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The Effects of Knockdown of CD24 and CD44 on Proteomics and N-glycomics in Colon Cancer

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Abstract

Clusters of differentiation (CDs) are glycoproteins which are expressed on the surface of different cells in the body. They are involved in the communication between cells and the induction of intracellular signalling. Among them, CD24 is correlated with tumorigenesis and tumour progression, while CD44 is closely associated with cancer cell metastasis and chemotherapy resistance. Both CD24 and CD44 are commonly used as markers for many cancer types and their overexpression is frequently associated with a poor prognosis. Despite the critical roles of CD24 and CD44 in cancer progression, little is known about proteome and glycome alterations derived from their knockdowns. To address this point, analysis by bottom-up proteomics and N-glycomics of colorectal cancer tissue under stable knockdowns of CD24 and CD44 were performed in this work. By the proteomic approach a number of proteins associated with cancer development, including cancer cell differentiation, migration, invasion and metastasis were identified. Some of the proteins can be used as indicators for evaluating treatment against CD24 or CD44. The regulation of some proteins was not coincided with references due to the certain compensatory changes that occurred. Further work focused on differentially abundant proteins (DAPs) and differentially abundant N-glycans which may provide valuable insights into the physiological and pathophysiological roles of CD24 and CD44 in colorectal cancer (CRC). Extracellular-matrix-organization and reactome-biological-oxidations were the main pathways involved in the development of CRC after knockdowns of CD24 and CD44, respectively. In addition, the differentially regulated N-glycans with N-acetylneuraminic acid (Neu5Ac) or N-glycolylneuraminic acid (Neu5Gc), especially Neu5Gc1Neu5Ac2HexNAc4Hex6Fuc2Red-HexNAc1, have a significant abundance on CRC after knockdowns of CD24 and CD44.

After comparison of both knockdowns against a wild type to generate lists of regulated proteins and N-glycans, these resulting lists were compared as well. While there is an overlap in the results of differentially regulated proteins and N-glycans for CD24kd and CD44kd, the number of regulated proteins and N-glycans exclusive for the CD24 or CD44 knockdown condition was higher.

In summary, the results generated in this work extend the knowledge of the molecular changes associated with CD24 and CD44 in cancer by analysing the respective knockdowns in colorectal cancer samples.

Zusammenfassung

Clusters of Differentiation (CDs) sind Glykoproteine, die auf der Oberfläche verschiedener Zellen im Körper exprimiert werden. Sie sind an der Kommunikation zwischen Zellen und der Induktion von intrazellulären Signalen beteiligt. Unter ihnen wird CD24 mit der Tumorigenese und Tumorprogression in Verbindung gebracht, während CD44 eng mit der Metastasierung von Krebszellen und der Resistenz gegen Chemotherapie assoziiert ist. Sowohl CD24 als auch CD44 werden häufig als Marker für viele Krebsarten verwendet und ihre Überexpression ist oft mit einer schlechten Prognose verbunden. Trotz der kritischen Rolle von CD24 und CD44 bei der Krebsprogression ist nur wenig über die Veränderungen des Proteoms und des Glykoms bekannt, die sich aus deren Knockdowns ergeben. Um diesen Punkt zu adressieren, wurden in dieser Arbeit Analysen mittels Bottom-up-Proteomik und N-Glykomik von kolorektalem Krebsgewebe unter stabilen Knockdowns von CD24 und CD44 durchgeführt.

Durch den proteomischen Ansatz wurde eine Reihe von Proteinen identifiziert, die mit der Krebsentwicklung assoziiert sind, einschließlich der Differenzierung von Krebszellen, Migration, Invasion und Metastasierung. Einige der Proteine können als Indikatoren für die Bewertung einer Behandlung gegen CD24 oder CD44 verwendet werden. Die Regulation einiger der Proteine stimmte nicht mit den Referenzen überein, da bestimmte kompensatorische Veränderungen auftraten. Weitere Arbeiten konzentrierten sich auf differentiell abundante Proteine (DAPs) und differentiell abundante N-Glykane, die wertvolle Einblicke in die physiologischen und pathophysiologischen Rollen von CD24 und CD44 bei kolorektalem Karzinom (CRC) liefern können. Die Extrazelluläre-Matrix-Organisation und reaktive biologische Oxidationen waren die Hauptwege, die an der Entwicklung von CRC nach Knockdowns von CD24 bzw. CD44 beteiligt waren. Darüber hinaus haben die differenziell regulierten N-Glykane mit N-Acetylneuraminsäure (Neu_5Ac) oder N-Glykolylneuraminsäure (Neu_5Gc), insbesondere $Neu_5Gc_1Neu_5Ac_2HexNAc_4Hex_6Fuc_2Red\text{-}HexNAc_1$, einen signifikanten Fülle auf CRC nach Knockdowns von CD24 und CD44.

Nach dem Vergleich beider Knockdowns gegen einen Wildtyp, um Listen von regulierten Proteinen und N-Glykanen zu generieren, wurden auch diese resultierenden Listen miteinander verglichen. Während es eine Überschneidung in den Ergebnissen

der differenziell regulierten Proteine und N-Glykane für CD24kd und CD44kd gibt, war die Anzahl der regulierten Proteine und N-Glykane, die ausschließlich für eine der CD24- oder CD44-Knockdown-Bedingung gefunden wurde, höher.

Zusammenfassend erweitern die in dieser Arbeit durch die Analyse der jeweiligen Knockdowns in kolorektalen Krebsproben generierten Ergebnisse das Wissen über die mit CD24 und CD44 assoziierten molekularen Veränderungen bei Krebs.

List of abbreviations

Abbreviation	Meaning
MS	Mass spectrometry
MS/MS (MS2)	Tandem mass spectrometry
ESI	Electrospray ionization
HCD	Higher-energy collision dissociation
CID	Collision-induced dissociation
ACN	Acetonitrile
DMSO	Dimethyl sulfoxide
FA	Formic acid
DDT	Dithiothreitol
IAA	Iodoacetamide
PTMs	Post-translational modifications
ATRA	All-trans retinoic acid
SDC	Sodium deoxycholate
TEAB	Triethylammonium bicarbonate
mL	Millilitre
µL	Microliter
mM	Milli Molar
µg	Microgram
rpm	Rotations per minute
AmbiCa	Ammonium bicarbonate
HPLC	High performance liquid chromatography
m/z	Mass to charge ratio
BCA	Bicinchoninic acid
DIA	Data independent acquisition
DDA	Data dependent acquisition
DAPs	Different abundant proteins

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1. Introduction

1.1 Cancer

Cancer is a type of disease that results when cellular changes cause the uncontrolled growth and division of cells at one or more locations in the body. There are more than 100 types of cancer and five major categories including carcinoma, sarcoma, myeloma, leukemia and lymphoma based on their histological characteristics. Cancer, as a leading cause of death, is still the main barrier to increasing life expectancy of humans. In 2020, there were 19.3 million new cases and about half of them dead (Fig.1) (https://gco.iarc.fr/).

A chemical substance, environmental agents, viral or genetic factors lead to a gene mutation in a cell, which can be created spontaneously and passed on through inheritance. When a normal population of cells sustains a genetic mutation which increases its propensity to proliferate, tumor development begins. The altered cell and its descendants seem normal, but they reproduce too much (hyperplasia). After years, other mutations are generated in these cells that further loosen controls on cell growth. The offspring of this cell appears abnormal in shape and orientation. Over time, these mutations induce the tumor to begin invading nearby tissues.[1] This type of cancer is malignant tumor, which often resistant to treatment, may spread to other tissues within the body and they sometimes recur after removal.

1.1.1 Colon cancer

Colorectal cancer (CRC) ranks the third most frequently diagnosed cancer among males and second among females. Over 1.9 million new cancer cases and 935,173 deaths worldwide were estimated in 2020 (Fig.1). CRC is heterogeneous, especially to the anatomic locations of tumors, and there are racial differences, genetic and dietary interactions that influence its progress. Approximately 95% of CRCs are evolved from adenomatous polyps. A variety of genetic and molecular changes occur as these polyps transform from benignity to malignancy. For colon cancer, the overall 5-year survival rate is 63%, while the survival rate is 90% for patients at localized stage. The 5-year survival rate is 71% when it has spread to surrounding tissues or organs and/or the regional lymph nodes. When colon cancer has spread to distant parts of the body, the 5-

year survival rate is 14% (https://www.cancer.net/cancer-types/colorectal-cancer/statistics). Patients suffering from CRC usually have rectal bleeding with bright red blood, a change in bowel habits, cramping or abdominal (belly) pain, losing weight without trying, constant tiredness. Treatment for colon cancer usually involves surgery for removal of tumors, radiation therapy and chemotherapy.

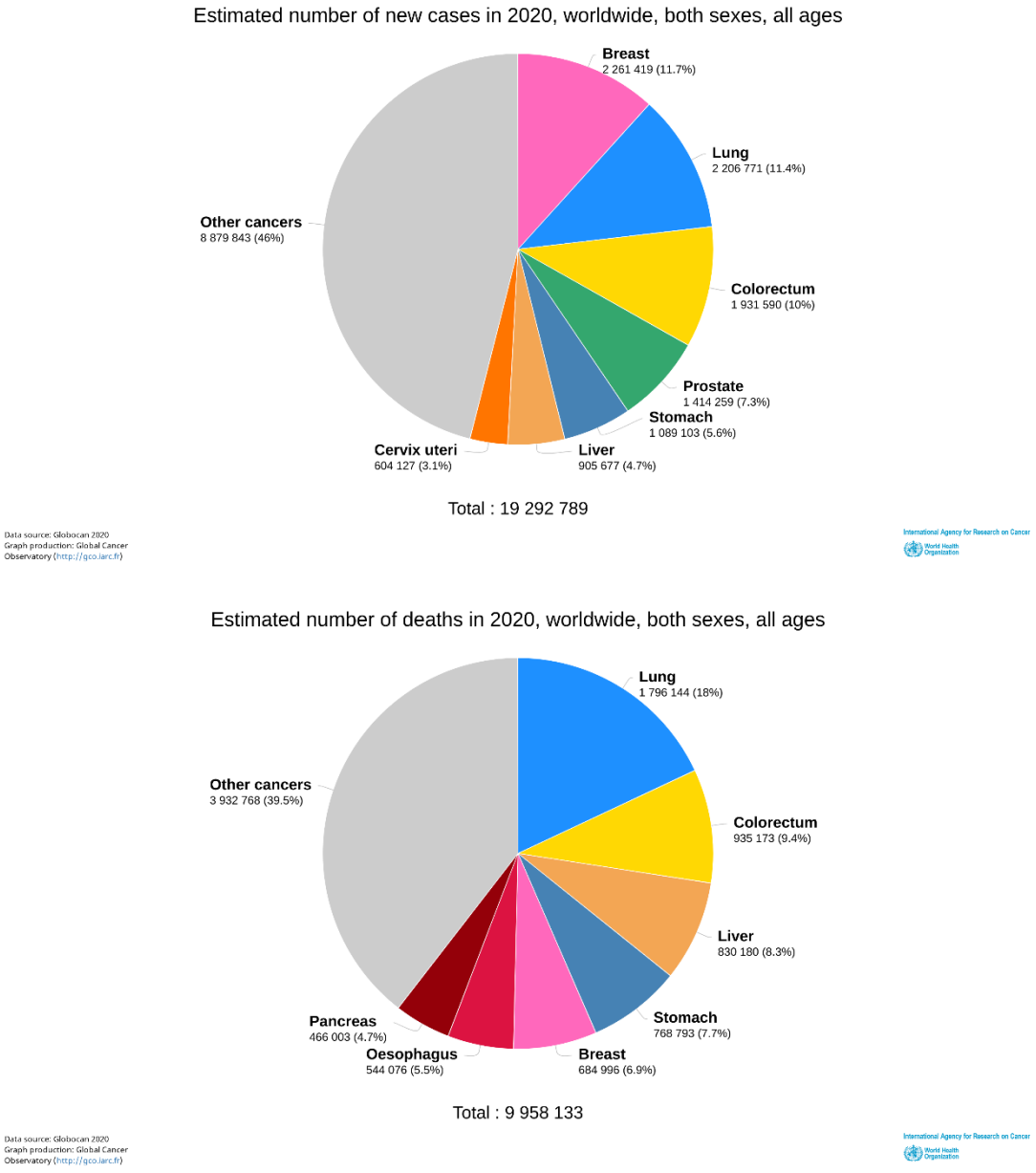


Fig. 1 The number of cancer new cases and deaths in 2020.

1.2 Cluster of differentiation (CD)

At the International Symposium on Human Leukocyte Differentiation Antigens in 1982, the initials "CD" was firstly established to stand for "cluster of differentiation"[2]. They are specific types of molecules found on the surface of cells that promote to differentiate

one defined cell type. CD molecules are involved into important functions, such as receptors or ligands to the cell and cell adhesion. The ectopic expression of CD markers has been discovered in many types of solid tumor.[3]

1.2.1 CD24

CD24, containing 27 amino acids, is a glycoprotein at the outer surface of the cell membrane and localized in lipid rafts through its glycosylphosphatidylinositol (GPI) anchor as cell adhesion molecule[4,5,6]. Many studies have described that CD24 over-expressed in various carcinomas such as renal cell carcinoma, non-small cell lung carcinoma, nasopharyngeal carcinoma, hepatocellular carcinoma, bladder carcinoma, ovarian cancer, breast cancer and colorectal cancer[7,8,9,10,11,12,13,14]. Several investigations confirmed that CD24 involve into cell adhesion[15], growth[16], proliferation[17], invasion[18] and metastasis[19] of tumor cells in vitro and vivo. However, the molecular mechanisms of CD24 in cancer development still are not clear. There are two potential mechanisms, glycosylation mediated mechanisms and Src kinases and integrins mediated mechanisms. Highly fucosylated glycans and sialylated epitopes are described as cancer related antigens. Sialyl-Lewis (x) antigen is necessary for CD24-P-selectin binding, which can facilitate the rolling and dissemination of malignant cells on platelets or endothelial cells.[20] The other mechanisms is that CD24 activates Src kinase, further Src kinase activates AKt, p38 MAPK, STAT3 and Mir-21. Activated STAT3 induces NANOG expression which drives cell self-renewal and tumor initiation. Also, CD24 activates EGFR signaling pathway, which induces the development of cancer through the activation of Akt and ERK[21] (Fig. 2).

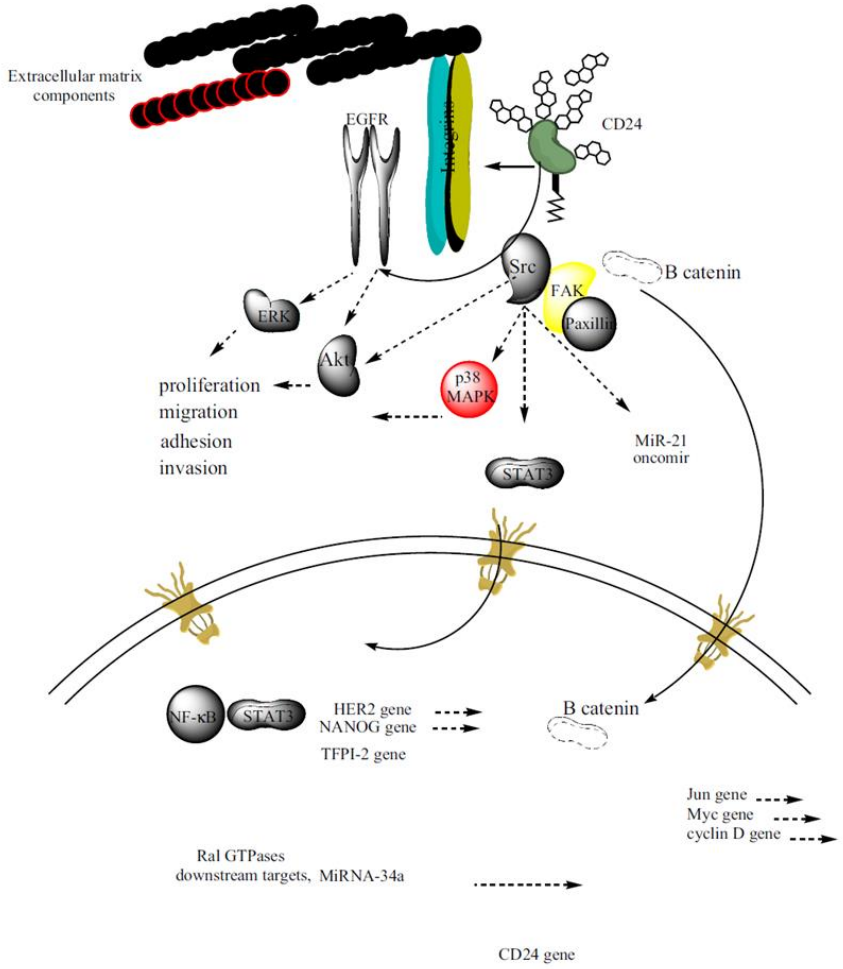


Fig. 2 Src kinases and integrins mediated mechanisms of CD24 in cancer[21].

1.2.2 CD44

CD44 is a single chain glycoprotein encoded by a single copy of a gene located on the short arm of chromosome 11 in humans and has a molecular weight of 85-200 kDa[22,23]. It is an acidic molecule (isoelectric point = 4.2 to 5.8), largely due to the attached sialic acids[24]. There are several ligands of CD44 including hyaluronic acid (HA), osteopontin (OPN), collagens and matrix metalloproteinases (MMPs)[25]. This interaction is thought to be responsible for cellular signaling and regulating other biological process within cells, including growth, survival, differentiation and motility[26,27,28,29]. CD44, as an adhesion molecule, enables cell communication by cell-cell signal transduction and plays a role in cancer cell migration and matrix adhesion in response to a cellular microenvironment, thus enhancing cellular aggregation and tumor cell growth[30]. Many researches indicate that CD44 expression is a promising prognostic indicator in solid tumors, including colon cancer[31], lung cancer[32], prostate cancer[33], breast cancer[34] and

gastric cancer[35]. Wu K et al. also showed that specific targeted knockdown of CD44 reduced cancer progression[36,37].

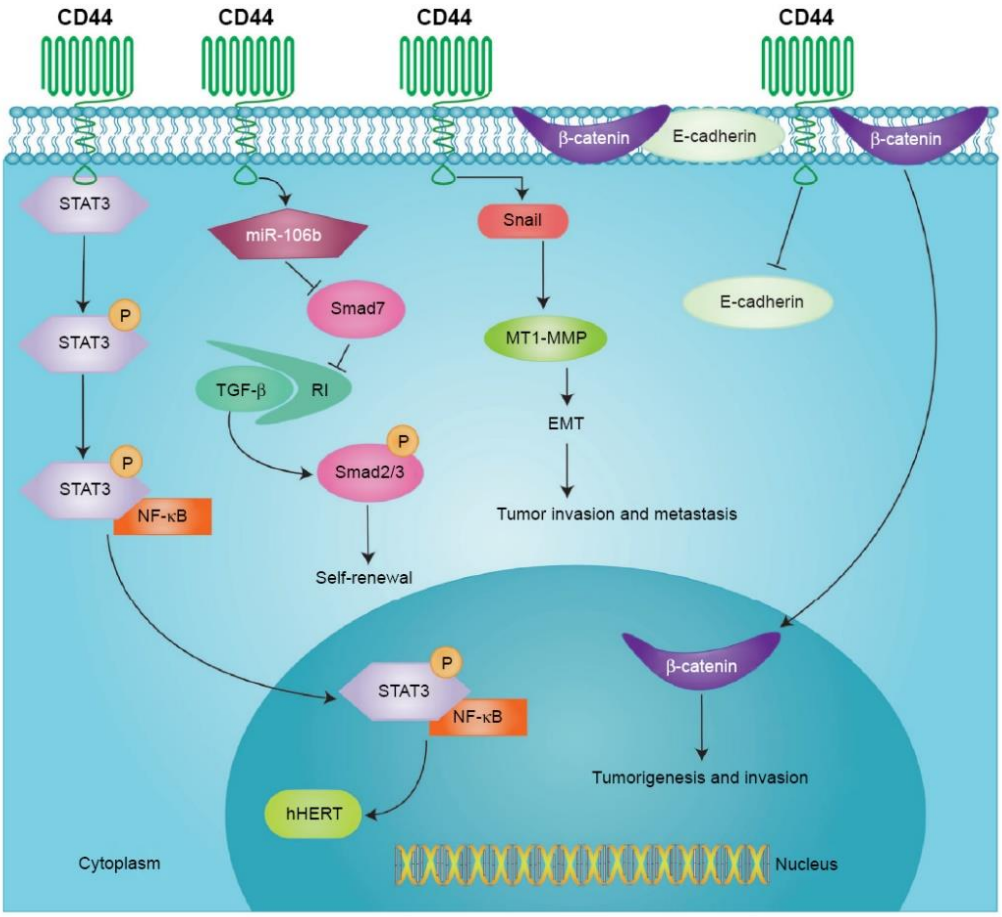


Fig. 3 Representative signal pathways induced by CD44[39].

CD44 expression is regulated by many extracellular or intracellular factors. CD44 promotes phosphorylation of STAT3 expression, leading to the nuclear translocation of pSTAT3 and activation of hHERT[38] and hHERT in turn increased CD44 expression. The CD44 has three pathways to affect the cancer cell development. One is that upregulation of miR-106b family represses inhibitory Smad7, which inhibits TGF-β/Smad2/3 signaling by suppressing RI and then enhances self-renewal of cancer cells. Another pathway is Snail upregulates the expression of MT1-MMP, which promotes tumor invasion and metastasis. In addition, CD44 prompts the separation of the membrane-associated E-cadherin and β-catenin complex. Afterwards, released β-catenin transfers into nucleus and then activates genes related to tumor[39] (Fig. 3).

1.2.3 Correlation between CD44 and CD24

The combined biomarkers of CD44 and CD24 have been identified as CSC surface markers in breast, prostate, pancreatic, nasopharyngeal and colorectal cancers[40,41,42,43]. In breast cancer, the $CD44^{(+)}/CD24^{(-)}$ phenotype had the best prognosis while the $CD44^{(-)}/CD24^{(+)}$ phenotype had the worst prognosis[44]. This result is not in line with the research which showed the expression of $CD44^{+}/CD24^{-}$ is a CSC marker for aggressive breast cancer types[45]. Min Hye et al. identified that CD44 expression was significantly associated with human epidermal growth factor receptor 2 (HER2)-negative status, while CD24 expression was significantly associated with HER2-positive status. CD44 and CD24 expression have no correlation with the age, tumor size, axillary lymph node metastasis status, tumor stage, histological grade, estrogen receptor status and progesterone receptor status of patients. There was also no statistical difference in overall survival according to the expression of CD44 and CD24[46]. In nasopharyngeal cancer, $CD44^{high}/CD24^{high}$ sufficiently represents CSC surface markers as physical markers. Besides, CD44 and CD24 are functionally capable of modulating stemness and EMT differentially through STAT3 activation. Wenzhe et al. demonstrated that high CD44/CD24 ratio correlates with strong proliferative capacity and tumorigenicity in breast cancer. When injected 4×10^{6} MDA-MB-231 cells into the mice with the highest CD44/CD24 ratio could generate tumors, which reached 670 mm^3 after 48 days culture[47].

2. Aim of this study

Many previous studies have shown that CD24 and CD44 play an important role in the development of cancer and can be used as markers and prognostic indicators for various cancers. There is no comprehensive study of proteome and glycome alterations derived from their knockdowns. In this study, we explore the effects of knockdown of CD24 and CD44 on proteomics and N-glycomics for colon cancer (CRC), and further study similarities and differences of their effects. To achieve this goal, three groups of colon tumor tissues were studied: Control (wildtype), CD24kd, which knockdown CD24 gene and CD44kd, which knockdown CD44 gene.

The first part of this study focuses on the proteomics of tumor tissue. The goal is to identify significant proteins which were regulated after knockdowns of CD24 and CD44 genes. Further step was that researched to gain insights on their role in cancer progression and figured out the pathway which mainly focus on cancer progression after knockdowns of CD24 and CD44 genes. Through the regulated proteins comparison, the CD24 and CD44 may have similar affections in colon cancer progression. The second part of the study focuses on identifying potential N-glycans by comparing the tumor samples to CD24kd-tumor tissue and CD44kd-tumor tissue. The differentially regulated N-glycans in either CD24kd or CD44kd tissues with respect to the Control-tumor tissues were then further investigated the relative abundance and fold change (FC) to confirm the most significantly differential N-glycans.

3. Results

3.1 Proteomic analysis of CD24kd, CD44kd with Control

In order to determine the influence of CD24 and CD44 gene in colon cancer, proteins from Control, CD24kd and CD44kd groups were analysed with differential bottom-up proteomics using label-free quantification (LFQ).

Totally 1879 proteins were identified across three groups. Based on log2 transformation and normalization of protein abundances, distinct clusters for all groups can be show by principal components analysis (PCA). X and Y-axis are the principal component 1 and principal component 2 and explained as 29.7% and 14.5% of the total variance, respectively. As the CD24kd and Control on the PCA plot are part of overlapping, indicating these two groups were not separated clusters and showing a higher similarity compared to CD44kd.

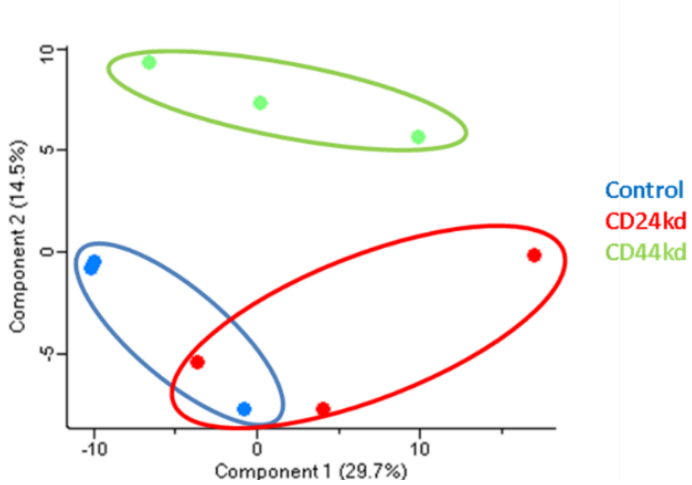


Fig. 4 Principal Component Analysis (PCA) for CD24kd, CD44kd and Control. The unsupervised clustering based on log2 transformed and normalized of proteins abundance.

3.1.1 Comparative proteomics of CD24kd and Control

The effects of CD24 knockdown in CRC was explored through comparing the protein abundance of the Colorectal cancer and Colorectal cancer with CD24 knockdown. As described in Materials and Methods, proteins being present in significantly different concentrations were obtained from the HT29 cell xenograft tissue. Using a two-samples student's tests with a significance level of 0.05 (P-value) and absolute fold-change (FC) 1.5, 50 proteins were differentially abundance in CD24kd. There were 37 proteins were significantly upregulated and 13 proteins were significantly downregulated (show in

Fig. 5 and Table S1).

