# OMICS: Current and future perspectives in reproductive medicine and technology

**Rocío Rivera Egea, Nicolás Garrido Puchalt, Marcos Meseguer Escrivá[1], Alex C. Varghese[2]**

Andrology Laboratory and Semen Bank, Instituto Universitario, [1]IVF Laboratory Instituto Universitario, IVI Valencia, Spain, [2]Dispur Polyclinic, Guwahati, Assam, India

**Address for correspondence:**
Dr. Rocío Rivera Egea, Andrology Laboratory and Semen Bank, Instituto Universitario IVI, Calle Guadassuar Bajo 1, 46015 Valencia, Spain.
E-mail: rocio.rivera@ivi.es

Received: 30.10.2013
Review completed: 14.01.2014
Accepted: 26.02.2014

**Access this article online**

Quick Response Code:

Website: www.jhrsonline.org

DOI: 10.4103/0974-1208.138857

## ABSTRACT

Many couples present fertility problems at their reproductive age, and although in the last years, the efficiency of assisted reproduction techniques has increased, these are still far from being 100% effective. A key issue in this field is the proper assessment of germ cells, embryos and endometrium quality, in order to determine the actual likelihood to succeed. Currently available analysis is mainly based on morphological features of oocytes, sperm and embryos and although these strategies have improved the results, there is an urgent need of new diagnostic and therapeutic tools. The emergence of the - OMICS technologies (epigenomics, genomics, transcriptomics, proteomics and metabolomics) permitted the improvement on the knowledge in this field, by providing with a huge amount of information regarding the biological processes involved in reproductive success, thereby getting a broader view of complex biological systems with a relatively low cost and effort.

**KEY WORDS:** Biotechnological technologies, molecular biomarkers, OMICS, reproductive medicine

## THE "OMICS" AND THEIR IMPORTANCE TO IMPROVE ASSISTED REPRODUCTION RESULTS

Fertility problems affect about 15% of couples at reproductive age,[1] being diagnosed about 25% as idiopathic (unknown origin) infertility. Assisted reproduction techniques (ART) may help to overcome infertility in order to achieve a pregnancy, although their effectiveness is still far from perfect.[2] One of the reasons is that molecular physiology of germ cells, embryos and endometrium, the three components conditioning reproductive results, remains yet poorly understood.[1] However, the therapeutic approach consists in repeating treatments, frequently without a real knowledge of the reasons behind the failure.

Unveiling all molecular factors involved in the reproductive function may help in these couple's infertility management, counseling and success chances. Up to now, several factors have been described as relevant in this process, leading to think that infertility may be caused by a multifactorial failure in one or more cell-type/tissue. This was a very complicated issue to study a decade ago, but fortunately, the emergence of OMICS technologies allow us to improve the knowledge in this field thereby getting a broader view of the complex biological system,[3] displaying the main advantage of obtaining a huge amount of information at a relatively low cost and effort.

These OMICS technologies are disciplines that include the study of the events and interactions of cellular structures and processes from deoxyribonucleic acid (DNA) to biological function, i.e., from DNA and genes to metabolites in a complex and global way. Therefore, epigenomics is the science that studies the heritable changes in gene expression that occur without any changes in gene sequence;[4] in the same way that genomics studies the complete set of genes expression of certain cell-types or tissues, proteomics studies the changes in all proteins expressed and translated from a single genome,[5] secretomics describes the proteins produced by the cells into the surrounding medium and metabolomics, studies simultaneously metabolites concentration and their fluctuations in a defined environment.

Using OMICS platforms, all classes of biological compounds, epigenetic marks, genes, messenger ribonucleic acid (mRNA), proteins and metabolites can be analyzed. In other words, the differences are that genomics/transcriptomics enables evaluation of potential information, proteomics permits assessing actually executed plans, and metabolomics will mostly display the results after these plans' execution.[3]

## OMICS TECHNOLOGIES

To analyze cells or tissues by their respective "omic" approach, very different biochemical and biotechnological technologies are employed in each case, and some examples are listed below:

- Epigenomics uses bisulfite sequencing to analyze DNA methylation that is the most common epigenetic marker,[6,7] and pyrosequencing[8-10]
- Genomics use fluorescence *in situ* hybridization (FISH),[11] comparative genome hybridization arrays (CGH),[12-15] bacterial artificial chromosome arrays (BAC), single nucleotide polymorphisms arrays (SNPs)[16-18]
- Transcriptomic uses mRNA microarrays[19-23] and real time polymerase chain reaction (RT-PCR)[24-27]
- Proteomics technologies include separation techniques such as one-dimensional sodium dodecyl-sulfate polyacrylamide gel (1D-SDS-PAGE),[28,29] two-dimensional (2D) PAGE[28-31] and 2D differential gel electrophoresis (2D-DIGE).[32,33] Other techniques are high-pressure liquid chromatography (HPLC) and ultra-pressure liquid chromatography,[34,35] reverse-phase liquid chromatography tandem mass spectrometry (RP-LC-MS/MS),[36] proteins' arrays and bioinformatics methods,[37] MS,[15,16] and matrix-assisted laser desorption ionization time-of-flight mass spectrometry[33,38,39]
- Metabolomics employ techniques such as gas chromatography-MS (GC-MS), LC-MS, HPLC,[40] Raman spectroscopy,[41,42] Near infra-red spectroscopy (NIR)[42] and H nuclear magnetic resonance (H-NMR).[43]

Once the results from these techniques are obtained, they are frequently validated by using another technique. For instance, in transcriptomics, mRNA microarrays were validated by RT-qPCR,[24,26,44] in proteomics 2D-DIGE results have been validated by immunostaining, the western blot[33,45] and enzyme-linked immunosorbent assay.[37,46]

Each omic technology described above can be put into practice in assisted reproduction field in order to define the optimal molecular traits of the cells and tissues involved in reproduction, namely spermatozoa, testis, oocyte, granulosa cells, embryos and endometrium, and also their metabolic products in seminal plasma, follicular fluid (FF), culture media, etc., This system biology approach can find the best spermatozoa and oocyte that can result in fertilization and the best embryo that can implant and result in a live birth, improving the assisted reproduction success. Hence, it is necessary to analyze these cells that play an important role in reproduction. This review is organized to provide a brief background on the types of OMICS technology and its application based on each target cells or tissues.

## EPIGENOMICS IN ASSISTED REPRODUCTION

Waddington[4] defined the epigenetics as "heritable changes in gene expression that occur without any changes in gene sequence." There are different types of epigenetic modifications such as DNA methylation, histone modifications as well as the presence of non-coding RNA but also we can find post-translational modifications as phosphorylation, ubiquitylation, nitrosylation and sumoylation in germ cells[47,48] being the most common the first one. These modifications are important to regulate cellular development, differentiation and function and are considered heritable because the chromatin preserves its epigenetic status during cell mitosis[49,50] and therefore might be transmitted to the next generation.

By definition, only one allele (maternal or paternal) is active, and the inactive one is epigenetically marked. DNA methylation is almost exclusively restricted to CpG dinucleotides.[51] In the human development, there are two periods of epigenetic modifications: Gametogenesis and early pre-implantation development. During these periods, female and male germ cells undergo a process where all imprinting marks are erased from the genome. The, methylation marks are reestablished before fertilization and early embryonic life respectively.[49] These methylation marks are essential for achieving cell-type specific gene expression patterns in different tissues even for sex differentiation (X-inactivation), since all cells of an organism exhibit the same genotype.[50]

The interest about the association between ART and imprinting disorders has increased in the recent years because of the results found in animal studies,[52] although their extrapolation to human individuals must be carefully confirmed. However, the incidence of children conceived by ARTs presenting rare genomic imprinting diseases seem increased.[49] Some examples are Beckwith-Wiedemann syndrome, that is related to hypomethylation of the maternal (KCNQ1OT1 differentially methylated regions [DMR]), Angelman's syndrome (AS), caused by a shortage of maternal UBE3A expression in the small nuclear ribonucleoprotein polypeptide N imprinting cluster, Silver-Russell Syndrome caused in most cases by histone 19 (H19) DMR hypomethylation and 5-10% by maternal uniparental disomy chromosome 17, retinoblastoma and

Prader-Willi syndrome. With regard to the last two diseases, in most cases the underlying molecular mechanism is a mutation.[50] However, it is not clear yet whether these adverse effects are the results of ARTs techniques *per se* or the consequence of parental subfertility.

