In search of tissue biomarkers for *BRCA1/2*-related breast cancer:

Investigating immunohistochemical, molecular, and 'gen-ethical' aspects

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About the cover:
Depicted are Japanese umbrellas (or breasts?), one of which is broken and has been repaired by Kintsugi (金継ぎ). Kintsugi, meaning "golden joinery", is the Japanese art of repairing broken pottery with lacquer mixed with gold or other noble metal. The underlying idea of Kintsugi relates to the Japanese philosophy and aesthetics of Wabi-sabi, centered on acceptance of transcience and embracing flawness or imperfection. Therefore, rather than disguising and throwing away broken objects, these items are valued and treasured for their history and imperfection.

In search of tissue biomarkers for *BRCA1/2*-related breast cancer:

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Een zoektocht naar weefselbiomarkers voor *BRCA1/2*-gerelateerde borstkanker:
Analyse van immuunhistochemische, moleculaire en 'gen-ethische' aspecten
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. H.R.B.M. Kummeling, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 3 oktober 2018 des ochtends te 10.30 uur

door

Shoko Vos

geboren op 4 oktober 1988 te Amersfoort

Promotoren:
Prof.dr. P.J. van Diest
Prof.dr. A.L. Bredenoord

“Een wijde blik verruimt het denken”

Loesje, 1989

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PART II:
'Gen-ethical' aspects

PART 1

Immunohistochemical and molecular aspects

Chapter 1

General introduction

INTRODUCTION ON *BRCA1/2*-RELATED BREAST CANCER

Breast cancer is the most frequent cancer type in women, estimated to affect one in eight women in the Western world [1]. A hereditary predisposition is one of the major risk factors for developing breast cancer [2–4]. In approximately 5-10% of cases, breast cancer occurs in such a hereditary setting, mostly due to germline mutations in the *BRCA1* (17q21.31, OMIM604370) or *BRCA2* (13q13.1, OMIM612555) genes [1, 5]. It has been estimated that 3% of all breast cancers are due to *BRCA1* germline mutations and 2% due to *BRCA2* germline mutations [1, 6]. Since the discovery of the *BRCA* genes in the 1990s [7, 8], other breast cancer susceptibility genes have been discovered like *CHEK2*, *PALPB2* and *MEN1*, but *BRCA1* and *BRCA2* germline mutations are still the main causes of hereditary breast cancer and are therefore the main targets for germline mutation screening [5, 9–13].

Women with *BRCA* germline mutations have in general a 50-60% and 6-39% lifetime risk of developing breast cancer and ovarian/tubal cancer, respectively [14–17]. These cancer risks have considerable therapeutic and preventive consequences for mutation carriers. First, they usually undergo therapeutic mastectomy instead of breast conserving therapy when diagnosed with breast cancer to lower the risk of ipsilateral breast recurrence [18]. Moreover, *BRCA* mutation status increasingly has consequences for (neo-)adjuvant systemic therapy, as it has been shown that *BRCA*-related tumors are very sensitive to platinum-based chemotherapy, relatively resistant to taxane-containing chemotherapy, and sensitive to treatment with Poly (ADP ribose) polymerase (PARP) inhibitors [19–30]. The high efficacy of traditional systemic breast cancer treatment has made it difficult to justify studies investigating platinum-based chemotherapy as well as PARP inhibitors, but currently several clinical trials are ongoing [31]. Second, to prevent the development of *BRCA*-related cancers, mutation carriers can undergo prophylactic surgery, i.e. prophylactic mastectomy (of the contralateral breast) and prophylactic adnectomy [32–35]. For breast cancer, it is also possible to choose for increased surveillance by mammography and MRI instead of prophylactic surgery. Third, identification of *BRCA* germline mutations also has implications for family members who may also be at risk. Identifying potential *BRCA* germline mutation carriers is therefore important, both in women who have already developed *BRCA*-related cancers and in pre-symptomatic women.

This thesis: a search for tissue biomarkers for *BRCA1/2*-related breast cancer

Currently, eligibility for genetic testing is largely based on clinical characteristics such as young age at tumor diagnosis (<35 years or triple-negative tumor <40 years), bilaterality of tumors with the first tumor <50 years, or a positive family history for breast, ovarian, Fallopian tube or prostate cancer [36]. Using these criteria, potential germline mutation carriers may be missed, since families are nowadays small, inheritance may occur through unaffected men, tumors may develop at an older age, and mutation penetrance is variable [37]. The estimated chance of missing *BRCA* carriers ranges from <5% up to 25% [38]. Moreover, about 25% of breast cancer patients eligible for germline mutation testing based on clinical criteria, were not referred to a geneticist by their surgeons [39]. Therefore, it would be valuable to be able to identify specific clues for an underlying *BRCA1* or *BRCA2* germline mutation in the tumor tissues themselves. In this way, the pathologist could predict the presence of an underlying *BRCA* germline mutation from the morphological, immunohistochemical and/or molecular characteristics of the tumor, similar to the use of immunohistochemistry for mismatch repair proteins and microsatellite instability analysis for Lynch syndrome.

The central question of this thesis is: to what extent can clinical pathology play a role in the early detection of *BRCA1/2* germline mutation carriers among breast cancer patients? More specifically: which tissue biomarkers could be of value in identifying potential *BRCA1/2* germline mutation carriers? The central question of this thesis will be addressed through the following sub-questions:

1) Which morphological and immunohistochemical characteristics may be of value in predicting the presence of an underlying *BRCA1* or *BRCA2* germline mutation in breast carcinomas?
2) Which molecular characteristics may be of value in predicting the presence of an underlying *BRCA1* or *BRCA2* germline mutation in breast carcinomas?

The aim of this thesis is thus to identify tumor characteristics which could be used to identify *BRCA1/2*-related breast cancers around the time of regular pathology diagnosis and which could be easily applied in formalin-fixed paraffin-embedded (FFPE) tissues and preferably, in any pathology laboratory. This could help in selecting patients for germline mutation testing. We hypothesize that specific *BRCA1/2*-related tumor characteristics can be identified, as these tumors are characterized by defective homologous recombination-mediated repair of double-strand DNA breaks resulting in genomic instability and accelerated tumorigenesis [40–42]. Homologous recombination is an important mechanism for protecting genome integrity [20, 41, 43]. Previous studies

have indeed yielded clues for *BRCA*-related breast cancers based on morphological, immunohistochemical, and molecular features of the cancer tissues, but few studies have attempted to translate this into clinical test applications so far, especially with respect to *BRCA2*-related breast cancer [44–61].

It is important to note however, that *BRCA* deficiency also occurs in sporadic breast carcinomas due to mechanisms other than *BRCA* germline mutations. As a consequence, sporadic breast carcinomas with *BRCA* deficiencies may show similar histopathological and molecular features to *BRCA1/2* germline mutation-related breast carcinomas. This concept is also referred to as *BRCA*ness [62]. *BRCA*ness may be caused by mechanisms, such as somatic *BRCA1* or *BRCA2* mutations (<5%) [63–66] and epigenetic *BRCA* gene silencing, for example by promoter hypermethylation (*BRCA1*: 11-14%; *BRCA2*: 0-9%) [67–72]. Genetic aberrations in other homologous recombination-related genes could also lead to *BRCA*ness [61, 62].

The technological possibilities in analyzing tumor tissues have tremendously increased in recent years. Notably, there is increased application of high-throughput DNA sequencing techniques in cancer research as well as in pathology practice, which has given rise to a new pathology subdiscipline called molecular pathology. These technological advances also impose several (new) moral responsibilities on researchers and pathologists as for instance, large amounts of potentially sensitive data are being generated [73–75]. Ethical reflection on these developments has however been lagging behind. The methods and techniques used to address above-mentioned research questions also bring about ethical issues. While working on this thesis, I realized that for responsible advancement of cancer research and clinical cancer care several ethical requirements need to be met. Therefore, I decided to also address 'gen-ethical' aspects of cancer research and modern molecular pathology practice in this thesis. These aspects will be addressed by the following sub-questions:

3) With which (new) moral duties are cancer researchers confronted with when using high-throughput DNA sequencing techniques? And how could they adequately deal with them?
4) Which ethical issues need to be considered with the emergence of molecular pathology? And how should they be dealt with for responsible advancement of personalized cancer care?

THESIS OUTLINE

This thesis consists of two parts in which a wide variety of research methodologies is used to answer the above-mentioned questions. In the first part of the thesis the value of several tissue biomarkers is analyzed. In **Chapter 2** an overview of the literature is given concerning hereditary breast cancer syndromes in general, including breast carcinomas related to *BRCA* germline mutations. In this chapter, the clinical and pathological characteristics of these hereditary breast cancers are reviewed. In order to answer question (1), we analyze differences in morphological characteristics and protein expression between *BRCA1/2*-related and sporadic breast carcinomas (**Chapter 3**). Protein expression is assessed by immunohistochemistry, a technique available in virtually all pathology laboratories. We then develop a prediction model for *BRCA1/2*-related breast carcinomas based on these differential characteristics (**Chapter 3**). To answer question (2), we evaluate the value of *BRCA1* and *BRCA2* methylation analysis by Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) as a tool to distinguish sporadic and *BRCA* germline mutation-related breast cancers (**Chapter 4**). MS-MLPA is a methylation analysis technique that can be easily applied in daily pathology practice using FFPE material. This is followed by a systematic review on the frequency of *BRCA* methylation in breast as well as ovarian carcinomas of *BRCA* germline mutation carriers. The aim of this review is to investigate whether or not *BRCA* methylation and germline mutations are mutually exclusive, as postulated by several individual studies in literature (**Chapter 5**). In **Chapter 6**, we further address question (2) by investigating miRNA expression patterns in normal breast tissue and invasive breast cancers of *BRCA* germline mutation carriers by miRNA microarray profiling analysis. This study aims to provide a comprehensive insight into the potential role of miRNA deregulation in *BRCA1/2*-related breast carcinogenesis as well as potential new diagnostic biomarkers and therapeutic targets. A comparison with sporadic breast cancer is also made by studying published miRNA expression data in sporadic breast cancers. miRNAs are relatively resistant to degradation caused by the formalin fixation process due to their small size. Therefore, potential miRNA biomarkers could be more rapidly translated into daily pathology practice.

In the second part of the thesis we investigate the 'gen-ethical' aspects of translational cancer research and the emergence of molecular pathology in addition to the biomedical research studies presented in the first part. This will be done by performing so-called ethical parallel research by which ethical issues related to new biomedical technologies are identified and evaluated as early as possible by a preferably multidisciplinary team, consisting of ethicists, biomedical researchers, medical doctors, etcetera. In this way,

new technologies could be implemented in biomedical research and/or clinical cancer care in a morally responsible manner. In order to answer (3), we identify and analyze the moral duties (cancer) genomics researchers are confronted with when using high-throughput DNA sequencing techniques by following a systematic review of reasons approach (**Chapter 7**). We also analyze how they may deal with these (new) duties for a responsible advancement of personalized genomic cancer care. To answer question (4), we identify and analyze the ethical issues that need to be considered with the emergence of molecular pathology (**Chapter 8**). Molecular pathology is expected to become an increasingly more important discipline in oncology, as particular tumor genetic aberrations will increasingly determine targeted clinical cancer care. This is changing the traditional role of pathologists in medical care from purely diagnostics to being actively involved in treatment decisions based on molecular disease characteristics. Also, it requires new skills, including awareness of ethical issues and attention to responsible research and innovation. **Chapter 9** contains a summary of the main findings, a general discussion as well as concluding remarks and future perspectives for these fields of research. In **Chapter 10**, a Dutch summary of this thesis is provided.

REFERENCES

1. Kleibl Z, Kristensen VN. Women at high risk of breast cancer: Molecular characteristics, clinical presentation and management. Breast. 2016;28:136–44.
2. Pérez-Solis MA, Maya-Nuñez G, Casas-González P, Olivares A, Aguilar-Rojas A. Effects of the lifestyle habits in breast cancer transcriptional regulation. Cancer Cell Int. 2016;16:7.
3. Gerber B, Müller H, Reimer T, Krause A, Friese K. Nutrition and lifestyle factors on the risk of developing breast cancer. Breast Cancer Res Treat. 2003;79:265–76.
4. Golubnitschaja O, Debald M, Yeghiazaryan K, Kuhn W, Pešta M, Costigliola V, et al. Breast cancer epidemic in the early twenty-first century: evaluation of risk factors, cumulative questionnaires and recommendations for preventive measures. Tumour Biol. 2016;37:12941–12957.
5. Stuckey AR, Onstad MA. Hereditary breast cancer: an update on risk assessment and genetic testing in 2015. Am J Obstet Gynecol. 2015;213:161–165.
6. Fraser JA, Reeves JR, Stanton PD, Black DM, Going JJ, Cooke TG, et al. A role for BRCA1 in sporadic breast cancer. Br J Cancer. 2003;88:1263–70.
7. Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science. 1994;266:66–71.
8. Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, et al. Identification of the breast cancer susceptibility gene BRCA2. Nature. 1995;378:789–92.
9. Dutch clinical guideline familial breast/ovarian carcinoma [http://oncoline.nl/hereditair-mamma-ovariumcarcinoom]
10. Paluch-Shimon S, Cardoso F, Sessa C, Balmana J, Cardoso MJ, Gilbert F, et al. Prevention and screening in BRCA mutation carriers and other breast/ovarian hereditary cancer syndromes: ESMO Clinical Practice Guidelines for cancer prevention and screening. Ann Oncol Off J Eur Soc Med Oncol. 2016;27(suppl 5):v103–v110.
11. Gadzicki D, Evans DG, Harris H, Julian-Reynier C, Nippert I, Schmidtke J, et al. Genetic testing for familial/hereditary breast cancer—comparison of guidelines and recommendations from the UK, France, the Netherlands and Germany. J Community Genet. 2011;2:53–69.
12. Daly MB, Pilarski R, Axilbund JE, Berry M, Buys SS, Crawford B, et al. Genetic/Familial High-Risk Assessment: Breast and Ovarian, Version 2.2015. J Natl Compr Canc Netw. 2016;14:153–62.
13. Bradbury AR, Olopade OI. Genetic susceptibility to breast cancer. Rev Endocr Metab Disord. 2007;8:255–267.
14. Chen S, Parmigiani G. Meta-analysis of BRCA1 and BRCA2 penetrance. J Clin Oncol. 2007;25:1329–33.
15. Mavaddat N, Peock S, Frost D, Ellis S, Platte R, Fineberg E, et al. Cancer Risks for BRCA1 and BRCA2 Mutation Carriers: Results From Prospective Analysis of EMBRACE. JNCI J Natl Cancer Inst. 2013;105:812–822.
16. Antoniou A, Pharoah PDP, Narod S, Risch HA, Eyfjord JE, Hopper JL, et al. Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. Am J Hum Genet. 2003;72:1117–30.
17. Brohet RM, Velthuizen ME, Hogervorst FBL, Meijers-Heijboer HEJ, Seynaeve C, Collée MJ, et al. Breast and ovarian cancer risks in a large series of clinically ascertained families with a high proportion of BRCA1 and BRCA2 Dutch founder mutations. J Med Genet. 2014;51:98–107.
18. Chiba A, Hoskin TL, Hallberg EJ, Cogswell JA, Heins CN, Couch FJ, et al. Impact that Timing of Genetic Mutation Diagnosis has on Surgical Decision Making and Outcome for BRCA1/BRCA2 Mutation Carriers with Breast Cancer. Ann Surg Oncol. 2016;23:3232–8.
19. Foulkes WD. BRCA1 and BRCA2: chemosensitivity, treatment outcomes and prognosis. Fam Cancer. 2006;5:135–42.
20. Lord CJ, Ashworth A. The DNA damage response and cancer therapy. Nature. 2012;481:287–294.

21. Byrski T, Gronwald J, Huzarski T, Grzybowska E, Budryk M, Stawicka M, et al. Response to neo-adjuvant chemotherapy in women with BRCA1-positive breast cancers. Breast Cancer Res Treat. 2008;108:289–296.
22. Byrski T, Gronwald J, Huzarski T, Grzybowska E, Budryk M, Stawicka M, et al. Pathologic complete response rates in young women with BRCA1-positive breast cancers after neoadjuvant chemotherapy. J Clin Oncol. 2010;28:375–9.
23. Kriege M, Jager A, Hooning MJ, Huijskens E, Blom J, van Deurzen CHM, et al. The efficacy of taxane chemotherapy for metastatic breast cancer in BRCA1 and BRCA2 mutation carriers. Cancer. 2012;118:899–907.
24. Rouleau M, Patel A, Hendzel MJ, Kaufmann SH, Poirier GG. PARP inhibition: PARP1 and beyond. Nat Rev Cancer. 2010;10:293–301.
25. Fong PC, Boss DS, Yap TA, Tutt A, Wu P, Mergui-Roelvink M, et al. Inhibition of Poly(ADP-Ribose) Polymerase in Tumors from *BRCA* Mutation Carriers. N Engl J Med. 2009;361:123–134.
26. Dizdar O, Arslan C, Altundag K. Advances in PARP inhibitors for the treatment of breast cancer. Expert Opin Pharmacother. 2015;16:2751–8.
27. Lee J-M, Ledermann JA, Kohn EC. PARP Inhibitors for BRCA1/2 mutation-associated and BRCA-like malignancies. Ann Oncol. 2014;25:32–40.
28. Livraghi L, Garber JE. PARP inhibitors in the management of breast cancer: current data and future prospects. BMC Med. 2015;13:188.
29. Farmer H, McCabe N, Lord CJ, Tutt ANJ, Johnson DA, Richardson TB, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature. 2005;434:917–21.
30. Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature. 2005;434:913–7.
31. Drost R, Jonkers J. Opportunities and hurdles in the treatment of BRCA1-related breast cancer. Oncogene. 2014;33:3753–63.
32. Rebbeck TR. Prophylactic oophorectomy in BRCA1 and BRCA2 mutation carriers. Eur J Cancer. 2002;38 Suppl 6:S15-7.
33. Rebbeck TR, Friebel T, Lynch HT, Neuhausen SL, van 't Veer L, Garber JE, et al. Bilateral prophylactic mastectomy reduces breast cancer risk in BRCA1 and BRCA2 mutation carriers: the PROSE Study Group. J Clin Oncol. 2004;22:1055–62.
34. Kauff ND, Satagopan JM, Robson ME, Scheuer L, Hensley M, Hudis CA, et al. Risk-reducing salpingo-oophorectomy in women with a BRCA1 or BRCA2 mutation. N Engl J Med. 2002;346:1609–15.
35. Nelson HD, Pappas M, Zakher B, Mitchell JP, Okinaka-Hu L, Fu R. Risk Assessment, Genetic Counseling, and Genetic Testing for BRCA-Related Cancer in Women: A Systematic Review to Update the U.S. Preventive Services Task Force Recommendation. Ann Intern Med. 2014;160:255–266.
36. Hereditary Tumors: Guidelines for Diagnosis and Prevention. Foundation for the Detection of Hereditary Tumors, Dutch Association of Clinical Genetics, Working Group Clinical Oncogenetics; 2010.
37. de Sanjosé S, Léoné M, Bérez V, Izquierdo A, Font R, Brunet JM, et al. Prevalence of BRCA1 and BRCA2 germline mutations in young breast cancer patients: a population-based study. Int J cancer. 2003;106:588–93.
38. Varesco L, Viassolo V, Viel A, Gismondi V, Radice P, Montagna M, et al. Performance of BOADICEA and BRCAPRO genetic models and of empirical criteria based on cancer family history for predicting BRCA mutation carrier probabilities: a retrospective study in a sample of Italian cancer genetics clinics. Breast. 2013;22:1130–5.
39. Hafertepen L, Pastorino A, Morman N, Snow J, Halaharvi D, Byrne L, et al. Barriers to genetic testing in newly diagnosed breast cancer patients: do surgeons limit testing? Am J Surg. 2016.
40. Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nat Rev Cancer. 2012;12:68–78.
41. Li ML, Greenberg R a. Links between genome integrity and BRCA1 tumor suppression. Trends Biochem Sci. 2012:1–7.
42. Mangia a, Malfettone a, Simone G, Darvishian F. Old and new concepts in histopathological characterization of familial breast cancer. Ann Oncol. 2011;22 Suppl 1(Supplement 1):i24–i30.

43. Schlacher K, Christ N, Siaud N, Egashira A, Wu H, Jasin M. Double-strand break repair-independent role for BRCA2 in blocking stalled replication fork degradation by MRE11. Cell. 2011;145:529–42.

44. Lakhani SR, Jacquemier J, Sloane JP, Gusterson BA, Anderson TJ, van de Vijver MJ, et al. Multifactorial analysis of differences between sporadic breast cancers and cancers involving BRCA1 and BRCA2 mutations. J Natl Cancer Inst. 1998;90:1138–45.

45. Bane AL, Beck JC, Bleiweiss I, Buys SS, Catalano E, Daly MB, et al. BRCA2 mutation-associated breast cancers exhibit a distinguishing phenotype based on morphology and molecular profiles from tissue microarrays. Am J Surg Pathol. 2007;31:121–8.

46. Palacios J, Honrado E, Osorio A, Cazorla A, Sarrio D, Barroso A, et al. Immunohistochemical characteristics defined by tissue microarray of hereditary breast cancer not attributable to BRCA1 or BRCA2 mutations: differences from breast carcinomas arising in BRCA1 and BRCA2 mutation carriers. Clin Cancer Res. 2003;9:3606-14.

47. Lynch BJ, Holden JA, Buys SS, Neuhausen SL, Gaffney DK. Pathobiologic characteristics of hereditary breast cancer. Hum Pathol. 1998;29:1140–4.

48. Pathology of familial breast cancer: differences between breast cancers in carriers of BRCA1 or BRCA2 mutations and sporadic cases. Breast Cancer Linkage Consortium. Lancet. 1997;349:1505–10.

49. Foulkes WD, Stefansson IM, Chappuis PO, Bégin LR, Goffin JR, Wong N, et al. Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. J Natl Cancer Inst. 2003;95:1482–5.

50. Lakhani SR, Gusterson BA, Jacquemier J, Sloane JP, Anderson TJ, van de Vijver MJ, et al. The pathology of familial breast cancer: histological features of cancers in families not attributable to mutations in BRCA1 or BRCA2. Clin Cancer Res. 2000;6:782–9.

51. Palacios J, Robles-Frías MJ, Castilla M a, López-García M a, Benítez J. The molecular pathology of hereditary breast cancer. Pathobiology. 2008;75:85–94.

52. Armes JE, Trute L, White D, Southey MC, Hammet F, Tesoriero A, et al. Distinct molecular pathogeneses of early-onset breast cancers in BRCA1 and BRCA2 mutation carriers: a population-based study. Cancer Res. 1999;59:2011–7.

53. Osin PP, Lakhani SR. The pathology of familial breast cancer: Immunohistochemistry and molecular analysis. Breast Cancer Res. 1999;1:36–40.

54. Mavaddat N, Barrowdale D, Andrulis IL, Domchek SM, Eccles D, Nevanlinna H, et al. Pathology of breast and ovarian cancers among BRCA1 and BRCA2 mutation carriers: results from the Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA). Cancer Epidemiol Biomarkers Prev. 2012;21:134–47.

55. Lakhani SR, Van De Vijver MJ, Jacquemier J, Anderson TJ, Osin PP, McGuffog L, et al. The pathology of familial breast cancer: predictive value of immunohistochemical markers estrogen receptor, progesterone receptor, HER-2, and p53 in patients with mutations in BRCA1 and BRCA2. J Clin Oncol. 2002;20:2310–8.

56. Bane AL, Pinnaduwage D, Colby S, Reedijk M, Egan SE, Bull SB, et al. Expression profiling of familial breast cancers demonstrates higher expression of FGFR2 in BRCA2-associated tumors. Breast Cancer Res Treat. 2009; 117:183–91.

57. Wessels LFA, Welsem T Van, Hart AAM. Molecular Classification of Breast Carcinomas by Comparative Genomic Hybridization : Molecular Classification of Breast Carcinomas by Comparative Genomic Hybridization : a Specific Somatic Genetic Profile for BRCA1 Tumors 1. 2002:7110–7117.

58. Jönsson G, Naylor TL, Vallon-Christersson J, Staaf J, Huang J, Ward MR, et al. Distinct genomic profiles in hereditary breast tumors identified by array-based comparative genomic hybridization. Cancer Res. 2005;65:7612–21.

59. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AAM, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature. 2002;415:530–6.

60. Hedenfalk I, Duggan D, Yidong C, Radmacher M, Bittner M, Simon R, et al. Gene-expression profiles in hereditary breast cancer. N Engl J Med. 2001;344:539–548.

61. Stefansson OA, Jonasson JG, Johannsson OT, Olafsdottir K, Steinarsdottir M, Valgeirsdottir S, et al. Genomic profiling of breast tumours in relation to BRCA abnormalities and phenotypes. Breast Cancer Res. 2009;11:R47.

62. Turner N, Tutt A, Ashworth A. Hallmarks of "BRCAness" in sporadic cancers. Nat Rev Cancer. 2004;4:814–819.

63. Futreal PA, Liu Q, Shattuck-Eidens D, Cochran C, Harshman K, Tavtigian S, et al. BRCA1 mutations in primary breast and ovarian carcinomas. Science. 1994;266:120–2.

64. Lancaster JM, Wooster R, Mangion J, Phelan CM, Cochran C, Gumbs C, et al. BRCA2 mutations in primary breast and ovarian cancers. Nat Genet. 1996;13:238–40.

65. Winter C, Nilsson MP, Olsson E, George AM, Chen Y, Kvist A, et al. Targeted sequencing of *BRCA1* and *BRCA2* across a large unselected breast cancer cohort suggests that one-third of mutations are somatic. Ann Oncol. 2016;27:1532–1538.

66. Li M, Zhang J, Ouyang T, Li J, Wang T, Fan Z, et al. Incidence of BRCA1 somatic mutations and response to neoadjuvant chemotherapy in Chinese women with triple-negative breast cancer. Gene. 2016;584:26–30.

67. Esteller M, Silva JM, Dominguez G, Bonilla F, Matias-Guiu X, Lerma E, et al. Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J Natl Cancer Inst. 2000;92:564–569.

68. Rice JC, Ozcelik H, Maxeiner P, Andrulis I, Futscher BW, J.C. R, et al. Methylation of the BRCA1 promoter is associated with decreased BRCA1 mRNA levels in clinical breast cancer specimens. Carcinogenesis. 2000;21:1761–1765.

69. Catteau A, Harris WH, Xu CF, Solomon E, A. C, W.H. H, et al. Methylation of the BRCA1 promoter region in sporadic breast and ovarian cancer: Correlation with disease characteristics. Oncogene. 1999;18:1957–1965.

70. Matros E, Wang ZC, Lodeiro G, Miron A, Iglehart JD, Richardson AL, et al. BRCA1 promoter methylation in sporadic breast tumors: Relationship to gene expression profiles. Breast Cancer Res Treat. 2005;91:179–186.

71. Turner NC, Reis-Filho JS, Russell AM, Springall RJ, Ryder K, Steele D, et al. BRCA1 dysfunction in sporadic basal-like breast cancer. Oncogene. 2007;26:2126–2132.

72. Dworkin AM, Spearman AD, Tseng SY, Sweet K, Toland AE. Methylation not a frequent "second hit" in tumors with germline BRCA mutations. Fam Cancer. 2009;8:339–346.

73. McGuire AL, Caulfield T, Cho MK, A.L. M, T. C, M.K. C. Research ethics and the challenge of whole-genome sequencing. Nat Rev Genet. 2008;9:152–156.

74. Rotimi CN, Marshall PA. Tailoring the process of informed consent in genetic and genomic research. Genome medicine. 2010:20.

75. Bredenoord AL, Kroes HY, Cuppen E, Parker M, van Delden JJM. Disclosure of individual genetic data to research participants: the debate reconsidered. Trends Genet. 2011;27:41–7.

Chapter 2

Hereditary breast cancer syndromes: molecular pathogenesis and diagnostics

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ABSTRACT

Breast cancer is a major cause of morbidity and mortality worldwide. Approximately 5-10% of breast cancer cases are due to a familial predisposition. Most of these familial breast cancers are due to mutations in well-known genes linked to breast cancer, such as *BRCA1, BRCA2, TP53, CHEK2, PTEN, CDH1, STK11/LKB1, RAD50, BRIP1,* and PALB2. Some of these cancers are however due to (a combination) of lower penetrance genes or show no relationship with known susceptibility genes. In this review, we describe the function of today's known breast cancer susceptibility genes, the molecular pathogenesis and histopathology of these hereditary breast cancer syndromes. This may provide a better understanding and classification of hereditary breast cancer and may offer tools for better diagnosis and selection of patients sensitive to specific targeted therapies.

KEY CONCEPTS

- Breast cancer is the most frequent cancer diagnosed in women worldwide.
- In 5-10%* of total breast cancer cases, there is familial breast cancer susceptibility.
- Hereditary breast cancer is familial breast cancer with a clear Mendelian inheritance pattern.
- Breast cancer susceptibility genes can be divided into high, intermediate and low risk genes, of which the high risk genes *BRCA1* and *BRCA2* are the best known.
- Breast cancer susceptibility genes most involve DNA repair mechanisms.
- The discovery of breast cancer susceptibility genes has improved our knowledge of breast carcinogenesis, including the morphological, immunohistochemical and molecular characterization of familial breast cancer.
- Moreover, knowledge of the biological processes underlying familial breast cancer offers tools for cancer screening, prevention and management

INTRODUCTION

Breast cancer is the most frequent cancer diagnosed in women worldwide, affecting one in eight women [1,2]. Established breast cancer risk factors include modifying life style factors such as alcohol consumption, obesity, physical inactivity, and hormone replacement therapy; reproductive factors such as early menarche, late menopause, low parity, and breast-feeding; and inherent genetic factors [3]. Clues towards an underlying inherited breast cancer predisposition are a family history of multiple affected relatives, an early age of disease onset, bilateral breast cancer, male breast cancer, and ovarian/ Fallopian tube cancer [2,4]. A positive family history is even the second largest risk factor after age [4,5]. Familial breast cancer is defined by a family history of one or more first or second-degree relatives with breast cancer that does not fit the more stringent definition of hereditary breast cancer. A family history that fits Mendelian inheritance patterns (usually autosomal dominant) is suggestive for hereditary breast cancer.

Familial breast cancer accounts for 5-10% of total breast cancer cases (see Figure 1) [6–10]. Within this group of familial breast cancers a subgroup of breast cancers can be made comprising patients with germline mutations in high susceptibility genes, either in *BRCA1* or *BRCA2* (25%) or other high susceptibility genes (5%)[10]. Furthermore, a category of patients with a germline mutation in intermediate susceptibility genes (about 5%) can be distinguished as well as a group of patients with mutations in low susceptibility genes (about 15%*). The last category consists of patients without a known genetic aberration at the moment, also referred to as *BRCAX* (about 50%) [10].

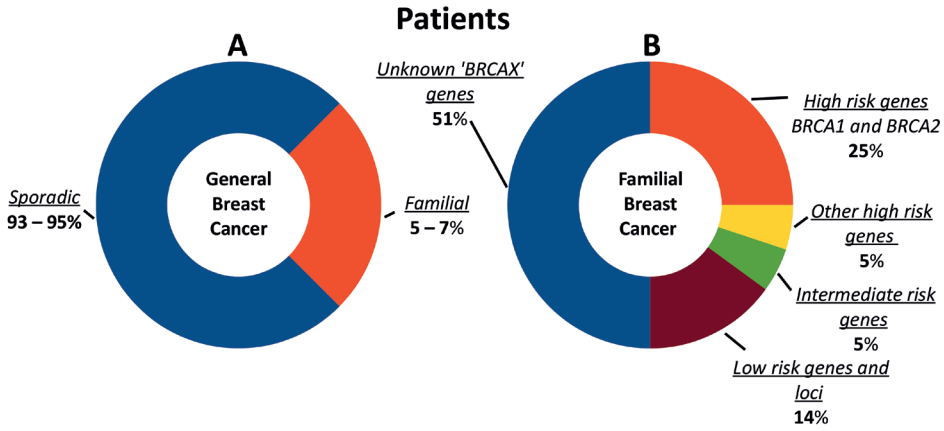

Figure 1. Distribution of breast cancer patients
Adapted from Melchor L and Benítez J (2010) [10].
A = general breast cancer; B = familial breast cancer

* Corrected from original publication.

The most important breast cancer susceptibility genes, *BRCA1* and *BRCA2*, have been discovered in 1991 and 1995, respectively [11–13]. Since then, a clearer understanding of the genetic susceptibility to breast cancer has been achieved through epidemiological studies, linkage analysis, mutational screening of candidate genes and association studies [14]. The development of the next-generation sequencing technique has contributed much to the discovery of new potential breast cancer susceptibility genes [3]. This has yielded rare high- and intermediate-penetrance genetic mutations and common low-penetrance genetic modifications [14]. Identification of new breast cancer susceptibility genes has been difficult due to genotypic and phenotypic heterogeneity of familial breast cancers, low penetrance alleles, polygenic mechanisms, and interaction with environmental factors [3,7,15].

Affected genes in hereditary breast cancer syndromes mostly involve DNA repair mechanisms and genomic integrity maintenance [3]. As a consequence, these patients are highly susceptible to the accumulation of even more mutations, affecting proto-oncogenes and tumor-suppressor genes that regulate important cellular processes, such as cell growth, differentiation and survival, and eventually to the development of cancer. The discovery of new breast cancer susceptibility genes and studying the genetic, protein, and histological features of hereditary breast cancer have not only improved our understanding of the molecular pathways important in breast carcinogenesis, but also improved patient counseling as well as cancer screening, prevention, and management [6,10,16].

In this review, we will discuss the most important hereditary breast cancer syndromes, focusing on well known breast cancer susceptibility genes and describe the complex genetic, protein, and histological features discovered so far [10]. Moreover, we will describe the relation with molecular breast cancer subtypes and development of a tissue classifier to predict *BRCA1* and *BRCA2* mutations.

CELLULAR PATHWAYS INVOLVED IN HEREDITARY BREAST CANCER

Most of the hereditary breast cancer syndromes are due to mutations in genes that play a role in genomic stability and are involved in processes such as DNA damage repair and cell cycle regulation (Table 1). A major pathway affected in hereditary breast cancer is that of DNA double strand break (DSB) repair. DSBs may occur due to exogenous factors such as radiation or endogenous factors including free oxygen radicals and are

Table 1. Overview of high and intermediate breast cancer risk genes

Gene	Syndrome	Relative risk of breast cancer	Carrier frequency / % of breast cancer	Breast cancer subtype	Associated diseases
BRCA1 *BRCA2*	Hereditary breast and ovarian cancer syndrome	65% life-time risk 39% life-time risk	25% of familial breast cancer	Invasive ductal carcinoma Basal-like (*BRCA1*) or luminal (*BRCA2*) molecular phenotype	Ovarian, Fallopian tube cancer (both); stomach, gallbladder, bile duct, pancreatic and prostate cancer (mostly *BRCA2*)
TP53	Li-Fraumeni syndrome	>90% by age 60	1:5000, <1% of total breast cancer	HER2 positive breast cancer (little evidence)	Sarcomas, leukemias, brain and adrenocortical cancer
PTEN	Cowden syndrome	50% life-time risk	1:200.000-250.000, <1% of total breast cancer	Similar to sporadic breast cancer (little evidence); apocrine differentiation	Hamartomas and malignant tumors of bowel, thyroid gland, and endometrium
CDH1	Hereditary diffuse gastric cancer syndrome	50% life-time risk		Lobular breast cancer	Diffuse gastric cancer
STK11 *LKB1*	Peutz-Jeghers syndrome	30-50% life-time risk		Similar to sporadic breast cancer (little evidence)	Mucocutaneous pigmentation, gastrointestinal hamartomatous polyps, fibrocystic breast disease, uterine leiomyomas, malignant tumors of bowel, pancreas, stomach, lung, ovarian, testis, endometrium, thyroid
CHEK2		37% life-time risk	<1% of total breast cancer	Invasive ductal carcinoma with luminal molecular phenotype; lobular breast cancer (I157T variant) (little evidence)	

ATM	Ataxia-telangiectasia	2-5 fold increased risk	0.5-1% carrier percentage	Similar to sporadic breast cancer (little evidence)	Cerebellar ataxia, immune deficiency, gliomas, medulloblastomas, lymphomas
Mismatch repair genes *(MLH1, MSH2, MSH6, PMS2)*	Lynch syndrome	2-18 fold increased risk	1:500	Unclear	Colorectal, stomach, endometrial, and ovarian cancer
RAD51C		1.5-5 fold increased risk (little evidence)	1% of all hereditary breast cancer cases	Usually invasive ductal carcinomas with luminal molecular phenotype (little evidence)	
PALB2 *BRIP1* and others	Fanconi anemia	2 fold increased risk	1% of all hereditary breast cancer cases	Often triple-negative breast carcinomas (little evidence)	Developmental anomalies, bone marrow failure, several malignant tumors
MEN1	MEN1 syndrome	2-3 fold increased risk		Luminal molecular phenotype (little evidence)	Parathyroid and pituitary adenomas, gastrointestinal and neuroendocrine tumors

extremely genotoxic as they may lead to chromosomal rearrangements and chromosomal instability. Several pathways exist for DSB repair, the most important pathways being homologous recombination (HR) and non-homologous end joining (NHEJ). If one of these pathways is disrupted, another pathway will take over. HR is an error-free process by which the sister chromatid is used as a template [17]. NHEJ, on the other hand, is error-prone as it uses no or very limited sequence homology [18].

DSBs are, like other types of DNA damage, recognized by DNA damage surveillance proteins, such as ATM and ATR. After activation of ATM or ATR, downstream targets are phosphorylated, including p53 and CHEK2, leading to cell cycle arrest and initiation of DNA repair [18]. BRCA1 is activated by ATM and ATR and plays a central role in the DNA damage response, as it links DNA damage sensors to DNA repair pathways [18–20].

BRCA1 activation leads to chromatin remodeling to make the damaged DNA become assessable for DNA repair and to activation of DSB repair pathways. BRCA1 is also able to activate other DNA repair pathways, such as mismatch repair or base excision repair mechanisms, depending on the type of damage [21]. DSB repair by HR is further mediated by the Rad family. BRCA1, being in a complex with BARD1, stimulates the Rad50 and MRN complex and is involved in whether DSBs will be repaired by either HR or NHEJ [18]. BRCA2 plays a more limited role in HR, downstream of BRCA1. BRCA2 is important for the recruitment of the essential recombinase Rad51 to the site of DSB. Together, they initiate DSB repair [18]. Some of the facilitators of the HR pathway include PALB2 (partner of BRCA2) and the BRCA1-interacting protein C-terminal helicase 1 (BRIP1), both of them being Fanconi anemia-associated proteins [22].

Germline mutations in many of the above-mentioned proteins may occur and result among other diseases in increased breast cancer risk (Table 1). They can be divided into highly, intermediately and low penetrant breast cancer risk genes and will be further discussed below. In the discussion below, it needs to be realized that very likely the various study groups are not comprised of pure hereditary cases, but will be diluted with some cancers that are due to sporadic carcinogenetic routes, since there is no reason to assume that germline-mutated patients are not also at baseline risk of sporadic cancers.

HIGHLY PENETRANT GENES - HIGH RISK CANCER SYNDROMES

High risk cancer syndromes result from germline mutations in highly penetrant genes that lead to a >5 times increased risk of developing breast cancer. These genes account for 30% of all familial breast cancer cases of which 25% is due to mutations in the most important breast cancer risk genes *BRCA1* and *BRCA2* and 5% is due to mutations in the remaining high risk genes [10].

BRCA1 and _BRCA2_ – Hereditary breast and ovarian cancer syndrome

Germline mutations in the *BRCA1* and *BRCA2* genes were the first discovered high-risk breast cancer genes and form the largest group within hereditary breast cancer syndromes, comprising 25% of all familial breast cancer cases [10,12,13]. *BRCA1* and *BRCA2* founder mutations have been found in Ashkenazi Jewish, Icelandic and Finnish populations [23,24].

Risks

BRCA1 germline mutation carriers have a 65% risk of developing breast cancer and 39% of developing Fallopian tube/ovarian cancer [25]. Moreover, these patients have an increased risk of developing stomach, pancreatic, colon, and prostate cancer [26]. *BRCA2* germline mutation carriers have a 39% risk of developing breast cancer and 11% risk of developing Fallopian tube/ovarian cancer [25]. Male patients have 6% risk of developing breast cancer [27,28]. *BRCA2* germline mutation carriers also have an increased risk of developing stomach, gallbladder, bile duct, pancreatic, and prostate cancer [29].

Genes and mutations

The *BRCA1* gene is located on chromosome 17q21 [11]. It is a large gene containing 5.592 nucleotides, leading to a protein of 1.863 amino acids [30]. Mutations have been found across the whole length of the gene and about 80% of the mutations lead to generation of a premature stop codon resulting in a non-functioning protein, mainly due to frame shift and nonsense mutations [30,31].

The *BRCA2* gene is located on chromosome 13q12-13 [30]. Similar to BRCA1, it is a large gene containing 10.485 nucleotides, leading to a protein of 3.495 amino acids. As for *BRCA1*, there are no hot spot mutations and the far majority of the mutations (90%) lead to premature protein truncation [30].

In >80% of the breast and other cancers that develop in *BRCA1* and *BRCA2* germline mutation carriers, the remaining wild-type allele is lost (loss of heterozygosity), resulting in complete loss of function of these important tumor suppressor genes [30,32,33]. In rare instances, small somatic mutations in the *BRCA* genes involving a few bases have been described [34]. *BRCA1* and *BRCA2* promoter hypermethylation, another mechanism leading to decreased protein expression, is very rare in cancers arising in *BRCA1* and *BRCA2* germline mutation carriers. However, *BRCA1* promoter hypermethylation has been found in 9-13% of sporadic breast carcinomas and 42% of hereditary breast carcinomas due to other causes than *BRCA1* and *BRCA2* germline mutations [35–39]. *BRCA1* hypermethylated tumors show a similar phenotype compared to breast carcinomas arising in *BRCA1* germline mutation carriers (see later) [17]. *BRCA2* promoter hypermethylation is also very rare in sporadic and hereditary breast carcinomas [40]. However, an alternative mechanism leading to *BRCA2* inactivation involves *EMSY* amplification, seen in 13% of sporadic breast cancer cases [41]. EMSY is able to directly interact with BRCA2, and EMSY overexpression inhibits *BRCA2* transcriptional activity [41–43].

Function and loss of function

As mentioned above, BRCA1 and BRCA2 are important proteins involved in different stages in the DNA damage response and DNA double-strand DSB repair by the very robust and error-free mechanism of HR, although they are involved at different stages in this pathway of genome protection [17]. BRCA1 or BRCA2 deficiency due to mutations in these genes leads to defective DSB repair. The reliable repair by HR is replaced by the error-prone mechanism of non-homologous end joining (NHEJ) and other less reliable mechanisms, resulting in chromosomal instability [44–46]. This predisposes to and accelerates tumor formation [47,48].

BRCA1 is a multi-functional protein with other functions besides DNA damage repair by HR, such as cell cycle control, transcription regulation, proliferation, protein ubiquitination, chromatin remodeling, and telomere maintenance [18,49–55]. The functions of BRCA2 are more restricted to DSB repair [18,49].

It has been suggested that loss of BRCA1 and BRCA2 function activates p53, an important factor in signaling DNA damage and activating DNA damage response, leading to cell-cycle arrest and apoptosis [31]. Loss of p53 is assumed to be essential for development of *BRCA*-associated breast tumors [56]. *BRCA1*- and *BRCA2*-associated breast carcinomas show a higher frequency of p53 mutations compared to sporadic breast carcinomas: 40% of *BRCA1*-associated, 30% of *BRCA2*-associated and 20% of

sporadic breast carcinomas [57–60]. The high incidence of p53 mutations in *BRCA1* related cancer is a possible explanation for the high grade and proliferation index seen in these cancers [60–62]. Indeed, it has been shown by several studies that *BRCA1*- and (to a lesser extent) *BRCA2*-associated breast carcinomas differ morphologically, immunophenotypically and molecularly from sporadic breast cancer in age-matched controls and from hereditary non-*BRCA*-associated breast carcinomas [31,63]. These differences likely reflect differences in tumor biology and could also be used in clinical practice in predicting which patients might have *BRCA1* or *BRCA2* germline mutations.

Pathology of BRCA1-associated breast cancer

The most common tumor type arising in *BRCA1* germline mutation carriers is invasive ductal carcinoma of no special type, similar to sporadic breast carcinomas. However, there is an increased proportion of medullary carcinomas (13% compared to 3% in *BRCA2*-associated and 2% in non-*BRCA*-associated breast carcinomas) [64]. *BRCA1*-associated breast carcinomas also frequently show a prominent lymphocytic infiltration and pushing tumor margins [65].

BRCA1-associated breast carcinomas are usually of high grade with little tubule formation, higher pleomorphism and high mitotic index (66-84% compared to 15-36%* in sporadic breast carcinomas) [64,66,67]. They are usually negative for ER (73-90%), PR (79%), and HER2 (97%-100%) [56,60,65,67–70]. Thus, about 69% of all *BRCA1*-associated breast carcinomas are triple negative [71]. Interestingly, the likelihood of ER negativity is 4.8 times higher in high-grade breast carcinomas in *BRCA1* germline mutation carriers than in sporadic cases [72]. Instead, they usually show a high expression of basal or myoepithelial cell markers, including CK5/6, CK14, and EGFR, and no expression of luminal-cell markers such as CK8/18 [73,74]. Therefore, they are typically of basal-like intrinsic type [73,74]. Moreover, these tumors are characterized by high expression of Ki-67, caspase-3, and c-MYC [69,75], and little Bcl2, p27, and cyclin D1 expression, which are ER associated genes [76–78]. Other proteins that are usually not expressed in *BRCA1*-associated breast carcinomas are CDK4, p16, and p21 [31]. *BRCA1*-associated breast carcinomas show overexpression of proteins that stimulate cell cycle progression, such as cyclin A, B1 and E [72,79,80]. Furthermore, a high expression of HIF1a caused by hypoxia and inducing angiogenesis by stimulating VEGF transcription has been found [81].

As breast cancer risks vary to some extent between families with *BRCA1* germline mutations, research has been done on potential gene modifiers of *BRCA1*-associated

* Corrected from original publication.

breast cancer risk. Several gene modifiers that encode proteins that interact with BRCA1 have been identified, including *ATM, BRCC36, BRCC45, BRIP1, CTIP, ABRA1, MERIT40, MRE11A, NBS1, PALB2, RAD50, RAD51, RAP80,* and *TOPB1* [82].

Pathology of BRCA2-associated breast cancer

The pathology of *BRCA2*-associated breast carcinomas is more heterogeneous and more comparable to sporadic breast cancer. Several studies showed no statistical differences with sporadic breast carcinomas [64,65,83]. Other studies reported that *BRCA2*-associated breast carcinomas are usually of intermediate to high grade and frequently show pushing tumor margins similar to *BRCA1*-associated breast cancer [84]. They show less tubule formation but similar cellular pleomorphism and mitotic activity compared to sporadic breast carcinomas [64,83].

BRCA2-associated tumors are usually ER positive (65%) and PR positive (40-60%), and HER2 negative (97-100%) [56,60,69]. According to grade, they are more often ER and PR positive, and negative for HER2 compared to sporadic breast carcinomas [56,69,85]. Only 16% of *BRCA2*-associated breast carcinomas are triple negative, much less than *BRCA1*-related and comparable to sporadic cancers. Moreover, *BRCA2*-associated breast carcinomas rarely express basal-cell markers. Therefore, *BRCA2*-associated breast carcinomas are typically of the luminal intrinsic type.

Furthermore, *BRCA2*-associated breast carcinomas show frequent cyclin D1 overexpression (27-55% vs. 5-33% of *BRCA1* and 35-100% of sporadic breast carcinomas) [60,78]. They also show frequent expression of Bcl2, which is also correlated with ER expression, and overexpression of FGF1 and FGFR2, resulting in upregulation of genes involved cell proliferation, cell adhesion and extracellular matrix interaction, and activation of the MAPK signaling pathway [85]. Furthermore, similar to *BRCA1*-associated carcinomas, *BRCA2*-associated carcinomas also overexpress HIF1α [86].

Precursor lesions

The development of *BRCA1*- and *BRCA2*-associated breast cancer from morphologically normal epithelium (via precursor lesions) to invasive breast cancer is not well understood [31]. In situ lesions, such as ductal and lobular carcinoma in situ, are infrequently found around invasive breast cancer in excision specimens of *BRCA1* and *BRCA2* mutation carriers [64]. The occurrence of in situ lesions in the absence of invasive breast cancer is not well known. In prophylactic mastectomy specimens, several precursor lesions have been reported (atypical ductal/lobular hyperplasia, lobular/ductal carcinoma in situ) and are found in up to 57% of cases, which is a higher incidence compared to

breast reduction specimens in non-affected women[87,88]. A faster progression from morphologically normal epithelium to invasive breast cancer has been suggested. Morphologically normal epithelium in *BRCA1*- or *BRCA2*-associated breast carcinomas already contains genetic alterations, including LOH and miRNA deregulation [89]. Furthermore, we have described an upregulation of hypoxia markers, such as HIF1α, Glut-1 and CAIX in ductal carcinoma in situ lesions of *BRCA1* or *BRCA2* mutation carriers, suggesting a role for these markers in the development of *BRCA*-associated breast cancer [86].

Prognosis
Limited research has been performed on the prognosis of *BRCA1*- and *BRCA2*-associated breast carcinomas. Most of the available data are derived from small, retrospective studies and are likely to contain several biases and lack of appropriate controls (matching for age, stage, grade, hormone receptors etc.). As far as is known today, there are no clear differences in prognosis between *BRCA1*- and *BRCA2*-associated and sporadic breast carcinomas [90–93].

_TP53_ - Li-Fraumeni syndrome
TP53 is a tumor suppressor gene, located on chromosome 17p13.1. It encodes a nuclear phosphoprotein (p53) that plays an important role in cell growth regulation [94]. p53 acts a transcription factor and is involved in regulation of DNA damage repair, genomic instability, cell cycle progression, senescence, and apoptosis [94,95]. Kinases such as ATM and CHEK2 are able to phosphorylate and stabilize the p53 protein [16].

