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Proteome Mining of Human Follicular Fluid Reveals a Crucial Role of Complement Cascade and Key Biological Pathways in Women Undergoing *in Vitro* Fertilization

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In vitro fertilization (IVF) is fraught with problems and currently proteomics approaches are being tried out to examine the microenvironment of the follicle in order to assess biological and immunological parameters that may affect its development. Additionally, better understanding of reproductive process may help increase IVF birth rate per embryo transfer and at the same time avoid spontaneous miscarriages or life threatening conditions such as ovarian hyperstimulation syndrome. The primary aim of this study was to search for specific differences in protein composition of human follicular fluid (HFF) and plasma in order to identify proteins that accumulate or are absent in HFF. Depletion of abundant proteins combined with multidimensional protein fractionation allowed the study of middle- and lower-abundance proteins. Paired comparison study examining HFF with plasma/serum from women undergoing successful IVF revealed important differences in the protein composition which may improve our knowledge of the follicular microenvironment and its biological role. This study showed involvement of innate immune function of complement cascade in HFF. Complement inhibition and the presence of C-terminal fragment of perlecan suggested possible links to angiogenesis which is a vital process in folliculogenesis and placental development. Differences in proteins associated with blood coagulation were also found in the follicular milieu. Several specific proteins were observed, many of which have not yet been associated with follicle/oocyte maturation. These proteins together with their regulatory pathways may play a vital role in the reproductive process.

Keywords: Human follicular fluid • plasma • assisted reproduction • IVF • proteomics • biomarkers • complement cascade • angiogenesis • blood coagulation

Introduction

Application of powerful proteomic technologies in reproductive medical research may significantly contribute to the comprehensive understanding of reproductive processes. Additionally, it may lead to the discovery and selection of specific biomarkers with diagnostic and prognostic values for a wide range of fertility problems and pregnancy related complications.[1]

Assisted reproduction refers to a number of advanced techniques that aid fertilization. Among these, the most used is *in vitro* fertilization (IVF) followed by embryo implantation into the woman's uterus. IVF was originally developed to help women with damaged or absent fallopian tubes which prevented the sperm from meeting the egg. It is now used to treat various fertility issues, and its effectiveness has improved in the past few years, but the chance of pregnancy is still only around 40%.[2] Controlled ovarian hyperstimulation is a key factor in the success of IVF. In the course of ovarian stimulation, the competent follicles start to grow, granulosa cells begin to divide, follicular basal lamina expands and the antrum fills up with follicular fluid (FF). FF provides a special microenvironment containing regulatory molecules which are important for the maturation of oocytes. The composition of FF results from the combination of secretions from the granulosa and thecal cells with minor contribution from the oocytes as well as from the transfer of blood plasma constituents that cross blood follicular barrier via theca capillaries.[3] FF is the resultant byproduct during aspiration of oocytes from mature ovarian follicle, and hence, it has been utilized in various studies focused on oocyte quality, fertilization success or pregnancy complications.

In 1993, Spitzer et al. for the first time used 2-DE to compare complex protein patterns of FF from mature and immature

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human follicles.[4] Since then, improved and advanced proteomic approaches have been applied to further reproductive research. The study performed by Anahory et al. aimed at profiling of human follicular fluid (HFF) of women undergoing IVF resulted in identification of three new proteins (thioredoxin peroxidase 1, transthyretin and retinol-binding protein) present in HFF.[5] In a similarly designed study, Lee et al. identified four other proteins, named hormone sensitive lipase, unnamed protein product 1, unnamed protein product 2, and apolipoprotein A-IV that were not yet reported in HFF.[6] Direct mass spectrometry (MS) based technique SELDI-TOF was applied by Schweigert et al. to compare peptide and protein profiles in serum and HFF of women undergoing IVF.[7] Among 186 individual MS signals, four were identified as haptoglobin alpha1- and alpha 2-chains, haptoglobin 1 and transthyretin. Specific peptide patterns were also analyzed by Liu et al. who reported peptide peaks that correlated with different developmental stages. Two proteins, apolipoprotein A-I and collagen type IV were verified using Western blot analysis.[8] Taking together such findings from these proteomic studies, it was evident that only limited information had been uncovered and that not all components of HFF had been identified. More importantly, their physiological roles in reproduction or pathologies in case of infertility remained unknown. In 2006, Angelucci et al. performed an in-depth study to determine the composition of HFF from women undergoing IVF for male associated infertility and to compare it with the plasma samples of the same women. Many proteins were identified in relatively high levels in HFF including mainly acute phase proteins and several proteins with antioxidant functionalities.[9] Subsequent studies utilized HFF of women with recurrent spontaneous abortion[10] or women that were less than 32 years old and failed to become pregnant after IVF.[11] The results indicated that coagulation factors may play an important role in response to IVF and maintaining normal pregnancy. More recently, Hanrieder et al. utilized combination of isoelectric focusing and reversed phase nanochromatography coupled with nano-LC MALDI TOF/TOF to analyze HFF samples of women undergoing IVF. This study revealed significantly increased numbers of proteins that had not been previously reported in HFF using proteomic techniques.[12] A majority of all identified proteins were plasma matched proteins mostly represented by acute phase proteins but some low copy proteins including sex hormone binding globulin and inhibin A were also found.

The primary aim of this study was to search for specific differences in protein composition of HFF and plasma in order to identify proteins that accumulate or are absent in HFF. These proteins may hold a key to the reproductive process and further evaluation may identify them as potential biomarkers of follicle/oocyte quality and successful IVF during assisted reproduction. To achieve this goal, we carried out for the first time the depletion of the 12 most abundant plasma/HFF matched proteins in paired samples of HFF and plasma obtained from women undergoing successful IVF. Following removal of abundant proteins, the samples were analyzed using combination of 2-D gel-based and 2-D liquid chromatography protein fractionation techniques. The protein identity of evaluated alterations in protein patterns was determined using MS and proteomic data were verified by specific immunoblot or biochemical assay (Figure 1). A majority of protein changes were found to belong to complement cascade and its regulatory proteins. Some other specific biological processes such as acute phase response, transport, lipid metabolism and blood coagulation were involved to a lesser extent.

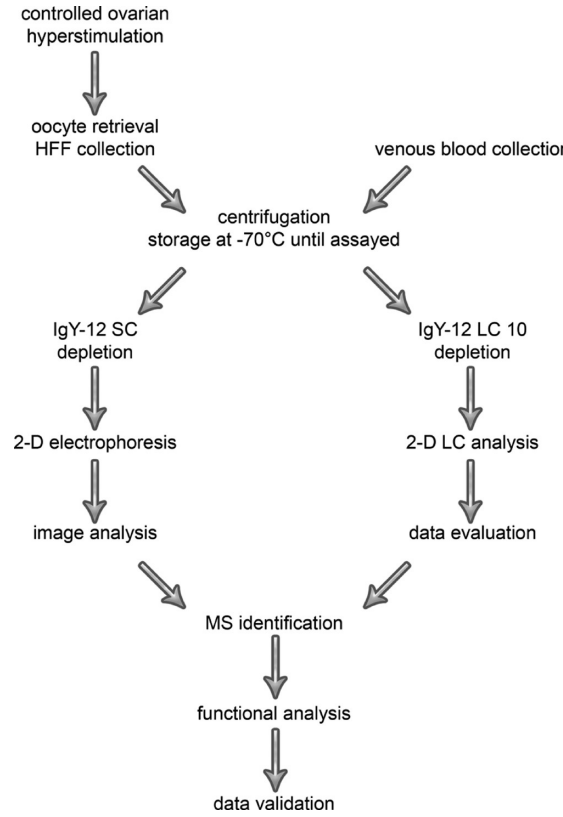

Figure 1. Schematic presentation of the IgY-12, 2-DE and 2-D HPLC workflows. The paired samples of follicular fluid and blood were retrieved from women undergoing controlled ovarian stimulation. The samples were depleted of the 12 most abundant plasma/HFF matched proteins and processed for 2-DE and 2-D HPLC analyses as indicated in the workflows. Subsequently, proteomic data were verified by specific immunoblot or biochemical assay.

