**Molecular Human Reproduction, Vol.25, No.9, pp. 527–537, 2019**
Advance Access Publication on June 7, 2019 doi:10.1093/molehr/gaz032

molecular human reproduct on

ORIGINAL RESEARCH

# Non-invasive mitochondrial DNA quantification on Day 3 predicts blastocyst development:a prospective, blinded,multi-centric study

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*Submitted on April 4, 2019; resubmitted on April 19, 2019; editorial decision on May 24, 2019*

**ABSTRACT:** In ART, embryo quality evaluation is routinely based on morphological criteria. We previously demonstrated that the mitochondrial DNA (mtDNA)/genomic DNA (gDNA) ratio in culture medium was significantly associated with embryo quality and viability potential. The purpose of this prospective, blinded, multi-centric study was to validate the use of mtDNA/gDNA ratio in Day 3 spent medium as a predictor of human embryo developmental competence. The mtDNA/gDNA ratio was assessed in Day 3 culture media ($n$=484) of embryos from 143 patients by quantitative PCR. A mixed effect logistic regression model was applied. We found that mtDNA/gDNA ratio in Day 3 culture medium combined with embryo morphology improves the prediction upon blastulation compared to morphology alone ($P < 0.0001$), independent of patient and cycle characteristics. With regard to routine use in clinics, we evaluated the ability of the novel, combined grading score to improve selection of developmentally competent embryos of a single cohort. Including embryos from 44 patients, the sensibility and specificity of the scoring system based on Day 3 morphological stage were 92% and 13%, respectively. Integration with the culture medium mtDNA/gDNA ratio increased the performance of the method (sensibility: 95%; specificity: 65%). The results of this study suggest the possibility of carrying out a non-invasive evaluation of embryonic mtDNA content through the culture medium. When combined with embryo morphology, it has the potential to help embryologists rank embryos and choose which embryo(s) has the greater development potential, and thus should be transferred on Day 3, among sibling embryos with the same morphological grade.

**Key words:** blastocyst / cell-free DNA / human embryos / mitochondrial DNA / non-invasive embryo evaluation

## Introduction

An effective strategy to identify embryos with optimum development potential is essential and it represents one of the major challenges in human ART. Traditionally, embryo quality assessment is based on morphological and developmental characteristics such as degree of fragmentation, blastomere size and multinucleation, embryo symmetry and cleavage rate, that are evaluated at distinct time points on Day 2 or 3 post insemination (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). However, individual observations are unable to detect dynamic processes that could have developmental significance (Salumets *et al.,* 2003), and morphological assessments are hampered by a lack of suitable standards and by inter- and intra-operator variability associated with a subjective grading system (Paternot *et al.,* 2011). Over recent years, time-lapse imaging has been proposed as a promising refinement in assessing morphological parameters that may noninvasively predict the developmental potential of embryos (Wong *et al.,* 2010; Meseguer *et al.,* 2011;

Cruz *et al.,* 2012; Chen *et al.,* 2013; Kirkegaard *et al.,* 2013). However, to date there is insufficient evidence of improvements in live birth, miscarriage, stillbirth or clinical pregnancy to indicate a real advantage of time-lapse system compared to conventional incubation and morphology evaluation (Armstrong *et al.,* 2018). Moreover, the available studies are too heterogeneous for firm conclusions to be drawn on the predictive value of morphokinetic characteristics for implantation and embryo aneuploidy screening (Rienzi *et al.,* 2015; Reignier *et al.,* 2018), and additional means of embryo competence assessment are necessary.

Elective single embryo transfer at blastocyst stage has been promoted to maximize implantation rates and decrease multiple gestations (Papanikolaou *et al.,* 2008; Veleva *et al.,* 2009; Gelbaya *et al.,* 2010; Glujovsky *et al.,* 2016). More recently, the application of preimplantation genetic testing (PGT) supports transferring single euploid blastocysts, which should lead to a reduction in risks of miscarriage, aneuploid conception and implantation failure (Scott *et al.,* 2013; Dahdouh *et al.,* 2015ab;Vaiarelli*et al.,* 2016). Moreover, mitochondrial DNA (mtDNA) copy number in embryos is associated with aneuploidy risk, female reproductive aging and embryo quality (Fragouli *et al.,* 2015; Fragouli and Wells, 2015; Diez-Juan *et al.,* 2015; Ravichandran *et al.,* 2017; Fragouli *et al.,* 2017; Ho *et al.,* 2018; Klimczak *et al.,* 2018), in spite of some discrepant studies related to technical issues in mtDNA quantification and/or data from a single IVF clinic (Seli *et al.,* 2016; Treff *et al.,* 2017; Victor *et al.,* 2017; Wells *et al.,* 2017; de Los Santos *et al.,* 2018).

Both PGT and embryo mtDNA assessment need embryo biopsy, which is highly skilled since it requires specific training of personnel, purchase and maintenance of a laser and it occupies time of experienced embryologists. Moreover, sampling cells from an embryo is not permitted in some countries. For these reasons, over the last decade developments in non-invasive embryo grading for the greatest viability have looked into metabolic parameters in culture media. These embryo selection methods are based on analysis of spent culture medium nutrient and metabolite content, such as glucose, lactate, pyruvate, amino acids, oxygen consumption by the embryo and analytical examination of the embryonic metabolome (Pearson *et al.,* 2006; Botros *et al.,* 2008; Bromer and Seli *et al.,* 2008; Nel-Themaat and Nagy *et al.,* 2011; Wang *et al.,* 2011; Katz-Jaffe and McReynolds *et al.,* 2013). To overcome biopsy, non-invasive and mini-invasive PGT approaches have also been proposed by collection and assessment of a culture medium drop, or of blastocoel fluid, respectively (Assou *et al.,* 2014; Galluzzi *et al.,* 2015; Wu *et al.,* 2015; Xu *et al.,* 2016; Shamonki *et al.,* 2016; Hammond *et al.,* 2017; Liu *et al.,* 2017; Yang *et al.,* 2017;

# Materials and Methods

## Study design

This was a prospective study performed in two independent IVF centers. Standard controlled ovarian stimulation protocols were used, with diversity in the type of stimulation protocol. Each laboratory used its own preferred culture medium in an effort to generate a generalizable prediction model that could be applied across centers.

