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Proteomic Analysis of Human Follicular Fluid Using an Alternative Bottom-Up Approach

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Abstract: Human follicular fluid (hFF) is the in vivo environment of oocytes during follicular maturation in the ovaries. It contains a huge variety of compounds such as, e.g., proteins that might play an important role in follicular development and oocyte growth. Previous proteomic studies on follicular fluid have isolated and already identified a certain number of proteins. Nevertheless, only a small part of proteins present in follicular fluid have been covered so far and a large number have still not been identified. Therefore, the need for new, more resolving, and sensitive approaches in proteome research is evident. We utilized a proteomic setup based on in solution isoelectric focusing (IEF) and reversed-phase nanoliquid chromatography coupled to matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (nano-LC MALDI TOF/TOF MS) for in depth protein analysis of human follicular fluid samples of patients undergoing controlled ovarian hyper stimulation (COH) for in vitro fertilization therapy (IVF). This approach led to the significant identification of 69 proteins, where 32 have not been reported before to be found in human follicular fluid with proteomic methods. Among these findings, at least two relevant compounds essentially involved in hormone secretion regulation during the folliculogenetic process were identified: sex hormone binding globulin (SHBG) and inhibin A (INHA). To confirm these results, both proteins were further validated by immunoassays.

Keywords: human follicular fluid (hFF) • proteomics • in solution isoelectric focusing (IEF) • matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI TOF/TOF MS) • sex hormone binding globulin (SHBG) • inhibin A (INHA)

1. Introduction

Human follicular fluid (hFF) constitutes the microenvironment for oocytes during their maturation. It contains a huge variety of substances that are essentially involved in reproductive processes. The chemical composition of hFF reflects the stage of oocyte development and the degree of follicle maturation. It is an indicator of secretory activities and metabolism of follicular cells.[1–3] However, the factors that are involved in the process of follicular growth are rarely known. Thus, the investigation of the hFF content might give a closer insight in the physiological processes controlling follicular development. Protein components in hFF may play an important role during follicular maturation. The investigation of hFF protein content can provide useful information about their function in the folliculogenetic process, e.g., in the case of in vitro fertilization (IVF) therapy.[2] In previous studies on protein profiling of hFF, a certain number of proteins have already been identified. Important contributions to help extend the knowledge about the protein content in hFF and their functionalities, e.g., related to reproductive disorders, have been made.[4–9] However, the general understanding about proteins and their functionalities in follicular growth processes is still quite limited. Due to its complexity, which is comparable to blood plasma, sensitive and high-resolving techniques for protein identification in follicular fluid are essential. The major challenge in proteome analysis in complex biological samples, such as plasma, CSF, or hFF, is to deal with the dynamic range of the present proteins. Therefore, high-resolving protein/peptide separation methods and highly accurate and high resolution mass spectrometric tools are essential for sensitive and significant protein identification.[10,11] The most common approach in proteomics is based on two-dimensional gel electrophoresis (2DGE) followed by in-gel protein digestion and final identification with mass spectrometry, e.g., MALDI TOF MS.[12] The main disadvantage of this approach is that it is quite work-intensive, is time-consuming, and holds a risk for sample loss due to less efficient in gel proteolysis. Nevertheless, 2DGE is nowadays the workhorse in proteomic research and has proven to be a reliable tool for protein screening.

To overcome some limitations of commonly used proteomic strategies, we chose an alternative approach that provides a quite fast and less work-intensive route to sensitive and absolute significant protein identification in complex biological matrices. The here presented strategy for protein identification in human follicular fluid is based on microscale solution

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isoelectric focusing (IEF) for protein prefractionation, followed by in solution digestion. The enzymatic cleavage products were separated by reversed-phase nanoliquid chromatography and were directly fractionated onto a MALDI target plate. Finally, the peptides were analyzed by MALDI TOF/TOF MS, and protein identification took place by a combined database search of all collected MS/MS data.