Materials and Methods

Chemicals. IgY-12 High Capacity SC Spin Column kit, IgY-12 High Capacity LC10 Proteome Partitioning kit and ProteomeLab PF 2D kit (includes chromatofocusing column, high-resolution reverse-phase column, start buffer and eluent buffer) were purchased from Beckman Coulter (Fullerton, CA). Amicon Ultra-15 Centrifugal Filter Device was from Millipore (Millipore Bedford, MA). Acrylamide, bis-acrylamide, urea, Tris-base, thiourea, SDS, bromophenol blue, amonium persulfate (APS), TEMED, *n*-octyl glucoside, Tris, (2-carboxyethyl) phosphine hydrochloride (TCEP), and iminodiacetic acid were obtained from Sigma-Aldrich (St. Louis, MO). Nonidet-40, 3-3(cholamidolpropyl)-dimethylammonio-1-propane sulfonate (CHAPS) and DTT were from USB Corporation (Cleveland, OH). Glycerol and β-glycerolphosphate were purchased from Penta (Prague, Czech Republic). Protease inhibitors cocktail were obtained from Roche (Mannheim, Germany). Immobiline DryStrip (18 cm, 3−10 NL), Ampholine pH 3−10, and PD-10 desalting columns were from GE Healthcare (Uppsala, Sweden). Silver Quest staining kit was purchased from Invitrogen (Carlsbad, CA). All other chemicals for protein fractionations were of HPLC or analytical grade and buffers were prepared using Mili-Q

water system (Millipore Bedford, MA). Unless otherwise specified, all chemicals used for MS were from Sigma (Steinheim, Germany).

Follicular Fluid and Plasma. Women undergoing stimulation for IVF were recruited for the study at the Centre of Assisted Reproduction, Department of Obstetric and Gynecology, General Teaching Hospital in Prague. In total, 38 women with the Body Mass Index ranged from 19.8 to 29.3 and age between 24 and 38 years were involved in the study. Paired HFF and plasma samples obtained from 12 women were used for proteomic analyses including Western blot (4 paired samples for 2-DE, 2 paired samples for 2-D HPLC and 8 paired samples for Western blot consisting of 2 samples used for 2-D analyses and 6 additional samples). In addition, paired samples of 29 women (including 3 paired samples from above-mentioned set) were analyzed for complement activity and concentrations of complement C3 and C4 components. All female patients gave their informed consent prior to sample collection. All samples used in this study were derived from the women with successful (100% rate) IVF. Patients suffering from the severe form of ovarian hyperstimulation syndrome (OHSS)[13] resultant from ovarian stimulation were excluded from the study.

To achieve stimulation, standard treatment protocol was applied including controlled ovarian follicle-stimulating hormone (FSH) hyperstimulation using gonadotropin-releasing hormone (GnRH) short antagonists and GnRH long agonists with human chorionic gonadotropin (hCG) administration to induce follicular/egg maturation. Oocyte transvaginal ultrasound retrieval was performed 36 h after hCG administration according to the strict procedure approved by Assisted Reproduction Centre. Each follicular fluid sample was obtained from puncture of dominant ovarian follicles (in diameter from 14 to 22 mm). Only macroscopically clear fluids, indicating lack of contamination, were considered in the study. After oocyte isolation, HFF was centrifuged to remove cellular components and debris and then transferred to sterile polypropylene tubes and frozen at −70 °C until further analysis.[14] In parallel, patient paired samples of venous blood (5 mL) were taken on the day of oocyte retrieval, collected in sterile plastic tubes containing EDTA as anticoagulant, cleared by centrifugation, and the resulting plasma samples were frozen at −20 °C and kept at −70 °C until assayed. Alternatively, for complement components C3, C4 and complement activity analyses, samples of blood were allowed to clot, cleared by centrifugation and the resulting sera were frozen at −20 °C and kept at −70 °C until assayed.

Depletion of Major Abundant Proteins and Sample Preparation. Protein concentrations in samples of HFF or plasma were determined using BCA Protein Assay kit (Thermo Scientific, Rockford, IL). Depletion of the 12 most abundant proteins (albumin, IgG, transferin, fibrinogen, IgA, α2-macroglobulin, IgM, α1-antitrypsin, haptoglobin, α1-acidic glycoprotein and apolipoproteins A-I a A-II) in plasma or HFF was carried out using multiple affinity ProteomeLab IgY-12 columns (Beckman Coulter, Fullerton, CA) as per manufacturer's instructions with a few modifications. Briefly, for follow-up 2-DE analysis, IgY-12 SC Spin Column with binding capacity of 10 μL of plasma was used. The 10 μL aliquots of original HFF samples and plasma were diluted in 490 μL of dilution buffer containing 0.15 M NaCl in 10 mM Tris-HCl, pH 7.4, and protease plus phosphatase inhibitors were added. The diluted sample was loaded onto affinity column and flow-through fraction was collected after 30 min by centrifugation. For each sample of HFF and plasma, the number of IgY-12 depletion cycles was adjusted according to sample protein concentration. With average concentrations of 50 and 75 mg/mL, 3 and 2 depletion cycles were obviously needed for HFF and plasma, respectively. The proteins in pools of flow-through fractions for each sample were precipitated by addition of 0.15% sodium deoxycholate for 10 min and 72% trichloroacetic acid (TCA) for 30 min (both in 1/10 of total volume). After washing with ice-cold acetone, pellets were resolubilized in 150 μL of the sample buffer containing 9 M urea, 3% (w/v) CHAPS, 2% (v/v) Nonidet 40, 70 mM DTT, pH 3−10 ampholytes (0.5% w/v), 10 mM beta-glycerol phosphate, 5 mM sodium fluoride, 0.1 mM sodium orthovanadate, and protease inhibitors.

For follow-up 2-D HPLC PF 2D analysis, we used pool of flow-through fractions collected from 6 and 4 cycles of ProteomeLab IgY-12 LC 10 column (binding capacity 250 μL) for HFF and plasma, respectively. For every cycle, an aliquot of sample containing 20 mg of proteins was diluted to final volume of 625 μL using dilution buffer containing 0.15 M NaCl in 10 mM Tris-HCl, pH 7.4. The diluted sample was cleaned using 0.45 μm membrane spin filters and loaded onto IgY-12 LC 10 column. Standard liquid chromatography protocol provided by manufacturer was carried out. Flow-through fractions of the same sample were pooled, concentrated using Amicon Ultra-15 centrifugal filter devices to 0.5 mL and diluted in denaturating buffer containing 7.5 M urea, 2.5 M thiourea, 12.5% glycerol, 62.5 mM Tris-HCl, 2.5% *n*-octylglucoside, and 1.25 mM EDTA to final volume of 2.5 mL.

Two-Dimensional Gel Electrophoresis and Image Analysis. Aliquots of samples of depleted HFF or plasma corresponding to 100 μg of protein were loaded in the first-dimension isoelectric focusing separation using active in gel rehydration of Immobiline DryStrips (IPG strip 18 cm 3−10 NL) in rehydration buffer containing 5 M urea, 2 M thiourea, 2% CHAPS, 2 mM TCEP, 40 mM Tris-base, and 0.003% bromophenol blue. Isoelectric focusing (IEF) was performed on IEF Cell (Bio-Rad, Hercules, CA) system using the following program: 1 h to 200 V, 1 h to 500 V, 1 h to 1000 V, 1 h to 3000 V, 1 h to 5000 V, and 5000 V until total of 55 kVh was reached. After IEF separation, the gel strips were equilibrated in 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 4% SDS, and 65 mM DTT for 15 min and then in the same buffer, except that the DTT was replaced by 4% iodacetamide and bromophenol blue, for 15 min. After equilibration, IPG strips were rinsed and applied to vertical 12%T acrylamide SDS-PAGE (18 × 18 × 1 mm gel). SDS-PAGE was carried out at a constant current of 40 mA per gel using two in series connected Protean II xi Cells (Bio-Rad, Hercules, CA) allowing simultaneous run of four gels. Gels were then stained with mass spectrometry compatible silver staining SilverQuest kit (Invitrogen, Carlsbad, CA). Stained gels were scanned and digitized at 400 dpi resolution using a GS800 scanner (Bio-Rad, Hercules, CA).

The images were evaluated using PDQuest version 7.1 software (Bio-Rad, Hercules, CA). 2-DE gels of four patient-paired samples of follicular fluids and plasma were included in the analysis. After automatic spot detection and matching, manual editing was performed and the results were in good agreement with those of the visual inspection. The relative abundance of each resolved protein spot was then quantified by fitting Gaussian curves in the X and Y dimension and performing additional modeling to create the final Gaussian spot and express a ppm value. Data were normalized, that is,

expressed as percentages of all valid spots, to account for any differences in protein loading and gel staining. Normalized data were analyzed using statistical procedures available within the PDQuest version 7.1 package which provides the table to determine minimum/maximal number of gels/samples per class of HFF or plasma to control procedure. The protein spots that were statistically significant with $P < 0.05$ according to Student's t tests were selected for identification by mass spectrometry.

