Research Article

Osteoprotegerin (OPG), The Endogenous Inhibitor of Receptor Activator of NF-κB Ligand (RANKL), is Dysregulated in BRCA Mutation Carriers

Martin Widschwendter a,⁎, Matthew Burnella, Lindsay Fraser a, Adam N. Rosenthal a, Sue Philpotta, Daniel Reisel a, Louis Dubaub, Mark Cline c, Yang Pand, Ping-Cheng Yid, D. Gareth Evans e, Ian J. Jacobs a,f, Usha Menona, Charles E. Wood c, William C. Dougall g

a Department of Women's Cancer, UCL EGA Institute for Women's Health, University College London, 74 Huntley Street, London WC1E 6AU, UK
b Department of Pathology, Keck School of Medicine, USC/Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, CA, USA
c Department of Pathology, Section on Comparative Medicine, Wake Forest School of Medicine, Winston-Salem, NC 27157, USA
d Department of Molecular Sciences, Amgen Inc, Seattle, WA 98119, USA
e Department of Genomic Medicine, Institute of Human Development, University of Manchester, St. Mary’s Hospital, Manchester M13 9WL, UK
f The University of New South Wales, UNSW, Sydney, NSW 2052, Australia
g Therapeutic Innovation Unit (TIU), Amgen Inc, Seattle, WA 98119, USA

⁎ Corresponding author at: Department of Women’s Cancer, UCL EGA Institute for Women’s Health, University College London, 74 Huntley Street, London WC1E 6AU, UK. E-mail addresses: MWidschwendter@ucl.ac.uk, http://www.instituteforwomenshealth.ucl.ac.uk/womens-cancer (M. Widschwendter).

A R T I C L E   I N F O

Article history:
Received 23 July 2015
Received in revised form 26 August 2015
Accepted 26 August 2015
Available online 9 September 2015

Keywords:
Breast cancer
BRCA1 and BRCA2 mutations
RANKL
OPG
Carcinogenesis
Cancer prevention

A B S T R A C T

Breast cancer development in BRCA1/2 mutation carriers is a net consequence of cell-autonomous and cell non-autonomous factors which may serve as excellent targets for cancer prevention. In light of our previous data we sought to investigate the consequences of the BRCA-mutation carrier state on RANKL/osteoprotegerin (OPG) signalling. We analysed serum levels of RANKL, OPG, RANKL-OPG complex, oestradiol (E2), and progesterone (P) during menstrual cycle progression in 301 BRCA1/2-mutation carriers and 782 noncarriers. These studies were complemented by analyses of RANKL and OPG in the serum and mammary tissues of female cynomolgus macaques (n = 88) and serum RANKL and OPG in postmenopausal women (n = 150). BRCA-mutation carriers had lower mean values of free serum OPG in particular in BRCA1-mutation carriers (p = 0.018) compared with controls. Among BRCA1/2 mutation carriers, lower OPG levels were associated with germline mutation locations known to confer an increased breast cancer risk (p = 0.003). P is associated with low OPG levels in serum and tissue, particularly in BRCA-mutation carriers (rho = −0.216; p = 0.002). Serum OPG levels were inversely correlated (rho = −0.545, p < 0.001) with mammary epithelial proliferation measured by Ki67 expression and increased (p = 0.01) in postmenopause. The P–RANKL/OPG system is dysregulated in BRCA-mutation carriers. These and previously published data provide a strong rationale for further investigation of antiprogestogens or an anti-RANKL antibody such as denosumab for breast cancer prevention.

© 2015 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Risk factors for breast cancer development include genetic predisposition and exposure to elevated sex steroid hormones. Germline mutations in BRCA1/2 account for elevated sex steroid hormones and 2–10% of breast cancer cases depending on ethnic origin (Fackenthal and Olopade, 2007). Hormonal risk factors include early menarche, late menopause or first full pregnancy, weight gain, and combined hormone replacement therapy (HRT) (Veronesi et al., 2005). Evidence suggests a potential relationship between BRCA mutations, sex hormone levels, and end-organ effects to hormones, and cancer risk (Kim and Oktay, 2013; Segev et al., 2015; Titus et al., 2013; Widschwendter et al., 2013). High breast cancer risk in BRCA-mutation carriers is particularly evident premenopausally (relative risk 32 and 10 for 40–49-year-old BRCA1/2-mutation carriers, respectively) (Robson and Offit, 2007), whilst removal of both ovaries in premenopausal BRCA1/2-mutation carriers markedly reduces breast cancer risk (Domchek et al., 2010). Studies in BRCA1/p53-deficient mice indicate a direct role for progesterone (P) in mediating mammary tumourigenesis (Poole et al., 2006). Specific signalling pathways mediating interactions between BRCA-associated risk and sex steroid exposure have not been identified.

