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Diagnosing Male Factor Infertility Using Microarrays

Review

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The largest component for the primary evaluation of the infertile couple remains focused on the woman. In part, this is because of two considerations. The first and primary consideration is that, initially, the woman typically pursues this issue on her own, with her gynecologist. Second, if a couple does present for evaluation, the female factor still dominates evaluation, as infertility has historically been considered principally a female problem. This is exacerbated in cases in which semen parameters are normal.

Approaches to the evaluation of the infertile couple differ from practitioner to practitioner. However, there are certain basic, generally accepted components for evaluating each member of the couple. The following will provide a brief overview; other resources can be consulted for a comprehensive discussion (Penzias, 2000; Brugh et al, 2002). The initial evaluation of the woman begins with a thorough medical history and a physical examination that focuses on physiological function, since ovulatory dysfunction and tubal/pelvic pathology each contribute to approximately 40% of infertility cases, while unusual and unexplained problems each contribute to about 10% of infertility cases (Speroff et al, 1999).

A basic medical history will help elicit any preexisting medical conditions that may affect infertility. The couple's coital habits should be discussed as well as whether prior sexually transmitted diseases are a factor. The history should also be evaluated for pelvic inflammatory disease and abdominal surgeries, since both may cause adhesion formation that can affect tubal patency (Westrom, 1980; Corfman and Badran, 1994). Information that is obtained from the patient's medical history is assessed in conjunction with her menstrual history. Women who are having regular cycles tend to ovulate, reducing the likelihood that they will be diagnosed with anovulation or oligo-ovulation. However, if either diagnosis is correct, it is important to determine the cause. For example, polycystic ovarian syndrome contributes to the majority of anovulation cases (ie, approximately 70%) (Knochenhauer et al, 1998), whereas hyperprolactinemia, hypothalamic dysfunction, premature ovarian failure, and extremes of body weight contribute to the remaining 30%. Ovulation function can be assessed by monitoring the surges in luteinizing hormone with ovulation predictors and by checking the basal body temperature and serum progesterone concentration. The initial physical examination may show undiagnosed anatomic abnormalities that preclude pregnancy, or it may yield clues to the underlying pathology (eg, endometriosis). The preliminary examination may disclose endocrine disorders such as hirsutism or profound thyroid dysfunction. The levels of follicle-stimulating hormone, serum androgen, and prolactin may also be assessed, and glucose screening may be required to exclude any undiagnosed medical condition(s).

Up to this point, the basic workup is relatively inexpensive and noninvasive. The male partner is then asked to submit a semen sample for analysis. If the semen analysis fulfills the normal criteria as described below, male factor issues are generally excluded from the differential diagnosis. In these cases of unexplained female infertility, a karyotype analysis may be necessary to exclude undiagnosed chromosomal abnormalities. If a diagnosis is still not forthcoming, women may elect to undergo further evaluation that usually includes more costly and invasive techniques.

The next step in evaluating the woman is to perform an evaluation of the uterine cavity and tubes, either by sonohysterography or hysterosalpingography. Although the former is less invasive, both procedures cause minimal discomfort. Neither is without risk. Endometrial sampling by biopsy to detect luteal phase defects has traditionally been considered part of the evaluation for infertility. The appropriateness of this test has recently been questioned (Coutifaris, 2002) even though this office procedure causes minimal discomfort and carries minimal

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risk. If the evidence gathered thus far is indicative of normal ovulation and suggests tubal patency, pregnancy can be attempted, or further evaluations for tuboperitoneal disease can be conducted. These may include hysteroscopy and laparoscopy. Hysteroscopy can detect intrauterine pathology such as polyps, fibroids, or an abnormal cavity, whereas laparoscopy can detect peritoneal disease. Both procedures are typically carried out in an outpatient setting, are costly, and have defined surgical risks. If no female abnormalities are identified using this battery of tests and if the semen analysis is normal, the couple is diagnosed with unexplained infertility. This is the case in approximately 10%–15% of infertile couples.

Male Evaluation

Given that male factor issues are causative in approximately 20% of infertile couples (Hull et al, 1985; Mosher and Pratt, 1990) and contributory in up to another 30%–40% (World Health Organization [WHO], 1987; Practice Committee of the ASRM and AUA, 2001), a thorough assessment of the male partner is essential but nevertheless often overlooked. Numerous reviews are available that explore male factor evaluation (de Kretser, 1997; Kim and Lipschultz, 1999; Spitz et al, 2000; Burrows et al, 2002). A brief overview follows.

