Cengiz Cinnioglu

Educational Conference & CRB Symposium

May 17-19, 2018

Disclosure

- Executive Laboratory Director at Igenomix-USA

Learning Objective

- Relationships between Mitochondrial DNA and embryo implantation
- MtDNA assays/platforms
- Review most recent published papers

Mitochondrial DNA

Still many unknowns and questions about mtDNA and embryo implantations

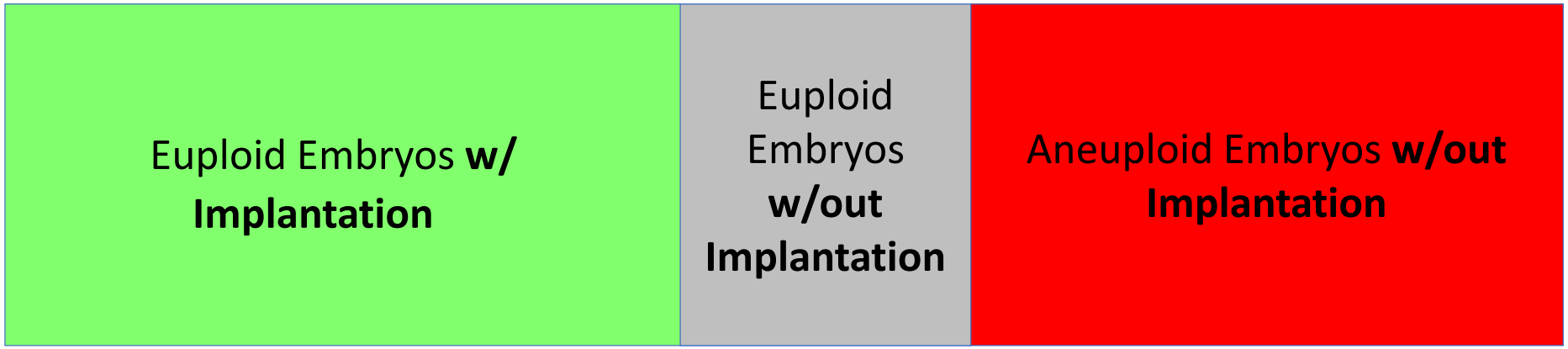
- Many positive and some negative papers on correlation between implantation and mtDNA content
- Correlation
  - mtDNDA and embryo morphology?
  - mtDNDA and pt age?
  - mtDNDA and Trophectoderm quality?
  - mtDNDA and embryo mosaicism?
- Does mtDNA helps to improve embryo implantation?

Embryo Implantation

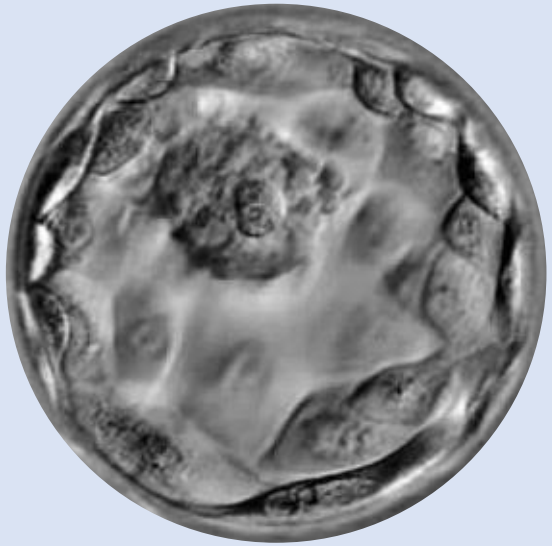
Not all embryos implant

Embryo Implantation

Not all ‘good’ embryos implant

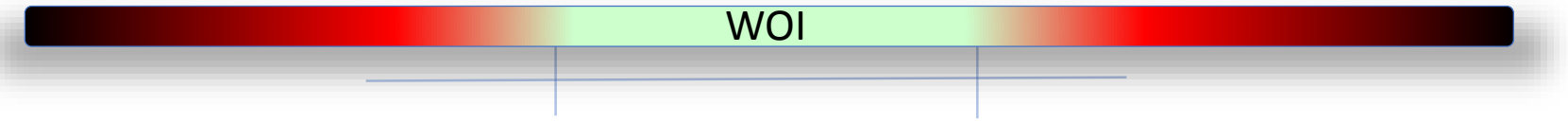


Why an Euploid Embryo DOES NOT IMPLANT?



THE ENERGY RESERVE OF THE EMBRYO.
A NEW PARADIGM

TIMING IS KEY

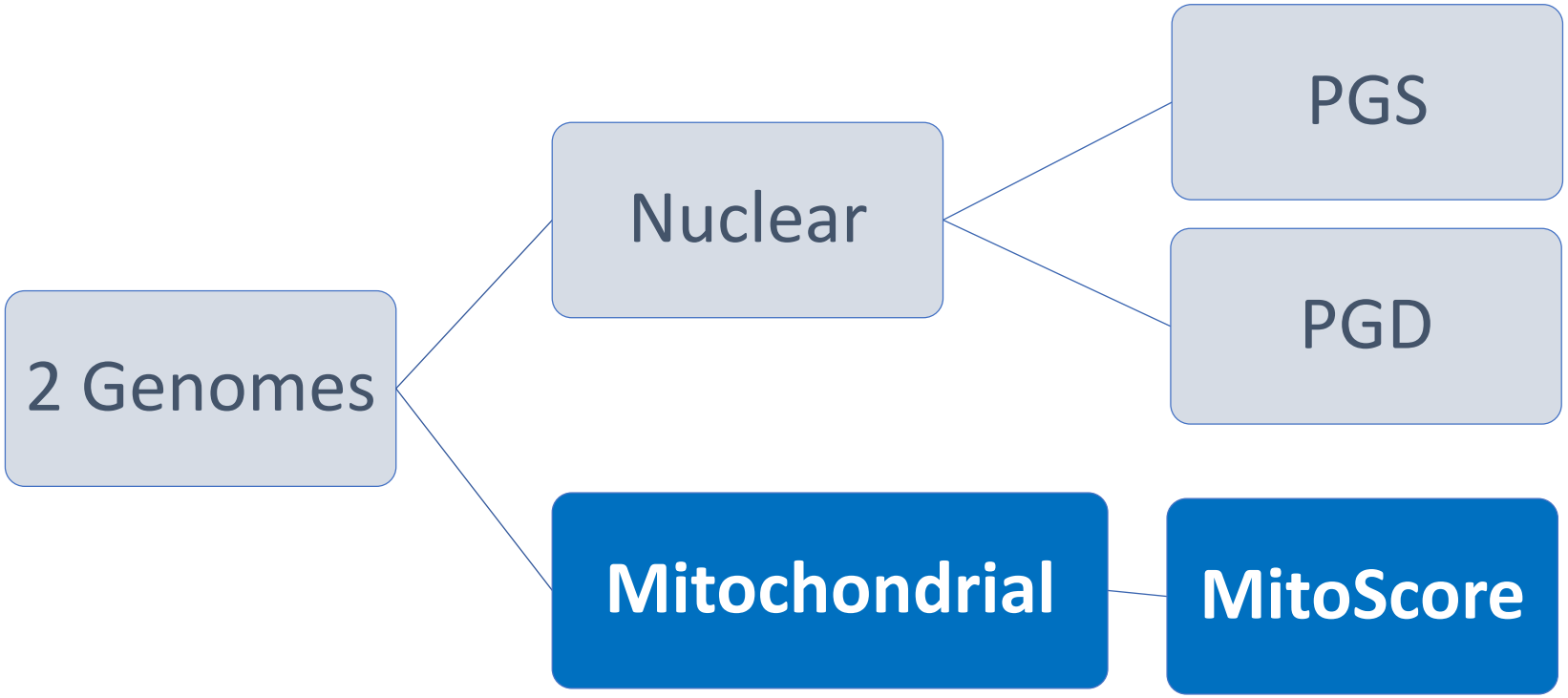


ARE WE DELIVERING THE EMBRYO AT THE WRONG TIME?

Endometrial Receptivity Assay (ERA)

Mitochondrial DNA

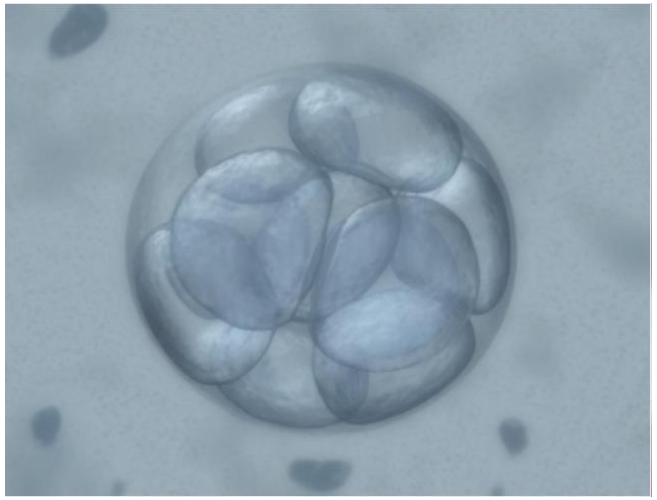
There are two genomes in the cell



Mitochondrial DNA

Mitochondrial Score (MitoScore)

