in vivo *28*: 683-690 (2014)

Review

Cell Adhesion Molecules and *In Vitro* Fertilization

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Abstract. *This review addresses issues regarding the need in the in vitro fertilization (IVF) field for further predictive markers enhancing the standing embryo selection criteria. It aims to serve as a source of defining information for an audience interested in factors related to the wide range of multiple roles played by cell adhesion molecules (CAMs) in several aspects of IVF ultimately associated with the success of an IVF cycle. We begin by stressing the importance of enriching the standing embryo selection criteria available aiming for the golden standard: "extract as much information as possible focusing on non-invasive techniques" so as to guide us towards selecting the embryo with the highest implantation potential. We briefly describe the latest trends on how to best select the right embryo, moving closer towards elective single embryo transfer. These trends are: frozen embryo transfer for all, preimplantation genetic screening, non-invasive selection criteria, and time-lapse imaging. The main part of this review is dedicated to categorizing and presenting published research studies focused on the involvement of CAMs in IVF and its final outcome. Specifically, we discuss the association of CAMs with conditions and complications that arise from performing assisted reproductive techniques, such as ovarian hyperstimulation syndrome, the state of the endometrium, and tubal pregnancies, as well as the levels of CAMs in biological materials available in the IVF laboratory such as follicular fluid, trophectoderm, ovarian granulosa cells, oocytes, and embryos. To conclude, since CAMs have been successfully employed as a diagnostic tool in several pathologies in routine clinical work, we suggest that their multi-faceted nature could serve as a prognostic marker in assisted reproduction, aiming to enrich the list of non-invasive selection and predictive criteria in the IVF setting. We propose that in light of the well-documented involvement of CAMs in the developmental processes of fertilization, embryogenesis, implantation, placentation, and embryonic development, further studies could contribute significantly to achieving a higher quality of treatment and management of infertility.*

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Key Words: In vitro fertilization (IVF), embryo selection criteria, cell adhesion molecules (CAMs), review.

From enabling fatherhood for azoospermic men through the innovation of intra-cytoplasmic sperm injection and offering solutions to infertile couples through gamete and embryo donation and surrogacy, to providing the basis enabling genetic embryo selection prior to implantation employing preimplantation genetic diagnosis (1), the world of *in vitro* fertilization (IVF) has progressed to cover a wide spectrum of infertility issues, extending to management of genetically inherited diseases.

In fact, according to the 13th European IVF-monitoring report, more than half a million cycles (537,463) were carried-out during 2009. In total, 109,239 Assisted Reproductive Technology (ART) infants were born in 2009 (2).

Embryo Selection Criteria

The importance of moving towards elective single embryo transfer (eSET) strengthens the need for effective selection criteria.

a) eSET. As implantation rates have improved, eSET is becoming increasingly common in IVF treatment as a means of reducing multiple pregnancy rates that lead to a higher incidence of medical, perinatal and neonatal complications (3, 4). Indeed, systematic reviews and meta-analyses show that pregnancies conceived employing eSET are associated with a decrease in preterm birth and low birth weight compared to double embryo transfer (5). Additionally, the eSET method can be applied even in older women (>40 years) with a good prognosis, resulting in high clinical pregnancy and live birth rates (6).

The aim of employing eSET is to diagnose and select healthier embryos in order to improve pregnancy rates and outcomes, while reducing the number of multiple and genetically abnormal pregnancies (7).

b) Frozen embryo transfer (FET) for all. The data of various studies show that much better perinatal outcomes can be achieved, with higher clinical pregnancy rates, opting for FET in comparison to fresh embryo transfer (8). Indeed, pregnancies achieved by FET appear to present a lower risk of various perinatal outcomes, namely low birth rate, or preterm birth (9).