2. Material and Methods

2.1. Chemicals and Reagents. Acetonitrile (ACN), methanol (MeOH), acetic acid (HAc), ammonium bicarbonate, ammonium-dihydrogen-phosphate ($NH_4H_2PO_4$), phosphoric acid (H_3PO_4), and sodium hydroxide (NaOH) were obtained from Merck (Darmstadt, Germany). Iodoacetamide (IAA), urea, and trifluoroacetic acid (TFA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT) was obtained from GE Healthcare (Uppsala, Sweden). High-purity water was taken from a Milli-Q (Millipore, Bedford, MA,USA) purification system.

2.2. Follicular Fluid Samples. Stimulated hFF was sampled from seven Swedish women attending IVF treatment in Uppsala University hospital, Uppsala, Sweden, because of male-factor, tubal-factor, nonovarian endometriosis, or unexplained infertility. The treatment protocol of IVF patients consisted of pituitary down regulation by a GnRH analogue (Buserelin; Hoechst, Frankfurt, Germany) employing the "long" protocol initiated at the midluteal phase (900 μg/day, intranasal administration). Recombinant FSH (Gonal-F, Serono S.A.) (SC) was injected daily (225 IU/day) starting on cycle day three. Transvaginal oocyte retrieval was performed under ultrasound guidance 36 h after HCG administration. Follicles larger than 15 mm in diameter were aspirated. The project was approved by an ethical committee on February 20th 1998 with diary number 98048.

2.3. In Solution Isoelectric Focusing. Protein separation experiments were carried out with a microrotofor solution isoelectric focusing device (BioRad, Hercules, CA, USA). A volume of 500 μL pooled hFF was diluted in 3.5 mL of milliQ water, and 100 μL of 40% (w/v) ampholyte solution (pH 3–11) (BioRad) was added resulting in a total ampholyte concentration of 1% (w/v). An amount of 2.5 mL of the prepared sample mixture was loaded to the separation cell by using a 1 mL syringe. The electrode buffers 0.1 M NaOH (cathode) and 0.1 M H_3PO_4 (anode) were loaded to the respective buffer reservoirs. Separation took place by the application of high-voltage (max. 3 kV) for 3 h where the output power was set to be constant at 1 W. After the separation voltage was switched off, the 10 fixed fractions were harvested by extraction out off the compartments with suction. The collected fractions were transferred in 0.5 mL tubes and stored in a freezer at −80 °C. For pI gradient validation, the pH values of all fractions were determined with a Rusell RL 150 pH-meter (Thermo Electron, Waltham, MA, USA).

2.4. Protein Digestion Procedure. An aliquot of 35 μL of each IEF fraction, corresponding to approximately 240 μg of total protein content, was dried down using a Speedvac system ISS110 (Thermo Savant, Holbrook, N.Y.) and redissolved in 100 μL of digestion buffer (8 M urea, 400 mM NH_4HCO_3). A volume of 10 μL of 45 mM dithiothreitol was added before incubation for 15 min (50 °C). Furthermore 10 μL of 100 mM iodoacetamide was added, followed by incubation at room temperature (15 min, darkness). Afterwards, 10 μg of trypsin (1:24 w/w) from bovine pancreas (Roche Diagnostics, Penzberg, Germany) dissolved in 100 μL of water was added, and the samples were incubated for 24 h at 37 °C, in darkness. The samples were desalted on ZipTip C_{18} columns (Eppendorf, Hamburg, Germany), and the procedure is described in detail elsewhere.[13]

