1 **Estropausal gut microbiota transplant improves measures of ovarian function in adult mice**

2 Minhoo Kim[1], Justin Wang[1,2,3], Steven E. Pilley[4], Ryan J. Lu[1,5], Alan Xu[1,6], Younggyun Kim[1,7], Minying Liu[8],

3 Xueyan Fu[8], Sarah L. Booth[8], Peter J. Mullen[1,4,9], and Bérénice A. Benayoun[1,9, 10,11,12]

4

5 1. Leonard Davis School of Gerontology, University of Southern California, Los Angeles, CA 90089, USA.

6 2. Department of Quantitative and Computational Biology, University of Southern California, Los Angeles,

7 CA 90089, USA.

8 3. Department of Chemistry, University of Southern California, Los Angeles, CA 90089, USA.

9 4. Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of

10 Southern California, Los Angeles, CA, USA

11 5. Graduate Program in the Biology of Aging, University of Southern California, Los Angeles, CA 90089,

12 USA.

13 6. Thomas Lord Department of Computer Science, USC Viterbi School of Engineering, Los Angeles, CA

14 90089, USA.

15 7. Alfred E. Mann Department of Biomedical Engineering, University of Southern California, Los Angeles,

16 CA 90089, USA.

17 8. Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA 02111,

18 USA

19 9. Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los

20 Angeles, CA, USA

21 10. Molecular and Computational Biology Department, USC Dornsife College of Letters, Arts and

22 Sciences, Los Angeles, CA 90089, USA.

23 11. Biochemistry and Molecular Medicine Department, USC Keck School of Medicine, Los Angeles, CA

24 90089, USA.

25 12. USC Stem Cell Initiative, Los Angeles, CA 90089, USA.

26 *Correspondence: berenice.benayoun@usc.edu

Abstract

Decline in ovarian function with age not only affects fertility but is also linked to a higher risk of age-related diseases in women (*e.g.* osteoporosis, dementia). Intriguingly, earlier menopause is linked to shorter lifespan; however, the underlying molecular mechanisms of ovarian aging are not well understood. Recent evidence suggests the gut microbiota may influence ovarian health. In this study, we characterized ovarian aging associated microbial profiles in mice and investigated the effect of the gut microbiome from young and estropausal female mice on ovarian health through fecal microbiota transplantation. We demonstrate that the ovarian transcriptome can be broadly remodeled after heterochronic microbiota transplantation, with a reduction in inflammation-related gene expression and trends consistent with transcriptional rejuvenation. Consistently, these mice exhibited enhanced ovarian health and increased fertility. Using metagenomics-based causal mediation analyses and serum untargeted metabolomics, we identified candidate microbial species and metabolites that may contribute to the observed effects of fecal microbiota transplantation. Our findings reveal a direct link between the gut microbiota and ovarian health.

Keywords

Microbiome, ovarian aging, menopause, 16S amplicon sequencing, ovarian RNA-seq

Main

Women are born with a definitive number of ovarian follicles and the gradual decrease in the number of the follicles ultimately leads to menopause[1-3]. The onset of menopause has been linked with an elevated risk of numerous age-related conditions, including osteoporosis and dementia[4-6]. Moreover, a later onset of menopause has been identified as a potential predictor of extended lifespan[7,8]. While the profound effects of menopause on the health- and lifespan of women are well-documented, the factors that contribute to ovarian aging and the strategies for its prevention remain poorly understood.

During the reproductive years, the hypothalamus-pituitary-ovarian hormone axis ensures the proper production and release of hormones necessary for ovulation and menstruation[9]. However, as menopause progresses, ovaries become less responsive due to reduced follicle numbers, ultimately leading to the hormonal imbalances characteristic of menopause[10]. Specifically, the serum concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) increase, whereas anti-Müllerian hormone (AMH) and Inhibin A (INHBA) decrease with menopause progression[11-13]. These hormonal adjustments are hallmarks of the menopausal transition and highlight the diminished function of the ovaries[10]. Importantly, this disrupted feedback mechanism has been attributed to the wide array of menopausal symptoms and health risks associated with this transition phase[14,15]. For instance, recent studies have shown FSH receptor activation by FSH in the brain increases susceptibility for Alzheimer's disease pathogenesis in women and blockade of FSH improves cognition in a mouse model of Alzheimer's disease[14,16].

The adult human gut microbiome is composed of approximately 10^{13} to 10^{14} micro-organisms[17,18]. The microbiome consists of bacteria, viruses and fungi, with bacteria representing the largest proportion of the microbial population[19]. Perturbations in the gut microbiota configuration are reported to lead to development and/or progression of serious disease, including diabetes and depression[20,21]. Interestingly, reported health effects of fecal microbiota derived from aged individuals have been inconsistent[22,23]. For example, one study demonstrated that fecal microbiota transplantation (FMT) from 24-month-old mice to 3-month-old mice led to the acceleration of age-associated phenotypes in the brain, retina, and gut of the recipient mice[22]. In contrast, another study found that grafting of fecal microbiota from 24-month-old mice to 5- to 6-week-old germ-free mice enhanced neurogenesis in the hippocampus and induced intestinal

72　growth[23]. These findings underscore the microbiome's complex, multifaceted effects on various organ

73　systems throughout life, significantly affecting host health.

74　　　A recent study discovered that women with premature ovarian insufficiency exhibited significantly

75　altered gut microbial profiles compared to healthy women[24]. Notably, alternations in the gut microbiome in

76　premature ovarian insufficiency patients were associated with changes in serum hormone levels of key

77　ovarian endocrine markers, including FSH, LH and AMH[24]. Similarly, the composition of the gut

78　microbiome has been implicated in the development of polycystic ovary syndrome (PCOS), a disorder

79　characterized by hyperandrogenism and polycystic ovaries[25]. Specifically, FMT of samples from PCOS

80　patients to mice resulted in ovarian dysfunction in otherwise healthy recipient mice[25]. Consistent with the

81　importance of a healthy microbiome for ovarian function, another study has shown that 17β-estradiol

82　levels are significantly lowered in the germ-free female mice[26]. Moreover, transcriptome analysis of sexual

83　development marker genes of the liver and histological studies of ovarian follicle development in germ-

84　free female mice indicated that sexual maturation is perturbed in microbiota-depleted mice[26]. Importantly,

85　the gut microbiota has been shown to modulate serum estrogen levels (through the "estrobolome"),

86　further highlighting the potential role of the gut microbiota in female reproductive health[27]. The gut

87　microbiota regulates action of estrogens through the secretion of β-glucuronidase, an enzyme that

88　deconjugates estrogens into their active forms[27-29]. Dysbiosis of the gut microbiota can impair this process,

89　leading to a reduction in circulating estrogens and potentially contributing to the development of estrogen-

90　modulated conditions[27]. Collectively, these findings highlight a significant causal relationship between the

91　gut microbiota and ovarian function and health, suggesting a pivotal role for microbial communities in

92　modulating female reproductive conditions.

93　　　In this study, we analyzed the gut microbial profiles of young and estropausal (post-reproductive

94　phase in rodents, analogous to human menopause) female mice via 16S rRNA amplicon sequencing and

95　explored how the distinct microbial communities affect ovarian health through FMT experiments in young

96　female mice. Unbiased RNA-seq analyses revealed that modifying the gut microbiota leads to profound

97　changes in the ovarian transcriptomic landscape. Surprisingly, heterochronic gut microbiota transplant

98　from estropausal mice to young female recipients led to a reduction in inflammation-related gene

99　expression in the ovaries, and dampened expression of genes that tend to be upregulated in aging

100   ovaries. Consistently, we observed improved ovarian function and health in the estropausal FMT

101   recipients, supporting a surprising finding whereby old gut microbiota has beneficial effects on ovarian

102   health. Lastly, we identified specific microbial genera and species, through 16S rRNA amplicon

103   sequencing and whole genome shotgun sequencing, respectively, that might drive the observed

104   transcriptomic changes in the ovaries via causal mediation analyses. Overall, our study shows that

105   manipulating the gut microbiota can directly influence ovarian function and health.

Results

Young and estropausal female mice show distinct fecal microbial profiles

To investigate the potential link between changes in the gut microbiome and ovarian aging in mice, we assessed (1) the ovarian health state and (2) microbial profiles of young (YF, 4-months) and naturally aged estropausal (EF, 20-months) C57BL/6JNia female mice[30] (Fig. 1A). To quantify the overall ovarian health state of the mice, we developed a composite "ovarian health index", modeled after the concept of frailty index[31] (Extended Data Fig. 1A). Our goal in constructing this index was to create a standardized, integrative tool that would enable comparative analysis of ovarian function across different datasets. The index combines ovarian follicle counts and serum levels of AMH, FSH, and Inhibin A, using a 3-tier scoring system based on the median values of the young and estropausal groups as reference points. Specifically, values beyond the young median are scored as 3, values between the medians of the young and estropausal groups are scored as 2, and values beyond the estropausal median are scored as 1 (Extended Data Fig. 1A). Given that ovarian follicle count and serum levels of key pituitary and ovarian hormones—AMH, FSH, and Inhibin A—are widely recognized as reliable indicators of ovarian health[32-35], we combined these parameters to form a comprehensive and objective evaluation of ovarian function (Fig.1A-D; see Methods). Additionally, to facilitate accessibility and broader use of the ovarian health index, we developed an R shiny application, allowing the public to analyze their own datasets using this tool (https://alanxu-usc.shinyapps.io/Ovarian_Health_Index_Calculator/).

Consistent with previous reports[30,36], we observed a significant reduction in ovarian follicle counts and serum levels of AMH and Inhibin A, as well as increased FSH levels in the estropausal group (Fig.1B,C and Extended Data Fig. 1B-D). Furthermore, the estropausal group exhibited a significantly lower ovarian health index score compared to the young group (p-value ~0.0117; Fig.1D), validating the subsequent use of our index as a measure of ovarian health and function.

We then collected fecal samples from four independent cohorts of mice and performed 16S rRNA V3-V4 amplicon sequencing to compare the gut microbial profiles of the young and estropausal female mice. 16S rRNA amplicon sequencing allows identification of bacteria at the genus level by amplifying and sequencing variable regions of the 16S rRNA gene (*e.g.* V3-V4 region), which contains unique sequences specific to different species[37]. Quantification and analysis of the sequences from the variable

134   regions, or amplicon sequence variants (ASVs), have been shown to provide important insights of the

135   microbial compositions and environment[38].

136   Our analysis focused on both beta diversity, which assesses differences between microbial

137   communities, and alpha diversity, which looks at diversity within a community[39,40]. Beta diversity was

138   evaluated using the Bray-Curtis dissimilarity and Jaccard index (Extended Data Fig. 2A). The analysis of

139   Bray-Curtis dissimilarity and Jaccard index, conducted by cohort, revealed a distinct separation between

140   the young and estropausal female groups (Extended Data Fig. 2A). Moreover, the results of the principal

141   component analysis, applied to center log-ratio (CLR)-transformed and batch-corrected ASV counts,

142   further highlighted clear clustering between the young and estropausal females across 4 independent

143   cohorts of animals (Fig. 1E). Batch correction was performed to account for technical variability across

144   cohorts[41]. Alpha diversity measures the diversity within a single microbial community or ecosystem,

145   focusing on the variety and abundance of ASVs in a specific environment[36]. Importantly, estropausal

146   females exhibited significantly higher scores in both observed features and Shannon entropy compared to

147   the young female group (Fig. 1F). Our results suggest that the estropausal females have a higher

148   biodiversity and increased species richness and evenness. This pattern of increased alpha diversity with

149   age in female mice aligns with findings from previous studies[42]. However, in humans, alpha diversity has

150   been shown variable trends with age, with both increases and decreases reported in different studies[43,44].

151   Additionally, a recent study reported elevated alpha diversity indices in PCOS model mice[45].

152   We next asked which specific microbial genera changed in abundance between young and

153   estropausal female mice recurrently across our 4 independent cohorts (Fig. 1G and Extended Data Fig.

154   2B, 3). Our analysis identified three genera that were more abundant ("UP in EF") and six that were less

155   abundant ("DOWN in EF") in the microbiota of estropausal females (Fig. 1G and Extended Data Fig. 2B, 3;

156   absolute effect size > 1, adjusted p-value < 0.05). Most of the differentially abundant genera belonged to

157   the *Lachnospiraceae* family, offering a potential lead for future research on their impact on ovarian health.

158   Previous studies have reported a relative decrease in abundance of microbial species from the

159   *Lachnospiraceae* family with age in humans[46,47]. Further studies will be required to elucidate the

160   molecular mechanisms through which these genera affect ovarian function and health. Additionally, we

161   conducted a predictive analysis of functional consequences of these changes in microbial abundances

162   using PICRUSt2[48] (Fig. 1H). This analysis suggested an increase in metabolic pathways such as

163   ketogluconate metabolism and the superpathway of glycerol degradation to 1,3-propanediol, while

164   pathways including the superpathway of fatty acid biosynthesis initiation and phospholipases were

165   predicted to decrease in the estropausal group (Fig. 1H). These predicted metabolic changes, including

166   an increase in ketoglutarate and alterations in lipid metabolism, have been previously associated with

167   ovarian cancers, underscoring the potential relevance of our findings to understanding ovarian health[49,50].

168   Our results collectively underscore the pronounced differences in the fecal microbial profiles between

169   young and estropausal females.

170

171   **Chemically-induced premature ovarian failure model mice show distinct fecal microbial profiles**

172   The 4-VinylCyclohexene Diepoxide (VCD)-injected mouse model is a follicle-deplete, ovary-intact

173   animal model that is known to recapitulate many key aspects of human ovarian aging[51,52]. VCD

174   accelerates atresia by causing selective loss of primary and primordial follicles in the ovary, while leaving

175   the rest of the ovarian structure intact for residual hormone production[51,52]. We assessed the ovarian

176   health index and fecal microbial profiles of vehicle- and VCD-exposed mice (CTL and VCD, respectively;

177   Extended Data Fig. 4A). Briefly, 4-month-old C57BL/6NTac female and male mice were subjected to daily

178   intraperitoneal injections of either vehicle (safflower oil) or VCD (at a dosage of 160 mg/kg/day) for 15

179   consecutive days. Then, the mice were maintained for an additional 100 days to ensure the complete

180   development of phenotypes associated with VCD exposure[53] (Extended Data Fig. 4A). To determine

181   whether the observed effects on microbial profiles in females were a direct consequence of VCD-induced

182   ovarian dysfunction rather than off-target effects of VCD administration, we also generated and analyzed

183   data from male mice.

184   For female mice, ovarian follicle depletion and alterations in crucial ovarian hormone levels (*i.e.*

185   AMH, FSH, and Inhibin A) were confirmed through ovarian histology analysis and serum hormone

186   concentration measurements, respectively (Extended Data Fig. 4B-E). In line with these findings, the

187   VCD-treated group exhibited a markedly lower ovarian health index (p-value ~0.0104; Extended Data Fig.

188   4F), consistent with overall depleted ovarian function.

189   Subsequently, we conducted 16S rRNA V3-V4 amplicon sequencing on vehicle- and VCD-treated

190   animals. Analysis through principal component analysis of CLR-transformed ASV counts, along with Bray-

191   Curtis dissimilarity and Jaccard index, demonstrated a distinct separation between the vehicle-treated

192   (CTL) and VCD-exposed groups (Extended Data Fig. 5A-B). Alpha diversity indices, on the other hand,

193   revealed no significant differences between these two groups (Extended Data Fig. 5C). These findings

194   suggest that the overall diversity within the group's communities may appear similar, but they may be

195   different in the species present, their relative abundances and the functions the species perform. Notably,

196   beta diversity analysis of microbial profiles in male mice showed no notable distinctions between vehicle-

197   and VCD-treated groups (Extended Data Fig. 5A, B). Additionally, there were no differences in alpha

198   diversity between these groups (Extended Data Fig. 5D). These findings indicate that the observed

199   effects on microbial profiles in females are causally induced by ovarian dysfunction and not off-target

200   effects of VCD administration.

201   Our analysis revealed differentially abundant genera from CTL vs. VCD groups (Extended Data

202   Fig. 5E and 6; absolute effect size > 1, adjusted p-value < 0.05). We detected several genera from the

203   *Lachnospiraceae* family, yet none of the unique ASV IDs corresponded with those identified as

204   differentially abundant in the estropausal female mice. Thus, further research to accurately identify the

205   species significant to ovarian aging and health will be important. Then, we utilized PICRUSt2 to predict

206   functional abundance between the CTL and VCD groups (Extended Data Fig. 5F). Notably, we detected

207   down-regulation of the phospholipases pathway in the VCD group, a finding consistent with observations

208   in the estropausal female group (Fig. 1H and Extended Data Fig. 5F). Our findings suggest that

209   physiological ovarian aging and chemically-induced ovarian failure lead to substantially different

210   outcomes on the gut microbiota.

211

Young and old male mice show distinct fecal microbial profiles

213   Next, as an important comparison point, we also characterized the fecal microbial profiles of

214   young (4-months) and naturally aged old (20-months) C57BL/JNia male mice (Extended Data Fig. 7A).

215   This comparative study among female and male aging mice, as well as the VCD-exposed mice, aimed to

216   identify differences in microbial profiles attributable to general aging and those specific to ovarian aging.

