

# Quantifying soluble HLA-G in supernatants of cultured embryos as a marker of implantation potential in an assisted reproduction program

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## CUANTIFICACIÓN DE HLA-G SOLUBLE EN SOBRENADANTES DEL CULTIVO DE EMBRIONES COMO UN INDICADOR DE SU CAPACIDAD DE IMPLANTACIÓN EN UN PROGRAMA DE REPRODUCCIÓN ASISTIDA

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### RESUMEN

HLA-G desempeña un papel tolerogénico en la interfase materno-fetal. En programas de reproducción asistida, en los que se cultivan embriones in vitro, ha cobrado interés la detección de las isoformas solubles de esta molécula (sHLA-G) en el medio en el que se han crecido los embriones. Aunque la determinación es compleja, tiene interés por su aparente relación con la idoneidad de dichos embriones. En el presente trabajo, se ha puesto a punto un ensayo ELISA amplificado para medir sHLA-G a las concentraciones esperables en dichos sobrenadantes. Como modelo comparativo se ha utilizado el cultivo a dilución límite de la línea de coriocarcinoma JEG-3. Con el ELISA desarrollado se han analizado retrospectivamente 111 sobrenadantes recogidos a las 44-48 horas de efectuada fecundación mediante inyección intracitoplasmática del espermatozoide, y los datos se han correlacionado con los resultados reproductivos de dichos embriones. En 22 de los sobrenadantes (19.8%) se ha detectado sHLA-G. No se ha encontrado relación entre los niveles de sHLA-G y el grado morfológico de los embriones. Los resultados reproductivos de los embriones de morfología normal que fueron transferidos al útero de las pacientes (2-3 por caso) fueron los siguientes: en el grupo de mujeres que recibió únicamente embriones sHLA-G negativos, las tasas de embarazo e implantación fueron, respectivamente, 29% (4 embarazos/14 mujeres) y 14% (5 sacos gestacionales/35 embriones transferidos). Por contra, en el grupo que recibió al menos un embrión sHLA-G positivo, las tasas de embarazo e implantación subieron al 60% (3 / 5) y 29% (4/14) respectivamente. En conclusión, es posible cuantificar niveles de sHLA-G en sobrenadantes de embriones y los resultados obtenidos sugieren asociación con la probabilidad de embarazo. La detección de sHLA-G, por tanto, podría ser un buen complemento de la selección morfológica de embriones útil para incrementar la tasa de implantación y reducir la de embarazos múltiples.

PALABRAS CLAVE: sHLA-G / ICSI / Cultivo de embriones / Embarazo.

### ABSTRACT

HLA-G plays a tolerogenic function at the maternal-fetal interface. Detection of the soluble isoforms of HLA-G (sHLA-G) in culture medium derived from embryos grown in vitro, although technically complex, has gained interest in assisted reproduction programs because of an apparent relationship with embryo competence. Here, an amplified ELISA was designed to measure sHLA-G at the concentrations expected in embryo supernatants using a limiting-dilution assay of the choriocarcinoma JEG-3 cell line as a surrogate model. With this ELISA approach, 111 single embryo culture supernatants, collected 44-48 hours after intracytoplasmic sperm injection, were retrospectively analysed and levels correlated with pregnancy results. The presence of sHLA-G was demonstrated in 22 (19.8%) of the embryo cultures. There was no relationship between sHLA-G levels and grading of embryo morphology. The reproductive outcome of the morphologically normal embryos that were transferred to the women uterus (2-3 per patient) was as follows: in the group of women in which all transferred embryos were sHLA-G negative, the pregnancy and implantation rates were 29% (4 pregnancies/14 women) and 14% (5 gestational sacs/35 embryos transferred), respectively. In contrast, in the group in which the embryo transfers included at least one sHLA-G positive the pregnancy and implantation rates increased to 60% (3/5) and 29% (4/14) respectively. In conclusion, sHLA-G levels in preimplantation embryo supernatants can be quantified and results suggest positive association with pregnancy likelihood. sHLA-G detection seems to be useful to complement morphology in selecting good quality embryos for increasing implantation rates and reducing multiple gestations.

