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Short Communication

Mitochondrial genome integrity in human oocytes: duplex sequencing explores endometriosis-associated germline mutagenesis



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Summary

The high mitochondrial content of oocytes highlights their essential role in reproduction. Mutations in mitochondrial DNA (mtDNA) can impair mitochondrial function and oocyte quality. Using highly accurate duplex sequencing, we found that mtDNA mutation frequency increases with age in oocytes from patients with endometriosis, whereas this trend was not observed in endometriosis-free women. This suggests that endometriosis-associated changes in the oocyte microenvironment may promote the accumulation of mtDNA mutations.

Introduction

Human oocytes are densely packed with mitochondria, reflecting their essential role in female reproduction and early embryonic development (Pakendorf, Stoneking 2005). Mitochondria harbor their own genome (mtDNA), where mutations can impair organelle function, potentially affecting fertility and offspring health (Habbane et al 2021). Since mtDNA is maternally inherited, mtDNA mutations in oocytes are of particular interest. A single oocyte contains hundreds of thousands of mtDNA copies, enabling the coexistence of wild-type and mutated alleles, a phenomenon known as heteroplasmy. While mtDNA variants above 1 % frequency have been extensively studied, far less is known about how new variants arise (Shoubridge, Wai 2007).

Investigating mtDNA mutagenesis in oocytes can improve our understanding of how aging and disease influence the mitochondrial genome in the human germline. Endometriosis is of particular interest in this context: This chronic inflammatory disease

affects approximately 10 % of women of reproductive age and not only causes pain, but also contributes to reduced fertility (Creed et al 2020). Previous studies have reported reduced mtDNA copy numbers and abnormal mitochondrial morphology in oocytes from patients with endometriosis, suggesting that the disease impairs mitochondrial function and may consequently reduce oocyte quality and fertility (Xu et al 2015).

Methods

To investigate the effects of age and disease on female germline mutagenesis, we adopted highly accurate duplex sequencing (DS) (Kennedy et al 2014) to analyze mtDNA in single human oocytes (Arbeithuber et al 2025; Kloimboeck et al 2026). Unlike conventional sequencing approaches that examine only one DNA strand, duplex sequencing analyzes both complementary strands of a DNA molecule. This enables robust discrimination of true *de novo* mutations from errors introduced during library preparation and sequencing and therefore allows reliable detection and analysis of ultra-low-frequency variants (<1 %). In addition, we established a workflow to reduce bias from nuclear mitochondrial sequences (NUMTs), which may become misaligned to the mitochondrial reference genome and result in false-positive mutation calls.

Oocyte collection

Women undergoing *in vitro* fertilization treatment at the Fertility Center of Kepler University Hospital Linz were enrolled (Ethics approval No. 1293/2020). Immature and morphologically abnormal oocytes, unsuitable for intracytoplasmic sperm injection, as well as unfertilized oocytes, were included in this study. Blood and/or saliva samples were also collected and prepared for DS to distinguish inherited heteroplasmies from *de novo* mutations.

mtDNA enrichment and duplex sequencing library preparation

Libraries were prepared as described before (Arbeithuber et al 2025; Kloimboeck et al 2026). In short, single oocytes were lysed and treated with exonuclease V to digest linear DNA derived from the nuclear genome, while circular mtDNA remained unaffected. Using ultrasound-shearing, the enriched samples were further mechanically fragmented before Y-shaped DS adapters, each carrying a unique 12 nt random tag, were ligated to both ends of a double strand DNA molecule. Following amplification and indexing, the libraries were sequenced on an Illumina NovaSeq X platform using 2×300 cycles.

Bioinformatic analysis

Duplex consensus sequences (DCSs) were generated using the Du Novo pipeline (Stoler et al 2016) implemented in Galaxy (The Galaxy Community, 2024) and mapped against the revised Cambridge Reference Sequence (rCRS; NC_012920). DCS reads containing more than two variants within a single read were considered potential nuclear mitochondrial DNA segments (NUMTs) and excluded from further analysis.

Variants detected in both somatic tissues and oocytes from the same donor were classified as inherited heteroplasmies. Variants absent from somatic tissues but detected in more than one oocyte from the same donor, where multiple oocytes were available, were classified as early germline heteroplasmies. Variants detected in only a single oocyte sample were classified as *de novo* mutations and included in the mutation frequency calculation.

Results and Discussion

A total of 66 oocytes from 11 control (endometriosis-free) and 21 endometriosis patients were analyzed (1–5 oocytes per donor, 2.1 on average). The mean age was 32.7 ± 4.1 years in the control cohort and 34.6 ± 4.1 years in the endometriosis cohort. Oocyte samples included on average 0.0006 % DCS reads identified as NUMTs, which were removed, resulting in a median DCS sequencing depth of 762× and 17.6 ± 13.2 variants detected per oocyte.