Two-Dimensional HPLC ProteomeLab PF 2D Chromatography and Image Analysis. Samples of depleted HFF or plasma in denaturating buffer were loaded on PD10 column equilibrated with 25 mL of the start buffer to exchange denaturing lysis buffer to the start buffer. The protein concentration in the sample collected from PD10 column was determined by direct measurement of absorbance at 280 nm (DU 7400 spectrophotometer, Beckman, Fullerton, CA). For the first-dimension chromatofocusing fractionation (HPCF) two buffers, a start buffer pH 8.5 and an elution buffer pH 4.0 both provided in the PF 2D kit were used to generate an internal linear pH gradient on the column. The HPCF column was equilibrated with 30 column volumes of start buffer and depleted sample of 2 mg of total protein was applied on HPCF column using 5 mL injection loop. The separation was performed at flow rate of 0.2 mL/min. Once the pH in the column achieved a stable pH at 8.5 (30 min), the linear gradient of elution buffer to pH of 4 was switched. The proteins remaining on the column at pH 4 were washed out by 1 M NaCl in 30% *n*-propanol solution. UV detection was performed at 280 nm and the pH of the effluent was monitored using a flow-through online pH probe. Fraction collection started when gradient reached pH 8.3 and individual fractions were collected in 0.3 pH intervals or with maximum time 8.5 min when the pH did not change. In every run, pH was monitored for 150 min, and UV was monitored for 220 min. The percentage of protein recovery from the column within pH gradient 8.5−4 was about 45%, while the remaining part of the loaded proteins was collected in either basic (flow-through) or acidic (wash out) fractions. In total, 35 fractions were collected during HPCF separation including the basic as well as acidic proteins out of the pH gradient. The pI fractions containing any proteins detected at 280 nm were further separated on reversed phase column packed with nonporous silica beads (HPRP). Solvent A was 0.1% trifluoroacetic acid (TFA) in water and solvent B was 0.08% TFA in acetonitrile (MeCN). The separation was done at 50 °C at a flow rate 0.75 mL/min. The gradient was run from 0% to 100% B in 30 min, followed by 100% B for 4 min and 100% A for 10 min. UV absorptions were monitored at 214 nm. The fractions were collected in 0.25 min time intervals into 96-deep well plates using the fraction collector Gilson FC204 (Immunotech a.s., Prague, Czech Republic) and stored at −80 °C until further use.

2-D protein expression maps of HFF and plasma displaying protein isoelectric point versus protein hydrophobicity were generated by ProteoVue software running on PF 2D system. ProteoVue software converts the UV peak intensity in the chromatograms from the second-dimension HPRP column of each p*I* fraction to a band and line format and provides the mean to view and quantify protein levels. The Viper software version 2.2.0 (Ludesi, Sweden) was used for PF 2D data evaluation of four patient pairs of HFF and plasma. The profiles of the second dimension were matched and quantitative data were analyzed using analysis of variance (ANOVA) statistical test implemented in Viper software. Only statistically significant (p-value < 0.05) protein peaks with reproducible profile were selected for mass spectrometric identification.

Enzymatic In-Gel Digestion. Silver nitrate stained protein spots were excised from the gel, cut into small pieces and washed with freshly prepared solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate (mixed in 1:1 ratio). After complete destaining, the gel was washed with water, shrunk by dehydration in MeCN and reswelled again in water. The supernatant was removed and the gel was partly dried in a SpeedVac concentrator. The gel pieces were then rehydrated in a cleavage buffer containing 25 mM 4-ethylmorpholine acetate, 5% MeCN and trypsin (5 ng/μL; Promega, Madison, WI), and incubated overnight at 37 °C. The digestion was stopped by addition of 5% TFA in MeCN and the aliquot of the resulting peptide mixture was desalted using a GELoader microcolumn (Eppendorf, Hamburg, Germany) packed with a Poros Oligo R3 material.[15] The purified and concentrated peptides were eluted from the microcolumn in several droplets directly onto MALDI plate using 1 μL of α-cyano-4-hydroxycinnamic acid (CCA) matrix solution (5 mg/mL in 50% MeCN/0.1% TFA).

The fractions from 2-D HPLC were dried completely using the SpeedVac concentrator and dissolved in 50 μL of the above-mentioned cleavage buffer. The digestion and desalting was performed as described for 2-DE protein spots.

MALDI Mass Spectrometry. Mass spectra were measured on an Ultraflex III MALDI-TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a smartbeam solid state laser and LIFT technology for MS/MS analysis. PMF spectra were acquired in the mass range of 700−4000 Da and calibrated internally using the monoisotopic $[M + H]^+$ ions of trypsin autoproteolytic fragments (842.5 and 2211.1 Da).

Protein Identification. For PMF database searching, peak lists in XML data format were created using flexAnalysis 3.0 program with SNAP peak detection algorithm. No smoothing was applied and maximal number of assigned peaks was set to 50. After peak labeling, all known contaminant signals were removed. The peak lists were searched using in-house MASCOT search engine against Swiss-Prot 57.0 database subset of human proteins with the following search settings: peptide tolerance of 30 ppm, missed cleavage site value set to two, variable carbamidomethylation of cysteine, oxidation of methionine and protein N-terminal acetylation. No restrictions on protein molecular weight and p*I* value were applied. Proteins with MOWSE score over the threshold 56 calculated for the used settings were considered as identified. If the score was lower or only slightly higher than the threshold value, the identity of protein candidate was confirmed by MS/MS analysis. In addition to the above-mentioned MASCOT settings, fragment mass tolerance of 0.6 Da and instrument type MALDI-TOF-TOF was applied for MS/MS spectra searching.

Immunoblot and Quantitative Analysis. Aliquots of the total nondepleted protein extracts of HFF and blood plasma (15 μg) were separated in 12% SDS-PAGE gels using Protean II xi Cell (Bio-Rad). Proteins were then transferred to Immobilon P (Millipore, Bedford, MA) membranes using a semidry blotting system (Biometra, Göttingen, Germany) and transfer buffer containing 48 mM Tris, 39 mM glycine and 20% methanol. The membranes were blocked for 1 h with 5% skimmed milk in Tris-buffered saline with 0.05% Tween 20 (TBST pH 7.4) and incubated overnight with primary antibodies raised against complement factor H (Santa Cruz Biot., CA, sc-59174; 1:500); clusterin (Abcam Inc., Cambridge, MA, ab16077; 1:2000) and

perlecan (C-terminus; Santa Cruz Biot., CA, sc-25848; 1:2000). Peroxidase-conjugated secondary anti-mouse or anti-rabbit IgG antibodies (Jackson Immunoresearch, Suffolk, U.K.), as appropriate, were diluted 1:10 000 in 5% skimmed milk in TBST, and the ECL+ chemiluminiscence (GE Healthcare, Uppsala, Sweden) detection system was used to detect specific proteins. The exposed CL-XPosure films (Thermo Scientific, Rockford, IL) were scanned by a calibrated densitometer GS-800 (Bio-Rad, Hercules, CA). The proteins bands of each sample were quantified as Trace Quantity (the quantity of a band as measured by the area under its intensity profile curve, units are intensity × mm) using Quantity One software (Bio-Rad, Hercules, CA).

Further immunoanalysis of clusterin and perlecan was carried out by separating nondepleted lysates of HFF samples containing 150 and 100 μg of protein, respectively, in 2-DE gels. Immobiline DryStrips 3−10 NL 13 cm were used to analyze microheterogeneity of clusterin and IPG 4−7 were used for perlecan analysis. Gels were then stained using SYPRO Ruby Protein Gel Stain kit (Bio-Rad, Hercules, CA) and stained gels were scanned and digitized at 800 dpi resolution using a Pharos FX scanner(Bio-Rad, Hercules, CA). Transfer of the proteins to membranes for immunodetection was performed as described above. Protein quantification was performed using ImageMaster Platinum 6.0 (GE Healthcare, Uppsala, Sweden) and data were expressed as relative spot volume of all spots representing a given protein.

Measurement of Total Complement Activity and Concentrations of C3 and C4 Components. The total hemolytic complement activity CH100 was assayed by hemaglutination method using Hemolytic Complement kit (The Binding Site Ltd., Birmingham, U.K.). The concentrations of C3 and C4 complement components in serum and follicular fluid were analyzed using the protein analysis system BNII (Siemens Healthcare Diagnostics, Inc., Deerfield, IL) and diagnostic kits Human C3C and C4C (Siemens Healthcare Diagnostics, Inc., Deerfield, IL). All the diagnostic kits were processed according to the manufacturer's instructions.

