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Glycomics as an innovative technology to identify biomarkers of aging

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Chapter 1

1. Glycomics

Glycobiology is the study of the structure, biosynthesis, biology, and evolution of saccharides (sugar chains or glycans) present on proteins and lipids, widely distributed in nature, and the proteins that recognize them.

Today, Glycobiology is reaching a broad relevance to many areas of basic research, biomedicine, and biotechnology. The field includes the chemistry of carbohydrates, the enzymology of glycan formation and degradation, the recognition of glycans by specific proteins (lectins and glycosaminoglycan-binding proteins), glycan roles in complex biological systems, and their analysis or manipulation by a variety of techniques ("Essential of Glycobiology", 2nd edition edited by Cold Spring Harbor Laboratory Press; 2009). Therefore, these studies are aimed to identify a relationship between the glycans structures and functionality of the proteins (Raman et al. 2005).

The interest in this type of study has led to many discoveries, in which it is shown that glycans play a decisive role in cell growth and differentiation (Hwang et al. 2003), in the cell communication and signalling (Collins et al. 2004), immune response (Miller 2005), tumor growth and metastasis and inflammation (Dube and Bertozzi et al. 2005) etc.. Moreover, it is demonstrated that the glycans of specific protein signals or cellular receptors are able to modulate the biological process in the development and function of multiple physiologic system (as organs and tissues), in part by regulating protein-protein and cell-cell interaction (Lowe et al. 2003).

The glycosylation of proteins is a post-translational event determined by a series of processes, which end with the assembling of a chain carbohydrate on a specific amino acid. The cellular compartments involved in this process are the rough endoplasmic reticulum (RER) and the Golgi apparatus, both holding several glycosyl- transferase and glycosidase.

The significance of protein glycosylation is manifold. In some cases the saccharide units play a structural role, for example, those present at the level of the cytoplasmic membrane are involved in cell adhesion. Another possible role might be to facilitate the folding of the protein in a particular conformation, and allow binding to specific membrane receptors or with the extracellular matrix. Although the study of changes in glycan structural composition is of great interest and promising, there are still many technical difficulties that render it quite cumbersome. Firstly, because the synthesis of glycans depends on the activity of a set of several coordinated, and not easily controllable, glycosyltransferases and glycosidase, many of which are tissue specific (Flukae et al. 2004). To date, it is still impossible to predict the structure(s) of the glycosylation side chain(s) based on the protein sequence.

Second, the cause of the heterogeneity of glycan chains is not only the different sequence of single sugars but it is also the bonding between two monosaccharide residues, that can have a variety of configurations and linkages. There are, in fact, two different stereochemical configurations of glycosidic bonds, an alpha linkage and a beta linkage. The only difference between the alpha and beta linkages is the orientation of the linked carbon atoms. The type of bond that exists between the different sugars greatly influences the degree of branching of a chain.

Therefore, this flexibility and complexity cannot be addressed by using conventional analytical approaches as in genomics and proteomics, and as consequence, in the "Omics" era, the concept of glycomics has evolved.

For definition, the terms "Glycome" refers to a repertoire of glycans in a tissue or a cell type, and the Glycomics is the study of Glycome.

Thanks to the progress in mass spectrometry (MS) and oligosaccharide analysis technologies, including techniques in derivatization, fluorescent labelling, capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC), has been made possible.

Similar to genomics and proteomics approaches, glycomics are supported by high-throughput screening in order to identify a panel of distinguishable glycomic structures simultaneously.

What is glycomic analysis good for? Glycomics should allow investigating on the global glycome within body fluids (blood, serum, plasma, seminal fluid, urine etc..) or tissues of interest, and this which could eventually lead to identify new type of biomarkers for cancer diagnosis, Alzheimer disease, cardiovascular diseases, or in the case of the present study, biomarkers of human aging studying specific models of aging.

Based on their chemical structure, the glycans, are classified in linear and branched sugars. Most of the linear sugars linear are glycosaminoglycans which contain repeat units of sulfated disaccharides, linked as polysaccharide chains to Ser or Thr residues (so called O-linked), to form aggregates of proteoglycans. The branched chains instead are classified into two groups: O-linked and N-linked. In particular, the N-linked glycans are attached to the amide group of asparagine (Asn) on the aminoacid sequence of the protein; those O-linked, however, are attached to the hydroxyl group of serine (Ser), threonine (Thr) or hydroxylysine (LysOH) (Fig.1)

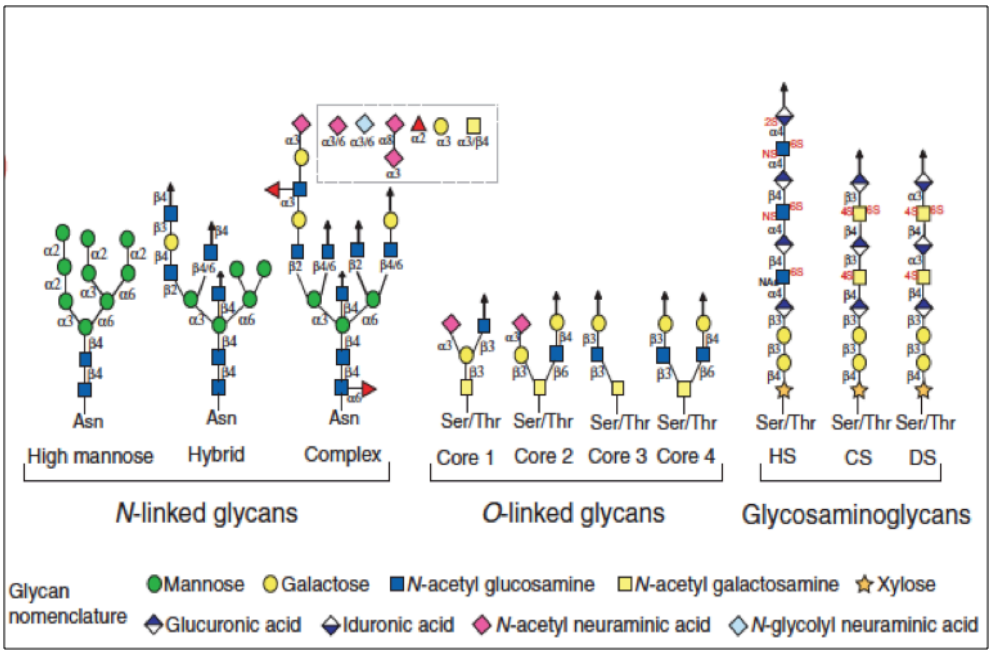


Figure 1 Chemical diversity of glycans. The directionality of assembling of glycans is from non reducing end at the top to the reducing end at the bottom with the arrows indicating the extension at the non reducing end. In the case of complex N-linked glycans, the common terminal motifs attached to Gal are shown in a dotted box. In the N-linked glycans, the assembling starts with the bound between the –OH group of the NAcGlc and the NH2 of the asparagine (Asn) Source: "Glycomics: an integrated systems approach to structure-function relationships of glycans" Raman R. et al 2005).

1.1 N-linked glycans

N-linked oligosaccharides can be subdivided into three main types: high mannose, hybrid and complex and they are formed mainly by the combination of five types of sugars: mannose (Man), N-acetylglucosamine, (NAcGlc), galactose (Gal), fucose (Fuc) and sialic or neuroaminic acid (S).

In the N-glycan structures, we recognize a "core invariant", and therefore peculiar to all the N-glycan chain, formed by a group of a N-N diacetylchitobiose and by a mannosidic core (Fig.2).

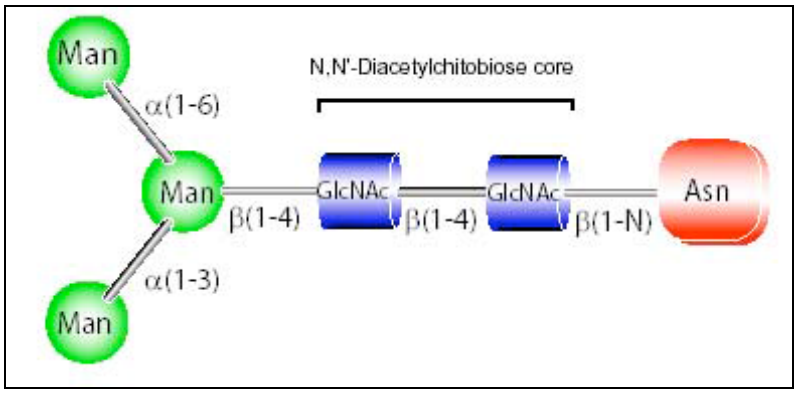


Figure 2 Invariant core of N-linked chains

The subsequent addition of other sugars, confers variability to the chain, in fact, from their combination it possible to generate about 35560 types of different tetrasaccharides.

In most cases, the mannose can be extended in all three positions by a residue of N-acetylglucosamine (GlcNAc). In particular, when GlcNAc is attached to the side mannose residues, determines the base of the branched glycans, while, when it is attached to the innermost mannose, the GlcNAc is referred to "bisecting GlcNac." and it not requires the addition of more sugars.

The chain can continue with the attachment of galactose on both ramifications and can be concluded with the addition of two sialic acids to form a complete biantennary N-glycan (Fig. 3).

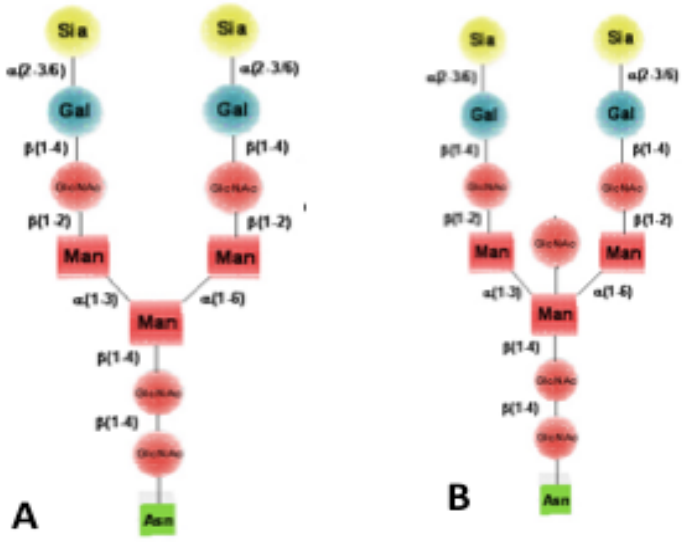


Figura 3 N-linkage glycan chain . a) biantennary N-glycan (H5N4S2); b) biantennary N-glycan with bisection GlcNAc (H5N5S2)

High-mannose type oligosaccharides refer to structures that have two to six mannose residues ligated to the internal core mannoses. On the other hand, complex oligosaccharides share the internal core mannoses, the outer two α-mannoses of this structure having N-acetyllactosamines (the disaccharide of GlcNAc and galactose) attached.

These N-acetyllactosamines can be further elongated by the addition of sialic or additional N-acetyllactosamines.

Hybride type oligosaccarides are exactly what their name indicates, they have more than three mannose residues and also have N-acetyllactosamine chain bound to the α-1-3 linked mannose. Hybrid molececules also usually contain a "bisecting" N-acetylglucosamine linked to the innermost mannose of the core (Fig.4)

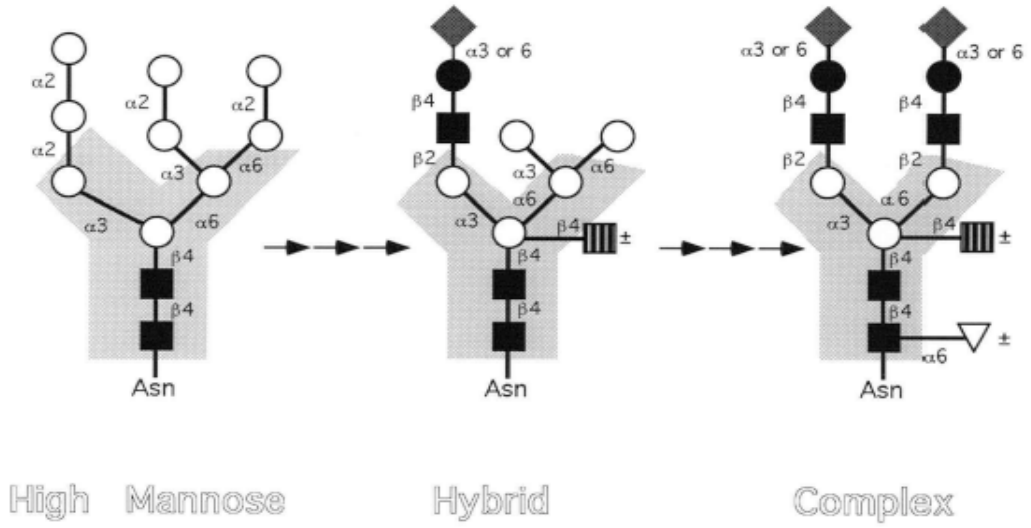


Figure 4 The three major types of asparagine.-linked oligosaccharides (N-linked glycans).

1.2 N-glycan Biosynthesis

In contrast to O-glycan synthesis, which initiates and progresses one saccharide moiety at a time, N-glycan formation begins in the lumen of the RER with the en bloc transfer of a lipid-linked oligosaccharide to asparagine cotranslationally (Kornfeld and Kornefeld, 1985; Fukada, 1992). The asparagine attached oligosaccharide is then trimmed giving rise to high mannose structures. After processing, high mannose structures are modified to yeld hybrid and complex oligosaccharides. The biosynthesis of complex N-linked branching proceeds with the linkage of GlcNAc(s) to the core mannose residues. In turn, these bound GlcNAc branches are extended by the sequential addition of monosaccharides. The following figure (Fig.5) gives a brief overview of this process.

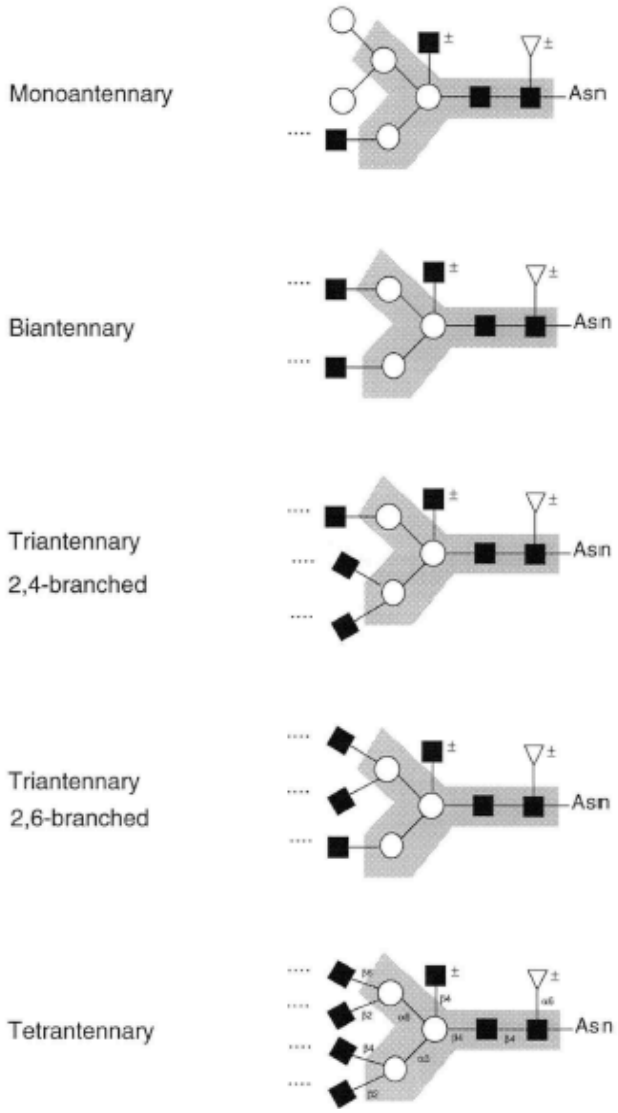


Figure 5 Branching of mammalian N-glycans

1.3 Formation of the Dolichol-Linked Precursor Sugar

Forming of sugar/lipid precursor begins on the cytosolic side of the RER and is completely autonomous from the protein that will be glycosylated subsequently. Synthesis of the lipid-linked oligosaccharide is initiated by the transfer of GlcNAc-1-phoshate to dolichol pyrophosphate (Dol-P) yielding GlcNAc-P-P-Dol. After the attachment of this GlcNAc, the remaining core sugars that are presented by the nucleotide donors, UDP-GlcNAc and GDP-

Man, become fastened by stepwise addition eventually producing $Man_5GlcNAc_2$-P-P-Dol.

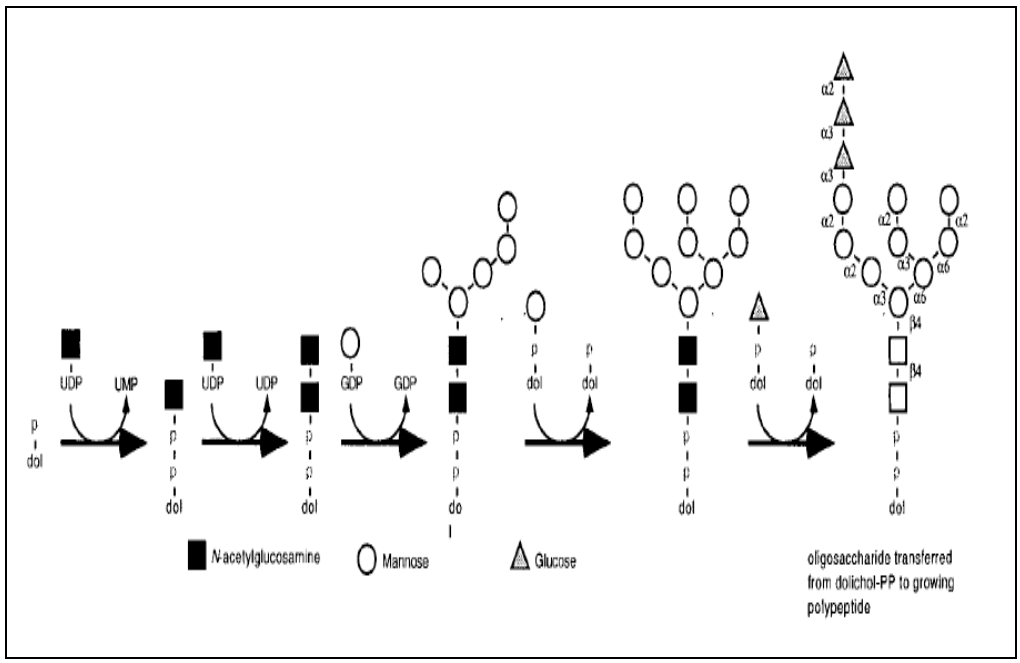


Figure 6 The synthesis of the oligosaccharide precursor. Sugars are added on one at time to the dolichol carrier molecule. The finished lipid-linked oligosaccharide is transfered en bloc to the nascent peptide by oligosaccharylthansferase. The dolichol product of this reaction is subsequently recycled.

This heptasaccharide-lipid intermediate is then translocated to the lumen of the RER where it is subjected to further processing. Although $Glc_3Man_9GlcNAc_2$-P-P-Dol is the full grown lipid oligosaccharide found in normal cells, a $Glc_3Man_5GlcNAc_2$-P-P-Dol structure, a glucosylated version of the heptasaccharide-lipid mentionated above, is the largest one found in a Dol-P-Man-deficient lymphoma cell line (Chapman et al. 1979). Taking into account the fact that this lipid-linked decasaccharide can also act as a donor substrate in protein glycosylation, it may play a role in the biosynthesis of N-glycans. This surrogate route has been termed the "alternative pathway".

The assembled precursor molecule is forwarded cotranslationally to asparagine residues with the consensus sequence Asn-X-Ser/Thr where X is any amino acid except proline or aspartic acid (Marshall, 1972).

1.4 Trimming of the Protein-Bound Oligosaccharide

Initial processing begins with the removal of the three glucose residues by two RER-membrane-bound –glucsosidases, α 1,2 glucosidase and α 1,3 glucosidase (Grinna and Robbins 1978; Elting et al. 1980). Prior to transiting to the cis-Golgi, an RER mannosidase cleaves off at least one α-mannosyl residue (Bischoff and Kornfeld, 1983). Additional trimming results in the conversion of the oligosaccharide to $Man_5GlcNAc_2$ by Golgi α1,2 mannosidase I. N-acetylglucosaminyltranferase I (GlcNAc-TI) modifies the high mannose structure to a hybride glycan as it traverses the medial-Golgi compartment. This is a decisive step as it is required for the synthesis of either hybrid or complex N-glycans. Cells deficient in GlcNAc-TI are blocked in this pathway and can only generate high mannose structures (Li and Kornfeld, 1978; Robertson, M.A et al.1978; Tabas et al. 1978).

