Chapter

Proteomics as a Future Tool for Improving IVF Outcome

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Abstract

New technical and methodical and more efficient approaches beyond preimplantation genetic screening (PGS) are needed to elevate success rates in in vitro fertilization (IVF). One new approach could be the characterization of the embryos' proteome during the IVF process. This means that specific proteins secreted by the embryo in the surrounding cultivating medium can be analyzed and compared between embryos in order to identify potential markers for a successful embryo transfer and resulting pregnancy. Furthermore, this procedure could result with understanding the processes during the whole time of incubation, from the moment of oocyte fertilization until embryo transfer and subsequently implantation by analyzing the culturing medium used in multiple culture medium exchange during the cultivation period. This procedure of embryo transfer to a new culture medium is essential for the embryo's development and is performed daily or at least when the embryos reached the stage of embryoblast at day 4. The remaining medium after embryo removal is routinely discarded. However, this medium still can be useful for a detailed analysis of proteins and lipids that were secreted by the embryo during the previous incubation process and could help gaining information on the embryos' current developmental status.

Keywords: in vitro fertilization, proteomics, pregnancy, oocyte, pregnancy outcome

1. Introduction

The European Society of Human Reproduction and Embryology (ESHRE) report from February 2018 shows that Europe continues to lead the world with approximately 50% of all reported in vitro fertilization (IVF) cycles. The latest figures are available for 2014, and these show that 39 European countries reported almost 800.000 cycles, which compare to 150.000 cycles in the USA or 65.000 cycles in Australia and New Zealand (https://www.eshre.eu/~/media/sitecore-files/Guidelines/ART-fact-sheet_vFebr18_VG.pdf?la=en). Although there are no official reports from China, it is estimated that this country performs more than 800.000 IVF cycles with a tendency to grow. In summary, the ESHRE estimates that around 2.5 million IVF cycles is performed every year and that about 500.000 babies are born following the IVF.

The pregnancy rate depends on the embryo quality, and in Europe in 2014, the mean pregnancy rate per embryo transfer was 35% after IVF, 33% after ICSI, 30% after frozen embryo transfer, and 59% after egg donation. It is observed that pregnancy rates are higher in younger patients (<35 years).

There are large differences between countries concerning the number of embryos transferred and the resulting multiple births that can occur following the multiple embryo transfer. Multiple pregnancies are classified as high-risk pregnancies and pose a significant health risk to both the mother and baby. However, regardless of that, the practice of multiple embryo transfer is still present, and it is being widely used in order to increase the chances of pregnancy. Currently, efforts are made to reduce the number of multiple embryo transfer, and a mean of 1.81 embryos per transfer is being reported. In Europe, according to the report of the European Society of Human Reproduction and Embryology, the number of multiple delivery rates for embryo transfers dropped from 26.9% in year 2000 to 17.5% in 2014 (latest data available), and it is expected to decline further. Some countries, e.g., Sweden, has reached a very low multiple delivery rate, and the single embryo transfer was performed in more than two-thirds of all transfers.

The importance of IVF in society is also highlighted by the 2010 Nobel Prize which has been awarded to Robert Edwards who is, together with Robert Steptoe, the "father" of the fist "IVF-Baby."

The IVF process is an utmost stressful, both physically and psychologically, for both parents but primarily for the mother-in-being. A number of physical examinations and medical procedures are being performed in order to ensure that the process will lead to a successful pregnancy.

During the IVF process, especially when decisions are made which embryo shall be transferred in order to ensure the greatest chance for success, the best possible decision, based on the fast and accurate information on the status of the embryo, is needed. Due to the increasing age of mothers-to-be, during the past years, a steady increase in pregnancies following artificial reproductive techniques (ART), namely, IVF and intra-cytoplasmatic sperm injection (ICSI), was observed. The reasons for this development must be seen in economic, educational, and social factors, which lead to steadily rising rates of elderly patients. With increasing age, fecundability and fertility decrease. However, the advances made in reproductive medicine itself might be one of the reasons, why an increasing number of women delay childbearing, hoping for late babies and consciously making decisions to use the help of reproductive medicine.

