Genetic variation in the immunosuppression pathway genes and breast cancer susceptibility: a pooled analysis of 42,510 cases and 40,577 controls from the Breast Cancer Association Consortium

Jieping Lei¹ · Anja Rudolph¹ · Kirsten B. Moysich² · Sabine Behrens¹ · Ellen L. Goode³ · Manjeet K. Bolla⁴ · Joe Dennis⁴ · Alison M. Dunning⁵ · Douglas F. Easton⁴,⁵ · Qin Wang⁶ · Javier Benitez⁶,⁷ · John L. Hopper⁸ · Melissa C. Southey⁹ · Marjanka K. Schmidt¹⁰ · Annegien Broeks¹⁰ · Peter A. Fasching¹¹,¹² · Lothar Haeberle¹¹ · Julian Peto¹³ · Isabel dos-Santos-Silva¹³ · Elinor J. Sawyer¹⁴ · Ian Tomlinson¹⁵ · Barbara Burwinkel¹⁶,¹⁷ · Frederik Marne¹⁶,¹⁸ · Pascal Guéné¹⁹,²⁰ · Thérèse Truong¹⁹,²⁰ · Stig E. Bojesen²¹,²²,²³ · Henrik Flyger²⁴ · Sune F. Nielsen²² · Børge G. Nordestgaard²²,²³ · Anna González-Neira⁶ · Primitiva Menéndez²⁵ · Hoda Anton-Culver²⁶ · Susan L. Neuhausen²⁷ · Hermann Brenner²⁸,²⁹,³⁰ · Volker Arndt²⁸ · Alfons Meindl³¹ · Rita K. Schmutzler³²,³³,³⁴ · Hiltrud Brauch³⁵,³⁶ · Ute Hamann³⁷ · Heli Nevanlinna³⁸ · Rainer Fagerholm³⁸ · Thilo Dörk³⁹ · Natalia V. Bogdanova⁴⁰ · Arto Mannermaa⁴¹,⁴²,⁴³ · Jaana M. Hartikainen⁴¹,⁴²,⁴³ · Australian Ovarian Study Group⁴⁴ · kConFab Investigators⁴⁵ · Laurien Van Dijck⁴⁶ · Ann Smeets⁴⁷ · Dieter Flesch-Janys⁴⁸,⁴⁹ · Ursula Elßner¹ · Paolo Radice⁵⁰ · Paolo Peterlongo⁵¹ · Fergus J. Couch⁵² · Emily Hallberg⁵³ · Graham G. Giles⁵⁴,⁵⁵ · Roger L. Milne⁵⁶,⁵⁷ · Christopher A. Haiman⁵⁸ · Fredrick Schumacher⁵⁴ · Jacques Simard⁵⁵ · Mark S. Goldberg⁵⁶,⁵⁷ · Vessela Kristensen⁵⁸,⁵⁹,⁶⁰ · Anne-Lise Borresen-Dale⁵⁸,⁵⁹ · Wei Zheng⁶¹ · Alicia Beeghly-Fadiel⁶¹ · Robert Winquist⁶²,⁶³ · Mervi Grip⁶⁴ · Irene L. Andruleit⁶⁵,⁶⁶ · Gord Glendon⁶⁷ · Montserrat García-Closas⁶⁸,⁶⁹ · Jonine Figueroa⁶⁸ · Kamila Czene⁶⁹ · Judith S. Brand⁶⁹ · Hatef Darabi⁶⁹ · Mikael Eriksson⁶⁹ · Per Hall⁶⁹ · Jingmei Li⁷⁰ · Angela Cox⁷⁰ · Simon S. Cross⁷¹ · Paul D. P. Pharoah⁷² · Mitul Shah⁵ · Maria Kabisch⁷³ · Diana Torres⁷⁰,⁷¹,³⁷,³⁸ · Anna Jakubowska⁷³ · Jan Lubinski⁷³ · Foluso Ademuyiwa⁷⁴ · Christine B. Ambrosone⁷⁴ · Anthony Swerdlow⁷⁵,⁷⁶ · Michael Jones⁷⁵ · Jenny Chang-Claude¹,⁷⁷

Received: 30 July 2015 / Accepted: 13 November 2015 / Published online: 30 November 2015 © The Author(s) 2015. This article is published with open access at Springerlink.com

Abstract Immunosuppression plays a pivotal role in assisting tumors to evade immune destruction and promoting tumor development. We hypothesized that genetic variation in the immunosuppression pathway genes may be implicated in breast cancer tumorigenesis. We included 42,510 female breast cancer cases and 40,577 controls of European ancestry from 37 studies in the Breast Cancer Association Consortium (2015) with available genotype data for 3595 single nucleotide polymorphisms (SNPs) in 133 candidate genes. Associations between genotyped SNPs and overall breast cancer risk, and secondarily according to estrogen receptor (ER) status, were assessed using multiple logistic regression models. Gene-level associations were assessed based on principal component

Jieping Lei and Anja Rudolph share the first authorship.

Electronic supplementary material The online version of this article (doi:10.1007/s00439-015-1616-8) contains supplementary material, which is available to authorized users.

Jenny Chang-Claude
j.chang-claude@dkfz-heidelberg.de

¹Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 581, 69120 Heidelberg, Germany

²Department of Cancer Prevention and Control, Roswell Park Cancer Institute, Buffalo, NY, USA

³Department of Health Sciences Research, Mayo Clinic, Rochester, MN, USA
analysis. Gene expression analyses were conducted using RNA sequencing level 3 data from The Cancer Genome Atlas for 989 breast tumor samples and 113 matched normal tissue samples. SNP rs1905339 (A>G) in the STAT3 region was associated with an increased breast cancer risk (per allele odds ratio 1.05, 95% confidence interval 1.03–1.08; \( p \) value = \( 1.4 \times 10^{-6} \)). The association did not differ significantly by ER status. On the gene level, in addition to TGFBR2 and CCND1, IL5 and GM-CSF showed the strongest associations with overall breast cancer risk (\( p \) value = \( 1.0 \times 10^{-3} \) and \( 7.0 \times 10^{-3} \), respectively). Furthermore, STAT3 and IL5 but not GM-CSF were differentially expressed between breast tumor tissue and normal tissue (\( p \) value = \( 2.5 \times 10^{-3} \), \( 4.5 \times 10^{-4} \) and 0.63, respectively). Our data provide evidence that the immunosuppression pathway genes STAT3, IL5, and GM-CSF may be novel susceptibility loci for breast cancer in women of European ancestry.

**Abbreviations**

BCAC Breast Cancer Association Consortium  
CCND1 Cyclin D1  
CI Confidence interval  
COGS Collaborative Oncological Gene-Environment Study  
DNA Deoxyribonucleic acid  
GM-CSF Granulocyte-macrophage colony stimulating factor  
EM Estimation maximization  
ENCODE Encyclopedia of DNA elements  
eQTL Expression quantitative trait loci  
ER Estrogen receptor  
GWAS Genome-wide association study  
HWE Hardy–Weinberg equilibrium  
IL5 Interleukin 5  
LD Linkage disequilibrium  
MAF Minor allele frequency  
MDSCs Myeloid-derived suppressor cells  
OR Odds ratio  
PCs Principal components  
PTRF Polymerase I and transcript release factor  
QQ Quantile–quantile  
RSEM RNA-Seq by expectation-maximization  
SD Standard deviation  
SNPs Single nucleotide polymorphisms  
STAT3 Signal transducer and activator of transcription 3  
TCGA The Cancer Genome Atlas  
TGFBR2 Transforming growth factor beta receptor II  
Treg cells Regulatory T cells  
TUBG2 Tubulin, gamma 2

