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Capturing and Identification of Differentially Expressed Fucone by a Gel Free and Label Free Approach

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Abstract

This research reports a proof-of-concept that describes an instrumental approach that is gel free and label free at both the separation and mass spectrometry ends for the capturing and identification of differentially expressed proteins (DEPs) in diseases, e.g., cancers. The research consists of subjecting/processing equalized and non-equalized (i.e., untreated) disease-free and hepatocellular carcinoma (HCC) human sera *via* a multicolumn platform for capturing/fractionating human serum fucone. The equalization was performed *via* the combinatorial peptide ligand library (CPLL) beads technology that ensured narrowing the protein concentration range, thus allowing the detection of low abundance proteins. The equalized and non-equalized disease-free and HCC sera were first fractionated online onto two lectin columns specific to fucose, namely *Aleuria aurantia* lectin (AAL) and *Lotus tetragonolobus* agglutinin (LTA) followed by the online fractionation of the lectin captured fucone by reversed phase chromatography. The online desalted fractions were first subjected to trypsinolysis and then to liquid chromatography-mass spectrometry (LC-MS/MS) analysis. In comparison with untreated serum, the CPLL treated serum is superior in terms of the total number of identified DEPs, which reflected an increased number of DEPs in a wide abundance range. The DEPs in HCC serum were found to be 70 and 40 in both LTA and AAL fractions for the serum treated by CPLL and untreated serum, respectively. In addition, the platform combined with the CPLL treatment was accomplished with virtually no sample loss and dilution as well as with no experimental biases and sample labeling when comparing the diseased-free and cancer sera using LC-MS/MS.

Keywords

Fucosylation; Glycoproteins; Hepatocellular carcinoma; Lectin Affinity Chromatography; Monolithic columns; ProteoMiner™

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1. Introduction

Glycosylation is an important post-translational modification involving the covalent linkages between oligosaccharides and proteins, which is regulated in various biological processes including birth, differentiation, growth, inflammation, carcinogenesis, and cancer metastasis [1–3]. Fucose is one of a few monosaccharides forming the oligosaccharide chains (i.e., glycans) attached to glycoproteins and glycolipids present in vertebrates, invertebrates, plants and bacteria. Fucosylation refers to the presence of a fucose residue in the *N*-glycans or *O*-glycans attached to glycoproteins [1, 4]. Several kinds of fucosyltransferases, guanosine diphosphate (GDP)-fucose synthetic enzymes and GDP-fucose transporters are involved in the regulation of fucosylation [1, 4]. The fucosylation level during liver carcinogenesis is relatively high compared to its low level in normal liver. An increase in fucosylated proteins or fucone in sera of the patients with cancer depends on cellular fucosylation of cancer tissues and/or changes in fucosylation states in the liver [1]. Hakomori *et al.* presented the first paper involving the study of fucosylation in cancer, comparing the fucosylation level of glycolipids in hepatoma cells and normal hepatocytes [5]. Alpha-fetoprotein (AFP) is a well-known tumor marker for hepatocellular carcinoma (HCC) [1, 4, 6]. Nevertheless, AFP is not specific only for HCC but an increase in the level of AFP is also observed in benign liver diseases such as chronic hepatitis and liver cirrhosis [1, 4, 7]. Whereas fucosylated AFP or AFP with core fucosylation (AFP-L3) is more specific tumor marker for HCC. Core-fucosylation, comprises the attachment of fucose to the innermost *N*-acetylglucosamine in *N*-glycans, which is catalyzed using alpha-1-6 fucosyltransferase (Fut8). Although AFP-L3 is more specific marker for HCC, expression of Fut8 during core fucosylation is increased in both HCC tissues and surrounding tissues with liver cirrhosis [8]. Block *et al.* have reported a fucosylated biomarker for HCC namely Golgi protein 73 (GP73) which has a positive predicative value equal to or greater than the AFP [6]. The use of AFP, AFP-L3 and GP73 as a primary screen for HCC has a limited utility for detecting HCC and more sensitive serum biomarkers for HCC are desired. Therefore, an in-depth profiling of changes in the fucone in HCC is needed for discovering more specific and powerful HCC candidate biomarkers.

Different analytical tools such as two dimensional polyacrylamide gel electrophoresis (2DE) [9, 10], two dimensional differential gel electrophoresis (2D-DIGE) [10–17], isotope coded affinity tags (ICAT) [18] and isobaric tags for relative and absolute quantification (iTRAQ) [19] have been developed for the identification of DEPs in complex biological sample of HCC. 2DE is a widely used proteomics technology to study proteome as well as differentially expressed proteins in HCC [9, 10]. The principle of 2DE is based on separation of proteins according to their charge in first dimension by isoelectric focusing and size in the second dimension by polyacrylamide gel electrophoresis [9]. However, the lack of reproducibility between gels is a major limitation of 2DE. Additional problems with 2DE include its low sensitivity and insufficient resolution to separate multi species originating from a single protein [9, 20]. 2D-DIGE has been introduced in order to address some of the drawbacks of 2DE technique. 2D-DIGE is one of the proteomic labeling techniques, which have been employed for separation and identification of DEPs in HCC [10–17]. 2D-DIGE is performed by sample pre-labeling with different fluorescent cyanine (Cy) dyes. These

labelled proteins are then mixed and separated simultaneously on the same 2D gel electrophoresis. The different protein extracts labelled with different CyDye DIGE fluor can then be visualized separately by exciting the different dyes at their specific excitation wavelengths [9, 20, 21]. 2D-DIGE has ability to reduce the effect of gel-to-gel variation, provides more accurate and reliable quantification information of protein abundance because the samples are separated together on the same gel. However, DIGE labeling suffers from the systematic variation because the phenomenon of protein-specific dye bias. Here, certain proteins are preferentially labelled with CyDye over others, despite their similar structures and identical reactive groups [22]. In addition, the labeling chemistry is required for attaching the dye to the proteins. For example, with the minimal dyes, high abundance protein spots in a conventional gel system could be a medium or low abundance protein spot in the DIGE system [20]. This is due to a low percentage of lysine residues in high abundance proteins. And also, 2D-DIGE is not applicable to those proteins without lysine (when labeling with the minimal dyes) or cysteine (when labeling with the saturation dyes) [20]. Non-gel based quantitative proteomic methods have been developed for studying DEPs in HCC, namely ICAT [18] and iTRAQ [19]. These methods are based on the similar physical and chemical properties of isotope labelled compounds to their unlabeled equivalents but with a different mass that can be recognized by mass spectrometer, and quantification is achieved by comparing their respective signal intensities [23, 24]. Limitations of using these stable isotope labeling methods include the potential for incomplete labeling, high cost of the labeling reagents, variable labeling efficiency, limitations of available labeling reagents for simultaneous quantification of proteins from multi samples and the requirement for specific quantification software. Furthermore, ICAT is only applicable to proteins containing cysteine [23–25].

