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INVITED REVIEW

Male Fertility

Seminal biomarkers for the evaluation of male infertility

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For men struggling to conceive with their partners, diagnostic tools are limited and often consist of only a standard semen analysis. This baseline test serves as a crude estimation of male fertility, leaving patients and clinicians in need of additional diagnostic biomarkers. Seminal fluid contains the highest concentration of molecules from the male reproductive glands, therefore, this review focuses on current and novel seminal biomarkers in certain male infertility scenarios, including natural fertility, differentiating azoospermia etiologies, and predicting assisted reproductive technique success. Currently available tests include antisperm antibody assays, DNA fragmentation index, sperm fluorescence *in situ* hybridization, and other historical sperm functional tests. The poor diagnostic ability of current assays has led to continued efforts to find more predictive biomarkers. Emerging research in the fields of genomics, epigenetics, proteomics, transcriptomics, and metabolomics holds promise for the development of novel male infertility biomarkers. Seminal protein-based assays of TEX101, ECM1, and ACRV1 are already available or under final development for clinical use. Additional panels of DNA, RNA, proteins, or metabolites are being explored as we attempt to understand the pathophysiologic processes of male infertility. Future ventures will need to continue data integration and validation for the development of clinically useful infertility biomarkers to aid in male infertility diagnosis, treatment, and counseling.

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INTRODUCTION

A couple's inability to conceive after 1 year of regular unprotected intercourse clinically defines infertility, a condition which affects approximately 15% of the reproductive-aged population.^{1,2} A contributing male factor may be found in over half of cases with up to 40% of those being secondary to male factors alone.^{2,3} Male factor infertility is often characterized by abnormalities on semen analysis such as low or absent sperm counts and low motility. The diagnostic ability of available male investigative tools is limited, however, and likely underestimates the true prevalence of male factors in infertile couples.

Due to the high frequency of contributing male fertility problems, a thorough male evaluation should be performed early in a couple's investigation. The male fertility evaluation should include a thorough medical history and physical examination and at least two semen analyses (SAs), as recommended by the American Urological Association (AUA) best practice statement.⁴ While the history and physical examination may provide some clues, semen analyses serve as a baseline marker of male fecundity with data regarding sperm quantity and quality. Multiple SAs are necessary to establish a trend, as substantial variability may exist between samples secondary to biological and laboratory factors. Men should also be questioned carefully about possible confounding factors such as abstinence length, recent illnesses, or testicular heat exposure when interpreting semen analysis results.

Unfortunately, the “gold-standard” SA provides limited information and cannot discriminate fertile from infertile men on an individual basis. Widely overlapping ranges of semen parameters among these groups have left clinicians in search of better seminal biomarkers.⁵ The continued broadening of “normal” SA ranges by the World Health Organization (WHO) in its guidelines, now in their fifth iteration, will additionally classify more men who may be struggling to conceive with normal SA results and in search of alternative tests.^{6,7}

Advancing research technology and techniques have allowed us to explore new potential biomarkers in the rapidly evolving fields of genomics, proteomics, transcriptomics, and metabolomics, together comprising the current “omics” era. Researchers are employing these novel methods to gather vast amounts of data on possible novel biomarkers of male fertility from blood plasma, urine, and semen. As a result of stringent cellular barriers between blood and the male reproductive organs, cell-free DNA, RNA and proteins typically found in the testicles and epididymis are absent or barely detectable in blood serum, however, and only appear in concentrated amounts in the semen.⁸

Several clinical areas in the field of male fertility are primed for development of seminal biomarkers due to the lack of alternative testing or need for invasive diagnostic procedures. This review will focus specifically on available seminal testing and advancements in biomarker research in the areas of natural male fertility, differentiating

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azoospermia etiologies, and predicting assisted reproductive technique (ART) success. Previous reviews have reported on general male health and fertility-related biomarkers;^{8,9} this review will serve as an update to prior reviews with specific focus on seminal markers in the clinical areas discussed.