Patients were required to be undergoing IVF treatment by ICSI using their own fresh eggs and ejaculated sperm. We included in the study only embryos that were cultured up to Days 5–7. We excluded embryos obtained from cryopreserved gametes, donor gametes and surgically removed sperm, in order to avoid potential biases due to cooling/defrosting procedures. Another exclusion criterion was fertilization by standard IVF, in order to exclude potential contaminating DNA from spermatozoa. Since both centers prevalently perform insemination by ICSI, this did not imply a selection of couples depending on andrological characteristics.

All couples that were eligible according to the above criteria were considered for participation in the study. The study was approved by the Ethical Committee of Regione Liguria (approval n. 234REG2016), and each couple signed a written informed consent.

## Standard embryo culture

Immediately following ICSI, each oocyte cohort from the same patient was individually cultured in the clinic's routine retrieval media (Sydney IVF Fertilization medium, Cook Medical, Bloomington, IN, USA) at center #1 and Sequential Fert™ (Sage®, Trumbull, CT, USA) at center #2 at 37◦C in a humidified atmosphere of 6% $CO_2$, 5% $O_2$ by using a standard incubator (Galaxy 48R incubator, New Brunswick Scientific, Edison, NJ, USA) at center #1 and MINC™ Benchtop Incubators (Cook Medical) or a time-lapse system (EmbryoScope, Vitrolife, Göteborg, Sweden) at center #2. Insemination of oocytes by ICSI was performed immediately after denudation. Embryos with two pronuclei were individually cultured into sequential media (from Day 1 to Day 3: Sydney IVF Cleavage medium (Cook Medical) at center #1 and Sequential Cleav™ (Sage®) at center #2; from Day 3 to Days 5–7: Sydney IVF Blastocyst medium (Cook Medical) at center #1 and Sequential Blast™ (Sage®) at center #2). In both centers, fertilization and embryo morphology grading was assessed 18–20 hours post-insemination and on Day 3, respectively. On Day 3, following removal of the embryos in preparation for transfer into the blastocyst medium, spent Day 1–3-culture media were collected, immediately frozen into sterile, DNA-, DNase-, RNase-, PCR inhibitorfree tubes (Eppendorf, Hamburg, Germany) and stored at −20◦C until nucleic acid purification. All

Feichtinger *et al.,* 2017; Vera-Rodriguez *et al.,* 2018; Ho *et al.,* 2018; Kuznyetsov *et al.,* 2018). Results are promising but further work is needed to improve this methodology.

First, we discovered that both genomic DNA (gDNA) and mtDNA are detectable in the secretome of human cleavage-stage embryos (Stigliani *et al.,* 2013). Analysis of DNA profiles of spent media in relation to embryo morphology demonstrated that mtDNA/gDNA ratio in Day 3 medium is significantly associated with embryo quality and degree of fragmentation, and it may be an early, non-invasive molecular marker of blastulation potential (Stigliani *et al.,* 2014). Based on such pilot analyses, the purpose of this prospective, blinded, multicentric study was to validate the hypothesis that the mtDNA/gDNA ratio in Day 3 spent culture medium may improve the prediction of blastocyst development compared to traditional morphology alone.

embryos were individually cultured to Days 5–7 in the same incubator (standard or time-lapse system) according to clinic's standard protocols. We collected data about outcome (blastocyst development) of all embryos included in the study and clinical information of the patients.

## Medium collection and quantification of mtDNA/gDNA ratio

All frozen medium samples were centralized at center #1 where the molecular analyses were performed, blinded to the sample type.

Starting volume of culture medium was 500 µl for samples collected at center #1 and 20 µl for those collected at center #2. PBS was added to each 20 µl sample up to a final volume of 500 µl. Cellfree DNA was concentrated and purified from embryo media by using the QIAamp® Circulating Nucleic Acid kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 50 µl Proteinase K, and 400 µl Buffer ACL were added to each embryo culture medium. After pulse-vortexing for 30 seconds, the lysis mixture was incubated at 60◦C for 30 minutes in a ThermoStat Comfort (Eppendorf). The highly denaturing conditions at elevated temperatures ensured inactivation of RNases and complete release of nucleic acids from any bound proteins. After adding 900 µl Buffer ACB to the lysate, the sample was incubated for 5 minutes on ice and then transferred onto a QIAamp Mini column. Cell-free DNA was adsorbed onto the silica membrane as the lysate was drawn through by centrifugation at 8000 g for 1 minute. Contaminants were efficiently washed away during three wash steps (in Buffer ACW1, Buffer ACW2, and absolute ethanol). Finally, DNA was eluted in 30 µl Buffer AVE and stored at −20◦C.