2.5. Nanoliquid Chromatography. Nano-RP-HPLC was performed with a 1100 nanoflow LC system (Agilent Technologies, Waldbronn, Germany), equipped with a fraction collector capable of direct fractionation onto MALDI Target plates. A volume of 10 μL, corresponding to approximately 400 ng of digestion products, was injected into a 10 μL sample loop. For separating the enzymatic cleavage products, a 15 cm × 180 μm Biobasic C_{18} column (Thermo Electron, Waltham, MA, USA) with 5 μm particle size together with an H_2O:ACN:TFA solvent system (H_2O, 0.1% TFA [A]; ACN, 0.1% TFA [B]) was used. A flow rate of 2 μL/min starting with isocratic elution at 2% B for 20 min, followed by gradient elution from 2% to 8% B in 5 min, then from 8% to 32% B within 86 min, then from 32% to 40% B in 5 min, and finally from 40% to 80% B in 1 min, was applied. The peptide elution was followed by online fractionation onto a MALDI target with a collection rate of four fractions a minute for 96 min within the elution period from 20 min (2% B) and 116 min (40% B) resulting in 384 fractions. For optimal MS results, disposable prespotted anchorchip targets (PAC-targets, Bruker Daltonics, Bremen, Germany) were chosen. After sample collection, the target plates were washed with 10 mM $NH_4H_2PO_4$/0.1% TFA before subjection to the mass spectrometer.

2.6. MALDI TOF/TOF MS. Mass data were acquired with an Ultraflex II MALDI TOF/TOF (Bruker Daltonics, Bremen, Germany) in reflector positive mode. A mass range of 700–4000 Da was analyzed with a sum of 600 shots/spot and 100 shots/position, respectively, in a hexagonal pattern. The laser frequency was set to 100 Hz. MALDI TOF/TOF tandem MS analysis was performed in LIFT mode by software-controlled data acquisition. Here, 400 shots were acquired in PARENT mode at a normal laser energy level but enabled precursor ion selection resulting in intact precursor ion spectra. A one-point calibration was performed before switching to FRAGMENT mode. Here 600 shots/spot with 30% increased laser energy were acquired to give the fragmentation spectra. Post LIFT motherion suppression was applied to deflect the precursor and elevate fragment ion intensity. Peptide monoisotopic signals were analyzed using the SNAP algorithm, implemented in the FlexAnalysis software (Bruker Daltonics).

2.7. Data Processing and Protein Identification. The spectra were calibrated externally using the prespotted calibrants adjacent to the sample spots with the following monoisotopic signals: Bradykinin, $[M + H]^+$, 757.38516; Angiotensin-II, $[M + H]^+$, 1046.54180; Angiotensin-I, $[M + H]^+$, 1296.68480; Neurotensin, $[M + H]^+$, 1673.91700; Renin-Substrate, $[M + H]^+$, 1758.93261; ACTH_clip (1–17), $[M + H]^+$, 2093.08620; ACTH_clip (18–39), $[M + H]^+$, 2465.19830; ACTH_clip (1–24), $[M + H]^+$, 2932.58787; ACTH_clip (7–38), $[M + H]^+$, 3657.92890. Real data acquisition was assisted by applying the WarpLC Software (Bruker Daltonics) for automatic TOF-MS spectra acquisition, background signal filtering, grouping of signals into a peptide profile with respect to their distribution and intensity, as well as optimized precursor ion selection for subsequent MS/MS experiments. For final protein identification, all collected MS/MS data were run in a comprehensive MS/MS ion search using the Mascot search engine version 2.2 (Matrix Science, Boston, MA, USA). The following specified parameters were applied for database search: database (SwissProt v. 51.6); taxonomy (*Homo*

