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REVIEW ARTICLE

Proteomics and metabolomics — Current and future perspectives in clinical andrology

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Abstract

Proteomics and metabolomics are emerging as promising tools to investigate the molecular mechanisms associated with male infertility. Proteins and metabolites play a pivotal role in regulating the molecular pathways associated with physiological functions of spermatozoa. Semen analysis, physical examination and laboratory work up cannot identify the etiology of infertility in 30%-40% of cases, which are classified as idiopathic. Therefore, the application of proteomics and metabolomics in the field of andrology will aid to overcome the limitations of the standard semen analysis. Understanding the molecular pathways associated with male infertility will help in planning ad hoc treatments, contributing to the clinical management of infertile patients. In this review, proteomics and metabolomics studies on spermatozoa and seminal plasma are discussed with a focus on molecular biomarkers associated with male infertility-related conditions.

KEYWORDS

biomarker, male infertility, mass spectrometry, metabolomics, proteomics

1 | INTRODUCTION

The evaluation of semen quality has been based on the results provided by the standard semen analysis since its introduction in the andrological practice. However, semen parameters do not determine the true fertility potential of an individual in 15% of cases (Hamada, Esteves, Nizza, & Agarwal, 2012), while physical examination, laboratory work and semen analysis fail to identify the etiology in 30%-40% of patients, those are classified as idiopathic male infertility (Bracke, Peeters, Punjabi, Hoogewijs, & Dewilde, 2018). Proteomics and metabolomics are the two new emerging -OMICS platforms used for the identification of new markers to define the male fertility potential, with an increasing number of publications over the last two decades (Agarwal, Baskaran, Panner Selvam, Barbarosie, & Master, 2020; Baskaran et al., 2019).

Complete profiling of proteins present in a tissue or a cell is defined as proteomics. Shotgun or bottom-up approaches are the most widely used in the field of proteomics, and they can detect more than 1,000 proteins in a given sample. In the field of male infertility, semen is the most commonly used biological fluid to evaluate the fertility status of men. Semen contains cellular and non-cellular components, that is spermatozoa and seminal plasma, which can be investigated by applying proteomics techniques (Jodar, Soler-Ventura, & Oliva, 2017; Panner Selvam & Agarwal, 2018; Sinha, Singh, & Yadav, 2017). Amaral et al. used a data mining approach to identify 6,198 proteins in human spermatozoa (Amaral, Castillo, Ramalho-Santos, & Oliva, 2014), whereas 2064 proteins were identified in the seminal plasma (Jodar et al., 2017). Considering that spermatozoa are transcriptionally and translationally inert, they rely on proteins for their functional activity. Defects in the normal physiological functions of spermatozoa are observed due to alterations in the spermatozoa and seminal plasma proteome, and they are associated with male infertility conditions such as varicocele (Agarwal, Sharma, Durairajanayagam, Ayaz et al., 2015; Agarwal, Sharma, Durairajanayagam, Cui, et al., 2015; Agarwal, Sharma, Harlev, & Esteves, 2016; Belardin et al., 2016; Mariana Camargo, Intasqui, & Bertolla, 2016; Samanta et al., 2018), idiopathic infertility (Herwig et al., 2013; Hetherington et al., 2016; Shen, Wang, Liang,

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& He, 2013), unexplained male infertility (UMI) (Azpiazu et al., 2014; Liu, Liu, et al., 2018; McReynolds et al., 2014; Xu et al., 2012), elevated oxidative stress (Ayaz et al., 2015; Hamada et al., 2013; Sharma et al., 2013) and testicular cancer (Agarwal, Tvrda, et al., 2015; Dias, Agarwal, Pushparaj, Ahmad, & Sharma, 2018). Key proteins associated with sperm functions such as hyperactivation, capacitation, acrosome reaction and the fertilization process can be used as non-invasive biomarkers in the pathophysiology of male infertility.

2 | TECHNIQUES IN PROTEOMICS

Sperm proteins are detected and identified using different proteomic techniques. The conventional approach includes the separation of the extracted spermatozoa or seminal plasma proteins based on properties such as molecular weight and isoelectric point using two-dimensional (2D) gel electrophoresis. A total of 98 human sperm proteins were separated using 2D-gel electrophoresis and identified by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) technique. These proteins were involved in transcription, energy production and protein synthesis (Martínez-Heredia, Estanyol, Ballescà, & Oliva, 2006). Difference gel electrophoresis (DIGE), an improved version of 2D-gel electrophoresis technique, is able to identify differentially expressed proteins (DEPs) with a minimum error of < 10% (Gupta, Ghulmiyyah, Sharma, Halabi, & Agarwal, 2014). The expression profile of the proteins is determined based on the intensity of Cy3 and Cy5 dyes using automated image analysis software.

Low sensitivity and the inability to detect less abundant proteins or low number of proteins are some of the limitations of conventional proteomic techniques. These limitations were overcome by using advanced high-throughput techniques such as MALDI-TOF and LC-MS/MS (liquid chromatography–tandem mass spectrometry). These instruments can even detect low abundance peptides present in a sample with low protein concentration. Johnston et al. used in-gel digestion-based LC-MS/MS approach to identify 1,760 sperm proteins (Johnston, Wooters, Kopf, Qiu, & Roberts, 2005), and the same approach was widely used to profile the spermatozoa of infertile men (Cao et al., 2018; Dias et al., 2018; Giacomini et al., 2015; Liu, Liu, et al., 2018).

Additional purification of seminal plasma is not required as they are rich in proteins and are readily available for proteomic experiments. Semen samples undergo purification and processing steps prior to the extraction of sperm proteins. Semen samples are subjected to different types of processing techniques such as single gradient centrifugation, double gradient centrifugation or multilayer gradient centrifugation to separate the spermatozoa from the seminal plasma. Several studies have used a density gradient centrifugation step to remove round cells and immature germ cells from semen samples to isolate a pure sperm fraction (Amaral, Paiva, et al., 2014; Intasqui et al., 2013; Martínez-Heredia et al., 2006; Wang et al., 2014, 2018). Further, proteomic studies on round cells and spermatozoa revealed that the influence of round cell proteins as contaminant in the sperm proteome was insignificant or very negligible (Panner Selvam, Agarwal, Dias et al., 2019; Panner Selvam, Agarwal, Dias, Martins, & Samanta, 2019). The presence of these round cell proteins does not interfere with the bioinformatics results related to the molecular pathways associated with sperm function.

In general, extraction of proteins using RIPA buffer provides maximum yield of proteins compared to other techniques such as sonication. Incubation of spermatozoa overnight with RIPA buffer results in the lysis of sperm membrane and release of proteins. Subsequently, the proteins are pelleted and washed 3 times with RIPA solution to remove cell debris. The concentration and purity of the sperm proteins are determined before they are subjected to proteomic analysis. A known amount of protein is separated using 4% to 15% SDS-PAGE, and in-gel digestion of the separated proteins is done using trypsin. Digested peptides are eluted from the gel and injected into mass spectrometry (Glish & Vachet, 2003). The instrument detects the proteins or peptides based on mass/charge ratio (m/z) with a very low false discovery rate. Enrichment of sperm proteins by immunoprecipitation is the most preferred protocol for purifying the acetylated, methylated and phosphorylated proteins for post-translational modification studies. Other advanced techniques such as MALDI-TOF and SELDI-TOF (surface-enhanced laser desorption/ionisation time-of-flight) are also used to detect the sperm proteins (Bracewell-Milnes et al., 2017; Rødgaard, Heegaard, & Callesen, 2015).

