Non-invasive analysis of embryonic aneuploidies in cell free DNA

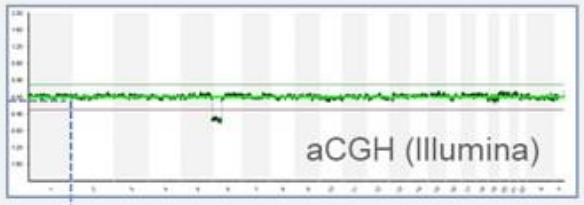
Carmen Rubio, Igenomix (Spain)

How to perform human embryo aneuploidy testing

1995

Fluorescence *In Situ* Hybridization (FISH)

Day-3 biopsies
2 blastomeres
PGT-A 1.0



Blastocysts
Deferred transfer
PGT-A 2.0

2008

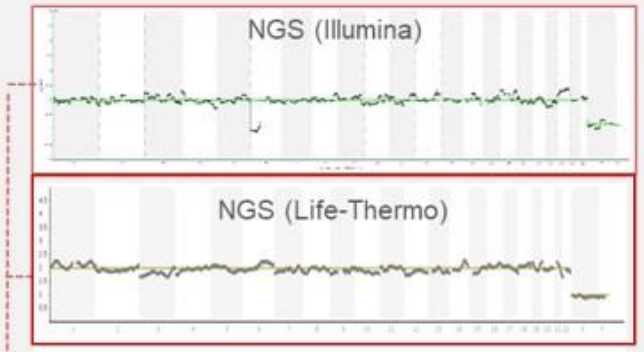
Array Comparative Genomic Hybridization (aCGH)

2010

Single-nucleotide polymorphism (SNP)

2012

Quantitative polymerase chain reaction (qPCR)



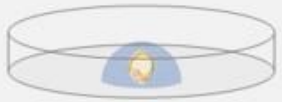
PGT-A 3.0

2013

Next Generation Sequencing (NGS)

2016-17

NGS with custom algorithm for mosaicism detection



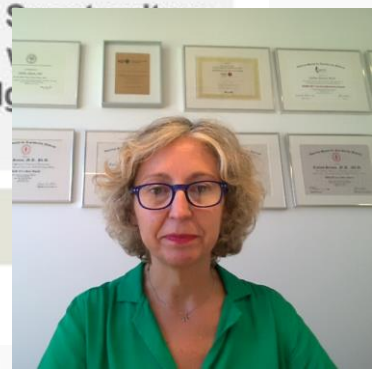
niPGT-A

2018-19

NGS in S
media
alg

≤12 chromosomes

24 chromosomes



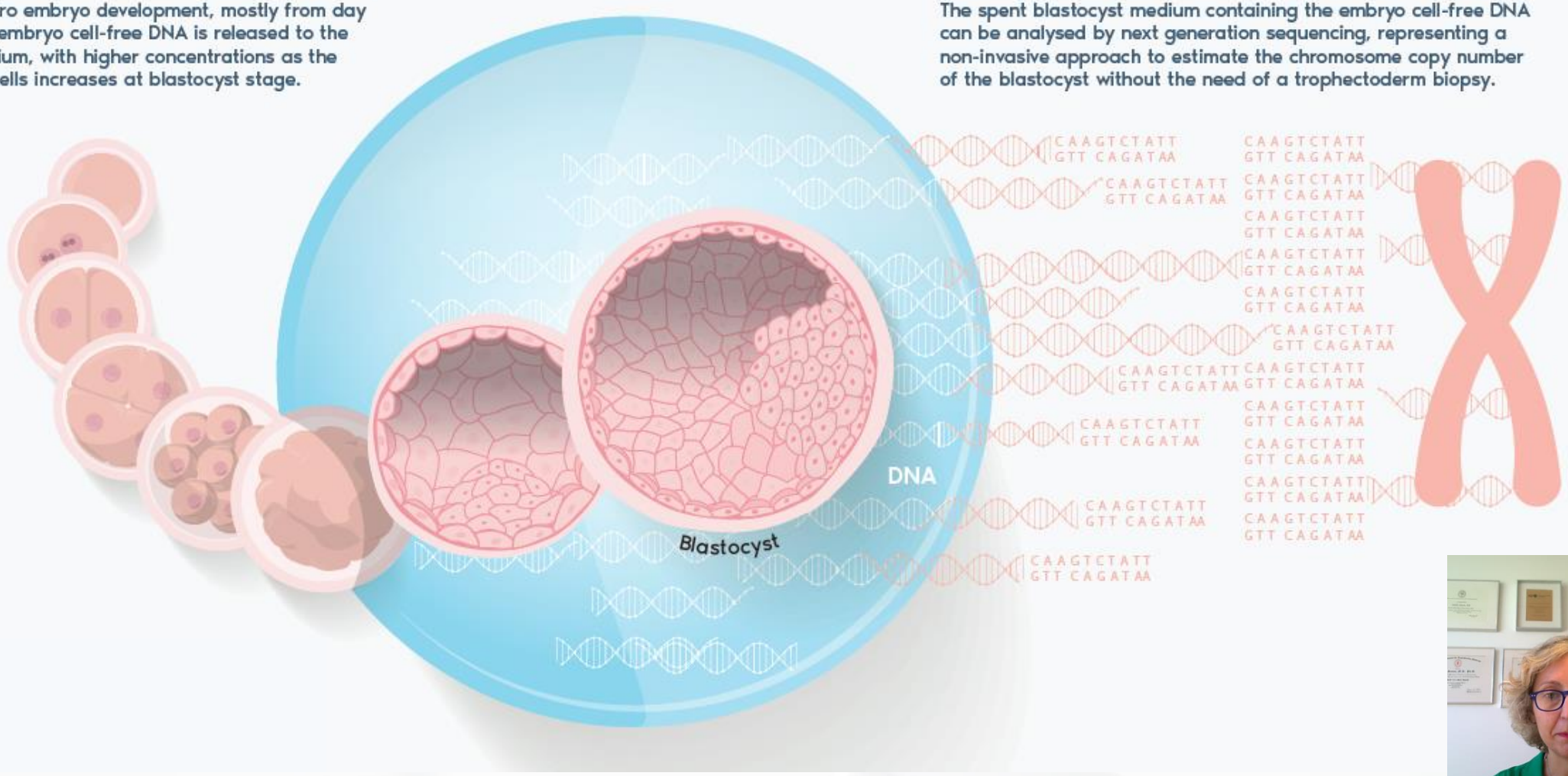
Embryo cell-free DNA is released during embryo development

1

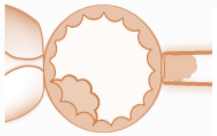
During in vitro embryo development, mostly from day 4 to day 6, embryo cell-free DNA is released to the culture medium, with higher concentrations as the number of cells increases at blastocyst stage.

2

The spent blastocyst medium containing the embryo cell-free DNA can be analysed by next generation sequencing, representing a non-invasive approach to estimate the chromosome copy number of the blastocyst without the need of a trophectoderm biopsy.



Updated publications on embryo cfDNA analysis

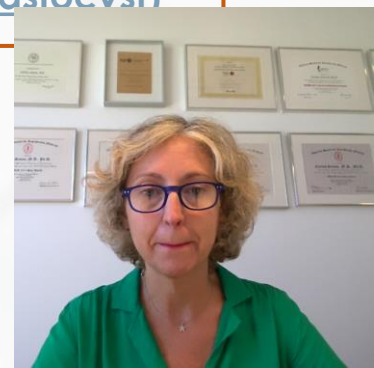
PGT-A  versus  **Embryo cfDNA**

Shamonki et al., Fertil Steril 2016	**3.5%**	concordance	(N=55) (+D3 AH, D5 biopsy)
Feichtinger et al., RBMonline 2017	**27%**	concordance	(N=22) (D1 AH, PB biopsy)
Ho et al., Fertil Steril 2018	**65.0%**	concordance	(N=61) (with and without AH, D5 biopsy)
Xu et al., PNAS 2016	**85.7%**	concordance	(N=42) (D3 AH, + vitrification, whole blastocyst)
Yeung et al., 2019 JARG	**62.1.%**	concordance	(N=167) (D3 AH, + vitrification, D5/6 biopsy)
Huang et al., 2019 PNAS	**82.0%**	concordance	(N=52) (Biopsy +vitrification, **concordance 93.8% whole blastocyst)**
Kuznyetsov et al., PlosOne 2018	**87.5%**	concordance	(N=47) (BF+SBM, **concordance 96.4% whole blastocyst)**
Jiao et al., Hum Reprod 2019	**75.0%**	concordance	(BF+SBM, D5 biopsy, **concordance 96.8% whole blastocyst)**

