Association of breast cancer risk in BRCA1 and BRCA2 mutation carriers with genetic variants showing differential allelic expression

Hamdi, Yosr; Soucy, Penny; Kuchenbaeker, Karoline B; Pastinen, Tomi; Droit, Arnaud; Lemaçon, Audrey; Adlard, Julian; Aittomäki, Kristiina; Andrlulis, Irene L; Arason, Adalgeir; Arnold, Norbert; Arun, Banu K; Azzollini, Jacopo; Bane, Anita; Barjhoux, Laure; Barrowdale, Daniel; Benítez, Javier; Berthet, Pascaline; Blok, Marinus J; Bobolis, Kristie A; Bonadona, Valérie; Bonanni, Bernardo; Bradbury, Angela R; Brewer, Carole; Buecher, Bruno; Buys, Saundra; Caligo, Maria A; Chiquette, Jocelyne; Chung, Wendy K; Claes, Kathleen B M; Daly, Mary B; Damiola, Francesca; Davidson, Rosemarie; de la Hoya, Miguel; De Leeneer, Kim; Diez, Orland; Ding, Yuan Chun; Dolcetti, Riccardo; Domchek, Susan M; Dorfling, Cecilia M; Eccles, Diana; Eeles, Ros; Einbeigi, Zakaria; Ejlsertsen, Bent; Engel, Christoph; Gareth Evans, D; Felibadelaló, Lidia; Foretova, Lenka; Fostira, Florentia; Foulkes, William D; Fountzilas, George; Friedman, Eitan; Frost, Debra; Ganschow, Pamela; Ganz, Patricia A; Garber, Judy; Gayther, Simon A; Gerdes, Anne-Marie; Glendon, Gord; Godwin, Andrew K; Goldgar, David E; Greene, Mark H; Gronwald, Jacek; Hahnen, Eric; Hamann, Ute; Hansen, Thomas V O; Hart, Steven; Hays, John L; Hogervorst, Frans B L; Hulick, Peter J; Imyanitov, Evgeny N; Isaacs, Claudine; Izatt, Louise; Jakubowska, Anna; James, Paul A; Janavicius, Ramunas; Jensen, Uffe Birk; John, Esther M; Joseph, Vijai; Just, Walter; Kaczmarek, Katarzyna; Karlan, Beth Y; Kets, Carolien M; Kirk, Judy; Krieger, Mieke; Laitman, Yael; Laurent, Maïté; Lázaro, Conxi; Leslie, Goska; Lester, Jenny; Lesueur, Fabienne; Liljegren, Annelie; Loman, Niklas; Loud, Jennifer T; Manoukian, Siranoush; Mariani, Milena; Mazoyer, Sylvie; McGuffog, Lesley; Meijers-Heijboer, Hanne E J; Meindl, Alfons; Miller, Austin; Montagna, Marco; Mulligan, Anna Marie; Nathanson, Katherine L; Neuhausen, Susan L; Nevanlinna, Hei; Nussbaum, Robert L; Olah, Edith; Olopade, Olufunmilayo I; Ong, Kai-ren; Oosterwijk, Jan C; Osorio, Ana; Papi, Laura; Park, Sue Kyung; Pedersen, Inge Sokilde; Peissel, Bernard; Segura, Pedro Perez; Peterlongo, Paolo; Phelan, Catherine M; Radice, Paolo; Rantal, Johanna; Rappaport-Fuerhauser, Christine; Rennert, Gad; Richardson, Andrea; Robson, Mark; Rodriguez, Gustavo C; Rookus, Matti A; Schmutzler, Rita Katharina; Sevenet, Nicolas; Shah, Payal D; Singer, Christian F; Slavin, Thomas P; Snape, Katie; Sokolowska, Joanna; Sønderstrup, Ida Marie Heeholm; Southey, Melissa; Spurdle, Amanda B; Stanler, Zosifa; Stoppa-Lyonnet, Dominique; Sukienicki, Grzegorz; Sutter, Christian; Tan, Yen; Tea, Muy-Kheng; Teixeira, Manuel R; Teulé, Alex; Teo, Soo-Hwang; Terry, Mary-Beth; Thomassen, Mads; Tihomirova, Laima; Tischkowitz, Marc; Tognazzo, Silvia; Toland, Amanda Ewart; Tung, Nadine; van den Ouweland, Ans M W; van der Luijt, Rob B; van Engelen, Klaartje; van Rensburg, Elizabeth J; Varon-Mateeva, Raymond; Wappenschmidt, Barbara; Wijnen, Juul T; Rebbeck, Timothy; Chenevix-Trench, Georgia; Offit, Kenneth; Couch, Fergus J; Nord, Silje; Easton, Douglas F; Antoniou, Antonis C; Simard, Jacques; EMBRACE

Published in:
Breast Cancer Research and Treatment

DOI:
10.1007/s10549-016-4018-2

Publication date:
2017

Document version
Publisher's PDF, also known as Version of record
Association of breast cancer risk in \textit{BRCA1} and \textit{BRCA2} mutation carriers with genetic variants showing differential allelic expression: identification of a modifier of breast cancer risk at locus 11q22.3

Yosr Hamdi$^1$ · Penny Soucy$^1$ · Karoline B. Kuchenbaeker$^{2,3}$ · Tomi Pastinen$^{4,5}$ · Arnaud Droit$^1$ · Audrey Lemaçon$^1$ · Julian Adlard$^6$ · Kristiina Aittomäki$^7$ · Irene L. Andrulis$^{8,9}$ · Adalgeir Arason$^{10,11}$ · Norbert Arnold$^{12}$ · Banu K. Arun$^{13}$ · Jacopo Azzollini$^{14}$ · Anita Bane$^{15}$ · Laure Barjhoux$^{16}$ · Daniel Barrowdale$^2$ · Javier Benitez$^{17,18,19}$ · Pascaline Berthet$^{20}$ · Marinus J. Blok$^{21}$ · Kristie Bobolis$^{22}$ · Valérie Bonadona$^{23}$ · Bernardo Bonanni$^{24}$ · Angela R. Bradbury$^{25}$ · Carole Brewer$^{26}$ · Bruno Buecher$^{27}$ · Sandra S. Buys$^{28}$ · Maria A. Caligo$^{29}$ · Jocelyne Chiquette$^{30}$ · Wendy K. Chung$^{31}$ · Kathleen B. M. Claes$^{32}$ · Mary B. Daly$^{33}$ · Francesca Damilano$^{16}$ · Rosemarie Davidson$^{34}$ · Miguel De la Hoya$^{35}$ · Kim De Leenheer$^{32}$ · Orland Diez$^{36}$ · Yuan Chun Ding$^{37}$ · Riccardo Dolcetti$^{38,39}$ · Susan M. Domchek$^{35}$ · Cecilia M. Dorfling$^{40}$ · Diana Eccles$^{41}$ · Ros Eeles$^{42}$ · Zakaria Einbi$^{43}$ · Bent Ejlertsen$^{44}$ · EMBRACE$^2$ · Christoph Engel$^{45,46}$ · D. Gareth Evans$^{37}$ · Lidia Felibadalo$^{48}$ · Lenka Foretova$^{49}$ · Florentia Fostira$^{50}$ · William D. Foulkes$^{51}$ · George Fountzilas$^{52}$ · Eitan Friedman$^{53,54}$ · Debra Frost$^{2}$ · Pamela Ganschow$^{55}$ · Patricia A. Ganz$^{56}$ · Judy Garber$^{57}$ · Simon A. Gayther$^{58}$ · GEMO Study Collaborators$^{59,60,61}$ · Anne-Marie Gerdes$^{62}$ · Gord Glendon$^8$ · Andrew K. Godwin$^{55}$ · David E. Goldgar$^{34}$ · Mark H. Greene$^{65}$ · Jacek Gronwald$^{66}$.

The content of this manuscript does not necessarily reflect the views or policies of the National Cancer Institute or any of the Collaborating Centers in the Breast Cancer Family Registry (BCFR), nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government or the BCFR. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Rita Katharina Schmutzler: On behalf of the German Consortium of Hereditary Breast and Ovarian Cancer (GC-HBOC).

Electronic supplementary material The online version of this article (doi:10.1007/s10549-016-4018-2) contains supplementary material, which is available to authorized users.

Jacques Simard
Jacques.Simard@crchudequebec.ulaval.ca

1 Genomics Center, Centre Hospitalier Universitaire de Québec Research Center and Laval University, 2705 Laurier Boulevard, Quebec, QC G1V 4G2, Canada
2 Centre for Cancer Epidemiology, Department of Public Health and Primary Care, University of Cambridge, Strangeways Research Laboratory, Worts Causeway, Cambridge, UK
3 The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus Hinxton, Cambridge CB10 1HH, UK
4 Department of Human Genetics, McGill University, Montreal, QC H3A 1B1, Canada
5 McGill University and Genome Quebec Innovation Centre, Montreal, QC H3A 0G1, Canada
6 Yorkshire Regional Genetics Service, Chapel Allerton Hospital, Leeds LS7 4SA, UK
7 Department of Clinical Genetics, Helsinki University Hospital, HUS, Meilahdentie 2, P.O. BOX 160, 00029 Helsinki, Finland
8 Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, ON M5G 1X5, Canada

Received: 5 October 2016 / Accepted: 8 October 2016 / Published online: 28 October 2016
© The Author(s) 2016. This article is published with open access at Springerlink.com

Abstract

Purpose Cis-acting regulatory SNPs resulting in differential allelic expression (DAE) may, in part, explain the underlying phenotypic variation associated with many complex diseases. To investigate whether common variants associated with DAE were involved in breast cancer susceptibility among BRCA1 and BRCA2 mutation carriers, a list of 175 genes was developed based of their involvement in cancer-related pathways.

