Non-invasive molecular biomarkers for assessment of oocyte and embryo in assisted reproduction

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Predicting viable oocyte and embryo

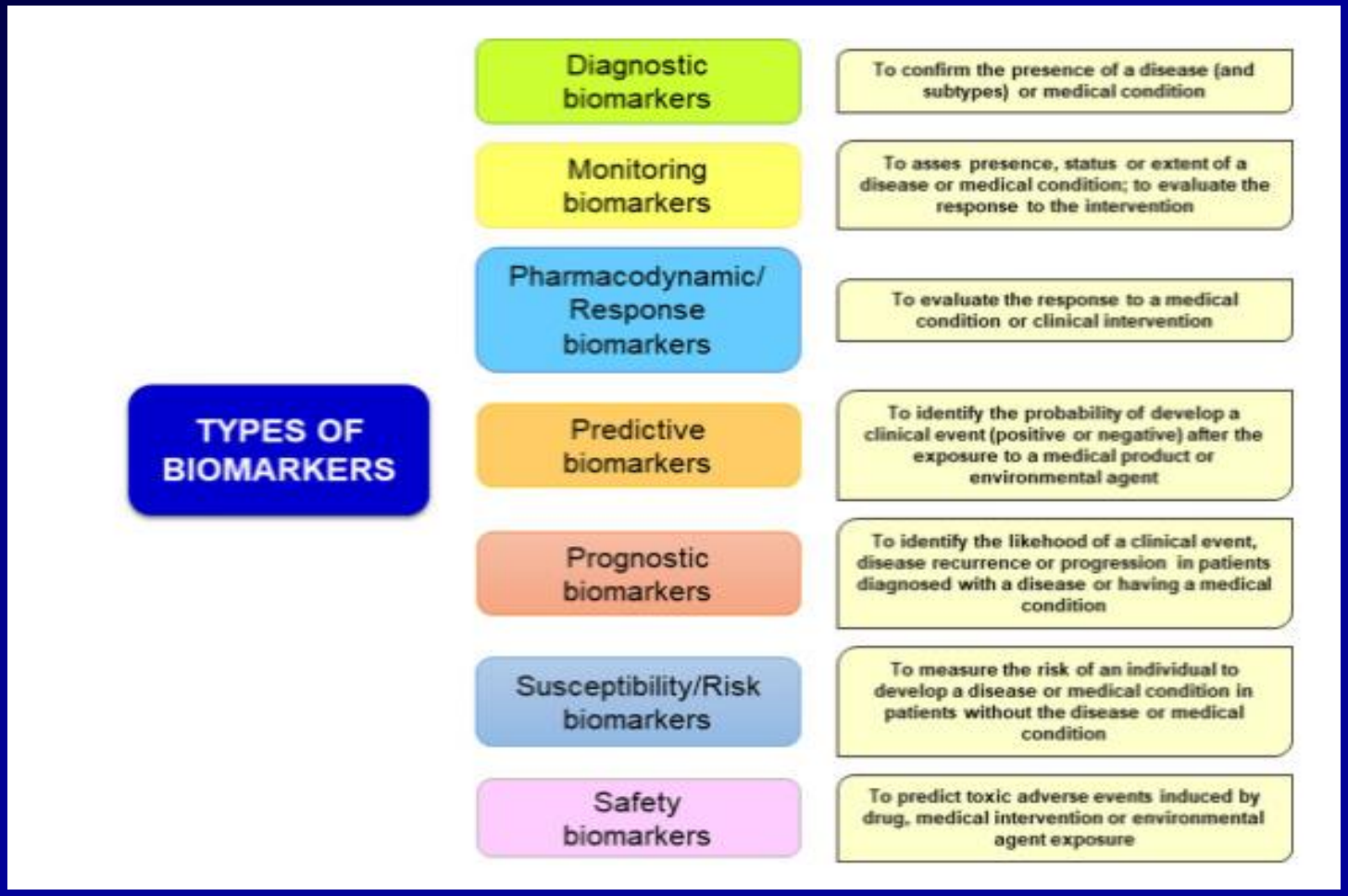
- **Predicting which oocyte and embryo has the highest viability (embryo's ability to implant and give rise) to a healthy baby has been a major challenge for embryologists since the introduction of ART.**
- **The predominant method for selecting viable embryos is morphological evaluation. However good embryo morphology may not necessarily identify a chromosomally normal embryo with the best implantation potential, and also it has interoperator and intraoperator variability.**
- **Consequently, several invasive and non-invasive approaches that overcome those handicaps were developed, such as:**
- **Preimplantation genetic Tests**
- **Morphokinetics evaluation of the embryo**
- **Extended culture to blastocyst**

