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**14. ABSTRACT**

We are testing the hypothesis that the risk of developing prostate cancer and the aggressiveness of the disease are influenced by protein glycosylation. We postulate that glycobiology contributes to the higher susceptibility of African American men. Newly optimized methods for the glycomic analysis of proteins fractionated into three components are in place. We have assembled the study population and completed the glycomic analysis in two fractions; analysis of the IgA/IgM fraction is under way. We have also evaluated mRNA datasets comparing prostate cancer in African American and Caucasian men. The analysis identified some connections to glycosylation. The final analysis and the interpretation of results is ongoing.

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Prostate cancer, glycomic analysis, health disparity

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**Progress Report, W81XWH-09-1-0202, Glycomic Analysis of Prostate Cancer, 07. 2012**

## INTRODUCTION

In this proposal, we are testing the hypothesis that the risk of developing prostate cancer and the aggressiveness of the disease are influenced by protein glycosylation. We postulate that glycobiology contributes to the higher susceptibility of African American men. A major goal of the study is to evaluate the glycosylation differences in prostate cancer of African American and Caucasian men living in the Baltimore-Washington metropolitan area.

**Aim1.** Quantify N-glycans in serum of men in a case-control study of prostate cancer with a focus on differences between Caucasians and African Americans.

**Aim2.** Evaluate prediction accuracy of select N-glycans for the detection of prostate cancer.

**Aim3.** Perform an exploratory study of N-glycans in urine of the participants and correlation of glycans with gene expression in existing array datasets.

## BODY

Our study consists of a comparison of healthy controls (men without any known prostate problems) and biopsy controls (men primarily with benign hypertrophy of the prostate, confirmed cancer free at biopsy) with prostate cancer cases. We have designed a pooled-unpooled study where initial discovery is conducted in smaller number of pooled samples followed by analysis of individual samples **(Table 1)**. The design of the study was further improved based on evidence obtained in our laboratory which shows that glycosylation of serum immunoglobulins differs significantly in type and response to disease compared to other serum proteins. This led to our decision to fractionate serum into three fractions: IgG fraction, other immunoglobulins (primarily IgA and IgM), and all the remaining serum proteins. We have now completed analysis of all three layers in pooled samples; we have also completed the analysis of the IgG and other proteins in individual samples. We will complete the analysis of individual samples in the "other immunoglobulin" fraction by November. We expect to have all data analyses completed by March and prepare manuscripts for publication by end of June 2013.

The optimized methodology for the analysis of N-glycans in serum separated into three fractions (IgG fraction, other immunoglobulins, and remaining serum proteins) is now published (Bekesova et al, Journal of Proteomics, 2012, , PMID ). We have reported in this publication preliminary findings on the reproducibility of these measurements in repeated samples of healthy controls (23 healthy controls, 39% African American, 35% Caucasian). For each of the participants, we have obtained four blood samples in the span of six months. Overall, we have identified consistently 82 N-glycans in the other protein fraction, 54 glycans in the IgG fraction, and 52 glycans in the other immunoglobulin fraction. The within subject variability was 15-34%. CVs were positively correlated with glycan mass and inversely correlated with intensity and tended to be higher among glycan structures that were fucosylated rather than sialylated. We have found that age, race and gender had a smaller effect on glycan measurement than lifestyle factors such as body mass index (BMI), use of non-steroidal anti-inflammatory drugs (NSAIDs), smoking status and education **(Figure 1)**. To carry out these measurements, the separation of immunoglobulins was carried out on protein A and G micro-columns. Glycosylation of enzymatically detached glycans in each protein layer has been quantified by mass spectrometric analysis of the permethylated N-glycans. This allowed us to analyze separately the N-glycans associated with immunoglobulins (immune system related glycosylation) and remaining proteins (non-immune glycosylation response).

We have now completed majority of the analyses of pooled samples and two individual fractions as described above. The mass spectrometric datasets were analyzed using an *in house* software developed for this purpose. This software tool allows us to convert mass spectra into quantities of individual N-glycans based on integration of normalized peak intensities. Depending on the layer and disease condition, we observe 40 to 89 different glycans per sample. Consistently detected glycans are compared across the disease groups using variety of statistical approaches (parametric and non-parametric comparisons, regression models etc) using SAS 9.2 software (SAS Institute, Cary, NC). We observe differences in glycan intensities by race and majority of the glycans found to differ between Caucasian and African Americans was confirmed in the analysis of individual samples **(Table 2)**. We have also observed differences by case status with the most interesting differences related to the "other immunoglobulin" fraction, which consists mainly of IgA and IgM classes of immunoglobulins **(Table 3)**. This layer is least abundant in protein of the three fractions analyzed and we are therefore accumulated

more starting material to complete the analysis of this fraction in individual samples. This analysis will be completed by November 2012 aqnd all data analyses are projected to complete in March 2013. We have also evaluated the analysis of glycans detached from urinary protein. This analysis is complicated by large variability of measurement. Instead of 14-34% CV, we observe CVs larger than 60% and large variation in samples obtained from the same individual at different time points. This is in contrast to the analysis of glycans detached from the proteins in serum of healthy controls and means that the technical methodology for glycan analysis is reproducible but the biological variability of urinary samples is larger than expected. This makes analysis of group differences in urinary samples impractical.

In addition, we have begun a comparative analysis of gene expression in African American (AA) and European American (EA) men in order to identify differentially expressed glycogenes. Glycogenes were defined as probes selected by the Consortium for Functional Glycomics (CFG) for inclusion on their mRNA array (http://www.functionalglycomics.org/static/consortium/resources/resourcecoree.shtml). The array contains probe sets for 1175 unique glycogenes. We looked for intersection of this gene set with the genes observed as differentially abundant in GSE6956 (Wallace et al, 2008, PMID: 18245496) and GSE17356 (Timofeeva et al, 2009, PMID: 19724911). These are the only two mRNA expression studies we identified comparing the expression in prostate cancer of AA and EA men. GSE6956 contains data of 89 samples; prostate tumor (n=69) and non-tumor tissue (n=20). We used the array data of 69 tumor samples for our study. Samples in GSE17356 are primary prostate cancer epithelial cell cultures (n=27). The authors compared the mRNA expression in prostate cancer samples isolated from AA and EA men.

By using the SAMR package and Bayesian regularized t test on re-annotation profile, we identified 28 glyco genes among the differentially expressed mRNA in GSE6956 and 40 glyco genes among the differentially expressed mRNA in GSE6956. We will further evaluate whether the glycosylation related genes identified in our analysis affect prostate cancer disparity and glycan profiles observed in our study. Comparison of mRNA expression in GSE6956 and GSE17356 showed 40 genes consistently up- or down- regulated in both sets (comparison of the AA and EA men). The genes are listed in **Table 4**. Two of the genes belong to the glycogene set **(Table 5)**. A strong association was found between the dysregulated genes and the insulin regulation of fatty acid metabolism.

## KEY RESEARCH ACCOMPLISHMENTS

1. Methods for the glycomic analysis of fractionated proteins are established
2. Analysis of pooled samples in three serum fractions and individual samples in two of the three fractions are completed
3. Data conversion is completed for the samples that have been analyzed
4. Data analyses of the completed datasets are under way and appropriate models were established
5. mRNA array informatics show 40 genes differentially abundant consistently in two existing datasets but these are the only two datasets comparing African American and Caucasian men we identified

## REPORTABLE OUTCOMES

Poster “Detection of prostate cancer using glycomic analysis: can differences in glycosylation explain the health disparity of the disease”; was presented at the IMPACT meeting, Orlando, FL in March 2011. One publication is published (below) and another publication is in preparation.

Bekesova S et al (2012) J Proteomics. 2012 Apr 3;75(7):2216-24. PMID: 22326963

## CONCLUSION

We have already completed majority of the proposed work under an optimized workflow which increases the analyses to three fractions of serum allowing an improved analysis of immunoglobulin-associated glycans. Completion of the remaining analyses, data interpretation, and manuscript writing is expected to complete by June 2013.

## REFERENCES

NA

## Appendices

NA

**Supporting Materials**

| Source | Cases | | Healthy controls | | Biopsy controls | |
|---|---|---|---|---|---|---|
| Race | CA | AA | CA | AA | CA | AA |
| Sample number | 70 | 70 | 50 | 50 | 38 | 38 |
| Pool number | 7 | 7 | 5 | 5 | 4 | 4 |

**Table 1.** Study design includes Caucasian American (CA) and African American (AA) men in each category of cancer cases, healthy population controls, and biopsy controls verified to be cancer free. Pools represent groups of 10 or 8 patient samples pooled for an exploratory analysis.