The emerging data also suggest that endocrine-disrupting chemicals (EDCs) (tobacco, pesticides, drugs, etc.)[48,50,53,54] are associated with perturbation of DNA methylation patterns, as well as stress, irradiations and nutrition problems.

### Cumulus cells, granulosa cells and the oocyte

Zama and Uzumcu[48] described the way by which female reproductive system may be sensitive to EDC exposure. They showed that the exposition of EDC in the ovary can lead to alterations in the epigenetics regulation in the oocyte, even causing transgenerational epigenetic effects.

The timing at the establishment of the epigenetic programming differs between male and female germ cell differentiation, taking place earlier in the male than female germ line (prospermatogonia stage and after the birth when oocytes grows respectively) and this status makes them more vulnerable to the effects of EDCs. The perinatal exposure to methoxychlor (MXC) has, indeed, been shown to cause a hypermethylation of the estrogen receptor beta (ESR2) ovarian ESR2 promoter and other ten genes in the ovary. In addition, diethylstilbestrol (non-steroidal synthetic estrogen), genistein (flavonoid phytoestrogen) and bisphenol A (plasticizer) also affect epigenetics mechanism in the oocyte. Therefore, it has been proved that perinatal EDC exposure affects adult ovaries and female reproductive tissues, inducing reproductive dysfunction. Moreover, these effects are mediated by steroid hormone receptor, ERβ in ovary and ERα in the uterus and perhaps both in the hypothalamus and pituitary. Hence, agonist action of ERβ lead to a precursor to premature ovarian failure and antagonist action showed inhibitory effects on follicular maturation reducing female fertility.[48]

The major problem about the studies involving imprinting-directed epigenetic reprogramming regards ethical reasons. Sato *et al*.[55] were the only authors publishing a study where the immature oocytes were of human origin. They reported that almost 50% of the maternally imprinted MEST, KCNQ1OT1 and PLAGL1DMR (DMR) alleles in primary follicle were methylated and increasing this level of methylation as follicle stage progress. However, the paternal imprinting H19 DMR was partially erased at primary follicles diminishing around 10%.

In ARTs, it is very common to perform ovulation induction to achieve the maximum number of oocytes. The problem in such a situation is related with the maternal age and suboptimal oogenesis, because aggressive stimulation can be confounded with potential effects on imprinting. These oocytes present different pattern of methylation mainly affecting at MEST DMR[55] and at H19, although paternal DMR also appears unmethylated in DLKI and MEG3.[56]

In Assisted conception treatments, the aim of controlled ovarian stimulation is to obtain the metaphase II (MII) oocytes, which can be fertilized either by conventional insemination or intracytoplasmic sperm injection (ICSI). Sometimes, aspiration of immature follicles followed by *in vitro* maturation (IVM) of oocytes is practiced to get the MII oocytes. However, this process can also affect imprint establishment or maintenance.

### Spermatozoa

Epigenetic processes are also involved in spermatogenesis, and failures may lead major adverse health and behavioral effects. In mature spermatozoa, the paternally imprinted DMRs H19 are completely methylated, while the maternally imprinted ones (MEST) are unmethylated.[47] The progression from diploid spermatogonia to haploid spermatozoa and testis specific gene involves testis specific gene expression, mitotic and meiotic division and histone-protamine transition. All of these developmental processes are regulated by epigenetic controls.[57]

Results from studies in sperm suggest that male infertility may contribute to epigenetic effects in pregnancies through ARTs.[49] Navarro-Costa *et al*.[6] in their study have reported that incorrect epigenetic marks (DNA methylation of the promoter CpGisland) in two germline regulator genes (DAZL and DAZ) may be correlated with male gametogenic defects causing a spermatogenic failure. These two genes remain unmethylated in germ cells where they are expressed. However, morphologically abnormal spermatozoa in oligoasthenoteratozoospermic (OAT) samples present increased levels of DAZL methylations defects. Moreover, they noted that there were OAT-intrinsic epigenetic disturbances that may undermine correct germ cell development in these patients. Hence, in OAT spermatozoa, methylation is drastically reduced.

In another study,[7] they have suggested the existence of moderate changes throughout the genome that may have a cumulative detrimental effect on fertilization. They were focused on histone modifications suggesting that it is probably an incomplete replacement of histones by protamines in the genome leading to an abnormal histone localization pattern in infertile men. This is supported by the finding that H3 Lysine 4 methylation (H3K4 me) and H3 Lysine 27 (H3K27 me) methylation patterns in infertile men were generally similar to those found on of fertile donors. However, the amount of these histones retained at

developmental transcription factors and imprinted genes were decreased. In conclusion, in comparison with fertile men, sperm from infertile men present H3K4 me enrichment in gametogenesis and H3K27 me represses loci in the future embryonic program.

Regarding DNA methylation, in the same article,[7] they reported that infertile men are more susceptible to these changes independently of the changes in chromatin in the mature sperm. Finally, they concluded that the genome packaging (histone modifications) and epigenomic alterations (DNA methylation) are altered in the gametes of infertile men, suggesting that the establishment of epigenetic marks in the spermatogonial stem cell remain largely intact in infertile men.

DMRs methylation also is related to sperm concentration, being the mainly cause of male infertility. In this case, sperm concentration is positively correlated with H19 methylation and negatively correlated with MEST methylation, that is normally absent.[8] Together, these data clearly indicate that DMR methylation defects are associated with poor spermatogenesis. However, sperm imprinting disorders seem to not affect the outcomes of ARTs. The same conclusion was obtained in other studies too.[9,10]

A relevant cause of spermatogenesis impairment and male infertility through methylation changes are the EDCs. Several studies have reported the effect of these disruptors in male gametes through epigenetic modifications of candidate genes regulating the spermatogenesis pathway.[47,53,58] Stouder and Paoloni-Giacobino[53] evaluated the possible deleterious effects of MXC on mice's imprinted genes considering that in human male spermatogenesis the effects of EDCs might be analogous although more complex. This study shows a consistent deleterious effect of EDCs on male gametogenesis and imprinting in the sperm while no effects were present on somatic cells. MXC induces transgenerational alterations in methylation pattern of paternally and maternally imprinted genes in the sperm from the F1 offspring, disappearing in F3 and leading to decrease sperm concentration.

Finally, another epigenetic modification named sumoylation[59] has been described in sperm. Sumoylation is a post-translational modification that is involved in the regulation of numerous cellular events. In sperm cells, the excessive sumoylation is a marker of defective spermatozoa and in these cells sumoylated proteins were localized in all sperm parts. High-level of sumoylation are related with non-motile and morphologically abnormal sperm. Numerous human sperm proteins have also been identified as targets of nitrosylation[60] and ubiquitination.[61]

Regarding the testicular tissues, the function of DNA methylation is different when compared somatic cells. Epigenetics has been related with gene deregulation causing the development of testicular cancer.[57]

### Embryos

Early embryonic developmental stages are considered sensitive to the effects of environmental influences on the establishment of epigenetic marks. After gamete's fusion, a global passive maternal demethylation takes place and at the end of the morula stage, and remethylation starts.[62] In blastocyst stage, trophoectoderm presents less methylation than the inner mass.[63]

Before being transferred back to the uterus, the embryos are grown *in vitro*. Low-quality and arrested embryos have been shown to harbor hypomethylation of H19 DMR. The first array-based analysis on CpG methylation at birth showed that the CpG sites are affected by ART procedures for a considerable frequency of sites, hypermethylating cord blood and hypomethylating placenta tissue.[50]

All epigenetic disorders can affect live birth. Several defects in fetus health have been reported as low-birth weight, diabetes, cardiovascular disease, glucose tolerance, systolic blood pressure.[50] Small *et al*.[64] have reported an imprinted gene KLF14 which could be the regulator in diabetes and adipocyte-related metabolic disease risk.

### Endometrium and placenta

In accordance with the rationale about EDCs, tobacco is associated with aberrant CpG dinucleotide specific DNA methylation in the adult, induced by interactions on regulation of placental gene expression and their changes in developmental regulation and fetal programming.[54] In this way, placenta global DNA methylation is decreased with tobacco smoke exposure which can produce genome instability and cancer.

Associations with genetic mutations of cancer-related genes have been shown, but to date haven't been completely explained.[65] Gene's silencing by DNA hypermethylation, a hereditary epimutation of DNA mismatch repair, may underlie carcinogenesis in endometrial cancer. Therefore, the new therapies incorporate histone deacetylase inhibitors.