Computational software such as SEQUEST, Mascot X!, Tandem and MaxQuant (Zhou, Zhou, & Guo, 2013) is used to identify the peptides and proteins. These computational tools perform a complete scan of proteins detected by MS and compare it with already available global databases for the human proteome. Further, the proteins are categorised as DEPs based on spectral counts (SC) and abundance of each protein. Bioinformatic analysis provides insight into the functional and molecular mechanisms based on the proteomic data (Lan et al., 2003). Gene ontology (GO) analysis of the proteins provides information about their localization, distribution and biological functions. Protein–protein interactions and defective molecular pathways can be identified using sophisticated programs such as Ingenuity Pathway Analysis (IPA) and Metacore™. STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis is commonly performed to display the link between the proteins (Agarwal et al., 2014).

3 | PROTEOMICS IN DECIPHERING KEY PROTEINS ASSOCIATED WITH MALE REPRODUCTION

Alteration in the expression profile of key proteins involved in the cellular and molecular pathways in the spermatozoa can be identified using proteomics (du Plessis, Kashou, Benjamin, Yadav, & Agarwal, 2011). Particularly, proteomics can be applied to analyse the whole sperm proteome as well as protein expression in subcellular compartments. Furthermore, the same approach can be used for

seminal plasma and testicular tissue, as explained in the subsequent sections.

3.1 | Whole sperm proteomics

Several investigators have characterised the proteome from whole sperm cells in normozoospermic men. In 2006, Martinez-Heredia et al. identified 98 proteins in normozoospermic men by using 2-DE and MALDI-TOF MS analysis, mainly structural proteins or involved in regulation of energy metabolism, protein synthesis, cellular cycle, apoptosis and cellular movement, as well as in the response to the oxidative stress (Martínez-Heredia et al., 2006). Nowicka-Bauer et al. identified 73 proteins involved in energy metabolism using 2-DE and MS (Nowicka-Bauer et al., 2018). They also reported 25 proteins for the first time in spermatozoa, including ANXA7, a calcium-dependent phospholipid binding protein localized in the sperm membrane, and c14orf105, still uncharacterized protein in the sperm nucleus. Proteomic profile of the spermatozoa differs among its subsets. Global proteomics has been conducted on different fractions of spermatozoa (mature and immature) from fertile and infertile men (Cui, Sharma, & Agarwal, 2016; Samanta, Sharma, Cui, & Agarwal, 2018). A higher expression of proteins involved in protein biosynthesis, transport and ubiquitination as well as in the cellular response to oxidative stress was observed in immature spermatozoa (Cui et al., 2016). In spermatozoa of infertile men, the most mature sperm fraction showed an altered expression of proteins involved in energy-related signalling cascade as well as protein folding (Samanta et al., 2018).

Whole sperm proteomics involves the characterization and profiling of proteins and peptides extracted from the spermatozoa. It has been investigated in several infertility-related conditions. Intasqui et al. analyzed the proteomic profile of spermatozoa in men with primary and secondary infertility (Intasqui, Agarwal, Sharma, Samanta, & Bertolla, 2018), and reported a total of 117 DEPs when compared to proven fertile men. Furthermore, BAG6 and HIST1H2BA were proposed as potential markers for male infertility. These proteins are involved in post-translational modifications and folding of proteins as well as in histones replacement by protamines, respectively (Intasqui et al., 2018).

3.1.1 | Subcellular proteomics

Proteomics studies on the whole cellular lysate do not provide sufficient information regarding function of the proteins localized in subcellular compartments or organelles of spermatozoa. Proteomic analysis of the proteins selectively extracted from the organelles of spermatozoa provides further information about the subcellular metabolism (Sadowski et al., 2006). In fact, the differential expression of proteins in subcellular compartments such as nucleus, acrosome and flagella is directly related to the function of spermatozoa (Amaral et al., 2013; Baker et al., 2013; Gu et al., 2011; Kim et al., 2007; de Mateo, Castillo, Estanyol, Ballescà, & Oliva, 2011; Naaby-Hansen & Herr, 2010; Stein, Go, Lane, Primakoff, & Myles, 2006). The sperm nuclear proteome is extracted by treatment of the sperm cells with the detergent cetyl trimethyl ammonium bromide (CTAB), to avoid any contamination from the flagella, mitochondria and acrosome region. De Mateo et al. analysed the nuclear proteins by 2D-PAGE and LC-MS/MS (de Mateo et al., 2011) and identified that histones and chromatin-related proteins are involved in the epigenetic regulation of spermatozoa, fertilisation and embryo development. A total number of 521 and 721 proteins were reported to be uniquely expressed in the head and tail of spermatozoa, respectively (Baker et al., 2013). Specifically, the flagellar subcompartment was enriched in metabolic proteins involved in pathways associated with the energy generation, such as glycolysis, gluconeogenesis, the Krebs cycle and the oxidative phosphorylation as well as metabolism of fatty acids and amino acids, in agreement with the role of sperm tail in motility (Amaral et al., 2013; Kim et al., 2007). Proteins localized in the sperm head were mainly proteases, kinases and proteins involved in signalling pathways (Baker et al., 2013), whereas proteins mediating capacitation, acrosomal reaction and oocyte binding were also described (Gu et al., 2011; Naaby-Hansen & Herr, 2010; Stein et al., 2006).

3.1.2 | Sperm surface proteomics

Sperm recognize the zona pellucida with the help of binding proteins expressed on sperm surface, which also regulate the induction of the acrosome reaction. Therefore, the proteins expressed on the membrane are essential in the zona penetration and fertilization. Furthermore, membrane composition is the result of the complex process involved in spermatogenesis and its analysis can be informative regarding the sperm differentiation and maturation. Sperm cells are first lysed to purify the plasma membrane proteins. This can be achieved by sonication or nitrogen cavitation techniques in combination with differential centrifugations to isolate the different cellular component (Zhou & Philips, 2017). It is possible to selectively extract peripheral membrane proteins by treating the lysate with high concentration of salt or specific detergents to extract the lipid drafts. Shetty et al. analyzed the sperm surface proteome in order to identify candidates for generating a contraceptive vaccine (Shetty et al., 2001). Expression of angiotensin-converting enzyme (ACE) on sperm membrane was identified by labelling proteins with sulfo-NHS-LC-biotin before 2D-gel electrophoresis and microsequencing. Domagala et al. applied proteomics techniques to identify immunoreactive antigens on sperm surface (Domagala, Pulido, Kamieniczna, Kurpisz, & Herr, 2011), in order to decipher the proteins involved in the generation of an autoimmune response against spermatozoa. They confirmed the expression of sperm antigens, such as L-lactate dehydrogenase C chain and GAPDH2, as well as other new intrinsic and surface antigens, such as the clathrin heavy chain.

Lipid drafts are domains in the plasma membrane where proteins involved in the same pathway co-localize in association with cholesterol and sphingolipids. Nixon et al. reported that these

proteins particularly mediate the binding to the zona pellucida and the further fusion of sperm membrane with the oolemma (Nixon et al., 2011). A total number of 124 proteins were identified in the sperm membrane lipid drafts by using a nano-LC/MS/MS approach. ACE, integrin beta-2, sperm adhesion molecule 1, SPA17 and arylsulphatase A proteins involved in sperm–oocyte binding and adhesion, while proteins involved in cellular signalling, protein folder and intracellular transfer were identified on sperm membrane (Nixon et al., 2011).