KEY WORDS: sHLA-G / ICSI / Embryo culture / Pregnancy.

## INTRODUCTION

From an immunological standpoint the embryo can be considered a semi-allogeneic graft, and even completely allogeneic in cases of oocyte or embryo donation. Contrary to artificial transplants, the conceptus is tolerated by maternal defence barriers. Several immunosuppressive mechanisms, some of them still pending clarification, contribute to prevent rejection. Specially protected is the extravillous cytotrophoblast, fetus-derived placental cells that migrate into the uterine wall and contact with maternal cells. Extravillous cytotrophoblast cells, besides lacking Human Leukocyte Antigens (HLA)-A, HLA-B and HLA class II, which are the major allograft rejection molecules, express HLA-G<sup>(1, 2)</sup>. This non-classical class Ib antigen is thought to be a critical tolerogenic molecule for the development of pregnancy.

HLA-G is minimally polymorphic (24 alleles identified so far) and can be found in seven isoforms resulting from alternative splicing of a single gene<sup>(3-6)</sup>. Isoforms HLA-G1, -G2, -G3 and -G4 exist as membrane anchored molecules, and although only the full-length -G1 isoform is easily detected at the cell surface, the others are revealed by intracellular staining<sup>(7-9)</sup>. The isoforms HLA-G5, -G6 and -G7 lack the exon 5, which codes the transmembrane domain, and would be consequently released by the cells. However, only -G5 and -G6 are detectable as soluble molecules, meanwhile -G7 has been only detected in extracts of transfected cells<sup>(10)</sup>. The HLA-G1 isoform can also be found as a soluble molecule after shedding or proteolytic cleavage contributing to the soluble pool of HLA-G (sHLA-G)<sup>(11)</sup>. Some researchers have been able to detect HLA-G on the surface of preimplantation embryos surplus from *in vitro* assisted reproduction techniques (ART), as well as sHLA-G in the surrounding culture media<sup>(12,13)</sup>. This finding, besides indicating the involvement of this molecule from the earliest stages of pregnancy, led these authors to suggest a potential utility of sHLA-G as indicator of embryo quality.

Currently, in ART, selection of embryos for transferring to the female uterus is based on morphology examination at day 2-3 post-fertilization. This procedure is not always reliable and 40% of patients who appear to have good quality embryos do not become pregnant during that *in vitro* fertilization cycle<sup>(14, 15)</sup>. Consequently, the development of an objective, quantitative and non invasive test to predict the implantation potential of an embryo is a main objective. The availability of this test would increase pregnancy rates reducing the number of embryos transferred and would decrease the prevalence of multiple gestations, which is the biggest medical problem in this area. Several groups have recently reported data on the correlation between levels of sHLA-G in embryo culture supernatants and fertility

success rates that support the measurement of this molecule as one of such a candidate test for embryonic quality<sup>(15-19)</sup>. However, the results of these studies are being questioned by prestigious researchers in fertility as: 1) it is unclear which of the splicing-derived isoforms of HLA-G mRNA and proteins are expressed by the preimplanted embryo; 2) for this reason, there is no agreement on which are the appropriate antibodies to capture the sHLA-G protein isoforms and, obviously, there is no suitable standard reference reagent; and 3) as a final consequence, some of the reported concentrations of sHLA-G in embryo-surrounding media seem artifactual, as they exceed the total expected protein content of a preimplantation embryo<sup>(20, 21)</sup>.

Here, a retrospective study was carried out trying to clarify the relationship between sHLA-G levels in embryo supernatants and the probability of pregnancy. A biotin-streptavidin amplified ELISA was designed to detect the full-length HLA-G1 and HLA-5 isoforms in the supernatant of limiting dilution cultures of the JEG-3 choriocarcinoma cell line as a surrogate model of single embryo culture. A protein standard was also prepared from HLA-G-transfected cells. With these tools, 111 embryo culture supernatants collected at 44-48 hours after fertilization by intracytoplasmic sperm injection (ICSI) were analyzed for sHLA-G. The results were correlated with morphology, implantation and pregnancy rates.