The epigenetic modifications are important, mainly in ARTs. Their alterations have been related to infertility and offspring problems. OMICS techniques may permit the analysis of imprinting modifications thereby improving fertility success and avoid associated problems.

## GENOMICS IN ASSISTED REPRODUCTION (DNA)

Multiple miscarriages are observed among infertile couples at a higher order and the current ART protocols are coupled with high first-trimester miscarriage rates.

### Embryos

Many studies indicate a significantly higher proportion of the aneuploid embryos as a result of *in vitro* fertilization (IVF) and the rate of aneuploidy is influenced by the patient's age.[66,67] The rate is about 25% of the embryos at the age of 30 but in as many as 60-70% of the embryos by the time a woman reaches 40 years of age.[68]

Since morphological scoring by itself cannot select the right embryos with euploidy, the transfer or cryostorage of apparently "normal looking" embryo carries considerable risk. Therefore, chromosome numbers and integrity have been effectively investigated as a determinant of embryo viability in assisted conception procedures as elective single embryo transfer is getting wider acceptance and regulatory mandates. These genetic approaches have recently been categorized as pre-implantation genetic screening (PGS) by the European Society for Human Reproduction and Embryology (ESHRE) to distinguish them from pre-implantation genetic diagnosis, done for infertile couples who are at high-risk of transmitting a genetic abnormality to their offspring, which include chromosomal anomalies and single gene defects.[69]

Screening embryos by FISH was a reasonable first answer to screen aneuploid embryos, but the approach was too limited because it could not screen all chromosomes at the same time. It was also shown that when embryos found to be aneuploid on day 3 are retested on day 5, about 20-30% of them may correct the problem and turn out to be euploid.[11] In about 20% of embryos, mosaicism can be found and this may influence the test results. Testing the entire genetic content and testing on day 5 at the blastocyst stage could improve the accuracy of the screening. Both American Society for Reproductive Medicine and ESHRE practice committee states that aneuploidy screening with PGS using FISH in IVF practice does not appear to be justified.[70,71]

CGH allows the testing of all 24 types of chromosomes; therefore, it should not miss aneuploidies. The clear advantage of CGH compared to FISH is that the copy number of all chromosomes can be determined. The copy number variations (CNVs) are general changes in the amount of a region of the genome, including duplications and deletions of around a 1000-5 million bases. Besides, CGH allows a detailed analysis of the entire length of each chromosome compared with FISH, which enable the detection of chromosome segments imbalance.[12]

To get the requisite embryonic DNA polar body, blastomere or trophectoderm (TE) can be used. Testing on day 5 allows a biopsy of the TE. Mosaicism is still an issue if the biopsy is done on day 3.[72-75] Because it takes longer to receive the test results in day 5 biopsies, this approach requires elective cryopreservation of the embryos and transfer at a later cycle. With many clinics nowadays getting proficient in vitrification technology, the biopsy and vitrification of biopsied embryos/blastocysts offers a viable option. In a recent prospective cohort study, Schoolcraft *et al*. (2010)[14] employed CGH in day 5 Blastocysts, which were then vitrified and transferred in a subsequent cycle. They achieved implantation and pregnancy rates of 68.9% and 82.2%, respectively.

Earliest report of conventional CGH applied to human early-stage embryos was reported around a decade ago.[13] To determine how copy numbers differ from a reference (control) sample: The sample and reference DNA are labeled with different colored fluorescent probes (green and red). Sampling larger human DNA segments (100-200 kb in size) incorporated into BAC clones results in BAC arrays, while smaller DNA segments (~60 nucleotides) constitute oligonucleotide (oligo) arrays. The two samples are applied to immobilize DNA on the array, and complementary sequences bind. Where there is no change in sequence copy number in the test sample, there will be equal binding of test and reference sample DNA, and equal amounts of green and red fluorescence will produce a net combined emission color (yellow).

For sequences where there has been a duplication in the test sample, there will be more green than red fluorescence and an overall green emission; conversely, deletions will result in a reduced level of green fluorescence relative to the red fluorescence from the reference sample, and a net emission of red light. By employing bioinformatics tools, the green-to-red fluorescence ratio for each DNA segment is mapped to the chromosome, resulting in an array profile. A variety of microarray-CGH platforms are available. As an example, the Cambridge-based company BlueGnome offers an array-based CGH protocol which allows analysis of biopsied polar body (PBs) within 11 h.[15] A proof-of-principle study was carried out by Geraedts *et al*.[76] and Magli *et al*.[77] to determine the reliability of an alternative form of PGS, i.e., PGS by PB biopsy, with whole genome amplification (WGA) and microarray-based CGH array analysis. Array CGH was applied on both first and second polar bodies to assess the copy numbers. The corresponding zygote was then also processed by array CGH for concordance analyses in cases of the PBs were

found to be aneuploid. The study concluded that the ploidy status of a zygote can be predicted with reliable accuracy by array CGH analysis on both PBs. One major drawback of polar body biopsy is that this approach will fail to detect aneuploidies that occur during meiosis II, and those arising from paternal origin. Alternatively, embryo biopsy and CGH analysis may be coupled with cryopreservation.

One cell from an embryo contains approximately 6 pg of DNA.[78] An initial input of hundreds of nanograms DNA is required for any array methods. Hence WGA method is employed to analyze single cell CNV of genome. WGA approaches can be either PCR-or non-PCR-based (isothermal) methods such as primer extension preamplification protocol, degenerate oligonucleotide primed-PCR, multiple displacement amplification (MDA) (non-PCR based). The later one appears to have several prominent advantages compared to the previous ones. The final product of MDA are of sufficient length and integrity, and the average product length is >10 kb. Other than the MDA method, OmniPlex is a useful approach for obtaining sufficient DNA from a limited amount of samples for genetic diagnosis.[79] GenomePlex (Sigma-Aldrich, St. Louis, MO 63103, USA) - WGA technology based on non-enzymatic random fragmentation of genomic DNA. The GenomePlex WGA allows for a rapid and highly representative, up to 1000-fold amplification of genomic DNA from trace samples as little as 10-100 ng. In this system, genomic DNA is subjected to random chemical fragmentation followed by a series of stepped, isothermal primer extensions to convert the resulting DNA fragments into an amplifiable library, called the OmniPlex Library. The OmniPlex Library is then subjected to traditional amplification using universal primers and a limited number of cycles. To meet the high-throughput requirements for amplification of genomic DNA samples, an automated method has been developed using the Biomek FX workstation. Recently a robust and reliable aCGH platform has been pioneered, BlueGnome CytoChip arrays - The CytoChip BlueGnome array which is available as whole-genome BAC array with a median resolution of 0.5-1 Mb.

A very recent study describes the clinical application of array-CGH technology to simultaneously screen embryos from both reciprocal and robertsonian translocation carriers for unbalanced translocation derivatives, as well as aneuploidy status of all 24 chromosomes.[80] The study involving 28 preimplantation genetic diagnosis (PGD) cycles, resulted in the establishment of chromosomally balanced pregnancies in 12 couples. Biopsied cells from the day 3 embryos were lysed and the DNA amplified by WGA. WGA products were then processed by array-CGH using 24sure arrays (BlueGnome, Cambridge). Euploid embryos were then selected for transfer on day 5 of the same cycle.

SNPs are common changes to a single base code in the DNA. Everyone has around ~10 million SNPs in their genome, some of which may predispose individuals to disease but most of which are benign. Rare single base changes are called mutations and may cause serious inherited diseases. CGH-microarray platforms involve simultaneous hybridization of differentially labeled DNAs to the same microarray. However, SNP-microarrays assess test and reference samples, separately, in parallel. The Affymetrix Genome-Wide Human SNP Array 6.0 features 1.8 million genetic markers, together with more than 906,600 SNPs and more than 946,000 probes for the detection of CNV. With the Affymetrix platform, analyses of 250,000 SNPs in first PB biopsies[16] and in day-3 embryos[17] have been reported.

In SNP method, a blood sample is taken from each parent and is screened in parallel with the cells from the embryo. The SNP results seems to be more reliable and precise than any other methods as it uses parent DNA as reference data to compare to the embryonic DNA. It can also detect whether the chromosomal abnormalities occurred before or after fertilization.

