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Journal

Human Mutation, 39(5)

ISSN

1059-7794

Authors

Colombo, Mara
López-Perolio, Irene
Meeks, Huong D
[et al.](#)

Publication Date

2018-05-01

DOI

10.1002/humu.23411

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Peer reviewed

The *BRCA2* c.68-7T > A variant is not pathogenic: A model for clinical calibration of spliceogenicity

Mara Colombo^{1*}  | Irene Lòpez-Perolio^{2*} | Huong D. Meeks³ | Laura Caleca¹ | Michael T. Parsons⁴ | Hongyan Li³ | Giovanna De Vecchi¹ | Emma Tudini⁴ | Claudia Foglia¹ | Patrizia Mondini¹ | Siranoush Manoukian⁵ | Raquel Behar² | Encarna B. Gómez Garcia⁶ | Alfons Meindl⁷ | Marco Montagna⁸ | Dieter Niederacher⁹ | Ane Y. Schmidt¹⁰ | Liliana Varesco¹¹ | Barbara Wappenschmidt^{12,13} | Manjeet K. Bolla¹⁴ | Joe Dennis¹⁴ | Kyriaki Michailidou^{14,15} | Qin Wang¹⁴ | Kristiina Aittomäki¹⁶ | Irene L. Andrulis^{17,18} | Hoda Anton-Culver¹⁹ | Volker Arndt²⁰ | Matthias W. Beckmann²¹ | Alicia Beeghly-Fadel²² | Javier Benitez^{23,24} | Bram Boeckx^{25,26} | Natalia V. Bogdanova^{27,28,29} | Stig E. Bojesen^{30,31,32} | Bernardo Bonanni³³ | Hiltrud Brauch^{34,35,36} | Hermann Brenner^{20,36,37} | Barbara Burwinkel^{38,39} | Jenny Chang-Claude^{40,41} | Don M. Conroy⁴² | Fergus J. Couch⁴³ | Angela Cox⁴⁴ | Simon S. Cross⁴⁵ | Kamila Czene⁴⁶ | Peter Devilee^{47,48} | Thilo Dörk²⁸ | Mikael Eriksson⁴⁶ | Peter A. Fasching^{21,49} | Jonine Figueroa^{50,51} | Olivia Fletcher⁵² | Henrik Flyger⁵³ | Marike Gabrielson⁴⁶ | Montserrat García-Closas⁵¹ | Graham G. Giles^{54,55} | Anna González-Neira²³ | Pascal Guénel⁵⁶ | Christopher A. Haiman⁵⁷ | Per Hall⁴⁶ | Ute Hamann⁵⁸ | Mikael Hartman^{59,60} | Jan Hauke^{12,13,61} | Antoinette Hollestelle⁶² | John L. Hopper⁵⁵ | Anna Jakubowska⁶³ | Audrey Jung⁴⁰ | Veli-Matti Kosma^{64,65,66} | Diether Lambrechts^{25,26} | Loid Le Marchand⁶⁷ | Annika Lindblom⁶⁸ | Jan Lubinski⁶³ | Arto Mannermaa^{64,65,66} | Sara Margolin⁶⁹ | Hui Miao⁵⁹ | Roger L. Milne^{54,55} | Susan L. Neuhausen⁷⁰ | Heli Nevanlinna⁷¹ | Janet E. Olson⁷² | Paolo Peterlongo⁷³ | Julian Peto⁷⁴ | Katri Pylkäs^{75,76} | Elinor J. Sawyer⁷⁷ | Marjanka K. Schmidt^{78,79} | Rita K. Schmutzler^{12,13,61} | Andreas Schneeweiss^{38,80} | Minouk J. Schoemaker⁸¹ | Mee Hoong See⁸² | Melissa C. Southey⁸³ | Anthony Swerdlow^{81,84} | Soo H. Teo^{82,85} | Amanda E. Toland⁸⁶ | Ian Tomlinson⁸⁷ | Thérèse Truong⁵⁶ | Christi J. van Asperen⁸⁸ | Ans M.W. van den Ouweland⁸⁹ | Lizet E. van der Kolk⁹⁰ | Robert Winqvist^{75,76} |

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Drakoulis Yannoukakos⁹¹ | Wei Zheng²² | kConFab/AOCS Investigators⁹² | Alison M. Dunning⁴² | Douglas F. Easton^{14,42} | Alex Henderson⁹³ | Frans B.L. Hogervorst⁹⁰ | Louise Izatt⁹⁴ | Kenneth Offitt⁹⁵ | Lucy E. Side⁹⁶ | Elizabeth J. van Rensburg⁹⁷ | Study EMBRACE⁹⁸ | Study HEBON⁹⁹ | Lesley McGuffog¹⁰⁰ | Antonis C. Antoniou¹⁰⁰ | Georgia Chenevix-Trench⁴ | Amanda B. Spurdle⁴ | David E. Goldgar³ | Miguel de la Hoya^{2*} | Paolo Radice^{1*}

¹Unit of Molecular Bases of Genetic Risk and Genetic Testing, Department of Research, Fondazione IRCCS (Istituto di Ricovero e Cura a Carattere Scientifico) Istituto Nazionale dei Tumori (INT), Milan, Italy

²Molecular Oncology Laboratory CIBERONC, Hospital Clinico San Carlos, IdISSC (Instituto de Investigación Sanitaria del Hospital Clínico San Carlos), Madrid, Spain

³Huntsman Cancer Institute, University of Utah, Salt Lake City, Utah

⁴Department of Genetics and Computational Biology, QIMR Berghofer Medical Research Institute, Brisbane, QLD 4006, Australia

⁵Unit of Medical Genetics, Department of Medical Oncology and Hematology, Fondazione IRCCS (Istituto di Ricovero e Cura a Carattere Scientifico) Istituto Nazionale dei Tumori (INT), Milan, Italy

⁶Department of Clinical Genetics and GROW, School for Oncology and Developmental Biology, MUMC, Maastricht, The Netherlands

⁷Department of Obstetrics and Gynecology, University Hospital, LMU Munich, Germany

⁸Immunology and Molecular Oncology Unit, Veneto Institute of Oncology IOV - IRCCS, Padua, Italy

⁹Department of Gynaecology and Obstetrics, University Hospital Düsseldorf, Heinrich-Heine University, Duesseldorf, Germany

¹⁰Center for Genomic Medicine, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

¹¹Hereditary Cancer Unit, IRCCS AOU San Martino -IST, Genova, Italy

¹²Center for Hereditary Breast and Ovarian Cancer, University Hospital of Cologne, Cologne, Germany

¹³Center for Integrated Oncology (CIO), Medical Faculty, University Hospital of Cologne, Cologne, Germany

¹⁴Centre for Cancer Genetic Epidemiology, Department of Public Health and Primary Care, University of Cambridge, Cambridge, UK

¹⁵Department of Electron Microscopy/Molecular Pathology, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus

¹⁶Department of Clinical Genetics, Helsinki University Hospital, University of Helsinki, Helsinki, Finland

¹⁷Fred A. Litwin Center for Cancer Genetics, Lunenfeld-Tanenbaum Research Institute of Mount Sinai Hospital, Toronto, Ontario

¹⁸Department of Molecular Genetics, University of Toronto, Toronto, Canada

¹⁹Department of Epidemiology, University of California Irvine, Irvine, California

²⁰Division of Clinical Epidemiology and Aging Research, German Cancer Research Center (DKFZ), Heidelberg, Germany

²¹Department of Gynaecology and Obstetrics, University Hospital Erlangen, Friedrich-Alexander University Erlangen-Nuremberg, Comprehensive Cancer Center Erlangen-EMN, Erlangen, Germany

²²Division of Epidemiology, Department of Medicine, Vanderbilt Epidemiology Center, Vanderbilt-Ingram Cancer Center, Vanderbilt University School of Medicine, Nashville, Tennessee

