(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 January 2008 (24.01.2008)

PCT

(10) International Publication Number
WO 2008/009705 A1

(51) International Patent Classification:
G01N 33/68 (2006.01)

(21) International Application Number:
PCT/EP2007/057430

(22) International Filing Date: 18 July 2007 (18.07.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/832,094 21 July 2006 (21.07.2006) US

(71) Applicant *(for all designated States except US)*: **MITHRA PHARMACEUTICALS NV/SA** [BE/BE]; Rue sur les Foulons 1, B-4000 Liège (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LÉDÉE, Nathalie** [FR/FR]; c/o Université Versailles St-Quentin en Yvelines, Service de Gynécologie-Obstétrique et Médecine dela Reproduction, CHI Poissy St-Germain en Laye, 10, rue du champs Gaillard, F-78300 Poissy (FR). **PICCINNI, Marie-Pierre** [IT/IT]; c/o University of Florence, Department of Internal Medicine, ImmunoallergologyUnit, Viale Morgagni 85, I-50134 Florence (IT). **LOMBROSO, Raoul** [FR/FR]; c/o Université Versailles St-Quentin en Yvelines, Unité clinique d'assistance médicale à la procréation (AMP), CHI Poissy St-Germain en Laye, 10, rue du champs Gaillard, F-78300 Poissy (FR).

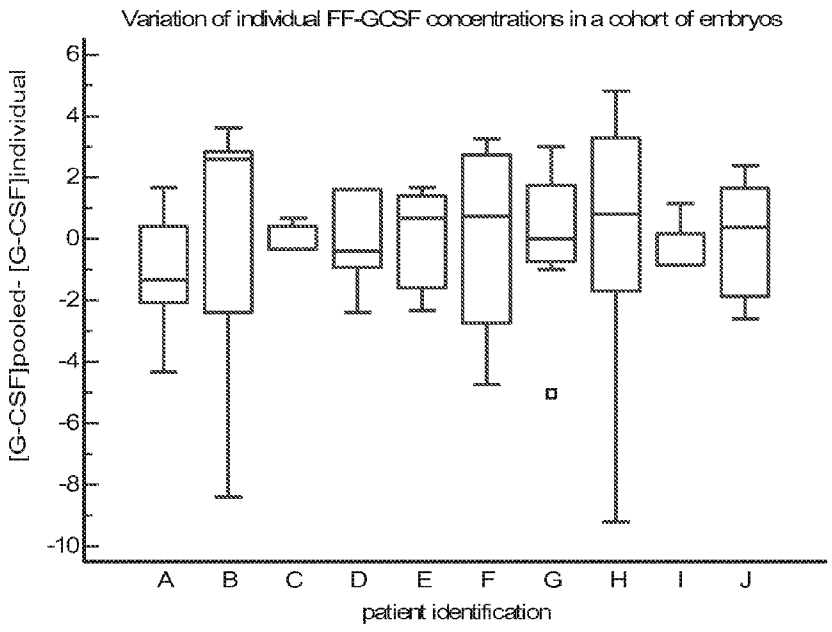
(74) Agents: **BRANTS, Johan Philippe Emile** et al.; De Clercq, Brants & Partners CV, E. Gevaertdreef 10a, B-9830 Sint-Martens-Latem (BE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: ASSAY AND KIT FOR PREDICTING IMPLANTATION SUCCESS IN ASSISTED FERTILISATION



(57) Abstract: The present invention relates to an assay for determining the implantation potential of a plurality of embryos each obtained or to be obtained by assisted fertilisation of an oocyte of a female subject, comprising measuring the levels of granulocyte-colony stimulating factor (G-CSF) in the follicular fluid (FF) present in the follicle from which each oocyte is derived, and determining the implantation potential of each embryo from the level of measured FF G-CSF. It also relates to a kit for carrying out the assay. It further relates to a method for assisted fertilisation.

WO 2008/009705 A1

Declaration under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

ASSAY AND KIT FOR PREDICTING IMPLANTATION SUCCESS IN ASSISTED FERTILISATION

Assisted fertilisation, such as *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) has been used in human patients with infertility problems successfully for three decades. Despite extensive research it is still a difficult and expensive procedure and a low implantation rate per transferred embryos (15 - 20%) is generally observed.

Hospitals and private centers providing an assisted fertilisation service, base their selection after fertilisation of the oocyte on characteristics of the embryo so produced. For example, selection may be based on the morphology of the embryo (Guerif F *et al.*, 2007, *Hum Reprod* **22(7):**1973), or on the production of soluble HLA-G by the embryos (Fuzzi B, *et al.*, 2002, *Eur J Immunol.* Feb;**32(2)**:311-5.). Both these techniques require interference with the embryo.

To increase the pregnancy success, the number of embryos transferred typically more than one. In Europe, it is normal practice to transfer two embryos to the uterine cavity. In the US, it is more, usually three or four embryos are transferred. The adverse effect of such a policy is to increase the number of multiple pregnancies and the subsequent related obstetrical pathologies, such as prematurity and low birth rate mainly.

Furthermore, assisted fertilisation is an expensive procedure and can also be psychologically traumatic for a patient. Surgical procedures are required to collect eggs for assisted fertilisation and following fertilization, further surgery is required to implant fertilised eggs in the womb. The recipient must then wait for a period of time before it can be determined whether or not pregnancy has been established. In some cases, pregnancy may never be established despite repeated attempts and these cases represent a considerable expense to society, both in financial and human terms.

Therefore, it would be desirable to provide an assay and kit which can indicate the potential for implantation of an an oocyte before fertilization, enabling the chances of successful implant of the embryo to be be maximised, and allowing indications of low success rates to be used to avoid the abovementioned trauma and costs of assisted fertilisation.

FIGURE LEGENDS

FIG. 1. ROC curve from a Luminex experiment to detect FF G-CSF using a Biorad Luminex kit. The true positive rate (Sensitivity) is plotted in function of the false positive rate (100-Specificity) for different cut-off points of FF-GCSF concentration. Each point on the ROC plot represents a sensitivity/specificity pair corresponding to a particular decision threshold. The area under the ROC curve is a measure of how well FF-G-CSF can distinguish between two main diagnostic groups (certain implantation / no implantation). Line 1: The Area under curve is 0.82, indicating that a randomly selected individual from the positive group has a test value larger than that for a randomly chosen individual from the negative group in 82 % of the time. Line 2: Area under the ROC curve is 0.5 representing the null hypothesis.

FIG. 2. ROC curve from a Luminex experiment to detect FF G-CSF using an R and D Luminex kit. Line 3: The Area under curve is 0.72, indicating that a randomly selected individual from the positive group has a test value larger than that for a randomly chosen individual from the negative group in 72 % of the time. Line 4: Area under the ROC curve is 0.5 representing the null hypothesis.

FIG. 3. Graph showing the variation in concentration of individual follicular fluid of a same cohort of embryos obtained from multiple subjects. Each box shows the variation of individual follicular fluids from the mean in a same cohort of embryos generated.

SUMMARY OF THE INVENTION

The invention is based on an unexpected finding by the inventors that a female subject providing a plurality of oocytes under ovarian hyperstimulation will exhibit a variation in the levels of several cytokines and growth factors present in the follicular fluid of the follicle from which each oocyte is derived. Furthermore, the inventors found there is a strong correlation between a high level of granulocyte-colony stimulating factor (G-CSF) present in the follicular fluid of the individual follicle from which an oocyte is derived and a high implantation potential of an embryo obtained by fertilisation of said oocyte. It has never been demonstrated before that, for the same subject, the follicular fluid surrounding each individual oocyte may vary in composition, and that said composition is indicative of the success of implantation of the subsequently fertilised oocyte. This finding allows a plurality of embryos obtained from a single patient to be ranked in order of implantation potential. For the first time, patients showing a borderline fertility potential using indicators that average oocyte fertility markers (*e.g.* 11-beta HSD) may be found to have oocytes showing a high implantation potential against a poor overall average; this offer new possibilities for previously infertile-indicating females. Furthermore, the method offers the

possibility to rate each oocyte individually and thus embryo individually, without interference to the embryo or oocyte.

