Proteomic and Metabolomic Fingerprinting in Male Infertility

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Key Points

- Semen proteomics is able to provide valuable information about the patho-physiological state of the spermatozoa.
- Integration of proteomics as a component of male infertility diagnosis in the clinical lab can help explain the underlying molecular etiology associated with specific infertility conditions.
- Metabolomics strategies could potentially be used to differentiate between fertile males and infertile patients or between two different infertility-related disorders.
- Male infertility-related disorders may each have specific metabolic profiles which could contribute toward the noninvasive diagnosis of infertility in males.
- Specific proteins or metabolites identified using the omics strategies may serve as potential biomarkers of a specific male infertility disorder and in the development of new therapeutic modalities.

9.1 Introduction

In the current scenario, infertility is a major concern among couples in the reproductive age group with a global prevalence of 9%. Infertility in 27% of these couples is due to both male and female factors while 38% is contributed by the female alone and 20% is attributed to the male factor. The remaining 15% of infertility problems are idiopathic [1]. An overall 50% of cases are contributed by male factor infertility [2]. Male infertility is a multifactorial disorder and it is evaluated based on the results of semen analysis tests. Conventional semen analysis is considered as the corner stone for the diagnosis of male infertility. It provides information about semen parameters such as sperm concentration, motility, morphology, and vitality. The World Health Organization (WHO) has provided well-established reference values for semen parameters to distinguish fertile men from infertile men [3]. Additionally, advanced tests are carried out along with basic semen analysis to determine the levels of oxidative stress and sperm DNA damage, which are a major cause of fertilization failure or male infertility [4, 5]. However, there are still other underlying mechanisms at a subcellular level of the spermatozoa that cannot be explained merely from the results of a conventional semen analysis.

On the other hand, omics studies are able to explain the molecular mechanisms underpinning male infertility using different approaches. The four main branches of omics include genomics, transcriptomics, proteomics, and metabolomics (Fig. 9.1). Among these omic studies, proteomics and metabolomics are being widely used in the field of male infertility. Semen proteomics and metabolomics are undertaken to understand the cellular pathways and metabolic pathways associated with normal gametogenesis and the role of proteins and metabolites in the fertilization process. Furthermore, proteomics and metabolomics analysis, with bioinformatic tools and metabolomic analysis along with chemometrics, serves as a promising tool in the identification of potential diagnostic and therapeutic biomarkers for the management of male infertility.

In recent years, the availability of advanced proteomic and metabolomic tools has increased the knowledge and understanding of the causes of male infertility. This chapter provides a brief overview of advanced proteomic and metabolomic techniques used in studies involving the sperm and seminal plasma of infertile males. It highlights the general steps involved in these omics approaches, including bioinformatic analysis of the proteomic data. Furthermore, pro-

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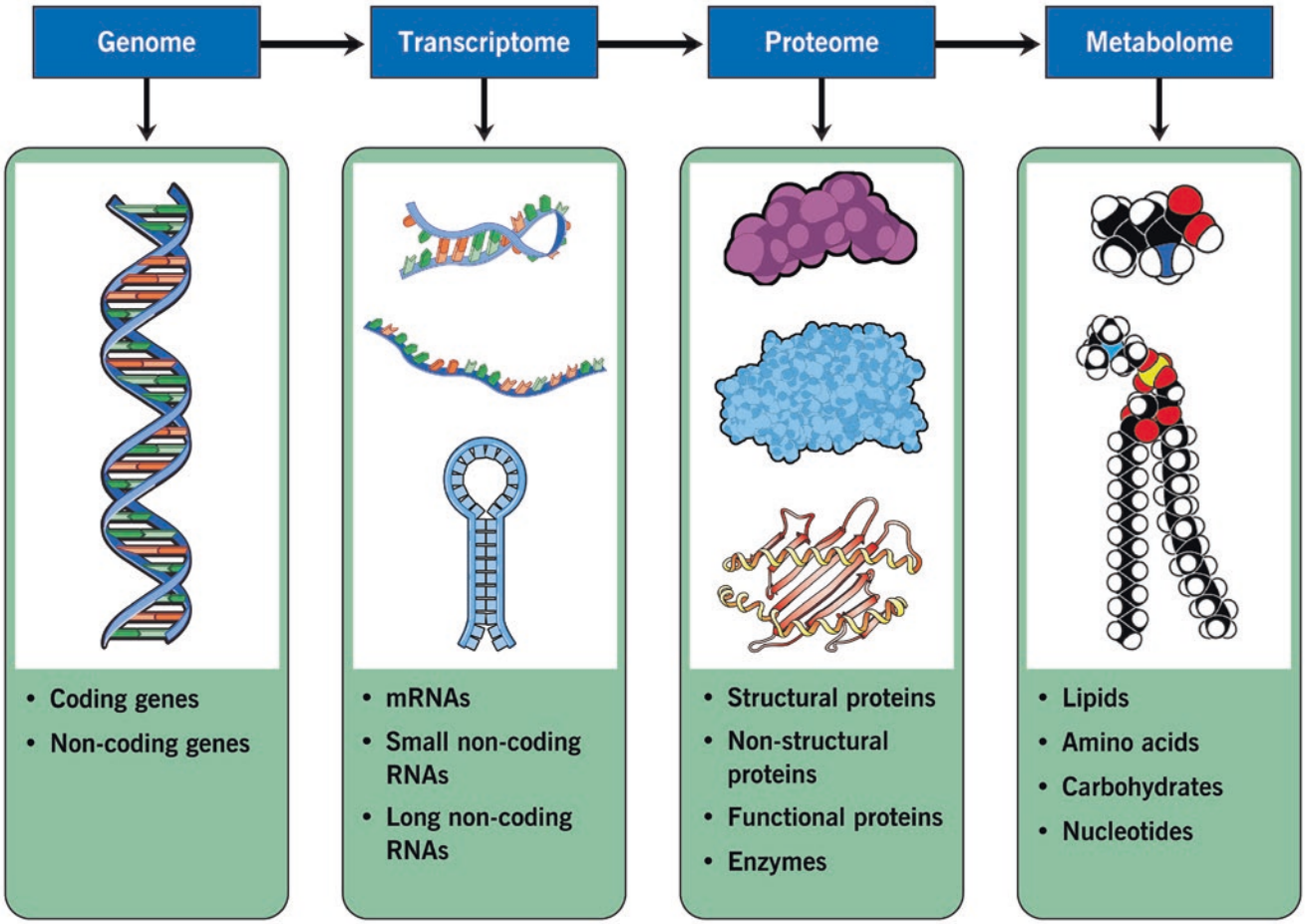

Fig. 9.1 Different classes of biomolecules detected in the spermatozoa using different omics techniques. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2018–2019. All Rights Reserved)

teomic- and metabolomic-based studies in sperm and seminal plasma are discussed in detail, along with the potential role of biomarkers in the prognosis and diagnosis of male infertility.