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4 Centre for Cancer Genetic Epidemiology, Department of Public Health and Primary Care, University of Cambridge, Cambridge, UK  
5 Centre for Cancer Genetic Epidemiology, Department of Oncology, University of Cambridge, Cambridge, UK  
6 Human Cancer Genetics Program, Spanish National Cancer Research Centre, Madrid, Spain  
7 Centro de Investigación en Red de Enfermedades Raras, Valencia, Spain  
8 Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, The University of Melbourne, Melbourne, Australia  
9 Department of Pathology, The University of Melbourne, Melbourne, Australia  
10 Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital, Amsterdam, The Netherlands  
11 Department of Gynaecology and Obstetrics, University Hospital Erlangen, Friedrich-Alexander University Erlangen-Nuremberg, Comprehensive Cancer Center Erlangen-EMN, Erlangen, Germany  
12 David Geffen School of Medicine, Department of Medicine Division of Hematology and Oncology, University of California at Los Angeles, Los Angeles, CA, USA  
13 Department of Non-Communicable Disease Epidemiology, London School of Hygiene and Tropical Medicine, London, UK  
14 Research Oncology, Guy’s Hospital, King’s College London, London, UK  
15 Wellcome Trust Centre for Human Genetics and Oxford NIHR Biomedical Research Centre, University of Oxford, Oxford, UK  
16 Department of Obstetrics and Gynecology, University of Heidelberg, Heidelberg, Germany  
17 Molecular Epidemiology Group, German Cancer Research Center (DKFZ), Heidelberg, Germany  
18 National Center for Tumor Diseases, University of Heidelberg, Heidelberg, Germany  
19 Environmental Epidemiology of Cancer, Center for Research in Epidemiology and Population Health, INSERM, Villejuif, France  
20 University Paris-Sud, Villejuif, France  
21 Copenhagen General Population Study, Herlev Hospital, Copenhagen University Hospital, Herlev, Denmark  
22 Department of Clinical Biochemistry, Herlev Hospital, Copenhagen University Hospital, Herlev, Denmark  
23 Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark  
24 Department of Breast Surgery, Herlev Hospital, Copenhagen University Hospital, Herlev, Denmark
Introduction

Breast cancer is the most frequent cancer among women and the second leading cause of cancer-related death after lung cancer in Europe. In addition to genetic variants with high and moderate penetrance, more than 90 common germline genetic variants contributing to breast cancer risk have been identified, comprising about 37% of the familial relative risk of the disease (Michailidou et al. 2013, 2015). This suggests that a substantial portion of inherited variation has not yet been identified. In addition, most of the known common susceptibility variants reside in non-coding regions and result in subtle regulation of gene expression. The biological mechanisms through which genetic variants exert their functions are still not entirely understood.

The ability to evade immune destruction has been increasingly recognized as a key hallmark of tumors (Hanahan and Weinberg 2011). Tumor cells may secrete immunosuppressive factors like TGF-β which hampers infiltrating cytotoxic T lymphocytes and natural killer cells (Yang et al. 2010). Inflammatory cells like regulatory T cells (Treg cells), a subset of CD4+ T lymphocytes, as well as myeloid-derived suppressor cells (MDSCs) may be recruited into the tumor environment, which are actively immunosuppressive (Lindau et al. 2013; Reisfeld 2013). Higher prevalence of Treg cells has been found in various cancers (Chang et al. 2010; Michel et al. 2008; Watanabe et al. 2002), including breast cancer (Bates et al. 2006). There is evidence that tumor infiltrating Treg cells endowed with immunosuppressive potential are associated with tumor progression and unfavorable prognosis, especially in estrogen receptor (ER)-negative breast cancer (Bates et al. 2006; Kim et al. 2013; Liu et al. 2012a). In addition, infiltrating MDSCs were also found in murine mammary tumor models (Aliper et al. 2014; Gad et al. 2014), but their relevance for breast cancer patients also in terms of prognosis is not well-understood. Furthermore, previous association studies have identified susceptibility alleles for breast cancer in two genes, TGFBR2 (transforming growth factor beta receptor II) (Michailidou et al. 2013) and CCND1 (cyclin D1) (French et al. 2013), which may be involved in immune regulation in cancer patients (Gabrilovich and Nagaraj 2009; Krieg and Boyman 2009), including those with breast cancer. We hypothesized that immunosuppression pathway genes, particularly those relevant to Treg cell and MDSC functions, may harbor further susceptibility variants associated with breast cancer tumorigenesis, with a possible differential association by ER status.

In this analysis, we investigated associations between breast cancer risk and single nucleotide polymorphisms (SNPs) in 133 candidate genes in the immunosuppression pathway in individual level data from the Breast Cancer Association Consortium (BCAC). We also assessed associations with breast cancer risk at the gene and pathway...
levels. Furthermore, we used publicly available datasets through the UCSC Genome Browser (2015) to examine the putative genetic susceptibility loci for potential regulatory function.

**Materials and methods**

**Study participants**

In this analysis, participants were restricted to 83,087 women of European ancestry from 37 case–control studies participating in BCAC, including 42,510 invasive breast cancer cases with stage I–III disease and 40,577 cancer-free controls. Of all breast cancer patients, 26,094 were known to have ER-positive disease and 6870 to have ER-negative disease. Details of included studies are summarized in Online Resource 1. All studies were approved by the relevant ethics committees and all participants gave informed consent (Michailidou et al. 2013).

**Candidate gene selection**

Candidate genes relevant to the Treg cell and MDSC pathways were identified through a comprehensive literature review in PubMed (DeNardo et al. 2010; DeNardo and Coussens 2007; Driessens et al. 2009; Gabrilovich and Nagaraj 2009; Krieg and Boyman 2009; Mills 2004; Ostrand-Rosenberg 2008; Poschke et al. 2011; Sakaguchi et al. 2013; Sica et al. 2008; Wilczynski and Duechler 2010; Zitvogel et al. 2006; Zou 2005), using the search terms “immunosuppression”/“immunosuppressive”, “regulatory T cells”/“Treg cells”/“FOXP3+ T cells”, “myeloid derived suppressor cells”/“MDSCs”, “immunosurveillance”, and “tumor escape”. The final candidate gene list included 133 immunosuppression-related genes (Online Resource 2). SNPs within 50 kb upstream and downstream of each gene were identified using HapMap CEU genotype data (2015) and dbSNP 126.

**SNP association analyses**

For the BCAC studies, genotyping was carried out using a custom Illumina iSelect array (iCOGS) designed for the Collaborative Oncological Gene-Environment Study (COGS) project (Michailidou et al. 2013). Of the 211,155 SNPs on the array, 4246 were located within 50 kb of the selected candidate genes. Centralized quality control of genotype data led to the exclusion of 651 SNPs. The exclusion criteria included a call rate less than 95 % in all samples genotyped with iCOGS, minor allele frequency (MAF) less than 0.05 in all samples, evidence of deviation from Hardy–Weinberg equilibrium (HWE) at $p$ value $<10^{-7}$, and concordance in duplicate samples less than 98 % (Michailidou et al. 2013). A total of 3595 SNPs passed all quality controls and was analyzed.
Per-allele associations with the number of minor alleles were assessed using multiple logistic regression models, adjusted for study, age (at diagnosis for cases or at recruitment for controls) and nine principal components (PCs) derived based on genotyped variants to account for European population substructure. We assessed the associations of SNPs with overall breast cancer risk as primary analyses, and then restricted to ER-positive (26,094 cases and 40,577 controls) and ER-negative subtypes (6870 cases and 40,577 controls) as secondary analyses. Differences in the associations between ER-positive and ER-negative diseases were assessed by case-only analyses, using ER status as the dependent variable. To determine the number of “independent” SNPs for adjustment of multiple testing, we applied the option “–indep-pairwise” in PLINK (Purcell et al. 2007). SNPs were pruned by linkage disequilibrium (LD) of $r^2 < 0.2$ for a window size of 50 SNPs and step size of 10 SNPs, yielding 689 “independent” SNPs. The significance threshold using Bonferroni correction corresponding to an alpha of 5% was $7.3 \times 10^{-5}$.