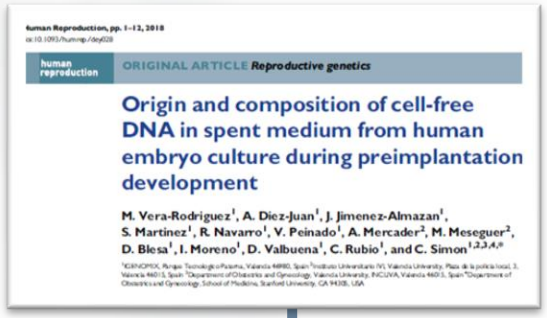
High variability between results

Embryo mosaicism

Maternal DNA contamination



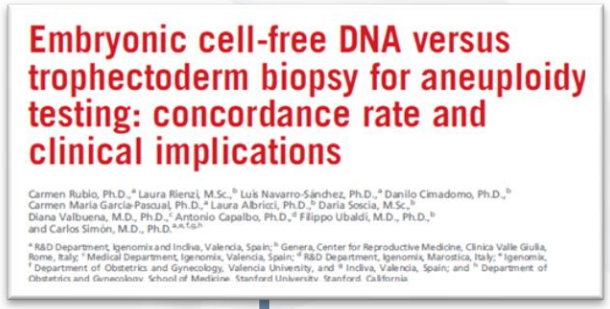
Igenomix Research and Experience


human reproduction, pp. 1–12, 2018

human reproduction ORIGINAL ARTICLE Reproductive genetics

Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development

M. Vera-Rodriguez[1], A. Diez-Juan[1], J. Jimenez-Almazan[1], S. Martinez[1], R. Navarro[1], V. Peinado[1], A. Mercader[2], M. Meseguer[2], D. Blesa[1], I. Moreno[1], D. Valbuena[1], C. Rubio[1], and C. Simon[1,2,3,4,5]


Embryonic cell-free DNA versus trophectoderm biopsy for aneuploidy testing: concordance rate and clinical implications

Carmen Rubio, Ph.D.,[a] Laura Rienzi, M.Sc.,[b] Luis Navarro-Sánchez, Ph.D.,[a] Danilo Cimadomo, Ph.D.,[b] Carmen María García-Pascual, Ph.D.,[a] Laura Albricci, Ph.D.,[b] Daria Soscia, M.Sc.,[b] Diana Valbuena, M.D., Ph.D.,[c] Antonio Capalbo, Ph.D.,[d] Filippo Ubaldi, M.D., Ph.D.,[b] and Carlos Simón, M.D., Ph.D.[a,e,f,g,h]

2018 2019 2020

Proof of concept
- Presence of cfDNA
- Concordance
- MCC

Pilot Study
- Improved Protocols
- Timing for collection
- Clinical Impact

Multicenter prospective study
- Concordance among centers
- Impact of culture conditions
- Concordance with ICM

Our experience in embryo cfDNA analysis: PROOF OF CONCEPT

Human Reproduction, pp. 1–12, 2018
doi:10.1093/humrep/dey028

human reproduction ORIGINAL ARTICLE *Reproductive genetics*

Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development

M. Vera-Rodriguez[1], A. Diez-Juan[1], J. Jimenez-Almazan[1], S. Martinez[1], R. Navarro[1], V. Peinado[1], A. Mercader[2], M. Meseguer[2], D. Blesa[1], I. Moreno[1], D. Valbuena[1], C. Rubio[1], and C. Simon[1,2,3,4,*]

[1]IGENOMIX, Parque Tecnologico Paterna, Valencia 46980, Spain [2]Instituto Universitario IVI, Valencia University, Plaza de la policia local, 3, Valencia 46015, Spain [3]Department of Obstetrics and Gynecology, Valencia University, INCLIVA, Valencia 46015, Spain [4]Department of Obstetrics and Gynecology, School of Medicine, Stanford University, CA 94305, USA

*Correspondence address. E-mail: carlos.simon@genomix.com

Submitted on October 29, 2017; resubmitted on December 27, 2017; accepted on January 26, 2018

STUDY QUESTION: What is the origin and composition of cell-free DNA in human embryo spent culture media?

SUMMARY ANSWER: Cell-free DNA from human embryo spent culture media represents a mix of maternal and embryonic DNA, and the mixture can be more complex for mosaic embryos.

WHAT IS KNOWN ALREADY: In 2016, ~300 000 human embryos were chromosomally and/or genetically analyzed using preimplantation genetic testing for aneuploidies (PGT-A) or monogenic disorders (PGT-M) before transfer into the uterus. While progress in genetic techniques has enabled analysis of the full karyotype in a single cell with high sensitivity and specificity, these approaches still require an embryo biopsy. Thus, non-invasive techniques are sought as an alternative.

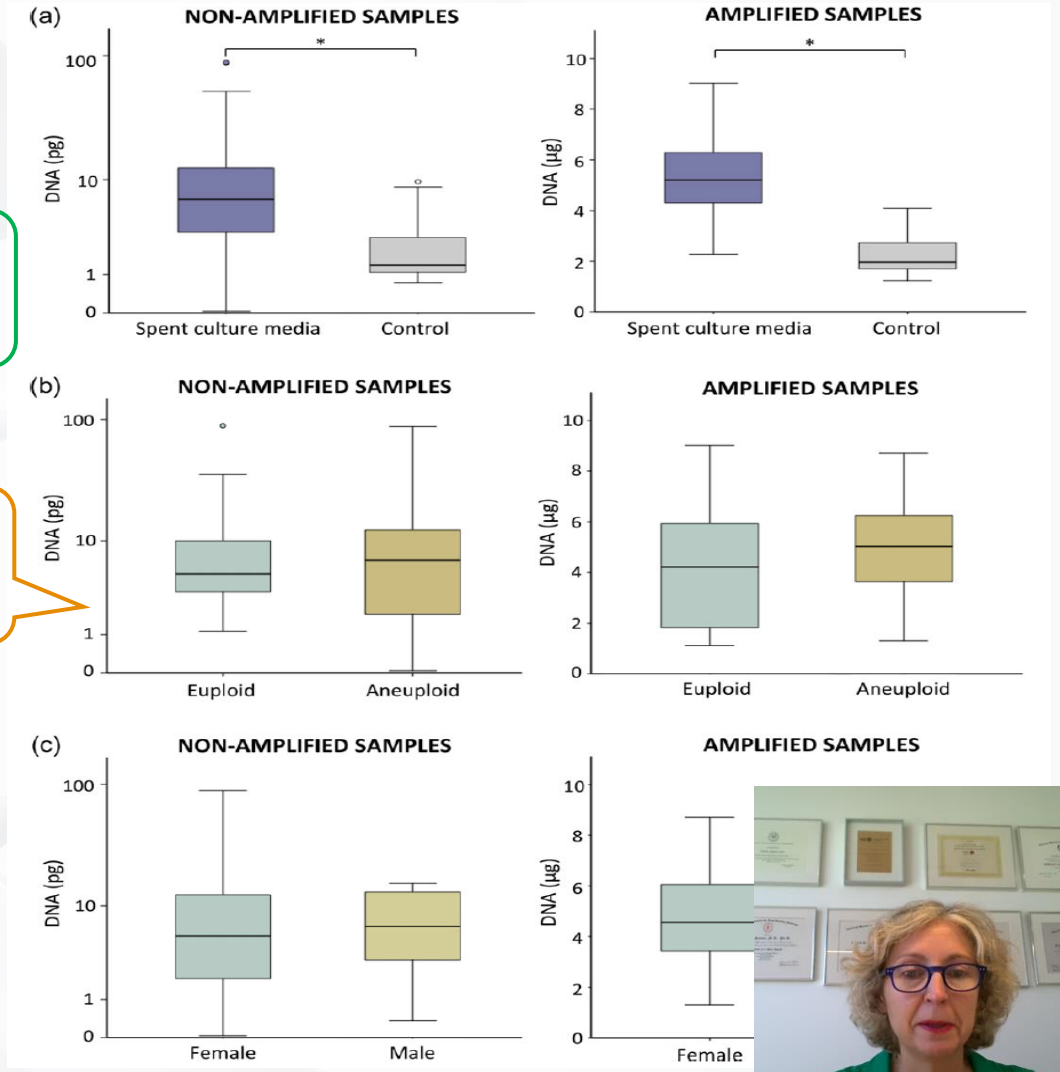
STUDY DESIGN, SIZE, DURATION: This study was based on a total of 113 human embryos undergoing trophectoderm biopsy as part of PGT-A analysis. For each embryo, the spent culture media used between Day 3 and Day 5 of development were collected for cell-free DNA analysis. In addition to the 113 spent culture media samples, 28 media drops without embryo contact were cultured in parallel under the same conditions to use as controls. In total, 141 media samples were collected and divided into two groups: one for direct DNA quantification (53 spent culture media and 17 controls), the other for whole-genome amplification (60 spent culture media and 11 controls) and subsequent quantification. Some samples with amplified DNA ($N = 56$) were used for aneuploidy testing by next-generation sequencing; of those, 35 samples underwent single-nucleotide polymorphism (SNP) sequencing to detect maternal contamination. Finally, from the 35 spent culture media analyzed by SNP sequencing, 12 whole blastocysts were analyzed by fluorescence *in situ* hybridization (FISH) to determine the level of mosaicism in each embryo, as a possible origin for discordance between sample types.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Trophectoderm biopsies and culture media samples (20 µl) underwent whole-genome amplification, then libraries were generated and sequenced for an aneuploidy study. For SNP sequencing, triads including trophectoderm DNA, cell-free DNA, and follicular fluid DNA were analyzed. In total, 124 SNPs were included with 90 SNPs distributed among all autosomes and 34 SNPs located on chromosome Y. Finally, 12 whole blastocysts were fixed and individual cells were analyzed by FISH using telomeric/centromeric probes for the affected chromosomes.

