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Common alleles at 6q25.1 and 1p11.2 are associated with breast cancer risk for *BRCA1* and *BRCA2* mutation carriers

Antonis C Antoniou[1,*], Christiana Kartsonaki[1], Olga M. Sinilnikova[3,4], Penny Soucy[5], Lesley McGuffog[1], Sue Healey[7], Andrew Lee[1], Paolo Peterlongo[9,11], Siranoush Manoukian[12], Bernard Peissel[12], Daniela Zaffaroni[12], Elisa Cattaneo[10], Monica Barile[14], Valeria Pensotti[15], Barbara Pasini[16], Riccardo Dolcetti[17], Giuseppe Giannini[18], Anna Laura Putignano[19,20], Liliana Varesco[21], Paolo Radice[9], Phuong L. Mai[22], Mark H. Greene[22], Irene L. Andrulis[23,24,25,27], Gord Glendon[27], Hilmi Ozcelik[23,26], Mads Thomassen[28], Anne-Marie Gerdes[29], Torben A. Kruse[28], Uffe Birk Jensen[30], Dorthe G. Crüger[31], Maria A. Caligo[32], Yael Laitman[33], Roni Milgrom[33], Bella Kaufman[34], Shani Paluch-Shimon[34], Eitan Friedman[33], Niklas Loman[35], Katja Harbst[35], Annika Lindblom[36], Brita Arver[37], Hans Ehrencrona[38], Beatrice Melin[39], SWE-BRCA[35,36], Katherine L. Nathanson[40], Susan M. Domchek[40], Timothy Rebbeck[40], Ania Jakubowska[41], Jan Lubinski[41], Jacek Gronwald[41], Tomasz Huzarski[41], Tomasz Byrski[41], Cezary Cybulski[41], Bohdan Gorski[41], Ana Osorio[42,43], Teresa Ramón y Cajal[44], Florentia Fostira[45], Raquel Andrés[46], Javier Benitez[42,43], Ute Hamann[47], Frans B. Hogervorst[48], Matti A. Rookus[49], Maartje J. Hooning[50], Marcel R. Nelen[51], Rob B. van der Luijt[52], Theo A.M. van Os[53], Christi J. van Asperen[54], Peter Devilee[55,56], Hanne E.J. Meijers-Heijboer[57], Encarna B. Gómez Garcia[58,59], HEBON[49], Susan Peock[1], Margaret Cook[1], Debra Frost[1], Radka Platte[1], Jean Leyland[1], D. Gareth Evans[60], Fiona Lalloo[60], Ros Eeles[61], Louise Izatt[62], Julian Adlard[63], Rosemarie Davidson[64], Diana Eccles[65], Kai-ren Ong[66], Jackie Cook[67], Fiona Douglas[68], Joan Paterson[69], M. John Kennedy[70], Zosia Miedzybrodzka[71], EMBRACE[1], Andrew Godwin[72], Dominique Stoppa-Lyonnet[73,74,75], Bruno Buecher[73], Muriel Belotti[73], Carole Tirapo[73], Sylvie Mazoyer[4], Laure Barjhoux[4], Christine Lasset[76,77], Dominique Leroux[78,79], Laurence Faivre[80,81], Myriam Bronner[82], Fabienne Prieur[83], Catherine Nogues[84], Etienne Rouleau[85], Pascal Pujol[86,87], Isabelle Coupier[86,88], Marc Frénay[89], CEMO Study Collaborators[3], John L. Hopper[90], Mary B. Daly[91], Mary B. Terry[92], Esther M. John[93,94], Saundra S. Buys[95], Yosuf Yassin[96], Alexander Miron[96], David Goldgar[97], Breast Cancer Family Registry, Christian F. Singer[98], Muy-Kheng Tea[98], Georg Pfeiler[98], Anne Catharina Dressler[98], Thomas v.O. Hansen[99], Lars Jønson[99], Bent Ejlertsen[100], Rosa Bjork Barkardottir[101,102], Tomas Kirchhoff[103], Kenneth Offit[103], Marion Piedmonte[104], Gustavo Rodriguez[106], Laurie Small[107], John Boggess[108], Stephanie Blank[109], Jack Basil[110], Masoud Azodi[111], Amanda Ewart Toland[112], Marco Montagna[113], Silvia Tognazzo[113], Simona Agata[113], Evgeny Imyanitov[114], Ramunas Janavicius[115,116], Conxi Lazaro[117],

[*]To whom correspondence should be addressed at: Strangeways Research Laboratory, University of Cambridge, Worts Causeway, Cambridge CB1 8RN, UK. Tel: +44 1223 740163; Fax: +44 1223 740159; Email: antonis@srl.cam.ac.uk

Ignacio Blanco[117], Paul D.P. Pharoah[2], Lara Sucheston[105], Beth Y. Karlan[118], Christine S. Walsh[118], Edith Olah[119], Aniko Bozsik[119], Soo-Hwang Teo[120,121], Joyce L. Seldon[122], Mary S. Beattie[123,124,125,126], Elizabeth J. van Rensburg[127], Michelle D. Sluiter[127], Orland Diez[128,129], Rita K. Schmutzler[130,131], Barbara Wappenschmidt[130,131], Christoph Engel[132], Alfons Meindl[133], Ina Ruehl[134], Raymonda Varon-Mateeva[135], Karin Kast[136], Helmut Deissler[137], Dieter Niederacher[138], Norbert Arnold[139], Dorothea Gadzicki[140], Ines Schönbuchner[141], Trinidad Caldes[142], Miguel de la Hoya[142], Heli Nevanlinna[143], Kristiina Aittomäki[144], Martine Dumont[5], Jocelyne Chiquette[145], Marc Tischkowitz[146,147], Xiaoqing Chen[7], Jonathan Beesley[7], Amanda B. Spurdle[7], kConFab investigators[8], Susan L. Neuhausen[148], Yuan Chun Ding[148], Zachary Fredericksen[149], Xianshu Wang[150], Vernon S. Pankratz[149], Fergus Couch[150], Jacques Simard[6], Douglas F. Easton[1,†], Georgia Chenevix-Trench[7,†] , on behalf of CIMBA[27]

[1]Department of Public Health and Primary Care, Centre for Cancer Genetic Epidemiology and [2]Department of Oncology, University of Cambridge, Cambridge, UK, [3]Centre Hospitalier Universitaire de Lyon/Centre Léon Bérard, Unité Mixte de Génétique Constitutionnelle des Cancers Fréquents, Lyon, France, [4]Equipe labellisée LIGUE 2008, UMR5201 CNRS, Centre Léon Bérard, Université de Lyon, Lyon, France, [5]Cancer Genomics Laboratory and [6]Canada Research Chair in Oncogenetics, Cancer Genomics Laboratory, Centre Hospitalier Universitaire de Québec and Laval University, 2705 Laurier Boulevard, T3-57, Quebec City, QC, Canada, [7]Queensland Institute of Medical Research, Brisbane, Australia, [8]kConFab: Kathleen Cuningham Consortium for Research into Familial Breast Cancer, Peter MacCallum Cancer Center, Melbourne, Australia, [9]Unit of Molecular Bases of Genetic Risk and Genetic Testing, Department of Preventive and Predictive Medicine and [10]Unit of Medical Genetics, Department of Preventive and Predicted Medicine, Fondazione IRCCS Istituto Nazionale Tumori (INT), Milan, Italy, [11]IFOM, Fondazione Istituto FIRC di Oncologia Molecolare, Milan, Italy, [12]Unit of Medical Genetics, Department of Preventive and Predicted Medicine, Fondazione IRCCS Istituto Nazionale Tumori (INT), Milan, Italy, [13]Department of Medicine, Surgery and Dentistry, Università degli Studi di Milano, Medical Genetics, Milan, Italy, [14]Division of Cancer Prevention and Genetics, Istituto Europeo di Oncologia (IEO), Milan, Italy, [15]Cogentech, Consortium for Genomics Technology, Milan, Italy, [16]Department of Genetics, Biology and Biochemistry, University of Turin, Turin, Italy, [17]Cancer Bioimmunotherapy Unit, Centro di Riferimento Oncologico, IRCCS, Aviano (PN), Italy, [18]Department of Experimental Medicine, University La Sapienza, Rome, Italy, [19]Department of Clinical Physiopathology, University of Florence, Florence, Italy, [20]Fiorgen Foundation for Pharmacogenomics, Sesto Fiorentino, Italy, [21]Unit of Hereditary Cancer, Department of Epidemiology, Prevention and Special Functions, Istituto Nazionale per la Ricerca sul Cancro (IST), Genoa, Italy, [22]Clinical Genetics Branch, Division of Cancer Epidemiology and Genetics, US National Cancer Institute, Rockville MD, USA, [23]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, ON, Canada, [24]Department of Molecular Genetics, [25]Department of Laboratory Medicine and Pathobiology and [26]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada, [27]Cancer Care Ontario, Toronto, ON, Canada, [28]Clinical Genetics, Odense University Hospital, Odense C, Denmark, [29]Clinical Genetics, Rigshospital, Copenhagen, Denmark, [30]Clinical Genetics, Aarhus University Hospital, Aarhus, Denmark, [31]Clinical Genetics, Vejle Hospital, Vejle, Denmark, [32]Section of Genetic Oncology, Department of Laboratory Medicine, University and University Hospital of Pisa, Pisa, Italy, [33]The Susanne Levy Gertner Oncogenetics Unit, Sheba Medical Center, Tel Hashomer, Israel, [34]The Breast cancer Unit, Institute of Oncology, Sheba Medical Center, Tel-Hashomer, Israel, [35]Department of Oncology, Lund University Hospital, Lund, Sweden, [36]Department of Clinical Genetics and [37]Department of Oncology, Karolinska University Hospital, S-17176 Stockholm, Sweden, [38]Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, S-751 85 Uppsala, Sweden, [39]Department of Radiation Sciences, Oncology, Umeå University, S-901 85 Umeå, Sweden, [40]University of Pennsylvania, Philadelphia, PA, USA, [41]International Hereditary Cancer Centre, Department of Genetics and Pathology, Pomeranian Medical University, Szczecin, Poland, [42]Human Genetics Group, Human Cancer Genetics Programme, Spanish National

[†]Joint senior authors.

