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The use of genomics, proteomics and metabolomics in identifying biomarkers of male infertility

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Abstract

Despite the fact that male factors contribute to approximately 50% of all infertility, the mechanisms underlying their origin are unknown. Currently, clinicians rely primarily on semen analyses to predict male reproductive potential and chart treatment success. Even when invasive procedures are performed, the causes of male infertility frequently remain elusive. Recently, the advent of new technologies has spurred the search for novel male infertility biomarkers, and the detection of genes, proteins or metabolites unique to the infertile male holds much promise. The concept that a cost-effective, non-invasive and accurate set of biomarkers can be identified to diagnose male infertility is tantalizing. This review focuses on the various methodologies employed in the discovery of novel biomarkers along with their findings. Specific attention is paid to recent advances in the fields of genetics, proteomics and metabolomics.

Keywords

male infertility; proteomics; genomics; biomarker

INTRODUCTION

Infertility affects ~15% of all couples with a male factor involved in ~50% (1–3). The complex nature of the condition is underscored by the fact that each individual contributes a complex array of genetic, proteomic, and metabolic differences that interact in unpredictable ways. The frustration that couples face is compounded by difficulties that clinicians have in both diagnosing and treating infertility, particularly in males. Indeed, many causes of male infertility are still defined as idiopathic (1, 3) and as such, most diagnoses tend to be descriptive rather than specific. For example, the simple classification of azoospermia into obstructive (OA) or non-obstructive (NOA) simply delineates a physical blockage from testicular failure. However, a more accurate taxonomy is not possible when the very origins of the condition are unknown.

Infertility is defined as the inability to conceive after 12 months of regular, unprotected intercourse (4). Clinical investigations should not begin until this amount of time has

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elapsed; with several exceptions including advanced maternal age. Once inquiry begins, a history and physical exam are performed and specific male factors addressed via semen analysis, karyotyping, Y-microdeletion analysis and endocrine profiling. Unfortunately, for many men, these tests are normal or inconclusive, leading to a diagnosis of idiopathic infertility. A definitive diagnosis must then be pursued via surgical intervention, in the form of a testicular biopsy, which carries with it inherent complications. As a result, the identification of alternative, non-invasive methods to diagnose specific etiologies of male infertility are essential.

The use of novel genetic, proteomic and metabolomic techniques may hold the key to more accurately diagnosing and treating male infertility. It is within these categories that the search for potential biomarkers can begin (5). A biomarker is a distinctive biological or biologically-derived indicator of a process, event or condition that can be objectively measured, evaluated and compared (6). The ideal biomarker should identify disease at an early stage, be easily detectable, cost-effective, and accurate while having minimal side effects. The discovery of such non-invasive, highly sensitive and specific biomarkers would be helpful in eliminating the need for invasive testing in the infertile male while allowing an expanded and more specific classification of male infertility (7, 8).

For the purposes of this review, the field of male fertility-related biomarkers is classified into several areas. First, basic laboratory analyses including semen analysis, endocrine laboratory investigations and antisperm antibodies (ASA) currently being used to assist clinicians in categorizing, diagnosing and treating male infertility are discussed. This is followed by a summary of the current knowledge underlying state-of-the-art genomics, proteomics, and metabolomics. The difficulty involved with bringing these biomarkers from the bench to the bedside is also discussed. While these areas are evolving and currently investigational, they have the potential to re-invent how male infertility is diagnosed and treated.

BASIC BIOMARKERS

A biomarker is any biological molecule that is measured and evaluated objectively and functions as an indicator of a physiological pathway (6). In the context of male infertility, biomarkers have the intent of evaluating, in an accurate and minimally invasive manner, a male's potential for fathering children. Currently, the most widely used biomarker to predict male fertility potential is the semen analysis.

Semen is a complex fluid comprised of a cellular element (i.e. spermatozoa) as well as a plasma element that functions as a nourishing and protective medium for spermatozoa (9). Semen is primarily derived from the seminal vesicles (65–75%) and prostate (25–30%), and its multi-focal origin makes the search for biomarkers more difficult (9). Indeed, while semen analysis yields basic, yet critical information, it is subject to significant modulation and is an overall poor predictor of male fertility (10). Several physiological factors (i.e. duration between ejaculates) as well as pathologies (i.e. diabetes, sarcoidosis (11)), systemic illnesses (i.e. flu) and environmental factors (i.e. smoking, alcohol) result in significant variability amongst semen samples (10). This leads to difficulties in the interpretation and management of males with abnormal findings (10).

