

***BRCA1* promoter methylation and clinical outcomes in ovarian cancer: an individual patient data meta-analysis**

Roshni D Kalachand, MBBCh

Medical Oncology Group, Department of Molecular Medicine, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin 9, Ireland

Britta Stordal, PhD*

Department of Natural Sciences, Middlesex University, Hendon, London NW4 4BT, United Kingdom

Stephen Madden, PhD*

Data Science Centre, Royal College of Surgeons in Ireland, Beaux Lane House, Dublin 4, Ireland

Benjamin Chandler, BSc

Department of Radiation Oncology, University of Michigan, Ann Arbor, Michigan

Julie Cunningham, PhD

Department of Laboratory Medicine and Pathology, Division of Experimental Pathology, Mayo Clinic, Rochester, Minnesota

Ellen L Goode, PhD

Department of Health Sciences Research, Division of Epidemiology, Mayo Clinic, Rochester, Minnesota

Ilary Ruscito, MD

Department of Gynecology, European Competence Center for Ovarian Cancer, Campus Virchow Klinikum, Charité - Universitätsmedizin Berlin, Corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, Berlin Institute of Health, Berlin, Germany.

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Cell Therapy Unit and Laboratory of Tumor Immunology, Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy.

Elena I Braicu, MD, PhD

Department of Gynecology, European Competence Center for Ovarian Cancer, Campus Virchow Klinikum, Charité - Universitätsmedizin Berlin, Corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, Berlin Institute of Health, Berlin, Germany.

Jalid Sehouli, MD, PhD

Department of Gynecology, European Competence Center for Ovarian Cancer, Campus Virchow Klinikum, Charité - Universitätsmedizin Berlin, Corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, Berlin Institute of Health, Berlin, Germany.

Atanas Ignatov, MD

Department of Gynecology and Obstetrics, University Medical Center, Regensburg, Germany

Herbert Yu, MD, PhD

University of Hawaii Cancer Centre, Honolulu, Hawaii

Dionyssios Katsaros, MD, PhD

AOU Citta della Salute and Department of Surgical Sciences, Gynecologic Oncology, University of Torino, Italy

Gordon B Mills, MD, PhD

Department of Cell, Development and Cancer Biology Knight Cancer Institute, Oregon Health and Sciences University, Portland Oregon

Karen H Lu, MD

University of Texas MD Anderson Cancer Center, Houston, Texas

Mark S Carey, MD

Department of Obstetrics and Gynaecology, Division of Gynecologic Oncology, University of British Columbia, Vancouver, British Columbia, Canada

Kirsten M Timms, PhD

Myriad Genetics, Inc., Salt Lake City, Utah

Jolanta Kupryjanczyk, MD, PhD

Department of Pathology and Laboratory Diagnostics, Maria Sklodowska-Curie Institute -
Oncology Center, Warsaw, Poland

Iwona K Rzepecka, PhD

Department of Pathology and Laboratory Diagnostics, Maria Sklodowska-Curie Institute -
Oncology Center, Warsaw, Poland

Agnieszka Podgorska, MSc

Department of Pathology and Laboratory Diagnostics, Maria Sklodowska-Curie Institute -
Oncology Center, Warsaw, Poland

Jessica N McAlpine, MD

Department of Gynecology and Obstetrics, Division of Gynecologic Oncology, University of
British Columbia, Vancouver, BC, Canada

Elizabeth M Swisher, MD

University of Washington School of Medicine, Seattle, Washington

Sarah S Bernards, BSc

University of Washington School of Medicine, Seattle, Washington

Ciaran O’Riain, MBBCh, FRCPath

Department of Histopathology, Trinity College Dublin, Central Pathology Laboratory, St. James's
Hospital, Dublin 8, Ireland

Sharon O’Toole, PhD

Department of Obstetrics and Gynaecology/Histopathology, Trinity College Dublin, Trinity
Centre for Health Sciences, St. James's Hospital, Dublin 8, Ireland

Emer Casey Research Laboratory, Molecular Pathology Laboratory, The Coombe Women and
Infants University Hospital, Dublin 8, Ireland

John J O’Leary, MD, PhD

Department of Histopathology, Trinity College Dublin, Central Pathology Laboratory, St. James's Hospital, Dublin 8, Ireland

Emer Casey Research Laboratory, Molecular Pathology Laboratory, The Coombe Women and Infants University Hospital, Dublin 8, Ireland

David D Bowtell, PhD

Peter MacCallum Cancer Centre, Melbourne, 3000, VIC, Australia

David M Thomas, MD, PhD, FRACP

Genomic Cancer Medicine, Cancer Division, Garvan Institute of Medical Research, The Kinghorn Cancer Centre, Darlinghurst, Australia

Katharina Prieske, MD

Department of Gynecology and Gynecologic Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Simon A Joosse, PhD

Department of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Linn Woelber, MD, PhD

Department of Gynecology and Gynecologic Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Parvesh Chaudhry, PhD

Department of Radiotherapy, Postgraduate Institute of Medical Education and Research, Chandigarh, India

Norman Häfner, PhD

Department for Gynaecology and Reproductive Medicine, Jena University Hospital—Friedrich Schiller University Jena, Jena, Germany

Ingo B Runnebaum, MD, MBA

Department for Gynaecology and Reproductive Medicine, Jena University Hospital—Friedrich Schiller University Jena, Jena, Germany

Bryan T. Hennessy, MD

Department of Medical Oncology, Beaumont Hospital, Dublin 9 and Our Lady of Lourdes Hospital, Drogheda, Ireland

Medical Oncology Group, Department of Molecular Medicine, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin 9, Ireland

** Joint second authors*

Corresponding author:

Dr Roshni Kalachand

Medical Oncology Group, Department of Molecular Medicine, Royal College of Surgeons in Ireland, Beaumont Hospital, Beaumont Road, Dublin 9, D09 YD60, Ireland

Phone: +353879016610

Email: rkalachand@rcsi.ie

ORCID ID: <https://orcid.org/0000-0003-3060-7877>

ABSTRACT

Background: *BRCA1* methylation has been associated with homologous recombination deficiency, a biomarker of platinum sensitivity. Studies evaluating *BRCA1*-methylated tubal/ovarian cancer (OC) do not consistently support improved survival following platinum chemotherapy. We examine the characteristics of *BRCA1*-methylated OC in a meta-analysis of individual participant data.

Methods: 2636 participants' data across 15 studies were analyzed. *BRCA1*-methylated tumors were defined according to their original study. Associations between *BRCA1* methylation and clinico-pathological characteristics were evaluated. The effects of methylation on overall survival (OS) and progression-free survival (PFS) were examined using mixed-effects models. All statistical tests were two-sided.

Results: 430 (16.3%) tumors were *BRCA1*-methylated. *BRCA1* methylation was associated with younger age and advanced-stage high-grade serous OC. There were no survival differences between *BRCA1*-methylated and non-*BRCA1*-methylated OC (median PFS = 20.0 vs 18.5 months, HR = 1.01, 95% CI = 0.87–1.16, $P=0.98$; median OS = 46.6 vs 48.0 months, HR = 1.02, 95% CI = 0.87–1.18, $P=0.96$). Where *BRCA1/2* mutations were evaluated ($n=1248$), *BRCA1* methylation displayed no survival advantage over *BRCA1/2* intact (*BRCA1/2* wild type non-*BRCA1*-methylated) OC. Studies used different methods to define *BRCA1* methylation. Where *BRCA1* methylation was determined using methylation-specific PCR and gel electrophoresis ($n=834$), it was associated with improved survival (PFS: HR = 0.80, 95% CI = 0.66–0.97, $P=0.02$; OS: HR = 0.80, 95% CI = 0.63–1.00, $P=0.05$) on mixed-effects modelling.

Conclusion: *BRCA1*-methylated OC displays similar clinico-pathological features to *BRCA1*-mutated OC, but is not associated with survival. Heterogeneity within *BRCA1* methylation

assays influences associations. Refining these assays may better identify cases with silenced *BRCA1* function and improved patient outcomes.