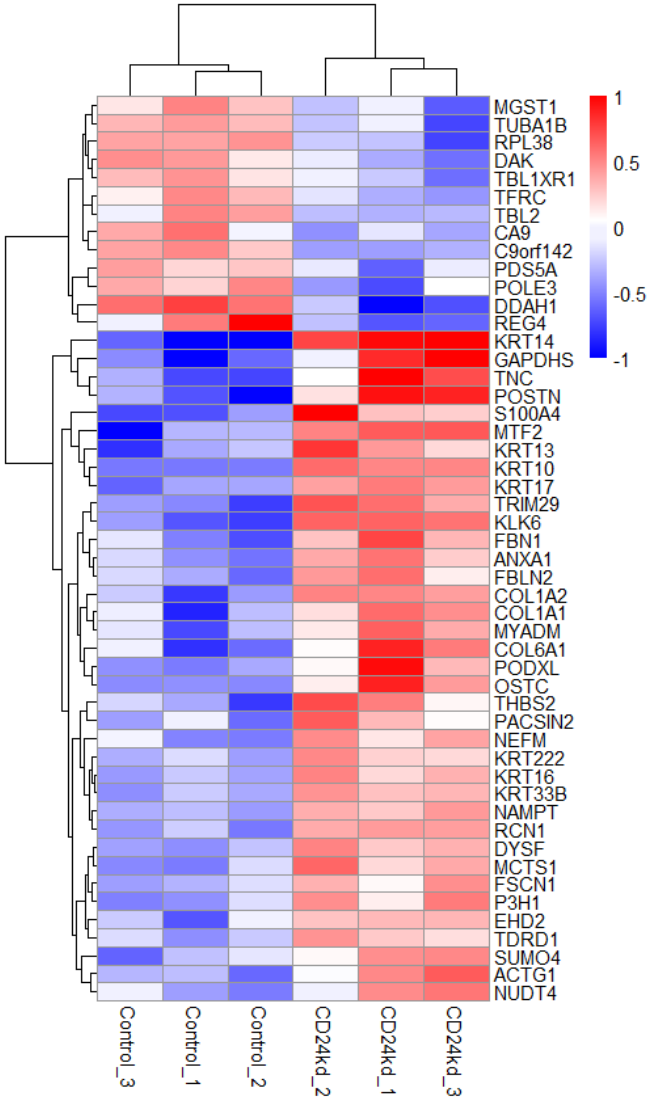


Fig. 5 Heat map of proteins with different abundances in CD24kd compared to control. Proteins are displayed with a |FC| larger than 1.5 and a P-value lower than 0.05.

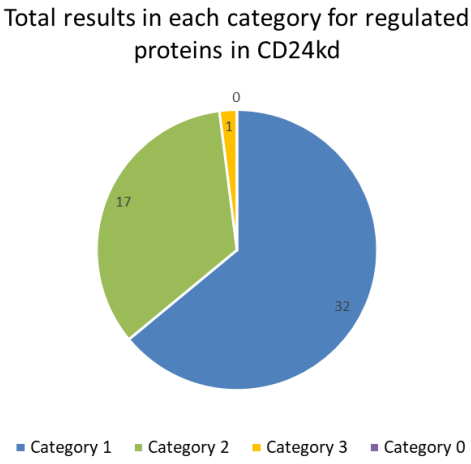


Fig. 6 Distribution of the different categories for 50 proteins analyzed by the OmixLitMiner tool.

OmixLitMiner was used to retrieve all PubMed listed publications that connect a protein to the keyword 'cancer' in title or abstract. The automated outputs from OmixLitMiner are shown in Table S2. A graphical overview of the categorization of retrieved literature from OmixLitMiner analysis of the 50 proteins is shown in Fig. 6. OmixLitMiner analysis for 32 proteins placed it in Category 1. 17 proteins and 1 protein were placed in category 2 and 3, respectively.

In order to figure out the relationship of regulated proteins and CD24 in colon cancer, we filtered literature manually about the regulated proteins in colon cancer from the outputs of OmixLitMiner. Table.1 and Table. 2 show the regulation of proteins in this study and in colon cancer versus normal according to the literature.

Table. 1 Significantly down-regulated proteins in CD24kd. Functions based on UniProt database search.

Gene symbol	Protein name	Function	Regulation in CD24kd	Regulation in CRC
C9orf142	Protein PAXX	DNA repair	down	up[48]
CA9	Carbonic anhydrase 9	morphogenesis of an epithelium, secretion, regulation of transcription from RNA polymerase II promoter	down	up[49]
REG4	Regenerating islet derived protein 4	heparin binding, mannan binding, signalling receptor activity	down	up[50]
TBL1XR1	F-box-like/WD repeat containing protein TBL1XR1	beta-catenin binding, multicellular organism growth, positive regulation of canonical Wnt signalling pathway, positive regulation of transcription	down	up[51]
TFRC	Transferrin receptor protein 1	negative regulation of apoptotic process, positive regulation of B cell proliferation, gene expression, T cell proliferation, protein phosphorylation	down	up[52]
DAK	Triokinase/FMN cyclase	ATP binding, innate immune response, negative regulation of MDA-5 signalling pathway	down	no
DDAH1	N(G), N(G)-dimethylarginine dimethylaminohydrolase 1	negative regulation of cell population proliferation, amino acid binding, positive regulation of angiogenesis	down	no
MGST1	Microsomal glutathione S-transferase 1	glutathione binding, identical protein binding, oxidation-reduction process	down	no
PDS5A	Sister chromatid cohesion protein PDS5 homolog A	cell division, DNA repair, negative regulation of DNA replication	down	no
POLE3	DNA polymerase epsilon subunit 3	DNA replication, protein heterodimerization activity	down	no

RPL38	60S ribosomal protein L38	RNA binding, regulation of translation	down	no
TBL2	Transducin beta-like protein 2	cellular response to glucose starvation and hypoxia, endoplasmic reticulum unfolded protein response	down	no
TUBA1B	Tubulin alpha-1B chain	cell division, microtubule cytoskeleton organization	down	no

*"Regulation in cancer" is refer to protein regulation in colon cancer versus normal according to the references; "Regulation in CD24kd" is refer to protein regulation in CD24kd versus Control in this study; "no" means no publication show the regulation in colon cancer when compared with normal.

In the Table. 1, C9orf142, CA9, REG4, TBL1XR1, TFRC were down-regulated in CD24kd group, while they were upregulated in colon cancer versus normal sample. There was no publication show the others' regulations in colon cancer when compare with normal. In the Table. 2, The proteins COL1A2, EHD2, FBLN2 were up-regulated in CD24kd group, while they were downregulated in colon cancer versus normal sample. There were 18 proteins were not reported in previous studies. The remaining 16 proteins ANXA1, COL1A1, FBN1, FSCN1, MCTS1, KLK6, KRT17, MYADM, NAMPT, PODXL, POSTN, RCN1, S100A4, THBS2, TNC, TRIM29 were both upregulation in two comparisons.

Table. 2 Significantly up-regulated Proteins in CD24kd compared to Control. Functions based on UniProt database search.

Gene symbol	**Protein name**	**Function**	**Regulation in CD24kd**	**Regulation in CRC**
COL1A2	Collagen alpha-2(I) chain	collagen fibril organization, regulation of immune response, leukocyte migration, blood vessel development	up	down[53]
EHD2	EH domain-containing protein 2	ATP binding, positive regulation of endocytic recycling, positive regulation of myoblast fusion	up	down[54]
FBLN2	Fibulin-2	positive regulation of cell-substrate adhesion, extracellular matrix organization	up	down[55]
ACTG1	Actin, cytoplasmic 2	positive regulation of cell migration, gene expression, regulation of focal adhesion assembly, angiogenesis, structural constituent of cytoskeleton	up	no
COL6A1	Collagen alpha-1(VI) chain	cell adhesion, collagen binding, endodermal cell and osteoblast differentiation	up	no
DYSF	Dysferlin	positive regulation of cell adhesion and endothelial cell proliferation, negative regulation of gene expression, and protein	up	no

		catabolic process		
KRT10	Keratin, type I cytoskeletal 10	keratinocyte differentiation, positive regulation of epidermis development, cornification	up	no
KRT13	Keratin, type I cytoskeletal 13	structural molecule activity, cornification, cytoskeleton organization	up	no
KRT14	Keratin, type I cytoskeletal 14	structural constituent of cytoskeleton, cornification, aging	up	no
KRT16	Keratin, type I cytoskeletal 16	negative regulation of cell migration, keratinocyte differentiation, keratinocyte migration, aging, cornification, cytoskeleton organization	up	no
KRT222	Keratin-like protein KRT222	structural molecule activity	up	no
KRT33B	Keratin, type I cuticular Ha3-II	structural molecule activity, aging, cornification, keratinization	up	no
OSTC	Oligosaccharyltransferase complex subunit OSTC	protein N-linked glycosylation via asparagine (transfer of a defined glycan from the lipid carrier dolichol-pyrophosphate to an asparagine residue within an Asn-X-Ser/Thr consensus motif in nascent polypeptide chains)	up	no
PACSIN2	Protein kinase C and casein kinase substrate in neurons protein 2	cadherin binding, cytoskeleton organization, regulation of endocytosis	up	no
SUMO4	Small ubiquitin-related modifier 4	negative regulation of transcription, DNA-templated, cellular response to oxidative stress, protein tag, regulation of DNA binding	up	no
TDRD1	Tudor domain-containing protein 1	multicellular organism development, gene silencing by RNA, DNA methylation involved in gamete generation	up	no
NUDT4	Diphosphoinositol polyphosphate phosphohydrolase 2	intracellular signal transduction, inositol phosphate metabolic process, alcium-mediated and cyclic-nucleotide-mediated signaling	up	no
P3H1	Prolyl 3-hydroxylase 1	negative regulation of cell population proliferation and post-translational protein modification, collagen metabolic process, protein folding, protein stabilization	up	no
GAPDHS	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	positive regulation of glycolytic process, NAD and NADP binding	up	no
MTF2	Metal-response element-binding transcription factor 2	stem cell differentiation, negative regulation of gene expression, positive regulation of transcription	up	no

		by RNA polymerase II		
NEFM	Neurofilament medium polypeptide	neurofilament bundle assembly, structural constituent of cytoskeleton, microtubule binding	up	no
MCTS1	Malignant T-cell-amplified sequence 1	positive regulation of cell population proliferation, regulation of growth, cell cycle, translation reinitiation	up	up[56]
ANXA1	Annexin A1	regulation of cell population proliferation, regulation of cell shape, positive regulation of cell migration involved in sprouting angiogenesis and T cell proliferation, negative regulation of apoptotic process	up	up[57]
COL1A1	Collagen alpha-1(I) chain	positive regulation of cell migration, transcription, DNA-templated, negative regulation of cell-substrate adhesion, cellular response to tumor necrosis factor, negative regulation of cell-substrate adhesion	up	up[58]
FBN1	Fibrillin-1	cell adhesion mediated by integrin, cellular protein metabolic process, negative regulation of osteoclast differentiation and development	up	up[59]
FSCN1	Fascin	cell migration, cell motility, cell-cell junction assembly, regulation of actin cytoskeleton organization	up	up[60]
KLK6	Kallikrein-6	regulation of cell differentiation, ollagen catabolic process, central nervous system development	up	up[61]
KRT17	Keratin, type I cytoskeletal 17	positive regulation of cell growth, translation, keratinization	up	up[62]
MYADM	Myeloid-associated differentiation marker	negative regulation of heterotypic cell-cell adhesion and gene expression, positive regulation of cell migration and substrate adhesion-dependent cell spreading	up	up[63]
NAMPT	Nicotinamide phosphoribosyltransferase	positive regulation of cell population proliferation, neuron death, aging, cell-cell signaling, negative regulation of autophagy and cellular senescence	up	up[64]
PODXL	Podocalyxin	cell adhesion, positive regulation of cell migration, negative regulation of cell-cell adhesion, positive regulation of cell-cell adhesion mediated by integrin	up	up[65]
POSTN	Periostin	cell adhesion, negative regulation of substrate adhesion-dependent cell spreading and cell-matrix adhesion, cellular response to tumor necrosis factor	up	up[66]
RCN1	Reticulocalbin-1	cellular protein metabolic process,	up	up[67]

		post-translational protein modification		
S100A4	Protein S100-A4	calcium ion binding, calcium ion binding, RNA binding, epithelial to mesenchymal transition	up	up[68]
THBS2	Thrombospondin-2	cell adhesion, negative regulation of angiogenesis, positive regulation of synapse assembly	up	up[69]
TNC	Tenascin	negative regulation of cell adhesion, positive regulation of cell population proliferation adn gene expression, post-translational protein modification	up	up[70]
TRIM29	Tripartite motif-containing protein 29	cadherin binding involved in cell-cell adhesion, negative regulation of transcription by RNA polymerase II, innate immune response	up	up[71]

*"Regulation in cancer" is refer to protein regulation in colon cancer versus normal according to the references; "Regulation in CD24kd" is refer to protein regulation in CD24kd versus Control in this study; "no" means no publication show the regulation in colon cancer when compare with normal.

Gene ontology (GO) analysis was used to investigate the functional associations of differentially abundant proteins. The results indicated that 'extracellular matrix organization', 'cell adhesion' and 'skeletal system development' were the most significant terms in the Biological process category. 'extracellular exosome', 'extracellular space' and 'extracellular region' were the most significant in Cellular component. The significant Molecular function terms were 'protein binding', 'structural molecule activity' and 'calcium ion binding' (Fig. 7).

Fig. 7 Gene ontology (GO) term enrichment analysis of differentially abundant protein (DAPs) in CD24kd.

3.1.2 Comparative proteomics between CD44kd and Control

Using the same analytical strategy as above, 51 significantly differential proteins in

CD44 kd group (Fig. 8 and Table S3), in which 21 species were downregulated while 30 species upregulated. The significantly changed proteins were also further analysed with OmixLitMiner and Gene Ontology (GO).

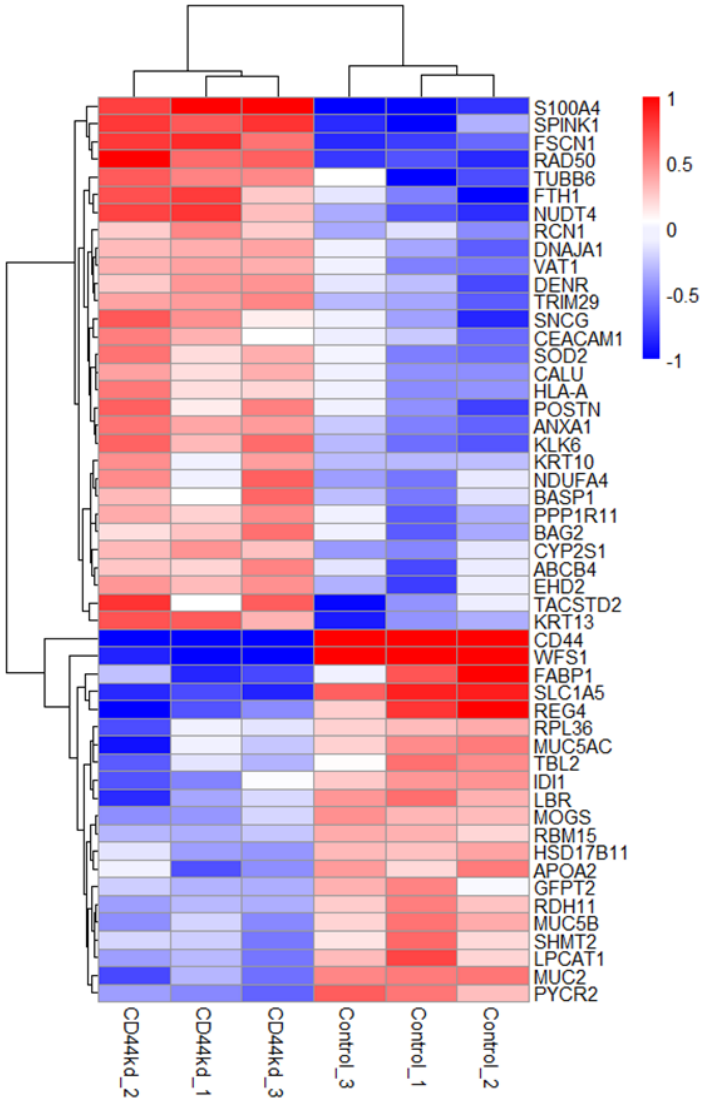


Fig. 8 Heatmap of proteins with different abundances in CD44kd compared to Control. Proteins are displayed with a |FC| larger than 1.5 and a P-value lower than 0.05.

The automated outputs from OmixLitMiner are shown in Table S4. A graphical overview of the categorisation of retrieved literature from OmixLitMiner analysis of the 51 proteins is shown in Fig. 9. OmixLitMiner analysis for 34 proteins placed it in Category 1. 16 proteins and 1 protein were placed in category 2 and 3, respectively.

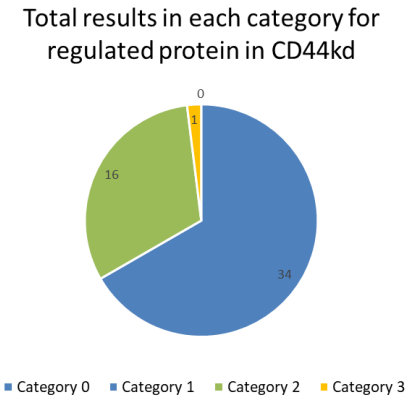


Fig. 9 Distribution of the different categories for 51 proteins analyzed by the OmixLitMiner tool.

In order to figure out the relationship of regulated proteins and CD24 in colon cancer, we filtered literature manually about the regulated proteins in colon cancer from the results of OmixLitMiner, the regulation showing in the Table.3 and 4 "Regulation in CRC" column.

Table. 3 Significantly down-regulated proteins in CD44kd compared to Control. Functions based on UniProt database search.

Gene symbol	Protein name	Function	Regulation in CD44kd	Regulation in CRC
FABP1	Fatty acid-binding protein, liver	negative regulation of apoptotic process, cellular response to hydrogen peroxide, positive regulation of hydrolase activity	down	down[72]
APOA2	Apolipoprotein A-II	cholesterol transport, lipoprotein metabolic process, lipase inhibitor activity, regulation of lipid metabolic process, regulation of protein stability,	down	down[73]
HSD17B11	Estradiol 17-beta-dehydrogenase 11	androgen catabolic process, estrogen biosynthetic process	down	no
IDI1	Isopentenyl-diphosphate Delta-isomerase 1	biosynthetic process of cholesterol, dimethylallyl diphosphate, isopentenyl diphosphate, isoprenoid	down	no
LBR	Delta(14)-sterol reductase LBR	cholesterol biosynthetic process, neutrophil differentiation, sterol biosynthetic process, lamin binding, oxidoreductase activity, acting on the CH-CH group of donors	down	no
MOGS	Mannosyl-oligosaccharide glucosidase	oligosaccharide metabolic process, protein folding, protein N-linked glycosylation	down	no
PYCR2	Pyrroline-5-carboxylate reductase 2	cellular amino acid biosynthetic process, cellular response to oxidative stress	down	no
RBM15	RNA-binding protein 15	negative regulation of myeloid cell differentiation, regulation of	down	no

		alternative mRNA splicing, regulation of megakaryocyte differentiation, RNA methylation		
RDH11	Retinol dehydrogenase 11	NADP-retinol dehydrogenase activity, oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	down	no
RPL36	60S ribosomal protein L36	RNA binding, translation	down	no
TBL2	Transducin beta-like protein 2	cellular response to glucose starvation and hypoxia, endoplasmic reticulum unfolded protein response	down	no
WFS1	Wolframin	negative regulation of neuron apoptotic process and programmed cell death, positive regulation of growth and protein metabolic process, post-translational protein modification, protein stabilization	down	no
SLC1A5	Neutral amino acid transporter B(0)	amino acid transport, signaling receptor activity, symporter activity	down	up[74]
SHMT2	Serine hydroxymethyltransferase, mitochondrial	positive regulation of cell population proliferation, amino acid binding, protein tetramerization	down	up[75]
CD44	CD44 antigen	cell adhesion, cell-cell adhesion, cell-matrix adhesion, cell migration, negative regulation of apoptotic process, positive regulation of heterotypic cell-cell adhesion,	down	up[76]
MUC5AC	Mucin-5AC	O-glycan processing, phosphatidylinositol-mediated signaling, stimulatory C-type lectin receptor signaling pathway	down	up[77]
MUC5B	Mucin-5B	O-glycan processing, stimulatory C-type lectin receptor signaling pathway	down	up[78]
MUC2	Mucin-2	maintenance of gastrointestinal epithelium, O-glycan processing, stimulatory C-type lectin receptor signaling pathway	down	up[79]
GFPT2	Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 2	carbohydrate derivative binding, glutamine-fructose-6-phosphate transaminase (isomerizing) activity	down	up[80]
LPCAT1	Lysophosphatidylcholine acyltransferase 1	positive regulation of protein catabolic process, phospholipid biosynthetic process	down	up[81]
REG4	Regenerating islet-derived protein 4	heparin binding, mannan binding, signalling receptor activity	down	up[82]

*"Regulation in cancer" is refer to protein regulation in colon cancer versus normal according to the references; "Regulation in CD44kd" is refer to protein regulation in CD44kd versus Control in this study; "no" means no publication show the regulation in colon cancer when compare with normal.

In the Table.3 FABP1 and APOA2 showed the downregulation in both two conditions.

CD44 was highly expressed in Control and the abundance of CD44 decreased 16-FC in CD44kd. Eight proteins (SLC1A5, SHMT2, MUC5AC, MUC5B, MUC2, GFPT2, LPCAT1, REG4) were all upregulated in colon cancer when compare with normal, which were downregulation in our data. From Table.4 ABCB4, CEACAM1, CYP2S1, EHD2, HLA-A also were opposite regulation compare to the results in group CD44kd, while 14 proteins (TACSTD2, SNCG, CALU, RAD50, DNAJA1, FSCN1, KLK6, POSTN, RCN1, S100A4, SOD2, SPINK1, TRIM29, ANXA1) were all upregulated both in the research of literature and our results. The regulation of other proteins was not reported in colon cancer in previous studies.

Table. 4 Significantly up-regulated proteins in CD44kd compared to Control. Functions based on UniProt database search.

Gene symbol	Protein name	Function	Regulation in CD44kd	Regulation in CRC
ABCB4	Phosphatidylcholine translocator ABCB4	phospholipid transporter activity, ATPase activity, lipid metabolic process	up	down[83]
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	cell adhesion, cell-cell adhesion via plasma-membrane adhesion molecules, cell migration, regulation of cell growth, regulation of sprouting angiogenesis	up	down[84]
CYP2S1	Cytochrome P450 2S1	organic acid metabolic process, oxidation-reduction process, xenobiotic metabolic process	up	down[85]
EHD2	EH domain-containing protein 2	ATP binding, positive regulation of endocytic recycling, positive regulation of myoblast fusion	up	down[86]
HLA-A	HLA class I histocompatibility antigen, An alpha chain	T cell receptor binding, regulation of immune response, signaling receptor binding	up	down[87]
TUBB6	Tubulin beta-6 chain	GTPase activity, GTP binding, structural constituent of cytoskeleton, microtubule cytoskeleton organization, mitotic cell cycle	up	no
DENR	Density-regulated protein	translation initiation factor activity, mRNA binding	up	no
BAG2	BAG family molecular chaperone regulator 2	microtubule-based process, microtubule cytoskeleton organization, mitotic cell cycle	up	no
BASP1	Brain acid soluble protein 1	mesenchymal to epithelial transition, negative regulation of transcription, DNA-templated, protein domain specific binding	up	no
FTH1	Ferritin heavy chain	ferric iron binding, iron ion transport, negative regulation of cell population proliferation and fibroblast	up	no

		proliferation, immune response		
NDUFA4	Cytochrome c oxidase subunit NDUFA4	mitochondrial electron transport, NADH to ubiquinone, proton transmembrane transport	up	no
PPP1R11	E3 ubiquitin-protein ligase PPP1R11	negative regulation of cytokine secretion and phosphoprotein phosphatase activity and protein dephosphorylation, ubiquitin-dependent protein catabolic process	up	no
VAT1	Synaptic vesicle membrane protein VAT-1 homolog	oxidoreductase activity, zinc ion binding, negative regulation of mitochondrial fusion	up	no
KRT10	Keratin, type I cytoskeletal 10	keratinocyte differentiation, positive regulation of epidermis development, cornification	up	no
KRT13	Keratin, type I cytoskeletal 13	structural molecule activity, cornification, cytoskeleton organization	up	no
NUDT4	Diphosphoinositol polyphosphate phosphohydrolase 2	intracellular signal transduction, inositol phosphate metabolic process, alcium-mediated and cyclic-nucleotide-mediated signaling	up	no
ANXA1	Annexin A1	regulation of cell population proliferation, regulation of cell shape, positive regulation of cell migration involved in sprouting angiogenesis and T cell proliferation, negative regulation of apoptotic process	up	up[88]
TACSTD2	Tumor-associated calcium signal transducer 2	negative regulation of cell motility, negative regulation of epithelial cell migration, positive regulation of stem cell differentiation and regulation of epithelial cell proliferation	up	up[89]
SNCG	Gamma-synuclein	cuprous ion binding, protein secretion, regulation of dopamine secretion, neuron death and neurotransmitter secretion	up	up[90]
CALU	Calumenin	calcium ion binding, cellular protein metabolic process, post-translational protein modification	up	up[91]
RAD50	DNA repair protein RAD50	DNA repair, protein-macromolecule adaptor activity, positive regulation of protein autophosphorylation	up	up[92]
DNAJA1	DnaJ homolog subfamily A member 1	regulation of apoptotic process, protein folding, regulation of protein transport, negative regulation of protein ubiquitination	up	up[93]
FSCN1	Fascin	cell migration, cell motility, cell-cell junction assembly, regulation of actin cytoskeleton organization	up	up[94]
KLK6	Kallikrein-6	regulation of cell differentiation, ollagen catabolic process, central nervous system development	up	up[95]
POSTN	Periostin	cell adhesion, negative regulation of substrate adhesion-dependent cell spreading and cell-matrix adhesion, cellular response to tumor necrosis	up	up[96]

		factor		
RCN1	Reticulocalbin-1	cellular protein metabolic process, post-translational protein modification	up	up[67]
S100A4	Protein S100-A4	calcium ion binding, calcium ion binding, RNA binding, epithelial to mesenchymal transition	up	up[97]
SOD2	Superoxide dismutase [Mn], mitochondrial	negative regulation of cell population proliferation, fat cell differentiation, fibroblast proliferation, neuron apoptotic process, manganese ion binding	up	up[98]
SPINK1	Serine protease inhibitor Kazal-type 1	endopeptidase inhibitor activity, serine-type endopeptidase inhibitor activity, negative regulation of calcium ion import	up	up[99]
TRIM29	Tripartite motif-containing protein 29	cadherin binding involved in cell-cell adhesion, negative regulation of transcription by RNA polymerase II, innate immune response	up	up[100]

*"Regulation in cancer" is refer to protein regulation in colon cancer versus normal according to the references; "Regulation in CD44kd" is refer to protein regulation in CD44kd versus Control in this study; "no" means no publication show the regulation in colon cancer when compare with normal.

The results of the GO analysis of the DAPs in CD44kd indicated that 'oxidation-reduction process', 'negative regulation of apoptotic process' and 'cell-cell adhesion' were the most significant terms in the Biological process category. 'extracellular exosome', 'cytoplasm' and 'membrane' were the most significant in Cellular component. The significant Molecular function terms were 'protein binding', 'calcium ion binding', 'calcium-dependent protein binding' (Fig. 10).