The relationship between higher serum sex steroid levels and increased breast cancer risk is well established in postmenopausal women (Fourkala et al., 2012; Key et al., 2011), but documenting a
premenopausal link is challenging due to hormonal fluctuations associated with menstrual cycle progression (Key et al., 2013; Kaaks et al., 2014). The temporal dynamics of oestradiol (E2), P, and other hormones during the menstrual cycle may be more important for breast cancer risk than absolute hormonal levels at a single time point (Brasken, 2013). Moreover, hormonal context, life stage, and reproductive status may significantly impact hormone-associated risk: transient exposure to high levels of P in women with multiple pregnancies has not been associated with increased breast cancer risk, whereas persistent exposure to relatively low progesterin levels postmenopausally has been associated with breast cancer in epidemiologic (Beral, 2003) and clinical (Chlebowski et al., 2003) studies.

BRCA mutations may lead to cell-autonomous defects including defects in chromosome duplication and cytokinesis (Venkitaraman, 2000). Although cell nonautonomous alterations such as hormonal alterations leading to aberrant growth of hormone-sensitive target cells may be particularly relevant to sporadic cancers (Veronesi et al., 2014). Although cell nonautonomous alterations such as hormonal effects in chromosome duplication and cytokinesis (Venkitaraman, 2000), studies have shown that the risk of breast cancer is significantly higher in women with BRCA1/2 mutations carriers meeting these criteria, and 782 noncarrier volunteers matched for age and day of menstrual cycle. All samples were stored in liquid nitrogen and tested at the same time. Additionally, we analysed serum from 50 and 150 healthy premenopausal and postmenopausal women (obtained from Cureline, S. San Francisco, CA), for which premenopausal criteria was defined as age ≤ 45 years or ≥ 3 consecutive normal menses immediately before blood collection; postmenopausal criteria was defined as age ≥ 52 years or last menses ≥ 12 months before blood collection. All women gave consent for their samples to be used for research.

2. Materials & Methods

2.1. Subjects and Design

2.1.1. Human

Subjects were participants in the UK Familial Ovarian Cancer Screening Study (UKFOCSS), recruited from 42 regional centres from June 2002–September 2010 after ethical approval (Eastern MREC 97/5/007). Included subjects were >35 years old with known BRCA status (Widschwendter et al., 2013), provided serum samples, no previous/subsequent history of cancer or intrauterine device, not used oral contraceptives during the collection period, and provided the dates of their last menstrual period. We enrolled 391 BRCA1/2-mutation carriers meeting these criteria, and 782 noncarrier volunteers matched for age and day of menstrual cycle. All samples were stored in liquid nitrogen and tested at the same time. Additionally, we analysed serum from 50 and 150 healthy premenopausal and postmenopausal women (obtained from Cureline, S. San Francisco, CA), for which premenopausal criteria was defined as age ≤ 45 years or ≥ 3 consecutive normal menses immediately before blood collection; postmenopausal criteria was defined as age ≥ 52 years or last menses ≥ 12 months before blood collection. All women gave consent for their samples to be used for research.

2.1.2. Animal

Archived samples were used from an experiment (Cline et al., 2002) involving 6–8-year-old, ovariectomised, adult female cynomolgus macaques (Macaca fascicularis) randomised to diets containing placebo (control; n = 31), 0.042 mg/kg conjugated equine oestrogens (CEE; n = 28), or CEE + 0.167 mg/kg medroxyprogesterone acetate (CEE + MPA; n = 29) over 2 years. Dose equivalents approximated standard HRT doses of CEE (0.625 mg/day) and MPA (2.5 mg/day). Serum and mammary tissue samples were collected at study end. Immunohistochemistry was performed as previously published for Ki67 and PgR (Cline et al., 2002) or RANK/RANKL antigens (Wood et al., 2013). H-scores, obtained by multiplying staining intensity (scales 0–3) with the number of positive cells (range, 0–300), were documented for epithelial tissues from each group for each antibody (Cline et al., 2002). Mammary gland expression of Ki67 (MKI67), RANKL (TNFSF11), and OPG (TNFRSF11B) mRNA was evaluated using real-time quantitative PCR, as previously described (Wood et al., 2013). All procedures involving macaques in the original study were conducted per state and federal laws and standards of the US Department of Health and Human Services and approved by the Wake Forest University Animal Care and Use Committee.