Methods Using data from a genome-wide map of SNPs associated with allelic expression, we assessed the association of ~320 SNPs located in the vicinity of these genes with breast and ovarian cancer risks in 15,252 BRCA1 and 8211 BRCA2 mutation carriers ascertained from 54 studies participating in the Consortium of Investigators of Modifiers of BRCA1/2.

Results We identified a region on 11q22.3 that is significantly associated with breast cancer risk in BRCA1 mutation carriers (most significant SNP rs2285959 p = 7 × 10^{-6}). This association was absent in BRCA2 carriers (p = 0.57). The 11q22.3 region notably encompasses genes such as ACAT1, NPAT, and ATM. Expression quantitative trait loci associations were observed in both normal breast and tumors across this region, namely for ACAT1, ATM, and other genes. In silico analysis revealed some overlap between top risk-associated SNPs and relevant biological features in mammary cell data, which suggests potential functional significance.

Conclusion We identified 11q22.3 as a new modifier locus in BRCA1 carriers. Replication in larger studies using estrogen receptor (ER)-negative or triple-negative (i.e., ER-, progesterone receptor-, and HER2-negative) cases could therefore be helpful to confirm the association of this locus with breast cancer risk.

Keywords Breast cancer · Genetic modifiers · Differential allelic expression · Genetic susceptibility · Cis-regulatory variants · BRCA1 and BRCA2 mutation carriers

Introduction

Pathogenic mutations in the BRCA1 and BRCA2 genes substantially increase a woman’s lifetime risk of developing breast and ovarian cancers [1–4]. These risks vary significantly according to (a) age at disease diagnosis in carriers of identical mutations, (b) the cancer site in the individual who led to the family’s ascertainment, (c) the degree of family history of the disease [1, 4, 5], and (d) the
type and location of BRCA1 and BRCA2 mutations [6]. These observations suggest that other factors, including lifestyle/hormonal factors [7] as well as other genetic factors, modify cancer risks in BRCA1 and BRCA2 mutation carriers. Direct evidence for such genetic modifiers of risk has been obtained through the association studies performed by the Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA), which have shown that several common breast cancer susceptibility alleles identified through population-based genome-wide association studies (GWASs) are also associated with breast cancer risk among BRCA1 and BRCA2 mutation carriers [8–10].

Global analysis of GWAS data has shown that the vast majority of common variants associated with susceptibility to cancer lie within genomic non-coding regions and are predicted to account for cancer risk through regulation of gene expression [11, 12]. A recent expression quantitative trait loci (cis-eQTL) analysis for mRNA expression in 149 known cancer risk loci performed in five tumor types (breast, colon, kidney, lung, and prostate) has shown that approximately 30 % of such risk loci were significantly associated with eQTLs present in at least one gene within 500 kb [13]. These results suggest that additional cancer susceptibility loci may be identified through studying genetic variants that affect the regulation of gene expression. In the present study, we selected genes of interest for their known involvement in cancer etiology, identified 320 genetic variants in the vicinity of these genes with evidence of differential allelic expression (DAE), and then investigated the associations of these variants with breast and ovarian cancer risks among BRCA1 and BRCA2 mutation carriers. These included variants in genes involved in DNA repair (homologous recombination and DNA interstrand crosslink repair), interaction with and/or modulation of BRCA1 and BRCA2 cellular functions, cell cycle control, centrosome amplification and interaction with AURKA, apoptosis, ubiquitination, as well as known tumor suppressors, mitotic kinases, and other kinases, sex steroid action, and mammographic density.

Materials and methods

Subjects

All study participants were female carriers of a deleterious germline mutation in either BRCA1 or BRCA2 and aged 18 years or older [14]. Fifty-four collaborating CIMBA studies contributed a total of 23,463 samples (15,252 BRCA1 mutation carriers and 8211 BRCA2 mutation carriers) to this study, including 12,127 with breast cancer (7797 BRCA1 and 4330 BRCA2 carriers) and 3093 with ovarian cancer (2462 BRCA1 and 631 BRCA2 carriers). The number of samples included from each study is provided in Online Resource 1. The recruitment strategies, clinical, demographic, and phenotypic data collected from each participant have been previously reported [14].
Ethics statement

BRCA1 and BRCA2 mutation carriers were recruited through the CIMBA initiative, following approval of the corresponding protocol by the Institutional Review Board or Ethics Committee at each participating center (Online Resource 2); written informed consent was obtained from all study participants [8, 9].

SNP selection and differential allelic expression

SNP selection was performed by first identifying a list of 175 genes of interest involved in cancer-related pathways and/or mechanisms. The list of genes was established by analyzing published results and by using available public databases such as the Kyoto encyclopedia of genes and genomes (http://www.genome.jp/kegg/). Next, DAE SNPs located within these gene regions were identified using previously reported data on allelic expression cis-associations, derived using (1) the Illumina Human1M-duo BeadChip for lymphoblastoid cell lines from Caucasians (CEU population) \((n = 53)\) [15], the Illumina Human 1M Omni-quad for primary skin fibroblasts derived from Caucasian donors \((n = 62)\) [13, 16], and the Illumina Infinium II assay with Human 1.2M Duo custom BeadChip v1 for human primary monocytes \((n = 188)\) [17]. Briefly, 1000 Genomes project data were used as a reference set (release 1000G Phase I v3) for the imputation of genotypes from HapMap individuals. Genotypes were inferred using algorithms implemented in IMPUTE2 [18]. The unrelated fibroblast panel consisted of 31 parent–offspring trios, in which the genotypes of offspring were used to permit accurate phasing. Mapping of each allelic expression trait was carried out by first normalizing allelic expression ratios at each SNP using a polynomial method [19] and then calculating average phased allelic expression scores across annotated transcripts, followed by correlation of these scores to local (transcript ± 500 kb) SNP genotypes in fibroblasts as described earlier [16]. A total of 355 genetic variants were selected on the basis of evidence of association with DAE in the selected 175 genes (see Online Resource 3 for a complete list of SNPs and genes). Following the selection process, SNPs were submitted for design and inclusion on a custom-made Illumina Infinium array (iCOGS) as previously described [8, 9]. Following probe design and post-genotyping quality control, 316 and 317 SNPs were available for association analysis in BRCA1 and BRCA2 mutation carriers, respectively. Genotyping and quality control procedures have been described in detail elsewhere [8, 9].

Statistical analysis

Associations between genotypes and breast and ovarian cancer risks were evaluated within a survival analysis framework, using a one degree-of-freedom score test statistic based on modeling the retrospective likelihood of the
observed genotypes conditional on the disease phenotypes [20, 21]. To estimate the magnitude of the associations [hazard ratios (HRs)], we maximized the retrospective likelihood, which was parameterized in terms of the per-allele HR. All analyses were stratified by country of residence and using calendar year and cohort-specific incidence rates of breast and ovarian cancers for mutation carriers. Given 320 tests, the cutoff value for significance after a Bonferroni adjustment for multiple testing was $p < 1.5 \times 10^{-4}$.

The associations between the genotypes and tumor subtypes were evaluated using an extension of the retrospective likelihood approach that models the association with two or more subtypes simultaneously [22]. Imputation was performed separately for \textit{BRCA1} and \textit{BRCA2} mutation carriers to estimate genotypes for other common variants across a ±50-kb region centered around the 12 most strongly associated SNPs (following the NCBI Build 37 assembly), using the March 2012 release of the 1000 Genomes Project as the reference panel and the IMPUTE v.2.2 software [18]. In all analyses, only SNPs with an imputation accuracy coefficient $r^2 > 0.30$ were considered [8, 9].

**Functional annotation**

Publicly available genomic data were used to annotate the SNPs most strongly associated with breast cancer risk at locus 11q22.3. The following regulatory features were obtained for breast cell types from ENCODE and NIH Roadmap Epigenomics data through the UCSC Genome Browser: DNase I hypersensitivity sites, chromatin hidden Markov modeling (ChromHMM) states, and histone modifications of epigenetic markers, more specifically commonly used marks associated with enhancers (H3K4Me1 and H3K27Ac) and promoters (H3K4Me3 and H3K9Ac). To identify putative target genes, we examined potential functional chromatin interactions between distal and proximal regulatory transcription factor-binding sites and the promoters at the risk loci, using the chromatin interaction analysis by paired end tag (ChiA-PET) and genome conformation capture (Hi-C, 3C, and 5C) datasets downloaded from GEO and from 4D-genome [23]. Maps of active mammary super-enhancer regions in human mammary epithelial cells (HMECs) were obtained from Hnisz et al. [24]. Enhancer–promoter specific interactions were predicted from the integrated method for predicting enhancer targets (IM-PETs) [25]. RNA-Seq data from ENCODE was used to evaluate the expression of exons across the 11q22.3 locus in MCF7 and HMEC cell lines. For MCF7 and HMEC, alignment files from 19 and 4 expression datasets, respectively, were downloaded from ENCODE using a rest API wrapper (ENCODExplorer R package) [26] in the bam format and processed using metagene R packages [27] to normalize in Reads per Millions aligned and to convert into coverages.