Results

The controlled ovarian hyperstimulation results in relatively synchronized maturation of several dominant follicles/oocytes, thus, reducing otherwise high impact of biological variation in samples for proteomic analyses. The paired samples of HFF and plasma obtained from women undergoing IVF and depleted of the 12 most abundant plasma matched proteins as described below were utilized in this study to search for relevant differences in protein composition of HFF and plasma.

Removal of Highly Abundant Proteins for Follow-Up Proteomic Analyses. To overcome limitations of commonly used proteomic techniques related to high dynamic range of protein concentration in a variety biological fluids and access rather middle- or low-abundance proteins (μg/mL to pg/mL), both HFF and plasma samples were depleted of the 12 most abundant proteins. Separation on IgY-12 SC removed around 90% of total loaded protein amount. Using column capacity of 10 μL of plasma resulted in yield of 75 and 50 μg of protein on average in flow-through fractions from plasma and HFF, respectively. Hence, to apply 100 μg of depleted proteins on analytical 2-DE gels, two and three IgY-12 spin column cycles were needed for each sample of plasma and FF, respectively. The removal of the 12 most abundant proteins was monitored using 2-DE fractionation of flow-through and bound fractions (Supplementary Figure 1) and determination of their protein content. The effectiveness of high capacity IgY-12 LC10 column with loading capacity up to 250 μL of plasma was slightly higher with removal of 95−98% of original protein amount. One cycle provided in average 630 and 930 μg of proteins of FF and plasma, respectively. In total, we performed six depletion cycles for FF and four for blood plasma to obtain 2 mg of depleted proteins of each individual sample for one run of 2-D LC PF 2D analysis.

Proteomic Changes Observed in Depleted Human Follicular Fluid Compared to Plasma Using Two Different Approaches: 2-DE and 2-D LC. A typical 2-DE separation performed in the pH 3−10 and the 10−200 kDa range resulted in separation of 477 ± 61 and 433 ± 73 protein spots on average in HFF and plasma, respectively, depleted of the 12 most abundant proteins (Figure 2A,B). Eight gels of four patient-paired samples were used for comparative analysis of protein profiles. A statistical comparison between the two groups of gels using Student's t test implemented in PDQuest software version 7.1 (Bio-Rad) identified 16 protein spots that were significantly ($p < 0.05$) increased or decreased in HFF compared to plasma (Figure 2A,B). Moreover, evaluation of the protein spots that appeared solely on gels from HFF or plasma (considered as qualitative changes based on the criterion of minimum differences being 10-fold stronger than background signals) did not reveal any protein spots that were typical of HFF. Most of the 16 quantitatively altered protein spots were decreased in HFF, but three spots, nos. 0103, 2004, and 2006, were significantly increased (Figure 2C). The protein identity in 11 of these 16 discriminate protein spots from silver nitrate-stained gels was satisfactorily determined using mass spectrometry. An abbreviated list of identified differentially expressed proteins and their functions is presented in Table 1. Comprehensive information about the proteins (SSP numbers, protein names, database accession numbers, protein MW, protein pI value and all MS identification data including Mascot scores, sequence coverage, matched peaks, unmatched peaks, and MS/MS confirmation) is presented in Supplementary Table 1. The presence of fibrinogen beta chain most probably results from incomplete removal of this protein in the course of depletion of major abundant proteins.

The purpose of 2-D LC PF 2D experiments was to complement observations from 2-DE using an advanced gel-independent fractionation technique. This method has the distinct advantage over the 2-DE based approach in that it overcomes many of its drawbacks and, importantly, the fractions of intact proteins can be directly utilized for mass spectrometric analysis. The PF 2D involves 2-D separation and mapping of the total protein expression. Proteins are fractionated by isoelectric points in pH gradient using the chromatofocusing at 0.3 pH intervals in the first dimension. Each of these pI protein fractions is further separated by hydrophobicity using nonporous silica reverse phase chromatography in the second dimension.[16] The global information about protein expression obtained by means of PF 2D separation has been depicted using ProteoVue software that enables the construction of 2-D protein map showing pI fractions versus protein bands according to their hydrophobicity (Figure 3A). In total, the samples of depleted HFF and plasma were separated on average into 1175 protein peaks and evaluation of qualitative and quantitative differences between 2-D protein maps using Viper software identified 96 differentially expressed protein bands with p-value ≤0.05 between HFF and plasma. Twelve of them with area

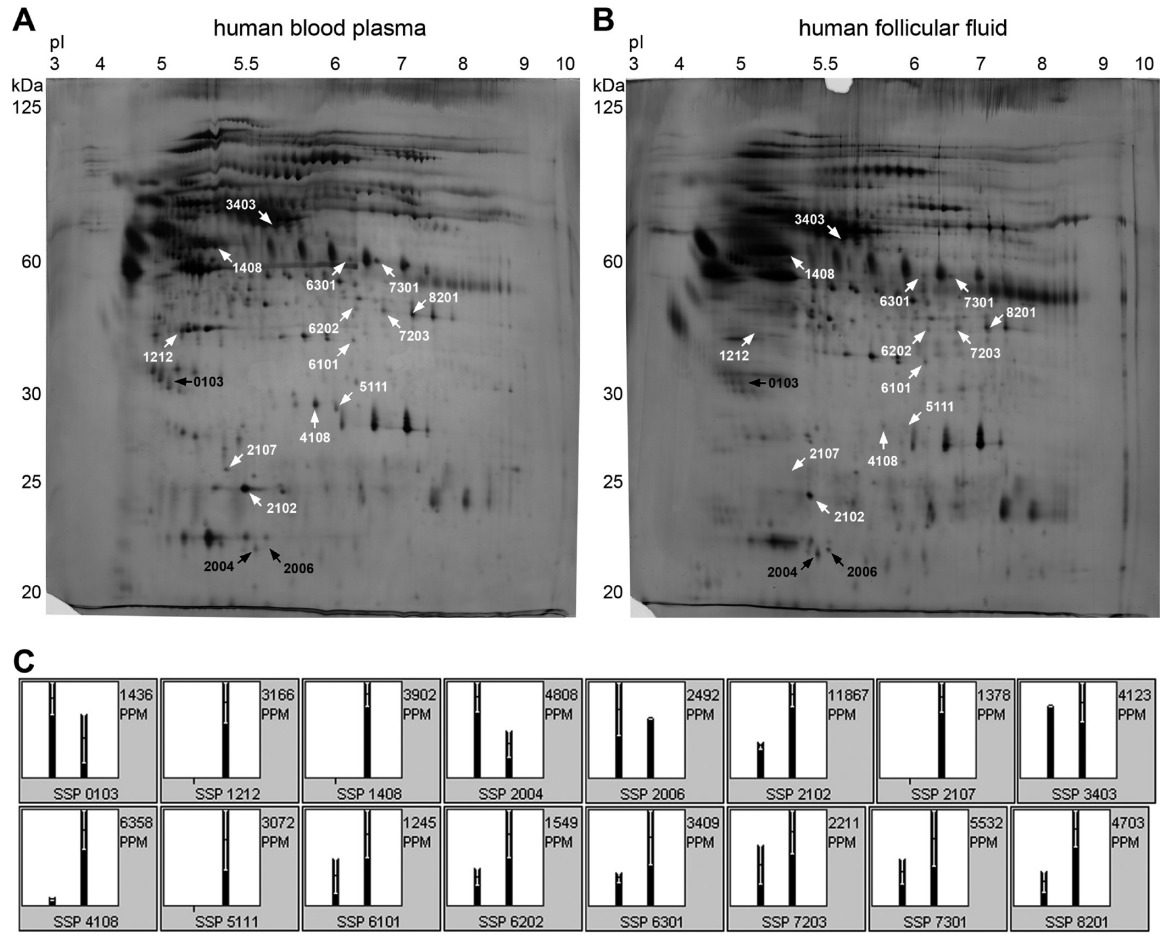

Figure 2. Two-dimensional electrophoretic protein maps of human plasma and follicular fluid depleted of the 12 most abundant plasma matched proteins and set of protein spots significantly different in follicular fluid. Protein lysates of paired samples of depleted human plasma and follicular fluid were subjected to 2-DE, followed by silver staining and image analysis using PDQuest software. The gels from eight liquid samples were evaluated. Panels (A) and (B) show representative gels from depleted plasma and follicular fluid, respectively, and protein spots that were differentially expressed ($P < 0.05$). Regulated proteins are indicated by their SSP numbers assigned by PDQuest software, black numbers indicate higher protein level in follicular fluid and white numbers denote lower level in follicular fluid compared to plasma. Panel (C) shows relative volume intensities of identified regulated protein spots calculated and graphically presented by PDQuest software. Left columns correspond to follicular fluid and right columns to blood plasma.

under curve higher than 1×10^{-5} at 214 nm were chosen for further mass spectrometry and the proteins in 6 of these discriminate protein bands were satisfactorily identified (Figure 3A,B). A summary of differentially expressed proteins and their functions is presented in Table 2. Supplementary Table 2 provides comprehensive information about the proteins (fraction numbers, protein names, database accession numbers, protein MW and all MS identification data including Mascot scores, sequence coverage, matched peaks, unmatched peaks, and MS/MS confirmation). Some of the protein bands collected after the second dimension contained more than one protein and this was reconfirmed by mass spectrometric analysis (Table 2). Most of the differentially expressed protein bands revealed by PF 2D approach and identified were present at significantly increased level in HFF (Figure 3B). These protein bands included apolipoprotein A-IV, alpha-1 antichymotrypsin, antithrombin-III, complement component C9, hemopexin, transthyretin, histidine-rich glycoprotein and complement factor H. The protein band no. 509 showing highly elevated increase in HFF and fulfilling the criteria of one protein/one band in a fraction corresponded to complement component C9 (Figure 3B and Table 2).