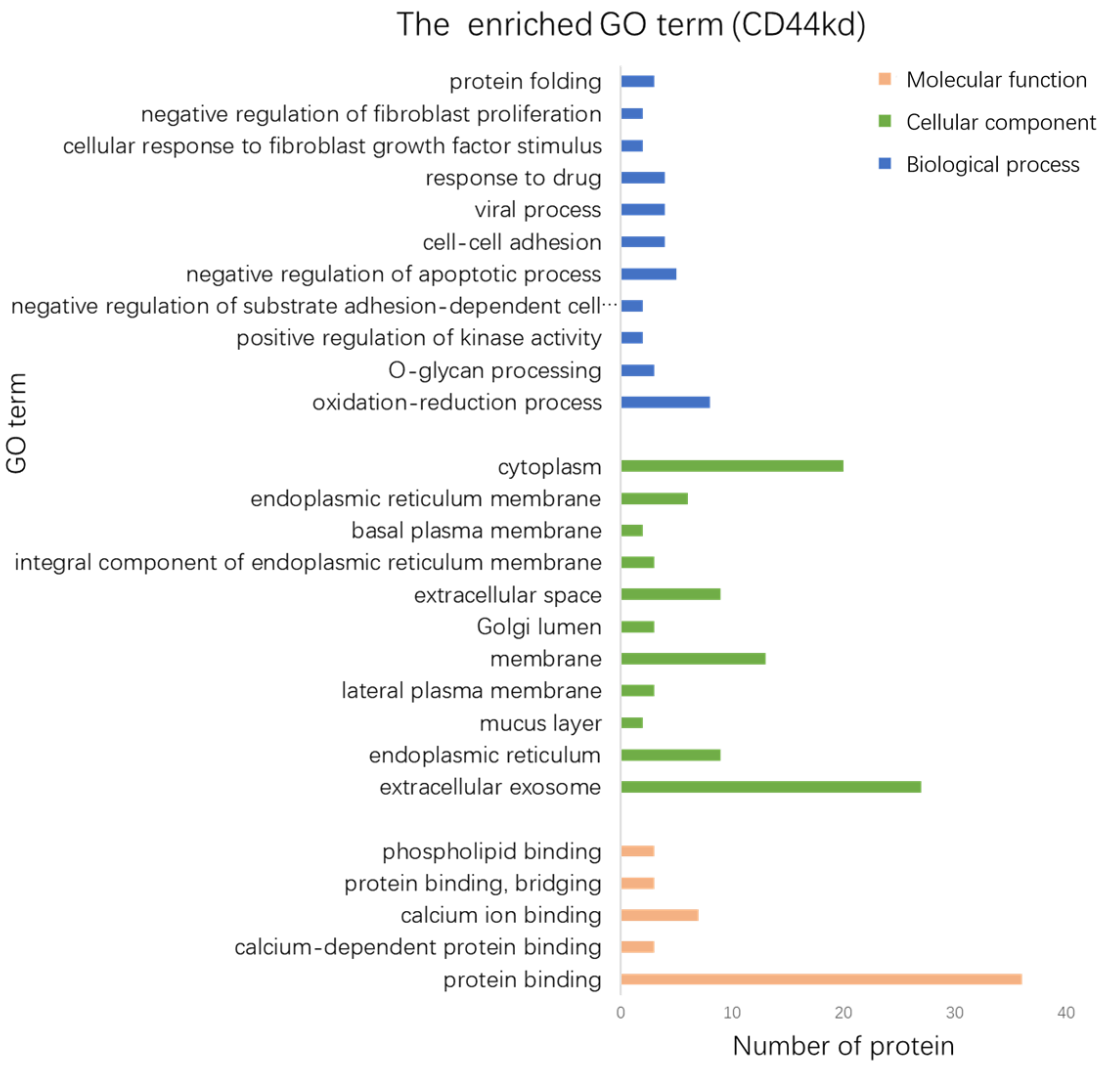


Fig. 10 Gene ontology (GO) term enrichment analysis of differentially abundant protein (DAPs) in CD44kd.

3.1.3 Gene Set Enrichment Analysis for different groups

A pathway controlling a particular cellular function is regulated by multiple genes. Small changes in many of those genes can have a strong effect on the final output, but are ignored by using the filter like fold-change or p-value before enrichment. To circumvent this problem, Gene set enrichment analysis (GSEA) directly compares gene abundance data to gene sets defined by previous studies. GSEA considers all genes in the matrix without arbitrary cut-off based on significance.

GSEA approach was used to further confirm enriched pathway data of regulated candidates from both Colorectal cancer and Colorectal cancer after knockdown CD24

and CD44. Totally 1879 genes were uploaded to analyse with GSEA. For CD24kd versus Control comparison, we found 71 gene sets that were significantly enriched (Nominal P-Value less than 0.05) for differentially expressed genes. Of these, 46 gene sets were significantly upregulated and 25 gene sets were downregulated in CD24kd. In the comparison between CD44kd and Control, 61 gene sets were significantly enriched at nominal p-value less than 5%, including upregulated 36 gene sets and downregulated 25 gene sets in CD44kd, respectively. A full list of the rank-ordered genes participating in the positively and negatively-enriched pathways are reported in Table S5 and S6.

'EXTRACELLULAR_MATRIX_ORGANIZATION' was identified, in accordance with the result of GO analysis. The enrichment score for this signaling pathway was positively correlated to CD24kd samples, while the enrichment score was lower in Control samples (Fig.11a). The GO and GSEA analysis both revealed that BIOLOGICAL_OXIDATIONS was enriched in Control group (Fig.11b) and the normalized enrichment score is 1.86.

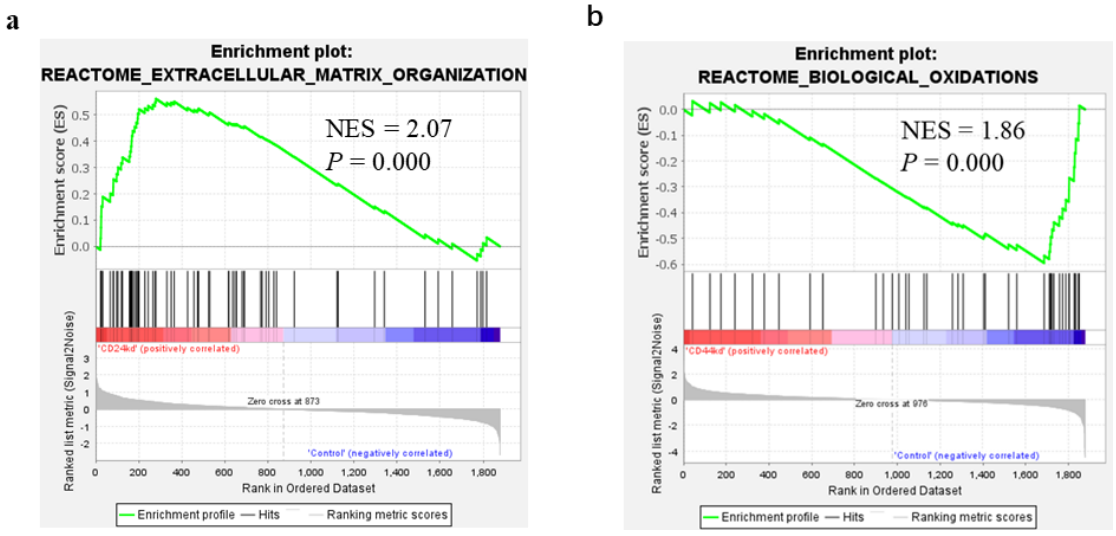


Fig. 11 GSEA Enrichment plot (score curves). The green curve corresponds to the ES (enrichment score) curve, which is the running sum of the weighted enrichment score obtained from GSEA software, while the normalized enrichment score (NES) and the corresponding P-value are reported within each graph. (**a**) denote the enriched (significant) pathway (i.e. Gene set) in accordance with GO analysis with CD24kd vs Control. (**b**) denote the enriched (significant) pathway (i.e. Gene set) in accordance with GO analysis with CD44kd vs Control.

3.1.4 DAPs comparison between CD24kd and CD44kd

To gain insight into potential overlapping roles of CD24 and CD44 in colon cancer, all the significant regulated proteins in CD24kd and CD44kd were compared in a Venn diagram (Fig. 12a) and this showed 13 proteins that were identified in both groups. REG4 and TBL2 were downregulated. Besides, REG4 abundance was less in CD44kd (2.93-FC) than in CD24kd (2.07-FC), TBL2 abundance is similar in both and downregulated by almost 3.20-fold. 11 proteins (EHD2, KRT10, ANXA1, TRIM29, POSTIN, RCN1, KLK6, NUDT4, FSCN1, KRT13 and S100A4) are upregulated (Fig. 12c). EHD2, ANXA1, KRT13, TRIM29, POSTN, RCN1, KLK6 have the similar abundance in CD24kd and CD44kd groups. KRT10 has higher amount in CD24kd. NUDT4, FSCN1and S100A4 amount are more in CD44kd (2.36, 2.79, 4-fold upregulated) than in CD24kd (1.59, 1.51, 2.31-fold upregulated), but KRT10 is less abundance in CD44kd (1.51-fold).

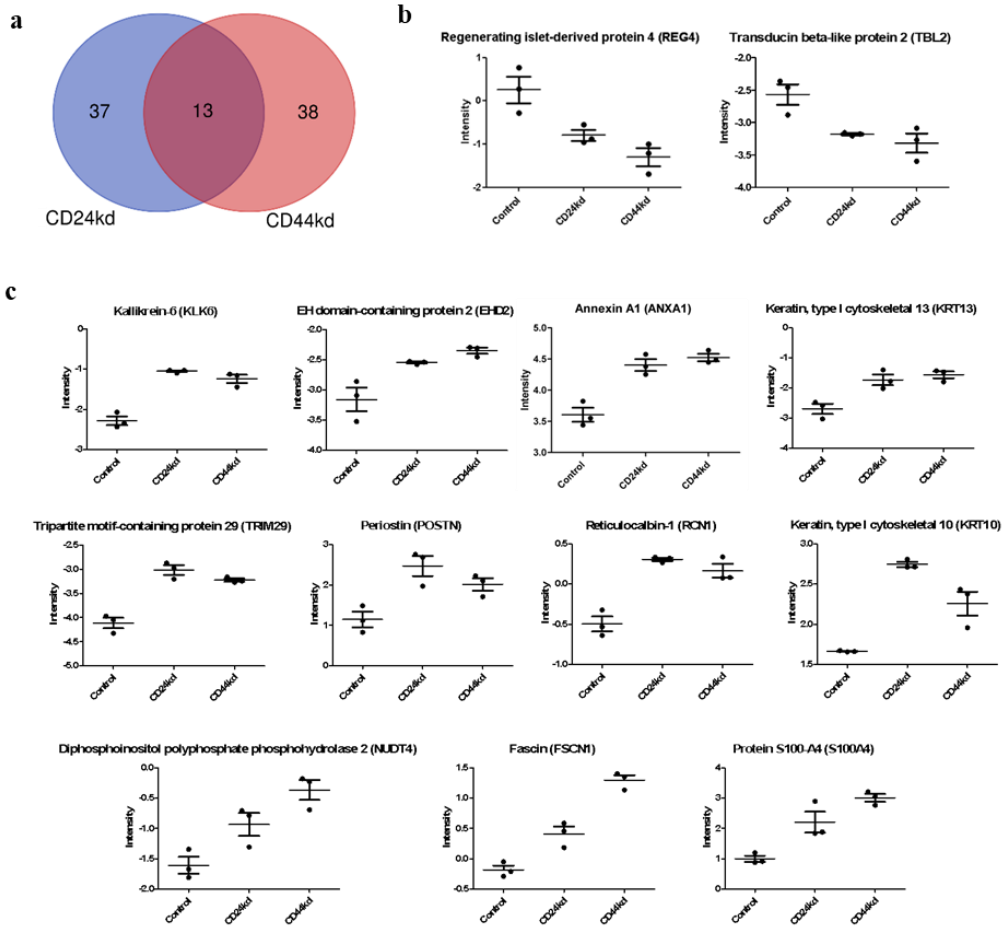


Fig. 12 (**a**) Venn diagram comparing DAPs obtained from two comparisons. (**b**) Down-regulated

proteins both in CD24kd and CD44kd. (**c**) Up-regulated proteins both in CD24kd and CD44kd. Intensity is transformed by log2 FLQ intensity after subtract.

3.2 N-glycomics comparison of CD24kd, CD44kd with Control

After the colon cancer cell lines were transplanted onto mice, the biosynthesis of N-glycans was incorporated in mouse tissue, which meant that the Neu5Gc was involved into this process[101]. In the three groups of tissue, 324 N-glycan compositions (647 N-glycan structures) were identified with 315 species in common (Fig.13 a) (Table S7). For the PCA, the first two principal components showed 49.0% of variance among three groups of tissue and CD24kd group had an outlier.

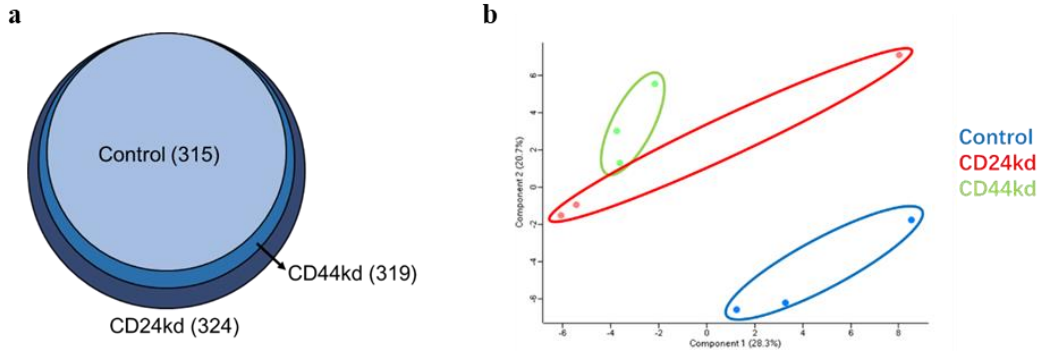


Fig. 13 (a) the numbers of N-glycans identified in three groups **(b)** Principal Component Analysis (PCA) for three groups. The unsupervised clustering based on log2 transformed and normalized of N-glycans abundance.

18 N-glycan compositions changed significantly in CD24kd tissue compared to Control tissue, including 9 down-regulated and 9 up-regulated species (Fig.14). Of them, 16 N-glycan have the Neu5Gc or Neu5Ac monosaccharide. The relative abundance of 18 regulated N-glycan shows in the Fig.15. The top 5 abundance N-glycans are $HexNAc_3Hex_3Fuc_2Red\text{-}HexNAc_1$, $Neu5Ac_2HexNAc_3Hex_5Red\text{-}HexNAc_1$, $Neu5Gc_1HexNAc_3Hex_6Fuc_1Red\text{-}HexNAc_1$, $Neu5Gc_1Neu5Ac_1HexNAc_4Hex_7Red\text{-}HexNAc_1$, $Neu5Gc_1Neu5Ac_1HexNAc_5Hex_8Red\text{-}HexNAc_1$, $Neu5Gc_1Neu5Ac_2HexNAc_4Hex_6Fuc_2Red\text{-}HexNAc_1$, $Neu5Gc_1Neu5Ac_2HexNAc_5Hex_6Fuc_1Red\text{-}HexNAc_1$. $Neu5Gc_1Neu5Ac_2HexNAc_4Hex_6Fuc_2Red\text{-}HexNAc_1$ expression in CD24kd was significantly higher than that in Control (31.3-FC).

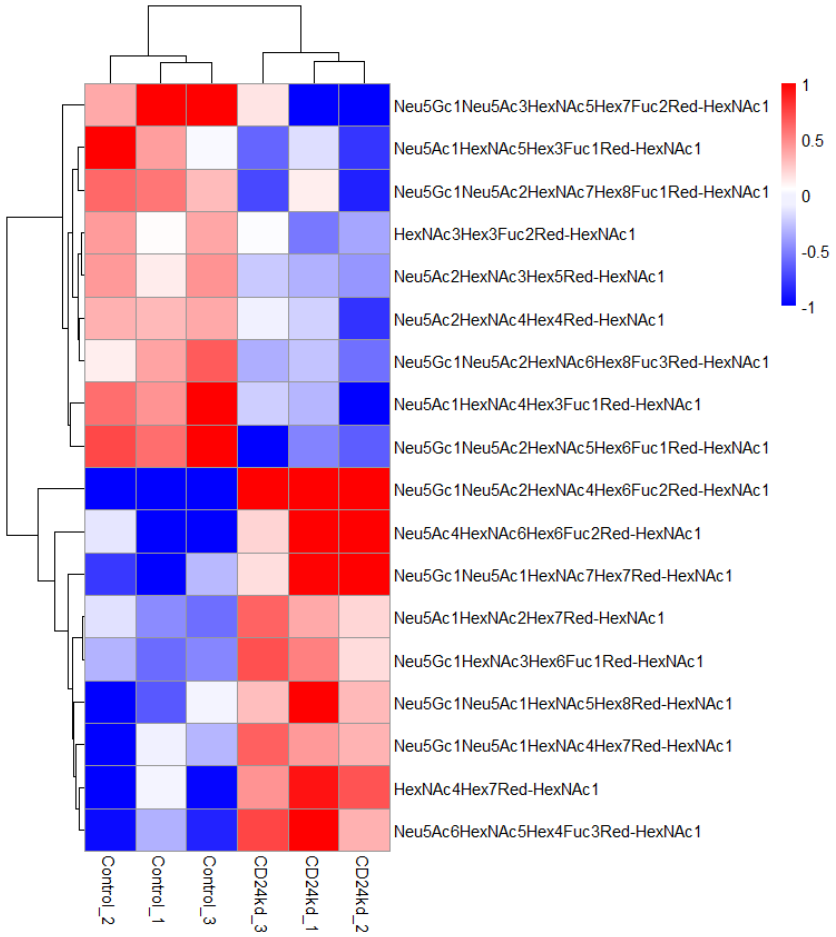


Fig. 14 Heat-map comparisons of differential N-glycans expression (CD24kd vs Control). N-glycans that displayed greater than a 1.5-|FC| and lower than 0.05 P-value in expression were identified and plotted in a heatmap.

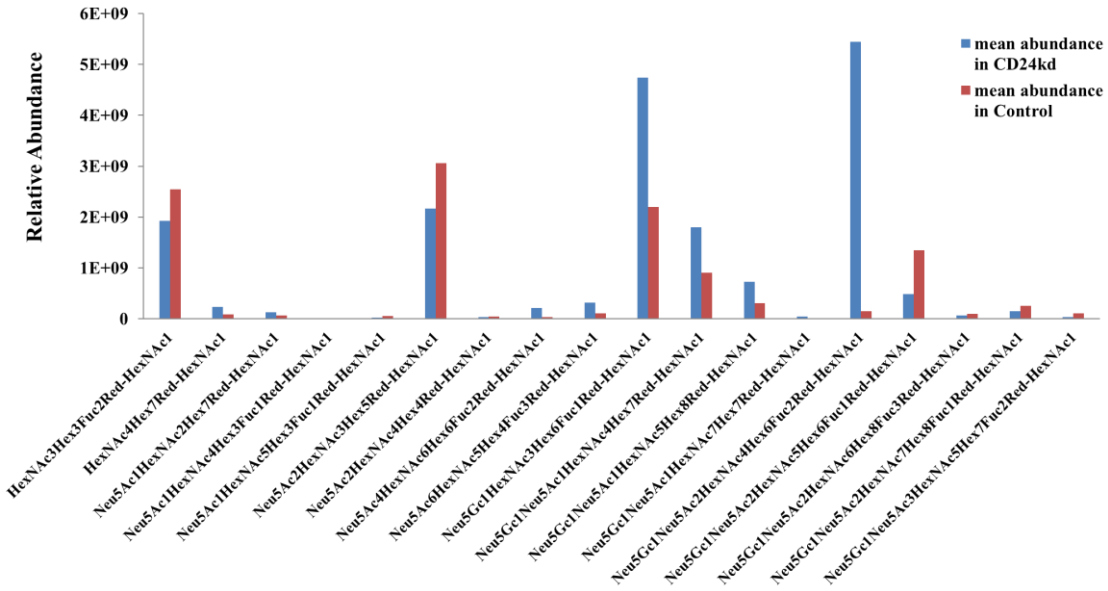


Fig.15 18 significant differential N-glycans mean value of abundance in CD24kd and Control.

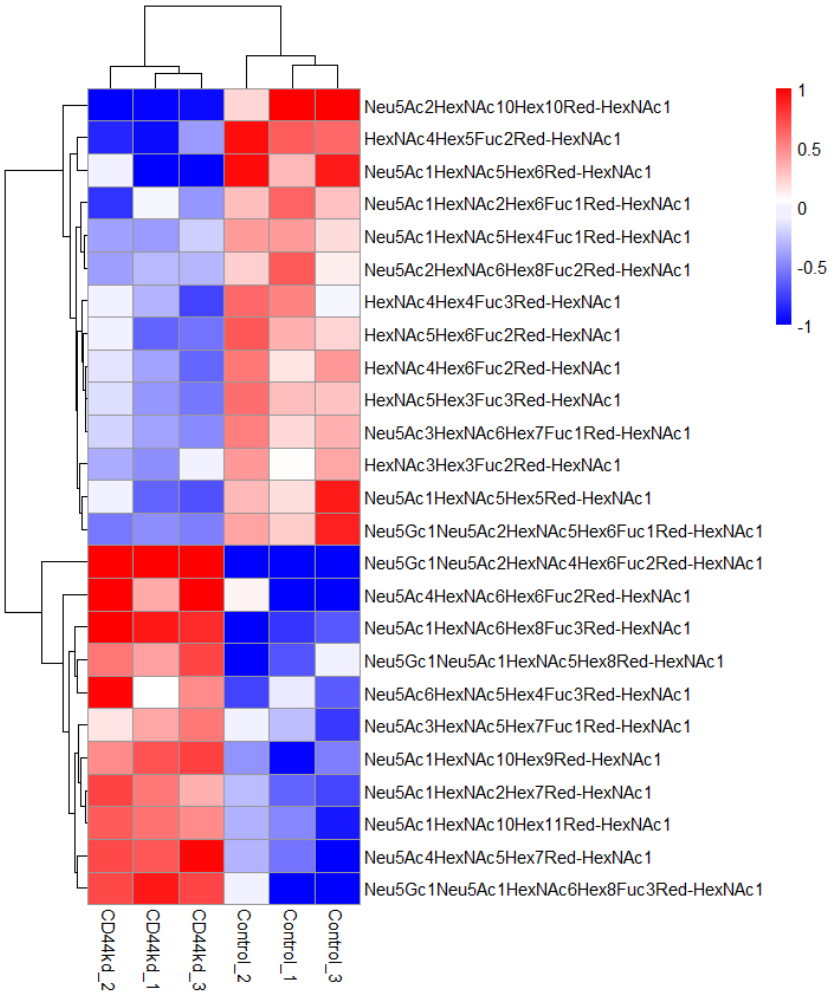


Fig. 16 Heat-map comparisons of differential N-glycans expression (CD44kd vs Control). N-glycans that displayed greater than a 1.5-FC and lower than 0.05 P-value in expression were identified and plotted in a heatmap.

25 N-glycan compositions changed significantly in CD44kd tissue compared to Control tissue, including 14 down-regulated and 11 up-regulated species (Fig. 16). 76% N-glycan compositions have the Neu5Gc or Neu5Ac monosaccharides. The relative abundance of 25 regulated N-glycan shows in the Fig. 17. The top 5 abundance N-glycans are $HexNAc_3Hex_3Fuc_2Red\text{-}HexNAc_1$, $HexNAc_5Hex_3Fuc_3Red\text{-}HexNAc_1$, $Neu5Ac_3HexNAc_6Hex_7Fuc_1Red\text{-}HexNAc_1$, $Neu5Ac_4HexNAc_5Hex_7Red\text{-}HexNAc_1$, $Neu5Gc_1Neu5Ac_2HexNAc_4Hex_6Fuc_2Red\text{-}HexNAc_1$.

$Neu5Gc_1Neu5Ac_2HexNAc_4Hex_6Fuc_2Red\text{-}HexNAc_1$ expression in CD44kd was significantly higher than that in control (23.75-FC).

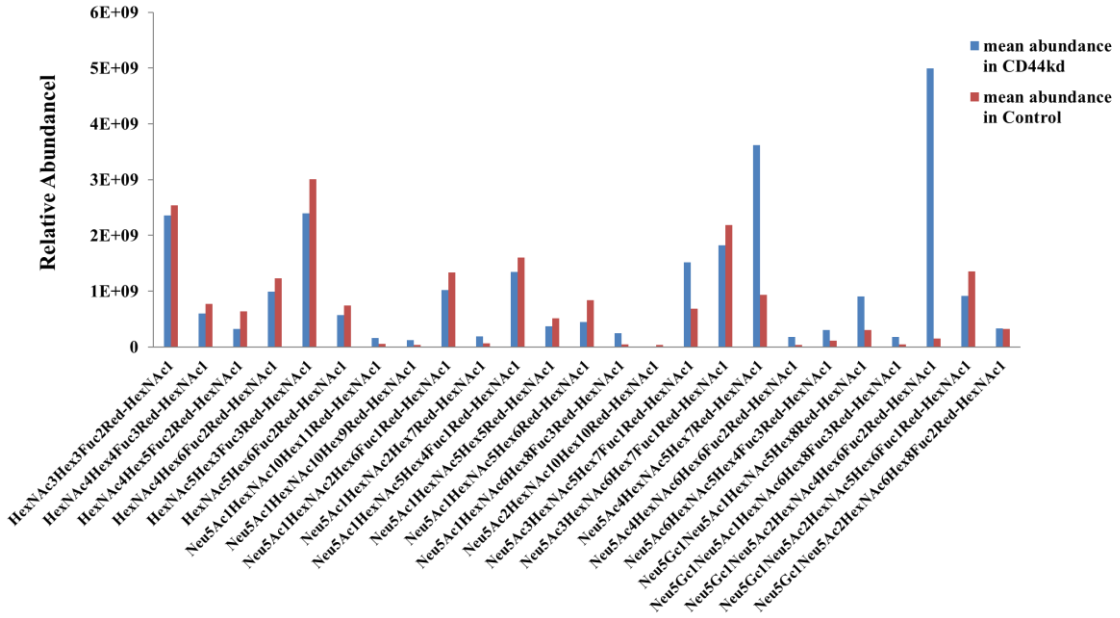


Fig. 17 The 25 significant regulated N-glycan mean value of abundance in CD44kd and Control.

All the regulated N-glycans in CD24kd and CD44kd are compared in a Venn diagram (Fig.18). There are 7 common regulated N-glycans present, $Neu5Gc_1Neu5Ac_2HexNAc_5Hex_6Fuc_1Red\text{-}HexNAc_1$, $HexNAc_3Hex_3Fuc_2Red\text{-}HexNAc_1$ are downregulated both in two groups and $Neu5Ac_6HexNAc_5Hex_4Fuc_3Red\text{-}HexNAc_1$, $Neu5Gc_1Neu5Ac_2HexNAc_4Hex_6Fuc_2Red\text{-}HexNAc_1$, $Neu5Gc_1Neu5Ac_1HexNAc_5Hex_8Red\text{-}HexNAc_1$, $Neu5Ac_1HexNAc_2Hex_7Red\text{-}HexNAc_1$, $Neu5Ac_4HexNAc_6Hex_6Fuc_2Red\text{-}HexNAc_1$ are upregulated in CD24kd and CD44kd.

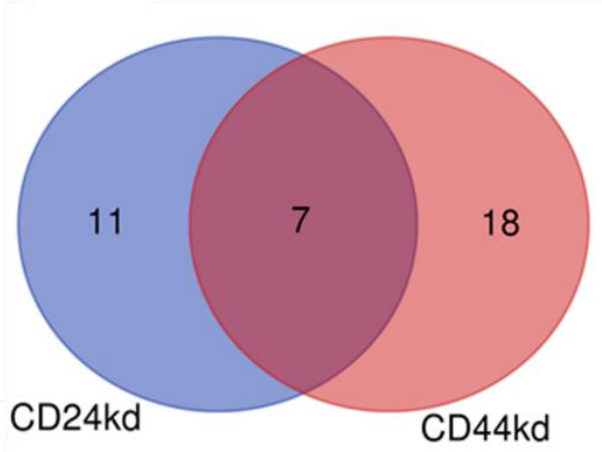


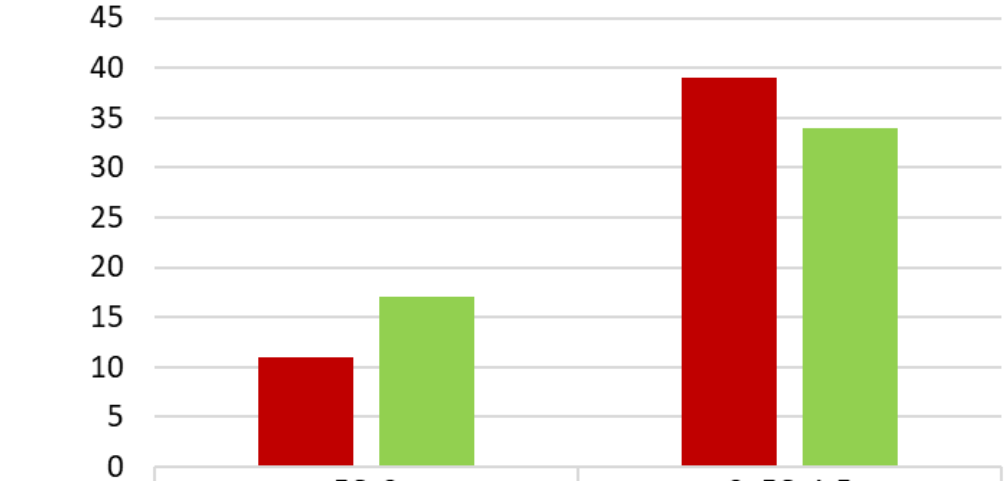
Fig. 18 Venn diagram comparing regulated N-glycans obtained from CD24kd and CD44kd

4. Discussion

Given that CD24 and CD44 mediate important cancer cellular functions and have been implicated in cancer cell development, mainly including proliferation, migration, adhesion, invasion and metastasis, they are potential therapeutic targets[102]. According to the overall results in previous studies, CD24 could be a potential target for a monoclonal antibody-mediated therapy [103]. Many inhibitors of CD44 have been proposed to have therapeutic benefits for multiple cancers. [104] In this study, we investigated the proteome and N-glycome changes resulting from gene knockdown in order to identify unique proteins and N-glycans regulated by the CD24 and CD44 genes.