The amount of mtDNA was measured by a relative copy number quantification: specific primers/probe mixes were used to amplify a fragment of the mitochondrial *MT-7S* D-loop region (FAM assay for Hs02596861_s1, Life Technologies, Carlsbad, CA, USA) and a fragment of the nuclear genomic *RNAse P* (FAM assay, Life Technologies). PCR efficiency of each assay was calculated from the given slopes in RealPlex software (Eppendorf). In order to have comparable efficiency rates, we used 0.5 µl and 0.4 µl of ×20 primers/probe mix for gDNA and mtDNA PCR, respectively. Reactions were set up in 96-whitewell Twin.tec® plates (Eppendorf). All reactions were performed in duplicate on the Mastercycler® epRealPlex$^2$ S system (Eppendorf). PCR reactions were performed according to standard conditions for TaqMan® assays: 95◦C for 10 minutes; 40 cycles at 95◦C for 15 seconds and 60◦C for 1 minute. A negative control (RNAse/DNAse free water) and a positive control (gDNA, Promega) were included in each run. For each sample the PCRs for mtDNA and gDNA were always performed on the same plate to avoid possible plate bias. The corresponding qPCR efficiencies (E) of one cycle in the exponential phase were calculated according to the equation: $E = 10^{[-1/\text{slope}]}$. Both investigated genes showed comparable, high efficiency rates (*MT7S*: 1.02; *RNAse P*: 0.91) in the range from 20 ng to 2 pg DNA input with high linearity (Pearson correlation coefficient $R^2$=0.999 for *MT-7S* and 0.987 for *RNAse P*). RealPlex software was used to determine Cq and for each reaction the duplicate Cq values were averaged. The relative amount of *MT-*

## Statistical methods

Power calculation of the study: if there is truly no difference between the standard [r~0.75 in a predictive model including female age, indication and Day 3 morphology (Dessolle *et al.,* 2010)] and experimental diagnostic tool to predict blastocyst development, then 400 samples (embryos) were required to be 80% sure that the upper limit of a one-sided 95% CI (or equivalently a 90% two-sided CI) excluded a difference in favor of the standard group of more than 10%.

In this study the endpoint was 'blastocyst development'. For each embryo whose culture medium was analyzed, we collected data about its morphology, cleavage stage and outcome (blastocyst development). Mann–Whitney test was performed to investigate statistically significant differences between the mtDNA/gDNA ratios in the culture media. The results were considered statistically significant at $P < 0.05$. Receiver operating characteristic (ROC) analysis (Hanley *et al.,* 1982) was performed to compare the accuracy in predicting embryo blastulation of the current morphological assessment to a novel one based on mtDNA/gDNA ratio in spent medium in combination with morphologic evaluation. These data analyses were performed using MedCalc® (Mariakerke, Belgium) software.

Because embryos generated from a patient do not provide independent information, we used a mixed effect logistic regression model in which different patients were assumed to be independent, but embryos within the same subject were correlated to each other (Park *et al.,* 2005). The variables collected and analyzed were: age of woman (two categories: 0 $\leq$ 35 years, 1 > 35 years), cause of infertility (endometriosis only, maternal advanced age only, anovulatory only, tubal only, male only, combination of known causes and idiopathic), type of pituitary suppression used (GnRH agonist or antagonist), type of gonadotrophin used (recombinant, urinary or a combination of both) and the Day 3 embryo morphology grade combined with the mtDNA/gDNA ratio in culture medium (Nelson *et al.,* 2011). We did not include other variables associated with mitochondrial dysfunctions, for example diabetes and obesity, because they occur with a low frequency in our ART population (<10%), not to the point of influencing embryo development in our cohort.

Only factors significantly associated with the outcome (blastocyst development) at univariate analysis ($P < 0.05$) were included in a multivariate model ($P$ for inclusion <0.05). All analyses were carried out using the SAS software version 9.3 (Institute Inc., Cary, NC, USA).

For cohort embryo scoring, we selected 44 patients with a minimum of two embryos (range 2–9) at the same stage on Day 3 and with different outcomes on Day 5 (yes/no blastocyst development). The predictive use of mtDNA/gDNA ratio in the

*7S* to *RNAse P* was determined using the equation $2^{-dCq}$, where dCq = ($Cq_{MT\text{-}7S} - Cq_{RNAse\ P}$).

## Morphological assessments of Day 3 embryos and blastocysts

Day 3 embryos were morphologically scored according to the current consensus system (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011) by combining the number and size of blastomeres, the degree of fragmentation and the cleavage rate. Standard blastocyst morphological assessment was carried out according to the criteria reported by Gardner and Schoolcraft (1999) and recently agreed by an expert panel of scientists
(Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

culture medium was explored by scoring embryos of each patient depending on Day 3 morphological grade only or their Day 3 morphological (M3) grade first and then, within each grade category, on mtDNA/gDNA ratio in the culture medium according to the criterion that 'more is better' (M3+mt rank). The predictive performance of the two scoring models was measured by calculating sensitivity and specificity. Sensitivity measured the proportion of actual positives that were correctly identified as such (that was the percentage of embryos which reached the blastocyst stage and were correctly identified as having such a potential). Specificity measured the proportion of actual negatives that were correctly identified as such (that was the percentage of embryos which arrested their development and were correctly identified as not having the potential to become blastocyst).

