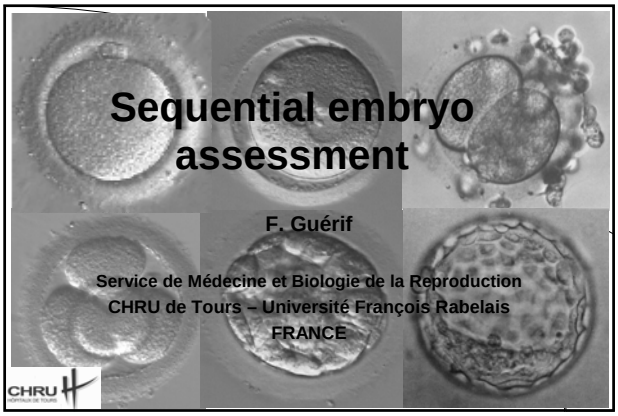

Sequential embryo assessment

F. Guérif

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Evaluation of embryo viability in IVF

- Selection of the best embryo (SET/SBT)
- Identification of viable embryos (only cryopreserved)

- Various methods (non-invasive)
- Common clinical practice (reliable / non-time consuming)

Evaluation of embryo viability in IVF

- Static

=observation at one point in time (snapshot)
D1 or D2 or D3

- Dynamic (sequential assessment)

=observation of embryo progression over time
D0 ± D1 ± D2 ± D3

Implantation rate

Single embryo transfer

In Vitro development

Individual embryo culture

Individual outcomes have more strength

Which useful parameters to select embryos ?

- **Oocyte morphology (day 0)**
- **Pronuclear morphology (day 1)**
- **Early cleavage (day 1)**
- **Embryo morphology (day 2 and/or day 3)**

Top quality embryos

Day 2

- **4-cell embryo**
- **Equal blastomeres**
- **No / minor fragmentation**
- **No multinucleation**

Day 3

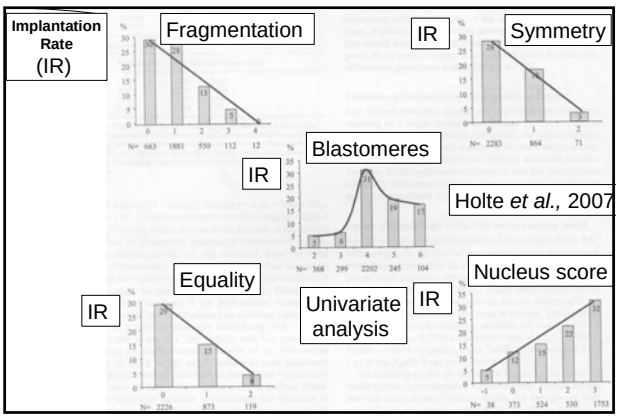
- **8-cell embryo**
- **Equal blastomeres**
- **No / minor fragmentation**
- **No multinucleation**

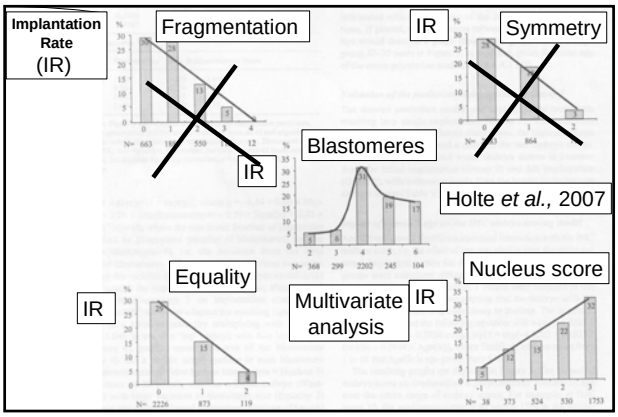
What is the weight of these parameters ?