One good reason supporting the advantages of FET is the state of the endometrium, since during FET cycles it is at a better receptive state, without the effects of controlled ovarian hyperstimulation protocols (10). It is true that during a FET cycle, the endometrial development can be controlled more precisely than in cycles with the use of controlled ovarian hyperstimulation. Furthermore, the elevated progesterone levels during fresh embryo transfer cycles have been associated with decreased implantation rates due to asynchrony between the embryo and the endometrium, something that could be avoided by the use of natural or artificial endometrial preparation during FET (11). It is the advent of vitrification, associated with excellent survival, developmental and pregnancy rates that has revolutionized treatment (12, 13). Vitrification has made it possible to consider offering FET for all as a valid option, since there is no longer an issue on compromising embryo/egg quality and implantation potential following vitrification. Moreover, on the matter of FET for all, vitrification of all embryos can further act as a selection process, separating compromised embryos that will not survive from embryos with good implantation potential (14).

c) Preimplantation genetic screening (PGS). PGS claims to further aid the "search" for the best possible embryos to transfer. PGS presents many challenges and is currently controversial as there is still no definitive evidence that it works towards improving pregnancy rates. Evidence suggests that PGS on cleavage stage embryos is ineffective but there is still debate as to whether it could prove beneficial when biopsy is performed at the polar body stage or at the blastocyst (15), coupled by application of DNA microarrays, single-cell array Comparative Genomic Hybridization (aCGH) and perhaps in the not too distant future, Next Generation Sequencing (NGS) and karyomapping (16, 17).

d) Non-invasive selection criteria. To date the most predominant criteria employed for embryo selection involve morphology of the oocyte, the early zygote (pronuclei score), and the cleavage stage embryos, mainly referring to the percentage of fragmentation and the number and architecture of blastomeres (18). Blastocyst culture is another possible strategy for selecting the best embryos.

The latest schools of thought believe that intensifying our investigation of the embryo's physiological state, and further defining new non-invasive strategies aiming to increase the accuracy of embryo selection in a clinical setting is the answer (18). The field of interest has been shown to shift towards the "omics" sciences, such as genomics, transcriptomics, proteomics and metabolomics, and back to non-invasive morphological criteria yet again (19). This trend, aiming for a comprehensive analysis of the biological markers of embryo viability, could represent a valuable adjunct to morphological criteria, which remain the traditional and customary tool for embryo selection (18).

e) Time-lapse imaging. Time-lapse imaging offers the advantage of being able to make multiple observations, providing better and stable culture conditions by the use of incubators with integrated microscopes (20). The embryologist is equipped with more and better comprehensive data about the kinetics of embryo development, something that may introduce new dynamic markers. Efforts have been made to upgrade time-lapse analysis by determining identifiable biomarkers and for which customized software platforms could be implemented (21). One such software platform combining time-lapse imaging and day 3 morphological assessment might lead to automated embryo selection claiming to improve implantation rates (22).

CAMs in IVF

It is well-documented that adhesion molecules influence directly and indirectly numerous aspects of cell behavior, such as proliferation, survival, migration, growth, angiogenesis, tumor invasion, and metastasis (23). Expression and modulation of CAMs play a key role in regulating cell–cell and cell–extracellular matrix interactions, the cascade of developmental processes implemented in the complex events of fertilization, embryogenesis, implantation, placentation, and embryonic development (23). Moreover, important studies revealed that with respect to the nature of neoplastic cells, indeed many of the morphological and behavioral features that characterize them could be the result of changes in the expression or function of CAMs (24, 25). Investigating and confirming their indisputable role in physiological and pathological mechanisms, it comes as no surprise that CAMs play an important role throughout the whole process of IVF and in its clinical outcome, and are therefore worth investigating in depth. It is of great essence to study and focus on the role of CAMs during the development of the early pre-implantation embryo that is generated and can only be studied within the frame of IVF.

