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

Amy Louise Winship ,[1] Chris Willson,[2,3] Karl R Hansen,[4] Karla J Hutt,[1] Martha Hickey[2,3]

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ALW and CW contributed equally.

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[1]Department of Anatomy and Developmental Biology, Monash University Monash Biomedicine Discovery Institute, Clayton, Victoria, Australia
[2]Department of Obstetrics and Gynaecology, The University of Melbourne, Melbourne, Victoria, Australia
[3]Gynaecology Research Centre, The Royal Women's Hospital, Melbourne, Victoria, Australia
[4]College of Medicine, University of Oklahoma, Oklahoma City, Oklahoma, USA

Correspondence to
Dr Martha Hickey;
hickeym@unimelb.edu.au

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 suggest 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 88 BRCA1 gene mutation carriers, 65 BRCA2 gene mutation carriers and 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. 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 and terminal deoxynucleotidyl transferase dUTP nick end labelling 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? (HREC12PMCC24-12/90) and Melbourne IVF.

Strengths and limitations of this study

- This will be the largest controlled study of primordial follicle density and serum anti-Müllerian hormone (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.

INTRODUCTION

Around 1 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 with women of the same age who do not carry a BRCA gene mutation.[4]

Female fertility potential largely depends on the ovarian reserve that describes the size of the primordial follicle pool that 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.[5] The clinical significance of decreased ovarian reserve extends beyond fertility to the long-term adverse health consequences of premature or early menopause.[6] 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.[7] 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.[8]

The initial suggestion that ovarian reserve may be reduced in BRCA1/2 mutation carriers came from observations of poor response to ovarian stimulation during in vitro fertilization (IVF) treatment compared with the general population.[9] However, larger studies of ovarian stimulation in BRCA mutation carriers have not confirmed these findings,[10] and subsequent reports indicate that BRCA gene mutation carriers produced greater numbers of mature oocytes for cryopreservation compared with age-matched women from the general population,[11] although these reports did not distinguish between women with BRCA1 versus BRCA2 mutations. Furthermore, other factors may influence responsiveness beyond ovarian reserve, including gonadotropin dose, protocol and degree of suppression.

Only two small studies have measured primordial follicle density in BRCA mutation carriers compared with population risk non-mutation carriers. Reduced follicle density was reported in one small cohort of 15 BRCA mutation carriers compared with population risk women undergoing bilateral salpingo-oophorectomy.[12] However, BRCA mutation carriers were older than non-carriers, and 4/15 had occult ovarian malignancy, which was independently associated with reduced follicle density, and results were not broken down by gene mutation type.[12] Another small study of ovarian sections from 18 BRCA1 and BRCA2 mutation carriers compared with 12 ovarian sections from organ donation cadavers showed primordial follicle density was significantly reduced in mutation carriers, and more DNA double-strand breaks were observed in BRCA mutations carriers, suggestive of defective DNA repair.[13]

Almost all published studies of ovarian reserve in BRCA mutation carriers have relied in circulating anti-Müllerian hormone (AMH) as an indirect measure. AMH is a member of the transforming growth factor-beta (TGF-β) superfamily produced by the granulosa cells of primary, preantral and small antral follicles[14–16] but not primordial follicles. Circulating AMH peaks during the early 20s and then declines over reproductive life. In an unselected population, serum AMH levels have been correlated with ovarian primordial follicle count.[17] Despite this, in a clinical setting, surrogate biomarkers of ovarian reserve including AMH and antral follicle count do not predict the clinically relevant outcomes of fertility, infertility or fecundity in the general population.[18–20] Studies measuring AMH in BRCA mutation carriers report conflicting findings. One report found no difference between serum AMH concentrations of 124 premenopausal women with BRCA1/2 mutations compared with 131 age-matched non-mutation carriers.[21] Larger studies indicate that circulating AMH concentrations may vary by gene mutation, with lower concentrations in BRCA1 gene mutation carriers compared with BRCA2 gene mutation carriers of similar ages.[22–24] This is supported by a clinical evidence that BRCA1 mutation carriers produced fewer mature oocytes following ovarian stimulation compared with BRCA2 mutation carriers of similar age.[25]

