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Early protein profile of human embryonic secretome

Carlo Foresta[1], Filippo Maria Ubaldi[2], Laura Rienzi[2], Cinzia Franchin[3,4], Micaela Pivato[4,5], Stefania Romano[2], Diego Guidolin[6], Raffaele De Caro[6], Alberto Ferlin[1], Luca De Toni[1]

[1]University of Padova, Department of Medicine & Andrology and Reproductive Medicine Unit, 35128, Padova, Italy, [2]Genera Centre, 00197, Roma, Italy, [3]University of Padova, Department of Biomedical Science, 35131, Padova, Italy, [4]Proteomics Center of Padova University, 35128, Padova, Italy, [5]DAFNAE; University of Padova, 35020, Legnaro (PD), Italy,[6]University of Padova, Department of Molecular Medicine, 35121, Padova, Italy

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1. ABSTRACT

Embryos obtained by *in vitro* fertilization are currently assessed by morphology, but displays limitations with over 70% of embryos failing to implant. In this study, we performed HPLC-MS/MS analysis on the conditioned medium obtained from 50 human embryos at the 3rd day of *in vitro* culture. 70 proteins were identified in the medium of 48 embryos. Validation by protein array on two pools of 11 and 9 conditioned media, showed a protein pattern overlap with HPLC-MS/MS of respectively 72% and 78%. Unsupervised hierarchical cluster analysis on protein spectra, allowed to divide embryos into 3 clusters. The first cluster selectively lacked of proteins involved in programmed cell death. The third cluster was devoid of proteins involved in cell development. Embryos taking the shortest time to develop into 5 cell morulas, featuring lower implantation rate, significantly segregated in the third cluster (P=0.047). Multiple linear regression analysis, identified 12 predictive proteins for transfer success (P<0.0.5). Proteomics of embryo secretome aids in understanding embryo physiology and in improving assisted reproductive technology.

2. INTRODUCTION

In vitro fertilization (IVF) treatments have long been known as a solution for infertility. Due to the low efficacy of the procedures and the need to maximize the chance of pregnancy, the practice of transferring more than one embryo has been frequently employed (1). Unfortunately, multiple births often associate to both increased maternal morbidity/mortality rates and costs to the health service (2). As a result, multiple birth pregnancy is now considered the most important risk deriving from IVF treatment (2,3). The adoption of a policy of single embryo transfer has been demonstrated to be an effective method to reduce the multiple pregnancy rate after IVF (4) but requires the identification of reliable methods to assess the overall embryo quality and competency to implant (5).

In most IVF centers, quality assessment of cultured embryos mainly relies on the morphological evaluation at cleavage stage, but this method displays many limitations with more than 70% of IVF embryos failing to implant (6). In order to identify other functional parameters with predictive values for high implantation

rates, new clues were derived from old observations. In fact, the speed rate of embryo development, otherwise known as "morphokinetics", has been shown to have a negative impact on the implantation rate whether too slow or too fast (7,8). In this regard, Meseguer *et al.* assessed embryo morphokinetics by the use a specific time-lapse photography system (9). In particular, for the time taken by the embryo to develop from a 2- to a 5-cell mass, authors demonstrated that a cleavage time falling into the lowest or the highest quartile was significantly associated with an unfavorable implantation rate (9). Subsequent studies confirmed this result (10), conferring to morphokinetics a value as cost-effective parameter to assist embryo selection (11,12).

Recent advances in "omics" technologies (genomics, transcriptomics, proteomics, and metabolomics), have enabled the investigation of new molecular methods for embryo selection. With particular regard to proteomics, knowledge of the human embryonic proteome remains very limited due to factors including limited template availability, low embryo protein concentrations and deficient protein database availability. On the contrary the secretome, defined as those proteins that are produced by embryos and secreted into the surrounding micro drop of culture medium, is of particular interest for the non-invasive identification of proteins involved in specific disease states (13). In this regard, pioneer studies attempted to identify protein markers of cell quality in the spent medium from embryo culture. In 2005, Yao *et al.* (14) suggested the dosage of soluble HLA-G in spent medium from embryo culture as predictor of implantation success. Moreover in a later study, Dominguez *et al.* (15) investigated the embryo secretome through a protein microarray assay. However, likely due to a insufficient platform sensitivity, these early evidences did not led to wider studies.

In this study, proteomic analysis of conditioned medium obtained at the third day of *in vitro* embryo culture was performed for 50 human embryos. Unsupervised statistical analysis of the embryonic protein profiles was applied in order to assess a possible correlation with morphology and morphokinetic features of embryos. Moreover, a regression analysis was applied to assess the prognostic value of secretome analysis for pregnancy outcome.

3. MATERIALS AND METHODS

3.1. Subjects

The study was conducted under approval of the Ethics Committee of the Padua University Hospital (protocol n. 2461) in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all study participants. Twenty infertile couples undergoing ICSI cycles at the GENERA Centre (Clinica Valle Giulia, Rome, Italy) were included in the study. Inclusion criteria were female age <45 years (mean age 38,7 years; range 35–44) and a good response to controlled ovarian stimulation (>10 MII oocytes retrieved). Exclusion criteria were: (i) patients presenting with abnormal karyotype; (ii) specific ovarian pathologies including polycystic ovary syndrome, endometriosis grade III or higher and premature ovarian failure and (iii) severe male factor infertility (motile sperm count of <500 000/ml after preparation of a fresh ejaculate).

3.2. *In vitro* fertilization

Controlled ovarian stimulation was performed using a GnRH-agonist long protocol as described previously (16) and oocyte collection was performed at 35 h post-hCG administration. Denudation of the oocyte from the cumulus oophorus was performed by a brief exposure to 40 IU/ml hyaluronidase solution in fertilization media (Sage In-Vitro Fertilization, Inc., Trumbull, CT, USA), followed by mechanical removal of all the corona radiata with the use of plastic pipettes of defined diameters (denuding pipette; COOK Ireland Ltd, Limerick, Ireland). The denudation procedure was performed in a controlled environment (6% CO2 at 37°) (IncuChamber L-323, Ksystems, Birkerod, Denmark) between 37 and 40 h post-hCG administration. During this procedure, particular attention was devoted to the removal of all adhering cumulus and coronal cells in order to lower the level of contamination attributable these cell populations. MII oocytes were then subjected to intracytoplasmatic sperm injection (ICSI), between 36 and 38 h post–hCG administration, using previously described techniques and instrumentation (17). 16–18 h post-ICSI, oocytes were assessed for the presence of pronuclei. Those displaying two pronuclei and a second polar body were cultured further. EmbryoSlide® was employed for separate culture of all embryos in 20 µl microdrops of cleavage medium under mineral oil (Sage) up to day 3 of embryo development, followed by blastocyst medium (Sage) up to day 5 or 6. 10 µl of conditioned cleavage medium was sampled on day 3 from each embryo culture, additioned with 1 µl of 10X protease inhibitor cocktail solution in PBS (I3911, Sigma-Aldrich, Milano, Italy) and stored at -80°C until further analysis. The time-lapse instrument EmbryoScopeTM tri-gas incubator with a built in microscope (Unisense FertiliTech, Aarhus, Denmark), was used to automatically acquire images during embryos development (9). Vitrification of blastocysts on day 5 or 6 was performed as described elsewhere (18). Endometrial preparation and transfer procedures were performed as previously described (16). Biochemical pregnancy was defined as elevated levels of hCG but ending in miscarriage before ultrasound confirmation. Clinical pregnancies was defined by observation of gestational sac or heartbeat at ultrasound evaluation.