The elevation of pregnancy rates for IVF procedures is certainly needed in order to reduce the burden on patients and lower costs affiliated with the procedures, which very often must be repeated until a pregnancy is reached and the embryo develops to successful birth. New technologies and methods more efficient than the current approach using PGS are needed. One new approach could be the analysis of the embryos' proteome during the IVF process. This means to analyze the specific proteins that are secreted by the embryo in the IVF surrounding of the culture medium. Routinely, the medium left after embryo transfer is being discarded. The multiple embryo transfer occurring during the IVF procedure is necessary after the fertilization step, and the spent media are a rich source of biological material that can be used for diagnostic purpose. This procedure of embryo transfer to a new culture medium is essential for the embryo's development and occurs daily or at least when the embryos have achieved the stage of embryoblast at day 4.

The spent medium can be useful for a detailed examination of proteins and lipids that were secreted by the embryo during each stage of development and can be used for the estimation of the "embryo quality" and selection of corresponding embryos for primary transfer.

An overview of technologies and approaches being used and a simplified experimental approach are presented in this chapter. The method used in our laboratory is used just as an example of the technologies used, and a number of different approaches will be discussed.

2. Materials and methods

2.1 Analyzed embryo cultivation medium: source of the material

Embryo cultivation medium is usually being discarded during the IVF procedure although it can be used for multiple diagnostic and prognostic procedures. Certainly, ethical and moral guidelines and procedures must be observed, and the ethical board or other corresponding bodies must approve of the use of this material. The following experiments have been performed by using cultivation media from IVF media upon approval from the ethical boards of the Medical University in Vienna and the University of Linz in Austria. Samples were collected during different stages of embryo development and analyzed using methods established for analysis of low samples amounts.

2.2 Proteomics sample preparation

Trypsin for protein digestion was purchased from Promega Inc. (Vienna, Austria). Solvents for HPLC—methanol (MeOH), acetonitrile (AcN), 2,2,2-trifluoroethanol (TFE), formic acid (FA), heptafluorobutyric acid (HFBA), iodoacetamide (IAA), triethylammonium bicarbonate (TEAB), and dithiothreitol—were purchased from Sigma-Aldrich (Vienna, Austria).

One of the most important steps when analyzing embryo cultivation media is the depletion of serum albumin present in these samples. Tarasova et al. [1] described the innovative method of using immobilized antihuman albumin antibodies for depletion of small sample volumes. Briefly, for depletion of culture media samples from selected embryos, the sample was diluted using the phosphate buffered saline buffer (pH 7.4) consisting of 0.01 M phosphate buffer, 0.0027 M KCl, and 0.14 M NaCl. This buffer was further also used as a washing buffer A upon the sample loading. In order to ensure full sample recovery from the depletion column, a ready-to-use elution buffer from Agilent (pH 2.25) (Agilent Technologies, CA, USA) was used as buffer B. We have developed a new column for the depletion of human albumin by immobilizing the antihuman albumin antibodies to the monolithic support disk, the CIMac-HSA column, especially for analysis of small sample amounts, which also occur in IVF samples. This column was used in an ICS-5000 inert HPLC system (Dionex-Thermo Scientific, Germering, Germany) for albumin depletion with a column flow rate of 0.3 mL/min. Upon sample injection, the loading and washing buffer A was pumped through the column for 5 min, and the flow-through fraction was collected (V = 350 μL). This fractions' volume corresponds to the full absorbance peak and contains all proteins that were not trapped on the column. The albumin was trapped by the interaction with the antibodies on the column's surface, and it was eluted by increasing the amount of the eluting mobile phase from 5 to 10 min. The column was, finally, flushed with the loading buffer A for additional 4 min, and this step was followed by an additional washing step with buffer B and, finally, equilibrating step using, again, the loading buffer A for 13 min. The total time for completing this depletion protocol is 30 min when applying the column flow rate of 0.3 mL/min. During this time, the very important column wash step and the complete re-equilibration of the column preceding the next depletion run is being perfromed. The flow rate used was selected for maximizing the protein's interaction time and was a compromise between the speed and efficacy of operation. If desired, higher column flow rates can be used without losing much of the column's performance [2], but this shall be carefully examined and optimized.

Proteins in both collected fractions and non-depleted samples were depleted using a standard protocol with trypsin. Due to a high concentration of phosphate buffer in collected fractions, 1 M triethylammonium bicarbonate (TEAB) was added to the fractions in order to reach a final concentration of 50 mM TEAB and to dilute the phosphate buffer. For protein denaturation, 10 μL of 0.1% (w/v) Rapigest in 50 m MTEAB were added to all fractions, and reduction of disulfide bonds was performed using dithiothreitol (final concentration of 5 mM DTT) and incubating the reaction mixture for 30 min at 60°C.

