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Deep sequencing shows that oocytes are not prone to accumulate mtDNA heteroplasmic mutations during ovarian ageing

L. Boucret[1,2], C. Bris[2,3], V. Seegers[4], D. Goudenège[2,3], V. Desquiret-Dumas[2,3], M. Domin-Bernhard[5], V. Ferré-L'Hotellier[1], P.E. Bouet[6], P. Descamps[6], P. Reynier[2,3], V. Procaccio[2,3], and P. May-Panloup[1,2,*]

[1]Laboratoire de Biologie de la Reproduction, Centre Hospitalier Universitaire d'Angers, 49933 Angers cedex 9, France [2]Institut MITOVASC, CNRS 6015, INSERM U1083, Université d'Angers, 49933 Angers, France [3]Département de Biochimie et Génétique, Centre Hospitalier Universitaire d'Angers, 49933 Angers cedex 9, France [4]Département de Biométrie, Institut de Cancérologie de l'Ouest, CRCINA—Unité Inserm U1232—Equipe 7, 49100 Angers, France [5]Département de Gynécologie Obstétrique et Reproduction Humaine, CHU de Rennes, F-35033 Rennes, France [6]Service de Gynécologie-Obstétrique, Centre Hospitalier Universitaire d'Angers, 49933 Angers cedex 9, France

*Correspondence address. Laboratoire de Biologie de la Reproduction, Centre Hospitalier Universitaire d'Angers, 49933 Angers cedex 9, France. E-mail: pamaypanloup@chu-angers.fr

STUDY QUESTION: Does ovarian ageing increase the number of heteroplasmic mitochondrial DNA (mtDNA) point mutations in oocytes?

SUMMARY ANSWER: Our results suggest that oocytes are not subject to the accumulation of mtDNA point mutations during ovarian ageing.

WHAT IS KNOWN ALREADY: Ageing is associated with the alteration of mtDNA integrity in various tissues. Primary oocytes, present in the ovary since embryonic life, may accumulate mtDNA mutations during the process of ovarian ageing.

STUDY DESIGN, SIZE, DURATION: This was an observational study of 53 immature oocyte–cumulus complexes retrieved from 35 women undergoing IVF at the University Hospital of Angers, France, from March 2013 to March 2014. The women were classified in two groups, one including 19 women showing signs of ovarian ageing objectified by a diminished ovarian reserve (DOR), and the other, including 16 women with a normal ovarian reserve (NOR), which served as a control group.

PARTICIPANTS/MATERIALS, SETTING, METHODS: mtDNA was extracted from isolated oocytes, and from their corresponding cumulus cells (CCs) considered as a somatic cell compartment. The average mtDNA content of each sample was assessed by using a quantitative real-time PCR technique. Deep sequencing was performed using the Ion Torrent Proton for Next-Generation Sequencing. Signal processing and base calling were done by the embedded pre-processing pipeline and the variants were analyzed using an in-house workflow. The distribution of the different variants between DOR and NOR patients, on one hand, and oocyte and CCs, on the other, was analyzed with the generalized mixed linear model to take into account the cluster of cells belonging to a given mother.

MAIN RESULTS AND THE ROLE OF CHANCE: There were no significant differences between the numbers of mtDNA variants between the DOR and the NOR patients, either in the oocytes ($P = 0.867$) or in the surrounding CCs ($P = 0.154$). There were also no differences in terms of variants with potential functional consequences. *De-novo* mtDNA variants were found in 28% of the oocytes and in 66% of the CCs with the mean number of variants being significantly different (respectively 0.321, SD = 0.547 and 1.075, SD = 1.158) ($P < 0.0001$). Variants with a potential functional consequence were also overrepresented in CCs compared with oocytes ($P = 0.0019$).

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: Limitations may be due to the use of immature oocytes discarded during the assisted reproductive technology procedure, the small size of the sample, and the high-throughput sequencing technology that might not have detected heteroplasmy levels lower than 2%.

WIDER IMPLICATIONS OF THE FINDINGS: The alteration of mtDNA integrity in oocytes during ovarian ageing is a recurring question to which our pilot study suggests a reassuring answer.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by the University Hospital of Angers, the University of Angers, France, and the French national research centers, INSERM and the CNRS. There are nocompeting interests.