3.2 | Seminal plasma proteomics

Seminal plasma is a heterogeneous fluid composed by secretions from organs and glands of the reproductive male tract. It has been estimated that 23% of the studies on proteomics in the male reproductive field in the last 20 years have been conducted on seminal plasma (Agarwal et al., 2020). The first study analyzing the seminal proteome in healthy men was published in 2006 (Pilch & Mann, 2006). By using LC-MS/MS analysis, authors reported the expression of 923 proteins, such as FN1, SEMG1 and 2, lactoferrin and laminin. When the seminal proteome was analysed by HPLC coupled with MS, 83 proteins were reported to be commonly expressed in 5 fertile men, all of them involved in the male fertility-related pathways, such as SEMG I and II (participating in the formation of coagulum after the ejaculation), lactoferrin (acting as an immune-modulatory protein and also having antibacterial and antioxidant properties) and clusterin (previously described to be involved in sperm agglutination) (Milardi et al., 2012). By classifying normozoospermic patients based on the values of sperm mitochondrial activity, acrosome integrity and sperm DNA fragmentation, Intasqui et al. proposed a set of seminal biomarkers associated with each of these alterations (Intasqui et al., 2016). Alterations of mitochondrial activity were associated with an increased expression of ANXA7, glutathione S-transferase Mu3 and endoplasmic reticulum resident protein 44; increased expression of the phospholipid transfer protein was associated with acrosome defects, while CRISPLD1, CRISPLD2 and retinoic acid receptor responder protein 1 were proposed as markers of sperm DNA damage.

A variation in seminal proteome was observed in normozoospermic patients classified according to the levels of semen lipid peroxidation, measured by thiobarbituric acid assay (Intasqui et al., 2015). By using LC-MS/MS analysis, oxidative stress was reported to influence seminal proteome, by inducing an increased expression of proteins related to the biosynthesis of unsaturated fatty acids, and response to oxidative, immune and heat stress. These preliminary observations have been further strengthen by the report of 44 DEPs in fertile men classified regarding the concentration of seminal ROS, detected by chemiluminescence (Dias et al., 2019). Particularly, fertile men having high ROS concentration expressed higher levels of SOD1 and PRDX4, antioxidants systems to counterbalance the oxidant levels and proteins involved in the degradation of defective proteins.

3.3 | Testicular proteomics

Proteomic research conducted on testicular tissue is very limited. Very few articles have been published using testicular tissue in the last 20 years (Agarwal et al., 2020). Testicular proteome provides substantial understanding about the cellular signalling pathways that are significantly altered in pathological conditions, such as obstructive and non-obstructive azoospermia, and testicular cancer (Alikhani et al., 2017; Leman et al., 2009; Liu et al., 2013). Future proteomics studies on testicular tissue will help in the investigation of proteins involved in spermatogenesis and to identify novel targets for treatment of male infertility (Li et al., 2011).

4 | THE IMPACT OF CRYOPRESERVATION ON SPERM PROTEOMICS

Semen cryopreservation is a procedure performed routinely to preserve the spermatozoa for future use in ART procedures. Cryopreservation has been correlated with reduced semen quality, particularly decreased motility, viability and acrosome integrity (Ozkavukcu, Erdemli, Isik, Oztuna, & Karahuseyinoglu, 2008). Bogle et al. also reported the alteration of sperm proteome depending on the use of cryoprotectant (protein-free cryosperm medium) and the freezing/thawing procedure itself, potentially due to the induction of protein degradation and osmotic stress (Bogle et al., 2017). Proteomics analysis of cryopreserved spermatozoa showed altered expression of proteins involved in reproductive function pathways, such as energy metabolism (ACO2, ENO1), the maintenance of cell surface integrity (vimentin) and the flagellar structural integrity (TEKT1) (Wang et al., 2014). Also, higher levels of global tyrosine phosphorylation were reported in frozen spermatozoa, in association with alteration of sperm motility, sperm functions and a premature induction of capacitation (Wang et al., 2014). The investigation of the altered pathways in spermatozoa after cryopreservation may be of outstanding importance for the creation of specific cryoprotectants having a low impact on sperm physiology and potentially improve the reproductive outcomes after ART.

5 | PROTEOMICS IN PROGNOSIS AND DIAGNOSIS OF MALE INFERTILITY

Over the last two decades, an increasing number of studies has been published about proteomics in male infertility-related clinical scenarios such as varicocele, idiopathic infertility, testicular cancer, UMI and sperm abnormalities-related conditions (Agarwal et al., 2020). Research is mainly aimed to decipher the molecular mechanisms affecting the reproductive function of infertile men and to identify potential biomarkers for the diagnosis and treatment in clinical setting.

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5.1 | Potential biomarkers of varicocele

Varicocele is a vascular disease characterised by the enlargement of the pampiniform venous plexus (Miyaoka & Esteves, 2012). Few global proteomic studies have reported the key proteins and the altered molecular mechanisms in male infertility-associated varicocele (Agarwal, Sharma, Samanta, Durairajanayagam, & Sabanegh, 2016; Ayaz et al., 2015; Panner Selvam & Agarwal, 2019; Panner Selvam, Agarwal, & Baskaran, 2019; Samanta et al., 2018). Reduced expression of proteins involved in mitochondrial (ATPase1A4) and cytoskeleton (SPA17) functionality and structure, as well as related to sperm functions (HSPA2, APOA1), was reported in varicocele sperm samples (Agarwal, Sharma, Samanta, et al., 2016; Hosseinifar et al., 2013; Samanta et al., 2018).

Panner Selvam & Agarwal (2019) showed an altered expression of 28 seminal plasma proteins in varicocele patients, mainly associated with oxidative stress response and sperm–oocyte membrane fusion, while other studies reported an alteration of seminal proteins related to sperm maturation, sperm motility and capacitation (Fariello et al., 2012; Zylbersztejn et al., 2013). Varicocele correction resulted in an improved expression of seminal plasma proteins involved in oxidative stress response, protein stabilization and gluconeogenesis (Fariello et al., 2012; Zylbersztejn et al., 2013; Camargo, Intasqui, & Pimenta Bertolla, 2016). Moreover, the expression of three proteins (HSPA5, SOD1 and ATP5D) involved in protein folding, oxidative stress response and energy generation increased after varicocele correction (Hosseinifar et al., 2014), with a shift towards the homeostasis (Camargo et al., 2013; Del Giudice et al., 2013).

A severe alteration of the sperm proteome was observed in bilateral varicocele compared to unilateral varicocele (Agarwal, Sharma, Durairajanayagam, Cui, et al., 2015) as well as proteins associated with inflammatory response such as IL-6 and Jak-STAT pathways (Panner Selvam, Agarwal, Pushparaj, Baskaran, & Bendou, 2019).

Proteins such as ENKUR, SEMG1, SEMG2, SPAM1 and CABYR, involved in calcium transduction machinery, capacitation, acrosome reaction and in the formation of semen coagulum, were significantly overexpressed in bilateral varicocele patients (Agarwal, Sharma, Durairajanayagam, Cui, et al., 2015). Moreover, the overexpressed PRDX2 and FASN and the reduced FN1 seminal plasma proteins were suggested to differentiate bilateral from unilateral patients (Panner Selvam et al., 2019). Sperm proteins such as CRISP2 and ARG2 were expressed uniquely in unilateral varicocele patients (Agarwal, Sharma, Durairajanayagam, Ayaz et al.,2015). CRISP2 is involved in calcium fluxes during the capacitation, while ARG2 was negatively associated with sperm concentration and positively with sperm motility (Cohen et al., 2011; Elgün, Kaçmaz, Sen, & Durak, 2000). Moreover, GNPDA1 was expressed only in unilateral patients, being involved in calcium oscillation triggering oocyte activation and embryo development (Agarwal, Sharma, Durairajanayagam, Cui, et al., 2015). The overexpression of proteins involved in antioxidant systems was also reported, suggesting the establishment of a reductive stress microenvironment in unilateral varicocele patients (Swain et al., 2019).