NATURAL MALE FERTILITY

The fertility evaluation is typically initiated for couples who have been trying to conceive naturally for some period of time without success. They should first undergo an evaluation to determine any barriers or available treatments for their best opportunity to conceive naturally. As mentioned, the initial laboratory investigation of male infertility includes at least two semen analyses to establish a trend in reproductive potential. Prior studies revealed substantial variability in seminal parameters between and within male patients with >70% variability between total motile sperm counts in some men.^{10–12} Most guidelines suggest an initial two SAs while others have recommended three tests to provide a better overview of fecundity due to the intrinsic variability.^{4,13} Men should be counseled regarding ideal collection guidelines including abstinence period, quick delivery to the laboratory, and proper transportation temperature to produce consistent and accurate results. Ideally, the same laboratories should be used for multiple tests as significant inter-laboratory variability may exist.

Following appropriate collection, SA testing and interpretation should be completed in accordance with WHO 5th edition guidelines.⁶ Following incubation, qualitative observations of color and viscosity, and quantitative measurements of total ejaculate volume and pH are made. An unstained preparation of semen is used for manual quantification of sperm count and motility with further calculation of the total motile count (TMC). Sperm morphology may be assessed based on criteria of variable strictness with an additional stained preparation.^{6,14} Some laboratories will perform sperm viability testing with dye exclusion or hypotonic swelling tests to better characterize immotile sperm. Chemical testing of semen for micronutrients such as zinc, selenium, and carnitine may additionally be performed. Although there is some evidence demonstrating defects in sperm count or function with deficiencies in these micronutrients,^{15,16} testing may be unnecessary given that we routinely recommend multivitamin supplementation including these compounds to men undergoing a fertility evaluation.

SA reports should include, at the minimum, specimen measurements of ejaculate volume, sperm concentration, motility, TMC, and morphology with a comparison to 2010 WHO 5th edition reference values. A SA, though easily performed, is not as easily correlated to a man's fertility potential. The results do not directly measure fertility but rather serve as a surrogate, predicting the likelihood of achieving a pregnancy.¹⁷ When creating SA reference values, the WHO interpreted SA results from nearly 2000 fertile men and defined "normal" values at the 5th percentile, which indicates that a percentage of normally fertile men will have "abnormal" SA results.⁶ Among the array of semen parameters, sperm concentration and motility appear to most consistently correlate with fertility potential when comparing proven fertile and infertile male cohorts.^{5,18} Other semen parameters must be cautiously interpreted in the evaluation and treatment of individual patients.

Sperm quality tests

Given the limitations of the parameters reported on a standard semen analysis, other sperm quality assays were historically developed to aid further in the male infertility evaluation. One such historical test is

the antisperm antibody (ASA) assay, which evaluates for the presence of immunoglobulins bound to a patient's sperm that may cause clumping with reduced sperm motility and function.¹⁹ Spermatozoa are normally located in an immunologically privileged site, protected from the systemic immune system via supporting cell tight junctions and physiologic processes which form the blood-testis barrier.²⁰ Breaches in the form of trauma, surgery, or chronic obstruction may expose the germinal epithelium, evoking an immune response.^{21,22} Testing for surface ASAs can be completed with immunobead-binding or mixed antiglobulin reaction assays.²³ The clinical ramifications of ASAs, however, remain controversial as they may not cause sperm agglutination and agglutination may be caused by factors other than ASAs.²⁴ Results of extensive studies and reviews on the presence of ASAs have concluded that they ultimately have little to no correlation with semen quality or natural pregnancy rates.^{25,26} Additionally, attempted treatment of immunologic infertility with corticosteroids has shown no benefit in pregnancy rates in blinded trials.^{27,28}