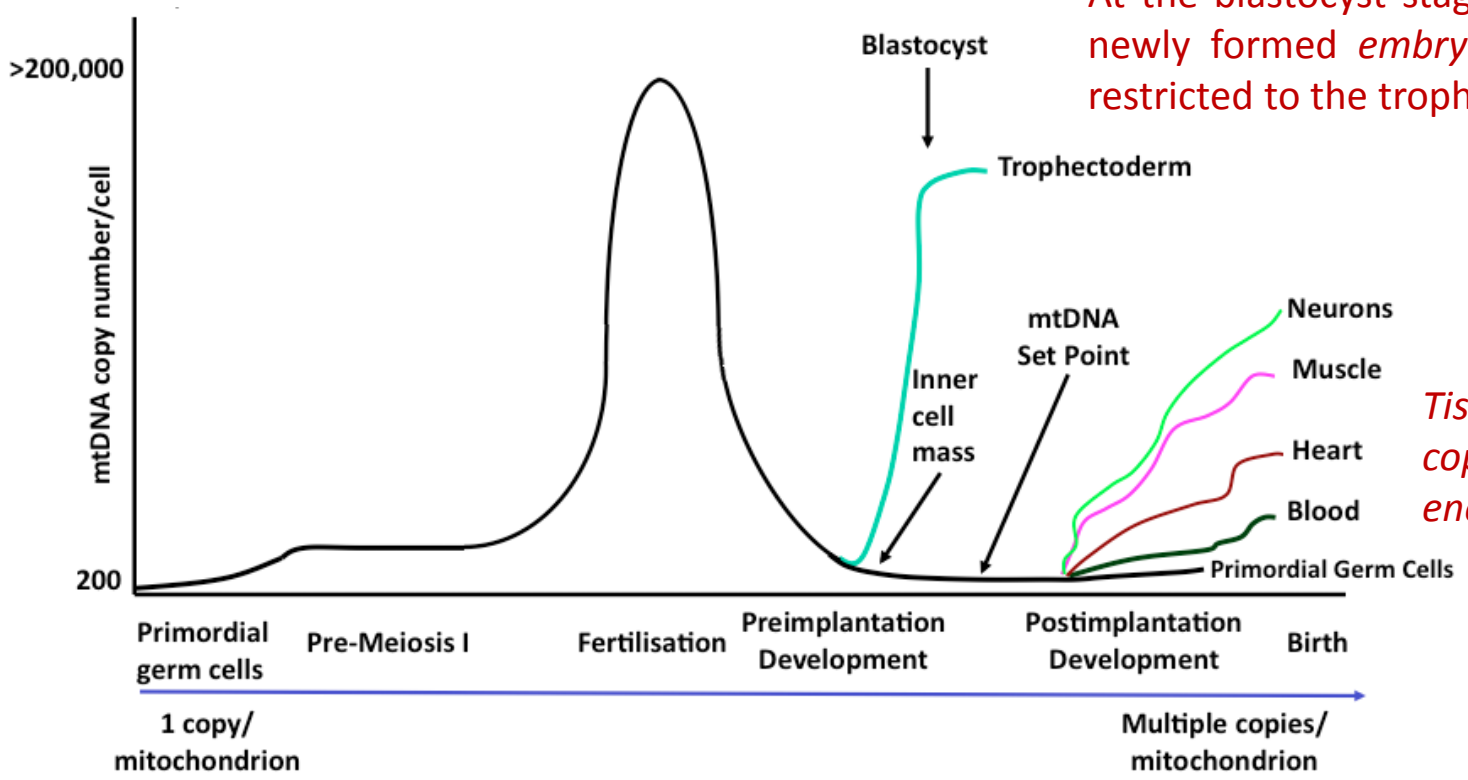
A new test to evaluate implantation potential



Mitochondrial DNA

Mitochondria in oocyte and embryo

Copy number of mtDNA increases significantly during oogenesis and then undergoes a rapid decrease during preimplantation development in larger mammals.



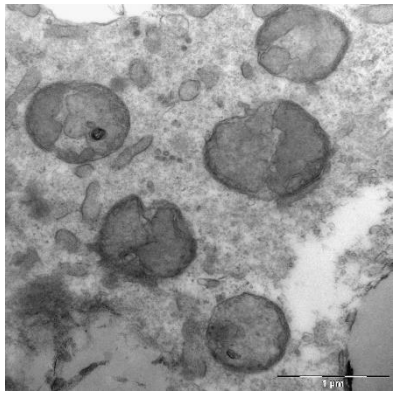
At the blastocyst stage, replication in the newly formed *embryo* is initiated but is restricted to the trophectoderm

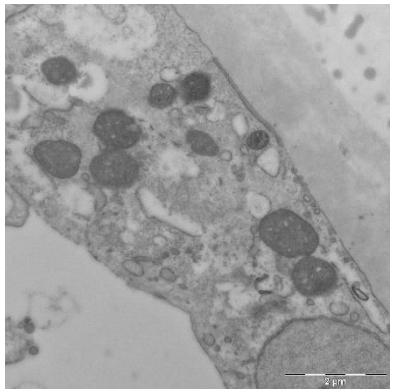
Tissue specific increase copy number to match energetic needs

Mitochondrial DNA

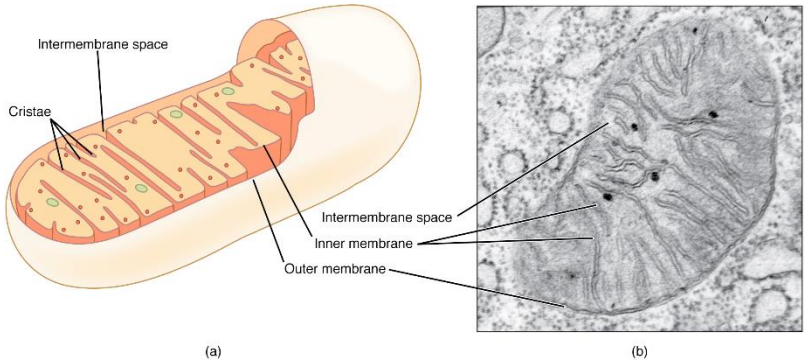
Mitochondria in the embryo

- ✓Each mitochondria in the embryo possess only one-two mtDNA molecules
- ✓Oocytes and embryos have a relatively low oxygen consumption.
- ✓Embryonic mitochondria have fewer and shorter cristae than mitochondria from metabolically active cells found in adult tissues.





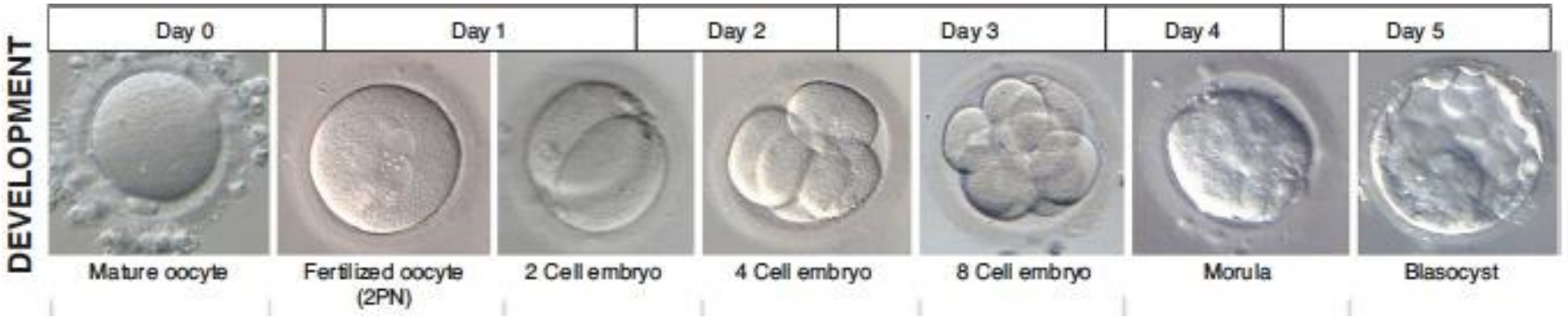
Blastocyst Mitochondria



Mature Mitochondria

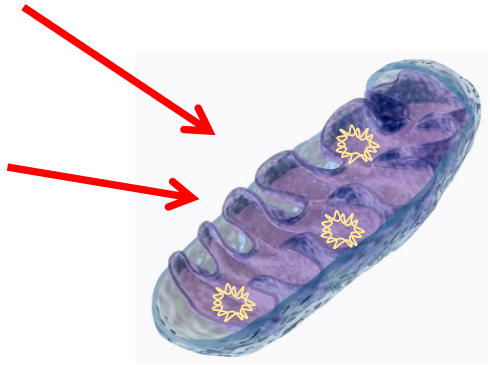
Mitochondrial DNA

Mitochondrial biogenesis is dispensable for early development



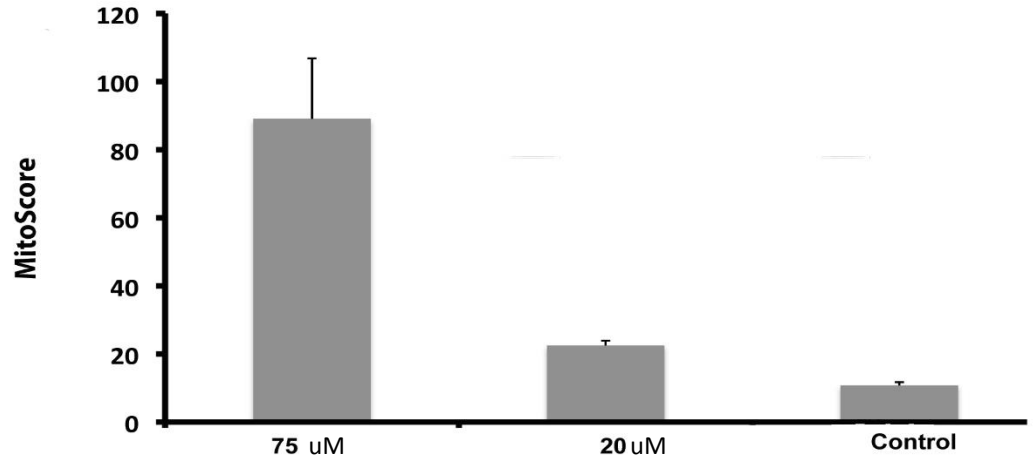
Inhibiting mitochondrial DNA replication in mouse embryos does not prevent development until around the time of implantation