5.2 | Potential biomarkers of unexplained (UMI) and idiopathic male infertility

UMI is defined as a condition in which the semen parameters are normal (according to WHO 5th edition guidelines) but the cause of infertility is unknown (Hamada et al., 2012). Idiopathic infertile patients are those with altered semen parameters with an unknown cause (Aktan et al., 2013). Proteomics analysis of spermatozoa from infertile normozoospermic men revealed underexpression of proteins (CRISP2, NME5, TSSK2, MYCBP, NDRG3, SPATA24, ROPN1L) involved in development/functions of male gametes (McReynolds et al., 2014). Moreover, proteins involved in response to the stress (HSPA2, HSPA5, STIP1, CLU) resulted to be overexpressed in case of poor blastocyst development and reduced implantation rate after in vitro fertilisation, suggesting them as potential molecular biomarkers for UMI (McReynolds et al., 2014). Other proteins such as the ANXA2, SPA17 and SERPINA5 were differentially expressed and proposed as potential biomarkers in UMI condition (Panner Selvam et al., 2019). SPA17 is involved in the acrosome reaction and oocyte fertilization (Chiriva-Internati et al., 2009), while SERPINA5 is mainly expressed in the head of spermatozoa, being involved in the sperm–oocyte binding (Yang & Geiger, 2017). Underexpression of both proteins (SPA17 and SERPINA5) may significantly affect the male fertility potential. On the other hand, ANXA2 is reportedly overexpressed during abnormal ubiquitination (Deng, Jing, Xing, Hou, & Yang, 2012) and may affect the sperm functions. The proteins clusterin, epididymal secretory protein E1 (HE1) and PSA were proposed as seminal biomarkers for success in unexplained infertile couples undergoing IVF (Kanannejad & Gharesi-Fard, 2019). These proteins are correlated with the sperm quality. Clusterin protects the spermatozoa from oxidative stress and DNA damage, and regulates the sperm motility and viability (Salehi et al., 2013). HE1 is secreted by epididymis and plays a role in cholesterol transport (Girouard, Frenette, & Sullivan, 2008), while the prostatic PSA regulates semen liquefaction (Balk, Ko, & Bubley, 2003).

Agarwal et al. reported the changes in the sperm proteome of idiopathic infertile men treated with antioxidant supplementation (FH PRO for Men) (Agarwal et al., 2019). Besides the beneficial effect of FH PRO for Men on semen quality, it is also predicted to enhance the metabolic pathways involved in the energy production, spermatogenesis, sperm functions and fertilization as well as increase cellular response to oxidative stress (Agarwal et al., 2019).

5.3 | Potential biomarkers of testicular cancer

Testicular cancer is common in men of reproductive age (15–40 years old) (Chia et al., 2010). It arises from malignant transformation of germ cells in 95% of cases, further subcategorized in seminomas (50%) and non-seminomas (50%) (Adra & Einhorn, 2017). Recently, Panner Selvam et al. reported an altered protein profile in spermatozoa of testicular cancer patients, particularly protein regulators of sperm motility, oocyte binding, capacitation and fertilization, and have suggested putative molecular biomarkers associated with each

dysfunctional pathway (Panner Selvam, Agarwal, & Pushparaj, 2019). Proteomic analysis of testicular cancer tissue has revealed proteins like PIWIL1 (involved in DNA methylation and RNA silencing) and TMPRSS12 (a serine protease) as markers of testicular tumour germ cells (Liu et al., 2013). Furthermore, glutathione S-transferase M3 was reported to be downregulated in case of seminoma and distinct polymorphisms associated with higher risk of cancer development (Zimmermann et al., 2006). Other putative markers identified in seminoma tissue by 2D-gel electrophoresis included the overexpression of GCP6 (microtubule nucleation) and CDK10 (cellular cycle regulator), as well as the underexpression of StarD7 (maintenance of the cellular shape) (Leman et al., 2009). Castillo et al. combined metal oxide affinity chromatography with LC-MS/MS to investigate testicular phosphoproteome and reported an overexpression of PAK4 protein in embryonal carcinoma (Castillo et al., 2019). PAK4 is involved in cellular proliferation, and its overexpression protects the cancer cells from the apoptosis.

5.4 | Potential biomarkers of sperm abnormalities

Proteomes have been analysed in several groups of sperm abnormalities. Protein pathways associated with sperm motility are compromised in asthenozoospermic patients (Hashemitabar, Sabbagh, Orazizadeh, Ghadiri, & Bahmanzadeh, 2015). The DEPs identified in asthenozoospermic patients were mainly associated with energy and metabolism, movement and structural organisation, stress response, signalling and antioxidant activity (Martinez-Heredia, de Mateo, Vidal-Tboada, Ballesca, & Oliva, 2008; Siva et al., 2010; Zhao et al., 2007). Hashemitabar et al. reported a decreased expression of proteins involved in the ATP synthesis, suggesting the oxidative phosphorylation as the main pathway in regulating sperm motility (Hashemitabar et al., 2015). Further, the absence of structural proteins such as ODF2 and tektin 4 in sperm flagella suggests cytoskeleton alteration as the main cause of asthenozoospermia (Shen et al., 2013). However, Zhao et al. reported higher levels of ODF proteins and a reduced expression of the 26S protease regulatory subunit 7 in asthenozoospermic Asian cohort (Zhao et al., 2007), whereas the subunit PSMB3 component of proteasome complex was overexpressed in the European population (Martinez-Heredia et al., 2008), underlining difference in protein expression due to geographical origin. Moreover, lower levels of GRP78 in idiopathic asthenozoospermic patients may be involved in a reduced sperm binding to zona pellucida, with consequences on sperm capacitation and fertilization (Shen et al., 2013). In addition to protein expression, post-translational modifications of proteins can adversely affect the sperm physiology (Baker, Witherdin, Hetherington, Cunningham-Smith, & Aitken, 2005). These include glycosylation, S-nitrosylation, sumoylation and phosphorylation (Lefièvre et al., 2007; Samanta, Swain, Ayaz, Venugopal, & Agarwal, 2016; Vigodner et al., 2013; Wang et al., 2013). Particularly, phosphorylation regulates sperm maturation and capacitation (Ficarro et al., 2003). Asthenozoospermic spermatozoa showed a reduced level of protein phosphorylation, resulting in the alteration of the mitochondrial energy metabolism and fibrous sheath/cytoskeletal structure, and lower sperm motility (Chan et al., 2009; Parte et al., 2012).

Seminal proteins such as ECM1, TEX101 and NPC2 have been proposed to discriminate obstructive from non-obstructive azoospermia (Drabovich et al., 2013; Yamakawa, Yoshida, Nishikawa, Kato, & Iwamoto, 2007). Freour et al. focused on the identification of biomarkers for the spermatozoa retrieval from testis in case of non-obstructive azoospermia and revealed higher expression of seminal LGALS3BP in men with successful TESE (Freour et al., 2013). Oligoasthenoteratozoospermic (OAT) patients showed altered seminal expression of proteins involved in metabolism and inflammation, stress response and proteins associated with binding, transporter, immune and hydrolase activities (Giacomini et al., 2015; Herwig et al., 2013; Liu, Wang, et al., 2018). Recently, Liu et al. proposed KLK3, LTF SERPINA1 and GAPDH as putative biomarkers to discriminate between OAT and normozoospermic patients (Liu, Wang, et al., 2018), besides the previously identified NPC2, M2BP and lipocalin-1 (Giacomini et al., 2015) (Table 1). Herwig et al. identified a panel of 46 proteins related to oxidative stress in patients with infertile idiopathic OAT (Herwig et al., 2013). These studies provided further insight into the molecular pathogenic mechanisms underlying OAT condition.

The proteome of globozoospermia, a rare genetic condition characterised by the absence of acrosome, has also been investigated. Comparative analysis of sperm proteome of globozoospermic patients against normozoospermic subjects revealed aberrant expression of 35 proteins involved in spermatogenesis, metabolism, cytoskeleton and sperm function (Alvarez Sedo', Rawe, & Chemes, 2012; Liao, Xiang, Zhu, & Fan, 2009). These included the overexpression of AKAP4 and actin, as well as the underexpression of enzymes of the Krebs cycle, tubulin and proteins associated with the outer dense fibres and acrosome membrane.