Another commonly reported assay, the DNA fragmentation index (DFI), measures sperm DNA integrity. Exposure to various cellular stress conditions may lead to broken or fragmented DNA, affecting fertilization and normal embryo development.²⁹ The most frequently employed tests of sperm DNA damage are the Comet, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), sperm chromatin dispersion, and sperm chromatin structure assays (SCSAs).³⁰ DNA fragmentation rates often correlate to SA parameters though a high abnormal DFI (>30%) may be found in up to 8% of infertile men with a normal SA, suggesting an adjunct role to the standard SA.³¹ In studies of natural pregnancy rates stratified by DFI, the rates of conception were statistically lower among couples with an elevated DFI.^{32–35} Additionally, an elevated DFI correlates to higher spontaneous miscarriage rates.³⁶ While DNA integrity testing may, therefore, aid in natural pregnancy counseling, reported treatment options such as antioxidants have shown little clinical benefit in reducing DFI and improving pregnancy rates.^{4,37} The unclear prognostic ability of individual testing and possible treatment options has led the American Society of Reproductive Medicine (ASRM) to not routinely recommend DFI testing in infertile couples.³⁸ We still recommend DFI testing in certain individual couples who are undecided regarding specific male infertility treatments such as varicocelectomy.

Reactive oxygen species (ROS) can produce an oxidative environment and have been implicated in DNA fragmentation. High seminal ROS levels may lead to spermatozoal damage or death with significantly higher levels found in infertile men.³⁹ ROS cannot be eliminated, however, as a low concentration is required for critical steps in fertilization including capacitation, the acrosome reaction, and sperm-oocyte fusion.⁴⁰ A lack of consensus on the physiologic versus pathologic ranges of seminal ROS has hampered its clinical applicability. In addition, studies demonstrating mild to no benefits with antioxidant therapy despite elevated ROS levels have prevented the ROS assay from becoming a routine clinical test for subfertile men.^{41,42}

It has long been recognized that many cases of fertilization or implantation failure occur as a result of genetic imbalances, prompting the development of fluorescence *in situ* hybridization (FISH) testing for sperm aneuploidy, or an abnormal number of chromosomes. FISH utilizes fluorescent tags to specific DNA elements to identify aneuploidies which typically result from spermatogenic meiotic errors.⁴³ The most commonly used tags report the frequency of numerical abnormalities involving chromosomes 13, 18, 21, X, and Y.⁴⁴ Tags to other chromosomes and genetic loci are commercially available but not routinely used. Early studies of chromosomal numerical

abnormalities established that most fertile men generally produce <2% aneuploid sperm.^{45,46} The clinical application of FISH has been studied in an array of infertile male populations including oligozoospermic, teratozoospermic, asthenozoospermic, and recurrent pregnancy loss.^{44,47} Although reduced SA parameters correlate with increased sperm aneuploidy rates, the cost of testing is somewhat prohibitive, and thus FISH tends to be used only in the most relevant clinical scenarios such as couples with recurrent miscarriages. Estimation of sperm aneuploidy for couples in this population may aid in patient counseling and treatment decisions, including *in vitro* fertilization (IVF) with preimplantation genetic determination or reproductive alternatives such as adoption or use of a sperm donor.

Sperm functional aspects have been previously studied as well including the sperm-mucus interaction, acrosome reaction (AR), and zona pellucida binding/penetration. The sperm-mucus interaction can be assessed with postcoital or *in vitro* tests although the ASRM no longer recommends postcoital testing due to poor reproducibility and patient inconvenience.⁴⁸ While *in vitro* sperm-mucus assays may demonstrate cervical infertility, the most common treatment, barring any severe male factors, would be to proceed with intrauterine insemination (IUI) regardless of test results. Many clinicians will now forgo testing and proceed directly to IUI in appropriately-selected couples. Testing of the AR and zona binding/penetration will be further discussed in the “Predicting ART Success” section.

Molecular and epigenetic markers

More than being a simple carrier of the male genetic complement, spermatozoa supply an epigenetically-modified genome with RNA and protein components critical for fertilization and embryonic development. The sperm epigenome is characterized by DNA methylation, which modifies the genetic material, and extensive protamination, or DNA repackaging. Protamines 1 and 2 (P1 and P2) replace histones during spermatogenesis, leading to a more compact chromatin packaging structure necessary for sperm function.⁴⁹ The relative concentrations of P1 and P2, normally equally expressed, may be abnormal in some groups of infertile men. Carrell and Liu found an undetectable P2 level in 17% of men requiring IVF with an associated reduced penetration capacity, possibly explaining their inability to conceive naturally.⁵⁰ Among a comparison group of 50 fertile men, all had measurable P2 concentrations. Similarly, aberrant DNA methylation, often in the form of hypermethylation, in several genes has been implicated as a contributing factor in male infertility cases.⁵¹ The cAMP response element modulator (*CREM*) gene is one example where hypermethylation was found to correlate negatively with sperm concentration, motility, and normal morphology.⁵² Other groups evaluating abnormally increased methylation of imprinted loci, such as mesoderm specific transcript (*MEST*), have likewise noted associations with abnormal semen parameters and male factor infertility.^{53–55}