In this work, which is a continuation to our recent investigations [26, 27], we intend to feature three new elements of the recently developed multicolumn platform: (i) the effectiveness of the platform in mapping the altered fuome in another cancer serum, namely human serum with HCC, (ii) reveal clearly the instrumental aspect of the platform for comparative proteomics that does not involve labeling chemistry and consequently requires much less labor than existing technology, and (iii) demonstrate the superiority of the described method in generating the whole fuome as compared to partial fuome by the combination of other methodologies. Regarding aim (i), since every cancer develops and behaves differently, it is therefore important to challenge the developed strategy in revealing similarities and differences in the altered fuome in HCC with respect to that in breast cancer. Aims (ii) and (iii) are justified by assessing the high throughput of the developed platform with respect to the existing technologies.

In short, this investigation describes the online selective capturing of fucosylated serum glycoproteins (i.e., the human fuome) by LAC followed by fractionation of the captured human fuome by reversed phase chromatography (RPC) in an instrumental multicolumn platform. The fuome in HCC serum was then analyzed with respect to disease free serum using LC-MS/MS. Recently, LAC has been shown very effective in the capturing and differential profiling of glycoproteins in biological matrices [26–30]. Our approach is a label free technique in both the separation stage using LAC and RPC and the identification stage using LC-MS/MS. Therefore, all of the above mentioned drawbacks associated with label-

based approaches have been eliminated. In fact, the platform eliminates all experimental biases, centrifugation, multistep dialysis, dilution and transfer from vessel-to-vessel. And also, the platform allows the transfer/processing of proteins from column-to-column in the liquid phase using high precision pumps and valves, and no sample loss as well as zero propagation of experiment errors. To further exploit the potentials of the platform and demonstrate its effectiveness in profiling the altered fuome in HCC, an off line protein equalization *via* the combinatorial peptide ligand library (CPLL) approach was performed prior to sample processing by the platform. The CPLL is a mixture of a multitude of linear hexapeptides, which has been shown to be very effective in the detection of novel proteins, due to the concomitant reduction of high abundance proteins and the concentration of low- or very low abundance proteins in many biological fluids and extracts [31–33], thus allowing an in-depth proteomics profiling. To capture the fuome, two lectin columns were incorporated in the platform. They consisted of immobilized fucose specific lectins namely, AAL and *Lotus tetragonolobus* agglutinin (LTA) onto the surface of glyceryl methacrylate (GMM)/ pentaerythritol triacrylate (PETA) monolith which was recently introduced by Gunasena and El Rassi for performing immuno affinity chromatography at reduced nonspecific interactions [34]. Immobilized AAL has a strong affinity towards glycoproteins with core fucosylated glycans [35], whereas immobilized LTA can bind to glycoproteins with glycans having fucose present in the outer arm. LTA also has an affinity for glycans containing the Le^x determinant [36]. The haptenic sugar for AAL and LTA is α -L-fucose. In order to establish the efficiency of the combination of CPLL technology with the multicolumn platform that facilitates the capturing, enrichment and fractionation of the human fuome prior to LC-MS/MS analysis, the aim of this work is to compare the differentially expressed glycoproteins in HCC with respect to disease free serum in both sera that were treated or untreated by CPLL beads (i.e., ProteoMiner™ treated or untreated serum).

2. Materials and methods

2.1 Materials

Two fucose specific lectins namely, AAL and LTA were purchased from Vector Laboratories (Burlingame, CA, USA). Pooled human HCC serum from five donors and pooled disease-free human serum from twenty donors (same age group and race as the cancer serum) were purchased from Bioreclamation (Westbury, NY, USA). Stainless steel tubing of 4.6 mm ID was purchased from Alltech Associates (Deerfield, IL, USA). The ProteoMiner™ bulk beads were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Glyceryl methacrylate (GMM) was supplied by Monomer-polymer & Dajac Labs (Feaster-Ville, PA, USA). The AcroSep™ SDR columns were obtained from Pall Life Sciences (Ann Arbor, MI, USA). 2,2'-Azobisisobutyronitrile (AIBN) was purchased from Aldrich Co. (Milwaukee, WI, USA). Cyclohexanol, pentaerythritol triacrylate (PETA), 1-dodecanol, sodium periodate, sodium cyanoborohydride, trifluoroacetic acid (TFA), tris(hydroxymethyl)aminomethane (Tris) and L-(-)-fucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile was supplied by Pharmaco-Aaper (Brookfield, CT, USA). The reversed phase chromatography column (ProSwift™ RP-1S) was purchased from Dionex Corporation (Sunnyvale, CA, USA).

2.2 Methods

2.2.1 Monolithic affinity columns—A polymerization mixture weighing 5 g was prepared from 7.6-wt% GMM, 7-wt% PETA, 59.1-wt% cyclohexanol, 22.9-wt%, dodecanol and 3.4-wt% water. This polymerization mixture containing 1.0-wt% AIBN with respect to monomers was sonicated for 15 min, and then purged with nitrogen for 5 min [34]. A stainless steel column of 25 cm × 4.6 mm ID was filled with the polymerization mixture. This column, which functions as a mold, was closed at both ends using screw caps and the mold was submerged in a 60 °C water bath for 15 h. The resulting monolithic column was washed with acetonitrile and then with water by using an HPLC pump. Thereafter, the monolith was transferred from the 25 cm column to a stainless steel column of 3 cm × 4.6 mm ID by connecting the two columns with a 1/4"-union and running water through the columns at a flow rate of 3.0 mL/min until the monolith is transferred. Two of 3 cm × 4.6 mm ID columns were prepared from each mold by using the just described procedure.

A freshly prepared 0.1 M NaIO₄ solution was passed through each 3 cm monolithic column using a syringe pump at a flow rate of 3.0 mL/h for 2 h at room temperature to convert the diol into aldehyde groups. The activated monolith thus obtained was washed with water for 10 min at a flow rate of 1.0 mL/min using an HPLC pump. Thereafter, 0.3 mL of 0.1 M sodium acetate solution pH 6.4 was passed through the column (as fast as possible). This was followed by passing through the column, using a micro-syringe pump, the lectin immobilization solution first at a flow rate of 0.3 mL/h for 1 h, then at a flow rate of 0.1 mL/h for 2 h and then left overnight (~16 h) at room temperature at a flow rate of 0.02 mL/h. Finally, passing the same immobilization solution for an extra 2h at a flow rate of 0.12 mL/h completed the immobilization of the given lectin. In this process, the same immobilization solution was passed through the column almost twice. The lectin immobilization solution consisted of 1 mg AAL or LTA in 0.5 mL of 0.1 M sodium acetate, pH 6.4, and containing 0.1 M fucose and 50 mM sodium cyanoborohydride. Sodium cyanoborohydride was replenished in between. Thereafter, a solution containing 0.4 M Tris/HCl, pH 7.2, 50 mM sodium cyanoborohydride was passed for 3 h at room temperature at a flow rate of 2 mL/h. 20 mM Tris/HCl, pH 7.4 containing 0.08% NaN₃ was used as a storage solution for three immobilized lectin columns, and the columns were kept at 4 °C until use.

