Do BRCA1 and BRCA2 gene mutation carriers have a reduced ovarian reserve?: Protocol for a prospective observational study

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Do BRCA1 and BRCA2 gene mutation carriers have a reduced ovarian reserve?: Protocol for a prospective observational study

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Abstract

Introduction: BRCA1/2 gene mutations increase risk of breast and/or ovarian cancer and may have implications for reproductive health. Indirect biomarkers of the ovarian primordial follicle pool (Anti-Müllerian hormone, AMH) and one small study in female cadavers suggests that ovarian reserve may be reduced in BRCA mutation carriers, but findings are conflicting and association between circulating AMH and primordial follicle number is not established. The aim of this study is to measure primordial follicle density in premenopausal ovarian tissue samples from women with BRCA1/2 gene mutations versus age-matched controls.

Methods and analysis: Prospective observational study measuring associations between BRCA gene mutation status, premenopausal ovarian primordial follicle density and serum AMH concentrations versus age matched premenopausal women from the general population. Primordial follicle density will be measured in cortical sections from ovarian tissue collected at the time of risk-reducing bilateral salpingo-oophorectomy (RRBSO) in n=88 BRCA1 gene mutation carriers, n=65 BRCA2 gene mutation carriers and n=157 controls. Primordial follicle density will be determined by counting follicles in a known volume of ovarian cortical tissue using light microscopy. Follicles will be identified by immunohistochemical staining for oocyte marker mouse vasa homologue (MVH). To inform the mechanisms underlying reduced ovarian reserve, the proportion of follicles containing oocytes with DNA damage will be determined by immunohistochemical staining for phosphorylated histone H2AX (γH2AX) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to identify apoptotic cells. Follicle density will be correlated with circulating AMH concentrations quantified in the same cohort, using an electrochemiluminescence immunoassay on an automated platform.

Ethics and dissemination: Ethics approval has been granted by Peter MacCallum Cancer Centre to access biobanks, including; The Kathleen Cuningham Foundation Consortium for Research into Familial Breast Cancer (kConFab-HREC#97_27) and the What Happens after Menopause? (WHAM) (HREC12PMCC24-12/90) and Melbourne IVF.

Article summary

Strengths and limitations of this study

- This will be the largest controlled study of primordial follicle density and serum AMH quantification in human BRCA mutation carriers and non-mutation carrier controls.

- This study will provide new information about ovarian reserve in BRCA mutation carriers, which could inform healthcare professionals and patients to guide decision making about fertility and family planning.

- This study will offer new information about the association between a widely used indirect measure of ovarian reserve (circulating AMH) and direct measures of ovarian reserve (primordial follicle count), which will inform the growing international debate about the veracity of AMH as a biomarker of ovarian reserve.

- The study samples representative areas of the human ovary, but not the entire ovary and this is an inherent limitation of studying the ovarian reserve in women.

Introduction

Around one in 350 women carry mutations on the BRCA1/2 gene which confer an increased risk of developing breast and ovarian cancer[1]. Ovarian cancer has a high mortality rate and because there is currently no effective screening tool, international guidelines consistently advise BRCA mutation carriers to undergo risk-reducing bilateral salpingo-oophorectomy (RRBSO) to reduce their ovarian cancer risk[2]. The recommended age for RRBSO varies according to family history and gene mutation but is generally before natural menopause (51 years). Hence, RRBSO will generally result in surgical menopause[3].

Surgical menopause leads to permanent infertility and BRCA gene mutation carriers commonly need to make complex reproductive decisions about whether and when to start and complete their families[4]. These decisions are further complicated by uncertainty about whether BRCA gene mutation carriers have reduced fertility compared to women of the same age who do not carry a BRCA gene mutation[4].

Female fertility potential largely depends on the ovarian reserve which describes the size of the primordial follicle pool which gives rise to all mature ovulatory oocytes[5]. The size of the primordial follicle pool is thought to be fixed by the time of birth. The primordial follicle pool declines during reproductive life, culminating in menopause[6]. The clinical significance of decreased ovarian reserve extends beyond fertility to the long-term adverse health consequences of premature or early menopause[7]. There is no established indirect method for measuring the primordial follicle pool and the gold standard for measuring ovarian reserve is to count the number of primordial follicles in whole ovaries or cortical sections[8]. Measuring the total number of primordial follicles requires entire ovaries, but primordial follicle density can be calculated from cortical sections, and this approach has been validated in fertile women against whole ovary data[9].

The initial suggestion that ovarian reserve may be reduced in BRCA1/2 mutation carriers came from observations of poor response to ovarian stimulation during IVF treatment compared to the general population[10]. However, larger studies of ovarian stimulation in BRCA mutation carriers have not confirmed these findings[11] and subsequent reports indicate that BRCA gene mutation carriers produced greater numbers of mature oocytes for cryopreservation compared to age matched women from the general population[12], although these reports did not distinguish between women with BRCA1 versus BRCA2 mutations. Furthermore, other factors may

136 influence responsiveness beyond ovarian reserve, including gonadotropin dose, protocol, and

137 degree of supression.

138

139 Only two small studies have measured primordial follicle density in BRCA mutation carriers

140 compared to population-risk controls. Reduced follicle density was reported in one small cohort

141 of 15 BRCA mutation carriers compared to population risk women undergoing BSO[13].

142 However, BRCA mutation carriers were older than non-carriers, and 4/15 had occult ovarian

143 malignancy, which was independently associated with reduced follicle density and results were

144 not broken down by gene mutation type[13]. Another small study of ovarian sections from 18

145 BRCA1 and BRCA2 mutation carriers compared to 12 ovarian sections from organ donation

146 cadavers, showed primordial follicle density was significantly reduced in mutation carriers and

147 more DNA double-strand breaks were observed in BRCA mutations carriers, suggestive of

148 defective DNA repair[14].