To provide further utility to semen analysis, the components are broken down into macroscopic and microscopic factors (12). Coagulation, color, viscosity, pH and volume are classified as macroscopic components while agglutination, sperm counts and concentration, motility, morphology and viability are microscopic (12). Sperm counts and concentration are primarily useful in the classification of male infertility into either azoospermic (absence of sperm) or oligospermic (<15 million sperm/mL) categories (12). Morphology is also an

important component of the semen analysis; however, its utility in determining male fertility potential is unclear.

While semen analysis is useful as a biomarker (13, 14), it is altered by a wide variety of environmental factors (15) including air pollution (16) and ozone levels (17). Organochlorines such as polychlorinated biphenyls (PCBs) used in motor oils and dichlorodiphenyltrichloroethane (DDT) used in pesticides have long half-lives that affect sperm counts and motility for years following exposure (18, 19). Phthalates present in industrial chemicals have also shown an inverse correlation with sperm motility, morphology and concentration (20–22). Further complicating the interpretation of a semen analysis are lifestyle factors such as cigarette smoking, alcohol consumption, psychological stress and caffeine, all of which negatively affect semen parameters (14). As such, while semen analysis is currently the main biomarker for male infertility used today, it is also the most unreliable (23, 24).

Antisperm antibodies (ASA) were one of the first biomarkers used in widespread clinical practice. These anitibodies develop after accidental or iatrogenic breach of the blood-testis barrier (i.e. due to torsion or trauma), accurately predict obstructive azoospermia (OA) (25) and affect fertility by numerous mechanisms, including altered sperm agglutination and cervical mucus penetration (26). No association has been found between ASA and semen quality (27), although infectious diseases like the human papillomavirus may alter both (28). Unfortunately, ASA do not correlate with spontaneous, *in-vitro* fertilization (IVF) or intra-cytoplasmic sperm injection (ICSI) pregnancy rates (29, 30) casting doubts on their importance as a biomarker.

The acrosome reaction has also been explored in the search for a biomarker of male infertility. Contained within the distal end of the sperm head, the acrosome contains the enzymes required for oocyte penetration (12). The Optimized Sperm Penetration Assay (SPA; zona-free hamster oocyte penetration assay) (31, 32) collectively assesses the acrosome reaction, sperm capacitation, and the ability of sperm to penetrate ova and undergo the initial steps of sperm head decondensation and fertilization. The SPA has a high positive predictive value; if positive, there is a high likelihood the sperm will fertilize an ova in IVF (assuming the sperm can penetrate the human zona pellucida) (31–33). For many years, the SPA was used to predict the fertilizing potential of sperm during IVF, but with the advent of ICSI, this was no longer a concern since the steps of acrosome reaction, capacitation, sperm zona- and ova membrane binding and penetration were bypassed (5, 34, 35). Nevertheless, the use of ICSI-SPA can accurately predict those patients whose sperm will fail to undergo decondensation in the ovum after ICSI, resulting in fertilization failure (34, 35).

GENOMIC BIOMARKERS

As technology has advanced, the approach to the treatment and diagnosis of male infertility has rapidly evolved. Indeed, moving beyond semen analysis to genetic testing is a part of this evolution. Recent estimates indicate that genetic abnormalities cause 15–30% of male factor infertility (36) and it is likely that this number will further increase as more genetic causes of infertility are identified. While genetic abnormalities were previously detectable only in the form of large structural chromosomal aberrations defined via karyotype analysis, much smaller genomic regions have been found to be responsible for infertility (37–40). In the near future, it is likely that single nucleotide changes leading to infertility will be identified, leading to more male infertility biomarkers (37).

Currently, one of the most common genetic tests used to evaluate the status of the infertile male is a karyotype analysis. Karyotyping was first utilized in 1959 to determine the genetic composition of Turner's (a single X and an absent Y chromosome; XO) as well Klinefelter's

(two X and one Y chromosome; XXY) syndromes (41, 42). With the use of light microscopy to evaluate the appearance of chromosomes, karyotyping resolves variations in the DNA complement of ≥4 megabases (Mb).