²³Human Cancer Genetics Program, Spanish National Cancer Research Centre, Madrid, Spain

²⁴Centro de Investigación en Red de Enfermedades Raras (CIBERER), Valencia, Spain

²⁵VIB Center for Cancer Biology, VIB, Leuven, Belgium

²⁶Laboratory for Translational Genetics, Department of Human Genetics, University of Leuven, Leuven, Belgium

²⁷Department of Radiation Oncology, Hannover Medical School, Hannover, Germany

²⁸Gynaecology Research Unit, Hannover Medical School, Hannover, Germany

²⁹N.N. Alexandrov Research Institute of Oncology and Medical Radiology, Minsk, Belarus

³⁰Copenhagen General Population Study, Herlev and Gentofte Hospital, Copenhagen University Hospital, Herlev, Denmark

³¹Department of Clinical Biochemistry, Herlev and Gentofte Hospital, Copenhagen University Hospital, Herlev, Denmark

³²Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

³³Division of Cancer Prevention and Genetics, Istituto Europeo di Oncologia, Milan, Italy

³⁴Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany

³⁵University of Tübingen, Tübingen, Germany

³⁶German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Heidelberg, Germany

³⁷Division of Preventive Oncology, German Cancer Research Center (DKFZ) and National Center for Tumor Diseases (NCT), Heidelberg, Germany

³⁸Department of Obstetrics and Gynecology, University of Heidelberg, Heidelberg, Germany

³⁹Molecular Epidemiology Group, C080, German Cancer Research Center (DKFZ), Heidelberg, Germany

⁴⁰Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Heidelberg, Germany

- ⁴¹Research Group Genetic Cancer Epidemiology, University Cancer Center Hamburg (UCCH), University Medical Center Hamburg-Eppendorf, Hamburg, Germany
- ⁴²Centre for Cancer Genetic Epidemiology, Department of Oncology, University of Cambridge, Cambridge, UK
- ⁴³Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, New York
- ⁴⁴Sheffield Institute for Nucleic Acids (SINFoNiA), Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK
- ⁴⁵Academic Unit of Pathology, Department of Neuroscience, University of Sheffield, Sheffield, UK
- ⁴⁶Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden
- ⁴⁷Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands
- ⁴⁸Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands
- ⁴⁹David Geffen School of Medicine, Department of Medicine Division of Hematology and Oncology, University of California at Los Angeles, Los Angeles, California
- ⁵⁰Usher Institute of Population Health Sciences and Informatics, The University of Edinburgh Medical School, Edinburgh, UK
- ⁵¹Division of Cancer Epidemiology and Genetics, National Cancer Institute, Rockville, Maryland
- ⁵²The Breast Cancer Now Toby Robins Research Centre, The Institute of Cancer Research, London, UK
- ⁵³Department of Breast Surgery, Herlev and Gentofte Hospital, Copenhagen University Hospital, Herlev, Denmark
- ⁵⁴Cancer Epidemiology & Intelligence Division, Cancer Council Victoria, Melbourne, Australia
- ⁵⁵Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, The University of Melbourne, Melbourne, Australia
- ⁵⁶Cancer & Environment Group, Center for Research in Epidemiology and Population Health (CESP), INSERM, University Paris-Sud, University Paris-Saclay, Villejuif, France
- ⁵⁷Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, California
- ⁵⁸Molecular Genetics of Breast Cancer, Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany
- ⁵⁹Saw Swee Hock School of Public Health, National University of Singapore, Singapore, Singapore
- ⁶⁰Department of Surgery, National University Health System, Singapore, Singapore
- ⁶¹Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany
- ⁶²Department of Medical Oncology, Family Cancer Clinic, Erasmus MC Cancer Institute, Rotterdam, The Netherlands
- ⁶³Department of Genetics and Pathology, Pomeranian Medical University, Szczecin, Poland
- ⁶⁴Translational Cancer Research Area, University of Eastern Finland, Kuopio, Finland
- ⁶⁵Institute of Clinical Medicine, Pathology and Forensic Medicine, University of Eastern Finland, Kuopio, Finland
- ⁶⁶Imaging Center, Department of Clinical Pathology, Kuopio University Hospital, Kuopio, Finland
- ⁶⁷Epidemiology Program, University of Hawaii Cancer Center, Honolulu, Hawaii
- ⁶⁸Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden
- ⁶⁹Department of Clinical Science and Education Södersjukhuset, Karolinska Institutet, Stockholm, Sweden
- ⁷⁰Department of Population Sciences, Beckman Research Institute of City of Hope, Duarte, California
- ⁷¹Department of Obstetrics and Gynecology, Helsinki University Hospital/University of Helsinki, Helsinki, Finland
- ⁷²Department of Health Sciences Research, Mayo Clinic, Rochester, New York
- ⁷³IFOM, The FIRC (Italian Foundation for Cancer Research) Institute of Molecular Oncology, Milan, Italy
- ⁷⁴Department of Non-Communicable Disease Epidemiology, London School of Hygiene and Tropical Medicine, London, UK
- ⁷⁵Laboratory of Cancer Genetics and Tumor Biology, Cancer and Translational Medicine Research Unit, Biocenter Oulu, University of Oulu, Oulu, Finland
- ⁷⁶Laboratory of Cancer Genetics and Tumor Biology, Northern Finland Laboratory Centre Oulu, Oulu, Finland
- ⁷⁷Research Oncology, Guy's Hospital, King's College London, London, UK
- ⁷⁸Division of Molecular Pathology, The Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital, Amsterdam, The Netherlands
- ⁷⁹Division of Psychosocial Research and Epidemiology, The Netherlands Cancer Institute – Antoni van Leeuwenhoek hospital, Amsterdam, The Netherlands
- ⁸⁰National Center for Tumor Diseases, University of Heidelberg, Heidelberg, Germany
- ⁸¹Division of Genetics and Epidemiology, The Institute of Cancer Research, London, UK
- ⁸²Breast Cancer Research Unit, Cancer Research Institute/University Malaya Medical Centre, Kuala Lumpur, Malaysia
- ⁸³Department of Pathology, The University of Melbourne, Melbourne, Australia
- ⁸⁴Division of Breast Cancer Research, The Institute of Cancer Research, London, UK
- ⁸⁵Cancer Research Malaysia, Subang Jaya, Selangor, Malaysia
- ⁸⁶Department of Molecular Virology, Immunology and Medical Genetics, Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio
- ⁸⁷Wellcome Trust Centre for Human Genetics and Oxford NIHR Biomedical Research Centre, University of Oxford, Oxford, UK
- ⁸⁸Department of Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands
- ⁸⁹Department of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands
- ⁹⁰Family Cancer Clinic, The Netherlands Cancer Institute - Antoni van Leeuwenhoek hospital, Amsterdam, The Netherlands
- ⁹¹Molecular Diagnostics Laboratory, INRASTES, National Centre for Scientific Research “Demokritos”, Athens, Greece
- ⁹²Peter MacCallum Cancer Center, Melbourne, Australia
- ⁹³Institute of Genetic Medicine, Centre for Life, Newcastle Upon Tyne Hospitals NHS Trust, Newcastle upon Tyne, UK

⁹⁴Clinical Genetics, Guy's and St. Thomas' NHS Foundation Trust, London, UK

⁹⁵Clinical Genetics Research Laboratory, Dept. of Medicine, Cancer Biology and Genetics, Memorial Sloan-Kettering Cancer Center, New York, New York

⁹⁶Wessex Clinical Genetics Service, Mailpoint 627, Princess Anne Hospital, Coxford Road, Southampton, SO16 5YA

⁹⁷Cancer Genetics Laboratory, Department of Genetics, University of Pretoria, Pretoria, South Africa

⁹⁸Centre for Cancer Genetic Epidemiology, Department of Public Health and Primary Care, University of Cambridge Strangeways Research Laboratory, Worts Causeway, Cambridge, UK

⁹⁹The Hereditary Breast and Ovarian Cancer Research Group Netherlands (HEBON), Coordinating center: Netherlands Cancer Institute, Amsterdam, The Netherlands

¹⁰⁰Centre for Cancer Genetic Epidemiology, Department of Public Health and Primary Care, University of Cambridge, Cambridge, UK

Correspondence

Mara Colombo, Fondazione IRCCS Istituto Nazionale dei Tumori, Department of Research, Unit of Molecular Bases of Genetic Risk and Genetic Testing, Milan, IT.
Email: mara.colombo@istitutotumori.mi.it

Funding information

the NHMRC Senior Research Fellowship Scheme; Spanish Instituto de Salud Carlos III funding, an initiative of the Spanish Ministry of Economy and Innovation partially supported by European Regional Development FEDER Funds; Associazione Italiana per la Ricerca sul Cancro, Grant/Award Number: N° 15547 to P.R.; the Cancer Council Queensland; NHMRC Project grant scheme

*These authors contributed equally to the work.