9.2 Proteomics in Male Infertility

Proteomics is defined as the complete profiling of proteins extracted from a tissue or cell. The most commonly used proteomic approaches, such as shotgun or bottom-up, can identify more than 1000 proteins in a short period of time. Semen is considered as the biological fluid used for the diagnosis of male infertility. Cellular component of semen is made up of sperm (5%) and seminal plasma (95%). Sperm are transcriptionally and translationally inert gametes and rely on proteins for their functional activity. Currently, proteomics is being widely used in the field of male infertility to study the protein profiles in spermatozoa and seminal plasma [6–8]. High throughput platforms such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and mass spectrometry coupled with liquid chromatography (LC-MS/MS) are used to profile a maximum number of proteins in the sperm and seminal plasma [9].

Advanced proteomic tools were used to identify more than 6000 sperm proteins. Using the data mining approach, 30 proteomic studies were analyzed and a total of 6198 proteins were identified in the spermatozoa [10]. A similar approach was used by Jodar et al., to identify 2064 proteins in the seminal plasma [7]. Any alteration in the sperm or seminal plasma proteome may have an adverse effect on the normal physiological function of the spermatozoa. Several proteomic studies have identified alterations in the sperm and seminal plasma proteome associated with male infertility conditions such as varicocele [11–16], idiopathic infertility [17–19], unexplained infertility [20–23], elevated oxidative stress [24–26], and testicular cancer [27, 28]. Moreover, the key proteins associated with vital sperm functions such as capacitation, hyperactivation, acrosome reaction, and fertilization process, are identified as potential noninvasive biomarkers to differentiate infertile men from normal healthy fertile men.

9.2.1 General Approach to Proteomics

Different proteomic techniques were used in the detection and identification of sperm proteins. The conventional approach includes the use of a two-dimensional (2D) gel electrophoresis of the extracted sperm or seminal plasma proteins. The proteins present in the sample are separated out based on the isoelectric focusing property and molecular weight of the peptides. Martinez-Heredia et al. identified a total of 98 distinct proteins in the human spermatozoa using 2D gel electrophoresis coupled with MALDI-TOF technique. The majority of these proteins were mainly involved in energy production, protein synthesis, and transcription process [29]. A modified version of the 2D gel electrophoresis technique, known as difference gel electrophoresis (DIGE), is used to identify differentially expressed proteins (DEPs) with a minimum error of <10% [30]. Based on the intensity of the different staining dyes (Cy3 and Cy5), the expression of the DEPs are determined on the same gel using automated image analysis software.

Conventional proteomic techniques have several limitations, such as decreased sensitivity, detection of a fewer number of proteins in a given sample, and the missing out in the detection of less abundant proteins. Investigators were able to overcome these limitations by using sophisticated and complex instruments such as MALDI-TOF and LC-MS/MS. These instruments can detect the maximum number of proteins even in samples that are of lesser concentration. Using the in-gel digestion-based LC-MS/MS approach, Johnston et al. identified 1760 sperm proteins and also reported the abundance of 26S proteasome complex [31]. Later on, several other studies also employed the LC-MS/MS-based proteomic profiling of spermatozoa in men with infertility disorders [22, 28, 32–34].

9.2.2 Assessment of Sperm and Seminal Plasma: Methods and Tools, Analysis, Bioinformatics

Proteomic analysis starts with the extraction of proteins either from the spermatozoa or seminal plasma. Seminal plasma are rich in proteins and are readily available for proteomic experiments without undergoing any purification process. However, prior to the extraction of proteins, sperm are subjected to several purification and processing steps. First, sperm are separated from the seminal plasma by the centrifugation technique. Apart from the sperm, semen also contains other cells such as round cells and immature germ cells. The round cells include both spermatogenic as well as non-spermatogenic cells. Investigators proposed that the use of sperm with round cells may contaminate the sperm proteome. Therefore, the density gradient centrifugation step was recommended and performed to isolate a pure fraction of the spermatozoa for proteomic analysis [35–39]. Recently, Paneer Selvam et al., conducted two proteomic studies to understand the role of contamination by round cell proteins in the proteome of sperm and their effect on biological pathways associated with sperm function [40, 41]. The presence of round cell proteins were masked by the sperm proteome, and the influence of non-spermatogenic round cell proteins was found to be very negligible or insignificant [40]. Moreover, the presence of these round cells and leukocyte proteins failed to show any effect on the molecular pathways associated with sperm function [41] (Fig. 9.2).

The isolated sperm are routinely washed for a minimum of three to four times with phosphate buffer saline to remove the remnants of seminal plasma. The sperm pellet, free from any contamination, is mixed with radioimmunoprecipitation assay (RIPA) buffer and left overnight. This results in the complete lysis of spermatozoa. Sonication of the spermatozoa suspended in an isotonic medium is also carried out to extract the sperm proteins. Extracted sperm proteins are checked for their purity and concentration, and then subjected to one-dimensional SDS-PAGE. Proteins separated by electrophoresis are subjected to in-gel digestion using trypsin. Digested proteins and peptides are eluted and injected into the mass spectrometry (MS) system. MS detects the peptides and proteins with an unbiased approach [42]. The proteins are identified with a very low false discovery rate based on their mass/charge ratio (m/z). To identify the post-translational modification such as acetylation, methylation, and phosphorylation in the sperm proteome, enrichment protocols are recommended. In addition, MS coupled with high performance liquid chromatography (HPLC) can simplify the detection of the complex proteins. Other techniques such as MALDI-TOF and SELDI-TOF (surface-enhanced laser desorption/ionization time-of-flight) are also successfully used to detect the sperm proteins [43, 44].

Initially, the complete scan of peptides detected by the MS is compared with the global database consisting of previously annotated and sequenced proteins. Computational software such as SEQUEST, Mascot and X! Tandem operating with different algorithms displays the complete list of proteins [45]. Furthermore, the proteins are categorized as DEPs based on spectral counts and abundance of each protein. These DEPs are used in the downstream bioinformatic analysis to understand the role of proteins in the molecular pathways [46]. Gene ontology (GO) analysis provides additional information such as localization and distribution of the proteins. Freely available bioinformatic tools such as STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) are used to understand the interaction between proteins [47]. In addition, commercially available sophisticated software such as Ingenuity Pathway Analysis (IPA) and Metacore™ are used to obtain a complete picture of the

