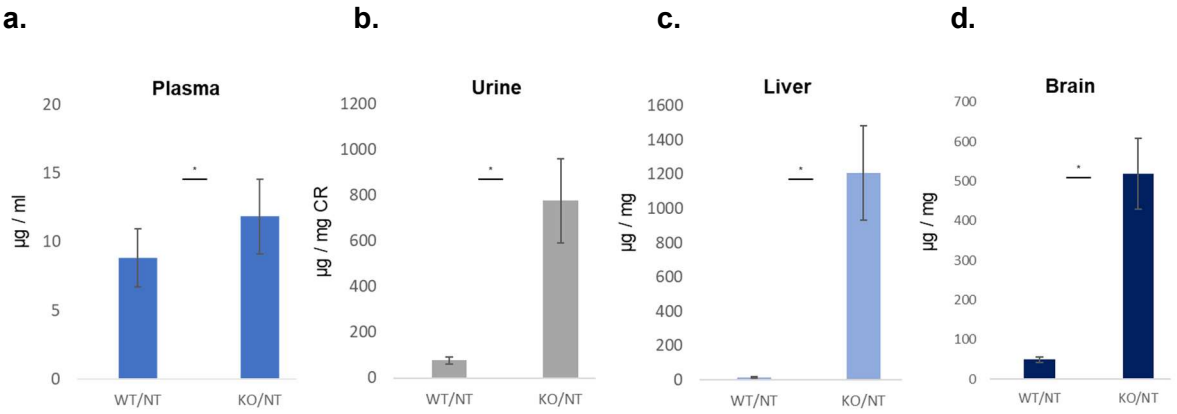
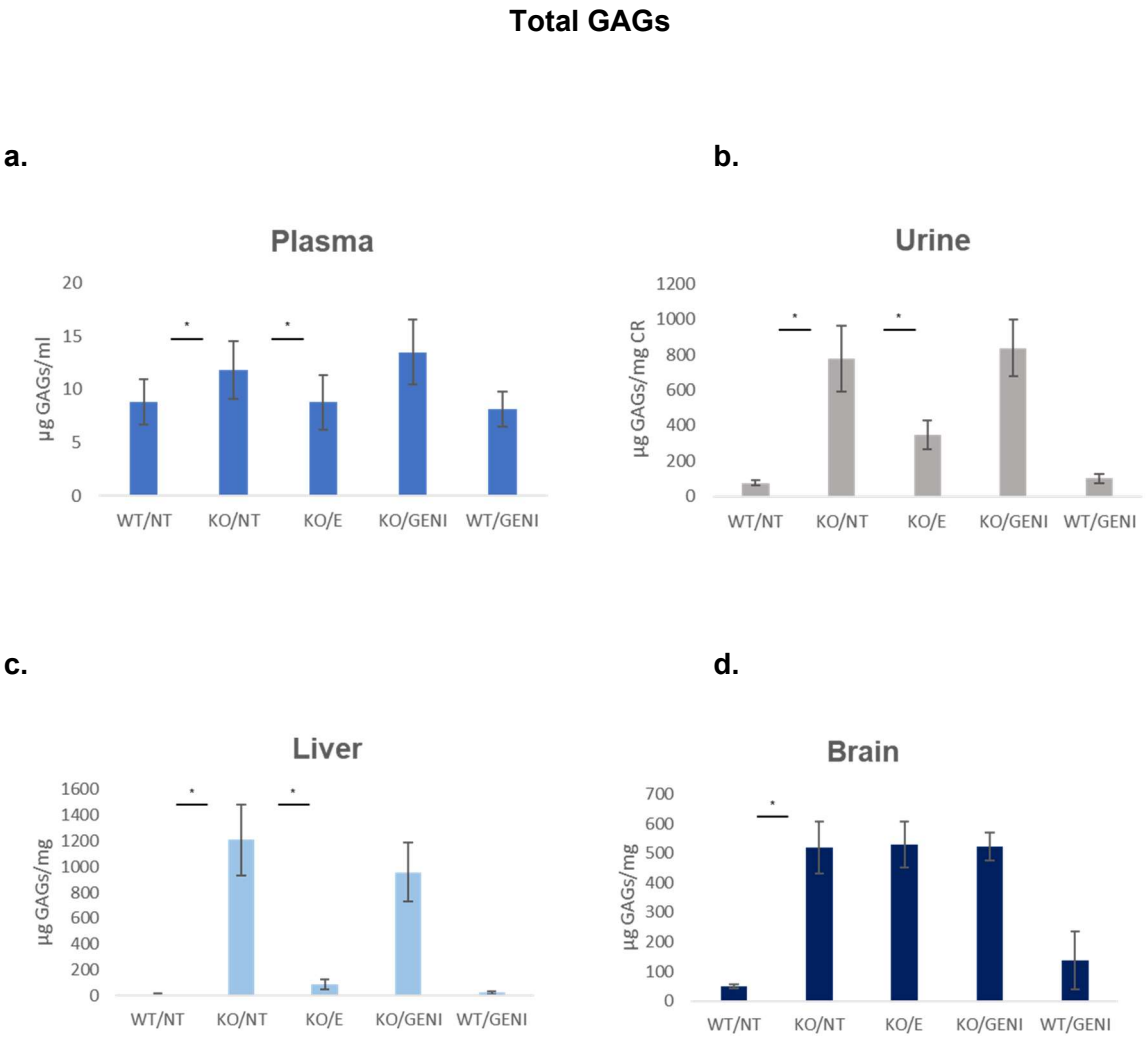


*Figure 5.1: Quantitation of total GAGs by CE-LIF, measured in plasma (a), urine (b), liver (c) and brain (d) of WT non treated (WT/NT) and I2S-KO non-treated (KO/NT) mice. All values are represented as mean ± SD. Asterisks indicate a statistically significant difference between I2S-KO and WT (Mann–Whitney U test; * p-value < 0.05).*

As for the therapies, we can see how ERT can considerably decrease total GAG content in plasma (- 26%), urine (- 55%) and liver (- 93%), compared to untreated I2S-KO samples (fig. 5.2). While there are no significant differences in brain. Despite the effectiveness of this treatment in reducing the total content of GAGs, it is not able to restore the WT condition. Instead, there appears to be no difference between GENI-treated I2S-KO samples and untreated I2S-KO samples. Also, GENI shows no effect on WT samples.



	a. Plasma µg GAGs/ml			b. Urine µg GAGs/mg CR			c. Liver µg GAGs/mg			d. Brain µg GAGs/mg		
	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%
WT/NT	8,81	4,26	48,4	77,03	32,27	41,9	15,05	10,62	70,6	49,33	12,59	25,5
KO/NT	11,85	5,44	45,9	776,20	368,08	47,4	1206,98	552,10	45,7	518,69	177,53	34,2
KO/E	8,79	5,15	58,6	348,11	164,83	47,3	86,98	77,11	88,7	529,93	155,12	29,3
KO/GENI	13,50	6,11	45,3	836,34	318,53	38,1	957,90	461,15	48,1	522,90	97,38	18,6
WT/GENI	8,16	3,25	39,8	100,82	49,53	49,1	23,22	17,62	75,9	136,06	196,84	144,7

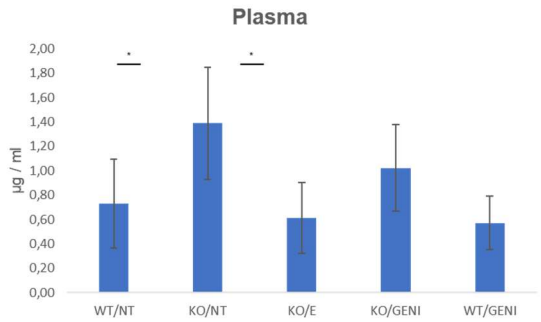
*Figure 5.2: Quantitation of total GAGs by CE-LIF, measured in plasma (a), urine (b), liver (c) and brain (d) of Wild Type non treated mice (WT/NT), I2S-KO non-treated mice (KO/NT), I2S-KO ERT-treated mice (KO/E), I2S-KO genistein-treated mice KO/GENI and Wild Type genistein-treated mice WT/GENI. All values are represented as mean ± SD. Asterisks indicate a statistically significant difference between I2S-KO and WT mice or between I2S-KO and I2S-KO ERT-treated mice (Mann–Whitney U test; * p-value < 0.05).*

5.2 Quantitation of HS, Galactosaminoglycans, and HA by CE-LIF

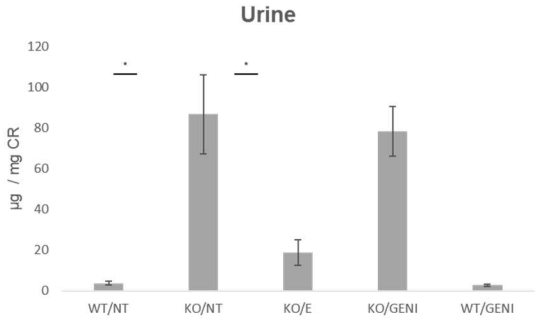
Quantitation of HS and galactosaminoglycans (CS + DS) by CE-LIF (Fig. 5.3) evidenced a variable increase in I2S-KO mouse samples compared to WT ones in all tissues. The variations of HS and galactosaminoglycans were more marked in urine, liver and brain than plasma. HS show a dramatic increase up to 2247% in urine, 8418% in liver and 1581% in brain, while galactosaminoglycans increased by 946% in urine and 2631% in liver. Analogously, the effect of ERT treatment was more visible in urine and liver than in plasma with higher per cent variations in HS values than CS + DS ones (- 79% in urine and - 93% in liver). On the contrary, ERT was not able to decrease HS or galactosaminoglycans values in brain. As expected, there is no difference between GENI-treated I2S-KO samples and untreated I2S-KO samples. Also, GENI shows no effect on WT samples.

