



**Mass spectrometric investigation into glycomes implicated in  
human reproductive biology**

A thesis submitted for the degree of Doctor of Philosophy at Imperial College London

Submitted by

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# Abstract

Glycosylation is a complex biological process where enzymes attach glycans to proteins, altering their structure and function. In reproduction, glycoproteins support sperm immunoprotection, modulate sperm-oocyte interactions, and influence implantation. Glycan sequences on cell surfaces, such as uterine epithelial cells, facilitate cell-to-cell communication, playing roles in both normal physiology and pathological conditions. Understanding glycan structures and functions enhances our knowledge of glycosylation in reproduction and its potential therapeutic and diagnostic applications.

This project examined the differential glycosylation of human chorionic gonadotrophin (hCG) in normal and abnormal pregnancies, as well as across pregnancy stages. Additionally, we analysed glycomic profiles of normal endometrial tissue and tissue affected by endometriosis. Structural characterisation of glycans was performed using Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS), Tandem Mass Spectrometry (MS/MS), and enzymatic digestion. Enzymatic digestion allowed for the breakdown of glycans into smaller components, facilitating their structural identification.

Our findings reveal that  $\beta$ hCG N-glycans exhibit high mannose, bi-, tri-, and tetraantennary structures. The novel identification of bisecting N-glycans in  $\beta$ -hCG, alongside their increased expression in late pregnancy, suggests a role in immune defence, possibly supporting immune tolerance. The data confirm that hCG undergoes differential glycosylation throughout pregnancy and that abnormal glycosylation is linked to pathological pregnancies.

Analysis of endometrial tissue N-glycans showed that endometriosis is associated with altered glycan profiles, likely due to hyposialylation. The N-glycan profiles presented here allow for direct comparisons between normal and pathological states. Changes in glycan composition and structure, particularly those with immunomodulatory potential, may serve as targets for non-surgical endometriosis management through diagnostic and therapeutic strategies.

## **Thesis Publications**

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Insights into the hyperglycosylation of human chorionic gonadotropin revealed by glycomics analysis. PLoS ONE 15(2): e0228507. <https://doi.org/10.1371/journal.pone.0228507>

## **Statement of Originality**

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## Abbreviations

### Abbreviations

|          |                                                                                 |
|----------|---------------------------------------------------------------------------------|
| AMBIC    | Ammonium bicarbonate                                                            |
| ART      | Assisted reproductive treatment                                                 |
| Asn      | Asparagine                                                                      |
| ATP      | Adenosine triphosphate                                                          |
| CA125    | Cancer antigen 125                                                              |
| CHAPS    | 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate                      |
| CHM      | Complete hydatidiform mole                                                      |
| CI       | Chemical Ionisation                                                             |
| CID      | Collision induced dissociation                                                  |
| CRD      | Carbohydrate recognition domains                                                |
| DC-SIGN  | Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin |
| DHB      | 2,5-dihydroxybenzoic acid                                                       |
| DMSO     | Dimethylsulphoxide                                                              |
| DNA      | Deoxyribonucleic acid                                                           |
| Dol-PP   | Dolichol pyrophosphate                                                          |
| DTT      | Dithiothreitol                                                                  |
| EDTA     | Ethylenediaminetetraacetic acid                                                 |
| EI-MS    | Electron Impact Mass Spectrometry                                               |
| EP-hCG   | Early pregnancy hCG                                                             |
| ER       | Endoplasmic Reticulum                                                           |
| ESI      | Electrospray ionisation                                                         |
| FAB      | Fast Atom Bombardment                                                           |
| FSH      | Follicle stimulating hormone                                                    |
| Fuc      | Fucose                                                                          |
| Gal      | Galactose                                                                       |
| GalNAc   | N-acetylgalactosamine                                                           |
| GBP      | Glycan-binding protein                                                          |
| GC-MS    | Gas Chromatography Mass Spectrometry                                            |
| GdA      | Glycodelin-A                                                                    |
| Glc      | Glucose                                                                         |
| GlcNAc   | N-acetylglucosamine                                                             |
| GlcNAc-T | N-acetylglucosamine transferase                                                 |
| GnRH     | Gonadotrophin-releasing hormone                                                 |
| GTD      | Gestational trophoblastic disease                                               |
| GTD-hCG  | Gestational trophoblastic disease-hCG                                           |
| GTN      | Gestational trophoblastic neoplasia                                             |
| HCA      | Hierarchical cluster analysis                                                   |
| hCG      | human chorionic gonadotropin                                                    |
| Hex      | Hexose                                                                          |
| HLA      | Human leukocyte antigens                                                        |
| HPG      | Hypothalamic-Pituitary-Gonadal Axis                                             |
| IAA      | Iodoacetic acid                                                                 |
| IL       | Interleukin                                                                     |

|            |                                                   |
|------------|---------------------------------------------------|
| IVF        | In vitro fertilisation                            |
| LacdiNAc   | N, N'-diacetyllactosamine                         |
| LacNAc     | N-acetyllactosamine                               |
| LC-MS      | Liquid chromatography                             |
| LH         | Luteinizing hormone                               |
| LP-hCG     | Late pregnancy hCG                                |
| m/z        | Mass to charge ratio                              |
| MAG        | Myelin-associated glycoprotein                    |
| MALDI      | Matrix Assisted Laser Desorption Ionisation       |
| MAN        | Mannosidase                                       |
| Man        | Mannose                                           |
| MBL        | Mannose-binding lectin                            |
| MHC        | Major histocompatibility complex                  |
| ml         | Millilitre                                        |
| MS         | Mass spectrometry                                 |
| MS/MS      | Tandem Mass Spectrometry                          |
| NeuAc      | N-acetylneuraminic acid                           |
| NeuGc      | N-glycolylneuraminic acid                         |
| NK         | Natural Killer cells                              |
| PCOS       | Polycystic ovary syndrome                         |
| PET        | Pre-eclamptic                                     |
| PNGase F   | Peptide N-glycosidase F                           |
| RER        | Rough endoplasmic reticulum                       |
| SDS-PAGE   | Sodium Dodecyl Sulphate PolyAcrylamide Gel        |
| Ser        | Serine                                            |
| Thr        | Threonine                                         |
| TM         | Transmembrane region                              |
| TOF        | Time-of-flight                                    |
| TRIS       | Tris(hydroxymethyl)aminomethane                   |
| UDP-GlcNAc | Uridine Diphosphate-activated N-acetylglucosamine |
| uNK        | Uterine natural killer cells                      |
| VEGF       | Vascular endothelial growth factor                |
| WFA        | Wisteria Floribunda agglutinin                    |

# 1 Chapter 1- Introduction

## 1.1 Glycobiology

Glycobiology is the study of the structure, biosynthesis, biology, and evolution of saccharides (sugar chains or glycans) that are widely distributed in nature in all life forms(1). Glycosylation is an enzymatic reaction that attaches glycans to proteins, lipids, or other organic molecules(2). It is estimated that the human genome consists of approximately 25,000 genes. Considering the complexity of the human body, this seems quite small. The human proteome is a lot more complex with single genes encoding for multiple proteins. Up to 80% of proteins encoded by the human genome are glycoproteins(2). Glycobiology is therefore vital as it looks at post-translational modifications that have a substantial impact on protein complexity. Glycosylation is a key feature of all living cells and glycosylation of proteins is recognised as one of the most important forms of post-translational modifications, affecting protein folding, conformation, distribution, stability, and activity as well as providing essential functional biodiversity by generating a wide range of phenotypes from a limited genotype(3). For example, the type and extent of glycosylation may differentially regulate the activity of a single gene product and play important roles in protein-protein interactions. The glycan profile of a cell or tissue type can reflect its inherent state of health(4). It is therefore interesting to study in this thesis the differential glycosylation of glycoproteins implicated in the reproductive system, which can help us better understand human reproduction and the pregnancy state.

Our understanding of the importance and role of glycobiology has expanded enormously in the last two decades. We know already that glycan recognition is a critical part of sperm-egg binding(5-8), with recent research identifying binding targets through Mass spectrometry (MS)

techniques(6, 9). There is also evidence that antibodies in uterine fluid can destroy sperm and the female immune response against sperm can cause reproductive incompatibility between individuals with mismatched cell-surface glycans(10). Once fertilisation has occurred, the process of implantation and placental function has been shown to involve glycans and glycan-binding proteins(11, 12). Many causal factors of infertility and early pregnancy loss have been implicated; defects in reproductive glycans and their glycosylation could account for a portion of unexplained reproductive failures, as well as abnormal cytotrophoblast invasion, associated with pregnancy complications such as preeclampsia(13).

This thesis focuses on robust structural characterisation of glycans involved in the human reproductive system using MS. Whilst this project cannot present a full comprehensive analysis of all glycans implicated in human reproduction, it focuses on a key reproductive glycoprotein, hCG, pivotal to pregnancy, as well as endometrial cell glycans, which are potentially involved in implantation, and on glycans in follicular fluid. A thorough structural analysis and report of these glycoproteins allows for a better understanding of the relationship between structure and function forming a basis for future modulation for beneficial effects.

## 1.2 Glycoproteins

Glycoproteins are synthesised by the covalent attachment of oligosaccharide chains (glycans) to amino acid residues of a protein molecule. This synthesis occurs in the endoplasmic reticulum and Golgi apparatus both co-translationally and post-translationally. Glycosylation in eukaryotic cells is mainly described by the nature of the linkage to the protein. Major types of protein glycosylation include N-linked glycans attached through the nitrogen atom of asparagine and O-linked glycans attached through an oxygen atom from serine or threonine(1). Fundamental to understanding the

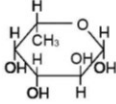
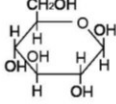
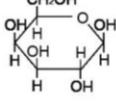
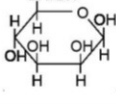
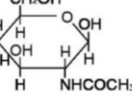
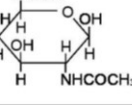
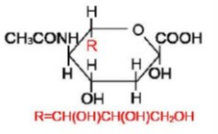
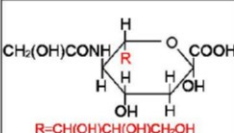
interaction between glycans and specific glycan binding proteins and establishing the roles glycans play in health and disease, is the determination of the glycan structure. The experimental work in this thesis focuses on human glycosylation in the reproductive tract.

A glycome is defined as the entire glycan population in the system being studied. The term glycomics is used to describe experimental approaches to studying the structure and function of glycomes. Glycomics can involve a variety of technologies including mass spectrometry. MS is the method of choice for defining the primary structures of glycans. It is able to characterise individual components within a complex mixture(14). The interpretation of MS data is based on knowledge of mammalian glycan biosynthetic pathways. Therefore, the formation and modification of N- and O-glycans will be described further in this chapter. Subsequent sections provide background information on the role glycans play in reproduction, paying particular attention to glycomic studies of hCG, endometrial tissue and follicular fluid.

### 1.2.1 Glycans

Glycans are compounds consisting of a series of glycosidically linked monosaccharides. The monosaccharides are represented by sugar symbols (15) depicted in Table 1-1. Linkage details can be added to the line connecting the symbols if required. All cells in nature are covered with a complex array of sugar chains, termed the glycocalyx(16). Glycans play a key role in communication between cells and their external environment.

Table 1-1: Commonly occurring monosaccharides in mammalian N- and O-glycans. Table shows the symbol nomenclature for graphical representations of glycans as well as the chemical structure and permethylated residue mass of each monosaccharide.

| Common name                                      |                                                   | Abbreviation | Symbolic representation                                                             | Permethylated residue mass | Chemical structure                                                                   |
|--------------------------------------------------|---------------------------------------------------|--------------|-------------------------------------------------------------------------------------|----------------------------|--------------------------------------------------------------------------------------|
| Deoxyhexose<br>$C_6H_{12}O_5$                    | Fucose                                            | Fuc          |    | 174                        |    |
| Hexose<br>(Hex)<br>$C_6H_{12}O_6$                | Glucose                                           | Glc          |    | 204                        |    |
|                                                  | Galactose                                         | Gal          |    |                            |    |
|                                                  | Mannose                                           | Man          |    |                            |    |
| N-acetylhexosamine<br>(HexNAc)<br>$C_8H_{15}O_6$ | N-acetylglucosamine                               | GlcNAc       |    | 245                        |   |
|                                                  | N-acetylgalactosamine                             | GalNAc       |  |                            |  |
| Sialic acid                                      | N-acetylneuraminic acid<br>$C_{11}H_{19}O_9$      | NeuAc        |  | 361                        |  |
|                                                  | N-glycolylneuraminic acid<br>$C_{11}H_{19}O_{10}$ | NeuGc        |  |                            |  |

#### 1.2.1.1 N-glycans

N-glycans share a pentasaccharide structure, the core, as shown in

Figure 1-1. This contains two N-acetylglucosamine and three mannose residues. N-glycans are grouped into three main classes, high mannose, hybrid, and complex types. The initial biosynthesis of N-glycans involves several enzymatic reactions occurring on both sides of the endoplasmic

reticulum (ER). Polypeptide chains of glycoproteins are synthesised by ribosomes on the surface of the rough endoplasmic reticulum (RER). The N-glycan pentasaccharide core is initially constructed on the dolichol pyrophosphate (Dol-PP) on the cytoplasmic side of the ER. This initial stage of N-glycan biosynthesis involves the sequential addition of activated monosaccharides to Dol-PP(17), forming  $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$ . As the protein chain extends, the  $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$  lipid-intermediate is simultaneously translocated into the lumen of the ER by a flippase. The remaining four mannose and three glucose residues are then added sequentially. In the final step, the resulting  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  is transferred en bloc to the polypeptide chain Figure 1-2.

Figure 1-1: N-linked oligosaccharides contain a common pentasaccharide core depicted above, containing two N-acetylglucosamine and three mannose residues. The reducing terminal N-acetylglucosamine residue is linked to the amide group of an asparagine residue in the amino acid sequence  $\text{Asn-X-Ser/Thr}$ , where X can be any amino acid except Proline. N-glycans are grouped into three main types based on their structure: high mannose, complex, and hybrid types.

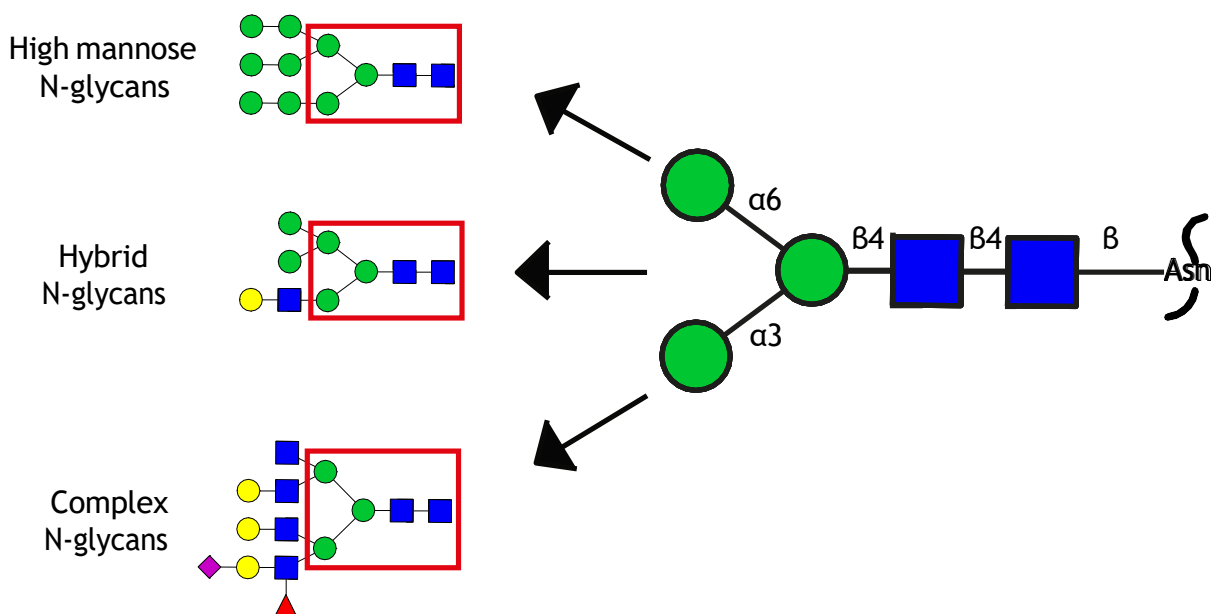
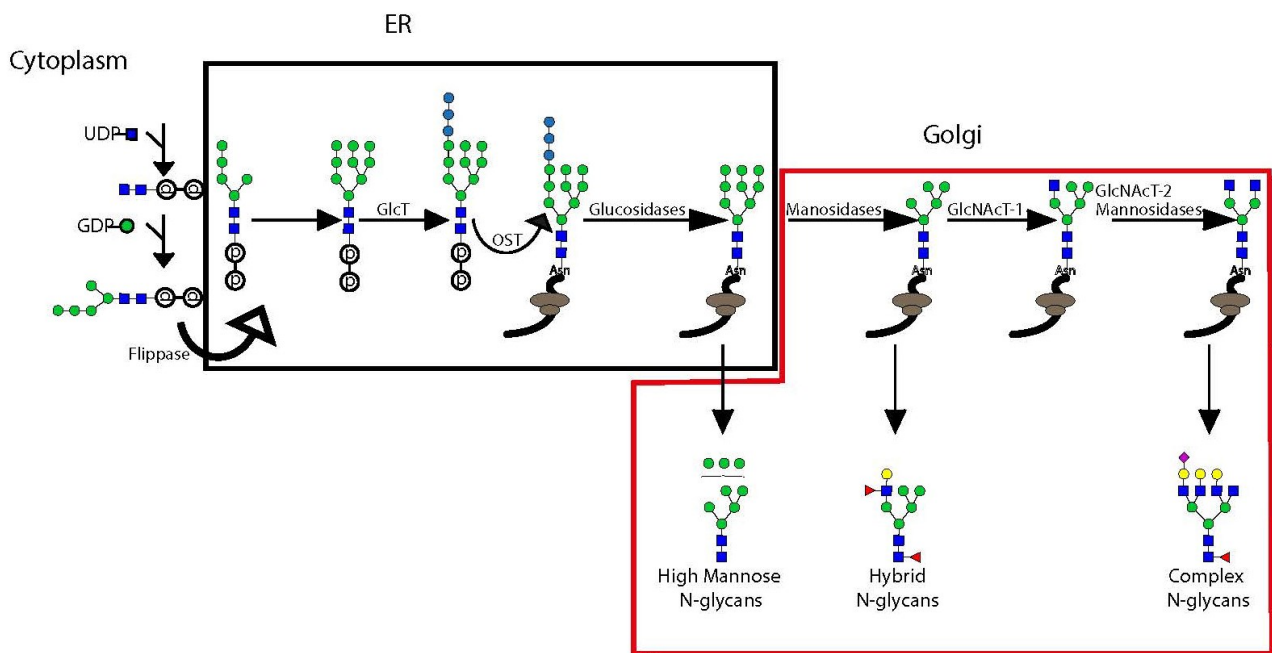


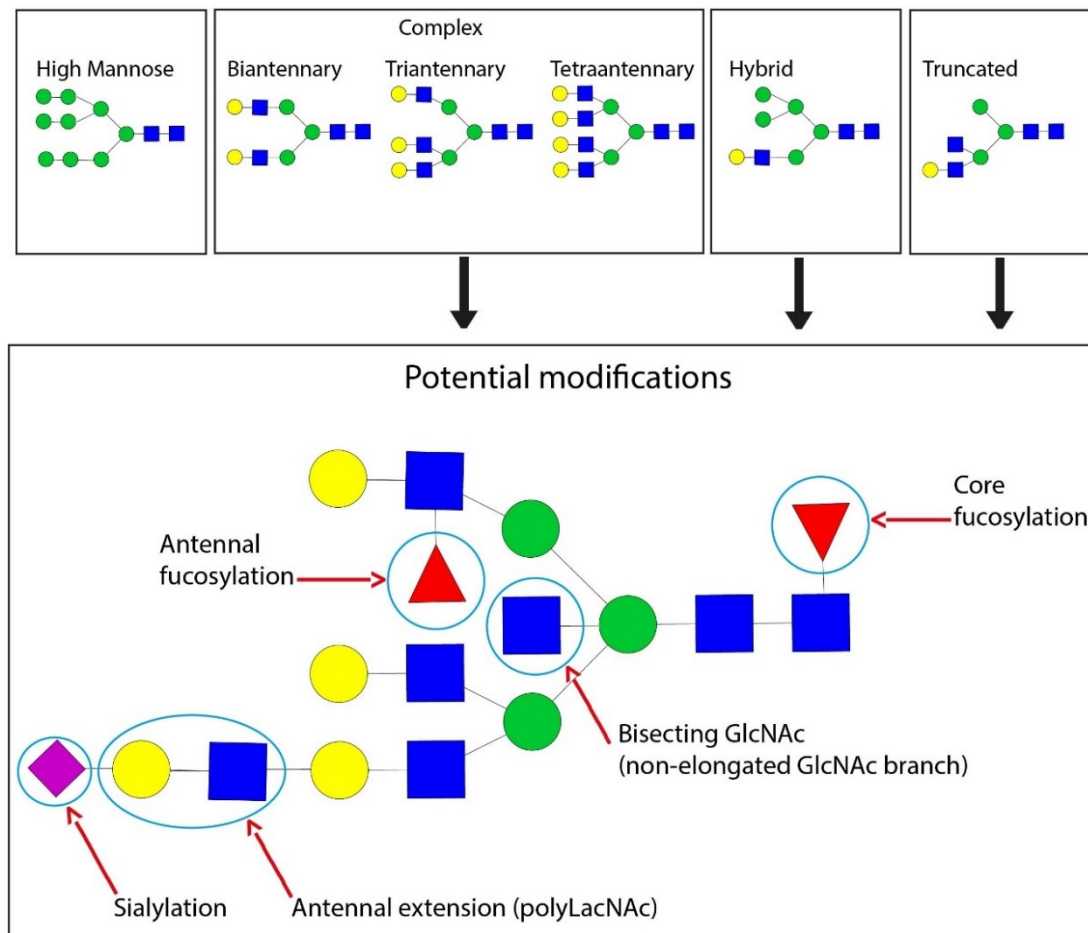
Figure 1-2: Schematic representation of the biosynthetic pathway for N-glycans. The biosynthesis of N-glycans begins with the transfer of a nucleoside activated N-acetylglucosamine (GlcNAc) to a dolichol membrane anchor (Dol-P-P). A fourteen-sugar precursor structure is created by elongation, in the ER lumen. This structure is then mass-transferred to an Asn residue of a newly synthesized protein within the ER lumen. Further processing is by glucosidases to remove glucose, and then by ER mannosidase I (MAN1) to remove mannose residues. Mannosidase II trims two Man residues from the intermediate to form the core GlcNAcMan3GlcNAc2 precursor of complex N-glycans. Lack of trimming by mannosidase II results in hybrid structures. Inhibition of mannosidase II results in high mannose type of N-glycans. Complex glycans are created in the Golgi by further cleavage of glycoproteins by mannosidase II (MAN2), then a series of transferases add GlcNAc, galactose, and other sugar residues to create complex N-glycans.



After the transfer to the polypeptide, a cascade of enzymatic reactions trims the N-glycan chain in the ER and Golgi compartments. Firstly, the three glucose residues (one  $\alpha$ 1,2-linked glucose and two  $\alpha$ 1,3-linked glucose) are removed by the action of glucosidases. This is followed by the action of ER  $\alpha$ -mannosidase I to remove the terminal  $\alpha$ 1-2Man from the central arm of Man<sub>9</sub>GlcNAc<sub>2</sub> to give a Man<sub>8</sub>GlcNAc<sub>2</sub> isomer. Further trimming by  $\alpha$ -mannosidase in the cis-Golgi results in Man<sub>5</sub>GlcNAc<sub>2</sub>, a crucial intermediate for hybrid and complex N-glycans. The action of subsequent glycosyltransferases and glycosidases determine whether complex or hybrid glycans are formed.

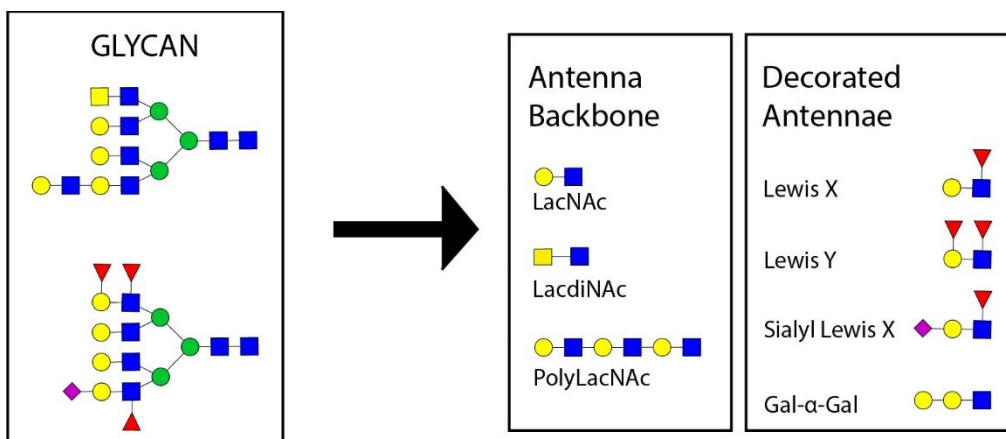
Not all N-glycans are fully trimmed, therefore resulting in between five and nine mannose residues, high mannose N-glycans(1), Figure 1-2. Processing of the N-glycan into the 3 major classes occurs in the Golgi. Complex and hybrid N-glycans can be modified by monosaccharide addition to the core, antennal extension, and capping of elongated branches as shown in **Error! Reference source not found..** In mammalian N-glycans the main core modification is the addition of fucose in  $\alpha$ 1-6 linkage to GlcNAc.

Figure 1-3: Enzymatic modification of N-glycans in the Golgi. Complex and hybrid N-glycans can be modified by monosaccharide addition to the core, antennal extension, and capping of elongated branches, depicted here.



The high mannose glycans are derived by limited processing and contain only  $\alpha$ -linked mannose residues in addition to the core. Complex glycans contain sugars on the 3-arm and 6-arm of the core, which are extended by the action of various glycotransferases to form bi-, tri- and tetra-antennary structures. Hybrid glycans have features of both high mannose and complex glycans, containing  $\alpha$ -mannose residues on the 6-arm and complex structures on the 3-arm. Complex and hybrid glycans undergo a final stage in their biosynthesis called capping. The main capping sugars are illustrated in **Error! Reference source not found..** These modifications are regulated biosynthetically. The capping structures act as ligands for lectins.

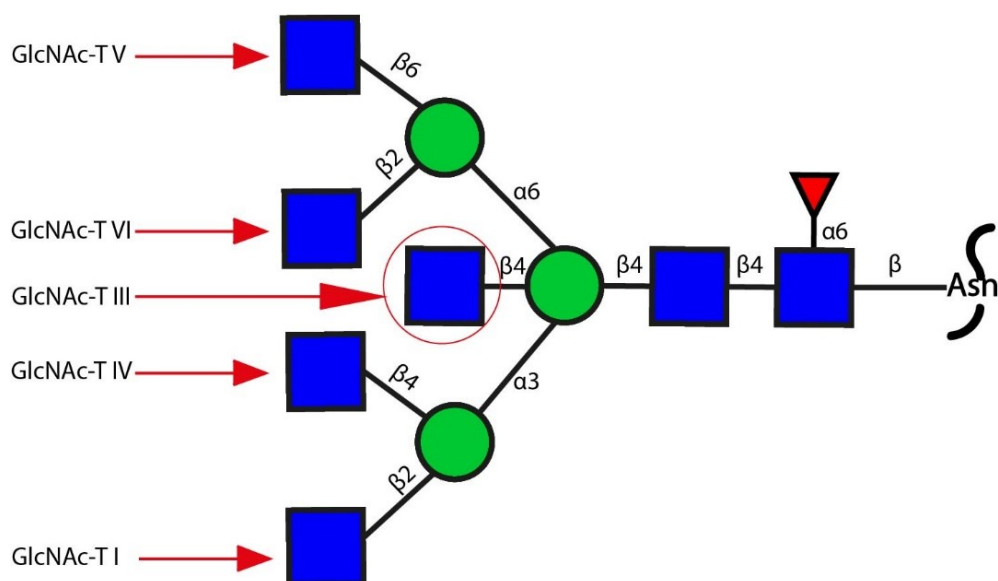
Figure 1-4: Main capping sugars are illustrated here. These modifications are regulated biosynthetically. The capping structures act as ligands and receptors recognised by lectins.



The biosynthesis of hybrid and complex glycans is initiated by GlcNAc-transferase I (GlcNAc-T-I) attaching GlcNAc in a  $\beta$  1-2 linkage to the  $\alpha$ 1-3 linked core Man in  $\text{Man}_5\text{GlcNAc}_2$  (Figure 1-2). The presence of the  $\beta$ 1-2 linked GlcNAc residue at the non-reducing end of the 3-arm is essential for subsequent action of other enzymes in the biosynthetic pathway.  $\alpha$ -Mannosidase II is responsible for trimming the  $\alpha$  3- and  $\alpha$  6-linked mannoses to allow the addition of a GlcNAc residues to the 2-position of the  $\alpha$ 1-6 core mannose by GlcNAc-T II, forming the basis for bi-antennary complex N-

glycans (Figure 3). Additional antennae are formed by the action of various GlcNAc-transferase (**Error! Reference source not found.**) which catalyse the attachment of GlcNAc to precise positions on the N-glycan core (18). A GlcNAc attached to the 4-position of the  $\beta$ -linked core Man by GlcNAc-T III is considered as a bisecting structure (**Error! Reference source not found.**). It is not considered an antenna and cannot be extended. The addition of this GlcNAc requires the previous action of GlcNAc-T I.

Figure 1-5: Highlighted below are the major enzymes involved in branching of complex N-glycans. Important to note here is the presence of the  $\beta$ 1-2 linked GlcNAc residue at the non-reducing end of the 3-arm is essential for subsequent action of other enzymes in the biosynthetic pathway. A GlcNAc attached to the 4-position of the  $\beta$ -linked core Man by GlcNAc-T III is considered as a bisecting structure.

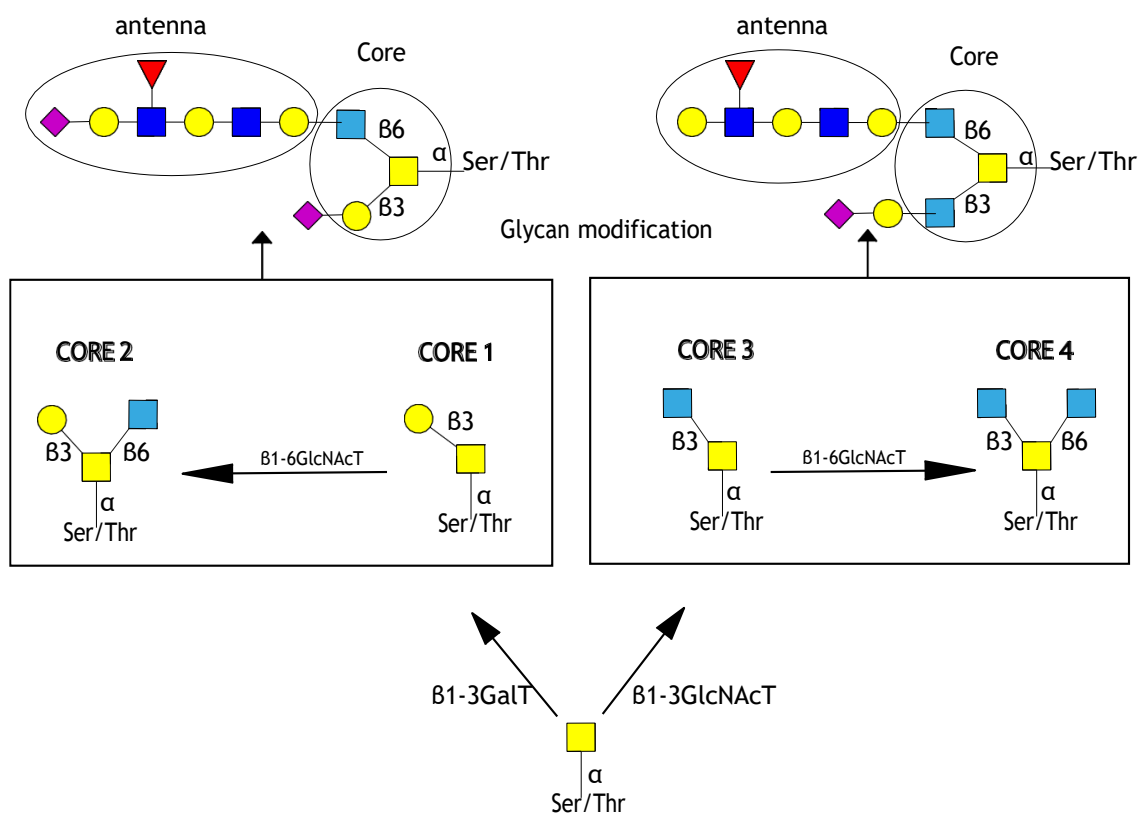


### 1.2.2 O-glycans

In this thesis, O-glycosylation refers to the attachment of monosaccharides to the oxygen atom of serine or threonine residues in a protein. Most mammalian O-linked glycans are formed by the transfer of GalNAc from UDP-GalNAc by a polypeptide N-acetylgalactosaminyltransferase (GalNAc

transferase) to serine or threonine residues in the protein chain. This is commonly referred to as mucin-type O-glycosylation. O-glycan biosynthesis is a multistep process occurring primarily in the Golgi apparatus and involves a series of glycosyltransferase enzymes (19). O-GalNAc is also termed the Tn-antigen. No amino acid consensus sequence is known for O-glycosylation although proline is usually present close to the glycosylation site. O-glycans are less branched than most N-glycans and are commonly biantennary(1). O-glycans can be divided into the core and antennae regions. Up to 8 mucin-type core structures have been identified (20). There are four common subtypes (Core 1-4) based on differential monosaccharide linkage reactions to the protein linked GalNAc (1). The biosynthesis of O-glycans is depicted in Figure 1-6.

Figure 1-6: Biosynthetic pathway for mucin type O-glycans. Biosynthesis of mucin type O-glycans is characterized by a stepwise sugar addition that occurs in the Golgi apparatus and involves a broad array of enzymes. It is initiated by the transfer of N-acetylgalactosamine (GalNAc) to Ser or Thr (S/T) of membrane-bound or secreted protein targets. The addition of other sugars in a gradual manner results in the formation of the common core O-glycan structures, core 1, core 2, core 3, and core 4.



Core 1 is formed by the addition of galactose in a  $\beta$ 1-3 linkage to GalNAc by the action of Core 1  $\beta$ 1-3 galactosyltransferase. Core 2 requires the Core 1 structure and is formed by the addition of GlcNAc to GalNAc of the Core 1 structure in a  $\beta$ 1-6 linkage. This is catalysed by one of several Core 2  $\beta$ 1-6 N-acetylglucosaminyltransferases. Additional biosynthesis of Core 1 and 2 can result in O-glycans bearing fucosylated and sialylated lactosamines. Core 3 is formed by the addition of GlcNAc in a 1-3 linkage to GalNAc. Core 3 may serve as a substrate for Core 4, where GlcNAc is added in a  $\beta$ 1-6 linkage to GalNAc. The formation of Core 3 and 4 is controlled by the activity of Core 3 GlcNAcT and Core 4 GlcNAcT respectively. Further biosynthesis can yield biantennary forms. O-glycans may vary in size from a single GalNAc residue to extended long chains, whose size can be comparable to complex N-glycans(21). Cores 5-8 are rare and are not discussed here. O-glycan biosynthesis can be modified and terminated with the addition of sialic acid residues (22). These sialic acid additions give rise to a series of O-glycan structures that generally restrict further biosynthetic steps (22).

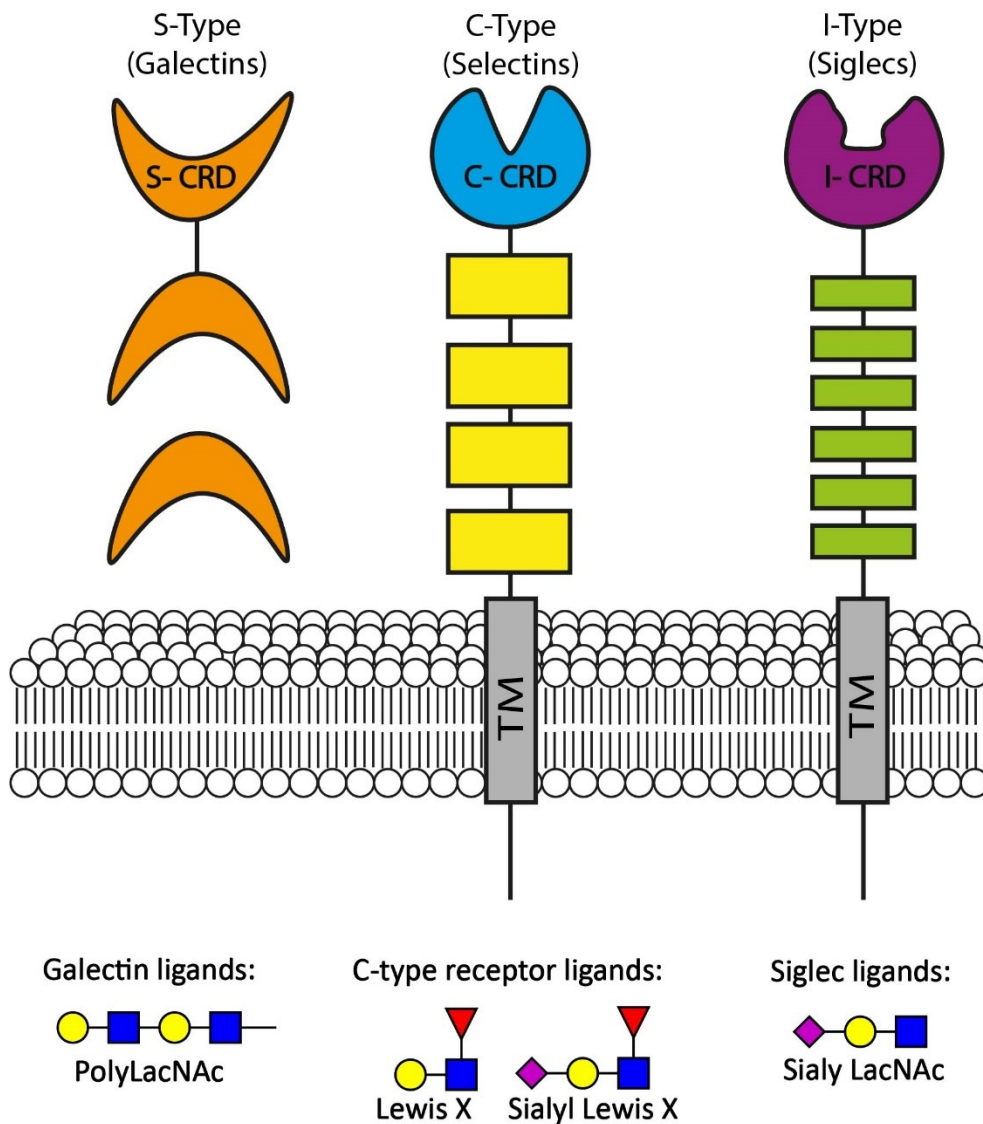
O-glycans have been implicated in the formation of the ABO blood group antigens, a system that has clinical relevance in blood transfusions. Various pathogens have also been reported to use oligosaccharide structures found on cell surface O-glycans as receptors for binding and infection of the host. O-glycans have been reported to function in sperm-egg binding later described in this chapter. Also later discussed in this chapter is the commonly referenced 'hyperglycosylated' isoforms of hCG and the clinical utility of its measurement. This is important to mention here as measurements of this 'hyperglycosylated' isoforms of hCG were all made using a unique antibody designated B152 (23). Antibody B152 has been mapped to a linear epitope around a single O-glycan on the beta-subunit of hCG hence the antibody can only measure isoforms of hCG that possess a core 2 type O-glycan. The Core-2 type O-glycan at Ser-121 on hCG has been previously

reported (24, 25). The core 2 isoforms are also of important use in cancer diagnosis and monitoring since their concentration appears to correlate with malignancy (23, 25)

### 1.3 Lectins

Lectins are glycan-binding proteins (GBP) that are highly specific for sugar moieties. GBP are found in all living things and their biological functions include trafficking, targeting and clearance of proteins; cell recognition and adhesion; and immune responses. Lectins mostly recognise the capping sugars on a glycan, mediating the glycan's biological function. The interaction between lectins and carbohydrates are comparable to enzyme-substrate or antigen-antibody interaction. The glycan-binding activity is determined by the independently folding domain of the polypeptide, specifically involved in binding to carbohydrate, referred to as the carbohydrate-recognition domains (CRDs). Lectins are classified by the structures of the CRDs they contain. Representative structures of common animal lectins are depicted in Figure 1-7. For the purposes of this thesis, the main lectins of interest are galectins, C-type lectins and siglecs.

Figure 1-7: Representative structures of common animal lectin families and examples of relevant carbohydrate ligands. Immune cells express lectin receptors such as galectins, C-type lectins and siglecs to name a few. They recognize glycan structures present on pathogens or host cells. Examples of capping sugars on a such glycan structures recognized by galectins, C-type lectins, and siglecs are shown here. CRD = Carbohydrate-recognition domain; S-CRD = S-type lectin CRD; C-CRD = C-type lectin CRD; I-CRD = I-type lectin CRD; TM = Transmembrane region



Galectins are one of the most widely expressed family of lectins (26). They bind  $\beta$ -galactosides (27). Galectins play an important role in development and tissue regeneration, cell apoptosis, cell-cell and cell-matrix interaction, cancer, and immune response (28, 29).

C-type lectins are the most diverse group of lectins. They have a characteristic calcium dependent CRD. This is found in over 1,000 proteins of diverse function and includes both soluble and membrane-bound proteins (30). They are grouped into subclasses based on the number and arrangement of the CRD. They include selectins, collectins, DC-SIGN and NK-cell receptors to name a few. Selectins are the best characterised and are involved in leukocyte trafficking (31). The three known selectins are L-selectin, E-selectin, and P-selectin. The three mammalian selectins are type-1 transmembrane glycoproteins that are expressed on platelets, endothelial cells, and leukocytes, hence the origin of their names (1). L-selectin is expressed constitutively on lymphocytes, E-selectin is expressed by activated endothelial cells, and P-selectin is expressed by activated platelets and endothelial cells (32). Selectins recognise sialyl Lewis X (SLe<sup>x</sup>) and its isomer sialyl Lewis a (SLe<sup>a</sup>).

Collectins play a role in innate immunity. Nine different collectins have been identified which include mannose-binding lectin (MBL). Binding of MBL and other collectins initiates the lectin pathway of complement activation(1). Collectins bind mannose, GlcNAc, glucose, N-acetylmannosamine and fucose residues but do not recognise galactose.

Siglecs are a family of sialic-acid-binding immunoglobulin-like lectins that are thought to promote cell-cell interactions and regulate the functions of cells in the innate and adaptive immune systems through glycan recognition (33). They bind to sialylated glycans. Siglecs are grouped into two subsets based on their sequence similarity and evolutionary conservation; one group contains sialoadhesin (Siglec-1), CD22 (Siglec-2), MAG- Myelin-Associated Glycoprotein (Siglec-4), and Siglec-15, and the other group contains CD33-related Siglecs. The main subset consists of the CD33-related subgroup, expressed mostly in the innate immune system and are important for

regulating cellular expansion and activation. The Siglec CD22 is also well-characterized and is an inhibitory receptor of B cells(33). Although all siglecs bind to sialic acid, linkage-specific variability exists between the different subgroups (33). CD22 interacts with  $\alpha$ 2–6-sialylated ligands, important for regulating signalling functions whereas sialoadhesin binds to NeuAc residues which are  $\alpha$ 2–3-linked to galactose (34-36).

### 1.3.1 Lectins as tools in reproductive glycobiology

As previously mentioned, glycosylation plays critical roles in many biological processes. Numerous studies have shown that aberrant glycosylation is associated with a variety of diseases, such as cancer and infertility (9, 37, 38), therefore, knowledge of the glycan composition, the structures, and the proteins to which they attach will help us understand their function. Whilst methods such as MALDI MS are highly sensitive in this type of analysis, they can be time-consuming and costly, requiring specialist handling. Furthermore, they are not suitable for rapid analysis of protein glycosylation. Therefore, once a comprehensive analysis has been achieved by rigorous methods such as MS, lectins derived from plants, animals and microbes can be employed for more rapid analysis of protein glycosylation. Lectin microarrays were first introduced in 2005(39, 40) and have been widely applied for various glycan analysis including sperm surface glycans (41), and cancer biomarker identification (42). It is important to note here that though lectins are useful for analysing glycosylation, they are not nearly as powerful as carbohydrate sequencing MS approaches. While lectins can distinguish between different types of sugars, they often cannot discern subtle differences in glycan structures. For example, a lectin might bind to a mannose residue but not distinguish between different mannose-containing glycans and are less precise in determining the exact structure and composition of glycans.

#### 1.4 Biological Importance of glycans and glycosylation in reproduction

Both male and female reproductive processes are affected by glycans and glycan-binding proteins. The series of events starting with ovulation, then fertilisation, implantation, and subsequently birth, is tightly controlled by several hormones and physiological events.

Several of these hormones are known glycoproteins, and glycosylation has been shown to play a vital role in the regulation of these events (3, 6, 9, 43-45). Many studies have suggested that glycans play a crucial role in reproduction. Specific glycans have now been identified as binding targets (5, 6, 45). Glycans are involved in the process of sperm egg binding (46). There is also evidence that glycans and glycan-binding proteins are involved in the processes of implantation and placental functions(11, 12). Seminal fluid and uterine secretions have a significant amount of glycoconjugates such as glycodelin. The female reproductive tract contains antibodies targeting glycans on sperm surface glycoproteins, such as CD52. Also, sperm manages immune evasion by being coated with highly sialylated glycopeptides such as b-defensin-126 (8, 47). Glycoproteins secreted by the female vary during the reproductive cycle and throughout the reproductive tract. For example, glycodelin (discussed later) has different glycoforms in the uterus, the oviduct, and the cumulus(48, 49). Studying the differential glycosylation of reproductive glycoproteins will help us improve our understanding of key determinants in human reproduction.

The role of glycosylation in reproductive hormone structure and function has been extensively studied. While the definition of a hormone and delineation of its function present little difficulty, the structure and functional roles of glycosylation in hormone action are much more elusive (50). Human chorionic gonadotropin (hCG), being the archetypal example of the classical glycoprotein hormones, is extensively discussed in this thesis, as well as glycans from the uterine endometrium and follicular fluid.

Abnormal glycosylation has been associated with many diseases, including congenital muscular dystrophies, rheumatoid arthritis and cancer(51). Studies have reported increased sLex level in both breast and ovarian cancer patients, as well as increased fucosylated structures in ovarian cancer(52). Elevated mannose levels have been shown in women with polycystic ovary syndrome (PCOS) (53). When combined with total testosterone levels, it revealed a high predictor for PCOS(53). Alterations in glycosylation patterns and their potential importance as biomarkers in gynaecological diseases are supported by several studies. There is a potential to develop less invasive diagnostic methods based on the measurement of glycosylation changes within the endometrium or even menstrual blood.

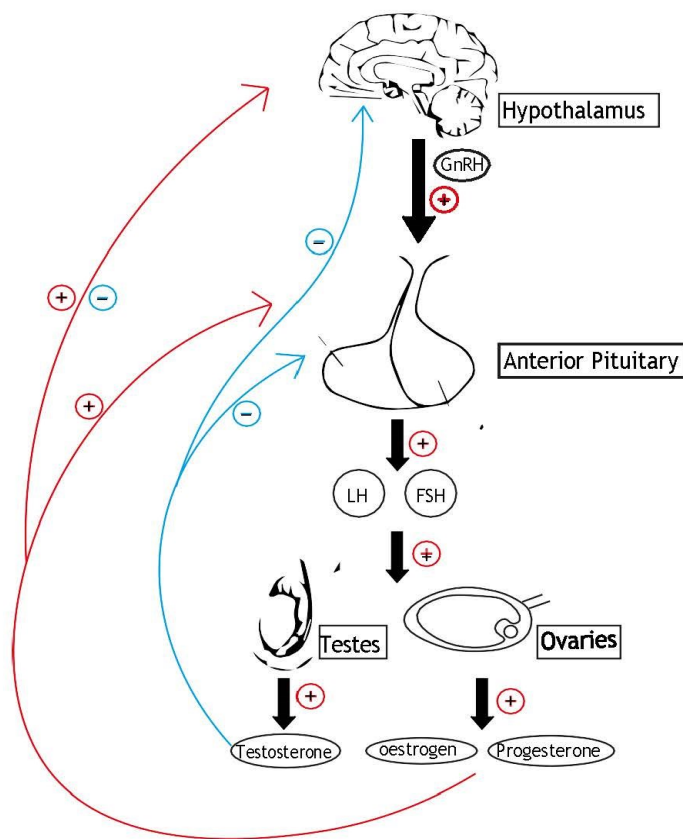
### 1.5 Human reproduction

Several diseases including cancers, endometriosis, infertility, miscarriage, and obstetrical syndromes contribute to the global burden of disease in reproductive medicine. Up until recent years, the focus of research has been on alterations in gene expression profiles with a lot less focus on glycoproteins and their differential glycosylation. Advances in techniques such as mass spectrometry has allowed for detailed identification and quantification of glycoproteins in the human body, allowing for comparison between normal and disease states with the aim of identifying diagnostic biomarkers and targeting treatment. We know that changes in glycan profiles can result in altered biological function as seen in cancers and diabetes (54, 55). To understand the changes that take place in disease states, we must first describe the normal.

The main features of human reproduction are ovulation, fertilization of the released egg by spermatozoa, transport of ovum to the uterus, implantation of the blastocyst, early embryo

development in the wall of the uterus supported by the corpus luteum, formation of a placenta to maintain and support the development of the fetus, and finally birth of the child and expulsion of the placenta. The reproductive cycle is controlled by a negative feedback mechanism between the hypothalamus, anterior pituitary, and gonads referred to as the Hypothalamic-Pituitary-Gonadal Axis (HPG) Figure 1-8 (56, 57) . The HPG axis is crucial for the normal function of the reproductive system in both males and females (58). The hypothalamus regulates the release of hormones from the pituitary gland. The decapeptide gonadotropin-releasing hormone (GnRH) is released into the hypophyseal portal system stimulating the production of the pituitary glycoprotein hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary, which in turn control gonad activity (58). The HPG axis undergoes several changes to promote the development of the reproductive system that are essential for propagation. At each level of the HPG axis, specific hormones are produced that regulate the next level through a complex feedback mechanism. Hypothalamic gonadotropin-releasing hormone (GnRH) has been recognized as the central regulator of the production and release of the pituitary gonadotropins that, in turn, regulate gonadal function and the production of sex steroids(57, 58). Even subtle alterations in the regulation of the different hormones or receptors involved in this process can be enough to cause infertility.

Figure 1-8: Hypothalamic-Pituitary-Gonadal Axis. The reproductive cycle is controlled by a feedback mechanism between the hypothalamus, anterior pituitary, and gonads; Hypothalamic-Pituitary-Gonadal Axis (HPG)(59)



The hypothalamus releases GnRH. GnRH triggers the anterior pituitary to release LH and FSH

FSH stimulates Sertoli cells in the testes to support and facilitate spermatogenesis. This process is regulated by a negative feedback mechanism involving inhibin and testosterone. LH stimulates the interstitial cells of Leydig to make and release testosterone. In females, FSH stimulates the development of follicles. LH plays a role in the development of ova, induction of ovulation, and stimulation of oestradiol and progesterone production by the ovaries.

Oestrogens and progesterone are released from the developing follicles. Oestrogen assists in endometrial regrowth, ovulation, and calcium absorption. Progesterone assists in endometrial growth and inhibition of FSH and LH release.

Testosterone stimulates spermatogenesis and is responsible for the secondary sexual characteristics that develop in the male during puberty. As testosterone levels rise the negative feedback mechanism triggers, acting on the hypothalamus and anterior pituitary to inhibit the release of GnRH, FSH, and LH.

In the male, FSH stimulates Sertoli cells in the testes to support and facilitate spermatogenesis. This process is regulated by a negative feedback mechanism involving inhibin and testosterone (59). FSH binds to receptors on Sertoli cells in the testes. This binding stimulates Sertoli cells to produce essential factors that support the development and maturation of sperm cells (spermatogenesis) (59-61). Sertoli cells produce inhibin, a hormone that specifically inhibits FSH

secretion from the anterior pituitary gland (60). This forms part of the negative feedback loop regulating FSH levels. LH stimulates the interstitial cells of Leydig to make and release testosterone into the testes and the blood(59, 60). Testosterone stimulates spermatogenesis and is responsible for the secondary sexual characteristics that develop in the male during puberty. High levels of testosterone inhibit the release of both LH and FSH from the anterior pituitary and GnRH from the hypothalamus. Sertoli cells produce the hormone inhibin, a hormone that specifically inhibits FSH secretion from the anterior pituitary gland. This forms part of the negative feedback loop regulating FSH levels (59).

In females, FSH plays a crucial role in the development of ovarian follicles and the ova within them. The follicular cells within these structures produce inhibin, which acts to inhibit FSH production through a feedback mechanism (62) . LH contributes to the development of ova, triggers ovulation, and stimulates the production of oestradiol and progesterone by the ovaries (63, 64) .

During the follicular phase of the menstrual cycle, rising levels of FSH and LH stimulate the growth of ovarian follicles, leading to the maturation of the ova (64). These developing follicles secrete increasing amounts of oestrogens and small quantities of progesterone. Oestrogen facilitates endometrial regrowth, ovulation, and calcium absorption, and is also responsible for the secondary sexual characteristics in females (63). Progesterone aids in endometrial development and suppresses further FSH and LH release through negative feedback (65, 66) .

As oestrogen levels rise during the follicular phase, they signal the pituitary gland to release a surge of LH, which triggers ovulation—the release of a mature egg from the ovary. The dominant

follicle then transforms into the corpus luteum, which secretes significant amounts of progesterone. This phase is known as the luteal phase (67).