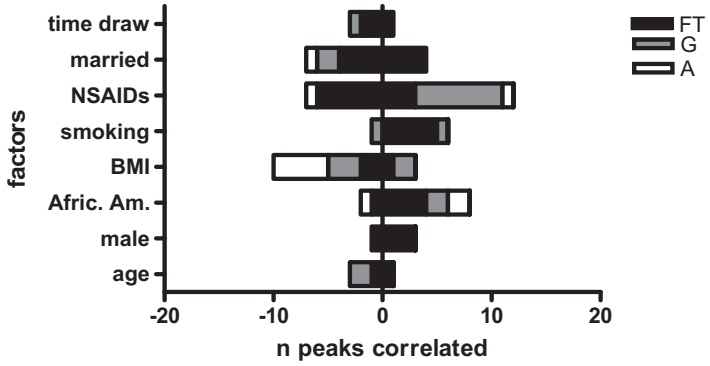



**Figure 1.** Number of glycans affected by host characteristics

| Pooled_FT_CA | | | | | | Pooled_FT_AA | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| m/z | N Obs | Mean | Median | Std Dev | N Obs | Mean | Median | Std Dev | P value | study |
| 2186 | 16 | 0.425 | 0.427 | 0.066 | 16 | 0.492 | 0.487 | 0.092 | 0.023 | pooled |
| 2390 | 16 | 0.631 | 0.667 | 0.105 | 16 | 0.708 | 0.695 | 0.081 | 0.027 | pooled |
| 2472 | 16 | 0.358 | 0.357 | 0.068 | 16 | 0.303 | 0.309 | 0.045 | 0.011 | pooled |
| 2646 | 16 | 0.048 | 0.046 | 0.031 | 16 | 0.027 | 0.024 | 0.026 | 0.046 | pooled |
| 2925 | 16 | 0.167 | 0.181 | 0.065 | 16 | 0.091 | 0.088 | 0.074 | 0.005 | pooled |
| 3228 | 16 | 0.374 | 0.349 | 0.092 | 16 | 0.435 | 0.422 | 0.066 | 0.039 | pooled |
| 4052 | 16 | 1.397 | 1.385 | 0.266 | 16 | 1.631 | 1.724 | 0.298 | 0.026 | pooled |
| 4413 | 16 | 1.343 | 1.322 | 0.388 | 16 | 1.664 | 1.776 | 0.389 | 0.027 | pooled |
| Unpooled__FT_CA | | | | | | Unpooled_FT_AA | | | | |
| 2186 | 95 | 0.483 | 0.470 | 0.117 | 95 | 0.541 | 0.529 | 0.147 | 0.039 | unpooled |
| 2390 | 95 | 0.726 | 0.704 | 0.135 | 95 | 0.839 | 0.805 | 0.205 | <.0001 | unpooled |
| 2925 | 95 | 0.063 | 0.000 | 0.090 | 95 | 0.131 | 0.170 | 0.099 | <.0001 | unpooled |
| 3228 | 95 | 0.351 | 0.358 | 0.102 | 95 | 0.406 | 0.380 | 0.159 | 0.005 | unpooled |
| 4413 | 95 | 1.007 | 0.929 | 0.579 | 95 | 1.228 | 1.136 | 0.740 | 0.019 | unpooled |

**Table 2.** Differences between Caucasian and African Americans in the glycan intensities in the pooled sample sets (top) and the confirmed differences in the individual unpooled samples (bottom).

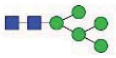
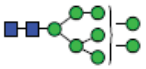
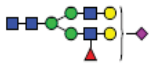
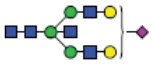
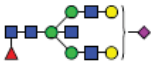
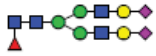
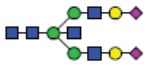
| Glycan | Dependent | Hypothesis | Source | DF | SS | MS | FValue | ProbF | Structure |
|---|---|---|---|---|---|---|---|---|---|
| 1579 | intensity | 3 | group1 | 2 | 31.64045 | 15.82023 | 8.05 | 0.0017 |  |
| 1783 | intensity | 3 | group1 | 2 | 26.30445 | 13.15222 | 3.48 | 0.0447 |  |
| 1835_ | intensity | 3 | group1 | 2 | 16.05139 | 8.025697 | 4.5 | 0.0202 |  |
| 2315 | intensity | 3 | group1 | 2 | 3.427402 | 1.713701 | 6.95 | 0.0035 |  |
| 2676 | intensity | 3 | group1 | 2 | 10.95956 | 5.479781 | 7.51 | 0.0024 |  |
| 2850 | intensity | 3 | group1 | 2 | 4.34334 | 2.17167 | 3.34 | 0.0501 |  |
| 2966 | intensity | 3 | group1 | 2 | 19.28809 | 9.644047 | 5.77 | 0.008 |  |
| 3037 | intensity | 3 | group1 | 2 | 2.626358 | 1.313179 | 6.26 | 0.0057 |  |
| 3211 | intensity | 3 | group1 | 2 | 3.717003 | 1.858501 | 7.08 | 0.0032 |  |

**Table 3.** Differences in “other immunoglobulin fraction” between prostate cancer and control groups; one-way anova of the pooled dataset.

| Gene symbol | FC_6956 | p_6956 | FC_17356 | p_17356 |
|---|---|---|---|---|
| ADI1 | 0.65 | 3.04E-05 | 0.46 | 3.04E-05 |
| AMFR | 1.68 | 4.29E-05 | 3.00 | 3.01E-07 |
| APIP | 0.83 | 5.49E-03 | 0.66 | 3.94E-03 |
| ATP11B | 0.83 | 6.23E-03 | 0.74 | 5.34E-03 |
| BIN2 | 1.17 | 5.62E-03 | 1.16 | 9.55E-03 |
| C14orf108 | 0.84 | 3.56E-04 | 0.72 | 7.39E-04 |
| C18orf10 | 1.16 | 8.56E-04 | 1.20 | 7.54E-03 |
| C3orf37 | 0.90 | 3.83E-03 | 0.75 | 6.45E-04 |
| C7orf49 | 0.89 | 2.60E-03 | 0.80 | 2.63E-03 |
| CLC | 0.91 | 2.34E-04 | 0.89 | 6.06E-03 |
| CNNM4 | 0.91 | 8.74E-03 | 0.80 | 1.55E-03 |
| CPSF4 | 0.92 | 6.05E-03 | 0.86 | 5.04E-03 |
| CRYBB2 | 1.93 | 7.80E-11 | 2.20 | 1.34E-04 |
| CTNNB1 | 1.37 | 7.18E-07 | 2.13 | 3.91E-07 |
| EBI2 | 1.55 | 4.06E-03 | 1.30 | 5.57E-03 |
| FAM128A | 0.76 | 1.36E-03 | 0.63 | 9.08E-03 |
| FASTKD3 | 0.86 | 8.46E-03 | 0.70 | 2.99E-03 |
| GOLPH4 | 1.18 | 1.33E-03 | 2.05 | 7.47E-10 |
| IL20RA | 0.71 | 2.15E-03 | 0.70 | 6.00E-04 |
| INDO | 1.39 | 2.17E-03 | 1.26 | 5.65E-04 |
| LEPROT | 1.19 | 6.06E-03 | 1.24 | 2.59E-03 |
| MAP3K15 | 1.20 | 9.75E-06 | 1.28 | 8.42E-03 |
| MAPK8 | 0.89 | 4.02E-03 | 1.16 | 8.05E-04 |
| MGAT1 | 1.07 | 8.32E-03 | 1.19 | 9.84E-03 |
| MRPL35 | 0.89 | 6.66E-03 | 0.81 | 8.53E-03 |
| MRPS7 | 0.89 | 5.97E-03 | 0.80 | 1.70E-03 |
| MTA1 | 0.89 | 5.05E-03 | 1.22 | 6.64E-03 |
| MXRA7 | 1.38 | 7.59E-04 | 1.35 | 8.08E-03 |
| NARS2 | 0.78 | 2.03E-04 | 0.75 | 3.12E-04 |
| PAPD1 | 0.88 | 1.29E-04 | 0.81 | 4.44E-03 |
| PRPSAP1 | 0.88 | 9.89E-04 | 0.80 | 4.45E-03 |
| PSPH | 2.34 | 1.70E-09 | 2.02 | 9.52E-03 |
| RFX5 | 1.10 | 1.53E-03 | 0.79 | 3.30E-03 |
| RPP38 | 0.87 | 6.80E-04 | 0.71 | 8.95E-06 |
| SFXN1 | 0.88 | 2.89E-03 | 0.56 | 3.49E-03 |
| SOS1 | 1.27 | 1.53E-03 | 1.67 | 9.72E-07 |
| TTC27 | 0.91 | 9.38E-03 | 0.74 | 2.70E-03 |
| VPS53 | 0.92 | 4.92E-03 | 1.24 | 1.06E-03 |
| VRK2 | 0.88 | 2.87E-03 | 0.80 | 1.50E-03 |
| ZNF227 | 0.87 | 1.87E-03 | 0.81 | 2.97E-03 |