6 | OVERVIEW OF METABOLOMICS

Metabolomics is one of most recent -OMICS techniques that was developed after genomics, transcriptomics and proteomics to analyse the entire metabolome, the final products of the metabolism within a biological system (Zhang, Sun, Wang, Han, & Wang, 2012). The metabolites are small molecules, less than 1kDa derived from metabolic pathways, and include hormones, amino acids, lipids, carbohydrates and nucleotides. The composition of the metabolome determines the current phenotypic state of a cell and actively changes in response to cellular and extracellular stimuli, making metabolomics studies more informative than the other -OMICS approaches, particularly in the investigation of cellular response to exogenous stimuli or in case of pathology (Nicholson, Lindon, & Holmes, 1999). The total estimate of metabolites is approximately 6,500, which is significantly lower than the number of genes (25,000), messenger RNAs (100,000) and proteins (1,000,000) present in a human cell. However, the development

TABLE 1 Potential protein biomarkers in different male infertility conditions

Condition	Sample	Results	Reference
Varicocele	Spermatozoa	Heat shock proteins, mitochondrial and cytoskeleton proteins are mainly affected by varicocele disease Proposed markers to discriminate between varicocele and healthy patients: PKAR1A, AK7, CCT6B, HSPA2, ODF2, ATPase1A4, HSPA2, SPA17 and APOA1	(Agarwal, Sharma, Samanta, Durairajanayagam, & Sabanegh, 2015; Hosseinifar et al., 2013)
		Proposed markers to discriminate between bilateral and unilateral varicocele: ENKUR, SEMG1, SEMG2, SPAM1, CABYR, PRDX2, FASN and FN1. Proteins CRISP2, ARG2 and GNPDA1 uniquely expressed in unilateral patients compared with fertile controls	(Agarwal, Sharma, Durairajanayagam, Cui, et al., 2015; Ayaz et al., 2015; Panner Selvam, Agarwal, & Baskaran, 2019)
		The expression of HSPA5, SOD1 and ATP5D improves after varicocelectomy	(Camargo et al., 2013; Del Giudice et al., 2013; Hosseinifar et al., 2014)
Unexplained male infertility (UMI)	Spermatozoa	Proposed markers to discriminate between UMI and fertile patients: SPA17, ANXA2 and SERPINA5	(Panner Selvam et al., 2019)
	Seminal plasma	Biomarkers for success in couples undergone IVF: clusterin, epididymal secretory protein E1 and PSA	(Kanannejad & Gharesi-Fard, 2019)
Testicular cancer	Spermatozoa	Altered proteins in asthenozoospermic testicular cancer spermatozoa: CCT3, ATP1A4, ATP5A1 and UQCRC2	(Panner Selvam et al., 2019)
	Testicular tissue	Proposed markers of testicular tumour germ cell: PIWIL1 and TMPRSS12	(Liu et al., 2013)
		Glutathione S-transferase M3 was reported to be downregulated in case of seminoma, while GCP6, CDK10 and StarD7 overexpressed	(Leman et al., 2009; Zimmermann et al., 2006)
		Overexpression of PAK4 protein in embryonal carcinoma	(Castillo et al., 2019)
Azoospermia	Seminal plasma	ECM1, TEX101 and NPC2 proteins were suggested for the discrimination of obstructive from non-obstructive azoospermia	(Drabovich et al., 2013; Yamakawa et al., 2007)
		Higher expression of seminal LGALS3BP in men with successful TESE	(Freour et al., 2013).
Oligoasthenozoospermia	Seminal plasma	Proposed markers to discriminate between oligoasthenoteratozoospermic (OAT) and normozoospermic patients: KLK3, LTF SERPINA1, GAPDH, NPC2, M2BP and lipocalin−1	(Giacomini et al., 2015; Liu, Wang, et al., 2018)
Globozoospermia	Spermatozoa	Overexpression of AKAP4 and actin, as well as the underexpression of enzymes of the Krebs cycle, tubulin, and proteins associated with the outer dense fibres and acrosome membrane	(Liao et al., 2009)

of new technologies facilitates the detection of maximum number of molecules (Wishart et al., 2013). Therefore, the investigation of a less number of molecules to be analysed makes metabolomics easier to be performed in a short period of time compared to other -OMICS approaches (Newgard, 2017). So far, the metabolomics studies were carried out using different human biological systems such as cells, tissues or fluids, including urine, blood plasma and serum, cerebrospinal fluid, saliva, follicular fluid, breast milk, tears and seminal plasma (Wishart et al., 2013; Zhang et al., 2012). Moreover, metabolomic studies have been conducted in several fields of human medicine such as pharmacology, toxicology, nutrition, cancer, metabolic diseases and reproduction (Čuperlović-Culf, Barnett, Culf, & Chute, 2010; Vivanco et al., 2011; Zhang et al., 2012).

In general, heterogeneous nature of the metabolites makes it challenging to perform metabolomics experiments, whereas the

heterogeneity of the molecules is comparatively less with other -OMICS approaches (Patti, Yanes, & Siuzdak, 2012). This can be partially overcome by using more efficient metabolite extraction and separation procedures, high-throughput platforms to scan and detect a large number of metabolites, and computational tools for storage, processing and analysis of large data sets (Zhang et al., 2012). To analyse intracellular metabolites, cells are completely lysed to extract the metabolites. The protocol used to extract the metabolites can introduce variability due to the selectively loss of particular molecules, while alteration of the metabolic profile can be due to mistakes induced by the operator or long processing time (Patti et al., 2012). However, metabolomic profiling of body fluids does not require pre-processing steps, which in turn reduces the sample preparation time and technical bias. Once sample has been collected, the metabolic activities in the cell must be arrested in order to reliably reflect the metabolomic profile at the time of collection. This is achieved by freezing the sample in liquid nitrogen, which is stored at −80°C, and further extraction of metabolites (Gika, Theodoridis, & Wilson, 2008). Most commonly employed separation techniques for metabolomic studies include high-performance liquid chromatography (HPLC) and capillary electrophoresis as well as gas chromatography (Fiehn, 2008; Juo, Chiu, & Shiao, 2008). The qualitative and quantitative analysis of metabolites can be performed by several advanced techniques such MS, nuclear magnetic resonance (NMR) spectroscopy, near infrared (NIR) and Raman spectroscopy (Deepinder, Chowdary, & Agarwal, 2007; Lindon & Nicholson, 2008). The metabolite identification/detection capacity of these techniques is limited and can univocally identify metabolites from one-third of the approximately 10,000 spectra obtained. However, these advanced techniques can be used complementarily to detect maximum number of metabolites starting from a very low amount of sample (Patti et al., 2012; Zamboni, Saghatelian, & Patti, 2015). Different databases such as HMDB, KEGG, PubChem, ChEBI, BioCyc/HumanCyc, LipidMAPS, ChemSpider, METLIN and Recon2 are used to identify the metabolites present in the given sample (Marco-Ramell et al., 2018). Analysis of the data can vary as fingerprinting, footprinting and profiling approaches based on the final goal (Vivanco et al., 2011). Metabolomic fingerprinting is a comparative analysis of metabolite profiles between two conditions rather than the identification of all metabolites present in the system. The footprinting aims to identify all the molecules which are released in the extracellular space, while metabolomics profiling analyses a pre-determined set of metabolites involved in specific pathways (Vivanco et al., 2011). In fact, the comparison with a placebo-treated system or a healthy control makes possible the investigation of metabolic pathways directly altered by the treatment/disease and the identification of more reliable molecular biomarkers of disease.