Progesterone from the corpus luteum makes the endometrium receptive to potential blastocyst implantation, a process termed decidualization, which occurs regardless of conception (63, 64). If pregnancy occurs, the embryo secretes hCG, maintaining the corpus luteum and its progesterone production, thus sustaining the decidua and preventing menstruation (62, 68). The placenta eventually assumes the role of progesterone production around 6-8 weeks of gestation. If pregnancy does not occur, the corpus luteum degenerates, leading to decreased levels of oestrogen and progesterone, which causes the shedding of the outer layer of the endometrium during menstruation (62)

Glycoconjugates from both the male and female reproductive tracts are critical components of the molecular mechanisms underlying the control of these reproductive processes. A good example of this is glycodelin, an immunosuppressive glycoprotein which is present in both male and female reproductive tracts (45, 49). Glycodelin glycosylation has been shown to be different in different areas of the female and the male reproductive tract (45, 49, 69, 70). Glycodelin A in the female reproductive tract and glycodelin S in the male share the same peptide backbone (49). Whilst glycodelin S can be protective towards sperm function, glycodelin A has been shown to have inhibitory effects on sperm-egg binding(69). The sperm surface antigen CD52 is another good example of the role glycosylation plays in reproduction (71). CD52 is a glycoprotein produced by both lymphocytes and epididymal epithelium, but it undergoes different glycosylation processes in these two cell types. In human spermatozoa, CD52 from epididymal epithelium is highly immunogenic, meaning it can elicit a strong immune response. Antibodies targeting this form of

CD52 can cause human sperm to clump together (agglutinate) and become completely immobile when complement proteins are present (71). Despite its potential to affect sperm function, CD52's presence on lymphocytes has significantly reduced its viability as a target for contraceptive development. The same peptide backbone of CD52 is expressed in lymphocytes, meaning that targeting CD52 could inadvertently affect the immune system, leading to undesirable side effects (71, 72).

### 1.5.1 Oocyte

The outer surface the ovary is covered by a layer of germinal epithelium (73). This outer cortex is occupied by primary oocytes surrounded by flattened epithelial cells called follicular cells and these form primordial follicles (73). Within the follicle, the ovum gradually matures. The early primary follicle is encapsulated by a single layer of cuboidal epithelial cells. With continued development, this lining becomes stratified epithelium composed of cuboidal granulosa cells surrounded by an outer layer of squamous theca cells (66, 73).

The immature primary oocyte stays in an arrested state from the completion of oogenesis during fetal development, and oocyte maturation is completed within antral stage follicles after puberty in response to the ovulatory surges in gonadotrophins (66, 73). The primary oocyte increases in size and a membrane, the zona pellucida, forms around it as the follicle matures. The zona pellucida is a thin band of glycoproteins that separates the oocyte from the surrounding granulosa cells (73). Proteins on the surface of sperm will bind to specific glycoproteins in the zona pellucida (5, 44, 72, 74). The follicle has a large fluid-filled antrum and is enveloped by follicular cells comprising the zona granulosa, as well as internal and external theca cells. The zona granulosa

produces oestrogen, whereas the theca interna produces androstenedione that is converted to oestrogen by the enzyme aromatase in the granulosa cells (66, 73).

Just before ovulation, the primary oocyte completes the first meiotic division. At ovulation, the nucleus of the secondary oocyte begins the second meiotic division progressing only to metaphase, then division arrests. If fertilization occurs, the second meiotic division is completed. During discharge of ovum from the graafian follicle (the matured dominant follicle), several layers of epithelial cells remain attached to the outer surface of zona pellucida and are arranged radially forming the corona radiata.

Follicular fluid is a liquid which fills the follicular antrum and surrounds the ovum in the follicle. Follicular fluid is a product of both the transfer of blood plasma constituents that cross the blood follicular barrier and of the secretory activity of granulosa and thecal cells(66). It contains a mixture of steroids, metabolites, polysaccharides, proteins and small peptides, reactive oxygen species and antioxidant enzymes(75). Follicular fluid provides an important microenvironment for the development of oocytes. It has a direct impact on oocyte quality, which is important in fertilization, implantation, and early embryo development(76). The protein content also reflects changes in the secretory processes of the granulosa and theca cells and their metabolic status during follicular growth (77). Thus, studying the glycoprotein profile of follicular fluid could provide insights into the follicular microenvironment and improve our understanding of the processes mediating folliculogenesis.

The accurate assessment of oocyte quality and potential is now of great significance in human in-vitro fertilization (IVF). As we move towards more controlled stimulation to avoid ovarian

hyperstimulation and overproduction of oocytes, and with the rapidly increasing demand for oocyte cryopreservation (78, 79), the challenge to identify criteria to select the best oocytes to inseminate is ever more evident.

### 1.5.2 Sperm

The egg is one of the largest cells in an organism, while the sperm is among the smallest. The sperm's primary role is to deliver paternal DNA to the egg. The end-product of spermatogenesis is a sperm cell, which consists of three morphologically and functionally distinct regions enclosed by a single plasma membrane: the head, which contains a condensed haploid nucleus and a specialised secretory vesicle known as the acrosomal vesicle; the midpiece, which contains mitochondria that generate ATP to provide the energy necessary for movement; and the tail, or flagellum, which propels the sperm towards the egg. The acrosome in the head contains hydrolytic enzymes that help the sperm penetrate the egg. Upon reaching the egg, the contents of the acrosomal vesicle are released by exocytosis in a process known as the acrosome reaction.

Spermatogenesis begins in the testes at puberty and continues in the seminiferous tubule. After release from the seminiferous epithelium in the testis, sperm are transported through the epididymis, where additional biochemical and functional modifications occur. They are then stored within the cauda epididymis, where they are in a functionally inactive state, immotile and incapable of interacting with eggs(80). Sperm are unable to fertilize eggs until they complete capacitation, within the female reproductive tract.

Sperm must withstand the female immune defence. Sperm are suspended in seminal plasma, which is made up of a complex mixture of proteins, glycoproteins, peptides, glycopeptides,

oligosaccharides, and prostaglandins secreted by the organs of the male reproductive tract (81, 82). Sperm is also covered in a complex sugar coat consisting of diverse glycan chains (6). Immune cells and secretions in the female reproductive tract in theory should make it difficult for sperm to survive. The female tract is well-equipped with innate immune receptors (83). The sperm glycocalyx may facilitate sperm survival in the female reproductive tract by binding to glycan-recognizing receptors along the female reproductive tract and thus contribute to successful fertilisation (84). A common feature of the sperm glycocalyx is the terminal monosaccharide sialic acid. Sialic acids can modulate the immune response by binding to Siglecs. It has been implied that sperm sialic acids interact with female expressed endometrial siglecs to modulate the leukocytic reaction and promote sperm survival within the female reproductive tract (85). Many human pathogens can express sialic acids that are recognised by several Siglecs. It is possible that Siglec-dependent recognition of these pathogen glycans leads to altered immune responses, either to the advantage or to the detriment of the pathogen (33).

It was reported over twenty years ago that surface expression of bisected-type N-glycans inhibits natural killer (NK) cell-mediated lysis (86). Recent studies have shown that human sperm display such bisected-type N-glycans on their surfaces(87, 88) as well as terminal Lewis<sup>x</sup> and Lewis<sup>y</sup> sequences on their glycoproteins which have been shown to play a role in the suppression of the adaptive immune response(43, 89, 90).

Investigations for subfertility include a semen analysis which consists of sperm count, motility, morphology, and vitality. This is, however, insufficient as a predictive factor for assisted reproductive outcomes and fertilisation potential of sperm. There is therefore a need to establish new biomarkers, helpful in estimation of male fertility.

### 1.5.3 Uterus

During the menstrual cycle, a complex series of physiological changes occur in the uterus resulting in the thickening of the endometrium to allow for implantation, should fertilisation occur. These changes are orchestrated by the Hypothalamic-Pituitary-Gonadal axis described earlier (Figure 8). They can be defined in three phases: the proliferative, secretory, and menstrual phases (74, 91). In the proliferative phase, the endometrium is exposed to increasing levels of oestrogen as a result of FSH and LH stimulating its production by the developing follicles (68, 92). This results in increasing endometrial thickness, vascularity, and the number of secretory glands. The next phase is the secretory phase which begins once ovulation has occurred. This phase is driven by progesterone produced by the corpus luteum, and optimises the endometrium, making the uterus more receptive for an embryo to implant. The menstrual phase occurs when the corpus luteum degenerates if implantation does not occur. The loss of the corpus luteum results in decreased progesterone production causing the spiral arteries in the functional endometrium to vasoconstrict. The loss of blood supply causes ischaemic changes in the functional layer of the endometrium and, as a result, the functional endometrium is shed as menstruation.

The process of embryo implantation has been linked to carbohydrate recognition. Studies have reported that sialyl-Lewis<sup>x</sup> is expressed on MUC-1 in the human endometrium (93), and that human trophoblasts express L-selectin, an adhesion molecule (11). It has been shown that uterine epithelial cells increase their expression of selectin ligands during the implantation window (11). Genbacev *et al* showed that implantation and placentation make use of the leukocyte emigration system, such as selectins and their carbohydrate-based ligands (11). On the maternal side, human uterine epithelial cells up-regulated selectin oligosaccharide-based ligands during the implantation

window. On the fetal side, human trophoblasts expressed L-selectin (11). This suggests that trophoblast L-selectin mediates interactions with the uterus and that this adhesion mechanism may be critical to establishing human pregnancy (11). Considering the numerous causes of infertility and early pregnancy loss put forward, defects in the selectin adhesion system could partially shed some light on unexplained reproductive failures, as well as deficient cytotrophoblast invasion, which is associated with pregnancy complications such as preeclampsia (13).

The heavily glycosylated surfaces of the implanting blastocyst and maternal uterine epithelium have been shown to interact in a highly controlled and specific manner unique to each mammalian species (94). Each has its own pattern of glycosylation. This mutual compatibility has been referred to as a glycode (94). In Clark's review of the functional glycosylation in the human and mammalian uterus, the characterisation of galectins with different carbohydrate binding specificities resulting in the concept that there is a glycode that directs appropriate functions under different physiological states in complex organisms is highlighted (44). The concept that there is specific recognition of a glycode that functions during the implantation of the mammalian embryo and that each mammalian species has its own unique pattern of glycosylation of the maternal uterine epithelium based on lectin binding, which they designated as a glycotype, is now widely supported (44).

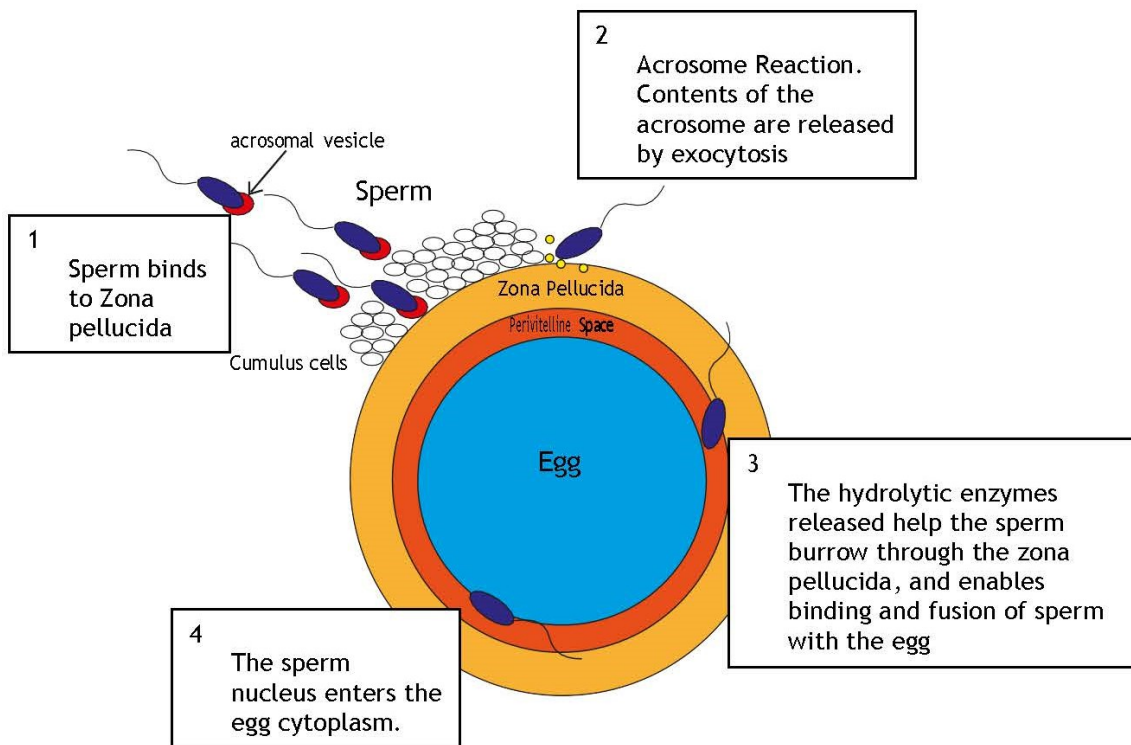
The main function of the uterus is to facilitate implantation and to maintain a controlled environment for the growing fetus. The environment in the pelvis can be altered by different pathological states such as endometriosis (95). Such pathologies can affect the ovaries and impact the developing egg, as well as alter the environment inside and outside the uterus, sometimes resulting in subfertility. The uterus is abundant with glycoproteins. These glycoconjugates interact

with sperm, play a significant role in implantation, and have predicted roles in the human fetoplacental defence system(45). Glycan sequences expressed on the outer surfaces of cells, such as uterine epithelial cells and their secretions play a role in cell-to-cell binding and important signaling events during both physiological and pathological states. With this in mind, we can propose that pathological states can affect the glycosylation of glycoconjugates associated with reproduction. However, there is very little information on the sequences of these glycans and the impact of differential glycosylation in different pathological states, such as endometriosis. In this project we utilise MS methods to analyse endometrial tissue glycomes, to increase our understanding of the biological function of sugars and the role of glycosylation in the uterus.

#### 1.5.4 Fertilisation

Fertilization begins with sperm approaching the egg within the oviduct. Fertilization involves the fusion of two haploid gametes to form a diploid zygote. Sperm capacitation is required prior to sperm-egg interaction. When sperm contacts the extracellular matrix, the zona pellucida, of the egg, the acrosome reaction is triggered and results in exocytosis of the acrosomal vesicle. The exposed sperm membrane fuses with the egg plasma membrane (Figure 1-9). Protein-carbohydrate interactions play a critical role in this complex process. Sperm-egg binding triggers the completion of meiosis. The sequence of events that result in mammalian fertilisation are depicted in Figure 1-9. Once fertilisation has occurred, the lack of an immune response against the developing embryo suggests that the immune and reproductive systems work together to prevent rejection of the developing embryo.

Figure 1-9: Events of fertilisation



A lot of what we know about the glycobiology of mammalian fertilisation is from animal studies. Whilst particularly useful insights are gained from animal research, it is essential to complement such studies with research on human samples because some aspects of glycosylation, for example types of capping sugars, are species specific. Research in this area has potential impact in modern medicine especially in relation to assisted reproductive techniques. The ex vivo maturation of a human oocyte was achieved in 1969(96) and in 1978 Steptoe and Edwards achieved a live birth after human IVF(97). Studies have shown that glycan recognition is essential for sperm-egg binding (46, 98). Glycans on the outer surface of the egg are recognised by lectin-like egg binding proteins on the surface of sperm. This model for specific cell-cell recognition relies on the strict regulation of glycan expression on both sperm and eggs. Several sugars and adhesion molecules have been

implicated in human gamete binding. In early work, the sialyl-Lewis<sup>x</sup> oligosaccharide was shown to negatively affect human sperm-egg binding and it was proposed as a sperm receptor (99). Almost two decades later, highly sensitive glycomic methodologies revealed that the glycoproteins in the zona pellucida that shrouds the human egg have abundant sialyl-Lewis<sup>x</sup> epitopes, as had been predicted (6). Human sperm-egg binding is also inhibited by glycodeclin-A (GdA) isolated from amniotic fluid although it is not yet known which glycan epitopes on GdA are responsible for the inhibition. GdA is rich in sialic acid and fucose but sialyl-Lewis<sup>x</sup> itself has not yet been confirmed in this glycoprotein (100). The mechanisms responsible for failed fertilisation are not fully understood. No study to date has looked at the glycan profile of follicular fluid, which develops with the maturing egg. It would be of interest to investigate glycosylation changes that may be associated with different pathological states.

## 1.6 Natural Killer cells

When investigating the role of glycans and glycosylation in human reproduction, it is essential to mention natural killer (NK) cells as they have been the topic of much research, especially in the field of assisted reproductive treatment and obstetrics, with some suggestion that they may be associated with reproductive failure in women with failed IVF, as well as a suggested role in the pathogenesis of pre-eclampsia (101-104). The glycan profile of a cell is pivotal for proper functioning of that cell and as mentioned previously in this chapter, aberrant glycosylation is associated with diseases. To communicate with other immune cells and apply their immunomodulatory role, NK cells often rely on N-glycans expressed on their surface and their receptors. Hence, the function of NK cells and consequently the progression of various inflammatory conditions is affected by altered N-glycosylation in innate immune cells due to chronic inflammation or disease(105). Aberrant glycosylation, which impairs recognition capability

of NK cells or modifies recognition pattern of target cells, has been reported and associated with cancer(106).

In human reproduction, uterine natural killer (uNK) cells have been reported to make up approximately 70% of CD45<sup>+</sup> leukocytes cells within the decidua (107, 108). They are found at the site of placentation close to infiltrating trophoblast cells and around the maternal spiral arteries(103). Although the function of uNK cells is not fully understood, several theories have been proposed. Studies of pre-eclampsia in pregnancy have suggested an association between uNK cells and regulation of the invasion of trophoblast cells, maintaining the balance between excessive and deficient trophoblast intrusion(109). Xiong et al suggests that recognition of fetal trophoblast cells activates the maternal NK cells, through certain ligand-receptor combinations, which could then enhance placentation (110).

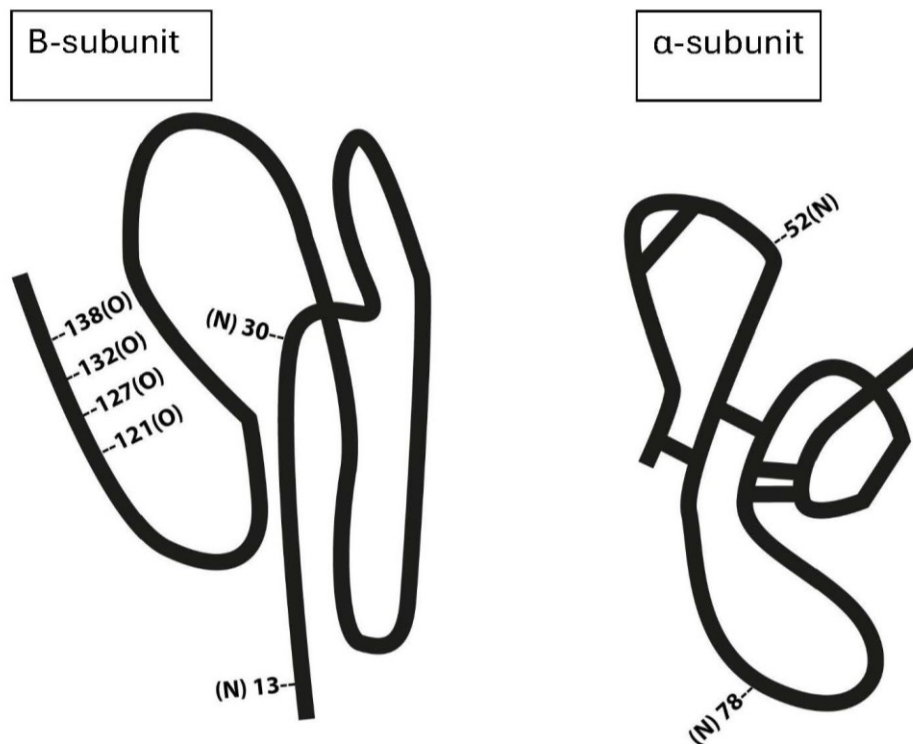
NK cells constitute part of the innate immune system and are fundamental for the destruction of tumour and virally infected cells. Major histocompatibility complex (MHC) molecules are cell surface proteins essential for the acquired immune system to recognise foreign molecules (102, 111, 112). It binds antigens and displays them on the cell surface for recognition by appropriate T-cells. In humans, MHC are referred to as human leukocyte antigens (HLA). We know that NK cells lyse cells lacking in HLA class I antigens. NK cell activation is regulated by a balance of activating and inhibitory signals mediated through cell-surface receptors which permit NK cells to kill infected or abnormal cells only. NK cells survey cells for the presence of MHC class I molecules, allowing them to discriminate between normal cells and those that have lost MHC class I expression, like tumour cells. Negative signals which prevent the cytotoxic activity of NK cells come from the inhibitory receptors that recognise MHC class I molecules. Positive signals that

activate NK cells come from stimulatory receptors that recognise ligands other than MHC class I allotypes.

### 1.7 hCG

hCG (Human chorionic gonadotropin) is a member of the glycoprotein hormone family that includes luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone. It consists of a non-covalently bound  $\alpha$ - and  $\beta$ -subunit. The  $\alpha$ -subunit is glycosylated at Asn 52 and 78(113) and the  $\beta$ -subunit has 2 N-linked glycans at Asn 13 and 30 and 4 O-linked glycans at Ser 121, 127, 132 and 138(114). The unique  $\beta$ -subunit of hCG determines its distinct biological activity. hCG is primarily secreted by the syncytiotrophoblast. hCG is excreted into urine predominantly as the hCG beta core fragment. It is an essential pregnancy glycoprotein hormone. The glycan structures and the maternal concentration change through pregnancy. Levels of hCG in the maternal blood increase progressively in early pregnancy until peak levels are reached at eight to eleven weeks of gestation. Thereafter the levels decline until around twenty weeks when they level off and remain comparatively low for the remainder of the pregnancy.

Figure 1-10 Intact hCG structure highlighting N- and O-glycan glycosylation sites. (N)= N-glycan, (O)- O-glycan.



hCG is needed for the maintenance of pregnancy; it mediates multiple placental, uterine, and fetal functions (111, 115-117). It is widely accepted that hCG may play a part in implantation, through its receptors on the endometrium (118). hCG is also thought to aid smooth muscle relaxation, maintaining quiescence of the myometrium in pregnancy(115). hCG induces relaxin secretion by the corpus luteum during the luteal phase and in early pregnancy. Both relaxin and progesterone play a key role in the maintenance of early pregnancy (119, 120). hCG may also contribute to maternal immunotolerance (111). As pregnancy progresses into the second and third trimester, its role becomes less clear. As well as secretion by placental cells in pregnancy, it can also be produced by trophoblastic cells in several trophoblastic diseases and malignancy, for which it is a sensitive biomarker (121). It can be expressed in gonadal and non-gonadal tumours (122, 123). Little is known about the regulation of hCG glycosylation in normal and abnormal pregnancy.

hCG is highly glycosylated with more than 30% of it constituted by carbohydrate residues (124). The glycosylation of hCG is important as it affects biological activity as well as its half-life in circulation (125). The glycosylation of hCG has not been fully appraised. The N-glycans of  $\beta$ hCG have been identified as consisting of biantennary complex type N-glycans with terminal sialic acids and variable content of fucose (126, 127). The structures have been characterised by matrix-assisted laser desorption ionization mass spectrometry (MALDI MS)(128, 129), and liquid chromatography electrospray ionization MS(24). Some isoforms have been associated with malignancy (130). A diverse range of structures have been associated with  $\beta$ hCG. Increase triantennary N-glycans have been linked to hCG from tumour cells (25). Abnormal biantennary N-glycans have also been linked with tumour derived hCG(131).

A major question in the study of reproductive glycobiology is whether changes in glycosylation seen in pregnancy play a role in the development of obstetric syndromes and can be used as diagnostic biomarkers. hCG is the most important pregnancy hormone that can be detected early on in pregnancy. Its glycosylation has been widely reported, however, there has been little clarity on the differential glycosylation associated with the various stages of pregnancy and changes seen in pathological states.

#### 1.7.1 Hyperglycosylated hCG

As well as the known isoforms of hCG, some studies have referred to a “Hyperglycosylated” hCG (25, 130, 132, 133). Hyperglycosylated hCG is said to contain more complex glycan moieties. It has been described as a family of glycoproteins with the same polypeptide structure as hCG but with larger N- and O-linked oligosaccharides (124). Hyperglycosylated hCG is believed to be produced

by cytotrophoblast cells while regular hCG is made by syncytiotrophoblast cells (134). The now regularly referenced B152 antibody, purported to recognise hyperglycosylated hCG, is thought to detect a single glycosylated epitope variant of hCG. This antibody is reported to detect the presence of O-linked biantennary oligosaccharides, at Ser residue 132 in the middle of the variable 3 O-linked oligosaccharides(134). Hyperglycosylated hCG refers, therefore, to hCG recognised by the B152 antibody. This antibody has been used to imply that early pregnancy hCG is mainly hyperglycosylated. Hyperglycosylated hCG has been associated with Down's syndrome (135) as well as other early pregnancy complications including early pregnancy loss (132). Cole et al have reported that hCG-h can discriminate benign from malignant GTN. This so-called hyperglycosylated hCG is likely to contain an array of molecular variants. Therefore, this term hyperglycosylated, with its description and claims, is rather misleading.

There is no direct evidence of early pregnancy (pregnancy < 12 weeks gestation) hCG being hyperglycosylated. This reflects the challenges of isolating hCG in early pregnancy in amounts sufficient for physicochemical analysis. Also, little is known of how different stages of pregnancy, and differing pregnancy complications impact on the glycosylation status of hCG. Better understanding of hCG glycosylation may open doors for its utilisation as a biomarker for various pregnancy related conditions. Pregnancy can be complicated by many different disorders, such as miscarriage, preeclampsia, and gestational diabetes. Detecting differential glycosylation patterns of hCG may allow us to accurately screen for these complications in early pregnancy. Getting an early and accurate diagnosis of these complications can help decrease the risk by enabling health care providers to diagnose, treat and manage conditions before they become serious.

To clarify the sugar structures of  $\beta$ hCG from normal and abnormal pregnancy and indeed demystify the referenced hyperglycosylated subtype, this thesis investigates the structural variations using MALDI TOF MS. A lot of studies to date were done using pooled urine samples, here we will use samples from individual patients and correlate findings to clinical presentation in pregnancy. Attention will be paid to changes between early and late pregnancy and abnormal pregnancy such as that seen with gestational trophoblastic disease (GTD).

### 1.8 Gestational Trophoblastic Disease

Gestational trophoblastic diseases (GTD) are a rare complication of pregnancy. The term refers to pregnancy-related disorders, consisting of the premalignant disorders of complete and partial hydatidiform mole, and the malignant disorders of invasive mole, choriocarcinoma, and the rare placental-site trophoblastic tumour. It is caused by defective differentiation of the trophoblast. Advances in medicine mean that overall cure rates of this condition are above 98% (136). The incidence and epidemiology of GTD differs worldwide. It is most common in Asia, while in Europe and North American the incidence is reported to be less than 1 per 1000 pregnancies (137, 138).

Currently, the management of trophoblastic disease relies on immunoassay determinations of serum hCG (139). Methotrexate is the treatment of choice for a selection of these women. In the context of GTD, methotrexate is particularly effective because the disease involves rapidly dividing trophoblastic cells that are highly dependent on folate for DNA synthesis and cell division. By targeting these processes, methotrexate can effectively reduce and eliminate the abnormal trophoblastic tissue (136, 139-141). hCG measurement is used in the diagnosis, monitoring, and assessment of successful treatment of GTD. Elevated hCG levels in a woman's blood or urine can be one of the first indicators of GTD, as trophoblastic cells produce large amounts of hCG. In cases

of molar pregnancy, hCG levels are typically much higher than those seen in a normal pregnancy at the same gestational age. After the evacuation of a molar pregnancy, serial hCG measurements are taken to ensure that hCG levels decline appropriately, indicating the removal of trophoblastic tissue. Persistent elevated hCG levels may suggest the presence of residual molar tissue or the development of gestational trophoblastic neoplasia (GTN). For patients undergoing chemotherapy for GTN, regular hCG measurements are taken to monitor the effectiveness of the treatment. A declining hCG level typically indicates a positive response to treatment, whereas plateauing or rising hCG levels may suggest treatment resistance or recurrence (136, 139, 141). This method is not fool proof. These assays do not differentiate the hormone made by gestational trophoblastic tumours from that produced during normal pregnancy. Furthermore, it does not differentiate between premalignant and malignant GTD and those patients with tumours that may be resistant to methotrexate treatment. In low-risk gestational trophoblastic neoplasia patients, a predictive marker for early identification of methotrexate resistance could be particularly useful in the diagnosis and long-term management of these patients.

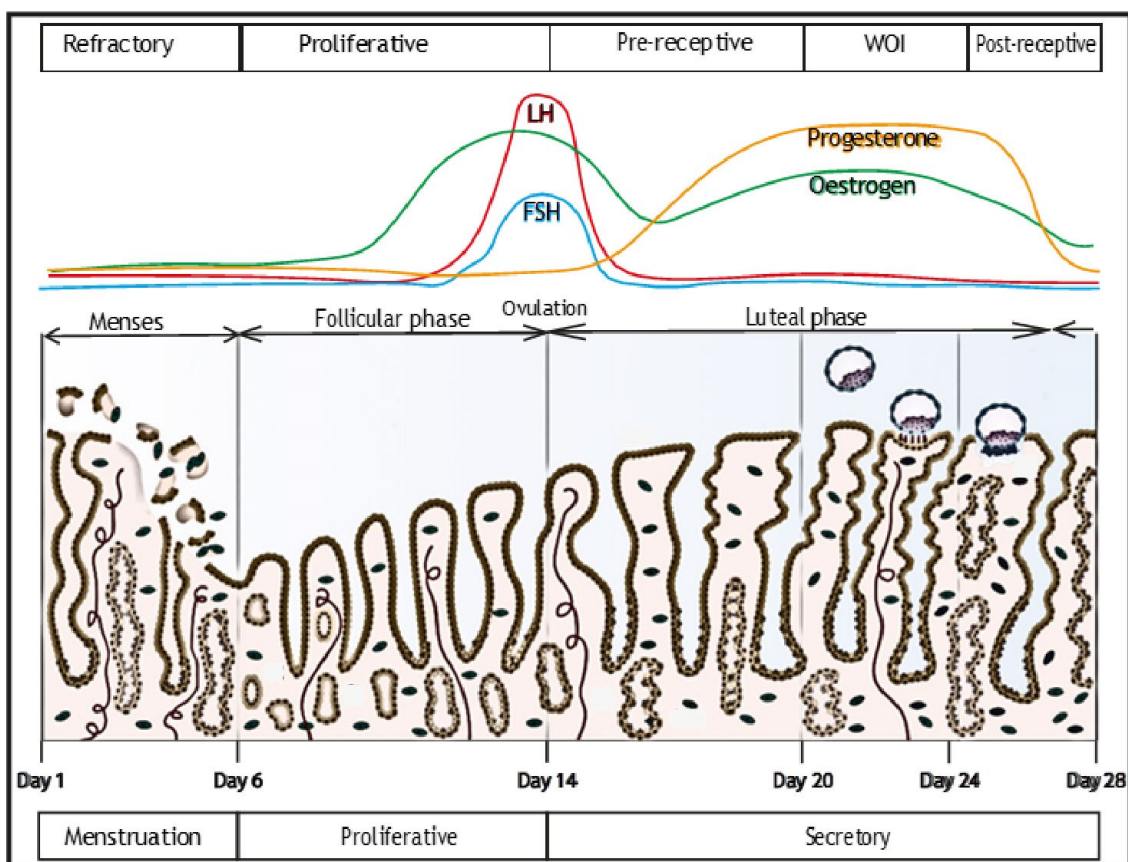
Aberrant protein glycosylation is a well-known event in various disease states including cancer (21, 142, 143). Understanding the glycosylation changes of pregnancy hCG in normal pregnancy versus disease states, will help in the development of an accurate biomarker, similar to the glycoprotein tumour marker CA 125, often found elevated in the blood of women with epithelial ovarian cancer (144-146).

### 1.9 The endometrium and the endometrial cycle

The endometrium is the mucosal layer that lines the inner wall of the uterus. It is made up of a basal layer, adjacent to the myometrium and a functional layer, adjacent to the uterine cavity. The

functional layer undergoes cyclical regeneration, thickening and then shedding during menstruation(62, 68, 74). These changes can be divided into three distinct phases that are tightly controlled by the steroid hormones oestrogen and progesterone, secreted by the ovaries: the menstrual phase; the proliferative phase; and the secretory phase, Figure 1-11.

Figure 1-11: The endometrial cycle can be divided into three distinct phases, depicted here. The period of endometrial maturation during which the trophectoderm of the blastocyst optimally attach to the endometrial epithelial cells is referred to as the window of implantation (WOI) and is of great importance in assisted reproductive treatment.



The menstrual phase, which lasts on average 5 days, is the beginning of the menstrual cycle. The withdrawal of ovarian hormones precipitated by the degeneration of the corpus luteum causes the endometrium to degenerate (62). The blood vessels become highly coiled reducing local blood flow and causing endometrial necrosis and menstruation. This is followed by the proliferative

phase. Under the influence of oestrogen produced by the growing follicle in the ovary, the endometrial cells proliferate, and the endometrial glands increase in size. By the end of this phase, at the point of ovulation, the endometrial thickness can measure up to 8mm (68, 92). Ovulation signals the start of the secretory phase. Progesterone produced by the corpus luteum results in continued growth of the stromal cells and blood supply to the endometrium. The endometrial glands increase in size and become actively secretory, ready to supply nutrition to the embryo were implantation to occur (62). As levels of progesterone rise in this phase, proliferation of the endometrium ceases and it causes endometrial stromal cell differentiation, commonly referred to as decidualisation(68, 91). There is also an influx of uterine natural killer cells (uNKs) after ovulation, which can make up a considerable portion of the cells in the stromal compartment (101). Various immune cells enter the endometrium, contributing to its dynamic nature throughout the menstrual cycle (91, 147). uNK cells play a crucial role in tissue remodelling, immune regulation, and promoting a conducive environment for embryo implantation and placental development (68, 147, 148). Macrophages are involved in tissue remodelling and immune regulation, while dendritic cells process antigens and maintain tolerance. T cells, including regulatory T cells, help with immune surveillance and tolerance to the semi-allogeneic fetus, and B cells, though less common, contribute to local antibody production(147). Neutrophils are active in the inflammatory response, particularly during menstruation, and eosinophils participate in immune responses, especially in allergic reactions and parasitic infections. Together, these immune cells help regulate the menstrual cycle, prepare for implantation, maintain pregnancy, and defend against infections, ensuring a balance between protection and tolerance. The main role of the endometrium is to achieve a successful pregnancy, facilitating implantation by providing an optimal environment for the growth and development of the embryo. When fertilisation has not occurred, the corpus luteum collapses resulting in a drop in levels of

progesterone and oestrogen that leads to the breakdown of the functional layer and the start of a new cycle.

#### 1.9.1 The window of implantation

The “implantation Window” refers to the period of endometrial maturation during which the trophoctoderm of the blastocyst can optimally attach to the endometrial epithelial cells and invade the endometrial stroma and vasculature(149). It usually occurs at around days 19-24 of the menstrual cycle(150) coinciding with the mid-luteal phase and 6-10 days after the LH surge (92) Figure 1-11.

Many studies have tried to establish biomarkers for endometrial receptivity including histological dating, immunohistochemistry, proteomic approaches, and microarray analysis(65, 92, 151-154). The human menstrual cycle can vary enormously in length, with the average being 28 days. The levels of circulating hormones are also highly variable and there is limited understanding of the amounts or timing of these hormones that are required for optimal receptivity.

Successful artificial reproductive treatments, such as in vitro fertilisation (IVF) and embryo transfer, have demonstrated that endometrial receptivity can be manipulated and enhanced through the administration of external hormones, specifically oestrogen and progesterone (155, 156). These hormones are crucial for preparing the endometrium to receive and support the embryo during implantation (92, 154, 157-159). Exogenous (externally administered) oestrogen and progesterone are used in various regimens to mimic the natural hormonal changes of the menstrual cycle (155, 156). During IVF cycles, these hormones are carefully controlled and timed

to ensure optimal endometrial thickening and receptivity. This preparation is essential because the success of embryo implantation largely depends on the endometrium being adequately primed to accept the embryo. While hormone preparation is crucial, the presence of a healthy blastocyst (embryo) is equally important. Ultimately, the success of implantation depends on the interaction between the prepared endometrium and the healthy blastocyst. The uterus, conditioned by the right hormonal environment, plays a significant role in facilitating this interaction (149). Therefore, factors which might affect the environment in the uterus, like endometriosis, can impact implantation. It is interesting to establish how these other factors can impact the endometrial environment despite optimal oestrogen and progesterone.

Assisted reproductive techniques have advanced over the last 30-40 years and are now widely used (160, 161). Their success rates are improving with advances in embryology such as pre-implantation genetic testing where embryos with chromosomal and genetic abnormalities can now be detected before transfer, and the introduction of new generation incubators that use time-lapse technology to allow us to continuously monitor embryos during their development with the potential advantage of improving embryo selection for assisted reproductive technology (ART) treatment (161-163). However, implantation remains one of the major limiting steps in ART success, especially in those with recurrent implantation failure after exclusion of embryonic factors (157-159). We know that while embryo-endometrial synchrony is important, it is not the only factor affecting implantation. Uterine pathologies such as endometrial polyps, uterine fibroids and adenomyosis and endometriosis are commonly found in women with infertility (164).

Some studies have proposed that these uterine pathologies alter molecular expression during the endometrial receptive phase, negatively impacting on implantation(152). Previous attempts to evaluate endometrial receptivity through histology dating of endometrial biopsy have been unsuccessful (165, 166). A study, looking at the accuracy of endometrial biopsy dating in relation to the midcycle luteinizing hormone peak showed that histologic dating of endometrial biopsies in relation to mid-cycle serum LH peak did not differ significantly between patients with tubal and male infertility and patients with unexplained infertility(167). The potential to accurately identify the implantation window and pathological endometrial states will impart valuable clinical benefit, especially in the field of ART.

#### 1.10 Endometriosis

Endometriosis is defined as the presence of endometrial-like tissue outside the uterus, which induces a chronic, inflammatory reaction. It is a chronic gynaecological disease with an unknown aetiology, and is associated with pelvic pain, dysmenorrhea, and infertility. The exact prevalence of endometriosis is unknown but estimates range from 2 to 10% of women of reproductive age, to 50% of infertile women(168). Diagnosis of endometriosis requires expensive invasive surgery; subsequent management may be surgical or medical with the aim of elimination of the lesions(169). Endometriosis cannot be cured by any known surgical or medical treatment and the lesions often re-occur over time.

Though the pathophysiology of endometriosis remains elusive, several causes have been proposed including retrograde menstruation through the fallopian tubes into the pelvis followed by attachment of endometrial tissue at ectopic locations within the peritoneal cavity(170). However, not all women with retrograde menstruation develop endometriosis. Immune system dysfunction

has also been suggested. Ectopic endometrium is usually cleared by immune cells such as macrophages, natural killer (NK) cells, and lymphocytes. Decreased NK cell cytotoxicity in endometriosis patients has been reported(104, 171). It has been suggested that the aetiology is most likely multifactorial involving genetic predisposition, prenatal exposure to endocrine-disrupting chemicals, the microbiome, the immune system, and sex hormones(172).

Endometriosis can result in tubal distortion and limited fimbrial motility due to pelvic adhesions. Active endometriotic lesions exhibit tumour-like characteristics such as intense vascularisation and invasive growth pattern (173). Assisted reproductive treatment has enabled some people living with endometriosis related infertility to achieve a family, however, even with the availability of ART, a negative impact can be seen on many parameters of IVF in women with endometriosis(174). These include a reduced ongoing pregnancy rate, suggesting an altered endometrial receptivity, a reduction in oocyte quality, reduced fertilisation rates, and poor embryo quality(175-177). The lack of a gold standard treatment means it remains a significant burden. Defining the cellular and molecular pathogenic mechanisms of endometriosis, specifically its impact on the pelvic environment, may hold the key to developing new non-surgical methods for diagnosis and treatment of this disorder. Endometriosis can often take years to diagnose due to the nonspecific nature of the symptoms and invasive methods of diagnosis. Patients often suffer for prolonged periods with multiple visits to their doctor to rule out other causes of pelvic pain and menorrhagia before they are diagnosed. Imaging has a role in severe disease with ovarian and deep infiltrating endometriosis, but the gold standard for diagnosis is still laparoscopic surgery with histological confirmation. Laparoscopy remains the gold standard for diagnosing endometriosis, and non-invasive tests should be used only in research settings. Cochrane studies have concluded that there are currently no non-invasive biomarker candidates that can replace

laparoscopic surgery in clinical practice. Additionally, several identified biomarkers have shown no diagnostic value for endometriosis (178). By studying the glycosylation patterns in the normal pelvis vs endometriosis affected pelvis, we may discover new biomarkers for the disease.

#### 1.10.1 Potential biomarker discovery in endometriosis

Cancer antigen (CA)-125 is a mucin-type glycoprotein, produced by the MUC16 gene, and associated with the cellular membrane(144). First described in the early 1980's (179) It is a component of the epithelium of the female reproductive tract. This biomarker is reported to be overexpressed in epithelial ovarian cancer(143). Although also raised in endometriosis, CA-125 has not been proven sensitive enough to be a viable biomarker in endometriosis. Increased levels of Glycodelin-A, produced by endometrial glands, have been reported in the serum of patients with endometriosis(180-182). Although unsuitable as a biomarker on its own, because of its low sensitivity, there may be a potential use in combination with other factors. Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein that has mitogenic activity specific for endothelial cells(183). VEGF is also an important mediator of angiogenesis. Several inhibitors of the VEGF/VEGF receptor system have been shown to prevent tumour growth via an antiangiogenic mechanism(184). Angiogenesis has been shown to play a role in the progression of ectopic lesions in endometriosis and VEGF is well studied as a pro-angiogenic factor overexpressed in endometriosis(182, 185). Its use as a biomarker in endometriosis, however, is not well defined.

Glycosylation changes have been shown in cancer, diabetes, immunological and infectious disorders(33, 186, 187). Endometriosis exhibits characteristics similar to those of cancer, such as cell proliferation, migration, invasion, and adhesion (188). Endometriosis has also been associated with altered inflammatory and immune processes (148, 188-191). It can, therefore, be suggested

that glycosylation might also play a role in the pathogenesis of this disease. It has been reported that the levels of glycoproteins such as CA125 and glycodelin are increased in serum, peritoneal fluid, and ectopic endometrium of patients with endometriosis(146, 192, 193). It is proposed that endometriosis, with its impact on the pelvic environment, can affect the environment in the ovaries and uterus and therefore affect egg maturation, fertilization, and implantation. The effects may be better understood by studying the glycosylation of follicular fluid and endometrial tissue in normal women and those affected with endometriosis. The glycans expressed on a cell can vary depending on the cell type and can reflect cellular conditions during development, differentiation, activation, inflammation, and even malignant transformation. Therefore, glycan profiling has the potential to provide us with a lot of information about endometriosis and its development.

### 1.11 Mass Spectrometry

Glycobiology requires an understanding of glycans and to achieve this it is essential to determine the structure of these glycans. Structural analysis of glycans is primarily carried out using mass spectrometry. The structure is determined by the monosaccharide sequence, positions of inter-glycosidic linkages (1-2, 1-3, 1-4 and 1-6) and anomeric configuration of the glycosidic linkages ( $\alpha$  and  $\beta$ ).

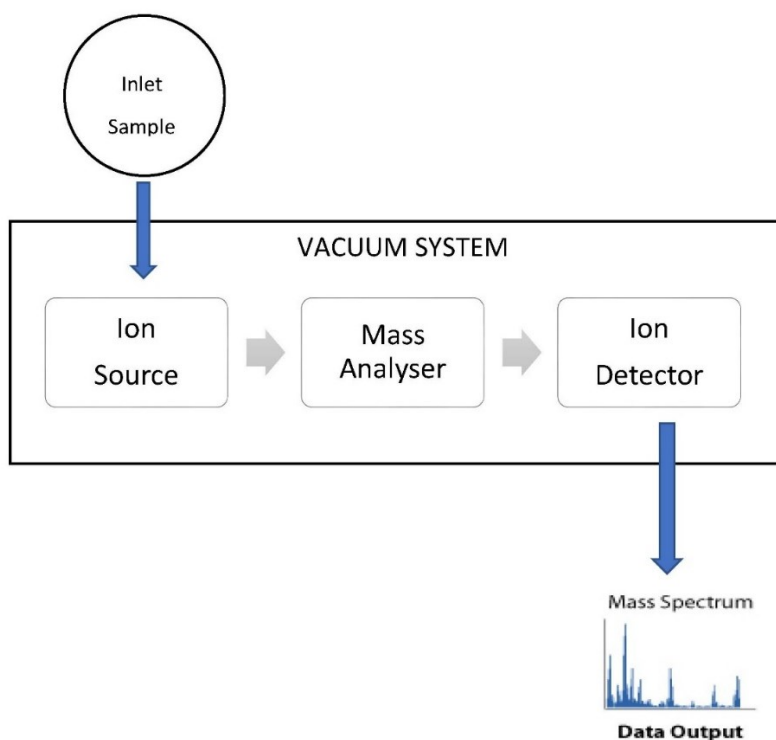
MS methods have been used to analyse glycans since the 1960s. Initially the technology could only be used to analyse low molecular weight molecules, less than 1000 Daltons. This was because the compound needed to be sufficiently volatile and increased molecular weight of the compound resulted in decreasing volatility. Also, the mass range of the mass spectrometer was limited. Today, with advances in technology, MS can analyse higher molecular weight molecules. MALDI-TOF MS enables ultrasensitive glycan profiling, MS/MS provides a means for further detailed

analysis of antennae sequences of specific glycan, and linkage analysis of glycans is possible by using GC-MS. Nano-LC/MS and Nano-LC/MS/MS are used to provide information on site occupancy and heterogeneity. A complete analysis of a glycoprotein will often require more than one MS approach. Here, we utilise MALDI-TOF MS and MS/MS to report the full glycan profile of endometrial tissue, follicular fluid, and Beta hCG.

A mass spectrometer converts individual molecules to ions so that they can be moved about and manipulated by external electric and magnetic fields Figure 1-12. Simplistically, a MS can be broken down to three components and essential functions:

1. The Ion Source: sample molecules are converted from neutral molecules into ions.
2. The Mass Analyser: The ions are sorted and separated according to their mass and charge.
3. The Detector: The separated ions are then measured, and the results displayed on a chart.

Figure 1-12: Primary elements of a mass spectrometer, the analyte must first be converted to Gas Phase IONS to allow manipulation by the magnetic and/or electric fields in the mass analyser. Three major ionisation techniques include electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI) and fast atom bombardment (FAB). Mass analysers include but are not limited to time-of-flight (TOF), Quadrupole and ion trap. The analyser used in this thesis, TOF, is based on recording the time of travel. There is no magnetic field, and the electrical fields are not directly involved in analysis but rather make the ions travel in the right direction at the right speed. Ions are sorted according to their mass to charge ratio ( $m/z$ ), which is then plotted against their intensity to generate a mass spectrum.

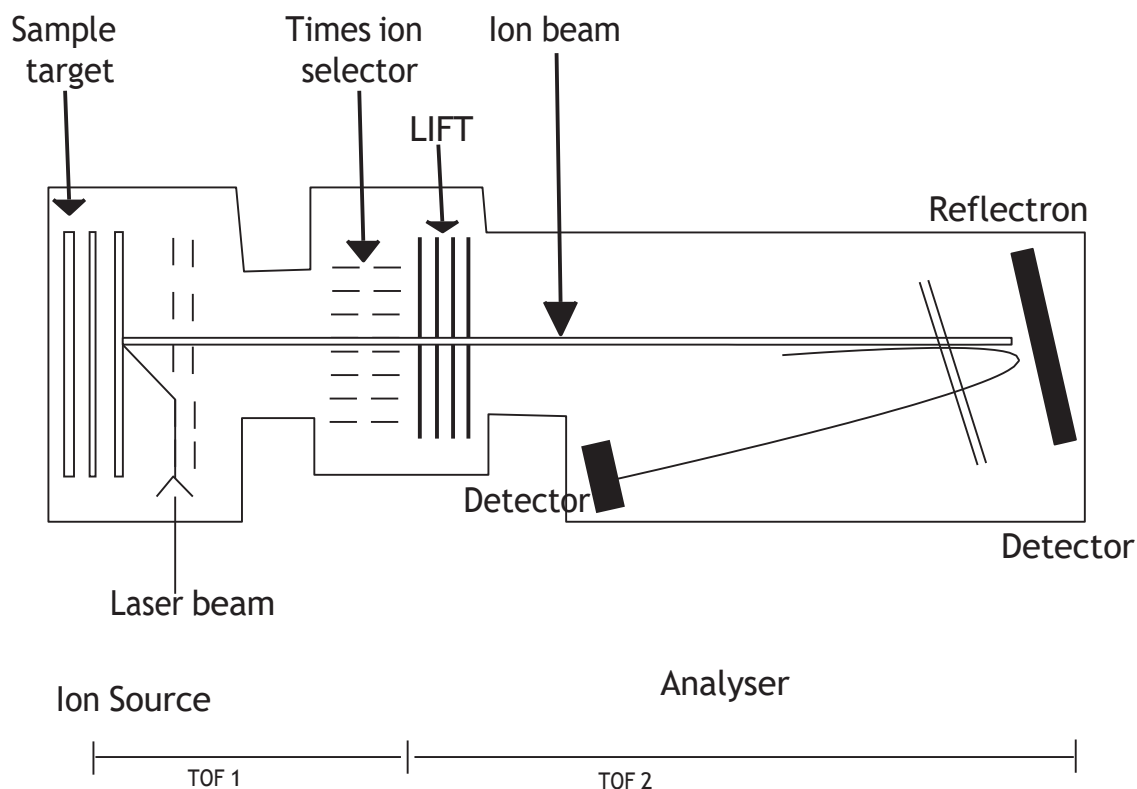


In MALDI, samples are co-crystallised with a low molecular weight ultraviolet absorbing matrix on a metal target. The target is then inserted into the MALDI source where the sample and matrix mixture are irradiated by laser pulses that result in desorption of ionised molecules. Once ions are formed the mass analyser separates the ions according to the  $m/z$ .

Ionisation of the sample is an essential part of MS experiments. This can be achieved by electron ejection, capture, protonation, or deprotonation. The type of ionisation mode used, depends on the type of compound being analysed and the information required. Electron ionization (EI) and

chemical ionisation (CI) were initially used in the '60s, but these methods were not suitable for high molecular weight and non-volatile molecules. Techniques like fast atom bombardment (FAB), electrospray ionisation (ESI) and MALDI, have allowed the analysis of non-volatile compounds and higher mass molecules. MALDI-MS is the most sensitive ionisation technique for glycan analysis and is the method applied in the glycomic analysis described in this thesis

Figure 1-13: Schematic diagram of MALDI TOF/TOF MS. The matrix and sample mixture are placed in the instrument on a target plate, brief UV laser pulses hit the mixture, desorbing and ionising molecules. The ionised molecules are accelerated into the first TOF analyser. TOF 1 ends with a selector. Only analytes of the selected mass and the derived fragments can pass to the ion source of the second TOF analyser (called LIFT). In the ion source of TOF 2 the selected parent and fragment ions are accelerated. Subsequently, these analyte ions are separated in the conventional TOF 2 analyser. This allows the mass analysis of the fragments, by measuring their arrival time on the detector. The reflectron in the second TOF analyser is used to enhance the resolution of the mass spectrum of the fragments.



#### 1.11.1.1 (14)MALDI

MALDI was discovered by Koichi Tanaka in 1988 (194). MALDI involves ionisation through pulsing a laser onto a low molecular weight ultraviolet absorbing matrix into which the sample is embedded. It forms predominantly singly charged ions. Once ions are formed the mass analysers separate the ions according to the  $m/z$ . The process produces very few fragment ions. MALDI MS is the method of choice for glycomics. It is a “softer” ionisation method compared to FAB or ESI. In contrast to FAB-MS, MALDI-MS uses a crystalline matrix, rather than a liquid one and a pulsed beam of photons, instead of a continuous beam of atoms or ions. The choice of matrix is vital for an efficient ionisation of the sample and many matrices have been developed, but one of the earliest, 2,5-dihydroxybenzoic acid (DHB) is used here. The matrix is essential in the MALDI technique as it absorbs strongly at the laser wavelength to minimise sample damage. It also increases the energy transferred to the analyte, enhancing the sensitivity of MALDI.

High concentrations of salts, detergent and other impurities disrupt the desorption and ionisation processes of MALDI. It is, therefore, important to take great care with sample purification and preparation in order to optimise the qualities of the MS.

The main drawback of MALDI MS analysis is that it is only semi-quantitative, therefore, the process of comparison is done by measuring and comparing peak heights in mass spectra, which correspond to relative abundance of ions in the sample, assuming that the ionisation and detection of different components in a sample are similar. The MALDI mass spectrum is a plot of intensities on the y-axis and the corresponding  $m/z$  values on the x-axis. The intensity scale is the

relative abundance of ions, but generally the region below about  $m/z$  500 is not shown because it is saturated with signals from matrix-derived ions.(195)

### 1.11.2 Mass analyser

The mass analyser sorts and separates the ionised molecules according to their mass to charge ratios, rather than by their mass alone. There are several types of mass analysers, utilising either an electric or magnetic field or a combination of both. The different types of mass analysers have their own special characteristics with benefits and limitations. The mass analyser used is based upon the application, cost, and performance desired.

A mass spectrum will usually be presented as collection of vertical lines, in which each line represents an ion having a specific mass-to-charge ratio ( $m/z$ ) and the length of the bar indicates the relative abundance of the ion. The most intense ion is assigned an abundance of 100%. Interpretation is carried out using the mass to charge ( $m/z$ ) value of a monoisotopic ion to deduce the molecular weight value from which it is possible to derive the number and type of monosaccharide. With knowledge of glycan biosynthetic pathways, we can suggest possible glycan structures based on the unique glycan composition for a given molecular weight.

Commonly used mass analysers include Time of Flight (TOF), quadrupole, ion trap devices and double focusing instruments which incorporate magnetic and electrostatic analysers. (196)Research in this thesis was carried out with TOF mass analysers which measure the time it takes ions of different masses to move from the ion source to the detector. They separate ions according to their differences in velocities as they move after acceleration in a field-free “flight tube” towards the detector. Ions of different  $m/z$  values move through the flight tube at different

velocities, reaching the detector at different times. The time at which the ions leave the ion source and enter the flight tube needs to be well-defined. Ions leaving the ion source of a TOF mass spectrometer have a spread of kinetic energies. To compensate for these differences, a reflectron or ion mirror is an ion optic device in which ions in a TOF mass spectrometer pass through a "mirror" or "reflectron" and their flight is reversed to decrease the spread in the ion flight times. It allows ions with higher kinetic energies to penetrate deeper into the reflectron than ions with lower kinetic energies. The ions that penetrate deeper will take longer to return to the detector, resulting in a reduction of the spread of initial kinetic energies of the ions produced and an improvement in resolution. Benefits include the high practical mass range, fast MS analyser and MS/MS information. In a linear TOF, unlike reflectron TOF, ions travel in a linear fashion and are detected by the detector at the opposite end. Linear TOF is limited in resolution leading to low mass accuracy, but it is capable of analysing much larger ions than reflectron TOF. The mass accuracy is substantially improved by the reflectron. The TOF is capable of separating ions that are produced in a pulsed manner rather than by continuous injection and, therefore, TOF is well suited to a desorption ionisation technique such as MALDI. The high sensitivity when coupled with a MALDI ionisation source, has made the MALDI-TOF mass spectrometer useful in the analysis of large molecules including proteins and glycans.