Holte *et al.*, 2007

- **Prospective study**
- **Individual embryo culture (3226 embryos)**
- **Investigation of the weight of five D2 parameters for implantation potential including no or twin implantation**
  - **Univariate analysis**
  - **Multivariate analysis**

Multivariate analysis is necessary:
- To find out which variable have independent power
- To find the correct power balance between such independent variables

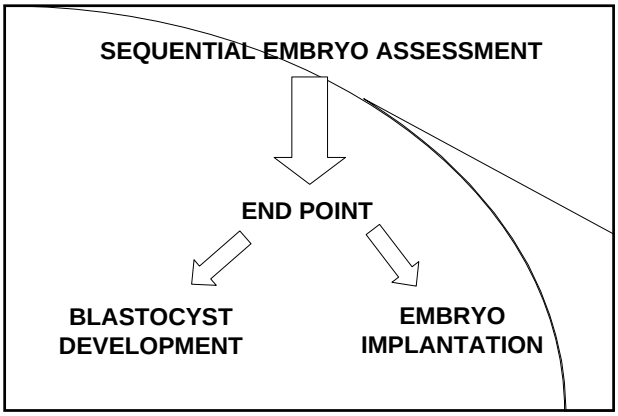




Various factors are involved with embryo development.

=> a single early (D0 or D1 or D2 or D3) static observation is probably inadequate to predict further development.

There are few studies where the efficacy of parameters, each or in combination were weighted against each other to ascertain their relative importance.



End point: blastocyst development

	D0	D1(PN)	D1(EC)	D2	D3
Fisch *et al.*, 2001		X	X		X
Neuber *et al.*, 2003		X	X	X	X
Rienzi *et al.*, 2005		X	X	X	X
Sjöblom *et al.*, 2006	X	X	X	X	
Guerif *et al.*, 2007		X	X	X	

The graduated embryo score (GES) predicts blastocyst formation and pregnancy rates from cleavage-stage embryos

Fisch *et al.*, 2001

Retrospective study

The outcome measure was to evaluate the correlation between a graduated embryo score (D1, D3) with blastocyst development

N=1245 individually cultured zygotes

Graduated embryo scoring (GES) of cleavage stage embryos.

Evaluation	Hours after insemination	Developmental milestone	Score
1	16-18	Nucleoli aligned along pronuclear axis	20
2	25-27	Cleavage regular and symmetrical	30
		Fragmentation[a]	
		Absent	30
		<20%	25
		>20%	0
3	64-67	Cell number and grade[b] 7CI, 8CI, 8CII, 9CI 7CII, 9CII, 10CI, 11CI, Compacting I	20 10
Total score			100

[a] If the embryo was not cleaved at 25–27 hours, grading of fragmentation should occur at the 64–67 hour evaluation if the embryo reached the seven-cell stage and had <20% fragmentation.
[b] Grade I = symmetrical blastomeres and absent fragmentation. Grade II = slightly uneven blastomeres and <20% fragmentation. Grade III = uneven blastomeres and >20% fragmentation. Grade A embryos are seven or more cells with <20% fragmentation.

Fisch *et al.*, 2001

Table III. Comparison of Graduated Embryo Scoring (GES) with blastocyst development

GES	Total embryos for extended culture	Blastocyst development [*n* (%)]
70-100	269	119 (44)[a]
90–100	110	70 (64)[b]
70–85	159	49 (31)[c]
0-65	714	61 (9)
30–65	443	49 (11)[d]
0–25	271	12 (4)
Total	983	180 (18)

[a] $P < 0.001$ compared with GES 0–65 and to GES 30–65.
[b] $P < 0.001$ compared with GES 70–85.
[c] $P < 0.001$ compared with GES 30–65.
[d] $P < 0.003$ compared with GES 0–25.