217 We collected fecal samples from three independent cohorts of mice and performed 16S rRNA V3-V4

218 amplicon sequencing to compare the gut microbial profiles of the young and old male mice. Analysis

219 using principal component analysis of CLR-transformed ASV counts, coupled with Bray-Curtis

220 dissimilarity and Jaccard index, showed a distinct divergence between the young and old male groups

221 (Extended Data Fig. 7B-C). However, this separation was less pronounced compared to what was

222 observed in the female cohorts (Fig. 1E). Alpha diversity indices indicated no significant disparities

223 between the two age groups of males (Extended Data Fig. 7D). Similarly, a previous study examining

224 microbial profiles in male mice aged 3-4 months and 19-20 months found comparable alpha diversity

225 index values between the two age groups[54]. Through differential abundance and functional abundance

226 prediction analyses, we identified several genera and functional terms that varied significantly between

227 young and old male mice (Extended Data Fig. 7E-F and 8). Importantly, we observed a few consistent

228 functional terms across female and male aging cohorts. In particular, the L-lysine biosynthesis I pathway

229 was found to be up-regulated, while the pyridoxal 5-phosphate biosynthesis I pathway and the

230 superpathway of pyridoxal 5-phosphate biosynthesis were down-regulated with age in both sexes (Fig.

231 1H and Extended Data Fig. 7F). These results suggest that these pathways may play roles in the general

232 aging processes, rather than being uniquely related to ovarian aging. Further research will be necessary

233 to elucidate the influence of these functional pathways on the aging process.

234

235 **Gut microbiota manipulation can induce changes in the ovarian transcriptome**

236 To investigate the potential effects of gut microbiota on ovarian health, we conducted FMT

237 experiments to directly assess how alterations in the gut microbiota after estropause may influence

238 ovarian function. Specifically, young adult female mice (4-months) were subjected to treatment with anti-

239 biotics/anti-mycotic (Ab/Am) treatment to eliminate their initial microbial environment, and then received

240 fecal microbiota grafts from either young (FMT-YF) or estropausal (FMT-EF) female mice (Fig. 2A).

241 Subsequently, the ovaries were harvested for bulk RNA-seq analysis (Fig. 2A). Based on previous

242 research that has shown adverse effects of gut microbiota from donors with ovarian dysfunction, such as

243 PCOS[25], our initial hypothesis was that transplanting fecal matter from estropausal mice could potentially

244 induce estropause-like conditions in the recipient mice.

245   Multi-dimensional scaling (MDS) analysis of the ovarian RNA-seq dataset revealed a clear

246   separation between the FMT-YF and FMT-EF groups (Fig. 2B). Additionally, we detected 2,131

247   differentially expressed genes between the FMT-YF and FMT-EF groups (FDR < 5%; Extended Data Fig.

248   9A). Then, we investigated the pathways that were distinctively regulated between the FMT-YF and FMT-

249   EF groups. Our gene set enrichment analysis (GSEA)[55] results highlighted that pathways related to

250   immune regulation were significantly down-regulated in the FMT-EF groups (Fig. 2C-E; highlighted in red).

251   To evaluate whether these changes were the results of substantial changes in immune cells infiltration in

252   the tissue, we leveraged a publicly available ovarian single-cell RNA-seq dataset[56] to conduct

253   deconvolution analysis, thus allowing us to deduce alterations in immune cell proportions within our

254   dataset. Using two distinct tools, CSCDRNA[57] and Granulator[58], we determined that the proportions of

255   immune cells did not significantly differ between the FMT-YF and FMT-EF groups (Extended Data Fig.

256   9B), consistent with the notion that the transcriptional changes that we observed may reflect cell

257   autonomous changes in gene expression profiles. In addition, our GSEA results revealed terms

258   associated with chromatin structure and dynamics, including kinetochore, chromatin silencing complex,

259   chromosome, centromeric region, and spindle pole (Fig. 2E). These findings suggest that transcriptional

260   changes in the ovaries may reflect alterations in chromatin organization and chromosome dynamics,

261   processes critical for maintaining oocyte quality[59,60]. Together, these results indicate that pathways

262   associated with both immune regulation and chromatin dynamics distinguish the FMT-YF and FMT-EF

263   groups.

264   To identify transcription factors potentially driving the transcriptomic alterations observed between

265   the FMT-YF and FMT-EF groups, we utilized Gene Transcription Regulation Database (GTRD)[61] with the

266   GSEA paradigm. While our analysis did not identify any transcription factors meeting the usual stringent

267   criteria of FDR < 5%, we found two transcription factors showing trends of up-regulation and eight

268   displaying down-regulation trends from the FMT-EF group, with an adjusted FDR < 10% threshold (Fig.

269   2F). Then, we identified publicly available ChIP-seq datasets for the trending transcription factors via the

270   Cistrome Data Browser[62,63], using corresponding *bona fide* peaks to determine potential enrichment by

271   GSEA. Our analysis showed significant negative enrichment scores of Ncoa1 and Usp7-regulated genes

272   within the FMT-EF group (p-value ~0.0138 and ~1e-04, respectively; Extended Data Fig. 9C). Moreover,

273   we noted that the expression levels of *Ncoa1* and *Usp7* remained consistent across FMT-YF and FMT-EF

274   groups (Extended Data Fig. 9D), suggesting potential post-transcriptional or post-translational regulation.

275   While research on the roles of Ncoa1 and Usp7 in ovarian function is sparse, one study highlighted that

276   Ncoa1 function is important in egg production traits in Shaobo hens[64]. Additionally, an inhibitor of Usp7,

277   P5091, was found to inhibit the proliferation of ovarian cancer cells in another study[65]. Together with our

278   findings, this suggests that Ncoa1 and Usp7 represent potential transcriptional regulators of ovarian

279   function.

280

281   **Heterochronic FMT may have rejuvenating effects on the ovarian transcriptome of the recipients**

282   Cumulative studies have indicated that aging leads to a pro-inflammatory shift in the ovarian

283   microenvironment, adversely affecting ovarian function and oocyte quality[66,67]. Based on our GSEA

284   results which indicated down-regulation of inflammatory pathways in the ovaries of the FMT-EF group, we

285   next asked whether changes in the ovarian transcriptome upon FMT-EF could represent a more general

286   trend of transcriptional rejuvenation. For this purpose, we analyzed two publicly available bulk RNA-seq

287   datasets profiling ovaries from young (2-3-months) and aged/estropausal (12-months) wild-type female

288   mice[68,69] to identify gene sets that are either up- or down-regulated with ovarian aging (Fig. 3A). To

289   evaluate whether the genes identified from publicly available RNA-seq datasets on ovarian aging ("UP

290   with ovarian aging" and "DOWN with ovarian aging") were significantly regulated in response to FMT-EF,

291   we conducted GSEA using significant age-regulated genes as input gene sets. Surprisingly (and contrary

292   to expectations based on previously reported observation at the somatic level for heterochronic FMT), our

293   findings revealed a significant negative enrichment score for genes that were up-regulated in the context

294   of ovarian aging (p-value ~0.0046; Fig. 3B), suggesting that the ovarian transcriptome of the

295   heterochronic FMT recipients (*i.e.* FMT-EF group) displays transcriptional profiles resembling those of

296   younger females.

297   As an orthogonal line of evidence, we next asked whether indicators of cellular senescence (as

298   measured by the highly curated senescent marker gene set, SenMayo[70]) were significantly impacted by

299   FMT-EF. Importantly, our results showed a significant negative enrichment score for the SenMayo gene

300   set in the FMT-EF group, consistent with an overall "rejuvenation" trend for the ovarian transcriptome of

301   FMT-EF mice (p-value ~0.0382; Fig. 3C).

302   A recently published study highlighted the role of Nicotinamide Adenine Dinucleotide (NAD+)

303   metabolism in ovarian aging, showing that increased NADase CD38 expression accelerated ovarian

304   aging [69]. CD38 deletion or pharmacological inhibition preserved fertility and follicle reserves in aged mice

305   [69]. Building on these findings, we examined the expression of *Cd38* as well as inflammation markers

306   discussed in the study, including *Cdkn1a* and *Il1a* (Fig. 3D). In our analysis, we observed reduced *Cd38*

307   expression in the FMT-EF group compared to the FMT-YF group, along with decreased expression of the

308   inflammation markers (Fig. 3D). These findings suggest the potential involvement of the CD38-NAD

309   pathway in the observed phenotypes of the FMT-EF group.

310

311   **FMT-EF mice show improved ovarian health**

312   Since we observed large ovarian transcriptome remodeling in the FMT-EF group consistent with

313   a less inflammatory, more youthful ovarian state, we next assessed the ovarian health state of the FMT-

314   YF *vs.* FMT-EF groups to determine whether FMT-EF led to enhanced ovarian function (Fig. 4). We

315   evaluated ovarian health function using a 2-pronged approach (Fig. 4A): (i) calculating the ovarian health

316   index, and (ii) evaluating effective fertility using litter size and time to first pregnancy after mating[71] (Fig. 4).

317   Consistent with our prediction from the ovarian transcriptomes, the FMT-EF mice exhibited

318   significantly higher scores on the composite ovarian health index than FMT-YF mice (p-value ~0.0255;

319   Fig. 4B, Extended Data Fig. 10A-D), supporting an enhanced ovarian health phenotype. The ovarian

320   health index incorporates multiple parameters, including serum levels of AMH, FSH, and Inhibin A, as well

321   as ovarian follicle counts (Extended Data Fig. 1A). Notably, for the FMT cohorts, FSH levels were

322   measured using two different assay kits during the study due to an update implemented by the core

323   facility conducting the assays. To ensure consistency and comparability of data across cohorts, we

324   applied a correction (see Methods). This adjustment ensured that FSH levels accurately reflected ovarian

325   health and were comparable between cohorts (Extended Data Fig. 10D). Next, we evaluated the result of

326   ovarian function – fertility – through measures of litter size and reproductive success rate. Specifically,

327   there was a suggestive trend for increased litter size in FMT-EF mice (p-value ~0.1726; Fig. 4C). In

328   addition, FMT-EF mice remained nulliparous for a shorter amount of time after pairing with a young health

329   male (p-value ~0.002; Fig. 4D), also suggestive of improved fertility. To determine whether the inclusion

330   of 0 pup count data influenced our findings, we re-analyzed the latency data after excluding animals with

331   0 pup counts. Importantly, the latency data remained statistically significant (p-value ~0.0159 without 0

332   pup counts vs. p-value ~0.002 with all data). Together, our findings are consistent with

333   improved/rejuvenated ovarian function after FMT-EF.

334   Importantly, no significant differences were observed in body weight between FMT-YF and FMT-

335   EF groups after Ab/Am treatment (Extended Data Fig. 10E and Extended Data Table 1). Additionally,

336   staging of the estrous cycle on the day of sample collection revealed no significant differences between

337   the groups (Extended Data Table 2).

338

339   **FMT-YF and FMT-EF mice show distinct microbial profiles**

340   Importantly, we next verified that fecal transplants from young or estropausal female mice led to

341   distinct microbial profiles in the recipient mice. For this purpose, we conducted and analyzed 16S rRNA

342   V3-V4 amplicon sequencing on the gut microbiota of FMT-YF and FMT-EF mice (Extended Data Fig. 11).

343   Principal component analysis of CLR-transformed ASV counts, complemented by Bray-Curtis

344   dissimilarity and Jaccard index, showed a clear segregation between the FMT-YF and FMT-EF groups

345   (Extended Data Fig. 11B). While no significant differences were noted across four alpha diversity indices

346   (Extended Data Fig. 11C), we identified several microbial genera with significant differential abundance

347   between the FMT-YF and FMT-EF groups (absolute effect size > 1, adjusted p-value < 0.05; Extended

348   Data Fig. 11D and 12). Notably, two genera that showed increased abundance in the FMT-EF recipient

349   group were also elevated in the donor estropausal females

350   (*Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae* and *Bacteria;Firmicutes;Clostridia;*

351   *Clostridiales;Lachnospiraceae;Coprococcus 2;uncultured organism ;* Extended Data Fig. 11E). These

352   results indicate that transplanting microbiota from young or estropausal female mice led to changes in the

353   microbial landscape of the recipient mice. Other studies have also reported the selective enrichment of

354   specific microbial species from donor microbiota in recipient subjects[22,72]. These observations may be

355 attributable to variations in the intestinal microenvironments, which allow the proliferation of certain

356 species.

357   Subsequently, we used PICRUSt2 to predict functional abundance differences between the FMT-

358 YF and FMT-EF groups (Extended Data Fig. 11F). We identified significant functional groups only with

359 up-regulated functional terms in the FMT-EF group, with no significant down-regulated terms. Notably,

360 there was a significant presence of terms related to the menaquinol biosynthesis pathway, including those

361 involved in 1,4-dihydroxy-6-naphthoate biosynthesis II, 1,4-dihydroxy-6-naphthoate biosynthesis I, and

362 the superpathway of menaquinol-8 biosynthesis II (Extended Data Fig. 11F). Menaquinols, the active

363 hydroquinone forms of vitamin K, also known as menaquinones (MKs), facilitate the carboxylation of

364 vitamin K-dependent proteins, which are crucial for biological processes such as blood clotting, bone

365 health, and heart health[73].

366   To pinpoint specific microbial species that may contribute to the observed changing in the ovarian

367 health state, we performed whole genome shotgun (WGS) metagenomics on the FMT cohort (Fig. 5A).

368 Consistent with the 16S amplicon sequencing results, PCA analysis of CLR-transformed data and beta

369 diversity analysis revealed distinct clustering between the FMT-YF and FMT-EF groups (Fig. 5B-C).

370 Similarly, no significant differences were observed in alpha diversity indices, reinforcing the similarity in

371 overall microbial diversity between the groups (Fig. 5D). WGS analysis identified 168 differentially

372 abundant microbial species (FDR < 5%; Fig. 5E-F and Extended Data Fig. 13), further confirming distinct

373 microbial compositions in the FMT-YF and FMT-EF groups. Intriguingly, microbial species previously

374 reported to be differentially enriched in the fecal microbiota of PCOS patients, such as *Bacteroides*

375 *xylanisolvens*, *Bacteroides thetaiotaomicron*, *Parabacteroides distasonis*, and *Bacteroides stercoris*[25],

376 were significantly depleted in the FMT-EF group (Extended Data Table 3).

377   Functional abundance analysis using HUMAnN3[74] revealed up-regulation of pathways related to

378 menaquinol biosynthesis, including PWY-7371: 1,4-dihydroxy-6-naphthoate biosynthesis II, consistent

379 with predictions from the 16S amplicon analysis (Fig. 5G and Extended Data Fig. 11F). Intriguingly, we

380 detected terms associated with the NAD biosynthesis pathway, such as PWY–7761: NAD salvage

381 pathway II (PNC IV cycle) and PYRIDNUCSYN–PWY: NAD de novo biosynthesis I (from

382   aspartate)|unclassified, which aligns with our ovarian RNA-seq data showing altered expression of *Cd38*

383   in the FMT-EF ovaries (Fig. 3D, 5G).

384   Given the stability of menaquinones and their primary synthesis by gut microbiota, we further

385   evaluated their levels in stool samples (MK6-MK13) to explore potential systemic effects post-absorption,

386   as they contribute significantly to the host's vitamin K status. However, no significant differences in MKs

387   concentrations in the stool samples of FMT-YF and FMT-EF animals were observed (Extended Data Fig.

388   14A,B), although this may be the result of high animal-to-animal variability and/or the high vitamin K

389   content of the standard mouse chow at the USC vivarium dietary (see Discussion).

390

391   **FMT-YF and FMT-EF mice show distinct serum metabolomic profiles**

392   It is well established that gut microbiota-derived molecules can enter the bloodstream and

393   influence health[75]. Additionally, metabolite levels in plasma have been shown to be affected by aging[76].

394   To assess whether shifts in the gut microbial profiles of the FMT animals induced changes in circulating

395   metabolites, we performed untargeted metabolomics on serum samples from two independent

396   experimental cohorts (Fig. 6A). First, we performed PCA of the identified metabolites to detect unbiased

397   separation between the FMT-YF and FMT-EF groups. Although non-linear batch effects were present and

398   could not be fully corrected, a clear separation was still evident within each batch (Fig. 6B). Subsequent

399   LDA analysis further showed the distinct metabolomic profiles of the two groups (Fig. 6C). Finally, we

400   extracted metabolites that changed consistently across both cohorts and selected the top 15 based on

401   the LDA loadings (Fig. 6D).

402   Among the metabolites identified, 3,4-dihydroxyphenylpropionic acid (3,4-DHPPA) and lactamide

403   were significantly decreased in FMT-EF animals at FDR < 0.1 (Fig. 6D, E). 3,4-DHPPA, a microbial

404   metabolite derived from polyphenol metabolism, has been linked to lipid metabolism and broader

405   metabolic regulation[77]. Given the critical role of lipid metabolism in hormonal balance and ovarian function,

406   its reduction in FMT-EF animals may reflect metabolic adaptations that contribute to improved ovarian

407   health. Additionally, while the direct role of lactamide in ovarian function is unclear, microbial-derived

408   lactic acid and its derivatives have been implicated in gut microbiota-driven regulation of reproductive

409   health[78]. For instance, lactic acid bacteria have been shown to influence sex hormone-related gut

410   microbiota composition, leading to beneficial effects on ovarian physiology in preclinical models[78]. The

411   observed decrease in lactamide levels may indicate broader microbiome-mediated metabolic changes

412   that support ovarian health.