Epithelial tubal, primary peritoneal and ovarian cancer, hereafter referred to as ovarian cancer (OC), is an aggressive disease with poor patient outcomes. High-grade serous cancer (HGSC) is the most common and lethal form of OC¹. Targeting homologous recombination deficiency (HRD), a molecular hallmark in approximately 50% of HGSC, could improve outcomes for a substantial number of women with OC. *BRCA1/2* germline and somatic mutations are observed in 15-20% of OC and account for ~1/3 of HRD tumors^{2,3}. These mutations are predictors of platinum and poly-ADP-ribose polymerase inhibitors (PARPi) response and are prognostic for improved outcomes in OC^{4,5}. Identifying other mechanisms producing HRD could expand the number of women with OC benefiting from PARPi. Another possible though less well characterised mechanism of HRD is *BRCA1* promoter methylation, occurring in approximately 10-15% of HGSC^{3,6-8}, although reported rates vary between 5-89.9%^{9,10}. By virtue of epigenetic silencing of *BRCA1*, *BRCA1*-methylated OC is postulated to compare to *BRCA1*-mutated OC in terms of HRD, platinum chemotherapy and PARPi sensitivity, clinical characteristics and survival outcomes.

Cell line models of *BRCA1*-methylated OC display specific sensitivity to platinum chemotherapy and PARPi¹¹. Analysis of *BRCA1*-methylated OC specimens, albeit in small cohorts, consistently display low *BRCA1* protein and mRNA expression^{7,12-14}. In the clinical setting, few retrospective studies have addressed the implication of *BRCA1* methylation on clinical characteristics and patient outcomes after platinum chemotherapy in OC, with inconsistent results. Recent large studies utilizing genome wide methylation arrays (GWMA) correlated to *BRCA1* mRNA expression to detect *BRCA1* methylation demonstrate no prognostic impact on survival^{3,15}. One study, however, shows similar hazard ratios for overall survival (OS) for both *BRCA1*-methylated (HR 0.74, 95% CI [0.49 – 1.14]) and *BRCA1*-mutated OC (HR 0.75, 95% CI [0.46 – 1.22])¹⁵ as compared to *BRCA1/2* intact disease, though neither was statistically significant. In contrast, smaller studies ($n=27$ to 332) utilizing methylation

sensitive or methylation specific PCR (MSP) as a diagnostic assay have conflicting findings with regards to associations with platinum sensitivity and survival. While some report statistically significant improvements in survival^{16,17}, as compared to non-*BRCA1*-methylated OC, others observe trends towards a worse outcome¹⁸. A comprehensive study of the clinical implications of *BRCA1*-methylated OC is required.

METHODS

Search strategy and study selection

The conduct of this meta-analysis followed the Meta-Analysis of Observational Studies in Epidemiology (MOOSE) guidelines. Original investigations were sought in PUBMED/Medline through 1 April 2018, with no restrictions on publication date or language. The search strategy followed the syntax: (BRCA*[Title/Abstract] OR BRCA1*[Title/Abstract]) AND methylat*[Title/Abstract] AND ovar*[Title/Abstract], and was performed independently by two investigators (R.K. and B.S.), who independently reviewed abstracts for eligibility. Additionally, abstracts from the 2009-2018 ASCO, ESMO and SGO annual meetings were searched. Eligible articles' reference lists were reviewed for further potential studies. The inclusion of published and unpublished studies regardless of publication language or date attempts to minimize publication bias.

Eligible studies needed to assess *BRCA1* methylation in fallopian tube, primary peritoneal or ovarian cancer specimens, report on participant and disease characteristics, report on progression-free survival (PFS) and/or OS, and provide suitable methodology on their *BRCA1* methylation assay. Clinical trials involving PARPi were not eligible. There were no restrictions on the *BRCA1* methylation assay used. As *BRCA2* methylation seldom occurs in

ovarian cancer, we did not investigate its role in OC. Studies reporting solely on *BRCA2* methylation in OC were therefore excluded.

Data acquisition

The following anonymized individual participant data (IPD) was requested from eligible studies' authors, using a pre-specified template and coding: *BRCA1* methylation assay details, participant and disease characteristics (age, histology, FIGO stage¹⁹, grade, HRD score, *BRCA1* methylation, *BRCA1/2* mutation), treatment details (receipt of adjuvant/neoadjuvant platinum chemotherapy, degree of surgical cytoreduction, platinum sensitivity) and survival outcomes (platinum-free interval (PFI), PFS and OS). Tumor HRD score is the unweighted sum of loss of heterozygosity (LOH), telomeric allelic imbalance, and large-scale state transitions²⁰. A score ≥ 42 defines a tumor as HR deficient²¹.

Data integrity and risk of bias assessment

IPD was checked for errors, missing data, and consistency with study publications. Study authors were contacted to resolve discrepancies or obtain missing data. Studies with partially missing data were analysed on the basis of available data.

Bias assessment for studies' internal validity was performed using the ROBINS-I tool²² recommended by the Cochrane collaboration for non-randomized studies.

Statistical analysis This meta-analysis aims to clinically characterize *BRCA1*-methylated OC. Its primary and secondary objectives were to determine the clinico-pathological characteristics associated with *BRCA1* methylation and ascertain the prognostic impact of *BRCA1* methylation on PFS and OS, respectively, in OC. A tumor was considered *BRCA1*-methylated if it was defined as such within its original study. OS was the time from diagnosis until death; participants were censored at the last known survival date. PFS was the time from

diagnosis until CA125 and/or RECIST disease progression, or death, whichever occurred first; participants were censored at the last known progression-free date.

Descriptive statistics were calculated for the combined IPD to summarize participants and disease characteristics for *BRCA1*-methylated OC versus non-*BRCA1*-methylated OC. The characteristics of interest were age, histotype, stage, grade, residual disease after surgical cytoreduction and platinum sensitivity. Comparisons between *BRCA1*-methylated and non-*BRCA1*-methylated OC were made using the generalized Cochran-Mantel-Haenzel test for repeated tests of independence with continuity correction to facilitate combining the multiple cohorts. Where available, HRD scores of *BRCA1*-methylated OC were compared to *BRCA1/2* intact, *BRCA1*-mutated and *BRCA2*-mutated OC using unpaired t-tests. The Kaplan Meier method (logrank test) was used to generate survival plots via the R package *survival*²³. Forest plots were generated using the R package *survcomp*²⁴. Univariate and multivariate analysis of PFS and OS were performed using Cox proportional hazards regression models, which estimated hazard ratios (HR) and 95% confidence intervals (CI) for each individual datasets. A mixed effects cox model was then used to perform univariate and multivariate analysis of the combined dataset, comparing *BRCA1*-methylated OC to non-*BRCA1* methylated OC. The assumptions of proportional hazards were tested using the Schoenfeld Residuals ensuring that they were independent of time. These models were generated using the R packages *survival* and *coxme*²⁵ respectively. Multivariate models were adjusted for the following clinical variables: age, grade, stage, residual disease after surgical cytoreduction. For cohorts with available germline and/or somatic *BRCA1/2* mutation data, the above analyses were repeated comparing *BRCA1*-methylated to *BRCA1/2* intact (*BRCA1/2*-wild type non-*BRCA1*-methylated) OC, in order to eliminate the potential survival bias attributed by *BRCA1/2*-mutated OC in the non-*BRCA1*-methylated population. *P*-values were adjusted for multiple testing (Benjamini-Hochberg method²⁶). Heterogeneity was quantified using the I^2 statistic²⁷, which provides a numerical

value ranging between 0% - 100%. This value and its 95% confidence interval were interpreted according to ranges described in the Cochrane Handbook for Systematic Review of Interventions, with values between 0 – 40%, 30 – 60%, 50 – 90% and 75 – 100% suggesting low, moderate, considerable and substantial heterogeneity, respectively²⁸. For all analyses, *P* values less than 0.05 (two-tailed) were considered statistically significant. All calculations were performed in the R statistical environment (<https://www.r-project.org/>).

RESULTS

Study selection

The literature search retrieved 159 records (Figure 1). Three were duplicated datasets, 8 were conference abstracts identified in the Pubmed search, 90 were irrelevant, 21 were reviews, 13 had no survival data, 1 had unsatisfactory methodology details, 1 concerned a PARPi clinical trial and 22 were eligible. Following contact with authors of eligible studies, 5 did not participate and 4 could not access or share the data (Supplementary Table 1)^{14,17,18,29-34}. In addition, we accessed 2 unpublished cohorts. One author provided data on additional patients not included in the original publication. Overall, 15 observational studies (430 cases; 2206 controls) were included^{3,13,15,16,35-43} (Supplementary Tables 2 – 4).