3.3. Embryo characteristics

Quality of cleaving embryos were evaluated at day 3 after ICSI with the use of a cumulative classification

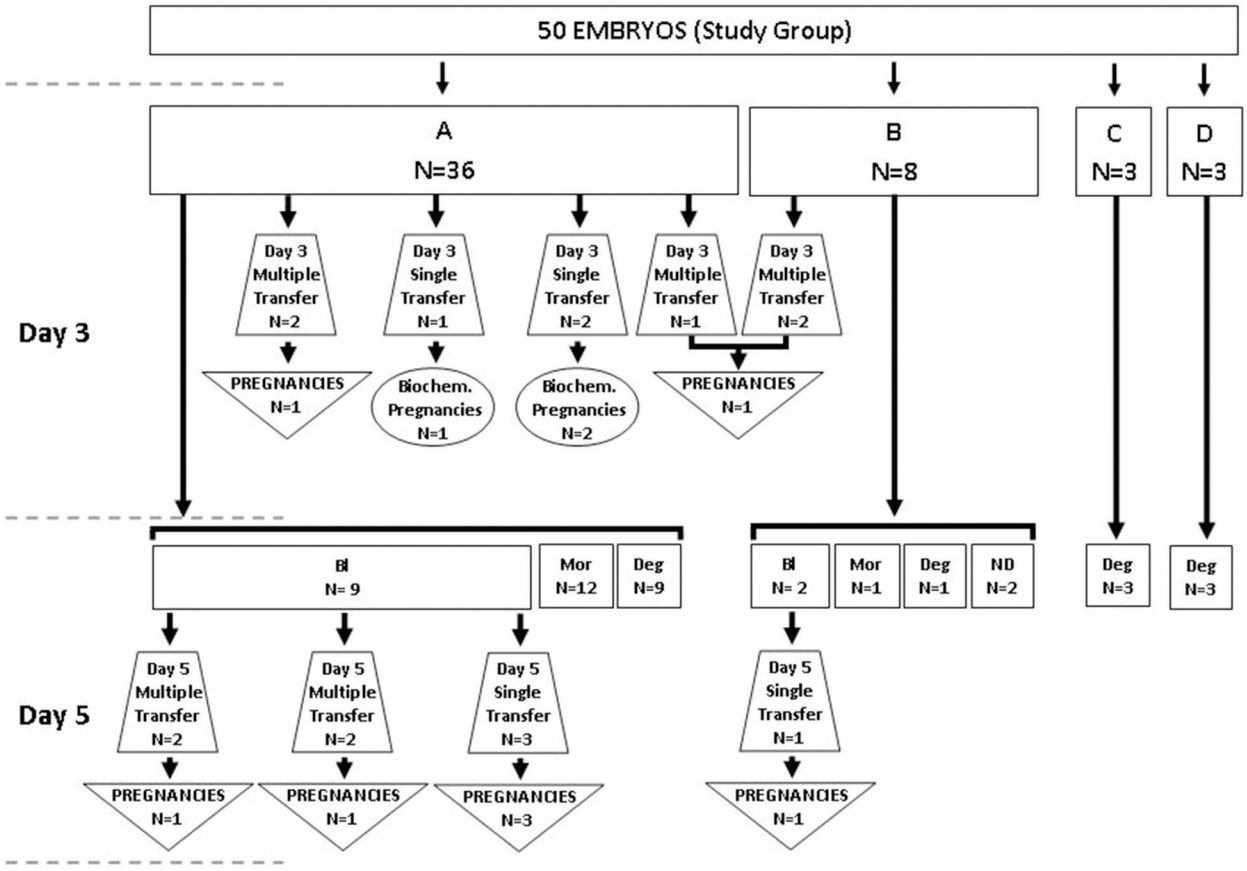

Figure 1. Embryos constituting the study cohort (N=50) are represented according to their quality-classes at day 3 of culture: 36 A (top quality), 8 B (good quality not for elective single embryo transfer), 3 C (impaired embryo quality) and 3 D (not recommend to transfer). Morphology of developing embryos at day 5 after ICSI were also scored as blast (Bl), morula (Mor), degenerated (Deg) or not detected (ND). Outcome of single or multiple embryo transfer was also recorded as clinical pregnancy (PREGNANCIES) or biochemical pregnancy (Biochem. Pregnancies).

scheme taking into account cleavage speed, blastomere symmetry, extent of fragmentation, presence/absence of multinucleated blastomers (5). Embryo quality-classes were: A (top quality), B (good quality not for elective single embryo transfer), C (impaired embryo quality) and D (not recommend to transfer). Morphology of developing embryos at day 5 after ICSI were also evaluated and recorded according to previously published scoring system (19).

Spent media of 50 embryos were obtained by fertilized oocytes from 9 female partners and underwent protein profiling by LC-MS/MS and called the “study group”. Morphological characteristics at both day 3 and day 5 and embryo transfers procedures featuring the study group are described in Figure 1. In summary, at day 3, 7 embryos underwent multiple transfer achieving 2 clinical pregnancies and 2 biochemical pregnancies whilst 1 embryo underwent single transfer and achieve a biochemical pregnancy (Figure 1). 8 embryos underwent 4 multiple and 4 single transfer at day 5 achieving respectively 2 and 4 clinical pregnancies (Figure 1).

Spent media of 20 embryos were obtained by fertilized oocytes of 11 female partners and were used for validation of LC-MS/MS analysis by protein array and called the “validation group”. Morphological features of both the validation group and the study group are summarized in Table 1.

30 aliquots of 20µl of unconditioned cleavage medium were maintained for three days in the same culture conditions and served as “blank” samples. 10 aliquots of 10µl each were used for blanking in LC-MS/MS analysis and further 20 aliquots of 10µl each were used for blanking in protein immunoassay (see below).

3.4. Time-lapse evaluation of morphokinetic parameters

Retrospective analysis of the acquired images of each embryo was made with an external EmbryoViewer workstation (Unisense FertiliTech, Aarhus, Denmark), using image analysis software in which all the developmental events of considered embryo were annotated, together with the corresponding

Table 1. Comparison of morphology and morphokinetics features of embryos between the study group and the validation group

	Study group (N=50) (%)	Validation group (N=20) (%)	P *vs* study group
Embryo morphology class at day 3 (nr)	A: 36 (72)	A: 15 (75)	0.6.8.
	B: 8 (16)	B: 3 (15)	0.9.1.
	C: 3 (6)	C: 1 (5)	0.8.7.
	D: 3 (6)	D: 1 (5)	0.8.7.
Embryo morphology class at day 5 (nr)	Bl: 11 (22)	Bl: 9 (55)	0.0.8.
	Mor: 13 (26)	Mor: 6 (20)	0.7.3.
	Deg: 16 (32)	Deg: 4 (20)	0.3.2.
	ND: 10 (20)	ND: 1 (5)	0.1.2.
Morphokinetics	**Study group (mean±SD)**	**Validation group (mean±SD)**	**P *vs* study group**
t2 (h)	28.0.8.±4.6.5.	27.4.6.±4.7.9.	0.3.2.
t3 (h)	38.4.8.±6.2.9.	36.9.5.±7.5.1.	0.2.0.
t4 (h)	41.0.3.±6.8.7.	40.1.2.±6.0.1.	0.6.3.
t5 (h)	51.2.8.±7.7.2.	49.8.0.±8.9.1.	0.4.1.
t8 (h)	62.4.3.±12.3.8.	62.1.5.±11.2.8.	0.8.5.
cc2= t3-t2 (h)	10.4.±4.6.4.	10.2.±4.2.1.	0.7.3.
s2= t4-t3 (h)	2.3.5.±3.9.9.	3.1.1.±4.0.3.	0.1.9.

SD: Standard deviation; Bl: Blastocyst. Mor: Morula; Deg: Degraded; ND: Not detected because of transfer or cryopreservation at day 3; t2: Time to 2 cell embryo; t3: Time to 3 cell embryo; t4: Time to 4 cell embryo; t5: Time to 5 cell embryo; t8: Time to 8 cell embryo; cc2: Second cell cycle; s2: Second synchrony; nr: Number; h: Hours

timing of the events after ICSI microinjection (expressed in hours). Subsequently, the precise timing of the first cell division (time to 2 cells, t2) was identified. Likewise, we annotated the second (time to 3 cells, t3), third (time to 4 cells, t4) and fourth (time to 5 cells, t5) cell division. We defined the duration of the second cell cycle (cc2) as t3-t2 and second synchrony s2, as t4-t3. The time of all events featuring both the study group and the validation group, expressed as hours post ICSI microinjection, are summarized in Table 1.