Communicated by Dominique Stoppa-Lyonnet

Abstract

Although the spliceogenic nature of the *BRCA2* c.68-7T > A variant has been demonstrated, its association with cancer risk remains controversial. In this study, we accurately quantified by real-time PCR and digital PCR (dPCR), the *BRCA2* isoforms retaining or missing exon 3. In addition, the combined odds ratio for causality of the variant was estimated using genetic and clinical data, and its associated cancer risk was estimated by case-control analysis in 83,636 individuals. Co-occurrence in trans with pathogenic *BRCA2* variants was assessed in 5,382 families. Exon 3 exclusion rate was 4.5-fold higher in variant carriers (13%) than controls (3%), indicating an exclusion rate for the c.68-7T > A allele of approximately 20%. The posterior probability of pathogenicity was 7.44×10^{-115} . There was neither evidence for increased risk of breast cancer (OR 1.03; 95% CI 0.86–1.24) nor for a deleterious effect of the variant when co-occurring with pathogenic variants. Our data provide for the first time robust evidence of the nonpathogenicity of the *BRCA2* c.68-7T > A. Genetic and quantitative transcript analyses together inform the threshold for the ratio between functional and altered *BRCA2* isoforms compatible with normal cell function. These findings might be exploited to assess the relevance for cancer risk of other *BRCA2* spliceogenic variants.

KEYWORDS

BRCA2, digital PCR, multifactorial likelihood analysis, quantitative real-time PCR, spliceogenic variants

1 | INTRODUCTION

BRCA1 (MIM# 113705) and *BRCA2* (MIM# 600185) are tumor suppressor genes and their inactivation promotes cancer development. Carriers of germline pathogenic variants in these genes are at high risk of developing breast and ovarian cancers, and *BRCA1/2* gene testing has become a widely used procedure in the clinical management of families suspected of hereditary susceptibility to these malignancies. The individuals within these families, identified as at-risk based on their genetic profile, may benefit from risk-reduction options. However, the usefulness of genetic testing relies on the ability to ascertain the pathogenic nature of the identified genetic variants, which is not necessarily straightforward for small in-frame deletions and insertions, variants in regulatory sequences, missense, synonymous and intronic changes, and variants introducing premature protein-truncating codons at the 3' end of the coding sequence.

The Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) has developed and documented criteria aimed at determining the clinical significance of sequence variants in

BRCA genes (<https://www.enigmaconsortium.org>). The classification, based on a five-class system (Plon et al., 2008), is intended to differentiate high risk variants (risk equivalent to that of protein-truncating pathogenic variants), including pathogenic and likely pathogenic variants (class 5 and 4, respectively), from variants with low or no risk, including not pathogenic and likely not pathogenic variants (class 1 and 2, respectively). Variants for which clinical significance is unclear are placed in class 3 and are referred to as variants of uncertain significance (VUSs).

One controversial variant in *BRCA2* is c.68-7T > A, which lies upstream of the acceptor splice site of exon 3. This variant (rs81002830) has been reported in several populations worldwide with an allelic frequency ranging from 0.02% in East Asians to 0.5% in non-Finnish Europeans (Lek et al., 2016). Several authors have reported c.68-7T > A being spliceogenic, that is, able to alter normal premRNA splicing. In particular, using semiquantitative approaches, it has been documented that the variant leads to an increase of the naturally occurring transcripts lacking exon 3 ($\Delta 3$) (Houdayer et al., 2012; Jarhelle, Riise Stensland, Maehle, & Van Ghelue, 2016; Sanz

et al., 2010; They et al., 2011; Vreeswijk et al., 2009). A competitive quantitative PCR (qPCR) analysis estimated that the proportion of the $\Delta 3$ transcript compared to full length was approximately 25% in variant samples versus 4% in normal samples (Muller et al., 2011). More recently, segregation analyses in two families indicated that the variant did not segregate in the affected branches (Santos et al., 2014). Although a few of the above studies tentatively classified the variant as benign or likely benign, they do not provide robust genetic evidence to justify this conclusion. Conversely, a recent article asserted that the variant was associated with breast cancer, based on a relatively limited case control association study in the Norwegian population (Møller & Hovig, 2017).

As a consequence, to date the classification of c.68-7T > A reported in databases aggregating information on genomic variations has remained inconclusive. In particular, ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>, last updated: Feb 1, 2018) reports conflicting interpretations classifying the variant as benign (seven entries), likely benign (nine entries) and of uncertain significance (four entries). Moreover, the BIC (Breast Cancer Information Core, <https://research.nhgri.nih.gov/bic/>) database presently annotates the variant as of unknown clinical importance, pending classification, while the BRCA ShareTM (UMD-BRCA2 mutations database) (<https://www.umd.be/BRCA2/>) classifies it as likely benign.

In the present study, we combined genetic approaches, including a large multicentre case-control study and segregation analysis in a sizeable number of families, with qualitative and quantitative analyses of the transcripts, and Mitomycin C growth inhibition test. Our findings provide a robust classification of the *BRCA2* c.68-7T > A variant with respect to its effect on cancer risk, and add evidence that splicing pattern alterations do not necessarily lead to pathogenicity.

2 | MATERIALS AND METHODS

2.1 | Nomenclature

The nucleotide numbering was based on the reference *BRCA2* complementary deoxyribonucleic acid (cDNA) sequence NM_000059.3. For the purposes of the study, we defined as $\nabla 3$ all *BRCA2* isoforms retaining exon 3 and as $\Delta 3$ all *BRCA2* isoforms missing exon 3, irrespective of additional alternative splicing events.

2.2 | Cell lines

Epstein-Barr virus (EBV)-immortalized human lymphoblastoid cell lines (LCLs) were obtained as previously described (Colombo et al., 2013). In this analysis 18 LCLs were considered, including six LCLs obtained from women carrying the *BRCA2* c.68-7T > A variant and 12 LCLs obtained from healthy female blood donors, recruited at the Istituto Nazionale dei Tumori (INT) of Milan. The c.68-7T > A carriers had been screened in all coding exons and corresponding intron-exon junctions of both *BRCA1* and *BRCA2*. Excluding common polymorphisms, none of them carried additional *BRCA* gene variants, with a single exception where a protein-truncating variant was detected in *BRCA1*

(c.1380dupA). Only *BRCA2* exon 3 was sequenced in the LCLs from normal controls and no pathogenic variants or VUS were observed. The two *BRCA2*-deficient cell lines, EUFA423 immortalized fibroblasts (*BRCA2*^{7691insAT/9900insA}) (Howlett et al., 2002) and pancreatic cancer cell line Capan1 (*BRCA2*^{-/6174delT}) (Goggins et al., 1996) were cultured as described elsewhere (Feng et al., 2011).