The present invention relates an assay for determining the implantation potential of a plurality of embryos each obtained or to be obtained by assisted fertilisation of an oocyte of a female subject, comprising measuring the levels of G-CSF in the follicular fluid present in the follicle from which each oocyte is derived, and determining the implantation potential of each embryo from the level of follicular fluid G-CSF. The oocyte from the follicle with the highest level of G-CSF in follicular fluid gives rise to an embryo with the greatest implantation potential.

The present invention to an assay kit which can be used to predict the outcome of assisted fertilisation in a female patient. The invention also relates to such assay and kit for use in a method of fertilisation treatment, to improve implantation.

One embodiment of the invention is an assay for determining for a female subject the implantation potential of embryos obtained or to be obtained by assisted fertilisation comprising:
(i) measuring, for a plurality of ooctyes collected from said subject, the level of follicular fluid granulocyte-colony stimulating factor (G-CSF) present in the follicular fluid (FF) of a follicle of each collected oocyte; and
(ii) determining from the levels of FF G-CSF measured, the implantation potentials of the embryos obtained or to be obtained by assisted fertilisation of the oocytes.

Another embodiment of the invention is an assay as described above, wherein oocytes having the highest levels of FF G-CSF have the highest potential of implantation.

Another embodiment of the invention is an assay as described above, wherein each sample of FF is obtained from a follicular aspirate.

Another embodiment of the invention is an assay as described above, wherein respective levels of FF G-CSF are measured within 20 hours of collection of the follicular aspirate.

Another embodiment of the invention is an assay as described above, wherein a level of FF-CSF equal to or less than 20.6 pg/ml determines no or a low potential of implantation.

Another embodiment of the invention is an assay as described above, wherein a level of FF-CSF equal to or greater than 24.0 pg/ml determines a high potential of implantation.

Another embodiment of the invention is an assay as described above, wherein the respective levels of FF G-CSF are measured using an immunoassay.

Another embodiment of the invention is an assay as described above, wherein the respective levels of FF G-CSF are measured using an a competitive or immunometric assay, such as RIA, IRMA, ELISA, or ELISPOT assay.

Another embodiment of the invention is an assay as described above, wherein the respective levels of FF G-CSF are measured using a Luminex assay.

Another embodiment of the invention is an assay as described above, wherein the Luminex assay employs a Biorad or R and D Luminex Kit.

Another embodiment of the invention is an assay as described above, wherein the respective levels of FF G-CSF are measured by determining the levels of FF G-CSF mRNA.

Another embodiment of the invention is an assay as described above, wherein the respective levels of FF G-CSF are measured by any of surface plasmon resonance, fluorescence resonance energy transfer, bioluminescence resonance energy transfer, fluorescence quenching fluorescence, fluorescence polarization, MS, HPLC, HPLC/SM, HPLC/MS/MS, capillary electrophoresis, rod or slab gel electrophoresis.

Another embodiment of the invention is a kit for use in performing the assay as described above, comprising at least one reagent suitable for detection of levels of FF G-CSF or FF G-CSF mRNA.

Another embodiment of the invention is a kit as described above, further comprising a set of concentration standards of FF G-CSF.

Another embodiment of the invention is a kit as described above, further comprising a plurality of aspirator tips for removing an oocyte and follicular fluid from a subject.

Another embodiment of the invention is a method for assisted fertilisation of a female subject comprising:
(i) collecting a plurality of oocytes from said subject,
(ii) determining the implantation potential for an embryo derived from each oocyte according to the assay as described above,
(iii) fertilising the oocytes corresponding to embryos having a high potential for implantation, and
(iv) implanting the embryo so obtained into the female subject.

Another embodiment of the invention is a method for assisted fertilisation of a female subject comprising:
(i) collecting a plurality of oocytes from said subject,
(ii) determining the implantation potential for an embryo derived from each oocyte according to the assay as described above,
(iii) fertilising the oocytes to obtain embryos, and
(iv) implanting the embryos having a high implantation potential.

DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art. All publications referenced herein are incorporated by reference thereto. All United States patents and patent applications referenced herein are incorporated by reference herein in their entirety including the drawings.

The articles "a" and "an" are used herein to refer to one or to more than one, *i.e.* to at least one of the grammatical object of the article. By way of example, "a sample" means one sample or more than one sample.

The recitation of numerical ranges by endpoints includes all integer numbers and, where appropriate, fractions subsumed within that range (*e.g.* 1 to 5 can include 1, 2, 3, 4 when referring to, for example, a number of samples, and can also include 1.5, 2, 2.75 and 3.80, when referring to, for example, concentrations). The recitation of end points also includes the end point values themselves (*e.g.* from 1.0 to 5.0 includes both 1.0 and 5.0)

As mentioned elsewhere present invention relates to an unexpected finding by the inventors that a female subject providing a plurality of oocytes under ovarian hyperstimulation will exhibit a variation in the levels of several cytokines and growth factors present in the follicular fluid of the follicle from which each oocyte is derived. Furthermore, the inventors found there is a strong correlation between a high level of granulocyte-colony stimulating factor (G-CSF) present in the follicular fluid of the individual follicle from which an oocyte is derived and a high implantation potential of an embryo obtained by fertilisation of said oocyte. It has never been demonstrated before that, for the same subject, the follicular fluid surrounding each individual oocyte may vary in composition, and that said composition is indicative of the success of implantation of the subsequently fertilised oocyte. This finding allows a plurality of embryos obtained from a single patient to be ranked in order of implantation potential. For the first time, patients showing a borderline fertility potential using indicators that average oocyte fertility markers (*e.g.* 11-beta HSD) may be found to have oocytes showing a high implantation potential against a poor overall average; this offer new possibilities for previously infertile-indicating females. Furthermore, the method offers the possibility to rate each oocyte individually and thus embryo individually, without interference to the embryo or oocyte.

The present invention thus relates to an assay method and assay kit which can be used to predict the outcome of assisted fertilisation in a female patient. The invention also relates to such assay and kit for use in a method of fertilisation treatment, to improve implantation. Although our invention described below has been developed from research on human female patients, it will be applicable to any mammalian female and can be used to increase the success of, for example, captive breeding programs of endangered species or commercial breeding by assisted fertilisation of livestock such as cattle or horses. Preferably the subject has undergone fertility pretreatment (*e.g.* ovarian hyperstimulation) to increase the number of eggs produced per monthly cycle. Assisted fertilisation, as used herein, refers to *ex vivo* fertilisation methods where the oocyte is fertilised outside the female body, such as *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI).

One embodiment of the invention is an assay for determining the implantation potential of a plurality of embryos each obtained or to be obtained by assisted fertilisation of an oocyte of a female subject, comprising measuring the levels of G-CSF in the follicular fluid present in the follicle from which each oocyte is derived, and determining the implantation potential of each embryo from the level of follicular fluid G-CSF.

Another embodiment of the present invention is an assay for determining for a female subject the implantation potential of embryos obtained or to be obtained by assisted fertilisation comprising:

(i) measuring, for a plurality of oocytes collected from said subject, the level of follicular fluid granulocyte-colony stimulating factor (G-CSF) present in the follicular fluid (FF) of a follicle of each collected oocyte; and

(ii) determining from the levels of FF G-CSF measured, the implantation potentials of the embryos obtained or to be obtained by assisted fertilisation of the oocytes.

The oocyte from the follicle with the highest level of G-CSF in follicular fluid gives rise to an embryo with the greatest implantation potential.