1.5 Formation of Hybrid and Complex N-Glycans

After the addition of GlcNAc to the Manα1,3Manβ1,4GlcNAc arm, one of two different reactions can occur. The bisecting GlcNAc can be added by GlcNAcTIII, thereby, shunting the route toward hybrid structures, or alternatively, Golgi α-mannosidase II can remove the two terminal mannoses on the Man Manα1,6Manβ1,4GlcNAc arm forming a monoantennary glycan. After the action of α-mannosidase II, a second GlcNAc can be added by GlcNAc-TII resulting in the conversion of the monoantennary glycan to a complex biantennary structure. Additional branches can be generated by GlcNAc-TIV and GlcNAC-TV, which add the β1,4 and β1,6 GlcNAc-linked residues respectively. Before the glycoprotein leaves the medial-Golgi, its branching has already been decided by the medial-Golgi GlcNAc-transferases.

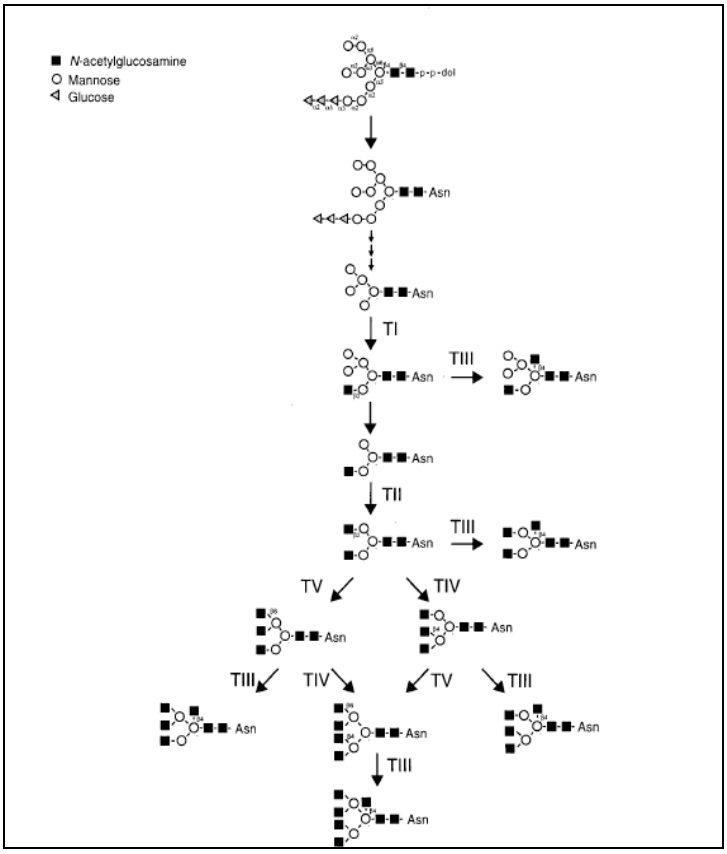


Figure 7 The antennae formation of N-glycans. After the transfer of the lipid-linked oligosaccharide to asparagine cotranslationally, trimming reactions in the Golgi and RER yeld a substrate for GlcNAC-Ti. The action of GlcNac-TII-V determined the number of antennae formed. The sugar are symbolized as follows: open circles, mannose: black squares, N-acetylglucosamine; shaded triangles, glucose. The GlcNAc-transferase I-V are denoted by TI-TV

After migration to the trans-Golgi, monosaccharides are added one at a time to complete the elongation of the antennas. Possible additions include galactose, fucose, sialic acid, GlcNAc and others. Moreover, the added sugar residues can undergo modifications such as phosphorylation, sulfation and acetylation.

1.6 Microheterogeneity

The study of a number of purified proteins has revealed that an identical protein can carry a variety of oligosaccharides even when produced in the same cell (Schachter, H, 1986). While this variance is quite restricted, the reason for its occurrence, termed “microheterogeneity” is presently unknown. Variant of a glycoprotein, called “glycoforms”, produced in two distinct cell types might

arise from differential glycosyltransferase gene expression and may be biologically relevant.

1.7 The Bisecting N-Acetylglucosamine of N-Glycans

An early step in complex and hybrid N-glycan biosynthesis may be initiated by GlcNAc-TIII which adds a GlcNAc monosaccharide in β1-4 linkage to the β1-4 linked mannose of the processed mannose core. Although the function of the resulting "bisecting" GlcNAc is not presently known, this modification can inhibit completely the action of the other enzymes in subsequent N-glycan biosynthesis (α-mannosidase-II, GlcNAc-TII, GlcNAc-TIV, GlcNAcTV, core α 1-6-fucosyltransferase) and inhibit partially UDP-Gal:GlcNAc β1-4 galactosyltransferase (Schachter H., 1986), suggesting a regulatory role in the formation and function of complex and hybrid N-glycans. While bisected N-linked oligosaccharides have not thus far been shown to specifically interact with endogenous lectin receptors, altered exogenous lectin binding has been reported (Cummings and Kornfeld, 1982; Narasimhan et al. 1986).

GlcNAc-TIII activity was first described in the hen oviduct (Narasimhan, 1982) and has subsequently been found in various systems including the rat liver during hepatocarcinogenesis (Narasimhan et al. 1988), rat brain (Nishikawa et al ,1988), human B lymphocytes (Narasimhan et al, 1988). The gene encoding GlcNAc-TIII has been isolated from human (MGAT3) and mouse (Mgat3) (Bhaumik et al. 1995).. Expression of Mgat3 RNA appears high in mouse brain and kidney tissue. In addition, high levels of GlcNAc-TIII activity and bisecting N-glycans have been reported in cells derived from patiens with chronic myelogenous leukemia in blast-crisis (Yoshimura et al. 1995).

1.8 The glycosylation modification of proteins

The glycobiology has led to many innovations in both the biomedical field and in basic research. The interest in this field of research is aroused by the fact

that the glycans not only influence the protein activity but also by the evidence that an aberrant composition of glycan chains is closely related to several diseases, such as autoimmune disease and the onset and progression of tumors.
In physiological condition, the glycans repertoire is involved in mechanism of molecular recognition, as in the case of bacterial or viral infections, cell adhesion, triggering of inflammatory processes and other events of cellular communication (Helenius et al. 2004).
The changes that can occur on a N-linked glycan chain, can be: an increasing in the number of branches; variations in the type of chemical linkage between the sugars and the attachment of new sugars within the chain. It is unclear, whether these changes are due to a malfunction of the enzymes responsible for the formation of the chain or if latters respond to a specific and a new genetic signal.
The changes of the mannosidic core, defined as increase in the number of branching constituting the glycan chains, however, are generally associated with cancer and autoimmune diseases.
A model system to understand how the state of glycosylation influences the activity of a protein is given by the IgGs. These have a single N-glycosylation site, but the IgGs not glycosylated, present in the circulation in rheumatoid arthritis, do not retain the ability to activate the complement (Yoshiki et al. 2006).
A sugar, instead, whose presence or absence within the glycan chains is strongly related to autoimmune diseases and cancer, is the fucose. In the complex N-linked glycan chains, the fucose can generally be linked to the first NAcGlc residue of dichitobiose or to the NAcGlc residue present on a branching, in this last case it forms a specific chain, defined Sialyl Lewis x(sLex) (Fig . 8).

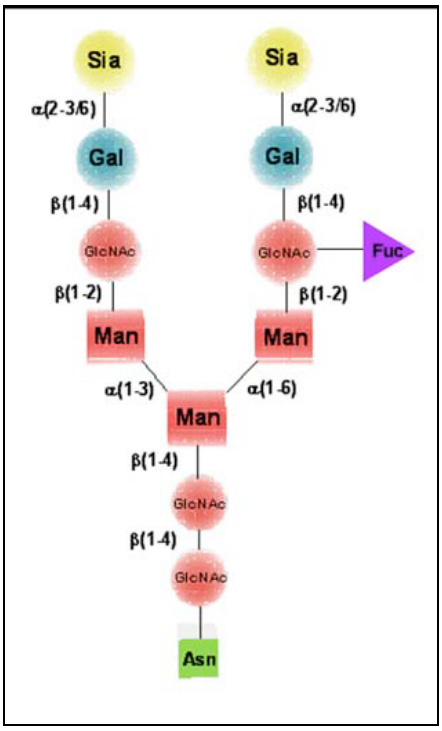


Figure 8 Sialyl Lewis x(sLex) glycan chain. The glucosidic linkage between NAGlc and Fuc is α 1-3 type.

This latter type of branching is present, on numerous proteins, in cases of acute and chronic inflammation (Van Dijk et al. 1995).

The expression of this chain counteracts the progression of pathologies; it has been observed in fact, that treatments with soluble sLex may counteract the inflammation-induced tissue damage (Mulligan et al. 1993). This is due to the fact that this sugar, present on the leukocyte membrane glycoproteins or metastatic cells, interacts with E selectins, present at the level of vascular endothelial walls. The recognition between sLex and E-selectin allows the leukocyte rolling, which precedes the extravasation of leukocytes from the blood stream (diapedesis). The increase of molecules with such glycan chain prevents the interaction of the endothelial cell receptor (ie the selectin E) with the specific ligand situated on the leukocyte membrane (Engers et al. 2000).

Regarding the variations in the type of chemical linkage between the sugars, for example, the sialic acids, in case of human glycosylation, can be attached to

a terminal galactose either by α 2,6 or α 2,3 glycosidic linkage, showing different functionality as a consequence. α2,3-linked N-acetylneuraminic acids for example, are specifically required for formation of sialyl Lewis X structures, which have shown to be indicative for metastasis of several types of cancer (Isozaki et al. 1998; Jorgensen, T., et al. 1995) whereas α2,6-linked sialic acids show roles in galectin inhibition, thereby promoting cell survival (Schultz et al. 2012; et al. 2006). Therefore, in this thesis we investigate the either α2,6 or α2,3 sialylation, indicating with (L) and (E), respectively the α2,3 glycosid linkage.

Chapter 2

2.1 Glycomics and Aging

As previously described, enzymatic glycosylation is the most common co/post-translational mechanisms of modification of protein involved in a variety of processes. Enzymatic glycosylation affects the biological function of the target protein: the sugar chains of glycoproteins play an important role in the regulation of the interaction between cells and between molecules, such as cell-cell communication, molecular signalling, antibody function, viral infection and bacteria colonization, and regulate the function of hormones and growth factors.

As the biosynthesis of glycan is not template dependent but is controlled by the concerned action of diverse enzymes, the sugar chain of glycoprotein is not rigorous defined as those of proteins of nucleic acids, but can be altered by the physiological or pathophysiological condition of the cell, as cancer, inflammation, immune diseases and aging. Though many reports elucidate the importance of alteration of glycan structure during development and disease [Freeze and Aebi, 2005] scarce information are available on the changes sugar moiety of glycoproteins coming during aging.

2.1 N-glycosylation during aging

It is well know that insulin and IGF-1 participate in growth and aging processes. IGF-1 synthesis and secretion is stimulated by Growth hormone (GH). IGF-1 is produced by in many tissues, most abundantly in the liver. IGF-1 mediates mainly actions of GH; it is transported in plasma bound to IGF Binding proteins (IGFBP) 1 to 6, but mostly to IGFBP-3. Growth hormone synthesis and secretion is influenced by sleep, age, exercise and nutritional states and is well documented to decrease with normal aging. Receptors for insulin and IGF-1 have similar structure and consist of two subunit linked by disulfide bonds that form a tetrameric structure. The receptor result highly

glycosilated in the mature and functional form (approximatly for the 25% of the final molecular weight; Duronio et al. 1986). Insulin-like growth factor-I (IGF-1) is for the most part bound in a ternary complex with IGF-binding protein-3 (IGFBP-3) and acid-labile subunit (ALS). The ternary complexes regulate IGF availability to the tissues and modulate the endocytosis of IGF-1 by cells. IGFBP-3 result N-glycosylated at 3 sites, and the modification is known to modulate the cell-binding activity of IGFBP-3 (Firth & Baxter, 1999). ALS present 7 sites of N-glycosylation that can modulate the affinity for IGFBP-3. It has been observed also that complete deglycosylation abolished the ability of ALS to associate with IGFBP-3 (Janosi et al. 1999). The alteration of N-glycosylation of IGF-1 and its binding partners during aging have important consequence in terms of cell-availability.

It has been demonstrated by Sato et al. that the N-linked glycans of glycoproteins in the brain of young adults and older rat change with age. Glycoproteins in the soluble fraction and in the membrane fraction of various portions of brains and spinal cords from 9-week-old rats and 29-month-old rats showed marked differences by the age of donors; in particular, glycoproteins with an apparent molecular weight of 123K and 115K are increased in old rats. These results indicate that the state of glycosylation of some proteins in the brain change during aging (Sato et al. 1998).

Alteration in N-glycosylation is particularly important in serum proteins. Immunoglobulin G (IgG) is an exceptional model of glycoprotein because its glycosylation has been well described and many important functional effects of alternative glycosylation have been designated (Gornik et al. 2012).

Recently developed high-throughput methods of analysis are now available to investigate the N-glycome, or the whole spectrum of N-linked glycans associate with proteins soluble in serum and body fluids of large number of people; these methods revealed that N-glycome in a way depended to aging. In particular, the N-glycome changes seem to be related with the sugar chains linked to Asn297 of immunoglobulin G. These aberrant glycosylated IgG can activate inflammatory response with ill-starred consequence for the organism. In fact, the N-linked sugar chain to Asn297 is located in the Fc portion of IgG

and forms the complex biantennary type (Arnold at al. 2007). Antennae are concluded by sialic acid and galactose residue (Tsuchiya et al. 1998), while the core structure can be replaced by bisecting GlcNAc and/or core-linked fucose.

The N-linked glycosylation of Asn297 mediate IgG stability and regulate its effector functions. In inflammation, autoimmune conditions and aging, the N-glycosylated Asn297 of IgG undergo characteristic glycosylation changes that can be reassumed as (i) reduced presence of galactose and sialic acid in the antennae, leaving GlcNAc in terminal positions, (ii) increased expression of bisecting GlcNAc and (iii) increased core fucosylation. The first condition is the most relevant; in fact the presence/absence of galactose on one or both arms of IgG can distinguish three subfamily called IgG-G0 (no galactose), IgG-G1 (galactose on one arm) or IgG-G2 (galactose on both arms; Jefferis et al., 1990). These modification appear to be associated with several disease as inflammation (IgG-G0), lung and ovarian cancers, rheumatoid arthritis and primary osteoarthritis.

A relationship between age and galactosylation of the N-linked chains of IgG is reported in a study (Parekh et al. 1988a) that investigated the alterations in the level of N-glycosylation of human IgGs during aging. The study involved 151 healthy individuals of both sex (age 1–70 years). Parekh et al. shown that the presence of agalactosyl N-glycans on total serum IgG-G0 decreased from birth until 25 years of age and then increased with aging. The relative incidence of digalactosyl structures (IgG-G2) showed a concomitant inverse relationship with age, whereas the relative incidence of monogalactosyl structures (IgG-G1) remained constant. These finding suggest that galactosylation of the N-glycans on the human serum IgGs of normal individuals is an age-related molecular parameter (Parekh et al. 1988b). Successively, a study on 403 individuals from 0 to 85 years old confirmed the increased expression of IgG-Go with aging, especially in females (Yamada et al. 1997).

Recently, three groups of researchers studied the age-dependent changes of serum N-glycome of large groups of individuals from different part of Europe.

The first group analysed the age-dependent N-glycomic changes in serum by DSA-FACE, in Italian and Belgian individuals (Vanhooren et al. 2007). They reported that in people over 60, in both population was there is an important increase or core-fucosylated, agalactosylated biantennary N-linked chains with or without a bisecting GlcNAc, and a concomitant decrease of core-fucosylated digalactosylated structure (Vanhooren et al. 2007, 2008, 2009), expecially in IgG and IgG-depleted glycoproteins.

The Italians' healthy centenary population, revealed that above 90 years old there is an increased agalactosylated structures, whereas in people aged 60-90 there is a decrease of digalactosylated structures (Vanhooren et al. 2007).

The second group of researchers established the Leiden Longevity Study which consists of of nonagenarian sibling pairs, their offspring, and partners of the offspring which serve as control (Ruhaak et al., 2010b). With this cohort group they evaluated the association of specific N-glycans with calendar age, but also their incidence in offspring, identifying markers for longevity. The analysis of tryptic peptides from IgG Fc glycopeptides confirmed the association of agalactosylated and monogalactosylated N-linked structures with age. Interestingly, in offspring aged less than 60, the level of agalactosylated structures with a bisecting GlcNAc was lower than in their partners, suggesting that low levels of these markers of aging in young people associate with longevity (Ruhaak et al. 2010b). This result was further confirmed by HPLC separation of N-glycans from total serum glycoproteins (Ruhaak et al. 2011). Two glycan features were found to be more highly expressed in the offspring than in controls; also if not fully confirmed, it seems that these markers are nonfucosylated biantennary glycans, that also correlated negatively with C-reactive protein levels which is a marker of inflammation (Ruhaak et al. 2011).

The third group investigate the N-glycome of 1008 individuals from the Croatian Adriatic island of Vis, by HPLC before and after sialidase treatment. The treatment give rise to 33 peaks contained different carbohydrate structures. The study confirmed the tendency to age-dependent increase of biantennary agalactosylated or mono-galactosylated structures with or without bisecting

Glc-NAc and/or core fucosylation, whereas digalactosylated structure finishing by sialic acid decrease with age (Knezevic et al. 2009).

In a genome wide association study, agalactosylated sugar structure presents in human blood was discovered to be associated with a single nucleotide polymorphism (SNP) located in the intron of the FUT8 locus that encode for the fucosyltransferase which mediate the enzymatic transfer of fucose to the core of the structure (Lauc et al. 2009, 2010). Linkage with agalactosylated biantennary N- linked chains was displayed also by the locus encoding estrogen receptor beta (ESR2).

These findings indicate that the human serum glycome is strongly influenced by a genetic basis even though environmental conditions can play a relevant role. Moreover, plasma glycan features reflect the age and the healthy status of the person and represent a reserve of disease biomarkers.

2.3 Pathogenic effects of altered IgGs

Numerous evidence supports the pathogenic role of IgG-G0 in inflammation through different but not mutually exclusive mechanisms. The differential glycosylation of IgG-G0 can modulate their effector function via four principal mechanisms, which can in turn mediate pathogenic effects: (i) interaction with the mannose binding lectin (MBL) for the complement activation; (ii) interaction with lectin receptors on the membrane of Antigen Presenting Cells (APC); (iii) interaction with Fc receptor on phagocytes and natural killer cells; (iv) formation of autoantibody aggregate.

The age-related accumulation and the differential glycosylation of IgG-G0 can contribute to inflammaging, a low-grade pro-inflammatory condition that would activate the immune system, exacerbating inflammation. (Dall'Olio et al. 2012). A systemic inflammatory condition is common to a variety of autoimmune diseases, infectious diseases, cancers and aging. This condition alters the glycosylation machinery of antibody-producing cells, resulting in the increased expression of agalactosylated and poorly sialylated IgG that, in turn, activate different effectors branches of the immune system (complement, APC,

phagocytes), resulting in the amplification of inflammatory signals. In this scenario, a related expression of sialylated IgG is observed, resulting in phagocyte activation.

The age-related alterations of the N-glycome are not only a strong biomarker of biological age, but also the cause of inflammation. Autoimmune diseases, cancer and aging are characterized by a low grade inflammatory status depending on various stimuli and sources, including the production of aberrantly glycosylated IgG that in turn fuel the inflammatory status. Thus, altered N-glycans are both powerful markers of aging and possible contributors to its pathogenesis (Dall'Olio et al. 2012)

Chapter 3

The Down Syndrome

3.1 The Down Syndrome, epidemiological aspects

Down syndrome (DS), or trisomy 21, is the most common and know chromosomal anomaly, caused by the presence in three copies of chromosome 21 (Hsa21) or, more rarely, parts of it, following a meiotic non-disjunction during ovogenesis. The origin of the supernumerary chromosome 21 is maternal in 93% of cases, while in 7% of cases is due to nondisjunction of the chromosome of paternal origin.