Inherited *TP53* mutations are the cause of Li-Fraumeni syndrome, a highly penetrant disorder resulting in autosomal dominant predisposition to several types of cancer [96]. These include sarcomas, leukemias, brain cancer, adrenocortical carcinomas, and breast cancer [96–98].

The *TP53* mutation carrier rate is about 1:5000 births [99]. The lifetime risk of cancer approaches 100% [96]. About 50% of *TP53* mutation carriers will develop one of the associated cancers by age 30 [96]. Although less than 1% of all breast cancer cases are due to inherited *TP53* mutations [100], breast cancer is the most common malignancy in female patients with Li-Fraumeni syndrome and include 1/3 of all cancers in Li-Fraumeni syndrome families [6]. Breast cancer risk is estimated to be 56% by age 45 rising to >90% by age 60. This is a 60 fold increased risk compared to the general population [6]. The average age of diagnosis for breast cancer is 35 years old [97,100]. Most common mutations include missense mutations and are localized to the DNA

binding domain, although deletions of the promoter or coding region also occur frequently [101]. These mutations all yield an ineffective p53 protein.

Very little is known about breast cancer characteristics in these patients, although it has been published that the majority of breast cancers in Li-Fraumeni syndrome patients are HER2 positive [102,103]. There is no clear genotype-phenotype correlation [101]. In contrast to *BRCA1* and *BRCA2*, somatic *TP53* mutations are regularly found in sporadic breast cancer (approximately 50%) [30]. However, in *BRCA*-associated breast carcinomas the frequency of somatic *TP53* mutations is higher compared to sporadic breast carcinomas [62,104].

***PTEN* - Cowden/*PTEN* hamartoma tumor syndrome**

PTEN (phosphatase tensin homolog) is a tumor suppressor gene, located on chromosome 10q23.3. It encodes a protein having phosphatidylinositol-3-kinase (PI3K) phosphatase activity, thereby regulating the pro-survival PI3K/Akt/mTOR pathway [105]. Loss of *PTEN* function leads to increased cellular proliferation, migration, and survival, although the precise mechanisms remain unclear [99,106,107]. *PTEN* germline mutations give rise to the rare autosomal dominant cancer syndrome with incomplete penetrance, called Cowden disease. The prevalence of Cowden disease is 1:200.000-1:250.000 [108]. Cowden disease is characterized by increased proliferation of ectodermal, mesodermal, and endodermal tissues, and a high risk of developing multiple hamartomas and carcinomas, such as in the breast, bowel, thyroid gland, and endometrium [109–111]. About 80% of patients with Cowden disease have a mutation in the *PTEN* gene [112]. Most frequent genetic alterations include missense and frame shift mutations, insertions, and deletions. In 20% of patients the mutation is located in the *PTEN* promoter region [112].

Breast cancer is the most frequently occurring malignant tumor in patients with Cowden disease (<1% of total breast cancer cases). A couple of studies have shown that breast cancer pathology in Cowden disease patients is similar to sporadic breast cancer [113,114], although an association with basal-like phenotype or apocrine differentiation has also been reported [115]. It is known that *PTEN* loss leads to increased androgen signalling, characteristic for apocrine cells [116,117]. The lifetime risk for breast cancer in Cowden disease patients is about 50% with an average age at diagnosis between 36 and 46 years old [118,119]. About 75% of patients with Cowden disease will develop benign breast disease (fibroadenomas, hamartomas, adenosis, apocrine metaplasia) [118,119]. Benign breast lesions in patients with Cowden disease also frequently show apocrine differentiation [113,114]. Somatic *PTEN* mutations are known to occur in breast cancer,

mainly occurring in basal-like tumors, and especially in *BRCA1*-associated carcinomas, although there was no association with apocrine differentiation [120,121].

***CDH1* - hereditary diffuse gastric cancer syndrome**

CDH1 (Cadherin 1, E-cadherin), located on chromosome 16q22.1, encodes for the E-cadherin protein, a calcium dependent cell-cell adhesion glycoprotein, junctioning epithelial cell together [122,123]. *CDH1* germline mutations are the cause of the autosomal dominant hereditary diffuse gastric cancer syndrome and predispose to diffuse gastric cancer. The risk of developing diffuse gastric cancer in patients with *CDH1* germline mutations is 70-80% [124]. Furthermore, women with *CDH1* germline mutations have an increased risk of developing early onset lobular breast cancer. The lifetime risk is estimated as high as about 50% [124,125].

Loss of E-cadherin expression is typical of lobular breast cancer. Lobular breast cancer is characterized by a lack of glandular differentiation and rather shows a growth pattern of small strands and/or solitary cells reflecting the loss of this cell-cell adhesion molecule. As far as is known today, lobular breast cancers arising in *CDH1* germline mutation carriers have a comparable immunohistochemical and molecular phenotype compared to their sporadic counterparts.

The most common genetic alterations in *CDH1* include frameshift mutations (28.7%), splice-site mutations (27.0%) and non-sense mutations (19.7%), resulting in a loss of E-cadherin expression [126]. The second allele is inactivated somatically. Hypermethylation or LOH are infrequent causes of down-regulation or inactivation of the second allele in *CDH1* germline mutation carriers [127,128]. E-cadherin is also absent in 85% of sporadic lobular breast cancer [129]. Somatic *CDH1* alterations have also been found in sporadic lobular cancer in about 56% [125,127], including non-sense mutations and *CDH1* hypermethylation [127,130]. So far, no other studies describing the histopathological or molecular characteristics of *CDH1* mutation-associated breast cancers.

***SKT-11/LKB1* - Peutz-Jeghers syndrome**

The *STK11/LKB1* (serine-threonine kinase 11) tumor suppressor gene is located on chromosome 19p13.3, encoding a serine-threonine kinase that functions as an negative regulator of the mTOR pathway [131,132]. Peutz-Jeghers syndrome is an autosomal dominant disorder characterized by inactivating germline *STK11/LKB1* mutations, resulting in mucocutaneous pigmentation, gastrointestinal hamartomatous polyps, multiple uterine leiomyomas, and fibrocystic breast disease [16,133]. Moreover, these

patients have an increased risk of several malignant tumors, including colorectal, small bowel, pancreatic, gastric, thyroid, lung, breast, endometrial, ovarian and testis tumors [47]. The lifetime risk of developing breast cancer in females is 30-50% [134–136]. The median age of breast cancer diagnosis is 45 years [136]. Limited research on the characteristics of these tumors has suggested a similar phenotype to sporadic breast carcinomas [6].

INTERMEDIATELY PENETRANT GENES – INTERMEDIATE RISK CANCER SYNDROMES

These cancer syndromes result from germline mutations in intermediate penetrant genes that lead to a 1.5 – 5 times increased risk of developing breast cancer and comprise about 5% of familial breast cancer [10].

CHEK2

The *CHEK2* (Checkpoint kinase 2) gene is located on chromosome 22q12.1 and encodes a cell cycle checkpoint kinase [137,138]. The CHEK2 protein is activated by DNA double strand breaks and activates downstream repair proteins including BRCA1 and p53, playing an important role in DNA damage response by activating cell-cycle checkpoints and stimulating DNA repair [137,139,140]. The most common genetic alteration is the 1000delC variant yielding an unstable CHEK2 protein, often even associated with complete loss of expression [141].

Breast cancers due to an underlying *CHEK2* germline mutation account for <1% of total breast cancer cases in females and 9% of all male breast cancer cases [138,142]. Female patients with *CHEK2* germline mutations have a 2-3 fold increased risk of developing breast cancer (life-time risk about 37%) [99]. Analysis of the pathology of *CHEK2*-associated breast carcinomas yielded contradictory results. Most of the tumors are of high-grade and ER and PR positive, and HER2 negative [141,143]. They have a less favorable clinical outcome compared to sporadic breast carcinomas [144]. However, a lower grade compared to breast carcinomas without *CHEK2* mutation has also been reported as well as no differences in ER and PR status [144]. An association with lobular breast carcinomas has also been reported, especially in patients with the missense I157T variant [145]. *CHEK2*-associated breast cancers are mostly of luminal phenotype. The I157T variant is found to be associated with a luminal A phenotype and *CHEK2* truncating mutations appears to be associated with a luminal B phenotype [146]. Therefore, differences in tumor pathology may be associated with different underlying *CHEK2* mutations.

***ATM* - Ataxia-telangiectasia**

The *ATM* (Ataxia-telangiectasia mutated) gene is located on chromosome 11q22.3. It encodes a cell cycle checkpoint kinase having important functions in DNA double strand break repair and cell cycle progression [6]. It activates important players in DNA damage response such as p53, BRCA1 and CHEK2 [147]. Homozygous *ATM* mutations give rise to the rare autosomal recessive Ataxia-telangiectasia syndrome, characterized by cerebellar ataxia, immune deficiency, and increased risk of developing gliomas, medulloblastomas, lymphomas, and breast cancer [148,149]. Heterozygous mutations give a 2-5 fold increased breast cancer risk [150,151]. *ATM* germline mutations are quite common in the general Western population (prevalence 0.5 – 1%) [149,150]. Moreover, decreased *ATM* expression is found in 50-70% of sporadic breast tumors [152] and is associated with worse prognosis [153]. The histopathology of breast tumors arising in *ATM* mutation carriers does not significantly differ from sporadic controls [154].

Mismatch repair genes - Lynch syndrome

The mismatch repair genes *MLH1*, *MSH2*, *MSH6* and *PMS2* play an important role in DNA repair by correction of base mismatches, insertions or deletions, maintaining microsatellite stability [155]. About 1:500 people carry germline mutations in one of these mismatch repair genes [156]. Germline mutations in these genes cause the autosomal dominant Lynch syndrome, which is characterized by an increased risk of colorectal, stomach, breast, endometrial, and ovarian carcinomas [157]. An increased risk of breast cancer is mainly described in families with *MLH1* and *MSH2* mutations [158] and varies between a 2-18 fold increased risk compared to the general population [159]. The pathology of these breast carcinomas and whether they differ from sporadic breast carcinomas is still unclear.

***MRN* complex**

The MRN complex (MRE11, RAD50, NBS1) plays a key role in DNA double strand break repair by recruiting ATM to double strand breaks [160]. Mutations in these genes result in a 2-3 fold increased risk of developing breast cancer [161]. Most genetic alterations include missense mutations and protein-truncating variants [161]. Homozygous germline mutations cause Ataxia-telangiectasia-like disorder and Nijmegen Breakage Syndrome [162]. The pathology of tumors with inherited mutations in one of the genes of the MRN complex has not been investigated systematically yet.

RAD51C

RAD51C is an important component of the DNA double strand break repair through homologous recombination. Homozygous mutations in *RAD51C* give a phenotype

similar to Fanconi anemia. Heterozygous mutations are associated with increased breast cancer risk and occur in about 1% of hereditary breast cancer cases [163,164]. Although little is known about the pathology of *RAD51C*-associated breast carcinomas, they are mostly invasive ductal carcinomas of no special type and usually ER and PR positive, and HER2 negative (80%) [165]. Moreover, they are usually positive for luminal cytokeratins and BCL2, thereby exhibiting a luminal A phenotype [165].

Fanconi anemia

Fanconi anemia is a rare autosomal recessive disorder characterized by a defect in homologous recombination DNA repair, resulting in developmental anomalies, bone marrow failure and increased risk of malignant tumors [166–168]. Mutations in several genes involved in the Fanconi anemia pathway can cause an increased risk of developing breast cancer, including *PALB2* and *BRIP1*. The PALB2 and BRIP1 proteins are both BRCA2 binding partners [169,170]. Mutations in these genes yield a 2 fold increased risk of developing breast cancer [163,170,171]. They each comprise about 1% of total hereditary breast cancer cases [163,171]. Little is known about the pathology of Fanconi anemia-associated breast carcinomas. They appear to be often ER, PR, and HER2 negative [172], although differences in tumor pathology as well as survival may be explained by differences in genotype [173].

***MEN1* – MEN1 syndrome**

MEN1 (multiple endocrine neoplasia type 1) is a tumor suppressor gene located on chromosome 11q13. The MEN1 syndrome, caused by *MEN1* germline mutations, is characterized by parathyroid adenomas, gastrointestinal neuroendocrine tumors, and pituitary adenomas [174]. Recently, it was discovered that female MEN1 patients have an increased risk for breast cancer with a relative risk of 2.83 and mean age at diagnosis of 48 years [174]. Little is known about the pathology of *MEN1*-associated breast carcinomas, but so far most of the tumors were invasive ductal carcinomas of no special type and were ER and PR positive, and HER2 negative [174]. The mechanism of increased breast cancer risk is thought to be through the encoding of menin, which is a co-regulator of the estrogen receptor [174].

LOW PENETRANT GENES – LOW RISK CANCER SYNDROMES

These cancer syndromes result from germline mutations in low penetrant genes that lead to a 1-1.5 times increased risk of developing breast cancer and comprise about 15% of familial breast cancer [10].

Discovery of remaining potential breast cancer risk genes has been difficult, because of reasons such as low penetrance and polygenic or recessive mechanisms. However, due to techniques such as genome-wide association studies novel rare breast cancer risk genes or loci have been identified.

Genes include *FGFR2* [175,176], *TNRC9* [175], *MRPS30*) [175], *MAPK3K1* [175], *CASP8* [177], and *LSP1* [175]. Risk loci that have been found are 2q35 [178], 5p12 [179], 6q25.1 [180,181], 8q24 [175], and 19p13 [182]. Some of these genes or loci are associated with ER positive disease (such as *FGFR2* and 5p12) [179], others are associated with triple-negative disease (such as 6q25.1 [180] and 19p13 [182]). For many of these genes and loci the relative risk of developing breast cancer is slightly above 1.0 and they may also act as modulators of breast cancer risk in patients carrying germline mutations in high and intermediate risk genes [163].

***BRCAX* families**

Families remain by which the frequent occurrence of breast cancer cannot be explained by above mentioned high, intermediate and low risk genes (about 50% of all breast cancer cases) and are referred to as '*BRCAX*' families [6–10]. These families may carry a mutation in breast cancer genes that still need to be identified or in which the increased breast cancer risk can be attributed by a combination of genetic alterations in several low-penetrant genes whether or not in combination with environmental factors [10,99,175].

CLINICAL APPLICATION

Early detection

Research from the last two decades has yielded important insights into the biological characteristics of hereditary breast cancers. This knowledge has to some extent already been translated into clinical practice and this will hopefully further expand in the near future.

Identifying potential hereditary breast tumors is important as it necessitates not only preventive interventions but also specific therapeutic measures, as chemosensitivity for these tumors is different (see also below). Currently, eligibility for genetic testing is based on clinical characteristics such as young age at tumor diagnosis, bilaterality of tumors and positive family history for breast, Fallopian tube/ovarian and/or some other types of cancer. Genetic testing is time consuming and expensive. For example,

BRCA1 and *BRCA2* are large size genes and are characterized by an absence of mutation hotspots. Moreover, potential germline mutation carriers may be missed, since families are nowadays small, the family history may be incomplete, and penetrance of mutations is variable. Furthermore, for some identified *BRCA1* and *BRCA2* mutations it is unclear whether they are pathogenic (so-called 'unclassified variants') [183]. This could eventually lead to incorrect treatment for the patients and their relatives. For example, patients aware of a possible hereditary breast cancer may choose for a therapeutic mastectomy and contralateral prophylactic mastectomy instead of breast conserving therapy. Therefore, it is highly relevant to identify specific clues in the tumor tissues for a potentially hereditary origin of breast cancer. These so-called tumor signatures could be used as tool for pre-selecting patients for genetic testing [3].

Previous studies have shown that morphological, immunohistochemical, and molecular signatures could have potential in early detection of hereditary breast carcinomas and interpretation of unclassified variants. Most research is performed on *BRCA1*- and *BRCA2*-associated breast carcinomas, as they comprise the largest group of hereditary breast cancers. Especially *BRCA1*-associated breast carcinomas form a quite distinctive group from sporadic breast carcinomas being typically of high grade and basal-like phenotype, whereas *BRCA2*- and other risk gene-associated breast carcinomas form a more heterogeneous group and show many similarities with sporadic breast cancer (i.e. lower grade and luminal phenotypes) [38,68].

Several studies have been performed investigating the association between molecular breast cancer subtypes and hereditary breast cancer. This association is strongest with triple-negative or basal-like breast cancer and *BRCA1* germline mutations. About 20% of triple-negative breast cancer patients have an underlying *BRCA1* germline mutation [184]. Using gene expression profiles, a prediction of *BRCA1* or *BRCA2* signature could be made with high accuracy (82-87%), using a panel of 100 genes for *BRCA1* and 110 genes for *BRCA2* [39]. Array CGH studies also yielded *BRCA1* and *BRCA2* signatures characterized by specific genomic gains and losses [185–188], yielding a sensitivity of 88% and specificity of 94% for *BRCA1* [187], and a sensitivity of 89% and specificity of 84% for *BRCA2* [188]. A combination of morphological and immunochemical characteristics has also been used to select patients for *BRCA1* genetic testing, using a combination of age, grade, ki67 proliferative activity, and expression of EGFR, MS110, lys27H3, and vimentin [189,190]. However, a standardized method for detecting hereditary breast carcinomas has not been developed yet.

Therapeutic consequences

Breast cancer surveillance takes place through regular imaging. The age to start screening depends on the risk level and varies between 25 and 40 years [191]. Screening mammographies in patients <30 years old are not advised as ionizing radiation itself is risk factor for breast cancer development and these patients usually have dense breasts that makes mammography unreliable [192]. In very young patients, screening is performed by MRI. Prophylactic treatment is advised from the age of 25 in high-risk patients and consists of bilateral mastectomy, which reduces the risk of breast cancer by almost 100% [193]. Especially in *BRCA1* and *BRCA2* germline mutation carriers, it is advised to also undergo prophylactic salpingo-oophorectomy from the age of 35 [193,194].

Treatment for breast cancer includes (a combination of) surgery, radiotherapy, chemotherapy, and hormonal therapy. Although usually mutation carriers choose to undergo mastectomy and prophylactic mastectomy of the contralateral breast, these patients can also be treated by conservative surgery (i.e. combination of lumpectomy and radiotherapy), although data are scarce [195]. It is known that *BRCA1*- and *BRCA2*-associated breast carcinomas show good radiosensitivity [195]. Above described hereditary breast cancer signatures could also aid in selecting patients sensitive to specific therapeutic options. For example, tumors with dysfunctional *BRCA1* or *BRCA2*, or *BRCA1/2*-like signature are very sensitive to specific DNA damaging agents that cause DNA cross-linking, such as cisplatin, mitomycin-c, and diepoxybutane [166,167,196,197]. This reflects the need of homologous recombination as a means to repair the damage caused by these agents [17].

Moreover, understanding of the molecular mechanisms underlying breast cancer predisposition genes may offer opportunities for new, targeted adjuvant and preventive therapies. For instance, poly (ADP ribose) polymerase (PARP) inhibitors have been shown to be especially effective in BRCA-deficient tumors with dysfunctional homologous recombination. PARPs are enzymes involved in base excision repair. PARP inhibition leads to single strand breaks that will turn into double strand breaks in replication forks. To repair these double strand DNA breaks, homologous recombination is required to be repaired, which in turns requires functional BRCA1 and BRCA2. Tumors cells deficient in BRCA1 or BRCA2 protein will therefore show severe chromosomal instability, cell cycle arrest and apoptosis when treated with PARP inhibitors [14,155]. Clinical trials have shown the efficacy of PARP inhibitors in BRCA-deficient carcinomas [198,199]. Moreover, they might be interesting as a preventive treatment tool. A pre-clinical mouse study has proven efficacy of PARP inhibitors in the prevention of breast carcinomas in BRCA-deficient mice (unpublished data by our group).

In the future, signatures based upon morphological, immunohistochemical and/or molecular tumor characteristics that reflect the status of *BRCA* and other important pathways in hereditary breast cancer may be developed that could aid in therapy selection [17]. Moreover, these assays may also be applicable to sporadic breast cancer as they may show deregulation of these pathways as well. For example, triple-negative sporadic breast carcinomas, sporadic breast tumors with *BRCA1* hypermethylation or EMSY amplification may also benefit from therapies such as DNA cross-linking agents and PARP inhibitors that require deficient homologous recombination.

CONCLUSION

In the past two decades, tremendous research has been performed on the histopathological, immunohistochemical and molecular characterization of hereditary breast cancer. This has yielded important knowledge on the biological processes underlying hereditary breast cancer. In this review, an overview has been given on the main breast cancer risk syndromes and their clinical and pathological characteristics. *BRCA1*-associated breast cancers appear to be the most distinctive group, which can nowadays fairly well be recognized. However, further research is needed, especially on non-*BRCA1* hereditary breast cancer risk syndromes to further improve detection of at risk patients, risk estimation and selection of patients who will benefit from newly developed targeted adjuvant and preventive therapies.

REFERENCES

1 Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013; 63: 11-30

2 Rich TA, Woodson AH, Litton J, et al. Hereditary breast cancer syndromes and genetic testing. J Surg Oncol 2015; 111: 66-80

3 Larsen MJ, Thomassen M, Gerdes A, et al. Hereditary Breast Cancer: Clinical, Pathological and Molecular Characteristics. Breast Cancer 2014; 8: 145-55

4 Lalloo F, Evans DG. Familial breast cancer. Clin Genet 2012; 82: 105-114

5 Claus EB, Schildkraut J, Iversen ES, et al. Effect of BRCA1 and BRCA2 on the association between breast cancer risk and family history. J Natl Cancer Inst 1998; 90: 1824-1829

6 Economopoulou P, Dimitriadis G, Psyrri a. Beyond BRCA: New hereditary breast cancer susceptibility genes. Cancer Treat Rev 2015; 41: 1-8

7 Lynch HT, Snyder C, Casey MJ. Hereditary ovarian and breast cancer: What have we learned. Ann Oncol 2013; 24: viii83-viii95

8 Filippini SE, Vega A. Breast cancer genes: beyond BRCA1 and BRCA2. Front Biosci (Landmark Ed) 2013; 18: 1358-1372

9 Carroll JC, Cremin C, Allanson J, et al. Hereditary breast and ovarian cancers. Can Fam Physician 2008; 54: 1691-1692

10 Melchor L, Benítez J. The complex genetic landscape of familial breast cancer. Hum Genet 2013; 132: 845-863

11 Hall JM, Lee MK, Newman B, et al. Linkage of early-onset familial breast cancer to chromosome 17q21. Science 1990; 250: 1684-1689

12 Miki Y, Swensen J, Shattuck-Eidens D, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 1994; 266: 66-71

13 Wooster R, Neuhausen SL, Mangion J, et al. Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. Science 1994; 265: 2088-2090

14 Turnbull C, Rahman N. Genetic predisposition to breast cancer: past, present, and future. Annu Rev Genomics Hum Genet 2008; 9: 321-345

15 Hedenfalk I, Ringner M, Ben-Dor A, et al. Molecular classification of familial non-BRCA1/BRCA2 breast cancer. Proc Natl Acad Sci U S A 2003; 100: 2532-2537

16 Nagy R, Sweet K, Eng C. Highly penetrant hereditary cancer syndromes. Oncogene 2004; 23: 6445-6470

17 Turner N, Tutt A, Ashworth A. Hallmarks of 'BRCAness' in sporadic cancers. Nat Rev Cancer 2004; 4: 814-819

18 Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nat Rev Cancer 2012; 12: 68-78

19 Cortez D, Wang Y, Qin J, et al. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. Science 1999; 286: 1162-1166

20 Tibbetts RS, Cortez D, Brumbaugh KM, et al. Functional interactions between BRCA1 and the checkpoint kinase ATR during genotoxic stress. Genes Dev 2000; 14: 2989-3002

21 Fleck O, Nielsen O. DNA repair. J Cell Sci 2004; 117: 515-517

22 Taniguchi T, Garcia-Higuera I, Andreassen PR, et al. S-phase-specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. Blood 2002; 100: 2414-2420

23 Abeliovich D, Kaduri L, Lerer I, et al. The founder mutations 185delAG and 5382insC in BRCA1 and 6174delT in BRCA2 appear in 60% of ovarian cancer and 30% of early-onset breast cancer patients among Ashkenazi women. Am J Hum Genet 1997; 60: 505-514

24 Thorlacius S, Olafsdottir G, Tryggvadottir L, et al. A single BRCA2 mutation in male and female breast cancer families from Iceland with varied cancer phenotypes. Nat Genet 1996; 13: 117-119

25 Antoniou A, Pharoah PDP, Narod S, et al. Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. Am J Hum Genet 2003; 72: 1117-1130

26 Thompson D, Easton DF. Cancer Incidence in BRCA1 mutation carriers. J Natl Cancer Inst 2002; 94: 1358-1365

27 Struewing JP, Hartge P, Wacholder S, et al. The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews. N Engl J Med 1997; 336: 1401-1408

28 Ford D, Easton DF, Bishop DT, et al. Risks of cancer in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. Lancet 1994; 343: 692-695

29 van Asperen CJ. Cancer risks in BRCA2 families: estimates for sites other than breast and ovary. J Med Genet 2005; 42: 711-719

30 Ellisen L, MD, PhD. Hereditary breast cancer. Annu Rev 1998: 697-709

31 Honrado E, Benítez J, Palacios J. The molecular pathology of hereditary breast cancer: genetic testing and therapeutic implications. Mod Pathol 2005; 18: 1305-1320

32 Collins N, McManus R, Wooster R, et al. Consistent loss of the wild type allele in breast cancers from a family linked to the BRCA2 gene on chromosome 13q12-13. Oncogene 1995; 10: 1673-1675

33 Cornelis RS, Neuhausen SL, Johansson O, et al. High allele loss rates at 17q12-q21 in breast and ovarian tumors from BRCAl-linked families. The Breast Cancer Linkage Consortium. Genes Chromosomes Cancer 1995; 13: 203-210

34 Khoo U-S, Ozcelik H, Cheung AN, et al. Somatic mutations in the BRCA1 gene in Chinese sporadic breast and ovarian cancer. Oncogene 1999; 18: 4643-4646

35 Esteller M, Silva JM, Dominguez G, et al. Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J Natl Cancer Inst 2000; 92: 564-569

36 Tapia T, Smalley S V, Kohen P, et al. Promoter hypermethylation of BRCA1 correlates with absence of expression in hereditary breast cancer tumors. Epigenetics 2008; 3: 157-163

37 Birgisdottir V, Stefansson OA, Bodvarsdottir SK, et al. Epigenetic silencing and deletion of the BRCA1 gene in sporadic breast cancer. Breast Cancer Res 2006; 8: R38

38 Honrado E, Osorio A, Milne RL, et al. Immunohistochemical classification of non-BRCA1/2 tumors identifies different groups that demonstrate the heterogeneity of BRCAX families. Mod Pathol 2007; 20: 1298-1306

39 Larsen MJ, Thomassen M, Tan Q, et al. RNA profiling reveals familial aggregation of molecular subtypes in non-BRCA1/2 breast cancer families. BMC Med Genomics 2014; 7: 9

40 Collins N, Wooster R, Stratton MR. Absence of methylation of CpG dinucleotides within the promoter of the breast cancer susceptibility gene BRCA2 in normal tissues and in breast and ovarian cancers. Br J Cancer 1997; 76: 1150-1156

41 Hughes-Davies L, Huntsman D, Ruas M, et al. EMSY links the BRCA2 pathway to sporadic breast and ovarian cancer. Cell 2003; 115: 523-535

42 Yao J, Polyak K. EMSY links breast cancer gene 2 to the 'Royal Family'. Breast Cancer Res 2004; 6: 201-203

43 Livingston DM. EMSY, a BRCA-2 partner in crime. Nat Med 2004; 10: 127-128

44 Nik-Zainal S, Alexandrov LB, Wedge DC, et al. Mutational processes molding the genomes of 21 breast cancers. Cell 2012; 149: 979-993

45 Yuan SS, Lee SY, Chen G, et al. BRCA2 is required for ionizing radiation-induced assembly of Rad51 complex in vivo. Cancer Res 1999; 59: 3547-3551

46 Zhong Q, Chen CF, Li S, et al. Association of BRCA1 with the hRad50-hMre11-p95 complex and the DNA damage response. Science 1999; 285: 747-750

47 Bradbury AR, Olopade OI. Genetic susceptibility to breast cancer. Rev Endocr Metab Disord 2007; 8: 255-267

48 Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. Nature 2004; 432: 316-323

49 O'Donovan PJ, Livingston DM. BRCA1 and BRCA2: breast/ovarian cancer susceptibility gene products and participants in DNA double-strand break repair. Carcinogenesis 2010; 31: 961-967

50 Venkitaraman AR. Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell 2002; 108: 171-182

51 Bertwistle D, Ashworth A. Functions of the BRCA1 and BRCA2 genes. Curr Opin Genet Dev 1998; 8: 14-20

52 Deng CX, Scott F. Role of the tumor suppressor gene Brca1 in genetic stability and mammary gland tumor formation. Oncogene 2000; 19: 1059-1064

53 Welcsh PL, Owens KN, King MC. Insights into the functions of BRCA1 and BRCA2. Trends Genet 2000; 16: 69-74

54 Ballal RD, Saha T, Fan S, et al. BRCA1 localization to the telomere and its loss from the telomere in response to DNA damage. J Biol Chem 2009; 284: 36083-36098

55 Narod SA, Foulkes WD. BRCA1 and BRCA2: 1994 and beyond. Nat Rev Cancer 2004; 4: 665-676

56 Lakhani SR, Van De Vijver MJ, Jacquemier J, et al. The pathology of familial breast cancer: predictive value of immunohistochemical markers estrogen receptor, progesterone receptor, HER-2, and p53 in patients with mutations in BRCA1 and BRCA2. J Clin Oncol 2002; 20: 2310-2318

57 Sensi E, Tancredi M, Aretini P, et al. p53 Inactivation is a Rare Event in Familial Breast Tumors Negative for BRCA1 and BRCA2 Mutations. Breast Cancer Res Treat 2003; 82: 1-9

58 Greenblatt MS, Chappuis PO, Bond JP, et al. TP53 mutations in breast cancer associated with BRCA1 or BRCA2 germ-line mutations: distinctive spectrum and structural distribution. Cancer Res 2001; 61: 4092-4097

59 Phillips KA, Andrulis IL, Goodwin PJ. Breast carcinomas arising in carriers of mutations in BRCA1 or BRCA2: are they prognostically different? J Clin Oncol 1999; 17: 3653-3663

60 Armes JE, Trute L, White D, et al. Distinct molecular pathogeneses of early-onset breast cancers in BRCA1 and BRCA2 mutation carriers: a population-based study. Cancer Res 1999; 59: 2011-2017

61 Crook T, Brooks LA, Crossland S, et al. p53 mutation with frequent novel codons but not a mutator phenotype in BRCA1- and BRCA2-associated breast tumours. Oncogene 1998; 17: 1681-1689

62 Holstege H, Joosse SA, van Oostrom CTM, et al. High Incidence of Protein-Truncating TP53 Mutations in BRCA1-Related Breast Cancer. Cancer Res 2009; 69: 3625-3633

63 Vargas AC, Da Silva L, Lakhani SR. The contribution of breast cancer pathology to statistical models to predict mutation risk in BRCA carriers. Fam Cancer 2010; 9: 545-553

64 Pathology of familial breast cancer: differences between breast cancers in carriers of BRCA1 or BRCA2 mutations and sporadic cases. Breast Cancer Linkage Consortium. Lancet 1997; 349: 1505-1510

65 Lakhani SR, Jacquemier J, Sloane JP, et al. Multifactorial analysis of differences between sporadic breast cancers and cancers involving BRCA1 and BRCA2 mutations. J Natl Cancer Inst 1998; 90: 1138-1145

66 Palacios J, Robles-Frías MJ, Castilla M a, et al. The molecular pathology of hereditary breast cancer. Pathobiology 2008; 75: 85-94

67 Lynch BJ, Holden J a, Buys SS, et al. Pathobiologic characteristics of hereditary breast cancer. Hum Pathol 1998; 29: 1140-1144

68 Lakhani SR, Gusterson BA, Jacquemier J, et al. The pathology of familial breast cancer: histological features of cancers in families not attributable to mutations in BRCA1 or BRCA2. Clin Cancer Res 2000; 6: 782-789

69 Palacios J, Honrado E, Osorio A, et al. Immunohistochemical characteristics defined by tissue microarray of hereditary breast cancer not attributable to BRCA1 or BRCA2 mutations: differences from breast carcinomas arising in BRCA1 and BRCA2 mutation carriers. Clin Cancer Res 2003; 9: 3606-3614

70 Osin PP, Lakhani SR. The pathology of familial breast cancer: Immunohistochemistry and molecular analysis. Breast Cancer Res 1999; 1: 36-40

71 Mavaddat N, Barrowdale D, Andrulis IL, et al. Pathology of breast and ovarian cancers among BRCA1 and BRCA2 mutation carriers: results from the Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA). Cancer Epidemiol Biomarkers Prev 2012; 21: 134-147

72 Foulkes WD, Metcalfe K, Sun P, et al. Estrogen receptor status in BRCA1- and BRCA2-related breast cancer: the influence of age, grade, and histological type. Clin Cancer Res 2004; 10: 2029-2034

73 Foulkes WD, Stefansson IM, Chappuis PO, et al. Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. J Natl Cancer Inst 2003; 95: 1482-1485

74 Lakhani SR, Reis-Filho JS, Fulford L, et al. Prediction of BRCA1 status in patients with breast cancer using estrogen receptor and basal phenotype. Clin Cancer Res 2005; 11: 5175-5180

75 Thompson ME, Jensen RA, Obermiller PS, et al. Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. Nat Genet 1995; 9: 444-450

76 Armes JE, Egan a J, Southey MC, et al. The histologic phenotypes of breast carcinoma occurring before age 40 years in women with and without BRCA1 or BRCA2 germline mutations: a population-based study. Cancer 1998; 83: 2335-2345

77 Freneaux P, Stoppa-Lyonnet D, Mouret E, et al. Low expression of bcl-2 in Brca1-associated breast cancers. Br J Cancer 2000; 83: 1318-1322

78 Osin P, Gusterson BA, Philp E, et al. Predicted anti-oestrogen resistance in BRCA-associated familial breast cancers. Eur J Cancer 1998; 34: 1683-1686

79 Chappuis PO, Donato E, Goffin JR, et al. Cyclin E expression in breast cancer: predicting germline BRCA1 mutations, prognosis and response to treatment. Ann Oncol 2005; 16: 735-742

80 Palacios J, Honrado E, Osorio A, et al. Phenotypic characterization of BRCA1 and BRCA2 tumors based in a tissue microarray study with 37 immunohistochemical markers. Breast Cancer Res Treat 2005; 90: 5-14

81 van der Groep P, Bouter A, Menko FH, et al. High frequency of HIF-1alpha overexpression in BRCA1 related breast cancer. Breast Cancer Res Treat 2008; 111: 475-480

82 Rebbeck TR, Mitra N, Domchek SM, et al. Modification of BRCA1-Associated Breast and Ovarian Cancer Risk by BRCA1-Interacting Genes. Cancer Res 2011; 71: 5792-5805

83 Agnarsson BA, Jonasson JG, Björnsdottir IB, et al. Inherited BRCA2 mutation associated with high grade breast cancer. Breast Cancer Res Treat 1998; 47: 121-127

84 Bane AL, Beck JC, Bleiweiss I, et al. BRCA2 mutation-associated breast cancers exhibit a distinguishing phenotype based on morphology and molecular profiles from tissue microarrays. Am J Surg Pathol 2007; 31: 121-128

85 Bane AL, Pinnaduwage D, Colby S, et al. Expression profiling of familial breast cancers demonstrates higher expression of FGFR2 in BRCA2-associated tumors. Breast Cancer Res Treat 2009; 117: 183-191

86 van der Groep P, van Diest PJ, Smolders YHCM, et al. HIF-1α overexpression in ductal carcinoma in situ of the breast in BRCA1 and BRCA2 mutation carriers. PLoS One 2013; 8: e56055

87 Hoogerbrugge N, Bult P, de Widt-Levert LM, et al. High prevalence of premalignant lesions in prophylactically removed breasts from women at hereditary risk for breast cancer. J Clin Oncol 2003; 21: 41-45

88 Hermsen BBJ, van Diest PJ, Berkhof J, et al. Low prevalence of (pre) malignant lesions in the breast and high prevalence in the ovary and Fallopian tube in women at hereditary high risk of breast and ovarian cancer. Int J Cancer 2006; 119: 1412-1418

89 Cavalli LR, Singh B, Isaacs C, et al. Loss of heterozygosity in normal breast epithelial tissue and benign breast lesions in BRCA1/2 carriers with breast cancer. Cancer Genet Cytogenet 2004; 149: 38-43

90 Goodwin PJ, Phillips K-A, West DW, et al. Breast Cancer Prognosis in BRCA1 and BRCA2 Mutation Carriers: An International Prospective Breast Cancer Family Registry Population-Based Cohort Study. J Clin Oncol 2011; 30: 19-26

91 Stoppa-Lyonnet D, Ansquer Y, Dreyfus H, et al. Familial invasive breast cancers: worse outcome related to BRCA1 mutations. J Clin Oncol 2000; 18: 4053-4059

92 Verhoog LC, Brekelmans CT, Seynaeve C, et al. Survival and tumour characteristics of breast-cancer patients with germline mutations of BRCA1. Lancet 1998; 351: 316-321

93 Robson ME, Chappuis PO, Satagopan J, et al. A combined analysis of outcome following breast cancer: differences in survival based on BRCA1/BRCA2 mutation status and administration of adjuvant treatment. Breast Cancer Res 2004; 6: R8-R17

94 Menendez D, Inga A, Resnick MA. The expanding universe of p53 targets. Nat Rev Cancer 2009; 9: 724-737

95 Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000; 408: 307-310

96 McBride KA, Ballinger ML, Killick E, et al. Li-Fraumeni syndrome: cancer risk assessment and clinical management. Nat Rev Clin Oncol 2014; 11: 260-271

97 Li FP, Fraumeni JF. Soft-tissue sarcomas, breast cancer, and other neoplasms. A familial syndrome? Ann Intern Med 1969; 71: 747-752

98 Malkin D, Li FP, Strong LC, et al. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science 1990; 250: 1233-1238

99 Yiannakopoulou E. Etiology of familial breast cancer with undetected BRCA1 and BRCA2 mutations: clinical implications. Cell Oncol (Dordr) 2014; 37: 1-8

100 Sidransky D, Tokino T, Helzlsouer K, et al. Inherited p53 gene mutations in breast cancer. Cancer Res 1992; 52: 2984-2986

101 Kamihara J, Rana HQ, Garber JE. Germline TP53 mutations and the changing landscape of Li-Fraumeni syndrome. Hum Mutat 2014; 35: 654-662

102 Melhem-Bertrandt A, Bojadzieva J, Ready KJ, et al. Early onset HER2-positive breast cancer is associated with germline TP53 mutations. Cancer 2012; 118: 908-913

103 Wilson JRF, Bateman AC, Hanson H, et al. A novel HER2-positive breast cancer phenotype arising from germline TP53 mutations. J Med Genet 2010; 47: 771-774

104 Chappuis PO, Nethercot V, Foulkes WD. Clinico-pathological characteristics of BRCA1- and BRCA2-related breast cancer. Semin Surg Oncol 2000; 18: 287-295

105 Blumenthal GM, Dennis PA. PTEN hamartoma tumor syndromes. Eur J Hum Genet 2008; 16: 1289-1300

106 Li J, Yen C, Liaw D, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 1997; 275: 1943-1947

107 Shen WH, Balajee AS, Wang J, et al. Essential role for nuclear PTEN in maintaining chromosomal integrity. Cell 2007; 128: 157-170

108 Nelen MR, Kremer H, Konings IB, et al. Novel PTEN mutations in patients with Cowden disease: absence of clear genotype-phenotype correlations. Eur J Hum Genet 1999; 7: 267-273

109 Nelen MR, Padberg GW, Peeters EA, et al. Localization of the gene for Cowden disease to chromosome 10q22-23. Nat Genet 1996; 13: 114-116

110 Hanssen AM, Fryns JP. Cowden syndrome. J Med Genet 1995; 32: 117-119

111 Liaw D, Marsh DJ, Li J, et al. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet 1997; 16: 64-67

112 Zhou X-P, Waite KA, Pilarski R, et al. Germline PTEN promoter mutations and deletions in Cowden/Bannayan-Riley-Ruvalcaba syndrome result in aberrant PTEN protein and dysregulation of the phosphoinositol-3-kinase/Akt pathway. Am J Hum Genet 2003; 73: 404-411

113 Schrager CA, Schneider D, Gruener AC, et al. Clinical and pathological features of breast disease in Cowden's syndrome: an underrecognized syndrome with an increased risk of breast cancer. Hum Pathol 1998; 29: 47-53

114 Schrager CA, Schneider D, Gruener AC, et al. Similarities of cutaneous and breast pathology in Cowden's Syndrome. Exp Dermatol 1998; 7: 380-390

115 Comprehensive molecular portraits of human breast tumours. Nature 2012; 490: 61-70

116 Lin H-K, Hu Y-C, Lee DK, et al. Regulation of androgen receptor signaling by PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor through distinct mechanisms in prostate cancer cells. Mol Endocrinol 2004; 18: 2409-2423

117 Nan B, Snabboon T, Unni E, et al. The PTEN tumor suppressor is a negative modulator of androgen receptor transcriptional activity. J Mol Endocrinol 2003; 31: 169-183

118 Brownstein MH, Wolf M, Bikowski JB. Cowden's disease: a cutaneous marker of breast cancer. Cancer 1978; 41: 2393-2398

119 Starink TM, van der Veen JP, Arwert F, et al. The Cowden syndrome: a clinical and genetic study in 21 patients. Clin Genet 1986; 29: 222-233

120 Bonneau D, Longy M. Mutations of the human PTEN gene. Hum Mutat 2000; 16: 109-122

121 COSMIC Catalogue of somatic mutations in cancer.

122 Graziano F, Humar B, Guilford P. The role of the E-cadherin gene (CDH1) in diffuse gastric cancer susceptibility: from the laboratory to clinical practice. Ann Oncol 2003; 14: 1705-1713

123 Becker KF, Atkinson MJ, Reich U, et al. E-cadherin gene mutations provide clues to diffuse type gastric carcinomas. Cancer Res 1994; 54: 3845-3852

124 Pharoah PD, Guilford P, Caldas C. Incidence of gastric cancer and breast cancer in CDH1 (E-cadherin) mutation carriers from hereditary diffuse gastric cancer families. Gastroenterology 2001; 121: 1348-1353

125 Schrader K a., Masciari S, Boyd N, et al. Hereditary diffuse gastric cancer: Association with lobular breast cancer. Fam Cancer 2008; 7: 73-82

126 Oliveira C, Pinheiro H, Figueiredo J, et al. E-cadherin alterations in hereditary disorders with emphasis on hereditary diffuse gastric cancer. Prog Mol Biol Transl Sci 2013; 116: 337-359

127 Pharoah PD, Guilford P, Caldas C. Incidence of gastric cancer and breast cancer in CDH1 (E-cadherin) mutation carriers from hereditary diffuse gastric cancer families. Gastroenterology 2001; 121: 1348-1353

128 Oliveira C, Sousa S, Pinheiro H, et al. Quantification of Epigenetic and Genetic 2nd Hits in CDH1 During Hereditary Diffuse Gastric Cancer Syndrome Progression. Gastroenterology 2009; 136: 2137-2148

129 Berx G, Van Roy F. The E-cadherin/catenin complex: an important gatekeeper in breast cancer tumorigenesis and malignant progression. Breast Cancer Res 2001; 3: 289-293

130 Asiaf A, Ahmad ST, Aziz SA, et al. Loss of Expression and Aberrant Methylation of the CDH1 (E-cadherin) Gene in Breast Cancer Patients from Kashmir. 2014; 15: 6397-6403

131 Collins SP, Reoma JL, Gamm DM, et al. LKB1, a novel serine/threonine protein kinase and potential tumour suppressor, is phosphorylated by cAMP-dependent protein kinase (PKA) and prenylated in vivo. Biochem J 2000; 345 Pt 3: 673-680

132 Corradetti MN, Inoki K, Bardeesy N, et al. Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. Genes Dev 2004; 18: 1533-1538

133 Hemminki A, Markie D, Tomlinson I, et al. A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. Nature 1998; 391: 184-187

134 Giardiello FM, Brensinger JD, Tersmette AC, et al. Very high risk of cancer in familial Peutz-Jeghers syndrome. Gastroenterology 2000; 119: 1447-1453

135 Hearle N. Frequency and Spectrum of Cancers in the Peutz-Jeghers Syndrome. Clin Cancer Res 2006; 12: 3209-3215

136 Lim W, Olschwang S, Keller JJ, et al. Relative frequency and morphology of cancers in STK11 mutation carriers. Gastroenterology 2004; 126: 1788-1794

137 Boardman LA, Thibodeau SN, Schaid DJ, et al. Increased risk for cancer in patients with the Peutz-Jeghers syndrome. Ann Intern Med 1998; 128: 896-899

138 Meijers-Heijboer H, van den Ouweland A, Klijn J, et al. Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations. Nat Genet 2002; 31: 55-59

139 Stracker TH, Usui T, Petrini JHJ. Taking the time to make important decisions: the checkpoint effector kinases Chk1 and Chk2 and the DNA damage response. DNA Repair (Amst) 2009; 8: 1047-1054

140 Chehab NH, Malikzay A, Appel M, et al. Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes Dev 2000; 14: 278-288

141 Nevanlinna H, Bartek J. The CHEK2 gene and inherited breast cancer susceptibility. Oncogene 2006; 25: 5912-5919

142 CHEK2 Breast Cancer Case-Control Consortium. CHEK2*1100delC and Susceptibility to Breast Cancer: A Collaborative Analysis Involving 10,860 Breast Cancer Cases and 9,065 Controls from 10 Studies. Am J Hum Genet 2004; 74: 1175-1182

143 Cybulski C, Huzarski T, Byrski T, et al. Estrogen receptor status in CHEK2-positive breast cancers: implications for chemoprevention. Clin Genet 2009; 75: 72-78

144 de Bock GH. Tumour characteristics and prognosis of breast cancer patients carrying the germline CHEK2*1100delC variant. J Med Genet 2004; 41: 731-735

145 Huzarski T, Cybulski C, Domagała W, et al. Pathology of breast cancer in women with constitutional CHEK2 mutations. Breast Cancer Res Treat 2005; 90: 187-189

146 Domagala P, Wokolorczyk D, Cybulski C, et al. Different CHEK2 Germline mutations are associated with distinct immunophenotypic molecular subtypes of breast cancer. Breast Cancer Res Treat 2012; 132: 937-945

147 Weischer M, Bojesen SE, Ellervik C, et al. CHEK2*1100delC genotyping for clinical assessment of breast cancer risk: meta-analyses of 26,000 patient cases and 27,000 controls. J Clin Oncol 2008; 26: 542-548

148 Ahmed M, Rahman N. ATM and breast cancer susceptibility. Oncogene 2006; 25: 5906-5911

149 Swift M, Morrell D, Massey RB, et al. Incidence of cancer in 161 families affected by ataxia-telangiectasia. N Engl J Med 1991; 325: 1831-1836

150 Thompson D, Duedal S, Kirner J, et al. Cancer risks and mortality in heterozygous ATM mutation carriers. J Natl Cancer Inst 2005; 97: 813-822

151 Renwick A, Thompson D, Seal S, et al. ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles. Nat Genet 2006; 38: 873-875

152 Hall J. The Ataxia-telangiectasia mutated gene and breast cancer: gene expression profiles and sequence variants. Cancer Lett 2005; 227: 105-114

153 Bueno RC, Canevari RA, Villacis RAR, et al. ATM down-regulation is associated with poor prognosis in sporadic breast carcinomas. Ann Oncol 2013; 25: 69-75

154 Mangia a, Malfettone a, Simone G, et al. Old and new concepts in histopathological characterization of familial breast cancer. Ann Oncol 2011; 22 Suppl 1: i24-30

155 van der Groep P, van der Wall E, van Diest PJ. Pathology of hereditary breast cancer. Cell Oncol (Dordr) 2011; 34: 71-88

156 Steinke V, Engel C, Büttner R, et al. Hereditary nonpolyposis colorectal cancer (HNPCC)/ Lynch syndrome. Dtsch Arztebl Int 2013; 110: 32-38

157 Weissman SM, Bellcross C, Bittner CC, et al. Genetic counseling considerations in the evaluation of families for Lynch syndrome--a review. J Genet Couns 2011; 20: 5-19

158 Shanley S, Fung C, Milliken J, et al. Breast cancer immunohistochemistry can be useful in triage of some HNPCC families. Fam Cancer 2009; 8: 251-255

159 Win AK, Lindor NM, Jenkins MA. Risk of breast cancer in Lynch syndrome: a systematic review. Breast Cancer Res 2013; 15: R27

160 Williams GJ, Lees-Miller SP, Tainer JA. Mre11-Rad50-Nbs1 conformations and the control of sensing, signaling, and effector responses at DNA double-strand breaks. DNA Repair (Amst) 2010; 9: 1299-1306

161 Damiola F, Pertesi M, Oliver J, et al. Rare key functional domain missense substitutions in MRE11A, RAD50, and NBN contribute to breast cancer susceptibility: results from a Breast Cancer Family Registry case-control mutation-screening study. Breast Cancer Res 2014; 16: R58

162 Rupnik A, Grenon M, Lowndes N. The MRN complex. Curr Biol 2008; 18: R455-R457

163 Rizzolo P, Silvestri V, Falchetti M, et al. Inherited and acquired alterations in development of breast cancer. Appl Clin Genet 2011; 4: 145-158

164 Meindl A, Hellebrand H, Wiek C, et al. Germline mutations in breast and ovarian cancer pedigrees establish RAD51C as a human cancer susceptibility gene. Nat Genet 2010; 42: 410-414

165 Gevensleben H, Bossung V, Meindl A, et al. Pathological features of breast and ovarian cancers in RAD51C germline mutation carriers. Virchows Arch 2014; 465: 365-369

166 D'Andrea AD, Grompe M. The Fanconi anaemia/BRCA pathway. Nat Rev Cancer 2003; 3: 23-34

167 Venkitaraman AR. Tracing the network connecting BRCA and Fanconi anaemia proteins. Nat Rev Cancer 2004; 4: 266-276

168 Alter BP, Greene MH, Velazquez I, et al. Cancer in Fanconi anemia. Blood 2003; 101: 2072

169 Xia B, Sheng Q, Nakanishi K, et al. Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. Mol Cell 2006; 22: 719-729

170 Seal S, Thompson D, Renwick A, et al. Truncating mutations in the Fanconi anemia J gene BRIP1 are low-penetrance breast cancer susceptibility alleles. Nat Genet 2006; 38: 1239-1241

171 Rahman N, Seal S, Thompson D, et al. PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. Nat Genet 2007; 39: 165-167

172 Kiiski JI, Pelttari LM, Khan S, et al. Exome sequencing identifies FANCM as a susceptibility gene for triple-negative breast cancer. Proc Natl Acad Sci U S A 2014; 111: 15172-15177

173 Barroso E, Pita G, Arias JI, et al. The Fanconi anemia family of genes and its correlation with breast cancer susceptibility and breast cancer features. Breast Cancer Res Treat 2009; 118: 655-660

174 Dreijerink KMA, Goudet P, Burgess JR, et al. Breast-Cancer Predisposition in Multiple Endocrine Neoplasia Type 1. N Engl J Med 2014; 371: 583-584

175 Easton DF, Pooley K a, Dunning AM, et al. Genome-wide association study identifies novel breast cancer susceptibility loci. Nature 2007; 447: 1087-1093

176 Hunter DJ, Kraft P, Jacobs KB, et al. A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer. Nat Genet 2007; 39: 870-874

177 Cox A, Dunning AM, Garcia-Closas M, et al. A common coding variant in CASP8 is associated with breast cancer risk. Nat Genet 2007; 39: 352-358

178 Stacey SN, Manolescu A, Sulem P, et al. Common variants on chromosomes 2q35 and 16q12 confer susceptibility to estrogen receptor-positive breast cancer. Nat Genet 2007; 39: 865-869

179 Ghoussaini M, Pharoah PDP, Easton DF. Inherited genetic susceptibility to breast cancer: the beginning of the end or the end of the beginning? Am J Pathol 2013; 183: 1038-1051

180 Zheng W, Long J, Gao Y-T, et al. Genome-wide association study identifies a new breast cancer susceptibility locus at 6q25.1. Nat Genet 2009; 41: 324-328

181 Hein R, Maranian M, Hopper JL, et al. Comparison of 6q25 breast cancer hits from Asian and European Genome Wide Association Studies in the Breast Cancer Association Consortium (BCAC). PLoS One 2012; 7: e42380

182 Antoniou AC, Beesley J, McGuffog L, et al. Common breast cancer susceptibility alleles and the risk of breast cancer for BRCA1 and BRCA2 mutation carriers: implications for risk prediction. Cancer Res 2010; 70: 9742-9754

183 Morris GJ. Assessing Genetic Variants of Uncertain Significance: The Example of Breast Cancer. eLS John Wiley Sons Ltd, Chichester 2013

184 Wong-Brown MW, Scott RJ. Genetic Susceptibility to Triple-Negative Breast Cancers. eLS John Wiley Sons Ltd, Chichester 2014

185 Jönsson G, Naylor TL, Vallon-Christersson J, et al. Distinct genomic profiles in hereditary breast tumors identified by array-based comparative genomic hybridization. Cancer Res 2005; 65: 7612-7621

186 Jönsson G, Staaf J, Vallon-Christersson J, et al. Genomic subtypes of breast cancer identified by array-comparative genomic hybridization display distinct molecular and clinical characteristics. Breast Cancer Res 2010; 12: R42

187 Joosse SA, van Beers EH, Tielen IHG, et al. Prediction of BRCA1-association in hereditary non-BRCA1/2 breast carcinomas with array-CGH. Breast Cancer Res Treat 2009; 116: 479-489

188 Joosse SA, Brandwijk KIM, Devilee P, et al. Prediction of BRCA2-association in hereditary breast carcinomas using array-CGH. Breast Cancer Res Treat 2012; 132: 379-389

189 van der Groep P, Bouter A, van der Zanden R, et al. Distinction between hereditary and sporadic breast cancer on the basis of clinicopathological data. J Clin Pathol 2006; 59: 611-617

190 Hassanein M, Huiart L, Bourdon V, et al. Prediction of BRCA1 Germ-Line Mutation Status in Patients with Breast Cancer Using Histoprognosis Grade, MS110, Lys27H3, Vimentin, and KI67. Pathobiology 2013; 80: 219-227

191 Dutch Association of Comprehensive Cancer Centers. Dutch clinical guideline familial breast/ovarian carcinoma.

192 Drooger JC, Hooning MJ, Seynaeve CM, et al. Diagnostic and therapeutic ionizing radiation and the risk of a first and second primary breast cancer, with special attention for BRCA1 and BRCA2 mutation carriers: a critical review of the literature. Cancer Treat Rev 2014; 41: 187-196

193 Dutch Association of Comprehensive Cancer Centers. Dutch clinical guideline hereditary breast/ovarian carcinoma.

194 Rebbeck TR. Prophylactic oophorectomy in BRCA1 and BRCA2 mutation carriers. Eur J Cancer 2002; 38 Suppl 6: S15-7

195 Bernier J, Poortmans P. Clinical relevance of normal and tumour cell radiosensitivity in BRCA1/BRCA2 mutation carriers: A review. The Breast 2015; 24: 100-106

196 Turner N, Tutt A, Ashworth A. Targeting the DNA repair defect of BRCA tumours. Curr Opin Pharmacol 2005; 5: 388-393

197 Vollebergh MA, Lips EH, Nederlof PM, et al. Genomic patterns resembling BRCA1- and BRCA2-mutated breast cancers predict benefit of intensified carboplatin-based chemotherapy. Breast Cancer Res 2014; 16: R47

198 Fong PC, Boss DS, Yap TA, et al. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. N Engl J Med 2009; 361: 123-134

199 Tutt A, Robson M, Garber JE, et al. Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial. Lancet 2010; 376: 235-244

FURTHER READING LIST

Aloraifi, F, Alshehhi, M, McDevitt, T, Cody, N, Meany, M, O'Doherty, A, Quinn, CM, Green, AJ, Bracken, A, Geraghty, JG. 2015. Phenotypic analysis of familial breast cancer: comparison of BRCAx tumors with BRCA1-, BRCA2-carriers and non-familial breast cancer. Eur J Surg Oncol. doi: 10.1016/j.ejso.2015.01.021.