3.5. Pre-Treatment of medium samples and LC-MS/MS analysis

All samples of conditioned medium from the study group as well as 10 blank samples of unconditioned cleavage medium underwent albumin removal using the SwellGel® Blue Albumin Removal Kit (Pierce-Thermo Scientific, Rockford, IL, U.S.A.) according to the manufacturer's indications for small volumes. Eluates were then treated with SDS and 2- mercaptoethanol, boiled for 10 min and loaded into an SDS-polyacrylamide gel (Bio-Rad, Milano, Italy) for gel aided-purification as described by Lawlor *et al*. (20). Briefly, the electrophoresis was stopped after the sample had entered into the gel, and a unique band for each sample was excised, containing the total protein sample. Subsequently, samples underwent alkylation, in-gel digestion, peptide extraction and LC-MS/MS analysis as described elsewhere (21). Peptides were then dissolved in 15 μL of 0.1.% formic acid and analyzed by LC-MS/MS using a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled with an Ultimate 3000 nano-HPLC system (Dionex – Thermo Fisher Scientific). Five μL of each sample were loaded onto a trap-column (C18, 300 Å, 3 mm, SGE Analytical Science) at a flow rate of 8 μL/min of solvent A (water/formic acid 0.1.%) and peptides were then transferred into a 10 cm picofrit chromatographic column (75 μm I.D., 10 μm tip, New Objectives) packed in-house with Reprosil C18 material (300Å, 3 μm). Peptides were eluted using a 3–40% linear gradient of solvent B (acetonitrile/0.1.% formic acid) in 19 min at a flow rate of 250 nL/min. Mass spectra were acquired in a data dependent mode: one full MS scan (300-1700 m/z) in the Orbitrap at 60000 resolution was followed by MS/MS spectra of the 10 most intense ions acquired in the linear ion-trap. To avoid sample cross-contamination, every LC-MS/MS analysis of a sample was followed by a blank injection. Raw data files were analyzed using Proteome Discoverer Software (version 1.2., Thermo Fisher Scientific, CA, USA) connected to a Mascot Search Engine server version 2.2.4. (Matrix Science, London, UK). Spectra were searched against the Uniprot database (release 2011_05; taxonomy restricted to Human (35846 entries)) using the following parameters: enzyme specificity was set to Trypsin with up to 2 missed cleavage events, peptide and fragment tolerance were set to 10 ppm and 0.6. Da respectively. Oxidation of Methionine was selected as variable modification, while carbamidomethylation of Cysteine was set as static modification. False Discovery Rates (FDR) of 5% and 1% were calculated by Proteome Discoverer based on the search against the corresponding randomized database. Protein matches were considered positive if at least two unique peptides were identified with medium confidence per protein. Proteins were grouped according to the principle of maximum parsimony and results are reported as single identified proteins.

3.6. Validation of LC-MS/MS

Expecting a broad range for protein spectrum in samples, an highly multiplexed protein immunoassays was chosen for validation of LC-MS/MS analysis (22). To this aim, L-493 RayBio® Label-based Human Antibody Array (successively called "protein array", Ray Biotech Inc, Norcross, GA USA). The assay relied on biotinylation of the whole proteins content of sample followed by incubation with membrane array, which was pre-printed with capture antibodies. Membranes were then incubated with HRP-Conjugated Streptavidin and signals were visualized by chemiluminescence.

Table 2. Proteins identified in day 3-conditioned medium from cultured human embryos of the study group

Acronym	Protein name	Frequency	Acronym	Protein name	Frequency
A2M	Alpha-2 macroglobulin	1/48	HIST1H4A	Histone H4	12/48
ACTB	Actin, cytoplasmic 1	8/48	HP	Haptoglobin	8/48
AMY2A	Alpha amylase	1/48	HPX	Hemopexin	3/48
ANXA2	Annexin A2	4/48	IGHA1	Ig alpha-1 chain C region	3/48
ANXA2P2	Putative annexin A2-like protein	1/48	IGHA2	Ig alpha-2 chain C region	3/48
APOA1	Apolipoprotein A-I	24/48	IGHG1	Ig gamma-1 chain C region	14/48
ARG1	Arginase-1	1/48	IGHG2	Ig gamma-2 chain C region	3/48
AZGP1	Zinc-alpha-2-glycoprotein	4/48	IGHG4	Ig gamma-4 chain C region	1/48
BPIFA1	BPI fold-containing family A member 1	1/48	IGJ	Immunoglobulin J chain	1/48
BPIFB1	BPI fold-containing family B member 1	2/48	IGKC	Ig kappa chain C region	18/48
C3	Complement C3	1/48	IGLC2	Ig lambda-2 chain C regions	6/48
CASP14	Caspase 14	3/48	JUP	Junction plakoglobin	20/48
CP	Ceruloplasmin	1/48	LRRC15	Leucine-rich repeat-containing protein 15	4/48
CSTA	Cystatin-A	1/48	LTF	Lactotransferrin	3/48
DCD	Dermcidin	9/48	LYZ	Lysozyme C	3/48
DEFA1	Neutrophil defensin 1	1/48	ORM1	Alpha-1-acid glycoprotein 1	1/48
DMBT1	Deleted in malignant brain tumors 1	1/48	PIGR	Polymeric immunoglobulin receptor	1/48
DP1	Desmoplakin	1/48	PKP1	Isoform 1 of Plakophilin-1	6/48
DP2	Isoform DPII of desmoplakin	1/48	PPIA	Peptidyl-prolyl cis-trans isomerase A	1/48
DSC1	Isoform 1B of Desmocollin-1	5/48	PRDX2	Peroxiredoxin-2	1/48
DSG1	Desmoglein 1	11/48	S100A8	Preotein S-100 A8	6/48
DUSP14	Dual specificity protein phosphatase 14	1/48	S100A9	Protein S100-A9	2/48
EEF1A1	Elongation factor 1-alpha 1	5/48	SELENBP1	Selenium-binding protein 1	3/48
FABP5	Fatty acid-binding protein, epidermal	1/48	SEMG1	Semenogelin I	1/48
FLG	Filaggrin	9/48	SERPINA1	Serpin 1	2/48
FLG2	Filaggrin 2	31/48	SERPINB12	Serpin B12	3/48
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	1/48	SERPINB5	Serpin B5	1/48
GAPDHS	Spermatogenic glyceraldehyde-3-phosphate dehydrogenase	1/48	SERPINC1	Antithrombin-III	2/48
H2AFZ	Histone H2A.Z	4/48	SFN	Isoform 2 of 14-3-3 protein sigma	5/48
H3F3C	Histone H3.3.C	5/48	TGM3	Protein-glutamine gamma-glutamyltransferase E	1/48
HBB	Hemoglobin subunit beta	1/48	TTR	Transthyretin	8/48
HIST1H1C	Histone H1.2.	3/48	TUBA1C	Tubulin alpha-1C chain	2/48
HIST1H2AH	Histone H2A type 1-H	7/48	UBC	Polyubiquitin-C	1/48
HIST1H2BK	Histone H2B type 1-K	10/48	VTDB	Vitamin D-binding protein	1/48
HIST1H3A	Histone H3.1.	3/48	YWHAE	14-3-3 protein epsilon	1/48
Number of cultured embryo with non null protein profile: 48					

Protein array was performed on two pools of respectively 11 and 9 spent media of the validation group and on two pools of 10 blank samples each according to manufacturer's instruction. Prior to pooling, both spent media and blank samples underwent individual albumin removal as described above. Protein signals on membranes were acquired with the Chemidoc XRS System (Bio-Rad). For each protein dot, the pixel density per square millimeter was calculated, and background pixel density was subtracted by means of Quantity One Software Version 4.6.9. (Bio-Rad). Protein dots signals whose intensity was greater than mean signal of negative controls + 2SD were considered as expressed in the sample.

3.7. Statistical analysis

3.7.1. Data treatment

Data were entered into an expression matrix of Np columns (one for each protein found) and Ns rows (one for each sample). The matrix was completed using binary values representing the presence (1) or absence (0) of the corresponding protein in the corresponding sample. Thus, samples were consistently coded as binary patterns in which each element records the presence or absence of a specific protein.

3.7.2. Cluster analysis

Sample classification criteria were defined using unsupervised clustering without any prior knowledge of grouping. SPSS 13.0. (IBM Corporation, Armonk, NY, USA) was used to obtain the hierarchical agglomerative data clustering, using Euclidean distance, with linkage defined by Ward's method. The number of substantive clusters was determined by identification of the dendrogram step showing the greatest change in the agglomeration coefficient (23).

The difference of morphological and morphoiynetic features between the study group and the validation group was evaluated by the Cramer's V correction for contingency tables larger than 2x2. The correlation between cluster membership and sample morphology was tested by χ^2-test. Differences in the morphokinetic parameters of the clusters of interest were assessed by one-way ANOVA, followed by Bonferroni's test for multiple comparisons. P<0.0.5. was always used as the limit for statistical significance.

3.7.3. Multiple linear regression analysis

As reported above, a subset (N=16) of the embryos included in the study underwent subsequent transfer, with the success of the procedure recorded. Data from these embryos were used in a first attempt to define a linear function able to predict the probability of implantation success from the observed protein expression profile by multiple linear regression analysis (MLRA). This technique provides coefficients (weights) sufficient to generate a linear combination of observed protein expression values, that will predict which outcome the sample will likely have (24). The analysis was performed by the specific routines available in SPSS 13.0. (IBM Corporation, Armonk, NY, USA).

4. RESULTS

4.1. Protein analysis

In order to identify proteins specifically attributable to embryo conditioning and to discard proteins of likely medium supplements/operator origin, the total protein profile of 10 blank samples was assessed and subtracted to that of each conditioned medium of the study group. In 2 out of 50 medium samples no specific protein attributable to embryo was found (null profile) and were then removed from subsequent analysis. 70 different proteins were detected at least once, in at least one sample and are summarized in Table 2 with the corresponding frequencies of detection.