---

[73] Comprehensive Cancer Center Arthur C. James Cancer Hospital and Richard J. Solove Research Institute Biomedical Research Tower, Room 588, 460 West 12th Avenue, Columbus, OH 43210, USA
[74] The Hereditary Breast and Ovarian Cancer Research Group Netherlands (HEBON), Coordinating Center: Netherlands Cancer Institute, Amsterdam, The Netherlands
[75] Family Cancer Clinic, Netherlands Cancer Institute, P.O. Box 90203, 1006 BE Amsterdam, The Netherlands
[76] Center for Medical Genetics, NorthShore University HealthSystem, University of Chicago Pritzker School of Medicine, 1000 Central Street, Suite 620, Evanston, IL 60201, USA
[77] N.N. Petrov Institute of Oncology, St. Petersburg, Russia 197758
[78] Lombardi Comprehensive Cancer Center, Georgetown University, 3800 Reservoir Road NW, Washington, DC 20007, USA
[79] Clinical Genetics, Guy’s and St. Thomas’ NHS Foundation Trust, London SE1 9RT, UK
[80] Familial Cancer Centre, Peter MacCallum Cancer Centre, Melbourne, VIC 3000, Australia
[81] Sir Peter MacCallum Department of Oncology, University of Melbourne, Melbourne, VIC 3010, Australia
[82] Hematology, Oncology and Transfusion Medicine Center, Department of Molecular and Regenerative Medicine, Vilnius University Hospital Santariskiu Clinics, Santariskiu st. 2, 08661 Vilnius, Lithuania
[83] State Research Institute Centre for Innovative Medicine, Zygymantu st. 9, Vilnius, Lithuania
[84] Department of Clinical Genetics, Aarhus University Hospital, Brendstrupgaardstræde 21C, Aarhus N, Denmark
[85] Department of Epidemiology, Cancer Prevention Institute of California, 2201 Walnut Avenue Suite 300, Fremont, CA 94538, USA
[86] Department of Health Research and Policy (Epidemiology) and Stanford Cancer Institute, Stanford University School of Medicine, Stanford, CA, USA
[87] Clinical Genetics Research Laboratory, Department of Medicine, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065, USA
[88] Institute of Human Genetics, University of Ulm, 89091 Ulm, Germany
[89] Women’s Cancer Program at the Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Suite 290W, Los Angeles, CA 90048, USA
eQTL analyses

The influence of germline genetic variations on gene expression was assessed using a linear regression model, as implemented in the R library eMAP (http://www.bios.unc.edu/~weisun/software.htm). An additive effect was inferred by modeling subjects’ copy number of the rare allele, i.e., 0, 1, or 2 for a given genotype. Only relationships in cis (defined as those for which the SNP is located at <1 Mb upstream or downstream from the center of the transcript) were investigated. The eQTL analyses were performed on both normal and tumor breast tissues (see Online Resource 4 for the list and description of datasets, as well as the sources of genotype and expression data). For all sample sets, the genotyping data were processed as follows: SNPs with call rates <0.95 or minor allele frequencies, MAFs (<0.05) were excluded, as were SNPs out of Hardy–Weinberg equilibrium with \( P < 10^{-13} \). All samples with a call rate <80% were excluded. Identity by state was computed using the R GenABEL package [28], and samples from closely related individuals whose identity by state was lower than 0.95 were removed. The SNP and sample filtration criteria were applied iteratively until all samples and SNPs met the set thresholds.

Results

From the 175 genes selected for their involvement in cancer-related pathways and/or mechanisms, we identified a set of 355 genetic variants showing evidence of association with DAE (see Online Resource 3 for the complete list of genes and SNPs). Of those, 39 and 38 SNPs were excluded because of low Illumina design scores, low call rates, and/or evidence of deviation from Hardy–Weinberg equilibrium (\( P \text{ value} <10^{-7} \)), for BRCA1 and BRCA2 analyses, respectively. A total of 316 and 317 SNPs (representing 227 independent SNPs with a pairwise \( r^2 <0.1 \)) were successfully genotyped in 15,252 BRCA1 and 8211 BRCA2 mutation carriers, respectively. Association results for breast and ovarian cancer risks for all SNPs are presented in Online Resource 5.

Breast cancer association analysis

Evidence of association with breast cancer risk (at \( p < 10^{-2} \)) was observed for nine SNPs in BRCA1 mutation carriers and three SNPs in BRCA2 mutation carriers (Table 1). The strongest association with breast cancer risk among BRCA1 carriers was observed for rs6589007, located at 11q22.3 in intron 15 of the NPAT gene.
Table 1  Associations with breast cancer risk in BRCA1 and BRCA2 mutation carriers for SNPs observed at $p < 10^{-2}$

<table>
<thead>
<tr>
<th>Locations</th>
<th>Positions</th>
<th>SNPs</th>
<th>Nearest genes</th>
<th>Unaffected (number)</th>
<th>Affected (number)</th>
<th>Unaffected (MAF)</th>
<th>Affected (MAF)</th>
<th>HR* (95% CI)</th>
<th>$p$ values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BRCA1</strong> mutation carriers</td>
<td>1q24.13</td>
<td>227,308,416</td>
<td>rs11806633</td>
<td>CDC42BPA</td>
<td>7455</td>
<td>7797</td>
<td>0.07</td>
<td>0.06</td>
<td>1.128 (1.039–1.225)</td>
</tr>
<tr>
<td>2p23.2</td>
<td>28,319,320</td>
<td>rs6721310</td>
<td>BRE</td>
<td>7454</td>
<td>7793</td>
<td>0.33</td>
<td>0.33</td>
<td>1.064 (1.018–1.111)</td>
<td>5.4 $\times 10^{-3}$</td>
</tr>
<tr>
<td>2q11.2</td>
<td>100,019,496</td>
<td>rs2305354</td>
<td>REVI1</td>
<td>7451</td>
<td>7796</td>
<td>0.44</td>
<td>0.44</td>
<td>1.057 (1.015–1.100)</td>
<td>7.1 $\times 10^{-3}$</td>
</tr>
<tr>
<td>4p15.33</td>
<td>14,858,341</td>
<td>rs1389999</td>
<td>CEBP</td>
<td>7454</td>
<td>7795</td>
<td>0.35</td>
<td>0.35</td>
<td>0.940 (0.901–0.982)</td>
<td>5.3 $\times 10^{-3}$</td>
</tr>
<tr>
<td>5q14.1</td>
<td>79,901,952</td>
<td>rs425463</td>
<td>DHFR, MSH3</td>
<td>7430</td>
<td>7755</td>
<td>0.33</td>
<td>0.35</td>
<td>1.058 (1.013–1.105)</td>
<td>9.5 $\times 10^{-3}$</td>
</tr>
<tr>
<td>11q22.3</td>
<td>108,040,104</td>
<td>rs6589007</td>
<td>NPAT, AAT, ATM</td>
<td>7451</td>
<td>7797</td>
<td>0.41</td>
<td>0.42</td>
<td>1.062 (1.019–1.107)</td>
<td>4.6 $\times 10^{-3}$</td>
</tr>
<tr>
<td>11q22.3</td>
<td>108,089,197</td>
<td>rs183459</td>
<td>NPAT, ATM</td>
<td>7447</td>
<td>7789</td>
<td>0.40</td>
<td>0.41</td>
<td>1.061 (1.018–1.105)</td>
<td>5.7 $\times 10^{-3}$</td>
</tr>
<tr>
<td>11q22.3</td>
<td>108,123,819</td>
<td>rs228592</td>
<td>ATM</td>
<td>7449</td>
<td>7792</td>
<td>0.42</td>
<td>0.41</td>
<td>1.061 (1.018–1.106)</td>
<td>5.5 $\times 10^{-3}$</td>
</tr>
<tr>
<td>12p13.33</td>
<td>986,004</td>
<td>rs7967755</td>
<td>WNK1, RAD52</td>
<td>7454</td>
<td>7797</td>
<td>0.16</td>
<td>0.152</td>
<td>0.927 (0.876–0.980)</td>
<td>7.5 $\times 10^{-3}$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Locations</th>
<th>Positions</th>
<th>SNPs</th>
<th>Nearest genes</th>
<th>Unaffected (number)</th>
<th>Affected (number)</th>
<th>Unaffected (MAF)</th>
<th>Affected (MAF)</th>
<th>HR* (95% CI)</th>
<th>$p$ values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BRCA2</strong> mutation carriers</td>
<td>6p22.1</td>
<td>28,231,243</td>
<td>rs9468322</td>
<td>NKAPL</td>
<td>3880</td>
<td>4329</td>
<td>0.04</td>
<td>0.05</td>
<td>1.235 (1.080–1.412)</td>
</tr>
<tr>
<td>8q11.21</td>
<td>48,708,742</td>
<td>rs6982040</td>
<td>PRKDC</td>
<td>3876</td>
<td>4327</td>
<td>0.006</td>
<td>0.002</td>
<td>0.497 (0.292–0.843)</td>
<td>2.7 $\times 10^{-3}$</td>
</tr>
<tr>
<td>16p13.33</td>
<td>1,371,154</td>
<td>rs2268049</td>
<td>UBE2I</td>
<td>3880</td>
<td>4325</td>
<td>0.14</td>
<td>0.16</td>
<td>1.116 (1.031–1.207)</td>
<td>4.5 $\times 10^{-3}$</td>
</tr>
</tbody>
</table>

CI confidence interval, HR hazard ratio, MAF minor allele frequency, SNP single-nucleotide polymorphism

* Hazard ratio per allele (one degree of freedom) estimated from the retrospective likelihood analysis

($p = 4.6 \times 10^{-3}$) at approximately 54 kb upstream of the ATM gene. Similar associations were observed for two other highly correlated variants ($r^2 > 0.8$) on chromosome 11, namely rs183459 ($p = 5.7 \times 10^{-3}$) also located within NPAT and rs228592 ($p = 5.5 \times 10^{-3}$) located in intron 11 of ATM. No association was observed between SNPs at this locus and breast cancer risk for BRCA2 carriers (Online Resource 5).

