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Serum Glycosylation Characterization of Osteonecrosis of the Femoral Head by Mass Spectrometry

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Abstract

Osteonecrosis of the femoral head (ONFH) is a recalcitrant and paralyzing disease often discovered in the end stage at the time of diagnosis, which is often performed by physical examination and diagnostic imaging. ONFH is typically caused by trauma or long-term steroid use. There are over 30 million patients in US taking steroids, and roughly 40% will develop ONFH. However, the exact pathophysiological process is not well understood. This study aims to examine the alteration in serum glycosylation of ONFH using the state-of-the-art analytical tool to provide more chemical data for pathophysiology research and possibly biomarker discovery. A training set containing 27 serum samples from steroid induced ONFH patients and 25 from gender and age matched controls were collected and analyzed. Glycosylation of whole serum and site-specific glycosylation of immunoglobulins are characterized using ESI-Q-TOF and ESI-Triple-Quadruple via multiple reaction monitoring (MRM), respectively. The whole serum glycosylation analysis yielded 14 N-glycan compositions and MRM yielded 8 glycopeptides that were altered between cases and controls with statistical significance. The increase of the non-sialylated, non-fucosylated N-glycans and decrease of the fucosylated N-glycans are associated with the development of ONFH. Glycosylation is a post-translational protein modification is apparently affected by ONFH. Future studies with a larger cohort and patients from earlier stage will be performed to assess these potential markers' value in disease onset.

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Supporting information

Complete table of all serum glycans monitored in ONFH with fold change, P value and AUC. Complete table of the proteins, protein subclasses, and glycopeptide monitored in ONFH with fold change, P value and AUC.

Disclosure statement

The authors have declared no conflicts of interest.

Keywords

Biomarker; liquid chromatography; mass spectrometry; N-glycan; Osteonecrosis of the femoral head

Introduction

Non-traumatic osteonecrosis of the femoral head (ONFH) is a common and devastating disease that primarily affects patients in the 3rd to 5th decades of their life. In the United States, 10,000 to 20,000 new cases are diagnosed every year, and around 200,000 new cases occur in China.(Lavernia et al., 1999; Mont et al., 2006; Li et al., 2010) ONFH accounts for 5–12% of total hip arthroplasties each year in the United States.(Safran et al., 2011) Steroid induced ONFH (SONFH) is believed to account for a high percentage of non-traumatic ONFH.(Zhang et al., 2008) Nagasawa et al. found that 33% of patients with systemic lupus erythematosus (SLE) progressed to ONFH after receiving steroid treatment.(Nagasawa et al., 2005) Vascular collapse and necrosis resulted from pressure increase in femoral head due to adipocytes hypertrophy and vascular occlusion due to fat embolism are the two proposed mechanisms for SONFH. However, the exact pathophysiological process of SONFH is still not well understood.(Callaghan et al., 2007) Steinberg classification is a commonly used system to evaluate the disease progression based on radiographic change including: Stage 0, I, II, III, IV, V, and VI. (Steinberg et al., 1995) Conservative treatments, both surgical and nonsurgical, can preserve the femoral head in early stages while a replacement of the hip joint is needed in late stages. A study shows a success rate of 86%, 61%, and 59% by conservative treatment in Steinberg stage I, II, and III respectively. (Castro and Barrack, 2000) Currently, magnetic resonance imaging (MRI) is believed to be effective in diagnosing ONFH by providing information on arthritic morphological changes. Unfortunately, MRI is very expensive and time-consuming and not useful in assessing the effectiveness of treatment.(Zhao et al., 2012; Kato et al., 2005) Early detection is critical in order to possibly prevent and delay the progression of the disease and avoid the eventual total hip arthroplasties (THA). Therefore, a less cumbersome, less intrusive but more efficient method is needed to observe and monitor the onset and pathological progression of ONFH. There have been biomarker studies reported for ONFH. Interleukin 33 and adiponectin using enzyme-linked immunosorbent assay method showed a significantly higher level of interleukin 33 in ONFH III/IV compared to I/II and a significant lower level of adiponectin in ONFH compared to healthy controls, however sensitivity and specificity were not evaluated.(Zheng et al., 2014; Shuai et al., 2010)

