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**Characterization of an oncofetal  
Chondroitin Sulfate epitope in Cancer**

Master thesis

Applied Biochemistry – Biomedicine

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## RESUMO

*Plasmodium falciparum* é um parasita da malária que pode infectar seres humanos. Quando o parasita replica dentro dos eritrócitos, eles tornam-se suscetíveis à eliminação pelo sistema imunológico. No entanto, para evitar esta eliminação, o parasita expressa proteínas de adesão na superfície de eritrócitos infectados. As proteínas de adesão ancoram os eritrócitos a receptores específicos no hospedeiro. Carboidratos designados de chondroitin sulfato (CS) estão presentes em diferentes tecidos no corpo humano. No entanto, na placenta humana está presente um CS com características muito específicas chamado oncofetal-CS (of-CS). Os eritrócitos infectados pelo parasita da malária expressam à superfície a proteína VAR2CSA que se liga especificamente a CS. A ligação entre VAR2CSA-CS provoca malária placentária, uma doença que pode ser mortal.

As células da placenta e as células de cancro têm algumas características semelhantes, tais como: crescimento rápido de células, migração, invasão celular e CS com características específicas. A malária placentar afeta uma grande parte da população mundial e por isso é muito importante encontrar uma possível vacina. A caracterização do CS presente especificamente na placenta e a caracterização da sua ligação à proteína VAR2CSA é fundamental, no entanto é muito difícil caracterizar as cadeias de CS através dos métodos comuns. O cancro também é uma doença mortal que afeta um grande número da população mundial. A caracterização de CS em células de cancro seria muito importante na patogênese, tratamento e diagnóstico.

No presente trabalho foi utilizada uma amostra de placenta humana a partir da qual o CS foi extraído através de vários métodos de purificação de tecidos. Posteriormente, a amostra de CS foi analisada por métodos analíticos, tais como High Performance Liquid Chromatography (HPLC) e por Liquid Chromatography-Mass Spectrometry (LC-MS). A cadeia CS é uma combinação de muitas características o que a torna muito específica e difícil de caracterizar. No entanto, através do trabalho realizado verificou-se que a sulfatação de 4-O na cadeia CS é necessária para a sua ligação à proteína VAR2CSA. Esta pesquisa demonstrou que o fragmento de ligação de CS à proteína VAR2CSA apresenta pouca sulfatação. Este trabalho tem como objetivo contribuir para novos estudos de compostos terapêuticos e para o diagnóstico de câncer, bem como a possível vacina anti-malária.

## **ABSTRACT**

*Plasmodium falciparum* is a malaria parasite that can infect humans. When the parasite replicates inside the erythrocytes, they become susceptible to elimination by the immune system. However, to avoid this elimination, the parasite expresses adhesion proteins on the surface of infected erythrocytes (iEs). The adhesion proteins anchor erythrocytes to specific receptors in the host. Carbohydrate called chondroitin sulfate (CS) is present in different tissues in the human body. However, in the human placenta is present a CS with very specific characteristics called oncofetal-CS (of-CS). Erythrocytes infected by the malaria parasite express to the surface the VAR2CSA protein that specifically binds to of-CS. The link between VAR2CSA-CS causes placental malaria (PM), a disease that can be deadly.

The placenta cells and cancer cells have some similar characteristics, such as: rapid cell growth, migration, cell invasion and specific CS. PM affects a large part of the world's population and so it is very important to find a possible vaccine. The characterization of the CS present specifically in the placenta and the characterization of its binding to the VAR2CSA protein is fundamental, however it is very difficult to characterize CS through the common methods. Cancer is also a deadly disease that affects a large number of the world's population. The characterization of CS in cancer cells would be very important in pathogenesis, treatment and diagnosis.

In the present work a human placenta sample was used from which CS was extracted through various methods of tissue purification. Posteriorly, the CS of the sample was analyzed by analytical methods, such as High Performance Liquid Chromatography (HPLC) and Liquid Chromatography-Mass Spectrometry (LC-MS). The CS chain is a combination of many characteristics that makes it specific and difficult to characterize. However, through the work performed it has been found that 4-*O* sulphation in the CS chain is required for its binding to the VAR2CSA protein. This search also demonstrated that the CS binding fragment to the VAR2CSA protein shows little sulfation. This work aims to contribute to new studies of therapeutic compounds and to the diagnosis of cancer, as well as to possible anti-malaria vaccine.

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## LIST OF ABBREVIATIONS

AMAC – 2-aminoacridone

C<sub>3</sub>H<sub>6</sub>O – Acetone

C4ST1L3 – GalNAc 4 sulfotransferases

CD36 – Cluster differentiation 36

ChABC – Chondroitinase ABC

ChCl<sub>3</sub> – Chloroform

CIDR – Cysteine-rich inter-region domains

CR1 – Complement receptor 1

CS – Chondroitin sulfate

CSA – Chondroitin sulfate A

CSGalNAcT-1 – *N*-acetylgalactosaminyltransferase-1

CSPG – Chondroitin sulfate proteoglycan

DMSO – Dimethyl sulfoxide

DS – Dermatan sulfate

ECM – Extracellular matrix

ELISA – Enzyme linked immunosorbent assay

EtOH – Ethanol

EXTL2 – Exostosin like glycosyltransferase 2

FD – Fluorescence detector

FGF – Fibroblast growth factor

FV2 – Full length variant surface antigen 2-Chondroitin sulfate A

GAG – Glycosaminoglycans

GalNAc – *N*-acetyl-D-galactosamine

GlcA – Glucuronic acid

GlcNAc – *N*-acetyl-D-glucosamine

HA – Hyaluronic acid

HCl – Hydrochloric acid

HPLC – High performance liquid chromatography

HS – Heparan sulfate



ICAM 1 – Intercellular adhesion molecule 1  
IdoUA – Iduronic acid  
iE – Infected erythrocyte  
KS – Keratan sulfate  
LBW – Low birth weight  
LC-MS – Liquid chromatography-mass spectrometry  
MeOH – Methanol  
MS – Mass spectrometry  
NaBH<sub>3</sub>CN – Sodium cyanoborohydride  
NaOAc – Sodium acetate  
NH<sub>4</sub>OAc – Ammonium acetate  
NHS – *N*-hydroxysuccinimidyl  
NTS – *N*-terminal segment  
Of-CS – Oncofetal CS  
PBS – Phosphate-buffered saline  
PfEMP-1 – *Plasmodium falciparum* erythrocyte membrane protein 1  
PGs – Proteoglycans  
PM – Placental malaria  
PTB – Preterm birth  
rVAR2CSA – Recombinant protein VAR2CSA  
VAR2CSA – Variant surface antigen 2 – Chondroitin sulfate A

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# 1. INTRODUCTION

## 1.1 Extracellular matrix (ECM) and structure

### 1.1.1 Proteoglycans (PGs) and glycosaminoglycans (GAGs)

The ECM is a dynamic, non-cellular structure that comprises a lot of macromolecules, including elastin, tenascin, fibronectin, thrombospondin, PGs and Hyaluronan [1]. PGs are proteins that are modified with GAG, which contribute to important biological functions, like: function of ECM, regulation of cell growth, enzyme activities [2]. GAGs are linear biopolymers composed of repeating disaccharide units of hexosamine (*N*-acetyl-D-galactosamine (GalNAc), *N*-acetyl-D-glucosamine (GlcNAc)) and uronic acid (D-glucuronic acid (GlcA) or L-iduronic acid (IdoUA)). In different subtypes of GAGs the repeating disaccharides can be differentially modified by *N*-acetylation and sulfation [3, 2], resulting in a highly heterogenous, negatively charged chains with a molecular weight which can be much higher than 100kDa. GAGs can be divided into two categories based on whether or not the chain is sulfated. The non-sulfated GAGs include only hyaluronic acid (HA). As for the sulfated GAGs, they include chondroitin sulfate (CS), keratan sulfate (KS), heparin and heparan sulfate (HS) and dermatan sulfate (DS) (Figure 1.1) [4, 5].

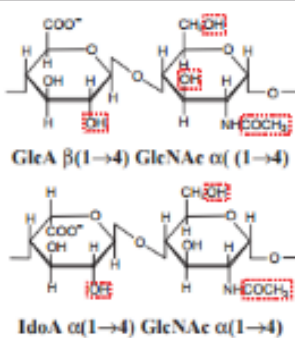
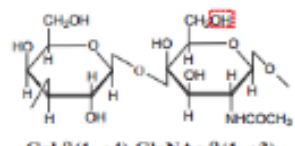
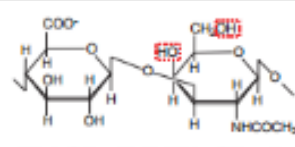
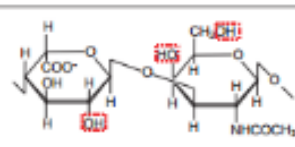
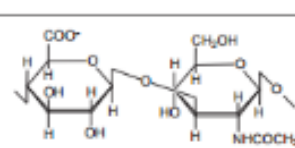
GAG	Hexuronic or Iduronic acid	Galactose	Hexosamine	Disaccharide composition
Heparan sulphate/ Heparin	D-glucuronic acid (GlcA) L-iduronic acid (IdoA)	-	D-glucosamine (GlcNAc)	 GlcA $\beta(1\rightarrow4)$ GlcNAc $\alpha(1\rightarrow4)$ IdoA $\alpha(1\rightarrow4)$ GlcNAc $\alpha(1\rightarrow4)$
Keratan sulphate	-	Galactose (Gal)	D-glucosamine (GlcNAc)	 Gal $\beta(1\rightarrow4)$ GlcNAc $\beta(1\rightarrow3)$
Chondroitin sulphate	D-glucuronic acid (GlcA)	-	D-galactosamine (GalNAc)	 GlcA $\beta(1\rightarrow3)$ GalNAc $\beta(1\rightarrow4)$
Dermatan sulphate	D-glucuronic acid (GlcA) L-iduronic acid (IdoA)	-	D-galactosamine (GalNAc)	 IdoA $\beta(1\rightarrow3)$ GalNAc $\beta(1\rightarrow4)$
Hyaluronic acid	D-glucuronic acid (GlcA)	-	D-glucosamine (GlcNAc)	 GlcA $\beta(1\rightarrow3)$ GlcNAc $\beta(1\rightarrow4)$

Figure 1.1 - Table representative of the composition and structures of the different GAGs. Possible sulfation sites are represented in the image with a red line [5]

The general structure of GAGs is linear, and is made up of a *O*-sulfated polysaccharide backbone of alternating residues of uronic acid and distinct hexosamine: CS contain GlcA and GalNAc; DS contain IdoA and GalNAc; HS contain IdoA or GlcA and GlcNAc (Figure 1.2) [4, 6].

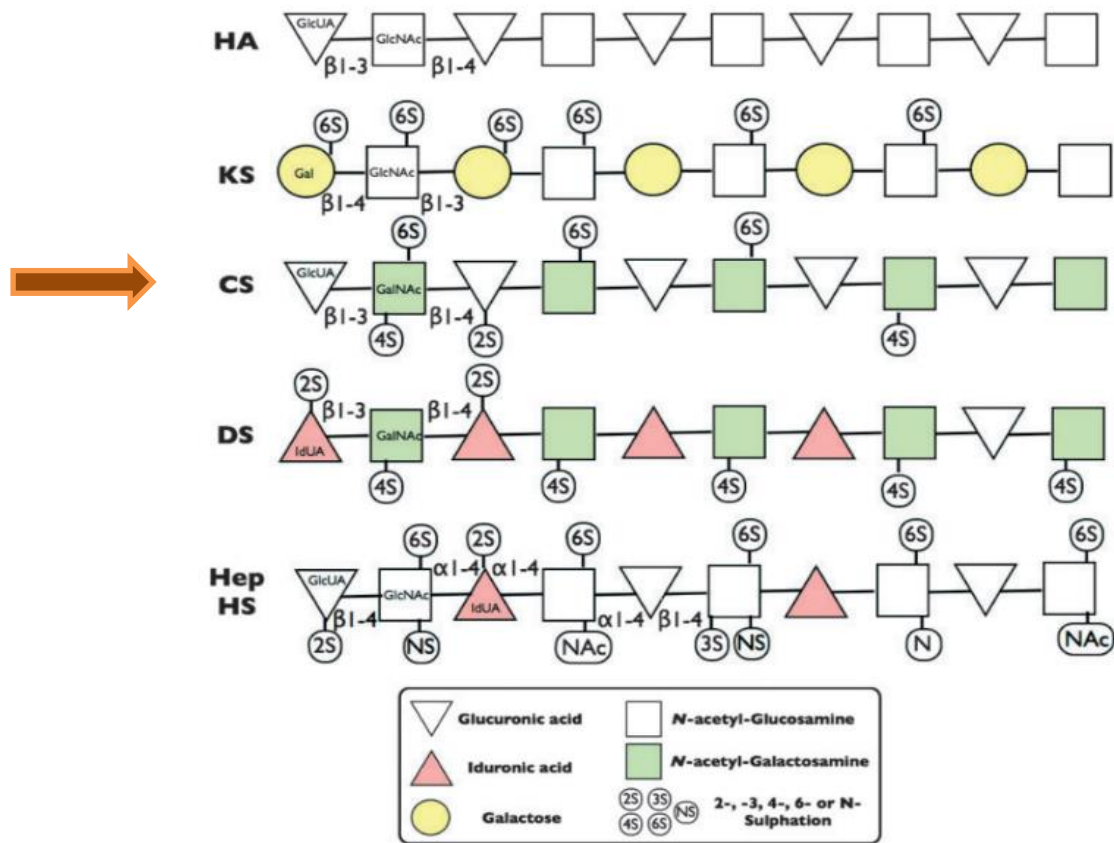


Figure 1.2 - Representation of the structural diversity of GAGs. Squares represent hexosamine (glucosamine or galactosamine), triangles represent uronic acid (GlcA or IdoUA), the circles represent galactose [7]

Different patterns of sulfation allow different interactions with protein ligands, usually through ionic interactions [5]. Depending on the type of sulfation, the different disaccharides are called 0, A, B, C, D and E. However, in most cases the GAG chain is a mix of different types of sulfations [7]. Chondroitin sulfate A (CSA, also called C4S) is a specific type of CS that is characterized by the presence of 4-*O*-sulfation on the majority of the GalNAC residues of a given CS chain (Figure 1.3) [8].

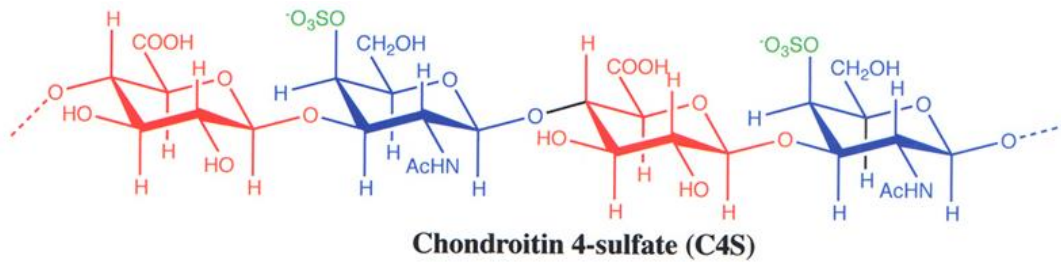


Figure 1.3 - C4S structure that represents repeating units of GAGs (red: D-Glucuronic acid, blue: *N*-acetyl-D-galactosamine, green: sulfate group). The GlcA residues link with *N*-acetylgalactosamine residues via  $\beta$  (1-3) glycosidic bonds, the *N*-acetylgalactosamine residues link to GlcA residues through  $\beta$  (1-4) linkages and also glycosidic [8]

### 1.1.2 Biosynthesis of different GAGs

CS, DS, HS and heparin, synthesis starts with the formation of a tetrasaccharide linker that contains xylose, galactose and GlcA (GlcA- $\beta$ 1,3-Gal- $\beta$ 1,3-Gal- $\beta$ 1,4-Xyl-O-) at a serine residue of the PG core protein (Figure 1.4) [5, 9].

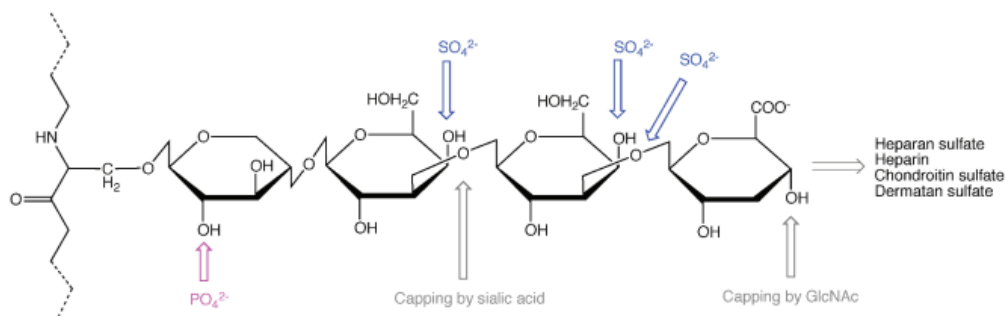


Figure 1.4 - HS, CS, DS modification site in a PG protein core with a tetrasaccharide linker sequence. In the figure are marked sulfation, phosphorylation and capping sites [9]

The fifth sugar added (acetylated amino sugar) to the tetrasaccharide linker decides whether the chain will be HS / heparin or CS / DS. HS is formed if GlcNAc is added to the tetrasaccharide linker and CS is formed if GalNAc is added [9, 10].

The CS *N*-acetylgalactosaminyltransferase-1 (CSGalNAcT-1) enzyme is responsible for catalyzing the transfer of a residue of GalNAc to form CS/DS and is very important in the chain initiation process of CS/DS. Exostosin like glycosyltransferase 2 (EXTL2) is in turn responsible for adding the first GlcNAc to form HS (Figure 1.5) [5, 11, 12]. The chain of CSA is elongated through the action of many enzymes and then the chain is sulfated through enzymes such as GalNAc 4 sulfotransferases (C4ST1L3) [13].

