

Mitochondrial Genome Integrity in Human Oocytes: Duplex Sequencing Reveals Endometriosis-Associated Germline Mutagenesis

Kloimboeck Teresa^{1*}, Samadpoor S¹, Moukble Ali Aldawla S², Ebner T³, Oppelt P³, Shebl O³, Arbeithuber B¹⁺

¹ Dept of Gynaecology, Johannes Kepler Univ, Linz, Austria; ² Dept of Biophysics, Johannes Kepler Univ., Linz, Austria; ³ Dept of Gynaecology, Obstetrics and Gynaecological Endocrinology, Kepler University Hospital, Linz, Austria

* Presenting author: teresa.kloimboeck@jku.at + Corresponding author: barbara.arbeithuber@jku.at

Contribution to [MiP2026](#) – Received 2026-09-24 – Online 2026-10-06

Cite: Kloimboeck T, Samadpoor S, Moukble Ali Aldawla S, Ebner T, Oppelt P, Shebl O, Arbeithuber B (2026) Mitochondrial Genome Integrity in Human Oocytes: Duplex Sequencing Reveals Endometriosis-Associated Germline Mutagenesis. BEC prep 2026.9
<https://doi.org/10.26124/becprep2026-0009>

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 but remains stable in endometriosis-free women. This suggests that endometriosis-associated changes in the oocyte microenvironment may promote the accumulation of mtDNA mutations.

Keywords – *single oocyte; mtDNA, duplex sequencing; germline mutagenesis, NUMTs*

1. Introduction

Human oocytes are densely packed with mitochondria, reflecting their essential role in female reproduction and early embryonic development (Pakendorf and 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 and 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).

2. 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.

2.1. 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.

2.2. 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 2x300 cycles.

2.3. Bioinformatic analysis

Duplex consensus sequences (DCSs) were generated on Galaxy (The Galaxy Community, 2024), using the Du novo pipeline (Stoler et al., 2016) and mapping

against the Revised Cambridge Reference Sequence (rCRS; NC_012920). DCS reads with more than 2 variants within one read were considered as potential NUMTs and excluded from further analysis.

3. Results and Discussion

A total of 66 oocytes from 11 control (endometriosis-free) and 21 endometriosis patients were analyzed. Patients had a mean age of 32.7 ± 5.2 years in the control and 34.0 ± 3.5 years in the endometriosis cohort.

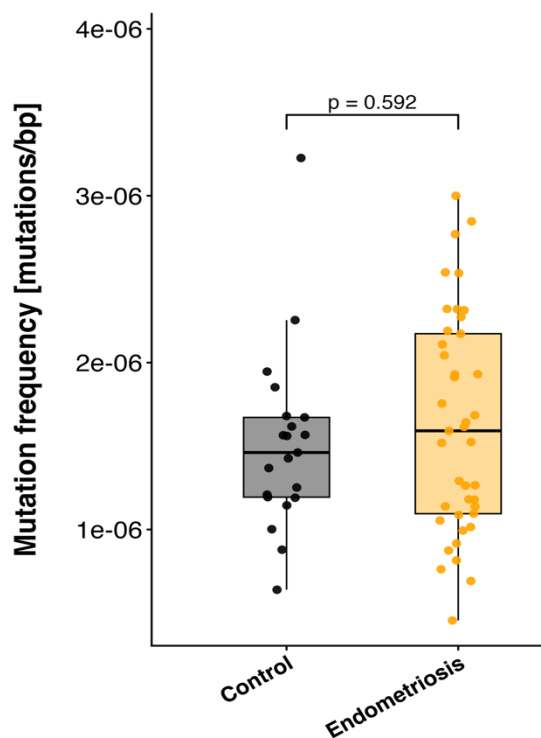


Figure 1. Endometriosis does not significantly affect total median mutation frequency in oocytes. Mutation frequency in mtDNA of single oocytes is shown as mutations/sequenced nucleotides. Wilcoxon Test; $N_{\text{control}} = 21$; $N_{\text{endometriosis}} = 45$.

In contrast, the mutation frequency remained stable in the control group (Figure 2). This suggests that endometriosis affects mtDNA mutagenesis in oocytes depending on the duration of their exposure to the endometriosis-associated chronic inflammatory environment.

Previous research has shown that, unlike in somatic tissues, mtDNA mutation frequency in human oocytes does not increase with maternal age (Arbeithuber et al., 2025). While we do not observe a significant difference in median mutation frequencies between the two groups, control and endometriosis (Figure 1), we show a significant positive correlation between age and mutational burden in endometriosis patients (Figure 2).

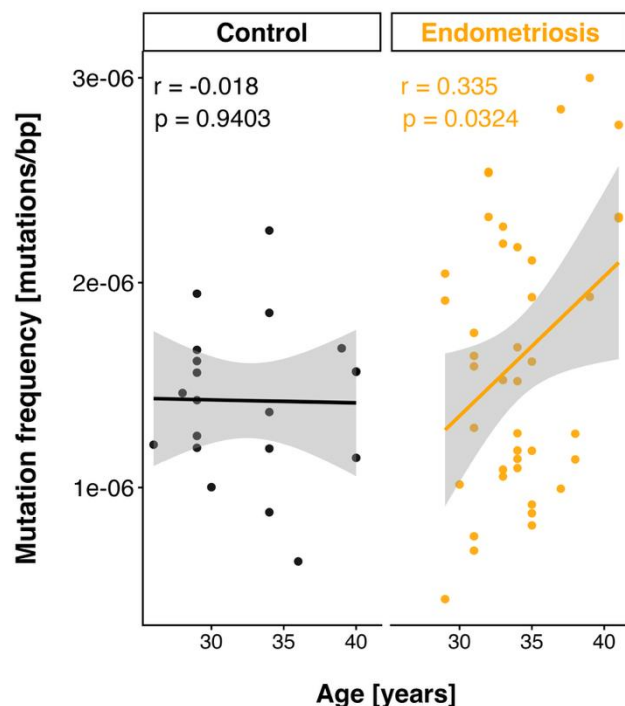


Figure 2. Mutational burden in endometriosis patient's oocytes correlates with age. Mutation frequency in mtDNA of single oocytes is shown as mutations/sequenced nucleotides. Linear regression; R^2 and p-value for the age-dependent slope.; $N_{\text{control}} = 21$; $N_{\text{endometriosis}} = 45$.

Abbreviations

DS	Duplex sequencing	DCS	Dublex consensus sequence
mtDNA	Mitochondrial DNA		

Acknowledgements

This work was supported by the Austrian Science Fund (FWF), Stand-Alone Project (DOI: 10.55776/P36928; BA).

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