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A minimally invasive methodology based on morphometric parameters for day 2 embryo quality assessment

Abstract

The risk to maternal-foetal health due to multiple pregnancies can be decreased by reducing the number of transferred embryos. Therefore, new tools for selecting embryos with the highest implantation potential should be developed. The aim of this study is to evaluate the predictive implantation ability of morphological and morphometric variables by analyzing images of embryos.

This is a retrospective study of 135 embryo photographs from 112 IVF-ICSI cycles performed between January and March 2011. The embryos were photographed immediately before transfer using "Cronus 3" software and their images were analyzed using the public program *ImageJ.*

Significant effects and higher discriminant power to predict implantation were observed for the morphometric embryo variables in comparison with morphological ones. The features for successfully implanted embryos were: 4-cells on day 2 of development; all blastomeres with circular shape (roundness factor greater than 0.9)), an average ZP thickness of 13 microns and an average of 17695.1 microns^2 for the embryo area. The size of the embryo, which is described by its area and the average roundness factor for each cell, provides two objective variables to consider when predicting implantation. This method would improve the current "*embryo classification systems".*

Key words: morphological and morphometric embryo parameters; image analysis; embryo grading systems; logit regression; ROC curve.

INTRODUCTION

The number of transferred embryos in IVF-ICSI cycles needs to be reduced due to the increased obstetric and perinatal risks involved in multiple pregnancies. Both the number and quality of transferred embryos are correlated with high multiple pregnancy rates, which is why the selection of one top quality embryo for transfer is proposed by Hu et al. (1998), Strandell et al. (2000) and Wright et al. (2006). Current embryo quality assessment, based on the morphological criteria of a transferred embryo, is highly subjective. Therefore, a scoring system for ranking implantation is essential when aiming for singleton pregnancies without a significant decrease in pregnancy rates (Catt et al. 2003; De Neubourg et al. 2004; Van Montfoort et al. 2005; Bergh 2005; De Neubourg and Gerris 2006). SET produces an unacceptably low pregnancy rate particularly in older patients and in those with a poor embryo quality (De Neubourg and Gerris 2006).

The woman's age and embryo quality are the variables with most influence on the implantation rate (Steer et al. 1992; Shulman et al. 1993; Giorgetti et al. 1995; Van Royen et al. 1999; Hardarson et al. 2001; Terriou et al. 2001; Hunault et al. 2002; Holte et al. 2007). The first variable is unchangeable, but when there is a sufficient number of embryos available we can select the embryos with the greatest implantation potential according to morphological criteria for transfer (Van Royen et al. 1999; Ebner et al. 2001; Van Royen et al. 2001; Scott et al. 2002; Ebner et al. 2003; Scott et al. 2003; Van Royen et al. 2003; Rienzi et al. 2005; Holte et al. 2007; Scott et al. 2007). Thus, it is important to increase knowledge about the characteristics of embryos with a high implantation potential as well as of non-top embryos (Debón et al, 2013).

Evaluation of the implantation potential of transferred embryos has generally been based on the construction of accumulated embryonic scores. Assumptions need to be made about the overall quality of transferred embryos and their subsequent implantation owing to ignorance about the exact quality of the embryo which is finally implanted (Cummins et al. 1986; Steer et al. 1992; Visser and Fourie 1993; Copperman et al. 1995; Giorgetti et al. 1995; Terriou et al. 2001; Laasch and Puscheck 2004). In more recent studies, logistic regression models have been used to predict the implantation rate of embryo, (Holte et al. 2004; 2006; 2007; Debón et al, 2013).

Traditionally, embryo quality assessment is based primarily on the morphological criteria of transferred embryos which is highly subjective and therefore very variable (Paternot et al. 2009; 2011a). As a result, embryonic classification systems based on the use of objective parameters of embryo morphology should be developed. That is, measurements should be taken directly from the embryo and used in place of the observer's opinion, thus totally avoiding the subjectivity of the measurements.

The use of morphometry in the standardization of elements and processes has been used for a long time in metallurgy, molecular biology and electron microscopy (Pertusa 2010). This study has been carried out linking morphometric embryo variables to embryo quality parameters such as embryo fragmentation and multinuclearity as well as embryonic segmentation and three-dimensional reconstruction (3D), (Hnida et al. 2004; Agerholm et al. 2008; Beuchat et al. 2008; Giusti et al. 2010; Santos et al. 2010). However, there are few studies evaluating

the predictive implantation ability of embryo morphometric parameters. Recently, Partenot demonstrated better prediction of implantation rates based on the number and size of blastomeres on day 3 and correlations between total embryo volume and clinical pregnancy (Paternot et al. 2011b; 2013).