2.2.2 Preparation of equalized and unequalized serum samples—Five mL of each serum samples (disease-free or cancer serum) were treated by the CPLL beads according the manufacturer instructions, and the procedure was also described very recently by Selvaraju and El Rassi [27]. The treated (i.e., equalized) disease-free or HCC serum was stored at -20 °C until use. The equalized protein solutions thus obtained were treated with AcroSep™SDR columns to remove urea and CHAPS according the manufacturer instructions. The resulting protein solutions were brought up to pH 7 by adding Tris to the solution. The final solution of 5.6 mL for each type of sera was ready to inject into the lectin columns. In the case of untreated sample, 40 µL of disease-free serum or HCC serum was prepared by diluting the original serum with binding mobile phase, containing 20 mM Tris/HCl, pH 7.4, to give 3-fold diluted serum.

2.2.3 Chromatographic setup and conditions for capturing of the fucosylated glycoproteins

A Model 590 programmable pump from Waters Corporation (Milford, MA, USA) and a solvent delivery system Model ConstaMetric 3500 from LDC Analytical (Riviera Beach, FL, USA) were used to deliver solvent through the multicolumn platform under investigation, see Fig. S1 in Supporting information. Sample injection was performed via an injector Model 7125 from Rheodyne (Cotati, CA, USA) equipped with a 1 mL or 20 μ L loop. The switching valves used to direct the flow in the multicolumn platform were also from Rheodyne. Detection was achieved with a Model Spectra 100 UV-Vis variable wavelength detector from Thermo Separation Products (Waltham, MA, USA). An eDAQ PowerChrom System ER280 (Denistone East, Australia) was used to process the detector signal and archive data using a personal computer. The Spectra/Chrom CF-1 fraction collector was from spectrum chromatography (Houston, TX, USA). All mass spectra were obtained using LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

The fucosylated glycoproteins from the equalized and untreated sera were enriched and fractionated by the following order of tandem columns, LTA (3 cm $\times$ 4.6 mm ID) $\rightarrow$ AAL (3 cm $\times$ 4.6 mm ID) $\rightarrow$ RPC column (5 cm $\times$ 4.6 mm ID), respectively, as shown in the chromatographic set up in Fig. S1 in the Supporting information. Acetonitrile was used as a storage solution for the RPC column. Prior to each daily analysis, the storage solution was removed by running a binary gradient from 100% mobile phase A to 100% mobile phase B at a flow rate of 1 mL/min for 8 min, then continued pumping mobile phase B for 8 min. Thereafter, the RPC column was equilibrated by returning to 100% mobile phase A in 1 min, and continued running 100% mobile phase A at a flow rate of 1 mL/min for 20 min, where the mobile phase A (aqueous rich mobile phase) consisted of H₂O/ACN (95:5 v/v) containing 0.1% TFA, while, the mobile phase B (eluting mobile phase) consisted of ACN/H₂O (95:5 v/v) containing 0.1% TFA. The binding mobile phase for the lectin columns consisted of 20 mM Tris containing 0.3 M NaCl, pH 7.4, while the eluting mobile phase consisted of 5 mM fucose in 20 mM Tris, pH 7.4.

The 5.6 mL of equalized serum (disease-free or cancer serum) was injected into the lectin columns by injecting 0.8 to 1.0 mL each time, followed by washing with the binding mobile phase for 15 min at a flow rate of 1 mL/min, and then re-injected until the rest of the equalized serum was finished. Firstly, the proteins from LTA column were transferred to the RPC column by passing the eluting mobile phase for 10 min at a flow rate of 1 mL/min, followed by washing with water for 1 min at the same flow rate. After that, the RPC column was washed with mobile phase A at a flow rate of 1 mL/min to remove the salts for 20 min. A linear gradient was applied through the RPC column by increasing ACN concentration in the mobile phase. The linear gradient was started by increasing the % of the mobile phase B from 0% to 75% (v/v) in 12 min. Then continued passing 75% mobile phase B for 2 min, and returning to the initial condition (0% mobile phase B or 100% mobile phase A) in 1 min. The proteins from the RPC column were collected every 30 s and evaporated to dryness in a SpeedVac from Savant Instruments, Inc. (Holbrook, NY, USA) and stored at -20°C until further use. Prior to running the next gradient, the RPC column was washed with 100% mobile phase A for 20 min. Thereafter, the accumulated proteins from the AAL

column were eluted to the RPC column and the elution and fractionation of the RPC column was performed using the same procedure of the LTA column. In the case of untreated serum, 40 μ L of 3-fold diluted disease-free serum or HCC serum were injected into the lectin columns by injecting 20 μ L of disease-free serum or HCC serum each time, followed by washing with the binding mobile phase for 15 min at a flow rate of 1 mL/min, and then re-injected the remaining 20 μ L of disease-free serum or HCC serum. The accumulated proteins from both lectin columns were eluted to the RPC column and the elution and fractionation of proteins from the RPC column was performed by the same procedure as that of the equalized serum.

2.2.4 Other methods—The digestion of protein fractions by trypsin, the LC-MS/MS methodology and the LC-MS/MS data analysis that were used in this work are given in the online Supplementary materials. Since the identification of proteins was based on the LC-MS/MS analysis of the peptides obtained from the tryptic digest of the proteins, the removal of glycans (i.e., the removal of carbohydrates from the glycoproteins) was not necessary prior to LC-MS/MS.

3. Results and Discussion

3.1 Protein capturing, fractionation and identification by LC-MS/MS

Figures 1 and 2 show the RPC fractionation of serum proteins captured by LAC using immobilized LTA and AAL columns, respectively. The online capturing and fractionating of the proteins from serum were achieved *via* the platform shown in Fig. S1 in Supporting information. The identified proteins by LC-MS/MS are listed in Tables S1, S2, S3 and S4 (see Supporting information). It should be noted that only proteins with protein and peptide identification probability not less than 99% and 95%, respectively, and also containing at least two unique peptides were considered. As can be seen in Table S1, the number of identified proteins in the RPC fractions that was captured by the LTA column from serum that was treated by CPLL beads (i.e., ProteoMiner™ treated serum) was found to be 140 and 143 in the disease-free and cancer sera, respectively. In contrast, 112 proteins from the untreated serum were identified in both disease-free and cancer sera (see Table S2). The RPC chromatograms for the LTA captured proteins from equalized and untreated serum (from disease-free and cancer sera) are shown in Fig. 1a and 1b, respectively. In the case of equalized serum, there were 17 and 20 proteins unique to disease-free and cancer sera, respectively, and 123 proteins common to both sera, while for the untreated serum, 11 proteins were unique to each of the disease-free and cancer sera with 101 proteins common to both sera. There were 37 proteins in equalized serum, which were reported in the range of a few ng to 2 μ g/mL [37]. These proteins are marked as low abundance (la) proteins in Table S1. In contrast, 15 proteins were reported as low abundance proteins for untreated serum (see Table S2). Some of the identified proteins were not listed in the human plasma proteome reference [37] and are marked as not listed (nl) in Tables S1 and S2.