The strongest evidence of association with breast cancer risk in BRCA2 mutation carriers was observed for
Table 2  Associations with breast cancer risk by tumor subtype in BRCA1 and BRCA2 mutation carriers

<table>
<thead>
<tr>
<th>Locations</th>
<th>Positions</th>
<th>SNPs</th>
<th>ER-positive</th>
<th>ER-negative</th>
<th>ER-diff</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HR (95 % CI)</td>
<td>p values</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRCA1 mutation carriers</td>
<td>1q42.13</td>
<td>227,308,416</td>
<td>rs11806633</td>
<td>1.10 (0.90–1.33)</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>2p23.2</td>
<td>28,319,320</td>
<td>rs6721310</td>
<td>1.00 (0.88–1.09)</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>2q11.2</td>
<td>100,019,496</td>
<td>rs2305354</td>
<td>0.98 (0.91–1.10)</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>4p15.33</td>
<td>14,858,341</td>
<td>rs1389999</td>
<td>0.94 (0.85–1.04)</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>5q14.1</td>
<td>79,901,952</td>
<td>rs425463</td>
<td>1.04 (0.94–1.15)</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>11q22.3</td>
<td>108,040,104</td>
<td>rs6589007</td>
<td>1.08 (0.99–1.19)</td>
<td>9.8 × 10⁻²</td>
</tr>
<tr>
<td></td>
<td>11q22.3</td>
<td>108,089,197</td>
<td>rs183459</td>
<td>1.08 (0.99–1.19)</td>
<td>9.3 × 10⁻²</td>
</tr>
<tr>
<td></td>
<td>11q22.3</td>
<td>108,123,189</td>
<td>rs228592</td>
<td>1.08 (0.96–1.19)</td>
<td>9.7 × 10⁻²</td>
</tr>
<tr>
<td></td>
<td>12p13.33</td>
<td>986,004</td>
<td>rs7967755</td>
<td>0.96 (0.84–1.09)</td>
<td>0.54</td>
</tr>
<tr>
<td>BRCA2 mutation carriers</td>
<td>6p22.1</td>
<td>28,231,243</td>
<td>rs9468322</td>
<td>1.30 (1.12–1.51)</td>
<td>5.0 × 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>8q11.21</td>
<td>48,708,742</td>
<td>rs6982040</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>16p13.3</td>
<td>1,371,154</td>
<td>rs2268049</td>
<td>1.10 (1.01–1.21)</td>
<td>4.0 × 10⁻²</td>
</tr>
</tbody>
</table>

CI confidence interval, HR hazard ratio, SNP single-nucleotide polymorphism, N/A not available
* Hazard ratio per allele (one degree of freedom) estimated from the retrospective likelihood analysis

rs6982040, located at 8q11.21 in intron 74 of the PRKDC gene \((p = 2.7 \times 10^{-3})\). However, this variant had a very low frequency in affected and unaffected individuals (MAF values of 0.002 and 0.006, respectively). No association was observed for this locus in BRCA1 carriers (Online Resource 5).

Of the nine SNPs associated with breast cancer risk in BRCA1 mutation carriers, three were primarily associated with estrogen receptor (ER)-negative breast cancer: rs11806633 at 1q42.13 in the CDC42BPA gene \((p = 9.0 \times 10^{-3})\), rs6721310 at 2p23.2 in the BRE gene \((p = 3.0 \times 10^{-3})\), and rs2305354 at 2q11.2 in the REV1 gene \((p = 1.0 \times 10^{-3})\), although the differences between ER-positive and ER-negative disease associations were not statistically significant (Table 2). Of the three BRCA2-associated loci, only rs9468322 at 6p22.1 was associated with...
ER-positive disease \((p = 5.0 \times 10^{-4})\), although the differences in HRs between ER-positive and ER-negative tumors were not statistically significant (Table 2).

Although evidence of association with breast cancer risk was observed for the above-described loci in \(BRCA1\) and \(BRCA2\) mutation carriers, none of these associations reached significance after a Bonferroni adjustment for multiple testing. Imputation using the 1000 Genomes data (encompassing \(\pm 50\) kb centered on each of the 12 associated variants, Online Resource 6) identified several SNPs included in the 1000 Genome Mar 2012 EUR release. Pairwise \(r^2\) values are plotted using a red scale, where white and red means \(r^2 = 0\) and 1, respectively. SNPs are plotted according to their chromosomal position: physical locations are based on the GRCh37/hg19 map. SNP rs228606 was genotyped in the iCOGS array but was not included in our original hypothesis of association with DAE. Gene annotation is based on the NCBI RefSeq gene descriptors from the UCSC genome browser.

**Fig. 1** Manhattan plot depicting the strength of association between breast cancer risk in \(BRCA1\) mutation carriers and all imputed and genotyped SNPs located across the 11q22.3 locus bound by hg19 coordinates chr11:107990104_108173189. Directly genotyped SNPs are represented as triangles and imputed SNPs \((r^2 > 0.3,\ MAF > 0.02)\) are represented as circles. The linkage disequilibrium \((r^2)\) for the most strongly associated genotyped SNP with each SNP was computed based on subjects of European ancestry that were included in the 1000 Genome Mar 2012 EUR release. Pairwise \(r^2\) values are plotted using a red scale, where white and red means \(r^2 = 0\) and 1, respectively. SNPs are plotted according to their chromosomal position: physical locations are based on the GRCh37/hg19 map. SNP rs228606 was genotyped in the iCOGS array but was not included in our original hypothesis of association with DAE. Gene annotation is based on the NCBI RefSeq gene descriptors from the UCSC genome browser.
with significant associations in BRCA1 mutation carriers at the 11q22.3 locus (with SNP rs228595 as the most significant, \( p = 7.38 \times 10^{-6} \)), and which were partly correlated with the genotyped SNPs (\( r^2 < 0.4 \), Fig. 1). After imputation, we also found associations (albeit not statistically significant after multiple testing adjustments), between one imputed SNP at locus 12p13 (rs2255390, \( p = 5.0 \times 10^{-4} \)) and breast cancer risk for BRCA1 carriers, and two SNPs and breast cancer risk for BRCA2 carriers, namely 6p22 (chr6:28226644:I, \( p = 9.0 \times 10^{-3} \)) and 8q11 (rs189286892, \( p = 2.0 \times 10^{-4} \)).

**Ovarian cancer association analyses**

Evidence of association with ovarian cancer risk (\( p < 10^{-2} \)) was observed for six SNPs in BRCA1 mutation carriers and three SNPs in BRCA2 mutation carriers (Table 3). The strongest association with ovarian cancer risk in BRCA1 carriers was observed for rs12025623 located at 1p36.12 (\( p = 7 \times 10^{-3} \)) in an intron of the ALPL gene. Another correlated variant (\( r^2 > 0.7 \)) on chromosome 1 was also genotyped, namely rs1767429 (\( p = 9 \times 10^{-3} \)), which was also located within ALPL. The strongest evidence of association with ovarian cancer risk in BRCA2 mutation carriers was observed for rs2233025 (\( p = 5 \times 10^{-3} \)), located at 1p32.22 within the MAD2L2 gene. None of these associations remained statistically significant after multiple testing adjustments. Imputed genotypes of SNPs in a region encompassing \( \pm 50 \) kb centered on each of the nine associated variants did not identify stronger associations.