### 1.11.3 Tandem mass spectrometry

Tandem MS involves two or more mass analysers coupled together. Thus, it involves two stages of MS. In the first stage, the first analyser isolates the ion for fragmentation. In the second stage, the second analyser separates the fragment ions, giving a MS/MS spectrum for each selected molecular ion. The fragmentation of the precursor ion happens in the "collision chamber", where a small amount of inert gas such as argon is present, which is located between the two analysers.

The precursor ions are fragmented with sufficient energy to break the glycosidic bonds in a process known as collision induced dissociation (CID). There are several different types of tandem instruments, and tandem MS can be performed using quadrupole and TOF instruments. Different mass analysers are often combined to form tandem instruments for specific applications. The MALDI TOF/TOF is utilised in this project and is therefore discussed in more detail below.

#### 1.11.3.1 MALDI TOF/TOF

Soft ionisation techniques such as MALDI and ESI produce very few fragment ions. This means that whilst the  $m/z$  of the sample is reported, there is little structural information available. The tandem MALDI-TOF/TOF mass spectrometer provides additional detailed structural information of molecules within a mixture, making it an essential analytical tool in the fields of glycomics and proteomics. The benefits of MALDI TOF include its speed, small sample volume required for analysis, broad mass range and easy automation. In this thesis, the Applied Biosystems 4800 MALDI-TOF/TOF mass spectrometer is used for glycan mass fingerprinting.

MALDI TOF MS combines two technologies in a two-phase process, the MALDI ion source and the TOF mass analyser. Additional elements incorporated for the MS/MS mode include a timed-ion-selector (TIS). The Applied Biosystems 4800 MALDI TOF/TOF analyser is a double focusing instrument with linear and reflector flight path options, permitting the selection of individual precursor ions for further fragmentation in the collision cell. Fragmentation can occur by application of laser induced dissociation or collision induced dissociation. Samples co-crystallised with a suitable matrix are spotted on a plate and placed in the ionisation chamber of the MALDI-TOF MS. Here, they are vaporised by repetitive pulsed laser into a vacuum while being ionised at the same time. The ions are accelerated through the application of a high voltage and

fragmentation can occur as they travel through the flight tube. Specific ion packages can be individually selected to continue through the flight tube.

In TOF/TOF, two time-of-flight mass spectrometers are used consecutively. The first TOF analyser is used as a mass filter and isolates precursor ions of choice, here the precursor ions and their fragments travel together. The first TOF ends with a selector. Only analytes of the selected mass and the derived fragments can pass to the ion source of the second TOF analyser. In the second TOF analyser the selected parent and fragment ions are accelerated and subsequently separated allowing the mass analysis of the fragments, by measuring their arrival time on the detector. The reflector in the second TOF analysers is used to enhance the resolution of the mass spectrum of the fragment. The ions travel toward an ion detector with small analytes traveling fastest, followed by progressively larger analytes as described earlier. The ions are separated by TOF according to their  $m/z$  ratio and a mass spectrum is produced.

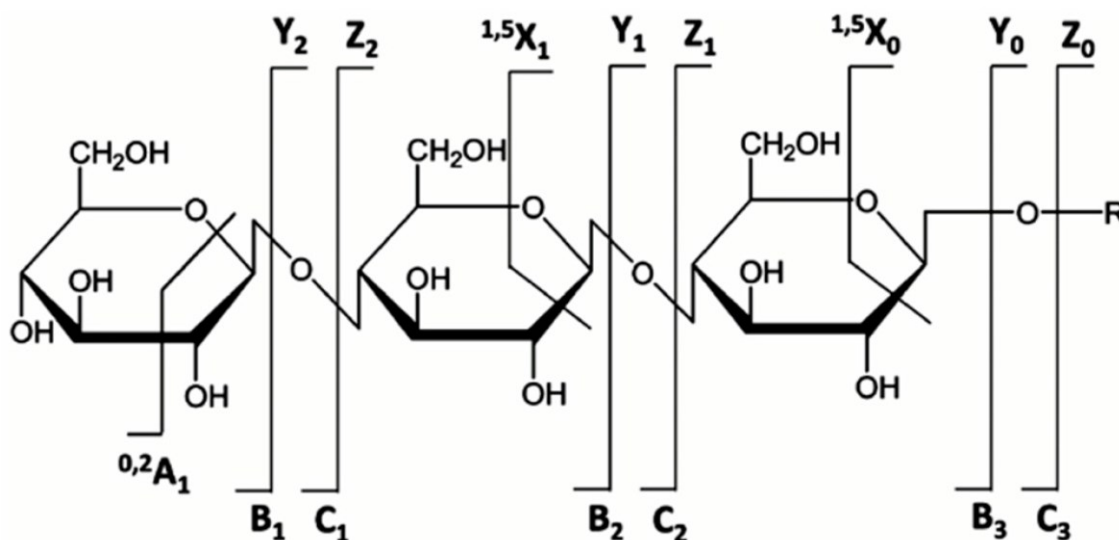
#### 1.11.4 Fragmentation of glycans

An understanding of carbohydrate fragmentation patterns is necessary for the interpretation of MS/MS spectra. In MS/MS, glycans are broken into fragments and the resulting  $m/z$  value of each fragment are displayed on the MS spectrum. Fragmentation involves the creation of an ion by cationisation (Protonation or sodiation) in ESI or MALDI. Characteristic peaks formed in MS/MS spectrum from fragmentation of glycopeptide ions enable detailed interpretation of structural information of that glycan. The nomenclature for oligosaccharide fragments is shown in

Figure 1-14. Glycan sequence isomers have the same molecular weight, the MS/MS fragmentation pattern generated will, however, be different. The detection of specific fragments can therefore be used to prove the presence of a glycan isomer.

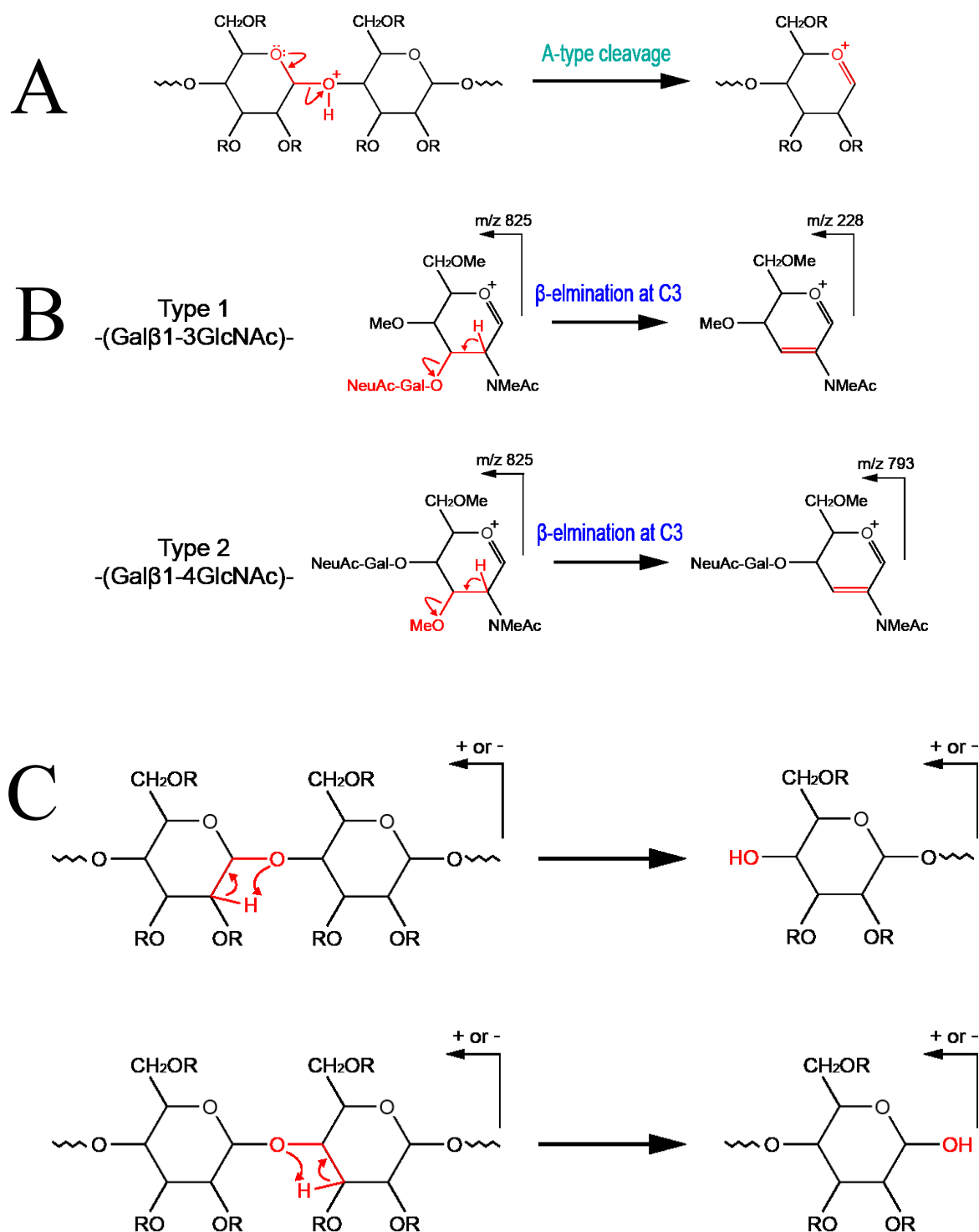
Derivatisation methods like permethylation is used to assist the ionisation of native oligosaccharides. This is a necessary step for obtaining structural information on carbohydrates by FAB MS, MALDI MS and ES MS. The process increases the sensitivity to detection by MS and offers a reliable and well-defined fragmentation allowing unambiguous sequencing (197). Methylated glycans ionize more efficiently than their native counterparts and are more easily purified. Permethylation replaces the protons on hydroxyl and amide groups of each carbohydrate with methyl groups enabling the generation of fragment ions that follow predetermined fragmentation pathway. This facilitates the identification of these ions. The fragmentation patterns of permethylated derivatives have been well reported (198-200). The nomenclature for glycan fragmentation, illustrated here in Figure 1-14, was first introduced by Domon and Costello in (201) and is still in use today. This is a detailed description where non-reducing terminal fragments are termed A, B, and C, and the intact reducing-end fragments are termed X, Y and Z. The subscripts refer to the number of sugar residues from the terminal, and the superscripts refer to the cleavage position of the cross-ring fragment.

Figure 1-14: Nomenclature for the fragmentation of glycans; A and X ions represent cross-ring fragments and B, C, Y and Z are glycosidic fragment ions. (Adapted from Domon and Costello (201)). Fragments containing the non-reducing ends are designated as A, B and C ions. Fragment with the reducing ends is named X, Y and Z ions. The ions are labelled by numbering each ring bond.



What we currently know about glycan fragmentation patterns comes from experiments using FAB MS (198, 199, 202) and can be applied to MALDI MS/MS data used here. In MS/MS, permethylated glycan fragmentation is mainly by breakage of the glycosidic bonds between the sugar units. The main fragmentation pathways of derivatised oligosaccharides are depicted in Figure 1-15. A-type cleavage occurs commonly on the non-reducing side, and involves cleavage across glycosidic bonds, producing an oxonium ion. A further fragmentation can also occur here, known as  $\beta$ -elimination, where the substituent at the 3-position of the oxonium ion is eliminated, forming a conjugated oxonium ion. This can be used to distinguish between type 1 and type 2 LacNAc units.  $\beta$ -cleavage is another common type of carbohydrate fragmentation, here a proton transfer accompanies glycosidic bond cleavage. The fragment ion can either be reducing or non-reducing.

Figure 1-15: Main Fragmentation pathways of derivatised oligosaccharides. A: A-type glycosidic bond cleavage. B: subsequent elimination of the C-3 substituent with corresponding mass losses; C: b-cleavage- glycosidic bond cleavages with hydrogen transfer.



## 1.12 Complementary methods for MS based glycomics

### 1.12.1 Linkage analysis

Linkage analysis is a method used to identify linkage sites in carbohydrate residues. This is achieved by derivatization of hydroxyl groups, breakage of glycosidic linkages by acid hydrolysis, (producing individual methylated monosaccharides with free hydroxyl groups at the positions that were previously involved in a linkage)(1), reduction and acetylation and analysis by gas chromatography (GC-MS). The location of acetyl groups indicates linkage positions, and the methyl groups indicate positions that were unsubstituted in the original glycan. Linkage analysis complements MS and MS/MS data as this method alone cannot give us a full picture of the linkage configuration.

### 1.12.2 Enzymatic digestion experiments

To further aid the structural analysis of glycans, enzymatic reactions can be used to complement the information derived from MS screening and MS/MS analysis. Examples of such enzymes include exoglycosidases that remove non-reducing end terminal sugar residues, endoglycosidases that cleave internal sugar residues, and glycotransferases that add sugar to non-reducing positions. The specific nature in which these enzymes act facilitates their use in glycan structural analysis. Specific enzymes recognise and cleave at specific linkages and monosaccharides of glycans. The samples are then characterised by MS and MS/MS analysis giving additional structural information by comparing the spectra before and after enzyme digestion.

In this thesis  $\beta$ -1,4 galactosyltransferase was used for further analysis of glycan structure.  $\beta$ -1,4 galactosyltransferase can catalyse the addition of 1,4-linked galactose onto GlcNAc on truncated antenna but not onto bisecting GlcNAc. Therefore, glycans with truncated antenna will be

galactosylated resulting in a mass shift, whereas signals for the bisected glycans remain unchanged.

### 1.13 Aim of thesis

The major aim of this thesis is to present a robust structural characterisation of glycans involved in the human female reproductive system using mass spectrometric strategies. The focus will be on the differential glycosylation of key reproductive glycoprotein, hCG, and endometrial cell glycans, involved in implantation and finally, glycans in follicular fluid.

The specific aims include:

1. Glycomic investigation of a urinary hCG in normal and abnormal pregnancy
2. Structural characterisation of early pregnancy hCG (1<sup>st</sup> trimester) with particular attention to describing changes in glycosylation that may occur as normal pregnancy progresses.
3. Using glycomic strategies to investigate changes in endometrial and follicular fluid environment in endometriosis.

## 2 Chapter 2- Materials and Methods

### 2.1 *Materials*

The following materials were obtained from the sources indicated:

#### 2.1.1 Chemicals and Reagents

- Ultrapure 18 MΩ·cm<sup>3</sup> distilled/deionised was used for all aqueous solutions produced by Purite Neptune ultrapure water purification system from Purite Ltd (Oxfordshire, UK).
- Methanol (MeOH) from Romil (Cambridge, UK)
- Acetonitrile (ACN) from Romil (Cambridge, UK)
- Ammonia bicarbonate (ambic) from Romil (Cambridge, UK)
- Dimethylsulphoxide (DMSO) from Romil (Cambridge, UK)
- Chloroform from Romil (Cambridge, UK)
- Propan-1-ol from Romil (Cambridge, UK)
- Sodium hydroxide (NaOH) from Romil (Cambridge, UK)
- Acetic acid from Romil (Cambridge, UK)
- Trifluoroacetic acid (TFA) Romil (Cambridge, UK)
- Dithioreitol (DTT) from Fluka (Poole, UK)
- Formic acid from Romil (Cambridge, UK)
- Potassium hydroxide (KOH) from Fluka (Poole, UK)
- Dowex 1-X8 (H) from Fluka (Poole, UK)
- Gentisic acid (2,5-dihydroxybenzoic acid) Romil (Cambridge, UK)
- Sodium hydroxide pallets from Romil (Cambridge, UK)

- $\alpha$  -cyano-4-hydroxycinnamic acid ( $\alpha$ -CHCA) from Sigma-Aldrich Corporation (Poole, UK)
- Ethylenediaminetetraacetic acid (EDTA) from Fluka (Poole, UK).
- Potassium borohydride ( $\text{KBH}_4$ ) from Sigma-Aldrich Corporation (Poole, UK)
- Iodoacetic acid (IAA) from Sigma-Aldrich Corporation (Poole, UK).
- Hexanes from Sigma-Aldrich Corporation (Poole, UK)
- 48% (v/v) hydrofluoric acid in water (HF) from Sigma-Aldrich Corporation (Poole, UK)
- Manganese (II) chloride tetrahydrate Sigma-Aldrich Corporation (Poole, UK)
- MOPS (3-[N-Morpholino] propanesulfonic acid) from Invitrogen (Paisley, UK)
- Potassium borohydride from Sigma-Aldrich Corporation (Poole, UK).
- Sodium borodeuteride ( $\text{NaBD}_4$ ) from Sigma-Aldrich Corporation (Poole, UK).
- Sodium chloride from Rose chemicals (London, UK).
- Uridine 5'-diphosphogalactose (UDP-Gal) were from Sigma-Aldrich Corporation (Poole, UK).
- Tris (hydroxymethyl) aminomethane (Tris) from Amresco (Solon, USA).
- 8 M guanidine-HCl solution obtained from Pierce (Rockford, USA).
- Snakeskin dialysis tubing (7kDa molecular weight cut off) from Pierce (Rockford, USA).
- Acetic anhydride Romil (Cambridge, UK). v Romil (Cambridge, UK).
- Methyl iodide was from Alfa Aesar (Lancashire, UK)
- 3-[(3-Cholamidopropyl) dimethyl-ammonio]-1-propane sulfonate (CHAPS) from Roche Applied Science (East Sussex, UK)
- Nitrogen supplied by BOC (Guildford, UK).

- Argon gases supplied by BOC (Guildford, UK).

### 2.1.2 Enzymes

- Trypsin for glycomic analysis was from Sigma-Aldrich (Poole, UK).
- Sequencing grade modified trypsin (porcine) from Promega (Hampshire, UK).
- Peptide-N-glycosidase F (PNGase F) containing 50% glycerol (*Flavobacterium meningosepticum*- recombinant from *E. coli*) from Roche Applied Science (East Sussex, UK)
- $\beta$ 1,4-Galactosyltransferase from Calbiochem/Merk Chemicals (Darmstadt, Germany).
- Sialidase S from Europa Bioproducts Ltd (Cambridge, UK)

### 2.1.3 Labware

- VC130PB (130 W) Vibra-Cell ultrasonic processor within a sound-abating enclosure from Sonics & Materials Inc (Meryin/Satigny, Switzerland). - used for sonication of cells
- Multi-tube vortexer
- Thermo Savant SPD122P SpeedVac concentrator which is connected to a RVT400 refrigerated solvent trap.
- Thermo Savant ModulyoD freeze dryer (Thermo Fisher Scientific Inc, Waltham, USA)
- Slide-A-Lyzer® dialysis cassettes (3.5 kDa molecular weight cut off), from Thermo Scientific (Basingstoke, UK).
- Screw-cap style Pyrex disposable culture tubes (7.5 ml) capped with disposable phenolic lids and plasticware, from Corning (New York, USA).

- Caps were lined with Teflon inserts from Owens Polyscience Ltd (Cheshire, UK).
- Reverse phase Classic C18 (360 mg, 55-150  $\mu$ m), Oasis HLB, C18 (Plus) Sep-Pak cartridges from Waters Ltd (Hertfordshire, UK)
- 10 ml glass syringes from Fisher Scientific Limited (Leicester, UK)
- Glass Pasteur pipettes (150 mm and 230 mm) from VWR International Ltd (Leicestershire, UK).
- Lo-bind® Eppendorf tubes from Sigma-Aldrich (Poole, UK)
- Applied Biosystems 4800 MALDI-TOF-TOFTM mass spectrometer.

## 2.2 Ethical Permission

Consent and Ethical approval for the research project was obtained from NHS National Research Ethics Service (NRES) Riverside Committee London (REC 14/LO/0199) and the Imperial College Healthcare Tissue Bank (ICTHB, reference no. Obs-TB-13-033). All subjects gave informed consent, and patient anonymity was preserved

## 2.3 Sample preparation

For the study of hCG glycosylation (Chapter 3), urinary hCG samples from individual patients were provided by Imperial College NHS trust early pregnancy unit and purified by Stewart Wilson from NHS Tayside. Endometrial and follicular fluid samples were collected from Chelsea and Westminster NHS trust assisted conception unit. All samples were frozen in dry ice immediately after collection and stored in -80 °C before analysis. All samples were analysed at Imperial College Mass spectrometry lab using MALDI. Methods for their preparations and analysis are presented in this chapter.

### 2.3.1 hCG

Samples were obtained from pregnant women presenting to the early pregnancy unit at weeks 6-9 and weeks 20-21. All GTD samples were from histologically confirmed complete hydatidiform mole pregnancies.

HCG was purified from 24hr urine collection obtained from individual patients. Purification was done using Phenyl-Sepharose. Urine samples were analysed for total hCG (hCG + hCG $\beta$  + other identified variant forms) using an in-house radioimmunoassay assay, which uses a polyclonal rabbit antiserum (Harvey et al. 2010). Urine samples were initially concentrated using Millipore Prep scale TFT Regenerated Cellulose 5 K Filter Cartridge (CDUF 001LC). The concentrated samples were then diluted in 0.6 M ammonium sulphate (pH 7.5) and loaded onto a Phenyl Sepharose chromatography column. Samples were firstly eluted with 50 mM ammonium bicarbonate at pH 7.5 (elutes nicked hCG) followed by a second elution with 40% (v/v) aqueous ethanol. The peak fractions from the 40% ethanol elution were pooled and exchanged into PBS, 0.1% sodium azide using GE PD10 desalting columns. Purified hCG samples were stored at -80 °C and lyophilised before preparation for glycomic analyses were carried out.

### 2.3.2 Endometrial tissue

The sample group included women between the age of 21-39 years old, undergoing assisted reproductive treatment. Samples were taken the cycle before hormone stimulation was commenced. Women were excluded if there had known retroviral infection. All samples were taken in the “fertile window” described in chapter 1. Samples were collected using an endometrial pipelle and stored at -80 °C until analysis. Patients presenting with male factor infertility were used as the control group.

Endometrial tissue was homogenised in ice cold homogenisation buffer to completely suspend the cells, (homogenisation buffer: 25 mM TRIS, 150 mM NaCl, 5 mM EDTA and 1% CHAPS\* at pH 7.4. To make up 50 ml, add 0.1514 g TRIS, 0.4383 g NaCl, 0.0731 g EDTA and 0.5 g CHAPS; after dissolving in a small volume of ultrapure water, complete to 50 ml with ultra-pure water and adjust to pH 7.4 with dilute acetic acid). The homogenates were transferred to high quality dialysis tubing and immersed in dialysis buffer at 4°C with constant stirring (Dialysis buffer: 50 mM AMBIC buffer. To make 4.5 L, add 17.6 g of AMBIC and complete with 4.5 L of ultrapure water. Adjust to pH 7.5 with dilute acetic acid). The samples were left to dialyse over a 48-hour period. The dialysis buffer was changed regularly. Homogenates were then transferred into a clean plastic falcon tube covered with perforated Parafilm and lyophilised before preparation for glycomic analyses were carried out.

### 2.3.3 Follicular Fluid

The sample group included women between the age of 21-39 years old, undergoing assisted reproductive treatment. Patients were stimulated with FSH/LH hormones for optimal follicular growth. hCG trigger was used to stimulate an artificial LH surge before egg collection 36 hrs post trigger. At egg collection, follicular fluid from follicles with viable eggs were collected for glycomic analysis. Women were excluded if there had known retroviral infection. Samples were stored at -80 °C until analysis. Patients presenting with male factor infertility were used as the control group. Samples were transferred into a clean plastic falcon tube covered with perforated Parafilm and lyophilised before preparation for glycomic analyses were carried out.

## 2.4 Method

### 2.4.1 Cleavage and blocking of disulphide bridges

Prior to reduction/carboxymethylation appropriate measures were taken to ensure that samples were as free as possible of detergents and involatile salts. Samples were lyophilised first to remove volatile contaminants that might interfere with the DTT reduction of S-S bridges.

#### 2.4.1.1 Reduction and Carboxymethylation

A solution of 10mg/ml DTT was prepared in 0.6M degassed TRIS (pH8.5): TRIS buffer: 0.6 M TRIS- 3.63 g of TRIS and complete to 50 ml with ultra-pure water. Adjust to pH 8.5 with HCl. Degas for 30 min by passing a slow stream of nitrogen (oxygen free) through the solution via a Pasteur pipette. Lyophilised samples were dissolved in 1ml of the DTT solution and 200µl 8M HCL to break disulphide bonds. The sample was then incubated for 1 hour at 37°C. Following this, 1.5ml of fresh 12mg/ml IAA in 0.6M Tris (pH 8.5) solution was added to block the reformation of the broken disulphide bonds and lysates were incubated in the dark for another 2 hours at room temperature. The mixtures were then dialysed against 4.5L of 50M ammonium bicarbonate buffer (pH 7.7) at 4°C for 24-48hrs with regular buffer changes. The sample was then lyophilised in a plastic falcon tube covered with perforated Parafilm.

### 2.4.2 Cleavage into glycopeptides

#### 2.4.2.1 Tryptic digestion

Lyophilised samples were dissolved in 300µl of ammonium bicarbonate buffer (50mM, pH8.4, described earlier). For every 1mg of homogenate, 1.5ml of 1mg/ml trypsin solution was added and incubated at 37°C for 14 hours. The reaction was then terminated by incubating the digests at 100°C for 3 minutes. Glycopeptides were then purified by Sep-Pak C<sub>18</sub> (Sep-Pak C18 cartridges

attached to glass syringes were conditioned with methanol, 5% acetic acid, 100%propan-1-ol and 5% acetic acid successively. The sample was then loaded onto the cartridge and eluted sequentially with acetic acid, 20% and 40% propan-1-ol in 5% acetic acid. The 5% acetic acid fraction which contained hydrophilic contaminants were discarded. The propan-1-ol fractions which contained the glycopeptides were collected in glass culture tube). The volume of the collected fractions was reduced in a Speed-Vac and subsequently lyophilised.

### 2.4.3 Cleavage of glycans from glycopeptides using PNGase-F digestion

#### 2.4.3.1 Peptide-N-glycosidase F (PNGase F) digestion:

Lyophilised glycopeptide fractions were dissolved in 200µl of ammonium buffer (50mM, pH8.4, described earlier). 10U of Peptide-N-glycosidase F (PNGase-F) was then added to each sample and incubated at 37°C for 24 hours. This was then lyophylised and dissolved again in 200µl of 5% acetic acid. The N-glycans are released as intact oligosaccharides. The N-glycans were separated from the mixture using Sep-Pak C18, as previously described, except here, the 5% acetic acid fractions contained released N-glycans, and therefore this was collected separately. The volumes collected were again reduced in glass tubes (covered with perforated Parafilm) in a Speed-Vac and subsequently Lyophilised. The purified native N-glycans were later permethylated prior to mass spectrometric analyses or subjected to appropriate (enzymatic/chemical) modifications.

### 2.4.4 Derivatisation of glycans and Sep-Pak® clean-up

#### 2.4.4.1 NaOH permethylation

The purified N-glycans were permethylated. The permethylation of the sample was carried out by adding sodium hydroxide dissolved in dimethylsulphoxide (DMSO) initially and then 0.6ml of methyl iodide: 3 ml of anhydrous DMSO was added to 5 pellets of NaOH in a dry mortar and

ground to form a slurry. 0.5-1 ml of the slurry was added to the sample then add 0.2-0.5 ml of methyl iodide. The sample was agitated for 20min. The reaction was terminated by drop wise addition of ultra-pure water, whilst constantly shaking the tube to reduce the effects of the highly exothermic reaction. This was followed by chloroform extraction. Permethylated samples were extracted with 1ml of chloroform and washed several times with ultra-pure water. The chloroform was then dried under a gentle stream of nitrogen gas. Permethylated samples were then purified by Sep-Pak C<sub>18</sub>.

#### 2.4.4.2 Purification of permethylated samples by Sep-Pak® C18 purification

This time, Sep-Pak C18 cartridges were attached to glass syringes and conditioned with methanol, ultra-pure water acetonitrile and ultra-pure water successively. The permethylated sample was dissolved in 1:1 methanol and ultra-pure water, a total volume of 200µl and loaded onto Sep-Pak C18 cartridge. The samples were eluted stepwise with 15%, 35%, 50% and 75% of aqueous acetonitrile. The 35%, 50% and 75% fractions, containing permethylated glycans) were collected. The volumes were reduced in a Speed-Vac and subsequently lyophilised.

#### 2.4.5 Enzymatic digestion experiments

##### 2.4.5.1 β-1,4 galactosyltransferase experiment

To define N-glycan structures with bisected GlcNAc (GlcNAcβ1,4Man), galactosylation using β1,4 galactosyltransferase was carried out (46). Lyophilised purified native N-glycans (non-permethylated N-glycans) were dissolved in 150 µl of 50 mM MOPS buffer containing 45 µM UDP-galactose (pH 7.4, adjusted with ammonia) before the addition of 20 mM manganese (II) chloride 4-hydrate. For the galactosylation enzymatic reaction, 10 µl of bovine milk β1,4 galactosyltransferase (Calbiochem) was added, and it was incubated for 20 h at 37 °C and

lyophilised. During the  $\beta$ 1,4 galactosyltransferase experiment, any terminal GlcNAc residue on the  $\alpha$ 1,3 and  $\alpha$ 1,6 mannose arms of a N-glycan is modified by the addition on a galactose residue, whereas the GlcNAc terminated residue attached to the mannose of the core chitobiose structure (bisected N-glycan) is not modified by the addition of a galactose residue.

#### 2.4.6 MALDI MS

Samples were each dissolved in 10 $\mu$ l of methanol. Next 1 $\mu$ l of sample was mixed with 1 $\mu$ l of matrix (DHB, (dihydroxybenzoic) 20mg/ml in 70:30 methanol: water). A 1  $\mu$ l aliquot of the mixture was spotted onto a 100 well sample plate. Analysis of glycans was performed using a Voyager MALDI-TOF instrument. The data was processed using Data Explorer 4.9 software and Glyco Workbench. MSMS data were acquired using a MALDI TOF/TOF MS.

##### 2.4.6.1 MALDI MS data interpretation

MALDI spectra of glycans usually contain peaks which correspond to singly charged molecular ions. Interpretation is carried out using the mass to charge (m/z) value of a monoisotopic ion to deduce the molecular weight value from which it is possible to derive the number and type of monosaccharide. With knowledge of glycan biosynthetic pathways, we can suggest possible glycan structures based on the unique glycan composition for a given molecular weight.

## 2.5 Troubleshooting and Development of MS Techniques

Considerable work was dedicated to troubleshooting and refining the mass spectrometry techniques employed in this study, although this detailed development process is not fully documented within the thesis. Initial challenges included optimising sample preparation protocols to ensure consistency and accuracy in glycan analysis. Several iterations of MS settings were

tested to enhance sensitivity and resolution, leading to the identification of optimal parameters for our specific analytes. Additionally, extensive calibration and validation steps were undertaken to confirm the reliability of the data obtained. These efforts were crucial in establishing the robust and precise MS methodologies that underpin the findings presented in this work.

A significant focus was also placed on optimising techniques to avoid contamination. Rigorous cleaning protocols for equipment and careful handling of samples were implemented to minimise the risk of introducing contaminants. Specific steps, such as using high-purity reagents, were incorporated to ensure the integrity of the samples and the accuracy of the results.

To ensure the hcG samples were indeed hCG, a MASCOT search was conducted. This involved using the MASCOT software to match MS data against known protein sequences, verifying the presence of hCG in our samples. This additional layer of validation was crucial for confirming sample identity and ensuring the accuracy of subsequent analyses.

## 2.6 Statistical analysis

All data acquired from the MS analysis were processed according to previous work (203, 204). For a given spectrum, data were normalised by dividing the relative intensity of an identified peak, corresponding to an N-glycan, by the sum of the relative intensities of all identified peaks. The total relative abundance for each structural feature (e.g., high mannose, mono-, bi-, tri- and tetra-antennary N-glycans, agalactosylated, core-fucosylated and bisected N-glycans, and LacNAc, NeuAc, LewisX terminal epitopes) was calculated by summing the normalised relative intensity of each identified N-glycan containing the corresponding structural feature. Specifically, the total relative abundance of terminal epitopes (LacNAc, NeuAc, LewisX) was calculated taking into

account the number of terminal epitopes on each identified peak. The total relative abundance of the antennae (mono-, bi-, tri- and tetra-antennary N-glycans) and the terminal epitopes (LacNAc, NeuAc, LewisX) was expressed as a percentage.

All statistical analyses were performed using SPSS version 25. A paired t-test was applied to analyse differences for paired hCG samples (EP-hCG versus LP-hCG), while an independent samples t-test was used for differences between EP-hCG and GTD-hCG. Significance was set at  $P < 0.05$ . For the paired t-test, the test of normality with Shapiro-Wilk's statistic and outliers was considered for the values resulting from the differences of the above variables (EP-hCG versus LP-hCG). For the independent samples t-test, the test of normality with Shapiro-Wilk's statistic, outliers, and variance according to Levene's test for equality of variances were considered. Variables not meeting the above assumptions were excluded from the analysis.

Hierarchical cluster analysis was applied using between-groups linkage as a clustering method and Euclidean distance as a measure interval (similar results were obtained with other clustering methods and measure intervals). For EP-hCG versus GTD-hCG samples, cluster analysis was performed for the variables found to be significant at least at the  $P < 0.05$  level (tri-antennary, tetra-antennary, core fucosylation, bisected, LacNAc, LewisX, NeuAc), while for the complete set of samples (EP-hCG, LP-hCG and GTD-hCG), hierarchical cluster analysis was performed using NeuAc and bisected N-glycans as variables.

## 3 Chapter 3- Comparative glycomic profiling of urinary hCG in normal and abnormal pregnancy states

### 3.1 Introduction to hCG Analysis

This chapter presents the glycomic analysis of urinary hCG carried out for this thesis.

The analysis was done firstly to describe the glycosylation of urinary hCG in normal pregnancy, secondly to highlight any changes in glycosylation that may occur as normal pregnancy progresses, and finally to compare glycosylation in normal pregnancy to that in abnormal pregnancy states, thereby highlighting any significant changes that may aid early pregnancy biomarker discovery. Purified hCG was provided by NHS Tayside (purification described in chapter 2). Samples were from the urine of eight pregnant women and the N-glycan content was analysed here. Four of the eight women had paired samples taken in the first trimester (weeks 6-9) and again in the 2<sup>nd</sup> trimester (weeks 20-21), to compare any changes in hCG glycosylation between early and late pregnancy. In this group, one of the women developed late onset preeclampsia (PET). Four samples were obtained from women diagnosed in early pregnancy with gestational trophoblastic disease (GTD); these were compared to normal early pregnancy hCG glycosylation content.

The analysis involved a comprehensive method described in chapter 2 of this thesis: hCG was purified, reduced, carboxymethylated, digested with trypsin, and treated to release and permethylate N-glycans. These were then purified and structurally analysed using MALDI-TOF MS and MALDI-TOF/TOF MS/MS, providing detailed glycan compositions and antennae sequences. Supplementary enzymatic galactosylation experiments helped characterise the structures further. The analysis identified significant variations in glycan expression between different pregnancy

stages and conditions, with changes noted in normal pregnancy progression and altered expression in PET and GTD.

Statistical analyses, including paired and unpaired t-tests, were performed on the data. The findings led to the hypothesis that hCG glycosylation changes reflect modifications in the pregnancy state. Supplementary experiments confirmed the presence of novel bisecting N-glycans, implicated in various biological processes and immune control. The results suggest that glycomic analysis of hCG can reveal significant insights into pregnancy progression and complications.

### 3.2 Sample details

Table 3-1 and Table 3-2 presents the detail of samples analysed in this chapter. Samples were processed using methods described in chapter 2.

Table 3-1: Details of hCG concentration from Early (EP-hCG) and late pregnancy (LP-hCG) urine samples collected from 4 individual healthy pregnant women. [hCG] denotes hCG concentration. Early pregnancy= first trimester, Late pregnancy=late 2<sup>nd</sup> trimester. \*\* Patient developed late onset pre-eclampsia in the third trimester.

| <b>Patient ID. (early/late pregnancy)</b> | <b>Gestational age of sample (weeks + days)</b> | <b>Sample [hCG] (IU/L)</b> | <b>[hCG] after purification (mg/L)</b> |
|-------------------------------------------|-------------------------------------------------|----------------------------|----------------------------------------|
| EP-hCG1                                   | 9+1                                             | 835,584                    | 1022                                   |
| LP-hCG1                                   | 20+1                                            | 75,568                     | 221                                    |
| EP-hCG2                                   | 7+4                                             | 369,648                    | 257                                    |
| LP-hCG2                                   | 20+6                                            | 63,107                     | 52                                     |
| EP-hCG3                                   | 7+1                                             | 120,101                    | 138                                    |
| LP-hCG3                                   | 20+1                                            | 11,468                     | 17                                     |
| EP-hCG4**                                 | 7+6                                             | 183,382                    | 389                                    |
| LP-hCG4**                                 | 20+3                                            | 96,163                     | 89                                     |

Table 3-2: Details of hCG concentration from urine samples collected from patients with GTD. All GTD pregnancies were subsequently histologically diagnosed as complete moles. \*Patient identity GTD-hCG4 had persistent trophoblastic neoplasia requiring multiagent chemotherapy to achieve normalisation of serum hCG and disease resolution, The remaining 3 patients had benign disease with serum hCG regressing to undetectable levels following molar evacuation.

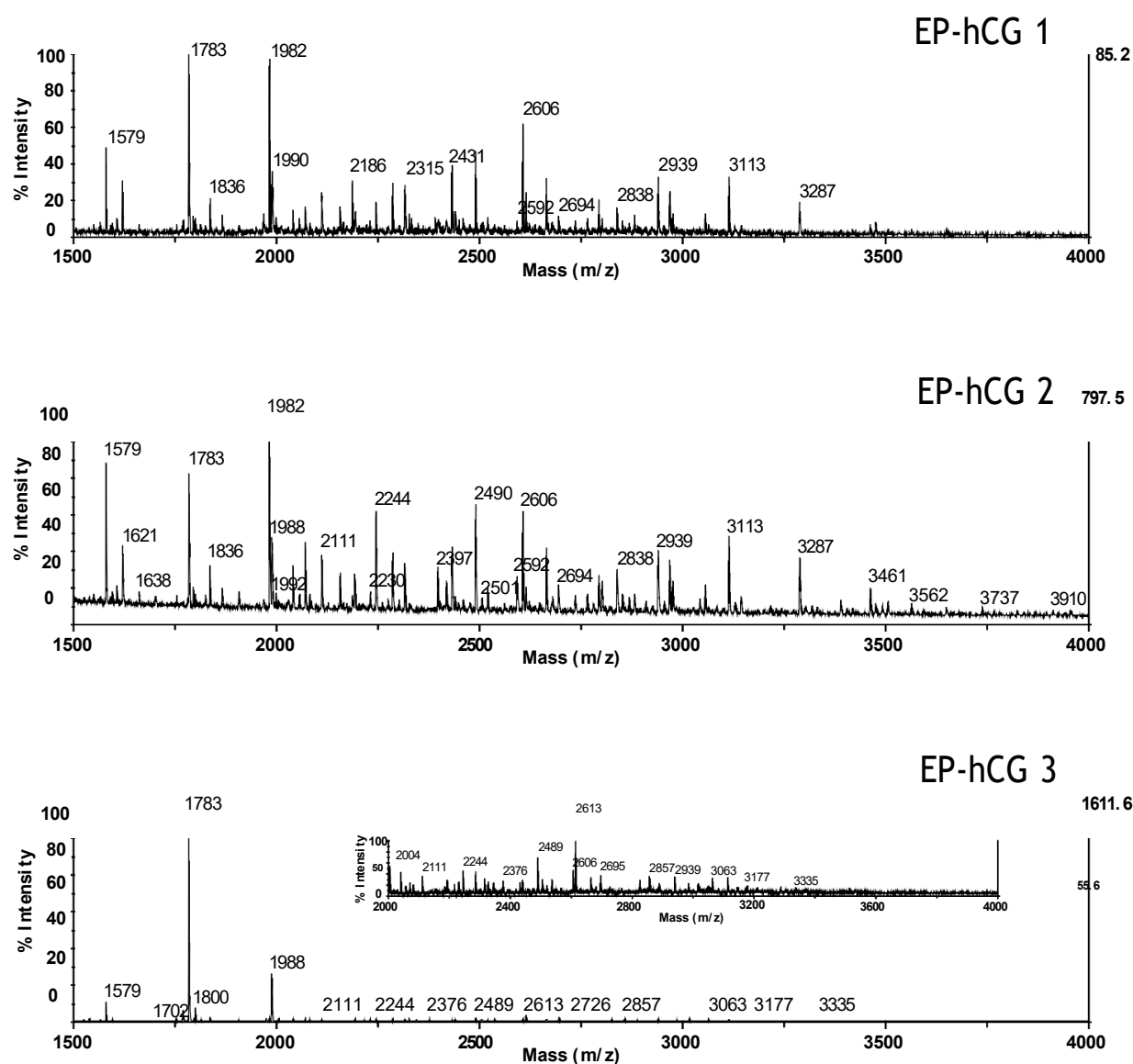
| <b>Patient Id. (Molar pregnancy)</b> | <b>Sample hCG (IU/L)</b> | <b>[hCG] after purification (mg/L)</b> | <b>Histology (Mole type)</b> | <b>Benign or Malignant?</b> |
|--------------------------------------|--------------------------|----------------------------------------|------------------------------|-----------------------------|
| GTD-hCG1                             | 693,492                  | 1297                                   | CHM                          | Benign                      |
| GTD-hCG2                             | 1,276 646                | 2978                                   | CHM                          | Benign                      |
| GTD-hCG3                             | 64,448                   | 361                                    | CHM                          | Benign                      |
| GTD-hCG4*                            | 7,704 768                | 11,544                                 | CHM                          | Malignant                   |

### 3.3 Results

#### 3.3.1 MALDI MS analysis EP-hCG

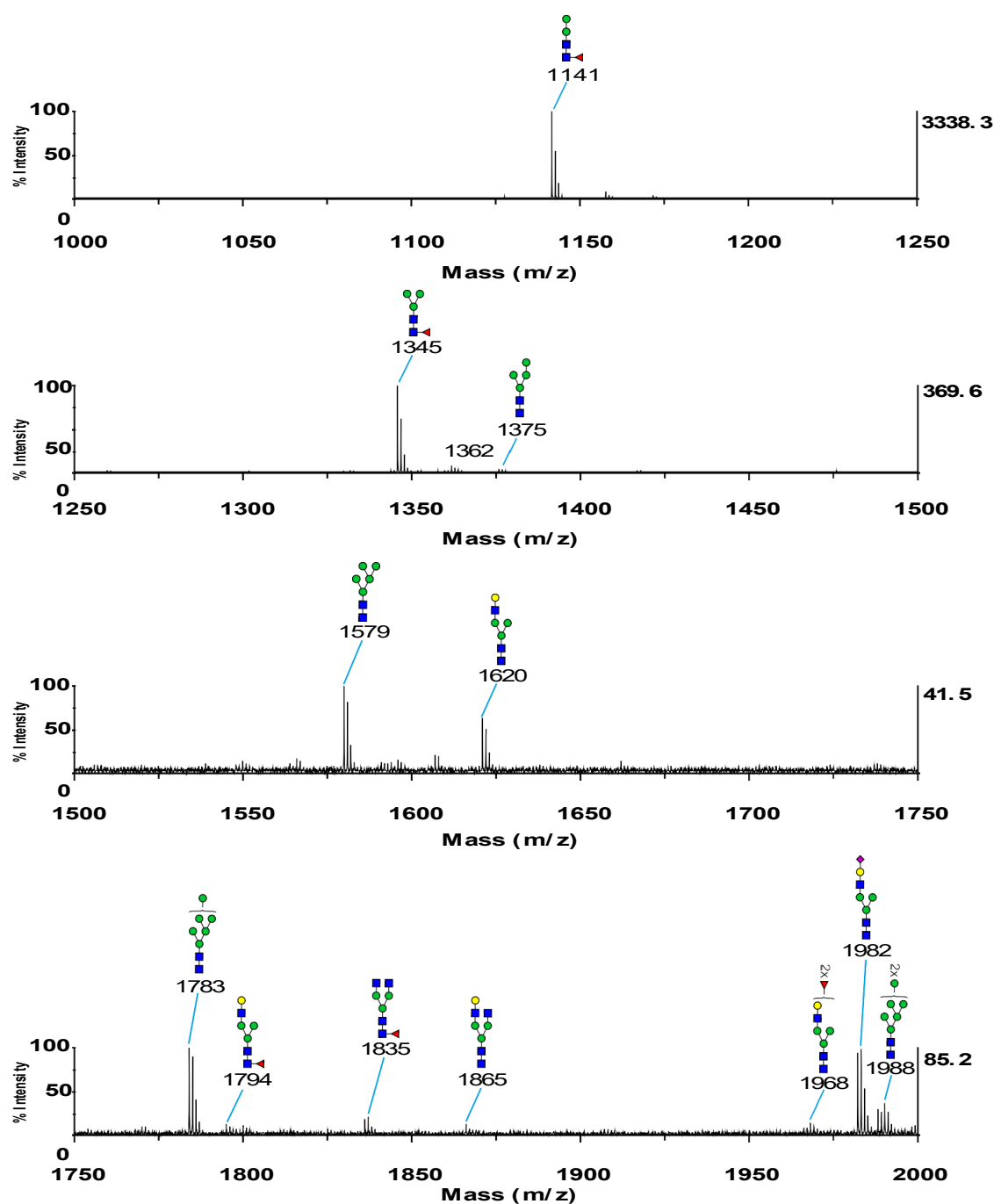
MALDI data were obtained from N-glycans released from hCG using methods described in chapter 2. MALDI-TOF MS spectra of permethylated early pregnancy hCG N-glycans for normal pregnancy from three different samples (EP-hCG1, EP-hCG2, EP-hCG3) are shown in Figure 3-1. All molecular ions are singly charged sodiated ions  $[M+Na]^+$ . The Putative structures for the monoisotopic peaks are illustrated in Figure 3-2 and are based on the molecular mass composition, tandem MS/MS data,  $\beta$ -galactosyltransferase experiments and knowledge of N-glycan biosynthetic pathways previously described in chapters 1 and 2.

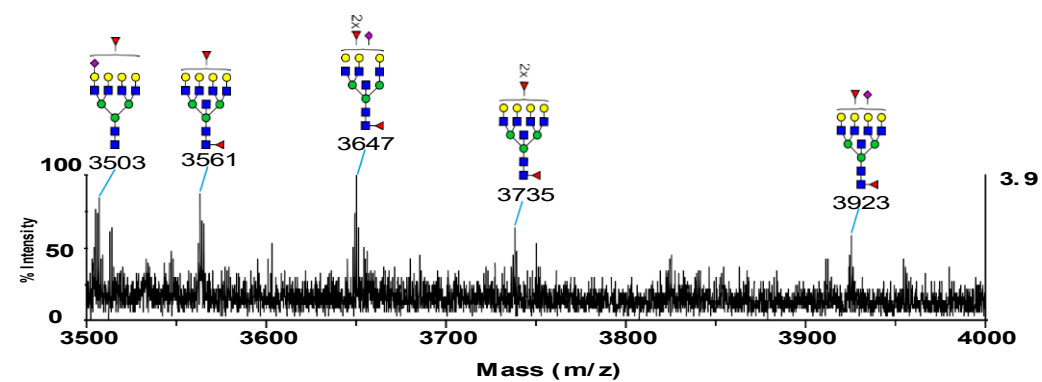
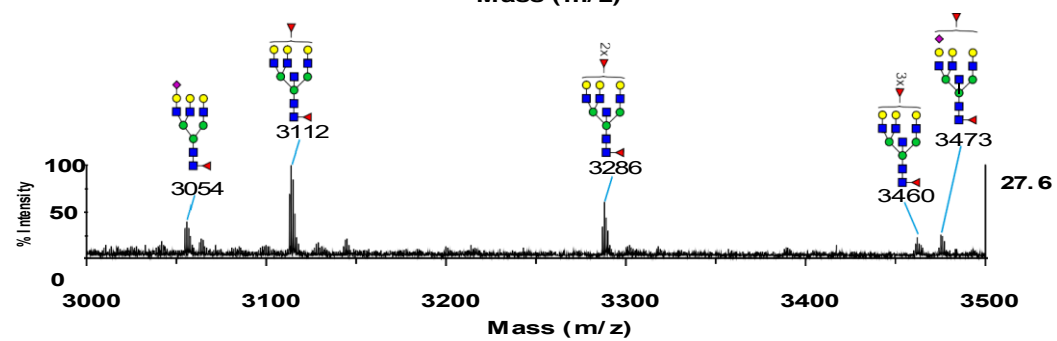
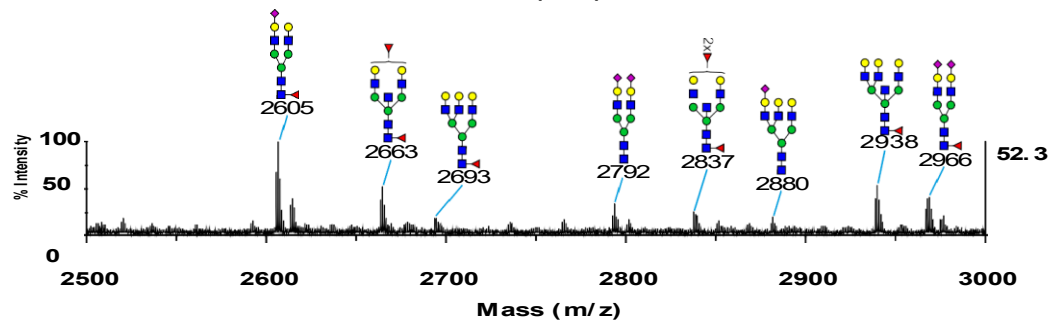
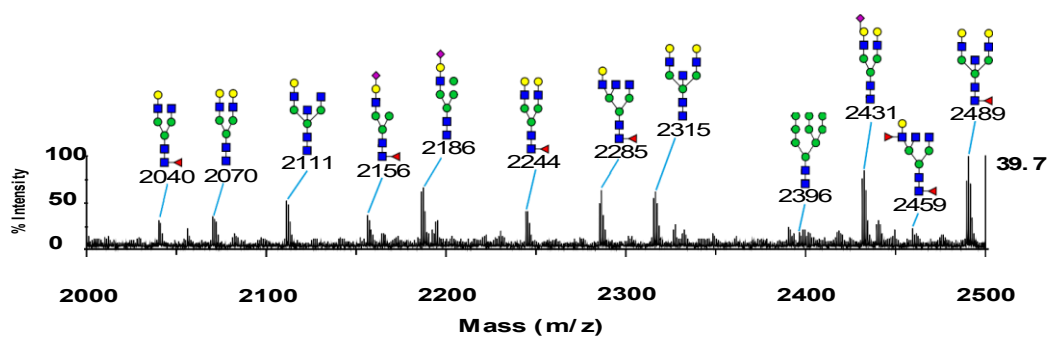
Figure 3-1: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) of permethylated N-glycans from human chorionic gonadotropin purified from pregnancy urine samples from 3 different patients in normal early pregnancy: EP-hCG1, -hCG2, -hCG3.



The MS shows that EP-hCG MS is dominated by peaks at the lower mass range at m/z 1579, 1783 and 1982, corresponding to high mannose structures and a monoantennary sialylated structure. The EP samples share the same glycan repertoire, albeit with some minor differences in the relative abundance of each glycan between the different EP-hCG samples.

Figure 3-2: Annotated MALDI-TOF MS spectra of permethylated N-glycans derived from EP-hCG 1 sample. Significant peaks are annotated with putative structures based on composition, tandem MS,  $\beta$ -galactosyltransferase experiment, and knowledge of biosynthetic pathways. All molecular ions are  $[M+Na]^+$ . Structures above a bracket were not unequivocally defined. The lower mass region was predominated by high mannose type N-glycans as well as monoantennary sialylated and biantennary type N-glycans. The higher mass regions were characterised by tri- and tetra-antennary fucosylated N-glycans. The dominant complex N-glycan in here is a monoantennary structure sialylated with NeuAc represented by a peak at  $m/z$  1982. This is typical of all EP-hCG samples studied.





### 3.3.2 MALDI MS analysis LP-hCG

MALDI-TOF MS spectra of permethylated late pregnancy hCG N-glycans for normal pregnancy from matched pairs of EP-hCG1, EP-hCG2 and EP-hCG3 are shown in **Error! Reference source not found.**, (LP-hCG1, LP-hCG2 and LP-hCG3). The Putative structures for the monoisotopic peaks are illustrated in Figure 3-4

Figure 3-3: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) of permethylated N-glycans from human chorionic gonadotropin purified from pregnancy urine samples from 3 different patients in normal late pregnancy: LP-hCG1, -hCG2, -hCG3. The MS shows that LP-hCG MS is dominated by peaks at both the lower mass range at  $m/z$  1579 and 1783 representing high mannose structure; as well as peaks at higher mass ranges at  $m/z$  2489, 2663 and 3112 representing novel bisected type N-glycans. The LP samples, like the EP samples also share the same glycan repertoire, with some minor differences in the relative abundance of each glycan between the different LP-hCG samples.