Gudmundsdottir, K, Ashworth, A. 2006. The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. Oncogene. 25: 5864-5874.

Jasin, M. 2002. Homologous repair of DNA damage and tumorigenesis: the BRCA connection. Oncogene. 21: 8981-8993.

Tutt, A, Ashworth, A. 2002. Trends Mol Med. The relationship between the roles of BRCA genes in DNA repair and cancer predisposition. 8: 571-576.

Chapter 3

Comprehensive proteomic profiling-derived immunohistochemistry-based prediction models for *BRCA1* and *BRCA2* germline mutation-related breast carcinomas

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ABSTRACT

Heredity, mostly due to *BRCA* germline mutations, is involved in 5-10% of all breast cancer cases. Potential *BRCA* germline mutation carriers may be missed following the current eligibility criteria for *BRCA* genetic testing. The purpose of this study was to therefore develop an immunohistochemistry-based model to predict likelihood of underlying *BRCA1* and *BRCA2* germline mutations in unselected female breast cancer patients. The study group consisted of 100 *BRCA1*-related, 46 *BRCA2*-related, and 94 sporadic breast carcinomas. Tumor expression of 44 proteins involved in (*BRCA*-related) breast carcinogenesis was assessed by immunohistochemistry. A prediction model for *BRCA*-related versus non-*BRCA*-related breast cancer was developed using Lasso logistic regression analysis with cross-validation. The model was assessed for its discriminative value and clinical usefulness. The optimal prediction model included 14 predictors (age, cyclinD1, ERα, ERβ, FGFR2, FGFR3, FGFR4, GLUT1, IGFR, Ki67, mitotic activity index (MAI), MLH1, p120, and TOP2A), and showed excellent discriminative performance (area under the receiving operating characteristic curve = 0.943 (95%CI: 0.909-0.978)), and reasonable calibration. To enhance possible implementation, we developed an alternative model only considering more widely available immunostains. This model included 15 predictors (age, BCL2, CK5/6, CK8/18, cyclinD1, E-cadherin, ERα, HER2, Ki67, MAI, MLH1, p16, PMS2, PR, and vimentin), and still showed very good discriminative performance (AUC = 0.853 (95%CI: 0.795-0.911)). We present a well-applicable and accurate tool to predict which breast cancer patients may have an underlying *BRCA* germline mutation, largely consisting of immunohistochemical markers independent of clinical characteristics. This may improve identification of potential *BRCA* germline mutation carriers and optimize referral for germline mutation testing.

INTRODUCTION

Five to 10% of breast cancer cases occur in a hereditary setting, mostly due to *BRCA1* (17q21.31) or *BRCA2* (13q13.1) germline mutations, causing a 40-80% lifetime risk of developing breast cancer [1–7]. Identifying potential hereditary breast tumors is important as it has preventive and therapeutic consequences. Currently, eligibility for *BRCA1* and *BRCA2* genetic testing is based on clinical criteria, including young age at tumor diagnosis, bilaterality of tumors, and positive family history for breast, ovarian/Fallopian tube and other *BRCA*-related cancers. However, potential germline mutation carriers may be missed using these criteria, since families are nowadays small, family history may be incomplete, and penetrance of the mutations is variable. Genetic testing is time-consuming and still quite expensive due to the large size of both genes, the absence of mutation hotspots and frequent identification of variants of uncertain significance (up to 20% of tests) [8–10]. The estimated chance of missing *BRCA* germline mutation carriers ranges from <5% up to 25% [11]. This could eventually also lead to incorrect diagnosis and under- (preventive) treatment for patients and their relatives.

The use of immunostains, whether or not in combination with clinical and/or morphological characteristics, may be a less expensive and faster method to predict the likelihood of underlying *BRCA* mutations and may be used as a pre-selection tool for genetic referral at the time of pathology diagnosis. Previous studies have yielded clues for the microscopic detection of *BRCA* germline mutation-related breast cancers on the basis of morphologic and immunohistochemical features of the tumor tissue, especially for *BRCA1*-related breast cancer. *BRCA1*-related breast carcinomas are frequently high grade ductal carcinomas, and negative for ERα, PR, and HER2 [12–21]. They frequently express basal markers, such as CK5, CK14 and EGFR, and hypoxia markers, like HIF1α, GLUT1, and CAIX [16,22,23]. They rarely express luminal markers, such as CK8/18, or ER-associated proteins, like BCL2, p27 and cyclinD1 [24–26]. Most *BRCA2*-related breast carcinomas, however, have a phenotype fairly similar to sporadic breast carcinomas. They are usually intermediate to high grade ductal carcinomas with expression of hormone receptors and rarely HER2 overexpression [12–15,17–21,27]. They frequently show expression of luminal cell markers and ER-associated proteins [18,25]. Overexpression of FGF1 and FGFR2 has also been reported [27]. Few studies have however tried to develop a prediction model based upon these differences between *BRCA*-related and sporadic breast carcinomas, especially with regard to *BRCA2*-related breast carcinomas [21,22,28–30]. Therefore, the aims of this study were to perform comprehensive immunohistochemical profiling of *BRCA*-related as well as sporadic female breast carcinomas and to use these data to develop a tool to predict likelihood of underlying *BRCA1* and *BRCA2* germline mutations in these patients.

MATERIAL AND METHODS

Patient samples

A (cross-sectional) case-control study was performed using a total of 100 *BRCA1*-related and 46 *BRCA2*-related breast carcinoma tissues, all formalin-fixed paraffin-embedded (FFPE). These tissues were obtained between 1981 and 2012, and collected from the archives of the UMC Utrecht, UMC Groningen, the Familial Cancer Clinic of the VUmc Amsterdam, and of local hospitals around Utrecht. *BRCA* status was confirmed through mutation analysis at a Medical Genetics department within the Netherlands. A set of 94 clinically sporadic breast cancer specimens (FFPE), randomly obtained between 1993 and 2005, was collected from the archive of the UMC Utrecht to serve as controls. Sporadic cases were unselected for age or family history. Since coded archival pathology specimens were used, no ethical approval or specific prior informed consent was required according to Dutch legislation (Human Tissue and Medical Research: Code of conduct for responsible use 2011, available from: https://www.federa.org/code-goed-gebruik-van-lichaamsmateriaal-2011). All tissues were revised and morphological features (e.g. tumor type and grade) were assessed by one experienced breast pathologist (P.J.v.D.), blinded to mutation status. Grading was performed according to the modified Bloom and Richardson grading system [31]. The mitotic activity index (MAI) was defined as the total number of mitotic figures counted in an area of 2 mm^2 [32].

Immunohistochemistry

The expression of 44 proteins selected for their importance in breast carcinogenesis, association with triple negativity of ERα, PR, HER2 or association with DNA repair mechanisms was investigated by immunohistochemistry. For this, two tissue microarray blocks containing *BRCA1/2*-related breast cancer cases and one tissue microarray containing sporadic cases were used. The *BRCA1/2*-related cases were supplemented by whole-slides. For each staining experiment, appropriate positive and negative controls were included. The scoring was performed by consensus of two observers (P.J.v.D and S.V.), blinded to mutation status. See Supplementary Information 1 for an overview of all immunohistochemical markers as well as staining and scoring protocols.

Statistical analysis

First, univariable associations between clinicopathological characteristics and mutation status were assessed by the Kruskal-Wallis test for continuous predictors. For categorical predictors either the Pearson Chi-square test or Fisher's exact test were used. For primary predictor selection, correlations between predictors were tested using the Kendall's Tau Rank correlation coefficient. Of highly correlated predictors

(correlation coefficient: > |0.75|), only one was selected for further multivariable modeling. The database had 5% missing data with 80% complete cases, originating from the fact that tissue blocks of some cases ran out of usable tumor tissue during the study. Missing data were handled by multiple imputation with 25 iterations and good convergence as before [33–36]. This yielded 10 imputed datasets. Least Absolute Shrinkage and Selection Operators (Lasso)-based logistic regression analysis was used to identify a panel for distinguishing *BRCA1/2*-related from sporadic breast carcinomas, as this method is suitable for regression with high-dimensional data with a relatively high number of predictors in comparison to cases and protects against overfitting [37–40]. Sporadic breast cancer cases were weighted with factor 30 to obtain a representative cohort with a ~5% prevalence of *BRCA1/2*-related breast cancers similar to the general breast cancer patient population. An optimal prediction model considering all primary selected predictors was developed as well as an alternative model using only clinical, morphological, and immunohistochemical stainings commonly available in pathology laboratories. Further details on the Lasso-based logistic regression analysis can be found in Supplementary Information 2. Based upon the logistic regression analysis results, immuno-based prediction tools were built to predict individual probability of *BRCA1/2* germline mutations. Calibration curves were plotted to assess whether model-derived predicted probabilities approximate actual probabilities. Accuracy measure curves were developed by applying this prediction tool to our full dataset to show the apparent effect of using different probability thresholds on identifying underlying *BRCA* mutations (i.e. sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV)). Results across multiple imputation datasets were combined using Rubin's rules. Data were analyzed in IBM SPSS statistics v23.0 (SPSS Inc, Chicago, Illinois, USA) and R software, v3.3.1. The following R packages were used: "mice" for multiple imputation, and "glmnet" for Lasso logistic regression analysis. Two-sided $P < 0.05$ was used as threshold for statistical significance. The reporting of this study was done according to the Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis (TRIPOD) statement guideline [41,42].

RESULTS

Patient characteristics

Characteristics of the investigated 100 *BRCA1*-, 46 *BRCA2*- and 94 sporadic breast carcinomas are shown in Table 1. *BRCA1/2*-related breast carcinomas were more frequently ductal breast cancers of high-grade compared to sporadic breast carcinomas (tumor type: $p = 0.037$; grade: $p = 0.001$) (Table 1). The majority of *BRCA1*-related breast

carcinomas were negative for ERα, PR, and HER2, whereas *BRCA2*-related and sporadic breast carcinomas were more often positive for ERα and PR and negative for HER2 (all $p < 0.05$) (Table 1). *BRCA1/2*-related breast carcinomas presented at a significantly lower median age compared to sporadic cancers ($p < 0.001$).

Table 1. Patient characteristics

	BRCA1		***BRCA2***		**Sporadic**		**p-value**
	n	%	n	%	n	%	
No.	100	41.7	46	19.2	94	39.2	
Age							<0.001*
Median	42		48.5		58		
Range	21-80		21-69		28-88		
Tumor type							0.037*
Ductal	88	94.6	41	91.1	75	81.5	
Lobular	4	4.3	3	6.7	9	9.8	
Ductulo-lobular	1	0.1	1	2.2	8	8.7	
Other	7[A]		1[B]		2[C]		
Grade							0.001*
1	3	3.0	0	0.0	9	9.6	
2	18	18.0	17	37.0	33	35.1	
3	79	79.0	29	63.0	52	55.3	
MAI							**0.016***
Median	21.5		17		14		
Range	0-134		0-85		0-196		
ERα							**<0.001***
Negative	74	74.0	12	26.1	16	17.0	
Positive	26	26.0	34	73.9	78	83.0	
PR							**<0.001***
Negative	83	83.0	26	56.5	38	40.4	
Positive	17	17.0	20	43.5	56	59.6	
HER2							**0.025***
Negative	97	98.0	43	93.5	83	88.3	
Positive	2	2.0	4	6.5	11	11.7	
Missing	1		0		0		

* Statistically significant (2-sided p-value < 0.05). MAI: mitotic activity index.
Tests used: Pearsons' Chi-Square test (ERα, PR); Fisher's exact test (grade, tumor type, HER2); Kruskal-Wallis test (age, MAI).
[A] 2 metaplastic carcinomas, 4 medullary carcinomas, 1 basaloid carcinoma. [B] 1 metaplastic carcinoma. [C] 1 metaplastic carcinoma, 1 mucinous carcinoma.

Primary predictor selection

Correlations between predictors were tested using the Kendall's Tau Rank correlation coefficient for predictor selection (Supplementary Information 3). Fourteen predictors were excluded (correlation coefficient > |0.75|). These consisted of predictors that were scored by 2 or more methods, of which 1 or 2 methods were excluded. For example, ERα was scored as a continuous predictor (absolute percentage of cells showing nuclear staining) and as a categorical predictor (negative when <10% of cells showed nuclear staining; positive when ≥10% of cells showed nuclear staining). The categorical predictor was excluded, and the continuous predictor was selected for prediction model development. Of note, no strong correlations between age and other predictors were seen in sporadic breast carcinomas. At the end, 48 predictors (4 clinicopathological characteristics and 44 immunostains) were selected for optimal prediction model development and 21 predictors (4 clinicopathological characteristics and 17 immunostains) for alternative prediction model development based on readily available predictors in clinical practice (Table 2A-B, Supplementary Information 3).

Table 2A. Variables selected for optimal prediction model development

Clinicopathological variables	Age	MAI
Immunostains	Grade	Tumor type
	BCL2	HER2
	BMI1	HIF1α
	CAIX	IGFR
	CK5/6	Ki67
	CK8/18	MLH1
	CK14	MSH2
	CyclinA	MSH6
	CyclinD1	p120
	CyclinE	p16
	E-cadherin	p21
	EGFR	p27
	ERα	p53
	ERβ	p63
	EZH2	P-cadherin
	FANCD2	PMS2
	FGF1	PPh3
	FGFR1	PR
	FGFR2	PTEN nuclear
	FGFR3	TOP1
	FGFR4	TOP2A
	GLUT1	Vimentin

Optimal prediction model

At the cross-validation-derived optimal penalty, Lasso logistic regression analysis of the full dataset identified 14/48 predictors with regression coefficients > |0.05|, which could distinguish *BRCA1/2*-related from sporadic breast carcinomas: age, cyclinD1, ERα, ERβ, FGFR2, FGFR3, FGFR4, GLUT1, IGFR, Ki67, MAI, MLH1, p120, and TOP2A (Figure 1A, Supplementary Information 4A). The 10 imputation datasets showed good agreement with respect to the weights of predictors (Supplementary Information 4A). The final model could discriminate *BRCA1/2*-related from sporadic breast carcinomas with a cross-validation area under the receiving operating characteristic curve (AUC) of 0.943 (95%CI: 0.909-0.978) (Figure 1A). In Supplementary Information 5, an Excel-tool based upon this prediction model is provided. In Figure 2 an example case is shown on which the prediction model is applied to predict the chance of the case being *BRCA1/2*-related. The cross-validation calibration curve for the probability of *BRCA1/2*-related breast cancer showed an overall reasonable agreement between prediction and observation (Figure 3A), with an overestimation of the risk of *BRCA* germline mutations at higher predicted probabilities, and a good agreement for patients with a predicted risk of less than 10%. In those patients, the average predicted *BRCA* germline mutation risk was 1.4% whereas the actual risk was 0.6%.

A.

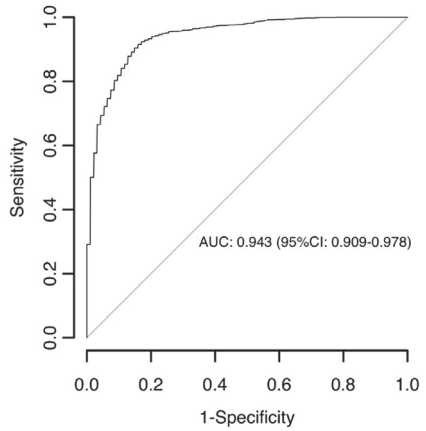


Figure 1. Area under the receiver operating characteristic curve (AUC) of the cross-validated optimal (A) and alternative (B) Lasso prediction models

Legend: The area under the receiver operating characteristic curve (AUC) of the cross-validated optimal (A) and alternative (B) Lasso model at the optimal lambda value. The black line is drawn at the optimal 10-imputation datasets averaged value.

B.

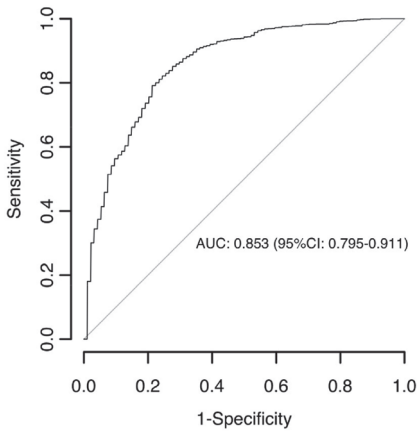


Figure 1. Area under the receiver operating characteristic curve (AUC) of the cross-validated optimal (A) and alternative (B) Lasso prediction models
Legend: The area under the receiver operating characteristic curve (AUC) of the cross-validated optimal (A) and alternative (B) Lasso model at the optimal lambda value. The black line is drawn at the optimal 10-imputation datasets averaged value.

When we applied the final model to our full dataset with a 10% predicted probability threshold, 10% of patients were deemed to be at high risk of a *BRCA* germline mutation, with a positive predictive value of 45%, a negative predictive value of 99%, and 90% sensitivity (Figure 4A). At a 20% threshold, these values were 5%, 76%, 99%, and 83% (Figure 4A). Thus, when a higher threshold is taken for, for instance, referral to clinical genetics, the negative predictive value stays as high as 99% while the sensitivity does no drop below 80%. In other words, the number of patients who will be referred will decrease while the yield for detecting *BRCA1/2* germline mutations would increase. Of note, a relaxed Lasso logistic regression analysis was also performed to investigate whether the number of predictors could be further decreased, but this yielded the same set of predictors (data not shown) [43].

Alternative prediction model

For this analysis, only clinical, morphological and immunohistochemical stainings commonly available in pathology laboratories were used (Table 2B). Lasso logistic regression analysis identified 15/21 predictors with regression coefficients > |0.05|, that could distinguish *BRCA1/2*-related from sporadic breast carcinomas: age, BCL2, CK5/6, CK8/18, cyclinD1, E-cadherin, ERα, HER2, Ki67, MAI, MLH1, p16, PMS2, PR, and

Table 2B. Variables selected for alternative prediction model development

Clinicopathological variables	Age	MAI
	Grade	Tumor type
Immunostains	BCL2	MSH2
	CK5/6	MSH6
	CK8/18	p16
	CyclinD1	p53
	E-cadherin	p63
	ERα	PMS2
	HER2	PR
	Ki67	Vimentin
	MLH1	

vimentin (Figure 1B, Supplementary Information 4B). The model that was developed based upon this regression analysis could discriminate *BRCA1/2*-related from sporadic breast carcinomas with a cross-validation AUC of 0.853 (95%CI: 0.795 -0.911) Figure 1B). In Supplementary Information 5, an Excel-tool based upon this prediction model is provided. In Figure 5 an example case is shown on which the prediction model is applied to predict the chance of the case being *BRCA1/2*-related. The cross-validation calibration curve indicated considerable overestimation of risk at higher predicted probabilities (Figure 3B), but – as for the optimal model – good agreement for patients with a predicted risk of less than 10% (average predicted versus observed risk: 1.9% versus 1.7%). *BRCA1*-related breast carcinomas can be distinguished more accurately with this model from sporadic breast carcinomas than *BRCA2*-related cancers.

When we applied this alternative model to our full dataset with a 10% predicted probability threshold, 12% of patients were deemed to be at high risk of a *BRCA* germline mutation, with a positive predictive value of 32%, a negative predictive value of 99%, and 78% sensitivity (Figure 4B).

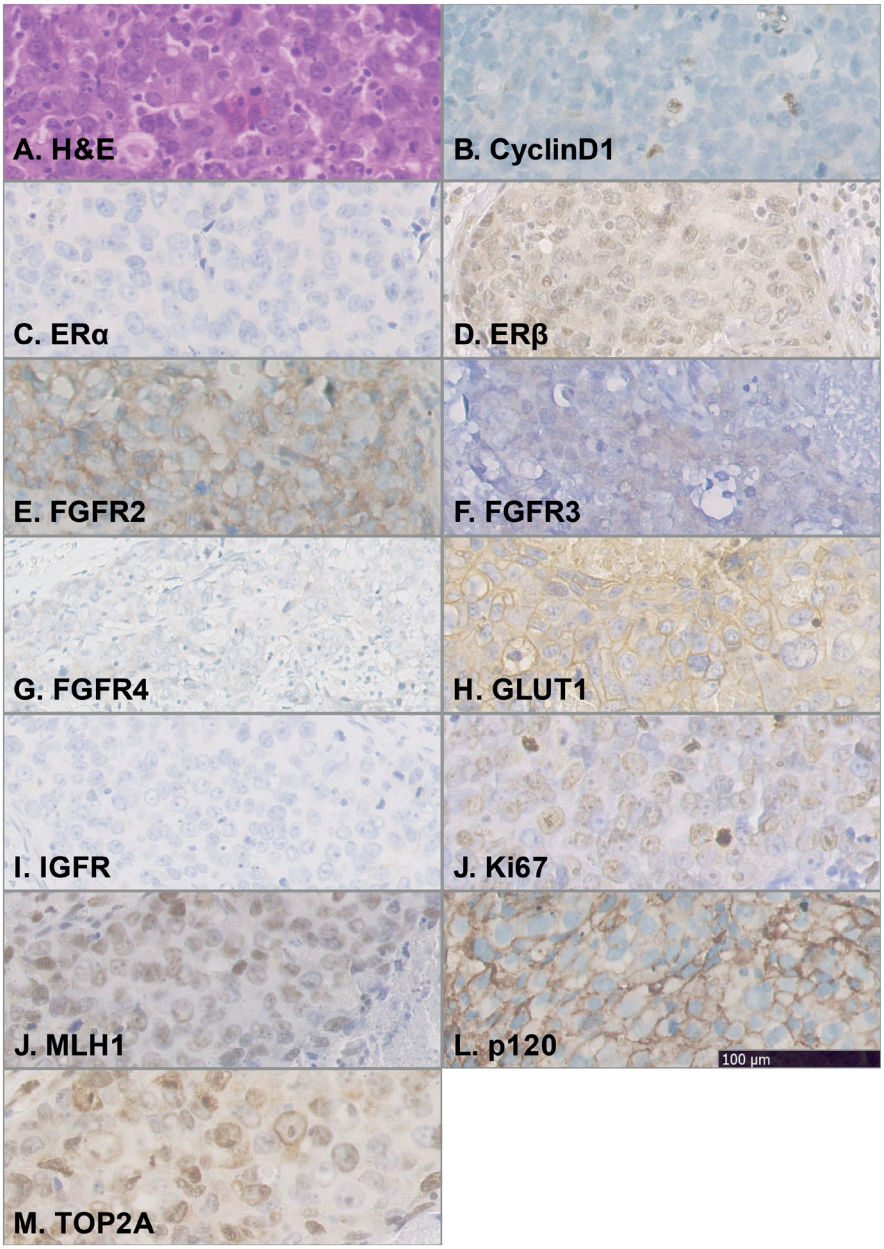
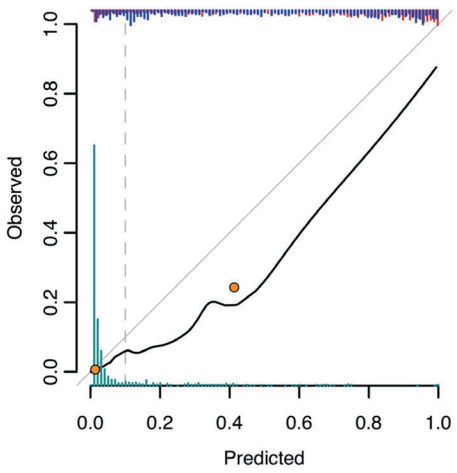


Figure 2. Example case using the optimal prediction model

Legend: Application of the optimal prediction model to one of our study cases, an invasive ductal carcinoma from a 39-year-old with a mitotic activity index of 37. A: Hematoxylin-and-eosin staining. B: CyclinD1 (1%). C: ERα (0%). D: ERβ(35%). E: FGFR2 (score 1). F: FGFR3 (score 1). G: FGFR4 (score 0). H: Glut1 (score 1). I: IGFR (score 0). J: Ki67 (75%). K: MLH1 (score 150: 75% * score 2). L: p120 (score 2). M: TOP2A (90%). Applying the prediction model, the predicted risk of this tumor to be *BRCA1/2* germline mutation-related was 0.95. This patient was known to have a *BRCA1* germline mutation.

A.



B.

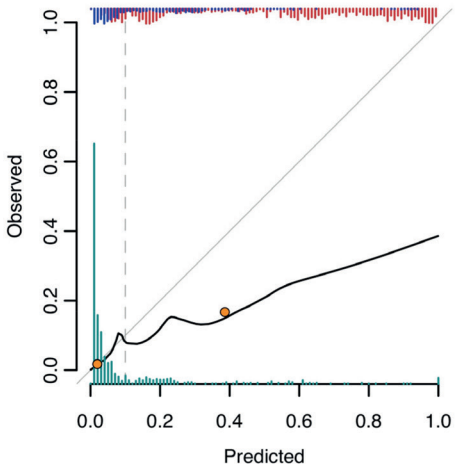
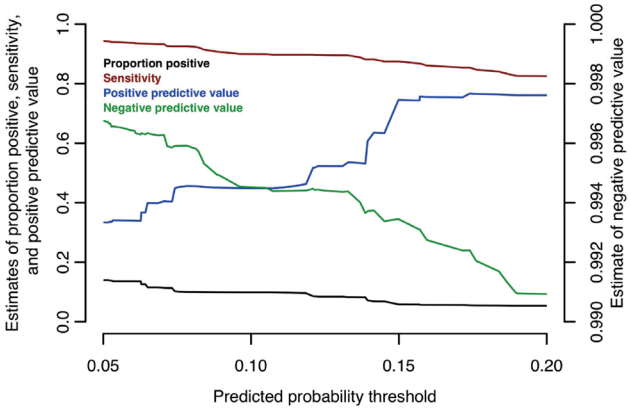


Figure 3. Calibration curve for the optimal (A) and alternative (B) prediction models.
Legend: The estimated probability of *BRCA1/2*-related breast cancer is plotted on the x-axis; the observed *BRCA1/2*-related breast cancer probability is plotted on the y-axis. The diagonal grey line represents perfect calibration. The solid black curved line depicts the cross-validated calibration of the optimal (A) and alternative (B) prediction model. The orange circles represent the average predicted and observed risks for those below and above the 10% threshold (shown as a vertical grey reference line), which would be a clinically reasonable threshold for selecting patients for further clinical genetics analysis. At the bottom, the histograms for sporadic cases are shown in green. At the top, the histograms for *BRCA1*-related cases (red) and *BRCA2*-related cases (blue) are shown.

A.



B.

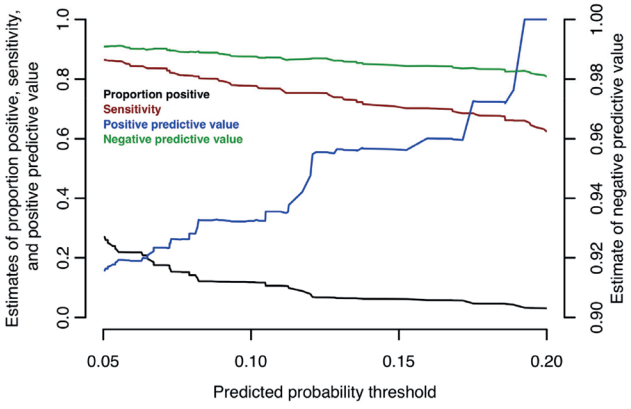


Figure 4. Accuracy measures curves for the optimal (A) and alternative (B) prediction models across various probability thresholds

Legend: The estimated proportion of positive cases, sensitivity, and positive predictive value (left y-axis) as well as negative predicted value (right y-axis) are plotted against different predicted probability thresholds (x-axis). The proportion of positive cases is depicted by the black line, the sensitivity by the brown line, the positive predictive value by the blue line, and the negative predictive value by the green line as observed in the development dataset.

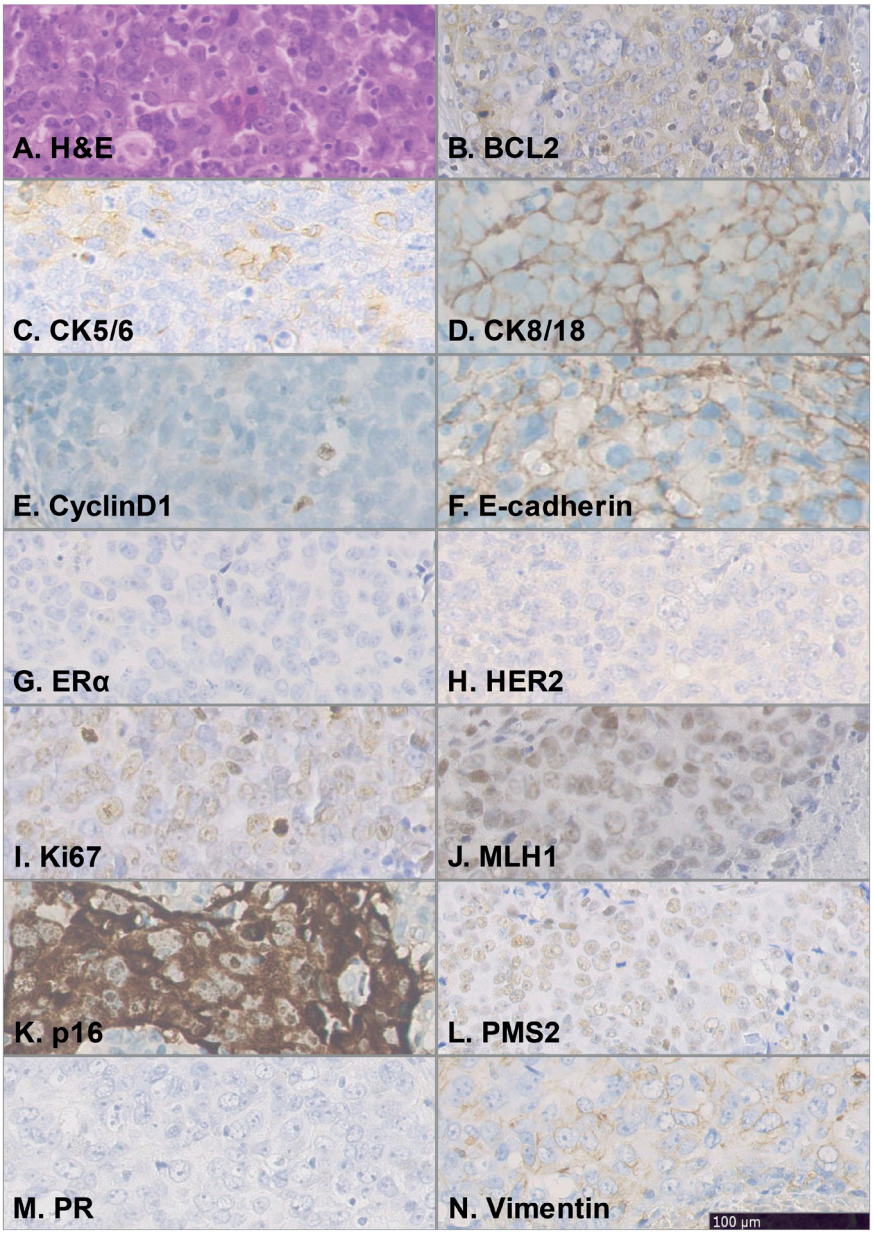


Figure 5. Example case applying the alternative prediction model
Legend: Application of the alternative prediction model to the same case with a *BRCA1* germline mutation as in figure 2. A: Hematoxylin-and-eosin staining. B: BCL2 (score 2). C: CK5/6 (score 1). D: CK8/18 (score 3). E: CyclinD1 (1%). F: E-cadherin (score 1). G: ERα (0%). H: HER2 (score 0). I: Ki67 (75%). J: MLH1 (score 150: 75% * score 2). K: p16 (score 3). L: PMS2 (score 150: 75% * score 2). M: PR (0%). N: Vimentin (score 1). Applying the alternative model, the predicted risk of this tumor to be *BRCA1/2* germline mutation-related was 0.58.

DISCUSSION

We developed an immunohistochemistry-based prediction model to identify *BRCA* germline mutation-related breast carcinomas, which is based upon 14 predictors (age, cyclinD1, ERα, ERβ, FGFR2, FGFR3, FGFR4, GLUT1, IGFR, Ki67, MAI, MLH1, p120, and TOP2A) with excellent discriminative performance (AUC = 0.943). This model could be used as a pre-selection tool for germline mutation testing. If of the immunohistochemical stainings, only the ones that are commonly available in pathology laboratories were preselected, the resulting prediction model still showed a very good discriminative performance (AUC = 0.853).

To our knowledge, this is the most comprehensive immunohistochemical profiling study in *BRCA1/2*-related breast carcinomas, resulting in the most accurate immuno-based prediction model so far (see Table 3 for comparison with models published in literature) [22,28–30,44]. Hassanein *et al.* (2013) performed regression analysis based on 21 immuno-markers. Their final model, including grade, hormone receptors, *BRCA1*, Lys27H3, vimentin, and Ki67 had a sensitivity of 0.83 and specificity of 0.85 [28]. Looking at the AUC, the discrimination of our model is better than models based on clinical predictors such as age and family history [45–47], comparable to prediction models based upon copy number alterations and gene expression [48–50], and slightly less discriminatory than a whole-genome sequencing-based model [51]. In line with previous literature, *BRCA1*-related breast carcinomas can be distinguished more accurately and easier from sporadic breast carcinomas than *BRCA2*-related cancers [21,22].

Although in recent years several molecular *BRCA* prediction models have been developed, we believe that there still is a niche for immunohistochemistry-based applications in the current era of advancing genomics or other molecular analysis applications. When compared to genomics or other molecular models, immunohistochemistry-based tools could be easier applied to archival FFPE patient material with degraded DNA/RNA. Currently and for the near future, immunohistochemistry-based models are less expensive compared to genetic analyses and could therefore be useful to apply as a first screen for *BRCA* germline mutations, in clinical as well as research settings. Another advantage is that immunohistochemistry does not have the risk of unsolicited genetic findings such as in large-scale genetic sequencing analyses.

Of several selected predictors (e.g. age, ERα, Ki67, MAI) it is well-known that they show differences between *BRCA*-related and sporadic breast carcinomas [13,16,18,20,52]. Moreover, lower levels of cyclinD1 have been reported in *BRCA1* and *BRCA2*-related breast cancers

compared to sporadic breast carcinomas [18]. ERβ expression is observed frequently in *BRCA1/2*-related breast carcinomas (in one study even more frequently compared to sporadic breast carcinomas), whereas most *BRCA1*-related breast carcinomas do not show ERα expression [53,54]. *BRCA2*-associated cancers showed higher FGFR2 expression compared to *BRCA1*-associated breast carcinomas [27]. GLUT1 expression is regulated by hypoxia via HIF-1α, which is known to be overexpressed in *BRCA1*-related breast carcinomas [23,55,56]. Other markers (e.g. FGFR3, FGFR4, IGFR, MLH1, p120) have been suggested to play a role in breast cancer development, progression, and/or survival, but associations with *BRCA* germline mutations remain unclear [57–67].

The current study looked at a broad, unselected group of *BRCA1/2*-related and sporadic breast carcinomas. It might be more efficient and (cost-)effective to use this tool for a more specific patient group that is however broader than the current eligibility criteria for genetic testing, e.g. breast cancer patients <50 years old instead of having no age restrictions. Of note, some of the cancers in the *BRCA1/2* germline mutation carriers may have been due to baseline sporadic carcinogenesis. Conversely, the presence of germline mutations cannot be ruled out in our sporadic breast cancer cases as they did not undergo germline mutation testing. In our study, we identified a few sporadic breast cancer cases with a predicted high probability of being *BRCA1/2*-related. Because of ethical concerns we could not investigate this further. Moreover, sporadic breast cancer cases could harbor mechanisms of *BRCA* deficiency other than germline mutations, such as promoter hypermethylation (*BRCA1*: 11-14%; *BRCA2*: 0-9%) [68–73] or somatic *BRCA1/2* mutations (<5%), also known as *BRCAness* [74–77]. Our model might be useful for predicting *BRCAness*, which is becoming more and more important in clinical practice with respect to therapy regimens (e.g. high dose chemotherapy and PARP-inhibitors). Further research is needed to find out for which group of breast cancer patients this prediction model will be (most) useful. Also, careful evaluation of the benefits and harms in decision making for *BRCA1/2* mutation testing is necessary. Its consequences and influence on the well-being of breast cancer patients, who eventually do not have a *BRCA* germline mutation, should not be taken lightly [78–80].

Limitations of this study relate to the limited sample size, especially in view to the number of predictors. Because of this, the Lasso regression analysis was used, to be able to select an optimal set of predictors in a relatively sparse data set. We performed multiple imputation because of 5% missing data. A potential bias that smaller tumors were more likely to have missing data cannot be ruled out. However, tumor size has not been reported to be associated with *BRCA* status. Furthermore, our model has not been externally validated, and especially our accuracy estimates that were based on the full

Table 3. Performance of our prediction model for *BRCA1/2*-related breast cancer in comparison with models published in literature

Prediction model type	Study	n *BRCA1*	n *BRCA2*	n non-*BRCA1/2* familial	n Sporadic	Predictors in final model	AUC	SE	SP	PPV	NPV	External validation
Clinical	Biswas 2016 Breast Ca Res Treat	701		3.356	NA	Age, family history (BRCAPRO, BRCAPROLYTE, BRCAPROLYTE-Plus, BRCAPROLYTE-Simple)	0.65-0.78	0.46-0.72	0.71-0.75	NA	NA	No
	Kang 2006 Brit J Cancer	34	18	328	NA	Age, family history (BRCAPRO, Manchester, Penn, Myriad-Frank models)	0.74-0.76	0.69-0.89	0.35-0.67	0.18-0.25	0.93-0.96	No
	Kang 2012 Breast Ca Res Treat	21	26	190	NA	Age, family history (BRCAPRO, Myriad II)	0.67	0.48-0.5	0.84-0.86	0.43-0.45	NA	No
Morphology/ immuno-histochemistry	Hassanein 2013 Pathobiology	27 + 28#	NA	NA	81 + 28#	Grade, hormone receptors, BRCA1, Lys27H3, Vimentin, Ki67	NA	0.83	0.81	NA	NA	Yes
	Honrado 2005 J Clin Oncol	33 + 41#	24 + 47#	46 + 62#	104 + 184#	RAD51, CHEK2	NA	0.81	0.76	NA	NA	No
	Lakhani 2005 Clin Cancer Res	182	63	NA	109	ER, CK5/6	0.77	0.56	0.97	0.28	0.99	No
						ER, CK5/6, CK14	0.87	NA	NA	NA	NA	No
	Miolo 2009 BMC Cancer	10	9	74	NA	ER, PR, HER2	NA	1	0.77	0.34	1	No
	Spurdle 2014 Breast Cancer Res	4.477	2.565	NA	47.565	Age, ER, PR, HER2	NA	0.57-0.67	0.82-0.87	NA	NA	No

	This study	100	46	NA	94	Age, CyclinD1, ERα, ERβ, FGFR2, FGFR3, FGFR4, GLUT1, IGFR, Ki67, MAI, MLH1, p120, TOP2A	0.94	..	..	..	..	No
						Age, BCL2, CK5/6, CK8/18, CyclinD1, E-cadherin, ERα, HER2, Ki67, MAI, MLH1, p16, PMS2, PR, Vimentin	0.86	..	..	..	..	No
Molecular	Davies 2017 Nature Med	5 + 1#	19 + 4#	0	536 + 75#	Whole-genome sequencing	0.98	0.86-0.99	NA	NA	NA	Yes
	Hedenfalk 2010 NEJM	7	7	NA	7	Gene expression profiling	0.95	NA	NA	NA	NA	No
	Lips 2011 Ann Oncol	40	NA	NA	185	Copy number analysis	NA	0.85	0.87	NA	NA	No
	Wessels 2002 Cancer Res	28 + 6#	NA	NA	42 + 19#	Array CGH	0.84	0.96	0.97	NA	NA	Yes

AUC: area under the receiver operating characteristic curve; BRCAPRO / BRCAPROLYTE / BRCAPROLYTE-Plus / BRCAPROLYTE-Simple models: genetic risk prediction model to calculate the probability of carrying a *BRCA1/2* germline mutation based upon patient and family cancer history; Manchester / Myriad - Frank / Penn models: models that determine the likelihood of identifying a *BRCA1/2* germline mutation based upon patient and family history; SE: sensitivity; SP: specificity; PPV: positive predictive value; NPV: negative predictive value; NA: not available; #: number of patients in development + validation sample set.

dataset at hand may be overoptimistic, but at least our internal cross-validation steps yielded promising results.

To conclude, this study presents an accurate tool to predict which breast cancer patients may have an underlying *BRCA* germline mutation, consisting largely of immunohistochemical markers, independent of clinical characteristics. This may improve identification of potential *BRCA* germline mutation carriers and optimize referral for germline mutation testing. Further research is however needed for validation of our model in the target population(s) and adequate implementation in clinical practice.

SUPPLEMENTARY FILES

Supplementary files are available online.

REFERENCES

1. Stuckey AR, Onstad MA. Hereditary breast cancer: an update on risk assessment and genetic testing in 2015. Am. J. Obstet. Gynecol. 2015;213:161–165. doi:10.1016/j.ajog.2015.03.003.
2. Kleibl Z, Kristensen VN. Women at high risk of breast cancer: Molecular characteristics, clinical presentation and management. Breast 2016;28:136–44. doi:10.1016/j.breast.2016.05.006.
3. Chen S, Parmigiani G. Meta-analysis of BRCA1 and BRCA2 penetrance. J. Clin. Oncol. 2007;25:1329–33. doi:10.1200/JCO.2006.09.1066.
4. Mavaddat N, Peock S, Frost D, et al. Cancer Risks for BRCA1 and BRCA2 Mutation Carriers: Results From Prospective Analysis of EMBRACE. JNCI J. Natl. Cancer Inst. 2013;105:812–822. doi:10.1093/jnci/djt095.
5. Antoniou A, Pharoah PDP, Narod S, et al. Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. Am. J. Hum. Genet. 2003;72:1117–30. doi:10.1086/375033.
6. Brohet RM, Velthuizen ME, Hogervorst FBL, et al. Breast and ovarian cancer risks in a large series of clinically ascertained families with a high proportion of BRCA1 and BRCA2 Dutch founder mutations. J. Med. Genet. 2014;51:98–107. doi:10.1136/jmedgenet-2013-101974.
7. Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nat. Rev. Cancer 2012;12:68–78. doi:10.1038/nrc3181.
8. Honrado E, Benítez J, Palacios J. Histopathology of BRCA1- and BRCA2-associated breast cancer. Crit. Rev. Oncol. Hematol. 2006;59:27–39. doi:10.1016/j.critrevonc.2006.01.006.
9. Eccles DM, Mitchell G, Monteiro ANA, et al. BRCA1 and BRCA2 genetic testing-pitfalls and recommendations for managing variants of uncertain clinical significance. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. 2015;26:2057–65. doi:10.1093/annonc/mdv278.
10. Cheon JY, Mozersky J, Cook-Deegan R. Variants of uncertain significance in BRCA: a harbinger of ethical and policy issues to come? Genome Med. 2014;6:121. doi:10.1186/s13073-014-0121-3.
11. Varesco L, Viassolo V, Viel A, et al. Performance of BOADICEA and BRCAPRO genetic

models and of empirical criteria based on cancer family history for predicting BRCA mutation carrier probabilities: a retrospective study in a sample of Italian cancer genetics clinics. Breast 2013;22:1130–5. doi:10.1016/j.breast.2013.07.053.