Research material in the form of human pre-implantation embryos is precious and difficult to obtain within the IVF laboratory due to its clinical nature. Therefore, any material related to the pre-implantation embryo is of research interest and value. To date, several studies have been performed on the role of CAMs in IVF, employing serum from patients during IVF treatment (26, 27), follicular fluid (28), granulosa cells (29), oocytes (30), endometrial biopsies (31, 32), and tubal biopsies (33). In the following paragraphs we discuss studies focusing on CAM involvement during the several phases and aspects of the treatment.

a) Ovarian hyperstimulation syndrome (OHSS). It is well-documented that CAMs are involved in ovarian physiology, and, along with the immune system, play a key role in all physiological ovarian processes (34-37). Soluble vascular endothelial–cadherin (sVE-cadherin) was studied by Villasante *et al*. (26) in an effort to characterize OHSS, and the implication of sVE-cadherin. OHSS is a life-threatening condition, classified as an iatrogenic complication of the ovarian stimulation induced in preparation for IVF and Intracytoplasmic Sperm Injection (ICSI) (26). The pathophysiological mechanism of OHSS is unknown and its treatment is empirical, therefore any information that could aid management of OHSS, or help to indicate markers to avoid it is precious. Serum levels of sVE-cadherin were recorded and evaluated in an effort to investigate whether the endothelium was a source and target of the vasoactive substances released in response to the conditions clinically- induced in women with OHSS (26). In accordance with a previous study by the same group (38), the results indicated with certainty the participation of this CAM in the pathophysiology and progression of OHSS, making sVE-cadherin a potential marker for indicating the *corpus luteum* function following controlled ovarian stimulation, as part of the standard procedure employed for IVF. A previous study correlating CAMs with the clinical and biological aspects of OHSS identified a significant positive correlation with soluble intercellular adhesion molecule-1 (s-ICAM-1) and a negative correlation with serum soluble E-selectin, linking the respective CAMs to the pathophysiology of capillary hyperpermeability in severe OHSS (39). The same CAMs were further investigated and their participation, especially in the severe forms of OHSS, was clearly elucidated in the work performed by Kovachev and colleagues in 2005 (40). Moreover, additional data have surfaced regarding the mechanism behind OHSS and the implication of certain CAMs. A specific model of steroidogenic–endothelial cell interaction in OHSS was used by Rodewald *et al*. (41). Through this model, Rodewald *et al*. determined that human Chorionic Gonadotropin (hCG) increases endometrial permeability through the increase of Vascular Endothelial Growth Factor (VEGF) and the decrease of claudin 5, an endothelial membrane protein. Recently, a number of soluble receptors for the VEGFs have been detected (sVEGFRs) and it has been shown that these sVEGFRs compete with the membrane-bound VEGFR to bind VEGFs (42). More precisely, sVEGF-R2 decreases in OHSS, and that decrease becomes more prominent as the severity of OHSS increases. Finally, VEGF and interleukin 8 (IL8) have an additive effect in the increase of endometrial permeability, and they also share a common receptor, the VEGFR-2. Dopamine can possibly block VEGF- and IL-8-induced endothelial permeability by inhibiting common VEGFR2-dependent signals (43).

b) Receptive endometrium. During IVF treatment there are numerous important points that can define success. One of the most important points to ensure a positive result is a receptive endometrium. Any abnormality in the structure and receptivity of the endometrium jeopardizes the whole treatment cycle (44). It is the combined actions of both estrogen and progesterone that control endometrial receptivity. Therefore, it is clear why the balance of the hormones is disturbed during IVF treatment due to ovulation induction, affecting the endometrial morphology and hence its receptivity (45). Integrins are a group of CAMs allowing cell to cell interaction. Within the endometrium, their expression is cyclical across the menstrual cycle but three integrins ($\alpha v\beta_3$, $\alpha_4\beta_1$, and $\alpha_1\beta_1$) are all expressed only during the implantation window and are therefore considered to be markers for endometrial receptivity (46-49). Their exact role remains controversial, with certain groups expressing their doubts as to their importance and relevance (50). Lessey *et al*., during their efforts to study the endometrial proteins expressed during the window of implantation, from endometrial biopsies during the luteal phase, hold that two different proteins, $\alpha v\beta 3$ integrin and osteopontin, are differentially expressed and play a key role in attachment and embryo signaling (51). It is possible that any disruption in the molecular pathways that regulate the expression of these proteins could lead to implantation failure. Additional data propose a different implication of $\alpha v\beta 3$ integrin in IVF failure (52). Endometrial $\alpha v\beta 3$ integrin, which plays a key role in the adhesion of the embryo, has the same $\beta 3$ subunit as $\alpha IIb/\beta 3$ integrin, which is associated with platelet aggregability. The main hypothesis is based on a known polymorphism in the b3 subunit gene sequence that increases the subunit's affinity to ligands. This polymorphism may affect not only platelet aggregation but also the adhesion of an embryo during implantation, leading to a probable recurrent pregnancy loss. Thomas and colleagues began investigating the expression of the three integrins on endometrial biopsies from oocyte donors and comparing them to fertile controls, in order to establish a relationship between them and infertility (32). In fact, the 2002 study demonstrated that integrin expression seems to be reduced in the glandular epithelium in the endometrium