Validation of LC-MS/MS analysis by protein array was performed on the two pools of the validation group. In terms of morphological and morphokinetic features, the validation group showed no significant difference compared to the study group (Table 1). Proteins profiles featuring blank samples were subtracted to the corresponding pool of spent media in order to detect specific protein attributable to embryo. Within each pool, a spectrum of respectively 11 and 9 proteins was recognized to be pertaining to embryo release. Among these, respectively 8 (72%) and 7 (78%) overlapped with LC-MS/MS results (Figure 2). In each pool, the signal density of overlapping proteins significantly correlated with the corresponding frequency found in protein profiling obtained by LC-MS/MS analysis in the study group (respectively p=0,77; p=0,025 and p=0,87; P=0,012 Figure 2).

4.2. Cluster analysis

Unsupervised cluster analysis, using Ward's linkage rule, was performed on the 48 samples of the study group with a not-null profile. Three main clusters were identified, consisting of 16, 23 and 9 samples respectively (Figure 3A). As expected, spectra of proteins owning to each cluster, defined as the frequency by which a single protein appeared in a given cluster (Figure 3B), showed a certain degree of redundancy. On the other hand, by focusing on proteins specifically absent in each cluster, a peculiar pattern was noted, as shown in Table 3. Using the g: Profiler program (25), a web server application for the interpretation and integration of gene lists in the context of biological evidence, a different functional profile of these latter protein profiles was determined. Indeed, cluster 1 lacked of proteins involved in nucleosome formation and programmed cell death, while cluster 3, the most distinct from cluster 1, lacked proteins required for modulation

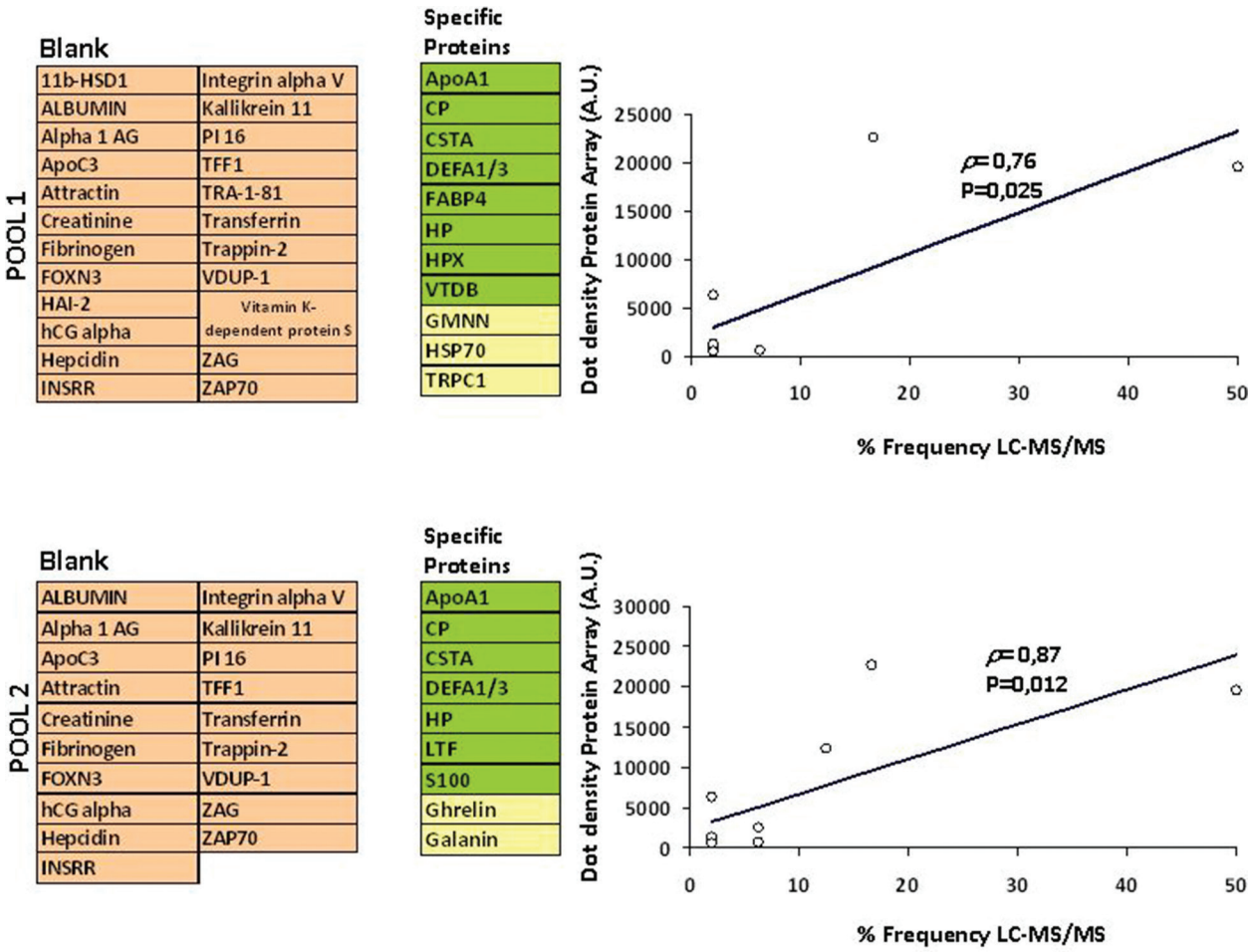

Figure 2. Protein array was performed on two pools of respectively 11 (POOL 1) and 9 (POOL 2) spent media of the validation group and on two pools of 10 blank samples each. Proteins profiles featuring blank samples (Blank) were subtracted to the corresponding pool of spent media in order to detect specific protein attributable to embryo. Within each pool, a spectrum of respectively 11 and 9 proteins was recognized to be pertaining to embryo release (Specific Proteins). Among these, respectively 8 and 7 overlapped with LC-MS/MS results (green in Specific Proteins). In each pool, the signal density of overlapping proteins (Dot density Protein Array) was plotted with the corresponding frequency found in protein profiling obtained by LC-MS/MS analysis (% Frequency LC-MS/MS).

of protease/preoteasome activity (Table 3). Cluster 2 showed the lowest number of proteins specifically absent, with a pertaining pathway generally involved in defense response (Table 3).

The possibility of a correlation between sample clustering and morphological and/or morphokinetic features, was also assessed (Table 1). Samples featured by higher morphology quality were more frequently segregated in cluster 1, despite this frequency being insufficient to demonstrate statistical significance (P = 0,474; Figure 4A). On the other hand, samples characterized by a t5 falling within the I quartile of the corresponding distribution range significantly segregated in cluster 3 (P = 0,047; Figure 4B). No significant segregation was found for all the other morphokinetic parameters assessed (data not shown).

4.3. Multiple linear regression analysis

We investigated the predicting value of implantation success on the bases of the observed protein profile of the 16 embryos that underwent transfer. Firstly, 2 embryos that were transferred in multiple and achieved biochemical pregnancies displayed a null protein profile were removed before any further analysis. Secondly, it is important to note that four patients underwent a transfer procedure involving multiple embryos as reported in Table 4. In the detail, three procedures transferred 2 embryos, and in one case 3 embryos were transferred. Moreover, in three patients embryo transfer was performed at day 3 post-ICSI, while in 6 patients embryo transfer happened at day 5 (Table 4). When multiple transfer was performed, each patient achieved no more than a single clinical pregnancy. In these cases it is not possible to directly assign a single embryo, and the corresponding protein profile, to a specific positive