2.3 | Cytoplasmic RNA isolation and first strand cDNA synthesis

Cytoplasmic RNA was isolated from fresh LCLs using the Cytoplasmic & Nuclear RNA Purification Kit (NORGEN BIOTEK CORPORATION, Canada), including the DNase I treatment according to manufacturer's instructions. The RNA purity and integrity was verified by measuring the A_{260}/A_{280} ratio and by electrophoresis on agarose gel. For capillary electrophoresis (CE), allele-specific expression analysis and reverse transcriptase quantitative PCR (RT-qPCR), first strand cDNA was generated using 1 μ g RNA, random hexamer primers and MaximaTM H Minus RT (Thermo Scientific), following the manufacturer's protocol in a final volume of 20 μ l. For digital PCR (dPCR), 1 μ g RNA was reverse transcribed with Prime-Script RT kit (TaKaRa Biotechnology, Japan) according to the manufacturer's protocol using a mixture of random and Oligo (dT) primers. No-RT controls, containing all reagents for the reverse transcription but the enzyme, were carried out.

2.4 | Capillary electrophoresis analysis

Multiplex fluorescently-labeled PCRs were performed with primers located upstream and downstream of exon 3, to simultaneously amplify both $\nabla 3$ and $\Delta 3$ transcripts, followed by CE analysis. A beta-2-microglobulin (*B2M*; MIM# 109700) cDNA fragment of 377 bp was co-amplified to normalize CE peaks and allow comparison between cases and controls. The sequences of the primers are listed in Supp. Table S1. PCR amplifications were performed in 20 μ l reaction volume containing 2 μ l of cDNA solution under end-point conditions. Cycling conditions were as follows: 95°C for 7 min, followed by 35 cycles at 95°C for 30'', 58°C for 30'' and 72°C for 30''. A final 7 min elongation step was performed at 72°C. The fluorescent amplification products were run on an ABI 3130 Genetic Analyzer (Applied Biosystems). GeneScanTM 500 ROXTM dye size standard was used as internal size-standard and size calling was performed with GeneMapper software v4.0 (Applied Biosystems).

2.5 | Assessment of allelic expression of $\nabla 3$ and $\Delta 3$ transcripts

The allelic origin of the $\nabla 3$ and $\Delta 3$ transcripts were ascertained by amplification and sequencing of the region containing the common c.-26G > A variant (rs1799943) in the 5'-UTR of *BRCA2*. PCR reactions were performed as described above. The forward primer was designed to anneal to a region upstream of c.-26G > A and the reverse primers to sequences in exon 3 and across the exon2-exon4 junction, specific of the $\nabla 3$ and $\Delta 3$ transcripts, respectively (Supp. Table S1).

Sequencing conditions were as previously described (Colombo et al., 2013).

2.6 | Quantitative PCR analysis

Specific quantitative assays were designed to capture the expression levels of the ∇ 3 and Δ 3 transcripts. The primer sets (Supp. Table S1) were validated with end-point PCR reactions, and the specificity of the amplification products were confirmed by sequencing.

The qPCR analysis were performed on the Eco real-time PCR system (Illumina) using SYBR[®] Green I dye chemistry (KAPA SYBR[®] FAST qPCR Kit, Kapa Biosystems). All reactions were carried out in a final volume of 10 μ l containing 1 μ l of cDNA and 200 nM of *GUSB* and ∇ 3 transcript specific primers, and 300 nM of Δ 3 transcript specific primers. The efficiency of qPCR assays was evaluated based on a relative standard curve, using threefold serial dilutions starting from pooled control cDNAs in triplicate. The thermal profile was the same for all assays (95°C for 3 min, followed by 40 cycles of 95°C for 3 sec and 62°C for 20 sec). The melting curve analysis was performed according to default conditions (95°C for 15 sec, 55°C for 15 sec and 95°C for 15 sec). All samples from both cases and controls were individually analyzed in triplicate, and the corresponding average values were considered. No template controls and no-RT controls were included in the analysis. The data, obtained in the form of quantification cycle (Cq), were normalized using the beta-glucuronidase gene (*GUSB*) (de Brouwer, van Bokhoven, & Kremer, 2006). The obtained values were used to compute, in both normal and mutated samples, *BRCA2* exon 3 exclusion rate, that is, the percentage of *BRCA2* mRNA isoforms missing exon 3 over the total amount of *BRCA2* transcripts, as follows:

$$[2^{-\Delta Cq_{\Delta 3}} / (2^{-\Delta Cq_{\Delta 3}} + 2^{-\Delta Cq_{\nabla 3}})] \times 100.$$

The distribution of transcript levels in control and mutant LCLs was calculated by normalization to that of pooled control cDNAs (reference sample) using the $\Delta\Delta Cq$ method (Livak & Schmittgen, 2001).

Statistical analysis was performed using GraphPad Prism software (version 5.02). The significance of the results was established using the F test.

2.7 | Digital PCR

The dPCR experiments were performed on a QuantStudio 3D dPCR 20K platform according to the manufacturer's instructions (Applied Biosystem, Foster City, CA). To detect *BRCA2* Δ 3 transcripts, we used a FAM-labeled custom designed TaqMan assay (Applied Biosystems) specific for the exon 2–4 junction (5'-CAAAGCAG-GAAGGAATG-3'). To detect ∇ 3 transcripts, we used a 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein labeled (VIC-labeled) predesigned TaqMan assay (Applied Biosystems, Hs00609076) specific for the exon 3–4 junction (5-AATTAGACTTAG-GAAGGAATGTTCC-3'). All relative quantification experiments were performed combining Δ 3 and ∇ 3 assays in individual chips. dPCR chips were analyzed in the QuantStudio 3D Analysis Suit Cloud software v2.0 (Applied Biosystem, Foster

City, CA), defining FAM as target. Default settings were used in all cases. After reviewing automatic assessment of the chip quality by the software, only green flag chips (data meet all quality thresholds, review of the analysis result not required) and yellow flag chips (data meet all quality thresholds, but manual inspection is recommended) were considered for further analyses. We used the target/total ratio, FAM/(FAM+VIC), calculated by the software as a proxy for *BRCA2* exon 3 exclusion rate. Different amounts of each sample were individually tested in 20K chips, but only data from the chip with the highest precision (according to the analysis software) was included in the expression analysis shown in Figure 3.

2.8 | Genotyping and sample sets

Direct genotyping of *BRCA2* c.68-7T > A was conducted as part of the Collaborative Oncological Gene-environment Study (COGS) detailed elsewhere (Michailidou et al., 2013). This study included genotype results from breast cancer cases and controls participating in the Breast Cancer Association Consortium (BCAC; <http://bcac.ccge.medschl.cam.ac.uk/>), and from the carriers of assumed pathogenic variants in *BRCA* genes, participating in the Consortium of Investigators of Modifiers of *BRCA1/2* (CIMBA; <http://cimba.ccge.medschl.cam.ac.uk/>). The BCAC and CIMBA datasets are described in de la Hoya et al., (2016). Information on breast tumor estrogen receptor and grade status were available for 189 variant carrier cases from BCAC. Via the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA; <https://enigmaconsortium.org/>) (Spurdle et al., 2012), we identified 16 families recruited through familial cancer clinics where at least one member tested positive for *BRCA2* c.68-7T > A, and test results (negative or positive) were available from at least one relative. All study participants had been previously enrolled into national or regional studies under ethically approved protocols.

2.9 | Statistical methods

The association of the *BRCA2* c.68-7T > A variant with breast cancer risk was evaluated in BCAC using logistic regression models, as previously detailed (de la Hoya et al., 2016).

In addition, multifactorial likelihood analysis was conducted as detailed in the Supp. Text. In brief, odds for causality were calculated based on carrier frequency and ages at diagnosis/interview in cases and controls, as previously described (Goldgar et al., 2004).

Bayes scores for segregation were derived as previously described (Thompson, Easton, & Goldgar, 2003).