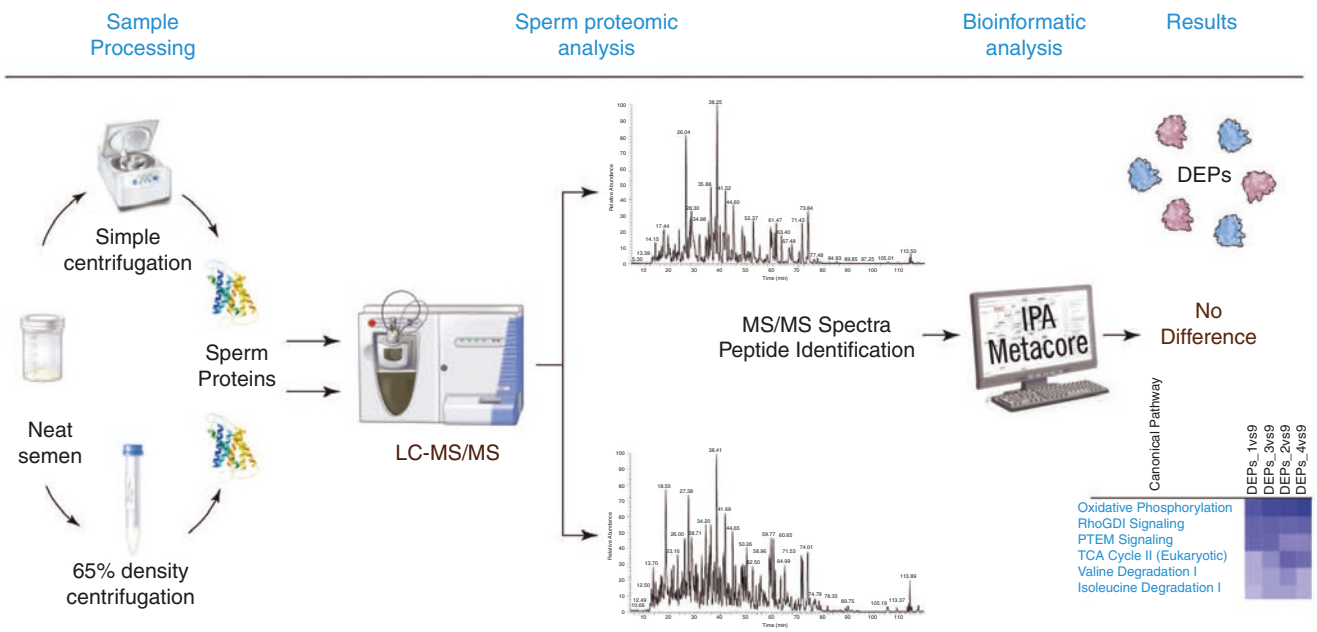

Fig. 9.2 LC-MS/MS analysis of sperm demonstrates the use of neat semen samples in proteomic/bioinformatic analysis. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2018–2019. All Rights Reserved)

interactions between the proteins and the transcriptional factors regulating their expression [47].

9.2.3 Sperm Proteomics

Any pathological state will alter the homeostasis and have a direct effect on the proteome of the tissue or cell. Changes in the proteome content of the spermatozoa may have deleterious effects on the fertilizing ability of spermatozoa. Several studies have identified the changes in the expression of the proteins associated with male infertility.

Proteomic profiling of sperm was performed on asthenozoospermic infertile men [48]. A total of 667 sperm proteins were identified in the asthenozoospermic men. These proteins were found to affect cellular pathways such as glycolysis, gluconeogenesis, and axoneme activation and nucleosome assembly [48]. Cao et al., demonstrated that proteins such as cytochrome c oxidase subunit 6B (COX6B), outer dense fiber 2 (ODF), and tubulin beta 2B (TUBB2B) involved in sperm motility were differentially expressed [32]. Similarly in another study on asthenozoospermic samples, Siva et al., identified that the proteins related to energy and metabolism, movement, and organization and protein turnover, folding, and stress response were significantly altered in their expression levels [49]. In addition to the proteins associated with sperm motility, the other protein components of proteasome complex were also differentially expressed [50, 51]. Moreover, phosphoproteome analysis of the asthenozoospermic samples showed that the dysregulation of these group of proteins were associated with cytoskeleton, fibrous sheath, and energy metabolism [52].

Globozoospermia is an abnormality of the spermatozoa that is associated with male infertility. Proteomic analysis of spermatozoa associated with globozoospermia revealed a total of 35 DEPs that play a vital role in spermatogenesis, cell skeleton, metabolism, and spermatozoa motility [53]. Also, perinuclear theca (PT) proteins were underexpressed and these were involved in acrosomal biogenesis, thus affecting the acrosome function in globozoospermic patients [54].

Proteomic analysis of sperm samples has identified the underlying changes or molecular pathology associated with varicocele condition. LC-MS/MS analysis of the sperm proteins in unilateral varicocele patients showed overexpression of 114 proteins and underexpression of 97 proteins. These DEPs were involved in sperm maturation, motility, capacitation, acrosome reaction, and fertilization [11]. Bioinformatics analysis demonstrated that small molecule biochemistry and post-translation modification proteins pathways were affected in infertile men with unilateral varicocele [11]. Agarwal et al. conducted a prospective study using the sperm samples from bilateral varicocele patients. A total of 73 DEPs were identified and the majority of these DEPs were involved in the regulation of functions such as metabolic processes, stress responses, and oxidoreductase activity. The remaining proteins were involved in sperm functions such as capacitation, motility, and sperm-zona binding [34]. Another proteomic study by the same researchers showed that the dysregulation of the mitochondrial proteins is a cause of male infertility in varicocele patients [16, 34]. The key DEPs

identified as biomarkers in different varicocele studies are presented in Table 9.1.

Testicular cancer has a deleterious effect on semen parameters and the fertilization potential of spermatozoa. The American Cancer Society estimated that there will be 9310 new cases and 400 deaths related to testicular cancer [55]. Several proteomic studies have been conducted for the diagnosis of testicular cancer [56–58]. These studies, however, did not examine the proteome of spermatozoa from testicular cancer patients prior to cancer treatment. Recently, Dias et al., profiled the sperm proteome in men with non-seminoma testicular cancer (NSTC) using the LC-MS/MS platform. They had identified a total of 189 DEPs in their study. Among the DEPs identified, NADH:Ubiquinone Oxidoreductase Core Subunit S1 (NDUFS1), ubiquinol-cytochrome C reductase core protein 2 (UQCRC2), and the testis-specific sodium/potassium-transporting ATPase subunit alpha-4 (ATP1A4) were proposed as the potential biomarker for NSTC patients. Furthermore, mitochondrial dysfunction is identified as the primary cause for the decrease in sperm concentration and motility [28].

9.2.4 Seminal Plasma Proteomics

Apart from sperm proteins, seminal plasma proteins are essential for sperm protection, maturation, and fertilization process. Male infertility conditions such as azoospermia, oligoasthenozoospermia (OAT), and varicocele showed alteration in the seminal plasma proteins [59].

