Role of metabolomic analysis of biomarkers in the management of male infertility

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Metabolomics is the systematic study of metabolites as small-molecule biomarkers that represent the functional phenotype in a cell, tissue or organism. Detection of crucial disturbances in the concentration of metabolites by metabolomic profiling of key biomarkers can be beneficial in the management of various medical conditions, including male-factor infertility. Recent studies have demonstrated the potential role of this rapid, noninvasive analysis in the investigation of infertile men. Differences in the concentration of oxidative stress biomarkers (–CH, –NH, –OH and ROH) have been found to be uniquely associated with semen plasma of healthy men compared with patients with idiopathic infertility, varicocele and vasectomy reversal. Furthermore, NMR spectra have shown significant differences in citrate, lactate, glycerylphosphorylcholine and glycerylphosphorylethanolamine among semen samples of men with spermatogenesis failure, obstructive azoospermia, oligoasthenoteratozoospermia and healthy donors. Evidence has also shown the value of ^{31}P-magnetic resonance spectroscopy in differentiating patients with testicular failure and ductal obstruction by utilizing phosphomonoester and β-adenosine triphosphate as biomarkers. In addition, metabolomics has shown promise in assisted reproductive techniques. Recent studies involving spectroscopic measurements of follicular fluid and embryo culture media have revealed an association between biomarkers of oxidative stress and pregnancy outcome of oocytes and embryos.

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Approximately 15–20% of all couples who attempt to conceive face the problem of infertility. Male-factor infertility is the sole or contributing factor in nearly half of these cases [1]. Despite improvements in both diagnostic assessment and treatment of infertile couples, management of male-factor infertility remains a challenge, largely because of two factors: the lack of a rapid, noninvasive test to evaluate semen quality and investigate the cause of male infertility; and the inability to predict gamete quality and embryo viability, which in turn lead to low success rates and a high incidence of multiple births after *in vitro* fertilization (IVF).

To overcome these limitations, a new science known as metabolomics has been conceived with an expectation that body-fluid analysis can be optimized to create a low-cost, informative and medically relevant means of measuring metabolic changes, even when standard clinical chemistry markers are within normal limits [2]. Metabolomics is the systematic study of the inventory of metabolites, as small-molecule biomarkers that represent the functional phenotype in a cell, tissue or organism [3,4]. The metabolome constitutes the dynamic, quantitative complement of all low-molecular-weight molecules (typically <3000 m/z) present in cells in a particular physiological or pathological state. Ultimately, metabolomics is the mining of the global population of biomarkers, which unveils the phenotype of the system (cell, tissue or organism).

Rationale behind metabolomics

The central dogma of molecular biology suggests that there is a unidirectional flow of information

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from gene to transcript to protein. However, it is now known that in addition to gene expression, post-transcriptional and post-translational events regulate metabolic fluxes. Hence, the metabolome is considered to be closer to the phenotype, rather than the transcriptome or proteome [5]. Although metabolomics is complementary to transcriptomics and proteomics, it provides us with a real-time snapshot of the downstream events that characterize gene expression [6]. While changes in the levels of individual enzymes have little impact on metabolic fluxes, they do have a significant effect on the concentration of a variety of individual metabolites [7]. Furthermore, with the downstream results of gene expression, changes in the metabolome are amplified relative to changes in the transcriptome and the proteome, which allows for increased sensitivity. Thus, metabolomics can provide us with better, more useful information with higher throughput at a lower cost than genomics, transcriptomics or proteomics and is therefore well suited for widespread investigations.

Biomarkers of disease

Approximately 3000 small-molecule metabolites make up the human metabolome. The metabolites arising from post-transcriptional and post-translational events that can be assessed systematically in the study of metabolomics serve as biomarkers [3]. Steroids, amino acids and various markers of oxidative stress (OS) have all been used as biomarkers by different researchers in the field of reproduction [8–11]. The current trend is to use multivariate biomarkers rather than a single biomarker [12]. The Metabolomics Study Group for Assisted Reproductive Techniques, which is credited with pioneering research in this field, is particularly focused on the biomarkers of OS [13–15]. OS arises as a consequence of excessive production of reactive oxygen species (ROS) and impaired antioxidant defense mechanisms [16]. OS biomarkers include –CH, –NH, –SH, C=C and –OH. These biomarkers have been found in both the male and female reproductive tracts and are known to affect sperm quality and function, oocyte quality and embryo viability [17,18]. Recent reports have found high levels of ROS in 25–40% of semen samples from infertile men [19]. Furthermore, enough evidence is available to suggest that ROS originating from embryo metabolism and the surrounding environment act on the cellular molecules of the embryo and block early embryo development [20–22].