Glycosylation is a post-translational modification of proteins that is common and abundant in blood, and has been shown to vary with various pathological disease states.(Ozcan et al., 2014; An et al., 2006; Kim et al., 2014; Maverakis et al., 2015; Parekh et al., 1985; Saldova et al., 2012; Vasseur et al., 2012; Barrabes et al., 2007; Christiansen et al., 2014) Significant improvements in glycomic and glycoproteomic methods have made it possible to identify and quantitate hundreds of glycoforms simultaneously.(Rosati et al., 2013; Hart and Copeland, 2010; Thaysen-Andersen and Packer, 2014; An et al., 2003; Krishnamoorthy and Mahal, 2009) The N-glycan analyses of human serum have also been reported using

hydrophilic interaction chromatography (HILIC).(Ruhaak et al., 2008; Saldova et al., 2014; Gornik and Lauc, 2008; Gornik et al., 2009) While these methods differ from that proposed here, they report many of the same structures that we have found in previous studies(Song et al., 2015) and in the current study. Saldova et al. and Gornik et al., the most abundant N-glycan composition in healthy control is consistent with our result which is A2G2S2 corresponding to N5402 in our study. The other major peaks in a HILIC chromatogram of serum N-glycans in the study from Gornik et al. contain A2G2S1 corresponding to N5401, A3F1G3S3 corresponding to N6513, A1G1 corresponding to N3300 are all observed in this study. However those glycans are reported with slight lower abundances both in this study and the study by Saldova et al. (Saldova et al., 2014) Cause of the difference is likely coming from the experimental settings, the separation mechanisms, and the detectors. In healthy individuals, the glycomic pattern is highly reproducible with relatively small variations.(Arnold et al., 2007) Thus, the serum glycomic profile has been the focus of several efforts in disease diagnosis, monitoring progression and pathogenesis.(Pierce and Taniguchi, 2013; Muthana and Gildersleeve, 2014; An et al., 2006; Kirmiz et al., 2007; An et al., 2009) Indeed, the glycomic signature has been shown altered in many diseases including in many types of cancers, rheumatoid arthritis and HIV. (Arthos et al., 2013; Ruhaak et al., 2013b)

There have been no glycomic analysis reported to monitor ONFH. However as a systemic disease, it is expected to similarly alter the patients' glycosylation. In this study, we mapped the serum glycomic pattern of SONFH (glucocorticoid) stage VI patients and compared them to age and gender-matched healthy controls. The comprehensive serum glycomic map include two types of analysis, namely profiling of released N-glycans using a microfluidic chip nano-LC system coupled to a time of flight (TOF) mass spectrometer and site-specific immunoglobulin glycoform monitoring using an ultra-performance liquid chromatography (UPLC) with a triple quadrupole (QqQ) mass spectrometer.(Ruhaak et al., 2013a; Hong et al., 2013b) This comprehensive serum glycomic analysis reveals serum glycans that can potentially be used as markers for the disease and may be of value toward understanding the pathological mechanism.

Results

The glycosylation of ONFH patient serum was investigated for its potential as biomarker. Two methods were employed for the glycomic analysis. First, a comprehensive profile was obtained yielding over 300 serum N-glycans. Identifications were based on searching the accurate masses against a combination of a theoretical and experimental database developed previously.(Kronewitter et al., 2009) The data base contains nearly 500 compositions. Second, site-specific glycoforms on IgG, IgA and IgM, the three major immunoglobulins in serum, were monitored using multiple reaction monitoring (MRM). The latter is an emerging technique that has been recently reviewed for its value in clinical practice.(Ruhaak and Lebrilla, 2015; Maverakis et al., 2015) The results represent the first serum glycan profile and site-specific glycoform determination of proteins for ONFH. Statistics tools including two-tailed unpaired t-test, partial least square and receiver operating characteristic (ROC) were used to evaluate the significance of the alteration, classification power, and sensitivity and specificity, respectively. Fourteen N-glycan compositions are significantly

changed between the control and ONFH groups. Eight site-specific N-glycan compositions are also significantly altered. Seven of the eight significant site-specific N-glycans do not show significant change in the released form, as the glycans may originate from many other proteins, thereby indicating the value of site-specific analysis.

Clinical samples and randomization

The sample information is summarized in Table 1. Each group was age and gender matched. Samples were randomized before sample preparation and analysis. One commercial standard human serum sample was inserted every ten randomized samples to monitor the sample preparation and instrument reproducibility.

Serum N-glycan profiling using nanoLC-Chip-TOF

Global serum N-glycomic profiles were acquired using nanoLC-Chip-TOF MS. N-Glycans are separated on a porous graphitized carbon column and detected by a time of flight mass analyzer. Figure 1 shows a representative total compound chromatogram for the ONFH and control groups. Each peak represents a distinct N-glycan structure. Abundances are calculated by integrating the ion count associated with each chromatographic peak. Relative abundances are obtained by normalizing the abundance of each N-glycan to the total N-glycan abundance. All comparisons were made based on relative abundances.

N-Glycans may be subdivided into several classes based on specific structural features: sialylated complex/hybrid (C/H-S), fucosylated complex/hybrid (C/H-F), sialylated fucosylated complex/hybrid(C/H-FS), high mannose (HM), and non-sialylated nonfucosylated complex/hybrid (C/H). Examination of the relative abundance of each N-glycan subclass indicates that C/H type glycans are up-regulated 20.1% significantly with a P value of 0.0018 while C/H-F type glycans are down-regulated 8.4% significantly with a P value of 0.0055 in the ONFH group (Figure 2). No significant differences were observed for the C/H-S, C/H-FS, and HM subclasses between the two groups. The relatively small error bars in Figure 2 representing the standard deviation indicates limited variation between the individuals for each subclass.