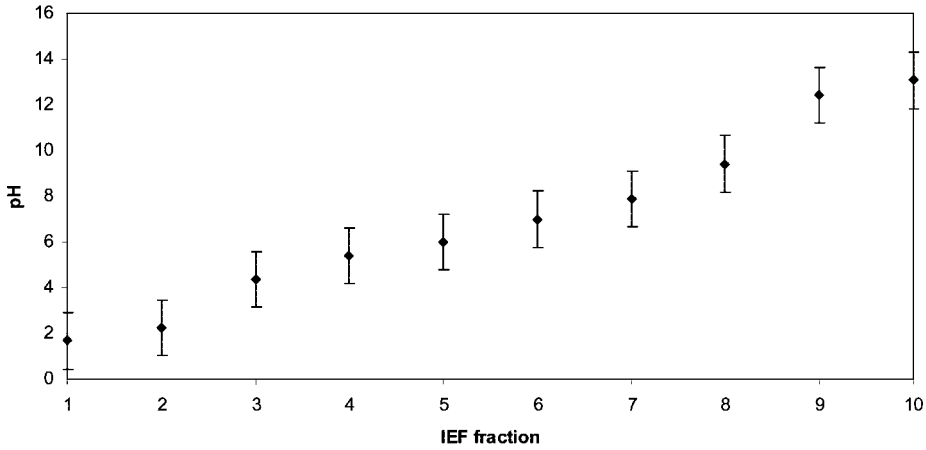

Figure 1. pH gradient evaluation of a single in solution IEF experiment. Gradient was established by adding 1% (w/v) ampholytes with a proposed pI range of 3–11. Error bars represent SD ($n = 3$).

sapiens); proteolytic enzyme (Trypsin); peptide mass tolerance (±25 ppm); fragment mass tolerance (±0.25 Da); global modification (Carbamidomethyl (Cys)); variable modification (Oxidation (Met)); peptide charge state (1+); and max missed cleavage (1).

2.8. Protein Validation and Quantification by Immunoassays. Samples were tested for SHBG using electrochemiluminescent immunoassay performed on a IMMULITE 2000 analyzer (Siemens Medical Solutions Diagnostics, PA) according to a protocol described in detail elsewhere.[14] Inhibin A was evaluated using a commercially available enzyme-linked immunoassay (Diagnostic Systems Laboratories, Inc., TX). The protocol is described in detail elsewhere.[15]

3. Results and Discussion

Proteomic analysis of barely explored biological samples like hFF is of great interest with potential clinical implications. In addition, the development of new analytical strategies to overcome limitations of commonly used techniques is a main concern in current proteome research. The here presented proteomic strategy based on solution-phase isoelectric focusing for protein prefractionation in conjunction with subsequent enzymatic digestion followed by nanoliquid chromatography coupled off line to MALDI TOF/TOF MS has been demonstrated to be a promising alternative approach for protein identification in complex biological samples. It proved to be a quite robust, easy to use, and less work-intensive strategy, especially with respect to the actual on-site (hands-on) workload of approximately 6 h for the whole workflow from sample preparation to final protein identification. The results show the overall identification of 69 proteins in all 10 IEF fractions, where the absolute number of protein matches prior to clearance of redundancies and isomeric distribution was 272. This is a quite remarkable result with respect to a 2DGE-based proteomic study of hFF reported by Angelucci et al., where in total 60 proteins have been identified in 183 different gel spots.[8] A number of 37 proteins that were identified in our experiments have been found in previously reported studies (Supporting Information, Table 1).[4–9] Furthermore, we found 32 proteins that have not been described to be found in hFF with proteomic methods so far (Supporting Information, Table 1Table 2). For further validation of the low-abundant protein identities of potential clinical interest, complementary immunoassays were performed.

Protein preseparation by in solution IEF proved to be a suitable approach for sample complexity reduction. Prior to the digestion step and final nano-LC MALDI TOF/TOF MS analysis, the separation efficacy could be roughly evaluated by pH value determination of the fractions (Figure 1). This provides information about the quality of the established pI gradient. The results show a steady and systematic pH increase among the fractions indicating a fair pI gradient establishment caused by the ampholytes. To obtain the best possible separation of the enzymatic cleavage products, gradient elution optimization is necessary to prevent suppression effects during TOF MS analysis. A satisfactory distribution of contingent precursor candidates for subsequent MS/MS experiments is essential to cover as many compounds as possible in the sample digest and to enhance the protein identification rate (Figure 2). The offline setup is hereby beneficial for optimal precursor ion selection for subsequent MS/MS experiments. First, TOF MS data from all 384 collected fractions were acquired. Afterward, a compound list of all contingent precursor candidates was created and a ranking of each detected ion for each spot was performed. Hereby, the distribution and abundance of each mass was taken into consideration to evaluate which precursor should be fragmented on which spot best to cover as many compounds as possible for following MS/MS experiments. The acquired tandem mass spectrometry data were combined in one data file and subjected to a comprehensive MS/MS ion search following the Mascot algorithm. Thereby, every single fragment spectrum was searched individually, and the results were merged afterward resulting in one protein list without redundancy. Each protein was considered to be a positive match if it was identified by at least one peptide fragment spectrum that fulfilled criteria of significance. In LC-MS/MS based proteomic studies, where protein identification by only one peptide is not uncommon, highly significant annotation scores are essential.[16] The significance threshold was therefore set to 99% ($p \leq 0.01$), corresponding to a Mascot ion score (M) of 28, and peptide uniqueness was required (bold red).