In prospective clinical study[18] employed a single SNP microarray - based method for simultaneous PGD of unbalanced inheritance of rearranged chromosomes and 24-chromosome aneuploidy screening. The study identified that the clinical pregnancy rate in the 12 patients receiving a transfer was 75% (six singleton deliveries and three on-going singleton pregnancies at the time of writing the article). Authors suggest this SNP array method is the first opportunity to improve outcomes by comprehensive identification of euploid embryos from translocation carrier couples. A case report by Brezina *et al*.[81] detailed the amplification of DNA so that both aneuploidy screening and single-gene testing was carried out. 10 blastocyst-stage embryos from a couple, both carriers of GM1 gangliosidosis, underwent TE biopsy. Developments in microarray technology for embryo testing 23 chromosome microarray analyses for aneuploidy and specific DNA sequencing for GM1 gangliosidosis mutations were performed and elective single embryo resulted in a viable pregnancy.

SNP-microarrays also provide genotype data, yielding a DNA fingerprint for each embryo tested. Such DNA fingerprints enable parental origin to be confirmed, thereby reducing lab related errors of transferring wrong embryos to patients and help identify the parental origin of aneuploidy. Since SNP array methods helps to identify the embryo which gave rise to implantation makes it possible to study additional factors associated with embryo viability and death.

A novel microarray platform that provides comprehensive aneuploidy screening of embryos while providing an

indication of mitochondrial DNA copy number and average telomere length was recently reported by Konstantinidis.[82]

## TRANSCRIPTOMICS IN ASSISTED REPRODUCTION (mRNA)

### CCs, GCs and the oocyte

Although using an invasive technology, microarray-based transcription profiles of oocytes at various stages of growth and maturation has provided a better understanding of the genes expressed during oocyte development. Disruption of transcription within an oocyte or a modification of their adequate transcriptomes (set of mRNAs expressed at a defined stage) could negatively affect its growth and development as well as the resulting embryos',[83] since oocyte mRNAs pool is correlated with the ability to develop until the blastocyst stage.[84]

For instance, transcriptomes of MII oocytes from normal women differ from the oocytes of women with polycystic ovaries (PCO). This may explain reduced fertility in women with PCO.[83] With the use of microarray platforms, oocyte quality can be estimated based on the expression profiles of at least 160 different genes,[85] and many of these markers are involved in the pathways of cell growth and death[86,87] being the expression of 29 genes the difference between good and bad quality oocytes.[84] There is great potential to find oocyte quality biomarkers among these transcripts, and gene expression levels of Pentraxin 3 (PTX3), hyaluronic acid synthase 2 (HAS2), cyclooxygenase 2 (COX-2) prostaglandin-endoperoxide synthase 2 (PTGS2) and gremlin 1 (GREM1), have already been studied for this purpose.[88] A study by Assidi *et al*.[89] identified several potential markers of oocyte competence. These are HAS2, inhibin beta A, epidermal growth factor receptor, GREM1, betacellulin (BTC), CD44, tumor necrosis factor-induced protein 6 (TNFAIP6), and PTGS2. The authors suggest that these biomarkers could be potential candidates to predict oocyte competence and to select higher-quality embryos for transfer.

Clinicians can utilize the information gained from the biomarkers associated with oocyte maturation to make informed choices regarding the selection of ovarian stimulation, thereby facilitating the selection of appropriate culture and oocyte manipulation techniques.[90]

It has been proposed that aberrant degradation or maintenance of certain classes of transcripts during oocyte maturation could be deleterious to oocyte quality, influencing developmental competence.[91,92] These events could lead to improper activation of the embryonic genome and an altered transcriptome that is incompatible with implantation. This kind of DNA fingerprinting approach may in near future identify a set of morphological characteristics, metabolic and biochemical non-invasive markers of viability.

As indicated above, oocyte mRNA microarrays are an invasive technique. Therefore, it is necessary to have another technique which can indirectly assess the oocyte quality without harming them. A non-invasive microarray approach has been adopted by investigating the GCs and/or CCs as a surrogate marker of oocyte potential in several studies[24,88,93-96] This approach has recently provided some interesting results in a study by Assou *et al*.[97] They found that CCs obtained from oocytes that developed into embryos with a good morphology had different gene expression profiles according to the pregnancy outcome of the embryo. They demonstrated that the expression of BCL2 L11, PCK1 and NFIB in CC is significantly correlated with embryo potential and successful pregnancy whereas, Gebhardt *et al*.[98] reported that VCAN, PTGS2, GREM1 and PFKP showed expression patterns indicative of good quality oocytes. A report by Bettegowda *et al*.[96] indicates a functional role for CC cathepsins in compromised oocyte competence suggesting that CC cathepsin mRNA abundance may be predictive of oocyte quality.

In a very recent study, Ouandaogo *et al*.[24] have reported that oocyte maturation and competence to development depends on its close relationship with CC and the CC transcriptomic profile is affected by the degree of oocyte nuclear maturation as well as by the oocyte maturation conditions. In this study,[24] they compared the transcriptome of CC of oocytes matured *in vivo* or *in vitro* and demonstrated that it varies according to maturation conditions and oocyte maturation stage because in IVM there exist a down-regulation in genes related to cumulus expansion (TNFAIP6, PTGS2 and PTX3) and oocyte maturation (LHCGR, EREG, AREG and BTC), and showing up-regulation in genes related to proliferation.

Somatic cells associated with the oocytes' function, such as CCs, are also related to embryo competence and pregnancy outcomes. In this sense, the transcriptomic signature of CC included 630 genes associated with pregnancy outcome, being those that were differentially expressed mainly up-regulated, suggesting that embryonic competence is acquired through necessary CC transcriptional activation.[93] Assou *et al*.[97] reported that among 630 genes, 45 were identified as biomarkers of embryo quality and pregnancy outcomes. Furthermore, Hamel *et al*.[99] suggested that 115 genes were differentially expressed between CCs from follicles that achieve pregnancy and CCs from follicles resulting in the early embryo arrested.

As MII oocyte (mature oocyte) is pivotal in ART scenario, it is interesting to study CC gene expression profile at

this specific stage because the transcriptomic profile vary according to the nuclear maturation stage of oocytes. Ouandaogo *et al*.[94] showed that there are only few genes differentially expressed in human CCs according to oocyte nuclear maturation stages. Among them, 25 genes were differentially over-expressed, 10 in CCs of Germinal Vesicle 4 in CCs of MI and 11 in CCs of MII oocytes.

As demonstrated, the dialogue between CC and oocyte is very important for the acquisition of oocyte and embryo competence, because CCs provides nutritional and metabolic support to the oocyte through the gap junctions and being essential for its growth, maturation and competence.[100] Indeed, inferior oocyte quality and poor embryo development have both been correlated with CC apoptosis[25] and aneuploidy oocytes represent less transcriptionally active CCs.[101] Therefore the cumulus corona cells can provide potential biomarkers to predict oocyte quality,[24] embryo quality[88] and pregnancy outcomes[97,99] and guide the optimization of process used in ARTs.[100]

On the other hand, GCs[95] also can be analyzed by microarray. As indicated above, the application of this technique in GCs is considered a non-invasive technique because the oocyte is not damaged. However, these cells have a drawback due to potential contamination by blood when the follicular aspiration is carried out affecting the gene expression profile.

GCs and CCs share many expressed genes, but GCs transcriptome also present specific genes expression. Maman *et al*.[102] showed that the expression of luteinizing hormone receptor mRNA was higher in GCs compared with CCs and reported that high-expression in GCs of MII oocytes was correlated with decreased fertilization rates, whereas lower levels were correlated with lower oocyte maturity.

### Spermatozoa and testis

In addition to the oocyte, microarray technology has been used to gain a more detailed understanding of the molecular basis of male infertility[103,104] by studying the spermatogenesis-related gene expression profiles between fertile and infertile males.[105] Uncovering the differences in mRNA profiles will yield a greater insight into potential markers for fertility as well as clues to its indirect causes or direct triggers.[106] The RNA amplification methods and microarray and RT-PCR technology allow us to analyze on a large scale male fertility status and will reveal much more information when compared with the techniques currently employed in fertility clinics.[104] But in this case the method employed is invasive as in the oocyte and we cannot use the spermatozoa analyzed, although the information can be retrieved from a small part of the ejaculate, while permitting the other part being utilized for ART.