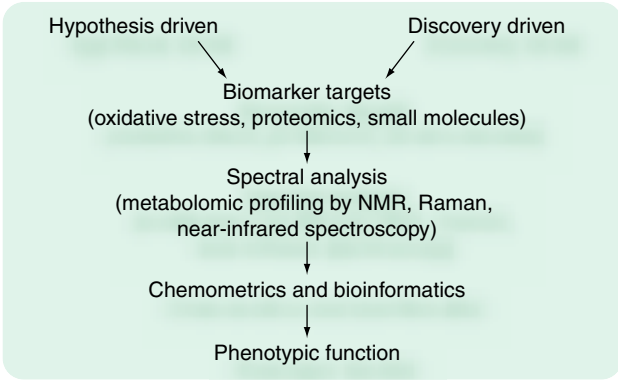

Figure 1. Pivotal steps that constitute the technology platform for biomarker analysis using metabolomic profiling.

Analysis of biomarkers

The identification of metabolites or biomarkers that are indicative of a disease is an active area of research. These biomarkers are quantified by various forms of analytical, biochemical and spectral analysis to establish the quantitative lists or signatures of the metabolites for healthy control population and test subjects with specific illnesses [3]. Unique metabolomic profiles describing differences in the concentration of specific biomarkers can be used to differentiate and grade test subjects from controls. To make the analysis as predictive as possible, stringent statistical validation is carried out using multivariate mathematical methods, known collectively as bioinformatics or computational biology (FIGURE 1) [23–25].

Biomarker analysis typically involves the use of following analytical technologies [26]:

- Gas chromatography (GC), high-performance liquid chromatography (HPLC) or capillary electrophoresis for separation of the biomarkers
- Mass spectrometry (MS) [7], NMR, Fourier-transform infrared, Raman or near-infrared spectroscopy (NIR) for identification and quantification of the biomarkers

GC-MS is the most widely used method in metabolomics research [27]. MS is both sensitive and specific and can even be utilized for both separation and detection of metabolites. Moreover, libraries of mass spectral fingerprints exist or can be developed, allowing the identification of metabolites according to their fragmentation pattern. However, the instrument is expensive and it consumes the sample for analysis. Although NMR can measure several molecules simultaneously and provides detailed structural information, it is relatively insensitive and one of the most expensive instruments to acquire and maintain [26]. The Metabolomics Study Group for Assisted Reproductive Techniques has focused on Raman and NIR spectroscopy and coined the term 'biospectroscopy-based metabolomics', which is the application of different forms of spectral analysis in human biology to identify, quantify and validate proteomic and metabolomic biomarkers [13]. NIR spectroscopy is the measurement of wavelength and intensity of the absorption of near-infrared light (800–2500 nm) by any sample. It is typically used for quantitative measurement of organic functional groups, especially O–H, N–H, and C=O. Raman spectroscopy relies on the inelastic scattering of monochromatic light and provides similar, yet complementary, information to infrared spectroscopy. Raman and NIR spectroscopy have been utilized because of their several added advantages [28]:

- Sample preparation is not necessary, and analysis does not destroy specimens
- They are less time-consuming because they analyze multiple biomarkers simultaneously