12. Lakhani SR, Jacquemier J, Sloane JP, et al. Multifactorial analysis of differences between sporadic breast cancers and cancers involving BRCA1 and BRCA2 mutations. J. Natl. Cancer Inst. 1998;90:1138–45.
13. Palacios J, Robles-Frías MJ, Castilla M a, et al. The molecular pathology of hereditary breast cancer. Pathobiology 2008;75:85–94. doi:10.1159/000123846.
14. Lynch BJ, Holden J a, Buys SS, et al. Pathobiologic characteristics of hereditary breast cancer. Hum. Pathol. 1998;29:1140–4.
15. Breast Cancer Linkage Consortium. Pathology of familial breast cancer: differences between breast cancers in carriers of BRCA1 or BRCA2 mutations and sporadic cases. Lancet 1997;349:1505–10.
16. Foulkes WD, Stefansson IM, Chappuis PO, et al. Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. J. Natl. Cancer Inst. 2003;95:1482–5.
17. Palacios J, Honrado E, Osorio A, et al. Immunohistochemical characteristics defined by tissue microarray of hereditary breast cancer not attributable to BRCA1 or BRCA2 mutations: differences from breast carcinomas arising in BRCA1 and BRCA2 mutation carriers. Clin Cancer Res. 2003;9:3606-14.
18. Armes JE, Trute L, White D, et al. Distinct molecular pathogeneses of early-onset breast cancers in BRCA1 and BRCA2 mutation carriers: a population-based study. Cancer Res. 1999;59:2011–2017.
19. Osin PP, Lakhani SR. The pathology of familial breast cancer: Immunohistochemistry and molecular analysis. Breast Cancer Res. 1999;1:36–40.
20. Mavaddat N, Barrowdale D, Andrulis IL, et al. Pathology of breast and ovarian cancers among BRCA1 and BRCA2 mutation carriers: results from the Consortium of Investigators of Modifiers of BRCA1/2 (CIMBA). Cancer Epidemiol. Biomarkers Prev. 2012;21:134–47. doi:10.1158/1055-9965.EPI-11-0775.
21. Lakhani SR, Van De Vijver MJ, Jacquemier J, et al. The pathology of familial breast cancer: predictive value of immunohistochemical markers estrogen receptor, progesterone receptor, HER-2, and p53 in patients with mutations in BRCA1 and BRCA2. J. Clin. Oncol. 2002;20:2310–8.
22. Lakhani SR, Reis-Filho JS, Fulford L, et al. Prediction of BRCA1 status in patients with breast cancer using estrogen receptor and basal phenotype. Clin. Cancer Res. 2005;11:5175–80. doi:10.1158/1078-0432.CCR-04-2424.
23. van der Groep P, Bouter A, Menko FH, et al. High frequency of HIF-1alpha overexpression in BRCA1 related breast cancer. Breast Cancer Res. Treat. 2008;111:475–80. doi:10.1007/s10549-007-9817-z.
24. Armes JE, Egan a J, Southey MC, et al. The histologic phenotypes of breast carcinoma occurring before age 40 years in women with and without BRCA1 or BRCA2 germline mutations: a population-based study. Cancer 1998;83:2335–45.
25. Osin P, Gusterson BA, Philp E, et al. Predicted anti-oestrogen resistance in BRCA-associated familial breast cancers. Eur. J. Cancer 1998;34:1683–6.
26. Freneaux P, Stoppa-Lyonnet D, Mouret E, et al. Low expression of bcl-2 in Brca1-associated breast cancers. Br. J. Cancer 2000;83:1318–22. doi:10.1054/bjoc.2000.1438.
27. Bane AL, Pinnaduwage D, Colby S, et al. Expression profiling of familial breast cancers demonstrates higher expression of FGFR2 in BRCA2-associated tumors. Breast Cancer Res. Treat. 2009;117:183–91. doi:10.1007/s10549-008-0087-1.
28. Hassanein M, Huiart L, Bourdon V, et al. Prediction of BRCA1 Germ-Line Mutation Status in Patients with Breast Cancer Using Histoprognosis Grade, MS110, Lys27H3, Vimentin, and KI67. Pathobiology 2013;80:219–227. doi:10.1159/000339432.
29. Spurdle AB, Couch FJ, Parsons MT, et al. Refined histopathological predictors of BRCA1 and BRCA2mutation status: a large-scale analysis of breast cancer characteristics from the BCAC, CIMBA, and ENIGMA consortia. Breast Cancer Res. 2014;16:3419. doi:10.1186/s13058-014-0474-y.
30. Honrado E, Osorio A, Palacios J, et al. Immunohistochemical expression of DNA

repair proteins in familial breast cancer differentiate BRCA2-associated tumors. J. Clin. Oncol. 2005;23:7503–11. doi:10.1200/JCO.2005.01.3698.

31. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991;19:403–10.
32. van Diest PJ, Baak JP, Matze-Cok P, et al. Reproducibility of mitosis counting in 2,469 breast cancer specimens: results from the Multicenter Morphometric Mammary Carcinoma Project. Hum. Pathol. 1992;23:603–7.
33. Rezvan PH, White IR, Lee KJ, et al. Evaluation of a weighting approach for performing sensitivity analysis after multiple imputation. BMC Med. Res. Methodol. 2015;15:83. doi:10.1186/s12874-015-0074-2.
34. Donders ART, van der Heijden GJMG, Stijnen T, et al. Review: a gentle introduction to imputation of missing values. J. Clin. Epidemiol. 2006;59:1087–91. doi:10.1016/j.jclinepi.2006.01.014.
35. Janssen KJM, Donders ART, Harrell FE, et al. Missing covariate data in medical research: to impute is better than to ignore. J. Clin. Epidemiol. 2010;63:721–7. doi:10.1016/j.jclinepi.2009.12.008.
36. van Buuren S, Groothuis-Oudshoorn K. Mice: multivariate imputation by chained equations in R. JStat Softw 2011;45:1–67.
37. Pavlou M, Ambler G, Seaman SR, et al. How to develop a more accurate risk prediction model when there are few events. BMJ 2015;351:h3868.
38. Huang Y, Liang C, He L, et al. Development and Validation of a Radiomics Nomogram for Preoperative Prediction of Lymph Node Metastasis in Colorectal Cancer. J. Clin. Oncol. 2016;34:2157–2164. doi:10.1200/JCO.2015.65.9128.
39. Wozniak MB, Scelo G, Muller DC, et al. Circulating MicroRNAs as Non-Invasive Biomarkers for Early Detection of Non-Small-Cell Lung Cancer. PLoS One 2015;10:e0125026. doi:10.1371/journal.pone.0125026.
40. Zhang J-X, Song W, Chen Z-H, et al. Prognostic and predictive value of a microRNA signature in stage II colon cancer: a microRNA expression analysis. Lancet. Oncol. 2013;14:1295–306. doi:10.1016/S1470-2045(13)70491-1.
41. Collins GS, Reitsma JB, Altman DG, et al. Transparent Reporting of a multivariable prediction model for Individual Prognosis Or Diagnosis (TRIPOD): The TRIPOD Statement. Ann. Intern. Med. 2015;162:55-63. doi:10.7326/M14-0697.
42. Moons KGM, Altman DG, Reitsma JB, et al. Transparent Reporting of a multivariable prediction model for Individual Prognosis Or Diagnosis (TRIPOD): Explanation and Elaboration. Ann. Intern. Med. 2015;162:W1. doi:10.7326/M14-0698.
43. Meinshausen N. Relaxed lasso. Comput. Stat. Data Anal. 2007;52:374–393.
44. Miolo G, Canzonieri V, De Giacomi C, et al. Selecting for BRCA1 testing using a combination of homogeneous selection criteria and immunohistochemical characteristics of breast cancers. BMC Cancer 2009;9:360. doi:10.1186/1471-2407-9-360.
45. Biswas S, Atienza P, Chipman J, et al. A two-stage approach to genetic risk assessment in primary care. Breast Cancer Res. Treat. 2016;155:375–383. doi:10.1007/s10549-016-3686-2.
46. Kang HH, Williams R, Leary J, et al. Evaluation of models to predict BRCA germline mutations. Br. J. Cancer 2006;95:914–920. doi:10.1038/sj.bjc.6603358.
47. Kang E, Park SK, Yang JJ, et al. Accuracy of BRCA1/2 mutation prediction models in Korean breast cancer patients. Breast Cancer Res. Treat. 2012;134:1189–1197. doi:10.1007/s10549-012-2022-8.
48. Lips EH, Mulder L, Hannemann J, et al. Indicators of homologous recombination deficiency in breast cancer and association with response to neoadjuvant chemotherapy. Ann. Oncol. 2011;22:870–876. doi:10.1093/annonc/mdq468.
49. Hedenfalk I, Duggan D, Chen Y, et al. Gene-expression profiles in hereditary breast cancer. N. Engl. J. Med. 2001;344:539–48. doi:10.1056/NEJM200102223440801.
50. Wessels LF, van Welsem T, Hart AA, et al. Molecular Classification of Breast Carcinomas

by Comparative Genomic Hybridization : a Specific Somatic Genetic Profile for BRCA1 Tumors. Cancer Res. 2002:627110–7117.

51. Davies H, Glodzik D, Morganella S, et al. HRDetect is a predictor of BRCA1 and BRCA2 deficiency based on mutational signatures. Nat. Med. 2017;23:517–525. doi:10.1038/nm.4292.
52. Da Silva L, Lakhani SR. Pathology of hereditary breast cancer. Mod. Pathol. 2010;23 Suppl 2:S46–S51. doi:10.1038/modpathol.2010.37.
53. Litwiniuk MM, Roznowski K, Filas V, et al. Expression of estrogen receptor beta in the breast carcinoma of BRCA1 mutation carriers. BMC Cancer 2008;8:100. doi:10.1186/1471-2407-8-100.
54. Daidone MG, Veneroni S, Cappelletti V, et al. Estrogen receptor-beta expression in hereditary breast cancer. J. Clin. Oncol. 2002;20:3752–3; author reply 3753. doi:10.1200/JCO.2002.99.116.
55. van der Groep P, van Diest PJ, Smolders YHCM, et al. HIF-1alpha overexpression in ductal carcinoma in situ of the breast in BRCA1 and BRCA2 mutation carriers. PLoS One 2013;8:e56055. doi:10.1371/journal.pone.0056055.
56. Vleugel MM, Greijer AE, Shvarts A, et al. Differential prognostic impact of hypoxia induced and diffuse HIF-1 expression in invasive breast cancer. J. Clin. Pathol. 2005;58:172–177. doi:10.1136/jcp.2004.019885.
57. Tenhagen M, Klarenbeek S, Braumuller TM, et al. p120-Catenin Is Critical for the Development of Invasive Lobular Carcinoma in Mice. J. Mammary Gland Biol. Neoplasia 2016;21:81–88. doi:10.1007/s10911-016-9358-3.
58. van de Ven RAH, Tenhagen M, Meuleman W, et al. Nuclear p120-catenin regulates the anoikis resistance of mouse lobular breast cancer cells through Kaiso-dependent Wnt11 expression. Dis. Model. Mech. 2015;8:373–84. doi:10.1242/dmm.018648.
59. Werner H, Bruchim I. IGF-1 and BRCA1 signalling pathways in familial cancer. Lancet Oncol. 2012;13:e537–e544. doi:10.1016/S1470-2045(12)70362-5.
60. Hudelist G, Wagner T, Rosner M, et al. Intratumoral IGF-I protein expression is selectively upregulated in breast cancer patients with BRCA1/2 mutations. Endocr. Relat. Cancer 2007;14:1053–1062. doi:10.1677/ERC-06-0075.
61. Peng M, Xie J, Ucher A, et al. Crosstalk between BRCA-Fanconi anemia and mismatch repair pathways prevents MSH2-dependent aberrant DNA damage responses. EMBO J. 2014;33:1698–1712. doi:10.15252/embj.201387530.
62. Tomlinson DC, Knowles MA, Speirs V. Mechanisms of FGFR3 actions in endocrine resistant breast cancer. Int. J. Cancer 2012;130:2857–66. doi:10.1002/ijc.26304.
63. Kuroso K, Imai Y, Kobayashi M, et al. Immunohistochemical detection of fibroblast growth factor receptor 3 in human breast cancer: correlation with clinicopathological/molecular parameters and prognosis. Pathobiology 2010;77:231–40. doi:10.1159/000314346.
64. Chin K, DeVries S, Fridlyand J, et al. Genomic and transcriptional aberrations linked to breast cancer pathophysiologies. Cancer Cell 2006;10:529–541. doi:10.1016/j.ccr.2006.10.009.
65. Moelans CB, de Weger RA, Monsuur HN, et al. Molecular profiling of invasive breast cancer by multiplex ligation-dependent probe amplification-based copy number analysis of tumor suppressor and oncogenes. Mod. Pathol. 2010;23:1029–1039. doi:10.1038/modpathol.2010.84.
66. Jaakkola S, Salmikangas P, Nylund S, et al. Amplification of fgfr4 gene in human breast and gynecological cancers. Int. J. Cancer 1993;54:378–82.
67. Meijer D, Sieuwerts AM, Look MP, et al. Fibroblast growth factor receptor 4 predicts failure on tamoxifen therapy in patients with recurrent breast cancer. Endocr. Relat. Cancer 2008;15:101–111. doi:10.1677/ERC-07-0080.
68. Esteller M, Silva JM, Dominguez G, et al. Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J. Natl. Cancer Inst. 2000;92:564–569.
69. Rice JC, Ozcelik H, Maxeiner P, et al. Methylation of the BRCA1 promoter is associated with decreased BRCA1 mRNA levels in clinical breast cancer specimens. Carcinogenesis 2000;21:1761–1765.
70. Catteau A, Harris WH, Xu CF, et al. Methylation of the BRCA1 promoter region in sporadic

breast and ovarian cancer: Correlation with disease characteristics. Oncogene 1999;18:1957–1965. doi:10.1038/sj.onc.1202509.

71. Matros E, Wang ZC, Lodeiro G, et al. BRCA1 promoter methylation in sporadic breast tumors: relationship to gene expression profiles. Breast Cancer Res. Treat. 2005;91:179–86. doi:10.1007/s10549-004-7603-8.
72. Turner NC, Reis-Filho JS, Russell AM, et al. BRCA1 dysfunction in sporadic basal-like breast cancer. Oncogene 2007;26:2126–2132. doi:10.1038/sj.onc.1210014.
73. Dworkin AM, Spearman AD, Tseng SY, et al. Methylation not a frequent "second hit" in tumors with germline BRCA mutations. Fam. Cancer 2009;8:339–346. doi:10.1007/s10689-009-9240-1.
74. Futreal PA, Liu Q, Shattuck-Eidens D, et al. BRCA1 mutations in primary breast and ovarian carcinomas. Science 1994;266:120–2.
75. Lancaster JM, Wooster R, Mangion J, et al. BRCA2 mutations in primary breast and ovarian cancers. Nat. Genet. 1996;13:238–40. doi:10.1038/ng0696-238.
76. Winter C, Nilsson MP, Olsson E, et al. Targeted sequencing of BRCA1 and BRCA2 across a large unselected breast cancer cohort suggests that one-third of mutations are somatic. Ann. Oncol. 2016;27:1532–1538. doi:10.1093/annonc/mdw209.
77. Li M, Zhang J, Ouyang T, et al. Incidence of BRCA1 somatic mutations and response to neoadjuvant chemotherapy in Chinese women with triple-negative breast cancer. Gene 2016;584:26–30. doi:10.1016/j.gene.2016.03.004.
78. Schwartz MD, Lerman C, Brogan B, et al. Impact of BRCA1/BRCA2 Counseling and Testing on Newly Diagnosed Breast Cancer Patients. J. Clin. Oncol. 2004;22:1823–1829. doi:10.1200/JCO.2004.04.086.
79. van Roosmalen MS, Stalmeier PFM, Verhoef LCG, et al. Impact of BRCA1/2 testing and disclosure of a positive test result on women affected and unaffected with breast or ovarian cancer. Am. J. Med. Genet. 2004;124A:346–355. doi:10.1002/ajmg.a.20374.
80. Schmidt MK, Vermeulen E, Tollenaar RAEM, et al. Regulatory aspects of genetic research with residual human tissue: Effective and efficient data coding. Eur. J. Cancer 2009;45:2376–2382. doi:10.1016/j.ejca.2009.03.008.

Chapter 4

BRCA promoter methylation in sporadic versus *BRCA* germline mutation-related breast cancers

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ABSTRACT

Background

In breast cancer, *BRCA* promoter hypermethylation and *BRCA* germline mutations are said to rarely occur together, but this property has not yet been translated into a clinical test. Our aim was to investigate the diagnostic value of *BRCA1/2* methylation in distinguishing breast carcinomas of *BRCA1* and *BRCA2* germline mutation carriers from sporadic breast carcinomas using a recently developed *BRCA* methylation assay based on Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA).

Methods

MS-MLPA was performed to assess *BRCA1* and *BRCA2* methylation in breast carcinoma tissues from 39 *BRCA1* and 33 *BRCA2* germline mutation carriers, and 80 sporadic breast cancer patients as well as normal breast tissues from 5 *BRCA1* and 4 *BRCA2* mutation carriers and 5 non-mutation carriers.

Results

Methylation frequencies varied considerably between CpG sites across the *BRCA1* and *BRCA2* promoters. Some CpG sites were methylated more frequently in *BRCA1/2*-related compared to sporadic carcinomas, whereas other CpG sites were methylated more frequently in sporadic carcinomas with large variances in sensitivity and specificity as a consequence.

Conclusions

The diagnostic value of *BRCA* promoter methylation analysis in distinguishing *BRCA1/2*-related from sporadic breast carcinomas seems to be considerably dependent on the targeted CpG sites. These findings are important for adequate use of *BRCA* methylation analysis as a pre-screening tool for *BRCA* germline genetic testing, or to identify *BRCA*ness patients who may benefit from targeted therapies such as PARP inhibitors.

BACKGROUND

Breast cancer is the most frequent cancer type in women worldwide [1]. In about 5-10%, breast cancer occurs in a hereditary setting, most commonly due to *BRCA1* or *BRCA2* germline mutations which lead to a 40-80% lifetime risk of developing breast cancer as well as a 30-40% lifetime risk of ovarian cancer development [2–8]. Promoter hypermethylation plays an important role in carcinogenesis of several organs, including the breast, as hypermethylation of CpG sites in promoter regions may lead to down-regulation of tumor suppressor genes [9–15]. It has been proposed in literature that *BRCA* promoter hypermethylation takes place almost exclusively in the sporadic setting and only rarely occurs in patients with an underlying *BRCA1* or *BRCA2* germline mutation [16–26]. This is potentially clinically important, since promoter methylation assays could then serve as a pre-screening test when a hereditary nature is suspected, obviating the need for germline mutation analysis in case of promoter methylation. However, for routine testing more confirmation is mandatory, e.g. with regard to the best CpG sites to target, and a robust assay needs to be at hand that works on small amounts of fragmented DNA from formalin-fixed paraffin-embedded (FFPE) tumor material. The latter is also important in view of the growing need to test for *BRCA1* and *BRCA2* (*BRCA1/2)* promoter methylation as a sign of *BRCA*ness, which may provide an indication for treatment with poly ADP ribose polymerase (PARP) inhibitors [27–31]. Methylation-Specific Multiplex Ligation-dependent Probe Amplification (MS-MLPA) is a rapid, robust, and inexpensive multiplex methylation test that works well on small amounts of DNA derived from FFPE tissues. The aim of this study was to investigate the diagnostic value of *BRCA1/2* promoter methylation in distinguishing breast carcinomas from *BRCA1* and *BRCA2* germline mutation carriers (*BRCA1/2*-related breast carcinomas) and sporadic breast carcinomas using a recently developed BRCA methylation MS-MLPA assay. In other words, to what extent can *BRCA1/2* promoter methylation be detected in *BRCA1/2*-related in comparison to sporadic breast carcinomas?

METHODS

Patient material

FFPE tissues of 39 *BRCA1* and 33 *BRCA2* germline mutation-related breast cancer resection specimens (*BRCA1/2*-C) were derived from the pathology archives at the University Medical Center Utrecht, University Medical Center Groningen, VU University Medical Center Amsterdam, and at local hospitals around Utrecht. Also, FFPE tissues

of prophylactic mastectomy specimens of 5 *BRCA1* and 4 *BRCA2* germline mutation carriers (*BRCA1/2*-N) were derived from the pathology archives of the University Medical Center Utrecht. *BRCA* status had been confirmed through mutation analysis at a Medical Genetics department within the Netherlands after informed consent. For comparison, FFPE tissue of 80 breast cancer resection (Sporadic-C) and 5 breast reduction samples (non-*BRCA*-related-N) from women not tested for a *BRCA* mutation were derived from the pathology archive of the University Medical Center Utrecht. These women did not receive *BRCA* germline mutation testing as there was no clinical suspicion of a hereditary nature. No further in- or exclusion criteria were applied. From the tissue blocks, 4 μm thick sections were cut and stained with haematoxylin & eosin. Tumor characterization, grading according to the modified Bloom and Richardson grading system [32], and scoring of immunohistochemical stainings were performed by an experienced breast pathologist (PJvD), blinded to mutation status. Estrogen receptor (ER) and progesterone receptor (PR) immunohistochemical stainings were considered positive when ≥10% of the tumor cells showed expression, regardless of intensity. Human Epidermal Growth Factor Receptor 2 (HER2) was scored according to the DAKO Herceptest scoring system for breast cancer with which only a 3+ score was considered positive (DAKO, Glostrup, Denmark). The clinicopathological characteristics are provided in Table 1.

DNA isolation

Normal breast and breast cancer tissues were harvested from 10x 10 μm thick and 4x 4 μm thick tissue sections, respectively. Areas with necrosis, pre-invasive lesions, and extensive inflammation were avoided. DNA isolation was performed by overnight incubation at 56° C in lysis buffer (50mM Tris-HCl, pH 8.0 and 0.5% Tween 20) with proteinase K (10 mg/ml, Roche, Basel, Switzerland). Proteinase K was deactivated by boiling for 10 min. After centrifugation for 2 min at 14.000 rpm, the supernatant was collected for further analysis. DNA content was measured using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA).

Methylation analysis

Five μl supernatant with a DNA concentration between 50 and 500 ng/μl was used for MS-MLPA analysis according to the manufacturer's instructions, using the ME053 *BRCA1-BRCA2* X1-0914 methylation assay (MRC-Holland, Amsterdam, the Netherlands). When the DNA concentration exceeded 500 ng/μl, the input volume was adjusted proportionally. The ME053 methylation assay contains 3 and 4 probes to detect *BRCA1* and *BRCA2* promoter methylation, respectively, enabling methylation status determination of 3 CpG sites in the *BRCA1* and 5 CpG sites in the *BRCA2*

Table 1. Clinicopathological characteristics of included breast samples

	***BRCA1*-C**		***BRCA2*-C**		**Sporadic-C**		***BRCA1*-N**		***BRCA2*-N**		**Non-*BRCA*-related-N**		**p-value[A]**
	n	%	n	%	n	%	n	%	n	%	n	%	
No.	39	23.5	33	19.9	80	48.2	5	3.0	4	2.4	5	3.0	
Age													**$9.56 \cdot 10^{-9}$*‡**
Median	43		46		58		31		36.5		22		**0.549[B]**
Range	30-80		21-69		29-86		29-33		35-38		18-52		
Grade[C]													**0.00007*‡‡**
1	0	0.0	1	3.0	20	25.3							
2	9	23.1	10	30.3	25	31.6							
3	30	76.9	22	66.7	34	43.0							
Tumor type													**0.230 ‡‡**
Ductal	34	87.2	29	87.9	69	86.3							
Lobular	2	5.1	2	6.1	10	12.5							
Other	3	7.7	2	6.1	1	1.3							
ER													**0.0004*‡‡**
Negative	26	66.7	8	24.2	23	28.8							
Positive	13	33.3	25	75.8	57	71.3							
PR													**0.001*‡‡**
Negative	29	74.4	17	51.5	30	37.5							
Positive	10	25.6	16	48.5	50	62.5							
HER2													**0.68‡‡**
Negative	38	97.4	32	97.0	75	93.8							
Positive	1	2.6	1	3.0	5	6.3							

[A] Testing *BRCA1*-C and *BRCA2*-C together against Sporadic-C. [B] Testing *BRCA1*-N and *BRCA2*-N together against non-*BRCA*-related-N. [C] Of 1/80 sporadic breast cancer cases, the grade was unknown. ‡ Mann-Whitney test; ‡‡ Fisher's Exact test; ‡‡‡ Pearson Chi-Square test; * statistically significant (2-sided p-value <0.05).

promoter region (see Table 2 and Figure 1 for further details). The MS-MLPA principle and analysis procedure have been described elsewhere [33] and the technique has been shown to be reliable for methylation assessment [33–37]. 100% methylated samples (SssI methyltransferase-treated MDA-MB-231 and A549 cells) were used as positive controls and normal peripheral blood samples as negative controls. No template controls were included as well. Moreover, the methylation assay included 2 digestion (methylation) control probes.

The Coffalyser.net software was used for methylation data analysis (MRC-Holland). Quality control showed that the results of the control probes and control samples

Table 2. Methylation probe characteristics of the MS MLPA assay ME053 *BRCA1-BRCA2* X1-0914 MRC-Holland

Gene	Probe ID	Length	Chromosome position	5' probe	3' probe	Start	End	CpG site	CpG loci ID
BRCA1	BRCA1.1	165	17q21.31	CCTCTGAGAGGCTGCTGCTT AGCGGTAGCCCCTT	GGTTTCCGTGGCAACGGAAAA GCGCGGGAATTACAGA	41277415	41277483	41277429	cg04110421
BRCA1	BRCA1.2	230	17q21.31	CATGCATCTGAGAAACCCCAC AGCCTGTCCCCCGTCCAGGAA	GTCTCAGCGAGCTCACGCCGC GCAGTCGCAGTTT	41277407	41321886	41277395	cg16630982
BRCA1	BRCA1.3	252	17q21.31	GTGGGGTTTCTCAGATAACT GGGCCCCTGC	GCTCAGGAGGCCTTCACCCTC TGCTCTGGGTAAAGGT	41277286	41277352	41277323	cg08993267
BRCA2	BRCA2.1	130	13q13.1	CCATCTTGTGGCGCGAGCTT CTGAAACTA	GGCGGCAGAGGCGGAGCCGC TGTGGCACTGCT	32889614	32889670	32889621	NA
BRCA2	BRCA2.2	149	13q13.1	TGCGGGTTAGTGGTGGTGGT AGTGGGTT	GGGACGAGCGCGTCTTCCGCA GTCCCAGTCCAGCGTGG	32889801	32889865	32889836	NA
BRCA2	BRCA2.3	160	13q13.1	CCTCTGAGAGGCTGCTGCTT AGCGGTAGCCCCTT	GGTTTCCGTGGCAACGGAAAA GCGCGGGAATTACAGA	32889665	32889746	32889672 + 32889683	NA
BRCA2	BRCA2.4	217	13q13.1	CTTCCGGGTGGTGCGTGTGC TGCGTGTCGC	GTCACGGCGTCACGTGGCCA GCGCGGGCTTGT	32889557	32889618	2889608	NA

The chromosomal locations are based upon GRCh37/hg19. The first nucleotides of the 5' probe may not be complementary to the target DNA as it contains the stuffer sequence. The CpG loci ID are as determined by the Infinium Human Methylation 450 Bead Chip Array (Illumina, San Diego, California, USA). NA = not available.

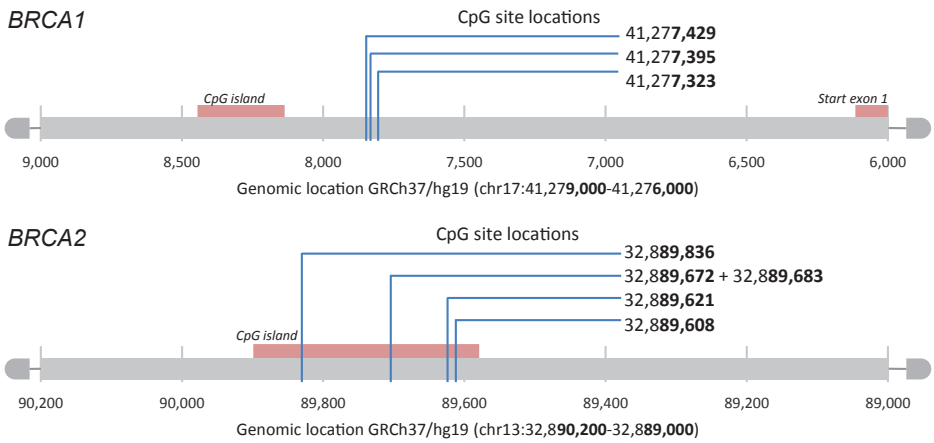


Figure 1. Overview of the targeted CpG sites in the *BRCA1* and *BRCA2* promoter region by MS-MLPA analysis

were adequate. The methylation percentage cut-off per probe was set at the highest methylation percentage value in normal breast tissues from non-mutation carriers (non-*BRCA*-related N), ranging from >15 to >19% (see also Figure 2). Moreover, the cumulative methylation index (CMI) was calculated as the sum of the methylation percentage of all methylation probes. MS-MLPA analysis was performed by SV and CBM, blinded to mutation status.

Statistical analysis

Statistical analyses were performed using IBM SPSS statistics v23.0 (SPSS Inc, Chicago, Illinois, USA). Associations between absolute methylation percentages, CMI or age, and mutation status (*BRCA1/2*-related carcinomas *versus* sporadic carcinomas) were assessed by the Mann-Whitney U test. Associations between dichotomized *BRCA* promoter methylation and mutation status or other clinicopathological characteristics were assessed by the Pearson Chi-square or Fisher's exact test. Sensitivity and specificity were calculated. Correlations between CMI and age were assessed using the Spearman rho's correlation coefficient. The level of significance used was set at two-sided $p < 0.05$.

Correlation between *BRCA1/2* methylation and mRNA expression

The Wanderer tool was used to assess the correlation between *BRCA1/2* methylation and mRNA expression. This tool was created based upon data from the TCGA Research Network [38]. The 450k Methylation Array was selected as methylation data type and Spearman correlation coefficient as correlation method.

RESULTS

***BRCA1* promoter methylation in *BRCA1/2*-related and sporadic breast carcinomas**

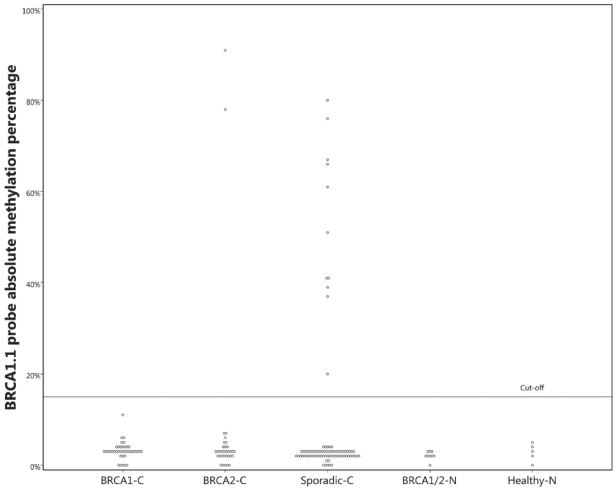
The absolute methylation percentages and their distribution varied considerably between the three *BRCA1* methylation probes (Table 3, Figure 2). For the BRCA1.2 and BRCA1.3 probes, *BRCA1/2*-C showed significantly higher median methylation percentages compared to Sporadic-C (p = 0.00006, and p = 0.00003, respectively). The dichotomized results are shown in Table 4. *BRCA1/2*-C showed significantly less frequent methylation with the BRCA1.1 probe (p = 0.019), but significantly more frequent methylation with the BRCA1.2 probe (p = 0.000009). Methylation of at least one of the three *BRCA1* methylation probes was seen in 46/72 *BRCA1/2*-C (63.9%), compared to 22/80 Sporadic-C (27.5%) (p = 0.000009). Sensitivity and specificity of the *BRCA1* methylation probes in distinguishing *BRCA1/2*-C from Sporadic-C are shown in Table 5. The calculation of the sensitivity and specificity differs between probes due to differences in methylation frequencies between *BRCA1/2*-C and Sporadic-C (Table 4 and explanation in Table 5). The BRCA1.1 and BRCA1.3 probes showed a good performance in ruling out *BRCA1/2* germline mutations when methylation was detected (sensitivity 97.2% and 90.3%, respectively), although the specificity was poor as many Sporadic-C did not show methylation with these probes either (specificity both 13.8%). The BRCA1.2 probe and the combination of the three *BRCA1* probes (*BRCA1* combined) showed moderate sensitivity (both 63.9%) and specificity (72.5%), when used to rule in *BRCA1/2* germline mutations when methylation is present.

Table 3. *BRCA* promoter methylation percentages in *BRCA1*/2-related and sporadic breast carcinomas by MS-MLPA

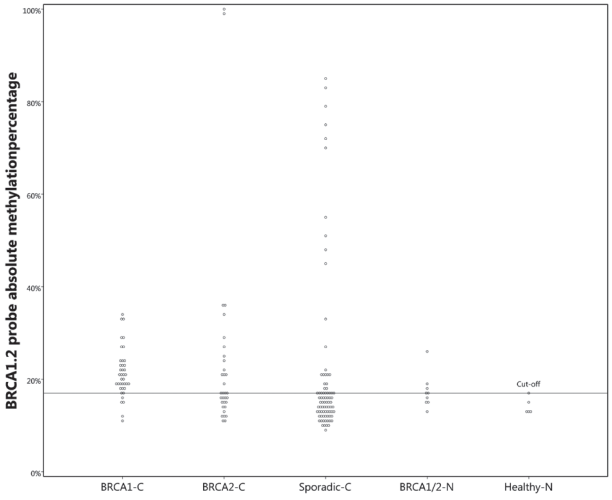
Probe	***BRCA1*** Median % (range)	***BRCA2*** Median % (range)	**Sporadic** Median % (range)	**Test statistic[A]**	**p-value**
BRCA1.1	3 (0-11)	3 (0-91)	3 (0-80)	2494.500	0.140
BRCA1.2	21 (11-34)	17 (11-100)	15 (9-85)	1795.500	0.00006*
BRCA1.3	11 (5-18)	9 (5-100)	7 (3-71)	1760.600	0.00003*
BRCA2.1	24 (14-56)	21 (8-100)	15 (8-43)	947.500	$9.85 \cdot 10^{-13}$*
BRCA2.2	11 (5-27)	10 (5-100)	5 (3-15)	596.000	$1.00 \cdot 10^{-13}$*
BRCA2.3	17 (9-33)	14 (8-100)	9 (5-18)	536.500	$1.00 \cdot 10^{-13}$*
BRCA2.4	9 (0-18)	8 (0-100)	5 (2-12)	984.500	$1.95 \cdot 10^{-12}$*

[A] Mann-Whitney U test, testing *BRCA1*-C and *BRCA2*-C together against Sporadic-C; * statistically significant (2-sided p-value <0.05).

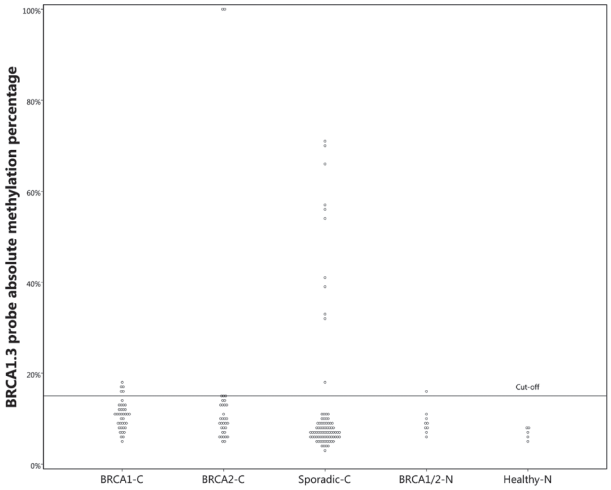
A.


BRCA1.1 probe absolute methylation percentage
100%
80%
60%
40%
20%
0%
Cut-off
BRCA1-C
BRCA2-C
Sporadic-C
BRCA1/2-N
Healthy-N

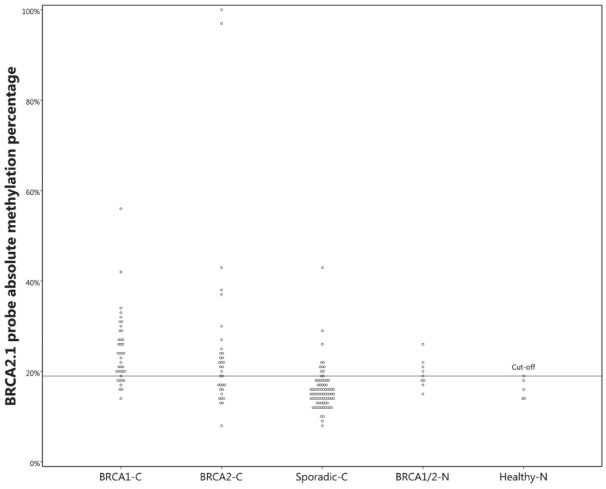
B.


BRCA1.2 probe absolute methylationpercentage
100%
80%
60%
40%
20%
0%
Cut-off
BRCA1-C
BRCA2-C
Sporadic-C
BRCA1/2-N
Healthy-N

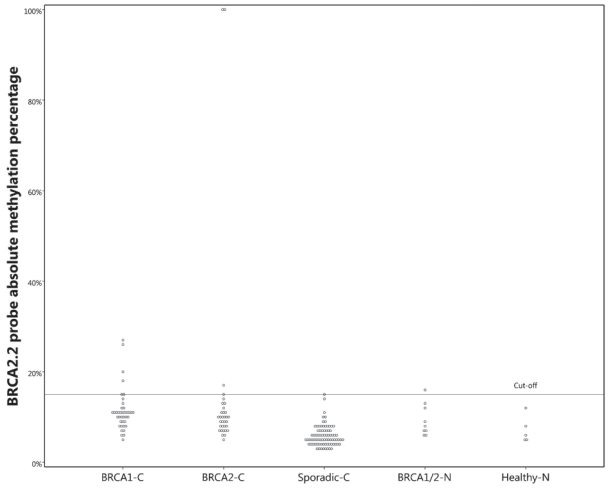
C.


BRCA1.3 probe absolute methylation percentage
100%
80%
60%
40%
20%
0%
Cut-off
BRCA1-C
BRCA2-C
Sporadic-C
BRCA1/2-N
Healthy-N

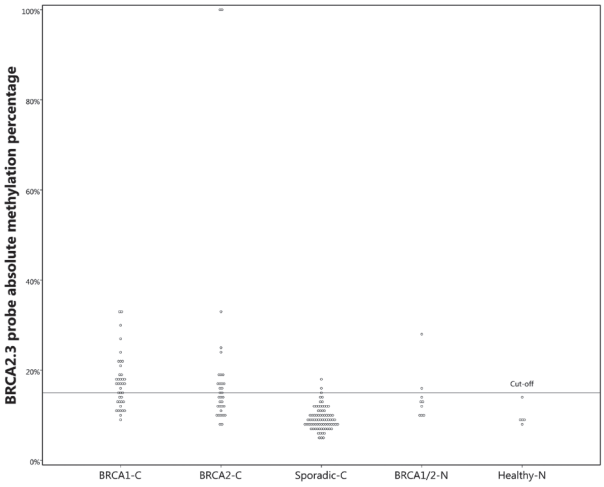
D.


BRCA2.1 probe absolute methylation percentage
100%
80%
60%
40%
20%
0%
Cut-off
BRCA1-C
BRCA2-C
Sporadic-C
BRCA1/2-N
Healthy-N

E.


BRCA2.2 probe absolute methylation percentage
100%
80%
60%
40%
20%
0%
Cut-off
BRCA1-C
BRCA2-C
Sporadic-C
BRCA1/2-N
Healthy-N

F.


BRCA2.3 probe absolute methylation percentage
100%
80%
60%
40%
20%
0%
Cut-off
BRCA1-C
BRCA2-C
Sporadic-C
BRCA1/2-N
Healthy-N

G.

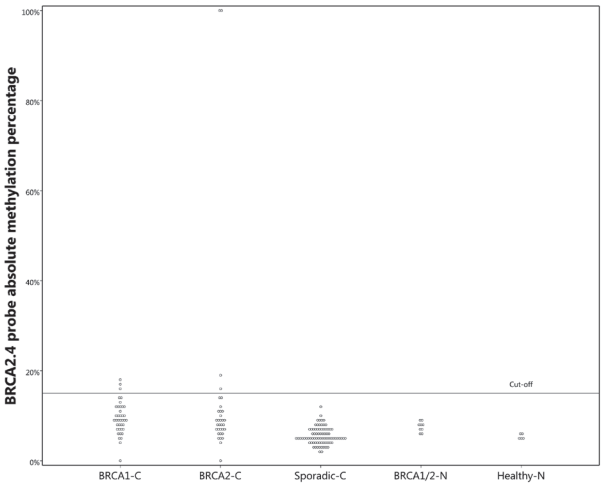


Figure 2. Distribution of absolute methylation percentages for all *BRCA1* and *BRCA2* methylation probes

To evaluate the robustness of the MS-MLPA assay, we compared the results of the ME053 assay with another MS-MLPA assay tested on Sporadic-C in our lab, the ME001 assay (MRC Holland) (CBM, unpublished observations, data not shown). One of the *BRCA1* methylation probes in this assay determines the methylation status of the same CpG site as the BRCA1.3 probe in the ME053 MS-MLPA assay. There was a strong correlation in dichotomized *BRCA1* promoter methylation results in Sporadic-C between the two assays (Spearman's rho correlation coefficient: 0.831; p-value: $1{,}000 \cdot 10^{-13}$). For absolute methylation percentages, the correlation was weaker but still significant (Spearman's rho correlation coefficient: 0.379; p-value: 0.001). In general, the Sporadic-C showed slightly higher *BRCA1* methylation percentages with the ME001 assay. In 4/80 cases, *BRCA1* was methylated according to the ME001 assay but unmethylated according to the ME053 assay. However, methylation percentages in these cases were only slightly above the threshold of 15% (17-20%) with the ME001 assay.

***BRCA2* promoter methylation in *BRCA1/2*-related and sporadic breast carcinomas**

BRCA1/2-C showed significantly higher median methylation percentages for all *BRCA2* methylation probes compared to Sporadic-C, although the absolute methylation percentages and their distribution varied considerably between the four *BRCA2* methylation probes (Table 3, Figure 2). Using dichotomized results, *BRCA1/2*-C showed significantly more frequent methylation in all four probes, as shown in Table 4. When

Table 4. Frequency of *BRCA* methylation (dichotomized results) in *BRCA1*/2-related and sporadic breast carcinomas by MS-MLPA

Probe	*BRCA1*	*BRCA2*	Sporadic	Test statistic[A]	P-value	Total (%)	Cut-off[B]
	Total: 39 (%)	Total: 33 (%)	Total: 80 (%)				
BRCA1.1	0 (0.0)	2 (6.1)	11 (13.8)	5.833‡‡	0.019*	13 (8.6)	>15%
BRCA1.2	32 (82.1)	14 (42.4)	22 (27.5)	20.296‡‡	0.000009*	68 (44.7)	>17%
BRCA1.3	5 (12.8)	2 (6.1)	11 (13.8)	0.589‡‡	0.465	18 (11.8)	>15%
BRCA1 total[C]	**32 (82.1)**	**14 (42.4)**	**22 (27.5)**	**20.296‡‡**	**0.000009***	**68 (44.7)**	
BRCA2.1	30 (76.9)	18 (54.5)	10 (12.5)	47.117‡‡	$2.93 \cdot 10^{-12}$*	58 (38.2)	>19%
BRCA2.2	4 (10.3)	3 (9.1)	0 (0.0)	8.153‡	0.005*	7 (4.6)	>15%
BRCA2.3	22 (56.4)	14 (42.4)	2 (2.5)	45.600‡‡	$1.65 \cdot 10^{-12}$*	38 (25.0)	>15%
BRCA2.4	3 (7.7)	4 (12.1)	0 (0.0)	8.153‡	0.005*	7 (4.6)	>15%
BRCA2 total[C]	**30 (76.9)**	**20 (60.6)**	**10 (12.5)**	**51.432‡‡**	**0.029***	**60 (39.5)**	

[A] Pearson Chi-square or Fisher's Exact test, testing *BRCA1*-C and *BRCA2*-C together against Sporadic-C. * statistically significant (2-sided p-value <0.05); ‡ Fisher's Exact test; ‡‡ Pearson Chi-square test. [B] Cut-off based upon highest methylation percentage detected in normal breast tissue from non-mutation carriers. [C] *BRCA1* and *BRCA2* total entails the number (and percentage) of samples showing methylation in at least one of the *BRCA1* or *BRCA2* probes, respectively.

Table 5. Sensitivity and specificity for each methylation probe in distinguishing *BRCA1/2*-related from sporadic breast carcinomas

Probe	Sensitivity	95% CI	Specificity	95% CI
BRCA1.1[A]	70/72 = 97.2%	90.3-99.6%	11/80 = 13.8%	7.1-23.3%
BRCA1.2[B]	46/72 = 63.9%	51.7-74.9%	58/80 = 72.5%	61.4-81.9%
BRCA1.3[A]	65/72 = 90.3%	90.0-96.0%	11/80 = 13.8%	7.1-23.3%
***BRCA1* combined[C]**	**46/72 = 63.9%**	**51.7-74.9%**	**58/80 = 72.5%**	**61.4-81.9%**
BRCA2.1[B]	48/72 = 66.7%	54.6-77.3%	70/80 = 87.5%	78.2-93.8%
BRCA2.2[B]	7/72 = 9.7%	4.0-19.0%	80/80 = 100%	95.5-100%
BRCA2.3[B]	36/72 = 50.0%	38.0-62.0%	78/80 = 97.5%	91.3-99.7%
BRCA2.4[B]	7/72 = 9.7%	4.0-19.0%	80/80 = 100%	95.5-100%
***BRCA2* combined[C]**	**50/72 = 69.4%**	**57.5-79.8%**	**70/80 = 87.5%**	**78.2-93.8%**

[A] Sensitivity and specificity calculated as if *BRCA1* promoter methylation would be performed to rule out *BRCA* germline mutations. True positive: *BRCA1/2*-related cancers without BRCA1.1 or BRCA1.3 methylation. True negative: sporadic cancers with BRCA1.1 or BRCA1.3 methylation.

[B] Sensitivity and specificity calculated as if *BRCA1* promoter methylation would be performed to rule in *BRCA* germline mutations. True positive: *BRCA1/2*-related cancers with BRCA1.2, BRCA2.1, BRCA2.2, BRCA2.3 or BRCA2.4 methylation. True negative: sporadic cancers without BRCA1.2, BRCA2.1, BRCA2.2, BRCA2.3 or BRCA2.4 methylation.

[C] Sensitivity and specificity calculated as if *BRCA1* promoter methylation would be performed to rule in *BRCA* germline mutations. True positive: *BRCA1/2*-related cancers with methylation of at least one of the *BRCA1* or *BRCA2* probes. True negative: sporadic cancers without methylation in any of the *BRCA1* or *BRCA2* probes.

combining the dichotomized results of the *BRCA2* methylation probes together, 50/72 *BRCA1/2*-C (69.4%) showed methylation of at least one of the four *BRCA2* methylation probes, compared to 10/80 Sporadic-C (12.5%) ($p = 0.029$). Sensitivity and specificity of the *BRCA1* methylation probes in distinguishing *BRCA1/2*-C from Sporadic-C are shown in Table 5. The BRCA2.2 and BRCA2.4 probes showed excellent specificity (both 100%) when used to rule in *BRCA1/2* germline mutations when methylation is detected, as no Sporadic-C were methylated with these probes. However, the sensitivity was poor (both 9.7%) as few *BRCA1/2*-C showed methylation. The BRCA2.1 and BRCA2.3 as well as the combination of all four *BRCA2* probes (*BRCA2* combined) showed moderate sensitivity (50.0-69.4%) and rather good specificity (87.5-97.5%) when used to rule in *BRCA1/2* germline mutations when methylation is detected.

Correlation with clinicopathologic variables

As shown in Table 1, the *BRCA1/2*-C and Sporadic-C differed significantly with respect to age, grade, and ER and PR status. We analysed whether the differences we observed in methylation frequencies between *BRCA1/2*-C and Sporadic-C may be related to these differences in clinicopathologic variables (Table 6A-C). In Sporadic-C, methylation of the BRCA1.1, BRCA1.2 and BRCA1.3 probes separately as well as combined (*BRCA1* combined) was significantly more frequently detected in grade 3 tumors compared to

Table 6A. Relationship between *BRCA1/2* methylation and clinicopathological variables in sporadic breast carcinomas

Sporadic carcinomas	**Grade 1-2 vs. 3**		**Ductal vs. lobular tumors**		**ER positive vs. negative**		**PR positive vs. negative**		**HER2 positive vs. negative**	
Probe	Test statistic[A]	p-value	Test statistic[A]	p-value	Test statistic[A]	p-value	Test statistic[A]	p-value	Test statistic[A]	p-value
BRCA1.1	12.230[‡]	0.001*	1.852[‡]	0.342	7.577[‡]	0.011*	6.753[‡]	0.016*	0.176[‡]	0.533
BRCA1.2	8.190[‡‡]	0.006*	0.026[‡]	1.000	6.689[‡‡]	0.014*	3.762[‡‡]	0.071	2.825[‡]	0.125
BRCA1.3	12.230[‡]	0.001*	1.852[‡]	0.342	7.577[‡]	0.011*	6.753[‡]	0.016*	0.176[‡]	0.533
***BRCA1* combined[‡‡]**	**8.190[‡‡]**	**0.006***	**0.026[‡]**	**1.000**	**6.689[‡‡]**	**0.014***	**3.762[‡‡]**	0.071	2.825[‡]	0.125
BRCA2.1	6.577[‡]	0.015*	1.659[‡]	0.345	0.706[‡]	0.462	0.274[‡]	0.736	3.688[‡]	0.115
BRCA2.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
BRCA2.3	2.775[‡]	0.178	0.297[‡]	1.000	5.084[‡]	0.080	0.137[‡]	1.000	0.137[‡]	1.000
BRCA2.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
***BRCA2* combined[‡‡]**	**6.577[‡]**	**0.015***	**1.659[‡‡]**	**0.345**	**0.706[‡‡]**	**0.462**	**0.274[‡‡]**	**0.736**	**3.688[‡‡]**	**0.115**

[A] Pearson Chi-square or Fisher's Exact test. * statistically significant (2-sided p-value <0.05); [‡] Fisher's Exact test; [‡‡] Pearson Chi-square test. NA: not applicable, as one of the two variables (either the methylation probe or the clinicopathological variable) was a constant.