In comparison with equalized serum, untreated serum had less number of unique proteins in both disease-free and cancer sera. In the case of disease-free serum, there were 57 unique proteins in equalized serum, which comprised 23 low abundance proteins whereas 29 unique proteins were found in untreated serum and only 5 proteins were low abundance proteins.

Similar results were found in HCC serum, whereby 59 unique proteins were identified in equalized serum, which comprised 24 low abundance proteins, whereas 28 unique proteins were identified in untreated serum with only 3 low abundance proteins. These results show the larger number of identified proteins in the equalized serum. The SWISSPROT database that provides the *N*- and *O*-glycosylation of proteins and NetNGlyc and NetOGlyc software, which could predict the potential *N*-glycosylation and *O*-glycosylation sites in proteins, respectively, were used to identify the percentage of glycoproteins captured by the lectin columns. The percentages of glycoproteins captured by the LTA column from equalized serum were found to be 85.7% and 87.4% in disease-free and HCC sera, respectively, while the remaining 14.3% and 12.6% were non-glycoproteins in disease-free and HCC sera, respectively. In the case of untreated serum, 86.6% and 88.4% of identified proteins were glycoproteins in disease-free and HCC sera, respectively, whereas the non-glycoproteins were found to be 13.4% and 11.6% in disease-free and HCC sera, respectively.

Using the same approach as in the preceding section, the identified proteins in equalized serum captured by the AAL column were found to be 119 and 123 for disease-free and cancer serum, respectively, while 167 and 146 proteins were identified in the untreated serum from disease-free and cancer sera, respectively. All identified proteins captured by the AAL column from equalized and untreated sera are listed in Table S3 and Table S4 (see Supporting information), respectively. The lower number of identified proteins captured by the AAL column from equalized serum may be due to the fact that most of the equalized proteins in the equalized serum have been captured by the first LTA column. Figures 2a and 2b show the RPC chromatograms for the fractionation of the AAL captured proteins from equalized and untreated serum, respectively. In the case of equalized serum, there were 20 and 24 proteins unique to disease-free and cancer sera, respectively, and 99 proteins common to both sera, while for the untreated serum, 34 and 13 proteins were unique to the disease-free and cancer sera respectively, with 133 proteins common to both sera. The number of identified proteins, which were reported in the literature as low abundance proteins, was found to be 32 and 43 for equalized and untreated sera, respectively (see Table S3 and Table S4). Also, some of the identified proteins were not listed in the reference [37], and these are marked as “nl” in Table S3 and Table S4.

In the AAL captured proteins, a lower number of unique proteins were identified in the equalized serum than the untreated serum for both disease-free and HCC sera. For the disease-free serum, there were 28 unique proteins in equalized serum, which comprised 9 low abundance proteins, whereas 76 unique proteins were found in untreated serum including 27 low abundance proteins. Similarly to disease-free serum, the 40 unique proteins found in equalized HCC serum comprised 13 low abundance proteins, while 63 unique proteins found in untreated HCC serum included 14 low abundance proteins. The lower number of unique proteins that was found in the equalized serum than the untreated serum may be due to the fact that most of the equalized proteins have been captured by the first LTA column. Therefore, there were a few remaining proteins that were captured by the AAL column. Although a lower number of unique AAL captured proteins were identified in the equalized serum than in the untreated serum for both disease-free and cancer serum, the

total number of the low abundance proteins from both LTA and AAL columns was larger in equalized serum (see Table 1) than in the untreated serum (see Table 2).

From the SWISSPROT database and using NetNGlyc and NetOGlyc software, the percentages of glycoproteins captured by the AAL column from the equalized disease-free and HCC sera were found to be 86.6% and 84.6%, respectively, while the remaining 13.4% and 15.4% were non-glycoproteins, respectively. In the case of untreated serum, 81.4% and 84.2% of identified proteins were glycoproteins in disease-free and HCC sera, respectively, whereas the non-glycoproteins were found to be 18.6% and 15.8% in disease-free and HCC sera, respectively.

3.2 Differentially expressed proteins in HCC serum

Figures 1 and 2 show the RPC chromatograms of the proteins captured by LTA and AAL columns, respectively, from equalized or untreated disease-free and HCC sera. As shown in these figures, a clear difference can be seen between the two protein RPC profiles captured by the LTA and AAL columns from equalized disease free and cancer sera (see Fig. 1a and 2a), as well as from untreated sera (see Fig. 1b and 2b). For both equalized and untreated sera, the DEPs in the HCC serum relative to the disease-free serum were considered if they could be established for at least 99.9% protein identification probability, 95% peptide identification probability, and contained at least 5 unique peptides [26, 27]. The DEPs were identified using the Q-Q scatterplot which plots the normalized spectral count for each protein found in the HCC serum versus the normalized spectral count of the same protein found in disease-free serum. The DEPs were considered from the proteins that were more than two standard deviations away from being the same in both categories, and all these proteins had a p-value < 0.05 using the t-test. As reported earlier by Selvaraju and El Rassi, the Q-Q scatterplots proved very reliable in the identification of DEPs [26, 27]. Some selected Q-Q scatterplots for the RPC fractions originating from both LTA and AAL lectin columns for equalized and untreated serum are provided in Supporting information (See Figs. S2, S3, S3 and S4).

From the Q-Q scatterplots, 53 DEPs were found to be either up or down regulated in the LTA captured proteins from the equalized serum (see Table 3). In these 53 DEPs, 10 proteins were previously reported to be low abundance proteins [37] including 8 glycoproteins and only 2 non-glycoproteins (78 kDa glucose-regulated protein and protein disulfide-isomerase). The low abundance protein thrombospondin-1, vitronectin, von Willebrand factor and the borderline low abundance protein coagulation factor XIII B chain have been reported to be potential cancer biomarkers [37]. To illustrate, the Q-Q plots for the RPC fractions #1, #2 and #5 of the LTA captured proteins from the equalized serum revealed 11, 15 and 13 DEPs (including one redundant DEP), respectively. This is well reflected in the intensity increment of the peaks corresponding to fractions #1, #2 and #5 shown in Fig. 1a for disease-free serum versus HCC serum. And also, the peak intensity of these three fractions from the disease-free serum is higher than those from the cancer serum (Fig. 1a). 18 and 20 non-redundant proteins were found to be down-regulated and up-regulated in these fractions, respectively. In the case of untreated serum, there were 21 DEPs in the RPC fractions captured by the LTA column (see Table 4). Out of these 21 DEPs, only