Because exhaustion of the primordial follicle pool is thought to result in menopause, age at menopause for BRCA mutation carriers has been investigated in cross-sectional studies. Most report no difference in age at menopause in BRCA gene mutation carriers compared with non-carrier relatives.[26 27] However, determining the age at menopause in BRCA mutations carriers is complicated by the risk of cancer treatments leading to ovarian failure, use of chemoprevention medication such as tamoxifen or RRBSO.[27]

BRCA1 and 2 have important roles in the repair of endogenously and exogenously induced DNA double-strand breaks. Importantly, DNA double-strand breaks are induced in oocytes during meiotic recombination and may also accumulate in oocytes as a consequence of normal metabolic processes or exposure to DNA damaging agents in the environment.[28 29] Reduced ability to repair DNA double-strand breaks in BRCA mutation carriers may increase oocyte apoptosis and reduce the number of primordial follicles initially established in the ovarian reserve at birth and potentially accelerate the depletion of primordial follicles during reproductive life.[30] Preclinical evidence from transgenic mice demonstrates that BRCA1 mutant mice have fewer primordial follicles at 5 days of life, produced fewer oocytes in response to ovarian stimulation and have smaller litter size compared with the wild-type mice.[31]

The objective of this study is to measure ovarian follicle density in premenopausal BRCA1 and BRCA2 mutation carriers compared with age-matched, population risk non-mutation carriers and to measure the association between primordial follicle density and circulating AMH concentrations. This will be the largest study of primordial follicle density in BRCA1 and BRCA2 mutation carriers versus age-matched women as a comparison group.

Additionally, we will assess the level of DNA damage in follicles in women with BRCA mutations by analysis of phosphorylated histone H2AX (γH2AX) immunohistochemical staining and apoptosis using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining analysis. The findings from this study will provide new data on the ovarian reserve in BRCA mutation carriers to inform clinical practice. These findings will also generate new information about the mechanism of oocyte loss during reproductive life, which has direct relevance for age-related infertility, the most common cause of infertility in resource-rich countries.

METHODS AND ANALYSIS

Project design

Prospective observational study measuring the association between BRCA gene mutation status, ovarian primordial follicle density and serum AMH concentrations compared with age-matched non-mutation carriers from the general population as the comparison group.

Research project setting

The University of Melbourne Department of Obstetrics and Gynaecology, Melbourne IVF (MIVF), Peter MacCallum Cancer Centre and Monash University Department of Anatomy and Developmental Biology.

Population

Premenopausal women known to be at high inherited risk of ovarian cancer (BRCA1/2 gene mutation carriers) prior to RRBSO to reduce their risk of ovarian cancer.

Patient involvement statement

Patients or the public were not involved in the design, or conduct, or reporting, or dissemination of this research.

Inclusion criteria

- Aged 18–45 years.
- Known to carry BRCA1 or BRCA2 gene mutation.
- Premenopausal at the time of oophorectomy.

Exclusion criteria (applies to cases and comparison group)

- Previous chemotherapy treatment likely to impact on ovarian reserve.
- Personal history of ovarian cancer.
- Using hormonal contraception at the time of oophorectomy.
- Ovarian cancer diagnosed at oophorectomy.
- Known to have polycystic ovary syndrome (associated with increased AMH).[32]

Age-matched non-mutation carrier comparison group

- Aged 18–45 years.
- Premenopausal women who have undergone premenopausal oophorectomy but do not carry BRCA gene mutations.
- Premenopausal women at population risk for ovarian cancer who have had ovarian tissue obtained at caesarean section.
- Premenopausal women who have undergone cryopreservation of ovarian tissue for fertility preservation and who have donated tissues to research at MIVF.

Sample size calculation

A sample size of 102 (34 for each of the BRCA1, BRCA2 and comparison groups) is required. This was calculated using the following criteria: 5% alpha, splitting 2.5% for the comparison of BRCA1 versus comparison group and 2.5% for the comparison of BRCA2 versus comparison group; two-sided t-test; 80% power; 1:1:1 allocation ratio; difference in mean follicle density of 6 with an SD of 8.