413   In addition to these significant metabolic changes, palmitoylcarnitine, a key metabolite involved in

414   fatty acid β-oxidation and mitochondrial energy production[79], was elevated in FMT-EF animals according

415   to LDA loadings. This suggests enhanced mitochondrial efficiency and metabolic flexibility, which are

416   essential for oocyte and follicular health[80]. Furthermore, palmitoylcarnitine has been implicated in immune

417   modulation[81], and its increased levels may indicate a metabolic shift supporting both energy homeostasis

418   and an anti-inflammatory ovarian environment. This aligns with our bulk RNA-seq analysis, which

419   revealed a downregulation of inflammatory pathways in the ovaries of FMT-EF animals (Fig. 2C-E).

420   Conversely, LDA loadings for β-estradiol suggested that serum levels are decreased in FMT-EF

421   mice. Based on the improved ovarian function observed in FMT-EF mice, this may reflect a more

422   physiologically regulated hormonal environment, rather than a pathological decline. Estradiol plays a

423   critical role in ovarian function, lipid metabolism, and mitochondrial activity[82], and its levels may be

424   influenced by gut microbiota-driven metabolic pathways[27]. The gut microbiome is known to modulate

425   steroid hormone metabolism[27], and FMT from EF donors may have contributed to a systemic

426   reprogramming of lipid and hormonal pathways that promote ovarian resilience.

427   Together, these findings suggest that FMT from estropausal donors induces distinct metabolic

428   shifts that may contribute to the observed improvements in ovarian health. Further studies will be needed

429   to determine the functional impact of these metabolites on ovarian physiology and their potential role in

430   reproductive aging.

431

432   **Causal mediation analyses reveal microbial species potentially influencing ovarian transcriptome**

433   Lastly, we performed causal mediation analyses to characterize microbial species that may

434   directly influence the ovarian transcriptome remodeling that we observed between FMT-YF and FMT-EF

435   mice (Fig. 7A). From the features exhibiting significant abundance variations between the groups, we

436   identified four microbial species (*Bacteroides zhangwenhongii, Shigella flexneri, Bacteroides stercoris*,

437   and *Bacteroides graminisolvens*) with a significant mediation effect on ovarian transcriptome changes

438   (Fig. 7B). Interestingly, *Bacteroides stercoris* was previously highlighted as a key contributor to the distinct

439   fecal microbiota profiles between healthy individuals and those with PCOS[25]. Further investigation into the

440   functional roles of these identified species in modulating ovarian function will be essential in future studies.

441   Similarly,   we   identified   four   ASV   IDs   (850e0d58c1aa35238538564a8010e0ff,

442   e4f32cdc3407edb8423447421078fdae,   55ebdcf041fb6b8b4632993423011372,   and

443   d4840c45eb37593108d020fb6a63f8e2) with a significant mediation effect on ovarian transcriptome

444   changes (Extended Data Fig. 16).

445   To further explore the relationship between gut microbiota and ovarian health, we performed

446   Spearman correlation analysis using WGS metagenomics data and serum hormone quantification data

447   (Fig. 7C). This analysis revealed significant correlations between multiple microbial species and hormone

448   levels, including *Shigella flexneri, Parabacteroides merdae,* and *Porphyromonas sp. oral taxon 275,*

449   suggesting these species as potential candidates influencing ovarian function. These findings provide a

450   strong foundation for future studies aimed at experimentally validating the roles of these microbial species

451   in modulating ovarian function and hormone levels.

452

453   **Discussion**

454   In this study, we examined the fecal microbial profiles of three distinct groups: (1) young and

455   naturally aged estropausal female mice, (2) mice with chemically-induced premature ovarian failure and

456   their control counterparts, and (3) young and naturally aged male mice. Through our comparative analysis,

457   we identified and differentiated between functional pathways that may be unique to ovarian aging (*i.e.*

458   phospholipases pathway) and those specific to general aging (*i.e.* L-lysine biosynthesis I, pyridoxal 5-

459   phosphate biosynthesis I, and superpathway of pyridoxal 5-phosphate biosynthesis). Furthermore,

460   through fecal microbiota transplantation assays, we discovered that alterations in the gut microbiota can

461   significantly impact the ovarian landscape, affecting both fertility and the health state of the ovaries. We

462   also identified specific microbial species as potential mediators of the observed effects on ovarian

463   function. The vitamin K2 and CD38-NAD pathways emerged as potential mechanisms to explore for

464   investigating the underlying causes of the observed phenotypes in our FMT experiments (Fig. 7D). Future

465   investigations focusing on these species hold significant promise for crafting novel therapeutic strategies

466   aimed at mitigating or potentially reversing ovarian aging.

467

468   **Ovarian RNA-seq data of the FMT recipients supports causal influence of the gut microbiota on**

469   **ovarian function**

470   Our analysis of ovarian RNA-seq data from the FMT recipients (FMT-YF vs. FMT-EF) revealed

471   significant changes in the ovarian transcriptome following alterations in the gut microbiota. While existing

472   research underscores the broad effects of the gut microbiome on host health, evidence detailing its direct

473   effects on the transcriptome of various tissues remains sparse. For instance, earlier research

474   demonstrated that mice raised in a germ-free environment exhibit a markedly distinct liver transcriptome

475   compared to their conventionally raised counterparts[26]. To the best of our understanding, the direct

476   influence of gut microbiota on the ovarian transcriptome has not yet been documented. Our analysis not

477   only uncovered significant variations in the ovarian transcriptome among FMT recipient mice (*i.e.* reduced

478   inflammatory pathway in the FMT-EF group), but we also noted the transmission of phenotypic alterations

479   (*i.e.* improved fertility and ovarian health). Collectively, these results offer substantial evidence of gut

480   microbiota's direct role in influencing ovarian function and health. Furthermore, the microbial species and

481   genera, and functional pathways we identified serve as valuable assets for future research aimed at

482   investigating potential therapeutic strategies.

483

484   **Unexpected advantageous effects of grafting estropausal female fecal microbiota may be due to**

485   **compensatory mechanisms**

486   Based on previous research that has shown detrimental effects of gut microbiota from donors

487   with ovarian dysfunction, such as PCOS[25], we initially hypothesized that transplanting fecal microbiota

488   from estropausal mice might induce estropause-like phenotypes in the recipient mice. Contrary to our

489   expectations, however, we observed an ovarian transcriptome in the recipients that resembled that of

490   young ovaries, alongside improvements in ovarian health and fertility following the transplantation of

491   microbiota from estropausal females. The unexpected advantageous effects observed could be attributed

492   to a compensatory mechanism. For instance, FSH, required for follicle maturation and ovulation, is found

493   in elevated levels in post-menopausal women and estropausal mice[2,83,84]. After reproductive senescence,

494   the ovaries no longer produce estrogen or mature eggs, so the pituitary gland compensates by producing

495   more FSH in an attempt to stimulate the now non-responsive ovaries[30]. Similarly, it is conceivable that the

496   gut microbiota in reproductively senescent female mice could be enriched with beneficial microbes and/or

497   metabolites that support ovarian health, perhaps as a compensatory response. Cumulative studies have

498   highlighted the role of gut microbiota in compensatory processes. For instance, one study demonstrated

499   that gut microbiota can influence the levels of peptides that regulate energy balance in the hypothalamus,

500   serving as a mechanism to decrease body fat[85]. Furthermore, an increase in microbes that produce

501   metabolites critical for the nervous system's function—such as serotonin, acetylcholine, histamine, and

502   dopamine—was observed in patients with mild cognitive impairment[86]. Therefore, when young adult

503   female mice received fecal microbiota transplants from estropausal females, it is possible that the

504   enriched gut microbial environment contributed to the manifestation of younger or more reproductively

505   active phenotypes in recipient mice.

506

507   **Potential involvement of MKs in ovarian health and aging**

508   Although our analysis of functional abundance from both WGS and 16S rRNA amplicon

509   sequencing datasets predicted potential shifts in the biosynthesis pathway of MKs in the FMT mice, we

510   observed no significant differences in the stool concentrations of MKs between the FMT-YF and FMT-EF

511   groups. This lack of observable difference might be attributed to the vitamin K content of the standard

512   chow used by the USC vivarium during our experiments (LabDiet, PicoLab Rodent Diet 20). Specifically,

513   the high vitamin K dietary content could have saturated the system, thus masking subtle variations in MK

514   production by the gut microbiome. Such nuances are typically more pronounced under conditions of a low

515   vitamin K diet[87]. Published studies have shown that vitamin K status influences MK production because

516   gut bacteria require vitamin K substrates to synthesize MKs[87]. In our study, the presence of vitamin K3

517   (menadione) in the diet, which is converted into MK4 in the body, likely contributed to the high basal MK

518   levels observed in the fecal matter of the recipient mice. While there have been limited studies, MKs have

519   demonstrated potential benefits for ovarian health in specific contexts, such as certain ovarian cancers

520   and in patients with PCOS[88,89]. Additionally, some studies have underscored the anti-inflammatory

521   properties of MKs[90]. Given our observations of decreased inflammatory function and enhanced ovarian

522   function in the FMT-EF mice, coupled with the documented benefits of MKs, MKs may be therapeutic

523   candidates for improving ovarian health and function. However, further research into the direct effects of

524   MKs on ovarian function will be necessary.

525

526   **Sex-differences in aging-associated gut microbial profiles**

527   In our comparative study, while we detected certain similarities in the microbial profiles of both

528   females and males with aging, our analysis primarily revealed notable differences between the sexes.

529   Specifically, while aging was associated with significant changes in alpha diversity in female mice, such

530   changes were not observed in males. Moreover, our analyses of differential abundance of microbial

531   genera and predictions of functional abundance revealed substantial differences in the gut microbial

532   profiles and their functions between females and males as they age. Indeed, extensive research has

533   established that aging manifests differently in females and males, a phenomenon known as sex

534   dimorphism in aging[91]. Particularly, the susceptibility to diseases associated with aging, including

535   Alzheimer's disease and obesity, varies significantly between the sexes[92-94]. Furthermore, the disparity in

536   lifespan between females and males is consistently observed across various species[95,96]. In agreement

537   with our findings in this study, sex differences in the gut microbiome associated with aging have

538   previously been documented[97,98]. While the limited number of existing studies suggest a possible

539   influence of sex hormones on the observed sex differences in the gut microbial profiles[99-101], these

540   findings do not negate the potential effects of non-hormonal factors, such as those related to sex

541   chromosomes, on the differences noted between sexes. Given the significant distinctions between sexes

542   highlighted in this study and their varied impacts, it is imperative to consider sex as a contributing factor in

543   future gut microbiome research, not only to deepen our understanding but also to address the specific

544   differences effectively.

545

546   **The potential role of the CD38-NAD pathway in mediating ovarian rejuvenation in the FMT-EF mice**

547   CD38, a primary NADase in mammalian tissues, plays a pivotal role in regulating NAD+ levels[102].

548   Emerging evidence implicates the CD38-NAD axis in aging, with increased CD38 expression contributing

549   to age-related declines in NAD+ levels, impairing mitochondrial function and cellular metabolism[103,104]. In

550   the context of ovarian aging, studies have shown that CD38 overexpression accelerates follicular

551   depletion and diminishes ovarian function, whereas CD38 inhibition preserves follicle reserves and fertility

552   in aged mice[69].

553   In this study, we observed reduced *Cd38* expression in the ovaries of the FMT-EF group,

554   alongside downregulation of inflammatory markers such as *Cdkn1a* and *Il1a* (Fig. 3D). Functional

555   pathway predictions from the metagenomics data further supported these findings, revealing enrichment

556   of pathways related to NAD biosynthesis in the FMT-EF group (Fig. 5G). Specifically, pathways such as

557   NAD salvage pathway II (PNC IV cycle) and NAD de novo biosynthesis I (from aspartate) were

558   upregulated (Fig. 5G). These results suggest that the gut microbiota established through estropausal

559   fecal microbiota transplantation may suppress CD38 activity, influencing ovarian NAD metabolism and

560   creating a more favorable ovarian environment. This aligns with previous research linking CD38-mediated

561   NAD+ depletion to inflammation and cellular senescence, processes that are detrimental to ovarian

562   function[105,106].

563   Together, these findings indicate that modulation of the CD38-NAD pathway by gut microbiota

564   may alleviate ovarian inflammation and promote a more youthful transcriptomic profile, contributing to the

565   enhanced ovarian health observed in FMT-EF mice.

566

567   **Limitations of the study**

568   One potential caveat in our chemically induced ovarian failure model is that VCD, while

569   selectively depleting ovarian follicles, is a systemically administered agent and could potentially affect

570   other biological systems. However, studies have shown that VCD's effects are confined to ovarian tissue,

571   and its impact on other organ systems have not been reported[107,108]. To further assess off-target effects,

572   we conducted control experiments in male mice injected with VCD or vehicle, and no significant

573   differences in gut microbial beta diversity indices (Bray-Curtis dissimilarity and Jaccard index) were

574   observed. These findings suggest that the observed microbial changes in female mice are primarily due

575   to ovarian dysfunction rather than direct effects of VCD, though we acknowledge the potential for non-

576   specific effects. Additionally, it is possible that the improved fertility outcomes in the FMT-EF group,

577　compared to the FMT-YF group, may reflect differences in how aged and young donor microbiota

578　recolonize the gut following antibiotics treatment. For example, the aged microbiota might better adapt to

579　recolonization, reducing the likelihood of artificially poor fertility outcomes in the recipients. Nonetheless,

580　the changes in the microbiota induced by FMT resulted in significant changes in ovarian health, as

581　evidenced by differences in ovarian transcriptomic profiles, fertility outcomes, and overall ovarian health

582　indices in the FMT-YF and FMT-EF groups. Finally, another limitation of the study involves the use of

583　PICRUSt2, which predicts functional abundances based on marker gene sequences. While PICRUSt2

584　provides insights into potential functional pathways affected by changes in the microbiota, it is a predictive

585　tool and does not directly measure functional abundances. Future studies will require additional functional

586　assays to validate these predictions.

587

588　　In summary, we performed a comparative analysis of microbial profiles among aging female,

589　aging male and a chemically-induced menopause model mice to characterize microbial genera that are

590　unique to general aging vs. ovarian aging. Our work highlights potential microbial species and genera,

591　and functional pathways that may play key roles in modulating ovarian function.

Acknowledgements

Author contributions

M.K. and B.A.B. designed the study. M.K. performed live animal experiments. M.K. and R.L.J. dissected mouse tissues. M.K., J.W. and Y.K analyzed ovarian histology data. A.X. generated R shiny application. M.L., X.F. and S.L.B quantified stool MK concentrations. S.E.P, M.K. and P.A.M performed and analyzed metabolomics data. M.K. and J.W. performed computational analyses. M.K. and B.A.B. wrote the manuscript with input from all authors. All authors edited and commented on the manuscript.

Declaration of interests

620   The authors declare no competing interests.

621 **Methods**

622

623 **Mouse husbandry**

624   All animals were treated and housed in accordance with the Guide for Care and Use of

625 Laboratory Animals. All experimental procedures were approved by the USC's Institutional Animal Care

626 and Use Committee (IACUC) and are in accordance with institutional and national guidelines. Samples

627 were derived from animals on approved IACUC protocol number 20770 and 21212.

628   For the "aging cohort," female and male C57BL/6JNia mice (4- and 20-month-old animals) were

629 obtained from the National Institute on Aging (NIA) colony at Charles River Laboratories. For the "VCD

630 cohort" and "fecal microbiota transplantation (FMT) cohort", female and male C57BL/6NTac mice (3.5-

631 month-old animals) were ordered from Taconic Biosciences. All animals were acclimated at the specific-

632 pathogen-free animal facility at USC for 2 weeks before any processing. All animals were fed PicoLab

633 Rodent Diet 20 (LabDiet, 5053). The facility is on a 12-h light/dark cycle and animal housing rooms are

634 maintained at 72 °F and 30 % humidity. All animals were euthanized between 8–11 am, by CO_2

635 asphyxiation followed by cervical dislocation.

636   For the "VCD cohort," animals were subjected to daily intraperitoneal injections of either vehicle

637 (safflower oil) or VCD (at a dosage of 160 mg/kg/day) for 15 consecutive days. All injections were

638 performed between 8:00 AM and 10:00 AM to minimize variability due to circadian effects. Lethality was

639 observed in male VCD-injected mice (4 out of 10 males in the experimental group within 16 days of the

640 start of injections). Intriguingly all lethality cases occurred in males that had to be single-housed prior to

641 injections due to fighting and wounds. Effects may have been aggravated due to lack of social

642 interactions and/or temperature regulation.

643

644 **Ovarian follicle counts**

645   Ovaries were fixed in Bouin's solution (Sigma, HT10132) for 24 hrs at room temperature and

646 transferred to 70 % ethanol for storage. Paraffin embedding, tissue sectioning and staining with

647 hematoxylin and eosin (H&E) were performed by the USC Norris Comprehensive Cancer Center

648 Translational Pathology Core Facility. H&E staining slides were imaged on the Keyence BZ-X All-in-One

649   Fluorescence Microscope platform. Three sections per ovary were used to count the number of primordial,

650   primary, secondary, and antral follicles and corpus luteum by three blinded observers. Median counts

651   across the 3 observers were used for data analysis. Statistical significance between groups was

652   assessed using the non-parametric Wilcoxon rank-sum test.