Assessment of released N-glycans as diagnostic markers

To determine the differentiating potential of the individual released N-glycans, t-test and receiving operating characteristics (ROC) curve analyses were performed. A ROC curve was constructed based on the regression of the relative abundances to sex and age. Over 330 N-glycan compositions were searched, and over 140 N-glycan compositions were observed. However, only 58 compositions were observed in over 75% of the samples (Supporting Table S1). Each composition is observed to have between one and five isomers to yield over 300 unique N-glycans throughout all samples.(Chu et al., 2009; Saldova et al., 2014) Table 2 shows the number of N-glycans that changed in ONFH with significant P values along with the AUC (area under the curve of ROC analysis), fold change, P value and structure. The remainder of the monitored N-glycans is listed in Supporting Table S2. There are 14 N-glycan compositions with statistically significant P values, 10 of which have AUC values greater than 0.7, making these potential biomarker candidates (Table 2). The compounds N3300, N3400, N3500, N4500, N4501, N5401, N5500, N5501, N5512, N9200 (N: N-

glycan, Four digits: Hex-HexNAc-Fuc-NeuAc, Hex: hexose, HexNAc: N-acetylhexoseamine, Fuc: fucose, NeuAc: N-acetylneuraminic acid) are found to be up-regulated in ONFH between 9% and 116%, with most around 50%. The compounds N3310, N4310, N4410, and N5410 are down-regulated in ONFH by 17%, 23%, 25% and 28% respectively. These results are consistent with the results obtained when only the N-glycan subclasses are considered. All N-glycans down-regulated are fucosylated, while most up-regulated glycans are complex/hybrid structures. The N-glycan that was most changed is N5501, which doubled in ONFH (Fold change: 2.16) with an AUC of 0.72. The compound N5410, the fucosylated di-galactosylated structure decreased 28% in ONFH and yielded the largest AUC (0.85).

A previous study on the association of serum glycosylation and the medication by Rudd and co-workers indicates that N5410, N5510, and N4411 decreased in the group taking glucocorticoids (P=0.0167), however did not look at ONFH patients.(Saldova et al., 2012) In this study, individuals with ONFH stopped glucocorticoid treatment 6–9 months before sample collection making it unlikely that the drug is still present in the subjects. To further investigate the relationship between the drug and the glycan, we compared the two groups with and without prior glucocorticoid regimen. In this analysis, N5410 decreased 28% (P<0.0001), while N5510 and N4411 did not change significantly. Their results may be indications of immediate and long term effects of the treatment on serum glycans.

Site-specific N-glycoforms of immunoglobulin

Immunoglobulins (Ig) G, A, and M are the most abundant glycoproteins in human serum and are associated with adaptive immune response.(Arnold et al., 2007) N-Glycosylation is known to have both profound and subtle effect on immune responses.(Arnold et al., 2007; Nechansky et al., 2007; Scallon et al., 2007; Rudd et al., 2001; Kaneko et al., 2006) Therefore, we monitored the N-glycans on specific glycosylation sites of Ig A, G and M to add to the global N-glycan analysis.(Hua et al., 2013) The glycoform and glyco-sites monitored in this work were chosen based on a previous comprehensive glycosite mapping of human serum. The MRM transitions used in this study are exactly the same and provided more comprehensively in two previous publications (Hong et al., 2015; Hong et al., 2013a) Upon tryptic digestion, glycopeptides were analyzed by triple quadrupole (QqQ) mass spectrometry using the MRM approach. The sensitivity and specificity of QqQ enabled the detection of less abundant glycopeptides (compared to peptides) in the complex serum tryptic digests. Moreover, the accuracy of its quantitation makes it a promising method in biomarker discovery. The relative abundance of each glycoform was obtained by normalizing the glycopeptide response to the peptide response of the glycoprotein as described previously.(Hong et al., 2013b) Differentially expressed glycopeptides in serum of ONFH patients were evaluated using a t-test, and the classification power was evaluated using ROC curves. Immunoglobulin glycopeptides with significant P values are listed along with the AUC, fold change, structures in Table 3. The remainder of the monitored glycopeptides is listed in Supporting Table S3. Eleven N-glycan compositions were monitored for the IgG1 subclass. Ten N-glycan compositions were monitored for the IgG2 subclass. Six N-glycan compositions were monitored for the IgG3 and 4 subclasses. All glycoforms on IgG reported in a previous IgG glycan mapping study were observed in this