Even if there is a debate regarding the roles of sperm RNA, a great number of articles refer to this topic[15,19-23,27,78-85,101-111] Currently, it has been proved that spermatozoa introduce some of their mRNA into the oocyte and these mRNAs that are necessary from the first embryo cleavages, remain stable until the activation of the embryonic genome, affecting phenotypic traits of embryos and offspring.[107] The amount of sperm mRNA inside oocyte is minimal in comparison with maternal mRNA[109] but its composition is very complex.

Microarrays and RT-PCR can be used in both testicular biopsy and sperm cells. In this way, it could analyze the global gene expression using testicular biopsies improving our knowledge about causes and diagnosing spermatogenic failure. Fox *et al*.[27] analyzed global gene in testicular tissue. They compared the global gene expression between patients with normal spermatogenesis and patients without spermatogenesis (no germ cells present). The results showed that the expressed genes in normal spermatogenesis patients are related to spermatogenesis pathway (meiosis, DNA repairmen, sperm development and spermiogenesis) whereas genes of patients without spermiogenesis were related to ubiquitous expression patterns. Therefore, in testes the method has revealed a reliable and reproducible gene expression profile in infertile males.

In the same way, microarray technology could be applied in epididymal, but the human anatomy has done that the available studies used animals' models.[111] Even so the obtained results can help to obtain fertility markers.

The increase in the knowledge about sperm mRNA in the last years has allowed getting results and relating them with assisted conception outcomes. García-Herrero *et al*.[19] studied about sperm transcriptomics in intrauterine insemination (IUI). They reported that sperm transcriptome differs between sperm samples that achieve pregnancy after IUI versus those do not. In their results the transcript B-cell lymphoma 2 related with apoptosis was found in non-pregnant group as well as genes related to cell signaling, ions channel-like chloride channel Kb, potassium inwardly-rectifying channel or sodium channel non-voltage-gated 1 beta that are exclusively expressed in this group, whereas interleukin 8 (IL-8) that is a chemotactic cytokine was overexpressed in pregnant group. Later the same group reported[21] microarrays results in ICSI treatments. In this study, they used fresh and frozen sperm and standardized female factor with oocyte donation program. Results demonstrated that among 2000 transcripts exclusively expressed in pregnant or non-pregnant, about

50 transcripts were differentially regulated in the fresh samples of both groups. No differential transcripts were found in frozen samples.

A very recent study[23] related sperm microarray with IUI results trying to find the sperm genetic markers related to reproductive outcomes and unexplained infertility. For that, they analyzed 68 normozoospermic donor samples by microarrays used for IUI and compared the gene expression results according to donors' pregnancy rates. The results showed differences in some genes' expression (RPL23A, RPS27A, RPS3, RPS8 and TOMM7) being underexpressed in the group with the lowest pregnancy rate.

In other studies[22] also a differential gene expression between donors and patients have been observed and important differences were found which can be a potential forecaster of fertility success.

Finally another study[20] found differences between infertile males and donors of proven fertility in Gene Ontology terms, being able to use as markers of fertility success and male infertility.

Hence, microarray technology is a powerful tool for detecting different gene expression profiles between fertile and infertile males and in the diagnosis of pathologies[106] since mRNA is considered as a molecular resource for infertility investigation. Furthermore, some variation in the mRNA populations of the same patients can be indicative of spermatozoa quality and fertility status.[110]

## Embryos

Two independent and distinct waves of transcription events have been demonstrated during early embryo development in mice. One occurs at zygotic genome activation (1-2 cell stage) and one which occurs at the morula-blastocyst transition.[112,113] Moreover, analysis of the gene expression profiles of mouse 4-cell, morula, and blastocyst stage embryos revealed differential regulation of genes implicated in the process of compaction and blastocoel formation.[114] These findings indicate that the molecules vital for implantation events may only be detectable at the blastocyst stage of development, questioning the relevance of data by blastomere biopsy from early cleavage stage embryos.

Reports from multiple transcriptional analyses of transferable blastocysts from various origins are starting to accumulate.[115-118] El-Sayed *et al.* addressed the relationship between transcriptome of embryos and pregnancy success by measuring the gene expression of blastocyst biopsies taken prior to transfer to recipients. Interestingly, several clusters of genes were found to be differentially expressed between biopsies derived from blastocysts that resulted in no pregnancy, resorption, or calf delivery. Further ontological classification of the genes revealed that biopsies resulting in calf delivery were enriched for transcripts necessary for implantation (COX-2 and CDX2), carbohydrate metabolism (ALOX15), growth factor (BMP15), signal transduction (PLAU), and placenta specific transcripts (PLAC8). Biopsies from embryos that were resorbed were enriched with transcripts involved in protein phosphorylation (KRT8), plasma membrane (OCLN), and glucose metabolism (PGK1, AKR1B1). Biopsies from embryos that resulted in no pregnancy were enriched with transcripts involved in inflammatory cytokines (TNF), protein amino acid binding (EEF1A1), transcription factors (MSX1, PTTG1), glucose metabolism (PGK1, AKR1B1), and CD9, which is an inhibitor of implantation.[119]

Similar study in human by Jones *et al*.[118] proved that viable blastocysts do express a different transcriptome that is compatible with implantation competence. When competent embryos produce signals to the endometrium, this surrounding (endometrial epithelial cells [EECs]) can switch on certain genes in the embryo that could induce pregnancy establishment.[120] The application of such DNA fingerprinting at the pre-implantation embryo stage and at birth could potentially identify the specific embryo that is responsible for the establishment of pregnancy from a cohort of blastocysts transferred.

A very recent study[26] reported the transcriptome of human TE cells from day 5 blastocysts compared to day 3 embryos. TE transcriptome included 2196 transcripts involved in different function but GATA2, GATA3 and GCM1 were TE-specific. In day 3 embryos, 1714 transcripts were up-regulated. Moreover, stemness genes (NANOG and DPPA2) and other genes were included. These results might also provide new biomarkers for the selection of viable and competent blastocysts thanks to the simultaneous analysis of the transcript level of thousands of genes.

## Endometrium

Successful implantation requires competent embryos,[26,118,120] a receptive endometrium[44,121,122] and a synchronized mother-embryo cross-talk.[123]

Histologic evaluation of endometrium based on morphology has been considered a standard technique for a clinical diagnosis during the last decade or so. This evaluation was guided by Noyes, Herting and Rock's criteria, but its accuracy, reproducibility and clinical utility has been questioned in several studies.[124]

The main difficulty of endometrial tissue is that it is a highly dynamic and can result in morphological and

functional changes as a consequence of the menstrual cycle phase,[121] but is important to determine the window of implantation as well as gene expression profiles that identify the mechanisms involved in the early dialogue between blastocyst and endometrium.[123] For this reason, several studies analyzed the transcriptome in different phases of the menstrual cycle.[121,125]

For these reasons, it is necessary to develop a new tool able to describe specific features of endometrium, its menstrual cycle changes and receptivity status. In this sense, a new tool, based on microarray has been developed and is clinically available,[44] which is considered more accurate and robust than one mentioned above.

The endometrial receptivity array (ERA)[44,122] is a customized array that analyzes the endometrial transcriptome providing per patient a transcriptomic signature as a description of human endometrial receptivity and also to predict endometrial pathologies. Díaz-Gimeno *et al*.[44] developed an ERA that included 238 genes differentially expressed and 134 of them have been defined as the transcriptomic signature employed in the test. The aim of this system is to identify and diagnose the endometrial receptivity status during the window of implantation, trying to avoid implantation failures, hence improving reproductive results.

## PROTEOMICS IN ASSISTED REPRODUCTION

Clinical proteomics is an emerging field that seeks to apply this science in the search for biomarkers and the generations of protein profiles that can help predict, diagnose and monitor human pathologies,[126] such as infertility where informative protein profiles linked to optimal reproductive results can help in the improvement of diagnosis, fertility prediction and the development of molecular strategies to select the best gametes and embryos and most receptive endometrium.

### CCs, GCs, oocyte and liquid fluid

Proteomics is a recent science and therefore, there is limited information related to protein available in databases for humans.[127] However, in the last year an increasing knowledge about this science in reproductive medicine has been produced by several research groups. Nevertheless there is still little information about CCs and GCs as well as oocyte due to the necessity of large numbers of oocytes to perform these analyses.[128] Hence, all available information has been obtained from experimental animal models.