149

150 Almost all published studies of ovarian reserve in BRCA mutation carriers have relied in

151 circulating AMH as an indirect measure. AMH is a member of the TGF-β superfamily

152 produced by the granulosa cells of primary, preantral and small antral follicles[15-17], but not

153 primordial follicles. Circulating AMH peaks during the early 20's and then declines over

154 reproductive life. In an unselected population, serum AMH levels have been correlated with

155 ovarian primordial follicle count[18]. Despite this, in a clinical setting, biomarkers of ovarian

156 reserve including AMH and antral follicle count do not predict the clinically relevant outcomes

157 of fertility, infertility or fecundity in the general population[19-21]. Studies measuring AMH in

158 BRCA mutation carriers report conflicting findings. One report found no difference between

159 serum AMH concentrations of 124 premenopausal women with BRCA1/2 mutations compared

160 to 131 age-matched non-mutation carriers[22]. Larger studies indicate that circulating AMH

161 concentrations may vary by gene mutation, with lower concentrations in BRCA1 gene mutation

162 carriers compared to BRCA2 gene mutation carriers of similar ages[23-25]. This is supported by

163 a clinical evidence that BRCA1 mutation carriers produced fewer mature oocytes following

164 ovarian stimulation compared to BRCA2 mutation carriers of similar age[26].

165

166 Because exhaustion of the primordial follicle pool is thought to result in menopause, age at

167 menopause for BRCA mutation carriers has been investigated in cross sectional studies. Most

168 report no difference in age at menopause in BRCA gene mutation carriers compared to non-

169 carrier relatives[27 28]. However, determining the age at menopause in BRCA mutations carriers

170   is complicated by the risk of cancer treatments leading to ovarian failure, use of

171   chemoprevention medication such as tamoxifen or, risk-reducing bilateral salpingo-

172   oophorectomy[28].

173

174   BRCA1 and 2 have important roles in the repair of endogenously and exogenously induced

175   DNA doublestrand breaks. Importantly, DNA doublestrand breaks are induced in oocytes

176   during meiotic recombination and may also accumulate in oocytes as a consequence of normal

177   metabolic processes or exposure to DNA damaging agents in the environment[29 30]. Reduced

178   ability to repair DNA doublestrand breaks in BRCA mutation carriers may increase oocyte

179   apoptosis and reduce the number of primordial follicles initially established in the ovarian

180   reserve at birth, and potentially accelerate the depletion of primordial follicles during

181   reproductive life[31]. Preclinical evidence from transgenic mice demonstrates that BRCA1

182   mutant mice have fewer primordial follicles at 5 days of life, produced fewer oocytes in

183   response to ovarian stimulation, and have smaller litter size compared with the wild-type (WT)

184   mice[32].

185

186   The objective of this study is to measure ovarian follicle density in premenopausal BRCA1 and

187   BRCA2 mutation carriers compared to age matched, population risk controls and to measure

188   the association between primordial follicle density and circulating AMH concentrations. This

189   will be the largest study of primordial follicle density in BRCA1 and BRCA2 mutation carriers

190   versus age-matched controls. Additionally, we will assess the level of DNA damage in follicles

191   in women with BRCA mutations by analysis of γH2AX immunohistochemical staining and

192   apoptosis using TUNEL staining analysis. The findings from this study will provide new data

193   on the ovarian reserve in BRCA mutation carriers to inform clinical practice. These findings

194   will also generate new information about the mechanism of oocyte loss during reproductive

195   life, which has direct relevance for age-related infertility, the most common cause of infertility

196   in resource-rich countries.

197

198

199   **Methods and analysis**

200   *Project design*

201   Prospective observational study measuring the association between BRCA gene mutation

202   status, ovarian primordial follicle density and serum AMH concentrations compared to age

203   matched controls from the general population.

Research project setting

205 The University of Melbourne Department of Obstetrics and Gynaecology, Melbourne IVF,

206 Peter MacCallum Cancer Centre and Monash University Department of Anatomy and

207 Developmental Biology.

Population

209 Premenopausal women known to be at high inherited risk of ovarian cancer (BRCA1/2 gene

210 mutation carriers) prior to risk-reducing bilateral salpingo-oophorectomy (RRBSO) to reduce

211 their risk of ovarian cancer.

Patient Involvement Statement

213 Patients or the public were not involved in the design, or conduct, or reporting, or dissemination

214 of this research.

Inclusion criteria

216 • Aged 18-45 years

217 • Known to carry BRCA1 or BRCA2 gene mutation

218 • Premenopausal at the time of oophorectomy

Exclusion criteria (applies to cases and controls)

220 • Previous chemotherapy treatment likely to impact on ovarian reserve

221 • Personal history of ovarian cancer

222 • Using hormonal contraception at the time of oophorectomy

223 • Ovarian cancer diagnosed at oophorectomy

224 • Known to have polycystic ovary syndrome (associated with increased AMH)[33]

Age-matched controls

226 • Aged 18-45 years

227 Pre-menopausal women who have undergone premenopausal oophorectomy but do not carry

228   BRCA gene mutations.

229 • Pre-menopausal women at population risk for ovarian cancer who have had ovarian tissue

230   obtained at Caesarean section.

231 • Premenopausal women who have undergone cryopreservation of ovarian tissue for fertility

232   preservation and who have donated tissues to research at Melbourne IVF.

Sample size calculation

234 A sample size of 102 (34 for each of the BRCA1, BRCA2, and control groups) is required.

235 This was calculated using the following criteria: 5% alpha, splitting 2.5% for the comparison

236 of BRCA1 vs. controls and 2.5% for the comparison of BRCA2 vs. controls; 2-sided t-test;

237 80% power; 1:1:1 allocation ratio; difference in mean follicle density of 6 with a SD of 8.

238 *Samples: Current status and recruitment sources*

239 *High-Risk Women*

240 *1. kConFab*

241 The Kathleen Cuningham Foundation Consortium for research into Familial Breast cancer

242 (kConFab Peter Mac HREC #97_27) biobank contains donated ovarian tissue sections from

243 women with a family history of breast cancer, including women with known BRCA gene

244 mutations. kConFab have already collected ovarian tissue sections (formalin fixed and/or fresh

245 frozen tissue) from women aged <45 years at the time of oophorectomy from BRCA1 gene

246 mutation carriers of whom several have already had AMH measured and the remaining have

247 stored serum available to measure circulating AMH. This cohort also has stored ovarian tissue

248 from BRCA2 gene mutation carriers of whom a number have already had AMH measured and

249 the remaining have stored serum for AMH testing. With signed consent, participants agree to

250 kConFab accessing clinical tissue samples (normal and tumour) and donate 18 ml of whole

251 blood and access to hospital, cancer registries, Australian Medicare data. At recruitment they

252 complete a comprehensive base line epidemiological questionnaire that includes information

253 about pregnancies, sex steroid use including hormonal contraception and risk-reducing

254 medication, age at menarche, previous menstrual patterns, infertility treatment and personal

255 cancer history and treatment. Routine follow up occurs to update these data points over time.

