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# Sperm and Seminal Plasma Proteomics: Molecular Changes Associated with Varicocele-Mediated Male Infertility

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Male infertility is a rising problem and the etiology at the molecular level is unclear. Use of omics has provided an insight into the underlying cellular changes in the spermatozoa of infertile men. Proteomics is one the promising omics techniques for biomarker screening that can provide complete information on molecular processes associated with male infertility. Varicocele is a pressing issue in the field of male infertility and the search for an appropriate diagnostic and therapeutic biomarker is still ongoing. In this review, we discuss the reports on proteomic profiles of sperm and seminal plasma in male infertility and provide an in-depth insight into varicocele studies associated with male infertility.

**Keywords:** Proteomics; Seminal plasma; Sperm; Varicocele

## INTRODUCTION

Currently, infertility is one of the top most concerns related to male reproductive dysfunction and male infertility contributes to nearly 50% of overall infertility cases [1]. The incidence of varicocele is about 21% to 41% in men with primary infertility and 75% to 80% in men with secondary infertility [2,3]. Varicocele is characterized by the enlargement of the pampiniform plexus, which may be due to the presence of malfunctioning valves. In these patients, testicular function is affected due to the retrograde flow of blood. Thus, varicocele has a detrimental effect on spermatogenesis by inducing a state of testicular hyperthermia, hypoxia and oxidative stress [4-6]. In addition, the reflux of metabolites and endocrine factors are associated with varicocele pathophysiology (Fig. 1). The mechanisms associated with the pathophysiology of varicocele have been reviewed in detail by Agarwal et al [7] and Cho et al [6]. Furthermore, varicocele drastically alters semen parameters [8,9] and these patients exhibit compromised fertility [10].

Laboratory evaluation of male infertility in varicocele patients is based on basic semen analysis (sperm concentration, motility, vitality, and morphology) as per the World Health Organization 2010 guidelines [11]. Additionally, advanced laboratory tests such as those for the quantification of reactive oxygen species (ROS) [12] and antioxidants in semen [12], oxidation–reduction potential in ejaculated semen by Male Infertility Oxi-

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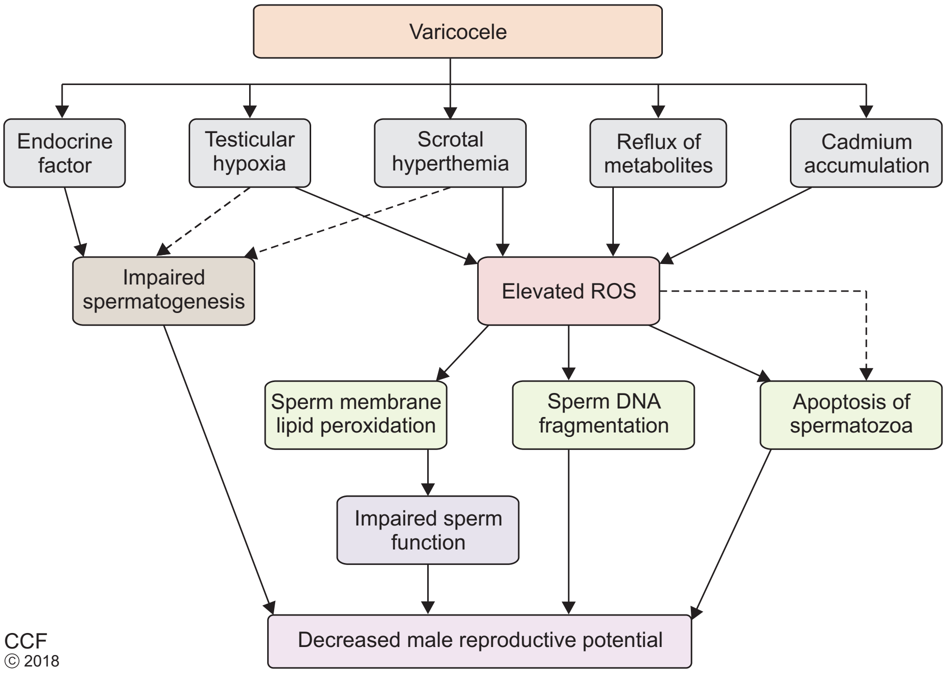

**Fig. 1.** Pathophysiology of varicocele. ROS: reactive oxygen species.

dative System (MiOXSYS®; Aytu BioScience Inc., Englewood, CO, USA) [13], and sperm DNA fragmentation (SDF) assessment by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay [13] and sperm chromatin structure assay [14] are used to identify the functionality of spermatozoa for further utilization in assisted reproductive technology. However, these tests lack the information on molecular changes at the subcellular level associated with the poor fertilizing ability of spermatozoa.