For a successful trypsinization, alkylation was performed using iodoacetamide at a final concentration of 15 mM IAA. Upon addition of IAA, the sample was incubated at room temperature, in the dark, for 30 min. An excess of iodoacetamide is inhibiting the trypsin action and needs to be neutralized, which was achieved by adding 2 μL of 50 mM DTT in 50 mM TEAB, vigorous mixing for 2 min at room temperature. Finally, 10 μL of 0.2 μg/μL trypsin solution were added, and samples were incubated for 16 h at 37°C in an incubation oven. Tryptic activity was stopped by acidifying the solution with 1% TFA solution.

For LC–MS/MS analysis, 20 μL of the digested and diluted sample (aqueous 0.1% TFA at a ratio of 2:3 (v/v)) were injected for LC–MS/MS analysis.

2.3 Chromatographic separation and mass spectrometry detection

A Dionex UltiMate 3000 RSLC nanoLC system (Dionex-Thermo Scientific) was used for the nanoHPLC separation of tryptic peptides. Mobile phases used for chromatographic separation of tryptic peptides were as follows: (A) 5% of acetonitrile in aqueous 0.1% formic acid and (B) methanol, trifluoroethanol, water and acetonitrile (30:10:10:50 (v/v/v/v) and 0.1% of formic acid). The sample was loaded onto the trap column for washing the residula salts and focusing of the analytes as a small sample front by using loading mobile phase consisting of 0.1% aqueous TFA chilled to 3°C.

The nonlinear gradient of 75 min total running time was used for HPLC separations of tryptic peptides. The gradient was composed using sequential linear steps:

1. 1.0% B min^{-1} for 7 min.

2. 0.5% B min^{-1} gradient for 38 min.

3. 1.5% B min – 1 for 20 min.

4. 3.0% B min^{-1} for 10 min.

The HPLC flow was introduced into the maXis Impact UHR TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), and the LC–MS/MS analysis was performed in triplicate for each sample. For peptide fragmentation (MS/MS), collision-induced dissociation was used, and all MS/MS data were acquired in a 300–2000 m/z range using a three-second cycle without further optimization of the instrument's parameters. In order to prevent unnecessary fragmentation of ions with low intensity, the lower threshold for precursor ions was set to 1000 counts. Due to tailing of some peptides and frequently observed co-elution of peaks, a dynamic exclusion was employed, and the duration was set to 120 s. In case where the precursor ion showed higher intensity after the exclusion period of 120 s, it was reconsidered for an additional MS/MS event.

Database search for protein identification was performed using the all-taxonomy Swiss-Prot database (http://www.uniprot.org). For this search, X! Tandem [3] with following parameters was used:

1. Precursor mass tolerance of ±10 ppm.

2. Fragment mass tolerance of ±0.3 Da.

3. Maximum of two missed cleavages were allowed.

Some posttranslational modifications of peptides due to the sample preparation and due to biological processes can be expected. For the experiment described, cysteine carbamidomethylation was selected as the fixed modification and methionine oxidation; phosphorylation of serine, threonine, and tyrosine residues, as well as the acetylation of lysine and N-terminal residues, were selected as variable modifications.

2.4 Data analysis

All data analysis for the experiments described in this manuscript was analyzed using the method described by Tarasova et al. [1]. The use of pyteomics.pepxmltk converter (https://pypi.python.org/pypi/pyteomics.pepxmltk) and search was described elsewhere [4]. Briefly, this platform was used to convert X! Tandem files to the standard pep.xml format and perform data analysis. All identifications, upon database search, were filtered to meet the requirement of the 1.0% FDR at peptide level. Search results were submitted for a post-search validation using the MPscore software, described earlier [5]. Quantitative information on identified proteins is a substantial requirement for determining the differences between biological samples, and all proteins fulfilling the identification and validation requirements were quantified using the label-free quantitation approach called normalized spectral index (SIn) (Griffin et al.) [6].