The majority of all proteins identified in this study was plasma proteins. This can be explained with the diffusion of plasma proteins over the blood−follicle barrier, which increases in its permeability during follicle maturation. Hence, plasma proteins with a molecular weight of up to 500 kDa can diffuse into the follicle. Most of the plasma matched proteins are acute

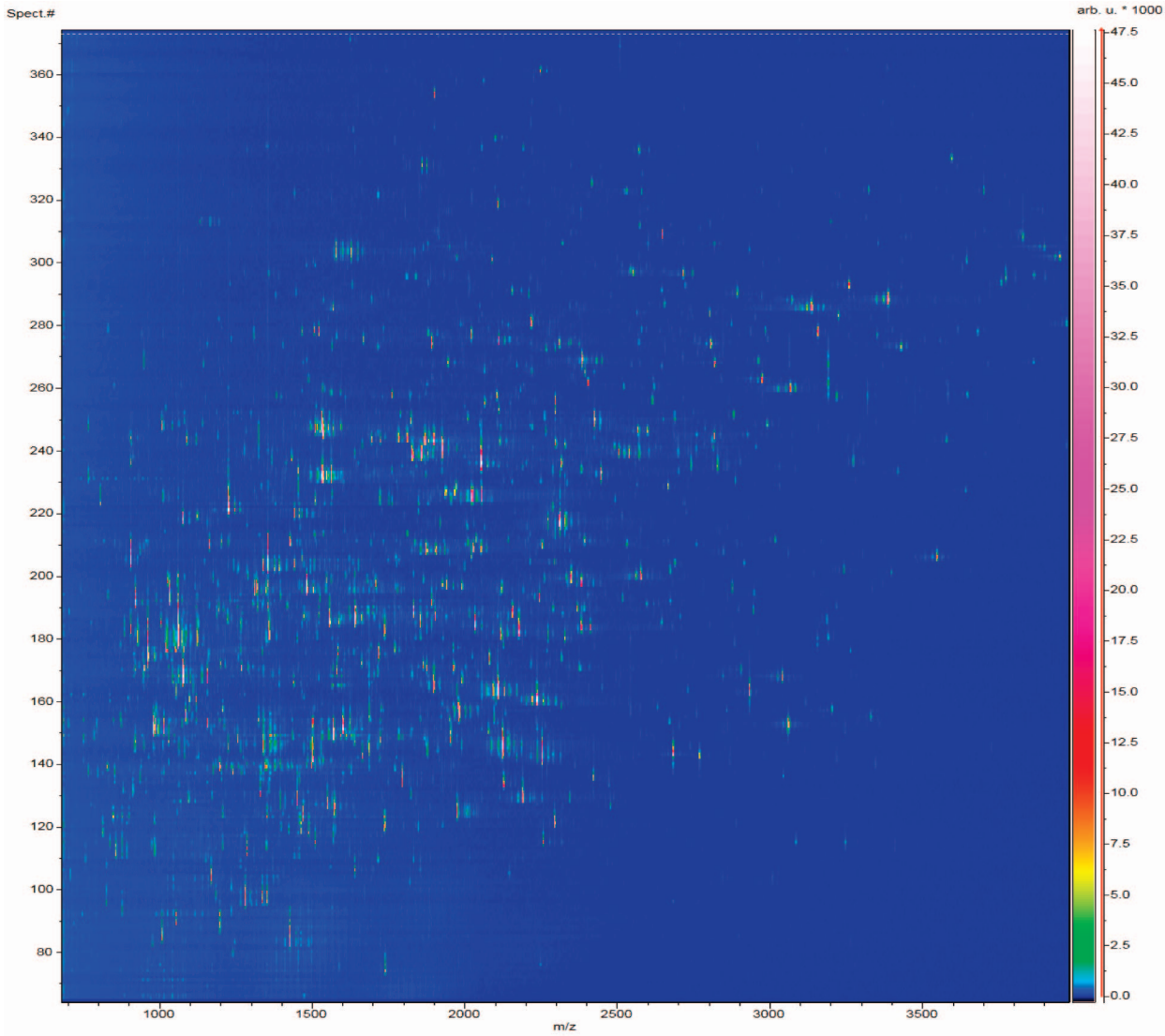

Figure 2. Mass chromatogram of one LC-MALDI MS experiment shown as a three-dimensional survey of all TOF MS spectra (*x*-axis) acquired from each collected fraction during peptide elution (*y*-axis). The values on the *z*-axis (rainbow-scale) indicate mass peak intensities in arbitrary units.

phase proteins (APP), secreted in excess mainly by the liver as a physiological response to inflammation. Representatives of up-regulated acute phase reactants that were found in our study are serum amyloid A-4, complement factors (C1S, C3, C4A, C7, C9, and factor B), coagulation factors (fibrinogen, plasminogen, prothrombin, factor XII), as well as α-1 antitrypsin, α-1 antichymotrypsin, α-2 antiplasmin, α-2 macroglobulin, ceruloplasmin, haptoglobin, heparin cofactor 2, and hemopexin.[17] The high levels of acute phase proteins that were identified in hFF support the assumption that the immune system is crucially involved in folliculogenesis and that, e.g., ovulation can be regarded as a suppressed inflammatory reaction.