Fisch *et al.*, 2001

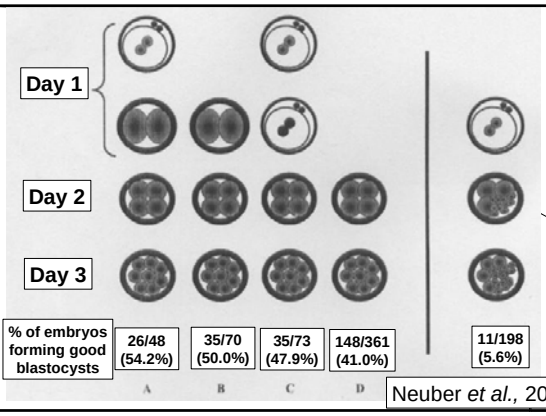
Sequential assessment of individually cultured human embryos as an indicator of subsequent good quality blastocyst development

Neuber *et al.*, 2003

Retrospective study

The outcome measure was to determine if developmental markers on day 1, 2 and 3 can predict good subsequent blastocyst development

N=1550 individually cultured zygotes



Neuber *et al.*, 2003

Significance of morphological attributes of the early embryo

Rienzi *et al.*, 2005

Retrospective study

The outcome measure was to determine the relationship between embryo score and blastocyst conversion

N=993 individually cultured zygotes

Feature evaluated	Day 1	Day 2	Day 3	Points
Pronuclear stage	Normal			0
	Pathological			2
Early cleavage	Yes			0
	No			1
Cleavage stage	–	4- to 5-cell	>6 cells	0
	–	2- to 3- and >5 cell	4- to 6-cell	2
Blastomere size	–	<2-cell	<4-cell	4
	–	Equal	Equal	0
	–		Unequal	1
	–	Unequal		2
Fragmentation (%)	–	<10	<10	0
	–	10–30	10–30	1
	–	30–50	30–50	2
	–	>50	>50	3
Multinucleation (%)	–	Absent	Absent	0
	–	≤50	≤50	2
		>50	>50	4

Rienzi *et al.*, 2005

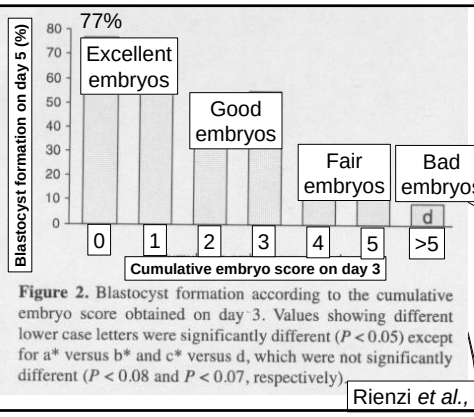


Figure 2. Blastocyst formation according to the cumulative embryo score obtained on day 3. Values showing different lower case letters were significantly different ($P < 0.05$) except for a* versus b* and c* versus d, which were not significantly different ($P < 0.08$ and $P < 0.07$, respectively).

Rienzi *et al.*, 2005

Prediction of embryo developmental potential and pregnancy based on early stage morphological characteristics

Sjöblom *et al.*, 2006

- **Retrospective study**
- **The outcome measure was the association of blastocyst development with early morphological characteristics (D0, D1, D2), alone or those in combination.**

N=431 individually cultured zygotes

Sjöblom *et al.*, 2006

D0 (oocyte) characteristics

	Description	Score
Polar body	Round/oval, unfragmented	1
	Other	0
Cytoplasm	Smooth, finely granulated	1
	Coarse, vacuoles, dark patch	0
Membrane	Smooth	1
	Jagged	0
Zona pellucida	Normal thickness and color, no debris	1
	Other	0
Maximum score		4
Aggregation of SER	Pancake-like diffuse structure	Deselected from transfer or freezing

D1 (zygote) characteristics

Polar bodies	<90° apart, <45° with axis of pronuclei	1
	Other	0
Cytoplasmic halo	Present, normal cytoplasm	1
	Absent	0
Membrane	Smooth	1
	Jagged	0
Nucleoli	≈Equal numbers and sizes, ≥3	1
	Other	0
Pronuclei	Equal size, central, apposed	1
	Other	0
Zona pellucida	Normal thickness and color, no debris	1
	Other	0
Maximum score		6
Syngamy	Breakdown of pronuclear membranes;	Noted and preferred when