HS

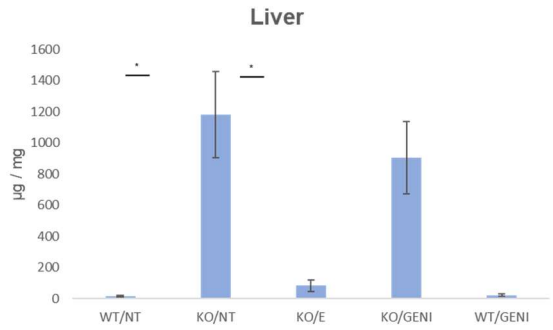
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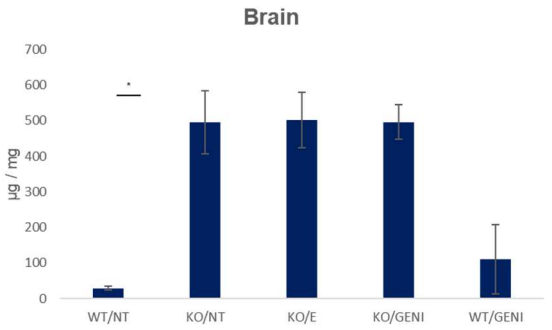
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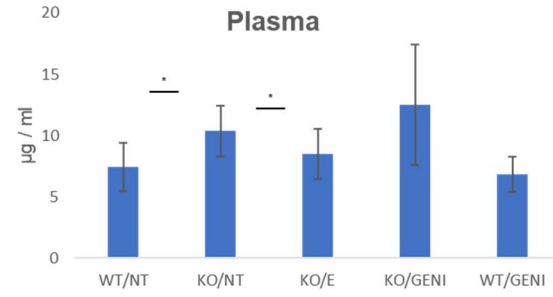
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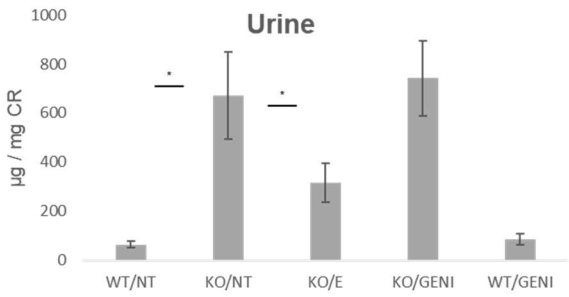
	a. Plasma µg HS/ml			b. Urine µg HS/mg CR			c. Liver µg HS/mg			d. Brain µg HS/mg		
	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%
WT/NT	0,73	0,73	100,0	3,70	2,08	56,2	13,86	10,37	74,8	29,48	9,92	33,6
KO/NT	1,39	0,92	66,2	86,83	38,78	44,7	1180,56	551,50	46,7	495,52	178,56	36,0
KO/E	0,61	0,58	95,1	18,57	12,62	68,0	83,35	75,18	90,2	502,18	154,88	30,8
KO/GENI	1,02	0,71	69,6	78,36	24,45	31,2	905,55	466,76	51,5	495,77	96,92	19,5
WT/GENI	0,57	0,44	77,2	2,69	1,29	48,0	22,30	16,87	75,7	110,24	194,77	176,7

CS+DS

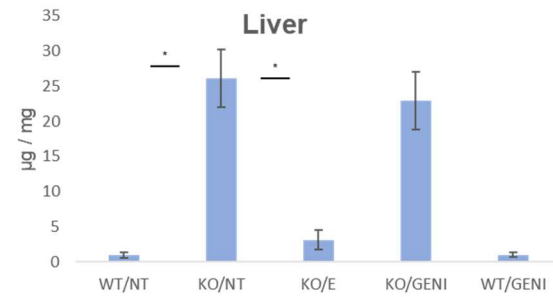
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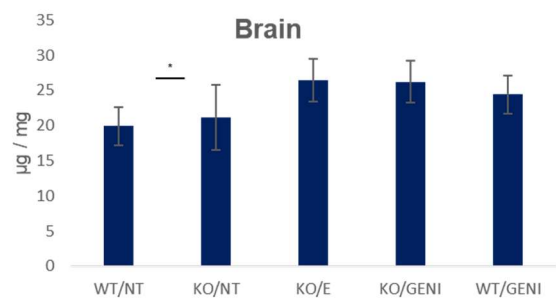
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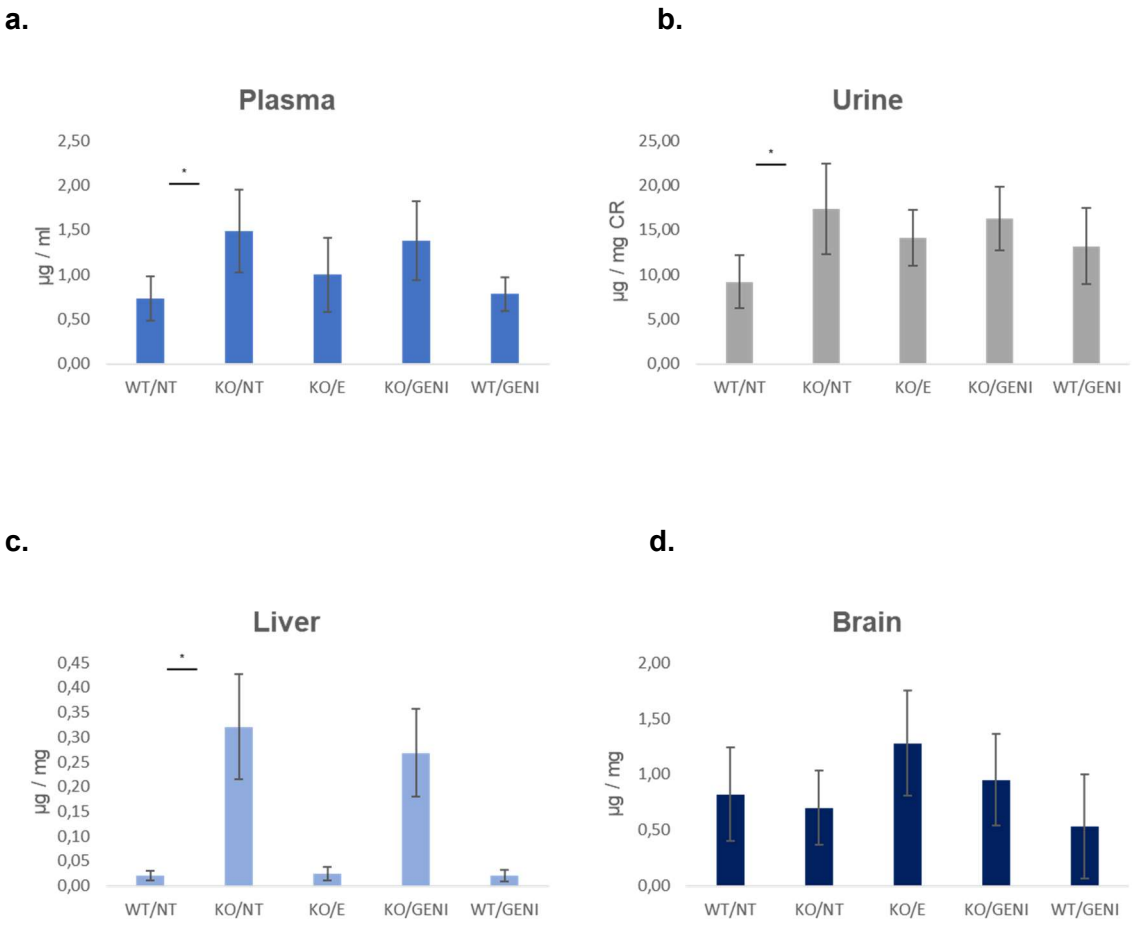


	a. Plasma µg CS+DS/ml			b. Urine µg CS+DS/mg CR			c. Liver µg CS+DS/mg			d. Brain µg CS+DS/mg		
	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%
WT/NT	7,45	3,98	53,4	64,10	28,31	44,2	0,96	0,71	74,3	19,92	5,39	27,1
KO/NT	10,37	4,19	40,4	670,34	356,36	53,2	26,08	8,22	31,5	21,14	9,14	43,2
KO/E	8,53	4,08	47,8	315,45	157,80	50,0	3,14	2,82	89,8	26,47	6,10	23,0
KO/GENI	12,50	9,84	78,7	741,69	303,46	40,9	22,94	8,22	35,8	26,23	5,88	22,4
WT/GENI	6,85	2,91	42,5	84,94	47,15	55,5	1,02	0,67	65,6	24,39	5,50	22,6

*Figure 5.3: Quantitation of HS and CS+DS by CE-LIF, measured in plasma (a), urine (b), liver (c) and brain (d) of Wild Type non treated mice (WT/NT), I2S-KO non-treated mice (KO/NT), I2S-KO ERT-treated mice (KO/E), I2S-KO genistein-treated mice KO/GENI and Wild Type genistein-treated mice WT/GENI. All values are represented as mean ± SD. Asterisks indicate a statistically significant difference between I2S-KO and WT mice or between I2S-KO and I2S-KO ERT-treated mice (Mann–Whitney U test; * p-value < 0.05).*

Moderate growth in plasma (+ 104%), urine (+ 89%) and liver (+ 1505%) samples of non-sulfated HA was also observed in I2S-KO mice. However, ERT and GENI effects are not statistically significant (Fig. 5.4).