3. Procedures

3.1. Serum Steroid Hormone Assays

Serum E2 and P from randomly mixed and masked batches of mutation carriers and controls were measured with automated immunoassays on an Elecsys 1010 analyser (Roche Diagnostics GmbH, Mannheim, Germany) (Widschwendter et al., 2013). E2 and P intra-assay coefficients of variability (CV) were 1.6%–5.7% and 1.5%–2.7%, while interassay CVs were 2.3%–6.2% and 3.7%–5.4%, respectively.

3.2. RANKL, OPG, and RANKL/OPG Serum Assays

Free soluble RANKL (sRANKL), free soluble OPG (sOPG), and sRANKL/OPG complex were measured by ELISA. The assays were validated for dilutional linearity, sample parallelism, accuracy, and precision. sRANKL capture was achieved with recombinant human OPG (R&D Systems, Minneapolis, MN, Cat# 185-OS-CF) precoated onto a microwell, while sOPG capture was achieved by precoating microplates with soluble recombinant huRANKL (R&D Systems, Cat# 390-TN-CF).
Postwashing, RANKL and OPG were detected by incubations with biotinylated monoclonal antibodies (Amgen Clones 80A9 & 78H10 for RANKL; PeproTech, Rocky Hill, NJ, Cat# 500-P149Bt for OPG) followed by washing and incubation with streptavidin peroxidase and a fluorogenic substrate solution (Thermo Scientific, Waltham, MA, Cat# 15,169). Free sRANKL and OPG were quantified by interpolation from standard curves (using Amgen recombinant huRANKL or Cat# 185-OS/CF from R&D) for each plate. The lower limit of quantitation for sRANKL assay was 1.5 pg/ml. Similar procedures were applied for detection of sRANKL/OPG complex captured with anti-huRANKL mAb 74F1 (Amgen Inc.).

4. Outcomes

4.1. Statistical Analysis

Differences between BRCA-mutation carriers and wild-type controls as a function of the menstrual cycle were assessed using linear regression models for each hormone and allowing hormone levels to vary differentially over time for cases and controls. Specifically, the log hormone levels were regressed on restricted cubic spline functions of time using three internal knots, interacted with case–control status and age. The knots were placed where a local polynomial model suggested P turning points (days 7 and 22) and where the luteal phase typically begins (day 13), although spline functions are largely insensitive to knot placement (Lambert and Royston, 2009). Likelihood ratio tests (LRTs) compared the full model with a nested model allowing no case–control interaction with time functions and to dynamically test the null hypothesis of no difference between BRCA-mutation carriers and controls. There were five degrees of freedom for the LRTs; the full model contained an additional four interaction terms with the four spline functions and one individual case–control factor term. Analyses were repeated using trigonometric functions (sine and cosine) of time, as the spline approach does not guarantee endpoint convergence, although trigonometric functions may not capture dynamic trends sufficiently. LRTs here had three degrees of freedom.

To visualise the spline (or trigonometric) functions, fitted mean curves for each mutational status type, fixed at the mean age of 42.2, were plotted and superimposed upon individual data points separately coded for BRCA1-carriers, BRCA2-carriers, and controls. Further, a mean difference curve (mean[log(value_{BRCA})] − mean[log(value_{control})]) was plotted for each hormone, comparing each group of interest. For both plot types, 95% confidence bands assumed normality of mean response and were calculated with Stata's margin command, using a factor or contrast operator, respectively, set to the necessary values of spline functions and age.

By summing the absolute deviations of the difference curves over the menstrual cycle, we estimated differential expression between cases and controls over vectors of the relevant hormones. Because all hormones were logged, difference estimates were based on a common scale, the log(ratio difference). This sum of absolute differences was estimated by fitting a multivariate regression model and using an LRT on

![Fig. 1. Serum analysis of hormones. Analysis of (log) (a) progesterone, (b) oestradiol, (c) RANKL, (d) OPG, and (e) RANKL/OPG complex in serum from women with and without a mutation in the BRCA1 or BRCA2 gene as a function of the menstrual cycle. The ranges of concentrations were: sRANKL (2.9 pg/ml–2135 pg/ml), sOPG (10.8 pg/ml–1414 pg/ml) and sRANKL/OPG complex (17.4 pg/ml–225,101 pg/ml). RANKL = receptor activator of NF-κB ligand, OPG = osteoprotegerin, and CI = confidence interval.](image)
20° of freedom to test the overall difference between cases and controls over the hormone vector. The vector of absolute differences follows a multivariate folded-normal distribution from which correct inferences can be difficult to draw. We therefore approximated the sum and associated 95% confidence band with 1000 bootstrap samples, using the 50th, 2.5th, and 97.5th centiles, respectively. The bootstrap median (or mean) was slightly larger than the simple sum of absolute differences calculated from each individual hormone because the absolute mean of a normal distribution is always less than that of its corresponding folded-normal distribution. Similarly, the appropriate null hypothesis value (based on a multivariate half-normal distribution) is not zero, but larger, ensuring positive values for the sum of absolute deviations. We estimated this null hypothesis value for each day of the cycle by simulation from a multivariate normal distribution \((n = 100,000)\) using a mean vector of zeroes and a covariance matrix containing the set of estimated variance-covariance terms from the multivariate model.

