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Peptide and protein profiles in serum and follicular fluid of women undergoing IVF

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BACKGROUND: Proteins and peptides in human follicular fluid originate from plasma or are produced by follicular structures. Compositional changes reflect oocyte maturation and can be used as diagnostic markers. The aim of the study was to determine protein and peptide profiles in paired serum and follicular fluid samples from women undergoing IVF. METHODS: Surface-enhanced laser desorption and ionization–time of flight–mass spectrometry (SELDI-TOF-MS) was used to obtain characteristic protein pattern. RESULTS: One hundred and eighty-six individual MS signals were obtained from a combination of enrichment on strong anion exchanger (110), weak cation exchanger (52) and normal phase surfaces (24). On the basis of molecular masses, isoelectric points and immunoreactivety, four signals were identified as haptoglobin (α_1- and α_2-chain), haptoglobin 1 and transthyretin (TTR). Immunological and MS characteristics of the TTR : retinol-binding protein (RBP) transport complex revealed no microheterogeneity differences between serum and follicular fluid. Discriminatory patterns arising from decision-tree-based classification and regression analysis distinguished between serum and follicular fluid with a sensitivity and specificity of 100%. CONCLUSIONS: Quantitative and qualitative differences indicate selective transport processes rather than mere filtration across the blood-follicle barrier. Identified proteins as well as characteristic peptide and/or protein signatures might emerge as potential candidates for diagnostic markers of follicle and/or oocyte maturation and thus oocyte quality.

Key words: human follicular fluid/peptide/protein/proteome/serum

Introduction

During follicular maturation, the follicular fluid constitutes the microenvironment for the oocyte. It contains substances implicated in oocyte meiosis, rupture of the follicular wall (ovulation), differentiation of the ovarian cells into the functional corpus luteum and finally fertilization (Richards, 1994; Tsafriri, 1995; Tsafriri and Reich, 1999). Thus, the composition of follicular fluid reflects stages of oocyte development and the degree of follicle maturation (Eppig *et al*., 2005; Sugiura *et al*., 2005). In consequence, certain components of follicular fluid might be of use as indicators for the maturation and thus the quality of the follicle (Mihm *et al*., 2000; Anahory *et al*., 2002; Mendoza *et al*., 2002).

Because follicular fluid is a product of both the transfer of blood plasma constituents that cross the blood follicular barrier and the metabolism of the granulosa cells, the concentration of specific peptides and proteins in follicular fluid can be seen as a reflection of this transfer and influenced by the metabolism of such follicular structures. With regard to the transfer of plasma proteins, the blood-follicle barrier was found to be permeable for proteins with molecular masses <500 kDa only. In addition to the size, the charge of the proteins effects the plasma protein transfer into follicular fluid (Hess *et al*., 1998). On the basis of these restrictions, the intrafollicular concentration of selected proteins is inversely related to their molecular mass, whereas the total concentration of proteins in follicular fluid is comparable with that in plasma (Shalgi *et al*., 1973; Andersen *et al*., 1976).

Numerous studies have investigated selected proteins in follicular fluid (Spitzer *et al*., 1996; Pellicer *et al*., 1999; Schweigert *et al*., 2003), but only a few so far have described the protein pattern and none the peptide pattern in follicular fluid (Pellicer *et al*., 1999; Wunder *et al*., 2005; Kim *et al*., 2006). The few studies that describe the proteome of the follicular fluid used 2D polyacrylamide gel electrophoresis (2D PAGE). This method, which is currently the golden standard for the elucidation of variations in the expression of proteins, is however time-consuming, expensive and difficult to reproduce, and only the more abundant proteins can be detected. Furthermore, the method is not able to separate peptides. This is of specific

importance because many hormones, growth factors and regulatory peptides belong to this size category. Until now, only three studies using MALDI–time of flight–mass spectrometry (MALDI-TOF-MS) are available for analysing follicular fluid (Anahory *et al*., 2002; Lee *et al*., 2005; Maniwa *et al*., 2005). Among the new technologies developed, the surface-enhanced laser desorption and ionization (SELDI)-TOF-MS approach combines the selectivity, through affinity chip surfaces, with the mass accuracy of MS (Issaq *et al*., 2002; Caputo *et al*., 2003). Different chromatographic surfaces are used to absorb proteins by various non-covalent interactions. Because of its high mass accuracy, post-translational modifications that occur as a result of such processes as limited proteolysis, glycosylation and phosphorylation can be identified (Schweigert, 2005). Protein and peptide signatures obtained from plasma of individuals with different disease states or treatments allow the description of differences without the absolute necessity for protein identification. Under ideal conditions, the pattern is very simple consisting of only a few proteins or peptides, which build the structure of a decision tree. Such an approach has been applied, for example, to single-out stage I ovarian cancer patients with a sensitivity of 100% and a specificity of 95%, in determining markers of prostate cancer in men or to characterize the effect of hormone replacement therapy on plasma proteins (Ardekani *et al*., 2002; Espina *et al*., 2004; Gericke *et al*., 2005; Xiao *et al*., 2005).

This study was thus conducted to compare the peptide and protein profiles of paired serum and follicular fluid samples of women undergoing IVF using the SELDI-TOF-MS to validate possible differences between serum and follicular fluid especially as a basis for a later evaluation of biomarkers of follicle and/or oocyte quality and thus as markers for fertilization success. Special emphasis was put into the molecular characterization of the protein complex of retinol-binding protein (RBP) and transthyretin (TTR) because both proteins showed quality-related changes in follicular fluid (Anahory *et al*., 2002).