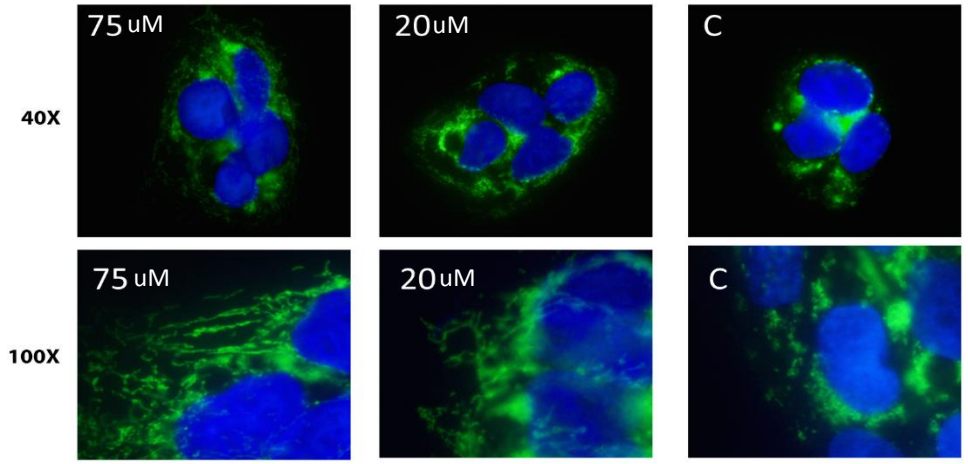
- ✓ With ethidium bromide (Piko and Chase,1973)
- ✓ Knocking out transcription factors required for mitochondrial biogenesis, (mTFA), (PGC-1) (NRF1) (Huo and Scarpulla, 2001; Johnson et al., 2003; Larssonet al., 1998).



Mitochondrial DNA

Energetic stress increases Ms and induces mitochondrial changes in a trophoblast cell line (JaR)





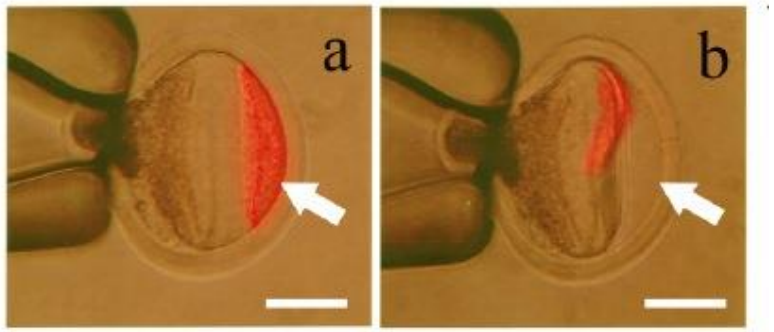
Energetic stress induced pharmacologically using 2,4-Dinitrophenol (DNP) increases the Ms and produces morphological mitochondrial changes.

After 48 hours, mtDNA content and mitochondrial morphology were analyzed, revealing a higher Ms and increased mitochondrial size.

Mitochondrial DNA

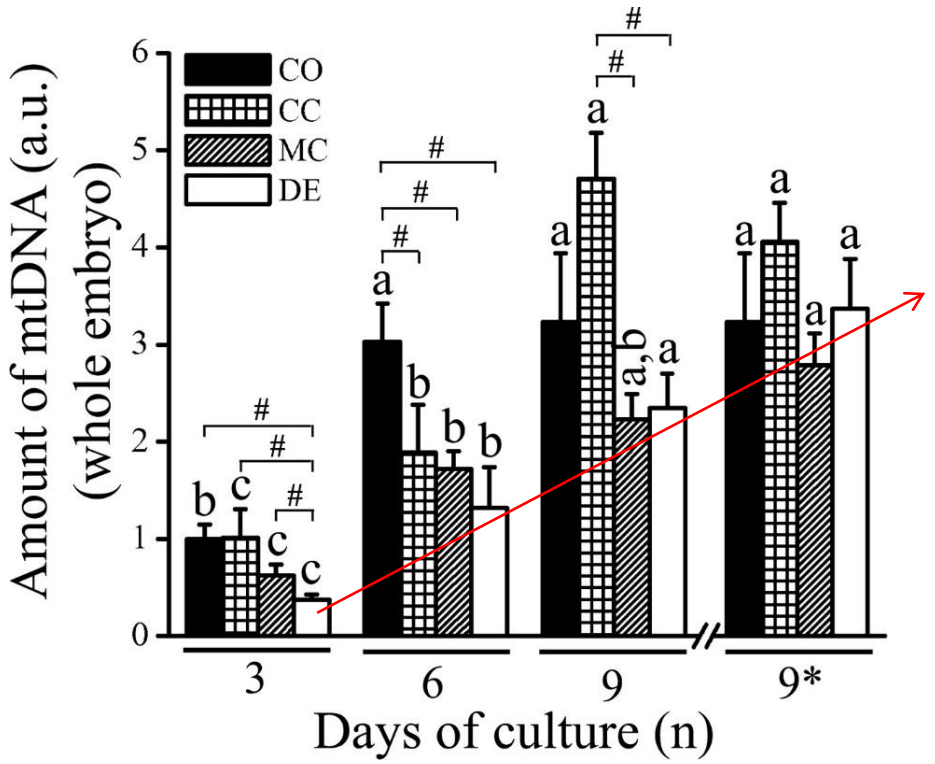
Embryos can activate mitochondrial biogenesis

Mitochondrial depleted embryos replenish mtDNA by increasing replication.



(Bovine oocytes)

Marcos R. Chiaratti et al.
Biol Reprod 2010;82:76-85

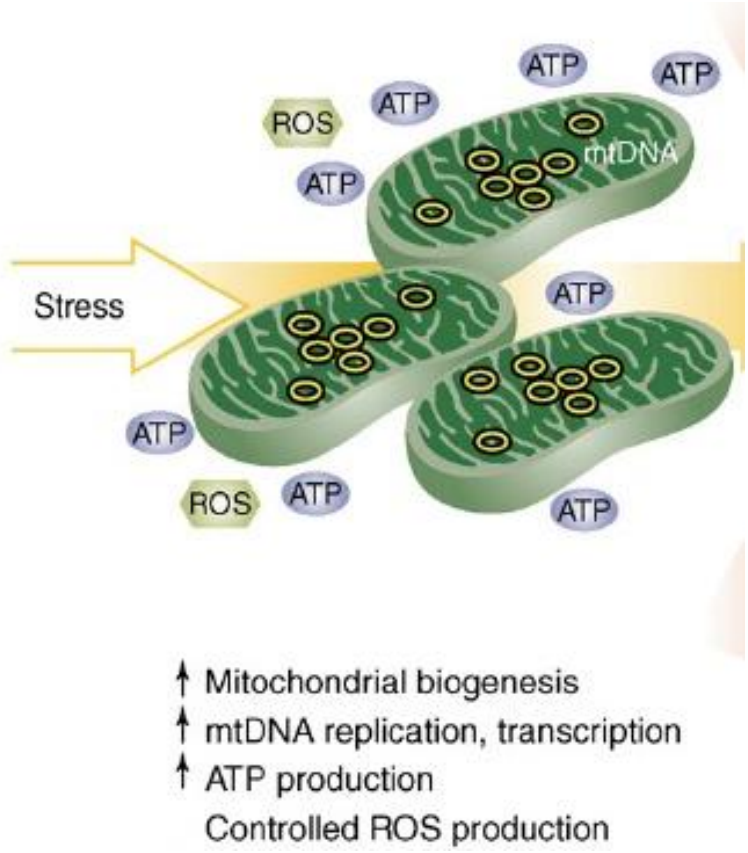


Female metabolic problems
Female age, oocyte maturation
Stress during embryo culture

(CC group) centrifuged but not micromanipulated
(CO group) without centrifugation or micromanipulation
(MC group) micromanipulated control
(DE group) mitochondrial depleted

Mitochondrial DNA

Mitochondria is a key components of the stress response



✓Mitochondrial biogenesis is an endogenous protective signal (under stress).

✓Acute stress results in increased mitochondrial biogenesis to meet the increased energy demands of the cell

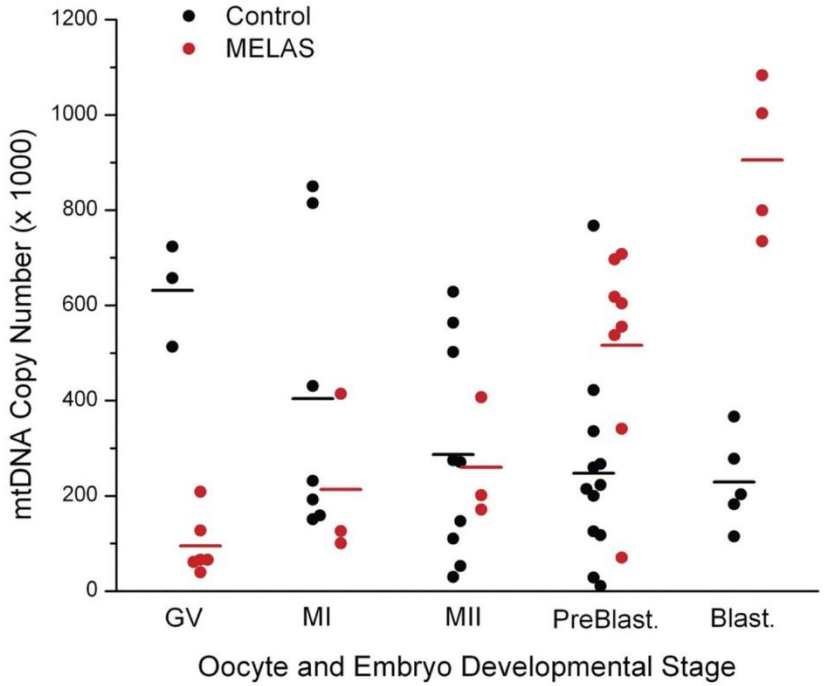
TRENDS in Endocrinology and Metabolism Vol.18 No.5, 2007

Mitochondrial DNA

Embryos can activate mitochondrial biogenesis

Mitochondrial mutations increase replication of mtDNA

mtDNA amount during late oogenesis and preimplantation development in MELAS m.3243A>G oocytes and embryos.