Azoospermia may be either obstructive or non-obstructive. The seminal plasma proteome of azoospermic subjects revealed extracellular matrix protein 1 (ECM1) as a biomarker to differentiate obstructive azoospermia (OA) from non-obstructive azoospermia (NOA) and a differential expression of testis-expressed protein 101 (TEX101) in distinct NOA subtypes [60, 61]. A proteomic study by Yamakawa et al., proposed a total of 4 and 1 biomarker for NOA and

Table 9.1 Key differentially expressed proteins (DEPs) identified in various clinical conditions associated with male infertility

Condition	Sample	Method	DEPs	Reference
Varicocele	Spermatozoa	1D PAGE LC-MS	TEKT3, TCP11	Agarwal et al. (2016) [13]
	Spermatozoa	1D PAGE LC-MS	PKAR1A, AK7, CCT6B, HSPA2, ODF2	Agarwal et al. (2016) [34]
	Spermatozoa	1D PAGE LC-MS/MS	GSTM3, SPANXB1, PARK7, PSMA8, DLD, SEMG1, SEMG2	Agarwal et al. (2015) [11]
	Spermatozoa	LC-MS/MS	LETM1, EFHC, MIC60, PGAM5, ISOC2,TOM22, NDFSU1, UQCRC2, COX5B, ATPase1A4, HSPA2, SPA17, APOA1	Samanta et al. (2018) [16]
	Seminal plasma	2D- LC-MS/MS	ZA2G, KCRB, ALBU, NPC2, FINC, PIP, SEMG1, SEMG2, KLK3, TRFL, PPAP, ANXA3, CATB, EP3B, PTGDS, SODE, A1AT, ASAH1, CALM, CRIS1	Fariello et al. (2012) [64]
	Seminal plasma	2D- LC-MS/MS	IBP-3, SMG1, BRE1B, NPC2, IDH, E3-beta	Zylbersztejn et al. (2013) [65]
Testicular cancer	Spermatozoa	1D PAGE MS	NDUFS1, UQCRC2, ATP1A4, ACR, ANXA 2	Dias et al. (2018) [28]
Azoospermia	Seminal plasma		ECM1, TEX101	Drabovich et al. (2013) [60]
	Seminal plasma	2D DIGE LC-MS/MS	STAB2, CP135, GNRP, PIP, NPC2	Yamakawa et al. (2017) [62]
Asthenozoospermia	Spermatozoa and seminal plasma	UPLC-MS	PLXNB2, POTEKP, NIN, PHF3, DYNLL1, PROCA1, FASCIN-3; LRRC37B, PLC	Saraswat et al. (2017) [48]
	Spermatozoa	2D PAGE MALDI MS/MS	TPIS, GKP2, OXCT1, TUBB2C, TEKT1, PSMA3, HSPA2	Siva et al. (2010) [49]
	Spermatozoa	UPLC-MS(E)	GRP78, HSP70–2, TUBA4A, TUBA3C, TUBA8, ODF1, AKAP3, AKAP4, GAPDHS, ROPN1B, SPANXB, CLU, PIP, ATP5B	Parte et al. (2012) [52]
Oligoasthenozoospermia	Seminal plasma	1D PAGE/LC-MS/MS	AACT, TBCB, ALDR	Herwig et al. (2013) [19]
	Seminal plasma	2D PAGE LC-MS/MS	NPC2, M2BP, LCN1, PIP	Giacomini et al. (2015) [33]
	Seminal plasma	2D chromatography LC-MALDI	LTF, PIP, ECM1, HE1, PTGDS, CD177, PSA	Liu et al. (2018) [63]
Globozoospermia	Spermatozoa	2D DIGE MALDI-TOF/TOF MS	SAMP1, ODF2, SPANXa/d, TUBA2, TPI1, PIP	Liao et al. (2009) [53]

OA, respectively. The NPC2 protein was suggested as a potential biomarker for OA patients [62].

OAT is a semen abnormality condition associated with male infertility. Proteomic analysis of seminal plasma exposed a total of 2489 proteins in subjects with OAT [19]. Twenty-four proteins, primarily involved in metabolism and inflammation, defense, and stress responses, were highly expressed in idiopathic OAT (iOAT). A comparative proteomic analysis of oligoasthenozoospermic and normozoospermic seminal plasma revealed epididymal secretory protein E1 (NPC2) and galectin-3-binding protein (M2BP) to be underexpressed, while lipocalin-1 and a form of prolactin-inducible protein to be overexpressed in iOAT [33]. A recent comparative proteomic analysis identified DEPs involved in multiple biological functions such as binding activity (lactotransferrin, LTF; Prolactin-induced protein, PIP; extracellular matrix protein 1, ECM1), transporter activity (human epididymis-specific *protein* 1, HE1; Prostaglandin D_2 synthase, PTGDS), immune activity (CD177), and hydrolase activity (prostate-specific antigen) were differentially expressed in the seminal plasma of OAT subjects [63].

Seminal plasma proteins were also studied in varicocele patients. The first report on seminal plasma proteins identified 95 DEPs in cigarette smoking, adult varicocele patients. Seminal plasma proteins involved in sperm maturation and sperm–oocyte fusion were dysregulated in these varicocele patients [64]. Whereas, the expression of proteins associated with sperm motility and capacitation were altered in seminal plasma of adolescents with varicocele [65]. A study by Belardin et al. reported that insulin-like growth factor-binding protein 7 (IGFBP7) and deoxyribonuclease-1 (DNASE1) were involved in the regulation of apoptosis as seminal plasma biomarkers in adolescents with varicocele [15]. The seminal plasma proteomic signature also varied from before and after varicocelectomy. Cellular pathways such as oxidative stress and protein stabilization were enriched in patients after varicocelectomy. Proteins related to homeostasis function such as DJ-1, S100-A9, SOD, ANXA1, G3P, and MDH were upregulated and proteins associated with oxidative stress (such as NELFE) were downregulated after varicocelectomy [65].

Proteomic studies identifying DEPs associated with various male infertility conditions are listed in Table 9.1.

9.3 Metabolomics in Male Infertility

Metabolomics is the latest of the omics technologies that has been gaining traction in male infertility research over the last decade. Metabolomics involves the unbiased identification and quantification of all low molecular weight metabolites (< 1 kDa) within a biological system. The resulting metabolome consists of a complete set of these metabolites, which include secondary metabolites as well as hormones and other signaling molecules present in a biological sample [66]. Metabolomics may be applied on various biological samples, including that of bodily fluids (e.g. urine, blood plasma or serum, seminal fluid, follicular, or endometrial fluid) and various tissues in the body [67, 68]. As intracellular metabolites are in a state of dynamic balance with the metabolites in the biological fluids that perfuse these cells, the composition of biological fluids could therefore provide useful insights into the present metabolic state of the body [69].

Moreover, the study of the metabolome is comparatively less complex, provides more real-time information, and could give direct impact of a certain condition/stimuli on the body. For example, while genes and mRNA transcripts may run up to hundreds of thousands in numbers, and proteins to millions, the downstream products of metabolism (i.e., metabolites) only amount to a few thousand within the human metabolome [44]. These metabolites resemble the current phenotypic state of the cell more closely than the transcriptome and proteome do. This is because following gene expression, post-transcriptional and post-translational modifications take place, and these changes are relatively augmented in the metabolome compared to the transcriptome or proteome [70]. As such, metabolomic profiling of a particular biofluid or tissue could thus reveal the current health status of the individual [71].