The aim of this study is to evaluate the predictive implantation reliability of the morphometric variables using image analysis of embryos that have already been transferred and whose fate is known (implanted or not implanted). The incorporation of these variables into the current embryo classification permits us to develop a new embryo classification system, based on a combination of morphological and morphometric variables, which would improve the quality of embryo selection prior to transfer.

MATERIALS AND METHODS

1-ART procedure MM

After oocyte retrieval, the oocytes were placed separately in 200 microliter drops of culture medium (IVF medium, Medicult, Denmark) under mineral oil (Mineral oil Medicult). Spermatozoa for the IVF procedure were prepared using standard swim-up procedures. Sperm samples for ICSI were diluted and centrifuged twice at 300g for 10 min. Standard IVF/ICSI procedures were performed 2–6 h after oocyte retrieval. In the IVF procedure, oocytes were inseminated with 300 000 progressively motile spermatozoa per oocyte.

In the case of an ICSI cycle, injected oocytes were incubated together in 20 microliter drops of culture medium (IVF medium, Medicult, Denmark) under mineral oil (Mineral oil Medicult). On Day 1 (16–20 h after insemination/injection) fertilization was evaluated. Only normally fertilized oocytes (2PN) were cultured

individually in a 20 microliter droplet of culture medium (IVF medium; Medicult) covered with mineral oil.

On day 2 (44–47 h after insemination/injection) embryo evaluation was carried out based on the assessment of cell number, size and degree of fragmentation. The best available embryos were chosen for transfer based on the standard embryo scoring system.

All the embryos were photographed immediately before transfer. The photographs were taken using "Cronus 3" software (*Research Instruments LTD*) implemented in a phase contrast inverted microscope (Nikon Eclipse) with a 20 x optic magnification and a Hoffman modulation contrast.

The images were analyzed using *ImageJ,* a public program developed by Wayne Rasband (http://rsb.info.nih.gov/ij/) and the available tools. This program was chosen for its accuracy in the assessment of embryonic characteristics and the large number of available tools allowing better embryo characterization by reducing the experimental error associated with the observer.

1. Patients and embryos.

In this study we studied the photographs of 135 embryos from 112 IVF-ICSI cycles performed from January to March 2011. A range of 1, 2 or 3 embryos were transferred on day 2 of development depending on their availability. In order to avoid adverse effects on embryo implantation, only the first cycle of IVF-ICSI were considered and cycles with endometriosis or a poor response were excluded.

2. Variables of the study.

Clinical variables of the couple, morphological embryo variables used in most IVF laboratories and morphometric embryo variables which provide a better understanding of all the characteristics of the embryo were considered.

Although the basic aim of this study was to evaluate the predictive implantation ability of morphological and morphometric variables, the issue of the clinical variables of the couple needs to be resolved as a possible confounding factor.

2.1. Clinical variables of the couple.

The clinical variables of the couple considered were age of the woman, severe male factor and number of transferred embryos.

2.2. Morphological embryo variables.

The morphological embryo variables number of cells, blastomere symmetry (Hardarson et al. 2001) and fragmentation, and structural abnormalities of the zona pellucida (ZP) were scored.

Number of cells was considered to be a quantitative variable with low number of values as it ranged from 2 to 6.

Symmetry was also analyzed as a qualitative variable according to the criteria described by Hardanson et al. 2001. Embryos with a difference in the blastomere size greater than 20% were considered to be asymmetric. Two levels were considered: symmetrical (1) and asymmetrical (0) embryos.

Blastomere fragmentation was studied by considering four embryo qualities depending on their fragmentation: less than 10%, between 11-25%, between 26-35%, and more than 35% (Alikani *et al.*, 1999), therefore the corresponding four

levels were established: 0, 1, 2 and 3. It was also analyzed as a qualitative variable.

The structural abnormalities of the ZP only included irregularities (bumps and thickening) (Alikani et al. 1999). Two levels were established: absence (0) and presence (1) of both ZP structural abnormalities.

Blastomere multinuclearities were not considered because, the embryos selected for subsequent transfer do not exhibit this feature, therefore we did not have enough images of this type of embryo.