Key words: diminished ovarian reserve / mitochondria / mtDNA / oocyte / ovarian ageing

Introduction

Mitochondria constitute the powerhouse of cells, producing the energy required for all cellular functions. In addition to their role in producing ATP, these organelles play a central role in apoptosis, calcium regulation, and thermogenesis, as well as in cell signaling pathways and gene expression (Gut and Verdin, 2013). Each mammalian somatic cell contains several hundred mitochondria that form a continuously dividing and recombining network containing hundreds of thousands of mitochondrial DNA (mtDNA) copies.

In humans, mtDNA is a double-stranded, circular 16 569 bp DNA molecule (Anderson *et al.*, 1981), coding for 13 essential subunits of the respiratory chain complexes, as well as 22 tRNAs, and 2 rRNAs, constituting part of the mitochondrial translation machinery. MtDNA is transmitted according to a non-Mendelian maternal mode with a genome exclusively transmitted through oocytes (Giles *et al.*, 1980).

The oocyte has the richest mtDNA content of all human cells, containing up to several hundred thousand copies (Otten and Smeets, 2015). This mtDNA content is formed by the stochastic distribution of the few (only about one hundred) mtDNA molecules present in primordial germ cells during oogonial divisions, combined with an amplification of selected molecules during oogenesis and folliculogenesis (Wai *et al.*, 2008; Mishra and Chan, 2014; Stewart and Chinnery, 2015). This process has led to the 'bottleneck theory' (Hauswirth and Laipis, 1985) according to which a very small number of mtDNA molecules may populate the oocyte and consequently the entire organism. This theory explains how mtDNA may be 'refreshed' and 'purified' from one generation to another (Jansen and de Boer, 1998; Mishra and Chan, 2014; Stewart and Chinnery, 2015). Whereas this bottleneck has been extensively studied for the transmission of mtDNA mutations carried by the mother, its protective role against de-novo mutations of the oocyte is much less known. MtDNA is particularly susceptible to accumulate mutations, with the mutation rate in humans being almost 25 times higher than that of nuclear DNA (Lynch *et al.*, 2006), mainly because of the proximity of mtDNA with the respiratory chain that produces reactive oxygen species (ROS) and the lack of protective histones and efficient repair mechanisms.

Ageing in mammals is correlated with a decline in the mitochondrial respiratory chain function and the accumulation of somatic mtDNA deletions and mutations (Krishnan *et al.*, 2007; Trifunovic and Larsson, 2008). In this study, we were specifically interested in the potential effect of ovarian ageing on the integrity of mtDNA in oocytes compared to the somatic cell compartment, i.e. for each oocyte, its corresponding CCs. Interestingly, in the ovary, the mtDNA molecules in the oocyte and the surrounding CCs are subject, in parallel, to two successive events: first, a long stagnation in the 'resting phase', and second, a multiplication during the growth phase of the oocyte and during the multiplication phase of the CCs during folliculogenesis.

Ovarian ageing is characterized by a diminished ovarian reserve (DOR), which combines the quantitative and qualitative alterations of the ovarian oocyte pool (te Velde and Pearson, 2002). Ovarian ageing occurs more or less early in women depending on the influence of environmental and genetic factors not yet well defined (te Velde and Pearson, 2002; Broekmans *et al.*, 2009). The great variability between women led us to consider the patient's ovarian reserve rather than merely the chronological age to evaluate the effect of ovarian ageing on mtDNA integrity.

We used the deep sequencing technique to evaluate heteroplasmic point mutations in 53 isolated oocytes and their corresponding CCs, obtained from two groups of women, one with a DOR and the other with a normal ovarian reserve (NOR).

Materials and Methods

Characteristics of the patients

Oocytes and CCs were obtained from 35 patients undergoing IVF with ICSI at the ART Center of the University Hospital of Angers, France, between March 2013 and March 2014. The Center proposes ICSI in cases of infertility, which represent about 50% of our IVF activity.

Among the 35 patients included, 19 presented a DOR whereas 16 had a NOR. The inclusion criteria used for classifying the patients in the DOR or NOR groups included the levels of the anti-Müllerian hormone (AMH), the FSH, and estradiol, together with the ultrasound measurement of the ovarian antral follicle count (AFC) (de Carvalho *et al.*, 2008). Patients were assigned to the NOR group if their AMH levels were above 2 ng/ml, FSH levels below 8 IU/l, estradiol levels below 60 pg/ml and the AFC > 4 per ovary. Patients were assigned to the DOR group if their AMH levels were below 2 ng/ml or if the AFC was <5 per ovary. All the women included in the study had both ovaries intact, with no history of ovarian surgery, autoimmune disease or gonadotoxic treatment that might have contributed to a DOR. Table I summarizes the characteristics of the 35 patients, as well as the procedures used for the suppression of pituitary gonadotrophin release [using GnRH antagonists, i.e. Cetrorelix (Cetrotide®; Merck-Serono, Geneva, Switzerland) or Ganirelix (Orgalutran®; Organon, Oss, Netherlands) or GnRH agonists, i.e. Triptoreline (Decapeptyl®; Ipsen Pharma, Paris, France)] and follicular growth stimulation [using FSH + LH, i.e. Menotropine (Menopur®; Ferring Pharmaceuticals, Copenhagen, Denmark) or Follitropine alpha + Lutropine alpha (Pergoveris®; Merck-Serono, Geneva, Switzerland) or rFSH, i.e. Follitropine alpha (Gonal-f®;Merck-Serono, Geneva, Switzerland) or