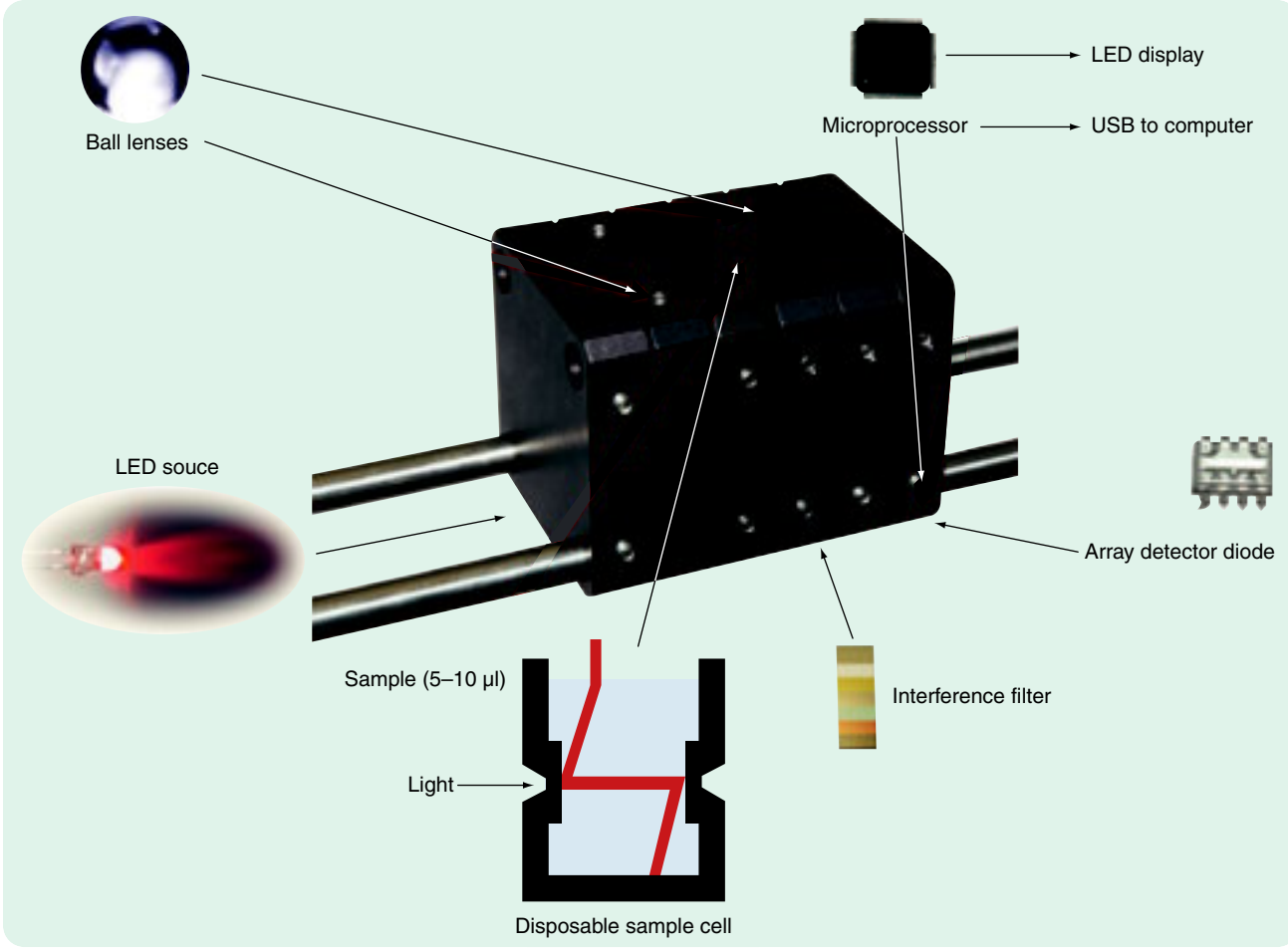

Figure 2. Instrument prototype design for obtaining spectral signatures of biomarkers. Reproduced with permission from Molecular Biometrics, LLC (Chester, NJ, USA).

- Less chemical bias
- Less expensive

Instrument prototype

A disposable sample cell containing 5–10 µl of specimen is inserted into a fixed position in the light path within the instrument. The stable light source passes light into the sample, which then travels through a wavelength filter to select a specified wavelength for the analysis. A light-detecting diode captures the photons of energy emerging from the filter and delivers them to the bioinformatics microprocessor chip. Here, biomarker profiles are calculated using proprietary and nonproprietary chemometrics and bioinformatics to determine the probability of normal cellular function versus the presence of a pathological state of cellular activity. The total analysis time is approximately 1 min. The instrument can be interfaced with any computer system to download data to patient files (FIGURE 2).