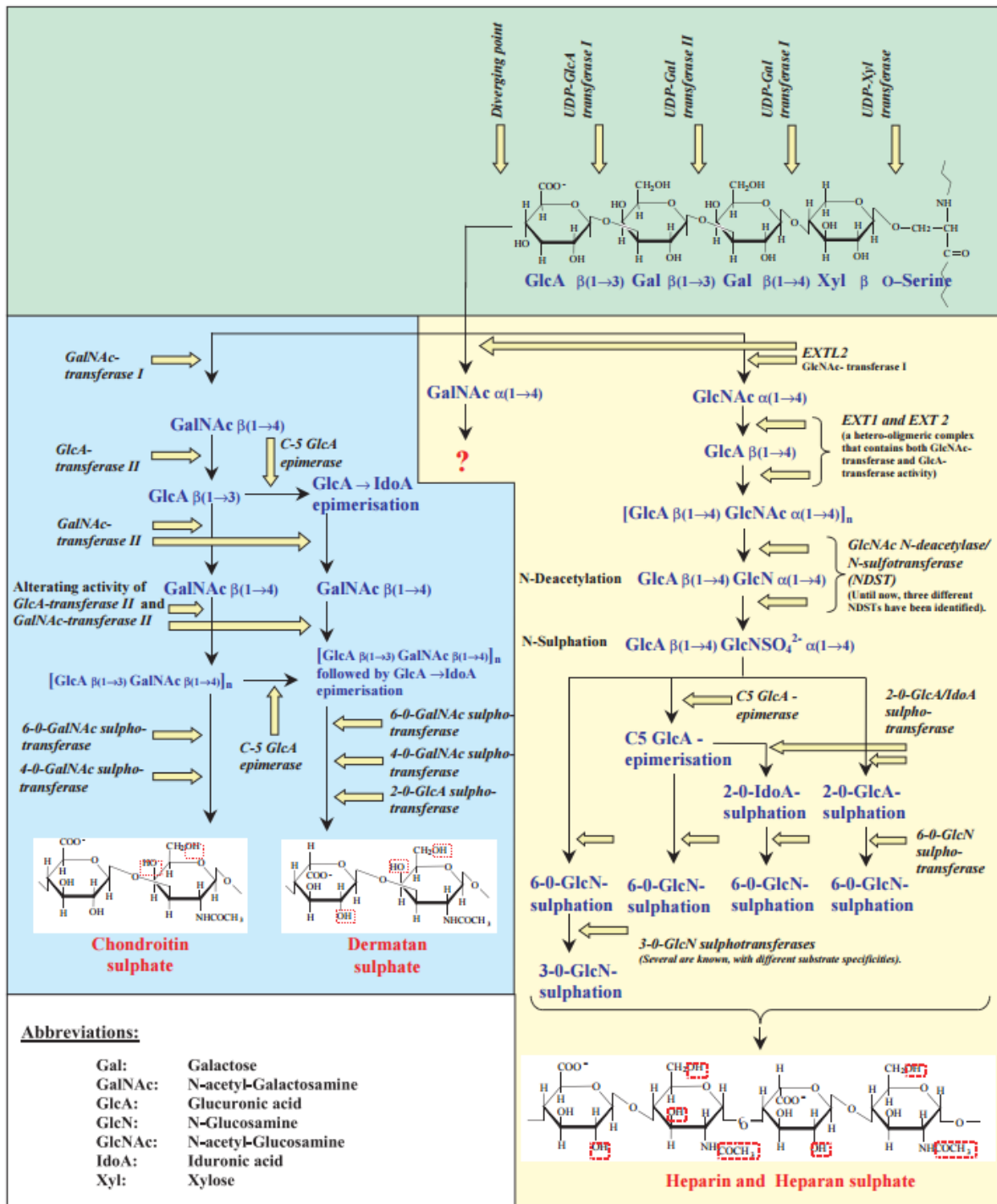


Figure 1.5 - Various steps in the synthesis of CS, DS, HS and heparin GAG chains [5]



### **1.1.3 Chondroitin sulfate proteoglycan (CSPG)**

GAGs are post-translational modifications to PGs present in the cellular membrane and ECM. Different PGs have been identified in different human tissues. CSPG are a family of very diverse proteins with a lot of functions. As a protein can have one or up to 200 CS chains, CSPGs can have different functions like regulation of cellular processes (e.g. differentiation, invasion, adhesion, growth) and providing structural integrity [14, 15]. The up/down regulation of CSPG expression is associated with pathological and physiological conditions. Some malignant tumors such as testicular, breast, pancreas and colon tumors overexpress two major CSPGs - versican, decorin [15]. The CS chain is very important to the functions of CSPG because it can act as a scaffold for sustained proliferative signaling by providing growth factors to the membrane receptors [16]. Several specific biological effects, such as binding to growth factors and their receptors, may be due to structural changes in the CS chains. In addition, the specificity of CS to the protein ligands is different than that of HS [15].

## **1.2 CS as a receptor**

### **1.2.1 Malaria parasite – *Plasmodium falciparum***

*Plasmodium falciparum* is a protozoan parasite [17] and this parasite can survive in different host organisms, such as vertebrates and invertebrates, both intracellularly and extracellularly. The life cycle of parasite is extraordinarily complex because it involves specialized protein expression [18]. So, the infection by the *Plasmodium falciparum* parasite can be very harmful.

*Plasmodium falciparum* is a malaria parasite that can infect the human being. Malaria can be a deadly disease and affects a large number of the world's population. According to the "The World Malaria report" in the year 2015 there were 214 million cases of malaria globally, 438,000 deaths due to malaria disease. The worst is in the African region where 90% of deaths occurred. Children (under 5 years) are very susceptible to infection, disease and death, and account for 70% of malaria deaths [19]. Pregnant women are also very susceptible to malaria. Each year, 125 million pregnancies are at risk of infection, with high levels of mortality rates. During pregnancy, malaria can cause different health complications, such as low birth weight (LBW) and intrauterine growth restriction, preterm birth (PTB) and other long-term effects [20, 21].

### 1.2.2 Life cycle of parasite

The life cycle of *Plasmodium falciparum* parasite has two stages: an exogenous sexual phase with multiplication of parasites in *Anopheles* mosquitoes; and an endogenous sexual stage, where there is a multiplication of the parasites in the human host. Male *Anopheles* mosquito eat only nectar and plant sap, but females eat blood. After the female *Anopheles* mosquito ingests the blood of the human host, the sexual phase of the parasite begins, and the zygote is formed in the stomach. The zygote passes through the mosquito stomach cell layer and, in a sporogony process, hundreds of infected forms are formed (sporozoites). The sporozoites go to the salivary glands, and when the mosquito stings a human body the sporozoites pass to the human host. Thirty to sixty minutes after entering the blood, the sporozoites penetrate the liver cells and start mitotic activity and nuclear division. The multinucleate liver-stage schizont bursts within 9 to 16 days and releases thousands of merozoites in the blood. The merozoites then invade erythrocytes cells and take 72 hours to develop trophozoites and start a new phase of mitotic division originating erythrocytic-stage schizonts. When the infected red blood cells rupture, each schizont liberates between 8 to 32 merozoites, which invade new erythrocytes. After a few cycles, some merozoites go to sexual stage called gametocytes. When gametocytes are ingested by feeding *Anopheles* mosquitoes, mature male and female gametes join to form a zygote in the midgut of the vector. The malaria parasite only has a diploid phase which is when there is the zygote formation and the only time there is meiosis shortly after formation of the zygote (Figure 1.6) [22, 23, 24, 25].

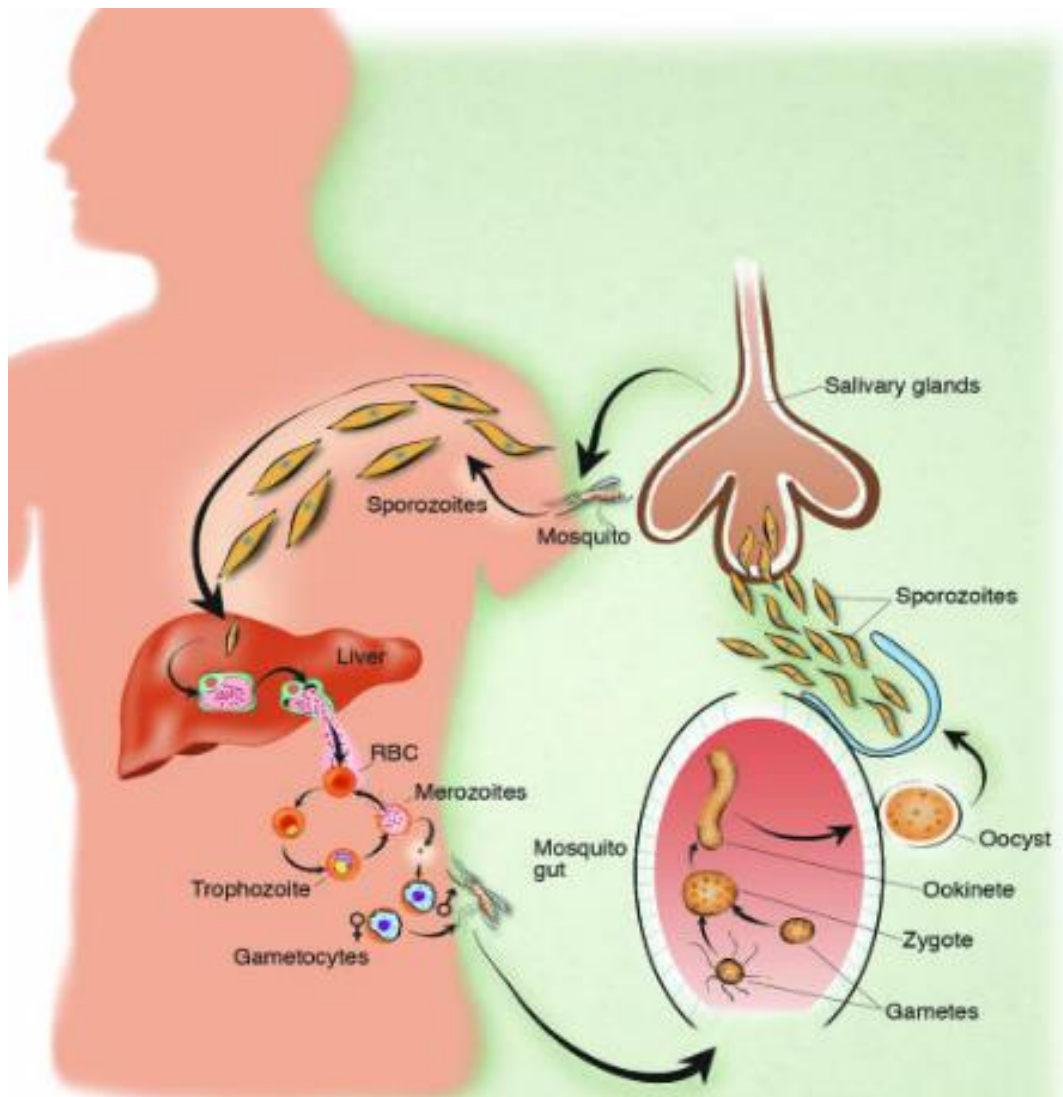


Figure 1.6 - Life cycle of malaria parasite [22]

### 1.2.3 Pathogenesis and Immunity

Var, Rif and stevor are three multigene families in the genome of the parasite *Plasmodium falciparum*. These genes encode proteins known as *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1), repetitive interspersed family (rifin) and sub-telomeric variable open reading frame (stevor), respectively. The products of the *var* and *rif* genes are transported to the surface of infected iEs. These proteins are involved in the adhesion to host endothelial receptors, allowing the iEs to be sequestered to different organs [26].

PfEMP1 is a family of proteins with a size between 200 and 350 kDa, encoded by the *var* genes. These proteins are known to be extremely important for the pathogenicity of the parasite, as it allows the iEs to

adhere to different receptors in the organism, preventing clearance by the spleen [27, 28]. This makes malaria caused by *Plasmodium falciparum* very difficult to eliminate.

When an organism is exposed to repeated infections that cause immunity. With repeated infections the host immunize the many PfEMP1 proteins [29]. The worst cases of the disease are in children up to five years of age. The antigens that the parasites express at the surface of iEs are essential in the process of infection, because most often the host organism do not have IgG antibodies specific to them. Immunity developed by accumulation of different antibodies specific for PfEMP1 in some episodes of disease. The big problem is the great genetic variability, as members of PfEMP1 family are expressed by 50 to 60 different genes [30].

#### **1.2.4 PM**

PM is caused by the parasite *Plasmodium falciparum*. This disease causes severe health complications both to the mother and the fetus, leading to LBW and severe maternal anemia. In Africa, around 200,000 infants and 10,000 mothers die every year from PM [21]. Previously acquired immunity makes malaria less frequent in adults. However, pregnant women are susceptible to malaria, despite the previously acquired immunity. Additionally, this susceptibility is higher in their first pregnancy as immunity to this type of malaria is only obtained in successive pregnancies [31].

Despite there being different types of PfEMP1 proteins, PM is characterized by a specific PfEMP-1 protein, named variant surface antigen 2-chondroitin sulfate A (VAR2CSA) [31]. During the course of pregnancy, the IEs accumulate in the intervillous space in the placenta, and bind to the syncytiotrophoblast. Through a transcriptional analysis of genes, the VAR2CSA protein was shown to be responsible for the IE binding to the placenta [32]. This binding was shown to be to a specific type of CS named CSA. Such binding is highly specific, as studies have shown that IEs do not bind to other receptors (like cluster differentiation 36 (CD36) and Intercellular Adhesion Molecule 1 (ICAM 1)) that normally support adhesion of other IE forms [33]. IEs are then retained in the placenta, leading to severe inflammation [34].

#### **1.2.5 VAR2CSA protein**

VAR2CSA belongs to the PfEMP1 family of proteins that are coded by *var* gene family, as previously mentioned. PfEMP1 proteins are made up of several Duffy domains (DBL) separated by Inter domains

(ID), and it is the DBL domains that cysteine rich. These domains can bind different receptors like CSA, CD36 and complement receptor 1 (CR1). The composition of PfEMP1 protein family is quite similar: all have a small acid intracellular domain and a large extracellular part. The sequence of PfEMP1 protein is very important since it influences the binding to their receptors. All proteins contain a small acidic intracellular domain and a large extracellular part with *N*-terminal segment (NTS), DBL, cysteine-rich inter-region domains (CIDR) and interdomains (ID) [35]. VAR2CSA is different because this protein has six DBL domain, with an amino acid in the *N*-terminal sequence distinct from others PfEMP1s. These domains are called: DBL1 $\alpha$ , DBL2 $\alpha$ , e DBL3 $\alpha$  (Figure 1.7). This protein have six Duffy binding-like domains, three DBL $\alpha$  and three DBL $\epsilon$ , and a cysteine-rich inter-domain region between DBL2 $\alpha$  and DBL3 $\alpha$ , [8, 36, 37]. VAR2CSA is a large protein (350 kDa) with a complex structure and is difficult to mass-produce because of genetic variability.

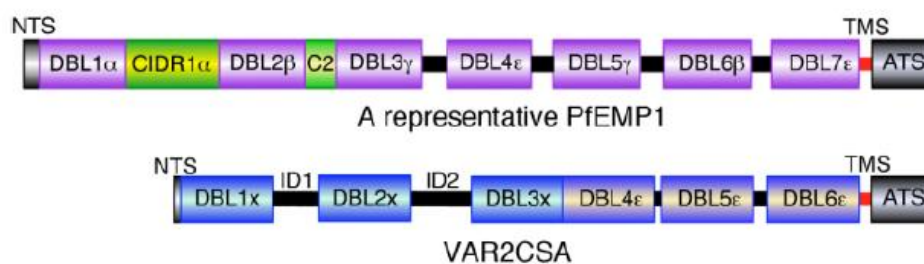


Figure 1.7 - Representative scheme of PfEMP1 and VAR2CSA. In the image are represented the domains DBL, the CIDR and the segments of interconnection (represented in black) [8]

During pregnancy, VAR2CSA-expressing IEs bind to CSA present in the placenta, leading to accumulation of IEs and causing PM. The severity of PM stresses the need to find a treatment for PM, and efforts have been made in this direction. One of the treatments that is under development is a vaccine. PfEMP1 proteins can be essential to the development of the vaccine, but isolates of *Plasmodium falciparum* have a high degree of genetic variation and a high degree of antigenic switching. This can make the whole development process of a specific vaccine more complicated. Fortunately, PM is an exception since the VAR2CSA protein is highly conserved and is the only antigen in PM. A strategy is also possible because the surface molecules expressed in IEs have conserved epitopes [38].

As mentioned before, VAR2CSA is a large and complex protein, making it extremely difficult to produce recombinantly for vaccine development. There may be a problem when using a large protein for the

development of a vaccine, since the high size may lead to the formation of non inhibitory antibodies. Targeting a structural epitope that is not involved in the binding increases the likelihood of escape mutations. Thus, it is essential to cut the CSA chain to find the true CSA binding site, so that it can be targeted and to act as a vaccine (to induce the production of antibodies capable of blocking the adhesion to CSA) [21].

The first studies to find the CSA binding region focused on producing individual DBL domains and studying them [39]. These studies showed that four VAR2CSA domains (DBL2x, DBL3x, DBL5E, DBL6E) bind specifically to CSA. The same DBL domains but from different VAR2CSA variants showed different affinities for binding. The recombinant protein with full length variant antigen 2-chondroitin sulfate A (FV2) proved to be highly specific in binding to CSA. This binding had much higher affinity than binding of single domains to CSA. So it is thought this evidence shows that VAR2CSA protein assumes a tertiary structure with all the domains interconnected. The interaction between them forms the true CSA binding site [37, 40, 41]. The study of FV2 was really important to understand the structure and binding properties of the VAR2CSA protein. Full length VAR2CSA ectodomain binds specifically to CSA and the CSA binding site was shown to be within the DBL2x-CIDRPAM region. Additionally, some fragments have been shown to possess the same affinity to CSA as the protein in its full length: two overlapping fragments – DBL1x-CIDR<sub>PAM</sub>, ID1-DBL3x and DBL1x-DBL3x. Currently, we lack information on the structure of full length VAR2CSA because no crystal structure has been obtained. However, different studies show that VAR2CSA protein has a compact structure and that the binding site to CSA depends on several domains and regions between domains. Mutations in classic sulfate-binding sites in VAR2CSA proved that the interaction between VAR2CSA and CSA is partially dependent on ionic interactions. Several small fragments of VAR2CSA can produce antibodies that can interfere with the adhesion of IEs to CSA [37].

GAGs that are found on the cell surface are used by many bacteria, parasites and viruses as adhesion receptors. Animal cells contain several types of GAGs but HS is the most studied GAG and is associated with pathogens. Some parasites are known to interact with CS chains including malaria parasites, herpes simplex virus, lymes disease spirochetes and *Toxoplasma gondii* [40, 41]. The VAR2CSA pathogen evolved to bind specifically to CSA, but we lack information on this specificity. A study demonstrated that parasites bind specifically to CSPGs present in the intervillous space in the placenta. The CSPG establishing the connection has specific characteristics, such as low sulfated and contains only 2-8% of 4-*O*-sulfated disaccharides [42]. The binding specificity of the IEs to the CSA chains is

dependent on units of non-sulfated disaccharides in combination with disaccharide units with 4-*O* sulfation. Then, dodecarides having four or five disaccharide units with 4-*O* sulfation and one or two units of non-sulphated disaccharides represent the minimum chain length to establish the specific binding between IEs and CSA [6, 43]. Information regarding the specific interaction between the VAR2CSA protein, expressed on the surface of the iEs, and CSA is still very incomplete. There are studies that explain the specificity of the interaction through a type of sulfation, conformation or specific modifications that the CSA can adopt. However, studies are very difficult because it is very difficult to use homogeneous CSA in some studies [6, 44, 45].

### **1.3 Cancer**

Cancer is one of the biggest threats to the world's population because it is one of the largest causes of death worldwide. In 2012 more than 8.2 million people died of cancer worldwide and in the coming decades these values are expected to increase [46, 47]. At this time the cancer treatment is mainly based on chemotherapy treatments, radiation therapy and surgery. Chemotherapy and radiotherapy are very nonspecific treatments for cancer cells and therefore have many unwanted side effects [48]. Surgery can not eliminate all metastatic cells, leading to relapse of cancer. Additionally, cancer is a heterogeneous disease which leads to an increased difficulty to find a universal therapy for all types of cancer. Thus, there is an urgent need for developing efficient and targeted therapies [49].