Advancement in the current omics techniques, especially proteomics have revolutionized the field of sperm molecular biology. Proteomics, a high throughput platform, is used to identify and select non-invasive biomarkers for the diagnosis of male infertility [15-17]. This has facilitated the identification of cellular and molecular pathways that are being dysregulated in the spermatozoa of infertile patients [18]. Post-translational modification (PTM) of sperm proteins provide valuable information pertaining to the biomolecules associated with the fertilization potential of spermatozoa [19,20]. Altered expression of sperm proteins in infertile patients indicate compromised spermatogenesis or defects in vital sperm functions, such as capacitation, hyperactivation and acrosome reaction, which are essential for the fertilization process [21,22]. Another important component of semen is seminal plasma, which is constituted by the secretions derived from testes, epididymis and accessory sex glands. Seminal plasma proteins interact with and modulate sperm functions, such as capacitation, hyperactivation and acrosome reaction required for fertilization [17,23]. In varicocele condition, these sperm functions are compromised, and sperm/seminal plasma proteomic analysis have reported altered expression of sperm and seminal plasma proteins [24-26].

In this review, we discuss the proteins involved in the regulation of sperm functions that are present in both the cellular (sperm) and fluidic component (seminal plasma) of semen. In addition, we have discussed the future of proteomics as a potential clinical tool for the diagnosis and management of varicocele patients.

## BACKGROUND OF SPERM AND SEMINAL FLUID PROTEOMICS

Semen is a highly complex biofluid that contains proteins and peptides with varied functions. The main components of semen include spermatozoa (cellular) and seminal fluid (enriched with proteins). Sperm proteomics came into the limelight because of the transcriptionally and translationally inert property of spermatozoa [27]. Characterization of proteins in spermatozoa and seminal plasma provides insight into the functions of specific proteins related to fertility [28]. Sperm proteins regulate the molecular pathways such as protein and energy metabolism, PTMs, DNA damage and oxidative stress response [19,29,30]. Spermatogenesis involves complex processes that ultimately produce the male gamete with specialized functions. The developmental process of spermatozoa is regulated by protein–protein interaction [31]. Amaral et al [30] identified 6,198 proteins in spermatozoa and 30% of these proteins originate from the testis. Protein characterization studies revealed that a total of 898, 984, and 532 proteins were present in the sperm head, tail and both locations, respectively [32]. Protein profiling using 2-dimensional (2D)-gel matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) detected that the sperm proteins were distributed in cytoplasm (37%), mitochondria (19%), nuclear (5%), tail and flagella (3%), and acrosome (2%) [33]. For the first time in human spermatozoa, 27 proteins present in the 26S proteasome complex was mapped from 1,760 proteins using 1-dimensional (1D)-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) combined with GeLC-tandem mass spectrometry (MS/MS) technique [34]. Bioinformatic tools predicted that the biological pathways, such as oxidative phosphorylation and glycolysis are influenced by sperm proteins [18,29,30].

Apart from sperm proteins, seminal plasma proteins are also essential for the maintenance of sperm functionality [35]. Seminal plasma secretions are derived from the testes and accessory sex glands (Fig. 2). Seminal plasma is rich in proteins (35–55 g/L) and semenogelins are present in high abundance (80%). Only 10% of the seminal plasma proteins are contributed by seminal extracellular vesicles (including epididymosomes and prostasomes) [15,32]. So far, 2,064 proteins have been identified in the seminal plasma [32]. About 70% of these proteins were also identified in spermatozoa [29,36]. Altered expressions of seminal plasma proteins have a direct effect on spermatozoa homeostasis and functions. Comparative proteomic approach allows the identification of the underlying molecular causes associated with the pathology of spermatozoa. Expression of semen proteins varies from one condition to another. Proteomics analysis demonstrated the differential expression of proteins in semen and its potential use as non-invasive biomarkers in infertile men with abnormal semen parameters. In azoospermic men, the proteins ACPP (prostatic acid phosphatase), KLK3 (prostate-specific antigen, PSA), CLU (clusterin), AZGP1 (zinc-alpha-2-glycoprotein), and PAEP (glycodelin) were absent in seminal plasma [37,38]. Drabovich et al [39] validated TEX101 (testis-expressed protein 101) as a biomarker in azoospermia and ECM1 (extracellular matrix protein 1) to distinguish non-obstructive azoospermia from vasectomy. In the case of asthenozoospermia, PTPN14 (tyrosine-protein phosphatase non-receptor type 14) was dysregulated [40], whereas CST3 (cystatin-C) was downregulated, and KLK3 and SEMG1 (semenogelin-1) were upregulated in oligoasthenozoospermia patients [41]. Other proteins associated with sperm function such as NPC2 (NPC intracellular cholesterol transporter 2), LGALS3BP (Galectin-3-binding