Materials and methods

Patients

Paired samples of follicular fluid and serum were obtained from 15 women (age 33 ± 5.2 years, mean ± SD) undergoing IVF at the Department of Obstetrics, Charité, University Medicine Berlin, Campus Virchow-Klinikum. Follicular fluid was obtained from the largest follicle present. The study protocol was approved by the ethics committee of the hospital. Informed consent for experimental use of serum and follicular fluid was obtained from all patients. IVF stimulation and sample collection were performed as described previously (Schweigert *et al*., 2003).

SELDI-TOF-MS analyses

Serum and follicular fluid samples were analysed using the SELDI-TOF-MS-based ProteinChip® System (PBS II, Ciphergen Biosystems, Fremont, USA) with ProteinChip arrays. Proteins were enriched on three different surfaces: (i) a strong anion exchange, with cationic, quaternary ammonium groups as spot surfaces that interact with the negative charges on the surface of target, (ii) a weak cation exchange, with anionc, carboxylate groups that interact with positive charges as spot surfaces and (iii) a normal phase surface, with active spots containing silicon oxide which allows proteins to bind via serine, threonine or lysine. The weak ionic spot surface was first equilibrated by the application of 300 µl of 0.1 M HCl per spot and incubated for 5 min at 30°C. Following equilibration, buffer was carefully aspirated without allowing spots to dry. Both types of surfaces were incubated for 5 min at 30°C with binding buffer (optimal conditions were reached with 0.1 M sodium phosphate (pH 6.5) for both ionic chip surfaces). Subsequently, 10 µl of sample was mixed with 190 µl of binding buffer and added to the spot surface. Peptides and proteins were captured on both surfaces after being incubated for 60 min at 30°C and shaken. Unbound proteins and other contaminants were removed from the spot surfaces by washing the spots for 15 min with 300 µl of binding buffer. Thereafter, the arrays were rinsed twice for a few seconds with de-ionized distilled water to remove salts. Excess water was subsequently removed. The normal phase chip surface was incubated for 15 min with 15 µl of sample at 30°C and rinsed twice with distilled water for a few seconds. Finally, although the surfaces were still moist, 1 µl of a saturated energy-absorbing molecule (EAM) solution [sinapinic acid dissolved in 50% acetonitrile and 0.5% trifluoracetic acid (TFA)] was applied to the spot surfaces, and the samples were allowed to dry. Follicular fluid and serum samples were run concurrently and intermingled on the same chip. The instrument was used in a positive ion mode, with an ion acceleration potential of 20 kV and a detector gain voltage of 2 kV. The chips were analysed manually under the following settings: laser intensity 230 (low) and 260 (high), detector sensitivity 9 and molecular mass range 1000–50 000 Da. All mass spectra were normalized to have the same total ion current. The m/z range from 0 to 3000 was eliminated from the analysis because this area contains adducts possibly from the chip surfaces and artefacts from matrix components. The peaks with S/N ratio >5 were chosen for analysis.

To surely identify proteins, which were putatively identified on the basis of their molecular weight and isoelectric point, we applied an immunoassay (Schweigert *et al*., 2004). The different antibodies (haptoglobin, prealbumin and transferrin; DakoCytomation, Germany) were coupled on the chip surface as has previously been described (Schweigert *et al*., 2004).

Cytochrome C (equine cardiac; 12 360.1 MW), myoglobin (equine cardiac; 16 951.5 MW), glyceraldehyde-3-phosphatedehydrogenase (GAPDH) (rabbit; 35 688 MW), albumin (bovine serum; 66 433 MW) and β-galactosidase (*Escherichia coli*; 116 351 MW) were used as calibrators. Mass resolution (defined as $m/\Delta m$) is routinely in the range of 300–400, and mass accuracy was within 0.1%. Peaks with amplitudes at least five times greater than the average background noise level were considered. The reproducibility was tested by depositing different aliquots of the same sample on eight different spots of the ProteinChip array.

Immunoprecipitation of RBP/TTR complex and MALDI-TOF-MS of TTR and RBP

Fifteen microlitres of serum or follicular fluid was treated with equal amounts of a polyclonal rabbit anti-human prealbumin or RBP (DakoCytomation, Denmark) and with 15 µl Sephadex G-15 and 1 mg/ml phosphate-buffered saline (PBS) (Pharmacia Fine Chemicals, Sweden). The mixture was incubated for 2 h at 37°C and then centrifuged at 15 000 × g for 15 min at room temperature. The supernatant was removed, and the immunoprecipitated complex of TTR and antibody or of antibody and RBP/TTR was then extensively washed (three times) with PBS, and the final precipitate was redissolved in 5 µl of PBS.

MALDI mass spectra of the precipitated RBP–TTR complex from serum and follicular fluid were obtained using a Reflex II MALDI-TOF mass spectrometer (Bruker-Daltonik, Bremen, Germany).

MALDI-TOF-MS of serum samples was performed in linear mode at 20 k acceleration voltage using sinapic acid as matrix. For ionization, a nitrogen laser (337 nm, 3-ns pulse width, 3 Hz) was used. The samples were prepared in a two-step procedure. First, 1 µl of immunoprecipitated sample was deposited on the target and dried. Second, 0.5 µl of saturated sinapinic acid solution was placed on the serum drop and also dried. This step was repeated. The matrix solution contained 1 mg of sinapinic acid and equal amounts (25 µl) of 1% TFA and acetonitrile. For the optimization of the mass spectra, the laser was aimed either at the central area of the sample or at the outmost edge of the crystal rim. All spectra were measured using external calibration.