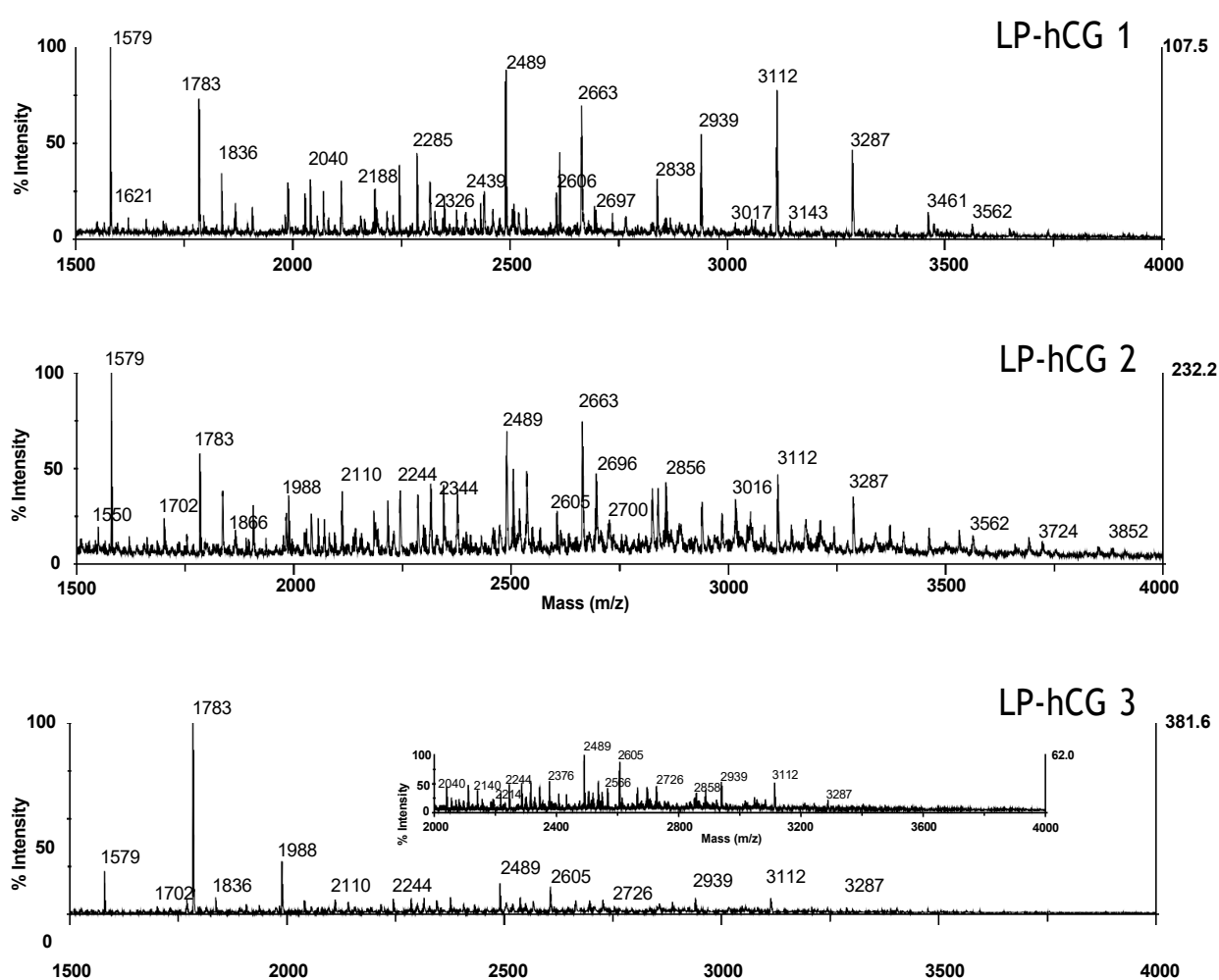
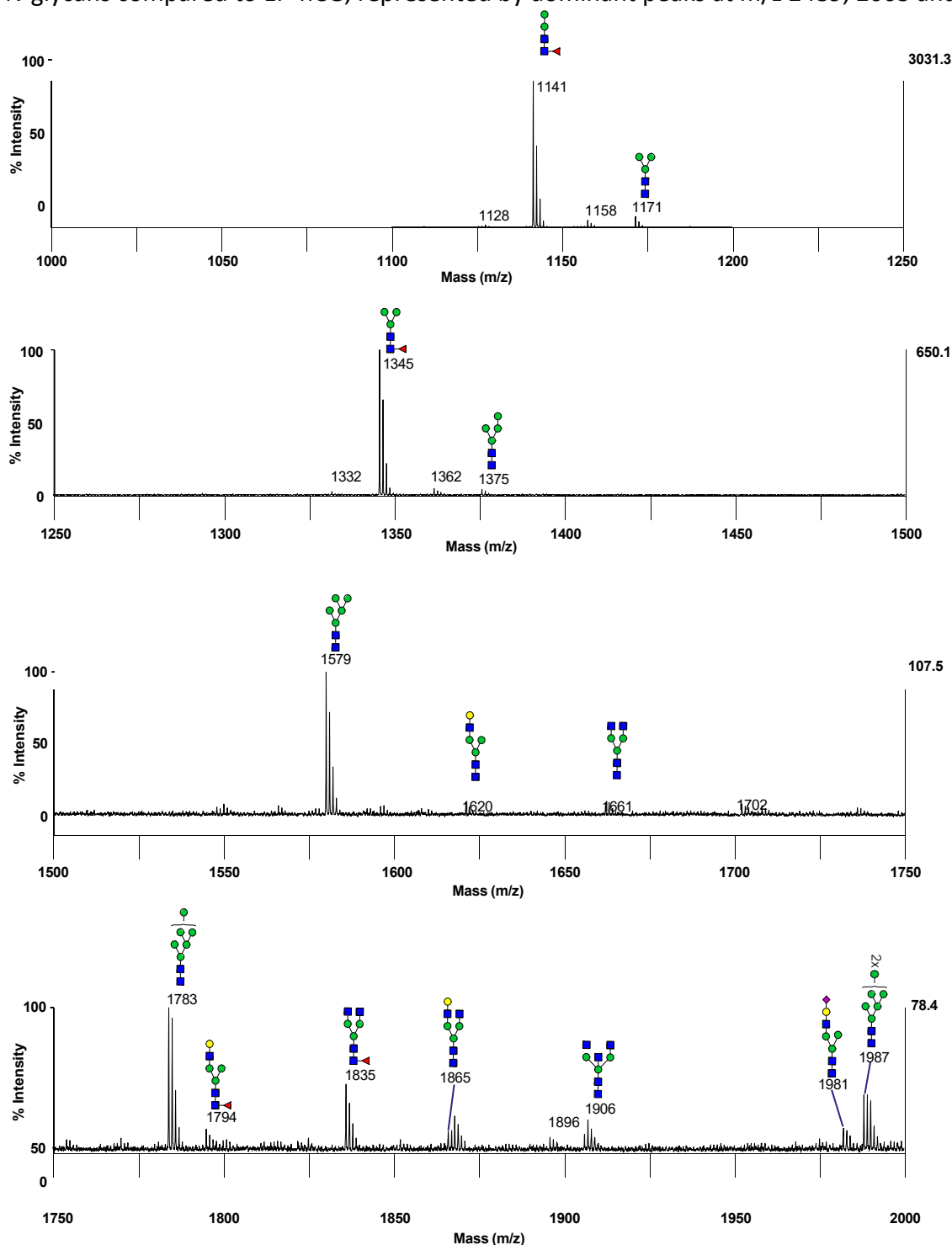
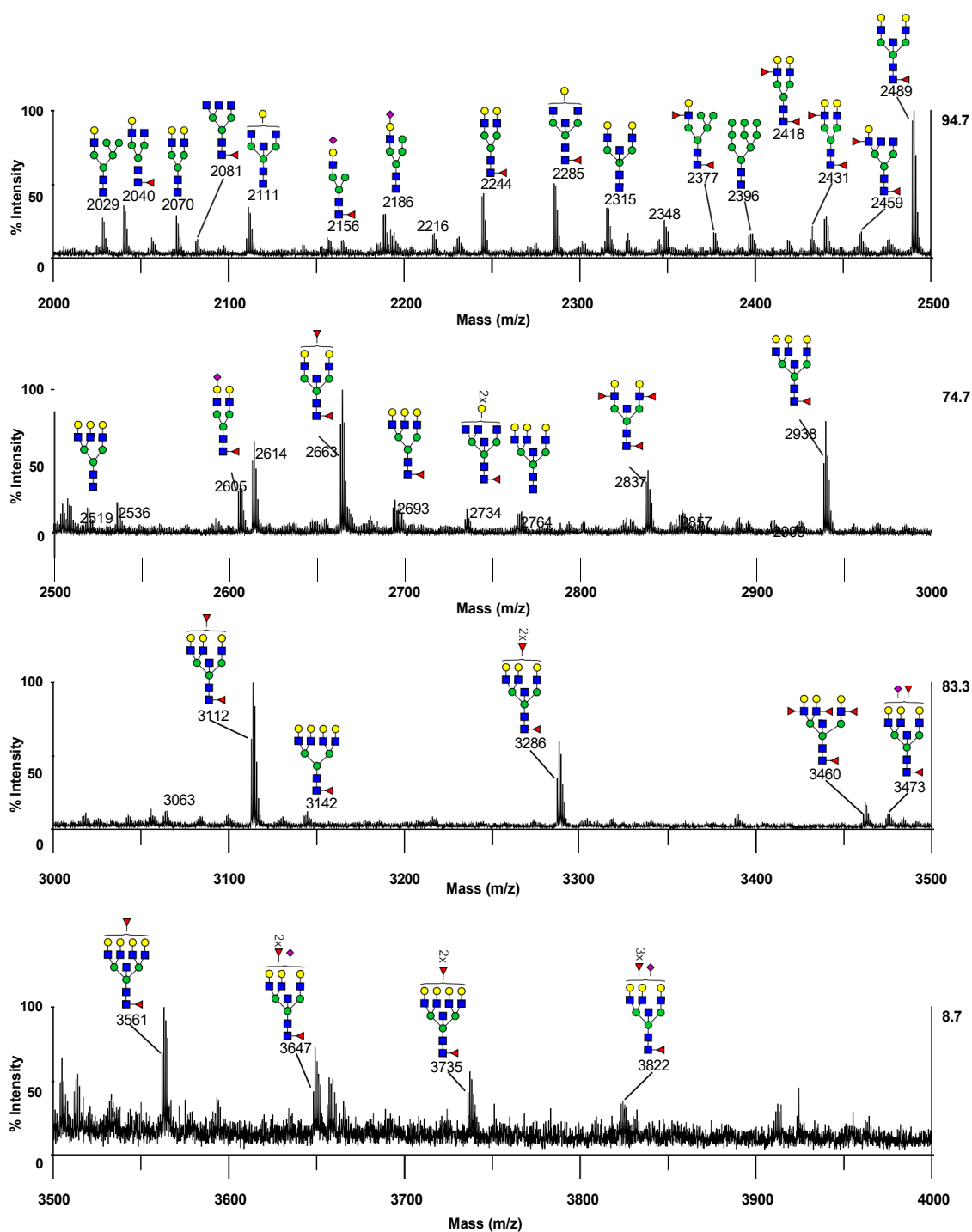


Figure 3-4: Annotated MALDI-TOF MS spectra of permethylated N-glycans derived from LP-hCG sample. Significant peaks are annotated with putative structures based on composition, tandem MS,  $\beta$ -galactosyltransferase experiments, and knowledge of biosynthetic pathways. All molecular ions are  $[M+Na]^+$ . Structures above a bracket were not unequivocally defined. The lower mass region was predominated by high mannose type N-glycans as well as monoantennary sialylated and biantennary type N-glycans. The higher mass regions were characterised by tri- and tetra-antennary fucosylated N-glycans. LP-hCG MS displayed increased expression of the novel bisected N-glycans compared to EP-hCG, represented by dominant peaks at  $m/z$  2489, 2663 and 3112.





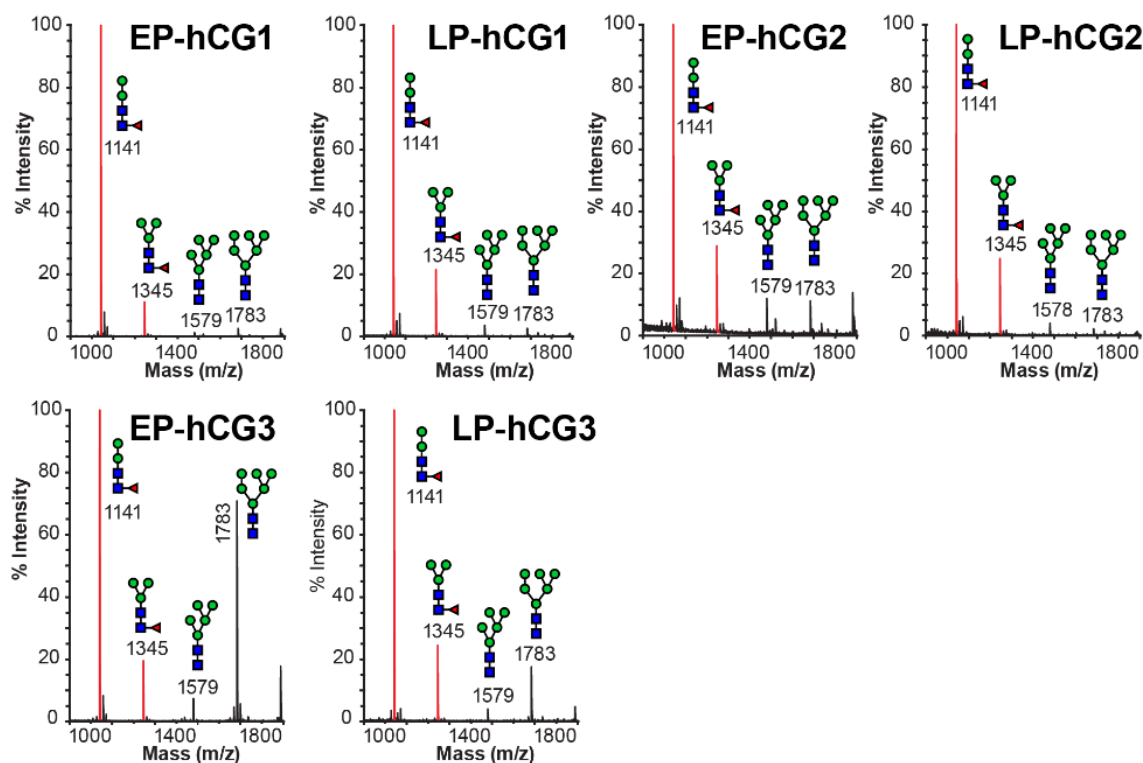
### 3.3.3 Glycomic characterisation of normal early pregnancy hCG and late pregnancy hCG

The MS data show that EP-hCG and LP-hCG possess a mixture of high mannose and complex type N-glycans. The high mannose peaks are represented by  $m/z$  1579 and 1783. The complex type N-glycans consisted of mono- ( $m/z$  1620, 1982), bi- ( $m/z$  2244, 2431, 2489, 2605), tri- ( $m/z$  2693, 2938, 3112, 3286) and tetra-antennary ( $m/z$  3142, 3561, 3735, 3923) structures with varying amounts of core fucosylation and antennae sialylation. This high mass end of the spectrum displayed more complex branched N-glycans and accounted for a smaller portion of the hCG N-glycans. Molecular ions were observed up to approximately  $m/z$  3923.

The lower mass region was dominated by high mannose type N-glycans as well as monoantennary sialylated and biantennary type N-glycans. The higher mass regions were characterised by tri- and tetra-antennary fucosylated N-glycans. The dominant complex N-glycan in EP-hCG is a monoantennary structure sialylated with NeuAc represented by a peak at  $m/z$  1982. In LP-hCG  $m/z$  2489 is the dominant MS peak and it corresponds to a complex N-glycan, a biantennary core fucosylated structure with no antennae sialylation.

In both EP and LP-hCG, complex structures were extended with LacNAc, NeuAc and Lewis<sup>x</sup> epitopes. Also observed in EP-hCG and LP-hCG samples were a high abundance of paucimannose type N-glycans at  $m/z$  1141 and 1345 Figure 3-5; the lower mass regions were dominated by these paucimannose and high mannose type structure as well as mono- and bi-antennary structures. The presence of molecular ions that were consistent with bisected glycans was confirmed by further experimental analysis detailed in chapter 2 and presented later in this chapter.

Figure 3-5: Paucimannose type N-glycans on EP and LP-hCG samples. MS spectra of permethylated N-glycans derived from EP-hCG, LP-hCG. Red peaks correspond to paucimannose type N-glycans. The high mannose N-glycans found at  $m/z$  1579 and 1783 are shown here for comparison of the relative intensity of the paucimannose type N-glycans.



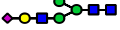
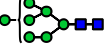
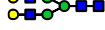
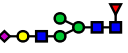
The EP samples shared the same glycan repertoire; however, some minor differences were noted in the relative abundance of each glycan group between the different EP-hCG samples. The molecular assignment and relative abundances of each significant peak is tabulated in Table 3-3.

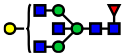
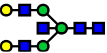
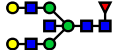
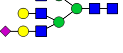
42% of EP-hCG were high mannose structures. The complex N-glycans were made up of 17.7% mono-, 56.7% bi-, 21.8% tri and 3.8% tetra-antennary N-glycans. 24.7% of these structures were agalactosylated. 60% of structures were core fucosylated and 23.6% were proven to be bisected structures. Notable terminal epitopes in EP-hCG included LacNAc, NeuAc and Lewis<sup>X</sup>. 62.4% of EP-hCG structures were capped with LacNAc, NeuAc residues were observed in 27.2% and Lewis<sup>X</sup> epitopes represented 10.4% of the capping sugars on the antennae in EP-hCG.

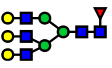
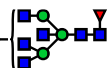
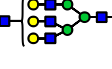
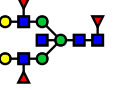
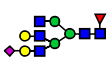
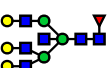
Similarly, LP-hCG samples also shared the same glycan repertoires with a small amount of inter-sample variation in the relative abundances as tabulated in Table 3-3. 28.5% of LP-hCG consisted of high mannose structures. The complex N-glycans were made up of 9% mono-, 62.6% bi-, 24.9% tri and 3.5% tetra-antennary structures. 30% of structures were agalactosylated. 66.7% of structures were core fucosylated. Compared to EP-hCG, a larger proportion of structures, 44.9% were proven to be bisected. LP-hCG displayed the same terminal epitopes as EP-hCG, though with differences in observed abundance. 67.7% of structures were capped with LacNAc, 17.6% NeuAc and 14.7% LewisX epitopes.

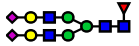
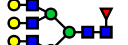
Table 3-3: Molecular assignment and relative abundance of N- glycans in each sample. The table presents the proposed structure for the observed signals as well as their relative abundance within each sample.

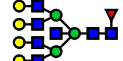
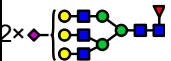
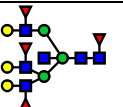
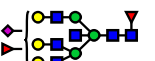
| structure                                                                           | m/z  | Relative intensity |             |             |             |             |             |             |             |       |       |       |       |
|-------------------------------------------------------------------------------------|------|--------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------|-------|-------|-------|
|                                                                                     |      | EP-<br>hCG1        | EP-<br>hCG2 | EP-<br>hCG3 | EP-<br>hCG4 | LP-<br>hCG1 | LP-<br>hCG2 | LP-<br>hCG3 | LP-<br>hCG4 | GTD 1 | GTD 2 | GTD 3 | GTD 4 |
|    | 1141 | 91.34              | 100         | 100         | 100         | 95.52       | 99.82       | 94.26       | 99.79       | 61.17 | 11.31 | 13.14 | 3.46  |
|    | 1345 | 10.15              | 29.5        | 0.11        | 17.9        | 20.18       | 25.01       | 23.15       | 16.25       | 16.43 | 3.75  | 3.50  | 1.93  |
|    | 1579 | 1.24               | 11.94       | 7.29        | 10.48       | 3.54        | 3.88        | 3.97        | 3.73        | 5.25  | 1.94  | 1.76  | 0.44  |
|  | 1620 | 0.78               | 5.42        | 0.45        | 2.12        | 0.40        | 0.44        | 0.27        | 0.55        | 3.25  | 1.56  | 1.47  | 1.14  |
|  | 1783 | 2.55               | 11.24       | 70.83       | 9.21        | 2.59        | 2.19        | 17.47       | 3.67        | 3.30  | 1.06  | 2.94  | 0.32  |
|  | 1835 | 0.45               | 3.96        | 1.45        | 2.51        | 1.22        | 1.41        | 1.43        | 1.41        | 1.83  | 0.74  | 0.00  | 0.27  |
|  | 1865 | 0.30               | 2.17        | 0.38        | 1.53        | 0.39        | 0.59        | 0.40        | 0.56        | 1.83  | 1.13  | 1.14  | 1.12  |
|  | 1906 | 0.15               | 1.84        | 0.38        | 0.73        | 0.34        | 0.93        | 0.82        | 1.61        | 1.15  | 0.00  | 6.31  | 0.00  |

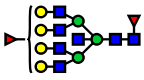
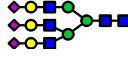
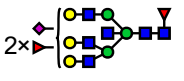
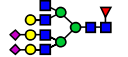
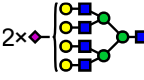
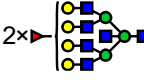
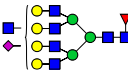
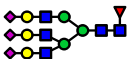
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|-------------------------------------------------------------------------------------|------|------|-------|-------|-------|------|------|------|------|-------|-------|-------|-------|
|    | 1982 | 2.49 | 12.65 | 1.13  | 29.68 | 0.41 | 0.96 | 0.64 | 4.12 | 38.36 | 32.19 | 49.27 | 52.81 |
|    | 1988 | 0.66 | 6.20  | 17.73 | 5.80  | 1.04 | 1.33 | 4.70 | 2.00 | 2.66  | 0.00  | 0.00  | 0.53  |
|    | 2040 | 0.33 | 4.04  | 1.01  | 1.69  | 1.11 | 0.95 | 1.22 | 0.86 | 1.55  | 0.82  | 0.00  | 0.43  |
|    | 2070 | 0.42 | 5.86  | 0.53  | 2.72  | 0.89 | 0.83 | 0.53 | 0.94 | 4.33  | 1.47  | 1.76  | 1.22  |
|   | 2081 | 0.14 | 1.58  | 0.47  | 1.28  | 0.36 | 0.46 | 0.62 | 0.70 | 1.16  | 0.00  | 2.44  | 0.29  |
|  | 2111 | 0.61 | 4.85  | 0.86  | 3.88  | 1.08 | 1.42 | 1.25 | 2.48 | 3.28  | 2.38  | 6.90  | 1.57  |
|  | 2156 | 0.43 | 3.24  | 0.29  | 2.70  | 0.44 | 0.46 | 0.59 | 0.87 | 2.25  | 4.02  | 3.48  | 4.78  |
|  | 2186 | 0.74 | 1.60  | 0.00  | 2.32  | 0.32 | 0.44 | 0.49 | 1.38 | 2.21  | 4.92  | 8.65  | 6.22  |
|  | 2244 | 0.49 | 8.01  | 0.94  | 2.69  | 1.31 | 1.27 | 1.35 | 0.99 | 2.50  | 1.07  | 0.00  | 0.56  |

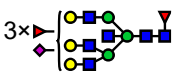
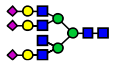
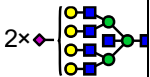
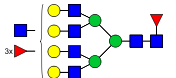
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|-------------------------------------------------------------------------------------|------|------|------|------|------|------|------|------|------|-------|------|------|------|
|    | 2285 | 0.57 | 4.50 | 0.91 | 3.06 | 1.58 | 1.37 | 1.23 | 1.96 | 3.29  | 1.31 | 2.15 | 0.51 |
|    | 2315 | 0.65 | 4.27 | 0.75 | 3.19 | 1.07 | 1.59 | 1.36 | 2.50 | 3.87  | 2.42 | 6.10 | 1.21 |
|    | 2396 | 0.21 | 4.11 | 0.20 | 1.61 | 0.46 | 0.66 | 0.36 | 0.70 | 0.96  | 0.48 | 0.00 | 0.36 |
|    | 2431 | 0.89 | 5.02 | 0.42 | 7.74 | 0.40 | 0.52 | 0.80 | 1.36 | 11.57 | 8.25 | 7.17 | 5.78 |
|  | 2489 | 0.87 | 7.80 | 1.21 | 5.03 | 2.91 | 2.18 | 2.40 | 3.80 | 5.79  | 1.67 | 2.77 | 0.80 |
|  | 2519 | 0.21 | 2.77 | 0.35 | 1.28 | 0.50 | 1.16 | 0.93 | 1.64 | 1.97  | 0.00 | 4.78 | 0.00 |
|  | 2605 | 1.04 | 6.79 | 0.85 | 6.29 | 0.77 | 0.99 | 1.97 | 2.99 | 5.25  | 4.81 | 4.76 | 3.10 |
|  | 2663 | 0.59 | 5.00 | 0.46 | 3.57 | 1.88 | 1.79 | 0.83 | 2.69 | 1.22  | 1.68 | 2.42 | 0.86 |
|  | 2676 | 0.15 | 1.51 | 0.00 | 1.55 | 0.00 | 0.00 | 0.44 | 0.85 | 2.51  | 3.28 | 2.51 | 1.75 |

|                                                                                              |      |      |      |      |       |      |      |      |      |       |       |       |       |
|----------------------------------------------------------------------------------------------|------|------|------|------|-------|------|------|------|------|-------|-------|-------|-------|
|                                                                                              |      |      |      |      |       |      |      |      |      |       |       |       |       |
|             | 2693 | 0.27 | 2.25 | 0.30 | 1.55  | 0.49 | 0.66 | 0.48 | 1.17 | 1.62  | 0.99  | 2.12  | 0.50  |
| $2 \times$  | 2734 | 0.16 | 1.57 | 0.25 | 1.63  | 0.30 | 0.51 | 0.00 | 0.74 | 2.01  | 1.12  | 0.00  | 0.73  |
|             | 2764 | 0.21 | 2.19 | 0.27 | 2.16  | 0.39 | 0.55 | 0.46 | 0.87 | 3.62  | 1.48  | 0.00  | 0.00  |
|            | 2792 | 0.31 | 2.67 | 0.00 | 27.93 | 0.21 | 0.46 | 0.32 | 5.08 | 74.63 | 76.31 | 73.95 | 75.65 |
|           | 2837 | 0.40 | 3.27 | 0.00 | 2.02  | 0.93 | 1.19 | 0.43 | 1.48 | 1.39  | 1.54  | 0.00  | 0.00  |
|           | 2850 | 0.19 | 1.98 | 0.00 | 1.66  | 0.00 | 0.61 | 0.58 | 1.05 | 2.08  | 1.92  | 0.00  | 0.00  |
|           | 2880 | 0.18 | 1.85 | 0.20 | 1.36  | 0.00 | 0.00 | 0.40 | 0.00 | 2.04  | 1.73  | 0.00  | 0.00  |
|           | 2938 | 0.61 | 4.28 | 0.73 | 3.59  | 1.23 | 1.14 | 0.89 | 2.10 | 5.17  | 1.68  | 0.00  | 1.43  |
|                                                                                              | 2966 | 0.42 | 3.14 | 0.00 | 16.35 | 0.00 | 0.00 | 0.27 | 5.98 | 20.60 | 51.85 | 53.52 | 49.45 |

|                                                                                     |      |      |      |      |      |      |      |      |      |      |      |      |      |
|-------------------------------------------------------------------------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|
|    |      |      |      |      |      |      |      |      |      |      |      |      |      |
|    | 3037 | 0.00 | 0.00 | 0.00 | 0.80 | 0.00 | 0.00 | 0.00 | 0.00 | 2.08 | 2.21 | 0.00 | 1.86 |
|    | 3054 | 0.26 | 1.92 | 0.25 | 1.87 | 0.22 | 0.00 | 0.49 | 1.00 | 1.80 | 1.71 | 1.70 | 0.76 |
|    | 3112 | 0.56 | 4.70 | 0.46 | 3.40 | 1.69 | 1.31 | 0.82 | 2.37 | 1.18 | 1.02 | 1.25 | 0.70 |
|  | 3142 | 0.10 | 1.53 | 0.24 | 1.03 | 0.25 | 0.38 | 0.22 | 0.59 | 1.10 | 0.00 | 0.00 | 0.00 |
|  | 3211 | 0.00 | 0.91 | 0.31 | 1.52 | 0.00 | 0.91 | 0.37 | 0.00 | 2.17 | 3.00 | 1.78 | 2.14 |
|  | 3241 | 0.00 | 1.14 | 0.15 | 1.37 | 0.00 | 0.46 | 0.26 | 0.00 | 2.57 | 1.83 | 1.37 | 1.05 |
|  | 3286 | 0.03 | 3.45 | 0.21 | 1.80 | 0.96 | 0.89 | 0.38 | 1.25 | 0.77 | 0.83 | 0.00 | 0.34 |

|                                                                                     |      |      |      |      |      |      |      |      |      |      |      |      |      |
|-------------------------------------------------------------------------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|
|    |      |      |      |      |      |      |      |      |      |      |      |      |      |
|    | 3299 | 0.00 | 1.21 | 0.12 | 0.95 | 0.00 | 0.00 | 0.16 | 0.00 | 1.49 | 0.76 | 0.00 | 0.24 |
|    | 3387 | 0.07 | 1.12 | 0.11 | 0.51 | 0.20 | 0.36 | 0.07 | 0.49 | 0.98 | 0.00 | 0.00 | 0.00 |
|    | 3415 | 0.00 | 0.92 | 0.10 | 1.18 | 0.00 | 0.00 | 0.00 | 0.55 | 1.61 | 1.49 | 1.12 | 0.64 |
|  | 3460 | 0.12 | 1.71 | 0.12 | 0.66 | 0.28 | 0.56 | 0.14 | 0.59 | 0.78 | 0.65 | 0.00 | 0.00 |
|  | 3473 | 0.09 | 1.05 | 0.11 | 1.18 | 0.21 | 0.00 | 0.19 | 0.79 | 0.81 | 0.65 | 0.00 | 0.28 |
|  | 3486 | 0.00 | 0.00 | 0.06 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.85 | 0.83 | 0.00 | 0.30 |
|  | 3503 | 0.00 | 1.38 | 0.13 | 0.94 | 0.14 | 0.00 | 0.17 | 0.43 | 0.95 | 0.70 | 0.00 | 0.20 |

|                                                                                     |      |      |      |      |      |      |      |      |      |      |      |      |      |
|-------------------------------------------------------------------------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|
|    | 3561 | 0.06 | 1.09 | 0.12 | 0.84 | 0.20 | 0.39 | 0.15 | 0.48 | 0.00 | 0.00 | 0.00 | 0.00 |
|    | 3603 | 0.06 | 0.00 | 0.00 | 2.34 | 0.00 | 0.00 | 0.00 | 0.90 | 6.38 | 5.93 | 2.51 | 5.84 |
|    | 3647 | 0.06 | 0.78 | 0.00 | 0.97 | 0.12 | 0.00 | 0.10 | 0.54 | 0.00 | 0.70 | 0.00 | 0.25 |
|   | 3660 | 0.00 | 0.00 | 0.09 | 0.62 | 0.00 | 0.41 | 0.00 | 0.00 | 0.47 | 0.46 | 0.00 | 0.00 |
|  | 3690 | 0.00 | 0.00 | 0.14 | 0.64 | 0.00 | 0.41 | 0.11 | 0.00 | 0.49 | 0.40 | 0.00 | 0.00 |
|  | 3735 | 0.04 | 0.82 | 0.00 | 0.58 | 0.12 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
|  | 3749 | 0.00 | 0.00 | 0.10 | 0.60 | 0.00 | 0.00 | 0.11 | 0.00 | 0.42 | 0.00 | 0.00 | 0.00 |
|  | 3776 | 0.00 | 0.65 | 0.00 | 1.30 | 0.00 | 0.00 | 0.00 | 0.56 | 2.17 | 4.50 | 2.33 | 5.36 |

|    | 3822 | 0.00 | 0.68 | 0.07 | 0.59 | 0.10 | 0.00 | 0.10 | 0.38 | 0.00 | 0.00 | 0.00 | 0.00 |
|-------------------------------------------------------------------------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|
|    | 3848 | 0.00 | 0.00 | 0.07 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.31 | 0.31 | 0.00 | 0.66 |
|    | 3864 | 0.00 | 0.70 | 0.05 | 0.69 | 0.00 | 0.00 | 0.00 | 0.30 | 0.42 | 0.40 | 0.40 | 0.44 |
|  | 3909 | 0.00 | 0.91 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
|  | 3923 | 0.03 | 0.68 | 0.00 | 0.59 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

### 3.3.4 MALDI MS analysis of GTD-hCG

MALDI MS analysis of N-glycans of GTD-hCG revealed fewer glycoforms than EP and LP-hCG with structures up to m/z 3864. The MS include minor abundances of high mannose structures at m/z 1579 and 1783, and a greater portion of complex type N-glycans made up of mono-, bi-, tri-, and tetra-antennary structures, with the most dominant peak at m/z 2792. Figure 3-6 depicts the

MALDI-TOF MS of permethylated GTD hCG N-glycans from GTD-hCG 1, GTD-hCG 2, GTD-hCG 3, GTD-hCG 4. The Putative structures for the monoisotopic peaks are illustrated in Figure 3-7.

Figure 3-6: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) of permethylated N-glycans from human chorionic gonadotropin purified from pregnancy urine samples from 4 different patients in with GTD: GTD-hCG1, GTD-hCG2, GTD-hCG3, GTD-hCG4. The MS shows dominant peaks at  $m/z$  1982, 2792 and 2966 representing mono and biantennary sialylated structures. The GTD-hCG samples share the same glycan repertoire, with very minor differences in the relative abundance of each glycan between the different GTD-hCG samples.

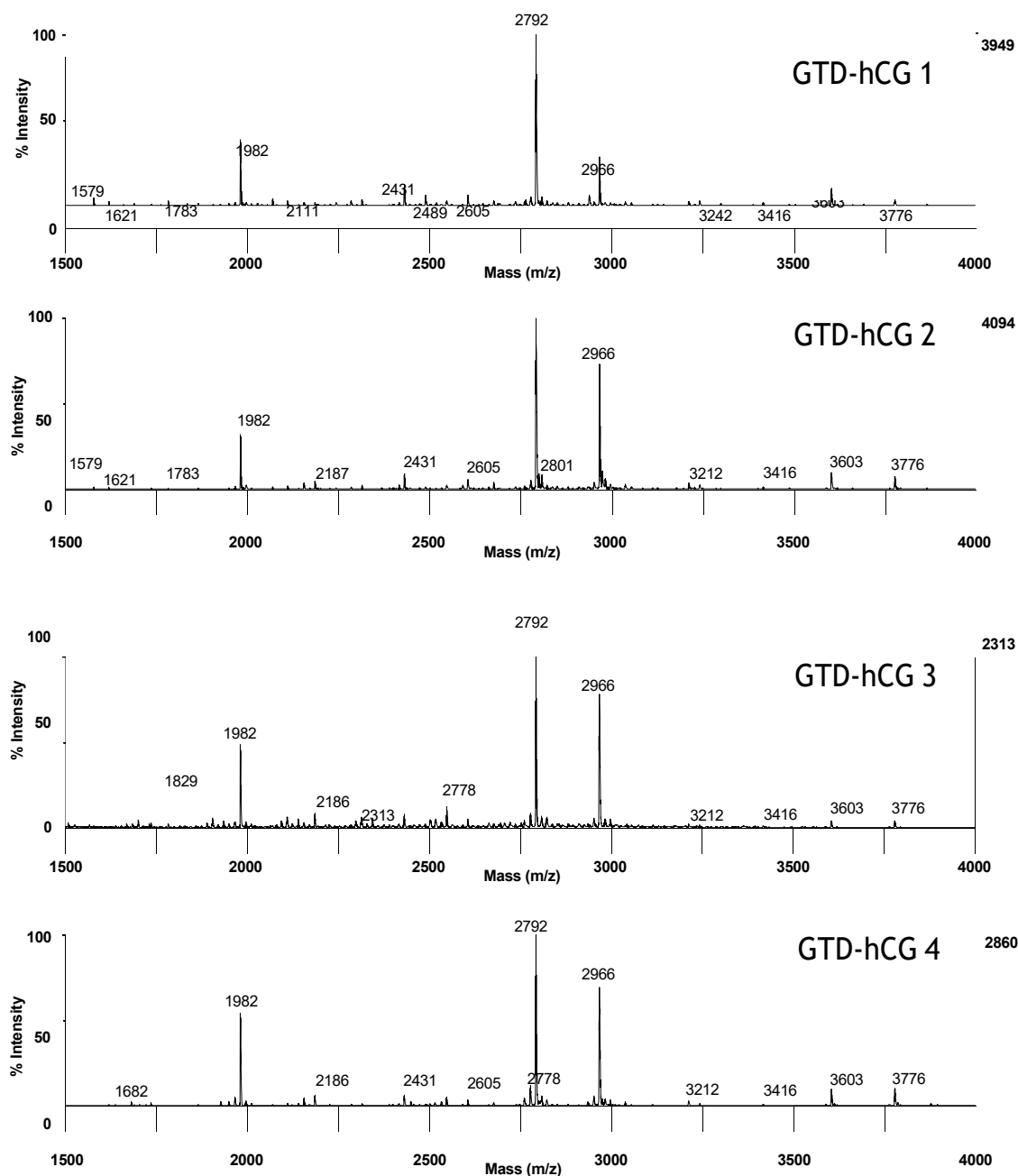
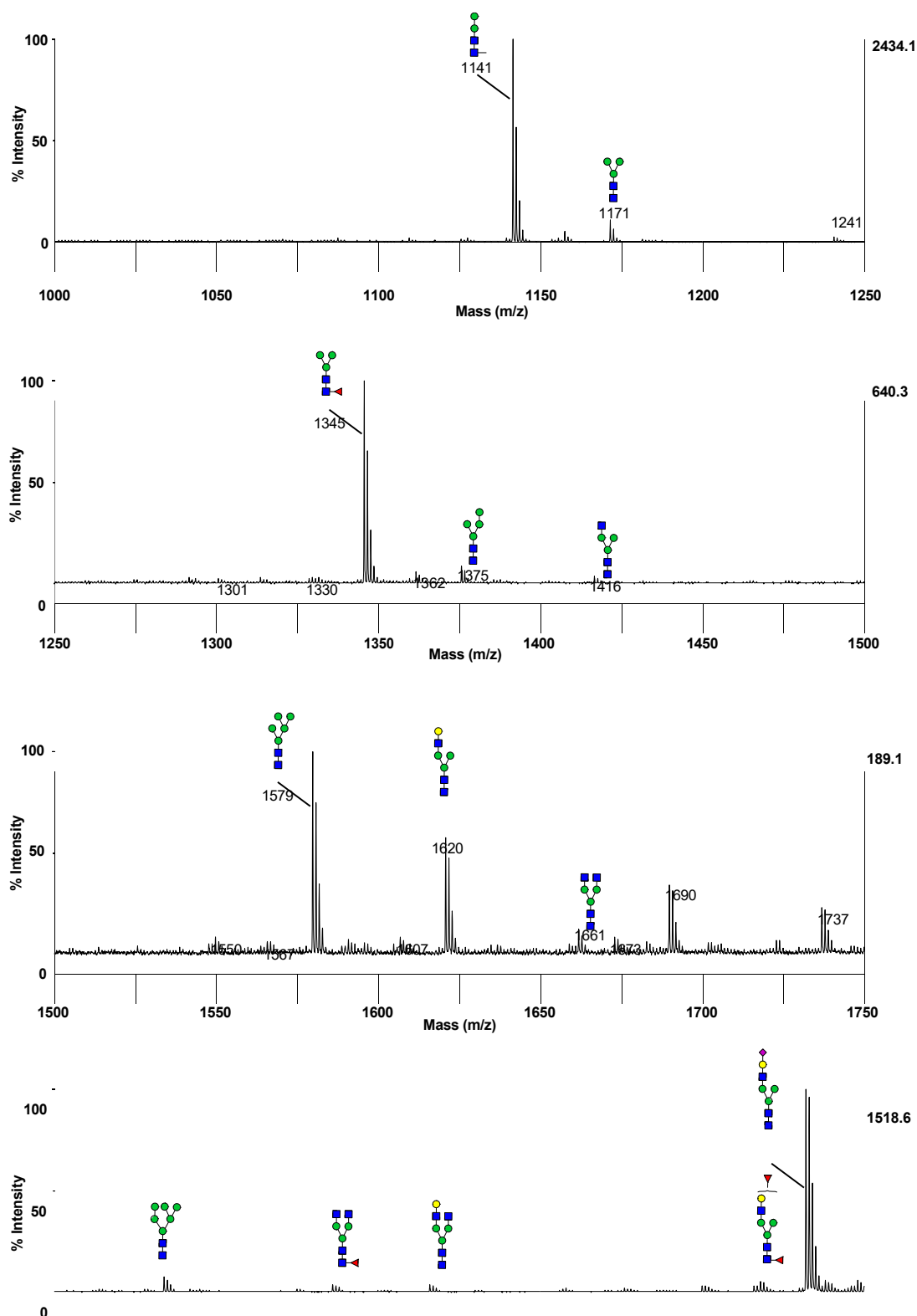
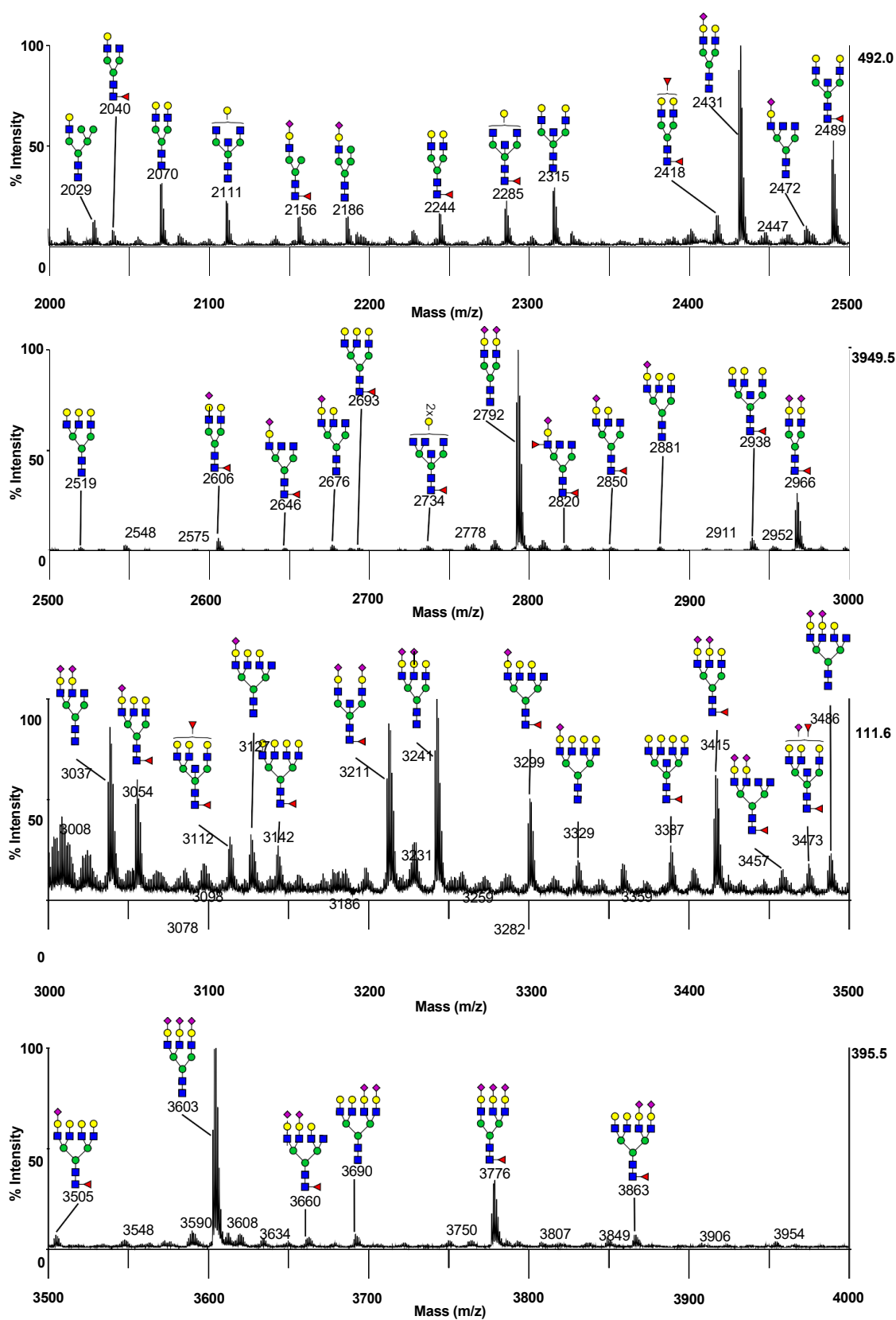


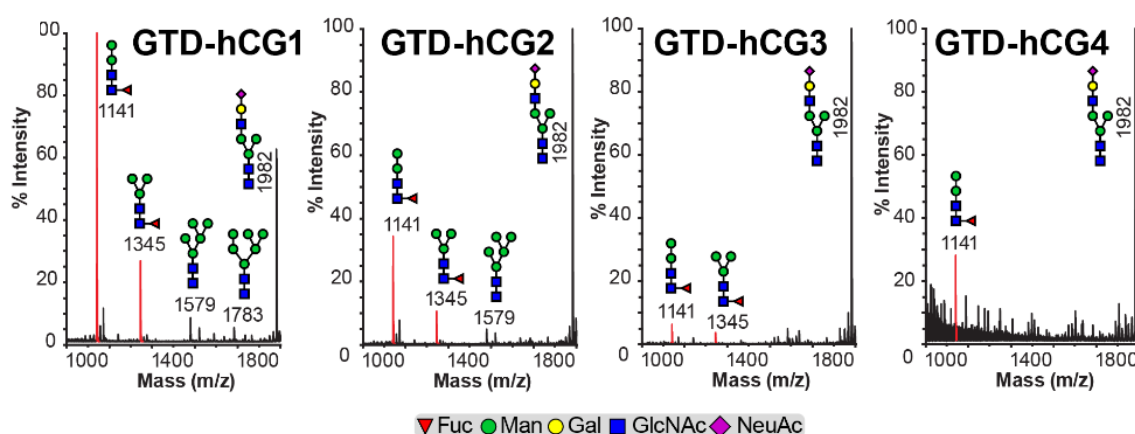
Figure 3-7: Annotated MALDI-TOF MS spectra of permethylated N-glycans derived from GTD-hCG sample. Significant peaks are annotated with putative structures are based on composition, tandem MS,  $\beta$ -galactosyltransferase experiments, and knowledge of biosynthetic pathways. All molecular ions are  $[M+Na]^+$ . Structures above a bracket were not unequivocally defined. There is a noticeable increase in sialylated structures here, compared to EP-hCG, with dominant peaks at  $m/z$  1982, 2792 and 2966 all terminating with NeuAc.





The main feature of the spectrum was mono- and bi-antennary complex N-glycans capped with NeuAc at  $m/z$  1982, 2792 and 2966. The lower mass region included high mannose structures as well as mono-antennary structures at  $m/z$  1579 and 1982, respectively. The high mass region mainly consisted of tri-antennary complex structures at  $m/z$  3241, 3415, 3603 and 3776. Observed to a lesser extent were tetra-antennary structures at  $m/z$  3864. Non-reducing terminals featured extensive sialylation. The sialylation consisted of NeuAc residues with some evidence of Lewis X epitopes (see ms/ms). Although less abundant, structures capped with LacNAc were also observed. Molecular ions consistent with bisected glycans also featured. Peaks for paucimannose type N-glycans at  $m/z$  1141 and 1345 were noted in all GTD-hCG samples as seen in Figure 3-8

Figure 3-8: Paucimannose type N-glycans on GTD-hCG samples. MS spectra of permethylated N-glycans derived from GTD-hCG. Red peaks correspond to paucimannose type N-glycans. The high mannose N-glycans found at  $m/z$  1579 and 1783 is shown here for comparison of the relative intensity of the paucimannose type N-glycans. The presence of paucimannose type N-glycans is a feature noted throughout all hCG sampled in this study.



The four GTD-hCG samples (GTD-hCG1, GTD-hCG2, GTD-hCG3 and GTD-hCG4) shared the same glycan repertoires, with minimal differences in the relative abundance of each glycan group between the different GTD-hCG samples.

GTD-hCG was made up of only 2% high mannose structures. The complex type N-glycans consists of 22% mono-, 66% bi-, 11% tri and 1% tetra-antennary N-glycans. 34% of structures were core fucosylated. 79% of complex N-glycans in GTD-hCG were capped with NeuAc, the rest were capped with either LacNAc (19%) or Lewis<sup>x</sup> (2%) epitopes. Also observed were bisected N-glycans which represented 6% of all structures observed in GTD-hCG.

### 3.3.5 hCG sample from pre-eclampsia

The N-glycomic profiles of the pre-eclampsia samples, EP-hCG4 and LP-hCG4, unusually displayed an MS profile which represented a mixture of features typical of normal pregnancy as well as pregnancy complicated by GTD. Figure 3-9 shows the MALDI-TOF mass spectra of permethylated early pregnancy hCG N-glycan for a pregnancy complicated by late onset preeclampsia (EP-hCG4). Figure 3-10 shows the MALDI-TOF MS matched LP- hCG pair of EP-hCG4 (LP-hCG4); pregnancy complicated by late onset preeclampsia. The structures were the same as those found in both the normal EP and LP-hCG, as well as GTD-hCG samples. EP-hCG4 and LP-hCG4 MS is characterised by the presence of significant peaks at m/z 1982, corresponding to a mono-antennary sialylated N-glycan, also significantly expressed in normal EP-hCG. Also, like normal pregnancy hCG, were the expression of bisected bi-antennary (m/z 2111, 2315, 2489, 2663) and tri-antennary (m/z 2938, 3112, 3286) N-glycans and Lewis<sup>x</sup> terminal epitopes. EP-hCG4 and LP-hCG4 MS displayed high abundance of sialylated bi-antennary N-glycans at m/z 2792 and 2966 which is characteristic of GTD-hCG.

Figure 3-9: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) of permethylated N-glycans from human chorionic gonadotropin purified from pregnancy urine samples from patient with late onset preeclampsia: EP-hCG4. For clarity, the top panel represents an overview of the MS data for sample EP-hCG4, which has been divided into three panels to allow clear annotation of significant peaks. The MS profile displays a mixture of features typical of normal pregnancy as well as pregnancy complicated by GTD, with significant peaks at  $m/z$  1982 as well as  $m/z$  2792 and 2966.

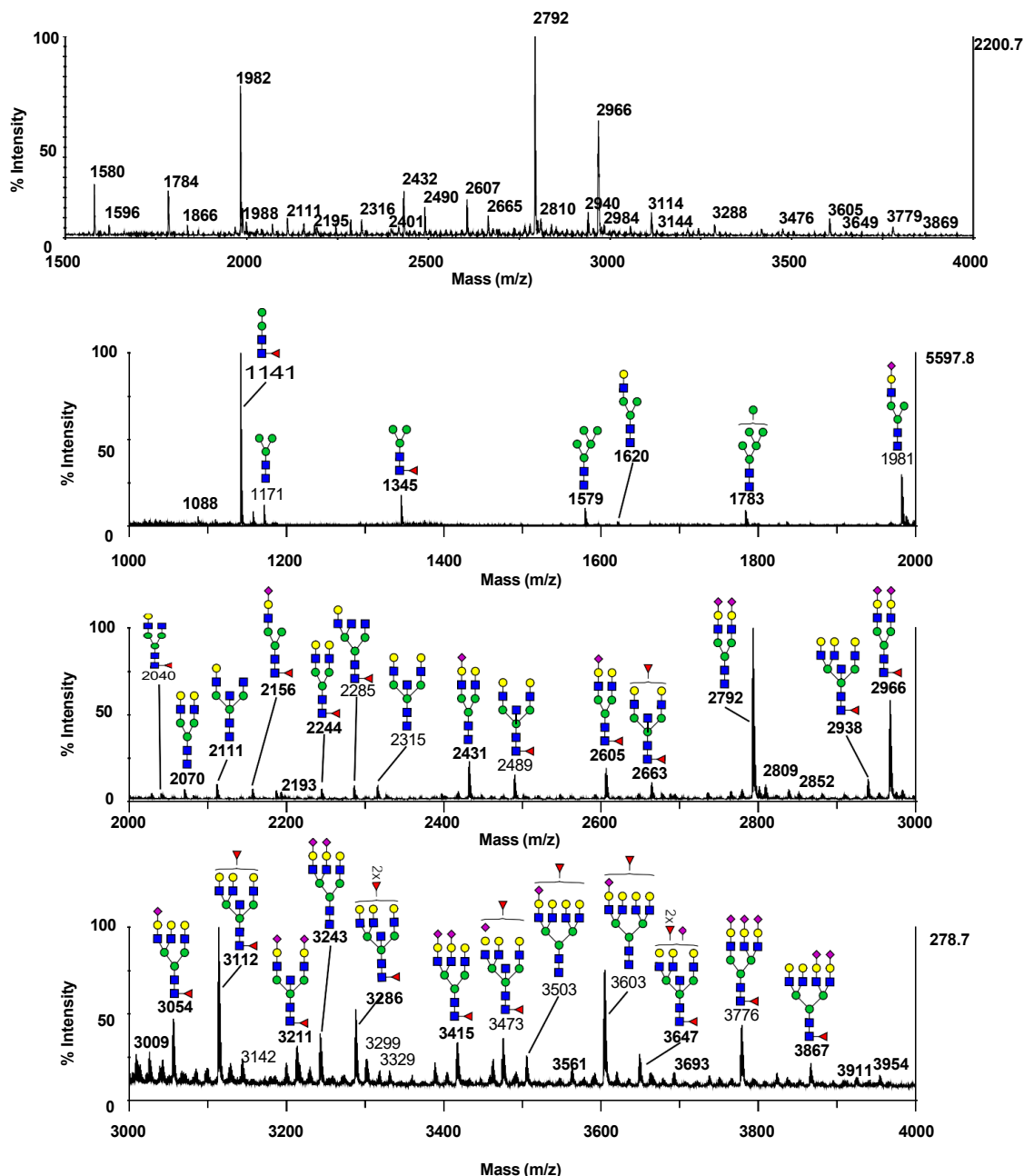
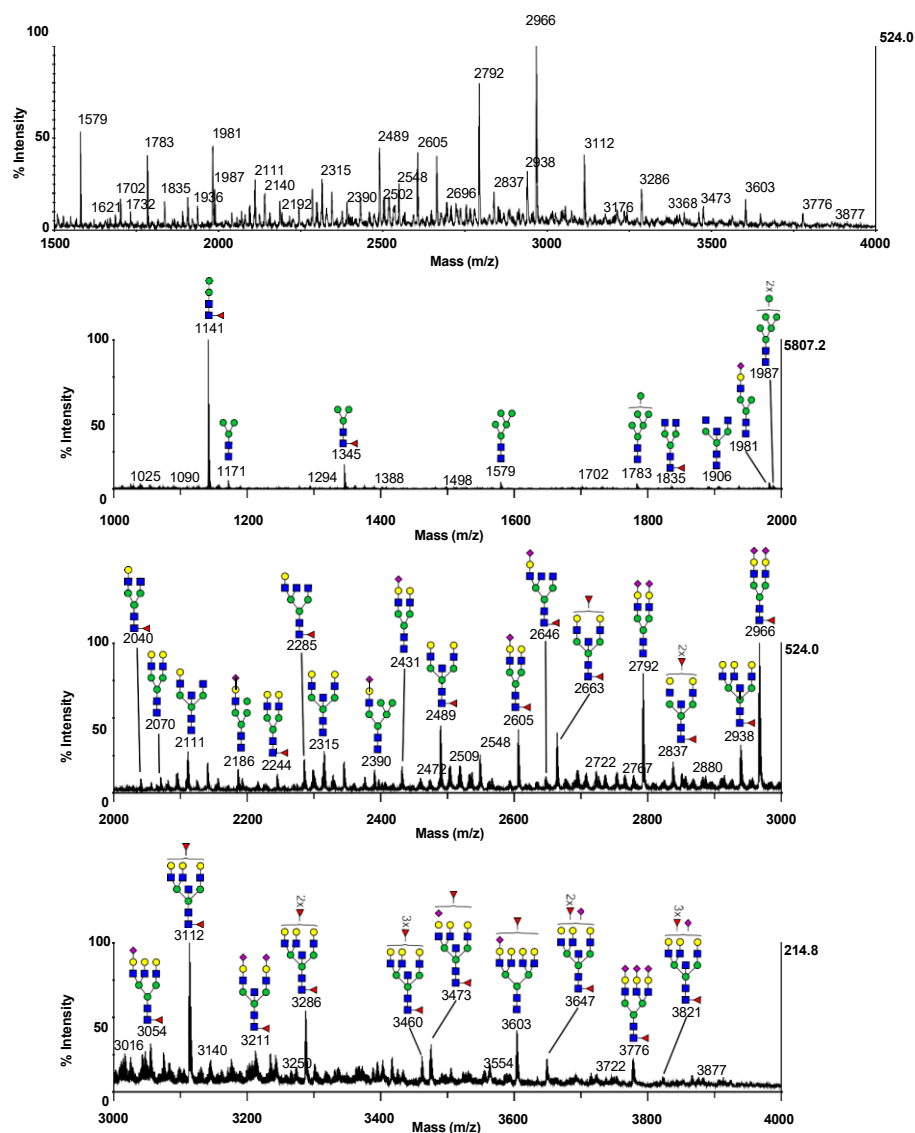


Figure 3-10: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) of permethylated N-glycans from human chorionic gonadotropin purified from pregnancy urine samples from patient with late onset preeclampsia: LP-hCG4. For clarity, the top panel represents an overview of the MS data for sample LP-hCG4, which has been divided into three panels to allow clear annotation of significant peaks. The MS profile, again here, displays a mixture of features typical of normal pregnancy as well as pregnancy complicated by GTD, with significant peaks at  $m/z$  1982 as well as  $m/z$  2792 and 2966. There is also a shift in the MS, with more significant peaks in the higher mass range when compared to EP-hCG4.