**Table 4.** mRNA expression of 40 genes consistently up- or down- regulated in the comparison of the AA and EA men in both GSE6956 and GSE17356 datasets.

| Probe | Gene Symbol | FC_6956 | p_6956 | FC_17356 | p_17356 |
|---|---|---|---|---|---|
| 202377_at | LEPROT | 1.19 | 6.06E-03 | 1.23 | 2.59E-03 |
| 201126_s_at | MGAT1 | 1.06 | 8.32E-03 | 1.19 | 9.84E-03 |

| Gene Symbol | Source1 | Source2 | Name | NM |
|---|---|---|---|---|
| LEPROT | xGrowth Factors & Receptors | Miscellaneous | LEPROT [leptin receptor gene-related protein] | NM_017526 |
| MGAT1 | Glycan-transferase | GlcNAc-T | MGAT1 (mannosyl (alpha-1,3-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase) | NM_002406 |

**Table 5.** Glycogenes selected from Table 2.

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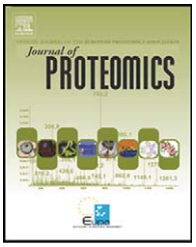

# N-glycans in liver-secreted and immunoglogulin-derived protein fractions

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ABSTRACT

N-glycosylation of proteins provides a rich source of information on liver disease progression because majority of serum glycoproteins, with the exception of immunoglobulins, are secreted by the liver. In this report, we present results of an optimized workflow for MALDI-TOF analysis of permethylated N-glycans detached from serum proteins and separated into liver secreted and immunoglobulin fractions. We have compared relative intensities of N-glycans in 23 healthy controls and 23 cirrhosis patients. We were able to detect 82 N-glycans associated primarily with liver secreted glycoproteins, 54 N-glycans in the protein G bound fraction and 52 N-glycans in the fraction bound to protein A. The N-glycan composition of the fractions differed substantially, independent of liver disease. The relative abundance of approximately 53% N-glycans in all fractions was significantly altered in the cirrhotic liver. The removal of immunoglobulins allowed detection of an increase in a series of high mannose and hybrid N-glycans associated with the liver secreted protein fraction.



## 1. Introduction

Chronic liver disease is on the rise in the United States and worldwide with viral hepatitis B or C infections, alcohol consumption, and nonalcoholic steatohepatitis (NASH) representing the main causes [1]. Progressive scaring of the liver leads eventually to cirrhosis, the major cause of death in chronic liver disease; people with liver cirrhosis have also an increased risk of developing hepatocellular carcinoma (HCC) [2]. Pathophysiology of cirrhosis is not fully understood but it is known that glycosylation of proteins changes in liver disease [3]. Glycosylation is a complex posttranslational modification [4,5] with a profound functional impact on biological processes [6,7]. Changes in N-glycosylation of proteins associated with premalignant liver diseases received an increased attention in recent years [8–10]. These studies strongly suggest that detailed characterization of N-glycans has the potential to provide improved tools for the management of liver diseases.

With a few exceptions, such as albumin and C-reactive protein, liver secreted proteins are N-glycosylated. The liver secreted N-glycoproteins are expected to reflect the changes in liver cirrhosis; however, recent literature points to changes in the glycosylation of immunoglobulins (Ig) [8–10], the most abundant class of glycoproteins in serum that originates in the cells of the immune system, as indicators of liver disease [11]. The function of Ig heavily depends on their glycosylation status [12]. It has been shown that the composition and glycosylation of IgA, IgM, and IgG change in chronic liver disease [13–16]. The results of Klein et al. show that the major differences associated with the development of cirrhosis are

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attributed to the N-glycosylation of Ig [16]. Mehta et al. used reactivity of fucosylated agalacto IgG to the AAL lectin as a test for fibrosis and cirrhosis [10]. Vanderschaeghe et al. showed that the bisecting fucosylated glycans of Fibro- and Cirrho-tests are associated with Ig [8]. However, the analysis of isolated liver secreted glycoproteins indicates that their glycosylation status changes in cirrhosis as well [17,18].

We have used MALDI-TOF analysis of permethylated N-glycans [19,20] for the study of liver diseases in our previous studies [21,22]. This method allows relative quantification of tens to hundreds of N-glycans in serum but does not distinguish N-glycans associated with Ig or the liver secreted glycoproteins. In this paper, we describe an optimized workflow which allowed us to compare N-glycans of 23 healthy individuals and 23 patients with liver cirrhosis, in proteins fractionated into two fractions of Ig and a fraction of liver secreted proteins. The results show that the depletion of Ig allows detection of changes in a series of hybrid and high mannose N-glycans associated with the enriched liver secreted protein fraction.

## 2. Experimental section

### 2.1. Materials

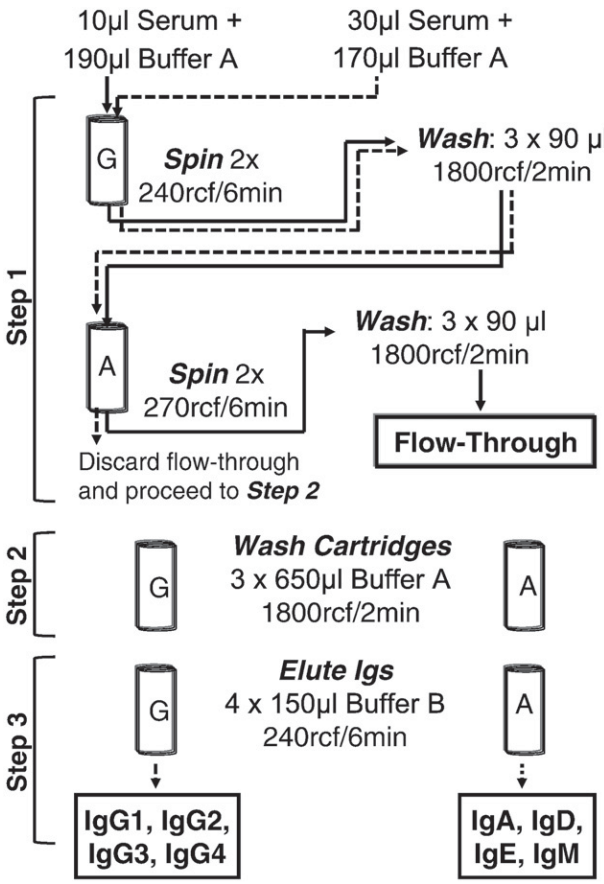
2,5-dihydroxybenzoic acid (39319), sodium hydroxide (01209BH), trifluoroacetic acid (T6508), acetonitrile (34998), chloroform (C-2432), iodomethane (06416ME), sodium chloride (D-5545) and water (270733) were purchased from Sigma-Aldrich (St. Louis, MO). Proteus protein G (PUR015, lot 221009) and A (PUR007, lot 281009) microspin columns were from AbD Serotech, Kidlington, UK, DMSO (327182500) was from Acros Organics (Pittsburgh, PA), tC18 Sep-Pak 50 mg cartridge (WAT054960) were obtained from Waters (Milford, MA). Charcoal solid-phase extraction column (744300) was from Harvard Apparatus (Hamden, CT). Protein N-Glycosidase F (PNGase F, P0705L, 0360907–7) was from New England BioLabs (Ipswich, MA).

### 2.2. Study population and sample collection

A total of 23 patients with liver cirrhosis and 23 healthy volunteers were analyzed. All participants were enrolled under protocols approved by the Georgetown University Institutional Review Board. Patients were enrolled as part of a study at Georgetown University Hospital, Department of Hepatology and Liver Transplantation, Washington DC. Basic demographic information such as age, race and gender was acquired through an administered questionnaire. Clinical data for the cirrhotic patients were extracted from medical charts. All subjects donated a blood sample and 20 of the 23 healthy controls provided 4 blood samples within a year, in 2–4 month intervals, to allow analysis of the variability of the N-glycans in the same person over time. The remaining 3 disease free subjects provided three samples. Serum samples were aliquoted and stored at −80 °C till analysis. All analyses were carried out at second thaw.

### 2.3. Fractionation of serum proteins

Serum was fractionated on Proteus protein G and A microspin columns according to manufacturer's suggestions with the following minor adjustments. Serum (10–30 μL) was diluted with binding buffer A (0.1 M $Na_2HPO_4$, pH 7.4, 1.5 M NaCl) as summarized in Scheme 1. The sample was loaded on the protein G spin column in two cycles, 6 minutes at 240 rcf each, washed with 3×90 μl buffer A (2 minutes, 1800 rcf), and the flow through (FT) and washes were combined for loading onto a protein A spin column. The protein A spin column was processed as above. The FT and washes from the protein A column were combined as the FT fraction (740 μl total volume). The protein G spin column was further loaded with 30 μl of serum in 170 μl Buffer A. The protein G FT and wash were loaded on the protein A column as above. The A and G spin columns were further washed three times with 650 μl buffer A and the bound proteins were eluted with elution buffer B (4×150 μl, 0.2 M Glycine pH 2.5) directly into a tube with 65 μl of neutralization buffer C to adjust the final pH to 7.5. The three elution cycles were combined and we refer to proteins bound to the protein G and A columns as 'G' and 'A' fractions, while the unbound FT fraction (from 10 μl of serum) is referred to as 'FT'. In addition to the fractionated serum samples, we have also carried out analysis of a 10 μl aliquot of unfractionated serum as described below.