HA



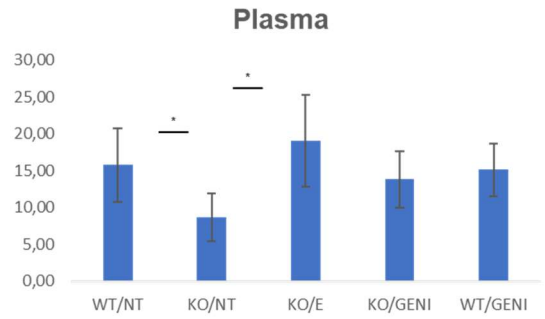
	a. Plasma µg HA/ml			b. Urine µg HA/mg CR			c. Liver µg HA/mg			d. Brain µg HA/mg		
	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%
WT/NT	0,73	0,50	68,5	9,17	5,91	64,5	0,02	0,02	100,0	0,82	0,84	102,4
KO/NT	1,49	0,93	62,4	17,32	10,19	58,8	0,32	0,21	66,4	0,70	0,66	94,3
KO/E	1,00	0,83	83,0	14,10	6,29	44,6	0,02	0,03	112,5	1,28	0,94	73,4
KO/GENI	1,38	0,89	64,5	16,30	7,12	43,7	0,27	0,18	66,0	0,95	0,82	86,3
WT/GENI	0,78	0,37	47,4	13,19	8,49	64,4	0,02	0,02	109,5	0,53	0,93	175,5

*Figure 5.4: Quantitation of HA by CE-LIF, measured in plasma (a), urine (b), liver (c) and brain (d) of Wild Type non treated mice (WT/NT), I2S-KO non-treated mice (KO/NT), I2S-KO ERT-treated mice (KO/E), I2S-KO genistein-treated mice KO/GENI and Wild Type genistein-treated mice WT/GENI All values are represented as mean ± SD. Asterisks indicate a statistically significant difference between I2S-KO and WT mice or between I2S-KO and I2S-KO ERT-treated mice (Mann–Whitney U test; * p-value < 0.05).*

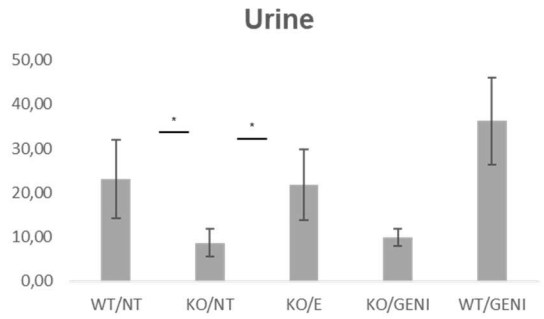
Being CS + DS substantially more abundant than HS in both plasma (about 10-fold) and urine (about 20-fold), the CS + DS/HS ratio allows us to easily evidence the fluctuation of HS with respect that of galactosaminoglycans. CS + DS / HS drop in I2S-KO vs WT mice was generally more marked in urine than in plasma (Fig. 5.5). Following treatment, CS + DS / HS ratio tended to restore to physiological values in both biological fluids, with statistically significant variation of + 120% in plasma and + 150% urine. Also in liver, we can see an increase of 93%. Again, there is no difference between GENI-treated I2S-KO samples and untreated I2S-KO samples.

(CS+DS)/HS Ratio

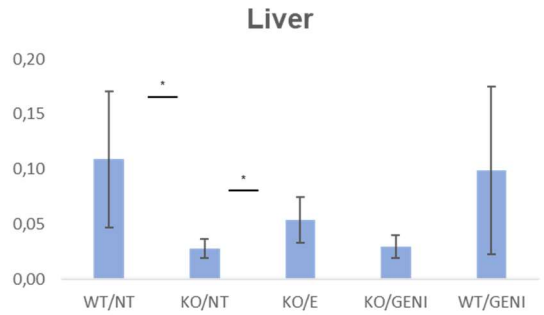
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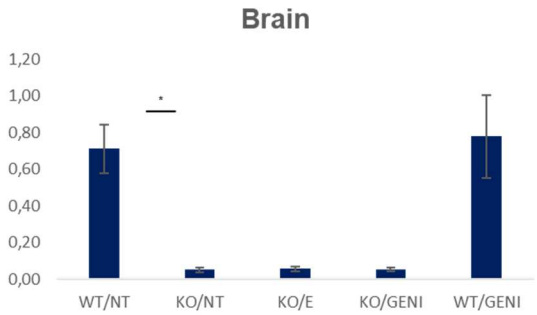
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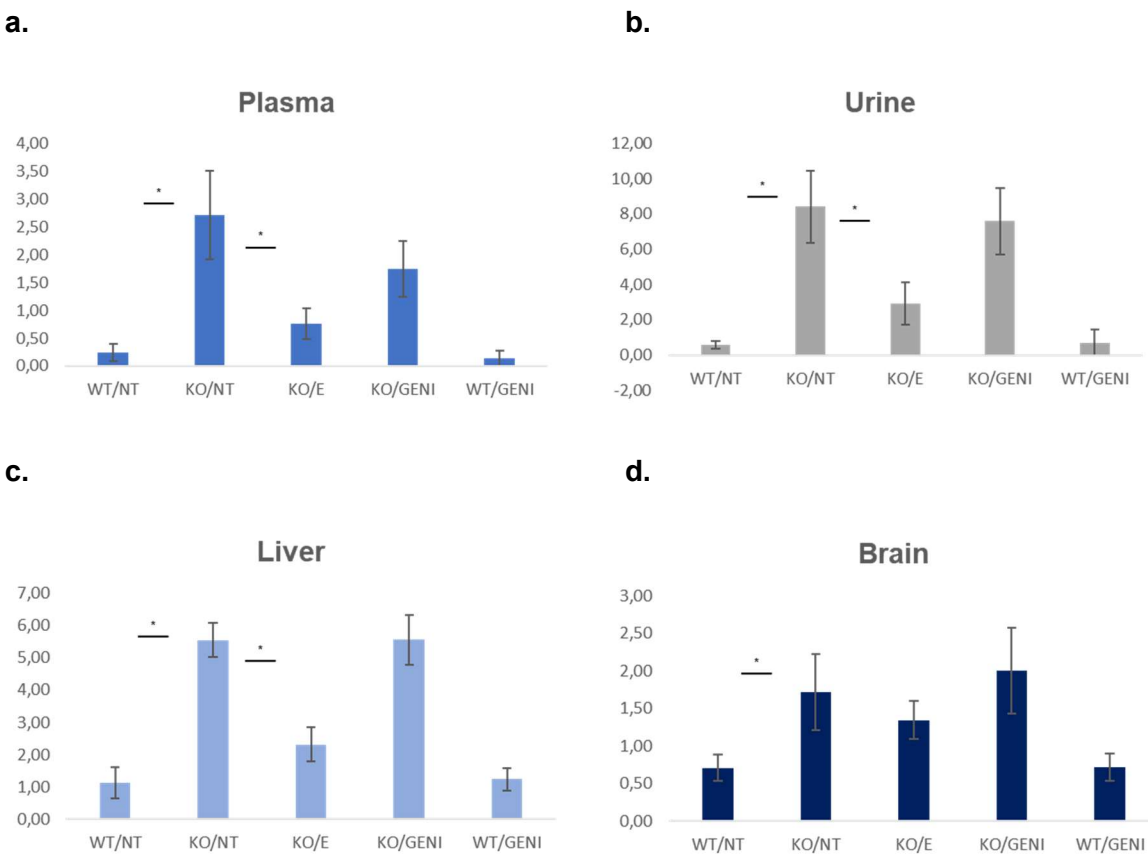


	a. Plasma (CS+DS)/HS Ratio			b. Urine (CS+DS)/HS Ratio			c. Liver (CS+DS)/HS Ratio			d. Brain (CS+DS)/HS Ratio		
	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%
WT/NT	15,75	9,97	63,3	23,05	17,65	76,6	0,11	0,12	113,8	0,712	0,26	36,9
KO/NT	8,64	6,52	75,5	8,68	6,28	72,4	0,03	0,02	60,7	0,052	0,03	50,0
KO/E	19,00	12,50	65,8	21,72	16,00	73,7	0,05	0,04	75,9	0,057	0,02	36,8
KO/GENI	13,79	7,56	54,8	9,96	3,86	38,8	0,03	0,02	70,0	0,055	0,02	30,9
WT/GENI	15,09	7,20	47,7	36,24	19,64	54,2	0,10	0,15	153,5	0,778	0,45	58,1

*Figure 5.5: (CS+DS)/HS ratio, measured in plasma (a), urine (b), liver (c) and brain (d) of Wild Type non treated mice (WT/NT), I2S-KO non-treated mice (KO/NT), I2S-KO ERT-treated mice (KO/E), I2S-KO genistein-treated mice KO/GENI and Wild Type genistein-treated mice WT/GENI. All values are represented as mean ± SD. Asterisks indicate a statistically significant difference between I2S-KO and WT mice or between I2S-KO and I2S-KO ERT-treated mice (Mann–Whitney U test; * p-value < 0.05).*

5.3 Structural Disaccharides Analysis of Galactosaminoglycans by CE-LIF

Structural analysis of galactosaminoglycans evaluated 8 differentially sulfated species in all tissues. Among them, we report (Fig. 5.6) the percentage of disaccharide disulfated in position 2 of uronic acid and 4 of GalNAc (disaccharide 2,4-dis). This percentage showed a statistically significant increase in I2S-KO mice compared to WT for all tissues (up to 1053% in plasma, 1352% in urine, 392% in liver and 142% in brain). Moreover, it significantly declined with ERT therapy in plasma (- 72%), urine (- 65%) and liver (- 58%). ERT therapy was not able to significantly decrease 2,4-dis values in brain and there is no difference between GENI-treated I2S-KO samples and untreated I2S-KO samples.