after ovulation induction, indicating that their reduced expression has an adverse effect on pregnancy rates (32). This sort of result should send clinicians a powerful message regarding good code of practice; it is not rarely that we sacrifice endometrium receptivity in order to ensure increased yield of oocytes, as high estrogen levels are desirable, but these high levels impair receptivity, reducing integrin expression and leading to reduced implantation rates. This fact is a driving force behind the latest trend of opting for FET for all, which is analyzed above. Such a practice enables a very practical dissociation of employing a protocol for a good yield of oocytes and running the risk of endometrial impairment, from ensuring a receptive properly prepared endometrium. A fine balance should be achieved through better understanding of how the endometrium becomes receptive (32). In less than a year, Thomas and colleagues revisited this topic from another point of view, studying the endometrial expression of integrins αvβ3, α4β1, and α1β1 on timed endometrial biopsies from patients undergoing IVF during the implantation window (31). The study demonstrated that the three integrins had a role in predicting IVF outcome, and in the future may potentially become markers on whether patients are eligible to opt for IVF treatment on the grounds of good prognosis. However, it should be noted that aiming to use integrin expression as a predictive marker is associated with certain drawbacks: firstly and foremost, assessing the endometrium prior to treatment could alone impair the implantation potential. Further work is required to consider this test in a clinical setting (31). More recent data submitted by Surrey *et al*. (53), Coughlan *et al*. (54), and Casals *et al*. (55), collected through the study of endometrial biopsies, strongly suggest that integrin αvβ3, which is the strongest candidate for being implicated in endometrial receptivity, actually has no prognostic value whatsoever. However, it has been suggested by Konac *et al*. (56), that at least at the transcriptional level, unexplained infertility has been connected with a marked decrease in the expression of vascular cell adhesion molecule 1 (VCAM1).

Indeed, the results regarding the role of integrins as a predictive marker of IVF outcome remain controversial. A recent project showed no difference in the expression of α1, α4, αvβ3 subunits comparing the levels between women with recurrent implantation failure and a control group, supporting the notion that there is no prognostic value of integrins (54).

c) Tubal pregnancy (TP). A very interesting study by Revel and colleagues pondered the paradox of the phenomenon of TP being twice as common following IVF treatment than with natural conception. This is a surprising fact, considering IVF embryo transfers aim for an accurate area in the uterine cavity (33). Perhaps the first IVF treatment resulting in TP should not be regarded as a random event (57). The pathology and mechanism behind TP is still unclear and the hypothesis raised is that either the embryo or the fallopian tube somehow participate in the pathological process leading to TP. E-Cadherin is presumed to be essential for embryo development and blastocystic implantation, therefore its potential abnormal overexpression in the fallopian tube could coax the blastocyst from where it is placed following the transfer of the embryo to the ectopic site (33). Additionally, recent results by Li *et al*. reinforce the involvement of E-cadherin in TPs, but also add the involvement of β-catenin and the Wingless-Type (Wnt) signaling pathway (58). More specifically, β-catenin expression is significantly increased, most likely through the activation of the WNT signaling pathway, while E-cadherin expression is reduced. Both the embryo and endometrium engage in a dialog involving adhesion molecule secretion, enabling all phases of implantation to take place (apposition, adhesion, penetration) (59); it is a valid hypothesis that over- or underexpression of certain CAMs involved in the process could initiate the pathology leading to TP (33), or failed implantation (60).