Immunoblotting Verification of Protein Changes Typical for Human Follicular Fluid. To validate the results of the proteomic analyses, immunoblot experiments were performed to confirm the identity of the proteins that were increased in HFF compared to plasma on 2-DE gels: clusterin and perlecan. Furthermore, complement factor H that was identified as one of three unambiguously identified proteins in band no. 1021 from 2-D LC with observed higher UV absorbance level in HFF was selected to demonstrate need of verification and contribution of a particular protein to UV absorbance level corresponding to protein amount. Eight paired samples of nondepleted HFF and plasma including six independent samples not previously used in proteomic analyses were separated using 1-D SDS-PAGE followed by protein transfer and specific immunodetection. The results shown in Figure 4 confirmed significantly higher level of clusterin in HFF versus plasma, while the level of complement factor H was lower in HFF. The total level of C-terminal fragment(s) of perlecan did not reveal significant difference between HFF and plasma.

Table 1. The List of Identified Significantly Different Proteins ($p < 0.05$) between HFF and Plasma Selected Using 2-DE[a]

spot no.	protein name	Swiss-Prot no.	upregulation/ fold change	functionality
0103	Clusterin precursor[b]	CLUS_HUMAN	HFF 2.69	Apoptosis Complement pathway Immune response Innate immunity
2006	Basement membrane-specific heparan sulfate proteoglycan core protein precursor (HSPG)[b]	PGBM_HUMAN	HFF 4.55	Cell adhesion
2102	Serum amyloid P-component precursor	SAMP_HUMAN	Plasma 2.61	Acute-phase response Chaperone-mediated protein complex assembly Protein folding
2107	Complement C4-A precursor[b]	CO4A_HUMAN	Plasma >100	Complement activation, alternative pathway Complement activation, classical pathway
4108	Ficolin-3 precursor	FCN3_HUMAN	Plasma 25.15	Complement activation, lectin pathway Signal transduction
5111	Ficolin-3 precursor	FCN3_HUMAN	Plasma >100	Complement activation, lectin pathway Signal transduction
6101	Complement C4-A precursor[b]	CO4A_HUMAN	Plasma 2.47	Complement activation, alternative pathway Complement activation, classical pathway
6301	Fibrinogen beta chain precursor	FIBB_HUMAN	Plasma 4.82	Platelet activation Protein polymerization Response to calcium ion Signal transduction
7203	Complement C3 precursor[b]	CO3_HUMAN	Plasma 3.54	Complement alternate pathway Complement pathway Immune response Inflammatory response Innate immunity
7301	Fibrinogen beta chain precursor	FIBB_HUMAN	Plasma 3.98	Platelet activation Protein polymerization Response to calcium ion Signal transduction
8201	Complement C3 precursor[b]	CO3_HUMAN	Plasma 3.24	Complement alternate pathway Complement pathway Immune response Inflammatory response Innate immunity

[a] The table shows SSP number, protein name, Swiss-Prot no., regulation/fold of the change and functionality based on search in UniProt/Gene Ontology/Biological Process. [b] Fragments only.

The microheterogeneity of clusterin that appeared as an approximate 37 kDa smear on immunoblots from reducing SDS-PAGE was better observed using 2-D immunoblotting of nondepleted samples. This was most likely related to the presence of two chains and their glycosylation as also observed by other researchers.[17] It was evident that protein forms with lower molecular weight and more basic p*I* corresponded to less glycosylated forms and these were observed to be present at higher level in FF (Figure 5A). Similarly, five protein spots corresponding to C-terminal truncated forms of perlecan were immunodetected using specific antibody raised against C-terminal part of the protein. On the basis of the measurements from 3 paired samples of HFF and plasma using Student's *t* test, relative volumes of two most basic spots, nos. 4 and 5, were significantly changed with $p < 0.06$ and $p < 0.02$, respectively, in HFF versus plasma (Figure 5). The level of immunodetected spot no. 4 was higher in HFF with ratio to plasma level corresponding to mean value of 3.96. The opposite was observed in plasma where the immunodetected form of spot no. 5 was higher with mean ratio levels of FF/plasma being 0.24.

Decreased Total Hemolytic Activity of Complement Cascade and Levels of C3 and C4 Component in Human Follicular Fluid Compared to Serum. On the basis of proteomic analyses presented above, many components of the complement cascade (complement C4-A, complement C3, complement component C9) as well as its regulatory proteins (clusterin, complement factor H, ficolin-3) were found in relatively different abundance in HFF compared to plasma. To demonstrate outcome of this dysregulation and possible impact on activity of complement cascade, we analyzed paired samples of HFF and serum for total complement activity as well as for native concentrations of two major complement components, C3 and C4, in order to justify functionality of this important immune process in the microenvironment of the growing follicle. The analysis performed with 29 paired samples showed significantly lower total hemolytic activity in HFF as compared to serum (43% decrease in average with significance of $P < 10^{-13}$, Student's *t* test, Table 3). In correlation with this observation the concentrations of complement components C3 and C4 were also significantly lower in FF compared to serum and their FF/serum ratios were 0.595 and 0.565, respectively (Table 3).

Discussion

Currently, couples having difficulties conceiving, resort to Assisted Reproductive Technology such as IVF in order to achieve pregnancy by artificial means. In IVF cycles, the serum levels of FSH and luteinizing hormone (LH) are determined by the amount of exogeneously administered hCG and by degree of pituitary suppression reducing the endogenous gonadotropin secretion which is regulated by administration of GnRH antagonist and later in final stage of follicular maturation by GnRH agonist. Recent studies demonstrated that administration of GnRH agonist results in less systemic inflam-