Granulocyte colony stimulating factor (G-CSF) is a naturally generated cytokine belonging to the family of hemopoietic growth factor (Clark, *et al.*, 1987, Science **236**(4806):1229). Its main role described is to act on proliferation, differentiation, and activation of hematopoietic cells of the neutrophilic lineage (Mielcarek *et al.*, 1996.,Blood **87(2)**:574, Visani *et al.*, 1995, **18(5-6)**:423). Primarily produced by hemopoietic cells, G-CSF is also produce by non-hemopoietic cells, such as in the reproduction tract: the human luteinized follicular granulosa cells (Salmassi A, *et al*, 2004, *Fertil Steril,* **81 Suppl 1**:786.), endometrial cells (Giacomini G, *et al.*, 1995, Hum Reprod **10(12):**3259.), decidua and placenta (Duan J.S., 1990, *Osaka City Med J* **36(2)**:81; Miyama M *et al.*, 1998, *Osaka City Med J.,* **44(1):**85) and various fetal tissue (Calhoun *et al.*, 1999. *Pediatr Res* **46(3)**:333). In the ovary, G-CSF protein and its receptor were located (western blot and immunohistochemistry) mainly in granulosa cells of the follicle and luteal cells (Salmassi, *et al.,* 2004).

The level of follicular fluid G-CSF (FF G-CSF) is preferably measured within the day of oocyte collection. As is known to the skilled person, follicular aspiration is guided using transvaginal sonography after local or general anaesthesia. Each follicular fluid corresponding to one ovarian follicle visualised through vaginal sonography is aspirated individually. The capture of each oocyte does not require any other manipulation because the follicular fluid, which surrounds the oocyte, is aspirated along with the oocyte. Inspection of the follicular fluid under microscope allows immediate identification of the presence of the oocyte. Instead of pooling the follicular fluids and respective oocytes, the oocyte is separated at the time of collection so the levels of FF G-CSF can be individually

measured. According to one aspect of the invention, the level of FF G-CSF is measured with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 hours of oocyte collection, of within a time between any two of the aforementioned values. Preferably the level of FF G-CSF is measured within 1 to 20 hours of oocyte collection.

The level of FF G-CSF associated with an oocyte can be measured using any suitable quantitative assay. The measuring may be performed, for example, using a method selected from biochemical assay (*e.g.,* solid or liquid phase immunoassay), surface plasmon resonance, fluorescence resonance energy transfer, fluorescence quenching, and fluorescence polarisation. Such techniques are well known in the art and are briefly described herein below.

Biochemical assays generally rely on the immobilisation of an analyte component, for example, to a membrane or other solid support, and exposure to a ligand. After washing away excess ligand, bound ligand is detected by immunoassay, or by using labelled ligand (*e.g.,* radio-labelled ligand, fluorescently labelled ligand, particulate labelled ligand etc.). Methods to determine and obtain ligands which bind with high affinity to a specific analyte in are also available in the art; see for example WO89/09088 entitled "Paralog Affinity Chromatography". In an example of an immunoassay, antibodies against G-CSF may be immobilised onto magnetic beads and exposed to a sample of follicular fluid. Bound G-CSF can be detected using primary and secondary antibody immunoassays to arrive at a concentration. Typically, an immunoassay is calibrated used a set of standards. Solid phase immunoassays are described for example in US 4,376,110. Variations of the immunoassays within the scope of the invention include any competitive or immunometric assay format using anti-G-CSF antibodies, for instance RIA (radio-immunoassay), ELISA (enzyme-linked immunosorbent assay), ELISPOT (enzyme-linked immunosorbent spot) or Luminex (bead-based multiplex sandwich immunoassay).

The levels FF G-CSF are preferably measured by using Luminex technology. Luminex is a highly sensitive method for measuring simultaneously the levels of specific components in a system. It makes use of solid phase, colour (dye) coded microspheres that are small enough to behave almost as a solution in a liquid. Each microsphere is coated with an antibody, or other ligand-binding reagent specific for the detected components (*e.g.* FF C-GSF). The components of the sample are captured and detected on the microspheres. Within an analyzer, lasers excite the internal dyes that identify each microsphere particle, and also any reporter dye captured during the assay. Many readings are made on each

bead set, to validate the results. In this way, a sensitive multiplex assay is made that is both rapid and precise. Preferably, the levels of FF C-GSF are measured using a kit(s) manufactured by Biorad ® or R and D ®. In a preferred embodiment the Biorad® kit is the Human Cytokine Fluorescent Bead Immunoassay Assay Kit, Bio-Plex™ (Hercules, CA, USA,17A11127). In another preferred embodiment, the R and D kit is the LUH000, LUH279, LUH270, LUH271, LUH278, LUH208, LUH214, LUH215B, LUH285, LUH200, LUH280, LUH201, LUH202, LUH204, LUH205, LUH206, LUH217, LUH317, LUH210, LUH293, LUB000, LUB320, LUB294, LUB219, and/or LUB213 kit.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes monoclonal antibodies, polyclonal antibodies, and fragments of whole antibodies which retain their binding activity for a target antigen. Such fragments include Fv, F(ab') and F(ab')2 fragments, as well as single chain antibodies. Furthermore, the antibodies and fragments thereof may be humanised antibodies, *e.g.* as described in EP-A-239400 (Winter).

Antibodies against FF G-CSF may be monoclonal or polyclonal antibodies. Monoclonal antibodies may be prepared by conventional hybridoma technology using the proteins or peptide fragments thereof, as an immunogen. Polyclonal antibodies may also be prepared by conventional means which comprise inoculating a host animal, for example a rat or a rabbit, with a peptide of the invention and recovering immune serum.

Alternatively, levels of FF G-CSF may be estimated by analysing the levels of FF G-CSF mRNA in the granulosa cells. Granulosa cells around the corona radiata may be stored at the stage of the decoronisation of each oocytes and be stored in RNA stabiliser (*e.g.* at 80°C) until assay . Probes for the FF G-CSF gene may be designed for use as probes, for example for use in a nucleic acid (PCR) amplification assay and/or hybridisation. Methods and conditions for performing a PCR and hybridisation reactions are known in the art, and can be found, for example, in Molecular Cloning: A Laboratory Manual (Third Edition) (Joseph Sambrook, Peter MacCallum, David Russell, Cold Spring Habor Laboratory Press) or could be performed by a quantigene plex assay, which is designed to quantitate multiple target-specific RNA molecules (Panomics).

A surface plasmon resonance assay may, alternatively, be used as a quantitative method to measure the level of G-CSF in a follicular fluid sample. A chip-bound anti-G-CSF antibody is challenged with a follicular fluid and the surface plasmon resonance

measured. Binding reactions are performed using standard concentrations to arrive at the levels of G-CSF of G-CSF in the follicular fluid.

FRET (fluorescence resonance energy transfer) may also be used to measure the level of G-CSF in a follicular fluid sample. The G-CSF and anti-G-CSF antibody are labelled with a complementary pair of donor and acceptor fluorophores. While bound closely together by the G-CSF : anti-G-CSF antibody interaction, the fluorescence emitted upon excitation of the donor fluorophore will have a different wavelength than that emitted in response to that excitation wavelength when the G-CSF and anti-G-CSF antibody are not bound, providing for quantitation of bound versus unbound molecules by measurement of emission intensity at each wavelength. Binding reactions can be compared with a set of standards to arrive at the level of G-CSF in the follicular fluid.

BRET (bioluminescence resonance energy transfer) may also be used to measure the level of G-CSF in a follicular fluid sample. Light is emitted by an acceptor when in close proximity to the donor, *i.e.*, when a G-CSF : anti-G-CSF antibody interaction complex is formed. By comparing the interaction with a set of standards, the level of G-CSF in the follicular fluid is determined.

Fluorescence quenching fluorescence similarly provides a measurement of G-CSF levels. Generally, a decrease in fluorescence of the labelled anti-G-CSF antibody is indicative that the G-CSF bearing the quencher has bound. Of course, a similar effect would arise when a G-CSF is fluorescently labelled and anti-G-CSF antibody bears the quencher. By comparing the interaction with a set of standards, the level of G-CSF in a follicular fluid sample can be measured.

Fluorescence polarisation measurement can also determine the level of G-CSF in a follicular fluid sample. Complexes, such as those formed by G-CSF associating with a fluorescently anti-G-CSF antibody, would have higher polarisation values than uncomplexed, labelled anti-G-CSF antibody. This form the basis for determining the levels of G-CSF in a follicular fluid sample, which measurements are typically performed concurrent with a set of standard G-CSF concentrations.

