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Predictive Roles of Proteomic Profiles in Assisted Reproduction-An Update

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Abstract

The prevalence of infertility is 12-15% in sexually active couples. When, a subgroup analysis is done, 50% of infertility can be ascribed to male factor or a combination of male and female factor. With the growing trend of male factor infertility and the desire for infertile couples to reproduce, assisted reproductive technology provides a means to overcome these challenges. Several techniques exist depending on the extent of the infertility. For cases of severe oligospermia or even azoospermia, a combination of in vitro fertilization (IVF) and intracytoplasmic injection is used. There has been birth of more than 4 million babies worldwide using the assisted reproductive techniques. In the United States alone 1% of the total babies born each year are conceived through ART However, there are very low rates of successful implantation and pregnancy per transferred embryo in in vitro fertilization. According to literature reports 70% of transferred embryos fail to implant. Also, several ART clinics perform multiple embryo transfer, rather than a single embryo transfer, which exposes patients to several complications. Proteomics has the potential to aid in selection of the best embryo. In this review, we present an appraisal; of existing scientific literature regarding the proteomic profiles of the key players impacting ART outcomes. We elaborate on the potential proteomic biomarkers that are a key to establishing a non-invasive, reliable, reproducible, and specific means of assessing endometrial receptivity, embryonic viability, aneuploidy, and fertilizing potential of the sperm. These biomarkers include a variety of both structural and functional proteins. The application of these potential biomarkers in the future will help in enhancing the ART outcomes by offering a personalized treatment for patients based upon their individualized signature proteomics profiles.

Keywords: Proteomics; Endometrium; Endometrial receptivity; Embryonic viability; Aneuploidy; Spermatozoa

Introduction

Infertility affects 15% of all couples around the world. Moreover, 50% of these couples are infertile due to causes related to the male. In 1981, Assisted Reproductive Technology (ART) was introduced. ART includes *in vitro* fertilization (IVF) and Intracytoplasmic Sperm Injection (ICSI). In IVF, collected eggs and sperm are fertilized in a petri dish before being exposed to the female's receptive endometrium in hopes of establishing implantation and a successful pregnancy. ICSI treats extreme cases of male infertility and involves injecting a selected sperm into an egg in order to ensure that fertilization occurs. In 2010, in recognition of the development and success of ART, Professor Sir Robert G. Edwards, the pioneer of human IVF, was awarded The Nobel Prize in Physiology or Medicine. Every year, 1% of all live births in the United States and 3% of all live births in Australia are attributed to ART [1,2]. In fact, in 2012, a total of 65,160 live born infants were conceived via ART and over 456 clinics in the United States utilized ART in 2012.

Despite the immense success of ART, there is great room for improvement. Rates of successful implantation and pregnancy per transferred embryo remain very low. In fact, 70% of transferred embryos fail to implant [3]. Also, when multiple embryos, rather than a single one, are transferred, patients are exposed to various complications such as an increased risk of a multiple pregnancy. Multiple pregnancies carry an increased risk of miscarriage(s), premature labor and premature birth; increased financial and emotional cost; increased incidence of hypertensive disorder in pregnancy (gestational hypertension and preeclampsia) and diabetes in women pregnant with more than one fetus and a likely need for prolonged hospitalization for the mother and babies after delivery. Another possibility is a tubal (ectopic) pregnancy and the chance of a combination of normal pregnancy and ectopic pregnancy. A tubal pregnancy may require laparoscopy or major surgery for treatment. In order to improve ART success rates and efficacy, it is important to establish a non-invasive, reliable, conclusive, specific, and efficacious means of assessing endometrial receptivity, embryonic viability, aneuploidy, and fertilizing ability of the spermatozoa via proteomics. These biomarkers will help increase ART success rates and replace multiple embryo transfer with single embryo transfer per ART procedure.

Proteomics is the study of the expression, localization, functions, post-translational modifications and interactions of proteins that are expressed by a genome at a specific condition and at a specific time. Proteomics identifies differentially expressed proteins utilizing robust techniques and search engines and data bases, quantifies their presence and also highlights the proteins that could serve as potential candidates by validation studies. Hence, proteomics provides us with information regarding the functionality of the cell as they represent proteins are the functional units of the cell [4]. They drive every cellular process after being transcribed, translated, and modified from their genomic origin. There are over 200 post-translational modifications, including splicing, glycosylation, phosphorylation, acetylation, etc. [5]. Due to post-translational modifications, multiple proteins can be derived from a single polypeptide which originates from a single, parent mRNA transcript, and hence, from the gene. Thus, the level of

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a specific protein within a cell does not necessarily correlate with the levels of its parent transcript [6]. Also, because of these alterations, the primary, secondary, and tertiary structure of a protein differs from the structure originally encoded by its parent transcript and gene [7]. As a result, applying proteomics to understand the functionality of a cell is far more advantageous than applying genomics, the study of the cellular genome, and transcriptomics, the study of the cellular transcriptome [8].

In comparative proteomics, proteomic techniques compare and contrast the proteomes of two different cells in order to examine their proteomic differences. These differences may consist of the presence or absence of a specific protein, differing levels of expression of a specific protein, or different qualitative characteristics of a specific protein [9]. For instance, if comparative proteomics was done on a normal cell and a pathological cell, then their proteomic differences could serve as a biomarker for the pathological state. "A biomarker is defined as a biological component, such as protein, miRNA or gene, whose concentration is altered according to the presence of a specific disease or outcome [10]. Biological functions, protein abundance and availability are some criteria that can be used to choose a protein to be validated. Validation of a protein is a complex process and can be done by finding proteins *in-silico* with bioinformatics or with validation techniques such as western blot and immunochemistry. Biomarker may have inherent reasons for variable expression [11]. In patients with endometriosis related infertility, biomarker expression can vary with menstrual cyclical phases, endometriosis stage and location of the endometriosis implant [11]. Lack of validation can result from limited numbers of cases and controls, lack of reproducibility and lack of robust statistical and bioinformatics approaches. Besides, globally standardized operating procedures and standardized clinical phenotyping should be achieved for characterization of a biomarker [12-14].

Many proteins have been studied in the female reproductive biologic windows. When referring to non-invasive diagnosis, urine, plasma, serum, peritoneal fluid, follicular fluid or even menstrual fluid can be considered. Menstrual fluid can be taken from the vagina's posterior fornix or from the cervix during speculum examination. Transcervical biopsy of the endometrium is considered a semi-invasive diagnosis, but is also useful. A good biomarker needs to have high specify and sensitivity and has to be affordable. Moreover, researcher have to have in mind that they need to achieve reproducible results and find proteins that are common between women of different ages, nationalities and cultures A biomarker is any biological molecule whose presence, quantity, or qualitative characteristics indicate a biological phenomenon, such as a pathological state. The ideal biomarker must be safe, easily, and accurately identified in a cost-effective and non-invasive or minimally-invasive manner [15]. In ART, biomarkers should have a significant potential of predicting fertilization, implantation and embryonic viability [16]. Proteomics is thus the key to improving ART success rates by identifying, quantifying, and characterizing protein profiles of the key players that determine ART success rates. These include the spermatozoa, oocyte, endometrium, uterine fluid, and embryonic secretome. The proteomic profiles can be diagnostically useful and capable of predicting sperm fertilizing potential, endometrial receptivity, embryonic viability, and aneuploidy in a reliable, non-invasive manner.