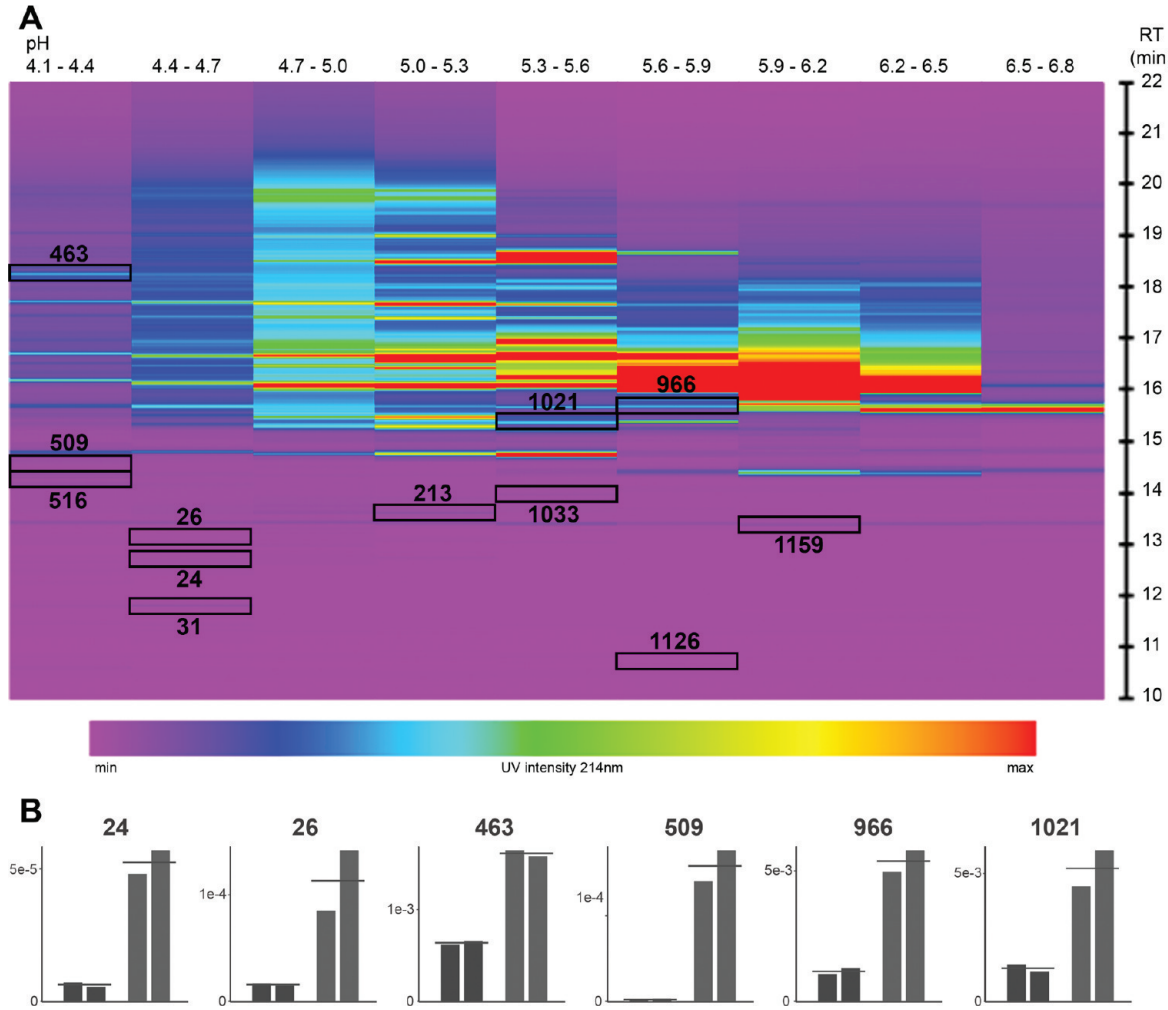

Figure 3. Two-dimensional liquid chromatography protein map of human follicular fluid depleted of the 12 most abundant plasma matched proteins and set of protein bands significantly different in follicular fluid compared to plasma. Protein lysates of paired samples of depleted human plasma and follicular fluid were subjected to 2-D liquid chromatography and protein fractionation/level was monitored using UV detection. 2-D protein expression maps were generated by ProteoVue software and their images were evaluated using Viper software. Panel (A) shows representative protein map from depleted follicular fluid and protein bands that were present in significantly different levels ($P < 0.05$) compared to plasma and were selected for follow-up identification using mass spectrometry. The colors of the protein bands correspond to UV intensity and provide information about protein quantification. Regulated proteins are indicated by their numbers assigned by Viper software. Panel (B) shows relative volume intensities of regulated protein bands calculated and graphically presented by Viper software. Two left columns correspond to two biological replicates of plasma and right columns to follicular fluid.

mation as reflected by level of C-reactive protein (CRP). This may help to prevent development of severe OHSS, the major complication of controlled ovarian hyperstimulation associated with presence of inflammatory cytokines, neutrophil activation and increased capillary permeability.[18,19] Treatment cycles are closely monitored and estradiol level and follicular growth are checked by gynecologic ultrasonography. Currently, there is a lack of assessment of oocyte quality or ability to predict success of the IVF treatment. Additionally, the diagnosis of OHSS is very limited and pathogenesis of this disease remains elusive; hence, its prevention is difficult, and currently, only symptomatic therapy can be applied. With current lack of diagnostic markers, the HFF represents a rich pool of proteins useful as a source of prognostic and/or diagnostic biomarker(s).

The oocyte matures in the milieu of FF rich in hormones, growth factors, cytokines, reactive oxygen and nitrogen species, antiapoptotic factors, polysaccharides and various proteins. Until now, study of single molecules such as FSH, LH, inhibins, insulin-like growth factor binding protein 3, pregnancy-associated plasma protein A and several cytokines has not revealed reliable markers of oocyte maturation, successful fertilization or pregnancy related complications or very early stage embryo development.[20] Implementation of *-omics* technologies, namely, metabolomics and proteomics may be extremely beneficial not only for monitoring complex regulatory networks involved in ovarian physiology and response to exogeneous stimulation, but additionally providing relevant group of candidate biomarkers.

Proteomic analyses of HFF mentioned above identified a variety of proteins present in HFF and most of them were matched to plasma proteins.[4−12] High proportion of acute phase proteins in human FF from women undergoing ovarian stimulation for IVF highlighted a possible involvement of the inflammatory process. Same could be true about the role of blood coagulation proteins in response to IVF treatment and impacting pregnancy or processes leading to miscarrage or abortion. To track down specific biomarkers in reproductive

Table 2. The List of Identified Differentially Expressed Proteins between HFF and Plasma Selected Using 2D-LC PF 2D[a]

peak no.	protein name	Swiss-Prot no.	upregulation/fold change	functionality
24	Apolipoprotein A-IV precursor[b]	APOA4_HUMAN	HFF	Cholesterol efflux
			8.46	Cholesterol homeostasis
				Cholesterol metabolic process
				Hydrogen peroxide catabolic process
				Innate immune response in mucosa
				Leukocyte adhesion
				Multicellular organismal lipid catabolic process
				Negative regulation of plasma lipoprotein oxidation
26	Apolipoprotein A-IV precursor[b]	APOA4_HUMAN	HFF	Phosphatidylcholine metabolic process
			7.32	Phopholipid efflux
				Protein−lipid complex assembly
				Regulation of cholesterol transport
				Removal of superoxide radicals
				Response to lipid hydroperoxide
				Reverse cholesterol transport
463	Alpha-1-antichymotrypsin precursor	AACT_HUMAN	HFF	Acute-phase response
			2.54	Regulation of lipid metabolic process
463	Antithrombin-III precursor	ANT3_HUMAN	HFF	Blood coagulation
			2.54	
509	Complement component C9 precursor	CO9_HUMAN	HFF	Complement alternate pathway
			99.74	Complement pathway
				Cytolysis Immune response
				Innate immunity
966	Hemopexin precursor	HEMO_HUMAN	HFF	Cellular iron ion homeostasis
			4.71	Heme transport
				Interspecies interaction between organisms
966	Transthyretin precursor	TTHY_HUMAN	HFF	Thyroid hormone generation
			4.71	Transport
1021	Histidine-rich glycoprotein precursor	HRG_HUMAN	HFF	unknown
			4.05	
1021	Complement factor H precursor	CFAH_HUMAN	HFF	Complement activation, alternative pathway
			4.05	
1021	Transthyretin precursor	TTHY_HUMAN	HFF	Thyroid hormone generation
			4.05	Transport

[a] The table shows peak number, protein name, Swiss-Prot no., regulation/fold of the change and functionality based on search in UniProt/Gene Ontology/Biological Process. [b] Fragments only.

medicine, depletion of highly abundant proteins enabled in-depth study of such proteins. This study was conducted after depletion of the 12 most abundant proteins from samples of HFF and plasma of women undergoing IVF followed by comparison of their 2-D protein patterns using two complementary techniques. The ultimate aim was to identify potential candidate proteins influencing follicle quality and development or impacting success of fertilization. Additionally, the understanding of any triggering of biochemical pathways affecting the final outcome of the reproductive process was of paramount interest in this study.

During folliculogenesis, follicles become more permeable to plasma proteins resulting in higher number of blood proteins crossing the blood−follicle barrier. Hence, there are notable similarities between protein composition of HFF and blood plasma/serum despite selective transport processes.[7,14] Comparison of fluids including plasma and serum can be facilitated by fractionation techniques such as immunoaffinity subtraction which provides an effective mean for simplifying the proteome while maintaining reasonable sample throughput.[21] Our study utilized an immunoaffinity system capable of removal of the 12 most abundant blood plasma proteins and human plasma and follicular fluid samples were processed using it. The removal of the 12 most abundant proteins was monitored using 2-DE fractionation of flow-through and bound fractions (Supplementary Figure 1) and determination of their protein content. The proteins of HFF and plasma depleted of major abundant proteins were fractionated using 2-DE and 2-D LC PF 2D and significant reproducible differences in protein composition of HFF versus plasma were then identified by mass spectrometry. A majority of these protein alterations, in contrast to the previous studies published by other authors,[5,6,9,12] were found to belong to the complement cascade (complement C4-A, complement C3, complement component C9) and its regulatory proteins (clusterin, complement factor H, ficolin-3) (Figure 6). Some other specific biological processes such as acute phase response (alpha1-antichymotrypsin, serum amyloid P-component), transport (hemopexin, transthyretin), blood coagulation (antithrombin-III), lipid metabolism (apolipoprotein A-IV) were affected to a lesser extent (Table 4).