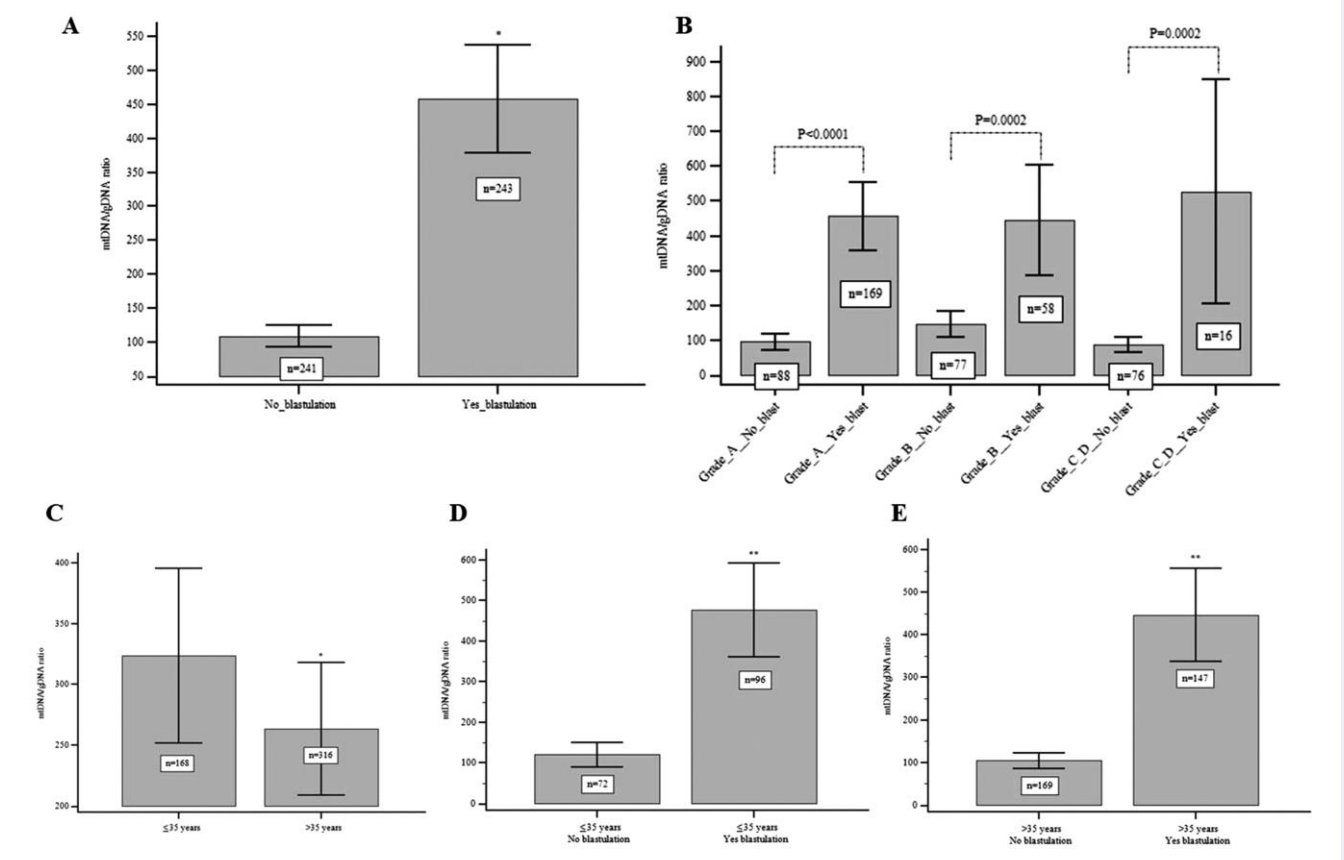



**Figure 1** **The mtDNA/gDNA ratio in spent media** Bar graphs comparing mtDNA/gDNA ratio between spent media of embryos that arrested or develop to blastocyst stage as a whole (**A**), after stratification of embryos according to their morphological grade on Day 3 (**B**), after stratification of embryos according to female age as a whole (**C**) and developmental outcome (**D–E**). Data are mean ± SE. Mann–Whitney test was used. $^{*}P < 0.05$. $^{**}P < 0.01$.

# Results

## Integration of DNA secretome and blastulation

We analyzed a total of 484 Day 3 spent media of embryos from 143 patients and cultured up to Days 5–7. Among them, 243 (50%) embryos developed up to blastocyst stage. Statistical analysis showed that DNA profile of the Day 3 secretome was correlated with embryo developmental potential. In fact, embryos that reached

(Fig. 1, panel A).

## Integration of cleavage-stage embryo morphology,mtDNA/gDNA ratio, blastulation and maternal age

in 62% (159/257) of grade A embryos, 43% (58/135) of grade B embryos, in 20% (14/71) of grade C embryos and in 9% (2/21) grade D embryos. The difference in mtDNA/gDNA ratio between spent blastocyst stage released significantly more mtDNA copies into the culture medium compared to embryos that arrested (*P*.

Regarding morphology on Day 3, 257 embryos were grade A, 135.

grade B, 71 grade C and 21 grade D. Blastulation was observed

media stratified according to their morphological grade and ability to become blastocysts was statistically significant ($P < 0.0001$ for grade A embryos, $P = 0.0002$ for grade B embryos and $P = 0.0001$ for grades C–D embryos; Fig. 1, panel B).

In the present study, 168 embryos were generated by women that were ≤35 years old (average: 31.8 years, range 22–35) and 316 embryos by women >35 years old (average: 39.1 years, range: 36–44). The rate of blastulation was significantly higher in younger women than in older ones (57% and 46% in young and old group, respectively, $P = 0.021$, chi-square test). Analysis of DNA profiles disclosed that

After data stratification based on ability to become blastocyst, the mtDNA/gDNA ratio was confirmed to be significantly higher in the culture medium of embryos that reached blastocyst stage compared to embryos that arrested, in both age classes of patients ($P < 0.0001$, Fig. 1, panels D–E).

The predictive performance of mtDNA/gDNA ratio was evaluated using ROC AUC. The AUC was equal to 0.713, with an optimal criterion (mtDNA/gDNA ratio) >184, associated with a sensitivity

the culture media of embryos from older women had less mtDNA copies than those from young women ($P = 0.0098$, Fig. 1, panel C).

## Blastulation prediction

(true positive rate) of 49.4% and a specificity (true negative rate) of 83.8% (Supplementary Fig. S1). The mtDNA/gDNA ratio predicted blastocyst development at $P < 0.001$.

Based on these results, we proposed an embryo grading that combined Day 3 standard morphology and the mtDNA/gDNA ratio (> or ≤184), as shown in Table I.

