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Extensive analysis of mitochondrial DNA quantity and sequence variation in human cumulus cells and assisted reproduction outcomes

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STUDY QUESTION: Are relative mitochondrial DNA (mtDNA) content and mitochondrial genome (mtGenome) variants in human cumulus cells (CCs) associated with oocyte reproductive potential and assisted reproductive technology (ART) outcomes?

SUMMARY ANSWER: Neither the CC mtDNA quantity nor the presence of specific mtDNA genetic variants was associated with ART outcomes, although associations with patient body mass index (BMI) were detected, and the total number of oocytes retrieved differed between major mitochondrial haplogroups.

WHAT IS KNOWN ALREADY: CCs fulfil a vital role in the support of oocyte developmental competence. As with other cell types, appropriate cellular function is likely to rely upon adequate energy production, which in turn depends on the quantity and genetic competence of the mitochondria. mtDNA mutations can be inherited or they can accumulate in somatic cells over time, potentially contributing to aging. Such mutations may be homoplasmic (affecting all mtDNA in a cell) or they may display varying levels of heteroplasmy (affecting a proportion of the mtDNA). Currently, little is known concerning variation in CC mitochondrial genetics and how this might influence the reproductive potential of the associated oocyte.

STUDY DESIGN, SIZE, DURATION: This was a prospective observational study involving human CCs collected with 541 oocytes from 177 IVF patients. mtDNA quantity was measured in all the samples with a validated quantitative PCR method and the entire mtGenome was sequenced in a subset of 138 samples using a high-depth massively parallel sequencing approach. Associations between relative mtDNA quantity and mtGenome variants in CCs and patient age, BMI (kg/m^2), infertility diagnosis and ART outcomes were investigated.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Massively parallel sequencing permitted not only the accurate detection of mutations but also the precise quantification of levels of mutations in cases of heteroplasmy. Sequence variants in the mtDNA were evaluated using Mitomaster and HmtVar to predict their potential impact.

MAIN RESULTS AND THE ROLE OF CHANCE: The relative mtDNA CC content was significantly associated with BMI. No significant associations were observed between CC mtDNA quantity and patient age, female infertility diagnosis or any ART outcome variable. mtGenome sequencing revealed 4181 genetic variants with respect to a reference genome. The *COXI* locus contained the least number of coding sequence variants, whereas *ATPase8* had the most. The number of variants predicted to affect the ATP production differed significantly between mitochondrial macrohaplogroups. The total number of retrieved oocytes was different between the H-V and J-T as well as the U-K and J-T macrohaplogroups. There was a non-significant increase in mtDNA levels in CCs with heteroplasmic mitochondrial mutations.

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: Although a large number of samples were analysed in this study, it was not possible to analyse all the CCs from every patient. Also, the results obtained with respect to specific clinical outcomes and macrohaplogroups should be interpreted with caution due to the smaller sample sizes when subdividing the dataset.

WIDER IMPLICATIONS OF THE FINDINGS: These findings suggest that the analysis of mtDNA in CCs is unlikely to provide an advantage in terms of improved embryo selection during assisted reproduction cycles. Nonetheless, our data raise interesting biological questions, particularly regarding the interplay of metabolism and BMI and the association of mtDNA haplogroup with oocyte yield in ovarian stimulation cycles.

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Key words: cumulus cells / mitochondria / assisted reproduction / BMI / mitochondrial genome sequencing

Introduction

The mitochondrion is an essential organelle that performs a range of vital functions required for appropriate cellular behaviour and viability, including key roles in bioenergetic, biosynthetic, apoptotic and signalling pathways (Chandel, 2014; Spinelli and Haigis, 2018). Not surprisingly, therefore, mitochondrial dysfunction is associated with a range of disorders of varying severity, which are often termed 'mitochondrial diseases' (Mayr *et al.*, 2015; Xia *et al.*, 2019; Martinez-Reyes and Chandel, 2020; Shukla and Mukherjee, 2020). Clinical characteristics of these diseases can include progressive neuropathy, cardiomyopathy, loss of vision, and premature death. In most cases, these features are a consequence of impaired oxidative phosphorylation (OXPHOS), a process that occurs within the mitochondria. This may be caused by mutations that impact critical components of the OXPHOS pathway or may result from a disruption of mitochondrial homeostasis (Taylor and Turnbull, 2005; Tuppen *et al.*, 2010; Wei and Chinnery, 2020).

Human mitochondria contain their own genome consisting of one or more circular DNA molecules of ~16.6 kb (NC_012920), encoding 13 polypeptides that comprise subunits of the respiratory chain apparatus, 22 tRNAs and 2 rRNAs (12S and 16S; Andrews *et al.*, 1999). Variants and mutations in the mitochondrial DNA (mtDNA) are responsible for most of the OXPHOS deficiencies that cause mitochondrial disease. Unlike nuclear DNA, there may be hundreds or thousands of copies of the mtDNA present in a cell and, apart from rare exceptions, the organelle and its genome are exclusively maternally inherited.

Although an individual mitochondrion may contain more than one copy of the mitochondrial genome (mtGenome) and levels of OXPHOS activity may vary, quantification of the number of mtDNA molecules has been used as a proxy for the measurement of mitochondrial function in some circumstances, and alteration in mtDNA copy numbers (mtDNA-cn) has been linked to certain disease states. Consequently, mtDNA-cn has been proposed as a potential biomarker that may have clinical value (Pyle *et al.*, 2016; Yue *et al.*, 2018; Eirin *et al.*, 2019; Fazzini *et al.*, 2019). The presence of mtDNA variants and mutations has also been linked to several human diseases, including cancers, as well as neurological and cardiological disorders (Taylor and Turnbull, 2005; Tuppen *et al.*, 2010; Alvarez-Mora *et al.*, 2020; Jahani *et al.*, 2020).

Although most clinical research studies have focused on the role of mitochondria in life-threatening diseases, the importance of the organelle in the context of infertility and assisted reproductive treatments (ART) should not be overlooked (Kristensen *et al.*, 2019). The presence of tens or even hundreds of thousands of mitochondria in mature human oocytes is a requirement for successful preimplantation development, supporting the energy demands of meiosis, fertilization and the first few mitotic divisions (Babayev and Seli, 2015). Relatively higher ATP levels in oocytes have been linked to improved embryo development, as well as superior implantation and pregnancy rates (Van Blerkom *et al.*, 1995; Zhao and Li, 2012).In addition, a correlation between mtDNA-cn and mitochondrial function in human embryos suggests that the quantity of mtDNA is associated with the organelle's performance during the early development (Hashimoto *et al.*, 2017). It is thus important to investigate potential correlations between embryo viability and mtDNA-cn in oocytes, embryos and the cells of other reproductively important tissues (Fragouli and Wells, 2015; Wells, 2017) and to establish whether mtDNA-cn can serve as a useful biomarker, predicting the outcome of ART (Kim and Seli, 2019).