CS has several functions in the organs where it is present. For example in the placenta it is responsible for rapid cell proliferation (attracting chemokines, nutrients), for the trophoblast invasion of uterine tissue, a high mitotic index, and to establish a complex and the vasculature of the uterine tissue [50, 51]. VAR2CSA binds to a specific type of CS exclusively expressed in the human placenta. Recently, the same specific type of CS was also shown to be present in cancer cells, thus allowing targeting with the recombinant VAR2CSA (rVAR2CSA). This binding can be used as a specific diagnosis and therapeutic strategy in cancer [52].

#### **1.3.1 CSPGs and cancer**

CSPGs have a lot of physiological importance, and its dysregulation can be associated with many kind of diseases such as cancer, Alzheimer's disease, atherosclerosis, among others [53, 54]. CS and its PGs are involved in some major features of cancer, such as apoptosis, adhesion, mounting the ECM,

invasion, angiogenesis, proliferation, and migration [55]. Some of these functions are provided individually or through the interaction of CSPGs with integrins, metalloproteases or RTK [56, 57]. The expression outside the normal CSPGs have been detected in various cancers such as testicular cancer, breast, colon [58, 59, 60]. For example, CSPG4 is abnormally expressed on melanoma, glioblastoma, and sarcomas [56, 57, 61]. The protein core of CSPGs has a great importance, but the CS chains are very critical in cancer development because they capture chemokines, cytokines, PGs and presents them to various membrane receptors functioning as a scaffold and oncogenic signaling [62, 16]. PGs that have CS/DS/HS may participate in the response that is given by basic fibroblast growth factor (FGF) that affects the growth of human melanoma and metastases [16, 63]. CS chains involved in the interaction between different CSPGs and cancer receptors as DS and CSE mediate binding of selectins (PDS, CSA, CS, CSD, CSE) that bind to CD44 [64, 65]. The creation of P selectin ligands on the cell surface of breast cancer is achieved by the action of CSPG4 and CHST11, which are involved in the formation of sulfation of GalNAc 4 (CSA) [66]. Other studies have shown that various types of changes to CS are associated in the development of cancer [15].

### **1.3.2 Targets for cancer therapy and VAR2CSA**

The presence of CS in most malignant situations places the CSPGs as an important target for cancer therapy. In fact, CSPG4 is currently one of the targets for cancer therapy. CSPG4 plays an important role in the growth and survival of cancer cells not only in melanoma cells but also in cells of various types of carcinoma and human sarcoma. Results demonstrated that CSPG4 is a suitable target for the monoclonal antibody (mAb) [67, 68, 69]. CS PGs are present in various tissues of the organism, chemical analysis has shown that the position and degree of chain sulfation allows them to be distinguished. The different sulfation composition can affect the properties of the GAG chain, and therefore interfere in the different associations and interactions with other components. Thus, there has been some difficulty in distinguishing CS with different types of sulfation in different tissues [70, 71]. Considering this, it is necessary to develop more efficient strategies for cancer diagnosis and therapy. The VAR2CSA protein binds specifically to the placenta, although little is known concerning the structure of the CS that mediates such binding. The most common features of cancer cells, such as rapid growth, invasion of surrounding tissues, and the capability to establish a new vasculature are common to trophoblastic cells and may be due to the CS expression [50, 51]. rVAR2CSA could be used



as a highly specific reagent for cancer and could be a strategic way to diagnosis and treatment, and realize the function of CS in cancer development and placenta.

#### **1.4 Purpose of this project**

The malaria parasite, *Plasmodium falciparum*, when it infects the erythrocytes of the human organism, causes the expression of the VAR2CSA protein on the surface of erythrocytes. Through the VAR2CSA protein, iEs adhere to a specific type of CS (of-CS) present in the human placenta. This binding allows iEs to escape from the body's elimination.

Placenta cells and cancer cells have many similarities and one of them is the presence of CS with very specific characteristics. CS is an extremely modified and heterogeneous molecule. Therefore, despite the enormous research towards its characterization and the characterization of the VAR2CSA-CS link, there are still many essential details to realize. The characterization of the CS chain and its binding to VAR2CSA is very important in the diagnosis and therapy of cancer and in the development of a vaccine for malaria. Some features are already known, for example that the VAR2CSA protein binds to a specific type of CS with a 4-*O* and 6-*O* sulfation mixture. However, much research work needs to be done to define the characteristics of the binding epitope.

The aim of the present work is to help define the structure of-CS and to understand what distinguishes it from the remaining CS present in other tissues of the human organism. In this work a human placenta sample was used from which CS was extracted by extraction / purification methods. Subsequently the CS sample was analyzed by HPLC and LC-MS methods. The study will provide information on a key target that may be of extreme importance in cancer and malaria.

## **2. EXPERIMENTAL PART**

### **2.1 Methods**

#### **2.1.1 Sample and digesting enzymes**

The placenta was obtained from healthy pregnant women delivering by caesarean section at term at Rigshospitalet University Hospital in Copenhagen, Denmark. The placenta was scrapped with forceps and distributed through different tubes with phosphate-buffered saline (PBS). The blood was removed with successive centrifugations (3700 Heraeus) at 1000g. CS was extracted from tissue by a series of protease digestion and separated from other GAGs by the action of hyaluronidase and heparin lyases.

In the work of purification of the CS a collagenase enzyme that is a protease was used. Collagenase has the function of the degradation of collagen fibers that are present in the connective tissues of the human organism [72]. According to its function it was the first enzyme to be used in the purification / extraction process. The benzonase enzyme is an endonuclease, its function is the degradation of DNA and RNA present in the sample. The enzyme degrades DNA and RNA, with single chain, double chain, linear or circular chain. Therefore, its use is essential for the removal of nucleic acids [73]. The pronase enzyme has the ability to digest denatured and native proteins into individual amino acids [74]. The hyaluronidase enzyme is specific for cleaving HA [75]. Heparinase enzymes are enzymes that have the ability to cleave the glycosidic bond between hexosamines and uronic acids in heparin and HS. When the heparinase I, II and III enzymes are used together, they cause a virtually complete degradation of heparin and HS [76]. The enzyme Chondroitinase ABC (ChABC) is used to digest the CS chains, acts efficiently on C4S, C6S and DS [94]. All enzymes used in the placenta sample had the common goal of extracting CS (Table 2.1) and therefore digestion of the remaining components of the tissue.

**Table 2. 1** Enzymes used in the purification of the placenta sample

Enzyme	Amount	From
Collagenase	200U/ml	ThermoFisher Scientific
Benzonase	250U/μl	Sigma-Aldrich
Pronase	50U/ml	Merck Millipore
Hyaluronidase	300U	Sigma-Aldrich
Heparinase I, II, III	50mU	Iduron
ChABC	50mU/ml	Sigma-Aldrich

## 2.2 Sample digestion processes

The placental CS was purified by various processes (Anexo I). Apart from digestion with enzymes (Table 2.1), the following steps were used: DEAE HiPrep column (from GE Healthcare Life Sciences), dialysis (from ThermoFisher Scientific), lyophilization and delipidization. All procedures were done to allow purification of CS from the placenta (Figure 2.1).

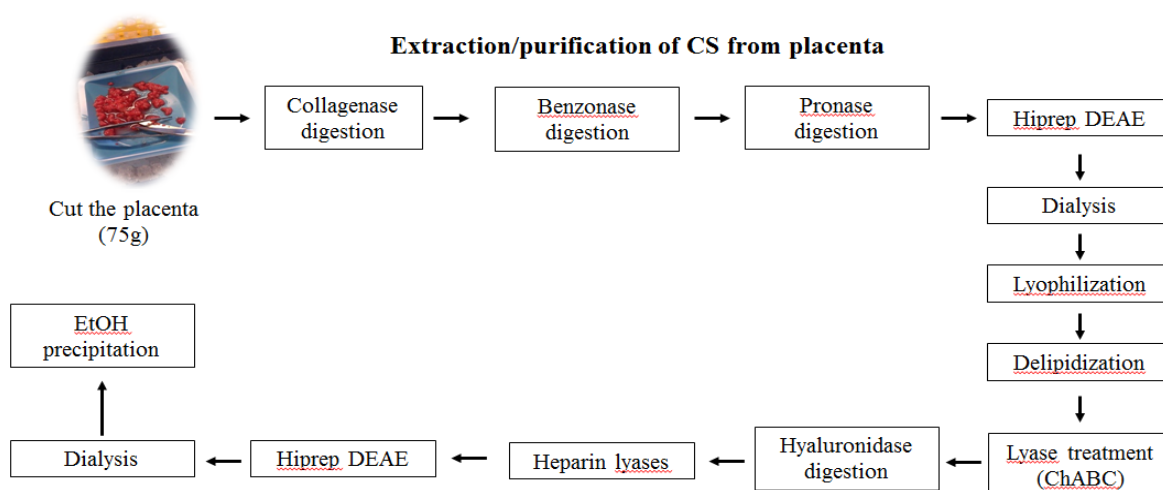


Figure 2.1 - Scheme illustration of the purification steps of placental CS

### **2.2.1 Separation of proteins and other biomolecules**

HiPrep DEAE FF 16/20 column is used to perform ion exchange chromatography, allowing the separation of proteins and other biomolecules [77].

The column is positively charged allowing binding of negatively charged compounds such as the GAG chains. During the first 10-15 minutes water was used with a low flow rate to allow removal of the ethanol (EtOH). The placenta sample was consecutively treated with 1xDPBS solution with 0.1% Triton X-100. The column was equilibrated with the same solution (1xDPBS with 0.1% Triton X-100, 1.5 mL / min). After equilibration of the column, the placenta was allowed to pass through the column overnight. When the entire sample from the placenta passed the column, the column was washed with DPBS for 10-15 minutes. The wash solution was prepared (20mM sodium acetate (NaOAc), pH 5, 0.1M NaCl, 1.5mL / min) and placed in the column to ensure that only the chains of negatively charged compounds are retained. Afterwards, the purified sample was eluted with 20mM NaOA, pH 5, 1M NaCl, 1.5mL / min.

### **2.2.2 Remove salts and small contaminants**

Slide-A-Lyser G2 dialysis cassettes (2K MWCO, 3mL) were used to remove salts and other small contaminants. The cassette is comprised of a cellulose membrane which has the ability to retain macromolecules like proteins, bigger than 2000 daltons [78].

Initially, the cassette was hydrated for 2 minutes by dipping it in water (water can be used because the goal of this step is the separation of sugars, which requires no special buffer). The sample was then placed inside the cassette (halfway) with a pipette. The air that was inside the cassette was previously removed so that the surface area / volume is maximum. The cassette was placed in water and stood overnight. Subsequently, the sample was carefully removed using a pipette and placed in a 50ml falcon tube.

### **2.2.3 Dehydration of the sample**

The lyophilization process aims to reduce the amount of water in the sample. Lyophilization is dehydration of the material by freezing and sublimation processes [79]. The placenta sample (50mL) was distributed in falcon tubes and frozen at -80 ° C. When frozen, the vials were covered with parafilm and placed in the lyophilizer for two days.

#### **2.2.4 Removal of lipids**

The lipid or lipid groups were removed from the sample through a placenta delipidization process. For this procedure were used different ratios of chloroform ( $\text{ChCl}_3$ ) and methanol (MeOH):

- 50mL (2:1 v/v)  $\text{ChCl}_3$ : MeOH, overnight  
(The supernant was removed)
- 50mL (1:1 v/v)  $\text{ChCl}_3$ : MeOH, overnight  
(The supernant was removed)
- 50mL (1:2 v/v)  $\text{ChCl}_3$ : MeOH, overnight  
(The supernant was removed)

The placenta sample was placed in a glass container with the first proportion  $\text{ChCl}_3$ : MeOH (2: 1). The solution was in complete rest overnight. The buffer was removed and the following ratio buffers added. The compound (CS) of interest will remain in the form of pellets. After the addition of different proportions ( $\text{ChCl}_3$ : MeOH) MeOH was added to the last wash. The sample was then dried at 55°C in falcon tubes.

#### **2.2.5 EtOH precipitation**

In order to further remove impurities, the delipidized sample was re-dissolved in 20mL ddH<sub>2</sub>O and subjected to EtOH precipitation. The placenta sample was precipitated by a solution of EtOH saturated with NaOAc. The EtOH solution saturated with NaOAc solid was prepared in a 50mL falcon tube and then added to the sample until precipitation of the placental CS (a volume ratio of 2:1). The sample was left at 4°C overnight. Then, it was centrifuged (Thermo Scientific Heraeus Fresh 21) at 4 ° C, 10000g for 30 minutes. The EtOH was removed and the sample was dried at 55°C again.

### **2.3 Enriching of-CSA on VAR2CSA columns**

CSA purification in the placenta sample was made possible with the use of an *N*-hydroxysuccinimidyl (NHS) activated column covalently coated with VAR2CSA proteins (Anexo II). This column is activated through the esters to capture primary amines. The retention of non-specific compounds is not significant because of the hydrophobic matrix properties. The column is packed with pre-NHS which is an ester that reacts directly with the amine group in the molecules [80]. In this purification procedure two columns were used: both columns were coated with VAR2CSA protein, but the second column contained ChABC after initial capture of CS on rVAR2. Thus, in the first column the entire GAG chain

will be retained but in the second column only the GAG binding segment to the VAR2CSA protein will be retained in the column because what is not in the binding site will be digested by the enzyme ChABC. The column (with a maximum volume of 1mL) was placed in an iron stand with a glass underneath. The cover that was on top of the column was removed and a drop of ice cold 1mM hydrochloric acid (HCl) was added to the top of the column. The application of solutions such as HCl was made with a syringe (2mL) and extreme care was taken not to let any air into the column. The snap-off end of the column out-let was removed. Before use the column was washed with 6mL of ice cold 1mM HCl, with a flow rate of 1mL / min (1/2 drop / sec) to remove the isopropanol used for storage. The washing process was performed in three parts using a 2ml syringe. The purification column was first loaded with a spycatcher (13kDa, 14mg / mL) in a volume of 1mL, which allows the binding of the VAR2CSA protein (121kDa) to the column, since the protein contains a spytag that binds directly to the spycatcher. The column was sealed with parafilm for 30 minutes at room temperature. The buffers A (0.5M Ethanolamine, 0.5M NaCl, pH 8.3) and B (0.1M Sodium acetate, 0.5M NaCl, pH 4) were prepared. Buffers were made to wash away the protein which has not been retained in the column and to deactivate any excess active groups that are not coupled. The first washing of the column was made in the following order: 6 ml Buffer A, 6 ml buffer B, 6 ml buffer A. The column was sealed for 30 minutes at room temperature and then another wash was made in the following order : 6 mL buffer B, 6mL buffer A, 6mL buffer B and 4mL of PBS. The placenta sample and the sample from Sigma CSA were prepared to be enriched. The placenta sample (100ug / mL) was diluted in 2.5mL of ammonium acetate (because it is a volatile substance). As for the Sigma CSA sample, 2mL of a 1.5mg / mL solution in ammonium acetate were prepared. Before the samples were placed inside the column, 100ul of each of the samples were stored for analysis (Input sample - sample that never into the column). The placenta sample was injected into the column with a 2mL syringe, and the run-through (i.e. the sample that did not bind to the column, that was not retained) was collected. This step was repeated five times and the last run-through was collected for further analysis. The column was washed with 10mL PBS and the run-through (PBS wash) was collected for further analysis. The sample was eluted with 2.5mL of elution buffer (0.25M, 1M, 2M of ammonium acetate (NH<sub>4</sub>OAc)). For each of the elution buffer the eluted samples were taken for further analysis. The column was washed with 10mL of PBS to be prepared for the Sigma CSA sample. The process was repeated in the same way as described above. At the end of this process the column was washed with 2mL of PBS. In parallel, CSA samples were passed through a different VAR2CSA column following the same procedure described. After the PBS

wash step, 10mU of ChABC was loaded into the column and allowed to digest the bound CSA for 2 h at room temperature. The column was then washed again with PBS and eluted in the same way described above.

### ***Separations of proteins and biomolecules (larger than 500Mr)***

PD MiniTrap G-10 column (from GE Healthcare) is used to make fast separations of proteins and biomolecules larger than 500Mr. However, the same column can also be used for removal of compounds with low weight and desalting. In this case the column was used to remove excess salt from the samples. Through the process of gel filtration chromatography, molecules can be separated based on their sizes [81]. The column was placed in an iron stand with a glass underneath. The top cap of the column was removed and the storage buffer in the column was removed. Column washing elution was performed with water and the flow-through discarded. The column was filled with water and the fixed instrument of the column was placed inside it to later add the samples. Samples were added and water was added to fill a final volume of 0.7mL (Table 2.2). The elution buffer was used, and the eluate was collected. The samples were placed in the speed vacuum over night.

Table 2. 2 Sample values added to the MiniTrap G10 column

<b>[NH<sub>4</sub>OAc]</b>	<b>Sample (μl)</b>	<b>H<sub>2</sub>O (μl)</b>	<b>Total (μl)</b>
100mM	270	430	700
250mM	250	450	700
1M	300	400	700
2M	300	400	700

## **2.4 Analysis processes**

### **2.4.1 HPLC**

The process of HPLC allows separation of chemical species that are present in the sample. Because of different molecular structures, functional groups and migration speeds, the different molecules can be

distinguished. The substance with higher affinity towards the column is eluted the last, and the one which elutes first has the lowest affinity to the column [82].

The standard samples of CS/DS disaccharides (from Iduron) – DOA0, DOA4, DOA6, DOA10 were used to quantify the placental sample and also as a standard sample for identification of the different disaccharide peaks of the placenta sample on HPLC chromatograms (Figure 2.2). The sample of CS from bovine trachea (Sigma CSA) (from Sigma-Aldrich) was used as a standard sample CS.

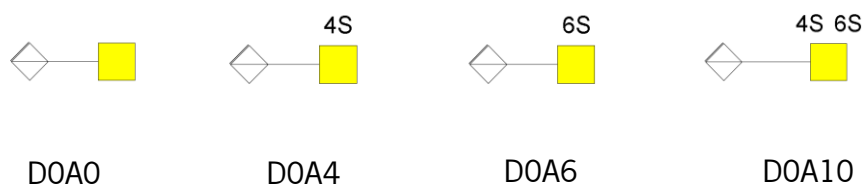


Figure 2.2 - Structure of CS/ DS disaccharides used as sample standards

HPLC analysis was first ran on the standard samples (DOA0, DOA4, DOA6, DOA10) and then on samples of purified placental CS and Sigma CSA. Before standard samples are placed in the HPLC (Thermo Scientific Dionex Ultimate 3000) they must be treated in order to achieve a good analysis (Anexo III). In this process we used the substance 2-aminoacridone (AMAC) to label the GAGs reducing ends (its adds to sensitivity because fluorescence is stronger than absorbance at 232nm). By marking the GAGs with AMAC, it is possible to separate them using a C18 X-Bridge column Shield column in HPLC. Separation of the AMAC-labeled CS disaccharides by reverse phase chromatography is possible. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase, i.e., the sorbent. The column used in HPLC shows Hydrophobic chains and AMAC, which is marking the CS, has hydrophobic characteristics. So it is possible to separate the disaccharides since these are retained on the HPLC column when the AMAC binds C18 chains (Figure 2.3).