With the use of the karyotype, initial reports suggested that ~2% of infertile men had chromosomal abnormalities, a rate 5 times higher than the normal population (43). Karyotypic anomalies vary with the severity of oligo- or azoospermia and a higher incidence of aberrations have been identified in azoospermic (18.71%) versus severely (14.55%) or moderately (2.37%) oligozoospermic men (44). With regards to specific karyotypic defects, Robertsonian translocation occurs in 1.5% of oligozoospermic and 0.2% of azoospermic men (45, 46), whereas reciprocal translocations occur in 0.7% of oligo- or azoospermic men (47). Other abnormalities that may occur in the infertile male include inversions, which are typically benign, and a 46, XX genotype, which does not produce sperm (48).

The concept that the Y chromosome determines maleness was initially put forth in 1976 by Tiepolo and Zuffardi (49). The Y chromosome was cloned in 1992 (50) and the first genetic marker of infertility, the Azoospermia Factor (AZF), was identified within its confines shortly thereafter (39, 40). These initial studies revealed that microdeletions in the Y chromosome (AZFa, b, and c) could act as biomarkers for male infertility. Indeed, the presence of sperm was dependent on which specific AZF sub-region was altered. For example, sperm were unlikely to be found in men with AZFa or AZFb deletions whereas men with AZFc deletions had a 75% chance of having sperm identified on testicular biopsy (51).

Since the identification of the Y chromosome's role in fertility, numerous other genes involved in male infertility have been identified (5, 52). The majority of these are located on autosomes and one of these, the cystic fibrosis transmembrane regulator (CFTR) gene, has become important in the evaluation of men with infertility in general, and congenital absence of the vas deferens (CBAVD) in particular (53). While the full genetic complexity of this biomarker has yet to be elucidated, there are currently >1000 known CFTR mutations that have yet to be explored (54).

Higher-resolution assessment of a sperm's genetic complement can be achieved using fluorescence in situ hybridization (FISH), a technique that utilizes fluorescently labeled DNA to identify specific genetic sequences (55). While not a first line investigation in the United States, sperm-FISH is used in cases of severe teratospermia or oligoasthenoteratospermia. Moreover, in situations of recurrent pregnancy loss, it defines meiotic defects in the form of aneuploid sperm (56). Its primary disadvantage as an investigative tool is that the sequence of interest must be known prior to determining whether a region of interest exists in a specific patient.

Microarray technologies, which evaluate men for copy number variations (CNV), gene expression levels and single nucleotide polymorphisms (SNPs) hold great promise for identifying highly sensitive and specific genetic biomarkers. Comparative genomic hybridization (CGH), a technique used to assess the relative quantities of DNA between samples, permits determination of gene copy number as a function of chromosomal location and can be applied to the entire genome using a microarray-based approach (array CGH). This approach was used in children with mental retardation, demonstrating that 10–20% have chromosomal abnormalities – a stark difference to the 3–5% detected using standard karyotype analysis (57). In the setting of male infertility, array CGH has identified Y-chromosomal microdeletions as well as additional CNVs outside the known AZF regions including CNVs in the PseudoAutosomal Regions (PARs) of the Y chromosome (specifically in *SHort stature HOmeoboX [SHOX]* gene in PAR1) (58, 59). Additional

candidate infertility genes have been identified using array CGH, although their roles remain to be elucidated (52, 60).

Microarray-based SNP analysis has also yielded numerous male fertility gene candidates and potential biomarkers. A recent genome-wide association study of family size and birth rates among 269 men evaluating ~250,000 SNPs revealed 41 SNPs with strong association to infertility and nine SNPs related to reduced sperm quality and function (61). The study implicated the genes *USP8, UBD, EPSTI1*, and *LRRC32* in male infertility, highlighting potential biomarkers (61). Other genes with SNPs associated in male fertility include: *PDE3A, EFCAB4B, COBL, ATP8A1, MASP1, PROK2* (62), *AHRR* (46, 47), *MTHFR* (58–60) and *UBE2B*, a homolog of the DNA repair gene *rad6* in mice, which results in male infertility when knocked out (61). The genes associated with these changes are listed in Table 1.