## MATERIALS AND METHODS

### Patients

Nineteen couples that underwent ICSI due to moderate/severe male factor were performed in our *in vitro* Fertilization Unit at Instituto Bernabeu Alicante. The requirements to enter the study were: maternal age <39, less than 3 previous embryonic transfers of fresh embryos, normal uterine cavity, an endometrial thickness  $\geq 9$  mm on the day of human chorionic gonadotropin (hCG) administration and at least two high quality embryos on the second day after fertilization [4 cells, less than 25% of cytoplasmic fragments, without blastomere multinucleation and equal or similar (<20% difference) cells].

### Stimulation protocol and ICSI procedure

In the previous cycle, oral contraceptives were given. A long protocol was used, including leuprolide acetate agonist (Gonapeptyl Depot; Ferring, Madrid, Spain) in the previous midluteal phase. After pituitary desensitization was obtained, a combined protocol, using human follicle stimulating hormone (FSH) (Gonal F; Serono, London, UK) and human

menopausal gonadotropin (hMG) (HMG-Lepori; Farma-Lepori, Barcelona, Spain), was given. Ovarian response was monitored by transvaginal ultrasound and plasmatic oestradiol levels. Ovulation was induced with 250 µg of recombinant hCG (Ovitrelle; Serono, London, UK). Oocytes were aspirated 36 hours after hCG administration by a transvaginal ultrasound-guided needle aspiration under sedation. Surrounding oocyte cumulus and corona radiata cells were removed by a brief exposure to 80 IU/mL of hyaluronidase (Hyase; Vitrolife, Göteborg, Sweden) followed by gentle pipetting. ICSI was carried out 4 hours after oocyte retrieval on a heated stage (Tokai Hit Thermoplate, Model MATS-U505R30, Japan) at 37°C, which was mounted on an inverted microscope (Nikon Eclipse TE200, Japan) equipped with Hoffmann modulation optics and a Narishige micromanipulation system (Narishige, Japan). Microinjection was performed according to Vansteirteghem et al<sup>(22)</sup>. Only metaphase II oocytes were injected and then incubated individually in 50 µl droplets of G1.3 medium (Vitrolife) covered with sterile equilibrated mineral oil (Ovoil; Vitrolife) at 37°C in an atmosphere of 6% CO<sub>2</sub>.

### Embryonic culture

16-18 hours after microinjection, the oocytes that showed two pronuclei and 2 polar bodies were considered fertilized and their progress was monitored microscopically until the day of transfer (see below). 44-48 hours after ICSI, the original culture media (G1.3) were collected and frozen at -20°C until tested for sHLA-G levels. Two-three high quality embryos were transferred intravaginally at day 2 or day 3 post-ICSI and the surplus embryos were frozen. Poor quality or developmentally arrested embryos were discarded.

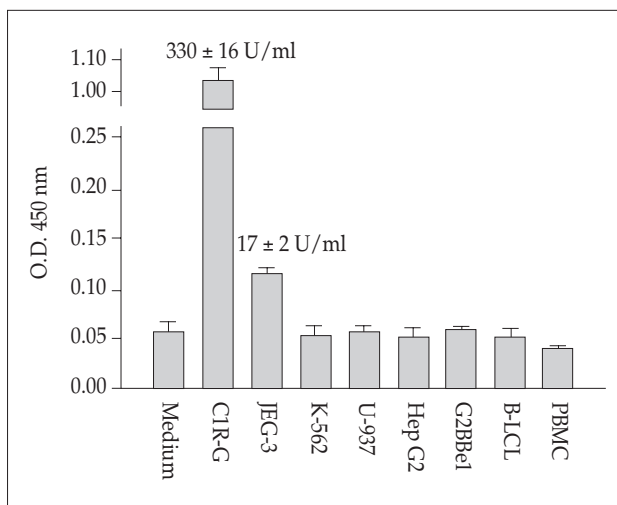
### ELISA for determination of sHLA-G levels