3. Results and discussion

3.1 Albumin depletion in IVF cultivation medium

The presence of albumin in cultivating medium is necessary for the normal development of embryos. However, its presence is a significant burden for proteomics analysis, and it must be removed prior to further analysis steps. The removal of albumin has been extensively discussed and described in a number of publications [1, 2, 7–9]. Different groups have used a number of methods such as immunodepleting chromatography, molecular weight cutoff filters, peptide libraries, size exclusion chromatography, etc. All these methods have some advantages but also show disadvantages. In case of depleting the albumin from IVF cultivating medium, the very low sample volume (max. of 40 μl) must be taken into consideration. Furthermore, depletion method must be performed fast and must be reproducible over a large number of samples, if intended to be used for fast analysis of clinical samples that shall help making the decision on which fertilized oocyte shall be transferred first and which ones shall be frozen for later procedures. The use of a novel immunoaffinity-based convective interaction media analytical columns (CIMac) for depletion of HSA (CIMac-HSA) was performed in this study, and it proved that it can be used for fast and reproducible albumin depletion from minute sample amounts. The column's architecture and the convective flow-through columns' channels enable a flow rate-independent binding capacity and excellent chromatographic resolution. These characteristics give CIMac-αHSA column some important analytical benefits like shorten time of analysis in comparison to common chromatographic depletion of albumin using silica-based columns, which

is an extraordinary important parameter for clinical use. The albumin content in different batches of cultivating medium differs strongly, and it also differs strongly between different suppliers. Therefore, the depletion method must be selected in a way that can be applied for a variety of samples. However, independently of the selected methods, the removal of albumin from the cultivating medium results with a number of identified proteins of which some can be of importance for embryo development and for later pregnancy development.

Figure 1 shows two SDS-gel lanes for the separation of two media upon embryo transfer. Since albumin originates from different donors and is being mixed at the manufacturing site, it will certainly contain a number of other proteins that may interfere with proteins secreted from the developing embryo. Also, secreted proteins might bind on albumin and, therefore, be "invisible" for proteomics analysis.

In addition to that, the human proteome, as any proteome, is dynamic, and it is constantly changing through internal and external stimuli. Proteins being translated from RNA are directly responsible for cellular function, but different gene expression studies have, unfortunately, shown that a reliable prediction of proteins' function or abundance cannot be made. As for the human proteome, significant advances have been made for the analysis of the whole proteome and of the proteome of different disease states [3, 5, 10–20]. Bearing all of it in mind, the analysis of proteins obtained from the cultivating media is challenging, and the results are not always conclusive.

Figure 2 shows the results obtained upon analyzing four different cultivating media used in routine IVF procedures and upon embryo transfer.

Dyrlund et al. [21] have performed the analysis of unconditioned commercial embryo culture media and have identified a number of proteins upon depletion and digestion of a total of 5 mL of media. The amount of 5 mL, however, will never be available if samples are retrieved during the actual IVF process. However, this analysis showed that this amount contains an astounding amount of 25 mg of albumin. Here, a total of 111 proteins with different concentrations in different batches of the media were identified in addition to albumin. The sample of unconditioned media also contained eight proteins previously suggested as possible markers of

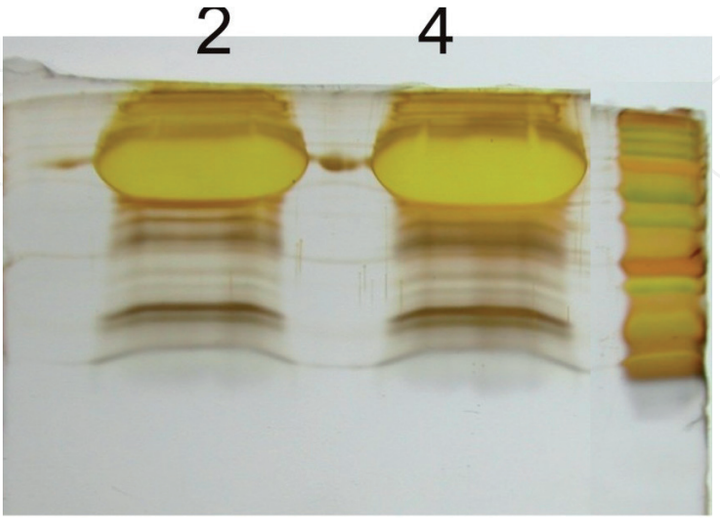

Figure 1.
Lanes 2 and 4 show an example of the separation of proteins secreted in culturing media on silver-stained SDS gel. As expected, large spots for human serum albumin (HSA) are observed for both samples with significantly lower amounts of other proteins. Since the culturing media have been declared to contain only HSA, the proteins below are secreted from the fertilized oocyte (lane 2) and unfertilized oocyte (lane 4).