There are several proteomic techniques that detect and characterize differentially expressed proteins between two samples (Figure 1), an example of which is the 2- dimensional gel electrophoresis (2-DE)

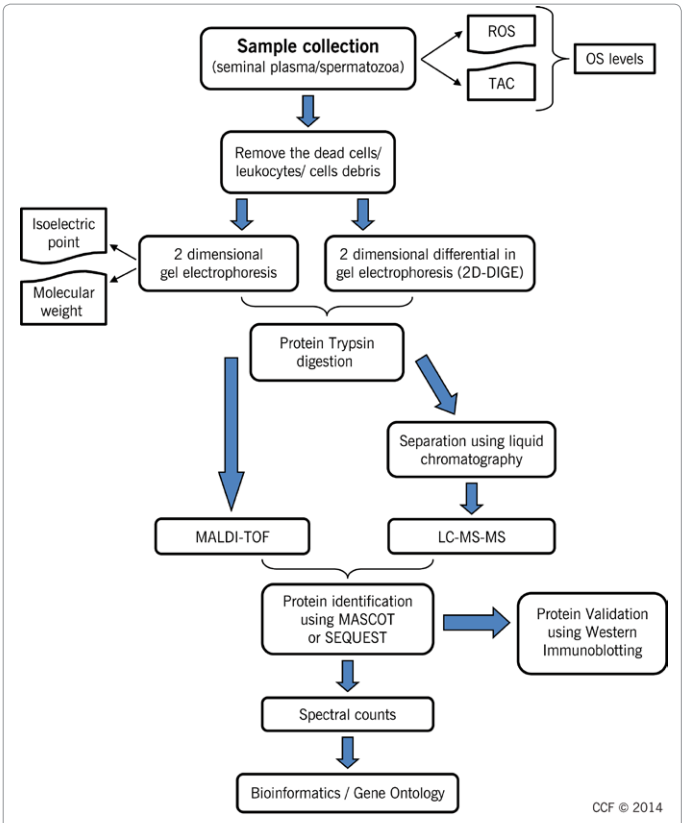

Figure 1: An overview of general methods used for protein isolation and identification in seminal plasma and spermatozoa.

Abbreviations: 2D-DIGE: 2 Dimensional-Differential In gel electrophoresis; 2D-GE: 2 Dimensional-gel electrophoresis; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; MALDI-TOF: Matrix Assisted Laser Desorption Ionization-Time of Flight MASCOT: A tandem mass spectrometry data analysis program from Matrix Science, which is used for protein identification; OS: Oxidative stress; ROS: Reactive oxygen species; SEQUEST: A tandem mass spectrometry data analysis program used for protein identification; TAC: Total antioxidant capacity.

which detects low levels of proteins present in biological samples. During the first step, proteins are separated in the first dimension according to their isoelectric points (pI), based on their charges. In the second dimension, the proteins are separated according to their molecular weight using sodium dodecyl sulphonate polyacrylamide gel electrophoresis (SDS-PAGE). However, 2-DE has limited sensitivity and poor reproducibility. 2-Dimensiomal difference in gel Electrophoresis (2-DIGE) was developed to surmount the limitations of 2-DE. In this technique, two protein samples are separately labeled with different fluorescent dyes. The labeled proteins are electrophoresed on the same 2-DE gel. 2-DIGE utilizes mass- and charge-matched, spectrally resolvable fluorescent dyes. The common dyes are Cy2, Cy3 and Cy5 and up to 3 different protein samples can be labeled prior to 2-D electrophoresis. Samples (control and the experimental) are run in a single polyacrylamide gel. The imaging however is separately visualized [17]. Differentially expressed proteins are detected, quantified and annotated using appropriate software. 2D-DIGE, therefore, is more sensitive and accurate technique that allows comparison between protein expressions among different samples. It can detect differences as small as 10% and therefore provides all the advantages of 2D-PAGE while overcoming the challenges inherently seen in a 2D-PAGE.

In gel-based proteomics, the gel of interest is excised and digested

with trypsin to break the proteins into peptides. These peptides are separated according to the mass-to-charge ratio by utilizing mass spectrometry [18]. Different modifications of mass spectrometry are available such as matrix assisted laser ionization/ desorption ionization (MALDI) and electrospray ionization (ESI). MALDI is a solid-state and pulsed process, whereas ESI is a liquid phase and induces continuous ionization. The most commonly used mass analyzers for protein biochemistry applications are time-of-flight (TOF), triple-quadrupole, quadrupole-TOF, ion trap instruments, and hybrid ion trap Orbitrap instruments (Figure 1). The TOF analyzer is conceptually the simplest spectrometer [18]. Typical configurations for biological applications are MALDI-TOF and ESI coupled to an ion trap, triple-quad, Quadrupole-TOF, or orbitrap. Another variant of MALDI-TOF is the surface-enhanced laser desorption/ionization (SELDI) –TOF. Post-translationally modified proteins can also be detected using SELDI-TOF-MS. There are other mass spectrometry methods that do not use TOF such as the Q-Exactive (Thermo). It utilizes a quadrupole-orbitrap hybrid mass spectrometer, and is the most widely used platform for shotgun proteomics. Similarly, Waters' MSE platform depends on a Quadrupole analyzer (hybrid) in line. Liquid chromatography–mass spectrometry (LC–MS) technology is the recognized standard for accurate mass and high-resolution measurement. LC–MS is a hyphenated technique, which combines the separation power of LC with the detection power of MS. LC-MS/MS (tandem MS) has helped in developing routine methods of high sensitivity, high specificity, high throughput, and high cost effectiveness in biochemical genetics/ newborn screening, drug and toxicology testing and endocrine testing of steroids and biogenic amines. Search programs such as Mascot, Sequest, X!Tandem, and Andromeda are widely used. MaxQuant, a computational proteomics platform is also helpful in comparing the experimentally observed MS/MS spectrum to the predicted peptide spectra and determine which peptides, and therefore which proteins, are the best matches. Coupling LC-MS/MS with database searches can result in thousands of protein identifications from a single experiment [19]. Common uses of MS based proteomics include: 1) Protein identification, 2) protein sequencing, 3) identification of post-translational modifications and 4) characterization of multi-protein complexes.

In this article, we highlight the most relevant studies; summarize their results, and briefly describe the potential of proteins as markers in predicting embryonic viability, successful implantation, successful pregnancy and aneuploidy in ART as well as enumerate the challenges and limitations.