the isoform C of fibulin-1 and von Willebrand factor were previously reported to be low abundance proteins [37], and both are glycoproteins. Furthermore, and as stated above, the von Willebrand factor has been reported to be potential cancer biomarkers [37]. To illustrate, the Q-Q plots of the LTA captured proteins from the untreated serum in the RPC fractions #1, #2 and #4 (see Fig. 1b) yielded 4, 10 and 2 DEPs (including one redundant DEP), respectively. This is consistent with the intensity of the peaks for disease-free versus cancer serum corresponding to fractions #1, #2 and #4 shown in Fig. 1b. In these fractions, a total of 7 and 8 non-redundant proteins were found to be up-regulated and down-regulated, respectively. When compared to untreated serum, the equalized serum yielded a larger number of DEPs, which is almost three times higher with 11 DEPs common to both equalized and untreated serum (see Table S5 in Supporting information). Also, a higher number of low abundance proteins (about five times higher) was found in the equalized serum than in the untreated serum (see Tables 1 and 2). This is somewhat reflected in Fig. 1b, which shows small differences in the peak intensity of the RPC collected fractions #1, #2 and #4 for both untreated disease free and cancer sera. In contrast, the difference in peak intensity of RPC collected fractions #1, #2 and #5 for the equalized serum in Fig. 1a is quite noticeable and significant. Thus, by a quick glance of the RPC protein profiles one can locate the fractions that might have DEPs, which would expedite the ensuing LC-MS/MS analysis of proteins, and in turn reduce the number of measurements by the rather lengthy LC-MS/MS process.

In the case of AAL column, 31 DEPs were found to be either up or down regulated in the equalized serum, see Table 3. Out of these 31 DEPs, 7 proteins were reported to be low abundance proteins [37] including 6 glycoproteins (chromogranin-A, vascular cell adhesion protein 1, nucleobindin-1, prothrombin, vitronectin and von Willebrand factor) and only one non-glycoprotein (protein disulfide-isomerase). The low abundance proteins, namely chromogranin-A, vascular cell adhesion protein 1, vitronectin and von Willebrand factor and the borderline abundance protein vitamin K-dependent protein C have been reported to be potential cancer biomarkers [37]. Moreover, the DEPs such as alpha-1-antitrypsin, alpha-2-HS-glycoprotein, ceruloplasmin, complement component C9, galectin-3-binding protein, histidine-rich glycoprotein, prothrombin, vitronectin and von Willebrand factor have been reported as core fucosylated glycoproteins [38]. The Q-Q plots for the RPC fractions #1, #2, #3 and #4 of the AAL captured proteins from the equalized serum (see Fig. 2a) revealed 6, 14, 10 and 9 DEPs (including 8 redundant DEPs), respectively, which are well reflected in the intensity difference of the RPC peaks corresponding to these fractions shown in Fig. 2a for disease-free versus HCC serum. And a total of 21 and 10 non-redundant proteins were found to be up-regulated and down-regulated, respectively, in these fractions. For the untreated serum, there were 23 DEPs in the RPC fractions of the proteins captured by the AAL column (see Table 4). In these 23 DEPs, 6 proteins were reported to be low abundance proteins [37] including 4 glycoproteins (fatty acid-binding protein epidermal, glyceraldehyde-3-phosphate dehydrogenase, mannan-binding lectin serine protease 1 and serpin B3) and two non-glycoproteins (protein S100-A7 and protein S100-A9). The Q-Q plots for the RPC fractions #1 #2, #3 and #4 of the AAL captured proteins from the untreated serum showed 5, 11, 3 and 5 DEPs (including one redundant DEP), respectively. A slightly higher intensity of the RPC peaks for the HCC serum than for the disease-free

serum was observed (see Fig. 2b). In these fractions, a total of 9 and 14 non-redundant proteins were found to be up-regulated and down-regulated, respectively. Similar to the results of the RPC protein fractions captured by the LTA column, the equalized serum yielded a larger number of DEPs than the untreated serum, with only one common DEP to both equalized and untreated sera (see Table S6 in Supporting information).

Overall, in the case of equalized sera, a total of 70 DEPs were found in both LTA and AAL fractions of which 39 and 17 proteins are unique to the LTA and AAL columns, respectively, and 14 DEPs are common to both lectins (see Table 3). In these 70 DEPs, the number of low abundance, borderline abundance, medium abundance, high abundance proteins and proteins that are not reported in the literature [37] were found to be 13, 19, 5, 17 and 16, respectively. For the untreated serum, 40 DEPs were found in both LTA and AAL fractions. Out of these 40 DEPs, there were 17 and 19 unique proteins in the LTA and AAL columns, respectively, and 4 DEPs common to both lectins (see Table 4). In these 40 DEPs, the number of low abundance, borderline abundance, medium abundance and high abundance proteins and proteins that are not reported in the literature [37] were found to be 8, 9, 1, 12 and 10, respectively. These results show that a larger number of total DEPs, low abundance and borderline abundance type DEPs were identified in both LTA and AAL fractions captured from serum that was treated by the CPLL beads.

Thus far, different approaches have been used for the identification of DEPs in complex biological samples of HCC. 2DE, which is a widely used proteomics technology to study proteome as well as DEPs in HCC [9, 10], permitted the isolation of 11 differentially expressed fucosylated proteins in HCC [39–43]. 2D-DIGE, which is a modification of 2DE allowed the identification of 28 differentially expressed fucosylated proteins in HCC samples when compared to normal samples [10–17]. Other label-based quantitative proteomic methods such as ICAT and iTRAQ have been developed for studying DEPs in HCC [18, 19]. Four and thirteen differentially expressed fucosylated proteins in HCC have been reported by ICAT [18] and iTRAQ [19] techniques, respectively. However, these approaches are gel-based and label-based techniques, which suffer from the limitations of gel-based and label-based approaches as discussed in the introduction section.

In this report, the label free and gel free approach has overcome the drawbacks associated with label-based and gel-based approaches and in turn yielded a wider range of fucosylated DEPs than those identified collectively in the earlier studies using gel-based and label-based approaches mentioned above [10–19, 39–43]. In fact, in the current study a total of 70 DEPs were identified in HCC serum with respect to disease free serum. It was found that there were 17 common DEPs and 53 and 24 unique DEPs to the current and earlier studies, respectively (see Table 5). In these 53 DEPs that were unique to the current study, there were 12, 15, 3 and 8 proteins reported previously [37] to be in low abundance range, borderline abundance range, medium abundance range and high abundance range, respectively. It should be noted that 31 of the 53 DEPs were identified as fucosylated or core fucosylated proteins, and 17 DEPs may be fucosylated proteins but their fucosylation have not been reported in the literature. Whereas, out of 24 DEPs that were unique to earlier studies, the low abundance, borderline abundance, medium abundance and high abundance DEPs were found to be 4, 6, 2 and 6, respectively, and all are fucosylated or core

fucosylated proteins. Owing to the larger number of DEPs that were obtained in our current study, and most of these proteins are in the low or borderline abundance range, one can state that the reported methodology here is far superior to the gel- and label-based methods.