Sophie Monnot et al. Hum. Mol. Genet. 2013;hmg.ddt040

Human Molecular Genetics

Mitochondrial DNA

Can mitochondrial biogenesis be an indicator of embryo stress?

Can embryo stress be an indicator of decrease implantation ability?

Mitochondrial DNA content as a viability score in human euploid embryos: less is better

Antonio Diez-Juan, Ph.D.,[a] Carmen Rubio, Ph.D.,[a,b] Carlos Marin, B.Sc.,[a] Sebastian Martinez, B.Sc.,[a] Nasser Al-Asmar, M.Sc.,[b] Marcia Riboldi, Ph.D.,[c] Patricia Díaz-Gimeno, Ph.D.,[d] Diana Valbuena, M.D., Ph.D.,[a] and Carlos Simón, M.D., Ph.D.[a,b,c,d]

Fertil Steril, 2015

Mitochondrial DNA

SurePlex amplificate

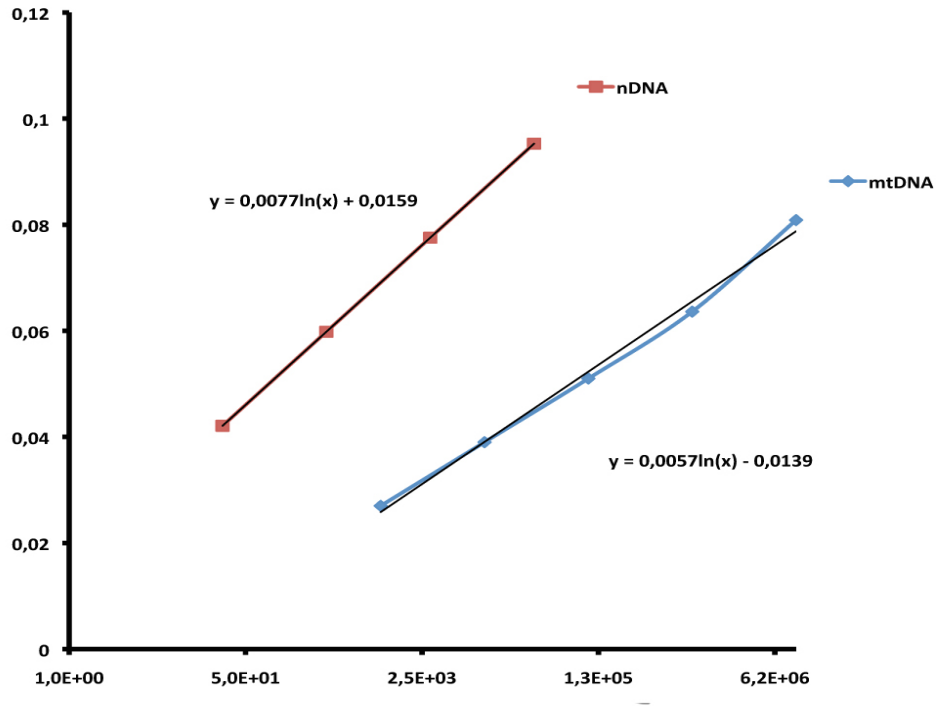
↓

Q-PCR
- ✓ Nuclear
- ✓ Mitochondrial

↓

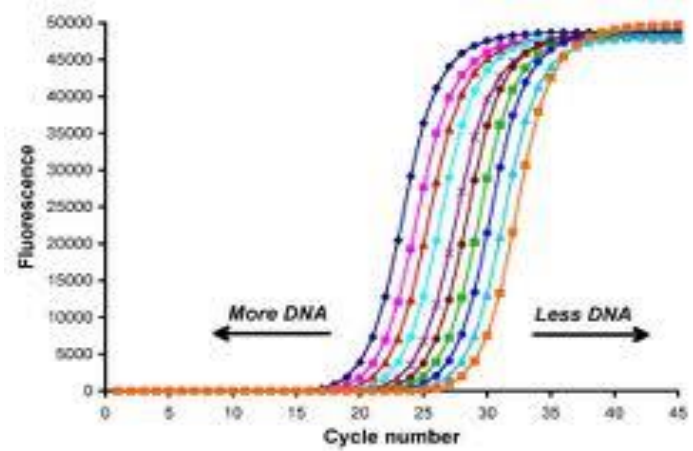
Normalization

MitoScore Methodology



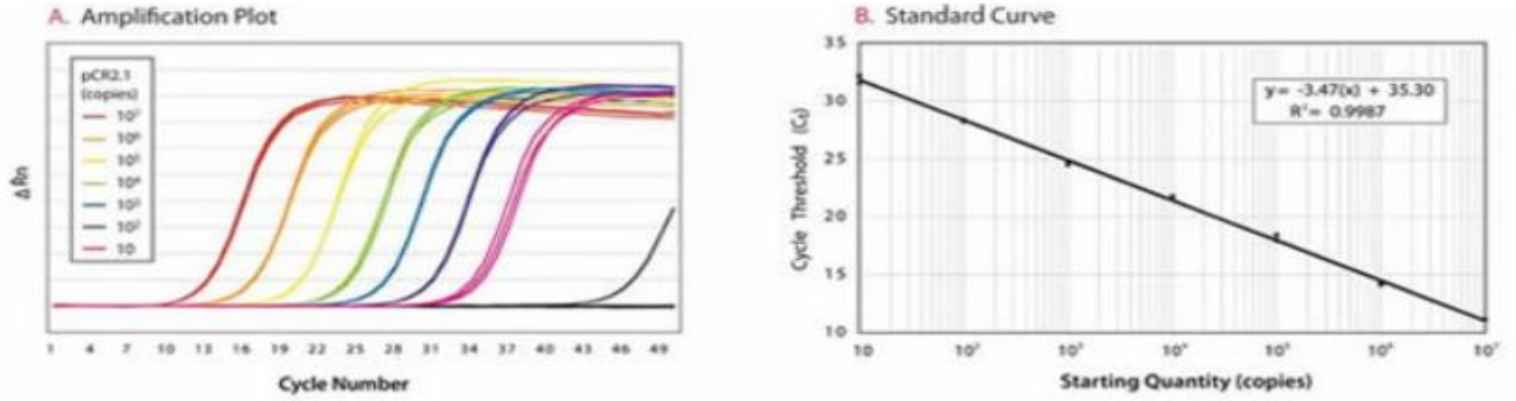
Quantitative PCR (qPCR)

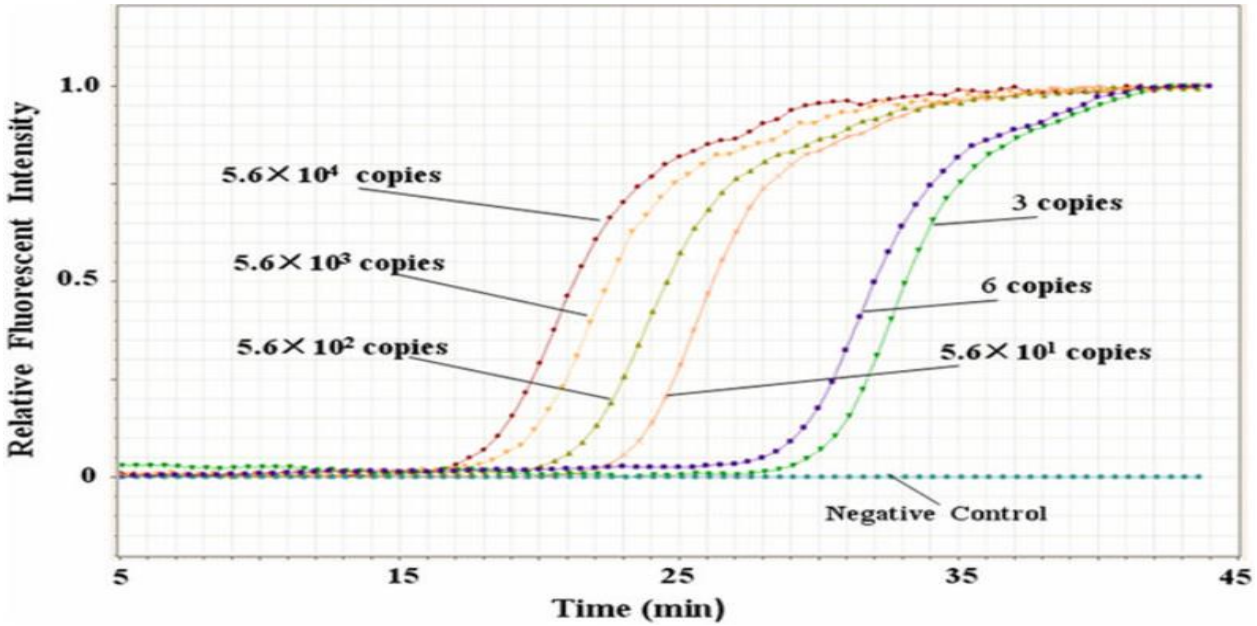
Quantitative PCR (qPCR)



Quantitative PCR (qPCR) continue

Quantitative PCR (qPCR)