### 3.3.6 MALDI MS/MS analysis

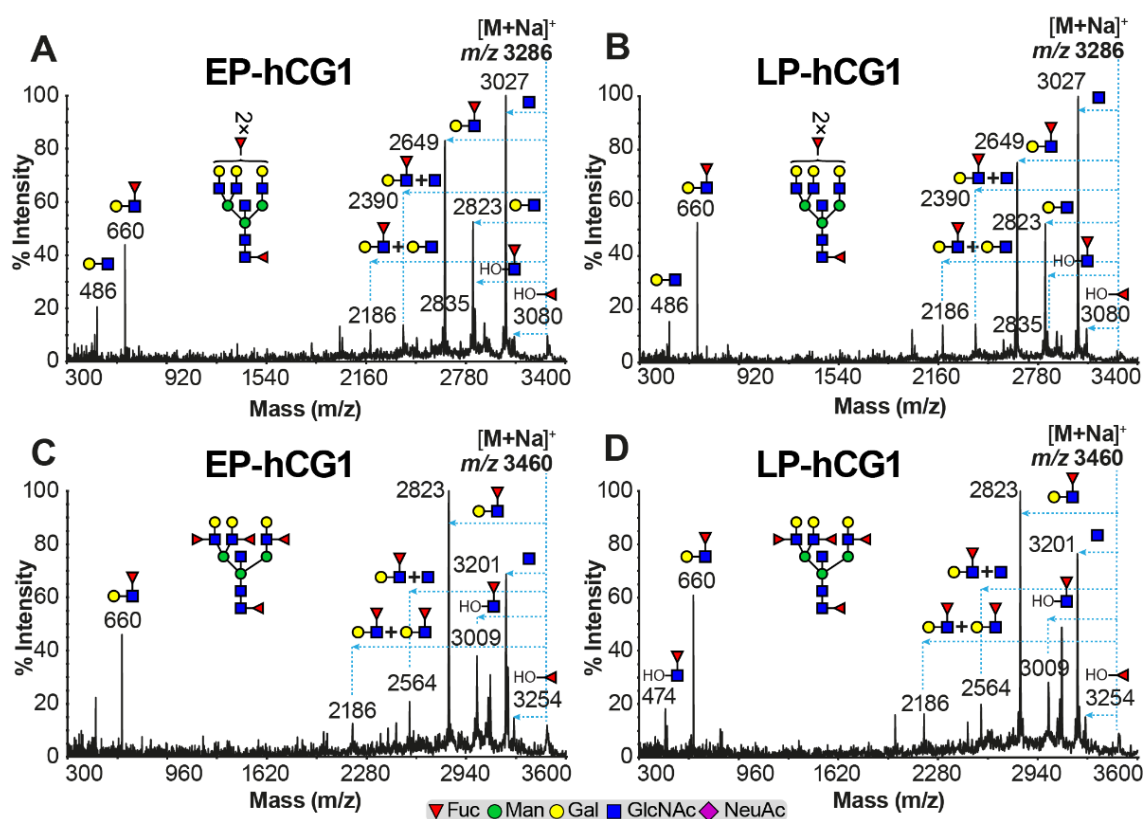
Common modifications of EP and LP-hCG included sialylation, and fucosylation. On the non-reducing end, the capping groups included LacNAc, NeuAc (sialylated LacNAc) and Lewis<sup>x</sup> epitopes.

MS/MS was used for further characterisation of structures to determine the sequence of glycans. MS/MS analysis confirmed the presence of suspected terminal epitopes and their relative abundance. Important peaks were analysed here to confirm the suspicion of the presence of Lewis<sup>x</sup> epitopes. Figure 3-11 contains MS/MS data used to characterise the peaks at *m/z* 3286 and 3460.

MS/MS analysis of *m/z* 3286 highlighted the presence of Lewis<sup>x</sup> epitopes seen in Figure 3-11. The formation of an ion at *m/z* 3080 corresponds to the elimination of a fucose residue, supporting the presence of a fucose on the GlcNAc residue. The second most abundant fragment ion at *m/z* 2649 and its concurrent ion at *m/z* 660 represents the fragmentation of a Lewis<sup>x</sup> epitope from the molecular ion at *m/z* 3286.

Similarly, MS/MS of *m/z* 3460 demonstrates a similar fragmentation at *m/z* 660, with the corresponding ion at *m/z* 2823, and again elimination of a fucose is noted by the peak at *m/z* 3254. Finally, the peak at *m/z* 474 confirms a fucose attached to GlcNAc representing the core fucosylation noted in a significant proportion of EP-hCG and LP-hCG N-glycan structures.

Figure 3-11: Evidence of Lewis x antigens. Representative MALDI/TOF/TOF MS/MS spectra of the molecular ions found at (A, B)  $m/z$  3286 and (C, D) 3460 for the EP- and LP-hCG1 samples respectively. Horizontal dashed lines correspond to indicated losses from the molecular ion  $[M+Na]^+$ . For (A and B), the fragment ion at  $m/z$  3080 corresponds to the elimination of a fucose residue from the molecular ion at  $m/z$  3286, indicative of a fucose residue being on the C3 of GlcNAc residue (Lewis1X). Similarly, for (C and D), the fragment ion at  $m/z$  3254, corresponds to the elimination of a fucose residue from the molecular ion at  $m/z$  3460.



### 3.3.7 Enzymatic experiments

Whilst the MALDI MS and MS/MS experiments defined the glycan composition and antennae sequences, further enzymatic experiments were needed to analyse some significant peaks of interest.

### 3.3.7.1 Gal transferase experiment

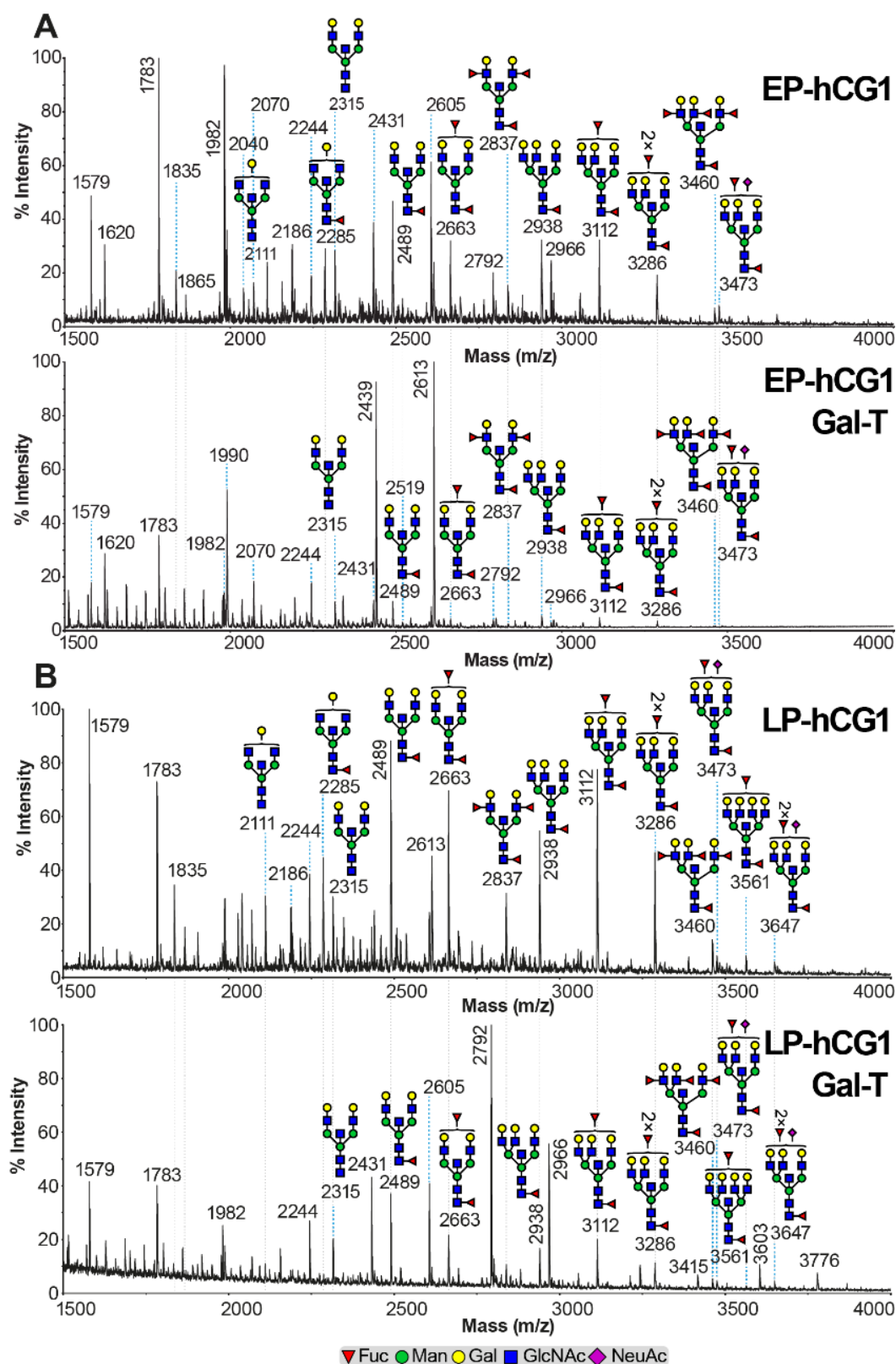
Of great interest, in hCG MS analysis, was the novel finding of glycans with masses consistent with bisected structures. These were observed at  $m/z$  1906, 2111, 2315, 2489, 2663, 2837, 2938, 3112, 3286, 3387 and 3460, 3473, 3561, 3647, 3735, 3822, and 3923. MS/MS analysis cannot prove the presence of bisected N-glycans, and for that reason samples were further analysed to confirm the presence of Bisecting-type N-Glycans.  $\beta$ -1,4 galactosyltransferase can catalyse the addition of 1,4-linked galactose onto GlcNAc on truncated antenna but not onto bisecting GlcNAc. Bisected GlcNAc residues are not accessible to  $\beta$ -galactosyltransferase and therefore are not capped with a galactose residue after treatment with  $\beta$ -galactosyltransferase and UDP-Gal as previously described in chapter 1. Therefore, glycans with truncated antenna will be galactosylated resulting in a mass shift, whereas signals for the bisected glycans remain unchanged.

The activity of the enzyme reaction is confirmed by the extension of truncated glycans such as that seen at  $m/z$  1835 and 1865.  $m/z$  1835 and 1865 correspond to agalactosylated N-glycans (GlcNAc terminated residues on mannose arms), no longer present in the MS after  $\beta$ -galactosyltransferase and UDP-Gal experiment Figure 3-12. The MS before  $\beta$ -galactosyltransferase and UDP-Gal experiment clearly shows peaks for  $m/z$  1835 and 1865. During the experiment, the  $\beta$ -galactosyltransferase facilitates the addition of 1,4-linked galactose onto GlcNAc on the truncated antennae resulting in a shift in the mass of the glycan corresponding to the addition of hexose to each terminal GlcNAc.

In contrast, there were no additions of galactose to suspected bisected N-glycans such as those seen at  $m/z$  1906, 2111, 2315, 2489, 2663, 2837, 2938, 3112, 3286, 3387 and 3460, 3473, 3561, 3647, 3735, 3822, and 3923 confirming the presence of bisected N-glycans in hCG. This is depicted

in Figure 3-12. Interestingly, bisected type N-glycans were the only significant discriminating variable between EP-hCG and LP-hCG ( $P=0.006$ ). Moreover, a certain number of them were found to be capped with Lewis<sup>x</sup> antigens, instead of the terminal sialylation which is characteristic of the non-bisected glycans.

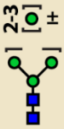
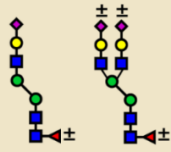
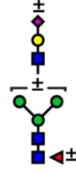
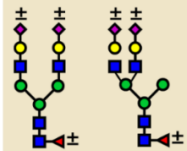
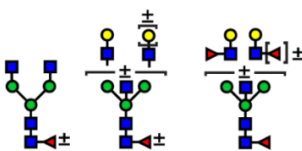
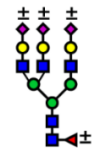
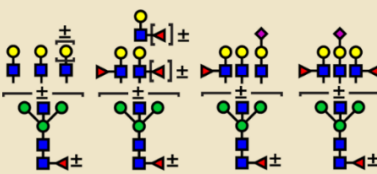
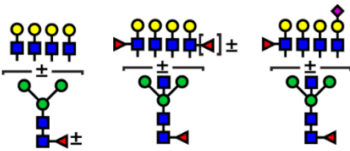
Figure 3-12: MALDI-TOF MS spectra of permethylated N-glycans derived from EP-hCG1 and LP-hCG1 sample before and after GalT experiment. Ions at  $m/z$  2315, 2489, 2663, 2837, 2938, 3112, 3286 3460, 3473 and 3647 are present on EP-hCG1 and LP-hCG1 spectra before the GalT experiment. The same ions were also detected on the EP-hCG1 and LP-hCG1 spectra after the GalT experiment (A and B, lower panels), indicating that those terminal GlcNAc residues are not subject to a GalT experiment mass shift and hence correspond to bisected N-glycan structures.



3.3.8 Structures identified.

A total of 62 N-glycan structures were identified from hCG. These are detailed in Figure 3-13 and Table 3-3.

Figure 3-13: Glycan structures of hCG: Bi-, tri- and tetra-antennary N-glycans detected in pregnancy hCG samples. The figure highlights the known structures of hCG as well as new structures revealed in this study. Plus, or minus sign ( $\pm$ ) above or to the right of each residue correspond to the presence or absence of the corresponding residue (204).

|              | Known Structures of hCG                                                             | New structures revealed in this study                                               |
|--------------|-------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| High Mannose |                                                                                     |    |
| Truncated    |   |  |
| Mono-        |  |                                                                                     |
| Bi-          |  |  |
| Tri-         |  |  |
| Tetra-       |                                                                                     |  |

### 3.4 Changes in hCG glycosylation as pregnancy progresses.

Although EP and LP hCG share the same glycan repertoire, there are subtle differences in the expression of the different glycans. The results here show a decrease in relative intensity of peaks representing high mannose structures in LP-hCG; from 40% in EP-hCG to 29% in LP-hCG. In both EP and LP, bi-antennary N-glycans represented the most abundant type N-glycans observed. The expression was slightly higher in late pregnancy, 63% vs 57%. EP-hCG featured a higher abundance of the mono-antennary N-glycans, 18%, vs 9% in LP-hCG.

All EP-hCG sample MS profiles were dominated by peaks representing high mannose and monoantennary sialylated structures. All LP MS profiles similarly were dominated by peaks representing high mannose structures with a lesser expression of the monoantennary sialylated structure, but rather, significant expression of peaks representing bisected type N-glycans. Of statistical significance was the difference in bisected structures. There is an increase in bisected structures in LP-hCG; 24% in EP vs 45% in LP ( $P= 0.006$ ). The differences observed are summarised in Table 3-4 and depicted in Figure 3-14.

Table 3-4: Comparison of the structural features (variables) used for the analysis of hCG samples. Mean values  $\pm$  standard error of the mean (SEM) of the structural features used for the analysis of hCG samples, t-test for paired EP-hCG vs LP-hCG samples, and t-test for independent EP-hCG vs GTD-hCG samples. Values in bold \* correspond to variables significant after correction for multiple comparison according to Bonferroni test.

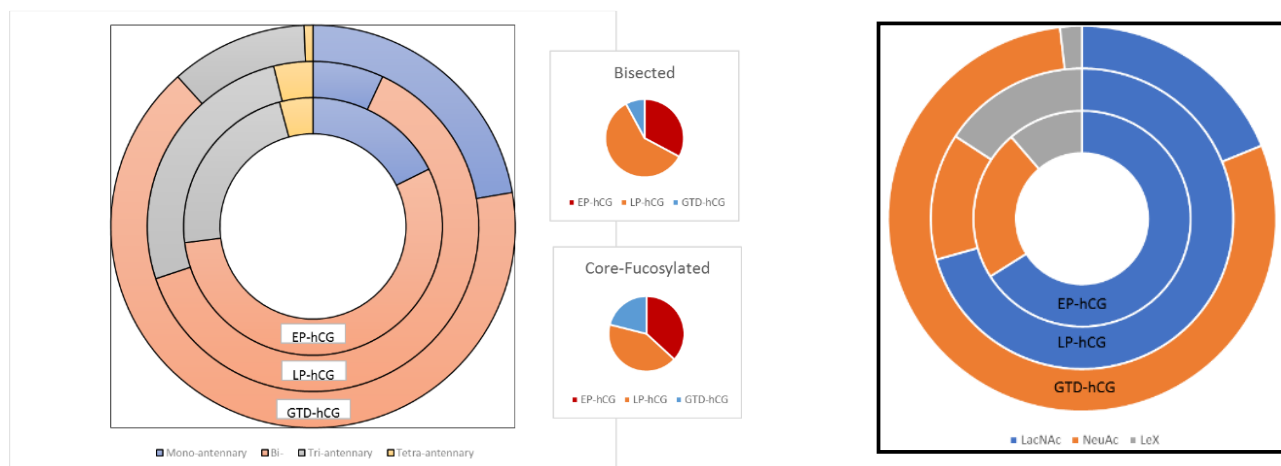
(1) Paired t-test of EP-hCG vs LP-hCG samples.

(2) Independent t-test of EP-hCG vs GTD-hCG samples.

(3) High mannose and bi-antennary N-glycans did not meet normality test in the independent t-test.

| Structural feature/<br>Variable | EP-hCG<br>mean $\pm$ SEM | LP-hCG<br>mean $\pm$ SEM | Paired t-test<br>(1) | GTD-hCG<br>mean $\pm$ SEM | Ind. T-test<br>(2) |
|---------------------------------|--------------------------|--------------------------|----------------------|---------------------------|--------------------|
| High mannose                    | 40.4 $\pm$ 21.1          | 28.5 $\pm$ 9.4           | 0.417                | 2.2 $\pm$ 0.9             | (3)                |
| Mono-antennary                  | 17.7 $\pm$ 5.0           | 9.0 $\pm$ 0.3            | 0.238                | 22.3 $\pm$ 2.4            | 0.402              |
| Bi-antennary                    | 56.7 $\pm$ 3.0           | 62.6 $\pm$ 1.2           | 0.113                | 66.0 $\pm$ 1.6            | (3)                |
| Tri-antennary                   | 21.8 $\pm$ 1.9           | 24.9 $\pm$ 0.9           | 0.299                | 10.9 $\pm$ 2.0            | 0.013              |
| Tetra-antennary                 | 3.8 $\pm$ 1.1            | 3.5 $\pm$ 0.5            | 0.766                | 0.7 $\pm$ 0.4             | 0.030              |
| Agalactosylated                 | 24.7 $\pm$ 6.6           | 30.0 $\pm$ 1.7           | 0.401                | 5.8 $\pm$ 2.1             | 0.092              |
| Bisected                        | 23.6 $\pm$ 1.4           | 44.9 $\pm$ 3.1           | <b>0.006 *</b>       | 6.4 $\pm$ 2.0             | <b>0.001 *</b>     |
| Core-fucosylated                | 60.4 $\pm$ 5.6           | 66.7 $\pm$ 2.2           | 0.473                | 34.1 $\pm$ 1.5            | <b>0.003 *</b>     |
| LacNAc                          | 62.4 $\pm$ 4.0           | 67.7 $\pm$ 1.0           | 0.344                | 18.8 $\pm$ 4.6            | <b>0.001 *</b>     |
| NeuAc                           | 27.2 $\pm$ 2.8           | 17.6 $\pm$ 2.9           | 0.232                | 79.4 $\pm$ 4.9            | <b>0.001 *</b>     |
| LewisX                          | 10.4 $\pm$ 2.1           | 14.7 $\pm$ 2.5           | 0.131                | 1.8 $\pm$ 0.4             | <b>0.005 *</b>     |

Figure 3-14: Comparison of the relative abundance of the glycan types and their antennae sequence found in EP-hCG, LP-hCG and GTD-hCG. There is an increase in the bisected structures as we move from EP to LP. Also evident is the high abundance of sialylated (NeuAc) structures in GTD-hCG.

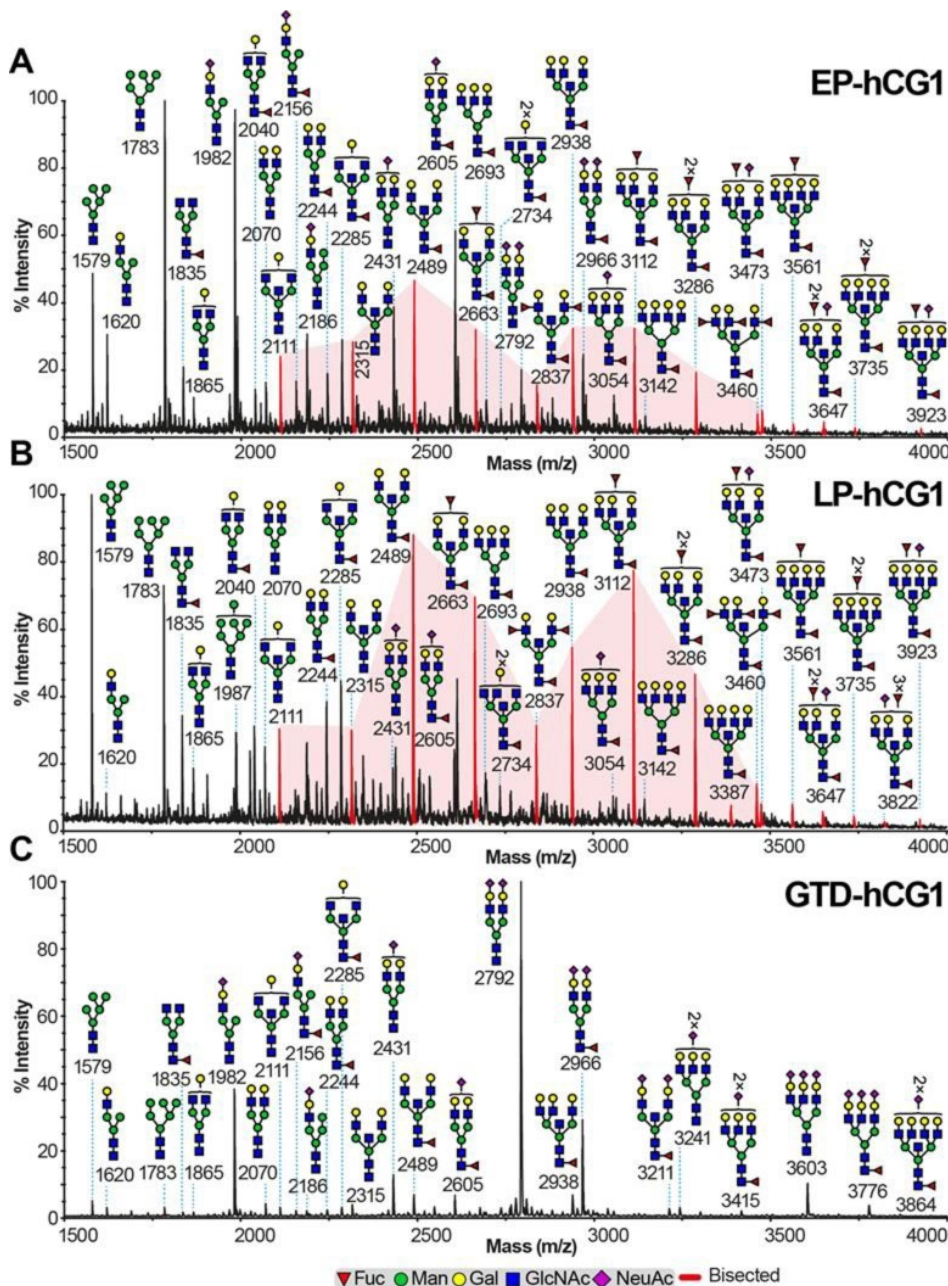


### 3.5 Changes between normal EP-hCG and GTD-hCG

GTD-hCG MS data displayed fewer glycoforms when compared to the MS from normal pregnancy hCG. The significant peaks corresponding to high mannose structures in normal pregnancy hCG, although present in GTD-hCG, were less prominent. EP was composed of 40% high mannose structures, vs only 2% in GTD-hCG. GTD-hCG were almost entirely made up of complex N-glycans with terminal sialylation. Structures with core- fucosylation were of minor abundance, representing only 34% compared to 60% and 67% in EP-hCG and LP- hCG, respectively. Again, in GTD-hCG, the significant peaks for paucimannose type structures found in normal pregnancy, were not consistently present in high abundance in GTD-hCG as seen in Figure 3-8. Other significant differences included a decrease in tri- and tetra-antennary structures when compared to normal EP-hCG, as the MS of GTD-hCG showed predominantly biantennary sialylated structures. With regards to the antennae decorations, a significant decrease in LacNAc and Lewis <sup>x</sup> epitopes was in GTD-hCG observed as tabulated in Table 3-4. Instead, however, what was evident

was a significant increase in expression of NeuAc residues from 27% in EP-hCG, to 79% in GTD-hCG. Finally, the novel presence of bisected N-glycans, observed in normal pregnancy, though present in GTD-hCG, was significantly less at 6% vs 24% in EP-hCG ( $P=0.001$ ). A representative MALDI-TOF MS profile of a sample from each group is depicted in Figure 3-15 to enable an easy visual comparison.

Figure 3-15: Comparison MS of the three groups: MALDI-TOF MS spectra of permethylated N-glycans derived from (A) EP-hCG1, (B) LP-hCG1 and (C) GTD-hCG1 samples. Structures above a bracket were not unequivocally defined. Putative structures are based on composition, tandem MS,  $\beta$ -galactosyltransferase experiments, and knowledge of biosynthetic pathways. All molecular ions are  $[M+Na]^+$ . EP-hCG and LP-hCG samples were characterised by high mannose (m/z 1579, 1783), mono- (m/z 1620, 1982), bi- (m/z 2244, 2431, 2489, 2605), tri- (m/z 2693, 2938, 3112, 3286) and tetra-antennary (m/z 3142, 3561, 3735, 3923) complex-type N-glycans all mainly core fucosylated (A and B). The main capping sugars on their antennae corresponded to undecorated N-acetylglucosamine (GlcNAc) units. Bisected N-glycans ranging from bi- to tetra-antennary structures (A and B) are highlighted in red peaks. Calculation of the relative intensities of the bisected N-glycans showed that their mean value shifted from 23.6% for the EP-hCG samples to 44.9% for the LP-hCG samples. GTD-hCG samples contained fewer glycoforms compared to those seen in EP-hCG and LP-hCG (C). GTD-hCG samples were characterised by the minor abundance of high mannose N-glycans and by the presence of complex N-glycans of which the most abundant were mono- and bi-antennary structures predominantly capped by NeuAc residues (204).



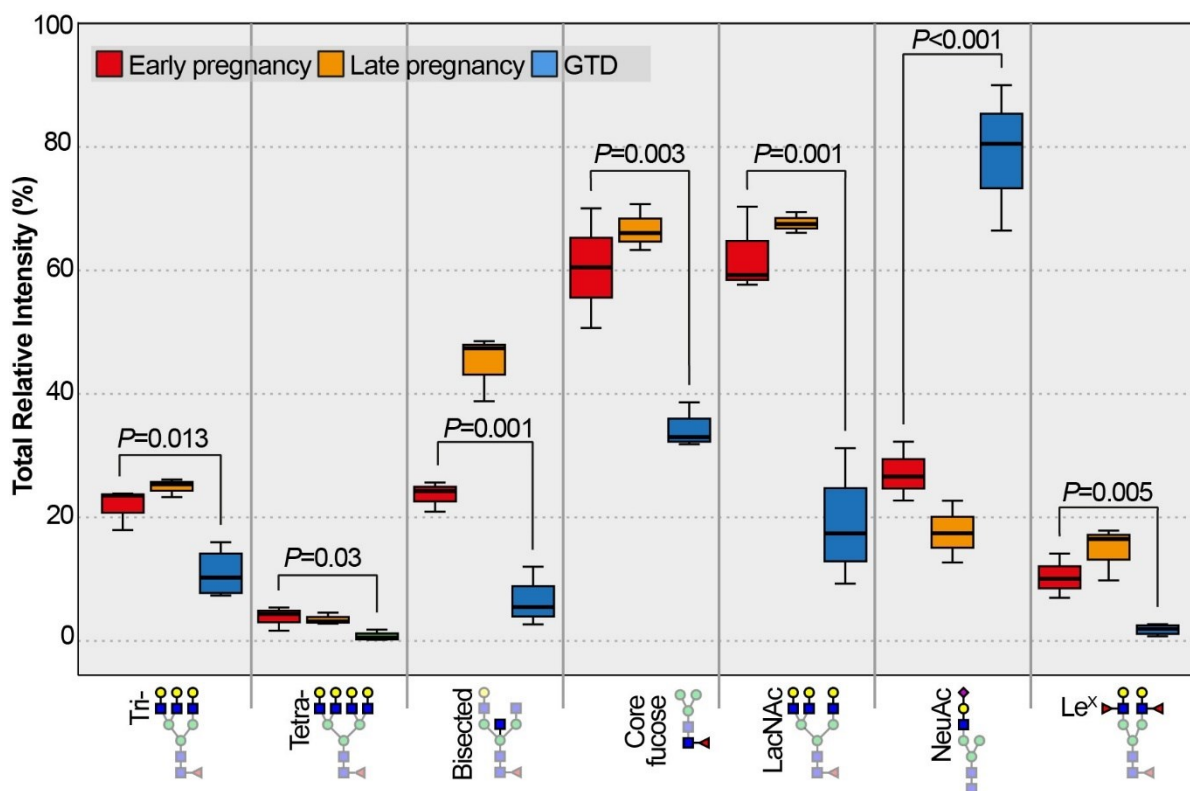
### 3.6 Statistical analysis

MALDI data enables a semi-quantitative analysis as the MS peak heights represent a relative abundance of individual permethylated N-glycan components found in the sample being analysed under the same MS conditions.

As previously described in chapter 2, Methods, using the relative intensity of each peak in a sample as a means for comparison between the groups, the relative intensity of each peak was calculated as a percentage of the total relative intensity in the sample. All structural features identified from MALDI TOF MS were used as variables and the relative intensity from each individual sample was calculated as a percentage of the total from that sample. The data were analysed using pair and unpaired t-tests carried out on SPSS. Statistical analysis of the means then allowed for a more quantitative analysis of differences. Figure 3-16

Through this method, Late pregnancy hCG is differentiated from early pregnancy hCG by a significant increase in bisected structures ( $P= 0.006$ ). Whilst EP and LP hCG displayed consistent differences in other structural features described earlier, the change in expression of the novel bisected structure was the only difference of statistical significance. Direct comparison of EP-hCG and GTD-hCG suggests a statistically significant decrease in bisected structures, core fucosylated structures, LacNAc and Lewis<sup>x</sup> terminal epitopes. The increases in expression of NeuAc terminal epitopes in GTP-hCG was statistically significant.

Figure 3-16: Box and whisker diagram showing statistical analysis of structural features of N-glycans on all hCG samples found some significant differences. For EP-hCG vs LP-hCG samples the only significant structural feature found was the bisected N-glycans. For EP-hCG vs GTD-hCG samples the significant structural features found were the bisected and core-fucosylated N-glycans, and the LacNAc, NeuAc and Lewis<sup>x</sup> terminal epitopes. For EP-hCG vs LP-hCG samples, paired *t*-test; for EP-hCG vs GTD-hCG samples, independent *t*-test. *P* values were corrected according to Bonferroni method.



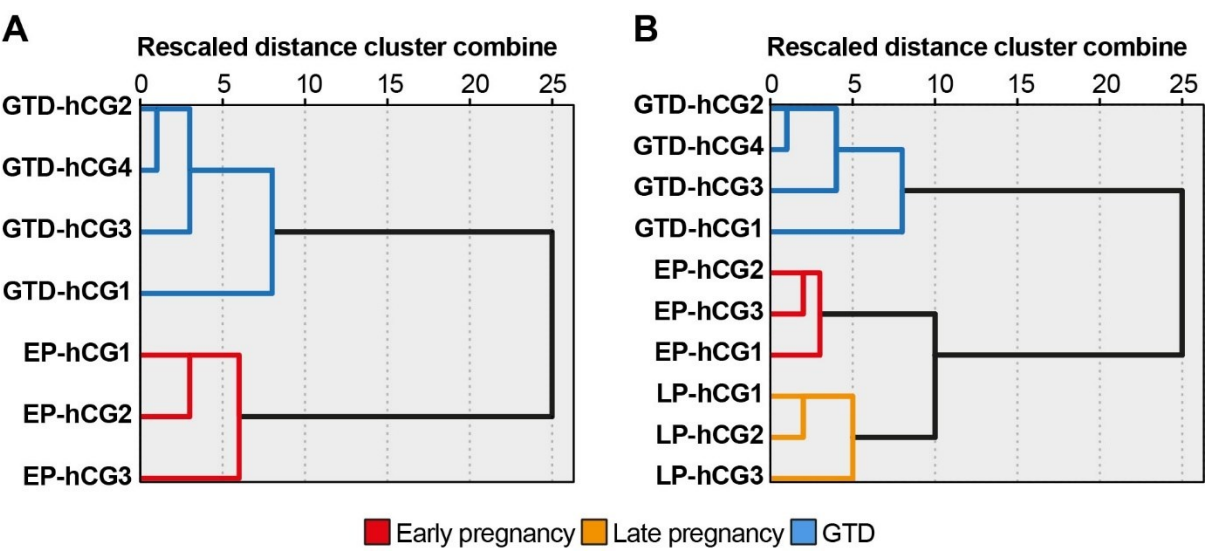
### 3.6.1 Hierarchical cluster analysis of hCG samples

Hierarchical cluster analysis (HCA) uses different variables to group samples with similar characteristics. The output is a dendrogram used to show the relationship between the groups. As the aim of this thesis was to identify possible areas for further investigation and use of pregnancy hCG as a biomarker, it was important to carry out HCA, to investigate whether the N-glycans identified here could be used to differentiate normal pregnancy from abnormal. In this thesis, the significant ( $P < 0.05$ ) structural features identified on MS analysis (Figure 3-16), were used as clustering variables. The HCA is depicted in **Error! Reference source not found..** HCA was carried

out on GTD-hCG and EP-hCG using bisected and core-fucosylated N-glycans, and LacNAc, Lewis<sup>x</sup> and NeuAc epitopes as clustering variables. HCA was also applied to differentiate GTD-hCG, EP-hCG and LP-hCG using as variables only the bisected N-glycans and the NeuAc epitope.

We can see from the output that EP-hCG and GTD-hCG were grouped into two distinct groups using bisected and core fucosylated N-glycans and LacNAc, Lewis X and NeuAc epitopes as clustering variables. When only bisected N-glycans and NeuAc epitopes were used as variables, the HCA was able to distinguish between the three pregnancy groups, EP-hCG, LP-hCG and GTD-hCG (Figure 3-17).

Figure 3-17: Hierarchical cluster analysis of hCG samples. (A) Hierarchical cluster analysis of EP-hCG and GTD-hCG samples using as clustering variables the total relative abundance of the structural features that were significant at the  $P < 0.05$  level (after correction for multiple comparison with Bonferroni test) as found from independent  $t$ -test. The variables bisected and core-fucosylated N-glycans, and LacNAc, NeuAc and Lewis<sup>x</sup> terminal epitopes. (B) Hierarchical cluster analysis of all hCG samples (EP, LP and GTD) using as clustering variables the total relative abundance of bisected N-glycans and NeuAc terminal epitope.



### 3.7 Discussion

In this chapter, the N-glycans of urinary hCG from pregnant women at different stages of pregnancy were characterised, including samples from women with gestational trophoblastic disease (GTD) and preeclampsia (PET). The MS analysis provides detailed information on the structural features of urinary hCG in normal pregnancies and highlights deviations in abnormal pregnancy states. Additionally, it characterises the differential glycosylation that occurs as normal pregnancy progresses.

Although the glycosylation of hCG has been previously described, these studies presented either very basic characterisations (25) or were based on hCG from trophoblastic tumours (205) (206) or pooled samples. Consequently, hCG glycosylation from healthy pregnant women has not been well-defined. Amongst the different hCG variants suggested, a “hyperglycosylated” variant of hCG (hCG-h) has been proposed, the nature of which is ill defined(134). This "hyperglycosylated" variant of hCG (hCG-h) has been proposed as a predictor of pregnancy outcomes including PET and gestational diabetes, as well as a tumour marker for gestational trophoblastic diseases(133, 135, 207). This highlights another gap in our knowledge of hCG glycosylation; not much is known about the glycosylated forms of hCG that are synthesised during obstetrical syndromes. This thesis not only presents the MS analysis from individual healthy women, but also paired EP-hCG and LP-hCG (Figure 3-15) to access the changes. The main aim of this study was to rigorously characterise normal healthy pregnancy hCG glycosylation patterns in EP (Figure 3-2) and LP (Figure 3-4) and abnormal GTD (Figure 3-7) pregnancy. Additionally, it aimed to shed some light on the different glycoforms of hCG that are synthesised during pregnancy complications such as PET (Figure 3-9, Figure 3-10). It also aimed to offer some clarity on the nature of the commonly referenced “hyperglycosylated” variant of hCG, addressing the question ‘is early pregnancy hCG

hyperglycosylated?’ as has been claimed by Cole et al (134). Finally, it explores the potential of glycosylation changes in hCG for biomarker discovery.

A total of 12 different hCG samples across four groups (early pregnancy, late pregnancy, GTD pregnancy and PET pregnancy) were analysed. Several key findings were observed. The first observation was that EP and LP hCG shared a similar glycan repertoire but showed significant differences in the relative abundance/expression of each glycan identified (Table 3-3, Figure 3-15). It was also observed that normal pregnancy hCG expressed novel bisected glycans, that significantly increased in expression as pregnancy progressed (Figure 3-12, Figure 3-14, Figure 3-15). Thus, bisected type N-glycans were quite abundant in LP-hCG. There were no statistically significant differences between the mono, bi-, tri- and tetra-antennary structures (Figure 1-1). The last observation contradicts previously suggested theory that hyperglycosylation in early pregnancy is associated with higher abundance of multi-antennary N-glycans compared with later in pregnancy (25, 124). The normal pregnancy hCG glycome is significantly more diverse than GTD-hCG (Figure 3-15), which displayed mainly sialylated complex N-glycans with significantly less bisected and core fucosylated structures and Lewis<sup>x</sup> epitopes (Figure 3-6). We observed that pregnancy complications could possibly be picked up in the early first trimester urine hCG MS analysis. EP-hCG4, was collected from a patient who went on to develop PET in their third trimester. The MS from this sample not only appeared different from normal EP-hCG but also, on further analysis, shared features of both normal EP and GTD hCG MS (Figure 3-9). This is a promising area of biomarker research and development. As mentioned earlier, very little is known about the glycoforms of hCG that are synthesised during obstetrical syndromes such as PET. The ability to diagnose these conditions as early as the first trimester may allow better patient care and management as well as the possibility to develop interventions to alter the pathway of the

disease/condition. Finally, we speculate that the presence of high mannose, bisected N-glycans and terminal Lewis<sup>x</sup> may play a role in immunosuppression via binding to lectins such as DC-SIGN reported to recognise high-mannose containing structures and Lewis antigens (31).

### 3.7.1 Early pregnancy vs. Late Pregnancy hCG MS profile

The normal pregnancy hCG N-glycome includes high levels of high mannose as well as bisected, bi-, tri- and tetra-antennary complex glycans, none of which has previously been identified on hCG. It is possible that the high mannose glycans may contribute to the well documented immunosuppressive state in pregnancy via binding to lectins such as DC-SIGN. All normal EP-pregnancy samples shared a similar MS profile as shown in Figure 3-1, even though there were some variations in relative abundances between the different samples. Despite this individual variability, all exhibited similar trends in glycosylation changes from early to late pregnancy; especially of note is the statistically significant increase in bisected type N-glycans. Early pregnancy hCG is primarily produced by the syncytiotrophoblasts (117). The syncytiotrophoblasts are the outer layer of the trophoblasts, which form the outer layer of the placenta. They are responsible for the production of hCG, which supports the corpus luteum in the ovary to maintain the pregnancy in its early stages by secreting progesterone (117). In late pregnancy, hCG continues to be produced primarily by the syncytiotrophoblasts of the placenta. While the levels of hCG peak in the first trimester and then decline, the syncytiotrophoblasts maintain its production throughout the pregnancy to support various functions, including the modulation of the maternal immune response and promotion of fetal development (117). The differences in glycosylation between early and late pregnancy hCG can be explained by several factors related to the changing physiological environment, the differentiation and function of trophoblast cells, and the specific needs of the developing fetus and maternal body during different stages of pregnancy. In early

pregnancy, hCG is primarily produced by cytotrophoblasts and syncytiotrophoblasts. Cytotrophoblasts are undifferentiated cells that later fuse to form the syncytiotrophoblast layer, which is the main source of hCG throughout pregnancy(117, 124, 208, 209). In late pregnancy, hCG production is predominantly maintained by the syncytiotrophoblasts. These cells are more differentiated and specialized compared to cytotrophoblasts, which may influence the glycosylation patterns of hCG. In early pregnancy, hCG plays a crucial role in supporting implantation and modulating the maternal immune response to prevent rejection of the embryo (117, 124, 209). This might require specific glycosylation patterns that facilitate these processes. In later stages, hCG supports the ongoing development of the fetus and the maintenance of the placenta. The glycosylation patterns may shift to support these functions, ensuring adequate nutrient transfer, hormonal regulation, and immune protection. It is possible that the glycosylation of hCG is a dynamic process influenced by the differentiation state of trophoblasts, the functional requirements of pregnancy, the hormonal environment, and the enzymatic activities within the placenta. Understanding these differences provides insights into how hCG supports pregnancy and highlights potential biomarkers for monitoring pregnancy health and complications.

Hormone levels in the various stages of pregnancy have previously been investigated (210). It has been suggested that hCG may have a role as an endogenous tocolytic in normal pregnancy by directly promoting relaxation of uterine contractions (210). We know that as pregnancy progresses, the environment in the uterus and the role of hCG changes. This is true up to the point of labour where it has been suggested that a significant decrease in serum hCG level before the spontaneous start of labour might contribute to increase contractility in the uterine muscle and gradually initiate the onset of labour (210). It is therefore interesting that we report a consistent

change in hCG glycosylation between early and late pregnancy which could explain the change in hCG function as the pregnancy progresses. It would be interesting to see what further changes may be observed in third trimester hCG samples from just before the onset of labour.

#### 3.7.1.1 Is EP-hCG hyperglycosylated?

The description of early pregnancy hCG as hyperglycosylated in comparison with hCG from later stages of pregnancy (130, 134, 211, 212) had previously not been subjected to thorough scrutiny. There is little evidence from the results here of early pregnancy hCG being hyperglycosylated. The findings here show that early pregnancy hCG displays the same glycan structures as late pregnancy hCG but there is a variation in the relative expression of these glycans. We have here attempted to quantify the extent of this variation in Table 3-3.

Hyperglycosylation in early pregnancy has been described as a higher abundance of multi-antennary N-glycans compared with later in pregnancy (25, 124). It has also been described as a family of glycoproteins with the same polypeptide structure as hCG but with larger N- and O-linked oligosaccharides (124). Despite these claims, the only evidence-based description of hyperglycosylation is its recognition by the B152 antibody which was raised against a choriocarcinoma-derived hCG (130, 135). Claims of larger N-glycans in hyperglycosylated hCG are not supported by the results here which showed that there were no statistically significant differences between the mono, bi-, tri- and tetra-antennary structures in EP-hCG and LP-hCG. In fact, there were similar amounts of multiantennary structures in EP-hCG and LP-hCG. The results did, however, show an interesting increase in sialylated structures in EP-hCG and GTD-hCG compared to LP-hCG (27% in EP-hCG and 79% in GTD-hCG vs 18% in LP-hCG). It is perhaps plausible that the “hyperglycosylated” glycoforms identified by the B152 antibody in early

pregnancy are enriched in sialylated mono- and bi-antennary structures. This conclusion is supported by literature evidence that sialylated glycoproteins migrate aberrantly on gels, often appearing to be much larger than their actual molecular weights (213, 214). This aberrant migration is due to the increased negative charge and altered conformation imparted by the sialic acid residues, which can affect the mobility of the glycoproteins during gel electrophoresis (213, 215). It is also consistent with the origin of the B152 antibody which was raised against a highly sialylated sample of choriocarcinoma hCG (130). The findings obtained in this study, therefore suggest that EP-hCG is not “hyperglycosylated” as previously claimed (116).

### 3.7.2 Normal Vs abnormal

The normal pregnancy hCG glycome was found to be substantially more diverse than GTD-hCG. The latter was characterised by complex N-glycans predominantly capped by NeuAc residues (79.4%), which is consistent with previous literature (23, 25). Compared with normal EP-hCG and LP-hCG, GTD-hCG MS displayed significant changes in various structural features as seen in Figure 3-16, including bisected glycans, core fucosylation, LacNAc, NeuAc and Lewis X epitopes, the most notable being the increase of NeuAc residues.

Sialic acid is usually the terminal monosaccharide in human N-glycans, and it affects the biological activities of glycoproteins. The relative amount of some sialic acid containing glycoproteins in amniotic fluid and maternal plasma has previously been reported as elevated during pregnancy and increases with advancing gestation (216-219). Earlier studies have inferred an immunomodulatory role of sialic acid in pregnancy (216, 219, 220). Sialylated glycoconjugates have been shown to modulate the host dendritic cell function by suppressing the production of the proinflammatory cytokine IL-12, as well as T cell activation and proliferation in response to

mitogens and antigens (221). Changes in the activity of sialic acid regulatory enzymes such as sialyltransferases and sialidase have been associated with diseased states in pregnancy such as gestational diabetes (217). High expression of sialyltransferase have been reported in different cancers and shown to correlate with increase motility, invasion, and metastatic potential of tumour cells (222). The statistically significant increase in sialylation in GTD-hCG, seen here, suggests that the degree of hCG sialylation could be of clinical significance.

Pre-eclampsia samples (EP-hCG4 and LP-hCG4) showed N-glycomic profiles consisting of structures found in both the EP/LP-hCG and GTD-hCG samples. They were characterised by the presence of bisected bi- and tri-antennary N-glycans and Lewis<sup>x</sup> terminal epitopes (as of EP/LP-hCG), but also by the high abundance of sialylated bi-antennary N-glycans (as of GTD-hCG, *m/z* 2792 and 2966). Again, here, increase sialylation can be linked to abnormality and possible association between high levels of hCG sialylation and risk of developing preeclampsia. Bisected and Lewis<sup>x</sup> structures are also present, but, although their abundance increases from EP to LP, their levels are below those observed in healthy pregnancies.

In abnormal pregnancies, such as GTD, we observe a larger variation in glycosylation compared to pregnancy associated hCG. Rigorous analysis of hCG glycosylation in other cases of the “Great Obstetrical Syndromes” and other types of problem pregnancies could provide additional diagnostic and prognostic tools in the clinical setting.

Siglecs (sialic acid-recognizing Ig superfamily lectins), briefly discussed in chapter 1 of this thesis, are a category of cell surface proteins that bind sialic acids. They are primarily expressed on immune cells and perform several different functions. Many Siglecs are inhibitory receptors that

mediate a variety of different inhibitory functions in the immune system, such as regulation of inflammation, promoting the tolerance of B lymphocytes and modulating the activation of dendritic cells and the activation of T cells (36, 223). Siglecs have immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytosolic tails, which suggests that they can perform inhibitory functions when they bind sialylated carbohydrates. Changes in the abundance or structure of sialic acids therefore could have significant clinical affects.

### 3.7.3 Significance of bisected N-glycans

Bisecting GlcNAc is a  $\beta$ 1–4-linked GlcNAc residue attached to a  $\beta$ -mannose of the *N*-glycan core (Figure 1-5), and the reaction is catalysed by *N*-acetylglucosaminyltransferase-III (GnT-III/Mgat-III). Bisecting GlcNAc modification can lead to the suppression of further processing and elongation of *N*-glycans(1) (Figure 3-12). As a result of this, bisecting GlcNAc appears to play an important control function since its presence could prevent the action of several enzymes involved in the synthesis of complex and hybrid oligosaccharides. Bisecting GlcNAc has been associated with cell growth control, and tumour progression. Studies have shown some evidence that bisecting GlcNAc of *N*-glycans regulates cellular signalling and tumour progression, through modulating *N*-glycan/galectin interactions (224, 225).

The significance of this novel finding of bisecting type *N*-glycans in hCG can be explained by what we already know of glycans and their role in immune evasion. Major histocompatibility complex (MHC) molecules (called human leukocyte antigen in humans) are cell surface proteins essential for the acquired immune system to recognise foreign molecules. They bind antigens to display on the cell surface for recognition by appropriate T-cells. We know that natural killer (NK) cells lyse cells lacking in human leukocyte antigen (HLA) class I antigens. Structural analysis of glycans derived from HLA class I molecules, confirm that 35-93% are biantennary bisecting type *N*-glycans

(226). HLA class I negative cells evade lysis by NK cells if they express high levels of bisecting type N-glycans (87, 88). We know that human sperm lack HLA class antigens (227), and a recent study has confirmed that they too possess a substantial amount of biantennary bisecting type N-glycans (228). The confirmation of bisecting type N-glycans in  $\beta$ -hCG and the increase in expression seen here in late pregnancy, may suggest a protective feature in the immunosuppressed pregnancy environment. It is also possible that tumour cells, to a certain extent, are protected in the same way from NK cell lysis. These bisecting type N-glycans, although significantly less, are expressed in GTD-hCG. Trophoblasts lack HLA class I expression, which could potentially make them more susceptible to NK cell-mediated cytotoxicity (112, 229). Recent glycomics analysis of human trophoblasts indicate that they express a much higher level of bisected type glycans compared to HLA class I positive cells and tissues, consistent with their ability to resist NK cell cytotoxicity (230).

It can be inferred that the presence of bisected type N-glycans in pregnancy hCG suggests a role for this hormone during the initial invasion of the endometrial lining by the early human embryo. Placental trophoblasts secrete monocyte inflammatory protein-1  $\alpha$  (MIP-1  $\alpha$ ) that induces the chemotaxis of NK cells and monocytes to their surface (231). NK cells represent about 70% of the lymphocytes in the endometrium prior to implantation (232). It is known that hCG derived from pregnancy urine inhibits NK cell cytotoxicity *in vitro* at nanomolar concentrations (233). This inhibitory activity was attributed to the hormonal influence of hCG. The results of the current glycomics analysis suggests the possibility that hCG employs its bisected type glycans as functional groups to suppress NK cytotoxicity *in vivo*. However, this assignment remains to be experimentally confirmed.

In addition to reporting on the novel bisecting N-glycans in hCG, it was noted that a proportion of these bisected N-glycans were capped with Lewis<sup>x</sup> antigens, instead of the terminal sialylation

which is characteristic of the non-bisected glycans here. These novel bisected N-glycans terminated with Lewis<sup>x</sup> antigens are rare in humans, previously only reported in human sperm where they have been implicated in the inhibition of immune responses(6, 70).

#### 3.7.4 Possibility for biomarker discovery and potential of glycobiology in clinical practice

The biological roles of the N-glycans from this study are yet to be determined, but we know that hCG plays a significant role in maintenance of pregnancy states. Many studies have presented the different effects and use of hCG in pregnancy. It is plausible that these effects can be partially ascribable to the glycans.

As previously touched on earlier in this thesis, glycosylation is one of the most important posttranslational modification of proteins and is involved in many pathological and physiological events. The properties and function of a glycoprotein are affected by glycosylation. The functional roles of many glycans can be determined by their non reducing end terminal structures. Here the significant differences in the terminal structures can represent an area for further research and exploitation. For instance, the core fucosylation of  $\alpha$ -fetoprotein is a well-known tumour marker for hepatocellular carcinomas.

The structure or modification of the sugars are determined by the activities of glycosyltransferases and glycosidases. Over 180 glycosyltransferase genes have been identified (234, 235). These can be exploited in order to modify the sugar structures and examine the effects of the modification (236, 237). By identifying the target proteins that are directly associated with the phenotypic changes, we can actually determine the function of a given oligosaccharide structure on the specific molecules (238). Modulation of the N-glycan could control many biological reactions such as cell signalling and cell adhesion.