Variations in the sperm epigenome may also contribute to male fertility via DNA protamination and methylation. Improper post-translational processing of protamine 2 (PRM2), one of the two proteins that replace histones in sperm, along with altered protamine expression, resulted in decreased sperm counts and function (63–66). In addition, lower levels of PRM1 and PRM2 mRNA were observed in asthenozoospermic men (67). In men with altered protamination, abnormal methylation of the *CREM* gene has also been observed (68). Methylation of genomic DNA, which regulates the accessibility of the DNA to histone binding, has also been shown to affect fertility. Indeed, DNA methylation studies in oligozoospermic men demonstrated altered methylation of imprinted genes in general (69–71) and *IGF2/H19* and *MEST* in particular (72, 73).

Sperm have been traditionally thought of as DNA storage and transport vehicles. However, the RNA complement of sperm, including both coding and non-coding RNAs, may also play a role in male infertility (74, 75). Full-length mRNAs and non-coding RNAs have been recently identified in sperm cytoplasm and play functional roles in fertilization and early zygote development (74, 75). Indeed, *TSPY1, CLU, PRM2, PSG1, HLA-E,* and *PLCD1* transcripts were identified and theorized to be potential biomarkers (74, 76), as highlighted in Table 1. Microarray technologies have enabled rapid evaluation of spermatozoal RNAs and studies using these techniques have identified mRNA patterns that correlate with spermatogenesis, sperm motility, germ cell anti-apoptotic processes, DNA repair, oxidative stress reduction and histone modification (77–79).

Small non-coding RNAs (sncRNAs) produced during the process of spermatogenesis and localized in spermatozoa are theorized to contribute to early embryonic function; however, their exact nature and classification remain controversial (75, 80, 81). Approximately 24,000 sncRNAs are believed to exist, with the best described being within the microRNA (miRNA) family that was identified in 2005 (82). A recent study by Krawetz *et al.* (75) found multiple classes of sncRNAs to be present in human spermatozoa. By examining small RNAs (<200 bases) isolated from three fertile donors, bioinformatics revealed human spermatozoa to contain miRNAs (~7%), *piwi*-interacting piRNAs (~17%), and repeat-associated small RNAs (~65%) (75). A small subset that framed the histone promoter-associated regions comprised ~11% and were termed quiescent RNAs (~11%) (75). When mouse oocyte and one-cell zygotes were compared, several paternally localized miRNAs were identified including *hsa-mir-34c*, *hsa-mir-375*, *hsa-mir-252*, and *hsa-mir-25* (75). The authors proposed that *hsa-mir-34c* was of particular interest owing to its place in a group of highly conserved miRNAs recently described to play a critical role during male spermatogenesis (75, 83). Interestingly, examination of the mRNA profiles of sperm after ICSI has found significant differences in the mRNA profiles from sperm that produced a viable pregnancy compared to those that did not (78). Specifically, sperm that resulted in

pregnancies had increased levels of 44 transcripts, yielding a panel of potential biomarkers including several cathepsins and metallothioneins, *ADD1, ACVRL1, AR*, and *ARNT* (Table 1) (78). It is important to note, however, that since not all RNA is translated, protein expression and its complement (see next section) also need to be examined.

The various genetic markers described above detail only a fraction of the genes, CNVs, SNPs and RNAs that may become important as biomarkers in the future. Thus, while the studies summarized provide insight into potential genetic biomarkers, many require validation and further experimentation.

PROTEOMIC BIOMARKERS

Proteomics joins the words “protein” and “genome” and is the study of both the structure and function of proteins with the goal of examining the total expressed protein complement of the genome, including modifications (5, 84, 85). This concept is critical, since not all RNA is translated and gene expression can differ from protein expression, particularly under different physiological states (i.e. OA versus NOA) (86–88). Indeed, proteomics is arguably more complex when compared to genomics, given that while a genome is relatively constant between cell types and organisms, the proteome differs from cell to cell as well as temporally.

Since distinct genes are expressed in a cell-dependent manner, and mRNA is not always translated to protein, some suggest that proteomic analyses has the potential to yield highly accurate biomarkers (9, 13, 86, 88). Currently, studies focused on proteomics in infertile men are in their infancy (5) but hold much promise. Indeed, analysis of semen has already produced several novel potential biomarkers for prostate cancer (89, 90). With respect to male infertility, recent work by Batruch *et al.* (91) compared the proteomes of pooled seminal plasma in men post-vasectomy to control men without vasectomy. Since men post-vasectomy lack testicular and epididymal effluent, the 32 unique proteins identified in control men were theorized to originate from these regions, serving as candidate biomarkers (91).