Accordingly, the grade of embryos was updated and two models were estimated, one including only Day 3 morphological grade (model 1) and the other including the combination of morphology grade and mtDNA/gDNA ratio (model 2). For this purpose, we analyzed the AUC for both models (Fig. 2). The AUC was equal to 0.696 for model 1 (sensitivity: 69.5%; specificity: 63.5%) and to 0.766 for model 2 (sensitivity: 85.6%; specificity: 51.0%), with a *P*-value for the difference = 0.0006. These data validated our preliminary results (Stigliani *et al.*, 2014) demonstrating that mtDNA/gDNA ratio in combination with morphological grade has the potential to be a better early, noninvasive, molecular classifier for blastulation of Day 3 embryos than morphology alone.

**Table I Novel human embryo grading combining Day 3 standard morphology and the mtDNA/gDNA ratio in culture medium.**

| Standard morphology grading | mtDNA/gDNA ratio | Combined grading (morphology + mtDNA) |
|---|---|---|
| 1 | <184 | 2 |
| 1 | ≥184 | 0 |
| 2 | <184 | 3 |
| 2 | ≥184 | 1 |
| 3 | <184 | 4 |
| 3 | ≥184 | 2 |
| 4 | <184 | 5 |
| 4 | ≥184 | 3 |

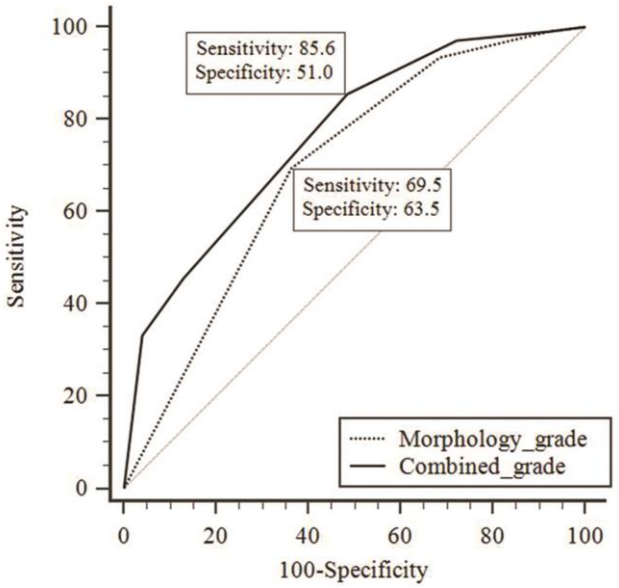
mtDNA/gDNA: mitochondrial DNA/genomic DNA

## Integration of mtDNA/gDNA ratio, blastulation and laboratory parameters

Since the two independent laboratories used their own preferred culture media and incubation systems, we wondered whether culture conditions could affect results. First of all, we determined that the blastocyst development rate at center #1 and center #2 was equal (53% and 48%, respectively, chi-square test not significant). Then we took into account the different culture media and incubators used in the two laboratories, and the performance of mtDNA/gDNA ratio in predicting embryo blastulation was good in both laboratories ($P < 0.0001$) (Fig. 3, panels A and B). Moreover, we demonstrated that at center #2 the association between mtDNA/gDNA ratio and blastocyst development was significant both under standard conditions of embryo incubation ($P < 0.0001$) and using a time-lapse system ($P = 0.0287$) (Fig. 3, panels C and D). These data were also confirmed by an equal blastocyst development rate under standard and time-lapse incubator systems (45% and 53%, respectively, chi-square test not significant) at center #2.

## . Logistic regression model

. The logistic regression analysis (Table II) showed that only the Day 3 embryo morphology grade combined with the mtDNA/gDNA ratio in culture medium was significantly associated with the blastocyst development. The higher the combined grading score, the less was the probability of the blastocyst development ($P < 0.0001$), without any




| Variable | AUC | SE [a] | 95% CI [b] |
|---|---|---|---|
| Morphology grade | 0.696 | 0.0240 | 0.650 to 0.734 |
| Combined grade | 0.766 | 0.0211 | 0.725 to 0.803 |

**Pairwise comparison of ROC curves**

| Morphology grade ≈ Combined grade | 95% CI [b] |
|---|---|
| Difference between areas | 0.0720 |
| Standard Error [a] | 0.0209 |
| 95% Confidence Interval | 0.0310 to 0.113 |
| Z statistic | 3.440 |
| Significance level | P=0.0006 |

[a] Hanley & McNeil, 1982
[b] Binomial exact

**Figure 2 Results of the ROC curve analysis.** Results of the ROC curve analysis displaying the predictive performances of day 3 morphological grade only and the combination of morphology grade and mtDNA/gDNA ratio.