To determine the disulphide linkage of TTR adducts, the immunoprecipitated TTR was treated with dithiothreitol (DTT). DTT solution, 100 mM in buffer (100 mM NH_4CO_3, pH 8.8), was added to the solution at a ratio of 1:1 (DTT solution volume/TTR solution volume). The mixture was incubated for 2 h at room temperature, and precipitated samples were subsequently subjected to MALDI-TOF-MS.

TTR and RBP immunoblot analysis

To assure the identification of TTR and to assess its association with RBP, we performed sodium dodecyl sulphate (SDS)–PAGE immunoblot analysis. For this purpose, we separated the protein complex of RBP and TTR from serum and follicular fluid by immunoprecipitation with an antibody against either RBP or TTR as described above. After precipitation, electrophoretic separation and blotting, TTR and RBP were visualized with the appropriate antibodies. Images were processed using the ChemiDoc™ XRS (Bio-Rad, Munich, Germany) and the Quantity One 1D Analysis Software (Bio-Rad).

Data analysis and statistics

All data are given as mean ± SD. Statistical analysis was accomplished by the use of non-parametric procedures. The Mann–Whitney *U*-rank test was used to test for significant differences between groups. Values of $P < 0.05$ were considered significant. Obtained spectra were subjected to bioinformatic analysis using Biomarker Patterns™ Software (Ciphergen Biosystems) for classification and regression tree analysis to identify the candidates that could be used to distinguish between follicular fluid and serum. Proteins were tentatively identified using the molecule mass and binding characteristics (isoelectric point) using Swiss-Prot release 41.10 and TrEMBL release 23.14 (http://usexpasy.org/sprot/). Searches were carried out using the 'homo sapiens' database. Additionally, haptoglobin α_1-chain, haptoglobin α_2-chain, haptoglobin1 and TTR were detected via MS-immunoassay (Schweigert *et al*., 2004).

Results

A total of 186 proteins and peptide mass signals were isolated from serum and follicular fluid when selectively enriched on three different affinity chip surfaces (24 on normal phase chip surface, 110 on strong anionic chip surface and 52 on weak cation chip surface). Table 1 summarizes these proteins that were used to distinguish between serum and follicular fluid. On the basis of this data sheet, 10 proteins were putatively identified based on molecular mass and isoelectric point (Figure 1). For four of them, haptoglobin α_1-chain, haptoglobin α_2-chain, haptoglobin1 and TTR, it was possible to identify the proteins by selective on-chip enrichment and coupled antibodies.

In Figure 2, significant differences in the protein patterns of serum and follicular fluid are shown as a scatter blot. Data show that individual peptides and proteins were highly variable with regard to correlation between serum and follicular fluid and follicular fluid ratio in percentage of serum.

Some peptide signals, such as for the peptides with molecular masses of 7666, 7746 and 7840 (strong anion exchange surface) or 7718 (weak cation exchange surface), are very low in follicular fluid despite their substantial intensities in serum. Others, albeit fewer peptides of the molecular masses (kDa) 6937 and 13 851 (strong anion exchange surface) or 6427 and 14 039 (normal phase chip surface) showed greater signals in follicular fluid. A classification and regression tree was built with the Biomarker Pattern Software and also a decision tree system based on the original CART code, to discriminate between serum and follicular fluid with sensitivity and specificity of 100% (Figure 3).

One protein with a greater signal in follicular fluid, namely TTR, was identified by MS immunoassay. As in serum, TTR in follicular fluid exists with characteristic microheterogeneity caused by a modification at the Cys10 position. No difference in this microheterogeneity was observed between serum and follicular fluid. The modification at the Cys10 position of the TTR molecule can be verified by the treatment of TTR with DTT, resulting in the single native form of TTR (data not shown). As in serum, the immunoprecipitation of RBP from follicular fluid resulted in an enrichment of the typical RBP/TTR complex, whereas the selective precipitation of TTR resulted in the enrichment of TTR only (Figure 4).

Table 1. Comparison of the molecular weights of proteins in serum and follicular fluid which were used to distinguish between both body fluids in recent studies and the determined molecular weights of the present study and additionally results from the Swiss-Prot database

Protein	SELDI-detected molecular weight (Da)	Literature	Swiss-Prot
Haptoglobin α_1-chain	9130	8–10 kDa[a]	9192.21
Transthyretin	13 763	13 800 Da[a]	13 761.41 Da
Haptoglobin α_2-chain	17 276	17 000 Da[a]	15 945.77 Da
AMBP protein	39 662	31–33.5 kDa[a]	38 999 Da
Haptoglobin 1	41 790	37.3–44.5 kDa[a]	38 452 Da
α_1-Antitrypsin	44 305	58–62 kDa[a]	44 324.55 Da
Serum albumin	66 479	66–70 kDa[a]	66 472.21 Da
Serotransferrin	78 876	7783 kDa[a]	75 181.44 Da
Gonadotrophin surge-attenuating factor (GnSAF)	59 565	60–66 kDa[b]	–
Sperm-attracting activity-associated protein	8560	8.6 kDa[c]	–

SELDI, surface-enhanced laser desorption and ionization.
[a]Anahory *et al*. (2002).
[b]Fowler *et al*. (2002).
[c]Serrano *et al*. (2001).