Novel biomarker research has associated spermatozoal RNA elements with natural fertility success.⁵⁶ In a pilot study comparing fertile to infertile men, Garrido *et al.* identified unique transcriptomes between cohorts, reporting 26 specific differentially expressed messenger RNAs.⁵⁷ Next generation sequencing (NGS) has more recently been applied to identify a larger population of coding and noncoding RNAs including thousands of microRNAs.^{58,59} MicroRNAs are delivered to the oocyte at the time of fertilization and believed to be involved in regulation of early embryogenesis. Utilizing NGS allowed Jodar and colleagues to characterize a larger group of 648 sperm required elements (SREs) for male fecundity.⁶⁰ Natural pregnancies (timed intercourse or intrauterine insemination) were achieved in 73% versus 27% of those couples with

and without the full complement of SREs, respectively. A recent study by Salas-Huetos *et al.* similarly demonstrated differences between microRNA profiles between fertile and infertile men and drilled down further, finding defect-specific altered microRNA profiles in groups of infertile men with isolated oligozoospermia, asthenozoospermia, and teratozoospermia.⁶¹ With increasingly complex descriptions of the complete spermatozoal transcriptome including microRNAs and Piwi-interacting piRNAs, efforts to integrate this data are needed to understand better the information quickly becoming available. Characterization and validation of the data with collaborative efforts across groups will be needed before these biomarkers may be available in the clinic.

Semen proteomics

Seminal fluid is made up of a small volume of sperm with additional secretions from other male reproductive organs including the epididymides, seminal vesicles, prostate gland, and bulbourethral glands (Figure 1). The total sperm count constitutes only approximately 10% of the total ejaculate volume while the remaining 90%, referred to as the seminal plasma, contains a diverse molecular composition. The high concentration of tissue-specific proteins within the seminal plasma provides a rich source of potential biomarkers in male fertility evaluation.⁸ Proteomic and biomarker discovery technologies have advanced greatly, progressing from basic electrophoresis techniques to liquid chromatography and mass spectrometry platforms, enabling high-throughput identification of thousands of high-, medium- and low-abundance proteins. Original investigations of seminal protein biomarkers in the 1940s identified only four unique protein separations.⁶² In 2011, Batruch *et al.* identified over 2300 seminal proteins in groups of infertile and fertile men using modern mass spectrometry techniques.⁶³ Researchers hope to develop natural fertility biomarker panels for male infertility diagnosis and treatment by studying these cohorts.

Comparing seminal plasma protein levels to routine semen analysis findings has been performed by a number of groups. In a comparison of normal and asthenozoospermic (AS) men, Wang *et al.* reported an increased expression of 45 proteins and downregulation of 56 in the AS

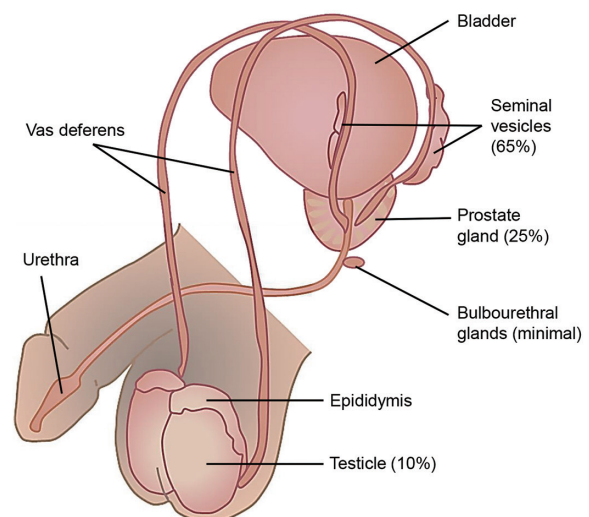


Figure 1: Male reproductive anatomy and relative contributions of individual organs to the total ejaculate volume (adapted from Drabovich *et al.*⁸ with permission).