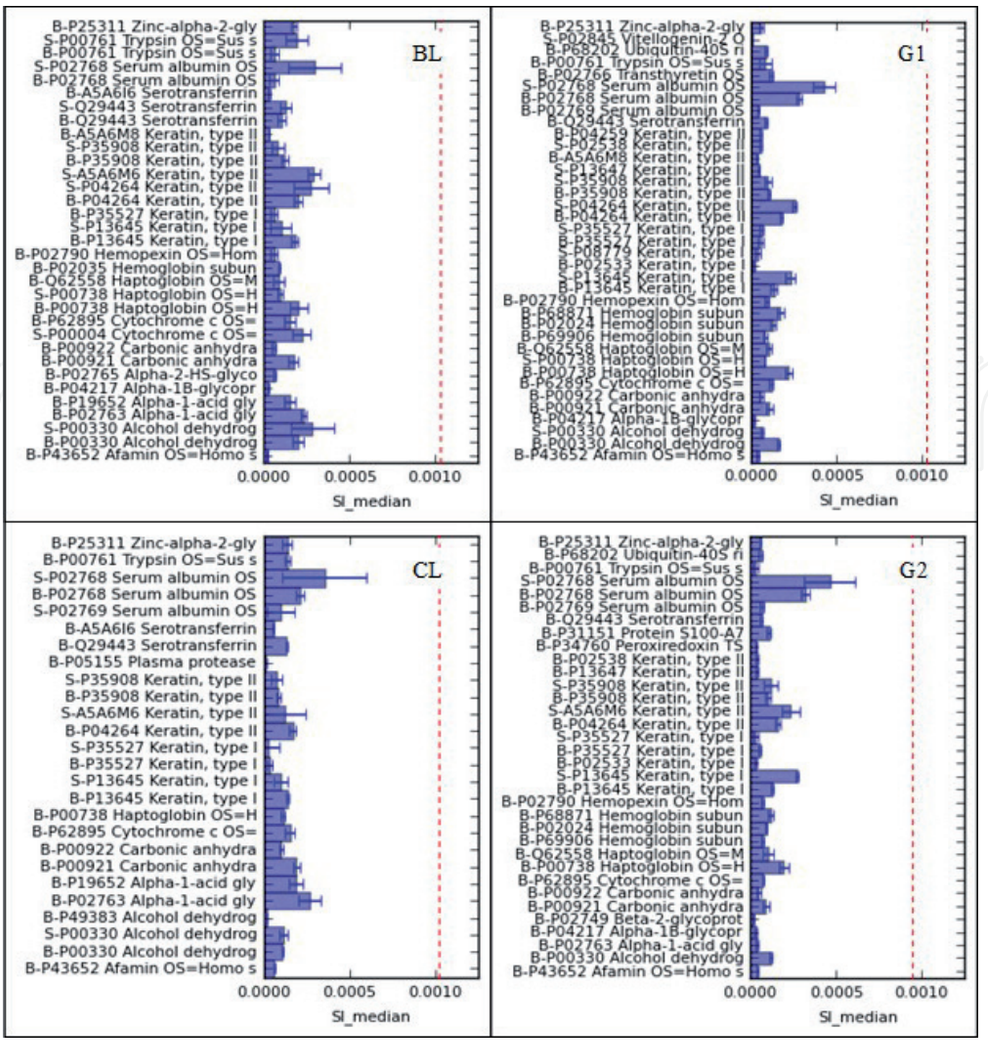

Figure 2.
Identified proteins and their relative quantities in albumin-free fractions after performing albumin depletion of culturing media samples on two different depletion columns. Proteins identified either in the CIMac-αHSA FT or the Seppro FT1 fractions are labeled with B and S, followed by Swiss-Prot protein ID. Median values of normalized spectral indexes (SI) and standard deviations are shown. Median SIHSA in non-depleted samples is marked with red dashes. More than two peptide-spectrum matches were assumed for each of the listed proteins according to Tarasova et al. [1].

embryonic viability, e.g., proteoglycan-4, serotransferrin, vitamin-D-binding protein, ubiquitin, etc.