Sjöblom *et al.*, 2006

D2/3 (embryo) characteristics

Cell numbers	Number of blastomeres	Noted
Zona pellucida	Normal thickness and color, no debris	1
	Other	0
Cytoplasm	Smooth, honey-colored	1
	Other	0
Membrane	Smooth	1
	Jagged	0
Cell size	Equal if 2^n blastomeres, otherwise in accord with cleavage stage	1
	Other	0
Cell shape	Spherical	1
	Other	0
Perivitelline space	Blastomeres fill the space under the zona	1
	Other	0
Fragmentation	<10% fragmented	2
	10%–30% fragmented	1
	>30% fragmented	0
Developmental rate	4 cells 42 hpi; 8 cells 66 hpi	2
	2, 3 or >4 cells 42 hpi; 6–7 or >8 cells 66 hpi	1

Association between morphological characteristics and blastocoel development in embryos cultured to D5 or D6, estimated by the Spearman rank order coefficient of correlation, r.

Blastocoel score vs.	n	r	*P* value
D0 polar body	222	0.044	NS
D0 cytoplasm	222	0.141 ↖	.035
D0 membrane	222	−0.083	NS
D0 zona pellucida	222	0.047	NS
D1 polar bodies	420	0.002	NS
D1 cytoplasm	420	0.020	NS
D1 membrane	420	0.082	NS
D1 nucleoli	420	0.105 ✗	.031
D1 pronuclei	420	0.146 ✗	.003
D1 zona pellucida	420	−0.001	NS
Syngamy	147	−0.007	NS
D2 zona pellucida	420	−0.020	NS
D2 cell size	420	0.042	NS
D2 cell shape	420	−0.076	NS
D2 perivitelline space	420	0.098	.044
D2 membrane	420	0.051	NS
D2 cytoplasm	420	0.101 ↘	.038
D2 fragments	420	−0.029	NS
D2 developmental rate	420	0.154 ✗	.002
D2 cell number	420	0.114 ✗	.019

The association between different embryo scores and blastocoel development in embryos cultured to D5 or D6, estimated by the Spearman rank order coefficient of correlation, r.

Blastocoel score vs.	n	r	*P* value
D0 score	222	0.095	.158
D1 score	420	0.136	.005
D2 score	420	0.086	.078
D2 corrected cumulative score	420	0.100	.040

Sjöblom *et al.*, 2006

Limited value of morphological assessment at days 1 and 2 to predict blastocyst development potential: a prospective study based on 4042 embryos

Guerif *et al.*, 2007

- **Prospective study**
- **4042 embryos were individually cultured**
  - **Univariate**
  - **Multivariate analysis**
- **The outcome measure was the association between usual D1-D2 morphological characteristics and blastocyst development**

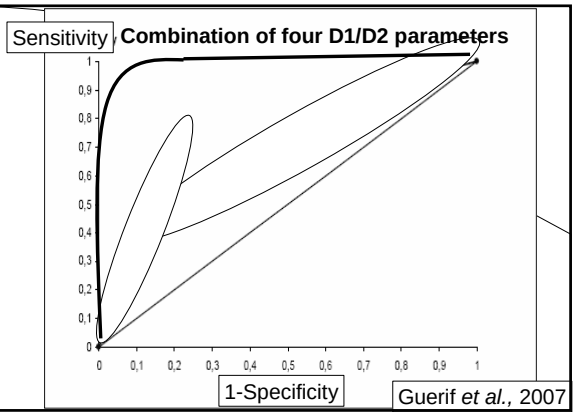
B3-B5 A/B (D5) and D1/D2 parameters

UNIVARIATE ANALYSIS	% B3-B5 (A/B) D5	*p*
Pattern 0 zygotes	**22.6%**	**0.0002**
Non-pattern 0 zygotes	**12.5%**	
Early cleavage embryos	**29.0%**	**<0.0001**
Non-early cleavage embryos	**7.3%**	
2-3 cell embryo on day 2	**4.3%**	**<0.0001**
4 cell embryo on day 2	**25.6%**	
5-8 cell embryo on day 2	**11.1%**	
<20% fragmentation on day 2	**18.0%**	**0.0019**
20-50% fragmentation on day 2	**12.3%**	
>50% fragmentation on day 2	**6.8%**	