256 Potentially eligible kConFab participants will be contacted by phone to determine whether they

257 were premenopausal at the time of oophorectomy, as determined by the presence of regular

258 menstrual cycles and no vasomotor symptoms along with polycystic ovarian syndrome (PCOS)

259 history if not already attained[34].

260 *2. WHAM (What Happens after Menopause?) Study*

261 WHAM is a prospective, controlled study of high-risk premenopausal women undergoing risk-

262 reducing bilateral salpingo-oophorectomy (RRBSO)[35]. Participants are premenopausal

263 BRCA1 and BRCA2 gene mutation carriers recruited prior to RRBSO and followed up for 2

264 years post RRBSO None of these women had ovarian cancer diagnosed at the time of surgery

265 and all have stored serum collected prior to oophorectomy for measurement of AMH. WHAM

266 participants have already consented to use of their stored serum and ovarian tissue for HREC-

267 approved studies (HREC12PMCC24 12/90).

268 *Age-matched controls*

269 *1. kConFab*

270 The Kathleen Cuningham Foundation Consortium for research into Familial Breast cancer

271 (kConFab, PeterMac HREC #97_27) biobank contains donated ovarian tissue sections from

272 women with a family history of breast cancer, including women who have been tested negative

273 for a BRCA gene. kConFab have already collected ovarian tissue from women aged <50 years

274 who have undergone oophorectomy and have tested negative for BRCA gene mutations. Stored

275 serum samples are available for measuring AMH. At recruitment, participants already agreed

276 to be routinely contacted by phone/email/post for general health updates and specific project

277 requests. For this study, kConFab will contact potentially eligible participants to determine

278 whether they were premenopausal at the time of oophorectomy, as determined by the presence

279 of regular menstrual cycles and no vasomotor symptoms along with PCOS history if not

280 already attained[34].

281 *2. WHAM study*

282 WHAM participants include women who have undergone bilateral oophorectomy but have

283 tested negative for BRCA1/2 gene mutations. All have stored ovarian tissue and serum samples

284 available for AMH testing.

285 *3. Premenopausal women at the time of Caesarean section*

286 Ovarian cortical biopsies have already been obtained from premenopausal women[9] and ovarian

287 follicle density has already been measured using the same protocol as the planned study. These

288 participants do not have stored serum samples for AMH measurements.

289 *4. Premenopausal women who have undergone ovarian tissue cryopreservation through*

290 *Melbourne IVF*

291 Ovarian tissue has already been collected from premenopausal women requesting fertility

292 preservation at Melbourne IVF due to malignant and some non-malignant disease. These

293 participants have already consented to their personal information and ovarian follicle count

294 data being included in ethically approved research projects.

295 *5. Premenopausal women who have undergone oophorectomy for benign conditions*

296 Ovarian cortical biopsies have already been obtained from premenopausal women[8] and ovarian

297 follicle density has already been measured using the same protocol as the planned study. These

298 participants do not have stored serum samples for AMH measurements.

299 *Data collection*

300 We will measure ovarian follicle density, DNA damage and apoptosis in existing ovarian tissue

301 samples and quantify AMH in existing stored serum samples. Ovarian follicle density has

302 already been measured in the participants from Scotland[9]. Participants are not expected to

303 undergo any additional visits or procedures and there will be no further follow-up. Data

304 Collection/Gathering for kConFab participants is largely complete. The only additional

305 information collected will be reproductive stage (pre or postmenopausal) and any history of

306   PCOS, where this information is not already available. These data have already been collected

307   for WHAM participants. The only additional testing for WHAM and kConFab participants will

308   be measurement of AMH in existing stored plasma samples.

309   **Study Procedures**

310   *Ovarian follicle density*

311   Each histological section will be stained for the oocyte marker MVH and evaluated by light

312   microscopy. Ovarian follicle density will be determined using previously established and

313   validated methodology[9][36]. Follicles will be classified into stages according to McLaughlin et

314   al[37]. Only follicles in which the nucleus is visible will be counted. The volume of the tissue

315   analysed per patient will be calculated (area of the tissue analysed x thickness of the section)

316   and follicle density will be expressed as follicles/mm^3. Tissues will be evaluated blind to gene

317   mutation status and patient age by assigning a code to each sample.

318   *Immunohistochemistry*

319   We will assess the level of DNA damage in follicles in women with BRCA mutations by

320   immunohistochemical analysis of γH2AX, known to form foci at DNA double strand break

321   sites. Every 9th ovarian tissue section (5 μm) will be assessed for DNA double strand breaks

322   using an antibody for Phospho-Histone (γ)H2AX (Ser139) (1:100; Cell Signaling Technology

323   #9718). Primordial follicles with nuclear γH2AX foci will be counted as positive and expressed

324   as a proportion of total primordial follicles assessed.

325   *TUNEL*

326   We will assess follicle apoptosis by performing TUNEL staining using the Apop Tag

327   Peroxidase *In Situ* Apoptosis Detection Kit (Merck) on every 10th ovarian tissue section (5 μm).

328   Follicles will be classified as positive if the oocyte and/or ≥2 granulosa cells are positive and

329   expressed as a proportion of total primordial follicles assessed.

330   *AMH Analysis*

331   AMH will be measured in existing stored plasma samples collected by kConFab or as part of

332   the WHAM study. All plasma samples were collected prior to oophorectomy in women who

333   were premenopausal. Plasma aliquots have been stored at -80 degrees within 48 hours of

334   collection. AMH measurements will be undertaken at the Melbourne IVF Endocrine

335   Laboratory utilizing the fully automated Elecsys® AMH assay on the Roche Cobas

336   electrochemiluminescence immunoassay platform[38][39]. The scientist conducting the assay will

337   be blinded to participant mutation status and patient age. The lowest level of detection of this

338   assay is 0.07 pmol/l and the intra-assay and inter-assay imprecision coefficients of variation at

339   7.0 pmol/l are 4.6 and 5.6% respectively[24]. Plasma samples from WHAM have been stored for

340 a mean of 3 years prior to AMH analysis and none had been previously thawed. Quality

341 assurance testing was performed on 30 non-study samples for which both plasma and serum

342 were available; AMH concentrations using serum samples were approximately 5% higher than

343 those using plasma. It was considered that this would not affect the study conclusions given

344 that plasma was used for both the comparison groups.