9.3.1 General Approach to Metabolomics

There are several approaches that could be utilized when applying the metabolomics strategy to biological samples. Metabolomic fingerprinting provides a high-throughput, global and rapid biochemical analysis, which serves as a screening tool to differentiate between samples from healthy controls and that of diseased patients [66]. Changes detected in patient samples from that of normal samples are then correlated with the severity status of the disease or used to assess how an intervention is faring. On the other hand, metabolomic profiling involves the identification and quantification of a selected number of pre-defined metabolites that are involved in a particular metabolic pathway [66], while untargeted metabolic profiling is commonly done as a comparative analysis between the control and treatment groups. Both metabolomic fingerprinting and profiling have been applied to studies dealing with infertile males. Yet another approach is metabonomics, which deals with the quantitative analysis of metabolites in response to either disease or therapeutic treatment or to genetic modification. No matter the approach utilized, all metabolomics strategies include the identification and quantitation of metabolites [72].

The detection of metabolites in human metabolomics studies may be approached as either targeted or untargeted (global) (reviewed in Agin et al. [73]). Studies that employ the targeted approach have a specific hypothesis which warrants the investigation of certain biochemical pathways. Therefore, pre-determined metabolite-specific signals and analytical standards are employed to quantify the concentrations of a specific number of known metabolites in a precise and accurate manner. On the other hand, the global approach aims to measure and compare as many signals as possible without knowing the nature and identity of the metabolites beforehand. Thus, complex datasets are generated along with metabolites that are yet to be characterized. Global metabolomics studies provide only qualitative and semi-quantitative data, however, these studies help identify unknown metabolites, new pathways and generate hypotheses [74–77]. The study of the metabolome is multi-disciplinary and involves disciplines such as analytical chemistry, chemometrics, and biology. Analytical chemistry is useful during sample preparation, generation of metabolic profiles, and elucidation of metabolic structure. Chemometrics is required to extract the most pertinent information from the large datasets generated, for example, in studies using the metabolic fingerprinting strategy. Biology is necessary for understanding the observations, underlying mechanisms of action, and metabolomics pathways of interest [78].

9.3.2 Analysis of the Metabolome

Analysis of the metabolome is generally performed in four main stages: (1) collection of samples (sampling, quenching, and storage), (2) preparation of samples (extraction of metabolites, dilution, and clean up), (3) acquisition of data, and (4) analysis of data to generate a metabolic profile [78, 79].

Samples used in studies pertaining to male infertility include testicular tissue, seminal plasma, spermatozoa, blood serum and plasma, and urine (Table 9.2). The sample chosen for a particular study is influenced by the type of sample that can provide the most information for the intended study, its feasibility, as well as ease of collection [80]. Moreover, each of these biofluids could provide different types of information. For example, urine has the highest amount of water-soluble molecules, whereas blood composition is less variable than urine [81].

Following sample collection, the sample has to be stabilized so that it represents the actual metabolome composition at the time of sample collection. This is done via a metabolism-quenching step in order to halt further metabolic reactions that could either generate or degrade metabolites. Samples are snap frozen in liquid nitrogen for this purpose and stored at −80 °C [82]. Next, the sample is prepared according to protocol. For example, solid matrices undergo an extraction step to transfer the metabolome compound into a liquid phase, whereas low volatile analytes that will be analyzed via gas chromatography (GC) will undergo a derivatization reaction step (e.g., alkylation, acylation, silylation) to increase its volatility and reduce its polarity [83, 84].

Analysis of the metabolome can be approached through several techniques including nuclear magnetic resonance spectroscopy (NMR), Fourier transform infrared spectroscopy (FTIR), near-infrared (NIR), Raman spectroscopy, liquid chromatography, or gas chromatography coupled with mass spectrometry (LC-MS or GC-MS, respectively). Each of these techniques has its own advantages and shortcomings that have been previously reviewed elsewhere [66, 68]. The data acquired are processed using software tools and analyzed using suitable statistical techniques to segregate fewer, relevant metabolites (i.e., potential biomarkers/differential metabolites) that could possibly differentiate the compared sub-groups. Experiments that follow would then focus on the potential biomarker metabolites and explanations of the changes observed [78].

9.3.3 Sperm Metabolomics

Back in 2009, Huser's group had used the micro-Raman spectroscopy technique on individual sperm cells of healthy males to examine if the Raman spectra of sperm chromatin packed within the heads of normal or abnormally shaped sperm cells showed any correlation with its protein content and DNA conformation [85]. While the efficiency of DNA packaging and relative protein content per cell differed between the morphologically normal and abnormal sperm, the study also highlighted the significant variation that existed in protein content and DNA packaging within sperm cells with normally shaped heads [85].

Some years later, Paiva and colleagues were the first to obtain a comprehensive metabolomic profile of mature human spermatozoa through the use of two complementary untargeted metabolomics strategies: proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy and GC-MS in normozoospermic and asthenozoospermic samples [86] (Table 9.2). The NMR and GC-MS techniques identified 42 and 27 endogenous metabolites, respectively, with an overlap of four metabolites. The bulk of the identified metabolites belonged to the super classes of amino acids, peptides, analogues, organic acids, and lipids [86]. NMR and MS strategies provide complementary results that may be applied toward completing the metabolome of the mature human sperm cell.

Using the proton magnetic resonance spectroscopy (^{1}H-MRS) technique, Reynolds et al. determined the molecular composition of a live human spermatozoon from healthy vol-

Table 9.2 Metabolomics studies on male fertility/infertility

Study population	Human body fluid/tissue	Analytical technique	Main study outcomes	Reference
Spermatogenic failure; obstructive azoospermia; oligoasthenozoospermic infertility	Seminal plasma	^{1}H-NMR spectroscopy	^{1}H-NMR spectroscopy could potentially be used to differentiate OA from NOA Peak areas for GPC, citrate and lactate in seminal plasma were smaller in azoospermic men Peak area ratios for citrate:lactate and GPC:lactate were altered in control vs. (i) spermatogenic failure, (ii) OA Peak intensity ratio for GPE:GPC was different between spermatogenic failure and OA subjects	Hamamah et al. (1993) [89]
Non-obstructive azoospermia	Seminal plasma	GC–MS	Proposed a novel diagnostic method for NOA patients using the TIC data of metabolites in seminal plasma 36 metabolites were identified as potential discriminatory biomarkers for different groups in NOA	Gilany et al. (2017) [91]
Non-obstructive azoospermia	Seminal plasma	Raman spectroscopy	The seminal plasma metabolome could be used to detect spermatogenesis in NOA patients This metabolomics technique can be applied to detect major metabolomic alterations in seminal plasma of NOA from TESE(+) vs. TESE(−) cases TESE(−) patients had severe oxidative imbalance compared to TESE(+)	Gilany et al. (2018) [92]
Non-obstructive azoospermia (maturation arrest or Sertoli-cell only); normozoospermia (vasectomy reversal)	Testicular tissue	^{1}H-NMR spectroscopy	Testicular concentrations of phosphocholine and taurine were significantly different between normal, and SCO tissue Phosphocholine concentrations were significantly higher in testes with spermatogenesis ^{1}H-NMR spectroscopy could be used to discern a distinct metabolic signature for spermatogenesis, thus contributing to the non-invasive diagnosis of sperm in men with NOA	Aaronson et al. (2010) [96]
Non-obstructive azoospermia and obstructive azoospermia	Testicular tissue	Raman spectroscopy; GC/TOF-MS	Raman spectroscopy could be used to discriminate between samples of NOA and OA testicular tissue Raman spectroscopy could be used to non-invasively differentiate seminiferous tubules with complete and incomplete spermatogenesis. Levels of 12 metabolites were altered between NOA and OA testes (increased: cis-Phytol, glutamine; decreased: S-carboxymethylcysteine, fructose, arachidonic acid, etc.)	Liu et al. (2014) [97]
Non-obstructive azoospermia	Blood serum	HPLC-MS/MS	Serum metabolomic profile could be used to differentiate NOA patients from fertile males 24 metabolites were identified as potential markers, many of which were involved in energy production, oxidative stress, and cell apoptosis in spermatogenesis Metabolic pathways of glycometabolism, lipid metabolism, and amino acid metabolism were disrupted in NOA males Serum metabolic disorders may contribute to the etiology of NOA	Zhang et al. (2017) [115]
Oligozoospermia; normozoospermic infertility	Seminal plasma	^{1}H-NMR spectroscopy	^{1}H-NMR spectroscopy could potentially be used to differentiate normozoospermia from oligozoospermia 10 biomarkers were detected Alanine, citrate, GPC, tyrosine, phenylalanine could be used to screen for MI	Gupta et al. (2011) [102]