**eQTL analysis in breast tissue**

To identify the genes influenced via the observed associations with breast cancer at locus 11q22.3, eQTL analysis was performed using gene expression data from tumor and normal breast tissues (for detailed descriptions of datasets, refer to Online Resource 4), and all genotyped as well as imputed SNPs within a 1-Mb region on either side of the most significant genotyped SNP. eQTL associations were observed in both normal and tumor breast tissues in this region, although none of those were correlated with our most significant risk SNPs (Online Resource 7). The strongest eQTL associations were observed in the breast cancer tissue dataset BC241 for the SLC35F2 gene (rs181187590, \( p = 1.4 \times 10^{-5}, r^2 = 0.08 \), i.e., \( 8 \) % of the variation in SLC35F2 expression was attributable to this SNP). Other eQTLs observed in this dataset included ELMO1 (rs181187590, \( p = 1.3 \times 10^{-4}, r^2 = 0.06 \), EXPH5 (rs181187590, \( p = 3 \times 10^{-4}, r^2 = 0.054 \)), and ATM (rs4987915, \( p = 3.7 \times 10^{-4}, r^2 = 0.05 \)). In The Cancer Genome Atlas (TCGA) BC765 breast cancer dataset, the strongest associations with gene expression were observed for the non-coding RNA lLOC643923 (rs183293362, \( p = 2.3 \times 10^{-4}, r^2 = 0.02 \), ATM (rs4987924, \( p = 8.3 \times 10^{-4}, r^2 = 0.015 \), and KDELCC2.

Table 3 Associations with ovarian cancer risk in BRCA1 and BRCA2 mutation carriers for SNPs observed at \( p < 10^{-2} \)

<table>
<thead>
<tr>
<th>Locations</th>
<th>Positions</th>
<th>SNPs</th>
<th>Nearest genes</th>
<th>Unaffected (number)</th>
<th>Affected (number)</th>
<th>Unaffected (MAF)</th>
<th>HR* (95 % CI)</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BRCA1 mutation carriers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1p36.12</td>
<td>21,889,340</td>
<td>rs1767429</td>
<td>ALPL, RAP1GAP</td>
<td>12,765</td>
<td>2460</td>
<td>0.42</td>
<td>1.092 (1.024–1.164)</td>
<td>9 ( \times 10^{-3} )</td>
</tr>
<tr>
<td>1p36.12</td>
<td>21,892,479</td>
<td>rs12025623</td>
<td>ALPL, RAP1GAP</td>
<td>12,789</td>
<td>2460</td>
<td>0.36</td>
<td>1.098 (1.027–1.173)</td>
<td>7 ( \times 10^{-3} )</td>
</tr>
<tr>
<td>6p21.32</td>
<td>32,913,246</td>
<td>rs1480380</td>
<td>BRD2, HLA-DMB, HLA-DMA</td>
<td>12,790</td>
<td>2462</td>
<td>0.07</td>
<td>1.178 (1.041–1.333)</td>
<td>9 ( \times 10^{-3} )</td>
</tr>
<tr>
<td>10p12.1</td>
<td>27,434,716</td>
<td>rs788209</td>
<td>ANKRD26, YME1L1, MASTL, ACBD5</td>
<td>12,754</td>
<td>2455</td>
<td>0.15</td>
<td>0.879 (0.804–0.961)</td>
<td>5 ( \times 10^{-3} )</td>
</tr>
<tr>
<td>17p13.1</td>
<td>8,071,592</td>
<td>rs3027247</td>
<td>MIR3676, C17orf59, AURKB, C17orf44, C17orf68, PFAS</td>
<td>12,786</td>
<td>2461</td>
<td>0.29</td>
<td>0.905 (0.844–0.970)</td>
<td>5 ( \times 10^{-3} )</td>
</tr>
<tr>
<td>17q22</td>
<td>53,032,425</td>
<td>rs17817865</td>
<td>MIR4315-1, TOMIL1, COX11, STXB4</td>
<td>12,790</td>
<td>2462</td>
<td>0.27</td>
<td>0.905 (0.842–0.971)</td>
<td>8 ( \times 10^{-3} )</td>
</tr>
<tr>
<td><strong>BRCA2 mutation carriers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1p32.22</td>
<td>11,735,652</td>
<td>rs2233025</td>
<td>MAD2L2, FBXO6</td>
<td>7574</td>
<td>631</td>
<td>0.18</td>
<td>0.777 (0.657–0.919)</td>
<td>5 ( \times 10^{-3} )</td>
</tr>
<tr>
<td>9p13.3</td>
<td>35,055,669</td>
<td>rs595429</td>
<td>VCP, FANCG, c9orf131</td>
<td>7579</td>
<td>631</td>
<td>0.46</td>
<td>0.856 (0.758–0.964)</td>
<td>6 ( \times 10^{-3} )</td>
</tr>
<tr>
<td>17q25.3</td>
<td>76,219,783</td>
<td>rs2293680</td>
<td>DHX29, SKIV2L2</td>
<td>7579</td>
<td>630</td>
<td>0.28</td>
<td>0.828 (0.722–0.948)</td>
<td>7 ( \times 10^{-3} )</td>
</tr>
</tbody>
</table>

CI confidence interval, HR hazard ratio, MAF minor allele frequency, SNP single-nucleotide polymorphism

* Hazard ratio per allele (one degree of freedom) estimated from the retrospective likelihood analysis
Functional annotation

In order to assess the potential functional role of the most significant risk SNPs in the 11q22.3 region, ENCODE chromatin biological features were evaluated in available breast cells, namely HMECs, breast myoepithelial cells, and MCF7 breast cancer cells. We observed some overlap between features of interest and candidate SNPs within the 11q22.3 region (Fig. 2). The most interesting variant was rs228606, which overlapped a monomethylated H3K4 mark in HMECs. Analysis of data from the Roadmap Epigenomisc project also showed overlap with a monomethylated H3K4 mark and an acetylated H3K9 mark in primary breast myoepithelial cells. From ChiA-PET data, chromosomal interactions were found in the NPAT and ATM genes in MCF7 cells, located mainly in the vicinity of the promoter regions of these genes, which encompassed a strongly associated imputed SNP at this locus, namely chr11:108098459_TAA-T. Lastly, although super-enhancers and predicted enhancer–promoter interactions mapped to the 11q22.3 locus in HMECs, none overlapped with our top candidate SNPs (Fig. 2).

Discussion

DAE is a common phenomenon in human genes, which represents a new approach to identifying cis-acting mechanisms of gene regulation. It offers a new avenue for the study of GWAS variants significantly associated with various diseases/traits. Indeed, the majority of GWAS hits localize outside known protein-coding regions [11, 12], suggesting a regulatory role for these variants. In the present study, we have assessed the association between 320 SNPs associated with DAE and breast/ovarian cancer risk among BRCA1 and BRCA2 mutation carriers. Using this approach, we found evidence of association for a region at 11q22.3, with breast cancer risk in BRCA1 mutation carriers. Analysis of imputed SNPs across a 185-kb region (±50 kb from the center of each of the three genotyped SNPs at this locus) revealed a set of five strongly correlated SNPs that were significantly associated with breast cancer risk. This region contains several genes including ACAT1, NPAT, and ATM. ACAT1 (acetyl-CoA acetyltransferase 1) encodes a mitochondrial enzyme that catalyzes the reversible formation of acetocacetyl-CoA from two molecules of acetyl-CoA. Defects in this gene are associated with ketothiolase deficiency, an inborn error of isoleucine catabolism [29]. NPAT (nuclear protein, co-activator of histone transcription) is required for progression through the G1 and S phases of the cell cycle, for S phase entry [30], and for the activation of the transcription of histones H2A, H2B, H3, and H4 [31]. NPAT germline mutations have been associated with Hodgkin lymphoma [32]. Finally, ATM (ataxia telangiectasia mutated) encodes an important cell cycle checkpoint kinase that is required for cellular response to DNA damage and for genome stability. Mutations in this gene are associated with ataxia telangiectasia, an autosomal recessive disorder [33]. ATM is also an intermediate-risk breast cancer susceptibility gene, with rare heterozygous variants being associated with increased risk of developing the disease [34]. Although several studies have assessed the role of the most common ATM variants in breast cancer susceptibility, the results obtained are inconsistent [35]. A recent study had identified an association between an ATM haplotype and breast cancer risk in BRCA1 mutation carriers with a false discovery rate-adjusted p value of 0.029 for overall association of the haplotype [36]. Four of the five SNPs making up the haplotype were almost perfectly correlated ($r^2 > 0.9$) with the three originally genotyped SNPs of the present study. These SNPs were, however, only moderately correlated ($r^2 > 0.4$) with the most significant risk SNPs ($p = 10^{-6}$), identified later through imputation.

Although eQTL analysis has identified cis-eQTL associations between several variants and ACAT1, ATM as well
as other neighboring genes in both breast carcinoma and normal breast tissues, none of these associations involved the most significantly associated risk SNPs. Furthermore, the correlation between eQTLs and the most significant risk SNPs was weak. The lack of consistency between the eQTL results and the allelic imbalance data originally used for SNP selection in the design of the present study can probably be explained by the differences between the cell types used in these analyses. The list of allelic imbalance-associated SNPs was selected from studies performed in lymphoblastoid cell lines [15], primary skin fibroblasts [13, 16], and primary monocytes [17], while eQTLs were analyzed in breast carcinoma and normal breast tissue. This tissue heterogeneity in the data sources used represents one of the limitations of this study, although no such data were available in mammary cells when this study was originally designed.