Table 6B. Relationship between *BRCA1/2* methylation and clinicopathological variables in *BRCA1*-related carcinomas

***BRCA1*-related carcinomas**	**Grade 1-2 vs. 3**		**Ductal vs. lobular tumors**		**ER positive vs. negative**		**PR positive vs. negative**		**HER2 positive vs. negative**	
Probe	Test statistic[A]	p-value	Test statistic[A]	p-value	Test statistic[A]	p-value	Test statistic[A]	p-value	Test statistic[A]	p-value
BRCA1.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
BRCA1.2	0.145[ǂ]	0.653	1.262[ǂ]	0.356	0.087[ǂ]	1.000	0.038[ǂ]	1.000	0.225[ǂ]	1.000
BRCA1.3	0.031[ǂ]	1.000	0.342[ǂ]	1.000	0.115[ǂ]	1.000	0.096[ǂ]	1.000	0.151[ǂ]	1.000
***BRCA1* combined[ǂǂ]**	**0.145[ǂ]**	**0.653**	**1.262[ǂ]**	**0.356**	**0.087[ǂ]**	**1.000**	**0.038[ǂ]**	**1.000**	**0.225[ǂ]**	**1.000**
BRCA2.1	0.005[ǂ]	1.000	1.694[ǂ]	0.310	0.000[ǂ]	1.000	0.363[ǂ]	0.669	0.308[ǂ]	1.000
BRCA2.2	0.009[ǂ]	1.000	0.265[ǂ]	1.000	3.482[ǂ]	0.099	0.001[ǂ]	1.000	0.117[ǂ]	1.000
BRCA2.3	0.003[ǂ]	1.000	3.328[ǂ]	0.144	0.209[ǂǂ]	0.740	0.070[ǂ]	1.000	0.793[ǂ]	1.000
BRCA2.4	0.975[ǂ]	1.000	0.193[ǂ]	1.000	1.625[ǂ]	0.253	1.121[ǂ]	0.556	0.086[ǂ]	1.000
***BRCA2* combined[ǂǂ]**	**0.005[ǂ]**	**1.000**	**1.694[ǂ]**	**0.310**	**0.000[ǂ]**	**1.000**	**0.363[ǂ]**	**0.669**	**0.308[ǂ]**	**1.000**

[A] Pearson Chi-square or Fisher's Exact test. * statistically significant (2-sided p-value <0.05); [ǂ] Fisher's Exact test; [ǂǂ] Pearson Chi-square test. NA: not applicable, as one of the two variables (either the methylation probe or the clinicopathological variable) was a constant.

Table 6C. Relationship between *BRCA1/2* methylation and clinicopathological variables in *BRCA2*-related carcinomas

***BRCA2*-related carcinomas**	**Grade 1-2 vs. 3**		**Ductal vs. lobular tumors**		**ER positive vs. negative**		**PR positive vs. negative**		**HER2 positive vs. negative**	
Probe	Test statistic[A]	p-value	Test statistic[A]	p-value	Test statistic[A]	p-value	Test statistic[A]	p-value	Test statistic[A]	p-value
BRCA1.1	1.065[ǂ]	0.542	0.147[ǂ]	1.000	0.769[ǂ]	0.432	0.002[ǂ]	1.000	0.067[ǂ]	1.000
BRCA1.2	0.062[ǂ]	1.000	0.057[ǂ]	1.000	0.248[ǂ]	0.695	0.730[ǂǂ]	0.491	0.760[ǂ]	1.000
BRCA1.3	1.065[ǂ]	0.542	0.147[ǂ]	1.000	0.769[ǂ]	0.432	0.002[ǂ]	1.000	0.067[ǂ]	1.000
***BRCA1* combined[ǂǂ]**	**0.062[ǂ]**	**1.000**	**0.057[ǂ]**	**1.000**	**0.248[ǂ]**	**0.695**	**0.730[ǂǂ]**	**0.491**	**0.760[ǂ]**	**1.000**
BRCA2.1	2.200[ǂǂ]	0.266	0.002[ǂ]	1.000	0.088[ǂ]	1.000	2.528[ǂǂ]	0.166	1.238[ǂ]	0.455
BRCA2.2	1.650[ǂ]	0.534	0.229[ǂ]	1.000	0.149[ǂ]	1.000	0.437[ǂ]	0.601	0.103[ǂ]	1.000
BRCA2.3	3.039[ǂ]	0.136	0.115[ǂ]	1.000	1.742[ǂ]	0.238	0.022[ǂǂ]	1.000	0.760[ǂ]	1.000
BRCA2.4	0.569[ǂ]	0.586	0.317[ǂ]	1.000	0.001[ǂ]	1.000	0.004[ǂ]	1.000	0.142[ǂ]	1.000
***BRCA2* combined[ǂǂ]**	**3.110[ǂ]**	**0.132**	**0.057[ǂ]**	**1.000**	**0.916[ǂ]**	**0.431**	**0.863[ǂǂ]**	**0.481**	**1.587[ǂ]**	**0.394**

[A] Pearson Chi-square or Fisher's Exact test. * statistically significant (2-sided p-value <0.05); [ǂ] Fisher's Exact test; [ǂǂ] Pearson Chi-square test. .

grade 1-2 tumors, and ER negative compared to ER positive tumors. Methylation of the BRCA1.1 and BRCA1.3 probes was also significantly more frequently detected in PR negative tumors. For *BRCA2* methylation in Sporadic-C, there was only a statistically significant association with grade: methylation of BRCA2.1 probe and of all four *BRCA2* probes combined was more frequently seen in grade 3 carcinomas. There were no statistically significant correlations between *BRCA1* or *BRCA2* methylation on the one hand, and tumor type (ductal versus lobular carcinomas) or HER2 status on the other hand. In *BRCA1*-C and *BRCA2*-C, there were no statistically significant associations between *BRCA1* or *BRCA2* methylation and clinicopathological variables. Moreover, no statistically significant correlation was found between CMI for *BRCA1* and/or *BRCA2* promoter methylation and age in *BRCA1*-C, *BRCA2*-C, Sporadic-C, *BRCA1/2*-N, and non-*BRCA*-related-N (Table 7).

Table 7. Correlation between age and CMI for *BRCA1/2* methylation

Age	CMI *BRCA1*		CMI *BRCA2*		CMI *BRCA1+2*	
	Spearman's rho	p-value	Spearman's rho	p-value	Spearman's rho	p-value
BRCA1-C	-0.297	0.066	-0.287	0.077	-0.275	0.090
BRCA2-C	-0.019	0.918	0.003	0.989	-0.035	0.846
Sporadic-C	-0.153	0.175	0.003	0.982	-0.120	0.289
BRCA1/2-N	-0.252	0.513	-0.467	0.205	-0.417	0.265
Non-*BRCA*-related-N	0.300	0.624	0.100	0.873	0.100	0.873

Correlation between age and CMI (cumulative methylation index) measured by Spearman's rho correlation coefficient. CMI is calculated as the sum of the methylation percentage of all *BRCA1* or *BRCA2* methylation probes.

Correlation between *BRCA1/2* methylation and mRNA expression

Methylation of the evaluated CpG sites within the *BRCA1* and *BRCA2* promoters showed weak correlations with mRNA levels by TCGA data extraction through the Wanderer viewer. The Spearman correlation coefficients between *BRCA1* methylation and mRNA expression were -0.203 for cg04110421 (targeted by the BRCA1.1 probe), -0.296 for cg16630982 (targeted by the BRCA1.2 probe), and -0.172 for cg08993267 (targeted by the BRCA1.3 probe). For *BRCA2*, the most closely located CpG loci IDs from the TCGA data to our MS-MLPA targets were used. Therefore, the correlation between *BRCA2* methylation and mRNA expression should be interpreted with caution. The Spearman correlation coefficients between *BRCA2* methylation and mRNA expression were -0.014 for cg20073910 (82 and 69 bp from the CpG sites targeted by the BRCA2.1 and BRCA2.4 probes, respectively), 0.067 for cg27253386 (80 and 69 bp from the CpG sites targeted

Table 8. *BRCA* promoter methylation percentages in normal breast from *BRCA1/2* germline mutation carriers and non-mutation carriers

Probe	***BRCA1/2*-N**		**Non-*BRCA*-related-N**		**Test statistic[A]**	**p-value**
	Median % (range)		Median % (range)			
BRCA1.1	2	(0-3)	3	(0-5)	15.500	0.325
BRCA1.2	17	(13-26)	13	(13-17)	8.500	0.057
BRCA1.3	9	(6-16)	7	(5-8)	8.000	0.050
BRCA2.1	19	(15-26)	16	(14-19)	8.500	0.060
BRCA2.2	8	(6-16)	6	(5-12)	12.000	0.158
BRCA2.3	13	(10-28)	9	(8-14)	6.500	0.031*
BRCA2.4	8	(6-9)	5	(5-6)	2.000	0.005*

[A] Mann-Whitney U test, testing *BRCA1/2*-N together against non-*BRCA*-related-N; * statistically significant (2-sided p-value <0.05).

by the BRCA2.3 probe), and -0.092 for cg08157964 (25 bp from the CpG site targeted by the BRCA2.2 probe).

***BRCA* promoter methylation in *BRCA1/2*-related and non-*BRCA*-related normal breast tissue**

BRCA1/2-N samples showed statistically significant higher absolute methylation percentages for the BRCA2.3 and BRCA2.4 probes ($p = 0.031$, and $p = 0.005$, respectively) (Table 8, Figure 2). There was a borderline significant trend of higher methylation percentages for the BRCA1.2, BRCA1.3, and BRCA2.1 probes in *BRCA1/2*-N samples compared to non-*BRCA*-related-N cases (Table 8, Figure 2). If methylation cut-offs per probe were based upon the highest methylation percentage found in non-*BRCA*-related-N cases, 40% (2/5) and 60% (3/5) of *BRCA1*-N cases would have at least one methylated *BRCA1* and *BRCA2* probe, respectively. *BRCA2*-N cases would have methylation of at least one *BRCA1* and *BRCA2* probe in 25% (1/4) and 50% (2/4) of cases, respectively (Table 9).

DISCUSSION

The aim of this study was to investigate the diagnostic value of *BRCA1/2* promoter methylation analysis using a new *BRCA* methylation MS-MLPA assay in distinguishing sporadic breast carcinomas from *BRCA1* and *BRCA2* germline mutation-related carcinomas, in order to arrive at a clinically applicable pre-screening test for *BRCA1/2* related cancers.

Table 9. Frequency of *BRCA* methylation (dichotomized) in prophylactic mastectomies of *BRCA1/2* germline mutation carriers by MS-MLPA

Probe	*BRCA1*	*BRCA2*	Total (%)	Cut-off*
	Total n = 5 (%)	Total n = 4 (%)	Total n = 9 (%)	
BRCA1.1	0 (0.0)	0 (0.0)	0 (0.0)	>15%
BRCA1.2	2 (40.0)	1 (25.0)	3 (30.0)	>17%
BRCA1.3	1 (20.0)	0 (0.0)	1 (11.1)	>15%
BRCA1* total*	**2 (40.0)**	**1 (25.0)**	**3 (30.0)**	
BRCA2.1	2 (40.0)	2 (50.0)	4 (44.4)	>19%
BRCA2.2	1 (20.0)	0 (0.0)	1 (11.1)	>15%
BRCA2.3	2 (50.0)	0 (0.0)	2 (22.2)	>15%
BRCA2.4	0 (0.0)	0 (0.0)	0 (0.0)	>15%
BRCA2* total*	**3 (60.0)**	**2 (50.0)**	**5 (55.5)**	

* Cut-off based upon highest methylation percentage detected in normal breast tissue from non-mutation carriers.
** *BRCA1* and *BRCA2* total entails the number and percentage of samples showing methylation in at least one of the *BRCA1* or *BRCA2* probes, respectively.

We observed considerably varying frequencies of *BRCA* promoter methylation between the targeted CpG sites across the *BRCA1* and *BRCA2* promoters. Some CpG sites were methylated more frequently in *BRCA1*/2-C compared to Sporadic-C (those targeted by the BRCA1.2, BRCA2.1, BRCA2.2, BRCA2.3, and BRCA2.4 probes), whereas other CpG sites were methylated more frequently in Sporadic-C (those targeted by the BRCA1.1 and BRCA1.3 probes). In general, we observed frequent *BRCA* promoter methylation in *BRCA1/2*-C. At least 63.8% (46/72) of *BRCA1/2*-C and 12.5% (10/80) of Sporadic-C showed methylation of at least one of the targeted CpG sites in the *BRCA1* or *BRCA2* promoter. Interestingly, several *BRCA1*-C showed *BRCA2* promoter methylation, and *vice versa*. Sensitivity and specificity varied considerably between the probes. The best probes to rule out Sporadic-C when methylation is detected were BRCA2.2 and BRCA2.4 (specificity 100%). However, many *BRCA1/2*-C would be missed as the sensitivity was poor (9.7%). The best probes to rule out *BRCA1/2*-C when methylation is not detected were BRCA1.1 and BRCA1.3 (sensitivity 97.2% and 90.3%, respectively). However, many Sporadic-C would be misclassified as potentially *BRCA1/2* germline mutation-related, as the specificity was poor (both 13.8%). Sensitivity and specificity were most balanced when using all four *BRCA2* probes to rule in *BRCA1/2* germline mutations when methylation is detected in at least one the *BRCA2* probes (sensitivity 69.4%, specificity 87.5%). *BRCA1* promoter methylation was more frequent in high-grade, ER and PR negative tumors. This finding is in line with literature, as *BRCA1* methylation has been more frequently described in triple-negative breast carcinomas [39, 40]. *BRCA2* promoter methylation was more frequent in high-grade tumors, but showed no other statistically significant clinicopathological associations.

In line with our findings, Daniels *et al.* (2016) recently demonstrated that DNA methylation levels vary between CpG sites in the *BRCA1* promoter [41]. However, our findings do not support the general assumption and previous findings in literature that *BRCA* promoter methylation and *BRCA* germline mutations are mutually exclusive. In most studies, none of the *BRCA*-related breast carcinomas showed *BRCA* promoter methylation [16, 21, 23–26]. Kontorovich *et al.* observed *BRCA1* promoter methylation in 3/48 *BRCA1*-related breast carcinomas (6.3%) and Tapia *et al.* observed *BRCA1* promoter methylation in 2/3 observed *BRCA1*-related breast carcinomas (66.7%) [17, 20]. Differences in observed methylation frequencies could be related to the technique and specific CpG sites targeted, the quality of input material and the determination of methylation cut-offs in subsequent analysis. It should be noted that some patients with a *BRCA* germline mutation may develop breast cancer through sporadic breast carcinogenetic mechanisms, which could affect methylation frequencies.

Whether *BRCA* promoter methylation may occur as a second-hit in *BRCA1/2*-related breast carcinomas is still unclear. The main question is whether methylation really drives carcinogenesis or whether it can be considered a bystander. Interestingly, in our study normal breast tissues from *BRCA1/2* germline mutation carriers showed higher *BRCA2* promoter methylation levels compared to normal breast tissues from patients without *BRCA* germline mutations, although the sample size was limited. Bijron *et al.* (2012) have described increased *BRCA2* promoter methylation in normal and precursor Fallopian tube tissues from *BRCA* germline mutation carriers compared to normal sporadic Fallopian tube tissues [42]. *BRCA* methylation might therefore play a role in carcinogenesis in a subset of *BRCA* germline mutation carriers.

To our knowledge, this is the largest study investigating both *BRCA1* and *BRCA2* promoter methylation in *BRCA1* as well as *BRCA2* germline mutation-related breast carcinomas. Moreover, this is the first MS-MLPA study to specifically test *BRCA* promoter methylation in *BRCA1* and *BRCA2*-related breast carcinomas compared to sporadic breast carcinomas and the first MS-MLPA study that investigated *BRCA* methylation levels in normal breast tissues of *BRCA* carriers. We validated our results for one of the *BRCA* methylation probes by comparing them with data obtained from a previous MS-MLPA experiment using the commercially available ME001 MS-MLPA assay.

Our findings may have important implications for clinical practice such as pre-screening for *BRCA* germline genetic testing or eligibility for certain therapeutic strategies. *BRCA1* promoter methylation analysis has been proposed as a cost-effective and reliable pre-

screening tool to exclude *BRCA1* germline mutations in breast cancer patients similar to *MLH1* promoter methylation and Lynch syndrome [22, 43]. Moreover, recent research shows that breast and ovarian carcinomas with *BRCA* deficiencies including *BRCA* methylation may also benefit from PARP inhibitor therapy [30, 31, 44–49].

Although MS-MLPA has been shown to be a reliable tool to assess methylation in general, it targets single specific sites targetable by the HhaI methylation-sensitive restriction enzyme. For MS-MLPA to be a reliable pre-screening tool for ruling in or ruling out *BRCA* germline mutations and/or determining sensitivity for targeted therapy, review of existing literature and further research, preferably assessing all CpG sites in the *BRCA* promoter regions for example by methylation-specific PCR, is needed to determine the most predictive CpG sites for each indication. The most predictive CpG sites should then be targetable by the HhaI methylation-sensitive restriction enzyme, as otherwise MS-MLPA may not be the preferred methylation analysis technique in this context.

CONCLUSIONS

In conclusion, the diagnostic value of *BRCA* promoter methylation analysis in distinguishing *BRCA1/2*-related and sporadic breast carcinomas is considerably dependent on the targeted CpG sites. These findings are important for adequate use of *BRCA* methylation analysis as a pre-screening tool for germline genetic testing or to identify patients who may benefit from targeted therapies such as PARP inhibitors, making their way to the clinic for breast cancer. Further research is needed to assess which other CpG sites are important in ruling in or ruling out *BRCA* germline mutations or determining sensitivity for targeted therapy.

REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J cancer. 2015;136:E359-86.
2. Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nat Rev Cancer. 2012;12:68–78.
3. Antoniou A, Pharoah PDP, Narod S, Risch HA, Eyfjord JE, Hopper JL, et al. Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. Am J Hum Genet. 2003;72:1117–30.
4. Begg CB, Haile RW, Borg A, Malone KE, Concannon P, Thomas DC, et al. Variation of breast cancer risk among BRCA1/2 carriers. JAMA. 2008;299:194–201.
5. Paul A, Paul S. The breast cancer susceptibility genes (BRCA) in breast and ovarian cancers. Front Biosci (Landmark Ed. 2014;19:605–18.
6. Venkitaraman AR. Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell. 2002;108:171–82.
7. Chen S, Parmigiani G. Meta-analysis of BRCA1 and BRCA2 penetrance. J Clin Oncol. 2007;25:1329–33.
8. Vos S, Van der Groep P, Van der Wall E, Van Diest PJ. Hereditary Breast Cancer Syndromes: Molecular Pathogenesis and Diagnostics. eLS. John Wiley & Sons, Ltd: Chichester; 2015.
9. Stefansson OA, Esteller M. Epigenetic modifications in breast cancer and their role in personalized medicine. Am J Pathol. 2013;183:1052–63.
10. Day TK, Bianco-Miotto T. Common gene pathways and families altered by DNA methylation in breast and prostate cancers. Endocr Relat Cancer. 2013;20:R215-32.
11. Nowsheen S, Aziz K, Tran PT, Gorgoulis VG, Yang ES, Georgakilas AG. Epigenetic inactivation of DNA repair in breast cancer. Cancer Lett. 2014;342:213–22.
12. Szyf M. DNA methylation signatures for breast cancer classification and prognosis. Genome Med. 2012;4:26.
13. Jovanovic J, Rønneberg JA, Tost J, Kristensen V. The epigenetics of breast cancer. Mol Oncol. 2010;4:242–54.
14. Suijkerbuijk KPM, van Diest PJ, van der Wall E. Improving early breast cancer detection: focus on methylation. Ann Oncol. 2011;22:24–9.
15. Widschwendter M, Jones PA. DNA methylation and breast carcinogenesis. Oncogene. 2002;21:5462–82.
16. Esteller M, Corn PG, Baylin SB, Herman JG, M. E, P.G. C, et al. A gene hypermethylation profile of human cancer. Cancer Res. 2001;61:3225–3229.
17. Tapia T, Smalley S V, Kohen P, Muñoz A, Solis LM, Corvalan A, et al. Promoter hypermethylation of BRCA1 correlates with absence of expression in hereditary breast cancer tumors. Epigenetics. 2008;3:157–63.
18. Dworkin AM, Spearman AD, Tseng SY, Sweet K, Toland AE, A.M. D, et al. Methylation not a frequent 'second hit' in tumors with germline BRCA mutations. Fam Cancer. 2009;8:339–346.
19. Goodheart MJ, Rose SL, Hattermann-Zogg M, Smith BJ, De Young BR, Buller RE, et al. BRCA2 alteration is important in clear cell carcinoma of the ovary. Clin Genet. 2009;76:161–167.
20. Kontorovich T, Cohen Y, Nir U, Friedman E. Promoter methylation patterns' of ATM, ATR, BRCA1, BRCA2 and P53 as putative cancer risk modifiers in Jewish BRCA1/BRCA2 mutation carriers. Breast Cancer Res Treat. 2009;116:195–200.
21. Rennstam K, Ringberg A, Cunliffe HE, Olsson H, Landberg G, Hedenfalk I, et al. Genomic alterations in histopathologically normal breast tissue from BRCA1 mutation carriers may be caused by BRCA1 haploinsufficiency. Genes Chromosom Cancer. 2010;49:78–90.
22. Lips EH, Mulder L, Oonk A, van der Kolk LE, Hogervorst FBL, Imholz ALT, et al. Triple-negative breast cancer: BRCAness and concordance of clinical features with BRCA1-mutation carriers. Br J Cancer. 2013;108:2172–2177.

23. Toffoli S, Bar I, Abdel-Sater F, Delree P, Hilbert P, Cavallin F, et al. Identification by array comparative genomic hybridization of a new amplicon on chromosome 17q highly recurrent in BRCA1 mutated triple negative breast cancer. Breast Cancer Res. 2014;16:466.
24. Severson TM, Peeters J, Majewski I, Michaut M, Bosma A, Schouten PC, et al. BRCA1-like signature in triple negative breast cancer: Molecular and clinical characterization reveals subgroups with therapeutic potential. Mol Oncol. 2015;9:1528–1538.
25. Tung N, Miron A, Schnitt SJ, Gautam S, Fetten K, Kaplan J, et al. Prevalence and predictors of loss of wild type BRCA1 in estrogen receptor positive and negative BRCA1-associated breast cancers. Breast Cancer Res. 2010;12:R95.
26. Lisowska KM, Dudaladava V, Jarzab M, Huzarski T, Chmielik E, Stobiecka E, et al. BRCA1-related gene signature in breast cancer: the role of ER status and molecular type. Front Biosci (Elite Ed). 2011;3:125–36.
27. Lee J-M, Ledermann JA, Kohn EC. PARP Inhibitors for BRCA1/2 mutation-associated and BRCA-like malignancies. Ann Oncol. 2014;25:32–40.
28. Livraghi L, Garber JE. PARP inhibitors in the management of breast cancer: current data and future prospects. BMC Med. 2015;13:188.
29. Dizdar O, Arslan C, Altundag K. Advances in PARP inhibitors for the treatment of breast cancer. Expert Opin Pharmacother. 2015;16:2751–8.
30. Ledermann JA. PARP inhibitors in ovarian cancer. Ann Oncol. 2016;27 Suppl 1:i40–i44.
31. Moschetta M, George A, Kaye SB, Banerjee S. BRCA somatic mutations and epigenetic BRCA modifications in serous ovarian cancer. Ann Oncol. 2016;27:1449–55.
32. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology. 1991;19:403–10.
33. Nygren AOH, Ameziane N, Duarte HMB, Vijzelaar RNCP, Waisfisz Q, Hess CJ, et al. Methylation-specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. Nucleic Acids Res. 2005;33:e128.
34. Leong KJ, Wei W, Tannahill LA, Caldwell GM, Jones CE, Morton DG, et al. Methylation profiling of rectal cancer identifies novel markers of early-stage disease. Br J Surg. 2011;98:724–34.
35. Paulsson K, An Q, Moorman A V, Parker H, Molloy G, Davies T, et al. Methylation of tumour suppressor gene promoters in the presence and absence of transcriptional silencing in high hyperdiploid acute lymphoblastic leukaemia. Br J Haematol. 2009;144:838–47.
36. Dikow N, Nygren AO, Schouten JP, Hartmann C, Krämer N, Janssen B, et al. Quantification of the methylation status of the PWS/AS imprinted region: comparison of two approaches based on bisulfite sequencing and methylation-sensitive MLPA. Mol Cell Probes. 2007;21:208–15.
37. Suijkerbuijk KPM, Pan X, van der Wall E, van Diest PJ, Vooijs M. Comparison of different promoter methylation assays in breast cancer. Anal Cell Pathol (Amst). 2010;33:133–41.
38. Díez-Villanueva A, Mallona I, Peinado MA. Wanderer, an interactive viewer to explore DNA methylation and gene expression data in human cancer. Epigenetics Chromatin. 2015;8:22.
39. Jacot W, Thezenas S, Senal R, Viglianti C, Laberenne A-C, Lopez-Crapez E, et al. BRCA1 promoter hypermethylation, 53BP1 protein expression and PARP-1 activity as biomarkers of DNA repair deficit in breast cancer. BMC Cancer. 2013;13:523.
40. Bal A, Verma S, Joshi K, Singla A, Thakur R, Arora S, et al. BRCA1-methylated sporadic breast cancers are BRCA-like in showing a basal phenotype and absence of ER expression. Virchows Arch. 2012;461:305–312.
41. Daniels SL, Burghel GJ, Chambers P, Al-Baba S, Connley DD, Brock IW, et al. Levels of DNA Methylation Vary at CpG Sites across the BRCA1 Promoter, and Differ According to Triple Negative and BRCA-Like Status, in Both Blood and Tumour DNA. PLoS One. 2016;11:e0160174.
42. Bijron JG, van der Groep P, van Dorst EB, Seeber LMS, Sie-Go DMDS, Verheijen RHM, et al. Promoter hypermethylation patterns in fallopian tube epithelium of BRCA1 and BRCA2 germ line mutation carriers. Endocr Relat Cancer. 2012;19:69–81.

43. Gausachs M, Mur P, Corral J, Pineda M, González S, Benito L, et al. MLH1 promoter hypermethylation in the analytical algorithm of Lynch syndrome: a cost-effectiveness study. Eur J Hum Genet. 2012;20:762–8.

44. Crafton SM, Bixel K, Hays JL. PARP inhibition and gynecologic malignancies: A review of current literature and on-going trials. Gynecol Oncol. 2016;142:588–96.

45. Alsop K, Fereday S, Meldrum C, deFazio A, Emmanuel C, George J, et al. BRCA mutation frequency and patterns of treatment response in BRCA mutation-positive women with ovarian cancer: a report from the Australian Ovarian Cancer Study Group. J Clin Oncol. 2012;30:2654–63.

46. Fong PC, Yap TA, Boss DS, Carden CP, Mergui-Roelvink M, Gourley C, et al. Poly(ADP)-ribose polymerase inhibition: frequent durable responses in BRCA carrier ovarian cancer correlating with platinum-free interval. J Clin Oncol. 2010;28:2512–9.

47. Banerjee S, Kaye SB, Ashworth A. Making the best of PARP inhibitors in ovarian cancer. Nat Rev Clin Oncol. 2010;7:508–19.

48. Konstantinopoulos PA, Spentzos D, Karlan BY, Taniguchi T, Fountzilas E, Francoeur N, et al. Gene expression profile of BRCAness that correlates with responsiveness to chemotherapy and with outcome in patients with epithelial ovarian cancer. J Clin Oncol. 2010;28:3555–61.

49. Veeck J, Ropero S, Setien F, Gonzalez-Suarez E, Osorio A, Benitez J, et al. BRCA1 CpG island hypermethylation predicts sensitivity to poly(adenosine diphosphate)-ribose polymerase inhibitors. J Clin Oncol. 2010;28:e563-4-6.

Chapter 5

A systematic review on the frequency of *BRCA* promoter methylation in breast and ovarian carcinomas of *BRCA* germline mutation carriers: mutually exclusive, or not?

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ABSTRACT

Background

A considerable number of breast and ovarian carcinomas are due to underlying *BRCA* gene aberrations. Of these, *BRCA* germline mutations and *BRCA* promoter methylation are thought to be mutually exclusive, which could be exploited in clinical practice. However, this paradigm has not been studied extensively and systematically.

Objective

To systematically investigate to what extent *BRCA* promoter methylation has been reported in breast and ovarian carcinomas of *BRCA* germline mutation carriers.

Methods

A comprehensive search on *BRCA* promoter methylation was performed in PubMed and Embase databases. Two authors independently selected studies, assessed study quality and extracted data according to PRISMA and QUADAS-2 guidelines.

Results

21 articles met the inclusion criteria. *BRCA1* methylation was found in at least 10/276 (3,6%) breast and 2/174 (1,1%) ovarian carcinomas of *BRCA* germline mutation carriers, and *BRCA2* methylation was found in at least 7/131 (5.3%) breast and 0/51 (0.0%) ovarian carcinomas of *BRCA* germline mutation carriers. Methylation frequencies varied between individual CpG sites. The selected studies showed important differences in methodology and performed in general a limited methylation and incomplete mutation analysis.

Conclusions

BRCA methylation is rare in breast and ovarian carcinomas of *BRCA* germline mutation carriers, although the frequency of *BRCA* promoter methylation may be underestimated. This could have major implications for clinical practice, including referral for genetic testing and *BRCA*ness analysis for treatment decision-making.

INTRODUCTION

Breast and ovarian cancer are among the most frequent cancer types in women worldwide. Breast cancer is the most frequent cancer with an estimated incidence of 1.7 million cancer cases and ovarian cancer is the seventh most common cancer with an estimated incidence of 239.000 cases [1]. In 5-15% of cases, breast and ovarian cancer occur in a hereditary setting, mostly due to germline mutations in the *BRCA1* (17q21.31) or *BRCA2* (13q13.1) genes. These *BRCA* germline mutations cause a 40-80% lifetime risk of developing breast cancer and 30-40% lifetime risk of ovarian cancer development [2–8]. However, other carcinogenic *BRCA* aberrations also occur in breast and ovarian cancer, including somatic mutations and promoter hypermethylation [9–13]. It has been proposed that *BRCA* promoter hypermethylation takes place almost exclusively in a sporadic setting and rarely occurs in patients with an underlying *BRCA1* or *BRCA2* germline mutation, although this has not been studied extensively and the evidence level of individual studies is limited. However, this paradigm could have major implications for clinical practice regarding for example a test that may obviate *BRCA1/2* germline mutation testing in case of promoter methylation. The purpose of this study was to investigate to what extent *BRCA1* and *BRCA2* promoter hypermethylation have been reported in breast and ovarian carcinomas of *BRCA1* and *BRCA2* germline mutation carriers, in order to establish how close *BRCA1/2* promoter methylation assays may be for implementation into clinical practice.

MATERIAL AND METHODS

Search strategy

The systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines [14,15]. A search string that combined synonyms for *BRCA* and methylation was applied to PubMed and Embase databases on 3 February 2016 (Supplementary Table 1).

Data collection and analysis

Studies that performed *BRCA1* and/or *BRCA2* methylation analysis in breast and/or ovarian carcinoma tissues from *BRCA1* and/or *BRCA2* (*BRCA1/2*) germline mutation carriers were selected. The exclusion criteria, applied to the title and abstract and full text of the studies, can be found in Supplementary Figure 1. From the selected studies, the following data were extracted: total number of patients; number of patients with *BRCA1/2* germline mutations; number of patients without *BRCA1/2* germline mutations

(if available); tumor type; method of methylation and mutation analysis including applied cut-offs, controls, targeted Cytosine phosphate Guanine (CpG) sites or exons, as well as the flow and timing of the analyses; and the frequency of observed *BRCA1/2* promoter methylation in breast and/or ovarian carcinomas from *BRCA1/2* mutation carriers and if available, from non-mutation carriers as well (Table 1 and Table 2A-D). Sensitivity and specificity values were calculated when possible. When the primer sequences of the methylation analysis were available, the targeted CpG sites could be identified by mapping them on the Human Genome version GRCh37/hg19, using the Genome Browser and Blat Tool from the University of California Santa Cruz (UCSC) (available from: https://genome.ucsc.edu/index.html). The primer sequences can be found in Supplementary Table 2. The quality, i.e. applicability and risk of bias of the selected studies was assessed according to a modified version of the Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2) guideline, which includes evaluation of patient selection, index test, reference standard and flow and timing [16](Table 3, Supplementary Table 3). Two review authors (S.V. and C.B.M.) independently selected the studies, performed data extraction and assessed study quality. Disagreements were resolved by discussion.

RESULTS

Study characteristics

The search strategy identified 779 unique articles, of which 21 met the inclusion criteria (Supplementary Figure 1). These studies have been published between 2001 and 2017. The characteristics of the selected studies can be found in Table 1.

Participants

The primary research question of 5 studies matched the aim of this review [17–21]. The remaining studies analysed genetic and epigenetic alterations (including *BRCA* mutation and methylation) in breast and ovarian carcinomas in general or in specific subgroups such as triple-negative breast carcinomas [22–24], high-grade serous ovarian carcinomas [25], clear cell ovarian carcinomas [26], or ovarian cancer resistant for platinum-based chemotherapy [27]. A couple of studies investigating ovarian carcinomas also included peritoneal or Fallopian tube cancer [27,28].

The selected studies show large differences in sample size. The total number of patients varied from 13-1063 [26,29], including 3-99 *BRCA1*-associated cancers [17,30] and 1-34 *BRCA2*-associated breast cancers [17,31]. Twelve studies used fresh frozen (FF) tissue

Table 1. Study characteristics

Study			**Material**					**Method**	
Author	Year	Journal	Br/Ov	FF/ FFPE	N total	N *BRCA1*	N *BRCA2*	Methylation	Mutation
Esteller	2001	Hum Mol Genet	Br	FF	268	99 T	34 T	*BRCA1*: MSP Primer sets: 1M/1U CpG sites: 7/7	For *BRCA1*: PTT (exon 10-11)/SSCP (rest) + second-line sequencing For *BRCA2*: first-line sequencing for exons 3,5,6,14,18,25; rest second-line sequencing Material: blood Mutation analysis in sporadic tumors: no
Gras	2001	Cancer	Ov	FF	52	NA	2 T	*BRCA2*: MSP Primer sets: 4M/4U CpG sites: 20/20	*BRCA2*: PTT (exon 10-11) /SSCP (exon 2-9 and 12-27) + second-line sequencing Material: tumor tissue + normal tissue + blood Mutation analysis in sporadic tumors: yes
Hilton	2002	J Nat Cancer Inst	Ov	FF	92	NA	5 T	*BRCA2*: MSP Primer sets: 1M/1U CpG sites: 2/1	*BRCA2*: PTT + second-line sequencing Material: tumor tissue + blood Mutation analysis in sporadic tumors: unclear
Press	2008	BMC Cancer	Ov	Unclear	49	8 T	2 T	*BRCA1*: qMSP (SYBR Green) Primer sets: 1M/0U CpG sites: 5/5 Cutoff: percentage of methylated reference ≥4	*BRCA1*: dHPLC + second-line sequencing + MLPA (deletions + duplications) Material: tumor tissue + blood Mutation analysis in sporadic tumors: yes
Tapia	2008	Epigenetics	Br	FFPE	49	3 T	2 T	*BRCA1*: MSP Primer sets: 1M/1U CpG sites: 7/7	*BRCA1*+2: heteroduplex analysis/PTT/SSCP + second-line sequencing Material: blood Mutation analysis in sporadic tumors: NA
Dworkin	2009	Fam Cancer	Br + Ov	FFPE	61	38 T	23 T	*BRCA1*+2: MSP Primer sets: 1M/1U (*BRCA1*), 1M/1U (*BRCA2*) CpG sites: 7/7 (*BRCA1*), 6/4 (*BRCA2*)	*BRCA1*+2: unclear Mutation analysis in sporadic tumors: NA
Goodheart	2009	Clin Genet	Ov	FF	13	NA	3 T	*BRCA2*: MSP Primer sets: 1M/1U CpG sites: 2/1	*BRCA2*: PTT + second-line sequencing Material: tumor tissue + blood Mutation analysis in sporadic tumors: yes
Kontorovich	2009	Breast Cancer Res Treat	Br	Unclear	230	26 T	22 T	*BRCA1*+2: qMSP (fluorogenic probe) Primer sets: 1M/0U (*BRCA1*), 1M/0U (*BRCA2*) CpG sites: 8/8 (*BRCA1*), 10/10 (*BRCA2*)	*BRCA1*+2: unclear Mutation analysis in sporadic tumors: yes

Study			Material					Method	
Author	Year	Journal	Br/Ov	FF/ FFPE	N total	N *BRCA1*	N *BRCA2*	Methylation	Mutation
Bol	2010	Histopathology	Ov	FFPE	75	25 T	NA	*BRCA1+2*: MS-MLPA (MRC Holland ME001b) Primer sets: 1 (*BRCA1*), 1 (*BRCA2*) CpG sites: 1/1 (*BRCA1*), 1/1 (*BRCA2*) Cutoff: >15% methylation	*BRCA1*: unclear Mutation analysis in sporadic tumors: no
Rennstam	2010	Genes Chrom Cancer	Br	FF	19	4 T	NA	*BRCA1*: pyrosequencing Primers: 2 CpG sites: 4/4	*BRCA1*: unclear Mutation analysis in sporadic tumors: NA
Tung	2010	Breast Cancer Res	Br	FFPE	88	88 T	NA	*BRCA1*: MSP Primer sets: 1M/1U CpG sites: 6/6	*BRCA1*: bidirectional first-line sequencing (missense and nonsense mutations) + DCP (insertions and deletions) Mutation analysis in sporadic tumors: NA
Lisowska	2011	Front Bioscience	Br	FF	35	12 T	1 T	*BRCA1*: MSP Primer sets: 1M/1U CpG sites: 6/6	*BRCA1+2*: multiplex PCR for most common mutations + allele-specific PCR (one additional mutation in each gene) Material: unclear Mutation analysis in sporadic tumors: no
Skytte	2011	ACTA Obs Gyn	Ov	FFPE	54	15 T	4 T	*BRCA1*: MS RT-PCR; for 3 samples additional bisulfite sequencing Primer sets: 1M/0U CpG sites: 4/4	*BRCA1*: TGCE/dHPLC/PTT/MLPA (combination) Material: unclear Mutation analysis in sporadic tumors: no
Yang	2011	JAMA	Ov	FF	316	27 T	20 T	*BRCA1+2*: genome-wide methylation assay (Illumina Infinium HumanMethylation27 arrays) CpG sites: 9/4 (*BRCA1*), 2/1 (*BRCA2*)	*BRCA1+2*: WES Material: tumor tissue + normal tissue Mutation analysis in sporadic tumors: yes
Rzepecka	2012	Cancer Genet	Ov	FF	257	38 T	NA	*BRCA1*: qMSP (SYBR Green) Primer sets: 1M/0U CpG sites: 8/8 Cutoff: percentage of methylated reference ≥4	*BRCA1*: SSCP/heteroduplex analysis + second-line sequencing for most common mutations Tumor tissue + normal tissue + blood Mutation analysis in sporadic tumors: yes
Lips	2013	Brit J Cancer	Br	FF	377	31 T	2 T	*BRCA1*: MS-MLPA (MRC Holland ME005 neoadjuvant series; ME001 familial series) Primer sets: 1 (ME001), unclear (ME005) CpG sites: 1/1 (ME001) Cutoff: 20% methylation	*BRCA1*: PTT (*BRCA1* exon 11, *BRCA2* exon 10 and 11)/DGGE (remaining exons)/ MLPA + second-line sequencing Material: blood Mutation analysis in sporadic tumors: yes

Cunningham	2014	Scientific Reports	Ov	FF	1063	32 T	28 T	*BRCA1+2*: genome-wide methylation assay, validation 1 CpG locus with pyrosequencing (Illumina Infinium Human Methylation 450 Bead Chip) CpG sites: 46/30 (*BRCA1*); 19/8 (*BRCA2*) Cutoff: >0.15	*BRCA1+2*: NGS (Illumina sequencing) Material: blood Mutation analysis in sporadic tumors: yes
Toffoli	2014	Breast Cancer Resear-ch	Br	FFPE	131	32 T	NA	*BRCA1*: qMSP (SYBR Green) Primer sets: unclear CpG sites: unclear Cutoff: unclear	*BRCA1*: unclear Mutation analysis in sporadic tumors: no
Severson	2015	Mol Oncol	Br	FF	112	10 T	NA	*BRCA1*: MS-MLPA (MRC Holland ME001) Primer sets: 1 CpG sites: 1/1 Cutoff: 20% methylation	*BRCA1*: WGS (Illumina TruSeq), validation by germline sequencing* Material: tumor tissue + matched normal DNA Mutation analysis in sporadic tumors: yes
Patch	2015	Nature	Ov	FF	99	13 T	0 T	*BRCA1+2*: genome-wide methylation assay (Illumina Infinium Methylation 450 Bead Chip) CpG sites: 46/30 (*BRCA1*); 19/8 (*BRCA2*) Cutoff: unclear	*BRCA1+2*: WGS (Illumina TruSeq) Material: blood + tissue Mutation analysis in sporadic tumors: yes
Vos	2017	Breast Cancer Res	Br	FFPE	166	39 T	33 T	*BRCA1+2*: MS-MLPA (MRC Holland ME053) Primer sets: 3 (*BRCA1*); 4 (*BRCA2*) CpG sites: 3/3 (*BRCA1*); 5/5 (*BRCA2*) Cutoff: based upon methylation levels of normal breast tissue from non-mutation carriers	*BRCA1+2*: unclear Material: blood Mutation analysis in sporadic tumors: no

1M/1U: 1 methylated primer/1 unmethylated primer; Br: Breast; *BRCA1*m: *BRCA1* promoter methylation; *BRCA1*T: *BRCA1*-associated tumor; DCP: Denaturing Capillary Analysis; DGGE: Denaturing Gradient Gel Electrophoresis; dHPLC: Denaturing High Performance Liquid Chromatography, FF: Fresh Frozen; FFPE: Formalin-Fixed Paraffin-Embedded;
LOH: Loss of Heterozygosity; MS-MLPA: Methylation-Specific Multiplex Ligation-dependent Probe Amplification; MS RT-PCR: Methylation-Specific RT-PCR; MSP: Methylation-Specific PCR; N: Normal tissue; NA: Not Applicable; NGS: Next-Generation Sequencing; Ov: Ovarian; PTT: Protein Truncation Test; qMSP: quantitative MSP; SSCP:
Single-Strand Conformation Polymorphism, T: Tumor tissue; TGCE: Temperature Gradient Capillary Electrophoresis; WES: Whole Exome Sequencing; WGA: Whole Genome Amplification; WGS: Whole Genome Amplification

Table 2A. Frequency of *BRCA1* promoter methylation in breast carcinomas of *BRCA* germline mutation carriers

Study	**Results**			
Author	*BRCA*	Sporadic	Test characteristics	
Esteller	*BRCA1*T: *BRCA1*m LOH+: 0/21 (0.0%), LOH-: 1/2 (50.0%) *BRCA2*T: *BRCA1*m NA	Sporadic: 13/106 *BRCA1*m (12.3%)	*BRCA1*m: *BRCA1*T vs sporadic SE: 22/23 = 95.7% SP: 13/106 = 12.3%	
Tapia	*BRCA1*T: 2/3 *BRCA1*m (66.7%) *BRCA2*T: 1/2 *BRCA1*m (50.0%)	Hereditary without BRCAmut: 21/42 *BRCA1*m (50.0%)	*BRCA1*m: *BRCA1*T vs sporadic SE: 1/3 = 33.3% SP: 21/42 = 50.0%	*BRCA1*m: *BRCA1*+2T vs sporadic SE: 2/5 = 40.0% SP: 21/42 = 50.0%
Dworkin	*BRCA1*T: 0/11 *BRCA1*m (0.0%)	NA	NA	
Kontorovich	*BRCA1*+2T: 3/48 *BRCA1*m (6.3%)	Sporadic: 3/52 *BRCA1*m (5.8%)		*BRCA1*m: *BRCA1*+2T vs sporadic SE: 45/48 = 93.8% SP: 3/52 = 5.8%
Rennstam	*BRCA1*T: 0/4 *BRCA1*m (0.0%)	NA	NA	
Tung	*BRCA1*T: 1/28 *BRCA1*m (3.6%) This case was LOH-	NA	NA	
Lisowska	*BRCA1*T: 0/12 *BRCA1*m (0.0%) *BRCA2*T: 0/1 *BRCA1*m (0.0%)	Sporadic: 5/14 *BRCA1*m (35.7%)	*BRCA1*m: *BRCA1*T vs sporadic SE: 12/12 = 100% SP: 5/14 = 35.7%	*BRCA1*m: *BRCA1*+2T vs sporadic SE: 13/13 = 100% SP: 5/14 = 35.7%
Lips	*BRCA1*T: 0/31 *BRCA1*m (0.0%)	Sporadic: 52/149 *BRCA1*m (34.9%) TNBC	*BRCA1*m: *BRCA1*T vs sporadic SE: 31/31 = 100% SP: 52/149 = 34.9%	
Toffoli	*BRCA1*T: 0/32 *BRCA1*m (0.0%)	Non-mutated TNBC: 7/21 *BRCA1*m (33.3%) Unscreened TNBC: 3/15 *BRCA1*m (20.0%)	*BRCA1*m: *BRCA1*T vs non-mut SE: 32/32 = 100% SP: 7/21 = 33.3%	*BRCA1*m: *BRCA1*T vs unscreened SE: 3/15 = 20.0% SP: 32/32 = 100%
Severson	*BRCA1*T: 0/9 *BRCA1*m (0.0%)	Sporadic: 14/79 *BRCA1*m (17.7%) TNBC	*BRCA1*m: *BRCA1*T vs sporadic SE: 9/9 = 100% SP: 14/79 = 17.7%	
Vos[‡]	*BRCA1*T: 0-32/39 *BRCA1*m (0.0-82.1%) *BRCA2*T: 2-14/33 *BRCA1*m (6.1-42.4%)	Sporadic: 11-22/80 *BRCA1*m (13.8-27.5%)	*BRCA1*m: *BRCA1*T vs sporadic Rule out *BRCA* mutation: SE: 39/39 = 100% SP: 11/80 = 13.8% Rule in *BRCA* mutation: SE: 32/39 = 82.1% SP: 58/80 = 72.5%	*BRCA1*m: *BRCA1*+2T vs sporadic Rule out *BRCA* mutation: SE: 70/72 = 97.2% SP: 11/80 = 13.8% Rule in *BRCA* mutation: SE: 46/72 = 63.9% SP: 58/80 = 72.5%

*BRCA1/2*T: *BRCA1/2*-associated tumors; *BRCA1/2*m: *BRCA1/2* promoter hypermethylation; LOH: Loss of Heterozygosity; NPV: Negative Predictive Value; PPV: Positive Predictive Value; SE: Sensitivity; SP: Specificity; TNBC: Triple-Negative Breast Carcinomas

[‡] Vos *et al.* analysed methylation frequencies for the targeted CpG sites separately. The range of methylation frequencies is presented in this table. The calculation of SE and SP differs between the probes due to differences in methylation frequencies between *BRCA1/2*T and sporadic tumors. For some CpG sites, the SE and SP are calculated as if *BRCA* promoter methylation analysis would be performed to rule out *BRCA* germline mutations when methylation is not detected, whereas for others the SE and SP are calculated as if *BRCA* promoter methylation analysis would be performed to rule in *BRCA* germline mutations when methylation is detected. The most balanced combination of SE and SP to rule out or to rule in *BRCA* germline mutations is presented in this table.

Table 2B. Frequency of *BRCA1* promoter methylation in ovarian carcinomas of *BRCA* germline mutation carriers

Study	**Results**			
Author	*BRCA*	Sporadic	Test characteristics	
Press	*BRCA1*T: 0/8 *BRCA1*m (0.0%) (2 LOH-, 6 LOH+) *BRCA2*T: 0/2 *BRCA1*m (0.0%) (both LOH+)	Sporadic: 9/40 *BRCA1*m (22.5%)	*BRCA1*m: *BRCA1*T vs sporadic SE: 8/8 = 100% SP: 9/40 = 22.5%	*BRCA1*m: *BRCA1*+2T vs sporadic SE: 10/10 = 100% SP: 9/40 = 22.5%
Bol	*BRCA1*T: 0/25 *BRCA1*m (0.0%)	Sporadic: 7/50 *BRCA1*m (14.0%)	*BRCA1*m: *BRCA1*T vs sporadic SE: 25/25 = 100% SP: 7/50 = 14.0%	
Skytte	*BRCA1*T: 1/15 *BRCA1*m (6.7%) *BRCA2*T: 0/4 *BRCA1*m (0.0%)	Unknown *BRCA*: 3/24 *BRCA1*m (12.5%)	*BRCA1*m: *BRCA1*T vs sporadic SE: 14/15 = 93.3% SP: 3/24 = 12.5%	*BRCA1*m: *BRCA1*+2T vs sporadic SE: 18/19 = 94.7% SP: 3/24 = 12.5%
Yang	*BRCA1*T: 0/37 *BRCA1*m (0.0%)	Sporadic: 33/269 *BRCA1*m (12.3%) Sporadic: 0/269 *BRCA2*m (0.0%)	*BRCA1*m: *BRCA1*T vs sporadic SE: 37/37 = 100% SP: 33/269 = 12.3%	
Rzepecka	*BRCA1*T: 1/38 *BRCA1*m (2.6%)	Sporadic: 32/203 *BRCA1*m (15.8%)	*BRCA1*m: *BRCA1*T vs sporadic SE: 37/38 = 97.4% SP: 32/303 = 10.6%	
Cunningham	*BRCA1*T: 0/32 *BRCA1*m (0.0%) *BRCA2*T: not analysed	Unclear Unclear		
Patch	*BRCA1*T: 0/13 *BRCA1*m (0.0%) *BRCA2*T: not analysed	Sporadic: 12/67 (17.9%) *BRCA1*m	*BRCA1*m: *BRCA1*T vs sporadic SE: 13/13 = 100% SP: 12/56 = 21.4%	

*BRCA1/2*T: *BRCA1/2*-associated tumors; *BRCA1/2*m: *BRCA1/2* promoter hypermethylation; LOH: Loss of Heterozygosity; NPV: Negative Predictive Value; PPV: Positive Predictive Value; SE: Sensitivity; SP: Specificity; TNBC: Triple-Negative Breast Carcinomas

Table 2C. Frequency of *BRCA2* promoter methylation in breast carcinomas of *BRCA* germline mutation carriers

Study Author	**Results** *BRCA*	Sporadic	Test characteristics	
Dworkin	*BRCA2*T: 1/11 *BRCA2*m (9.1%) (Br+Ov) This case was LOH-	NA	NA	
Kontorovich	*BRCA1*+2T: 0/48 *BRCA2*m (0.0%)	Sporadic: 0/52 *BRCA1*m (0.0%)		*BRCA2*m: *BRCA1*+2T vs sporadic SE: 48/48 = 100% SP: 0/52 = 0.0%
Vos[‡]	*BRCA1*T: 3-30/39 *BRCA2*m (7.7-76.9%) *BRCA2*T: 3-18/33 *BRCA2*m (9.1-54.5%)	Sporadic: 0-10/80 *BRCA2*m (0.0-12.5%)	*BRCA2*m: *BRCA2*T vs sporadic Rule in *BRCA* mutation: SE: 30/39 = 76.9% SP: 70/80 = 87.5%	*BRCA2*m: *BRCA1*+2T vs sporadic Rule in *BRCA* mutation: SE: 50/72 = 69.4% SP: 70/80 = 87.5%

*BRCA1/2*T: *BRCA1/2*-associated tumors; *BRCA1/2*m: *BRCA1/2* promoter hypermethylation; LOH: Loss of Heterozygosity; NA: Not Applicable; NPV: Negative Predictive Value; PPV: Positive Predictive Value; SE: Sensitivity; SP: Specificity

[‡] Vos *et al*. analysed methylation frequencies for the targeted CpG sites separately. The range of methylation frequencies is presented in this table. The SE and SP are calculated as if *BRCA* promoter methylation analysis would be performed to rule in *BRCA* germline mutations when methylation is detected. The most balanced combination of SE and SP to rule out or to rule in *BRCA* germline mutations is presented in this table.

