Immunological biomarkers of implantation in individual follicular fluids and embryo supernatants

Nathalie Lédée, MD PhD

Potentiality of implantation of IVF/ICSI generated embryos

- Assessing the potential of implantation of the embryo(s) to be transferred is crucial

- To increase the success rates of IVF-ICSI cycles while reducing the risk of multiple pregnancies.
- To promote the "single embryo transfer" (SET) policy
- To decrease the maternal and foetal morbidity and mortality associated with assisted reproductive technologies (ART) (De Neubourg and Gerris 2003, De Sutter, et al. 2003, Fiddelers, et al. 2006, Gerris, et al. 2004, Pinborg 2005)

- The analysis of the morphology of the pre-implantation embryo, although important, is generally not sufficiently informative (Guerif, et al. 2007)

If any immunological biomarker would be informative ?

- Individual Follicular fluids: Cytokines /chemokines

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, and MIP-1-beta, LIF

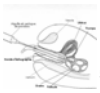
- Embryo supernatants: soluble HLA-G

<u>TRACEABILITY of each sample analysed</u>

- until delivery
- Documentation of the corresponding embryo

Cytokines and chemokines in follicular fluids

Randomised ICSI population
(n=71)



Oocytes retrieval: follicle by follicle
530 individual follicular fluids with mature oocytes

Retrospective selection of individual follicular fluids (132) for which an embryo was obtained after fertilization and subsequently transferred

Anonymous samples were analysed
to determine their cytokine and chemokine contents using the Luminex EMBIC plaform
(bead-based multiplex sandwich immunoassay - MP piccinni, Florence)

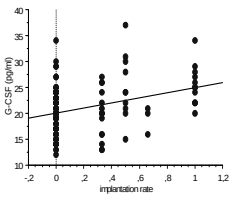
Implantation and delivery rates

Embryo staging on day-2

Almost all the cytokines, growths factors and chemokines analysed were present in follicular fluids
Important variations from a sample to another

Cytokines/ chemokines pg/ml	*Mean*	*Std. deviation*	*Std error*
IL-1Ra	225	530	46
IL-2	8	5.8	0.5
IL-4	**1.8**	**0.7**	**0.06**
IL-6	21.2	79	6.8
IL-8	399	2785	241
IL-9	9.9	13.4	1.16
IL-10	**4.6**	**4.6**	**0.4**
IL-12	**15.3**	**6.2**	**0.53**
IL-13	**4.5**	**0.73**	**0.064**
IL-15	1.77	3.76	0.32
IFN-gamma	32.9	43.1	3.7
G-CSF	**21.06**	**4.64**	**0.40**
GM-CSF	**25.4**	**11.1**	**0.96**
VEGF	126.16	13565	1176
PDGF	248.8	1388	120
FGF	19	47.6	4.1
IP-10	2083	1948	168.9
MCP-1	256.6	1560	135
RANTES	449	1087	94
EOTAXIN	138	103	8.9
MIP-1 beta	266	1989	172
LIF	954	1150	103

The level of granulocyte colony stimulating factor (G-CSF) in individual FF samples was correlated with the implantation potential of the corresponding embryo



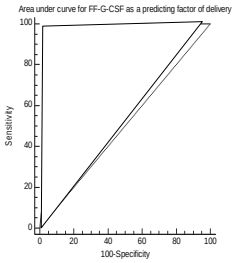
- Correlation coefficient
  R= 0,30 (P=0,0005)
- Mean: 21 +/- 0.4 (12-37) pg/ml
- Standard deviation: 4,6 pg/ml
- Intratest SD for G-CSF from 0.18 to 0.79 pg /ml.

Is FF G-CSF able to distinguish embryos that would lead to certain delivery versus to those to No pregnancy ?

- The area under the ROC curve Methodology

AuROC: 0.84 [0.75-0.90] (p=0.0001)

- The true positive rate (sensitivity) is plotted as a function of the false-positive rate (100-specificity) for different cut-off points of FF-GCSF concentration.
- A randomly selected embryos which lead to a birth has a test value larger for their FF G-CSF than those which failed to lead to a sucessful implantation **84% of the time**.



Human reprod, in revision, 2008

Implantation and delivery rates as a function of the follicular fluid G-CSF concentrations in individual FF

From the AUC-ROC curve

- **The lower threshold**

100% Negative predictive value of (less than 20 pg/ml).

- **The higher threshold**

The highest positive predictive value (over 24 pg/ml).