2.3. Morphometric embryo variables.

Embryo area and perimeter, equivalent circle radius of the embryo, embryonic roundness, zona pellucida thickness, blastomeres area and perimeter, equivalent circle radius of the blastomeres and blastomeric roundness were evaluated from the photographs taken immediately before transfer. **All measurements were repeated at least 3 times taking into account 3 different reference points to avoid errors associated with deformities of the maximum projection circle.**

The ZP envelope converts the embryo into a spheroid therefore the overall size of the embryo would be an almost circular projection of the maximum plane in any position. The embryos were photographed at 200X. This microscopic magnification produced a complete image of the whole embryo showing all of its cells by transparency. It is possible that the orientation of the embryo should be considered as an other factor. In future studies reconstruction techniques such as con-focal microscopy will be used to calculate the volume from the voxel value. At present, this biological

material is very sensitive and has to be exposed to sub-optimal culture conditions the minimum time necessary for a single photograph.

Embryo evaluation was based on the assessment of cell number, size and the degree of fragmentation. The best available embryos were chosen for transfer based on this standard embryo scoring system. All the embryos were selected and photographed immediately before transfer (44–47 h after insemination/injection). The time interval between the embryo selection, photography and embryo transfer never exceeded 20 minutes. Consequently, our measures should not be affected by embryo timing.

-Embryo area and perimeter: To measure these variables, the "Elliptical or brush selection" tool was used. To increase measurement accuracy, after marking the 2 diameters (the major and minor diameter), the ellipse described by the program, was interactively adjusted to the embryo boundary, so that both shapes coincided in as many points as possible.

-Embryo radius: To measure this, the "*Three Point Circular ROI*" plug was used (Landini, 2001;http://www.dentistry. ham.ac.uk/landinig/software/software.html). In order to calculate the radius with minimal error, three points on the embryo perimeter were selected and a circle which fitted the embryo perimeter as well as possible was drawn.

-Embryonic blastomeres area: To carry out the measurement of the embryonic blastomeres area, the "*Polygon Selections*" and "*Freehand selections"* tools were used. To calculate the blastomere area, some points that delimit the contour of each cell were marked. The "*Elliptical or brush selection*" tool was not used because blastomeres have an irregular boundary that does not fit the feature of an

ellipse as in the case of the whole embryo. Thus the setting is much more accurate, since the analyzer is the one that defines the exact contours, point by point.

-ZP thickness: The tool used in this case was "Straight"; a tool which permits the drawing of segments, the thickness variation of the ZP was measured at three different points for each embryo.

-Circularity or roundness factor is a rate defined as $4\pi(\text{Area})/(\text{Perimeter})^2$, with a value of 1.0 indicating a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated shape (Russ 1992).

-Equivalent circle radius: Equivalent circle radius is the radius of a circle having the same area as the studied object, but avoiding embryo irregularities. This radius is measured both for the embryo and its blastomeres.

All morphometric variables were considered as quantitative variables.

3. Statistics

The embryonic variables (morphological and morphometric) were compared in relation to implantation. For the implantation study, only embryos whose fates are known (implanted or not implanted) were considered:

-0% implantation group: 108 embryos from 56 cycles with transference of one, two or three embryos which gave a negative pregnancy test.

-100% implantation group: 27 embryos from the 56 cycles which gave a positive pregnancy test in which the number of gestational sacs observed by ultrasound coincided with the number of transferred embryos.

Cycles with fewer gestational sacs than transferred embryos (implantation rate of 33.3% or 50%) were not considered in the implantation study because in

those cases we did not know the morphological and morphometric variables of the embryos that were successfully implanted.

The first step in understanding our data is to establish the kinds of variables, a descriptive analysis and an analysis to contrast the effect of each of these variables on embryo implantation. This statistical analysis was performed using Statgraphics Centurion XV. In the case of quantitative variables, an average comparison test (t-test) was performed. **The normality of the quantitative variables were tested using QQ-plot in the stats package for R. Q-Q Plot is a plot of the percentiles of a standard normal distribution against the corresponding percentiles of the observed data. If the observations approximately follow a normal distribution, the resulting plot should be a roughly straight line with a positive slope.**

The qualitative variables or quantitative variables with a low number of values were compared using crosstabs, and the Chi-square test was used to assess the statistical significance of the independence of the variables. A p-value <0.05 was considered statistically significant.

These results are in tables which show the description of variables by columns:

- In the case of quantitative variables: mean and standard error of the mean by means of a confidence interval for each group, the corresponding p-value and statistical test for the comparison of the morphological embryo parameters between the 0% implantation group and 100% implantation group.

- In the case of qualitative variables or quantitative variables with a low number of values: values, frequency (percentage) for each group, the corresponding p-value and statistical test for the comparison of the morphological embryo parameters between the 0% implantation group and 100% implantation group.