Table 2D: Frequency of *BRCA2* promoter methylation in ovarian carcinomas of *BRCA* germline mutation carriers

Study	**Results**		
Author	*BRCA*	Sporadic	Test characteristics
Gras	*BRCA2*T: 0/2 *BRCA2*m (0.0%)	Sporadic: 0/14 *BRCA2*m (0.0%)	*BRCA2*m: *BRCA2*T vs sporadic SE: 2/2 = 100% SP: 0/14 = 0.0%
Hilton	*BRCA2*T: 0/1 *BRCA2*m (0.0%) All 5 *BRCA2*T were LOH+	Sporadic: 1/11 *BRCA2*m (9.1%)	*BRCA2*m: *BRCA2*T vs sporadic SE: 1/1 = 100% SP: 1/11 = 9.1%
Dworkin	*BRCA2*T: 0/2 *BRCA2*m (0.0%)	NA	NA
Goodheart	*BRCA2*T: 0/1 *BRCA2*m (0.0%)	Sporadic: 1/2 *BRCA2*m (50.0%)	*BRCA2*m: *BRCA2*T vs sporadic SE: 1/1 = 100% SP: 1/2 = 50.0%
Bol	*BRCA1*T: 0/25 *BRCA2*m (0.0%)	Sporadic: 0/50 *BRCA2*m (0.0%)	*BRCA2*m: *BRCA1*T vs sporadic SE: 25/25 = 100% SP: 0/50 = 0.0%
Yang	*BRCA2*T: 0/20 *BRCA2*m (0.0%)	Sporadic: 0/269 *BRCA2*m (0.0%)	*BRCA2*m: *BRCA2*T vs sporadic SE: 20/20 = 100% SP: 0/269 = 0.0%

*BRCA1/2*T: *BRCA1/2*-associated tumors; *BRCA1/2*m: *BRCA1/2* promoter hypermethylation; LOH: Loss of Heterozygosity; NA: Not Applicable; NPV: Negative Predictive Value; PPV: Positive Predictive Value; SE: Sensitivity; SP: Specificity

samples [17,22,33,34,24–29,31,32], whereas 7 used formalin-fixed paraffin-embedded (FFPE) tissue samples [18,20,21,23,30,35,36]. Of 2 studies it was unclear whether they used FF or FFPE tissue samples [19,37].

METHODS

Ten studies investigated *BRCA* promoter methylation in breast cancer (8 *BRCA1* promoter methylation, 2 both *BRCA1* and *BRCA2* promoter methylation). Another 10 studies investigated *BRCA* promoter methylation in ovarian cancer (5 *BRCA1* promoter methylation, 3 *BRCA2* promoter methylation, and 2 both *BRCA1* and *BRCA2* promoter methylation). 1 study investigated both *BRCA1* and *BRCA2* promoter methylation in breast as well as ovarian carcinomas.

Several techniques were used to study BRCA promoter methylation, targeting variable numbers of CpG sites in the *BRCA1* and *BRCA2* genomic region (1-46 CpG sites) (Table 1) [20,22,24,27,29]. Methylation-specific PCR (MSP) was the most frequently applied technique (8 studies) [17,18,26,28,30–32,35]. From 20 studies, the primer sequences could be derived by which the targeted CpG sites could be identified [17,18,28–37,19–22,24–27]. For *BRCA1*, 37 CpG sites of a total of 95 relevant CpG sites (38.9%) were analysed when taking the 17 applicable studies together (Figure 1A, Supplementary Table 4A). CpG sites were considered relevant when located in the first CpG island upstream of the transcription start site (TSS) (containing 24 CpG sites), in the promoter region or the first exon [38,39]. The most extensive studies have analysed 30 relevant CpG sites (31.6%) [27,29]. Most of the CpG sites (n = 24) were analysed in 2 independent studies. The most frequently studied CpG site was located at chr17:41277445, analysed in 11 studies. Interestingly, there is overlap in targeted CpG sites between studies that showed *BRCA1* promoter methylation in *BRCA*-associated cancers and those that did not. For *BRCA2*, 28 CpG sites of a total of 70 relevant CpG sites (40.0%) were analysed when taking the selected studies together (Figure 1B, Supplementary Table 4B). The most extensive study analysed 20 relevant CpG sites (28.6%) [32]. Most of the CpG sites (n = 17) were analysed in only one study. The most targeted CpG site was located at chr13:32889608, analysed in 4 studies. Again, there is overlap in targeted CpG sites between studies that showed *BRCA2* promoter methylation in *BRCA*-associated cancers and those that did not, although the study of Vos and Moelans *et al.* (2017) investigated relatively unique CpG sites [21]. This is also the only study that has investigated methylation frequencies per CpG site and found that these varied considerably (Table 2A + 2C).

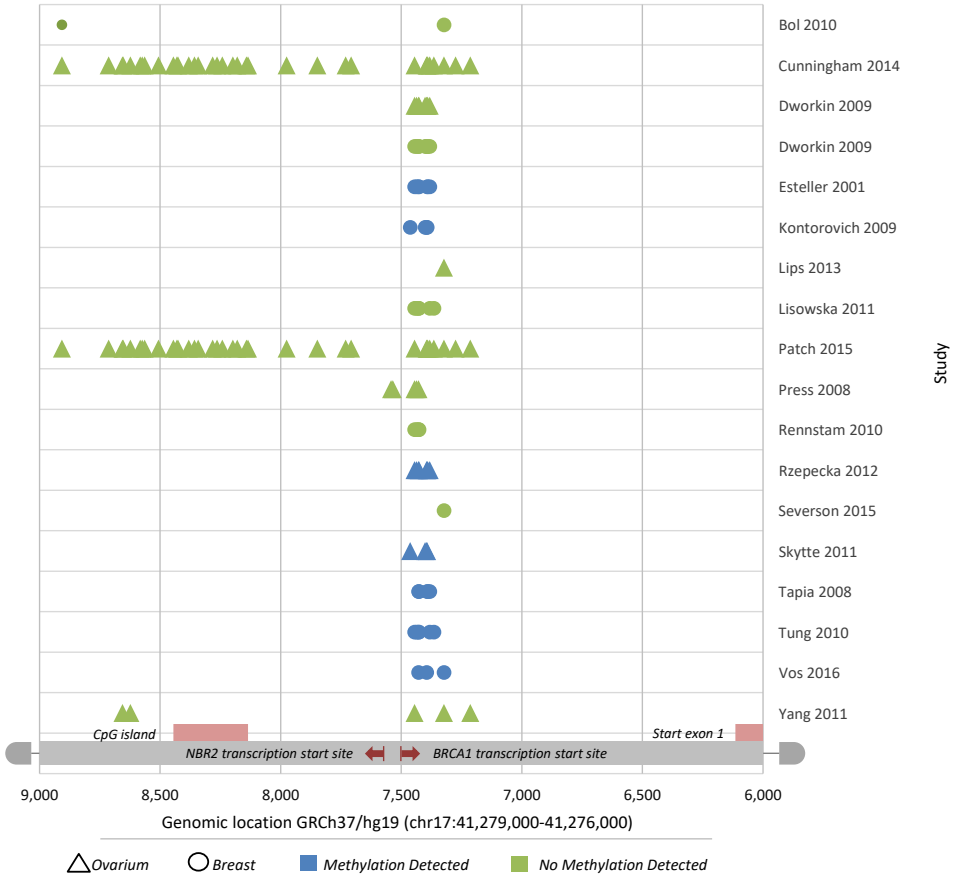

Figure 1A. Overview of the targeted CpG sites by *BRCA1* methylation analysis of the selected studies

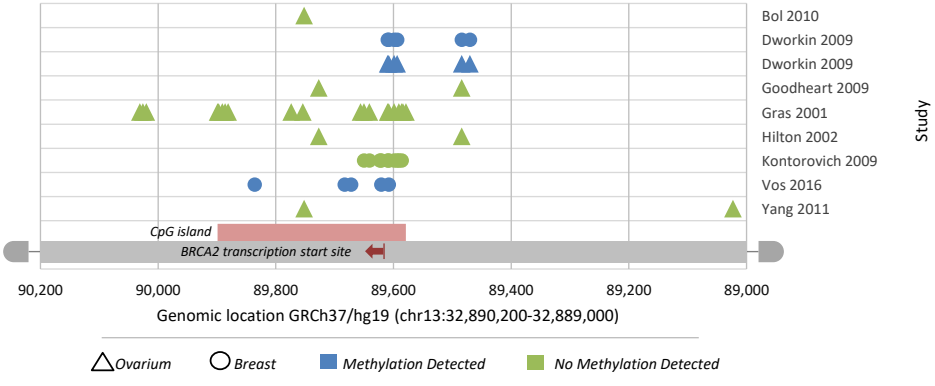

Figure 1B. Overview of the targeted CpG sites by *BRCA2* methylation analysis of the selected studies

For mutation analysis, several techniques on several types of material such as blood, tumor tissue, and normal tissue were used (Table 1). Many studies used (a combination of) rough screening techniques such as Protein Truncation Test (PTT) and Single-Strand Conformation Polymorphism (SSCP) analysis, usually followed by second-line sequencing [17,22,26,28,30,32,34,36,37]. For 6 studies, the method of mutation analysis was unclear. In 10 studies, mutation analysis was also applied in (clinically) sporadic tumors.

Frequency of *BRCA1* promoter hypermethylation in breast carcinomas from *BRCA1/2* germline mutation carriers

BRCA1 methylation was reported in 0.0-63.9% of breast carcinomas from *BRCA1/2* germline mutation carriers, based upon 11 studies (Table 2A) [17–19,21,22,24,30,31,33,35]. Most of the studies reported low frequencies of *BRCA1* promoter methylation in *BRCA1/2*-associated breast carcinomas (<5%), while some studies reported higher frequencies [19,21,30]. The largest study found *BRCA1* promoter methylation in 2-46/72 cases (2.8-63.9%), depending on the targeted CpG site [21]. Taking the results of all studies together, *BRCA1* promoter hypermethylation was found in at least 10/276 (3.6%) breast carcinomas of *BRCA1/2* germline mutation carriers.

Seven studies also investigated *BRCA1* promoter methylation in sporadic breast carcinomas and found *BRCA1* methylation in 5.8-35.7% of these cases [17,19,21–24,31]. The presence of *BRCA1* promoter methylation seems to be higher in triple-negative breast carcinomas (17.7-34.9%) [22–24]. If the presence of *BRCA1* promoter methylation would be used to rule out *BRCA1/2* germline mutations when methylation is detected (true positives: *BRCA1/2*-associated breast carcinomas without methylation; true negatives: sporadic carcinomas without methylation), the pooled results would yield a sensitivity of at most 96.4% (266/276) and a specificity of at most 23.1% (119/516) [17,19,21–24,31]. Interestingly, the study by Vos and Moelans *et al.* (2017) showed 1 CpG site (located at chr17:41277395) that was more frequently methylated in *BRCA1/2*-associated compared to sporadic breast carcinomas [21]. Methylation at this CpG site might be used to rule in *BRCA1/2*-associated breast carcinomas when methylation is detected (Table 3A). Two studies also investigated Loss of Heterozygosity (LOH). The tumors with *BRCA1* promoter methylation were all LOH negative in these studies [17,35].

Frequency of *BRCA1* promoter hypermethylation in ovarian carcinomas from *BRCA1/2* germline mutation carriers

BRCA1 methylation was observed in 0.0-5.3% of ovarian carcinomas from *BRCA1/2* germline mutation carriers, based upon 7 studies (Table 2B) [20,25,27,29,34,36,37]. Five studies reported a frequency of 0.0% of which the largest study contained 37 *BRCA1/2*-associated ovarian carcinomas. Rzepecka and Szafron *et al.* (2012) reported a frequency of 2.6% (1/38 tumors) and Skytte and Waldstrom *et al.* (2011) reported a frequency of 6.7% (1/15 tumors) [34,36]. Taking the results together, *BRCA1* promoter hypermethylation was found in 2/174 (1.1%) ovarian carcinomas of *BRCA1/2* germline mutation carriers.

From 5 studies, the frequency of *BRCA1* promoter methylation in sporadic ovarian carcinomas could be obtained, varying from 12.3 to 22.5% [20,25,27,34,37]. When the presence of *BRCA1* promoter methylation would be used to rule out *BRCA1/2* germline mutations when methylation is detected, the pooled sensitivity would then be 98.9% (172/174), and the specificity would be 14.7% (96/653) [20,25,27,34,37].

Frequency of *BRCA2* promoter hypermethylation in breast carcinomas from *BRCA1/2* germline mutation carriers

BRCA2 promoter methylation has been investigated in 3 studies and was observed in 0.0-66.7% of *BRCA1/2*-associated breast carcinomas (Table 2C) [18,19,21]. The largest study by Vos and Moelans *et al.* (2017) found *BRCA2* promoter methylation in 6-48/72 cases (8.3-66.7%), depending on the targeted CpG site [21]. Taking the results together, *BRCA2* promoter hypermethylation was found in at least 7/131 (5.3%) breast carcinomas of *BRCA1/2* germline mutation carriers. Two studies investigated *BRCA2* promoter methylation in sporadic breast cancers as well and found frequencies of 0.0-12.5% [19,21]. When the presence of *BRCA2* promoter methylation would be used to rule out *BRCA1/2* germline mutations when methylation is detected, the pooled results would yield a sensitivity of at most 58.3% (70/120), and a specificity of at most 7.6% (10/132) [19,21]. Interestingly, the study by Vos and Moelans *et al.* (2017) found more frequent methylation of CpG sites located at chr13:32889621, chr13:32889836, chr13:32889672, chr13:32889683, and chr13:2889608 in *BRCA1/2*-associated compared to sporadic breast carcinomas [21]. Methylation at these CpG sites might be used to rule in *BRCA1/2*-associated breast carcinomas when methylation is detected (Table 2C). One study investigated LOH, and the only case with *BRCA2* promoter methylation demonstrated LOH- [18].

Frequency of *BRCA2* promoter hypermethylation in ovarian carcinomas from *BRCA1/2* germline mutation carriers

BRCA2 promoter methylation in *BRCA1/2*-associated ovarian carcinomas was found in 0.0% (0/51) of cases, when taking the results of 6 studies together with a largest sample size of 25 cases (Table 2D) [18,20,25,26,28,32]. Five of these studies also investigated *BRCA2* promoter methylation in sporadic ovarian carcinomas, which was detected in 0.0-50.0% of cases [20,25,26,28,32]. However, the study with the highest percentage contained only 2 sporadic tumors of which 1 showed *BRCA2* promoter methylation. When the presence of *BRCA2* promoter methylation would be used to rule out *BRCA1/2* germline mutations when methylation is detected, the pooled results would yield a sensitivity of 100% (51/51), and a specificity of 0.6% (2/346) [20,25,26,28,32].

Quality assessment

Applicability

The results of the applicability assessment according to the QUADAS-2 guideline are shown in Table 3 and Supplementary Table 3. There were very limited concerns regarding the applicability of the studies. Only 2/21 studies (9.5%) were judged to have a moderate applicability concern for the patient selection domain, as they investigated either clear cell ovarian carcinomas [26], which are typically not associated with *BRCA* germline mutations, or ovarian carcinomas resistant for platinum-based chemotherapy [27], which is atypical for *BRCA*-associated ovarian carcinomas. Therefore, these studies might have investigated a set of atypical (*BRCA*) cases.

Risk of bias

The risk of bias assessment results according to the QUADAS-2 guideline are shown in Table 3 and Supplementary Table 3. The risk of bias was unclear for many studies. This was most extreme for the patient selection (18/21 studies, 85.7%) and index test (13/21 studies, 61.9%) domains, mainly due to a lack of information with respect to consecutive or random inclusion, inappropriate exclusions, blinding, thresholds, and controls that were used. Three studies (14.3%) were judged to have a low risk of bias for patient selection as they consecutively included patients and did not have inappropriate exclusions [26,32,37]. 19/21 studies (90.5%) were judged as having either a high or unclear risk of bias regarding the index test (i.e. methylation analysis) due to no use of or unclear information on blinding, thresholds and controls. Regarding the reference standard (i.e. mutation analysis), 20/21 studies (95.2%) were judged as having a high or unclear risk as they did not perform state-of-the-art mutation analysis in the form of first-line sequencing on blood for all exons of the *BRCA* genes. Many studies used

Table 3. Critical Appraisal

Study		**Tumor type**	**Methylation**	**Applicability concerns**			**Risk of bias**			
Author	Year	Breast/ovarian	analysis	Patient selection	Index test	Reference standard	Patient selection	Index test	Reference standard	Flow and timing
Esteller	2001	Breast	*BRCA1*	Low	Low	Low	Unclear	Unclear	High	High
Gras	2001	Ovarian	*BRCA2*	Low	Low	Low	Low	Unclear	High	Unclear
Hilton	2002	Ovarian	*BRCA2*	Low	Low	Low	Unclear	Unclear	High	Moderate
Press	2008	Ovarian	*BRCA1*	Low	Low	Low	Low	Unclear	Unclear	Moderate
Tapia	2008	Breast	*BRCA1*	Low	Low	Low	Unclear	Unclear	High	High
Dworkin	2009	Breast+ovarian	*BRCA1+2*	Low	Low	Low	Unclear	Unclear	Unclear	High
Goodheart	2009	Ovarian	*BRCA2*	Moderate	Low	Low	Low	High	High	Moderate
Kontorovich	2009	Breast	*BRCA1+2*	Low	Low	Low	Unclear	High	Unclear	Moderate
Bol	2010	Ovarian	*BRCA1+2*	Low	Low	Low	Unclear	Unclear	Unclear	Moderate
Rennstam	2010	Breast	*BRCA1*	Low	Low	Low	Unclear	High	Unclear	Moderate
Tung	2010	Breast	*BRCA1*	Low	Low	Low	Unclear	Unclear	Unclear	High
Lisowska	2011	Breast	*BRCA1*	Low	Low	Low	Unclear	Unclear	High	Low
Skytte	2011	Ovarian	*BRCA1*	Low	Low	Low	Unclear	High	Unclear	Moderate
Yang	2011	Ovarian	*BRCA1+2*	Low	Low	Low	Unclear	Moderate	Low	Low
Rzepecka	2012	Ovarian	*BRCA1*	Low	Low	Low	Unclear	Unclear	High	Moderate
Lips	2013	Breast	*BRCA1*	Low	Low	Low	Unclear	Unclear	High	Moderate
Cunningham	2014	Ovarian	*BRCA1+2*	Low	Low	Low	Unclear	Unclear	Unclear	Moderate
Toffoli	2014	Breast	*BRCA1*	Low	Low	Low	Unclear	High	Unclear	High
Severson	2015	Breast	*BRCA1*	Low	Low	Low	Unclear	Unclear	High	Moderate
Patch	2015	Ovarian	*BRCA1+2*	Moderate	Low	Low	Unclear	High	Unclear	Low
Vos	201?	Breast	*BRCA1+2*	Low	Low	Low	Unclear	Low	Unclear	High

(a combination of) rough mutation analysis techniques, including PTT and SSCP. As a consequence, *BRCA* germline mutations could have been missed and false negatives may have occurred. This is especially a concern for studies that used a study group of triple-negative breast carcinomas, as these tumors have a higher baseline risk of containing *BRCA* germline mutations [22]. Moreover, 2/21 (9.5%) studies only checked for mutations common in the Polish population [31,34]. The risk of false positive *BRCA* germline mutations is considered to be small, as many studies performed second-line sequencing for validation.

For flow and timing, most studies (11/21, 52.4%) were judged as having a moderate risk of bias, because either the methylation and mutation analysis were not performed in every case or the cases did not receive the same reference standard. The most common causes of missing values were that sporadic tumors did not receive mutation analysis, there was a pre-selection for methylation analysis (based upon LOH or mRNA data) or there was insufficient DNA for methylation analysis. This may have caused false negatives for both *BRCA* germline mutations and *BRCA* promoter methylation. The application of different mutation analysis techniques can be explained by differences in delay between mutation and methylation analysis. Seven studies (33.3%) were judged as having a high or unclear risk of bias and 3 studies (14.3%) as having a low risk of bias. In addition, there are two studies with a risk of reporting bias, as they should have obtained data on *BRCA2* promoter methylation but did not report the results [27,29]. The risk of publication bias was considered to be relatively low. One could imagine a risk of publication bias at the expense of studies not showing *BRCA* promoter methylation in *BRCA* germline mutation carriers, as this would be less newsworthy. However, most of the latest papers show no *BRCA* promoter methylation in *BRCA* mutation carriers [23,24,27,29].

DISCUSSION

This study is the first systematic review that has investigated to what extent *BRCA1* and *BRCA2* promoter hypermethylation has been reported in breast and ovarian carcinomas of *BRCA1/2* germline mutation carriers and what the diagnostic accuracy would be if *BRCA* promoter methylation analysis were to be used to rule out *BRCA* germline mutations when methylation would be detected. Our comprehensive literature search identified 21 eligible studies. Overall, *BRCA1* hypermethylation was found in at least 10/265 (3.6%) breast and 2/174 (1.1%) ovarian carcinomas of *BRCA* germline mutation carriers, whereas *BRCA2* hypermethylation was found in at least 7/131 (5.3%) breast

and 0/51 (0.0%) ovarian carcinomas of *BRCA* germline mutation carriers. Our review shows that *BRCA* promoter hypermethylation is rare in breast and ovarian carcinomas of *BRCA* germline mutation carriers, but is relatively more frequent in breast carcinomas. Moreover, *BRCA2* promoter methylation, although being less studied, occurs more frequently than *BRCA1* promoter methylation in breast cancer. When the presence of *BRCA1* promoter methylation would be used to rule out *BRCA1/2* germline mutations, the pooled results would yield a sensitivity of at most 96.4% and 98.9%, and specificity of at most 23.1% and 14.7% for breast and ovarian carcinomas, respectively. For *BRCA2* promoter methylation, the sensitivity would be at most 58.3% and 100%, specificity of at most 7.6% and 0.6%, for breast and ovarian carcinomas, respectively.

The results of this review should, however, be interpreted with caution as it is challenging to pool the results of the individual studies which vary significantly in setting (e.g. population, tumor type), sample size, methylation and mutation analysis methods (e.g. differences in quality input material, cutoffs, targeted CpG sites or exons), and quality and risk of bias. The selected studies show in general a limited extent of methylation analysis with differences in targeted CpG sites and incomplete mutation analysis. Interestingly, the studies with the most extensive methylation and mutation analyses and a relatively large sample size show no *BRCA* promoter methylation in the *BRCA*-associated ovarian carcinomas [25,27,29]. Moreover, these studies have relatively limited risks of bias in comparison to studies that show a number of *BRCA*-associated cancers with *BRCA* promoter hypermethylation using limited methylation and mutation analyses.

As a result of the limited extent of methylation and mutation analysis together with missing variables such as no mutation analysis in clinically sporadic tumors or debatable prerequisites for methylation analysis such as decreased *BRCA* mRNA levels, the frequency of *BRCA* promoter hypermethylation in *BRCA* germline mutation carriers with breast or ovarian carcinomas may have been underestimated. Currently, no specific CpG sites could be identified that are more or less likely to be methylated in *BRCA*-associated cancers due to limited data. Vos and Moelans *et al*. (2017), and Daniels and Burghel *et al*. (2016, not included for this review) have shown that methylation frequencies vary considerably between CpG sites in *BRCA1* as well as *BRCA2* promoter regions and that some CpG sites seem to be more frequently methylated in *BRCA1/2*-associated compared to sporadic breast carcinomas [21,40]. Despite the apparent influence on methylation frequency, the other selected studies did not specify at which CpG locus methylation was detected, if possible.

Correlation between *BRCA* methylation and gene expression

Several studies have demonstrated that *BRCA1* promoter methylation could lead to downregulation of gene expression with reduction of *BRCA1* mRNA and protein expression in breast and ovarian carcinomas as a mechanism of somatic *BRCA1* inactivation, functionally equivalent to harbouring a *BRCA1* germline mutation [12,25,47–54,30,36,41–46]. However, according to The Cancer Genome Atlas (TCGA) Research Network data, methylation of specific CpG sites within the *BRCA1* and *BRCA2* promoters showed in general weak correlations with mRNA levels (highest Spearman's correlation coefficient of -0.333 and -0.124 for *BRCA1* and *BRCA2*, respectively) [55]. In breast cancer, somatic inactivation of *BRCA1* by hypermethylation has been shown to be correlated with a *BRCA1*-like (basal-like) phenotype and homologous recombination deficiency [10,23,59,24,30,41,49,52,56–58]. For *BRCA2* methylation, the relationship with gene expression and homologous recombination deficiency is less well studied [60,61].

***BRCA* methylation and germline mutations paradigm**

A proposed explanation for the lower levels of methylation generally observed in *BRCA*-associated cancer is that *BRCA*-associated tumors show more overall genomic instability due to functional loss of *BRCA1* or *BRCA2*. *BRCA* haploinsufficiency has been shown to result in decreased DNA repair capacity and increased genomic instability [62–65]. Moreover, increased proliferation and copy number alterations have been observed in normal breast tissues from healthy *BRCA* germline mutation carriers, whereas methylation was rarely detected [33]. Therefore, *BRCA*-associated carcinogenesis is thought to be mainly driven by mutations whereas methylation of tumor suppressor genes is thought to play only a secondary role or to occur as a side-effect [66]. However, other studies have shown increased methylation levels of *BRCA* and other genes in normal breast and Fallopian tube tissues from *BRCA* germline mutation carriers [21,67]. Moreover, *BRCA* promoter hypermethylation has been observed in breast carcinomas with retention of heterozygosity, although data are limited [17,18,35]. Thus it may be possible that *BRCA* promoter methylation drives carcinogenesis in a subset of *BRCA* carriers.

Limitations

At the study and outcome level, the main limitation of this review concerns the heterogeneity, poor or incomplete reporting, as well as risk of bias of the included studies with respect to setting and patient population, methylation and mutation analyses and outcome definitions. At a review level, the risk of incomplete retrieval of studies is considered to be small as a broad search strategy was applied and the data

extraction process and analysis were systematic and comprehensive. However, studies that performed methylation analysis solely on samples other than tissue, such as blood, ascites or nipple fluid were not included.

Implications for practice and further research

This study's results have important implications for clinical practice. At first, methylation analysis has been proposed as a cost-effective pre-screening tool to rule out *BRCA* germline mutations, similar to current practice for Lynch syndrome (i.e. *MLH1* methylation analysis) [22,68]. *BRCA* methylation is said to occur at higher frequencies in sporadic compared to *BRCA* germline mutation-related carcinomas, at least for ovarian carcinomas, although large differences in incidence of *BRCA* methylation in sporadic carcinomas have been reported. *BRCA1* methylation has been observed in 5-36% of breast carcinomas and 11-89% of ovarian carcinomas in general [18,19,69–74,20,23,27,30,34,44,46,49]. Interestingly, it has recently been shown that mechanisms of homologous recombination deficiency may differ between ethnicity. In Caucasians, *BRCA1* mutations have been found to predominate, whereas in black people *BRCA1* methylation was more frequent [57]. *BRCA2* methylation has been reported in 0-44% of breast and 0-98.7% of ovarian carcinomas in general [18–21,25,26,28,32,61,75]. However, it should be noted that the data on *BRCA2* methylation are more controversial as they are not reported or confirmed by TCGA studies, which are often regarded as the 'gold standard' in this field. Moreover, *BRCA* methylation occurs at higher frequencies than *BRCA* somatic and germline mutations in breast and ovarian carcinomas and its analysis is quicker and less expensive compared to mutation analysis [12,76,77]. However, this review shows that, although the evidence and the quality thereof are limited, *BRCA* germline mutations cannot be completely ruled out when *BRCA* promoter methylation is detected in ovarian, but especially breast carcinomas. It is nevertheless important to note that 100% sensitivity cannot be expected as some patients with *BRCA* germline mutations may develop breast or ovarian cancer through sporadic carcinogenetic mechanisms. These tumors may thereby more likely show *BRCA* methylation.

Secondly, *BRCA*ness analysis, including *BRCA* germline and somatic mutation analysis, is increasingly being performed for treatment purposes, such as chemotherapy and poly ADP ribose polymerase (PARP) inhibitor treatment, in ovarian and breast carcinomas [12,78–84]. Response to PARP inhibition has been found to be dependent upon homologous recombination deficiency in general and not restricted to *BRCA1/2* germline mutations [81,85–87]. The indications of PARP inhibitor treatment in clinical ovarian and breast cancer care are expanding and may increasingly require *BRCA* promoter methylation analysis as it has been shown that ovarian carcinomas with *BRCA1*

methylation are sensitive to PARP inhibitors [12,78,89–93,79–85,88]. As to response to platinum-based chemotherapy in ovarian carcinomas and *BRCA1* methylation, there have been contradictory reports [71,94–98]. For breast carcinomas as well as *BRCA2* methylation, the sensitivity to PARP inhibitor treatment and platinum-based chemotherapy is under-examined and needs further clarification [74,99].
To summarize, *BRCA* methylation analysis might be used as a cost-effective pre-screening tool to rule out *BRCA* germline mutations and for treatment indication. However, as methylation frequencies vary considerably between CpG sites, further research is needed to clarify which CpG sites are optimal for distinguishing sporadic from *BRCA*-associated carcinomas, and which CpG sites are best predictive of treatment response.

CONCLUSION

This systematic review shows that *BRCA* promoter methylation is rare in breast and ovarian carcinomas of *BRCA* germline mutation carriers, although being relatively more frequent in breast carcinomas, and varying between CpG sites. However, the selected studies show differences in methodology and performed in general a limited methylation and incomplete mutation analysis. Therefore, the frequency of *BRCA* promoter methylation in *BRCA* germline mutation carriers with breast or ovarian carcinomas may have been underestimated and thereby less mutually exclusive than often thought. This could have major implications for clinical practice, where *BRCA*ness analysis for treatment management is increasingly being used.

SUPPLEMENTARY FILES

Supplementary files are available online.

REFERENCES

1 Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J cancer 2015; **136**: E359-86

2 Venkitaraman AR. and the Functions of BRCA1 and BRCA2. Cell 2002; **108**: 171-182

3 Antoniou A, Pharoah PDP, Narod S, et al. Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies. Am J Hum Genet 2003; **72**: 1117-1130

4 Chen S, Parmigiani G. Meta-analysis of BRCA1 and BRCA2 penetrance. J Clin Oncol 2007; **25**: 1329-1333

5 Begg CB, Haile RW, Borg A, et al. Variation of breast cancer risk among BRCA1/2 carriers. JAMA 2008; **299**: 194-201

6 Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. Nat Rev Cancer 2012; **12**: 68-78

7 Paul NW, Banerjee M, Michl S. Captious certainties: makings, meanings and misreadings of consumer-oriented genetic testing. J Community Genet 2014; **5**: 81-87

8 Vos S, Van der Groep P, Van der Wall E, et al. Hereditary Breast Cancer Syndromes: Molecular Pathogenesis and Diagnostics. eLS. John Wiley & Sons, Ltd: Chichester; 2015.

9 Chiang JW, Karlan BY, Cass L, et al. BRCA1 promoter methylation predicts adverse ovarian cancer prognosis. Gynecol Oncol 2006; **101**: 403-410

10 Bal A, Verma S, Joshi K, et al. BRCA1-methylated sporadic breast cancers are BRCA-like in showing a basal phenotype and absence of ER expression. Virchows Arch 2012; **461**: 305-312

11 Jacot W, Thezenas S, Senal R, et al. BRCA1 promoter hypermethylation, 53BP1 protein expression and PARP-1 activity as biomarkers of DNA repair deficit in breast cancer. BMC Cancer 2013; **13**: 523

12 Moschetta M, George A, Kaye SB, et al. BRCA somatic mutations and epigenetic BRCA modifications in serous ovarian cancer. Ann Oncol 2016; **27**: 1449-1455

13 Sun C, Li N, Ding D, et al. The role of BRCA Status on the prognosis of patients with epithelial ovarian cancer: A systematic review of the literature with a meta-analysis. PLoS One 2014; **9**: e95285

14 Liberati A, Altman DG, Tetzlaff J, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. PLoS Med 2009; **6**: e1000100

15 Moher D, Liberati A, Tetzlaff J, et al. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. PLoS Med 2009; **6**: e1000097

16 Whiting PF, Rutjes AWS, Westwood ME, et al. QUADAS-2: a revised tool for the quality assessment of diagnostic accuracy studies. Ann Intern Med 2011; **155**: 529-536

17 Esteller M, Fraga MF, Guo M, et al. DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet 2001; **10**: 3001-3007

18 Dworkin AM, Spearman AD, Tseng SY, et al. Methylation not a frequent 'second hit' in tumors with germline BRCA mutations. Fam Cancer 2009; **8**: 339-346

19 Kontorovich T, Cohen Y, Nir U, et al. Promoter methylation patterns of ATM, ATR, BRCA1, BRCA2 and P53 as putative cancer risk modifiers in Jewish BRCA1/BRCA2 mutation carriers. Breast Cancer Res Treat 2009; **116**: 195-200

20 Bol GM, Suijkerbuijk KPM, Bart J, et al. Methylation profiles of hereditary and sporadic ovarian cancer. Histopathology 2010; **57**: 363-370

21 Vos S, Moelans CB, van Diest PJ. BRCA promoter methylation in sporadic versus BRCA germline mutation-related breast cancers. Breast Cancer Res 2017; **19**: 64

22 Lips EH, Mulder L, Oonk A, et al. Triple-negative breast cancer: BRCAness and concordance of clinical features with BRCA1-mutation carriers. Br J Cancer 2013; **108**: 2172-2177

23 Toffoli S, Bar I, Abdel-Sater F, et al. Identification by array comparative genomic hybridization of a new amplicon on chromosome 17q highly recurrent in BRCA1 mutated triple negative breast cancer. Breast Cancer Res 2014; **16**: 466

24 Severson TM, Peeters J, Majewski I, et al. BRCA1-like signature in triple negative breast cancer: Molecular and clinical characterization reveals subgroups with therapeutic potential. Mol Oncol 2015; **9**: 1528-1538

25 Yang D, Khan S, Sun Y, et al. Association of BRCA1 and BRCA2 Mutations With Survival, Chemotherapy Sensitivity, and Gene Mutator Phenotype in Patients With Ovarian Cancer. JAMA 2011; **306**: 1557

26 Goodheart MJ, Rose SL, Hattermann-Zogg M, et al. BRCA2 alteration is important in clear cell carcinoma of the ovary. Clin Genet 2009; **76**: 161-167

27 Patch A-M, Christie EL, Etemadmoghadam D, et al. Whole-genome characterization of chemoresistant ovarian cancer. Nature 2015; **521**: 489-494

28 Hilton JL, Geisler JP, Rathe JA, et al. Inactivation of BRCA1 and BRCA2 in ovarian cancer. J Natl Cancer Inst 2002; **94**: 1396-1406

29 Cunningham JM, Cicek MS, Larson NB, et al. Clinical characteristics of ovarian cancer classified by BRCA1, BRCA2, and RAD51C status. Sci Rep 2014; **4**: 4026

30 Tapia T, Smalley S V, Kohen P, et al. Promoter hypermethylation of BRCA1 correlates with absence of expression in hereditary breast cancer tumors. Epigenetics 2008; **3**: 157-163

31 Lisowska KM, Dudaladava V, Jarzab M, et al. BRCA1-related gene signature in breast cancer: the role of ER status and molecular type. Front Biosci (Elite Ed) 2011; **3**: 125-136

32 Gras E, Cortes J, Diez O, et al. Loss of heterozygosity on chromosome 13q12-q14, BRCA-2 mutations and lack of BRCA-2 promoter hypermethylation in sporadic epithelial ovarian tumors. Cancer 2001; **92**: 787-795

33 Rennstam K, Ringberg A, Cunliffe HE, et al. Genomic alterations in histopathologically normal breast tissue from BRCA1 mutation carriers may be caused by BRCA1 haploinsufficiency. Genes Chromosom Cancer 2010; **49**: 78-90

34 Rzepecka IK, Szafron L, Stys A, et al. High frequency of allelic loss at the BRCA1 locus in ovarian cancers: clinicopathologic and molecular associations. Cancer Genet 2012; **205**: 94-100

35 Tung N, Miron A, Schnitt SJ, et al. Prevalence and predictors of loss of wild type BRCA1 in estrogen receptor positive and negative BRCA1-associated breast cancers. Breast Cancer Res 2010; **12**: R95

36 Skytte A-B, Waldstrom M, Rasmussen AA, et al. Identification of BRCA1-deficient ovarian cancers. Acta Obstet Gynecol Scand 2011; **90**: 593-599

37 Press JZ, De Luca A, Boyd N, et al. Ovarian carcinomas with genetic and epigenetic BRCA1 loss have distinct molecular abnormalities. BMC Cancer 2008; **8**: 17

38 Rhee J-K, Kim K, Chae H, et al. Integrated analysis of genome-wide DNA methylation and gene expression profiles in molecular subtypes of breast cancer. Nucleic Acids Res 2013; **41**: 8464-8474

39 Brenet F, Moh M, Funk P, et al. DNA Methylation of the First Exon Is Tightly Linked to Transcriptional Silencing. Papavasiliou N, ed. PLoS One 2011; **6**: e14524

40 Daniels SL, Burghel GJ, Chambers P, et al. Levels of DNA Methylation Vary at CpG Sites across the BRCA1 Promoter, and Differ According to Triple Negative and BRCA-Like Status, in Both Blood and Tumour DNA. PLoS One 2016; **11**: e0160174

41 Joosse SA, Brandwijk KIM, Mulder L, et al. Genomic signature of BRCA1 deficiency in sporadic basal-like breast tumors. Genes Chromosomes Cancer 2011; **50**: 71-81

42 Hughes-Davies L, Huntsman D, Ruas M, et al. EMSY links the BRCA2 pathway to sporadic breast and ovarian cancer. Cell 2003; **115**: 523-535

43 Dobrovic A, Simpfendorfer D. Methylation of the BRCA1 gene in sporadic breast cancer. Cancer Res 1997; **57**: 3347-3350

44 Hedenfalk I, Duggan D, Chen Y, et al. Gene-expression profiles in hereditary breast cancer. N Engl J Med 2001; **344**: 539-548

45 Wilcox CB, Baysal BE, Gallion HH, et al. High-resolution methylation analysis of the BRCA1 promoter in ovarian tumors. Cancer Genet Cytogenet 2005; **159**: 114-122

46 Baldwin RL, Nemeth E, Tran H, et al. BRCA1 promoter region hypermethylation in ovarian carcinoma: a population-based study. Cancer Res 2000; **60**: 5329-5333

47 Swisher EM, Gonzalez RM, Taniguchi T, et al. Methylation and protein expression of DNA repair genes: association with chemotherapy exposure and survival in sporadic ovarian and peritoneal carcinomas. Mol Cancer 2009; **8**: 48

48 Rice JC, Ozcelik H, Maxeiner P, et al. Methylation of the BRCA1 promoter is associated with decreased BRCA1 mRNA levels in clinical breast cancer specimens. Carcinogenesis 2000; **21**: 1761-1765

49 Esteller M, Silva JM, Dominguez G, et al. Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. J Natl Cancer Inst 2000; **92**: 564-569

50 Matros E, Wang ZC, Lodeiro G, et al. BRCA1 promoter methylation in sporadic breast tumors: relationship to gene expression profiles. Breast Cancer Res Treat 2005; **91**: 179-186

51 Yamashita N, Tokunaga E, Kitao H, et al. Epigenetic inactivation of BRCA1 through promoter hypermethylation and its clinical importance in triple-negative breast cancer. Clin Breast Cancer 2015; **15**: 498-504

52 Kawazu M, Kojima S, Ueno T, et al. Integrative analysis of genomic alterations in triple-negative breast cancer in association with homologous recombination deficiency. PLOS Genet 2017; **13**: e1006853

53 Ledermann J, Harter P, Gourley C, et al. Olaparib Maintenance Therapy in Platinum-Sensitive Relapsed Ovarian Cancer. N Engl J Med 2012; **366**: 1382-1392

54 Rigakos G, Razis E. BRCAness: finding the Achilles heel in ovarian cancer. Oncologist 2012; **17**: 956-962

55 Díez-Villanueva A, Mallona I, Peinado MA. Wanderer, an interactive viewer to explore DNA methylation and gene expression data in human cancer. Epigenetics Chromatin 2015; **8**: 22

56 Foulkes WD, Smith IE, Reis-Filho JS. Triple-Negative Breast Cancer. N Engl J Med 2010; **363**: 1938-1948

57 Polak P, Kim J, Braunstein LZ, et al. A mutational signature reveals alterations underlying deficient homologous recombination repair in breast cancer. Nat Genet 2017; **49**: 1476-1486

58 Alvarez S, Diaz-Uriarte R, Osorio A, et al. A predictor based on the somatic genomic changes of the BRCA1/BRCA2 breast cancer tumors identifies the non-BRCA1/BRCA2 tumors with BRCA1 promoter hypermethylation. Clin Cancer Res 2005; **11**: 1146-1153

59 Stefansson OA, Jonasson JG, Olafsdottir K, et al. CpG island hypermethylation of BRCA1 and loss of pRb as co-occurring events in basal/triple-negative breast cancer. Epigenetics 2011; **6**: 638-649

60 Li Z, Heng J, Yan J, et al. Integrated analysis of gene expression and methylation profiles of 48 candidate genes in breast cancer patients. Breast Cancer Res Treat 2016; **160**: 371-383

61 Pradjatmo H. Methylation Status and Expression of BRCA2 in Epithelial Ovarian Cancers in Indonesia. Asian Pac J Cancer Prev 2015; **16**: 8599-8604

62 Konishi H, Mohseni M, Tamaki A, et al. Mutation of a single allele of the cancer susceptibility gene BRCA1 leads to genomic instability in human breast epithelial cells. Proc Natl Acad Sci U S A 2011; **108**: 17773-17778

63 Feilotter HE, Michel C, Uy P, et al. BRCA1 haploinsufficiency leads to altered expression of genes involved in cellular proliferation and development. PLoS One 2014; **9**: e100068

64 Sedic M, Skibinski A, Brown N, et al. Haploinsufficiency for BRCA1 leads to cell-type-specific genomic instability and premature senescence. Nat Commun 2015; **6**: 7505

65 Vaclová T, Gómez-López G, Setién F, et al. DNA repair capacity is impaired in healthy BRCA1 heterozygous mutation carriers. Breast Cancer Res Treat 2015; **152**: 271-282

66 Suijkerbuijk KPM, Fackler MJ, Sukumar S, et al. Methylation is less abundant in BRCA1-associated compared with sporadic breast cancer. Ann Oncol 2008; **19**: 1870-1874

67 Bijron JG, van der Groep P, van Dorst EB, et al. Promoter hypermethylation patterns in fallopian tube epithelium of BRCA1 and BRCA2 germ line mutation carriers. Endocr Relat Cancer 2012; **19**: 69-81

68 Gausachs M, Mur P, Corral J, et al. MLH1 promoter hypermethylation in the analytical algorithm of Lynch syndrome: a cost-effectiveness study. Eur J Hum Genet 2012; **20**: 762-768

69 Bell D, Berchuck A, Birrer M, et al. Integrated genomic analyses of ovarian carcinoma. Nature 2011; **474**: 609-615

70 Comprehensive molecular portraits of human breast tumours. Nature 2012; **490**: 61-70

71 Ruscito I, Dimitrova D, Vasconcelos I, et al. BRCA1 gene promoter methylation status in high-grade serous ovarian cancer patients--a study of the tumour Bank ovarian cancer (TOC) and ovarian cancer diagnosis consortium (OVCAD). Eur J Cancer 2014; **50**: 2090-2098

72 Pradjatmo H, Dasuki D, Anwar M, et al. Methylation status and immunohistochemistry of BRCA1 in epithelial ovarian cancer. Asian Pac J Cancer Prev 2014; **15**: 9479-9485

73 Ignatov T, Poehlmann A, Ignatov A, et al. BRCA1 promoter methylation is a marker of better response to anthracycline-based therapy in sporadic TNBC. Breast Cancer Res Treat 2013; **141**: 205-212

74 Brianese RC, Nakamura KDM, Almeida FGDSR, et al.BRCA1 deficiency is a recurrent event in early-onset triple-negative breast cancer: a comprehensive analysis of germline mutations and somatic promoter methylation. Breast Cancer Res Treat November 2017

75 Ramalho EA, Silva-Filho JL, Cartaxo MF, et al. Assessment of changes in the BRCA2 and P53 genes in breast invasive ductal carcinoma in northeast Brazil. Biol Res 2014; **47**: 3

76 Turner N, Tutt A, Ashworth A. Hallmarks of 'BRCAness' in sporadic cancers. Nat Rev Cancer 2004; **4**: 814-819

77 Burgess M, Puhalla S. BRCA 1/2-Mutation Related and Sporadic Breast and Ovarian Cancers: More Alike than Different. Front Oncol 2014; **4**: 19

78 Banerjee S, Kaye SB, Ashworth A. Making the best of PARP inhibitors in ovarian cancer. Nat Rev Clin Oncol 2010; **7**: 508-519

79 Fong PC, Yap TA, Boss DS, et al. Poly(ADP)-Ribose Polymerase Inhibition: Frequent Durable Responses in BRCA Carrier Ovarian Cancer Correlating With Platinum-Free Interval. J Clin Oncol 2010; **28**: 2512-2519

80 Konstantinopoulos PA, Spentzos D, Karlan BY, et al. Gene expression profile of BRCAness that correlates with responsiveness to chemotherapy and with outcome in patients with epithelial ovarian cancer. J Clin Oncol 2010; **28**: 3555-3561

81 Veeck J, Ropero S, Setien F, et al. BRCA1 CpG Island Hypermethylation Predicts Sensitivity to Poly(Adenosine Diphosphate)- Ribose Polymerase Inhibitors. J Clin Oncol 2010; **28**: e563-e564

82 Alsop K, Fereday S, Meldrum C, et al. BRCA mutation frequency and patterns of treatment response in BRCA mutation-positive women with ovarian cancer: a report from the Australian Ovarian Cancer Study Group. J Clin Oncol 2012; **30**: 2654-2663

83 Crafton SM, Bixel K, Hays JL. PARP inhibition and gynecologic malignancies: A review of current literature and on-going trials. Gynecol Oncol 2016; **142**: 588-596

84 Ledermann JA. PARP inhibitors in ovarian cancer. Ann Oncol 2016; **27 Suppl 1**: i40-i44

85 Ibragimova I, Cairns P. Assays for hypermethylation of the BRCA1 gene promoter in tumor cells to predict sensitivity to PARP-inhibitor therapy. Methods Mol Biol 2011; **780**: 277-291

86 Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature 2005; **434**: 917-921

87 Fong PC, Boss DS, Yap TA, et al. Inhibition of Poly(ADP-Ribose) Polymerase in Tumors from BRCA Mutation Carriers. N Engl J Med 2009; **361**: 123-134

88 Drew Y, Mulligan EA, Vong W-T, et al. Therapeutic potential of poly(ADP-ribose) polymerase inhibitor AG014699 in human cancers with mutated or methylated BRCA1 or BRCA2. J Natl Cancer Inst 2011; **103**: 334-346

89 Cai F, Ge I, Wang M, et al. Pyrosequencing analysis of BRCA1 methylation level in breast cancer cells. Tumor Biol 2014; **35**: 3839-3844

90 Lee J-M, Ledermann JA, Kohn EC. PARP Inhibitors for BRCA1/2 mutation-associated and BRCA-like malignancies. Ann Oncol 2014; **25**: 32-40

91 Dizdar O, Arslan C, Altundag K. Advances in PARP inhibitors for the treatment of breast cancer. Expert Opin Pharmacother 2015; **16**: 2751-2758

92 Livraghi L, Garber JE. PARP inhibitors in the management of breast cancer: current data and future prospects. BMC Med 2015; **13**: 188

93 Ohmoto A, Yachida S. Current status of poly(ADP-ribose) polymerase inhibitors and future directions. Onco Targets Ther 2017; **10**: 5195-5208

94 Prieske K, Prieske S, Joosse SA, et al. Loss of BRCA1 promoter hypermethylation in recurrent high-grade ovarian cancer. Oncotarget 2017; **8**: 83063-83074

95 Sun T, Ruscito I, Dimitrova D, et al. Genetic Versus Epigenetic BRCA1 Silencing Pathways: Clinical Effects in Primary Ovarian Cancer Patients: A Study of the Tumor Bank Ovarian Cancer Consortium. Int J Gynecol Cancer 2017; **27**: 1658-1665

96 Ignatov T, Eggemann H, Costa SD, et al. BRCA1 promoter methylation is a marker of better response to platinum–taxane-based therapy in sporadic epithelial ovarian cancer. J Cancer Res Clin Oncol 2014; **140**: 1457-1463

97 Bernards SS, Pennington KP, Harrell MI, et al. Clinical characteristics and outcomes of patients with BRCA1 or RAD51C methylated versus mutated ovarian carcinoma. Gynecol Oncol 2018; **148**: 281-285

98 Chaudhry P, Srinivasan R, Patel FD. Utility of Gene Promoter Methylation in Prediction of Response to Platinum-Based Chemotherapy in Epithelial Ovarian Cancer (EOC). Cancer Invest 2009; **27**: 877-884

99 Silver DP, Richardson AL, Eklund AC, et al. Efficacy of neoadjuvant cisplatin in triple-negative breast cancer. J Clin Oncol 2010; **28**: 1145-1153

Chapter 6

miRNA expression patterns in normal breast tissue and invasive breast cancers of *BRCA1* and *BRCA2* germline mutation carriers

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ABSTRACT

miRNA deregulation has been found to promote carcinogenesis. Little is known about miRNA deregulation in hereditary breast tumors as no miRNA expression profiling studies have been performed in normal breast tissue of *BRCA1* and *BRCA2* mutation carriers. miRNA profiles of 17 *BRCA1*- and 9 *BRCA2*-associated breast carcinomas were analyzed using microarrays. Normal breast tissues from *BRCA1* and *BRCA2* mutation carriers (both n = 5) and non-mutation carriers (n = 10) were also included. Candidate miRNAs were validated by qRT-PCR. Breast carcinomas showed extensive miRNA alteration compared to normal breast tissues in *BRCA1* and *BRCA2* mutation carriers. Moreover, normal breast tissue from *BRCA1* mutation carriers already showed miRNA alterations compared to non-mutation carriers. Chromosomal distribution analysis showed several hotspots containing down- or up-regulated miRNAs. Pathway analysis yielded many similarities between the *BRCA1* and *BRCA2* axes with miRNAs involved in cell cycle regulation, proliferation and apoptosis. Lesser known pathways were also affected, including cellular movement and protein trafficking. This study provides a comprehensive insight into the potential role of miRNA deregulation in *BRCA1/2*-associated breast carcinogenesis. The observed extensive miRNA deregulation is likely the result of genome-wide effects of chromosomal instability caused by impaired *BRCA1* or *BRCA2* function. This study's results also suggest the existence of common pathways driving breast carcinogenesis in both *BRCA1* and *BRCA2* germline mutation carriers.

