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
human follicles. 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. 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. 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. 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. Taking mental stages. Two proteins, apolipoprotein A-I and collagen 

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. 

phase response, transport, lipid metabolism and blood coagulation were involved to a lesser extent.

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, amonion persulfate (APS), TEMED, n-octyl glycoside, Tris, (2-carboxyethyl) phosphine hydrochloride (TCEP), and iminodiacetic acid were obtained from Sigma-Aldrich (St. Louis, MO). Nonidet-40, 3-(cholamidopropyl)-dimethylammonio-1-propane sulfonate (CHAPS) and DTT were from USB Corporation (Cleveland, OH). Glycerol and β-glycerophosphate were purchased from Penta (Prague, Czech Republic). Protease inhibitors cocktail were obtained from Roche (Mannheim, Germany). Immobilon 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.
follicular fluid and plasma. Women undergoing stimulation for IVF were recruited for the study at the Centre of Assisted Reproduction, Department of Obstetrics 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) 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 centrifugated to remove cellular components and debris and then transferred to sterile polypropylene tubes and frozen at −70 °C until further analysis. 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, transferrin, 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) allowing simultaneous run of four gels. Gels were then equilibrated, 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 dimension chromatofocusing fractionation (HPCF) two buffers, 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 autotryptolytic 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 $pI$ value were applied. Proteins with MOWSE score over the threshold 50 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 Protein II xi Cell (Bio-Rad). Proteins were then transferred to Immobilon P (Millipore, Bedford, MA) membranes using a semidy 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+ chemiluminescence (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 protein 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 Pharus 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 CH110 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
under curve higher than $1 \times 10^{-5}$ 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.
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. It was evident that protein forms with lower molecular weight and more basic pI 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 perlecain 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 exogenously 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-
Information 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. 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. 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. 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 miscarriage or abortion. To track down specific biomarkers in reproductive
proteins, however, are complex, and numerous components, including lipids, carbohydrates, and proteins, are present in the blood plasma. 

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

<table>
<thead>
<tr>
<th>peak no.</th>
<th>protein name</th>
<th>Swiss-Prot no.</th>
<th>upregulation/fold change</th>
<th>functionality</th>
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<tbody>
<tr>
<td>24</td>
<td>Apolipoprotein A-IV precursor</td>
<td>APOA4_HUMAN</td>
<td>8.46</td>
<td>Cholesterol efflux</td>
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<td>Innate immune response in mucosa</td>
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<td>Multicellular organismal lipid catalytic process</td>
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<td>Negative regulation of plasma lipoprotein oxidation</td>
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<td>463</td>
<td>Alpha-1-antichymotrypsin precursor</td>
<td>AACT_HUMAN</td>
<td>2.54</td>
<td>Regulation of lipid metabolic process</td>
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<tr>
<td>463</td>
<td>Antithrombin-III precursor</td>
<td>ANT3_HUMAN</td>
<td>2.54</td>
<td>Blood coagulation</td>
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<td>Complement component C9 precursor</td>
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<td>99.74</td>
<td>Complement alternate pathway</td>
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<td>1021</td>
<td>Histidine-rich glycoprotein precursor</td>
<td>HRG_HUMAN</td>
<td>4.05</td>
<td>Thyroid hormone generation</td>
</tr>
<tr>
<td>1021</td>
<td>Complement factor H precursor</td>
<td>CFAH_HUMAN</td>
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<td>Transport</td>
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<td>TTHY_HUMAN</td>
<td>4.05</td>
<td>Thyroid hormone generation</td>
</tr>
</tbody>
</table>

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. Fragments only.

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, 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 (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.  

Similar to blood serum, the level of complement factor H in HFF is supposed to regulate complement cascade by inactivat-
ing C3 convertase and alternative pathway activation important for tissue homeostasis.\textsuperscript{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\textsuperscript{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 inhibition 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.\textsuperscript{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.\textsuperscript{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 placentation development, that was captured by high levels of soluble VEGF receptor 1. Inhibition of complement activation prevented these angiogenesis failure and rescued pregnancies.\textsuperscript{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\textsuperscript{31} and clusterin mRNA level in testicular biopsies was significantly lower in azoospermic patients with constitutive or idiopathic spermatogenic failure.\textsuperscript{32} However, there are some discrepancies among studies showing either decreased level of clusterin in plasma of women carrying Down syndrome fetus\textsuperscript{33} or increased level of clusterin.\textsuperscript{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.\textsuperscript{35} Perlecan expression and function is controlled at the level of transcription and
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, hence, it may be possible that its presence in HFF can contribute to regulation of vasculature in follicle. 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. 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, 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. 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 controlled ovarian stimulation for 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. 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              | $P < 10^{-13}$      |                     |                     |                     | $P < 10^{-9}$       |                     |                     | $P < 10^{-7}$       |                     |
sin, heparin and heparan sulfate, plasminogen, plasmin, fibrinogen, thrombospodin, immunoglobulins and strong bond 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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