Clinicians currently rely on semen analysis for the diagnosis of male infertility, although semen analysis alone is usually insufficient for diagnosis. Proteomic analysis can confirm the presence of a given protein, measure its quantity and detect activated, phosphorylated, ubiquitinated, methylated, acetylated, glycosylated, oxidated and/or nitrosylated protein forms (9, 13, 86, 88). Given these variables, it is easy to see how proteomic technology can be a powerful tool in identifying potential biomarkers for both diagnosis and treatment (88). Indeed, using proteomics to measure changes in global protein levels while monitoring specific protein interactions, heralds a new approach to the diagnosis of male infertility (84, 85). The challenge in identifying ideal biomarkers will be to distinguish relevant proteins from their chemically modified forms within the setting of such a complex milieu.

The use of semen for proteomic analysis is complicated by the fact that it contains both sperm as well as seminal plasma. The seminal plasma is composed of products from the prostate, seminal vesicles and bulbourethral glands (9) and offers sperm a nutritional environment while assisting in capacitation, sperm-zona pellucida interaction and sperm-egg fusion (88, 92, 93). Unfortunately, proteomic analysis of semen is complex given the variations between and within individuals as well as seasonal and age-related fluctuations (94, 95). Sperm also undergo distinct physiological changes following ejaculation that further complicate proteomic analysis. For example, different proteins are activated at distinct phases of coagulation and liquefaction during fertilization (9). Following ejaculation, the semen coagulates and approximately 30 minutes later, liquefies via the release of proteolytic enzymes (9). Several factors regulate this process including

semenogelins 1 and 2 (9, 96) which undergo proteolytic digestion by PSA, causing liquefaction of the semen coagulum and release of motile spermatozoa (9). All of these complicating aspects serve to make the identification of a common set of proteins a challenge.

Most proteomic investigations to date targeted the seminal plasma, which comprises ~90% of the semen and is the supernatant remaining after centrifugation of semen (9). The search for biomarkers in seminal plasma began in the 1940s (97, 98) with advances being limited by the technology available. In the 1980's, one of the first proteins found to be secreted by human Sertoli cells, transferrin, was also the first protein with binding sites identified on germ cells (99). The discovery that transferrin concentrations within the semen of NOA and oligospermic patients were significantly lower than controls pointed to a critical role in male fertility (99–101). While small gains were made in the intervening decades (102, 103), one of the first modern characterizations of pooled seminal plasma from healthy individuals was made in 2004 (104). In that study, 42 distinct proteins were identified including PSA, prostatic acid phosphatase and semenogelin (104). This list of potential biomarkers was expanded in 2006 by Pilch and Mann (90) who identified 923 unique seminal plasma proteins in a single individual. The most abundant proteins identified included fibronectin, lactoferrin, laminin, albumin as well as semenogelin 1 and 2 (90). The most prevalent molecular function of the identified proteins (~60%) was in the realm of catalytic enzymatic activity (90).

Another subset of proteins which have been theorized to act as male infertility biomarkers are the heparin-binding proteins (HBPs) (105). These glycosaminoglycans are potent enhancers of sperm capacitation in other animals (106). Within the seminal plasma, HBPs attach to the sperm surface, particularly to phosphoryl-choline-containing lipid groups, facilitating progress and capacitation in the female reproductive tract (105). Initial work reported purification of seven HBPs from human seminal plasma with the significant HBPs being lactoferrin, semenogelin 1 and 2, PSA, bovine seminal plasma-proteins, zinc finger protein and fibronectin (107). A more recent study using two-dimensional gel electrophoresis and mass spectrometry identified 40 proteins, including those previously mentioned (105, 107). Based on functional clustering, these proteins were suggested to be mostly involved with protein metabolism (38%), RNA processing/transcription (20%), cell transport/structure (18%) and signal transduction (16%) (105). Further work remains to better elucidate the value of HBPs as biomarkers of male infertility.

Prolactin inducible protein (PIP) is another component of seminal plasma which has been theorized to act as a biomarker for male infertility (108, 109). PIP was found to be one of eight proteins with increased levels of expression in azoospermic men (108). The others were: fibronectin, prostatic acid phosphatase, proteasome subunit alpha type-3, beta-2-microglobulin, galectin-3-binding protein, and cytosolic nonspecific dipeptidase (108). PIP is a 17 kDa glycoprotein known to be a marker of breast carcinoma (110) which has recently been found to interact and form complexes with human serum albumin (HSA) (109). HSA is an abundant seminal plasma protein found to have a role in capacitation while also functioning to improve sperm motility. As such, it is tempting to speculate that PIP, HSA and/or the PIP-HSA complex could serve as biomarkers. While previous studies have failed to show a correlation between PIP and fertility status (111), the ability of these proteins to affect immunoglobulins and immunoreactivity makes them tempting targets (108, 109, 111, 112).