**Scheme 1 – Separation of serum into a liver secreted protein fraction (FT) and two Ig fractions bound to protein A (A) and protein G (G), respectively.**

### 2.4. Analysis of N-glycans

The handling of the three fractions, or unfractionated serum, was performed according to a published protocol with slight modifications [20]. Briefly, sample volumes were adjusted with 25 mM ammonium bicarbonate, reduced with dithiothreitol and alkylated with iodoacetamide. N-glycans were detached with PNGase F, 600 U, overnight at 37 °C. The deglycosylated proteins were precipitated at 90 °C for 10 minutes and further removed on a C18 cartridge (tC18, 100 mg, Waters, Milford, MA). The N-glycans were further cleaned on an activated charcoal solid-phase extraction column (Harvard Apparatus, Hamden, CT, USA). The N-glycans trapped on the activated charcoal were eluted in four volumes of a 250-μl of 50% acetonitrile/water (v/v) containing 0.1% TFA. The combined eluents were dried under vacuum and the released N-glycans were permethylated on sodium hydroxide beads (reagent grade, Sigma-Aldrich, St. Louis, MO) in a solution consisting of 79.3% DMSO, 19.5% iodomethane, and 1.2% water. The optimum amounts of water and iodomethane was determined experimentally. The reaction was carried out in a capped vial at room temperature for 20 minutes and reagents were collected by a brief 10 second centrifugation at 5 000 rpm that removes the hydroxide beads. The permethylated samples were immediately extracted by a liquid–liquid extraction using 400-μL aliquots of chloroform and water. The chloroform layer was further back-extracted with three 1-ml volumes of water and dried under vacuum.

### 2.5. MALDI-TOF mass spectrometry

The dried permethylated samples were resuspended in a 50:50 methanol:water solution. Each sample (0.5 μl) was spotted directly on a MALDI plate and mixed with an equal volume of DHB matrix (10 mg/ml DHB in 50:50 methanol:sodium acetate, 2 mM). The sample spots were dried under vacuum to achieve uniform crystallization. Spectra were acquired on an Applied Biosystems 4800 Mass Analyzer (AB Sciex, Framingham, MA) equipped with a Nd:YAG 355-nm laser. MALDI spectra were recorded in the positive ion mode as permethylation eliminates the negative charge normally associated with sialylated glycans.

### 2.6. Data processing and analysis

Raw spectra were exported as text files and processed using an *in house* software modifying our previously published spectral processing methods [22,23]. The MALDI-TOF spectra were calibrated on masses of a set of previously identified N-glycans [20,21,24] and exported as text files for further processing. We eliminated a binning step which was found to distort intensity-ratios of the N-glycan isotope clusters. Instead, the spectra were smoothed by the Savitsky–Golay algorithm [25], de-noised by Daubechies D20 wavelet transform [25,26], and the baseline was corrected by removing low frequency nodes using FFT convolution/deconvolution. Similar to recent publications, the presence of previously identified N-glycans was determined by matching the theoretical distribution of their isotopic clusters to the observed spectra and resolving peak integral overlaps using iterative prediction, correction procedure [24,27]. The detected N-glycans were subtracted from the spectrum and the remaining isotopic clusters, with intensity above a predefined cutoff and present in more than 20% of the analyzed spectra, were interpreted as unknown N-glycans. The identified peaks were normalized by scaling the total peak intensities to 100.

All analyses were performed using SAS software, version 9 (SAS Institute Inc., Cary, NC). We used *t*-test and Wilcoxon rank sum test to determine differences in glycan abundance between healthy individuals and patients with cirrhosis. All *p* values were two-sided, adjusted by Holm's multiple comparison procedure as needed. Pearson correlation was used to estimate correlations between glycan intensities and globulin. We have used Mixed procedure for repeated measures, adjusted by Bonferroni method, to compare intensities of 9 glycans with highest fold difference between cirrhosis and control in the FT fraction; the healthy controls were sampled and analyzed at four different time points. The association of peak intensities with age, gender, and race was analyzed by the Generalized estimating equations (GEE) method.

## 3. Results

The goal of our study was to evaluate changes in the N-glycosylation of liver secreted proteins. To this end, we have analyzed serum samples of 23 disease free controls and 23 patients with liver cirrhosis (Table 1). Majority of the cirrhotic patients were of viral HCV etiology and had a mean Model of end stage liver disease (MELD) score of 13. Our results show that N-glycans detached from the unfractionated serum proteins are dominated by decrease in the biantennary sialylated glycan *m/z* 2792.4 and increase in the biantennary agalacto core fucosylated glycan *m/z* 1835.9, as reported previously [9] (Fig. 1). We have also observed an increase in the bisecting fucosylated glycans, the type of N-glycans selected previously as part of the GlycoFibro and GlycoCirrho tests [8]. It was pointed out that the above N-glycans are associated primarily with immunoglobulins [16].

More than half of the cirrhotic patients in our study have elevated globulins (Table 1). When we stratify the cirrhotic

**Table 1 – Basic characteristics of the study population.**

| | Healthy (*n*=23) | Cirrhotic (*n*=23) |
|---|---|---|
| Age, mean (SD) | 48 (9) | 57 (8) |
| Male (%) | 65% | 78% |
| Race (%) | | |
| Caucasian | 35% | 52% |
| African American | 39% | 17% |
| Other | 26% | 31% |
| Etiology (%) | | |
| Viral (HCV, HBV) | N/A | 57% |
| Alcoholic | | 17% |
| Other | | 26% |
| **Globulin (gm/dl)** | | |
| Normal (2.3–3.5) | N/A | 35% |
| High (3.6–4.8) | | 55% |
| Very high (>4.8) | | 10% |
| MELD score, mean (SD) | N/A | 12.7 (5.1) |