The identification of a region at 11q22.3 that is associated specifically with breast cancer risk in BRCA1 mutation carriers may explain why association studies performed using breast cancer cases from the general population have so far yielded conflicting results with regard to common variants at this locus. The majority of tumors arising in BRCA1 carriers show either low or absent ER expression, while the majority of BRCA2-associated tumors are ER positive, as in most sporadic cancers arising in the general population. Large-scale studies using only ER-negative or triple-negative (i.e., ER-, progesterone receptor-, and HER2-negative) cases could therefore be helpful to confirm the association of this locus with breast cancer risk.

Acknowledgments Silje Nord was financed by a Carrier Grant from the Norwegian Regional Health authorities (Grant Number 2014061). BCFR-AU: Maggie Angelakos, Judi Maskili, Gillian Dite, Helen Tsimiklis. BCFR-NY: we wish to thank members and participants in the New York site of the Breast Cancer Family Registry for their contributions to the study. BCFR-ON: we wish to thank the members and participants in the Ontario Familial Breast Cancer Registry for their contributions to the study. BFBOCC-LT thank Valius Rudaitis and Laimonas Giriškevičius. BFBOCC-LV thank Drs. Janis Eglitis, Anna Krišova, and Atvars Stengrevics. BMBSA wish to thank the families who contribute to the BMBSA study. BRICOH: we wish to thank Yuan Chun Ding and Linda Steele for their work in participant enrollment and biospecimen and data management. CBCS: we wish to thank Bent Ejlersen and Anne-Marie Gerdes for the recruitment and genetic counseling of participants. CNIO: we thank Alicia Barroso, Rosario Alonso, and Guillermo Pita for their assistance. CONSI T TEAM: Daniela Zaffaroni of the Fondazione IRCCS Istituto Nazionale Tumori (INT), Milan, Italy; Monica Barile and Irene Feroco of the Istituto Europeo di Oncologia, Milan, Italy; Maria Grazia Tibilietti of the Ospedale di Circolo-Università dell’Insubria, Varese, Italy; Liliana Varesco of the IRCCS AOU San Martino: IST Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy; Alessandra Viel of the CRO Aviano National Cancer Institute, Aviano, Italy; Gabriele Capone of the University of Florence, Florence, Italy; Laura Ottini and Giuseppe Giannini of the “Sapienza” University, Rome, Italy; Antonella Savarese and Aline Martayan of the Istituto Nazionale Tumori Regina Elena, Rome, Italy; Stefania Tommasi and Brunella Pilato of the Istituto Nazionale Tumori “Giovanni Paolo II,” Bari, Italy; and the personnel of the Cogentech Cancer Genetic Test Laboratory, Milan, Italy. CORE: the CIMBA data management and analysis was funded through Cancer Research: UK Grant C12292/ A11174. ACA is a Senior Cancer Research: UK Research Fellow. We wish to thank Sue Healey for her enormous contribution to CIMBA, in particular taking on the task of mutation classification with Olga Sinilnikova. EMBRACE: RE was supported by NIHR support to the Biomedical Research Centre at The Institute of Cancer Research and The Royal Marsden NHS Foundation Trust. FCCC: we thank Ms. JoEllen Weaver and Dr. Betsy Bove for their technical support. GC-HB: we would like to thank LIFE: Leipzig Research Centre for Civilization Diseases (Markus Loefller, Joachim Thiery, Matthias Nièchter, Ronny Baber). Genetic Modifiers of Cancer Risk in BRCA1/2 Mutation Carriers (GEMO) Study: National Cancer Genetics Network «UNICANCER Genetic Group», France. We wish to pay a tribute to Olga M. Sinilnikova, who with Dominique Stoppa-Lyonnet initiated and coordinated GEMO until she sadly passed away on the 30th June 2014, and to thank all the GEMO Collaborating Groups for their contribution to this study. GEMO Collaborating Centers are as follows: Coordinating Centres, Unité Mixte de Génétique Constitutionnelle des Cancers Fréquents, Hospices Civils de Lyon: Centre Léon Bérard, and Équipe «Génétique du cancer du sein», Centre de Recherche en Cancérologie de Lyon: Olga Sinilnikova‡, Sylvie Mazoyer, Francesca Damiola, Laure Barjhoux, Carole Verry-Pierre, Mélanie Léone, Nadia Boutry-Krzyza, Alain Calender, Sophie Giraud; and Service de Génétique Oncologique, Institut Curie, Paris: Dominique Stoppa-Lyonnet, Marion Gauthier-Villars, Bruno Bécuecher, Claude Houdayer, Etienne Rouleau, Lisa Golmard, Agnès Collet, Virginie Moncoutier, Muriel Belotti, Antoine de Pauw, Camille Elan, Catherine Nogues, Emmanuelle Fourmè, Anne-Marie Birot. Institut Gustave Roussy, Villejuif: Brigitte Bressac-de-Paillerets, Olivier Caron, Marine Guillaud-Bataille. Centre Jean Perrin, Clermont-Ferrand: Yves-Jean Bignon, Nancy Uhrhammer. Centre Léon Bérard, Lyon: Christine Lasset, Valérie Bonadona, Sandrine Handallaud. Centre François Baclesse, Caen: Agnès Hardoun, Pascale Berthet, Dominique Vaur, Laurent Castéra. Institut Paoli Calmettes, Marseille: Hagay Sobol, Violaine Bourdon, Tetsuro Noguchi, Audrey Remenieres, François Eisinger. CHU Arnaud-de-Villeneuve, Montpellier: Isabelle Coupier, Pascal Pujol. Centre Oscar Lambret, Lille: Jean-Philippe Peyrat, Joëlle Fournier, Françoise Revillion, Philippe Vennin‡, Claude Adenis, Centre Paul Strauss, Strasbourg: Danielle Migner, Jean-Pierre Meller, Jean-Pascal Buffet, Isabelle Moreux. CHU Hôpital d’Enfants, Université de Lyon: Emmanuelle Barouk-Simonet, Françoise Bonnet, Virginie Bubien, Nicolas Severet, Michel Longy. Institut Claudius Regaud, Toulouse: Christine Toulou, Rosine Guimbaut, Laurence Gladieff, Viviane Feillél. CHU Grenoble: Dominique Leroux, Hélène Dreyfus, Christine Rebischung, Magalie Peysselom. CHU Dijon: Fanny Coron, Laurence Faivre. CHU St-Etienne: Fabienne Prieur, Marine Lebrun, Caroline Kientz. Hôtel Dieu Centre Hospitalier, Chambéry: Sandra Ferr-Fetrier. Centre Antoine Lacassagne, Nice: Marc Frémy. CHU Limoges: Laurence Vénat-Bouvet. CHU Nantes: Capucine Delnate. CHU Bretonneau, Tours: Isabelle Mortemousque. Groupe Hospitalier Pitié-Salpêtrière, Paris: Florence Colet, Chrystelle Colas, Florent Soubrier, Mathilde Warcoin. CHU Vandoeuvre-les-Nancy: Johanna Sokolowska, Myriam Bronner. CHU Besançon: Marie-Agnès Col-longe-Rame, Alexandre Damette. Creighton University, Omaha, USA: Henry T. Lynch, Carrie L. Snyder. G-FAST: we wish to thank the technical support of Ilse Coene en Brecht Crombez. HCSC: we acknowledge the technical assistance of Alicia Tosar and Paula Diaque. HEBCS would like to thank Drs. Sofía Khan, Tara A. Muranen, Carl Blomqvist and RNs Irja Erkkilä and Virpi Palola for their help with the HEBCS data and samples. The Hereditary Breast and Ovarian Cancer Research Group Netherlands (HEBON) consists of the following Collaborating Centers: Coordinating Center: Netherlands Cancer Institute, Amsterdam, NL: M.A. Rookus, F.B.L.