653

654   **Quantification of serum AMH, FSH and Inhibin A concentrations**

655   After euthanasia, blood was collected directly from the heart. Blood was allowed to clot at room

656   temperature for 1 hour, and serum was separated using MiniCollect® Serum Tube (Greiner, 450472),

657   then stored at -80 °C until further processing. Quantification of serum AMH (Rat and Mouse Anti-Müllerian

658   hormone (AMH) ELISA kit, Ansh Labs, AL-113), FSH (Millipore Pituitary Panel Multiplex kit, RPT86K and

659   Ultra-Sensitive Mouse & Rat FSH, UVA Ligand Core, in-house[109]), and Inhibin A (Inhibin A ELISA kit, Ansh

660   Labs, AL-161) levels were performed by the University of Virginia Center for Research in Reproduction

661   Ligand Assay and Analysis Core. Standard normalized values were provided by the core. Statistical

662   significance between groups was assessed using the non-parametric Wilcoxon rank-sum test.

663   For FSH quantification, two different kits were used during the course of the study, necessitating

664   correction of the dataset generated by the ultra-sensitive kit. To ensure consistency, we generated data

665   using both kits with the same serum samples and devised a polynomial model to fit the relationship

666   between the two datasets ($R^2 = 0.90$). The trained polynomial model was then applied to correct the data

667   generated by the Ultra-Sensitive Mouse & Rat FSH assay. This correction process was specifically

668   applied to the data for the FMT cohort. The raw data used to train the model has been uploaded to Github

669   (https://github.com/BenayounLaboratory/Ovarian_Aging_Microbiome).

670

671   **Ovarian health index calculation**

672   The ovarian health index was calculated by integrating two key components: (1) ovarian hormone

673   levels (AMH, FSH, and Inhibin A) and (2) follicle counts (combined counts of primordial, primary,

674   secondary, antral follicles, and corpus luteum). A 3-tier scoring system was used for each parameter.

675   Values beyond the young medians were assigned a score of 3, values between the young and

676   estropausal medians were assigned a score of 2, and values beyond the estropausal medians were

677   assigned a score of 1. The average hormone score (calculated from the AMH, FSH, and Inhibin A scores)

678   was then combined with the follicle score in a 1:1 ratio to generate the overall ovarian health index. This

679   combined score was scaled to a 0-100 range for ease of interpretation.

680   Statistical significance between groups was assessed using the Wilcoxon rank-sum test. Scripts

681   used to calculate the ovarian health index are available on the Benayoun lab GitHub at

682   https://github.com/BenayounLaboratory/Ovarian_Aging_Microbiome.

683

684   **DNA extraction for 16S rRNA amplicon and shotgun metagenomics analyses**

685   Fecal matter for 16S rRNA V3-V4 amplicon and shotgun metagenomics analyses were collected

686   aseptically post-euthanasia. Samples were immediately snap frozen in sterile tubes and stored at -80 °C.

687   DNA extraction was performed using QIAamp Fast DNA Stool Mini Kit (QIAGEN, 51604), following the

688   manufacturer's protocol.

689   V3-V4 region targeted amplification and sequencing were performed by Novogene Inc.

690   Amplification primers used were CCTAYGGGRBGCASCAG (FWD_341F) and

691   GGACTACNVGGGTWTCTAAT (REV_806R). Amplicon libraries were sequenced on the Illumina

692   NovaSeq 6000 machine. Shotgun metagenomics library preparation and sequencing were performed by

693   Novogene Inc. on the Illumina NovaSeq X machine. De-multiplexed FASTQ files were provided by

694   Novogene Inc. for data analysis.

695

696   **16S rRNA V3-V4 amplicon sequencing data analysis**

697   Data pre-processing, quality control and denoising

698   Data pre-processing, quality control and denoising were performed within the QIIME2[110] (v.

699   2023.7) platform. Each cohort was analyzed independently for memory and computing efficiency. FASTQ

700   files were imported to QIIME2 and denoised using DADA2[111]. Forward and reverse reads were truncated

701   at 226 bp and 224 bp, respectively, to obtain reads with quality scores above 25 and to ensure coverage

702   of the V3-V4 region (–p-trunc-len-f 226 –p-trunc-len-r 224).

703

704   Batch correction and principal component analysis of amplicon sequence variants

705   Previous studies have shown that effectiveness of batch correction tools are dataset-dependent[41].

706 For batch correction, a previously described benchmarking pipeline[41] was used to select the most

707 effective batch correction tool for our dataset. removeBatchEffect function from limma[112] (v. 3.50.1),

708 Combat from sva[113] (v. 3.42.0) and FAbatch from bapred[114] (v. 1.1) were benchmarked. Denoised feature

709 tables were imported to R using qiime2R[115] package (v. 0.99.6). Then, features with low counts (less than

710 0.01% of all the counts) were removed and filtered counts were centered log ratio (CLR)-transformed.

711 Based on principal component analysis results (method described below) and variance calculations,

712 FAbatch-corrected values were chosen for downstream analyses.

713

714 <u>Principal component analysis of amplicon sequence variants</u>

715   For principal component analysis (PCA), pca function from mixOmics[116] (v. 6.18.1) was used in R.

716 For female and male aging cohorts, FAbatch-corrected values were used for PCA. For VCD and FMT

717 cohorts, CLR-transformed values were used for PCA.

718

719 <u>Beta diversity and alpha diversity analyses</u>

720   For beta and alpha diversity analyses, phylogenetic trees were generated using the 'qiime

721 phylogeny align-to-tree-mafft-fasttree' command. Rooted phylogenetic trees were used to run the 'qiime

722 diversity core-metrics-phylogenetic' command for beta and alpha diversity analyses. Diversity analysis

723 results were imported to R using qiime2R[115] package (v. 0.99.6) for further plot generation and

724 significance tests. Bray-Curtis dissimilarity and Jaccard index principal coordinate analysis results were

725 used to generate beta diversity plots using ggplot2 (v. 3.4.2). To ensure comparability across different

726 cohorts, we normalized alpha diversity metrics within each cohort by dividing the values of each sample

727 by the median value of the corresponding metric (e.g., observed features, Shannon entropy) within that

728 cohort (e.g., CTL vs. VCD, FMT-YF vs. FMT-EF). This normalization allowed for combined cohort analysis.

729 The statistical significance of differences in alpha diversity between groups was determined using the

730 Wilcoxon rank-sum test.

731

732 <u>Differential abundance analysis of microbial genera</u>

733   Differential abundance analysis of microbial genera was performed using the ALDEx2[117] plugin

734   within the QIIME2[110] platform. Taxonomic classifier was generated using the SILVA[118] (132 release)

735   reference. Reference reads were extracted for CCTAYGGGRBGCASCAG (FWD_341F) and

736   GGACTACNVGGGTWTCTAAT (REV_806R) primer set and a classifier was trained using the 'qiime

737   feature-classifier extract-reads' and 'qiime feature-classifier fit-classifier-naive-bayes' commands,

738   respectively. Differential abundance analysis results and taxonomic classifier were imported to R using

739   qiime2R[115] for comparative analysis and plot generation. For female and male aging cohorts, differential

740   abundance analysis results for each cohort for each sex was combined based on the directionality of the

741   differential abundance measures, identifying features that consistently exhibited either an increase or

742   decrease across all cohorts for each sex. Using Fisher's method and Benjamini-Hochberg (BH) correction

743   method, p-values for each feature's differential abundance were combined and corrected, respectively.

744   Additionally, the mean of effect size across cohorts for each feature was used for analysis. For combined

745   female and aging cohorts, as well as VCD and FMT cohorts, features with an adjusted p-value < 0.05 and

746   an absolute effect size > 1 were identified as significantly different in abundance.

747

748   <u>Functional abundance prediction analysis</u>

749   Functional abundance prediction analysis was performed via the PICRUSt2[48] plugin within the

750   QIIME2[110] platform using default parameters. PICRUSt2 results were imported to R using qiime2R. For

751   differential abundance and statistical significance analyses, ALDEx2[117] was used (500 Monte Carlo

752   samples, Wilcoxon test for statistical testing, effect size calculation enabled, and the interquartile log ratio

753   (iqlr) for denominator calculation). To combine independent cohorts for female and male aging cohorts,

754   Fisher's method and Benjamini-Hochberg multiple testing correction were used to combine and correct

755   the p-values. Also, the mean values of effect sizes were calculated for each feature. Adjusted p-value less

756   than 0.05 and absolute effect size greater than 1 were used as criteria to filter functional pathways that

757   were significantly different in abundance.

758

759   **Fecal microbiota transplantation**

760   For fecal microbiota transplantation (FMT), animals underwent a 10-day course of

761   antibiotics/antimycotic (Ab/Am) treatment before transplantation. The regimen included ampicillin in

762   drinking water at 1 g/L (Sandoz, 00781), and oral gavage of amphotericin-B (Sigma, A9528) at 1

763   mg/kg/day, metronidazole (MFR VIONA PHARMACEUTICALS, 096988) at 100 mg/kg/day, vancomycin

764   (Alvogen, Vet-Rx-MW 090160) at 50 mg/kg/day, and neomycin (MFR VETONE, 510570) at 100

765   mg/kg/day. Starting on the third day after the end of the Ab/Am treatment, FMT was performed biweekly

766   for three weeks via oral gavage. Donor stool samples from more than 30 young or estropausal female

767   mice were pooled and prepared by suspending in saline (20 mg/ml), followed by vortexing and

768   centrifugation to obtain the supernatant. Each recipient animal received 100 µl of the fecal suspension.

769   Two independent cohorts were enrolled in the study, each using independently prepared pools of FMT

770   materials. Sample collection for subsequent processing and analysis occurred three days after the final

771   FMT session.

772

773   **Ovarian RNA-seq sample preparation**

774   Ovaries were harvested and immediately snap-frozen in sterile tubes, and then stored at -80 °C.

775   For RNA extraction, 600 µl of Trizol reagent (Ambion, 15596018) was added to the ovaries in Lysing

776   Matrix D tubes (MP, 6913500) and tissues were homogenized with a BeadBug 6 microtube homogenizer

777   (Benchmark Scientific, D1036) at 3,500 rpm for 30 seconds for 3 cycles in the cold room. Total RNA was

778   purified using the Direct-Zol RNA Miniprep (Zymo Research, R2052), following the manufacturer's

779   instructions. Integrity and quality of the purified total RNA were assessed on the 4200 TapeStation system

780   (Agilent, G2991A) using High Sensitivity RNA ScreenTapes (Agilent, 5067–5579) to determine the RNA

781   Integrity Number (RIN). Subsequently, mRNA-seq libraries were prepared and sequenced by Novogene

782   Inc. to obtain PE150 sequencing reads on the NovaSeq X Plus platform.

783

784   **FMT cohort ovarian RNA-seq data analysis**

785   <u>Data quality control and pre-processing</u>

786   Paired-end 150-bp reads were hard-trimmed to remove the first 6 bases and keep up to 100bp

787   using Fastx Trimmer[119] (v. 0.0.14) and processed using Trim Galore[120] using default parameters (v. 0.6.6).

788   Trimmed reads were mapped to the mm39 genome reference using STAR[121] (v. 2.7.9a). Read counts

789   were assigned to genes from the mm39 genome reference using subread[122] (v. 2.0.3). After assigning

790   read counts to genes, downstream processing was performed in R (v. 4.1.2). Low count genes (genes

791   with less than 1 count in at least 6 out of 14 RNA-seq libraries) were removed to improve memory and

792   speed efficiency of DESeq2[123], as recommended by the developers. We used surrogate variable analysis

793   to estimate and correct for unwanted experimental noise. R package sva[113] (v. 3.42.0) was used to

794   estimate surrogate variables and the removeBatchEffect function from limma[112] (v. 3.50.1) was used to

795   regress out the effects of surrogate variables and RNA integrity differences (RNA integrity number scores)

796   from raw read counts. To remove extreme values within the read counts, Winsorize function from

797   DescTools[124] (v. 0.99.50) was used to adjust the values above the 99.9th percentile to the values at the

798   99.9th percentile. DESeq2[123] package (v. 1.34.0) was used for downstream analyses of the RNA-seq data.

799   Genes with FDR < 5% were considered statistically significant. For the multidimensional scaling (MDS)

800   analysis, we calculated the distance between samples using the inverse of Spearman's rank correlation

801   coefficient (1-Rho) as the distance metric. These distances were then input into the cmdscale function in

802   R to compute the MDS coordinates.

803

804   Gene ontology analysis of RNA-seq dataset

805   We used the GSEA[55] approach through the phenoTest package. GO term annotations

806   (ENS.GO.BP, ENS.GO.MF, ENS.GO.CC and GTRD gene sets) were sourced from ENSEMBL (Ensembl

807   108) and Molecular Signature Database[125]. The t-statistic from DESeq2[123] was used to rank genes for

808   functional enrichment analysis. For readability, only the top ten most-significant pathways with negative

809   NES and top ten most-significant pathways with positive NES are shown in figures if more than that

810   passed the FDR < 5% significance threshold for GO terms and FDR < 10% for GTRD transcription

811   factor targets (see main text for explanation).

812

813   Deconvolution analysis of immune cell type proportion

814   To estimate any differences in the immune cell proportions between FMT-YF and FMT-EF groups

815   of the FMT cohort, single-cell RNA-seq reference-based deconvolution analyses were performed, using

816   CSCDRNA[126] and Granulator[127]. We downloaded the cell type annotated Seurat object of a publicly

817   available murine ovarian single-cell RNA-seq dataset (Open Science Framework dataset ID 924fz)[56]. In

818   the CSCDRNA analysis, the min.p parameter was set to 0.3 following the authors' recommendation[126], to

819   efficiently exclude the 30% least significant portion of marker genes for each cell type. This threshold was

820   chosen to increase efficiency and reduce noise. For Granulator, the qprogwc method[128] was used with

821   default parameters to estimate the immune cell proportions within the ovarian bulk RNA-seq dataset. Cell

822   proportions were tested for significance using a Wilcoxon test rank-sum test.

823

824   Pre-processing and filtering candidate transcription factor-bound genes

825   For potential transcription factors identified from GSEA[55] analysis against GTRD[61] transcription

826   factor targets, peak information was obtained for Cistrome.org. Peak annotation was performed using

827   HOMER[129] (v. 4.11.1). Genes with peaks annotated in TSS-promoter of protein coding genes were

828   considered direct target genes. Obtained gene lists were used to perform GSEA through the phenoTest (v.

829   1.42.0) package.

830

831   Pre-processing of public ovarian aging datasets

832   To evaluate the correlation between ovarian aging and FMT gene expression profiles of the

833   ovaries, we pre-processed and analyzed two publicly available ovarian aging datasets (2-3-month-old vs.

834   12-month-old female mice; BioProject dataset PRJNA100222 and GSA dataset CRA003645)[68,69]. For

835   each dataset, reads were pre-processed and aligned to the mm39 genome reference using the same

836   methods as for the FMT cohort ovarian RNA-seq dataset. Low count genes (genes with less than 1 count

837   in at least 4 out of 7 RNA-seq libraries) were removed. sva[113] (v. 3.42.0) was used to estimate surrogate

838   variables and the removeBatchEffect function from limma[112] (v. 3.50.1) was used to regress out the

839   effects of surrogate variables from raw read counts. DESeq2[123] package (v. 1.34.0) was used for

840   downstream analyses of the RNA-seq data. p-values for common genes between the two datasets were

841   combined and corrected using Fisher's method and BH correction method, respectively. FDR < 1e-5 was

842   used to catalog gene sets that are robustly up- or down-regulated with aging. Obtained gene lists were

843   used to perform GSEA through the phenoTest package.

844

Fertility assay

846   Fertility assay was performed as previously described[71]. FMT female mice were paired with 4-

847   month-old males at a 1:1 ratio 3 days after the last day of FMT administration until the birth of the first

848   litter. The number of pups in the first litter and the latency, defined as the duration between the pairing

849   date and the birth date of the first litter, were recorded and analyzed. Statistical difference in latency in the

850   1st litter after pairing was evaluated using a log-rank test. For pairs that did not produce pups within the

851   60-day timeframe, the latency was recorded as 60 days.

852

Whole genome shotgun metagenomics analysis

Data quality control and pre-processing

855   Paired-end 150-bp reads were pre-processed to remove sequencing adapters and low-quality

856   reads using Trimmomatic (v. 0.39)[130]. Trimmed reads were then mapped to the mouse reference genome

857   (mm10) using Bowtie2 (v. 2.4.4)[131] to remove host DNA contamination. Unmapped reads from paired-end

858   files were extracted using the --un-conc-gz option in Bowtie2 and used for subsequent analyses. Quality-

859   controlled and host-depleted reads were then processed for taxonomic classification and functional

860   profiling.

861

Taxonomic classification, abundance estimation and functional profiling

863   Kraken2 (v. 2.1.2)[132] was used for taxonomic classification of unmapped reads. Reads were

864   classified against a pre-built standard Kraken2 database containing bacterial, archaeal, and viral

865   sequences. Classification outputs were summarized using Bracken (v. 2.7)[133] to refine species- and

866   genus-level abundance estimates. Bracken was configured with a read length of 150 bp and default

867   settings for abundance profiling. Functional profiling was conducted using HUMAnN3 (v. 3.0)[74]. Reads

868   were processed in paired-end mode. Pathway and gene family tables were further merged and

869   normalized using human_join_tables and human_renorm_table functions.