Numerous studies have been undertaken with the aim of determining the utility of mtDNA analyses for the evaluation of oocyte and embryo quality. However, the results have been contradictory. There has been particular controversy over the quantification of mtDNA in cells biopsied from blastocyst stage embryos and its use as a predictor of embryo implantation potential. Even if the analysis of mtDNA in cells sampled from preimplantation embryos proved to be clinically useful, the invasive nature of the biopsy can be seen as undesirable because it requires extensive training and adds to the cost of IVF procedures. Non-invasive approaches are attractive, because they avoid any risk of damage to the embryo and have the potential to be delivered at lower cost.

The cumulus cells (CCs), specialized somatic cells that enclose the oocyte, are considered a possible source of biological information relevant to the competence of the oocyte that they surround (Feuerstein *et al.*, 2012). Because a small sample of CCs can be safely removed from around the oocyte without compromising it, they may provide a means of non-invasive assessment. CCs are intimately associated with the oocyte, exchanging molecules via filamentous projections that pierce through the encapsulating zona pellucida, and connecting with its cytoplasm via gap junctions (Huang and Wells, 2010; Feuerstein *et al.*, 2012; Li and Albertini, 2013; Dumesic *et al.*, 2016). The bidirectional communication established between CCs and oocytes is essential for oocyte viability. Disruption of this communication by the

inhibition of gap junctions has been shown to lead to a reduction in intracellular ATP in the oocyte, indicating a role for CCs in the maintenance of oocyte energy levels (Dalton *et al.*, 2014). As with other cells, appropriate CC function is likely to rely upon adequate energy production, which in turn depends on the quantity and genetic competence of mitochondria within the cells. Given the importance of mitochondria for CC function, and the vital role of CCs in promoting oocyte competence, it has been proposed that mtDNA-cn quantification might provide information concerning the quality of the oocyte, which is of value in the context of embryo selection during IVF treatment.

In addition to the quantitative analysis of mtDNA, qualitative assessment of the mtGenome, identifying single-nucleotide variants (SNVs), rearrangements (deletions, duplications, insertions), and point mutations, is of great interest with respect to their potential relationship with oocyte quality (Ma *et al.*, 2020). The mtGenome is more prone to genetic damage than the nuclear genome, due to the absence of protective histones, a lack of robust DNA repair mechanisms (decreased DNA polymerase gamma, POLG fidelity) and its exposure to free radicals produced by the respiratory transport chain. In addition, single-nucleotide substitutions in the mtGenome are, on average, more likely to have an impact than those in the nuclear genome because a much higher proportion of the mtDNA is functionally important (e.g. there are no introns). Mitochondrial DNA mutations can be inherited, or they can accumulate in cells over time, potentially contributing to ageing (Barritt *et al.*, 2000) as well as several disease phenotypes (Schon *et al.*, 2012). Mitochondrial genotypes within a cell can be classified as homoplasmic where all copies of the mtGenome are identical or heteroplasmic where there is a mixture of two or more distinct mitochondrial genotypes.

Currently, little is known concerning the associations between mitochondrial genetic variation in CCs and oocyte competence and ART outcomes. Recently, a study comparing patients with normal ovarian reserve to those with diminished ovarian reserve found no differences with respect to mtGenome variants in CCs or their corresponding oocytes (Boucret *et al.*, 2017). In the present study, we determined relative mtDNA levels and the frequency of mitochondrial genetic variations in a large number of human CC samples and searched for associations with a range of clinically important ART outcomes.

Materials and methods

Study population

CC clusters were donated for research under consent by patients undergoing IVF treatment for infertility at Brigham and Women's Hospital, Boston, USA. The study protocol was approved by the Institutional Review Board of Partner's Healthcare (Partner's IRB # 2014P000874). The only criterion for excluding patients was if they were undergoing an oocyte freeze-all cycle. Additional patient information is summarized in Table I.

Sample collection

A small cluster of CCs from each of up to four randomly selected cumulus–oocyte complexes was collected immediately after completion of an oocyte retrieval following ovarian stimulation as previously described (Bakkensen *et al.*, 2019). Clusters were vitrified on Cryolocks (FUJIFILM Irvine Scientific, USA) using the Irvine Vit-Kit (Cat # 90133-SO) and then stored at −196°C in preparation for shipment to the Juno Genetics Laboratory in the UK for subsequent mitochondrial and genomic analyses. Patient age, body mass index (BMI; kg/m^2), clinical diagnosis with respect to fertility and ART outcomes (e.g. oocyte maturity, fertilization, embryo morphology, implantation) were recorded for each cumulus complex.

Table I Demographic and clinical characteristics of patients.

Clinical characteristics	Values*
Age	36.8 ± 4.4 (36.1, 37.4)
BMI (kg/m^2)	27.0 ± 6.4 (26.1, 28.0)
Normal weight (18.5–24.9)	22.3 ± 1.5 (22.1, 22.5)
Pre-obesity (25.0–29.9)	27.1 (26.9, 27.4)
Obese (≥30.0)	36.7 (35.8, 37.6)
AMH (ng/ml)	2.8 ± 2.4 (2.4, 3.2)
Number of oocytes retrieved	16.6 ± 10.7 (15.0, 18.2)
% of mature oocytes of total retrieved	74.7±17.9 (72.0, 77.3)

AMH, Anti-Müllerian hormone; CI, confidence interval; BMI, body mass index; SD, standard deviation.
*Mean ± SD (CI) for all variables.

Clinical laboratory protocols

The standard protocols used in the IVF laboratory at Brigham and Women's Hospital were used as previously described (Bakkensen *et al.*, 2019). Immediately after CC sample collection, clusters and their corresponding cumulus-oocyte complexes (COCs) were assigned numbers for tracking purposes. Briefly, the COCs were incubated separately in 25 µl drops of GLB-Fert (IVFOnline, Guelph, Ontario) under mineral oil in Miri Esco bench-top incubators in a dry atmosphere of 5% O_2, 6–7% CO_2, balanced with N_2. Oocyte maturity was determined immediately after removing the CCs for oocytes destined for ICSI. For oocytes that underwent standard insemination for IVF, an oocyte was considered mature if, at the fertilization check, it exhibited the first polar body but no pronuclei, or if it exhibited any number of pronuclei. Those that were conventionally inseminated were inseminated with ∼50 000 motile sperm, whereas those for ICSI were stripped of remaining cumulus-corona cells following exposure to hyaluronidase using standard procedures. Zygotes exhibiting two pronuclei (2PN) at the fertilization check were moved to 25 µl drops of GLB-Total under mineral oil for culture.

The morphology of embryos was evaluated on Day 3 between 66 and 69 hr post-insemination (PI) and again on Day 5 (between 112 and 117 hr PI) for embryos in extended culture. The variables used for evaluations were as previously described (Bakkensen *et al.*, 2019). Embryos were classified as good, fair and poor quality according to the grades assigned as previously described. The decision to transfer, cryopreserve or discard an embryo was based on its morphological grade.