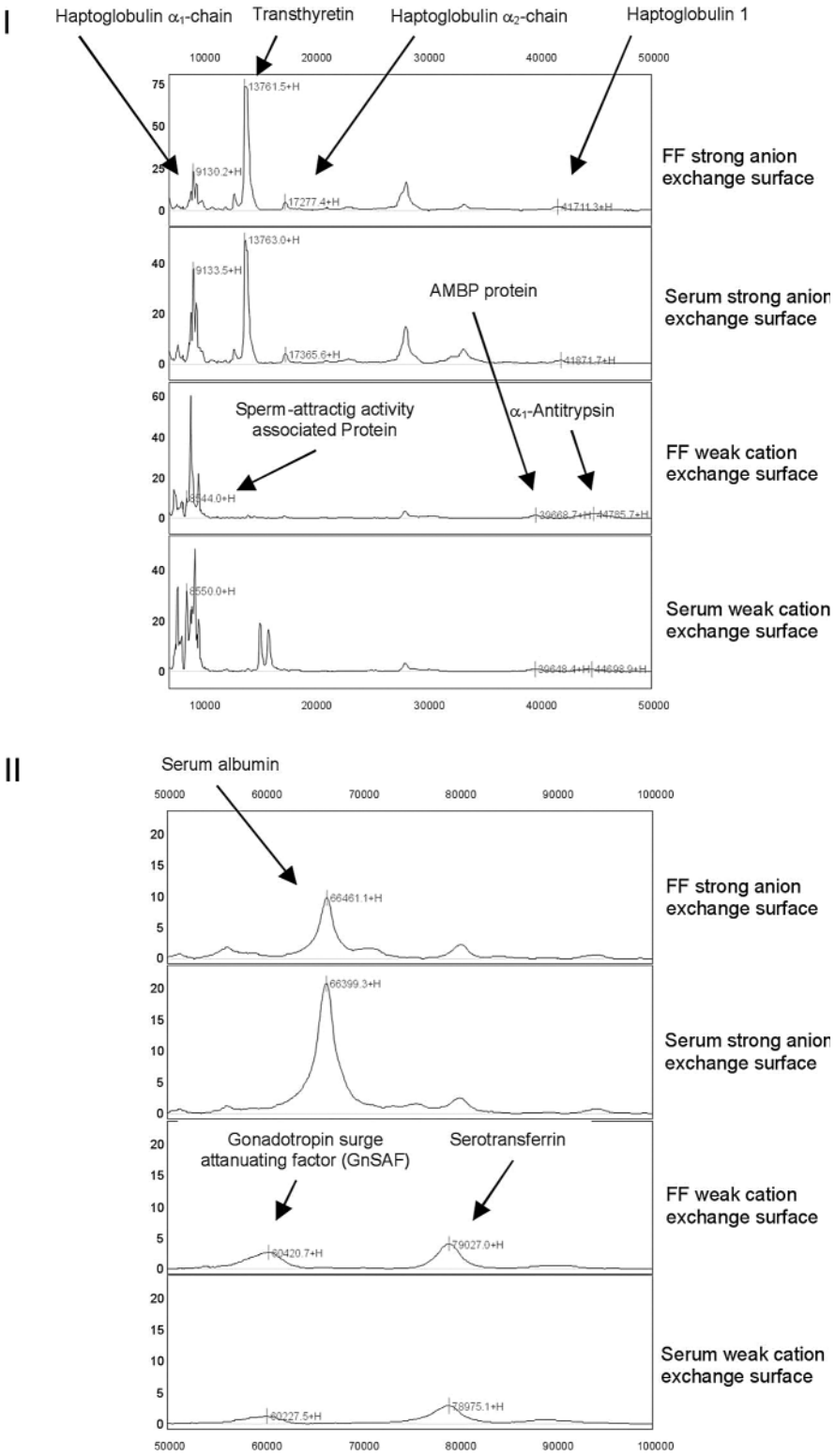
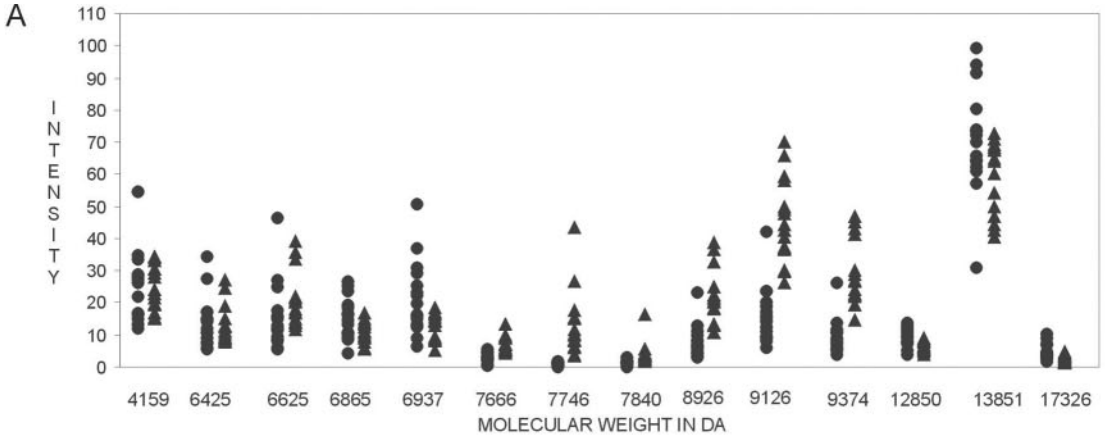
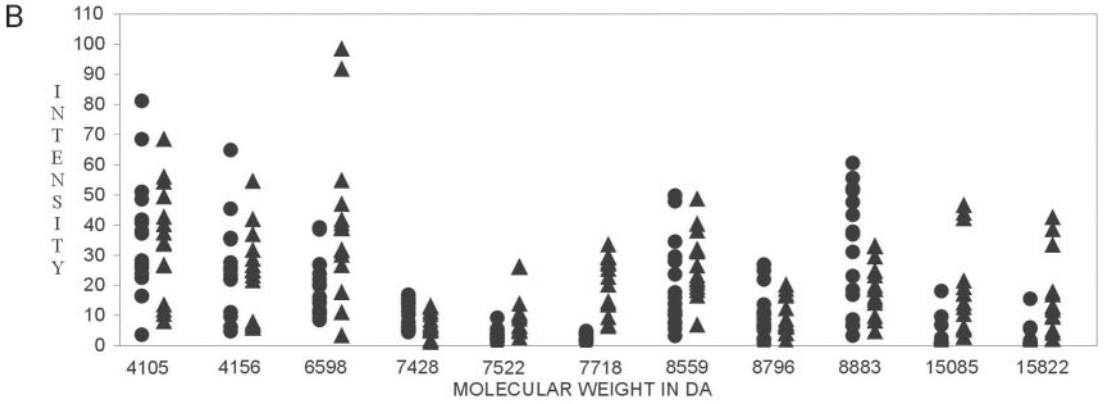