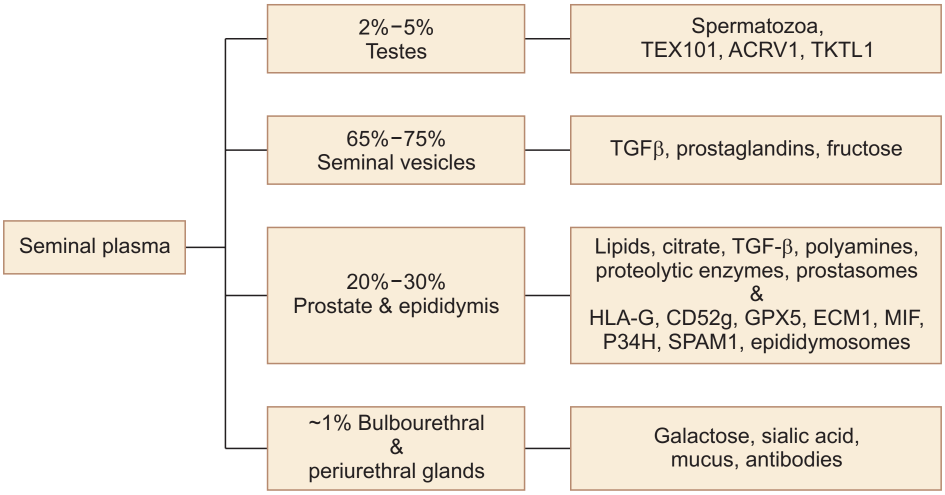

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**Fig. 2.** Seminal plasma: contributions of the testes and accessory sex glands, and its composition/constituents. TGF: transforming growth factor.

protein), LCN1 (lipocalin-1), and PIP (prolactin-inducible protein) were downregulated in oligoasthenoteratozoospermia [42]. Proteomic studies conducted by Sharma et al [41] demonstrated the involvement of differentially expressed proteins (DEPs) in stress response and regulatory pathways in men with high seminal ROS. The MME (membrane metallo-endopeptidase) protein was detected in the seminal plasma of ROS positive men (≥20 relative light units (RLU)/s/×$10^6$ sperm) but was absent in ROS negative men (<20 RLU/s/×$10^6$ sperm). However, proteins FN1 (fibronectin 1) and MIF (macrophage migration inhibitory factor) were present only in the ROS negative group [41]. Intasqui et al [43] also reported sperm nuclear DNA damage markers using bioinformatic analysis of proteomic data. SLC2A14 (solute carrier family 2, facilitated glucose transporter member 14), PGK2 (phosphoglycerate kinase 2), ODF1 (outer dense fiber protein 1), CLU, VDAC2 (voltage-dependent anion-selective channel protein 2), VDAC3 (voltage-dependent anion-selective channel protein 3), ZPBP2 (zona pellucida-binding protein 2), and PGC (progastricsin) have been reported as potential biomarkers of sperm DNA damage.

## SHOTGUN PROTEOMIC APPROACH

Advanced proteomic techniques are used to identify the complete proteome of a cell. Integration of proteomic data with computational bioinformatic analysis helps in understanding the function of peptides and proteins in cellular pathways. In the current era of proteomics, sperm proteins that are associated with infertility are widely studied [18]. Sophisticated and complex instruments such as liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and MALDI-TOF mass spectrometry are integrated with conventional 2D-gel electrophoresis to overcome the drawbacks of using the later technique alone. The high sensitivity and specificity of these techniques enable the detection of the maximum number of proteins in spermatozoa [44].

Prior to subjecting the protein samples to proteomic analysis, sperm and seminal plasma proteins are separated and processed for protein extraction. The extracted proteins are resolved either on 1D-gel electrophoresis or 2D-gel electrophoresis. Later, the gels are cut into pieces and the proteins are digested using trypsin. The sample is then injected into the high-throughput instrument and spectral counts are used to identify and relatively quantify the proteins. Expression of the proteins are measured by comparing the NSAF (normalized spectral abundance factor) of each protein [45]. A typical workflow involving the processing of semen samples for proteomics is shown in Fig. 3.

Besides the gel-based proteomic approach, gel-free techniques are widely used in proteome profiling. The extracted proteins are resolved either by peptide fractionation, ion-exchange chromatography, reverse-phase chromatography, 2D-LC or off-gel electrophoresis [46]. Currently, in sperm proteomics conventional 2D-gel separation techniques are replaced by LC-based methods. The extracted proteins are digested by trypsin. Further, these proteins are passed through reverse phase columns and are separated based on their hydrophobicity [32]. The most advanced ultraperformance LC are used to resolve the proteins in both sperm and seminal plasma fractions [43,47,48]. The peptides separated by LC are identified using a MS instrument [49,50].