group.⁶⁴ They identified significantly decreased expression of DJ-1-a, a protein implicated in oxidative stress regulation, in the AS men, which may indicate a loss of oxidative stress regulation in men with low sperm motility. Diamandis *et al.* chose a panel of candidate proteins to analyze in 202 ejaculates noting a positive correlation between seminal prostaglandin D synthase (PTGDS) concentration and SA concentration, motility, and normal morphology.⁶⁵ Additionally, a progressive decline in PTGDS was seen from the normal to oligozoospermic to azoospermic to postvasectomy men, suggesting a relation to testicular obstruction. A similar study by Rolland *et al.* characterized the seminal proteomes among a group of men with normozoospermia, azoospermia, and postvasectomy, identifying a total of 699 proteins.⁶⁶ They then compared testis-specific proteins identified on proteomic analysis, finding that transketolase-like protein 1 (TKTL1), L-lactate dehydrogenase C chain (LDHC), and phosphoglycerate kinase 2 (PGK2), which were easily detected in the normal SA men, were undetectable or barely detected in the azoospermia and postvasectomy groups. Azoospermic men separately demonstrated upregulation of eight proteins (fibronectin, prostatic acid phosphatase, prolactin-inducible protein, beta-2-microglobulin, proteasome subunit alpha type-3, galectin-3-binding protein, and cytosolic nonspecific dipeptidase) in another cohort comparison.⁶⁷ Results of these studies augment the proteomic understanding of male subfertility but have yet to add diagnostic abilities above and beyond the currently-available semen analysis.

Milardi and colleagues took this approach one step further, evaluating the seminal protein profiles among a pool of five men with proven fertility and hoping to identify a set of commonly expressed proteins required for fecundity.⁶⁸ Over 900 proteins were identified in each patient with 83 proteins present in samples from all men. The authors concluded that some of the proteins identified, such as olfactory receptor 5R1, lactoferrin, hCAP18, spindlin 1, and clusterin, may be required in male fertility. No subfertile population was available for comparison, however. Another small cohort study by Cadavid *et al.* observed increased concentrations of 10 seminal proteins in nine infertile men versus seven fertile individuals.⁶⁹ Though they did not finalize the identification of the proteins, the group suggested that several of them are involved in the ubiquitination pathway. It is important to note that significant intra-group variation may exist in seminal protein concentrations, making the development and interpretation of future biomarker assays more difficult.⁷⁰ Moving forward, big data studies involving multi-institutional cohorts will likely be needed to determine diagnostic panels of fertility required seminal proteins and adequately power their validation.

Proteins detected in the previously mentioned research studies represent the soluble protein composition of seminal plasma and do not account for the approximately 3% contained within secreted microvesicles, such as epididymosomes and prostasomes.⁷¹ This burgeoning area of male fertility research is just beginning to characterize the diversity of proteins contained within these membranous vesicles.^{72,73} In a mass spectrometry analysis of epididymosomes collected during vasovasostomy, Thimon and colleagues reported the presence of 146 individual intravesicular proteins.⁷⁴ Further studies are being designed to determine the applicability of seminal secreted microvesicles in male disorders such as infertility, benign prostatic hyperplasia, and prostate cancer.⁷⁵

DIFFERENTIATING AZOOSPERMIA ETIOLOGIES

Emerging proteomic technologies promised to discover biomarkers for the noninvasive diagnosis of multiple urological disorders and

male infertility.^{76,77} This included differentiation of azoospermia forms, obstructive (OA) versus nonobstructive (NOA), and histopathological subtypes of the nonobstructive azoospermia (hypospermatogenesis, maturation arrest, and Sertoli cell-only syndrome), as well as prediction of sperm retrieval outcomes by surgical techniques.