7 | METABOLOMICS AND MALE REPRODUCTION

In male reproduction, metabolomic studies have been conducted to shed light on the altered pathways in male infertility scenarios. The results from investigation of metabolites in sperm cells, seminal plasma and testicular tissue are described in the next sections.

7.1 | Sperm metabolomics

Metabolomics analysis of the spermatozoa is challenging due to the presence of seminal plasma and use of reagents for sperm washing that potentially interfere with metabolomics analysis. The first metabolomics study on spermatozoa was conducted in 2015 by Paiva et al. using spermatozoa selected by density gradient. The metabolome of spermatozoa was analysed by combining results from proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy and gas chromatography–mass spectrometry (GC-MS) (Paiva et al., 2015), detecting a maximum number of metabolites (n = 69). For the first time, authors reported intracellular metabolome of the spermatozoa such as amino acids, peptides and analogues as the most representative molecules, followed by lipids organic acids, aliphatic acyclic compounds, carbohydrates and nucleotides. The presence of lactate, carnitine and acetylcarnitine in important metabolomic pathways such as glycolysis and fatty acid oxidation and its association with sperm physiology provided foundation for further studies. Two years later, Reynolds et al. used ^{1}H-NMR to profile the metabolome of spermatozoa selected by density gradient. Metabolome profiles of mature and immature fraction of spermatozoa revealed differences in lipid composition with a higher concentration of lactate, choline and glycerophosphocholine in immature fraction due to the presence of morphological defects in the selected sperm population (Reynolds, Calvert, Paley, & Pacey, 2017).

Metabolomic profile of spermatozoa investigated by GC-MS in idiopathic asthenozoospermic patients revealed the under- and overexpression of 27 and 6 metabolites, respectively, when compared to the healthy subjects (Zhao et al., 2018). Molecular pathways related to the metabolism of nucleosides, amino acids and sugars were altered in asthenozoospermic samples. Some essential amino acids, such as tryptophan and leucine, were significantly underexpressed, suggesting that sperm motility of asthenozoospermic patients can be improved by exogenous amino acid supplementation. Further, reduced levels of 3-phosphoglycerate and lactic acid suggested a disturbance in glycolysis and Krebs cycle pathways, with consequences on energy production. Targeted metabolomic experiments on semen before and after cryopreservation showed disturbance in sugar metabolism associated with reduced semen motility following freezing/thawing processes (Fu et al., 2019). Metabolomics analysis revealed dysfunction of glycolysis pathway in cryopreserved spermatozoa, also supported by the underexpression of the hexokinases (HK2) enzyme, detected by Western blotting. Finally, intracellular metabolic profile has been correlated with semen parameters by analysing semen samples from healthy volunteers (Engel et al., 2019). Specifically, alterations of the intracellular metabolites' concentration such as acylcarnitines, lysophosphatidylcholines, phosphatidylcholines,

sphingomyelins and sugars have been closely associated with sperm motility. Although further studies are needed to identify the physiological concentration of such metabolites, these preliminary results laid the foundation for the identification of more reliable molecular markers of semen quality.

7.2 | Seminal plasma metabolomics

The first metabolomics study on seminal plasma was published by Hamamah et al. in 1993, and analysed metabolomics profile of seminal plasma in infertile patients with obstructive azoospermia, oligoasthenoteratozoospermia and spermatogenic failure (Hamamah et al., 1993) (Table 2). The results, obtained by ^{1}H-NMR, were compared to those from a control group of normozoospermic men. Hamamah et al suggested that a combination of biomarkers, such as glycerylphosphorylethanolamine—GPC, citrate and lactate, might be used to differentiate obstructive azoospermic patients, while the ratios between citrate:lactate and GPC:lactate were altered in both oligoasthenoteratozoospermic and spermatogenic failure patients (Hamamah et al., 1993). The other metabolites such as alanine, tyrosine and phenylalanine were also reported to be altered in infertile normozoospermic and oligozoospermic patients (Gupta et al., 2011). Xu et al. used LC-MS technique to identify the difference in seminal metabolome between fertile and infertile patients (Xu, Lu, Wang, Zhang, & Wu, 2020). A total of 63 metabolites were identified as potential biomarkers of infertility, and 17 of these were correlated with semen parameters. The relationship was established between acylcarnitines and sperm concentration, sperm motility and antioxidant levels in semen. Furthermore, carnitines involved in the sperm maturation process (Niederberger, 2005) also has an impact on oxidative stress (Dobrakowski et al., 2017).

The metabolic seminal profile was also analysed in male infertility-associated conditions such as spinal cord injury and obesity by MALDI-TOF MS, and Kidney Young deficiency syndrome (KYDS) by LC/QTOF-MS (Chen, Hu, Dai, & Chen, 2015; Guo et al., 2017; da Silva et al., 2011). In case of spinal cord injury, altered metabolites were associated with metabolism and biosynthesis of lipids, nucleotides, response to oxidative stress and vitamins (da Silva et al., 2011), whereas KIDS seminal plasma metabolome showed altered biosynthesis and metabolism of aromatic amino acids, sugars and lipids (Chen et al., 2015). Spermidine and spermine levels were increased in obese patients with abnormal semen quality, highlighting the role of these polyamines in the testicular development and spermatogenesis (Guo et al., 2017).

Metabolomics of seminal plasma was also investigated in several conditions related to sperm abnormalities. Jayaraman et al. used ^{1}H-NMR to analyse seminal plasma metabolome of oligozoospermic, asthenozoospermic, teratozoospermic, azoospermic and idiopathic infertile men (Jayaraman et al., 2014). Besides the altered metabolites, a unique metabolic profile was identified in idiopathic infertility, characterised by altered levels of fructose, citrate and amino acids (lysine, arginine, tyrosine and proline). These metabolic dysfunctions in seminal plasma may explain the origin of idiopathic infertility (Jayaraman et al., 2014). Similarly in the case of UMI, amino acid catabolism was increased, which indicates that oxidative stress is associated with the UMI aetiology (Jafarzadeh et al., 2015; Qiao et al., 2017).

Since 2014, different techniques have been used to profile the metabolome of asthenozoospermic patients. By performing ^{1}H-NMR, metabolomic dysregulations related to sugars, lipids and amino acids were reported in seminal plasma of asthenozoospermic patients and indicate that oxidative stress mechanism is disturbed in asthenozoospermia (Jayaraman et al., 2014; Zhang, Diao, Zhu, Li, & Cai, 2015). Moreover, GC-MS identified increased levels of oleic and palmitic acids and reduced valine concentration that may cause membrane dysfunction and poor sperm motility, respectively (Tang et al., 2017). Furthermore, HPLC-ESI-MS/MS techniques was able to report the alteration of metabolites related to arachidonic acid metabolism (arachidonic acid, 15-HETE, 8,9-EET, PGE_2, 14,15-DHET, 14,15-EET, PGD_2, 5-HETE, $PGF_{2\alpha}$, Tetranor-PGEM, 11,12-DHET, 20-HETE) (Yu et al., 2019), whereas metabolomic fingerprinting of seminal plasma assessed by Raman spectroscopy was able to discriminate asthenozoospermic from normozoospermic patients (Gilany, Moazeni-Pourasil, Jafarzadeh, & Savadi-Shiraz, 2014).