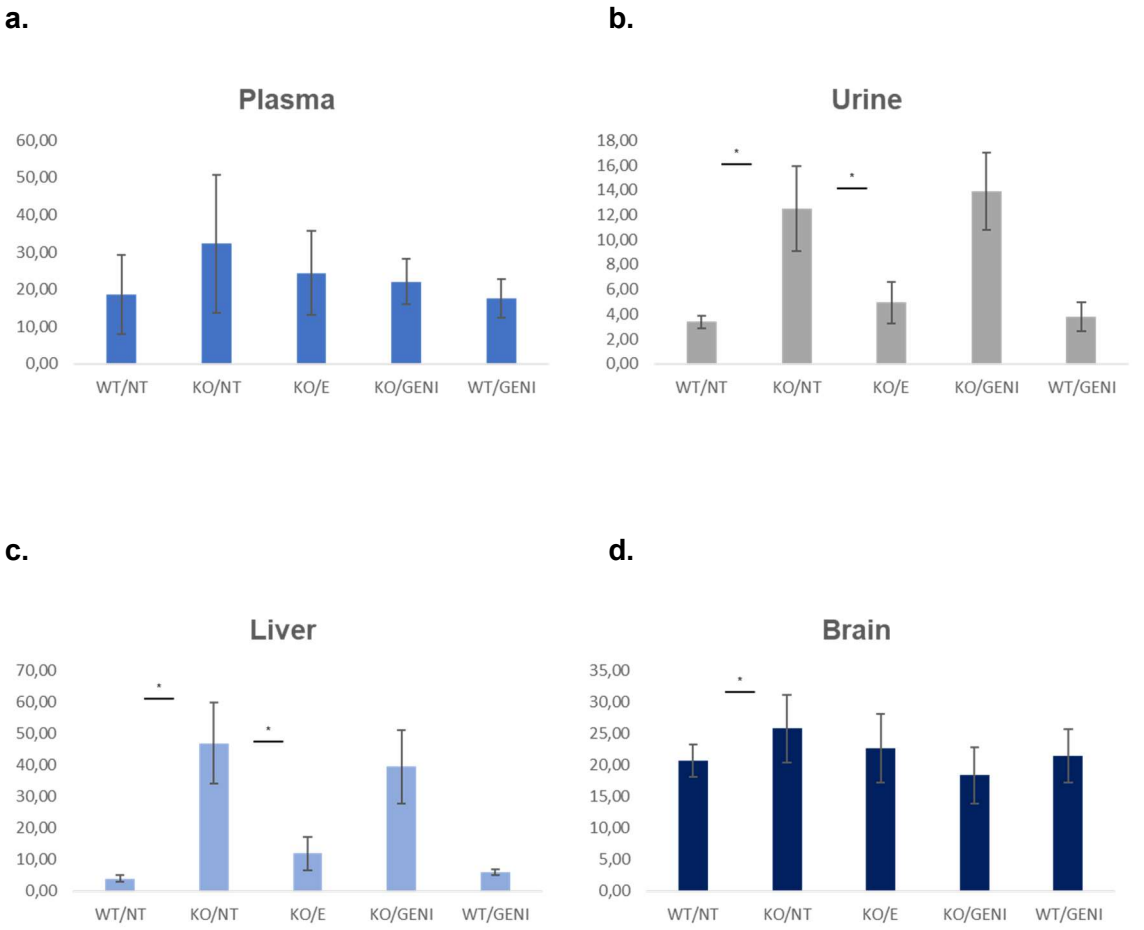
	a. Plasma % 2,4-dis (DS)			b. Urine % 2,4-dis (DS)			c. Liver % 2,4-dis (DS)			d. Brain % 2,4-dis (DS)		
	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%
WT/NT	**0,24**	0,31	132,3	**0,58**	0,48	82,8	**1,13**	0,97	86,2	**0,71**	0,34	48,5
KO/NT	**2,71**	1,60	59,0	**8,42**	4,09	48,6	**5,55**	1,04	18,8	**1,72**	1,01	58,8
KO/E	**0,76**	0,54	71,1	**2,92**	2,41	82,5	**2,31**	1,08	46,6	**1,35**	0,50	37,2
KO/GENI	**1,74**	1,00	57,5	**7,59**	3,74	49,3	**5,56**	1,53	27,4	**2,00**	1,14	56,8
WT/GENI	**0,14**	0,28	200,0	**0,70**	1,47	210,0	**1,24**	0,69	55,8	**0,72**	0,37	51,3

*Figure 5.6: Structural analysis of galactosaminoglycans by CE-LIF. Content of the disaccharide disulfated in position 2 of uronic acid and 4 of N-acetyl-galactosamine (2,4-dis) measured in plasma (a), urine (b), liver (c) and brain (d) of Wild Type non treated mice (WT/NT), I2S-KO non treated mice (KO/NT), I2S-KO ERT-treated mice (KO/E), I2S-KO genistein-treated mice KO/GENI and Wild Type genistein-treated mice WT/GENI. All values are represented as mean ± SD. Asterisks indicate a statistically significant difference between I2S-KO and WT mice or between I2S-KO and I2S-KO ERT-treated mice (Mann–Whitney U test; * p-value < 0.05).*

Another structural parameter is 4s/6s, the ratio between disaccharide sulfated in position 4 of uronic acid and disaccharide sulfated in position 6 of uronic acid (4s/6s). Thanks to this analysis we can see how the ratio increased in favor of the 4s disaccharide. We can see a significant increase in urine (+ 272%), liver (+ 1083%) and brain (+ 25%) in I2S-KO mice compared to WT. Moreover, it significantly declined with ERT therapy in urine (- 60%) and

liver (- 75%). There is no difference between GENI-treated I2S-KO samples and untreated I2S-KO samples.