study.(Hong et al., 2015; Hong et al., 2013a) The abundances of each IgG subclass and total IgG were also measured. The results show that the IgG subclasses and the total absolute abundances of IgG remained unchanged between the ONFH and control groups (Supporting Table S3). However, there were important changes in the glycosylation. The compounds N5510 and N5411 on IgG1 and N4410 on IgG3 and 4 were down-regulated with P values of 0.0478, 0.0366 and 0.0392 respectively. However, the analysis of the corresponding released glycans of N5510 and N5411 in serum did not alter significantly (Supporting Table S2) between the two patient groups. The results are not totally unexpected as there are many other proteins that can be a potential source for these two glycans. The N-glycan N4410 on IgG3&4 was down regulated as in accordance with the liberated glycan in serum, (P= 0.039 and 0.0004, respectively). Interestingly, N4410 on IgG1 and IgG2 did not change significantly between the patient groups, suggesting differential behavior among the same glycan compositions with different protein subclasses. Compounds N3410 and N4400 on IgG2 were up-regulated significantly with P values of 0.0352 and 0.0252, respectively while remained unchanged in serum (Table 3). A decrease of fucosylation on IgG1 was correlated with a stronger binding affinity to Fc gamma receptor IIIa on natural killer cell thereby increasing the antibody-dependent, cell-mediated cytotoxicity (ADCC) effect. (Jefferis, 2009; Shields et al., 2002) In this study, the fucosylated N-glycan N5411 and N5510 on IgG1 were down-regulated significantly indicating that the ADCC of IgG1 in ONFH may be induced to a higher level compared to the control group.

Nine N-glycan compositions were monitored for site 144 of IgA. This tryptic peptide is the same for IgA1 and IgA2. Eight N-glycan compositions were monitored for site 205 of IgA2 (**Table 3,** Supporting Table S3). Six N-glycan compositions were monitored for IgM site 46 along with four N-glycan compositions for IgM site 209, five N-glycan compositions for IgM site 439, and three N-glycan compositions for the J chain (**Table 3,** Supporting Table S3). The absolute protein abundances of IgA and the relative abundances of the subclasses were also measured. IgA1 was up-regulated in the ONFH group significantly while IgA2 did not change significantly. Consequently, total IgA protein was up-regulated significantly in the ONFH group. N-Glycans N5400 and N5401 on IgA site 144 were both down-regulated significantly with P values of 0.0016 and 0.0037 respectively. J chain and IgM protein levels were not altered in ONFH serum. The glycan N4511 on site 209 of IgM was down-regulated in the ONFH group with a P value of 0.0485, while the same composition did not change in the released glycan analysis. AUC values from ROC curves were evaluated for the site-specific N-glycans (**Table 3,** Supporting Table S3). N-Glycans with both a significant P value and an AUC greater than 0.7 included N3410 on IgG2, N5400 and N5401 on IgA site 144. The largest AUC value is 0.781 corresponding to N5400 on site 144 of IgA.

Discussion

This study examined the serum glycosylation associated with ONFH to discover potential biomarkers for monitoring steroid users who are under the risk developing ONFH. In general, we find that ONFH is associated with a general decrease in the fucosylation of serum. The individual glycans and the site specific analysis however are more complicated but appear to yield several very promising candidate markers.

Non-fucosylated glycans N4501, N4500, N3500, N5501, N5500, N3300, N3400 and N5401 are all up-regulated significantly in ONFH group in serum glycan analysis. Fucosylated glycan N5410, N4410, N4310 and N3310 are down-regulated in ONFH group, which correlates well with the increase of non-fucosylated glycans. The decrease of fucosylation further correlates with the decrease of some site-specific fucosylated glycan of immunoglobulins: N5411 and N5510 on IgG1, N4410 on IgG3&4, and N4511 on IgM site 209.

Specific glycans, namely N4501 and N3400, can differentiate the ONFH group from control group and are both up-regulated in serum but did not change significantly in site-specific analysis of the immunoglobulins. However, their fucosylated versions (N4511 on IgM site 209, N3410 on IgG2) on the immunoglobulins can differentiate the disease. The fucosylation pathways of immunoglobulins are potentially affected by ONFH.

Leave-one-out cross validation (LOOCV) partial least squares (PLS) regression was used to evaluate the classification power of the 10 potential N-glycan biomarkers with significant P value and AUC greater than 0.7 (Figure 3a). The AUC of the 10 N-glycan classifier is 0.846 with a 0.148 false positive rate (FPR). N5410, a fucosylated N-glycan, carries a similar AUC (0.85) compared to the 10 N-glycan classifier. They both exhibit a great potential in distinguishing ONFH stage VI from healthy group. With a prevalence of up to 40% steroid users develop ONFH, a candidate biomarker with a 0.85 AUC value of ROC curve shows a promising potential in continuing study to give a satisfactory predictive value.(Zhang et al., 2013; Rifai et al., 2006)

LOOCV PLS was also used for those three site-specific N-glycans with significant P values and AUC greater than 0.7 (Figure 3b). The three N-glycan together shows an AUC of 0.742, which is a smaller value compared to N5400 on IgA 144 alone. As shown in **Figure 3,** the combined 10 global N-glycan classifier (**3a**) exhibits a better separation compared to the three site-specific N-glycan classifier (**3b**). Based on this study, it appears that the global N-glycans are more effective in distinguishing ONFH from healthy group and therefore potentially better biomarkers.