Pathology likelihood ratios (LRs) were applied as indicated in Spurdle et al., (2014). The segregation scores, pathology LRs and case-control LRs are mutually independent and were combined to derive a combined odds for causality as described previously (Goldgar et al., 2004; Goldgar et al., 2008). Prior probability of pathogenicity was assigned based on predicted effect of the variant on splicing, as derived in Vallee et al., (2016). Variant classification was based on the IARC 5-tier scheme (Plon et al., 2008).

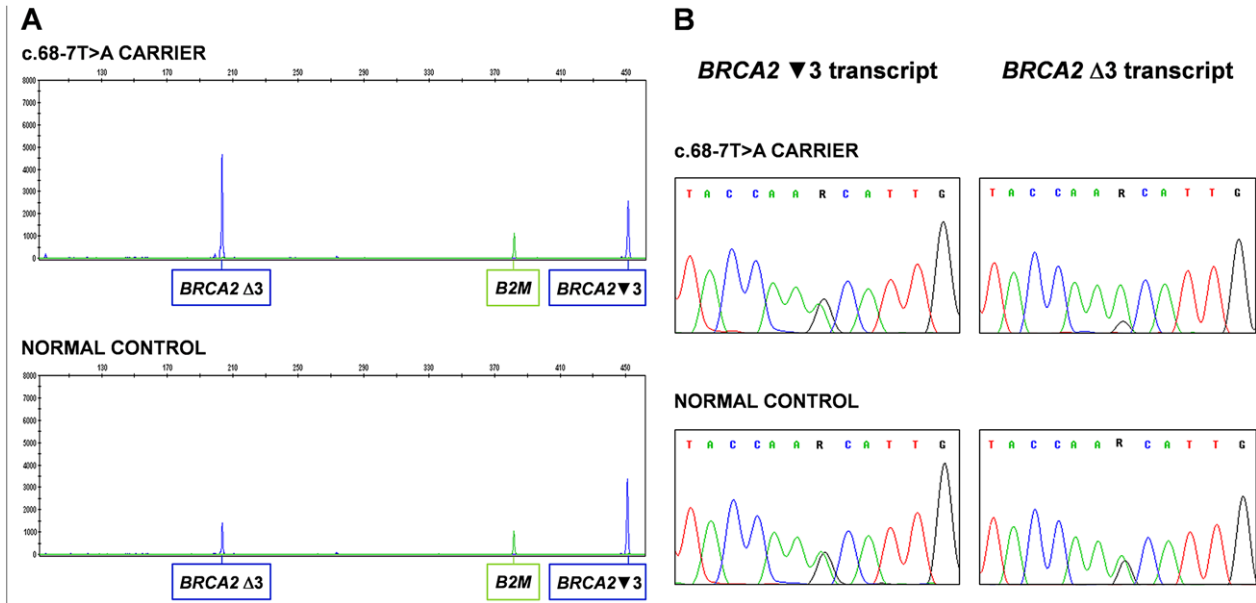


FIGURE 1 Evaluation of the effects of the *BRCA2* c.68-7T > A variant at mRNA level. (A) Capillary electrophoresis analysis of *BRCA2* cDNA showing the relative increase of $\Delta 3$ transcript and decrease of $\nabla 3$ transcript in c.68-7T > A carriers compared to normal controls *B2M* reference transcript. Since the PCR assays were performed under end-point conditions, the results of these assays were not used to quantify the fold-change of $\Delta 3$ versus $\nabla 3$ transcript ratio in cases compared to controls. (B) Assessment of allele-specific expression of the $\nabla 3$ and $\Delta 3$ transcripts in c.68-7T > A carriers and normal controls by analysis of the common c.-26G > A variant. The sequencing of the RT-PCR products obtained by selectively amplifying the $\nabla 3$ and $\Delta 3$ transcripts in separate reactions (left panels and right panels, respectively) shows that the variant allele, which is in linkage with the A allele of the common variant, retained the ability to synthesize the $\nabla 3$ transcript

2.10 | Mitomycin C (MMC) growth inhibition test and statistical analyses

A total of 3×10^6 cells/ml were seeded in triplicate in 25 ml flasks and grown for 72 hr in the absence or in the presence of MMC (Sigma-Aldrich) at a final concentration of 170 ng/ml. Percentage of viable cells was determined using trypan blue dye exclusion assay, following the manufacturer's instruction (Sigma-Aldrich). Statistical differences in cell viability after exposure to MMC compared to controls were determined by two-tailed Student *t*-test using GraphPad Prism software.

3 | RESULTS

3.1 | Transcript analyses

3.1.1 | Confirmation of $\Delta 3$ transcripts increase in variant carriers

The effect of the *BRCA2* c.68-7T > A variant at the mRNA level was evaluated by fluorescently-labeled end-point RT-PCR on cDNAs derived from six LCLs obtained from women carrying the investigated variant and from 12 nonvariant carrier females. The visual inspection of the CE outputs confirmed the increase of the $\Delta 3$ transcripts and the corresponding decrease of the $\nabla 3$ transcripts in variant carriers compared to controls (a representative example is shown in Fig. 1A), in agreement with previous studies (Houdayer et al., 2012; Jarhelle et al., 2016; Sanz et al., 2010; Thery et al., 2011; Vreeswijk et al., 2009).

The allelic-specific expression of both the $\nabla 3$ and $\Delta 3$ transcripts was assessed by investigating the c.-26G > A variant, verified to be in linkage with the c.68-7T > A, in heterozygous samples (five controls and three cases). Each transcript was selectively amplified in separate reactions and sequenced. Even considering that transcript quantification by sequencing analysis is not entirely accurate, it was apparent that, while in normal samples the levels of the $\Delta 3$ transcripts originating from the two alleles were comparable, in carriers the contribution of the variant allele was higher than that of the wild-type allele. In addition, it was also observed that in carriers the variant allele retained the ability to synthesize the $\nabla 3$ transcripts. A representative example is shown in Figure 1B.

3.1.2 | Quantitative mRNA analyses

To quantify the relative amount of *BRCA2* $\nabla 3$ and $\Delta 3$ transcripts in LCLs from both normal individuals ($n = 12$) and variant carriers ($n = 6$), a qPCR analysis was performed. The analysis showed a 3.1-fold increase in the relative level of $\Delta 3$ transcripts ($p < 10^{-4}$) in carriers (average 2.98; range 1.28–4.31) compared to controls (average 0.97; range 0.79–1.23) and a 0.5-fold not statistically significant ($p = 0.4$) decrease in the relative level of $\nabla 3$ transcripts in carriers (average 0.44; range 0.27–0.66) compared to controls (average 0.86; range 0.49–1.11), (Fig. 2).

The relative quantification of $\Delta 3$ and $\nabla 3$ transcripts in each sample allowed us to compare the exon 3 exclusion rates (see methods) in carriers and controls and to obtain a quantitative score reflecting the magnitude of the splicing alteration induced by the variant. The exclusion rate in LCLs carrying the variant allele was 5.2-fold higher

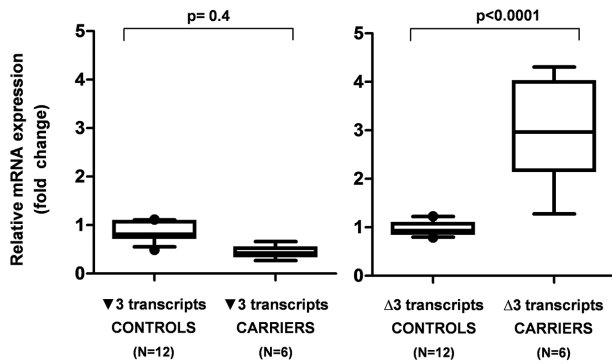


FIGURE 2 Relative expression of *BRCA2* ▼3 and Δ3 transcripts in six c.68-7T > A carriers and 12 normal controls by quantitative PCR. The boxplots (displaying low, Q1, median, Q3, and high values) show qPCR levels of ▼3 and Δ3 transcripts in carriers and controls. Values are normalized to *GUSB* mRNA and expressed as fold difference relative to pooled control cDNAs using the $\Delta\Delta Cq$ method (see Materials and Methods). The analysis shows in carriers a statistically significant increase of the relative level of Δ3 transcripts compared to controls (2.98 vs. 0.97; $p < 0.0001$). Conversely, the decrease observed in the relative level of ▼3 transcripts (0.44 vs. 0.86) is not statistically significant ($p = 0.4$)