345 *Statistical analysis*

346 Mean follicle density will be plotted against age for non-carriers, BRCA1 and BRCA2

347 mutation carriers. Groups will be compared using t-test or Wilcoxon test (if data is not normally

348 distributed) to assess the primary objective. Correlation between AMH and ovarian follicle

349 density will be measured by calculating the Pearson product-moment correlation coefficient.

350 The mean difference between groups, and limits of agreement at one and two standard

351 deviations from mean will be derived from Bland-Altman analysis. Proportional error will be

352 calculated as the correlation between the means and differences derived from the Bland-Altman

353 plot, with absence of correlation indicating low proportional error.

354

355 **Outcome measures**

356 1. Ovarian primordial follicle density according to BRCA mutation status (BRCA1/2/no gene

357   mutation or population risk)

358 2. Association between ovarian primordial follicle density and circulating AMH

359   concentrations obtained prior to RRBSO

360

361 **Expected outcome significance**

362 Largest prospective study of primordial follicle density (ovarian reserve) in premenopausal

363   BRCA mutation carriers and the first to be adequately powered to distinguish between

364   ovarian reserve in BRCA1 and BRCA2 mutation carriers and to compare findings with age

365   matched premenopausal controls.

366 • New information about ovarian reserve in BRCA mutation carriers which will guide

367   decision making by high-risk women about fertility and family planning and inform

368   healthcare professionals managing these patients.

369 • New information about the association between a widely used indirect measure of ovarian

370   reserve (circulating AMH) and direct measures of ovarian reserve (primordial follicle count)

371   which will inform the growing international debate about the veracity of AMH as a

372   biomarker of ovarian reserve.

373

374 **Data management**

375 Patient data will be kept strictly confidential according to the National Statement on Ethical

376 Conduct in Human Research 2007 and the Australian Code for Responsible Conduct of

377 Research 2007. Patient research data will be kept onsite at the Women's Research Precinct at

378 The Royal Women's Hospital. Data concerning ovarian follicle numbers and density will be

379 kept onsite at Monash University. The results from AMH assays will be kept at MIVF. Patient

380 research data will only be accessed by the named investigators and named statistician.

381 Electronic records of research data will be retained on password protected computer(s) in

382 databases requiring password access. These data will be stored separately from the patient

383 name in order to recontact potential participants for follow-up interviews. Any hard copies of

384 data will be kept in locked facilities of the Royal Women's Hospital in the Department of

385 Obstetrics and Gynaecology and at Monash University in the Department of Anatomy and

386 Developmental Biology. All computers will be password-protected and electronic records

387 stored on it will be coded and in databases requiring password access. Patient data will be only

388 be transferred in a non–identifiable form. Individual patients will not be identifiable from the

389 information stored, presented or published material. All data will be stored for 15 years. After

390 this time the files will be destroyed by erasure and/or incineration unless further approval for

391 retention is obtained.

392

393 **Discussion**

394 Accurate measures of ovarian reserve in BRCA gene mutation carriers are important for two

395 reasons. First, because pregnancy planning for BRCA gene mutation carriers is already

396 complicated by their gene status and they should be informed if they have reduced fertility

397 compared to women of a similar age and secondly because BRCA1/2 are tumour suppressive

398 genes that act to ensure the integrity of the genome though repair of DNA double-strand

399 breaks[40]. Hence, the relationship between BRCA gene mutations and ovarian reserve may

400 inform the mechanisms underlying ovarian ageing due to reduced primordial follicle reserve.

401

402 We acknowledge some minor limitations to this study design. The controls are premenopausal

403 women who have been tested for BRCA gene mutations and found to be negative, or women

404 at population risk of ovarian cancer who have undergone oophorectomy or ovarian biopsy for

405 benign indications and are unlikely to carry BRCA mutations. Benign indications for

406 oophorectomy (adnexal masses, torsion, pelvic pain, endometriosis, and hysterectomy) are

407 generally not associated with reduced ovarian reserve. Aged matched controls from 2 sources

408 have been tested and are negative for BRCA1/2 mutations. Controls from the remaining 3

409 sources have not been tested. The prevalence of BRCA1/2 mutations in the general population

410 is very low, estimated at 0.1%. It is therefore, unlikely that BRCA mutations are found in

411 samples from these sources. The project team expect no confounding factors from these

412 controls. Additionally, there is no gold standard assay for the measurement of AMH. However,

413 the automated Roche electrochemiluminescent method has the equivalent highest sensitivity

414 and specificity to any other routinely used platform. As a large number of samples have already

415 been assessed on the same instrument, the continued use in the posterior analysis will prevent

416 the introduction of cross platform variability.

417

418 **Conclusion**

419 This is the first large scale study aimed at exploring the measurement of ovarian reserve (as

420 follicle density) in both BRCA1 and BRCA2 mutation carriers compared to age-matched

421 controls and to compare follicle density with circulating levels of AMH. Improving the

422 accuracy of association between circulating AMH and follicle density will enlighten the

423 mechanism of oocyte loss during reproductive life particularly for women who are thought to

424 have reduced ovarian reserve, such as BRCA mutation carriers.

425

426

427 **Competing interests**

428 Nil

429 **Author contributions**

430 AW and CW wrote the manuscript, CW, KJH and MH designed the study, KRH provided

431 critical evaluation of the study design and all authors edited the manuscript.

432 **Funding**

439

440

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BMJ Open

Do BRCA1 and BRCA2 gene mutation carriers have a reduced ovarian reserve?: Protocol for a prospective observational study

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1   Do BRCA1 and BRCA2 gene mutation carriers have a reduced ovarian reserve?:
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5   **2   Protocol for a prospective observational study**
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37   21 **Keywords:** Oocyte / follicle / BRCA / germline mutation / DNA repair / fertility
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Abstract

Introduction: BRCA1/2 gene mutations increase risk of breast and/or ovarian cancer and may have implications for reproductive health. Indirect biomarkers of the ovarian primordial follicle pool (Anti-Müllerian hormone, AMH) and one small study in female cadavers suggests that ovarian reserve may be reduced in BRCA mutation carriers, but findings are conflicting and association between circulating AMH and primordial follicle number is not established. The aim of this study is to measure primordial follicle density in premenopausal ovarian tissue samples from women with BRCA1/2 gene mutations versus age-matched comparison group.