The second step is to avoid multicolinerity because the independent variables could be correlated, and then we should find predictor variables of real interest when independent variables are all considered at the same time. Collinearity, or excessive correlation between explanatory variables, can complicate or prevent the identification of an optimal set of explanatory variables for a statistical model. A simple approach to identify collinearity among explanatory variables is the use of variance inflation factors (VIF). VIF calculations are straightforward and easily comprehensible; the higher the value, the higher the collinearity.

Therefore, **in the third step**, we used a generalized linear model (GLM) to study embryo traceability from transfer to implantation: treatments resulting in 0% implantation or 100% implantation and the selection of variables with “stepwise”.

Finally, the model was validated using ROC curves by including cycles with a lower number of sacs than transferred embryos. ROC curves provided comprehensive representation of the accuracy of each model and also allowed an easy comparison with other models. These last statistical analyses were performed using the R environment for statistical computing (R Development Core Team, 2005).

RESULTS

The women´s average age was 34.25±0.75 with an average of 11.0±4.2 retrieved metaphase II oocytes. All the transferred embryos were photographed immediately before the transfer and the supernumerary good quality embryos were vitrified.

The QQ-plots of the quantitative variables were drawn, all of them showed approximately a straight line that supports normality and consequently the corrected application of the t-test.

Clinical variables of the couple related to implantation.

Table 1 shows the description and comparison of the clinical variables of the couple related to implantation. From results of the comparison by means of t-test and chi-squared which were not significant (p-values>0.05) for all the variables, we were able to say that there were no significant differences between women in both groups as regards age, severe male factor and number of transferred embryo.

Table 1. Statistical tests used in the comparison of the clinical variables of the couple between 0% implantation group and 100% implantation group.

Variable		0% Implantation Group	100% Implantation Group	P-value	Statistics
AGE		34.5±0.85	33.4±1.67	0.2278	t-test
SEMEN	0	6 (8.95%)	1 (1.49%)	0.9487	Chi-Square
	1	46(68.66%)	14 (20.89%)		

NET	**1**	**8 (11.94%)**	**4 (5.97%)**	**0.5341**	**Chi-Square**
	2	**44 (65.67%)**	**11(16.41%)**		**Chi-Square**

AGE: Woman age; SEMEN: Severe male factor; NET: number of transferred embryos.

Embryo variables related to implantation.

1. Morphological embryo variables

Table 2 shows the description and comparison of the morphological embryo parameters. 0% implantation group: 108 embryos rom 56 cycles with transfers of one (8), two (44) or three (1) embryos which gave a negative pregnancy test. 100% implantation group: 27 embryos from cycles in which the number of gestational sacs observed by ultrasound coincided with the number of transferred embryos, 5 cycles with transference of only 1 embryo resulting in a singleton pregnancy and 11 transfers of 2 embryos which resulted in a double pregnancy. Significant differences between both groups were observed for FRAG and ZP bumps as their corresponding p-values were lesser than 0.05. Although these variables didn't have significantly lower values in embryos of the 100% implantation group than the 0% implantation group as expected.

Table 2. Statistical tests used in the comparison of the morphological embryo variables between 0% implantation group and 100% implantation group.

Variable		0% Implantation Group 108 embryos	100% Implantation Group 27 embryos	P-value	Statistics
	2	15 (13.89%)	0 (0.00%)	0.4243	Chi- Square
	3	12 (11.11%)	3 (7.40%)		
	4	73 (67.59%)	23 (85.18%)		
	5	7 (6.48%)	1 (3.70%)		
	6	1 (0.92%)	0 (0.00%)		
SYM	0	21 (19.44%)	6 (22.22%)	0.1113	Chi- Square
	1	87 (80.56%)	21 (77.78%)		
FRAG	0	69 (63.89%)	11 (40.74%)	0.0002	Chi- Square
	≤10%	28 (25.92%)	11 (40.74%)		
	11-25%	11 (10.18%)	5 (18.52%)		
	26-35%	0 (0.00%)	0 (0.00%)		
ZP	0	86 (79.63%)	26 (96.30%)	0.0256	Chi-Square with Yates correction
	1	22 (20.37%)	1 (3.70%)		

CN: Number of cells; **SYM:** Blastomere symmetry; **FRAG:** Blastomere fragmentation; **ZP:** Structural abnormalities of the Zona Pellucida.

2. Morphometric embryo variables

Table 3 shows the description and comparison of the morphometric embryo and blastomere parameters between the 0% implantation group and 100%

implantation group. Highly significant differences (p-value<0.05) were observed for embryo perimeter, ZP thickness, equivalent circle radius of the embryo and for all the measurements related to the blastomere: area, perimeter, equivalent circle radius and roundness.