MAIN RESULTS AND THE ROLE OF CHANCE: We found a higher quantity of cell-free DNA in spent culture media co-cultured with embryos versus control media samples ($P \leq 0.001$). The presence of cell-free DNA in the spent culture media enabled a chromosomal diagnosis, although results differed from those of trophectoderm biopsy analysis in most cases (67%). Discordant results were mainly attributable to a high percentage of maternal DNA in the spent culture media, with a median percentage of embryonic DNA estimated at 8%. Finally,

Higher [DNA] in spent culture media than in controls → DNA secreted by the embryo

[DNA] is similar in both euploid aneuploid TE → No apoptotic origin?



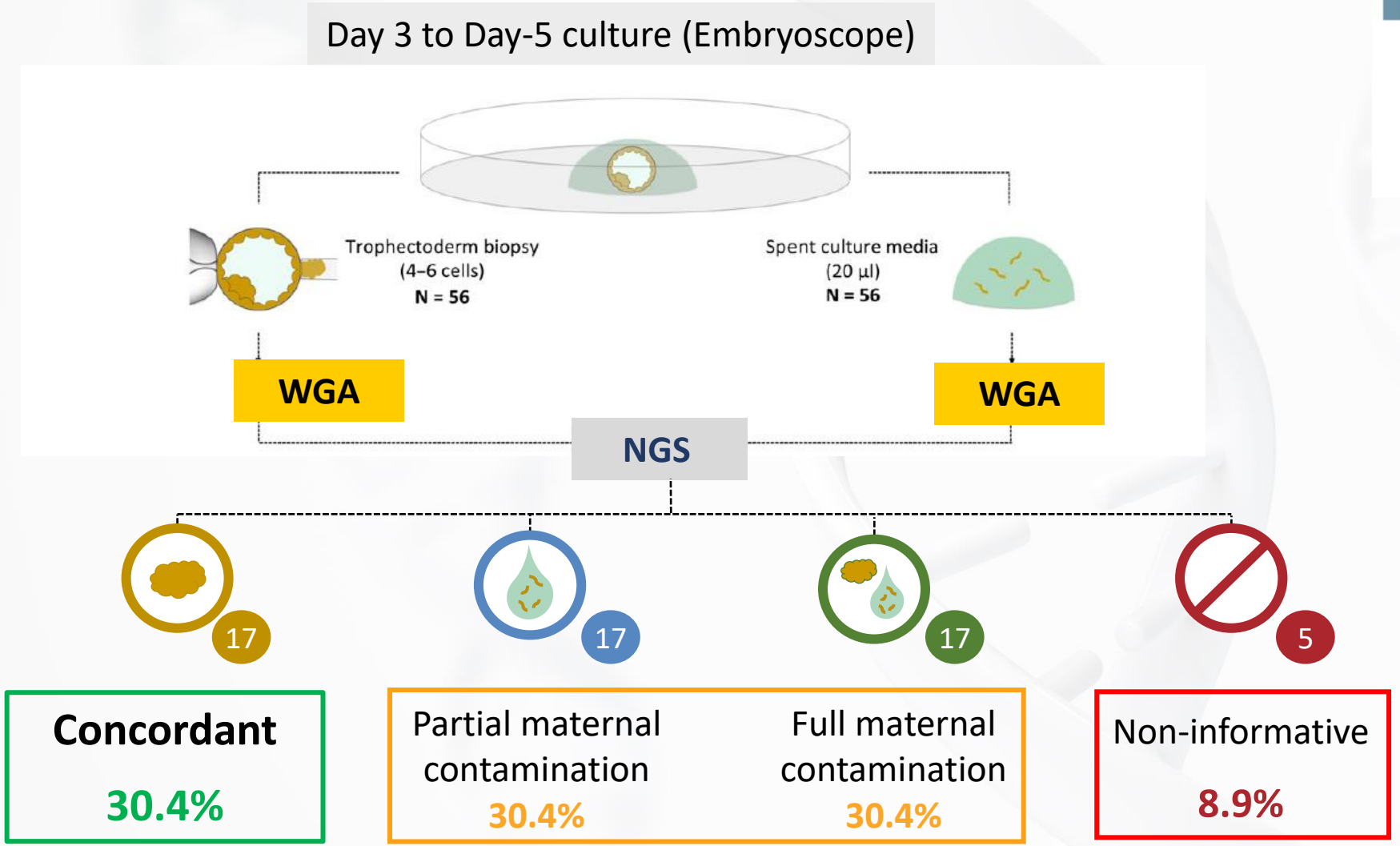
Our experience in embryo cfDNA analysis: PROOF OF CONCEPT

Human Reproduction, pp. 1–12, 2018
doi:10.1093/humrep/dey028

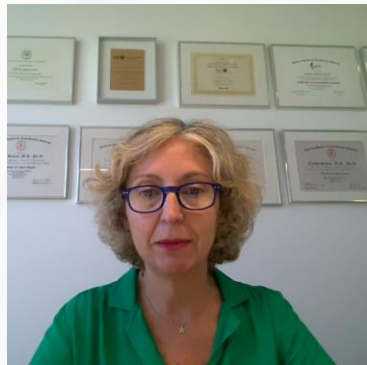
human reproduction ORIGINAL ARTICLE *Reproductive genetics*

Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development

M. Vera-Rodriguez[1], A. Diez-Juan[1], J. Jimenez-Almazan[1], S. Martinez[1], R. Navarro[1], V. Peinado[1], A. Mercader[2], M. Meseguer[2], D. Blesa[1], I. Moreno[1], D. Valbuena[1], C. Rubio[1], and C. Simon[1,2,3,4,*]



WGA: Whole Genome Amplification
NGS: Next Generation Sequencing



Our experience in embryo cfDNA analysis: PILOT STUDY

Check for updates

Embryonic cell-free DNA versus trophectoderm biopsy for aneuploidy testing: concordance rate and clinical implications

Carmen Rubio, Ph.D.,[a] Laura Rienzi, M.Sc.,[b] Luis Navarro-Sánchez, Ph.D.,[a] Danilo Cimadomo, Ph.D.,[b] Carmen María García-Pascual, Ph.D.,[a] Laura Albricci, Ph.D.,[b] Daria Soscia, M.Sc.,[b] Diana Valbuena, M.D., Ph.D.,[c] Antonio Capalbo, Ph.D.,[d] Filippo Ubaldi, M.D., Ph.D.,[b] and Carlos Simón, M.D., Ph.D.[a,e,f,g,h]

[a] R&D Department, Igenomix and Incliva, Valencia, Spain; [b] Genera, Center for Reproductive Medicine, Clinica Valle Giulia, Rome, Italy; [c] Medical Department, Igenomix, Valencia, Spain; [d] R&D Department, Igenomix, Marostica, Italy; [e] Igenomix, [f] Department of Obstetrics and Gynecology, Valencia University, and [g] Incliva, Valencia, Spain; and [h] Department of Obstetrics and Gynecology, School of Medicine, Stanford University, Stanford, California

Objective: To study whether embryonic cell-free DNA (cfDNA) in spent blastocyst media is representative of the chromosomal constitution of a blastocyst.
Design: Pilot prospective blinded study.
Setting: In vitro fertilization center and genetics laboratory.
Patient(s): A total of 115 trophectoderm (TE) biopsies and spent blastocyst media (SBM) from 46 patients with ages ranging from 32 to 46 years, whose indications for preimplantation genetic testing of aneuploidy (PGT-A) were advanced maternal age, recurrent miscarriage, or recurrent implantation failure.
Intervention(s): Spent blastocyst media collection and TE biopsy.
Main Outcome Measure(s): Concordance rates, sensitivity, and specificity between TE biopsies and SBM. Clinical outcomes in cases with euploid TE biopsies and euploid SBM compared with cases with euploid TE and aneuploid SBM.
Result(s): In general, the total concordance rate for ploidy and sex was 78.7%, and sensitivity and specificity were 94.5% and 71.7%, respectively. A significant increase for all parameters was observed for day 6/7 samples compared with day 5 samples, with day 6/7 samples showing total concordance for ploidy and sex of 84%, and sensitivity and specificity of 95.2% and 82.1%, respectively. Ongoing implantation rates in euploid TE/euploid SBM showed a threefold increase compared with euploid TE/aneuploid SBM (52.9% vs. 16.7%, respectively), without reaching significant differences. Interestingly, no miscarriages were observed when TE and SBM were euploidy concordant.
Conclusion(s): These results offer a better understanding of the dynamics of cfDNA during embryo development and despite more basic research being needed, they are reassuring to consider in the future this noninvasive approach as an alternative to TE biopsy for PGT-A. (Fertil Steril® 2019;112:510–9. ©2019 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Embryo, spent blastocyst media, trophectoderm biopsy, aneuploidy, noninvasive PGT-A

Received February 26, 2019; revised April 24, 2019; accepted April 25, 2019; published online June 11, 2019.
C.R. has nothing to disclose. L.R. has nothing to disclose. L.N.-S. has nothing to disclose. D.C. has nothing to disclose. C.M.G.-P. has nothing to disclose. L.A. has nothing to disclose. D.S. has nothing to disclose. D.V. has nothing to disclose. A.C. has nothing to disclose. F.U. has nothing to disclose. C.S. reports personal fees from Igenomix as head of Scientific Advisory Board.
C.R. and L.R. and F.U. and C.S. should be considered similar in author order.
Supported by Igenomix and Genera Centers for Reproductive Medicine.
Reprint requests: Carmen Rubio, Ph.D., Igenomix Valencia, Ronda Narciso Monturiol, 11 B, Parque Tecnológico Paterna, 46980, Paterna, Valencia, Spain (E-mail: carmen.rubio@igenomix.com).