Another study performed on IVF medium examined the batch-to-batch variations and showed that variation in both protein amounts and protein identifications can be expected [22]. When studying these media, it is important to pay attention to the fact that both media, the control and the media where embryos grow, must be from the same batch in order to make valid comparison.

Spent IVF medium is a valuable source for proteins that can be used as putative biomarkers in IVF. The major focus of researchers has been on secreted proteins, but the proteins already present in the medium can be equally valuable and must be considered in the pursuit for biomarkers of embryos' "quality." It is very possible that proteins already present in the medium may be necessary for embryo development, and the uptake or degradation of specific proteins might correlate to ascertain embryo development or lack thereof. Therefore, these media must be evaluated for their potential to differentiate embryos' success rates and track the proteins, which can be potential biomarkers. Additionally, the safety of these proteins in culture media for the offspring should also be evaluated.

3.2 Identification of proteins in IVF cultivation medium and their possible role for embryo development

Proteins identified by Dyrlund et al. were also reported by Katze-Jaff upon analysis of the secretome of the individually cultured human embryos, and a hint was made that these proteins could be related to embryo's morphology and thus its viability [23–25]. They have reported that ubiquitin was upregulated in developing blastocytes as compared to degenerating blastocytes. However, there was no correlation between the ubiquitin upregulation and the observed pregnancy rates.

Cortezzi et al. [26] reported the identification of proteins in both positive and negative groups of embryos, i.e., embryos termed viable for transfer and does who were not selected. For positive-implantation group, protein, called Jumonji (JARID2), was reported. This protein composes a histone methyltransferase complex called polycomb repressive complex 2 (PRC2), which modifies chromatin methylation to silence many embryonic patterning genes, acting as a negative regulator of cell proliferation signaling. This results with a restricted gene expression to an appropriate cell population that is essential for development, differentiation, and maintenance of cell fates.

In the same study, the testis-specific gene 10 protein (TSGA10) was identified only in negative samples. TSGA10 is a perinuclear protein, and it was described to participate in actively dividing and fetal differentiating tissues in mice embryos.

Katz-Jaffe et al. reported the identification of heparin-binding epidermal growth factor EGF-like growth factor precursor (HB-EGF) [24]. This growth factor precursor belongs to the EGF family growth factors, and it is found in the membrane-anchored form (proHB-EGF) and the soluble form (sHB-EGF). The soluble form is produced from the proteolytic cleavage of proHB-EGF at the extracellular domain. Furthermore, soluble HB-EGF has been observed to be a growth stimulator, and it does significantly improve the blastocyst development and hatching when added to serum-free medium. However, other studies from an in vitro model system indicate that proHB-EGF might function in cell-to-cell signaling by a juxtacrine mechanism inhibiting growth activity [27]. Since this protein was identified in degenerating blastocytes, its upregulation might contribute to the lack of further development.

A cystatin-like precursor is needed for successful mammalian implantation for a controlled trophoblastic invasion of the maternal uterine epithelium. It must be available and functional [28] since this invasion involves the extracellular degradation of the uterine matrix by a variety of proteinases, and one of the crucial ones are cysteine proteinases. Cystatins are known to inhibit cysteine proteinases, and their upregulation will most probably contribute to failure of implantation of degenerating blastocysts. Therefore, it is not only of importance to identify these proteins, the possible biomarkers, but also to validate these findings in the human.

4. Conclusion

Proteomics is a promising technology for the identification and validation of possible biomarkers for embryo selection in ART. As listed by Dyrlund et al., a growing list of secreted proteins has been identified that could further contribute to this field [21]. However, the challenge ahead of the research still includes the reliable and reproducible identification of a proteomics secretome signature. This signature shall be directly associated with embryo viability and the success of the procedure, i.e., successful pregnancy, and child's birth. This is a very challenging task not only due to the complexity, heterogeneity, and diversity of human embryos

but also because of irreproducibility of used culturing media and contaminant proteins therein.

Another challenge for the clinical use of proteomics methods is the speed of the proteomics analysis. Sample preparation, measurement, and data analysis of a sample is currently not feasible within the time window needed for IVF. Current sample analysis methods require at least half a day for the fastest proteomics method available, which is too long. Nevertheless, proteomics methods can contribute to identification and validation of putative biomarkers, which once clinically confirmed, can be analyzed using other, faster, methods upon building corresponding antibodies.

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