Bioinformatic analysis provides meaningful results from the proteomic data [51]. Gene ontology (GO)

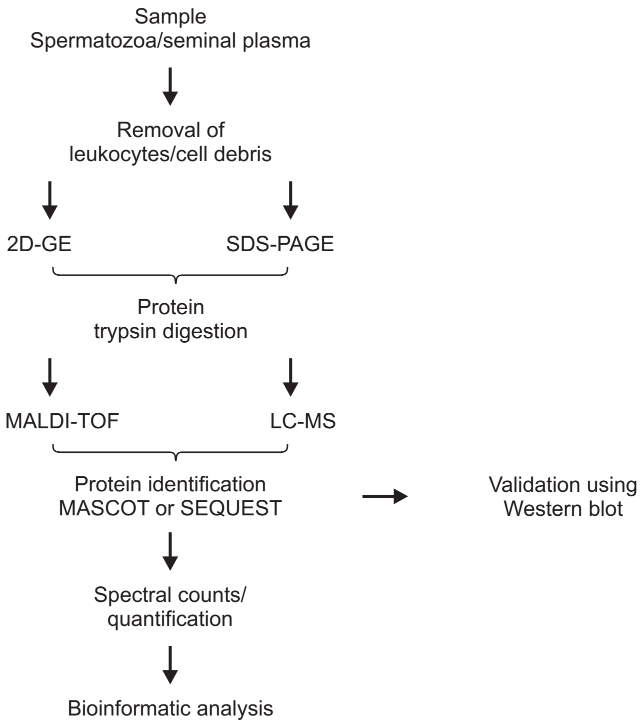

Fig. 3. Typical workflow involving the processing of semen samples for proteomics. 2D-GE: 2-dimensional gel electrophoresis, SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis, MALDI-TOF: matrix-assisted laser desorption/ionization time-of-flight, LC-MS: liquid chromatography mass spectrometry.

analysis of the identified proteins provides information about their localization, distribution and biological functions. Sophisticated programs such as Ingenuity Pathway Analysis (IPA) and Metacore$^{TM}$ can demonstrate the interaction between proteins and the pathways dysregulated due to differential expression of proteins. STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis is commonly performed to display the link between the proteins [52].

## VARICOCELE AND SPERM PROTEOMICS

There are only few reports on sperm proteomics in varicocele patients that are available in the literature. Protein profiling in normozoospermic men without varicocele and oligozoospermic men with varicocele using 2D-gel electrophoresis resulted in the identification of only 15 DEPs. The authors reported that the molecular pathways involving mitochondrial proteins, cytoskeleton proteins and heat shock proteins were affected in these varicocele patients [53]. Another proteomic study conducted by the same group demonstrated changes in the expression of sperm proteins in pre- and post-varicocelectomy patients. Varicocele repair in these patients led to a significant increase in the expression of mitochondrial function protein (ATP5D), antioxidant protein (SOD1) and heat shock protein (HSPA5) [54]. It was noted that the use of the conventional 2D-gel electrophoresis was a major limitation in these studies. However, several molecular mechanisms and subcellular pathways involved in the pathophysiology of varicocele have been elucidated by employing a global proteomic approach (*via* a LC-MS/MS platform) and in-depth bioinformatic analysis (reviewed by Swain et al [55]).

Varicocele associated male infertility manifests as a consequence of a high state of oxidative stress and mitochondrial dysfunction [56]. The expression of mitochondrial proteins in varicocele patients were found to be altered and linked to the pathophysiology of spermatozoa with mitochondrial dysfunction [55,57]. The sperm proteomic profile of varicocele patients revealed that 87% of DEPs involved in sperm function and energy metabolism were downregulated in both unilateral and bilateral varicocele patients [58]. Using high throughput proteome analysis (LC-MS/MS), Samanta et al [57] reported that 141 mitochondrial proteins were present in spermatozoa of varicocele patients, of which 22 DEPs were related to mitochondrial structure and function. Underexpression of the ATPase1A4, HSPA2, SPA17, and APOA1 proteins were associated with impaired mitochondrial function. Mitochondrial electron transport chain (ETC) proteins are regulated by the nuclear transcription factors and there exists a cross-talk between the same (Fig. 4). Also, mitochondrial proteins (NDUFS1, ACO2, OGDH, UQCRC2, and IDH3B) interact functionally with each other and are co-expressed in varicocele patients. Underexpression of Complex-III of the ETC (Cytochrome bc I complex subunit) in varicocele condition indicates hypoxia-induced oxidative stress [58]. Other ETC (NDUFS1: NADH-ubiquinone oxidoreductase 75 kDa subunit, UQCRC2: ubiquinol-Cytochrome C Reductase Core Protein 2 and COX5B: cytochrome C oxidase subunit 5B) and testis-specific protein PDH have been suggested as potential non-invasive biomarkers of mitochondrial dysfunction in varicocele patients [57].