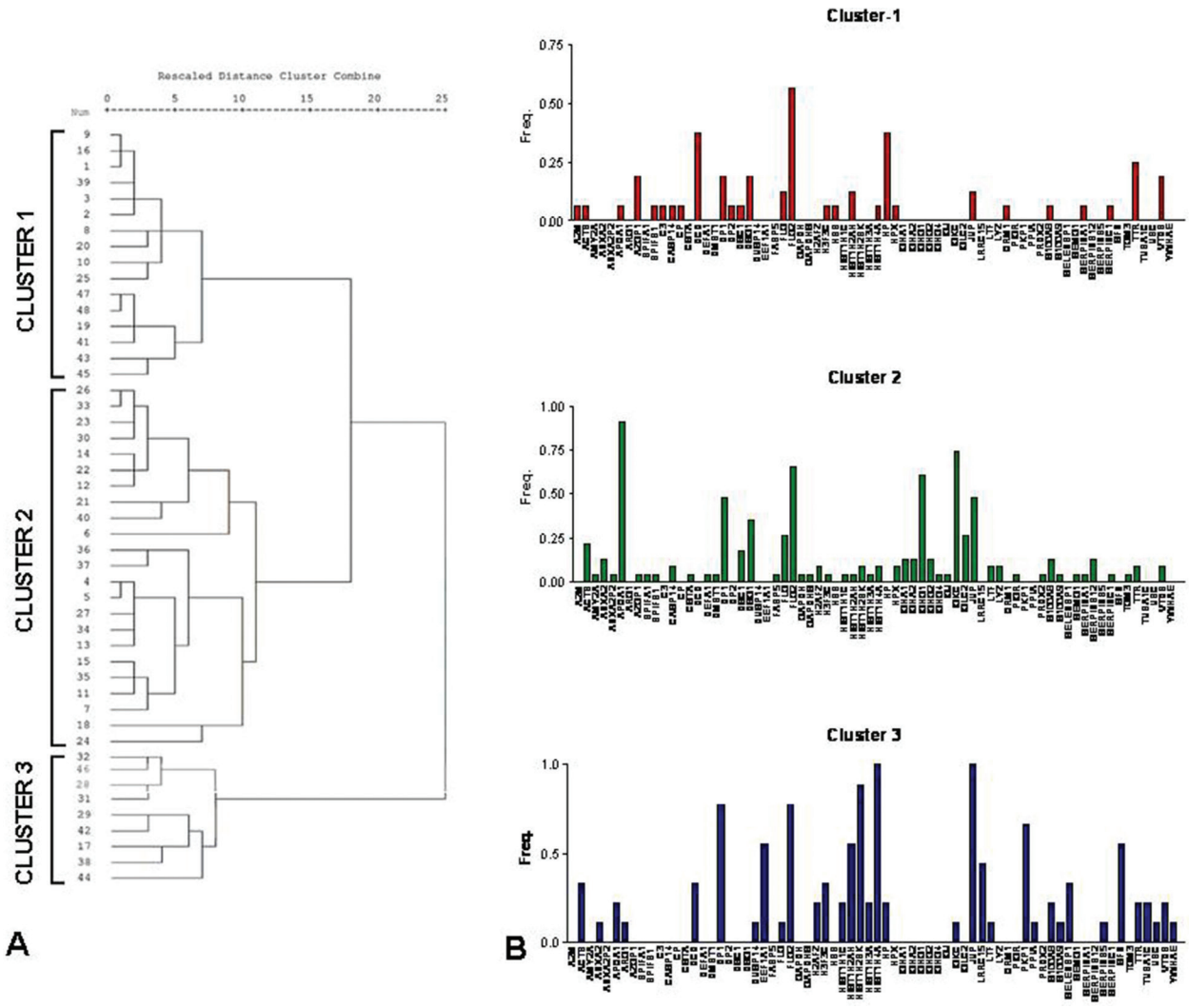

Figure 3. Ward's method-hierarchical agglomerative clustering of embryos, according to the protein profile of conditioned medium at day 3 of *in vitro* culture, identified 3 main clusters (A) characterized by a distinctive spectrum of protein frequencies (B).

(pregnancy) or negative (miscarriage) outcome. In order to identify a parameter describing of the overall efficacy of the transfer procedure, we defined the probability of pregnancy as the ratio between the number of clinical pregnancies achieved and the transferred embryo in a given patient. According to this definition, the higher is the number of gestational sacs per transferred embryos and the higher is the overall efficacy of the procedure. Consequently, pregnancies obtained by single embryo transfer had the highest efficacy or probability (equal to 1). Vice versa, the only biochemical pregnancy derived by the single transfer of an embryo displaying not-null profile, evidenced a null efficacy (0). Intermediate situations had a probability ranging from 0 to 1. With this convention, MLRA was applied to the 14 samples characterized by a known pregnancy outcome and not-null proteomic profile, further divided for transfer at day 3 and day 5. As a result were found a total set of 12 proteins, 5 for transfer at day 3 and 7 for transfer at day 5, to be of higher interest since their linear combination, in terms of presence (value "1") or absence (value "0") and weighted by a specific coefficient, showed a significant predictive value ($p<0.0.5.$, Table 5).

5. DISCUSSION

Proteomic analysis of embryo development is considered a promising platform aimed to improve the success of artificial reproductive technology (26). In this study, the individual protein profile of 48 embryos was identified and compared by the analysis of 10 μl of the corresponding spent medium at the third day of culture. Samples were pre-treated exclusively to remove albumin, an additive typically present at high concentrations in culture medium, that could act as a masking agent (27). In this regard, a further blanking strategy was adopted by

Table 3. List of functional pathways requiring proteins found to be absent within each cluster

Absent proteins	Pathway(s)
Cluster 1	
H2AFZ	Nucleosome, systemic lupus erythematosus, programmed cell death
HIST1H1C	Nucleosome, programmed cell death
HIST1H3A	Nucleosome, programmed cell death
HIST1H2BK	Nucleosome, systemic lupus erythematosus, defense response, programmed cell death
LTF	Defense response
S100A9	Defense response
Cluster 2	
DCD	Extracellular region, defense response
HP	Defense response, response to hydrogen peroxide
Cluster 3	
DSC1	Desmosome
DSG1	Desmosome
HPX	Heme metabolic process, endocytic vesicle
SERPINA1	Negative regulation of hydrolase activity, protease binding
SERPINC1	Negative regulation of hydrolase activity, protease binding

subtracting contaminants deriving from culture medium supplements and/or sample manipulation. Moreover, the accurate removal of cumulus cells during oocyte denudation rendered unlikely a possible conditioning of culture medium by cells of non embryonic origin. As a result, a total number of 70 proteins specifically ascribable to embryo were detected. Protein array, used for the validation of LC-MS/MS data and performed two pool of spent media of the validation group, was able to detect respectively 11 and 9 proteins of specific embryo origin showing a lower sensitivity than the chromatographic method. Nonetheless, we could observe a high degree of overlap between the two methods since respectively 8/11 (72%) and 7/9 (78%) proteins detected by protein array were also identified by LC-MS/MS. Moreover the signal intensity observed by protein array on each pool of the validation group, correlating with the concentration of a given protein in the pool, was significantly associated with the frequency by which the same protein was observed the singular protein profiles of study group. Our results are similar to that reported by Cortezzi *et al.* (28). In that study, a bottom-up, label-free, mass spectrometric proteomic approach was used to evaluate the protein profile of the human embryonic secretome (28). Two pools, derived respectively from 8 medium samples obtained from embryos with positive implantation outcomes and 8 with negative outcomes, underwent whole protein precipitation and analysis. The authors identified 64 proteins ascribable to embryo production, of which 15 proteins were found to be uniquely associated

Table 4. Summary of embryo information for samples selected for multiple linear regression analysis

Transfer day and modality	Sample ID	Morphology at day 3	Pertaining cluster	Pregnancies (number of gestational sacs with heartbeat at ultrasound evaluation)	Assigned probability
3/single	17	A	2	0	0.0.0.
3/multiple	48	A	2	1	0.5.0.
	15	A	3		0.5.0.
3/multiple	1	A	1	1	0.3.3.
	2	B	1		0.3.3.
	40	B	3		0.3.3.
5/single	10	B	1	1	1.0.0.
5/single	12	A	2	1	1.0.0.
5/single	25	A	3	1	1.0.0.
5/single	31	A	1	1	1.0.0.
5/multiple	5	A	2	1	0.5.0.
	42	A	2		0.5.0.
5/multiple	26	A	1	1	0.5.0.
	27	A	2		0.5.0.
Number of cultured embryo of the study group featured by with non null protein profile and undergoing transfer: 14					