In 95% of cases, trisomy 21 is free form, which is the presence of the entire surplus Hsa21 not translocated to other chromosomes: in most cases, the trisomy 21 is homogeneous, i.e. it is present in all cells analysed, while in 3,5% of patients trisomy 21 is in the mosaic, only present in some cells. In 4% of cases, the trisomy 21 is due to a translocation of Hsa21 on another chromosome (Robertsonian translocation), and finally in 1% of patients trisomy 21 is partial. From the clinical point of view the effect is the same, while the reproductive consequence (risk of recurrence) for the parental couple are related to type of cytogenetic trisomy 21. Therefore for the diagnosis of DS is needed the presence of trisomy 21 in association with the clinical picture.

The estimated incidence of Ds is approximately 1/700- 1/1000 live births, with a slight predominance in males (Canfield et al. 2006); the annual number of children born with DS appears to be decreasing mainly because of the voluntary interruption of pregnancy (VIP); VIP logs show a progressive increase in the years of voluntary terminations for DS and It is estimated that the percentage of abortions after diagnosis of DS is about 58%.

The only established risk factor for the DS is advanced maternal age, responsible for changes in the process of meiosis (M) of oocytes, in particular the meiotic nondisjunction of Hsa21 (Ghosh et al. 2009) during both the phase

I (MI) and the phase II (MII), the error rate varies according to maternal age: they are lower among mothers aged<19 and >40 years and higher in the intermediate group and, in women aged > 40 years, the MII nondisjunction is more common (Allen et al. 2009).

Trisomy 21 results in a high risk of spontaneous abortion and neonatal morbidity and mortality: in the period between the time of chorionic villus sampling an the period of gestation, the percentage of miscarriages is 43%, while between the time of amniocentesis and the term of gestation is 23% (Morris et al. 1999). The literature has shown a correlation between maternal age and a directly proportional incidence of spontaneous abortion of fetuses with DS, with the increasing of the incidence of spontaneous abortions associated to the increasing maternal age (from 23% for the mothers at the age of 25 years to 45% for those 45 years) (Savva et al. 2006).

In recent years the quality and life expectancy of patients with DS were significantly improved, thanks to continued progress in medical treatment: in particular has increased survival in early childhood, following the improvement of cardiac surgery, and most people with DS reach over 60 years of age; life expectancy for males is greater of about 3.3 years compared females; life extension, however, lead to an increase of percentage of diseases associated with aging (Glasson et al. 2002).

3.2. The Down Syndrome: cytogenetic aspects

Many studies were designed to identify phenotypic and clinical consequences related to the triple gene dosage due to trisomy 21.

Chromosome 21 has been completely sequenced in the late ‘90s (Hattori et al. 2000). The genes od Hsa21 so far identified are more than 400 (Gardiner et al. 2006), 20-50 of which are located at the terminal part of the long arm (21q22.2), the so-called Down Syndrome Critical Region (DSCR). Although this number is high, it is not an intractable one, and the number of genes that are involved in DS is likely to be considerably less than their total number on the chromosome. The main question in whether the DS phenotype is

determined by only a few genes with a major phenotypic effect or whether the phenotype results from the interaction of several genes of modest effect.
Based on studies in animal models, there is reason to hope that the former might be the case.
Furthermore DS is a disorder of gene dosage in which the genes that are involved are normal and their gene products are also normal. The genetic abnormality is, therefore, qualitative rather than qualitative and involves the production of increased amounts of the products of the genes on chromosome 21. The simplest expectation was that the degree of overexpression of the unbalanced gene would be about 50% (Hattori et al. 2000) and it now seems to be generally true (Amano et al. 2004, Dauphinot et al. 2005). This raises the question of how such a relatively modest increase in gene expression can have such deleterious consequences. The genes in triplicate Hsa21 have an effect on the entire integrated genome and affect the expression of many other genes, activating or inhibiting, depending on the individual genetic constitution (genetic polymorphism): this could explain the variability of phenotypic expression and diversity disease in individual patients with DS.
Finally trisomy for functional non-protein-coding DNA elements might be involved in some of the abnormal phenotypes. Comparative analysis of human with other vertebrate genomes revealed the presence of conserved regions that are not part of the annotated genes (Dermitzakis et al. 2005) .
The sequence conservation strongly indicates that these elements are functional, but the precise function of each one of them is unknown. It is however possible that three copies of some of the conserved elements contribute to the DS pathology.

3.3 The Down Syndrome: pathogenic aspects

Similar to almost all conditions that result from chromosome imbalance, DS affects multiple systems and produces both structural and functional defects (Epstein, 2001). In addition, the evolution of the phenotype has a temporal

dimension so the issues concerning affected individual, their families and society change age with age.

The clinical diagnosis of DS can be made at birth on the basis of the presence of marked hypotonia and a constellation of minor dysmorphic features that involve all parts of the body but are especially visible in the craniofacies, hands and feet. Nevertheless, these dysmorphic features have little effect on function. Anyhow, there are two groups of major congenital abnormalities associated with DS, congenital heart disease and several types of gastrointestinal- tract obstruction or dysfunction, that can cause morbidity or even death if untreated.

The recent years considerable progress has been made in understanding and explanation of the genetic mechanisms underlying the main phenotypic characteristic of DS: developmental abnormalities, susceptibility to certain diseases, disability. In particular, through an logarithm to identify non-coding RNAs, five microRNAs (miRNAs) have been identified on Hsa21 (Kuhn et al. 2008, Sethupathy et al. 2007). MicroRNAs regulated the expression of the other genes (Bartel, 2004) and their role in DS not fully understood.

3.4 Phenotypic variability

Almost every aspect of the phenotype of DS is subject to a high degree of variability (Antonarakis and Epstein, 2006). No two patients are likely to have exactly the same combination of features and none of the minor dysmorphic features is present in >80% of the cases. Nevertheless, even when superimposed upon familial features, the overall pattern of minor anomalies ad distinct, and enables clinical recognition (Epstein, 2001).

Furthermore, life expectancy of DS Individuals has been rapidly increasing over the past 20 years, with a doubling of the median age of death in white populations (up to 50 years in 1997), even if environmental effects that result from social disparities seem to have deprived various ethnic groups of similar benefits (Yang et al. 2002, Leonard et al. 2000).

3.5 Congenital Heart defects and Gastrointestinal system Malformations

Approximately half of all children with DS are born a signification congenital heart defect (CHD), the most common of which is an atrioventricular septal defect (AVSD) (Freeman et al.1998).

Typically developing children with CHD have been shown to have neurocognitive and psychomotor deficits (Malec et al. 1999). For instance, school aged children with hypoplastic left heart syndrome have a mean full-scale IQ of 86, which is below the population normative value, approximately one standard deviation (Mahle et al. 2006. Visootsak et al. (2001) have documented that there may be possible developmental differences in children with DS+AVSD compared to children with DS without CHDs. Issues that remain largely unknown in children with DS+AVSD compared to the children with DS without CHDs. Issues that remain largely unknown in children with DS+AVSD are the magnitude of their cognitive, language, motor, social, and adaptive deficits; the patient-related characteristics and perioperative factors may influence their neurodevelopmental outcomes and the effects of home environmental variables on their development.

Gastrointestinal defects are second to heart defects among the common serious structural birth defects associated with DS (Fabia and Drolette, 1970; Kallen et al. 1996) and atresia/stenosis of the small intestine were most common (Cleves e al. 2007).

3.6 Immune and Hematopoletic Systems

In addition to the effects on function of the heart and gastrointestinal system, trisomy 21 also produces many changes in cellular function (Epstein, 2001). Among these, those involving the immune and hematopoietic systems. The impact of infection has been greatly reduced with the availability of hematopoietic systems. The impact of infection has been greatly reduced with the availability of immunizations for various viral and bacterial pathogens improved hygiene and antibiotics, however infection is still a major cause of

death in DS at any age, and has been suggested that this susceptibility to infection depends on abnormal maturation of the thymus and impaired function of T lymphocytes (Philip et al. 1986, Murphy et al. 1995), Franceschi et al. (1978) found a decreased percentage of T lymphocytes and an increased percentage of "null cells" (non-T, non-B lymphocytes), so the immunologic defect seems to be a congenital feature of DS, and the antigen overloading could worsen the intrinsic immunological derangement and lead to a stress-immunodeficiency. In particular subjects with DS showed normal responsiveness in allogeneic mixed lymphocyte reactions, but they response to phytohemagglutinin and in autologous mixed lymphocyte reactions were severely impaired; moreover, the blood concentration of serum thymic factor in the majority of DS subjects was much lower than that found in age matched healthy controls (Franceschi et al. 1981).

Furthermore, infants with DS develop acute leukaemia, most commonly acute megakaryoblastic (M7) leukemia , with a frequency twenty times higher than that in the normal population (Hitzler et al. 2003). This is often preceded by a neonatal leukemic reaction (transient myeloproliferative disorder), which might be a form of transient leukaemia. A unique non–activating mutation in the GATA binding protein 1 (GATA1) gene (on the X chromosome) has been found in association with both the leukaemia and the leukemic reaction (Hitzler et al.2003, Wechsler et al. 2002), and children with DS seem to have a better prognosis and require less chemotherapy than children without DS (Gamis, 2005).

3.7 Central Nervous System

The trisomic state hits the central Nervous Systems (CNS), which results vulnerable throughout life. The brain is grossly normal with the exception of some degree of hypoplasia of the cerebellum (Aylward et al. 1997). Nevertheless, perhaps the most frequent sign of DS is a central hypotonia, followed by delayed cognitive abilities in adulthood and the development of Alzheimer's disease (AD) in later years (Busciglio et al. 2002).

The literature shows that the 8-13,6% of individuals with DS develop epilepsy, (Pueschel et al.1991; Goldberg-Dtern et al. 2001), that cognitive development is in the range of mental retardation (usually grade medium-mild) (Brown et al.1990) and that there is a frequency of autistic disorder, 7-11% (Knit et al.1999; Kroger and Nelson, 2006).

In general, the stages of development follow the normal sequence, with a particular deficit in the production of language (Chapman et al. 1998), in childhood have been reported behavioural disorders conduct (5,4%) or aggressive behaviour (6,5%) while it can take as an adult major depression (6,1%) or aggressive behaviour (6,15) (Myers and Pueschel, 1991).

All domains of intellectual function do not have uniformly cognitive impairment, and there are both strengths and weaknesses in intellectual ability (Nedel 2003). The forms of learning that depend on the hippocampus are particularly affected, and there is also evidence for impairment of prefrontal cortex and cerebellar functions (Nedel 2003). Speech and articulation are particularly affected. Numerous anatomical, biochemical and physiological studies have shed little light on the basis for cognitive impairment (Epstein, 2001). Several years ago have been observed morphological abnormalities of the dendritic spines, which are the sites of synapse formation, and this originally non-specific finding has been considerable reappraised with the discovery of similar abnormalities in the brains of Ts65Dn mice (Belichencko et al. 2005). Hippocampal neurons in TsD65D brains also exhibit major abnormalities in long-term potentiation (LPTD) and long-term depression (LTD), which are electrophysiological phenomena associated with learning and memory (Kleschevnikov et al. 2004). The finding of similar abnormalities in the brain of people with DS might increase the understanding of the basis of cognitive impairment in DS and might provide potential therapeutic targets (Antonarakis and Epstein, 2006). Moreover, another abnormality in the Ts65Dn mouse brain might be directly relevant to humans, i.e. the progressive loss and atrophy of cholinergic neurons in the basal forebrain (Salehi et al. 2006; Granholm et al. 200), a neuronal population that is particularly vulnerable in AD.

The diagnosis of dementia in DS requires an understanding of baseline cognitive and emotional functioning in the disorder. For example, most healthy people with DS prior to the onset of dementia would score below detectable levels on the Mini Mental State Examination, a screening test for dementia administered to risk individuals in the general population. In the child and adult with DS, measures od IQ, general cognitive functioning, and mental age are at least two standard deviations below the norm and these global anchor measures appear to exceed performance in language, phonology, and memory (Miller, 1988). Auditory verbal memory is selectively impaired beyond global baseline functioning (Varnhagen et al. 1987). These findings can become confounding factors for the determination of dementia in DS and have led to a consensus statement for testing individuals at risk (Aylward et al. 1997). One alternative to traditional direct testing of adults with developmental disabilities involves looking to sources other than the patient for information (Burt et al. 1998).

Aim of the study

Previous studies, performed in the laboratory where my thesis was elaborated, demonstrated that there is a linkage between successful/unsuccesful ageing and alterations of serum glycome, suggesting that measurements of the log ratio of two glycans (NGA2F and NA2F), named GlycoAge test (Vanhooren et al. 2007, 2010), could provide a non invasive biomarker of aging. As discussed in the previous chapters, Down syndrome (DS) is a genetic disorder in which multiple major aspects of senescent phenotype occur much earlier than in healthy age-matched subjects and has been often defined as an accelerated aging syndrome.

Therefore the questions this study was aimed at finding a possible answer are:

- Is DS characterized by a specific N-glycomic signature? Data will be presented comparing the plasma N-glycome of patients affected by DS with age- and sex matched non-affected controls, represented by their siblings (DSS). Since DS and DSS subjects share or have shared for many years the same environment, this comparison will be informative on the effect of the disease on the plasma N-glycosylation changes.
- What is the contribution of ageing within DS? Data will be presented comparing DS of different age, as well as DS with their mothers (DSM) who also shared a genetic background.
- What is the contribution of epigenetic or environmental factors? Data will be presented comparing DS singletons (DSW) with unrelated age-sex matched controls.

Glycomic analysis has been performed on blood serum by means of two different techniques:

i. a high-sensitive protocol based on a MALDI-TOF-MS approach. A special focus on α2-3 and α2-6 specific linkage of sialic acids of the plasma N-glycome will be made;
ii. the DSA-FACE technology, in order to validate and go deeper the first approach.

Material and Methods

1. Study design

The rationale of this study was to compare the plasma N-glycome of patients affected by Down Syndrome (DS) with age and sex matched non-affected controls, represented by their siblings (DSS), in order to assess if Down Syndrome is characterized by a specific N-glycomic pattern and to study glycosylation changes of human blood proteins which are associated with Down syndrome.

Since DS and DSS subjects share or have shared for many years the same environmental factors, this family-based study model is particularly useful to investigate the effect of disease on the plasma N-glycosylation changes, while reducing the between-group difference in familial and epigenetic variables. Moreover, we enrolled a group of DS singletons, (DSW) i.e without brothers/sisters, that have a different age respect the DS and DSS group. This group is important not only to investigate the effect of aging on the plasma N-glycans changes within the disease but also to validate the specific N-glycomic signature of disease, reducing all other factors (epigenetic, aging, genetic, etc..) that could impact and influence the pattern discrimination.

Lastly, in order to investigate if the N-glycans changes that occur in DS were able to reveal an accelerated aging in DS patients, we enrolled, when it was possible, the mothers (DSM) of the DS and DSS, representing the non-affected control group with a different chronological age respect to DS.

2. Samples

The study population is represented by the following three groups of subjects (158 subjects in total) i) 75 subjects affected with Down Syndrome (DS), in particular, in this group, are present two subgroups: a. the first, is constituted by 38 Down syndrome subjects (DS) with a mean age of 27.2 years, belonging

to families where it is present also their respective brother/sister with similar age; b. the second, is constituted by 37 Down syndrome patients, singletons (DSW); with a mean age of 38.5 years, i.e without brothers/sisters, and characterized to be older than DS. ii) 38 age- and sex-matched non-affected controls (DSS) represented by the siblings of DS patients, with a mean age of 30.9 years; iii) 45 subjects represented by the mothers (DSM) of the DS and DSS, with a mean age of 57.1 years.

We recruited in total 35 complete family composed by the triad DS-DSS-DSM, 3 families where are only present the brother/sister of the DS; 37 DS subjects singletons, i.e, without brother/sisters. In this last group are present 27 DS without siblings and mothers, and 10 DS without siblings but their mother have been recruited.

After informed consent obtained from the family or the guardians, plasma samples were collected from EDTA-treated blood of all subjects. DS diagnosis was confirmed by karyotype. In the study were excluded subjects with an acute pathology in place; patients with hepatic, renal, or cardiac deficiency; patients who have taken two months before the recruitment multivitamin or anti-inflammatory drugs. The study was approved by the local ethics committee.

For this study we applied two different N-glycomics approaches:

- first, in order to study the complete plasma N-glycome and the glycosylation changes of human blood proteins which are associated with Down syndrome, we applied a high-sensitive protocol based on a MALDI-TOF-MS approach. Thanks to the specificity of this protocol particular attention was focused on the linkage specificity α2-3 and α2-6 of sialic acids of the plasma N-glycome.
- second, we used, on the same samples, a second approach based on the DSA-FACE technology in order to validate and to deep with the first approach the plasma DSA-FACE fingerprinting and the derived variables, as the GlycoAge test.

3. Plasma N-Glycome with MALDI-TOF/MS approach.

The analysis of the samples, performed with the MALDI-TOF/MS approach, were conducted during my PhD visiting research at the laboratory of Glycomic

and Glycoproteomics in Leiden (Netherlands). This new MALDI-TOF/MS protocol is in paper submission.

3.1 Enzymatic N-glycan release

N-glycans were released from the plasma of all subjects. 10 µL of plasma was denatured by adding 20 µL 2% sodium dodecyl sulphate (SDS) and incubating for 10 minutes at 60°C. The release step was subsequently performed by adding 20 µl release mixture containing 2% Nonidet P-40 (NP-40) and 0.5 mU recombinant peptide-N-glycosidase F (PNGase F) in 2.5x PBS and incubating overnight at 37°C.

3.2 Ethyl esterification of unpurified PNGase F released plasma N-glycome samples

A robust, high-throughput MALDI-TOF-MS method for profiling human plasma N-glycans with linkage-specific derivatization of sialic acid residues was assessed according as the described in *Reiding et al, 2013 (submitted)*. Reaction conditions were optimized to achieve ethyl-esterification at the carboxyl group of 2,6-linked sialic acid residues, whilst 2,3-linked sialic acid underwent lactone formation. (Fig.9).

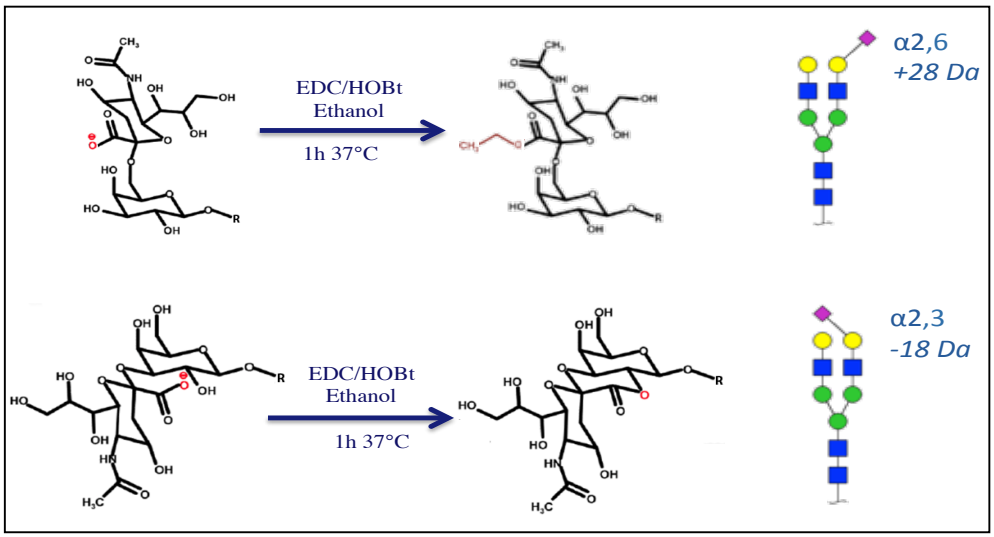


Figure 9. Linkage-specific ethyl esterification of sialic acid residues in released plasma N-glycans

Shortly, for each sample, 1 µL released plasma N-glycome (approximating 14 µg of plasma protein) was added to 20 µL of ethanol, containing reagent

combinations 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) with hydroxybenzotriazole (HOBt), each one at the final concentration of 0.25 M. All the samples, distributed in 96-wells PCR plate format, were incubated 1h at 60°C, sealing the plate to prevent evaporation.

3.3. Hydrophilic interaction liquid chromatography (HILIC)-SPE of unpurified N-glycans

A sample clean-up was deemed necessary for enrichment and purification of the glycans from the reaction mixture, and we chose for hydrophilic interaction liquid chromatography (HILIC)-SPE using cotton as stationary phase, as described in Selman et al.2011.