DNA extraction from CCs

Total DNA extraction from individual CC samples was achieved using the QIAmp DNA Micro kit according to the manufacturer's instructions (QIAGEN, USA). Briefly, lysis was achieved by incubating the samples in buffer ATL, AL and proteinase K at 56°C for 10 min. Ethanol was added to the lysate to precipitate the DNA that was then bound to the silica membrane in the QIAamp MinElute columns. The binding process was reversible and specific to nucleic acids. Two different washes were performed using AW1 and AW2 buffers to remove any contamination. Genomic DNA was finally eluted in 55 µl of AE elution buffer at room temperature (15–25°C). The concentration of the extracted DNA was measured using the Qubit™ 1× dsDNA HS Assay Kit (Q33231; Invitrogen).

Relative mtDNA quantification

Relative quantification of mtDNA in the CC samples was accomplished using a published protocol previously validated in our laboratory (Fragouli *et al.*, 2015). Custom-designed TaqMan assays (ThermoFisher, USA) amplifying distinct human mtDNA sites were used. Briefly, each CC DNA sample was diluted 1:10. A quantitative PCR (qPCR) was then performed using 10 µl of TaqMan Universal Mastermix II (2×) no UNG, 1 µl of the Taq Man assay (20×) and 9 µl of diluted DNA per reaction. Samples were analysed in triplicate using a Viia7 real-time PCR machine (ThermoFisher, USA). As previously described (Fragouli *et al.*, 2015), a nuclear multicopy Alu sequence was also amplified from each sample and used as an internal standard, allowing the normalization of data with respect to the number of cells in the sample, DNA extraction efficiency and differences in PCR performance. In addition, for every qPCR experiment, the same DNA sample was used to act as a reference to maintain consistency across different runs.

Next-generation sequencing of mtGenome: mtDNA long-range PCR amplification and paired-end sequencing

The entire mtGenome was sequenced from 138 CC samples with a protocol that involved amplification of the whole mtGenome, followed by the next-generation sequencing using a protocol validated for this purpose (Human mtDNA Genome Guide; 15037958; Illumina, USA). Briefly, mtDNA from CC samples was amplified by long-range PCR using two pairs of overlapping primers, giving rise to 9065- and 11 170-bp fragments. PCR was set up using TaKaRa LA Taq polymerase (RR002M; TaKaRa) in a total volume of 50 µl. Amplified mtDNA was subjected to electrophoresis and tapestation analysis to confirm the successful amplification. The concentration of each amplicon was measured using Qubit™ dsDNA BR assay kit (Q32853; Invitrogen) and the concentration was normalized to 0.2 ng/µl for each amplicon. For each amplicon, 10 µl was mixed to constitute the whole mtDNA for each sample. Then, 1 µg (5 µl of mixed amplicons) of DNA was converted into dual-indexed sequencing libraries using the Nextera XT DNA Sample Preparation and Index Kits according to the validated Human mtDNA Genome protocol (Illumina). The cleaned and normalized libraries were then subjected to paired-end sequencing: 2×150 cycles with dual indexing on an Illumina MiSeq using the MiSeq Reagent Kit v3 (Illumina). Sequenced reads were aligned to the human genome (hg19) generating FASTQ and BAM files. The *mtDNA variant processor* Illumina Basespace app was used to create VCF files, which were subsequently analysed by another app, *mtDNA variant Analyzer*. Default and suggested analysis parameters for *mtDNA variant processor* are as follows: base call quality score of 30, minimum read count value of 10 (number of reads required at the mtGenome reference coordinate to produce a base call), analysis threshold of 10% (the nucleotide call is not visible or reported as part of the call if it is less than the value of the analysis threshold) and interpretation threshold of 25% (a nucleotide contributes to the call for the coordinate if it exceeds the total number of reads greater than the interpretation threshold percentage). For each sequencing run, a sequencing control was analysed in parallel to confirm the consistency of sequencing results in terms of the number and types of variants detected.

Statistical analysis

All the quantitative variables, including discrete (numbers of SNVs) and continuous (age, BMI, mtDNA, Anti-Müllerian hormone (AMH)) variables are presented as mean values and standard deviation. The binary and qualitative variables (oocyte nuclear maturity, fertilizability, embryo quality, variant number) are described with their percentage or counts. The correlation between relative mtDNA level and age, BMI and AMH was assessed using the linear regression model. Similar comparisons between the total number and types of SNVs with mtDNA level, age and BMI were also determined using a Poisson linear regression model. The association between haplogroups and available ART outcomes was tested using Fisher's exact test, as were haplogroup data with respect to other categorical variables. Pairwise test of independence was used to assess the BMI difference between macrohaplogroups.

To compare the clinical characteristics of oocytes corresponding to their associated CCs and the ART outcomes, a linear mixed model (LMM) with random effect was used to take account of individual patients with multiple CC samples. All of the statistical analyses were performed in R version 4.0.3. LMM was specifically computed using the *nlme* packages of R (Lindstrom and Bates, 1988). All the statistical graphs and figures were derived using R packages: ggplot2 and ggstatsplot. The differences in all the statistical analyses were considered statistically significant at $P < 0.05$.

Results

Study population

A total of 541 CC clusters from 177 patients were collected. Patient age ranged from 24.8 to 44.9 (36.8), their BMI ranged from 17.8 to 49.5 (27.0) and their AMH levels ranged from 0.1 to 12.0 (2.8). Most common infertility diagnoses were represented in our study population. The average number of oocytes retrieved was 16.6, of which 12.4 were mature (74.7%). Of the 541 oocytes associated with the collected CC clusters, 371 were inseminated by ICSI and 161 by conventional IVF (Table II). Of the 2PN zygotes obtained from the trimmed COCs (n = 331), 105 were transferred fresh, 119 were cryopreserved of which 30 were subsequently thawed and transferred and 4 were discarded. Overall, of those embryos transferred, 111 were

Table II Number of samples in each statistics analysis.

Statistical comparison	Number of patients*	Number of samples*	Number of samples in studied groups
Relative CC mtDNA levels	177	541	–
Age and relative CC mtDNA levels	176	541	–
BMI and relative CC mtDNA levels	176	541	–
BMI categories and CC mtDNA levels	172	529	252 (≥18.5 and ≤24.9) 151 (≥25.0 and ≤29.9) 126 (≥30.0)
Female diagnosis and CC mtDNA levels	173	530	–
ART types	176	537	371 (ICSI) 161 (IVF) 05 (no insemination)
Oocyte maturity and CC mtDNA levels	177	534	439 (MII-mature) 47 (germinal vesicle) 26 (MI) 13 (abnormal) 06 (degraded) 03 (between MI and MII)
Fertilization and CC mtDNA levels	169	443	331 (2 PN) 51 (0 PN) 28 (3 PN or more) 16 (degraded) 12 (1 PN) 05 (abnormal)
Day 3 embryo morphology and CC mtDNA levels	145	315	95 (excellent) 84 (good) 83 (poor) 53 (fair)
Day 5 embryo morphology and CC mtDNA levels	106	215	117 (very poor) 38 (poor) 37 (fair) 18 (good) 05 (excellent)
CC mtDNA levels between discarded and fresh and cryopreserved embryos	176	533	309 (discarded) 224 (transferred/ cryopreserved embryos)
CC mtDNA levels between fate of embryo transfers	74	111	60 (failed implantation) 51 (consists of viable, ectopic, abortions and chemical pregnancy)

ART, assisted reproductive technology; BMI, body mass index; CC, cumulus cell; mtDNA, mitochondrial DNA; PN, pronuclei.
*The number of samples used in the statistical model to compare the differences between mtDNA levels and clinical and ART outcomes.

traceable to the developmental fate (a viable foetus at >7 weeks gestation or no viable foetus).