Maxisorp flat-bottom 96-well microtiter modules (Nunc, Roskilde, Denmark) were coated with 50 µl/well of monoclonal antibody (mAb) MEM-G/9 (Exbio, Praha, Czech Republic) at a concentration of 10 µg/ml in 0.05 M carbonate/bicarbonate buffer pH 9.6, for 1 h at 37 °C in a humidified chamber (covered tray) and then overnight at 4 °C. After this one and the following incubation steps, wells were washed four times with 300 µl of phosphate buffered saline (PBS) containing 0.5% caseine (Sigma, St Luis, MO) and blotted dry by inversion on clean paper towels. After washing, the wells were blocked with 100 µl of PBS containing 5% bovine serum albumin (BSA) (Sigma) and after 15 min at RT with shaking the wells were aspirated off, blocking step repeated and washed. Undiluted supernatant samples and properly diluted controls were added to each well (50 µl) and incubated in a humidified chamber for 2h at 37 °C with shaking. After

washing, biotin conjugated mAb W6/32 (Serotec, Oxford, UK) at a concentration of 0.2 µg/ml in PBS containing 1% BSA was added (50 µl/well) and incubated overnight at 4 °C. Wells were washed and streptavidin-horseradish peroxidase (HRP) (Biosource, Camarillo, CA), at a concentration of 0.1 µg/ml in PBS containing 1% BSA was added (50 µl/well) and incubated in a humidified chamber at 37 °C with shaking. Plates were washed three times with 300 µl/well of PBS containing 0.05 % Tween 20 (Sigma) and once with 300 µl/well of PBS. During washes the wells were soaked for 1 min intervals to reduce background. A solution of 3,3',5,5'-tetramethylbenzidine (TMB) HRP substrate (Sigma) was added (50 µl/well) and incubated for 30 min in the dark at RT. Reaction was stopped by the addition of 50 µl/well of 2N H<sub>2</sub>SO<sub>4</sub> and the optical density (O.D.) determined at a wavelength of 450 nm with a 690 nm reference filter using a microplate reader (Asys Hitech, Eugendorf, Austria). A supernatant from the human B lymphoblastoid cell line C1R transfected with HLA-G serially diluted from 100 or 50 to 0.1 U/ml in culture media was used for the generation of standard calibration curves. The sHLA concentrations of the samples were determined by logistic four-parameter curve fitting using the public domain software Elisa for Windows<sup>(23)</sup>, downloaded from the Center for Disease Control web site (<http://www.cdc.gov>). Mean O.D. values of supernatant from untransfected cells and embryo culture media (background) were subtracted to generate standard calibration curves and to estimate sHLA-G concentration of supernatants, respectively. The minimum detectable concentration was 0.1 U/ml.

### Cells

Human cell lines Hep G2 (hepatocellular carcinoma), C2BBe1 (colorectal adenocarcinoma), U-937 (promonocytic) and K-562 (erythroleukemia) were obtained from ATCC (Manassas, VA). The human choriocarcinoma JEG-3 was provided by Dr. Castrillo (Centro de Biología Molecular Severo Ochoa, Madrid, Spain). The HLA-G transfected C1R [B-cell lymphoblastoid line (B-LCL) lacking surface HLA A and B antigens] was provided by Dr. P. Aparicio (University of Murcia, Murcia, Spain). The B-LCL SuUn was obtained after Epstein-Barr virus transformation. Peripheral blood mononuclear cells (PBMC) were obtained from a healthy donor. All the cells were grown in RPMI 1640 with Glutamax (Gibco, UK) supplemented with 10% heat-inactivated fetal bovine serum (PAA, Pasching, Austria) and penicillin-streptomycin (Biowhittaker, Walkersville, MD). Supernatants were harvested after 3-4 days of cell culture (or until confluence for JEG-3) and frozen at -20 °C. Membrane HLA-G expression was confirmed by flow



**Figure 1.** Soluble HLA-G detection in culture supernatants of HLA-G-expressing and HLA-G negative control cell lines. All supernatants were tested at least in duplicate with a MEM-G/9 (capture mAb) and W6/32 biotin (detection mAb) ELISA. O.D. values + SD are shown. Positive supernatants show the estimated U/ml of sHLA-G.