Quality assessment

Individual study assessments were made on the basis of raw IPD, study manuscript and any necessary clarifications with study authors (Supplementary Table 5). Studies' internal validity was deemed overall good, with a likely low risk of bias on the meta-analysis results. Of the 15 studies, 13 had an overall moderate risk of bias, mainly owing to the potential for confounding baseline factors that were nevertheless adjusted for appropriately. In addition, 4 studies had a moderate risk in patient selection with regards to determining the

clinicopathological characteristics of *BRCA1*-methylated OC, as only HGSC were included. A moderate risk was also found for 5 studies where intended *BRCA1* methylation assessment failed due to inadequate tumor tissue/DNA. Two studies had an overall serious risk of bias, owing to missing data. One did not collect tumor grade and OS data for their entire cohort ($n=35$)³⁹. The other provided IPD for 61.0% ($n=147/241$) of study participants due to time constraints in data collection and provision³⁶. Neither of these two studies collected data pertaining to *BRCA1/2* mutation status or HRD score. The exclusion of these two studies from the entire meta-analysis cohort did not alter the results of the meta-analysis for any of the endpoints measured (data not shown). It was therefore deemed reasonable to include these two studies in this meta-analysis of individual patient data.

Participant characteristics

Data was obtained on 2645 participants. 9 participants with dual aberrations (*BRCA1* methylation and/or *BRCA1/2* mutations) were excluded (Supplementary Table 2), leaving 2636 participants within the analysis. Amongst participants with known *BRCA1/2* mutation status ($n=1257$), *BRCA1* methylation and *BRCA1* mutation were mutually exclusive (odds ratio: 0.18, $P=0.003$), as were *BRCA1* methylation and *BRCA2* mutation (odds ratio 0.33, $P=0.04$). In the entire cohort, the median age was 59 years old. Fallopian tube and primary peritoneal cancers comprised 0.4% ($n=7/2022$) and 1.3% ($n=26/2022$) of the entire cohort, respectively. 85.4% ($n=2247/2630$) participants presented with advanced stage disease (FIGO Stage III/IV). 95.6% ($n=2396/2506$) participants received (neo)adjuvant platinum-based therapy. 79.7% ($n=2065/2592$) were HGSC. Stage III/IV HGSC comprised 73.9% participants ($n=1904/2576$). Surgical cytoreduction below 1 cm residual disease was achieved in 71.9% ($n=1757/2444$) participants (Supplementary Table 6).

Association of *BRCA1* methylation with participants, disease and molecular characteristics

The *BRCA1* methylation rate varied in studies from 6.2% to 73.7% (Supplementary Table 2), with a pooled rate of 16.3% ($n=430/2636$). *BRCA1*-methylated OC was statistically significantly associated with younger age ($P=0.005$), and high grade disease ($P=0.03$) (Table 1). No other statistically significant clinico-pathological correlations were observed.

Germline and/or somatic *BRCA1/2* mutation status was available for 1248 participants from 7/15 included studies. Of these, 10.6% ($n=132/1248$) were *BRCA1*-mutated, 6.5% ($n=81/1248$) were *BRCA2*-mutated and 10.3% ($n=128/1248$) were *BRCA1*-methylated. Within this cohort with known germline and/or somatic *BRCA1/2* mutation status, *BRCA1* methylation was also associated with younger age ($P=0.007$) and high grade disease ($P=0.005$), when compared to patients with *BRCA1/2* intact OC. Furthermore, *BRCA1*-methylated OC was associated with advanced stage ($P=0.01$) and serous histology ($P=0.009$), compared to *BRCA1/2* intact OC. *BRCA1* mutation was associated with younger age ($P<0.001$), high grade ($P=0.006$), serous histology ($P=0.005$), advanced stage ($P=0.02$) and platinum sensitivity ($P=0.008$), when compared to *BRCA1/2* intact disease. The clinico-pathological profile of *BRCA1*-methylated OC did not differ statistically significantly from that of *BRCA1*-mutated OC (Supplementary Table 7).

Tumor HRD score was available for 447 participants (MDACC 2010 and TCGA 2011 cohorts). HRD scores were statistically significantly higher in *BRCA1*-methylated OC (median 68, interquartile range (IQR) 62- 74), compared to *BRCA1/2* intact disease (median 26, IQR 18-38.8), *BRCA1*-mutated (median 63, IQR 56- 70) and *BRCA2*-mutated (median 56, IQR 44.5-65.5) disease (Figure 2).

Association of *BRCA1* methylation with survival

The median follow-up time was 2.8 years (range 0-18.3; IQR 1.5-4.8). There was a statistically significant constituent study effect on the cox regression model first used to associate *BRCA1* methylation with PFS and OS ($P < 0.001$ for both PFS and OS). Statistically significant moderate to substantial heterogeneity was observed between studies for PFS ($I^2 = 62.0\%$ (CI 26.0%-86.0%), $Q = 36.4$, $df = 14$, $P < 0.001$). While not statistically significant, we note low heterogeneity between studies for the assessment of OS ($I^2 = 37.0\%$ (CI 0.0%-75.0%), $Q = 20.7$, $df = 13$, $P = 0.08$), though the 95% confidence interval is wide. Clinically, heterogeneity is expected given observed differences between cohorts with regards to patient/disease clinical characteristics and study characteristics. A mixed-effect model was therefore employed to adjust for study heterogeneity. In order to justify our acceptance of the null hypothesis of this meta-analysis, we performed a power calculation utilizing observed information (study heterogeneity) and assumptions (predicted effect size). Using this information, we calculated that we have an 84.0% power to detect a modest effect size (Cohen's d of 0.2), across the 15 studies with an average *BRCA1* methylation rate of 29 samples per study and an average non-*BRCA1* methylation rate of 147 samples per study with an alpha value of 0.05 and a moderate level of heterogeneity.

In the combined population, there was no statistically significant difference in PFS and OS between *BRCA1*-methylated and non-*BRCA1*-methylated OC (median PFS = 20.0 vs 18.5 months, HR = 1.01, 95% CI = 0.87-1.16, $P = 0.98$; median OS = 46.6 vs 48.0 months, HR = 1.02, 95% CI = 0.87-1.18, $P = 0.96$, respectively). This lack of association persisted in a multivariate model adjusted for age, stage, grade and cytoreduction (Figures 3-4A).

Within the subgroup with known *BRCA1/2* mutation status, *BRCA1*-methylated OC was associated with a worse PFS than *BRCA1/2* intact OC on univariate analysis (median PFS = 15.7 vs 18.0 months, HR = 1.26, 95% CI = 1.02-1.56, $P = 0.03$), though this statistical significance was lost on multivariate analysis. There was no OS difference on univariate or multivariate analyses between the *BRCA1*-methylated and the *BRCA1/2* intact groups (median

OS = 43.5 vs 47.5 months, univariate HR = 1.05, 95% CI = 0.83-1.32, $P=0.70$). *BRCA1*-mutated OC was only associated with a statistically significant improvement in PFS and OS, as compared to *BRCA1/2* intact OC, on multivariate analysis (PFS: median = 17.3 months, univariate HR = 0.94, 95% CI = 0.75-1.17, $P=0.57$; multivariate HR = 0.78, 95% CI = 0.62-0.99, $P=0.04$; OS: median = 47.4 months, univariate HR = 0.81, 95% CI = 0.64-1.03, $P=0.09$; multivariate HR = 0.76, 95% CI = 0.58-0.98, $P=0.03$). *BRCA2* mutation conferred a clear PFS and OS benefit compared to *BRCA1/2* intact OC on univariate and multivariate analyses (median PFS = 28.6 months, univariate HR = 0.58, 95% CI = 0.44-0.78, $P<0.001$; median OS = 87.0 months, univariate HR = 0.55, 95% CI = 0.40-0.77, $P<0.001$) (Figure 4B, Table 2).