Fertility and Sterility® Vol. 112, No. 3, September 2019 0015-0282/$36.00

https://doi.org/10.1016/j.fertnstert.2019.04.038

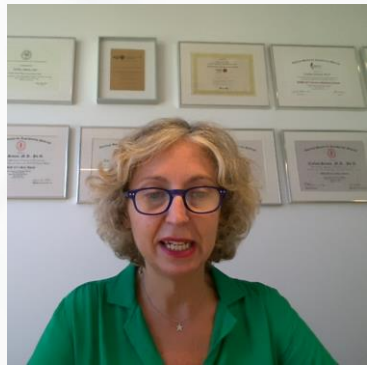
The high incidence of aneuploid embryos in IVF (ranging from 20% to 100%) is an important biologic burden [1–3]. Preimplantation genetic testing for aneuploidy (PGT-A) is, at present, the most reliable method to assess the chromosomal status of preimplantation embryos. Currently, DNA isolated and amplified from trophectoderm (TE) biopsies and

AIMS:

1) To improve IVF conditions and NGS protocol

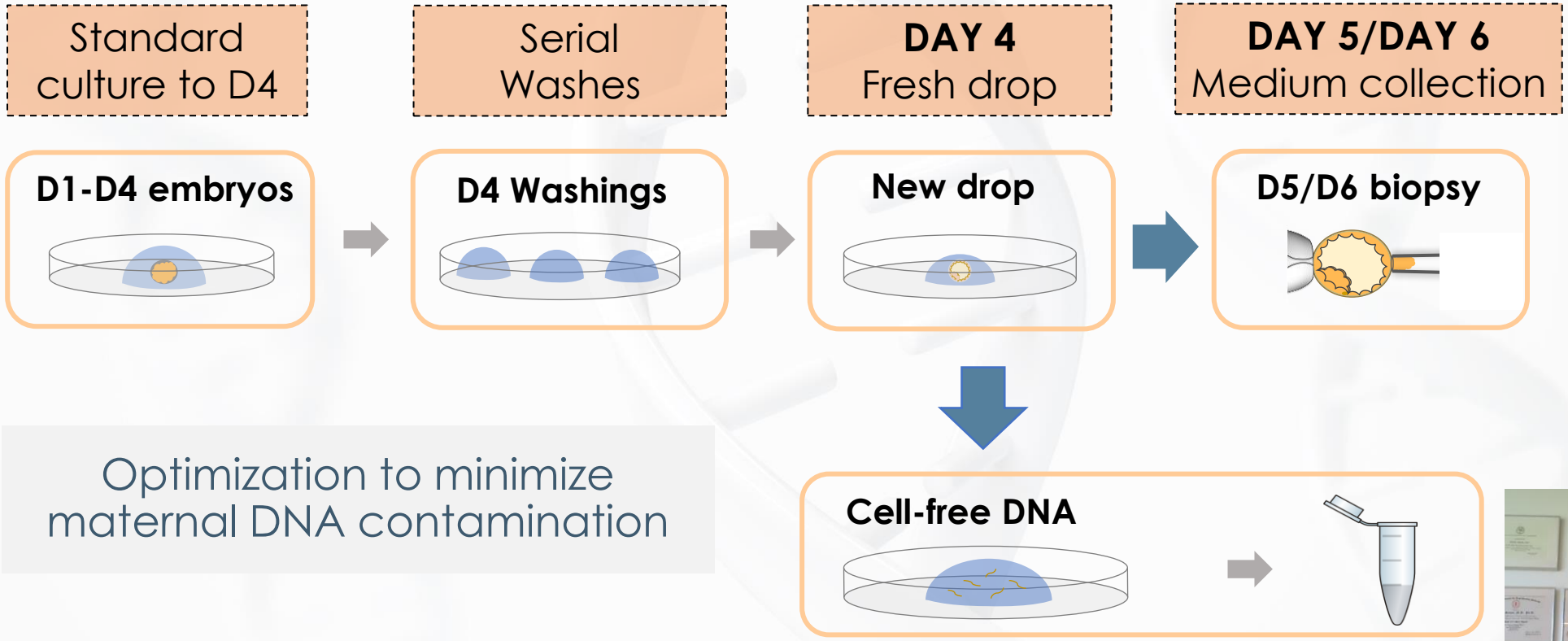
2) To estimate the optimal time for media collection

3) To assess the impact in clinical outcome



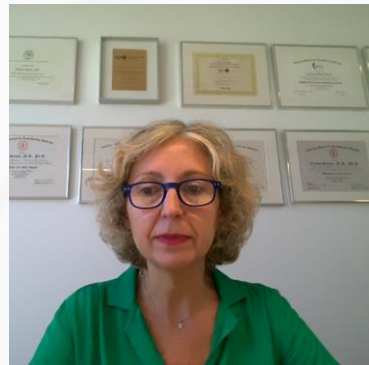
Our experience in embryo cfDNA analysis: PILOT STUDY

Prospective pilot study by Igenomix-Genera (November 2017 - March 2018)
in **115** blastocysts to define optimum time for media collection with the new protocol



Optimization to minimize maternal DNA contamination

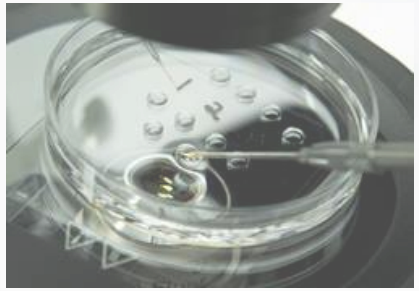
Rubio & Rienzi et al., Fertil Steril 2019



Our experience in embryo cfDNA analysis: PILOT STUDY

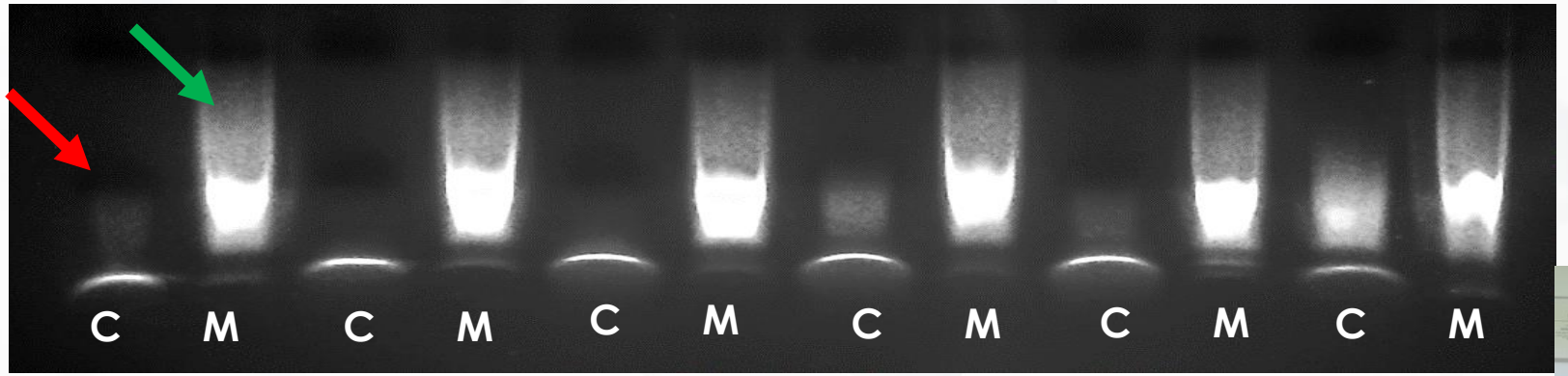
Modified Reproseq protocol <15 h
Ion Reporter 5.4

Ion Torrent™ Technology (S5)

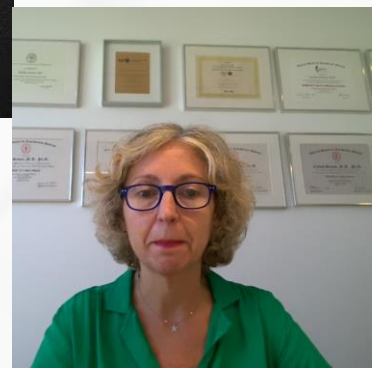


Elecrophoresis Gel

WGA with conventional and modified protocol



Time in culture: 30h | 30h | 30h | 48h | 48h | 72h

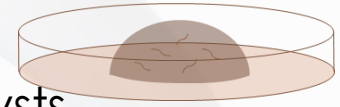


Rubio & Rienzi et al., Fertil Steril 2019

Our experience in embryo cfDNA analysis: PILOT STUDY

Concordance rates of TE biopsies versus cfDNA

N=108 blastocysts



Consistent results with media collected on D6

RESULTS DAY 5

TE BIOPSY vs. SBM

(N=27)

INFORMATIVITY	CONCORDANCE
81.8%	63%

RESULTS DAY 6

TE BIOPSY vs. SBM

(N=81)