Methods and analysis: Prospective observational study measuring associations between BRCA gene mutation status, premenopausal ovarian primordial follicle density and serum AMH concentrations versus age matched premenopausal women from the general population. Primordial follicle density will be measured in cortical sections from ovarian tissue collected at the time of risk-reducing bilateral salpingo-oophorectomy (RRBSO) in n=88 BRCA1 gene mutation carriers, n=65 BRCA2 gene mutation carriers and n=157 non-mutation carriers. Primordial follicle density will be determined by counting follicles in a known volume of ovarian cortical tissue using light microscopy. Follicles will be identified by immunohistochemical staining for oocyte marker mouse vasa homologue (MVH). To inform the mechanisms underlying reduced ovarian reserve, the proportion of follicles containing oocytes with DNA damage will be determined by immunohistochemical staining for phosphorylated histone H2AX (γH2AX) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to identify apoptotic cells. Follicle density will be correlated with circulating AMH concentrations quantified in the same cohort, using an electrochemiluminescence immunoassay on an automated platform.

Ethics and dissemination: Ethics approval has been granted by Peter MacCallum Cancer Centre to access biobanks, including; The Kathleen Cuningham Foundation Consortium for Research into Familial Breast Cancer (kConFab-HREC#97_27) and the What Happens after Menopause? (WHAM) (HREC12PMCC24-12/90) and Melbourne IVF.

Article summary

Strengths and limitations of this study

- This will be the largest controlled study of primordial follicle density and serum AMH quantification in human BRCA mutation carriers and non-mutation carriers as a comparison group.

- This study will provide new information about ovarian reserve in BRCA mutation carriers, which could inform healthcare professionals and patients to guide decision making about fertility and family planning.

- This study will offer new information about the association between a widely used indirect surrogate measure of ovarian reserve (circulating AMH) and direct measures of ovarian reserve (primordial follicle count), which will inform the growing international debate about the veracity of AMH as a biomarker of ovarian reserve.

- The study samples representative areas of the human ovary, but not the entire ovary and this is an inherent limitation of studying the ovarian reserve in women.

102

Introduction

103

104 Around one in 350 women carry mutations on the BRCA1/2 gene which confer an increased

105 risk of developing breast and ovarian cancer[1]. Ovarian cancer has a high mortality rate and

106 because there is currently no effective screening tool, international guidelines consistently

107 advise BRCA mutation carriers to undergo risk-reducing bilateral salpingo-oophorectomy

108 (RRBSO) to reduce their ovarian cancer risk[2]. The recommended age for RRBSO varies

109 according to family history and gene mutation but is generally before natural menopause (51

110 years). Hence, RRBSO will generally result in surgical menopause[3].

111

112 Surgical menopause leads to permanent infertility and BRCA gene mutation carriers commonly

113 need to make complex reproductive decisions about whether and when to start and complete

114 their families[4]. These decisions are further complicated by uncertainty about whether BRCA

115 gene mutation carriers have reduced fertility compared to women of the same age who do not

116 carry a BRCA gene mutation[4].

117

118 Female fertility potential largely depends on the ovarian reserve which describes the size of the

119 primordial follicle pool which gives rise to all mature ovulatory oocytes[5]. The size of the

120 primordial follicle pool is thought to be fixed by the time of birth. The primordial follicle pool

121 declines during reproductive life, culminating in menopause[6]. The clinical significance of

122 decreased ovarian reserve extends beyond fertility to the long-term adverse health

123 consequences of premature or early menopause[7]. There is no established indirect method for

124 measuring the primordial follicle pool and the gold standard for measuring ovarian reserve is

125 to count the number of primordial follicles in whole ovaries or cortical sections[8]. Measuring

126 the total number of primordial follicles requires entire ovaries, but primordial follicle density

127 can be calculated from cortical sections, and this approach has been validated in fertile women

128 against whole ovary data[9].

129

130 The initial suggestion that ovarian reserve may be reduced in BRCA1/2 mutation carriers came

131 from observations of poor response to ovarian stimulation during IVF treatment compared to

132 the general population[10]. However, larger studies of ovarian stimulation in BRCA mutation

133 carriers have not confirmed these findings[11] and subsequent reports indicate that BRCA gene

134 mutation carriers produced greater numbers of mature oocytes for cryopreservation compared

135 to age matched women from the general population[12], although these reports did not distinguish

136   between women with BRCA1 versus BRCA2 mutations. Furthermore, other factors may

137   influence responsiveness beyond ovarian reserve, including gonadotropin dose, protocol, and

138   degree of supression.

139

140   Only two small studies have measured primordial follicle density in BRCA mutation carriers

141   compared to population-risk non-mutation carriers. Reduced follicle density was reported in

142   one small cohort of 15 BRCA mutation carriers compared to population risk women

143   undergoing BSO[13]. However, BRCA mutation carriers were older than non-carriers, and 4/15

144   had occult ovarian malignancy, which was independently associated with reduced follicle

145   density and results were not broken down by gene mutation type[13]. Another small study of

146   ovarian sections from 18 BRCA1 and BRCA2 mutation carriers compared to 12 ovarian

147   sections from organ donation cadavers, showed primordial follicle density was significantly

148   reduced in mutation carriers and more DNA double-strand breaks were observed in BRCA

149   mutations carriers, suggestive of defective DNA repair[14].

150

151   Almost all published studies of ovarian reserve in BRCA mutation carriers have relied in

152   circulating AMH as an indirect measure. AMH is a member of the TGF-β superfamily

153   produced by the granulosa cells of primary, preantral and small antral follicles[15-17], but not

154   primordial follicles. Circulating AMH peaks during the early 20's and then declines over

155   reproductive life. In an unselected population, serum AMH levels have been correlated with

156   ovarian primordial follicle count[18]. Despite this, in a clinical setting, surrogate biomarkers of

157   ovarian reserve including AMH and antral follicle count do not predict the clinically relevant

158   outcomes of fertility, infertility or fecundity in the general population[19-21]. Studies measuring

159   AMH in BRCA mutation carriers report conflicting findings. One report found no difference

160   between serum AMH concentrations of 124 premenopausal women with BRCA1/2 mutations

161   compared to 131 age-matched non-mutation carriers[22]. Larger studies indicate that circulating

162   AMH concentrations may vary by gene mutation, with lower concentrations in BRCA1 gene

163   mutation carriers compared to BRCA2 gene mutation carriers of similar ages[23-25]. This is

164   supported by a clinical evidence that BRCA1 mutation carriers produced fewer mature oocytes

165   following ovarian stimulation compared to BRCA2 mutation carriers of similar age[26].