In order to identify more strongly associated variants, genotypes were imputed for SNPs at the locus for which strongest evidence of association was observed, via a two-stage procedure involving SHAPEIT (Howie et al. 2012) and IMPUTEv2 (Howie et al. 2009), using the 1000 Genomes Project data as the reference panel (Abecasis et al. 2012). Details of the imputation procedure are described elsewhere (Michailidou et al. 2015). Models assessing associations with imputed SNPs were adjusted for 16 PCs based on 1000 Genome imputed data to further improve adjustment for population stratification. To determine independent signals within imputed SNPs at STAT3, we ran a stepwise forward multiple logistic regression model including the most significant genotyped SNP rs1905339 and all imputed SNPs, adjusted for study, age and 16 PCs.

Gene-level and pathway association analyses

Gene-level associations were determined by a subset of PCs, which were derived from a linear combination of SNPs in each gene explaining 80% of the variation in the joint distribution of all relevant SNPs. Associations with derived PCs were assessed within a logistic regression framework (Biernacka et al. 2012), for overall breast cancer, ER-positive and ER-negative diseases, respectively. Pathway association of the immunosuppression pathway was assessed based on a global test of association by combining the gene-level $p$ values via the Gamma method (Biernacka et al. 2012). For gene-level associations, associations with $p$ value $<3.8 \times 10^{-4}$ (Bonferroni correction) were considered statistically significant. To gain empirical $p$ values for gene-level associations of $TGFBR2$ and $CCND1$ as well as for the pathway association, a Monte Carlo procedure was used with up to 1,000,000 randomizations (Biernacka et al. 2012). An exact binomial test based on the results of the single SNPs association analyses was carried out to estimate enrichment of association in the immunosuppression pathway. Gene-level and pathway association analyses were carried out in R (version 3.1.1) using the package ‘GSAgm’ version 1.0.

Haplotype analyses

To follow up the interesting gene associations observed, haplotype analyses were performed to identify potential susceptibility variants. Haplotype frequencies were determined with the use of the estimation maximization (EM) algorithm (Long et al. 1995) implemented in PROC HAPLOTYPE in SAS 9.3 (Cary, NC, USA). Haplotypes with frequency more or equal than 1% were examined and the most common haplotype was used as the reference. Rare haplotypes with frequency less than 1% were grouped into one category. Haplotype-specific odds ratios
(ORs) and 95% confidence intervals (CIs) were estimated within a multiple logistic regression framework, adjusted for the same covariates as in the single SNP association analyses. Global \( p \) values for association of haplotypes with breast cancer risk were computed using a likelihood ratio test comparing models with and without haplotypes of the gene of interest.

**Gene expression analyses**

In order to examine whether potential causative genes influence RNA expression in breast tumor tissue, we downloaded RNA sequence level 3 data from The Cancer Genome Atlas (TCGA) (2015). We retrieved the RNA expression level as the form of RNA-Seq by expectation–maximization (RSEM) based on the IlluminaHiSeq_RNASeqV2 array. Gene expression differences in RNA levels between 989 invasive breast cancer tissues and 113 matched normal tissues for four genes of interest (\( STAT3, PTRF, IL5, \) and \( GM-CSF \)) were analyzed using a two-sided Wilcoxon–Mann–Whiney test. In addition, data from 183 breast tissues in the GTEx (V6) (2015) publically available online databases were evaluated to obtain information on whether the most interesting variants (\( rs1905339, rs8074296, rs146170568, \) chr17:40607850:1 and \( rs77942990 \)) were expression quantitative trait loci (eQTL) for any gene. Also, GTEx was queried to obtain information on whether the five variants were eQTL for \( STAT3 \) or \( PTRF \).

**Functional annotation**

To investigate potential regulatory functions of interesting polymorphisms, we used the Encyclopedia of DNA Elements (ENCOD\( E \)) database through the UCSC Genome Browser as well as Haploreg v4 (Ward and Kellis 2012).

**Results**

Selected characteristics of the study population are described in Table 1. The controls and breast cancer patients included in this study had comparable mean reference ages of 54.8 and 55.9 years and also the proportion of postmenopausal women was similar (68% in controls and 69% in breast cancer patients). The proportion of women indicating a family history of breast cancer in first degree relatives was as expected greater in breast cancer patients (25%) than in controls (12%).

**Single SNP associations**

Excluding the known \( TGFBR2 \) and \( CCND1 \) breast cancer susceptibility loci, the quantile–quantile (QQ) plot for associations with overall breast cancer risk for the genotyped SNPs of the other candidate genes indicated deviation from expected \( p \) values and thus evidence of further SNPs associated with breast cancer risk (Online Resource 3). Genetic associations with overall breast cancer risk for all assessed 3595 SNPs are summarized in Online Resource 4.

Four independent genotyped SNPs (\( LD^2 < 0.3 \) were significantly associated with breast cancer risk at \( p \) value \(< 7.3 \times 10^{-5} \), accounting for the multiple comparisons (Table 2). The four significant SNPs were located in or near \( TGFBR2, STAT3 \) and \( CCND1 \). Since \( TGFBR2 \) and

| Characteristic                      | Controls | Cases |
|-------------------------------------|----------|-------|
| No. | % | No. | % |
|-------------------------------------|----------|-------|
| Total number                        | 40,577   | 42,510|
| Age (mean, SD)                      | 54.8     | 12.0  | 55.9 | 11.6 |
| Family history of breast cancer     |          |       |
| No                                   | 20,940   | 88    | 24,397| 75  |
| Yes                                  | 2829     | 12    | 7971  | 25  |
| Unknown/missing                      | 16,808   |       | 10,142|     |
| Menopausal status                   |          |       |
| Pre/perimenopausal                  | 9174     | 32    | 9296  | 31  |
| Postmenopausal                      | 19,753   | 68    | 20,714| 69  |
| Unknown/missing                     | 11,650   |       | 12,500|     |
| Estrogen receptor status            |          |       |
| Negative                            | 6870     | 21    |       |
| Positive                            | 26,094   | 79    |       |
| Unknown/missing                     | 9546     |       |       |
| Progesterone receptor status        |          |       |
| Negative                            | 9299     | 33    |       |
| Positive                            | 19,017   | 67    |       |
| Unknown/missing                     | 14,194   |       |       |
| Triple-negative cancer              |          |       |
| No                                  | 13,675   | 84    |       |
| Yes                                 | 2600     | 16    |       |
| Unknown/missing                     | 26,235   |       |       |
| Stage                               |          |       |
| 0                                   | 25       | 0.1   |       |
| I                                   | 12,044   | 50    |       |
| II                                  | 9711     | 40    |       |
| III                                 | 1975     | 8     |       |
| IV                                  | 496      | 2     |       |
| Unknown/missing                     | 18,259   |       |       |
| Grade                               |          |       |
| Well differentiated                 | 6125     | 21    |       |
| Moderately differentiated           | 14,092   | 48    |       |
| Poorly/un-differentiated            | 8937     | 31    |       |
| Unknown/missing                     | 13,356   |       |       |