Varicocele is encountered on the left side in 90% of unilateral varicocele cases [59]. A comparative proteomic study reported a total of 369 sperm proteins that are differentially expressed in fertile men and unilateral varicocele patients. The majority of these DEPs were involved in important cellular molecular functions including ion binding (44.85%), and oxidoreductase activity (13.65%); as well as biological processes such as small molecule metabolic process (43.73%), response to stress (32.87%), signal transduction (29.25%), and cellular protein modification process (20.33%) [60]. Moreover, altered expression of the sperm proteins impacted the molecular pathways, *via* PTM, free radical scavenging, protein ubiquitination, and mitochondrial dysfunction, which could then affect the normal physiological functions of spermatozoa. A profile of 29 proteins associated with reproductive functions (sperm maturation, motility, hyperactivation, capacitation, and acrosome reaction), which are essential for the fertilization process, were found to be altered in the spermatozoa of unilateral varicocele patients. Based on the coverage of peptides, nine proteins (CABYR: calcium binding tyrosine phosphorylation regulated, AKAP: A-Kinase anchoring protein 5, APOPA1: apolipoprotein A-I, SEMG1: semenogelin-1, ACR: acrosin, SPA17: sperm surface protein Sp17, RSPH1: radial spoke head 1 homolog, RSPH9: radial spoke head protein 9 homolog and DNAH17: dynein heavy chain 17) associated with

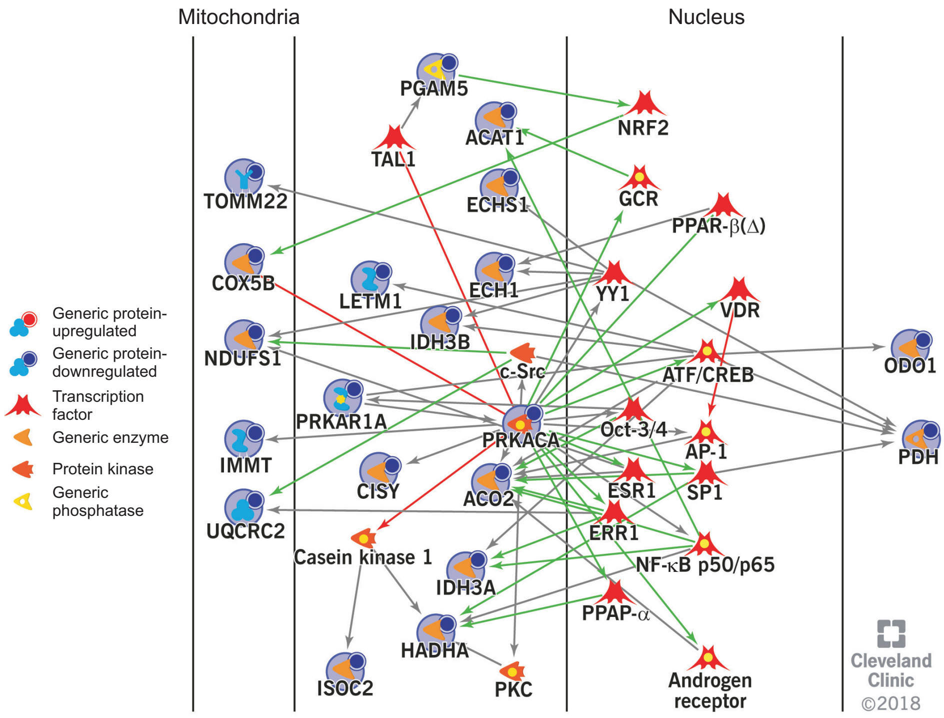

Fig. 4. Interaction between the differentially expressed proteins and transcriptional factors in varicocele patients with mitochondrial dysfunction. TOMM22: mitochondrial import receptor subunit TOM22 homolog, COX5B: cytochrome c oxidase subunit 5B, mitochondrial, NDUFS1: NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial, IMMT: NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial, UQCRC2: cytochrome b-c1 complex subunit 2, mitochondrial, PGAM5: serine/threonine-protein phosphatase PGAM5, mitochondrial, TAL1: T-cell acute lymphocytic leukemia protein 1, ACAT1: acetyl-CoA acetyltransferase, mitochondrial, ECHS1: enoyl-CoA hydratase, mitochondrial, ECH1: delta(3,5)-delta(2,4)-dienoyl-CoA isomerase, mitochondrial, LETM1: mitochondrial proton/calcium exchanger protein, IDH3B: isocitrate dehydrogenase [NAD] subunit beta, mitochondrial, c-Src: proto-oncogene tyrosine-protein kinase Src, PRKAR1A: cAMP-dependent protein kinase type I-alpha regulatory subunit, PRKACA: cAMP-dependent protein kinase catalytic subunit alpha, CISY: citrate synthase, mitochondrial, ACO2: aconitate hydratase, mitochondrial, IDH3A: isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial, HADHA: trifunctional enzyme subunit alpha, mitochondrial, ISOC2: isochorismatase domain-containing protein 2, PKC: protein kinase C, NRF2: nuclear factor erythroid 2 (NFE2)-related factor 2, GCR: glucocorticoid receptor, PPAR-β (Δ): peroxisome proliferator-activated receptor beta or delta, YY1: Yin Yang 1, VDR: vitamin D receptor, ATF: activating transcription factor, CREB: cAMP response element binding, OCT-3/4: octamer-binding transcription factor 3/4, AP-1: activator protein 1, SP1: specificity protein 1, ESR1: estrogen receptor 1, ERR1: steroid hormone receptor ERR1, NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells.