Furthermore, the current study, which is an extension to the previous report from our laboratory about the fucome in breast cancer, has proved that the altered fucome in two different cancers differ widely. In fact, when compared to the previous work from our laboratory [27] that involved the capturing of human fucome from disease-free and breast cancer sera using similar multicolumn platform in combination with the off line protein equalization *via* the CPLL approach, a total of 70 DEPs in HCC serum were found in the current study versus 58 DEPs in breast cancer serum were found in the previous study. There were 35 and 23 unique DEPs to the current and previous study, respectively, see Table S7 in Supporting information. A larger number of DEPs was identified in HCC serum than in the breast cancer serum with 35 DEPs common to both studies (see Table S7 in Supporting information).

4. Conclusions

The above results demonstrated that the proposed multicolumn fractionation platform with an off line protein treatment *via* the CPLL approach allowed for efficient capturing/fractionating the human serum fucome thus facilitating the identification of DEPs in HCC serum with respect to disease-free serum *via* LC-MS/MS analysis. In addition, using this gel free and label free approach eliminated all the limitations of gel-based and label-based approaches, and proved effective in facilitating the LC-MS/MS detection of DEPs over a wide range of abundance spanning from low to high abundance proteins. The identified DEPs reported in this research article could be viewed as “candidate biomarkers” for HCC that merit further investigation. This in principle would lead to the discovery of potential HCC biomarkers, which may facilitate the diagnosing of this cancer at an early stage and may contribute to evaluate therapy treatment and guide new drug discovery. Furthermore, it will also be interesting to use the described approach for studying other cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard abbreviations

AAL	<i>Aleuria aurantia</i> lectin
AIBN	2,2'-azobis(isobutyronitrile)
CPLL	combinatorial peptide ligand library

DEPs	differentially expressed proteins
GMM	glyceryl methacrylate
HCC	hepatocellular carcinoma
ICAT	isotope coded affinity tags
iTRAQ	isobaric tags for relative and absolute quantification
LAC	lectin affinity chromatography
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LTA	<i>Lotus tetragonolobus</i> agglutinin
PETA	pentaerythritol triacrylate
RPC	reversed phase chromatography
TFA	trifluoroacetic acid
2D-DIGE	two dimensional differential gel electrophoresis

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at ...

HIGHLIGHTS

- A gel free and label free approach incorporating CPLL and a multicolumn platform was introduced
- The approach allowed the capturing of differentially expressed fucosylated proteins (DEPs) in HCC
- 70 DEPs were identified in HCC serum treated by CPLL.

Fig. 1. RPC chromatograms of the LTA captured proteins from equalized (in panel a) and untreated (in panel b) disease-free and HCC sera. Linear gradient elution from 0% to 75% mobile phase B in mobile A in 12 min; mobile phase A, H₂O/ACN (95:5 v/v) containing 0.1% TFA; mobile phase B, ACN/H₂O (95:5 v/v) containing 0.1% TFA; column, ProSwiftTM RP-1S, 5 cm × 4.6 mm ID; flow rate, 1 mL/min; detection wavelength, 214 nm.

Fig. 2.
RPC chromatograms of the AAL captured proteins from equalized (in panel a) and untreated (in panel b) disease-free and HCC sera. Conditions are the same as Fig. 1

Table 1

Identified low abundance (la) proteins in both LTA and AAL fractions captured from equalized serum treated by CPLL beads.

No.	Low abundance proteins unique to LTA fractions	Low abundance proteins common to both LTA and AAL fractions	Low abundance proteins unique to AAL fractions
1	14-3-3 protein gamma (la)	Complement factor H-related protein 5 (la)	14-3-3 protein zeta/delta (la)
2	45 kDa calcium-binding protein (la)	Extracellular superoxide dismutase [Cu-Zn] (la)	Angiopoietin-related protein 3 (la)
3	78 kDa glucose-regulated protein (, * , la)	Isoform C of Fibulin-1 (la)	Chromogranin-A (la)
4	Actin, cytoplasmic 2 (la)	Keratin, type I cytoskeletal 10 (la)	Dickkopf-related protein 3 (la)
5	Beta-Ala-His dipeptidase (la)	Keratin, type I cytoskeletal 14 (, * , la)	Fetuin-B (la)
6	Biotinidase (la)	Keratin, type II cytoskeletal 5 (la)	Follistatin-related protein 1 (la)
7	Cadherin-5 (la)	Mannan-binding lectin serine protease 1 (la)	Galectin-1 (la)
8	Calreticulin (la)	Nucleobindin-1 (la)	Hepatocyte growth factor activator (la)
9	Endoplasmin (la)	Protein convertase subtilisin/kexin type 9 (la)	Histone H4 (, * , la)
10	Fibulin-5 (la)	Protein disulfide-isomerase (, * , la)	Insulin-like growth factor-binding protein 1 (, * , la)
11	Hepatocyte growth factor-like protein (la)	Prothrombin (la)	Isoform Smooth muscle of Myosin light polypeptide 6 (la)
12	Isoform 2 of C4b-binding protein beta chain (la)	Secreted phosphoprotein 24 (, * , la)	Keratin, type I cuticular Ha3-II (la)
13	Isoform 2 of Mannan-binding lectin serine protease 1 (la)	Selenoprotein P (la)	Keratin, type I cuticular Ha6 (la)
14	Lactotransferrin (la)	SPARC (la)	Keratin, type I cytoskeletal 16 (, * , la)
15	Oncoprotein-induced transcript 3 protein (la)	Vitronectin (la)	Phospholipid transfer protein (la)
16	Phosphatidylcholine-sterol acyltransferase (la)	von Willebrand factor (la)	Vascular cell adhesion protein 1 (la)
17	Procollagen C-endopeptidase enhancer 1 (la)		
18	Proteasome subunit alpha type-5 (la)		
19	Serum paraoxonase/lactonase 3 (la)		
20	SPARC-like protein 1 (la)		
21	Thrombospondin-1 (la)		

* Nonglycoprotein.

The protein concentrations were based on the published or predicted values from the literature [37].

Table 2

Identified low abundance (la) proteins in both LTA and AAL fractions captured from untreated or unequilized serum (i.e., serum not treated by CPLL beads).