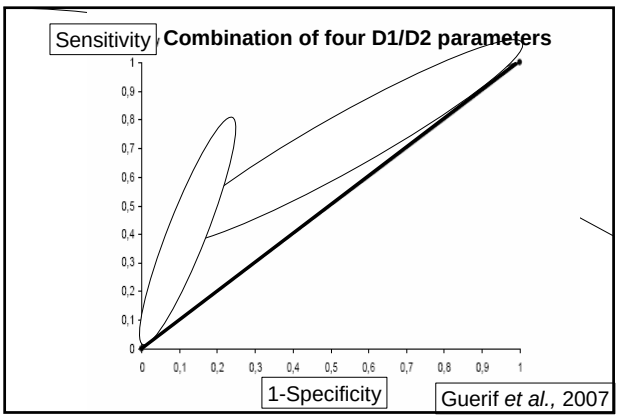
B3-B5 A/B (D5) and D1/D2 parameters

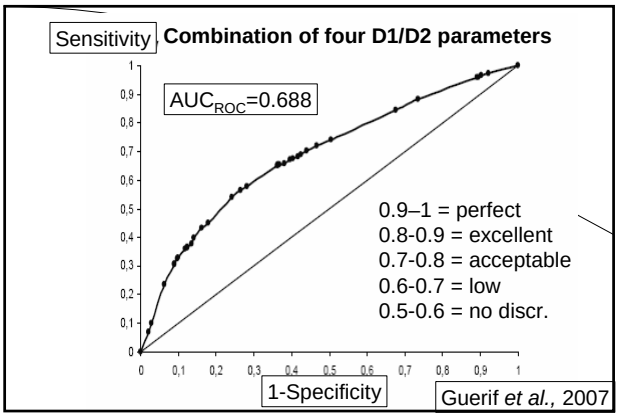
MULTIVARIATE ANALYSIS	**OR**	**95% CI**	*P-value*	*Overall p*
Early cleavage (Day 1)				
Early-cleavage embryos	**1.00**			
Non-early cleavage embryos	**0.40**	**0.32 - 0.50**	**<0.0001**	
Number of cells (Day 2)				**<0.0001**
4 cells (Day 2)	**1.00**			
2- 3 cells (Day 2)	**0.30**	**0.22 - 0.40**	**<0.0001**	
5-8 cells (Day 2)	**0.54**	**0.41 - 0.72**	**<0.0001**	

Guerif *et al.*, 2007



Guerif *et al.*, 2007





End point: implantation

	D0	D1(PN)	D1(EC)	D2	D3
De Placido *et al.*, 2002		X		X	X
Nagy *et al.*, 2003		X			X
Sjöblom *et al.*, 2006	X	X	X	X	
Scott *et al.*, 2007	X	X		X	X
Rehman *et al.*, 2007		X		X	X

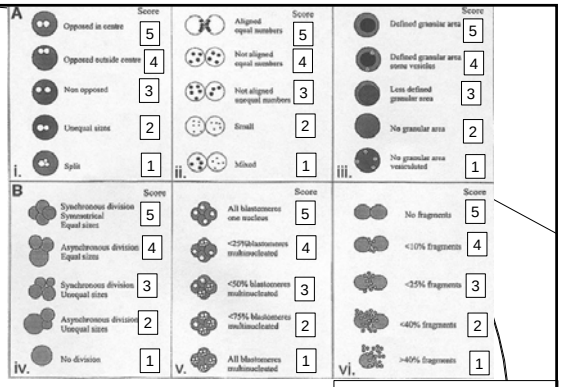
High outcome predictability after IVF using a combined score for zygote and embryo morphology and growth rate

De Placido *et al.*, 2002

Retrospective study

The outcome measure was to determine utility of a combination of scoring systems (D1 and D2/3) to predict IVF outcome

N=154 cycles



De Placido *et al.*, 2002

Score = zygote score X (embryo score X number blastomeres)