As already previously shown (Arbeithuber et al 2025), unlike in somatic tissues, we did not observe an increase in mtDNA mutation frequency in human oocytes with maternal age. Although no significant difference in median mutation frequency was observed between the control and endometriosis cohorts (Figure 1), a positive trend between age and mutational burden was observed in the endometriosis cohort, whereas no association was detected in the control cohort (Figure 2). These findings raise the possibility that endometriosis may influence mtDNA mutagenesis in oocytes, potentially depending on the duration of exposure to the chronic inflammatory environment associated with the disease. To further study the effect of age on mutation frequency, we built a generalized mixed-effects linear model considering each individual and each condition (endometriosis and controls) separately (each oocyte was also considered separately), following our approach in Arbeithuber et al 2025. The model predicted the probability of having a mutation at a site as a function of age and included the mutation frequency as a response, the number of sequenced nucleotides per sample as weight, and age, condition, and their interactions as fixed-effect predictors. The model indicated no significant difference in mutation frequency with age in control oocytes (Z test P = 0.657) and endometriosis oocytes (Z test P = 0.391). The donor ID was used as a random effect and did not show a significant effect (likelihood ratio test P = 0.071).

Although the model did not confirm a statistically significant association, the observed trend in the endometriosis cohort warrants further investigation. The control cohort in this pilot study was relatively small (21 oocytes) compared with the endometriosis cohort (45 oocytes), highlighting the need to include additional control samples to improve the statistical power of the analysis.

Furthermore, analyzing oocytes from younger patients (aged <30 years) and older patients (aged >38 years) may help clarify whether prolonged exposure to the inflammatory environment associated with endometriosis contributes to increased mtDNA mutagenesis in oocytes.

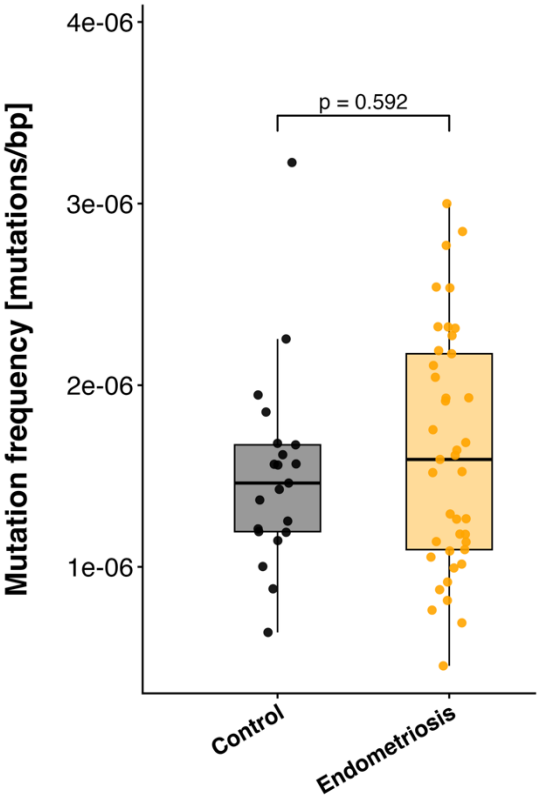


Figure 1. Endometriosis does not significantly affect total median mutation frequency in oocytes. Mutation frequency in mtDNA of single oocytes is shown as mutations/sequenced nucleotides. Wilcoxon Test; $N_{control}$ = 21; $N_{endometriosis}$ = 45.

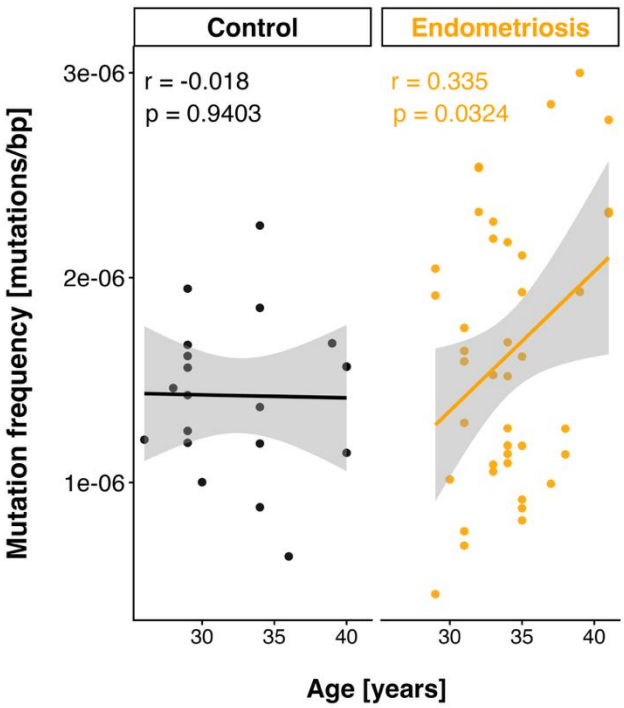


Figure 2. Mutational burden in endometriosis patient's oocytes correlates with age. Mutation frequency in mtDNA of single oocytes is shown as mutations/sequenced nucleotides. Linear regression; r and p-value for the age-dependent slope.; $N_{control}$ = 21; $N_{endometriosis}$ = 45.

Abbreviations

DS	Duplex sequencing	DCS	Duplex consensus sequence
mtDNA	Mitochondrial DNA	NUMTs	nuclear mitochondrial sequences

Conflicts of interest: All authors declare no conflict of interest.

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Keywords: single oocyte; mtDNA; duplex sequencing; germline mutagenesis; NUMTs

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