Male fertility assessment begins with a thorough medical history as well as a physical examination that focuses on any historical causes of infertility. The medical history should be checked for peripubertal mumps, which cause sterility in 13% of affected individuals (Beard et al, 1977), and a unilaterally/bilaterally undescended testis, which, in 30%–80% of individuals (Grasso et al, 1991; Lee, 1993), yields abnormal semen parameters. Any chronic medical conditions that could alter fertility, such as diabetes or pulmonary disease, should be explored. Exposure to chemotherapeutic agents or radiation therapy due to malignancy may also contribute to male factor infertility. Medications and exposure to environmental agents as well as a history of pelvic, spinal cord, or direct groin trauma may also affect fertility. A couple's coital habits, including the use of lubricants known to be spermatotoxic (Kutteh et al, 1996), should also be explored.

The physical examination should focus on generalized evidence of endocrine disorders including immature secondary sex characteristics and other evidence of hypogonadism. Penile or testicular abnormalities are then considered. For example, testicular atrophy can be assumed by noting smaller than normal testicles (Sigman and Jarow, 2002). Signs of varicocele, the most common identifiable anatomic cause of male factor infertility, should be excluded. Palpation of the scrotum may identify congenital absence of the vas deferens. Anatomic investigation can be further delineated using imaging techniques such as transrectal ultrasound to detect ejaculatory duct obstruction (Kim and Lipschultz, 1996). The presumptive diagnosis offered by the physical examination dictates the utility of additional imaging techniques.

Normal values for semen analysis[a]

Volume:	≥2 mL
pH:	≥7.2
Sperm concentration:	≥20 × 10^6 sperm/mL
Total sperm number:	≥40 × 10^6 sperm/ejaculate
Motility:	≥50% with grade A + B motility or ≥ 25% with grade A motility
Morphology:	≥15% by strict criteria
Viability:	≥75% of viable sperm
White blood cells:	<1 million/mL

[a] WHO (1999) criteria.

The laboratory investigation of the man begins with the semen analysis. If the analysis meets WHO (1999) criteria for ''normal,'' shown in the Table, the male factor is typically excluded as the cause of the couple's infertility. If the analysis is suboptimal, then repeat analyses are performed and, if confirmed, the man is usually referred for urologic evaluation. The urologic assessment involves a complete medical history and a physical examination in another attempt to identify any previous injuries or exposures that may have altered sperm production. Physical examination findings consistent with anatomic defects or varicocele may help delineate male factor issues. Spermatozoa function is further assessed using a battery of tests. These include the hypo-osmotic swelling test, sperm capacitation assays, the postcoital test, the acrosome reaction assay, the sperm penetration assay, and the reactive oxygen species assay. Evaluation of gonadotropins, testosterone, estrogen, prolactin, and, occasionally, thyroid function may be warranted since endocrine disorders may contribute to male factor infertility in approximately 20% of the cases (Sigman and Jarow, 1997).

Genetic evaluation of the infertile man often depends on the findings of the physical examination and semen analysis. For example, men with congenital absence of the vas deferens should be tested for mutations in the cystic fibrosis *CFTR* gene (Lissens et al, 1996; Patrizio and Salameh, 1998), as a clear association has been demonstrated. Azoospermic or oligospermic men should also be offered genetic evaluation, including routine karyotyping, to identify aneuploidy, sex chromosome abnormalities, translocations, and inversions as well as deletions of the azoospermia factor region on the Y chromosome (Hargreave, 2000; Dohle et al, 2002).

If the diagnosis is indicative of azoospermia or severe oligospermia, the man may be encouraged to undergo a testicular biopsy to evaluate testicular function and spermatogenesis. This anxiety-provoking and painful procedure is typically carried out by one of three methods (Goldstein, 2002). These include open testicular biopsy,

percutaneous testicular biopsy, and percutaneous testicular aspiration. Open testicular biopsy is the ''gold standard,'' as it provides an optimal amount of tissue. A surgical incision is made within the testes to recover testicular tissue for pathologic sampling. In contrast, the percutaneous testicular biopsy uses a large-bore biopsy needle, and percutaneous testicular aspiration uses a smaller-gauge needle to obtain the sample. Neither procedure is without associated risks. Each requires extreme precision to ensure that the needle is guided to a seminiferous tubule. All 3 methods are painful and require local or regional anesthesia. The complications associated with these methods include inadvertent biopsy of the epididymis and hematoma.

New Modality for Male Evaluation

A diagnosis of male factor infertility is reached in only 40% of the affected males seeking assistance. Clearly, the current approaches for assessing male factor infertilities are limited. The clinical utility of semen analysis in diagnosing the infertile man has been under review (Guzick et al, 2001; Menkveld et al, 2001). A recent study by Menkveld et al (2001) demonstrated that the values for determining normality may actually be lower than those set forth by WHO criteria. In addition, the need to standardize protocols for semen analysis, including the use of computer-aided evaluation, continues to be explored (Cooper et al, 2002). Nevertheless, when couples seek assistance, an initial semen analysis remains the current standard for male evaluation.