Figure 1. Representation of the surface-enhanced laser desorption and ionization–time of flight–mass spectrometry (SELDI-TOF-MS) on different chip surfaces in the molecular weight range of 10 000–50 000 Da (I) and 50 000–100 000 Da (II) and identification of individual proteins.

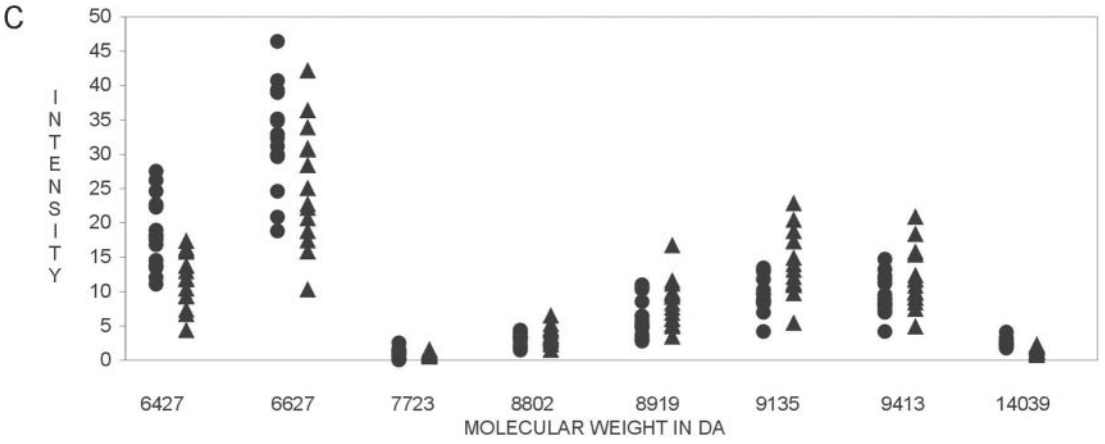
With the use of MALDI-TOF-MS, we detected TTR and RBP in serum and follicular fluid (Figure 5). Four major mass signals were observed for TTR from follicular fluid at 13 776 ± 18 Da (serum: 13 756 ± 11 Da), 13 900 ± 16 Da (serum: 13 881 ± 9 Da), 13 961 ± 20 Da (serum: 13 934 ± 6 Da) and 14 101 ± 18 Da (serum: 14 077 ± 6 Da) after internal calibration. These signals represented native, S-cysteinylated, S-cysteinglycinylated and S-glutathionylated TTR, respectively. The RBP in follicular fluid shows three mass signals: the dominant RBP 21 090 ± 29 Da (serum: 21 072 ± 16 Da) and the two post-translational



M/Z	4159.58	6425.11	6625.28	6865.44	6937.70	7666.57	7746.18	7840.97	8926.40	9126.54	9374.20	12850.49	13851.50	17326.90
p	0.9399	0.7062	0.0382	0.0348	0.0261	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0029	0.0316	0.0083
correlation	0,3934	0,3064	0,2264	0,3870	0,5580	0,1553	-0,4292	-0,1156	0,3451	0,3147	0,3127	0,4376	0,4010	-0,3817
ratio in % of serum	113,17	121,06	81,06	153,01	167,99	44,34	14,97	48,28	37,79	36,38	36,15	146,38	121,44	227,13