Role of metabolomics in the investigation of infertile men

One of our recent studies, which was presented at the 2006 American Society for Reproductive Medicine (ASRM) meeting, demonstrated a potential role of metabolomic profiling of biomarkers of OS as a diagnostic tool to evaluate semen function and quality [13]. In this study, we collected seminal plasma from a group of patients seeking infertility evaluation and a group of healthy donors. The subjects were divided into four different groups: idiopathic male infertility (n = 15); varicocele (n = 70); vasectomy reversal (n = 9); and healthy donors (n = 30). The semen specimens from all four groups showed unique NIR spectral signatures that were statistically different from one another, indicating differences in the concentration of OS biomarkers (–CH, –NH, –OH and ROH). The –CH to ROH content, which is reflective of OS status, was also found out to be different on quantification of each metabolomic profile using a direct exponential curve resolution algorithm and logistic regression analysis. The healthy donors and vasectomy reversal groups were well defined within the self-organized map. However, the profile from the varicocele patients was found to be broadly distributed among all the groups and did not segregate as a separate population with uniquely identifiable biomarker characteristics. The idiopathic male-infertility patients were represented as two regions in the map (FIGURE 3). The total analysis time per sample was approximately 1 min, and only 10 µl of seminal plasma was required to perform the

analysis. The sensitivity and specificity of the different spectroscopic measurements reproducibly exceeded 80%. This study revealed that different levels of OS biomarkers are uniquely associated with semen plasma of normal men compared with different forms of male-factor infertility. Hence, metabolomic profiling of semen using NIR spectroscopy and proprietary chemometrics and bioinformatics may provide a rapid, noninvasive and cost-effective diagnostic method of analyzing semen for abnormalities related to ROS damage and OS.

Recently, De Iuliis and coworkers demonstrated the nonmitochondrial generation of superoxide in spermatozoa by analyzing a superoxide-specific product, 2-hydroxyethidium, utilizing HPLC, NMR, MS and spectrofluorometry. Spontaneous production of superoxide by human spermatozoa was found to be negatively correlated with sperm motility. They validated the use of dihydroethidium as a probe for investigating the origin of OS in a male germline using spectroscopy techniques and the significance of superoxide detection in human spermatozoa for investigating male infertility [29].

In a small study conducted more than a decade ago, testicular metabolic integrity was investigated using ^{31}P-magnetic resonance (MR) spectroscopy in 23 patients with azoospermia and six healthy controls. Significant differences were found in the ^{31}P-MR spectra ratios of phosphomonoester (PM)–β-adenosine, PM–phosphodiester and inorganic phosphate–PM in normal and azoospermic testicles. Furthermore, among the azoospermia group, there were significant differences in the same peak area ratios between patients with testicular failure and ductal obstruction [30]. This showed the potential role of ^{31}P-MR spectroscopy as a noninvasive technique in the diagnosis of male infertility by differentiating testicular failure and ductal obstruction, and to predict the chances of pregnancy in patients planning for vasovasostomy for correcting a prior vasectomy [31].