This study represents an early example where both whole serum N-glycosylation and site-specific immunoglobulin N-glycosylation were simultaneously examined in a clinical set. The results indicate that the two methods have complementary power in biomarker discovery. While in this case released N-glycans yielded good candidate markers, glycosylation of immunoglobulins may lead to better mechanistic understanding of the disease. It further illustrates the potential of glycans as biomarkers for diseases.

Materials and methods

Chemicals and reagents

Pooled human serum standard and human serum proteins (Immunoglobulin G, Immunoglobulin A, Immunoglobulin M) were purchased from Sigma-Aldrich (St. Louis, MO); Peptide: N-Glycosidase F (PNGase F) was obtained from New England Biolabs (Ipswich, MA); Sequencing grade modified trypsin, porcine was purchased from Promega

(Madison, WI); Sodium borohydride was purchased from Sigma-Aldrich (St. Louis, MO). All solvents used are of analytical or HPLC grade.

Clinical sample collection

The blood samples were collected from patients and healthy controls under approval by the Research Ethics Committee of Institute of First Affiliated Hospital of Guangzhou University of Chinese Medicine, China. Informed consent was obtained from all patients and healthy controls. All the subjects are genetically unrelated. Patients were diagnosed using anteroposterior and lateral pelvic radiographs and magnetic resonance images. SONFH was defined by a history of a mean daily dose of ≥16.6 mg or highest daily dose of 80 mg of prednisolone equivalent within 1 year prior to the development of symptoms or radiological diagnosis in asymptomatic cases. 27 glucocorticoid-induced ONFH stage VI patients and 25 healthy individuals were included in this study. The patients all stopped the glucocorticoid treatment 6–9 months before sample collection.

Blood fluid were drained into blood tubes without anticoagulants and allowed to clot overnight at 4°C. Subsequently, the serum was separated by centrifugation at 2,000g for 15 minutes. The serum was aliquoted into 1.5ml Eppendorf tubes and stored at −80 °C.

N-Glycan sample preparation

N-Glycans were released from the serum of the ONFH group, control group and the standard serum sample using PNGase F, purified and enriched by solid phase extraction (SPE) using porous graphitized carbon (PGC) on a 96-well plate based on an established protocol.(Ruhaak et al., 2013a) The serum standard was aliquoted on the sample plate every 10 samples as the sample preparation control. The starting volume for each sample was 20 μL. Released N-glycan samples were then dried and reconstituted in 50 μL de-ionized water for nanoLC-CHIP-TOF MS analysis.

NanoLC-Chip-TOF profiling of overall glycosylation

A 1 μL solution of each reconstituted N-glycan sample was injected into an Agilent 6220 nanoLC-Chip-TOF mass spectrometer (MS) for global N-glycan profiling. A standard serum N-glycan pool (obtained from the standard serum) was injected every 10 samples as the instrument control in addition to the sample preparation control standard serum. N-glycans were separated on a chip containing a PGC analytical column (Particle size 5 μm, 75 μm x 43 mm) and PGC enrichment column (Particle size 5 μm, 40 nL). The 60-minute binary gradient consisted of solvent A of 3% acetonitrile, 0.1% formic acid; solvent B of 90% acetonitrile, 0.1% formic acid in nano-pure water (v/v) at a flow rate of 0.3 μL/min.

Glycopeptide preparation

20 μL of IgG (5 mg/mL), 20 μL of IgA (1.1 mg/mL), 20 μL of IgM (1.1 mg/mL) and 2 μL of each serum sample were reduced and alkylated prior to overnight trypsin digestion in a 37°C water bath. The resulting peptide and glycopeptide mixture was directly used for triple quadrupole (QqQ) MS analysis.

UPLC-ESI-QqQ analysis of site-specific glycosylation

Immunoglobulin peptides and glycopeptides were analyzed using an Agilent 1290 infinity UPLC system coupled to an Agilent 6490 electrospray ionization (ESI) QqQ MS. The UPLC separation was performed on an Agilent Eclipse Plus C18 column (RRHD 1.8 μm, 2.1×100 mm) capped with a Agilent Eclipse Plus C18 guard column with a 10-minute binary gradient consisting of solvent A of 3% acetonitrile, 0.1% formic acid; solvent B of 90% acetonitrile, 0.1% formic acid in nano-pure water (v/v) at a flow rate of 0.5 mL/min.