SD standard deviation
Table 2 TGFBR2, CCND1 and STAT3 SNPs associated with overall breast cancer risk in women of European ancestry after Bonferroni correction (p value < 7.3 × 10^{-5})

| SNP          | Chr. | Position* | Gene     | Minor allele | MAF cases | MAF controls | Cases    | Controls | OR (95 % CI)** | p value |
|--------------|------|-----------|----------|--------------|-----------|--------------|----------|----------|----------------|---------|
| rs13431131   | 3    | 30,675,880| TGFBR2   | A            | 0.37      | 0.36         | 42,508   | 40,574   | 1.06 (1.04–1.08) | 2.6 × 10^{-8}|
| rs11924422   | 3    | 30,677,484| TGFBR2   | C            | 0.40      | 0.41         | 42,491   | 40,572   | 0.95 (0.94–0.97) | 6.9 × 10^{-6}|
| rs7177       | 11   | 69,466,115| CCND1    | C            | 0.46      | 0.47         | 42,411   | 40,486   | 0.96 (0.94–0.98) | 2.7 × 10^{-5}|
| rs1905339    | 17   | 40,582,296| STAT3    | G            | 0.34      | 0.33         | 42,504   | 40,576   | 1.05 (1.03–1.08) | 1.4 × 10^{-6}|

SNP: single nucleotide polymorphism, Chr: chromosome, MAF: minor allele frequency, OR: odds ratio, CI: confidence interval, TGFBR2: transforming growth factor beta receptor II, CCND1: cyclin D1, STAT3: signal transducer and activator of transcription 3

* Build 37
** OR per minor allele, adjusted for age, study and nine European principal components

CCND1 have been identified as breast cancer susceptibility loci in previous studies (French et al. 2013; Michailidou et al. 2013; Rhie et al. 2013), we focused on the association of the SNP at STAT3. The variant rs1905339 (A>G) at STAT3 was positively associated with overall breast cancer risk (per allele odds ratio (OR) 1.05, 95 % confidence interval (CI) 1.03–1.08, p value = 1.4 × 10^{-5}). It showed similar associations with ER-positive and ER-negative cancers (Online Resource 5). We did not observe further SNPs that were significantly associated with ER-positive or ER-negative disease (data not shown).

To identify additional susceptibility variants at STAT3, we further investigated 707 SNPs that spanned a ±50 kb window around STAT3. Seven independent signals at STAT3 were found through the stepwise forward selection procedure. The genotyped SNP rs1905339 was not selected. The imputed SNP rs8074296 (A>G), which was in high LD with rs1905339 (r^2 = 0.99), showed a comparable OR for the association with overall breast cancer risk with a more extreme p value (per allele OR 1.05, 95 % CI 1.03–1.08, p value = 8.6 × 10^{-7}, Table 3). A second imputed SNP rs146170568 (C>T), associated with a per allele OR of 1.32 (95 % CI 1.16–1.50, p value = 2.1 × 10^{-5}), was still strongly associated at a p value of 3.2 × 10^{-4} after accounting for rs8074296 (Table 3). None of the independently associated imputed SNPs besides rs8074296 were correlated with rs1905339 or with each other (r^2 ≤ 0.01, Fig. 1). As rs8074296 and rs1905339 are located closer to PTRF than to STAT3, we additionally analyzed data of 178 imputed variants located within ±50 kb of PTRF. Associations of most additional variants in the PTRF region with breast cancer risk were attenuated in analyses conditioning on rs8074296 (Table 4). The variants chr17:40607850:I and rs77942990 still showed a strong association with breast cancer risk (per allele OR 1.16–1.50, p value = 2.3 × 10^{-5})

Table 3 Associations with overall breast cancer risk for seven independent imputed SNPs at STAT3 in women of European ancestry

| SNP          | Chr. | Position* | Counted allele | AFb | Cases | Controls | Single SNP analysis | Conditional analysis | OR (95 % CI)** | p value |
|--------------|------|-----------|----------------|-----|-------|----------|--------------------|---------------------|----------------|---------|
| rs8074296    | 17   | 40,583,421| G              | 0.336| 42,510| 40,577   | 1.05 (1.03–1.08)   | 0.01                | 1.05 (1.03–1.07) | 2.3 × 10^{-5}|
| rs146170568  | 17   | 40,517,716| T              | 0.005| 42,510| 40,577   | 1.32 (1.16–1.50)   | 0.16                | 1.27 (1.11–1.44) | 3.2 × 10^{-4}|
| rs141732716  | 17   | 40,469,832| A              | 0.005| 42,510| 40,577   | 1.38 (1.14–1.68)   | 0.001               | 1.33 (1.09–1.62) | 0.004   |
| rs138391971  | 17   | 40,505,106| G              | 0.003| 42,510| 40,577   | 0.60 (0.43–0.83)   | 0.002               | 0.61 (0.44–0.85) | 0.003   |
| rs12952342   | 17   | 40,553,640| G              | 0.119| 42,510| 40,577   | 1.07 (1.03–1.12)   | 0.002               | 1.07 (1.02–1.11) | 0.005   |
| rs190765034  | 17   | 40,428,622| G              | 0.026| 42,510| 40,577   | 1.14 (1.03–1.25)   | 0.010               | 1.17 (1.06–1.29) | 0.002   |
| rs190137766  | 17   | 40,422,371| T              | 0.002| 42,510| 40,577   | 0.68 (0.50–0.94)   | 0.018               | 0.66 (0.48–0.90) | 0.009   |

SNP: single nucleotide polymorphism, Chr: chromosome, OR: odds ratio, CI: confidence interval, STAT3: signal transducer and activator of transcription 3

* Build 37
b OR per minor allele, adjusted for age, study and 16 European principal components
c Each SNP was tested adjusting for rs8074296, age, study and 16 European principal components. Estimate for rs8074296 is based on model including rs146170568

SNP rs146170568 (C>T), associated with a per allele OR of 1.32 (95 % CI 1.16–1.50, p value = 2.1 × 10^{-5}), was still strongly associated at a p value of 3.2 × 10^{-4} after accounting for rs8074296 (Table 3). None of the independently associated imputed SNPs besides rs8074296 were correlated with rs1905339 or with each other (r^2 ≤ 0.01, Fig. 1). As rs8074296 and rs1905339 are located closer to PTRF than to STAT3, we additionally analyzed data of 178 imputed variants located within ±50 kb of PTRF. Associations of most additional variants in the PTRF region with breast cancer risk were attenuated in analyses conditioning on rs8074296 (Table 4). The variants chr17:40607850:I and rs77942990 still showed a strong association with breast cancer risk (per allele OR 1.16–1.50, p value = 2.3 × 10^{-5})
and 0.07, respectively) while all other variants in Table 4 were at least in moderate LD with rs8074296 \( (r^2 \geq 0.46) \) and independent of the other six imputed SNPs \( (r^2 \leq 0.01) \) at \( STAT3 \). LD was estimated based on control data.

Fig. 1 Linkage disequilibrium plot showing \( r^2 \) values and color schemes for the genotyped SNP rs1905339 and seven independent imputed SNPs as well as imputed SNP rs18188151 within ±50 kb of \( STAT3 \). The linkage disequilibrium (LD) plot shows that SNP rs1905339 is in strong LD with the imputed SNP rs8074296 \( (r^2 = 0.99) \), and independent of the other six imputed SNPs \( (r^2 \leq 0.01) \) at \( STAT3 \). LD was estimated based on control data.

Gene-level and pathway associations

Gene-level associations with risks of overall breast cancer, ER-positive and ER-negative diseases, respectively, for the 133 candidate genes in the immunosuppression pathway are summarized in Online Resource 17. \( TGFBR2 \) and \( CCND1 \) showed significant associations with overall breast cancer risk (\( p \) value \(<10^{-6} \) and \( 3.0 \times 10^{-4} \), respectively). In addition, \( IL5 \) and \( GM-CSF \) may be further potential susceptibility loci of breast cancer (\( p \) value = \( 1.0 \times 10^{-3} \) and \( 7.0 \times 10^{-3} \), respectively). \( STAT3 \) showed a less significant association with overall breast cancer risk (\( p \) value = 0.033). The immunosuppression pathway as a whole yielded a significant association with overall breast cancer risk (\( p \) value \(<10^{-6} \)). Similar gene-level and pathway associations were found for ER-positive but not for ER-negative breast cancer (Online Resource 17). We found significant enrichment of association in the immunosuppression pathway based on the results of the single SNPs association analyses (313 of 3595 tests significant at \( \alpha = 0.05 \), exact binomial test \( p \) value = \( 2.2 \times 10^{-16} \)).