870

Alpha and beta diversity and differential abundance analysis

872   Raw taxonomic abundance tables from Bracken (v. 2.7)[133] were loaded in R and filtered to

873   remove low-abundance and low-prevalence features. Features with total counts <20, relative abundance

874   <0.01%, or prevalence in fewer than 10% of samples were excluded. Samples with high host DNA

875   alignment rates (i.e., MHK271) were also removed. Centered log-ratio (CLR) transformation of filtered

876   relative abundances was performed. Shannon entropy was calculated using the mia[134] (v. 1.12.0) R

877   package. CLR-transformed relative abundance data were analyzed using mixOmics[116] (v. 6.28.0) and

878   Bray-Curtis and Jaccard distance matrices were calculated using vegan (v. 2.6-8). Differential abundance

879   of species was assessed using ALDEx2[117] (v. 1.36.0). Features with adjusted p-values of <0.05 were

880   deemed significant.

881

882   Functional pathway analysis

883   Relative abundance data for microbial pathways from HUMAnN3[74] (v. 3.9) was loaded in R and

884   low-abundance pathways with total abundance below 0.001 across all samples were removed.

885   Differential abundance of microbial pathways between groups was analyzed using ANCOMBC2[135] (v.

886   2.0.0). Significant pathways with Q-values below 0.1 were identified, and log2-fold change values were

887   used to assess the direction and magnitude of differential abundance.

888

889   **Stool menaquinone concentration quantification**

890   Frozen stool samples were shipped to the Vitamin K Laboratory at the Tufts University Human

891   Nutrition Research Center on Aging (HNRCA), where stool menaquinones concentrations were measured

892   using the LC/MS, as detailed elsewhere[136].

893

894   **Serum untargeted metabolomics**

895   Sample processing

896   Serum samples were stored at -80°C until they were thawed on ice for analysis. Metabolites were

897   extracted by adding 80 µL of ice-cold methanol to 20 µL of serum, followed by incubation on ice with

898   vortexing every 5 minutes. The samples were then centrifuged at 21,300 × g for 10 minutes at 4°C.

899

900   Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

901   Untargeted metabolomics was performed at the USC Alfred E. Mann Multi-Omics Mass

902   Spectrometry Core. A volume of 10 µL from each sample was injected onto a SeQuant ZIC-pHILIC

903   Column (5 µm, 2.1 mm × 150 mm; Millapore Sigma) using a Vanquish Flex UHPLC System (Thermo

904   Fisher Scientific). Chromatography was conducted at a flow rate of 100 µL/min with an optimized gradient

905   of solvent A (water/20 mM ammonium carbonate, pH 9.8) and solvent B (acetonitrile) as follows: 0 min/97%

906   B, 1 min/97% B, 28 min/75% B, 39 min/50% B, 44 min/20% B, 45 min/20% B, 45 min/97% B, 60 min/97%

907   B. The chromatography system was coupled to a heated electrospray ionization (HESI) source and a

908   hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific). Mass spectra were

909   acquired in Full MS/data-dependent MS2 mode, alternating between positive and negative ion polarities.

910   Source parameters were set as follows: a source voltage of 3.5 kV, source temperature of 275°C,

911   sheath gas of 45, auxiliary gas of 10, sweep gas of 2, and capillary temperature of 320°C. Full scans

912   were performed across an m/z range of 70 - 1050 with the following settings: an automated gain control

913   (AGC) target of $5 \times 10^{\square}$, a maximum injection time of 100 ms, and a resolution of 70,000 at m/z 200. Up

914   to five consecutive MS/MS scans were acquired per full scan with the following parameters: quadrupole

915   isolation of charge states 1–7, isolation window of 0.6 m/z, normalized collision energies of 20, 30, and 40,

916   dynamic exclusion of 30 s, AGC target of $1 \times 10^{\square}$, maximum injection time of 100 ms, and resolution of

917   17,500 at m/z 200.

918

919   Data analysis

920   Raw data were processed using Compound Discoverer (Version 3.3 SP3, Thermo Fisher

921   Scientific) for metabolite identification, retention time alignment, and unknown compound detection across

922   all samples. Metabolites were identified by matching exact masses (delta ppm of >10 or <-10) and

923   fragmentation spectra to the mzCloud database (ddMS2; 'mzCloud Best Match' > 50). For quality control,

924   only ions with a peak rating > 4 in at least six samples within a group were retained. VSN normalized

925   filtered metabolic matrix via normalizeVSN function of limma[112] (v. 3.50.1) was used for PCA analysis. The

926   filtered metabolic matrix was then scaled, and Linear Discriminant Analysis (LDA) was performed via the

927   MASS (v. 7.3-60.2) R package. Metabolites with a consistent direction of $\log_2$ fold change ($\log_2$FC)

928   between two batches were subsequently retained for abundance analysis.

929

Causal mediation analysis

931   Causal mediation analysis to identify specific features and amplicon sequence variants (ASVs)

932   from WGS and 16S rRNA amplicon sequencing datasets from the FMT cohort acting as mediators

933   between the treatment (FMT-YF vs. FMT-EF) and outcome (changes in the ovarian transcriptome profiles)

934   was performed using mediation package[137] (v. 4.5.0) in R. Feature table, taxonomic reference and

935   differential abundance analysis results generated from QIIME2[110] were imported to R using qiime2R[115].

936   ASVs identified as significant in differential abundance analyses were tested. CLR-transformed ASV

937   counts were used for the mediation analyses. For the outcome variable, the first dimension from the

938   multidimensional scaling (MDS) analysis of the FMT ovarian mRNAseq dataset was used. Linear models

939   were constructed for both the mediator and the outcome, incorporating the treatment as an independent

940   variable in each. The mediate function from the mediation package was then employed to estimate the

941   Average Causal Mediation Effects (ACME), Average Direct Effects (ADE), and Total Effects.

942

Quantification and statistical analysis

944   All statistical analysis was performed using the R software, version 4.2.4. For all boxplots, the

945   data is shown with the median, the 25th and 75th percentile of the data, and the whiskers represent 1.5 ∗

946   the inter-quartile range (IQR). Individual datapoints are always overlayed for transparency and rigor.

947   Specific statistical tests used, the number of biological replicates and how many animals they are

948   indicated in the corresponding figure legends and associated method section.

949

Data availability

951   The raw FASTQ files have been deposited to the Sequence Read Archive under accession

952   PRJNA1076509. Raw microscopy pictures of ovarian hematoxylin and eosin staining are available on

953   Figshare   (Figshare   DOIs: 10.6084/m9.figshare.25749588,   10.6084/m9.figshare.25749594,

954   10.6084/m9.figshare.25749591, 10.6084/m9.figshare.28462274). Processed serum metabolomics data

955   can be found in Extended Data Table 4. Raw data can be found on Metabolomics Workbench, project

956   ST003750. All R code was run using R 4.1.2. Any additional information required to reanalyze the data

957   reported in this work paper is available from the corresponding author upon request.

958

959   **Code availability**

960   All scripts used to analyze the datasets are available on the Benayoun lab GitHub at

961   https://github.com/BenayounLaboratory/Ovarian_Aging_Microbiome.

Figure legends

Fig 1. Characterization of ovarian health state and fecal microbial profiles of young and estropausal female mice. (A) Schematic diagram of the experimental setup of female aging cohorts. Resources from Flaticon.com were used. (B) Boxplot of combined follicle count of young and estropausal mice. (C) Boxplot of serum concentrations of AMH. (D) Boxplot of ovarian health index of young and estropausal mice. (E) Principal component analysis result of CLR-transformed and batch-corrected ASV counts from young and estropausal female mice. Animals from four independent cohorts (C1-C4), n=19-20 per group (variation due to animal death prior to experiment; n=20 for young females; n=19 for estropausal females). (F) Boxplots of Observed features and Shannon entropy indices of young and estropausal female mice. Medians from young females for each cohort were used to normalize indices per cohort. (G) Differential abundance analysis results of microbial genera of young vs. estropausal female mice using ALDEx2. (H) Functional abundance prediction analysis of young vs. estropausal female mice using PICRUSt2. Significance in nonparametric two-sided Wilcoxon rank-sum tests are reported for (B) and (D). YF: Young female; EF: Estropausal female. C: Cohort.

Fig 2. Bulk RNA-seq analysis of ovaries from young and estropausal female mice fecal microbiota transplantation recipient mice. (A) Schematic diagram of the experimental setup of fecal microbiota transplantation (FMT) cohort. Fecal microbiota from either young (4 months old) or estropausal (20 months old) female mice were transplanted into young (4 months old) female recipient mice. FMT recipient groups are referred to as FMT-YF (receiving young donor microbiota) and FMT-EF (receiving estropausal donor microbiota). (B) Multidimensional scaling (MDS) analysis result of RNA expression between young and estropausal female mice FMT recipient mice (FMT-YF and FMT-EF, respectively). (C-F) Top GSEA enriched terms from (C) Gene Ontology Biological Process (GO BP), (D) Gene Ontology Molecular Function (GO MF), (E) Gene Ontology Cellular Component (GO CC) and (F) Gene Transcription Regulation Database (GTRD – TF targets). Only up to top ten most-upregulated and top ten most-downregulated gene sets are plotted for readability.

990   **Fig 3. Analysis of public ovarian aging bulk RNA-seq datasets.** (A) Schematic diagram of the analysis

991   setup of public ovarian aging bulk RNA-seq datasets, CRA003645 and PRJNA1002222, from young (2-3-

992   months) and estropausal (12-months) female mice. (B-C) GSEA enrichment score plots of (B) genes that

993   are up-regulated with ovarian aging ("UP with ovarian aging", left panel) and down-regulated with ovarian

994   aging ("DOWN with ovarian aging, right panel), and (C) SenMayo gene set. In both (B) and (C), the black

995   vertical lines indicate the positions of the genes from the gene set within the ranked list of genes. The

996   curve represents the running enrichment score (ES), with the peak indicating the point of maximum

997   enrichment for the gene set. The color scale at the bottom of the plot reflects the ranking metric, where

998   red represents upregulated genes and blue represents downregulated genes. The p-value shown is

999   calculated based on GSEA and adjusted for multiple testing using the Benjamini-Hochberg method. DGE:

1000   Differential gene expression. (D) Boxplots of DESeq2 normalized $\log_2$ counts of *Cd38*, *Cdkn1a* and *Il1a*.

1001

1002   **Fig 4. Evaluation of fertility and ovarian health state of fecal microbiota transplantation recipient**

1003   **mice.** (A) Schematic diagram of the experimental setup. (B) Boxplot of ovarian health index of FMT-YF

1004   and FMT-EF mice. Ovarian health index was calculated for two independent cohorts (n=5 or 7 per group

1005   and 10 or 14 per cohort). (C) Boxplot of pup counts of first litters of young and estropausal female mice

1006   fecal microbiota transplantation recipient mice (FMT-YF and FMT-EF, respectively). Pup counts were

1007   measured from two independent cohorts (n=8 or 10 per group and 16 or 20 per cohort). (D) Reproductive

1008   success rate analysis result shown as percentages of nulliparous between FMT-YF and FMT-EF.

1009   Significance in nonparametric two-sided Wilcoxon rank-sum tests are reported for (B) and (C), and

1010   logrank test for (D).

1011

1012   **Fig 5. Characterization of fecal microbial profiles of fecal microbiota transplantation recipient mice.**

1013   (A) Schematic diagram of the experimental setup. (B) Principal component analysis result of CLR-

1014   transformed and batch-corrected counts from young and estropausal female mice fecal microbiota

1015   transplantation recipient mice (FMT-YF and FMT-EF, respectively). (C) Principal coordinate analysis

1016   results of Bray-Curtis dissimilarity (left panel) and Jaccard (right panel) indices. (D) Boxplots of Observed

1017   features and Shannon entropy indices of FMT-YF and FMT-EF mice. (E) Volcano plot of differential

1018   abundance analysis results of microbial species of FMT-YF vs. FMT-EF mice using ALDEx2. (F) Bubble

1019   plot of top 10 up- and down-regulated microbial species in FMT-EF. (G) Functional abundance analysis of

1020   FMT-YF vs. FMT-EF mice using HUMAnN3 (Q-value < 0.1). Significance in nonparametric two-sided

1021   Wilcoxon rank-sum tests are reported for (D).

1022

1023   **Fig 6. Characterization of serum metabolic profiles of fecal microbiota transplantation recipient**

1024   **mice.** (A) Schematic diagram of the experimental setup. (B) PCA plots of identified serum metabolites

1025   from two independent FMT cohorts. (C) LDA2 projection scatter plot displaying LDA1 scores for the FMT-

1026   YF and FMT-EF groups. (D) Top 15 metabolites contributing to LDA-based separation, ranked by their

1027   positive and negative contributions to the LDA axis. (E) Boxplots of 3,4-dihydroxyphenylpropionic acid

1028   (3,4-DHPPA) and lactamide serum levels between FMT-YF and FMT-EF groups. Significance in

1029   nonparametric two-sided Wilcoxon rank-sum tests are reported for (E).

1030   **Fig 7. Causal mediation analysis of microbial species and ovarian transcriptome changes in fecal**

1031   **microbiota transplantation recipient mice.** (A) Graphical representation of causal mediation analysis.

1032   (B) Causal mediation analysis results of whole genome shotgun metagenomics counts that showed

1033   significant mediation effects on changes in the ovarian transcriptome in fecal microbiota transplantation

1034   recipient mice. Nonparametric bootstrap confidence intervals with the percentile method are reported. (C)

1035   Heatmap of Spearman correlation analysis between CLR-transformed WGS count data and serum

1036   hormone quantification data. #: p-value < 0.1; *: p-value < 0.05. (D) Summary diagram. The estropausal

1037   female fecal microbiota exhibits increased levels of commensal microbes and metabolites through

1038   compensatory mechanisms. As a result, fecal transplantation of estropausal microbiota enhances ovarian

1039   health measures. Potential mechanisms, including NAD and vitamin K synthesis pathways, should be

1040   further examined. Generic Diagramming Platform (GDP) was used to generate the summary diagram[138].

1041   ACME: Average Causal Mediated Effect; ADE: Average Direct Effect.

1042 **Extended Data Fig. legend**

1043

1044 **Extended Data Fig. 1. Characterization of ovarian health state of young and estropausal female**

1045 **mice.** (A) Schematic diagram of ovarian health index calculation. (B) Representative images of ovarian

1046 hematoxylin-eosin stained sections. Scale bar: 50 μm. (C) Boxplots of ovarian follicle counts per section.

1047 The schematic diagrams of the follicles were created using the Generic Diagramming Platform (GDP)[138].

1048 (D) Boxplots of serum concentrations of FSH and Inhibin A. Significance in nonparametric two-sided

1049 Wilcoxon rank-sum tests are reported for (C) and (D). YF: Young female; EF: Estropausal female.

1050

1051 **Extended Data Fig. 2. Characterization of fecal microbial profiles of young and estropausal female**

1052 **mice.** (A) Principal coordinate analysis results of Bray-Curtis dissimilarity (top panel) and Jaccard (bottom

1053 panel) indices. (B) Boxplots of base abundance of differentially abundant microbial genera. YF: Young

1054 female; EF: Estropausal female.

1055

1056 **Extended Data Fig. 3. Characterization of fecal microbial profiles of young and estropausal female**

1057 **mice.** Boxplots of base abundance of differentially abundant microbial genera. YF: Young female; EF:

1058 Estropausal female.

1059

1060 **Extended Data Fig. 4. Characterization of ovarian health state of VCD cohort mice.** (A) Schematic

1061 diagram of the experimental setup of VCD cohort. (B) Representative images of ovarian hematoxylin-

1062 eosin stained sections. Scale bar: 50 μm. (C-F) Boxplots of (C) ovarian follicle counts per section, (D)

1063 combined follicle counts, (E) ovarian health index, and (E) serum concentrations of AMH, FSH and Inhibin

1064 A of control and VCD-injected mice. The schematic diagrams of the follicles were created using the

1065 Generic Diagramming Platform (GDP)[138]. Significance in nonparametric two-sided Wilcoxon rank-sum

1066 tests are reported for (C-F).

1067

1068 **Extended Data Fig. 5. Characterization of fecal microbial profiles of VCD cohort mice.** (A) Principal

1069 component analysis result of CLR-transformed and batch-corrected ASV counts from control and VCD-

1070 injected mice. (B) Principal coordinate analysis results of Bray-Curtis dissimilarity (top panel) and Jaccard

1071 (bottom panel) indices. (C, D) Boxplots of normalized observed features and Shannon entropy indices of

1072 (C) females, and (D) males. Median from control-injected mice was used to normalize indices. (E)

1073 Differential abundance analysis results of microbial genera of control vs. VCD-injected female mice using

1074 ALDEx2. (F) Functional abundance prediction analysis of control vs. VCD-injected female mice using

1075 PICRUSt2. Significance in nonparametric two-sided Wilcoxon rank-sum tests are reported for (C,D).

1076

1077 **Extended Data Fig. 6. Differential abundance analysis of 16S amplicon sequencing data of VCD**

1078 **cohort female mice.** Boxplots of base abundance of differentially abundant microbial genera between

1079 control and VCD-injected female mice.