CC mitochondrial DNA

Relative mtDNA levels in CCs

The overall mean number of COCs for relative CC mtDNA quantification per patient was 3.1. To determine the intra-individual variability, an intraclass correlation coefficient (ICC: a measure of the reliability of measurements for clusters sorted into groups) score was computed. In our study, an ICC value of 0.60 indicated less variance of mtDNA of CCs for individual patients than between different patients. The observed average relative quantity of mtDNA in 541 CC samples from 177 patients is shown in Fig. 1. Patient age was not related to CC mtDNA levels ($P = 0.987$). However, while controlling for age in the mixed model, BMI of women was significantly associated with the CC mtDNA content: women with a higher BMI had a higher quantity of mtDNA in CCs than those with a lower BMI ($P = 0.026$, Fig. 2). Moreover, a significant difference was also detected between the group categorized as normal weight (BMI 18.5–24.9) and those classified as 'obese' (BMI ≥ 30.0, $P = 0.028$, Fig. 3). Regarding classification of the cause of infertility, no statistically significant differences were

observed for CC mtDNA level and diagnosis. In relative terms, the quantity of CC mtDNA was similar regardless of the cause of infertility.

CC mtDNA and ART outcomes: oocyte maturity and fertilizability and embryo quality

This study included 439 CC samples associated with MII oocytes that were mature at the time of denudation. A further 95 CC samples were associated with oocytes that were either immature or abnormal (Table II). No association was found between oocyte maturity and the levels of mtDNA in the corresponding CC (GV [n = 47] versus MII [n = 439], $P = 0.879$; MI [n = 26] versus MII [n = 439], $P = 0.829$; GV + MI [n = 73] versus MII [n = 439], $P = 0.938$). Neither were there any associations between CC mtDNA levels for zygotes exhibiting 2PN (n = 331) compared with those displaying an abnormal fertilization outcome ($P = 0.431$). Similarly, no significant difference was seen between the mtDNA levels of CCs associated with oocytes that were activated (2PN + 3PN, n = 359) versus those that showed no evidence of fertilization (0PN, n = 51, $P = 0.683$). No associations were found between the CC mtDNA content and any other clinical parameter related to the patients or their treatment. The relative quantity of CC mtDNA showed no significant difference with respect to the morphology of the resulting embryo on either Day 3 (embryos graded 'excellent', 'good' or 'fair' [n = 232] versus those categorized as 'poor' [n = 83; $P = 0.912$]) or Day 5 (embryos graded 'excellent', 'good' or 'fair' [n = 60] versus those categorized as 'poor' or 'very poor' [n = 155; $P = 0.109$]). No association between CC mtDNA levels and embryo fate was detected. Specifically, the mtDNA levels in CCs associated with oocytes that produced embryos suitable for transfer or cryopreservation, with a view to potential future transfer (n = 224), were not significantly different from levels in CCs associated with poor-quality embryos that were subsequently discarded (n = 309; $P = 0.317$).

CC mtDNA, implantation and viable pregnancy

Of 111 embryos transferred with the known developmental fate, 19 embryos achieved a viable pregnancy, 16 resulted in chemical pregnancy, 6 resulted in ectopic pregnancies, 10 resulted in miscarriage and 60 failed to implant. CC mtDNA levels did not differ significantly between those embryos resulting in a confirmed intrauterine implantation (i.e. a group including ongoing pregnancy and miscarriage (0.005231 ± 0.003862) compared with cases in which there was no implantation; 0.004425 ± 0.003121, $P = 0.309$).

Variants of CC mtGenome

Homoplasmic variants in CC mtDNA

With respect to the revised Cambridge mitochondrial reference genome (Andrews *et al.*, 1999), analysing the entire mtGenome of CCs from 138 patients revealed 4181 genetic variations: 3633 transitions, 85 transversions, 229 deletions and 234 insertions located in both the coding and non-coding regions including the mtDNA control region as well as tRNAs and rRNAs (Fig. 4). Within the non-coding compartment of the mtGenome, a majority of variants were located in the D-loop region. The mean number of paired reads (paired-end depth) for identified bases, A, T, G, C (per sample), was ≈3000, with an average of 3–4 million reads per sample.

Location of variants within the mtGenome

The distribution of variants in the mtGenome was examined by normalizing the number of variants detected in each region to the size of the region (Yeung *et al.*, 2014). The probability of detecting a variant was greater in the non-coding region (0.168) than the coding region (0.047). In the non-coding region, variants were most likely to be observed in the D-loop region. Within genes, variants were least likely to be found in Cytochrome C Oxidase I (*COX I*; 0.032), whereas they were most common in *ATPase8* (0.063), with the difference between the two genes being almost double.

Synonymous and non-synonymous variants

With respect to the reference genome (Andrews *et al.*, 1999), we detected a total of 1207 synonymous SNVs (i.e. no amino acid change), which are presumed to be without functional consequences, and another 775 non-synonymous SNVs (potentially impacting normal function). Among these, 1913 were transitions and 69 were transversions (Ti/Tv ratio is ≈29:1). There were 137 tRNA variants found in our study cohort, of which 136 were transitions and 1 was a transversion. The most frequent was mt.12308A>G, followed by mt.15924A>G, both of which are predicted to be damaging in nature. The most frequent base substitutions in the coding compartment of the CC mtGenome were mt.15326A>G, mt.14766C>T, mt.11719G>A, mt.8860A>G, mt.7028C>T and mt.4769A>G. No correlation was observed between the CC mtDNA level and mtDNA variant types (deletions [$P = 0.264$], insertions [$P = 0.955$], transitions [$P = 0.718$] and transversions [$P = 0.665$]). Also, no significant difference was observed for these mtDNA variant types between activated oocytes (2PN + 3PN) and those that failed to activate (0PN) and between those with normal fertilization (2PN) and the unfertilized oocytes (0PN). Similarly, the number of CC mtDNA variant types was same between usable (transferred and cryopreserved) and discarded embryos.

Heteroplasmic variants in CC mtDNA

Using the *mtDNA variant analyser*, heteroplasmy was identified at 28 different positions from 22 different patients. For those patients with heteroplasmy, the number of distinct heteroplasmic variants observed in different samples ranged from one to four (Supplementary Table SI). The majority of heteroplasmic patients (81%) carried only one mtDNA variant. CC mtDNA heteroplasmy was detected in both the coding (86.3%) and non-coding regions of the mtGenome. The greatest number of heteroplasmic variants was identified in *ATP6* with four variants followed by *ND3* with three variants. Of the instances of heteroplasmy detected, nine involved non-synonymous changes, five of which were predicted to be pathogenic, affecting the *ATP6* and *ND5* genes. The relative mtDNA levels were higher on average in CCs with heteroplasmic variants but did not reach statistical significance ($P = 0.220$) compared with the non-heteroplasmic group.