As with semen analysis, the utility of the diagnostic testicular biopsy has come under scrutiny, particularly in the era of in vitro fertilization-intra cytoplasmic sperm injection (ICSI). In some cases, it may not be necessary. It has recently been shown that follicle-stimulating hormone levels are significantly increased in men with nonobstructive azoospermia when compared to men without obstruction (Sasagawa et al, 2001; Schoor et al, 2002). Thus, these patients can be diagnosed using noninvasive measures. In such cases, the practitioner directly proceeds to retrieving the specimen with the intention of employing ICSI. The efficacy of the testicular biopsy has also been brought into question with regard to site of biopsy, number of biopsies, and methodology. Recent studies have shown that, for diagnostic purposes, multiple bilateral biopsies are necessary to obtain an adequate specimen, regardless of the technique used to recover the tissue (Plas et al, 1999; Altay et al, 2001). In part, the requirement for multiple biopsies may be alleviated with the use of cutting-needle and fine-needle aspirations. Both have now been shown to yield adequate specimens in a less invasive manner than traditional testicular biopsies (Kessaris et al, 1995; Meng et al, 2001; Rosenluned et al, 2001).

Given the limitations of our current ability to diagnose male factor infertility, the need for new and improved techniques is evident. One potential method for evaluating male factor infertility that could surpass the limitations of current techniques is the use of spermatozoal RNA profiles (Ostermeier et al, 2002b). With this method, semen samples are collected using noninvasive techniques. Spermatozoa are directly isolated from the ejaculate, yielding a ready source of RNAs that provide a historical record of spermatogenesis. Once the RNA is obtained, an objective portrait of the spermatozoal transcripts can be constructed using microarray-based transcriptional profiling. This noninvasive testing modality is poised to yield greater information regarding male fertility status than current examination techniques.

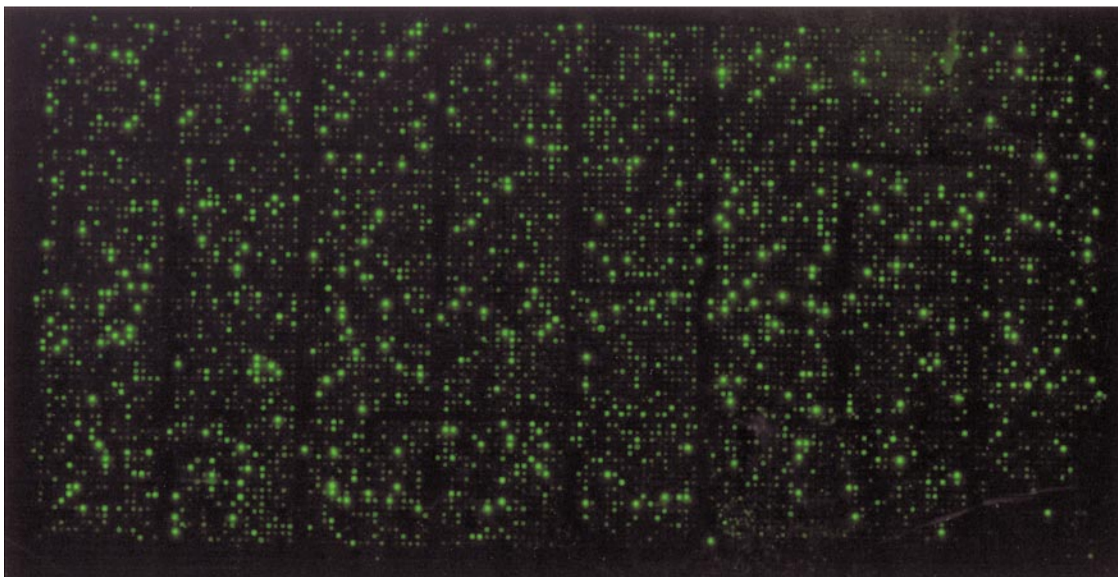
Transcriptional Profiling

With the completion of the human genome project (International Human Genome Consortium, 2001; Venter et al, 2001), we are undergoing a revolution in molecular medicine (Krawetz et al, 1999; Clarke et al, 2001; Gerling et al, 2003). One of the results of this revolution has enabled genome-wide transcriptional profiling studies to investigate the response of cells to changes in environment or conditions that alter messenger RNA (mRNA) expression. This allows investigators to monitor cells in both normal and diseased states and their response to various stimuli.

Transcriptional profiling can be classified as either an open or a closed technology. In open methods such as differential display, the purpose is to identify the mRNA species that have the most notable change in expression under certain conditions. After the set of differentially regulated mRNAs is identified, its sequence is determined. Previous knowledge of the genomic sequence is not necessary. This presents a series of advantages; however, it is quite time-consuming and expensive.