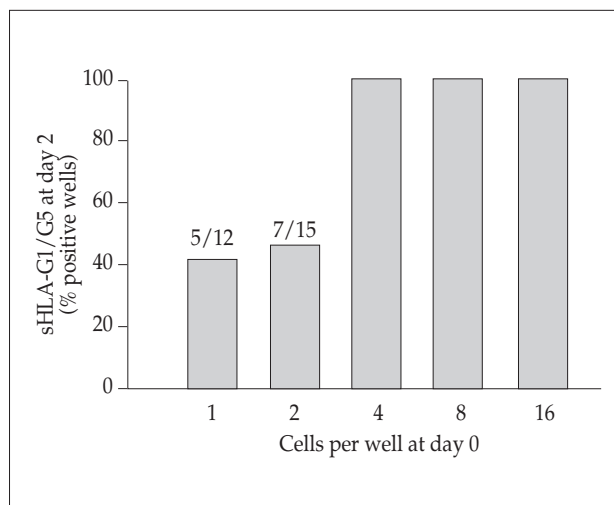
cytometry using the HLA-G-specific mAb MEM-G/9 and a secondary goat anti mouse-PE Conjugated (Dako, Glostrup, Denmark) as described<sup>(24)</sup>.

## RESULTS

### Performance of the sHLA-G ELISA

To determine the specificity and detection limit of our sHLA-G ELISA detection method, the supernatants of different HLA-G expressing- and HLA-G negative cell lines were tested. Results (Figure 1) showed a clear signal for C1R-HLA-G transfected and JEG-3 cell lines supernatants, whereas the HLA-class I negative cell line K-562 and the HLA-class I positive-HLA-G negative cell lines U-937, Hep G2, C2BBel, B-LCL SuUn, as well as PBMC showed an O.D. similar or even below the negative control (medium). For quantification, a calibration standard was prepared from a diluted supernatant of C1R-HLA-G and was assigned 100 U/ml (reference units) to which all the results were referred.

Given the disparity of reported results concerning the presence of sHLA-G in the culture medium of IVF-ICSI embryos, we next asked whether our ELISA system was able to detect sHLA-G secretion from a smaller number of cells. The JEG-3 cell line, which synthesises all HLA-G isoforms<sup>(25)</sup>, was seeded using limiting dilution methods at a range of 1 to 16 cells per well in 100 µl cultures. The results

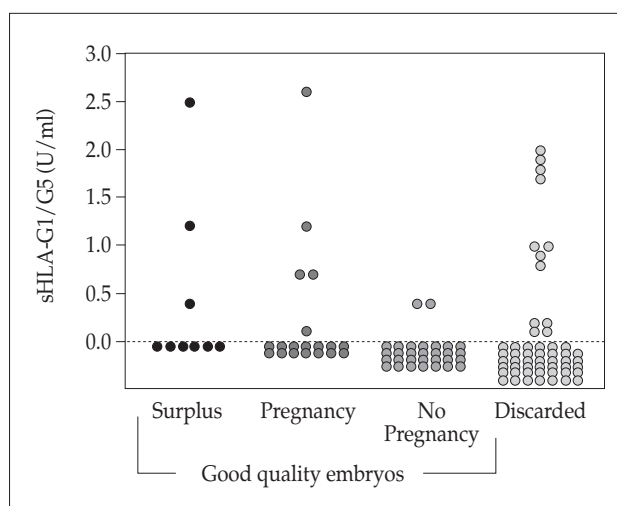


**Figure 2.** Detection of sHLA-G in culture supernatants of small numbers of JEG-3 cells as a sensitivity test for the ELISA method. Cells of the JEG-3 choriocarcinoma cell line were seeded by limiting dilution plating in volumes of 100 µl/well on day 0. Supernatants were collected 48 h later and quantified for sHLA-G. As most of the O.D. signals were either lower than the background (culture medium) or in between the background and that of the 0.1 U/ml standard concentration (i.e. the detection limit of the assay), wells were considered sHLA-G positive only if their O.D. values exceeded the mean O.D. + 3 SD values of background wells.