166

167   Because exhaustion of the primordial follicle pool is thought to result in menopause, age at

168   menopause for BRCA mutation carriers has been investigated in cross sectional studies. Most

169   report no difference in age at menopause in BRCA gene mutation carriers compared to non-

170 carrier relatives[27 28]. However, determining the age at menopause in BRCA mutations carriers
171 is complicated by the risk of cancer treatments leading to ovarian failure, use of
172 chemoprevention medication such as tamoxifen or, risk-reducing bilateral salpingo-
173 oophorectomy[28].

174

175 BRCA1 and 2 have important roles in the repair of endogenously and exogenously induced
176 DNA doublestrand breaks. Importantly, DNA doublestrand breaks are induced in oocytes
177 during meiotic recombination and may also accumulate in oocytes as a consequence of normal
178 metabolic processes or exposure to DNA damaging agents in the environment[29 30]. Reduced
179 ability to repair DNA doublestrand breaks in BRCA mutation carriers may increase oocyte
180 apoptosis and reduce the number of primordial follicles initially established in the ovarian
181 reserve at birth, and potentially accelerate the depletion of primordial follicles during
182 reproductive life[31]. Preclinical evidence from transgenic mice demonstrates that BRCA1
183 mutant mice have fewer primordial follicles at 5 days of life, produced fewer oocytes in
184 response to ovarian stimulation, and have smaller litter size compared with the wild-type (WT)
185 mice[32].

186

187 The objective of this study is to measure ovarian follicle density in premenopausal BRCA1 and
188 BRCA2 mutation carriers compared to age matched, population risk non-mutation carriers and
189 to measure the association between primordial follicle density and circulating AMH
190 concentrations. This will be the largest study of primordial follicle density in BRCA1 and
191 BRCA2 mutation carriers versus age-matched women as a comparison group. Additionally,
192 we will assess the level of DNA damage in follicles in women with BRCA mutations by
193 analysis of γH2AX immunohistochemical staining and apoptosis using TUNEL staining
194 analysis. The findings from this study will provide new data on the ovarian reserve in BRCA
195 mutation carriers to inform clinical practice. These findings will also generate new information
196 about the mechanism of oocyte loss during reproductive life, which has direct relevance for
197 age-related infertility, the most common cause of infertility in resource-rich countries.

198

199

200 **Methods and analysis**

201 *Project design*

202   Prospective observational study measuring the association between BRCA gene mutation

203   status, ovarian primordial follicle density and serum AMH concentrations compared to age

204   matched non-mutation carriers from the general population as the comparison group.

205   *Research project setting*

206   The University of Melbourne Department of Obstetrics and Gynaecology, Melbourne IVF,

207   Peter MacCallum Cancer Centre and Monash University Department of Anatomy and

208   Developmental Biology.

209   *Population*

210   Premenopausal women known to be at high inherited risk of ovarian cancer (BRCA1/2 gene

211   mutation carriers) prior to risk-reducing bilateral salpingo-oophorectomy (RRBSO) to reduce

212   their risk of ovarian cancer.

213   *Patient Involvement Statement*

214   Patients or the public were not involved in the design, or conduct, or reporting, or dissemination

215   of this research.

216   *Inclusion criteria*

217   • Aged 18-45 years

218   • Known to carry BRCA1 or BRCA2 gene mutation

219   • Premenopausal at the time of oophorectomy

220   *Exclusion criteria (applies to cases and comparison group)*

221   • Previous chemotherapy treatment likely to impact on ovarian reserve

222   • Personal history of ovarian cancer

223   • Using hormonal contraception at the time of oophorectomy

224   • Ovarian cancer diagnosed at oophorectomy

225   • Known to have polycystic ovary syndrome (associated with increased AMH)[33]

226   *Age-matched non-mutation carrier comparison group*

227   • Aged 18-45 years

228   Pre-menopausal women who have undergone premenopausal oophorectomy but do not carry

229   BRCA gene mutations.

230   • Pre-menopausal women at population risk for ovarian cancer who have had ovarian tissue

231   obtained at Caesarean section.

232   • Premenopausal women who have undergone cryopreservation of ovarian tissue for fertility

233   preservation and who have donated tissues to research at Melbourne IVF.

234   *Sample size calculation*

235 A sample size of 102 (34 for each of the BRCA1, BRCA2, and comparison groups) is required.

236 This was calculated using the following criteria: 5% alpha, splitting 2.5% for the comparison

237 of BRCA1 vs. comparison group and 2.5% for the comparison of BRCA2 vs. comparison

238 group; 2-sided t-test; 80% power; 1:1:1 allocation ratio; difference in mean follicle density of

239 6 with a SD of 8.

240 *Samples: Current status and recruitment sources*

241 *High-Risk Women*

242 *1. kConFab*

243 The Kathleen Cuningham Foundation Consortium for research into Familial Breast cancer

244 (kConFab Peter Mac HREC #97_27) biobank contains donated ovarian tissue sections from

245 women with a family history of breast cancer, including women with known BRCA gene

246 mutations. kConFab have already collected ovarian tissue sections (formalin fixed and/or fresh

247 frozen tissue) from women aged <45 years at the time of oophorectomy from BRCA1 gene

248 mutation carriers of whom several have already had AMH measured and the remaining have

249 stored serum available to measure circulating AMH. This cohort also has stored ovarian tissue

250 from BRCA2 gene mutation carriers of whom a number have already had AMH measured and

251 the remaining have stored serum for AMH testing. With signed consent, participants agree to

252 kConFab accessing clinical tissue samples (normal and tumour) and donate 18 ml of whole

253 blood and access to hospital, cancer registries, Australian Medicare data. At recruitment they

254 complete a comprehensive base line epidemiological questionnaire that includes information

255 about pregnancies, sex steroid use including hormonal contraception and risk-reducing

256 medication, age at menarche, previous menstrual patterns, infertility treatment and personal

257 cancer history and treatment. Routine follow up occurs to update these data points over time.