Exploratory analysis of methylation methodology on survival

We explored PFS and OS comparing *BRCA1*-methylated OC to non-*BRCA1*-methylated OC within 3 subgroups of the meta-analysis' entire cohort divided according to methylation assay type: those studies utilising methylation-specific PCR (MSP) with gel electrophoresis (GE) (7 studies, $n=765$), those utilising quantitative analysis of MSP or methylation sensitive restriction endonuclease digestion (MRED; 5 studies, $n=828$), and those utilising genome wide methylation arrays (GWMA; 3 studies, $n=1043$). Methylation assays are further detailed in Supplementary Table 2 and Supplementary Figure 1. In the combined cohorts utilising MSP-GE, *BRCA1*-methylated OC was associated with an improved PFS and OS (univariate HR = 0.80, 95% CI = 0.66-0.97, $P=0.02$; univariate HR = 0.80, 95% CI = 0.63-1.00, $P=0.05$, respectively) as compared to non-*BRCA1*-methylated OC, though statistical significance was lost for OS on multivariate analysis ($P=0.08$). Amongst cohorts utilising MSP/MRED with quantitative analysis, *BRCA1*-methylated OC was associated with a worse PFS (HR = 1.47, 95%CI = 1.10-1.96, $P=0.008$) and OS (HR = 1.45, 95%CI = 1.05-2.00, $P=0.02$), when compared to non-*BRCA1*-methylated OC. This persisted on multivariate analysis. There were no survival

differences observed between *BRCA1*-methylated OC and non-*BRCA1*-methylated OC in the subgroup employing GWMA (Figure 5, Table 3).

DISCUSSION

Dysfunctional *BRCA1* and *BRCA2* proteins as a result of *BRCA1/2* mutations render OC particularly susceptible to therapies targeting the homologous recombination DNA repair pathway. While the association of *BRCA1/2*-mutated OC with improved survival resulting from sensitivity to (neo)adjuvant platinum-based chemotherapy is well established, the clinical and therapeutic implications of other *BRCA1* dysfunction mechanisms is less understood. In a recent meta-analysis, loss of *BRCA1* expression by immunohistochemistry was also associated with a statistically significant improved survival⁴⁴, although the mechanisms behind absent *BRCA1* protein expression were not specified. Gene silencing through promoter methylation is one such mechanism, though other potential indirect or post translational mechanisms leading to *BRCA1* inactivation or reduced expression require investigation. Whilst *BRCA1* methylation is a recognized event in OC, promoter methylation of *BRCA2* appears to be a rare occurrence in OC, if at all. Amongst 6 studies investigating *BRCA2* methylation in OC^{15,45-49}, only 2 *BRCA2*-methylated cases were identified amongst 612 cases tested. This meta-analysis was therefore limited to studies investigating *BRCA1* methylation. Reports on *BRCA1*-methylated OC have been conflicting in terms of clinico-pathological associations, with smaller cohorts observing associations with FIGO stage I/II disease³¹ or a lack of association with any histotype¹⁸. Other cohorts limited their assessment of *BRCA1* methylation to homogeneous HGSC cohorts, thereby precluding the detection of histopathological associations. Our study population comprised heterogeneous OC subtypes, though dominated by advanced stage HGSC. We

show that features of *BRCA1*-methylated OC mirror that of *BRCA1*-mutated disease in terms of advanced stage, high grade, serous disease, and a younger age at diagnosis.

The expectation is therefore that *BRCA1*-methylated disease will be sensitive to platinum and PARPi by virtue of HRD. We provide evidence of HRD in *BRCA1*-methylated OC, as defined by the HRD score, albeit in a limited subgroup of participants derived from 2 studies. Cell line and patient-derived xenograft models of *BRCA1*-methylated OC demonstrate clear sensitivity to platinum and/or PARPi therapy^{11,50,51}. Recent data from the ARIEL2 phase II clinical trial demonstrate an encouraging 63% ($n=12/19$) RECIST response rate amongst *BRCA1*-methylated recurrent HGSC to the PARPi rucaparib, as compared to response rates of 79% ($n=23/29$) and 13.5% ($n=7/52$) observed in *BRCA1*-mutated and *BRCA1* intact/low genome-wide LOH recurrent HGSC, respectively⁵². However, large clinical cohorts (e.g. TCGA) show no difference in PFI by *BRCA1* methylation status. Within this meta-analysis, PFI data was unavailable for 56.9% ($n=1539/2636$) of participants, thereby precluding a representative assessment. Nevertheless, we found no association between *BRCA1*-methylated OC and platinum sensitivity. Moreover, we observed no PFS difference between *BRCA1*-methylated and non-*BRCA1*-methylated OC in the entire cohort. When evaluated against the more appropriate comparator population that is *BRCA1/2* intact OC, albeit within a smaller cohort of 1248 patients, *BRCA1*-methylated OC once again did not display improved survival. In contrast, both *BRCA1* and *BRCA2* mutations were prognostic of improved PFS/OS on multivariate analysis, as compared to *BRCA1/2* intact OC, despite the limited cohort size of 1248 patients. Reasons for the discrepancy in survival between *BRCA1*-mutated OC and *BRCA1*-methylated OC, relative to *BRCA1/2* intact OC, are unclear and warrant further investigation. Compared to *BRCA2* mutations, the survival benefit conferred by *BRCA1* mutations is of a lesser magnitude, echoing recent reports evaluating survival in *BRCA1/2*-mutated OC. Some studies find no survival difference between *BRCA1*-mutated and *BRCA1/2* wild type cancers^{15,49}. A pooled

cohort (>6500 participants) demonstrated the expected positive prognostic effect of *BRCA1* mutation on OS, which was to a markedly lesser degree than observed with *BRCA2* mutation (*BRCA1* mutation HR = 0.83, 95% CI = 0.74-0.93, $P < 0.001$; *BRCA2* mutation HR = 0.55, 95% CI = 0.47-0.65, $P = 0.002$)⁵³. It is likely the survival benefit is diluted by heterogeneity within *BRCA1*-mutated disease, whereby some cases assigned a *BRCA1*-mutated status actually behave in HR proficient manner. Similarly, the difference in survival patterns between *BRCA1* mutation and *BRCA1* methylation, despite sharing similar clinico-pathological features, could be explained in part by heterogeneity within *BRCA1*-methylated OC, as discussed further below. Moreover, methylation as a rule is a more dynamic mechanism relative to mutation, and subject to change depending on specific characteristics of the tumor microenvironment. The survival benefit observed in *BRCA1/2*-mutated OC results from their marked sensitivity to platinum-based chemotherapy, used as standard of care in the (neo)adjuvant treatment of OC. Potentially, chemotherapy induces changes affecting the methylation levels of the *BRCA1* promoter, causing earlier and perhaps more frequent resistance to platinum-based chemotherapy than is observed in *BRCA1*-mutated OC. This may manifest as methylation loss in relapsed *BRCA1*-methylated OC, observed in 16.7% - 80.0% of small mostly retrospective cohorts of 6 to 13 paired primary/recurrent *BRCA1*-methylated HGSC^{43,52,54,55}.

The pooled *BRCA1* methylation rate was 16.3%. While this reflects most reports of *BRCA1* methylation frequency, there is marked variability in the reported occurrence of *BRCA1* methylation in OC (5%-89.9%)^{9,39}. Amongst studies included in this meta-analysis, *BRCA1* methylation frequency ranged from 6.2% to 73.6%, with an interquartile range of 10.1% - 18.9%. To some extent, this could be explained by cohort sampling bias, in terms of size, populations and histological subtypes included, with slightly higher rates often observed when cohorts are restricted to HGSC (Supplementary Table 2). In breast cancer, *BRCA1* methylation is more frequent amongst Asians, as compared to Caucasians⁵⁶, however ethnicity data was not

available in this meta-analysis. Moreover, tissue sampling size and content introduces bias owing to variations in sampling sites (ovary versus metastatic), neoplastic cell content and intratumor heterogeneity. Four studies report particularly high *BRCA1* methylation rates: 21.4%³⁵, 33.6%¹⁶, 42%³⁹ and 73.7%⁴³. This latter cohort, which confirmed all its MSP-GE determined *BRCA1*-methylated OC cases with Sanger sequencing, consisted solely of relapsed cases, which may account to some extent for this variation. We also observed that these 4 studies determined *BRCA1* methylation using MSP-GE, whilst studies utilising quantitative methodologies or high throughput microarrays reported rates varying between 8.2% and 16.0%. In a meta-analysis evaluating *BRCA1* promoter methylation as a risk for the development of breast cancer, studies utilising MSP were statistically significantly more likely to report higher frequencies of *BRCA1* methylation⁵⁶. Non-specific primer binding or incomplete bisulfite conversion have linked MSP with false positive results, and may account for this observation to some extent⁵⁷.