of ELISA measurements in the supernatants collected after 44-48 h culture (Figure 2) showed that the assay was able to detect sHLA-G in all the wells from 4 cell cultures and in approximately half of the cultures containing 1-2 cells per well. Although the protein secretion rate of a human preimplantation embryo can be rather different from that of the tumour JEG-3 cells (just for comparison, the diameter of a human preimplantation embryo can be more than ten times the diameter of a single JEG-3 cell), these results suggest that the ELISA procedure is appropriate to detect sHLA-G from a single (48h) preimplantation embryo (average 4 blastomeres).

### Detection of sHLA-G in supernatants of preimplantation embryos

Supernatants from 111 embryo cultures, collected 44-48 h after ICSI, were analysed for sHLA-G, and the results were correlated with morphology. The cut-off value to consider a supernatant as sHLA-G positive was 0.1 U/ml. sHLA-G could be detected in 22 supernatants (19.8 % of total), with sHLA-G levels ranging from 0.1 to 2.6 U/ml (mean  $0.99 \pm 0.78$  U/ml) (Figure 3). Ten out of 58 good quality embryos, as determined by morphology evaluation, were sHLA-G positive (mean  $1.02 \pm 0.88$  U/ml), and twelve out of 53 poor quality, discarded embryos were also found to secrete



**Figure 3.** sHLA-G levels in embryo supernatants. Samples were grouped into 'good quality' and 'discarded' embryos according to the morphology and fate of the embryo they belonged to. Good quality embryos were transferred (2-3 per woman) and surplus embryos were frozen. Transferred embryos were in turn grouped in 'pregnancy' or 'no pregnancy' depending on the final gestational result. Biochemical pregnancies were included in the 'no pregnancy' group. All the supernatants below the zero line represent undetectable sHLA-G concentrations.

sHLA-G (mean  $0.97 \pm 0.73$  U/ml). This means that there is no relationship between sHLA-G levels and grading of

embryo morphology. Interestingly, some of the higher sHLA-G values were found in two opposite situations: embryos with advanced cleavage status (i.e. initiating cavitation) which appear as surplus frozen embryos in Figure 3, and in development arrested, discarded embryos.

#### sHLA-G in embryo supernatants and reproductive outcome

The results of sHLA-G quantification of embryos that were transferred to the women uterus were next correlated with pregnancy and implantation rates (Table I). In the group of 5 women in which the embryo transfers included at least one sHLA-G positive, 3 patients became pregnant (pregnancy rate 60%), in contrast, in the group of 14 women in which all transferred embryos were sHLA-G negative, 4 women became pregnant (pregnancy rate 29%). Considering the number of gestational sacs per embryo transferred, in the first group of women, which received an average of 2.8 embryos per patient including at least one sHLA-G positive, 4 sacs resulted from a total of 14 embryos. This means an implantation rate of 28.6 %. In opposition, in the group receiving only sHLA-G negative embryos (mean 2.5 embryos per patient) only 5 sacs were seen from 35 embryos transferred (implantation rate 14.3 %). These results strongly suggest that presence of sHLA-G in embryo supernatants correlated with gestation.

**TABLE I.** sHLA-G levels in culture supernatants of transferred embryos in pregnant and non-pregnant patients (biochemical pregnancies are not considered)

Group	Patient	sHLA-G1/G5 in embryo supernatants (U/ml)	Day of transfer (day post-ICSI)	Clinical pregnancy
At least one sHLA-G positive embryo	3	0.4 / 0 / 0	3	–
	4	0.7 / 1.2 / 0	3	Twin
	5	0 / 0.4	2	–
	10	0.7 / 2.6 / 0	2	Single
	17	0 / 0.1 / 0	2	Single
sHLA-G negative embryos	1	0 / 0 / 0	2	Single
	2	0 / 0 / 0	3	–
	6	0 / 0 / 0	2	–
	7	0 / 0	3	–
	8	0 / 0 / 0	3	–
	9	0 / 0	2	–
	11	0 / 0	2	–
	12	0 / 0	3	Twin
	13	0 / 0 / 0	3	–
	14	0 / 0	3	–
	15	0 / 0	5	–
	16	0 / 0 / 0	3	Single
	18	0 / 0	3	Single
	19	0 / 0 / 0	3	–