Hogervorst, F.E. van Leeuwen, S. Verhoef, M.K. Schmidt, N.S. Russell, D.J. Jenner; Erasmus Medical Center, Rotterdam, NL: J.M. Collée, A.M.W. van den Ouweland, M.J. Hooring, C. Seynave, C.H.M. van Deurzen, I.M. Obdeijn; Leiden University Medical Center, NL: C.J. van Asperen, J.T. Wijnen, R.A.E.M. Tollenaar, P. Devilee, T.C.T.E.F. van Cronenburg; Radboud University Nijmegen Medical Center, NL: C.M. Kets, A.R. Mensenkamp; University Medical Center Utrecht, NL: M.G.E.M. Ausems, R.B. van der Luijt, C.C. van der Pol; Amsterdam Medical Center, NL: C.M. Aalfs, T.A.M. van Os; VU University Medical Center, Amsterdam, NL: J.J.P. Gille, Q. Waisfisz, H.E.J. Meijers-Heijboer; University Hospital Maastricht, NL: E.B. Gómez-Garcia, M.J. Blok; University Medical Center Groningen, NL: J.C. Oosterwijk, A.H. van der Hout, M.J. Mourtis, G.H. de Bock; The Netherlands Foundation for the Detection of Hereditary Tumours, Leiden, NL: H.F. Vasen; The Netherlands Comprehensive Cancer Organization (IKNL): S. Siesling, J. Verloopen; The Dutch Pathology Registry (PALGA): L.I.H. Overbeek. The HEBON study was supported by the Dutch Cancer Society Grants NK1998-1854, NK2004-3088, NK2007-3576, the Netherlands Organization of Scientific Research Grant NWO 91109024, the Pink Ribbon Grants 110005 and 2014-187.W076, the BBMRI Grant NWO 184.021.007/CP46, and the Transcan Grant JTC 2012 Cancer 12-054. HEBON thanks the registration teams of IKNL and PALGA for their help in part of the data collection. HRBCP: we wish to thank Hong Kong Sanatorium and Hospital for their continued support. HUN-BOCS: we wish to thank the Hungarian Breast and Ovarian Cancer Study Group Members (Janos Papp, Tibor Vaszko, Aniko Bozskik, Judit Franko, Maria Balogh, Gabriella Domokos, Judit Ferenczi, Department of Molecular Genetics, National Institute of Oncology, Budapest, Hungary) and the clinicians and patients for their contributions to this study. HVH: we wish to thank the Oncogenetics Group (VHIO) and the High Risk and Cancer Prevention Unit for their contributions to this study. The University Hospital Vall d’Hebron. Thanks to the Cellex Foundation for providing research facilities and equipment. ICO: we wish to thank the ICO Hereditary Cancer Program Team led by Dr. Gabriel Capella. INHERIT: we would like to thank Dr. Martine Dumont for sample management and skillful assistance. J.S. is Chairholder of the Canada Research Chair in Oncogenetics. J.S. and P.S. were part of the QC and Genotyping Coordinating Group of the iCOGS (BCAC and CIBMA). IPOBCS: we wish to thank Drs. Ana Peixoto, Catarina Santos, and Pedro Pinto for their skillful contribution to the study. KCONFAB: we wish to thank Heather Thorne, Eveline Niedermayr, all the high risk nurses, research nurses and staff, the heads and staff of the Family Cancer Clinics, and the Clinical Follow Up Study [which has received funding from the NHMRC, the National Breast Cancer Foundation, Cancer Australia, and the National Institute of Health (USA)] for their contributions to this resource, and the many families who contribute to iConFab. Modifier Study of Quantitative Effects on Disease (MODSQUAD) thank ModSQuAD members Csilla Szabo (National Human Genome Research Institute, National Institutes of Health,Bethesda, MD, USA); Lenka Foretova and Eva Machackova (Department of Cancer Epidemiology and Genetics, Masaryk Memorial Cancer Institute and MF MU, Brno, Czech Republic); and Michal Zitan, Petr Pohliech, and Zdenek Kleibl (Oncogynecologic Center and Department of Biochemistry and Experimental Oncology, First Faculty of Medicine, Charles University, Prague, Czech Republic). MSKCC: Anne Lincoln, Lauren Jacobs. NICCC: we wish to thank the NICCC National Familial Cancer Consultation Service Team led by Sara Dison, the Lab Team led by Dr. Flavio Lejbkowicz, and the Research Field Operations Team led by Dr. Mila Pinchey. NRG Oncology: we thank the investigators of the Australia New Zealand NRG Oncology Group. OCGN: we wish to thank the members and participants in the Ontario Cancer Genetics Network for their contributions to the study. OSUCCG: Leigha Senter, Kevin Sweet, Caroline Craven, Julia Cooper, and Michelle O’Conor were instrumental in accrual of study participants, ascertainment of medical records, and database management. SEABASS: we would like to thank Yip Cheng Har, Nur Aishah Mohd Taib, Phuah Sze Yee, Norhashimah Hassan, and all the research nurses, research assistants, and doctors involved in the MyBrCa Study for their assistance in patient recruitment, data collection, and sample preparation. In addition, we thank Philip Iau, Sng Jen-Hwei, and Sharifah Nor Akmal for contributing samples from the Singapore Breast Cancer Study and the HUKM-HKL Study. The Malaysian Breast Cancer Genetic Study was funded by Research Grants from the Malaysian Ministry of Science, Technology and Innovation, Ministry of Higher Education (UM.C/HIR/MOHE/06), and charitable funding from Cancer Research Initiatives Foundation. SMC Team wishes to acknowledge the assistance of the Meirav Comprehensive Breast Cancer Center Team at the Sheba Medical Center for their assistance in this study. SWE-BRCA: Swedish scientists participating as SWE-BRCA Collaborators are from Lund University and University Hospital: Åke Borg, Håkan Olsson, Helena Jerström, Karin Henriksson, Katja Harbst, Maria Soller, Ulf Kristofferson; from Gothenburg Sahlgrenska University Hospital: Anna Överholm, Margareta Nordling, Per Karlsson, Zakaria Einbeigi; from Stockholm and Karolinska University Hospital: Anna von Wachenfeldt, Annelie Liljegren, Annika Lindblom, Brita Arver, Gisela Barbany Bustinza, Johanna Rantalu; from Umeå University Hospital: Beatrice Melin, Christina Edwinsdotter Ardnor, Monica Emanuelsen; from Uppsala University: Hans Ehrencren, Maritta Hellström Pigg, Richard Rosenquist; and from Linköping University Hospital: Marie Stenmark-Askmal, Sigrun Liedgren. UCHICAGO: we wish to thank Cecilia Zvoecc, Qun Niu, physicians, genetic counselors, research nurses and staff of the Cancer Risk Clinic for their contributions to this resource, and the many families who contribute to our program. UCLA: we thank Joyce Seldon MSGC and Lorna Kwan, MPH, for assembling the data for this study. UCSF: we would like to thank Dr. Robert Nussbaum and the following genetic counselors for participant recruitment: Beth Crawford, Kate Loranger, Julie Mak, Nicola Stewart, Robin Lee, Amie Blanco, and Peggy Conrad. And thanks to Ms. Salina Chan for her data management. UKFOCR: we thank Susan Ramus, Carole Pye, Patricia Harrington, and Eva Wozniak for their contributions toward the UKFOCR. VFCTG: Geoffrey Lindeman, Marion Harris, Martin Delaytcky of the Victorian Familial Cancer Trials Group. We thank Sarah Sawyer and Rebecca Driessen for assembling the data and Ella Thompson for performing all DNA amplification.