Several principles have been put forward to account for the adaptation in pregnancy to accommodate the foreign fetus. The role of glycosylation here, is commonly disregarded or not considered. We know of several glycoproteins that have been connected to this adaptation, including glycodelin-A and CA125 (239). HLA-G is perhaps the best-known immune modulator in the pregnant human uterus (102). Previous glycomic analysis of extra-villous cytotrophoblasts in combination with the sensitivity of uterine HLA-G to endo- $\beta$ -galactosidase digestion confirms that uterine HLA-G expresses N-glycans with extended linear polylactosamine sequences (208, 240). Glycans bearing these polylactosamine sequences bind to galectins that are prevalent in the pregnant human uterus (241, 242). Galectins have been documented to mediate many immune modulatory effects in the pregnant human uterus (243). HLA-G in the pregnant uterus could bind to these galectins, which manifest specific immunomodulatory effects that protect the developing fetus in utero. This model for the induction of tolerance mediated primarily by carbohydrate recognition was put forward by Clark, et al and is known as the human feto-embryonic defence system (hu-FEDS) hypothesis (45, 239). With further evidence to confirm the glycans act as functional groups to suppress NK cell cytotoxicity, hCG could potentially have a place in this hypothesis.

### 3.8 Concluding remarks

This work has characterised the N-glycome of hCG using MALDI MS. The data confirm that hCG is differentially glycosylated as pregnancy advances, and aberrant glycosylation changes are associated with abnormal pregnancy such as GTD and PET. The data also go further to show that hCG expresses bisecting type N-glycans that are like those linked to classical MHC class I molecules.

Currently, serum samples are the preferred sample for quantitative analysis of hCG and screening, whilst urine samples are used mainly for pregnancy tests using a monoclonal anti-hCG antibody. The facility to quantitate hCG levels is useful in the management of germ cell and trophoblastic tumours as well as the clinical management of ectopic pregnancies and miscarriage. For example, a sub-optimally rising hCG can be indicative of an ectopic or failing pregnancy; hCG levels are also used to monitor response to treatment in cases of trophoblastic tumours and used in early pregnancy screening for trisomy. With this in mind, we can appreciate the potential application of the findings presented here in clinical practice. Bisected and Lewis<sup>x</sup> structures are potential targets for diagnostics. They are also likely to be important for hCG function. hCG isolated from pregnancy urine inhibits NK cell cytotoxicity *in vitro* at nanomolar levels and bisected type glycans have previously been implicated in the suppression of NK cell cytotoxicity, suggesting that hCG-related bisected type N-glycans may directly suppress NK cell cytotoxicity.

Overall, it may be said that little is known of how different stages of pregnancy, and differing pregnancy complications impact on the glycosylation changes in hCG. A better understanding of hCG glycosylation may open doors for its utilisation as a biomarker for various pregnancy related conditions. Pregnancy can be complicated by many different disorders, such as miscarriage, preeclampsia, and gestational diabetes. Detecting differential glycosylation patterns of hCG may allow us to accurately screen for these complications in early pregnancy. Getting early and accurate diagnosis of these complications can help decrease the risk by enabling health care providers to diagnose, treat and manage conditions before they become serious.

## 4 Chapter 4- Mass spectrometric characterisation of endometrial tissue N-glycans in endometriosis

### 4.1 Introduction to endometriosis

Endometriosis is the growth of endometrial-like tissue outside the uterine cavity. The condition is often associated with pelvic pain, dysmenorrhea, and infertility. It is a chronic gynaecological disease with an unknown aetiology affecting approximately 10% of women of reproductive age and up to 30% of women who have subfertility (244). Despite its prevalence and impact in women's health, the pathology is not fully understood, and treatment options are only able to offer symptomatic relief.

Several hypotheses for the aetiology of endometriosis have been presented but none of these have been substantiated, in part due to the likelihood that endometriosis is a condition of multifactorial aetiology. The devastating impact of this condition on women's health is largely because endometriosis has a high latency and the diagnostic methods available are limited, invasive and often delayed taking an average of 7 years to diagnose. Diagnosis, therefore, is one of the main challenges, as the symptoms are broad and not specific to the condition, and in order to provide definitive diagnosis, laparoscopic surgery with histologic examination after biopsy is required. Imaging techniques can be used but they are mainly effective in diagnosing severe disease with ovarian and deep infiltrating endometriosis. As a result, patients go many years undiagnosed and sub-optimally managed. There is therefore a demand for an accurate less invasive diagnostic biomarker.

At present, Cochrane studies have confirmed that there are no viable non-invasive alternatives that can replace laparoscopic surgery in clinical practice (178). Some well-known markers have been shown to be raised in endometriosis. Cancer antigen (CA)-125 is one such example. It is a membrane glycoprotein found in the female reproductive tract. It is effective for indicating inflammatory, benign, and malignant transformations of the genital tract. Currently it is used as a biomarker to calculate the risk of epithelial ovarian cancer and to monitor treatment responses (143). Despite levels being raised in endometriosis, CA-125 is poorly specific as a marker for endometriosis and has not been proven sensitive enough to be a viable biomarker in early endometriosis(145, 245, 246).

Glycodelin-A is another glycoprotein found in the female reproductive tract which has an immunosuppressive function and is involved in the regulation of angiogenesis and apoptosis (49). Glycodelin-A levels have been shown to increase in patients with endometriosis (180, 181). Despite this, it has not been proven a suitable biomarker candidate due to the poor specificity to the condition. The suggestion of a combination of glycodelin-A with other factors to form an effective biomarker has been put forward (181, 182).

Endometriosis is strongly associated with infertility and many women with endometriosis undergo assisted reproduction treatments (ART) to achieve a viable pregnancy. Although a viable option, ART outcomes can be negatively impacted by the presence of endometriosis (174, 176, 177). Associated negative impacts reported include a reduced oocyte and embryo quality, as well as reduced fertilisation (175-177). The process by which endometriosis impacts ART outcomes is still up for discussion. One suggestion is that the reduced ongoing pregnancy rate in those with

endometriosis is due to an altered endometrial receptivity in these patients (176). It is likely that the effects of endometriosis on fertility and outcome of ART is multifactorial.

Despite numerous studies into the effect of endometriosis on fertility and women's health, there is little knowledge of how the condition effects glycosylation in the reproductive tract. Glycans, as discussed in Chapter 1 of this thesis, play a significant role in signalling and metabolic processes. They are also important in determining the properties of proteins and types of immunological responses within the body (1, 3).

Impaired or deficient glycosylation has been associated with disease states, including congenital muscular dystrophies, rheumatoid arthritis and ovarian cancer to name a few (51).

Alterations in glycosylation patterns and their potential importance as biomarkers in gynaecological diseases have been suggested by several studies. The role of monosaccharide biosynthesis in the pathogenesis of polycystic ovary syndrome (PCOS) has been reported by Izumi *et al* (53). A study analysing peritoneal fluid obtained during laparoscopy also reported significantly higher levels of mannose receptors in cases where endometriosis was found at laparoscopy versus the non-endometriosis control group(247). A further study analysed glycan expression profiles in primary cultured human endometriotic cyst stromal cells against normal endometrial stromal cells, using lectin microarray analysis. It found that levels of WFA-binding glycans were decreased in endometriotic cyst stromal cells, suggesting that reduced expression of N-glycans with WFA-binding properties is a novel characteristic of endometriosis (248). Glycomics clearly provides an opportunity to discover new glyco-biomarkers. Endometriosis-associated glycomic profiling opens potential viable targets for the development of diagnostics in this poorly understood disease.

Little is published on endometriosis and glycosylation. The published data to date, whilst helping to paint the picture of the role glycosylation plays in endometriosis, is still a way off from having a complete catalogue description of the glycans seen in normal states versus in endometriosis. Furthermore, we need other studies investigating ways to use tissues retrieved by less invasive methods, as this will give rise to less invasive, definitive diagnosis of the disease and possibly its management.

Whilst diagnosis is a major challenge in the management of endometriosis, the associated subfertility in many of these patients is another significant obstacle that may be linked to glycosylation pattern and needs to be further investigated/examined. Deficiency of endometrial epithelial differentiation and glycosylation have been related to endometriosis associated infertility (249-251). Additionally, blastocyst implantation has been shown to be closely related to the adequate epithelial glycocalyx of endometrial cells. Any alteration here may contribute to infertility in this group of women.

The number of studies examining glycosylation changes during endometriosis is small. The data available is often from animal studies or tissue from invasive operative biopsy of endometriotic lesions. Studies have examined tissue or peritoneal fluid glycosylation (obtained by invasive surgical techniques) but so far, none of the biomarker candidates have proven sensitive enough to compete with laparoscopic diagnosis. The results, none the less, are exciting and promising, and highlight the need for further research on the subject. Endometrial tissue is easily retrieved without need for surgery or anaesthesia or a hospital setting. Since endometrial cell glycans have not been fully characterised to determine the effects of the disease, it opens the possibility of a less invasive method for diagnosis.

To test the hypothesis that glycan profiles in the reproductive tract can be affected by disease states and the possibility of manipulation of these changes to benefit diagnosis and enable better treatment, glycomic analysis was performed to determine whether protein glycosylation is modified and to what extent in endometriosis patients. The N-glycomes in endometrial tissue and follicular fluid from healthy patients and patients with endometriosis have been rigorously characterised and described in this chapter. Both structural data and comparative data are presented here.

## 4.2 Results

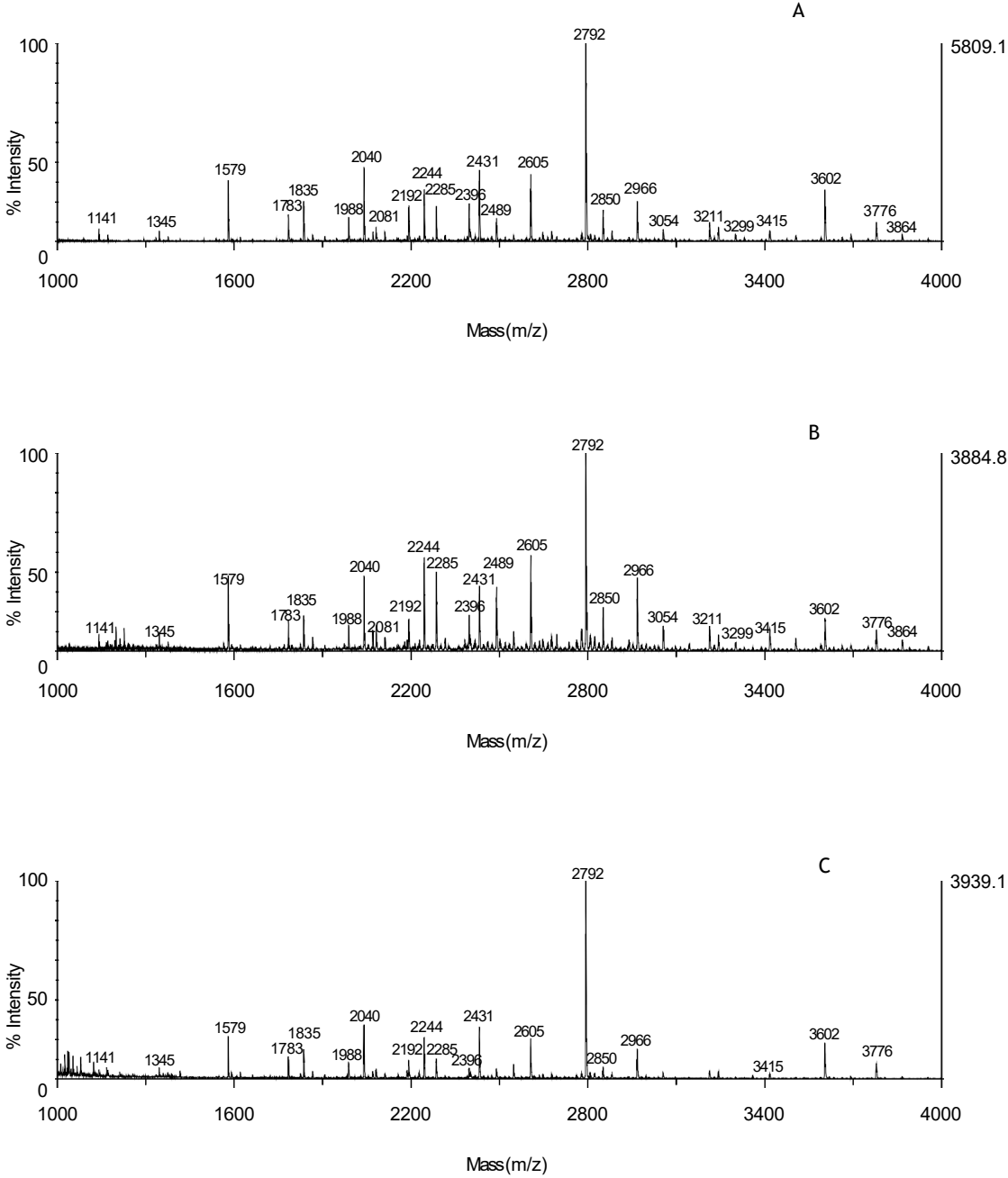
### 4.2.1 Endometrial tissue

Endometrial cell N-glycans were enzymatically released, permethylated and analysed using MALDI-TOF MS as described in Chapter 2. The resulting MALDI spectra are presented here. Proposed putative structures for the monoisotopic peaks of the  $[M+Na]^+$  molecular ions are based on the molecular mass composition, tandem MS/MS data, and knowledge of N-glycan biosynthetic pathways described in Chapter 1 and 2 in more detail. Semi-quantitative analysis is based on the relative abundances of individual components drawn from the MALDI MS data.

#### 4.2.1.1 *MS profiling of endometrial tissue N-glycans*

Figure 4-1 presents the MALDI spectrum of PNGase F released N-glycans from six different samples of normal endometrial tissue. The MS analysis was from individual samples of patients with no medical pathology. Significant structures are presented in Figure 4-2 and Table 4-1, along with the relative abundances.

Figure 4-1 : MALDI TOF MS of permethylated N-glycans from endometrial tissue from normal controls with no medical history of endometriosis. Six individual patients labelled A-F. m/z 2792 is consistently the dominant peak, corresponding to a biantennary sialylated N-glycan with no core fucosylation. The different samples share the same glycan repertoire, with some minor differences in the relative abundance of each glycan.



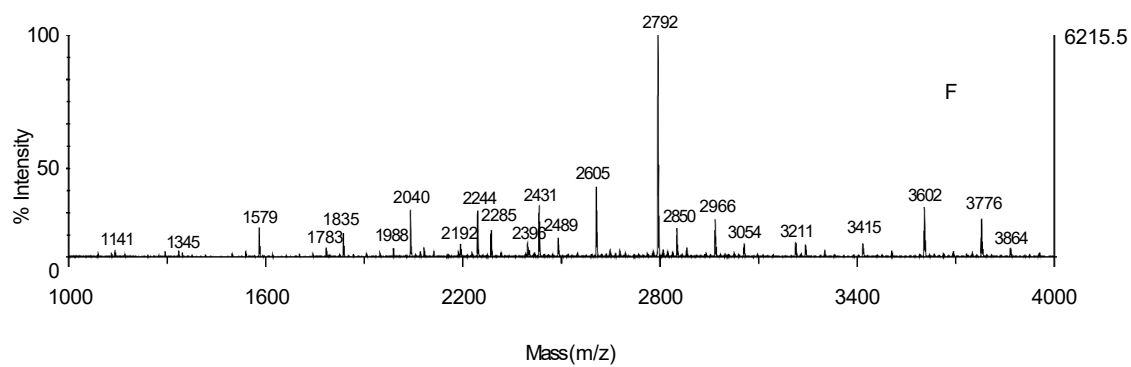
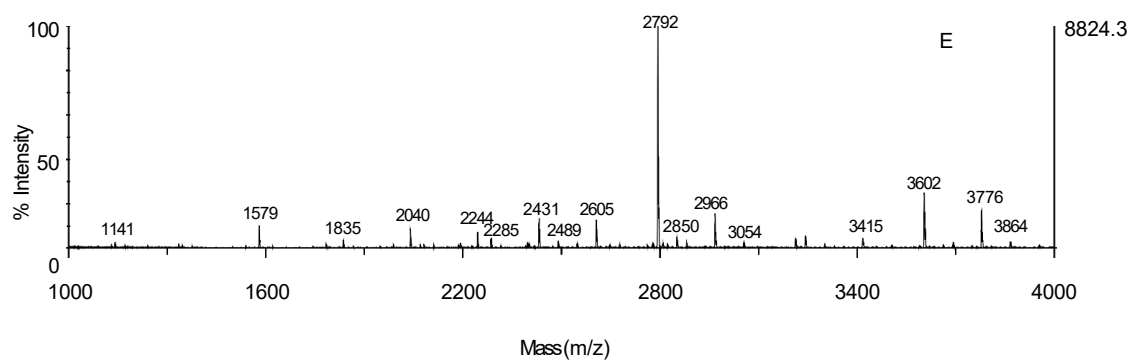
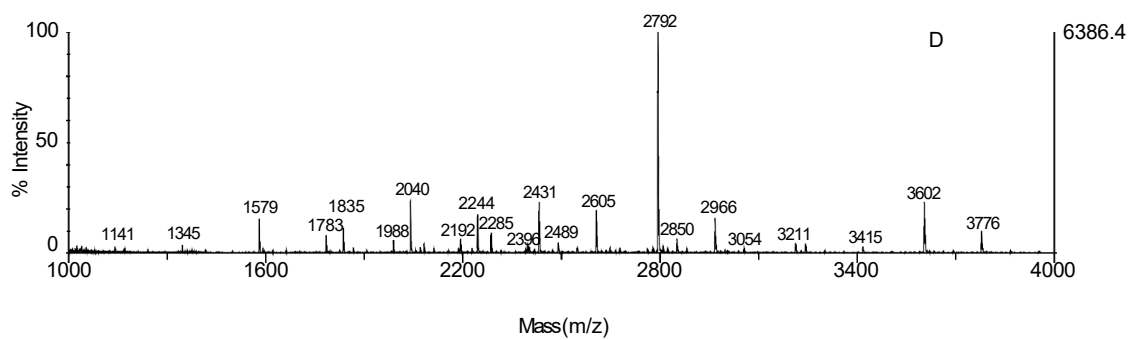
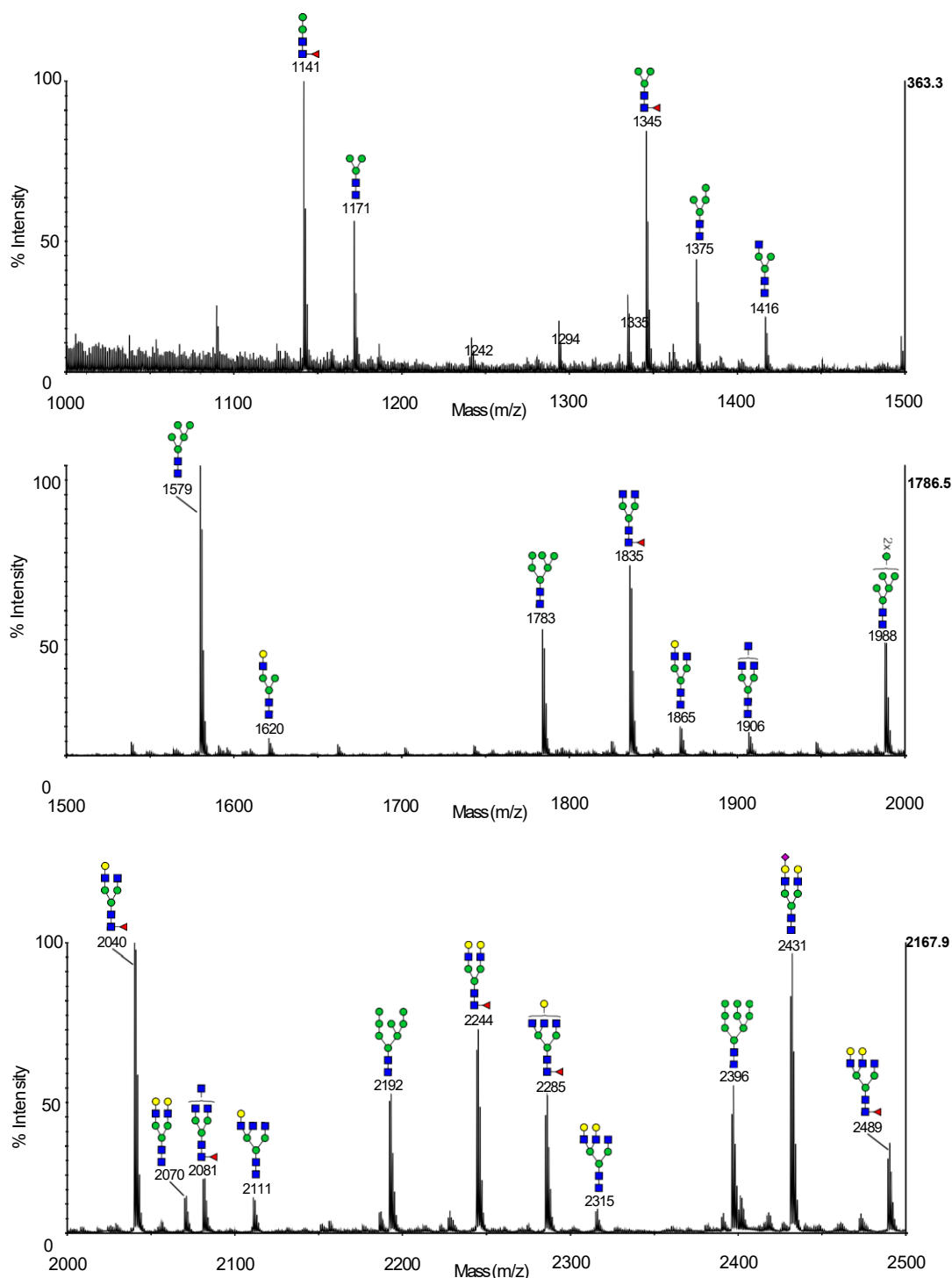
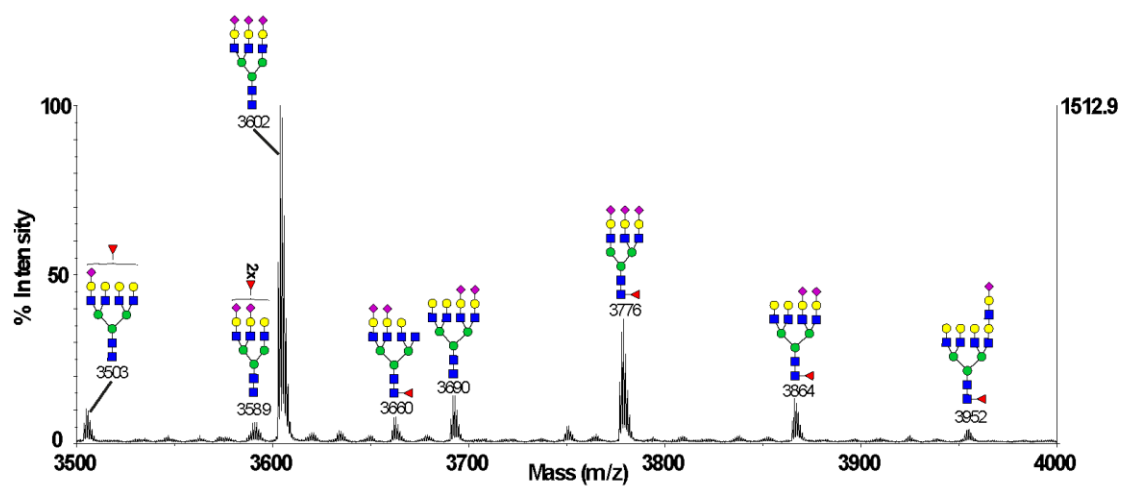
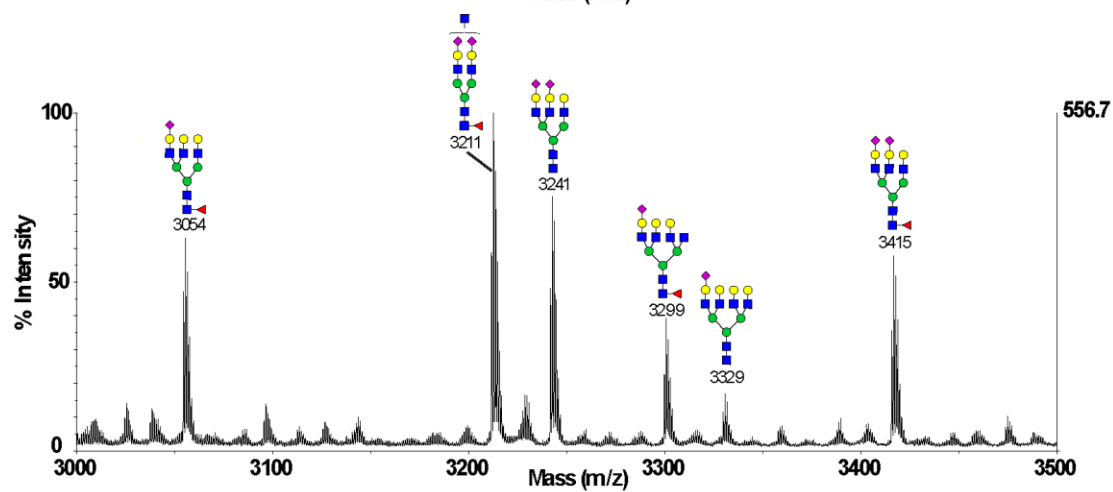
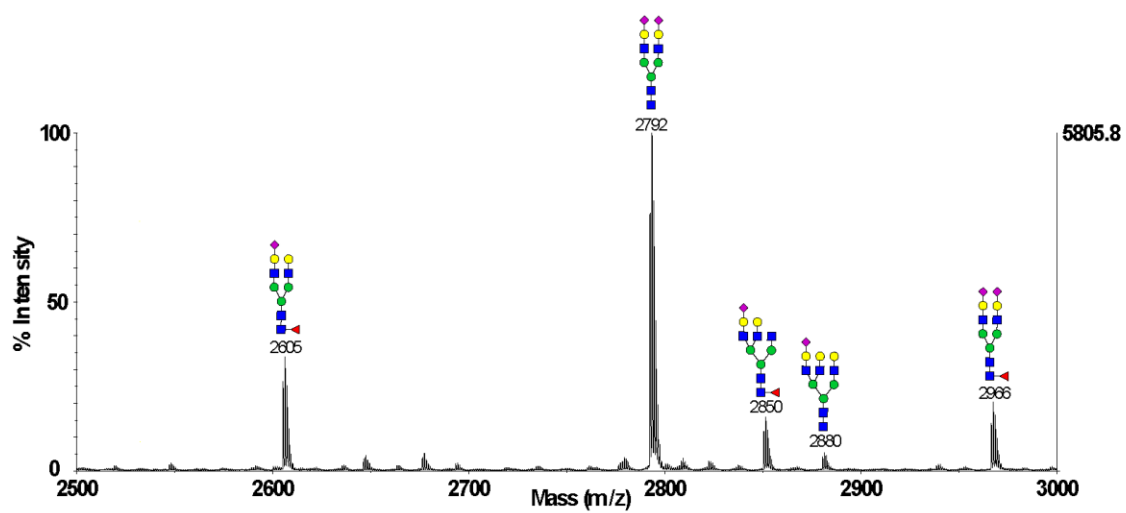


Figure 4-2: Annotated MALDI-TOF MS spectra of permethylated N-glycans derived from sample A, representing normal endometrial tissue with no evidence of endometriosis. Significant peaks are annotated with putative structures based on composition, tandem MS, and knowledge of biosynthetic pathways. All molecular ions are  $[M+Na]^+$ . Structures above a bracket were not unequivocally defined. The lower mass spectral region was predominated by high mannose type N-glycans such as that seen at  $m/z$  1579. The dominant peak at  $m/z$  2792 corresponds to a biantennary sialylated type glycan, with no fucosylation. The higher mass regions were characterised by tri- and tetra-antennary sialylated N-glycans.





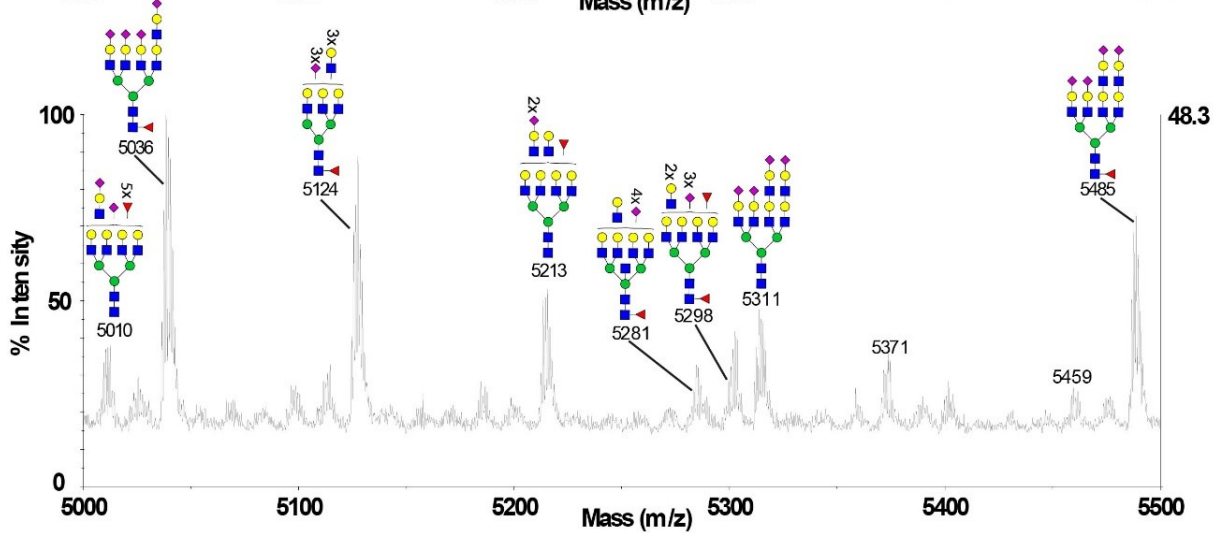
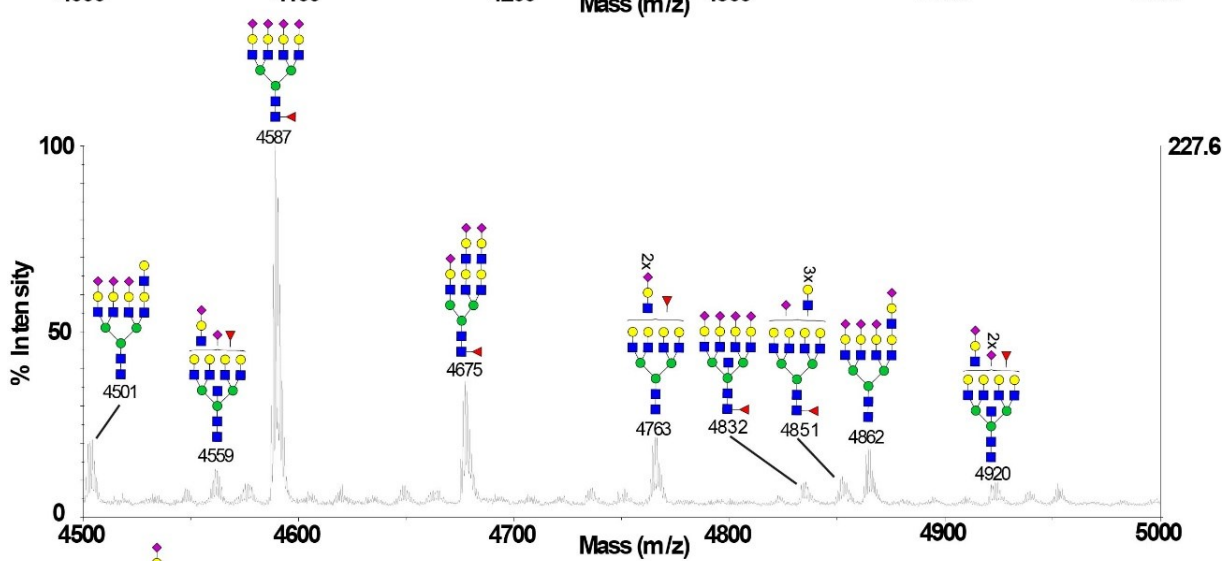
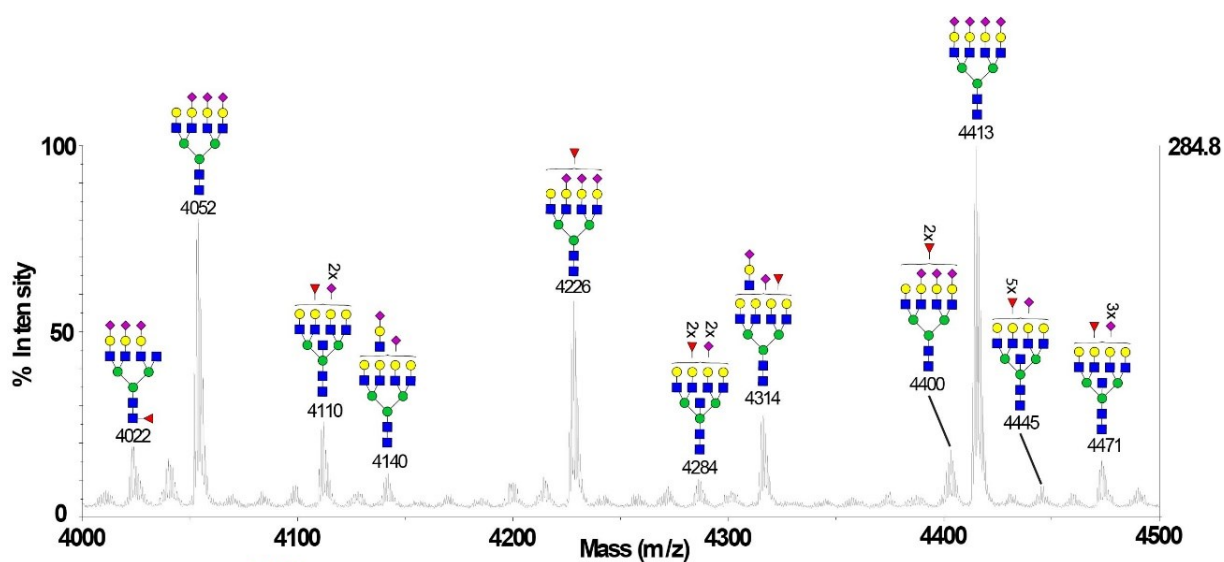


Table 4-1: Proposed glycan structures observed in MALDI-MS of permethylated N-glycans derived from endometrial tissue. The relative intensity is presented for the m/z values. A-F represent data from normal controls and G-I represent data from endometriosis samples.

| structure                                                                           | m/z  | Relative intensity |       |       |       |       |       |  |       |       |       |
|-------------------------------------------------------------------------------------|------|--------------------|-------|-------|-------|-------|-------|--|-------|-------|-------|
|                                                                                     |      | A                  | B     | C     | D     | E     | F     |  | G     | H     | I     |
|    | 1141 | 6.31               | 8.59  | 2.93  | 2.97  | 2.57  | 2.95  |  | 5.87  | 15.01 | 11.0  |
|    | 1171 | 3.37               | 5.30  | 2.12  | 2.13  | 1.24  | 1.18  |  | 2.58  | 3.18  | 6.33  |
|    | 1345 | 5.25               | 9.05  | 3.62  | 3.69  | 1.32  | 1.95  |  | 10.32 | 8.99  | 13.40 |
|    | 1375 | 2.49               | 4.70  | 1.38  | 1.63  | 0.90  | 0.83  |  | 3.52  | 4.70  | 5.18  |
|    | 1416 | 1.22               | 2.20  | 1.41  | 1.69  | 0.39  | 0.58  |  | 1.20  | 1.96  | 6.45  |
|    | 1579 | 30.61              | 38.0  | 13.79 | 15.49 | 10.10 | 13.21 |  | 66.84 | 56.88 | 77.58 |
|    | 1620 | 1.92               | 1.18  | 1.09  | 1.25  | 0.58  | 0.92  |  | 3.38  | 3.39  | 4.33  |
|  | 1661 | 1.25               | 1.01  | 0.75  | 1.13  | 0.34  | 0.45  |  | 1.70  | 2.67  | 5.55  |
|  | 1783 | 13.42              | 15.92 | 5.03  | 8.03  | 1.75  | 0.25  |  | 27.21 | 20.76 | 26.34 |
|  | 1794 | 0.82               | 1.35  | 0.81  | 0.44  | 0.33  | 0.57  |  | 4.57  | 3.85  | 3.60  |
|  | 1824 | 1.61               | 1.15  | 0.32  | 1.20  | 0.55  | 1.06  |  | 5.0   | 3.86  | 4.33  |
|  | 1835 | 0.45               | 1.63  | 7.86  | 0.33  | 3.46  | 0.28  |  | 27.26 | 29.38 | 53.20 |
|  | 1865 | 3.17               | 1.39  | 0.38  | 0.34  | 0.85  | 1.02  |  | 7.39  | 7.52  | 13.50 |
|  | 1906 | 0.71               | 1.28  | 0.60  | 0.43  | 0.35  | 0.96  |  | 3.11  | 10.01 | 4.48  |
|  | 1988 | 12.13              | 13.16 | 4.24  | 5.81  | 1.65  | 4.0   |  | 20.65 | 15.06 | 21.31 |
|  | 2040 | 37.26              | 38.07 | 12.45 | 23.29 | 8.43  | 20.56 |  | 74.34 | 85.84 | 99.05 |
|  | 2070 | 4.52               | 10.62 | 1.99  | 2.44  | 1.10  | 2.31  |  | 14.37 | 13.31 | 14.75 |
|  | 208  | 6.98               | 11.90 | 2.44  | 3.90  | 1.45  | 4.08  |  | 12.71 | 24.56 | 16.91 |
|  | 2111 | 4.56               | 6.95  | 1.19  | 2.15  | 1.17  | 2.59  |  | 7.58  | 9.91  | 8.14  |
|  | 2192 | 17.02              | 15.71 | 3.79  | 5.78  | 1.92  | 5.61  |  | 26.78 | 19.40 | 21.15 |

|  |      |       |       |       |       |       |       |  |       |       |       |
|--|------|-------|-------|-------|-------|-------|-------|--|-------|-------|-------|
|  | 2244 | 23.86 | 44.43 | 9.24  | 16.76 | 6.75  | 19.15 |  | 90.92 | 87.50 | 70.16 |
|  | 2285 | 15.14 | 35.06 | 5.00  | 8.25  | 3.83  | 10.35 |  | 47.63 | 57.13 | 34.69 |
|  | 2315 | 2.80  | 6.10  | 0.65  | 1.08  | 0.95  | 1.91  |  | 7.89  | 7.42  | 5.63  |
|  | 2396 | 15.43 | 15.56 | 2.53  | 3.56  | 1.96  | 5.60  |  | 26.79 | 20.99 | 12.92 |
|  | 2431 | 30.46 | 28.97 | 16.06 | 18.97 | 11.02 | 19.66 |  | 4.36  | 11.81 | 13.77 |
|  | 2489 | 9.69  | 26.92 | 3.43  | 3.71  | 2.75  | 7.05  |  | 44.52 | 37.11 | 19.93 |
|  | 2519 | 1.54  | 4.30  | 0.58  | 0.66  | 0.42  | 1.09  |  | 3.89  | 3.85  | 3.00  |
|  | 2592 | 1.60  | 3.45  | 0.66  | 0.73  | 0.45  | 0.87  |  | 1.65  | 2.44  | 3.25  |
|  | 2605 | 26.43 | 39.04 | 12.09 | 14.95 | 9.79  | 24.09 |  | 7.02  | 16.29 | 10.76 |
|  | 2663 | 1.64  | 3.95  | 1.70  | 1.05  | 0.38  | 1.46  |  | 5.91  | 6.01  | 3.26  |
|  | 2693 | 2.19  | 6.96  | 0.90  | 0.84  | 0.56  | 1.42  |  | 9.42  | 7.35  | 4.11  |
|  | 2734 | 1.29  | 3.80  | 0.59  | 0.82  | 0.54  | 1.14  |  | 3.23  | 3.56  | 2.29  |
|  | 2764 | 0     | 3.24  | 0.68  | 0.77  | 0.47  | 0.77  |  | 1.02  | 1.60  | 0.98  |
|  | 2792 | 76.29 | 76.69 | 72.46 | 73.27 | 73.01 | 71.57 |  | 1.49  | 13.26 | 4.55  |
|  | 2837 | 1.72  | 4.35  | 1.12  | 0.76  | 0.47  | 1.85  |  | 4.13  | 6.62  | 3.25  |
|  | 2850 | 11.48 | 16.81 | 4.37  | 4.55  | 3.48  | 9.72  |  | 3.09  | 9.44  | 4.11  |
|  | 2867 | 1.34  | 2.75  | 0.56  | 0.61  | 0.42  | 1.04  |  | 1.80  | 2.16  | 1.27  |
|  | 2880 | 4.11  | 5.36  | 1.10  | 1.38  | 1.80  | 3.05  |  | 1.20  | 3.03  | 1.60  |
|  | 2938 | 1.52  | 4.62  | 0.60  | 0.58  | 0.60  | 1.13  |  | 4.71  | 4.43  | 2.35  |
|  | 2966 | 13.95 | 26.42 | 8.72  | 9.61  | 10.27 | 12.06 |  | 0.62  | 4.74  | 1.53  |
|  | 2968 | 16.53 | 30.44 | 10.87 | 12.42 | 0     | 0     |  | 1.09  | 0     | 1.83  |
|  | 3024 | 0.97  | 2.65  | 0.71  | 0.35  | 0.44  | 1.23  |  | 0.79  | 4.08  | 0.71  |
|  | 3041 | 0.93  | 1.82  | 0.61  | 0.44  | 0.25  | 0.67  |  | 0.94  | 1.59  | 1.04  |
|  | 3054 | 4.47  | 9.09  | 1.54  | 1.60  | 1.21  | 4.08  |  | 1.37  | 3.62  | 1.71  |

|  |      |       |      |      |       |       |       |  |      |      |      |
|--|------|-------|------|------|-------|-------|-------|--|------|------|------|
|  | 3112 | 0.61  | 1.33 | 0.36 | 0     | 0     | 0.44  |  | 1.39 | 1.42 | 0.91 |
|  | 3142 | 0.77  | 2.91 | 0    | 0.58  | 0.47  | 0.78  |  | 2.23 | 2.81 | 1.31 |
|  | 3211 | 5.61  | 8.50 | 2.94 | 2.94  | 3.00  | 4.40  |  | 0.40 | 3.64 | 0.58 |
|  | 3228 | 1.63  | 2.55 | 0.79 | 0.76  | 0.54  | 1.12  |  | 1.12 | 1.38 | 0.70 |
|  | 3241 | 4.57  | 5.53 | 2.07 | 2.85  | 3.45  | 3.26  |  | 0    | 1.21 | 0.45 |
|  | 3286 | 0     | 1.17 | 0    | 0     | 0.19  | 0.47  |  | 0.86 | 1.22 | 0.70 |
|  | 3299 | 2.17  | 3.07 | 0.47 | 0.48  | 0.88  | 1.38  |  | 0.33 | 1.42 | 0.61 |
|  | 3316 | 0.59  | 1.10 | 0    | 0     | 0     | 0     |  | 0.56 | 0.64 | 0.57 |
|  | 3329 | 1.11  | 1.41 | 0    | 0     | 0.44  | 0.66  |  | 0    | 0.44 | 0    |
|  | 3387 | 0.59  | 1.64 | 0    | 0     | 0.29  | 0.69  |  | 1.53 | 1.47 | 0.86 |
|  | 3402 | 0.75  | 1.57 | 0    | 0.27  | 0.35  | 0.50  |  | 0.36 | 0.84 | 0.57 |
|  | 3415 | 3.31  | 6.90 | 1.29 | 1.57  | 2.59  | 3.33  |  | 0    | 1.52 | 0    |
|  | 3460 | 0.56  | 0.95 | 0.46 | 0     | 0     | 0.71  |  | 0.64 | 1.00 | 0    |
|  | 3503 | 1.54  | 0.74 | 0.44 | 0.43  | 0.75  | 1.43  |  | 0.52 | 1.24 | 0.62 |
|  | 3561 | 0.39  | 0.60 | 0    | 0.25  | 0     | 0.42  |  | 0.91 | 0.82 | 0.52 |
|  | 3589 | 1.53  | 2.50 | 0.69 | 0.49  | 0     | 0     |  | 0    | 0.53 | 0.24 |
|  | 3591 | 1.62  | 2.79 | 0.73 | 0.66  | 0     | 1.00  |  | 0.67 | 1.40 | 0    |
|  | 3602 | 13.73 | 1.18 | 7.80 | 12.10 | 13.20 | 11.64 |  | 0    | 0.76 | 0.23 |
|  | 3660 | 1.0   | 0.44 | 0.19 | 0.32  | 0.74  | 0.17  |  | 0    | 0.48 | 0    |
|  | 3664 | 0     | 1.84 | 0.49 | 0.41  | 0     | 0     |  | 0.34 | 0.57 | 0    |
|  | 3690 | 1.85  | 0    | 0.44 | 0.60  | 1.38  | 1.43  |  | 0    | 0.42 | 0    |
|  | 3735 | 0.34  | 0    | 0    | 0     | 0     | 0     |  | 0.64 | 0.86 | 0.24 |
|  | 3748 | 0.75  | 0    | 0.22 | 0     | 0     | 0.98  |  | 0.33 | 0.61 | 0.38 |
|  | 3776 | 4.7   | 0    | 2.06 | 4.58  | 0     | 8.60  |  | 0    | 1.11 | 0    |

|  |      |      |      |      |      |      |      |  |      |      |      |
|--|------|------|------|------|------|------|------|--|------|------|------|
|  | 3836 | 0    | 0.78 | 0    | 0.18 | 0    | 0.44 |  | 0.47 | 0    | 0    |
|  | 3864 | 1.74 | 0    | 0.32 | 0.53 | 0    | 1.90 |  | 0    | 0    | 0    |
|  | 3909 | 0    | 0    | 0    | 0    | 0    | 0.47 |  | 0.52 | 0.45 | 0.30 |
|  | 3952 | 0.70 | 0.80 | 0    | 0.39 | 0.42 | 0.88 |  | 0    | 0.33 | 0    |
|  | 4022 | 0.51 | 0.48 | 0.27 | 0    | 0.53 | 0    |  | 0    | 0.24 | 0    |
|  | 4052 | 1.81 | 1.10 | 0.37 | 1.26 | 2.26 | 1.38 |  | 0    | 0.27 | 0    |
|  | 4110 | 0.61 | 0.67 | 0    | 0    | 0.45 | 0.60 |  | 0    | 0    | 0    |
|  | 4140 | 0.43 | 0    | 0    | 0    | 0.18 | 0.29 |  | 0    | 0    | 0    |
|  | 4226 | 1.13 | 1.18 | 0.34 | 0.50 | 2.02 | 1.42 |  | 0    | 0    | 0    |
|  | 4284 | 0.14 | 0.41 | 0    | 0    | 0.11 | 0.46 |  | 0    | 0.28 | 0    |
|  | 4314 | 0.55 | 0.87 | 0    | 0.25 | 0.45 | 0.53 |  | 0    | 0    | 0    |
|  | 4400 | 0.56 | 0.41 | 0    | 0    | 0.48 | 0.36 |  | 0    | 0    | 0    |
|  | 4413 | 1.77 | 0.57 | 0.63 | 0.72 | 3.35 | 1.23 |  | 0    | 0.18 | 0    |
|  | 4445 | 0.36 | 0    | 0    | 0    | 0    | 0.23 |  | 0    | 0    | 0    |
|  | 4471 | 0.34 | 0    | 0    | 0    | 0.41 | 0.33 |  | 0    | 0    | 0    |
|  | 4501 | 0.36 | 0    | 0    | 0.23 | 0.35 | 0.29 |  | 0    | 0    | 0    |
|  | 4559 | 0    | 0    | 0    | 0    | 0    | 0.46 |  | 0    | 0    | 0    |
|  | 4587 | 1.14 | 0.51 | 0.55 | 0.42 | 3.26 | 1.18 |  | 0    | 0    | 0    |
|  | 4675 | 0.57 | 0.37 | 0    | 0.24 | 0.60 | 0.41 |  | 0    | 0.20 | 0    |
|  | 4763 | 0.34 | 0.53 | 0    | 0.23 | 0    | 0    |  | 0    | 0    | 0    |
|  | 4862 | 0.26 | 0    | 0    | 0.15 | 0.43 | 0.09 |  | 0    | 0    | 0    |
|  | 4920 | 0.16 | 0    | 0    | 0    | 0.33 | 0    |  | 0    | 0    | 0    |
|  | 5010 | 0.21 | 0    | 0    | 0    | 0.25 | 0    |  | 0    | 0    | 0    |

|  |      |      |   |   |      |      |      |  |   |   |   |
|--|------|------|---|---|------|------|------|--|---|---|---|
|  | 5036 | 0.31 | 0 | 0 | 0.17 | 0.07 | 0.20 |  | 0 | 0 | 0 |
|  | 5124 | 0.25 | 0 | 0 | 0    | 0.3  | 0.16 |  | 0 | 0 | 0 |
|  | 5485 | 0.12 | 0 | 0 | 0    | 0.30 | 0.17 |  | 0 | 0 | 0 |
|  | 5573 | 0.09 | 0 | 0 | 0    | 0.14 | 0.09 |  | 0 | 0 | 0 |
|  | 5659 | 0    | 0 | 0 | 0    | 0.07 | 0    |  | 0 | 0 | 0 |
|  | 5934 | 0.11 | 0 | 0 | 0    | 0    | 0.07 |  | 0 | 0 | 0 |
|  | 6023 | 0.12 | 0 | 0 | 0    | 0.07 | 0.11 |  | 0 | 0 | 0 |

typical MALDI spectrum from PNGase F released N-glycans of normal endometrial tissue, is dominated by a signal at m/z 2792 corresponding to a biantennary sialylated type glycan with no fucosylation, as illustrated in Figure 4-2. Additional signals of interest are noted at m/z 1579, consistent with high mannose type N-glycans, m/z 2040, 2244, 2605 corresponding to fucosylated biantennary type N-glycans, and m/z 3602 representing a triantennary sialylated N-glycan. The biantennary sialylated N-glycans at m/z 2792 was consistently dominant in all samples. The lower intensity N-glycans varied between samples, however.

The MS shows that normal endometrial tissue N-glycans are made up of a mixture of high mannose and complex type N-glycans. The high mannose peaks are represented by m/z 1579, 1783, 1987, 2192, and 2396. The complex type N-glycans consisted of bi-, tri and tetra-antennary structures. Molecular ions were observed up to m/z 5485. The antennae were capped with LacNAc, NeuAc, Sialyl Lewis<sup>x</sup> and Lewis<sup>x</sup> epitopes, and there was evidence of polyLacNAc extensions in some of the larger glycans.

Approximately 13% of structures were high mannose type N-glycans and 85% were complex N-glycans. A small proportion of paucimannose type structures were also observed. Of the complex type N-glycans, the majority, 71%, were biantennary and 41% of structures were core fucosylated. Structures were mostly capped with NeuAc; this represented approximately 61% of structures. 54% had LacNAc epitopes and Lewis<sup>x</sup> and Sialy Lewis<sup>x</sup> were observed in 3% and 1% of structures respectively.

#### *4.2.1.2 MS profiling of endometrial tissue N-glycans in the presence of endometriosis*

In Figure 4-3 the MS profile of endometrial tissue from patients with histologically diagnosed endometriosis is presented. Significant structures are presented in Figure 4-4 along with the relative abundances. The MS is characterised by significant peaks at m/z 1579, 2040 and 2244, corresponding to a high mannose and biantennary fucosylated structure. The MS shows that in the presence of endometriosis, endometrial tissue N-glycans consist of high mannose and complex type N-glycans, as seen in normal endometrial tissue without pathology. In the presence of endometriosis, however, high mannose N-glycans represent 25% of structures and complex N-glycans 71%. Also present are a small number of paucimannose type structures. Of the complex type N-glycans, the majority, 84%, were biantennary and 57% of structures were core fucosylated.

Figure 4-3: MALDI TOF MS of permethylated N-glycans from endometrial tissue from endometriosis samples. Three individual patients' samples labelled G-I are presented here. The MS are persistently dominated by peaks at  $m/z$  1579, 2040 and 2244, corresponding to a high mannose structure and a biantennary core fucosylated structure with no sialylation. The different samples share the same glycan repertoire, with some minor differences in the relative abundance of each glycan.