1080

1081 **Extended Data Fig. 7. Characterization of fecal microbial profiles of young and old male mice.**

1082 (A) Schematic diagram of the experimental setup of male aging cohorts. (B) Principal component analysis

1083 result of CLR-transformed and batch-corrected ASV counts from young and old male mice. Animals from

1084 three independent cohorts, n=14 per group (variation due to animal death or abnormal health issues prior

1085 to experiment). (C) Principal coordinate analysis results of Bray-Curtis dissimilarity (top panel) and

1086 Jaccard (bottom panel) indices. (D) Boxplots of observed features and Shannon entropy indices of young

1087 and old male mice. Medians from young males for each cohort were used to normalize indices per cohort.

1088 (E) Differential abundance analysis results of microbial genera of young and old male mice using ALDEx2.

1089 (F) Functional abundance prediction analysis of young vs. old male mice using PICRUSt2. Significance in

1090 nonparametric two-sided Wilcoxon rank-sum tests are reported for (D). YM: Young male; OM: Old male.

1091

1092 **Extended Data Fig. 8. Differential abundance analysis of 16S amplicon sequencing data of young**

1093 **and old male mice.** Boxplots of base abundance of differentially abundant microbial genera between

1094 young and old male mice.

1095

1096 **Extended Data Fig. 9. Bulk RNA-seq analysis of ovaries from young and estropausal female mice**

1097 **fecal microbiota transplantation recipient mice.** (A) Heat map of significant (FDR < 5%) genes

differentially expressed in FMT-YF vs. FMT-EF mice. (B) Dot plots of immune cell proportions in percentages between young and estropausal fecal microbiota transplantation recipient mice (FMT-YF and FMT-EF, respectively) predicted by deconvolution analyses using CSCDRNA (left panel) and Granulator (right panel). (C) GSEA enrichment score plots Ncoa1-peak (left panel) and Usp7-peak (right panel) genes. (D) Bar plots of normalized $\log_2$ counts of *Ncoa1* and *Usp7* expression.

Extended Data Fig. 10. Characterization of ovarian health state of FMT cohort mice. (A) Representative images of ovarian hematoxylin-eosin stained sections. Scale bar: 50 μm. (B-E) Boxplots of (B) combined ovarian follicle counts, (C) ovarian follicle counts per section, (D) serum concentrations of AMH, FSH (Multiplex assay), FSH (Ultra-sensitive assay), FSH combined and INHBA, and (E) weight percentage difference between day 0 and 16 of FMT regimen. The schematic diagrams of the follicles were created using the Generic Diagramming Platform (GDP)[138].

Extended Data Fig. 11. Characterization of fecal microbial profiles of FMT cohort mice. (A) Principal component analysis result of CLR-transformed and batch-corrected counts from young and estropausal female mice fecal microbiota transplantation recipient mice (FMT-YF and FMT-EF, respectively). (B) Principal coordinate analysis results of Bray-Curtis dissimilarity (left panel) and Jaccard (right panel) indices. (C) Boxplots of observed features and Shannon entropy indices of FMT-YF and FMT-EF mice. (D) Differential abundance analysis results of microbial species of FMT-YF vs. FMT-EF mice using ALDEx2. (E) Venn diagram of overlapping microbial genera between genera that increase in abundance with female aging ("UP in EF") and increase in abundance in FMT-EF recipient mice ("UP in FMT-EF"). (F) Functional abundance prediction analysis of FMT-YF vs. FMT-EF mice using PICRUSt2. Significance in nonparametric two-sided Wilcoxon rank-sum tests are reported for (C). YF: Young female; EF: Estropausal female.

Extended Data Fig. 12. Differential abundance analysis of 16S amplicon sequencing data of FMT cohort mice. Boxplots of base abundance of differentially abundant microbial genera between FMT-YF and FMT-EF mice.

1126

1127 **Extended Data Fig. 13. Differential abundance analysis of WGS data of FMT cohort mice.** Boxplots

1128 of base abundance of differentially abundant microbial species between FMT-YF and FMT-EF mice.

1129

1130 **Extended Data Fig. 14. Stool vitamin K quantification of FMT cohort mice.** (A) Stool concentrations

1131 of combined vitamin K (MK6-MK13) of FMT-YF and FMT-EF. (B) Fold change in stool concentrations of

1132 vitamin K (MK6-MK13) of FMT-YF and FMT-EF.

1133

1134 **Extended Data Fig. 15. Serum untargeted metabolomics analysis of FMT cohort mice.** Boxplots of

1135 metabolite abundances of top 15 metabolites contributing to LDA separation between FMT-YF and FMT-

1136 EF.

1137

1138 **Extended Data Fig. 16. Causal mediation analysis of microbial species and ovarian transcriptome**

1139 **changes in fecal microbiota transplantation recipient mice.** (A) Graphical representation of causal

1140 mediation analysis. (B) Causal mediation analysis results of 16S amplicon ASVs that showed significant

1141 mediation effects on changes in the ovarian transcriptome in fecal microbiota transplantation recipient

1142 mice. Nonparametric bootstrap confidence intervals with the percentile method are reported. ACME:

1143 Average Causal Mediated Effect; ADE: Average Direct Effect.

1144 **Extended Data Table**

1145

1146 Extended Data Table 1. Weight measurements of FMT cohort.

1147 Extended Data Table 2. Estrous cycle staging data of FMT cohort.

1148 Extended Data Table 3. ALDEx2 differential abundance analysis result of metagenomics data of FMT

1149 cohort.

1150 Extended Data Table 4. MS2-filtered metabolite abundance matrix.

1151

References

1   Davis, S. R. *et al.* Menopause. *Nat Rev Dis Primers* **1**, 15004, doi:10.1038/nrdp.2015.4 (2015).

2   Broekmans, F. J., Soules, M. R. & Fauser, B. C. Ovarian aging: mechanisms and clinical consequences. *Endocr Rev* **30**, 465-493, doi:10.1210/er.2009-0006 (2009).

3   Faddy, M. J. & Gosden, R. G. A model conforming the decline in follicle numbers to the age of menopause in women. *Hum Reprod* **11**, 1484-1486, doi:10.1093/oxfordjournals.humrep.a019422 (1996).

4   Fu, C. *et al.* Association of reproductive factors with dementia: A systematic review and dose-response meta-analyses of observational studies. *EClinicalMedicine* **43**, 101236, doi:10.1016/j.eclinm.2021.101236 (2022).

5   Shuster, L. T., Rhodes, D. J., Gostout, B. S., Grossardt, B. R. & Rocca, W. A. Premature menopause or early menopause: long-term health consequences. *Maturitas* **65**, 161-166, doi:10.1016/j.maturitas.2009.08.003 (2010).

6   Muka, T. *et al.* Association of Age at Onset of Menopause and Time Since Onset of Menopause With Cardiovascular Outcomes, Intermediate Vascular Traits, and All-Cause Mortality: A Systematic Review and Meta-analysis. *JAMA Cardiol* **1**, 767-776, doi:10.1001/jamacardio.2016.2415 (2016).

7   Ossewaarde, M. E. *et al.* Age at menopause, cause-specific mortality and total life expectancy. *Epidemiology* **16**, 556-562, doi:10.1097/01.ede.0000165392.35273.d4 (2005).

8   Hong, J. S. *et al.* Age at menopause and cause-specific mortality in South Korean women: Kangwha Cohort Study. *Maturitas* **56**, 411-419, doi:10.1016/j.maturitas.2006.11.004 (2007).

9   Knobil, E. The neuroendocrine control of the menstrual cycle. *Recent Prog Horm Res* **36**, 53-88, doi:10.1016/b978-0-12-571136-4.50008-5 (1980).

10   Santoro, N., Roeca, C., Peters, B. A. & Neal-Perry, G. The Menopause Transition: Signs, Symptoms, and Management Options. *J Clin Endocrinol Metab* **106**, 1-15, doi:10.1210/clinem/dgaa764 (2021).

11   Randolph, J. F., Jr. *et al.* Change in follicle-stimulating hormone and estradiol across the menopausal transition: effect of age at the final menstrual period. *J Clin Endocrinol Metab* **96**, 746-754, doi:10.1210/jc.2010-1746 (2011).

12   Kelsey, T. W., Wright, P., Nelson, S. M., Anderson, R. A. & Wallace, W. H. A validated model of serum anti-mullerian hormone from conception to menopause. *PLoS One* **6**, e22024, doi:10.1371/journal.pone.0022024 (2011).

13   Weiss, G., Skurnick, J. H., Goldsmith, L. T., Santoro, N. F. & Park, S. J. Menopause and hypothalamic-pituitary sensitivity to estrogen. *JAMA* **292**, 2991-2996, doi:10.1001/jama.292.24.2991 (2004).

14   Xiong, J. *et al.* FSH blockade improves cognition in mice with Alzheimer's disease. *Nature* **603**, 470-476, doi:10.1038/s41586-022-04463-0 (2022).

15   Mao, L., Wang, L., Bennett, S., Xu, J. & Zou, J. Effects of follicle-stimulating hormone on fat metabolism and cognitive impairment in women during menopause. *Front Physiol* **13**, 1043237, doi:10.3389/fphys.2022.1043237 (2022).

1195   16   Xiong, J. *et al.* FSH and ApoE4 contribute to Alzheimer's disease-like pathogenesis via
1196   C/EBPbeta/delta-secretase in female mice. *Nat Commun* **14**, 6577, doi:10.1038/s41467-
1197   023-42282-7 (2023).
1198   17   Luckey, T. D. Introduction to intestinal microecology. *Am J Clin Nutr* **25**, 1292-1294,
1199   doi:10.1093/ajcn/25.12.1292 (1972).
1200   18   Savage, D. C. Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol* **31**, 107-
1201   133, doi:10.1146/annurev.mi.31.100177.000543 (1977).
1202   19   Sender, R., Fuchs, S. & Milo, R. Are We Really Vastly Outnumbered? Revisiting the Ratio
1203   of Bacterial to Host Cells in Humans. *Cell* **164**, 337-340, doi:10.1016/j.cell.2016.01.013
1204   (2016).
1205   20   Biagi, E. *et al.* Ageing and gut microbes: perspectives for health maintenance and
1206   longevity. *Pharmacol Res* **69**, 11-20, doi:10.1016/j.phrs.2012.10.005 (2013).
1207   21   Zapata, H. J. & Quagliarello, V. J. The microbiota and microbiome in aging: potential
1208   implications in health and age-related diseases. *J Am Geriatr Soc* **63**, 776-781,
1209   doi:10.1111/jgs.13310 (2015).
1210   22   Parker, A. *et al.* Fecal microbiota transfer between young and aged mice reverses
1211   hallmarks of the aging gut, eye, and brain. *Microbiome* **10**, 68, doi:10.1186/s40168-022-
1212   01243-w (2022).
1213   23   Kundu, P. *et al.* Neurogenesis and prolongevity signaling in young germ-free mice
1214   transplanted with the gut microbiota of old mice. *Sci Transl Med* **11**,
1215   doi:10.1126/scitranslmed.aau4760 (2019).
1216   24   Wu, J. *et al.* Association between premature ovarian insufficiency and gut microbiota.
1217   *BMC Pregnancy Childbirth* **21**, 418, doi:10.1186/s12884-021-03855-w (2021).
1218   25   Qi, X. *et al.* Gut microbiota-bile acid-interleukin-22 axis orchestrates polycystic ovary
1219   syndrome. *Nat Med* **25**, 1225-1233, doi:10.1038/s41591-019-0509-0 (2019).
1220   26   Weger, B. D. *et al.* The Mouse Microbiome Is Required for Sex-Specific Diurnal Rhythms
1221   of Gene Expression and Metabolism. *Cell Metab* **29**, 362-382 e368,
1222   doi:10.1016/j.cmet.2018.09.023 (2019).
1223   27   Lombardi, P., Goldin, B., Boutin, E. & Gorbach, S. L. Metabolism of androgens and
1224   estrogens by human fecal microorganisms. *J Steroid Biochem* **9**, 795-801,
1225   doi:10.1016/0022-4731(78)90203-0 (1978).
1226   28   Adlercreutz, H., Martin, F., Jarvenpaa, P. & Fotsis, T. Steroid absorption and
1227   enterohepatic recycling. *Contraception* **20**, 201-223, doi:10.1016/0010-7824(79)90094-5
1228   (1979).
1229   29   Eriksson, H. & Gustafsson, J. A. Steroids in germfree and conventional rats. Distribution
1230   and excretion of labelled pregnenolone and corticosterone in male and female rats. *Eur J
1231   Biochem* **15**, 132-139, doi:10.1111/j.1432-1033.1970.tb00987.x (1970).
1232   30   Finch, C. E. The menopause and aging, a comparative perspective. *J Steroid Biochem Mol
1233   Biol* **142**, 132-141, doi:10.1016/j.jsbmb.2013.03.010 (2014).
1234   31   Whitehead, J. C. *et al.* A clinical frailty index in aging mice: comparisons with frailty index
1235   data in humans. *J Gerontol A Biol Sci Med Sci* **69**, 621-632, doi:10.1093/gerona/glt136
1236   (2014).
1237   32   Wallace, W. H. & Kelsey, T. W. Human ovarian reserve from conception to the
1238   menopause. *PLoS One* **5**, e8772, doi:10.1371/journal.pone.0008772 (2010).

1239   33   Hirshfield, A. N. Development of follicles in the mammalian ovary. *Int Rev Cytol* **124**, 43-
1240   101, doi:10.1016/s0074-7696(08)61524-7 (1991).
1241   34   Tehrani, F. R., Mansournia, M. A., Solaymani-Dodaran, M. & Azizi, F. Age-specific serum
1242   anti-Mullerian hormone levels: estimates from a large population-based sample.
1243   *Climacteric* **17**, 591-597, doi:10.3109/13697137.2014.912262 (2014).
1244   35   Broer, S. L., Broekmans, F. J., Laven, J. S. & Fauser, B. C. Anti-Mullerian hormone: ovarian
1245   reserve testing and its potential clinical implications. *Hum Reprod Update* **20**, 688-701,
1246   doi:10.1093/humupd/dmu020 (2014).
1247   36   Felicio, L. S., Nelson, J. F. & Finch, C. E. Prolongation and cessation of estrous cycles in
1248   aging C57BL/6J mice are differentially regulated events. *Biol Reprod* **34**, 849-858,
1249   doi:10.1095/biolreprod34.5.849 (1986).
1250   37   Johnson, J. S. *et al.* Evaluation of 16S rRNA gene sequencing for species and strain-level
1251   microbiome analysis. *Nat Commun* **10**, 5029, doi:10.1038/s41467-019-13036-1 (2019).
1252   38   Gupta, S. *et al.* Amplicon sequencing provides more accurate microbiome information in
1253   healthy children compared to culturing. *Commun Biol* **2**, 291, doi:10.1038/s42003-019-
1254   0540-1 (2019).
1255   39   Knight, R. *et al.* Best practices for analysing microbiomes. *Nat Rev Microbiol* **16**, 410-422,
1256   doi:10.1038/s41579-018-0029-9 (2018).
1257   40   Willis, A. D. Rarefaction, Alpha Diversity, and Statistics. *Front Microbiol* **10**, 2407,
1258   doi:10.3389/fmicb.2019.02407 (2019).
1259   41   Wang, Y. & LeCao, K. A. Managing batch effects in microbiome data. *Brief Bioinform* **21**,
1260   1954-1970, doi:10.1093/bib/bbz105 (2020).
1261   42   Gamez-Macias, P. E. *et al.* Intestinal Permeability, Gut Inflammation, and Gut Immune
1262   System Response Are Linked to Aging-Related Changes in Gut Microbiota Composition: A
1263   Study in Female Mice. *J Gerontol A Biol Sci Med Sci* **79**, doi:10.1093/gerona/glae045
1264   (2024).
1265   43   de la Cuesta-Zuluaga, J. *et al.* Age- and Sex-Dependent Patterns of Gut Microbial
1266   Diversity in Human Adults. *mSystems* **4**, doi:10.1128/mSystems.00261-19 (2019).
1267   44   Ghosh, T. S., Shanahan, F. & O'Toole, P. W. Toward an improved definition of a healthy
1268   microbiome for healthy aging. *Nat Aging* **2**, 1054-1069, doi:10.1038/s43587-022-00306-
1269   9 (2022).
1270   45   Luo, M. *et al.* E. coli Nissle 1917 ameliorates mitochondrial injury of granulosa cells in
1271   polycystic ovary syndrome through promoting gut immune factor IL-22 via gut
1272   microbiota and microbial metabolism. *Front Immunol* **14**, 1137089,
1273   doi:10.3389/fimmu.2023.1137089 (2023).
1274   46   Biagi, E. *et al.* Gut Microbiota and Extreme Longevity. *Curr Biol* **26**, 1480-1485,
1275   doi:10.1016/j.cub.2016.04.016 (2016).
1276   47   Wang, N. *et al.* Enriched taxa were found among the gut microbiota of centenarians in
1277   East China. *PLoS One* **14**, e0222763, doi:10.1371/journal.pone.0222763 (2019).
1278   48   Douglas, G. M. *et al.* PICRUSt2 for prediction of metagenome functions. *Nat Biotechnol*
1279   **38**, 685-688, doi:10.1038/s41587-020-0548-6 (2020).
1280   49   Murali, R. *et al.* Deregulated Metabolic Pathways in Ovarian Cancer: Cause and
1281   Consequence. *Metabolites* **13**, doi:10.3390/metabo13040560 (2023).