## DISCUSSION

In ART, the selection of good quality embryos for transferring to the female uterus is crucial in order to increase both implantation rates and the number of singleton pregnancies. Recent studies have reported that sHLA-G in the supernatants from 2 to 3-day embryos may be the key predictive marker to decide which embryos to transfer or cryopreserve<sup>(15, 17, 18, 26)</sup>. The results we describe here support the potential utility of quantifying sHLA-G for improving embryo selection for two reasons. First, it was possible to detect sHLA-G in 19.8 % of embryo cultures. Second, our data demonstrate a positive correlation of sHLA-G with reproductive success.

Systematic investigations claim, however, that sHLA-G is technically undetectable in 2 to 6-day embryo cultures and consequently has no utility for improving embryo selection<sup>(20, 21, 27)</sup>. These contradictory results raise the question of whether technical differences in the detection procedures and/or sHLA-G standards may be critical for quantification of sHLA-G. In this regard, we and all the groups that have detected the presence of sHLA-G in embryo supernatants, except one<sup>(17)</sup>, have used the ELISA or Luminex format with the pair of antibodies validated by the recent workshop for measurement of sHLA-G in plasma samples<sup>(28)</sup>. This format includes the use of MEM-G/9 as capture antibody (specific for native  $\beta_2$ -microglobulin associated shed and cleaved HLA-G1 and -G5) with either W6/32 (a pan-HLA class I mAb) or an anti- $\beta_2$ -microglobulin as the reporter antibody. With this ELISA format, we demonstrate the technical possibility of detecting sHLA-G in 48 h supernatants of 1-16 cell cultures of the choriocarcinoma cell line JEG-3, which could resemble the expected levels in the media surrounding embryos 44-48 h post fertilization. Here it should be noted that supernatants of JEG-3 cells have been reported to be sHLA-G negative, and weakly positive after 7-fold concentration, using ELISA procedures different from the workshop consensus and with supposed detection limits of 0.15 ng/ml<sup>(29)</sup>. This proves the importance of selecting the right antibody combination for sHLA-G research.

The percent of sHLA-G positive embryos in our series is almost equal to the 19.9 % recently reported by Rebmann et al.<sup>(19)</sup> but considerably lower than the 36.2 % observed by Noci et al.<sup>(16)</sup> or the 43 % reported by Desai et al.<sup>(18)</sup> in spite of using similar ELISA tests. A possible explanation can be found in the use of distinct sHLA-G preparations to establish the cut-off of positivity. Because no accessible, accepted standard reagent exists, every group has used a different calibrator purified from the supernatant of diverse transfected cells. The poor accuracy of the sHLA-G concentrations reported is obvious in that groups that perform embryo cultures of

the same duration and settings and that use the same ELISA approach, but differ in the calibrator, published either the complete absence of sHLA-G in all the embryo cultures<sup>(21)</sup> or levels ranging from 1.05 to 37.0 ng/ml<sup>(16, 18)</sup>. What is even more important, if embryo culture conditions were similar but a different antibody combination was used for the ELISA, and the calibrator was an HLA-G pattern purified from placental tissues, the mean concentration found rose to 165 ng/ml<sup>(17)</sup>. In view of these results, some authors have reminded that a proportion of the reported levels of sHLA-G exceed by far the maximum expected total protein content of a preimplantation embryo (i.e. 45-50 ng)<sup>(20)</sup>, that would mean that the detection assays are not calibrated to detect real amounts of sHLA-G. In this regard, Sargent et al.<sup>(30)</sup> have proposed that the overestimation in sHLA-G concentrations would result from the significant amounts of contaminating proteins present in some of the calibrators currently in use. The sHLA-G concentrations measured in our study are referred to a standard in units prepared from the C1R HLA-G transfected cell line, using as background culture media or supernatant from untransfected cells. This semi quantitative strategy rules out the necessity of determining total protein content and contamination concerns for routine measurements. The standard in units has been subsequently compared with a plasma sample quantified in ng/ml, which indicates that 1 Unit is equivalent to 1.05 ng (data not shown). We have found levels of sHLA-G ranging from 0.1 to 2.6 U/ml (i.e. 0.1-2.7 ng/ml), comparable to the levels reported by Rebmann et al.<sup>(19)</sup> using Luminex technology (i.e. 0.04-5.6 ng/ml).