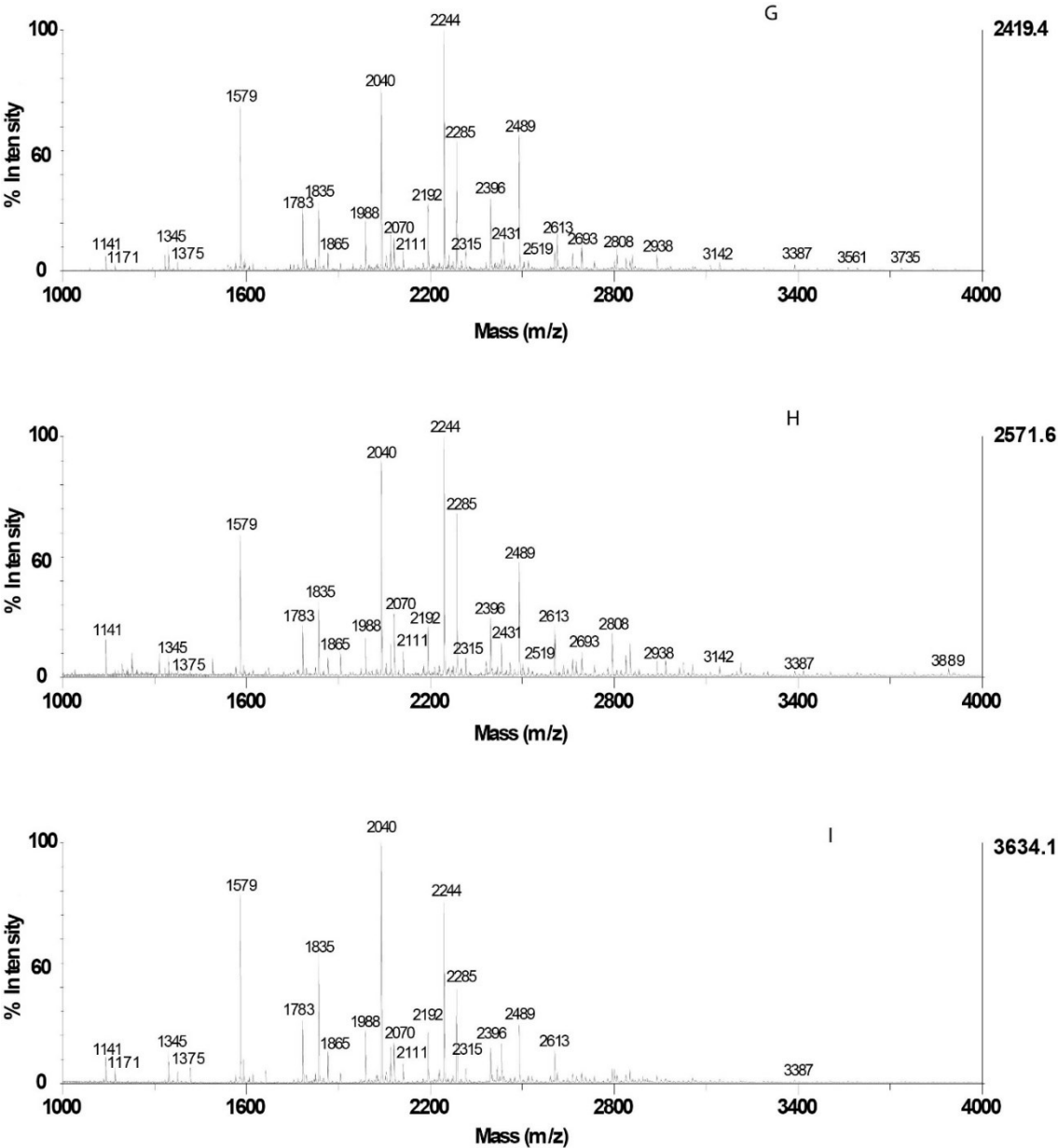
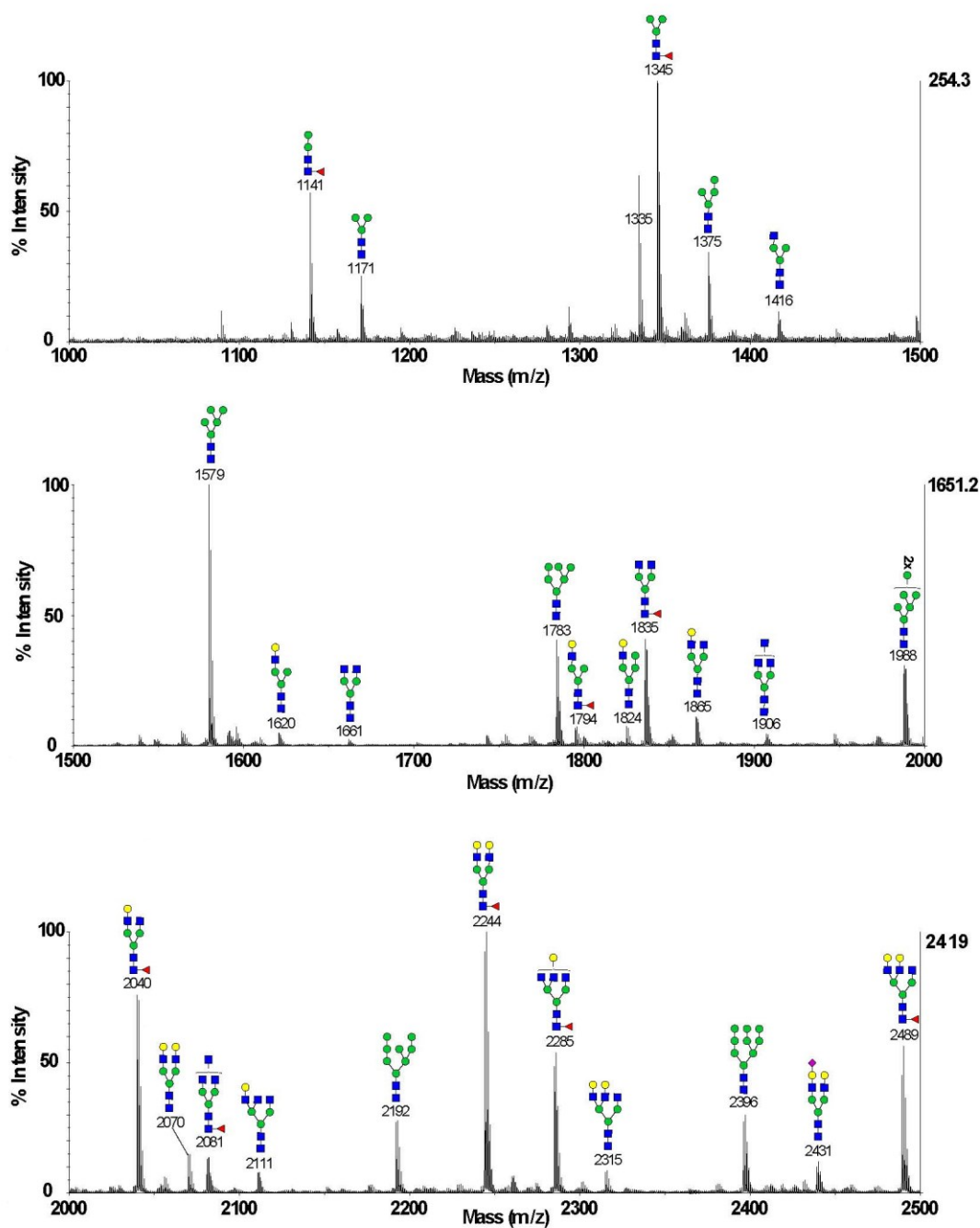
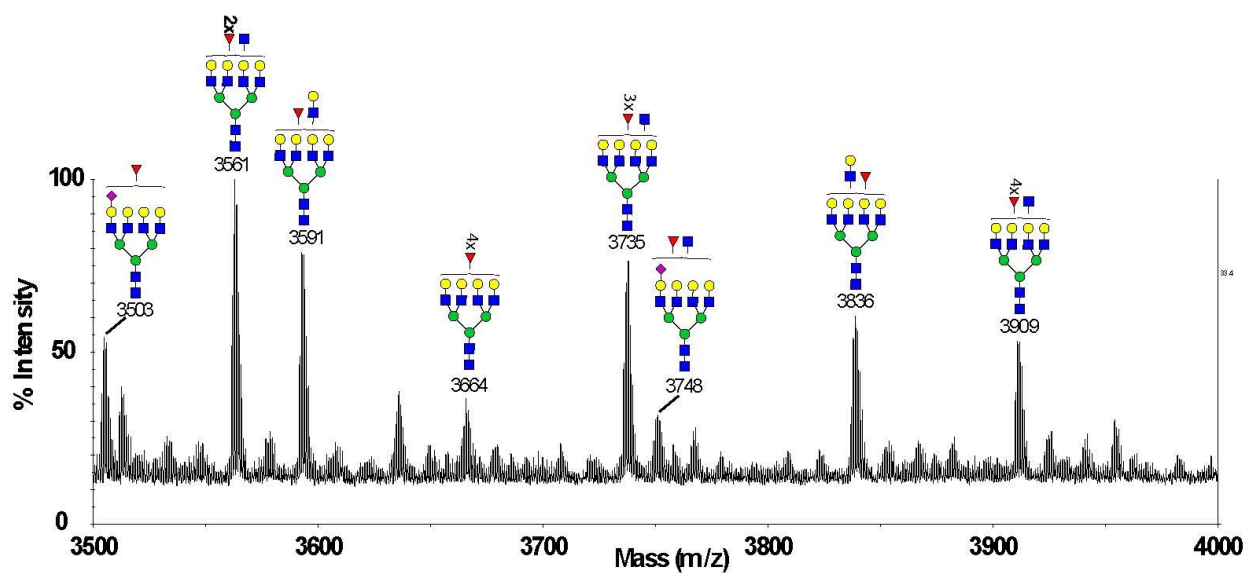
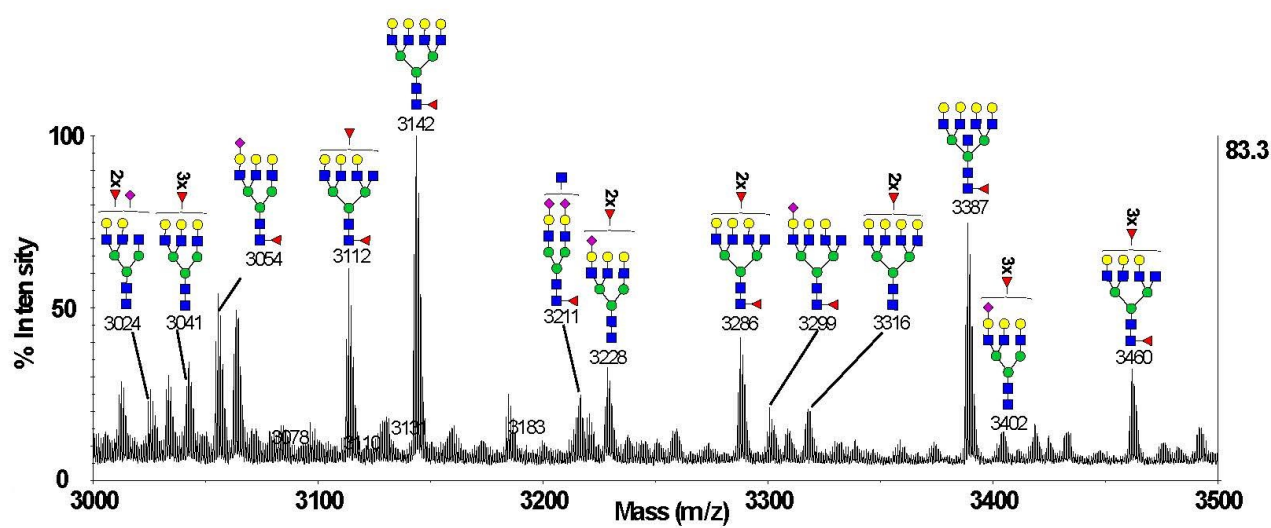
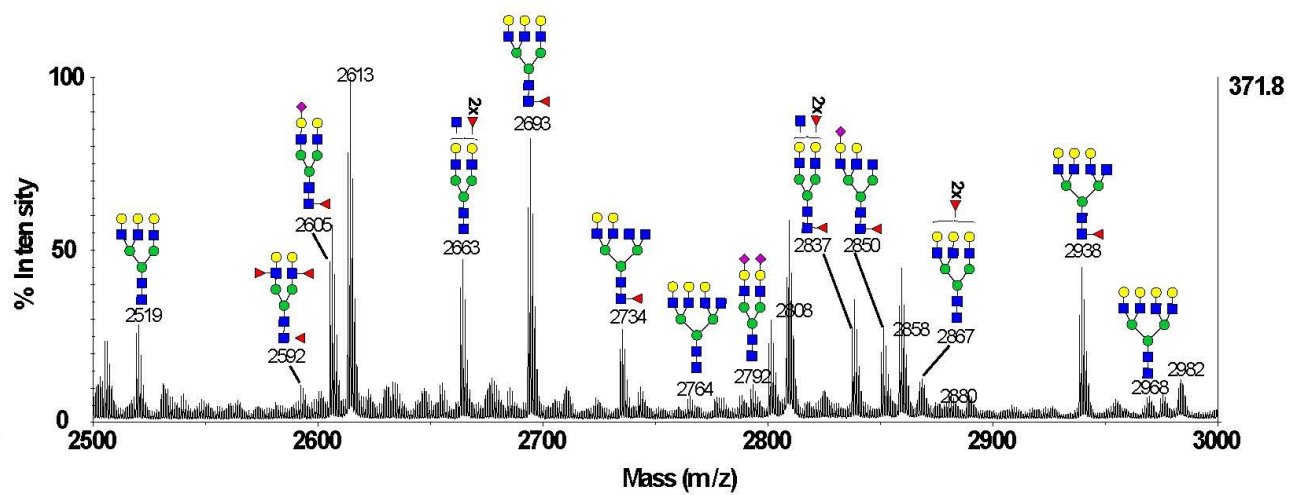


Figure 4-4: Annotated MALDI-TOF MS spectra of permethylated N-glycans derived from sample G, a patient with endometriosis. Significant peaks are annotated with putative structures based on composition, tandem MS, and knowledge of biosynthetic pathways. All molecular ions are  $[M+Na]^+$ . Structures above a bracket were not unequivocally defined. The lower mass region was predominated by high mannose type N-glycans such as that seen at  $m/z$  1579. The higher mass regions were characterised by complex type N-glycans with no sialylation. The dominant peaks are at  $m/z$  2040 and 2244, corresponding to a core fucosylated biantennary type glycan with no sialylation.





#### 4.2.2 Follicular fluid

Follicular fluid N-glycans were also enzymatically released using PNGase F, permethylated and analysed using MALDI-TOF MS as described in Chapter 2. This was done to assess whether any changes seen in endometrial tissue, were consistent throughout the female pelvis. The MALDI spectra presented are made up of singly charged sodiated ions  $[M+Na]^+$ , and the proposed putative structures for the monoisotopic peaks are based on the molecular mass composition, tandem MS/MS data, and knowledge of N-glycan biosynthetic pathways described in Chapter 1 and 2. Semi-quantitative analysis is based on the relative abundances of individual components drawn from the MALDI MS data.

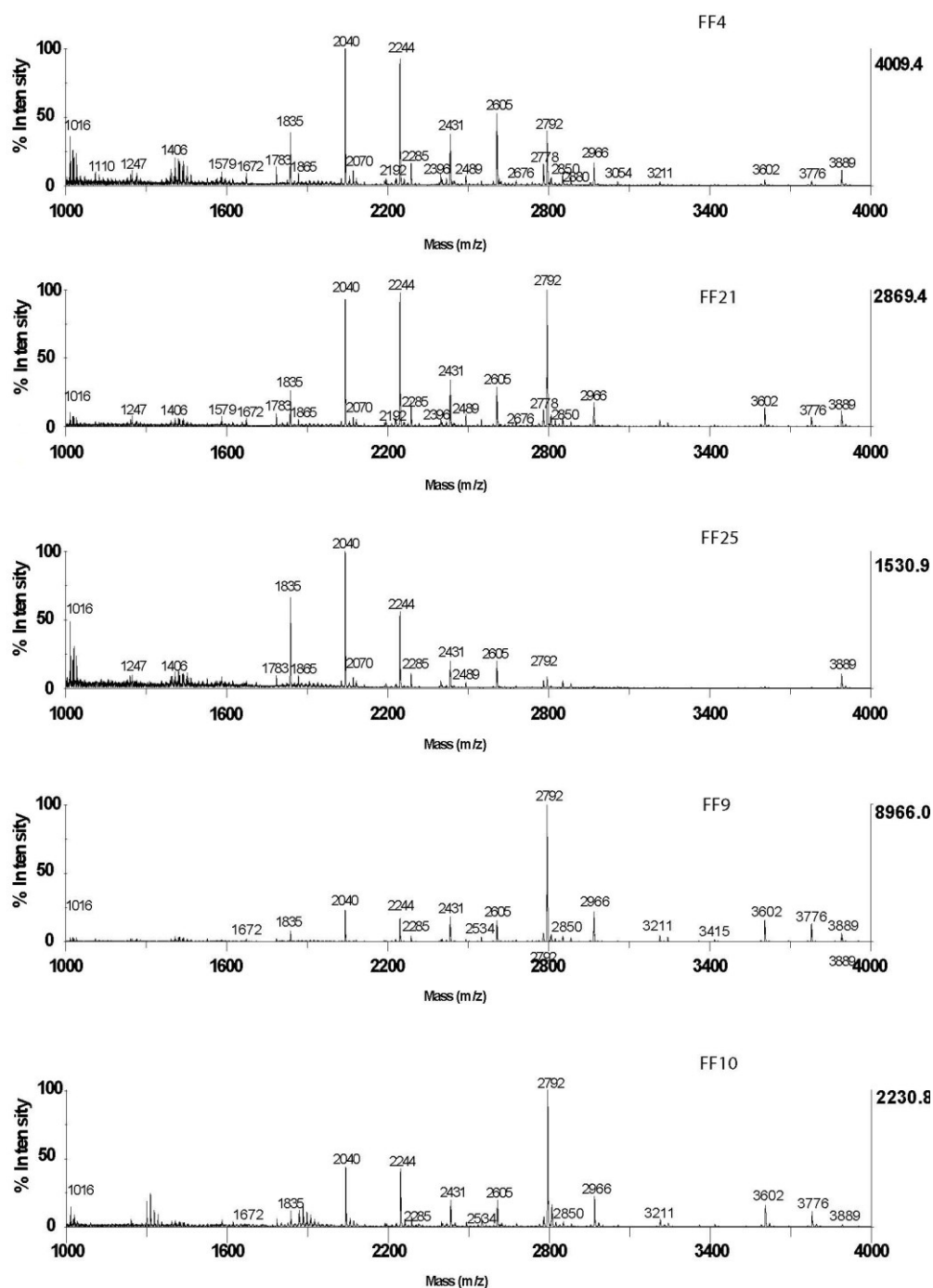
##### 4.2.2.1 MS profiling of follicular fluid N-glycans

The resulting MALDI spectra from enzymatically release N-glycans from follicular fluid samples is presented in Figure 4-5. The MS show that follicular fluid N-glycans, like endometrial tissue, are made up of high mannose and complex type N-glycans. The complex type N-glycans consisted of bi-, tri- and tetra-antennary structures. As reported with normal endometrial tissue, the follicular fluid N-glycan structures from normal samples were capped with LacNAc, NeuAc, polyLacNAc, Sialy Lewis<sup>x</sup> and Lewis<sup>x</sup> epitopes.

The spectra were dominated by a signal at  $m/z$  2792 for the normal samples and  $m/z$  2040 and 2244 in the endometriosis samples. The glycan profile of normal follicular fluid was similar to that of normal endometrial tissue. It is noted that in the absence of endometriosis, the spectra are dominated by a biantennary sialylated N-glycan at  $m/z$  2792. The changes seen between normal women and women with endometriosis is also consistent. There was, however, a reduction in the

high mannose peaks detected in endometrial tissue samples at  $m/z$  1579, 1783, 1987, 2192, and 2396, in both normal and endometriosis group of follicular fluid samples.

Figure 4-5: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) of permethylated N-glycans from follicular fluid samples. Panel FF9 and FF10 represent the MS from normal samples. FF4, FF21 and FF25 represent the MS from endometriosis samples. The MS is persistently dominated by peaks at  $m/z$  2040 and 2244 in the endometriosis samples and  $m/z$  2792 in the normal controls.



#### 4.2.2.2 *MS profiling of follicular fluid N-glycans in the presence of endometriosis*

The MALDI data from the follicular fluid of endometriosis patients presented in Figure 4-5 suggests that the changes seen in the endometrial tissue glycan profile between normal and endometriosis patients are consistent throughout the female pelvis. The main observation was a decreased expression of complex sialylated structures in the endometriosis patients.

The MS is mostly made up of complex type un-sialylated N-glycans with core fucosylation. The most dominant peaks were consistently at  $m/z$  1835, 2040, and 2244, all corresponding with a biantennary core fucosylated type structures, as depicted in Table 4-1. Capping sugars observed were mainly LacNAc, and Lewis<sup>x</sup> epitopes.

#### 4.3 *Glycosylation changes seen in reproductive tract N-glycans in diseased states*

Comparison of MS data from healthy versus endometriosis donors revealed that biantennary sialylated N-glycans were the most abundant glycan present in healthy donors versus a biantennary core fucosylated N-glycan without sialic acid in cases with endometriosis.

Table 4-2, Table 4-3, and Table 4-4 sets out the structural features derived from endometrial samples from normal controls and endometriosis. The data suggests that endometriosis is associated with increased mannose structures and a significant reduction in sialylated structures. In both cohorts, complex N-glycans make up most of the N-glycome, with biantennary fucosylated N-glycans as the predominant structure. Lewis<sup>x</sup> and Sialy Lewis<sup>x</sup> make up a small portion of the capping sugars. Figure 4-6 presents an annotated MS from the two groups for ease of comparison. These changes were found in both endometrial tissue and follicular fluid.

Table 4-2: Comparison of the glycan composition of endometrial tissue in all samples. A-F are data from normal controls and G-I are data from endometriosis samples. The average for each group is shown in red. Endometriosis is associated with an increase in mannose type structures. Both endometriosis and normal controls are made up of a mixture of high mannose and complex type glycans, with the majority observed as complex type N-glycans.

|          | High<br>Mannose % | Complex N-<br>glycan % |
|----------|-------------------|------------------------|
| Sample A | 18.79             | 78.14                  |
| Sample B | 15.87             | 80.64                  |
| Sample C | 9.73              | 87.53                  |
| Sample D | 13.60             | 83.33                  |
| Sample E | 8.41              | 89.23                  |
| Sample F | 9.12              | 89.00                  |
| Average  | 12.59             | 84.65                  |
| Sample G | 27.90             | 69.05                  |
| Sample H | 20.09             | 75.95                  |
| Sample I | 25.90             | 69.26                  |
| Average  | 24.61             | 71.42                  |

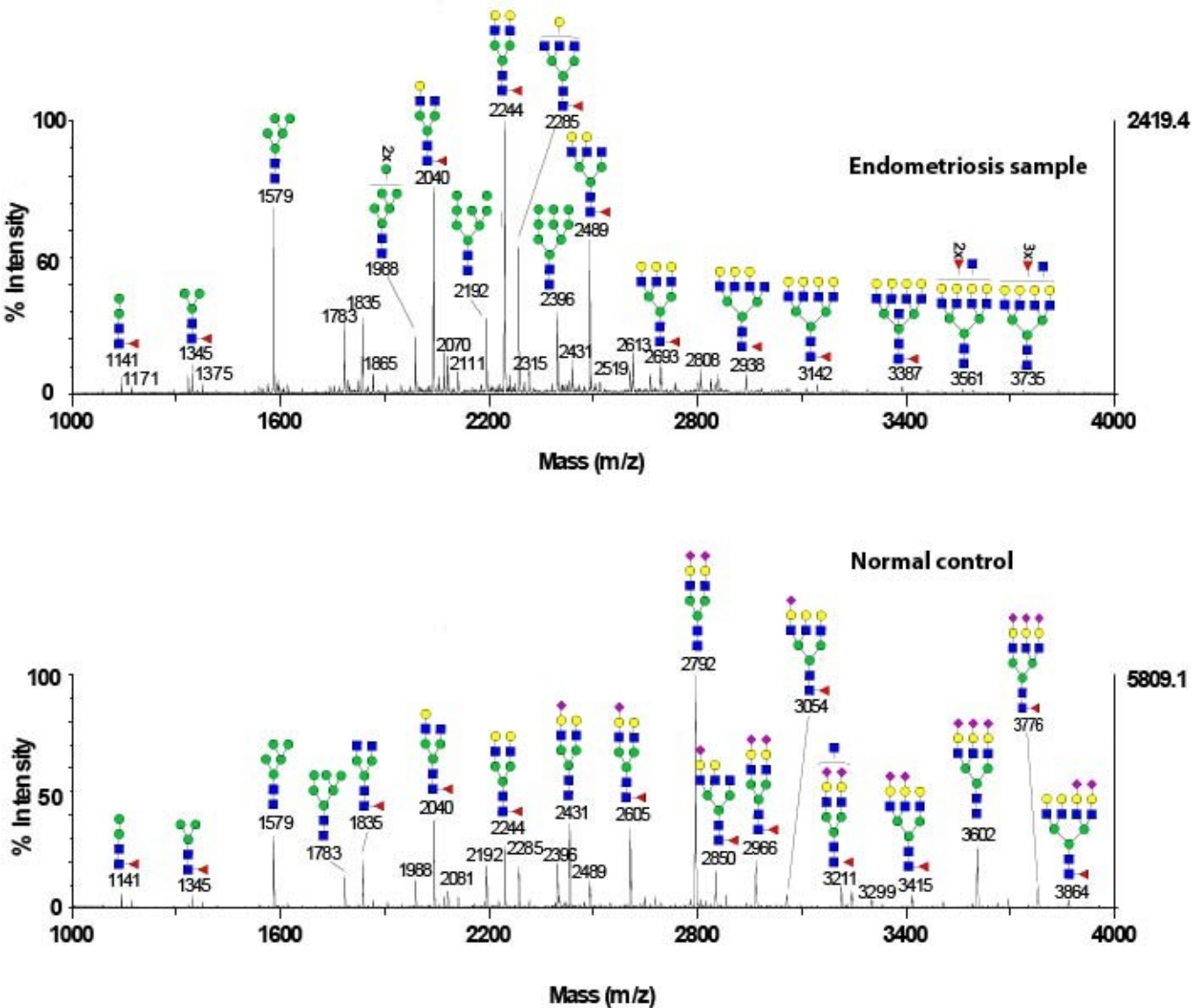
Table 4-3: The comparison of the relative intensities of the different complex glycans from the analysis of endometrial tissue. A-F represent data from normal controls and G-I represent data from endometriosis samples. The average for each group is shown in red. Endometrial tissue N-glycans are dominated by biantennary type N-glycans.

|          | Mono- | Bi-   | Tri-  | Tetra- | Core-F |
|----------|-------|-------|-------|--------|--------|
| Sample A | 1.04  | 72.35 | 14.18 | 10.54  | 42.07  |
| Sample B | 0.90  | 73.14 | 13.79 | 9.89   | 51.03  |
| Sample C | 1.20  | 59.25 | 33.01 | 5.66   | 27.91  |
| Sample D | 1.37  | 76.09 | 12.84 | 8.25   | 37.77  |
| Sample E | 0.67  | 73.42 | 15.24 | 9.93   | 36.10  |
| Sample F | 0.72  | 74.11 | 16.61 | 7.14   | 49.40  |
| Average  | 0.98  | 71.39 | 17.61 | 8.57   | 40.71  |
| Sample G | 1.76  | 69.16 | 6.35  | 1.88   | 57.75  |
| Sample H | 2.09  | 96.83 | 10.99 | 2.96   | 60.45  |
| Sample I | 3.27  | 86.04 | 5.34  | 1.51   | 53.98  |
| Average  | 2.37  | 84.01 | 7.56  | 2.12   | 57.39  |

Table 4-4: Comparison of the structural features from glycans identified in the analysis of endometrial tissue. A-F represent data from normal controls and G-I represent data from endometriosis samples. The average for each group is shown in red. Endometriosis is associated with decreased sialylation evident from the drop in NeuAc capped glycans.

|          | LacNAc | NeuAc | Le <sup>x</sup> | sLe <sup>x</sup> | Agalacto    |
|----------|--------|-------|-----------------|------------------|-------------|
| Sample A | 59.11  | 58.86 | 3.32            | 1.47             | 23.41688654 |
| Sample B | 65.78  | 45.96 | 5.34            | 1.85             | 25.424668   |
| Sample C | 55.52  | 49.65 | 2.41            | 0.79             | 15.87238501 |
| Sample D | 48.72  | 63.07 | 1.97            | 0.76             | 21.9757085  |
| Sample E | 39.49  | 79.19 | 1.90            | 0.91             | 10.95071149 |
| Sample F | 56.58  | 67.40 | 3.72            | 1.23             | 14.62313303 |
| Average  | 54.20  | 60.69 | 3.11            | 1.17             | 18.71       |
| Sample G | 86.29  | 4.88  | 5.04            | 0.53             | 43.47007904 |
| Sample H | 79.07  | 14.79 | 5.90            | 1.36             | 44.77411431 |
| Sample I | 76.40  | 9.35  | 3.77            | 0.50             | 55.58814168 |
| Average  | 80.59  | 9.67  | 4.90            | 0.80             | 47.94       |

Figure 4-6: MALDI-TOF N-glycomic profiles of endometrial tissue samples from normal control women and women with endometriosis. Putative structures are based on composition, tandem MS, and knowledge of biosynthetic pathways. All molecular ions are  $[M+Na]^+$ .



#### 4.4 Discussion

Our study is the first of its kind to present the results from structural characterisation of N-glycans in endometrial tissue from women with and without endometriosis using MALDI MS analysis. In the past, lectin and antibody binding have been commonly employed to define the expression of carbohydrate sequences. This work has generated detailed information about glycan structure to produce a conclusive report of glycosylation changes in endometriosis. This study has analysed endometrial samples obtained by pipelle biopsy during the fertile window of the menstrual cycle. Samples were obtained from patients with histologically diagnosed endometriosis as well as normal controls and analysed using MALDI MS. The relative abundance of the glycan peaks was compared between the study groups.

From the MS analysis, we were able to report a significant decrease of sialic acid containing N-glycans and an increase in high mannose type N-glycans in samples from women with endometriosis. The observed alterations may be related to altered glycosylation of endometrial cell N-glycans in the presence of endometriosis. These changes could provide novel insights into the pathophysiology of the disease and the alterations in sialic acid levels coupled with the elevated mannosylation level may serve as a possible target of a diagnostic tests. This has the potential of providing an opportunity to develop new less invasive diagnostic methods based on the measurement of glycosylation changes.

Endometriosis displays a variable scale of inflammatory reaction, and even though it's a benign condition, the inflammation, infiltration, and invasion of distant organs are comparable to that of malignant cancer. The role of glycosylation in the pathogenesis of various cancers is well documented (37, 252). Since endometriosis is linked with altered inflammatory and immune

processes, and shows similarities with cancer, one might expect that altered glycosylation might also play a role in the pathogenesis and progression of the disease. The data presented here show that in endometriosis, glycan structures are modified. This finding has been validated in the case of gynaecological diseases by several studies (53, 217, 253). Feng et al recruited a total of 132 women, 71 patients with PCOS and 61 control subjects, mannose levels were measured in their serum samples and multivariate logistic regression analysis was used to assess whether serum mannose levels were associated with PCOS after adjusting for other co-variables. The results showed that serum mannose levels were significantly increased in PCOS patients compared with control subjects. Lee et al purified Glycodelin-A (GdA) in amniotic fluid from women with normal and gestational diabetes affected pregnancies. They were able to report that gestational diabetes affected the glycosylation of GdA. In the presence of diabetes, GdA had a lower abundance of  $\alpha$ 2-6-sialylated and high-mannose glycans and a higher abundance of glycans with Sda (NeuAc $\alpha$ 2-3[GalNAc $\beta$ 1-4]Gal) epitopes compared with GdA from normal pregnancies.

Finally, subfertility in women with endometriosis remains unexplained and the impact in women's health is dramatic. Various unverified hypotheses exist. In addition to suggesting an abnormality in endometrial cell glycosylation associated with endometriosis, the findings here may be relevant to implantation failure and subfertility associated with endometriosis. Previous literature has linked sperm egg binding and blastocyst attachment with the glycocalyx (8, 9, 228).

#### 4.4.1 Impact of endometriosis on glycosylation

To understand the significance of the changes in sialylation reported in this thesis, we must firstly define the function of sialic acid in humans, then in cancer cells and finally in the natural history of endometriosis.

In humans, sialic acid exists solely as Neu5Ac. Sialylation catalysed by sialyltransferases is a crucial glycosylation modification. Sialylation is known to play a role in mediating signalling, immunological response, and cell-cell interaction in normal cells. Aberrant expression of terminal sialic acid has been detected in various carcinoma cells (254). For decades, aberrant sialylation has been linked with malignancies, tumour invasion and metastasis (255-258). Abnormal activity of sialyltransferases has been linked to cancer cells and the characteristic adhesion and invasion of cancer cells (37). Alterations in sialyl Lewis<sup>x</sup> antigens, as well as in terminal  $\alpha$ 2,6-sialylated structures have been documented in cancer cells. In fact, increased overall sialylation as a result of altered expression of glycosyltransferases is one of the most common changes in glycosylation associated with cancer (252, 258). With this in mind, it is pertinent to highlight the correlation between cancer cell activity and endometriosis. Endometriosis is characterised by inflammation, proliferation, migration, invasion, and adhesion (188). Endometrial cells can invade into the surrounding tissue and grow locally, forming endometriotic lesions in the abdominal cavity. The most commonly accepted hypothesis for the pathogenesis of endometriosis is that of retrograde menstruation whereby reflux of endometrial tissues during menstruation results in adhesion and ectopic endometriosis lesion formation (259). Additionally, altered expression of cytokines and growth factors allow for adhesion of the endometrium to the peritoneum in endometriosis (188). Endometriosis is unique given that it is a benign condition that displays characteristics similar to those of cancer cells. Interestingly, despite the similarities with cancer cell activity, the sialylation pattern of endometriotic cells, and the effect of sialic acid glycan epitopes on the development of endometriosis has not received the high level of scrutiny that sialylation has been subjected to in cancer research. As mentioned earlier, in cancer cells, sialylation has been reported to promote metastasis (142, 256). In endometriosis, the effects of altered sialylation on the genesis of

endometriosis, particularly on the adhesion between endometrial cells and peritoneal mesothelial cells, remain largely undefined. However, we know that abnormal or altered sialylation of glycoproteins by defective or altered expression of enzymes involved in their metabolism such as sialyltransferases, sialidases, have been associated with diseases (255). It can be inferred from the many studies on cancer cells that there is a correlation between increased sialylation and cancer metastatic potential, and prognosis. This is contrary to our observations here, where decreased sialylation was demonstrated. Perhaps it is the fact that endometriosis is a benign disease, despite its similarities to cancer cell activity, that determine the glycosylation profile here particularly in the case of sialylation.

Only a handful of studies have reported on the sialylation pattern of endometriotic cells (249, 250, 260-262). A study by Miller *et al.* (2010) compared endometrial tissue from 12 women with subfertility and advanced endometriosis with 11 healthy controls, using light microscopy to analyse lectin histochemistry. It reported lower levels of  $\alpha$ -2,3 sialylation in endometrial biopsies from women with endometriosis compared to healthy controls (250). Another study by Maignien *et al.* (2019) collected peritoneal fluids and endometrial and endometriotic biopsies from 102 endometriosis patients and 71 normal controls. The expression of  $\alpha$ -2,6-sialyltransferase was studied and the  $\alpha$ -2,6 sialylation levels measured. The study reported reduced  $\alpha$ -2,6 sialylation patterns in the peritoneal fluid of endometriosis-affected women (260). None of these studies gave a detailed report of the glycan profile of endometrial tissue in endometriosis and normal control. This was made possible in this thesis by the use of ultrasensitive MALDI MS analysis.

Maignien *et al.* (2019) reported that the expression of sialyltransferases was down-regulated in the ectopic endometrium obtained from endometriosis patients, and treatment with a sialidase

enzyme increased the *in-vitro* ability of endometrial stromal cells of endometriosis patients to migrate. This suggested that the hyposialylation reported here in this thesis, in endometrial cells of endometriosis patients, may be associated with enhanced migratory abilities, which might contribute to the establishment of early endometriotic implants (260). These observations need to be confirmed and their therapeutic potential explored.

#### 4.4.2 Role of glycosylation in endometriosis associated subfertility.

There are a few theories that account for endometriosis associated subfertility. These range from possible distortion of pelvic architecture and abnormal endometrial maturation to abnormal gene expression to mention a few. None of these have been unequivocally proved. The cause is likely to be multifactorial but the changes in endometrial glycosylation in this group of patients may be directly relevant to implantation failure. ART is currently the most effective treatment of endometriosis associated infertility; however, the outcomes are still significantly worse than in ART in non-endometriosis groups. Studies have suggested that women with endometriosis have poor ovarian reserve, low oocyte and embryo quality, and poor implantation (95, 263). The inflammatory effects seen in endometriosis have been shown to affect both oocyte production in ART and ovulation (264). There is also evidence of a luteal phase disruption in endometriosis (264, 265). Aberrant gene expression, during the window of implantation in women with endometriosis, has also been put forward as an explanation for the associated infertility (266).

The experiments presented in this thesis, showed that endometrial tissue glycosylation is different in the endometriosis patient group compared to controls. Glycosylation has been shown to be an important factor in the establishment of pregnancy (94, 267). Adhesion molecules such as integrins and selectin ligands are up regulated in normal endometrium during the implantation

window, possibly playing a critical role in implantation (11, 157, 268, 269). Glycans may be involved in ligand-receptor interactions between the blastocyst and the endometrium(94, 250, 267). Fucosylated glycoconjugates have been implicated in uterine receptivity(94), as well as specific glycoproteins, such as MUC-1, expressed during the implantation window(270) (271, 272), expression of which is altered in endometriosis(251). Aberrant glycosylation has been reported in cases of unexplained infertility(273) and recurrent miscarriage, providing further evidence for the role of glycosylation in the establishment of pregnancy. Studies have also reported that sialylation influences fertilization. In addition to indicating abnormalities associated with endometriosis, the glycosylation changes presented in this chapter, may also play a role in implantation failure and endometriosis associated subfertility.

Altered endometrial glycosylation has been associated with hormonal changes and the menstrual cycle. (250, 274). A study has shown the expression of Glycodelin-A, a well-known glycoprotein associated with endometrial function, is significantly increased in luteal phase of endometriosis tissue, and displayed a different N-glycan profile. The expression of various glycans has been shown to change over the course of the menstrual cycle in animal models (274). Studies have suggested that endometriosis is associated with changes in the gland architecture and biochemistry, resulting in dyssynchrony within the window of uterine receptivity, which may result in the reduced fertility associated with this disease (275). It is therefore possible, that our endometriosis samples display an MS glycosylation profile closer to that seen outside of the implantation window of the menstrual cycle, especially in cases associated with failed ART. It would be interesting therefore to investigate this further. These alterations may contribute to infertility in women with endometriosis, as blastocyst implantation requires the adequate epithelial glycocalyx of endometrial cells.

#### 4.4.3 Possibility for biomarker discovery and implication for future management of the disease

Based on current knowledge, endometriosis development is associated with an inflammatory process. The sialic acid-dependent anti-inflammatory effect is significantly reduced as seen by the loss of its expression in the endometriosis samples. Also reported here is an increase in high mannose type N-glycans in samples from women with endometriosis. The elevated mannosylation level could be an additional possible target for biomarker discovery. Elevated serum mannose levels have been shown to be a good diagnostic marker in women with polycystic ovary syndrome (53). Considering the above information and the results obtained in the present study, the analysis of the MS profile of these glycans may be very promising in the diagnostics of endometriosis. Characterizing the glycan composition using MALDI MS will enable further studies of the biological functions of each glycan, and the glycan-binding proteins, to help identify possible glycan biomarkers.

With increasing cost and time pressures on healthcare, it is highly advantageous to develop diagnostic tests that are not only accurate but less invasive. We were able to show significant changes here from samples retrieved using minimally invasive pipelle endometrial biopsy. This is not only less invasive than the current surgical method for diagnosing endometriosis, but it can also be carried out in a non-hospital setting. The ability to offer this group of patients an earlier effective diagnosis will significantly improve their quality of life and relieve pressure on the healthcare system.

#### 4.5 Concluding remarks

Glycans derived from endometrial tissue have been described here. In an effort to understand the role glycosylation plays in the pathology of endometriosis, the glycan structures in normal and disease states were compared. We found significant differences in the glycosylation pattern in endometriosis which opens the door to further analysis and discussion. Possible causes of the endometriosis-associated glycosylation changes that we have observed include altered glycosyltransferase expression, and availability and abundance of the sugar nucleotide donors (276, 277). In future work, establishing the causes and identifying the glycoproteins that are aberrantly glycosylated will further our understanding of this elusive condition and aid in the development of potential diagnostic markers and therapeutic treatments for endometriosis.

## 5 Chapter 5: Final Observations

### 5.1.1 Research question revisited

This thesis investigates the role of glycans in reproductive health through a robust structural characterisation using MS strategies. The study focuses on two main research questions, aiming to demonstrate the potential of glycomic studies in advancing biomarker discovery and its potential clinical utilisation.

The first question explores how the glycan profile of hCG differ between early, late, and abnormal pregnancies? This question explores the variations in the glycosylation patterns of hCG across different stages of pregnancy, including early, late, and those complicated by conditions such as gestational trophoblastic disease and preeclampsia. By comparing these profiles, the study aims to identify specific glycan structures that could serve as biomarkers for normal and abnormal pregnancy states.

The second question examines the glycosylation differences in endometrial tissue between healthy controls and patients with endometriosis? The N-glycan profile in endometrial tissues of healthy individuals is compared to those with endometriosis. The goal is to uncover distinct glycosylation patterns that may be associated with the pathophysiology of endometriosis, potentially leading to new biomarkers for diagnosis and therapeutic targets.

By addressing these questions, the thesis seeks to highlight the significant role of glycosylation in reproductive health and its implications for clinical applications, particularly in the context of disease diagnosis and management.

### 5.1.2 Key findings

The findings here highlight the role of glycans in reproductive health through detailed structural characterisation using mass spectrometry (MS) strategies. The key findings can be summarised as follows:

Firstly, the analysis of hCG glycosylation revealed notable differences between early, late, and abnormal pregnancies (Figure 3-15). In early pregnancy, hCG displayed a unique glycan profile, including the presence of novel bisecting GlcNAc (Figure 3-2). The glycosylation pattern in abnormal pregnancies, such as GTD and PET (Figure 3-7 and Figure 3-10), also showed distinct variations compared to normal pregnancies, suggesting that the glycan profile of hCG could potentially serve as a biomarker for identifying and distinguishing between normal and abnormal pregnancy states.

The characterisation of endometrial tissue N-glycans highlighted differences between healthy controls and patients with endometriosis. The study identified variations in glycosylation patterns, with a notable association between hyposialylation and endometriosis. This finding provides valuable insights into the pathogenesis of endometriosis and underscores the importance of glycosylation in reproductive health. The study suggests that alterations in glycan sialylation could serve as potential biomarkers for endometriosis, paving the way for future therapeutic interventions.

Overall, these findings contribute to the field of glycobiology by offering a comprehensive characterisation of hCG and endometrial N-glycomes. By elucidating the glycosylation patterns associated with normal and abnormal reproductive health, this thesis advances our understanding of the role of glycans in obstetrical syndromes and reproductive pathologies. Furthermore, the identification of novel glycan structures and the association with disease states underscore the

potential for glycomic studies in biomarker discovery and clinical applications. For instance, the presentation of the novel bisecting type N-glycan in hCG further adds to the field of glycobiology as we are the first to describe it in this setting. The expression of these bisecting N-glycans, implicated in the suppression of NK cell cytotoxicity, supports the human embryonic/fetal defence system hypothesis, and opens the window for further investigation into the mechanisms underlying its action and possible roles as a therapeutic target and/or biomarkers. Again, the discovery of a possible role of sialylation acts as a basis for further investigation. The importance of sialylation in immune cell development and functional regulation is well reported and alterations in glycan sialylation has been observed in a variety of inflammatory conditions (105, 255, 278, 279). The findings here broadened the scope of sialylation investigations in the management of endometriosis

### 5.1.3 Interpretation of results and implications

The glycosylation profile of hCG shows significant variation between early and late pregnancy, as well as in abnormal pregnancies (Figure 3-15, Figure 3-16). In early pregnancy, hCG exhibits unique glycan structures, including novel bisecting GlcNAc, which are less prevalent in late pregnancy. This differentiation in glycan structures suggests a functional adaptation of hCG to the specific needs of early pregnancy, such as immunomodulation and fetal-maternal interface support. This supports the hypothesis that specific glycosylation patterns play crucial roles in maintaining pregnancy. The distinct glycosylation observed in abnormal pregnancies indicates that deviations from the normal glycan profile of hCG can be indicative of pathological conditions, highlighting their potential for early detection and differentiation of pregnancy complications such as PET.

The characterisation of endometrial tissue N-glycans revealed interesting differences between healthy controls and patients with endometriosis (Figure 4-6). Notably, the study identified an association between hyposialylation and endometriosis. This suggests that altered sialylation of glycans may contribute to the pathophysiology of endometriosis, potentially affecting cell adhesion, immune recognition, and inflammatory responses within the endometrial environment.

The observed hyposialylation in endometriosis patients may reflect a disruption in the normal regulatory mechanisms of glycan synthesis and processing, which could influence the disease's progression and severity. This insight opens new avenues for investigating the molecular mechanisms underlying endometriosis and highlights the potential for targeting glycosylation pathways in therapeutic interventions.

Overall, the findings underscore the critical role of glycosylation in reproductive health and disease. The identification of specific glycan structures associated with normal and pathological conditions provides a foundation for the development of glycan-based biomarkers. Such biomarkers could improve the early detection and management of pregnancy complications and reproductive pathologies.

The discovery of novel bisecting GlcNAc in hCG and the association of hyposialylation with endometriosis represent significant advancements in the field of glycobiology. These findings not only enhance our understanding of the molecular mechanisms involved in reproductive health but also pave the way for future research aimed at developing glycan-targeted diagnostics and therapies.

In conclusion, the results of this thesis highlight the potential of glycomic analyses in elucidating the complex roles of glycans in reproduction. The detailed characterisation of hCG and

endometrial N-glycomes provides valuable insights that could lead to improved clinical outcomes through the development of novel diagnostic tools and therapeutic strategies

#### 5.1.4 Limitations

While this research provides a comprehensive characterisation of N-glycomic profiles of hCG and the endometrium, it does not address all the questions posed, leaving some issues unresolved and prompting further investigation in this field. The underlying mechanisms for the observed changes in glycosylation remain unclear. Given that glycosylation in the reproductive tract can be modified by hormonal changes(280), and hormones are the mainstay of non-surgical management for endometriosis, it would be beneficial to investigate how hormonal treatments affect endometrial glycosylation in both the presence and absence of endometriosis. Confirming changes in sialylation could potentially serve as a new biomarker for endometriosis.

The study was also limited by the lack of details on O-glycans due to the limited availability of samples. The focus on N-glycans was justified as nearly all membrane and extracellular proteins in humans are glycosylated, with N-glycans being the more common variant essential for multicellular life. However, understanding O-glycans could provide a more complete picture of the role of glycosylation in reproductive health.

Despite remarkable advances in MS technologies over the past decade, challenges persist.

Accurate quantification by MS is complex and, in this study, quantitation was semi-quantitative, achieved through comparison of glycan peaks and relative abundance. The quantity of specific glycans can be influenced by different physiological states of the producing cell type (281, 282).

While exact quantification was not essential for this study, as characterisation and semi-

quantitation have been shown to be remarkably informative (203, 281, 282) , it may benefit certain analytical questions in the future.

Another significant challenge encountered was the impact of sample contamination on data interpretation. A considerable number of samples did not produce clear, high-quality MS output due to contamination or insufficient quantity, which only became apparent once the spectra were produced. Nevertheless, with expert knowledge in glycomics, meaningful data could still be extracted.

#### 5.1.5 Recommendations for future work

Further research should focus on elucidating the mechanisms underlying the observed glycosylation changes. Since glycosylation in the reproductive tract can be influenced by hormonal changes, it is imperative to investigate how hormonal treatments affect endometrial glycosylation, both in the presence and absence of endometriosis. This could help clarify the role of hormonal regulation in glycosylation and its implications for disease management.

Additionally, future studies should aim to confirm the changes in sialylation identified in this research and explore their potential as biomarkers for endometriosis. Given the crucial role of sialylation in immune cell development and regulation, understanding its alterations in reproductive pathologies could provide valuable insights into disease mechanisms and highlight new therapeutic targets.

The current study was limited to N-glycans due to sample availability, but expanding the scope to include O-glycans would offer a more comprehensive understanding of glycosylation in reproductive health. O-glycans play significant roles in various biological processes, and their characterisation could reveal important functional implications for reproductive diseases.

Advancements in mass spectrometry (MS) technologies have significantly improved our ability to analyse glycosylation. However, the complexity of accurate quantification by MS remains a challenge. Developing and refining MS methodologies to achieve more precise quantification of glycans would enhance the robustness of glycomic studies and their applications in clinical diagnostics.

Exploring alternative methods for high-throughput glycan analysis, such as lectin microarrays, is also recommended. These methods, which allow rapid screening of glycan patterns(283), could complement MS techniques and facilitate large-scale studies. Lectin microarrays have shown promise in biomarker discovery (284-287) and could be particularly useful in identifying glycan alterations associated with endometriosis and other reproductive disorders.

In summary, future research should aim to clarify the hormonal regulation of glycosylation, validate potential biomarkers, expand the analysis to include O-glycans, refine MS quantification methods, and explore high-throughput glycan analysis techniques. These efforts will advance our understanding of glycosylation in reproductive health and its potential clinical applications.

#### 5.1.6 Concluding summary

This thesis underscores the critical role of glycosylation in reproductive health, highlighting the importance of glycomic studies for advancing our understanding and potential clinical applications in this field. Through the comprehensive characterisation of N-glycans in human chorionic gonadotropin (hCG) and endometrial tissue, significant insights into the glycosylation patterns associated with normal, abnormal pregnancies, and endometriosis were revealed.

The identification of differential glycosylation in hCG between early, late, and abnormal pregnancies enhances our knowledge of pregnancy-related glycosylation changes and opens new

avenues for biomarker discovery. Similarly, the characterisation of N-glycans in endometrial tissue provides valuable insights into the pathophysiology of endometriosis and highlights potential targets for therapeutic intervention.

Despite the challenges encountered, such as sample contamination and the limitations of current MS technologies, the findings presented here lay a robust foundation for future research. By addressing these limitations and exploring new methodologies, we can further our understanding of glycosylation in reproductive health and improve diagnostic and therapeutic strategies.

The advancements in mass spectrometry and glycan analysis techniques hold great promise for the future. Continued research in this area, with a focus on hormonal regulation, validation of biomarkers, and high-throughput glycan analysis, will significantly contribute to the field of reproductive health. The integration of structural and functional glycomic data will enhance our ability to diagnose, manage, and treat reproductive disorders, ultimately improving patient outcomes and healthcare practices.

In conclusion, this thesis demonstrates the profound impact of glycosylation on reproductive health and underscores the potential of glycomic studies to drive innovations in biomarker discovery and clinical applications. The knowledge gained from this research provides a solid platform for future investigations and highlights the importance of continuing to explore the complex and dynamic world of glycans in reproductive biology

## References

1. Varki A. Essentials of glycobiology. Essentials of glycobiology. 1999:xvii+653p.
2. Taylor ME, Drickamer K. Introduction to glycobiology. Introduction to glycobiology. 2003:i.
3. Varki A. Biological roles of glycans. Glycobiology. 2017;27(1):3-49.
4. Kobata A. A retrospective and prospective view of glycopathology. Glycoconjugate J. 1998;15(4):323-31.
5. Mengerink KJ, Vacquier VD. Glycobiology of sperm-egg interactions in deuterostomes. Glycobiology. 2001;11(4):37R-43R.
6. Pang PC, Chiu PCN, Lee CL, Chang LY, Panico M, Morris HR, et al. Human sperm binding is mediated by the sialyl-lewis(x) oligosaccharide on the zona pellucida. Science. 2011;333(6050):1761-4.
7. Wassarman PM, Litscher ES. Towards the molecular basis of sperm and egg interaction during mammalian fertilization. Cells Tissues Organs. 2001;168(1-2):36-45.
8. Diekman AB. Glycoconjugates in sperm function and gamete interactions: how much sugar does it take to sweet-talk the egg? Cellular and Molecular Life Sciences. 2003;60(2):298-308.
9. Clark GF. A role for carbohydrate recognition in mammalian sperm-egg binding. Biochem Biophys Res Commun. 2014;450(3):1195-203.
10. Ghaderi D, Springer SA, Ma F, Cohen M, Secrest P, Taylor RE, et al. Sexual selection by female immunity against paternal antigens can fix loss of function alleles. Proc Natl Acad Sci U S A. 2011;108(43):17743-8.
11. Genbacev OD, Prakobphol A, Foulk RA, Krtolica AR, Ilic D, Singer MS, et al. Trophoblast L-selectin-mediated adhesion at the maternal-fetal interface. Science. 2003;299(5605):405-8.
12. Linden E, Hurtado-Ziola N, Hayakawa T, Wiggleton L, Benirschke K, Varki A, et al. Human-specific expression of Siglec-6 in the placenta. Glycobiology. 2007;17(9):922-31.
13. Zhou Y, Damsky CH, Fisher SJ. Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype - One cause of defective endovascular invasion in this syndrome? Journal of Clinical Investigation. 1997;99(9):2152-64.
14. Dell A, Morris HR. Glycoprotein structure determination mass spectrometry. Science. 2001;291(5512):2351-6.
15. Varki A, Cummings RD, Aebi M, Packer NH, Seeberger PH, Esko JD, et al. Symbol Nomenclature for Graphical Representations of Glycans. Glycobiology. 2015;25(12):1323-4.
16. Varki A. Glycan-based interactions involving vertebrate sialic-acid-recognizing proteins. Nature. 2007;446(7139):1023-9.
17. Burda P, Aebi M. The dolichol pathway of N-linked glycosylation. Biochim Biophys Acta-Gen Subj. 1999;1426(2):239-57.
18. Yamashita K, Kamerling JP, Kobata A. STRUCTURAL STUDY OF THE CARBOHYDRATE MOIETY OF HEN OVOMUCOID - OCCURRENCE OF A SERIES OF PENTA-ANTENNARY COMPLEX-TYPE ASPARAGINE-LINKED SUGAR CHAINS. J Biol Chem. 1982;257(21):2809-14.
19. Rapoport TA, Jungnickel B, Kutay U. Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. Annu Rev Biochem. 1996;65:271-303.
20. Wopereis S, Lefeber DJ, Morava E, Wevers RA. Mechanisms in protein O-glycan biosynthesis and clinical and molecular aspects of protein O-glycan biosynthesis defects: A review. Clin Chem. 2006;52(4):574-600.
21. Brockhausen I. Mucin-type O-glycans in human colon and breast cancer: glycodynamics and functions. EMBO Rep. 2006;7(6):599-604.

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23. Valmu L, Alfthan H, Hotakainen K, Birken S, Stenman UH. Site-specific glycan analysis of human chorionic gonadotropin beta-subunit from malignancies and pregnancy by liquid chromatography-electrospray mass spectrometry. *Glycobiology*. 2006;16(12):1207-18.
24. Liu CL, Bowers LD. Mass spectrometric characterization of the beta-subunit of human chorionic gonadotropin. *J Mass Spectrom*. 1997;32(1):33-42.
25. Elliott MM, Kardana A, Lustbader JW, Cole LA. Carbohydrate and peptide structure of the alpha -and beta-subunits of human chorionic gonadotropin from normal and aberrant pregnancy and choriocarcinoma. *Endocrine*. 1997;7(1):15-32.
26. Liu F-T, Rabinovich GA. Galectins as modulators of tumour progression. *Nature Reviews Cancer*. 2005;5(1):29-41.
27. Barondes SH, Cooper DN, Gitt MA, Leffler H. Galectins. Structure and function of a large family of animal lectins. *J Biol Chem*. 1994;269(33):20807-10.
28. Camby I, Le Mercier M, Lefranc F, Kiss R. Galectin-1: a small protein with major functions. *Glycobiology*. 2006;16(11):137r-57r.
29. Liu FT, Rabinovich GA. Galectins: regulators of acute and chronic inflammation. *Ann N Y Acad Sci*. 2010;1183:158-82.
30. Robinson MJ, Sancho D, Slack EC, LeibundGut-Landmann S, Sousa CRE. Myeloid C-type lectins in innate immunity. *Nat Immunol*. 2006;7(12):1258-65.
31. van Kooyk Y. C-type lectins on dendritic cells: key modulators for the induction of immune responses. *Biochem Soc Trans*. 2008;36:1478-81.
32. Cummings RD, Smith DF. THE SELECTIN FAMILY OF CARBOHYDRATE-BINDING PROTEINS - STRUCTURE AND IMPORTANCE OF CARBOHYDRATE LIGANDS FOR CELL-ADHESION. *Bioessays*. 1992;14(12):849-56.
33. Crocker PR, Paulson JC, Varki A. Siglecs and their roles in the immune system. *Nat Rev Immunol*. 2007;7(4):255-66.
34. Collins BE, Paulson JC. Cell surface biology mediated by low affinity multivalent protein-glycan interactions. *Curr Opin Chem Biol*. 2004;8(6):617-25.
35. Nitschke L, Floyd H, Crocker PR. New functions for the sialic acid-binding adhesion molecule CD22, a member of the growing family of Siglecs. *Scand J Immunol*. 2001;53(3):227-34.
36. Pillai S, Netravali IA, Cariappa A, Mattoo H. Siglecs and immune regulation. In: Paul WE, editor. *Annual Review of Immunology*, Vol 30. *Annual Review of Immunology*. 30. Palo Alto: Annual Reviews; 2012. p. 357-92.
37. Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. *Nature Reviews Cancer*. 2015;15(9):540-55.
38. Sutton-Smith M, Dell A. *Cell biology: A laboratory handbook* 2005. 415-25 p.
39. Pilobello KT, Krishnamoorthy L, Slawek D, Mahal LK. Development of a lectin microarray for the rapid analysis of protein glycopatterns. *ChemBioChem*. 2005;6(6):985-9.
40. Kuno A, Uchiyama N, Koseki-Kuno S, Ebe Y, Takashima S, Yamada M, et al. Evanescent-field fluorescence-assisted lectin microarray: a new strategy for glycan profiling. *Nat Methods*. 2005;2(11):851-6.
41. Xin AJ, Cheng L, Diao H, Wang P, Gu YH, Wu B, et al. Comprehensive profiling of accessible surface glycans of mammalian sperm using a lectin microarray. *Clinical Proteomics*. 2014;11:9.
42. Yamashita K, Kuno A, Matsuda A, Ikehata Y, Katada N, Hirabayashi J, et al. Lectin microarray technology identifies specific lectins related to lymph node metastasis of advanced gastric cancer. *Gastric Cancer*. 2016;19(2):531-42.