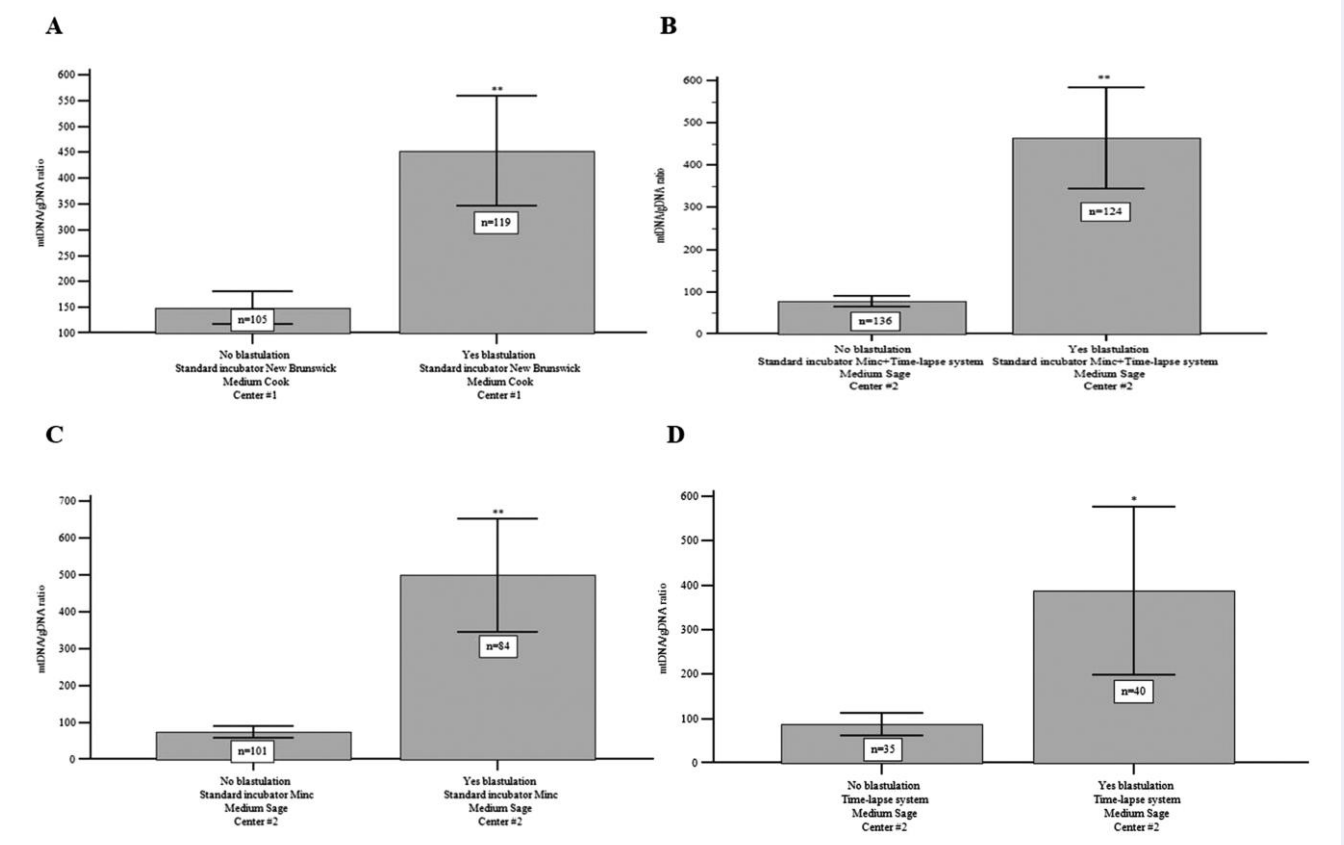



**Figure 3 Comparing the mtDNA/gDNA ratios between embryos.** Bar graphs comparing the mtDNA/gDNA ratio between spent media of embryos that arrested or develop to blastocyst stage at Center #1 (**A**) and Center #2 (**B**), and at Center #2 after stratification of embryos according to standard conditions of embryo incubation (**C**) or time-lapse system (**D**). Data are mean ± SE. Mann–Whitney test was used. $^{*}P < 0.05$. $^{**}P < 0.01$.

influence from patient and treatment-related factors. The age of the patient was close to significance in the univariate analysis ($P = 0.051$). However, in the multivariable analysis including the Day 3 embryo morphology grade combined with the mtDNA/gDNA ratio in culture medium and age, only the combined score remained significantly associated with the outcome, corroborating the hypothesis that the fittest model was the one with only the combined grading.

### Cohort embryo selection from the same woman

From the perspective of routine use in clinics, we evaluated the ability of the novel, integrated M3+mt rank to improve selection of developmentally competent embryos of a single cohort compared to traditional Day 3 morphology. Figure 4 shows a representative application of M3+mt rank of embryos with different day 3 morphological grades and development outcome from three patients. Taking into account embryo cohorts of 44 patients with a minimum of two embryos at the same stage on Day 3 and with different outcomes on Day 5, sensitivity and specificity of the scoring system based on Day 3 morphological stage was 92% and 13%, respectively. The integration with the information about mtDNA/gDNA ratio in culture medium increased the performance of the method (sensibility: 95%, +3%; specificity: 65%, +80%).

## Discussion

We previously identified a relationship between mtDNA/gDNA ratio in embryo culture medium and quality and blastulation potential of Day 3 embryos (Stigliani *et al.,* 2013; Stigliani *et al.,* 2014). Our pilot studies are confirmed in this prospective, blinded analysis of spent culture media from two independent centers using their own stimulation protocols, preferred culture media and incubation systems. Furthermore, we demonstrated that there is no influence from patient and treatmentrelated factors on combined Day 3 grade as a putative predictor of viability.

It is a widespread belief that mtDNA content in oocytes increases significantly during oogenesis and then undergoes a rapid decrease in embryo cells during preimplantation development, without any mtDNA replication between fertilization and morula stage (St John *et al.,* 2012; Hashimoto *et al.,* 2017). The total amount of mtDNA is split between blastomeres during each division, so that the mtDNA content of oocytes should be enough to sustain embryo development before implantation occurs. Consistently, the oocyte and cumulus cells mtDNA content positively correlates with both fertilization success and embryo viability (Santos *et al.,* 2006; Chappel, 2013; Ogino *et al.,* 2016; Desquiret-Dumas *et al.,* 2017; Reynier *et al.,* 2001). The mitochondrial content of embryos has been proposed as an indicator of embryo viability: embryos with euploidy status and greater