Others methods than can be used for quantitatively assaying G-CSF in the FF include mass spectrometry (MS), high performance liquid chromatography (HPLC) , HPLC/MS, HPLC/MS/MS, capillary electrophoresis and rod or slab gel electrophoresis associated

with image analysis. Such techniques are well known in the art as described, for example, in *Modern HPLC for Practicing Scientists* (Dong, M, Wiley-Interscience, June 2006), *Tandem Mass Spectrometry* (McLafferty F.W. John Wiley & Sons Inc, November, 1983), *Mass Spectrometry for Biotechnology* (Siuzdak, G., Academic Press, February 1996), *Clinical Applications of Capillary Electrophoresis* (Methods in Molecular Medicine) (Palfrey S. M., Humana Press, June 1999), *Handbook of Capillary Electrophoresis, Second Edition*, (Landers J.P. CRC; December 1996), *High-Resolution Electrophoresis and Immunofixation: Techniques and Interpretation* (Keren, D. F. Hodder Arnold, January, 1994).

Once the levels of FF G-CSF have been measured in a plurality of oocytes from a single patient, the results may be used to establish the relative implantation potential of embryos obtained by fertilization of said oocytes *i.e.* a ranking order. The level of FF G-CSF may be used to determine whether all, some or none of the oocytes will after fertilisation establish implantation in a female subject undergoing assisted fertilisation treatment. In addition, the level of FF G-CSF may be used to determine whether all, some or none of the embryos will implant in a female subject undergoing assisted fertilisation treatment.

In our studies, we have measured levels of FF G-CSF using immunoassays, in particular using Luminex technology from Biorad and R&D. We have found that those embryos derived from oocytes having a concentration of FF-CSF equal to or less than 20.0 pg/ml show a reduced or no implantation success. In contrast, embryos derived from oocytes having a concentration of FF-CSF above 24 pg/ml show a certain implantation.

Those of skill in the art will appreciate that although in our research we have determined a "threshold" level of FF G-CSF below which embryos are not implanted (and above which patients have significantly improved probability of implantation), the value is a statistical measure and other measurements and thresholds can be used. In practicing the invention, it is most important to achieve consistency of assay, and so each individual practitioner (or assisted fertilisation team) will be capable of establishing their own particular assay method and determining their own threshold level. This could be established by first conducting a historical study on samples from previous patients.

Thus, the level of FF G-CSF mentioned above represents the measure we have used in our studies as a suitable limit. However, if levels of FF G-CSF were to be measured in any of the other ways mentioned above, it would be desirable to conduct, using routine

procedures, a control using our method of assay in order to determine the relationship between our results and the results of other methods, in order to make direct comparisons.

According to one aspect of the invention, an embryo derived from an oocyte where a level of FF G-CSF in its follicle is equal to or less than 21.6 pg/ml, 21.4 pg/ml, 21.2 pg/ml, 21.0 pg/ml, 20.8 pg/ml, 20.6 pg/ml, 20.4 pg/ml, 20.2 pg/ml, 20.0 pg/ml, 19.8 pg/ml, 19.6 pg/ml, 19.4 pg/ml, 19.2 pg/ml, 19.0 pg/ml, 18.8 pg/ml, 18.6 pg/ml, 18.4 pg/ml, 18.2 pg/ml, 18.0 pg/ml 17.8 pg/ml, 17.6 pg/ml, 17.4 pg/ml, 17.2 pg/ml, 17.0 pg/ml, 16.8 pg/ml, 16.6 pg/ml, 16.4 pg/ml, 16.2 pg/ml, 16.0 pg/ml, 15.8 pg/ml, 15.6 pg/ml, 15.4 pg/ml, 15.2 pg/ml, 15.0 pg/ml or a level between any two of the aforementioned values, is predicted to have a low implantation potential. Preferably, a level of FF-CSF equal to or less than 15.0 pg/ml to 20.0 pg/ml, more preferably equal to or less than 19.8 to 20.6 pg/ml, most preferably less than 20.6 pg/ml is predicted to have no or a low implantation potential. The levels of this embodiment are considered threshold levels for a method of assisted fertilization (below). A low level of implantation is a probability of implantation of 10 %, 9%, 8% or less.

According to one aspect of the invention, an embryo derived from an oocyte where a level of FF G-CSF in its follicle is equal to or less than 34.0 pg/ml, 33.5 pg/ml, 33.0 pg/ml, 32.5 pg/ml, 32.0 pg/ml, 31.5 pg/ml, 31.0 pg/ml, 30.5 pg/ml, 30.0 pg/ml, 29.5 pg/ml, 29.0 pg/ml, 28.5 pg/ml, 28.0 pg/ml, 27.5 pg/ml, 27.0 pg/ml, 26.5 pg/ml, 26.0 pg/ml, 25.5 pg/ml, 25.0 pg/ml, 24.5 pg/ml, 24.0 pg/ml, 23.5 pg/ml, 23.0 pg/ml, 22.5 pg/ml, 22.0 pg/ml, 21.5 pg/ml, 21.0 pg/ml, 20.5 pg/ml, 20.0 pg/ml, 19.5 pg/ml, 19.0 pg/ml, 18.5 pg/ml, 18.0 pg/ml, 17.5 pg/ml, 17.0 pg/ml, 16.5 pg/ml, 16.0 pg/ml, 15.5 pg/ml, 15.0 pg/ml or a level between any two of the aforementioned values, is predicted to have a likely implantation success. Preferably, a level of FF-CSF in the range 15.0 pg/ml to 34.0 pg/ml, more preferably in the range 20.0 to 24.0 pg/ml predicted to be likely to be implanted. Likely to be implanted means a higher chance of success that no certainty of implantation; a likely potential of implantation means a probability of implantation of 15 % to 25 %. The levels of this embodiment are considered threshold levels for a method of assisted fertilization (below).

According to one aspect of the invention, an embryo derived from an oocyte where a level of FF G-CSF in its follicle is equal to or higher than 22.0 pg/ml, 22.1 pg/ml, 22.2 pg/ml, 22.3 pg/ml, 22.4 pg/ml, 22.5 pg/ml, 22.6 pg/ml, 22.7 pg/ml, 22.8 pg/ml, 22.9 pg/ml, 23.0 pg/ml, 23.1 pg/ml, 23.2 pg/ml, 23.3 pg/ml, 23.4 pg/ml, 23.5 pg/ml, 23.6 pg/ml, 23.7 pg/ml, 23.8 pg/ml, 23.9 pg/ml, 24.0 pg/ml, 24.1 pg/ml, 24.2 pg/ml, 24.3 pg/ml, 24.4 pg/ml, 24.5

pg/ml, 24.6 pg/ml, 24.7 pg/ml, 24.8 pg/ml, 24.9 pg/ml, 25.0 pg/ml, 25.1 pg/ml, 25.2 pg/ml, 25.3 pg/ml, 25.4 pg/ml, 25.5 pg/ml, 25.6 pg/ml, 25.7 pg/ml, 25.8 pg/ml, 25.9 pg/ml, 26.0 pg/ml, 26.1 pg/ml, 26.2 pg/ml, 26.3 pg/ml or a level between any two of the aforementioned values, is predicted to have a high implantation potential. Preferably, a level of FF-CSF equal to or higher than 24.0 pg/ml, more preferably higher than 35 pg/ml is predicted to have a high implantation potential. The levels of this embodiment are considered threshold levels for a method of assisted fertilization (below). A high level of implantation is a probability of implantation of 30 %, 35%, 40%, 43%, 44% or more.

If levels of FF G-CSF in such patients is significantly below the level associated with likely or certain implantation in all collected oocytes, then there would be a saving in time, money and stress to the patient not to undertake implantation. In such cases, it will be possible for the practitioner (or assisted fertilisation clinic) to decide whether or not to even attempt a first implantation. On the other hand, if one or more oocytes indicate a high or complete certainty of implantation, these oocytes alone may be fertilized and the embryos so obtain implanted, so saving money and resources by fertilizing only those oocytes likely to become established as embryos. Alternatively, all the oocytes may be fertilized, and only those embryos derived from oocytes indicating a high or complete certainty of implantation, are implanted; this allows a higher chance of success as the indication of implantation does not necessarily correlated with chances of fertilisation.