The marked variation in the methodology used to determine *BRCA1* promoter methylation is also likely to contribute to differences in reported *BRCA1* methylation rates (Supplementary Figure 1). This epigenetic phenomenon is characterized by the methylation of CpG dinucleotides within a ~2.7 kB 5'CpG island containing 96 CpG dinucleotides and involving the bidirectional *BRCA1* promoter and its adjacent alternative first exons (exon 1a/1b)^{58,59}. An essential regulatory area 202 bp downstream and 20 bp upstream of the *BRCA1* transcription start site at exon 1a (according to GenBank U37574) contains sequence specific transcription factor binding sites that prevent transcription when methylated⁶⁰. However, the individual contribution of CpG dinucleotides within this area (or elsewhere in the 2.7 kb CpG island) to the regulation of *BRCA1* transcription has yet to be comprehensively evaluated in OC. Commonly used assays include MRED, MSP, methylation sensitive multiplex ligation probe amplification (MS-MLPA), bisulfite sequencing and, more recently, GWMA. In contrast to the other

methodologies, the latter correlates *BRCA1* mRNA expression to CpG dinucleotides' methylation status (9-46 assessed), to enable the selection of relevant CpG probes^{3,15}. Despite this common rationale, 2 studies^{15,42} using the same GWMA assay (Illumina Infinium HumanMethylation 450k Beadchip) selected 8 and 21 CpG probes to determine *BRCA1* methylation status within their samples. Were both assays applied to the same samples, different results may ensue depending on the level of methylation in tumor samples at these CpG sites. PCR-based assays use different primers which assess often overlapping though different regions of the *BRCA1* promoter, resulting in different CpG dinucleotides being interrogated between assays (Supplementary Table 2, Supplementary Figure 1). A study using 2 sets of primers, each targeting a different region within the *BRCA1* promoter, reported different methylation levels within the same OC sample: 17.5% (region 1) and 3.3% (region 2)⁶¹. Using an arbitrary cut-off of 10% to define methylation, and had only one set of primers been used, this sample may or may not be defined as *BRCA1*-methylated. Moreover, methylation of individual CpG dinucleotides, which may or may not be involved in regulation of *BRCA1* transcription, cannot be distinguished within a PCR-based assay. The importance of CpG site selection to determine *BRCA1* methylation status was underscored in a study demonstrating a correlation between high methylation levels at the *BRCA1* promoter and the triple negative breast cancer subtype, as determined by pyrosequencing. However the level of methylation was variable across the 11 CpG sites evaluated, with 4 CpG sites displaying low/lack of methylation⁶². Differences also exist with regards to assay interpretation, which includes GE (used in 8 of the included studies) or quantitative analyses. The latter use varying methodologies to determine non-methylated reads and quantify the percentage methylated reads within a sample. Quantitative analyses arbitrarily define low thresholds (4-10%) to define methylation, without critically evaluating the threshold required for *BRCA1* inactivation. Arguably, a low threshold is selected to account for potential dilution of methylated fragments in specimens with low neoplastic cell content or tumor heterogeneity, though may result in

labelling non-*BRCA1*-methylated samples with high neoplastic cell content/homogeneous tumor as *BRCA1*-methylated. Finally, whilst technically valid, these assays have not been compared to one another in the same OC dataset. A study compared methylation status of 4 genes using MSP-GE, quantitative MS-MLPA and quantitative multiplex MSP in 40 breast cancers, and found high discrepancies between MSP-GE and the quantitative assays' results⁶³. Methylation assay heterogeneity was evident between studies included in this meta-analysis. In an exploratory analysis, we identified a survival benefit within a subset of *BRCA1*-methylated OC identified with MSP-GE, as compared to non-*BRCA1*-methylated OC. This subset was particularly homogeneous as 5 of the 7 cohorts included^{13,16,35,41,43} evaluated the same 7 CpG sites, whilst 4 CpG sites were common to 6 cohorts (CpG details for 1 cohort were unavailable). ~~This~~ Our findings may give credence to these particular CpG sites in terms of their essential role on *BRCA1* transcription. However, only 2 of these 7 cohorts individually reported an improved survival for *BRCA1*-methylated OC, compared to non-*BRCA1*-methylated OC, as did 1 of 5 eligible studies using identical PCR primers excluded from this analysis (Supplementary Table 1)^{14,17,29,30}. While this observation could be related to individual studies' small sample size, our findings are exploratory and should be interpreted with caution.

Factors other than diagnostic methylation assays may contribute to heterogeneity within *BRCA1*-methylated OC, as is observed in *BRCA1*-mutated disease. Indeed, studies suggest resistance to platinum and/or PARPi therapy with *BRCA1* mutations occurring within the *BRCA1* RING domain⁶⁴. Moreover, mono-allelic *BRCA1* mutations do not display an HR-deficient phenotype when *BRCA1*-locus LOH is absent, as evaluated in a cohort of 52 *BRCA1*-mutated OC⁶⁵. In this study, 7% of *BRCA1*-mutated OC had absent *BRCA1*-locus LOH and a worse survival, compared to *BRCA1*-mutated OC with *BRCA1*-locus LOH ($P=0.02$). Similarly, the discrepancy between preclinical findings and our analysis results with regards to platinum sensitivity and survival may lie in heterogeneity within *BRCA1*-methylated OC. This has been

illustrated in *BRCA1*-methylated HGSC patient-derived xenograft models, whereby one model was cisplatin sensitive, and the other cisplatin resistant, using the same MSP assay⁵¹. Potentially, mono-allelic *BRCA1* methylation with absent *BRCA1*-locus LOH would result in transcription of an intact *BRCA1* on the non-methylated allele, resulting in a functioning BRCA1 protein. By pooling studies evaluating LOH in *BRCA1*-methylated OC, we found that 19.6% (n=18/92) of *BRCA1*-methylated OC have absent LOH, as determined by analyzing microsatellites near *BRCA1* (Supplementary Table 8). Current methylation assays do not routinely examine *BRCA1* locus LOH, nor do they differentiate between mono/bi-allelic methylation. In quantitative assays, greater than 50% methylation at the *BRCA1* promoter may assume bi-allelic methylation. A recent study estimated percentage *BRCA1* promoter methylation (adjusted for *BRCA1* locus LOH, neoplastic cellularity and *BRCA1* copy number) to differentiate homozygous (>50% methylation) and heterozygous (<50% methylation) *BRCA1* methylation in 21 *BRCA1*-methylated tumors from the ARIEL2 phase II clinical trial. Encouragingly, homozygous *BRCA1*-methylated OC (n=6) was associated with a longer PFS than *BRCA1/2* intact OC (n=143), though this was not statistically significant (median PFS 14.5 months (95% CI 4.8-18.3 months) vs 5.5 months (95% CI 5.0-6.2 months), $P=0.06$)⁶⁶. The development of allele-specific methylation methodologies that include LOH assessment, confirmed by absent/low *BRCA1* mRNA expression, and ideally using specimens with 100% neoplastic cell content should minimize heterogeneity and permit a more accurate determination of *BRCA1* epigenetically silenced tumors, or 'true' *BRCA1*-methylated OC.

This is the most extensive meta-analysis to date evaluating the clinical characteristics of *BRCA1*-methylation in OC. The inclusion of published and unpublished studies without publication language restrictions, along with the use of IPD from studies assessed to be of overall good quality further strengthens our results.

Limitations include incomplete inclusion of all eligible studies identified through our search strategy, and statistically significant heterogeneity between cohorts in the assessment of PFS and OS, which was mitigated by the use of a mixed-effects model. Moreover, heterogeneity within the methylation assays utilised in the included studies was evident. Finally, the availability of *BRCA1/2* mutation status, which enables a more thorough assessment of the prognostic effect of *BRCA1* methylation on survival by using *BRCA1/2* intact OC as a comparator, was limited to n=1248/2636 patients.

In the largest meta-analysis on this topic, we show that *BRCA1*-methylated OC has a clinico-pathological profile similar to that of *BRCA1*-mutated OC, presenting at a younger age as advanced stage HGSC. However, *BRCA1* methylation does not predict for platinum sensitivity nor is it prognostic of survival. While early and/or frequent platinum resistance mechanisms may account for this observation, there is marked heterogeneity between methylation assays used to detect *BRCA1*-methylated OC, in terms of the exact CpG sites assessed and the interpretation of the observed result. Moreover, these assays are not allele-specific and do not account for *BRCA1*-locus LOH. Potentially, a comprehensive assay that examines CpG dinucleotides critical to *BRCA1* transcription in OC specimens in an allele-specific manner, combined with assessment of *BRCA1*-locus LOH may permit a better selection of 'true' *BRCA1*-methylated OC. Defined as such, *BRCA1*-methylated OC may represent a smaller subset, yet permit a more accurate selection of patients with OC that would derive clear benefit from PARPi and other novel therapies targeting HR-deficient OC.