Cancer Research Centre, Madrid, Spain, [43]Spanish Network on Rare Diseases (CIBERER), [44]Oncology Service, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain, [45]Molecular Diagnostics Laboratory, I.R.R.P., National Center for Scientific Research 'Demokritos', Athens, Greece, [46]Oncology unit, Hospital clinico Universitario "Lozano Blesa", Zaragoza, Spain, [47]Molecular Genetics of Breast Cancer, Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany, [48]Family Cancer Clinic and [49]Department of Epidemiology, Netherlands Cancer Institute, Amsterdam, The Netherlands, [50]Department of Medical Oncology, Family Cancer Clinic, Erasmus University Medical Center, Rotterdam, The Netherlands, [51]Department of Human Genetics 849, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, [52]Department of Medical Genetics, University Medical Center Utrecht, Utrecht, The Netherlands, [53]Department of Clinical Genetics, Academic Medical Center, Amsterdam, The Netherlands, [54]Department of Clinical Genetics, [55]Department of Human Genetics and [56]Department of Pathology, Leiden University Medical Center Leiden, Leiden, The Netherlands, [57]Department of Clinical Genetics, VU Medical Center, Amsterdam, The Netherlands, [58]Department of Clinical Genetics and GROM, School for Oncology and [59]Developmental Biology, MUMC, Maastricht, The Netherlands, [60]Genetic Medicine, Manchester Academic Health Sciences Centre, Central Manchester University Hospitals NHS Foundation Trust, Manchester, UK, [61]Oncogenetics Team, The Institute of Cancer Research and Royal Marsden NHS Foundation Trust, Surrey, UK, [62]Clinical Genetics, Guy's and St. Thomas' NHS Foundation Trust, London, UK, [63]Yorkshire Regional Genetics Service, Leeds, UK, [64]Ferguson-Smith Centre for Clinical Genetics, Yorkhill Hospitals, Glasgow, UK, [65]Wessex Clinical Genetics Service, Princess Anne Hospital, Southampton, UK, [66]West Midlands Regional Genetics Service, Birmingham Women's Hospital Healthcare NHS Trust, Edgbaston, Birmingham, UK, [67]Sheffield Clinical Genetics Service, Sheffield Children's Hospital, Sheffield, UK, [68]Institute of Human Genetics, Centre for Life, Newcastle Upon Tyne Hospitals NHS Trust, Newcastle upon Tyne, UK, [69]Department of Clinical Genetics, East Anglian Regional Genetics Service, Addenbrookes Hospital, Cambridge, UK, [70]Academic Unit of Clinical and Molecular Oncology, Trinity College Dublin and St James's Hospital, Dublin, Eire, [71]North of Scotland Regional Genetics Service, NHS Grampian & University of Aberdeen, Foresterhill, Aberdeen, UK, [72]Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, Kansas City, USA, [73]Service de Génétique Oncologique and [74]Unité INSERM U830, Institut Curie, Paris, France, [75]Faculté de Médecine, Université Paris Descartes, Paris, France, [76]CNRS UMR5558, Université Lyon 1, Lyon, France, [77]Unité de Prévention et d'Epidémiologie Génétique, Centre Léon Bérard, Lyon, France, [78]Department of Genetics, Centre Hospitalier Universitaire de Grenoble, Grenoble, France, [79]Institut Albert Bonniot, Université de Grenoble, Grenoble, France, [80]Centre de Génétique, Centre Hospitalier Universitaire de Dijon, France, [81]Centre de Lutte Contre le Cancer Georges François Leclerc, Dijon, France, [82]Laboratoire de Génétique Médicale, Nancy Université, Centre Hospitalier Régional et Universitaire, Vandoeuvre-les-Nancy, France, [83]Service de Génétique Clinique Chromosomique et Moléculaire, Centre Hospitalier Universitaire de St Etienne, St Etienne, France, [84]Oncogénétique Clinique and [85]Laboratoire d'Oncogénétique, Hôpital René Huguenin/Institut Curie, Saint-Cloud, France, [86]Unité d'Oncogénétique, CHU Arnaud de Villeneuve, Montpellier, France, [87]INSERM 896, CRCM Val d'Aurelle, Montpellier, France, [88]Unité d'Oncogénétique, CRLCC Val d'Aurelle, Montpellier, France, [89]Centre Antoine Lacassagne, Nice, France, [90]The University of Melbourne, Melbourne, Australia, [91]Women's Cancer Program, Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA, USA, [92]Columbia University New York, New York, NY, USA, [93]Cancer Prevention Institute of California (formerly the Northern California Cancer Center), Fremont, CA, USA, [94]Stanford Cancer Center, Stanford University School of Medicine, Stanford, CA, USA, [95]Huntsman Cancer Institute, University of Utah Health Sciences Centre, Salt Lake City, UT, USA, [96]Dana-Farber Cancer Institute, Boston, MA, USA, [97]Department of Dermatology, University of Utah, Salt Lake City, UT, USA, [98]Department of Obstetrics/Gynaecology, Medical University of Vienna, Vienna, Austria, [99]Genomic Medicine, Department of Clinical Biochemistry and [100]Department of Oncology, Rigshospitalet, Copenhagen University Hospital, Allerød, Denmark, [101]Laboratory of Cell Biology, Department of Pathology, Landspitali-University Hospital, Tromsø, Norway, [102]Faculty of Medicine, University of Iceland, Reykjavik, Iceland, [103]Clinical Genetics Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY, USA, [104]GOG Statistical and Data Center and [105]Department of Cancer Prevention and Control, Roswell Park Cancer Institute, Buffalo, NY 14263, USA, [106]Evanston Northwestern Healthcare, Evanston, IL, USA, [107]Maine Medical Center, Scarborough, ME, USA, [108]University of North Carolina, Chapel Hill, NC, USA, [109]New York University School of Medicine, New York, NY, USA, [110]St. Elizabeth Medical Center, Edgewood, KY, USA, [111]Yale University School of Medicine, New Haven, CT,