M/Z	4105.85	4156.91	6598.04	7428.44	7522.50	7718.23	8559.76	8796.05	8883.65	15085.35	15822.48
p	0.9369	0.8125	0.0050	0.0361	0.0000	0.0000	0.0819	0.9056	0.1138	0.0000	0.0000
correlation	-0,5782	-0,0389	0,3909	0,3309	-0,0501	0,1021	0,1489	-0,0311	0,2711	0,0500	0,0939
ratio in % of serum	208,91	200,44	79,16	272,79	45,07	19,98	82,78	164,95	198,66	21,90	19,49



M/Z	6427,35	6627,04	7723,72	8802,49	8919,04	9135,46	9413,16	14039,86
p	0,0007	0,0401	0,7557	0,3297	0,0538	0,0030	0,1013	0,0000
correlation	-0,1269	-0,1894	0,1089	-0,3204	-0,0481	0,1755	0,3274	-0,4210
ratio in % of serum	184,92	144,87	85,26	101,00	86,22	76,24	89,26	205,81

Figure 2. Scatter blot of peptides and proteins in follicular fluid (Δ) and serum (□) with molecular masses, significances of difference, correlation coefficient and ratio between serum and follicular fluid separated on different affinity chip surfaces such as (**A**) a strong anion exchange surface, (**B**) a weak cation exchange surface and (**C**) a chip with normal phase surface.

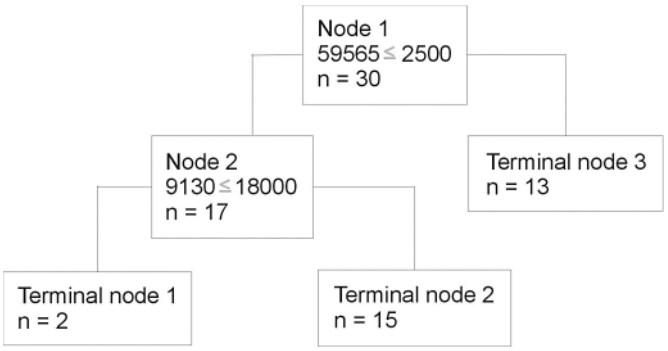


Figure 3. Classification and regression tree for a protein pattern to discriminate between serum and follicular fluid (sensitivity and specificity 100%). The Biomarker Pattern Software is a decision tree system based on the original CART code developed by Stanford University and University of California at Berkeley [Statisticians Breiman, Friedman, Olshen and Stone (Biomarker Pattern Software, Users Guide)]. If the answer to a question in a node of the tree is yes, one proceeds down to the left node, whereas after a negative answer, one proceeds down to the right node. The molecular mass is given in dalton. The high sensitivity and specificity is achieved after putting together all data obtained from surface-enhanced laser desorption and ionization–time of flight–mass spectrometry (SELDI) analysis.

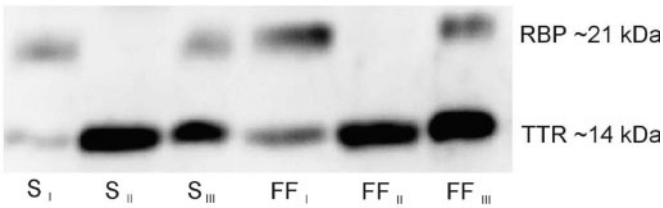


Figure 4. Western blot of transthyretin (TTR) and retinol-binding protein (RBP) after sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using a polyclonal antibody in the paired serum and follicular fluid of six representative women. FF, follicular fluid; S, serum; I, untreated fluid; II, pellet after immunoprecipitation with TTR antibody; III, pellet after immunoprecipitation with RBP antibody.

processed forms RBP_1 20 988 ± 33 Da (serum: 20 956 ± 33 Da) and RBP_2 20 758 ± 5 Da (serum: 20 843 ± 9 Da).

Discussion

Determination of the protein and peptide composition of follicular fluid may not only lead to an improved understanding of ovarian physiology, especially in regard to follicular development and oocyte maturation, but also offers new insights into the possibility of using selected peptides or proteins as markers of development to optimize selection processes during IVF. In this study, characteristic peptides and proteins were identified using a novel proteomic technology, the SELDI-TOF-MS. This method needs only small amounts of sample, describes the peptide patterns and delivers reliable and reproducible data. Many proteins formerly masked by others in the crude sample are visible after this separation for the very first time, therefore increasing the chances of finding novel biomarkers. Furthermore, proteins <25 kDa and those with extremes of hydrophobicity or isoelectric point are more easily accessible as with the 2D-gel approach.

The number of proteins detected in serum and follicular fluid by SELDI-TOF-MS screening is within the range reported in studies for follicular fluid as observed using high-resolution 2D PAGE (Spitzer *et al*., 1996; Anahory *et al*., 2002). Differences between paired follicular fluid and serum samples in peptide and protein profiles were significant for many peptides and proteins. Furthermore, our results are in contrast to studies showing a close inverse relationship between molecular mass and intrafollicular concentration (Andersen *et al*., 1976). In this study, however, proteins were of molecular masses >20 kDa. The greater variability of this study for the correlation between follicular fluid and serum and follicular fluid ratio in percentage of serum might indicate that individual peptides and proteins are not exclusively filtered but also locally synthesized. This is further supported by the observation that in some instances, levels of selected peptides are present in very low concentrations in follicular fluid despite substantial amounts in serum, whereas for another smaller group, such peptides are present in greater amounts in the follicular fluid.