BROUND tends significantly to the value 1, which corresponds to a perfect circle, in the case of the 100% implantation group. By contrast, cells from the non-implantation group had a roundness factor lower than 0.9, which means that cell shape was more elongated with an elliptical appearance. **Although this shape could be assessed by optical observation only, computerized image analysis is recommended.** The measurements related to the whole embryo, EP and ECRE, also had significant differences. The embryos that successfully implanted had an equal and circular blastomere shape with a lower radius and derived parameters (area and perimeter).

Table 3. Mean, confidence intervals, relative frequencies, p-value and statistical tests used in the comparison of the morphometric embryo variables between Group 0% implantation and Group 100% implantation.

Variable	**Group 0% Implantation 108 embryos**	**Group 100% Implantation 27 embryos**	**p-value**	**Statistics**
EA	18743.2 ± 2973.28	17695.1 ± 1787.31	0.082	T- Test
EP	486.375 ± 32.52	472.398 ± 24.01	0.038	T- Test
ER	76.862 ± 4.88	75.097 ± 3.86	0.083	T- Test
ZPT	16.186 ± 2.66	13.006 ± 1.74	0.000	T-Test

EROUND	0.992 ± 0.003	0.994 ± 0.003	0.457	T- Test
ECRE	77.075± 5.08	74.956 ± 3.83	0.045	T-Test
BA	3513.8 ± 987.168	3234.12 ± 678.278	0.006	T-Test
BP	220.060 ± 29.084	210.666 ± 22.379	0.002	T-Test
ECRB	33.153 ± 4.403	31.905 ± 3.414	0.050	T-Test
BROUND	0.897 ± 0.041	0.906 ± 0.044	0.007	T-Test

EA: Embryo Area; **EP:** Embryo Perimeter; **ER:** Embryo radius; **ZPT:** Zona pellucida thickness; **EROUND:** embryonic roundness; **ECRE:** equivalent circle radius of the embryo; **BA:** blastomeres area; **BP:** Blastomeres perimeter; **ECRB:** equivalent circle radius of the blastomeres; **BROUND:** blastomeric roundness

3. Logit model.

The aim of our model was to predict the way in which implantation potential varies due to characteristics of the embryos and of their corresponding blastomeres**.** Analytical limitations related to multicollinearity required us to think carefully about the variables we chose to model by means of VIF. Several packages in R provide functions to calculate VIF but we used function "*vif_function"* which is available and explained in detail on the web http://beckmw.wordpress.com/2013/02/05/collinearity-and-stepwise-vif-selection/.

Therefore, we were able to fit a logit model under Bernoulli distribution for binary response variable "correct" or "failed" implantation (i.e., 1 or 0). **GLM allowed us to analyse binary data and logit models, with categorical and continuous predictors, a detailed description is available in Debon et al (2013). Therefore, qualitative or quantitative variables with a low number of**

values were considered as categorical predictors often called factors, and quantitative variables, in general, were considered continuous predictors. The coefficients in logit models are used to study the impact of an independent variable on implantation probabilities but in the case of factors are used to study the differences in probabilities between different factor values. Within the framework of GLMs, least squares (LS) parameter estimation is replaced by maximum likelihood estimation (MLE).

The next step is to select the most relevant variables for fitting. **Model with all possible variables without multicolinearity is used as the initial model in the stepwise search.** Our criterion here was based on AIC (Akaike information criterion) which is a measure of the relative quality of a statistical model. Hence AIC not only rewards goodness of fit, but also includes a penalty that is an increasing function of the number of estimated parameters.

The results of the logit model for selected blastomere characteristics are shown in Table 4, which includes the name of the variables, the value of the parameter estimate for each of the variables, the standard error (SE), the t-value, and the p-value or significance for each of the coefficients.

Table 4. Estimates for the parameters of the logit model based on blastomere variables.

Variable	Parameter estimate	SE	t-value	p-value
Intercept	-0.5221	0.7657	-0.682	0.4953
BP	-0.0069	0.0013	-2.949	0.0000
BROUND	1.5867	0.7913	2..005	0.0449

Similarly the results of the logit model for selected embryo characteristics, including the average of blastomere variables of each embryo, are shown in Table 5. **It is important to note that although age of the woman and the rest of embryo characteristics were analyzed, Table 5 only includes the final variables without muticolinearity, and selected in stepwise.**

Some of the predictors were introduced as factors such as fragmentation (FRAG) and ZP bumps (ZP)**.** Therefore, parameters for the reference values, which are the lowest value, for these factors do not appear in this table. Table 5 includes name of the factors or variables, the value of the coefficients for each of the factor values or the value of the parameter estimate for each of the variables, the standard error (SE), the t-value and the p-value or significance for each of the coefficients.