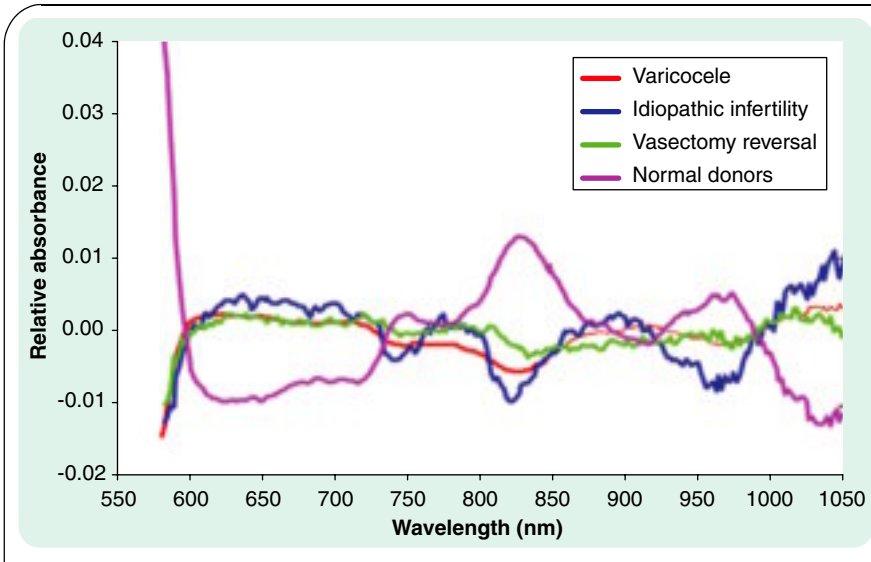

Figure 3. Near-infrared spectra from seminal plasma of four groups of men using metabolomic analysis. The groups include: varicocele patients (n = 70); idiopathic male infertility patients (n = 15); vasectomy reversal patients (n = 9); and healthy donors (n = 30). The difference between the groups was statistically significant.

In another study, Hamamah and coworkers analyzed the content of citrate, lactate, glycerylphosphorylcholine (GPC) and glycerylphosphorylethanolamine (GPE) in human seminal plasma (n = 60) utilizing ^{1}H-NMR. They observed that peak areas of ^{1}H NMR spectra for GPC, citrate and lactate were significantly smaller for patients with oligoasthenoteratozoospermia than for healthy controls. Furthermore, the peak area ratios for citrate–lactate and GPC–lactate were significantly different between patients with spermatogenic failure or obstructive azoospermia and healthy controls, and GPE–GPC between spermatogenesis failure and obstructive azoospermia. This study hence demonstrated the potential role of some quantitative biomarkers in the investigation of male infertility using ^{1}H-NMR spectroscopy [32].

The ability to quantify differences in the metabolomic profiles of various types of male-infertility patients could thus prove as a diagnostic tool to evaluate semen quality and function and investigate infertile men.

Metabolomics & assisted reproduction

Assisted reproductive techniques (ART) are one of the most important treatment options available to infertile couples diagnosed with male-factor infertility. A negative correlation has been observed between the ROS levels in follicular fluid and embryo culture media and ART outcomes [17]. The recent introduction of metabolomics into the field of ART might help predict pregnancy outcomes and improve success rates. Studies have been conducted to correlate the levels of various biomarkers present in the follicular fluid and the embryo culture media with pregnancy outcomes using metabolomic profiling.

In a recent study, 83 follicular fluid samples were analyzed for biomarkers of OS at specific wavelengths using NMR, Raman and NIR spectroscopy. The metabolomic profiles were quantified using proprietary chemometrics, and bioinformatics and were correlated to the pregnancy outcome. The CH–ROH ratio content in the follicular fluid was different between the pregnant and nonpregnant groups. Spectral regions obtained from all spectroscopic measurements revealed that an association existed between the OS biomarkers (–CH, –NH, –OH and ROH) and pregnancy outcome, with a sensitivity and specificity of 80% (FIGURE 4) [33]. The study demonstrated for the first time the potential role of metabolomics in oocyte selection for ART procedures. Further studies are needed to confirm these observations.

A prospective multicenter trial presented at ASRM 2006 involving two academic centers and a private ART center explored the role of metabolomics in embryo selection. In this study, the day-3 culture media of 108 embryos were collected and

analyzed using Raman and NIR spectroscopy. The spectral profiles revealed that the concentrations of –CH, –NH, –OH and ROH in the culture media of embryos that resulted in pregnancy were different from the concentrations of the culture media from the embryos that did not. The ratio of –CH to ROH was also different between the two groups. Results from the logistic regression analysis using Raman spectroscopy demonstrated a sensitivity of 95% and specificity of 80%, whereas NIR resulted in a 73% sensitivity and 83% specificity [15]. Scott and coworkers confirmed the results of the above study by performing a blinded evaluation utilizing a similar model. A total of 35 embryos were evaluated. The spent media were collected on day 3 cleavage stage after 44 h of embryo culture and evaluated using Raman and NIR spectroscopy. Changes in the hydroxyl modifications of various molecular constituents in the media were analyzed using the selective genetic algorithm, and a viability score was obtained for each sample based on its unique spectral profiles. A significant difference was found in the spectral profiles between the embryos that had the capability of implantation (and thus were reproductively competent) and the incompetent embryos [14]. Another study found unique metabolomic profiles of OS biomarkers in the discarded culture media (n = 228) of day-3 and day-5 embryos resulting in pregnancy and those that did not. These observations were consistent with all the methods of spectroscopy used (NMR, NIR and Raman), and the spectra from day-3 embryos were significantly different from that of the day-5 embryos [UNPUBLISHED DATA].