Statistical analysis

Abundances of the released N-glycans were normalized to the total N-glycan abundance and abundances of site-specific N-glycans were normalized to the peptide response of the corresponding protein before statistical analysis. Glycans with a detection frequency less than 75 % among all samples were removed. The remaining unobserved values were replaced with one half of the observed minimum value of the corresponding N-glycan. The data satisfied parametric assumptions. Unpaired t-test and receiver operating characteristic (ROC) curve analysis were applied using JMP Pro 11 (Cary, NC). Leave one out cross validation partial least squares regression was performed in DeviumWeb (reference: Dmitry Grapov. (2014) DeviumWeb: Dynamic Multivariate Data Analysis and Visualization Platform. v0.4).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ONFH	Osteonecrosis of the femoral head
MRM	multiple reaction monitoring
SLE	systemic lupus erythematosus
MRI	magnetic resonance imaging
THA	total hip arthroplasties
TOF	time of flight
UPLC	ultra-performance liquid chromatography
QqQ	triple quadrupole
PNGase F	N-Glycosidase F
SPE	solid phase extraction

PGC	porous graphitized carbon
MS	mass spectrometer
ESI	electrospray ionization
ROC	receiver operating characteristic
C/H-S	sialylated complex/hybrid
C/H-F	fucosylated complex/hybrid
C/H-FS	sialylated fucosylated complex/hybrid
HM	high mannose
C/H	non-sialylated nonfucosylated complex/hybrid
Ig	Immunoglobulins
LOOCV	Leave one out cross validation
PLS	partial least squares
FPR	false positive rate

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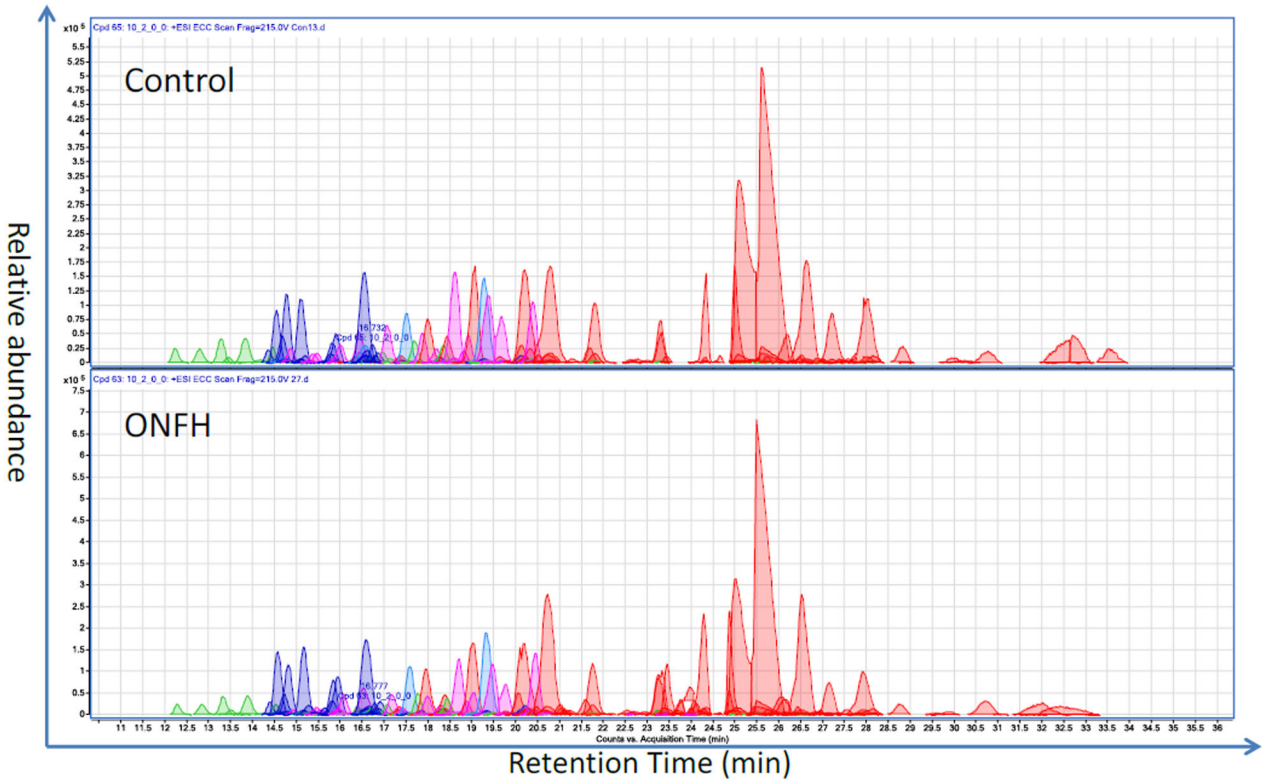


Figure 1.

Total compound chromatogram of released N-glycans from serum of control and ONFH patients.