Assessment of embryo viability

Assessment	Advantages	Disadvantages
Morphological phenotypes in every stage of development *(classical selection method)*	- Non-invasive - Consensus	-Subjective -Static evaluation -Limited accuracy: good quality does not ensure implantation
Morphokinetic evaluation (using time-lapse)	- Non-invasive - Objective (better if automated)	-No universal algorithms -Potential risk of wasting low-scored viable embryos -Limited accuracy
Extended culture up to blastocyst	- Non-invasive - Embryo 'self-selection'	-Usually applicable to large embryo cohort -Potential secondary or deleterious effects -Limited accuracy: development does not ensure implantation
Preimplantation genetic screening (PGS)	The most effective if any aneuploidy present (correlation between abnormal embryos and poor viability)	-Invasive -Still applied in poor prognosis patients -Potential false positive due to mosaicism -Limited accuracy: normal ploidy does not ensure implantation

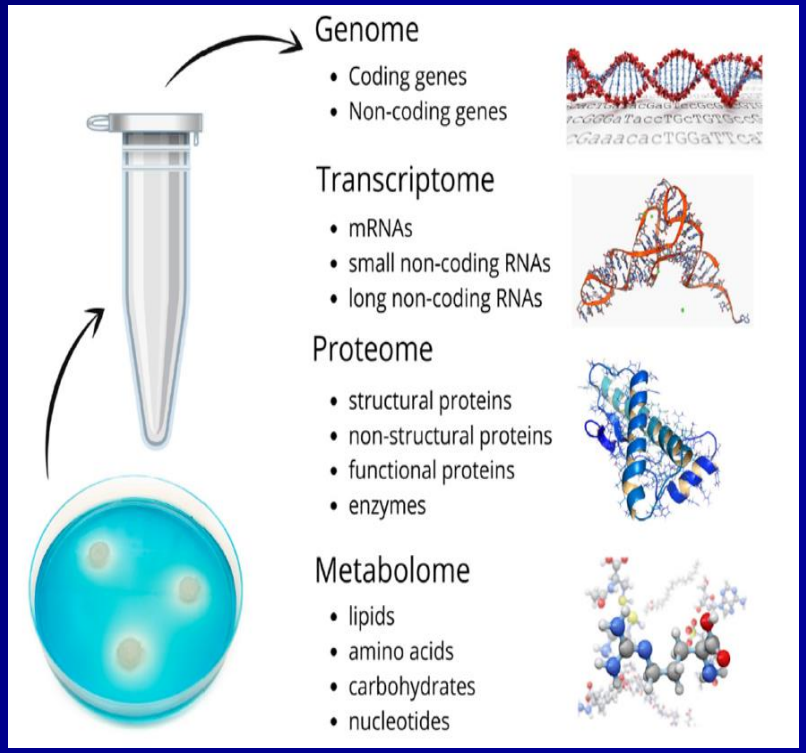
What are biomarkers?