**Table II Influence of different possible predictors on blastocyst development.**

| | Odds ratio (95%CI) | *P*-value |
|---|---|---|
| **Age (>35 versus ≤35 years)** | | |
| >35 years | 1 | |
| ≤35 years | 0.62 (0.38–1.00) | 0.051 |
| **Cause of infertility** | | |
| Tubal only | 1 | |
| Endometriosis | 2.38 (0.94–6.02) | 0.068 |
| Maternal age | 0.85 (0.38–1.90) | 0.695 |
| Anovulatory | 2.03 (0.64–6.40) | 0.225 |
| Idiophatic | 1.69 (0.79–3.63) | 0.175 |
| Male factor | 1.56 (0.69–3.51) | 0.286 |
| Other/combination | 0.70 (0.13–3.66) | 0.669 |
| **Pituitary suppression** | | |
| Antagonist | 1 | |
| Agonist | 0.90 (0.53–1.52) | 0.686 |
| **Gonadotrophin used** | | |
| Recombinant | 1 | |
| Urinary | 1.13 (0.69–1.85) | 0.631 |
| Both | 0.81 (0.33–1.95) | 0.642 |
| **Combined grading** | | |
| 0 | 1 | |
| 1 versus 0 | 0.18 (0.08–0.38) | <0.0001 |
| 2 versus 0 | 0.14 (0.07–0.28) | <0.0001 |
| 3 versus 0 | 0.06 (0.03–0.13) | <0.0001 |
| 4 versus 0 | 0.01 (0.004–0.04) | <0.0001 |
| 5 versus 0 | 0.01 (0.003–0.06) | <0.0001 |

Data compared with mixed effect logistic regression model.
*Statistically significant differences, with $P < 0.05$

activation. In such a scenario, our idea is that the mtDNA/gDNA ratio in the embryo secretome would inversely mirror the overall embryonic mtDNA content.

We also examined the effect of maternal age on mtDNA/gDNA ratio in culture media. The significant decrease of medium mtDNA/gDNA ratio with advancing maternal age may reflect the low development competence of embryos from older women. Research has identified an oocyte age-associated deterioration in both the quality and quantity of mitochondria, i.e. mitochondrial swelling, vacuolization, alteration of cristae and mitochondrial membrane potential, that together with chromosomal aneuploidies and incidence of apoptosis may cause arrest of pre-implantation embryos (Muller-Hocker *et al.,* 1996; Wilding *et al.,* 2001; Van Blerkom *et al.,* 2011). However, logistic regression analysis corroborated the hypothesis that the combined score was significantly associated with embryo outcome, both in young and old patients.

Intriguingly, evidence that a high mtDNA/gDNA ratio predicts blastulation leads to the prospect of carrying out a non-invasive evaluation of the embryonic mtDNA content through spent Day 3 culture medium. Similarly, non-invasive PGS has been recently discussed as a potential future technique for embryo chromosome assessment (Cohen *et al.,* 2013; Feichtinger *et al.,* 2017). The most recent studies concluded that blastocoel fluid and spent media, as sources of gDNA for genetic analyses, do not provide sufficiently reliable results due

implantation potential and, therefore, of better quality have a small mtDNA content (Fragouli *et al.,* 2015; Diez-Juan *et al.,* 2015; Fragouli *et al.,* 2015; Ravichandran *et al.,* 2017). The implantation potential of euploid embryos inversely correlates with mtDNA copy number not only for blastocysts, but also for cleavage stage embryos (Diez-Juan *et al.,* 2015). According to the 'quiet embryo' hypothesis (Leese, 2002), in healthy embryos with a normal energy reserve the mitochondrial biogenesis is low, whereas embryos under stress and with reduced developmental potential activate mitochondrial biogenesis and mtDNA replication in an attempt to meet their energy demand. In this study we found that embryos that reached blastocyst stage showed a significantly higher mtDNA/gDNA ratio in the culture medium compared to embryos that arrested. On the contrary, less viable embryos, that are known to have mostly a high mtDNA content, a slower cell division and an increased degradation of gDNA over time, are those embryos that we found with a lower mtDNA/gDNA ratio in the culture medium. In line with the flexible nature of mtDNA content needed in early embryogenesis (Lin *et al.,* 2004), those embryos with a reduced developmental potential would slightly expel mtDNA in the medium, and they would preserve most of the oocyte-derived mtDNA in order to support fertilization, cell cleavage and embryonic genome to embryo mosaicism and maternal DNA presence (Tobler *et al.,* 2015; Capalbo *et al.,* 2018; Vera-Rodriguez *et al.,* 2018). We believe that in a non-invasive mtDNA/gDNA ratio evaluation the potential maternal mtDNA contamination would not confound prediction of embryo viability, since we found that the mtDNA/gDNA ratio is higher in the culture medium of embryos that reached blastocyst stage compared to embryos that arrested, regardless of maternal age, and, indirectly, of oocyte mtDNA content. Undoubtedly, a comparison between embryo mtDNA/gDNA ratio and that in the medium would provide support for this potential, non-invasive approach for embryo mtDNA analysis and development prediction.

Consequently, our future experiments will be also focused on the challenge of identifying the source(s) of mtDNA and gDNA in the culture medium.

Regarding a clinical translation of this work, we proposed a rank for embryos from the same patients based on Day 3 morphology grade and culture medium mtDNA/gDNA ratio, as an embryo blastulation marker. In our cohort of samples we demonstrated that our novel, non-invasive, integrated M3+mt rank is able to improve the selection of developmentally competent embryos of a single cohort compared to traditional day 3 morphology alone. The great increase of the specificity in predicting blastocyst development by combining the mtDNA/gDNA ratio in the culture medium with Day 3 morphology may diminish the probability that an embryo destined to arrest its development is classified as an embryo with a high viable potential and, thus, is transferred on Day 3. At the same time, the added value of the medium mtDNA/gDNA ratio in embryo score would not increase the risk of wrongly classify embryos that have the potential to become a blastocyst. The mtDNA/gDNA ratio analysis in culture medium in addition to routine morphologic classification would help the embryologist to rank embryos and choose which embryo(s) of a single cohort has the greater development potential, and thus should be transferred on Day 3, among sibling embryos available with the