INTRODUCTION

Breast cancer, the most common cancer as well as the leading cause of death in women worldwide [1,2], can occur both in sporadic and hereditary settings. Germline mutations in the *BRCA1* or *BRCA2* genes are the most common causes of breast cancer predisposition resulting in a 70% and 60% lifetime risk of developing breast cancer, respectively [3,4]. These *BRCA1/2*-associated breast carcinomas account for 5-7% of all breast cancer cases [5]. Major progress in the classification of human breast tumors has been made by gene expression (mRNA) profiling using microarray analysis defined by luminal A, luminal B, basal-like and HER-2 subtypes [6–8].

More recently, miRNA expression profiling has been given much attention for further classification. miRNAs are small non-coding RNAs of approximately 22 nucleotides in length, that play an important role in post-transcriptional gene regulation, causing translational repression or mRNA degradation of their target mRNAs [9]. Quantitative and qualitative changes in miRNAs have been found to promote carcinogenesis, as they could lead to increased expression of oncogenes and decreased expression of tumor suppressor genes [10–18]. A global decrease in mature miRNA levels is found in tumors compared to normal tissues [19,20], possibly attributed to the fact that many miRNAs have tumor suppressor functions. miRNA expression profiles of sporadic breast tumors show several differentially expressed miRNAs compared to normal breast tissue [21–24]. Differences in miRNA expression can partially explain breast cancer heterogeneity, such as estrogen receptor (ER) [22,24] and progesterone receptor (PR) [22,24] expression and presence of HER2 amplification [24]. Moreover, miRNA expression patterns can predict therapy response and resistance [25]. These findings suggest that deregulated miRNA expression is important in sporadic breast carcinogenesis. An advantage of miRNAs is that they are more resistant to degradation caused by the formalin fixation process of tissues [26]. Therefore, the opportunity to use miRNAs as biomarkers in formalin-fixed paraffin-embedded (FFPE) samples, the usual processing method applied in pathology, can be more rapidly translated to clinical practice [27].

Little is known about miRNA expression in *BRCA1/2*-associated breast carcinomas. The identification of target genes and pathways affected by deregulated miRNAs in *BRCA1/2*-associated breast carcinomas is important for attaining a better understanding of *BRCA1/2*-specific breast tumorigenesis and could yield new diagnostic biomarkers and therapeutic targets. The aims of this study were: (1) to analyze differences in miRNA expression profiles between *BRCA1/2*-associated breast carcinomas, normal breast tissue from *BRCA1* and *BRCA2* germline mutation carriers, and normal breast

tissue from non-mutation carriers; (2) to obtain more insight into *BRCA1/2*-associated carcinogenesis by identification of target genes and pathways regulated by miRNAs.

RESULTS

Clinicopathologic characteristics of tissue samples

The patient samples consisted of 5 classes: *BRCA1*-associated breast carcinomas (*BRCA1*-C) (n = 17); *BRCA2*-associated breast carcinomas (*BRCA2*-C) (n = 9); normal breast tissue from *BRCA1* germline mutation carriers (*BRCA1*-N) (n = 5) and *BRCA2* germline mutation carriers (*BRCA2*-N) (n = 5), both derived from prophylactic mastectomies; and normal breast tissue from non-mutation carriers derived from mammoplasty specimens (healthy-N) (n = 10). For external validation of specific miRNAs by qRT-PCR, a second, independent, cohort of patient samples was used. This cohort consisted of a total of 60 FFPE samples, obtained from the same archives. The patient samples also consisted of 5 classes: *BRCA1*-C (n = 15); *BRCA2*-C (n = 15); *BRCA1*-N (n = 10); *BRCA2*-N (n = 10); and Healthy-N (n = 10). Patient characteristics are shown in Tables 1a and 1b for the first and second cohorts, respectively. Details on the characterization of patient samples are given in the Supplementary methods.

Average age at diagnosis in the *BRCA1*-associated breast carcinomas was 46.1 years (range 21 – 81) in the first cohort, and 41.3 years (range 28 – 56) in the second cohort. The tumors were mainly of ductal type (58.8% and 93.3% in the first and second cohorts, respectively), and ER, PR, and HER2 negative (58.8%, 76.5% and 82.4%, respectively in the first cohort; and 80%, 86.7% and 80%, respectively in the second cohort). The patients with *BRCA2*-associated breast cancer had an average age at diagnosis of 46.7 years (range 21 – 66) in the first cohort, and 45.8 years (range 27 – 67) in the second cohort. These tumors were also mainly of ductal type (88.9% and 100% in the first and second cohorts, respectively), ER positive (55.6% and 80% in the first and second cohorts, respectively), PR negative (66.7%) in the first cohort and more PR positive (53.3%) in the second cohort, and HER2 negative (100%) in the first cohort and more HER2 positive (46.7%) in the second cohort. The average age of patients of whom normal breast tissues were used, was 33.6, 36.2, and 30.4 years for *BRCA1*-N, *BRCA2*-N, and Healthy-N in the first cohort, respectively; and 35.5, 41.0, and 40.4 years in the second cohort, respectively.

Table 1a. Patient characteristics, initial cohort

Characteristics	n (%)				
	BRCA1 cancers	*BRCA2* cancers	*BRCA1* normal tissue	*BRCA2* normal tissue	Healthy normal tissue
Age in years (mean ± SD)	46.1 ± 16.4 (range 21 - 81)	46.7 ± 14.8 (range 21 - 66)	33.6 ± 8.4 (range 26 - 49)	36.2 ± 7.7 (range 27 - 49)	30.4 ± 6.4 (range 21 - 38)
Grade					
I	1 (5.9)	0 (0.0)			
II	5 (29.4)	4 (44.4)			
III	11 (64.7)	5 (55.6)			
Tumor type					
Ductal	10 (58.8)	8 (88.9)			
Lobular	3 (17.6)	1 (11.1)			
Medullary	3 (17.6)	0 (0.0)			
Metaplastic	1 (5.9)	0 (0.0)			
Estrogen receptor					
Negative	10 (58.8)	4 (44.4)			
Positive	7 (41.2)	5 (55.6)			
Progesterone receptor					
Negative	13 (76.5)	6 (66.7)			
Positive	4 (23.5)	3 (33.3)			
HER2 status					
Negative	14 (82.4)	9 (100)			
Positive	3 (17.6)	0 (0.0)			

miRNA microarray analysis

Of the 2006 human miRNAs on the microarray, 862 miRNAs remained after filtering on low expression variation. Differential expression analysis between the five classes (*BRCA1*-C, *BRCA2*-C, *BRCA1*-N, *BRCA2*-N, and Healthy-N) was performed. The numbers of differentially expressed miRNAs with fold change (FC) ≥ |1.5| and statistical significance (False Discovery Rate (FDR) < 0.05) for each comparison are shown in Table 2. The *BRCA2*-C vs. Healthy-N comparison yielded many more differentially expressed miRNAs (n = 600) compared to the *BRCA1*-C vs. Healthy-N comparison (n = 269). Moreover, the *BRCA2*-C vs. Healthy-N comparison yielded mainly up-regulated miRNAs in contrast to down-regulated miRNAs in the *BRCA1*-C vs. Healthy-N comparison. 317 miRNAs were differentially expressed in the *BRCA1*-C vs. *BRCA2*-C comparison. There were 150 differentially expressed miRNAs between *BRCA1*-N and Healthy-N. However, the *BRCA2*-N vs. Healthy-N comparison yielded no significant

Table 1b. Patient characteristics, second cohort

Characteristics	**n (%)**				
	BRCA1 cancers	*BRCA2* cancers	*BRCA1* normal tissue	*BRCA2* normal tissue	Healthy normal tissue
Age in years (mean ± SD)	41.3 ± 9.1 (range 28 - 56)	45.8 ± 10.1 (range 27 - 67)	35.5 ± 6.5 (range 26 - 40)	41.0 ± 18.4 (range 28 - 54)	40.4 ± 12.5 (range 21 - 60)
Grade					
I	0 (0.0)	0 (0.0)			
II	1 (6.7)	3 20.0)			
III	13 (86.7)	12 (80.0)			
Missing	1 (6.7)	0 (0.0)			
Tumor type					
Ductal	14 (93.3)	15 (100)			
Missing	1 (6.7)	0 (0.0)			
Estrogen receptor					
Negative	12 (80.0)	3 (20.0)			
Positive	3 (20.0)	12 (80.0)			
Progesterone receptor					
Negative	13 (86.7)	7 (46.7)			
Positive	2 (13.3)	8 (53.3)			
HER2 status					
Negative	12 (80.0)	7 (46.7)			
Positive	2 (13.3)	7 (46.7)			
Missing	1 (6.7)	1 (6.7)			

results. Potential correlations between biological differences (irrespective of *BRCA* status) and miRNA expression, which could have influenced above mentioned results, were also investigated. miRNA expression did not show any significant correlations with age (≤50 versus >50 years), grade (2 versus 3), the presence of lymph node metastases, or expression of PR, ER, CK5/6, CK14, EGFR, or Ki-67 (<20 versus ≥20%), irrespective of *BRCA* status (data not shown). However, HER2 expression and tumor type were associated with differences in miRNA expression, irrespective of *BRCA* status. 85 miRNAs were significantly differentially expressed between HER2 positive and negative tumors (see Supplementary table SI). One miRNA (miR-4633-5p) was significantly differentially expressed between ductal and lobular breast carcinomas (FC = 1.71, FDR = 0.0318, data not shown).

Table 2. Number of differentially expressed miRNAs between classes

Class	Class compared to	Total differentially expressed miRNAs	Up-regulated miRNAs	Down-regulated miRNAs
BRCA1 cancers	Healthy normal tissue	269	90	179
BRCA1 cancers	*BRCA1* normal tissue	145	41	104
BRCA1 normal tissue	Healthy normal tissue	150	55	95
BRCA2 cancers	Healthy normal tissue	600	360	240
BRCA2 cancers	*BRCA2* normal tissue	96	8	88
BRCA2 normal tissue	Healthy normal tissue	0	0	0
BRCA1 cancers	*BRCA2* cancers	317	121	196
BRCA1 normal tissue	*BRCA2* normal tissue	0	0	0

miRNAs with with fold change ≥ |1.5| and statistical significance (False Discovery Rate < 0.05) are included in this analysis using unpaired *t*-test for unequal variance.

Table 3. Top 10 differentially expressed miRNAs shared between the *BRCA1* and *BRCA2* axis

miRNA	Chromosomal location	Cancer vs. normal in *BRCA1* carriers			Cancer vs. normal in *BRCA2* carriers		
		Unadjusted p-value	FDR	FC	Unadjusted p-value	FDR	FC
Up-regulated							
hsa-miR-3676-5p	Chr17: 8090493-8090577 [+]	0.0034	0.0212	1.67	0.0000	0.0212	2.47
hsa-miR-937-5p	Chr8q24.3: 144895127-144895212 [-]	0.0052	0.0286	1.55	0.0046	0.0286	1.64
Down-regulated							
hsa-miR-99a-3p	Chr21q21.1: 17911409-17911489 [+]	0.0000	0.0000	25.29	0.0000	0.0048	25.79
hsa-miR-204-5p	Chr9q21.12: 73424891-73425000 [-]	0.0000	0.0000	67.98	0.0000	0.0048	227.67
hsa-miR-4328	ChrX: 78156691-78156746 [-]	0.0000	0.0000	17.93	0.0001	0.0048	33.96
hsa-miR-136-3p	Chr14q32.2: 101351039-101351120 [+]	0.0000	0.0000	58.28	0.0001	0.0048	67.82
hsa-miR-99a-5p	Chr21q21.1: 17911409-17911489 [+]	0.0000	0.0000	6.76	0.0000	0.0048	13.60
hsa-miR-125b-5p	Chr11q24.1: 121970465-121970552 [-]	0.0000	0.0000	5.35	0.0001	0.0058	11.97
hsa-miR-100-5p	Chr11q24.1: 122022937-122023016 [-]	0.0000	0.0001	4.27	0.0001	0.0082	10.01
hsa-miR-4770	ChrX: 6301947-6302004 [-]	0.0000	0.0001	14.09	0.0000	0.0031	35.44
hsa-miR-195-5p	Chr17p13.1: 6920934-6921020 [-]	0.0000	0.0001	4.65	0.0001	0.0053	10.82
hsa-miR-199b-5p	Chr9q34.11: 131007000-131007109 [-]	0.0000	0.0001	5.77	0.0006	0.0195	10.57

Ranking of miRNAs based upon FDR (smallest to largest) and FC (largest to smallest). Chromosomal locations are based upon NCBI Gene results and GRCh37.p5 coordinates. miRNAs marked in yellow are selected for qRT-PCR validation. This selection is based upon above described ranking and availability of qRT-PCR primers. FDR = false discovery rate. FC = fold change.

We focused on *BRCA1*-C vs. *BRCA1*-N and *BRCA2*-C vs. *BRCA2*-N comparisons, as differentially expressed miRNAs from these comparisons could play a role in *BRCA1/2*-associated breast carcinogenesis. The *BRCA1*-C vs. *BRCA1*-N comparison yielded 145 miRNAs compared to 96 in the *BRCA2*-C vs. *BRCA2*-N comparison. These comparisons had 53 miRNAs in common. The shared miRNAs, and the *BRCA1* and *BRCA2* axis-specific miRNAs were ranked based upon FDR and subsequently on fold change, and the top 10 miRNAs are shown in Tables 3-5. Of these, the following miRNAs were selected for qRT-PCR validation, based on assay availability: miR-99a, miR-210, miR-21, miR-183, miR-378, miR-153, miR-4443, miR-1287, let-7b and miR-551b.

Table 4. Top 10 differentially expressed miRNAs specifically altered between normal tissues and cancers of *BRCA1* carriers

miRNA	Chromosomal location	Unadjusted p-value	FDR	FC
Up-regulated				
hsa-miR-1307-3p	Chr10: 105154010-105154158 [-]	0.0000	0.0001	1.70
hsa-miR-210	Chr11p15.5: 568089-568198 [-]	0.0000	0.0002	4.61
hsa-miR-3162-3p	Chr11: 59362550-59362631 [-]	0.0000	0.0002	2.41
hsa-miR-155-5p	Chr21q21.3: 26946292-26946356 [+]	0.0000	0.0003	3.59
hsa-miR-21-5p	Chr17q23.1: 57918627-57918698 [+]	0.0000	0.0004	3.62
hsa-miR-4306	Chr13: 100295313-100295403 [+]	0.0000	0.0004	1.83
hsa-miR-183-5p	Chr7q32.2: 129414745-129414854 [-]	0.0000	0.0007	7.19
hsa-miR-185-5p	Chr22q11.21: 20020662-20020743 [+]	0.0000	0.0009	2.47
hsa-miR-574-5p	Chr4: 38869653-38869748 [+]	0.0000	0.0009	2.71
hsa-miR-4455	Chr4: 185859537-185859594 [-]	0.0001	0.0012	3.27
Down-regulated				
hsa-miR-378a-5p	Chr5q32: 149112388-149112453 [+]	0.0000	0.0000	29.07
hsa-miR-153	Chr2q35: 220158833-220158922 [-]	0.0000	0.0000	18.90
hsa-miR-29a-5p	Chr7q32.3: 130561506-130561569 [-]	0.0000	0.0004	7.32
hsa-miR-1258	Chr2q31.3: 180725563-180725635 [-]	0.0000	0.0004	12.82
hsa-miR-335-3p	Chr7q32.2: 130135952-130136045 [+]	0.0000	0.0006	14.98
hsa-miR-6500-3p	Chr1: 51525690-51525775 [+]	0.0000	0.0006	10.69
hsa-let-7i-3p	Chr12q14.1: 62997466-62997549 [+]	0.0000	0.0007	47.60
hsa-miR-411-5p	Chr14q32.31: 101489662-101489757 [+]	0.0000	0.0007	47.60
hsa-miR-219-5p	Chr6p21.32: 33175612-33175721 [+]	0.0000	0.0009	15.36
hsa-miR-139-5p	Chr11q13.4: 72326107-72326174 [-]	0.0000	0.0009	11.10

Ranking of miRNAs based upon FDR (smallest to largest) and FC (largest to smallest). Chromosomal locations are based upon NCBI Gene results and GRCh37.p5 coordinates. miRNAs marked in grey are selected for qRT-PCR validation. This selection is based upon above described ranking and availability of qRT-PCR primers. FDR = false discovery rate. FC = fold change.

Table 5. Top 10 differentially expressed miRNAs specifically altered between normal tissues and cancers of *BRCA2* carriers

miRNA	Chromosomal location	Unadjusted p-value	FDR	FC
Up-regulated				
hsa-miR-4778-5p	Chr2: 66585381-66585460 [-]	0.0002	0.0105	1.62
hsa-miR-4443	Chr3: 48238054-48238106 [+]	0.0005	0.0169	2.05
hsa-miR-5010-5p	Chr17: 40666206-40666325 [+]	0.0022	0.0333	1.62
hsa-miR-1287	Chr10q24.2: 100154975-100155064 [-]	0.0039	0.0434	1.91
hsa-miR-663b	Chr2: 133014539-133014653 [-]	0.0045	0.0459	2.05
hsa-miR-4688	Chr11: 46397952-46398034 [+]	0.0060	0.0489	1.61
Down-regulated				
hsa-miR-664b-5p	ChrX: 153996871-153996931 [+]	0.0000	0.0048	2.72
hsa-let-7b-5p	Chr22q13.31: 46509566-46509648 [+]	0.0005	0.0169	4.37
hsa-miR-29b-1-5p	Chr7q32.3: 130562218-130562298 [-]	0.0007	0.0195	17.92
hsa-miR-551b-3p	Chr3q26.2: 168269642-168269737 [+]	0.0009	0.0242	63.99
hsa-let-7g-5p	Chr3p21.1: 52302294-52302377 [-]	0.0010	0.0255	4.21
hsa-miR-650	Chr22q11.22: 23165270-23165365 [+]	0.0011	0.0264	1.97
hsa-miR-29a-3p	Chr7q32.3: 130561506-130561569 [-]	0.0012	0.0268	3.81
hsa-miR-1234-3p	Chr8: 145625476-145625559 [-]	0.0012	0.0268	1.86
hsa-miR-224-3p	ChrXq28: 151127050-151127130 [-]	0.0016	0.0295	45.54
hsa-miR-148a-3p	Chr7p15.2: 25989539-25989606 [-]	0.0017	0.0295	4.97

Ranking of miRNAs based upon FDR (smallest to largest) and FC (largest to smallest). Chromosomal locations are based upon NCBI Gene results and GRCh37.p5 coordinates. miRNAs marked in grey are selected for qRT-PCR validation. This selection is based upon above described ranking and availability of qRT-PCR primers. FDR = false discovery rate. FC = fold change.

miRNA validation by qRT-PCR

miRNA validation by qRT-PCR was performed in the same samples as used for microarray analysis together with an independent cohort of samples, yielding a combination of internal and external validation of the initial miRNA microarray results. By doing this, more well founded results would be obtained that are also generalizable to other cases. All miRNAs selected for qRT-PCR validation, except miR-1287, were differentially expressed in the same direction in qRT-PCR as in microarray analysis between the invasive breast carcinomas and the asymptomatic normal breast tissues of *BRCA1* and *BRCA2* germline mutation carriers (see Table 6). However, qRT-PCR analysis appeared to be more sensitive compared to microarray analysis. miRNAs miR-210, miR-21, miR-183 and miR-153 were specifically differentially expressed between the *BRCA1*-C and *BRCA1*-N stages in microarray analysis and were confirmed by qRT-PCR analysis. Only miR-378 was not significantly differentially expressed between the *BRCA1*-C and *BRCA1*-N stages in qRT-PCR analysis. Noteworthy, all the

Table 6. miRNA validation by qRT-PCR: test statistics

Invasive breast carcinomas *vs.* asymptomatic normal breast tissues of *BRCA1* and *BRCA2* germline mutation carriers										
	Let-7b	miR-153	miR-183	miR-210	miR-378	miR-4443	miR-551b	miR-1287	miR-21	miR-99a
Kruskal-Wallis test	31.904	26.434	76.684	74.067	26.808	23.231	31.321	5.655	77.210	63.637
Signifance level	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.226	0.000	0.000

microarray specifically differentially miRNAs between *BRCA1*-C and *BRCA1*-N were also significantly differentially expressed between the *BRCA2*-C and *BRCA2*-N stages in qRT-PCR analysis.

Of miRNAs specifically differentially expressed between the *BRCA2*-C and *BRCA2*-N stages in microarray analysis (let-7b, miR-4443, miR-551b, miR-1287), miR-4443 and miR-1287 were not significantly differentially expressed between these two classes in qRT-PCR analysis. Let-7b was found to be specifically deregulated between invasive breast carcinomas and asymptomatic normal breast tissues in *BRCA2* germline mutation carriers. miR-551b was found to be significantly differentially expressed between the *BRCA2*-C and *BRCA2*-N stages as well as between the *BRCA1*-C vs. *BRCA1*-N stages. miR-4443 was however found to be significantly differentially expressed between the *BRCA1*-C and *BRCA1*-N stages by qRT-PCR. qRT-PCR analysis of miR-99a confirmed the microarray results showing that it was down-regulated in both *BRCA1*- and *BRCA2*-associated breast carcinomas compared to their normal breast tissue counterparts.

Chromosomal distribution

Chromosomal distribution of the differentially expressed miRNAs from the *BRCA1*-C vs. *BRCA1*-N (n = 145) and *BRCA2*-C vs. *BRCA2*-N (n = 96) comparisons were also investigated (Figure 1). Chromosomes 4, 7, 10, 12, 17, and 19 showed a higher number of deregulated miRNAs in the *BRCA1* axis, while chromosomes 6 and 13 showed a higher number of miRNAs deregulated in the *BRCA2* axis. However, the chromosomal distribution between the two axes was not significantly different (Fischer's exact test: p = 0.989). A more detailed view is given in Figure 2, showing the differentially expressed miRNAs at their exact localization on the chromosomes, their direction of change, and whether these miRNAs are shared between the *BRCA1* and *BRCA2* axis. Only miRNAs of which the exact localization within the chromosomes was known are included in this figure. The amount of miRNAs of which the localization was not known was n = 44 for the *BRCA1*-C vs. *BRCA1*-N comparison and n = 20 for the *BRCA2*-C vs. *BRCA2*-N comparison). Within chromosomes, a mixture of up- and down-regulated miRNAs was seen, although within hotspots (≥ 4 miRNAs at the same locus) the miRNAs showed

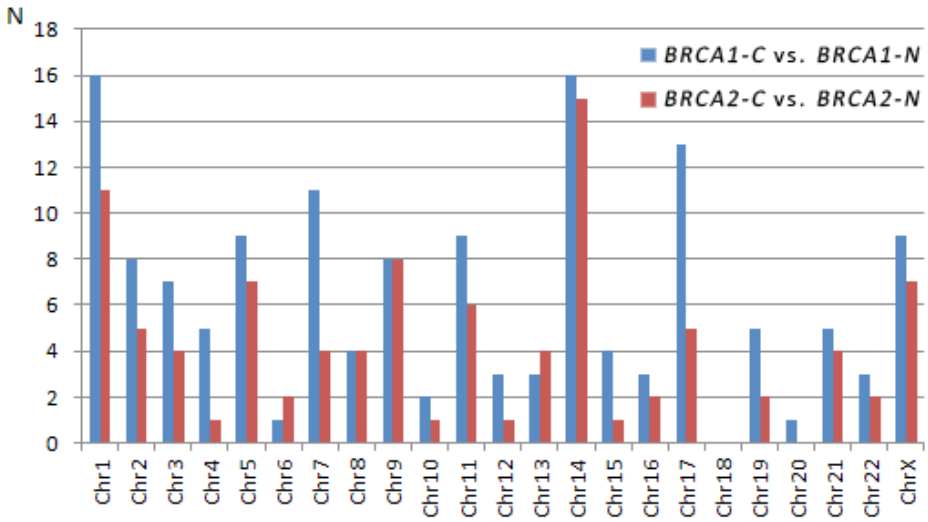
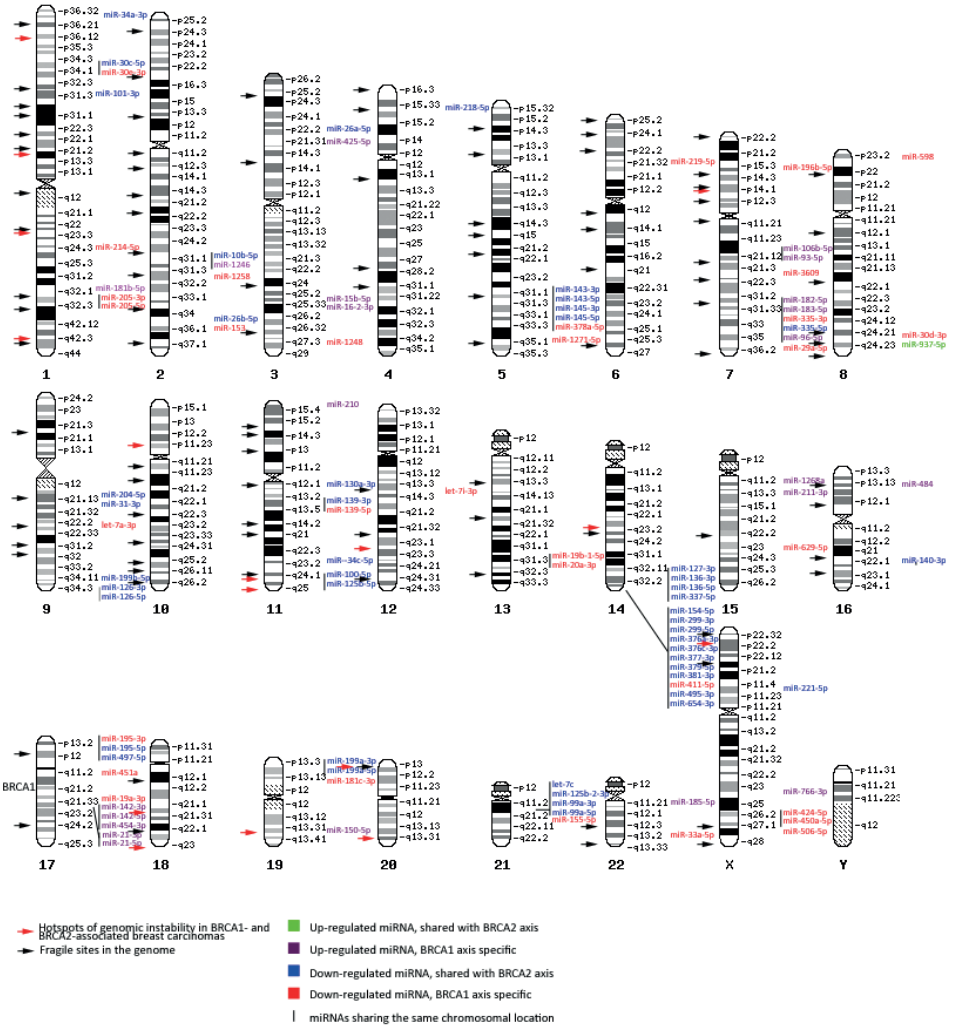


Figure 1. Number of differentially expressed miRNAs per chromosome from the comparison between cancers and normal tissue from *BRCA1* and *BRCA2* carriers, respectively
Explanation: Fisher's exact test: 10.200, p-value: 0.989. *BRCA1*-C = *BRCA1*-associated breast carcinomas; *BRCA2*-C = *BRCA2*-associated breast carcinomas; *BRCA1*-N = normal breast tissue from *BRCA1* germline mutation carriers; *BRCA2*-N = normal breast tissue from *BRCA2* germline mutation carriers.

a similar direction of deregulation. Shared hotspots between the *BRCA1* and *BRCA2* axes were 5q32, 14q32.2, 14q32.31, and 21q21.1 (all down-regulated). A *BRCA1*-specific hotspot was 7q32.2 (mainly up-regulated miRNAs). The chromosomal location of 3 deregulated miRNAs (miR-100-5p, miR-125b-5p, and miR-150-5p) matches hotspot regions of genomic instability in *BRCA1/2*-associated breast carcinomas [28]. Several more miRNAs are located at fragile sites in the genome [29], 20.8% and 22.4% for the *BRCA1* and *BRCA2* axis, respectively (Figure 2).

Unsupervised clustering

The most distinguishing parts between the classes of the full heatmap are shown in Figure 3. Seven individual clusters in two main groups were seen, largely separating breast cancers (*BRCA2*-C more than *BRCA1*-C) from the *BRCA1/2*-N and Healthy-N normal breast tissue samples. In general, most *BRCA1/2*-N tissues clustered with healthy-N tissue. However, some of them clustered with *BRCA1/2*-C. miRNAs showing a significantly different expression pattern between these two groups are surrounded with a box (Figure 3). These miRNAs, which might distinguish *BRCA1* and *BRCA2* germline carriers with a higher risk from those with a lower risk of developing breast cancer, are involved in pathways such as integrin signaling, estrogen receptor signaling,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X
Y
BRCA1
Hotspots of genomic instability in BRCA1- and BRCA2-associated breast carcinomas
Fragile sites in the genome
Up-regulated miRNA, shared with BRCA2 axis
Up-regulated miRNA, BRCA1 axis specific
Down-regulated miRNA, shared with BRCA2 axis
Down-regulated miRNA, BRCA1 axis specific
miRNAs sharing the same chromosomal location

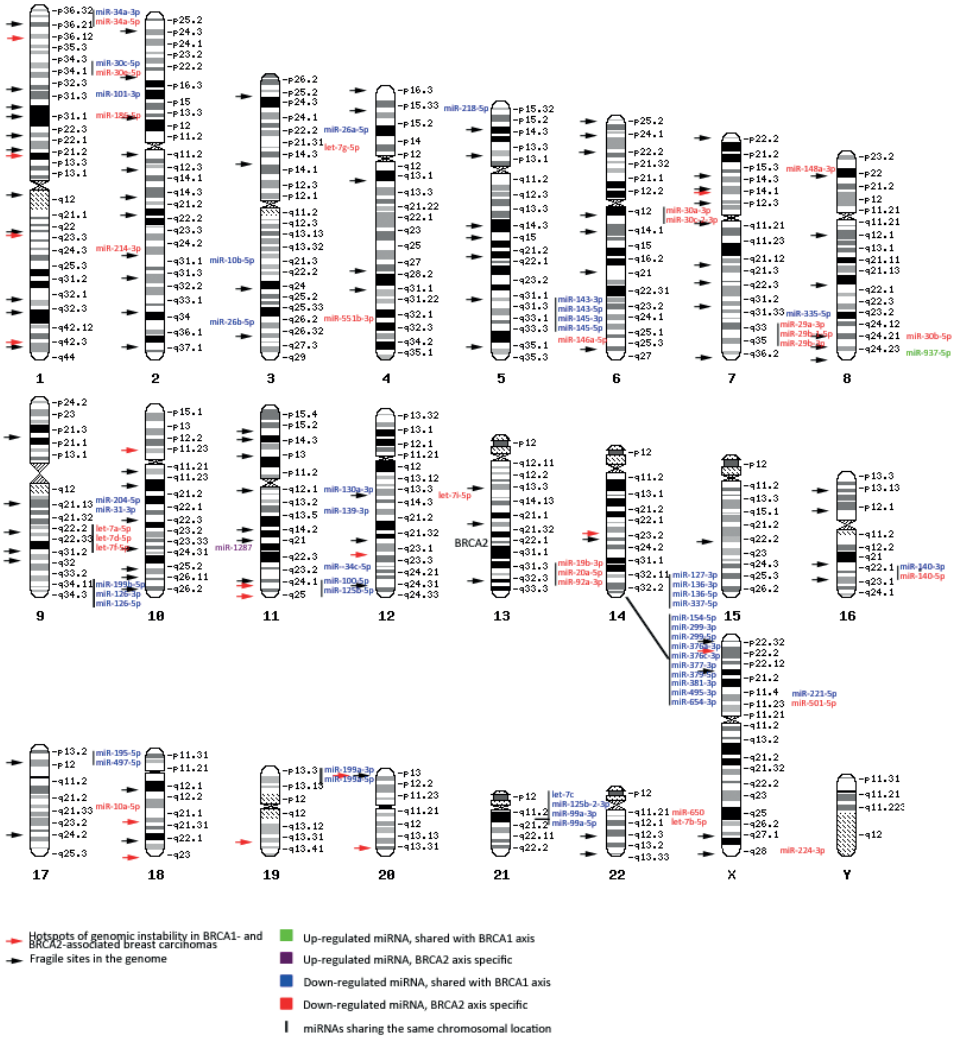
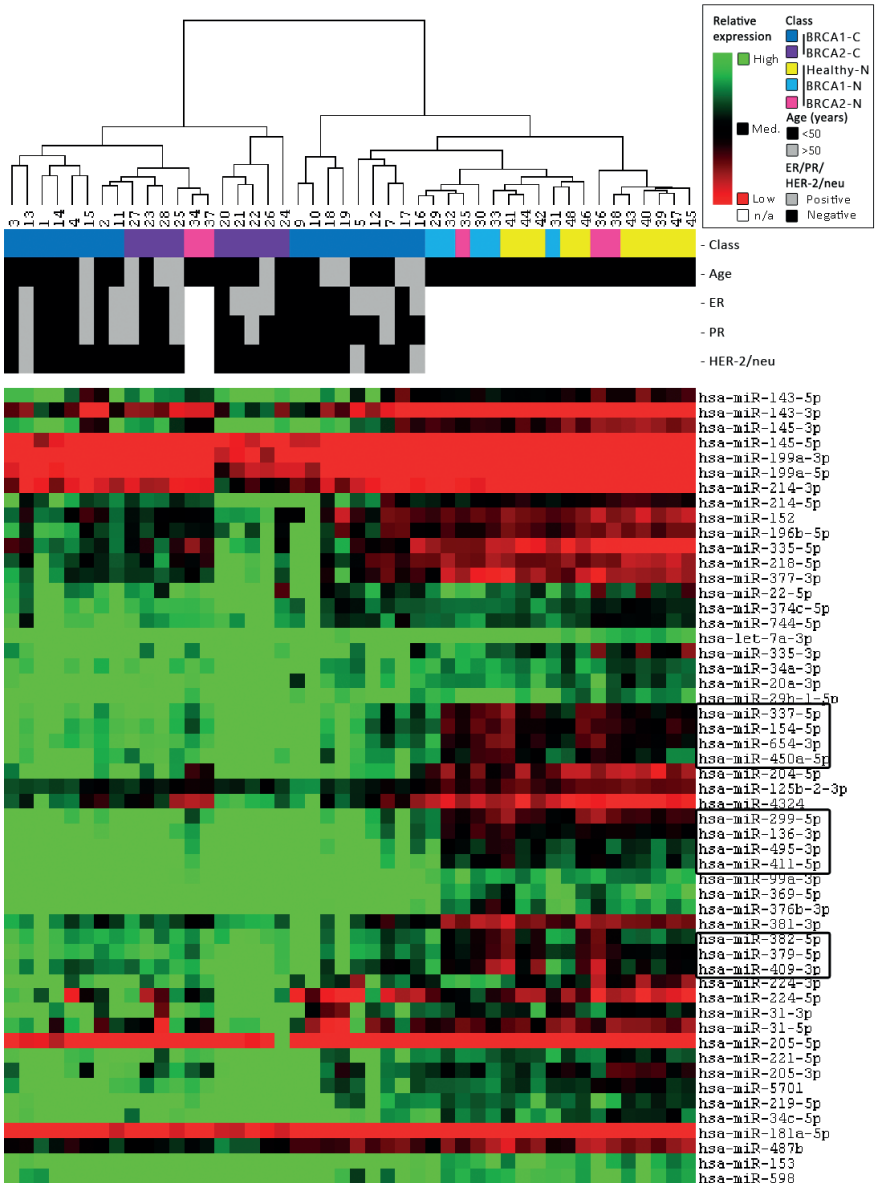


Figure 2. Chromosomal location of differentially expressed miRNAs between the normal tissue and cancers of *BRCA1* and *BRCA2* carriers, respectively

I. Chromosomal distribution of miRNAs differentially expressed between normal tissue and cancers of *BRCA1* carriers. II. Chromosomal distribution of miRNAs differentially expressed between normal tissue and cancers of *BRCA2* carriers. All differentially expressed miRNAs from both comparisons with fold change ≥ |1.5| and false discovery rate < 0.05 and known exact chromosomal location are presented. Within chromosomes a mixture of up- and down-regulated miRNAs can be seen. Several hotspots (≥ 4 miRNAs at the same locus) can be seen, in which the miRNAs show a similar direction of deregulation. The miRNA locations partly overlap with known hotspots of chromosomal instability in *BRCA1*- and *BRCA2*-associated carcinomas and fragile sites in the genome, in which miRNAs are often located.


Relative expression
High
Med.
Low
n/a
Class
BRCA1-C
BRCA2-C
Healthy-N
BRCA1-N
BRCA2-N
Age (years)
<50
>50
ER/PR/ HER-2/neu
Positive
Negative
3 13 1 14 4 15 2 11 27 23 28 25 34 37 20 21 22 26 24 9 10 18 19 5 12 7 17 16 29 32 35 30 33 41 44 42 31 48 46 36 38 43 40 39 47 45
- Class
- Age
- ER
- PR
- HER-2/neu
hsa-miR-143-5p
hsa-miR-143-3p
hsa-miR-145-3p
hsa-miR-145-5p
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hsa-miR-152
hsa-miR-196b-5p
hsa-miR-335-5p
hsa-miR-218-5p
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hsa-miR-22-5p
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hsa-miR-744-5p
hsa-let-7a-3p
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hsa-miR-34a-3p
hsa-miR-20a-3p
hsa-miR-29b-1-5p
hsa-miR-337-5p
hsa-miR-154-5p
hsa-miR-654-3p
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hsa-miR-495-3p
hsa-miR-411-5p
hsa-miR-99a-3p
hsa-miR-369-5p
hsa-miR-376b-3p
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hsa-miR-5701
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hsa-miR-487b
hsa-miR-153
hsa-miR-598

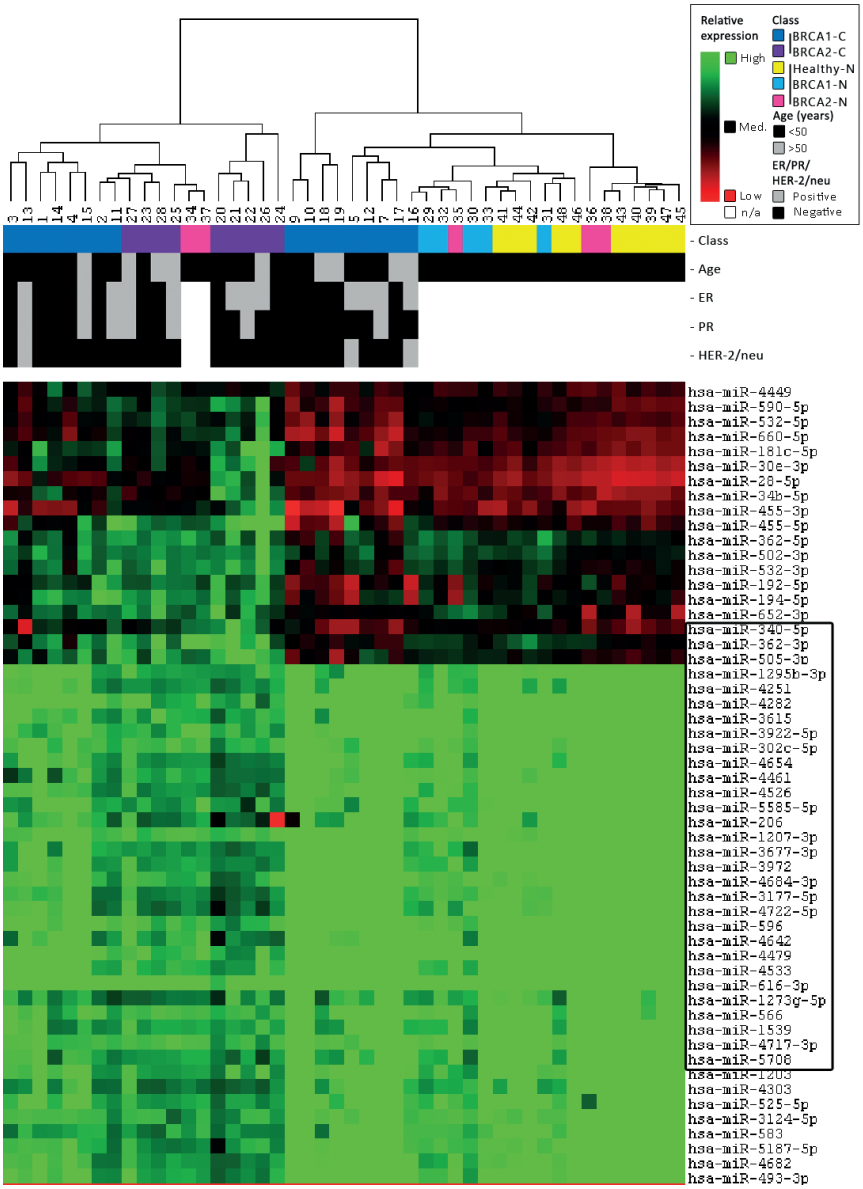

Figure 3. Unsupervised clustering results

Clustering was performed on both all samples and all miRNAs using a Self-Organizing Map algorithm. From the total heatmap, the most distinguishing parts between the classes are shown in this figure. For further information on the figure, see the legend in the top right corner. The clustering indicates that miRNAs can separate carcinomas (*BRCA2* > *BRCA1*) from the normal breast tissue of both *BRCA1/2* and non-mutation carriers. In general, normal breast tissue of *BRCA1/2*-mutation carriers clusters more with normal breast tissue from non-carriers. However, some of them cluster better with *BRCA1/2*-associated breast carcinomas.

breast cancer regulation by Stathmin1, HIF1α Signaling, Wnt/β-catenin Signaling, and p53 signaling (IPA analysis). Patient samples similar in age, ER, PR, and HER2 status did not cluster together.

Target gene and pathway analysis by DIANA-mirPath

Differentially expressed miRNAs between the *BRCA1*-C and *BRCA1*-N with fold change ≥ |2.0| and FDR < 0.05 yielded 216 significantly enriched pathways. These miRNAs target multiple genes in pathways involved in cell proliferation, apoptosis, protein ubiquitination, gene transcription, and extra-cellular matrix (ECM-) receptor signaling. There were 208 significantly enriched pathways for the *BRCA2*-C vs. *BRCA2*-N comparison, showing many pathways specific for the *BRCA1* or *BRCA2* axis (data not shown). The top 10 enriched pathways and a number of biologically interesting pathways ($p < 0.05$) are shown in Tables 7-8. A more detailed overview containing the gene names and miRNA entities for each enriched pathway is given in Supplementary table SII. Specific target analysis for the miRNAs selected for qRT-PCR was performed using IPA ($p < 0.05$) (Tables 9-10). The number of targeted genes varied from 0 (hsa-miR-99a-3p) to 19 (hsa-miR-21-5p and hsa-miR-4443).

Table 7. DIANA-mirPath pathway enrichment analysis for miRNAs differentially expressed between normal tissues and cancers of *BRCA1* carriers

Rank	KEGG pathway	*p*-value	N of genes	N of miRNAs
1	Prion diseases	0.0000	3	3
2	Pathways in cancer	0.0000	154	20
3	PI3K-Akt signaling pathway	0.0000	175	21
4	Prostate cancer	0.0000	58	23
5	Wnt signaling pathway	0.0000	100	31
6	Neurotrophin signaling pathway	0.0000	70	21
7	Axon guidance	0.0000	69	18
8	MAPK signaling pathway	0.0000	120	18
9	TGF-beta signaling pathway	0.0000	47	16
10	Ubiquitin mediated proteolysis	0.0000	76	13
15	ErbB signaling pathway	0.0000	52	15
17	Transcriptional misregulation in cancer	0.0000	78	15
26	ECM-receptor interaction	0.0000	22	8
32	p53 signaling pathway	0.0000	37	12

Table showing significantly enriched pathways ($p < 0.05$) and the number of genes targeted by how many of the top miRNAs differentially expressed between *BRCA1*-C and *BRCA1*-N conditions with fold change ≥ |2.0| and FDR < 0.05 using DIANA-mirPath pathway enrichment analysis. The ranking is based upon a combination of *p*-value, N of genes and N of miRNAs.

Table 8. DIANA-mirPath pathway enrichment analysis for miRNAs differentially expressed between normal tissues and cancers of *BRCA2* carriers

Rank	KEGG pathway	p-value	N of genes	N of miRNAs
1	Prion diseases	0.0000	1	4
2	Protein digestion and absorption	0.0000	33	13
3	Amoebiasis	0.0000	44	15
4	Axon guidance	0.0000	70	17
5	ECM-receptor interaction	0.0000	31	17
6	Small cell lung cancer	0.0000	46	17
7	Long-term potentiation	0.0000	43	18
8	ErbB signaling pathway	0.0000	53	19
9	Ubiquitin mediated proteolysis	0.0000	76	19
10	Insulin signaling pathway	0.0000	70	19
14	MAPK signaling pathway	0.0000	126	25
15	Pathways in cancer	0.0000	176	26
19	PI3K-Akt signaling pathway	0.0000	197	36
20	Wnt signaling pathway	0.0000	101	38
22	TGF-beta signaling pathway	0.0000	49	18
27	p53 signaling pathway	0.0000	40	19
31	Transcriptional misregulation in cancer	0.0000	76	20
53	HIF-1 signaling pathway	0.0010	46	10

Table showing significantly enriched pathways ($p < 0.05$) and the number of genes targeted by how many of the top miRNAs differentially expressed between *BRCA2*-C and *BRCA2*-N conditions with fold change ≥ |2.0| and FDR < 0.05 using DIANA-mirPath pathway enrichment analysis. The ranking is based upon a combination of *p*-value, N of genes and N of miRNAs.

Targeted genes of miRNAs specific for the *BRCA1*-N to *BRCA1*-C transition are involved in several cellular processes associated with *BRCA1* function, such as cell cycle regulation (BTG2, BTRC, CDK6, and E2F1), proliferation (ACTVR1B, BTRC, and DVL3), apoptosis (BCL2, FAS, TNF, and PTEN), but also less expected processes, including epithelial junctions and ECM interaction (ACTA2, ACVR1B, FGF1, PRKACB, and PTEN), cellular movement (FGF9 and PIK3R1), protein trafficking (DNM3, FGF1, FGF9, and RAB7A), and metabolism (FOXO3 and PTPN1).