The complement system, composed of over 30 proteins, responds by means of recognition and activating mechanisms to foreign proteins, tissue injury, apoptosis and necrosis. The three complement activation pathways, classical, lectin and alternative, converge on the C3 component which results in common effector pathway and functions. Complement activation initiates inflammation via recruitment and activation of inflammatory cells[22] (Figure 6). The low levels of complement components C3 and C4-A (or their fragments) together with high level of C9 in HFF observed in this study clearly indicated distinctive regulation of complement cascade in HFF. Additionally, low level of ficolin 3 in HFF can significantly limit complement activation via reduction of lectin-mediated pathway which is used for recognition of self (altered)/nonself.[23,24] Similar to blood serum, the level of complement factor H in HFF is supposed to regulate complement cascade by inactivat-

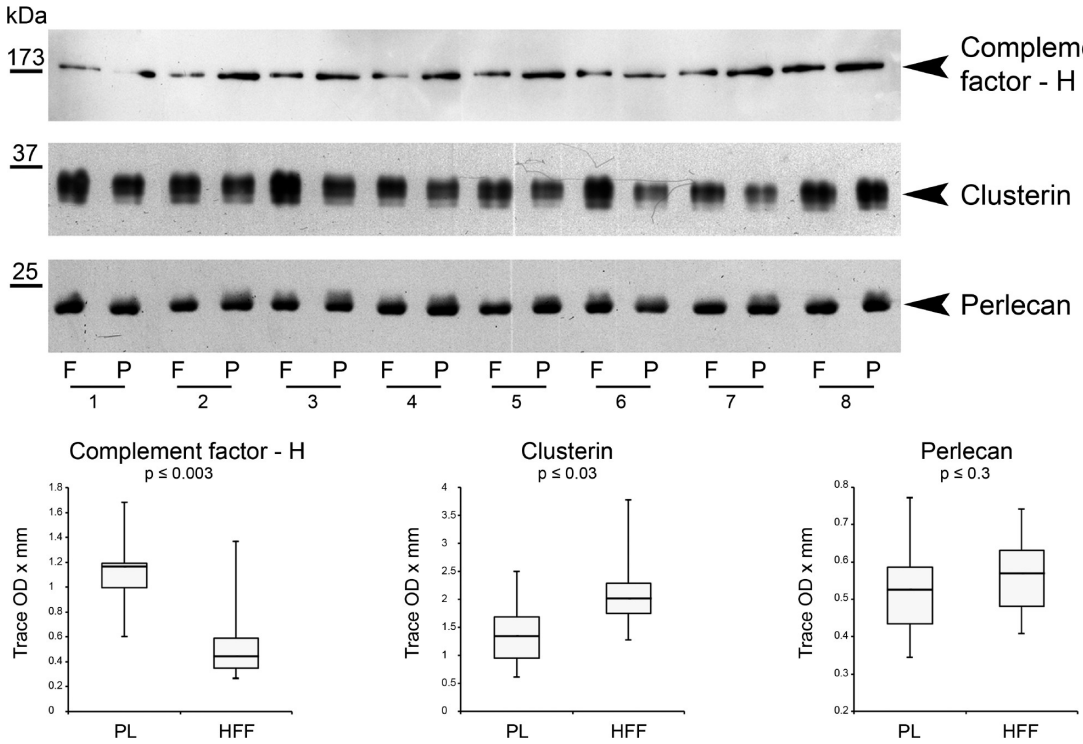

Figure 4. Immunoblot analysis of complement factor H, clusterin and perlecan (C-terminal fragment) in paired samples of nondepleted follicular fluid and plasma of women undergoing IVF. Protein lysates prepared from eight paired samples of follicular fluid (F) and plasma (P) were examined on immunoblots using specific antibodies recognizing complement factor H, clusterin and perlecan (C-terminal fragment). The protein bands were quantified using Quantity One software and distribution of the values was illustrated using boxplot. Significance of differences was calculated by Student *t* test (*p*-value).

ing C3 convertase and alternative pathway activation important for tissue homeostasis.[25,26] We observed low levels of complement factor H in HFF in this study which may be allowing the alternative pathway to remain active. The third complement regulatory protein highlighted in this study was clusterin. It plays an active role in inhibition of complement-mediated cell damage[27] and may also play protective role in reproduction. This study demonstrated high level of clusterin in HFF, which might contribute to the inhibition of cytolytic activity of complement-mediated membrane attack. Analysis of data obtained from our study and extrapolating its relationship to the schematic representation of complement cascade (Figure 6) provides an understanding of the complement cascade inhibiton in HFF of women undergoing ovarian stimulation for IVF. It appears that controlled complement activity in follicular fluid across several different levels of regulation may significantly contribute to optimized oocyte maturation and IVF success leading to higher pregnancy rates. Murine studies supporting the role of complement cascade in control of reproductive process in antiphospholipid syndrome characterized by pregnancy loss that occurs in the presence of antiphospholipid antibodies have also alluded to similar outcome. These studies identified a novel role for complement cascade linking abortion with inappropriate complement activation.[22,28] Additional data from such animal model studies have shown the important role played by complement regulatory proteins in prevention of harmful amplification of complement cascade and this too was evident in our study using human samples.[29] Interestingly, direct link between complement activation and angiogenesis was demonstrated in antibody independent mouse model of spontaneous miscarriage. Complement activation caused deficiency of free vascular endothelial growth factor (VEGF), the angiogenic factor required for normal placental development, that was captured by high levels of soluble VEGF receptor 1. Inhibition of complement activation prevented these angiogenesis failure and rescued pregnancies.[30] Our current ongoing studies using bead-based multiplex assays from the Endocrine and Cytokine Panels (Millipore, www.millipore.com/analytes) and Luminex 200 Instrumentation (data not shown) indicate that low level of complement activity in HFF described in this study is associated with increased level of VEGF compared to serum samples of women undergoing IVF which has important role in perifollicular angiogenesis and may significantly affect oocyte maturation and quality.

Besides the role of clusterin in complement inactivation, it is a multifunctional protein that is up-regulated during many different pathophysiological states and studies have focused on its role in reproductive complications. Clusterin expression in the placental tissues of the preeclampsia group was significantly higher than in the normal pregnancy group[31] and clusterin mRNA level in testicular biopsies was significantly lower in azoospermic patients with constitutive or idiopathic spermatogenic failure.[32] However, there are some discrepancies among studies showing either decreased level of clusterin in plasma of women carrying Down syndrome fetus[33] or increased level of clusterin.[34] The problem may be related to the necessity to target specific form or modification of clusterin to particular biological process and cellular or extracellular localization.

Among other proteins increased in HFF, we found perlecan, highly conserved multidomain heparan sulfate proteoglycan. This multifunctional molecule supports cell adhesion, growth factor binding, regulates apoptosis and it is responsible for charge selective ultrafiltration properties.[35] Perlecan expression and function is controlled at the level of transcription and

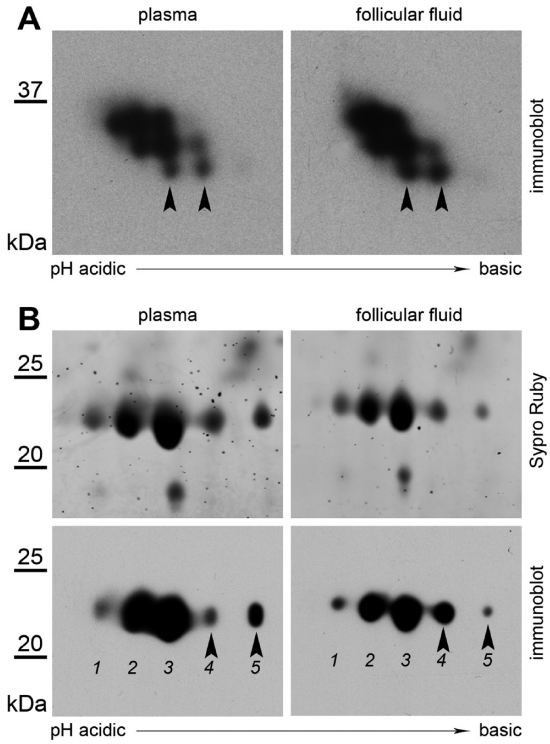

Figure 5. 2-D immunoblot analysis of clusterin and perlecan (C-terminal fragment) in samples of nondepleted follicular fluid and plasma of women undergoing controlled ovarian stimulation for IVF. Protein lysates prepared from paired samples of follicular fluid and plasma were examined on 2-D immunoblots using specific antibodies recognizing clusterin (A) and perlecan (C-terminal fragment) (B). The arrows indicate the positions of the differentially regulated proteins. The figure is representative of three different pairs of samples.