Table 5. Estimates for the parameters of the logit model based on embryo variables.

Variable	**Parameter estimate**	**SE**	**t-value**	**p-value**
Intercept	-10.26	2.455	-4.181	0.000
EA	0.0001	0.00002	6.222	0.000
FRAG	0.4714	0.1769	2.665	0.007
(1,2,3 vs 0)	1.308	0.2057	6.357	0.000
	1.509	0.4055	3.721	0.000
ZPT	-0.347	0.0033	-10.360	0.000
ZP (1 vs 0)	-1.225	0.2673	-4.583	0.000

BROUND(average)	12.860	2.703	4.760	0.000

The interpretation of the positive coefficient which corresponds to some factors can be interpreted as that the logit implantation rate (i.e, implantation rate) is higher in the embryos with this value than in the reference value. On the contrary, the interpretation of the negative coefficient is that the implantation rate is lower in this value than in the reference value. Similarly, if we consider the continuous variables the meaningfulness of the positive coefficient which corresponds to some of them can be interpreted thus the implantation rate increases with the increment of variable values. On the contrary, the meaningfulness of the negative coefficient which corresponds to others can be interpreted thus: the implantation rate is higher for the lower variable values.

From the results of the fitted model in Table 4 we are able to say that the perimeter of the blastomere decreases the implantation rate while blastomere roundness, increases this rate. Table 5 shows that embryo area and average blastomere roundness increases the implantation rate, while zona pellucida thickness decreases this rate. In addition, Table 5 shows that fragmentation increases the rate while structural abnormalities decreases it. We would like to point out that the behaviour for the fragmentation is not logical.

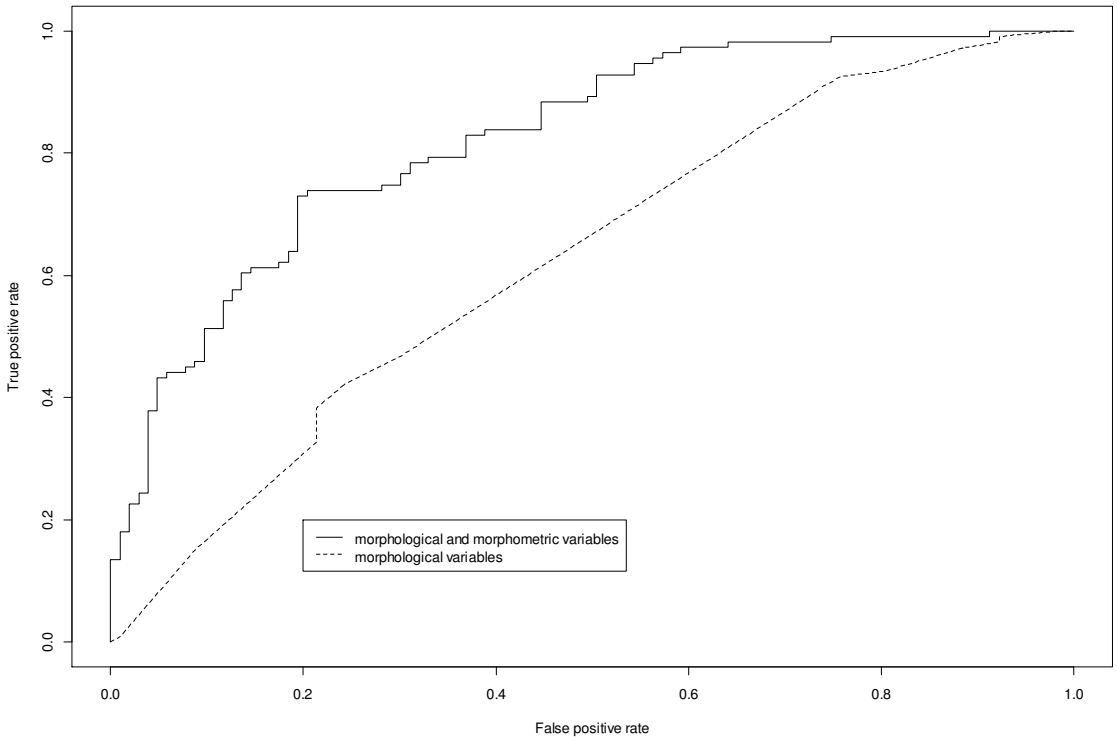
4. ROC curve.