258 Potentially eligible kConFab participants will be contacted by phone to determine whether they

259 were premenopausal at the time of oophorectomy, as determined by the presence of regular

260 menstrual cycles and no vasomotor symptoms along with polycystic ovarian syndrome (PCOS)

261 history if not already attained[34].

262 *2. WHAM (What Happens after Menopause?) Study*

263 WHAM is a prospective, controlled study of high-risk premenopausal women undergoing risk-

264 reducing bilateral salpingo-oophorectomy (RRBSO)[35]. Participants are premenopausal

265 BRCA1 and BRCA2 gene mutation carriers recruited prior to RRBSO and followed up for 2

266 years post RRBSO None of these women had ovarian cancer diagnosed at the time of surgery

267 and all have stored serum collected prior to oophorectomy for measurement of AMH. WHAM

268   participants have already consented to use of their stored serum and ovarian tissue for HREC-

269   approved studies (HREC12PMCC24 12/90).

270   *Age-matched non-mutation carrier comparison group*

271   *1. kConFab*

272   The Kathleen Cuningham Foundation Consortium for research into Familial Breast cancer

273   (kConFab, PeterMac HREC #97_27) biobank contains donated ovarian tissue sections from

274   women with a family history of breast cancer, including women who have been tested negative

275   for a BRCA gene. kConFab have already collected ovarian tissue from women aged <50 years

276   who have undergone oophorectomy and have tested negative for BRCA gene mutations. Stored

277   serum samples are available for measuring AMH. At recruitment, participants already agreed

278   to be routinely contacted by phone/email/post for general health updates and specific project

279   requests. For this study, kConFab will contact potentially eligible participants to determine

280   whether they were premenopausal at the time of oophorectomy, as determined by the presence

281   of regular menstrual cycles and no vasomotor symptoms along with PCOS history if not

282   already attained[34].

283   *2. WHAM study*

284   WHAM participants include women who have undergone bilateral oophorectomy but have

285   tested negative for BRCA1/2 gene mutations. All have stored ovarian tissue and serum samples

286   available for AMH testing.

287   *3. Premenopausal women at the time of Caesarean section*

288   Ovarian cortical biopsies have already been obtained from premenopausal women[9] and ovarian

289   follicle density has already been measured using the same protocol as the planned study. These

290   participants do not have stored serum samples for AMH measurements.

291   *4. Premenopausal women who have undergone ovarian tissue cryopreservation through*

292   *Melbourne IVF*

293   Ovarian tissue has already been collected from premenopausal women requesting fertility

294   preservation at Melbourne IVF due to malignant and some non-malignant disease. These

295   participants have already consented to their personal information and ovarian follicle count

296   data being included in ethically approved research projects.

297   *5. Premenopausal women who have undergone oophorectomy for benign conditions*

298   Ovarian cortical biopsies have already been obtained from premenopausal women[8] and ovarian

299   follicle density has already been measured using the same protocol as the planned study. These

300   participants do not have stored serum samples for AMH measurements.

301   *Data collection*

302 We will measure ovarian follicle density, DNA damage and apoptosis in existing ovarian tissue

303 samples and quantify AMH in existing stored serum samples. Ovarian follicle density has

304 already been measured in the participants from Scotland[9]. Participants are not expected to

305 undergo any additional visits or procedures and there will be no further follow-up. Data

306 Collection/Gathering for kConFab participants is largely complete. The only additional

307 information collected will be reproductive stage (pre or postmenopausal) and any history of

308 PCOS, where this information is not already available. These data have already been collected

309 for WHAM participants. The only additional testing for WHAM and kConFab participants will

310 be measurement of AMH in existing stored plasma samples.

311 **Study Procedures**

312 *Ovarian follicle density*

313 Each histological section will be stained for the oocyte marker MVH and evaluated by light

314 microscopy. Ovarian follicle density will be determined using previously established and

315 validated methodology[9 14 36]. Follicles will be classified into stages according to McLaughlin

316 et al[37]. Only follicles in which the nucleus is visible will be counted. The volume of the tissue

317 analysed per patient will be calculated (area of the tissue analysed x thickness of the section)

318 and follicle density will be expressed as follicles/mm^3. Tissues will be evaluated blind to gene

319 mutation status and patient age by assigning a code to each sample.

320 *Immunohistochemistry*

321 We will assess the level of DNA damage in follicles in women with BRCA mutations by

322 immunohistochemical analysis of γH2AX, known to form foci at DNA double strand break

323 sites. Every 10th ovarian tissue section (5 μm) will be assessed for DNA double strand breaks

324 using an antibody for Phospho-Histone (γ)H2AX (Ser139) (1:100; Cell Signaling Technology

325 #9718). Primordial follicles with nuclear γH2AX foci will be counted as positive and expressed

326 as a proportion of total primordial follicles assessed.

327 *TUNEL*

328 We will assess follicle apoptosis by performing TUNEL staining using the Apop Tag

329 Peroxidase *In Situ* Apoptosis Detection Kit (Merck) on every 10th ovarian tissue section (5 μm).

330 Follicles will be classified as positive if the oocyte and/or ≥2 granulosa cells are positive and

331 expressed as a proportion of total primordial follicles assessed.

332 *AMH Analysis*

333 AMH will be measured in existing stored plasma samples collected by kConFab or as part of

334 the WHAM study. All plasma samples were collected prior to oophorectomy in women who

335 were premenopausal. Plasma aliquots have been stored at -80 degrees within 48 hours of

336 collection. AMH measurements will be undertaken at the Melbourne IVF Endocrine
337 Laboratory utilizing the fully automated Elecsys® AMH assay on the Roche Cobas
338 electrochemiluminescence immunoassay platform, in singleton[38 39]. The scientist conducting
339 the assay will be blinded to participant mutation status and patient age. The lowest level of
340 detection of this assay is 0.07 pmol/l and the intra-assay and inter-assay imprecision
341 coefficients of variation at 7.0 pmol/l are 4.6 and 5.6% respectively[24]. Since the automated
342 electrochemiluminescence method excludes manual handling, the requirement to run samples
343 in duplicate or triplicate is not necessary. However, testing will be repeated if the result is less
344 than 2 pmol/L for confirmation, if there are processing errors, or if the result exceeds the
345 reference range and a dilution is required. Samples are run in batches and prior to each batch
346 run, Biorad quality control samples will be included in each run at clinically relevant targets of
347 7, 34 and 105 (pmol/L). Plasma samples from WHAM have been stored for a mean of 3 years
348 prior to AMH analysis and none had been previously thawed. Quality assurance testing was
349 performed on 30 non-study samples for which both plasma and serum were available; AMH
350 concentrations using serum samples were approximately 5% higher than those using plasma.
351 It was considered that this would not affect the study conclusions given that plasma was used
352 for both the comparison groups.