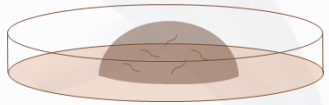
INFORMATIVITY	CONCORDANCE
100%	84%

Rubio & Rienzi et al., Fertil Steril 2019

Our experience in embryo cfDNA analysis: PILOT STUDY

Discordant results between TE biopsies and cfDNA

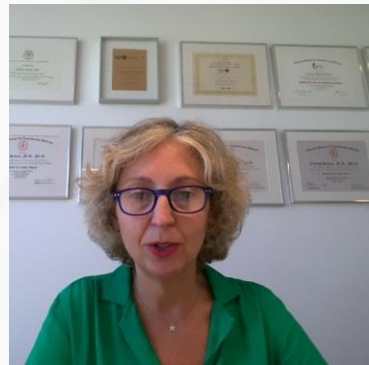
N=81 blastocysts



Day 6 concordance: TE biopsy vs. SBM

CONCORDANT RESULTS	CONCORDANT RESULTS With different gender
84%	5%

FALSE NEGATIVES: Aneuploid biopsy →Euploid media	FALSE POSITIVES: Euploid biopsy →Aneuploid media
2.5%	8.5%



Rubio & Rienzi et al., Fertil Steril 2019

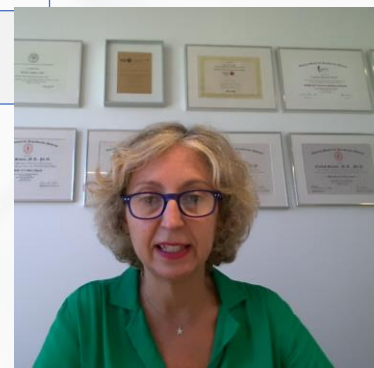
Our experience in embryo cfDNA analysis: PILOT STUDY

Clinical outcome after SET of euploid versus aneuploid SBM

Clinical Outcome	Euploid TE/ Euploid SBM	Euploid TE/ Aneuploid SBM	TOTAL
Number of transfers	17	12	29
Mean maternal age (SD)	37.5 (2.5)	37.4 (2.3)	37.5 (2.4)
Positive pregnancy test	11 (64.7)	4 (33.3)	15 (51.7)
Biochemical pregnancy loss	2 (18.2)	0	2 (13.3)
Clinical pregnancy rate (%)	9 (52.9)	4 (33.3)	13 (44.8)
Clinical miscarriage (%)	0	2 (50.0)	2 (15.4)
Ong implantation rate (%)	9 (52.9)	2 (16.7)	11 (37.9)

Lower miscarriage rates with euploid cfDNA

Rubio & Rienzi et al., Fertil Steril 2019



Our experience in embryo cfDNA analysis: MULTICENTER STUDY

Original Research ajog.org

OBSTETRICS

Multicenter prospective study of concordance between embryonic cell-free DNA and trophectoderm biopsies from 1301 human blastocysts

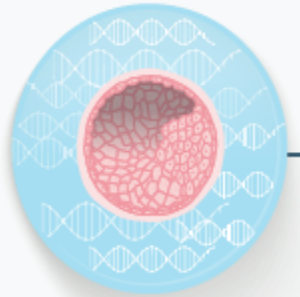
Carmen Rubio, PhD[1]; Luis Navarro-Sánchez, PhD[1]; Carmen M. García-Pascual, PhD; Olcay Ocali, BS; Danilo Cimadomo, PhD; William Venier, MSc; Gerardo Barroso, MD; Laura Kopcow, MD; Mustafa Bahçeci, MD; Marcos Iuri Roos Kulmann, BSc; Lourdes López, MD; Emilio De la Fuente, MSc; Roser Navarro, MSc; Diana Valbuena, MD, PhD; Denny Sakkas, PhD; Laura Rienzi, MSc; Carlos Simón, MD, PhD

1,301

Spent blastocyst media

This is the largest study to date assessing ploidy concordance per embryo between invasive and non-invasive PGT-A.

The study was designed with two main objectives:



Embryo cell-free DNA

Trophectoderm DNA

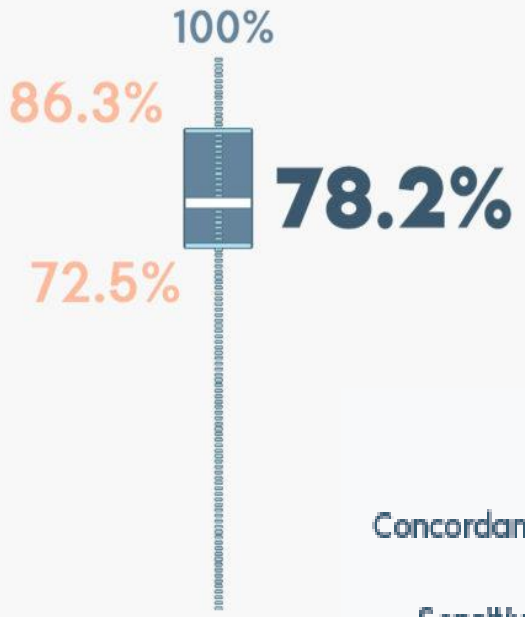
To evaluate the concordance and reproducibility of testing embryo cell-free DNA versus trophectoderm DNA obtained from the same embryo in a large sample of 1,301 day 6 and day 7 human blastocysts,

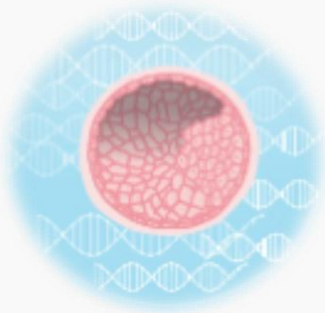
Inner cell mass DNA

and to assess the concord
with the inner cell mass of
in a subset of 81 aneuploi
donated for research.

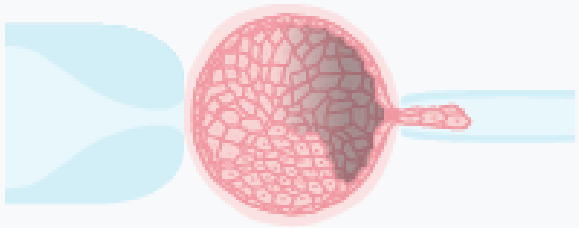
Our experience in embryo cfDNA analysis: MULTICENTER STUDY

Concordance rates of 1,301 embryo cell-free DNA and trophectoderm DNA





VS.

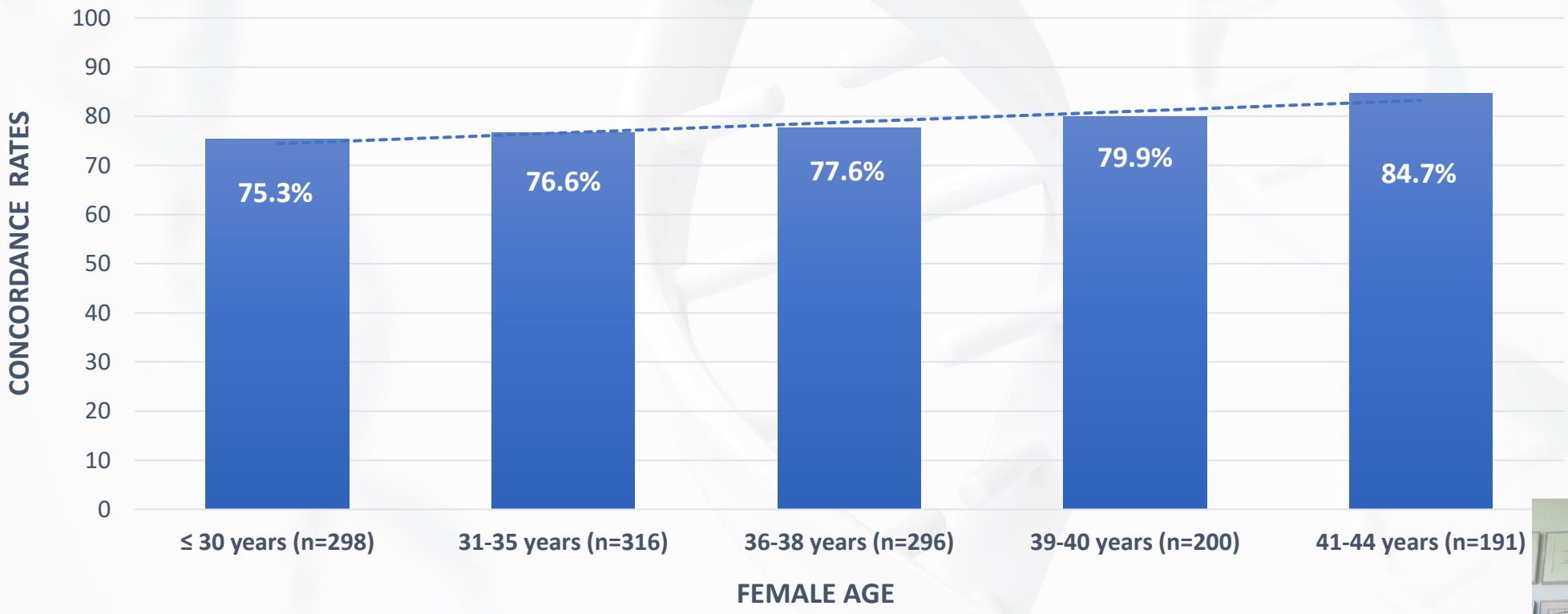


	Center 1	Center 2	Center 3	Center 4	Center 5	Center 6	Center 7	Center 8	TOTAL
Concordance	75.6	77.1	81.8	86.3	84.2	85.0	72.5	77.0	**78.2**
Sensitivity	80.5	84.8	88.2	86.7	91.3	76.7	76.5	78.9	**81.7**
Specifity	69.9	72.7	85.2	87.5	80.0	93.3	64.7	78.1	

Rubio et al: Multicenter prospective study of concordance between embryo cell-free DNA and trophectoderm biopsies from 1,301 human blastocyst. American Journal of Obstetrics and Gynecology.