Table I Characteristics of the 35 patients studied.

	Overall	NOR	DOR	P-value
	N = 35	N = 16	N = 19	
Age, median (IQR)	34 (29.5–36)	31 (29–32.5)	36 (32.5–37)	**0.0144**
Age range	25–42	28–38	25–42	
Mean (SD)	33.1 (3.9)	31.4 (2.8)	34.5 (4.2)	
BMI, median (IQR)	20.8 (20.2–23.1)	21.2 (20.6–22.9)	20.6 (19.6–23.1)	0.4658
Tobacco				
Non-smokers	24 (72.7%)	10 (30.3%)	14 (42.4%)	0.4636
Smokers	5 (15.2%)	2 (6.1%)	3 (9.1%)	
Former smokers	4 (12.1%)	3 (9.1%)	1 (3.0%)	
Information missing		1	1	
E2 (pg/ml), median (IQR)	31.5 (23.1–47.8)	33 (25–57)	30 (20.5–46)	0.5706
FSH (UI/l), median (IQR)	8 (6.4–9.5)	7.0 (5.2–7.4)	8.8 (8.1–11.2)	**0.0008**
LH (UI/l), median (IQR)	4.9 (3.9–6.6)	4.7 (4.2–5.3)	5.7 (3.9–6.6)	0.3002
AMH (ng/ml), median (IQR)	1.71 (1.4–2.35)	2.9 (2.16–3.9)	1.4 (0.98–1.53)	**<0.0001**
AFC, median (IQR)	11 (8.5–21)	22 (19–24.5)	9 (7–10)	**<0.0001**
Initial dose of FSH, median (IQR)	300 (200–300)	200 (150–225)	300 (300–325)	**<0.0001**
Total dose of FSH administered (UI), median (IQR)	2512 (1800–3200)	1744 (1575–2081)	3150 (2850–3338)	**<0.0001**
Number of OCCs retrieved	8 (5–10)	10.5 (8.8–13)	5 (4–7.5)	**0.0003**
% of mature oocytes, median (IQR)	77 (60–82)	80 (76.5–88.5)	67 (50–80)	**0.0086**
LH suppression				
Agonist	9 (25.7%)	6 (37.5%)	3 (15.8%)	0.282
Antagonist	26 (74.3%)	10 (62.5%)	16 (84.2%)	
Stimulation				
FSH	25 (71.4%)	12 (75%)	13 (68.4%)	0.9572
FSH + LH	10 (28.6%)	4 (25%)	6 (31.6%)	

IQR = interquartile range: 25th–75th percentiles.
The differences in bold were considered significant at $P < 0.05$.

Follitropine beta (Puregon®; Organon, Oss, Netherlands)]. In each case, ovulation was induced with Ovitrelle® (Choriogonadotropine alfa; Merck-Serono, Geneva, Switzerland), and the oocytes were retrieved transvaginally under ultrasound guidance.

Isolation of oocytes and cumulus cells

A total of 53 oocyte–cumulus complexes (OCCs) were collected, 27 among the NOR group, 26 among the DOR group. For each of the OCCs retained, we collected the oocyte, on one hand, and the corresponding CCs, on the other, for individual studies. Among the 53 OCCs, 3 were obtained from each of 4 women, 2 from each of 10 women and 1 from each of 21 women. The OCCs were retrieved 36 h after treatment with human chorionic gonadotrophin treatment, and washed in multiple dishes with Flushing medium (Origio-France, Limonest, France) to eliminate the remaining mural granulosa cells, blood cells, and cellular debris. The OCCs were subsequently incubated for 1 h in Ferticult culture media (Fertipro, Beernem, Belgium). For each patient, the oocytes were individually stripped using hyaluronidase (80 IU; Fertipro) and gentle pipetting with a 125 μm diameter stripper pipette (Origio, France). Metaphase II oocytes were used for the ICSI procedure. Oocytes which had not extruded their first polar body 2 h after removal of the CCs, i.e. germinal vesicles blocked at prophase I (GVs) or oocytes blocked after prophase I (GVBDs), were retained for our study. There were 25 oocytes in prophase I, and 28 were