Hereafter, 20 µL acetonitrile (ACN) was added and all samples were incubated 15 min at 20°C before proceeding with glycan enrichment and analysis by MALDI-TOF-MS. To perform HILIC SPE, 200 µg of cotton was inserted into tips, washed with 3x 20 µL Milli-Q water (MQ) and 3x 20 µL 85% ACN, loaded with sample by pipetting 20x into the reaction mixture, washed with 3x 20 µL 85% ACN 1% trifluoroacetic acid (TFA,) 3x 20 µL 85% ACN, and eluted with 10 µL MQ. Careful pipetting was necessary to prevent clotting of the tips.

For MALDI-TOF-MS analysis, 1 µL of glycan sample purified by cotton HILIC SPE was spotted on a MTP AnchorChip 800/384 TF MALDI target (Bruker Daltonik, Bremen, Germany), mixed on plate with 1 µL 2.5-dihydroxybenzoic acid (2.5-DHB) (5 mg/mL) in 50% ACN and 1 mM NaOH, and left to dry. The matrix crystals were homogenized by adding 0.2 µL ethanol, for a rapid recrystallization.

3.4 Processing of MALDI-TOF-MS data

Analysis was performed by UltraFlextreme MALDI-TOF/TOF-MS (Bruker Daltonik, Bremen, Germany) in reflectron positive (RP) ion mode, using Flexcontrol 3.4 Build 119. Before sample measurement, the spectra were calibrated on the known masses of a peptide calibration standard (Bruker Daltonik, Bremen, Germany). All samples were ionized by a smartbeam-II

laser, and accelerated with 25 kV after 140 ns delayed extraction. . For each spectrum, 20000 laser shots were accumulated at a laser frequency of 2000 Hz, using a complete sample random walk with 200 shots per raster spot. Using flexAnalysis v3.3 build 65 (Bruker Daltonik), the acquired MALDI-TOF-MS spectra were internally recalibrated using varying calibration masses and the masses were calculated as $[M+Na]^+$. Abbreviations used for glycan annotation are: hexose (H), N-acetylhexosamine (N), fucose (F) and N-acetylneuraminic acid with unspecified linkage (S), with the accompanying number indicating the number of residues. Glycan signals were integrated, normalized to the sum of intensities and the relative signals calculated. Starting from 10 ul of human plasma, glycan profiles were achieved allowing the differentiation of 114 glycan species (**Fig.10)**

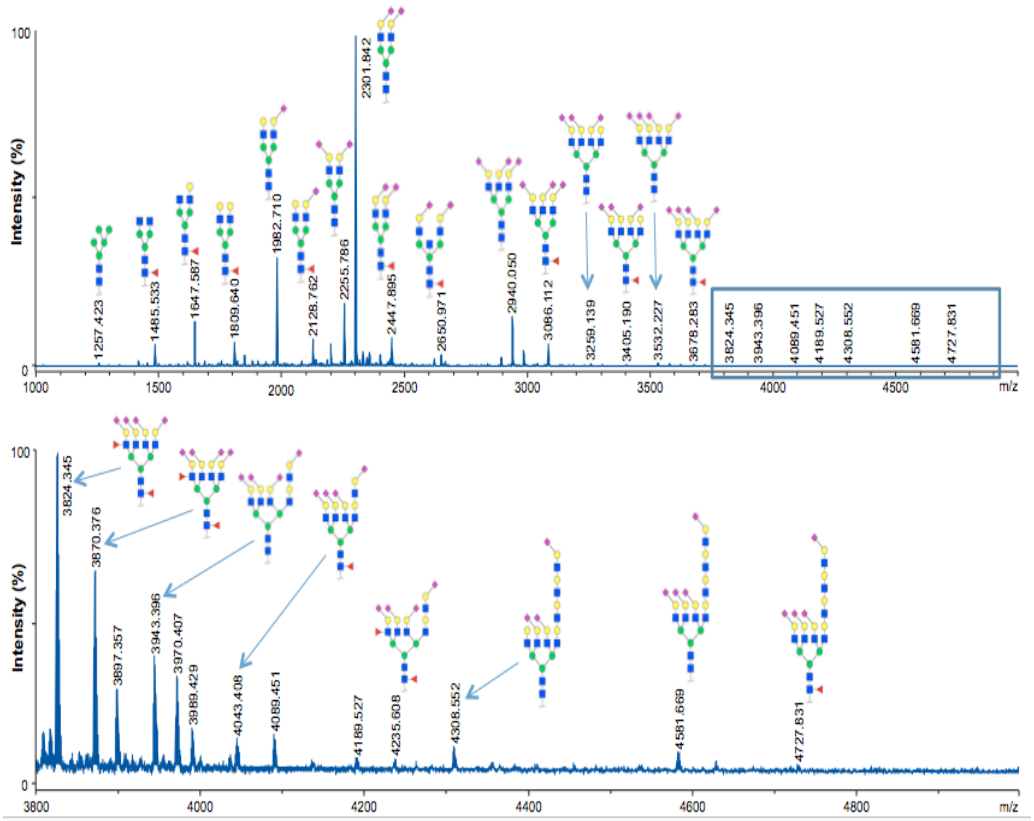


Figure 10 RP MALDI-TOF-MS spectrum after applying EDC+HOBt ethyl esterification protocol for plasma N-glycome analysis. A) Complete spectrum overview showing relative intensities from 1000 Da to 5000 Da.. B) High range spectrum from 3800 Da to 5000 Da showing glycan derivable masses up to 4727.640 Da. While the mass precision makes most compositional assignments likely, the structures shown are based on literature and may not reflect the actual sample.

Plasma profiles studied using the ethyl esterification protocol showed glycans [M+Na]+ ranging in mass from 1257.423 Da (H5N2) to 4727.640 Da (H10N9F1 with four lactonized sialic acids (abbreviated as L) indicating α2,3-linkage).

Reproducibility of human plasma N-glycome profiling employing 1 h 37°C EDC + HOBt ethyl esterification was demonstrated by multiple repeat analyses on three different days on 24 samples controls.

Average relative intensity values for the highest peak (H5N4 with two esterified sialic acids (abbreviated as E) indicating α2,6-linkage) were around 54.7% (SD ± 2.3%) across all measurements, with the CV averaging around 3.8%. (**Fig. 11**)

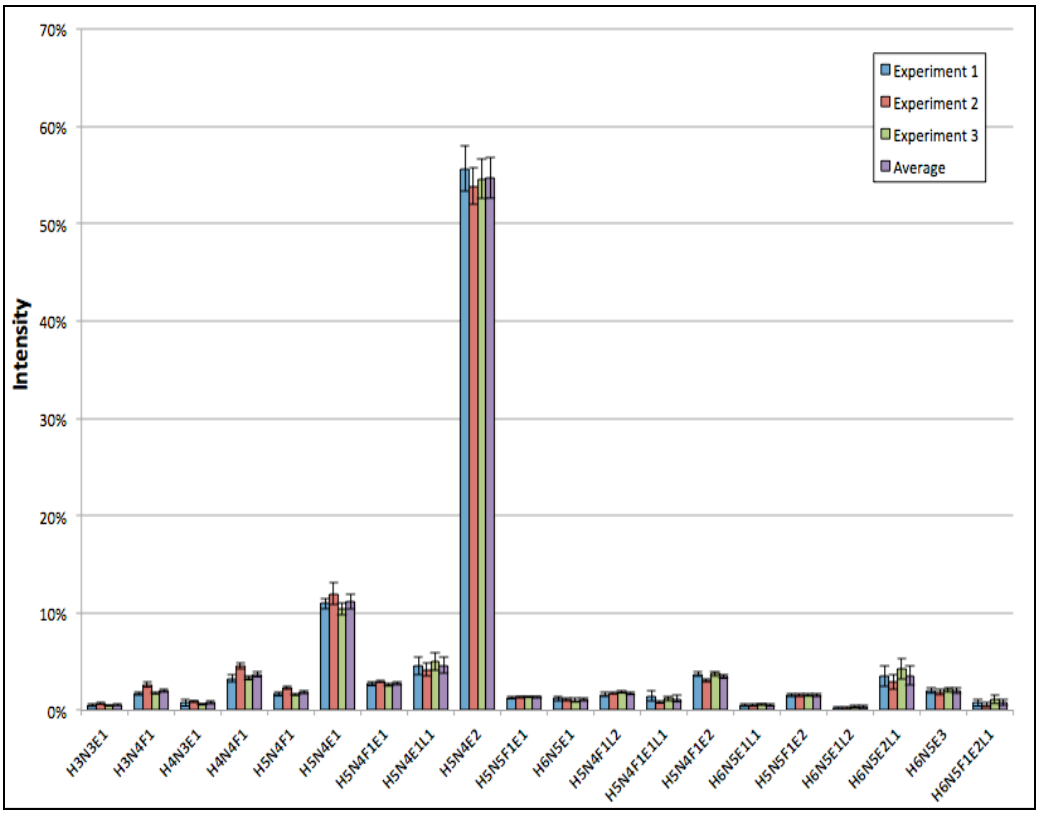


Figure 11 Reproducibility assay of 1h 37°C EDC + HOBt ethyl esterification. 24 samples originating from a common stock of plasma were independently released by PNGase F, ethyl esterified, purified by HILIC SPE, crystallized with matrix, recrystallized with ethanol, and analyzed by RP MALDI-TOF-MS. The experiment was performed three times on separate days, indicated as experiment 1-3. The graph shows the average relative intensities observed (normalized to the sum of intensities), with error bars for standard deviation

For our analysis, we used all peaks, that had signal intensity higher than background noise, selecting 98 peaks N-glycans. The complete list of 98 N-glycans features, with the assigned composition is indicated in the Table 1.

CODE PEAK	COMPOSITION	m/z
1	H3N3	1136,4
2	H5N2	1257,42
3	H3N3F1	1282,45
4	H4N3	1298,45
5	H3N4	1339,48
6	H3N3l1	1409,48
7	H6N2	1419,48
8	H4N3F1	1444,51
9	H3N3e1	1455,52
10	H5N3	1460,5
11	H3N4F1	1485,53
12	H4N4	1501,53
13	H3N5	1542,56
14	H3N3F1l1	1555,54
15	H4N3l1	1571,53
16	H7N2	1581,53
17	H3N3F1e1	1601,58
18	H5N3F1	1606,56
19	H4N3e1	1617,58
20	H6N3	1622,55
21	H4N4F1	1647,59
22	H5N4	1663,58
23	H3N5F1	1688,61
24	H4N5	1704,61
25	H5N3l1	1733,59
26	H8N2	1743,58
27	H4N3F1e1	1763,63
28	H4N4l1	1774,61
29	H5N3e1	1779,63
30	H5N4F1	1809,64
31	H4N4e1	1820,66
32	H6N4	1825,63
33	H4N5F1	1850,67
34	H5N5	1866,66
35	H9N2	1905,63
36	H5N4l1	1936,67
37	H6N3e1	1941,68
38	H4N4F1e1	1966,71
39	H5N4e1	1982,71
40	H5N5F1	2012,72
41	H4N5e1	2023,73
42	H5N4F1l1	2082,72
43	H6N4l1	2098,72
44	H4N7	2110,77
45	H5N4F1e1	2128,77
46	H4N5F1e1	2169,79
47	H5N5e1	2185,79
48	H5N4l2	2209,75
49	H5N4e1l1	2255,79

CODE PEAK	COMPOSITION	m/z
50	H5N4e2	2301,83
51	H5N5F1e1	2331,85
52	H6N5e1	2347,84
53	H5N4F1l2	2355,81
54	H4N6F1e1	2372,87
55	H5N4F1e1l1	2401,85
56	H3N6F1l2	2437,86
57	H5N4F1e2	2447,89
58	H6N5F1e1	2493,9
59	H5N5e2	2504,91
60	H5N5F1e1l1	2574,92
61	H6N5e1l1	2620,93
62	H5N5F1e2	2650,97
63	H6N5e2	2666,97
64	H6N5F1l2	2720,94
65	H6N5F1e1l1	2766,98
66	H6N5F1e2	2813,02
67	H8N6F2	2848,01
68	H6N5e1l2	2894,01
69	H6N8l1	2911,04
70	H6N5e2l1	2940,05
71	H6N5e3	2986,09
72	H6N5F1e1l2	3040,07
73	H6N5F1e2l1	3086,11
74	H6N5F1e3	3132,15
75	H6N5F2e1l2	3186,13
76	H6N5F2e2l1	3232,17
77	H7N6e1l2	3259,14
78	H7N6e2l1	3305,18
79	H7N6e3	3351,23
80	H7N6F1e1l2	3405,2
81	H7N6F1e2l1	3451,24
82	H7N6e1l3	3532,23
83	H7N6e2l2	3578,27
84	H7N6e3l1	3624,31
85	H7N6F1e1l3	3678,28
86	H7N6F1e2l2	3724,33
87	H7N6F1e3l1	3770,37
88	H7N6F2e1l3	3824,34
89	H7N6F2e2l2	3870,38
90	H8N7e1l3	3897,36
91	H8N7e2l2	3943,4
92	H7N6F3e1l3	3970,4
93	H8N7F1e1l3	4043,42
94	H8N7F1e2l2	4089,46
95	H9N8F1e1l2	4135,46
96	H7N6F2e3l2	4189,51
97	H9N8e2l2	4308,53
98	H11N9F2e1l1	4535,62

Table 1 Complete list of the total 98 neutral N-glycans of plasma N-glycome detected using ethyl esterification protocol. In the table is indicated for each N-glycans feature the corrisponding code peak, into the MALDI spectrum, the composition and the m/z value.

4. Plasma N-Glycome with DSA-FACE approach

The department for Molecular Biomedical Research of Ghent University (Belgium) developed an ultra-sensitive technique to profile and sequence N-linked glycans using standard DNA-sequencing equipment (Callewaert et al. 2001; Laroy et al. 2006). This technology is named "DNA Sequencer Adapted-Fluorophore Assisted Carbohydrate Electrophoresis" (**DSA-FACE**), thanks to which it possible to obtain an accurate fingerprint of the glycan mixture (serum or plasma). Subsequently, Valerie Vanhooren and Chitty Chen of the Ghent University, adapted DSA-FACE technique for the study of human aging, publishing several works in collaboration with the laboratory, directed by Prof. Claudio Franceschi (Vanhooren V. et al, 2007 and 2010).

During my PhD period I set up the same technique in our laboratory, and I applied the protocol to analyse the samples of this study. A schematic visualization of the N-glycan analysis using DSA-FACE technique is illustrated in figure 12.

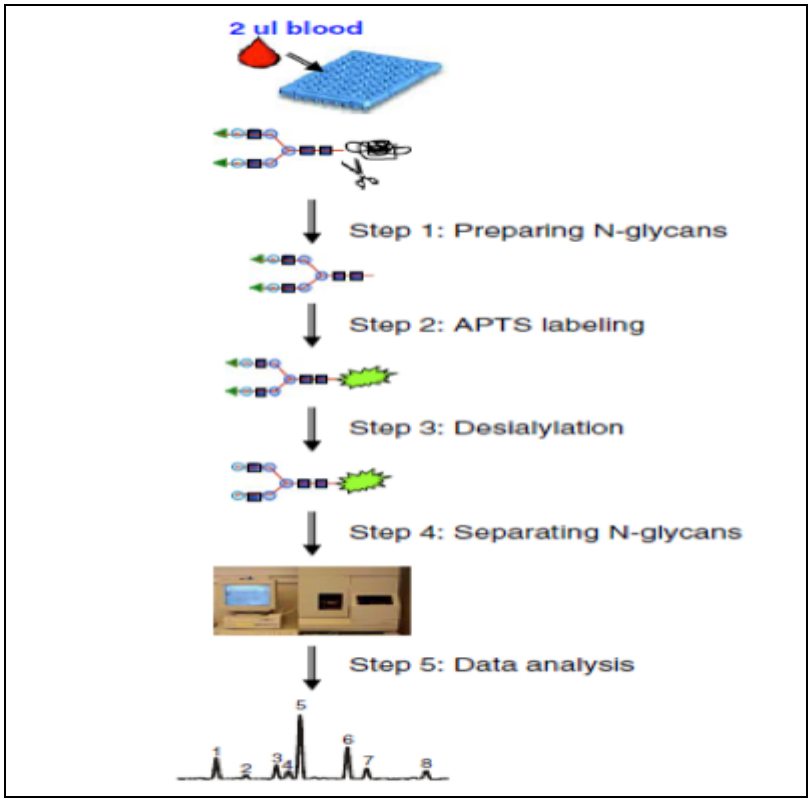


Figure 12. Schematic visualization of the N-glycans analyis via DSA_FACE techique. (Image was taken from VanHooren et al. 2008)

4.1 Preparing N-glycans

The total protein pool present in 2 ul of plasma is incubated with 2 ul of Milli-Q water (MQ) and 2 ul of denaturing buffer (5% SDS in 10 mM NH4HCO3) in a 96-well plate, for the protein denaturation. The plate is sealed and incubated for 5 min at 95 °C and then cool down for 15 min at 4°C. The N-glycans are cleaved by enzymatic digestion with 3 µl of a release mixture containing 3.33% Nonidet P-40 (NP-40) and 6.6 units of recombinant peptide-N-glycosidase F (PNGase F) (New England Biolabs) in denaturing buffer and incubating for 3 h at 37 °C. The reaction is stopped by adding 100 µl of water.

4.2 Labelling of released plasma N-glycans

The released plasma N-glycan were then derivatized with the fluorophore 8-amino-1,3,6-pyrenetrisulfonic acid (APTS). Briefly, 6 µl of released N-glycans mixture are dried completely into a new plate, at 60 °C for 1 h, and then, on the dried pellet were added 2 µl of APTS labeling buffer, constituted by a 1:1 mixture of 20 mM APTS in 1.2 M citric acid and 1M NaCNBH3 in Dimethylsulfoxide (DMSO). The labelling reaction is followed by an overnight incubation at 37 °C, sealing the plate to prevent the evaporation, and then stopped by adding 200 µl of water for well.

4.3 Desialylation of labelled plasma N-glycans.

Since most of the plasma/serum glycans terminate with sialic acids, which contain negative charges, their removal, before of the electrophoretic run, makes the final profiling clearer. In this step, the N-glycans were desialylated by adding into 2 µl of APTS labelled sugars, 0.25 µl 100 mM NH4AC, 0.2 µl neuraminidase of Arthrobacter ureafaciens (Roche) and 2.55 µl water, with a overnight incubation at 37°C. The desialylation reaction was stopped with 160 µl of water added in each wells.

4.4. Separating N-glycans with DNA-sequencer and data processing

10 µl of APTS labeled and digested glycans were analysed using a capillary electrophoresis (CE)-based ABI 3130 DNA sequencer (Applied Biosystem) equipped with a standard 36-cm capillary array filled with the POP-7

polyacrylamide linear polymer. The data was processed with the GeneMapper software (Applied Biosystem) using the factory-provided settings for amplified fragment length polymorphism (AFLP) analysis.

Each structure of N-glycans, identified with a peak into a typical DNA sequencing electropherogram was numerically described by normalizing its height to the sum of the heights of all peaks.

4.5 Plasma N-glycan profiling

10-12 peaks generally constitute the characteristic DSA-FACE N-glycome profiling for total plasma/serum glycoproteins, and each one identifies a specific N-glycan structure. Compared with mass spectrometric methods, this protocol resolves isobaric glycan stereoisomers, but the possibilities of characterization of an unknown compound are limited to the use of standars for comigration and to enzymatic degradations (Klein A., Adv Clin Chem. 2008) Indeed, the structures of these peaks had been characterized previously by Laroy W. et al., Nat Protoc, 2006, using specific exoglycosidases. The structures of the N-glycans peaks are shown in figure 13. In particular Peak 1 is an agalactosylated,core-α-1,6-fucosylated biantennary glycan (NGA2F), peak 2 is an agalactosylated, core-α-1,6-fucosylated bisecting biantennary (NGA2FB), peaks 3 and 4 are mono galactosylated, core-α-1,6-fucosylated biantennary (NG1A2F), peak 5 is a bigalactosylated, biantennary glycan (NA2), peak 6 is a bigalactosylated, core- α-1,6-fucosylated biantennary (NA2F), peak 7 is a bigalactosylated, core-α-1,6-fucosylated bisecting biantennary (NA2FB), peak 8 is a trigalactosylated, tri-antennary (NA3), peak 9 is a branching α-1,3-fucosylated trigalactosylated tri-antennary (NA3F(b)), peak 9’ is a core-1,6 fucosylated trigalactosylated tri-antennary (NA3F), peak 10 is a tetra-galactosylated, tetra antennary (NA4).