Tests of mean difference between each treatment group of experimental animals were performed with a Dunnett-type adjustment for multiple comparisons to a control.

We used the reported hazard ratios (HR) for breast cancer based on the nucleotide position of the \(BRCA1\) and \(BRCA2\) mutation (Rebbeck et al., 2015). The estimated HRs indicating the risk for breast cancer at specific regions of the \(BRCA1/2\) mutation was regressed on the log of free serum OPG (pg/ml), adjusted for age at sample at cycle day. The use of fractional polynomials indicated that a linear fit was appropriate.

5. Role of the Funding Source

The work was in part sponsored by Amgen Inc. Amgen Inc. employees (YP, PY, and WCD) were involved in the study design, data collection, and data analysis (ie RANKL and OPG assays in human and animal tissues), data interpretation, and writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication. He is accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

6. Results

The mean log measurements of serum P, E2, free RANKL, free OPG, and RANKL/OPG complex were fitted as a function of time, with separate mean curves for \(BRCA\)-mutation carriers vs controls. Overlays of curves illustrating individual data points of mutation carriers, and controls showed typical menstrual cycle dynamics for P and E2, but free RANKL, free OPG and RANKL/OPG complex did not vary substantially throughout the menstrual cycle (Fig. 1).

We examined potential hormone level variations between \(BRCA\)-mutation carriers and controls from difference curves over the menstrual cycle with 95% confidence bands (Fig. 2; Supplemental Fig. 1). Overall, the LRT for P was not significantly different between cases and controls.

![Fig. 2. Case–control differences in hormone values. Mean differences in (log) (a–c) RANKL and (d–f) OPG in serum between women with and without a mutation in the \(BRCA1\) and/or \(BRCA2\) gene as a function of the menstrual cycle, with 95% confidence bands. RANKL = receptor activator of NF-\(κB\) ligand, LRT = likelihood ratio test, df = degrees of freedom, and OPG = osteoprotegerin.](image-url)
(p = 0.1114, Supplemental Table 1), although the difference plots showed significantly higher levels in BRCA-mutation carriers around days 6 and 24 (especially BRCA1). The LRT for E2 showed no difference between cases and controls (p = 0.4623); however, levels tended to be higher in the follicular phase, mostly for BRCA2. All mutation carriers had lower mean values of free serum RANKL than controls (p = 0.0006), particularly in the early- to mid-follicular phase. Overall difference in the mean curves (p = 0.0649) for free serum OPG was indicated, while OPG levels were significantly lower throughout the menstrual cycle in BRCA1-mutation carriers. Serum RANKL/OPG complex values were consistently lower in BRCA carriers (p = 0.008) throughout the cycle. This was the only variable for which the spline function-fitted mean curves did not display endpoint-convergence. Overall conclusions based on LRT p-values (Supplemental Table 1) and mean curve shapes were broadly similar using trigonometric functions. Stratification by BRCA1/2 status revealed no significant difference, although differences vs controls appeared greater for BRCA1-mutation carriers, most notably for P and OPG.

Supplemental Fig. 2a depicts the sum of absolute differences for all serum hormones analysed, with an approximate null hypothesis curve for inferential comparison. There was strong evidence of differential levels across the four distinct hormones analysed between BRCA1/2-mutation carriers and controls (multivariate LRT p = 0.0007), most noticeable during days 4–11, with the peak at 7 days corresponding to a 16.0% (95% CI: 7.1%–26.2%) geometric mean difference in hormone expression vs the null (Supplemental Fig. 2b). No serum RANKL peak was apparent in the luteal phase (Fig. 1), but a clear inverse pattern of expression vs the null (Supplemental Fig. 2a). No serum RANKL peak was observed (Fig. 2d–f; Supplemental Fig. 1a–c). Correlation of serum P with serum RANKL, OPG, and RANKL/OPG in the luteal phase separately for controls and mutation carriers showed no association between serum P and RANKL or RANKL/OPG (Supplemental Fig. 3). Serum P and serum OPG demonstrated a negative association that was much more significant in BRCA-mutation carriers (rho = −0.216; p = 0.002) vs controls (rho = −0.098; p = 0.06; Supplemental Fig. 4).