4.1 Differential proteomics

By means of the PCA model, a relative separation between the Control, CD24kd and CD44kd groups was found, and the proteome expression patterns in the CD24kd samples demonstrated greater similarity with the control samples, as compared with the CD44kd samples, which indicates that the proteome profile is sensitive to CD44 knockdown, thus making it possible to discriminate between CD44kd from those with Control. Even though the CD44kd group has only one more DAP than CD24kd with 1.5-fold, the number of DAPs with 2-fold between each of the two groups that Control vs CD24kd had 11 DAPs, followed by Control vs CD44kd had 17 DAPs, which matched with the above conclusion (show in Fig. 19 and Table S1, S3).



	FC>2	2>FC>1.5
CD24kd	11	39
CD44kd	17	34

Fig. 19 The number of DAPs with different FC (Fold Change) filter

4.1.1 Differential proteomics between CD24kd and Control group

The results from this study confirmed 13 DAPs downregulated with Control vs CD24kd. DDAH1, REG4 showed 2-fold lower abundance with CD24kd. DDAH1 is a key enzyme involved in the metabolism of the endogenous nitric oxide synthase (NOS) inhibitors asymmetric dimethylarginine (ADMA) and monomethyl arginine (L-NMMA). It is involved in regulation of cell population proliferation and angiogenesis. DDAH1 overexpression conveys tumor growth and angiogenesis in prostate cancer[105], while overexpression of DDAH1 in the gastric cancer cells suppressed migration, invasion and metastatic potential[106]. REG4 is associated with the growth, survival, adhesion and resistance to apoptosis of tumor cells. Furthermore, REG4 has been identified as one of the genes upregulated in colon cancer cells displaying invasive properties[107]. RPL38, belonging to the L38E family of ribosomal proteins, was highly expressed in the pancreatic ductal epithelium[108] and might be useful as tumor markers. C9orf142 (now called PAXX) is a characterized protein associated with the classical NHEJ pathway and it facilitates the assembly of the core NHEJ complex at the DNA damage site. TBL1XR1 mediate the transcription activity of various transcription factors. Its expression is involved in malignancy development.[109,110,111,112] C9orf142 and TBL1XR1 overexpression emerged as an independent prognostic marker for poor overall in colon cancer. CA9 catalyzes the rapid conversion of carbon dioxide and water into carbonic acid to control of intracellular pH and further protects tumor cells from hypoxia-induced apoptosis.[113] Moreover, CA9 overexpression induces weakening of cell adhesions and augmented cell motility. TUBA1B is a protein-coding gene and a member of the human consensus coding sequence. The α- and β-tubulin heterodimers that form the microtubules reversibly and dynamically aggregate into microtubules: the cytoskeletal elements that regulate the cell shape, cell adhesion, cell movement, replication and division, and drive mitosis and transport within the cells.[114] MGST1 protein are localized to the endoplasmic reticulum and outer mitochondrial membrane, where they protect the membranes from lipid peroxidation and oxidative stress.[115] TFRC has been relevant its role in cellular proliferation and is higher in rapidly dividing cells (including malignant cells).[116] TBL2, DAK, POLE3 and PDS5A remained to be elucidated the influence with cancer development.

37 upregulated proteins were shown in the Fig. 5 and Table S1, Table 2. Proteins of 2-

fold higher expression are KRT10, TRIM29, TNC, S100A4, KLK6, MTF2, POSTN, GAPDHS, KRT14. TRIM29 functions as an oncogene or a tumor suppressor depending on the cell type, levels of expression, posttranslational modifications, and compartmentalization.[117,118] Tao J et al. have indicated that TRIM29 expression was much higher in colorectal cancer tissue and significantly associated with the depth of tumor invasion, lymph node metastasis, distant metastasis, histological differentiation, vascular invasion and advanced tumor stage[119]. However, it is a tumor suppressor in some types of breast and bone cancers.[120] TNC is an extracellular matrix protein that is widely expressed in the stromal fibroblasts of various cancers. TNC expression was markedly upregulated in colorectal cancer tissues, and TNC silencing reduced the proliferation, migration and invasion of colorectal cancer cells.[121] S100A4 is a Ca ($^{2+}$)-binding protein and highly associated with components of the cytoskeleton. it changes the cell's morphology and makes it more susceptible to invasion from proteins, such as cathepsin B and cyclin B1, which contribute to metastasis.[122] KLK6 is a secreted trypsin-like serine protease and its expression in colon cancer can be induced by oncogenic K-RAS and also increased through caveolin 1 (CAV1) activating.[123] Previous studies mentioned that KLK6 has been implicated in angiogenesis, migration and invasion. In addition, overexpression of KLK6 correlated with a poor prognosis.[124] POSTN has been reported to affect cell adhesion during cancer development.[125] POSTN expression prevents stress-induced apoptosis in the cancer cells and augment endothelial cell survival to promote angiogenesis and then enhanced metastatic growth of colon cancer.[126] COL1A1, COL1A2 and COL6A1 are involved in collagen chain trimerization, assembly of collagen fibrils, other multimeric structures, collagen degradation. They are dysregulated in cancer cells and influenced cell metastasis and migration. EHD2 is an important member of the EHD family, which appears to regulate receptors in a broad range of human organs and tissues[127]. EHD2 overexpression suppresses the proliferation, migration, and invasion in human colon cancer. ACTG1, involved in various types of cell motility, is ubiquitously expressed in all eukaryotic cells. ACTG1 has been reported to exhibit high expression levels in skin cancer tissue, where it may regulate cell proliferation and migration via the Rho-associated protein kinase signaling pathway.[128] FBLN2 is a secreted extracellular matrix glycoprotein that is involved in tissue development and remodeling. Li D et al have proved that FBLN2 is an important tumor suppressor. FBN1, as an important and intricate lattice protein in extracellular matrixes, is involved in cancer pathogenesis.[129] TDRD1 was negative in

all cancer tissue (https://www.proteinatlas.org/ENSG00000095627-TDRD1/pathology), decreasing in CD24kd according to our study. NEFM participates in the generation of neurofilaments, which are structural components of the cytoskeleton in mature neurons. Literatures reported the deregulation of the protein in several malignancies.[130] ANXA1 is considered to be associated with cancer development in colorectal cancer because of its functions: apoptosis, anti-inflammatory effects and the regulation of cellular differentiation and proliferation.[131,132,133] FSCN1 is an actin-binding protein, whose overexpression promoted cancer cell migration, invasion, and metastasis in vitro and vivo. Alajez et al. data revealed poor overall survival and disease free survival in CRC patients when FSCN1 overexpressing in CRC.[134] PODXL is an anti-adhesive transmembrane protein belonging to the CD34 family and inhibits cell-cell interaction through charge-repulsive effects. Overexpression of PODXL is an independent factor of poor prognosis in colorectal cancer. RCN1 is a Ca^{2+}-binding protein which exists during the whole secretory pathway of mammalian cells. RCN1 overexpression has been observed in invasive cancer cells (including colorectal cancer cells), suggesting a role for RCN1 in tumorigenesis and invasion.[135] THBS2 is a member of extracellular matrix (ECM) glycoproteins and participates in diverse biological such as angiogenesis, apoptosis, cytoskeletal organization, cell proliferation, cell motility and adhesion. THBS2 was demonstrated that it can be utilized as a novel prognostic marker in colorectal cancer. NAMPT is essential for NAD biosynthesis and further affects ATP synthesis. Inhibition of NAMPT causes the attenuation of cancer cell proliferation and death. MCTS1 may play a role in promoting lymphoid tumor development, and contribute to the pathogenesis and progression of breast cancer via promotion of angiogenesis and inhibition of apoptosis. According to Gavin Stewart study, MCT1 was expressed with high MCT1 protein abundance in the HT-29 cell line under control conditions. The expression of P3H1, MTF2, NUDT4, MYADM, DYSF, PACSIN2, SUMO4, GAPDHS and OSTC need further investigation about cancer. The rest proteins, belonging to the keratin, play a role in structural and protective functions and also regulate key cellular activities, such as cell growth and protein synthesis.

C9orf142, CA9, REG4, TBL1XR1, TFRC are up-regulated in colon cancer compare with normal. However, these proteins were down-regulated after CD24 knocking down in our study. The expression of COL1A2, EHD2, FBLN2 were lower in colon cancer

than in normal sample while their abundances were higher in CD24kd versus Control. These evidences indicate that CD24 knockdown may be a viable therapeutic direction for the treatment of colon cancer and the proteins (C9orf142, CA9, REG4, TBL1XR1, TFRC, COL1A2, EHD2, FBLN2) can be the indicators for evaluating treatment against CD24 in colon cancer. However, 16 proteins have the same regulation in both comparisons. These proteins are highly expressed in colon cancers (compared with normal), and some are used as biomarker or prognostic indicators for colon cancer. These proteins' regulation shows knocking down of CD24 can further induced colon cancer development.

GO and GSEA approach was utilized to confirm enriched pathway in CD24kd. 'EXTRACELLULAR_MATRIX_ORGANIZATION' was identified. It displays weak association between CD24 and the 'extracellular matrix organization' gene set. Extracellular matrix (ECM) is the main structural component of the tumor microenvironment and it is composed of a unique network of biochemical components, including fibrin, glycoprotein, proteoglycan and polysaccharide. The increase of ECM protein deposition and cross-linking in cancer tissues promote the proliferation, migration and invasion.[136] This pathway was reported to upregulated in distinct populations of circulating cancer cells[137] and $CD24^+$ cells[138], our results was not in line with the literature.

4.1.2 Differential proteomics between CD44kd and Control group

Totally, 51 significant DAPs were identified in CD44kd. The abundance of CD44 is the lowest, which decreased 16-fold in CD44kd. ASCT2 mediates the exchange of amino acid substrates and accounts for glutamine transport and its upregulation in the cancer supports cancer cell growth and survival.[139] FABP1, belonging to the FABP family, is involved in lipid metabolism but also plays a role in the regulation of inflammation and cellular metabolism via interaction with peroxisome proliferator-activated receptors (PPARs). Studies showed that FABP1 play a role in development of colorectal cancer as differential marker which is downregulated at the adenoma stage. MUC2, MUC5AC and MUC5B belong to gel-forming MUCs that covers on the intestinal mucosal surface and participates in the processes of intercellular adhesion, signal transduction and immune regulation.[140] LPCAT1 has been an independent predictor of early tumor recurrence and represents a novel prognostic biomarker for many types of cancers.

Morita Y et al. performed cell line experiments, LPCAT1 overexpression enriched phosphatidylcholines and promoted cell proliferation, migration and invasion, while LPCAT1 knockdown did viceversa in hepatocellular carcinoma[141]. SHMT2 is linked to several aspects of metabolism that are important for the survival and reproduction of cancer cells. Due to the differences between cells, the expression of SHMT2 shows multiple regulations in cancers.[142] RPL36 has been strongly associated with development of tumors. RPL36 expression may be involved in early hepatocarcinogenesis and RPL36 is a promising biomarker for prediction of prognosis in hepatocellular carcinoma.[143] GFPT2 plays important roles in cell life activities, and its upregulation promoted the proliferation, invasion and metastasis of CRC cells. Apolipoprotein A2 (APOA2) is the second major protein of the HDL-C particles and comprises about 20% of the total HDL-C protein content.[144] APOA2 has been used in clinical research of various types of cancer: screening for high-risk status of pancreatic cancer[145], potential bladder cancer markers in urine[146], one of the combinatorial markers in colorectal carcinoma with blood tests[147]. WFS1, PYCR2, LBR, IDI1, MOGS, RDH11, HSD17B11 and RBM15 are further investigated in cancer tissue (colon cancer).

RAD50 is mainly involved in DNA repairing to prevent mutations of chromosomal aberration and suppress tumorigenesis. Chen et al have reported that RAD50 was upregulated in CRC cancerous tissue samples compared to non-cancerous adjacent tissues and elevated RAD50 expression was associated with poor patient survival in CRC. SPINK1 plays a role in stimulating mucosal repair at the site of injury and protection of the mucus layer from excessive digestion in the gastrointestinal tract. Knocking down SPINK1 significantly decreases cell proliferation and invasion in the colon adenocarcinoma WiDr cells. FTH1 is a 21 kDa subunit of the ferritin complex. The ferritin complex captures intracellular ferrous iron (Fe^{2+}) and converts it into ferric iron (Fe^{3+}) by the ferroxidase activity of FTH1, which potentially reduces DNA damage caused by Fe^{2+} induced reactive oxygen species (ROS) and protects cancer cells from cell death. FTH1 may suppress tumor metastasis and therefore serve as a potential therapeutic target for Triple Negative Breast Cancer.[148] The dismutase SOD2 localizes in the matrix and is a major antioxidant to alter the amount of H_2O_2. SOD2 overexpression enhances invasiveness and migration of cancer cells. TUBB4B is critical for microtubule-vimentin interaction. Furthermore, TUBB4B could be a marker

for detection of the preinvasive stages of the colon cancer cells since it has been shown that the protein decrease is accompanied by cell elongation and increased number of matured focal adhesion sites, which is a characteristic of the cell metastatic stage. TACSTD2, as a cell-surface glycoprotein, plays a role in regulating the growth of carcinoma cells. TACSTD2 has been reported to be highly expressed in invasive ductal breast cancer with a poor prognosis in invasive ductal breast cancer.[149] SNCG is one member of the synuclein family and has reported as a critical player in cancer metastasis. The upregulated SNCG mediated cell proliferation and metastatic ability via activating the PI3K/AKT pathway of high-grade serous ovarian cancer and SNCG silencing inhibited the ovarian cancer cell migratory capacity.[150] NDUFA4 participates in glycolysis in tumor cells. The downregulation of NDUFA4 was observed in in renal cell carcinoma by Ellinger J group and discovered that NDUFA4 can be a novel biomarker to help to identify RCC and monitor treatment response.[151] CYP2S1 is involved in the metabolism of toxic and carcinogenic compounds. Yang et al. demonstrated that CYP2S1 knockdown elevate cell proliferation in colorectal HCT116 cells in vitro.

DNAJA1 participates in various pathological conditions including cancers. DNAJA1 upregulation in CRC tissues has been reported closely related with the increase of serosa invasion, lymphatic metastasis and poor survival of CRC patients. BAG2 plays important roles in the regulation of apoptosis, cell survival and the stress response. Proteomics reflects the high expression of BAG2 in different types of tumors, including ovarian cancer, papillary thyroid carcinoma, fibrosarcoma and multiple myeloma.[152] ABCB4 is also involved in the cancer resistance and growth. Knockdown of ABCB4 suppresses the caspase-dependent apoptosis pathway, in returns overexpression of the ABCB4 induced the apoptotic response in the HCT8R cells. BASP1 functions in transcriptional regulation, apoptosis, and differentiation have been reported. Lung tumors exhibited higher BASP1 expression than adjacent normal lung tissues and associated with tumor progression and poor outcomes.[153] CEACAM1 is a cell adhesion molecule of the immunoglobulin family behaving as a tumor inhibitory protein in colon cancers. CEACAM1 downregulation significantly inhibited cell proliferation and promoted cell apoptosis. HLA-A is part of leukocyte antigen class I molecules (HLA-I), which together with a peptide and costimulatory molecules are presented to cytotoxic T lymphocytes. VAT-1 is a largely uncharacterized enzyme involved in cell migration. CALU (Calumenin) contains a N-terminal signal peptide (19 amino-acids) and multiple

EF-hand domains.[154] It has been demonstrated to be associated with invasiveness and metastasis in some malignancies. The relationship between cancer and proteins of PPP1R11 and DENR are still unclear.

FABP1 and APOA2 have the downregulation in both comparisons. SLC1A5, CD44, SHMT2, MUC5AC, MUC5B, MUC2, GFPT2, LPCAT1 and REG4 are up-regulated in colon cancer compare to normal. However, these proteins were downregulated after CD24 knocking down in our study. The abundance of ABCB4, CEACAM1, CYP2S1, EHD2, HLA-A were lower in colon cancer than in normal sample while their abundances were higher in CD24kd versus control and the proteins can be the indicators for evaluating treatment against CD44 in colon cancer. TACSTD2, SNCG, CALU, RAD50, DNAJA1, FSCN1, KLK6, POSTN, RCN1, S100A4, SOD2, SPINK1 and TRIM29 have the same upregulation in both comparisons. According to above discussion, the proteins that have the different regulation and same regulation predicts CD24 knockdown leads to reduced and induced for colon cancer development respectively.

The GO and GSEA approach confirm pathway 'REACTOME_BIOLOGICAL_OXIDATIONS' was enriched in CD44kd vs Control. Redox imbalance is resulted from the destruction of balance between oxidants and antioxidants. The dominant oxidants are reactive oxygen species (ROS) and high level of ROS can positively affect cancer cell growth, metabolism, invasion and metastasis through gene mutation, DNA damaging, protein conformation transition and so on.[155] The expression of CD44 (CD44v) contributes to ROS defense, which also enhances cancer development and increases chemotherapy resistance.[156] The pathway was negatively correlated to CD44kd samples in our study, that is consistent with the literatures mentioned above.

4.1.3 DAPs in CD24kd and CD44kd

After respective knockdowns of CD24 and CD44, comparison of regulated proteins in a Venn-diagram revealed an overlap of 14.8% (Fig. 13a). This low overlap indicates that the effect from CD24 and CD44 knockdown differ considerably. 13 common regulated proteins have consistent regulation in CD24kd and CD44kd compared with Control. KRT10, NUDT4, FSCN1 and S100A4 have the different fold change and

others show similar fold change. CD24 or CD44 has a number of similar effects in CRC.

4.2 Differential N-glycomics

Changes in glycosylation, which is one of the most common protein post-translational modifications, are considered to be a hallmark of cancer.[157] N-glycans can modulate cell migration, cell-cell adhesion, cell signaling, growth and metastasis.[158] According to our results, most of the regulated N-glycans contained Neu5Ac or Neu5Gc residues either in CD24kd vs Control or CD44kd vs Control.

In general, sialylation is frequently observed in tumor tissue compared to corresponding normal tissue[159]. The total amount of sialic acids in serum or glycolipid-bound sialic acids is significantly elevated in multiple cancers such as ovarian cancer, leukemia, colorectal cancer and breast cancer[160]. It was reported that sialic acid metabolism was upregulated in highly metastatic breast tumors, while knocking out CMAS gene, a key node in sialic acid metabolism, inhibited the synthesis of the activated form of sialic acid and decreased the formation of lung metastases in vivo[161]. On the other hand, the aberrant expression of sialyltransferases and sialidases accelerated and sustained sialylation status on glycoconjugates. The sialylation further facilitates immune escape, enhances tumor proliferation and metastasis, helps tumor angiogenesis and assists in resisting apoptosis and cancer therapy (Fig. 20)[162]. Despite the fact that humans are genetically unable to produce Neu5Gc, this molecule is detectable on surfaces of human epithelia and endothelia and in higher amounts in malignant tissue[163]. The only possible source for incorporation is dietary intake, like red meat and milk products[164] (Fig.21). For humans, Neu5Gc-containing antigens are immunogenic and this may promote tumor growth. Low doses of anti-Neu5Gc antibodies sustain Neu5Gc-positive tumors in Neu5G deficient mice by triggering a chronic inflammation that helped tumor growth[165]. Thus, Neu5Gc maybe the link between red meat consumption and the colorectal cancer.

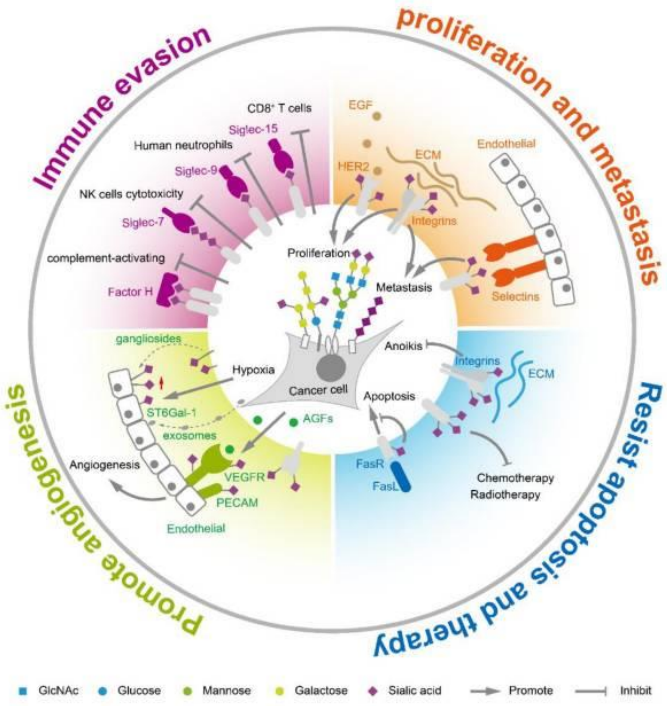


Fig. 20 The functions of sialic acids in tumor biology[162].

$Neu5Gc_1Neu5Ac_2HexNAc_4Hex_6Fuc_2Red\text{-}HexNAc_1$ is the most significantly upregulated N-glycan in two comparisons. This glycan may have closed relationship with CD24 and CD44 in CRC, and figure out this relationship may have a clearer understanding for future cancer treatments by CD24 and CD44 or be a factor.

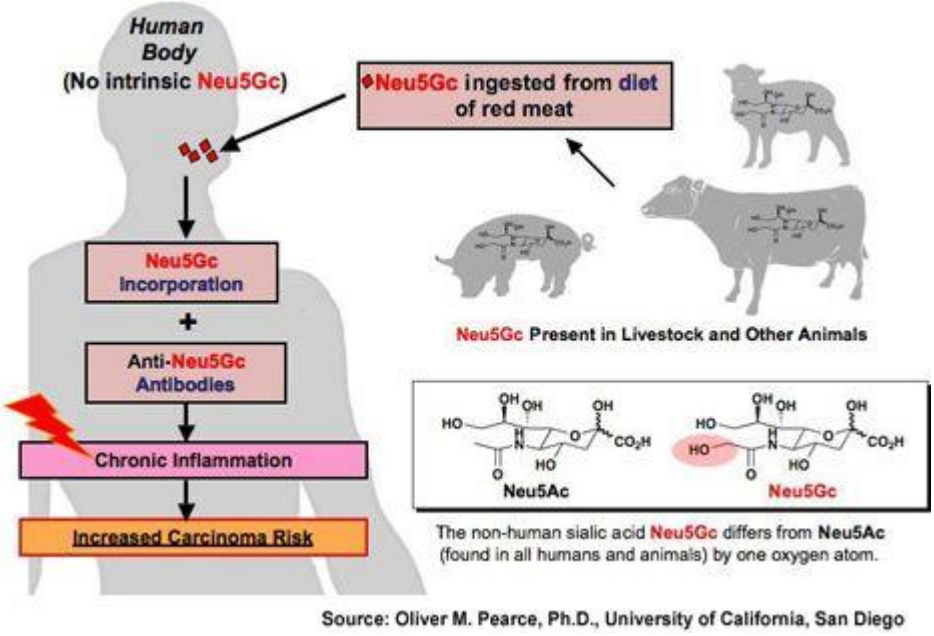


Fig. 21 The possible source of Neu5Gc for human[164].

5. Conclusion

In summary, N-glycomics and proteomic approaches were used to investigate the effects after the knockdowns of CD24 and CD44 in CRC. A number of DAPs are associated with cancer development (metastasis, invasion et al.). As opposed to primary effects of gene knockdown, certain compensatory changes occur over time, and lead that the regulation of some DAPs are not coincide with references. We cannot get a clear conclusion about whether knockdown of CD24 or CD44 has a negative effect on cancer development. Further studies focused on DAPs may provide valuable insights into the physiological and pathophysiological roles of CD24 and CD44 in CRC. But some of the DAPs still can be the indicators for evaluating treatment against CD24 and CD44 in colon cancer. EXTRACELLULAR_MATRIX_ORGANIZATION and REACTOME_BIOLOGICAL_OXIDATIONS are the main pathways involved in the development of CRC after knockdown CD24 and CD44, respectively. In addition, the regulated N-glycans containing Neu5Ac and Neu5Gc especially $Neu5Gc_1Neu5Ac_2HexNAc_4Hex_6Fuc_2Red\text{-}HexNAc_1$, which was most affected by CD24 and CD44 knockdown on CRC. Finally, while there is an overlap in the results of differentially regulated proteins and N-glycans for the CD24 or CD44 knockdown condition, the differences are far greater. Altogether, these results extend our knowledge on the molecular changes associated with CD24 and CD44 knockdown in colon cancer.

6. Materials and Methods

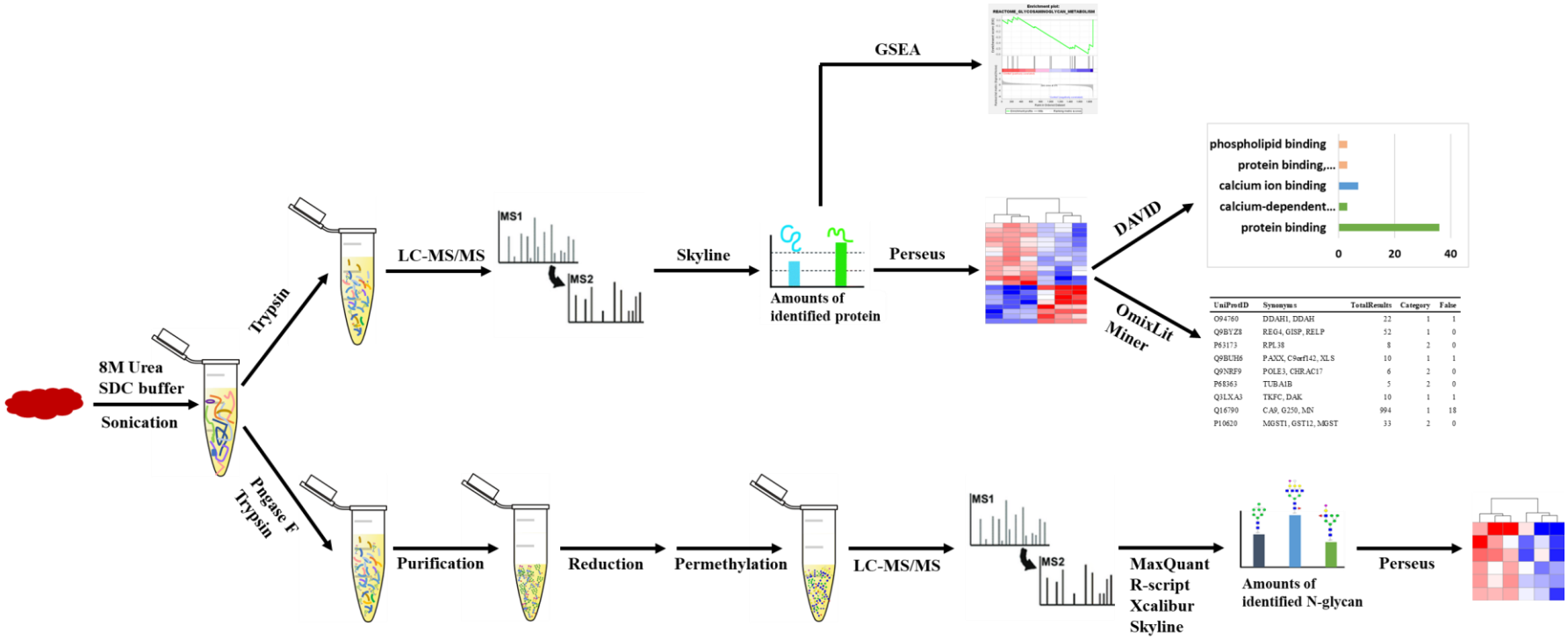


Fig. 22 The workflow from sample preparation to data analysis for proteomics and N-glycomics.