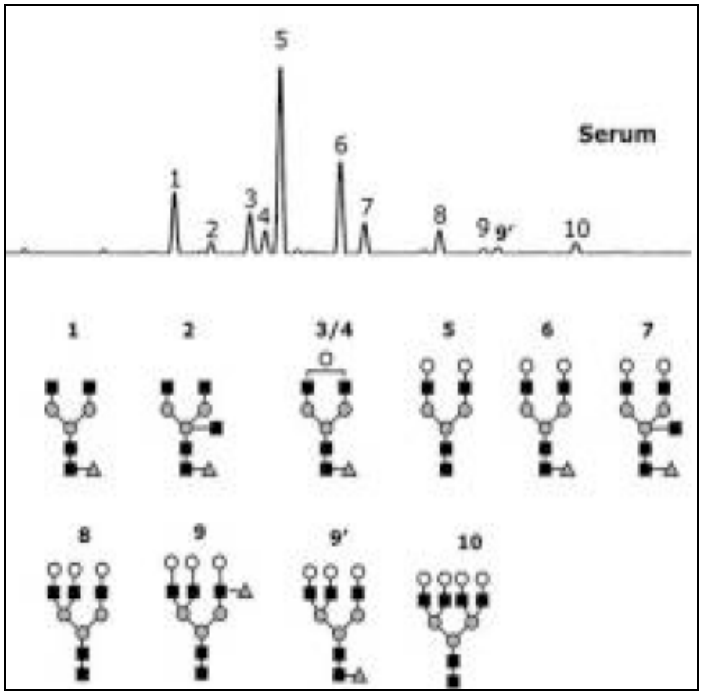


Figure 13 A typical desialylated N-glycan profile for total serum/plasma proteins, For each peak is indicated the corrisponding N-glycan structure. The symbols used in the structural formulas are: n N-acetyl-glucosamine (GlcNAc); o β-linked galactose; Δ α -1,3/6-linked fucose; □ α/β-linked mannose.

5. Statistical Analysis

The pattern of plasma N-glycome of Down Syndrome, both for the analysis performed with MALDI-TOF/MS and with DSA-FACE approach, was investigated by partial least squares discriminant analysis (PLS-DA), which is a supervised multivariate data analysis method that is part of the SIMCA software (Umetrics). The results from the PLS-DA analysis were visualized by plotting two principal components of the model against each other in a scatterplot. The plot illustrates the degree of separation explained by the components. Each point in the scatterplot represents a subject. Each principal component receives a Q2 value, which describes the statistical significance and the predictive power of that component.

The PLS-DA allows us to plot the structures according to their importance for separating the groups. Structures receive a variable of importance (VIP) value. Values > 1 indicate that the structure is involved above average in the

separation of groups.. To assess the individual discriminant ability of each component of the signature, we applied the non parametric Wilcoxon Rank sum tests among the compared groups (SPSS).

Results were corrected for multiple comparisons by a Benjiamin Hoocberg correction (R free online software). A P value <0.05 was considered significant after the FDR (false discovered rate) correction.

Results

4. Samples

In this study we analysed in total 158 samples, constituted by the following three groups of subjects (see **Table 2**):

i) 75 total patients affected by Down Syndrome (DS), constituted by 2 subgroups: 38 Down syndrome subjects (DS) with a mean age of 27.2 years, belonging to families where it is present also their respective brother/sister with similar age; b. 37 Down syndrome patients, singletons (DSW); with a mean age of 38.5 years, i.e without brothers/sisters, and characterized to be older than DS.

ii) 38 non-affected controls represented by the siblings of 38 DS patients, with a mean age of 30.0 years (range:11-65);

iii) 45 subjects represented by the mothers (DSM) of the DS and DSS, with a mean age of 56.9 years (range:12-76).

These subjects are distributed this way: 35 complete families in which is present the triad DS,DSS and DSM; 3 families where only the DS subject and his/her brother/sister have been recruited; 37 DS Singletons (DSW), i.e subjects without brothers/sisters. In this group are present 27 DS without siblings and mothers, and 10 DS without siblings but their mother have been recruited.

Status Group	Total Down Syndrome Patients (DS) N=75		Non -affected Siblings (DSS)	Mothers (DSM)
	DS N=38	DS "Singletons"(DSW) N=37	N=38	N=45
Males (N, %)	21	18	11	-
Females (N,%)	17	19	27	45
Age both sex: *mean* ±SD, years (Age Range)	27.2 ± 11.1 (10-58)	38.5± 14.5 (13-66)	30.9 ± 10.9 (12-52)	57.1 ± 9.6 (41-83)
P value[a]	<0.001[1]		0.152[2] 0.015[3]	<0.001[4] <0.001[5]

a. A parametric T-test was performed to compare Ds and DSW with the other groups. *[1]=DSvsDSW; [2]=DSvsDSS; [3]=DSWvsDSS;[4]=DSvsDSM;[5]=DSWvsDSM.*

Table 2: Sample distribution and general characteristics of study participants

5. Plasma N-glycome analysis with MALDI-TOF/MS technology

As described previously in Material and Method, using MALDI-TOF mass spectrometry we detected a set of 98 plasma N-glycans that was chosen for a quantitative analysis. Their intensities were normalized to the sum of all those peaks.

Fig 14 presents an example of three representative N-glycan spectrum of a complete family, composed by DS, DSS and their DSM. We quantified 98 glycan ions (see Table 1 in the Material method for the completed list) and confirmed assigned glycan structures by MS^n sequencing.

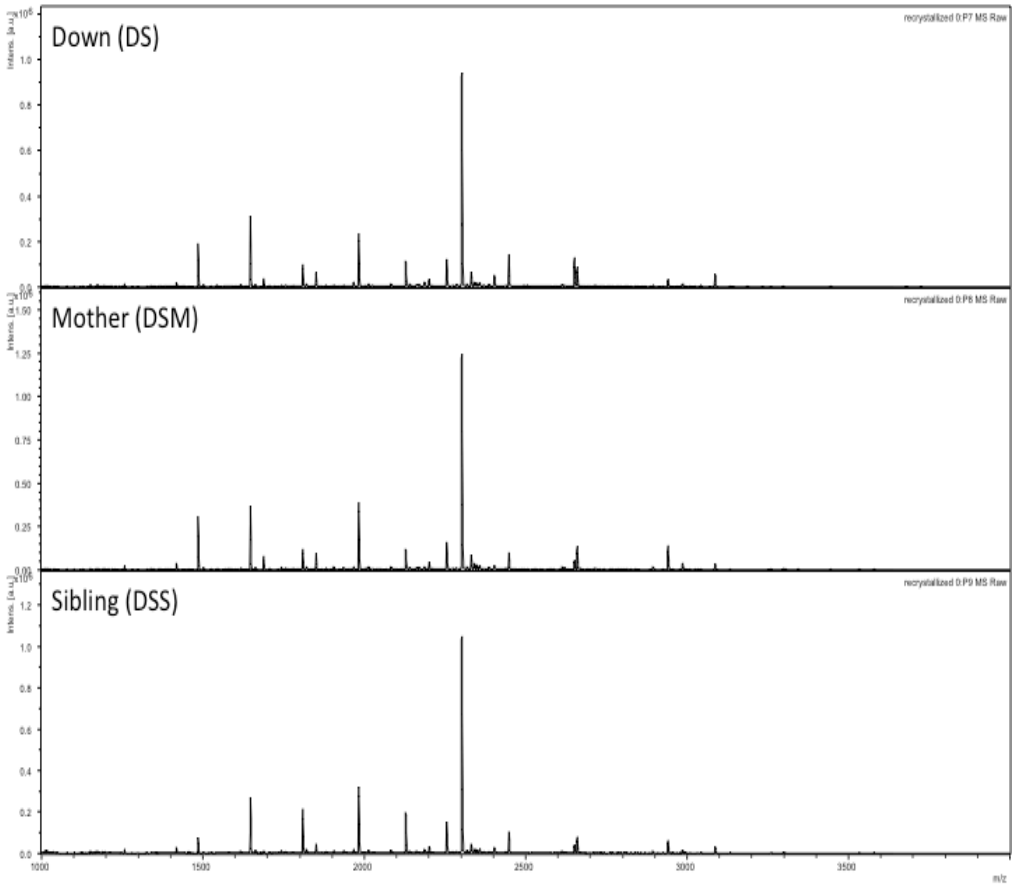


Figure 14 Representative plasma N-glycan profile of a triad family composed by DS, DSS, DSM. All plasma N-glycan (see Table 1 in Material and Method) were confirmed by MSn fragmentation. For clarity of the images, only a selection of glycans is shown. GlcNAc (f), mannose (F), Gal (E), NeuNAc (), and Fuc (Š) moieties are indicated

In order to identify a specific N-glycomic signature of the Down syndrome, we directed our analysis in the identification of the plasma N-glycans that were

able to discriminate the group of DS from those of the respective non-affected DSS, by focusing, initially, on the complete pairs DS-DSS. This comparison should be representative of the effect of disease on the plasma N-glycosylation changes.

Therefore, we extracted the N-glycan structures with potential discriminative power, performing a partial lest squares discriminant analysis (PLS-DA) (see the statistical analysis in Materials and Method for details) using as variables for the model only the 98 totals N-glycans (the variables as gender and age were not considered into the analysis).

The multivariate discriminant analysis (see the statistical analysis in Materials and Method for details) performed comparing DS and DSS is shown in the PLS-DA scatterplot (Fig 15)

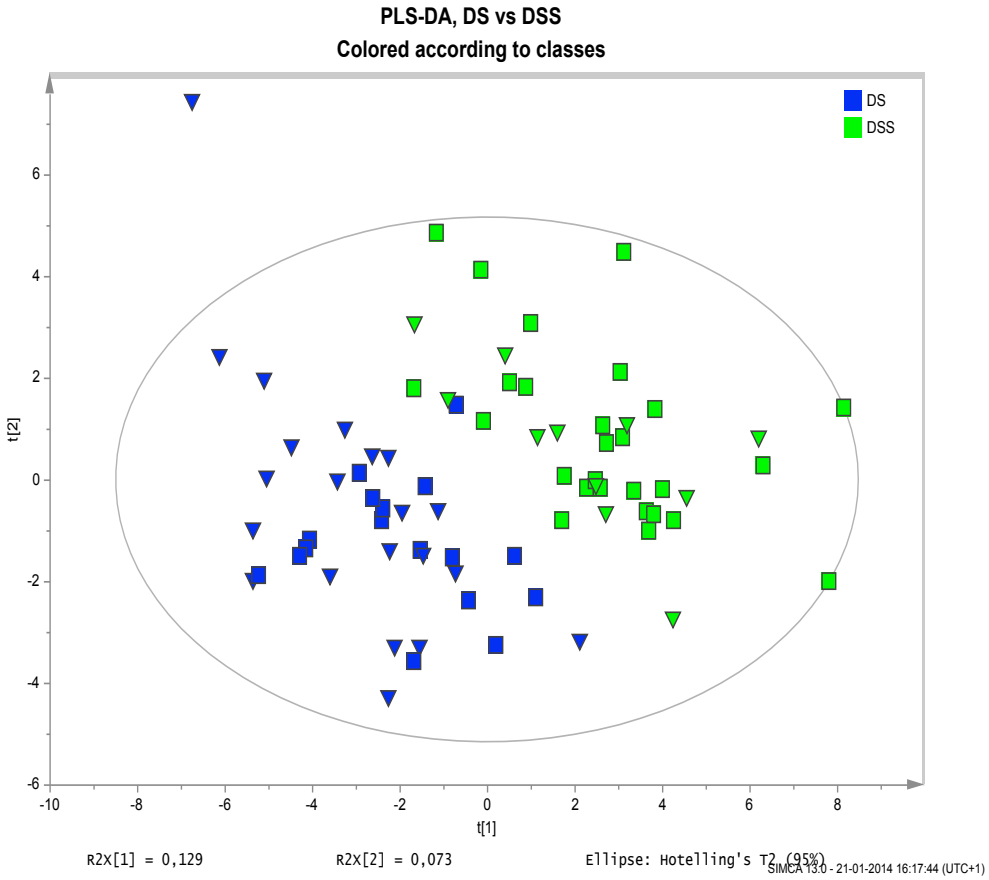


Fig15. Scatterplots of the PLS-DA models assessed comparing DS with DSS. The plot illustrates the degree of separation between groups. Each plot represent a sample where, in blue are coloured the DS and in green their DSS. The triangle indicate the males, the square the females. The horizontal scale refers to the first component (t[1]), whereas the vertical scale refers to second component (t[2]).

The analysis revealed an evident clustering of the two groups, in particular a large part of the variation within the data (72% R2Ycum) could be explained by the two-component PLS-DA model and a good prediction ability Q2(cum) of 85% was achieved. The corresponding loading plot of the PLS-DA model is shown in Fig 16.

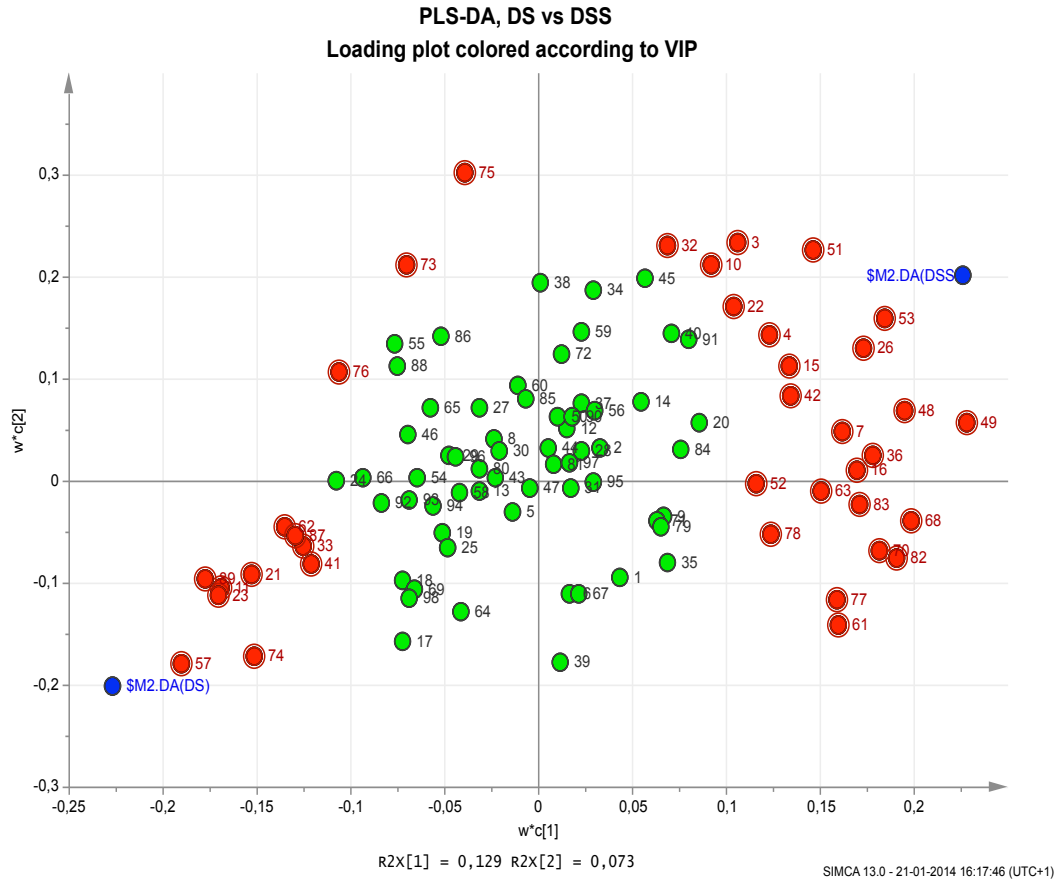


Fig.16 Loading plot of the PLS-DA models assessed comparing DS with DSS. In this figure is shown the loading plot of the PLS-DA model based on MALDI-TOF/MS data with DS and DSS as class identities, which indicates the N-glycans variables (data points) contributing most to the separation observed in the score plots. In red are highlighted all the VIP variables with a value >1. The numbers indicated on each plot are referred to the code of the peak in the Maldi spectrum.

Then, in order to investigate the effect of aging within the Down syndrome disease we compared the DS singletons (DSW) (i.e the subjects unrelated with

the DSS group) with the rest of DS subjects, belonging to two different age groups but both affected by Down's syndrome (Pvalue=<0.001).

Interestingly, the PLS-DA analysis of this aging model within the disease showed a clear clustering of the two groups, separated purely on the basis of plasma N-glycosylation changes. The overlapping of some samples is given by the fact that some subjects of the two groups had a similar age (fig 17).

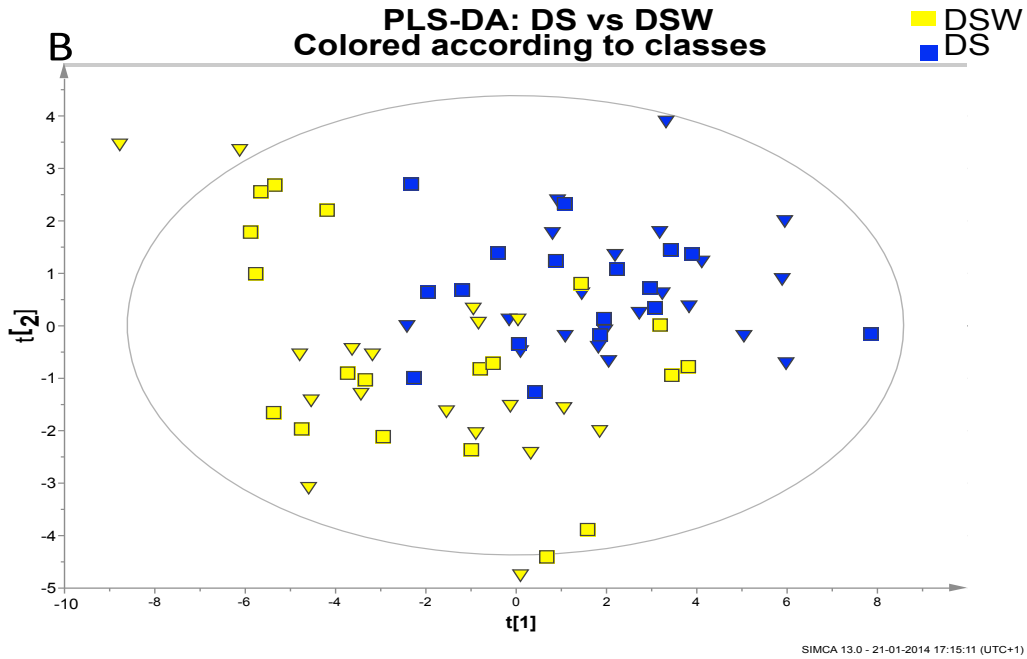


Figure 17 Scatterplots of the PLS-DA models assessed comparing DS with DSW. The plot illustrates the degree of separation between groups. Each plot represent a sample where, in blue are coloured the DS and in yellow the DSW. The triangle indicate the males, the square the females. The horizontal scale refers to the first component (t[1]), whereas the vertical scale refers to second component (t[2]).

Lastly, we compared the DS Singletons (DSW) with the group of DSS in order to validate the previous models, reducing the number of variables to those that should be associated only with the disease or/and aging. In this comparison we should have a model that takes into account the effect of disease (because we compared DSW with the non affected DSS controls) together with the aging effect (because we compared two different age-groups, since that DSW were older than DSW) (Fig.18)

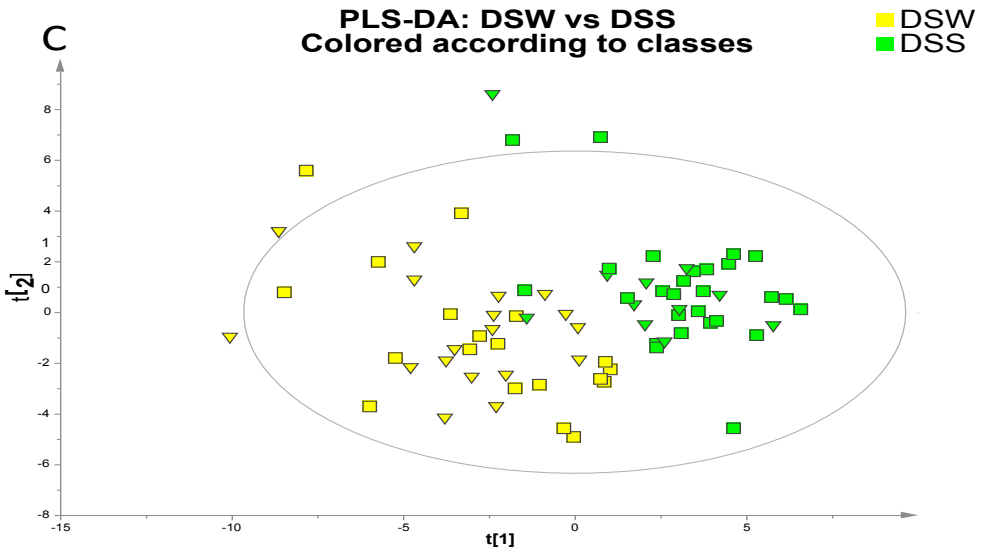


Figure 18 Scatterplots of the PLS-DA models assessed comparing DS with DSW. The plot illustrates the degree of separation between groups. Each plot represent a sample where, in blue are coloured the DS and in yellow the DSW. The triangle indicate the males, the square the females. The horizontal scale refers to the first component (t[1]), whereas the vertical scale refers to second component (t[2]).