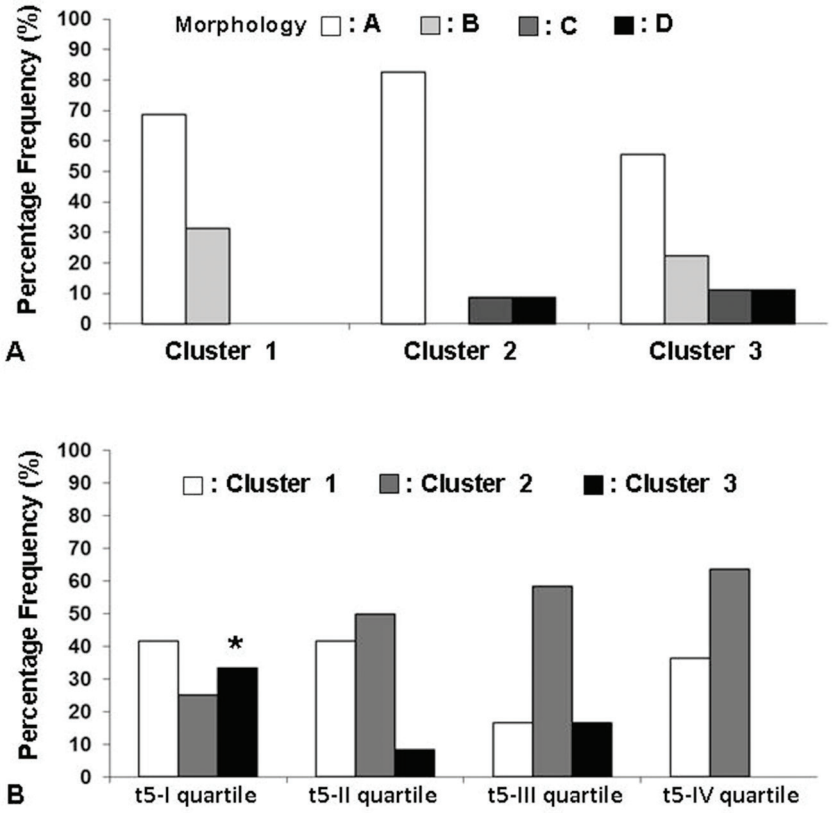

Figure 4. (A) Segregation analysis of embryo morphological classes within clusters obtained by protein profile comparisons. Data are expressed as percentage of embryos, of a given morphological class, within the cluster considered. (B) Segregation analysis of embryo-t5 within clusters obtained by protein profile comparisons. Data are expressed as percentage of embryos of a given cluster, pertaining to the t5-quartile considered. Significance; * =P<0.0.5.

Table 5. Proteins of significant importance in outcome prediction as determined by multiple linear regression analysis

Protein	Coefficient
Transfer at day 3	
DP2	0.3.6.
H3F3C	0.5.5.
IGLC2	0.5.5.
S100A8	0.3.6.
UBC	0.3.6.
Transfer at day 5	
ANXA2P2	0.2.3.
IGHG1	0.3.2.
LYZ	−0.2.3.
SERPINA1	0.4.6.
SERPINB12	0.6.5.
SERPINC1	0.2.3.
Table refers to embryo features reported in Table 4	

with a positive implantation outcome. A direct comparison with the results here presented, however, it is not possible given the major methodological differences between the two studies, both in terms of embryo culture conditions and protein isolation method.

An assessment of possible correlations between the secretome pattern, morphology, morphokinetics and pregnancy outcome of the cultured embryos was performed in the present study by applying unsupervised statistical analysis of the proteomic results. Initially, by the use of hierarchical clustering analysis of the medium samples based on protein profile similarity, three main clusters were identified. As expected, given the number of samples assessed and the breadth of the total protein spectrum, a redundancy of protein representation was found. Therefore, an alternative strategy focused on the panel of proteins specifically absent in each cluster was adopted. Moreover, in an attempt to identify the involvement these panels in a functional pathways, the g: Profiler program was used. g: Profiler is a web server conceived to interpret gene lists, ranked lists and gene list collections by automated interpretative analysis algorithms that take advantage of systematized databases including Gene Ontology (29), Human

Phenotype Ontology (30), KEGG (31), Reactome(32), BioGrid (33), MicroCosm (34) and Transfac (35). The results of this analysis found that cluster 1 was characterized by the absence of histone proteins H2AFZ, HIST1H1C, HIST1H3A and HIST1H2BK, which take part in nucleosome formation (36) and are released during the late phases of the apoptotic process (37). On the other hand, cluster 3 was found to lack DSC1, DSG1 and SERPINA1-C1, which are involved in desmosome formation, modulation of protease activity and, in particular for the SERPIN family, stem cell development and embryo/endometrium interactions (38,39). Differently from clusters 1 and 3, cluster 2 was specifically devoid of very few proteins, in particular DCD and HP. The biological function of these two proteins have been widely studied in developmental context. DCD is reported to be a human homologue of mouse proteolysis-inducing factor with cachectic functions (40), whilst HP has been recently suggested to be a “switch-on” marker of early-onset neonatal sepsis in preterm newborns (41). In the light of this differential involvement in important functional pathways, it could be hypothesized that samples belonging to cluster 1 possessed a functional status different from cluster 3. In accordance with this idea, a consistent segregation of functional parameters of embryonic development were observed between the two clusters. Indeed, even if not statistically significant, morphological classes differentially distributed among clusters and embryos of a higher morphological class found more frequently in cluster 1. Moreover, embryos for which time to 5 cells fell in the lowest quartile, significantly segregated to cluster 3. Previous studies have indicated that the timing of embryo development could represent an important functional parameter for implantation success, since embryos that cleaved at intermediate time points were shown to have a significantly improved chance of implantation when compared with embryos that either developed faster or more slowly (11). In particular, time of cleavage to the 5 cell stage appeared to be a good indicator of implantation potential (9). Furthermore, it has been documented that early cleavage divisions during of embryo development are featured by an intense gene activation that allows the substitution of maternal molecular machinery with the embryonic one and that dramatically influences morphological changes observed during the late-preimplantation phase (42). Accordingly, the early pattern of proteins released by day 3-cultured embryos was found to correlate significantly with the functional evolution of embryos. However, further studies are required to clarify whether the embryo secretome represents a stand-alone paracrine signaling or is formed by simple diffusion of cytosolic constituents.

Finally, the prognostic value of the embryo secretome protein profile for implantation efficiency was assessed. Several studies aimed to identify a secreted protein that could be indicative of embryo fate after transfer. In this regard, apolipoprotein-A1 obtained from the supernatant of high-quality blastocysts (43) have generated a great deal of interest. However very recent data from Nyalwidhe *et al*. (44) showed that fragments of this protein are significantly reduced in the embryo media samples from embryo transfer cycles resulting in viable pregnancies, rising concerns about the prognostic value of apolipoprotein-A1. On the other side, a 8.5.-kDa protein was observed to have increased expression in the protein secretome of developing blastocysts, potentially indicating an association between this protein and developmental competence. Protein identification using MS/MS with peptide sequencing indicated that the most likely candidate for this 8.5.-kDa protein was ubiquitin (45). However, it has to be considered the multifactorial nature of human embryos, and it is very unlikely that only one molecule would be able to predict developmental competence and/or implantation potential (26). In accordance, by applying MLRA to the protein profile of embryos with known implantation outcome, few proteins were found to be of significant predicting importance among the 70 proteins identified in this study very. In particular, 5 proteins showed prognostic value for transfer at day 3 and 7 for transfer ad day 5. Interestingly, polyubiquitin-C was identified as a significant early predictor, confirming previous data from Katz-Jaffe *et al*. about the key role of ubiquitination process in embryo development (45,46). It is intriguing to note that proteins of the ubiquitin–proteasome system are generally detectable at both cytoplasmic, nuclear and membranes compartments, with the expression greatly depending on the stage of cells differentiation (47). Nevertheless, it is shared opinion that enzymes are somehow released into the extracellular environment and may function as paracrine factors (48). In particular, extracellular ubiquitin was found to influences a wide range of cell events: from the differentiation of pro-B- and pro-T-lymphocytes (49), to antimicrobial activity of hematopoietic cells (50,51), induction of apoptosis (52) and influence on the LPS induced production of TNF-α in macrophages (53). In line with this hypothesis other factors known to influence protease activity and interacting with the proteasome system (54), such as SERPINA1 and SERPINC1, showed prognostic value for implantation efficacy after transfer at day 5, reinforcing their functional role already noted in cluster analysis.

The main limitation of the present study is represented by the reduced number of samples analyzed, particularly for samples deriving from embryos with known outcome. Moreover, considering the complexity of the whole proteomic assessment, some analytical factors may have likely influenced our results. Firstly, we used a qualitative liquid chromatography-mass spectrometry technique for protein detection. Perhaps, the application of a more quantitative method would allow to identify the most important proteins secreted by the embryo related to its implantation. In addition albumin removal, performed to lower medium supplements, may have resulted in the failed detection of occasional proteins bound to

albumin (55). Finally, possible medium conditioning attributable to adhering cumulus and coronal cells cannot be excluded. However, all the possible efforts to lower this possibility have been implemented, such as devoting particular attention during oocyte denudation.

In conclusion, proteomic analysis of the embryo secretome appears to be both a promising tool for the comprehension of embryo physiology and an aid to improvement the success of assisted reproductive technology. However, further studies are required to improve the analysis of secretory patterns, and to validate the prognostic potential of this method by performing studies on larger groups of implanted embryos in standardized culture conditions.