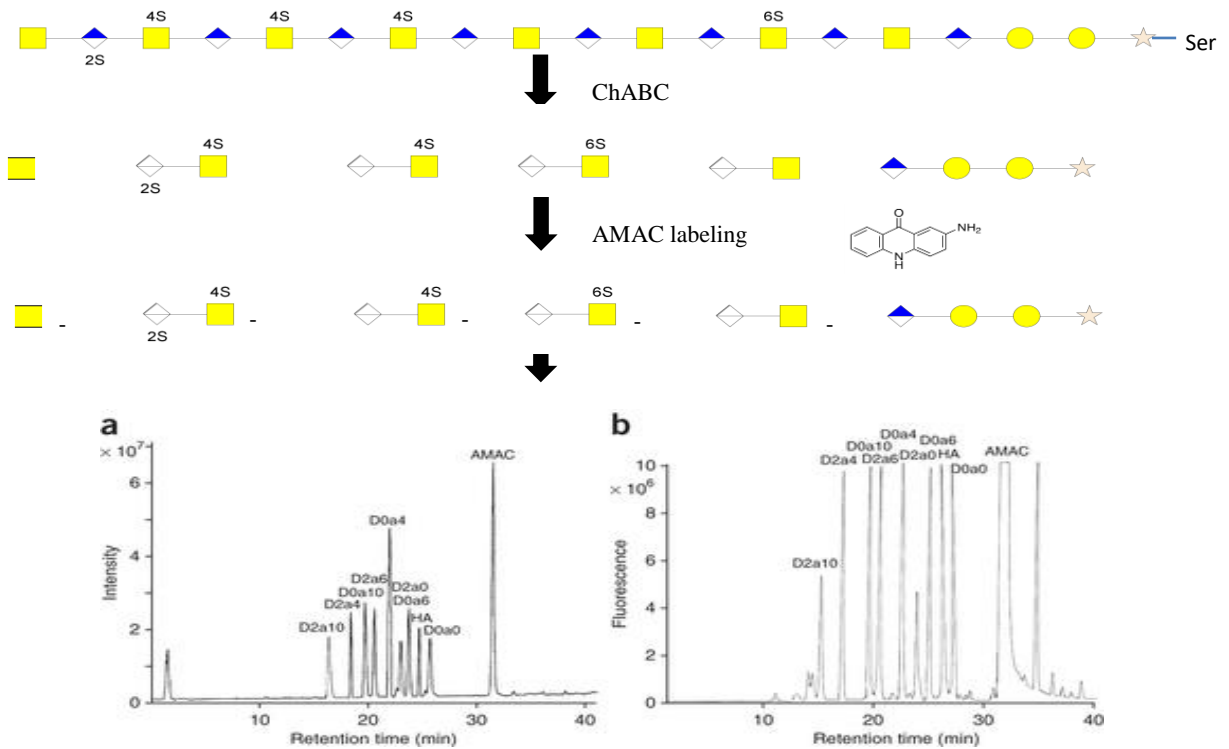


Figure 2.3 - AMAC labeling scheme after ChABC activity. The AMAC labeling allows visualization of the disaccharide peaks in HPLC (Anexo III)

The buffers (A and B) that were prepared previously were placed on the HPLC and the column and a UV detector were also placed. During the first 50 minutes it is necessary to balance the machine with a gradient in a range of 2-30% of the solvent B (equilibration solution) (Table 2.3).

Buffer A: 60mM NH<sub>4</sub>OAc, pH 5.6 and Buffer B: Acetonitrile

Table 2. 3 Description of the solvents used in HPLC and equilibrium gradients

	<b>CS/DS/HA</b>
<b>Column</b>	C18 column X-Bridge Shield
<b>Solvent A</b>	60mM NH <sub>4</sub> OAc, pH 5.6
<b>Solvent B</b>	Acetonitrile
<b>Equilibrate solution</b>	2% solvent B
<b>Gradient</b>	2-30% solvent B over 50 min

The wavelength to be used is decided after measuring the absorption spectrum of the AMAC. It was found that it had a maximum absorption wavelength of 487nm. Before running the standard samples, a blank test with water only was made. The standard samples were then placed in HPLC in solution, both together and also separately. The procedure was done for the CS from Sigma CSA (10ug). About 10µg of Sigma CSA was digested by ChABC in a 80µl, 12.5mM NaOAc and 80mU of ChABC. The reaction was incubated overnight at 37°C. After the enzyme was inactivated with a boiling water bath for 10 minutes. The solution was centrifuged at 12,000g for 10 minutes, and the supernant was used for the following steps. The supernant was placed on dry ice for 50 minutes to freeze. Initially, a solution of 0.1M AMAC in glacial acetic acid: dimethyl sulfoxide (DMSO) 3:17 (v/v) (25mg of AMAC, 0.225mL of acetic acid: 1.275mL of DMSO) was prepared. After 50 minutes to freeze it was placed on the lyophilizer for 5 hours to later proceed with the addition of 5µl of AMAC labelling reagents dissolved glacial acetic acid and DMSO in a volume ratio of 3:17 (v/v). The solution was incubated at room temperature for 15 minutes. Then a 1M solution of sodium cyanoborohydride (NaBH<sub>3</sub>CN) was prepared and added. The mixture was centrifuged at 10,000g for 1 minute at room temperature. The reaction mixture was incubated at 45°C for 4 hours. On dilution mixture was made into 200ng /µl using DMSO and ultrapure water at 1: 1 ratio (v/v). First test with Sigma CSA sample was made without acetone (C<sub>3</sub>H<sub>6</sub>O) precipitation but afterwards C<sub>3</sub>H<sub>6</sub>O was used to remove excess of AMAC after lyophilization. The placenta samples, Sigma CSA purified on HiTrap NHS columns subjected to the same procedures. The remaining samples followed the same procedure, taking into account the following values:

<p>Placenta CS input, Placenta CS (0.25M, 1M, 2M), Sigma CSA (0.25, 1M, 2M), Digested samples (100mM, 250mM, 1M, 2M): 25µl of 0.2M NaOAc, 5µl um/µl ChABC, 70µl HPLC water</p>
<p>Sigma CSA input: 14.5µl of sample, 25µl of 0.2M NaOAc, 5µl um/µl ChABC, 55.5µl HPLC water</p>
<p>Placenta CS RT, Placenta CS PBS wash, Sigma CSA RT, Sigma PBS wash: 5µl of sample, 25µl of 0.2M NaOAc, 5µl um/µl ChABC, 65µl HPLC water</p>

### **2.4.2 LC-MS**

LC-MS is a method that combines the HPLC technique with mass spectrometry (MS). It is an analytical technique that contains the ability to separate HPLC with the mass detection capabilities of MS. The LC-MS technique has several applications in the separation, in the ability to identify products with very specific masses because it has a very high sensitivity. Through the LC-MS process it is possible to identify non-reducing disaccharide and with only HPLC is not possible to identify this [83, 84]. Samples were analyzed using this method. The same HPLC systems described above was used to deliver the AMAC labelled samples into a Orbitrap Fusion mass spectrometer (Thermo Scientific). The eluents were sprayed using a Thermo Scientific Ion Max interface. The spray voltage was set at 3000 V in the negative mode and the transfer tube temperature was set at 300°C. Typically 0.1 to 0.5µg of GAG samples prior to digestion were injected in a volume of 30µL.

## **2.5 Results**

The HPLC separation method was applied to the Sigma CSA, standards of CS and placenta samples. This method allows the separation of the various compounds that are present in the samples. By doing so, it is possible to identify several peaks corresponding to the different CS disaccharides. The first sample to be analyzed on HPLC was the Sigma CSA sample, which was used as a control CS sample (100pmol of sample were injected). After labelling of the Sigma CSA sample with the AMAC fluorescent marker it is possible to identify peaks corresponding to DOA10, DOA4, DOA6, DOA0. The designations DOA10, DOA4, DOA6 and DOA0 correspond to different disaccharides with their respective sulfation at different sites in the chain. DOA0 represents a disaccharide which has no sulfation, DOA4 has a disaccharide having a sulfation at the 4-*O* position, DOA6 represents a disaccharide having a sulfation at 6-*O* and DOA10 represents a disaccharide with two sulfation at the 4,6-*O*. The different disaccharides are identified because they have different retention times according to their greater or lesser affinity to the column (with C18 chains) used in this method. The chromatogram corresponding to the Sigma CSA sample, as well as all other samples, was analyzed at a wavelength of 428nm (Figure 2.4).

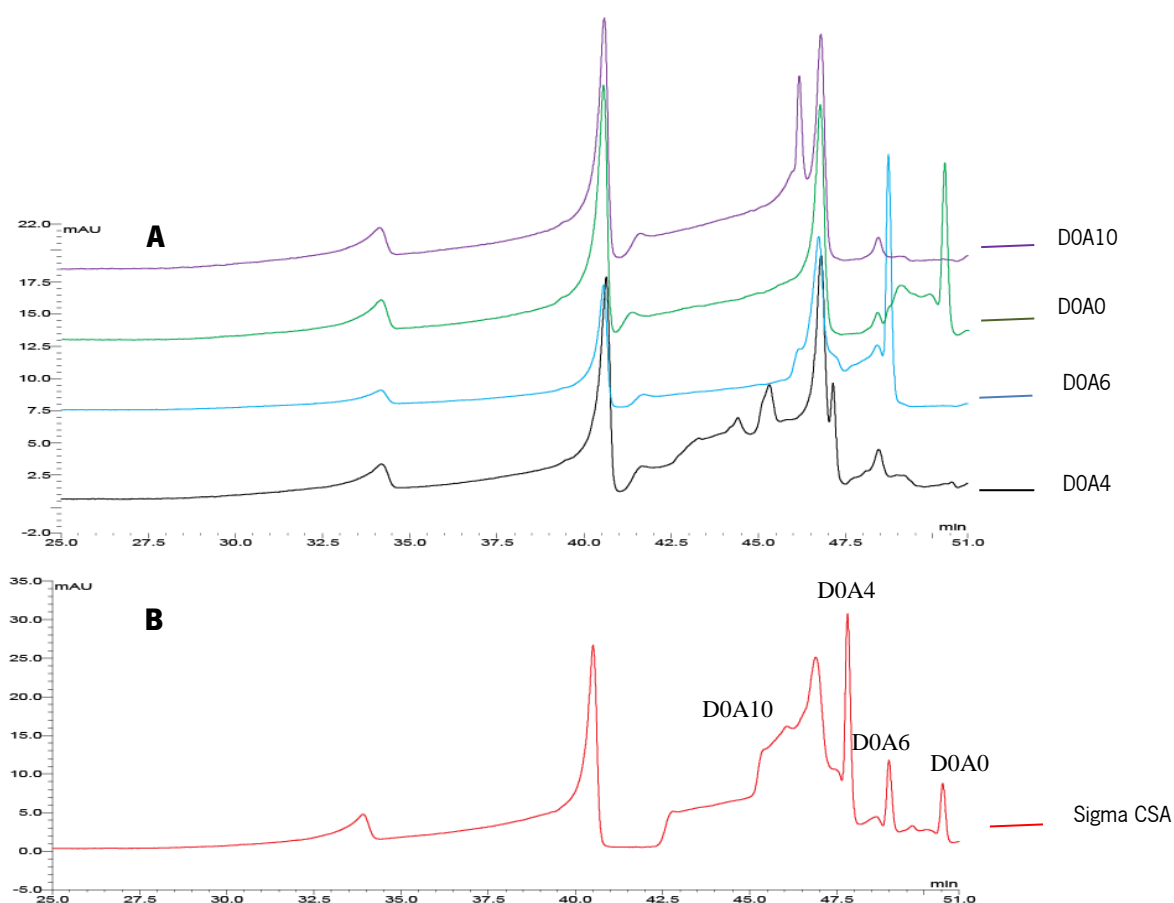


Figure 2.4 - HPLC chromatogram of the standard samples (A) and chromatogram of Sigma CSA (B). The peaks D0A10, D0A4, D0A6, D0A0 were identified in the figure by UV absorption at 428nm. In the HPLC chromatogram on the x-axis the retention time corresponding to each peak (minutes) is shown, and on the y-axis is shown mini absorption units (ABS) (mAU), which is a dimensionless unit based on the Beers' law

The peaks corresponding to the disaccharides present in the Sigma CSA sample were identified. The disaccharide D0A10 $\approx$ 46.5 minutes, D0A4 $\approx$ 47minutes, D0A6 $\approx$ 48.5 minutes and D0A0 $\approx$ 50minutes. Further analysis of the chromatogram allows the identification of peaks that represent noise. These peaks are beyond the peaks of the disaccharides of interest and do not represent sugars. It is possible to identify noise picks in the remaining chromatograms of the other samples. However, the same sample was subjected to C<sub>3</sub>H<sub>6</sub>O precipitation after the lyophilization process and before being placed on the HPLC. This procedure aimed to remove the excess of AMAC that could be present in the sample and, consecutively, allow a better identification of the peaks. When comparing the two HPLC chromatograms (Figure 2.5 B, C) it was possible to identify the same peaks (D0A0, D0A4, D0A6, D0A10). However, the identification of the peaks is more clear in Figure 2.5 C. As expected, the retention times of the different disaccharides were the same with or without C<sub>3</sub>H<sub>6</sub>O precipitation. Since

the precipitation process proved to be quite effective with regard to the removal of excess AMAC from the sample, this step was applied to the remaining samples before analysing them on the HPLC.

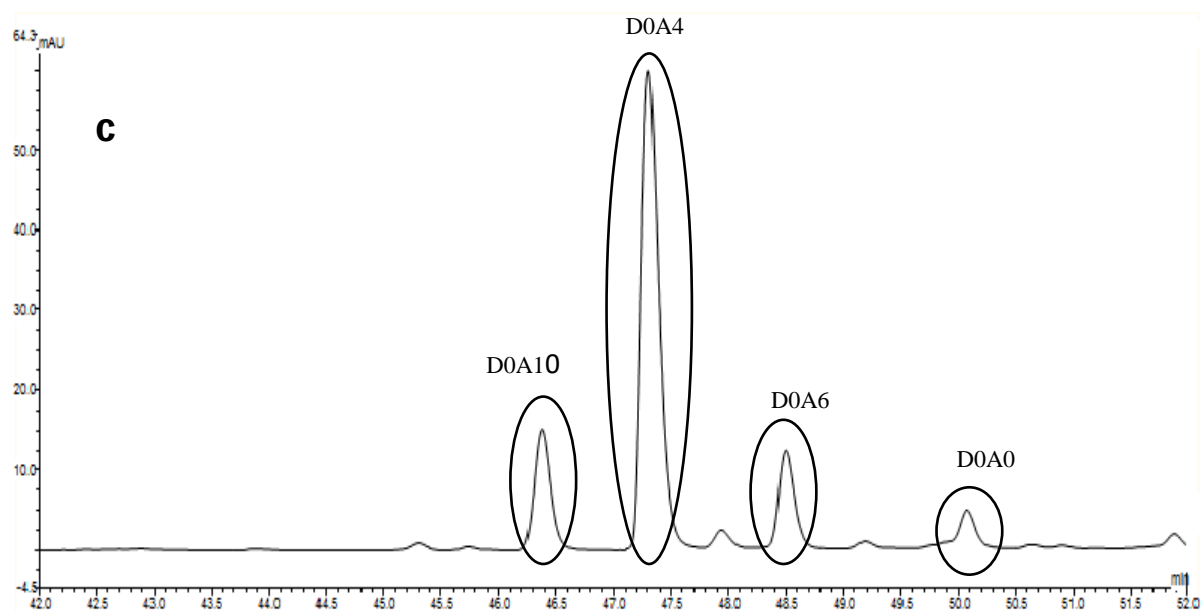
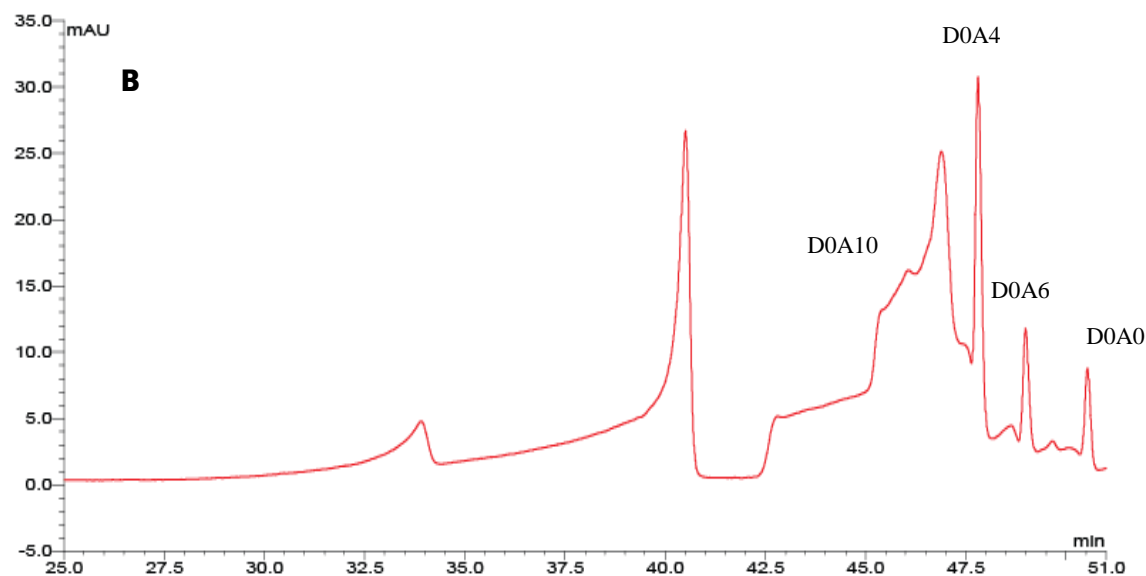


Figure 2.5 - HPLC chromatogram of the Sigma CSA without  $C_3H_6O$  precipitation (B) and with  $C_3H_6O$  precipitation (C) after lyophilisation with the precipitation is possible to see de peaks more clean. The peaks D0A0, D0A4, D0A6, D0A10 were identified in the figure by UV absorption at 428nm. In the HPLC chromatogram on the x-axis the retention time corresponding to each peak (minutes) is shown, and on the y-axis is shown mini absorption units (ABS) (mAU), wich is a dimensionless unit based on the Beers' law

The addition of  $C_3H_6O$  to the samples to be analyzed in HPLC results in the formation of a precipitate corresponding to the labeled oligosaccharide. The  $C_3H_6O$  precipitation reduces the excess AMAC that was present in the sample solution [85]. The precipitation result is visible in the chromatogram of the Sigma CSA sample after precipitation with  $C_3H_6O$  (Figure 2.5 C). When comparing the two chromatograms before and after precipitation (Figure 2.5 B, C) it is found that in the latter, the same peaks are identified more clearly and practically without noise. The  $C_3H_6O$  precipitation process was repeated for standard samples of CS/DS disaccharides and for the placenta sample. The chromatograms of all the samples were analyzed at a wavelength of 428nm, since it was the value at which a better identification of the peaks was obtained.