353 *Statistical analysis*

354 Mean follicle density will be plotted against age for non-carriers, BRCA1 and BRCA2
355 mutation carriers. Groups will be compared using t-test or Wilcoxon test (if data is not normally
356 distributed) to assess the primary objective. Correlation between AMH and ovarian follicle
357 density will be measured by calculating the Pearson product-moment correlation coefficient.
358 The mean difference between groups, and limits of agreement at one and two standard
359 deviations from mean will be derived from Bland-Altman analysis. Proportional error will be
360 calculated as the correlation between the means and differences derived from the Bland-Altman
361 plot, with absence of correlation indicating low proportional error.

362

363 **Outcome measures**

364 1. Ovarian primordial follicle density according to BRCA mutation status (BRCA1/2/no gene
365   mutation or population risk)
366 2. Association between ovarian primordial follicle density and circulating AMH
367   concentrations obtained prior to RRBSO

368

369 **Expected outcome significance**

370   Largest prospective study of primordial follicle density (ovarian reserve) in premenopausal

371   BRCA mutation carriers and the first to be adequately powered to distinguish between

372   ovarian reserve in BRCA1 and BRCA2 mutation carriers and to compare findings with age

373   matched premenopausal <u>non-mutation carrier comparison group</u>.

374   • New information about ovarian reserve in BRCA mutation carriers which will guide

375   decision making by high-risk women about fertility and family planning and inform

376   healthcare professionals managing these patients.

377   • New information about the association between a widely used indirect measure of ovarian

378   reserve (circulating AMH) and direct measures of ovarian reserve (primordial follicle count)

379   which will inform the growing international debate about the veracity of AMH as a

380   biomarker of ovarian reserve.

381

382 **Data management**

383 Patient data will be kept strictly confidential according to the National Statement on Ethical

384 Conduct in Human Research 2007 and the Australian Code for Responsible Conduct of

385 Research 2007. Patient research data will be kept onsite at the Women's Research Precinct at

386 The Royal Women's Hospital. Data concerning ovarian follicle numbers and density will be

387 kept onsite at Monash University. The results from AMH assays will be kept at MIVF. Patient

388 research data will only be accessed by the named investigators and named statistician.

389 Electronic records of research data will be retained on password protected computer(s) in

390 databases requiring password access. These data will be stored separately from the patient

391 name in order to recontact potential participants for follow-up interviews. Any hard copies of

392 data will be kept in locked facilities of the Royal Women's Hospital in the Department of

393 Obstetrics and Gynaecology and at Monash University in the Department of Anatomy and

394 Developmental Biology. All computers will be password-protected and electronic records

395 stored on it will be coded and in databases requiring password access. Patient data will be only

396 be transferred in a non–identifiable form. Individual patients will not be identifiable from the

397 information stored, presented or published material. All data will be stored for 15 years. After

398 this time the files will be destroyed by erasure and/or incineration unless further approval for

399 retention is obtained.

400

401 **Discussion**

402 Accurate measures of ovarian reserve in BRCA gene mutation carriers are important for two

403 reasons. First, because pregnancy planning for BRCA gene mutation carriers is already

404 complicated by their gene status and they should be informed if they have a reduced fertile

405 lifespan compared to women of a similar age and secondly because BRCA1/2 are tumour

406 suppressive genes that act to ensure the integrity of the genome though repair of DNA double-

407 strand breaks[40]. Hence, the relationship between BRCA gene mutations and ovarian reserve

408 may inform the mechanisms underlying ovarian ageing due to reduced primordial follicle

409 reserve. Interestingly, there is limited information regarding oocyte quality of BRCA mutation

410 carriers and this should be the subject of future investigations.

411

412 We acknowledge some inherent limitations to this study design. The comparison group are

413 premenopausal women who have been tested for BRCA gene mutations and found to be

414 negative, or women at population risk of ovarian cancer who have undergone oophorectomy

415 or ovarian biopsy for benign indications and are unlikely to carry BRCA mutations. Benign

416 indications for oophorectomy (adnexal masses, torsion, pelvic pain, endometriosis, and

417 hysterectomy) are generally not associated with reduced ovarian reserve. Despite this, it would

418 be interesting for future studies to evaluate potential differences in the ovarian reserve between

419 this type of non-mutation carrier comparison group in our protocol, with 'healthy' individuals,

420 although these samples are difficult to obtain. Aged matched women from 2 sources have been

421 tested and are negative for BRCA1/2 mutations. The comparison group from the remaining 3

422 sources have not been tested. The prevalence of BRCA1/2 mutations in the general population

423 is very low, estimated at 0.1%. It is therefore, unlikely that BRCA mutations are found in

424 samples from these sources. The project team expect no confounding factors from these

425 comparison groups. Additionally, there is no gold standard assay for the measurement of AMH.

426 However, the automated Roche electrochemiluminescent method has the equivalent highest

427 sensitivity and specificity to any other routinely used platform. As a large number of samples

428 have already been assessed on the same instrument, the continued use in the posterior analysis

429 will prevent the introduction of cross platform variability.

430

431 **Conclusion**

432 This is the first large scale study aimed at exploring the measurement of ovarian reserve (as

433 follicle density) in both BRCA1 and BRCA2 mutation carriers compared to age-matched non-

434 mutation carriers and to compare follicle density with circulating levels of AMH. Improving

435 the accuracy of association between circulating AMH and follicle density will enlighten the

436 mechanism of oocyte loss during reproductive life particularly for women who are thought to

437 have reduced ovarian reserve, such as BRCA mutation carriers.

Competing interests

There are no competing interests.

Author contributions

AW and CW wrote the manuscript, CW, KJH and MH designed the study, KRH provided critical evaluation of the study design and all authors edited the manuscript.

Ethics and dissemination: Ethics approval has been granted by Peter MacCallum Cancer Centre to access biobanks, including; The Kathleen Cuningham Foundation Consortium for Research into Familial Breast Cancer (kConFab-HREC#97_27) and the What Happens after Menopause? (WHAM) (HREC12PMCC24-12/90) and Melbourne IVF.

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