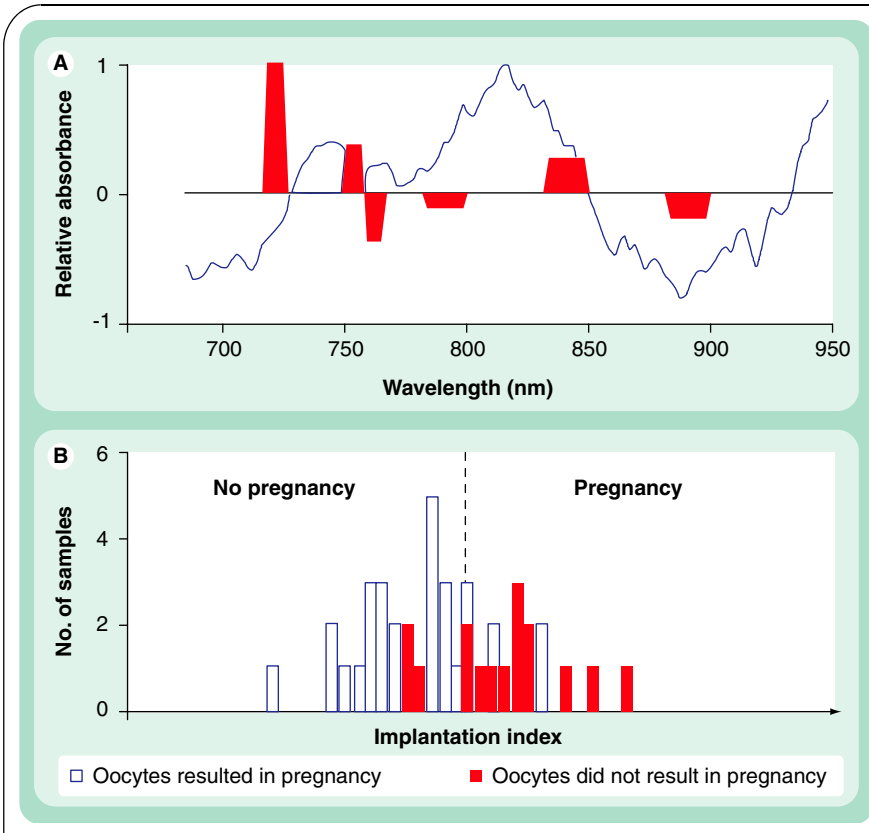

Figure 4. (A) Near-infrared spectra of a single follicular fluid specimen. The six distinct biomarker regions are illustrated. **(B)** Distribution of implantation index scores (calculated by subjecting the spectral data to bioinformatics algorithms) of the follicular fluid specimens, illustrating pregnancy outcome of the respective oocytes. The oocytes resulting in pregnancy and those that did not are shown both illustrated.

Houghton and coworkers investigated the amino acid turnover of embryo culture media by measuring the physiological mixture of 18 amino acids in the *in vitro* culture media using HPLC. They observed that the amino acid flux patterns of alanine, arginine, glutamine, methionine and asparagine from the embryos developing into the blastocyst stage exhibited a pattern distinct from the growth-arrested embryos, despite having similar morphological appearances [9]. Lopes and coworkers observed oxygen consumption of bovine embryos and discussed the use of embryonic respiration rate for the assessment of embryo quality [10].

Thus far, these preliminary studies have demonstrated the usefulness of noninvasive metabolomic profiling of biomarkers to select viable embryos for ART.