In an attempt to diagnose azoospermia subtypes and predict sperm retrieval outcomes, multiple studies thoroughly examined a variety of blood serum proteins. Proteins studied included follicle-stimulating hormone,⁷⁸ anti-Müllerian hormone,⁷⁹ and inhibin B.⁸⁰ The most promising biomarker, follicle-stimulating hormone, had a sensitivity of only around 77% and specificity of 85% to predict spermatogenesis.⁸¹ Due to their relatively low diagnostic specificities and sensitivities, blood-based markers may require more invasive confirmatory testing methods.

In a search for biomarkers of azoospermia, recent proteomic studies also profiled thousands of semen and seminal plasma proteins, a number of which have already been mentioned above.^{63,82-84} Some protein biomarkers, such as PTGDS,^{85,86} ACRV1,^{87,88} LGALS3BP,⁸⁹ ECM1,⁸⁶ and TEX101^{86,90} were further verified and validated (**Table 1**). Lateral flow immunochromatographic assay of ACRV1 protein was recently implemented into commercially available home tests (SpermCheck Fertility® and SpermCheck Vasectomy®)^{87,88} while commercial TEX101- and ECM1-based immunodiagnostic assays are currently under development.⁹¹

Even though some biomarkers already deliver near-absolute diagnostic sensitivities and specificities (**Table 1**), there is still a need for novel biomarkers with better diagnostic sensitivity to predict testicular sperm extraction (TESE) outcome in NOA patients and to differentiate between hypospermatogenesis, maturation arrest, and Sertoli cell-only syndrome. Accounting for etiologies of OA (absence of vas deference or physical obstruction of the vas deferens) and considering all the glands which secrete proteins into semen, we may suggest that epididymis-specific proteins would emerge as biomarkers for differentiation between NOA and OA. ECM1, a protein secreted into semen predominantly by epididymis supports this hypothesis.⁹⁰ Likewise, proteins with a specific expression in testis, such as TEX101, would emerge as biomarkers for the prediction of TESE outcome and differentiation between NOA subtypes of hypospermatogenesis, maturation arrest, and Sertoli cell-only syndrome.⁹⁰ Identification of both testis-specific and germ cell type-specific proteins secreted into semen exclusively by spermatocytes, spermatids, or spermatozoa should provide markers to accurately pinpoint the stage of spermatogenesis

Table 1: Differentiating azoospermia etiologies with seminal plasma-based protein biomarkers

Diagnosis with a positive test	Biomarker	Sensitivity (%)	Specificity (%)	Threshold
Normozoospermia (vs azoospermia)	TEX101	100	100	>120 ng per ml ⁹⁰
	LDHC	100	100	>160 ng per ml ⁹⁰
	ACRV1	93	97	Positive test ⁸⁸
NOA (vs OA)	ECM1	100	73	>2.3 µg per ml ⁹⁰
	PTGDS	28.6	50	>100 ng per ml ⁸⁵
HS (vs SCO)	TEX101	67	100	>5 ng per ml ⁹⁰
MA (vs SCO)	TEX101	54	100	>5 ng per ml ⁹⁰
Favorable TESE outcome in NOA	LGALS3BP	45	100	>153 ng per ml ⁸⁹

NOA: nonobstructive azoospermia; OA: obstructive azoospermia; HS: hypospermatogenesis; MA: maturation arrest; SCO: sertoli cell-only; TESE: testicular sperm extraction; LDHC: L-lactate dehydrogenase C chain; PTGDS: prostaglandin D synthase; ECM1: extracellular matrix protein 1; TEX101: testis expressed 101; ACRV1: acrosomal vesicle 1; LGALS3BP: lectin galactoside-binding soluble 3 binding protein



failure and thus predict TESE outcome with a better diagnostic performance.