CC mtDNA variants and haplogroup analysis

Mitochondrial haplogroup classification was achieved using HaploGrep 2, based on Phylotree (van Oven and Kayser, 2009; Weissensteiner *et al.*, 2016). All of the predicted haplogroups were assigned with a quality score of ~80%. There were 120 distinct haplogroups predicted in the set of samples sequenced. Due to the scarcity of some haplogroups in the present cohort, these were grouped into macro

haplogroups (H-V, J-T, U-K) for the purpose of statistical analysis. H-V dominated the haplogroup classification with a frequency of 32.6%, followed by U-K (26.8%) and J-T (21.0%). Other less common haplogroups accounted for the remaining 19.6% of patients. None of the macrorhaplogroups showed any association with BMI. This remained the case when BMI was classified into groups: healthy, overweight and obese (H-V versus J-T, $P = 0.567$; H-V versus U-K, $P = 0.277$; J-T versus U-K, $P = 0.721$; Fig. 5).

The total number of retrieved oocytes differed significantly for patients of the H-V macrohaplogroup in comparison with those of J-T ($P = 0.003$), and between J-T and U-K ($P < 0.0001$), but not between H-V and U-K ($P = 0.078$; Fig. 6). However, no significant differences were observed when comparing the proportion of mature MII oocytes (from the total number of retrieved oocytes) for patients of different macrohaplogroups: J-T versus H-V ($P = 0.400$) and U-K versus H-V ($P = 0.125$). Also, the fertilization outcome, as defined by the formation of two pronuclei ($P = 0.911$) and the fate of embryo transfer (fresh transfer or cryopreserved for potential future use versus unsuitable for clinical use/discarded), was not different for each macrohaplogroup ($P = 0.784$). Although not statistically significant, the relative mtDNA levels detected in CCs were lowest in the H-V group and highest in the U-K group (Fig. 7).

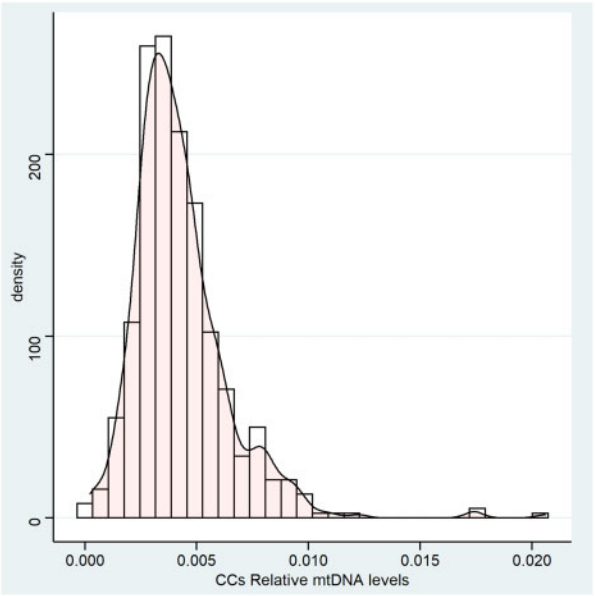

Figure 1. Distribution of relative mtDNA levels in the CCs ($n = 541$) included in the study. The distribution of mtDNA quantity CCs shows a positive asymmetry. Density (instead of counts) on *y*-axis represents density points in bin, scaled to integrate to 1. CCs, cumulus cells; mtDNA, mitochondrial DNA.

Pathogenic variants

The potential pathogenicity of homoplasmic non-synonymous variants was predicted using HmtVar (Preste *et al.*, 2019). There were 135 variants, detected from 75 patients, classed as potentially pathogenic, with a likely impact on ATP production. We did not find any association between ART outcome and the presence of *in silico* predicted pathogenic variants. A significant difference was observed between the macrohaplogroups regarding the number of predicted mtDNA pathogenic variants (P: H-V versus J-T: 0.037; HV-U-K: <0.0001).

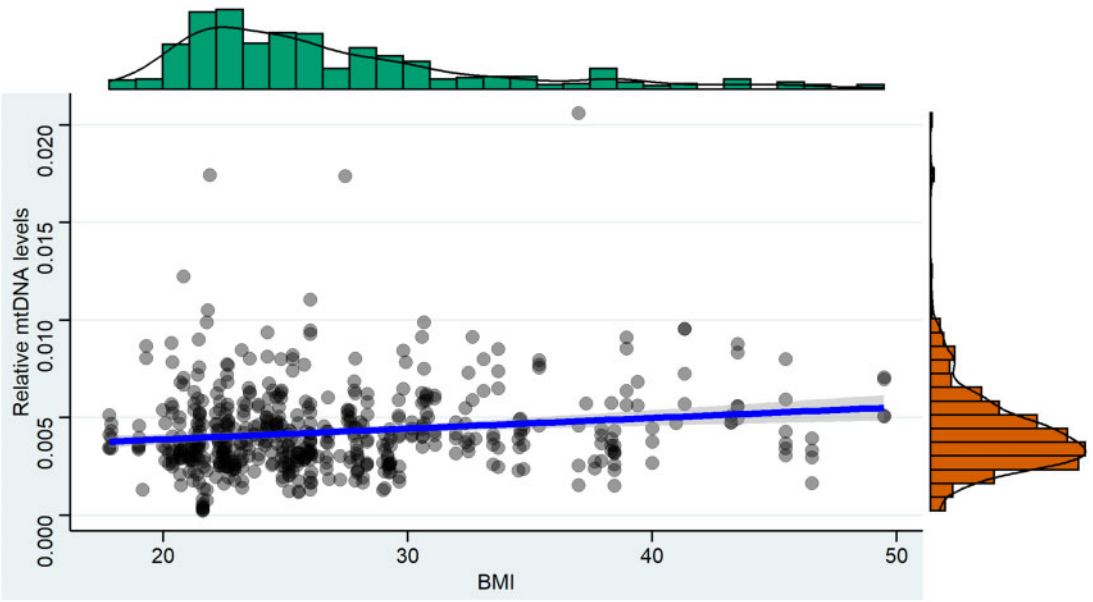

Figure 2. The correlation between relative mtDNA levels in CCs and BMI in 541 samples from 176 patients. CCs, cumulus cells; mtDNA, mitochondrial DNA.