Haplotype analyses

Despite the evidence for a possible role of \( IL5 \) and \( GM-CSF \) in breast cancer susceptibility from the gene-level analysis, no individual SNPs at \( IL5 \) or \( GM-CSF \) yielded significant genetic associations. To identify potential susceptibility haplotypes, haplotype-specific associations were assessed based on seven SNPs in or near \( IL5 \) (rs4143832, rs2079103, rs20706399, rs743562, rs739719, rs2069812 and rs2244012) and nine SNPs in or near \( GM-CSF \) (rs11575022, rs2069616, rs25881, rs25882, rs25883, rs27349, rs27438, rs40401 and rs743564). The LD structures for these SNPs at \( IL5 \) and \( GM-CSF \) are shown in Online Resource 18 and 19, respectively. In our study sample of women of European ancestry, 11 and 7 common haplotypes with frequency \( >1 \% \) were observed at \( IL5 \) and \( GM-CSF \), respectively. The haplotype AAAACGG in \( IL5 \) was associated with a decreased overall breast cancer risk (\( OR = 0.96, 95 \% CI 0.93–0.99 \) \( p \) value = \( 5.0 \times 10^{-3} \), Table 5). In \( GM-CSF \), the haplotype AAGAGCGAA was
### Table 4: Associations with overall breast cancer risk for 19 imputed variants near PTRF in women of European ancestry

| SNP | Chr | Position | Counted allele | Cases | Controls | Single SNP analysis | Conditional analysis | p value | p value |
|-----|-----|----------|----------------|-------|----------|--------------------|----------------------|---------|---------|
|     |     |          |                 |       |          | OR (95 % CI)       | OR (95 % CI)         |         |         |
| rs8074296 | 17 | 40,583,421 | G       | 0.336 | 42,510   | 40,577     | 1.05 (1.03–1.08) | 8.6 $\times$ 10^{-7} | 1.04 (1.02–1.06) | 0.0006 |
| rs1032070 | 17 | 40,618,251 | T       | 0.269 | 42,510   | 40,577     | 1.06 (1.04–1.09) | 1.5 $\times$ 10^{-7} | 1.04 (1.00–1.09) | 0.0359 |
| rs34460267 | 17 | 40,615,865 | C       | 0.269 | 42,510   | 40,577     | 1.06 (1.04–1.09) | 1.9 $\times$ 10^{-7} | 1.04 (1.00–1.09) | 0.0424 |
| rs34807589 | 17 | 40,624,656 | T       | 0.264 | 42,510   | 40,577     | 1.06 (1.04–1.09) | 2.0 $\times$ 10^{-7} | 1.04 (1.00–1.09) | 0.0423 |
| rs36005199 | 17 | 40,597,555 | G       | 0.268 | 42,510   | 40,577     | 1.06 (1.04–1.09) | 2.1 $\times$ 10^{-7} | 1.04 (1.00–1.09) | 0.0490 |
| rs12603201 | 17 | 40,595,927 | T       | 0.581 | 42,510   | 40,577     | 0.95 (0.93–0.97) | 3.1 $\times$ 10^{-7} | 0.97 (0.93–1.00) | 0.0662 |
| chr17:40607850:I | 17 | 40,607,850 | CT      | 0.055 | 42,510   | 40,577     | 1.13 (1.07–1.18) | 7.0 $\times$ 10^{-7} | 1.09 (1.04–1.15) | 0.0005 |
| rs4796662 | 17 | 40,594,882 | C       | 0.576 | 42,510   | 40,577     | 0.95 (0.93–0.97) | 1.8 $\times$ 10^{-6} | 0.98 (0.94–1.01) | 0.2217 |
| rs34349578 | 17 | 40,598,129 | A       | 0.195 | 42,510   | 40,577     | 1.07 (1.04–1.10) | 2.1 $\times$ 10^{-6} | 1.04 (1.00–1.08) | 0.0809 |
| rs62075801 | 17 | 40,593,921 | T       | 0.576 | 42,510   | 40,577     | 0.95 (0.93–0.97) | 2.1 $\times$ 10^{-6} | 0.98 (0.94–1.01) | 0.2385 |
| rs12951640 | 17 | 40,594,298 | A       | 0.253 | 42,510   | 40,577     | 1.06 (1.03–1.08) | 2.1 $\times$ 10^{-6} | 1.03 (0.98–1.07) | 0.2269 |
| rs77942990 | 17 | 40,622,538 | A       | 0.046 | 42,510   | 40,577     | 1.13 (1.07–1.19) | 2.2 $\times$ 10^{-6} | 1.09 (1.04–1.15) | 0.0007 |
| rs35111218 | 17 | 40,595,572 | T       | 0.252 | 42,510   | 40,577     | 1.06 (1.03–1.08) | 2.3 $\times$ 10^{-6} | 1.03 (0.98–1.07) | 0.2311 |
| rs650704 | 17 | 40,592,253 | A       | 0.253 | 42,510   | 40,577     | 1.06 (1.03–1.08) | 2.3 $\times$ 10^{-6} | 1.03 (0.98–1.07) | 0.2413 |
| rs12943498 | 17 | 40,593,901 | C       | 0.253 | 42,510   | 40,577     | 1.06 (1.03–1.08) | 2.5 $\times$ 10^{-6} | 1.02 (0.98–1.07) | 0.2529 |
| rs12951549 | 17 | 40,593,502 | T       | 0.253 | 42,510   | 40,577     | 1.06 (1.03–1.08) | 2.6 $\times$ 10^{-6} | 1.02 (0.98–1.07) | 0.2537 |
| chr17:40593802:I | 17 | 40,593,802 | GTTTC   | 0.251 | 42,510   | 40,577     | 1.06 (1.03–1.08) | 3.5 $\times$ 10^{-6} | 1.02 (0.98–1.07) | 0.2943 |
| rs6503703 | 17 | 40,592,207 | T       | 0.261 | 42,510   | 40,577     | 1.06 (1.03–1.08) | 6.5 $\times$ 10^{-6} | 1.02 (0.98–1.06) | 0.3775 |
| chr17:40595896:D | 17 | 40,595,896 | C       | 0.211 | 42,510   | 40,577     | 1.06 (1.03–1.09) | 9.0 $\times$ 10^{-6} | 1.02 (0.98–1.07) | 0.2373 |

SNP: single nucleotide polymorphism, Chr: chromosome, OR: odds ratio, CI: confidence interval, STAT3: signal transducer and activator of transcription 3

*| Build 37 |
| Allele frequency (AF) of counted allele |
| OR per counted allele, adjusted for age, study and 16 European principal components |
| Each SNP was tested adjusting for rs8074296, age, study and 16 European principal components. Estimate for rs8074296 was based on model including chr17:40607850:I |

Fig. 2: Regional association plot for the genotyped SNP rs1905339 and 885 imputed SNPs within ±50 kb of STAT3 and PTRF. Each dot represents an SNP. The color of each dot reflects the extent of linkage disequilibrium ($r^2$) with SNP rs1032070 (in purple diamond). Genomic positions of SNPs were plotted based on hg19/1000 Genomes Mar 2012 European. Association is represented at the $-\log_{10}$ scale. cM/Mb: centiMorgans/megabase.