Endometrial Receptivity

The endometrial cycle consists of three phases, i.e. the menstrual, proliferative, and secretory phases. The endometrium undergoes various dynamic changes throughout each phase. These changes are orchestrated by several proteins, including growth factors, cytokines, phosphatases, and kinases [20]. The highest amounts of proteins are secreted during the secretory phase as the endometrium prepares itself to accept an embryo. In fact, endometrium becomes receptive during the mid-secretory phase, after ovulation, during the 19^{th}-23^{rd} day of the menstrual cycle [21]. The window of implantation takes place during the progesterone peak. Thus, progesterone drives receptivity by regulating genetic and proteomic expression.

In order for implantation and pregnancy to occur, transferred embryos must be received by a receptive endometrium. Unfortunately, there is no single specific test for endometrial receptivity [22]. Instead, endometrial status is detected via inconclusive, clinically-insignificant morphological and histological data attained via various imaging techniques [23,24]. Morphological data is clinically not significant because it is cannot detect abnormal endometrial receptivity and fails to distinguish receptive from non-receptive endometrium [25].

Protein biomarkers of endometrial receptivity can be identified by differentiating the proteomes of endometrial tissue or uterine fluid, representing the endometrial secretome during the receptive and non-receptive phases of the menstrual cycle. It was recently reported that the endometrial tissue is obtained via biopsy or curettage while uterine fluid is collected via aspiration or flushing of the fluid. Conducting proteomic studies on uterine fluid is more advantageous than endometrial tissue for various reasons [26,27]. For one, aspiration and lavage are less invasive than biopsy and curettage, and hence, expose the patients to fewer complications [23]. The endometrium is not harmed and implantation rates are not altered after aspiration of uterine fluid via an embryo catheter [28].

In addition, endometrial tissue is morphologically dynamic. The cellular composition and structure not only vary throughout the menstrual cycle, but also amongst different individuals. Furthermore, the endometrium is a mosaic as different structural parts of the same endometrium vary in composition at a given time [29]. As a result, it is difficult to obtain a standardized endometrial biomarker. Laser capture can reduce this endometrial mosaic structure and complexity. This may alter protein concentration and further complicate the proteomic study [30]. Furthermore, tissue biopsy does not represent the entire endometrium. Instead, biopsy picks up a limited amount of tissue that represents a random 2-4% of the overall morphologically dynamic tissue [31]. Thus, specific biomarkers identified via biopsy cannot be apSTATH characterize the whole dynamic STAsue [32]. Moreover, proteomic studies on endometrial tissue usually detect proteins that are in high abundance, most of which have structural functions [30]. In order to detect less abundant, yet more functionally significant proteins, endometrial samples have to be pre-fractionated before and analyzed in gel-free methods such as liquid chromatography/capillary electrophoresis [33].

On the other hand, proteomic profile of the uterine fluid is less complicated than endometrial tissue proteome [27]. Furthermore, uterine fluid reflects the microenvironment of the uterus during the implantation period [23]. Thus, any molecular alterations within uterine fluid reflect alterations in the microenvironment of implantation. However, a widely accepted, standardized means of collecting uterine fluid is lacking, which results in variability and discrepancy amongst similar samples.

The two main methods of collecting uterine fluid are by aspiration and flushing, both of which present many advantages and disadvantages. Aspiration is more convenient because of the complications associated with the latter. Furthermore, it is quite difficult to quantify biomarkers present at low levels because flushing involves the use of 1-2 mL of saline thereby significantly diluting the uterine fluid [28]. However, in aspiration, only a maximum amount of 10 microliter can be collected due to the small amount of fluid naturally present in females.

Proteomics and Endometrial Receptivity

Various studies have identified potential proteomic biomarkers of endometrial receptivity by discriminating between the proteomes of receptive and non-receptive endometrium. Li et al. analyzed endometria collected from four fertile women undergoing the same

phases of the menstrual cycle at the same time. After comparing the proteomes of the receptive and non-receptive endometria, they identified 31 proteins related to implantation [34]. 29 out of the 31 proteins identified were further examined – of these 5 were involved in cell migration or assimilation, 9 had enzymatic activity, 9 were involved in signal transduction and gene regulation, 4 were immunoregulators, and 2 were involved in vascularization (fibrinolysis or blood clotting) [34].

Higher levels of Annexin (A) were identified within the receptive endometria [34,35]. Li et al. detected Annexin 4 (A4) to be 2.1-fold higher, while Dominguez et al. [35] discovered Annexin 2 (A2) levels to be 1.9 times higher respectively in the receptive endometria [28,35] (Table1). In addition, refractory endometria also lacked functional levels of A2. These results were validated by western blot and immunohistochemistry [35]. Upregulated levels of the A4 transcript were reported by Li et al. [36], where they also reported increases in progesterone levels associated with increase in A4 transcript levels [34]. Progesterone drives endometrial receptivity and A4 may have a crucially important role in mediating endometrial cells from the pre-receptive to receptive state because it is involved in apoptosis [34]. A2 promotes cellular adhesion required for blastocysts to adhere to the endometrium [35]. Studies have shown A2 stimulating the expression of cell adhesion molecules in endothelial cells [37,38].

Using differential in-gel electrophoresis and MALDI-MS techniques, Dominguez et al. [35] identified differential levels of stathmin 1, a protein important for cytoskeletal reorganization, in receptive and pre-receptive endometrial tissue collected from eight fertile females of ages 23-29 years (Table 1). They collected pre-receptive tissue two days after the LH surge and receptive tissue seven days after the LH surge. High levels of stathmin1 were shown to be present in pre-receptive endometria and low levels of stathmin1 are present in receptive endometria, while dysregulated levels were present in refractory endometria [35]. High levels of stathmin1 stimulate stromal proliferation and prepare cells for implantation. Low levels of stathmin1 destabilize microtubules, preparing cells for decidualization [39]. However, dysregulated levels of stathmin 1 prevent implantation and decidualization from occurring [40].

Dominguez et al. compared the proteomic profiles of endometrial stromal cells (ESC) which were decidualized *in-vitro* versus the non decidualized ESC. Utilizing 2D-DIGE and matrix assisted laser desorption/ionization time of flight mass spectrometry, 60 differentially expressed proteins were identified. These proteins included existing biomarkers for decidualization such as cathepsin B and newer biomarkers like transglutamine 2, peroxiredoxin 4 and ACTB protein. The secretome analysis conducted by this group also identified well known existing biomarkers such as IGF binding protein 1 and prolactin and newfound markers such as myeloid progenitor inhibitory factor 1 [41].

Scotchie et al. [30] examined the proteome of uterine fluid collected from 10 fertile females between the ages of 18 and 34. They collected samples in the pre-receptive phase, 4 days after the LH surge, and receptive phase, 9 days after the LH surge. Using 2-dimensional gel electrophoresis and mass spectrometry, they detected 152 proteins, 82 of which were differentially expressed between the phases [30]. The proteins detected the most were immunoglobulins, which support the endometrium having an immune function. In addition, proteins involved in apoptosis regulation, stress response, host defense, immune/inflammatory response, molecule transport, and ion homeostasis were frequently identified. Proteins that had the greatest frequency of varied expression were associated with host defense, coagulation, apoptotic regulation, and stress response [30].