USA, [112]Comprehensive Cancer Center, Department of Molecular Virology, Immunology and Medical Genetics, The Ohio State University, Columbus, OH, USA, [113]Immunology and Molecular Oncology Unit, Istituto Oncologico Veneto IOV - IRCCS, Padua, Italy, [114]N.N. Petrov Institute of Oncology, St.-Petersburg, 197758, Russia, [115]Department of Molecular and Regenerative Medicine, Vilnius university hospital Santariskiu Clinics, Hematology, Oncology and Transfusion Medicine Center, Vilnius, Lithuania, [116]State Research Institute Inovative medicine center, Vilnius, Lithuania, [117]Hereditary Cancer Program, Catalan Institute of Oncology, Barcelona, Spain, [118]Women's Cancer Research Institute at the Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, CA, USA, [119]Department of Molecular Genetics, National Institute of Oncology, Budapest, Hungary, [120]Cancer Research Initiatives Foundation, Sime Darby Medical Centre, Subang Jaya, Malaysia, [121]University Malaya Cancer Research Institute, University Malaya, Kuala Lumpur, Malaysia, [122]UCLA Cancer Genetics Program, CLA Jonsson Comp Cancer Center, Santa Monica, CA, USA, [123]UCSF Cancer Risk Program, University of California, San Francisco, CA, USA, [124]UCSF Department of Medicine, [125]Department of Epidemiology and [126]Department of Biostatistics, University of California, San Francisco, CA, USA, [127]Department of Genetics, University of Pretoria, Pretoria, South Africa, [128]Oncogenetics Laboratory, University Hospital Vall d'Hebron, Barcelona, Spain, [129]Vall d'Hebron Institute of Oncology (VHIO), Barcelona, Spain, [130]Centre of Familial Breast and Ovarian Cancer, Department of Gynaecology and Obstetrics and [131]Centre for Integrated Oncology (CIO), University hospital of Cologne, Cologne, Germany, [132]Institute for Medical Informatics, Statistics and Epidemiology, University of Leipzig, Leipzig, Germany, [133]Department of Gynaecology and Obstetrics, Division of Tumor Genetics, Klinikum rechts der Isar, Technical University Munich, Munich, Germany, [134]Department of Gynaecology and Obstetrics, Ludwig-Maximillians University Munich, Munich, Germany, [135]Institute of Human Genetics, Campus Virchov Klinikum, Charite Berlin, Germany, [136]Department of Gynaecology and Obstetrics, University Hospital Carl Gustav Carus, Technical University Dresden, Dresden, Germany, [137]Department of Gynaecology and Obstetrics, University Hospital Ulm, Ulm, Germany, [138]Department of Gynaecology and Obstetrics, University Hospital Düsseldorf, Heinrich-Heine University Düsseldorf, Düsseldorf, Germany, [139]Department of Gynaecology and Obstetrics, University Hospital of Schleswig-Holstein, Campus Kiel, Christian-Albrechts University Kiel, Kiel, Germany, [140]Institute of Cell and Molecular Pathology, Hannover Medical School, Hannover, Germany, [141]Centre of Familial Breast and Ovarian Cancer, Department of Medical Genetics, Institute of Human Genetics, University Würzburg, Würzburg, Germany, [142]Molecular Oncology Laboratory, Hospital Clinico San Carlos, Madrid, Spain, [143]Department of Obstetrics and Gynecology and [144]Department of Clinical Genetics, Helsinki University Central Hospital, Helsinki, Finland, [145]Unité de recherche en santé des populations, Centre des maladies du sein Deschênes-Fabia, Centre de recherche FRSQ du Centre hospitalier affilié universitaire de Québec, Québec, QC, Canada, [146]Program in Cancer Genetics, Departments of Human Genetics and Oncology, McGill University, Montreal, QC, Canada, [147]Lady Davis Institute, Segal Cancer Centre, Jewish General Hospital, Montreal, QC, Canada, [148]Department of Population Sciences, Beckman Research Institute of the City of Hope, Duarte, CA, USA, [149]Department of Health Sciences Research, Mayo Clinic, Rochester, MN, USA and [150]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA

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Two single nucleotide polymorphisms (SNPs) at 6q25.1, near the *ESR1* gene, have been implicated in the susceptibility to breast cancer for Asian (rs2046210) and European women (rs9397435). A genome-wide association study in Europeans identified two further breast cancer susceptibility variants: rs11249433 at 1p11.2 and rs999737 in *RAD51L1* at 14q24.1. Although previously identified breast cancer susceptibility variants have been shown to be associated with breast cancer risk for *BRCA1* and *BRCA2* mutation carriers, the involvement of these SNPs to breast cancer susceptibility in mutation carriers is currently unknown. To address this, we genotyped these SNPs in *BRCA1* and *BRCA2* mutation carriers from 42 studies from the Consortium of Investigators of Modifiers of *BRCA1/2.* In the analysis of 14 123 *BRCA1* and 8053 *BRCA2* mutation carriers of European ancestry, the 6q25.1 SNPs ($r^2 = 0.14$) were independently associated with the risk of breast cancer for *BRCA1* mutation carriers [hazard ratio (HR) = 1.17, 95% confidence interval (CI): 1.11–1.23, *P*-trend = 4.5×10^{-9} for rs2046210; HR = 1.28, 95% CI: 1.18–1.40, *P*-trend = 1.3×10^{-8} for

rs9397435], but only rs9397435 was associated with the risk for *BRCA2* carriers (HR = 1.14, 95% CI: 1.01–1.28, *P*-trend = 0.031). SNP rs11249433 (1p11.2) was associated with the risk of breast cancer for *BRCA2* mutation carriers (HR = 1.09, 95% CI: 1.02–1.17, *P*-trend = 0.015), but was not associated with breast cancer risk for *BRCA1* mutation carriers (HR = 0.97, 95% CI: 0.92–1.02, *P*-trend = 0.20). SNP rs999737 (*RAD51L1*) was not associated with breast cancer risk for either *BRCA1* or *BRCA2* mutation carriers (*P*-trend = 0.27 and 0.30, respectively). The identification of SNPs at 6q25.1 associated with breast cancer risk for *BRCA1* mutation carriers will lead to a better understanding of the biology of tumour development in these women.

INTRODUCTION

Genome-wide association studies (GWASs) have identified multiple common alleles that are associated with breast cancer risk in the general population (1–7). Such alleles provide plausible candidates as modifiers of cancer risk for *BRCA1* and *BRCA2* mutation carriers. Nine of these polymorphisms have been investigated as risk modifiers to date (8–10); single nucleotide polymorphisms (SNPs) in *FGFR2*, *TOX3*, *MAP3K1*, *LSP1*, 2q35, *SLC4A7* and 5p12 have been shown to be associated with breast cancer risk for *BRCA2* mutation carriers, but only SNPs in *TOX3* and 2q35 were associated with the risk for *BRCA1* mutation carriers. The differential patterns of associations between *BRCA1* and *BRCA2* mutation carriers appear to be in line with the differential effects of these polymorphisms for oestrogen receptor-positive and oestrogen receptor-negative breast cancer in the general population (10,11). More recently, a GWAS restricted to *BRCA1* mutation carriers identified a locus at 19p13 which modified breast cancer risk for *BRCA1* mutation carriers and the risk of oestrogen receptor (ER) negative and triple negative (oestrogen, progesterone receptor (PR) and Human Epidermal growth factor receptor 2 (HER2) negative) breast cancer in the general population (12). A separate GWAS in *BRCA2* mutation carriers suggested that another locus at *ZNF365* may modify the risk of breast cancer for *BRCA2* mutation carriers (13). Candidate gene studies have also suggested that a SNP in *CASP8* is also associated with the risk of breast cancer for *BRCA1* mutation carriers (14). Each of these polymorphisms confers modest relative risks for breast cancer, but evidence so far suggests that they interact multiplicatively on the breast cancer risk for mutation carriers and the range of the combined risks of these SNPs is ~6-fold (10). Since *BRCA1* and *BRCA2* mutations confer high risks of breast cancer, these relative risks result in substantial differences in the absolute risk of developing breast cancer between SNP genotype categories, and such differences could potentially influence the clinical management of mutation carriers (15). However, several other variants identified through population-based GWAS have not yet been evaluated as modifiers of cancer risk for *BRCA1* and *BRCA2* mutation carriers. Identifying further modifiers of risk could enhance risk prediction and will lead to a better understanding of the biology of tumour development in *BRCA1* and *BRCA2* mutation carriers.

Using data from the Shanghai Breast Cancer Study, Zheng *et al*. (7) identified a polymorphism at 6q25.1 through a GWAS on the risk of breast cancer among Chinese women. SNP rs2046210 was located upstream of the gene encoding for ER α-*ESR1:* 29 kb upstream of the first untranslated exon and 180 kb upstream of the first coding exon. Each copy of the minor allele of the SNP was estimated to confer an Odds Ratio (OR) of 1.29 among Chinese women and the authors reported a stronger association with ER-negative than ER-positive breast cancer. The same study also found an association between rs2046210 and the risk of breast cancer for European women, but a subsequent larger study among Europeans suggested that the association in Europeans is primarily due to another weakly correlated SNP in the region (rs9397435) (16). In a separate GWAS, Thomas *et al.* identified two further SNPs associated with the risk of the breast cancer in the Cancer and Genetic Markers of Susceptibility (CGEMS) study: rs11249433 at 1p11.2 in a linkage disequilibrium block neighbouring *NOTCH2* and *FCGR1B*, and rs999737 at 14q24.1 in *RAD51L1* (6). SNP rs11249433 was mainly associated with ER-positive disease.

To evaluate the associations between these four SNPs and breast cancer risk for *BRCA1* and *BRCA2* mutation carriers, we genotyped these SNPs in *BRCA1* and *BRCA2* mutation carriers from the Consortium of Investigators of Modifiers of *BRCA1/2* (CIMBA).

RESULTS

Characteristics of the eligible mutation carriers, after quality control exclusions, are summarised in Table 1. The primary analysis included only mutation carriers of self-reported white European ancestry, and included data from 11 604 women considered affected (first breast cancer diagnosis) and 10 572 considered as unaffected (censored at bilateral prophylactic mastectomy, ovarian cancer or age at last observation).