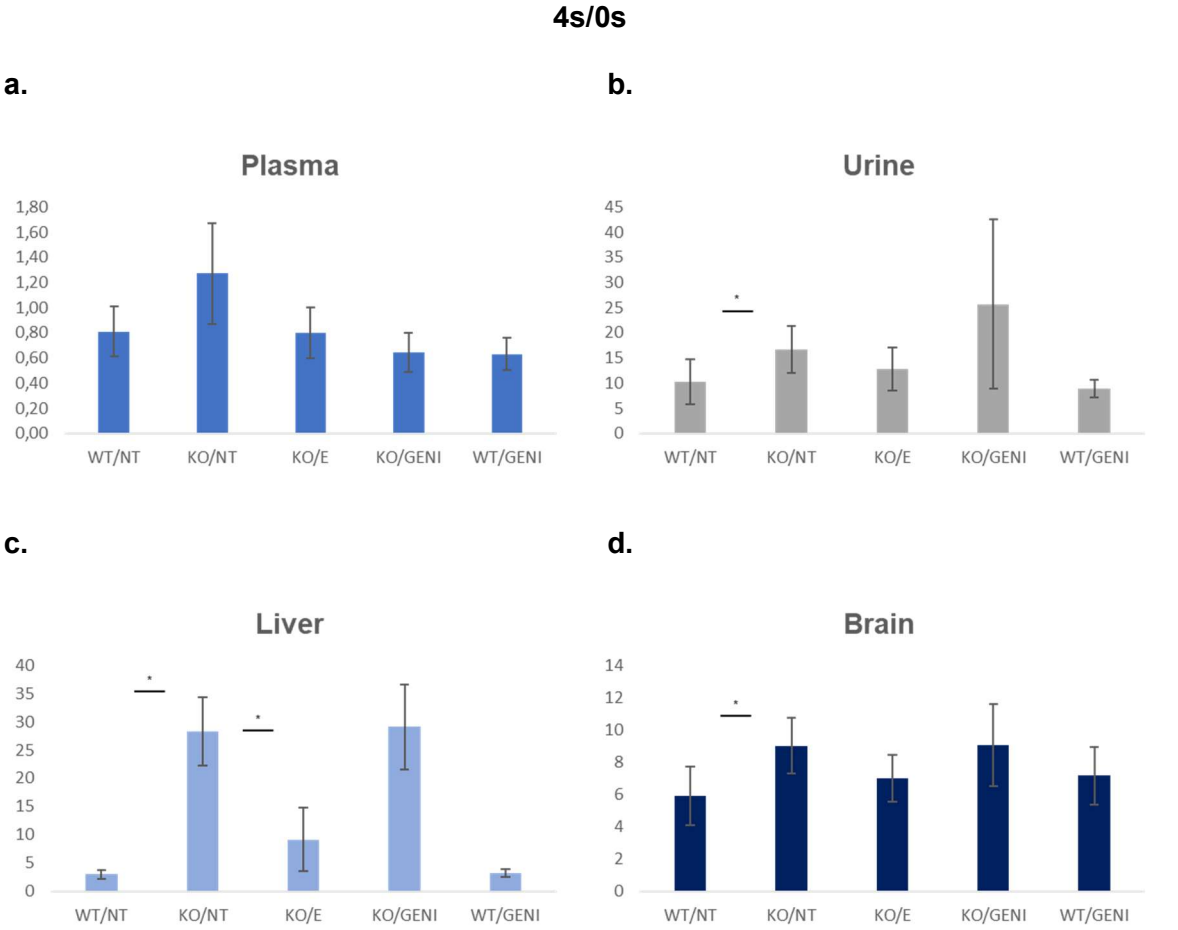
4s/6s



	a. Plasma 4s/6s			b. Urine 4s/6s			c. Liver 4s/6s			d. Brain 4s/6s		
	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%
WT/NT	18,67	21,23	113,7	3,36	0,95	28,3	3,96	2,38	60,1	20,62	5,09	24,7
KO/NT	32,25	74,46	230,9	12,50	6,80	54,4	46,84	25,80	55,1	25,77	10,68	41,4
KO/E	24,40	22,40	91,8	4,95	3,34	67,5	11,87	10,42	87,8	22,65	10,82	47,8
KO/GENI	22,02	12,12	55,0	13,89	6,28	45,2	39,40	23,29	59,1	18,37	8,97	48,8
WT/GENI	17,54	10,30	58,7	3,81	2,31	60,6	5,93	2,11	35,6	21,41	8,42	39,3

*Figure 5.7: Structural analysis of galactosaminoglycans by CE-LIF. CS+DS 4s/6s disaccharides ratio. The ratio between disaccharide 4s and disaccharide 6s (4s/6s) measured in plasma (a), urine (b), liver (c) and brain (d) of Wild Type non treated mice (WT/NT), I2S-KO non-treated mice (KO/NT), I2S-KO ERT-treated mice (KO/E), I2S-KO genistein-treated mice KO/GENI and Wild Type genistein-treated mice WT/GENI. All values are represented as mean ± SD. Asterisks indicate a statistically significant difference between I2S-KO and WT mice or between I2S-KO and I2S-KO ERT-treated mice (Mann–Whitney U test; * p-value < 0.05).*

For 4s/0s, the ratio between disaccharide sulfated in position 4 of uronic acid and non-sulfated disaccharide (4s/0s), we can note again how the ratio increased in favor of the 4s disaccharide. We can see a significant increase in urine (+ 62%), liver (+ 854%) and brain (+ 52%) in I2S-KO mice compared to WT. Moreover, it significantly declined with ERT therapy in liver (- 68%). There is no difference between GENI-treated I2S-KO samples and untreated I2S-KO samples.

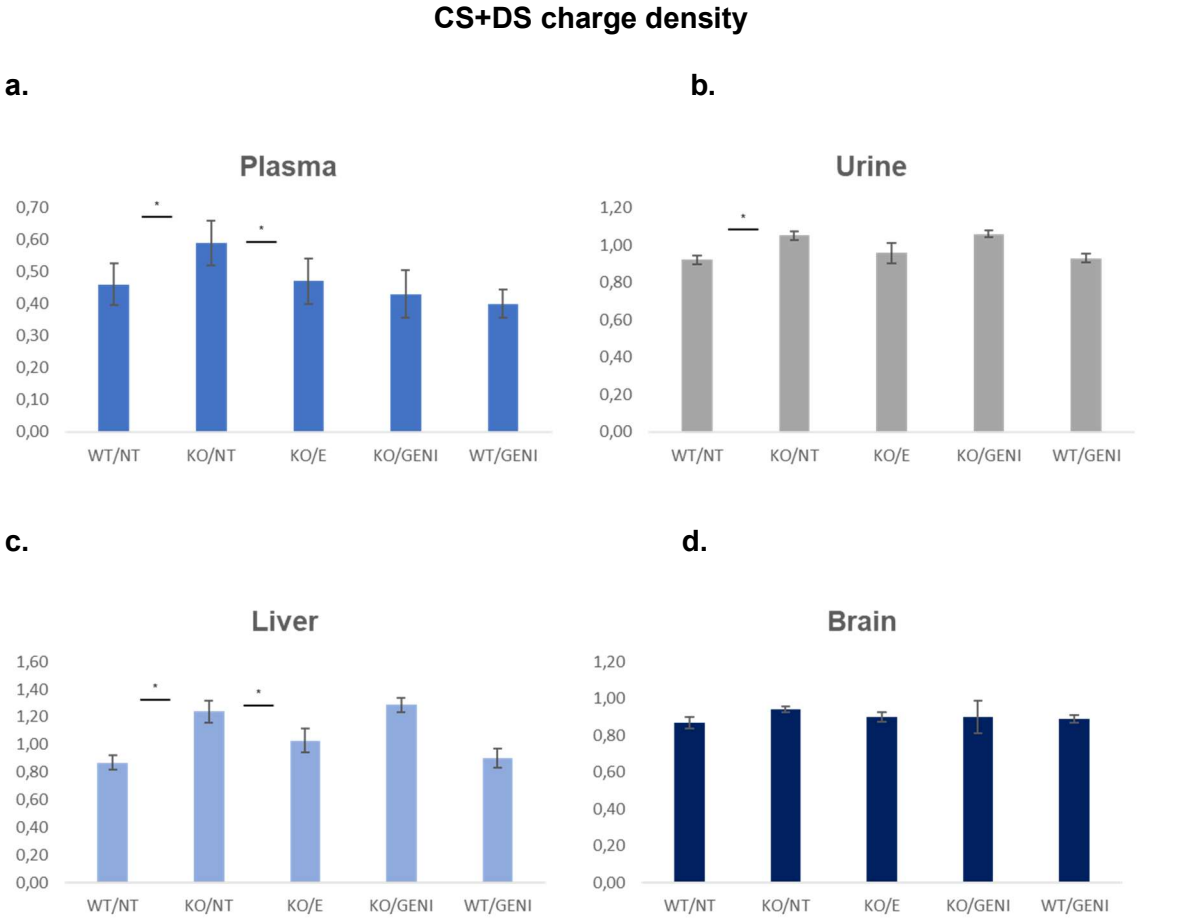


	a. Plasma 4s/0s			b. Urine 4s/0s			c. Liver 4s/0s			d. Brain 4s/0s		
	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%
WT/NT	0,81	0,40	49,4	10,27	8,96	87,2	2,96	1,70	57,4	5,91	3,67	62,1
KO/NT	1,27	0,80	63,0	16,61	9,31	56,1	28,24	12,06	42,7	9,00	3,45	38,3
KO/E	0,80	0,40	50,0	12,82	8,61	67,2	9,17	11,11	121,2	7,00	2,89	41,3
KO/GENI	0,64	0,31	48,4	25,70	33,83	131,6	29,10	15,04	51,7	9,05	5,13	56,7
WT/GENI	0,63	0,26	41,3	8,90	3,61	40,6	3,17	1,35	42,6	7,17	3,58	49,9

Figure 5.8: Structural analysis of galactosaminoglycans by CE-LIF. CS+DS 4s/0s disaccharides ratio. The ratio between disaccharide 4s and non-sulfated disaccharide 0s (4s/0s) measured in plasma (a), urine (b), liver (c) and brain (d) of Wild Type non treated mice (WT/NT), I2S-KO non treated mice (KO/NT), I2S-KO ERT-treated mice (KO/E), I2S-KO genistein-treated mice KO/GENI and Wild Type genistein-treated mice WT/GENI. All values are represented as mean ± SD.

*Asterisks indicate a statistically significant difference between I2S-KO and WT mice or between I2S-KO and I2S-KO ERT-treated mice (Mann–Whitney U test; * p-value < 0.05).*

The analysis of CS + DS charge density evidenced notable variations mostly in plasma (+ 28%), urine (+ 14%) and liver (+ 42%); the reductions in ERT-treated mice vs untreated in plasma (- 20%) and liver (- 17%) resulted statistically significant (Fig. 4.3.2). No differences were found in GENI treated samples.



	a. Plasma CS+DS charge density			b. Urine CS+DS charge density			c. Liver CS+DS charge density			d. Brain CS+DS charge density		
	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%
WT/NT	0,46	0,13	28,3	0,92	0,05	5,1	0,87	0,10	11,7	0,87	0,06	6,9
KO/NT	0,59	0,14	23,7	1,05	0,05	4,4	1,24	0,16	13,1	0,94	0,03	3,2
KO/E	0,47	0,14	29,8	0,96	0,11	11,1	1,03	0,18	17,1	0,90	0,05	5,6
KO/GENI	0,43	0,15	34,9	1,06	0,04	3,4	1,29	0,11	8,3	0,90	0,18	4,5
WT/GENI	0,40	0,09	22,5	0,93	0,05	5,3	0,90	0,14	15,1	0,89	0,04	20,0

*Figure 5.9: CS+DS charge density measured in plasma, urine, liver and brain of Wild Type non treated mice (WT/NT), I2S-KO non-treated mice (KO/NT), I2S-KO ERT-treated mice (KO/E), I2S-KO genistein-treated mice KO/GENI and Wild Type genistein-treated mice WT/GENI. All values are represented as mean ± SD. Asterisks indicate a statistically significant difference between I2S-KO and WT mice or between I2S-KO and I2S-KO ERT-treated mice (Mann–Whitney U test; * p-value < 0.05).*

5.4 Structural Disaccharides Analysis of HS by CE-LIF

Analogously to CS + DS, HS structural analysis allowed the quantitation of 8 species with a different pattern of sulfation/ acetylation. Among them, we report the analysis of N-acetyl groups, N-sulfo, 2-sulfo, 6-sulfo groups and the whole charge density of HS measured in different tissues and biological fluids.