While Scotchie et al. [30] examined uterine fluid, Desouza et al. [41] examined endometrial tissue. Desouza et al. [41] identified 119 proteins differentially expressed between the proliferative and secretory phases of the endometrium (Table1). Of these 119 proteins, 28 are identical to the ones discovered by Scotchie et al. [30], including actin, alcohol dehydrogenase, α-2-HS-glycoprotein, β-2-glycoprotein I, cofilin, complement C3 precursor, creatine kinase B, enolase, fibrinogen γ chain, gelsolin, glutathione S-transferase, glyceraldehyde 3-phosphate dehydrogenase, haptoglobin, heat shock cognate 71 kd protein, hemoglobin β, hemopexin precursor, immunoglobulin (Ig) γ 2 chain, Igκ chain, phosphatidylethanolamine-binding protein, polymeric Ig receptor precursor, transferrin, albumin, superoxide dismutase, triosephosphate isomerase, tropomyosin α 4 chain, tubulin β chain, and vitamin D-binding protein precursor. In addition, both studies identified increased levels of fibrinogen γ chain [30]. Desouza et al. [41] dentified a 1.2-fold increase while Scotchie et al. [30] discovered a 4- to 5-fold increase between the early and mid-secretory phase. The two studies highlight the need for utilizing the proteomic profiles of the endometrium as well as the secretome for determining optimal endometrial receptivity. The variation of protein expression between receptive and non-receptive endometrium was attributed to the protein source.

In addition, Parmar et al. [42] detected higher levels of alpha 1 antitrypsin in the early secretory versus mid-secretory endometria. These investigators also reported high levels of heat shock protein B1 (HSPB1) and transferrin in endometrial tissue, uterine fluid, and mid-secretory secretions. Scotchie et al. [30] on the other hand, identified

Study (Year)	Proteomes Analyzed	Biomarker(s) Identified	Changes in Biomarker(s)	Biomarker(s) Function
Li et al. [36]	Receptive vs. non-receptive endometria	Annexin A4	2.1 times higher in receptive compared to non-receptive endometria	Apoptosis; mediates endometrial cells from pre-receptive to receptive state
Dominguez et al. [35]	i) Receptive vs. non-receptive endometria; ii) refractory endometria	Annexin A2	i) 1.9 times higher in receptive compared to non-receptive endometria; ii) low levels in refractory endometria	Cellular adhesion; implantation
Dominguez et al. [35]	i) Receptive vs. pre-receptive endometria	Stathmin1	i) High levels in pre-receptive endometria; ii) low levels in receptive endometria; iii) dysregulated levels in refractory endometria	High levels stimulate stromal proliferation for implantation; low levels destabilize microtubules for decidualization; dysregulated levels inhibit decidualization
Scotchie et al. [30]	Proliferative vs. secretory endometria	Fibrinogen y chain	Increased levels in proliferative endometria	Vascularization
Parmar et al. [42]	Early secretory vs. mid-secretory endometria	Alpha 1 trypsin	Increased in early secretory endometria	Protease inhibitor

Table 1: Potential Biomarkers of Endometrial Receptivity.

two different forms of both HSPB1 and transferrin in endometrial secretomes using mass spectrometry [30]. One form was up-regulated, while the other was down-regulated, suggesting posttranslational modification [30].

Chen et al. [31] identified 196 proteins expressed at different levels in mid-proliferative and mid-secretory endometria. Most of the proteins differentially expressed were those that were not differentially expressed at the genomic level. Hannan et al. [43] found 7 proteins that varied between the proteome of mid-secretory and mid-proliferative endometria. Hannan et al. [43] also found 12 proteins that varied within fertile and infertile mid-secretory endometria.

Endometrial stromal cells are important for implantation. Undifferentiated, yet decidualized endometrial stromal cells (ESC) prevent the implantation of developmentally abnormal embryos by inhibiting the release of mediators of implantation. These mediators of implantation include interleukin 1 beta, heparin binding hormone, epidermal growth factor, and immunomodulators (interleukin 5, 6, 10, and 11, and eotaxin). Cheong et al. [28] assessed the migratory activity of ESCs in low quality and high quality embryos. They discovered that in the presence of chromosomally-abnormal embryos, decidualized ESCs did not migrate and inhibited implantation. However, in the presence of high quality embryos, ESCs migrated basally, permitting implantation [28].

Apolipoprotein H (ApoH) binds to phospholipids on decidual and trophoblast cells [44,45]. If anti-phospholipid antibodies attack ApoH, pregnancy complications and fetal death can occur [45]. Studies have shown ApoH levels to be 1.7 higher in receptive versus non-receptive endometria [30,35,36]. Furthermore, secretory endometria have increased levels of proprotein convertase 6, an enzyme critical for implantation, which is reduced in the mid-secretory phase in a cohort of infertile women [46]. If PC6 is inhibited in mice, implantation does not occur [46]. Thus, this protein along with the other ones mentioned previously, have the potential to be biomarkers of endometrial receptivity.

Proteomics and Embryonic Viability

In order for ART to be successful, viable embryos should be transferred into the uterus. At present, viable embryos with high implantation potential are determined based on embryonic morphological criteria [47,48]. These parameters are limited in assessing embryonic viability and often provide inconclusive data, which is why most embryos do not implant [49]. Proteomic studies aim to improve methods of assessing embryonic viability by discovering potential biomarkers [50,51] (Table 2).

Improving assessment methods of embryonic viability will not only improve ART success rates, but will also lead to the transfer of single, rather than multiple embryos. Transferring multiple embryos in ART exposes patients to various maternal and fetal morbidities [16,48,52].

Katz-Jaffe et al. [47] were the first to characterize the embryonic secretome. They discovered high levels of ubiquitin within developing blastocysts using tandem MS and database peptide sequence identification. Ubiquitin is involved in proliferation, apoptosis, and implantation and is important for blastocyst development (Table 2).

Cortezzi et al. [53] used the bottom-up label-free proteomics to analyze the embryonic secretome. They compared and contrasted secretomes of 8 embryos that successfully implanted and established pregnancy (the positive implantation group) and 4 embryos that did not implant (the negative implantation group) (Table 2). They examined and quantified the proteins specifically expressed in each group using 2D nano-UPLC chromatography separation *via* the nanouplc-MS_E technique. 15 proteins were exclusively present in the positive implantation group, and 10 were exclusively present in the negative implantation group [53]. Most of the proteins detected in both groups were involved in binding, while others had catalytic activity, served as transcription factors, or had unknown cellular functions. Jumonji (JARID2) was exclusively present in the positive implantation group (JARID2). Jumonji synthesizes polycomb repressive complex 2 (PRC2), a histone methyl-transferase complex that silences genes by affecting histone methylation levels [54,55]. It silences genes involved in development, differentiation, and cell maintenance [56]. On the other hand, testis-specific gene 10 protein (TSGA10) is the most abundant protein exclusively detected in the negative implantation group [53].