The variable importance in the projection (VIP) ranks the variables (N-glycans identified by m/z values) according to their significance for the model, helping to reveal discriminative structures contributing mainly to the separation. Therefore, based on the VIP analysis, 67 isotopic peaks of plasma N-glycan structures with discriminative potential were extracted, considering in total the three PLS-DA models above described (Fig 19).

In order to understand which variables were exclusive to a N-glycomics signature of Down syndrome and what were associated with aging within Down syndrome, we have grouped all the 67 VIP variables in an Euler Venn diagram, and studied the intersections of the three sets, each representing a PLS-DA model (A,B and C)(Fig 19).

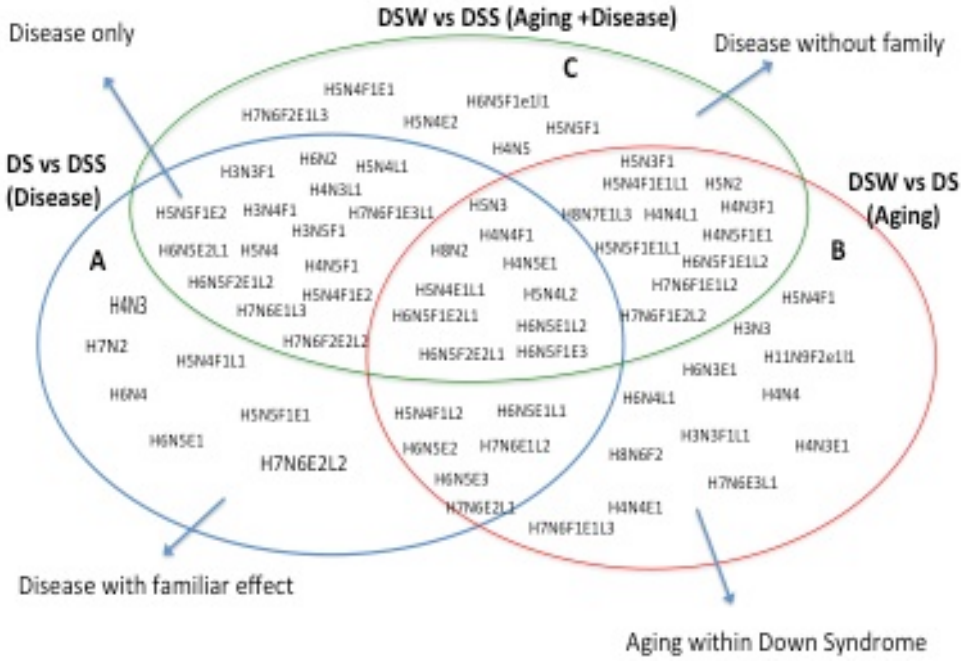


Figure 19 Description of the three PLS-DA models analyzed with an Euler Venn diagram. Each of the three circles/sets includes the VIP variables derived from the three PLS-DA models (A,B and C). The blue circle includes all the VIP discriminatory variables derived from model A, in which we compared the DS subjects with their siblings (DSS) of the same family. The red circle identifies VIP variables from the model B, in which we compared the DS singletons (DSW) with the rest of the Down subjects (DS). The green circle identifies the VIP variables of model C, which represents a validation model of the two previous models, it takes into account both disease effect and the aging effect. The arrows indicate the groups of variables that have been isolated from the analysis of the intersection of the three models/sets.

Interestingly we noticed as the N-glycan species associated with the only effect of the disease have been reduced from 37 species to 15 species (derived from the models intersection [(A∩C)-B]) and how many of them were influenced by the aging effect (see the intersection of the models: A∩B), or by familiarity (A\{B∪C}, or other factors that were different from the familiar genetic factors (epigenetic factors).

Subsequently, after having isolated the "N-glycomics signature" of the Down syndrome disease from all other confounding effects (aging, familiarity and other unknown factors) in order to assess the individual discriminant ability of each single component, we applied Wilcoxon Rank sum tests comparing the two groups DS versus DSS (Table 3).

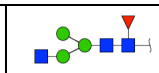
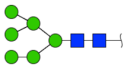
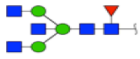
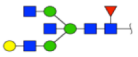
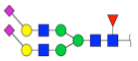
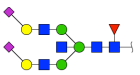
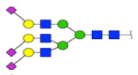
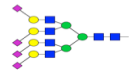
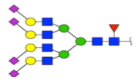
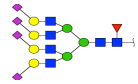
m/z [M+Na]$^+$	Assigned structure	Composition	Category	R.I (%) Median (IQR) DS	R.I (%) Median (IQR) DSS	R.I (%) Median (IQR) DSM	VIP DSvsDSS	P value	Change	Code Peak
1282.45		H3N3F1	1 Fucose, Truncated	0.15 (0.17)	0.31 (0.33)	0.12 (0.14)	1.39	**0.00068**	↓	**3**
1419.48		H6N2	Oligomannosidic	0.26 (0.07)	0.34 (0.10)	0.33 (0.11)	1.43	**0.00001**	↓	**7**
1485.53		H3N4F1	1 Fucose, Truncated	2.58 (1.40)	1.37 (1.21)	1.94 (1.61)	1.50	**<0.00001**	↑	**11**
1571.53		H4N3L1	Afucosylated α 2-3 Linkage	0.011 (0.006)	0.017 (0.019)	0.010 (0.007)	1.27	**0.00032**	↓	**15**
1663.58		H5N4	Biantennary	0.13 (0.06)	0.19 (0.08)	0.16 (0.06)	1.30	**0.00034**	↓	**22**
1688.61		H3N5F1	1 Fucose, Truncated, Bisected	0.58 (0.31)	0.33 (0.22)	0.47 (0.34)	1.55	**<0.00001**	↑	**23**
1850.67		H4N5F1	1 Fucose, Bisected	0.85 (0.38)	0.71 (0.36)	0.72 (0.41)	1.22	**0.00453**	↑	**33**
1936.67		H5N4L1	Afucosylated, α 2-3 Linkage	0.23 (0.07)	0.30 (0.09)	0.24 (0.06)	1.63	**<0.00001**	↓	**36**
2447.89		H5N4F1E2	1 Fucose, α 2-6 Linkage	4.71 (1.55)	3.44 (1.81)	3.10 (1.14)	1.96	**<0.00001**	↑	**57**
2650.97		H5N5F1E2	1 Fucose, α 2-6 Linkage, Bisected	1.81 (1.14)	1.27 (0.95)	1.36 (0.76)	1.05	**0.00032**	↑	**62**
2894.01		H6N5E1L2	Afucosylated, α 2-3 Linkage	0.35 (0.16)	0.52 (0.25)	0.46 (0.16)	1.58	**<0.00001**	↓	**68**
2940.05		H6N5E2L1	Afucosylated, α 2-6 Linkage	2.90 (2.00)	4.07 (1.94)	4.12 (1.52)	1.40	**0.00004**	↓	**70**
3532.23		H7N6E1L3	No Fucose, α 2-3 Linkage	0.10 (0.07)	0.16 (0.07)	0.11 (0.07)	1.56	**0.00004**	↓	**82**
3724.33		H7N6F1E2L2	1 Fucose, α 2-3 Linkage	0.042 (0.03)	0.036 (0.02)	0.033 (0.02)	1.20	**0.01831**	↑	**86**
3770.37		H7N6F1E3L1	1 Fucose, α 2-6 Linkage	0.0077 (0.008)	0.0045 (0.005)	0.0072 (0.007)	1.00	**0.00114**	↑	**87**

Table 3. Discriminators based on multivariate statistical analyses of the PLS/DA, assessed comparing Down Syndrome patients (DS) with their non-affected siblings (DSS), resulting Plasma carboxy-ethylated N-glycans were analyzed by MALDI-TOF/MS. Each N-glycan was identified with its m/z and with the corresponding code peak of the MALDI-spectum. The median of the percentage of the relative intensity (R.I) of each N-glycans structure was calculated for DS and DSS group The arrows are indicating a decrease or increase of the relative intensity of the DS respect non-affected DSS. The p-values were obtained by a Wilcoxon's signed rank test after Benjamin-Hoochberg correction (the significant p-values (<0.05) are highlighted in bold), in order to validate the predictive value of the singles variables. Sialylation categories assigned as follows: for N-glycans where only α 2-3 or α 2-6 occur then categorization is straighforward. When α 2-3≥ α 2-6 then the category is assigned as α 2-3.

All compounds that were considered as potential discriminators have a VIP value of 1 or higher for at least two consecutive isotopic peaks. In addition, the composition of these structures has been elucidated according the literature or MS fragmentation, and each structure was categorized according to its chemicals features. In this table, for the qualitative categorization of N-glycans carrying sialic acids with both α2,6 and α2,3 glycosidic linkage in their terminals galactoses , we considered as α2,3 all those N-glycans having a number of α2,3-linked N-acetylneuraminic acids $\geq$ α 2-6.

Interesting, two of the top three discriminators based on the MS data were found to be decreased in Down Syndrome patients: the H5N4L1 (m/z 1936.67[M+Na]+; (VIP value=1.63, Pvalue=<0.00001) as well as the N-glycan H5N4E1L1 (m/z 2255.79 [M+Na]+; (VIP value=1.86, Pvalue=<0.00001). These peaks identify two bigalactosylated and afucosylated biantennary N-glycans (G2), respectively, the first is α2-3 mono-sialylated, the second is α2-3 and α 2-6 sialylated. Instead, the H5N4F1E2 N-glycan (m/z 2255.79 [M+Na]+) with the highest VIP value (VIP value=1.96, Pvalue=<0.00001) was found to be increased in the DS subjects. This N-glycan is bigalactosylated, core-α-1,6 fucosylated biantennary, and mono α 2-6 syalylated (G2F).

Moreover, we observed that in the list of potential discriminators were present 5 tri-and-tetragalactosylated N-glycan species.

Furthermore, we observed mainly in these 15 potential discriminators species, that when was present one or more fucoses in their structure a significant increasing in DS, conversely, the corresponding species without fucose were decreased in DS respect DSS.

In DS, most of these N-glycan species, showed similar values to those of mothers or even higher.

In general, this N-glycomic pattern of disease revealed that fucosylated glycans showed a tendency toward increased levels in DS respect non-affected age-matched controls (DSS).

Interestingly, we found that 3 structures of this specific pattern, are bisecting GlcNAc features. In particular: H3N5F1 (m/z 1688.61); H4N5F1 (m/z

1850.67); H4N5E1 (m/z 2023.73); H5N5F1E2 (m/z 2650.97) increased significantly in DS respect DSS.

On the basis of this "N-glycomic signature", we focused on the general plasma N-glycosylation changes that occur in the disease, and we created a set of N-glycosylation categories, including in analysis all the peaks of total plasma N-glycome and all subjects affected by Down syndrome (DS+DSW, n=75). Moreover, in order to investigate if a premature aging in DS respect to DSS is revealed, we included in the analysis also the group of the mothers (DSM), representing a control group for age (Table 4)

N-glycosylation categories included the following glycosylation features: level of fucosylation, distinguishing in afucosylated, monofucosylated, and structures with more of >1 fucose; level of bisecting; proportion of oligomannosidic, truncated and hybrid type glycans; level of sialylation, taking into account either alfa 2-3 and alfa 2-6 linkage sialylation. For each feature, we summed the relative ion intensities for all glycan peaks presenting that feature.

Truncated structures included all complex N-glycans mono or biantennary without galactose (G0) or with one galactose (G1) without a terminal sialic acid (NeuNac), that may carry also a core fucose and bisecting GlcNac. The level of total alfa 2-3 and total alfa 2-6 linkage sialylation was calculated weighting the number of alfa 2-3 or/and alfa 2-6 linked sialic acids on the total number of galactoses of each sialylated N-glycan.

The quantitative analysis of the fucosylation status of the total DS subjects revealed a general increasing of fucosylated structures, both in the monofucosylated structures than in those with more of one fucose. Instead, the afucosylated species decreased in DS respect the non affected subjects. This phenomena could be indicate that the fucosylation changes are a peculiar aspect of Down Syndrome disease, independently by the aging and gender effect (see Table 3 and Fig. 20).

Subjects	DS (n=75)	DSS (n=38)	DSM (n=45)	Group comparison	
Age	*Mean Age, ys* (SD)	*Mean Age, ys* (SD)	*Mean Age, ys* (SD)	DS vs DSS P-value	DS vs DSM P-value
	32.7 (14.1)	30.0 (12.3)	56.9 (9.5)	0.728872	**<0.000001**
N-glycosylation category	R.I (%) *Median* (IQR)	R.I (%) *Median* (IQR)	R.I (%) *Median* (IQR)	P-value	P-value
Monofucosylated	28.42 (7.45)	24.74 (5.58)	22.41 (4.90)	**0.000179**	**<0.000001**
Fucose >1	0.150 (0.10)	0.104 (0.08)	0.103 (0.06)	**0.000486**	**0.000002**
Afucosylated	71.42 (7.38)	75.16 (5.59)	77.51 (4.81)	**0.000138**	**<0.000001**
Bisection (H≤N)	5.55 (2.18)	4.86 (2.04)	5.08 (1.72)	**0.003721**	0.073240
Oligomannosidic	1.04 (0.29)	1.26 (0.37)	1.22 (0.38)	**0.000006**	**0.000044**
Truncated*	10.01 (4.32)	7.49 (3.36)	7.97 (4.41)	**0.000248**	**0.004389**
Hybrid type	0.401 (0.14)	0.457 (0.15)	0.406 (0.11)	**0.008819**	0.810819
α 2-3 linkage (L)	8.73 (1.43)	10.46 (1.74)	7.73 (1.31)	**<0.000001**	**0.008727**
α 2-6 linkage (E)	76.81 (6.11)	77.11 (3.43)	81.14 (5.35)	0.883286	**0.016799**

Subjects	*Male* DS (n=39)	*Male* DSS (n=13)	Group comparison
Age	*Mean Age, ys* (SD)	*Mean Age, ys* (SD)	DS vs DSS P-value
	33.4 (12.02)	28.4 (9.6)	0.234289
N-glycosylation category	R.I (%) *Median* (IQR)	R.I (%) *Median* (IQR)	P-value
Monofucosylated	28.98 (6.59)	25.37 (5.48)	**0.007512**
Fucose >1	0.165 (0.09)	0.118 (0.08)	**0.004785**
Afucosylated	70.87 (6.56)	74.52 (5.48)	**0.007052**
Bisection (H≤N)	5.55 (1.94)	4.72 (1.43)	**0.005820**
Oligomannosidic	1.04 (0.21)	1.21 (0.38)	**0.005820**
Truncated*	10.33 (5.20)	8.00 (3.14)	**0.025783**
Hybrid type	0.415 (0.19)	0.408 (0.21)	0.403870
α 2-3 linkage (L)	7.24 (1.16)	8.48 (1.36)	**0.000550**
α 2-6 linkage (E)	78.09 (5.36)	78.56 (3.31)	0.791661

Subjects	*Female* DS (n=36)	*Female* DSS (n=27)	*Female* DSM (n=45)	Group comparison	
Age	*Mean Age, ys* (SD)	*Mean Age, ys* (SD)	*Mean Age, ys* (SD)	DS vs DSS P-value	DS vs DSM P-value
	30.5 (16.1)	33.3(13.2)	56.9 (9.5*)*	0.617364	**<0.000001**
N-glycosylation category	R.I (%) *Median* (IQR)	R.I (%) *Median* (IQR)	R.I (%) *Median* (IQR)	DS vs DSS P-value	DS vs DSM P-value
Monofucosylated	28.12 (7.21)	24.44 (5.46)	22.41 (4.90)	**0.025344**	**0.000064**
Fucose >1	0.138 (0.10)	0.099 (0.09)	0.103 (0.06)	0.104377	**0.002740**
Afucosylated	71.76 (7.24)	75.48 (5.46)	77.51 (4.81)	**0.023581**	**0.000062**
Bisection (H≤N)	5.72 (2.42)	5.02 (2.21)	5.08 (1.72)	0.062728	0.188855
Oligomannosidic	0.93 (0.40)	1.34 (0.37)	1.22 (0.38)	**0.000602**	**0.000570**
Truncated*	9.79 (4.30)	6.89 (3.39)	7.97 (4.41)	**0.013958**	0.124906
Hybrid type	0.386 (0.10)	0.469 (0.18)	0.406 (0.11)	**0.000067**	0.290652
α 2-3 linkage (L)	7.32 (1.10)	8.32 (1.77)	7.73 (1.31)	**0.000044**	**0.041363**
α 2-6 linkage (E)	80.29 (6.07)	79.1 (3.82)	81.14 (5.35)	0.728424	0.134199

Table 4: Multiple comparison between Down patients (DS) with age-matched non-affected siblings (DSS) and Mothers (DSM) for different category of N-glycans, stratified for sex. For each couple-comparison a non parametric test (Mann-Whitney test) was assessed with the Benjamini–Hochberg correction. If the FDR-corrected p-value (q-value) is <=0.05(q-values not reported in the table), the p-values are considered significative and highlighted in bold. *In the truncated category we have included the asialylated mono or biantennary N-glycans with 0 or 1 galactose that may carry core fucose and bisecting GlcNAc

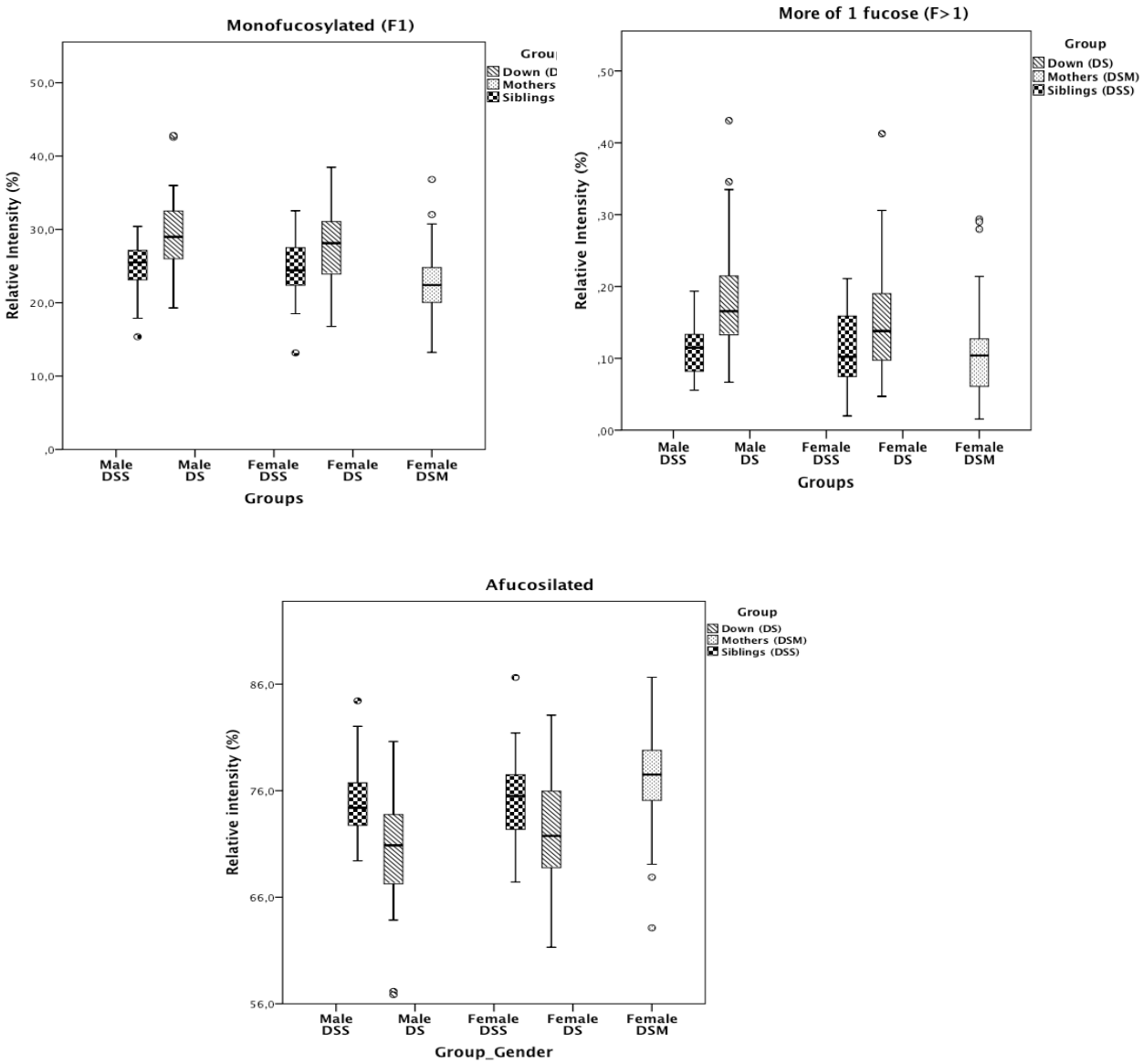


Fig. 20 Comparison of fucosylation levels in the plasma N-glycome between total DS, DSS, and DSM. On the top of the figure are shown the box plots of the relative intensity (%) of the N-glycan structures with more one fucose, mono-fucosylated and without fucose. At the right the loading plot of the PLS-DA model assessed comparing DS vs DSS, where are shown in green the total afucosylated N-glycans, in blue the monofucosyltated, and in red and yellow the structures with more of one fucose**.**

Then, we took into account the relative abundance of total bisecting structures in DS disease, and we found a significant increase of these species. In particular, we found that their concentration is significantly higher compared to the age- and sex matched DSS group (P value=0.003721), but not in the comparison between DS and DSM (P value=0.073240) (Table 4).