The present invention significantly increase the implantation rate while decreasing the number of embryos replaced. It also allows a specialist to become more efficient in preventing multiple pregnancies and all the related fetal and maternal morbidity. The oocyte and thus the embryo with the highest potential can be implanted implant, therefore, allows a policy of single embryo transfer while not decreasing the overall pregnancy rate.

The assay described herein may also be employed in a method of assisting the fertilisation of a female subject. One embodiment of the invention is a method for assisted fertilisation of a female subject comprising:

(i) collecting a plurality of oocytes from said subject,

(ii) determining the level of FF G-CSF in the follicle of each collected oocyte,

(iii) fertilising the oocytes having the highest FF G-CSF levels, and

(iv) implanting the embryo so obtained into the female subject.

The number of oocyes subjected to further fertilization may be 1, 2, 3, 4 or 5 or more. Alternatively, 50 %, 40 %, 30 %, 20 %, or 10 % of the oocytes are fertilized, which percentage have the highest levels of FF G-CSF.

Another embodiment of the invention is a method for assisted fertilisation of a female subject comprising:
(i) collecting a plurality of oocytes from said subject,
(ii) determining the level of FF G-CSF in the follicle of each collected oocyte,
(iii) fertilising the oocytes to obtain embryos,
(iv) implanting the embryos derived from oocytes having the highest FF G-CSF levels.
The number of embryos implanted may be 1, 2, 3, 4 or 5 or more. Alternatively, 50 %, 40 %, 30 %, 20 %, or 10 % of the embryos are implanted, which percentage have the highest levels of FF G-CSF.

Another embodiment of the invention is a method for assisted fertilisation of a female subject comprising:
(i) collecting a plurality of oocytes from said subject,
(ii) determining the level of FF G-CSF in the follicle of each collected oocyte,
(iii) fertilising the oocytes to obtain embryos,
(iv) implanting the embryos derived from oocytes having a FF G-CSF level above a predetermined threshold.
The number of embryos implanted may be 1, 2, 3, 4 or 5 or more. Alternatively, 50 %, 40 %, 30 %, 20 %, or 10 % of the embryos are implanted, which percentage have the highest levels of FF G-CSF.

Another embodiment of the invention is a method for assisted fertilisation of a female subject comprising:
(i) collecting a plurality of oocytes from said subject,
(ii) determining the implantation potential for an embryo derived from each oocyte according to the assay as defined above,
(iii) fertilising the oocytes corresponding to embryos having a high potential for implantation, and
(iv) implanting the embryo so obtained into the female subject.

Another embodiment of the invention is a method for assisted fertilisation of a female subject comprising:

(i) collecting a plurality of oocytes from said subject,
(ii) determining the implantation potential for an embryo derived from each oocyte according to the assay as defined above,
(iii) fertilising the oocytes to obtain embryos, and
(iv) implanting the embryos having the highest implantation potential.

The embodiments described above in regard of the assay apply to corresponding embodiments of the method of assisted fertilisation. The threshold values are indicated elsewhere herein. The skilled person will understand that intervening steps may be present such as freezing after oocyte collection. By use of the present invention, it will be possible for assisted fertilisation clinics to allocate resources more efficiently, so that patients with low levels of FF G-CSF in the follicle of a recovered oocyte who are unlikely to become pregnant by assisted fertilisation treatment are not treated.

Kits for use in performing the assay of the invention may be provided. Such kits include at least one reagent useful for the detection of FF G-CSF. Suitable reagents include antibodies, or other suitable ligand-binding reagents, against FF G-CSF optionally linked to a label. Typical labels are those commonly used in immunoassay procedures, for example horse radish peroxidase. The kit may also contain standards, for examples predetermined amounts of FF G-CSF (*e.g.* protein or RNA) may be labelled with a detectable label. The kit may also contain disposable aspirator tips for use in extracting the oocytes and follicular fluid.

The kit may be used for the measurement of FF G-CSF for use in a method of diagnosis, prognosis, and/or assisted fertilisation treatment of a female subject. The invention further provides the use of a reagent for the detection of FF G-CSF for the prognosis of the likelihood of establishing pregnancy by assisted fertilisation in a female subject.

The abovementioned antibodies, fragments and variants thereof, and other suitable ligand-binding reagents, which may optionally be labelled with a detectable label, may be used for the manufacture of a diagnostic kit for use in the treatment or diagnosis of suitability for assisted fertilisation.

Levels of FF G-CSF may also be assayed *via* analysis of the levels of FF G-CSF mRNA present in samples obtained. In order to achieve this, FF G-CSF or fragments thereof may be used as a probe to determine levels of G-CSF in the follicular fluid. Alternatively, levels

of FF G-CSF may be estimated by analysing the levels of FF G-CSF mRNA expressed in the follicular fluid or in the granulosa cells. Granulosa cells around the corona radiate may be stored at the stage of the decoronisation of each oocytes and stored in RNA stabiliser (*e.g.* at 80°C) until assay. Such probes may also be formulated into kits in a manner analogous to those described for antibodies, and may contain control nucleic acids. Probes for the FF G-CSF gene may be designed for use as probes, for example for use in a nucleic acid amplification assay.

EXAMPLES

The following non-limiting examples illustrate certain embodiments of the invention.

Example 1: Experimental design

Patients

280 female patients presenting with infertility and included in an ICSI program were recruited between January 2005 to March 2007. The reason for inclusion in ICSI was predominantly male infertility but also previous IVF failure or previous low fertilization rate in conventional IVF. We proceeded to a randomization at the time of inclusion to not introduce biases in the clinical patient selection. Each patient was included one time within the study period. All patients were fully informed, and the Institutional Review Board approved this investigation (Comité Consultatif de Protection des personnes Poissy- St germain en Laye,).

Pre-Treatment

Patients underwent the classical procedure of ovarian hyperstimulation. We applied the protocol referred by their physician. Response to stimulation was controlled by serial blood tests and ultrasonic evaluation to control follicles and endometrial growth. Criteria for triggering the ovulation was obtained when at least 5 follicles reached 16 mm.

The oocytes retrieval took place 35 to 36 hours after the triggering of ovulation. The oocyte aspiration was performed under general or local anaesthesia with vaginal ultrasonography using individual 10ml syringe for each follicle in the studied group. We thus adaptated the classical method of oocyte aspiration in order to individualise the follicular fluid of each oocyte collected.

Follicular fluid samples

The presence or absence of an oocyte in each follicle was immediately assessed and the follicles devoid of an oocyte were discarded. In the studied group, individual follicular fluid

sample, each corresponding to one mature ovocyte was collected. The volume and the aspect (citrin, orange or hematic) of each follicular fluid sample was recorded. Individual follicular fluid samples were centrifuged and the supernatant aliquoted after proceeding to the anonymisation of each sample according to a database in order to blind subsequent analysis. Samples were initially stored at -20°C, then at -80°C until assay. All the clinical and biological information were recorded in real-time on the database (Medifirst).

Oocyte fertilization and embryo culture until day 2

Oocytes were collected and cumuls and corona cells removed with hyluronidase 80 IU (Fertipro). Oocyte were injected in a 5µl drop of flushing medium (JCD), with a sperm sample slowed by PVP medium (fertopto). Injected oocytes were cultured in singly 40µl microdroplet of ISM1 (Medicult, france) under oil at 37°C. Pronuclei number and aspect were assesses after 20 hours according to Gianaroli criteria. On day 2, the number, fragmentation and regularity of each blastomere were recorded. Embryo transfer was scheduled on day 2.

We divided the transferred embryos in two categories for analysis:

1. the best quality embryos defined by 4-5 cells on Day 2, 8-9 on Day 3, and less than 10% of fragmentation and regular cells (high quality embryos),

or

2. any other patterns (low quality embryos)

Only, the follicular fluids corresponding to the embryos transferred were analysed using the Luminex method.

Evaluation of the potential of implantation

Each sample was related to a probability of implantation, described here as the implantation rate. The clinical Implantation rate of an embryo is defined for each tested sample as the number of yolk sac / number of embryos transferred. Clinical implantation was defined at 8 weeks of amenorrhea by the ultrasonic visualization of a yolk sac.