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also associated with a decreased overall breast cancer risk (OR 0.92, 95% CI 0.87–0.96, \( p \) value = \( 2.7 \times 10^{-4} \), Table 6). The global \( p \) value for haplotype association was significant for both \( IL5 \) (\( p \) value = 0.005) and \( GM-CSF \) (\( p \) value = 0.007).

### Gene expression analyses

Using TCGA RNA sequencing level 3 data, we found that RNA expression levels of \( STAT3 \) and \( IL5 \) were significantly higher in 113 normal tissue samples compared to 989 breast tumor samples (\( p \) value = \( 1.3 \times 10^{-3} \) and \( 7.0 \times 10^{-4} \), respectively, Online Resources 20 and 21), while overall expression of \( IL5 \) was low in both tissues. Also expression levels of \( PTRF \) were significantly higher in normal tissue compared to tumor tissue samples (\( p \) value ≤ 0.0001, Online Resource 22). \( GM-CSF \) expression was very low and did not differ between breast tumor samples and normal tissue samples (\( p \) value = 0.49, Online Resource 23). Among 183 mammary tissues in the GTEx database, SNPs rs1905339, rs8074296 and rs77942990 were not significantly correlated with \( STAT3 \) (\( p \) values = 0.36, 0.36, and 0.2, respectively; Online Resource 24 to 26) or \( PTRF \) expression (\( p \) values = 0.4, 0.4, and 0.39 Online Resource 27 to 29). The SNPs rs1905339 and rs8074296 were significant eQTL for \( TUBG2 \) (both \( p \) values = \( 9.9 \times 10^{-7} \), Online Resource 30 and 31). The \( STAT3/PTRF \) variants rs146170568 and chr17:40607850:I were not available in the GTEx database.

### Discussion

Our comprehensive examination of associations between polymorphisms in the immunosuppression pathway genes and breast cancer risk revealed that \( STAT3 \), \( IL5 \), and \( GM-CSF \) may play a role in overall breast cancer susceptibility among women of European ancestry.

The in silico functional analysis revealed that within a ±50 kb window of \( STAT3 \), several polymorphisms are located in regulatory regions that could actively affect DNA transcription (Fig. 3). The SNP rs181888151, which is in complete LD with rs146170568 (\( r^2 = 1 \)) but independent of rs1905339 (\( r^2 = 0.01 \), Fig. 1) was significantly associated with increased risk for overall breast cancer (per allele OR 1.31, 95% CI 1.16–1.49, \( p \) value = \( 2.8 \times 10^{-5} \)). Together with a further independently associated imputed SNP rs141732716, these polymorphisms reside in strong DNase I hypersensitivity and transcription regulatory sites (Fig. 3). This suggests that they may be functional polymorphisms, but further experimental work is required for confirmation.

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**Table 5** Haplotype associations with overall breast cancer risk for seven SNPs at \( IL5 \) in women of European ancestry

| Haplotype | Frequency | OR (95% CI) | \( p \) value |
|-----------|-----------|-------------|---------------|
| C C G G C G A | 0.42 | 1.00 | 0.62 |
| C C A A C A A | 0.42 | 0.96 (0.93–0.99) | 0.005 |
| A A C G G A A | 0.42 | 0.96 (0.93–0.99) | 0.005 |
| C C G G C G G | 0.42 | 0.96 (0.93–0.99) | 0.005 |
| A A C A A A A | 0.42 | 0.96 (0.93–0.99) | 0.005 |
| C C G G C A A | 0.42 | 0.96 (0.93–0.99) | 0.005 |
| C C A A C G A | 0.42 | 0.96 (0.93–0.99) | 0.005 |
| A A C G C A G | 0.42 | 0.96 (0.93–0.99) | 0.005 |
STAT3 encodes the signal transducer and activator of transcription 3, which is a member of the STAT protein family. Activated by corresponding cytokines or growth factors, STAT3 can be phosphorylated and translocate into the cell nucleus, acting as a transcription activator. In addition, STAT3 plays a key role in regulating immune response in the tumor microenvironment (Yu et al. 2009). STAT3 signaling is required for immunosuppressive and tumor-promoting functions of MDSCs (Cheng et al. 2003, 2008; Kortylewski et al. 2005, 2009; Kujawski et al. 2008; Ostrand-Rosenberg and Sinha 2009; Yu et al. 2009), as well as for Treg cell expansion (Kortylewski et al. 2005, 2009; Matsumura et al. 2007).

STAT3 has been reported in several previous genome-wide association studies (GWAS) to be associated with immune relevant diseases such as Crohn’s disease (Barrett et al. 2008; Franke et al. 2008; Yamazaki et al. 2013), inflammatory bowel disease (Jostins et al. 2012), and multiple sclerosis (Jakkula et al. 2010; Patsopoulos et al. 2011; Sawcer et al. 2011). Additionally, expression of STAT3 was suggested to be enriched in triple-negative breast cancer, and negatively associated with lymph node involvement and breast tumor stage in a study based on an in silico network approach (Liu et al. 2012b). However, the association of rs1905339 with triple-negative breast cancer risk in our study (N triple-negative breast cancer = 2600) was similar and not stronger compared to the association observed for overall breast cancer risk (per allele OR 1.06, 95% CI 0.99–1.14, p value = 0.11).

The genotyped SNP rs1905339 is also located at 7 kb 5' of PTRF, which encodes the polymerase I and transcript release factor, and is not known to be directly involved in immunosuppression. In addition, two independently associated imputed SNPs rs8074296 and rs12952342 (r² = 0.99 and 0 with rs1905339, respectively, Fig. 1) are located at 8 kb 5' and 0.8 kb 3' of PTRF, respectively (Fig. 3). PTRF is known to contribute to the formation of caveolae, small membrane caves involved in cell signaling, lipid regulation, and endocytosis (Chadda and Mayor 2008). Recently, down-regulation of PTRF was observed in breast cancer cell lines and breast tumor tissue, suggesting that PTRF expression might be an indicator for breast cancer progression (Bai et al. 2012). The SNPs rs1905339 and rs8074296 were also found to be eQTL for TUBG2 (tubulin, gamma 2) in the GTEx database, the expression of TUBG2 decreased with each variant allele (Online Resources 30 and 31, respectively). TUBG2 encodes γ-tubulin, a protein required for the formation and polar orientation of microtubules in cells. It is currently unknown, whether TUBG2 plays a role in breast cancer development or progression.

The other two potential susceptibility loci, IL5 and GM-CSF, are both located in a known cytokine gene cluster at 5q31. IL5 encodes interleukin 5, a cytokine secreted by CD4+ T helper 2 cells (Mills 2004; Parker 1993). IL5 is a
with breast cancer susceptibility. The strongest candidates variation in the immunosuppression pathway is associated Overall, our data provide strong evidence that common }

Conclusions

Overall, our data provide strong evidence that common variation in the immunosuppression pathway is associated with breast cancer susceptibility. The strongest candidates for mediating this association were STAT3, ILS, and GM-CSF, but we cannot exclude the possibility of multiple alleles each with effects too small to confirm.