No.	Low abundance proteins unique to LTA fractions	Low abundance proteins common to both LTA and AAL fractions	Low abundance proteins unique to AAL fractions
1	Cathepsin D (la)	Beta-Ala-His dipeptidase (la)	14-3-3 protein zeta/delta (la)
2	Hepatocyte growth factor-like protein (la)	Carboxypeptidase N catalytic chain (la)	Beta-2-microglobulin (la)
3	N-acetylglucosamine-1-phosphotransferase subunit gamma (la)	Cartilage oligomeric matrix protein (la)	Calmodulin-like protein 5 (la)
4	Protein Z-dependent protease inhibitor (la)	Fetuin-B (la)	Complement C1r subcomponent-like protein (la)
5		Isoform C of Fibulin-1 (la)	Cystatin-B (*, la)
6		Keratin, type I cytoskeletal 10 (la)	Elongation factor 1-alpha 1 (la)
7		Phosphatidylcholine-sterol acyltransferase (la)	Fatty acid-binding protein, epidermal (la)
8		Prothrombin (la)	Galectin-7 (la)
9		Selenoprotein P (la)	Glyceraldehyde-3-phosphate dehydrogenase (la)
10		Vitronectin (la)	Heat shock protein beta-1 (la)
11		von Willebrand factor (la)	Hepatocyte growth factor activator (la)
12			Histone H4 (*, la)
13			Intercellular adhesion molecule 2 (la)
14			Isoform 2 of Neural cell adhesion molecule L1-like protein (la)
15			Keratin, type I cytoskeletal 14 (*, la)
16			Keratin, type I cytoskeletal 16 (*, la)
17			Keratin, type I cytoskeletal 17 (*, la)
18			Keratin, type II cytoskeletal 5 (la)
19			Lactotransferrin (la)
20			Mannan-binding lectin serine protease 1 (la)
21			Peptidyl-prolyl cis-trans isomerase A (la)
22			Peroxiredoxin-1 (*, la)
23			Protein S100-A11 (la)
24			Protein S100-A7 (*, la)

No.	Low abundance proteins unique to LTA fractions	Low abundance proteins common to both LTA and AAL fractions	Low abundance proteins unique to AAL fractions
25			Protein S100-A8 (*, Ia)
26			Protein S100-A9 (*, Ia)
27			Receptor-type tyrosine-protein phosphatase eta (Ia)
28			Serpin B3 (Ia)
29			Serpin B4 (Ia)
30			Tubulin alpha-4A (Ia)
31			Ubiquitin (*, Ia)
32			Vascular cell adhesion protein 1 (Ia)

* Nonglycoprotein.

The protein concentrations were based on the published or predicted values from the literature [37].

Table 3

DEPs in the LTA and AAL fractions from the equalized serum.

No.	DEPs unique to LTA fractions	DEPs common to both LTA and AAL fractions	DEPs unique to AAL fractions
1	78 kDa glucose-regulated protein (, la)	Alpha-1-antitrypsin (ha, F)	Alpha-1-antitrypsin (ha, CF, F)
2	Afamin (bla, CF, F)	Coagulation factor V (bla, F)	Alpha-2-HS-glycoprotein (ha, CF, F)
3	Alpha-1B-glycoprotein (ha, F)	Complement C1s subcomponent (bla, F)	Apolipoprotein D (Fragment) (, nl)
4	Alpha-2-antiplasmin (bla, F)	Complement C3 (ha, F)	Ceruloplasmin (ha, CF, F)
5	Alpha-2-macroglobulin (ha, CF, F)	Complement component C9 (bla, CF, F)	Chromogranin-A (la)
6	Angiotensinogen (bla, F)	Galectin-3-binding protein (bla, CF, F)	Coagulation factor IX (bla)
7	Antithrombin-III (ha, F)	Histidine-rich glycoprotein (ma, CF, F)	Complement component C8 alpha chain (bla, F)
8	Apolipoprotein B-100 (ha, F)	Inter-alpha-trypsin inhibitor heavy chain H2 (ha, F)	Complement factor H-related protein 4 (, nl)
9	Apolipoprotein D (ma, CF, F)	Inter-alpha-trypsin inhibitor heavy chain H4 (ma, F)	Ig gamma-3 chain C region (nl, F)
10	Apolipoprotein E (bla, F)	Nucleobindin-1 (la)	Isoform 2 of Clusterin (nl)
11	C4b-binding protein alpha chain (ha, F)	Plasminogen (ma, F)	Isoform Short of Latent-transforming growth factor beta-binding protein 1 (nl)
12	Coagulation factor XIII B chain (bla)	Protein disulfide-isomerase (, la)	Prothrombin (la, CF, F)
13	Complement C1r subcomponent (nl, F)	Vitronectin (la, CF, F)	Serum albumin (, ha)
14	Complement C4-A (nl, CF, F)	von Willebrand factor (la, CF)	Serum paraoxonase/arylesterase 1 (nl, F)
15	Complement C5 (nl, F)		Vascular cell adhesion protein 1 (la, F)
16	Complement component C6 (bla, F)		Vimentin (bla)
17	Complement factor H (bla, CF, F)		Vitamin K-dependent protein C (bla, F)
18	Fibrinogen alpha chain (ha, F)		
19	Fibulin-1 (nl, CF)		
20	Ficolin-3 (bla, CF, F)		
21	Haptoglobin (ha, CF)		
22	Hepatocyte growth factor-like protein (la)		
23	Ig lambda-2 chain C regions (nl)		
24	Inter-alpha-trypsin inhibitor heavy chain H1 (ma, CF, F)		
25	Isoform 10 of Fibronectin (nl)		

No.	DEPs unique to LTA fractions	DEPs common to both LTA and AAL fractions	DEPs unique to AAL fractions
26	Isoform 2 of Mannan-binding lectin serine protease 1 (la)		
27	Isoform 3 of Vitamin D-binding protein (nl)		
28	Isoform 5 of Clusterin (nl)		
29	Isoform LMW of Kininogen-1 (nl)		
30	Lumican (bla, F)		
31	Plasma protease C1 inhibitor (ha, CF, F)		
32	Plasma serine protease inhibitor (bla)		
33	Selenoprotein P (la, CF, F)		
34	Serotransferrin (ha, CF)		
35	Serum amyloid P-component (bla, F)		
36	SPARC-like protein 1 (la)		
37	Thrombospondin-1 (la, F)		
38	Transferrin (ha, F)		
39	Vitamin K-dependent protein C heavy chain (nl)		

la, low abundance (few ng/mL to < 2 µg/mL level); **bla**, borderline between low and medium abundance proteins (2 µg/mL to 0.1 mg/mL); **ma**, medium abundance (0.1 mg/mL to 0.2 mg/mL); **ha**, high abundance (0.2 mg/mL to > 1 mg/mL); **nl**, not listed. **CF**, core fucosylated; **F**, fucosylated;

* nonglycoprotein.

The protein concentration based on the published or predicted values from the literature [37]. Data on fucosylation are from Refs [38, 44–46] based on glycan analysis and/or the reactivity of proteins with fucose specific lectins. All other glycoproteins listed in the table may be fucosylated but their fucosylation have not been reported in the literature.

Table 4

DEPs in the LTA and AAL fractions from the untreated serum.