It is also intriguing to speculate if any of the aforementioned protein biomarkers in seminal plasma (**Table 1**) would also be informative in the blood serum and thus facilitate blood-based diagnostics of male infertility and azoospermia. Careful consideration of the male urogenital anatomy reveals that all reproductive glands are sequestered from the systemic circulation by the stringent tissue-blood barriers and are thus immune privileged.²⁰ As a result, reproductive gland-specific proteins are typically not found in the blood serum, and it is unlikely that seminal plasma-based biomarkers will be informative in blood. However, if male infertility is associated with the destruction of the blood-testis barrier due to inflammation, presence of testis-specific proteins in the blood can be assumed. In addition, such proteins leaked even in negligible amounts can lead to an autoimmune reaction, and the presence of the corresponding autoantibodies may facilitate the development of blood-based diagnostics of male infertility.⁹²

PREDICTING ART SUCCESS

Assisted reproductive techniques employ artificial methods in an attempt to augment a couple's chances at conception and include intrauterine insemination and *in vitro* fertilization with or without intracytoplasmic sperm injection (ICSI). IUI may slightly improve pregnancy rates over natural attempts, especially if ovarian stimulation is added during a cycle.⁹³ IVF/ICSI is often recommended for couples in certain clinical situations (e.g., severe oligozoospermia or Fallopian tube obstruction) or when IUI has failed. Despite the weighty costs and advanced technology of IVF/ICSI, success rates may remain lower than patients' expectation. According to the Centers for Disease Control 2012 IVF data, only 36% and 29.4% of the IVF cycles resulted in pregnancies and live births, respectively.⁹⁴ Age significantly impacts the success rates with live births resulting from 40% of IVF cycles in women <35-year-old compared to <10% of cycles in women 42 and older. Despite these limitations, over 150 000 IVF procedures were performed in the United States that year.⁹⁵ In total, IVF procedures resulted in the birth of over 65 000 infants or 1.5% of all US childbirths. Some European countries have reported up to 5% of the live births conceived using assisted reproduction.⁹⁶

Before ICSI, additional sperm tests were frequently used in an effort to assess the functional capacity of sperm to complete the final steps of fertilization necessary for IVF, namely capacitation, the acrosome reaction, and oocyte zona pellucida binding/penetration.⁹⁷ Sperm capacitation normally occurs during transport through the female genital tract with the acrosome reaction taking place as the sperm approaches the oocyte.⁹⁸ *In vitro* testing of this process may employ a calcium ionophore or natural agonists such as progesterone or zona pellucida proteins to induce the acrosome reaction and may aid in predicting fertilization success with IVF.^{99,100} The spermatozoa penetration assay (SPA) measures the ability of human spermatozoa to bind and penetrate a zona-free hamster oocyte, thereby collectively assessing capacitation, acrosome reaction, sperm-oocyte binding, penetration, and sperm chromatin decondensation.¹⁰¹ SPA was frequently used in the pre-ICSI era though interpretation of the test remained challenging. While a few may advocate for ICSI-SPA to directly assess chromatin decondensation,¹⁰² the clinical application of ICSI has largely replaced the need for SPA testing.

As previously discussed, ASA testing provided little benefit in predicting natural pregnancy rates. Additional reviews of ASA presence among couples undergoing IVF with or without ICSI revealed no relationship to ART success rates.¹⁰³ DFI, on the other hand, was found

to have a minimal but significant negative correlation with IVF results among meta-analyses, although individual studies demonstrate a great deal of heterogeneity.^{104–106} The small predictive value of DFI with ART may not be clinically significant enough to warrant DFI testing in all couples undergoing IVF. ICSI pregnancy rates, on the other hand, do not appear to be affected by DNA fragmentation levels.¹⁰⁷ There is also evidence that couples with an elevated sperm DFI may be at a greater risk of pregnancy loss with IVF and ICSI.¹⁰⁸ These studies collectively suggest that bypassing natural barriers to conception with IVF/ICSI may improve pregnancy rates among couples with an elevated DFI, but concerns are now being expressed about possible genomic effects on offspring.¹⁰⁹ We need to adequately capture the results of DFI testing, IVF outcomes, and birth defects/developmental outcomes in properly-collected databases to determine the true implications of using sperm with compromised DNA for assisted reproductive techniques.