Apolipoprotein A1 (ApoA1) is a potential biomarker of embryonic viability [57]. Mains et al. [51] were the first to report that embryos synthesize ApoA1. According to their investigations, embryos of different qualities express different levels of ApoA1. Good quality blastocysts and mitotically-arrested embryos had highly varied levels of ApoA1. In addition, good quality blastocysts and poor quality embryos that developed into blastocysts had significantly different levels of ApoA1 expression [51]. However, blastocysts that implanted and those that failed to implant had insignificant or no difference in ApoA1 levels. Another study did not find a correlation between ApoA1 levels and IVF success rates [57].

Furthermore, Dominguez et al. discovered decreased levels of

Study (Year)	Biomarker(s) Identified	Changes in Biomarker(s)	Biomarker(s) Function
Dominguez et al. [6,58]	CXCL13, IL-6	Differential expression amongst implanted vs. non-implanted blastocysts	Chemo-attractant used by implanted blastocysts. IL-6 favors implantation
González et al. [67]	Leptin	Present at higher levels in viable blastocysts vs. arrested embryos	Binds to leptin receptors to establish fetal/maternal communication
Katz-Jaffe et al. [47]	Ubiquitin	Higher levels in developing blastocysts vs. non-implanted blastocysts.	Involved in proliferation, apoptosis, and implantation.
Cortezzi et al. [53]	Jumonji	1/15 proteins exclusively present in embryos that lead to implantation	Synthesizes PRCS2, which silences differentiation, developmental genes via methylation
Cortezzi et al. [53]	TSGA10	1/10 proteins exclusively present in embryos that did not lead to implantation	Involved in cell division, differentiation, and migration of murine embryos
Katz-Jaffe et al. [50]	IL6, CXCL13	Present at higher levels in implanted blastocysts vs. blastocysts that did not implant with the EEC co-culture	Involved in development and implantation
Cheong et al. [28]	Leucine	Highest amino acid important nutrients for blastocysts	Essential, branched amino acid required for embryonic nutrition

Table 2: Potential Biomarkers of Embryonic Viability.

C-X-C motif chemokine 13 (CXCL13) and granulocyte macrophage colony stimulating factor (GM-CSF) in implanted blastocysts compared to those that failed to implant [6]. In fact, when added to human blastocysts, GM-CSF promoted embryonic development and implantation [53]. Moreover, they discovered that interleukin 6 (IL6) is utilized by viable blastocysts more than it is used by blastocysts that failed to establish pregnancy [6]. Hence, IL6 might have role in blastocyst development and implantation. They also observed increased levels of IL6, placental growth factor, and CXCL13 and decreased levels of fibroblast growth factor 4, IL12, vascular endothelial growth factor, and urokinase receptor in endometrial epithelial cell co-culture systems [58].

Many studies suggest human leukocyte antigen G (HLA-G) to serve as a potential predictive biomarker of viability if assessed along with morphological parameters [59-61]. However, many studies have failed to observe a relationship between HLA-G and pregnancy [62]. In fact, in some studies, embryos that lacked HLAG implanted and established successful pregnancies [63,64]. These discrepant results may be attributed to multiple factors that affect embryonic levels of HLAG, including the day the media was collected and the composition of the media [62].

High microRNA levels have been detected in murine blastocysts [65,66]. Moreover, according to Gonzalez et al. [67] higher levels of leptin are present within viable blastocysts than arrested embryos. Leptin binds to endometrial receptors to stimulate fetal/ maternal communication [68]. Homeobox A10 has also been shown to mediate fetal/ maternal communication within viable embryos. In addition, embryos synthesize platelet activating factor (PAF) to affect maternal immunity and activate maternal platelets [69].

Embryonic viability may be indicated by the metabolic status of an embryo. A study by Leese [70] provided evidence that the less metabolically active an embryo, the higher its viability. According to their results, the most viable pre-implantation embryos are those with the lowest overall metabolism, glycolytic rates, and amino acid turnover. Furthermore, leucine, an essential amino acid, is the only amino acid significantly consumed by embryos that developed into blastocysts two or three days after intrauterine insemination. Leucine is detected in high concentration in uterine fluid and is involved in the embryonic transport of amino acids and insulin. Hence, leucine regulates embryonic energy levels [28].

Proteomics and Aneuploidy

More than 5% of early miscarriages are due to aneuploid fetuses. Aneuploidy is an abnormal condition in which a person either has an additional chromosome or lacks a chromosome. The older a women's age is, the higher her chances of having an aneuploid child. Currently, aneuploidy is diagnosed via invasive procedures, including amniocentesis and choronic villi sampling [71]. These tests have the potential to cause fetal defects and miscarriages. Aneuploidy can be non-invasively detected via fetal ultrasound and maternal serum analysis. Although the diagnostic tests detect 85% of aneuploid cases, they carry a 5% false positive rate. Replacing these methods with diagnostic biomarkers of aneuploidy will improve the selection and hence, the transfer of euploid rather than aneuploid embryos in ART.

To discover a biomarker for aneuploidy, we must compare the proteomic profile of amniotic fluid from a healthy pregnancy with matched aneuploid cases. Amniotic fluid's molecular and proteomic content varies throughout the duration of pregnancy due to developmental fetal and maternal changes. For instance, during early pregnancy, amniotic fluid is composed of water more than protein [72]. However, after eleven weeks of pregnancy, as fetal kidneys mature, it consists of high amounts of proteins excreted from the fetal kidneys [73].

McReynolds et al. discriminated between the secretomes of morphologically similar aneuploid and euploid blastocysts and discovered 9 proteins differentially expressed between the two using MS analysis. Of the 9 proteins, the most differentially expressed protein was lipocalin-1 [74] (Table 3). It was present at higher levels within aneuploid blastocysts. In addition, pattern of expression of lipocalin-1 within viable, euploid blastocysts that resulted in pregnancy was similar to expression pattern in euploid blastocysts that failed to implant [74]. Hence, lipocalin-1's expression reflects aneuploidy rather than failed implantation. In fact, Lipocalin-1 binds to hypdrophobic ligands and inhibits cysteine proteases, which are involved in implantation and embryo hatching [75,76]. Lipocalin-1 is highly expressed within states of stress, infection, and inflammation. Proteases and anti-proteases must be balanced in order for trophoblasts to invade [50]. Overexpression