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7. REFERENCES

1. Malizia, B. A., Dodge, L. E., Penzias, A. S., and M.R. Hacker: The cumulative probability of liveborn multiples after in vitro fertilization: a cohort study of more than 10,000 women. *Fertil Steril* 99:393-399 (2013) DOI: 10.1016/j.fertnstert.2012.10.018
2. Zollner, U., and J. Dietl: Perinatal risks after IVF and ICSI. *J Perinat Med* 41:17-22 (2013) DOI: 10.1515/jpm-2012-0097
3. ESHRE: Task Force on Ethics and Law Ethical issues related to multiple pregnancies in medically assisted procreation. *Hum Reprod* 18:1976-1979 (2003) DOI: 10.1093/humrep/deg357
4. Lukassen, H., Braat, D., Wetzels, A., Zielhuis, G., Adang, E., Scheenjes, E., and J. A. Kremer: Two cycles with single embryo transfer versus one cycle with double embryo transfer: a randomised controlled trial. *Hum Reprod* 20:702-708 (2005) DOI: 10.1093/humrep/deh672
5. Alpha Scientists in Reproductive medicine and ESHRE Special Interest Group of Embryology: The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Hum Reprod* 26:1270-1283 (2011) DOI: 10.1093/humrep/der037
6. Ebner, T., Moser, M., Sommergruber, M., and G. Tews: Selection based on morphological assessment of oocytes and embryos at different stages of preimplantation development: a review. *Hum Reprod Update* 9:251-262. (2003) DOI: 10.1093/humupd/dmg021
7. Ziebe, S., Petersen, K., Lindenberg, S., Andersen, A. G., Gabrielsen, A., and A. N. Andersen:. Embryo morphology or cleavage stage: how to select the best embryos for transfer after in-vitro fertilization. *Hum Reprod* 12:1545-1549 (1997) DOI: 10.1093/humrep/12.7.1545
8. Desai, N. N., Goldstein, J., Rowland, D. Y., and J. M. Goldfarb: Morphological evaluation of human embryos and derivation of an embryo quality scoring system specific for day 3 embryos: a preliminary study. *Hum Reprod* 15:2190-2196 (2000) DOI: 10.1093/humrep/15.10.2190
9. Meseguer, M., Herrero, J., Tejera, A., Hilligsøe, K. M., Ramsing, N. B., and J. Remohí: The use of morphokinetics as a predictor of embryo implantation. *Hum Reprod* 26:2658-2671 (2011) DOI: 10.1093/humrep/der25
10. Basile, N., Nogales, M. D., Bronet, F., Florensa, M., Riqueiros, M., Rodrigo, L., García-Velasco, J., and M. Meseguer: Increasing the probability of selecting chromosomally normal embryos by time-lapse morphokinetics analysis. *Fertil Steril* 101:699-704 (2014) DOI: 10.1016/j.fertnstert.2013.12.005
11. Wong, C. C., Loewke, K. E., Bossert, N. L., Behr, B., De Jonge, C. J., Baer, T. M., and R. A. Reijo Pera:. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nature Biotech* 28:1115-1121 (2010) DOI: 10.1038/nbt.1686
12. Meseguer, M., Rubio, I., Cruz, M., Basile, N., Marcos, J., and A. Requena: Embryo incubation and selection in a time-lapse monitoring system improves pregnancy outcome compared with a standard incubator: a retrospective cohort study. *Fertil Steril* 98:1481-1489 (2012) DOI: 10.1016/j.fertnstert.2012.08.016

13. Kulasingam, V., and E. P. Diamandis: Proteomics analysis of conditioned media from three breast cancer cell lines: a mine for biomarkers and therapeutic targets. *Mol Cell Proteomics* 6:1997-2011 (2007)
DOI: 10.1074/mcp.M600465-MCP200

14. Yao, Y. Q., Barlow, D. H., and I. L. Sargent:. Differential expression of alternatively spliced transcripts of HLA-G in human preimplantation embryos and inner cell masses. *J Immunol* 175:8379-8385 (2005)
DOI: 10.4049/jimmunol.175.12.8379

15. Domínguez, F., Gadea, B., Esteban, F. J., Horcajadas, J. A., Pellicer, A., and C. Simón: Comparative protein-profile analysis of implanted versus non-implanted human blastocysts. *Hum Reprod* 23:1993-2000 (2008)
DOI: 10.1093/humrep/den205.

16. Ubaldi, F., Anniballo, R., Romano, S., Baroni, E., Albricci, L., Colamaria, S., Capalbo, A., Sapienza, F., Vajta, G., and L. Rienzi: Cumulative ongoing pregnancy rate achieved with oocyte vitrification and cleavage stage transfer without embryo selection in a standard infertility program. *Hum Reprod* 5:1199-1205 (2010)
DOI: 10.1093/humrep/deq046

17. Rienzi, L., Ubaldi, F., Anniballo, R., Cerulo, G., and E. Greco: Preincubation of human oocytes may improve fertilization and embryo quality after intracytoplasmic sperm injection. *Hum Reprod* 4:1014-1019 (1998)
DOI: 10.1093/humrep/13.4.1014

18. Rienzi, L., Romano, S., Albricci, L., Maggiulli, R., Capalbo, A., Baroni, E., Colamaria, S., Sapienza, F., and F. Ubaldi: Embryo development of fresh 'versus' vitrified metaphase II oocytes after ICSI: a prospective randomized sibling-oocyte study. *H*um *Reprod* 25:66-73 (2010)
DOI: 10.1093/humrep/dep346

19. Gardner, D. K., and WB Schoolcraft:. In-vitro culture of human blastocyst. In Jansen R., and Mortimer, D. (eds), Toward Reproductive Certainty: Fertilyty and Genetics Beyond. Parthenon Press, Carnforth, pp 378-388 (1999)
DOI not Found

20. Lawlor, K., Nazarian, A., Lacomis, L., Tempst, P., and J. Villanueva. Pathway-based biomarker search by high-throughput proteomics profiling of secretomes. *J Proteome Res* 8:1489-503 (2009)
DOI: 10.1021/pr8008572.

21. Berggård, T., Arrigoni, G., Olsson, O., Fex, M., Linse, S., and P. James: 140 mouse brain proteins identified by Ca2+-calmodulin affinity chromatography and tandem mass spectrometry. *J Proteome Res* 5:669-687 (2006)
DOI: 10.1021/pr050421l

22. Solier, C., and H. Langen: Antibody-based proteomics and biomarker research - current status and limitations. *Proteomics* 14:774-83 (2014)
DOI: 10.1002/pmic.201300334.

23. R. Burns: Business research methods and statistics using SPSS. London: SAGE Publications Ltd. (2009)
DOI not Found

24. Kutner, M. H., Nactsheim, C. J., Neter, J., and L. William: Applied linear statistical models. Scarborough: Irwin Professional Pub (2004)
DOI not Found

25. Reimand, J, Arak, T. and J. Vilo: g: Profiler--a web server for functional interpretation of gene lists (2011 update). *Nucl Ac Res* 39:(Web Server issue) W307-315 (2011)
DOI: 10.1093/nar/gkr378

26. Katz-Jaffe, M. G., and S. McReynolds: Embryology in the era of proteomics. *Fertil Steril* 99:1073-1077 (2013)
DOI: 10.1016/j.fertnstert.2012.12.038

27. Holewinski, R. J., Jin, Z., Powell, M. J., Maust, M. D., and J. E. Van Eyk: A fast and reproducible method for albumin isolation and depletion from serum and cerebrospinal fluid. *Proteomics* 13:743-750 (2013)
DOI: 10.1002/pmic.201200192

28. Cortezzi, S. S., Garcia, J. S., Ferreira, C. R., Braga, D. P., Figueira, R. C., Iaconelli, A. Jr., Souza, G. H., Borges, E. Jr., and M. N. Eberlin: Secretome of the preimplantation human embryo by bottom-up label-free proteomics. *Anal Bioanal Chem* 401:1331-1339 (2011)
DOI: 10.1007/s00216-011-5202-1

29. Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T., Harris, M. A., Hill, D. P., Issel-Tarver,

L., Kasarskis, A., Lewis, S., Matese, J. C., Richardson, J. E., Ringwald, M., Rubin, G. M., and G. Sherlock: Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nature Gen* 25:25-29 (2000) DOI: 10.1038/75556