d) Follicular fluid. Benifla *et al*. performed a study recording concentrations of VEGF, platelet endothelial cell adhesion molecule-1 (PECAM1), and VCAM1, in the follicular fluid of women treated with assisted reproductive technology (28). The results indicated that sVCAM1 can be considered as a valid biochemical marker of fertilization, with elevated sVCAM1 concentrations in the follicular fluid being associated with a high fertilization rate (28). sVCAM1, as well as sICAM1, have been known to fluctuate cyclically during the menstrual cycle and may reflect remodeling events in the endometrium. If so, they could also prove viable markers for disease state (61). Contrary to the results obtained by previous studies by Friedman and colleagues (62), according to which VEGF can act as a predictive marker of conception rate, with high levels of VEGF associated with poor outcome and decreased ovarian reserve, in the Benifla study, VEGF, CD31, or sVCAM1 did not hold any predictive value for the clinical outcome.

A study on serum levels of sVCAM1 in patients undergoing IVF treatment, demonstrated that its expression is affected significantly following gonadotropin stimulation for IVF due to the changes in E_2 levels, although the exact mechanism through which E_2 suppresses sVCAM expression is unknown (27).

e) Trophectoderm. Employing novel DNA microarrays, Assou *et al*. tried to define the gene expression profile in trophectoderm cells from day 5 human blastocysts compared to endometrial cells in stimulated cycles for IVF (63). More specifically, through the study of endometrial biopsies during the implantation window, they managed to specify part of

that dialog. In summary, they found a differential expression of various molecules and tissue-specific differences, suggesting their possible role during the early stages of blastocyst attachment and implantation. They determined that in that window, the melanoma cell adhesion molecule (MCAM), integrin alpha E (ITGAE) (a member of the integrin family) and collectin sub-family member 12 (COLEC12) (a member of the selectin family) were overexpressed in the trophectoderm, whereas activated leukocyte cell adhesion molecule (ALCAM), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), platelet endothelial cell adhesion molecule-1 and CD44 (the receptor for hyaluronic acid) were overexpressed in the endometrium (64).

Indeed the role of cell–cell adhesion molecules of the cadherin superfamily in implantation is intriguing. Since E-cadherin expression is essential in trophoblastic invasion, it is safe to hypothesize that abnormal expression could lead to unsuccessful pregnancies (65). It is worth noting that even though there are studies on E-cadherin expression, there is a gap bibliographically with respect to the regulation of its expression, and information could be drawn and extrapolated from the cancer literature on parallel neoplastic cells (23). The loss of the adhesive properties of trophoblast cells, mediated by E-cadherin, was verified as being associated with spontaneous abortions by Batistatou and colleagues, who also soundly point the need for cytogenetic analysis investigating karyotypic abnormalities and E-cadherin expression, in order to couple and strengthen the relevant research (23).

f) Ovarian granulosa cells (GCs). These are unique endocrine cells. In growing follicles, GCs proliferate and achieve functional maturation, secreting estrogens and regulating oocyte maturation. Clavero *et al*. set out to investigate the expression of integrin fraction and adhesion molecules on human GCs, aiming to define the relation with oocyte maturity, and follicular steroidogenesis. This study found that the expression pattern for integrin fractions and adhesion molecules could be of predictive value in assessing oocyte maturity, and therefore could function as a predictive marker for IVF outcome, through ovarian stimulation response and its prognosis (29).