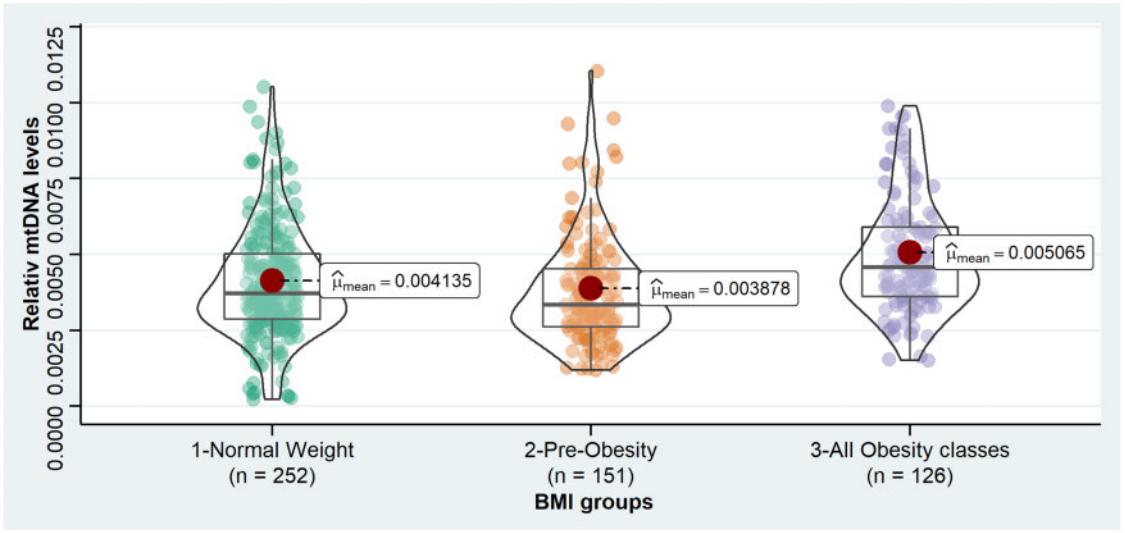

Figure 3. Relative level of mtDNA in CC in BMI classified groups: normal weight, pre-obese and all obese groups. BMI, body mass index; CCs, cumulus cells; mtDNA, mitochondrial DNA.

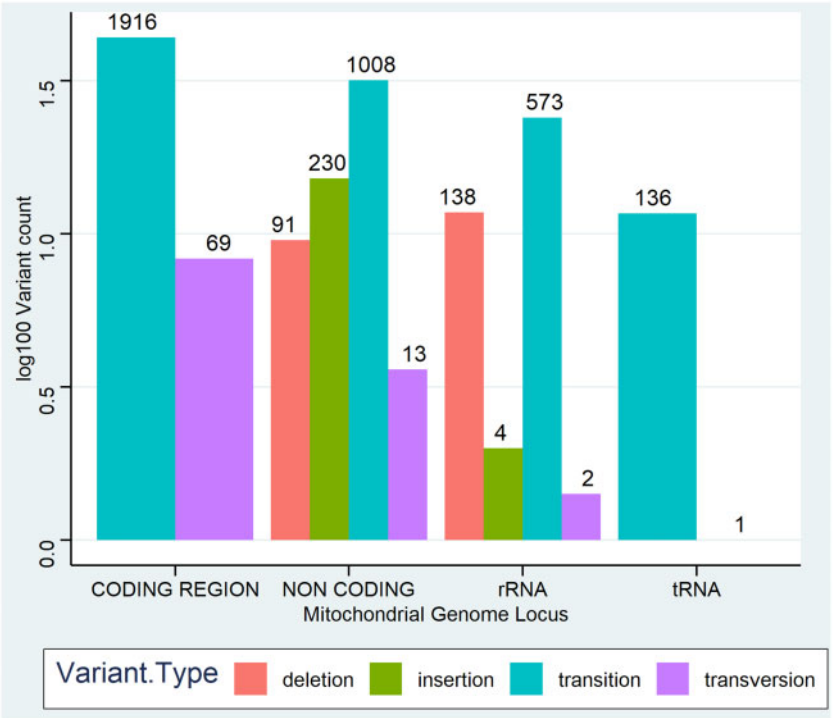

Figure 4. Distribution of mutation types in human mitochondrial genome in sequenced samples from the current study.

Discussion

In this study, we examined whether the mitochondrial genetics of human CCs is associated with the outcome of ART. To our knowledge, the present study is the most comprehensive undertaken to date, involving a large number of patients, the majority of whom provided several CC samples. The investigation assessed the mtDNA qualitatively

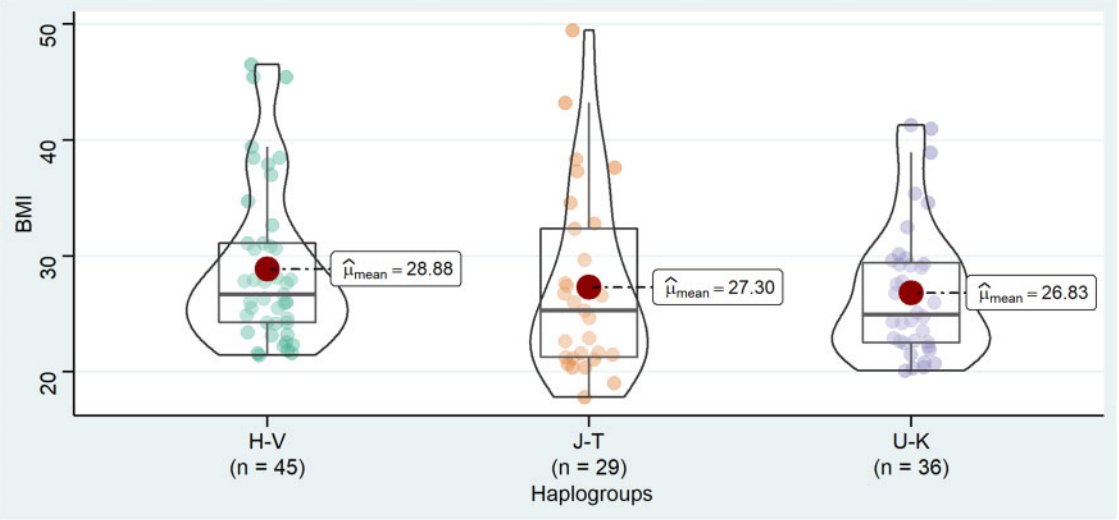

Figure 5. Box plot representation of BMI in different macrohaplogroups as observed in the study. BMI, body mass index.

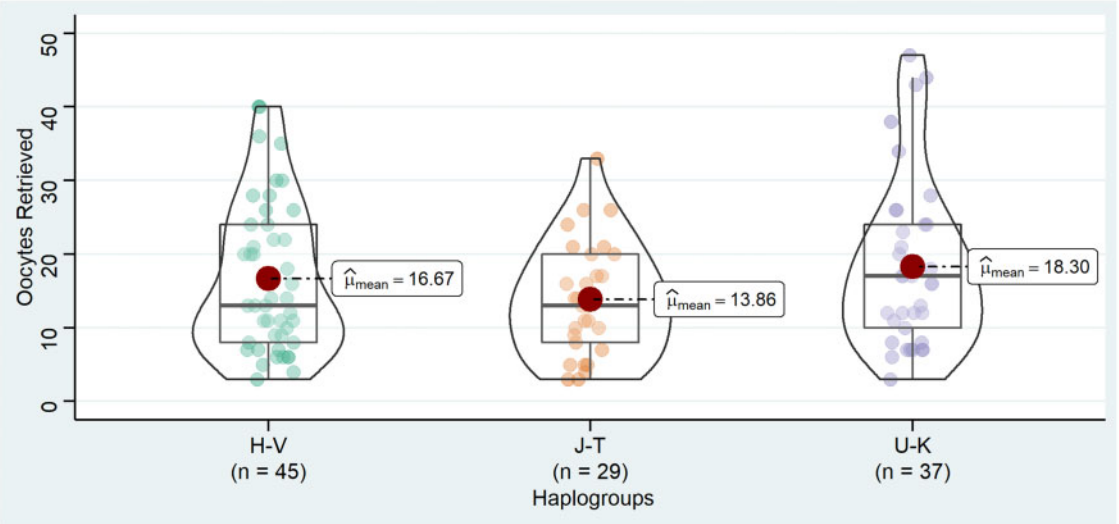

Figure 6. Distribution of total retrieved oocytes and macrohaplogroups: H-V, J-T and U-K.