the fertilization potential of spermatozoa were identified as potential biomarkers for unilateral varicocele patients [60].

Agarwal et al [61] demonstrated the differences in the sperm proteome profile of bilateral varicocele patients and fertile men. The sperm proteome profile was able to decipher the subcellular role of proteins responsible for infertility associated with bilateral varicocele. In total, 73 proteins were differentially expressed in these patients. Absence of APOA1, under expression of mitochondrial import receptor subunit TOM22 homolog (TOM22) and over expression of protein-glutamine gamma-glutamyl transferase 4 (TGM4) were associated with the molecular pathological changes related particularly to oxidative stress and SDF [61]. Also, proteins linked with the reproductive function (such as ODF2: Outer dense fiber protein 2; TEKT3: Tektin-3; TCP11: T-complex protein 11 homolog; CLGN: Calmegin) were aberrantly expressed in bilateral varicocele patients, thus affecting the fertilization potential of the sperm. Differential expression of sperm proteins ENKUR: enkurin, SEMG1, SEMG2: semenogelin-1, SPAM1: sperm adhesion molecule 1 and CABYR are indicators of poor semen quality in bilateral varicocele patients [60].

Semen quality is more severely compromised in bilateral varicocele patients compared to that of unilateral varicocele patients. Comparative protein profiling was able to address the pathophysiology associated with the extensive damage caused by bilateral varicocele [60]. The 253 DEPs identified between the unilateral and

**Table 1.** Sperm and seminal plasma protein biomarkers in varicocele patients

| Function | Sample | Potential biomarker | Study |
|---|---|---|---|
| Fertilization | Sperm | APOPA1, ACR, SPA17, TGM4, HIST1H2BA | Agarwal et al (2015) [63] |
| | | CRISP2, CALGN, SPAM1 | Agarwal et al (2015) [60] |
| | | TCP11 | Agarwal et al (2016) [61] |
| | | HSPA2 | Agarwal et al (2016) [58] |
| | | TALDO1, HIST1H2B, GNPDA1 | Selvam et al (2017) [62] |
| | Seminal plasma | PSA | Zylbersztejn et al (2013) [25] |
| | | ACR | Panner Selvam et al (2019) [65] |
| Motility | Sperm | CABYR, AKAP3, SEMG1, DNAH17, ODF2 | Agarwal et al (2015) [63] |
| | | TEKT3 | Agarwal et al (2016) [61] |
| | | AK7 | Agarwal et al (2016) [58] |
| Morphology | Sperm | RSPH1, RSPH9 | Agarwal et al (2015) [63] |
| | | SPANXB1 | Agarwal et al (2015) [60] |
| | | CCT6B | Agarwal et al (2016) [58] |
| DNA damage | Sperm | HSP90AB1, PPP5C, RUVLB | Selvam et al (2017) [62] |
| | Seminal plasma | DNASE1 | Belardin et al (2016) [26] |
| | | BCL2 and BAX | Mostafa et al (2014) [67] |
| | | SMG1, IGFBP-3 | Zylbersztejn et al (2013) [25] |
| | | FASN | Panner Selvam et al (2019) [66] |
| | | APOA2 | Panner Selvam et al (2019) [65] |
| Oxidative stress | Sperm | PARK7 | Agarwal et al (2015) [63] |
| | | SOD1 | Hosseinifar et al (2014) [54] |
| | Seminal plasma | NELFE | Del Giudice et al (2013) [64] |
| | | PRDX2 | Panner Selvam et al (2019) [65] |
| Mitochondrial dysfunction | Sperm | NDUFS1, UQCRC2, COX5B, PDH | Samanta et al (2018) [57] |
| | | PKAR1A, AK7, CCT6B, HSPA2, ODF2 | Agarwal et al (2016) [58] |
| | | DLD | Agarwal et al (2015) [63] |
| | | ATP5D | Hosseinifar et al (2014) [54] |
| | | SDHA, PRDX1, GSHR | Selvam et al (2017) [62] |