1282   50   Ji, Z. *et al.* Deregulation of Lipid Metabolism: The Critical Factors in Ovarian Cancer. *Front*
1283   *Oncol* **10**, 593017, doi:10.3389/fonc.2020.593017 (2020).
1284   51   Borman, S. M., Christian, P. J., Sipes, I. G. & Hoyer, P. B. Ovotoxicity in female Fischer rats
1285   and B6 mice induced by low-dose exposure to three polycyclic aromatic hydrocarbons:
1286   comparison through calculation of an ovotoxic index. *Toxicol Appl Pharmacol* **167**, 191-
1287   198, doi:10.1006/taap.2000.9006 (2000).
1288   52   Van Kempen, T. A., Milner, T. A. & Waters, E. M. Accelerated ovarian failure: a novel,
1289   chemically induced animal model of menopause. *Brain Res* **1379**, 176-187,
1290   doi:10.1016/j.brainres.2010.12.064 (2011).
1291   53   Mayer, L. P., Devine, P. J., Dyer, C. A. & Hoyer, P. B. The follicle-deplete mouse ovary
1292   produces androgen. *Biol Reprod* **71**, 130-138, doi:10.1095/biolreprod.103.016113 (2004).
1293   54   Boehme, M. *et al.* Microbiota from young mice counteracts selective age-associated
1294   behavioral deficits. *Nat Aging* **1**, 666-676, doi:10.1038/s43587-021-00093-9 (2021).
1295   55   Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for
1296   interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* **102**, 15545-
1297   15550, doi:10.1073/pnas.0506580102 (2005).
1298   56   Morris, M. E. *et al.* A single-cell atlas of the cycling murine ovary. *Elife* **11**,
1299   doi:10.7554/eLife.77239 (2022).
1300   57   Jew, B. *et al.* Accurate estimation of cell composition in bulk expression through robust
1301   integration of single-cell information. *Nat Commun* **11**, 1971, doi:10.1038/s41467-020-
1302   15816-6 (2020).
1303   58   Newman, A. M. *et al.* Robust enumeration of cell subsets from tissue expression profiles.
1304   *Nat Methods* **12**, 453-457, doi:10.1038/nmeth.3337 (2015).
1305   59   Lovasco, L. A. *et al.* Accelerated ovarian aging in the absence of the transcription
1306   regulator TAF4B in mice. *Biol Reprod* **82**, 23-34, doi:10.1095/biolreprod.109.077495
1307   (2010).
1308   60   Baumann, C. *et al.* Acute irradiation induces a senescence-like chromatin structure in
1309   mammalian oocytes. *Commun Biol* **6**, 1258, doi:10.1038/s42003-023-05641-0 (2023).
1310   61   Yevshin, I., Sharipov, R., Valeev, T., Kel, A. & Kolpakov, F. GTRD: a database of
1311   transcription factor binding sites identified by ChIP-seq experiments. *Nucleic Acids Res*
1312   **45**, D61-D67, doi:10.1093/nar/gkw951 (2017).
1313   62   Zheng, R. *et al.* Cistrome Data Browser: expanded datasets and new tools for gene
1314   regulatory analysis. *Nucleic Acids Res* **47**, D729-D735, doi:10.1093/nar/gky1094 (2019).
1315   63   Mei, S. *et al.* Cistrome Data Browser: a data portal for ChIP-Seq and chromatin
1316   accessibility data in human and mouse. *Nucleic Acids Res* **45**, D658-D662,
1317   doi:10.1093/nar/gkw983 (2017).
1318   64   Huang, H. Y. *et al.* Association of polymorphisms for nuclear receptor coactivator 1 gene
1319   with egg production traits in the maternal line of Shaobo hens. *Br Poult Sci* **52**, 328-332,
1320   doi:10.1080/00071668.2011.577057 (2011).
1321   65   Wang, M. *et al.* The USP7 Inhibitor P5091 Induces Cell Death in Ovarian Cancers with
1322   Different P53 Status. *Cell Physiol Biochem* **43**, 1755-1766, doi:10.1159/000484062 (2017).
1323   66   Briley, S. M. *et al.* Reproductive age-associated fibrosis in the stroma of the mammalian
1324   ovary. *Reproduction* **152**, 245-260, doi:10.1530/REP-16-0129 (2016).

1325   67   Zhang, Z., Schlamp, F., Huang, L., Clark, H. & Brayboy, L. Inflammaging is associated with
1326   shifted macrophage ontogeny and polarization in the aging mouse ovary. *Reproduction*
1327   **159**, 325-337, doi:10.1530/REP-19-0330 (2020).
1328   68   Zhou, Z. *et al.* Temporal transcriptomic landscape of postnatal mouse ovaries reveals
1329   dynamic gene signatures associated with ovarian aging. *Hum Mol Genet* **30**, 1941-1954,
1330   doi:10.1093/hmg/ddab163 (2021).
1331   69   Yang, Q. *et al.* NADase CD38 is a key determinant of ovarian aging. *Nat Aging* **4**, 110-128,
1332   doi:10.1038/s43587-023-00532-9 (2024).
1333   70   Saul, D. *et al.* A new gene set identifies senescent cells and predicts senescence-
1334   associated pathways across tissues. *Nat Commun* **13**, 4827, doi:10.1038/s41467-022-
1335   32552-1 (2022).
1336   71   Mehalko, K. *et al.* Lack of accelerated ovarian aging in a follicle-stimulating hormone
1337   receptor haploinsufficiency model. *Transl Med Aging* **7**, 1-8,
1338   doi:10.1016/j.tma.2023.01.001 (2023).
1339   72   Wrzosek, L. *et al.* Transplantation of human microbiota into conventional mice durably
1340   reshapes the gut microbiota. *Sci Rep* **8**, 6854, doi:10.1038/s41598-018-25300-3 (2018).
1341   73   Gong, X., Gutala, R. & Jaiswal, A. K. Quinone oxidoreductases and vitamin K metabolism.
1342   *Vitam Horm* **78**, 85-101, doi:10.1016/S0083-6729(07)00005-2 (2008).
1343   74   Beghini, F. *et al.* Integrating taxonomic, functional, and strain-level profiling of diverse
1344   microbial communities with bioBakery 3. *Elife* **10**, doi:10.7554/eLife.65088 (2021).
1345   75   Dekkers, K. F. *et al.* An online atlas of human plasma metabolite signatures of gut
1346   microbiome composition. *Nat Commun* **13**, 5370, doi:10.1038/s41467-022-33050-0
1347   (2022).
1348   76   Pilley, S. E. *et al.* A metabolic atlas of mouse aging. *bioRxiv*, 2024.2005.2004.592445,
1349   doi:10.1101/2024.05.04.592445 (2024).
1350   77   Marquez Campos, E., Stehle, P. & Simon, M. C. Microbial Metabolites of Flavan-3-Ols and
1351   Their Biological Activity. *Nutrients* **11**, doi:10.3390/nu11102260 (2019).
1352   78   He, Y. *et al.* Lactic acid bacteria alleviate polycystic ovarian syndrome by regulating sex
1353   hormone related gut microbiota. *Food Funct* **11**, 5192-5204, doi:10.1039/c9fo02554e
1354   (2020).
1355   79   Dikalov, S., Panov, A. & Dikalova, A. Critical Role of Mitochondrial Fatty Acid Metabolism
1356   in Normal Cell Function and Pathological Conditions. *Int J Mol Sci* **25**,
1357   doi:10.3390/ijms25126498 (2024).
1358   80   Wang, Z. H. *et al.* Targeting mitochondria for ovarian aging: new insights into
1359   mechanisms and therapeutic potential. *Front Endocrinol (Lausanne)* **15**, 1417007,
1360   doi:10.3389/fendo.2024.1417007 (2024).
1361   81   Zhang, I. W. *et al.* Palmitoylcarnitine impairs immunity in decompensated cirrhosis. *JHEP*
1362   *Rep* **6**, 101187, doi:10.1016/j.jhepr.2024.101187 (2024).
1363   82   Xu, X. L. *et al.* Estrogen Biosynthesis and Signal Transduction in Ovarian Disease. *Front*
1364   *Endocrinol (Lausanne)* **13**, 827032, doi:10.3389/fendo.2022.827032 (2022).
1365   83   Stachenfeld, N. S. Hormonal changes during menopause and the impact on fluid
1366   regulation. *Reprod Sci* **21**, 555-561, doi:10.1177/1933719113518992 (2014).

84   Liu, T., Bowen, R. L., Wilson, A. C. & Atwood, C. S. Estropause, Sex Hormones and Metal Homeostasis in the Mouse Brain. *Front Neurol* **13**, 841822, doi:10.3389/fneur.2022.841822 (2022).

85   Schele, E. *et al.* The gut microbiota reduces leptin sensitivity and the expression of the obesity-suppressing neuropeptides proglucagon (Gcg) and brain-derived neurotrophic factor (Bdnf) in the central nervous system. *Endocrinology* **154**, 3643-3651, doi:10.1210/en.2012-2151 (2013).

86   Fan, K. C. *et al.* Altered gut microbiota in older adults with mild cognitive impairment: a case-control study. *Front Aging Neurosci* **15**, 1162057, doi:10.3389/fnagi.2023.1162057 (2023).

87   Ellis, J. L. *et al.* Dietary vitamin K is remodeled by gut microbiota and influences community composition. *Gut Microbes* **13**, 1-16, doi:10.1080/19490976.2021.1887721 (2021).

88   Tarkesh, F., Namavar Jahromi, B., Hejazi, N. & Tabatabaee, H. Beneficial health effects of Menaquinone-7 on body composition, glycemic indices, lipid profile, and endocrine markers in polycystic ovary syndrome patients. *Food Sci Nutr* **8**, 5612-5621, doi:10.1002/fsn3.1837 (2020).

89   Sibayama-Imazu, T. *et al.* Induction of apoptosis in PA-1 ovarian cancer cells by vitamin K2 is associated with an increase in the level of TR3/Nur77 and its accumulation in mitochondria and nuclei. *J Cancer Res Clin Oncol* **134**, 803-812, doi:10.1007/s00432-007-0349-z (2008).

90   Harshman, S. G. & Shea, M. K. The Role of Vitamin K in Chronic Aging Diseases: Inflammation, Cardiovascular Disease, and Osteoarthritis. *Curr Nutr Rep* **5**, 90-98, doi:10.1007/s13668-016-0162-x (2016).

91   Sampathkumar, N. K. *et al.* Widespread sex dimorphism in aging and age-related diseases. *Hum Genet* **139**, 333-356, doi:10.1007/s00439-019-02082-w (2020).

92   Podcasy, J. L. & Epperson, C. N. Considering sex and gender in Alzheimer disease and other dementias. *Dialogues Clin Neurosci* **18**, 437-446, doi:10.31887/DCNS.2016.18.4/cepperson (2016).

93   Vina, J. & Lloret, A. Why women have more Alzheimer's disease than men: gender and mitochondrial toxicity of amyloid-beta peptide. *J Alzheimers Dis* **20 Suppl 2**, S527-533, doi:10.3233/JAD-2010-100501 (2010).

94   Varghese, M. *et al.* Female adipose tissue has improved adaptability and metabolic health compared to males in aged obesity. *Aging (Albany NY)* **12**, 1725-1746, doi:10.18632/aging.102709 (2020).

95   Austad, S. N. & Fischer, K. E. Sex Differences in Lifespan. *Cell Metab* **23**, 1022-1033, doi:10.1016/j.cmet.2016.05.019 (2016).

96   Lemaitre, J. F. *et al.* Sex differences in adult lifespan and aging rates of mortality across wild mammals. *Proc Natl Acad Sci U S A* **117**, 8546-8553, doi:10.1073/pnas.1911999117 (2020).

97   Markle, J. G. *et al.* Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science* **339**, 1084-1088, doi:10.1126/science.1233521 (2013).

1410 98   Steegenga, W. T. *et al.* Sexually dimorphic characteristics of the small intestine and colon
1411   of prepubescent C57BL/6 mice. *Biol Sex Differ* **5**, 11, doi:10.1186/s13293-014-0011-9
1412   (2014).
1413 99   Org, E. *et al.* Sex differences and hormonal effects on gut microbiota composition in
1414   mice. *Gut Microbes* **7**, 313-322, doi:10.1080/19490976.2016.1203502 (2016).
1415 100   Vemuri, R. *et al.* The microgenderome revealed: sex differences in bidirectional
1416   interactions between the microbiota, hormones, immunity and disease susceptibility.
1417   *Semin Immunopathol* **41**, 265-275, doi:10.1007/s00281-018-0716-7 (2019).
1418 101   Kaliannan, K. *et al.* Estrogen-mediated gut microbiome alterations influence sexual
1419   dimorphism in metabolic syndrome in mice. *Microbiome* **6**, 205, doi:10.1186/s40168-
1420   018-0587-0 (2018).
1421 102   Hogan, K. A., Chini, C. C. S. & Chini, E. N. The Multi-faceted Ecto-enzyme CD38: Roles in
1422   Immunomodulation, Cancer, Aging, and Metabolic Diseases. *Front Immunol* **10**, 1187,
1423   doi:10.3389/fimmu.2019.01187 (2019).
1424 103   Camacho-Pereira, J. *et al.* CD38 Dictates Age-Related NAD Decline and Mitochondrial
1425   Dysfunction through an SIRT3-Dependent Mechanism. *Cell Metab* **23**, 1127-1139,
1426   doi:10.1016/j.cmet.2016.05.006 (2016).
1427 104   Gomes, A. P. *et al.* Declining NAD(+) induces a pseudohypoxic state disrupting nuclear-
1428   mitochondrial communication during aging. *Cell* **155**, 1624-1638,
1429   doi:10.1016/j.cell.2013.11.037 (2013).
1430 105   Chini, C. C. S. *et al.* CD38 ecto-enzyme in immune cells is induced during aging and
1431   regulates NAD(+) and NMN levels. *Nat Metab* **2**, 1284-1304, doi:10.1038/s42255-020-
1432   00298-z (2020).
1433 106   Covarrubias, A. J. *et al.* Senescent cells promote tissue NAD(+) decline during ageing via
1434   the activation of CD38(+) macrophages. *Nat Metab* **2**, 1265-1283, doi:10.1038/s42255-
1435   020-00305-3 (2020).
1436 107   Wright, L. E. *et al.* Comparison of skeletal effects of ovariectomy versus chemically
1437   induced ovarian failure in mice. *J Bone Miner Res* **23**, 1296-1303,
1438   doi:10.1359/jbmr.080309 (2008).
1439 108   National Toxicology, P. Toxicology and Carcinogenesis Studies of 4-Vinyl-1-cyclohexene
1440   Diepoxide (CAS No. 106-87-6) in F344/N Rats and B6C3F1 Mice (Dermal Studies). *Natl
1441   Toxicol Program Tech Rep Ser* **362**, 1-249 (1989).
1442 109   Ongaro, L. *et al.* Development of a Highly Sensitive ELISA for Measurement of FSH in
1443   Serum, Plasma, and Whole Blood in Mice. *Endocrinology* **162**,
1444   doi:10.1210/endocr/bqab014 (2021).
1445 110   Bolyen, E. *et al.* Reproducible, interactive, scalable and extensible microbiome data
1446   science using QIIME 2. *Nat Biotechnol* **37**, 852-857, doi:10.1038/s41587-019-0209-9
1447   (2019).
1448 111   Callahan, B. J. *et al.* DADA2: High-resolution sample inference from Illumina amplicon
1449   data. *Nat Methods* **13**, 581-583, doi:10.1038/nmeth.3869 (2016).
1450 112   Ritchie, M. E. *et al.* limma powers differential expression analyses for RNA-sequencing
1451   and microarray studies. *Nucleic Acids Res* **43**, e47, doi:10.1093/nar/gkv007 (2015).
1452 113   Leek, J. T. & Storey, J. D. Capturing heterogeneity in gene expression studies by surrogate
1453   variable analysis. *PLoS Genet* **3**, 1724-1735, doi:10.1371/journal.pgen.0030161 (2007).

114   Hornung, R., Boulesteix, A. L. & Causeur, D. Combining location-and-scale batch effect adjustment with data cleaning by latent factor adjustment. *BMC Bioinformatics* **17**, 27, doi:10.1186/s12859-015-0870-z (2016).

115   Bisanz, J. <https://github.com/jbisanz/qiime2R> (

116   Rohart, F., Gautier, B., Singh, A. & Le Cao, K. A. mixOmics: An R package for 'omics feature selection and multiple data integration. *PLoS Comput Biol* **13**, e1005752, doi:10.1371/journal.pcbi.1005752 (2017).

117   Fernandes, A. D. *et al.* Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. *Microbiome* **2**, 15, doi:10.1186/2049-2618-2-15 (2014).

118   Yilmaz, P. *et al.* The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. *Nucleic Acids Res* **42**, D643-648, doi:10.1093/nar/gkt1209 (2014).

119   Gordon, A. <http://hannonlab.cshl.edu/fastx_toolkit/> (

120   Krueger, F. <https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/> (

121   Dobin, A. *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15-21, doi:10.1093/bioinformatics/bts635 (2013).

122   Liao, Y., Smyth, G. K. & Shi, W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923-930, doi:10.1093/bioinformatics/btt656 (2014).

123   Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* **15**, 550, doi:10.1186/s13059-014-0550-8 (2014).

124   Signorell, A. <https://andrisignorell.github.io/DescTools/> (

125   Liberzon, A. *et al.* The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst* **1**, 417-425, doi:10.1016/j.cels.2015.12.004 (2015).