Recent evidence indicates that BRCA1/2 mutation triggered breast cancer risk also depends on the actual site of the germline mutation (Rebeck et al., 2015). In order to test whether the breast cancer risk within the BRCA-mutation carriers might be associated with serum OPG levels we performed a linear regression analysis (adjusted for age and menstrual cycle day) of serum OPG levels and reported breast cancer risk associated with specific sites of mutations in the 222 samples for which we had exact information on the site of the mutation (Rebeck et al., 2015). We observed a significant decrease in reported HR for breast cancer risk with increasing serum OPG levels (beta = −0.058; 95% CI: −0.020, −0.096; p = 0.003; Fig. 3).

Because P was negatively associated with serum OPG, especially in BRCA-mutation carriers, but the well-established P-mediated increase in RANKL in breast tissue was not mirrored in serum, we examined serum and mammary gland tissues from ovariectomised adult female cynomolgus macaques treated with no hormones (control), oestrogen only (CEE) or oestrogen/progestin (CEE + MPA) for 2 years. Whereas progestin treatment led to higher RANKL expression in the mammary gland (Fig. 4a), no differences in serum RANKL were observed between the three groups (Fig. 4b). In contrast, OPG was significantly lower post-progestin treatment in both breast (Fig. 4c) and serum (Fig. 4d).

We then evaluated whether serum OPG was associated with increased mammary epithelial proliferation, and found a significant inverse correlation (rho = −0.545, p < 0.001; Fig. 5a) between serum OPG and mammary gland Ki67 mRNA expression. Although we cannot distinguish the effects of OPG from direct effects of P on proliferation, the significant inverse correlations between serum OPG and mammary gland proliferation with either oestrogen alone or oestrogen/progestin (Fig. 5a), plus the significant inverse correlations between serum OPG and Ki67 expression within alveolar (Fig. 5b) or ductal (Fig. 5c) epithelia for each treatment group (p < 0.05 for CEE or CEE + MPA), suggest an effect of OPG independent of PgR signalling (Supplemental Fig. 5).

**Fig. 3.** Association between the nucleotide position of the BRCA1/2 germline mutation and serum OPG levels. The estimated hazard ratio (HR) indicating the risk for breast cancer depending on the nucleotide position of the BRCA1/2 mutation was regressed on the log of free serum OPG (pg/ml) adjusted for age and menstrual cycle day at sample donation (a). Serum samples from human volunteers have been split according to the HR (<0.9; 0.9–1.1; >1.1) given by the volunteers nucleotide position of the BRCA mutation and serum OPG levels blotted for each group. OPG = osteoprotegerin.
A strong association was noted between mammary tissue RANKL and Ki67 expression (Fig. 6a), but not with RANK (Fig. 6b). In animals expressing RANKL in the mammary gland, low (median) serum OPG levels trended (p = 0.09) towards higher mammary gland proliferation (Fig. 6a).

Finally, evaluation of OPG levels in postmenopause (a state known to protect from breast cancer) suggested that serum OPG levels are significantly (p = 0.01) higher (Fig. 7a) in postmenopause vs premenopause; the opposite tendency (p = 0.0001) was true for serum RANKL (Fig. 7b).

7. Discussion

Hormonal factors, particularly exposure to P, may influence breast cancer risk associated with BRCA mutations (Poole et al., 2006; Widschwendter et al., 2013). We found that dysregulation of OPG may mediate this relationship. We examined serum RANKL and OPG levels across the menstrual cycle and their associations with hormonal responsiveness in the mammary gland. OPG was dysregulated in BRCA-mutation carriers and inversely associated with breast cancer risk and mammary epithelial proliferation, as suggested by the aberrantly low OPG through most of the menstrual cycle in mutation carriers (in particular in those at extremely high risk for breast cancer), an inverse correlation between serum OPG and luteal-phase P levels that was more marked in BRCA-mutation carriers, low serum OPG levels in the animal model associated with increased mammary epithelial cell proliferation, and significantly higher OPG levels in the absence of functional ovaries (i.e. postmenopause). Interestingly, OPG levels in breast and serum were both decreased in presence of P. However, serum levels of RANKL do not appear to reflect local increases at the tissue level, including breast (this study) and bone (Eghbali-Fatourechi et al., 2003). These data suggest that the net magnitude of RANK signalling in the breast upon P exposure may be regulated by local increases of RANKL along with decreases in local and systemic (serum) OPG thereby increasing the RANKL:OPG ratio in this tissue (Wood et al., 2013).