In contrast to the methodology used in open techniques, closed techniques require previous knowledge of the elements being studied. Using these methods, the expression of known genes is studied under different conditions, allowing insight into the mechanisms of disease. The prime example of this method is the DNA microarray, also known as the gene chip (Duggan et al, 1999; Clarke et al, 2001; Ostermeier et al, 2002b), an example of which is shown in the Figure.

Gene chips are constructed by affixing oligonucleotides, complementary DNAs (cDNAs), or other nucleic acids onto glass slides, nylon membranes, or other solid supports. The oligonucleotides are synthesized by standard methods and are typically much smaller than the cDNA samples. The arrays can be assembled using cloned, polymerase chain reaction (PCR)-amplified, or synthesized molecules. The elements on the array are then interrogated using either radioactive or fluorescently la-



Typical spermatozoal fingerprint. Total RNA was isolated from the ejaculate spermatozoa of a normal fertile male. The RNA probe was hybridized to the MWG 10 K human oligonucleotide pan array. Hybrids were detected using the Digene HC Express array kit then the array scanned using a typhoon 9210 microarray scanning system.

beled probes corresponding to the RNA sample being investigated. The probe will only bind to its appropriate complement on the array through a process termed hybridization. When the probe has bound to the target, a positive signal will be detected. Compiling these lists of both intense and weak signals allows investigators to construct transcript profiles or RNA fingerprints from each treatment group. Subsequent analysis can then be used to discern functional status.

In spite of the advantages of microarray technologies, they still have limitations. First, it must be established that the RNA sample under investigation represents the tissue or cell type of interest, since microarrays are extremely sensitive. This is critical when preparing spermatozoal RNAs, because ejaculates can have numerous somatic contaminants. However, stringent precautions can be used to avoid such contamination. For example, spermatozoa can be purified using sequential centrifugations through discontinuous Percoll gradients (Ostermeier et al, 2002b). This approach removes immotile spermatozoa and somatic contaminants from the population. In addition, crude semen preparations or Percoll-purified samples can be treated with Triton X-100. Such treatment lyses somatic cells (Mortimer, 1981), effectively removing any nonspermatozoal RNAs. The efficacy of these treatments has been validated in many ways, including microscopic evaluation, electrophoresis, reverse transcription-PCR, and microarray technologies (Miller et al, 1994, 1999; Ostermeier et al, 2002b). In all cases, evidence was provided to show that essentially pure spermatozoal RNA populations can be obtained. Microarrays produce an inordinate amount of data, which at times can seem rather daunting. However, new bioinformatic tools and software programs are continually being developed to address these needs (Khatri et al, 2002; Ostermeier et al, 2002a; Draghici et al, 2003). Although microarrays present challenges, overcoming them is worthwhile because of the wealth of information this technology can provide.

Microarray Analysis and Male Factor Infertility

The observation that mammalian spermatozoa carry mRNA has revolutionized the investigation of male infertility (Kramer and Krawetz, 1997; Miller et al, 1999; Miller, 2000; Wykes et al, 2000). Using spermatozoal mRNA functional profiles as a tool, the diagnosis of male infertility may be simplified. There are 2 main factors that limit male fertility. The first factor is the inability of the spermatozoa to fertilize oocytes. Several characteristics in these abnormal spermatozoa can be noted, including primary and/or secondary abnormalities (Azfelius et al, 1975; Kruger et al, 1986; Chemes et al, 1987, 1998; Oehninger et al, 1992; Liu and Baker, 1994; Garrett et al, 1997; Esterhuizen et al, 2001). The second factor that limits fertility is the inability of the male gamete to initiate zygotic, embryonic, or fetal development (McGrath and Solter, 1984; Ostermeier et al, 2002b). For example, once the spermatozoon penetrates the egg, it must deliver a signal sufficient to activate the oocyte, which will promote the development of the zygote. Since spermatozoa are transcriptionally dormant (Miller, 1997), all of these structures and signals must be properly packaged within the spermatozoa prior to spermiation. Thus, any error in spermatogenesis is likely to influence fertility. It has been demonstrated that the RNA profiles observed in sperma-

tozoa coincide with those observed in the testis. In effect, they echo spermatogenic gene expression (Dix et al, 2002; Ostermeier et al, 2002b). These concordant profiles should permit the development of a noninvasive testing protocol to assess the functional capacity of human spermatozoa.