The association results with breast cancer risk are summarized in Table 2. The minor allele of SNP rs2046210, at 6q25.1, was associated with an increased risk of breast cancer for *BRCA1* mutation carriers (per-allele HR = 1.17, 95% CI: 1.11–1.23, *P*-trend = 7.5×10^{-9}). In contrast, there was little evidence of association with the risk of breast cancer for *BRCA2* mutation carriers (per-allele HR = 1.06, 95% CI: 0.99–1.14, *P*-trend = 0.09). There was no evidence for heterogeneity in the HRs across studies for *BRCA1* mutation carriers (*P*-heterogeneity = 0.47), but there was marginal evidence for heterogeneity for *BRCA2* mutation carriers (*P*-heterogeneity = 0.03; Fig. 1). This was mainly due to data from the HUNBOCS study. After excluding this study from the analysis, there was no longer evidence for heterogeneity

Table 1. Summary characteristics for the 22 176 eligible *BRCA1* and *BRCA2* carriers[a] used in the analysis

Characteristic	*BRCA1* Unaffected	*BRCA1* Breast cancer	*BRCA2* Unaffected	*BRCA2* Breast cancer
Number	6930	7193	3642	4411
Person-years follow-up	294 555	296 222	160 459	194 052
Median age at censure (IQR)	41 (34–50)	40 (34–47)	43 (34–52)	43 (37–50)
Age at censure, *N* (%)				
<30	1016 (14.7)	603 (8.4)	510 (14.0)	209 (4.7)
30–39	1990 (28.7)	2816 (39.1)	978 (26.9)	1366 (31.0)
40–49	2075 (29.9)	2528 (34.1)	1005 (27.6)	1687 (38.3)
50–59	1218 (17.6)	940 (13.1)	647 (17.8)	812 (18.4)
60–69	448 (6.5)	244 (3.4)	344 (9.5)	274 (6.2)
70+	183 (2.6)	62 (0.9)	158 (4.3)	63 (1.4)
Year of birth, *N* (%)				
<1920	31 (0.5)	38 (0.5)	24 (0.7)	46 (1.0)
1920–1929	143 (2.1)	220 (3.1)	111 (3.1)	191 (4.3)
1930–1939	419 (6.1)	577 (8.0)	255 (7.0)	484 (11.0)
1940–1949	935 (13.5)	1522 (21.2)	502 (13.8)	1018 (23.1)
1950–1959	1590 (22.9)	2256 (31.4)	760 (20.9)	1361 (30.9)
1960+	3812 (55.0)	2580 (35.9)	1990 (546.8)	1311 (29.7)
Mutation class, *N* (%)				
Class 1[b]	4581 (66.1)	4363 (60.7)	3426 (94.1)	4092 (92.8)
Class 2[b]	1964 (28.3)	2252 (31.3)	75 (2.0)	115 (2.6)
Other	385 (5.6)	578 (8.0)	141 (3.9)	204 (4.6)

IQR, interquartile range.
[a]Carriers of self-reported European ancestry only.
[b]See methods for definitions.

($P = 0.14$) and the estimated HR for *BRCA2* mutation carriers was virtually unchanged (per-allele HR = 1.05, 95%CI: 0.98–1.13, *P*-trend = 0.13). There was no evidence that the HR for *BRCA2* mutation carriers varied by age ($P = 0.87$), but there was evidence that the per-allele HR for *BRCA1* mutation carriers decreased with age (*P*-trend = 0.0036). To investigate this further, we fitted models allowing for a separate HR for each decade of age (Supplementary Material, Table S2). This analysis revealed significant associations between SNP rs2046210 and the risk of breast cancer for *BRCA1* mutation carriers at ages <50 years (HR for the age group 20–29 = 1.24, 95% CI: 1.08–1.42; HR for the age group 30–39 = 1.24, 95% CI: 1.16–1.33; HR for the age group 40–49 = 1.11, 95% CI: 1.04–1.21) but not for ages 50 or over (HR for the age group 50–59 = 1.02, 95% CI: 0.90–1.15; HR for the age group 60–69 = 1.19; 95% CI: 0.96–1.47; HR for the age group 70–79 = 0.82, 95% CI: 0.50–1.36).

SNP rs9397435 was associated with the risk of breast cancer for both *BRCA1* and *BRCA2* mutation carriers, but the evidence of association was stronger for *BRCA1* (per-allele HR = 1.28, 95% CI: 1.18–1.40, *P*-trend = 1.3×10^{-8}) than for *BRCA2* (HR = 1.14, 95% CI: 1.14, 95% CI: 1.01–1.28, *P*-trend = 0.031). There was some evidence of heterogeneity in the HRs across studies when considered individually (*P*-heterogeneity = 0.023). However, this was mainly due to studies with small numbers of mutation carriers and the low frequency of the minor allele of this SNP (minor allele frequency among unaffected = 6.7%). Repeating the analysis by grouping all studies within each country, there was no evidence of heterogeneity in the country-specific HRs (*P*-heterogeneity = 0.26; Fig. 2). Similarly, there was no evidence of heterogeneity in the HRs across countries for *BRCA2* mutation carriers (*P*-heterogeneity = 0.12; Fig. 2). There was no evidence that the HR for *BRCA1* mutation carriers varied by age (*P*-trend = 0.34), but there was evidence that the HR for *BRCA2* mutation carriers decreased with age (*P*-trend = 0.0025). The estimated age-specific HRs for rs9397435 among *BRCA2* mutation carriers were all >1 for ages <50, but there was no evidence of an increased risk for ages >50 years (Supplementary Material, Table S2).

SNPs rs9397435 and rs2046210 are located in the same region at 6q25.1 and were only weakly correlated (pair-wise $r^2 = 0.14$ based on the current data set). In an analysis for the joint effects of these SNPs on breast cancer risk for *BRCA1* mutation carriers (based on 9347 carriers with genotypes at both SNPs), the most parsimonious model included the effects of both SNPs (*P* for inclusion = 1.4×10^{-5} and 0.0037 for rs2046210 and rs9397435, respectively; 2-degree of freedom (df) $P = 5.8 \times 10^{-10}$ for the inclusion of both SNPs compared with the null model).

The minor allele of SNP rs11249433 at 1p11.2 was associated with the risk of breast cancer for *BRCA2* mutation carriers (HR = 1.09, 95% CI: 1.02–1.17, *P*-trend = 0.015), but was not associated with the risk of breast cancer for *BRCA1* mutation carriers (HR = 0.97, 95% CI: 0.92–1.02, *P*-trend = 0.20). There was no evidence that these HRs varied across the study groups for either *BRCA1* or *BRCA2* mutation carriers (p-heterogeneity = 0.10 and 0.14, respectively, Fig. 3) or that the HRs varied by age ($P = 0.41$ for *BRCA1*; $P = 0.93$ for *BRCA2*).

Carriers in each study were genotyped for either rs999737 or rs10483813 in the *RAD51L1* region, but none were genotyped for both SNPs. Since rs999737 and rs10483813 are perfectly correlated (pair-wise $r^2 = 1$), the genotypes across

Table 2. SNP genotype distributions and associations with breast cancer risk

Mutation	Genotype	Unaffected, *N* (%)	Affected[a], *N* (%)	HR	95% CI	*P*-value
6q25.1 (rs2046210)						
BRCA1	CC	2282 (43.0)	2067 (37.5)	1		
	TC	2361 (44.5)	2669 (48.4)	1.23	1.14–1.33	
	TT	659 (12.4)	779 (14.1)	1.32	1.18–1.47	
	2-df test					7.5×10^{-9}
	Per-allele			1.17	1.11–1.23	4.5×10^{-9}
BRCA2	CC	1144 (40.8)	1321 (39.1)	1		
	TC	1312 (46.7)	1574 (46.5)	1.02	0.92–1.13	
	TT	351 (12.5)	486 (14.4)	1.16	1.00–1.34	
	2-df test					0.13
	Per-allele			1.06	0.99–1.14	0.09
6q25.1 (rs9397435)						
BRCA1	AA	5361 (86.5)	5282 (82.9)	1.00		
	AG	802 (12.9)	1043 (16.4)	1.31	1.19–1.43	
	GG	38 (0.6)	49 (0.8)	1.37	0.92–2.06	
	2-df test					5.3×10^{-8}
	Per-allele			1.28	1.18–1.40	1.3×10^{-8}
BRCA2	AA	2786 (84.1)	3141 (82.6)	1.00		
	AG	510 (15.4)	631 (16.6)	1.11	0.98–1.26	
	GG	17 (0.5)	32 (0.8)	1.56	0.91–2.67	0.077
	2-df test					0.077
	Per-allele			1.14	1.01–1.28	0.031
1p11.2 (rs11249433)						
BRCA1	TT	1833 (34.4)	1961 (35.1)	1		
	CT	2584 (48.5)	2732 (48.9)	1.00	0.90–1.10	
	CC	911 (17.1)	890 (15.9)	0.92	0.83–1.03	
	2-df test					0.21
	Per-allele			0.97	0.92–1.02	0.20
BRCA2	TT	1016 (35.9)	1135 (33.2)	1		
	CT	1377 (48.7)	1698 (49.6)	1.07	0.96–1.19	
	CC	434 (15.4)	590 (17.2)	1.20	1.04–1.38	
	2-df test					0.05
	Per-allele			1.09	1.02–1.17	0.015
RAD51L1 (rs999737[b]/rs10483813[c])						
BRCA1	CC/TT	2725 (62.3)	2849 (63.6)	1		
	TC/AT	1461 (33.4)	1439 (32.1)	0.93	0.86–1.01	
	TT/AA	186 (4.3)	195 (4.3)	1.01	0.84–1.22	
	2-df test					0.25
	Per-allele			0.96	0.90–1.03	0.27
BRCA2	CC/TT	1609 (61.1)	1950 (62.2)	1		
	TC/AT	869 (33.0)	1039 (33.1)	0.98	0.88–1.09	
	TT/AA	154 (5.9)	147 (4.7)	0.86	0.69–1.08	
	2-df test					0.43
	Per-allele			0.96	0.88–1.04	0.30

Analysis restricted to mutation carriers of European ancestry.
[a]Breast cancer.
[b]Genotyped using iPLEX.
[c]Genotyped using Taqman, pair-wise $r^2 = 1$ between rs999737 and rs10483813 based on HapMap data.

studies were combined and analysed as a single locus. There were no significant associations between this SNP and the risk of breast cancer for either *BRCA1* or *BRCA2* mutation carriers (*BRCA1*: per-allele HR = 0.96, 95% CI: 0.90–1.03, *P*-trend = 0.27; *BRCA2*: per-allele HR = 0.96, 95% CI: 0.88–1.04, *P*-trend = 0.30). The HR estimates were consistent across the studies for both *BRCA1* (*P*-heterogeneity = 0.11) and *BRCA2* mutation carriers (*P*-heterogeneity = 0.42). There was no evidence that the HRs varied by age for *BRCA1* ($P = 0.50$) or *BRCA2* ($P = 0.60$) mutation carriers.