blocked between prophase I and metaphase II. Each oocyte was placed in 5 μl Ferticult culture media and immediately frozen in liquid nitrogen until extraction of the nucleic acid. CCs were recovered in 600 μl of Ferticult culture media and centrifuged at 2000 g for 10 min. The supernatant was removed and the CC pellets were immediately frozen in liquid nitrogen until extraction of the nucleic acid.

Extraction of DNA

Total DNA was extracted from isolated oocytes and the corresponding CC fractions using the Nucleospin® tissue Kit (Macherey-Nagel®, Hoerdt, France) according to the manufacturer's recommendations. Genomic DNA was finally eluted in 60 μl of elution buffer.

Quantification of mtDNA copy numbers

To determine the mtDNA copy number, real time quantitative PCR (Q-PCR) was performed using the Chromo4 System (Biorad®, Hercules, CA, USA) in a 20 μl reaction volume containing ×1 IQ SYBR Green Supermix (Biorad®) and a final concentration of 0.5 μM of each gene-specific primer and 3 μl of template. The pairs of primers selected were, respectively, F1R1 (forward 1 and reverse 1; nucleotide positions 8342–8359 and 8546–8559) to quantify the mtDNA in oocytes and in CCs, and *NCOA3* F/R (nuclear receptor coactivator three forward: 5′-AAAGTTAATGGAGTTTCCTG-3′ and reverse: 5′-AGGACTGGCGTTTATGTCTT-3′) to quantify the

nuclear DNA in CCs. The reactions were performed as described in Supplementary Table SI.

The presence of the common 4977 bp mtDNA deletion (from nucleotide 8469 to nucleotide 13 447) was excluded in the tested samples (Poe et al., 2007). Briefly, the non-deleted mtDNA was amplified by the F1R1 method, and the deleted mtDNA was amplified with the F1R2 method (forward 1 and reverse 2; nucleotide positions 8342–8359 and 15 727–15 749) by quantitative PCR. The common deletion was considered absent for a ratio of <0.1% between the mtDNA copy numbers found with the F1R1 and F1R2 methods.

Ion proton sequencing

Compilation of the mtDNA library

The whole mitochondrial genome was amplified in two overlapping fragments of 8009 bp (amplicon A) and 8994 bp (amplicon B). The primer pairs used for both PCRs, tested on DNA extracted from cells devoid of mtDNA-(Rho Zero cells) to avoid pseudogene amplification, were located on the cytochrome b and cytochrome c oxidase one genes: 5′-TACGTTG TAGCCCACTTCCACT-3′ and 5′-GCCCGATGTGTAGGAAGAG-3′ for amplicon A; 5′-AACTTCGGCTCACTCCTTGG-3′ and 5′-AGTAAC GTCGGGGCATTCCG-3′ for amplicon B. Long-range PCR was performed with KapaLongRange DNA polymerase according to the manufacturer's recommendations (Kapa Biosystems, Boston, MA, USA). Agarose gel electrophoresis (1%) was used to confirm the presence of PCR products. The concentrations of PCR products were quantified by the Qubit® 2.0 Fluorometer using the Qubit® dsDNA HS Assay Kit (Thermo Fischer technologies, Waltham, MA, USA). For each sample, amplicons A and B were pooled in an equimolar manner. Libraries were compiled from 50 to 200 ng of pooled PCR products with the enzymatic fragmentation method of the Library Builder Automate using the dedicated Ion Xpress™ Plus Fragment Library Kit (Thermo Fischer technologies). All purification procedures were performed using Agencourt AMpure XP beads (Beckman Coulter, Brea, CA, USA).

Emulsion PCR, enrichment and sequencing

Emulsion PCR and enrichment were performed on pooled libraries diluted to 70–100 ppM using the Ion Chef Automate (Thermo Fischer technologies). The enriched mtDNA samples were sequenced using an ion proton high-throughput sequencing platform according to the manufacturer's protocol (Thermo Fischer technologies). The possibility of contamination was excluded by the use of negative controls with water instead of the amplified mtDNA.