30. Robinson, P. N., and S. Mundlos: The human phenotype ontology. *Clin Gen* 77:525-534 (2010) DOI: 10.1111/j.1399-0004.2010.01436.x
31. Kanehisa, M., Goto, S., Furumichi, M., Tanabe, M., and Hirakawa, M:. KEGG for representation and analysis of molecular networks involving diseases and drugs. *Nucl Ac Res* 38:D355-D336 (2010) DOI: 10.1093/nar/gkp896
32. Matthews, L., Gopinath, G., Gillespie, M., Caudy, M., Croft, D., de Bono, B., Garapati, P., Hemish, J., Hermjakob, H., Jassal, B., Kanapin, A., Lewis, S., Mahajan, S., May, B., Schmidt, E., Vastrik, I., Wu, G., Birney, E., Stein, L., and P. D'Eustachio: Reactome knowledgebase of human biological pathways and processes. *Nucl Ac Res* 37:D619-D622 (2009) DOI: 10.1093/nar/gkn863
33. Stark, C., Breitkreutz, B. J., Chatr-Aryamontri, A., Boucher, L., Oughtred, R., Livstone, M. S., Nixon, J., Van Auken, K., Wang, X., Shi, X., Reguly, T., Rust, J. M., Winter, A., Dolinski, K., and M. Tyers: The BioGRID Interaction Database: 2011 update. *Nucl Ac Res* 39:698-704 (2011) DOI: 10.1093/nar/gkq1116
34. Griffiths-Jones, S., Saini, H. K., van Dongen, S., and A. J. Enright: miRBase: tools for microRNA genomics. *Nucl Ac Res* 36:D154-D158 (2008) DOI: 10.1093/nar/gkm952
35. Matys, V., Kel-Margoulis, O. V., Fricke, E., Liebich, I., Land, S., Barre-Dirrie, A., Reuter, I., Chekmenev, D., Krull, M., Hornischer, K., Voss, N., Stegmaier, P., Lewicki-Potapov, B., Saxel, H., Kel, A. E., and E. Wingender: TRANSFAC and its module TRANSCompel: transcriptional gene regulation in eukaryotes. *Nucl Ac Res* 34(Database issue):D108-D110 (2006) DOI: 10.1093/nar/gkj143
36. Zentner, G. E., and S. Henikoff: Regulation of nucleosome dynamics by histone modifications. *Nature Struct Mol Biol* 20:259-166 (2013) DOI: 10.1038/nsmb.2470
37. Gabler, C., Kalden, J. R., and H. M. Lorenz: The putative role of apoptosis-modified histones for the induction of autoimmunity in Systemic Lupus Erythematosus. *Biochem Pharmacol* 66:1441-1446. (2003) DOI: 10.1016/S0006-2952(03)00495-7
38. Getsios S., Amargo, E. V., Dusek, R. L., Ishii, K., Sheu, L., Godsel, L. M., and K. J. Green: Coordinated expression of desmoglein 1 and desmocollin 1 regulates intercellular adhesion. *Differentiation* 72:419-433 (2004) DOI: 10.1111/j.1432-0436.2004.07208008.x
39. Pak, S. C., Kumar, V., Tsu, C., Luke, C. J., Askew, Y. S., Mills, D. R., Brömme, D., and G. A. Silverman: SRP-2 is a cross-class inhibitor that participates in postembryonic development of the nematode Caenorhabditis elegans: initial characterization of the clade L serpins. *J Biol Chem* 279:15448-15459 (2004) DOI: 10.1074/jbc.M400261200
40. Lee Motoyama, J. P., Kim-Motoyama, H., Kim, P., Nakagama, H., Miyagawa, K., and K. Suzuki: Identification of dermcidin in human gestational tissue and characterization of its proteolytic activity. *Biochem Biophysl Res Commun* 357:828-833 (2007) DOI: 10.1016/j.bbrc.2007.03.112
41 Buhimschi, C. S., Bhandari, V., Dulay, A. T., Nayeri, U. A., Abdel-Razeq, S. S., Pettker, C. M., Thung, S., Zhao, G., Han, Y. W., Bizzarro, M., and I. A. Buhimschi IA: Proteomics mapping of cord blood identifies haptoglobin "switch-on" pattern as biomarker of early-onset neonatal sepsis in preterm newborns. *PLoS One* 6:e26111 (2011) DOI: 10.1371/journal.pone.0026111.
42. Hamatani, T., Ko, M. Sh., Yamada, M., Kuji, N., Mizusawa, Y., Shoji, M., Hada, T., Asada, H., Maruyama, T., and Y. Yoshimura: Global gene expression profiling of preimplantation embryos. *Hum Cell* 19:98-117 (2006) DOI: 10.1111/j.1749-0774.2006.00018.x
43. Nyalwidhe, J., Burch, T., Bocca, S., Cazares, L., Green-Mitchell, S., Cooke, M., Birdsall, P., Basu, G., Semmes, O. J., and S. Oehninger: The search for biomarkers of human embryo developmental potential in IVF: a comprehensive proteomic approach. *Mol Hum Reprod* 19:250-263 (2013) DOI: 10.1093/molehr/gas063
44. Katz-Jaffe, M. G., Schoolcraft, W. B.,

and D. K. Gardner: Analysis of protein expression (secretome) by human and mouse preimplantation embryos. *Fertil Steril* 86:678–685 (2006)
DOI: 10.1016/j.fertnstert.2006.05.022

45. Naujokat, C., and T. Sarić: Concise review: role and function of the ubiquitin-proteasome system in mammalian stem and progenitor cells. *Stem Cells* 25:2408-2418 (2007)
DOI: 10.1634/stemcells.2007-0255

46. Rivett, A. J., Palmer, A., and E. Knecht: Electron microscopic localization of the multicatalytic proteinase complex in rat liver and in cultured cells. *J Histochem Cytochem* 40:1165–1172 (1992).
DOI: 10.1177/40.8.1619280

47. Sixt, S. U., and B. Dahlmann: Extracellular, circulating proteasomes and ubiquitin - incidence and relevance. *Biochim Biophys Acta* 1782:817-823 (2008)
DOI: 10.1016/j.bbadis.2008.06.005.

48. Goldstein, G., Schedi, M., Hammerling, U., Schlesinger, D. H., Niall, H. D., and E. A. Boyse: Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. *Proc Natl Acad Sci USA* 72:11–15. (1975)
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC432229/pdf/pnas00044-0017.pdf

49. Majetschak, M., Krehmeier, U., Bardenheuer, M., Denz, C., Quintel, M., Voggenreiter, G., and U. Obertacke. Extracellular ubiquitin inhibits the TNF-alpha response to endotoxin in peripheral blood mononuclear cells and regulates endotoxin hyporesponsiveness in critical illness. *Blood* 101:1882–1890 (2003)
DOI: 10.1182/blood-2002-03-0918

50. Kieffer, A. E., Goumon, Y., Ruh, O., Chasserot-Golaz, S., Nullans, G., Gasnier, C., Aunis, D., and M. H. Metz-Boutigue: The N- and C-terminal fragments of ubiquitin are important for the antimicrobial activities. *FASEB J* 17:776–778 (2003).
DOI: 10.1096/fj.02-0699fje

51. Daino, H., Matsumura, I., Takada, K., Odajima, J., Tanaka, H., Ueda, S., Shibayama, H., Ikeda, H., Hibi, M., Machii, T., Hirano, T., and Y. Kanakura: Induction of apoptosis by extracellular ubiquitin in human hematopoietic cells: possible involvement of STAT3 degradation by proteasome pathway in interleukin 6-dependent hematopoietic cells. *Blood* 95:2577–2585 (2000)
http://bloodjournal.hematologylibrary.org/content/95/8/2577

52. Mains, L. M., Christenson, L., Yang, B., Sparks, A. E., Mathur, S., and B. J. Van Voorhis: Identification of apolipoprotein A1 in the human embryonic secretome. *Fertil Steril* 96:422-427 (2011).
DOI: 10.1016/j.fertnstert.2011.05.049

53. Nabika, T., Terashima, M., Momose, I., Hosokawa, Y., Nagasue, N., and Y. Tanigawa: Synergistic effect of ubiquitin on lipopolysaccharide-induced TNF-alpha production in murine macrophage cell line RAW 264.7. cells. *Biochim Biophys Acta* 1450:25–34 (1999)
DOI: 10.1016/S0167-4889(99)00024-5

54. Boncela, J., Przygodzka, P., Papiewska-Pajak, I., Wyroba, E., Osinska, M., and C. S. Cierniewski:. Plasminogen activator inhibitor type 1 interacts with alpha3 subunit of proteasome and modulates its activity. *J Biol Chem* 286:6820-6831 (2011).
DOI: 10.1074/jbc.M110.173781.

55. U. Kragh-Hansen: Molecular and practical aspects of the enzymatic properties of human serum albumin and of albumin-ligand complexes. *Biochim Biophys Acta* 12:5535-5544 (2013)
DOI: 10.1016/j.bbagen.2013.03.015.

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Send correspondence to:. Carlo Foresta, Department of Molecular Medicine, Section of Clinical Pathology and Centre for Human Reproduction Pathology, University of Padova, Via Gabelli 63, 35121 Padova, Italy. Tel 39-49-8218517, Fax 39-49-8218520, E-mail: carlo.foresta@unipd.it