Expert commentary

The purpose of this article was to discuss the potential role of metabolomic profiling of biomarkers in the field of reproductive health. The tests available for analyzing semen quality are deficient and no consistent methodologies are currently available that can assist in gamete and embryo selection to ensure efficacy and safety of the IVF procedure. A number of recent studies have demonstrated the potential role of metabolomics in rapid, noninvasive testing of semen in infertile men, and oocyte and embryo selection for ART procedures.

Metabolomics testing in the field of reproductive health is still in its infancy. The technique has not yet been standardized and requires further research to validate the observations from preliminary studies. However it is likely to lead to:

- More men seeking infertility evaluation
- Enhanced success rates in ART procedures
- Reduction in the incidence of multiple births due to feasibility of single embryo transfer technique
- Reduced healthcare costs associated with providing medical care to multiple premature infants

Thus, this new technology paradigm is expected to fuel extensive research in near future.

Five-year view

Given the potential role of metabolomics in the management of infertility, several trials are underway to confirm the preliminary results achieved by earlier investigators. Currently, a multicenter, multinational study is being conducted to demonstrate

the relationship between an embryo's metabolomic profile and its implantation potential. Other potential applications of metabolomics in the field of reproduction are:

- Gamete selection: metabolomic profiling of biomarkers of OS can be developed as a routine method for assessing sperm function in ART.
- Functional genomic testing: aneuploidy screening and testing for other genetic conditions responsible for male infertility is a promising application of metabolomics.
- Endometrial receptivity: the role of metabolomics in the noninvasive examination of the endometrial lining of the uterus just prior to embryo transfer is being investigated and it is expected to increase the success rates of IVF.
- Fetal monitoring: metabolomic analysis of biomarkers in amniotic fluid has a potential role in assessing the development of the fetus.

The discovery of biomarkers is also gaining a great deal of interest worldwide as alterations of metabolites are involved in many reproductive disorders, and the identification of key metabolite markers will likely lead to greater diagnostic and therapeutic interventions. However, there are a number of challenges in developing the science of metabolomics [3,34]:

- Active metabolic pathways responsible for changes in the concentrations of various metabolites need to be identified.
- More than half of the metabolites that are detected need identification of their chemical structures. A naming protocol has been suggested by Bino and coworkers in order to name the metabolites with unknown chemical natures so that they can be recognized between different laboratories [35].
- As metabolite data are multivariate and hence complex, it is essential that the data are validated prior to being uploaded. In order for metabolomics to develop, standards must be adopted that will allow the integration of large amounts of data generated in metabolomics experiments and enhance the reproducibility and credibility of the data.

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Key issues

- Metabolomics is the systematic study of the inventory of metabolites, as small-molecule biomarkers that represent the functional phenotype in a cell, tissue or organism.
- Metabolomics may provide us with better, more useful information with higher throughput at a lower cost than genomics, transcriptomics or proteomics.
- Metabolomic profiling of semen, using near-infrared spectroscopy and proprietary chemometrics and bioinformatics, may provide a rapid, noninvasive and cost-effective diagnostic tool to evaluate semen quality and function.
- Differences in the concentration of oxidative stress biomarkers (–CH, –NH, –OH and ROH) have been found to be uniquely associated with semen plasma of healthy men compared with patients with idiopathic infertility, varicocele and vasectomy reversal.
- ^{31}P-mass resonance spectroscopy has shown a potential role as an alternative for testicular biopsy for differentiating testicular failure and ductal obstruction by utilizing phosphomonoester and β-adenosine triphosphate as biomarkers.
- NMR spectra have shown significant differences in citrate, lactate, glycerylphosphorylcholine and glycerylphosphorylethanolamine among semen samples of men with spermatogenetic failure, obstructive azoospermia, oligoasthenoteratozoospermia and healthy donors.
- Metabolomic analysis of follicular fluid and embryo culture media has revealed an association between biomarkers of oxidative stress (–CH, –NH, –OH and ROH) and pregnancy outcome of oocytes and embryos and thus has shown promise in assisted reproduction.
- Further research is needed to confirm all the preliminary observations and validate the usefulness of these tests before they can be put into clinical practice.

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