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Table 1. Association between *BRCA1* methylation status and clinico-pathological factors*

Parameter	BRCA1-methylated (n=430)	Non-BRCA1 methylated (n=2206)	Adjusted p-value†
Age, No. (%)			0.005
<59	245 (57.0)	1090 (49.6)	
≥59	185 (43.0)	1109(50.4)	
Missing	0	7	
Grade, n= (%)			0.03
Low	25 (6.0)	198 (9.1)	
High	389 (94.0)	1979 (90.9)	
Missing	16	29	
FIGO stage, n= (%)			0.47
I/II	48 (11.2)	339 (15.4)	
III/IV	381 (88.8)	1862 (84.6)	
Missing	1	5	
Histology, n= (%)			0.97
Serous	354 (82.3)	1799 (81.6)	
Non-serous	76 (17.7)	407 (18.4)	
Missing	0	0	
Residual disease post cytoreduction, n= (%)			0.47
Macro <1cm	306 (73.6)	1451 (71.5)	
Macro ≥1cm	110 (26.4)	577 (28.5)	
Missing	14	178	
Platinum sensitivity, n= (%)			0.34
<6 months	66 (19.4)	463 (28.2)	
≥6 months	275 (80.6)	1177 (71.8)	
No platinum	17	106	
Missing	72	460	

* percentages reflect percentage of total non-missing data

† Two-sided Cochran-Mantel-Haenzel test with *p* value adjusted for study

Table 2. Univariate and multivariate analysis for PFS and OS according to BRCA1/2 aberrations

Variable	Univariate analysis				Multivariate analysis*			
	PFS		OS		PFS		OS	
	HR (95%CI)	P†	HR (95%CI)	P†	HR (95%CI)	P†	HR (95%CI)	P†
Entire cohort (n=2636)								
Age	--	--	--	--	1.10 (1.00 – 1.25)	0.05	1.22 (1.10 – 1.37)	<0.001
Grade	--	--	--	--	2.38 (1.84 – 3.07)	<0.001	1.80 (1.35 – 2.41)	<0.001
Stage	--	--	--	--	2.89 (2.36 – 3.47)	<0.001	2.76 (2.19 – 3.48)	<0.001
Residual disease	--	--	--	--	1.59 (1.41 – 1.80)	<0.001	1.79 (1.57 – 2.03)	<0.001
BRCA1 methylation	1.01 (0.87 – 1.16)	0.98	1.02 (0.87 – 1.18)	0.96	1.01 (0.88 – 1.15)	0.92	1.00 (0.86 – 1.17)	0.97
Cohort with known BRCA1/2 mutations (n=1248)								
Age	--	--	--	--	1.08 (0.93 – 1.25)	0.33	1.18 (1.00 – 1.37)	0.04
Grade	--	--	--	--	2.30 (1.48 – 3.57)	<0.001	1.97 (1.19 – 3.27)	0.01
Stage	--	--	--	--	4.07 (3.00 – 5.52)	<0.001	3.10 (2.18 – 4.42)	<0.001
Residual disease	--	--	--	--	1.37 (1.15 – 1.62)	<0.001	1.54 (1.29 – 1.83)	<0.001
BRCA1 methylation	1.26 (1.02-1.56)	0.03	1.11 (0.88-1.41)	0.35	1.06 (0.85 – 1.34)	0.59	0.97 (0.75 – 1.24)	0.78
BRCA1 mutation	0.99 (0.80-1.23)	0.91	0.87 (0.69-1.10)	0.24	0.78 (0.62 – 0.99)	0.04	0.76 (0.58 – 0.98)	0.03
BRCA2 mutation	0.58 (0.43-0.77)	<0.001	0.57 (0.41-0.80)	<0.001	0.51 (0.37 – 0.69)	<0.001	0.51 (0.35 – 0.73)	<0.001

†Two-tailed mixed-effects Cox proportional hazards regression model with p value adjusted for study

*All clinical variables within the multivariate model are binary, as follows: Age: 0 for <60 (median age) and 1 for ≥60; Grade: 0 for low grade, 1 for high grade; 0 for Stage I/II, 1 for Stage III/IV; Residual disease: 0 for < 1cm, 1 for ≥1cm.

PFS: progression-free survival; OS: overall survival

Table 3. Association of BRCA1 methylation with PFS and OS according to methylation assay subgroup (methylated relative to non-methylated)

Methylation assay subgroup	PFS			OS		
	HR (95%CI)	P*	n (events)	HR (95%CI)	P*	n (events)
Univariable						
Genome wide methylation array correlated to BRCA1 mRNA expression	1.12 (0.89 – 1.41)	0.33	976 (767)	1.09 (0.85 – 1.39)	0.51	1043 (690)
MRED or MSP with quantitative analysis	1.47 (1.10 – 1.96)	0.008	781 (590)	1.45 (1.05 – 2.00)	0.02	826 (382)
MSP with gel electrophoresis	0.80 (0.66 – 0.97)	0.02	706 (518)	0.80 (0.63 – 1.00)	0.05	730 (411)
Multivariable†						
Genome wide methylation array correlated to BRCA1 mRNA expression	1.11 (0.87 – 1.42)	0.40	838 (657)	1.04 (0.80 – 1.35)	0.78	896 (591)
MRED or MSP with quantitative analysis	1.36 (1.00 – 1.84)	0.04	733 (556)	1.40 (1.01 – 1.94)	0.04	774 (354)
MSP with gel electrophoresis	0.82 (0.68 – 1.00)	0.05	655 (482)	0.81 (0.64 – 1.03)	0.08	724 (407)

* Two-tailed mixed-effects Cox proportional hazards regression model with p value adjusted for cohort

†Adjusted for binary clinical variables as follows: Age: 0 for <60 (median age) and 1 for ≥60; Grade: 0 for low grade, 1 for high grade; Stage: 0 for Stage I/II, 1 for Stage III/IV; Residual disease: 0 for < 1cm, 1 for ≥1cm.

MRED: methylation-sensitive restriction endonuclease digest; MSP: methylation-specific PCR; PFS: progression-free survival; OS: overall survival

FIGURE LEGENDS

Figure 1. Flow chart of study

Figure 2. HRD score as assessed on 447 tumor samples obtained from the TCGA 2011 and MDACC 2010 cohorts, according to underlying *BRCA1/2* aberration

Box plots depict the median and 95% confidence intervals of the HRD scores according to the underlying *BRCA1/2* aberration in the TCGA 2011 and MDACC 2010 cohorts. The dotted line represents the threshold value of 42 above which samples are considered to be homologous recombination deficient as per the HRD score assay. Stars depict the level of statistical significance between sets of groups (unpaired t-tests). Numbers in each subgroup are as follows: Wild type n=286; *BRCA1* methylation n=65; *BRCA1* mutation n=57; *BRCA2* mutation n=39.

Figure 3. Univariate and multivariate analyses of *BRCA1* methylation on progression-free survival (PFS) and overall survival (OS) by study and for the combined dataset

Forest plots of the association of *BRCA1* methylation with progression-free survival (PFS) and overall survival (OS) on univariate and multivariate analyses.. A: Univariate analyses of PFS and OS; B: Multivariate analyses of PFS and OS, adjusted for binary clinical variables as follows: age: 0 for <60 (median age) and 1 for ≥60; Grade: 0 for low grade, 1 for high grade; 0 for Stage I/II, 1 for Stage III/IV; Residual disease: 0 for < 1cm, 1 for ≥1cm. Numbers in 'non-*BRCA1*-methylated' and '*BRCA1*-methylated' columns represent number of events/total numbers. Squares determine study-specific estimates and horizontal lines indicate 95% confidence interval; diamond depicts summary estimate with its associated 95% confidence interval. Hazard ratios (HR) and 95% confidence intervals were determined using a Cox proportional hazards mixed effects model. All statistical tests were two-sided. CI = confidence interval.