Meng *et al*.[129] identified 156 proteins composing a mouse mature oocyte protein profile using 2D electrophoresis coupled to MS. In another study,[130] the same technology was used resulting in 380 unique proteins identified in MII oocyte. Zhang *et al*.[36] reported with 1D-SDS-PAGE and RP-LC-MS/MS the presence of 625 proteins in mouse oocyte. This great number of identified proteins as a result of assessing all oocyte stages, obtained information associated with early development. In a recent study,[131] 3699 proteins were identified in MII oocyte.

As with mRNA profiles, oocyte-CC dialogue is important for oocyte competence and the identification of CC markers could be a good tool to assess oocyte quality in a clinical environment, since this experimental approach preserves oocytes viability.[132] CCs present a total number of 1423 proteins among which we can find several involved in metabolism, oxidative phosphorylation and post-transcriptional mechanism. McReynolds *et al*.[133] revealed that 110 proteins were differentially expressed in oocytes of advanced maternal age.

All this information is important to understand oocyte maturation mechanism as well as fertilization and embryo development.

FF presents much interest given that the proteomic analysis of this sample is easier and non-invasive.

FF presents a simpler protein pattern than somatic cells making proteomic analysis easier.[127] However, it has a drawback related to the abundance of albumin, immunoglobulin and other abundant serum proteins. These proteins mask the less abundant proteins and make the analysis difficult. Consequently, a preliminary removal step must be implemented.

Estes *et al*.[46] analyzed the FF proteome in women ≤32 years old in order to find protein markers that predict ovarian response and live birth. They found 11 potential protein markers. Among them, eight proteins had increased expression in the group achieving live birth and three proteins had decreased expression. The aim of another study[45] was to identify protein markers in FF to detect patients at risk of ovarian hyperstimulation syndrome. They identified 19 potential protein markers differentially expressed. Among them, kininogen-1 plays an important role because it mediates interactions with the other identified proteins. Since IVF success depends on ovarian hyperstimulation they hypothesized that a combined cluster of multiple biomarkers would be more valuable. A similar study[134] reported that those oocytes that resulted in pregnancy presented high-amount of proteins with biosynthesis functions, and those oocytes that resulted in no pregnancy presented high-amount of ubiquitinated peptides, being the most abundant protein alpha 2-globulin that correspond to oocytes that resulted in miscarriages.

One of the causes of anovulatory infertility is the PCO syndrome. Therefore, is interesting to analyze different protein expression in FF retrieved from these women.[135] In this study, 20 unique proteins were identified, 13 upregulated and 7 downregulated, involved in cellular metabolism and physiological processes.

In a very recent study,[35] 246 proteins of FF involved in coagulation and immune-response pathway were identified. This much number of proteins is higher than reported in other studies due to the improvement in proteomic techniques' sensitivity.

### Spermatozoa, testis and seminal fluids

The spermatozoon is a good cell to be evaluated by proteomic analysis because it is an accessible cell,[28] can be easily purified, and the number of spermatozoa that are needed to perform the analysis is not a limiting factor.

Several studies[28,32] have reported sperm protein profile in order to decipher potential biomarkers that can aid in the detection of sperm physiological impairment and to develop diagnostic tools for infertile couples.[32] Thus, these studies have been focused on the assessment of male fertility defects[136,137] as well as failed fertilization in ARTs[31] and comparisons between fertile and infertile males.[38]

Some studies reported proteomic differences in asthenozoospermic[136] and oligozoopermic[137] sperm samples. In the first one[136] variations of 17 proteins were detected in asthenozoospermic samples, but none of them alone is univocally associated with this spermatic state. In oligozoospermic males,[136] variations of 14 proteins were detected in comparison with control groups. These proteins are grouped in three groups in terms of their functions: Estrogen production proteins, structural proteins and signaling and regulatory proteins. These proteins could be involved in oligospermic phyisio-pathogenic mechanisms or however their alteration could be the result of a general protein alteration in these patients. Moreover, a case with globozoospermia was also studied using this technique[128] showing that spermatozoal acrosome membrane associate proteins were down-regulated in this condition.

Other studies[31] compared the sperm proteome from patients with failed fertilization in classical IVF versus controls and they found alterations in at least 20 proteins in infertile men. Moreover, de Mateo *et al*.[30] tried to correlate proteomic expression, protamine content and DNA integrity. They analyzed 47 sperm samples from infertile patients and 10 from semen donor and they identified 101 spots that correspond to 58 proteins differentially expressed in infertile individual's sperm samples, 8 proteins correlated with DNA integrity and 7 correlated with protamine content.

However, Thacker *et al*.[38] identified four unique proteins predominantly present in semen of healthy men, prostate-specific antigen isoform 1 pre-protein, prolactin-induced protein (PIP), clusterin isoform 1, and semenogelin II precursor of which only the former two were identified in infertile men.

Regarding seminal plasma, the fluid contains several proteins originating from the various internal accessory organs (prostate gland, ejaculatory ducts, seminal vesicles, and bulbourethral glands). A very recent study[138] have reported proteomic profiles of seminal plasma from adolescent males presenting varicocele concluding that these proteins are potential markers for an early diagnosis of this disease. Indeed, Fu-Jun and Xiao-Fang[139] analyzed the proteome in order to find candidate proteins of sperm maturation and they found 270 proteins. Among them, 34 epididymal milieu proteins and 274 prostatic milieu proteins contributed to the composition of seminal fluid proteome.

Finally four candidate markers, stabilin 2, 135-kDa centrosomal protein (CP135), guanine nucleotide-releasing protein, and PIP have been identified as markers for non-obstructive azoospermia.[128]

Furthermore, the protein content present in epididymal fluid and testicular tissue have been tried to be characterized[28,111] Rolland *et al*.[29] identified multiple potential biomarkers in reproductive tissue, concretely 83 proteins in testis, 42 in epididymis, 7 within seminal vesicle and 17 in prostate. Their relevance is based on their participation in secretions present in seminal plasma, their effect on sperm's quality, and their potential use as reproductive disorders markers.

### Embryos and culture media

An in-depth understanding of the embryonic proteome should lead to a true indication of cellular function and metabolism during mammalian pre-implantation development. Apoptotic and growth-inhibiting pathways are theoretical candidates to be closely involved in this process.[140] These biomarkers provide a potential diagnostic platform for improving IVF procedures including *in vitro* culture conditions (supplementing media), stimulation protocols or cryopreservation techniques.[141]

Identification of biomarkers will provide a mechanistic insight into the biological processes occurring at the cellular level during pre-implantation embryonic development. From a clinical perspective, quantification of embryonic viability potential will result in an increase in IVF pregnancy rates and live births while reducing the number of embryos transferred.[140] This team also reported significant alterations in the expression of proteins related to morphological development of human blastocysts.

Protein analysis of individual blastocysts will further increase our understanding about its interaction with the maternal uterine epithelium.[140,142] Indeed, Katz-Jaffe *et al*. have proposed that viable embryos possess a unique proteome and that some of these proteins are potentially secreted into the surrounding culture medium, contributing to the secretome. This non-invasive technique to assess embryonic development has increased knowledge of embryo physiology and thus new methods to predict embryo competence and viability can be developed allowing the elective single embryo transfer and reducing the risk involved in multiple births.[143] Domínguez *et al*.[144] studied the secretome of human blastocysts that implant versus those that do not. In this study, proteins such as granulocyte macrophage colony-stimulating factor (GM-CSF) and chemokine ligand 13 (CXCL13) were found lower in implanted blastocysts' secretome. More recently Cortezzi *et al*.[34] have identified a total 15 proteins belonging to positive implantation group being the most representative Jumonji protein (JARID2).

Katz-Jaffe and Gardner[5] have proposed that viable embryos possess a unique proteome and some of these proteins are potentially secreted into the surrounding culture medium, contributing to the secretome.

Platelet activating factor[145] and leptin[146] were also produced and secreted by pre-implantation embryos. Survivin is an inhibitor of apoptosis protein[147] that is expressed by oocytes and embryos but also is secreted by them playing an important role in oogenesis and embryogenesis.