More recent work by Milardi *et al.* (88) examined the seminal proteins of five fertile men who had all conceived within 3 months of providing a semen sample. The authors found between 919 to 1487 unique proteins within each individual with 83 present in all five

samples (88). Two proteins, human cationic antimicrobial protein (hCAP18) and spindlin1, were previously implicated in human reproduction (88). Present in the human epididymis and seminal plasma, hCAP18 has a key role in innate immunity and is closely associated with sperm (113, 114). It is thus tempting to speculate that hCAP18 may play a role in protecting against infection during fertilization (88, 113, 114). Spindlin1, is also involved with spermatogenesis and localizes to the tails of murine sperm, suggesting a role in sperm motility (88, 115). As such, both hCAP18 and spindlin1 are potential proteomic biomarkers.

In order to further classify proteins contained in semen, Batruch *et al.* (91) evaluated the seminal plasma of controls and men post-vasectomy. Those proteins most likely to be derived from the testes/epididymides were TEX101, PGK2, HIST1H2BA, SLC2A14, SPACA3, GAPDHS and AKAP4 (91). As such, these proteins are leading candidates for male infertility biomarkers (Table 1). Further work from the same group compared the seminal plasma proteomes of controls to men with NOA and identified two proteins, SPAG11B and TEX101, as key male fertility biomarkers (86). While both were elevated in normal controls and only SPAG11B was increased in men with NOA (86), setting the course for further investigations into this protein as a biomarker.

While the seminal plasma has been examined in the search for protein-based fertility biomarkers, the proteomes of spermatozoa have also been evaluated towards the goal of identifying contraceptive targets for drug development (116–118). These experiments are typically challenging given the limited amount of specimen involved (87). Very recent work in the field of biomarkers has begun to focus on the failure of sperm-egg recognition during fertilization. Redgrove *et al.* (119) recently identified a key role for heat shock protein 2 (HSPA2) in this process. HSPA2 was found to have significantly reduced expression when the proteomic profiles of sperm from fertile men were compared to those of men whose sperm exhibited impaired fertilization capacity (119). HSPA2, present in the sperm acrosome, was also noted to exist in close interaction with sperm adhesion molecule 1 (SPAM1) and arylsulfatase A (ASRA). As such, the authors proposed that SPAM1, ASRA and HSPA2 interacted together in a multimeric complex to mediate sperm-egg fusion (119). Any of these molecules could act as potential biomarkers of male fertility.

Moreover, recent reviews have highlighted four to six sperm surface antigens that may act as biomarkers of male fertility (116, 117). In addition, a study by Hao *et al.* (61) on sperm extracts and surface proteins identified several protein products with common peptide sequences. Interestingly, the authors found that these proteins were the product of a single gene - *SAMP32* (118). Moreover, this gene was found to have a testis-specific expression pattern and acrosomal localization, making it a very enticing potential biomarker for male infertility (118). In an excellent example of the use of proteomics in the identification of fertility-related proteins, SAMP32 (currently named SPACA1 or sperm acrosome membrane-associated protein), was recently found to code for a sperm membrane protein that functions in sperm-egg fusion (120) (Table 1). Fujihara and colleagues (120) then furthered this work with the creation of a SPACA1 murine knockout in which males have both abnormally shaped sperm heads (similar to human globozoospermia) as well as absent acrosomes (120). The pathway of discovery for the SPACA1 biomarker is prototypical for how proteomics can lead the way to novel innovations.

METABOLOMIC BIOMARKERS

The study of cellular metabolic products (metabolomics) as potential male fertility biomarkers is currently in its infancy and is the least advanced of the three topics covered in this review. Metabolomics is the study of small, low molecular weight molecular metabolites which are the products of metabolism (121, 122). The physiological functions of

these ~3000 molecules span a diverse range and include growth, development and reproduction (121). Metabolites can be either 'intrinsic', resulting from normal cellular physiology, or 'extrinisic', resulting from the influences of exogenously administered pharmaceuticals (121). Metabolomics reflects events downstream of gene expression and is considered to be closer to the actual phenotype than either proteomics or genomics (123–125).