For N-acetyl groups, we can evidence (figure 5.10) how the percentage significantly decrease for I2S-KO mice in both plasma (- 24%) and urine (- 15%). While ERT therapy was able to partially restore the WT condition (+ 18% in plasma and + 10% in urine).

Instead, the percentage of Ns groups increase in plasma (+ 123%), urine (+ 38%) and liver (+ 25%) of I2S-KO mice. Again, samples treated with ERT showed significant differences in plasma (- 29%) and urine (- 17%) Ns groups.

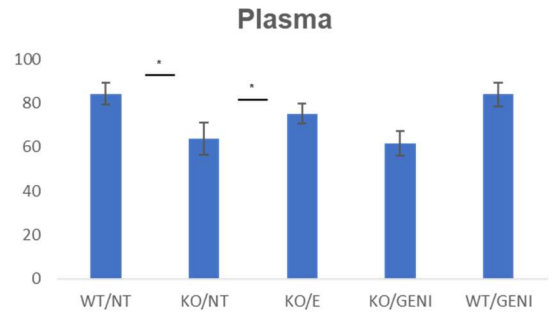
The comparison of I2S-KO vs WT revealed significant differences in the 2-sulfo group's percentage in plasma (+ 148%), urine (+ 85%) and liver (+ 110%). Considering the effect of treatment, this was more evident in plasma, urine and liver with a significant drop to 38% in urine.

The last analyzed parameter was the percentage of 6s groups, for which we found a significant variation only in I2S-KO urine samples (+29%) and ERT-treated urine samples (-22%).

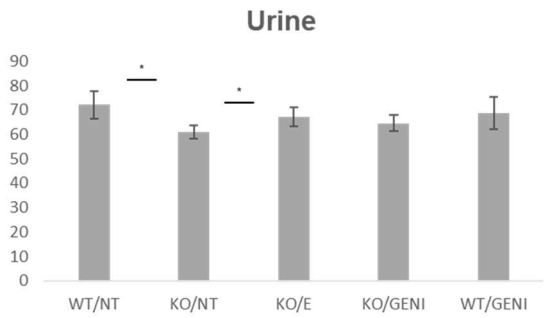
For all the different disaccharide species, GENI therapy did not show any significant differences in the comparison between I2S-KO vs GENI- treated mice.

% HS N-acetyl groups

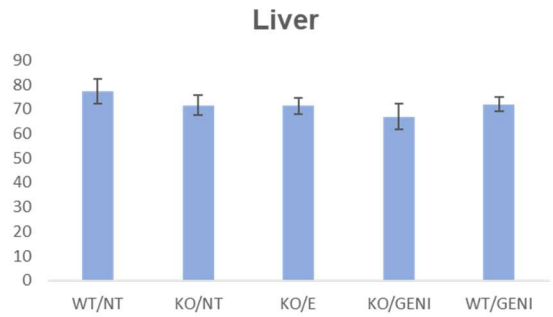
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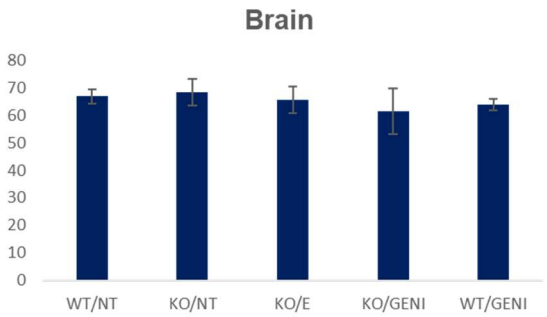
b.



c.



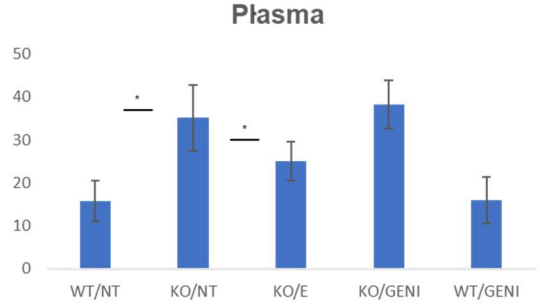
d.



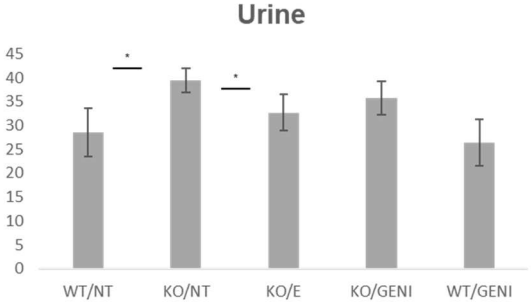
	a. Plasma % HS N-acetyl groups			b. Urine % HS N-acetyl groups			c. Liver % HS N-acetyl groups			d. Brain % HS N-acetyl groups		
	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%
WT/NT	84,27	9,67	11,5	72,10	11,25	15,61	77,45	10,08	13,01	66,93	5,23	7,8
KO/NT	63,82	14,90	23,3	60,99	5,74	9,41	71,65	7,84	10,94	68,38	9,65	14,1
KO/E	75,08	9,12	12,1	67,29	7,63	11,34	71,28	6,68	9,37	65,52	9,66	14,7
KO/GENI	61,77	11,23	18,2	64,54	6,58	10,19	66,88	10,66	15,94	61,40	16,59	27,0
WT/GENI	83,93	10,89	13,0	68,86	13,35	19,39	71,99	5,60	7,78	63,83	4,15	6,5

% HS N-sulfo groups

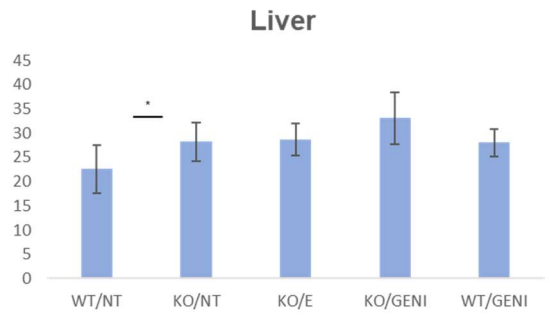
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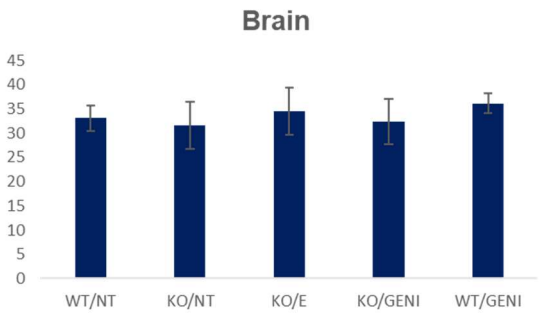
b.



c.



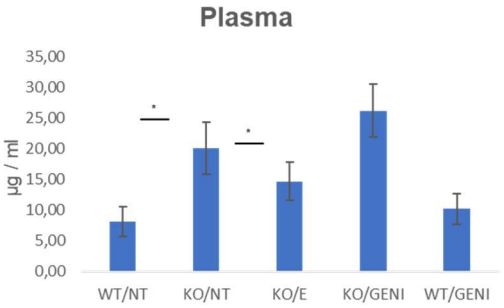
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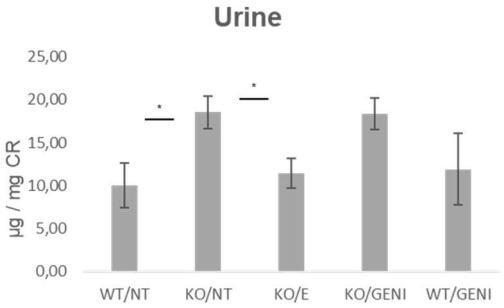
	a. Plasma % HS N-sulfo groups			b. Urine % HS N-sulfo groups			c. Liver % HS N-sulfo groups			d. Brain % HS N-sulfo groups		
	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%
WT/NT	15,75	9,67	61,4	28,51	10,11	35,46	22,56	10,09	44,74	33,07	5,23	15,8
KO/NT	35,11	15,41	43,9	39,47	5,03	12,74	28,20	7,91	28,06	31,62	9,65	30,5
KO/E	24,95	9,13	36,6	32,72	7,68	23,48	28,68	6,72	23,41	34,48	9,66	28,0
KO/GENI	38,24	11,22	29,3	35,76	7,01	19,62	33,11	10,67	32,23	32,35	9,37	29,0
WT/GENI	15,97	10,91	68,3	26,40	9,69	36,71	28,04	5,60	19,98	36,17	4,15	11,5

% HS 2-sulfo groups

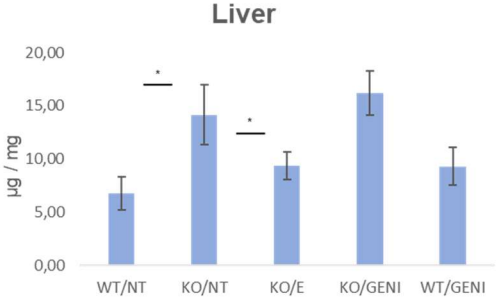
a.