A general overview of the samples and the workflow employed in the experiments in this study is as depicted in Fig. 22 above. All the samples used in this study were provided by Dr. Tobias Lange from the Institute of Anatomy and Experimental Morphology at the University Medical center Hamburg Eppendorf (UKE). They were all processed and analyzed at the laboratory of Prof. Schlüter, the Section Mass Spectrometry Proteomics at the UKE.

6.1 Materials

Phosphate-buffered saline (PBS) was purchased from Thermo Fisher Scientific (Bremen, Germany). Sodium hydroxide, dimethylsulfoxide (DMSO), iodomethane, sodium deoxycholate (SDC) and tetraethylammonium bromide (TEAB) were purchased from Sigma (Darmstadt, Germany). All other chemicals were also purchased from Sigma unless otherwise stated. Sequencing grade modified trypsin and PNGase F were obtained from Promega (Madison, WI, USA). 0.5 mL centrifugal filters (3k and 10k devices) were purchased from Merck KGaA (Darmstadt, Germany). Solid-phase-extraction (SPE) columns containing reversed-phase (RP) materials (C_{18} Sep-Pak cartridges) were obtained from Waters (Miford, MA, USA). All experiments were approved by the local ethical committee and conducted according to the guidelines for the Care and Use of Laboratory Animals.

6.2 Tryptic digestion of the proteins extract

Colon cancer cell lines HT29 were cultured into three groups, including Control, CD24 knockdown and CD44 knockdown cells. These three groups of cell lines were transplanted onto mice to obtain tumor tissue, named as Control, CD24kd and CD44kd respectively.

At 4°C room, tissue was firstly cut into species and then grinded with cooling by dry ice. The powders of tissue were dissolved into 8 M urea and SDC buffer (1% SDC in 0.1M TEAB), followed by sonication with 25% of normal energy at 3 cycles for 30 sec on ice.[166] The protein concentration was measured using the Pierce™ BCA Protein Assay Kit, following the manufacturer's instructions (Thermo Fisher Scientific, Bremen, Germany).

For trypsin digestion, Filter-Aided Sample Preparation (FASP) protocol was performed.[167] Briefly, 200 µg extracted proteins were transferred into 10 k centrifugal filters, reduced by 20 mM dithiothreitol (DTT) at 60°C for 30 min and alkylated by 40 mM iodoacetamide (IAA) at room temperature for 30 min in the dark. DTT and IAA were prepared in 100 mM ammonium bicarbonate (AmbiCa) buffer. Then the samples were desalted by centrifugation at 12,000 rpm using a Centrifuge 5424 (Eppendorf AG, Hamburg, Germany) and exchanged by 100 mM ABC buffer. Trypsin (1/50, w/w) was added at 37°C for 20 h in the centrifugal filters. After centrifugation, the peptides were lyophilized and stored at -20° until further analysis by MS-based data independent acquisition (DIA).

6.3 DIA MS analysis for peptides

Tryptic peptides were dissolved in 0.1% (v/v) FA, transferred to an autosampler and injected into a Dionex Ultimate 3000 UPLC system (Thermo Fisher Scientific, Bremen, Germany). Peptides were purified and desalted using an RP C_{18} trapping column (Thermo Scientific™ Acclaim PepMap™, 100 µm×2 cm, 5 µm, 100Å) at a flow rate of 15 µL/min with 1% solvent B (0.1% (v/v) FA in ACN) and 99% solvent A (0.1% (v/v) FA) and then transferred to an analytical RP C_{18} column (Thermo Scientific™ Acclaim PepMap™ RSLC, 75 µm×50 cm, 2 µm, 100Å) after 10 min at a flow rate of 0.3 µL/min with 3% solvent B. A gradient elution phase then followed with an increase in the concentration of solvent B to 30% in 70 minutes followed by an increase to 90% in a 0.1 minute, 90% in 5 minutes, a 0.1minute hold phase and back to 3% for 15 minutes for column equilibration. The column temperature was maintained at 45 °C. The eluted peptides were ionized via electrospray-ionization in positive mode. DDA method with following settings was used for library generation, MS1 scan: 120,000, AGC target: 1×10^5, max injection time: 120 ms; scan range: 400 to 1300 m/z, the spectra data type: profile, charge state: 2-5. MS2 scan: isolation mode: Quadrupole, isolation window: 1.6 m/z, activation type: HCD, HCD collision energy: 30%, Orbitrap resolution: 15,000, AGC target: 2×10^5. For DIA method the same LC was employed as described above. And the MS scan range (m/z) from 390 to 1210, the resolution of 60,000 with automatic gain control (AGC) target at 200,000. The maximum injection time was 50ms. The MS/MS scans isolation windows (m/z) was 20 to generate DIA segments from m/z 350 to 1,800 according to the m/z distribution of all precursors. The HCD collision energy

of was set to 28%, AGC target of $1x10^5$. Protein

6.4 Protein Quantification & statistical analysis

For DDA-MS files, the Proteome Discoverer 2.0 (Thermo Fisher Scientific) search engine was used with Sequest HT and Percolator. Raw files were searched against the SwissProt database downloaded from Uniprot in June 2019. The setting parameters were as follows: Enzyme, trypsin (Full); maximum missed cleavage sites, 2; precursor mass tolerance, 10 ppm; fragment mass tolerance, 0.02 Da. Methionine oxidation (+15.995 Da) and acetylation (+42,01057 Da) at serine and lysine as dynamic modifications, and also carbamidomethylation (+57.021 Da) on cysteine as static modification. All spectra were validated by Percolator Node with the false discovery rate of 1%. All other settings were set to default. The DDA result files were generate from PD output files. Imported the results to Skyline 20.1 (MacCoss Lab, University of Washington) and set the cutoff score to 0.99. The skyline will start reading the files and building the spectral library which includes their observed chromatographic retention time, their mass, and their fragmentation pattern from tandem mass spectra. And then import the DIA raw data to identify the proteins and quantify the area of the peptide peaks. The setting parameters matches the accuracy of the Proteome Discoverer, MS instrument. Other settings were in accordance with the previous literature[168].

Uniprot IDs were converted to human gene names, delete the duplicate, blank and mice gene name. The statistical software used was Perseus version 1.5.1.6[169] for T tests, and filtering for valid values. Only the proteins identified with p-value less than 0.05 and |FC| more than 1.5 were considered as statistically significant differential abundant among the compared groups. Heatmaps were generated by the R environment.

6.5 DAPs researching (OmixLitminer) and Gene Ontology (GO) enrichment

To evaluate the regulated proteins in CD24kd or CD44kd in cancer, a guide database search was performed using OmixLitminer. OmixLitMiner is a bioinformatics tool for prioritizing biological leads from 'omics data using literature retrieval and data mining[170]. In the input .xlsx file, “accession” was chosen as the ID Type, TaxID is 9606 (human), “cancer” is keyword and KeywordInTitelOnly fill in “no”. All scientific articles containing all query terms above can be found either in the title or the abstract of a publication. The tool assigns the proteins into three main categories (1–3) and an

additional Category 0. Category 1 hits are proteins/genes which show at least one review paper where the synonyms and the selected keywords are found together in the article title and in the abstract. Category 2 hits are proteins/genes where at least one publication was found, but no review article, in which the synonyms and the selected keywords are both present. Category 3 represents proteins/genes where no publication was found which mentions both the synonyms and the keywords together. Category 0 is used for proteins/genes where the tool could not find any synonyms. Further manually explored the results from OmixLitminer with "colon cancer". According to the literatures, some proteins regulations were figured out in comparison of colon cancer and normal.

In order to deepen the understanding of the significantly DAPs, Gene Ontology (GO) enrichment was retrieved using DAVID. The significantly enriched GO terms in biological process, molecular function and cellular component branches are presented.

6.6 Gene set enrichment analysis (GSEA)

Subramanian et al. suggested a novel approach for gene expression data analysis, called Gene Set Enrichment Analysis (GSEA)[171]. It is a computational method that determines whether an a priori defined set of genes shows statistically significant, concordant differences between two biological states. We used GSEA (version 4.1.0) to analyze microarray data from the colon cancer tissue. Perseus was used to transform, normalize quantitative protein data. Expression dataset and phenotype labels' file format did according to the GSEA User Guide (https://www.gsea-msigdb.org/gsea/doc/GSEAUserGuideFrame.html). A gene set is a group of genes that shares pathways, functions, chromosomal localization or other features. In this study, we chose the C2 cured REACTOME and KEGG gene sets deposited in the molecular signature database (https://www.gsea-msigdb.org/gsea/msigdb/collections.jsp#C2). The minimum and maximum criteria for selection of gene sets from the collection were 15 and 500 genes, respectively. Only gene sets identified as enriched with a nominal p-value less than 0.05 were considered with statistical significance.

6.7 N-glycan release, purification and permethylation

To obtain the free N-glycans, the extracted proteins were denatured, reduced and alkylated as mentioned above. Then desalting and buffer exchange were performed

using 3k centrifugal filters to ammonium bicarbonate (ABC) buffer. Thirty units of PNGase F was added and incubated at 37°C for 24 h to release N-glycans, followed by trypsin digestion for another 20 h. N-glycans and tryptic peptides were separated as described by Morelle et al. using a RP-SPE C_{18} cartridge.[172] The RP cartridge was conditioned with 5 mL methanol and equilibrated with 10 mL 5% (v/v) acetic acid respectively. Then each digested sample was loaded into the cartridge and N-glycans were eluted with 5 mL 5% (v/v) acetic acid. The N-glycan solvent was evaporated using a SpeedVac™ vacuum concentrator. Then the optimized solid-phase permethylation was performed, which has been developed in our previous study.[173] Briefly, 10 μg/μL borane-ammonia complex was added to dried N-glycans and incubated at 60°C for 1 h. After evaporation, 110 μL of water/DMSO (10/100, v/v) and 100 μL iodomethane was added and transferred into sodium hydroxide beads (200 mg) in glass vial by rotation for 10 min. The solution was transferred into a new vial with one more addition of 150 μL DMSO into the sodium hydroxide beads. After addition of 200 μL 5% (v/v) acetic acid, permethylated N-glycans were purified using chloroform-water extraction and dried by a SpeedVac™ vacuum concentrator, followed by MS analysis.

6.8 Nano LC-MS/MS measurement for permethylated N-glycans

Permethylated N-glycans were first dissolved into 0.1% (v/v) FA, transferred to an autosampler and injected into a Dionex Ultimate 3000 UPLC system (Thermo Fisher Scientific, Bremen, Germany). Permethylated N-glycans were purified and desalted using an RP C_{18} trapping column (Thermo Scientific™ Acclaim PepMap™, 100 μm×2 cm, 5 μm, 100Å) at a flow rate of 3 μL/min with 2% solvent B (0.1% (v/v) FA in ACN) and 98% solvent A (0.1% (v/v) FA) and transferred to an analytical RP C_{18} column (Thermo Scientific™ Acclaim PepMap™ RSLC, 75 μm×50 cm, 2 μm, 100Å), at a flow rate of 0.2 μL/min, for chromatographic separation. For permethylated N-glycans, a 90 min gradient was used, starting with 10% solvent B. Solvent B increased to 30% in 5 min followed by a linear gradient elevating the concentration of 75% in 70 min and finally increased to 95% in 80 min. Eluted N-glycans were ionized using a nano spray ion source for electrospray ionization at a capillary voltage of 1.8 kV. Derivative N-glycan ions were transferred to a tribrid quadrupole-orbitrap-ion trap mass spectrometer (Fusion, Thermo Fisher Scientific, Bremen, Germany). For MS1 scanning, an orbitrap mass analyzer was used with an orbitrap resolution of 120,000 FWHM at m/z 200; the

maximum injection time was 120 ms, to an AGC target of 2×10^5; m/z scan range was from 450 to 2,000. Data dependent acquisition was used in the top speed mode. For CID-MS/MS, the most intense precursor ions were selected for fragmentation and isolated using an isolation window of 3; the normalized collision energy of CID was set to 35%; fragment ions were injected to an ion trap with maximum injection time was 20 ms at an AGC target of 1×10^5. The data were visualized and analyzed using Xcalibur software.

6.9 Identification and quantification for N-glycomics

An improved informatics strategy has been developed in our previous study. Briefly, MaxQuant in the version 1.6.2.3 (http://www.maxquant.org) was used to extract masses of all tentative glycan precursors in an "allPeptides.txt" file from the MS raw data[174]. In this "allPeptides.txt" file, all the masses from the "Mass" column are extracted as "Mass.csv" and matched to the possible monosaccharide compositions using designed R-scripts (https://github.com/guan181992/Glyco-informatics), outputting the result file named as "Monosaccharide composition.csv". Three tissue groups derived N-glycan MS raw data were processed by mouse derived R-script. After matching to monoisotopic m/z, bundled sequencing algorithm is used to illustrate the N-glycan structure with the MS2 data using GlycoWorkbench in the version 2.1 stable build 146 (https://download.cnet.com/GlycoWorkbench-64-bit/3000-2383_4-75758804.html)[175]. The N-glycan compositions identified from CD24kd and CD44kd species were compared to the Control groups, respectively. The result that are exported from Skyline included the monosaccharide composition and Total Area MS1 (the abundance of each monosaccharide composition) to input into Perseus in the version 1.6.2.1 (http://www.perseus-framework.org). Normalization and two-sample student's T-test were performed to compare the N-glycome quantification profile, in which $p < 0.05$ and a FC more than 1.5 were defined as the minimum requirements to show statistical significance. Heatmap were made as the same processing as above.

7. References

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8. Supplement

Table S1. Table showing the significant (p-value < 0.05, |fold-change| > 1.5) regulated proteins after CD24 knockdown.

Uniprot ID	Gene symbol	$-Log_{10}$(p-value)	Log_2(FC)
O94760	DDAH1	2.34902537	-1.2862148
Q9BYZ8	REG4	1.47400983	-1.0466143
P63173	RPL38	2.12827506	-0.8288091
Q9BUH6	C9orf142	3.32553727	-0.7614091
Q9NRF9	POLE3	1.43266793	-0.7322712
P68363	TUBA1B	1.60064133	-0.7059174
Q3LXA3	DAK	1.80788786	-0.6909218
Q16790	CA9	1.53570637	-0.6424383
P10620	MGST1	1.35725092	-0.6245111
P02786	TFRC	1.92234825	-0.6148993
Q9BZK7	TBL1XR1	1.65015146	-0.612208
Q9Y4P3	TBL2	1.72739691	-0.6118946
Q29RF7	PDS5A	1.48319297	-0.5881685
Q9BXT4	TDRD1	2.1284419	0.58858554
Q16658	FSCN1	1.89740725	0.59388034
Q9NZN4	EHD2	1.45006775	0.60957845
Q8N1A0	KRT222	2.17984673	0.61441485
Q9NZJ9	NUDT4	1.35076492	0.67484919
P07197	NEFM	1.55082989	0.68603198
Q6EEV6	SUMO4	1.59871417	0.69232877
P43490	NAMPT	3.43270993	0.69677544
Q9UNF0	PACSIN2	1.37726808	0.69929441
P08779	KRT16	2.55542336	0.70042165
Q14525	KRT33B	2.95732646	0.70880254
O75923	DYSF	2.85093382	0.74842135
Q32P28	P3H1	1.95498012	0.75806491
P98095	FBLN2	1.81791709	0.76424027
Q96S97	MYADM	1.53456458	0.76727422
Q9ULC4	MCTS1	2.0736397	0.79755275
P04083	ANXA1	2.24829241	0.79760106
P63261	ACTG1	1.69531997	0.79988098
Q15293	RCN1	2.96983353	0.8034757
P02452	COL1A1	1.4808126	0.83557065
P35442	THBS2	1.57887363	0.89254252
P35555	FBN1	1.78876577	0.89585622
O00592	PODXL	1.52396979	0.90177027
Q04695	KRT17	3.20803202	0.91972987
Q9NRP0	OSTC	1.87197913	0.94518407
P08123	COL1A2	2.34799019	0.95553462
P13646	KRT13	1.75333275	0.95804405
P12109	COL6A1	1.43701832	0.99353727
P13645	KRT10	5.33204416	1.07890256
Q14134	TRIM29	2.76988282	1.09833908

P24821	TNC	1.69154586	1.19603984
P26447	S100A4	1.54529997	1.20826976
Q92876	KLK6	3.40886743	1.23197683
Q9Y483	MTF2	1.79086118	1.23989105
Q15063	POSTN	1.8689527	1.32606761
O14556	GAPDHS	1.43718813	1.48770968
P02533	KRT14	2.80382555	1.97423999

Table S2. Extract from OmixLitMiner from the list of 50 significant proteins in the comparison of Control and CD24kd using the keyword "Cancer".

UniProtID	Synonyms	TotalResults	Category
Q16790	CA9, G250, MN	994	1
P26447	S100A4, CAPL, MTS1	661	1
Q9ULC4	MCTS1, MCT1	320	1
P02452	COL1A1	310	1
P43490	NAMPT, PBEF, PBEF1	295	1
P04083	ANXA1, ANX1, LPC1	216	1
P24821	TNC, HXB	183	1
Q16658	FSCN1, FAN1, HSN, SNL	181	1
P08123	COL1A2	162	1
Q15063	POSTN, OSF2	152	1
Q92876	KLK6, PRSS18, PRSS9	128	1
P35442	THBS2, TSP2	116	1
Q15293	RCN1, RCN	111	1
P02786	TFRC	82	1
O00592	PODXL, PCLP, PCLP1	74	1
Q14134	TRIM29, ATDC	74	1
P35555	FBN1, FBN	58	1
Q9NRP0	OSTC, DC2, HDCMD45P, HSPC307	54	1
Q9BYZ8	REG4, GISP, RELP	52	1
Q04695	KRT17	51	1
Q9BZK7	TBL1XR1, IRA1,TBLR1	49	1
P12109	COL6A1	47	1
P63261	ACTG1, ACTG	46	1
P07197	NEFM, NEF3, NFM	31	1
O94760	DDAH1, DDAH	22	1
Q9NZN4	EHD2, PAST2	18	1
Q29RF7	PDS5A, KIAA0648, PDS5, PIG54	14	1
Q9BUH6	PAXX, C9orf142, XLS	10	1
Q3LXA3	TKFC, DAK	10	1
O75923	DYSF, FER1L1	6	1
O14556	GAPDHS, GAPD2, GAPDH2, GAPDS, HSD-35, HSD35	5	1
Q6EEV6	SUMO4, SMT3H4	3	1
P02533	KRT14	71	2
P10620	MGST1, GST12, MGST	33	2

P13646	KRT13	23	2
P08779	KRT16, KRT16A	22	2
Q9BXT4	TDRD1	18	2
P13645	KRT10, KPP	16	2
P98095	FBLN2	14	2
Q9Y483	MTF2, PCL2	9	2
P63173	RPL38	8	2
Q9NRF9	POLE3, CHRAC17	6	2
P68363	TUBA1B	5	2
Q32P28	P3H1, GROS1, LEPRE1, PSEC0109	5	2
Q8N1A0	KRT222, KA21, KRT222P	4	2
Q96S97	MYADM, UNQ553/PRO1110	4	2
Q9Y4P3	TBL2, WBSCR13, UNQ563/PRO1125	3	2
Q9UNF0	PACSIN2	1	2
Q14525	KRT33B, HHA3-II, HKA3B, KRTHA3B	1	2
Q9NZJ9	NUDT4, DIPP2, KIAA0487, HDCMB47P	0	3

Table S3. Table showing the significant (p-value < 0.05, |fold-change| > 1.5) regulated proteins after CD44 knockdown.

Uniprot ID	**Gene symbol**	**-Log_{10}(p-value)**	**Log2(FC)**
P16070	CD44	3.342537	-3.96562
O76024	WFS1	3.779966	-2.29902
Q15758	SLC1A5	4.157663	-1.61567
Q9BYZ8	REG4	1.87499	-1.55324
P07148	FABP1	1.408138	-1.23606
Q02817	MUC2	3.048606	-1.07539
Q96C36	PYCR2	2.885186	-1.009
Q14739	LBR	1.961205	-0.92801
Q8NF37	LPCAT1	2.044615	-0.84365
P98088	MUC5AC	1.406428	-0.84163
P02652	APOA2	1.558014	-0.77523
Q13907	IDI1	1.554285	-0.76814
Q9HC84	MUC5B	2.314593	-0.76668
Q9Y4P3	TBL2	1.575454	-0.75008
Q13724	MOGS	2.713591	-0.73089
Q8TC12	RDH11	2.722612	-0.70558
Q8NBQ5	HSD17B11	2.6029	-0.6586
P34897	SHMT2	1.582854	-0.64324
Q96T37	RBM15	3.336705	-0.6108
Q9Y3U8	RPL36	1.336549	-0.59798
O94808	GFPT2	1.798366	-0.59367
P13645	KRT10	1.771463	0.590436
P13688	CEACAM1	1.44306	0.623613
P04439	HLA-A	1.53341	0.62421
O43852	CALU	1.76908	0.62878
P80723	BASP1	1.488443	0.652574
Q15293	RCN1	2.193449	0.661809

P21439	ABCB4	1.415046	0.664063
O95816	BAG2	1.440115	0.688251
P31689	DNAJA1	1.715314	0.697185
Q96SQ9	CYP2S1	2.329388	0.698802
O60927	PPP1R11	1.769095	0.71953
O00483	NDUFA4	1.360587	0.721718
Q99536	VAT1	1.888481	0.729941
P04179	SOD2	1.558876	0.741538
O43583	DENR	1.810989	0.767087
Q9NZN4	EHD2	1.793245	0.805621
O76070	SNCG	1.345026	0.840888
Q15063	POSTN	1.611939	0.868334
Q14134	TRIM29	2.849908	0.889154
P04083	ANXA1	2.699199	0.919167
P09758	TACSTD2	1.383949	1.013875
Q92876	KLK6	2.641101	1.039861
Q9BUF5	TUBB6	1.594979	1.123413
P13646	KRT13	2.266326	1.127933
P02794	FTH1	1.629084	1.162494
Q9NZJ9	NUDT4	2.35727	1.242449
Q16658	FSCN1	3.780708	1.481589
P00995	SPINK1	2.537347	1.523335
Q92878	RAD50	3.201943	1.544224
P26447	S100A4	3.585211	2.008324

Table S4. Extract from OmixLitMiner from the list of 51 significant proteins in the comparison of Control and CD44kd using the keyword “Cancer”.

UniProtID	Synonyms	TotalResults	Category
P16070	CD44, LHR, MDU2, MDU3, MIC4	994	1
O76024	WFS1	12	1
Q15758	SLC1A5, ASCT2, M7V1, RDR, RDRC	199	1
Q9BYZ8	REG4, GISP, RELP	52	1
P07148	FABP1, FABPL	36	1
Q02817	MUC2, SMUC	586	1
Q14739	LBR	37	1
Q8NF37	LPCAT1, AYTL2, PFAAP3	28	1
P98088	MUC5AC, MUC5	448	1
Q9HC84	MUC5B, MUC5	93	1
P34897	SHMT2	70	1
Q96T37	RBM15, OTT, OTT1	189	1
P13688	CEACAM1, BGP, BGP1	247	1
P04439	HLA-A, HLAA	970	1
P80723	BASP1, NAP22	25	1
Q15293	RCN1, RCN	111	1
P21439	ABCB4, MDR3, PGY3	79	1
O95816	BAG2	18	1

P31689	DNAJA1, DNAJ2, HDJ2, HSJ2, HSPF4	28	1
O00483	NDUFA4	9	1
P04179	SOD2	468	1
O43583	DENR, DRP1, H14	244	1
Q9NZN4	EHD2, PAST2	18	1
O76070	SNCG, BCSG1, PERSYN, PRSN	98	1
Q15063	POSTN, OSF2	152	1
Q14134	TRIM29, ATDC	74	1
P04083	ANXA1, ANX1, LPC1	216	1
P09758	TACSTD2, GA733-1, M1S1, TROP2	145	1
Q92876	KLK6, PRSS18, PRSS9	128	1
P02794	FTH1, FTH, FTHL6, OK/SW-cl.84, PIG15	67	1
Q16658	FSCN1, FAN1, HSN, SNL	181	1
P00995	SPINK1, PSTI	282	1
Q92878	RAD50	417	1
P26447	S100A4, CAPL, MTS1	662	1
Q96C36	PYCR2	5	2
P02652	APOA2	25	2
Q13907	IDI1	7	2
Q9Y4P3	TBL2, WBSCR13, UNQ563/PRO1125	3	2
Q13724	MOGS, GCS1	4	2
Q8TC12	RDH11, ARSDR1, PSDR1, SDR7C1, CGI-82	7	2
Q8NBQ5	HSD17B11, DHRS8, PAN1B, SDR16C2, PSEC0029, UNQ207/PRO233	5	2
Q9Y3U8	RPL36	6	2
O94808	GFPT2	9	2
P13645	KRT10, KPP	16	2
O43852	CALU	426	2
Q96SQ9	CYP2S1, UNQ891/PRO1906	15	2
O60927	PPP1R11, HCGV, TCTE5	4	2
Q99536	VAT1	3	2
Q9BUF5	TUBB6	12	2
P13646	KRT13	23	2
Q9NZJ9	NUDT4, DIPP2, KIAA0487, HDCMB47P	0	3

Table S5. Statistics of the enrichment analysis of the ES gene sets in CD24kd vs. Control. Enrichment pattern with NOM p-val lower than 5% was considered significant. Gene sets with the positive ES value upregulated in CD24kd and ones with the negative ES value upregulated in Control.

