Scientific Rationale: Using a glycoproteomic methodology that we developed, we have discovered a set of serum biomarker that have shown great promise as biomarkers of hepatocellular carcinoma (HCC). This simple methodology first identifies changes in N-linked glycosylation that occur with the disease. This change acts as a "tag" so that we can extract out those specific proteins that contain that glycan structure(Block *et al.* 2005). Our initial work lead to the discovery of a protein, GP73, that is 3 times more sensitive at detecting HCC than the current marker, AFP (Marrero et al., 2005). Indeed in our initial blinded study, GP73 had an AUROC of 0.79 as compared to 0.61 for AFP at the detection of Stage 1 or 2 tumors versus cirrhosis (Marrero et al.,2005). Subsequent glycoproteomics has discovered over 50 fucosylated glycoproteins that are altered in patients with HCC(Comunale 2006).Many of these proteins, like GP73, have shown promise as biomarkers of HCC (Semmes et al., 2006 and preliminary evidence). This initial work was performed in an effort to assess the ability of our biomarker(s) to distinguish subjects with cancer from those with cirrhosis. The use of the samples from the HALT-C study will allow us to determine the usefulness of our lead biomarker candidates (GP73 and fucosylated kininogen)in a proper retrospective longitudinal study, where the primary aim is to evaluate the capacity of the biomarker to detect preclinical disease.

Specific Aims: The specific aims of this application are to 1) determine the level of GP73 and fucosylated kininogen as a function of HCC in the HALT-C study group and 2) to determine the levels of GP73 and fucosylated kininogen as a function of HCC in a retrospective longitudinal study using the HALT-C samples.

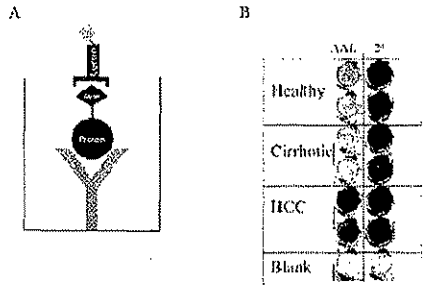

Figure 1:Lectin-FLISA for the measurement of fucosylated glycoproteins. A) Periodate oxidized mouse anti-human alpha-1 anti-trypsin (AAT) was used as the capture antibody and the level of fucosylated protein determined by a biotin conjugated lectin (*AAL*) and detected using IRDye™ 800 Conjugated Streptavidin. Signal intensity was measured using the Odyssey® Infrared Imaging System. In all cases sample intensity was compared to commercially purchased human serum (Sigma Chemicals). **B) Example of Lectin-ELISA for the detection of fucosylated AAT.** Left column shows results of Lecin-FLISA for fucosylated AAT. Right column shows the level of total AAT. The column labeled AAL represents the amount of AAT reactive towards the fucose binding lectin AAL. The column labeled 2° represents the total amount of AAT captured as detected through the use of a secondary AAT antibody.

Methods and Preliminary Data: In the work proposed, we will examine 2 markers either by a simple ELISA (for GP73) or by a lectin ELISA (for fucosylated kininogen). The analysis of total GP73 utilizes a simple ELISA that is detailed below and is straightforward. However, the development of assays to examine the fucosylated glycoforms of several serum proteins has proven difficult and is described here in more detail. In our previous work, we analyzed the levels of fucosylated GP73 and fucosylated hemopexin in patients with HCC via immunoblotting of lectin enriched fractions(19). This method involved the depletion of immunoglobulin from serum samples followed by lectin extraction of all fucosylated proteins. Subsequently, proteins were resolved through polyacrylamide gels and proteins of interest detect via immunoblotting. As this technique was not suitable for the analysis of larger sample numbers a lectin-Fluorophore-Linked Immunosorbent Assay (FLISA) was developed. Although simple in design, this assay (shown in figure 1) was initially hampered by the interference of heterophilic antibodies(21). As the antibodies used to capture the proteins of

Table I: Sample population characteristics

Obtained From	St. Louis University[1]		University of Michigan[2]	
Disease Diagnosis	HCC[3]	Cirrhosis[4]	HCC[3]	Cirrhosis[4]
Number	65	32	99	81
Etiology% (HBV/HCV/crypto/alcohol/other)[5]	14/52/6/20/8	0/100/0/0/0	11/51/24/10/4	5/48/20/18/9
Age	58.04±11	50±8	58.6±12	58±3
Gender M:F%	71:29	84:16	75/25	60/40
MELD Score[6]	11.8±5	N/A	10.3±4	9±2
Child Class (A/B/C/NA%)[7]	52:29:9:10	88:8:4:0	48/42/10/0	40/54/6/0
Tumor Stage (1/2/3/4) %[8]	26:48:12:14	NA	21/40/26/13	NA