[18] Further findings were, e.g., vitronectin, a glycoprotein that plays a significant role in a number of physiological processes such as cell adhesion, cell migration, modulation of the immune system, and regulation of the plasminogen activation system;[19,20] zinc α-2 glycoprotein, a soluble fat-depleting factor related to major histocompatibility complex proteins;[21] and Mac 2 binding protein, a cell-adhesive protein of the extracellular matrix which binds β1 integrins, collagens, and fibronectin.[22] Furthermore, gelsolin, an actin and fibronectin binding protein that is involved in various biological processes, F-box only protein 6, a member of the SCF protein ligase complex that promotes protein ubiquitination and degradation, and basement membrane-specific heparan sulfate proteoglycan core protein (perlecan), an integral component of basement membranes that is responsible for the fixed negative electrostatic charge and which is involved in the charge-selective ultrafiltration properties,[23] were identified. Further protein identities detected in this study that have been found in hFF before and whose relevancies in follicular growth have been extensively discussed in previous reports are the insulin growth factor-binding protein acid labile subunit,[24,25] transferrin,[5,26,27] and transthyretin.[5,28–30]

Among our findings, several protein identities that are known to be particularly involved in hormone regulation during reproductive processes could be detected with a proteomic approach for the first time. Those include vasopressin-neurophysin 2 copeptide (VP), sex hormone binding globulin (SHBG), and inhibin A (INHA). The secretion of the peptide hormone vasopressin (VP) from the posterior pituitary is strongly influenced by estrogens,[31–33] and it has been suggested to effect