GS	SIZE	ES	NES	NOM p-val
REACTOME_DEGRADATION_OF_THE_EXTRACELLULAR_ MATRIX	30	0.69	2.15	0
REACTOME_ECM_PROTEOGLYCANS	20	0.74	2.1	0
REACTOME_REGULATION_OF_INSULIN_LIKE_GROWTH_ FACTOR_IGF_TRANSPORT_AND_UPTAKE_BY_INSULIN_LIKE _GROWTH_FACTOR_BINDING_PROTEINS_IGFBPS_	40	0.63	2.08	0

REACTOME_EXTRACELLULAR_MATRIX_ORGANIZATION	63	0.56	2.07	0
REACTOME_COLLAGEN_BIOSYNTHESIS_AND_MODIFYING_ENZYMES	18	0.71	2	0
REACTOME_BINDING_AND_UPTAKE_OF_LIGANDS_BY_SCAVENGER_RECEPTORS	20	0.7	1.98	0
KEGG_FOCAL_ADHESION	49	0.56	1.96	0
KEGG_ECM_RECEPTOR_INTERACTION	22	0.66	1.91	0
REACTOME_COLLAGEN_FORMATION	22	0.66	1.9	0.002
REACTOME_KERATINIZATION	23	0.63	1.87	0.004
REACTOME_FORMATION_OF_THE_CORNIFIED_ENVELOPE	23	0.63	1.85	0
REACTOME_SMOOTH_MUSCLE_CONTRACTION	17	0.66	1.84	0.008
REACTOME_SIGNALING_BY_RECEPTOR_TYROSINE_KINASES	82	0.47	1.79	0
REACTOME_SIGNALING_BY_BRAF_AND_RAF_FUSIONS	21	0.58	1.69	0.006
KEGG_COMPLEMENT_AND_COAGULATION_CASCADES	20	0.6	1.68	0.01
REACTOME_MUSCLE_CONTRACTION	42	0.5	1.67	0.004
REACTOME_CLASS_I_MHC_MEDIATED_ANTIGEN_PROCESSING_PRESENTATION	67	0.45	1.67	0.004
REACTOME_STRIATED_MUSCLE_CONTRACTION	15	0.62	1.63	0.017
KEGG_ANTIGEN_PROCESSING_AND_PRESENTATION	20	0.57	1.62	0.011
REACTOME_ONCOGENIC_MAPK_SIGNALING	24	0.54	1.62	0.03
REACTOME_NON_INTEGRIN_MEMBRANE_ECM_INTERACTIONS	16	0.6	1.61	0.018
REACTOME_IRON_UPTAKE_AND_TRANSPORT	16	0.6	1.59	0.032
KEGG_VIRAL_MYOCARDITIS	21	0.57	1.59	0.018
KEGG_CALCIUM_SIGNALING_PATHWAY	23	0.53	1.59	0.031
REACTOME_SIGNALING_BY_MODERATE_KINASE_ACTIVITY_BRAF_MUTANTS	17	0.58	1.58	0.012
REACTOME_COMPLEMENT_CASCADE	16	0.58	1.57	0.033
REACTOME_SIGNALING_BY_MET	15	0.59	1.55	0.044
REACTOME_ADAPTIVE_IMMUNE_SYSTEM	133	0.37	1.53	0.002
REACTOME_ANTIGEN_PROCESSING_UBIQUITINATION_PROTEASOME_DEGRADATION	47	0.44	1.51	0.024
REACTOME_VESICLE_MEDIATED_TRANSPORT	162	0.36	1.51	0.008
REACTOME_MAPK_FAMILY_SIGNALING_CASCADES	68	0.4	1.49	0.006
REACTOME_POST_TRANSLATIONAL_PROTEIN_MODIFICATION	249	0.33	1.47	0.002
KEGG_OOCYTE_MEIOSIS	26	0.49	1.46	0.047
KEGG_VASCULAR_SMOOTH_MUSCLE_CONTRACTION	17	0.54	1.45	0.047
REACTOME_INTEGRIN_CELL_SURFACE_INTERACTIONS	26	0.48	1.43	0.046
REACTOME_CYTOKINE_SIGNALING_IN_IMMUNE_SYSTEM	150	0.33	1.42	0.012
REACTOME_ER_TO_GOLGI_ANTEROGRADE_TRANSPORT	56	0.4	1.41	0.038
REACTOME_PLATELET_ACTIVATION_SIGNALING_AND_AGGREGATION	70	0.39	1.41	0.023
REACTOME_SIGNALING_BY_GPCR	55	0.39	1.41	0.043
REACTOME_TRANSPORT_TO_THE_GOLGI_AND_SUBSEQUENT_MODIFICATION	56	0.4	1.4	0.041
REACTOME_DISEASES_OF_SIGNAL_TRANSDUCTION_BY_GROWTH_FACTOR_RECEPTORS_AND_SECOND_MESSENGERS	80	0.36	1.4	0.028
REACTOME_RRNA_PROCESSING	107	-0.52	-2.07	0
REACTOME_RESPONSE_OF_EIF2AK4_GCN2_TO_AMINO_ACID_DEFICIENCY	78	-0.52	-1.99	0
KEGG_RIBOSOME	72	-0.52	-1.97	0
REACTOME_EUKARYOTIC_TRANSLATION_ELONGATION	78	-0.49	-1.88	0
REACTOME_SELENOAMINO_ACID_METABOLISM	82	-0.48	-1.85	0
REACTOME_NONSENSE_MEDIATED_DECAY_NMD_	82	-0.47	-1.8	0
REACTOME_COMPLEX_I_BIOGENESIS	18	-0.61	-1.7	0.011
REACTOME_BIOLOGICAL_OXIDATIONS	40	-0.52	-1.7	0.004
REACTOME_PHASE_II_CONJUGATION_OF_COMPOUNDS	26	-0.56	-1.68	0.008

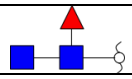
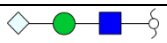
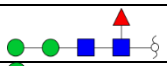
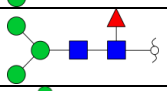
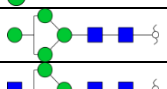
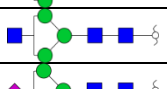
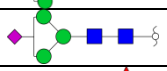
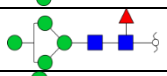
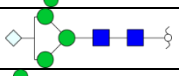
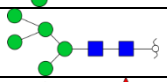
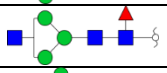
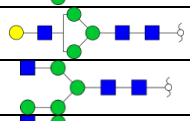
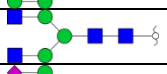
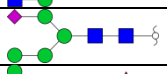
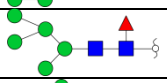
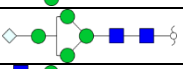
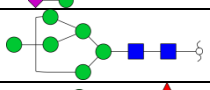
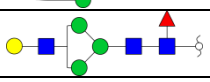
REACTOME_RESPIRATORY_ELECTRON_TRANSPORT	39	-0.49	-1.63	0.002
REACTOME_METABOLISM_OF_RNA	253	-0.36	-1.62	0
KEGG_GLUTATHIONE_METABOLISM	20	-0.56	-1.6	0.018
REACTOME_EUKARYOTIC_TRANSLATION_INITIATION	96	-0.4	-1.6	0.004
REACTOME_RRNA_MODIFICATION_IN_THE_NUCLEUS_AND_CYTOSOL	24	-0.53	-1.57	0.019
REACTOME_DEADENYLATION_DEPENDENT_MRNA_DECAY	16	-0.56	-1.53	0.035
REACTOME_RESPIRATORY_ELECTRON_TRANSPORT_ATP_SYNTHESIS_BY_CHEMIOSMOTIC_COUPLING_AND_HEAT_PRODUCTION_BY_UNCOUPLING_PROTEINS_	40	-0.45	-1.52	0.015
REACTOME_SRP_DEPENDENT_COTRANSLATIONAL_PROTEIN_TARGETING_TO_MEMBRANE	88	-0.38	-1.51	0.006
KEGG_PARKINSONS_DISEASE	43	-0.43	-1.48	0.03
REACTOME_INFLUENZA_INFECTION	100	-0.37	-1.47	0.012
REACTOME_TRANSLATION	144	-0.34	-1.43	0.006
KEGG_SPLICEOSOME	61	-0.39	-1.42	0.038
REACTOME_PROCESSING_OF_CAPPED_INTRON_CONTAINING_PRE_MRNA	86	-0.36	-1.42	0.021

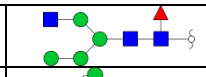
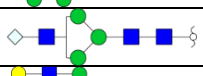
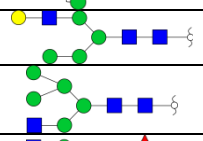
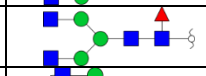
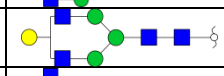
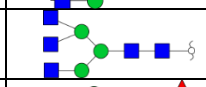
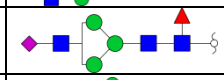
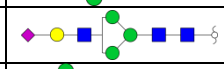
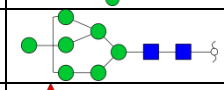
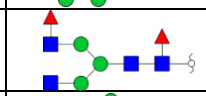
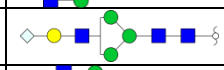
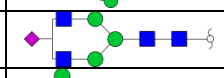
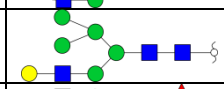
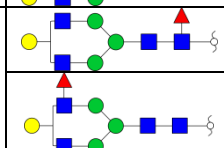
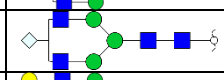
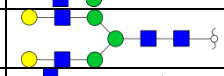
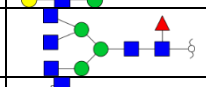
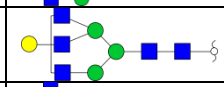
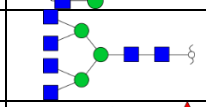
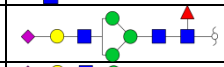
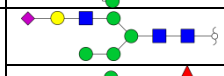
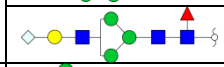
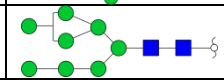
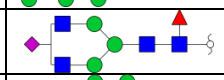
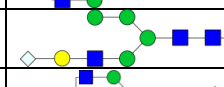
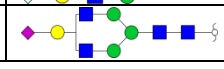
Table S6. Statistics of the enrichment analysis of the ES gene sets in CD44kd vs. Control. Enrichment pattern with NOM p-val lower than 5% was considered significant. Gene sets with the positive ES value upregulated in CD44kd and ones with the negative ES value upregulated in Control.

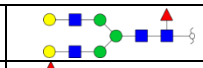
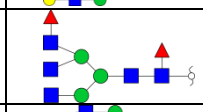
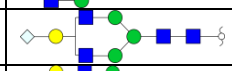
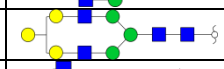
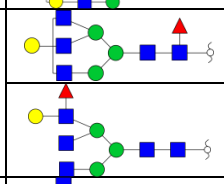
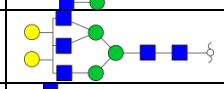
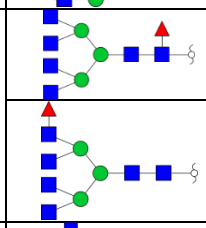
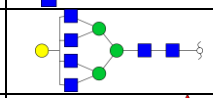
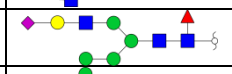
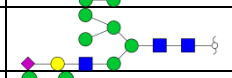
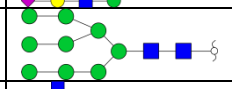
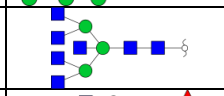
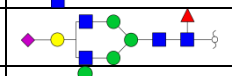
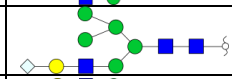
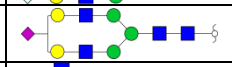
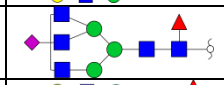
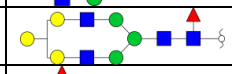
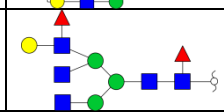
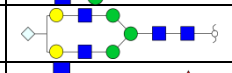
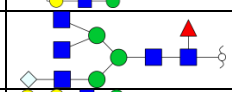
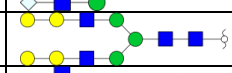
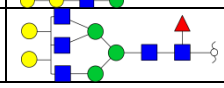
GS	SIZE	ES	NES	NOM p-val
KEGG_ANTIGEN_PROCESSING_AND_PRESENTATION	20	0.67	1.82	0
REACTOME_KERATINIZATION	23	0.63	1.76	0.004
REACTOME_FORMATION_OF_THE_CORNIFIED_ENVELOPE	23	0.63	1.76	0.007
KEGG_VIRAL_MYOCARDITIS	21	0.62	1.69	0.007
KEGG_DILATED_CARDIOMYOPATHY	19	0.62	1.69	0.01
REACTOME_SMOOTH_MUSCLE_CONTRACTION	17	0.65	1.68	0.004
REACTOME_TRANSCRIPTIONAL_REGULATION_BY_TP53	53	0.49	1.67	0
REACTOME_COLLAGEN_BIOSYNTHESIS_AND_MODIFYING_ENZYMES	18	0.62	1.65	0.01
REACTOME_INTERLEUKIN_12_FAMILY_SIGNALING	31	0.55	1.63	0.011
REACTOME_GENE_AND_PROTEIN_EXPRESSION_BY_JAK_STAT_SIGNALING_AFTER_INTERLEUKIN_12_STIMULATION	28	0.55	1.62	0.012
KEGG_GAP_JUNCTION	17	0.62	1.62	0.01
REACTOME_INTERLEUKIN_12_SIGNALING	29	0.55	1.6	0.024
REACTOME_SIGNALING_BY_INTERLEUKINS	91	0.42	1.55	0.008
REACTOME_PROTEIN_FOLDING	34	0.5	1.55	0.025
REACTOME_SELECTIVE_AUTOPHAGY	23	0.55	1.55	0.032
REACTOME_IRON_UPTAKE_AND_TRANSPORT	16	0.59	1.55	0.025
REACTOME_COLLAGEN_FORMATION	22	0.57	1.53	0.029
REACTOME_ECM_PROTEOGLYCANS	20	0.57	1.52	0.038
KEGG_TIGHT_JUNCTION	39	0.48	1.51	0.03
REACTOME_CHROMOSOME_MAINTENANCE	15	0.59	1.51	0.038
REACTOME_SIGNALING_BY_RECEPTOR_TYROSINE_KINASES	82	0.41	1.47	0.014
REACTOME_HOST_INTERACTIONS_OF_HIV_FACTORS	68	0.41	1.47	0.023

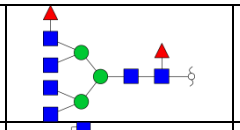
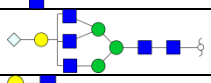
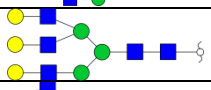
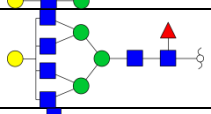
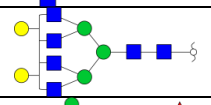
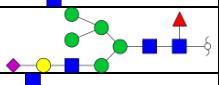
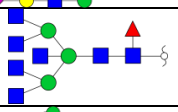
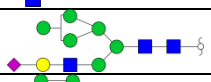
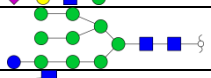
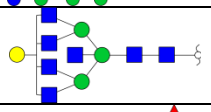
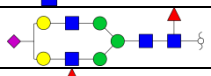
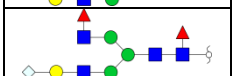
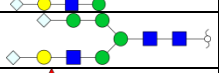
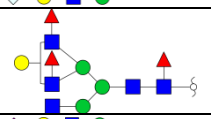
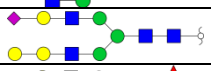
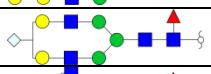
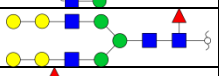
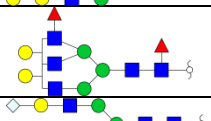
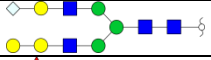
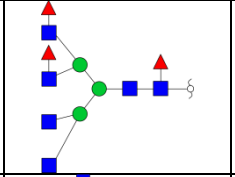
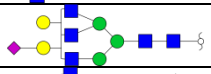
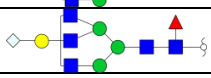
REACTOME_SIGNALING_BY_HEDGEHOG	46	0.44	1.45	0.031
REACTOME_MUSCLE_CONTRACTION	42	0.46	1.45	0.039
REACTOME_RNA_POLYMERASE_II_TRANSCRIPTION	139	0.36	1.44	0.008
REACTOME_SIGNALING_BY_WNT	64	0.41	1.4	0.031
REACTOME_MITOTIC_G2_G2_M_PHASES	63	0.4	1.39	0.026
REACTOME_HIV_INFECTION	79	0.37	1.37	0.027
REACTOME_BIOLOGICAL_OXIDATIONS	40	-0.59	-1.86	0
REACTOME_SLC_MEDIATED_TRANSMEMBRANE_TRANSPORT	19	-0.68	-1.8	0.002
REACTOME_METABOLISM_OF_VITAMINS_AND_COFACTORS	35	-0.59	-1.79	0.004
KEGG_FATTY_ACID_METABOLISM	19	-0.62	-1.69	0.006
KEGG_AMINO_SUGAR_AND_NUCLEOTIDE_SUGAR_METABOLISM	16	-0.66	-1.65	0.019
REACTOME_METABOLISM_OF_STEROIDS	34	-0.54	-1.65	0.016
REACTOME_DISEASES_OF_METABOLISM	36	-0.53	-1.64	0.012
KEGG_VALINE_LEUCINE_AND_ISOLEUCINE_DEGRADATION	19	-0.6	-1.6	0.026
REACTOME_CELL_SURFACE_INTERACTIONS_AT_THE_VASCULAR_WALL	29	-0.54	-1.58	0.01
REACTOME_PLASMA_LIPOPROTEIN_ASSEMBLY_REMODELING_AND_CLEARANCE	19	-0.56	-1.52	0.049
REACTOME_DISEASES_OF_GLYCOSYLATION	21	-0.55	-1.52	0.03
REACTOME_PHASE_II_CONJUGATION_OF_COMPOUNDS	26	-0.52	-1.51	0.036
REACTOME_GLYCOSAMINOGLYCAN_METABOLISM	15	-0.59	-1.5	0.045
REACTOME_RESPONSE_OF_EIF2AK4_GCN2_TO_AMINO_ACID_DEFICIENCY	78	-0.41	-1.49	0.014
KEGG_ARGININE_AND_PROLINE_METABOLISM	21	-0.53	-1.46	0.049
KEGG_RIBOSOME	72	-0.41	-1.46	0.02
REACTOME_EUKARYOTIC_TRANSLATION_ELONGATION	78	-0.4	-1.42	0.027
REACTOME_EUKARYOTIC_TRANSLATION_INITIATION	96	-0.38	-1.42	0.041
REACTOME_INFLUENZA_INFECTION	100	-0.38	-1.42	0.022
REACTOME_METABOLISM_OF_LIPIDS	100	-0.37	-1.37	0.03

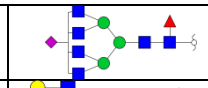
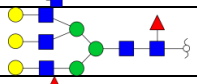
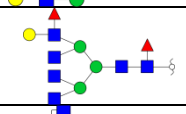
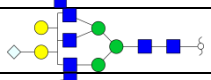
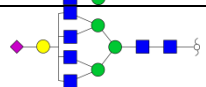
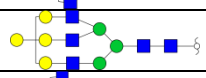
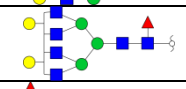
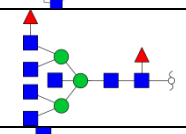
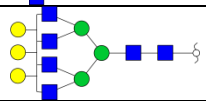
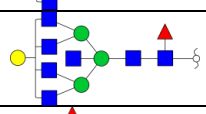
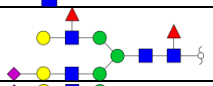
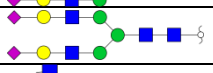
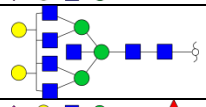
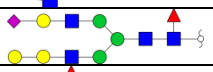
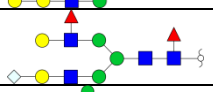
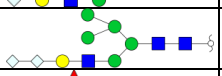
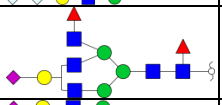
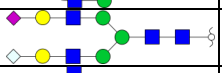
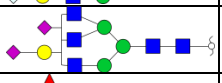
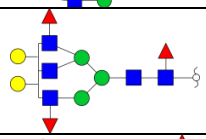
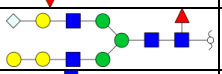
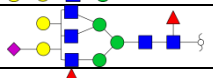
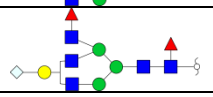
Table S7. All the identified N-glycans from three groups of tissue, Control, CD24kd and CD44kd tissue, at MS1 and MS2 levels (324 N-glycan compositions and 647 N-glycan structures).

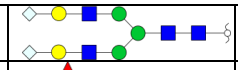
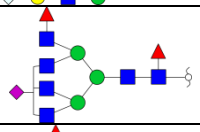
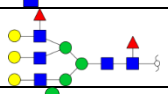
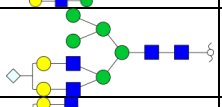
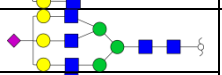
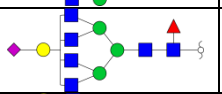
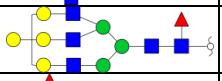
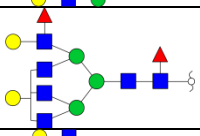
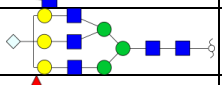
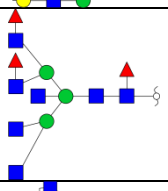
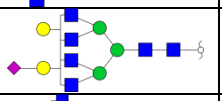
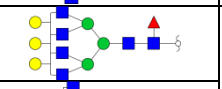
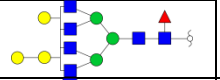
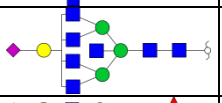
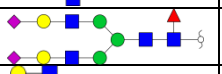
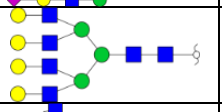
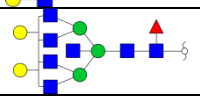
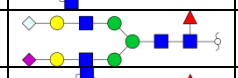
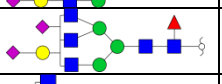
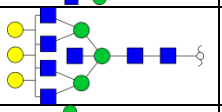
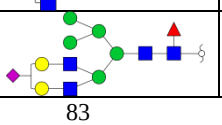
N-glycan composition	No. of isomeric structures	Preferred N-glycan structures	Permethylation monoMW		Deviation (p.p.m.)
			Experiment	Theory	
$Fuc_1Red\text{-}HexNAc_1$	1		481.2888	481.2887	0.17
$Hex_1Red\text{-}HexNAc_1$	1		511.2990	511.2993	0.61
$Hex_2Red\text{-}HexNAc_1$	3		715.3991	715.3990	0.11
$HexNAc_1Fuc_1Red\text{-}HexNAc_1$	1		726.4151	726.4153	0.18
$HexNAc_1Hex_1Red\text{-}HexNAc_1$	1		756.4260	756.4256	0.49
$Neu5Ac_1Hex_1Red\text{-}HexNAc_1$	3		872.4730	872.4729	0.05
$Neu5Gc_1Hex_1Red\text{-}HexNAc_1$	3		902.4828	902.4835	0.78
$Hex_3Red\text{-}HexNAc_1$	1		919.4995	919.4988	0.77
$HexNAc_1Hex_2Red\text{-}HexNAc_1$	2		960.5256	960.5254	0.29
$HexNAc_1Hex_2Fuc_1Red\text{-}HexNAc_1$	2		1134.6156	1134.6146	0.92
$HexNAc_1Hex_3Red\text{-}HexNAc_1$	3		1164.6258	1164.6251	0.58
$HexNAc_1Hex_3Fuc_1Red\text{-}HexNAc_1$	2		1338.7145	1338.7143	0.13
$HexNAc_1Hex_4Red\text{-}HexNAc_1$	3		1368.7254	1368.7249	0.37
$HexNAc_2Hex_3Red\text{-}HexNAc_1$	2		1409.7519	1409.7515	0.33
$Neu5Ac_1HexNAc_1Hex_3Red\text{-}HexNAc_1$	1		1525.7995	1525.7988	0.47
$HexNAc_1Hex_4Fuc_1Red\text{-}HexNAc_1$	2		1542.8137	1542.8141	0.26
$Neu5Gc_1HexNAc_1Hex_3Red\text{-}HexNAc_1$	3		1555.8096	1555.8094	0.16
$HexNAc_1Hex_5Red\text{-}HexNAc_1$	2		1572.8256	1572.8247	0.60
$HexNAc_2Hex_3Fuc_1Red\text{-}HexNAc_1$	3		1583.8419	1583.8407	0.79
$HexNAc_2Hex_4Red\text{-}HexNAc_1$	7		1613.8516	1613.8512	0.24
$HexNAc_3Hex_3Red\text{-}HexNAc_1$	6		1654.8786	1654.8778	0.51
$Neu5Ac_1HexNAc_1Hex_4Red\text{-}HexNAc_1$	1		1729.8988	1729.8986	0.14
$HexNAc_1Hex_5Fuc_1Red\text{-}HexNAc_1$	1		1746.9156	1746.9139	0.99
$Neu5Gc_1HexNAc_1Hex_4Red\text{-}HexNAc_1$	2		1759.9105	1759.9091	0.78
$Neu5Ac_1HexNAc_2Hex_3Red\text{-}HexNAc_1$	1		1770.9275	1770.9251	1.36
$HexNAc_1Hex_6Red\text{-}HexNAc_1$	1		1776.9260	1776.9245	0.88
$HexNAc_2Hex_4Fuc_1Red\text{-}HexNAc_1$	5		1787.9412	1787.9404	0.44

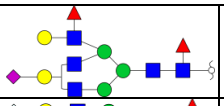
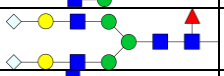
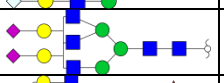
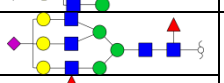
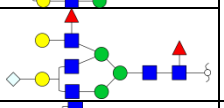
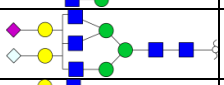
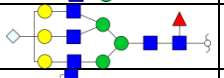
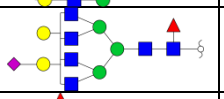
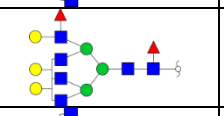
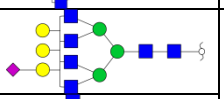
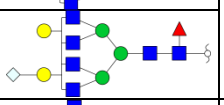
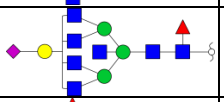
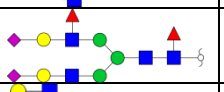
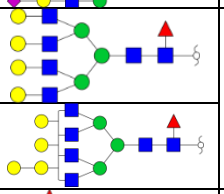
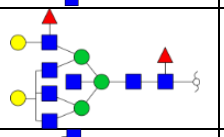
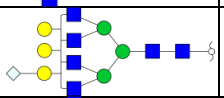
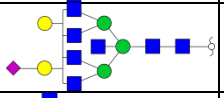
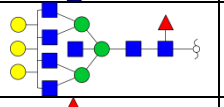
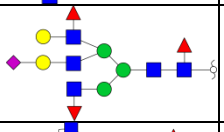
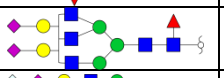
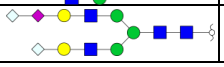
					
Neu5Gc$_1$HexNAc$_2$Hex$_3$Red-HexNAc$_1$	3		1800.9360	1800.9357	0.18
HexNAc$_2$Hex$_5$Red-HexNAc$_1$	4		1817.9513	1817.9510	0.18
HexNAc$_3$Hex$_3$Fuc$_1$Red-HexNAc$_1$	6		1828.9682	1828.9670	0.67
HexNAc$_3$Hex$_4$Red-HexNAc$_1$	6		1858.9782	1858.9776	0.36
HexNAc$_4$Hex$_3$Red-HexNAc$_1$	4		1900.0048	1900.0041	0.38
Neu5Ac$_1$HexNAc$_2$Hex$_3$Fuc$_1$Red-HexNAc$_1$	1		1945.0139	1945.0143	0.21
Neu5Ac$_1$HexNAc$_2$Hex$_4$Red-HexNAc$_1$	2		1975.0267	1975.0249	0.93
HexNAc$_1$Hex$_7$Red-HexNAc$_1$	2		1981.0247	1981.0242	0.25
HexNAc$_3$Hex$_3$Fuc$_2$Red-HexNAc$_1$	1		2003.0577	2003.0562	0.76
Neu5Gc$_1$HexNAc$_2$Hex$_4$Red-HexNAc$_1$	5		2005.0357	2005.0355	0.13
Neu5Ac$_1$HexNAc$_3$Hex$_3$Red-HexNAc$_1$	1		2016.0521	2016.0514	0.34
HexNAc$_2$Hex$_6$Red-HexNAc$_1$	1		2022.0517	2022.0508	0.47
HexNAc$_3$Hex$_4$Fuc$_1$Red-HexNAc$_1$	4		2033.0668	2033.0668	0.03
Neu5Gc$_1$HexNAc$_3$Hex$_3$Red-HexNAc$_1$	2		2046.0618	2046.0620	0.09
HexNAc$_3$Hex$_5$Red-HexNAc$_1$	8		2063.0776	2063.0773	0.15
HexNAc$_4$Hex$_3$Fuc$_1$Red-HexNAc$_1$	5		2074.0934	2074.0933	0.05
HexNAc$_4$Hex$_4$Red-HexNAc$_1$	3		2104.1048	2104.1039	0.45
HexNAc$_5$Hex$_3$Red-HexNAc$_1$	3		2145.1308	2145.1304	0.19
Neu5Ac$_1$HexNAc$_2$Hex$_4$Fuc$_1$Red-HexNAc$_1$	2		2149.1151	2149.1141	0.47
Neu5Ac$_1$HexNAc$_2$Hex$_5$Red-HexNAc$_1$	4		2179.1256	2179.1247	0.44
Neu5Gc$_1$HexNAc$_2$Hex$_4$Fuc$_1$Red-HexNAc$_1$					
HexNAc$_1$Hex$_8$Red-HexNAc$_1$	2		2185.1230	2185.1240	0.44
Neu5Ac$_1$HexNAc$_3$Hex$_3$Fuc$_1$Red-HexNAc$_1$	1		2190.1416	2190.1407	0.44
Neu5Gc$_1$HexNAc$_2$Hex$_5$Red-HexNAc$_1$	2		2209.1350	2209.1352	0.10
Neu5Ac$_1$HexNAc$_3$Hex$_4$Red-HexNAc$_1$	4		2220.1511	2220.1512	0.04