Mitochondrial DNA

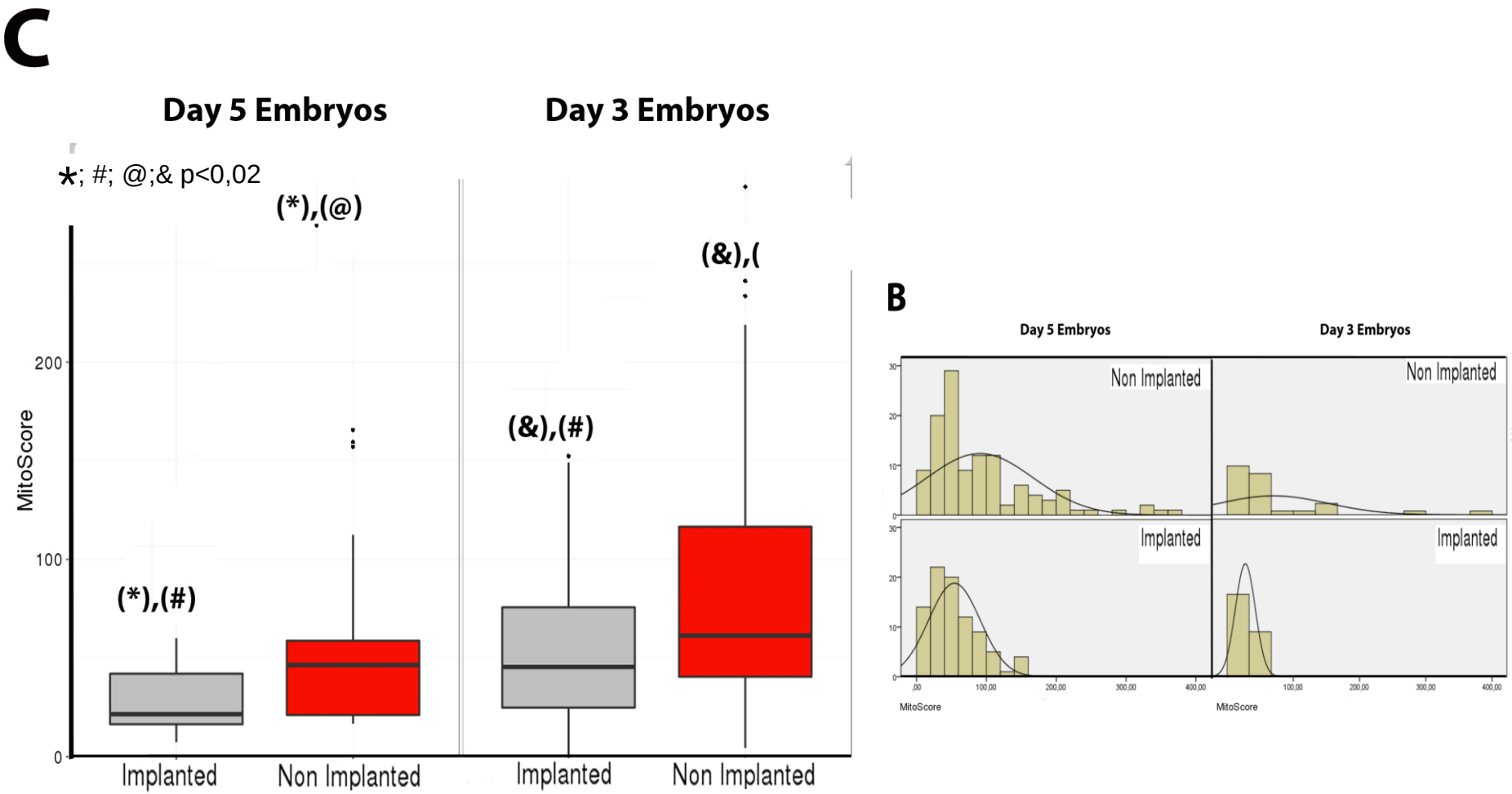
- **Objective:** To investigate the clinical relevance of mitochondrial mtDNA content as a viability score in human euploid embryos.

- **Design:** Retrospective analysis of mtDNA content of transferred euploid embryos.

- **Patients:** Single embryo transfer in 270 patients who underwent preimplantation genetic screening (205 day-3 blastomere biopsies, and 65 day-5 trophectoderm biopsies), and 10 patients with double embryo transfer (male-female).

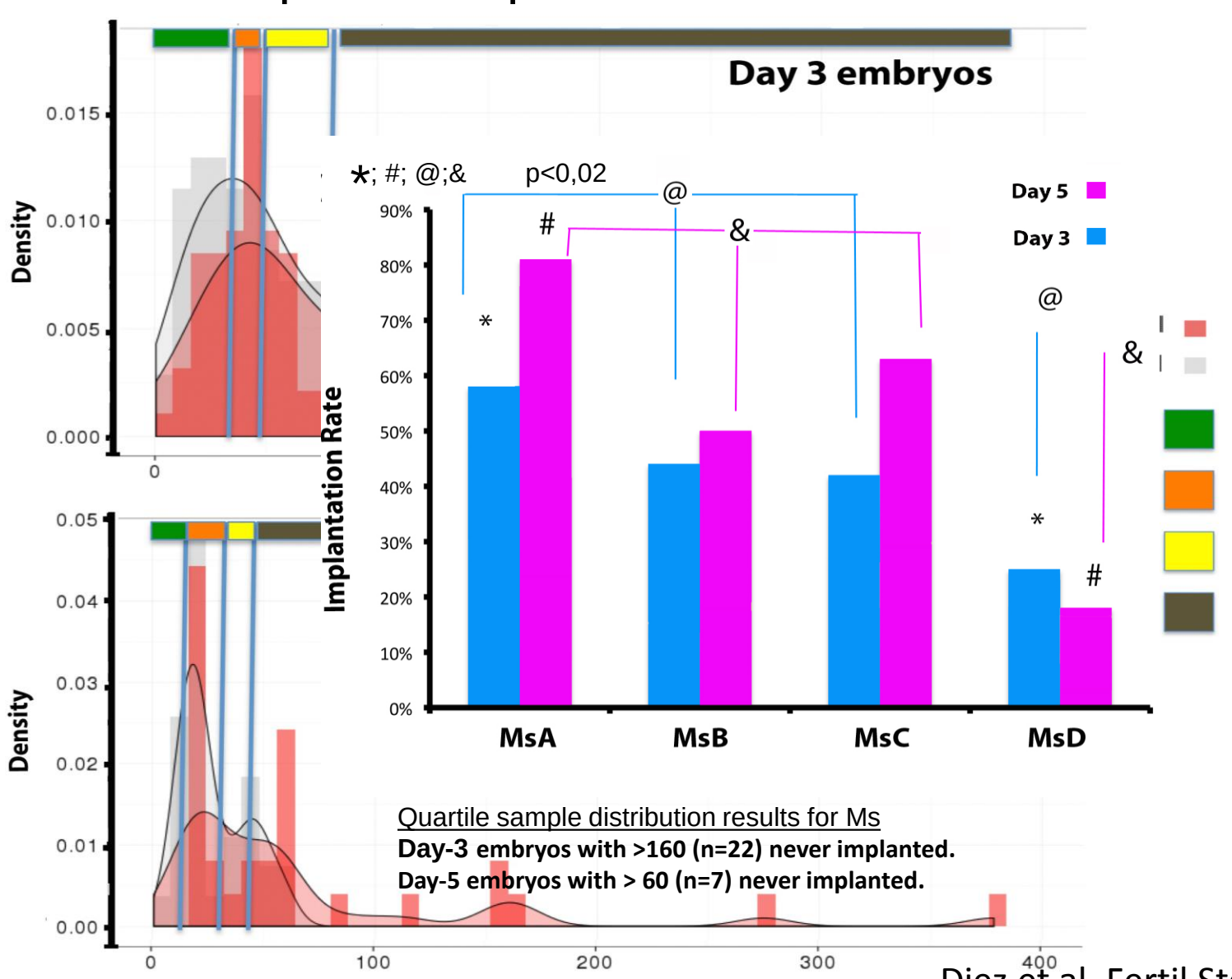
- **Main Outcome Measures:** Normalized mtDNA content versus nDNA from transferred euploid embryos.

Reduced Relative mtDNA in Implanted embryos



Diez et al, Fertil Steril 2015

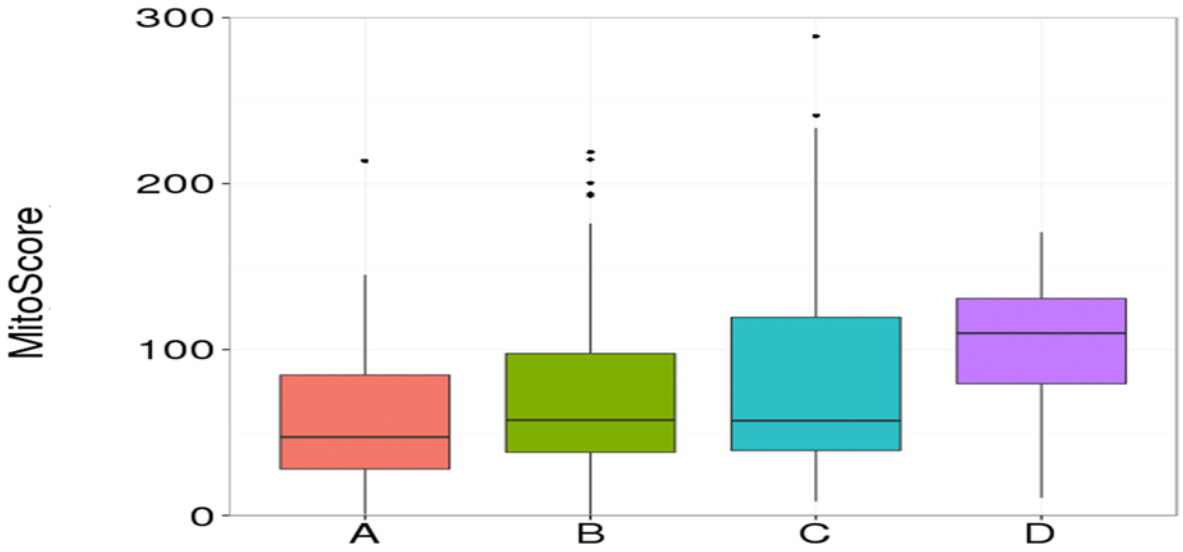
Mitochondrial DNA threshold values required to predict implantation outcome