During folliculogenesis, follicles become more permeable to plasma proteins, resulting in a progressive increase in the number of serum proteins capable of passing through the blood-follicle barrier. As a result, there are significant similarities between proteins in follicular fluid and serum (Manarang-Pangan *et al*., 1971). In addition to these serum proteins, follicular fluid also contains proteins produced by both the granulosa and the thecal cells (Nandedkar *et al*., 1992). Using Biomarker Patterns Software for the three chip surfaces and optimized measurement, we found a classification and regression tree for two of these proteins, allowing the classification of serum and follicular fluid with a sensitivity and specificity of 100% (Figure 3). Both the parent nodes are the proteins gonadotrophin surge-attenuating factor (GnSAF) and the glycoprotein haptoglobin α_1-chain.

Discovered in 1939 (Polonovski and Jayle, 1939), the protein haptoglobin which is synthesized in the liver is a tetramer in its active form, comprising two α- and two β-subunits and is known to bind free haemoglobin. It is produced as an acute phase reactant during infection, inflammation, traumatic damage and malignant proliferation. Bottini *et al*. (1999) pointed to a significant impact of haptoglobin on the fertility of women. Haptoglobin transport in the follicle depends on the integrity of the blood-follicle barrier and might be associated with oocyte quality, possibly by interfering with the role of apolipoprotein A-1 in cholesterol or vitamin E exchange between high-density lipoproteins and granulosa cells (Porta *et al*., 1999). In human follicular fluid, haptoglobin inhibits the reverse transport of cholesterol by preventing the ApoA1 stimulation of the LCAT activity (Balestrieri *et al*., 2001). In water buffalo, haptoglobin can be used as a molecular marker to assess the physiological state of the blood-follicle barrier or to discriminate between atretic and healthy follicles (Bergamo *et al*., 1995).

A study describing the human follicular fluid proteome by 2D electrophoresis singled out four proteins in follicular fluid as markers of follicular quality. Two of these were RBP and TTR (Anahory *et al*., 2002). TTR belongs to a group of proteins,