Prior to the realization of using spermatozoal RNAs to diagnose male infertility, the spermatozoal RNA profile that defines the normal fertile man must be deciphered. In the recent work of Ostermeier et al (2002a,b), 3281 transcripts in spermatozoa obtained from a pool of 9 normal fertile men were identified. Further, it was established that spermatozoal transcripts were indeed concordant with those from the testis, lending further credence to the use of microarray profiling for infertility testing in men.

Other studies have compared the gene expression profiles of fertile and infertile men. Altered expression patterns produced by the spermatozoa of the infertile men were observed (Patrizio et al, 2001). Perhaps this is the foundation necessary to commence building a new modality for diagnosing male factor infertility.

The lack of a direct link between monogenic disorders and spermatozoal heterogeneity suggests that a majority of male factor infertilities are more complex than single gene mutations (Ostermeier et al, 2002). Many known monogenic disorders that act on the testis affect other organ systems of equal or greater consequence (Van Assche et al, 1996; Cooke, 1999; Lahn and Page, 1999; Lissens, 1999; Hargreave, 2000). Accordingly, it is reasonable to assume that most male factor infertilities result from oligogenic and/or polygenic effects on spermatozoa production (Hsiung et al, 1991; Mieusset and Bujan, 1995; Lindbohm, 1999; Sharpe, 2000; Telisman et al, 2000). If correct, these types of mutations should be reflected by changes in the presence or absence of multiple transcripts within and among various spermatogenic pathways. For example, a heterozygous CREM^{-} man presents as subfertile and is likely classified as oligozoospermic. On the basis of current mouse CREM/microarray expression data (http://www.dkfz-heidelberg.de/tbi/crem/affydiff.html), this phenotype is characterized by the greater than fivefold up-regulation of 16 genes, including laminin beta 3, C-ros proto-oncogene, spermidine/spermine N1-acetyltransferase, smooth muscle calponin, and acidic epididymal glycoprotein, and greater than fivefold down-regulation of 119 genes, including *STAT4,* RAR-related orphan receptor alpha, outer dense fiber of sperm tails 1, inositol polyphosphate-1-phosphatase, and fibrous sheath component 1. The up-regulation and down-regulation of each member of the affected pathways define the lesion. The results of this simple profiling study yield potential management strategies that could be targeted to the various affected pathway members.

It is well known that even semen obtained from normal fertile individuals is remarkably heterogeneous (Tomlinson et al, 1999). This heterogeneity is likely a result of nondeleterious oligogenic and/or polygenic modifiers of spermatogenesis. Thus, it is thought that an invariant universal core of transcripts necessary for the production of viable male gametes exists within the ejaculated spermatozoa of normal fertile men. This population of transcripts can easily be defined by creating scatterplots of intensity profiles from two different microarrays. Genes that share similar patterns of expression are often coregulated and will show a strong linear relationship within such scatterplots. Scatterplots can be constructed for any number of expression profile pairs, and regression analyses can be used to define their association. Once this association is identified, 95% prediction limits can be constructed. The transcripts within these limits can be considered possible candidates for the universal core. These candidates can be compared across all sample pairs, and those that are consistently found will define the invariant universal core of gene transcripts that is always present in spermatozoa obtained from normal fertile ejaculates. Genes that are either up-regulated or down-regulated in one sample compared to another will exceed the 95% prediction limits and will represent those transcripts that vary in spermatozoa obtained from normal fertile ejaculates. The invariant universal core of spermatozoal gene transcripts could then serve as the standard to compare profiles from infertile men. Using a similar approach, van't Veer et al (2002) determined that breast cancer prognosis could be reliably based on differences in as few as 70 specific expressed sequence tags (ESTs). Given that spermatozoal RNA fingerprints from normal fertile men can reliably be distinguished by as few as 98 ESTs (Ostermeier et al, 2002b), it is evident that microarray technologies can be employed to diagnose male factor infertility.

Where Do We Go From Here?

With the information that we have gained, we find significant evidence that favors the potential use of the microarray to evaluate and diagnose the infertile man. This technique is relatively inexpensive and noninvasive in comparison to what the woman must bear. More importantly, it may offer more information concerning the male's contribution to conception and early pregnancy than the semen analysis alone.

This may become a technique that enables the identification of men at high risk for infertility due to environmental factors. Men employed in certain occupations associated with increased rates of abnormal spermatogenesis could undergo pretesting to evaluate sperm function. Planning assistance could then be offered for their reproductive years. Perhaps couples with multiple, recurrent spontaneous abortions would also benefit from the use of

microarrays. Continued research in this area may uncover the functional role that spermatozoa play in the pathology of recurrent birth losses. The potential applications for DNA microarrays in infertility management are unbounded.

References

Altay B, Hekimgil M, Cikili N, Turna B, Soydan S. Histopathological mapping of open testicular biopsies in patients with nonobstructive azoospermia. *BJU Int.* 2001;87:834–837.