Processing the sequencing data

Sequencing was performed using Ion Torrent Proton, and the signal processing and base calling was done by the pre-processing embedded pipeline. Demultiplexed reads were mapped on an mtDNA reference sequence (NC_012920) before being analysed with a dedicated in-house pipeline integrating various modules for coverage analysis, variant calling, annotation and prioritization. The calling module uses a consensus-based approach and integrates the prediction of six callers (Torrent Suite Variant Caller, GATK [PMID:20644199], VarScan [PMID:25553206], SNVer [PMID:21813454], LoFreq [PMID:23066108] and Platypus [PMID:25017105]). All of the variant calling formats (VCFs) generated were normalized and decomposed before launching the annotation-prioritization module which combines the NCBI Variant Reporter for gene annotation and ANNOVAR [PMID:25017105] for variant prioritization. ANNOVAR allowed the inclusion of several databases, i.e. Mitomap [PMID:25489354] and MitImpact2 [PMID:25516408] and prioritization tools, i.e. Polyphen2 [PMID:23315928], SIFT [PMID:19561590] and MutationTaster [PMID:24681721]. All the results were then included in a private database that may be interrogated via a dedicated graphical user interface for reducing the number of candidate variants by applying prioritization filters. Putative variants were further filtered using the following criteria: at least three callers predicting the variant, including the Torrent Suite Variant Caller, coverage greater than ×500, quality score >150 and a minimum mtDNA heteroplasmy level of 2%, was validated as a sensitive threshold for the detection of variants. Only substitution variants were studied.

Statistical analysis

For the characteristics of patients, categorical variables were described using percentages and were compared using Chi-squared tests or Fisher's exact tests as appropriate. Quantitative and discrete variables were described using the median and the interquartile range (IQR), i.e. values between the 25th and 75th percentiles, and were compared using the Mann–Whitney test. The association between age and the number of variants was assessed using a linear regression model.

To compare the characteristics of the oocytes and the surrounding CCs, we also computed a mixed generalized linear regression model (GLMM) to take into account the cluster of oocytes and CCs belonging to a given mother (with a random effect), and recorded the P-values corresponding to the factors studied. All the statistical tests were bilateral and the differences were considered significant at $P < 0.05$. All analyses were computed using R version 3.3.2 (R Core Team, 2016) and the lme4 packages (Bates et al., 2015).

Ethical approval

All participants gave their written informed consent, and the collection of OCCs was authorized by the Ethical Committee of the University Hospital of Angers, France (Number DC-2014–2224).

Results

Patients

As expected, the AMH and AFC levels were significantly lower and the FSH levels significantly higher in the DOR group than in the NOR group of patients since these were precisely the criteria used for classifying the patients into the two groups. Not surprisingly, the doses of FSH used for the stimulation phase were higher in the DOR group than in the NOR group of patients. However, there was no significant difference with respect to the drugs used for suppressing LH and to the ovarian stimulation between the two groups of patients (Table I).

The age of the patients was significantly lower in the NOR group (median 31 years; IQR: 29–32.5 years) than in the DOR group (median 36 years; IQR: 32.5–37 years). However, there were no significant differences between the two groups in terms of the BMI or addiction to tobacco smoking, both of which are likely to adversely affect ovarian reserve (Table I).

The mean number of OCCs retrieved was lower in the DOR group than in the NOR group, and the percentage of immature oocytes was significantly different between the two groups of patients (Table I). However, the numbers of GVs and GVBDs studied was similar in the DOR and the NOR groups (Table II).

Analysis of OCCs

The overall mean number of OCCs (oocytes and corresponding CCs) studied per patient was 1.51 (SD = 0.7), with no significant difference between the NOR and DOR patients for which the mean numbers were respectively 1.69 (SD = 0.87) and 1.37 (SD = 0.5), $P = 0.36$.

Table II MtDNA copy numbers in the oocytes and CCs of NOR and DOR patients.

| | Overall | NOR | DOR | P value |
	N = 53	N = 27	N = 26	
Number of GV/GVBD studied	25/28	14/13	11/15	0.6740
mtDNA in oocytes median (IQR)	114 100 (85 820–138 200)	125 200 (93 320–137 100)	93 650 (77 360–139 000)	0.233
mtDNA per CC median (IQR)	161 (135–194)	169 (142.5–199)	146 (122.8–173.5)	0.0953
mtDNA in the totality of CCs studied median (IQR)	135 200 (68 180–172 300)	135 200 (75 040–186 600)	134 300 (67 500–165 400)	0.588

P-value from Mann–Whitney test for mtDNA.
The differences in bold were considered significant at *P* < 0.05.