Implantation Rate (%)	Group1 TQ scoring	Group 2 Medium scoring	Group 3 Low scoring
Zygote morphology	**13.2%**	**15.7%**	**7.1%**
Embryo morphology	**14.9%**	**15.4%**	**4.0%**
Weighted Score D2	**11.9%**	**13.3%**	**10.4%**
Weighted Score D3	**24.5%**	**13.4%**	**4.2%**

De Placido *et al.*, 2002

Pronuclear morphology evaluation with subsequent evaluation of embryo morphology significantly increases implantation rates

Nagy *et al.,* 2003

Prospective study

The outcome measure was to determine the correlation of pronuclear morphology with embryo morphology and implantation rates

N=290 cycles

Method of embryo selection for embryo transfer	Cycles	Implantation rate
2 PN morphology	95	12.1%[b]
Day 3 morphology	98	15.1%[a]
Day 3 morphology + 2 PN morphology	97	21.1%[ab]

a,b=p<0.05

Nagy *et al.,* 2003

Prediction of embryo developmental potential and pregnancy based on early stage morphological characteristics

Sjöblom *et al.,* 2006

- **Retrospective study**
- **A differentially weighted scoring system was developed and its relationship to implantation rates was analysed**

N=268 cycles

D1 (oocyte) characteristics

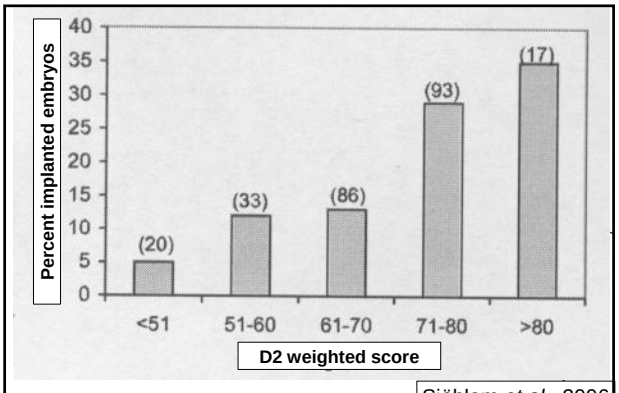
Characteristic	Description	Score
(day after oocyte pick-up)		
Polar body orientation	<45° with axis of pronuclei	3
	Other	0
Cytoplasmic halo	Present	3
	Absent	0
Cytoplasmic texture	Normal	6
	Slightly granular	3
	Vacuoles, dark patch, very granular	0
Membrane	Smooth	3
	Jagged	0
Nucleoli	Equal number (<8) and polarized	18
	Equal number and scattered	6
	Other	0
Pronuclei, size	Equal	3
	Unequal	0
Pronuclei, position	Central	3
	Eccentric	0
Pronuclei, apposition	Apposed	3
	Apart	0
Syngamy at 25 hpi	Cleavage to the 2-cell stage	8
	Breakdown of pronuclear membranes	4
	Intact PNs	0
	Maximum score	50
Aggregation of smooth endoplasmic reticulum on D0	No freezing or transfer, total score 0	

Sjöblom *et al.*, 2006

D2 (embryo) characteristics

42 hpi (2 days after oocyte pick-up)		
Zona pellucida thickness	Variable	3
	Uniform	0
Cytoplasm	Clear	3
	Granular, vacuoles	0
Membrane	Smooth	3
	Jagged	0
Blastomere size	Equal if 2^n blastomeres, otherwise in accord with cleavage stage	3
	Other	0
Cell shape	Spherical, regular	3
	Other	0
Perivitelline space	Blastomeres fill the space under the zona	5
	Large space between cells and zona	0
Fragmentation	<10% fragmented	10
	10%–30% fragmented	5
	>30% fragmented	0
Developmental rate	4 cells	20
	2, 3 or >4 cells	10
	Other	0
	Maximum score	50

Sjöblom *et al.*, 2006



Sjöblom *et al.*, 2006

Morphologic parameters of early cleavage-stage embryos that correlate with fetal development and delivery: prospective and applied data for increased pregnancy rates