Azfelius BA, Eliasson R, Johnson D, et al. Lack of dynein arms in immotile human spermatozoa. *J Cell Biol.* 1975;66:225–232.

Beard CM, Benson RC, Kelalis PP, et al. The incidence and outcome of mumps orchitis in Rochester, Minnesota, 1935–1974. *Mayo Clin Proc.* 1977;52:3.

Brugh VM III, Nudell DM, Lipshultz LI. What the urologist should know about the female infertility evaluation. *Urol Clin North Am.* 2002;29:983–992.

Burrows PJ, Schrepferman CG, Lipschultz LI. Comprehensive office evaluation in the new millennium. *Urol Clin North Am.* 2002;29:873–984.

Chemes HE, Brugo S, Carrere C, et al. Ultrastructural pathology of the sperm flagellum: association between flagellar pathology and fertility prognosis in severely asthenozoospermic men. *Hum Reprod.* 1998;13:2521–2526.

Chemes HE, Brugo S, Zanchetti F, et al. Dysplasia of the fibrous sheath. An ultrastructural defect of human spermatozoa associated with sperm immotility and primary infertility. *Fertil Steril.* 1987;48:664–669.

Clarke PA, te Poele R, Wooster R, Workman P. Gene expression microarray analysis in cancer biology, pharmacology, and drug development: progress and potential. *Biochem Pharmacol.* 2001;62:1311–1336.

Cooke HJ. Y chromosome and male infertility. *Rev Reprod.* 1999;4:5–10.

Cooper TG, Bjomdahl L, Vreeburg J, Nieschlag E. Semen analysis and external quality control schemes for semen analysis need global standardization. *Int J Androl.* 2002;25:306–311.

Corfman RS, Badran O. Effect of pelvic adhesions on pelvic pain and fertility. *Infertil Reprod Clin North Am.* 1994;5:405–411.

Coutifaris C. The reproductive medicine network: the endometrial biopsy as a diagnostic tool in the evaluation of the infertile patient. In: *58th Annual Meeting of the American Society for Reproductive Medicine.* Seattle, Wash: Fertility and Sterility; 2002.

de Kretser DM. Male infertility. *Lancet.* 1997;349:787–790.

Dix DJ, Thompson KE, Rockett JC, Schmid JE, Goodrich RJ, Miller D, Ostermeier GC, Krawetz SA. Testis and spermatozoal RNA profiles of normal fertile men. *Biol Reprod.* 2002;66(suppl 1):194.

Dohle GR, Halley DJJ, Van Hemel JO, van den Ouweland AMW, Pieters MHEC, Weber RFA, Govaerts LCP. Genetic risk factors in infertile men with severe oligospermia and azoospermia. *Hum Reprod.* 2002;17:13–16.

Draghici S, Khatri P, Martins RP, Ostermeier GC, Krawetz SA. Global functional profiling of gene expression. *Genomics.* 2003;81:98–104.

Duggan DJ, Bittner M, Chen Y, Meltzer P, Trent JM. Expression profiling using cDNA microarrays. *Nat Genet.* 1999;21(suppl):10–14.

Esterhuizen AD, Franken DR, Lourens JGH, van Rooyen LH. Clinical importance of zona pellucida induced acrosome reaction (ZIAR test) in cases of failed human fertilization. *Hum Reprod.* 2001;16:138–144.

Garrett C, Liu DY, Baker HWG. Selectivity of the human sperm–zona pellucida binding process to sperm head morphometry. *Fertil Steril.* 1997;67:362–371.

Gerling IC, Solomon SS, Bryer-Ash M. Genomes, transcriptomes, and proteomes. Molecular medicine and its impact on medical practice. *Arch Intern Med.* 2003;163:190–198.

Goldstein M. Surgical management of male infertility and other scrotal disorders. In: Walsh P, ed. *Campbell's Urology.* 8th ed. Philadelphia, Pa: WB Saunders; 2002:1532–1587.

Grasso M. Bounaguidi A, Lania C, et al. Postpubertal cryptorchidism: review and evaluation of fertility. *Eur Urol.* 1991;20:126–128.

Guzick DS, Overstreet JW, Factor-Litvak P, et al. Sperm morphology, motility, and concentration in fertile and infertile men. *N Engl J Med.* 2001;345:1388–1393.

Hargreave TB. Genetics and male infertility. *Curr Opin Obstet Gynecol.* 2000;12:207–219.

Hsiung R, Nieva H, Clavert A. Scrotal hyperthermia and varicocele. *Adv Exp Med Biol.* 1991;286:241–244.

Hull MG, Glazener CM, Kelly NJ, et al. Population study of causes, treatment, and outcome of infertility. *Br Med J.* 1985;291:1693–1697.