bilateral varicocele were involved in metabolism, apoptosis and signal transduction pathways. Dysregulation of sperm functions (capacitation, hyperactivation, and acrosome reaction) and reproductive functions (zona pellucida binding and fertilization) in bilateral varicocele patients were more pronounced due to differential expression of proteins, such as GSTM3: glutathione S-transferase Mu 3, SPANX1: sperm protein associated with nucleus X chromosome, CYB5R2: cytochrome B5 reductase 2, CALGN: calmegin and PARK7 (also known as DJ-1 proteins) [60]. The majority of these DEPs (>50% of proteins) were involved in the acetylation process and suggest that the downregulation of proteasome complex proteins is a predisposing factor for increased DNA damage in bilateral varicocele patients [62]. Acetylation-associated proteins involved in fertilization and acrosome reaction (TALDO1: transaldolase 1, HIST1H2B: histone cluster 1 H2B family member B, GNPDA1: glucosamine-6-phosphate isomerase 1), apoptosis and DNA damage (HSP90AB1: heat shock protein 90 alpha family class B member 1, PPP5C: protein phosphatase 5 catalytic subunit, RUVBL: RuvB-like proteins), mitochondrial dysfunction and oxidative stress (SDHA: succinate dehydrogenase, PRDX1: peroxiredoxin 1 and GSHR: glutathione reductase) have been proposed as post-translational protein biomarkers in varicocele patients [62].

The list of potential sperm biomarkers in varicocele patients based on fertilization, motility and morphology, DNA damage, oxidative stress, and mitochondrial dysfunction are presented in Table 1 [25,26,54,57,58,60-67].

## VARICOCELE AND SEMINAL PLASMA PROTEOMICS

In addition to sperm proteins, the seminal plasma proteome also plays a key role in determining the fertilization capacity of the sperm [23]. The seminal plasma serves as a potential source for protein biomarkers, as the DEPs involved in the pathophysiology of male infertility can be used as predictive biomarkers for its diagnosis [17]. The first report on seminal plasma proteomics in adult varicocele patients using the 2D gel electrophoresis (2D SDS-PAGE) technique was published in 2012 [68]. The study reported of 20 proteins that were differentially expressed in the seminal plasma of cigarette smoking adult varicocele patients. Moreover, proteins involved in the inflammatory response, proteolysis and regulation of apoptosis, sperm maturation and sperm-oocyte fusion were dysregulated in these patients [68]. Nitric oxide metabolism and tetratricopeptide repeat domain-binding functions were also more enhanced in adult varicocele patients [24]. These alterations in the seminal plasma proteome mark the deleterious effects of varicocele on semen quality and the functional integrity of sperm in adult males.

Varicocele occurs with a prevalence of 6% to 26% in adolescents [69]. In adolescents with varicocele having poor semen quality, the seminal plasma proteins associated with normal physiological function of spermatozoa are differentially expressed. For example, proteins associated with sperm motility and capacitation such as SEMG I and PSA, were found to be overexpressed and underexpressed respectively in the seminal plasma of adolescent varicocele patients [25]. Belardin et al [26] reported that the proliferative or apoptotic equilibrium of seminal plasma is altered in varicocele patients. Insulin-like growth factor-binding protein 7 (IGFBP7) associated with the proliferative process was overexpressed, whereas deoxyribonuclease-1 (DNASE1) involved in the regulation of apoptosis was underexpressed in the seminal plasma of adolescents with varicocele [26]. Furthermore, proteomic analysis by the same group revealed that seminal plasma was enriched with immune response proteins leading to a chronic inflammatory reaction in adolescents with varicocele [26]. These changes reflect the alterations in testicular functions leading to decreased semen quality in adolescents with varicocele.

A recently published study on seminal plasma proteomics in varicocele patients discusses in detail the functional pathways affected in varicocele patients. A total of 486 proteins were detected in the varicocele patients. Proteins associated with molecular pathways such as response to oxidative stress (PRDX1 and PRDX2) and sperm-oocyte interaction (CCT4 and CCT8) are dysregulated in the seminal plasma of varicocele patients. PRDX2, HSPA2 and APOA2 were proposed as potential biomarkers to understand the molecular pathology associated with varicocele mediated male infertility [65]. Another proteomic report in seminal plasma demonstrated that inflammatory response pathways were dysregulated, especially interleukin 6 signaling and Janus kinase signal transducer and activator of transcription (Jak-STAT) pathways, in bilateral varicocele patients [66].