g) Oocytes/embryos. On the same topic of oocyte maturation and its prediction by CAMs serving as biochemical markers, Borgatti and colleagues presented an important study where the material employed, analyzed and used to provide information was IVF-generated oocytes and embryos. The analysis of oocyte maturation is of great importance in predicting successful fertilization and embryo development (30). Oocyte selection is an important part of the treatment that can define the end result. The assessment of oocyte maturation and quality and subsequent selection is based on morphological criteria, which have been claimed to correlate with the outcome, such as polar body morphology (66), cytoplasm appearance (67), zona pellucida thickness (68), and the position and shape of the spindle (69). Even though molecular approaches have been proposed, such as ploidy, and chromosome/chromatin status (70, 71), morphology is still the method of choice in the average IVF laboratory. The results of the current study proved a negative correlation between the concentration of sICAM1 released from oocytes and the degree of maturation and grade, with the mature and better graded oocytes releasing lower concentrations of sICAM1 when compared to immature oocytes of lower grade. In light of the reduction of the number of fertilized oocytes and transferred embryos being the main target of assisted reproductive medicine, this study suggests that sICAM1 should be a marker for oocyte maturation and grading (30).

Concluding Remarks

The method of choice in identifying the most viable embryo and therefore the one with higher implantation potential in the average IVF laboratory, is still through to be the use of morphological criteria, such as cell size, number, the phenomenon of multinucleation, type and percentage of fragmentation, and cleavage rate (72-75). However, based on morphology alone, important aspects of embryo viability remain excluded (76). IVF studies constantly aim to provide new markers, enriching and validating embryo selection criteria. The focus is targeted on the genetic constitution of the embryo and its metabolomic/secretomic profile.

With respect to investigating the chromosomal complement of the embryo, the most validated route involves the step of embryo biopsy in order to obtain the genetic representation of the embryo. Even though such a step is classified as being invasive, there is proof that removing an appropriate number of cells from an embryo at a certain developmental level is totally compatible with a positive outcome (77). However, in the context of PGS and not preimplantation genetic diagnosis, and in order to move away from a technique potentially hazardous to the embryo but at the same time ensure sufficient genetic material, the current trend is to opt for biopsy at the blastocyst stage when performing PGS (16, 78).

Always concerned with obtaining maximum information within the IVF treatment, the weight is being slightly shifted from genomics to transcriptomics, to proteomics, and ultimately to metabolomics, defining a new era in the preimplantation embryo physiology. There are approximately 25,000 genes in the human genome, however, the way genes are transcribed to proteins, of which there are estimated to be around a million, is not uncomplicated. Proteomics

describes the protein content within a cell, expressed and coded from the genome, while metabolomics identifies small molecules produced in biological fluids as an end result of protein actions. Investigating biological fluids produced by the embryo, follicle, or oocyte in an IVF laboratory, is currently a way to obtain valid information on the embryos' identity and potential, in a safe and non-invasive fashion, and more work needs to performed employing the preimplantation embryo as its focal point.

In view of the generic importance of CAMs well-documented in literature, and their involvement in all pivotal stages of embryogenesis, development and implantation, it is only natural to suggest and promote the focus and design of studies aiming to delineating the connection of CAMs and their contribution to and participation in all critical stages in order to assess further their potential as biochemical markers of predictive value within the field of reproductive medicine. It has long been apparent that adhesion molecules can be employed as diagnostic tools in tumor pathology, given the appropriate continuous investigation suited to such a clinical setting (79). From their involvement in such a routine clinical setting, we could extrapolate that they might similarly act as prognostic markers of the preimplantation embryo and IVF treatment, granted that their employment will be implemented based on relevant investigation. A broad-spectrum approach to determine key factors that act on the morphological, genetic, proteomic, transcriptomic, and metabolomic levels, and employing histological, cytogenetic, and molecular techniques, will help us acquire critical information to achieve a higher level of treatment and management of infertility.

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Received March 19, 2014
Revised May 27, 2014
Accepted May 29, 2014