Scott *et al.*, 2007

- **Prospective study**
- **The outcome measure was to evaluate the usefulness of morphological characteristics (D0, D1, D2, D3, D5) in predicting implantation**

Scoring parameters analysed

- **D0 (Oocyte morphology)**
  - **Size of the 1st PB**
  - **Shape of the 1st PB**
  - **Size of the PVS**
  - **Thickness of the ZP**
  - **Texture of the cytoplasm**
- **D1 (PN morphology)**
  - **Number of the NPB**
  - **Alignment of the NPB**
- **D2 (Embryo morphology)**
  - **Blastomere number**
  - **Equality of cell size**
  - **State of nucleation**
- **D3 (Embryo morphology)**
  - **Blastomere number**
  - **Degree of fragmentation**
  - **Cell size**
- **D5 (Blastocyst morphology)**
  - **ICM**
  - **Trophectoderm**

- **D1-D2 parameters are stronger predictive of implantation than D3 morphology or ability to achieve the blastocyst stage of development.**
- **Most significant D1/D2 factors:**
  - **PN morphology and NPB ratio**
  - **Day 2 cell number, blastomere symmetry, nucleation**
  - **Ability to cleave from D2 to D3**

N=155 cycles

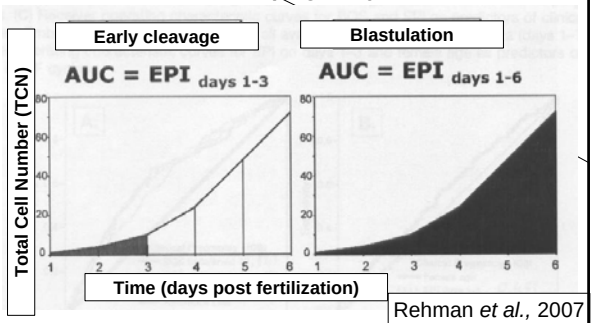
Later stages of embryo progression are a much better predictor of clinical pregnancy than early cleavage in ICSI and IVF cycles with blastocyst-stage transfer

Rehman *et al.*, 2007

- **Retrospective study**
- **Determination of Embryo Progression Index (EPI) by the Area Under the Curve (AUC) of total cell number over time**

N=2134 cycles

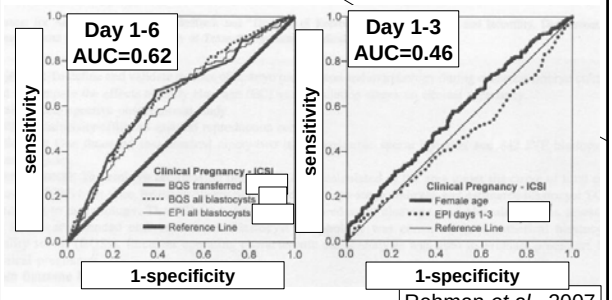
Comparison of D1-D3 stages vs blastocyst stages on clinical pregnancy



Rehman *et al.*, 2007

ICSI cycles

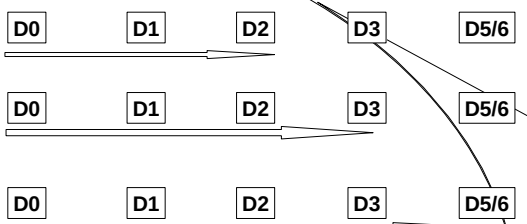
ROC Curves in the prediction of clinical PR



Rehman *et al.*, 2007

CONCLUSIONS

Sequential embryo assessment



Gardner and Sakkas 2003

18-19 h post insemination / ICSI

The fertilized embryo is examined for:

(a) Equal size and symmetry of PN	10
(b) Alignment between the PN and polar bodies	5
(c) Lack of heterogeneity and granularity in cytoplasm	5
(d) Presence of PN with both polarized or both not-polarized NPB	10
(e) A difference of less than 3 in the number of NPB in the PN	10
(f) Polar bodies are not displaced from each other	10