A biomarker (short for biological markers) is a substance that is measured in a biological system as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention



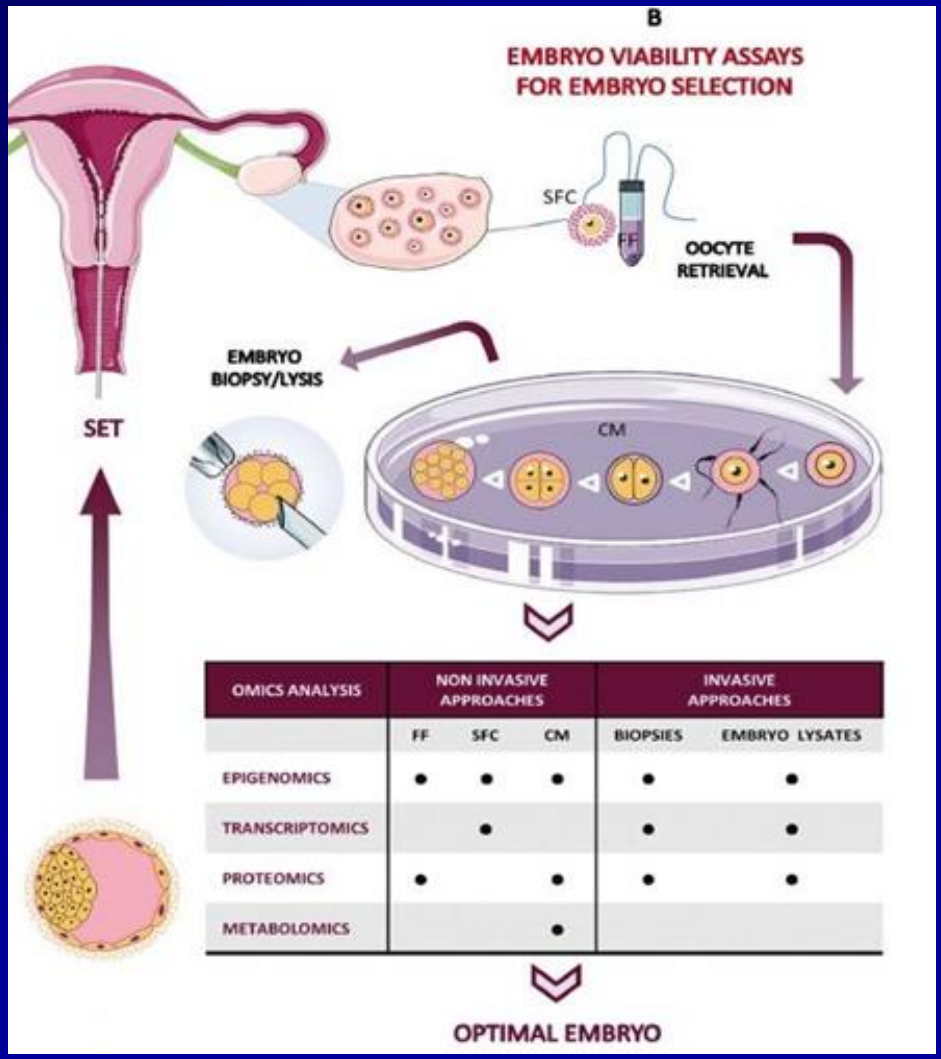
Omics

- **In the last two decades, advances in biotechnology have led to the emergence of new high-throughput techniques, generally grouped under the term of 'omics' approaches. These approaches offer a global view of biological processes owing to the simultaneous analysis of thousands of molecules in a single biological sample**
- **Using OMICS platforms, all classes of biological compounds such as, epigenetic marks, genes, messenger ribonucleic acid (mRNA), proteins and metabolites can be analyzed.**
- **The field of omics is ever expanding**
- **Exomics (analysis of exons)**
- **Epigenomics (assessment of epigenetic modifications)**
- **Secretomics (analysis of secreted products)**
- **Lipidomics (large-scale analysis of the whole lipid species)**
- **Interactome (the complete set of molecular interactions to understand the system and its functions as *a whole* rather than as separate components)**



Non-invasive sampling for assessment of embryo viability

- Different omics approaches are currently being applied to embryo viability assessment for embryo selection:
- Somatic follicular cells (SFCs) (granulosa and cumulus cells, GC and CC)
- Culture media (CM)
- Follicular fluid



Genomic biomarkers

No specific DNA sequences (independent of known lethal mutations) have been associated with increased viability, indicating that genome-wide association studies (GWAS) are not a likely strategy for embryo viability assessments.