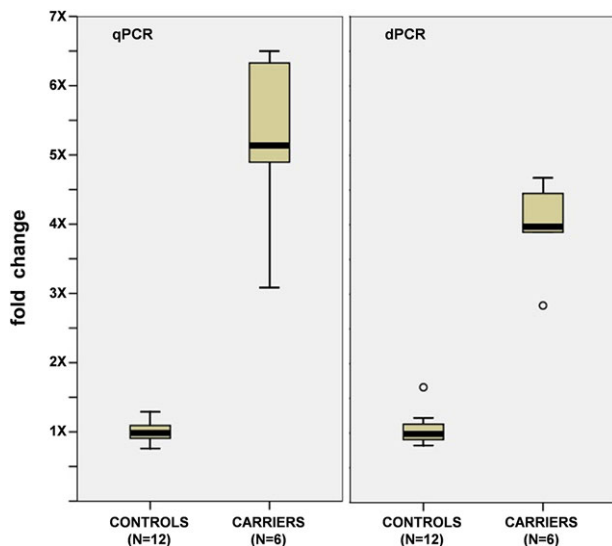


FIGURE 3 *BRCA2* exon 3 exclusion rate in LCLs from *BRCA2* c.68-7T > A carriers and controls. The boxplots (displaying low, Q1, median, Q3, and high values) show qPCR (left panel) and dPCR (right panel) measures of exclusion rate. The data is expressed as the fold-increase relative to the average of 12 controls. Outliers (> 1.5 inter quartile range, IQR) are displayed as small circles. On average, a 5.2-fold increase is observed in carriers according to qPCR data and a 4.2-fold increase according to dPCR data (3.8-fold increase if outliers are included in the analysis)

than in normal LCLs ($p = 3.9 \times 10^{-4}$) (Fig. 3), with an average exclusion rate of 12.4% (range 6.3%–16.0%) in carriers and 2.4% (range 1.8%–3.4%) in controls (Supp. Figure S1).

Subsequently, an independent dPCR-based quantitative analysis was performed to measure *BRCA2* exon 3 exclusion rates directly in the same sample set. After excluding two outliers, we found that the exclusion rate in LCLs carrying the variant allele (15.5%; range

14.4%–17.2%) was 4.2-fold higher than in normal LCLs (3.7%; range 3.0%–4.5%; $p < 10^{-4}$) (Fig. 3 and Supp. Figure S1).

3.2 | Genetic analyses

BRCA2 c.68-7T > A was identified in 242/41,890 (0.58%) invasive breast cancer cases and 216/41,746 (0.52%) controls of reported European ancestry recruited through BCAC studies. Standard case-control analysis adjusted for six principle components yielded an odds ratio (OR) of 1.03 (95% CI 0.86–1.24). However, some studies indicated that they had performed *BRCA1/2* mutation screening of cases and might have excluded cases with *BRCA1/2* VUS. This could have created a bias due to preferential exclusion of c.68-7T > A carrier cases but not controls. However, the OR was similar after exclusion of four studies that performed such genetic testing, (OR 1.09; 95% CI 0.89–1.33). The odds for causality based on carrier frequency and ages at diagnosis/interview in these cases and controls was 9.44×10^{-93} . There was also strong evidence against causality from segregation analysis (6.39×10^{-9}) and breast tumor pathology (2.40×10^{-14}). Considering all data together, and assigning prior probability of 0.34 based on splicing prediction, the posterior probability of pathogenicity was calculated to be 7.44×10^{-115} (see Supp. Text for further details).

3.3 | Co-occurrence of the c.68-7T > A with pathogenic variants

Overall 15 female individuals from 13 apparently unrelated families with clear evidence of the c.68-7T > A being in trans with a pathogenic variant in *BRCA2* were assessed. Thirteen individuals from 11 families were detected through the genotyping of the CIMBA sample set, one was reported via the ENIGMA consortium, and another one was ascertained at INT (Supp. Table S2). None of these cases was included in the RNA analyses described above.

3.4 | Evaluation of the effect of the *BRCA2* c.68-7T > A on cellular sensitivity to mitomycin C

Carriers of bi-allelic *BRCA2* inactivating variants are affected with Fanconi Anemia (FA), complementation group D1. FA is characterized by congenital defects, including anatomical abnormalities, congenital disabilities and increased risk of cancer, most often acute myelogenous leukemia (Auerbach, 2009). In addition, the cells of FA patients exhibit hypersensitivity to DNA interstrand cross-links (ICLs) caused by agents such as mitomycin C (MMC) (Godthelp et al., 2006). A breast cancer-affected woman, with no clinical signs of FA, was found by segregation analysis to carry the truncating *BRCA2* c.5722_5723delCT variant in trans with the c.68-7T > A variant (Supp. Table S2). To exclude an FA phenotype at the cellular level, we evaluated the sensitivity to MMC of an LCL derived from this patient. An LCL carrying one copy of the c.68-7T > A, without an additional *BRCA2* pathogenic variant or VUS (*BRCA2*^{wt/c.68-7T>A}), the MMC hypersensitive EUFA423 and Capan1 *BRCA2*-null cell lines and an LCL from a normal donor (*BRCA2*-proficient) were included in the assay as controls. The sensitivity to MMC was evaluated by comparing the viability of MMC-treated cells

with that of the untreated cells. As shown in Supp. Figure S2, EUFA423 (FA-D1) and Capan1 cells showed a significant decrease of the cellular viability ($p < 0.01$) after exposure to MMC, while no differences were observed in LCLs from normal donor and carriers of *BRCA2* c.68-7T > A, either in heterozygosity or in trans with the pathogenic variant.

4 | DISCUSSION

In the present study, we analyzed the *BRCA2* c.68-7T > A variant, located in the proximity of the acceptor site of exon 3, in order to establish its clinical relevance and association with breast cancer risk. In accordance with previous studies (Houdayer et al., 2012; Jarhelle et al., 2016; Sanz et al., 2010; They et al., 2011; Vreeswijk et al., 2009), we observed that this variant leads to a modest increased expression of the transcript lacking exon 3 ($\Delta 3$) in carriers compared to controls. Moreover, we found that in LCLs of carriers of the variant the exon 3 exclusion rate (i.e., the relative amount of *BRCA2* $\Delta 3$ transcripts) was approximately 4- to 5-fold higher than in LCLs of controls and the total amount of $\nabla 3$ transcripts in carriers was approximately 50% compared to controls. The latter finding would seem to contradict the observation that the variant allele maintains the ability to express a transcript coding for a normal (full-length) protein. The apparent discrepancy may be explained comparing the overall expression of *BRCA2* transcripts in cases and controls. In fact, summing up in each sample the amount of $\nabla 3$ and $\Delta 3$ transcripts assessed by qPCR, and setting as 1 the average expression of *BRCA2* mRNA observed in our cohort, we observed notable inter-individual variability (ranging from 0.43 to 1.50), with many control samples clustering above the average (Supp. Figure S3). Hence, it is very much possible that the strong reduction in the amount of $\nabla 3$ transcripts observed in carriers simply reflects random inter-individual variability in *BRCA2* gene expression levels.

Although the above findings were confirmed using two complementary assays (qPCR and dPCR), it must be noted that the outcomes of transcript quantification analyses may be influenced by the nature of examined biological material. Therefore, the magnitude of changes in transcript ratio associated with the c.68-7A > T should be verified also in samples other than LCLs.