Figure 4. Survival in the entire pooled dataset and a pooled subset with known *BRCA1/2* mutation status

Estimates of progression-free survival (PFS) and overall survival (OS) from Kaplan Meier curves, with tests of differences by two-sided logrank test. (A) PFS and OS in the entire pooled cohort (n=2636). Kaplan Meier curves for *BRCA1* methylation (orange) and no *BRCA1* methylation (grey) curves are depicted. (B) PFS and OS in a pooled subset of the entire cohort with known *BRCA1/2* mutation status (n=1248). Kaplan Meier curves for *BRCA1* methylation (orange), *BRCA1* mutation (dark blue), *BRCA2* mutation (light blue) and *BRCA1/2* intact (non-*BRCA1*-

methylated *BRCA1/2* wild type) (grey) curves are depicted.. In all graphs, univariate hazard ratios, 95% confidence interval (CI) and *p* value for *BRCA1* methylation vs *BRCA1/2* intact, adjusted for study, are given.

Figure 5. Survival analyses in subgroups combined according to methylation assay

Estimates of progression-free survival (PFS) and overall survival (OS) from Kaplan Meier curves, in subgroups combined according to methylation assay, with tests of differences by two-sided logrank test. A) PFS and OS in cohorts where *BRCA1* methylation was determined using genome wide methylation assays correlated to *BRCA1* mRNA expression represents B) PFS and OS in cohorts where *BRCA1* methylation was determined using MSP or MRED analyzed using quantitative methods C) PFS and OS in cohorts where *BRCA1* methylation was determined using MSP and gel electrophoresis. In all graphs, univariate hazard ratios (HR) and 95% confidence interval (CI) and *p* value for *BRCA1* methylation (orange), as compared to no *BRCA1*-methylation (grey), adjusted for study, are given. MRED = methylation-sensitive restriction endonuclease digestion, MSP = methylation specific PCR; CI = confidence interval; *BRCA1* meth = *BRCA1* methylation; non-*BRCA1*-meth=no *BRCA1* methylation

Figure 1

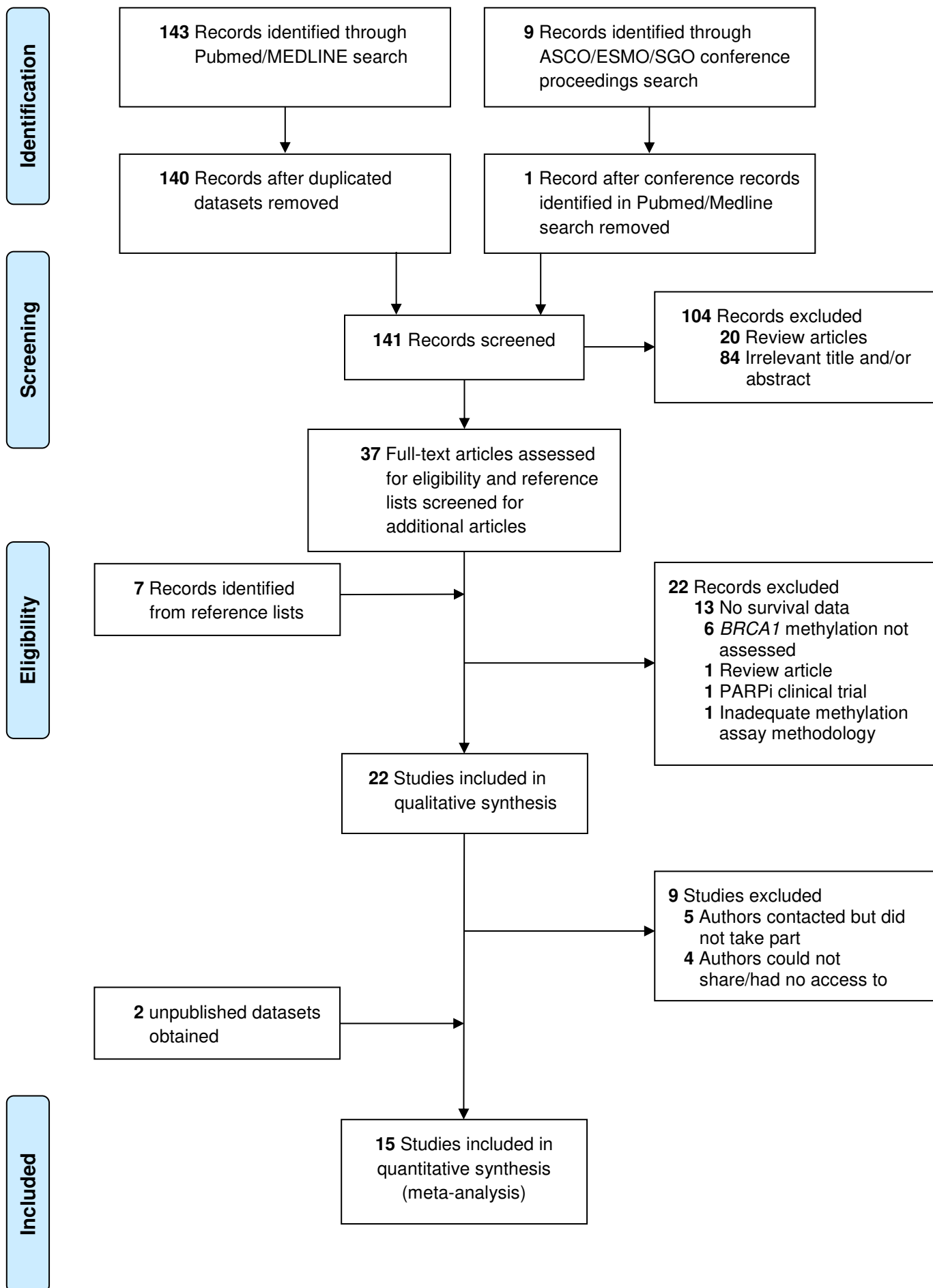


Figure 2

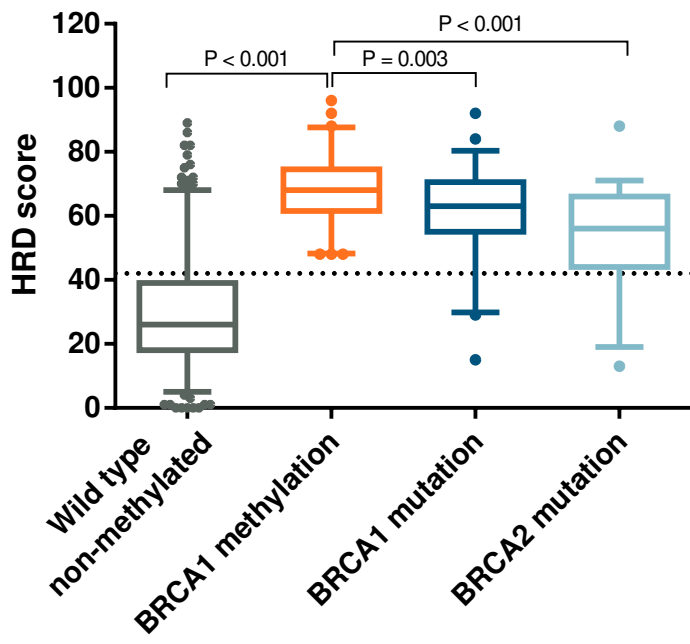
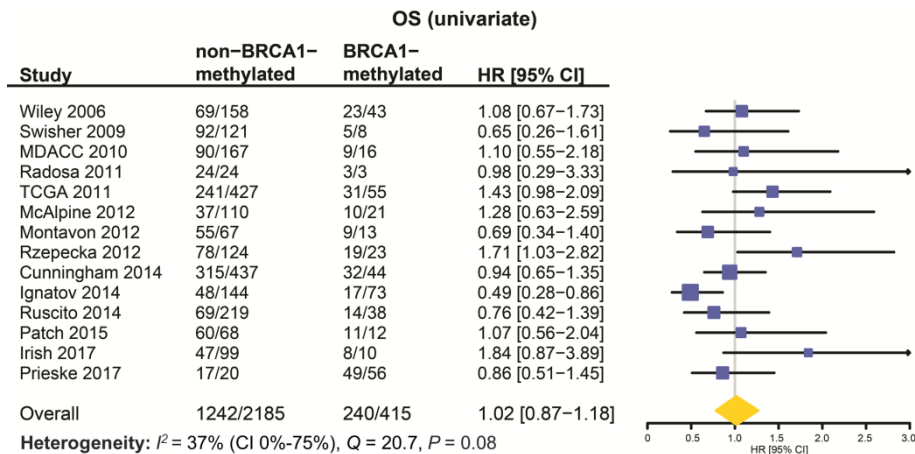
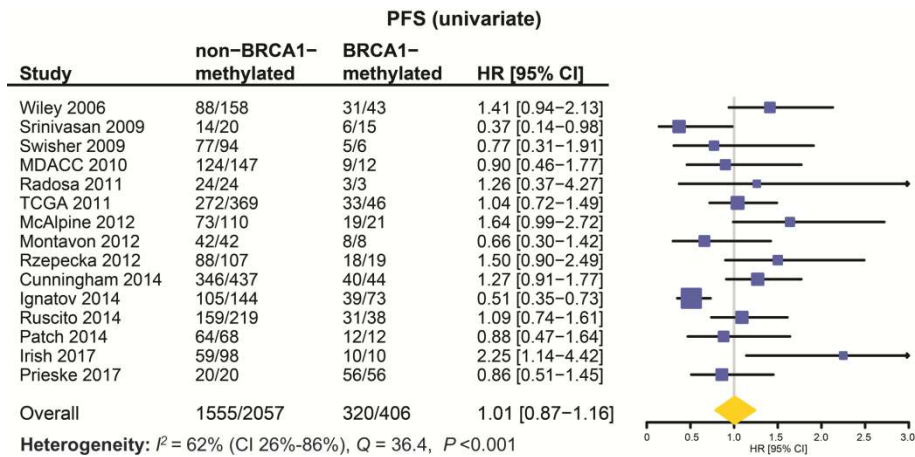