Our experience in embryo cfDNA analysis: MULTICENTER STUDY

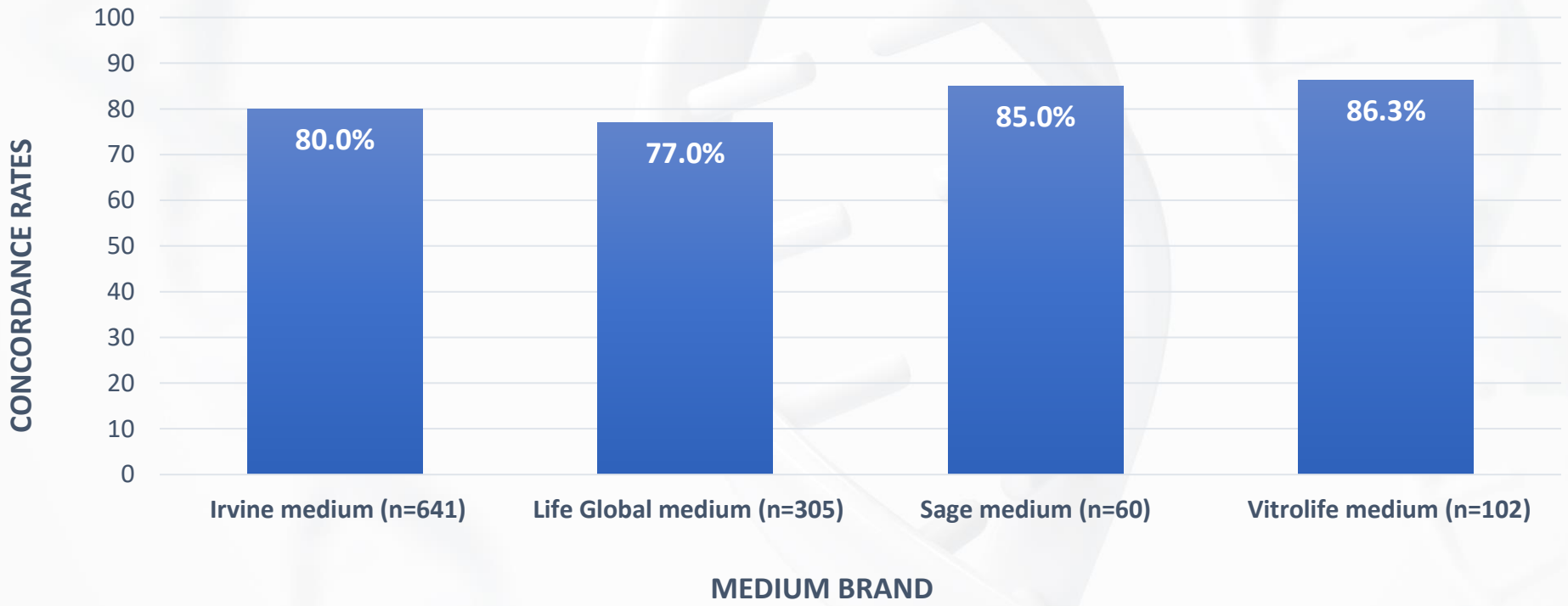
Higher concordance with higher female age: significant linear trend



Rubio et al: Multicenter prospective study of concordance between embryo cell-free DNA and trophectoderm biopsies from 1,301 human blastocyst. American Journal of Obstetrics and Gynecology.

Our experience in embryo cfDNA analysis: MULTICENTER STUDY

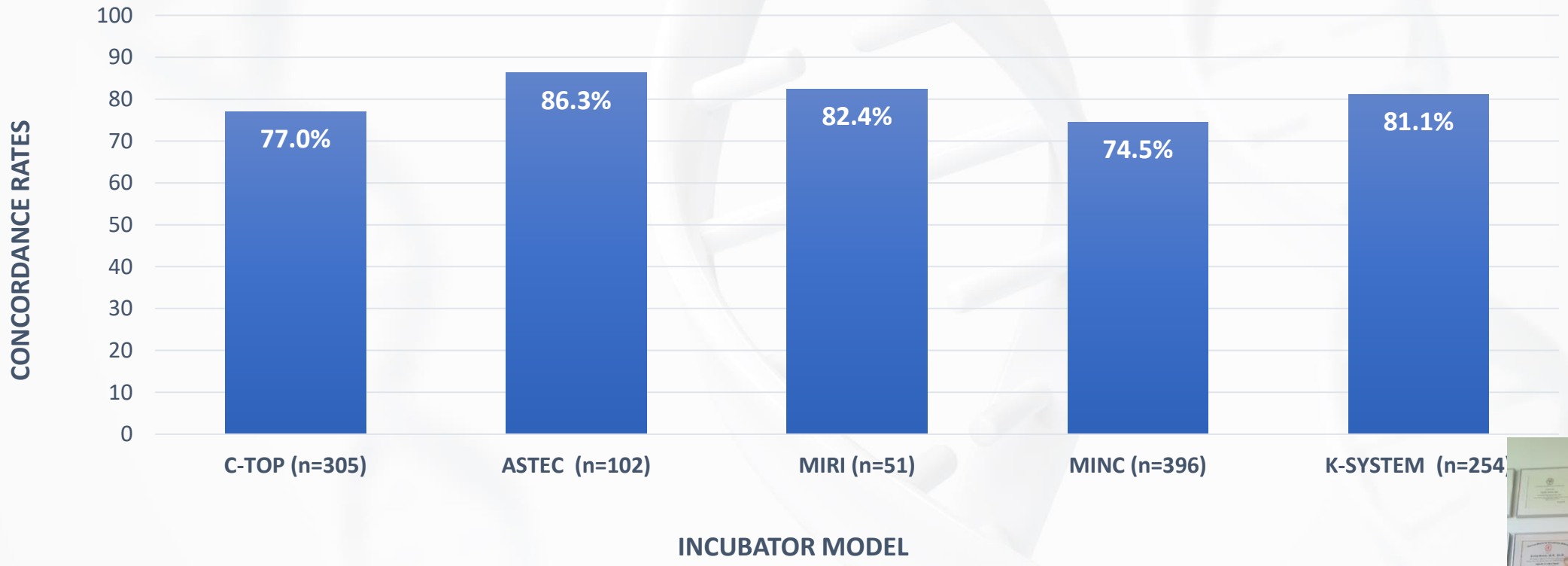
No significant differences according to the culture media



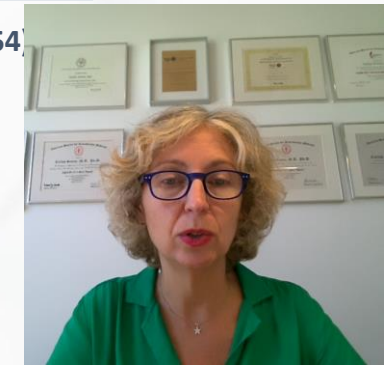
Rubio et al: Multicenter prospective study of concordance between embryo cell-free DNA and trophectoderm biopsies from 1,301 human blastocyst. American Journal of Obstetrics and Gynecology.

Our experience in embryo cfDNA analysis: MULTICENTER STUDY

No significant differences according to the incubator model

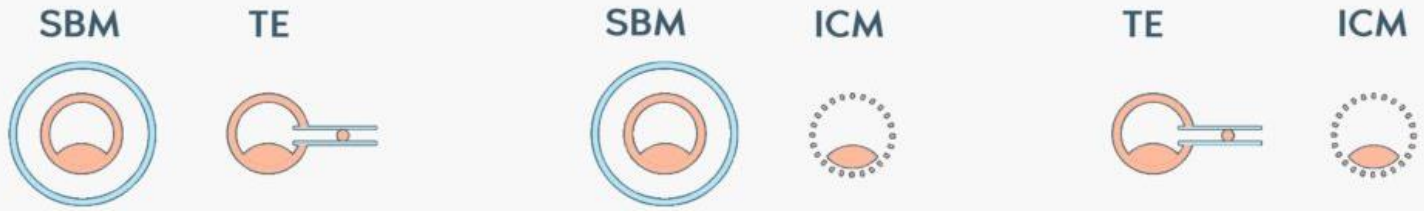


Rubio et al: Multicenter prospective study of concordance between embryo cell-free DNA and trophectoderm biopsies from 1,301 human blastocyst. American Journal of Obstetrics and Gynecology.