Diez et al, Fertil Steril 2015

Mitochondrial DNA

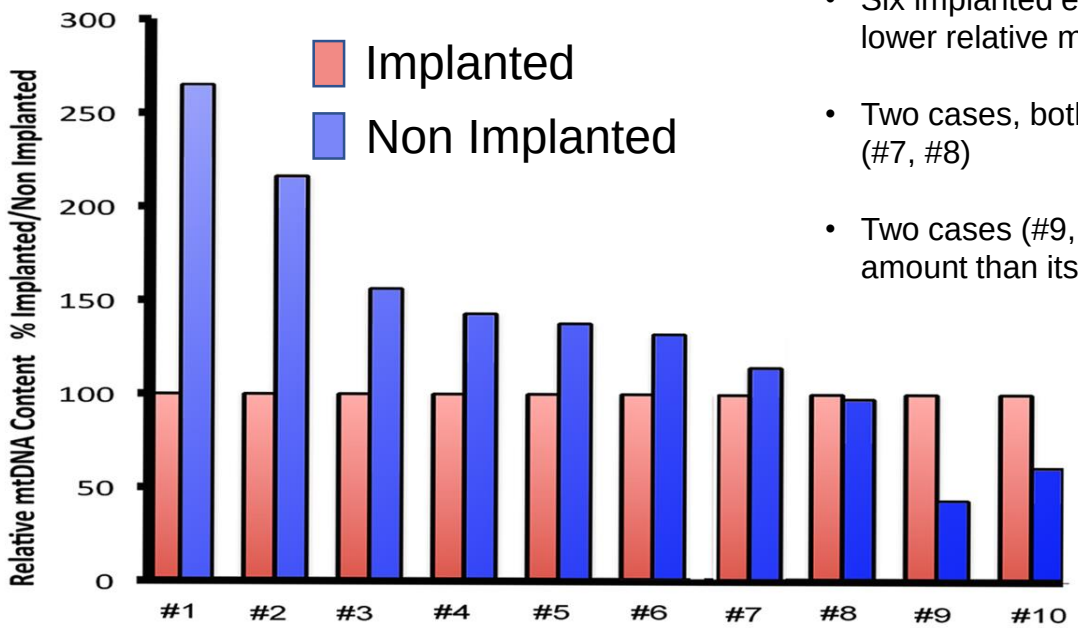
MitoScore and embryo Quality



Diez et al, Fertil Steril 2015

Mitochondrial DNA

MtDNA content and single embryo implantation in DET



- Six implanted embryos corresponded to those that had the lower relative mtDNA content (#1, #2, #3, #4, #5, #6).
- Two cases, both embryos had an equivalent amount of mtDNA (#7, #8)
- Two cases (#9, #10), the implanted embryo had a higher amount than its sibling

mtDNA content in pairs of transferred euploid embryos (one male and one female), which resulted in single pregnancies where the gender of the newborn was reported.

Diez-Juan and Rubio Fertil Steril, 2015

PGS: Mitochondrial DNA

RESEARCH ARTICLE

Altered Levels of Mitochondrial DNA Are Associated with Female Age, Aneuploidy, and Provide an Independent Measure of Embryonic Implantation Potential

Elpida Fragouli[1]*, Katharina Spath[2], Samer Alfarawati[1], Fiona Kaper[3], Andrew Craig[4], Claude-Edouard Michel[4], Felix Kokocinski[4], Jacques Cohen[5], Santiago Munne[5], Dagan Wells[1,2]

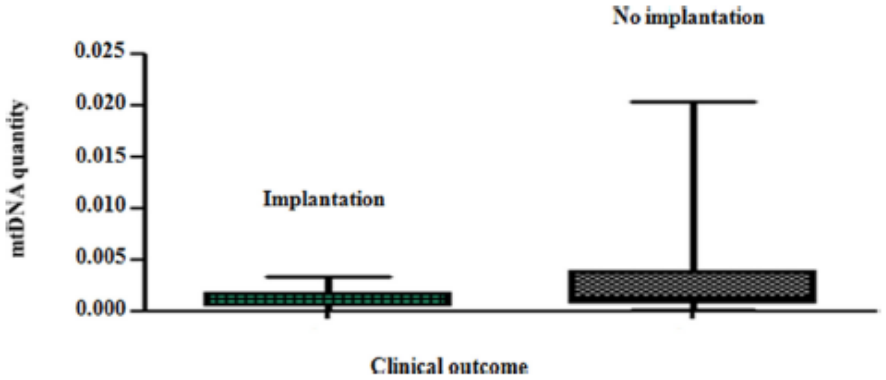
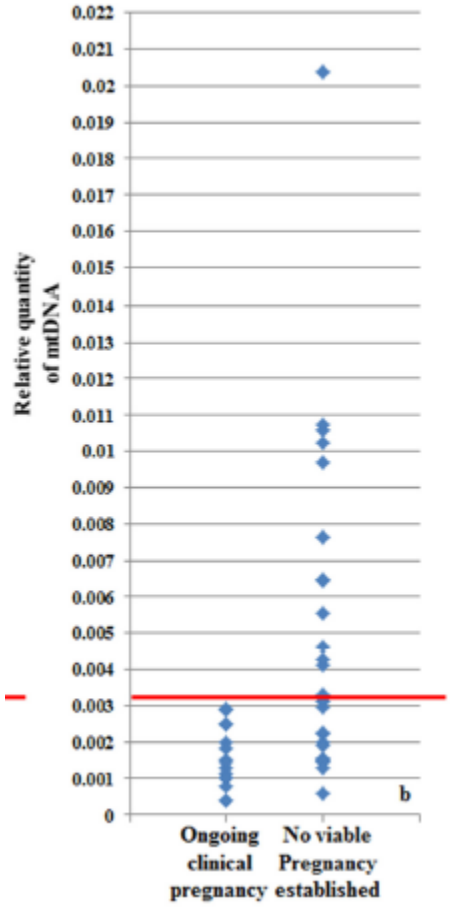


Fig 3. The mtDNA content of chromosomally normal blastocysts in relation to clinical outcome. On average, chromosomally normal blastocysts capable of establishing a clinical pregnancy contained significantly (P = 0.007) lower levels of mtDNA compared to chromosomally normal blastocysts that failed to do so.



Plos Genetics, 2015

Published Literature

LOWER EMBRYONAL MITOCHONDRIAL DNA CONTENT IS ASSOCIATED WITH BETTER QUALITY EMBRYOS

- **Retrospective study**
  - 2013 and June 2016.
  - Total of 259 subjects with 1510 blastocyst biopsies

- **Results**
  - Grade 1 (N=951; 62%)
  - Grade 2 (N=331; 21%)
  - Grade 3 (N=228; 15%)
- Embryos with high mtDNA content were found to be of poorer quality (Grade 3) relative to grades 1 and 2. (RR1.03 [95%CI 1.01-1.05]; P=0.003).
- No correlation between mtDNA content and the subjects age
- Non-statistically significant trend was observed where poor quality embryos had higher mtDNA content

- **CONCLUSIONS:**
  - A higher quantity of embryonal mtDNA
  - Poorer quality embryo, possibly due to greater oxidative stress.
  - Lower mtDNA content suggests a higher quality embryo

A. M. Klimczak et al. F&S (2017)

Published Literature

Day 5 blastocysts contain higher mitochondrial DNA content compared to Day-6 blastocysts

- Objective: Day-5 vs Day 6 mt DNA content
  - Total of 1460 embryos between June 2013- June 2016
- Results:
  - Day 5 blastocysts contain higher mtDNA compare to Day 6 blastocysts
  - Day 5 blastocysts determined to be high quality than Day 6 blastocysts
  - Day 5 blastocysts had less aneuploidy compare to Day 6 blastocysts
  - High quality (Grade 1) Day 5 blastocysts contain significantly higher mtDNA compare to Day 6 blastsocysts
- Conclusion:
  - Compared to day 6, Day 5 blastocysts contained contain significantly higher mtDNA and lower rate of aneuploidy and more likely to be high quality of embryos

A. M. Klimczak et al. PCRS (2018)

Published Literature

Mitochondrial DNA copy number measured by mitoscore is associated to trophectoderm quality

- Retrospective study
- Total of 1572 embryos 421 PGS cycles (June2016–January2017)
- **Indications for PGS**
  - AMA (n = 265),
  - RIF (n=51)
  - Severe MF(n=32)
  - RPL (n=36)
  - Previous Aneuploidy(n=6)
  - Monogenic disease(n=22)
  - others(n=8)

Embryo Grade

- 2.48 % graded A
- 53.18% graded B
- 43.08% grade C
- 0.25% garde D

Median values of Mitoscore

- A=19.02
- B=21.07
- C=21.43;
- D=31.5 (p=0.0459)

Results

No differences

- interms of age (38.9±3.7,38.8±3.9)
- days of stimulation(10.6±3.7,11.1±3.6) total doses of gonadotrophins (2664±1444 UI vs 2471±1584UI)
- Total number of oocytes retrieved (10.5±7.8 vs 11.5±7.9) were observed between patients

Conclusions: The relationship between quality of trophectoderm and Mitoscore may indicate that alterations in mitochondrial biogenesis may negatively affect trophoblast proliferation capacity. This situation may therefore impair further trophoblast differentiation which is necessary for the adhesion and invasion steps during implantation.