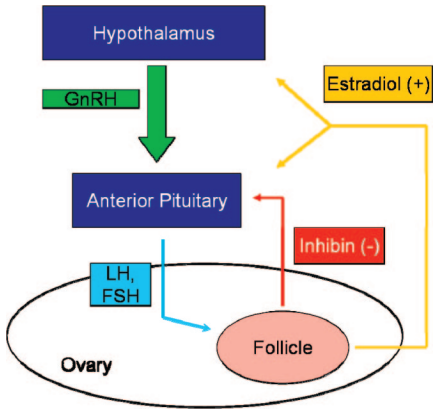

Figure 3. Schematic illustration of sex hormone secretion regulation. SHBG and INHA are key compounds in the human hormone regulatory system. While SHBG indirectly influences LH and GnRH levels by being the essential carrier for estradiol, INHA directly mediates FSH synthesis and secretion.

the contractility of the nonpregnant human myometrium.[34] The main function of sex hormone binding globulin (SHBG), a 90 kDa glycoprotein mainly secreted in the liver, is to regulate the bioavailability of steroid hormones like, e.g., estradiol that mediate LH and GnRH secretion at the anterior pituitary and the hypothalamus, respectively (Figure 3). Therefore, the concentration of SHBG in plasma is the most important known indicator for biological active steroid sex hormones in circulation.[35–38] SHBG has been known to be present in the human ovary for a long time and was proposed to have an important role in the local regulation of ovarian functions.[39] During hormonal treatment for IVF therapy[40–44] that is characterized by producing periovulatory supraphysiological estradiol levels, a more than 2-fold increase of SHBG concentration in hFF from 35 to 87.6 nmol/L has been observed.[45] To validate the identification of SHBG in the sample and to determine its level of abundance, we performed an electrochemiluminescent immunoassay.[14] Here, the protein was found in an elevated concentration of 77 nmol/L which is in strong consistence with previously reported studies.[45] The proteohormone inhibin A (INHA) is a multifunctional glycoprotein that is secreted by the granulosa- and thecal cells in the ovary. Its main function is the negative feedback control of FSH secretion at the pituitary gland.[46] There are significantly different INHA levels in plasma and hFF during the menstrual cycle.[47,48] The evaluation of inhibin concentration is especially suited for monitoring follicular growth during controlled ovarian stimulation for assisted reproduction. Inhibin concentration levels provide information about the number of oocytes retrieved and fertilized, the probability of pregnancy, or ovarian hyperstimulation syndrome.[49–51] For further validation and quantitative evaluation of INHA in hFF, an enzyme-linked immunoassay was performed.[15] The results show an INHA concentration of 73.5 ng/mL (1.85 nmol/L). In a previously reported immunoassay based study on INHA concentration evaluation in hFF from patients undergoing hormonal treatment in the early phase of IVF therapy, a variation of INHA between 10 and 30 ng/mL was observed which reflected changes in follicle size.[52] Changes of inhibin secretion are also indications for ovarian disorders such as polycystic ovarian syndrome (PCOS) or premature ovarian failure (POF) [53–56] as well as for Down's syndrome where INHA quantification is part of the prenatal quad screen during early pregnancy.[57]

4. Conclusion

In this report, we present a study on proteome profiling of human follicular fluid in women undergoing controlled ovarian hyperstimulation for in vitro fertilization therapy by using an alternative bottom-up approach. The here presented proteomic workflow, based on miniaturized in solution IEF and nano-LC coupled offline to MALDI TOF/TOF MS, proved to be a suitable approach for sensitive protein identification in complex biological samples and offers an alternative to other more commonly used proteomic setups. The results show the significant MS/MS data based identification of 69 different proteins in hFF, where 32 proteins have not been described before to be found in hFF with proteomic methods. Among the found matches, two proteins, SHBG and INHA that are essentially involved in reproductive processes could be identified. Complementary immunoassay based validation experiments confirmed these findings. In addition, elevated levels of abundance were observed for both proteins which are consistent with earlier reported clinical studies. SHBG and INHA are the main mediators in sex hormone level regulation and therefore potential markers for future clinical proteomic-based studies that might give further insight to understand ongoing processes on a molecular level during follicular development.

Supporting Information Available: Tables 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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