The mtDNA common deletion was not present in our cohort of patients

No 4977 bp common deletion was found either in the oocytes or in the CCs of the patients studied.

The mtDNA copy numbers were not significantly different in the groups studied

The mtDNA copy numbers per cell, in the oocytes and the CCs, were not significantly different in the DOR and the NOR groups (Table II).

The mtDNA copy numbers per cell, in the oocytes and the CCs, were not significantly different between GVs and GVBDs (Supplementary Table SII).

The mtDNA copy numbers on which our analyses of variants were performed were similar between the oocytes and the totality of the corresponding CCs studied (respectively, median: 114 100; IQR: 85 820–138 200 and median: 135 200; IQR: 68 180–172 300, *P* = 0.716) and between the DOR and the NOR groups in the totality of the CCs studied (Table II).

MtDNA variants

Variants identified both in the oocytes and in the corresponding CCs
Eight variants were found in six oocytes and in the corresponding CCs (Fig. 1 and Supplementary Table SIII), with average proportions of heteroplasmy of 38.7% (σ = 29.9%, range: 2–94%) for the oocytes and 36.9% (σ = 29.8%, range: 2–91%) for the CCs.

Variants specifically identified in one of the two cellular types (specific variants)
There were 17 variants found in the oocytes only (Fig. 1 and Supplementary Table III), with an average proportion of heteroplasmy of 10.3% (σ = 6.6%, range: 3.8–20.6%); these variants were found in 15 of the 53 oocytes studied (28%) (Fig. 2).

There were 57 variants found in the CCs only (Fig. 1 and Supplementary Table III), with an average proportion of heteroplasmy of 9.6% (σ = 8.7%, range: 3.1–45.5%); these variants were found in 35 of the 53 CCs studied (66%) (Fig. 2).

There was a significant difference between the mean number of variants in the oocytes and in the corresponding surrounding CCs in all of the patients and in each of the DOR and NOR groups of patients (Table III).

There was no significant difference between the mean number of specific variants in the DOR and NOR patients either in the oocytes or in the CCs (Table III).

There was also no relationship between chronological age and the number of variants in the oocyte (*P* = 0.320) or in CCs (*P* = 0.570).

No difference was found in the proportion of oocytes or CCs bearing variants according to the immaturity state of the oocyte (GV or GVBD): for oocytes, 5/15 GV and 10/28 VGBD (*P* = 0.2049) and for CCs, 19/25 VG and 16/28 GVBD (*P* = 0.1479).

Different types of specific variants
We distinguished between the two main categories of variants without potential functional consequences, i.e. variants with non-coding regions or synonymous variants, and those with potential functional consequences affecting mRNAs, tRNAs and rRNAs in the oocytes and in the surrounding CCs (summarized in Fig. 2 and Supplementary Table SIII).

The proportion of variants with potential functional consequences was lower in the oocytes than in the corresponding surrounding CCs in all of the patients and in each group of the DOR and NOR patients (Table IV).

There was no significant difference between the proportions of variants with potential functional consequences in the DOR and NOR groups of patients, either in the oocytes or in the surrounding CCs (Table IV).

Pathogenic mutations
Among the variants identified, two pathogenic mutations have been definitely associated with mitochondrial diseases in the literature (for details, see Mitomap database http://www.mitomap.org). One is the m.14459G > A mutation in the ND6 gene associated with the Leigh syndrome and Leber's Hereditary Optic Neuropathy (LHON) (3% heteroplasmy), and the other is the m.586G > A mutation in a tRNA (phe) gene responsible for a neurodegenerative disorder identified in two women with, respectively, 4 and 12% heteroplasmy (Supplementary Table III). These three variants were seen only in the CCs, and in only one CC when several OCCs from the same patient were studied.

Discussion

Ovarian ageing may affect oocyte competence by targeting cytoplasmic components, including mitochondria, thereby entailing serious cellular consequences including nuclear chromosomal abnormalities (Meldrum *et al.*, 2016). The effects of ovarian ageing on mitochondria in oocytes and in the corresponding CCs have attracted much attention in recent years and one of the most recurrent findings is the

Figure 1 Representation of mtDNA, mitochondrial genes and variants found in oocytes or CCs. The gray dots symbolize the variants found in both the oocytes and in the corresponding CCs. The arrows symbolize the variants specifically found in one or the other cell types (green: non-coding variants or synonyms; red: coding tRNA or rRNA). RNR: rRNA; ND = NADH dehydrogenase, complex I; CYB = cytochrome b, complex III; CO = cytochrome oxidase, complex IV; and ATP = ATPase, complex V. The blue lines symbolize the 22 tRNAs.