Seminal reactive oxygen species are often a causal factor in DNA fragmentation and, therefore, may additionally affect ART success rates. In a meta-analysis by Agarwal and colleagues, the seminal ROS concentration was found to inversely correlate with IVF fertilization rates.¹¹⁰ They concluded that assessing ROS level may aid in predicting ART success and counseling patients. A review of patients undergoing IVF or ICSI by Hammadeh *et al.* conversely found that, despite significantly higher ROS concentrations among the IVF cohort, fertilization rates were similar between groups.¹¹¹ A better understanding of the "normal" and "abnormal" reference values is needed before additional studies will be able to determine the relationship between ROS as well as antioxidant therapies on assisted reproductive outcomes.

For couples failing multiple attempts at IVF/ICSI, additional testing with sperm fluorescence *in situ* hybridization may be warranted. Petit and colleagues noted elevated sperm aneuploidy rates among couples with repeated ICSI failures.¹¹² Discovery of increased sperm chromosomal numerical abnormalities with FISH analysis may aid in counseling these couples regarding preimplantation genetic diagnosis or alternative reproductive options. Other genomic factors including the epigenome have been shown to impact assisted reproduction outcomes in small series. As previously discussed, protamines 1 and 2 are necessary for compact chromatin packaging and the two proteins are typically expressed in similar concentrations. In a review of men undergoing standard IVF, those with abnormal protamine 1/protamine 2 ratios were found to have reduced fertilization rates compared to men with a normal ratio.¹¹³ Significantly lower pregnancy rates were seen among men who had an abnormally reduced protamine ratio.

In addition to the genetic material it delivers to the oocyte at fertilization, the spermatozoon also provides a complement of proteins necessary for early embryo development. IVF pregnancy rates may thus be affected by the seminal plasma proteome, as reported by Zhu and colleagues.¹¹⁴ They analyzed the seminal proteome from 12 men, six who conceived with ART and six who did not. Out of a total of 2045 proteins identified, 21 were differentially expressed between the groups. With confirmatory analyses, three proteins (A2LD1, ATP1B3, and FBXO2) were shown to be significantly differently expressed. Azpiazu *et al.* similarly reviewed the seminal proteome in 31 men subdivided based on IVF success.¹¹⁵ They observed differential expression of 66 proteins with confirmatory testing of one protein, SRSF protein kinase 1. Based on functional clustering, many of the proteins were involved in lipoprotein metabolism and chromatin assembly. Again, additional validation of sperm and seminal plasma proteomes with respect to ART outcomes in big data multi-institutional cohorts may

provide better diagnostic tools for counseling couples considering assisted reproduction.

FUTURE OF MALE INFERTILITY BIOMARKERS IN THE CLINIC

Recent research holds promising results for the development of novel, clinically useful male fertility biomarkers to better inform our patients and possibly avoid the need for more invasive testing. Several clinically relevant areas still need to be explored including markers that predict success of sperm retrieval and provide insight to the health of offspring conceived via ART with male factor infertility. Challenges will be faced in more advanced proteomic applications given the variety of chemically modified protein forms that can be present and the evolving proteome as ejaculated semen progresses from coagulated to liquefied states.¹¹⁶

As we have discovered, a number of promising molecules and panels are currently being explored to aid in the assessment of men's fertility potential, azoospermia etiologies, and predicting ART success. This research in the field of male fertility biomarkers has led to an accumulation of diverse genomics, proteomics, transcriptomics, and metabolomics data, as discussed in this review. Individual studies may over-simplify the human body by analyzing one "omics" field with resulting collections of disjointed individual data sets. A few groups are beginning to integrate data across multiple fields and will need to be a focus of collaborations moving forward to better define biomarkers of male fertility.¹¹⁷ With a better understanding of the pathophysiologic processes of male infertility, we can further translate the results of biomedical research into future clinical diagnostic tools. Additionally, though ELISA assays for many protein biomarkers are quite affordable (\$2–5 per sample), further studies will need to address costs across the array of novel testing for cost-effectiveness comparisons to our standard semen analysis.¹¹⁸

AUTHOR CONTRIBUTIONS

The manuscript design, literature review, drafting, and final approval were performed by all authors.

COMPETING INTERESTS

All authors declare no competing financial interests.

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