The International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. *Nature.* 2001;409:860–921.

Kessaris DN, Wasserman P, Mellinger BC. Histopathological and cytopathological correlations of percutaneous testis biopsy and open testis biopsy in infertile men. *J Urol.* 1995;153:1151–1155.

Khatri P, Draghici S, Ostermeier GC, Krawetz SA. Profiling gene expression utilizing onto-express. *Genomics.* 2002;79:266–270.

Kim ED, Lipschultz LI. Role of ultrasound in the assessment of male infertility. *J Clin Ultrasound.* 1996;24:437–453.

Kim ED, Lipschultz LI. Evaluation and imaging of the infertile male. *Infertil Reprod Med Clin North Am.* 1999;10:377–409.

Knochenhauer ES, Key TJ, Kahsar-Miller M, et al. Prevalence of the polycystic ovary syndrome in unselected black and white women of the southeastern United States: a prospective study. *J Clin Endocrinol Metab.* 1998;83:3078–3082.

Kramer JA, Krawetz SA. RNA in spermatozoa: implications for the alternative haploid genome. *Mol Hum Reprod.* 1997;3:473–478.

Krawetz SA, Kramer JA, McCarrey JR. Reprogramming the male gamete genome: a window to successful gene therapy. *Gene.* 1999;234:1–9.

Kruger TF, Menkveld R, Stand FSH, et al. Sperm morphologic features as a prognostic factor in in vitro fertilization. *Fertil Steril.* 1986;46:1118–1123.

Kutteh WH, Chao CH, Ritter JO, et al. Vaginal lubricants for the infertile couple: effect on sperm activity. *Int J Fertil Menopaus Stud.* 1996;41:400–404.

Lahn BT, Page DC. Retroposition of autosomal mRNA yielded testis-specific gene family on human Y chromosome. *Nat Genet.* 1999;21:429–433.

Lee PA. Fertility in cryptorchidism: does treatment make a difference? *Endocrinol Metab Clin North Am.* 1993;22:479.

Lindbohm ML. Effects of occupational solvent exposure on fertility. *Scand J Work Environ Health.* 1999;25:44–46.

Lissens W. Genetics of male reproductive dysfunction. In: Fauser BCJM, ed. *Molecular Biology in Reproductive Medicine.* New York, NY: Parthenon Publishers; 1999:479–504.

Lissens W, Mercier B, Tournaye H. Cystic fibrosis and infertility caused by congenital bilateral absence of the vas deferens and related clinical entities. *Hum Reprod.* 1996;11:55–80.

Liu DY, Baker HWG. Disordered acrosome reaction of spermatozoa bound to the zona pellucida: a newly discovered sperm defect causing infertility with reduced sperm–zona penetration and reduced fertilization in vitro. *Hum Reprod.* 1994;9:1694–1700.

McGrath J, Solter D. Completion of mouse embryogenesis requires both maternal and paternal genomes. *Cell.* 1984;37:179–183.

Meng MV, Cha I, Ljung BM, Turek PJ. Testicular fine-needle aspiration in infertile men. *Am J Surg Pathol.* 2001;25:71–79.

Menkveld R, Wong WY, Lombard CJ, Wetzels AMM, Thomas CMG,

Merkus HMWM, Steegers-Theunissen RPM. Semen parameters, including WHO and strict criteria morphology, in a fertile and subfertile population: an effort towards standardization of in-vivo thresholds. *Hum Reprod.* 2001;16:1165–1171.

Mieusset R, Bujan L. Testicular heating and its possible contributions to male infertility: a review. *Int J Androl.* 1995;18:169–184.

Miller D. RNA in the ejaculate spermatozoon: a window into the molecular events in spermatogenesis and a record of the unusual requirements of haploid gene expression and post-meiotic equilibration. *Mol Hum Reprod.* 1997;3:669–676.

Miller D. Analysis and significance of messenger RNA in human ejaculated spermatozoa. *Mol Reprod Dev.* 2000;56:259–264.

Miller D, Briggs D, Snowden H, Hamlington J, Rollinson S, Lilford R, Krawetz SA. A complex population of RNAs exists in human ejaculate spermatozoa: implications for understanding molecular aspects of spermiogenesis. *Gene.* 1999;237:385–392.

Miller D, Tang PZ, Skinner C, Lilford RJ. Differential RNA fingerprinting as a tool in the analysis of spermatozoal gene-expression. *Hum Reprod.* 1994;9:864–869.

Mortimer D. The assessment of human-sperm morphology in surface replica preparations for transmission electron-microscopy. *Gamete Res.* 1981;4:113–119.

Mosher WD, Pratt WF. *Fecundity and Infertility in the United States, 1965–88.* Hyattsville, Md: National Center for Health Statistics; 1990.