Several fractions of the Sigma CSA and placenta samples were collected during the purification process, which were subsequently analyzed by HPLC. Standard samples of the CS disaccharides were also analysed by HPLC (Figure 2.6).

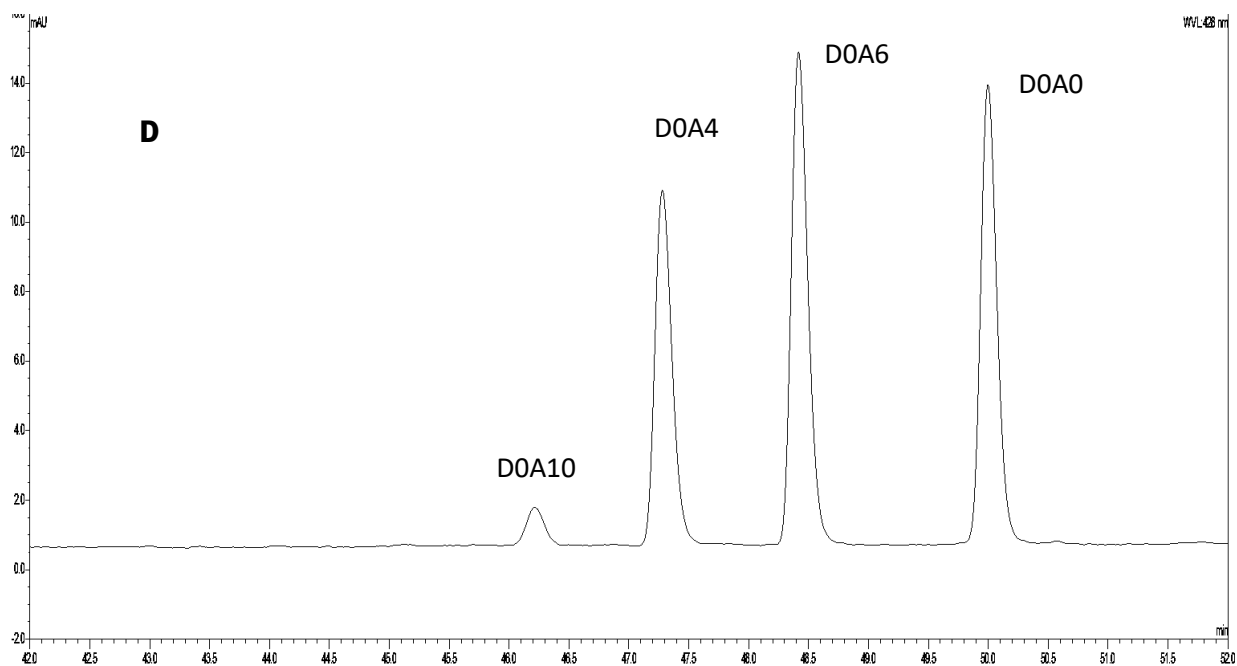
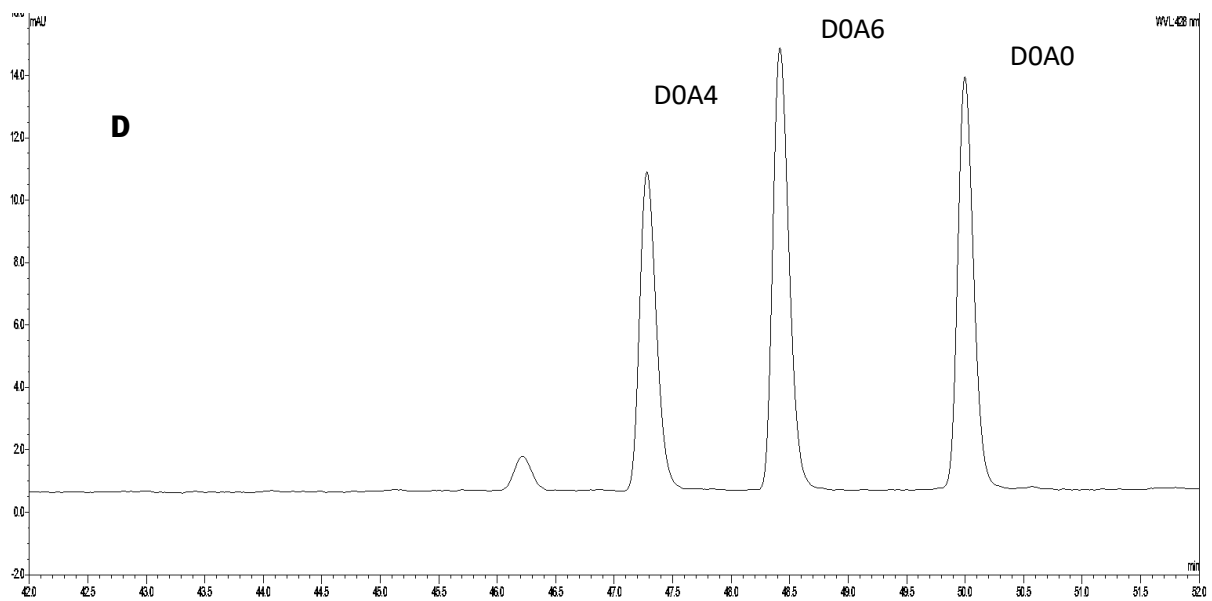


Figure 2.6 - HPLC chromatogram of standard samples. The peaks D0A0, D0A4, D0A6, D0A10 were identified in the figure by UV absorption at 428nm. In the HPLC chromatogram on the x-axis the retention time corresponding to each peak (minutes) is shown, and on the y-axis is shown mini absorption units (ABS) (mAU), which is a dimensionless unit based on the Beers' law

After the Sigma CSA sample, standard disaccharide samples of CS/DS were analyzed on HPLC. These samples were used to quantify the placenta sample and also as standard samples. The chromatogram

of the standard sample was read at a wavelength of 428 nm, as in the Sigma CSA sample and the placenta samples. Through its analysis (Figure 2.6 D) it is possible to identify the peaks D0A4, D0A6, D0A0 by their different retention times. Comparison of the chromatogram of the standard samples with the chromatogram of the placenta sample facilitates identification of the different picks.

Purification of CSA from the placental sample in NHS columns gave rise to different sample fractions which were analyzed on HPLC. The different fractions of analyzed samples provided different information, the sample of placenta input represents the sample before the purification and so when analyzed we perceive which disaccharides make the sample of the placenta before the purification. In the input fraction of the placenta sample it was possible to identify the peaks D0A4, D0A6, D0A0. This identification of the peaks is facilitated by the comparison of the chromatogram of standard disaccharide samples of CS (Figure 2.7 D) with the chromatogram of the placenta input sample (Figure 2.7 E). The retention times of each of the peaks is approximately the same as that of the Sigma CSA sample chromatogram (Figure 2.5 C).



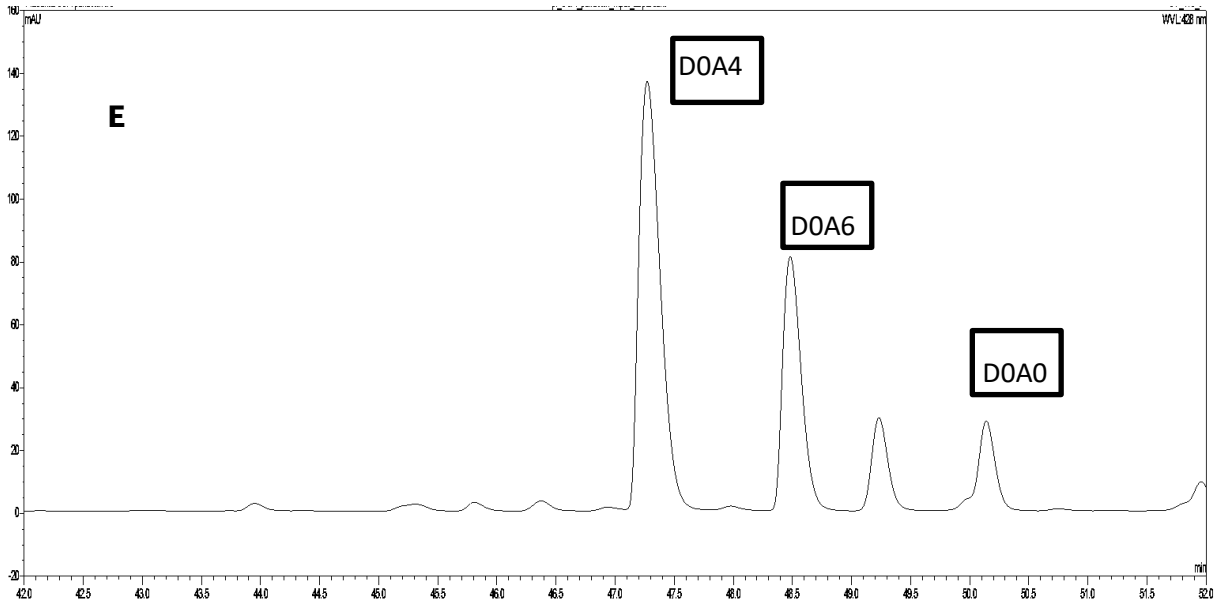
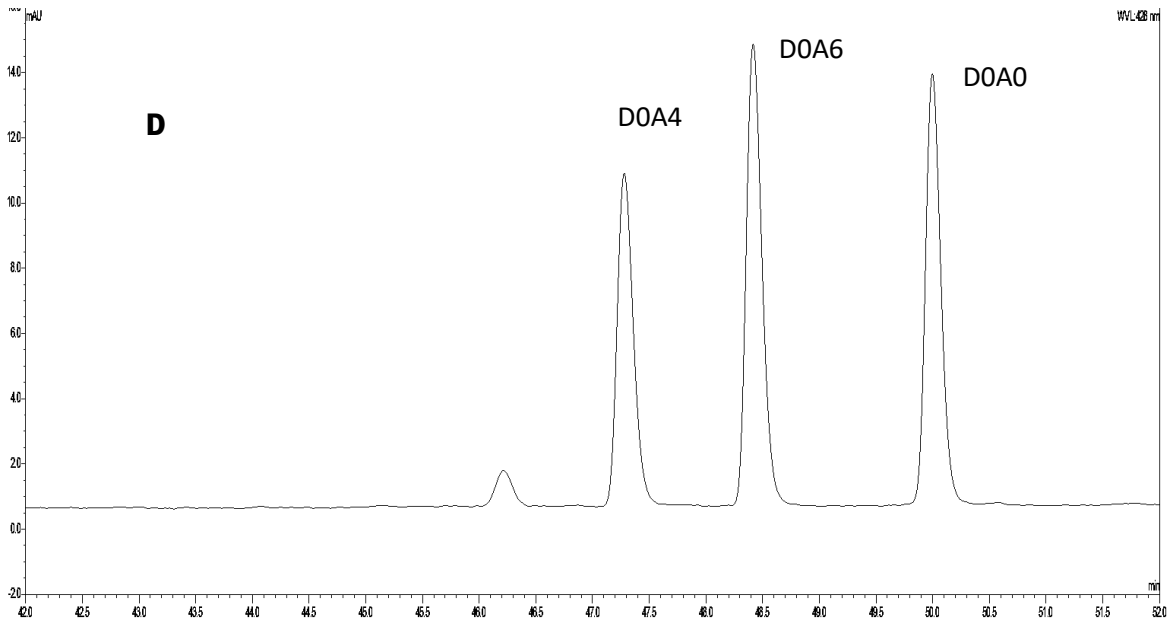


Figure 2.7 - HPLC chromatogram of standard sample (D) and the placenta input (E). The peaks DOA0, DOA4, DOA6 were identified in the figure by UV absorption at 428nm. The peaks of the disaccharides from the placenta input sample are identified by comparison with the chromatogram peaks of the standard sample samples. In the HPLC chromatogram on the x-axis the retention time corresponding to each peak (minutes) is shown, and on the y-axis is shown mini absorption units (ABS) (mAU), which is a dimensionless unit based on the Beers' law

The run-through fraction of the placenta sample (i.e. the sample that did not bind in the column) was analyzed on HPLC. When comparing the chromatogram of the standard sample with the chromatogram of the run-through sample it was possible to identify all the different peaks DOA0, DOA4, DOA6, DOA10 (Figure 2.8).





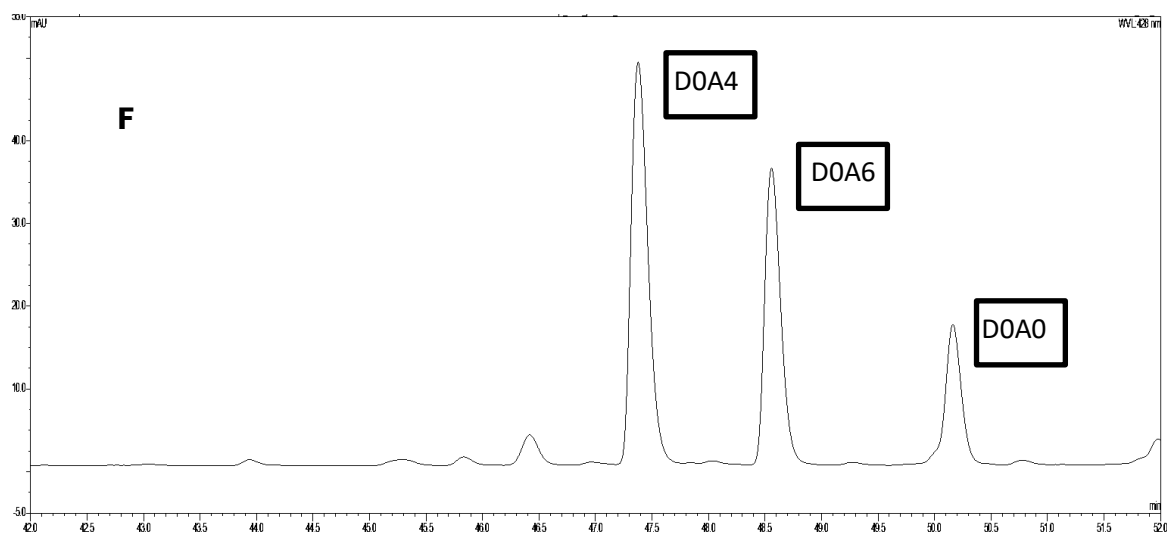


Figure 2.8 - HPLC chromatogram of the standard sample (D) and placenta run-through (F). The peaks D0A0, D0A4, D0A6 were identified in the figure by UV absorption at 428nm. The peaks of the disaccharides from the placenta run-through sample are identified by comparison with the chromatogram peaks of the standard sample samples. In the HPLC chromatogram on the x-axis the retention time corresponding to each peak (minutes) is shown, and on the y-axis is shown mini absorption units (ABS) (MAU), which is a dimensionless unit based on the Beers' law

In the NHS column, used for the enrichment of the of-CSA from the samples, was added spycatcher (13kDa, 14mg / mL) which allows the binding of the VAR2CSA protein (121kDa) to the column. Subsequently, the placenta sample was passed on the NHS column and the run-through sample was collected. When analyzing this sample in the HPLC, the peaks of the disaccharides D0A4, D0A6 and D0A0 were identified in the chromatogram (Figure 2.8 F). Identification was facilitated by comparison with the standard sample chromatogram (Figure 2.8 D, F). Once the same peaks were identified in the placental input sample (Figure 2.7 E), the chromatograms of the placenta sample input were compared with the run-through sample chromatogram (Figure 2.9 E, F). It can be verified that the amount of D0A4 disaccharide decreases by decreasing the area of the peak in the last chromatogram.

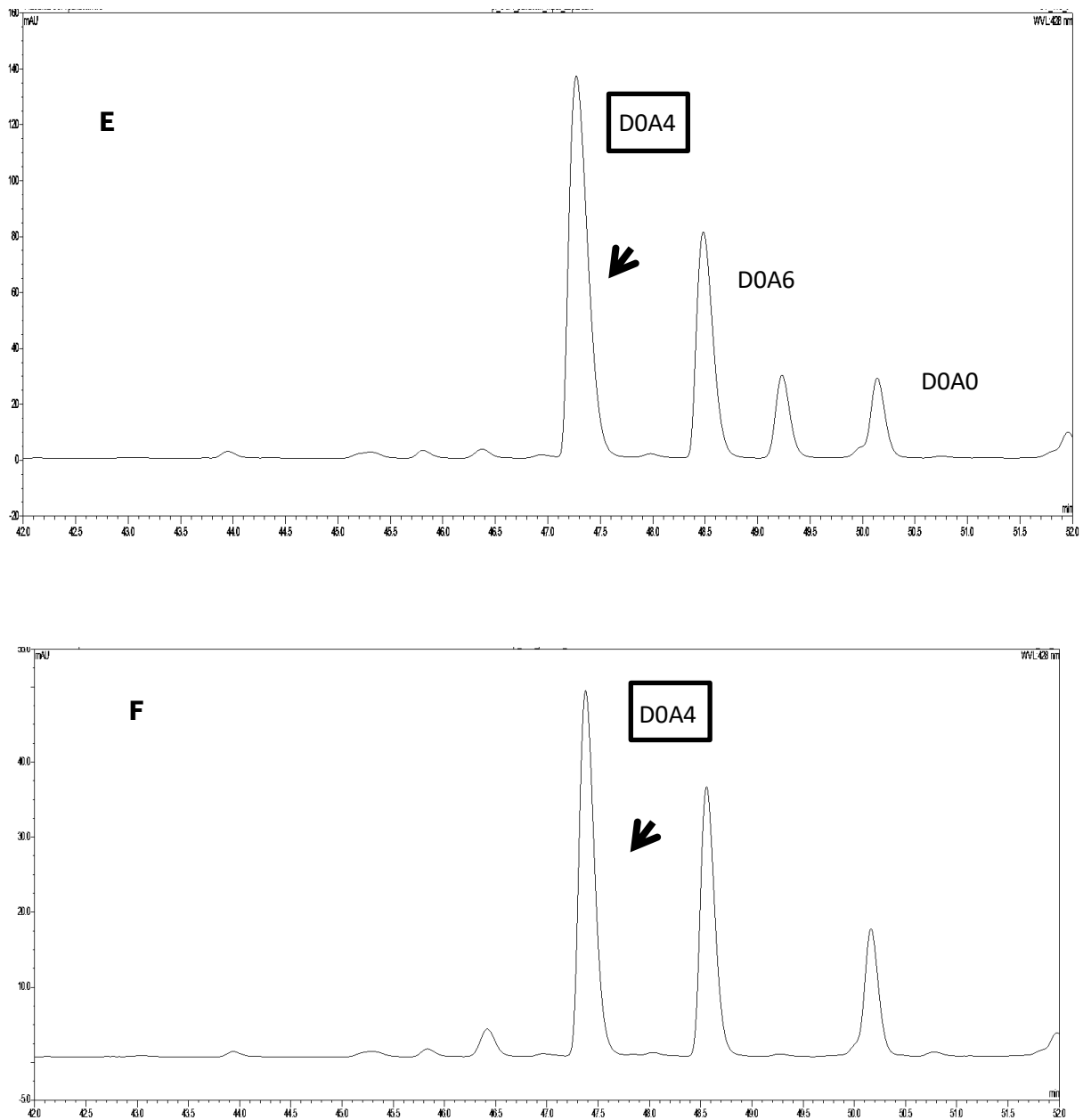
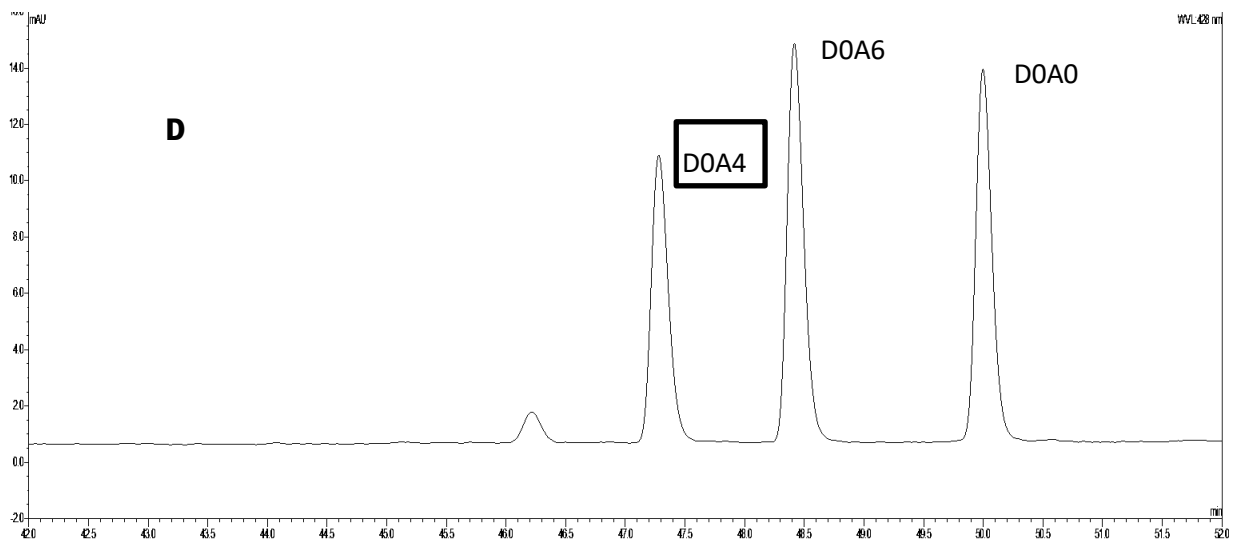