The pathogenic implication of *BRCA2* exon 3 deletion has been long debated. Exon 3 is 249-bp long and its deletion does not alter the open reading frame. In addition, the $\Delta 3$ isoform has been described as one of the major naturally occurring alternatively splicing events in *BRCA2* (Fackenthal et al., 2016). However, the predicted protein product is expected to be lacking important functional activities. In fact, this exon codes for *BRCA2* amino acids 23 to 106, including the C-terminal portion of a primary transactivating domain (PAR, amino-acid residues 18–60) and an auxiliary transactivating domain (AAR, residues 60–105) (Milner, Ponder, Hughes-Davies, Seltmann, & Kouzarides, 1997), whose activity may be regulated by phosphorylation (Milner, Fuks, Hughes-Davies, & Kouzarides, 2000). Interestingly, the region spanning residues 21–39 mediates the interaction with PALB2, a nuclear protein that promotes the stable intranuclear localization and accumulation of *BRCA2*, making possible its DNA recombinational repair

and checkpoint functions, eliciting tumor suppression (Oliver, Swift, Lord, Ashworth, & Pearl, 2009; Xia et al., 2006). Moreover, the PALB2-binding site directly overlaps that of EMSY, another nuclear protein that has endogenous transcriptional repressor activity (Hughes-Davies et al., 2003).

Several *BRCA2* alterations causing the complete loss of exon 3 and the exclusive synthesis of $\Delta 3$ transcripts have been ascertained, including c.316 + 5G > C (Bonnet et al., 2008), c.316 + 3delA and c.68-925_316 + 2889del (Muller et al., 2011) and c.156_157insAlu, a variant reported as a founder Portuguese mutation (Peixoto et al., 2009).

The characterization of the above variants supports the hypothesis that the exclusive synthesis of the $\Delta 3$ transcripts from one allele has a pathogenic effect. On the contrary, the association with cancer risk of variants that, like the c.68-7T > A, increase the relative amount of $\Delta 3$ isoforms but maintain the ability of transcribe a full-length mRNA, is presently unclear. Indeed, the classification of the variants with incomplete effects at the transcript level represents an important and challenging question. According to current ENIGMA criteria, splicing variants leading to in-frame deletions, but maintaining the ability to produce mRNA transcript(s) predicted to encode intact full-length protein, cannot be assumed as pathogenic or likely pathogenic, even if targeting clinical relevant domains. Such alterations require further investigation to assess pathogenicity.

To address the issue, we performed a multifactorial-likelihood analysis combining the odds for causality derived from a large case-control study, using the datasets of BCAC, pathology likelihood based on breast tumor phenotype, and co-segregation data from ENIGMA. Overall, the posterior probability of c.68-7T > A being pathogenic was 7.44×10^{-115} . This value is well below the threshold established by ENIGMA for a *BRCA1/2* variant to be classified as class 1, that is, not pathogenic (probability of pathogenicity < 0.001), when considered against characteristics of the average truncating pathogenic variant. In addition, the confidence interval of the odds ratio estimate (OR 1.09; 95%CI 0.89–1.33) excludes even moderate breast cancer risk (Hollestelle, Wasielewski, Martens, & Schutte, 2010).

Additional evidence of the non-pathogenicity of c.68-7T > A was provided by the observation of its occurrence in trans with a *BRCA2* pathogenic variant in 15 unrelated individuals, including 13 from 11 of 5,284 families recruited by CIMBA and genotyped for the variant. If c.68-7T > A were pathogenic, the frequency of unrelated FA affected individuals among CIMBA *BRCA2* mutation carriers would be approximately 2.1 in 1,000, which is inconsistent with the frequency observed in the general population, that is, two to six in 1,000,000 (Bogliolo & Surrallés, 2015). Finally, no evidence of hypersensitivity to DNA ICL agents, a characteristic of FA patients, was detected in an LCL derived from one of the individuals carrying a pathogenic variant in trans with the c.68-7T > A. Together, these findings indicate that carriers of the *BRCA2* c.68-7T > A variant should not be counseled to undergo the clinical interventions recommended to carriers of high risk *BRCA* gene variants.

While the present article was under review, a study was published claiming that the *BRCA2* c.68-7T > A variant was associated with breast cancer (Møller & Hovig, 2017). This conclusion was based on

the detection of the variant in 17 out of 714 (2.4%; 95%CI 1.4%–3.8%) Norwegian unrelated breast cancer kindreds, a frequency significantly higher ($p < 0.0001$) compared to the prevalence of the variant in a sample of the Norwegian population ($3/1588 = 0.2\%$). Segregation data based on a single family was inconclusive (LR 0.36), and the estimate of prospective incidence rate in 24 variant carriers overlapped that for the general population. The authors concluded (assumedly based on their case-control findings alone) that carriers of the *BRCA2* c.68-7T > A variant have increased risk for breast cancer in families selected due to aggregation of breast cancer, and state in their discussion "...carriers of the variant should be informed that they probably have a clinically actionable pathogenic variant and referred to health care accordingly". We believe that the conclusion of Moller and Hovig (2017) is unjustified, and disagree with their recommendation on clinical action. Our much larger study (sample size 59x for cases and 26x for controls) including individuals from multiple different countries provide no evidence for increased risk of breast cancer in familial cases carrying this variant: the OR was 1.03 (95% CI 0.86–1.24) including all studies, and the risk estimate was nominally greater although not significantly different (OR 1.09, 95% CI 0.89–1.33) after excluding familial breast cancer cohorts.

The difference between the findings from our much larger case-control study and that of Møller & Hovig, (2017) need for caution when utilizing case-control data for clinical interpretation of rare variants, such that significant differences in frequency can nonetheless be unreliable due to random error and bias arising from small sample size, incomplete matching of cases and controls, and when considering familial cases, co-occurrence of (other) risk-related genetic factors as acknowledged by the authors themselves.

Different hypotheses, not necessarily mutually exclusive, can be proposed to explain the lack of pathogenicity of c.68-7T > A despite it being spliceogenic. First, the reduction in full-length *BRCA2* mRNAs in variant carriers compared to normal controls, which was not statistically significant, might not be enough to affect cellular tumor suppressor ability. Second, the $\Delta 3$ transcripts are predicted to lead to the synthesis of an unstable and nonfunctional protein product and, therefore, unlikely to interfere with the activity of the normal protein due to the loss of the PALB2 interaction domain, whose binding stabilizes the *BRCA2* protein (Xia et al., 2006). Assuming that in the examined samples, the overall *BRCA2* expression level from both alleles is similar, and that in carrier samples the accompanying normal alleles contribute on average an exclusion rate of approximately 3% as assessed by our quantitative analyses, we estimated, based on an average cumulative exclusion rate of both alleles in variant carriers of 13%, that the average exclusion rate (x) for the c.68-7T > A allele is close to 23% [$(x\% + 3\%)/2 = 13\%$]. Therefore, the present study strongly suggests that *BRCA2* spliceogenic alleles demonstrating up to approximately 20% exon 3 exclusion rates are not associated with high or even moderate risk of cancer.

The classification of variants based on mRNA splicing data alone is problematic for spliceogenic variants that lead to equivocal or "leaky" transcript profiles. The quantitative in vitro transcript and genetic analyses conducted for *BRCA2* c.68-7T > C provide important data to inform the threshold for ratio between functionally proficient and

altered *BRCA2* isoforms compatible with normal cell function. These findings might facilitate the future classification of rare spliceogenic variants whose relevance for cancer risk cannot easily be ascertained through multifactorial likelihood analyses.

ENIGMA COLLABORATORS

We thank Bent Ejlersen, Department of Oncology, and Anne-Marie Gerdes, Department of Clinical Genetics, Rigshospitalet, Copenhagen, Denmark for recruitment and genetic counselling of breast cancer patients.