RANK pathway activation by P-mediated RANKL upregulation plays an important role in breast carcinogenesis (Gonzalez-Suarez et al., 2010; Schramek et al., 2010), in part by increasing mammary stem cell proliferation (Schramek et al., 2010; Joshi et al., 2010). Moreover, deleting RANK from the mammary epithelium decreases incidence and delays onset of PgR-mediated mammary cancer (Schramek et al., 2011), indicating that RANK signalling suppression might be an excellent strategy for breast cancer prevention. Whereas anti-P treatment inhibits mammary tumourigenesis in BRCA1/p53-deficient mice (Schramek et al., 2011), long-term treatment of premenopausal women with selective PgR modulators has not been tested sufficiently and would probably lead to substantial side-effects, including adverse effects on the endometrium (Benagiano et al., 2014). Because OPG was not regulated throughout the menstrual cycle and luteal-phase P showed a much stronger inverse association in BRCA-mutation carriers vs controls, cell-autonomous factors might be involved in P-OPG regulation. Hence, our study provides strong support for the direct targeting of RANKL (ie replacing low OPG levels in mutation carriers), an idea so far only investigated in mice because of the influence of the menstrual cycle on RANKL signalling in premenopausal humans (Haynes et al., 2014; Hu et al., 2014).
Fig. 5. Serum OPG and mammary gland proliferation. Correlation between serum OPG and mammary gland Ki67 (a) mRNA and IHC labelling in (b) alveolar epithelial cells and (c) ductal epithelial cells in ovariectomised adult female cynomolgus macaques treated for two years with placebo (control), CEE at 0.042 mg/kg CEE or CEE + MPA at 0.167 mg/kg (CEE + MPA). IHC = immunohistochemical, OPG = osteoprotegerin, CEE = conjugated equine oestrogens, and MPA = medroxyprogesterone acetate.

Fig. 6. Mammary gland proliferation stratified by RANKL and RANK expression in the mammary gland and OPG levels in the serum. Cynomolgus macaques were divided according to expression of (a) RANKL and (b) RANK in their mammary gland and then substratified according to the serum OPG level (high/low, above and below the median). OPG = osteoprotegerin, RANKL = receptor activator of NF-κB ligand, IHC = immunohistochemical, and RANK = receptor activator of NF-κB.
Recent evidence suggests OPG deficiency may promote breast cancer development. Serum OPG analysis in >6000 healthy subjects (Vik et al., 2015) showed an inverse relation in younger women (<60 years) between serum OPG and risk of incident cancer at all sites, but primarily in the breast and reproductive tract (Vik et al., 2015). High OPG levels protected against breast cancer and mortality (Vik et al., 2015). An independent analysis of ~4500 primary breast cancers showed no prognostic value for RANK/RANKL expression, but a substantially better prognosis in oestrogen receptor-positive breast cancer cases showing high OPG expression (Sanger et al., 2014). A "suppressed" RANK pathway was likewise associated with better prognosis in another large breast cancer set (Santini et al., 2011).

Denosumab, an antibody against RANKL that mimics the physiological role of OPG, is currently in clinical use for osteoporosis and for the treatment of skeletal related events in patients with bone metastasis resulting from solid tumours. In patients with breast cancer, denosumab has recently been shown to reduce the risk of clinical fractures in postmenopausal women with breast cancer receiving aromatase inhibitors and is currently being studied for any effect on prolonging bone metastasis free survival in women with early-stage breast cancer who are at high risk for disease recurrence (Gnant et al., 2015). While our previous data highlighted the potential for antiprogestogens for breast cancer prevention, the current data additionally support a clinical breast cancer prevention trial using denosumab to compensate for the low OPG levels throughout the menstrual cycle in BRCA-mutation carriers.

**Contributors**

MW had the idea, did the literature search, designed the study, was involved in data collection and data interpretation, wrote the first draft of the manuscript and together with MB did the statistical analysis and prepared the Figures. WCD made substantial contributions to the conception and design of the work. LF, ANR, SP, DR, DGE, UM, and IJJ collected, analysed and interpreted the human data. LD interpreted the data. MC, CEW, and WCD contributed the animal data. YP, PY, and WCD developed the serum RANKL, OPG, and RANKL/OPG assays and analysed the human and animal serum samples. All authors were involved in revising the manuscript for important critical content. All authors approved the final version of this manuscript.

**Declaration of Interests**

MW, MB, LF, SP, DR, LD, MC, DGE, IJJ and CEW have no conflict of interests to declare.

ANR received honoraria from Roche, provided expert testimony for AstraZeneca, and consulted for Myriad and Abcodia.