43. Pang P-C, Tissot B, Drobnis EZ, Sutovsky P, Morris HR, Clark GF, et al. Expression of bisecting type and Lewisx/Lewisy terminated N-glycans on human sperm. *J Biol Chem*. 2007;282(50):36593-602.
44. Clark GF. Functional glycosylation in the human and mammalian uterus. *Fertility research and practice*. 2015;1:17-.
45. Clark GF, Oehninger S, Patankar MS, Koistinen R, Dell A, Morris HR, et al. A role for glycoconjugates in human development: The human feto-embryonic defence system hypothesis. *Hum Reprod*. 1996;11(3):467-73.
46. Pang PC, Drobnis E, Sutovsky P, Morris H, Lattanzio F, Sharpe-Timms K, et al. The expression of bisecting type n-glycans and ligands for dc-sign on human sperm. *Glycobiology*. 2006;16(11):1161-.
47. Tollner TL, Bevins CL, Cherr GN. Multifunctional glycoprotein DEFB126-a curious story of defensin-clad spermatozoa. *Nat Rev Urol*. 2012;9(7):365-75.
48. Focarelli R, Luddi A, De Leo V, Capaldo A, Stendardi A, Pavone V, et al. Dysregulation of GdA Expression in Endometrium of Women With Endometriosis: Implication for Endometrial Receptivity. *Reproductive Sciences*. 2018;25(4):579-86.
49. Seppala M, Taylor RN, Koistinen H, Koistinen R, Milgrom E. Glycodelin: A major lipocalin protein of the reproductive axis with diverse actions in cell recognition and differentiation. *Endocr Rev*. 2002;23(4):401-30.
50. Varki A. BIOLOGICAL ROLES OF OLIGOSACCHARIDES - ALL OF THE THEORIES ARE CORRECT. *Glycobiology*. 1993;3(2):97-130.
51. Reily C, Stewart TJ, Renfrow MB, Novak J. Glycosylation in health and disease. *Nature Reviews Nephrology*. 2019;15(6):346-66.
52. Saldo R, Wormald MR, Dwek RA, Rudd PM. Glycosylation changes on serum glycoproteins in ovarian cancer may contribute to disease pathogenesis. *Disease Markers*. 2008;25(4-5):219-32.
53. Feng D, Shi B, Bi F, Sagnelli M, Sun X, Jiao J, et al. Elevated Serum Mannose Levels as a Marker of Polycystic Ovary Syndrome. *Frontiers in Endocrinology*. 2019;10.
54. Qian Y, Wang Y, Zhang X, Zhou L, Zhang Z, Xu J, et al. Quantitative Analysis of Serum IgG Galactosylation Assists Differential Diagnosis of Ovarian Cancer. *J Proteome Res*. 2013;12(9):4046-55.
55. Yang GL, Zhang JX, Liu CM, Guan F. Quantitative analysis of n-glycome of different bladder cancer cells. *Prog Biochem Biophys*. 2015;42(12):1144-54.
56. Auchus RJ. Chapter 8 - Human Steroid Biosynthesis. In: Plant TM, Zeleznik AJ, editors. *Knobil and Neill's Physiology of Reproduction (Fourth Edition)*. San Diego: Academic Press; 2015. p. 295-312.
57. Barrett KE, Barman SM, Brooks HL, Yuan JXJ. *Ganong's Review of Medical Physiology*, 26e. New York, NY: McGraw-Hill Education; 2019.
58. *Textbook of Endocrine Physiology*. Griffin JE, Ojeda SR, editors: Oxford University Press; 2004 31 Oct 2023.
59. Johnson L, Thompson DL, Jr., Varner DD. Role of Sertoli cell number and function on regulation of spermatogenesis. *Anim Reprod Sci*. 2008;105(1-2):23-51.
60. Cheng CY, Mruk DD. The biology of spermatogenesis: the past, present and future. *Philos Trans R Soc Lond B Biol Sci*. 2010;365(1546):1459-63.
61. Cheng CY, Wong EW, Yan HH, Mruk DD. Regulation of spermatogenesis in the microenvironment of the seminiferous epithelium: new insights and advances. *Mol Cell Endocrinol*. 2010;315(1-2):49-56.
62. Knobil E, Neill JD. *Knobil and Neill's Physiology of Reproduction*: Elsevier; 2006.

63. Fritz MA, Speroff L. Clinical Gynecologic Endocrinology and Infertility: Wolters Kluwer Health; 2012.
64. Speroff L, Fritz MA. Clinical Gynecologic Endocrinology and Infertility: Lippincott Williams & Wilkins; 2005.
65. Paulson RJ. Hormonal induction of endometrial receptivity. *Fertility and Sterility*. 2011;96(3):530-5.
66. Fortune JE. OVARIAN FOLLICULAR-GROWTH AND DEVELOPMENT IN MAMMALS. *Biology of Reproduction*. 1994;50(2):225-32.
67. Hall JE. Guyton and Hall Textbook of Medical Physiology: Elsevier Health Sciences; 2015.
68. Gellersen B, Brosens JJ. Cyclic Decidualization of the Human Endometrium in Reproductive Health and Failure. *Endocrine Reviews*. 2014;35(6):851-905.
69. Morris HR, Dell A, Easton RL, Panico M, Koistinen H, Koistinen R, et al. Gender-specific glycosylation of human glycodelin affects its contraceptive activity. *J Biol Chem*. 1996;271(50):32159-67.
70. Saraswat M, Joenvaara S, Tomar AK, Singh S, Yadav S, Renkonen R. N-glycoproteomics of human seminal plasma glycoproteins. *Journal of Proteome Research*. 2016;15(3):991-1001.
71. Kirchhoff C, Schroter S. New insights into the origin, structure and role of CD52: A major component of the mammalian sperm glycocalyx. *Cells Tissues Organs*. 2001;168(1-2):93-104.
72. Schroter S, Osterhoff C, McArdle W, Ivell R. The glycocalyx of the sperm surface (vol 5, pg 302, 1999). *Hum Reprod Update*. 1999;5(5):561-.
73. Orsi NM, Baskind NE, Cummings M. Anatomy, Development, Histology and Normal Function of the Ovary. In: Wilkinson N, editor. *Pathology of the Ovary, Fallopian Tube and Peritoneum*. Cham: Springer International Publishing; 2023. p. 1-33.
74. Myers KM, Elad D. Biomechanics of the human uterus. *Wiley Interdisciplinary Reviews-Systems Biology and Medicine*. 2017;9(5):20.
75. Ambekar AS, Nirujogi RS, Srikanth SM, Chavan S, Kelkar DS, Hinduja I, et al. Proteomic analysis of human follicular fluid: A new perspective towards understanding folliculogenesis. *Journal of Proteomics*. 2013;87:68-77.
76. Revelli A, Delle Piane L, Casano S, Molinari E, Massobrio M, Rinaudo P. Follicular fluid content and oocyte quality: from single biochemical markers to metabolomics. *Reprod Biol Endocrinol*. 2009;7.
77. Edwards RG. FOLLICULAR-FLUID. *J Reprod Fertil*. 1974;37(1):189-+.
78. Cobo A, García-Velasco JA, Coello A, Domingo J, Pellicer A, Remohí J. Oocyte vitrification as an efficient option for elective fertility preservation. *Fertil Steril*. 2016;105(3):755-64.e8.
79. Authority HFaE. Egg freezing in the UK: 2018 report. *Human Fertilisation and Embryology Authority*; 2018.
80. Wassarman PM, Florman HM. Cellular mechanisms during mammalian fertilization. *Comprehensive Physiology*. 2010:885-938.
81. Mann T, Lutwak-Mann C. Male reproductive function and semen: themes and trends in physiology, biochemistry and investigative andrology: Springer Science & Business Media; 2012.
82. Robertson SA, Sharkey DJ, editors. The role of semen in induction of maternal immune tolerance to pregnancy. *Seminars in immunology*; 2001: Elsevier.
83. Zandieh Z, Ashrafi M, Jameie B, Amanpour S, Mosaffa N, Yazdi RS, et al. Evaluation of immunological interaction between spermatozoa and fallopian tube epithelial cells. *Andrologia*. 2015;47(10):1120-30.
84. Springer SA, Gagneux P. Glycan Evolution in Response to Collaboration, Conflict, and Constraint. *J Biol Chem*. 2013;288(10):6904-11.

85. Tecle E, Reynoso HS, Wang RX, Gagneux P. The female reproductive tract contains multiple innate sialic acid-binding immunoglobulin-like lectins (Siglecs) that facilitate sperm survival. *J Biol Chem.* 2019;294(31):11910-9.
86. Yoshimura M, Ihara Y, Ohnishi A, Ijuhin N, Nishiura T, Kanakura Y, et al. Bisecting N-acetylglucosamine on K562 cells suppresses natural killer cytotoxicity and promotes spleen colonization. *Cancer Res.* 1996;56(2):412-8.
87. El Ouagari K, Teissié J, Benoist H. Glycophorin A Protects K562 Cells from Natural Killer Cell Attack ROLE OF OLIGOSACCHARIDES. *J Biol Chem.* 1995;270(45):26970-5.
88. Yoshimura M, Ihara Y, Ohnishi A, Ijuhin N, Nishiura T, Kanakura Y, et al. Bisecting n-acetylglucosamine on k562 cells suppresses natural killer cytotoxicity and promotes spleen colonization. *Cancer Research.* 1996;56(2):412-8.
89. Bergman M, Del Prete G, van Kooyk Y, Appelmelk B. *Helicobacter pylori* phase variation, immune modulation and gastric autoimmunity. *Nature Reviews Microbiology.* 2006;4(2):151-9.
90. Bergman MP, Engering A, Smits HH, Van Vliet SJ, Van Bodegraven AA, Wirth H-P, et al. *Helicobacter pylori* modulates the T helper cell 1/T helper cell 2 balance through phase-variable interaction between lipopolysaccharide and DC-SIGN. *Journal of Experimental Medicine.* 2004;200(8):979-90.
91. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: Mechanisms, functions, and clinical perspectives. *Semin Reprod Med.* 2007;25(6):445-53.
92. Achache H, Revel A. Endometrial receptivity markers, the journey to successful embryo implantation. *Human Reproduction Update.* 2006;12(6):731-46.
93. Hey NA, Aplin JD. Sialyl-Lewis x and Sialyl-Lewis a are associated with MUC1 in human endometrium. *Glycoconjugate J.* 1996;13(5):769-79.
94. Jones CJP, Aplin JD. Glycosylation at the fetomaternal interface: does the glyocode play a critical role in implantation? *Glycoconjugate J.* 2009;26(3):359-66.
95. Herzog M, Pavslar K, Vogler A. The impact of endometriosis on in vitro fertilization outcome. *Zdravniški Vestnik-Slovenian Medical Journal.* 2012;81(6):453-60.
96. Edwards RG, Bavister BD, Steptoe PC. Early stages of fertilization in vitro of human oocytes matured in vitro. *Nature.* 1969;221(5181):632-5.
97. Steptoe PC, Edwards RG. BIRTH AFTER REIMPLANTATION OF A HUMAN EMBRYO. *Lancet.* 1978;2(8085):366-.
98. Monroy A. *Chemistry and physiology of fertilization*: New York, Chicago, San Francisco, Toronto and London: Holt, Rinehart & Winston.; 1965. x + 150 pp. p.
99. Clark GF, Patankar MS, Hinsch KD, Oehninger S. NEW CONCEPTS IN HUMAN SPERM-ZONA PELLUCIDA INTERACTION. *Hum Reprod.* 1995;10:31-7.
100. Oehninger S, Coddington CC, Hodgen GD, Seppala M. FACTORS AFFECTING FERTILIZATION - ENDOMETRIAL PLACENTAL PROTEIN-14 REDUCES THE CAPACITY OF HUMAN SPERMATOZOA TO BIND TO THE HUMAN ZONA-PELLUCIDA. *Fertil Steril.* 1995;63(2):377-83.
101. Croy BA, van den Heuvel MJ, Borzychowski AM, Tayade C. Uterine natural killer cells: a specialized differentiation regulated by ovarian hormones. *Immunol Rev.* 2006;214:161-85.
102. Ferreira LMR, Meissner TB, Tilburgs T, Strominger JL. HLA-G: At the Interface of Maternal-Fetal Tolerance. *Trends Immunol.* 2017;38(4):272-86.
103. Moffett A, Shreeve N. First do no harm: uterine natural killer (NK) cells in assisted reproduction. *Human Reproduction.* 2015;30(7):1519-25.
104. Wilson TJ, Hertzog PJ, Angus D, Munnery L, Wood EC, Kola I. Decreased natural killer cell activity in endometriosis patients: relationship to disease pathogenesis. *Fertil Steril.* 1994;62(5):1086.

105. Radovani B, Gudelj I. N-Glycosylation and Inflammation; the Not-So-Sweet Relation. *Frontiers in Immunology*. 2022;13:24.
106. Benson V, Grobarova V, Richter J, Fiserova A. Glycosylation regulates NK cell-mediated effector function through PI3K pathway. *International Immunology*. 2010;22(3):167-77.
107. King A, Wellings V, Gardner L, Loke YW. IMMUNOCYTOCHEMICAL CHARACTERIZATION OF THE UNUSUAL LARGE GRANULAR LYMPHOCYTES IN HUMAN-ENDOMETRIUM THROUGHOUT THE MENSTRUAL-CYCLE. *Human Immunology*. 1989;24(3):195-205.
108. Vento-Tormo R, Efremova M, Botting RA, Turco MY, Vento-Termo M, Meyer KB, et al. Single-cell reconstruction of the early maternal-fetal interface in humans. *Nature*. 2018;563(7731):347-+.
109. Moffett A, Colucci F. Uterine NK cells: active regulators at the maternal-fetal interface. *Journal of Clinical Investigation*. 2014;124(5):1872-9.
110. Xiong S, Sharkey AM, Kennedy PR, Gardner L, Farrell LE, Chazara O, et al. Maternal uterine NK cell-activating receptor KIR2DS1 enhances placentation. *Journal of Clinical Investigation*. 2013;123(10):4264-72.
111. Bansal AS, Bora SA, Saso S, Smith JR, Johnson MR, Thum MY. Mechanism of human chorionic gonadotrophin-mediated immunomodulation in pregnancy. *Expert Review of Clinical Immunology*. 2012;8(8):747-53.
112. Karre K. NK cells, MHC class I molecules and the missing self. *Scand J Immunol*. 2002;55(3):221-8.
113. Bellisario R, Carlsen RB, Bahl OP. Human chorionic gonadotropin - linear amino-acid sequence of alpha subunit. *J Biol Chem*. 1973;248(19):6796-809.
114. Carlsen RB, Bahl OP, Swaminathan N. Human chorionic gonadotropin - linear amino-acid sequence of beta subunit. *J Biol Chem*. 1973;248(19):6810-27.
115. Ambrus G, Rao CV. Novel regulation of pregnant human myometrial smooth-muscle cell gap-junctions by human chorionic-gonadotropin. *Endocrinology*. 1994;135(6):2772-9.
116. Cole LA. New discoveries on the biology and detection of human chorionic gonadotropin. *Reprod Biol Endocrinol*. 2009;7:37.
117. Cole LA. Biological functions of hcg and hcg-related molecules. *Reproductive Biology and Endocrinology*. 2010;8:14.
118. Berndt S, d'Hauterive SP, Blacher S, Pequeux C, Lorquet S, Munaut C, et al. Angiogenic activity of human chorionic gonadotropin through lh receptor activation on endothelial and epithelial cells of the endometrium. *Faseb Journal*. 2006;20(14):2630-+.
119. Islami D, Bischof P, Chardonens D. Modulation of placental vascular endothelial growth factor by leptin and hCG. *Mol Hum Reprod*. 2003;9(7):395-8.
120. Keay SD, Vatish M, Karteris E, Hillhouse EW, Randeve HS. The role of hCG in reproductive medicine. *BJOG*. 2004;111(11):1218-28.
121. Stenman UH, Alfthan H, Hotakainen K. Human chorionic gonadotropin in cancer. *Clin Biochem*. 2004;37(7):549-61.
122. Bellet D, Lazar V, Bieche I, Paradis V, Giovangrandi Y, Paterlini P, et al. Malignant transformation of nontrophoblastic cells is associated with the expression of chorionic gonadotropin beta genes normally transcribed in trophoblastic cells. *Cancer Res*. 1997;57(3):516-23.
123. Marcillac I, Troalen F, Bidart JM, Ghillani P, Ribrag V, Escudier B, et al. Free human chorionic-gonadotropin beta-subunit in gonadal and nongonadal neoplasms. *Cancer Res*. 1992;52(14):3901-7.
124. Fournier T. Human chorionic gonadotropin: Different glycoforms and biological activity depending on its source of production. *Ann Endocrinol*. 2016;77(2):75-81.

125. Lustbader JW, Lobel L, Wu H, Elliott MM. Structural and molecular studies of human chorionic gonadotropin and its receptor. *Recent Progress in Hormone Research*, Vol 53. 1998;53:395-425.
126. Kessler MJ, Reddy MS, Shah RH, Bahl OP. Structures of n-glycosidic carbohydrate units of human chorionic-gonadotropin. *J Biol Chem*. 1979;254(16):7901-8.
127. Weisshaar G, Hiyama J, Renwick AGC, Nimtz M. Nmr investigations of the n-linked oligosaccharides at individual glycosylation sites of human lutropin. *Eur J Biochem*. 1991;195(1):257-68.
128. Laidler P, Cowan DA, Hider RC, Keane A, Kicman AT. Tryptic mapping of human chorionic-gonadotropin by matrix-assisted laser-desorption ionization mass-spectrometry. *Rapid Commun Mass Spectrom*. 1995;9(11):1021-6.
129. Jacoby ES, Kicman AT, Laidler P, Iles RK. Determination of the glycoforms of human chorionic gonadotropin beta-core fragment by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Clin Chem*. 2000;46(11):1796-803.
130. Birken S, Krichevsky A, O'Connor J, Schlatterer J, Cole L, Kardana A, et al. Development and characterization of antibodies to a nicked and hyperglycosylated form of hcg from a choriocarcinoma patient - generation of antibodies that differentiate between pregnancy hcg and choriocarcinoma hcg. *Endocrine*. 1999;10(2):137-44.
131. Mizuochi T, Nishimura R, Derappe C, Taniguchi T, Hamamoto T, Mochizuki M, et al. Structures of the asparagine-linked sugar chains of human chorionic-gonadotropin produced in choriocarcinoma - appearance of triantennary sugar chains and unique biantennary sugar chains. *J Biol Chem*. 1983;258(23):4126-9.
132. Kovalevskaya G, Birken S, Kakuma T, Ozaki N, Sauer M, Lindheim S, et al. Differential expression of human chorionic gonadotropin (hcg) glycosylation isoforms in failing and continuing pregnancies: Preliminary characterization of the hyperglycosylated hcg epitope. *J Endocrinol*. 2002;172(3):497-506.
133. Cole LA. Hyperglycosylated hcg and pregnancy failures. *J Reprod Immunol*. 2012;93(2):119-22.
134. Cole LA. Hyperglycosylated hcg, a review. *Placenta*. 2010;31(8):653-64.
135. Cole LA, Shahabi S, Oz UA, Bahado-Singh RO, Mahoney MJ. Hyperglycosylated human chorionic gonadotropin (invasive trophoblast antigen) immunoassay: A new basis for gestational down syndrome screening. *Clin Chem*. 1999;45(12):2109-19.
136. Seckl MJ, Sebire NJ, Berkowitz RS. Gestational trophoblastic disease. *Lancet*. 2010;376(9742):717-29.
137. Loukovaara M, Pukkala E, Lehtovirta P, Leminen A. Epidemiology of hydatidiform mole in finland, 1975 to 2001. *Eur J Gynaecol Oncol*. 2005;26(2):207-8.
138. Lybol C, Thomas CMG, Bulten J, van Dijck J, Sweep F, Massuger L. Increase in the incidence of gestational trophoblastic disease in the netherlands. *Gynecol Oncol*. 2011;121(2):334-8.
139. Ngan HYS, Seckl MJ, Berkowitz RS, Xiang Y, Golfier F, Sekharan PK, et al. Update on the diagnosis and management of gestational trophoblastic disease. *International Journal of Gynecology & Obstetrics*. 2015;131:S123-S6.
140. Horowitz NS, Goldstein DP, Berkowitz RS. Management of gestational trophoblastic neoplasia. *Semin Oncol*. 2009;36(2):181-9.
141. Lurain JR. Gestational trophoblastic disease I: epidemiology, pathology, clinical presentation and diagnosis of gestational trophoblastic disease, and management of hydatidiform mole. *Am J Obstet Gynecol*. 2010;203(6):531-9.

142. Bassaganas S, Carvalho S, Dias AM, Perez-Garay M, Ortiz MR, Figueras J, et al. Pancreatic Cancer Cell Glycosylation Regulates Cell Adhesion and Invasion through the Modulation of alpha 2 beta 1 Integrin and E-Cadherin Function. *Plos One*. 2014;9(5):14.
143. Bottoni P, Scatena R. The Role of CA 125 as Tumor Marker: Biochemical and Clinical Aspects. *Advances in Cancer Biomarkers: from Biochemistry to Clinic for a Critical Revision*. 2015;867:229-44.
144. Dochez V, Caillon H, Vaucel E, Dimet J, Winer N, Ducarme G. Biomarkers and algorithms for diagnosis of ovarian cancer: CA125, HE4, RMI and ROMA, a review. *Journal of Ovarian Research*. 2019;12.
145. Hirsch M, Duffy JMN, Deguara CS, Davis CJ, Khan KS. Diagnostic accuracy of Cancer Antigen 125 (CA125) for endometriosis in symptomatic women: A multi-center study. *European Journal of Obstetrics & Gynecology and Reproductive Biology*. 2017;210:102-7.
146. Nakagawa N, Koda H, Nitta N, Nakahara Y, Uno J, Hashimoto T, et al. Reactivity of CA19-9 and CA125 in histological subtypes of epithelial ovarian tumors and ovarian endometriosis. *Acta Medica Okayama*. 2015;69(4):227-35.
147. Brosens JJ, Salker MS, Teklenburg G, Nautiyal J, Salter S, Lucas ES, et al. Uterine selection of human embryos at implantation. *Sci Rep*. 2014;4:3894.
148. Baldi A, Campioni M, Signorile PG. Endometriosis: Pathogenesis, diagnosis, therapy and association with cancer (Review). *Oncology Reports*. 2008;19(4):843-6.
149. Tabibzadeh S, Babaknia A. The signals and molecular pathways involved in implantation, a symbiotic interaction between blastocyst and endometrium involving adhesion and tissue invasion. *Mol Hum Reprod*. 1995;1(4):179-202.
150. Harper MJK. THE IMPLANTATION WINDOW. *Baillieres Clin Obstet Gynaecol*. 1992;6(2):351-71.
151. Haouzi D, Dechaud H, Assou S, De Vos J, Hamamah S. Insights into human endometrial receptivity from transcriptomic and proteomic data. *Reprod Biomed Online*. 2012;24(1):23-34.
152. Munro MG. Uterine polyps, adenomyosis, leiomyomas, and endometrial receptivity. *Fertil Steril*. 2019;111(4):629-40.
153. Paulson RJ. Introduction: Endometrial receptivity: evaluation, induction and inhibition. *Fertility and Sterility*. 2019;111(4):609-10.
154. Craciunas L, Gallos I, Chu J, Bourne T, Quenby S, Brosens JJ, et al. Conventional and modern markers of endometrial receptivity: a systematic review and meta-analysis. *Hum Reprod Update*. 2019;25(2):202-23.
155. Alper MM, Fauser BC. Ovarian stimulation protocols for IVF: is more better than less? *Reprod Biomed Online*. 2017;34(4):345-53.
156. Lambalk CB, Banga FR, Huirne JA, Toftager M, Pinborg A, Homburg R, et al. GnRH antagonist versus long agonist protocols in IVF: a systematic review and meta-analysis accounting for patient type. *Hum Reprod Update*. 2017;23(5):560-79.
157. Aplin JD. Embryo implantation: the molecular mechanism remains elusive. *Reproductive Biomedicine Online*. 2006;13(6):833-9.
158. Kliman HJ, Frankfurter D. Clinical approach to recurrent implantation failure: evidence-based evaluation of the endometrium. *Fertility and Sterility*. 2019;111(4):618-28.
159. Sharkey AM, Smith SK. The endometrium as a cause of implantation failure. *Best Practice & Research Clinical Obstetrics & Gynaecology*. 2003;17(2):289-307.
160. Bosch E, De Vos M, Humaidan P. The Future of Cryopreservation in Assisted Reproductive Technologies. *Front Endocrinol (Lausanne)*. 2020;11:67.
161. Casper R, Haas J, Hsieh TB, Bassil R, Mehta C. Recent advances in in vitro fertilization. *F1000Res*. 2017;6:1616.

162. Doody KJ. Infertility Treatment Now and in the Future. *Obstet Gynecol Clin North Am.* 2021;48(4):801-12.
163. Viotti M. Preimplantation Genetic Testing for Chromosomal Abnormalities: Aneuploidy, Mosaicism, and Structural Rearrangements. *Genes (Basel).* 2020;11(6).
164. Hinckley MD, Milki AA. 1000 office-based hysteroscopies prior to in vitro fertilization: feasibility and findings. *JSLs : Journal of the Society of Laparoendoscopic Surgeons.* 2004;8(2):103-7.
165. Balasch J, Fabregues F, Creus M, Vanrell JA. THE USEFULNESS OF ENDOMETRIAL BIOPSY FOR LUTEAL PHASE EVALUATION IN INFERTILITY. *Hum Reprod.* 1992;7(7):973-7.
166. Coutifaris C, Myers ER, Guzick DS, Diamond MP, Carson SA, Legro RS, et al. Histological dating of timed endometrial biopsy tissue is not related to fertility status. *Fertil Steril.* 2019;112(4):E116-E24.
167. Koninckx PR, Goddeeris PG, Lauweryns JM, De Hertogh RC, Brosens IA. Accuracy of endometrial biopsy dating in relation to the midcycle luteinizing hormone peak. *Obstetrical & gynecological survey.* 1977;32(7):613-5.
168. Eskenazi B, Warner ML. Epidemiology of endometriosis. *Obstet Gynecol Clin N Am.* 1997;24(2):235-+.
169. Brosens IA. Endometriosis - current issues in diagnosis and medical management. *J Reprod Med.* 1998;43(3):281-6.
170. Sampson JA. Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. *American Journal of Obstetrics & Gynecology.* 1927;14(4):422-69.
171. Kang Y-J, Jeung IC, Park A, Park Y-J, Jung H, Kim T-D, et al. An increased level of IL-6 suppresses NK cell activity in peritoneal fluid of patients with endometriosis via regulation of SHP-2 expression. *Hum Reprod.* 2014;29(10):2176-89.
172. Garcia-Penarrubia P, Ruiz-Alcaraz AJ, Martinez-Esparza M, Marin P, Machado-Linde F. Hypothetical roadmap towards endometriosis: prenatal endocrine-disrupting chemical pollutant exposure, anogenital distance, gut-genital microbiota and subclinical infections. *Hum Reprod Update.* 2020;26(2):214-46.
173. Nisolle M, Casanasroux F, Anaf V, Mine JM, Donnez J. Morphometric study of the stromal vascularization in peritoneal endometriosis. *Fertil Steril.* 1993;59(3):681-4.
174. Horton J, Sterrenburg M, Lane S, Maheshwari A, Li TC, Cheong Y. Reproductive, obstetric, and perinatal outcomes of women with adenomyosis and endometriosis: a systematic review and meta-analysis. *Hum Reprod Update.* 2019;25(5):593-633.
175. Tanbo T, Fedorcsak P. Endometriosis-associated infertility: aspects of pathophysiological mechanisms and treatment options. *Acta Obstetrica Et Gynecologica Scandinavica.* 2017;96(6):659-67.
176. Sanchez AM, Pagliardini L, Cermisoni GC, Privitera L, Makieva S, Alteri A, et al. Does Endometriosis Influence the Embryo Quality and/or Development? Insights from a Large Retrospective Matched Cohort Study. *Diagnostics.* 2020;10(2).
177. Xu B, Guo N, Zhang X-m, Shi W, Tong X-h, Iqbal F, et al. Oocyte quality is decreased in women with minimal or mild endometriosis. *Scientific Reports.* 2015;5.
178. Gupta D, Hull ML, Fraser I, Miller L, Bossuyt PMM, Johnson N, et al. Endometrial biomarkers for the non-invasive diagnosis of endometriosis. *Cochrane Database of Systematic Reviews.* 2016(4).
179. Bast RC, Feeney M, Lazarus H, Nadler LM, Colvin RB, Knapp RC. REACTIVITY OF A MONOCLONAL-ANTIBODY WITH HUMAN OVARIAN-CARCINOMA. *Journal of Clinical Investigation.* 1981;68(5):1331-7.

180. Koninckx PR, Riittinen L, Seppala M, Cornillie FJ. CA-125 AND PLACENTAL PROTEIN-14 CONCENTRATIONS IN PLASMA AND PERITONEAL-FLUID OF WOMEN WITH DEEPLY INFILTRATING PELVIC ENDOMETRIOSIS. *Fertil Steril*. 1992;57(3):523-30.
181. Drosdzol-Cop A, Skrzypulec-Plinta V. Selected cytokines and glycodelin A levels in serum and peritoneal fluid in girls with endometriosis. *Journal of Obstetrics and Gynaecology Research*. 2012;38(10):1245-53.
182. Vodolazkaia A, El-Aalamat Y, Popovic D, Mihalyi A, Bossuyt X, Kyama CM, et al. Evaluation of a panel of 28 biomarkers for the non-invasive diagnosis of endometriosis. *Hum Reprod*. 2012;27(9):2698-711.
183. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. VASCULAR ENDOTHELIAL GROWTH-FACTOR IS A SECRETED ANGIOGENIC MITOGEN. *Science*. 1989;246(4935):1306-9.
184. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, et al. INHIBITION OF VASCULAR ENDOTHELIAL GROWTH FACTOR-INDUCED ANGIOGENESIS SUPPRESSES TUMOR-GROWTH INVIVO. *Nature*. 1993;362(6423):841-4.
185. Kressin P, Wolber EM, Wodrich H, Meyhofer-Malik A, Buchweitz O, Diedrich K, et al. Vascular endothelial growth factor mRNA in eutopic and ectopic endometrium. *Fertil Steril*. 2001;76(6):1220-4.
186. Ohtsubo K, Marth JD. Glycosylation in cellular mechanisms of health and disease. *Cell*. 2006;126(5):855-67.
187. Marth JD, Grewal PK. Mammalian glycosylation in immunity. *Nat Rev Immunol*. 2008;8(11):874-87.
188. Burney RO, Giudice LC. Pathogenesis and pathophysiology of endometriosis. *Fertility and sterility*. 2012;98(3):511-9.
189. Le NXH, Loret de Mola JR, Bremer P, Groesch K, Wilson T, Diaz-Sylvester P, et al. Alteration of systemic and uterine endometrial immune populations in patients with endometriosis. *Am J Reprod Immunol*. 2021;85(3):e13362.
190. Sharpe-Timms KL, Nabli H, Zimmer RL, Birt JA, Davis JW. Inflammatory cytokines differentially up-regulate human endometrial haptoglobin production in women with endometriosis(dagger). *Human Reproduction*. 2010;25(5):1241-50.
191. Vallvé-Juanico J, Houshdaran S, Giudice LC. The endometrial immune environment of women with endometriosis. *Hum Reprod Update*. 2019;25(5):564-91.
192. Berkes E, Mužinić A, Rigo Jr J, Tinneberg H-R, Oehmke F. The analysis of the human plasma N-glycome in endometriosis patients. *European Journal of Obstetrics & Gynecology and Reproductive Biology*. 2013;171(1):107-15.
193. Kocbek V, Hevir-Kene N, Bersinger NA, Mueller MD, Rižner TL. Increased levels of biglycan in endometriomas and peritoneal fluid samples from ovarian endometriosis patients. *Gynecological endocrinology*. 2014;30(7):520-4.
194. Tanaka K, Waki H, Ido Y, Akita S, Yoshida Y, Yoshida T, et al. Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom*. 1988;2(8):151-3.
195. Duncan MW, Roder H, Hunsucker SW. Quantitative matrix-assisted laser desorption/ionization mass spectrometry. *Briefings in Functional Genomics & Proteomics*. 2008;7(5):355-70.
196. Nier AO. THE DEVELOPMENT OF A HIGH-RESOLUTION MASS-SPECTROMETER - A REMINISCENCE. *J Am Soc Mass Spectrom*. 1991;2(6):447-52.
197. Dell A. PREPARATION AND DESORPTION MASS SPECTROMETRY OF PERMETHYL AND PERACETYL DERIVATIVES OF OLIGOSACCHARIDES. McCloskey, J a (Ed) *Methods in Enzymology*, Vol

- 193 Mass Spectrometry Xxx+960p Academic Press, Inc: San Diego, California, USA; London, England, Uk Illus. 1990:647-60.
198. Egge H, Peterkatalinic J. FAST-ATOM-BOMBARDMENT MASS-SPECTROMETRY FOR STRUCTURAL ELUCIDATION OF GLYCOCONJUGATES. *Mass Spectrom Rev.* 1987;6(3):331-93.
199. Dell A. FAB-MASS SPECTROMETRY OF CARBOHYDRATES. *Adv Carbohydr Chem Biochem.* 1987;45:19-72.
200. Analysis of carbohydrates by GLC and MS. *Analysis of carbohydrates by GLC and MS.* 1989:292 pp.
201. Domon B, Costello CE. A SYSTEMATIC NOMENCLATURE FOR CARBOHYDRATE FRAGMENTATIONS IN FAB-MS MS SPECTRA OF GLYCOCONJUGATES. *Glycoconjugate J.* 1988;5(4):397-409.
202. Dell A, Morris HR, Egge H, Vonnicolai H, Strecker G. FAST-ATOM-BOMBARDMENT MASS-SPECTROMETRY FOR CARBOHYDRATE-STRUCTURE DETERMINATION. *Carbohydrate Research.* 1983;115(APR):41-52.
203. Dewal MB, DiChiara AS, Antonopoulos A, Taylor RJ, Harmon CJ, Haslam SM, et al. Xbp1s links the unfolded protein response to the molecular architecture of mature n-glycans. *Chemistry & Biology.* 2015;22(10):1301-12.
204. Ibeto L, Antonopoulos A, Grassi P, Pang PC, Panico M, Bobdiwala S, et al. Insights into the hyperglycosylation of human chorionic gonadotropin revealed by glycomics analysis. *Plos One.* 2020;15(2):19.
205. Li DY, Zhang P, Li F, Chi LQ, Zhu DY, Zhang QY, et al. Recognition of n-glycoforms in human chorionic gonadotropin by monoclonal antibodies and their interaction motifs. *J Biol Chem.* 2015;290(37):22715-23.
206. Nemansky M, Thotakura NR, Lyons CD, Ye S, Reinhold BB, Reinhold VN, et al. Developmental changes in the glycosylation of glycoprotein hormone free alpha subunit during pregnancy. *J Biol Chem.* 1998;273(20):12068-76.
207. Cole LA. Proportion hyperglycosylated hcg a new test for discriminating gestational trophoblastic diseases. *Int J Gynecol Cancer.* 2014;24(9):1709-14.
208. Chen QS, Pang PC, Cohen ME, Longtine MS, Schust DJ, Haslam SM, et al. Evidence for differential glycosylation of trophoblast cell types. *Mol Cell Proteomics.* 2016;15(6):1857-66.
209. Fournier T, Guibourdenche J, Evain-Brion D. Review: Hcgs: Different sources of production, different glycoforms and functions. *Placenta.* 2015;36:S60-S5.
210. Edelstam G, Karlsson C, Westgren M, Lowbeer C, Swahn ML. Human chorionic gonadatropin (hCG) during third trimester pregnancy. *Scand J Clin Lab Invest.* 2007;67(5):519-25.
211. Amano J, Sato S, Nishimura R, Mochizuki M, Kobata A. Sialic acids, but not their linkage to galactose residues, are required for full expression of the biological-activity of human chorionic-gonadotropin. *Journal of Biochemistry.* 1989;105(3):339-40.
212. Chuan S, Homer M, Pandian R, Conway D, Garzo G, Yeo L, et al. Hyperglycosylated human chorionic gonadotropin as an early predictor of pregnancy outcomes after in vitro fertilization. *Fertility and Sterility.* 2014;101(2):392-8.
213. Gam LH, Latiff A. Sds-page electrophoretic property of human chorionic gonadotropin (hcg) and its beta-subunit. *Int J Biol Sci.* 2005;1(3):103-9.
214. Segrest JP, Jackson RL, Andrews EP, Marchesi VT. Human erythrocyte membrane glycoprotein - re-evaluation of molecular weight as determined by sds polyacrylamide gel electrophoresis. *Biochem Biophys Res Commun.* 1971;44(2):390-&.
215. Thaysen-Andersen M, Larsen MR, Packer NH, Palmisano G. Structural analysis of glycoprotein sialylation – Part I: pre-LC-MS analytical strategies. *RSC Advances.* 2013;3(45):22683-705.

216. Orczyk-Pawłowicz M, Augustyniak D, Hirnle L, Kątnik-Prastowska I. Lectin-based analysis of fucose and sialic acid expressions on human amniotic IgA during normal pregnancy. *Glycoconj J*. 2013;30(6):599-608.
217. Lee CL, Chiu PCN, Pang PC, Chu IK, Lee KF, Koistinen R, et al. Glycosylation failure extends to glycoproteins in gestational diabetes mellitus evidence from reduced alpha 2-6 sialylation and impaired immunomodulatory activities of pregnancy-related glycodelin-a. *Diabetes*. 2011;60(3):909-17.
218. Orczyk-Pawłowicz M, Floriański J, Zalewski J, Katnik-Prastowska I. Relative amounts of sialic acid and fucose of amniotic fluid glycoconjugates in relation to pregnancy age. *Glycoconj J*. 2005;22(7-9):433-42.
219. Orczyk-Pawłowicz M, Augustyniak D, Hirnle L, Kątnik-Prastowska I. Degree of sialylation and fucosylation of plasma and amniotic immunoglobulin G changes progressively during normal pregnancy. *Prenat Diagn*. 2012;32(5):432-9.
220. Abeln M, Albers I, Peters-Bernard U, Fläcksig-Schulz K, Kats E, Kispert A, et al. Sialic acid is a critical fetal defense against maternal complement attack. *J Clin Invest*. 2019;129(1):422-36.
221. Erdmann H, Steeg C, Koch-Nolte F, Fleischer B, Jacobs T. Sialylated ligands on pathogenic trypanosoma cruzi interact with siglec-e (sialic acid-binding ig-like lectin-e). *Cell Microbiol*. 2009;11(11):1600-11.
222. Wu X, Zhao JD, Ruan YY, Sun L, Xu CJ, Jiang H. Sialyltransferase st3gal1 promotes cell migration, invasion, and tgf-beta 1-induced emt and confers paclitaxel resistance in ovarian cancer. *Cell Death Dis*. 2018;9:14.
223. Mahajan VS, Pillai S. Sialic acids and autoimmune disease. *Immunol Rev*. 2016;269(1):145-61.
224. Miwa HE, Song YH, Alvarez R, Cummings RD, Stanley P. The bisecting GlcNAc in cell growth control and tumor progression. *Glycoconjugate J*. 2012;29(8-9):609-18.
225. Song YH, Aglipay JA, Bernstein JD, Goswami S, Stanley P. The Bisecting GlcNAc on N-Glycans Inhibits Growth Factor Signaling and Retards Mammary Tumor Progression. *Cancer Res*. 2010;70(8):3361-71.
226. Barber LD, Patel TP, Percival L, Gumperz JE, Lanier LL, Phillips JH, et al. Unusual uniformity of the N-linked oligosaccharides of HLA-A, -B, and -C glycoproteins. *Journal of Immunology*. 1996;156(9):3275-84.
227. Hutter H, Dohr G. HLA expression on immature and mature human germ cells. *J Reprod Immunol*. 1998;38(2):101-22.
228. Pang PC, Tissot B, Drobnis EZ, Sutovsky P, Morris HR, Clark GF, et al. Expression of bisecting type and lewis(x)/lewis(y) terminated n-glycans on human sperm. *J Biol Chem*. 2007;282(50):36593-602.
229. Blaschitz A, Hutter H, Dohr G. HLA Class I protein expression in the human placenta. *Early Pregnancy*. 2001;5(1):67-9.
230. Chen Q, Pang PC, Cohen ME, Longtine MS, Schust DJ, Haslam SM, et al. Evidence for Differential Glycosylation of Trophoblast Cell Types. *Molecular & cellular proteomics : MCP*. 2016;15(6):1857-66.
231. Drake PM, Gunn MD, Charo IF, Tsou CL, Zhou Y, Huang L, et al. Human placental cytotrophoblasts attract monocytes and CD56(bright) natural killer cells via the actions of monocyte inflammatory protein 1alpha. *The Journal of experimental medicine*. 2001;193(10):1199-212.
232. Moffett A, Loke C. Immunology of placentation in eutherian mammals. *Nature reviews Immunology*. 2006;6(8):584-94.

233. Sulke AN, Jones DB, Wood PJ. Hormonal modulation of human natural killer cell activity in vitro. *Journal of reproductive immunology*. 1985;7(2):105-10.
234. Narimatsu H. Human glycogene cloning: focus on beta 3-glycosyltransferase and beta 4-glycosyltransferase families. *Current Opinion in Structural Biology*. 2006;16(5):567-75.
235. Taniguchi N, Honke K, Fukuda M. Handbook of glycosyltransferases and related genes. Edition 1. *Handbook of glycosyltransferases and related genes Edition 1*. 2002:i-xviii, 1-670.
236. Taniguchi N, Miyoshi E, Gu J, Honke K, Matsumoto A. Decoding sugar functions by identifying target glycoproteins (vol 16, pg 561, 2006). *Current Opinion in Structural Biology*. 2006;16(6):796-.
237. Lowe JB, Marth JD. A genetic approach to mammalian glycan function. *Annual Review of Biochemistry Volume 72*. 2003;Volume 72:643-91.
238. Takahashi M, Kuroki Y, Ohtsubo K, Taniguchi N. Core fucose and bisecting GlcNAc, the direct modifiers of the N-glycan core: their functions and target proteins. *Carbohydrate Research*. 2009;344(12):1387-90.
239. Clark GF. The role of glycans in immune evasion: the human fetoembryonic defence system hypothesis revisited. *Mol Hum Reprod*. 2014;20(3):185-99.
240. McMaster M, Zhou Y, Shorter S, Kapasi K, Geraghty D, Lim KH, et al. HLA-G isoforms produced by placental cytotrophoblasts and found in amniotic fluid are due to unusual glycosylation. *Journal of Immunology*. 1998;160(12):5922-8.
241. Than NG, Romero R, Kim CJ, McGowen MR, Papp Z, Wildman DE. Galectins: guardians of eutherian pregnancy at the maternal-fetal interface. *Trends Endocrinol Metab*. 2012;23(1):23-31.
242. Hirabayashi J, Hashidate T, Arata Y, Nishi N, Nakamura T, Hirashima M, et al. Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. *Biochim Biophys Acta-Gen Subj*. 2002;1572(2-3):232-54.
243. Rabinovich GA, Toscano MA. Turning 'sweet' on immunity: galectin-glycan interactions in immune tolerance and inflammation. *Nat Rev Immunol*. 2009;9(5):338-52.
244. Giudice LC, Kao LC. Endometriosis. *Lancet*. 2004;364(9447):1789-99.
245. McKinnon B, Mueller MD, Nirgianakis K, Bersinger NA. Comparison of ovarian cancer markers in endometriosis favours HE4 over CA125. *Mol Med Rep*. 2015;12(4):5179-84.
246. Fassbender A, Burney RO, Dorien FO, D'Hooghe T, Giudice L. Update on Biomarkers for the Detection of Endometriosis. *Biomed Res Int*. 2015;2015:14.
247. Izumi G, Koga K, Takamura M, Makabe T, Nagai M, Urata Y, et al. Mannose receptor is highly expressed by peritoneal dendritic cells in endometriosis. *Fertility and Sterility*. 2017;107(1):167-+.
248. Hirakawa T, Nasu K, Kai K, Aoyagi Y, Ishii T, Uemura T, et al. Wisteria floribunda agglutinin-binding glycan expression is decreased in endometriomata. *Reproductive Biology and Endocrinology*. 2014;12.
249. Jones CJP, Inuwa IM, Nardo LG, Litta P, Fazleabas AT. Eutopic Endometrium From Women With Endometriosis Shows Altered Ultrastructure and Glycosylation Compared to That From Healthy Controls-A Pilot Observational Study. *Reproductive Sciences*. 2009;16(6):559-72.
250. Miller DL, Jones CJP, Aplin JD, Nardo LG. Altered glycosylation in peri-implantation phase endometrium in women with stages III and IV endometriosis. *Human Reproduction*. 2010;25(2):406-11.
251. Burney RO, Talbi S, Hamilton AE, Vo KC, Nyegaard M, Nezhat CR, et al. Gene expression analysis of endometrium reveals progesterone resistance and candidate susceptibility genes in women with endometriosis. *Endocrinology*. 2007;148(8):3814-26.

252. Hakomori S. Glycosylation defining cancer malignancy: New wine in an old bottle. *Proceedings of the National Academy of Sciences of the United States of America*. 2002;99(16):10231-3.
253. Sogabe M, Nozaki H, Tanaka N, Kubota T, Kaji H, Kuno A, et al. Novel Glycobiomarker for Ovarian Cancer That Detects Clear Cell Carcinoma. *Journal of Proteome Research*. 2014;13(3):1624-35.
254. Cui HX, Lin Y, Yue LL, Zhao XM, Liu JC. Differential expression of the alpha 2,3-sialic acid residues in breast cancer is associated with metastatic potential. *Oncology Reports*. 2011;25(5):1365-71.
255. Vajaria BN, Patel KR, Begum R, Patel PS. Sialylation: an Avenue to Target Cancer Cells. *Pathology & Oncology Research*. 2016;22(3):443-7.
256. Oliveira-Ferrer L, Legler K, Milde-Langosch K. Role of protein glycosylation in cancer metastasis. *Seminars in Cancer Biology*. 2017;44:141-52.
257. Holmes EH, Ostrander GK, Clausen H, Graem N. ONCOFETAL EXPRESSION OF LEX CARBOHYDRATE ANTIGENS IN HUMAN COLONIC ADENOCARCINOMAS - REGULATION THROUGH TYPE-2 CORE CHAIN SYNTHESIS RATHER THAN FUCOSYLATION. *Journal of Biological Chemistry*. 1987;262(23):11331-8.
258. Kim YJ, Varki A. Perspectives on the significance of altered glycosylation of glycoproteins in cancer. *Glycoconjugate Journal*. 1997;14(5):569-76.
259. Sundqvist J, Andersson KL, Scarselli G, Gemzell-Danielsson K, Lalitkumar PGL. Expression of adhesion, attachment and invasion markers in eutopic and ectopic endometrium: a link to the aetiology of endometriosis. *Human Reproduction*. 2012;27(9):2737-46.
260. Maignien C, Santulli P, Chouzenoux S, Gonzalez-Foruria I, Marcellin L, Doridot L, et al. Reduced alpha-2,6 sialylation regulates cell migration in endometriosis. *Human Reproduction*. 2019;34(3):479-90.
261. Berkes E, Muzinic A, Rigo J, Tinneberg HR, Oehmke F. The analysis of the human plasma N-glycome in endometriosis patients. *European Journal of Obstetrics & Gynecology and Reproductive Biology*. 2013;171(1):107-15.
262. Kocbek V, Hevir-Kene N, Bersinger NA, Mueller MD, Rizner TL. Increased levels of biglycan in endometriomas and peritoneal fluid samples from ovarian endometriosis patients. *Gynecological Endocrinology*. 2014;30(7):520-4.
263. Brosens I. Endometriosis and the outcome of in vitro fertilization. *Fertility and Sterility*. 2004;81(5):1198-200.
264. Holoch KJ, Lessey BA. Endometriosis and Infertility. *Clinical Obstetrics and Gynecology*. 2010;53(2):429-38.
265. Giudice LC. Endometriosis. *New England Journal of Medicine*. 2010;362(25):2389-98.
266. Zanatta A, Rocha AM, Carvalho FM, Pereira RMA, Taylor HS, Motta ELA, et al. The role of the Hoxa10/HOXA10 gene in the etiology of endometriosis and its related infertility: a review. *Journal of Assisted Reproduction and Genetics*. 2010;27(12):701-10.
267. Aplin JD. GLYCANS AS BIOCHEMICAL MARKERS OF HUMAN ENDOMETRIAL SECRETORY DIFFERENTIATION. *J Reprod Fertil*. 1991;92(2):525-41.
268. Singh H, Aplin JD. Adhesion molecules in endometrial epithelium: tissue integrity and embryo implantation. *J Anat*. 2009;215(1):3-13.
269. Fukuda MN, Sugihara K. An integrated view of L-selectin and trophinin function in human embryo implantation. *Journal of Obstetrics and Gynaecology Research*. 2008;34(2):129-36.
270. Aplin JD. MUC-1 glycosylation in endometrium: possible roles of the apical glycocalyx at implantation. *Hum Reprod*. 1999;14:17-25.

271. Hey NA, Graham RA, Seif MW, Aplin JD. THE POLYMORPHIC EPITHELIAL MUCIN MUC1 IN HUMAN ENDOMETRIUM IS REGULATED WITH MAXIMAL EXPRESSION IN THE IMPLANTATION PHASE. *J Clin Endocrinol Metab.* 1994;78(2):337-42.
272. Meseguer M, Aplin JD, Caballero-Campo P, O'Connor JE, Martin JC, Remohi J, et al. Human endometrial mucin MUC1 is up-regulated by progesterone and down-regulated in vitro by the human blastocyst. *Biology of Reproduction.* 2001;64(2):590-601.
273. Graham RA, Seif MW, Aplin JD, Li TC, Cooke ID, Rogers AW, et al. AN ENDOMETRIAL FACTOR IN UNEXPLAINED INFERTILITY. *British Medical Journal.* 1990;300(6737):1428-31.
274. Jones CJP, Fazleabas AT, McGinlay PB, Aplin JD. Cyclic modulation of epithelial glycosylation in human and baboon (*Papio anubis*) endometrium demonstrated by the binding of the agglutinin from *Dolichos biflorus*. *Biology of Reproduction.* 1998;58(1):20-7.
275. Jones CJP, Denton J, Fazleabas AT. Morphological and glycosylation changes associated with the endometrium and ectopic lesions in a baboon model of endometriosis. *Hum Reprod.* 2006;21(12):3068-80.
276. Kumamoto K, Goto Y, Sekikawa K, Takenoshita S, Ishida N, Kawakita M, et al. Increased expression of UDP-galactose transporter messenger RNA in human colon cancer tissues and its implication in synthesis of Thomsen-Friedenreich antigen and sialyl Lewis A/X determinants. *Cancer Research.* 2001;61(11):4620-7.
277. Kellokumpu S, Sormunen R, Kellokumpu M. Abnormal glycosylation and altered Golgi structure in colorectal cancer: dependence on intra-Golgi pH. *Febs Letters.* 2002;516(1-3):217-24.
278. Brazil JC, Parkos CA. Finding the sweet spot: glycosylation mediated regulation of intestinal inflammation. *Mucosal Immunology.* 2022;15(2):211-22.
279. Kori M, Aydin B, Gulfidan G, Beklen H, Kelesoglu N, Iscan AC, et al. The Repertoire of Glycan Alterations and Glycoproteins in Human Cancers. *Omics-a Journal of Integrative Biology.* 2021;25(3):139-68.
280. Campo S, Andreone L, Ambao V, Urrutia M, Calandra RS, Rulli SB. Hormonal Regulation of Follicle-Stimulating Hormone Glycosylation in Males. *Frontiers in Endocrinology.* 2019;10:8.
281. Duncan MW, Roder H, Hunsucker SW. Quantitative matrix-assisted laser desorption/ionization mass spectrometry. *Briefings in Functional Genomics.* 2008;7(5):355-70.
282. Bucknall M, Fung KYC, Duncan MW. Practical quantitative biomedical applications of MALDI-TOF mass spectrometry. *Journal of the American Society for Mass Spectrometry.* 2002;13(9):1015-27.
283. Zhang L, Luo S, Zhang BL. The use of lectin microarray for assessing glycosylation of therapeutic proteins. *Mabs.* 2016;8(3):524-35.
284. Gupta G, Surolia A, Sampathkumar SG. Lectin Microarrays for Glycomic Analysis. *Omics-a Journal of Integrative Biology.* 2010;14(4):419-36.
285. Hirabayashi J, Yamada M, Kuno A, Tateno H. Lectin microarrays: concept, principle and applications. *Chemical Society Reviews.* 2013;42(10):4443-58.
286. Huang WL, Li YG, Lv YC, Guan XH, Ji HF, Chi BR. Use of lectin microarray to differentiate gastric cancer from gastric ulcer. *World Journal of Gastroenterology.* 2014;20(18):5474-82.
287. Kobayashi Y, Masuda K, Banno K, Kobayashi N, Umene K, Nogami Y, et al. Glycan profiling of gestational choriocarcinoma using a lectin microarray. *Oncology Reports.* 2014;31(3):1121-6.