BCAC STUDIES AND COLLABORATORS

ABCFS: Maggie Angelakos, Judi Maskiell, Gillian Dite. **ABCS:** Blood bank Sanquin, The Netherlands. **BBCS:** Eileen Williams, Elaine Ryder-Mills, Kara Sargus. **BIGGS:** Niall McInerney, Gabrielle Collieran, Andrew Rowan, Angela Jones. **BSUCH:** Peter Bugert, Medical Faculty Mannheim. **CGPS:** staff and participants of the Copenhagen General Population Study, and Dorthe Uldall Andersen, Maria Birna Arnadottir, Anne Bank, Dorthe Kjeldgård Hansen for the excellent technical assistance. The Danish Cancer Biobank is acknowledged for providing infrastructure for the collection of blood samples for the cases. **CNIO-BCS:** Guillermo Pita, Charo Alonso, Nuria Álvarez, Pilar Zamora, Primitiva Menendez, the Human Genotyping-CEGEN Unit (CNIO). **CTS:** the CTS Steering Committee includes Leslie Bernstein, James Lacey, Sophia Wang, Huiyan Ma, and Jessica Clague DeHart at the Beckman Research Institute of City of Hope, Dennis Deapen, Rich Pinder, and Eunjung Lee at the University of Southern California, Pam Horn-Ross, Peggy Reynolds, Christina Clarke Dur and David Nelson at the Cancer Prevention Institute of California, Argyrios Ziogas, and Hannah Park at the University of California Irvine, and Fred Schumacher at Case Western University. **ESTHER:** Hartwig Ziegler, Sonja Wolf, Volker Hermann, Christa Stegmaier, Katja Butterbach. **GC-HBOC:** Stefanie Engert, Heide Hellebrand, Sandra Kröber and LIFE - Leipzig Research Centre for Civilization Diseases (Markus Loeffler, Joachim Thiery, Matthias Nüchter, Ronny Baber). **GENICA Network:** Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, and University of Tübingen, Germany [HB, Wing-Yee Lo, Christina Justenhoven], German Cancer Consortium (DKTK) and German Cancer Research Center (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, Deutsches Krebsforschungszentrum (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:** Sofia Khan, Johanna Kiiski, Carl Blomqvist, Rainer Fagerholm, Kirsimari Aaltonen, Karl von Smitten, Irja Erkkilä. **HMBCS:** Peter Hillemanns, Hans Christiansen and Johann H. Karstens. **KBCP:** Eija Myöhänen, Helena Kemiläinen. **kConFab/AOCS:** 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 NHMRC, the National Breast Cancer Foundation, Cancer Australia, and the National Institute of Health (USA)) for their contributions to this resource, and the many families who contribute to kConFab. **LMBC:** Gilian Peuteman, Thomas Van Brussel, Evy-Vanderheyden and Kathleen Corthouts. **MARIE:** Petra Seibold, Dieter Flesch-Janys, Judith Heinz, Nadia Obi, Alina Vrieling, Sabine Behrens, Ursula Eilber, Muhabbet Celik, Til Olchers and Stefan Nickels. **MBCSG:** Bernard Peissel, Jacopo Azzollini, Daniela Zaffaroni and Milena Mariani of the Fondazione IRCCS Istituto Nazionale dei Tumori (INT); Monica Barile and Irene Feroce of the Istituto Europeo di Oncologia (IEO) and the personnel of the Cogentech Cancer Genetic Test Laboratory. **MYBRCA:** study participants and research staff (particularly Patsy Ng, Nurhidayu Hassan, Yoon Sook-Yee, Daphne Lee, Lee Sheau Yee, Phuah Sze Yee and Norhashimah Hassan) for their contributions and commitment to this study. **NBHS:** study participants and research staff for their contributions and commitment to this study. **OBCS:** Arja Jukkola-Vuorinen, Mervi Grip, Saila Kauppila, Meeri Otsukka, Leena Keskitalo and Kari Mononen for their contributions to this study. **OFBCR:** Teresa Selander and Nayana Weerasooriya. **ORIGO:** E. Krol-Warmerdam, and J. Blom for patient accrual, administering questionnaires, and managing clinical information. The LUMC survival data were retrieved from the Leiden hospital-based cancer registry system (ONCDOC) with the help of Dr. J. Molenaar. **PBCS:** Louise Brinton, Mark Sherman, Neonila Szeszenia-Dabrowska, Beata Peplonska, Witold Zatonski, Pei Chao, Michael Stagner. **pKARMA:** the Swedish Medical Research Counsel. **RBCS:** Petra Bos, Jannet Blom, Ellen Crepin, Elisabeth Huijskens, Anja Kromwijk-Nieuwlaat, Annette Heemskerk, the Erasmus MC Family Cancer Clinic. **SASBAC:** the Swedish Medical Research Counsel. **SBCS:** Sue Higham, Helen Cramp, Dan Connley, Ian Brock, Sabapathy Balasubramanian and Malcolm W.R. Reed. **SEARCH:** the SEARCH and EPIC teams. **SGBCC:** the participants and research coordinator Ms Tan Siew Li. **SZBCS:** Ewa Putresza. **UKBGS:** 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. We acknowledge NHS funding to the Royal Marsden/ICR NIHR Biomedical Research Centre.

CIMBA STUDIES AND COLLABORATORS

HEBON: The Hereditary Breast and Ovarian Cancer Research Group Netherlands (HEBON) consists of the following Collaborating Centers: Netherlands Cancer Institute (coordinating center), Amsterdam, NL: M.A. Rookus, F.B.L. Hogervorst, F.E. van Leeuwen, L.E. van der Kolk, M.K. Schmidt, N.S. Russell, J.L. de Lange, R. Wijnands; Erasmus Medical Center, Rotterdam, NL: J.M. Collée, A.M.W. van den Ouweland, M.J. Hooning, C. Seynaeve, C.H.M. van Deurzen, I.M. Obdeijn; Leiden University Medical Center, NL: C.J. van Asperen, J.T. Wijnen, R.A.E.M. Tollenaar, P. Devilee, T.C.T.E.F. van Cronenburg; Radboud University Nijmegen Medical Center, NL: C.M. Kets, A.R. Mensenkamp; University Medical Center Utrecht, NL: M.G.E.M. Ausems, R.B. van der

Luijt, C.C. van der Pol; Amsterdam Medical Center, NL: C.M. Aalfs, T.A.M. van Os; VU University Medical Center, Amsterdam, NL: J.J.P. Gille, Q. Waisfisz, H.E.J. Meijers-Heijboer; Maastricht University Medical Center, NL: E.B. Gómez-García, M.J. Blok; University of Groningen, NL: J.C. Oosterwijk, A.H. van der Hout, M.J. Mourits, G.H. de Bock; The Netherlands Comprehensive Cancer Organisation (IKNL): S. Siesling, J.Verloop; The nationwide network and registry of histo- and cytopathology in The Netherlands (PALGA): L.I.H. Overbeek. HEBON thanks the study participants and the registration teams of IKNL and PALGA for part of the data collection. The HEBON study is supported by the Dutch Cancer Society grants NKI1998-1854, NKI2004-308, NKI2007-3756, the Netherlands Organisation of Scientific Research grant NWO 91109024, the Pink Ribbon grants 110005 and 2014-187.WO76, the BBMRI grant NWO 184.021.007/CP46 and the Transcan grant JTC 2012 Cancer 12-054.

ACKNOWLEDGMENTS

We thank Cristina Lecchi (Dipartimento di Medicina Veterinaria, Università di Milano, Milano, Italy) for technical advices.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Mara Colombo  <http://orcid.org/0000-0001-5465-354X>

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How to cite this article: Colombo M, Lòpez-Perolio I, Meeks HD, et al. The BRCA2 c.68-7T > A variant is not pathogenic: a model for clinical calibration of spliceogenicity. *Human Mutation*. 2018;39:729–741. <https://doi.org/10.1002/humu.23411>