Engel et al. reported that the metabolomic profile of seminal plasma correlated with the sperm concentration and morphology in healthy donors using LC-MS/MS approach (Engel et al., 2019). Huang et al. reported an association between sperm concentration/sperm count and products of fatty acid, lipid and amino acid metabolism (Huang et al., 2019). Metabolomics platform was used to evaluate the relationship between the metabolites in seminal plasma and normal sperm morphology. Reduced levels of metabolites, such as amino acids, lactate, citrate, creatinine, α-ketoglutaric acid, spermine and putrescine, were noticed in the seminal plasma of oligoasthenozoospermic patients (Mumcu, Karaer, Dogan, & Tuncay, 2019). On the other hand, amino acids, citric acid, choline, D-glucose, myo-inositol, lactate and pyruvate were significantly overexpressed in infertile teratozoospermic patients (Mehrparvar et al., 2020). In oligozoospermic patients, ^{1}H-NMR detected altered levels of fructose, myo-inositol, aspartate and choline, involved in the sperm maturation, energy metabolism and cell membrane composition (Murgia et al., 2020). Future in-depth studies are required to better investigate the seminal metabolome and identify biomarkers associated with sperm abnormalities.

Metabolomics has been recently applied to the field of assisted reproductive techniques (ART) to predict spermatogenesis in non-obstructive azoospermic (NOA) patients (Gilany et al., 2017, 2018). Techniques such as GS-MS and Raman spectroscopy were able to identify 36 discriminatory metabolites in seminal plasma as well as to classify NOA patients based on the TESE outcome. Although the field of metabolomics in male reproductive research is still in emerging phase, metabolites are very promising non-invasive biomarkers for the diagnosis and management of male infertility.

TABLE 2 Metabolomics studies in seminal plasma

References	Studied population	Metabolomics techniques	Main findings
Hamamah et al. (1993)	Spermatogenic failure (21) OA (14) OAT (7) Normozoospermia (18)	^{1}H-NMR	Glycerylphosphorylethanolamine, citrate and lactate varied in azoospermic men, while citrate:lactate ratios and GPC:lactate ratios were altered in spermatogenic failure and OA
Gupta et al. (2011)	Infertile normozoospermic (65) Infertile oligozoospermic (60) Healthy fertile men (60)	^{1}H-NMR	Alanine, citrate, glycerylphosphorylcholine, tyrosine and phenylalanine suggested as biomarkers for screening of male infertility
da Silva et al. (2011)	Spinal cord injury (6) Healthy controls (6)	MALDI-TOF MS	Altered pathways in spinal cord injury patients: biosynthesis of nucleotides, sterol biosynthesis, arachidonic acid metabolism and response to hydrogen peroxide, steroid hormone and vitamins.
Jayaraman et al. (2014)	Idiopathic male infertility (17) Oligozoospermia (20) Asthenozoospermia (20) Teratozoospermia (20) Azoospermia (20) Normozoospermia (6)	^{1}H-NMR	Idiopathic infertile men profile differed from that of fertile controls and other infertile groups due to either up- or downregulation of lysine, fructose, arginine, tyrosine, citrate and proline
Gilany et al. (2014)	Asthenozoospermia (13) Normozoospermia (13)	Raman spectroscopy	Metabolomic fingerprinting could be used to discriminate between asthenozoospermic and normozoospermic males
Zhang et al., 2015	Asthenozoospermia (33) Healthy men (30)	^{1}H-NMR	Asthenozoospermia associated with metabolic dysregulation of amino acids, lipids, phospholipids (choline), cholesterol, nucleosides, sugar and energy generation. Overexpression of oxysterols indicated the role of oxidative stress in the mechanism of asthenozoospermia.
Chen et al. (2015)	Infertile males with Kidney Yang Deficiency Syndrome (KYDS) (18) Fertile men (18)	LC/QTOF-MS	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
Jafarzadeh et al. (2015)	UMI (19) Fertile men (15)	Raman spectroscopy	Men with unexplained infertility have an imbalance of oxidative stress, increased biomarkers of oxidative stress and absence of a functional antioxidant system
Tang et al. (2017)	Asthenozoospermia (30) Healthy donors (30)	GC/MS	Levels of oleic acid and palmitic acid were higher in asthenozoospermic men, which may signify a metabolic disorder of the sperm membrane. Deficiency of valine may contribute to poor sperm motility
Qiao et al. (2017)	UMI (80) Fertile men (80)	GC/MS	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
Guo et al. (2017)	Normal BMI, normal semen quality (55) Obese, abnormal semen quality (9)	MALDI-TOF MS	Spermidine and spermine significantly higher in obese men with abnormal semen quality. They were likely to play a vital role in the spermatogenesis progress.
Gilany et al. (2017)	NOA with TESE-negative (11) NOA with TESE-positive (9) Fertile healthy men (10)	GS-MS	36 discriminatory metabolites for different groups in NOA condition
Gilany et al. (2018)	NOA with TESE-negative (10) NOA with TESE-positive (10) Fertile healthy men (15)	Raman spectroscopy	The seminal plasma metabolome could be used to detect spermatogenesis in NOA patients. TESE-negative patients have severe oxidative imbalance compared to TESE-positive
(Yu et al., 2019)	Asthenozoospermia (30) Healthy men (33)	HPLC-ESI-MS/MS	A significant disorder of arachidonic acid metabolism was observed in asthenozoospermic samples.
Engel et al. (2019)	Healthy donors (20)	LC-MS/MS	Seminal plasma metabolites are closely related to sperm concentration and morphology

(Continues)

TABLE 2 (Continued)

References	Studied population	Metabolomics techniques	Main findings
Mumcu et al. (2019)	OAT (31) Normozoospermia (28)	^{1}H NMR spectroscopy t	The differences were based on the metabolites lactate, citrate, lysine, arginine, valine, glutamine, creatinine, a-ketoglutaric acid, spermine, putrescine and tyrosine. Except the tyrosine, levels of the above metabolites were significantly decreased in patients with OAT compared to the controls.
Huang et al. (2019)	Chinese men (98)	UPLC-MS/MS	16 and 22 seminal plasma metabolites were related to sperm concentration and count, respectively, and they are mainly involved in fatty acid, lipid and amino acid metabolism.
Xu et al. (2020)	Infertile men (140) Healthy men (35)	LC-MS/MS	17 metabolites were found to be significantly correlated with sperm parameters. All the potential biomarkers were involved in 14 metabolic pathways playing important role in energy production, antioxidation, hormone regulation and sperm membrane.
Mehrparvar et al. (2020)	Infertile teratozoospermia (14) Fertile men (15)	^{1}H-NMR	18 deregulated metabolites were identified in fertile men compared to teratozoospermia patients.
Murgia et al. (2020)	Oligozoospermia (18) Normozoospermia (29)	^{1}H-NMR	Fructose, myo-inositol, aspartate and choline were altered in oligozoospermia and also correlated with sperm motility profile

Abbreviations: BMI, body mass index; NOA, non-obstructive azoospermia; OA, obstructive azoospermia; OAT, oligoasthenoteratozoospermia; TESE, testicular sperm extraction.

7.3 | Testicular metabolomics

Metabolomics has been used as a non-invasive tool to investigate the occurrence of spermatogenesis in the testicular tissue of non-obstructive azoospermic patients (Aaronson, Iman, Walsh, Kurhanewicz, & Turek, 2010; Liu et al., 2014). Analysis of frozen testicular tissue with ^{1}H-NMR revealed increased phosphocholine (PC) in patients with normal spermatogenesis undergoing vasectomy, in comparison with patients showing maturation arrest or Sertoli cell only syndrome (Aaronson et al., 2010). Hence, PC can be used as a putative screening biomarker of spermatogenesis, as its concentration is indicative of cellular proliferation and membrane synthesis (Aaronson et al., 2010). Liu et al., (2014) identified 12 differentially expressed metabolites using GS-MS in fresh seminiferous tubules of non-obstructive and obstructive azoospermic patients, with increased levels of cis-Phytol and glutamine, and decreased levels of saccharides and amino acids in non-obstructive testes. Further, Raman spectroscopy was able to distinguish the non-obstructive and obstructive azoospermic patients with high sensitivity (90%) and specificity (85.71%), emerging as a potential non-invasive tool to predict spermatogenesis in the seminiferous tubules (Liu et al., 2014).