1&2) Samples were provided coded from St. Louis University Medical School or from the University of Michigan. See text for more details. **3&4**). Disease diagnosis was determined by MRI or by liver biopsy. **5**) For Etiology: HBV, hepatitis B virus; HCV, hepatitis C virus; crypto, cryptogenic liver disease; alcohol, alcohol induced liver disease; other, liver disease of unknown origin. **6)** MELD: Model for end stag liver disease. N/A, not available. **7)** The percent of patients with each Child-Pugh score is given as a percentage in each group. **8)** Tumor staging was determined using the United Network of Organ Sharing-modified TNM staging system for HCC. The percent of patients within each stage is given.

interest are made in animals, they contain epitopes or saccharide structures that are considered foreign and can be reactive tohuman antibodies. Hence, they can bind to the capture antibody and lead to signal interference. This is particularly applicable in our situation as we have recently determined that heterophilic antibodies reactive towards oligosaccharide Gal□-1-3Gal□1-(3)4GlcNAc-R (referred to as the alpha-gal epitope) are increased and become reactive to fucose binding lectins with the development of fibrosis and cirrhosis(21). As almost all patients develop HCC in the background of fibrosis/cirrhosis, these alpha-gal antibodies must be neutralized before analysis of specific protein of interest could be determined.

An example of the lectin-FLISA is shown in Fig. 1A. Briefly, fucosylated glycoproteins of interest are detected by incubating serum with wells coated with periodate oxidized antibody, followed by incubation with biotinylated, fucose specific lectin. Bound lectin is than detected using IRDye™ 800 Conjugated streptavidin and the signal intensity measured using the Odyssey® Infrared Imaging System. In all cases, signal intensity was compared to signals detected with commercially purchased human serum (Sigma Chemicals). It is noted that the lectin-FLISA detects the amount of fucosylation present on an equal amount of captured molecules from each patient sample and is performed in a manner that is independent of the total amount of protein in any given patient. As shown in figure 1B, using this system we were able to detect fucosylated alpha-1-anti-trypsin in patients with HCC. The left panels show the reactivity of fucose binding lectin to captured alpha-1 anti-trypsin from healthy patients, patients with biopsy confirmed cirrhosis or cirrhosis and HCC. As this panel shows, positive lectin binding was observed primarily in the HCC sample even though, as the right column shows, equal amounts of AAT were captured regardless of the disease state. This assay was used for the analysis of fucosylated kininogen. An assay for the analysis of fucosylated GP73, while in

Table II. Sensitivities and specificities of markers at the detection of stage 1 or 2 cancer.

	FC-AAT[1]	FC-Kin[2]	GP73[3]	AFP[4]	GP73, AFP& Fc-kin†
AUROC	0.74	0.79	0.89	0.83	**0.94**
SE	0.04	0.03	0.02	0.03	0.02
95% CI	0.67-0.81	0.73-0.85	0.85-0.93	0.77-0.88	**0.91-0.97**
***p* value**	< 0.0001	< 0.0001	< 0.0001	< 0.0001	<0.0001
%Specificity at 50% Sensitivity	81	84	97	98*	**99**
%Specificity at 75% Sensitivity	64	67	86	74	**95***
%Specificity at 90% Sensitivity	41	47	68	36	**77***
%Specificity at 95% Sensitivity	28	42	43	28	**70***
%Specificity at 100% Sensitivity	4	23	25	4	**36***

1&2) Analysis of fucosylated alpha-1 anti-trypsin (FC-AAT) or fucosylated kininogen via lectin-FLISA as described in figure 1; 3) GP73 was analyzed by immunoblot; 4) AFP was measured using a commercially available AFP ELISA kit. The best values for each category are given in bold. *Statistically different then the other values in the given group (p<0.5).

development, is currently not available and hence a typical sandwich ELISA for total GP73 is used.