Finally, Receiver Operating Characteristics (ROC) was used to assess the accuracy of this model. ROC curves provide an overall representation of accuracy, they are well-described by Fawcett (2006). If the test does not allow discrimination

between classes, the ROC curve is the diagonal joining the vertices from bottom left to top right. The accuracy of the test increases as the curve moves towards the top left corner. To evaluate the discriminative performance of the logistic model with morphological and morphometric variables and to compare the classifiers, we wanted to reduce ROC performance to a single scalar value representing expected performance. Calculating the area under the ROC curve of the classifier, in short AUC, is a common method. Since the AUC is a portion of the area of the square unit, its value is always between 0 and 1, so random guessing procedures have an area of 0.5. Therefore, when the area under the ROC curve (AUC) increases, the classifier power also increases.

This study validates the model by taking into account all the embryos: 0%, 50% and 100% implantation, in order to discriminate between pregnancy or not. Figure 1 illustrates the ROC curves for the two models whose comparison allows us to assert that model including morphometric variables assigns scores that discriminate better between embryos which are implanted or not than the other model with only classical morphometrical variables (number of cells, fragmentation and symmetry) which provides a curve closer to the diagonal.

Figure1: Comparison of the ROC curves for models



In addition, in this study the AUC was 0.8241 for our model with morphological and morphometric variables. In order to compare our model with those used with classical morphological variables such as number of cells, fragmentation and symmetry we calculated the AUC for our data. The corresponding AUC was 0.6263, which was lower than our value. Therefore, comparison of ROC curves and the corresponding AUC allow us to assert that our model assigns scores that discriminate better between embryos which are implanted or not.

DISCUSSION

Although there is general agreement among embryologists as to which morphological features are characteristic of a top quality embryo in the cleavage stage, evidence is still lacking for the ranking of implantation potential of top and

non-top quality embryos based on morphometric parameters. The need to increase knowledge of embryo quality variables and thus construct reliable scoring systems is evident. The available scientific data to date is based on studies containing a limited number of treatments with a traceable association between embryo and implantation (Giorgetti et al. 1995; Ziebe et al. 1997; Van Royen et al. 1999; 2001; Holte et al. 2007**)** due to the lack of large databases and the difficulties in following the fate of an individual embryo. The prevailing clinical practice of transferring more than one embryo makes deduction from embryo quality variables unreliable when the resulting pregnancy contains fewer sacs than the number of transferred embryos.

The ideal approach to studying the morphological determinants of a single embryo's implantation would be to analyse single-embryo transfers exclusively. However, in most single-embryo transfer programmes only 'top' quality embryos are transferred, and thus an optimal span of variables for statistical evaluation cannot be obtained by this approach. Alternatively, data from treatments which result in only a single embryo being available for transfer should be analysed. Although this has been done, producing important information, the evaluation of such data is hampered by the fact that these treatments mainly involve women with a poor response, poor embryo quality and low implantation figures, again not permitting a wide span of morphological variation (Giorgetti et al. 1995; Holte et al. 2007**).**

A variety of evaluation techniques have been described to assess the viability of embryos in assisted reproduction techniques (ARTs). These evaluations are mostly based on the morphological characteristics of the embryos

(Backzkowski et al. 2004), which are basically evaluated by an embryologist in a fast but subjective way (Partenot et al. 2009). Embyonic classification systems based on the use of objective parameters of embryo morphology should be developed. That is, measurements are taken directly from the embryo that might be used in an external observer's opinion, totally avoiding the subjectivity of the measurement. Computer-assisted scoring systems in combination with the automation of embryo visualization can improve embryo assessment (Paternot et al. 2011b; 2013). These systems give additional information on embryo characteristics that cannot be evaluated by manual scoring.

The number of cells is the morphological marker strongly associated with implantation potential (Giorgetti et al. 1995; Ziebe et al. 1997; Van Royen et al. 1999; 2001; Holte et al. 2007). Optimal results are achieved when four cells embryos are transferred on day 2. Embryo implantation potential decreases when the number of cells is other than four. The results of Debon et al (2013) coincided with those previously obtained by many research groups (Van Royen et al. 1999; Van Montfoort et al. 2004; Guerif et al. 2007). But in this study, Table 5 shows that the number of cells is an objective morphological embryo variable **which is not significant in the presence of embryo and blastomere morphometric parameters.**

In relation to embryo fragmentation, the fragment percentage over which the embryo`s ability to implant could be compromised is unknown (Ziebe et al. 1997). The results obtained in this study, show that fragmentation of less than 25% of the embryo volume should not compromise implantation. These results agreed with those proposed by other authors. (Alikani and Cohen 1995; Van Royen et al. 1999;