Maria José De Los Santos et al. RBO (2018)

Published Literature

CLINICAL APPLICATION OF MITOCHONDRIAL DNA QUANTIFICATION FOR EMBRYO VIABILITY ASSESSMENT: A BLINDED PROSPECTIVE NON-SELECTION STUDY

- Prospective blinded clinical study.
  - N:337 blastocysts/chromosomally normal. (195 couples)
  - average female age 36.7 years

- **Results**
  - 25/336 (7.4%) of embryos contained elevated mtDNA levels
  - 109 (69%) of these led to ongoing pregnancies, (100%) had mtDNA levels in the normal range.
  - 50 (31%) blastocysts failed to implant.
  - 8 (16%) of these non-viable embryos were found to have elevated quantities of mtDNA. ($P<0.0001$)

- No embryos with elevated mtDNA levels implanted

E. Fragouli et al. F&S 2016

Published Literature

IMPACT OF MATERNAL AGE AND DIFFERENT ANEUPLOIDY PATTERNS ON MITOCHONDRIAL DNA CONTENT.

- Retrospective study
  - 5,328 trophectoderm biopsies (October 2016 to March 2017)
  - Ion ReproSeq PGS (Thermofisher Scientific).

- Results
  - MitDNA was not related to maternal age.
  - However, MitDNA was significantly increased in blastocysts with uniform aneuploidies compared to euploid embryos, and displayed an intermediate pattern in mosaics and partial dup/del.

  - These results can indicate a potential energetic effort in embryos depending on the type of chromosomal abnormalities and independently of maternal age. This energetic exhaustion could have and additional effect in the lower implantation rates reported when mosaic embryos are selected for transfer.

TABLE 1. Mit DNA (Mean, SD).

Maternal age	Euploid Embryos	Uniform Aneuploidy	Mosaic Aneuploidy	Partial dup/del
<=35yr	22.5 (7.6)	29.3(14.1)	25.3 (8.4)	25.6 (8.1)
36yr	21.8 (6.2)	27.7 (14.5)	26.1 (12.4)	27.7 (11.1)
37yr	24.1 (8.8)	27.5 (12.4)	26.4 (8.5)	27.1 (11.3)
38yr	23.8 (9.1)	26.4 (12.1)	27.7 (5.5)	26.1 (13.8)
39yr	23.1 (8.7)	26.2 (11.6)	23 (6.4)	26.7 (8.5)
40yr	23.3 (9.4)	26.2 (11.6)	24.8 (10.1)	22.8 (7.0)
41yr	23.2 (8.3)	27.7 (12.6)	24.9 (8.1)	29.1 (13.2)
42yr	22.1 (6.9)	26.2 (9.6)	26.5 (8.8)	27 (9.1)
43yr	22.8 (6.2)	27.4 (12.4)	21.3 (7.5)	28.6 (7.3)
44yr	23.2 (10.3)	28.1 (10.9)	23.4 (9.8)	17 (12.1)
TOTAL	22.9 (8.1)[a]	28.0 (12.9)[b]	25.1 (8.5)[c]	25.7 (9.4)[d]

C. Rubio et al. F&S (2017)

Published Literature

Mouse study on mtDNA

- Ethidium bromide mouse cell line (Coriell Cell Repository ID GM05384) WGA/ Ion Proton (PGM)
- Increased mtDNA content was associated with elevated aneuploidy risk
- Aneuploid blastocysts (n=19) possessed a significantly higher (1.8-fold) quantity of mtDNA compared to euploid blastocyst (n=98) ($P<0.001$).

X. Tao et al F&S 2016)

Published Literature

- Retrospective observational study
  - Between 2013 and June 2016.
  - A total of 1,396 embryos derived from 259 patients

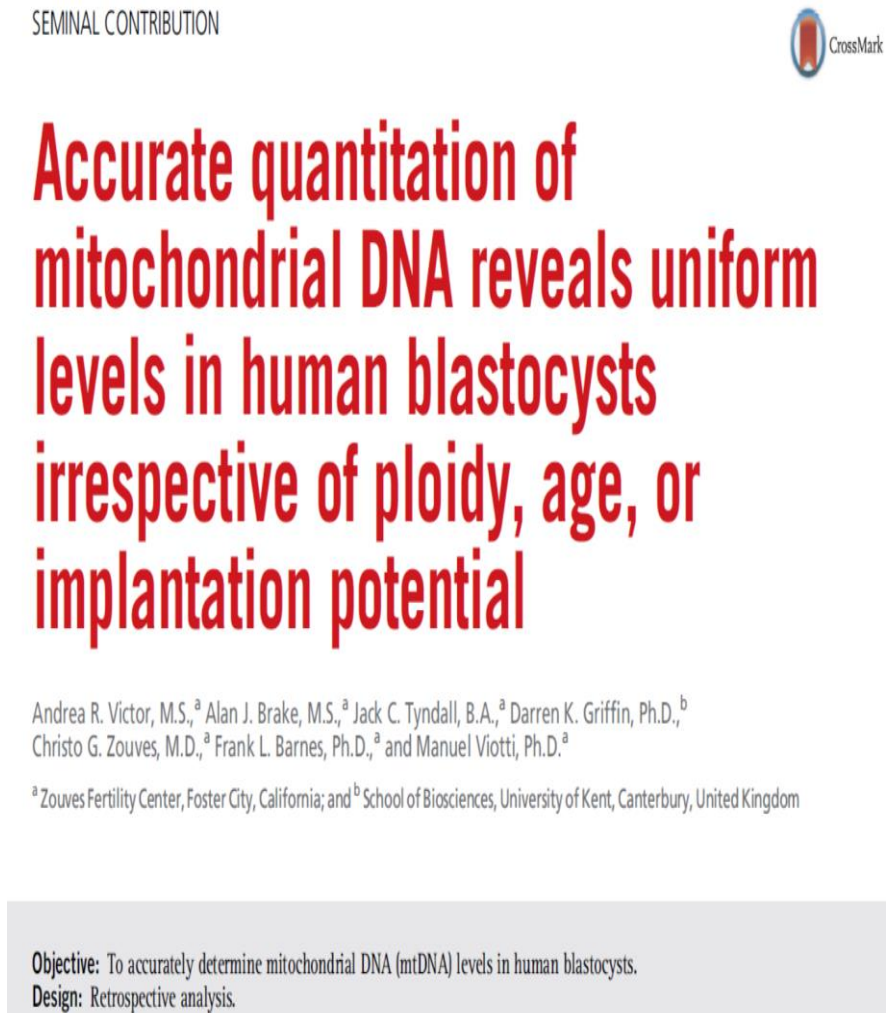
Results

- Levels of mtDNA in euploid and aneuploid embryos showed a statistically insignificant difference by NGS (euploids n=775, aneuploids n=621) and by qPCR (euploids n = 100, aneuploids n = 50).

- Blastocysts derived from younger or older patients had comparable mtDNA levels by NGS (‘‘young’’ age group n =874, ‘‘advanced’’ age group n = 514) and by qPCR (‘‘young’’ age group n =92, ‘‘advanced’’ age group n = 58).

- Viable blastocysts did not contain significantly different mtDNA levels compared with unviable blastocysts when analyzed by NGS (implanted n = 101, nonimplanted n=140) and by qPCR (implanted n = 49, nonimplanted n = 51).

- CONCLUSIONS
  - Overall levels of mtDNA are largely equal between blastocysts stratified by ploidy, age, or implantation potential



SEMINAL CONTRIBUTION

CrossMark

Accurate quantitation of mitochondrial DNA reveals uniform levels in human blastocysts irrespective of ploidy, age, or implantation potential

Andrea R. Victor, M.S.,[a] Alan J. Brake, M.S.,[a] Jack C. Tyndall, B.A.,[a] Darren K. Griffin, Ph.D.,[b] Christo G. Zouves, M.D.,[a] Frank L. Barnes, Ph.D.,[a] and Manuel Viotti, Ph.D.[a]

[a] Zouves Fertility Center, Foster City, California; and [b] School of Biosciences, University of Kent, Canterbury, United Kingdom

Objective: To accurately determine mitochondrial DNA (mtDNA) levels in human blastocysts.
Design: Retrospective analysis.

Andrea R. Victor et al. F&S (2017)

Why are we getting different conclusion?

- **METHODOLOGICAL CONSIDERATIONS**

➢ Allele drop out (ADO)

- At least half of biopsied pecimens will experience ADO in one or more of the constituent cells and over one third are likely to suffer ADO in two of the five cells.
- If one cell in a 5-cell biopsy specimen suffers ADO normalized mtDNA levels are increased by 12%, if two cells are affected the increase is 25%. multi-copy sequence (or combines data from multiple distinct sequences) is essential

➢ Degradation of the DNA during storage (cycles of freezing and thawing)

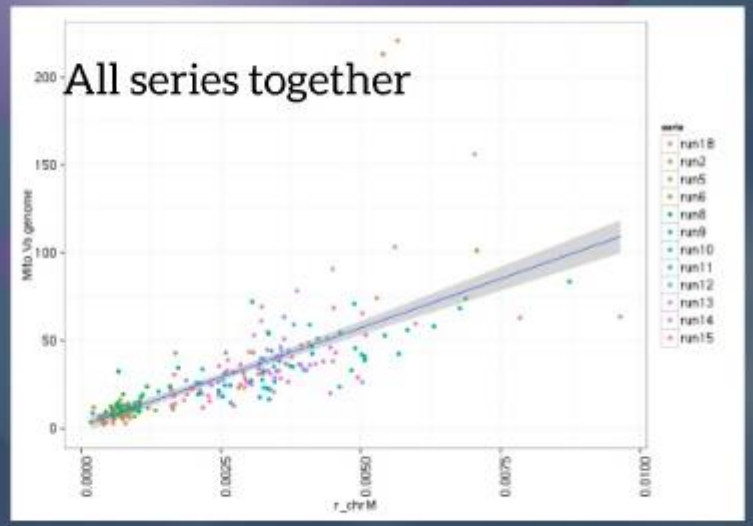
➢ Next Generation Sequencing (NGS) gives uneven coverage of the mitochondrial genome and generate insufficient numbers of mtDNA sequences for reliable quantification.