In contrast, two PGS methods based on omics technologies, the comparative genomic hybridisation array and, more recently, next-generation sequencing (NGS), are widely used to screen the entire embryonic genome with a high accuracy, preventing the transfer of aneuploid (and perhaps non-viable embryos) in poor-prognosis patients.

Genomic biomarkers

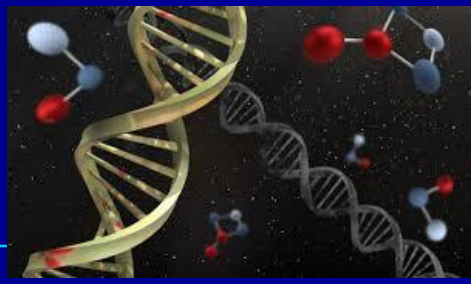
Target Molecule	Sample	Omics & validation method	Biomarker & Findings	Reference
Cell-free DNA	FF	Q-PCR	• FF concentration cfDNA was not associated with oocyte's maturity stage	Scalici et al. (2014)
Cell-free DNA	FF	Q-PCR	• cfDNA did not seem to have any direct role in the IVF outcome	Dimopoulou et al. (2014)
Cell-free DNA	FF	Q-PCR	• Decreased level of cfDNA is associated with top quality embryos • FF cfDNA level was an independent and significant predictive factor for pregnancy outcome	Traver et al. (2015)
Cell-free DNA	FF	Q-PCR	• cfDNA level was negatively correlated with embryo quality and pregnancy rates	Guan et al. (2017)

Genomic biomarkers

Expression profiles of the metabolic gene PFKP, and extracellular matrix genes HAS2, TNFIP6, versican, PTGS2 and PTX3 in CC have been associated with subsequent embryo development and/or live birth rate (McKenzie et al., 2004; Gebhardt et al., 2011).

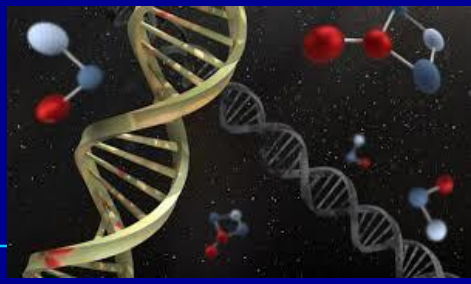
No reliable oocyte metabolic biomarkers that predict ART outcomes are identified so far. This is because studies to date have had limitations, such as small sample size and confounding factors caused by varying practices across treatment clinics, and the lack of randomized controlled trials to validate potential targets (Richani et al., 2020).

Genomic biomarkers



It has been reported that the ratio of mtDNA to gDNA in day-3 SCM was positively associated with subsequent blastulation rates, trophectoderm quality and implantation outcomes (Stigliani et al., 2014).

Genomic biomarkers



Cell-free DNA in CM is primarily employed for the identification of embryo ploidy, but the technique was also used to correlate other outcomes such as pregnancy rate and embryo quality.

A recent systematic review concluded that even though genetic material was successfully detected and amplified reliability due to arising discrepancies is still debated. The main discrepancy sources identified were a low amount of DNA, the varying selection of reference sources for concordance studies, or potential contamination with exogenous DNA (Brouillet et al., 2020).

Epigenetic biomarkers

Some **methylation-regulated genes** are also involved in human trophoblast differentiation, potentially affecting the invasiveness of trophoblast.

lncRNA has a regulatory role during embryo development.