Figure 3

A



B

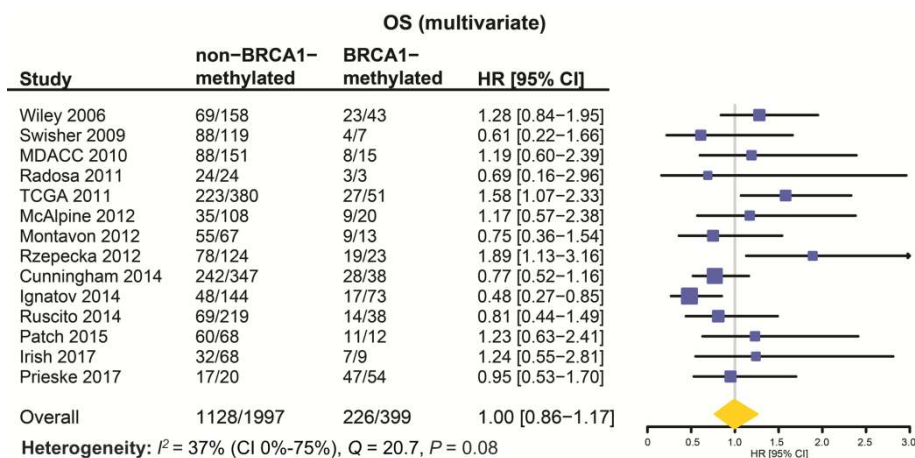
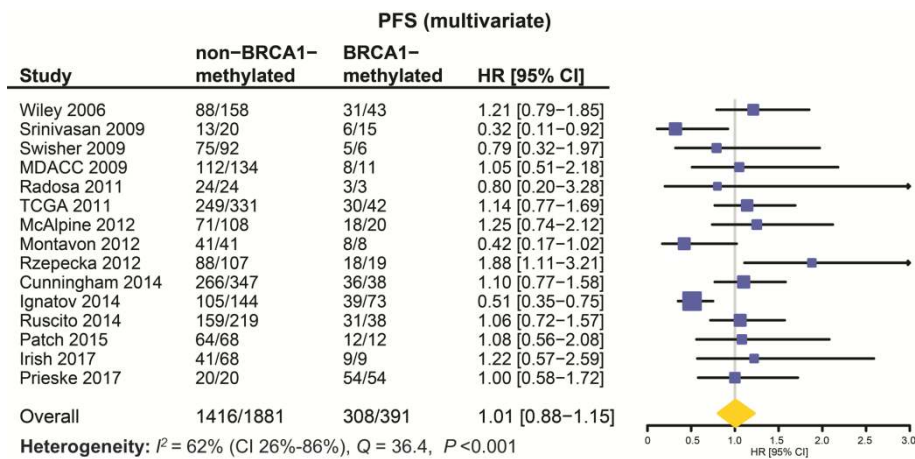


Figure 4

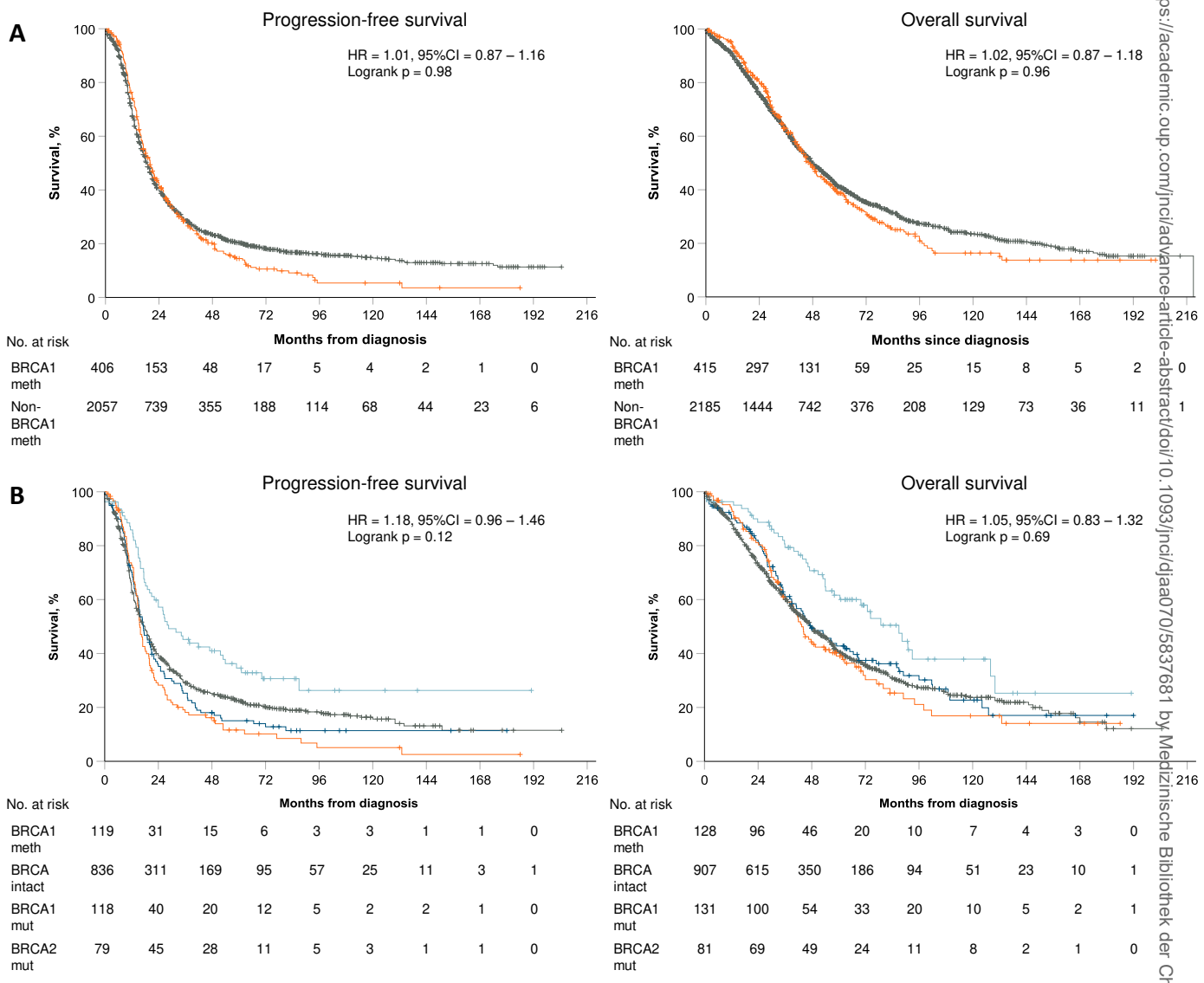




Figure 5