SAMPLES: CURRENT STATUS AND RECRUITMENT SOURCES

High-risk women

Kathleen Cuningham Foundation Consortium for research into Familial Breast cancer (kConFab)

The kConFab (kConFab Peter Mac HREC #97_27) biobank contains donated ovarian tissue sections from women with a family history of breast cancer, including women with known BRCA gene mutations. kConFab has already collected ovarian tissue sections (formalin fixed and/or fresh frozen tissue) from women aged <45 years at the time of oophorectomy from BRCA1 gene mutation carriers of whom several have already had AMH measured and the remaining have stored serum available to measure circulating AMH. This cohort also has stored ovarian tissue from BRCA2 gene mutation carriers of whom a number have already had AMH measured and the remaining have stored serum for AMH testing. With signed consent, participants agree to kConFab accessing clinical tissue samples (normal and tumour) and donate 18 mL of whole blood and access to hospital, cancer registries, Australian Medicare data. At recruitment they complete a comprehensive baseline epidemiological questionnaire that includes information about pregnancies, sex steroid use including hormonal contraception and risk-reducing medication, age at menarche, previous menstrual patterns, infertility treatment and personal cancer history and treatment. Routine follow-up occurs to update these data points over time. Potentially eligible kConFab participants will be contacted by phone to determine whether they were premenopausal at the time of oophorectomy, as determined by the presence of regular menstrual cycles and no vasomotor symptoms along with polycystic ovarian syndrome (PCOS) history if not already attained.[33]

What Happens after Menopause? (WHAM) study

WHAM is a prospective, controlled study of high-risk premenopausal women undergoing RRBSO.[34] Participants are premenopausal BRCA1 and BRCA2 gene mutation carriers recruited prior to RRBSO and followed up for 2 years post-RRBSO. None of these women had

ovarian cancer diagnosed at the time of surgery, and all have stored serum collected prior to oophorectomy for measurement of AMH. WHAM participants have already consented to use of their stored serum and ovarian tissue for HREC-approved studies (HREC12PMCC24 12/90).

Age-matched non-mutation carrier comparison group

Kathleen Cuningham Foundation Consortium for research into Familial Breast cancer

The kConFab (Peter Mac HREC #97_27) biobank contains donated ovarian tissue sections from women with a family history of breast cancer, including women who have been tested negative for a BRCA gene. kConFab has already collected ovarian tissue from women aged <50 years who have undergone oophorectomy and have tested negative for BRCA gene mutations. Stored serum samples are available for measuring AMH. At recruitment, participants already agreed to be routinely contacted by phone/email/post for general health updates and specific project requests. For this study, kConFab will contact potentially eligible participants to determine whether they were premenopausal at the time of oophorectomy, as determined by the presence of regular menstrual cycles and no vasomotor symptoms along with PCOS history if not already attained.[33]

WHAM study

WHAM participants include women who have undergone bilateral oophorectomy but have tested negative for BRCA1/2 gene mutations. All have stored ovarian tissue and serum samples available for AMH testing.

Premenopausal women at the time of caesarean section

Ovarian cortical biopsies have already been obtained from premenopausal women,[8] and ovarian follicle density has already been measured using the same protocol as the planned study. These participants do not have stored serum samples for AMH measurements.

Premenopausal women who have undergone ovarian tissue cryopreservation through MIVF

Ovarian tissue has already been collected from premenopausal women requesting fertility preservation at MIVF due to malignant and some non-malignant disease. These participants have already consented to their personal information and ovarian follicle count data being included in ethically approved research projects.

Premenopausal women who have undergone oophorectomy for benign conditions

Ovarian cortical biopsies have already been obtained from premenopausal women,[7] and ovarian follicle density has already been measured using the same protocol as the planned study. These participants do not have stored serum samples for AMH measurements.