The associations were not altered after excluding long-term survivors (Supplementary Material, Table S3) and there was no evidence of differences in the associations between class 1 and class 2 *BRCA1* mutation carriers (*P* for difference > 0.15 for all SNPs, Supplementary Material, Table S3).

BRCA1 and *BRCA2* mutations also confer high risks of ovarian cancer. To determine whether the three polymorphisms modify ovarian cancer risk in mutation carriers, we analysed the associations within a competing risk analysis framework by estimating simultaneously the HRs for breast and ovarian cancer. There was no evidence of association with the risk of ovarian cancer for any of the SNPs (Table 3). The estimated HRs for breast cancer were similar to those from the primary analysis. SNPs rs2046210 and rs9397435 remained significantly associated with the risk of breast cancer for *BRCA1* mutation carriers (*P*-trend = 6.7×10^{-8} and 7.8×10^{-7}) and there was a slightly stronger evidence of association between SNP rs11249433 and the risk of breast cancer for *BRCA2* mutation carriers (*P*-trend = 0.0052).

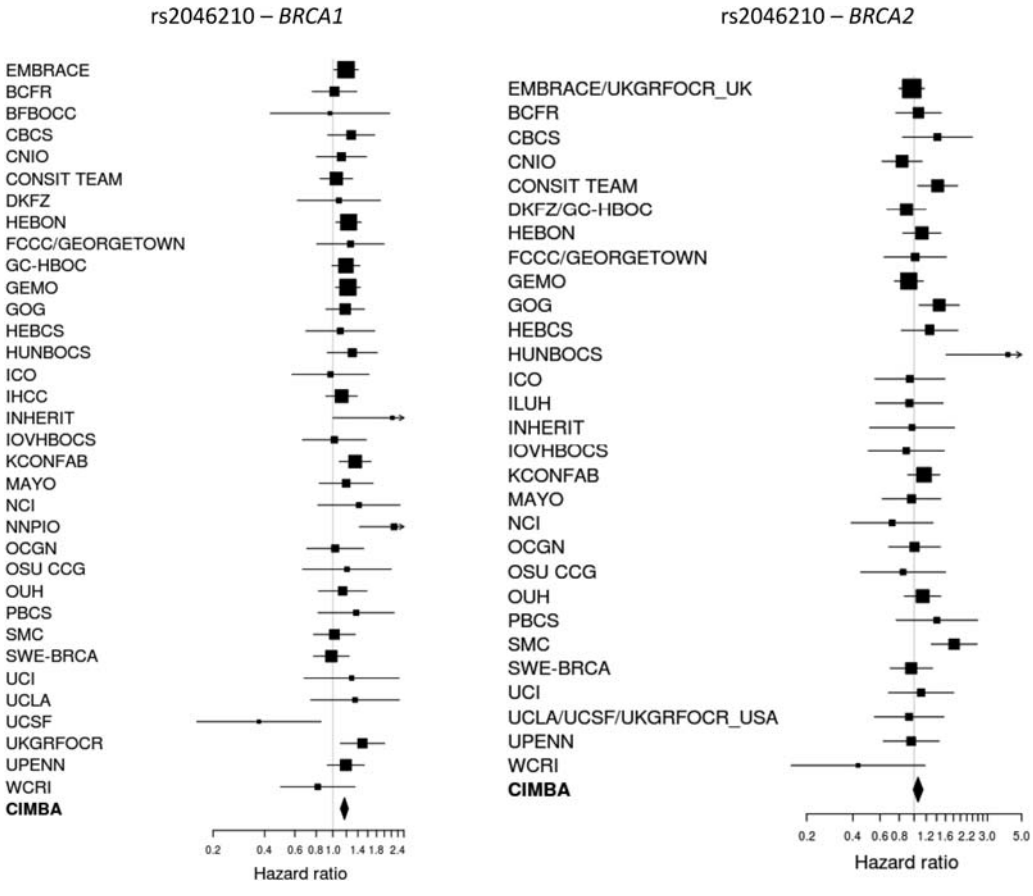

Figure 1. Study-specific per-allele HR estimates for *BRCA1* and *BRCA2* mutation carriers for SNP rs2046210 at 6q25.1 near *ESR1*. The area of the square is proportional to the inverse of the variance of the estimate. Horizontal lines indicate 95% CIs.

DISCUSSION

Several common variants identified through GWASs in the general population and *BRCA1* and *BRCA2* mutation carriers have been demonstrated to be associated with the risk of breast cancer for *BRCA1* and/or *BRCA2* mutation carriers (8–10,12,13). In this study, we evaluated the associations of four additional variants, identified through population-based GWAS or subsequent follow-up mapping studies, with the risk of breast and ovarian cancer for *BRCA1* and *BRCA2* mutation carriers.

We found strong evidence that SNP rs2046210 at 6q25.1 was associated with the risk of breast cancer for *BRCA1* mutation carriers, but there was no clear evidence of association with the risk of breast cancer for *BRCA2* mutation carriers (P for difference: 0.027). The observed association with *BRCA1* breast cancer risk was unaltered after the exclusion of prevalent breast cancer cases, and did not vary by the predicted functional effect of the mutations. This polymorphism was identified through a GWAS in Chinese women (7), in whom the authors estimated a per-allele OR of 1.29 (95% CI: 1.21–1.37) for breast cancer in this population. This OR estimate was greater than our estimated HR for *BRCA1* mutation carriers. However, the Chinese study reported a further replication of their findings among women of European ancestry for whom the per-allele OR was estimated to be 1.15, similar to the HR based on our analysis of *BRCA1* mutation carriers who were also of self-reported European ancestry. A more recent study by the same group also found evidence for association with breast cancer in an independent sample of European-ancestry American cases and controls (17). Both studies (7,17) also reported a stronger association with ER-negative disease than ER-positive in particular among Asian women, although the SNP was associated with both disease subtypes. This is consistent with our finding that this SNP is predominantly associated with the risk of breast cancer for *BRCA1* mutation carriers, the majority of whom present with ER-negative tumours (18), and hence conforms to the general pattern we have observed previously that the breast cancer susceptibility SNPs confer a similar relative risk in carriers to that in the general population, once receptor status is taken into account (10,11). A more recent study by Stacey *et al*. (16) evaluated the associations of SNP rs2046210 in a larger set of women of European ancestry, but failed to replicate the association. The authors concluded that this SNP does not confer a substantial risk of breast cancer in Europeans and they postulated that this is due to the different linkage disequilibrium structures between the causal variant and SNP rs2046210 in Europeans. They found a different SNP, rs9397435 (pair-wise $r^2 = 0.08$ based on CEU HapMap data, $r^2 = 0.71$ based on CHB + JPT HapMap data), accounting for the association in both the