Aberrant **miRNA** expression are linked to implantation failures (McCallie et al. (2010)). miRNAs might be early indicators of the prognosis for human embryos from the very beginning of their development (Rosenbluth et al. (2013)).

Epigenetic biomarkers

Some **secreted miRNAs** in IVF **CM**, whose expression was related not only to chromosomal status but also to pregnancy outcomes, supporting miRNAs as promising markers of human embryo potential (Rosenbluth et al., 2014).

miR-142-3p to be significantly correlated with implantation failure; thus, embryos producing this miRNA could be excluded from uterine transfer (Borges et al., 2016).

Epigenetic biomarkers

In addition, different miRNA profiles have recently been identified in FF according to the oocyte maturation or fertilization competence, and to embryo quality (Moreno et al., 2015; Martinez et al., 2018); thus, there is potential to find embryo viability biomarkers in that easily accessible fluid.

Transcriptomic biomarkers

Microarray technology and, more recently, NGS have advanced the complete transcriptional analysis of individual embryos.

Specific transcriptomes are mainly related to cell proliferation and differentiation as well as to pathways activated for implantation.

Little information connecting the embryonic transcriptome with its viability, referred to as implantation potential, has been obtained until recently.

Transcriptomic biomarkers

In recent years, several studies have explored the transcriptome of SFC with the intention to identify novel biomarkers predictive of oocyte and embryo competence and/or pregnancy outcome

Some recent omics studies have gone further, generating pregnancy or live birth predictive models for prospective human embryo selection based upon the expression of several SFC genes

Transcriptomic biomarkers

- Some transcriptomic human CC biomarkers predictive of oocyte and embryo competence and also pregnancy outcome have been proposed recently, but further studies are still required to confirm their predictive value (Assidi et al., 2011; Feuerstein et al., 2012).
- Parallel studies also identified some potential pregnancy markers within the transcriptome of mural GC (Hamel et al., 2008; Hamel et al., 2010).

Proteomic biomarkers

Some proteins secreted from the human embryo into the CM (the protein secretome) , such as ubiquitin (Katz-Jaffe et al., 2006), apolipoprotein A1 (Mains et al., 2011) or different hCG isoforms (Butler et al., 2013) have been differentially identified in the secretome of ongoing blastocysts when compared to arrested embryos.

Nevertheless, since the association between those proteins levels and implantation outcomes were not observed or analysed in all cases. further validation studies are needed before including their analysis in a non-invasive embryo viability assay.

sCD146 and sHLA-G in blastocyst CM has been reported to identify the most competent embryos for implantation (Bouvier, et al., 2019; Díaz, et al., 2019)

Proteomic biomarkers

Interleukin (IL)-6 has been proposed as a potential predictor of blastocyst selection for proper implantation.

Recently, more sensitive proteomic nanotechnologies in Day 3 spent media, found up to 15 proteins uniquely expressed in embryos that implanted and 10 specific proteins in those that failed to implant, confirming that competent embryos secrete unique putative biomarker proteins into the surrounding media.

Proteomic biomarkers

Another on embryonic secretom study has been showed a set of nine potential biomarkers of chromosome aneuploidy in a cohort of human transferable-quality blastocysts (Katz-Jaffe *et al.* (2009))**.**

Proteomic biomarkers

While different transcriptomic biomarkers have been identified in both CC and GC, no link between their protein products and oocyte or embryo competence has been established.

In FF, on the other hand, a recent proteomic investigation of samples from women undergoing IVF has identified a large number of differentially expressed proteins associated with oocytes leading to different clinical outcomes (no pregnancy, miscarriages or baby born) (Kushnir et al., 2012)).

Proteomic biomarkers

Despite their promise, neither embryo secretom analysis nor other proteomic technologies have been implemented clinically. Other challenges in the search for proteomic biomarkers, including the lack of standardised methods and the prospective validation of biomarkers from individual embryos, must be overcome before implementation of these promising platforms.