Acknowledgments We thank all the individuals who took part in these studies and all the researchers, clinicians, technicians, and administrative staff who have enabled this work to be carried out. This analysis would not have been possible without the contributions of the following: Per Hall (COGS); Douglas F. Easton, Paul Pharoah, Kyriaki Michaelidou, Manjee K. Bolla, Qin Wang (BCAC), Andrew Berchuck (OCAC), Rosalind A. Eeles, Douglas F. Easton, Ali Amin Ol Alama, Zsofia Kote-Jarai, Sara Benlloch (PRACTICAL), Georgia Chenevix-Trench, Antonis Antoniou, Lesley Mcguflf, Fergus Couch and Ken Orift (CIMBA), Joe Dennis, Alison M. Dunning, Andrew Lee, and Ed Dicks, Craig Luccarini and the staff of the Centre for Genetic Epidemiology Laboratory, Javier Benitez, Anna Gonzalez-Neira and the staff of the CNIO genotyping unit, Jacques Simard and Daniel C. Tessier, Francois Bacot, Daniel Vincent, Sylvie LaBoissière and Frederic Robidoux and the staff of the McGill University and Genome Quebec Innovation Centre, Stig E. Bojesen, Sune F. Nielsen, Borge G. Nordestgaard, and the staff of the Copenhagen DNA Laboratory, and Julie M. Cunningham, Sharon A. Windelbank, Christopher A. Hilker, Jeffrey Meyer and the staff of Mayo Clinic Genotyping Core Facility. ABCFS would like to thank Maggie Angelakos, Judi Marshell, and Gillian Dite. ABCS would like to thank Sanquin Amsterdam, the Netherlands. BBCS thanks Eileen Williams, Elaine Ryder-Mills, and Karol Sargs. BIGGS thanks Niall Mcnernery, Gabrielle Colleran, Andrew Rowan, and Angela Jones. BSUCH would like to thank Peter Bugert and Medical Faculty Mannheim. CGPS thanks Staff and participants of the Copenhagen General Population Study, as well as excellent technical assistance from Dorthe Uldall Andersen, Maria Birna Arnadottir, Anne Bank, and Dorthe kjeldgard Hansen. CNIO-BCS would like to thank Guillermo Pita, Charo Alonso, Daniel Herrero, Nuria Alvarez, Pilar Zamora, Primitiva Menendez, and the Human Genotyping-CGEN Unit. CTS would like to thank the CTS Steering Committee including Leslie Bernstein, Susan Neuhusen, James Lacey, Sophia Wang, Huiyan Ma, Yani Lu, and Jessica Clague Dehart at the Beckham Research Institute of City of Hope, Dennis Deapen, Rich Pinder, Eunjuung Lee, and Fred Schumacher at the University of Southern California, Pam Horn-Ross, Peggy Reynolds, Christina Clarke Dur and David Nelson at the Cancer Prevention Institute of California, and Hoda Anton-Culver, Argyros Ziogas, and Hannah Park at the University of California Irvine. ESTHER thanks Hartwig Ziegler, Christa Stegmaier, Sonja Wolf, and Volker Hermann. GC-HBOC thanks Stefanie Engert, Heide Helebrand, and Sandra Kober. GENICA would like to thank the GENICA Network, including Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, and University of Tuebingen, Germany (HB, Wing-Yee Lo,
Christina Justenhoven), German Cancer Consortium (DKTK) and Deutsches Krebsforschungszentrum (DKFZ) (HB), Department of Internal Medicine, Evangelische Kliniken Bonn gGmbH, Johanniter Krankenhaus, Bonn, Germany (Yon-Dschun Ko, Christian Baisch), Institute of Pathology, University of Bonn, Germany (Hans-Peter Fischer), Molecular Genetics of Breast Cancer, DKFZ, Heidelberg, Germany (UH), Institute for Prevention and Occupational Medicine of the German Social Accident Insurance, Institute of the Ruhr University Bochum (IPA), Bochum, Germany (Thomas Brüning, Beate Pesch, Sylvia Rabstein, Anne Lotz), and Institute of Occupational Medicine and Maritime Medicine, University Medical Center Hamburg-Eppendorf, Germany (Volker Harth).

HEBCS would like to thank Kirstimari Aaltonen, Karl von Smitten, Sofia Khan, Tuomas Heikkinen, and Irya Erkkili. HMBCS would like to thank Peter Hillemans, Hans Christiansen, and Johann H. Karstens.

NBHS would like to thank study participants and research staff for their contributions and commitment to this study. OBCS thanks Meeri Otsuka and Kari Mononen. OFBCR thanks Teresa Selander and Nayana Weerasooriya. PBCS thanks Louise Brinton, Mark Sherman, Neolina Szeszenia-Dabrowska, Beata Peplonska, Witold Zatonski, Pei Chao, and Michael Stagner. SASBAC would like to thank the Swedish Medical Research Council. SBCS would like to thank Sue Higham, Helen Cramp, Ian Brock, Sabapathy Balasubramanian, and Dan Connelly. SEARCH thanks the SEARCH and EPIC teams. SKKDKFZS thanks all study participants, clinicians, family doctors, researchers and technicians for their contributions and commitment to this study. TNBCC thanks Robert Pilaris and Charles Shapiro who were instrumental in the formation of the OSU Breast Cancer Tissue Bank, and also thanks the Human Genetics Sample Bank for processing of samples and providing OSU Columbus area control samples. UKBGS would like to thank Breast Cancer Now and the Institute of Cancer Research for support and funding of the Breakthrough Generations Study, and the study participants, study staff, and the doctors, nurses and other health care providers and health information sources who have contributed to the study, and acknowledge the NHS funding to the Royal Marsden/ICR NIHR Biomedical Research Centre. kConFab/AOCS wish to thank Heather Thorne, Eveline Niedermayr, all the kConFab 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 NHRMC, the National Breast Cancer Foundation, Cancer Australia, and the National Institute of Health (USA)) for their contributions to this resource, and many families who contribute to kConFab.