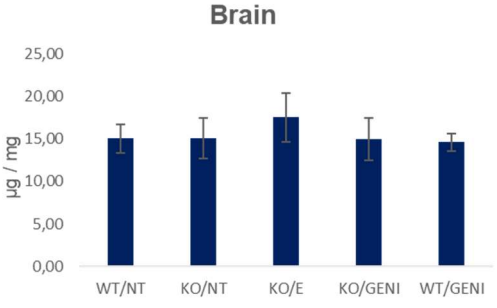
b.



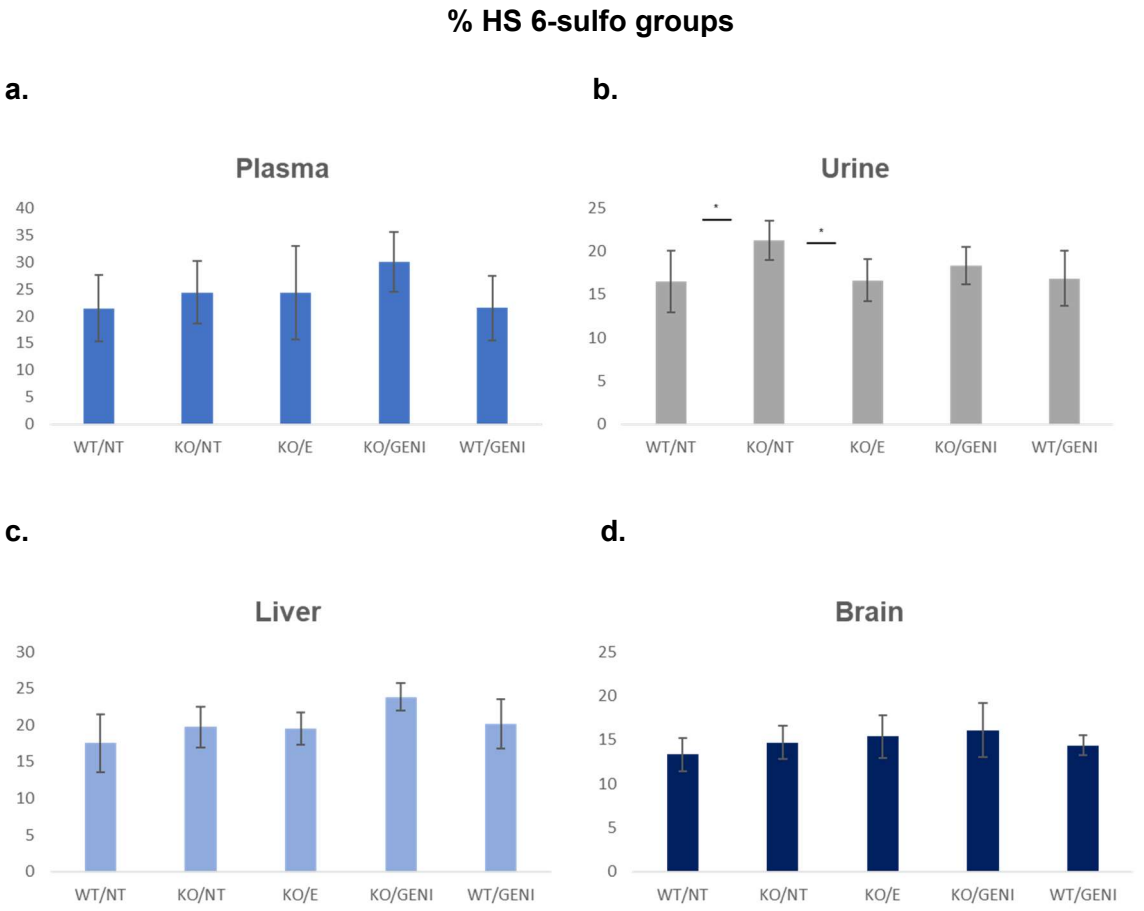
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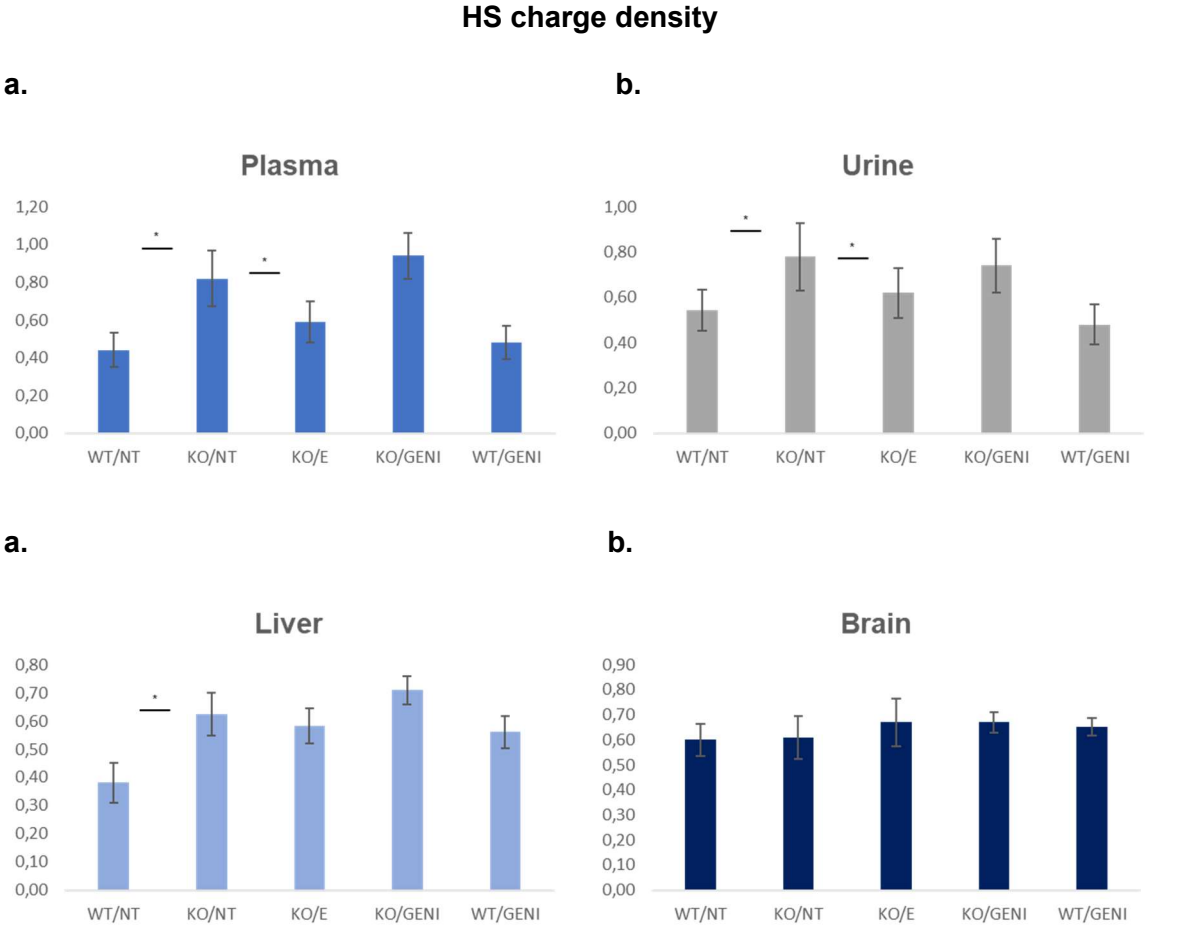
	a. Plasma % HS 2-sulfo groups			b. Urine % HS 2-sulfo groups			c. Liver % HS 2-sulfo groups			d. Brain % HS 2-sulfo groups		
	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%
WT/NT	8,10	4,90	60,5	10,00	5,20	52,0	6,76	3,15	46,6	15,03	3,36	22,4
KO/NT	20,10	8,50	42,3	18,51	3,82	20,6	14,17	5,61	39,6	15,11	4,75	31,4
KO/E	14,70	6,20	42,2	11,42	3,45	30,2	9,37	2,65	28,3	17,53	5,76	32,9
KO/GENI	26,20	8,70	33,2	18,34	3,60	19,6	16,19	4,15	25,6	14,97	5,05	33,7
WT/GENI	10,20	5,00	49,0	11,89	8,34	70,1	9,30	3,54	38,1	14,63	2,07	14,1



	a. Plasma % HS 6-sulfo groups			b. Urine % HS 6-sulfo groups			c. Liver % HS 6-sulfo groups			d. Brain % HS 6-sulfo groups		
	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%
WT/NT	21,40	12,27	57,3	16,51	7,10	43,0	17,56	7,95	45,3	13,34	3,80	28,5
KO/NT	24,38	11,62	47,7	21,25	4,45	20,9	19,73	5,66	28,7	14,69	3,83	26,1
KO/E	24,32	17,22	70,8	16,65	4,81	28,9	19,50	4,36	22,4	15,37	4,82	31,4
KO/GENI	30,05	10,99	36,6	18,35	4,26	23,2	23,84	3,76	15,8	16,10	6,16	38,3
WT/GENI	21,47	11,90	55,4	16,87	6,38	37,8	20,21	6,79	33,6	14,37	2,18	15,2