Oehninger SC, Acosta AA, Toner JP, et al. Sperm zona binding: predicting fertilization potential of human gametes by hemizona assay. *Arch Androl.* 1992;3:1–13.

Ostermeier GC, Dix D, Krawetz S. A bioinformatic approach to sequencing cDNA libraries. *Bioinformatics.* 2002a;18:949–952.

Ostermeier GC, Dix DJ, Miller D, Khatri P, Krawetz SA. Spermatozoal RNA profiles of normal fertile men. *Lancet.* 2002b;360:772–777.

Patrizio P, Hecht N, Rockett J, Schmid J, Dix D. DNA microarrays to study gene expression profiles in testis of fertile and infertile men. *Fertil Steril.* 2001;76:S40.

Patrizio P, Salameh WA. Expression of the cystic fibrosis transmembrane conductance regulator (CFTR) mRNA in normal and pathological adult human epididymis. *J Reprod Fertil.* 1998;53(suppl):261–270.

Penzias AS. Infertility: contemporary office based evaluation and treatment. *Obstet Gynecol Clin North Am.* 2000;27:473–485.

Plas E, Riedl CR, Engelhardt PF, Muhlbauer H, Pfluger H. Unilateral or bilateral testicular biopsy in the era of intracytoplasmic sperm injection. *J Urol.* 1999;162:2010–2013.

Practice Committee of the ASRM and Male Infertility Best Practice Policy Committee of the AUA. *Report on Optimal Evaluation of the Infertile Male.* Baltimore, Md: American Urological Association Best Practice Policy; 2001.

Rosenluned B, Kvist U, Ploen L, Ekstrom U, Hovatta O. Percutaneous cutting needle biopsies for histopathological assessment and sperm retrieval in men with azoospermia. *Hum Reprod.* 2001;16:2154–2159.

Sasagawa I, Yazawa H, Suzuki Y, Tateno T, Ichiyanagi O, Kobayashi T, Matsuki S, Nakada T. Reevaluation of testicular biopsies of males with nonobstructive azoospermia in assisted reproductive technology. *Arch Androl.* 2001;46:79–83.

Schoor RA, Elhanbly S, Niederberger CS, Ross LS. The role of testicular biopsy in the modern management of male infertility. *J Urol.* 2002; 167:197–200.

Sharpe RM. Environment, lifestyle and male infertility. *Baillieres Best Pract Res Clin Endocrinol Metab.* 2000;14:489–503.

Sigman M, Jarow JP. Medical evaluation of infertile men. *Urology.* 1997; 50:659–664.

Sigman M, Jarow JP. Male infertility. In: Walsh P, ed. *Campbell's Urology.* 8th ed. Philadelphia, Pa: WB Saunders; 2002:1475–1531.

Speroff L, Glass RH, Kase NG, eds. Female infertility. In: *Clinical Gynecologic Endocrinology and Infertility.* 6th ed. Philadelphia, Pa: Lippincott Williams and Wilkins; 1999:1013–1042. Society for Reproductive Medicine Practice Committee Report, 2001.

Spitz A, Kim ED, Lipschultz LI. Contemporary approach to the male infertility evaluation. *Obstet Gynecol Clin North Am.* 2000;27:487–516.

Telisman S, Cvitkovic P, Jurasovic J, Pizent A, Gavella M, Rocic B. Semen quality and reproductive endocrine function in relation to biomarkers of lead, cadmium, zinc, and copper in men. *Environ Health Perspect.* 2000;108:45–53.

Tomlinson MJ, Kessopoulou E, Barratt C. The diagnostic and prognostic value of traditional semen parameters. *J Androl.* 1999;20:588–593.

Van Assche E, Bonduelle M, Tournaye H, Joris H, Verheyen G, Devroey P, Van Stierteghem A, Liebaers I. Cytogenetics of infertile men. *Hum Reprod.* 1996;11:1–26.

van't Veer LJ, Dai H, van de Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature.* 2002;415:530–536.

Venter JC, Adams MD, Myers EW, et al. The sequence of the human genome. *Science.* 2001;291:1304–1351.

Westrom I. Incidence, prevalence and trends of acute pelvic inflammatory disease and its consequences in industrialized countries. *Am J Obstet Gynecol.* 1980;138:880.

World Health Organization. Towards more objectivity in diagnosis and management of male infertility. *Int J Androl.* 1987;7(suppl):1–53.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interactions.* 4th ed. Cambridge, United Kingdom: Cambridge University Press; 1999: 4–33.

Wykes SM, Miller D, Krawetz SA. Mammalian spermatozoal mRNAs: tools for the functional analysis of male gametes. *J Submicrosc Cytol Pathol.* 2000;32:77–81.