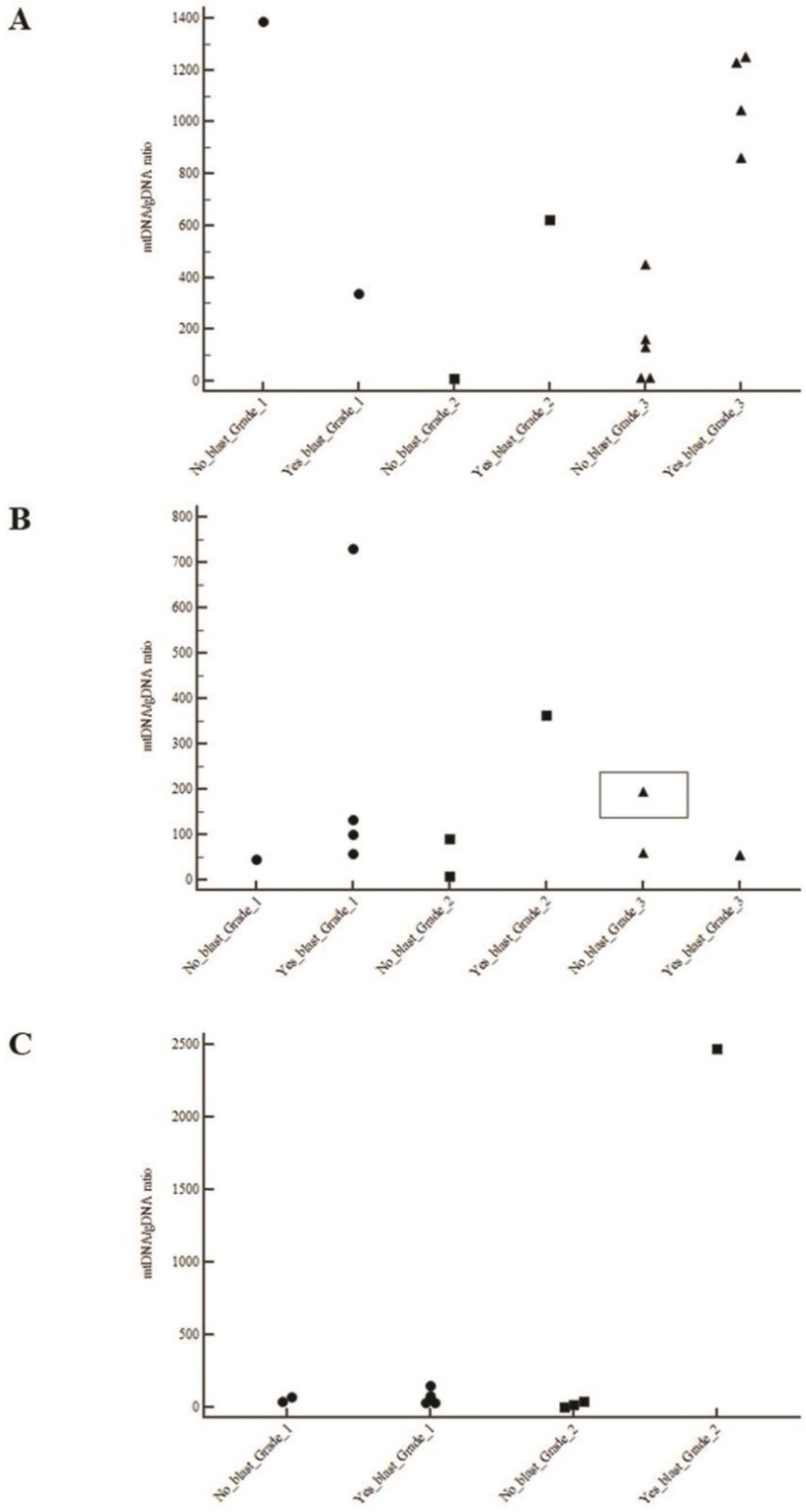



**Figure 4 Scatter plots of embryo cohorts from three representative patients showing medium mtDNA/gDNA ratio after stratification of embryos according to their Day 3 morphological grade.** On the X axis, the development outcome of embryos is specified (No/Yes blastulation). Panel **A** refers to a patient with two grade 1 embryos, two grade 2 embryos and nine grade 3 embryos. Panel **B** refers to a patient with five grade 1 embryos, three grade 2 embryos and three grade 3 embryos. Panel **C** refers to refers to a patient with six grade 1 embryos and four grade 2 embryos. The box in Panel B shows one grade 3 embryos wrongly predicted as having potential to reach the blastocyst stage. •: grade 1 embryos; : grade 2 embryos; : grade 3 embryos.

same morphological grade. Improved selection of developmentally competent embryos would reduce the time to pregnancy. Before routine use, the challenge is validation of this embryo ranking method in randomized perspective studies performed in independent IVF laboratories.

The added value of this corroboratory study is that results were consistent between two centers, where there may be different cellular stress sources, such as the choice of culture medium, the type of the incubator, gas composition, extent of embryo handling (e.g. number of times the embryo is taken out of the incubator for observation, media changes, etc.) or even differences in hormonal stimulation regimens. From the technical point of view, it is conceivable that in this work all the PCRs and the subsequent analyses have been performed at center #1, so that the logical clinical translation should consider a standardization of the procedure within each IVF laboratory to be applicable to its own embryos.

In conclusion, the mtDNA/gDNA ratio in the culture medium represents a possible marker for a non-invasive evaluation of embryo quality.

## Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

## Authors' roles

S.S. performed experiments and contributed to critical discussion. G.O. and I.C. performed embryo culture and contributed to medium collection. C.M. and F.B. performed statistical analyses. P.A., F.M.U., V.R. and L.R. contributed to critical discussion. P.S. performed study design, analyzed and interpreted the data, drafted the manuscript and contributed to critical discussion. All authors read and approved the manuscript.

## Funding

Gedeon Richter (FORWARD Grant).

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## Conflict of interest

The sponsor had no role in study design, data collection, data analysis, data interpretation and writing of the paper. Authors declare no conflicts of interest.

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