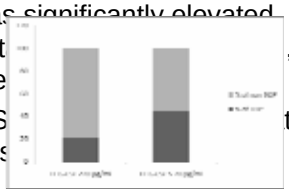
G-CSF concentrations (Luminex Biorad)	Number of embryos concerned	Mean implantation rate	Mean delivery rate
Low G-CSF (<20 pg/ml)	45	9%	6%
Intermediate G-CSF (Between 20 to 24 pg/ml)	62	18%*	15.6%*
High G-CSF (>24 pg/m)l	25	44% **	44%**

*p=0.0005 and 0.001 between intermediate and low G-CSF for implantation and delivery rates

** p<0.0001 between high and low G-CSF for both implantation and delivery rates

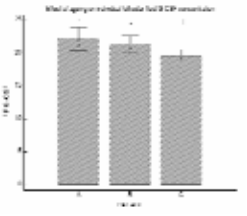
Day-2 embryo morphology and Cytokines/ chemokines content

- More TOP embryo if FF G-CSF was over 20 pg/ml (20% versus 44%, p=0.007)
- IL-12 was significantly elevated in high fragment , et al. 2002; Be
- RANTES ted in TOP vers



FACTORS AFFECTING THE FF G-CSF CONTENT

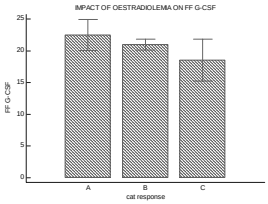
Maternal Age



- A< 30 Y (n=38)
- B: 31 -36 Y (n=57)
- C: over 37 Y (n=57)

P=0.03 between category A and C

Ovarian hyperstimulation



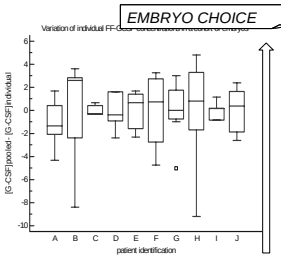
A <1500 B 1500-3500 C >3500

G-CSF, IL-8, IL-15, IL-17, IFN-gamma, VEGF, IP-10, RANTES, and MIP-beta WERE AFFECTED BY HSO

The variation in concentration of individual FF in the same cohort of embryos obtained from 10 patients

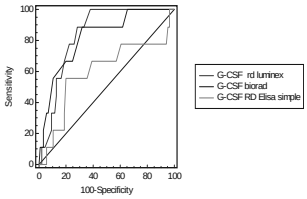
- The mean value of G-CSF in the pooled FF did not reflect the variations observed in individual FF samples.
- Embryos generated are not equally correlated with FF G-CSF and differ in their individual implantation potential

EMBRYO CHOICE



Comparison of two Luminex assay and a standard G-CSF Elisa assay to verify if the result was reproducible

- To develop an ELISA assay adaptated to IVF/ICSI that will reach the sensibility of the luminex method
- Prototype of assay is in construction
- (PCT WO-2008/009705)



Unsubmitted, in preparation

WHAT IS KNOWN ON G-CSF ?

- G-CSF is a member of the colony stimulating factor family (Clark and Kamen 1987).
- Western blotting and immunohistochemistry have located the G-CSF protein and its receptor in the ovary — mainly in the **granulosa cells of the follicle and in luteal cells** (Salmassi, et al. 2004).
- G-CSF concentrations are much higher at ovulation in FF than in serum (Salmassi, et al. 2005).
- Previous studies of G-CSF in serum provided some evidence that it is **involved in implantation**:

  Serum levels rise at implantation in the case

- of successful natural cycles (Yanagi, et al. 2002)
- After successful IVF/ICSI attempts.(Salmassi, et al. 2005).

Mechanism by which FF G-CSF is predictive of implantation ?

- If we consider the pregnancy as a semi-allograft, the question of maternal immune tolerance is essential.
- It has been reported that the pre-treatment of mice with G-CSF before an allograft promotes T cell tolerance towards these grafts.
- [FF]G-CSF may inform on the mRNA content of the oocyte itself and its immune potential of tolerance (adhesion molecules at the oocyte cell surface)
- Another hypothesis is that the [FF] G-CSF provides the embryo with crucial information on how to repair itself.
- G-CSF has been described in various models (heart, liver) as an agent promoting endogenous repair by enhancing the endogenous stem cells per se or either through the mobilisation of multipotent progenitor cells.

Soluble HLA-G in Day-2/3 embryo supernatants

sHLA-G and Embryo potential of implantation

- Some authors reported that sHLA-G detection in culture embryo supernatants was informative on the potential of the embryo to implant
- **Retrospective Multicentric** EMBIC analysis (Poissy, Toulouse, Liege)
- Embryo supernatants of **1360 embryos** singly cultured in 40 µl microdroplet
- All the samples were anonymised at the time of the collection to be blindly analysed (EMBIC database)
- Analysis: Toulouse (J. Tabiasco, N. Kozma, Ph Le Bouteiller)
- Outcome: implantation rate of each embryo, embryo quality, IVF/ICSI conditions.