A drastic change in the expression profile of proteins has been observed in the seminal plasma of post-varicocelectomy patients. The proteome profile of seminal plasma in post-varicocelectomy men revealed 38 proteins to be uniquely expressed. Molecular pathways such as response to oxidative stress, gluconeogenesis and protein stabilization were enriched in post-varicocelectomy patients. Overexpression of DJ-1: parkinsonism associated deglycase, S100-A9: S100 calcium binding protein A9, SOD: superoxide dismutase 1, ANXA1: annexin A1, G3P: glyceraldehyde-3-phosphate dehydrogenase, and MDH: malate dehydrogenase in seminal plasma could help retain the homeostasis post-varicocelectomy [24]. Decreased expression of negative elongation factor E (NELFE) indicates a decreased state of oxidative stress, whereas increased expression of transglutaminase-4 suggests (TGM4) that the sperm-binding activity was retained in post-varicocelectomy patients [25]. The same group of investigators evaluated the seminal plasma proteins in 25 varicocele patients before and after varicocelectomy. Receiver operating characteristic curve analysis with area under curve of 84.5% ($p=0.014$) predicted the tripeptidyl peptidase-1 (TPP1) protein as a positive outcome predictor for varicocelectomy in patients. Expression of TPP1 protein had increased to three-folds in the seminal plasma of men with positive outcome of varicocelectomy when compared to patients that had failed to show any improvement in semen parameters post-varicocelectomy [47].

Several other proteomic studies have been conducted to identify noninvasive biomarkers for the diagnosis of varicocele associated male infertility. Expression of apoptotic markers B-cell lymphoma 2 protein (BCL2) and BCL2-Associated X Protein (BAX) were decreased and increased, respectively, in the seminal plasma of varicocele patients. BAX has been negatively correlated with sperm concentration, motility and normal sperm morphology [67].

## FUTURE OF PROTEOMICS

Rapid progress in the proteomic (LC-MS/MS, MALDI-TOF) techniques over the last five years has geared the ’omics research to validate the proteins as potential diagnostic and therapeutic biomarkers for male infertility. Initially, several challenges were faced in sperm and seminal plasma proteomics pertaining to the complexity of the sample, processing of sample for mass spectrometry analysis, quantification of proteins and identification of PTMs [70]. However, simplification of the proteomic techniques by employing protein enrichment strategies and targeted proteomic approach facilitated the detection of the low abundance proteins and PTMs (glycosylation, phosphorylation, acetylation, and methylation) effectively in sperm and seminal plasma.

One of the major limitations of sperm proteomic studies is the complexity of the semen sample which is highly heterogenous. The presence of abundant proteins in the seminal plasma (such as SMEGs) tend to mask the detection of other low abundant proteins which may have a vital role in the spermatozoa [24,43]. To address these issues, better analytical and enrichment procedures must be adapted for accurate detection of low abundant proteins present in the semen sample [71,72]. Apart from sperm and seminal plasma protein studies, the focus has currently shifted to understand the physiological function of the exosomes. In 2017, Yang et al [73] profiled the seminal exosomal proteins in fertile donors. Exosomal proteins were associated with protein metabolism, energy pathway and transport. Differential screening of exosomal proteins in infertile male patients may serve as a potential biomarker in assessing the functional status of exosomes in seminal plasma. Recently, we have identified the alterations in seminal plasma proteins associated with exosome functions in varicocele patients. Exosome-associated proteins ANXA2 and KIF5B may serve as potential protein biomarkers of exosomal dysfunction and exosome-mediated infertility in varicocele patients [74].

Proteomics is technology-driven field that rely on bioinformatic analysis. Advancement in computational tools and user compatible data analysis tools such as IPA, Metacore, Cytoscape, and Reactome make the interpretation of results more versatile and feasible. Implementation of these techniques into a clinical set up depends on the powerful meta-analysis of biomarker validation results. It is anticipated that the future of male infertility diagnostics and therapeutics depends on the effective integrated analysis of all the ‘omics (genomic, proteomic, and metabolomic) data to identify accurate and reliable biomarkers for a specific infertility condition.

## CONCLUSIONS

Besides the advanced tests performed to determine oxidative stress and DNA fragmentation, molecular biomarkers can be promising in the non-invasive diagnosis of various pathologies associated with male infertility. Proteomics must be considered as a complementary ‘omics’ tool to investigate the biomarkers of male infertility. Although the proteomics result seems promising, validation of the biomarkers in larger sample sizes using western blot or ELISA will definitely strengthen the significance of these findings. In depth omics studies on seminal exosomes, can help in developing new diagnostics and therapeutic strategies for treating exosome dysfunction in infertile men with varicocele.

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### Conflict of Interest

The authors have nothing to disclose.

### Author Contribution

Conceptualization: all authors. Data curation: MKPS. Formal analysis: all authors. Supervision: AA. Writing–original draft: MKPS. Writing–review & editing: all authors.

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