Figure 2.9 - HPLC chromatogram of the placenta input (E) and placenta run-through (F). The peak D0A4 was identified in the figure by UV absorption at 428nm. In the chromatogram of the run-through placenta sample, a decrease in the D0A4 disaccharide peak is observed. In the HPLC chromatogram on the x-axis the retention time corresponding to each peak (minutes) is shown, and on the y-axis is shown mini absorption units (ABS) (mAU), which is a dimensionless unit based on the Beers' law

This comparison shows that in the chromatogram of the placenta run-through sample there is a decrease in the percentage of D0A4 disaccharide. The decrease can be observed by the reduction of peak area corresponding to D0A4 in the chromatogram of the placental run-through sample (Figure 2.9

F). Decreasing DOA4 disaccharide after passage into the NHS column, enriched with VAR2CSA protein, was expected since the run-through placenta sample is the sample that passed through the column but was not retained. The DOA4 disaccharide was retained, thus showing VAR2CSA protein binding specificity.

After passing the placenta and Sigma CSA samples through the purification column, the sample that was trapped inside the column was eluted with elution buffer. The elution buffer used contained three different salt ( $\text{NH}_4\text{OAc}$ ) concentrations (0.25M, 1M, 2M). The first concentration (0.25M) is too low to elute the sample efficiently, the latter concentration (2M) is too high. So, was chosen a lower concentration (1M) to minimize the salt contamination. When comparing the chromatogram of the placenta sample that was eluted with 1M salt (Figure 2.10 G), with the standard sample chromatogram, (Figure 2.10 D) it was possible to notice that the peak that is visible after elution, corresponds to the DOA4 disaccharide.



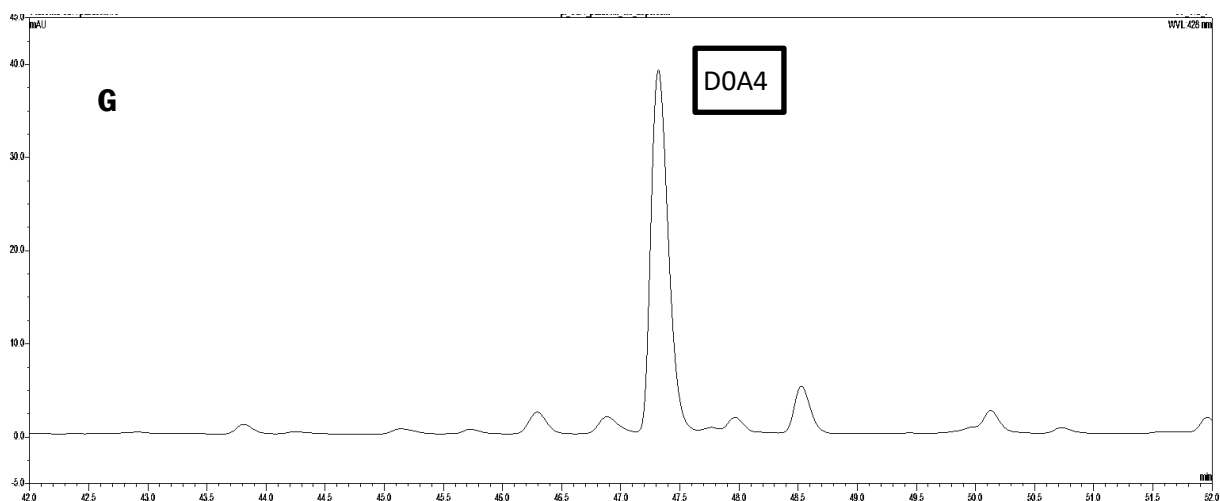


Figure 2.10 - HPLC chromatogram of the standard sample (D) and the placenta CSA, 1M of  $\text{NH}_4\text{OAc}$  (G). The peaks DOA4 was identified in the figure G by UV absorption at 428nm. The DOA4 disaccharide peak in the chromatogram of the CSA placental sample eluted with a 1 M of  $\text{NH}_4\text{OAc}$  solution was identified by comparison with the chromatogram of the standard samples. In the HPLC chromatogram on the x-axis the retention time corresponding to each peak (minutes) is shown, and on the y-axis is shown mini absorption units (ABS) (mAU), which is a dimensionless unit based on the Beers' law

The elution solution contains the  $\text{NH}_4\text{OAc}$  salt because it breaks the bonds between the VAR2CSA protein, which is in the column, and the disaccharide binding element to the protein in the column. Thus, it is possible to elute only the sample retained in the VAR2CSA protein (with the 1M of  $\text{NH}_4\text{OAc}$ ) and to analyze which type of disaccharide has specificity for this binding. When the chromatogram of the standard sample was compared with the chromatogram of the placenta sample eluted at the concentration of 1M of  $\text{NH}_4\text{OAc}$ , it was possible to identify a signal for the DOA4 disaccharide (Figure 2.10 D, G). The same was done for the Sigma CSA sample after it passed the purification column. The chromatogram of the Sigma CSA sample which was also eluted from the column at a salt concentration of 1M showed high similarity to the chromatogram of the placenta sample eluted at the same concentration (Figure 2.11 G, H).

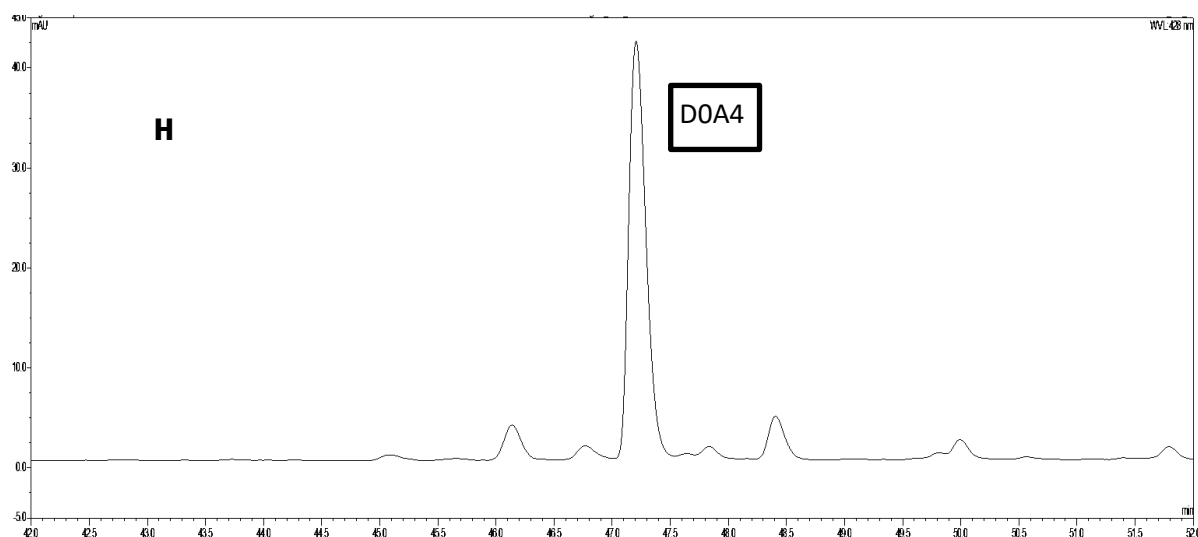
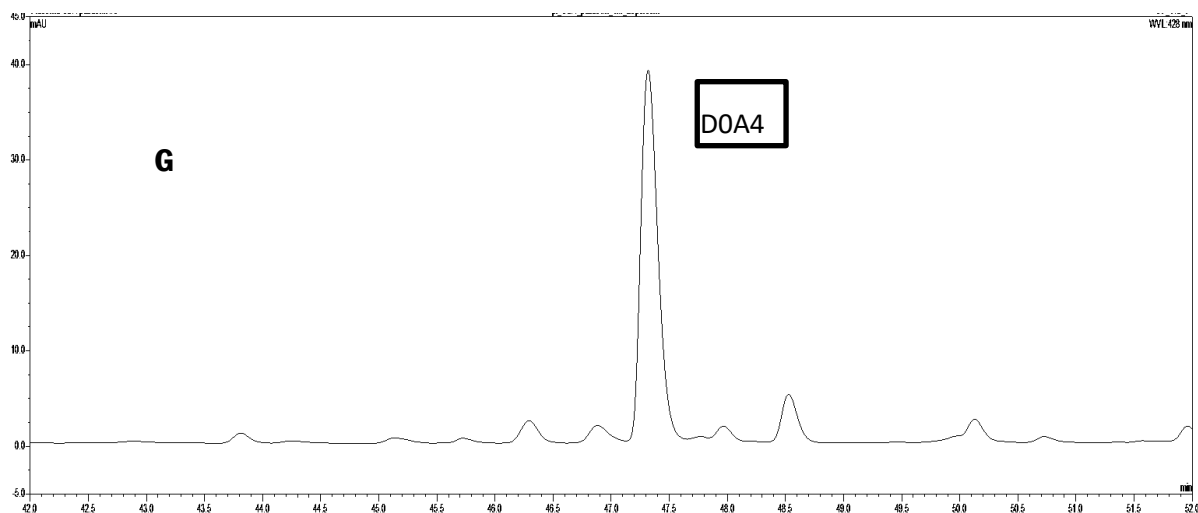


Figure 2.11 - HPLC chromatogram of the placenta CSA, 1M of NH<sub>4</sub>OAc (G) and sigma CSA, 1M of NH<sub>4</sub>OAc (H). The peak DOA4 was identified in the figure by UV absorption at 428nm. Both chromatograms show the same peak corresponding to the disaccharide DOA4. In the HPLC chromatogram on the x-axis the retention time corresponding to each peak (minutes) is shown, and on the y-axis is shown mini absorption units (ABS) (mAU), which is a dimensionless unit based on the Beers' law

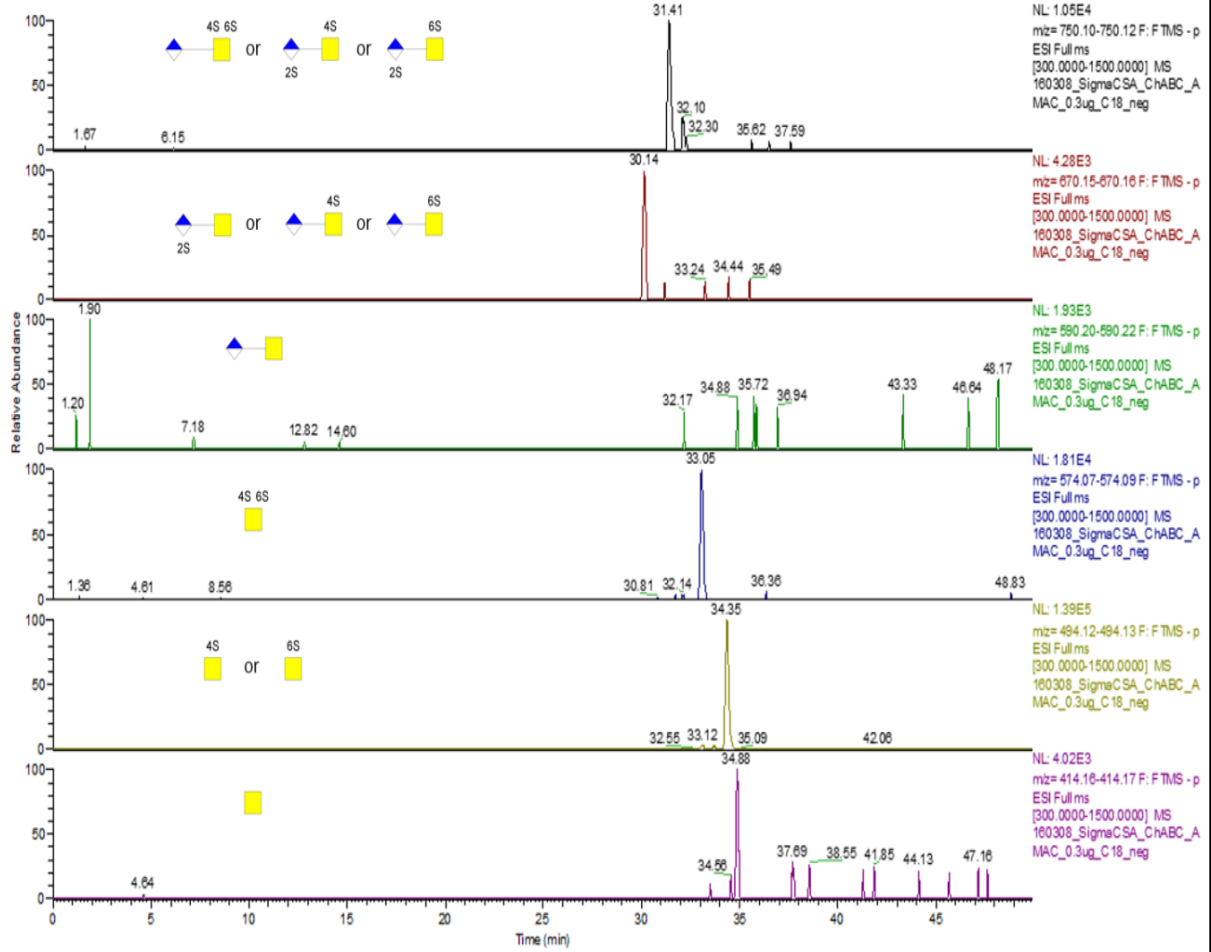
In addition to the HPLC analysis, the samples were analyzed by LC-MS. This method combines HPLC analysis with mass chromatography analysis. Initially, the Sigma CSA sample was analysed (Figure 2.12). By analyzing with the LC-MS method it is possible to identify non-reducing end disaccharides, which would not be possible with HPLC alone. However, it is difficult to accurately match the mass to a specific type of disaccharide because some have the same mass value.

# Non-reducing end disaccharide and monosaccharide profiles

160308\_SigmaCSA\_ChABC\_AMAC\_0.3ug\_C18\_neg

03/08/16 16:42:19

RT: 0.00 - 49.90 SM: 7G



## Internal disaccharide profiles

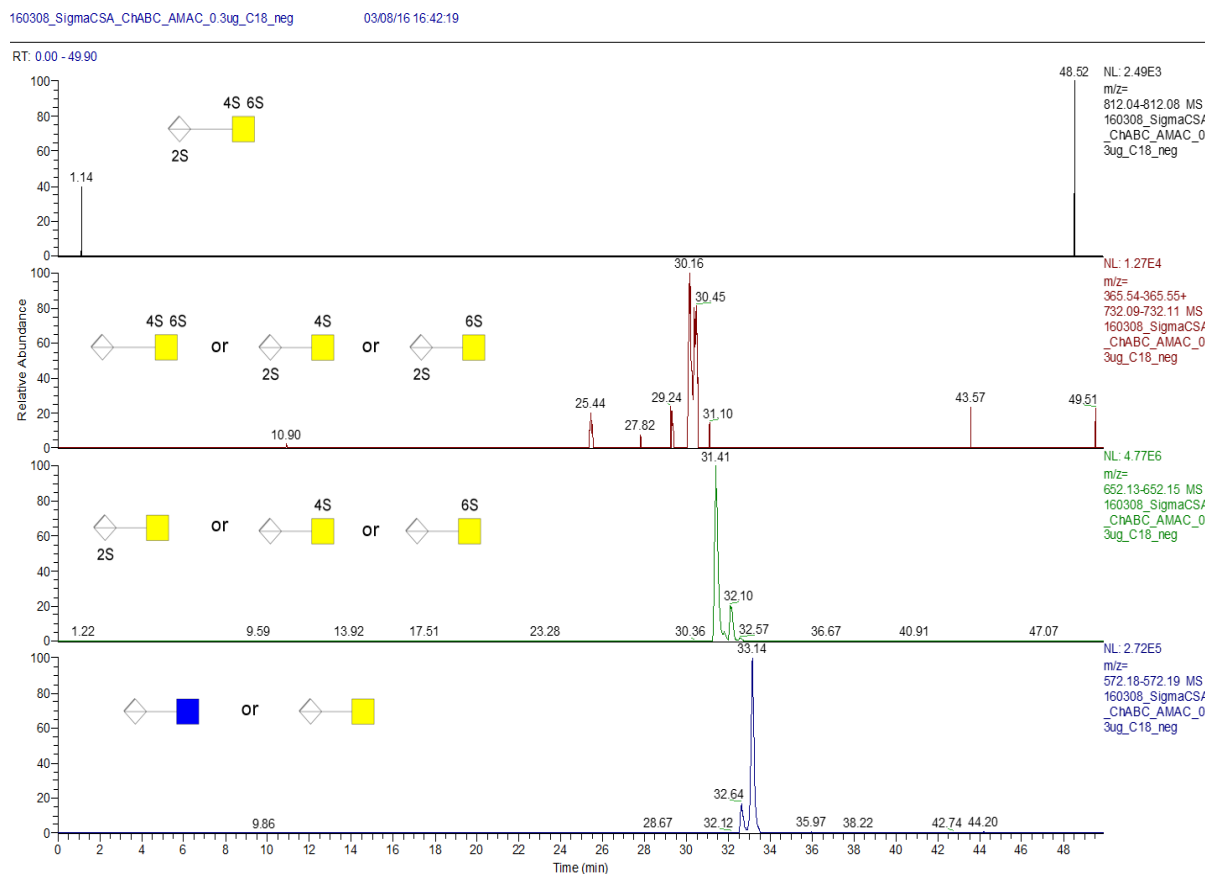


Figure 2.12 - Chromatograms corresponding to the Sigma CSA sample at LC-MS analysis. In the LC-MS chromatogram it is possible to identify non-reducing end disaccharides. In the figure, the different values of mass corresponding to the different disaccharides are observed

Afterwards, the placenta sample was analyzed by the same LC-MS method. Analysis was performed on the sample after the purification of CSA in the first column, without digestion enzymes (Figure 2.13 I), and the placenta sample that was used in the column with digestion enzyme (ChABC) (Figure 2.13 J).