Measurement of soluble human leukocyte antigen-G (sHLA-G) in embryo culture supernatants have been proposed as a good marker of embryos that can give rise to pregnancies.[148] It is suggested that measurement of sHLA-G on day 5-6 of culture would be more fruitful due to an increase in HLA-G mRNA and protein during pre-implantation development period of day 3 onwards.[149] However, a meta-analysis study by Vercammen *et al*.[150] indicate the necessity of further research involving single embryo culture, single embryo transfer and more sensitive HLA-G detection techniques so as to establish the accuracy of sHLA-G for predicting pregnancy among women undergoing IVF. In ICSI, the effects of sHLA-G are more apparent.[148]

On the other hand, culture media is susceptible to be analyzed by proteomic techniques and the obtained protein profile can provide proteins biomarkers. Performing proteomic analysis of the culture media permits de identification of embryo's secretome.[127] Among secreted proteins, survivin appears in the 94% of culture media being correlated this secretion with embryo cleavage rates.[147] Dominguez *et al*.[151] studied the secretome profile of blastocysts that were grown in two different culture media, sequential and EEC coculture media. They identified differences in the protein secretion/consumption profiles between both media, presenting relative abundance of proteins the EEC coculture media. The most abundant proteins were IL-6 and PIGF being IL-6 utilization essential for blastocyst growth and implantation processes. The same group[144] compared blastocysts' conditioned media with control medium reporting the increase expression of soluble TNF receptor 1 and IL-10 and the decreased expression of macrophage-stimulating protein, stem cell factor, CXCL13 among others, being CXCL13 and GM-CSF decreasing in implanted blastocysts media.

## Endometrium

As indicated above blastocysts' reproductive competence[152] is important for successful implantation but endometrium also play an important role in this process. Proteomics can be applied on the endometrial tissue in order to detect proteins that serve as receptivity markers of endometrium.

Several studies are focused on the identification of the proteome of the different phases of the menstrual cycle. Li *et al*.[33] identified the proteomic patterns of prereceptive (day 2 after LH surge) and receptive (day 7 after LH surge) phases, finding 31 proteins supposedly involved in implantation process. Five up-regulated proteins (annexin A4, annexin A2, Vimentin, coagulation factor XIII A chain and collage VI alpha-2 chain) had the same tendency as previously found on previous papers[153] while DJ-1 protein was found to be differentially present in an opposite direction. Annexin A4 plays a crucial role in receptive process. Domínguez *et al.* also observed annexin A-2 and stathmin-1 being involved. In proliferative and secretory phase endometrium, Rai *et al*.[154] found 194 proteins, of which 7 were differentially expressed proteins. Others studies[128] have found downregulated proteins as calecticulin, fibrinogen adenylatelinase isoenzyme-5 and transferrin and up regulated as annexin 5, alpha-1-atitypsin, peroxiding-6 and creatinekinaseinin the mid-secretory (receptive) phase.

Regarding decidualization of endometrium,[37] 60 differentially expressed proteins were identified. Among them, 36 were over expressed and 24 were under expressed and included decidualization markers as cathepsin B, tranglutaminase 2, peroxiredoxin 4 and ACTB protein. In this study, they also analyzed the secretomic profile obtaining 11 secreted proteins up regulated and 2 down regulated and among them IGF binding protein-1, prolactin, myeloid progenitor inhibitory factor-1 and platelet endothelial cell adhesion molecule-1 as markers.

Brosens *et al*.[39] reported that recurrent implantation failure (RIF) was associated with a characteristic protein

profile different of fertile women. They identified apolipoprotein A-I as an endometrial anti-implantation factor. In midsecretory eutopic endometrial tissue from patients with endometriosis, this protein was highly expressed and its regulation was dysregulated and might result in RIF. In fact, endometriosis has a characteristic protein profile that included heat shock protein 90-alpha and beta being down regulated.[155]

Currently, endometrial cancer is the seventh most common cancer worldwide among females. Identification of protein profile in this case might be interesting as molecular markers for improved diagnostic and therapeutic intervention. Habermann *et al*.[156] identified two proteins AKR7A2 and ANXA2 that showed translational alterations and therefore transcriptional changes. Differentially expressed proteins were involved in the same functions as cancer, cell death and cellular assembly and organization.

Most of these studies[33,37,39,152-155,157] focused in the analysis of human endometrium uses endometrial biopsies. However, although the analysis of tissue biopsies has some benefits, since you can get information directly from the cells that compose the tissue, have also many drawbacks,[157] as for example the changes in endometrial structure and cellular composition, and diversity in its morphology produced by menstrual cycle phases.

## METABOLOMICS IN ASSISTED REPRODUCTION

Metabolic profiling or metabolomics is the analysis of various molecular metabolites within cells and fluids using various forms of spectral and analytical approaches, and it attempts to determine metabolites associated with physiologic and pathologic states.[158,159] It offers a significant advantage over the use of the two related fields of study. Smaller variations in gene expression and protein synthesis result in an amplified change in the metabolite profile known as the metabolome, and this information can be used to detect subtle cellular events.[160]

The aims of metabolomic analysis[42] are to help in the selection of viable embryo or gamete to improve ARTs success, to pick out embryos with implantation potential to facilitate single embryo transfer and to estimate the overall viability of the cohort of embryos.

### CCs, GCs and oocytes

The available information about metabolomics in CCs, GCs and oocytes is very limited unlike other OMICS because metabolomics analysis in FF and culture media to select the best oocyte or the best embryo is easier and non-invasive technique. Therefore, there are many articles related to oocyte quality are found which studied FF and culture media metabolomics.

### Spermatozoa, testis and seminal fluid

In this case, the available information is also limited because few articles studied sperm metabolome because metabolomics is a very recent throughput analysis method. Related to this item, Deepinder *et al*.[160] found differences in seminal plasma oxidative stress biomarkers concentration (-CH, -NH, -OH and ROH) between fertile men and idiopathic infertility, varicocele and vasectomy. Furthermore differences in citrate, lactate, glycerylphosphorylcholine and glycerylphosphorylethanolamine between donors and infertile males have been also found. On the other hand, Deepinder used phosphomonoester and beta-adenosine triphosphate as biomarkers to assess testicular failure and ductal obstruction.

In another study Gupta *et al*.[161] used H-NMR spectroscopy to analyze seminal fluid metabolome in order to determine biomarkers of infertility. Among 10 detected metabolites, alanine, citrate, glycerophosphocholine, tyrosine and phenylalanine can be used to determine male infertility. This technique is also non-invasive and rapid results can be obtained.

More studies are necessary to identify sperm metabolome and infertility markers that complete the spermatozomic study.

### Embryo

A large number of articles are available exploring the embryo metabolome by analyzing the culture media where they are grown *in vitro*.

Seli *et al*.[162] analyzed 69 pre-implantation embryos' spent media samples from 30 patients with known outcome (0 or 100% sustained implantation rates) using Raman and NIR spectroscopy. The study indicated that individual samples could be analyzed in approximately 1 min using 15 μL of media. Viability indices calculated by Raman and NIR spectroscopy were higher for embryos that implanted and resulted in a delivery, compared with those that failed to implant. Metabolic profiling indicates that as embryos develop *in vitro* the chemical mileu of the culture media get differentiated based on the quality of the zygotes. Numerous functional groups including -SH, -CH, -NH, and -OH which are biomarkers of oxidative stress have been identified as spectral signatures affecting embryo viability based on their relative amounts. In one study, Vergouw *et al*.[163] showed that NIR spectral analysis produced unique metabolic profiles that correlated to an embryo's reproductive potential. Resulting relative viability scores between positive and negative pregnancy outcomes were statistically significant ($P < 0.03$). A logistic regression of

factors correlated to pregnancy outcomes showed that maternal age, percent fragmentation and relative viability scores all demonstrated a relationship. The extent of the correlation was determined by accuracy computation. They concluded that NIR metabolomic profiling of spent embryo culture media was able to distinguish viable embryos from non-viable embryos for reproduction. Of particular interest is that a viability score calculated from the metabolic profile is able to provide a second tier of information above that of morphology and that this technique used in addition to morphology may be able to better distinguish more viable embryos.[43] Non-invasive and highly sensitive metabolic profiling may provide a more comprehensive analysis of pre-implantation embryos, thereby facilitating single embryo transfers.

### Endometrium

The unique existing data about endometrial metabolomics analysis has been focused on lipidomic analysis of endometrial receptivity. Lipidomics is defined as the massive study of lipid species existing in a cell or biological system and metabolic pathways and networks related.

Unlike ERA test, where it is necessary to perform an endometrial biopsy and the embryo transfer cannot be performed in such cycle; lipidomic analysis of the endometrium is a non-invasive technique, given that endometrial fluid is obtained and analyzed.