Dagan Wells. F&S 2016

MitoScore2016

NGS

Correlation between NGS and mitoscore



Same reaction PGS-NGS

MitoScore: Methodology

Embryo Biopsy

PGS by NGS:
Nuclear reads ➔ Euploid (normal) / Aneuploid (abnormal)

Mitochondrial Reads ➔ MitoScore

MitoScore Don't need more sampling
MitoScore Don't need more time

NGS-based PGS (HR)

Embryonic nDNA and mt DNA is amplified (WGA)

Millions of short sequences produced

Amplified embryonic DNA Fragmentation into a Library of smaller fragments (100-200 bp)

Barcoding Samples

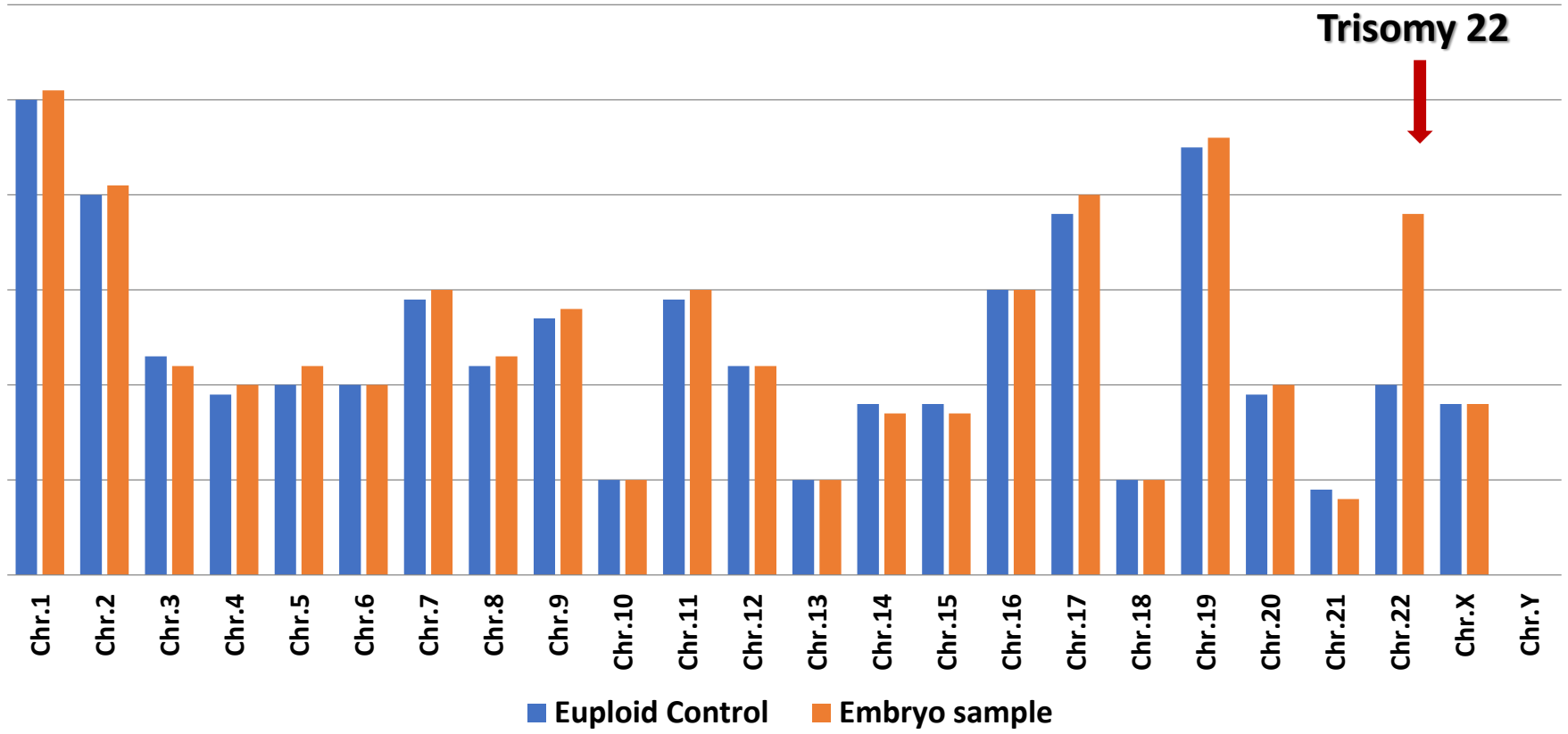
Specific barcode sequences are attached to DNA fragments

Libraries from each sample are pooled and sequenced in parallel

Barcode sequences are used to differentiate reads from each sample

Each set of reads is aligned to the reference sequence

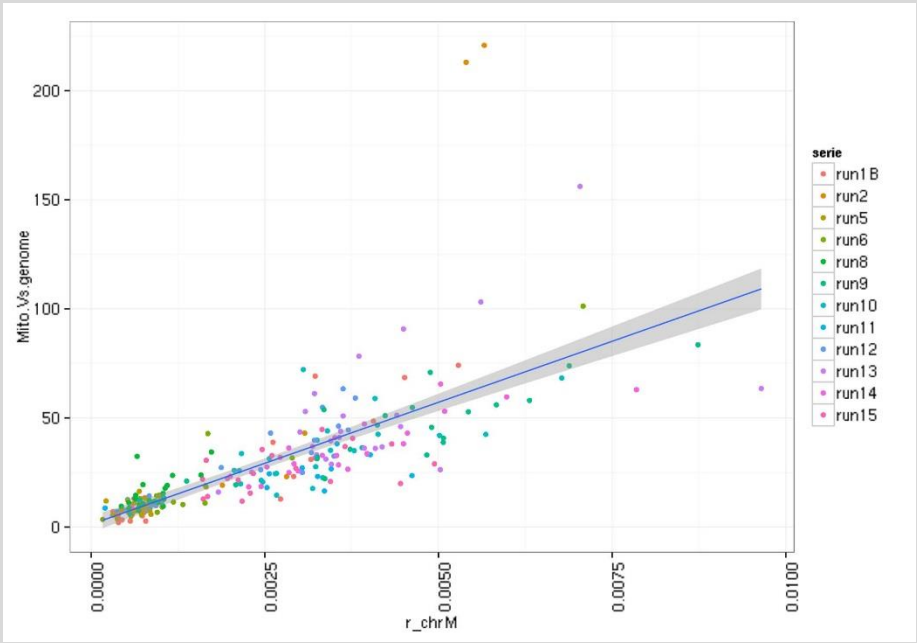
Data Analysis



Sequences from each chromosome are counted using software then compared with a chromosomally normal reference DNA

Mitochondrial DNA with NGS

- ✓ Linear regression study QPCR vs. NGS to develop and algorithm for accurate mitDNA quantification.
- ✓ Day of biopsy should be considered, 3, 5, 6
- ✓ Run QC parameters



Mitochondrial DNA-RCT

Randomized multi-centric prospective study for the evaluation of "MitoScore" marker in the diagnosis of embryo viability in euploid blastocyst

To evaluate the potential of analyzing mitochondrial DNA content of an euploid embryo as a biomarker compared to standard morphology parameters.

Mitochondrial DNA-RCT

Inclusion criteria

- PGS cycles for different indications
- Maternal age: ≤40 years old (FIV/ICSI patients)
- Maternal age: <50 years old (Ovum donation patients)
- Sperm concentration: > 2 million of sperm/ml
- Single embryo transfer
- ≥ 8 oocytes MII
- Number of Antral Follicules (AFC: ≥10 MII)

Mitochondrial DNA

Take home message

- Detection rates may vary depending on platform, laboratory and algorithm
- Standardization
- Randomized clinical trial (RCT)
- Embryo morphology is still the gold the standard for embryo transfer

Acknowledgements

Scientists

Carlos Simón

Al-Assmar Nasser
Blesa David
Bandeira Carla
Campos-Galindo Inma
Cervero Ana
Chopra Rupali
Cinnioglu Cengiz
Chiu Yatfung
Díaz-Juan Antonio
Dizon Bautista Abelard
Elshaikh Noon
Garcia-Herrero Sandra
Garcia Carmen
Gómez Eva
Jiménez Jorge
Kayali Refik
Khajuria Rajni
López Pilar
Mae Hoover Larissa
Marín Carlos
Marín Lucía
Martín Julio
Martínez José Antonio
Mateu Emilia
Milán Miguel
Mir Pere
Miravet José
Moreno Inma
Navarro Roser
Peinado Vanesa
Poo María Eugenia
Riboldi Marcia
Rincón Alejandro
Rodrigo Lorena
Rodríguez Beatriz
Rubio Carmen
Ruiz María
Sánchez Maribel
Sanz Lucía
Sen Gurkan
Uehara Mariane
Valbuena Diana
Vera María
Vilella Felipe
Whittenburg Alex

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Gómez María
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Martinez Lucia
Martinez Sebastian
Martinez Tantra
Mateos Pablo
Moles Sara
Morata María Jesus
Nguyen Tuan
Nieto Jessica
Peris Laura
Pozo Ana
Singh Vinita

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Thank you!!

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