Our experience in embryo cfDNA analysis: MULTICENTER STUDY

In addition, in a subgroup of 81aneuploid blastocysts donated for research, the comparison of the inner cell mass with the embryo cell-free DNA and the trophectoderm biopsies has shown similar concordance rates, 84.4% and 87.5% respectively (64 informative for the three sample types).



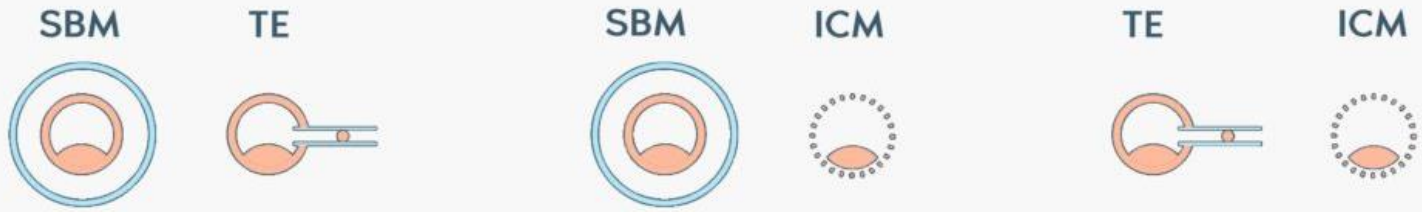
GLOBAL CONCORDANCE	**87.5%**	**84.4%**	**87.5%**
False positives	**0%**	**6.3%**	**10.9%**
False negatives	**12.5%**	**9.4%**	**1.6%**

WC: Whole Chromosome; LM: Low Mosaic; SC: Segmental Chromosome

Rubio et al: Multicenter prospective study of concordance between embryo cell-free DNA and trophectoderm biopsies from 1,301 human blastocyst. American Journal of Obstetrics and Gynecology.

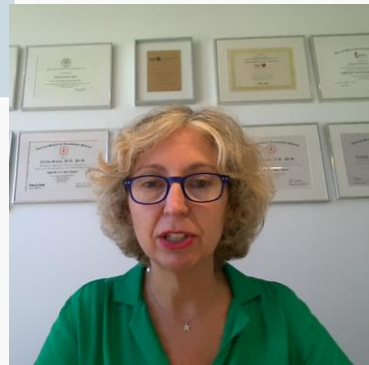
Our experience in embryo cfDNA analysis: MULTICENTER STUDY

If only whole chromosome aneuploidies of meiotic origin are considered, the comparison of the inner cell mass with the embryo cell-free DNA and the trophectoderm biopsies has shown concordance rates of 85.9% and 93.7% respectively.



Only WC Aneuploidies	**92.2%**	**85.9%**	**93.7%**
False positives	**0%**	**4.7%**	**4.7%**
False negatives	**7.8%**	**9.4%**	**1.6%**

WC: Whole Chromosome; LM: Low Mosaic; SC: Segmental Chromosome



Rubio et al: Multicenter prospective study of concordance between embryo cell-free DNA and trophectoderm biopsies from 1,301 human blastocyst. American Journal of Obstetrics and Gynecology.

Open questions? Work in progress

BASIC RESEARCH (Plachta Lab)

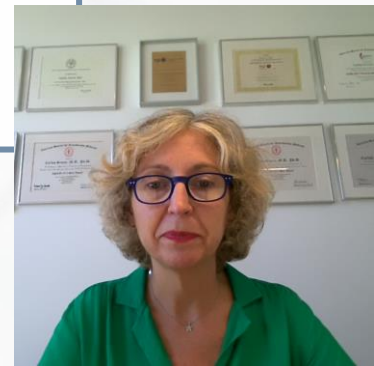
Origin of the DNA? From inner cell mass and trophectoderm cells?

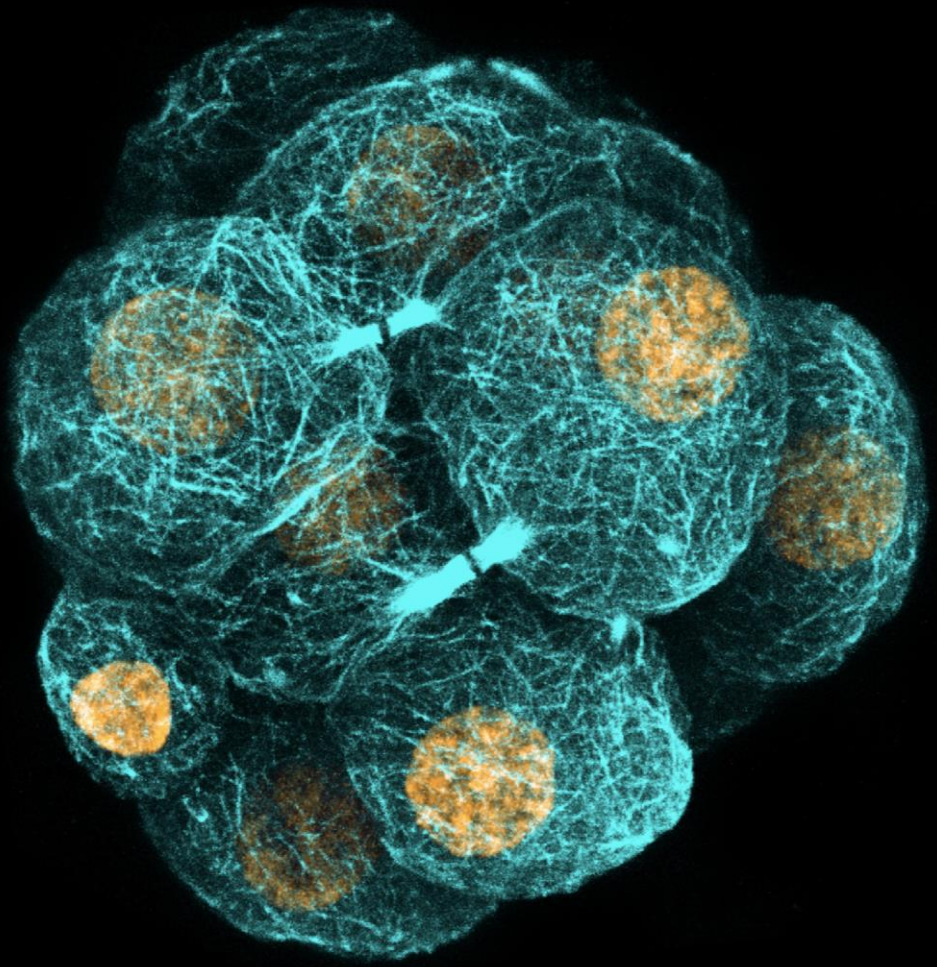
Mechanism? Different embryonic DNA replication? Tubulin bridges?

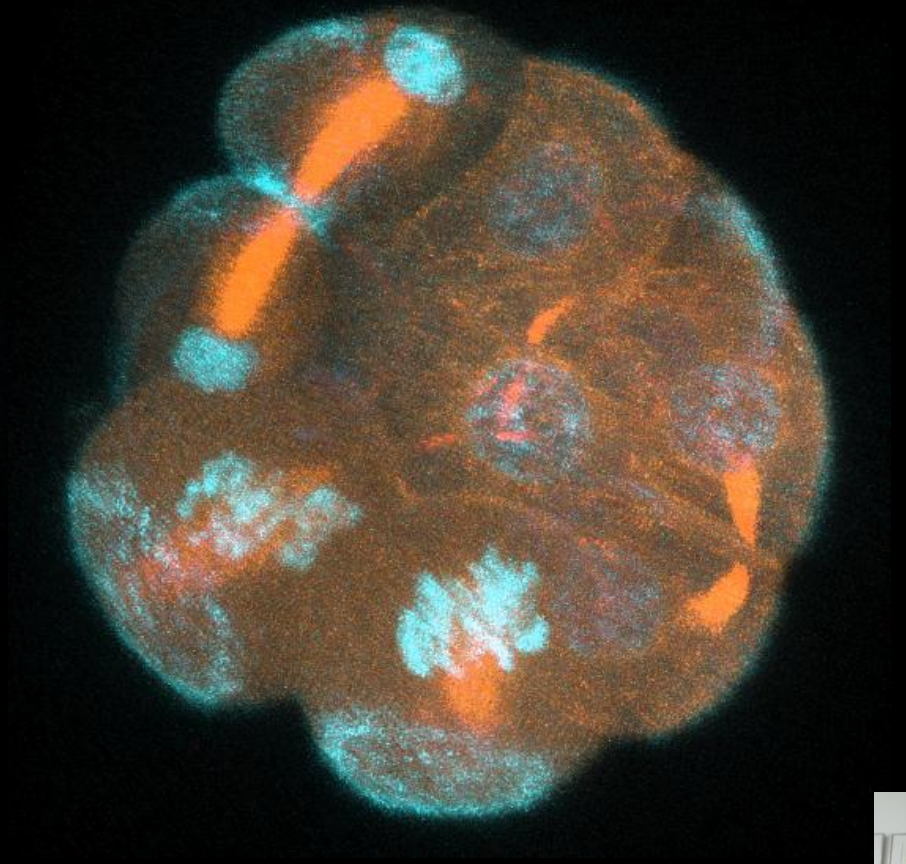
RCT STUDY on Clinical Outcome

Could be applied with the aim of improving implantation for all IVF patient?