The fucosylation of two proteins, alpha-1-anti-trypsin (AAT) and low molecular weight kininogen were tested in a two cohort coded study comprising of 113 patients (see tables 1&2) with biopsy confirmed cirrhosis, 108 patients with cirrhosis plus stage 1 or 2 HCC, and 51 patients with cirrhosis plus stage 3 or 4 HCC. The method used was the lectin-FLISA as described in figure 1. For both fucosylated AAT and fucosylated kininogen, relative levels were compared to commercially purchased serum. There was a substantial increase in the level of both fucosylated AAT and fucosylated kininogen in patients with HCC. The level of fucosylated AAT was 1.8 fold elevated in cirrhotic patients (compared to purchased serum), 2.9 fold elevated in patients with stage 1 or 2 HCC, and 3.6 fold in patients with stage 3 or 4 HCC. Statistical significance was observed between the cirrhosis group and all HCC groups($P<0.0001$) but not between the HCC groups ($p=0.05$). Similar alterations were observed with fucosylated kininogen, which had a mean increase of 1.1 fold in patients with cirrhosis, 2.3 fold in patients with stage 1 or 2 HCC, and 2.9 in patients with stage 3 or 4 HCC. As with Fc-AAT, statistical significance was observed between the cirrhosis group and all HCC groups ($P<0.0001$) but not between the individual HCC groups ($P=0.32$). The marker GP73, which has been previously been reported was also tested in these patients (17, 20). GP73 had a mean increase of 2.4 fold in the patients with cirrhosis, 8.4 fold in patients with stage 1 or 2 HCC and 8.1 fold in patients stage 3 or 4 HCC. Again, while no statistical significance was observed between the two HCC groups ($p=0.79$), statistical significance was observed between the cirrhosis group and the HCC groups ($P<0.0001$).For comparison, the levels the currently used marker of HCC, AFP, were also measured in these samples. In these samples, AFP had a mean level 10 ng/ml in patients with cirrhosis, 8356 ng/ml in patients with stage 1 or 2 HCC and 944 ng/ml fold in patients stage 3 or 4 HCC.

Changes in glycosylation and more importantly, fucosylation are known to occur with the development of cancer. The possibility that the increase in fucosylation would be observed in

patients with cancer was tested through the examination of serum samples from patients with prostate cancer *(n=20)*, ovarian cancer *(n=22)*, lung cancer *(n=10)*, cervical cancer *(n=16)* and colorectal cancer *(n=10)*. These patients had values similar of fucosylated AAT, fucosylated kininogen and total GP73 to those obtained with serum from control subjects, suggesting specificity of this marker for liver disease (data not shown).

Receiver operator characteristic (ROC) curves were plotted to determine overall performance and to identify the sensitivity and specificity for each marker in differentiating HCC from cirrhosis. As a major goal of our biomarker discovery work is the development of a more sensitive marker of early cancer, the specificity of each marker was determined at fixed points of sensitivity (see table II). As detailed in table II, in differentiating cirrhosis from stage 1 or 2 HCC, the AUROC curve for fucosylated kininogen was 0.79 with a specificity of 42% at a fixed sensitivity of 95%. Comparable results were obtained when comparing cirrhosis to all HCC stages. Similarly, Fc-AAT had an AUROC of 0.74 with a specificity of 28% at a fixed sensitivity of 95%. Like fucosylated kininogen, results were similar when comparing cirrhosis to all HCC stages.

The marker GP73 had the best individual performance characteristics. As shown in table II, GP73 had an AUROC of 0.89 with a specificity 43% at a fixed sensitivity of 95%, in differentiating cirrhosis from stage 1 or 2 HCC. The addition of stage 3 or 4 HCC patients did not alter the performance of GP73. For comparison, as table II shows, AFP had a similar performance as GP73 with specificity of 28%, at a fixed sensitivity of 95%, and an AUROC of 0.83.

The performance of these markers when used in combination was also tested (table II). This was done using either a combination of any two to four markers using logistic regression analysis. ROC analysis is presented in figure 4 for the analysis of the markers used in the study set. The combination of GP73, fucosylated kininogen and AFP gave the best overall results with an AUROC of 0.94 with a specificity of 70% at a fixed sensitivity of 95%. This was much greater than any maker alone, as shown in table II or III ($P<0.05$). For all markers used in combination, performance was similar in both early tumors (stage 1 or 2) or with the analysis of all cases of HCC.