Table 9.2 (continued)

Study population	Human body fluid/tissue	Analytical technique	Main study outcomes	Reference
Oligozoospermia	Urine	LC/QTOF–MS	Oligozoospermic infertile men could be differentiated from fertile controls based on altered urinary metabolomic profiles Oligozoospermia seems to be associated with energy consumption and antioxidant defenses in spermatogenesis The combined pattern of acetylcarnitine, carnitine C3:1, and aspartic acid provided moderate diagnostic power for oligozoospermic infertility	Zhang et al. (2014) [95]
Asthenozoospermia	Seminal plasma	Raman spectroscopy	Metabolomic fingerprinting could be used to discriminate between asthenozoospermic and normozoospermic males	Gilany et al. (2014) [90]
Asthenozoospermia	Seminal plasma	^{1}H-NMR spectroscopy	Nineteen metabolites were either up- or down-regulated Asthenozoospermia-related changes of metabolites in lipid, phospholipid (choline), cholesterol, nucleoside, Krebs cycle, and energy metabolic pathways were detected Oxysterols (5α-cholesterol and 7-ketocholesterol) were raised in seminal plasma of patients with asthenozoospermia Oxidative stress is an underlying mechanism in the development of asthenozoospermia	Zhang et al. (2015) [93]
Asthenozoospermia	Seminal plasma	GC–MS	Of the 25 metabolites identified, 7 were significantly different in asthenozoospermic samples compared to controls Levels of oleic acid and palmitic acid were higher in seminal plasma of asthenozoospermic men, which may signify a metabolic disorder of the sperm membrane Deficiency of valine in seminal plasma of asthenozoospermic males may contribute to poor sperm motility	Tang et al. (2017) [116]
Asthenozoospermia	Spermatozoa	^{1}H-NMR spectroscopy and/or GC-TOF/MS	69 metabolites were identified in human sperm extracts: (i) 42 using NMR, (ii) 27 using GC-MS, (iii) 4 by both techniques (carnitine, L-threonine, gamma-aminobutyric acid, oxoglutaric acid) The majority of metabolites identified belonged to the amino acids, peptides, and analogues super class	Paiva et al. (2015) [86]
Idiopathic asthenozoospermia	Spermatozoa	GC-MS	33 metabolites were identified in spermatozoa 27 metabolites (e.g., 3-phosphoglycerate, lactic acid, glutamic acid, tryptophan, leucine, cysteine, guanosine, cytidine) were decreased in IAS group compared to controls 6 metabolites (zymosterol, dithioerythritol, orotic acid, 2-deoxyerythritol, benzoic acid, ethanolamine) were increased in IAS group compared to controls Pathways for nucleoside, amino acid and energy metabolism, and the Krebs cycle were either up- or downregulated) in IAS	Zhao et al. (2018) [88]
Idiopathic infertility, oligozoospermia, asthenozoospermia, teratozoospermia and azoospermia	Seminal plasma	NMR spectroscopy	Seminal plasma metabolomic profile of infertile males differed significantly from that of fertile males The metabolomic profile of idiopathic infertile men were clearly segregated from that of (i) fertile controls and (ii) other infertile groups The biomarker profile of idiopathic infertile patients differed from that of the other groups due to either up- or down-regulation of lysine, fructose, arginine, tyrosine, citrate, and proline	Jayaraman et al. (2014) [109]

(continued)

Table 9.2 (continued)

Study population	Human body fluid/tissue	Analytical technique	Main study outcomes	Reference
Unexplained male infertility	Seminal plasma	Raman spectroscopy	Metabolomic fingerprinting could be used as (i) a screening tool to diagnose male infertility and (ii) to study oxidative stress Men with unexplained infertility have (i) an imbalance of oxidative stress, (ii) increased biomarkers of oxidative stress, and (iii) absence of a functional antioxidant	Jafarzadeh et al. (2015) [94]
Unexplained male infertility	Seminal plasma	GC–MS	153 metabolites were identified in the seminal plasma of UMI subjects, of which 44 metabolites were differentially expressed in UMI compared to fertile controls Tryptophan, phenylalanine, glycine, serine, threonine, isoleucine, proline, and valine were decreased significantly; while urea and glutamine were increased significantly in UMI seminal plasma The major metabolic signature of UMI seminal plasma is the increased catabolism of various amino acids 4-Hydroxyphenylacetic acid is a key metabolite in differentiating between UMI and controls, and its seminal plasma concentration related positively to sperm counts	Qiao et al. (2017) [108]
Idiopathic normozoospermic infertility	Urine	LC/MicrOTOF–Q II MS	The urinary metabolomic profile could be used to differentiate normozoospermic infertile males from fertile controls 37 potential biomarkers were identified with functional roles in energy production, antioxidation, and hormone regulation in spermatogenesis A combination of the top 5 negative biomarkers (i.e. xanthosine, leukotriene E4, methoxytryptophan, 3-hydroxypalmitoylcarnitine, and aspartate) had the best diagnostic ability for detecting normozoospermic infertility	Zhang et al. (2014) [95]
Spinal cord injury-derived infertility	Seminal plasma	MALDI-TOF MS	85 ions were differentially present in seminal plasma of fertile and infertile men The lipids identified were mostly glycerolipids and were related to pathways for biosynthesis of CTP, UTP, and GTP Other metabolic pathways involved were sterol biosynthesis, arachidonic acid metabolism, and response to (i) hydrogen peroxide, (ii) steroid hormone, and (iii) vitamin Men with SCI-related infertility are likely to have altered signal transduction	Da Silva et al. (2011) [117]
Kidney yang deficiency syndrome-associated infertility (erectile dysfunction/premature ejaculation, weak ejaculation/orgasm disorder, oligozoospermia, asthenozoospermia)	Seminal plasma	LC/QTOF–MS	41 metabolites were differentially present in the seminal plasma of fertile men and infertile males with KYDS 7 metabolites were related to the 5 potential metabolic pathways Changes in metabolic pathways of biosynthesis and metabolism of aromatic amino acids, citric acid cycle, and sphingolipid metabolism may contribute to the development of KYDS-associated infertility	Chen et al. (2015) [118]