Characteristics of the collected samples

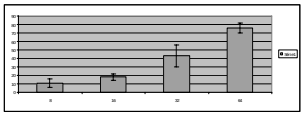
	POISSY UNIT	*TOULOUSE UNIT*	*LIEGE UNIT*
No. of patients	78	196	82
ART type	ICSI only	IVF and ICSI	IVF and ICSI
Analyzed samples	360 embryo supernatants and 197 corresponding individual follicular fluids	450 embryo supernatants	595 embryo supernatants and 40 unfertilized oocyte supernatants
Embryo transferred	Fresh transferred embryos only	Fresh transferred embryos only	Fresh and freeze-thaw transferred embryos
No. of embryos transferred: Fresh Freeze-Thaw	164	405	132 44
Day of transfer or freezing	Day-2	Day-2	Day-3

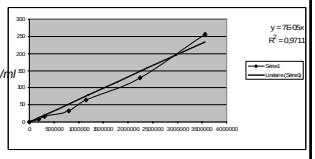
sHLA-G Detection

- **Chemioluminescent** Elisa Assay
- Coating Antibody: anti-HLA-G MEMG/9
- Capture Antibody anti-MHC I: W6/32

(julie Tabiasco, Ph Le bouteiller)

- Negative control: medium without Embryo
- Detection limit was established by spike recovery and less than 1FP/60





sHLA-G in embryo culture supernatants and implantation

REPRODUCTIVE UNITS	**Percentage of detectability Mean (ng/ml)**	**Implantation rate sHLA-G positive**	**Implantation rate sHLA-G negative**	*p*
Poissy Centre *(n=146)*	19% 3.3 ng/ml	34%	19%	*0.0379
Toulouse Centre *(n=404)*	34% 18 ng/ml	17%	18%	NS
Liège Centre *(n=176)*	44% 16 ng/ml	17%	18%	NS

sHLA-G and embryo quality

	LIEGE CENTER (n=595)			***POISSY CENTER (n=360)***		
Embryo quality	TOP	Non TOP		TOP	Non TOP	
No. of embryos observed	147	448		166	183	
sHLA-G (+) embryo supernatants	39%	46%		18%	20%	
Embryo destiny following embryologist decision	Destruction (including cleavage failure)	Freezing	Fresh transfer	Destruction (excluding cleavage failure)	Freezing	Fresh transfer
No. of embryos observed	170	288	137	74	126	148
sHLA-G (+) embryo supernatants	48%	45%	39%	26%	15%	18%

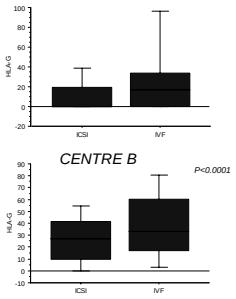
Influence of the culture media on sHLA-G detection in embryo culture supernatants

	LIEGE CENTER *595 embryo supernatants*		*P* values*
Media	Batch of culture medium 1	Batch of culture medium 2	
sHLA-G (+) embryo supernatants	23.4 % (77/328)	70 % (187/267)	<0.0001
sHLA-G (+) supernatants among transferred embryos	27.8% (34/122)	61 % (33/54)	<0.0001
Implantation rate	15.6 %	23.1 %	0.18

sHLA-G: argument for a maternal origin

- 60% of detectability
- immature oocytes
- unfertilized oocytes

- Higher detectability in IVF versus ICSI (participation of granulosa cells)



sHLA-G in D-2 and D-3 embryo supernatants

- NO consistent correlation with the potential of implantation
- Variations in function of culture condition especially the medium used for fertilization
- Its detection could be useful for setting personnalized optimal Embryo culture condition
- Detectability for immature and unfertilized oocytes, the differences between IVF and ICSI, high detection level in the follicular fluid and the absence of mRNA in day-2 embryo suggest a maternal (oocyte) origin of secretion

FF G-CSF appears as an immunological biomarker of the oocyte immune tolerance and is predictive of implantation

- FF G-CSF may help to promote the single embryo policy by helping to distinguish even before fertilization the oocyte with a good or a bad potential of implantation.
- FF G-CSF may also be helpful to evaluate individually the oocyte immune tolerance (women with a low ovarian reserve) and identify the best protocol of ovarian stimulation to apply
- A prospective randomised study need to confirm the hypothesis and should begin as soon as the sensitive and specific prototype would be set up

aknowlegment

CHI poissy St Germain/UVSQ
- Yves Ville
- Raoul Lombroso
- Marc Bailly
- Robert Wainer
- Pierre Oger
- Ibrahim Hammoud
- Jacqueline Selva
- Marianne Bergère

CHR de liège
- Sophie Perrier Hauterive
- Jean-michel Foidard
- Fabienne Thonon
- Francis frankennes

INSERM Clamart
- *Gerard Chaouat*
- *Sylvie Dubanchet*
- *Marie petitbarat*

University of Florence
LUMINEX analysis
- Marie pierre Piccinni
- Letizia Lombardelli

INSERM Toulouse
- Philippe le Bouteiller
- Julie tabiasco
- Noemie kozma
- Jean Parinaud