Figure 2 Flow chart showing the numbers of specific mtDNA variants in oocytes and CCs.

decrease in mtDNA copy numbers in oocytes with ovarian ageing (see review by May-Panloup *et al.*, 2016).

Concerning the qualitative aspects, the link between oocyte mtDNA integrity and ovarian ageing remains controversial. Studies on oocytes from older women, have reported a greater number of mtDNA abnormalities, such as the accumulation of the common 4977-bp mtDNA

deletion (Keefe *et al.*, 1995; Chan *et al.*, 2005), or some point mutations (Barritt *et al.*, 2000), whereas other studies have found no difference in the number of deletions (Brenner *et al.*, 1998; Barritt *et al.*, 1999; Chan *et al.*, 2005) or that of mutations (Fragouli *et al.*, 2015). However, in these studies, the mutational burden was very low and did not appear to be responsible for any direct functional outcome (Brenner *et al.*, 1998). In effect, a pathogenic mtDNA mutation manifests itself only when the mutant load exceeds a certain threshold, leading to the appearance of symptoms. Nevertheless, the effects of low levels of heteroplasmy in the oocytes of older mothers may appear in the offspring (Rebolledo-Jaramillo *et al.*, 2014). Thus, maternally inherited mtDNA mutations, associated with the combination of acquired somatic mutations, have been shown to be responsible for age-related disorders and defective brain development in mice (Ross *et al.*, 2013). Thus, it would be important to study oocyte heteroplasmy and its variation with ageing, reflected here by pool size.

Next-generation sequencing offers the great sensitivity required for the detection of low levels of heteroplasmy. To our knowledge, this is the first study carried out on humans using this technique to compare the variants present in oocytes to those in CCs. The sequencing was performed on an mtDNA copy number which was similar between the different groups compared, thus rendering the quantification of variants fully comparable.

Table III Specific variants in oocytes and CCs, in DOR and NOR patients.

	Oocytes and CCs	Oocyte	CCs	*P* value GLMM model
Overall mean number (SD) of specific variants in NOR and DOR patients	0.698 (0.9776)	0.321 (0.547)	1.075 (1.158)	<0.0001
Mean number (SD) of specific variants in NOR patients	0.593 (0.714)	0.333 (0.480)	0.852 (0.818)	0.0064
Mean number (SD) of specific variants in DOR patients	0.808 (1.189)	0.308 (0.618)	1.308 (1.408)	0.0013
P-value GLMM model	0.259	0.867	0.154	

The GLMM regression takes into account clusters of oocytes or CCs belonging to a given mother.
The differences in bold were considered significant at $P < 0.05$.

Table IV Specific variants with potential functional consequences (PFCs) in oocytes and CCs, in DOR and NOR patients.

Variants with PFCs	Oocytes and CCs with specific variant (*N* = 50*)	Oocyte (*N* = 15 oocytes with specific variants)	CCs (*N* = 35 CCs with specific variants)	*P*-value GLMM model
In NOR and DOR patients (*N* = 50* OCCs)	34/50 (68.0%)	5/15 (33.3%)	29/35 (85.3%)	**0.0019**
In NOR patients (*N* = 26 OCCs with specific variants)	15/26 (57.7%)	2/9 (22.2%)	13/17 (76.5%)	**0.0135**
In DOR patients (*N* = 24 OCCs with specific variants)	19/24 (79.2%)	3/6 (50%)	16/18 (88.9%)	0.0785
P-value GLMM model	0.1859	0.3287	0.4018	

The GLMM regression takes into account clusters of oocytes or CCs belonging to a given mother.
*Three OCCs were without any specific variants.
The differences in bold were considered significant at $P < 0.05$.

Heteroplasmic mtDNA variants found both in oocytes and corresponding CCs

The eight variants in both oocytes and corresponding CCs (Fig. 1 and Supplementary Table SIII) potentially correspond to the variants pre-existing in the mother, which could be transmitted via the oocyte to the offspring without elimination through the mitochondrial bottleneck. When several OCCs obtained from a given woman were studied, these eight variants were found in all of the oocytes and the CCs, except for one variant that was found in only one of the three OCCs. However, the level of heteroplasmy of this variant was only 3% in the oocyte, and it is possible that the variant escaped detection in the other OCCs because of the threshold of sensitivity of the technique used.