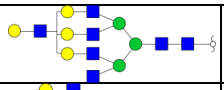
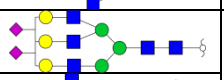
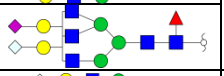
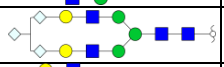
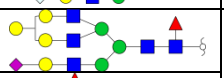
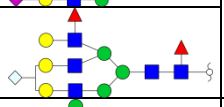
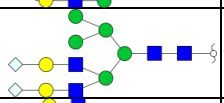
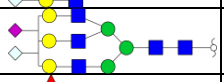
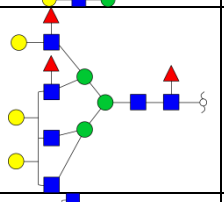
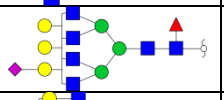
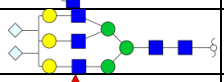
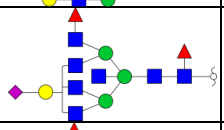
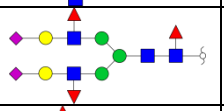
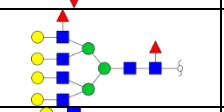
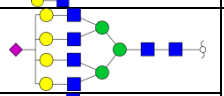
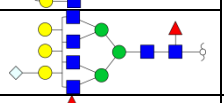
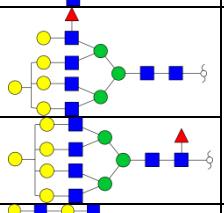
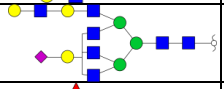
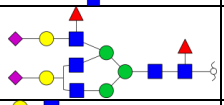
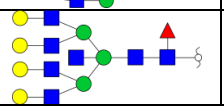
$HexNAc_3Hex_5Fuc_1Red\text{-}HexNAc_1$	2		2237.1657	2237.1665	0.36
$HexNAc_4Hex_3Fuc_2Red\text{-}HexNAc_1$	1		2248.1829	2248.1825	0.18
$Neu5Gc_1HexNAc_3Hex_4Red\text{-}HexNAc_1$	4		2250.1611	2250.1618	0.29
$HexNAc_3Hex_6Red\text{-}HexNAc_1$	1		2267.1795	2267.1771	1.07
$HexNAc_4Hex_4Fuc_1Red\text{-}HexNAc_1$	4		2278.1928	2278.1931	0.11
$HexNAc_4Hex_5Red\text{-}HexNAc_1$	4		2308.2034	2308.2036	0.10
$HexNAc_5Hex_3Fuc_1Red\text{-}HexNAc_1$	5		2319.2194	2319.2196	0.09
$HexNAc_5Hex_4Red\text{-}HexNAc_1$	3		2349.2310	2349.2302	0.35
$Neu5Ac_1HexNAc_2Hex_5Fuc_1 Red\text{-}HexNAc_1$	2		2353.2141	2353.2139	0.11
$Neu5Ac_1HexNAc_2Hex_6Red\text{-}HexNAc_1$	2		2383.2246	2383.2244	0.08
$HexNAc_1Hex_9Red\text{-}HexNAc_1$	3		2389.2262	2389.2238	1.03
$HexNAc_6Hex_3Red\text{-}HexNAc_1$	1		2390.2580	2390.2567	0.54
$Neu5Ac_1HexNAc_3Hex_4Fuc_1 Red\text{-}HexNAc_1$	4		2394.2395	2394.2404	0.38
$Neu5Gc_1HexNAc_2Hex_6Red\text{-}HexNAc_1$	3		2413.2349	2413.2350	0.03
$Neu5Ac_1HexNAc_3Hex_5Red\text{-}HexNAc_1$	6		2424.2503	2424.2510	0.27
$Neu5Ac_1HexNAc_4Hex_3Fuc_1 Red\text{-}HexNAc_1$	1		2435.2677	2435.2670	0.31
$HexNAc_3Hex_6Fuc_1Red\text{-}HexNAc_1$	1		2441.2659	2441.2663	0.16
$HexNAc_4Hex_4Fuc_2Red\text{-}HexNAc_1$	3		2452.2812	2452.2823	0.44
$Neu5Gc_1HexNAc_3Hex_5Red\text{-}HexNAc_1$	2		2454.2620	2454.2616	0.19
$Neu5Gc_1HexNAc_4Hex_3Fuc_1 Red\text{-}HexNAc_1$	2		2465.2771	2465.2775	0.17
$HexNAc_3Hex_7Red\text{-}HexNAc_1$	1		2471.2783	2471.2769	0.59
$HexNAc_4Hex_5Fuc_1Red\text{-}HexNAc_1$	4		2482.2926	2482.2929	0.09

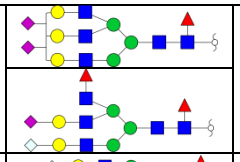
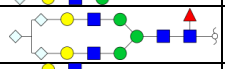
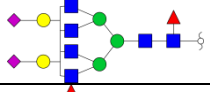
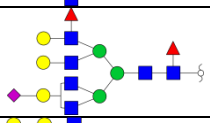
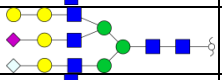
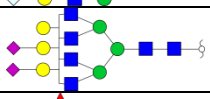
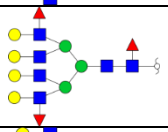
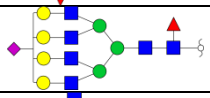
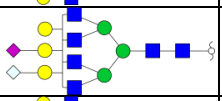
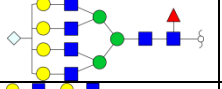
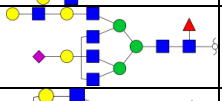
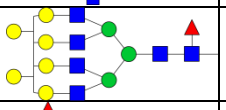
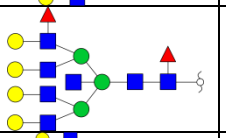
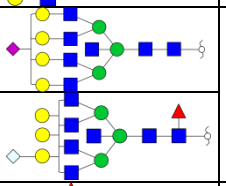
$HexNAc_5Hex_3Fuc_2Red\text{-}HexNAc_1$	2		2493.3101	2493.3088	0.51
$Neu5Gc_1HexNAc_4Hex_4Red\text{-}HexNAc_1$	1		2495.2895	2495.2881	0.57
$HexNAc_4Hex_6Red\text{-}HexNAc_1$	4		2512.3033	2512.3034	0.04
$HexNAc_5Hex_4Fuc_1Red\text{-}HexNAc_1$	4		2523.3199	2523.3194	0.21
$HexNAc_5Hex_5Red\text{-}HexNAc_1$	3		2553.3302	2553.3300	0.10
$Neu5Ac_1HexNAc_2Hex_6Fuc_1$ $Red\text{-}HexNAc_1$	2		2557.3141	2557.3137	0.19
$HexNAc_6Hex_3Fuc_1Red\text{-}HexNAc_1$	1		2564.3461	2564.3460	0.07
$Neu5Ac_1HexNAc_2Hex_7Red\text{-}HexNAc_1$	1		2587.3240	2587.3242	0.07
$HexNAc_1Hex_{10}Red\text{-}HexNAc_1$	1		2593.3262	2593.3235	1.04
$HexNAc_6Hex_4Red\text{-}HexNAc_1$	1		2594.3561	2594.3565	0.15
$Neu5Ac_1HexNAc_3Hex_5Fuc_1$ $Red\text{-}HexNAc_1$	4		2598.3403	2598.3402	0.05
$Neu5Gc_1HexNAc_3Hex_4Fuc_2$ $Red\text{-}HexNAc_1$					
$Neu5Gc_2HexNAc_2Hex_5Red\text{-}HexNAc_1$	2		2600.3200	2600.3195	0.22
$HexNAc_4Hex_4Fuc_3Red\text{-}HexNAc_1$	1		2626.3705	2626.3705	0.37
$Neu5Ac_1HexNAc_3Hex_6Red\text{-}HexNAc_1$	5		2628.3511	2628.3508	0.14
$Neu5Gc_1HexNAc_3Hex_5Fuc_1$ $Red\text{-}HexNAc_1$					
$Neu5Ac_1HexNAc_4Hex_4Fuc_1$ $Red\text{-}HexNAc_1$	6		2639.3663	2639.3667	0.16
$HexNAc_3Hex_7Fuc_1Red\text{-}HexNAc_1$	1		2645.3681	2645.3661	0.77
$HexNAc_4Hex_5Fuc_2Red\text{-}HexNAc_1$	2		2656.3819	2652.3821	0.05
$Neu5Gc_1HexNAc_3Hex_6Red\text{-}HexNAc_1$	2		2658.3618	2658.3613	0.19
$HexNAc_5Hex_3Fuc_3Red\text{-}HexNAc_1$	2		2667.3988	2667.3980	0.29
$Neu5Ac_1HexNAc_4Hex_5Red\text{-}HexNAc_1$	4		2669.3779	2669.3773	0.23
$Neu5Gc_1HexNAc_4Hex_4Fuc_1$ $Red\text{-}HexNAc_1$					

$Neu5Ac_1HexNAc_5Hex_3Fuc_1$ Red-$HexNAc_1$	1		2680.3949	2680.3933	0.61
$HexNAc_4Hex_6Fuc_1$Red-$HexNAc_1$	3		2686.3932	2686.3926	0.22
$HexNAc_5Hex_4Fuc_2$Red-$HexNAc_1$	1		2697.4076	2697.4086	0.37
$Neu5Gc_1HexNAc_4Hex_5$Red-$HexNAc_1$	2		2699.3906	2699.3879	1.02
$Neu5Ac_1HexNAc_5Hex_4$Red-$HexNAc_1$	3		2710.4034	2710.4039	0.16
$HexNAc_4Hex_7$Red-$HexNAc_1$	1		2716.4049	2716.4032	0.64
$HexNAc_5Hex_5Fuc_1$Red-$HexNAc_1$	4		2727.4193	2727.4192	0.05
$HexNAc_6Hex_3Fuc_2$Red-$HexNAc_1$	1		2738.4350	2738.4352	0.05
$HexNAc_5Hex_6$Red-$HexNAc_1$	3		2757.4304	2757.4297	0.25
$HexNAc_6Hex_4Fuc_1$Red-$HexNAc_1$	1		2768.4463	2768.4457	0.22
$Neu5Ac_1HexNAc_3Hex_5Fuc_2$ Red-$HexNAc_1$	1		2772.4293	2772.4294	0.03
$Neu5Ac_2HexNAc_3Hex_5$Red-$HexNAc_1$	5		2785.4250	2785.4247	0.14
$HexNAc_6Hex_5$Red-$HexNAc_1$	1		2798.4557	2798.4563	0.20
$Neu5Ac_1HexNAc_3Hex_6Fuc_1$ Red-$HexNAc_1$	3		2802.4406	2802.4400	0.24
$Neu5Gc_1HexNAc_3Hex_5Fuc_2$ Red-$HexNAc_1$					
$Neu5Gc_2HexNAc_2Hex_6$Red-$HexNAc_1$	2		2804.4209	2804.4192	0.60
$Neu5Ac_1HexNAc_4Hex_4Fuc_2$ Red-$HexNAc_1$	1		2813.4575	2813.4560	0.56
$Neu5Gc_1Neu5Ac_1HexNAc_3$ Hex_5Red-$HexNAc_1$	3		2815.4327	2815.4352	0.88
$Neu5Ac_2HexNAc_4Hex_4$Red-$HexNAc_1$	1		2826.4506	2826.4512	0.20
$HexNAc_4Hex_5Fuc_3$Red-$HexNAc_1$	1		2830.4718	2830.4713	0.19
$Neu5Gc_1HexNAc_3Hex_6Fuc_1$ Red-$HexNAc_1$	1		2832.4474	2832.4505	1.09
$Neu5Ac_1HexNAc_4Hex_5Fuc_1$ Red-$HexNAc_1$	4		2843.4663	2843.4665	0.07
$Neu5Gc_1HexNAc_4Hex_4Fuc_2$ Red-$HexNAc_1$					

$Neu5Gc_2HexNAc_3Hex_5Red\text{-}HexNAc_1$	3		2845.4474	2845.4458	0.58
$Neu5Ac_1HexNAc_5Hex_3Fuc_2Red\text{-}HexNAc_1$	1		2854.4834	2854.4825	0.32
$HexNAc_4Hex_6Fuc_2Red\text{-}HexNAc_1$	3		2860.4801	2860.4818	0.60
$Neu5Gc_1HexNAc_3Hex_7Red\text{-}HexNAc_1$	2		2862.4544	2862.4611	2.33
$Neu5Ac_1HexNAc_4Hex_6Red\text{-}HexNAc_1$	5		2873.4770	2873.4771	0.02
$Neu5Ac_1HexNAc_4Hex_5Fuc_1Red\text{-}HexNAc_1$					
$Neu5Ac_1HexNAc_5Hex_4Fuc_1Red\text{-}HexNAc_1$	2		2884.4920	2884.4931	0.36
$HexNAc_4Hex_7Fuc_1Red\text{-}HexNAc_1$	2		2890.4932	2890.4924	0.29
$HexNAc_5Hex_5Fuc_2Red\text{-}HexNAc_1$	1		2901.5068	2901.5084	0.54
$Neu5Gc_1HexNAc_4Hex_6Red\text{-}HexNAc_1$	3		2903.4891	2903.4877	0.51
$HexNAc_6Hex_3Fuc_3Red\text{-}HexNAc_1$	2		2912.5215	2912.5244	0.98
$Neu5Ac_1HexNAc_5Hex_5Red\text{-}HexNAc_1$	3		2914.5037	2914.5036	0.03
$HexNAc_5Hex_6Fuc_1Red\text{-}HexNAc_1$	7		2931.5192	2931.5190	0.10
					
$Neu5Ac_1HexNAc_6Hex_4Red\text{-}HexNAc_1$	1		2955.5275	2955.5302	0.90
$Neu5Ac_2HexNAc_3Hex_5Fuc_1Red\text{-}HexNAc_1$	5		\	2959.5139	0.33
$HexNAc_5Hex_7Red\text{-}HexNAc_1$	1		2961.5294	2961.5295	0.03
$HexNAc_6Hex_5Fuc_1Red\text{-}HexNAc_1$	1		2972.5470	2972.5455	0.51
$Neu5Gc_1Neu5Ac_1HexNAc_3Hex_5Fuc_1Red\text{-}HexNAc_1$	3		2989.5258	2989.5244	0.47
$Neu5Ac_2HexNAc_4Hex_4Fuc_1Red\text{-}HexNAc_1$	1		3000.5384	3000.5404	0.66
$HexNAc_6Hex_6Red\text{-}HexNAc_1$	1		3002.5578	3002.5561	0.59
$Neu5Ac_1HexNAc_3Hex_7Fuc_1Red\text{-}HexNAc_1$	1		3006.5374	3006.5397	0.77

$Neu5Ac_1HexNAc_4Hex_5Fuc_2$ Red-$HexNAc_1$	1		3017.5558	3017.5557	0.03
$Neu5Gc_2HexNAc_3Hex_5Fuc_1$ Red-$HexNAc_1$	3		3019.5356	3019.5350	0.21
$Neu5Ac_2HexNAc_4Hex_5$Red-$HexNAc_1$	3		3030.5520	3030.5510	0.35
$Neu5Ac_1HexNAc_4Hex_6Fuc_1$ Red-$HexNAc_1$	4		3047.5653	3047.5663	0.32
$Neu5Gc_1HexNAc_4Hex_5Fuc_2$ Red-$HexNAc_1$					
$Neu5Gc_1Neu5Ac_1HexNAc_4$ Hex_5Red-$HexNAc_1$	1		3060.5617	3060.5615	0.06
$Neu5Gc_1HexNAc_4Hex_6Fuc_1$ Red-$HexNAc_1$	8		3077.5770	3077.5769	0.06
$Neu5Ac_1HexNAc_5Hex_5Fuc_1$ Red-$HexNAc_1$	4		3088.5920	3088.5928	0.26
$HexNAc_5Hex_6Fuc_2$Red-$HexNAc_1$	2		3105.6069	3105.6082	0.40
$Neu5Ac_1HexNAc_5Hex_6$Red-$HexNAc_1$	4		3118.6030	3118.6034	0.12
$Neu5Gc_1HexNAc_5Hex_5Fuc_1$ Red-$HexNAc_1$					
$Neu5Ac_1HexNAc_6Hex_4Fuc_1$ Red-$HexNAc_1$	1		3129.6188	3129.6194	0.18
$Neu5Ac_2HexNAc_3Hex_5Fuc_2$ Red-$HexNAc_1$	1		3133.6045	3133.6031	0.47
$HexNAc_5Hex_7Fuc_1$Red-$HexNAc_1$	5		3135.6191	3135.6187	0.13
$HexNAc_6Hex_5Fuc_2$Red-$HexNAc_1$	1		3146.6339	3146.6347	0.25
$Neu5Gc_1HexNAc_5Hex_6$Red-$HexNAc_1$	2		3148.6144	3148.6140	0.15
$Neu5Ac_1HexNAc_6Hex_5$Red-$HexNAc_1$	1		3159.6314	3159.6300	0.47
$HexNAc_6Hex_6Fuc_1$Red-$HexNAc_1$	2		3176.6468	3176.6453	0.49
$Neu5Ac_1HexNAc_4Hex_5Fuc_3$ Red-$HexNAc_1$	1		3191.6460	3191.6449	0.34
$Neu5Ac_2HexNAc_4Hex_5Fuc_1$ Red-$HexNAc_1$	3		3204.6392	3204.6402	0.30
$Neu5Gc_2Neu5Ac_1HexNAc_3$ Hex_5Red-$HexNAc_1$	2		3206.6203	3206.6195	0.27

$HexNAc_6Hex_7Red\text{-}HexNAc_1$	1		3206.6532	3206.6558	0.81
$Neu5Ac_2HexNAc_4Hex_6Red\text{-}HexNAc_1$	4		3234.6494	3234.6508	0.40
$Neu5Gc_1Neu5Ac_1HexNAc_4Hex_5Fuc_1Red\text{-}HexNAc_1$					
$Neu5Gc_3HexNAc_3Hex_5Red\text{-}HexNAc_1$	2		3236.6319	3236.6300	0.59
$Neu5Ac_1HexNAc_4Hex_7Fuc_1Red\text{-}HexNAc_1$	2		3251.6668	3251.6661	0.24
$Neu5Gc_1HexNAc_4Hex_6Fuc_2Red\text{-}HexNAc_1$					
$Neu5Gc_2HexNAc_3Hex_7Red\text{-}HexNAc_1$	1		3253.6487	3253.6453	1.04
$Neu5Gc_1Neu5Ac_1HexNAc_4Hex_6Red\text{-}HexNAc_1$	1		3264.6609	3264.6613	0.12
$HexNAc_5Hex_6Fuc_3Red\text{-}HexNAc_1$	1		3279.6988	3279.6974	0.45
$Neu5Ac_1HexNAc_5Hex_6Fuc_1Red\text{-}HexNAc_1$	3		3292.6934	3292.6926	0.25
$Neu5Gc_2HexNAc_4Hex_6Red\text{-}HexNAc_1$	3		3294.6737	3294.6719	0.56
$Neu5Ac_1HexNAc_6Hex_4Fuc_2Red\text{-}HexNAc_1$	1		3303.7074	3303.7086	0.35
$Neu5Ac_2HexNAc_3Hex_5Fuc_3Red\text{-}HexNAc_1$	1		3307.6916	3307.6923	0.20
$HexNAc_5Hex_7Fuc_2Red\text{-}HexNAc_1$	1		3309.7096	3309.7079	0.51
$Neu5Ac_1HexNAc_5Hex_7Red\text{-}HexNAc_1$	5		3322.7035	3322.7032	0.11
$Neu5Gc_1HexNAc_5Hex_6Fuc_1Red\text{-}HexNAc_1$					
$HexNAc_5Hex_8Fuc_1Red\text{-}HexNAc_1$	2		3339.7165	3339.7185	0.59
$Neu5Ac_1HexNAc_6Hex_6Red\text{-}HexNAc_1$	3		3363.7294	3363.7297	0.09
$Neu5Ac_2HexNAc_4Hex_5Fuc_2Red\text{-}HexNAc_1$	1		3378.7297	3378.7294	0.10
$HexNAc_6Hex_7Fuc_1Red\text{-}HexNAc_1$	1		3380.7430	3380.7450	0.59

$Neu5Ac_2HexNAc_4Hex_6Fuc_1$ Red-$HexNAc_1$	3		3408.7401	3408.7400	0.05
$Neu5Gc_1Neu5Ac_1HexNAc_4$ Hex_5Fuc_2Red-$HexNAc_1$					
$Neu5Gc_3HexNAc_3Hex_5Fuc_1$ Red-$HexNAc_1$	1		3410.7207	3410.7192	0.44
$Neu5Gc_1Neu5Ac_1HexNAc_4$ Hex_6Fuc_1Red-$HexNAc_1$	1		3438.7499	3438.7505	0.17
$Neu5Ac_2HexNAc_5Hex_5Fuc_1$ Red-$HexNAc_1$	4		3449.7657	3449.7665	0.22
$Neu5Ac_1HexNAc_5Hex_6Fuc_2$ Red-$HexNAc_1$	1		3466.7841	3466.7818	0.67
$Neu5Gc_1Neu5Ac_1HexNAc_4$ Hex_7Red-$HexNAc_1$	2		3468.7626	3468.7611	0.45
$Neu5Ac_2HexNAc_5Hex_6$Red-$HexNAc_1$	2		3479.7769	3479.7771	0.04
$HexNAc_5Hex_7Fuc_3$Red-$HexNAc_1$	1		3483.7965	3483.7971	0.18
$Neu5Ac_1HexNAc_5Hex_7Fuc_1$ Red-$HexNAc_1$	3		3496.7925	3496.7924	0.04
$Neu5Gc_1Neu5Ac_1HexNAc_5$ Hex_6Red-$HexNAc_1$	1		3509.7898	3509.7876	0.63
$Neu5Gc_1HexNAc_5Hex_7Fuc_1$ Red-$HexNAc_1$	2		3526.8027	3526.8030	0.06
$Neu5Ac_1HexNAc_6Hex_6Fuc_1$ Red-$HexNAc_1$	3		3537.8159	3537.8189	0.85
$HexNAc_5Hex_9Fuc_1$Red-$HexNAc_1$	3		3543.8219	3543.8183	1.04
$HexNAc_6Hex_7Fuc_2$Red-$HexNAc_1$	1		3554.8335	3554.8343	0.20
$Neu5Ac_1HexNAc_6Hex_7$Red-$HexNAc_1$	3		3567.8321	3567.8295	0.74
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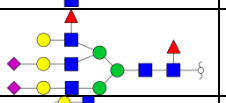
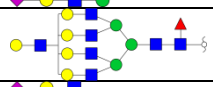
$Neu5Ac_2HexNAc_4Hex_6Fuc_2$ Red-$HexNAc_1$	2		3582.8307	3582.8292	0.44
$HexNAc_6Hex_8Fuc_1$Red-$HexNAc_1$	1		3584.8476	3584.8448	0.79
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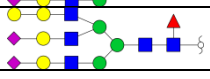
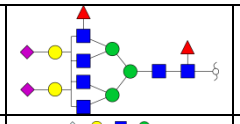
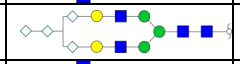
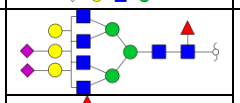
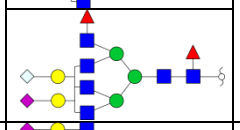
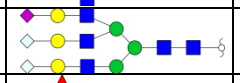
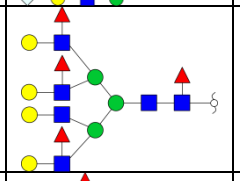
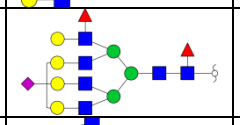
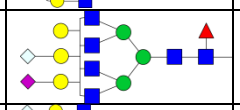
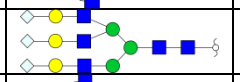
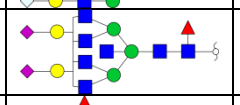
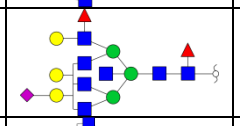
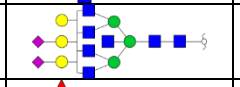
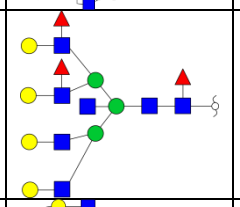
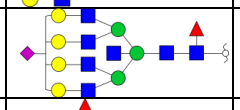
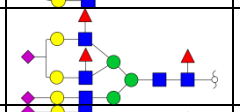
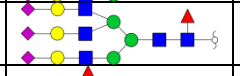
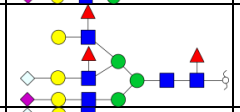
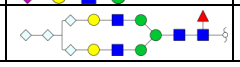
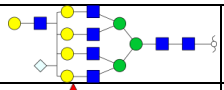
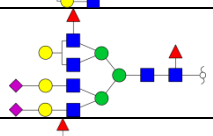
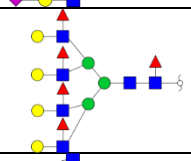
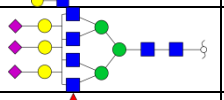
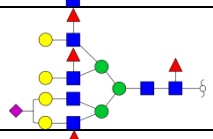
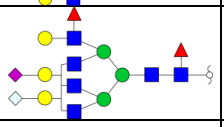
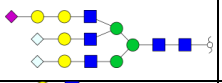
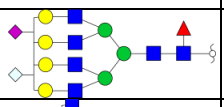
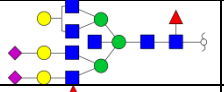
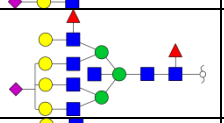
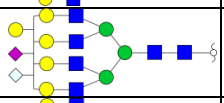
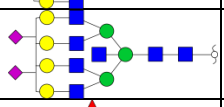
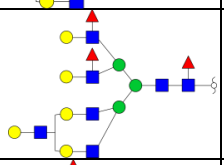
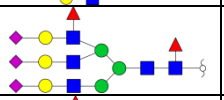
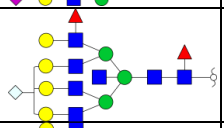
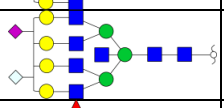
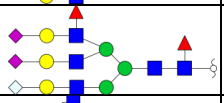
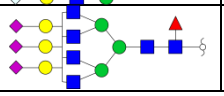
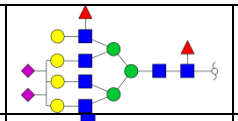
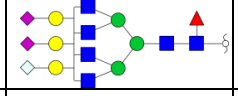
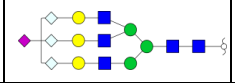
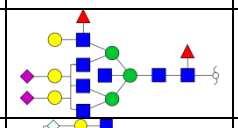
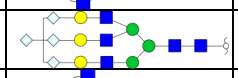
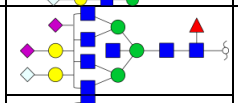
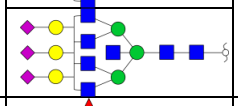
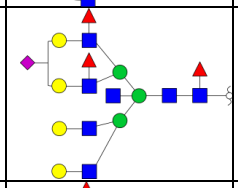
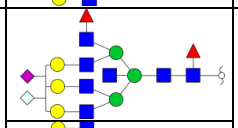
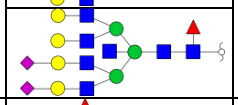
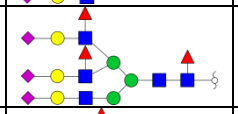
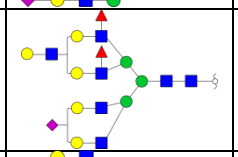
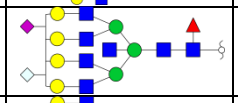
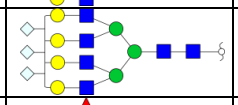
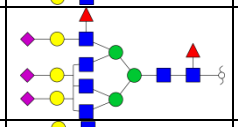
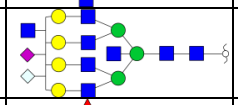
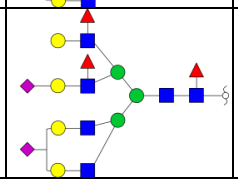
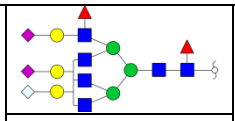
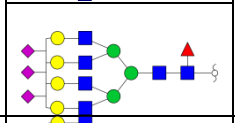
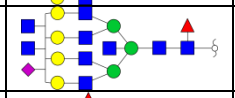
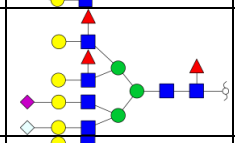
$Neu5Ac_2HexNAc_4Hex_7Fuc_1$ Red-$HexNAc_1$	2		3612.8390	3612.8397	0.19