An association of sHLA-G levels with morphological quality of the embryos has not been demonstrated so far<sup>(16, 19)</sup>. Here, similar proportions of sHLA-G positive embryos and average sHLA-G concentrations were found in normal and abnormal embryos. This finding suggests that measurement of sHLA-G complements but does not replace morphological selection of good quality embryos. Of note, we detected some of the higher values for sHLA-G in development-arrested embryos. This, the lack of correlation with morphology, and the fact that transcription of embryonic genome does not take place earlier than 70 h after oocyte fertilization<sup>(31, 32)</sup>, points towards an oocitary origin of at least part of the HLA-G protein released by the embryo at the 4-8 cell stage. In this regard, several studies have reported HLA-G protein expression by oocytes and an important presence in the follicular fluid that contributes to their maturation<sup>(12, 33, 34)</sup>. We are tempted to speculate whether different hormonal status and/or hormonal stimulation protocols of the fertility patients could influence HLA-G mRNA and/or protein content of the oocyte and of the preimplantation embryo, contributing to the final reproductive result. In this regard, a recently

identified progesterone response element has been involved in upregulation of HLA-G gene expression in the JEG-3 cell line<sup>(35)</sup>.

Regarding the reproductive outcome of the embryos analyzed in the present study, 28.6% of the embryos successfully implanted when the transfers included at least one embryo positive for sHLA-G, in contrast, the proportion fell to 14% when all the embryos were sHLA-G negative. A possible bias induced by female infertility can be ruled out because the infertility of the couples was caused by male factors. These results are in line with the implantation rates observed in retrospective studies that grouped embryos according to the same criteria (38% and 19% implantation rates, respectively)<sup>(18)</sup>. Interestingly, a prospective cohort study in which patients received either all sHLA-G positive or all negative embryos (2-3 embryos per patient in both groups), showed a higher implantation rate (44%) for the 'positive' group but a comparable rate (14%) for the 'negative' group<sup>(15)</sup>. Additionally, and as otherwise expected from these results, our pregnancy rates (i.e. 60% in the group of patients transferred with at least one positive embryo and 29% with negative embryos) lie in between the pregnancy rates reported after ICSI and morphology plus sHLA-G positive or negative classification: 75-64% for sHLA-G positives compared to 36-23% for sHLA-G negatives<sup>(15, 18)</sup>.

Thus, with all the previous technical considerations, our data supports the idea that whenever a study is able to detect sHLA-G in embryo supernatants, much better success rates are found for sHLA-G positive embryos than for negative ones. Importantly, several embryos whose culture supernatants, in our hands, did not reveal any sHLA-G were able to implant and achieve pregnancies. Various studies except one<sup>(16)</sup> have also reported this finding in their embryo series<sup>(15, 17, 18)</sup>. This would suggest that detectable HLA-G expression or secretion at the 4-8 cell stage is not a prerequisite to embryo implantation, but it might be later on. The presence of sHLA-G at this cleavage stage may be then a useful epiphenomenon indicating active protein synthesis, assembly, export to membrane and secretion, all of them mandatory activities for the conceptus to survive in the female uterus.

In summary, the results presented here indicate that sHLA-G detection in supernatants of embryos conceived after ICSI correlates with successful pregnancy and its objective quantification, along with morphological score, can be of value when selecting suitable embryos for transfer.

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## DISCLOSURES

The authors have no financial conflict of interest.

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