In one further case, the same variant was retrieved in several CCs but not found in the corresponding oocytes. It is interesting to note that this variant corresponded to a non-synonymous mutation (m.6027G > A; G42S) whereas the other variants were five non-coding variants, one synonymous variant that did not result in an amino acid change, and two variants involving either a tRNA gene or a rRNA gene. Indeed, it has been shown that the purifying selection that occurs during the bottleneck process preferentially targets protein-coding genes rather than tRNA genes (Stewart and Larsson, 2014).

Heteroplasmic mtDNA variants found only either in oocytes or corresponding CCs

Among the 53 oocytes studied, 15 contained a total of 17 variants, i.e. 28% of the oocytes carried at least one mtDNA variant (Fig. 2). These variants (Fig. 1 and Supplementary Table SIII), found only in the oocytes, are unlikely to have been inherited from the mother, since they were found neither in the corresponding CCs nor in the other oocytes or CCs from the same women. More probably, they correspond to mutations acquired during germ line quiescence or development. This result is in accordance with previous studies showing mtDNA mutations in 27% of human oocytes (Jacobs et al., 2007), and de novo variants in 20% of zebra fish oocytes (Otten et al., 2016).

In contrast, mtDNA variants were significantly more frequent in CCs with 57 variants found in 35 of the 53 OCCs studied, i.e. 66% of CCs carried at least one variant (Fig. 2). These CC variants (Fig. 1 and Supplementary Table SIII) were either mutations that were counter-selected in the corresponding oocytes by the bottleneck mechanism or acquired during folliculogenesis. The former hypothesis appears less likely since all these variants, with the exception of the one discussed above, i.e. m.6027G > A; G42S, were found in only one CC when several OCCs from the same patient were studied.

Despite the similarity of the kinetics of mtDNA replication and the environmental conditions of the oocytes and the surrounding CCs, twice as many CCs as oocytes carried at least one mtDNA variant, and the mean number of variants was significantly higher in CCs than in oocytes ($P < 0.0001$) (Table III). Interestingly, the percentage of variants potentially affecting gene expression, i.e. the non-synonymous, tRNA and rRNA variants was significantly higher in the CCs than in the oocytes ($P = 0.0019$) (Table IV). Moreover, the two pathogenic mutations associated with mitochondrial diseases, identified in our study, were found only in the CCs. Overall, oocytes seem spared from mtDNA damage in comparison to CCs. This could reflect either the

protective role of CCs against oxidative stress in oocytes (Shaeib *et al.*, 2016) or the action of the bottleneck effect in eliminating acquired variants. Interestingly, this phenomenon has been shown to continue during the growth phase of the oocyte, i.e. after the quiescent phase and up to a few months before ovulation (Wai *et al.* 2008).

There were no significant differences between the numbers of mtDNA variants between the DOR and the NOR patients, either in the oocytes or in the surrounding CCs (Table III). There were also no differences between the DOR and NOR patients in terms of variants with potential functional consequences (Table IV). Moreover, the number of variants was not correlated with the chronological age of the patients. Our findings concord with those of a recent study on a model of cloned cows, 3 and 10 years old, which did not find any significant accumulation of heteroplasmic mtDNA point mutations associated with ageing, although it did show an age-related increase in the number of mtDNA deletions in oocytes (Hammond *et al.*, 2016). In our study, it could have been expected that the number of mutations in the DOR group may have been underestimated because of an increase in the 4977pb deletion both in oocytes and in the surrounding CCs. Indeed, this age-related deletion may eliminate a portion of the mitochondrial genome potentially carrying mutations (Tsai *et al.*, 2010). However, we did not identify the common deletion in our series of DOR patients.

This above results suggest that oocytes are not prone to accumulating mtDNA heteroplasmic mutations during ovarian ageing.

In this study, we only used immature oocytes (because of ethical considerations) and cannot confirm that the over-representation of mtDNA variants in CCs compared to oocytes also exists in mature oocytes. Indeed, due to the importance of mitochondrial functions in the cumulus with respect to the terminal maturation of the oocyte (Dalton *et al.*, 2014), the mitochondrial dysfunction and mtDNA mutations in the CCs could be directly related to the lack of oocyte maturity. In contrast, in immature oocytes the absence of accumulation of mutations with ovarian aging is likely to be also found in mature oocytes. Moreover, our study may be also limited by the relatively small number of OCCs studied, and finally, because the high-throughput sequencing technology used may have failed to measure levels of heteroplasmy lower than 2% with sufficient reliability.

Nevertheless, our pilot study argues against the notion of the accumulation of mtDNA mutations in oocytes with ovarian ageing, although it remains a recurring and highly debated topic.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

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Conflict of interest

None declared.

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