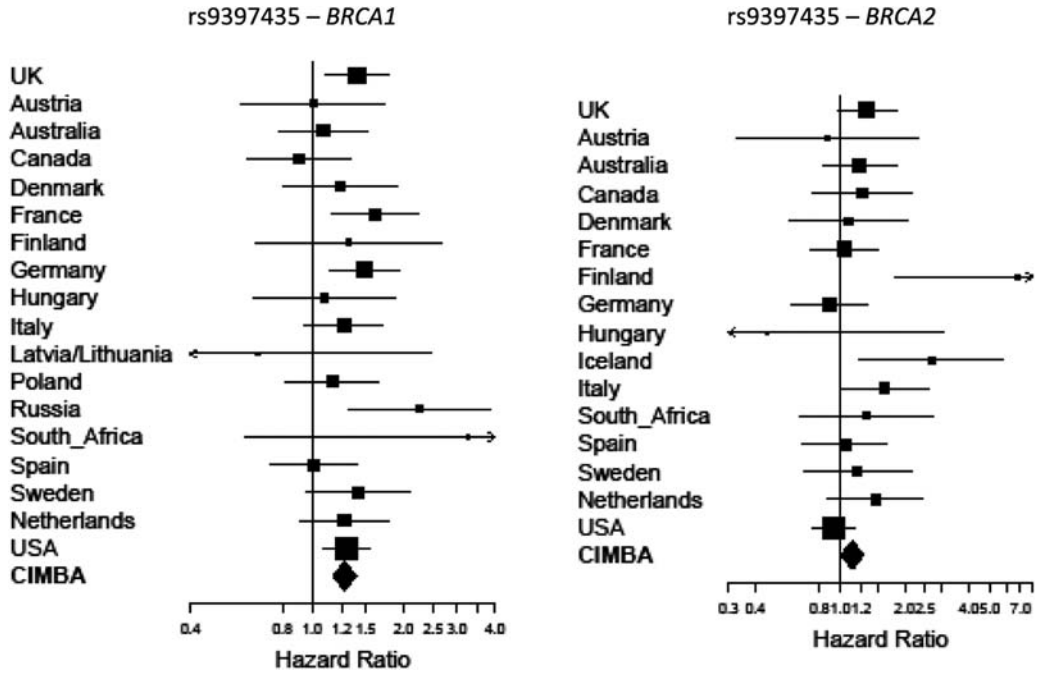

Figure 2. Country-specific per-allele HR estimates for *BRCA1* and *BRCA2* mutation carriers for SNP rs9397435 at 6q25.1. The area of the square is proportional to the inverse of the variance of the estimate. Horizontal lines indicate 95% CIs. Due to the low minor allele frequency at this SNP and small study sample we were unable to obtain study-specific estimates for all studies. Studies were therefore grouped by country of origin.

Europeans and Chinese. SNP rs9397435 was also strongly associated with the risk of breast cancer for *BRCA1* mutation carriers in our data set and exhibited weak association with the risk for *BRCA2* mutation carriers (*P* for difference between *BRCA1* and *BRCA2* = 0.10). Our joint analysis of SNPs rs9397435 and rs2046210 among *BRCA1* mutation carriers demonstrated that a model that includes both SNPs fits significantly better than a model that includes either SNP on its own, and are not therefore consistent with the conclusions of Stacey *et al*. (16) who suggest that the association is primarily due to SNP rs9397435. Our results suggest that either the observed associations are driven by another causative variant that is partially associated with both SNPs, or that more than one causative variant is located in this region. Further comprehensive genotyping of variants across the region will be required to determine which of these hypotheses is correct. A potential explanation for the observed differences between our study and that of Stacey *et al*. (16) could be the fact the *BRCA1* tumours are predominantly ER-negative, whereas the majority of cases in Stacey *et al.* were ER-positive. This result could have been observed if rs2046210 was mainly associated with ER-negative breast cancer and rs9397435 was associated with both ER-negative and ER-positive breast cancer. Stacey *et al*. (16) did not present the associations of rs2046210 by tumour ER status, but reported that rs9397435 was associated with both ER-positive and ER-negative disease, with a higher per-allele OR for ER-negative breast cancer consistent with our observation of a larger HR estimate for *BRCA1* mutation carriers than *BRCA2* carriers for rs9397435. Although we found no significant evidence of association between rs2046210 and the risk of breast cancer for *BRCA2* mutation carriers, the estimated association was in the same direction.

Our results also suggest that rs2046210 is associated with higher relative risks of breast cancer at younger ages among *BRCA1* mutation carriers. A similar pattern was observed for rs9397435 among *BRCA2* mutation carriers. Stacey *et al.* (16) also found that rs9397435 was associated with an earlier age at diagnosis in Europeans from the general population.

SNPs rs2046210 and rs9397435 are located close to *ESR1*, which encodes ER α mediator of oestrogen action (19). Elevated oestrogen levels have been associated with increased breast cancer risk (20), and although it is assumed that the action of oestrogen is via ER in ER-positive tumours, two studies have recently provided evidence that the size and repopulating ability of the mammary stem cell compartment in mice are controlled by17β-estradiol and progesterone via a paracrine-signalling mechanism from steroid receptor-positive luminal cells to steroid receptor-negative stem cells (21,22). This may explain the apparently paradoxical observation that a SNP in *ESR1* could modify the risk of breast cancer in *BRCA1* carriers, in which the tumour phenotype is usually ER-negative. The cell of origin of basal ER-negative tumours in BRCA1 mutation carriers is likely to be a luminal progenitor cell that is dependent on steroid hormone signalling (23). There is also indirect evidence that steroid hormones regulate breast cancer stem cells in humans where the same paracrine regulation probably occurs, perhaps mediated via the Receptor Activator of NF-κB) (RANK) ligand (24). Other studies have also provided evidence that oophorectomy decreases the risk of breast cancer in BRCA1 mutation carriers (26,27) and tamoxifen treatment may decrease the risk of contralateral breast cancer for *BRCA1* mutation carriers. Both of these findings suggest the potential ER involvement in *BRCA1* associated disease (28).

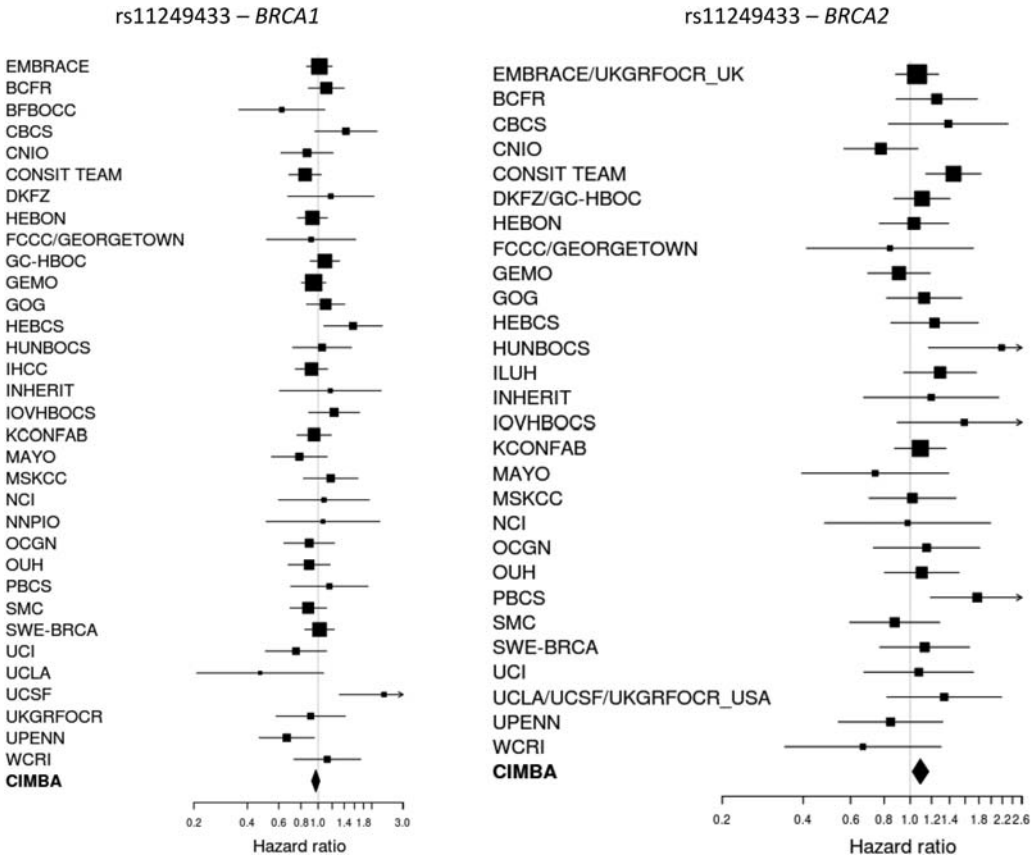

Figure 3. Study-specific per-allele HR estimates for *BRCA1* and *BRCA2* mutation carriers for SNP rs11249433 at 1p11.2. The area of the square is proportional to the inverse of the variance of the estimate. Horizontal lines indicate 95% CIs.

There are currently limited data in the literature on the impact of these variants on expression levels of *ESR1* in breast tumour samples. Stacey *et al.* found some evidence that breast tumours from 11 GG homozygote carriers of rs9397435 expressed higher mean levels of *ESR1* compared with tumours from over one thousand carriers of the 'A' allele (16). However, Dunbier *et al*. (29) recently reported that *ESR1* is co-expressed in tumour biopsies along with three uncharacterized open reading frames located upstream of *ESR1*. It is therefore currently uncertain whether rs9397435 or correlated causal variant(s) affect breast cancer risk through modulating *ESR1* expression levels or those of additional genes in the region. If the *ESR1* gene is found to be the target, this would provide direct evidence that ER signalling is important in the development of ER-negative breast cancer (and breast cancer in *BRCA1* carriers in particular).