Data collection

We will measure ovarian follicle density, DNA damage and apoptosis in existing ovarian tissue samples and quantify AMH in existing stored serum samples. Ovarian follicle density has already been measured in the participants from Scotland.[8] Participants are not expected to undergo any additional visits or procedures, and there will be no further follow-up. Data collection/gathering for kConFab participants is largely complete. The only additional information collected will be reproductive stage (premenopausal or postmenopausal) and any history of PCOS, where this information is not already available. These data have already been collected for WHAM participants. The only additional testing for WHAM and kConFab participants will be measurement of AMH in existing stored plasma samples.

Study procedures

Ovarian follicle density

Each histological section will be stained for the oocyte marker MVH and evaluated by light microscopy. Ovarian follicle density will be determined using previously established and validated methodology.[8 13 35] Follicles will be classified into stages according to McLaughlin *et al*.[36] Only follicles in which the nucleus is visible will be counted. The volume of the tissue analysed per patient will be calculated (area of the tissue analysed × thickness of the section), and follicle density will be expressed as follicles/mm^3. Tissues will be evaluated blind to gene mutation status and patient age by assigning a code to each sample.

Immunohistochemistry

We will assess the level of DNA damage in follicles in women with BRCA mutations by immunohistochemical analysis of γH2AX, known to form foci at DNA double-strand break sites. Every 10th ovarian tissue section (5 µm) will be assessed for DNA double-strand breaks using an antibody for Phospho-Histone (γ)H2AX (Ser139) (1:100; Cell Signalling Technology #9718). Primordial follicles with nuclear γH2AX foci will be counted as positive and expressed as a proportion of total primordial follicles assessed.

Terminal deoxynucleotidyl transferase dUTP nick end labelling

We will assess follicle apoptosis by performing TUNEL staining using the Apop Tag Peroxidase In Situ Apoptosis Detection Kit (Merck) on every 10th ovarian tissue section (5 µm). Follicles will be classified as positive if the oocyte and/or ≥2 granulosa cells are positive and expressed as a proportion of total primordial follicles assessed.

AMH analysis

AMH will be measured in existing stored plasma samples collected by kConFab or as part of the WHAM study. All plasma samples were collected prior to oophorectomy in women who were premenopausal. Plasma aliquots have been stored at −80° within 48 hours of collection. AMH measurements will be undertaken at the MIVF Endocrine Laboratory using the fully automated Elecsys AMH assay on the Roche Cobas electrochemiluminescence immunoassay platform in singleton.[37 38] The scientist conducting the assay will be blinded to participant mutation status

and patient age. The lowest level of detection of this assay is 0.07 pmol/L and the intra-assay and interassay imprecision coefficients of variation at 7.0 pmol/L are 4.6% and 5.6%, respectively.[23] Since the automated electrochemiluminescence method excludes manual handling, the requirement to run samples in duplicate or triplicate is not necessary. However, testing will be repeated if the result is less than 2 pmol/L for confirmation, if there are processing errors or if the result exceeds the reference range and a dilution is required. Samples are run in batches, and prior to each batch run, Biorad quality control samples will be included in each run at clinically relevant targets of 7, 34 and 105 (pmol/L). Plasma samples from WHAM have been stored for a mean of 3 years prior to AMH analysis, and none had been previously thawed. Quality assurance testing was performed on 30 non-study samples for which both plasma and serum were available; AMH concentrations using serum samples were approximately 5% higher than those using plasma. It was considered that this would not affect the study conclusions given that plasma was used for both the comparison groups.

Statistical analysis

Mean follicle density will be plotted against age for non-carriers, BRCA1 and BRCA2 mutation carriers. Groups will be compared using t-test or Wilcoxon test (if data are not normally distributed) to assess the primary objective. Correlation between AMH and ovarian follicle density will be measured by calculating the Pearson product-moment correlation coefficient. The mean difference between groups, and limits of agreement at 1 and 2 SDs from mean will be derived from Bland-Altman analysis. Proportional error will be calculated as the correlation between the means and differences derived from the Bland-Altman plot, with absence of correlation indicating low proportional error.

Outcome measures

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

Expected outcome significance

Largest prospective study of primordial follicle density (ovarian reserve) in premenopausal BRCA mutation carriers and the first to be adequately powered to distinguish between ovarian reserve in BRCA1 and BRCA2 mutation carriers and to compare findings with age-matched premenopausal non-mutation carrier comparison group.