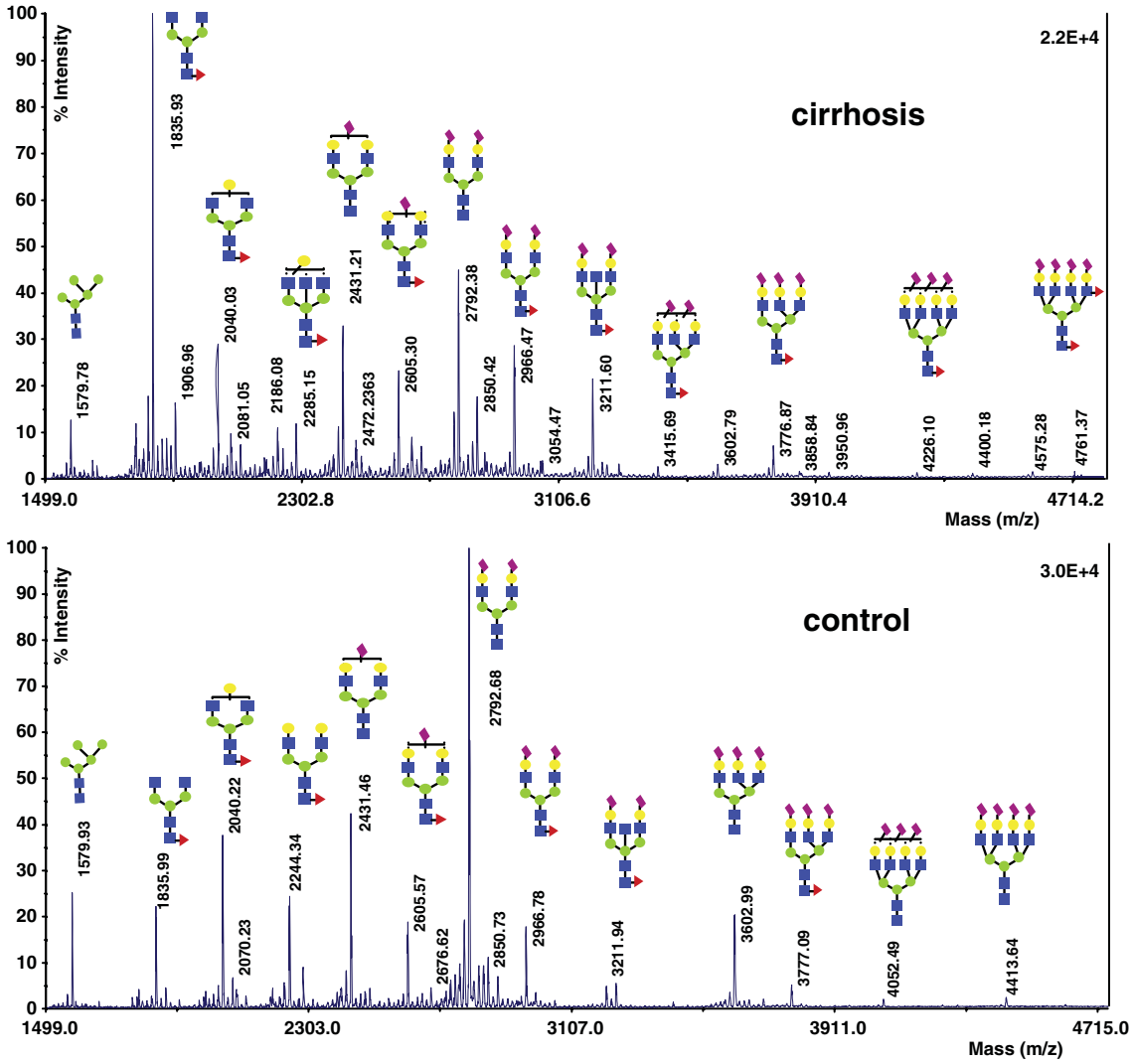



**Fig. 1 – Major N-glycans observed in a typical MALDI-TOF spectrum of the cirrhosis and control groups.**

patients into the groups of normal (2.3–3.5 g/dl), elevated (3.6–4.8 g/dl), and high (>4.8 g/dl) globulin, we observe a strong association of the glycans with the titers (Fig. 2). Overall, 28 of the 85 N-glycan peaks in the unfractionated serum correlate with globulin. We have therefore used a combined protein G and A depletion strategy to enrich the liver secreted glycoproteins (Scheme 1). This expansion of the previously described MALDI-TOF analysis of permethylated N-glycans [20] allows examination of the glycosylation changes associated with liver secreted proteins otherwise dominated by the N-glycans associated with Ig [16]. Our results show that the glycans detected in the combined Ig fractions substantially differ from the glycans in the FT fraction and glycans in the unfractionated serum (Fig. 3).

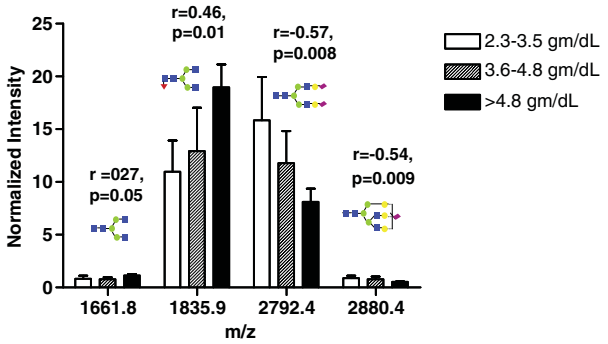



**Fig. 2 – Association of N-glycans with immunoglogulins.**

The Ig associated glycans represent a significant portion of the N-glycans in the unfractionated serum with some N-glycans detected only in the Ig fraction, as reported recently [9,28].

Specifically, the biantenary core fucosylated agalacto (*m/z* 1835.9) and monogalactosylated (*m/z* 2040.0) glycans were virtually undetectable in the spectra of the FT fraction of serum which supports the efficiency of the protein G and A double depletion strategy. The glycans with *m/z* 1835.9 and 2040.0 were also differentially abundant in the G fraction of healthy and cirrhotic subjects with *m/z* 1835.9 increasing relative to 2040.0 as the core fucosylated glycans shift with cirrhosis toward agalacto forms.

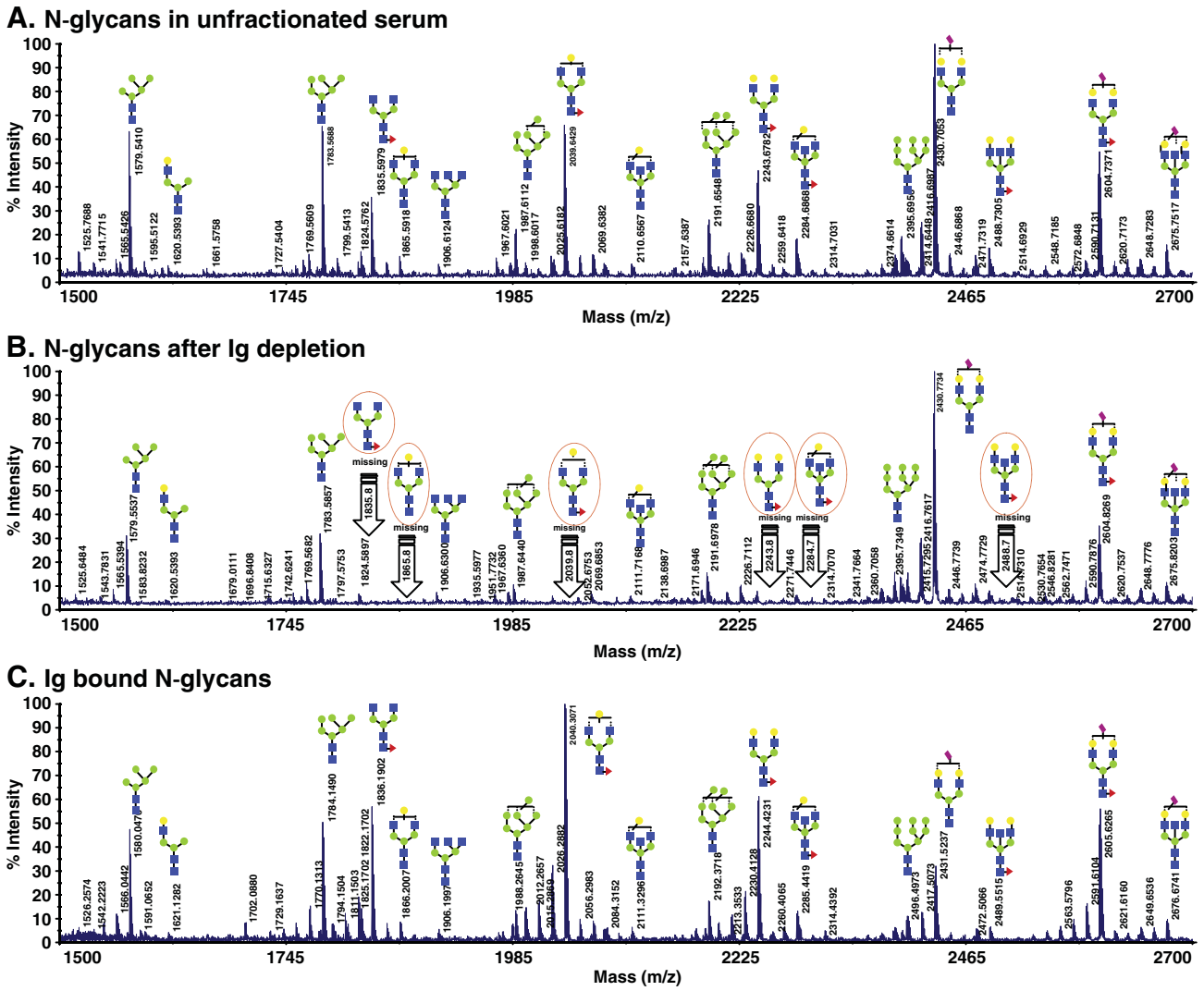



**Fig. 3 – N-glycans isolated from A. unfractionated serum; B. serum depleted of immunoglobulins; C. immunoglobulins isolated on protein A and G. The arrows point to structures depleted by the removal of immunoglobulins.**

Because the literature points out that IgM and IgA classes of globulins, besides IgG, are important in the development of liver cirrhosis, we further fractionated the globulins into two layers. This is achieved by successive passage of the serum proteins through the protein G and protein A affinity resins which generates the FT fraction (primarily liver secreted) and two Ig fractions bound to the G and A proteins (Scheme 1). Protein G binds IgG1-4; protein A binds all the IgG subclasses, except IgG3, and IgA, IgD, IgE and IgM. The sequential trapping of Ig on protein G and protein A resins minimizes the carryover of Ig into the FT fraction and allows a separate analysis of the Ig fractions. Since the order of loading in our analysis is protein G followed by the A spin column, the protein G binds predominantly IgGs and protein A binds the remaining Ig classes. Based on the known concentrations of globulins in serum, we expect that the majority of Ig bound in the A fraction are IgMs and IgAs. Because IgMs have also multiple glycosylation sites, they are likely to represent the major contribution to the A layer especially in the case of disease with an HCV etiology [29]. The throughput of approximately 96 samples in one week is sufficient for such studies of medium throughput as the one reported here. In addition, the two individual fractions of Ig can be examined at the same time.