There is therefore three main categories in function of the outcome:

- No implantation: Implantation Rate=0
- Certain implantation: The number of embryos replaced was equal to the number of yolk sac observed by ultrasound at 8 weeks of amenorrhea (1 embryo replaced and single pregnancy, two embryos replaced and twin pregnancy): Implantation Rate =1

- Likely implantation which is a probability of implantation since the number of yolk sac is lower than the number of embryos replaced (*e.g* 1 out of 2, 1 out of 3, 2 out of 3, hence Implantation Rate = 0.5, 0.33, 0.66).

To construct the ROC curve (Receiver Operating Charateristics), we only took in account the two categories

- No implantation,
- Certain implantation: The number of embryos replaced was equal to the number of yolk sac observed by ultrasound at 8 weeks of amenorrhea (1 embryo replaced and single pregnancy, two embryos replaced and twin pregnancy)

The discrimination attained between no implantation and certain implantation in function of the concentration of G-CSF in each sample was evaluated with ROC curve analysis (MedCalc Software, Mariakerke, Belgium). Sensitivity, specificity and the area under curve (AUC) ROC were obtained for the three methods of detection. In a ROC curve the true positive rate (Sensitivity) is plotted in function of the false positive rate (100-Specificity) for different cut-off points. Each point on the ROC plot represents a sensitivity/specificity pair corresponding to a particular decision threshold. A test with perfect discrimination (no overlap in the two distributions) has a ROC plot that passes through the upper left corner (100% sensitivity, 100% specificity). Therefore, the closer the ROC plot is to the upper left corner, the higher the overall accuracy of the test (Zweig & Campbell, 1993).
Calculation of the AUC-ROC provides the quantitative of accuracy, *i.e.* the ability of G-CSF to discriminate between implantation and no implantation.

However, most of the embryos that implant were defined by a probability of implantation. For example, if two embryos were transferred and only one implanted, each sample is characterized by a probability of 50% of implantation.

We thus defined for each method

- Lower threshold of implantation defined by a negative predictive value of 100% from the AUC-ROC curve.
- Higher threshold of implantation defined by the highest positive predictive value for implantation.

G-CSF Assays performed on the FF samples

Two Luminex methods of detection were successively applied for individual follicular fluid samples from which transferred embryos were issue.

From January, 2005 to June 2005

- The Luminex technology was applied with the kit Biorad (Hercules, Ca, USA,17A11127, human cytokines, 27-plex kit).

From September 2005 to March 2007

80 of the samples were common to the previous Luminex biorad, and 120 were new samples collected

- The Luminex technology was applied with the R and D kit (Minneapolis, MN, USA ,LUH000, LUH279, LUH270, LUH271, LUH278, LUH208, LUH214, LUH215B, LUH285, LUH200, LUH280, LUH201, LUH202, LUH204, LUH205, LUH206, LUH217, LUH317, LUH210, LUH293, LUB000, LUB320, LUB294, LUB219, LUB213)

Multivariate and univariate analysis were performed. A p value below 0.05 was considered as significant. Table 1 summarizes the population and the number of samples analysed successively using the two of the methods of investigation applied.

Parameter measured	***Luminex BIORAD***	***Luminex R&D***
Number of patients included	71	121
Number of individual follicular fluids analysed corresponding to an transferred embryo	132	200
Mean Clinical Pregnancy Rate	31.5%	27.3%
Mean implantation rate	20%	18%

TABLE 1: Population and the number of samples analysed successively using two of the methods of investigation applied.

Evaluations using the Biorad® and R and D® Luminex kits are elaborated in Examples 2 and 3.

Example 2: Evaluation using the Luminex Kit manufactured by Biorad®

132 follicular fluid samples corresponding to the subsequent 132 transferred embryos were analysed for levels of certain cytokines and chemokines. In particular, concentrations of IL-1beta, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IFN alpha, TNF-alpha, G-CSF, GM-CSF, VEGF, PDGF, FGF, IP-10, MCP-1, RANTES,

EOTAXIN, MIP-1 alpha, MIP-1 beta were evaluated using Luminex technology, utilising a a Biorad ® Luminex ® Kit.

The following results were obtained:

1) LIF, IL-1ra, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, G-CSF, VEGF, IP-10, MCP-1, Eotaxin and MIP-beta were detected in all the follicular fluid samples,

2) IL-1 beta, IL-5, IL-7, IL-17, TNF alpha, MIP-alpha were detected not detected in any follicular fluid sample,

3) IL-15, GM-CSF, Rantes, PDGF, IFN-gamma, IL-9, IL-2, IL-15, FGF were detected respectively in 95%, 94%, 88%, 81%, 76%, 65%, 60%, 48% and 22% of the follicular fluid samples.

G-CSF was not related to the embryo morphology if compared in regard the two categories best embryo quality versus others. Therefore, there was no correlation between G-CSF and embryo morphological quality (High quality versus other quality transferred embryos)

Cytokines, growth factors and implantation rates

Only one cytokine was associated in either univariate and multivariate analysis with the potential of the corresponding embryo to implant, which was the Granulocyte- Colony Stimulating factor (G-CSF)

Embryos were classified according to their implantation rates.

To construct the AUC-ROC for G-CSF, we took into account embryos that did not implant (n=89) and the ones that exhibited implantation (n=13). Certain implantation is defined when all the embryos replaced leads to a yolk sac.

The area under the ROC curve was 0.82 [0.73-0.89] and highly significant (p=0.0001) (Fig 1). Thus, G-CSF is correlated with the implantation rate ($r=0.40$ $p<0.0001$).

We also found a significant difference between embryos with certain implantation and no implantation (p=0.0002) and between embryos with certain implantation and likely implantation (p=0.001) (Table 2)

Implantation	*Number of embryos concerned*	*FF G-CSF +/- standard error (pg/ml)*	*FF IL-1ra +/- standard error (pg/ml)*
Certain implantation	13	25.3 +/-1	764 +/-373
Likely implantation	30	21.6 +/- 1	225+/-106
No implantation	89	20.2 +/-0.4	148+/-17

TABLE 2 Correlation between implantation success and levels of FF G-CSF and FF IL-1ra measured using a Luminex kit manufactured by Biorad

According to the AUC-ROC curve, we defined a lower threshold and upper threshold for G-CSF to evaluate if G-CSF concentration may be used to evaluate for each embryo a "potential of implantation" in order to decide the number of embryos we should replace.

The lower threshold was defined by the stronger negative predictive value of implantation. If G-CSF is lower than 20 pg/ml, negative predictive value is at 100% from AUC-ROC. If G-CSF is over 24, positive predictive value reaches its maximum: 40%

If all the embryos replaced are evaluated according to the level of G-CSF, we can observe the subsequent differences of the implantation rate (Table 3).

G-CSF (Luminex biorad)	*Number of embryos concerned*	*Mean implantation rate*
Low G-CSF (Below 20 pg/ml)	45	9%
Medium G-CSF (Between 20 to 24 pg/ml)	62	18%*
High G-CSF (Over 24 pg/m)l	25	44% **

TABLE 3 Correlation between implantation success and levels of FF G-CSF measured using a Luminex kit manufactured by Biorad; *p=0.003 between medium and low G-CSF; ** p<0.001 between high and low G-CSF

Example 3: Evaluation of follicular fluids using the Luminex Kit manufactured by R and D

200 follicular fluid samples corresponding to the subsequent 200 transferred embryos were analysed. The concentrations of the following cytokines and chemokines IL-1 alpha, IL-1beta, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17, IFN alpha, TNF-alpha, G-CSF, GM-CSF, MIP-1 alpha, MIP-1 beta, RANTES,MCP-1, VEGF, were evaluated using Luminex technology utilizing the kit made by R&D.