Table 9.2 (continued)

Study population	Human body fluid/tissue	Analytical technique	Main study outcomes	Reference
Kidney yang deficiency syndrome-associated infertility (erectile dysfunction/premature ejaculation, oligozoospermia, asthenozoospermia, azoospermia, teratozoospermia)	Blood plasma	GC–MS	Metabolomics profiles of infertile males with KYDS were identified 10 potential biomarkers (e.g., 1,5-anhydroglucitol, hydroxyvaleric acid) and 6 metabolic pathways (galactose glucitol, phenylalanine, glutamic acid, L-isoleucine, ornithine, lysine) could be used to discriminate between infertile males with KYDS and healthy controls Infertility in males with KYDS is probably associated with energy consumption and antioxidant defenses	Zheng et al. (2017) [119]
Erectile dysfunction; demen abnormalities	Blood plasma	GC–MS	Males with ED and semen abnormalities could be differentiated from fertile controls based on altered plasma metabolomic profiles 1,5-anhydro-sorbitol and α-hydroxyisovaleric acid are potential biomarkers to differentiate between infertile and fertile males Lactate, glutamate, and cholesterol could be used to discern between subjects with ED or semen abnormalities	Zhou et al. (2016) [120]
Young Danish men presenting with different sperm concentrations: (i) low (>0–20 × 10^6/mL), (ii) intermediate (45–75 × 10^6/mL), or (iii) high (>100 × 10^6/mL)	Blood serum	LC-MS	The serum metabolic profiles differed significantly between males presenting with different sperm concentrations Metabolites identified were mainly amino acids and carboxylic acids Peptides related to protein complement C3f (involved in innate immunity) were down regulated in men with low sperm concentrations and may serve as potential markers of fertility	Courant et al. (2013) [78]

unteers [87]. Sperm were obtained from fresh ejaculates using simple centrifugation or density gradient centrifuge (DGC) with either one or two washes. The DGC yielded sperm populations of 40% or 80%, which was then used to obtain the ^{1}H-MRS spectra. The lactate, lipid, and choline/glycerophosphocholine (GPC) peaks differed significantly between the ^{1}H spectrum of 40% and 80% sperm populations with greater differences evident in the 40% sperm population, which was more likely to possess greater structural defects [87].

Zhao and colleagues were the first to perform an untargeted metabolomics study utilizing GC-MS spectroscopy to compare the metabolic profile of spermatozoa from idiopathic asthenozoospermic and normozoospermic males [88] (Table 9.2). Results of this exploratory study showed dysregulation in amino acid and nucleotide metabolism pathways as well as disruption of glycolysis, Krebs cycle, and energy metabolism in patients with idiopathic asthenozoospermia compared to that of healthy males. Not only were the levels of amino acids (e.g., cysteine, leucine, tryptophan, and glutamic acid) found to be downregulated, guanosine and cytidine levels were also significantly lower in asthenozoospermic patients [88].

9.3.4 Seminal Plasma Metabolomics

A biochemical exploratory study of human seminal plasma in infertile males has been reported as far back as 25 years ago. Utilizing the ^{1}H-NMR method, the study measured the peak areas for glycerylphosphorylcholine (GPC), glycerylphosphorylethanolamine (GPE), citrate, and lactate in human seminal plasma to determine if these metabolites could act as biomarkers to differentiate between azoospermic and normozoospermic males [89] (Table 9.2). The study showed that azoospermic patients had significantly smaller peak areas for GPC, citrate, and lactate in seminal plasma compared to controls, while patients with spermatogenic failure and obstructive azoospermia differed significantly in their GPE to GPC peak intensity ratio [89].

Gilany et al. applied Raman spectroscopy in combination with chemometrics to differentiate between the seminal plasma metabolomic profile of asthenozoospermic and normozoospermic patients [90] (Table 9.2). Based on the Raman spectra obtained from the two groups, the researchers came up with a diagquadratic model that was able to predict between normal and asthenozoospermic samples with a validity of 83% [90]. Gilany's group went on to

examine the seminal plasma metabolome in patients with NOA using two different strategies: untargeted metabolomic profiling using GC-MS and advanced chemometrics [91] as well as metabolic fingerprinting via the Raman spectroscopy approach [92] (Table 9.2). Commonly, males with NOA undergo the invasive testicular sperm extraction (TESE) procedure for detection of sperm in their testes. However, in the untargeted metabolic profiling study, the researchers proposed the use of multivariate models as a new noninvasive diagnostic method to differentiate between fertile and NOA men who were either TESE positive or TESE negative [91]. Their 2018 study also proposed the use of the seminal plasma metabolome as a noninvasive method to detect spermatogenesis in NOA males who were either TESE positive or TESE negative. They also found that patients who were TESE negative had higher reactive oxygen species (ROS) levels compared to TESE positive males [92].

Similarly, Zhang's group conducted an untargeted metabolomic profiling study of seminal plasma using the H^1-NMR spectroscopy approach in asthenozoospermic males. They found that these patients had raised levels of oxysterols (i.e., 5α-cholesterol, 7-ketocholesterol), which suggests that oxidative stress is an underlying mechanism in their asthenozoospermic condition [93]. The role of oxidative stress in unexplained male infertility was also evident when Jafarzadeh and colleagues reported an increase in the –CH functional group (an oxidative stress biomarker) coupled with the absence of the –SH group (a functional antioxidant) that was detected in the Raman spectra of seminal plasma metabolome of these men [94] (Table 9.2).

9.3.5 Urine Metabolomics

In other studies, urine samples have been used to conduct a differential diagnosis between healthy men and diseased patients. For example, the urinary metabolome of normozoospermic infertile patients has been used to discern between normozoospermic infertile men and fertile controls [95] (Table 9.2). The group proposed that the potential negative changes in the citric acid cycle and hormonal activity during spermatogenesis, as well as oxidative stress are among the underlying events that could lead up to normozoospermic infertility [95].

In another study, Zhang's group showed that the urinary metabolomic profile of oligozoospermic patients (sperm concentration <20 M/mL) differed significantly from that of normozoospermic males [95] (Table 9.2). A stronger risk of oligozoospermia seemed to be indicated when biomarkers related to sperm concentration and amplitude of lateral head displacement was altered. Moreover oligozoospermia, a common indication in most male subfertility cases, was potentially associated with disruptions in fatty acid metabolism and antioxidant defenses in spermatogenesis [95].