Study (Year)	Proteomes Analyzed	Biomarker(s) Identified	Changes in Biomarker(s)	Biomarker(s) Function
McReynolds et al. [74]	Secretomes of morphologically similar aneuploidy vs. euploidy blastocysts	Lipocalin-1	Differentially expressed between aneuploidy and euploidy blastocysts; reflects aneuploidy rather than failed implantation; if overexpressed, causes protease/ anti-protease imbalance inhibiting implantation	Binds to hydrophobic ligands; inhibits cysteine proteases; high levels signify stress, infection, and inflammation
Tsangaris et al. [73]	Amniotic fluid of Down Syndrome fetuses (cases) vs. healthy fetuses (controls)	Insulin-like growth factor binding proteins	40% less in Down Syndrome cases than in controls	Transport
Wang et al. [79]	Amniotic fluid of Down Syndrome fetuses (cases)vs. healthy fetuses (controls)	Pre-albumin, transferrin, trypsin	Higher in Down Syndrome cases than in controls	Transport, serine protease
Tsangaris et al. [73]	Turner Syndrome fetuses (cases) vs. healthy fetuses (controls)	Serotransferrrin, lumican, plasma retinol-binding protein, apolipoprotein A1 kiniogen, prothrombin, apolipoprotein A-IV	a. Higher in cases than controls b. Higher in controls than cases	Fetal growth and development Nutrient cofactors and hormone carriers
Wang et al. [79]	Trisomy 18 fetuses (cases) vs. healthy fetuses (controls)	a. Apolipoprotein A1, phaeodactylum tricornumtum, antitrypsin b. placental protein 14	a. Higher in controls than cases b. Higher in cases than controls	Immunity, skin pigmentation, platelet disorders

Table 3: Potential Biomarkers of Aneuploidy.

of lipocalin-1 increases levels of proteases, causing a protease/anti-protease imbalance, which may have prevented implantation from taking place [77].

One out of eight thousand babies born in the world are born with Down Syndrome (DS), Trisomy 21 [71] (Table 3). Cho et al. analyzed DS fetuses. Cho et al. discovered several changes within their proteomes, including alterations in amino acids, metabolic pathways, and purines [78]. On the other hand, Tsangaris et al. discovered 7 differentially expressed proteins between DS fetuses and healthy ones. Moreover, insulin-like growth factor-binding proteins levels were 40% less in DS cases than the controls [73] (Table 3). DS cases also only had one arginine/serine-rich splicing factor and higher levels of alpha-1-microglobulin, collagen alpha one chain, collagen alpha-3(IV) chain, and basement membrane-specific heparin sulfate proteoglycan core protein. Wang et al. demonstrated higher levels of trypsin, pre-albumin, and transferrin present within the amniotic fluid of DS cases in contrast to controls. DS cases also expressed a differential level of APOA1, serprina3, pre-albumin, and transferrin [79]. Levels of pre-albumin and transferrin, along with trypsin, was higher in DS cases than in controls. In addition, their differentially expressed proteins were involved in dysfunctional lipid and cholesterol metabolism, processes of metal ion transport, adenosine triphosphate metabolism, and energy-coupled protein transport [79].

The proteomes of maternal serum within pregnant women carrying DS fetuses and normal, healthy ones were compared and contrasted [80]. Both cases and controls were gestationally age-matched, and their serum was collected during the first and second trimesters. According to their results, many proteins were differentially expressed: 19 proteins during the first trimester, 16 during the second trimester, and 10 in both trimesters.

Kolialexi et al. [71] also analyzed the proteomes of maternal serum pregnant cases carrying DS fetuses and controls carrying healthy ones. They discovered 9 proteins differentially expressed amongst both cases and controls. Their cases expressed lower levels of one protein and higher levels of 8 proteins. Out of the nine proteins, 3 were associated with the DS phenotype, whereas 6 were involved in fetal growth and development.

One out of every two thousand babies is born with Turner Syndrome per year. Females with Turner Syndrome only have a single X chromosome. Tsangaris et al. [73] discovered Turner fetuses have higher levels of serotransferrin, lumican, plasma retinol-binding protein, and apolipoprotein 1 (APO A1) [69], but lower levels of kininogen, prothrombin, and apolipoprotein A-IV when compared to controls [81].

One in 3000 babies are born with Trisomy 18. Wang et al. discovered that Trisomy 18 fetuses had lower levels of APOA1, Phaeodactylum tricornutum, and antitrypsin, but higher levels of placental protein-14 when compared to controls [79]. Also, most of the proteins in Trisomy 18 were associated with immune processes, dysfunction of skin pigmentation, and platelet disorders.

Proteomics and Male Infertility

50% of infertile cases are due to male factors. However, most cases are classified as idiopathic because there is no reliable, specific, non-invasive method of assessing male fertility or infertility. Instead, fertility is assessed via history taking, physical examination, endocrinological tests, and semen analysis. If these tests yield normal or inconclusive results, a risky, invasive surgical biopsy is conducted.

Semen consists of spermatozoa and seminal plasma. Semen is a poor predictor of male fertility because its composition varies from individual to individual, in different pathological states, and in different environments [82]. Semen parameters can also vary within the same individual at different ages and during different seasons [82,83]. Semen analysis involves the characterization of its macroscopic (coagulation, color, viscosity, pH, and volume) and microscopic parameters (agglutination, sperm counts, concentration, motility, morphology, and viability). Azoospermia is defined as the complete absence of sperm, and oligozoospermia is defined as the presence of low concentration of spermatozoa in semen [84]. Both azoospermia and oligospermia can be detected by semen analysis (sperm count and concentration). Azoospermia can also be diagnosed by the presence of antisperm antibodies that break through the blood-testis barrier and cause infertility [85]. The antibodies are insignificant in detecting ART success rates [86,87].

The acrosome reaction serves as a potential biomarker of male fertility based on the sperm penetration assay [51,88]. Positive SPA results indicate that fertilization will occur if the sperm penetrates the zona pellucida. However, SPA can only assess IVF outcomes and not ICSI outcomes because fertilization is induced in ICSI [89]. By comparing the proteome of normal sperm with pathological sperm, comparative proteomic studies have identified potential protein biomarkers indicative of male fertility. Further validation of these biomarkers will not only improve ART success rates, but may open doors to uncovering the etiologies behind most cases of idiopathic male infertility.

Seminal plasma makes up 90% of seminal ejaculate. It is a rich source of proteins and therefore serves as a better investigative source of proteomic biomarkers of fertility than spermatozoa. Batruch et al. [90] discovered 32 proteins only found within the seminal plasma of fertile males. Milardi et al. [91] found 83 proteins common in samples collected from five fertile men at three months before conception. Two of these proteins are human cationic antimicrobial protein (hCAP) and spindlin1. HCAP is a part of innate immunity, and thus, may prevent infection during fertilization [91,92]. Murine studies have shown Spindlin1 attach to sperm tails and may be involved in sperm motility and spermatogenesis [93].