**Funding** Funding for the iCOGS infrastructure came from the European Community’s Seventh Framework Programme under Grant Agreement No. 223175 (HEALTH-F2-2009-223175) (COGS), Cancer Research UK (C1287/A10118, C1287/A10710, C12292/A11174, C1281/A12014, C5047/A8384, C5047/A15007, C5047/A10692, C19179/A16565), the National Institutes of Health (CA128978) and Post-cancer GWAS Initiative (1U19 CA148537, 1U19 CA148065, and 1U19 CA148112: the GAME-ON Initiative), the Department of Defence (W81XWH-10-1-0341), the Canadian Institutes of Health Research (CIHR) for the CIHR Team in Familial Risks of Breast Cancer, Komen Foundation for the Cure, the Breast Cancer Research Foundation, and the Ovarian Cancer Research Fund. BFBOCC: this work was supported by Grant UM1 CA164920 from the National Cancer Institute. BFBOCC was supported by Lithuania (BFBOCC- LT): Research Council of Lithuania Grant SEN-18/2015. BIDMC was supported by the Breast Cancer Research Foundation. BRCA gene mutations and breast cancer in South African women (BMBSA) was supported by Grants from the Cancer Association of South Africa (Cansa) to Elizabeth J. van Rensburg. BRICOH: SLN was partially supported by the Morris and Horowitz Families Endowed Professorship. CNIO: this work was partially supported by Spanish Association Against Cancer (AECC08), RTI06/0020/1060, FIS/P08/1120, Mutua Madrileña Foundation (FMMF), and SAF2010-20493.
COH-CCGCRN: patients were recruited for this study from the City of Hope Clinical Cancer Genomics Community Research Network, supported in part by Award Number RC4CA153828 (PI: J. Weitzel) from the National Cancer Institute and the Office of the Director, National Institutes of Health. CONSIT Team: Associazione della Italiana Ricerca sul Cancro (AIRC) to P. Peterlongo (IG 2012 Id.12821) and P. Radice (IG 2014 Id.15547). Funds from Italian citizens who allocated the 5 × 1000 share of their tax payment in support of the Fondazione IRCCS Istituto Nazionale Tumori, according to Italian laws (INT-Institutional Strategic Projects “5x1000”) to S. Manoukian. FiorGen Foundation for Pharmacogenomics to L. Papi. CORE: the CIMBA Data Management and Data Analysis were supported by Cancer Research: UK Grants C12292/A20861, C12292/A11174. ACA is a Cancer Research UK Senior Cancer Research Fellow. GCT is an NHMRC Senior Principal Research Fellow. J. Lecarpentier has been financially supported by the Fondation ARC (FONDATION ARC, 9 rue Guy Môquet 94803 Villejuif; France), Grant Number SAE20131200623. This work was supported by the Canadian Institutes of Health Research for the “CIHR Team in Familial Risks of Breast Cancer” Program: Grant No. CRN-87521 and the Ministry of Economic Development, Innovation and Export Trade: Grant No. PSR-SIIRI-701. The PERSPECTIVE Project was supported by the Government of Canada through Genome Canada and the Canadian Institutes of Health Research, the Ministère de l’Économie, Innovation et Exportation du Québec through Genome Quebec, and The Quebec Breast Cancer Foundation. This work was also supported by the Ministère de l’Économie, Innovation et Exportation du Québec: Grant No. PSR-SIIRI-701. DEMOKritos: this research has been co-financed by the European Union (European Social Fund: ESF) and Greek National Funds through the Operational Program “Education and Lifelong Learning” of the National Strategic Reference Framework (NSRF): Research Funding Program of the General Secretariat for Research and Technology: SYNa11_10_19 NBCA. Investing in Knowledge Society through the European Social Fund. The DKFZ Study was supported by the DKFZ. EMBRACE was supported by Cancer Research UK Grants C1287/A10118 and C1287/A11990. D. Gareth Evans and Fiona Lalloo were supported by an NIH Grant to the Biomedical Research Centre, Manchester. The Investigators at The Institute of Cancer Research and The Royal Marsden NHS Foundation Trust were supported by an NIH Grant to the Biomedical Research Centre at The Institute of Cancer Research and The Royal Marsden NHS Foundation Trust: Ros Eeles and Elizabeth Bianco were supported by Cancer Research UK. Grants SG0472 A8385. Ros Eeles was also supported by NIH support to the Biomedical Research Centre at The Institute of Cancer Research and The Royal Marsden NHS Foundation Trust. FCCC: the authors acknowledge the support from The University of Kansas Cancer Center (P30 CA168524) and the Kansas Bioscience Authority Eminent Scholar Program. A.K.G. was funded by 5U01CA113916, R01CA140323, and by the Chancellors Distinguished Chair in Biomedical Sciences Professorship. The German Consortium of Hereditary Breast and Ovarian Cancer (GC-HBOC) was supported by the German Cancer Aid (Grant No. 110837, Rita K. Schmutzler). This work was supported by the European Regional Development Fund and Free State of Saxony, Germany (LIFE: Leipzig Research Centre for Civilization Diseases, Project Numbers 713-241202, 713-241202, 14505/2470, 14575/2470). GEMO: the study was supported by the Ligue Nationale Contre le Cancer; the Association “Le cancer du sein, parlons-en!” Award; the Canadian Institutes of Health Research for the “CIHR Team in Familial Risks of Breast Cancer” Program and the French National Institute of Cancer (INCa). GEORGETOWN: CI received support from the Non-therapeutic Subject Registry Shared Resource at Georgetown University (NIH/NCI Grant P30-CA051008), the Fisher Center for Hereditary Cancer and Clinical GenomicsResearch, and Swing Fire the Cure. G-FAST: Bruce Poppe is a Senior Clinical Investigator of FWO. Mattias Van Heetvelde obtained funding from IWT. HCSC was supported by a Grant RD12/0036/0006 and 15/00059 from ISCIII (Spain), partially supported by European Regional Development FEDER Funds. The HEBCS was financially supported by the Helsinki University Hospital Research Fund, Academy of Finland (266528), the Finnish Cancer Society and the Sigrid Juselius Foundation. HEBON Study was supported by the Dutch Cancer Society Grants NKI1998-1854, NK2004-3088, and NKZ2007-3756, the Netherlands Organization of Scientific Research Grant NWO 91109024, the Pink Ribbon Grants 110005 and 2014-187.W076, the BMMRI Grant NWO 184.021.007/CP46, and the Transcan Grant JTC 2012 Cancer 12-054. HEBON thanks the Registration Teams of Dutch Cancer Registry (IKNL; S. Siesling, J. Verloop) and the Dutch Pathology database (PALGA; L. Overbeck) for their help in part of the data collection. HRBCP was supported by the Hong Kong Sanatorium and Hospital, Dr. Ellen Li Charitable Foundation, The Kerry Group Kuok Foundation, National Institute of Health I 03CA130065, and North California Cancer Center. Hungarian Breast and Ovarian Cancer Study (HUNBOCS) was supported by Hungarian Research Grants KTIA-OTKA CK-80745 and OTKA K-112282. ICO: Contract Grant Sponsor: Asociación Española Contra el Cáncer, Spanish Health Research Fund; Carlos III Health Institute: Catalan Health Institute and Autonomous Government of Catalonia. Contract Grant Numbers: ISICIIETIC RD06/00020/1051, RD12/0036/008, P10/01422, P10/00748, PI13/00285, PIE13/00022, 2009GSR290, and 2014SGR364, The IHCC was supported by Grant PBZ_KBN_122/POS/2004. The ILU Group was supported by the Icelandic Association “Walking for Breast Cancer Research” and by the Landsfari University Hospital Research Fund. INHERIT: this work was supported by the Canadian Institutes of Health Research for the “CIHR Team in Familial Risks of Breast Cancer” Program: Grant No. CRN-87521 and the Ministry of Economic Development, Innovation and Export Trade: Grant No. PSR-SIIRI-701. The PERSPECTIVE Project was supported by the Government of Canada through Genome Canada and the Canadian Institutes of Health Research (Grant GPH-129344), the Ministère de l’Économie, Science et Innovation du Québec through Genome Québec, and the Quebec Breast Cancer Foundation. IOVHBOCS was supported by Ministero della Salute and “5x1000” Istituto Oncologico Veneto Grant. IPoBCS: this study was in part supported by Liga Portuguesa Contra o Cancro. kConFab was supported by a Grant from the National Breast Cancer Foundation, and previously by the National Health and Medical Research Council (NHMRC), the Queensland Cancer Fund, the Cancer Councils of New South Wales, Victoria, Tasmania, and South Australia, and the Cancer Foundation of Western Australia. KOBRA was supported by a Grant from the National R&D Program for Cancer Control, Ministry for Health, Welfare and Family Affairs, Republic of Korea (1020350). MAYO was supported by NIH Grants CA116167, CA192393, and CA176785, an NCI Specialized Program of Research Excellence (SPRE) in Breast Cancer (CA116210), and a Grant from the Breast Cancer Research Foundation. MCGILL: Jewish General Hospital Weekend to End Breast Cancer, Quebec Ministry of Economic Development, Innovation and Export Trade. MODSQUAD was supported by MH CZ: DRO (MMCI, 00298085) and by the European Regional Development Fund and the State Budget of the Czech Republic (RECAMO, CZ.1.05/2.1.00/03.0101) to LF, and by Charles University in Prague Project UNCE204024 (MZ). MSKCC was supported by Grants from the Breast Cancer Research Foundation, the Robert and Kate Niehaus Clinical Cancer Genetics Initiative, and the Andrew Sabin Research Fund. NAROD: 1R01 CA149429-01. NIC: the research of Drs. MH Greene and PL Mai was supported by the Intramural Research Program of the US National Cancer Institute, NIH, and by Support Services Contracts N02-CP-11019-50 and N02-CP-65504 with Westat, Inc., Rockville, MD. NICC was supported by Clalit Health Services in Israel. Some of its activities were supported by the Israel Cancer Association and the Breast Cancer
Research Foundation (BCRF), NY. **NNPIO:** this work has been supported by NRG Oncology Operations Grant Number U10 CA180868 as well as NRG SDMC Grant U10 CA180822, Gynecologic Oncology Group (GOG) Administrative Office and the GOG Tissue Bank (CA 27469), and the GOG Statistical and Data Center (CA 37517). Drs. Greene, Mai, and Savage were supported by funding from the Intramural Research Program, NCI. **OSUCCG** was supported by the Ohio State University Comprehensive Cancer Center. **PBCC:** this work was supported by the Italian Association of Cancer Research (AIRC) [IG 2013 N.14477] and Tuscany Institute for Tumors (ITT) Grant 2014–2015–2016. **SEABASS:** Ministry of Science, Technology and Innovation, Ministry of Higher Education (UM.C/HIR/MOE/06), and Cancer Research Initiatives Foundation. The **SMC** Team was in part sponsored by a Grant from the Israeli Cancer Association to the Israeli Consortium of Hereditary Breast Cancer. **SWE-BRCA** Collaborators were supported by the Swedish Cancer Society. **UCHICAGO** was supported by NCI Specialized Program of Research Excellence (SPOR) in Breast Cancer (CA125183), R01 CA142996, U01CA161032, and by the Ralph and Marion Falk Medical Research Trust, the Entertainment Industry Fund National Women’s Cancer Research Alliance, and the Breast Cancer Research Foundation. **OIO** is an ACS Clinical Research Professor. **UCLA:** Jonsson Comprehensive Cancer Center Foundation; Breast Cancer Research Foundation. **UCSF:** UCSF Cancer Risk Program and Helen Diller Family Comprehensive Cancer Center. **UKFOCR** was supported by a Project Grant from CRUK to Paul Pharoah. **UPENN:** National Institutes of Health (NIH) (R01-CA102776 and R01-CA083855; Breast Cancer Research Foundation; Susan G. Komen Foundation for the Cure, Bassier Research Center for BRCA. **VFCGT:** Victorian Cancer Agency, Cancer Australia, National Breast Cancer Foundation. **WCP:** Dr. Karlan was funded by the American Cancer Society Early Detection Professorship (SIOP-06-258-01-COUN) and the National Center for Advancing Translational Sciences (NCATS), Grant UL1TR000124.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** Study participants were recruited through the CIMBA Initiative, following the approval of the corresponding protocol by the Institutional Review Board or Ethics Committee at each participating center. Written informed consent was obtained from all study participants.

**Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

**References**