We also found evidence that SNP rs11249433 at 1p11.2 was associated with the risk of breast cancer for *BRCA2*, but not *BRCA1* mutation carriers (*P* for difference: 0.007). The estimated HR was slightly greater (1.15 versus 1.09) when *BRCA2* mutation carriers with prevalent breast cancer were excluded from the analysis, although the difference in the estimates was small. This could potentially arise if the SNP is also associated with survival after breast cancer diagnosis. In this case, the inclusion of prevalent cases could lead to an attenuation of the HR. Future studies will aim to evaluate the associations of this SNP with breast cancer prognosis. The observed association with the risk for *BRCA2* mutation carriers is consistent with the observation of Thomas *et al.* (6) that this SNP is mainly associated with the risk for ER-positive breast cancer.

We found no significant evidence of association between the *RAD51L1* locus and the risk of breast cancer for *BRCA1* or *BRCA2* mutation carriers. However, the OR estimate from the original breast cancer GWAS (0.94) is only slightly different from the HR estimates for both *BRCA1* and *BRCA2* carriers (0.96) and is included in the CIs for both estimates (5). If the relative risk associated with each copy of the minor allele of this SNP is between 0.90 and 1.00, we have limited power to detect these associations given our sample size (30). *RAD51L1* is known to be essential to DNA repair via homologous recombination; therefore, if the breast cancer association seen in the general population was mediated through *RAD51L1*, an absence of an association in *BRCA1* and *BRCA2* mutation carriers (i.e. a 'negative interaction' with BRCA status) could also be plausible. It is interesting to note, however, that a rare allele in the *RAD51* gene, in the same pathway, was previously associated with an increased risk of breast cancer for *BRCA2* mutation carriers (25). Future studies with a larger number of mutation carriers, and analysis of the causal variant once it has been identified, may help to clarify the involvement of this locus in breast cancer for mutation carriers.

Including the SNPs from the present study, five loci are now known to modify the risk of breast cancer for *BRCA1* mutation

Table 3. Competing risk analysis

	Genotype	Unaffected, *N* (%)	Breast cancer, *N* (%)	Ovarian cancer, *N* (%)	Breast cancer HR	95% CI	*P*-value	Ovarian cancer HR	95% CI	*P*-value
6q25.1 (rs2046210)										
BRCA1	CC	1658 (42.5)	2046 (37.4)	645 (44.7)	1			1		
	TC	1740 (44.6)	2648 (48.4)	642 (44.5)	1.23	1.13–1.33		0.98	0.86–1.12	
	TT	505 (12.9)	777 (14.2)	156 (10.8)	1.30	1.16–1.47		0.89	0.72–1.10	
	2-df test						9.8×10^{-8}			0.54
	Per-allele				1.16	1.10–1.23	6.7×10^{-8}	0.95	0.87–1.05	0.31
BRCA2	CC	988 (40.8)	1317 (39.1)	160 (40.4)	1			1		
	TC	1130 (46.6)	1567 (46.5)	189 (47.7)	1.01	0.91–1.13		0.99	0.78–1.27	
	TT	305 (12.6)	485 (14.4)	47 (11.9)	1.14	0.98–1.33		0.88	0.61–1.26	
	2-df test						0.22			0.76
	Per-allele				1.05	0.98–1.13	0.15	0.95	0.81–1.12	0.57
6q25.1 (rs9397435)										
BRCA1	AA	4116 (86.1)	5245 (82.9)	1282 (87.5)	1.00			1.00		
	AG	633 (13.2)	1034 (16.3)	178 (12.1)	1.28	1.16–1.41		0.90	0.75–1.08	
	GG	33 (0.7)	49 (0.8)	5 (0.3)	1.25	0.81–1.94		0.47	0.18–1.18	
	2-df test						2.8×10^{-6}			0.15
	Per-allele				1.25	1.14–1.37	7.8×10^{-7}	0.87	0.73–1.03	0.10
BRCA2	AA	2410 (83.9)	3131 (82.5)	386 (85.4)	1.00			1.00		
	AG	466 (15.5)	631 (16.6)	64 (14.2)	1.11	0.97–1.26		0.93	0.69–1.26	
	GG	15 (0.5)	32 (0.8)	2 (0.4)	1.50	0.85–2.62		0.65	0.16–2.68	
	2-df test						0.12			0.75
	Per-allele				1.13	1.00–1.27	0.047	0.92	0.70–1.20	0.52
1p11.2 (rs11249433)										
BRCA1	TT	1363 (34.8)	1945 (35.1)	486 (33.5)	1			1		
	CT	1863 (47.5)	2714 (49.0)	739 (50.9)	1.02	0.94–1.11		1.12	0.97–1.29	
	CC	695 (17.7)	880 (15.9)	226 (15.6)	0.91	0.81–1.01		0.91	0.75–1.11	
	2-df test						0.08			0.05
	Per-allele				0.96	0.91–1.02	0.18	0.98	0.90–1.08	0.74
BRCA2	TT	889 (36.4)	1129 (33.1)	133 (33.7)	1			1		
	CT	1182 (48.4)	1695 (49.7)	198 (50.1)	1.10	0.98–1.22		1.12	0.88–1.44	
	CC	372 (15.2)	588 (17.2)	64 (16.2)	1.23	1.06–1.42		1.23	0.88–1.72	
	2-df test						0.02			0.44
	Per-allele				1.11	1.03–1.19	0.0052	1.11	0.94–1.31	0.20
RAD51L1 (rs999737/rs10483813)										
BRCA1	CC/TT	2014 (61.9)	2828 (63.6)	732 (63.4)	1			1		
	TC/AT	1100 (33.8)	1426 (32.1)	374 (32.4)	0.92	0.84–1.01		0.95	0.81–1.10	
	TT/AA	138 (4.2)	194 (4.4)	49 (4.2)	1.02	0.83–1.24		1.00	0.69–1.45	
	2-df test						0.17			0.76
	Per-allele				0.96	0.89–1.03	0.21	0.97	0.85–1.10	0.60
BRCA2	CC/TT	1384 (61.1)	1942 (62.1)	233 (62.0)	1			1		
	TC/AT	753 (33.2)	1036 (33.2)	119 (31.6)	0.98	0.87–1.09		0.95	0.74–1.21	
	TT/AA	130 (5.7)	147 (4.7)	24 (6.4)	0.88	0.70–1.11		1.16	0.71–1.90	
	2-df test						0.56			0.72
	Per-allele				0.96	0.88–1.05	0.35	1.01	0.83–1.23	0.92

Associations with breast and ovarian cancer risk for *BRCA1* and *BRCA2* mutation carriers. Analysis restricted to mutation carriers of European ancestry.

carriers (*CASP8*, *TOX3*, 2q35, 19p13 and 6q25.1) (8–10, 12,14) and nine loci are known to modify the risk of breast cancer for *BRCA2* mutation carriers (*FGFR2*, *TOX3*, *MAP3K1*, *LSP1*, 2q35, *SLC4A7*, 5p12, *ZNF365* and 1p11.2) (8–10,13). These loci are estimated to account for ~3.0% of the genetic variance in the risk of breast cancer in *BRCA1* mutation carriers and 5.6% of the variance in *BRCA2* mutation carriers. Although these variants account for a small proportion of the variability in risk, it has been demonstrated that these SNPs have implications for the absolute risk prediction in mutation carriers (10), and could therefore be relevant in the genetic counselling of women carrying mutations (15). There are also suggestions from candidate gene studies that other variants may modify cancer risks for mutation carriers, which are currently being investigated in larger sample sizes (31,32). The three associated polymorphisms presented here, in conjunction with previously identified risk-modifying polymorphisms and other risk- modifying factors, can be used to improve risk prediction in *BRCA1* and *BRCA2* mutation carriers.

Data from the general population indicate that chemopreventive agents have different effects on the risk of ER-positive and ER-negative breast cancer (33). Ongoing and future CIMBA studies will aim to clarify the involvement of these polymorphisms in ER-positive and ER-negative breast cancer risk, as well as other tumour subtypes, in *BRCA1* and *BRCA2* mutation carriers, which should lead to further improvements in risk prediction. Since *BRCA1* and *BRCA2* mutations confer high risks of breast cancer, these SNPs, taken together with other risk factors such as mammographic breast density (34), will result in substantial differences in the absolute risk of developing breast cancer between combined SNP and risk factor categories

(10,35). These will enable preventive therapies, including chemoprevention and prophylactic surgery, to be targeted at mutation carriers most likely to benefit.

MATERIALS AND METHODS

Subjects

All carriers participated in clinical or research studies at the host institutions under ethically approved protocols and data were analysed anonymously. Subjects were *BRCA1* and *BRCA2* mutation carriers recruited by 42 study centres in 22 countries through the CIMBA initiative (Supplementary Material, Table S1). The large majority of carriers were recruited through cancer genetics clinics offering genetic testing, and enrolled into national or regional studies. Some carriers were identified by population-based sampling of cases, and some by community recruitment (e.g. in Ashkenazi Jewish populations). Eligibility to participate in CIMBA is restricted to female carriers of pathogenic *BRCA1* or *BRCA2* mutations who were 18 years old or older at recruitment. Information collected included the year of birth; mutation description, including nucleotide position and base change; age at the last follow-up; ages at breast and ovarian cancer diagnoses; and age or date at bilateral prophylactic mastectomy. Information was also available on the country of residence, which was defined to be the country where the carrier family was recruited to the study. Related individuals were identified through a unique family identifier. Women were included in the analysis if they carried mutations that were pathogenic according to generally recognized criteria (25) (Breast Cancer Information Core). Further details of the CIMBA initiative can be found elsewhere (36).