pKARMA would like to thank the Swedish Medical Research Council.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Financial supports Funding for the iCOGS infrastructure came from: the European Community’s Seventh Framework Programme under grant agreement number 223175 (HEALTH-F2-2009-223175) (COGS), Cancer Research UK (C1287/A10118, C1287/A10710, C12292/A11174, C1281/A12014, C5047/A8384, C5047/A15007, C5047/A10962, C8197/A16565), the National Institutes of Health (NIH, CA128978, CA122443) and Post-Cancer GWAS initiative (C12292/A10710, C1287/A10118, C1287/A12014) and by the European Community’s Seventh Framework Programme under grant agreement number 223175 (grant number HEALTH-F2-2009-223175) (COGS). The ABCFS study was supported by grant UM1 CA164920 from 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. BCAC is funded by Cancer Research UK (C1287/A10118, C1287/A12014) and by the European Community’s Seventh Framework Programme under grant agreement number 223175 (grant number HEALTH-F2-2009-223175) (COGS). The ABCFS study was supported by grant UM1 CA164920 from 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. BCAC is funded by Cancer Research UK (C1287/A10118, C1287/A12014) and by the European Community’s Seventh Framework Programme under grant agreement number 223175 (grant number HEALTH-F2-2009-223175) (COGS). The ABCFS study was supported by grant UM1 CA164920 from 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. BCAC is funded by Cancer Research UK (C1287/A10118, C1287/A12014) and by the European Community’s Seventh Framework Programme under grant agreement number 223175 (grant number HEALTH-F2-2009-223175) (COGS). The ABCFS study was supported by grant UM1 CA164920 from 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. BCAC is funded by Cancer Research UK (C1287/A10118, C1287/A12014) and by the European Community’s Seventh Framework Programme under grant agreement number 223175 (grant number HEALTH-F2-2009-223175) (COGS). The ABCFS study was supported by grant UM1 CA164920 from 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. BCAC is funded by Cancer Research UK (C1287/A10118, C1287/A12014) and by the European Community’s Seventh Framework Programme under grant agreement number 223175 (grant number HEALTH-F2-2009-223175) (COGS). The ABCFS study was supported by grant UM1 CA164920 from 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. BCAC is funded by Cancer Research UK (C1287/A10118, C1287/A12014) and by the European Community’s Seventh Framework Programme under grant agreement number 223175 (grant number HEALTH-F2-2009-223175) (COGS). The ABCFS study was supported by grant UM1 CA164920 from 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.
BioMolecular resources Research Infrastructure—Netherlands (BBMRI-NL), which is a Research Infrastructure financed by the Dutch government (NWO 184.021.007). The work of the BBBC was partly funded by ELAN-Fond of the University Hospital of Erlangen. The BBCS study was funded by Cancer Research UK and Breakthrough Breast Cancer and acknowledges National Health Service (NHS) funding to the National Institute for Health Research (NIHR) Biomedical Research Centre, and the National Cancer Research Network (NCRN). The BIGGS study was supported by NIHR Comprehensive Biomedical Research Centre, Guy’s & St. Thomas’ NHS Foundation Trust in partnership with King’s College London, United Kingdom. It was supported by the Oxford Biomedical Research Centre. The BSUCH study was supported by the Dietmar-Hopp Foundation, the Helmholtz Society and the Deutsches Krebsforschungszentrum (DKFZ). The CECILE study was funded by Fondation de France, Institut National du Cancer (INCa), Ligue Nationale contre le Cancer, Ligue contre le Cancer Grand Ouest, Agence Nationale de Sécurité Sanitaire (ANSES), Agence Nationale de la Recherche (ANR). The CGPS study was supported by the Chief Scientist Johan Boserup and Lise Boserup Fund, the Danish Medical Research Council and Herlev Hospital. The CNIO-BCS study was supported by the Instituto de Salud Carlos III, the Red Temática de Investigación Cooperativa en Cáncer and grants from the Asociación Española Contra el Cáncer and the Fondo de Investigación Sanitario (PI11/00923 and PI12/00070). The CTS study was initially supported by the California Breast Cancer Act of 1993 and the California Breast Cancer Research Fund (contract 97-10500) and is currently funded through the NIH (R01 CA77398). Collection of cancer incidence data (GLOBOCAN 2012) was supported by the California Department of Public Health as part of the statewide cancer reporting program mandated by California Health and Safety Code Sect. 103885. HAC received support from the Lon V Smith Foundation (LVS39420). The ESTHER study was supported by a grant from the Baden Württemberg Ministry of Science, Research and Arts. Additional cases were recruited in the context of the VERDI study, which was supported by a grant from the German Cancer Aid (Deutsche Krebshilfe). The GC-HBC study was supported by the German Cancer Aid (grant no 110837, coordinator: Rita K. Schmutzler). The GENICA study was funded by the Federal Ministry of Education and Research (BMBF) Germany grants 01KW99755/5, 01KWW9768, 01KWW9770 and 01KWW114, the Robert Bosch Foundation, Stuttgart, DKFZ, Heidelberg, the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance, Institute of the Ruhr University Bochum (IPA), Bochum, as well as the Department of Internal Medicine, Evangelische Kliniken Bonn gGmbH, Johanniter Krankenhaus, Bonn, Germany. The HEBCS study was financially supported by the Helsinki University Central Hospital Research Fund, Academy of Finland (266528), the Finnish Cancer Society, the Nordic Cancer Union and the Sigrid Juselius Foundation. The HMBCS study was supported by a grant from the Friends of Hannover Medical School and by the Rudolf Bartling Foundation. The KBCP study was financially supported by the special Government Funding (EVO) of Kuopio University Hospital grants, Cancer Fund of North Savo, the Finnish Cancer Organizations, and by the strategic funding of the University of Eastern Finland. The LMBC study was supported by the ‘Stichting tegen Kanker’ (232-2008 and 196-2010). The MARIE study was supported by the Deutsche Krebshilfe e.V. (70-2892-BR 1, 106332, 108253, 108419), the Hamburg Cancer Society. DKFZ and the Federal Ministry of Education and Research (BMBF) Germany (01KH0402). The MBCSG study was supported by grants from the Italian Association for Cancer Research (AIRC) and by funds from the 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 “5 x 1000”). The MCBCS study was supported by the NIH grants CA128978, CA116167, CA176785 and NIH Specialized Program of Research Excellence (SPORE) in Breast Cancer (CA116201), and the Breast Cancer Research Foundation and a generous gift from the David F. and Margaret T. Grohne Family Foundation and the Ting Tsung and Wei Fong Chao Foundation. The MCCS cohort recruitment was funded by VicHealth and Cancer Council Victoria. This study was further supported by Australian NHMRC grants 209057, 251553 and 504711 and by infrastructure provided by Cancer Council Victoria. Cases and their vital status were ascertained through the Victorian Cancer Registry (VCR) and the Australian Institute of Health and Welfare (AIHW), including the National Death Index. The MEC study was supported by NIH grants CA63464, CA54281, CA98758 and CA132839. The work of MTLGBCMS was supported by the Quebec Breast Cancer Foundation, the Canadian Institutes of Health Research (CIHR) for the “CIHR Team in Familial Risks of Breast Cancer” program—grant # CRN-87521 and the Ministry of Economic Development, Innovation and Export Trade—grant # PSR-SIRI-701. The NBCS study has received funding from the K.G. Jebsen Centre for Breast Cancer Research, the Research Council of Norway grant 193387/V50 (to A-L Børresen-Dale and V.N. Kristensen) and grant 193387/H10 (to A-L Børresen-Dale and V.N. Kristensen), South Eastern Norway Health Authority (grant 39346 to A-L Børresen-Dale) and the Norwegian Cancer Society (to A-L Børresen-Dale and V.N. Kristensen). The NBHS study was supported by NIH grant R01CA100374. Biological sample preparation was conducted the Survey and Biospecimen Shared Resource, which is supported by P30 CA68485. The OBBS study was supported by research grants from the Finnish Cancer Foundation, the Academy of Finland (grant number 250083, 122715 and Center of Excellence grant number 251314), the Finnish Cancer Foundation, the Sigrid Juselius Foundation, the University of Oulu, the University of Oulu Support Foundation and the special Governmental EVO funds for Oulu University Hospital-based research activities. The OFBRC study was supported by grant UM1 CA164920 from the National Cancer Institute (USA). The PBS study was funded by Intramural Research Funds of the National Cancer Institute, Department of Health and Human Services, USA. The SASBAC study was supported by funding from the Agency for Science, Technology and Research of Singapore (A*STAR), the US National Institute of Health and the Susan G. Komen Breast Cancer Foundation. The SBCS study was supported by Yorkshire Cancer Research S295, S299, S305PA and Sheffield Experimental Cancer Medicine Centre. The SEARCH study was funded by a programme grant from Cancer Research UK (CA490/ A1004424) and supported by the UK National Institute for Health Research Biomedical Research Centre at the University of Cambridge. The SKKDKFZS study was supported by the DKFZ. The SZBCS study was supported by Polish State Committee for Scientific Research Grant PBZ, KBN, 122/P05/2004. The TNBCC study was supported by: a Specialized Program of Research Excellence (SPORE) in Breast Cancer (CA116201), a grant from the Breast Cancer Research Foundation, a generous gift from the David F. and Margaret T. Grohne Family Foundation, the Stefanie Spielman Breast Cancer fund and the OSU Comprehensive Cancer Center, the Helmer Cooperative Oncology Group research grant (HR R_BG/04) and the Greek General Secretary for Research and Technology (GSRT) Program, Research Excellence II, 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)—ARISTEIA. The UKBOGS study was funded by Breast Cancer Now and the Institute of Cancer Research (ICR), London. ICR acknowledged NHS funding to the NIHR Biomedical Research Centre. The kConFab study 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. Financial support for the
AOCs was provided by the United States Army Medical Research and Materiel Command (DAMD17-01-1-0729), Cancer Council Victoria, Queensland Cancer Fund, Cancer Council New South Wales, Cancer Council South Australia, the Cancer Foundation of Western Australia, Cancer Council Tasmania and the National Health and Medical Research Council of Australia (NHMRC; 400413, 400281, 199600). The pKARMA study was supported by Märit and Hans Rausings Initiative Against Breast Cancer.

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