9.3.6 Testicular Tissue Metabolomics

Metabolomics studies on testicular tissue have proposed noninvasive methods to diagnose either the presence of spermatogenesis in NOA men [96] or to differentiate between seminiferous tubules with complete or incomplete spermatogenesis cycles [97] (Table 9.2). In the latter, testicular tissue from azoospermic males (maturation arrest or Sertoli cell only) was snap frozen and subjected to ^{1}H-MRS. This method is potentially an alternative to testis biopsy as a diagnostic test to detect normal or abnormal spermatogenesis in azoospermic males [96]. In the former study, Raman spectroscopy was used to scan the seminiferous tubules within fresh testicular tissues retrieved from OA and NOA patients with a sensitivity of 90% and specificity of 85.71% in order to discern between seminiferous tubules that had complete spermatogenesis cycles and those that did not [97].

9.4 Potential Biomarkers of Male Infertility

By definition, a biomarker is identified as a characteristic biological marker that represents a condition, event, or process that can be quantitatively assessed, measured, and studied [98]. The biomarker should be highly sensitive, specific, and ideally be easily accessible in order to minimize the need for invasive and often inconvenient tests in the infertile male. In addition, such a biological molecule should facilitate a more detailed and precise classification of the infertile male [99]. Research studies that are evolving in the areas of genomics, proteomics, and metabolomics could potentially lead to the development of novel male infertility biomarkers [100].

The most commonly used biomarker to gauge the male fertility potential is semen analysis, which despite providing critical fundamental information, is highly variable and thereby a poor predictor of fertility [101]. In fact, some infertile males present with normal semen parameters in spite of their poor fertility potential [102]. As semen analysis alone is clearly inadequate to diagnose the infertile male, proteomic and metabolomic technologies are fast becoming potentially vital tools in identifying appropriate biomarkers for use in diagnosis, prognosis, and treatment of male infertility [103, 104]. Moreover, advances in bioinformatics and analytical technologies in the omics have helped further develop protein and metabolite profiling as a useful tool in biomarker discovery [59, 105].

Seminal fluid is made up mainly of seminal plasma and only a small volume of spermatozoa. Seminal plasma has a varied molecular composition and contains a high concentration of tissue-specific proteins which acts as an abundant source of potential biomarkers in the assessment of male fertility [61, 100]. Proteins such as the testis-specific TKTL1 (transketolase-like protein 1), LDHC (lactate dehydrogenase C), and PGK2 (phosphoglycerate kinase 2) seem to be able to serve as a biomarker to distinguish semen from fertile and infertile men [106]. TEX101, a cell membrane protein expressed specifically by testicular germ cells, is among the most promising biomarkers of male infertility [107]. TEX101 (testis-expressed protein 101) could act as a biomarker to predict TESE outcome and to differentiate between Sertoli cell-only syndrome and the other NOA subtypes (maturation arrest, hypospermatogenesis). In addition, the epididymis-expressed protein ECM1 (extracellular matrix protein 1) appears to be able to differentiate between NOA and OA [60]. Other examples of DEPs that could serve as potential biomarkers in the infertile male are shown in Table 9.1.

Metabolomics too shows great promise as a useful tool in disease diagnosis among infertile males [108]. Metabonomic profiling has been proposed as a tool to detect idiopathic infertility, as lysine concentration in the seminal plasma detected using ^{1}H-NMR was found to give a good indication of idiopathic infertility [109]. Qiao's group reported that patients with unexplained male infertility have increased catabolism of several amino acids that could impact male reproduction. Using a GC-MS-based metabolite profiling platform, the study had identified 4-hydroxyphenylacetic acid as a significant metabolite in seminal plasma that could help differentiate between males with unexplained infertility from those who were healthy [108]. Among asthenozoospermic males, deficiency in valine along with high levels of oleic and palmitic acids detected via GM-MS in their seminal plasma may serve as potential biomarkers of asthenozoospermia [108]. While 37 potential biomarkers were identified when comparing the urinary metabolome of infertile normozoospermic and fertile men, it seemed that the best diagnostic ability for the detection of normozoospermic infertility was obtained when a combination of the top five negative biomarkers (i.e., xanthosine, leukotriene E4, methoxytryptophan, 3-hydroxypalmitoylcarnitine, and aspartate) was applied [95].

9.5 Current Challenges and Future Outlook

Proteomics and metabolomics studies in the infertile male have to date identified a number of putative biomarkers for a variety of conditions related to male infertility, which can be used to distinguish between healthy fertile men and patients with a specific infertility disorder [6, 68]. Biomarkers that are identified using an omics approach may be associated with disease pathogenesis and could therefore offer novel therapeutic targets for the management of disease [110]. It is hoped that these omics studies would eventually pave the way for the development of biomarker molecules or panels of natural fertility in the diagnosis and treatment of male infertility [100]. In fact, a combination of biomarkers has the potential to deliver a higher predicative power than would a single biomarker [95, 111].

While differential proteomic and metabolomics studies have identified an extensive set of sperm proteins and metabolites that are present either in varied quantity or state in infertile males, further research is required before it can actually be utilized in a clinical setting [7, 68, 112]. An inherent challenge from all the datasets obtained is in developing clinically relevant biomarkers [112]. Discovery of potential biomarkers must be followed by analytical validation and evaluation of clinical utility, before it can actually be utilized clinically [113]. Despite the increase in the number of studies and the rapid advances in the strategies and analytics for biomarker discovery, translation of these biomarkers from bench to bedside remains at a slow pace [114]. However, as technology and analytics of omics studies improves, it is hoped that the cost of performing wider-scale omics studies also becomes concurrently more affordable in order to facilitate greater advancements in biological knowledge of human fertility.

9.6 Conclusion

There are a multitude of causes underlying male infertility which remain undiscovered. Greater understanding of the molecular and genetic mechanisms underpinning the male fertility potential is needed to determine possible intervention strategies for the management of the subfertile male. In addition to the advanced tests that aid in the determination of oxidative stress and DNA fragmentation in infertile patients, biomarker discovery promises of a viable alternative for the noninvasive diagnosis of male infertility-related pathologies. Proteomics and metabolomics strategies are rapidly growing analytical tools that complement each other in the goal to discover the biomarkers of infertility in the male. These biomarkers may aid in the evaluation of the male fertility potential, and to differentiate between the various etiologies of infertility, and perhaps even help predict successful outcomes of assisted reproduction technologies. Outcomes of studies utilizing omics approaches and bioinformatics would eventually yield in greater understanding of the spermatogenesis process, sperm function, as well as the events that follow fertilization. Awareness of the molecular and genetic basis of male infertility would immensely aid the clinician in the management of the infertile male.

9.7 Review Criteria

An extensive search of studies examining the relationship between proteomics and male infertility along with metabolomics and male infertility was performed using search engines such as PubMed, MEDLINE, OVID, Science Direct, and Google Scholar. The start and end dates for these searches were July 2018 and Dec 2018, respectively. The overall strategy for study identification and data extraction was based on the following key words: "omics", "proteomics", "proteins", "metabolomics", "metabolites", "male infertility", "infertility", "spermatozoa", "spermatogenesis", "seminal plasma", "semen", "seminal fluid", "urine", "serum", "blood", "testicular tissue", "biomarkers", "bioinformatics". Articles published in languages other than English were also considered, provided the abstract was in English. Data that were solely published in conference or meeting proceedings, websites, or books were not included.

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