Women who carried pathogenic mutations in both *BRCA1* and *BRCA2* were excluded from the current analysis. The primary analysis was restricted to women self-reported as 'white of European ancestry', but additional analyses were performed which were restricted to mutation carriers of non-European ancestry. We investigated possible overlap of carriers between studies by comparing the year of birth, exact mutation description, and the reported ages, to identify potential duplicate individuals. Where possible we also used SNP genotype data available within the CIMBA database to find hidden duplicates. When a potential duplicate was identified, we contacted the relevant centres for further information about these individuals, in a manner that protected the identity of the individuals in question, in order to determine precisely the extent of true overlap in subjects and families appearing more than once in the data set. Duplicated mutation carriers were included only once in the analysis. To avoid inclusion of families extending over several studies, we included only the individual with the most complete version of the family history in the study.

Genotyping

The genotyping platforms used by each study are shown in Supplementary Material, Table S1. Genotyping for the four SNPs was performed in two stages. Stage 1 involved SNPs rs2046210, rs11249433 and the *RAD51L1* SNPs rs999737 and rs10483813. DNA samples from 11 studies were genotyped using the iPLEX Mass Array platform at a single genotyping centre. All remaining studies used the 5′ endonuclease assay (Taqman), with reagents supplied by Applied Biosystems and tested centrally. A Taqman assay could not be adequately designed for SNP rs999737 and studies using this platform genotyped the surrogate SNP rs10483813 (pair-wise $r^2 = 1$ with rs999737 based on HapMap data). Stage 2 involved SNP rs9397435 and all samples were genotyped using the iPLEX Mass Array platform at four genotyping centres. All centres included at least 2% of the samples in duplicate, no template controls in every plate and a random mixture of affected and unaffected carriers. Samples that failed for more than two of the SNPs genotyped (or $\geq$20% of the SNPs typed if more than three SNPs were analysed using multiplex genotyping) were excluded from the analysis. A study was included in the analysis only if the call rate was over 95% after samples that failed at multiple SNPs had been excluded. The concordance between duplicates had to be at least 98%. To assess the accuracy of genotyping across genotyping centres, all centres genotyped 95 DNA samples from a standard test plate (Coriell Institute) for all three SNPs. If the genotyping was inconsistent for more than one sample in the test plate, the study was excluded from the analysis of that SNP. On the basis of these criteria, two studies were excluded from the analysis of rs2046210, eight studies were excluded from the analysis of rs999737/rs10483813 and three studies were excluded from the analysis of rs11249433. As an additional genotyping quality-control check, we also evaluated the deviation from Hardy–Weinberg equilibrium (HWE) for unrelated subjects separately for each SNP and study. Seven studies had HWE *P*-values in the range 0.003–0.05 (one study for the rs2046210 SNP, two for rs9397435 and four studies for rs11249433). Upon examination of the cluster plots for these studies and SNPs, none revealed any unusual patterns and these studies were included in all the analyses. After the above exclusions, a total of 22 176 unique mutation carriers (14 123 *BRCA1* and 8053 *BRCA2*) from 42 studies had an observed genotype for at least one of the SNPs and were therefore included in the primary analysis (Supplementary Material, Table S1).

Statistical analysis

The aim of the primary analysis was to evaluate the association between each genotype and the risk of breast cancer. The phenotype of each individual was therefore defined by their age at diagnosis of breast cancer or their age at the last follow-up. For this purpose, individuals were censored at the age of the first breast cancer diagnosis, ovarian cancer diagnosis or bilateral prophylactic mastectomy or the age at the last observation. Mutation carriers censored at ovarian cancer diagnosis were considered unaffected. Since mutation carriers were not sampled randomly with respect to their disease status, standard methods of survival analysis (such as Cox regression) may lead to biased estimates of the hazard ratios (HRs) (37). We therefore conducted the analysis by modelling the retrospective likelihood of the observed genotypes conditional on the disease phenotypes as previously described (25). The effect of each SNP was modelled either as a per-allele HR (multiplicative model) or as separate HRs for

heterozygotes and homozygotes, and these were estimated on the logarithmic scale. The HRs were assumed to be independent of age (i.e. we used a Cox proportional-hazards model). The assumption of proportional hazards was tested by adding a 'genotype × age' interaction term to the model in order to fit models in which the HR changed with age. Where there was significant evidence of a 'genotype × age' interaction, we fitted models that allowed for age-specific HRs. These allowed for age-specific HRs to be estimated simultaneously in 10-year intervals (20–29, 30–39, . . . , 70–79). Thus, these models included six log-HR parameters. We examined between-study heterogeneity by comparing the models that allowed for study-specific log-HRs against models in which the same log-HR was assumed to apply to all studies. Analyses were carried out with the pedigree analysis software MENDEL (38), and details of this approach have been described previously (25). Under the retrospective likelihood approach, the baseline age-specific incidence rates in the Cox proportional-hazards model were chosen such that the overall breast cancer incidence rates, averaged over all genotypic categories, agree with external estimates of incidence for *BRCA1* and *BRCA2* mutation carriers. All analyses were stratified by study group and country of residence and used calendar-year- and cohort-specific breast cancer incidence rates for *BRCA1* and *BRCA2* (39).

To evaluate the combined effects of the *ESR1* SNPs on the risk of breast cancer, we fit retrospective likelihood models where the breast cancer incidence $\lambda(t)$was assumed to be of the form $\lambda(t) = \lambda_0(t) \exp(\beta_1 x_1 + \beta_2 x_2)$, where $\lambda_0(t)$ is the baseline incidence, β_1 is the per-allele log-HR for SNP1, β_2 is the per-allele log-HR for SNP2, and x_1 and x_2 represent the number of minor alleles at SNP 1 and 2, respectively (0,1,2), while allowing for linkage disequilibrium between the loci. To test whether the fit of the model is significantly improved by the inclusion of a locus into the model, we tested for the significance of parameters β_1 and β_2.

To investigate whether our results were influenced by any of our assumptions, we performed additional sensitivity analyses. If any of the SNPs were associated with disease survival, the inclusion of prevalent cases may influence the HR estimates. Current data indicate that 5-year survival after a breast cancer diagnosis is now over 80% (Cancer Research—UK, Breast cancer survival statistics). We therefore repeated our analysis by excluding mutation carriers diagnosed more than 5 years prior to the age at recruitment into the study. To examine whether SNP associations differed by type of mutations, we classified *BRCA1* mutations according to their potential functional effect (40–42). Class 1 mutations comprised loss-of-function mutations, expected to result in a reduced transcript or protein level due to mRNA nonsense-mediated decay and/or degradation or instability of truncated proteins, translation re-initiation but no production of stable protein, or the absence of expression because of the deletion of transcription regulatory regions. Class 2 mutations were those likely to generate potentially stable mutant proteins that might have dominant negative action, partially preserved normal function or loss of function. Class 2 mutations include missense substitutions, in-frame deletions and insertions, as well as truncating mutations with premature stop codons occurring in the last exon. Mutations whose consequences at the transcript or protein level could not be inferred were not considered for this classification. These were mainly mutations located in splice sites but not characterized for their effect at the transcript level, or large deletions or insertions with undetermined boundaries.

We further evaluated the associations of these SNPs with the risk of ovarian cancer within a competing risk analysis framework (12,43), by estimating HRs simultaneously for breast and ovarian cancers. In this model, each individual was at risk of developing either breast or ovarian cancer, by assuming that the probabilities of developing each disease were independent conditional on the underlying genotype. A different censoring process was used in this case, whereby individuals were followed up to the age of the first breast or ovarian cancer diagnosis and were considered to have developed the corresponding disease. No follow-up was considered after the first cancer diagnosis. Individuals were censored for breast cancer at the age of bilateral prophylactic mastectomy and for ovarian cancer at the age of bilateral oophorectomy and were assumed to be unaffected for the corresponding disease. The remaining individuals were censored at the age at the last observation and were assumed to be unaffected for both diseases.

All analyses were stratified by study group and country of residence and used calendar-year- and cohort-specific cancer incidences for *BRCA1* and *BRCA2* (39). For sensitivity analyses, strata with a small number of mutation carriers were grouped. We used a robust variance-estimation approach to allow for the non-independence among related carriers (44). Data on the two completely correlated SNPs (rs999737 and rs10483813) were combined and treated as a single locus in the analysis of associations.

WEB RESOURCES

Breast Cancer Information Core: http://research.nhgri.nih.gov/bic/.

Cancer Research—UK, Breast cancer—survival statistics: http://info.cancerresearchuk.org/cancerstats/types/breast/survival/.

SUPPLEMENTARY MATERIAL

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