126   Karimnezhad, A. More accurate estimation of cell composition in bulk expression through robust integration of single-cell information. *Bioinform Adv* **2**, vbac049, doi:10.1093/bioadv/vbac049 (2022).

127   Pfister, S., Kuettel, V., Ferrero, E. <https://github.com/Novartis/granulator> (

128   Gong, T. & Szustakowski, J. D. DeconRNASeq: a statistical framework for deconvolution of heterogeneous tissue samples based on mRNA-Seq data. *Bioinformatics* **29**, 1083-1085, doi:10.1093/bioinformatics/btt090 (2013).

129   Heinz, S. *et al.* Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol Cell* **38**, 576-589, doi:10.1016/j.molcel.2010.05.004 (2010).

130   Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114-2120, doi:10.1093/bioinformatics/btu170 (2014).

131   Langmead, B., Wilks, C., Antonescu, V. & Charles, R. Scaling read aligners to hundreds of threads on general-purpose processors. *Bioinformatics* **35**, 421-432, doi:10.1093/bioinformatics/bty648 (2019).

132   Wood, D. E., Lu, J. & Langmead, B. Improved metagenomic analysis with Kraken 2. *Genome Biol* **20**, 257, doi:10.1186/s13059-019-1891-0 (2019).

1497   133   Lu, J. *et al.* Metagenome analysis using the Kraken software suite. *Nat Protoc* **17**, 2815-
1498   2839, doi:10.1038/s41596-022-00738-y (2022).
1499   134   mia: Microbiome analysis (2025).
1500   135   Lin, H. & Peddada, S. D. Multigroup analysis of compositions of microbiomes with
1501   covariate adjustments and repeated measures. *Nat Methods* **21**, 83-91,
1502   doi:10.1038/s41592-023-02092-7 (2024).
1503   136   Karl, J. P., Fu, X., Dolnikowski, G. G., Saltzman, E. & Booth, S. L. Quantification of
1504   phylloquinone and menaquinones in feces, serum, and food by high-performance liquid
1505   chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* **963**,
1506   128-133, doi:10.1016/j.jchromb.2014.05.056 (2014).
1507   137   Tingley, D., Yamamoto, T., Hirose, K., Keele, L. & Imai, K. mediation: R Package for Causal
1508   Mediation Analysis. *Journal of Statistical Software* **59**, 1 - 38, doi:10.18637/jss.v059.i05
1509   (2014).
1510   138   Jiang, S. *et al.* Generic Diagramming Platform (GDP): a comprehensive database of high-
1511   quality biomedical graphics. *Nucleic Acids Res* **53**, D1670-D1676,
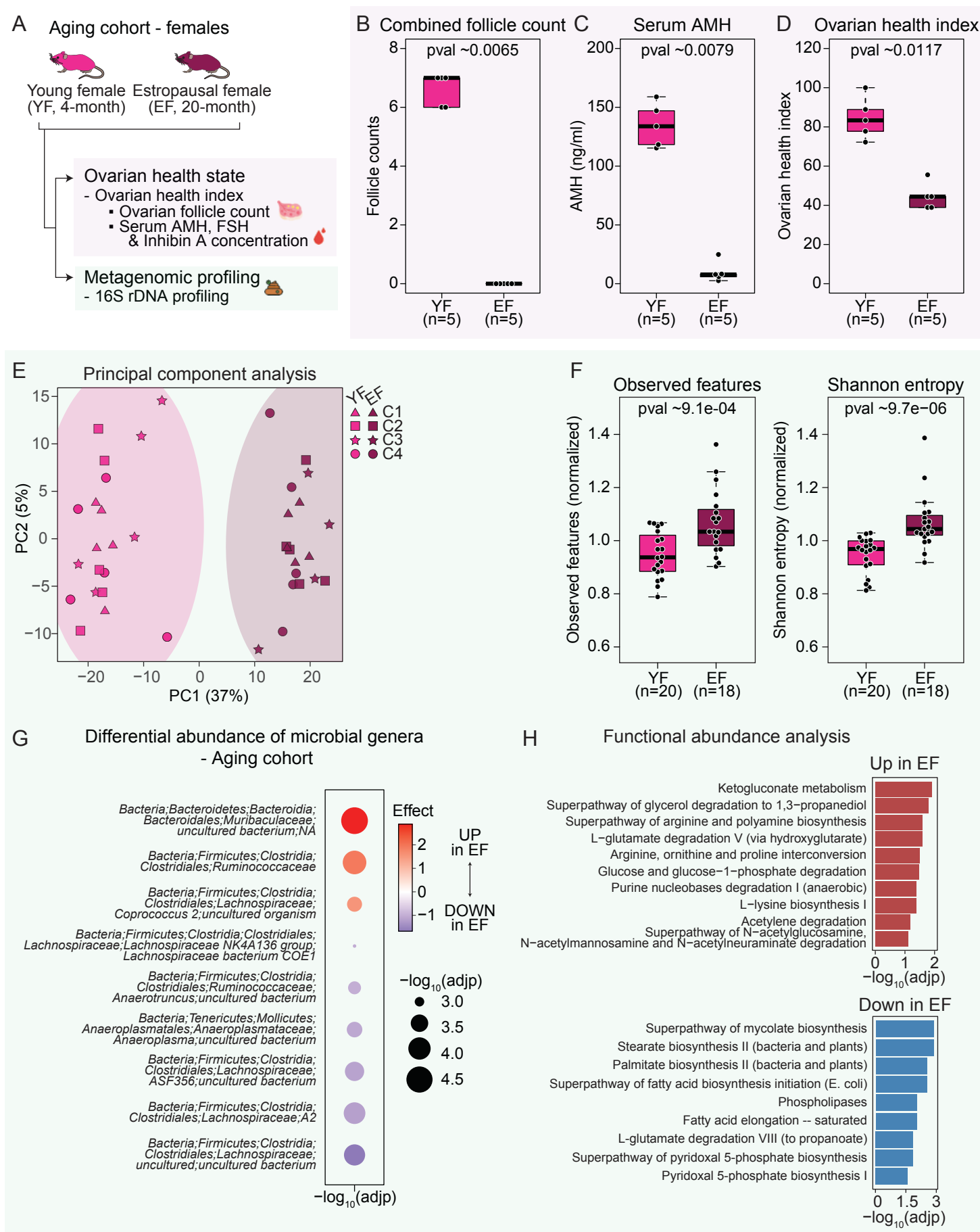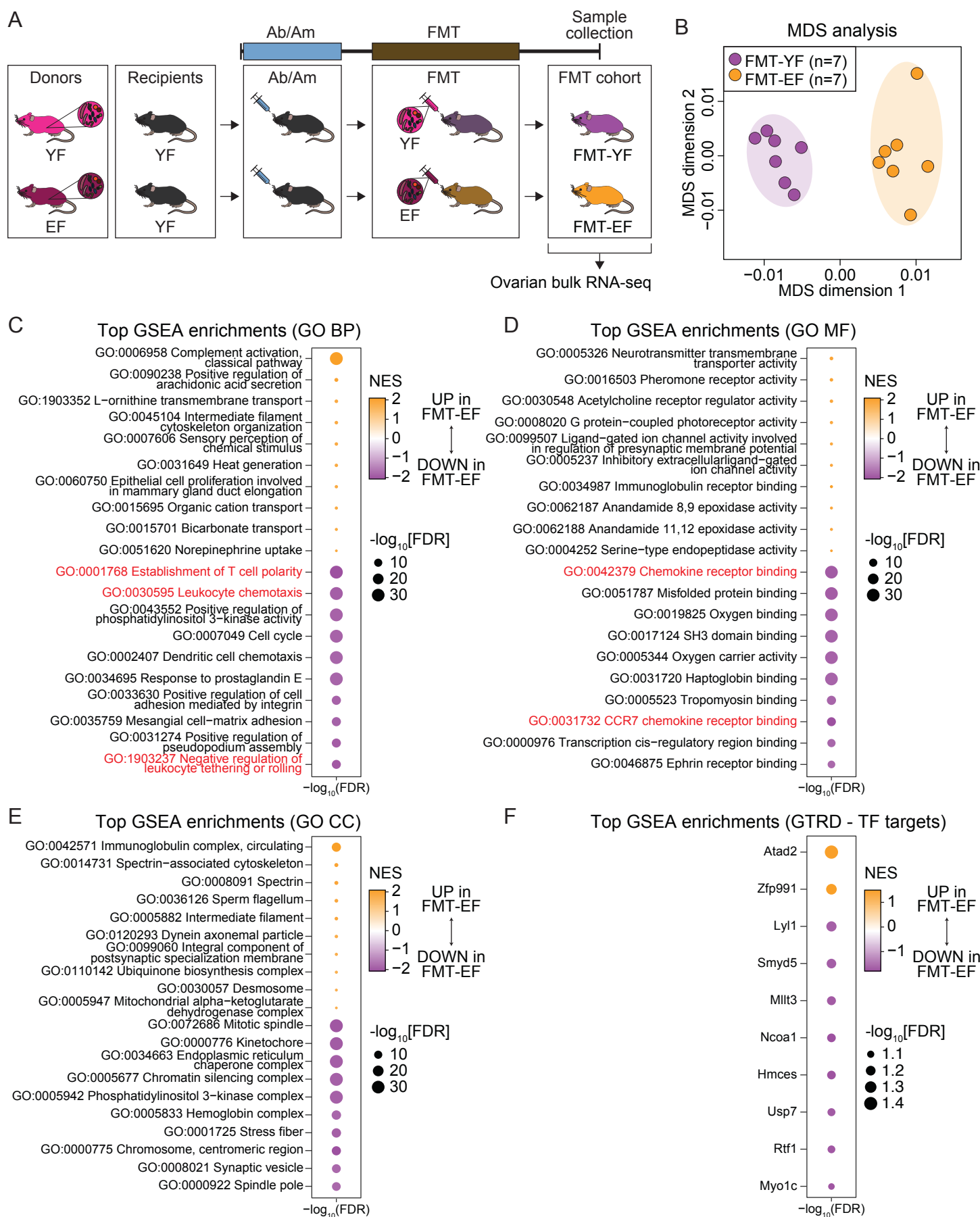1512   doi:10.1093/nar/gkae973 (2025).
1513
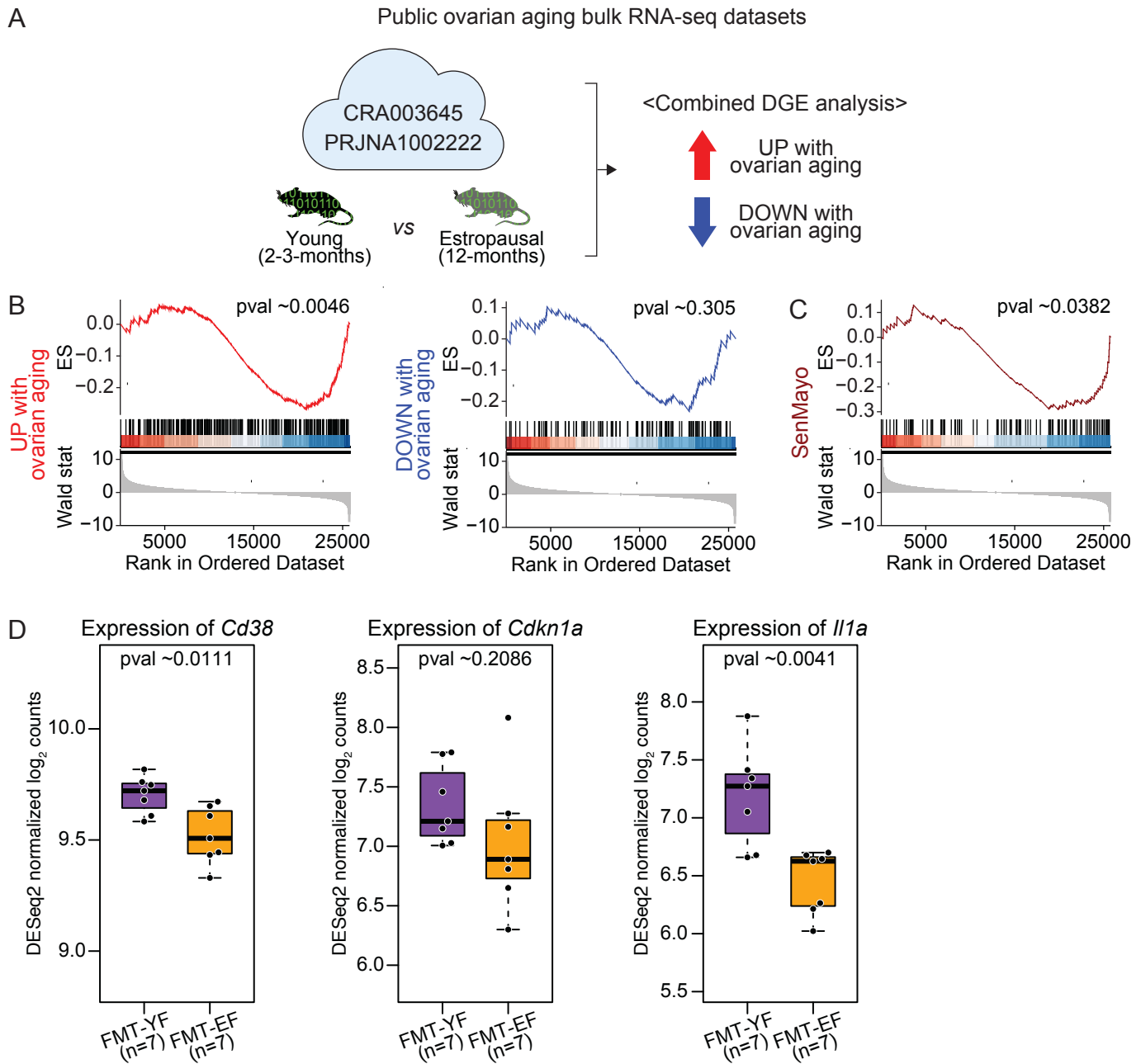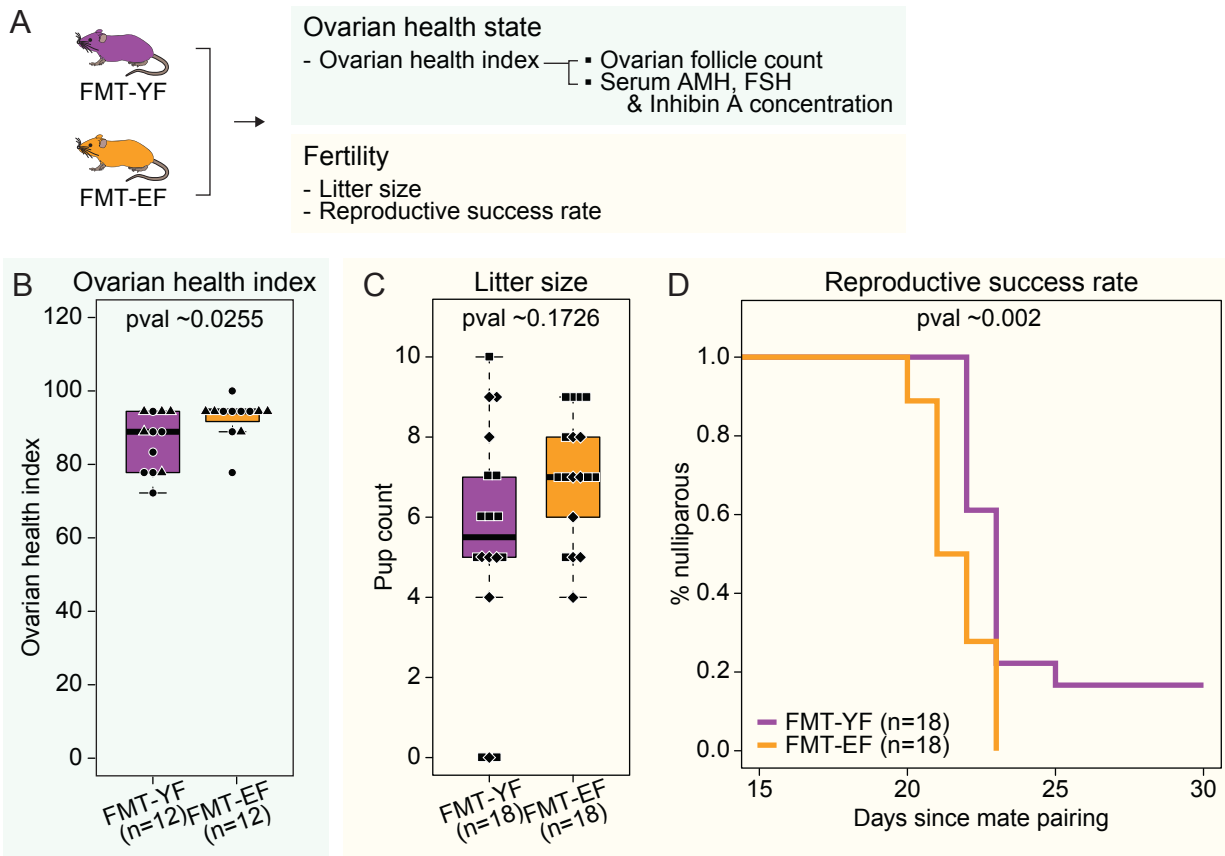
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7