- New information about ovarian reserve in BRCA mutation carriers that will guide decision making by high-risk women about fertility and family planning and inform healthcare professionals managing these patients.
- 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) that will inform the growing international debate about the veracity of AMH as a biomarker of ovarian reserve.

Data management

Patient data will be kept strictly confidential according to the National Statement on Ethical Conduct in Human Research 2007 and the Australian Code for Responsible Conduct of Research 2007. Patient research data will be kept onsite at the Women's Research Precinct at The Royal Women's Hospital. Data concerning ovarian follicle numbers and density will be kept onsite at Monash University. The results from AMH assays will be kept at MIVF. Patient research data will only be accessed by the named investigators and named statistician. Electronic records of research data will be retained on password-protected computer(s) in databases requiring password access. These data will be stored separately from the patient name in order to recontact potential participants for follow-up interviews. Any hard copies of data will be kept in locked facilities of the Royal Women's Hospital in the Department of Obstetrics and Gynaecology and at Monash University in the Department of Anatomy and Developmental Biology. All computers will be password protected, and electronic records stored on it will be coded and in databases requiring password access. Patient data will be only be transferred in a non-identifiable form. Individual patients will not be identifiable from the information stored, presented or published material. All data will be stored for 15 years. After this time the files will be destroyed by erasure and/or incineration unless further approval for retention is obtained.

DISCUSSION

Accurate measures of ovarian reserve in BRCA gene mutation carriers are important for two reasons. First, because pregnancy planning for BRCA gene mutation carriers is already complicated by their gene status and they should be informed if they have a reduced fertile lifespan compared with women of a similar age and second because BRCA1/2 are tumour suppressive genes that act to ensure the integrity of the genome through repair of DNA double-strand breaks.[39] Hence, the relationship between BRCA gene mutations and ovarian reserve may inform the mechanisms underlying ovarian ageing due to reduced primordial follicle reserve. Interestingly, there is limited information regarding oocyte quality of BRCA mutation carriers, and this should be the subject of future investigations.

We acknowledge some inherent limitations to this study design. The comparison group are premenopausal women who have been tested for BRCA gene mutations

and found to be negative or women at population risk of ovarian cancer who have undergone oophorectomy or ovarian biopsy for benign indications and are unlikely to carry BRCA mutations. Benign indications for oophorectomy (adnexal masses, torsion, pelvic pain, endometriosis and hysterectomy) are generally not associated with reduced ovarian reserve. Despite this, it would be interesting for future studies to evaluate potential differences in the ovarian reserve between this type of non-mutation carrier comparison group in our protocol, with 'healthy' individuals, although these samples are difficult to obtain. Aged-matched women from two sources have been tested and are negative for BRCA1/2 mutations. The comparison group from the remaining three sources have not been tested. The prevalence of BRCA1/2 mutations in the general population is very low, estimated at 0.1%. It is, therefore, unlikely that BRCA mutations are found in samples from these sources. The project team expect no confounding factors from these comparison groups. Additionally, there is no gold standard assay for the measurement of AMH. However, the automated Roche electrochemiluminescent method has the equivalent highest sensitivity and specificity to any other routinely used platform. As a large number of samples have already been assessed on the same instrument, the continued use in the posterior analysis will prevent the introduction of cross platform variability.

CONCLUSION

This is the first large-scale study aimed at exploring the measurement of ovarian reserve (as follicle density) in both BRCA1 and BRCA2 mutation carriers compared with age-matched non-mutation carriers and to compare follicle density with circulating levels of AMH. Improving the accuracy of association between circulating AMH and follicle density will enlighten the mechanism of oocyte loss during reproductive life particularly for women who are thought to have reduced ovarian reserve, such as BRCA mutation carriers.

Contributors ALW 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.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval 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? (HREC12PMCC24-12/90) and Melbourne IVF.

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ORCID iD

Amy Louise Winship http://orcid.org/0000-0001-7848-1447

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