In the fractionated samples, we have detected overall 82 N-glycans in the FT fraction, 54 in the G fraction, and 52 in the A fraction (Supplemental Table 1). The Ig layers contain primarily glycans $m/z < 3000$, mostly biantennary complex structures and high mannose glycans. The G fraction in cirrhotics is dominated by the appearance of agalacto core fucosylated glycans. The A layer is in our hands characterized by the monosialylated biantennary glycan $m/z$ 2431.2 and high mannose glycans (Supplemental Table 1). A comparison of the N-glycans of the 3 glycoprotein layers (FT, G and A) in healthy versus cirrhotic participants showed significant differences in the abundance of glycans across the three fractions. We have observed a major shift in the distribution of relative intensities ranging from 68% of N-glycans in the FT layers to 33% of the N-glycans in the G layer (Table 2). The focus of this presentation is on the description of the procedure

**Table 2 – Number of N-glycans up- and down- regulated in cirrhosis in each of the FT, G, and A fractions; *p*-values adjusted by Holm's multiple comparison procedure.**

| | FT ($n$=82 glycans) N, % | | G ($n$=54 glycans) N, % | | A ($n$=52 glycans) N, % | |
|---|---|---|---|---|---|---|
| Change | Up | Down | Up | Down | Up | Down |
| <2 fold | 6 (7%) | 9 (11%) | 2 (4%) | 2 (4%) | 1 (2%) | 3 (6%) |
| 2- to 5-fold | 6 (7%) | 25 (30%) | 1 (2%) | 12 (22%) | 1 (2%) | 15 (28%) |
| >5 fold | 0 | 10 (12%) | 0 | 1 (2%) | 0 | 12 (23%) |
| Total | 58 (68%) | | 18 (33%) | | 32 (61%) | |

allowing the analysis of the immunoglobulin depleted glycoproteins which are primarily secreted by the liver. The flow through fraction is therefore discussed further.

With the immunoglobulins removed, we begin to observe glycosylation changes in the liver secreted fraction. The most interesting observation is an increase in high mannose and hybrid glycans in the FT (liver secreted) protein fraction (Fig. 4). Decreases in other glycans summarized in the supplemental tables may be equally important; however, we find the increase in an entire series of high mannose and hybrid glycans most interesting and focus on this novel finding. We have compared N-glycans in the cirrhosis group ($n$=23) to the healthy controls ($n$=23) sampled at four different time points. This demonstrates that the observed increase in the cirrhotic group is not due to random fluctuation in the N-glycosylation of liver secreted glycoproteins. The repeat measurements in the disease free groups are consistent with median intra person coefficient of variation (across the four repeats) of 17% and inter person coefficient of variation of 46% (among 23 participants). The intensities of the nine N-glycans are not significantly different between the draws in healthy controls except for two of the 45 comparisons (Fig. 4). In contrast, all nine N-glycans are significantly elevated in the cirrhotic group. Because the cirrhotics are a slightly older group with more Caucasian males, we have used the GEE models to investigate the effects of age, gender and race on the nine N-glycans from the FT fraction. The analysis shows only two significant associations; glycan $m/z$ 2029.0 is associated with gender ($p$=0.009) and glycan $m/z$ 2390.2 is associated with age ($p$=0.048). The intensity of 2029.0 increases 1.3 times ($p$<0.0001) in connection with cirrhosis when adjusted for gender and the influence of gender becomes insignificant ($p$=0.54). The $m/z$ 2390.0 increases 1.5 times ($p$<0.0001) with cirrhosis when adjusted for age and the intensity increases only 1. 004 times with age when adjusted for cirrhosis ($p$=0.05). The analysis shows that for these nine glycans the age, race, and gender are of marginal significance compared to the disease group. This demonstrates that the series of hybrid and high mannose N-glycans are elevated in liver secreted glycoproteins in connection with cirrhosis.

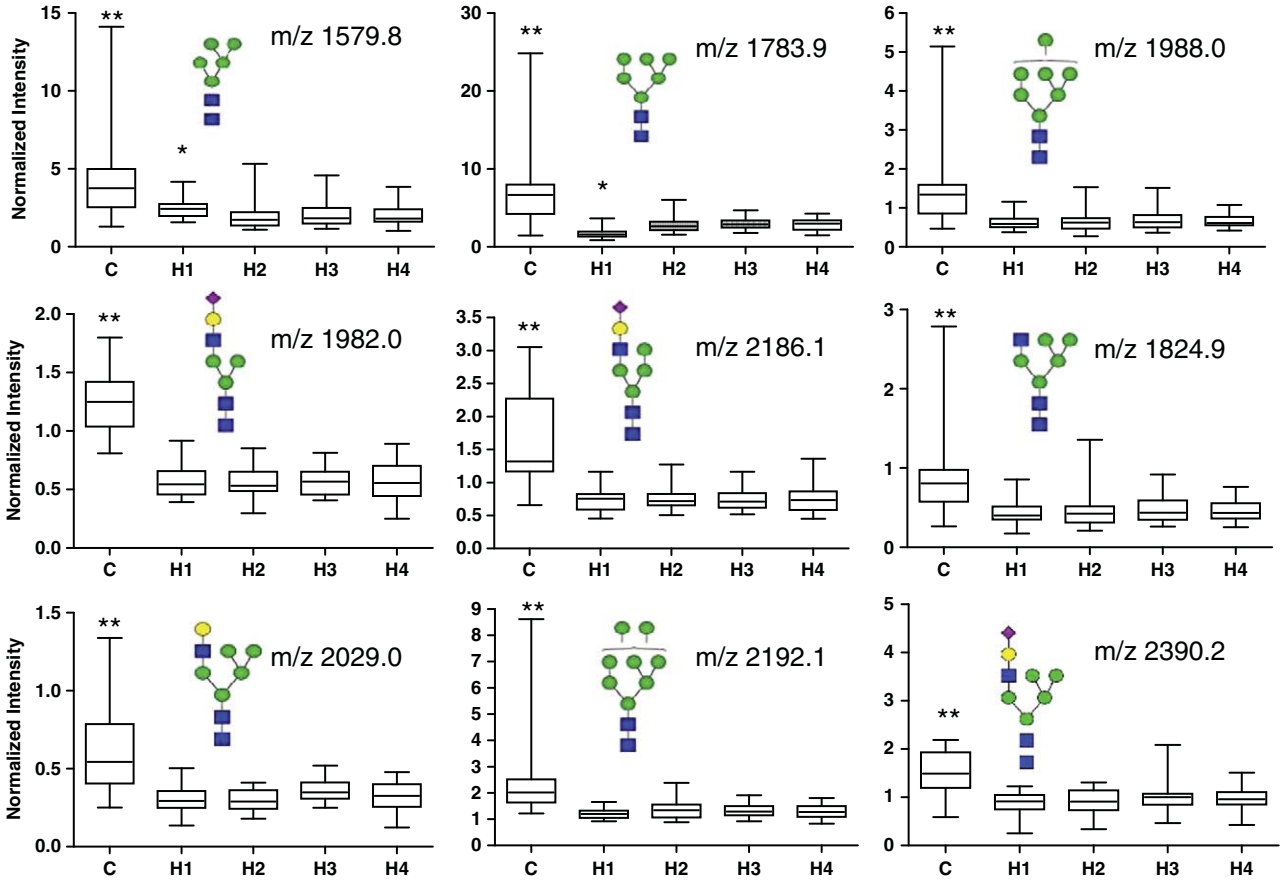



**Fig. 4 – Boxplots of nine N-glycans with highest differences between cirrhosis and controls in the FT fraction; serum samples of the controls were collected at four different time points. **Significantly different between cirrhosis and controls; *Significantly different among one of the control groups.**

## 4. Discussion

Chronic liver disease is a growing worldwide problem [1]. Progressive changes in liver diseases of various etiologies lead to cirrhosis, the major cause of death and a risk factor for the development of HCC [2]. Complications of liver biopsy support the need for non-invasive diagnosis of the liver diseases. The quantification of N-glycosylation of Ig [10] and detached N-glycans [8] represent an important step in this direction. The goal of our study was to evaluate changes in the N-glycosylation of liver secreted proteins using an optimized workflow for the analysis of detached N-glycans.

Considerable evidence indicates that changes in the N-glycosylation of proteins occur in liver diseases [30]. Early studies of the protein N-glycosylation showed changes in sialylation [31,32]. Increased fucosylation of haptoglobin was reported in alcoholic liver disease [33,34] and α1-acid glycoprotein and serum cholinesterase fucosylation status were reported to diagnose liver cirrhosis [35,36]. Using mass spectrometric techniques, Morelle et al. identified three groups of N-glycan modifications in alcoholic liver cirrhosis [28]. The major changes included the presence of bisecting GlcNAc, the increase in α-1,6 fucosylated structures and the presence of neutral agalactosylated oligosaccharides [28]. A quantitative analysis of select N-glycans showed convincingly that the agalacto fucosylated N-glycans, associated primarily with Ig, can be used to detect cirrhosis [8]. We decided to examine further the N-glycans associated with liver secreted N-glycoproteins, because studies of isolated proteins show that the glycosylation cascade is modified in the development of liver cirrhosis [17,18].