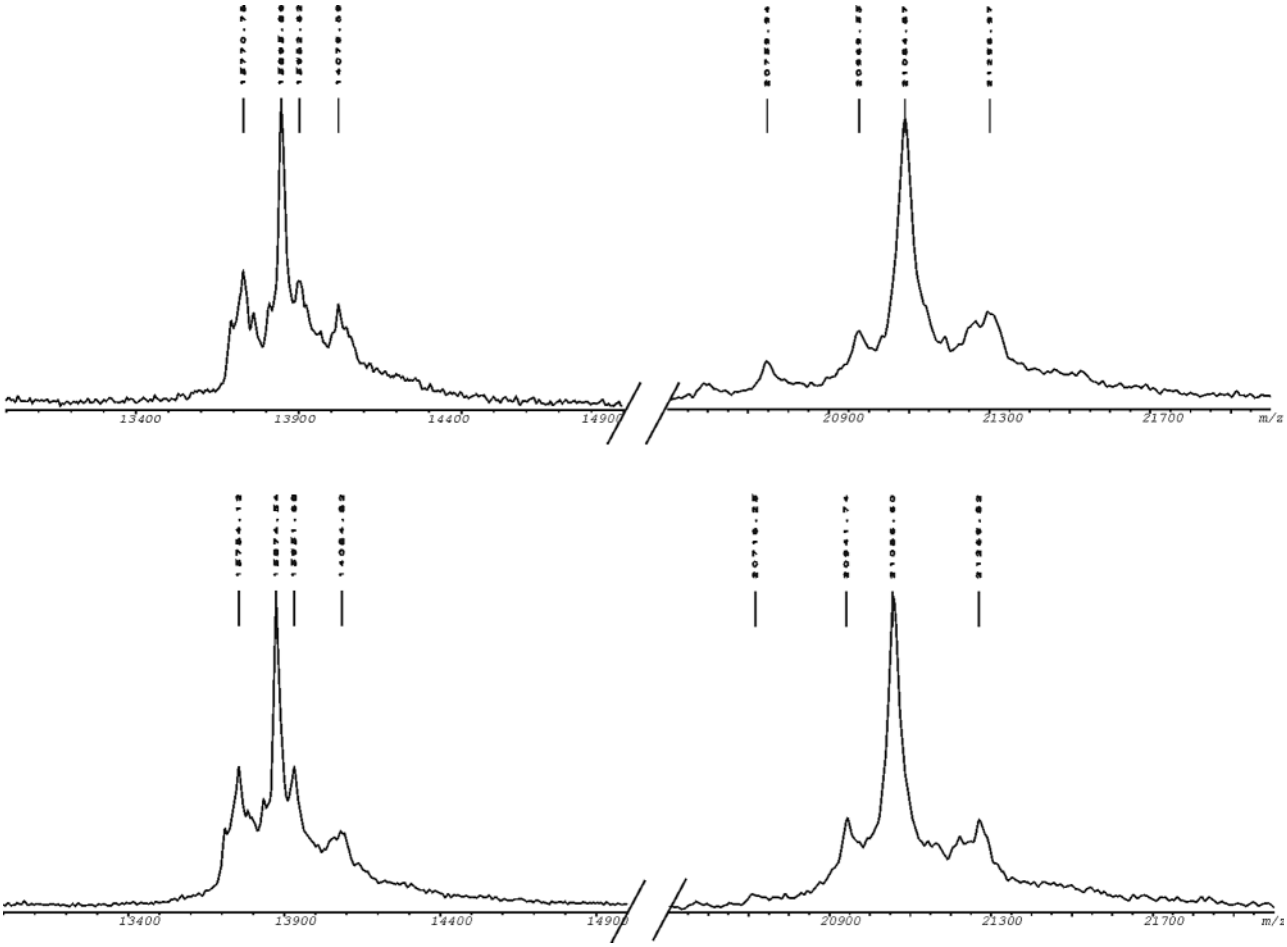


Figure 5. Microheterogeneity of transthyretin (TTR) (left) and retinol-binding protein (RBP) (right) in serum (I) and follicular fluid (II) detected via MALDI-TOF-MS after the immunoprecipitation of RBP. Isoforms of TTR result when the Cys^{10} residue of the native TTR (~13 740 Da) makes a mixed disulphide with the amino acid cysteine (~13 880 Da), the peptide cysteinyl-glycine (~13 930 Da) or the peptide glutathione (14 055 Da). Isoforms of RBP result from the full-length molecule C-terminally truncated by one or forms des(^{182}Leu)RBP and des(^{182}Leu–^{183}Leu)RBP.

which includes thyroxine-binding globulin and albumin, that bind to and transport thyroid hormones and is also indirectly implicated in the carriage of vitamin A. Retinol is essential for reproduction, and retinoids have been suggested to play a role in ovarian steroidogenesis, oocyte maturation and early embryonic development. Schweigert and Zucker (1988) and more recently Brown *et al*. (2003) reported the presence of retinol or RBP in the follicular fluid of bovine follicles. Both studies observed retinol and RBP concentrations to be the highest in large non-atretic follicles and lowest in small atretic follicles. Additionally, in the later study, both the mRNA and the immunoreactive RBP were detected in granulosa cells, theca cells and the blood vessels lining the follicle. Mohan *et al*. (2003) detected mRNA for RBP in cumulus cells. All these findings indicate that retinol is available to the follicle either via passive filtration from serum across the blood-follicular barrier into the follicular fluid or by the local secretion of blood-derived retinol associated with locally synthesized RBP. In favour of a passive transfer is the observation that in humans and in cattle, retinol and RBP in follicular fluid are lower than in serum. The concentration ratio observed is in the order of magnitude as expected from the molecular mass of the complex (Schweigert and Zucker, 1988; Schweigert *et al*., 2003).

For the evaluation of the importance of retinol in follicular fluid for follicle and oocyte development and for the possible application of RBP for diagnostic purposes, we used different immunological approaches to characterize the complex of RRP and TTR in serum and follicular fluid. In this study, only TTR was detected by mass screening in both body fluids. An explanation for the lack of RBP detection by SELDI screening might lie in its lower concentration and selected binding with the washing conditions applied for the screening. We were however able to show its presence immunologically. Both proteins in serum and follicular fluid were identified with certainty by immunoprecipitation, immunoblot and on-chip immunoassay by SELDI-TOF-MS. Using different antibodies against RBP and TTR for the immunoprecipitation of the complex, we observed obvious differences in the precipitation pattern. Whilst the RBP antibody extracted both RBP and TTR from serum and follicular fluid, the TTR antibody selectively enriched only TTR. This might be due to differences in binding affinity for the complexed and the non-complexed forms of TTR. The comparison of serum with follicular fluid showed

however that in both body fluids, RBP and TTR are present in the characteristic complex.

This similarity was further strengthened by the characterization of the complex with MALDI-TOF-MS. Both RBP and TTR showed a very similar pattern of molecule microheterogeneity. The four variants of the TTR molecule correspond to previous studies (Schweigert *et al*., 2004; Schweigert, 2005; Shimizu *et al*., 2006). The mixed disulphides with amino acids are bound by the single cystein residue on position 10 of the TTR molecule. The modification at this position can be validated by the treatment of TTR with DTT, which results in original TTR in its native SH form (data not shown). The masses for RBP correspond with the results from Jaconi *et al*. (1995), who described three different molecular forms of RBP, the full-length form and the two C-terminally truncated forms des(^{182}Leu)RBP and des(^{182}Leu^{-183}Leu)RBP, which are named RBP_1 and RBP_2, respectively.

On the basis of the similarity of the behaviour of the transport complex and the similarity in its microheterogeneity for retinol, it might be assumed that both retinol and the RBP–TTR complex in follicular fluid originate from serum and that possible quality-dependant changes are then observed in follicular fluid (Schweigert and Zucker, 1988; Anahory *et al*., 2002; Brown *et al*., 2003). The quantitative changes might be attributed to changes in the permeability of the blood-follicular fluid barrier during maturation (Suchanek *et al*., 1990). However, although both these aspects appear in favour of filtration, local modulation of the concentration of the complex should not be excluded because the different locations of synthesis, such as liver or granulosa cells, need not automatically result in differences of the microheterogeneity.

In conclusion, despite the fact that the question concerning the origin of the retinol–RBP–TTR complex in follicular fluid remains unresolved, the study enriches our understanding and points to the possibility of a combination of both passive transfer and local synthesis in the granulosa cells. The study further shows that a high throughput screening of peptide and protein profiles gives important and valuable results necessary for a deeper understanding of physiological processes. Vital for this purpose is also an intensive characterization of specific peptides and proteins including protein–protein interactions as well as the microheterogeneity possibly resulting from post-translational changes as seen in the case of RBP and TTR.

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