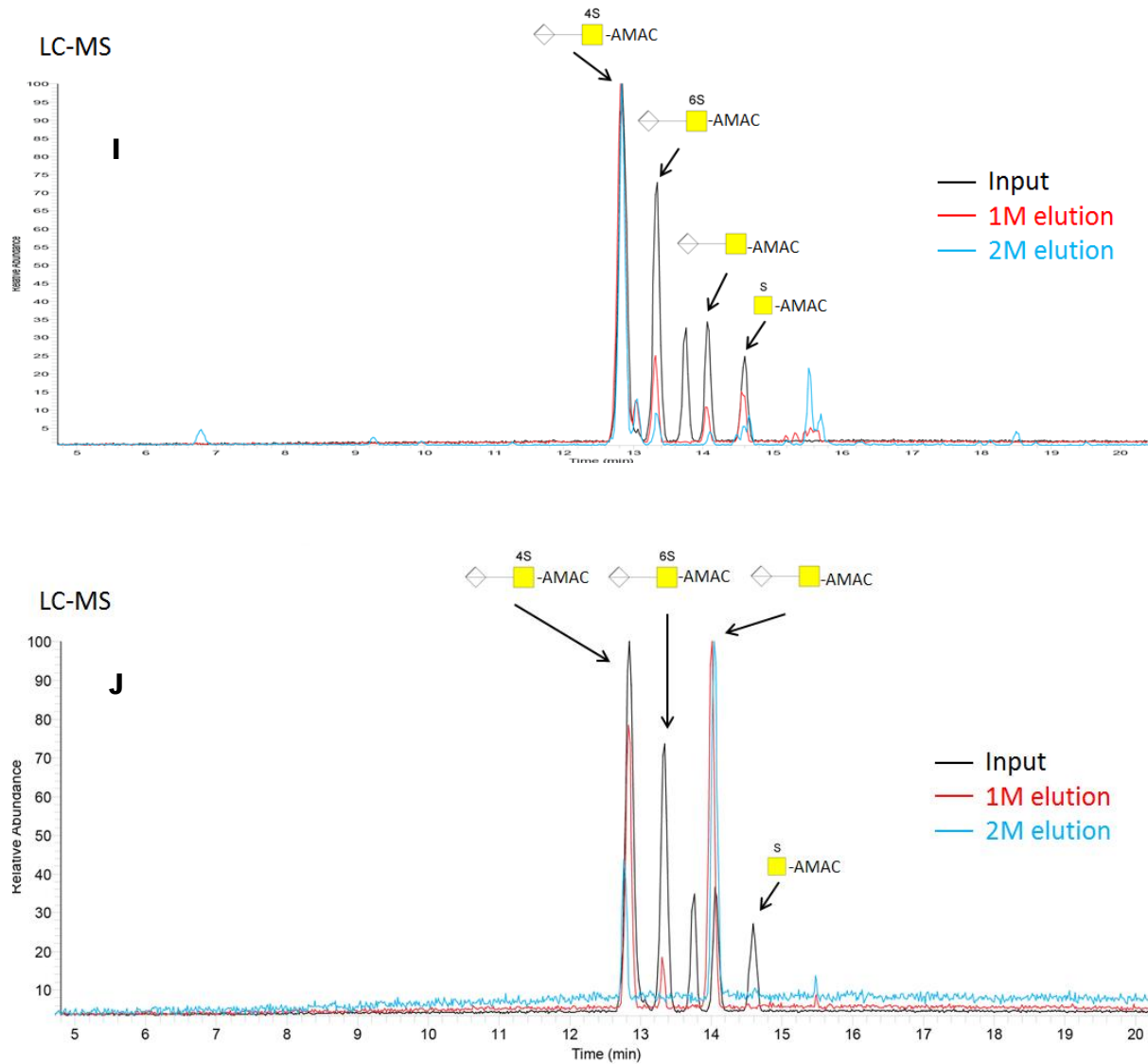


Figure 2.13 - Chromatograms corresponding to the placenta sample at LC-MS analysis. The I - after the purification of CSA in the first column and J- placenta sample that was used in the column with digestion enzyme. In I increased the percentage of the peak corresponding to the disaccharide DOA0 and decreases the percentage of the peak corresponding to the disaccharide DOA6 and the corresponding peak to the unsulfated disaccharide. In J the peak corresponding to the poorly sulfated disaccharide resisted the salt wash and was eluted

Thus, it was concluded by HPLC analysis that the disaccharide retained on the column when the Sigma CSA sample and placenta sample were passed was DOA4. In addition to HPLC analysis, samples were analyzed by LC-MS. This process combines the properties of HPLC and of mass MS. When the LC-MS chromatogram of the Sigma CSA sample was analyzed, it was concluded that it was possible to identify different CS disaccharides present in the sample and to identify non-reducing monosaccharides. The correspondence of the exact disaccharide to each identified peak is not easy, since different



disaccharides have equal mass values (Figure 2.12). When analyzing the LC-MS chromatograms of the sample which was eluted from NHS columns with elution buffer with and without digestion enzyme enrichment (ChABC) some data was obtained. In the sample eluted in the column without digestion enzyme. The percentage of DOA4 disaccharide was increased and the percentage of DOA6 disaccharide and non-sulphated disaccharides (DOA0) was decreased. This information is in accordance with the information collected in the HPLC analysis. However, in the analysis of the sample eluted in the NHS column with digestion enzymes, it is perceived that it was a fraction of low sulfated CS that was retained in the column and later eluted. In the second NHS column only the binding fragment of CS to VAR2CSA protein was retained, since the remaining disaccharide chain was digested by the enzyme.

## 2.6 Discussion

PM causes severe health complications for the mother and the fetus, such as: LBW, maternal anemia, abortion, pre-eclampsia and death [37]. The disease is caused by the binding of the VAR2CSA protein, expressed on the surface of erythrocytes infected by the *Plasmodium falciparum* malaria parasite, to the human placenta [86, 87, 32, 88]. This protein interacts specifically with CSA, which is expressed in the intervillous space of the placenta and in the syncytiotrophoblast [42, 89]. It is known that the VAR2CSA protein is the main intervenient in the protective immunity that is acquired during the multiple pregnancy [32, 90]. Therefore, the creation of a malaria vaccine is possible based on the VAR2CSA protein.

CS is a sulfated GAG that is present in several tissues of the human body. The placenta and cancer cells show some similarities in terms of proliferation, migration and invasion [50, 51]. It was recently shown that cancer cells express the same modification of of-CS where as normal cells do not [52]. It is important to define the exact structure of of-CS and characterize the VAR2CSA-CS binding because it may be a specific target of cancer in pathogenesis, treatment and diagnosis. Despite extensive research regarding the nature of the interaction between VAR2CSA-CS, the structure of the binding epitope remains unclear. However, data point to the of-CSA as a long structure with mostly 4-*O*-sulfation [8, 91]. The exact determination of the specificity of VAR2CSA allows a better understanding of this epitope and may be very important in the prevention and treatment of PM and cancer. The human placenta contains three types of CSPGs: CSPGs that are associated with the cells, extracellular CSPGs that are located in the intervillous spaces, and DS / CSPGs that are located in the matrix [42]. In the intervillous

spaces are CS chains with specific characteristics, sulfated in 4-*O* (CSA). These specific chains establish the binding between erythrocytes infected by the malaria parasite which express the VAR2CSA binding protein to the surface [32, 33, 42].

The present work aims to characterize the CS that was isolated from the human placenta [91]. Several purification techniques were used for the isolation of the CS chains. The purification process proved to be very specific and time-consuming because it takes several strenuous steps. After purification of the CS chains, these were analyzed by HPLC and LC-MS methods. The isolated disaccharide chains were labeled with a fluorescent label (AMAC) at the reducing end (is a disaccharide which has the ability to act as a reducing agent because it consists of an aldehyde group or a free ketone group). The aromatic fluorescent label provides better detection on HPLC. After the use of HPLC, the sulfated disaccharides were analyzed by LC-MS, a method that combines the properties of HPLC and MS in the same line of analysis (Anexo III) [92].

Initially, HPLC was used with Sigma CSA samples and the results were compared later with the results of the placenta sample, because both samples are made up of CS. In the HPLC chromatogram corresponding to the Sigma CSA sample, it was possible to identify the peaks corresponding to the disaccharides DOA10, DOA4, DOA6 and DOA0 (Figure 2.4). The different disaccharides represented by the different peaks have different retention times, which allows their identification in the chromatograms. In this work a UV detector was used in the HPLC. However, as the disaccharides were labeled with a fluorescent marker, the use of a fluorescence detector (FD) would allow the visualization of the peaks more clearly. In the current work, a sufficient amount of sample was injected into the HPLC, which makes it possible to visualize the peaks even with the noise of other peaks. Although it is possible to identify the peaks, it is not possible to quantify the different amounts of disaccharides (DOA10, DOA4, DOA6, DOA0). The amount of disaccharide is calculated by the value of the area of the peak, which in this case, is not possible to calculate because of noise interference. The quantification is done as follows: a small fraction of the placenta sample is taken (for example 0.01%), and disaccharide analysis is performed. After identification of the DOA10, DOA4, DOA6 and DOA0 peaks, the area of those peaks 0.01% of the placenta sample is calculated, and is divided by the signal of 100pmol of disaccharide standard. This allows the calculation of how many mg there was in the beginning with the mass of those disaccharide standards.

In order to improve the visualization of the peaks in the chromatogram and to be able to quantify the disaccharides using a UV detector, the samples were precipitated before being placed on the HPLC for

analysis. We first did the precipitation of the Sigma CSA sample. The detection sensitivity of the peaks, corresponding to the different disaccharides, depends on the reaction and the quality of the sample being used. Excessive fluorescent marker (AMAC) may negatively interfere with peak visualization in the chromatogram. Considering this, sample precipitation was done with  $C_3H_6O$  [85] to remove excess AMAC that could be present in the sample.  $C_3H_6O$  is an organic, polar compound that is often used in the precipitation of sugars.

The run-through placenta sample is the sample that passed through the purification column but was not retained inside, thus providing information on disaccharides that are not retained (Figure 2.8). The placental sample retained on the column was eluted through a 1M solution of  $NH_4OAc$ , this sample provides information on the disaccharide which was retained in the column by binding to the VAR2CSA protein that was inside. The first fraction to be studied was the placenta input, which is the sample that has never entered the column and therefore was not enriched for the VAR2CSA epitope. In the chromatogram corresponding to the placenta input sample the peaks of the DOA4, DOA6 and DOA0 disaccharides were identified (Figure 2.7 E). The comparison with the chromatogram of the standard sample allowed the identification of the different disaccharides mentioned (Figure 2.7 D, E).

During purification of CSA (in NHS columns) from the placenta and Sigma CSA samples, two purification columns were used. The first column was enriched just with VAR2CSA protein, and second column was enriched with VAR2CSA protein and digestion enzymes (ChABC). After passage of the placenta sample into the column. These enzymes allowed the long chain disaccharide to be digested, with only the disaccharide binding element being retained in the column to the VAR2CSA protein. After elution of the sample on the column with an elution buffer (100mM, 0.25M, 1M, 2M of  $NH_4OAc$ ) it would be possible to characterize the disaccharide binding element to the VAR2CSA protein. In addition to identifying the disaccharide retained in the VAR2CSA containing column, it is important to characterize the specificity of the binding epitope. Figure 2.10 shows that the disaccharide retained in the column corresponds to DOA4.

For analyzing the samples, HPLC followed by an LC-MS analysis method, as described before. First, the Sigma CSA sample was analyzed by the LC-MS method (Figure 2.12). When analyzing the LC-MS chromatogram of the Sigma CSA sample it was found that the method is capable of extracting different types of CS and non-reducing monosaccharides. However, it is difficult to match each peak to a specific disaccharide, since different disaccharides have equal mass values. It is only possible to assign correspondence possibilities to each peak (Figure 2.12). By comparing the results obtained with

standard samples corresponding to the LC-MS method the identification of the peaks could be facilitated and more rigorous. Next, the placenta sample that was eluted from the first NHS column (without digestion enzymes) and the placenta sample that was eluted after passage through the second NHS column (with digestion enzymes) were analyzed on LC-MS. Both columns were enriched with the VAR2CSA protein and both were eluted at a concentration of 1M of  $\text{NH}_4\text{OAc}$ . The extracted ion chromatograms of the input and eluted samples show the compositions of the CSA changed after the enrichment. Without on-column digestion, simple enrichment increased the 4-*O* sulfation percentage and decreased 6-*O* sulfation and unsulfated disaccharides. Surprisingly, after the on-column digestion with digestion enzymes (ChABC), a lowly-sulfated CS fraction survived the salt wash and got eluted (Figure 2.13). In this work salt was used to disrupt the GAG/ protein interaction, which is ionic in nature, highly-sulfated chains have a tendency to stay on the VAR2CSA column longer. In other words, highly-sulfated chains might not have a very specific interactions with VAR2CSA, the 4-*O* sulfation might only provide a weak interaction, which adds up in a long, undigested CS chain. When CS was digested in column it was expected the VAR2CSA binding motif will be protected by VAR2CSA during the ChABC digestion. Also, the accumulation of weak/ non-specific interaction is no longer important for the digested oligosaccharides. Therefore, it is believed this method, if proved to be working, should give us more information of the VAR2CSA binding site. Thus, it was observed that in the NHS column with ChABC enzyme a fraction of CS with low sulfation content was retained in the column. This purification column only retains the CS binding fragment to the VAR2CSA protein, since the rest of the chain was digested by the enzyme. This type of analysis provides information relatively to the CS binding epitope. The results obtained through the processes of digestion, extraction and analysis of the placenta proved to be very important for the characterization of the specific binding that exists between VAR2CSA-CS. The study of this link is of extreme importance for the development of an antimalarial vaccine and for a specific target in cancer, pathogenesis, treatment and even diagnosis. The research on this subject is vast but still many details are to be analyzed.

## **3. GENERAL CONCLUSION**

### **3.1 Conclusion**

This work aimed to help define the structure of of-CS and to perceive what distinguishes this CS from other CS located in several tissues in the human body. The characterization of this specific type of CS can become very important in the development, control and prevention of two deadly diseases: PM and cancer. These two diseases, in addition to sharing other characteristics, share the presence of the same specific CS. Therefore, understanding the structure and binding epitope of this CS could lead to the development of an anti-malarial vaccine, and also provide information about a specific cancer target [93].

Throughout the various methods and techniques used in the current work it was possible to extract of-CS from the placenta tissue. This extraction process proved to be very meticulous and long, since it was necessary to resort to different digestion enzymes and specific separation techniques. After extracting the CS from the placenta, an NHS column was used to enrich the of-CS. For this work, two NHS columns were used: the first one was coupled with VAR2CSA protein, and the second one coupled with VAR2CSA protein and with a later on-column digestion with ChABC. After enrichment in the VAR2CSA columns, the different fractions of the CS were analyzed by HPLC and LC-MS. With the HPLC analysis it was possible to identify the types of disaccharides that were present in the sample being analyzed. In the Sigma CSA sample, the DOA0, DOA4 and DOA6 disaccharides were identified, like in the placenta input sample. In the placenta run-through sample, the same disaccharides, DOA0, DOA4 and DOA6 were identified. However, when comparing the chromatogram of the placenta input sample with the chromatogram of the placental run-through sample, it was verified that the amount of DOA4 disaccharide decreased in the placenta run-through sample. This is due to the retention of the DOA4 disaccharide within the column and consecutively to the VAR2CSA protein. The sample that was retained in the inside of the purification column was analyzed by HPLC. The chromatogram showed that the disaccharide retained in the column by binding to the VAR2CSA protein had been DOA4. Interestingly, after the enriched CS was subjected to on-column digestion of ChABC, only a low-sulfated fraction was survived and got eluted from the VAR2CSA column. Assuming the binding of the of-CS to the VAR2CSA protein protected the binding motif from being digested by ChABC, this result might suggest the CS epitope of VAR2CSA has a low-sulfated composition.

The present work was important to help characterize the specific CS found in the placenta. However, much work still needs to be done to completely elucidate the specificity of this CS that allows VAR2CSA protein binding. After the laboratory work done in this study, further work is necessary to fully understand the nature of VAR2CSA binding. First of all, further efforts are necessary to characterize the exact structures of the VAR2CSA-binding motif, including the size of the epitope, the position of the sulfate groups in the sequence, and the epimerization of the uronic acids. The CS fraction surviving the on-column digestion will be subjected to further MS analysis and potentially NMR studies. Second, it would be of interest to test binding capacity of identified oligosaccharides by inhibition of binding in enzyme linked immunosorbent assay (ELISA), flow cytometry and biosensors, and once an optimal oligosaccharide has been selected this will be used in co-crystallization with rVAR2. It is also of extreme importance to understand how CS is produced and how its regulation is made in order to understand what leads to the formation of the specific type of CS in most cancer cells. With regard to malaria, the anti-malaria vaccine taking into account the VAR2CSA-CS binding specificity would be one of the positive developments with regard to this deadly disease.