Detailed methods:Lectin FLISA: Monoclonal antibodies are fucosylated and are reactive with fucose binding lectins. Hence they must be modified prior to analysis via the Lectin-FLISA. Briefly, to remove the fucosylation of the capture antibody (Mouse anti-human AAT or rabbit anti-human LMW kininogen, Bethyl Laboratories, Montgomery, TX), antibody was incubated with 10mM sodium periodate for 1 hour at 40C. An equal volume of ethylene glycol was added and the oxidized antibody brought to a concentration of 10µg/mL with sodium carbonate buffer, pH 9.5. Antibody (5 µg/well) was added to the plate and following incubation washed with 0.1% Tween 20/PBS 7.4 and blocked overnight with 3% BSA/PBS. For analysis, 5 µl of serum was diluted in 95 µL of Heterophilic Blocking tubes (Scantibodies Laboratory, Inc. Santee, CA 92071 USA) and incubated at room temperature for 1 hour. Subsequently, samples were added to the plates for 2 hours and washed 5 times in lectin incubation buffer (10mM Tris pH 8.0, 0.15M NaCl, 0.1%Tween 20) before fucosylated protein was detected with a biotin conjugated Aleuria aurantia (AAL) lectin (Vector Laboratories, Burlingame, CA). Bound lectin was detected using IRDye™ 800 Conjugated streptavidin and signal intensity measured using the Odyssey® Infrared Imaging System (LI-COR Biotechnology, Lincoln, Nebraska). In all cases sample intensity was compared to commercially purchased human serum (Sigma Inc., St Louis, MO.).

GP73 ELISA: Briefly, polyclonal anti-GP73(5 µg/well) in sodium carbonate buffer, pH 9.5, is added to the wells of a 96 well plate and following overnight incubation washed with 0.1% Tween 20/PBS 7.4 and subsequently blocked overnight with 3% BSA/PBS. For analysis, 5 µl of serum was diluted in 95 µL of Heterophilic Blocking tubes (Scantibodies Laboratory, Inc.

Santee, CA 92071 USA) and incubated at room temperature for 1 hour. Subsequently, samples were added to the plates for 2 hours and washed 5 times in lectin incubation buffer (10mM Tris pH 8.0, 0.15M NaCl, 0.1%Tween 20) before GP73 detected with a biotin conjugated anti-GP73 monoclononal antibody (developed in house). Bound monoclonal anti-GP73 was detected using IRDye™ 800 Conjugated streptavidin and signal intensity measured using the Odyssey® Infrared Imaging System (LI-COR Biotechnology, Lincoln, Nebraska). In all cases sample intensity was compared to commercially purchased human serum (Sigma Inc., St Louis, MO.).

Patient sample size and suggested data analysis – In an effort to determine the appropriate sample size required for analysis, we have assumed that the data structure obtained from the HALT-C samples will be similar to those obtained in our previous work using samples from the University of Michigan, St. Louis University, Thomas Jefferson University and Fox Chase Cancer Center. Based on our previous data, we have determined that for the analysis of GP73 and fucosylated kininogen we will require at minimum 70 HCC serum samples and 70 cirrhosis (control) serum samples to test GP73 and Fc-kininogen. This sample number will allow us to perform the two-tailed hypothesis test at a 5% level of significance, with 90% power for both markers (Desu and Raghavarao, 1990). This number will also allow for the inclusion of AFP if necessary to improve marker performance without leading to bias.

For data analysis, all samples will be compared to commercially purchased serum, and relative values assigned as "fold over" the commercially purchased serum (Sigma Chemicals). Receiver operative characteristic (ROC) curves will be constructed to judge the discriminatory ability of GP73, Fc-kininogen, and the combination of GP73 and Fc-Kininogen.

Based on our previous studies, a multiple logistic regression model proved to be a relatively robust method for use with multiple markers (Wang et al., 2008). While this analysis could (and should initially) be used in the HALT-C patient cohort, we also propose to combine a bootstrap method with logistic regression as in described in Swanepoel and Fraggos(1994). We expect that with the assistance of a re-sampling technique, we will obtain a more accurate estimation of the area under the ROC curve(AUC) to judge each marker and their combination. Estimations of parameters, such as mean, standard deviation and confidence intervals will be improved in accuracy with aid of bootstrap approach(Davison and Hinkley,1997).

Conclusion: The goal of this application is to determine the performance of our lead biomarkers candidates, GP73 and fucosylated kininogen, in the HALT-C patient cohort. It is expected that these markers, either individually or in combination will outperform AFP, in the detection of HCC.