In the search for male fertility biomarkers, metabolomics was initially focused on the changes seen in cases of oxidative stress. Due to excessive production and formation of reactive oxygen species (ROS) along with impaired antioxidant defense mechanism, oxidative stress results in spermatogenic abnormalities (122, 126). Previous studies have shown that oxidative stress markers (-CH, -NH, -OH, -SH) affect sperm and oocyte quality as well as embryo viability (123, 127). Indeed, ROS are elevated in a significant portion of semen samples from infertile men (122). While few studies examining the role of ROS as biomarkers exist, their importance is well recognized. One recent study identified the spontaneous generation of superoxide in spermatozoa and observed a negative correlation with sperm motility (128). Furthermore, seminal plasma levels of citrate, lactate and glycerylphosphorylcholine are altered in men with azoospermia compared to healthy controls, suggesting a possible involvement of ROS with infertility (129).

ROS are also generated by advanced glycosylation end products (AGEs) which accumulate in a wide variety of environments including the male reproductive tract (130–134). AGEs may cause a wide range of cellular effects and are key pathogenic initiators of diabetic complications (130). Sperm DNA is susceptible to attack from ROS due to its high unsaturated fatty acid composition (131). Indeed, sperm from men with type 1 diabetes have a 1.6 fold increase in fragmented sperm nuclear DNA (nDNA) (131). While most components of the semen analysis are similar between diabetics and non-diabetics, the most prominent AGE, carboxymethyl-lysine, has been shown to be significantly higher in the sperm of non-diabetics (133), suggesting a possible role in male infertility.

Other work examining the associations between small metabolite molecules in the testes has identified potential metabolomic alterations between type 1 diabetics and controls (135). For example, Mallidis *et al.* (135) examined the effects of diabetes on small molecular metabolites in a streptozotocin-induced diabetic mouse model. Diabetic mice exhibited decreased creatine, choline and carnitine levels along with increased lactate, alanine and myo-inositol (135). These studies provided support to the notion that diabetes effected changes in the metabolome of the testicle itself. Furthermore, considering that the decreases observed were in metabolites with antioxidant qualities, the authors (135) speculated a link between ROS and the metabolomic changes seen in the testes of diabetics – intriguing concepts which require further investigation.

Other work on metabolomics in the realm of infertility has focused on assisted reproductive technology (ART) by forging an association between ART outcomes and levels of ROS in follicular fluid and embryo culture media (122, 123). Indeed, a recent study examining follicular fluid samples and ROS has suggested a link between the metabolic markers of oxidative stress (-CH, -NH, -OH, and ROH) and pregnancy outcomes (122, 123). Previous *in-vitro* studies examining 35 embryos and their spent media noted different hydroxyl modifications in embryos which implanted successfully compared to those that did not (122). While further work is required, the field of metabolomics and its potential to contribute to identification of male fertility-related biomarkers is great.

BIOMARKER DEVELOPMENT

The identification of novel biomarkers is a laborious and demanding process. Given that a biomarker may be a gene, protein, or messenger RNA (136), the possibilities are nearly endless. Once a biomarker has been identified and shown to be relevant, it must be brought into the clinical realm. However, in spite of the multitude of biomarkers which have been recently discovered, progress moving from the bench to the bedside has been slow. The most significant advances in the field have come from the field of oncology (137). The ideal cancer biomarker would be detectable only in malignant tissue while eliciting a biological signal that is distinct from the surrounding normal tissues (137). One example of a biomarker used in oncology is prostate specific antigen, or PSA (137). The difficulties surrounding PSA are well known and significant given its inability to differentiate cancer from other conditions such as urinary tract infection and prostatitis. It is the existence of these types of issues that makes the search for an ideal biomarker so difficult.

Furthermore, gene and protein expression data has identified thousands of potential markers which are differentially expressed between benign and malignant tissues (137). This ‘information overload’, however, is only the start of the problem. The pathways of several candidate biomarkers are intrinsic to both normal and malignant cells, while some biomarkers exhibit such low levels they are clinically useless. The fact that a majority of cancers exhibit significant mutagenic heterogeneity has currently precluded a single biomarker from being identified in any cancer.