Alikani et al. 2000**).** However, the fragmentation percentage that could affect embryo implantation cannot be determined, as they are selected embryos for subsequent transfer, we do not have enough images of embryos with more than 25% fragmentation. On the other hand, as it is a rather subjective variable, the fragmentation percentage should be included in the embryo classification system as a morphometric variable. In the paper by Paternot el al. (2009), the criteria for distinguishing between a blastomere and a fragment were based on the findings by Hnida et al. (2005) and Johansson et al. (2003), who reported that the diameter of a blastomere should be ≥45 mm on Day 2 and ≥40 mm on Day 3. The "Cell Counter" tool should perhaps be used in this case.

In relation to the ZP characteristics, the evaluation of only the ZP surface irregularities is a subjective and inaccurate procedure. The use of the morphometric ZP thickness variable seems to be more accurate and better in predicting embryo implantation. From the results obtained in this study, embryos with a lower ZP thickness have a higher chance of successfully implanting. These results coincide with those proposed by the research group that studied ZP thickness and its relationship with embryo implantation morphometricly (Roux et al. 1995). ZP thickness **is associated with** the embryo's ability to both develop and implant **(**Bertrand et al. 1995; **Garside et al 1997**, Veeck 1999; Gabrielsen et al. 2000, 2001**, Shiloh and Dirnfeld 2001, Nawroth et al 2001 and Sun et al 2005**). **Although more recently Balakier et al (2012) found no relationship between ZP thickness and implantation. In addition, Balakier et al (2012) report no significant correlation between the ZPT and the patient' age while the results**

of Janny and Menezo (1996) give a clear indication of decline in the quality of human embryos arising from aging oocytes.

For the other morphometric variables analyzed, highly significant differences were observed only for the embryo measurements: area and roundness when they were compared in relation to implantation. These results agree with those obtained by Partenot et al. (2009; 2011a), which demonstrated better prediction of the implantation rate based on the number and size of blastomeres and on the total embryo volume on day 3 embryos.

One of the analyzed morphometric variables with greatest statistical significance for implantation is the roundness factor of cells. This variable indicates the similarity to a perfect circle and therefore the regularity of the embryonic cells. The average of this variable was slightly more than 0.9 for successfully implanted embryos. Whereas, in the case of embryos that failed to implant, this value was lower than 0.9**.** The maximum value which corresponds to a perfect circle is 1. Therefore, the fact that the embryos that were successfully implanted had a roundness factor of 0.9 means that the embryos' cells have a shape close to a circle. In summary, blastomere roundness could be less subjective and more accurate than embryo equality and symmetry, which have long been used for embryo classification (Hardarson et al. 2001; Holte et al. 2007; Debon et al 2013). Therefore, the embryo features for successful implantation would be: 4-cell embryos, a fragmentation percentage lower than 25%, equal sized blastomeres with a circular shape (a roundness factor greater than 0.9), an average ZP thickness of 13 microns and an average of 17695.1 microns^2 for the embryo area.

In conclusion, morphometric variables are more accurate and less subjective than the morphological ones which have been used to date. The blastomeric roundness variable could replace the blastomeric symmetry and equality variable. The size of the embryo and its cells, described by use of the embryo area, is a less subjective variable to consider when predicting implantation. Consequently, we propose a new characterization of day 2 human embryos with the highest implantation potential taking into account the following embryo parameters: number of blastomeres and fragmentation, embryo area, blastomere roundness and ZP thickness. The incorporation of these morphometric variables into the current embryo classification will significantly improve embryo selection prior to transfer. This embryo characterization is a quick, objective, efficient and accurate tool to optimize embryo selection for day 2 transfers.

Finally, our results indicate that in terms of key statistical measurements of interest for the quality of embryos, especially in a SET context, threshold discrimination based in more than one variable is ideal. This is where ROC curves are useful. Therefore, we have also proposed the ROC curve as a graphical tool and the AUC as a numerical value for validation and comparison of the different models.

The major shortcomings are the retrospective nature of the work, the very small sample size (considering the complexity of the multifactorial issue of implantation and morphology). On the other hand, morphometric analysis of embryo variable and their cells is time consuming. Therefore, in this specific situation this study could not yet confirm the clinical utility of these variables, but could be indicative of a general trend. This study describes

new, minimally invasive methodology (morphometrics and statistical analysis) that promises to improve laboratories' ability to select the embryos with better prognosis although the predictive power of the significant variables identified should be confirmed with a prospective study.

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