To achieve this goal, we adjusted a workflow for the analysis of permethylated N-glycans [20] used previously in our laboratory to study glycosylation in HCC [21]. Analysis of detached N-glycans has become an established tool for the characterization of protein glycosylation [37,38]. Chemical or enzymatic methods for glycan detachment were described but N-glycans are typically harvested from isolated proteins [19,20] or complex protein mixtures [21] with PNGaseF. The selection of methods for subsequent glycan analysis depends on the starting material, sensitivity of detection, need for characterization of structural details, and quantitative aims. Fluorescent labeling of glycans in combination with HILIC chromatography [39] or capillary electrophoresis [40] allows an efficient fractionation and quantification. Structural characterization of the glycans is achieved by exoglycosidase digestion [41] and mass spectrometric analysis [42]. Mass spectrometry, in general, has become very useful for both structural analysis and quantification [11,43]. Mass spectrometry allows analysis of small quantities of unmodified N-glycans [44] but derivatization is often used to improve the stability, ionization efficiency, and fragmentation of the oligosaccharides [20,45]. A combination of methods is typically needed to achieve complete characterization of a sample. Extensive efforts from a number of groups have established *in house* libraries of glycans in specific tissue and disease context [46–49].

In this study, we have separated liver secreted N-glycoproteins from IG by a two-step Protein G and A enrichment (Scheme 1). The N-glycans known to be associated with Ig are efficiently removed (Fig. 3). We have shown that some glycan structures (*m/z* 1835.9, 2040.0, 2244.1, 2285.1, 2489.2) are detected predominantly in the Ig fractions. The G fraction was significantly enriched with glycans of *m/z* 1835.9 and 2040.0, the N-glycans recently described as IgG specific in connection with the alcoholic cirrhosis of the liver [16,50]. Our population of cirrhotics consists primarily of HCV etiology. With the limited sample size, it was not possible that we carry out a conclusive analysis by etiology but the increase in the N-glycan *m/z* 1835.9 dominates the unfractionated cirrhotic spectra as well. While the above is the most striking change, the protein A bound and the FT (liver secreted) fractions change substantially as well. This is most clearly seen after removal of the IgG on the protein G resin.

We do not have information on the exact class of Ig associated with the differences in the A fraction. Both IgM and IgA are glycosylated, present in this fraction, and modified in the progression of liver disease. Klein et al. reported minor changes in N-glycans detached from the serum fraction depleted of immunoglobulins; major differences in their analysis were attributed to the changes in the glycosylation of IgA [9,16]. We do not know at this point whether changes in our population are associated with IgA or IgM but we have detected the most relevant differences in the liver secreted N-glycoprotein fraction. The difference, compared to the study of Klein et al., could be possibly related to the dominant HCV etiology in our study.

Two classes of N-glycans, hybrid and high mannose, are significantly increased in liver secreted glycoproteins in cirrhosis (Fig. 4). It is possible that the reorganization of the liver structure and the influence of HCV infection on the host glycosylation apparatus lead to the release of a higher portion of N-glycoproteins associated with these incompletely developed forms. The N-glycans of liver secreted N-glycoproteins are mainly of the complex type but it is known that some abundant proteins, like alpha-2-macroglobulin or complement 3 and 4, carry high mannose glycans [51,52]. It is also plausible that the hybrid glycans representing a partially complete N-glycosylation are higher in the cirrhotic liver cells. The fact that we observe an increase of an entire series of the high mannose and hybrid glycans further points to a consistent effect on these pathways. The approximately 2-fold increase is quite high and outside the range of variability of our measurements and, more importantly, outside the variability of repeat sampling in the disease free controls (Fig. 4).

The influence of demographic factors such as age, gender, and race is not well understood. The only study we are aware of described the variability and heritability of N-glycan structures in the plasma proteins [53]. The analysis of the sources of variability in N-glycosylation in this population identified small (up to 10%) but significant effect of age and gender on the N-glycan distribution [53]. We have therefore examined whether the increases in the high mannose and hybrid N-glycans could be affected by age, race, or gender differences. We have observed only minor influence of the demographic factors and conclude that the high mannose and hybrid glycans are upregulated in connection with the development of cirrhosis. This expands the changes in N-glycosylation of proteins reported to develop in the context of liver damage and

will allow further exploration of the mechanisms that lead to the progression of the disease.

## 5. Conclusion

In conclusion, we have established a method for the analysis of enzymatically detached permethylated N-glycans in serum fractionated into liver secreted and Ig derived fractions. We have shown fraction-specific serum alterations in N-glycosylation. A consistent upregulation of a series of high mannose and hybrid N-glycans was observed in the fraction of liver secreted glycoproteins. This is in line with the fact that majority of serum proteins are liver secreted N-glycoproteins that reflect the pathophysiology of the organ. We demonstrate that the separation of Ig and liver secreted fractions provides a rich source of information that has the potential to improve our understanding of liver disease progression.

Supplementary data to this article can be found online at doi:10.1016/j.jprot.2012.01.024.

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## REFERENCES

[1] Williams R. Global challenges in liver disease. Hepatology Sep. 2006;44(3):521–6.
[2] Kuper H, Ye W, Broome U, Romelsjo A, Mucci LA, Ekbom A, et al. The risk of liver and bile duct cancer in patients with chronic viral hepatitis, alcoholism, or cirrhosis. Hepatology Oct. 2001;34(4 Pt 1):714–8.
[3] Turner GA. N-glycosylation of serum proteins in disease and its investigation using lectins. Clin Chim Acta Jun. 30, 1992;208(3):149–71.
[4] Cummings RD. The repertoire of glycan determinants in the human glycome. Mol Biosyst Oct. 2009;5(10):1087–104.
[5] Dube DH, Bertozzi CR. Glycans in cancer and inflammation—potential for therapeutics and diagnostics. Nat Rev Drug Discov Jun. 2005;4(6):477–88.
[6] Kiessling LL, Splain RA. Chemical approaches to glycobiology. Annu Rev Biochem 2010;79:619–53.
[7] Varki NM, Strobert E, Dick Jr EJ, Benirschke K, Varki A. Biomedical differences between human and nonhuman hominids: potential roles for uniquely human aspects of sialic acid biology. Annu Rev Pathol Feb. 28, 2011;6:365–93.
[8] Vanderschaeghe D, Laroy W, Sablon E, Halfon P, Van HA, Delanghe J, et al. GlycoFibroTest is a highly performant liver fibrosis biomarker derived from DNA sequencer-based serum protein glycomics. Mol Cell Proteomics May 2009;8(5):986–94.
[9] Klein A, Carre Y, Louvet A, Michalski JC, Morelle W. Immunoglobulins are the major glycoproteins involved in the modifications of total serum N-glycome in cirrhotic patients. Proteomics Clin Appl Apr. 2010;4(4):379–93.
[10] Mehta AS, Long RE, Comunale MA, Wang M, Rodemich L, Krakover J, et al. Increased levels of galactose-deficient anti-Gal immunoglobulin G in the sera of hepatitis C virus-infected individuals with fibrosis and cirrhosis. J Virol Feb. 2008;82(3):1259–70.
[11] Adamson JT, Hakansson K. Electron capture dissociation of oligosaccharides ionized with alkali, alkaline Earth, and transition metals. Anal Chem Apr. 1, 2007;79(7):2901–10.
[12] Arnold JN, Radcliffe CM, Royle L, Dwek RA, Rudd PM, Sim RB. Immunoglobulin glycosylation: the search for MBL binding targets. Mol Immunol Jun. 2004;41(2–3):203–4.
[13] Lee FI. Immunoglobulins in viral hepatitis and active alcoholic liver-disease. Lancet Nov. 20, 1965;2(7421):1043–6.
[14] Becker W. Variations of immunoglobulins in disease. J Clin Pathol Suppl (Assoc Clin Pathol) 1975;6:92–101.
[15] Thiele GM, Freeman TL, Klassen LW. Immunologic mechanisms of alcoholic liver injury. Semin Liver Dis Aug, 2004;24(3):273–87.
[16] Klein A, Michalski JC, Morelle W. Modifications of human total serum N-glycome during liver fibrosis-cirrhosis, is it all about immunoglobulins? Proteomics Clin Appl Apr. 2010;4(4):372–8.
[17] Comunale MA, Rodemich-Betesh L, Hafner J, Wang MJ, Norton P, Di Bisceglie AM, et al. Linkage specific fucosylation of alpha-1-antitrypsin in liver cirrhosis and cancer patients: implications for a biomarker of hepatocellular carcinoma. PLoS One Aug. 25, 2010;5(8).
[18] Zhang S, Shu H, Luo KX, Kang XN, Zhang Y, Lu HJ, et al. N-linked glycan changes of serum haptoglobin beta chain in liver disease patients. Mol Biosyst 2011;7(5):1621–8.
[19] Kang P, Mechref Y, Klouckova I, Novotny MV. Solid-phase permethylation of glycans for mass spectrometric analysis. Rapid Commun Mass Spectrom 2005;19(23):3421–8.
[20] Kang P, Mechref Y, Novotny MV. High-throughput solid-phase permethylation of glycans prior to mass spectrometry. Rapid Commun Mass Spectrom 2008;22(5):721–34.
[21] Goldman R, Ressom HW, Varghese RS, Goldman L, Bascug G, Loffredo CA, et al. Detection of hepatocellular carcinoma using glycomic analysis. Clin Cancer Res Mar. 1, 2009;15(5):1808–13.
[22] Ressom HW, Varghese RS, Goldman L, An Y, Loffredo CA, bdel-Hamid M, et al. Analysis of MALDI-TOF mass spectrometry data for discovery of peptide and glycan biomarkers of hepatocellular carcinoma. J Proteome Res Feb. 2008;7(2):603–10.
[23] Tang Z, Varghese RS, Bekesova S, Loffredo CA, Hamid MA, Kyselova Z, et al. Identification of N-glycan serum markers associated with hepatocellular carcinoma from mass spectrometry data. J Proteome Res Jan. 2010;9(1):104–12.