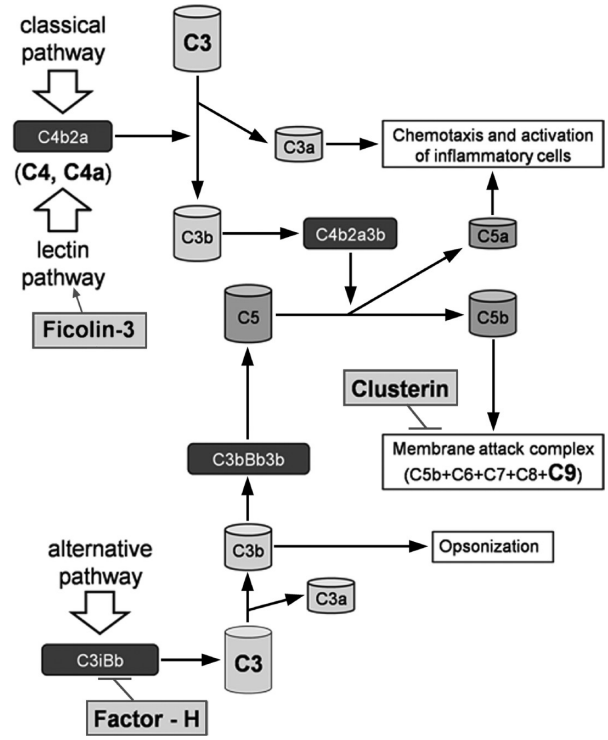

Figure 6. Complement cascade and complement regulatory proteins: proposed mechanism of complement inhibition in follicular fluid. The three complement activation pathways, the classical, lectin and alternative, converge on the C3 component which results in a common effector pathway and functions. Complement activation initiates inflammation via recruitment and activation of inflammatory cells. The changes in the levels of complement components C3, C4, C4a, and C9 (in bold) as well as complement regulatory proteins ficolin-3 complement factor H and clusterin (in box) were identified in this study. These changes may subsequently contribute to the inhibition of complement cascade activity in follicular fluid of women undergoing controlled ovarian stimulation for IVF. It appears that controlled complement activity in follicular fluid coming across several different levels of regulation is favorable for intrafollicular milieu and it may influence maturation of oocyte and its competence for successful fertilization. On the other hand, disregulation of this milieu may contribute to the pathologies associated with ovarian stimulation.

alternative splicing, but significant contribution comes also from extracellular proteolysis. It appears that perlecan fragments may have distinct activities than original intact molecule. In support of this, C-terminal fragment of perlecan has been shown to inhibit angiogenesis;[36] hence, it may be possible that its presence in HFF can contribute to regulation of vasculature in follicle.[37] It was demonstrated that selective degradation of perlecan occurred during ovulation in the focal intraepithelial matrix that develops between granulosa cells and the follicular basal lamina in ovarian follicles.[38] Using specific immunoblot, we confirmed the presence of at least five forms of C-terminal fragment of perlecan with two of them having distinct levels in HFF compared to plasma. Perlecan was for the first time identified in HFF by Hanrieder et al in 2007,[12] but distinctive perlecan forms were not defined in this study.

Histidine-rich glycoprotein was found in fraction collected in 2-D LC together with complement factor H and transthyretin. By UV quantification, protein content in this fraction was higher in HFF compared to plasma, but Western blot confirmed lower level of complement factor H in HFF, and with dissimilarities to other studies reporting transthyretin,[7,9,33] it is therefore likely that histidine-rich glycoprotein, described for the first time in this study to be present in HFF, is increased in HFF of women undergoing IVF. The physiological function of this protein is not yet known; however, on the basis of its homology with high molecular weight kininogen, the His-rich region of this protein may mediate the contact activation phase of intrinsic blood coagulation cascade.[39] Histidine-rich glycoprotein interacts with many ligands including Zn^{2+}, tropomyo-

Table 3. Total Hemolytic Activity of Complement Cascade in Hff and Serum and Concentration of Complement Components C3 and C4

	CA Serum (U/mL)	CA FF (U/mL)	CA FF/S ratio	C3 FF (g/L)	C3 serum (g/L)	C3 FF/S ratio	C4 FF (g/L)	C4 serum (g/L)	C4 FF/S ratio
Mean	803	465	0.574	0.594	1.000	0.595	0.115	0.207	0.565
SD	144	164	0.156	0.192	0.166	0.171	0.037	0.062	0.135
N	29	29	29	22	22	22	22	22	22
t test		$P < 10^{-13}$			$P < 10^{-9}$			$P < 10^{-7}$	

Table 4. Summary of the Differentially Expressed Proteins between HFF and Plasma Revealed by Complementary Proteomic Techniques and Their Classification According to the Biological Processes

protein name	Swiss-Prot no.	methodology	increase	functionality
Alpha-1-antichymotrypsin precursor	AACT_HUMAN	2D-LC	HFF	acute-phase response
Antithrombin-III precursor	ANT3_HUMAN	2D-LC	HFF	blood coagulation
Apolipoprotein A-IV precursor	APOA4_HUMAN	2D-LC	HFF	lipid transport
Perlecan (HSPG2)	PGBM_HUMAN	2D-E	HFF	cell adhesion
Clusterin precursor	CLUS_HUMAN	2D-E	HFF	complement pathway apoptosis
Complement C3 precursor	CO3_HUMAN	2D-E	PL	complement pathway innate immunity
Complement C4-A precursor	CO4A_HUMAN	2D-E	PL	complement pathway innate immunity
Complement component C9 precursor	CO9_HUMAN	2D-LC	HFF	complement pathway innate immunity
Complement factor H precursor	CFAH_HUMAN	2D-LC	HFF	complement pathway innate immunity
Fibrinogen beta chain precursor	FIBB_HUMAN	2D-E	PL	blood coagulation
Ficolin-3 precursor	FCN3_HUMAN	2D-E	PL	complement pathway innate immunity
Hemopexin precursor	HEMO_HUMAN	2D-LC	HFF	transport
Histidine-rich glycoprotein precursor	HRG_HUMAN	2D-LC	HFF	not yet known
Serum amyloid P-component precursor	SAMP_HUMAN	2D-E	PL	acute-phase response
Transthyretin precursor	TTHY_HUMAN	2D-LC	HFF	transport

sin, heparin and heparan sulfate, plasminogen, plasmin, fibrinogen, thrombospondin, imunoglobulins and strong bound to several complement proteins. Therefore, the maintenance of immune functions as well as coagulation may be extensively influenced in the presence of histidine-rich glycoprotein and such possible links deserve further investigation.

Conclusion

Paired comparison study examining HFF with plasma/serum from women undergoing successful IVF revealed important protein differences which may improve our knowledge of the follicular microenvironment and its biological role. This study showed involvement of innate immune function of complement cascade in HFF of women undergoing ovarian stimulation for IVF. Complement inhibition and the presence of C-terminal fragment of perlecan also suggested possible links to angiogenesis, a process paramount to follicle and embryo development. Additionally, differences in proteins associated with blood coagulation were observed and may influence follicular milieu. Depletion of abundant proteins combined with multidimensional protein fractionation was instrumental in allowing the study of middle- and lower-abundance proteins, many of which have not yet been associated with follicle/oocyte maturation. These proteins together with their regulatory pathways may play a vital role in reproductive process. We propose a set of key proteins as potential biomarker candidates to aid a successful IVF therapy in women desperate to have a child.

Abbreviations: ANOVA, analysis of variance; BCA, bicinchoninic acid; CCA, α-cyano-4-hydroxycinnamic acid; CRP, C-reactive protein; FF, follicular fluid; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; hCG, human chorionic gonadotropin; HFF, human follicular fluid; HPCF, high performance chromatofocusing; HPLC, high performance liquid chromatography; HPRP, high performance reverse phase; IEF, isoelectric focusing; IVF, *in vitro* fertilization; LC, liquid chromatography; LH, luteinizing hormone; MALDI, matrix-assister laser desorption/ionization; MeCN, acetonitrile; MS, mass spectrometry; MW, molecular weight; OHSS, ovarian hyperstimulation syndrome; PF 2D, protein fractionation 2D; PMF, peptide mass fingerprinting; SELDI, surface-enhanced laser desorption/ionization; TOF, time-of-flight; VEGF, vascular endothelial growth factor.

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Supporting Information Available: Figure of 2-DE fractionation of flow-through and bound fractions. Tables of differentially expressed proteins identified from 2-DE experiment and 2D-HPLC experiment. This material is available free of charge via the Internet at http://pubs.acs.org.

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