(sequencing the entire mtGenome) and quantitatively (evaluating relative mtDNA-cn) and evalua0ted the results for associations with various important clinical outcomes (e.g. oocyte maturity, fertilization, embryo development and implantation) as well as patient characteristics (e.g. age, cause of infertility, BMI).

There has been considerable interest in the potential of mtDNA to reveal clinically useful information in the context of ART (Diez-Juan *et al.*, 2015; Fragouli *et al.*, 2017; Ravichandran *et al.*, 2017). However, these findings are considered controversial and typically involve invasive methods in which cells are biopsied from preimplantation embryos (Treff *et al.*, 2017; Victor *et al.*, 2017; Scott *et al.*, 2020). Whether differing conclusions concerning the value of mtDNA measurement from embryonic cells are a consequence of variation between studies in technical, biological or clinical factors remains unclear at this time (Barnes *et al.*, 2017; Wells *et al.*, 2017).

The notion that the mtDNA content of CCs might provide an indication of the status of the oocyte with which they are associated is attractive, because embryo viability during the first few days of development is largely dependent on the oocyte and because the CCs can be collected safely. CCs play a vital role in support of the oocyte,

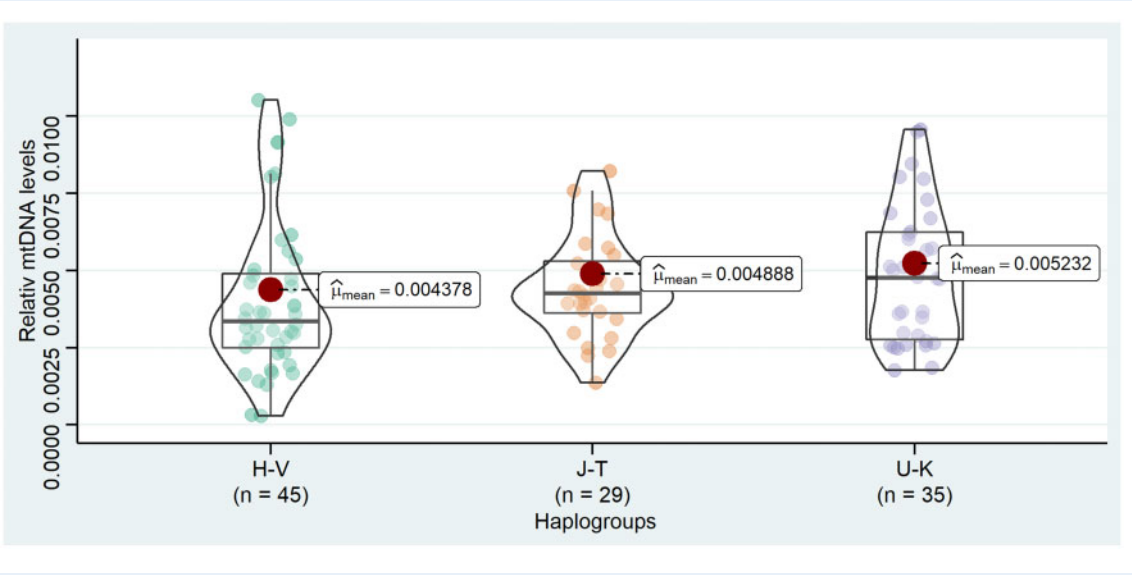

Figure 7. Box plot showing the levels of CC relative mtDNA between different macrohaplogroups observed in the study. CC, cumulus cell; mtDNA, mitochondrial DNA.

exchanging macromolecules and transducing signals, and it therefore seems likely that adequate metabolic function of CCs would be required for the associated oocyte to achieve competence. In addition, the quantity of mtDNA within CCs has been reported to be correlated with levels observed in the corresponding oocyte in some patients, suggesting that inferences about mtDNA-cn in the oocyte could be made by analysing the surrounding CCs (Boucret *et al.*, 2015). Recent publications have added further information concerning the value of mtDNA assessment in CCs and oocytes, although such studies have often had limited sample sizes and/or have included the analysis of patients with confounding factors. It is noteworthy that certain patient and treatment characteristics, such as age (Tatone and Amicarelli, 2013; Al-Edani *et al.*, 2014), BMI (Robker *et al.*, 2009; Pantasri and Norman, 2014), cause of infertility and stimulation protocol, all appear to have the capacity to affect the metabolic function of CCs.

Age was not associated with mtDNA-cn in CCs in the current study. This is in agreement with a recent investigation in which CC mtDNA quantity was unaffected by age and did not correlate with assisted reproduction outcomes (Liu *et al.*, 2021). Consistent with this previous study, we detected no differences in the mtDNA content related to morphological measures of embryo quality or likelihood of implantation (Liu *et al.*, 2021). Interestingly, in the present study, a significant positive relationship between BMI and relative CC mtDNA quantity was observed. This relationship remained significant even after controlling for age.

A major function of the cumulus complex is the protection of the oocyte from oxidative stress by acting as an antioxidant buffer (Shaeib *et al.*, 2016). CCs provide a variety of antioxidants (not synthesized by oocyte) that can effectively convert reactive species into a non-reactive state. The protective nature of CCs is evident from comparisons of IVF and ICSI techniques, the latter of which involves the routine removal of CCs from around the oocyte prior to fertilization. Greater oxidative damage has been reported for ICSI fertilized oocytes (Bedaiwy *et al.*, 2004). In obese women, oxidative stress in CCs leads to reduced mitochondrial activity (mitochondrial potential index [MPI]) affecting the overall cumulus–oocyte complex (Gorshinova *et al.*, 2017). Moreover, in mural granulosa cells (the cells lining the antral cavity), the MPI significantly declines with age, whereas the relative mtDNA-cn has been reported to increase (Liu *et al.*, 2017). The positive association between BMI and mtDNA content in our study might suggest a compensatory mechanism to overcome the increased ROS and altered redox biology and maintain mitochondrial output. Adipose tissue, a crucial player in obesity has been shown to display a positive correlation between cellular mtDNA-cn and BMI (Skuratovskaia *et al.*, 2018). However, our finding is inconsistent with one previous study where a negative correlation was observed between CC mtDNA and BMI (Desquiret-Dumas *et al.*, 2017).