Until date, it is only available in mouse models. These studies have demonstrated that several lipids such as triglycerides, prostaglandins (PG), thromboxanes, endocanabinnoids and sphingolipids, play an important role in reproductive biology during early pregnancy, including pre-implantation embryo formation and development, implantation and post-implantation growth.[164-168]

PG that are the result of arachidonic acid oxidation by cyclooxygenases (COX-1 and COX-2) and PG synthases action are the most studied lipid in endometrium.

In mice,[164] the simultaneous inhibition of COX-1 and COX-2 did not produce pregnancy and therefore PGs had an important role in pregnancy because defects in cyclooxygenases enzymes can be corrected with the addition of PGI (involved in mice embryo implantation) and PGE to a lesser extent. Wang and Dey[165] showed that PG are essential to ovulation, fertilization and implantation whereas endocannabinoids are important for the synchronization between pre-implantation embryo development and endometrial receptivity. Therefore in cases of RIF[166] PGs synthesis was disturbed suggesting that poor endometrial receptivity is related to low PG synthesis. The biopsy analysis revealed[167] that PGE2 is the most abundant PG in human endometrium and that levels of PGE2 and PGF are decreased in proliferative phase rising PGF in the luteal phase whereas PGE2 levels remain lower. Then in menstrual phase the levels of PGE2 increased.

Another lipid mediator, leukemia inhibitor factor (LIF) has been related to human embryo implantation and endometrial receptivity indirectly given that LIF knockout mice did not present embryo implantation.[168] This factor acts together with its receptor and gp 130. They are expressed along the menstrual cycle, but their expression increased in mid-secretory phase and therefore in the endometrial receptivity phase (window of implantation). However, in infertile women's endometrium the expression was less.

### FF and culture media

In contrast to genomics and proteomics where only one class of the compound is analyzed, metabolomics-based analysis have to deal with diverse classes of molecules.

The chemical[169] constituents of FF have been grouped in: Hormones, transforming growth factor beta, other growth factors and IL, reactive oxygen species, anti-apoptotic factor, proteins, peptides and amino-acids, sugars and prostanoids. These metabolites are involved in the physiology of the oocyte and therefore can provide information about oocyte state and can help to select the best oocyte with fertilizing capacity. Metabolomics are potentially more informative than genomics, transcriptomics or proteomics because it represents the final products of cell regulatory process.

In FF,[170] the metabolomics profile is clustered in large antral follicles and heterogeneous in small follicles reflecting differences in their maturational stage. In a very recent metabolomic study[171] based on H-NMR, demonstrated differences between FF metabolome with developmental competence of the oocytes. Therefore, competent oocyte must have glucose (increased in FF from failure cleavage oocyte), lactate (decrease in FF from failure cleavage oocyte and in non-pregnancy group), choline (decreased in FF from failure cleavage oocyte), phosphocholine, proline, leucine/isoleucine, glutamine (aminoacids in general increased in FF of positive pregnancy group) and high-density lipoprotein (increased in FF from failure cleavage oocyte). Piñero-Sagredo *et al*.[172] also used NMR analysis to FF. They identified 42 metabolites and correlated the existence of an important anaerobic glycolytic metabolism in follicles with fatty acids synthesis and with more successful fertilization. However, they did not relate this metabolome with successful IVF outcomes. Another study that used NMR analysis[43] has demonstrated a statistically significant increase in glutamate concentration in the culture media of embryos which resulted in a positive pregnancy.

Pacella *et al*.[173] identified the metabolites present in FF

of women with reduced ovarian reserve at advanced maternal age. In these women follicular cell metabolism, FF metabolome and progesterone production are affected. Indeed, glucose levels decreased and lactate and progesterone increased although GC and CC metabolism is altered. This situation can affect oocyte and embryo development. It has seen that metabolic alterations in the serum are reflected in the FF irrespective of body mass index (BMI).[174] BMI only affect C-reactive protein, triglycerides, insulin and insulin growth factor-1 in FF.

Metabolic turnover is crucial for a pre-implantational embryo to grow and reach a successful pregnancy. Accordingly, nutrients and metabolites within the culture media, they have been studied as potential predictors of embryo quality by non-invasive measurements.

Several techniques are used in metabolomic analysis of culture media. Microfluorometric enzymatic assays[127] exhibit several limitations. Microfluidics systems, however, allow simultaneous measurements of metabolites in small volumes. But NMR and MS are the most commonly employed techniques for metabolomics analysis.[159] Other techniques discussed above are GC-MS, LC-MS, HPLC analysis, NIR, Raman and capillary electrophoresis-MS.

In early stages of pre-implantational development when pyruvate and lactate are the main sources of energy embryos present a carboxylic acid metabolism. However, when embryo progresses from the zygote, glucose uptake increase and its metabolism predominates in blastocysts stage. Amino-acids are essential for embryonic development and lower uptake of glutamine, arginine and methionine is correlated with successful development of blastocysts. Therefore, changes in these metabolites can lead to problems in the development of embryos.[127]

Raman metabolomic analysis[41,42] has demonstrated the correlation between spent culture media and clinical outcomes. Glucose, lactate, pyruvate and amino acids, among others, were the culture medium components analyzed. In addition, Nagy *et al*.[175] demonstrated that NIR spectroscopic analysis presented higher viability indices of oocytes that result in implanted embryos than those that did not.

Regard to carbohydrates low glycolytic activity[176] and glucose utilization[127] that increases in the transition from the morula to blastocyst, are related to more viable blastocysts.

Brison *et al*.[40] analyzed amino acids turn over in order to select viable embryos. They found that elevated asparagine and decreased glycine and leucine being significantly correlated with clinical pregnancy and live births. As we know, amino acids can be subdivided into essential and non-essential, being the last one those which must be supplied exogenously by the diet.[177] Therefore, amino acid supplementation of embryo culture medium has been recognized but still remains unclear. However, glutamine, aspartate and methionine probably are the most common amino acid added to embryo culture.

Seli *et al*.[162] described differences in metabolomics profile between embryos that resulted in pregnancy and those that did not, being the oxidative stress biomarkers (-CH, -NH and -OH groups) the most predictive factors of pregnancy.

Lipids' role has also been described by using metabolomics analysis.[178] It has been reported that embryos developed beyond 4-cell stage had greater concentrations of unsaturated fatty acids (linoleic and oleic acids) and lower concentration of saturated fatty acids.

## CONCLUSION

OMICS, the high-throughput measurement technologies, in which aspects of cellular structure or function, such as proteins or RNA transcripts, or metabolites are studied on a global scale, are opening wider and wider doors into reproductive medicine and technology. It is likely that information obtained using OMICS will change the way we perform the current IVF procedures. Therefore, the OMICS technologies are suitable diagnostic tools to explore differences among follicles, human gametes and embryos. Since single embryo transfer is getting momentum across the IVF clinics, such platforms will be inevitable to select the embryos for transfer.

Oocyte ageing leading to increased aneuploidy and associated pregnancy loss has already been established. Since the average age of women attending fertility clinics continues to increase, a safe and simple method to identify oocytes having anomalies would be extremely beneficial. Clinical results in enhancing live birth rate with PGS on embryos have been promising. The results from several ongoing randomized controlled trials, performed at different cell biopsy stage and categories of patients, will provide the data on which type of array method (aCGH/SNP) prove useful in ART set up. Several research groups are working on the designs to develop a novel embryo culture system such as microfluidic platforms that will culture multiple single embryos under simulated physiological conditions while simultaneously performing real-time monitoring on biochemical markers of embryo quality. Once such an integrated system is introduced, merging analysis and culture competencies this can perform metabolic profiling of embryos as well as *in vitro* culture simulating *in vivo*

conditions.

Thanks to these techniques and their application in assisted reproduction field, novel molecular biomarkers related to infertility problems have been described, allowing the increase of our knowledge in order to design new diagnostic or selection tests aiming to improve the success rates in ARTs.

Currently, there are several tools available as a result yielded from the - OMICS approach in this area, as the ERA test, while others as sperm fertility array is under development.

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**How to cite this article:** Egea RR, Puchalt NG, Escrivá MM, Varghese AC. OMICS: Current and future perspectives in reproductive medicine and technology. J Hum Reprod Sci 2014;7:73-92.
**Source of Support:** Nil, **Conflict of Interest:** None declared.