Metabolomic biomarkers

Studies of the metabolic products of the preimplantation embryo on spent CM at different stages of development reveal their influence on the implantation process (Gardner andWale, 2013; Uyar and Seli, 2014).

Although carbohydrate metabolism seem to correlate with development to blastocyst stage, their relation to implantation or pregnancy outcome remains uncertain (reviewed in Gardner andWale, 2013; Uyar and Seli, 2014).

Metabolomic biomarkers

Caproate and androsterone sulphate, were identified to differentiate between trisomy/monosomy 21 and euploid embryos.

Metabolomic biomarkers

Amino acids have also been proposed for selecting a competent embryo owing to the different metabolic profile observed between embryos that grew into blastocysts and those that arrested (Houghton et al., 2002; Houghton and Leese, 2004). Further, some particular amino acids in CM, or the relative amino acid concentration, are correlated with the ploidy status of human embryos (Picton et al., 2010) or with clinical pregnancy and live birth (Brison et al., 2004; Seli et al., 2008; Marhuenda-Egea et al., 2010).

Metabolomic biomarkers

Twelve metabolites were identified and analyzed by 1H-NMR (proton-NMR), identifying that the increase in format to glycine ratio and the decrease in citrate to alanine ratio was indicative of intrauterine pregnancy.

Metabolomic biomarkers

Other less common metabolic biomarker tests proposed to evaluate embryo developmental competence are based on stem cell factor (SCF), interferon (IFN) 2, Na+/K+ consumption by sodium pumps; oxidative stress, which negatively affects embryo quality; embryo respiratory rates, which have been correlated with both morphology and embryo viability; and fatty acid correlation with implanting embryos (Houghton and Leese, 2004; Haggarty et al., 2006; Cortezzi et al., 2013).

Metabolomic biomarkers

However, none of those metabolic approaches have been implemented as standard embryo selection procedures in the fertility clinics, probably because the expensive and accurate platforms required for analysis are not practical in the clinical setting.

Metabolomic biomarkers

It has been suggested that women who became pregnant after ART had lower levels of glucose and higher proline, lactate, leucine and isoleucine in their FF (Wallace et al., 2012).

Metabolic profiles of spent CM from oocytes and from FF were also related to fertilization rates (Pinero-Sagredo et al., 2010) or pregnancy outcomes (Nagy et al., 2009;Wallace et al., 2012). Unfortunately, subsequent RCTs using commercial devices did not support the previous results (Hardarson et al., 2012; Vergouw et al., 2012; Sfontouris et al., 2013).

Metabolomic biomarkers

NMR or electrospray ionisation MS (ESI-MS) for spectrometry fingerprinting (Seli et al., 2008; Pinero-Sagredo et al., 2010; Sanchez-Ribas et al., 2012; Nadal-Desbarats et al., 2013; Pudakalakatti et al., 2013; Cortezzi et al., 2013), are providing new candidates in the embryo secretom. Conversely, the ability of NMR metabolomics profiles to identify implantable embryos has been also questioned (Rianudo et al., 2012; Kirkegaard et al., 2014).

Finally, a recent systematic review concluded that there is no evidence for the use of metabolomics in clinical practice to improve fertility outcomes (Bracewell-Milnes et al., 2017).

Conclution

In summary, despite the predictive value of embryo implantation competence demonstrated by omics technologies, more prospective clinical trials comparing them with conventional morphological selection criteria are needed.

Additionally, understanding the networks between embryo gene product expression (transcriptome, proteome and metabolome), morphokinetics or karyotype and embryo physiology requires a more global exploration of the major mechanisms involved in embryo competence.

Conclution

With current developments in both high-throughput, clinically applicable hardware and bioinformatic tools, this goal is getting closer to being reached. Nevertheless, there is still some work needed to translate the pilot studies into the clinical arena.