UM has stocks and received research funding from Abcodia Ltd. which has an interest in cancer screening and biomarkers.

YP, PY, and WCD are employed by and own Amgen Inc. stocks.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.08.037.

**Acknowledgements**

This work was funded by Amgen Inc. and the Eve Appeal (http://www.eveappeal.org.uk/) and undertaken at UCLH/UCL, which received a proportion of its funding from the Department of Health NIHR Biomedical Research Centres (BRC) funding scheme. The UKFOCSS was core funded by Cancer Research UK (grants C315/A2621 and C1005/A6383), the UK Department of Health and the Eve Appeal with additional support from grants MRC8804/A/A7058, CA152990 & CA086381 from the NCI Early Detection Research Network. This work was aided by grant number R01 CA133117 from the US National Institutes of Health, by Organon Pharmaceuticals, and the Martin and Sharleen Cohen Foundation for Biomedical Research. Editing and formatting support was provided by Albert Y. Rhee (Amgen Inc).

**References**

Benagiano, G., Bastianelli, C., Farris, M., Brosens, I. 2014. Selective progesterone receptor modulators: an update. Expert. Opin. Pharmacother. 15, 1403–1415.

Beral, V., 2003. Breast cancer and hormone-replacement therapy in the million women study. Lancet 362, 419–427.
Branden, C., 2013. Progesterone signalling in breast cancer: a neglected hormone coming into the limelight. Nat. Rev. Cancer 13, 385–396.
Chlebowski, R.T., Hendrix, S.L., Langer, R.D., et al., 2003. Influence of estrogen plus proges-
terin on breast cancer and mammography in high-risk postmenopausal women: the women's health initiative randomized trial. JAMA 289, 3243–3253.
Chodankar, R., Kwang, S., Sangiorgi, F., et al., 2005. Cell-nonautonomous induction of ovarian and uterine serous cystadenomas in mice lacking a functional Brca1 in ovar-
ian granulosa cells. Curr. Biol. 15, 561–565.
Cline, J.M., Register, T.C., Clarkson, T.B., 2002. Effects of tibolone and hormone replace-
ment therapy on the breast of cynomolgus monkeys. Menopause 9, 422–429.
Cruz, J., Sestak, I., Forbes, J.F., et al., 2014. Anastrozole for prevention of breast cancer in high-risk postmenopausal women (IBIS-B): an international, double-blind, randomised placebo-controlled trial. Lancet 383, 1041–1048.
Dovek, C., 2013. Progesterone signalling in breast cancer: a neglected hormone coming into the limelight. Nat. Rev. Cancer 13, 385–396.
Fackenthal, J.D., Olopade, O.I., 2007. Breast cancer risk associated with BRCA1 and BRCA2 in diverse populations. Nat. Rev. Cancer 7, 937–948.
Findlay, D., Chehade, M., Tsangari, H., et al., 2008. Circulating RANKL is inversely related to RANKL mRNA levels in bone in osteoarthritic males. Arthritis Res. Ther. 10, R2.
Forbes, J.F., Cruz, J., Buzdar, A., Howell, A., Tobias, J.S., Baum, M., 2008. Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 100-month analysis of the ATAC trial. Lancet Oncol. 9, 45–53.
Foulkes, W.D., Metcalfe, K., Sun, P., et al., 2004. Estrogen receptor status in BRCA1- and BRCA2-related breast cancer: the influence of age, grade, and histological type. Clin. Cancer Res. 10, 2029–2034.
Fournkala, E.O., Zakin, A., Burnell, M., et al., 2012. Association of serum sex steroid receptor bioactivity and sex steroid hormones with breast cancer risk in postmenopausal women. Endocr. Relat. Cancer 19, 137–147.
Gaunt, M., Pfeifer, G., Dubsky, P.C., et al., 2015. Adjuvant denosumab in breast cancer (ABC5G–18): a multicentre, randomised, double-blind, placebo-controlled trial. Lancet.
Gonzalez-Suarez, E., Jacob, A.P., Jones, J., et al., 2010. RANK ligand mediates progestin-in-
duced mammary epithelial proliferation and carcinogenesis. Nature 468, 103–107.
Haynes, B.P., Vieal, G., Galimberti, V., et al., 2014. Differences in expression of proliferation-associated genes and RANKL across the menstrual cycle in estrogen receptor-
positive primary breast cancer. Breast Cancer Res. Treat. 148, 327–335.
Hong, H., Yen, H.Y., Brockmeyer, A., et al., 2010. Changes in the mouse estrus cycle in re-