In agreement with our findings, Desquiret-Dumas *et al.* (2017) found no relationship between the CC mtDNA content and either oocyte maturity or fertilizability. Another study also reported no differences between mtDNA-cn in CCs and stage of oocyte maturation (Lan *et al.*, 2020). We were unable to identify any relationship between the diagnosed cause of infertility and the relative mtDNA content of CCs. The analysis was further extended, comparing women with no apparent ovarian involvement in their infertility (e.g. referred for ART for reasons of male infertility or due to tubal occlusion) with those who had diminished ovarian reserve. Again, no differences were detected. This is in accordance with a previous study where similar results were obtained in such patient groups (Boucret *et al.*, 2017).

Previous studies have suggested that mtDNA quantities in CCs might correlate with clinically relevant factors. Separate studies have reported a positive association between embryo morphological grade and CC mtDNA level (Ogino *et al.*, 2016; Desquiret-Dumas *et al.*, 2017; Yang *et al.*, 2021), whereas Taugourdeau *et al.* (2019) detected a significant increase in CC mtDNA-cn associated with implantation in

patients treated with ICSI. Categorically Ogino *et al.*, while studying 60 oocyte CC complexes from 30 women, determined a threshold (86) CC mtDNA-cn to predict the good and bad (median mtDNA content: 140 versus 57) embryos. Similarly, CC mtDNA content (median mtDNA content: 738 versus 342) and embryo quality were further correlated; however, predicting the general threshold value was not possible as CC mtDNA was apparently affected by patient specificity (Desquiret-Dumas *et al.*, 2017). In the current study, the morphology of embryos was graded on Day 3 and also on Day 5 (as excellent, good, fair or poor). Outcomes, such as implantation and pregnancy rate, were known for a subset of 111 transferred embryos including both fresh and frozen transfers. No significant association was detected between morphology at the cleavage or blastocyst stages and outcome, although a non-significant trend towards an increased level of mtDNA was observed for good-quality Day 5 embryos compared with poorer ones. The lack of association between CC relative mtDNA-cn and embryo quality or embryo developmental potential is consistent with another recent publication and argues that the examination of mtDNA quantity in CCs is likely to be of little clinical value in the context of embryo selection (Liu *et al.*, 2021).

To explore qualitative aspects of the mtDNA, we sequenced the entire mtGenome in CCs using massively parallel sequencing. To our knowledge, this is the largest study to perform mtDNA sequencing in CCs, providing a deeper insight into the frequency of nucleotide variants, their potential association with ART outcomes and patient demographic data. mtGenome mutations have been implicated in altered reproductive potential and may influence the outcome of assisted conception treatments (Steffann *et al.*, 2015; Zou *et al.*, 2020). To date, most studies have focused on analysing mtGenome mutations in the oocyte or at early embryonic stages (De Fanti *et al.*, 2017; Lledo *et al.*, 2018). The accumulation of mitochondrial mutations in mammalian oocytes with advancing age has been noted (Arbeithuber *et al.*, 2020), although whether this has functional significance is unclear. In the current study, we sought to ascertain whether the variation in the mtGenome of CCs was associated with the potential of the corresponding oocyte to undergo fertilization. We also examined mtGenome variation with respect to embryo quality (whether considered suitable for transfer, cryopreservation or discarded). We found that the likelihood of finding a sequence variant in different regions of the mtGenome was comparable with that reported in previous studies (Yeung *et al.*, 2014; Tsai *et al.*, 2016).

When all mtGenome variants were considered together, including the homoplasmic variants that are more likely to have been inherited than to have arisen by mutation in somatic cells, there was no apparent association with the age of the patient. This result agrees with the previous research, in which no relationship between chronological age and the number of variants in CCs mtGenome was observed (Boucret *et al.*, 2017). However, four patients were detected to have two or more distinct heteroplasmic mutations in their CC samples (range 2–4); the average age of these patients was 39.6, ∼3 years older than the cohort as a whole. Mitochondrial mutations that occur spontaneously over time are likely to be found in a heteroplasmic state, with each mutation confined to a single CC sample; thus, the finding of such mutations in an older group of patients is in line with expectations and suggests that an accumulation of mtDNA mutations with time can occur. Such mutations may impair mitochondrial function with potential ramifications for the competence of the associated oocyte. However, potentially pathogenic heteroplasmic mutations were not common in our study cohort, suggesting that their impact on the likelihood of successful IVF treatment, if any, may be limited. The number of samples affected by acquired mtDNA mutations was too small to undertake a meaningful analysis of their influence on clinical outcomes.

Previous publications have postulated a possible relationship between the mtGenome haplogroup and reproductive capacity. In the current study, we found a significant association between macrohaplogroups and the number of retrieved oocytes. The difference was observed when comparing H-V and J-T macrohaplogroups, and the J-T and U-K groups, but not between the H-V and U-K groups. The number of retrieved oocytes is generally considered an indicator of good prognosis (Sermondade *et al.*, 2018). A study of mtDNA haplogroups (from blood samples) indicated that the J-T macrohaplogroup (associated with the fewest retrieved oocytes in the current study) has a protective effect with respect to premature diminished ovarian reserve (May-Panloup *et al.*, 2014). Similarly, in pigs, mitochondrial haplogroups are correlated with the efficiency of reproduction, with the A and B haplogroups having relatively lower reproductive potential (Tsai *et al.*, 2016). Given that the number of individuals in each haplogroup was relatively modest, this interesting observation should not be viewed as definitive and requires further confirmation. Haplogroup analysis also revealed a significant difference in the number of predicted pathogenic variants, potentially explaining the variation in reproductive capabilities related to the maternal macrohaplogroup. However, the presence of *in silico* predicted pathogenic variants alone is not sufficient to confirm an impact on mitochondrial function (Bolze *et al.*, 2020). In the present study, none of the macrohaplogroups was associated with other ART endpoints. When considering mitochondrial macrohaplogroups, no association with BMI was identified, a result consistent with the findings of a previous study in which analysis of the mitochondrial haplogroups in patients with weight gain showed no correlation with their weight change (Mittal *et al.*, 2017).

Conclusion

Despite extensive evaluation of mtDNA-cn and sequence in CCs, few significant associations were detected with patient characteristics or clinically relevant outcomes at different stages of the ART procedure. These findings suggest that analysis of the sequence or quantity of mtDNA in CCs is unlikely to provide an advantage in terms of improved embryo selection during assisted reproduction cycles. Nonetheless, our data raise interesting biological questions, particularly regarding the interplay of mitochondria, metabolism and BMI.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material. The raw sequence data files will be shared on reasonable request to the corresponding author.

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Authors' roles

C.R., D.W., D.N. conceived the study. K.K. performed the experiments, analysed the data and drafted the manuscript. M.V. assisted in collecting the samples and maintaining the patient database. All authors participated in the study, approved the final version of the manuscript and agreed to publish the results.

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Conflict of interest

D.J.N. and C.R. co-hold patent US20150346100A1 and D.J.N. holds US20170039415A1, both for metabolic imaging methods. The remaining authors have no conflicts of interest to declare.

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