Challenges also exist in developing and bringing biomarkers to market once they have been scientifically validated (137). For example, the use of PCA3 in prostate cancer requires special handling, thus limiting dissemination of the assay (137). From a business point of view, the majority of the research in biomarkers is currently reported in the academic literature which exists in the public domain (137). The difficulty of establishing, and protecting, intellectual property is significant. If a test is eventually developed for a biomarker, it would require government approval which is expensive and time consuming. All of these factors, and many more, currently limit the ability of a biomarker to move from discovery to application.

CONCLUSION

Many causes of male infertility are unknown, related in large part to the lack of understanding of the molecular and genetic mechanisms responsible for fertility defects. However, many advances are currently being made at a rapidly increasing pace. While reliance on semen analysis for diagnosis is a significant limiting factor, the dawn of novel genomic, proteomic and metabolomic advances holds great promise. It is important to remember however, that while the molecular technologies continue to advanced, the clinical segment of the research must not be forgotten. Clinicians should be vigilant when seeing patients with male infertility and accurately document all previous medical and family history as well as phenotype. Awareness of the genetic underpinnings of male infertility will hopefully soon develop a combined molecular and clinical picture of the infertile male. The concepts, ideas and studies detailed in this review highlight the most meaningful advances in the fields of genetics, proteomics and metabolomics as they relate to male infertility.

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Abbreviations

AGEs	advanced glycosylation end products
ASA	antisperm antibodies
AZF	azoospermia factor
CGH	comparative genomic hybridization
CNV	copy number variation
COX	cyclo-oxygenase
HBPs	heparin-binding proteins
NOA	nonobstructive azoospermia
OA	obstructive azoospermia
ROS	reactive oxygen species
SNP	single nucleotide polymorphisms
sncRNAs	small non-coding RNAs
SPA	sperm penetration assay

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TABLE 1

Gene	Name	Location
AZF	Azoospermia Factor	Yq11
CFTR	Cystic Fibrosis Transmembrane Regulator	7q31.2
SHOX	Short Stature Homeobox	Xp22.33; Yp11.3
USP8	ubiquitin specific peptidase 8	15q21.2
UBD	Ubiquitin D	6p21.3
EPSTI1	Epithelial-Stromal Interaction 1	11q13.5-q14
LRRC32	leucine rich repeat containing 32	11q13.5-q14
PDE3A	Phosphodiesterase 3A	12p12
EFCAB4B	EF-hand calcium binding domain 4B	12p13.32
COBL	cordon-bleu WH2 repeat protein	7p12.1
ATP8A1	ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1	4p13
MASP1	mannan-binding lectin serine peptidase 1	3q27-q28
PROK2	prokineticin 2	3p13
AHRR	aryl-hydrocarbon receptor repressor	5p15.3
MTHFR	methylenetetrahydrofolate reductase	1p36.3
UBE2B	ubiquitin-conjugating enzyme E2B	5q31.1
CREM	cAMP responsive element modulator	10p11.21
TSPY1	testis specific protein, Y-linked 1	Yp11.2
CLU	clusterin	8p21-p12
PRM2	protamine 2	16p13.2
PSG1	pregnancy specific beta-1-glycoprotein 1	19q13.2
HLA-E	major histocompatibility complex, class I, E	6p21.3
PLCD1	phospholipase C, delta 1	3p22-p21.3
ADD1	adducin 1 (alpha)	4p16.3
ACVRL1	activin A receptor type II-like 1	12q13.13
AR	androgen receptor	Xq12
ARNT	aryl hydrocarbon receptor nuclear translocator	1q21
hCAP18	CAMP cathelicidin antimicrobial peptide	3p21.3
SPIN1	Spindlin 1	9q22.1
TEX101	testis expressed 101	19q13.31
PGK2	phosphoglycerate kinase 2	6p12.3
HIST1H2BA	histone cluster 1, H2ba	6p22.2
SLC2A14	solute carrier family 2 (facilitated glucose transporter), member 14	12p13.31
SPACA3	sperm acrosome associated 3	17q11.2
GAPDHS	glyceraldehyde-3-phosphate dehydrogenase, spermatogenic	19q13.12
AKAP4	A kinase (PRKA) anchor protein 4	Xp11.2
SPAG11B	sperm associated antigen 11B	8p23.1
SAMP32/SPACA1	sperm acrosome associated 1	6q15