sponse to BRCA1 inactivation suggest a potential link between risk factors for familial and sporadic ovarian cancer. Cancer Res. 70, 221–228.
Hu, H., Wang, J., Gupta, A., et al., 2014. RANKL expression in normal and malignant breast tissue responds to progesterone and is up-regulated during the luteal phase. Breast Cancer Res. Treat. 146, 515–523.
Joshi, P.A., Jackson, H.W., Beristain, A.G., et al., 2010. Progesterone induces adult mamma-
ry stem cell expansion. Nature 465, 803–807.
Kaaks, R., Tikka, K., Sookthai, D., et al., 2014. Premenopausal serum sex hormone levels in relation to breast cancer risk, overall and by hormone receptor status — results from the EPIC cohort. Int. J. Cancer 134, 1947–1957.
Key, T.J., Appleby, P.N., Reeves, G.K., et al., 2011. Circulating sex hormones and breast can-
cer risk factors in postmenopausal women: reanalysis of 13 studies. Br. J. Cancer 105, 709–722.
Key, T.J., Appleby, P.N., Reeves, G.K., et al., 2013. Sex hormones and risk of breast cancer in premenopausal women: a collaborative reanalysis of individual participant data from seven prospective studies. Lancet Oncol. 14, 1009–1019.
Kim, J., Oktay, K., 2013. Baseline E2 levels are higher in BRCA2 mutation carriers: a poten-
tial target for prevention? Cancer Causes Control 24, 421–426.
Lambert, P.C., Royston, P., 2009. Further development of flexible parametric models for survival analysis. StatA J. 9, 265–290.
Metcalfe, K., Lynch, H.T., Foulkes, W.D., et al., 2015. Effect of oophorectomy on survival after breast cancer in BRCA1 and BRCA2 mutation carriers. JAMA Oncol. 1, 306–313.
Poole, A.J., Li, Y., Kim, Y., Lin, S.C., Lee, W.H., Lee, E.Y., 2006. Prevention of BRCA1-mediated mammary tumorigenesis in mice by a progesterone antagonist. Science 314, 1467–1470.
Robson, M., Offit, K., 2007. Clinical practice. Management of an inherited predisposition to breast cancer. N. Engl. J. Med. 357, 154–162.
Sangera, N., Ruckhaberle, E., Bianchini, C., et al., 2014. OPG and PGF show similar cohort specific effects as prognostic factors in ER positive breast cancer. Mol. Oncol. 8, 116–1207.
Santin, D., Schiavon, G., Vincenzi, B., et al., 2011. Receptor activator of NF-κB (RANK) ex-
pression in primary tumors associates with bone metastasis occurrence in breast cancer patients. PloS One 6, e19234.
Schrämke, D., Leibbrandt, A., Sigl, V., et al., 2010. Osteoclast differentiation factor RANKL controls development of progestin-driven mammary cancer. Nature 468, 98–102.
Schrämke, D., Sigl, V., Penninger, J.M., 2011. RANKL and RANK in sex hormone-induced breast cancer and breast cancer metastasis. Trends Endocrinol. Metab 22, 188–194.
Segev, Y., Rosen, B., Lubinski, J., et al., 2015. Risk factors for endometrial cancer among women with a BRCA1 or BRCA2 mutation: a case control study. Fam. Cancer (April 3 [Epub ahead of print]).
Titus, S., Li, F., Stobezki, R., et al., 2013. Impairment of BRCA1-related DNA double-strand break repair leads to ovarian aging in mice and humans. Sci. Transl. Med. 5 [172ra21].
Venkitaraman, A.R., 2014. Cancer suppression by the chromosome custodians, BRCA1 and BRCA2. Science 343, 1470–1475.
Veronesi, U., Boyle, P., Goldhirsch, A., Orecchia, R., Viale, G., 2005. Breast cancer. Lancet 365, 1727–1741.
Vik, A., Brodin, E.E., Mathiesen, E.B., et al., 2015. Serum osteoprotegerin and future risk of cancer and cancer-related mortality in the general population: the Tromsø study. Eur. J. Epidemiol. 30, 219–230.
Widschwendter, M., Rosenthal, A.N., Philpott, S., et al., 2013. The sex hormone system in carriers of BRCA1/2 mutations: a case–control study. Lancet Oncol. 14, 1226–1232.
Wood, C.E., Branstetter, D., Jacob, A.P., et al., 2013. Progestin effects on cell proliferation pathways in the postmenopausal mammary gland. Breast Cancer Res. 15, R62.
Yen, H.Y., Cahet, Y., Liu, Y., et al., 2012. Alterations in Brca1 expression in mouse ovarian granulosa cells have short-term and long-term consequences on estrogen-responsive organs. Lab. Investig. 92, 802–811.