[24] Tang H, Mechref Y, Novotny MV. Automated interpretation of MS/MS spectra of oligosaccharides. Bioinformatics Jun. 2005;21(Suppl. 1):i431–9.
[25] Yang C, He Z, Yu W. Comparison of public peak detection algorithms for MALDI mass spectrometry data analysis. BMC Bioinformatics 2009;10:4.
[26] Coombes KR, Tsavachidis S, Morris JS, Baggerly KA, Hung MC, Kuerer HM. Improved peak detection and quantification of mass spectrometry data acquired from surface-enhanced laser desorption and ionization by denoising spectra with the undecimated discrete wavelet transform. Proteomics Nov. 2005;5(16):4107–17.
[27] Goldberg D, Sutton-Smith M, Paulson J, Dell A. Automatic annotation of matrix-assisted laser desorption/ionization N-glycan spectra. Proteomics Mar. 2005;5(4):865–75.
[28] Morelle W, Flahaut C, Michalski JC, Louvet A, Mathurin P, Klein A. Mass spectrometric approach for screening modifications of total serum N-glycome in human diseases: application to cirrhosis. Glycobiology Apr. 2006;16(4):281–93.
[29] Arnold JN, Wormald MR, Suter DM, Radcliffe CM, Harvey DJ, Dwek RA, et al. Human serum IgM glycosylation—identification of glycoforms that can bind to mannan-binding lectin. J Biol Chem Aug. 12, 2005;280(32):29080–7.
[30] Blomme B, Van SC, Callewaert N, Van VH. Alteration of protein glycosylation in liver diseases. J Hepatol Mar. 2009;50(3):592–603.
[31] Martinez J, Barsigian C. Carbohydrate abnormalities of N-linked plasma glycoproteins in liver disease. Lab Invest Sep. 1987;57(3):240–57.
[32] Gravel P, Walzer C, Aubry C, Balant LP, Yersin B, Hochstrasser DF, et al. New alterations of serum glycoproteins in alcoholic and cirrhotic patients revealed by high resolution two-dimensional gel electrophoresis. Biochem Biophys Res Commun Mar. 7, 1996;220(1):78–85.
[33] Thompson S, Matta KL, Turner GA. Changes in fucose metabolism associated with heavy drinking and smoking: a preliminary report. Clin Chim Acta Sep. 14, 1991;201(1–2): 59–64.
[34] Mann AC, Record CO, Self CH, Turner GA. Monosaccharide composition of haptoglobin in liver diseases and alcohol abuse: large changes in glycosylation associated with alcoholic liver disease. Clin Chim Acta Jun. 1994;227(1–2):69–78.
[35] Hada T, Kondo M, Yasukawa K, Amuro Y, Higashino K. Discrimination of liver cirrhosis from chronic hepatitis by measuring the ratio of *Aleuria aurantia* lectin-reactive serum cholinesterase to immunoreactive protein. Clin Chim Acta Mar. 1999;281(1–2):37–46.
[36] Ryden I, Pahlsson P, Lindgren S. Diagnostic accuracy of alpha(1)-acid glycoprotein fucosylation for liver cirrhosis in patients undergoing hepatic biopsy. Clin Chem Dec. 2002;48(12):2195–201.
[37] Marino K, Bones J, Kattla JJ, Rudd PM. A systematic approach to protein glycosylation analysis: a path through the maze. Nat Chem Biol Oct. 2010;6(10):713–23.
[38] Mechref Y, Novotny MV. Miniaturized separation techniques in glycomic investigations. J Chromatogr B Analyt Technol Biomed Life Sci Sep. 1, 2006;841(1–2):65–78.
[39] Bones J, Mittermayr S, O'Donoghue N, Guttman A, Rudd PM. Ultra performance liquid chromatographic profiling of serum N-glycans for fast and efficient identification of cancer associated alterations in glycosylation. Anal Chem Dec. 15, 2010;82(24):10208–15.
[40] Szabo Z, Guttman A, Rejtar T, Karger BL. Improved sample preparation method for glycan analysis of glycoproteins by CE-LIF and CE-MS. Electrophoresis Apr. 2010;31(8):1389–95.
[41] Royle L, Dwek RA, Rudd PM. Determining the structure of oligosaccharides N- and O-linked to glycoproteins. Curr Protoc Protein Sci Mar. 2006 Chapter 12:6.1–45.
[42] Ahn J, Bones J, Yu YQ, Rudd PM, Gilar M. Separation of 2-aminobenzamide labeled glycans using hydrophilic interaction chromatography columns packed with 1.7 mu m sorbent. J Chromatogr B Analyt Technol Biomed Life Sci Feb. 1, 2010;878(3–4):403–8.
[43] Harvey DJ. Structural determination of N-linked glycans by matrix-assisted laser desorption/ionization and electrospray ionization mass spectrometry. Proteomics May 2005;5(7): 1774–86.
[44] Madi A, Lakhdari O, Blottiere HM, Guyard-Nicodeme M, Le RK, Groboillot A, et al. The clinical Pseudomonas fluorescens MFN1032 strain exerts a cytotoxic effect on epithelial intestinal cells and induces Interleukin-8 via the AP-1 signaling pathway. BMC Microbiol 2010;10:215.
[45] Ashline D, Singh S, Hanneman A, Reinhold V. Congruent strategies for carbohydrate sequencing. 1. Mining structural details by MSn. Anal Chem Oct. 1, 2005;77(19):6250–62.
[46] Stumpo KA, Reinhold VN. The N-glycome of human plasma. J Proteome Res Sep. 2010;9(9):4823–30.
[47] Stanta JL, Saldova R, Struwe WB, Byrne JC, Leweke FM, Rothermund M, et al. Identification of N-glycosylation changes in the CSF and serum in patients with schizophrenia. J Proteome Res Sep. 2010;9(9):4476–89.
[48] Wu S, Grimm R, German JB, Lebrilla CB. Annotation and structural analysis of sialylated human milk oligosaccharides. J Proteome Res Feb. 4, 2011;10(2):856–68.
[49] Lattova E, McKenzie EJ, Gruwel MLH, Spicer V, Goldman R, Perreault H. Mass spectrometric study of N-glycans from serum of woodchucks with liver cancer. Rapid Commun Mass Spectrom Sep. 2009;23(18):2983–95.
[50] Harada H, Kamei M, Tokumoto Y, Yui S, Koyama F, Kochibe N, et al. Systematic fractionation of oligosaccharides of human immunoglobulin G by serial affinity chromatography on immobilized lectin columns. Anal Biochem Aug. 1, 1987;164(2):374–81.
[51] Arnold JN, Wallis R, Willis AC, Harvey DJ, Royle L, Dwek RA, et al. Interaction of mannan binding lectin with alpha(2) macroglobulin via exposed oligomannose glycans—a conserved feature of the thiol ester protein family? J Biol Chem Mar. 17, 2006;281(11):6955–63.
[52] Ritchie GE, Moffatt BE, Sim RB, Morgan BP, Dwek RA, Rudd PM. Glycosylation and the complement system. Chem Rev Feb. 2002;102(2):305–19.
[53] Knezevic A, Polasek O, Gornik O, Rudan I, Campbell H, Hayward C, et al. Variability, heritability and environmental determinants of human plasma N-glycome. J Proteome Res Feb. 2009;8(2):694–701.