If this is the case, could transfer prioritization be an acceptable alternative?

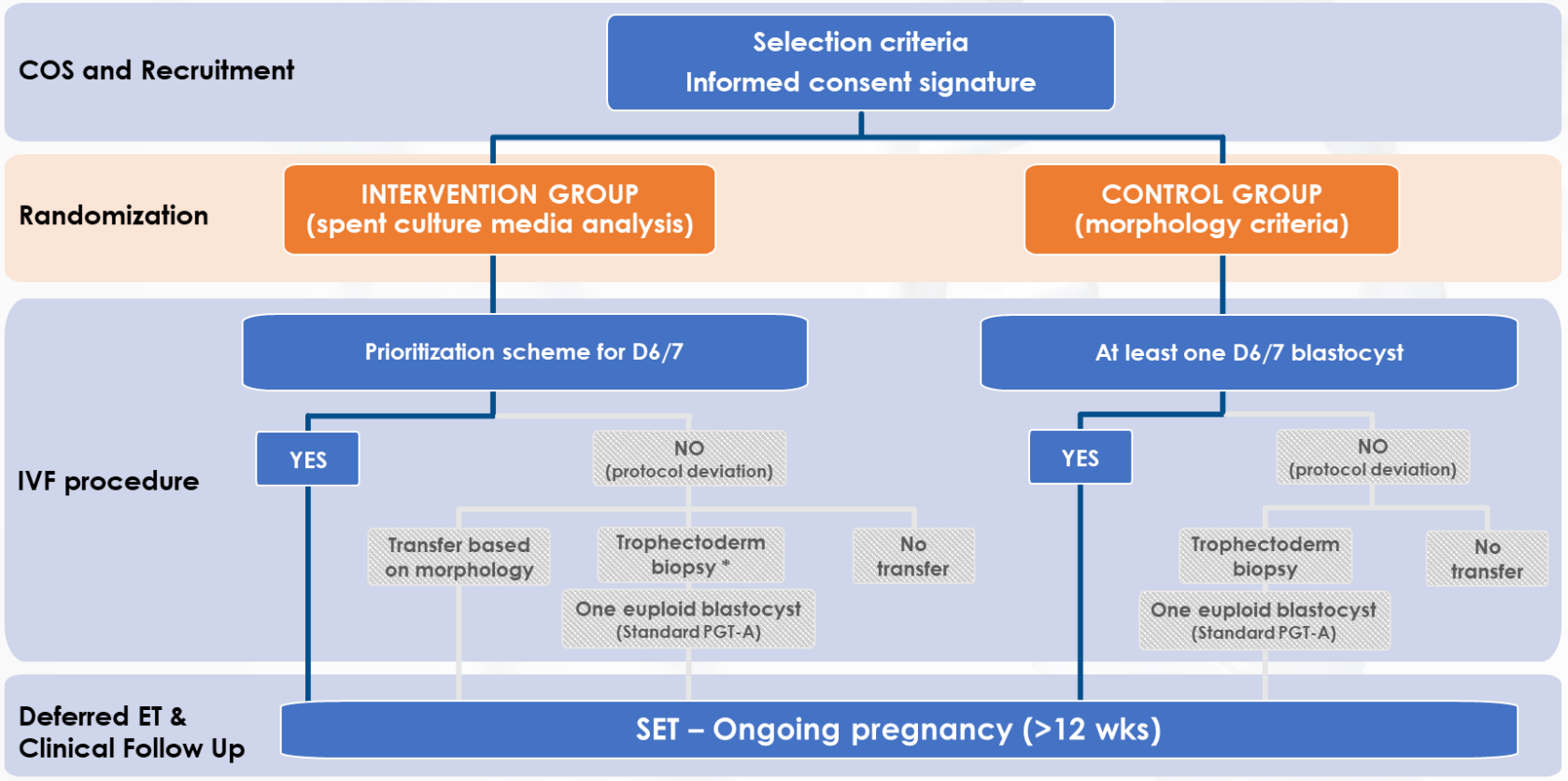






RCT (15 centers involved, n=1108 SETs)

Aim: To investigate if niPGT-A can improve ongoing pregnancy rate (≥12 gestational weeks) in the first SET compared to embryo selection based on morphology (10% improvement).



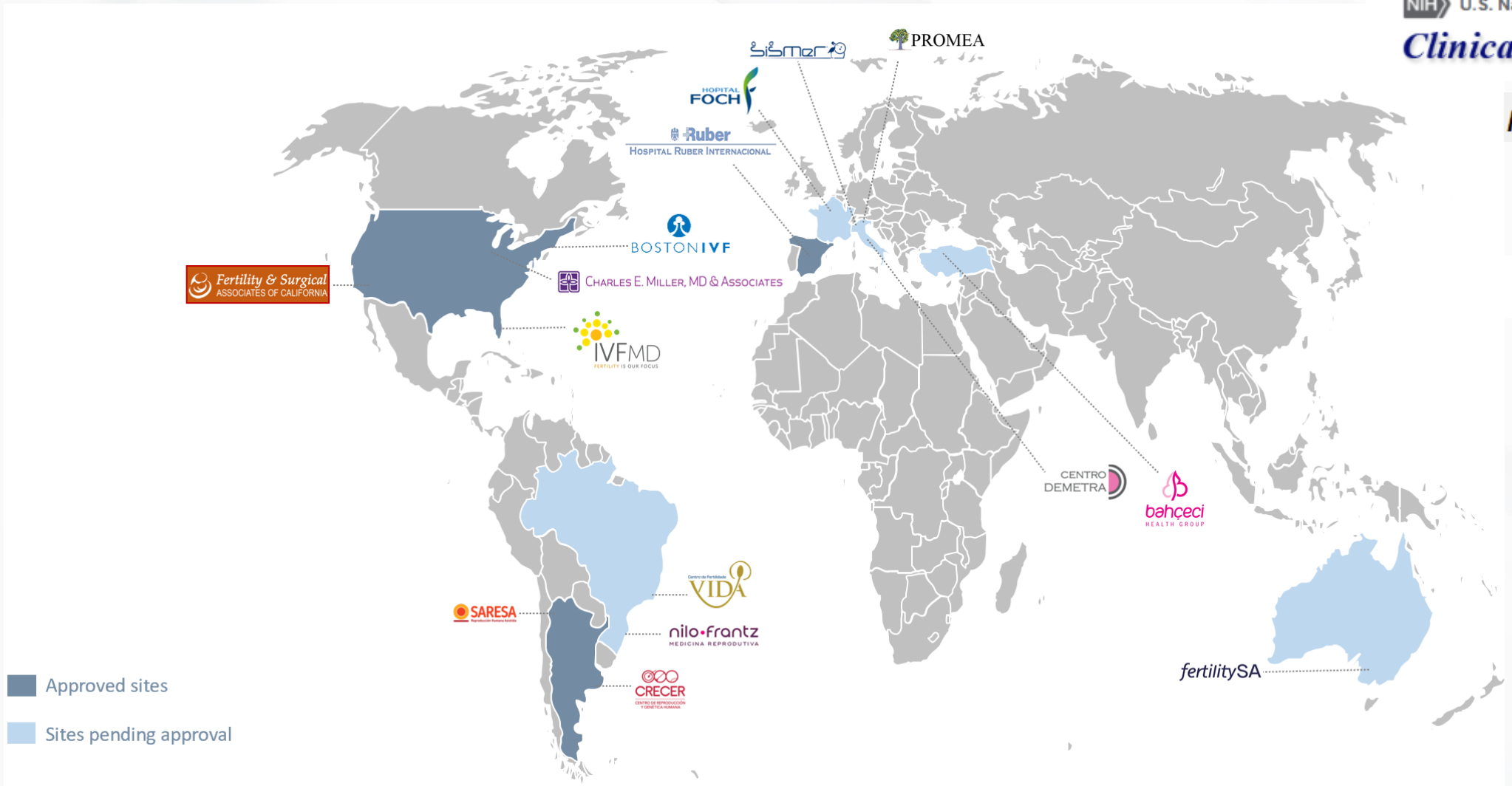
COS: Controlled ovarian stimulation
SET: Single embryo transfer
ET: Embryo transfer

*Free PGT-A by request, if trophectoderm biopsy performed

NIH U.S. National Library of Medicine
ClinicalTrials.gov

NCT04000152

RCT (15 centers involved, n=1108 SETs)



Conclusions

- The cfDNA can be amplified and analysed by NGS and it is representative of embryo chromosomal content.
- Concordance rates with TE biopsies and ICM have been found as high as 84-85% at D6 of development.
- High concordance rates of the cell-free DNA in the culture media with inner cell mass.
- This approach shows robust results with different culture conditions and incubator systems, therefore can be applied in any IVF lab without the need of additional equipment.
- This new strategy would avoid embryo biopsy and the conflict of discarding potentially euploid embryos in PGT-A.

Acknowledgements

Head of the Scientific Advisory Board of Igenomix
Carlos Simón

Clinical Studies
Carlos Gómez
Emilio De la Fuente
Marta Soledad

Research ni PGT-A
Luis Navarro
Carmen Garcia
Lucía Martínez
Carmen Rubio

Nicolás Plachta Lab