In addition, Pilch et al. [94] identified 923 proteins in the seminal plasma of a single fertile male. Various heparin binding proteins (HBPs) including seminogelin 1, seminogelin 2, lactoferrin, fibronectin, laminin, and albumin were amongst the proteins discovered. Kumar et al. identified 40 HBPs within the seminal fluid, including those involved in metabolism, transcription, cellular transport, cellular structure, and signal transduction [95]. These HBPs release motile sperm from coagulum and trap spermatozoa in the gel to protect them from mechanical damage [96]. Many HBPs including seminogelin 1, seminogelin 2, and fibronectin, are differentially expressed in fertile and infertile males. Lactoferrin, the main HBP present in sperm, which quantitatively varies between fertile and infertile males [97,98]. It protects sperm from microbes in the female reproductive tract [99].

Male infertility can be caused by changes in sperm DNA integrity. Sperm cells are highly susceptible to changes in their DNA. After spermatogenesis, histones are replaced by protamines. As a result, nucleosomes only make up 5-15% of spermatic chromatin [100]. Infertile sperm exhibit overexpressed levels of histones (sperm-specific histone 2B) and underexpressed levels of protamines, in comparison to fertile sperm [101]. As a result of this imbalance, their nuclei acquire a hydrodynamic shape, which threatens the integrity of their

DNA, predisposing them to infertility [102]. There are four types of protamines: P1, P2, P3, and P4. Fertile sperm should have equivalent levels of P1 and P2 [103]. However, sperm from infertile men have a higher P1/P2 ratio because of the underexpressed levels of P2. As a result, DNA fragmentation occurs and causes infertility [104].

de Mateo et al. [105] also detected high levels of histones and low levels of protamines in their study. They identified chromatin proteins by analyzing isolated spermatic nuclei collected from four normozoospermic males after three days of sexual abstinence. They identified 403 proteins, 212 of which were not identified in other studies. In addition, they detected 159 nuclear proteins, 72 of which had not been detected in other studies. The nuclear proteins detected were histones, representing 9.5% of all the proteins identified in their study. They identified 39 histone proteins. Histone proteins have various fertility-related functions. Some histones are involved in spermiogenesis when replaced by protamines, and mediate nucleosome eviction. Histone H2B is involved in forming a pronucleus after gamete fusion and is required for chromatin determination after fertilization. Moreover, de Mateo et al. detected levels of protamine 2, but not protamine 1, although they are both common spermatic nuclear proteins [100]. A reason for this discrepancy may be due to the limitations of mass spectrometry in detecting small peptide fragments.

Furthermore, fertile and infertile males express different levels of many peripheral spermatic proteins. Thus, these peripheral proteins may relate to fertility. One of these proteins is eppin, an epididymal protease inhibitor [106]. If antibodies attack and silence eppin, the acrosome reaction fails to occur [96]. Guanyl cyclase receptor G [107] levels also differ among fertile and infertile males. HGCG is present in the human testis and is associated with spermatic binding to the zona pellucida [66].

Many proteins required for the oocyte-sperm interaction can also serve as potential biomarkers of fertility [108]. In murine studies, mice that lacked angiotensin-converting enzymes could not bind to the zona pellucida and thus, became infertile [109]. In addition, ADAM3-null murine sperm abnormally migrated and bound to the zona pellucida, predisposing them to infertility. ADAM3 is a membrane protein in human and murine sperm that binds to the zona pellucida. Redgrove et al. [110] detected lower levels of heat shock protein 2 (HSP2) within fertile men compared to men with impaired fertilization. HSP2 mediates the fusion of sperm and egg along with arylsulfatase A and sperm adhesion molecule 1, two potential biomarkers of sperm fertility [110].

Proteomics and Asthenozoospermia, Globozoospermia, Oligozoospermia and Azoospermia

Various biomarkers have been related to and identified in asthenozoospermic, globozoospermic, oligozoospermic, and azoospermic sperm and seminal plasma. They can serve as diagnostic and therapeutic means of assessing these causes of infertility. Asthenozoospermic sperm are characterized by low motility. 34 proteins are differentially expressed between normal and asthenozoospermic sperm [96]. These proteins have enzymatic, structural, and signaling functions. The enzymatic proteins are involved in the synthesis of ATP via various metabolic pathways. Energy is required for motility. Isocitrate dehydrogenase subunit a, an enzyme involved in the Krebs cycle, was suppressed within asthenozoospermic sperm [111]. In addition, phosphoglycerate mutase 2, triosephosphate isomerase, and oxaloacetate transaminase 1 are overexpressed within asthenozoospermic cases. Patients with mild asthenozoospermia lacked Tat1, septin4, and septin7 in 97% of their sperm. Septins are GTPases of cellular membranes and microtubules [112]. Mice that lacked septin4 developed bent/detached flagella and mitochondrial dysfunction, causing them to become asthenozoospermic [113]. Wang et al. [79] detected overexpressed levels of two epididymal proteins (E1 and E4) and low levels of oxidative stress regulator DJ-1 in asthenozoospermic patients.

Globozoospermic sperm lack acrosomes. ICSI was first used to treat globozoospermia in 1994. Despite successful pregnancies from globozoospermic patients, the success rates of ICSI in globozoospermic patients are less than the general ICSI success rates [114,115]. In contrast to normozoospermic sperm, globozoospermic infertile sperm have less expressed levels of actin and tubulin [116]. Actin and tubulin are cytoskeletal proteins involved in cell motility, signal transduction, and which help maintain cell membrane shape [96]. In addition, globozoospermic sperm have lower levels of outer dense fiber protein 2 (ODF2). ODF2 is required for flagellar elasticity and strength, and cellular motility [117]. Sperm immobility can also result from abnormalities in flagellar proteins (AKAPS and outer dense fibers) and axonemal proteins (tektin and dyneins) [116]. Liao et al. [116] conducted a study on globozoospermic sperm only to discover downregulated levels of sperm protein associated with the nucleus in the X chromosome A-C (SPANX A-C) and a lack of SPACA1, a protein involved in sperm-oocyte fusion. Moreover, seminal plasma secretory actin-binding protein (SABP), the main cytoskeletal protein in sperm, was upregulated in globozoospermic men. SABP prevents motility and the acrosome reaction from taking place.

Oligozoospermia is a condition characterized by low sperm concentration. Oligozoospermic males have lower levels of transferrin than healthy, fertile males. On the other hand, azoospermic patients, who lack motile sperm, highly express prolactin inducible protein (PIP) [118]. Prolactin inducible protein binds and forms a complex with human serum albumin (HAS) which is involved in capacitation and motility [119]. Despite the lack of correlation detected between PIP and fertility in some studies, PIP and HSA together may serve as biomarkers [120]. Azoospermic cases also express fibronectin, prostatic acid phosphatase, proteasome subunit alpha type 3, beta 2 microglobulin, galectin 3 binding protein, and cytosolic nonspecific dipeptidase [15].