Detection of cytokines and chemokines

G-CSF, IL-1 Ra; IL-6, IL-8, MIP-beta, RANTES, MCP-1, VEGF were detected in 95 to 100% of the follicular fluid samples tested:

- IL-4, TNF-alpha, GM-CSF, IL-5 were detected in 75% to 94% of the follicular fluid samples tested,
- IL-1 alpha, IL-1 beta, IL-10, MIP-alpha were detected in 50 to 74% of the follicular fluid samples tested,

IL-2, IFN-gamma and IL-17 were detected in less than 50% of the follicular fluid samples tested.

Cytokines, chemokines and implantation rates

To construct the AUC-ROC for G-CSF, we took in account embryos that did not implant (n=146) and the ones that certainly implantated (n=16) The area under the ROC curve was at 0.72 [0.65-0.79] and highly significant (p=0.0025) (**FIG. 2**)

A significant difference was observed for G-CSF between embryos with certain implantation and no implantation (p=0.01) success and between embryos with likely implantation success and certain implantation success (p=0.03) (**Table 4**)

Implantation	***Number of embryos concerned***	***G-CSF(pg/ml) +/- standard error***
Certain implantation	16	28 +/-2.3
Likely implantation	37	20.5 +/- 1.7
No implantation	146	20.7 +/-0.9

TABLE 4: Correlation between implantation success and levels of FF G-CSF measured using a Luminex kit manufactured by R and D.

G-CSF was detected in all fluids and with low standard variations from a sample to another which is a strong requirement to identify a biomarker. According to the AUC-ROC curve, we defined a lower threshold and upper threshold for G-CSF to evaluate if G-CSF concentration may be used to predict for each embryo a “potential of implantation” that would help in the decision of the number of embryos we should replace. The lower threshold was defined by the stronger negative predictive value of implantation. If G-CSF is lower than 15 pg/ml, negative predictive value was 100% from AUC-ROC. If G-CSF was over 34, positive predictive value reaches it maximum of 27.8%.

If all the embryos replaced are evaluated according to level category defined by the AUC-ROC of G-CSF, we can observe the subsequent differences of the implantation potentiality (**TABLE 5**).

G-CSF	*Number of embryos concerned*	*Mean implantation rate*
Low G-CSF (Below 15 pg/ml)	61	9% **
Medium G-CSF (Between 15 to 34 pg/ml)	117	22%**
High G-CSF (Over 35 pg/m)l	22	43.5% **

TABLE 5 Correlation between implantation success and levels of FF G-CSF measured using a Luminex kit manufactured by R and D; ** p <0.001 between low, median, and high G-CSF

Example 4: The mean of G-CSF in pooled follicular fluid do not reflect the variations observed in individual follicular fluids.

Among 15 patients, all the follicular fluids that lead to an embryo independently of its outcome were evaluated in the same cohort. 76 samples were evaluated using the Luminex kit manufactured by Biorad.

For each sample, we evaluated the following ratio. Mean G-CSF in pooled Follicular fluid Less G-CSF concentration in individual FF (n=76).

FIG. 3 is graph showing the variation in concentration of individual follicular fluid of a same cohort of embryos obtained from 10 multiple subjects. Each box shows the variation of individual follicular fluids from the mean in a same cohort of embryos generated. These observation suggests that all the embryos generated are not equal in regard of FF-GCSF and hence in their potential of implantation.

CLAIMS

1. An assay for determining for a female subject the implantation potential of embryos obtained or to be obtained by assisted fertilisation comprising:
(i) measuring, for a plurality of ooctyes collected from said subject, the level of follicular fluid granulocyte-colony stimulating factor (G-CSF) present in the follicular fluid (FF) of a follicle of each collected oocyte; and
(ii) determining from the levels of FF G-CSF measured, the implantation potentials of the embryos obtained or to be obtained by assisted fertilisation of the oocytes.

2. Assay according to claim 1, wherein oocytes having the highest levels of FF G-CSF have the highest potential of implantation.

3. Assay according to claim 1 or 2, wherein each sample of FF is obtained from a follicular aspirate.

4. Assay according to any of claims 1 to 3, wherein respective levels of FF G-CSF are measured within 20 hours of collection of the follicular aspirate.

5. Assay according to any of claims 1 to 4 wherein a level of FF-CSF equal to or less than 20.6 pg/ml determines no or a low potential of implantation.

6. Assay according to any of claims 1 to 4 wherein a level of FF-CSF equal to or greater than 24.0 pg/ml determines a high potential of implantation.

7. Assay according to any of claims 1 to 6, wherein the respective levels of FF G-CSF are measured using an immunoassay.

8. Assay according to any of claims 1 to 7, wherein the respective levels of FF G-CSF are measured using an a competitive or immunometric assay, such as RIA, IRMA, ELISA, or ELISPOT assay.

9. Assay according to any of claims 1 to 7, wherein the respective levels of FF G-CSF are measured using a Luminex assay.

10. Assay according to claim 9, wherein the Luminex assay employs a Biorad or R and D Luminex Kit.

11. Assay according to any of claims 1 to 6, wherein the respective levels of FF G-CSF are measured by determining the levels of FF G-CSF mRNA.

12. Assay according to any of claims 1 to 6, wherein the respective levels of FF G-CSF are measured by any of surface plasmon resonance, fluorescence resonance energy transfer, bioluminescence resonance energy transfer, fluorescence quenching fluorescence, fluorescence polarization, MS, HPLC, HPLC/SM, HPLC/MS/MS, capillary electrophoresis, rod or slab gel electrophoresis, .

13. A kit for use in performing the assay according to any of claims 1 to 12, comprising at least one reagent suitable for detection of levels of FF G-CSF or FF G-CSF mRNA.

14. Kit according claim 13, further comprising a set of concentration standards of FF G-CSF.

15. Kit according claim 13 or 14, further comprising a plurality of aspirator tips for removing an oocyte and follicular fluid from a subject.

16. A method for assisted fertilisation of a female subject comprising:
(i) collecting a plurality of oocytes from said subject,
(ii) determining the implantation potential for an embryo derived from each oocyte according to the assay of any of claims 1 to 12,
(iii) fertilising the oocytes corresponding to embryos having a high potential for implantation, and
(iv) implanting the embryo so obtained into the female subject.

17. A method for assisted fertilisation of a female subject comprising:
(i) collecting a plurality of oocytes from said subject,
(ii) determining the implantation potential for an embryo derived from each oocyte according to the assay of any of claims 1 to 12,
(iii) fertilising the oocytes to obtain embryos, and
(iv) implanting the embryos having a high implantation potential.