$Neu5Ac_2HexNAc_5Hex_5Fuc_2$ Red-$HexNAc_1$	2		3623.8539	3623.8557	0.49
$Neu5Gc_4HexNAc_3Hex_5$Red-$HexNAc_1$	1		3627.8160	3627.8143	0.49
$Neu5Ac_2HexNAc_5Hex_6Fuc_1$ Red-$HexNAc_1$	4		3653.8671	3653.8663	0.24
$Neu5Gc_1Neu5Ac_1HexNAc_5$ Hex_5Fuc_2Red-$HexNAc_1$					
$Neu5Gc_2Neu5Ac_1HexNAc_4$ Hex_6Red-$HexNAc_1$	3		3655.8466	3655.8456	0.30
$HexNAc_5Hex_7Fuc_4$Red-$HexNAc_1$	1		3657.8861	3657.8864	0.06
$Neu5Ac_1HexNAc_5Hex_7Fuc_2$ Red-$HexNAc_1$	1		3670.8806	3670.8816	0.26
$Neu5Gc_1Neu5Ac_1HexNAc_5$ Hex_6Fuc_1Red-$HexNAc_1$	2		3683.8778	3683.8769	0.27
$Neu5Gc_3HexNAc_4Hex_6$Red-$HexNAc_1$	3		3685.8573	3685.8561	0.33
$Neu5Ac_2HexNAc_6Hex_5Fuc_1$ Red-$HexNAc_1$	1		3694.8909	3694.8928	0.51
$Neu5Ac_1HexNAc_6Hex_6Fuc_2$ Red-$HexNAc_1$	1		3711.9066	3711.9082	0.41
$Neu5Ac_2HexNAc_6Hex_6$Red-$HexNAc_1$	2		3724.9035	3724.9034	0.04
$HexNAc_6Hex_7Fuc_3$Red-$HexNAc_1$	1		3728.9238	3728.9235	0.10
$Neu5Ac_1HexNAc_6Hex_7Fuc_1$ Red-$HexNAc_1$	1		3741.9191	3741.9187	0.11
$Neu5Ac_2HexNAc_4Hex_6Fuc_3$ Red-$HexNAc_1$	1		3756.9155	3756.9184	0.76
$Neu5Ac_3HexNAc_4Hex_6Fuc_1$ Red-$HexNAc_1$	5		3769.9146	3769.9136	0.27
$Neu5Gc_1Neu5Ac_1HexNAc_4$ Hex_6Fuc_3Red-$HexNAc_1$	2		3786.9277	3786.9289	0.32
$Neu5Gc_1Neu5Ac_2HexNAc_4$ Hex_6Fuc_1Red-$HexNAc_1$	4		3799.9252	3799.9242	0.28
$Neu5Gc_4HexNAc_3Hex_5Fuc_1$ Red-$HexNAc_1$	1		3801.9041	3801.9035	0.18

$Neu5Gc_1HexNAc_6Hex_8Red\text{-}HexNAc_1$	1		3801.9419	3801.9398	0.55
$Neu5Ac_2HexNAc_5Hex_6Fuc_2Red\text{-}HexNAc_1$	1		3827.9539	3827.9555	0.40
$HexNAc_5Hex_7Fuc_5Red\text{-}HexNAc_1$	1		3831.9739	3831.9756	0.43
$Neu5Ac_3HexNAc_5Hex_6Red\text{-}HexNAc_1$	3		3840.9500	3840.9507	0.18
$Neu5Ac_1HexNAc_5Hex_7Fuc_3Red\text{-}HexNAc_1$	1		3844.9712	3844.9708	0.11
$Neu5Gc_1Neu5Ac_1HexNAc_5Hex_6Fuc_2Red\text{-}HexNAc_1$	3		3857.9642	3857.9661	0.47
$Neu5Gc_2Neu5Ac_1HexNAc_4Hex_7Red\text{-}HexNAc_1$	3		3859.9460	3859.9453	0.19
$Neu5Gc_1Neu5Ac_1HexNAc_5Hex_7Fuc_1Red\text{-}HexNAc_1$	2		3887.9798	3887.9766	0.83
$Neu5Ac_2HexNAc_6Hex_6Fuc_1Red\text{-}HexNAc_1$	2		3898.9928	3898.9926	0.06
$Neu5Ac_1HexNAc_6Hex_7Fuc_2Red\text{-}HexNAc_1$	1		3916.0094	3916.0079	0.39
$Neu5Gc_1Neu5Ac_1HexNAc_5Hex_8Red\text{-}HexNAc_1$	1		3917.9891	3917.9872	0.50
$Neu5Ac_2HexNAc_6Hex_7Red\text{-}HexNAc_1$	3		3929.0053	3929.0032	0.55
$HexNAc_6Hex_8Fuc_3Red\text{-}HexNAc_1$	1		3933.0222	3933.0232	0.25
$Neu5Ac_3HexNAc_4Hex_6Fuc_2Red\text{-}HexNAc_1$	2		3944.0048	3944.0028	0.51
$Neu5Gc_1HexNAc_6Hex_7Fuc_2Red\text{-}HexNAc_1$	1		3946.0210	3946.0185	0.65
$Neu5Gc_1Neu5Ac_1HexNAc_6Hex_7Red\text{-}HexNAc_1$	1		3959.0162	3959.0137	0.63
$Neu5Gc_1Neu5Ac_2HexNAc_4Hex_6Fuc_2Red\text{-}HexNAc_1$	2		3974.0151	3974.0134	0.44
$Neu5Ac_3HexNAc_5Hex_6Fuc_1Red\text{-}HexNAc_1$	3		4015.0411	4015.0400	0.30

$Neu5Ac_2HexNAc_5Hex_7Fuc_2$ Red-$HexNAc_1$	2		4032.0550	4032.0553	0.05
$Neu5Gc_1Neu5Ac_2HexNAc_5$ Hex_6Fuc_1Red-$HexNAc_1$	2		4045.0480	4045.0505	0.61
$Neu5Gc_3Neu5Ac_1HexNAc_4$ Hex_6 Red-$HexNAc_1$	1		4047.0278	4047.0298	0.48
$Neu5Ac_2HexNAc_6Hex_6Fuc_2$ Red-$HexNAc_1$	2		4073.0795	4073.0818	0.56
$Neu5Gc_4HexNAc_4Hex_6$Red-$HexNAc_1$	5		4077.0424	4077.0403	0.51
$Neu5Gc_1Neu5Ac_2HexNAc_6$ Hex_5Fuc_1Red-$HexNAc_1$	2		4086.0771	4086.0771	0.00
$Neu5Ac_3HexNAc_6Hex_6$Red-$HexNAc_1$					
$Neu5Ac_1HexNAc_6Hex_7Fuc_3$ Red-$HexNAc_1$	1		4090.0981	4090.0971	0.25
$Neu5Gc_1Neu5Ac_1HexNAc_6$ Hex_6Fuc_2Red-$HexNAc_1$	3		4103.0940	4103.0924	0.41
$Neu5Ac_2HexNAc_6Hex_7Fuc_1$ Red-$HexNAc_1$					
$Neu5Ac_3HexNAc_4Hex_6Fuc_3$ Red-$HexNAc_1$	1		4118.0946	4118.0920	0.63
$Neu5Ac_1HexNAc_6Hex_8Fuc_2$ Red-$HexNAc_1$	2		4120.1062	4120.1077	0.35
$Neu5Gc_1Neu5Ac_1HexNAc_6$ Hex_7Fuc_1Red-$HexNAc_1$	2		4133.1031	4133.1029	0.05
$Neu5Gc_3HexNAc_5Hex_7$Red-$HexNAc_1$	2		4135.0839	4135.0822	0.42
$Neu5Ac_3HexNAc_5Hex_6Fuc_2$ Red-$HexNAc_1$	3		4189.1287	4189.1292	0.10
$Neu5Gc_1Neu5Ac_1HexNAc_7$ Hex_7 Red-$HexNAc_1$	1		4204.1435	4204.1401	0.83
$Neu5Ac_2HexNAc_5Hex_7Fuc_3$ Red-$HexNAc_1$	1		4206.1446	4206.1445	0.04

$Neu5Gc_1Neu5Ac_2HexNAc_5Hex_6Fuc_2Red\text{-}HexNAc_1$	3		4219.1409	4219.1397	0.29
$Neu5Ac_3HexNAc_5Hex_7Fuc_1Red\text{-}HexNAc_1$					
$Neu5Ac_1HexNAc_8Hex_7Fuc_1Red\text{-}HexNAc_1$	1		4232.1730	4232.1714	0.40
$Neu5Gc_1Neu5Ac_1HexNAc_5Hex_7Fuc_3Red\text{-}HexNAc_1$	2		4236.1587	4236.1550	0.88
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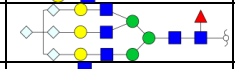
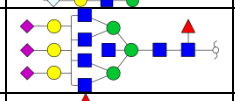
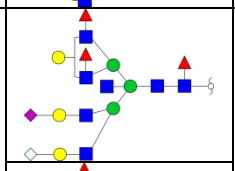
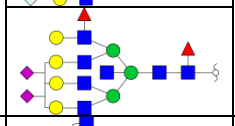
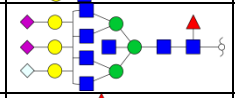
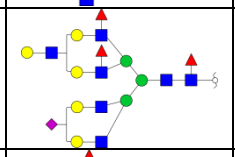
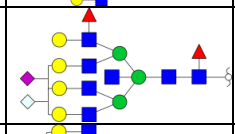
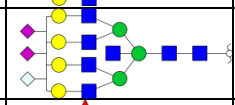
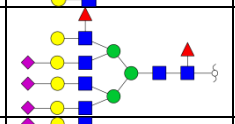
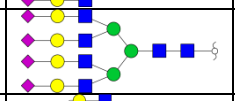
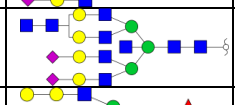
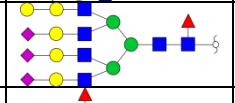
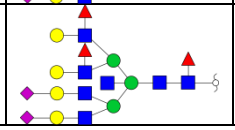
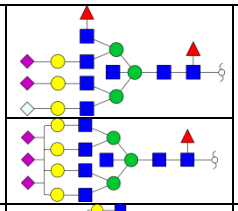
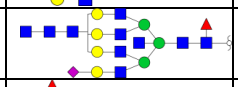
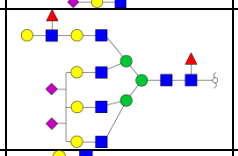
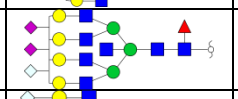
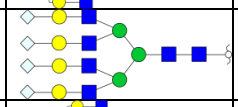
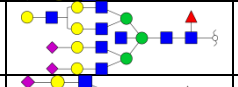
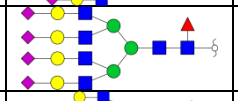
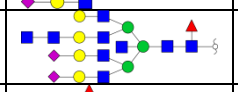
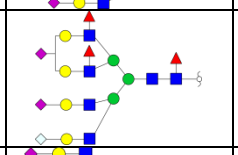
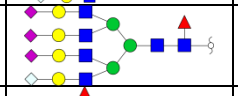
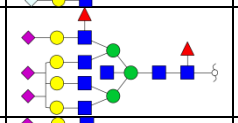
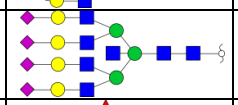
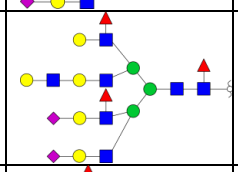
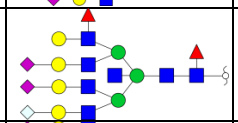
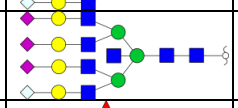
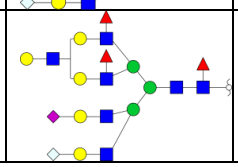
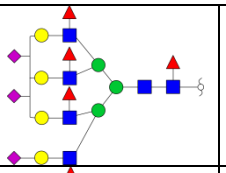
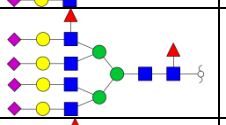
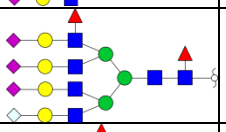
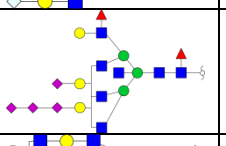
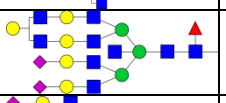
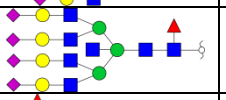
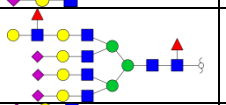
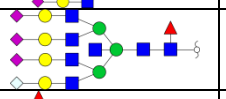
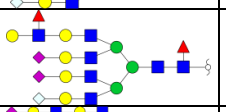
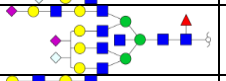
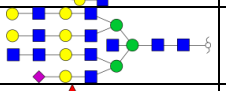
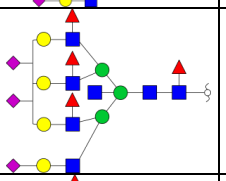
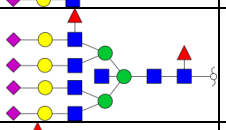
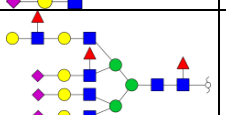
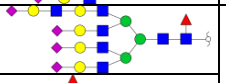
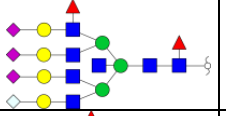
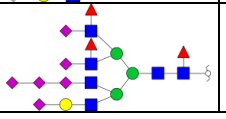
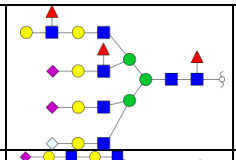
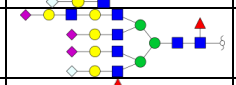
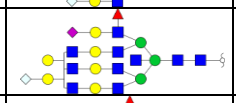
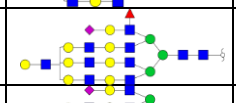
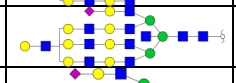
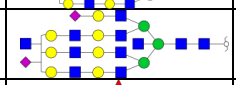
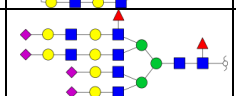
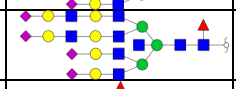
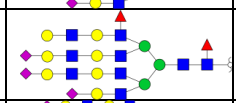
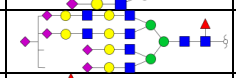
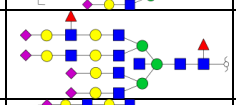
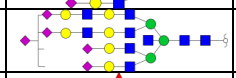
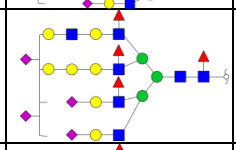
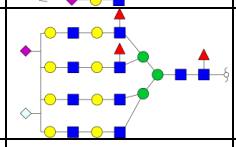
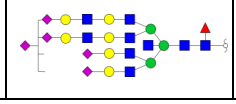
$Neu5Gc_4HexNAc_4Hex_6Fuc_1Red\text{-}HexNAc_1$	1		4251.1330	4251.1296	0.82
$Neu5Ac_3HexNAc_6Hex_6Fuc_1Red\text{-}HexNAc_1$	3		4260.1667	4260.1663	0.11
$Neu5Gc_1Neu5Ac_1HexNAc_6Hex_6Fuc_3Red\text{-}HexNAc_1$	3		4277.1851	4277.1816	0.83
$Neu5Ac_2HexNAc_6Hex_7Fuc_2Red\text{-}HexNAc_1$					
$Neu5Gc_1Neu5Ac_2HexNAc_6Hex_6Fuc_1Red\text{-}HexNAc_1$	2		4290.1780	4290.1768	0.28
$Neu5Ac_1HexNAc_6Hex_8Fuc_3Red\text{-}HexNAc_1$	1		4294.1980	4294.1969	0.27
$Neu5Gc_1Neu5Ac_1HexNAc_6Hex_7Fuc_2Red\text{-}HexNAc_1$	1		4307.1956	4307.1922	0.81
$Neu5Gc_1Neu5Ac_2HexNAc_6Hex_7Red\text{-}HexNAc_1$	1		4320.1908	4320.1874	0.80
$Neu5Ac_3HexNAc_5Hex_7Fuc_2Red\text{-}HexNAc_1$	2		4393.2298	4393.2289	0.21
$Neu5Ac_4HexNAc_5Hex_7Red\text{-}HexNAc_1$	2		4406.2239	4406.2242	0.05
$Neu5Ac_2HexNAc_8Hex_7Red\text{-}HexNAc_1$	1		4419.2595	4419.2558	0.85
$Neu5Ac_3HexNAc_5Hex_8Fuc_1Red\text{-}HexNAc_1$	1		4423.2431	4423.2395	0.83
$Neu5Ac_2HexNAc_6Hex_7Fuc_3Red\text{-}HexNAc_1$	1		4451.2709	4451.2708	0.03

$Neu5Gc_1Neu5Ac_2HexNAc_6Hex_6Fuc_2Red\text{-}HexNAc_1$ $Neu5Ac_3HexNAc_6Hex_7Fuc_1Red\text{-}HexNAc_1$	4		4464.2674	4464.2660	0.32
$Neu5Ac_1HexNAc_9Hex_7Fuc_1Red\text{-}HexNAc_1$	2		4477.3008	4477.2977	0.71
$Neu5Ac_2HexNAc_6Hex_8Fuc_2Red\text{-}HexNAc_1$	2		4481.2811	4481.2814	0.05
$Neu5Gc_1Neu5Ac_2HexNAc_6Hex_7Fuc_1Red\text{-}HexNAc_1$	2		4494.2744	4494.2766	0.48
$Neu5Gc_4HexNAc_5Hex_7Red\text{-}HexNAc_1$	2		4526.2695	4526.2664	0.68
$Neu5Ac_2HexNAc_7Hex_8Fuc_1Red\text{-}HexNAc_1$	1		4552.3169	4552.3185	0.33
$Neu5Ac_4HexNAc_5Hex_7Fuc_1Red\text{-}HexNAc_1$	2		4580.3148	4580.3134	0.32
$Neu5Ac_2HexNAc_8Hex_7Fuc_1Red\text{-}HexNAc_1$	2		4593.3477	4593.3450	0.59
$Neu5Gc_1Neu5Ac_2HexNAc_5Hex_7Fuc_3Red\text{-}HexNAc_1$	1		4597.3248	4597.3287	0.84
$Neu5Gc_1Neu5Ac_3HexNAc_5Hex_7Fuc_1Red\text{-}HexNAc_1$	2		4610.3269	4610.3240	0.65
$Neu5Ac_3HexNAc_6Hex_7Fuc_2Red\text{-}HexNAc_1$	1		4638.3589	4638.3553	0.80
$Neu5Ac_4HexNAc_6Hex_7Red\text{-}HexNAc_1$	1		4651.3502	4651.3505	0.05
$Neu5Ac_2HexNAc_6Hex_8Fuc_3Red\text{-}HexNAc_1$	1		4655.3705	4655.3706	0.00
$Neu5Gc_1Neu5Ac_2HexNAc_6Hex_7Fuc_2Red\text{-}HexNAc_1$	3		4668.3671	4668.3658	0.29
$Neu5Gc_1Neu5Ac_3HexNAc_6Hex_7Red\text{-}HexNAc_1$	1		4681.3649	4681.3611	0.83
$Neu5Gc_1Neu5Ac_1HexNAc_6Hex_8Fuc_3Red\text{-}HexNAc_1$	1		4685.3806	4685.3811	0.10

$Neu5Ac_3HexNAc_5Hex_7Fuc_4$ Red-$HexNAc_1$	2		4741.4049	4741.4074	0.51
$Neu5Ac_4HexNAc_5Hex_7Fuc_2$ Red-$HexNAc_1$	1		4754.4021	4754.4026	0.09
$Neu5Gc_1Neu5Ac_3HexNAc_5$ Hex_7Fuc_2Red-$HexNAc_1$	1		4784.4155	4784.4132	0.50
$Neu5Ac_4HexNAc_6Hex_6Fuc_2$ Red-$HexNAc_1$	1		4795.4269	4795.4292	0.46
$Neu5Ac_2HexNAc_8Hex_8Fuc_1$ Red-$HexNAc_1$	2		4797.4465	4797.4448	0.37
$Neu5Ac_4HexNAc_6Hex_7Fuc_1$ Red-$HexNAc_1$	1		4825.4396	4825.4397	0.01
$Neu5Ac_3HexNAc_6Hex_8Fuc_2$ Red-$HexNAc_1$	1		4842.4555	4842.4550	0.11
$Neu5Gc_1Neu5Ac_3HexNAc_6$ Hex_7Fuc_1Red-$HexNAc_1$	1		4855.4549	4855.4503	0.97
$Neu5Gc_1Neu5Ac_2HexNAc_6$ Hex_8Fuc_2Red-$HexNAc_1$	1		4872.4635	4872.4656	0.42
$Neu5Gc_1Neu5Ac_2HexNAc_7$ Hex_8Fuc_1Red-$HexNAc_1$	1		4943.5079	4943.5027	1.06
$Neu5Ac_1HexNAc_{10}Hex_9$ Red-$HexNAc_1$	1		4956.5334	4956.5343	0.18
$Neu5Ac_3HexNAc_6Hex_7Fuc_4$ Red-$HexNAc_1$	1		4986.5364	4986.5337	0.56
$Neu5Ac_4HexNAc_6Hex_7Fuc_2$ Red-$HexNAc_1$	1		4999.5335	4999.5289	0.93
$Neu5Ac_3HexNAc_6Hex_8Fuc_3$ Red-$HexNAc_1$	2		5016.5401	5016.5442	0.81
$Neu5Ac_4HexNAc_6Hex_8Fuc_1$ Red-$HexNAc_1$	3		5029.5433	5029.5395	0.77
$Neu5Gc_1Neu5Ac_3HexNAc_6$ Hex_7Fuc_2Red-$HexNAc_1$					
$Neu5Ac_6HexNAc_5Hex_4Fuc_3$ Red-$HexNAc_1$	1		5038.5389	5038.5398	0.17

$Neu5Gc_1Neu5Ac_2HexNAc_6Hex_8Fuc_3Red\text{-}HexNAc_1$	1		5046.5579	5046.5548	0.63
$Neu5Gc_1Neu5Ac_3HexNAc_6Hex_8Fuc_1Red\text{-}HexNAc_1$	1		5059.5561	5059.5501	1.21
$Neu5Gc_1Neu5Ac_1HexNAc_9Hex_9Fuc_1Red\text{-}HexNAc_1$	1		5276.6828	5276.6815	0.27
$Neu5Ac_1HexNAc_9Hex_{11}Fuc_1Red\text{-}HexNAc_1$	1		5293.7004	5293.6968	0.70
$Neu5Ac_1HexNAc_{10}Hex_{11}Red\text{-}HexNAc_1$	1		5364.7355	5364.7339	0.31
$Neu5Ac_2HexNAc_{10}Hex_{10}Red\text{-}HexNAc_1$	1		5521.8098	5521.8078	0.38
$Neu5Ac_4HexNAc_7Hex_9Fuc_2Red\text{-}HexNAc_1$	1		5652.8537	5652.8548	0.18
$Neu5Ac_4HexNAc_8Hex_9Fuc_1Red\text{-}HexNAc_1$	1		5723.8927	5723.8919	0.15
$Neu5Ac_3HexNAc_8Hex_{10}Fuc_2Red\text{-}HexNAc_1$	1		5740.9115	5740.9072	0.76
$Neu5Ac_5HexNAc_7Hex_9Fuc_1Red\text{-}HexNAc_1$	1		5839.9429	5839.9393	0.64
$Neu5Ac_4HexNAc_8Hex_9Fuc_2Red\text{-}HexNAc_1$	1		5897.9791	5897.9811	0.33
$Neu5Ac_5HexNAc_8Hex_9Red\text{-}HexNAc_1$	1		5910.9802	5910.9764	0.66
$Neu5Ac_4HexNAc_6Hex_{10}Fuc_4Red\text{-}HexNAc_1$	1		5960.0010	5960.0067	0.94
$Neu5Gc_1Neu5Ac_1HexNAc_9Hex_{11}Fuc_3Red\text{-}HexNAc_1$	1		6033.0540	6033.0594	0.89
$Neu5Ac_5HexNAc_8Hex_9Fuc_1Red\text{-}HexNAc_1$	2		6085.0712	6085.0656	0.94

*N-glycan compositions: Hex (hexose: ○ galactose/ ● mannose), ■ HexNAc (N-acetylhexosamine), ◆Neu5Ac (N-acetylneuraminic acid), ◇Neu5Gc (N-Glycolylneuraminic acid) and ▲ Fuc (fucose).

9. Risks and safety statements

According to Globally Harmonized System of Classification and Labeling of Chemicals (GHS), a list of potentially hazardous chemicals with the respective hazard and precautionary statements is given as follows:

Compound	GHS symbol	GHS hazard	Hazard statements	Precautionary statements
Methanol (LiChrosolv®)		GHS02 GHS06 GHS08	H225-H301+H311 +H331-H370	P210-P240-P280-P302+P352 -P304+P340-P308+P310-P403+P233
Ammonium bicarbonate		GHS07	H302	P301+P312+P330
Dithiothreitol		GHS07	H302-H315-H319-H335	P261-P305+P351+P338
Iodoacetamide		GHS06 GHS08	H301-H317-H334	P261-P280-P301+P310-P342+P311
Formic acid		GHS02 GHS06 GHS05	H226-H302-H314-H331-EUH071	P210-P280-P301+P330+P331-P304+P340-P305+P351+P338-P308+P310

Trifluoroacetic acid		GHS05 GHS07	H314-H332-H412	P273-P280-P305+P351+P338-P310
Acetonitrile (LiChrosolv®)		GHS02 GHS07	H225-H302+H312+H332-H319	P210-P240-P302+P352-P305+P351+P338-P403+P233
Trypsin		GHS07 GHS08	H315-H319-H334-H317-H335	P264-P272-P280-P302+P352-P305+P351+P338-P312
Iodomethane		GHS06 GHS08	H301-H312-H315-H331-H335-H351	P261-P280-P301+P310-P311
Sodium deoxycholate		GHS07	H302	P301+P312+P330
Tetraethylammonium borohydride		GHS02 GHS07	H261-H315-H319-H335	P231+P232-P261-P305+P351+P338-P422

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11. Declaration

I hereby declare on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids. The submitted written version corresponds to the version on the electronic storage medium. I hereby declare that I have not previously applied or pursued for a doctorate (Ph.D. studies).

City and date:
Hamburg, 01.07.2021

Signature: Min Zhang