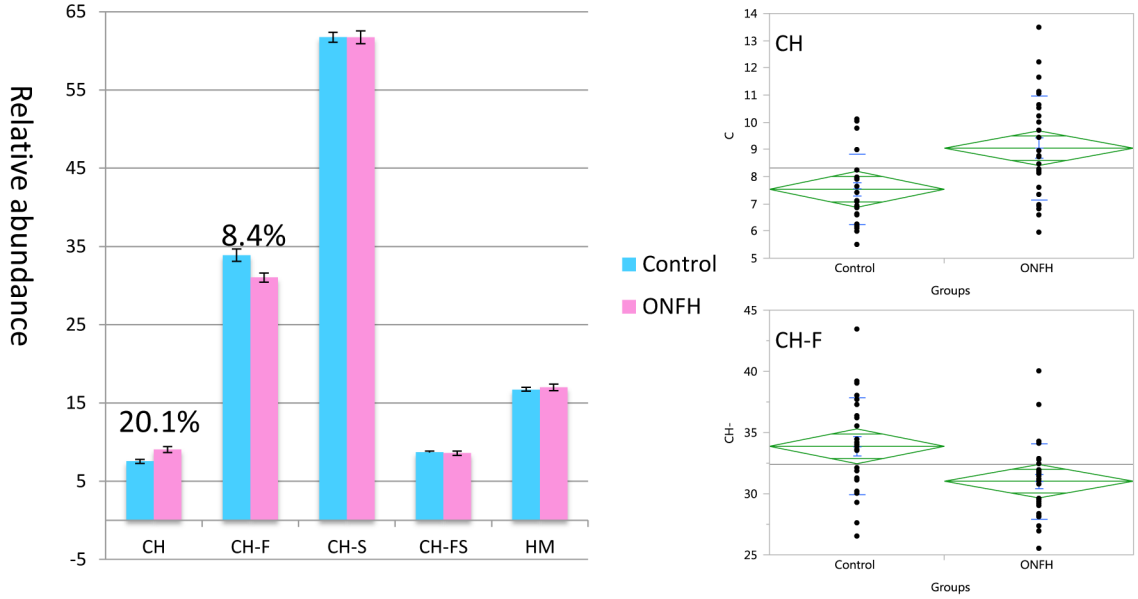
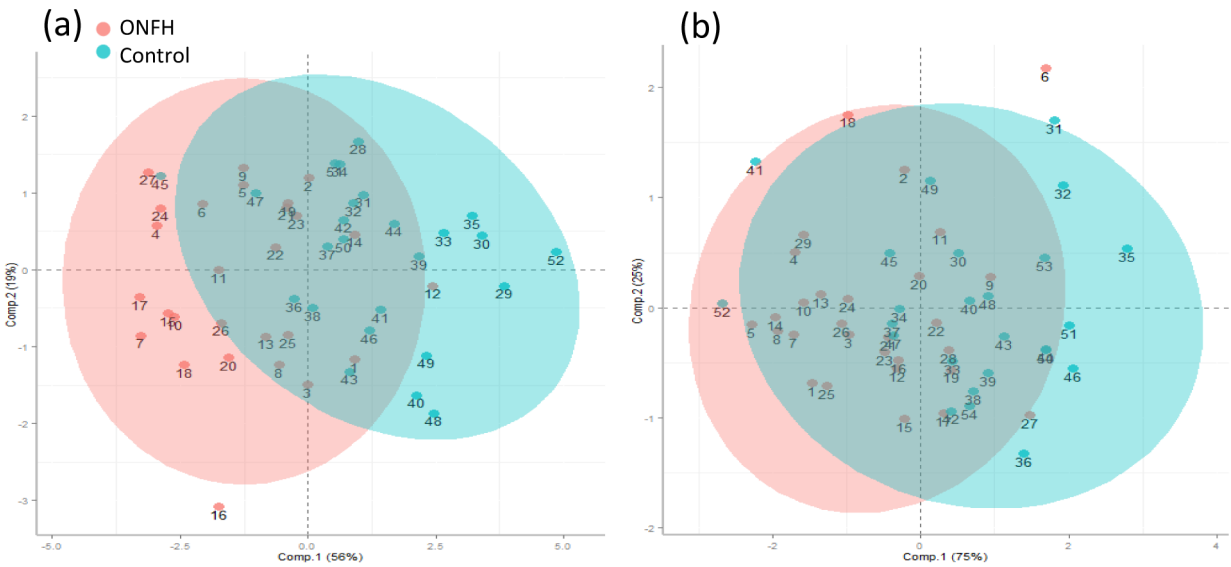


Figure 2.