8 | METABOLOMICS IN PROGNOSIS AND DIAGNOSIS OF MALE INFERTILITY

The identification of biomarkers for prognosis, diagnosis and severity, as well as to predict the success of a therapeutic approach, is one of the main goal of metabolomics studies. An ideal biomarker should have high sensitivity and specificity, and its detection should be easy and noninvasive as well as fast and economically sustainable (Bieniek, Drabovich, & Lo, 2016). Moreover, using a combination or panel of biomarkers may increase their power as a prognostic and diagnostic tool. Metabolites present in the seminal plasma can serve as predictive biomarkers in several conditions associated with male infertility (Table 3). A group of metabolites (alanine, citrate, GPC, tyrosine and phenylalanine) was able to discriminate patients based on the fertility status as well as oligozoospermic from normozoospermic patients with the accuracy of 92.4% and 92.9%, respectively (Gupta et al., 2011). Lysine was proposed as a potential biomarker to discriminate idiopathic infertile men from normozoospermic men (Jayaraman et al., 2014). Qiao et al. developed a model based on 153 differentially expressed metabolites in seminal plasma to discriminate UMI patients from fertile healthy men with a specificity of 92% (Qiao et al., 2017). Particularly, 4-hydroxyphenylacetic acid positively correlated with the sperm count and considered as decisive in screening UMI patients. A group of metabolites composed by lactate, citrate, lysine, arginine, valine, glutamine, creatinine, α-ketoglutaric acid, spermine, putrescine and tyrosine was also recommended to discriminate normozoospermic and oligoasthenoteratozoospermic samples (Mumcu et al., 2019), while altered levels of aspartate, choline, fructose and myo-inositol were reported in oligozoospermic cases (Murgia et al., 2020). Metabolomics study conducted on sperm cells isolated by density gradient centrifugation suggested that 33 altered metabolites may be used as a molecular signature of idiopathic asthenozoospermia (Zhao et al., 2018). Metabolites concentration in blood and urine samples is used to predict the fertility status of men. Combination of urinary metabolites was used to predict the sperm abnormalities. Carnitines and aspartic acid were

able to predict oligozoospermia with moderate sensitivity (65%) and specificity (71%) (Zhang et al., 2014). Idiopathic normozoospermic infertile men can be predicted using a combination of 5 biomarkers (leukotriene E4, carnitine, aspartate, xanthosine and methoxytryptophan) (Zhang et al., 2014). In fact, higher sensitivity (85.7%) and specificity (86.8%) were noticed when they were all included in the predictive model in comparison with using the single markers. Like urine and seminal plasma, blood metabolites are analyzed and used as a non-invasive tool for differential disease diagnosis. A recent multivariate analysis identified a set of 38 metabolites from blood plasma to discriminate healthy controls from infertile men with semen abnormalities (sensitivity: 78.69%, specificity: 84.09%) and infertile men with erectile dysfunction (sensitivity: 80.33%, specificity: 100%) (Zhou et al., 2016). Metabolomics is emerging as a new promising tool for the diagnosis and prognosis of male infertility, and its application to the semen and other biological fluids will provide evidence on the metabolic dysfunctions associated with the male infertility.

9 | CHALLENGES AND FUTURE PERSPECTIVES OF PROTEOMICS AND METABOLOMICS IN CLINICAL ANDROLOGY

Adjunct use of biomarker screening along with standard semen analysis may increase the diagnostic power in evaluating the male

TABLE 3 Studies showing the diagnostic/prognostic power of metabolomics

References	Sample/ Studied population	Metabolomics techniques	Main findings
Gupta et al. (2011)	Seminal plasma Infertile normozoospermic (65) Infertile oligozoospermic (60) Healthy fertile men (60)	^{1}H-NMR	Alanine, citrate, GPC, tyrosine and phenylalanine can be used to determine infertility. High accuracy in differentiating healthy controls from infertile patients (92.4%) and normozoospermic from oligozoospermic samples (92.9%)
Jayaraman et al. (2014)	Seminal plasma Idiopathic male infertility (17) Oligozoospermia (20) Asthenozoospermia (20) Teratozoospermia (20) Azoospermia (20) Normozoospermia (6)	^{1}H-NMR	Lysine has the potential to aid in the detection and diagnosis of idiopathic infertility
Zhang et al. (2014)	Urine Normozoospermic infertile cases (*n* = 71) and fertile controls (*n* = 47)	LC/ MicrOTOF–Q II MS	Leukotriene E4, 3-hydroxypalmitoylcarni- tine, aspartate, xanthosine and methoxytryptophan can discriminate normozoospermic infertile patients from fertile controls
Zhang et al. (2014)	Urine Oligozoospermic infertile men (135) Fertile volunteers (158)	LC/QTOF–MS	The combined pattern of acetylcarnitine, carnitine C3:1 and aspartic acid provided moderate diagnostic power for oligozoospermic infertility
Zhou et al. (2016)	Blood sample Erectile dysfunction (26) Semen abnormalities (44) Healthy controls (61)	GC-MS	Set of metabolites from plasma samples was proposed to discriminate healthy controls from infertile men with semen abnormalities and infertile men with erectile dysfunction
Qiao et al. (2017)	Seminal plasma UMI (80) Fertile men (80)	GC/MS	153 seminal plasmatic metabolites were able to distinguish 82% of the UMI patients from healthy controls with a specificity of 92%
Zhao et al. (2018)	Spermatozoa Idiopathic asthenozoospermia (30) Healthy subjects (30)	GC-MS	Altered expression of metabolites was used to discriminate between idiopathic asthenozoospermic patients and healthy controls
Mumcu et al. (2019)	Seminal plasma OAT (31) Normozoospermia (28)	^{1}H NMR spectroscopy t	A PLS-DA model built on the NMR data achieved 89.29% sensitivity and 93.55% specificity results in a leave-one-out cross-validation process
Murgia et al. (2020)	Seminal plasma Oligozoospermia (18) Normozoospermia (29)	^{1}H-NMR	NMR-based metabolomics allowed the identification of a specific metabolic fingerprint of the seminal fluids of patients affected by oligozoospermia

infertility, particularly in case of idiopathic male infertility (Jungwirth et al., 2016). Male infertility is a multifactorial disorder and the use of a set of biomarkers instead of a single biomarker may significantly enhance the predictive power of pathological conditions (Zhang et al., 2014). Longo et al. (2018) reported that volatile metabolites (volatilome) were able to demonstrate a relationship between sperm metabolism and male infertility in astheno- and oligozoospermic patients. The identification of altered metabolic processes in male infertility scenarios by proteomics and metabolomics studies will open the avenue for the identification of novel therapeutic targets. Further studies based on larger and heterogeneous population are required to validate the preliminary markers identified in the clinical practice. The presence of confounding factors, such as age, gender, nationality, exposure to environmental factors, should be taken into consideration during the analysis.

10 | CONCLUSION

Proteomics and metabolomics are emerging as powerful tools to investigate the molecular causes of male infertility. The application of high-throughput platforms for the detection of proteins and metabolites, combined with the use of sophisticated bioinformatic software for their analysis, will enable the identification of biomarkers for prognosis and diagnosis of male infertility and prediction of reproductive outcomes in the ART. The understanding of the molecular changes associated with male infertility will help in planning ad hoc treatments, with significant contribution in the clinical management of infertile patients.

11 | TAKE HOME MESSAGE

- Proteomics and metabolomics can decipher the molecular etiologies associated with male infertility conditions.
- Proteomics and metabolomics may allow the identification of more reliable markers of diagnosis and prognosis of male infertility.

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