Recent studies and reviews on sperm proteome highlight the future direction of research in our understanding of the underlying pathology of male infertility [74,121-130]. The role of oxidative stress in male infertility has also been recently highlighted in our studies. Figure 1 shows the general schematics of the proteomic analysis for identifying the oxidative stress related proteins of interest in spermatozoa or in the seminal plasma. We identified 74 proteins in spermatozoa of which 10 DEP proteins showed a 2-fold difference and were overexpressed compared to 5 DEP that were under-expressed in ROS positive group [123]. One of the proposed signaling pathways in the testis that may be influenced by the overexpressed and under-expressed proteins in the ROS positive group is shown in Figure 2. Using the 2-DIGE technique we demonstrated a significantly different expression of proteins conferring protection against the oxidative stress. Of the 1,343 protein spots detected in gel 1, 1,265 spots were detected in gel 2. 31 spots were differentially expressed and 6 spots were significantly decreased and 25 increased in ROS negative samples compared with ROS positive samples [119]. Similarly in the seminal plasma of the same group of men exhibiting oxidative stress we identified 14 proteins of which 7 were common and 7 were unique in the ROS positive and ROS negative

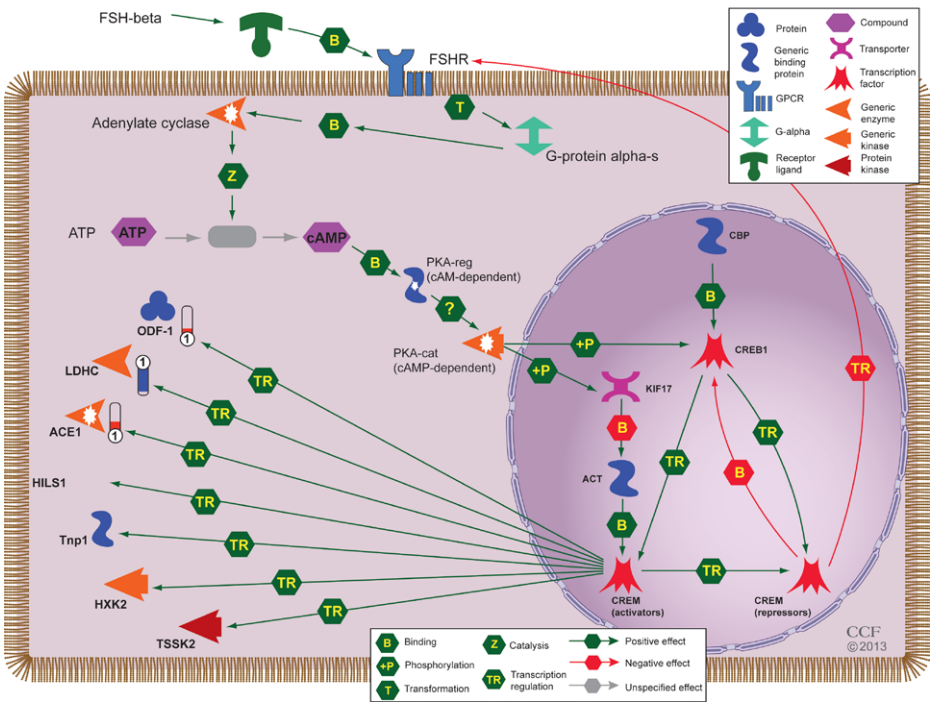

Figure 2: CREM signaling in the testis. Proteins in red are the ones overexpressed while those in blue are underexpressed in spermatozoa from the ROS positive compared to ROS negative group. The color levels in the tubes reflect their expression levels (red-overexpressed and blue underexpressed).

Abbreviation: B=Binding; Z=catalysis; T=transformation and TR=transcription regulation. The figure shows the generic enzymes (Adenylate cyclase, LDHC, ACE1, HXK2); Protein kinases (PKA-cat (cAMP –dependent and TSSK2); CREM activators and repressors; generic binding proteins (ACT, CPB and Tnp1 and PKA-reg (cAMP–dependent) and proteins (HILS1 and ODF1) involved in the CREM signaling in the testis. CREM=cAMP responsive element modulator; ACE=Angiotensin I converting isoform 1 precursor; ODF1=outer dense fiber of sperm tails 1; LDHC=L-lactate dehydrogenase C.

group [118]. We demonstrated how oxidative stress may modify or down-regulate some of these proteins that could contribute to male infertility.

Proteomics is not without inherent challenges like any other technology. These include cost of conducting proteomic studies, availability of advanced instruments, time restraints, lack of reproducibility and validation of the identified proteins. The challenges are at several steps such as, the sample preparation level, protein extraction, protein elution, data collection, database analysis and analysis of the protein functional implications. There is tremendous variability in protocols for protein digestion, protein elution and protein selection across different laboratories. The mass spectrometers have high mass accuracy but suffer from lack of reproducibility. There is a need to have well defined protocols to select peptides upon elution with chromatography (Wasinger, 2013 #198). Techniques such as targeted MS are being proposed to accomplish this goal (Schmidt et al. [14]). Global standardization of the methodologies and database analysis of the identified proteins is required.

Validation of differentially expressed proteins in serum/ peritoneal fluid of endometriosis patients, co-culture fluid from oocyte–spermatozoa incubation media, or spermatozoa/ seminal plasma can be carried out using different techniques depending on the source of the proteome or the secretome. Western blot immunochemistry, ELISA and RT-PCR techniques can be applied to validate the proteins. If spermatozoa markers are being validated all except RT-PCR can be applied. Spermatozoa are transcriptionally inert and hence RT-PCR is not suitable. Western blot requires the use of appropriate concentration of the primary antibody against the protein marker of choice and appropriate secondary antibody. Many proteins are novel and have been reported for the first time. Therefore, their purification, characterization and chromosomal localization need to be established before undertaking the validation of their function and expression.

Conclusion

Proteomics has the potential to revolutionize reproductive medicine and ART procedures. The application of proteomics to ART procedures is still a growing field. Although many studies have discovered potential biomarkers for endometrial receptivity, embryonic viability, aneuploidy, and spermatic fertility/infertility, more research and standardization needs to be conducted before any of these biomarkers can be used for standardized diagnostic and therapeutic purposes. While many proteins are described in this review, several proteins have to be validated in order to select the most likely candidate biomarkers. Most of these biomarkers at present do not have any proven clinical value. This is an innovative article with a focus on Proteomics and ART. Our manuscript elaborates on the potential proteomic biomarkers that are key to establishing a non-invasive, reliable, reproducible, and specific means of assessing endometrial receptivity, embryonic viability, aneuploidy, and fertilizing potential of the sperm. The application of these potential biomarkers in the future may help in enhancing the ART outcomes by offering a personalized treatment for patients based upon their individualized signature

proteomics profiles. Studies with larger sample sizes are necessary for a better understanding of the proteomic profiles as well as identifying the potential biomarkers proteins in predicting embryonic viability, successful implantation of the embryo, successful establishment of pregnancy and aneuploidy in ART. This will aid in the selection of the best embryo and enhance the ART outcomes.

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