Moreover, we investigated if these changes were associated with age. We found a strong association between the relative intensity (%) of plasma bisection and age (Pvalue=<0.0001), but only in Down subjects (Fig.21). Then, stratifying the DS subjects in two age groups (<30 years and >30 years), we evidentiated that increasing of bisection was significant only in the subjects with more than of 30 years (Pvalue=<0.001) both in males and females (Fig.22)

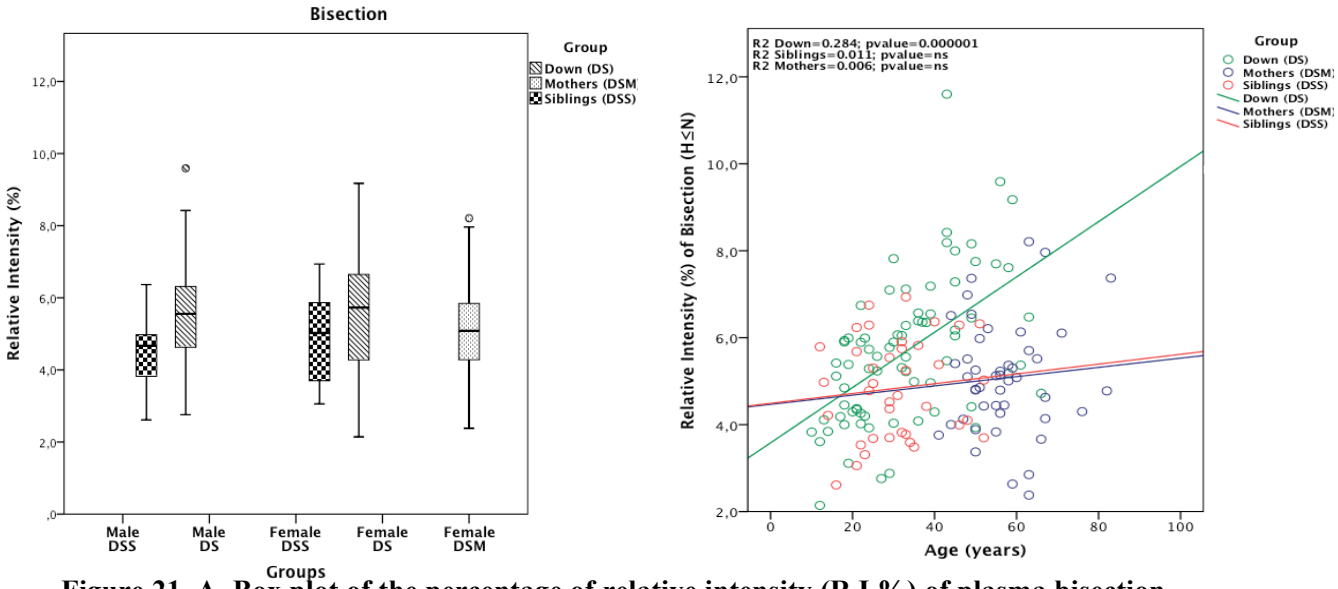


Figure 21. A. Box plot of the percentage of relative intensity (R.I %) of plasma bisection in the DS, DSS, DSM group, stratified for gender. B. Scatterplot of R.I (%) in function of age.

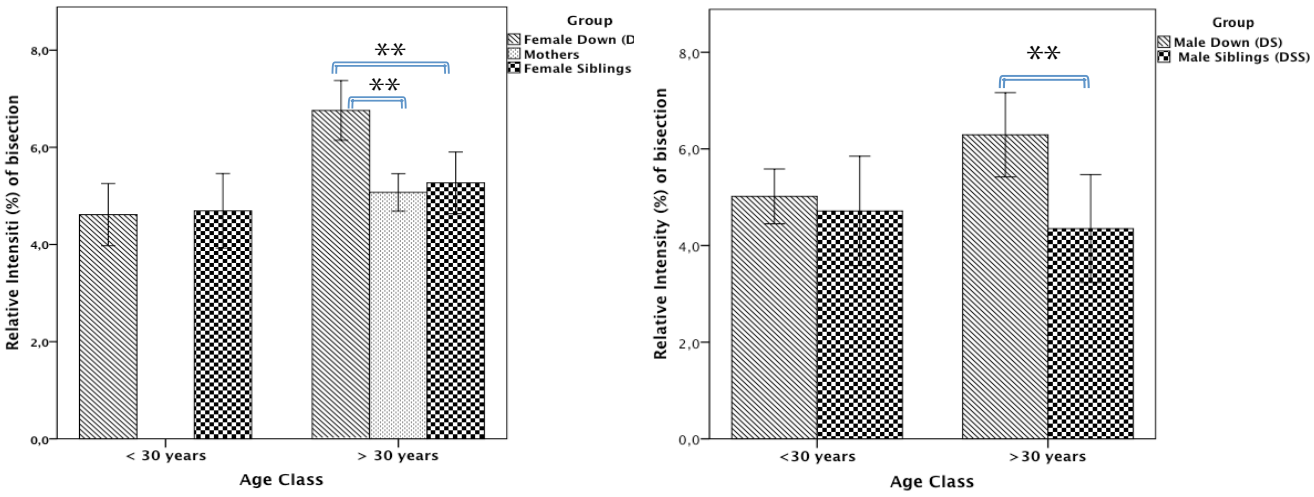


Figure 22 Bar plot of the percentage of relative intensity of bisection (%) in subjects stratified for age groups (<30 and >30 years old) . In A are indicated females and in B males. ** indicate a P-value <0.001.

In addition, we took into account the truncated glycan species, in order to study all those N-glycans, mono or biantennary with 0 or with 1 galactose without a terminal sialic acid (NeuNac), that may carry also a core fucose and bisecting GlcNac. Briefly, we focused on the category, so-called, G0 and G1(asialylated). As shown in Table 3 and Fig 23, truncated species in DS subjects showed a significant increase, compared to both non affected age- and sex matched controls (DSS) (Pvalue=0.000248) and mothers group (DSM) (Pvalue= 0.004389). Moreover, in DS group, both gender, showed an increase in truncated plasma N-glycans compared to age-matched controls DSS (Females Pvalue=0.013958; Males P value=0.025783). Interestingly, truncated species were also different between DSM and DSS subjects, the decrease took on an extreme level in DS subjects while DSM mother showed an intermediate value between DSS and DS individuals.

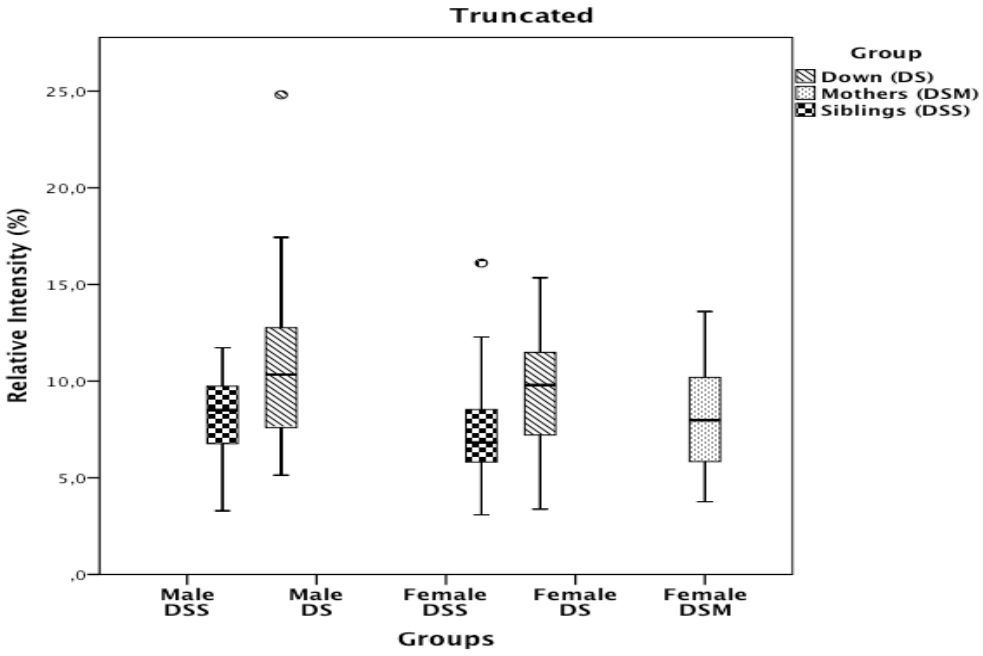


Figure 23 A. Box plot of the percentage of relative intensity (R.I %) of plasma truncated N-glycans. in the DS, DSS, DSM group, stratified for gender.

In addition, as mentioned in materials and methods, we took into account not only the total levels of sialylation of human N-glicome. but also the specific alfa 2-3 and alfa 2-6 glycosidic linkage of the sialic acids attached to a terminal galactose. Interestingly, we found a significant decrease of alfa 2-3 sialylation levels in DS compared to the non-affected controls (DSS), both in males and in females. However, we observed a trend towards a decrease in alfa 2-3 sialylation levels from DSS to DSM, with DS subjects, showing intermediate values, similar to mother groups (table 3 and fig 24).

No significant difference was observed in the levels of alpha 2-6 sialylation.

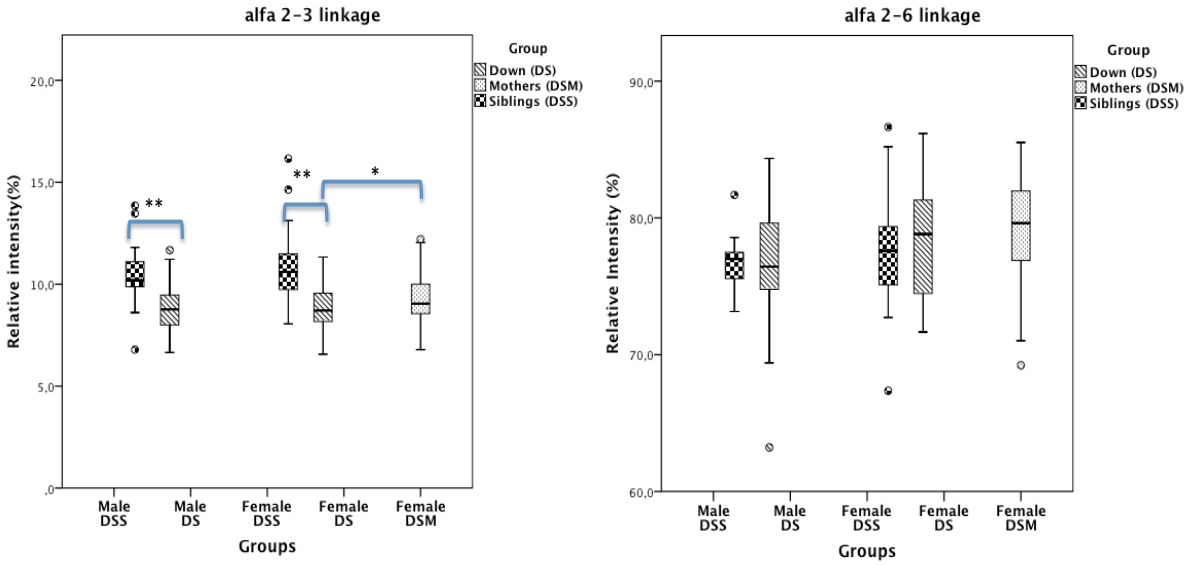


Figure 24 Box plot of the percentage of relative intensity (R.I %) of plasma alfa 2-3 and alfa 2-6 linkage sialylation levels. in the DS, DSS, DSM group, stratified for gender.

6. Plasma N-glycome analysis with DSA-FACE technology

As described previously in Material and Method, using DSA-FACE technic we detected a set of 10 plasma N-glycans that was chosen for a quantitative analysis. Their intensities were normalized to the sum of all those peaks.

Similarly to the previous analysis performed on MALDI data, we extracted N-glycan structures with potential discriminative power, performing a partial lest

squares discriminant analysis (PLS-DA) (see the statistical analysis in Materials and Method for details) using as variables for the model the 10 totals N-glycans (the variables as gender and age were not considered into the analysis).

The multivariate discriminant analysis performed comparing DS and DSS is shown in the PLS-DA scatterplot (Fig 25).

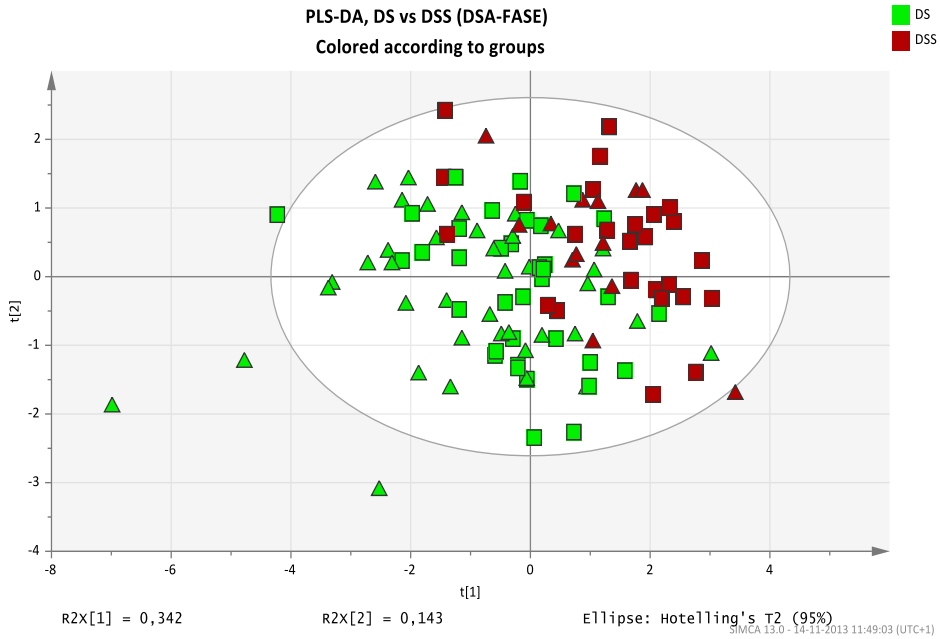
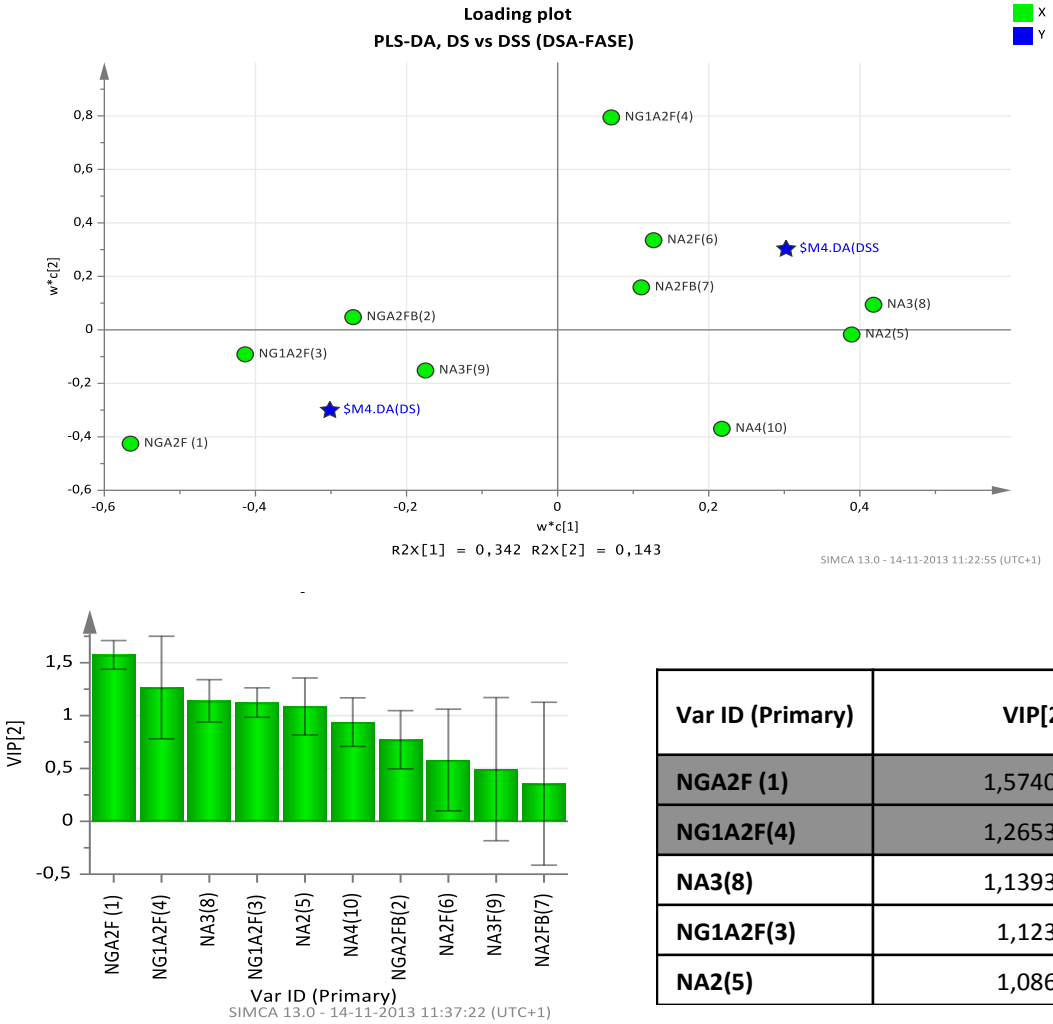


Figure 25 Scatterplots of the PLS-DA models assessed comparing DS with DSS. The plot illustrates the degree of separation between groups. Each plot represent a sample where, in green are coloured the DS and in red their DSS. The triangle indicate the males, the square the females. The horizontal scale refers to the first component (t[1]), whereas the vertical scale refers to second component (t[2]).

The analysis revealed an evident clustering of the two groups, also applying this different technological approach in particular a large part of the variation within the data (48% R2Ycum) could be explained by the two-component PLS-DA model and a good prediction ability was achieved. The corresponding loading plot of the PLS-DA model and the variables important in the projection (VIP) with a value >1 are shown in fig 26.

Interestingly, we found Peak 1 with the highest VIP value (1.57) , but also Peak 3 (1.12) and Peak 4 (1.26). These last two peaks have the same chemical structure, but have a different stereoisomerism about the terminal galactose positioned in the branches, and it is interesting as they are placed in a different position in the loading plot: peak 3 much more towards the DS group and the peak 4 to the DSS group.