25-26 h post insemination / ICSI

(a) Embryos that have already cleaved to form a 2-cell embryo with even blastomeres and no fragments	15
(b) Zygotes that have progressed to nuclear membrane breakdown	5

42-44 h post insemination / ICSI

(a) Number of blastomeres should be greater or equal to 4	10
(b) Fragmentation of less than 20 per cent	10
(c) No multinucleated blastomeres	5

66-68 h post insemination / ICSI

(a) Number of blastomeres should be greater or equal to 8	10
(b) Fragmentation of less than 20 per cent	10
(c) No multinucleated blastomeres	5

94-96 h post insemination / ICSI

(a) Compaction	10
(b) Signs of blastocoel formation	15

106-108 h post insemination / ICSI

(a) Full blastocoel cavity	10
(b) Inner cell mass with tightly packed numerous cells	15
(c) Trophectoderm with many cells forming epithelium	10

Possible concerns with sequential embryo assessment

- **Potential damage done to the embryo by performing multiple viewvings.**
- **It necessitates culture of single embryos in droplets which is more time consuming.**
- **There is no consensus about some parameters (D0, D1)**
- **The weight of studied parameters remains IVF center dependent.**

Possible concerns with sequential embryo assessment

- **It should be underlined that all visual real time scoring procedures are affected by varying inherent difficulties. Intra and inter observer variations are likely to be larger for some variables than others.**
- **Thus such qualities in a parameter may diminish its prognostic power, even if the variable is of significant biological importance.**

Need to identify new markers of embryo viability ?

TOP QUALITY EMBRYOS AND BLASTOCYST DEVELOPMENT

	No Emb	Characteristics	Blastocyst dev.D5 TQ Embryos
Fisch *et al.*,2001	1245	PN + EC + D3	64 % blastocysts
Lan *et al.*, 2003	1894	PN + D3	92 % blastocysts
Neuber *et al.*, 2003	1550	PN + EC + D2 + D3	54 % blastocysts
Rienzi *et al.*, 2005	993	PN + EC + D2 + D3	77 % blastocysts
Guerif *et al.*, 2007	4042	PN + EC + D2	59 % blastocysts

	Rijnders and Jansen, 1998	Milki *et al.*, 2002	IVF Unit, Tours-France
Number of cycles	48	100	140
Day of observ.	Day 3	Day 3	Day 2
No. Emb transfer.	2-3	2	1
Total agreement	20%	23%	32%
Partial agreement	56%	38%	34%
No Agreement	24%	39%	34%
Blastocyst IR	30%	NA	41.5%

Predictive value of embryo morphology on day 2/3 for subsequent blastocyst formation seems limited

Early parameters and blastoc. implantation ?

	Della Ragione *et al.*, 2007 SBT Day 5 (B3-B5 A/B)		IVF Unit, Tours, France SBT Day 5 (B3-B5 A/B)	
	100% Implant.	Non Implant.	100% Implant.	Non Implant.
n	93	110	83	97
% Pattern 0 zygotes	NA	NA	20.5%	20.6%
% Early cleavage	NA	NA	43.5%	58.6%
% 4-cell embryos	86.0%	86.4%	78.3%	78.4%
% <10% fragment.	88.2%*	76.4%*	75.9%	69%
	IR=45.8%		IR=46.1%	

- **Reports suggest that cleavage stage scoring have a limited ability to predict blastocyst development.**
- **Good quality blastocysts can develop from embryos classified as suboptimal (Hadarson *et al.*, 2003).**
- **Even within a cohort of blastocyts with the same alphanumeric score, there exists considerable differences in their metabolics activity (Gardner and Sakkas).**

There is a need to identify new markers of embryo viability !

Service de Médecine et Biologie de la Reproduction CHU Tours – Unité FIV

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- **Dr F Guérif**
- **R Bidault**
- **V Cadoret**
- **O Gasnier**
- **C Jamet**
- **MH Saussereau**
- **M Lemseffer**
- **P Feuerstein**