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## 5. ANEXOS

### ANEXO I

#### Protocol - Purification of CS in placenta sample

##### **Sample preparation**

One piece of human placenta: ~75g

Soaked with 1x DPBS (without Ca, Mg) on ice ON

Discard supernant

(Repeat a few times to bleed the tissue. Press the tissue with a forceps to help the bleeding)

The tissue was then minced into small pieces by cutting, transferred into four 50mL falcon tubes, soaked in 1xDPBS (without Ca, Mg) 20mL per tube. Centrifuged at 1000g, 5min, 4 degrees, discard supernant

(Repeat a few times to further bleed the tissue)

##### **Collagenase digestion**

Then the tissue was soaked in 1x DPBS (with Ca, Mg) 20mL per tube, add ~0.74mg/mL collagenase (~200U/mL)

(37 degrees, end-to-end mix or rock, ON 16h)

The cells were pelleted down at 3000g, 15min, collect the supernant

The pellet was washed with 1x DPBS (without Ca, Mg) 20mL per tube

The pellet was then pelleted down again and the supernant was combined with the collagenase supernant

##### **Trypsinization**

The pellet was treated with 1x Trypsin/EDTA without phenol red (20mL per tube) 37 degrees, end-to-end mix or rock, 2h

The cells were pelleted down at 4000g, 10min (high speed if available, our centrifuge can only reach 4000 g maximum)

The pellet was washed with 5~10mL DPBS (without Ca, Mg), then pelleted down at 4000g, 15min

All the supernants were combined

Add Triton X-100 to final concentration 1%.

Add Mg<sup>2+</sup> to final concentration 2mM

##### **Benzonase digestion**

Add 10uL 250U/uL benzonase

37 degrees, incubate, ON

##### **Pronase digestion**

Add in 50U/mL

37 degrees, incubate, ON.

Heat inactivation, boil, 10min

Centrifugation: high speed >4500rpm, 30min, remove pellet



Adjust to 60mM NaOAc, pH6.0, 100mM NaC

### **Hiprep DEAE**

Column: Hiprep DEAE FF 16/20

Sample Preparation: filter the sample on a 0.45 um filter

Column equilibration: 1mL/min 1x DPBS (without Ca, Mg) with 1% Triton X-100

Load samples: 0.5~1mL/min by peristaltic pump

Wash: 1. 60mL 1x DPBS (without Ca, Mg) with 1% Triton X-100 at ~1.5mL/min

2. 60mL 20mM NaOAc, pH6, 100mM NaCl at ~1.5mL/min

Elute: 40mL/min 20mM NaOAc, pH6, 1M NaCl at ~1.5mL/min

### **Dialysis**

Dialyze against ddH<sub>2</sub>O using two of the following 30mL dialysis cassettes.

change water every 12 hours for a total of three times to get rid of all the salts.

### **Lypholization**

Collect the dialyzed sample into two 50mL falcon tubes

Freeze at -80 degrees

Lypholize to dryness

### **Delipidization**

Transfer the dried sample into a glass separatory funnel

Use MeOH to wash the tubes to make sure everything gets transferred.

Add 50mL 2:1 v:v  $\text{CHCl}_3$ : MeOH , shake a few minutes, and let it sit overnight

Remove the supernant

Add 50mL 1:1 v:v  $\text{CHCl}_3$ : MeOH , shake a few minutes, and let it sit overnight

Remove the supernant

Add 50mL 1:2 v:v  $\text{CHCl}_3$ : MeOH , shake a few minutes, and let it sit overnight

Remove the supernant

The sample was then dried at 55 degrees.

### **Hyaluronidase digestion**

The sample was re-dissolved into 40mL 20mM NaOAc, pH6.0, then add 30U of the following hyaluronidase.

37 degrees, incubate ON

### **Heparin lyases treatment**

Adjust to in 40 mM Tris, pH7.0, 4mM  $\text{CaCl}_2$

then added 50mU heparinase I, 50mU heparinase II, and 50mU heparinase III

37 egress, incubate, ON.

### **Hiprep DEAE**

Column: Hiprep DEAE 16/20

Column equilibration: 20mM NaOAc, pH6.0, 100mM NaCl

Load: 0.5~1mL/min by peristaltic pump

Wash: 60mL 20mM NaOAc, pH6.0, 100mM NaCl

Elute: HPLC flow rate: 1.5mL/min

A: 20mM NaOAc, pH6.0, 100mM NaCl

B: 20mM NaOAc, pH6.0, 1M NaCl

Gradient: 0-30min, 0%B

30-90min, 0~100% B linear gradient

90-120min, 100%B

120-130min, 100~0%B linear gradient

130-160min, 0%B

Combine fractions from 55 to 120min

### **EtOH precipitation**

Aliquotted the combined fractions into centrifuge tubes

Add 3 volume of EtOH saturated with NaOAc

4 degrees, ON (~16h)

Centrifugation, 4 degrees, >10,000 g, 30min

After removing the EtOH, the sample was dried at 55 degree oven

### **Finishing up**

Dissolve the pellet into 1.6mL ddH<sub>2</sub>O

Centrifugation, 15000rpm for 10min,

remove whatever precipitate that might show up.

Store the sample at -20 degrees

Yang Mao

## ANEXO II

### Protocol - CS purification using NHS column

NHS-activated HP columns are designed for covalent coupling of ligands containing primary amino group. Non-specific adsorption of proteins to HiTrap columns are negligible due to the hydrophobic properties of the base matrix.

#### **Buffers**

- Coupling Buffer: 0.2M NaHCO<sub>3</sub>, 0.5M NaCl, pH 8.3 (coupling can be done at a pH range of 6.5-9, but optimal coupling at pH8);
- Buffer A: 0.5M Ethanolamine (Light sensitive), 0.5M NaCl, pH 8.3;
- Buffer B: 0.1M Sodium Acetate, 0.5M NaCl, pH 4;
- PBS;
- Elution Buffer: 0.25M, 0.5M, 1M and 2M NaCl;
- Neutralization buffer: 1M HCl, pH 9.0;
- Storage buffer: 0.05M Na<sub>2</sub>HPO<sub>4</sub>, 0.1% NaN<sub>3</sub>, pH7.

#### **Protein Buffer Exchange using PD-10 Desalting Column**

##### 1. PD-10 Desalting column preparation

- Remove the top cap and pour off the column storage solution;
- Cut the sealed end of the column at notch.

##### 2. Column equilibration

- Fill up the column with equilibration buffer (=coupling buffer) and allow the equilibration buffer to enter the packed bed completely;
- Repeat 4 times;
- Discard the flow-through.

Note: about 25mL equilibration buffer should be used in total for all three steps.

##### 3. Sample application

- Add maximum 2.5 ml of protein (1.5mg protein) to the column;
- For sample volumes less than 2.5mL, add equilibration buffer to adjust the volume up to 2.5mL after the sample has entered the packed bed completely;
- Let the sample or equilibration buffer enter the packed bed completely;

- Discard the flow-through.

#### 4. Elution

- Place a test tube for sample collection under the column;
- Elute with 3.5mL buffer (=coupling buffer) and collect the eluate.

### **Up-concentration of the protein**

1. Add 1mL PBS to the 10.000MWO spin column (mark where is 1mL);
2. Spin for 5 minutes at 3700 rcf;
3. Remove PBS just before adding sample (do not allow column to dry!!);
4. Add protein and spin for 5 minutes at 3700 rcf each time, until volume lowers to 1mL (pipette up and down between each spin. If volume goes beneath 1mL, add coupling volume to a final volume of 1mL);
5. Check protein concentration in nano-drop.

### **Coupling of protein to column**

6. Place the NiTrap NHS-activated HP column (1mL) in an iron stand and place a glass underneath;
7. Remove the top cap of the column and apply a drop of ice cold 1mM HCl to the top of the column;
8. Screw on the supplied HiTrap luer connector (red with a hole going through);
9. Remove the snap-off end at the column out-let;
10. Wash out the isopropanol (storage buffer) with 6mL 1mM HCl, ice cold. Use a 2mL syringe and be sure not to exceed a flow-rate of 1mL/min (1/2 drop/sec). This can irreversibly damage the column. Remember this throughout the entire procedure;
11. Immediately inject 1mL of the protein solution onto the column;
12. Seal the column with parafilm and let it stand for 30 minutes at RT (or 4hours at 4C).

### **Washing and deactivation of the column**

13. Deactivate any excess active groups that have not coupled to the ligand and wash out the non-specifically bound protein by injecting:
  - 6mL Buffer A;
  - 6mL Buffer B;
  - 6mL Buffer A.
14. Seal the column and let it stand for 30 minutes at RT (or 4hours at 4C);
15. Wash column by injecting:

6mL Buffer B;  
6mL Buffer A;  
6mL Buffer B;  
4mL PBS

### **Purification**

16. Prepare samples to be purified:

- First we will purify the placental CS. We will dilute the samples to 2.5mL in PBS (if necessary). As for the Sigma CSA, we want to add 3mg into the column. So we will prepare 2mL of a 1.5mg/ml solution in PBS. Save 100ul of Input for each sample!
17. Inject sample Placental CS solution to the column using a 2mL syringe. Collect the run-through in a tube and re-run the sample 5 times. The last run-through is saved for later analysis;
18. Wash the column by injecting 10mL PBS. Collect the wash run-through for later analysis;
19. Elute the samples with 2.5mL of each of the elution buffers (0.25M, then 0.5M, then 1M and lastly 2M). For each elution buffer, collect the run-through in a tube for later analysis. Remember that the first 0.5mL are from the previous elution!!
20. Wash the column by injecting 10mL PBS in order to prepare the column for a new sample;
21. Repeat steps 2-5 with Sigma CSA;
22. In order to store the column, inject 5mL storage buffer.

### **Sample storage**

23. After the purification, add 5M NaCl to a final concentration of 0.5M NaCl to the 0.25M, wash and run-through samples;
24. Add 2 volumes of 96% EtOH to let the samples precipitate, either for 1hour at RT or O.N. +4C;
25. Spin samples at 10000 rcf, 30 minutes;
26. Remove supernatant and let pellet dry O.N.

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## ANEXO III

### Nature Protocol - Analysis of glycosaminoglycan-derived, precolumn, 2-aminoacridone-labeled disaccharides with LC-fluorescence and LC-MS detection

#### PROCEDURE

##### Enzyme treatment and production of GAG-derived disaccharides ● TIMING ~5–10 h

**1|** In contrast to GAG-derived oligosaccharides that are prepared on the basis of the partial controlled enzymatic degradation<sup>46</sup> (**Table 2**), the production of GAG-derived disaccharides relies on the exhaustive enzymatic depolymerization of the GAG chains using specific enzymes. Disaccharides of various structures bearing a different number of sulfo groups in various positions (**Table 1**) may be produced, depending on the substrate, the enzyme specificity and the site of action. The exhaustive action of GAG lyases<sup>47–49</sup> on GAGs catalyzes their  $\beta$ -eliminative cleavage to disaccharide (and oligosaccharides still resistant to their activity) products having a 4,5-unsaturated UA residue ( $\Delta$ UA) at their nonreducing ends. The resulting disaccharides, before or after fluorescent tagging, can be separated by various analytical approaches. Set up the reactions for HA/CS/DS treatment with chondroitin lyase ABC (option A), HS/Hep treatment with a cocktail of Hep lyases (option B) or HA/CS/DS and HS/Hep with both chondroitin lyase ABC and Hep lyases (option C). Multiple replicate samples ( $n \geq 3$ ) can be treated with a single or multiple options if the GAG composition is not known beforehand. It is very useful to include various GAG standards (**Table 1**) in amounts bracketing the concentrations of GAGs in the unknown samples, as measured by the carbazole assay<sup>78</sup>, as such as in known concentration for an accurate evaluation of the enzymatic treatment owing to the necessity to have an exhaustive and reproducible GAG depolymerization to generate disaccharides. The biological starting material is the lyophilized biological fluid, cell or tissue extract, prepared as described in Box 1 in ref. 46 ([http://www.nature.com/nprot/journal/v5/n6/box/nprot.2010.48\\_BX1.html](http://www.nature.com/nprot/journal/v5/n6/box/nprot.2010.48_BX1.html)) and in **Boxes 1** and **2** of this protocol.

##### **(A) HA/CS/DS treatment**

- (i) Add 0.1 mg of HA and CS and DS standard (in 100  $\mu$ l) to 700  $\mu$ l of 50 mM Tris/60 mM sodium acetate (pH 8.0) in a 1.5-ml centrifuge tube. Dissolve the lyophilized sample in 800  $\mu$ l of 50 mM Tris/60 mM sodium acetate (pH 8.0) in a 1.5-ml centrifuge tube.
- (ii) Add 50 mU of chondroitin lyase ABC (in 200  $\mu$ l) to the 1.5-ml centrifuge tube.

##### **(B) HS/Hep treatment**

- (i) Add 0.1 mg of HS and Hep standard (in 100  $\mu$ l) to 850  $\mu$ l of 50 mM sodium phosphate buffer (pH 7.0) in a 1.5-ml centrifuge tube. Dissolve the lyophilized sample in 850  $\mu$ l of 50 mM sodium phosphate buffer (pH 7.0) in a 1.5-ml centrifuge tube.
- (ii) Add 10 mU of Hep lyase I (10  $\mu$ l), 10 mU of Hep lyase II (20  $\mu$ l) and 10 mU of Hep lyase III (20  $\mu$ l) to the 1.5-ml centrifuge tube.

### (C) Simultaneous HA/CS/DS and HS/Hep treatment

- (i) Add 0.1 mg of each GAGs standard, HA and CS and DS and HS and Hep (in a total of 100  $\mu$ l), to 200  $\mu$ l of distilled water in a 1.5-ml centrifuge tube. Dissolve the lyophilized sample in 300  $\mu$ l of distilled water in a 1.5-ml centrifuge tube.
- (ii) Add 50 mU of chondroitin lyase ABC (200  $\mu$ l) to the 1.5-ml centrifuge tube.
- (iii) Incubate the reaction mixture in a water bath at 37 °C for 5 h.
- (iv) Heat the reaction mixture in a boiling water bath for 10 min.
- (v) Remove the denatured protein by centrifugation at 12,000g for 10 min at room temperature and recover the supernatant.
- (vi) Add 450  $\mu$ l of 50 mM sodium phosphate buffer (pH 7.0).
- (vii) Add 10 mU of Hep lyase I (10  $\mu$ l), 10 mU of Hep lyase II (20  $\mu$ l) and 10 mU of Hep lyase III (20  $\mu$ l).

- 2| Incubate the reaction mixture in a water bath at 37 °C for 5 h.
- 3| Heat the reaction mixture in a boiling water bath for 10 min to inactivate the enzyme and to stop the reaction.
- 4| Remove the denatured protein by centrifugation at 12,000g for 10 min at room temperature. Use the supernatant for derivatization with AMAC and disaccharide characterization by HPLC-FD-ESI-MS.  
■ **PAUSE POINT** Samples may be stored at 4 °C for 1–2 d or at –20 °C for long-term storage before further AMAC derivatization. The samples generated via Step 1A, 1B and 1C are referred to as sample types A, B and C, respectively.

### Fluorotagging GAG-derived disaccharides (and residual oligosaccharides) by AMAC ● **TIMING** ~9–10 h

- 5| Completely freeze-dry all of the solution before derivatization. About 5 h is sufficient enough to freeze-dry 1 ml of solution.  
? **TROUBLESHOOTING**

- 6| Add 5  $\mu$ l of 0.1 M AMAC in glacial acetic acid:DMSO 3:17 (vol/vol) solution per estimated lyophilized  $\mu$ g of disaccharide or oligosaccharide standard, or sample based on the carbazole micro-assay.
- 7| Incubate the above reaction solution at room temperature for 15 min.
- 8| Add 1 M NaBH<sub>3</sub>CN solution to the reaction solution.
- 9| Briefly centrifuge at 10,000g for 1 min at room temperature.
- 10| Incubate the reaction mixture at 45 °C for 4 h.
- 11| Make up the reaction solution to ~ 200 ng/ $\mu$ l with DMSO-ultrapure water at a 1:1 ratio (vol/vol).  
? **TROUBLESHOOTING**

### GAG disaccharide analysis and characterization by RP-HPLC on-line with FD and ESI-MS ● **TIMING** ~1–2 h

▲ **CRITICAL** Steps 13 and 14 describe how to set up the HPLC-MS instrument for analysis of the disaccharide standards and for the disaccharides derived from the samples (types A, B and C) generated in Step 3. Both disaccharide standards and disaccharides derived from the samples are separately derivatized with AMAC in Steps 6–11 by HPLC-FD-ESI-MS. After conditioning, the system is ready to perform sample separation (Steps 15–18).

- 12| Equilibrate the appropriate HPLC or UPLC column for 20 min with the corresponding ratio of ammonium acetate to acetonitrile/methanol, as shown in the table below:

	HA/CS/DS (sample type A)	HS/Hep (sample type B)	HA/CS/DS and HS/Hep (sample type C)
Column	C18 column X-Bridge Shield	Discovery C18	Acquity UPLC BEH C18 column
Ammonium acetate (vol%)	60 mM, pH 5.6, 98%	60 mM, pH 5.6, 95%	80 mM, 88%
Acetonitrile (vol%)	2%	5%	—
Methanol (vol%)	—	—	12%
Flow rate	1.0 ml/min	1.0 ml/min	0.1 ml/min

**13|** Equilibrate the electrospray interface on-line with HPLC (sample types A and B) by flushing it with conditioning solvents at the related flow rates for 30 min, as previously reported. Set the mass analyzer in negative ionization mode with the skimmer potential set at  $-43.2$  V, capillary exit set at  $-120.4$  V and temperature set at  $350$  °C in full-scan spectra ( $100$ – $2,000$  Da, ten full scans per s) with a maximum accumulation time of 300 ms and an ion charge control (ICC) target of 20,000. Set nitrogen at 12 liters/min and nebulizing gas at 4.14 bars. Equilibrate the electrospray interface on-line with UPLC (sample type C) by flushing with conditioning solvents at the related flow rates for 30 min. Set the mass analyzer in negative ionization mode with the skimmer potential set at  $-40.0$  V, capillary exit set at  $-40.0$  V and a temperature of  $350$  °C in full-scan spectra ( $150$ – $1,200$  Da, ten full scans/s). Set nitrogen at  $8$  liters  $\text{min}^{-1}$  and nebulizing gas at 2.76 bars.

**14|** Inject GAG disaccharide standard at a concentration of about  $0.2$ – $0.5$   $\mu\text{g}$ , and then inject samples at a concentration of about  $1$ – $2$   $\mu\text{g}$  onto the injection loop by using a Hamilton syringe.

**15|** Elute the disaccharides with the appropriate gradient, as shown in the table below:

	CS/DS/HA (sample type A)	HS/hep (sample type B)	CS/DS/HA and HS/hep (sample type C)
Column	C18 column X-Bridge Shield	Discovery C18	Acquity UPLC BEH C18 column
Solvent A	60 mM ammonium acetate, pH 5.6	60 mM ammonium acetate, pH 5.6	80 mM ammonium acetate
Solvent B	Acetonitrile	Acetonitrile	Methanol
Equilibrate solution	2% solvent B	5% solvent B	12% solvent B
Gradient	2–30% solvent B over 50 min	5–50% solvent B over 65 min	12% solvent B for 5 min, 12–15% solvent B over 5–15 min, 15–30% solvent B over 15–30 min, 30–100% solvent B over 30–60 min

**16|** Monitor the eluent by using the on-line FD detector at excitation  $\lambda = 428$  nm and emission  $\lambda = 525$  nm; gain is set at 100 for unsaturated disaccharides derivatized with AMAC. Simultaneously monitor the eluent by using the on-line mass detector setup in series.

**17|** Identify the disaccharide peaks based upon the elution order and by using available standards and/or by means of ESI-MS characterization (see, for example, **Fig. 1**).

#### ? TROUBLESHOOTING