Relative abundances of each N-glycan subclass for ONFH (Pink) and control (Blue). Statistically significant increase was obtained with the non-sialylated, non-fucosylated Complex/Hybrid (CH) structures and decrease with the fucoslylated Complex/Hybrid (FCH) group. Inset on the right side is the JMP diamond plot of CH and CH-F of control and ONFH. The center line of the diamond represents the mean and the top and bottom blue line represent the standard deviation.



Glycans in a classifier	AUC	Sensitivity	Specificity	FPR	Youden index
10 N-glycans in serum	0.846	0.84	0.852	0.148	0.692
3 site specific N-glycans	0.742	0.76	0.724	0.276	0.484

Figure 3.

(a) LOOCV PLS analysis using a classifier of 10 N-glycans with both significant P value and AUC greater than 0.7. (b) LOOCV PLS analysis using a classifier of three site-pecific N-glycans with both significant P value and AUC greater than 0.7. They are N5400 and N5401 on IgA1/2 site 144, and N3410 on IgG2 site 297 respectively. The AUC, sensitivity, specificity, false positive rate (FPR) and Youden index are listed.

Table 1.

Patient demographics and clinical profiles.

Diagnostic Category	N	Mean Age±SD	Sex	
			M	F
ONFH	27	34.9±9.7	12	15
Control	25	34.5±8.8	14	11
Total	52	34.9±9.2	26	26

Table 2.

Released N-glycans with fold change between control and ONFH. Symbolic N-glycan structures correspond to (

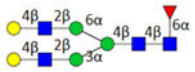
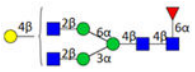
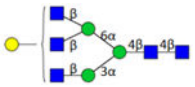
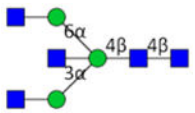
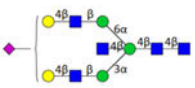
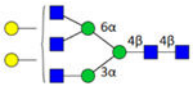
) N-acetylglucosamine (

) mannose (

) galactose (

) fucose (

) N-acetylneuraminic acid. Monosaccharides without linkage annotation means the location is putative. For example, N-glycan 3500 is the sum of all isomers including bisecting and lactosamine.

N-glycan	AUC	Fold Change	P	Structure
5410	0.85	0.721	<0.0001	
4501	0.816	1.491	0.0002	
4410	0.773	0.747	0.000	
4500	0.779	1.422	0.0008	
3500	0.721	1.435	0.0032	
5501	0.72	2.156	0.0053	
5500	0.716	1.197	0.0138	

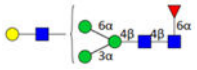
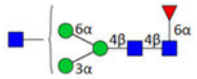
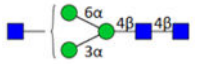
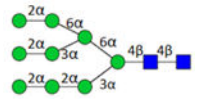
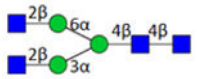
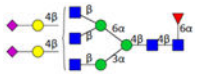
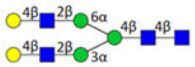
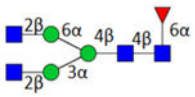
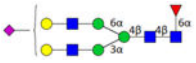
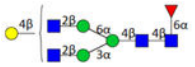
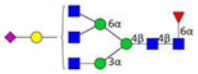
N-glycan	AUC	Fold Change	P	Structure
4310	0.714	0.770	0.0199	
3310	0.67	0.831	0.021	
3300	0.71	1.479	0.0338	
9200	0.641	1.132	0.0373	
3400	0.714	1.475	0.0378	
5401	0.637	1.094	0.0459	
5512	0.652	1.209	0.05	

Table 3.

Proteins, protein subclasses, and site-specific glycans altered in ONFH with P values. Symbolic N-glycan structures correspond to () N-acetylglucosamine () mannose ( ) galactose () fucose () N-acetylneuraminic acid. Concentration: Conc.

N-glycan	AUC	Fold Change	P	Structure	Peptide
5400_IgA1/2_144	0.781	0.757	0.002		LSLHRPALEDLLLGSEA**N**^{144}LT CTLTGLR
5401_IgA1/2_144	0.756	0.903	0.004		LSLHRPALEDLLLGSEA**N**^{144}LT CTLTGLR
IgA1	0.712	1.443	0.011	NA	TPLTATLSK
IgA_Conc.	0.697	1.388	0.012	NA	WLQGSQELPR
4400_IgG2	0.640	1.442	0.025		EEQF**N**^{176}STFR
3410_IgG2	0.708	1.325	0.035		EEQF**N**^{176}STFR
5411_IgG1	0.674	0.804	0.037		EEQY**N**^{180}STYR
4410_IgG3&4	0.632	0.735	0.039		EEQY**N**^{231}STFR/EEQF**N**^{173}STYR
5510_IgG1	0.643	0.817	0.048		EEQY**N**^{180}STYR

N-glycan	AUC	Fold Change	P	Structure	Peptide
4511_IgM_209	0.639	0.802	0.049		GLTFQQ**N**[209]ASSMCVPDQDTAIR