No.	DEPs unique to LTA fractions	DEPs common to both LTA and AAL fractions	DEPs unique to AAL fractions
1	Alpha-2-HS-glycoprotein (ha, CF, F)	Alpha-1-antitrypsin (ha, CF, F)	Actin, cytoplasmic 1 (nl)
2	Apolipoprotein A-IV (ha)	Complement factor H (bla, CF, F)	Antithrombin-III (ha, F)
3	Apolipoprotein B-100 (ha, F)	Haptoglobin (ha, CF)	Carboxypeptidase N subunit 2 (bla)
4	Apolipoprotein D (ma, CF, F)	Isoform LMW of Kininogen-1 (nl)	Coagulation factor XIII B (bla)
5	Apolipoprotein E (bla, F)		Complement C4-B (nl, F)
6	Ceruloplasmin (ha, CF, F)		Complement factor H-related protein 1 (bla, F)
7	Coagulation factor XIII B chain (bla)		Fatty acid-binding protein, epidermal (la)
8	Complement C1r subcomponent (nl, F)		Fibrinogen alpha chain (ha, F)
9	Complement factor H-related protein 4 (*, nl)		Glyceraldehyde-3-phosphate dehydrogenase (la)
10	Fibrinogen beta chain (ha, F)		Hemoglobin subunit alpha (bla, F)
11	Isoform 2 of Vitamin D-binding protein (nl)		Hemoglobin subunit beta (bla, F)
12	Isoform C of Fibulin-1 (la)		Ig gamma-2 chain C region (nl, CF, F)
13	Isoform Gamma-A of Fibrinogen gamma chain (nl)		Isoform 4 of Extracellular matrix protein 1 (nl)
14	Plasma kallikrein heavy chain (Fragment) (nl)		Mannan-binding lectin serine protease 1 (la)
15	Plasma protease C1 inhibitor (ha, CF, F)		Phosphatidylinositol-glycan-specific phospholipase D (bla)
16	Serotransferrin (ha, CF)		Protein S100-A7 (*, la)
17	von Willebrand factor (la, CF)		Protein S100-A9 (*, la)
18			Serpin B3 (la)
19			Serum albumin (*, ha)

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Table 5

DEPs that were unique and common to the current and previous studies.

No.	DEPs unique to the current study (treated with CPLL beads)	DEPs common to the current and previous studies	DEPs unique to previous studies [10–19, 39–43]
1	78 kDa glucose-regulated protein (*, la)	Alpha-1-antitrypsin (ha, CF, F)	Apolipoprotein A-I (ha, F)
2	Afamin (bla, CF, F)	Alpha-1B-glycoprotein (ha, F)	Apolipoprotein A-II (ha, F)
3	Alpha-1-antichymotrypsin (ha, F)	Alpha-2-HS-glycoprotein (ha, CF, F)	Apolipoprotein C-II (bla, F)
4	Alpha-2-antiplasmin (bla, F)	Alpha-2-macroglobulin (ha, CF, F)	Apolipoprotein C-III (ma, F)
5	Apolipoprotein B-100 (ha, F)	Angiotensinogen (bla, F)	Apolipoprotein L-I (bla, F)
6	Apolipoprotein D (Fragment) (*, nl)	Antithrombin-III (ha, F)	Calumenin (unk, CF)
7	C4b-binding protein alpha chain (ha, F)	Apolipoprotein D (ma, CF, F)	Clusterin: complement-associated protein SP-40 (ma, F)
8	Chromogranin-A (la)	Apolipoprotein E (bla, F)	Collagen alpha 1/ Collagen alpha 1 (I) chain (la, F)
9	Coagulation factor IX (bla)	Ceruloplasmin (ha, CF, F)	Complement factor B (nl, F)
10	Coagulation factor V (bla, F)	Complement component C9 (bla, CF, F)	Complement factor H-related protein 1, FHR-1 (bla, F)
11	Coagulation factor XIII B chain (bla)	Haptoglobin (ha, CF)	Fibrinogen beta chain (ha, F)
12	Complement C1r subcomponent (nl, F)	Plasma protease C1 inhibitor (ha, CF, F)	Fibrinogen gamma chain (PRO2061) (ha, CF, F)
13	Complement C1s subcomponent (bla, F)	Plasminogen (ma, F)	Fibronectin (unk, CF, F)
14	Complement C3 (ha, F)	Serotransferrin (ha, CF)	Hemoglobin subunit beta (bla, F)
15	Complement C4-A (nl, CF, F)	Serum amyloid P-component (bla, F)	Hemopexin (ha, CF, F)
16	Complement C5 (nl, F)	Serum paraoxonase/arylesterase 1 (nl, F)	Ig alpha-1 chain C region (nl, CF, F)
17	Complement component C6 (bla, F)	Vitronectin (la, CF, F)	Ig gamma-1 chain C region (nl, CF, F)
18	Complement component C8 alpha chain (bla, F)		Leucine-rich alpha-2-glycoprotein (bla, CF, F)
19	Complement factor H (bla, CF, F)		Mannose-binding protein C (la, F)
20	Complement factor H-related protein 4 (*, nl)		Proteasome subunit beta type 4 (Proteasome beta chain) (la, CF)
21	Fibrinogen alpha chain (ha, F)		Thioedoxin (la, F)
22	Fibulin-1 (nl, CF)		Tropomyosin beta chain (unk, F)
23	Ficolin-3 (bla, CF, F)		Vitamin D-binding protein (ha, F)
24	Galectin-3-binding protein (bla, CF, F)		Zinc-alpha-2-glycoprotein (bla, CF, F)
25	Hepatocyte growth factor-like protein (la)		

No.	DEPs unique to the current study (treated with CPLL beads)	DEPs common to the current and previous studies	DEPs unique to previous studies [10–19, 39–43]
26	Histidine-rich glycoprotein (ma, CF, F)		
27	Ig gamma-3 chain C region (nl, F)		
28	Ig lambda-2 chain C regions (nl)		
29	Inter-alpha-trypsin inhibitor heavy chain H1 (ma, CF, F)		
30	Inter-alpha-trypsin inhibitor heavy chain H2 (ha, F)		
31	Inter-alpha-trypsin inhibitor heavy chain H4 (ma, F)		
32	Isoform 10 of Fibronectin (nl)		
33	Isoform 2 of Clusterin (nl)		
34	Isoform 2 of Mannan-binding lectin serine protease 1 (la)		
35	Isoform 3 of Vitamin D-binding protein (nl)		
36	Isoform 5 of Clusterin (nl)		
37	Isoform LMW of Kininogen-1 (nl)		
38	Isoform Short of Latent-transforming growth factor beta-binding protein 1 (nl)		
39	Lumican (bla, F)		
40	Nucleobindin-1 (la)		
41	Plasma serine protease inhibitor (bla)		
42	Protein disulfide-isomerase (* , la)		
43	Prothrombin (la, CF, F)		
44	Selenoprotein P (la, CF, F)		
45	Serum albumin (* , ha)		
46	SPARC-like protein 1 (la)		
47	Thrombospondin-1 (la, F)		
48	Transferrin (ha, F)		
49	Vascular cell adhesion protein 1 (la, F)		
50	Vimentin (bla)		
51	Vitamin K-dependent protein C (bla, F)		
52	Vitamin K-dependent protein C heavy chain (nl)		
53	von Willebrand factor (la, CF)		

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