Var ID (Primary)	VIP[2]
NGA2F (1)	1,57402
NG1A2F(4)	1,26534
NA3(8)	1,13931
NG1A2F(3)	1,1233
NA2(5)	1,0861

Figure 26 Loading plot of the PLS-DA models assessed comparing DS with DSS. In this figure is shown the loading plot of the PLS-DA model based on DSA-FACE data with DS and DSS as class identities, which indicates the N-glycans variables (data points) contributing most to the separation observed in the score plots. In red are highlighted all the VIP variables with a value >1. The numbers indicated on each plot are referred to the code of the peak in the Maldi spectrum.

Based on this first PLS-DA analysis, and in order to deep the discriminatory value of GlycoAge test (see introduction for details), which is a variable derived from the log ratio between Peak 1 and Peak 6, we performed a second PLS-DA analysis, including as additional variables, the glycoAge test, and also the ratio between Peak3/Peak 4 (Fig.27).

This second analysis confirmed the clear clustering of the two groups (DSvsDSS) but observing the values of discriminatory VIP variables, we found that GlycoAge test had not only the highest VIP value, but it is also

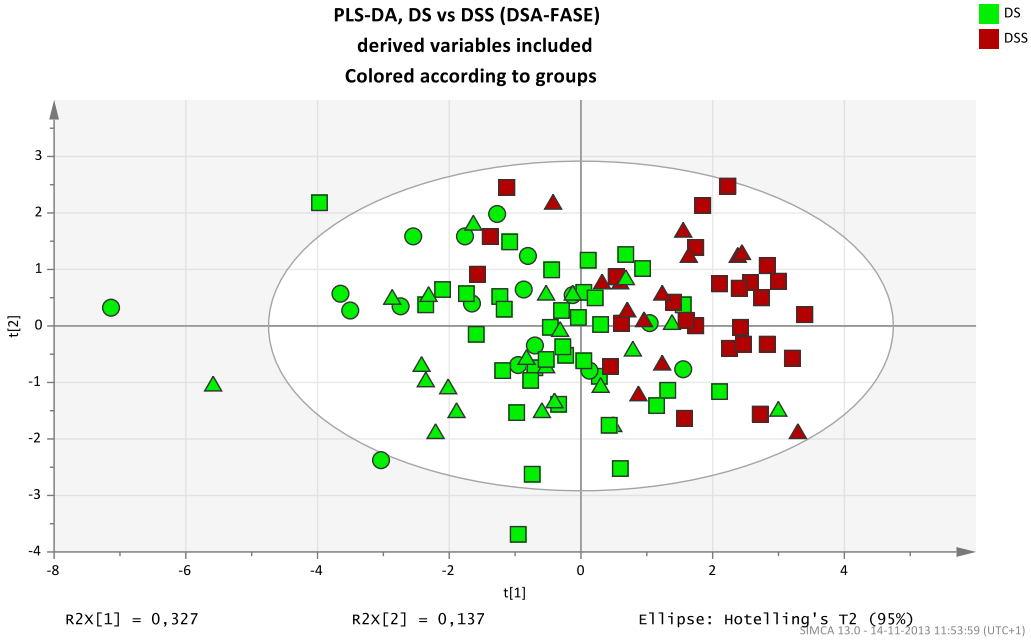


Figure 27 Scatterplots of the PLS-DA models assessed comparing DS with DSS, with derived variables included. The plot illustrates the degree of separation between groups. Each plot represent a sample where, in green are coloured the DS and in red their DSS. The triangle indicate the males, the square the females. The horizontal scale refers to the first component (t[1]), whereas the vertical scale refers to second component (t[2]).

higher compared to Peak 1 individually considered. Moreover, also the ratio between peak 3/4 had a high discriminatory power, and its VIP value is higher than peak 3.(table 5).

Looking at the N-glycomics "signature" achieved by using this different approach, we noticed how fucosylated species (such as P1, P3) increase in DS compared to non-affected controls (both DSM and DSS), and conversely, the

species without fucose (such as P5 and P8) decrease. In addition, the Peak 1 is confirmed as a strong discriminator of disease also from the analysis in MALDI-TOF. For the other peaks is difficult to confirm the same glycoform in either protocols, because the desialylation process before the analysis does not allow to take into account the sialylated species. Interestingly, even try-galactosylate species, similar to the analysis in MALDI-TOF, showed significant differences in the disease. This could mean that the N-glycosylation changes is not only restricted at the biantennary glycans, more frequently present on the plasmatic igG

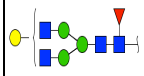
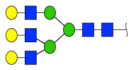
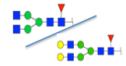
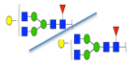
Peak/Variable	Assigned structure	Composition	R.I (%) Median (IQR) DS	R.I (%) Median (IQR) DSS	R.I (%) Median (IQR) DSM	VIP DSvsDSS	P value	Change
1		H3N4F1	9.11 (2.9)	5.58 (2.6)	7.70 (4.2)	1.421	**<0.000001**	↑
3		H4N4F1	8.13 (2.4)	6.46 (1.4)	6.58 (2.6)	1.075	**0.000060**	↑
5		H5N4	39.1 (5.5)	42.4 (4.3)	41.73 (6.3)	1.028	**0.000369**	↓
8		H6N5	6.11 (2.33)	7.82 (2.78)	8.16 (2.15)	1.067	**0.000103**	↓
GlycoAge test		H3N4F1 / H5N4F1	-0.34 (0.17)	-0.58 (0.23)	-0.35 (0.30)	1.468	**<0.000001**	↑
Peak3/Peak4		H4N4 / H4N4	0.30 (0.12)	0.21 (0.12)	0.20 (0.11)	1.355	**0.000003**	↑

Table 5. Discriminators based on multivariate statistical analyses of the PLS/DA, assessed comparing Down syndrome patients (DS) with their non-affected siblings (DSS). Plasma N-glycans were analyzed by DSA-FACE technology. The median of the percentage of the relative aboundance of 10 total peaks, and two derivated variables of these peaks (GlycoAge test and the ratio between peak3 and peak4) was calculated for DS and DSS group. The arrows are indicating a decrease or increase of the relative intensity of the DS respect non-affected DSS. The p-values were obtained by a Wilcoxon's signed rank test after Benjamin-Hoochberg correction (the significant p-values are highlighted in bold), in order to validate the predictive value of the singles variables.

We found that the GlycoAge, evaluated in DS subjects, was similar to that of their mothers, chronologically older, and was significantly higher compared to age-matched subjects. This confirmed that the GlycoAge test is able to indicate a premature aging in DS. In addition, we found that the ratio between peak 3

and peak 4, was able to clearly distinguish the DS from the total not affected controls (DSS and DSM), pointing as a marker associated with the disease (Fig.27).

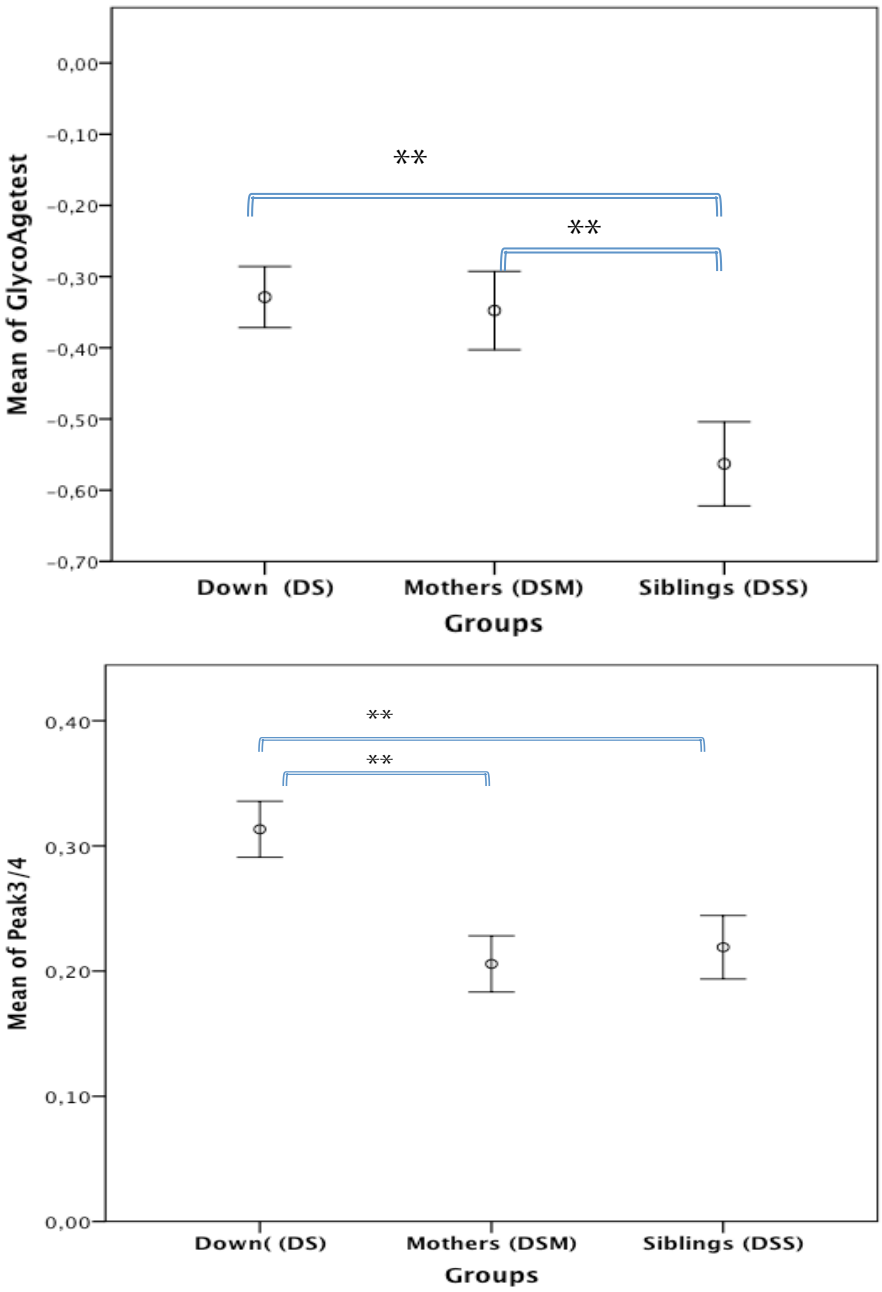


Figure 27 Values of GlycoAge test and the ratio between Peak3/4 from the three groups of the study (DS-DSM-DSS). The vertical axis represents the 95% confidence interval for mean of the percentage of the toal N-glycan peak height.

Discussion

The glycosylation of proteins is a very complicate and diversified phenomenon, due to the intrinsic complexity of the ramified glycan structures. In addition, the glycome results to be in a dynamic equilibrium: within an individual the glycans pattern remains highly reproducible in a given physiological state, but can steadily change in response to altered conditions, for example during aging or disease (Ruhaak L, et al, 2010). Therefore, the study of protein glycosylation might help identifying potential biomarkers of diseases, and contribute to understand their biology/pathogenesis (Pareck et al., 2006). Recently, the research on human aging, using the modern tools of glycomics, has led to the discovery of glycan biomarkers for both longevity and aging that may reflect health status and predict the healthy aging (Vanhooren V et al., 2007, Ruhaak L. et al., 2010; Krištić J, et al., 2013).

It has been hypothesised that these biomarkers could be indicative of the biological age of individuals, which do not always correspond to the chronological age. It is not yet clear whether this is true or not, therefore we addressed the question by studying a model of premature human aging such as the Down Syndrome. Down Syndrome subjects (DS) indeed undergo premature skin wrinkling and hair greying, visual and hearing impairments, thyroid dysfunction, diabetes, obesity, immune senescence and Alzheimer-like dementia (Esbensen, 2010). We thus reasoned that, if the age-related changes of the glycome should be present also in relatively young DS, this could be a demonstration that these markers are indicative of the biological age of the individuals; on the contrary if no difference is found with non trisomic age-matched subjects, this could indicate that these markers are related to the pure chronological age. Finally, it is also possible that Down syndrome per se is characterised by a glycomic signature with respect to non trisomic subjects. Therefore, the research project was aimed at studying DS subjects from a glycomic point of view by using the most advanced techniques available at present.

There are several approaches to glycosylation analysis, all of which have their strengths and limitations, including mass spectrometry (MS), high performance liquid chromatography (HPLC), capillary electrophoresis (CE) and lectin binding (Arnold, J.N et al.,2004). MS and HPLC analysis of sugars released are very sensitive methods of analysis of glycans , and can give all the structural information. Lectin can be easily incorporated in tests based on ELISA, but these reagents generally have a low specificity and affinity for glycan epitopes and can not be used to provide a complete structural analysis. The identification and validation of new biomarkers requires detailed analysis of glycans, quantitative and sensitive. Currently these requirements are best met by a combination of HPLC and/or MS (Arnold, J.N et al.,2008). Recently, the Gent University developed a quantitative N-glycan analysis system based on DSA-FACE, that is a robust, reproducible and high-throughput technology platform. However, this technique has a limitation as it works on desialylated N-glycans, thus misses information about the sialic acid distribution (Vanhooren et al, 2008).

To overcome this limitation, a new protocol based on MALDI-TOF mass spectrometry was developed by the LUMC Glycomics and Glycoproteomic Laboratory (Reiding K, et al, 2013, submitted), that is sensitive and quantitative and it can be used to analyse 112 N-glycans identified in human plasma, and it is able to analyse the specific alfa 2,3 and alfa 2,6 glycosidic linkage of sialic acids. In this study we applied both methods to our samples.

We focused on N-glycosylation changes of plasma proteins that occur in Down syndrome subjects, as mentioned, and in particular we studied a familiar model of disease, composed by: i) DS subjects (DS); ii) their age matched siblings (DSS). At the same time we investigated if N-glycans were able to predict a premature aging on DS, comparing DS with their mothers (DSM) i.e subjects of different chronological age and with a similar genetic background. This familiar model allowed us to minimize the effects of genetic individual variability and environment.

The analysis in mass spectrometry revealed that Down syndrome impacts significantly on the N-glycosylation changes of plasma proteins, generating a characteristic "signature" of disease. In particular, we identified by comparing the DS with their age- and sex matched siblings, 37 glycan species that changed significantly in the two groups.
The analysis was further deepened considering which of these N-glycan species were influenced by the effect of age. This was achieved by comparing DS subjects of different ages. The analysis led to the identification that 16 N-glycan species changed during aging of subjects affected by disease. Finally, the comparison between DS unrelated with non-affected controls allowed us to further refine the signature of the syndrome by excluding 7 species (that could indicate a individual/familial contribution) from the original pool of 37 glycans associated with DS. From these analyses we have, therefore, identified the final N-glicomics signature of disease consisting of 15 N-glycans species.

Within this N-glicomic signature, we identified, H3N4F1, an agalactosyl (G0) N-glycan, corresponding to the "Peak1" of DSA-FACE fingerprinting, which we found, in our previous studies, to be increased with age. Interestingly, the relative abundance of this N-glycan resulted not only increased compared to DSS, but also to DSM, who are chronologically older than DS. Most of this N-glycan species had similar values to those of DSM or even higher, in agreement with the hypothesis that Down syndrome, also from the point of view of glycomics, shows the characteristics of a premature aging.
Among them appears also H5N4F1E2, a core-fucosylated biantennary (G2) and alpha 2-6 sialylated N-glican, which should be included in the G2 N-glycans pool, grouped by "Peak 6," through analysis in the DSA-FACE. Interestingly, we found in DS a significant increase of this N-glycan compared to DSS and DSM. Generally, our previous studies demonstrated a decrease of this glycan with age.
Looking at the N-glycan signature we noticed that in DS a general increase of fucosylation occurs, since many of the glycan species were fucosylated, while

the non fucosylated decreased; moreover, all those afucosylated species were also galactosylated.

We therefore wanted to investigate the mechanisms that underlie the N-glycosylation changes, and we created some N-glycans categories, which take into account all detectable plasma N-glycans, grouped in order to share the same chemical characteristic.

Therefore, we took into account the fucosylation process, discriminating between monofucosylated, polyfucosylated and afucosylated. The species with more than one fucose could give an indication of the fucosylation process of antennae, as for sure a fucose is localized in the core and the other in the antennae.

This analysis confirmed our hypothesis that all fucosylated species are more expressed in DS, and fucosylation affects both biantennary and tri and tetra antennary species.

The presence or absence of fucose within the glycan chains is strongly related to autoimmune diseases and cancer. Moreover, when linked to the NAcGlc and a sialic acid alfa 2-3 linked fucose forms a specific chain, defined Sialyl Lewis x(sLex). sLex is present on numerous proteins, in cases of acute and chronic inflammation (Van Dijk W. et al. Glycoconj. J. 1995).

The expression of this chain counteracts the progression of pathologies; it has been observed in fact, that treatments with soluble sLex may counteract the inflammation-induced tissue damage (Mulligan MS. Et al., Nature 1993).

Moreover, in vitro studies have shown that during short-term acute inflammation, alfa1-acid glycoprotein contains increased biantennary structures, but this shifts to an increase in tri- and tetra-antennary structures with long term chronic inflammation (De Graaf, T et al, 1993; Fassbender, K et al, 1991).

Increased tri and tetra-antennary glycans with SLex attached to haptoglobin have been reported in chronic inflammatory conditions such as alcoholic liver disease (Grevel, P. et al.; 1996), rheumatoid arthritis (RA) (Thompson, S.,et

al., 1993), ovarian cancer, stomach cancer and Crohn's disease (Goodarzi, M.T et al., 1998).
The increase in tri and tetra-antennary glycans and Slex in the serum may reflect a systemic side effect of the inflammatory cytokines on the hepatocytes of the liver (Arnolod J.N et al, 2008).
Therefore, the increase in fucosylation that we have found in individuals with Down syndrome might be related to a higher abundance of these sLex structures, which would feed a state characteristic of chronic inflammation, involving multiple mechanisms, both hepatic and immunological.

Unlike the biantennary truncated species, (like GO and the asialylated G1), we found an increase of these species in DS compared to age matched controls, and they reached similar values (mainly in females) to their mothers or even more elevated.
In literature, IgG-G0 are elevated in cancer patients and have been shown to increase with tumour progression. Hyperfucosylation has also been identified, and this is a result of an increase in the glycan structure F(6)A2, which forms part of IgG-G0 glycoform population. F(6)A2 in the serum is almost exclusively attached to IgG molecules, and a small proportion is present on IgA (Comunale, M.A et al, 2006, Arnolod J.N 2008). The serum increase in IgG-G0 has classically been described in relation to the chronic inflammatory condition, RA, however serum elevations of IgG-G0 are also found in other inflammatory chronic conditions such as Crohn's disease, juvenile onset chronic arthritis, systemic lupus erythematosus complicated by Sjögren's syndrome and tuberculosis (Parekh et al, 1985; Bond, A,: 1997).
IgG-G0 can also be considered to be a pro-inflammatory molecule since the G0 glycans are epitopes for lectin binding (Dong, X, et al, 1999; Malhotra, R, et al, 1995) and activate the complement cascade.

A relationship between agalactosylated G0/and less sialylated igG, with "inflammaging" i.e the low-grade pro inflammatory status that characterizes elderly (Franceschi C, et al,. 2000), has been conceptualized in a recent review

by our group (Dall'Olio F et al, 2012). The Down Syndrome could have an higher grade of "inflammaging", respect the non affected subjects, and the concentration of GO could be an efficient marker of inflammaging.

Finally, the N-glycome profiling performed with the DSA-FACE technology has shown to be sensitive enough to distinguish the DS from DSS. We have found, similarly to the analysis in MALDI TOF, that the peak 1 is able to discriminate between DS and non affected controls, reaching the highest values in DS. Also the DSA-FACE profiling, indicated that afucosylated and galactosylated peak decreased in DS, and the fucosylated species decreased, as the peak 1 and 3.
Moreover, we found that the GlycoAge, evaluated in DS subjects, was similar to that of their mothers, chronologically older, and was significantly higher compared to age-matched subjects. This confirms that the GlycoAge test is able to indicate a premature aging in DS. In addition, we found that the relationship between peak 3 and peak 4, is able to clearly distinguish the DS from the non affected controls (DSS and DSM), pointing as a marker associated with the syndrome.

Further studies will be needed to clarify the precise role of these N-glycans in the aging process of the immune system and of the organism as a whole, especially in the context of the Down syndrome that has been tackled in this study.

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