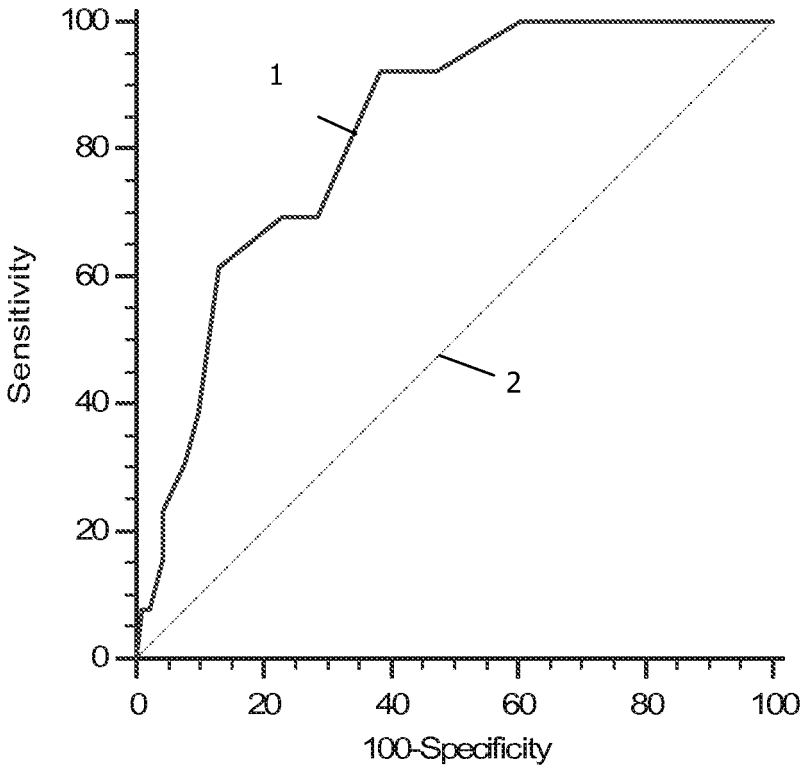

100
80
60
40
20
0
Sensitivity
1
2
0
20
40
60
80
100
100-Specificity

FIG. 1

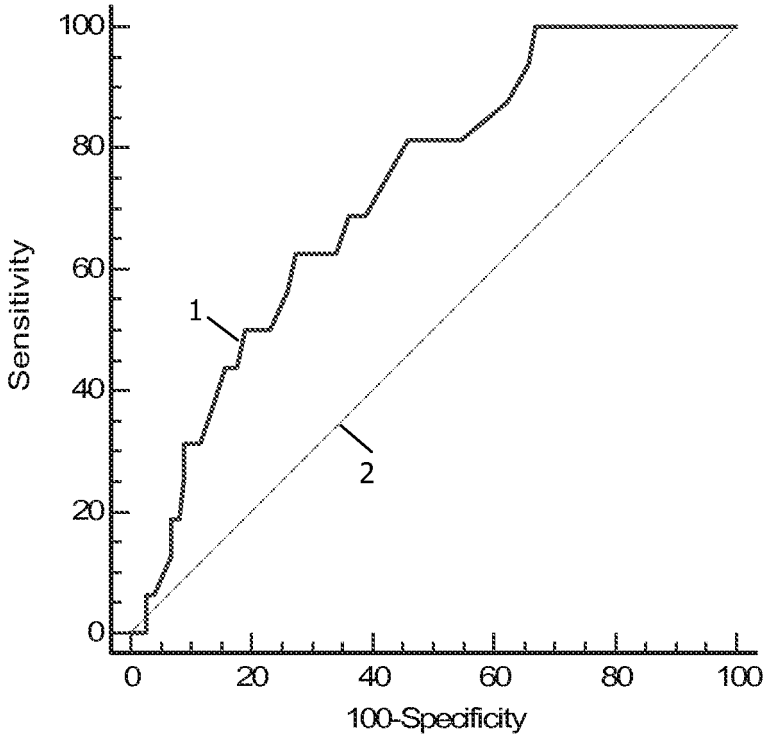

100
80
60
40
20
0
Sensitivity
1
2
0
20
40
60
80
100
100-Specificity

FIG. 2

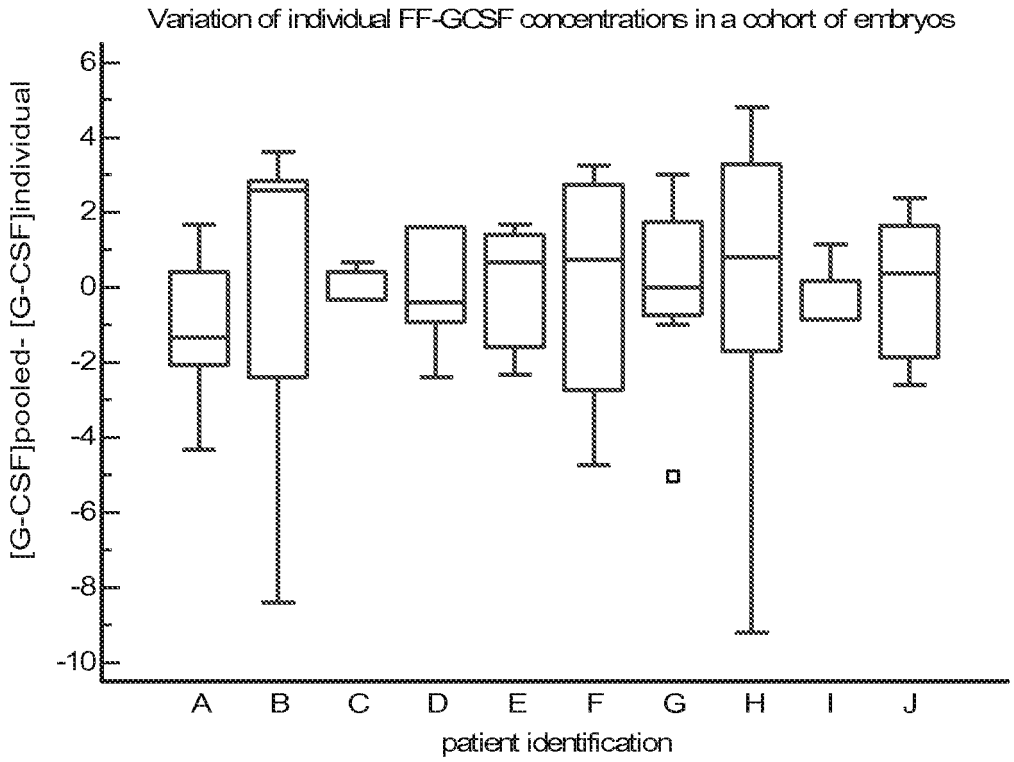

Variation of individual FF-GCSF concentrations in a cohort of embryos
[G-CSF]pooled- [G-CSF]individual
6
4
2
0
-2
-4
-6
-8
-10
A
B
C
D
E
F
G
H
I
J
patient identification

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/057430

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SALMASSI A ET AL: "Is granulocyte colony-stimulating factor level predictive for human IVF outcome?" HUMAN REPRODUCTION (OXFORD), vol. 20, no. 9, September 2005 (2005-09), pages 2434-2440, XP002453576 ISSN: 0268-1161 the whole document in particular: abstract page 2435, left-hand column, line 5 - right-hand column, line 16 page 2439, right-hand column, line 4 - line 6 page 2439, right-hand column, last paragraph	1-8, 13-17
Y		9-12

-/--

[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 October 2007	16/10/2007

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Tuynman, Antonin

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SALMASSI A ET AL: "Success of COH and IVF correlated with granulocyte-colony-stimulating factor" HUMAN REPRODUCTION (OXFORD), vol. 21, no. Suppl. 1, June 2006 (2006-06), page I159, XP002453577 & 22ND ANNUAL MEETING OF THE EUROPEAN-SOCIETY-OF-HUMAN-REPRODUCTION-AND EMBRYOLOGY; PRAGUE, CZECH REPUBLIC; JUNE 18 -21, 2006 ISSN: 0268-1161 the whole document	1-8, 13-17
Y		9-12
Y	GIAVEDONI L D: "Simultaneous detection of multiple cytokines and chemokines from nonhuman primates using luminex technology" JOURNAL OF IMMUNOLOGICAL METHODS, ELSEVIER SCIENCE PUBLISHERS B.V.,AMSTERDAM, NL, vol. 301, no. 1-2, June 2005 (2005-06), pages 89-101, XP004976653 ISSN: 0022-1759 the whole document in particular: abstract	9,10
Y	YANAGI K ET AL: "Cyclic changes of granulocyte colony-stimulating factor (G-CSF) mRNA in the human follicle during the normal menstrual cycle and immunolocalization of G-CSF protein." HUMAN REPRODUCTION (OXFORD), vol. 17, no. 12, December 2002 (2002-12), pages 3046-3052, XP002453578 ISSN: 0268-1161 the whole document in particular: abstract	11
Y	WANG J: "Proteomic identification of inflammatory markers in cerebrospinal fluid from infectious and non - infectious human brain diseases." SOCIETY FOR NEUROSCIENCE ABSTRACT VIEWER AND ITINERARY PLANNER, vol. 2003, 2003, pages Abstract No. 105.3 URL-http://sf, XP002453579 & 33RD ANNUAL MEETING OF THE SOCIETY OF NEUROSCIENCE; NEW ORLEANS, LA, USA; NOVEMBER 08-12, 2003 the whole document	12

-/--

2

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/057430

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SALMASSI ALI ET AL: "Detection of granulocyte colony-stimulating factor and its receptor in human follicular luteinized granulosa cells." FERTILITY AND STERILITY, vol. 81, no. Suppl. 1, March 2004 (2004-03), pages 786-791, XP008084388 ISSN: 0015-0282 the whole document	1-17