*Figure 5.10: HS structural disaccharides analysis. The percentage content of N-acetyl, N-sulfo, 2-sulfo and 6-sulfo groups measured in plasma(a), urine (b), liver (c) and brain (d) of Wild Type non treated mice (WT/NT), I2S-KO non treated mice (KO/NT), I2S-KO ERT-treated mice (KO/E), I2S-KO genistein-treated mice KO/GENI and Wild Type genistein-treated mice WT/GENI. All values are represented as mean ± SD. Asterisks indicate a statistically significant difference between I2S-KO and WT mice or between I2S-KO and I2S-KO ERT-treated mice (Mann–Whitney U test; * p-value < 0.05).*

Consequently, these changes in concentration of the different sulfated/acetylated forms of disaccharides determined a variation of the whole charge density of HS (Fig. 3.4.2). A statistically significant growth of HS charge density in I2S-KO vs WT mice was observed in plasma (+ 86%), urine (+ 43%) and liver (+ 64%). Following ERT, HS charge density slightly declined, tending towards WT values, with a statistical significance only in plasma and urine. No significant differences were found in GENI-treated mice vs I2S-KO non-treated mice.



	a. Plasma HS charge density			b. Urine HS charge density			c. Liver HS charge density			d. Brain HS charge density		
	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%	MEAN	SD	CV%
WT/NT	0,44	0,18	40,9	0,54	0,18	33,1	0,38	0,14	37,5	0,60	0,13	21,7
KO/NT	0,82	0,30	36,6	0,78	0,30	38,5	0,63	0,15	24,3	0,61	0,17	27,9
KO/E	0,59	0,22	37,3	0,62	0,22	35,5	0,58	0,13	21,4	0,67	0,19	28,4
KO/GENI	0,94	0,24	25,5	0,74	0,24	32,4	0,71	0,10	14,1	0,67	0,08	11,9
WT/GENI	0,48	0,18	37,5	5,39	0,18	3,3	0,56	0,11	20,1	0,65	0,07	10,8

*Figure 5.11: HS charge density measured in plasma (a), urine (b), liver (c) and brain (d) of Wild Type non treated mice (WT/NT), I2S-KO non-treated mice (KO/NT), I2S-KO ERT-treated mice (KO/E), I2S-KO genistein-treated mice KO/GENI and Wild Type genistein-treated mice WT/GENI. All values are represented as mean ± SD. Asterisks indicate a statistically significant difference between I2S-KO and WT mice or between I2S-KO and I2S-KO ERT-treated mice (Mann–Whitney U test; * p-value < 0.05).*

6 DISCUSSION

The main aim of the present project was to quantify and structurally characterize plasma, urine, liver and brain GAGs composition in a I2S-KO mouse model compared to WT animals, to validate this experimental model for the MPS II study. The second aim was to evaluate GAGs composition changes in I2S-KO mice treated with ERT or GENI.

According to the enzyme defect, the lack of functional I2S in MPS II does not permit the cleavage of chemical sequences in which the sulfate groups are located in the C2 position of the terminal non-reducing end of iduronate residues in HS and DS, causing their accumulation in tissues and organs [88].

In this study, we used CE-LIF as a specific analytical procedure to analyze GAG, an approach that can potentially provide absolute GAG species quantitation independently from calibration curves, age of patients, or creatinine levels for urine. Moreover, CE-LIF, due to its high resolving power, sensitivity, and high-throughput capacity, is useful in the analysis of complex and simple carbohydrates. It permits the analysis of many samples in about 48–50 h at relatively low costs.

Finally, along with modifications of total and single GAG content, this analysis offers a method able to measure changes in GAG composition and structure, making it possible to identify potential biomarkers. A similar analytical procedure was previously adopted in a large cohort of healthy newborns of 2–3 days of age and in a small group of normal adult subjects, to obtain a basic profile of the total content of urine GAGs, their composition, and structural signatures [82]. A comparable analysis was also performed in dried blood spots obtained from 600 healthy newborns and from a small group of MPS subjects matched for age, confirming that the plasma modifications of GAG composition and structure would be useful for a possible early diagnosis of various MPS subtypes [56]. Consistently with the enzyme deficit, in this study, we observed in I2S-KO mice a statistically significant increase of HS and CS + DS levels in all analyzed tissues. Notably, we can see marked GAGs increase in liver, in particular, HS values are drastically higher. This reflects the wild type

subjects, where the liver is naturally richer in HS, but is amplified by the lack of I2S enzyme causing a huge accumulation of HS in the tissue, suggesting an important GAGs accumulation in solid organs. In addition, ERT treatment allowed a drastic reduction of HS and a considerable decline of galactosaminoglycans in plasma, urine and liver, while in brain, there was no significant change. A reduction of both CS + DS and HS is also reported by Menkovic et al. [89] by UPLC–MS/MS method in urine and plasma collected during a 16-week follow-up of ERT-treated and untreated I2S-KO mice. We also observed in I2S-KO mice a moderate increase in plasma, urine and liver of the non-sulfated HA, as already observed in several organs of an MPS IIIA mouse model [90], possibly accumulated through a secondary storage mechanism.

The relative proportion of the main sulfated GAGs, galactosaminoglycans (CS + DS) and glycosaminoglycans (HS), is altered in all analyzed tissues of I2S-KO animals compared to WT. Indeed, both DS and HS increase in KO animals due to the lack of I2S, but the percentage of increase of HS is higher than that of DS; thus, the galactosaminoglycans/ HS ratio decreases. As this ratio is an absolute value calculated through the absolute content of sulfated GAGs and is restored to physiological values in both biological fluids after ERT treatment, we can likely consider it a potential biomarker of MPS II.

The determination of the disaccharide pattern by the present analytical procedure also permits the evaluation of the sulfation level of the different GAGs and the overall charge density. The I2S deficit causes an overall increase in the sulfation level, due to the undegraded HS and DS, accumulated in the different organs and tissues and released in the biological fluids. This effect on the variation of the sulfation status, depending on the pathological conditions and the ERT treatment, was registered as similar in blood and urine. The CS+DS charge density of I2S-KO mice was higher than WT mainly due to the increase of 4s disaccharide, which was also evident from the greater 4s/0s ratio compared to the healthy group. This is indicative of an increase of DS that is more sulfated in position 4 of GalNAc than CS [91] according to the lack of I2S. Among the differentially sulfated CS+DS disaccharides analyzed, also the percentage of the 2,4dis (DS or disaccharide B) could be

reasonably considered a candidate biomarker for MPS II. Indeed, it is substantially increased in I2S-KO mice in all analyzed tissues, and it tends to considerably decline after ERT, especially in plasma, urine and liver. Notable alterations of the disaccharide B concentrations were previously registered in biological fluids of patients affected by Hurler-Scheie syndrome [92].

In healthy conditions, besides DS, I2S also cleaves the sulfate group linked in C2 position of the iduronate residues in HS. Consequently, as expected, a detailed disaccharide mapping showed in the I2S-KO mouse a deep modification of HS structure in all analyzed tissues. Among the different HS species quantified, the change in the percentage of disaccharides sulfated in position 2 of iduronate (HS 2s) was the most significant in the comparison of both untreated vs WT and ERT treated vs untreated, as HS 2s is the main product of the failed cleavage of the sulfate group in position C2. Consequently, this species could be reasonably added to the list of potential biomarkers of disease and therapeutic efficacy for MPS II. Moreover, the variations of N-acetyl and N-sulfo forms of HS are also noteworthy, especially in plasma, although to a lower extent. Consequently, these changes in concentration of the different sulfated/acetylated forms of disaccharides determined a variation of the whole charge density of HS with relevant differences in plasma, urine and liver.

Already after a few weeks of infusion, ERT was effective in rapidly reducing GAGs content and partially restoring some chemical features, to levels that remained rather steady during the entire 24 weeks of treatment. Despite this strong reduction, we never observed a complete normalization, in particular for some of HS and DS structural parameters. This is consistent with what was previously observed following ERT administration in MPS patients. Fujitsuka et al. evidenced this in the blood of MPS II patients, treated with ERT for at least 1 month [93]. The inability of ERT to normalize GAGs further supports the presence of pathogenetic mechanisms other than the primary GAG storage, and the consequent impairment of autophagic process, contributing to the complex clinical manifestations of MPSs [94]. Among them, the alteration of different signalling pathways, like fibroblast

growth factor and sonic hedgehog, has been reported [95] as well as the impairment of ion homeostasis and other intra- and extracellular processes [96].

Regarding the effects of ERT treatment on brain samples, no significant differences were found between the ERT-treated samples and the untreated I2S-KO ones. This lack of GAGs content reduction in the brain is explained by the inability of the recombinant enzyme to cross the blood-brain barrier and to determine effects in this organ [59]. This is also confirmed by the inability of this therapy to induce cognitive improvement in MPS types involving the nervous system [59].

Concerning the treatment with GENI, it was not possible to identify statistically significant differences for any of the analyzed parameters. Not only GENI does not reduce GAGs accumulation in the different tissues, but it seems to increase it in some WT samples, even if no significant differences were found between the untreated I2S mice and the GENI-treated samples. Therefore, this therapy would not seem to benefit the patient.

In conclusion, the illustrated analytical procedure was able to measure changes in plasma and urine GAGs concentration, composition, and chemical structure due to the enzyme defect in the MPS II mouse model, which could be significantly reduced with enzyme treatment. These modifications are therefore potentially useful for precocious diagnosis and as markers of therapeutic efficacy.

In the next stage of the study, we will analyze GAGs from different organs samples to have more information on possible GAGs structural changes in other tissues, which could lead to differences in the molecular effectiveness of these therapies.

7 REFERENCES

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