Targeted genes of miRNAs specific for the *BRCA2*-N to *BRCA2*-C transition are involved in many similar processes, including epithelial junctions and ECM interaction (ACTA11, ACVR1B, CDLDN19, and FGF11), apoptosis (CASP2, and FAS), protein trafficking (FGF11, and ITGA2B), proliferation (CDH5, DACVR1B, DVL3, ERBB3, LRP6, and RPS20), cell cycle regulation (BTG2, CDK6, E2F2, PPP2R2A, and RBL2), and cellular movement (FGF11). Some of the targeted genes are shared with the *BRCA1* axis (ACVR1B, BTG2, CDK6, DVL3, and FAS) or belong to the same family (e.g. FGFs or E2F), although they are regulated by different miRNAs. However, miRNAs

Table 9. IPA target gene analysis on miRNAs specifically altered between normal tissue and cancers of *BRCA1* carriers and selected for qRT-PCR

miRNA	Targeted genes	Associated pathways
hsa-miR-153	ACVR1B	Epithelial adherens junction, PPARα/RXRα activation, TGF-β signaling, Wnt/β-catenin signaling
	BCL2	Apoptosis, glucocorticoid receptor signaling, p53 signaling, PEDF signaling, PI3K/AKT signaling, PTEN signaling, TGF-β signaling, VEGF signaling
	CBX5	ATM signaling
	DNM3	Clathrin-mediated endocytosis signaling, remodeling of epithelial adherens junctions
	DVL3	Embryonic stem cell pluripotency, regulation of the epithelial-mesenchymal transition pathway, Wnt/β-catenin signaling
	FOXO3	Glucocorticoid receptor signaling, IGF-1 signaling, insulin receptor signaling, PI3K/AKT signaling, PTEN signaling, PXR/RXR activation, VEGF signaling
	RAB7A	Clathrin-mediated endocytosis signaling, remodeling of epithelial adherens junctions
	SNAI1	Epithelial adherens junction signaling, ILK signaling, regulation of the epithelial-mesenchymal transition pathway
hsa-miR-183-5p	BTRC	Cyclins and cell cycle regulation, NF-κB signaling, Wnt/β-catenin signaling
	FGF9	Actin cytoskeleton signaling, clathrin-mediated endocytosis signaling, FGF signaling, regulation of the epithelial-mesenchymal transition pathway
	FOXO1	ErbB signaling, IGF-1 signaling, PI3K/AKT signaling, PTEN signaling, VEGF signaling
	PRKACB	Breast cancer regulation by Stathmin1, CDK5 signaling, NF-κB signaling PPARα/RXRα activation, tight junction signaling, cAMP-mediated signaling, eNOS signaling
	RAD50	ATM signaling, DNA double-strand break repair by homologous recombination, DNA double-strand break repair by non-homologous end joining, hereditary breast cancer signaling, role of *BRCA1* in DNA damage response, role of CHK proteins in cell cycle checkpoint control, telomere extension by telomerase
hsa-miR-21-5p	ACTA2	Actin cytoskeleton signaling, gap junction/integrin/tight junction signaling, VEGF signaling
	BMPR2	BMP signaling, adherens junction signaling, PPARα/RXRα activation, TGF-βsignaling
	BTG2	Cell cycle regulation
	CDK6	Cyclins and cell cycle regulation, HER-2 signaling, hereditary breast cancer signaling
	CDKN1A	See CDK6, role of *BRCA1* in DNA damage response, p53 signaling, PI3K/AKT signaling, PTEN signaling
	CFL2	Actin cytoskeleton signaling, role of tissue factor in cancer
	E2F1	Breast cancer regulation by Stathmin1, cyclins and cell cycle regulation, hereditary breast cancer signaling, p53 signaling, role of *BRCA1* in DNA damage response
	FAS(LG)	Apoptosis, p38 MAPK signaling, p53 signaling, PEDF signaling, PTEN signaling
	FGF1	Actin cytoskeleton signaling, clathrin-mediated endocytosis signaling, epithelial adherens junction signaling, FGF signaling, regulation of the epithelial-mesenchymal transition pathway
	JAG1	Notch signaling, regulation of EMT pathway
	PIK3R1	Actin cytoskeleton signaling, breast cancer regulation by Stathmin1, HER-2 signaling in breast cancer, hereditary breast cancer signaling,p53 signaling, PTEN signaling, regulation of EMT pathway, VEGF signaling
	PTEN	Adherens junction signaling, ErbB signaling, hereditary breast cancer signaling, integrin signaling, p53 signaling, PI3K/AKT signaling, PTEN signaling, role of tissue factor in cancer, tight junction signaling
	SERPINB5	p53 signaling
	TGFBR2	Adherens junction signaling, glucocorticoid receptor signaling, p38 MAPK signaling, PPARα/RXRα activation, TGF-βsignaling, tight junction signaling, Wnt/β-catenin signaling
	TNF	Apoptosis, glucocorticoid receptor signaling, p38 MAPK signaling, PPAR signaling, tight junction signaling
hsa-miR-210	ACVR1B	Adherens junction signaling, PPARα/RXRα activation, TGF-βsignaling, Wnt/βcatenin signaling
	E2F3	Breast cancer regulation by Stathmin1, cyclins and cell cycle regulation, estrogen-mediated S-phase entry, role of *BRCA1* in DNA damage response
	FGFRL1	FGF signaling, NF-βB signaling, PTEN signaling, regulation of EMT pathway
	PTPN1	Caveolar-mediated endocytosis signaling, insulin receptor signaling, JAK/Stat signaling, protein kinase A signaling
hsa-miR-378a-5p	SUFU	Molecular mechanisms of cancer

All $p < 0.05$, using IPA.

Table 10. IPA target gene analysis on miRNAs specifically altered between normal tissue and cancers of *BRCA2* carriers and selected for qRT-PCR

miRNA	Targeted genes	Associated pathways
hsa-let-7b-5p	ACTA1	ILK signaling, integrin signaling, regulation of actin-based motility by Rho, remodeling of epithelial adherens junctions, tight junction signaling, VEGF Signaling
	ACVR1B	Epithelial adherens junction signaling, PPARα/RXRα activation, TGF-β signaling, Wnt/β-catenin signaling
	BTG2	Cell cycle regulation
	CDK6	Cyclins and cell cycle regulation, HER-2 signaling, hereditary breast cancer signaling
	DVL3	Embryonic stem cell pluripotency, regulation of the epithelial-mesenchymal transition pathway, Wnt/β-catenin signaling
	E2F2	Breast cancer regulation by Stathmin1, cyclins and cell cycle regulation, estrogen-mediated S-phase entry, role of *BRCA1* in DNA damage response, role of CHK proteins in cell cycle checkpoint control
	FAS(LG)	Apoptosis, p38 MAPK signaling, p53 signaling, PEDF signaling, PTEN signaling
	FGF11	Actin cytoskeleton signaling, clathrin-mediated endocytosis signaling, FGF signaling regulation of the epithelial-mesenchymal transition pathway
	PPP2R2A	Breast cancer regulation by Stathmin1, cyclins and cell cycle regulation, ERK/MAPK signaling, ILK signaling, telomerase signaling, tight junction signaling, Wnt/β-catenin signaling
hsa-miR-1287	ERBB3	ErbB signaling
	HTR2B	G-protein coupled receptor signaling, gap junction signaling
	RBL2	G1/S checkpoint regulation, role of *BRCA1* in DNA damage response
	RPS20	EIF2 signaling, mTOR signaling
hsa-miR-4443	CDH5	Wnt/β-catenin signaling
	CLDN18	Tight junction signaling
	DLL4	Notch signaling
	F2RL2	Tight junction signaling
	FAS	Apoptosis, p38 MAPK signaling, p53 signaling, PEDF signaling
	IL1RN	NF-κB signaling, p38 MAPK signaling, PPAR signaling
	ITGA2B	Caveolar-mediated endocytosis signaling, integrin signaling
	LRP6	Wnt/β-catenin signaling
	NCOA1	Androgen signaling, estrogen receptor signaling, HIF1α signaling, PPAR signaling
	NTRK3	NF-κB signaling, PTEN signaling
	PCK1	Estrogen receptor signaling
	PLCL1	Gap junction signaling, PPARα/RXRα activation, protein kinase A signaling
	PRKAA2	AMPK signaling, eNOS signaling, glucocorticoid receptor signaling, mTOR signaling, PPARα/RXRα activation
	RARB	RAR activation, Wnt/β-catenin signaling
	SMO	Protein kinase A signaling, regulation of EMT pathway, Wnt/β-catenin signaling
	THBS1	Inhibition of TSP1, p53 signaling
	TNS1	FAK signaling
	TRPC5	Breast cancer regulation by Stathmin1
hsa-miR-551b	CASP2	Apoptosis, TNFR1 signaling
	ERBB4	ErbB signaling
	HES7	Notch signaling
	MEF2C	Calcium signaling, ERK5 signaling, p38 MAPK signaling, phospholipase C signaling, PPARα/RXRα activation
	NTRK2	NF-κB signaling, PTEN signaling

All $p < 0.05$, using IPA.

deregulated in the *BRCA2* axis show more targeted genes involved in estrogen receptor signaling compared to the *BRCA1* axis. A more detailed overview of the targeted genes and associated pathways can be found in Supplementary table SIII.

Comparison with published miRNA expression data

The systematic search yielded 1739 articles in PubMed. Six articles met the inclusion and exclusion criteria [22,27,30–33]. An overview of the selected studies is given in Table 11. Five studies used sporadic breast carcinomas, whereas the study of Tanic et al. (2012) [32] investigated miRNA expression in "familial" breast cancer, including patients with proven *BRCA1* or *BRCA2* germline mutations and non-*BRCA1/2*-associated familial carcinomas. Tumor-adjacent normal breast tissue, normal breast tissue from mammoplasty surgeries and normal breast tissue from prophylactic mastectomies were used as normal breast tissue controls in the different studies. Unfortunately, many patient characteristics could not be derived. The number of differentially expressed miRNAs was lower in all studies compared to the present study. Moreover, four out of six studies reported more up-regulated miRNAs compared to down-regulated miRNAs. The studies show little overlap in miRNA expression patterns: only 15 out of 92 (16.3%) up-regulated miRNAs and 15 out of 101 (14.9%) down-regulated miRNAs were reported by at least two studies (Table 12). Most frequently reported deregulated miRNAs were miR-21 (up-regulated in five studies), miR-155 (up-regulated in three studies), miR-145 (down-regulated in three studies) and miR-143 (down-regulated in three studies). Other up-regulated miRNAs, reported by two studies, were miR-181b, miR-98, miR-20a, miR-183, miR-141, miR-200b, miR-106b, miR-425, miR-149, miR-210, miR-1280, miR-29b and let-7f. Other down-regulated miRNAs, reported by two studies, were miR-205, miR-125b, miR-99a, miR-100, miR-195, miR-10b, miR-320c, miR-130a, miR-575, let-7d, miR-486-5p, miR-140-3p, and miR-335. Several of these miRNAs were also deregulated in our dataset (See Table 12, highlighted (colored) miRNAs), including some of the top 10 deregulated in miRNAs in *BRCA1/2*-associated breast carcinomas, as well as other miRNAs. Further explanations on the similarities and differences between miRNAs deregulated in sporadic and *BRCA1/2*-associated breast carcinomas will be made below.

Table 11. Overview breast cancer miRNA expression profiling studies in literature

Author	Year	Journal	Country	N of samples	miRNA analysis					
					Platform	Total # miRNAs on array	GEO accession number	Cut-off criteria	# differentially expressed miRNAs	# up-regulated miRNAs	# down-regulated miRNAs
Chen	2013	PLoS One	USA	8 paired sporadic BC and pre-invasive/normal adjacent tissue; 16 unpaired sporadic BC	Human miRNA Microarray V3 (Agilent)	866	NA	$p <= 0.05$ (paired analysis); FDR <= 0.01 (unpaired analysis)	25	15	10
Iorio	2005	Cancer Res	Italy, USA	76 sporadic BC; 34 NBT	miRNA microarray V1.0 (KCI)	161	NA	FDR < 0.05	29	17	12
Ouyang	2014	PLoS One	China	3 triple-negative BC; 3 adjacent NBT	miRCURY LNA Array 16.0	1513	NA	$p <= 0.05$	41	18	23
Tahiri	2014	Carcinogenesis	Norway	29 sporadic BC; 29 NBT	Human miRNA Microarray V3 (Agilent)	866	E-MTAB-779	FDR < 0.001	63	31	32
Tanic	2012	PLoS One	Spain	22 familial BC; 14 NBT*	miRCURY LNA microRNA Array Kit (Exiqon)	1276	GSE32922	FDR < 0.05	19	17	2
Yan	2008	RNA	China	8 paired sporadic BC normal adjacent tissue	CapitalBio	435	NA	FDR = 0; FC > 2.0	16	9	7

BC = breast cancer; NBT = normal breast tissue; NA = not available; FDR = false discovery rate. * 22 hereditary tumors (3 *BRCA1*, 5 *BRCA2*, 14 *BRCAX*) and 14 normal breast tissues (3 *BRCA1*, 5 *BRCA2*, and 1 *BRCAX* from prophylactic surgery; 5 from breast reductions).

Table 12. Results miRNA expression data comparison from breast cancer miRNA expression profiling studies already available in literature

miRNA	Number of studies that consistently reported miRNA	Studies by author	Total number of samples	Average FC	Range FC
Up-regulated miRNAs					
hsa-miR-21	5	Chen, Iorio, Tahiri, Tanic, Yan	236	5.27	1.55-14.69
hsa-miR-155	3	Iorio, Tahiri, Yan	176	2.22	1.28-2.29
hsa-miR-210	2	Iorio, Tahiri	168	2.23	1.43-3.03
hsa-miR-149	2	Iorio, Tahiri	168	1.94	1.08-2.8
hsa-miR-183	2	Chen, Tahiri	82	6.50*	NA
hsa-miR-200b	2	Chen, Tahiri	82	4.18*	NA
hsa-miR-141	2	Chen, Tahiri	82	3.04*	NA
hsa-miR-425	2	Chen, Tahiri	82	2.31*	NA
hsa-miR-106b	2	Chen, Tahiri	82	2.07*	NA
hsa-miR-20a	2	Chen, Tahiri	82	1.68*	NA
hsa-miR-98	2	Tahiri, Yan	66	2.01	1.88-2.13
hsa-miR-181b	2	Tahiri, Yan	66	1.43*	NA
hsa-miR-1280	2	Ouyang, Tahiri	64	1.74*	NA
hsa-let-7f	2	Chen, Yan	32	2.39*	NA
hsa-miR-29b-3p	2	Chen, Yan	32	2.27*	NA
Down-regulated miRNAs					
hsa-miR-145	3	Iorio, Tahiri, Tanic	204	3.31	2.38-4.24
hsa-miR-143	3	Iorio, Tahiri, Tanic	204	1.48	1.10-1.85
hsa-let-7d	2	Iorio, Tahiri	168	1.53	1.12-1.94
hsa-miR-99a	2	Tahiri, Tanic	94	3.57*	NA
hsa-miR-125b	2	Tahiri, Tanic	94	3.42*	NA
hsa-miR-10b	2	Tahiri, Tanic	94	2.57*	NA
hsa-miR-100	2	Tahiri, Tanic	94	2.54*	NA
hsa-miR-205	2	Tahiri, Tanic	94	2.48*	NA
hsa-miR-195	2	Tahiri, Tanic	94	2.37*	NA
hsa-miR-130a	2	Tahiri, Tanic	94	2.01*	NA
hsa-miR-320c	2	Tahiri, Tanic	94	1.93*	NA
hsa-miR-575	2	Chen, Tahiri	82	2.26*	NA
hsa-miR-486-5p	2	Ouyang, Tahiri	64	4.86*	NA
hsa-miR-140-3p	2	Ouyang, Tahiri	64	3.15*	NA
hsa-miR-335	2	Tanic, Yan	44	2.43	1.40-3.45

*FC based upon a single value. NA = not applicable.
Marked in blue: miRNA also found differentially expressed in the same direction in *BRCA1*-C vs. *BRCA1*-N comparison in our analysis.
Marked in yellow: miRNA also found differentially expressed in the same direction in *BRCA1*-C vs. *BRCA1*-N and *BRCA2*-C vs. *BRCA2*-N comparisons in our analysis.
Marked in red: miRNA also found differentially expressed in the same direction in *BRCA2*-C vs. *BRCA2*-N comparison in our analysis.
Marked in green: miRNA also found differentially expressed in the opposite direction in *BRCA1*-C vs. *BRCA1*-N and *BRCA2*-C vs. *BRCA2*-N comparisons in our analysis.
Marked in purple: miRNA also found differentially expressed in the opposite direction in *BRCA2*-C vs. *BRCA2*-N comparison in our analysis.

DISCUSSION

In the present study, miRNA expression profiles were investigated by miRNA microarray between normal and cancer tissue from *BRCA1* and *BRCA2* germline mutation carriers, in comparison with normal tissue from non-carriers. This yielded several biologically interesting findings.

First, many more miRNAs were found to be differentially expressed between the carcinomas and asymptomatic normal breast tissue in *BRCA1* and *BRCA2* germline mutation carriers compared to the number of differentially expressed miRNAs between sporadic breast carcinomas and normal breast tissue as derived from the literature. This may be due to the extensive chromosomal instability seen in *BRCA1/2*-associated breast carcinomas, leading to loss of chromosomal regions and consequently, miRNA genes. miRNAs deregulated in the *BRCA1* and *BRCA2* axes showed a similar chromosomal distribution, and several hotspots of down-regulated miRNAs were found in both axes. Indeed, about 21% of deregulated miRNAs matched reported locations of chromosomal instability in *BRCA1/2*-associated breast carcinomas and fragile sites in the genome [28]. The amount of miRNAs found at fragile sites in the genome was however lower than the >50% reported previously [29]. This could be explained by the fact that the chromosomal location of a considerable number of miRNAs from our analysis is still unclear. We found no clear indications whether miRNA deregulation in *BRCA1/2*-associated breast carcinomas could be due to direct effects of impaired *BRCA1/2* function. For instance, pathway analysis did not yield specifically enriched pathways for the *BRCA1* and *BRCA2* axes. However, the *BRCA2*-C vs. *BRCA2*-N comparison yielded many more deregulated miRNAs compared to the *BRCA1*-C vs. *BRCA1*-N comparison. It was recently discovered that *BRCA1* accelerates miRNA processing via interaction with Drosha [34]. Impaired *BRCA1* function could therefore lead to less miRNA production.

We found several deregulated miRNAs in *BRCA1/2*-associated breast carcinomas that were also reported in studies investigating sporadic breast tumors, similar to the study by Tanic et al. (2012) [32]. This suggests the existence of miRNAs which are important in regulating oncogenes and tumor suppressor genes in both the hereditary and sporadic settings [32]. These miRNAs have been shown to play a role in cell proliferation and invasion, acting on HER signaling (miR-143, miR-145, miR-205) [35,36], cell cycle regulation (miR-195) [37], epithelial to mesenchymal transition (miR-145, miR-205) [38,39], and tumor angiogenesis (miR-145) [40]. Interestingly, several miRNAs that were up-regulated in sporadic breast carcinomas were also up-regulated in *BRCA1*-

associated breast carcinomas from our analysis compared to normal breast tissues from *BRCA1* germline mutation carriers. *BRCA2*-associated breast carcinomas did not show any similarities in up-regulated miRNAs with sporadic breast carcinomas, which is remarkable since *BRCA2*-associated carcinomas otherwise strongly resemble sporadic carcinomas. Mechanisms underlying these differences are currently unclear.

However, we also found many differentially expressed miRNAs between *BRCA1/2*-associated and sporadic carcinomas. This was mainly the case for up-regulated miRNAs, including miR-141 and miR-1280 (up-regulated in sporadic breast carcinomas and not deregulated in *BRCA1/2*-associated breast carcinomas); miR-1307-3p, miR-3162-3p, miR-155-5p, miR-4306, miR-185-5p, miR-574-5p, and miR-4455 (up-regulated in *BRCA1*-associated breast carcinomas compared to their normal counterparts, and not consequently deregulated in sporadic breast carcinomas); miR-4778-5p, miR-4433, miR-5010-5p, miR-1287, miR-663b, and miR-4688 (up-regulated in *BRCA2*-associated breast carcinomas compared to their normal counterparts, and not consequently deregulated in sporadic breast carcinomas); miR-3676-5p and miR-937-5p (up-regulated in *BRCA1/2*-associated breast carcinomas and not deregulated in sporadic breast carcinomas); and miR-320c and miR-486-5p (down-regulated in sporadic breast carcinomas and not deregulated in *BRCA1/2*-associated breast carcinomas). The function of most of these miRNAs in breast cancer is still unclear. However, miR-155 deregulation has been associated with drug resistance in breast cancer by repression of FOXO3a, stimulation of epithelial-to-mesenchymal transition and MAPK signaling [41]. Elevated miR-155 expression levels have been found in HER2-positive breast carcinomas [42]. Moreover, several miRNAs were deregulated in the opposite direction between *BRCA1/2*-associated breast carcinomas and sporadic breast carcinomas. These mostly entailed up-regulated miRNAs in sporadic breast carcinomas and down-regulated in *BRCA2*-associated breast carcinomas (miR-20a-5p, let-7f-5p, and miR-29b-3p). miR-20a-5p has been associated with triple-negative tumors that showed enhanced expression compared to luminal A tumors [43]. miR-29b has been negatively associated with HER2 expression [44]. The function of let-7f is still unclear. Apart from potential underlying biological mechanisms, differences found in miRNA expression between *BRCA1/2*-associated and sporadic breast carcinomas are also to some extent likely to be due to technical differences (see below).

Second, several miRNAs were found to be already deregulated in the normal breast tissue from *BRCA1* germline mutation carriers. Of these, miR-140-3p, miR-335, miR-320c and miR-486-5p have been previously described as deregulated in sporadic breast cancer. Recently, it has been reported that most miRNA deregulation occurs at a very

early stage in sporadic breast carcinogenesis, during the transition from normal breast tissue to atypical ductal hyperplasia [27]. In case of an underlying *BRCA1* or *BRCA2* germline mutation, impaired DNA repair could already lead to aberrant miRNA expression in epithelial cells before these alterations lead to morphological changes. Additionally, miRNA deregulation at this stage could also be explained as the result of stromal changes. Deregulation of the microenvironment and intercellular interactions have an important role in breast neoplastic transformation [28]. It has been shown that the asymptomatic breast of *BRCA1* and *BRCA2* germline mutation carriers shows epithelial and stromal changes, including less differentiated lobules and a more dense and fibrotic intra-lobular stroma [28]. No miRNAs were deregulated in the normal breast tissue from *BRCA2* germline mutation carriers compared to non-carriers. This could mean that miRNA deregulation along the *BRCA2* axis occurs at a later stage compared to the *BRCA1* axis. However, the *BRCA2*-C vs. Healthy-N comparison yielded 600 differentially expressed miRNAs and the *BRCA2*-C vs. *BRCA2*-N comparison 'only' 96 miRNAs. An explanation for the uneven distribution of differentially expressed miRNAs along the *BRCA2* axis could be that subtle changes in miRNA deregulation could not be identified due to a small sample size.

Third, no significant correlations were found between miRNA expression profiles on the one hand and biological differences (such as age and ER status) on the other hand. This is in contrast with previously reported findings based on sporadic carcinomas [22,24]. The miRNA changes caused by impaired *BRCA1* or *BRCA2* function might overrule the effect of these parameters on miRNA expression. However, we did not apply matching for sample selection and the sample size was relatively small. Therefore, a relation between age and ER status may have been missed.

Fourth, pathway analysis yielded several less expected processes in which deregulated miRNAs are predicted to be involved, such as epithelial junctions and ECM interaction, cellular movement, and protein trafficking. Dacheux *et al.* [45] recently discovered cellular movement and protein trafficking as possible new functions of *BRCA1* as well. Weber *et al.* [28] showed extensive genomic instability in the cancer stroma in *BRCA1/2*-associated breast carcinomas. A genetically unstable stroma might facilitate neoplastic transformation in the breast epithelium. Moreover, it has recently been published that stromal components also show miRNA deregulation and are affected by miRNAs secreted from tumor cells [46–49]. However, the role of these less expected processes in *BRCA1/2*-associated breast carcinogenesis needs to be further examined.

Two miRNAs had a similar differential expression pattern in microarray and qRT-PCR analysis: miR-99a and let-7b. miR-99a was down-regulated in breast carcinomas compared to normal breast tissues in both *BRCA1* and *BRCA2* germline mutation carriers. miR-99a deregulation has been reported in several human cancers, including breast cancer, and is involved in apoptosis and epithelial-to-mesenchymal transition by regulation of the mTOR, Akt and TGF-β pathway [50–55]. TGF-β pathway activity is decreased by loss of miR-99a, resulting in increased proliferation and decreased migration [51]. Let-7b was found to be specifically down-regulated in invasive breast carcinomas of *BRCA2* germline mutation carriers. Our target gene analysis showed that let-7b is involved in cellular pathways, including those of p38 MAPK, p53, Wnt/β-catenin, apoptosis, tight junction, integrin and actin cytoskeleton.

A few studies have shown let-7b down-regulation in human cancers, including breast, gastric and lung cancer [56–59]. In breast cancer, low let-7b expression has been associated with poor prognosis [57], whereas high let-7b expression has been observed in luminal A tumors and is associated with a favorable prognosis [56]. Further research is needed to investigate whether let-7b might be able to distinguish *BRCA2*-associated from sporadic breast carcinomas. This would be very interesting as *BRCA2*-associated and sporadic breast carcinomas show many similarities in histology and protein expression.

There are some limitations inherent to the techniques used in this study. Whole tissue and not laser-captured micro-dissected tissue samples have been used for miRNA profiling. This could have influenced the results, as miRNA profiling on whole tissue samples reflect changes in both epithelial and stromal cells [60]. However, we used maximally enriched samples by scraping off specific relevant regions from sections marked by a pathologist, so we do not believe that this has played a major role. Further, microarray technology does not show a distinct boundary between up-regulated and down-regulated miRNAs, but rather shows a broad distribution of miRNA levels. Until now, it is unclear which level is biologically functional for each miRNA. Lastly, the complex nature of microarray data makes the analysis highly dependent on bio-informatics and statistics [24]. This could, together with other factors such as the use of different microarray platforms, explain the little overlap between the findings of different miRNA profiling studies as shown in the systematic literature analysis [24].

In conclusion, this study revealed multiple deregulated miRNAs in *BRCA1/2*-associated breast carcinomas, some shared with sporadic breast carcinomas, but several possibly specific to *BRCA1/2* carcinogenesis. Specific deregulated miRNAs in *BRCA1/2*-

associated carcinomas appear to target similar pathways. This suggests the existence of common targetable miRNA regulated pathways driving *BRCA1/2*-associated breast carcinogenesis. These findings warrant further studies on the role of these miRNAs in *BRCA1/2*-associated breast carcinogenesis.

MATERIALS AND METHODS

Patient samples

For miRNA microarray analysis and initial qRT-PCR validation, a total of 46 FFPE patient samples were obtained from the archives of the University Medical Center Utrecht (UMCU), University Medical Center Groningen, the Familial Cancer Clinic of the VU University Medical Center Amsterdam, and of local hospitals around Utrecht. Since we used archival pathology material which does not interfere with patient care and does not involve the physical involvement of the patient, no ethical approval is required according to Dutch legislation: the Medical Research Involving Human Subjects Act (Wet Medisch Wetenschappelijk Onderzoek met Mensen) [61]. Use of anonymous or coded left-over material for scientific purposes is part of the standard treatment contract with patients and therefore informed consent procedure was not required according to our institutional medical ethical review board. This has also been described by van Diest et al. [62]. The patient samples have already been described under results and are shown in Tables 1a and 1b.

RNA isolation

Whole tissue sections (10 μm thick) were cut from the paraffin tissue blocks for RNA isolation. Total RNA was extracted using the miRNeasy FFPE Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's protocol. For the tumor samples, the tumor area in five sections was scraped off from the slides, guided by haematoxylin and eosin stainings of the tissue samples. For the normal breast tissue samples, the whole area of the 10 tissue sections was scraped off. RNA concentration and integrity were determined using the 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA).

miRNA microarray analysis

miRNA microarray analysis was performed at the Sidney Kimmel Comprehensive Cancer Center Microarray Core Facility at the Johns Hopkins University, Baltimore, USA. The Human miRNA Microarray v19.0 (Agilent Technologies), containing 2006 human miRNAs from the miRBase database (release 19.0), was used. Sample preparation

and hybridization were done according to manufacturer's instructions (for details, see Supplementary methods). Raw data were processed and analyzed using GeneSpring GX v12.5 (Agilent Technologies). Median fluorescence intensity values smaller than a threshold of 1 were set equal to 1. Probe level data were log2 transformed and normalized to the 75th percentile based upon the distribution of signal intensities. miRNA probes were included if 100 percent of the samples in any 1 out of the 5 classes had normalized expression values within the 50th and 100th percentile in order to remove miRNAs showing low expression and little variation between the samples. To correlate miRNA expression with BRCA status or other clinicopathologic characteristics, the unpaired *t*-test for unequal variance or ANOVA for unequal variance were used, depending on the number of groups to compare. In order to correct for multiple comparisons, adjusted *p*-values were obtained by using Benjamini and Hochberg's FDR. Level of significance was set at FDR < 0.05.

Chromosomal distribution analysis

The chromosomal distribution of differentially expressed miRNAs between classes was analyzed using Fisher's exact test, performed in SPSS v20 (IBM, Armonk, NY, USA). Only miRNAs of which the localization in the genome was known were included in this analysis.

qRT-PCR

All patient samples were diluted to a total RNA concentration of 2 ng/µl. For reverse transcription of specific miRNAs, the TaqMan microRNA Reverse Transcription kit together with microRNA Assays was used according to the manufacturer's protocol (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The following thermal cycler conditions were used for reverse transcription: 30 min at 16 ^{0}C, 30 min at 42 ^{0}C, 5 min at 85 ^{0}C, followed by 4 ^{0}C. miRNA expression was investigated using the TaqMan Universal PCR Master Mix II without UNG together with microRNA assays (Applied Biosystems). The following thermal cycler conditions were used for qRT-PCR: 10 min at 95 ^{0}C, followed by 40 cycles (15 s at 95 ^{0}C, and 60 s at 60 ^{0}C). The microRNA Assays (Applied Biosystems) consisted of the following primer sets (5X primer for reverse transcription, 20X primer for qRT-PCR): hsa-miR-99a-3p, hsa-miR-210, hsa-miR-21-5p, hsa-miR-183-5p, hsa-miR-378a-5p, hsa-miR-153, hsa-miR-4778-5p, hsa-miR-4443, hsa-let-7b-5p, hsa-miR-551b-3p. All samples were tested in duplicate. Reverse transcription and qRT-PCR for samples of the initial cohort were done on the IQ5 Multicolor Real-Time PCR Detection System and C1000 Touch thermal cycler with CFX96 Real-Time system (both Bio-Rad, Hercules, CA, USA), respectively. For the second cohort, the Veriti 96 Well Thermal Cycler (Applied Biosystems) and the ViiA™ 7 Real Time PCR System (Life Technologies) were used for reverse transcription and

qRT-PCR, respectively. The qRT-PCR data of the first cohort were normalized to the second cohort by the use of replicates. Expression levels were calculated based on the comparative threshold cycle (ΔΔ Ct) method and were normalized to miR-125a-5p. The statistical analysis, *i.e.* the Kruskal-Wallis test to compare the median of the normalized Ct values between the classes, was performed in SPSS v20 (IBM). Level of significance was set at $p \leq 0.01$, due to multiple comparisons.

Cluster analysis

Clustering was carried out on both samples and miRNAs using a Self-Organizing Map in Cluster 3.0 (Eisen Lab, University of California, Berkeley) using all samples and all miRNAs that passed the filtering criteria. Default settings were used: a grid of 6x6, 100.000 iterations, and an initial learning rate of 0.02 for clustering genes; a grid of 3x3, 20.000 iterations, and an initial learning rate of 0.02 for clustering samples. The Self-Organizing Map was carried out using the Pearson centered distance algorithm with complete linkage rule. For visualization of the dendrogram, Java Treeview (http://jtreeview.sourceforge.net/) was used.

Target gene and pathway analysis

Target gene and pathway analysis were performed using the web-based computational tool DIANA-mirPath v2.0 (http://www.microrna.gr/miRPathv2) and Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, http://www.ingenuity.com/products/ipa). DIANA-mirPath was used to investigate the combinatorial effect of multiple miRNAs on pathways. Differentially expressed miRNAs between invasive breast carcinomas and normal breast tissues from prophylactic mastectomies for both *BRCA1* and *BRCA2* germline mutation carriers with FC ≥ |2.0| and FDR < 0.05 (unpaired *t*-test for unequal variance) were selected for this analysis. The Pathways Union mode was used with micro-T-threshold of 0.8 and level of significance was set at $p < 0.05$ with FDR correction applied. miRNAs selected for qRT-PCR validation were subjected to IPA, which combines the results from several prediction tools, including TarBase, miRecords, and TargetScan. Only experimentally validated and highly predicted targets were considered. Level of significance of the Fisher's exact test was set at $p < 0.05$.

Comparison with miRNA expression data available in literature

Breast cancer miRNA expression profiling studies were identified using the PubMed database. The search syntax is shown in Table 13. Inclusion and exclusion criteria were used to screen the articles and are mentioned in Figure 4. The following data were extracted from the studies: author, year of publication, journal, location of study, selection and characteristics of patient samples, miRNA microarray platform, cut-off criteria used for

Table 13. Overview search strategy for breast cancer miRNA expression profiling studies

Database	Search syntax
PubMed	(((((((mirna[Title/Abstract]) OR mirnas[Title/Abstract]) OR microrna[Title/Abstract]) OR micrornas[Title/Abstract]) OR mir[Title/Abstract]) OR mirs[Title/Abstract])) AND (breast[Title/Abstract])) AND ((((((cancer[Title/Abstract]) OR cancers[Title/Abstract]) OR tumor[Title/Abstract]) OR tumors[Title/Abstract]) OR tumour[Title/Abstract]) OR tumours[Title/Abstract])
Date:	Nov 23, 2014

statistical analysis, and lists of up- and down-regulated miRNAs including p-value and fold change (if available). The differentially expressed miRNAs reported by the studies were ranked according to the following protocol [63–65]: (1) number of studies that reported the miRNA and with a consistent direction of expression change; (2) total number of samples from studies in agreement upon which the differential expression of the miRNA is based; (3) average fold change from studies in agreement. For the ranking, an online bio-informatics tool for comparing lists (http://bioinfogp.cnb.csic.es/tools/venny/) was used.

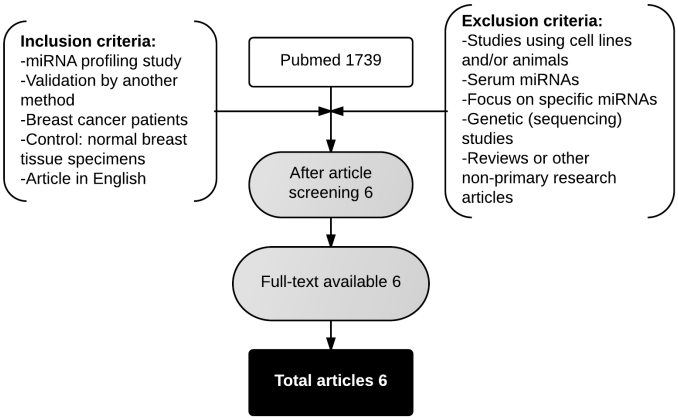

Figure 4. Flowchart showing the article selection strategy to attain breast miRNA expression profiling studies

SUPPLEMENTARY FILES

Supplementary files are available online.

REFERENCES

1. Jemal A, Bray F, Ferlay J, Center MM, Ward E, Forman D. Global Cancer Statistics. CA. Cancer J. Clin. 2011; 61: 69–90.
2. GLOBOCAN. Most frequent cancers: women [Internet]. Sect. Cancer Inf. 2008. Available from: http://globocan.iarc.fr/factsheets/populations/factsheet.asp?uno=900#WOMEN
3. Narod SA. BRCA mutations in the management of breast cancer: the state of the art. Nat. Rev. Clin. Oncol. 2010; 7: 702–707.
4. Venkitaraman AR. and the Functions of *BRCA1* and *BRCA2*. Cell. 2002; 108: 171–182.
5. Roy R, Chun J, Powell SN. *BRCA1* and *BRCA2*: different roles in a common pathway of genome protection. Nat. Rev. Cancer. 2012; 12: 68–78.
6. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, et al. Molecular portraits of human breast tumours. Nature. 2000; 406: 747–752.
7. Hu Z, Fan C, Oh DS, Marron JS, He X, Qaqish BF, Livasy C, Carey LA, Reynolds E, Dressler L, Nobel A, Parker J, Ewend MG, et al. The molecular portraits of breast tumors are conserved across microarray platforms. BMC Genomics. 2006; 7: 96.
8. Comprehensive molecular portraits of human breast tumours. Nature. 2012; 490: 61–70.
9. Bartel DP, Lee R, Feinbaum R. MicroRNAs : Genomics , Biogenesis , Mechanism , and Function Genomics : The miRNA Genes. Cell. 2004; 116: 281–297.
10. Lee C-H, Subramanian S, Beck AH, Espinosa I, Senz J, Zhu SX, Huntsman D, van de Rijn M, Gilks CB. MicroRNA profiling of *BRCA1/2* mutation-carrying and non-mutation-carrying high-grade serous carcinomas of ovary. PLoS One. 2009; 4: e7314.
11. Lowery AJ, Miller N, McNeill RE, Kerin MJ. MicroRNAs as prognostic indicators and therapeutic targets: potential effect on breast cancer management. Clin. Cancer Res. 2008; 14: 360–365.
12. Samantarrai D, Dash S, Chhetri B, Mallick B. Genomic and epigenomic cross-talks in the regulatory landscape of miRNAs in breast cancer. Mol. Cancer Res. 2013; 11: 315–328.
13. Jukic DM, Rao UNM, Kelly L, Skaf JS, Drogowski LM, Kirkwood JM, Panelli MC. Microrna profiling analysis of differences between the melanoma of young adults and older adults. J. Transl. Med. 2010; 8: 27.
14. Schmeier S, Schaefer U, Essack M, Bajic VB. Network analysis of microRNAs and their regulation in human ovarian cancer. BMC Syst. Biol. 2011; 5: 183.
15. Croce CM, Causes and consequences of microRNA dysregulation in cancer. Nat. Rev. Genet. 2009; 10: 704–714.
16. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat. Rev. Cancer. 2006; 6: 857–866.
17. Cimmino A, Calin GA, Fabbri M, Iorio M V, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, et al. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc. Natl. Acad. Sci. U. S. A. 2005; 102: 13944–13949.
18. Voorhoeve PM, le Sage C, Schrier M, Gillis AJM, Stoop H, Nagel R, Liu Y-P, van Duijse J, Drost J, Griekspoor A, Zlotorynski E, Yabuta N, De Vita G, et al. A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. Cell. 2006; 124: 1169–1181.
19. Dvinge H, Git A, Gräf S, Salmon-Divon M, Curtis C, Sottoriva A, Zhao Y, Hirst M, Armisen J, Miska EA, Chin S-F, Provenzano E, Turashvili G, et al. The shaping and functional consequences of the microRNA landscape in breast cancer. Nature. 2013; 497: 378–382.
20. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, et al. MicroRNA expression profiles classify human cancers. Nature. 2005; 435: 834–838.
21. Volinia S, Calin GA, Liu C-G, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc. Natl. Acad. Sci. U. S. A. 2006; 103: 2257–2261.

22. Iorio MV, Ferracin M, Liu C-G, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M, Ménard S, Palazzo JP, Rosenberg A, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 2005; 65: 7065–7070.

23. Farazi TA, Spitzer JI, Morozov P, Tuschl T. miRNAs in human cancer. J. Pathol. 2011; 223: 102–115.

24. Lowery AJ, Miller N, Devaney A, McNeill RE, Davoren PA, Lemetre C, Benes V, Schmidt S, Blake J, Ball G, Kerin MJ. MicroRNA signatures predict oestrogen receptor, progesterone receptor and HER2/neu receptor status in breast cancer. Breast Cancer Res. 2009; 11: R27.

25. Iorio M V, Casalini P, Tagliabue E, Ménard S, Croce CM. MicroRNA profiling as a tool to understand prognosis, therapy response and resistance in breast cancer. Eur. J. Cancer. 2008; 44: 2753–2759.

26. Xi Y, Nakajima GO, Gavin E, Morris CG, Kudo K, Hayashi K, Ju J. Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. RNA. 2007; 13: 1668–1674.

27. Chen L, Li Y, Fu Y, Peng J, Mo M-H, Stamatakos M, Teal CB, Brem RF, Stojadinovic A, Grinkemeyer M, McCaffrey TA, Man Y, Fu SW. Role of deregulated microRNAs in breast cancer progression using FFPE tissue. PLoS One. 2013; 8: e54213.

28. Weber F, Shen L, Fukino K, Patocs A, Mutter GL, Caldes T, Eng C. Total-genome analysis of *BRCA1/2*-related invasive carcinomas of the breast identifies tumor stroma as potential landscaper for neoplastic initiation. Am. J. Hum. Genet. 2006; 78: 961–972.

29. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M, Croce CM. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc. Natl. Acad. Sci. U. S. A. 2004; 101: 2999–3004.

30. Ouyang M, Li Y, Ye S, Ma J, Lu L, Lv W, Chang G, Li X, Li Q, Wang S, Wang W. MicroRNA profiling implies new markers of chemoresistance of triple-negative breast cancer. PLoS One. 2014; 9: e96228.

31. Tahiri A, Leivonen S-K, Lüders T, Steinfeld I, Ragle Aure M, Geisler J, Mäkelä R, Nord S, Riis MLH, Yakhini Z, Kleivi Sahlberg K, Børresen-Dale A-L, Perälä M, et al. Deregulation of cancer-related miRNAs is a common event in both benign and malignant human breast tumors. Carcinogenesis. 2014; 35: 76–85.

32. Tanic M, Yanowsky K, Rodriguez-Antona C, Andrés R, Márquez-Rodas I, Osorio A, Benitez J, Martinez-Delgado B. Deregulated miRNAs in hereditary breast cancer revealed a role for miR-30c in regulating KRAS oncogene. PLoS One. 2012; 7: e38847.

33. Yan LX, Huang XF, Shao Q, Huang MY, Deng L, Wu QL, Zeng YX, Shao JY. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. RNA. 2008; 14: 2348-2360.

34. Kawai S, Amano A. *BRCA1* regulates microRNA biogenesis via the DROSHA microprocessor complex. J. Cell Biol. 2012; 197: 201–208.

35. Yan X, Chen X, Liang H, Deng T, Chen W, Zhang S, Liu M, Gao X, Liu Y, Zhao C, Wang X, Wang N, Li J, et al. miR-143 and miR-145 synergistically regulate ERBB3 to suppress cell proliferation and invasion in breast cancer. Mol. Cancer. 2014; 13: 220.

36. Iorio M V, Casalini P, Piovan C, Di Leva G, Merlo A, Triulzi T, Ménard S, Croce CM, Tagliabue E. microRNA-205 regulates HER3 in human breast cancer. Cancer Res. 2009; 69: 2195–2200.

37. Li D, Zhao Y, Liu C, Chen X, Qi Y, Jiang Y, Zou C, Zhang X, Liu S, Wang X, Zhao D, Sun Q, Zeng Z, et al. Analysis of MiR-195 and MiR-497 expression, regulation and role in breast cancer. Clin. Cancer Res. 2011; 17: 1722–1730.

38. Hu J, Guo H, Li H, Liu Y, Liu J, Chen L, Zhang J, Zhang N. MiR-145 regulates epithelial to mesenchymal transition of breast cancer cells by targeting Oct4. PLoS One. 2012; 7: e45965.

39. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat. Cell Biol. 2008; 10: 593–601.

40. Zou C, Xu Q, Mao F, Li D, Bian C, Liu L-Z, Jiang Y, Chen X, Qi Y, Zhang X, Wang X, Sun Q, Kung H-F, et al. MiR-145 inhibits tumor angiogenesis and growth by N-RAS and VEGF. Cell Cycle. 2012; 11: 2137–2145.

41. Yu D, Lv M, Chen W, Zhong S, Zhang X, Chen L, Ma T, Tang J, Zhao J. Role of miR-155 in drug resistance of breast cancer. Tumour Biol. 2015; 36: 1395–1401.

42. Zeng H, Fang C, Nam S, Cai Q, Long X. The clinicopathological significance of microRNA-155 in breast cancer: a meta-analysis. Biomed Res. Int. 2014; 2014: 724209.

43. Calvano Filho CMC, Calvano-Mendes DC, Carvalho KC, Maciel GA, Ricci MD, Torres AP, Filassi JR, Baracat EC. Triple-negative and luminal A breast tumors: differential expression of miR-18a-5p, miR-17-5p, and miR-20a-5p. Tumour Biol. 2014; 35: 7733–7741.

44. Qin L, Li R, Zhang J, Li A, Luo R. Special suppressive role of miR-29b in HER2-positive breast cancer cells by targeting Stat3. Am. J. Transl. Res. 2015; 7: 878–890.

45. Dacheux E, Vincent A, Nazaret N, Combet C, Wierinckx A, Mazoyer S, Diaz J-J, Lachuer J, Venezia ND. *BRCA1*-Dependent Translational Regulation in Breast Cancer Cells. PLoS One. 2013; 8: e67313.

46. Bullock MD, Pickard KM, Nielsen BS, Sayan a E, Jenei V, Mellone M, Mitter R, Primrose JN, Thomas GJ, Packham GK, Mirenzami a H. Pleiotropic actions of miR-21 highlight the critical role of deregulated stromal microRNAs during colorectal cancer progression. Cell Death Dis. 2013; 4: e684.

47. Chou J, Werb Z. MicroRNAs play a big role in regulating ovarian cancer-associated fibroblasts and the tumor microenvironment. Cancer Discov. 2012; 2: 1078–1080.

48. Mitra AK, Zillhardt M, Hua Y, Tiwari P, Murmann AE, Peter ME, Lengyel E. MicroRNAs reprogram normal fibroblasts into cancer-associated fibroblasts in ovarian cancer. Cancer Discov. 2012; 2: 1100–1108.

49. Nishida N, Nagahara M, Sato T, Mimori K, Sudo T, Tanaka F, Shibata K, Ishii H, Sugihara K, Doki Y, Mori M. Microarray analysis of colorectal cancer stromal tissue reveals upregulation of two oncogenic miRNA clusters. Clin. Cancer Res. 2012; 18: 3054–3070.

50. Hu Y, Zhu Q, Tang L. MiR-99a antitumor activity in human breast cancer cells through targeting of mTOR expression. PLoS One. 2014; 9: e92099.

51. Turcatel G, Rubin N, El-Hashash A, Warburton D. MIR-99a and MIR-99b modulate TGF-β induced epithelial to mesenchymal plasticity in normal murine mammary gland cells. PLoS One. 2012; 7: e31032.

52. Yang Z, Han Y, Cheng K, Zhang G, Wang X. miR-99a directly targets the mTOR signalling pathway in breast cancer side population cells. Cell Prolif. 2014; 47: 587–595.

53. Yu S-H, Zhang C-L, Dong F-S, Zhang Y-M. miR-99a Suppresses the Metastasis of Human Non-Small Cell Lung Cancer Cells by Targeting AKT1 Signaling Pathway. J. Cell. Biochem. 2015; 116: 268–276.

54. Wu D, Zhou Y, Pan H, Zhou J, Fan Y, Qu P. microRNA-99a inhibiting cell proliferation, migration and invasion by targeting fibroblast growth factor receptor 3 in bladder cancer. Oncol. Lett. 2014; 7: 1219–1224.

55. Wang L, Chang L, Li Z, Gao Q, Cai D, Tian Y, Zeng L, Li M. miR-99a and -99b inhibit cervical cancer cell proliferation and invasion by targeting mTOR signaling pathway. Med. Oncol. 2014; 31: 934.

56. Quesne J Le, Jones J, Warren J, Dawson S-J, Ali HR, Bardwell H, Blows F, Pharoah P, Caldas C. Biological and prognostic associations of miR-205 and let-7b in breast cancer revealed by in situ hybridization analysis of micro-RNA expression in arrays of archival tumour tissue. J. Pathol. 2012; 227: 306–314.

57. Ma L, Li G-Z, Wu Z-S, Meng G. Prognostic significance of let-7b expression in breast cancer and correlation to its target gene of BSG expression. Med. Oncol. 2014; 31: 773.

58. Kang W, Tong JHM, Lung RWM, Dong Y, Yang W, Pan Y, Lau KM, Yu J, Cheng AS, To KF. let-7b/g silencing activates AKT signaling to promote gastric carcinogenesis. J. Transl. Med. 2014; 12: 281.

59. Jusufović E, Rijavec M, Keser D, Korošec P, Sodja E, Iljazović E, Radojević Z, Košnik M. let-7b and miR-126 are down-regulated in tumor tissue and correlate with microvessel density and survival outcomes in non--small--cell lung cancer. PLoS One. 2012; 7: e45577.

60. Squadrito ML, Etzrodt M, De Palma M, Pittet MJ. MicroRNA-mediated control of macrophages and its implications for cancer. Trends Immunol. 2013; 34: 350–359.

61. Central Committee on Research involving Human Subjects. Medical Research Involving Human Subjects Act [Internet]. 2015. Available from: http://www.ccmo.nl/nl/niet-wmo-onderzoek

62. Van Diest PJ. No consent should be needed for using leftover body material for scientific purposes. BMJ. 2002; 325: 648–651.

63. Chan SK, Griffith OL, Tai IT, Jones SJM. Meta-analysis of colorectal cancer gene expression profiling studies identifies consistently reported candidate biomarkers. Cancer Epidemiol. Biomarkers Prev. 2008; 17: 543–552.

64. Guan P, Yin Z, Li X, Wu W, Zhou B. Meta-analysis of human lung cancer microRNA expression profiling studies comparing cancer tissues with normal tissues. J. Exp. Clin. Cancer Res. 2012; 31: 54.

65. Griffith OL, Melck A, Jones SJM, Wiseman SM. Meta-analysis and meta-review of thyroid cancer gene expression profiling studies identifies important diagnostic biomarkers. J. Clin. Oncol. 2006; 24: 5043–5051.

PART 2

‘Gen-ethical’ aspects

Chapter 7

Moral duties of genomics researchers: why *personalized* medicine requires a *collective* approach

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ABSTRACT

Advances in genome sequencing together with the introduction of personalized medicine offer promising new avenues for research and precision treatment, particularly in the field of oncology. At the same time, the convergence of genomics, bioinformatics and the collection of human tissues and patient data creates novel moral duties for researchers. After all, unprecedented amounts of potentially sensitive information are being generated. Over time, traditional research ethics principles aimed at protecting individual participants have become supplemented with social obligations related to the interests of society and the research enterprise at large, illustrating that genomic medicine is also a social endeavor. In this review, we provide a comprehensive assembly of moral duties that have been attributed to genomics researchers and offer suggestions for a responsible advancement of personalized genomic cancer care.

GENOMICS' PROGRESS CAUSING MORAL DILEMMAS FOR RESEARCHERS

The development of genomic techniques has markedly increased our knowledge on the genetic origins of diseases, especially cancer, and has led to the introduction of personalized medicine [1–3]. At the same time, these developments create ethical challenges for researchers performing these studies. The scope is changing from single-gene sequencing studies to large-scale, whole exome or whole genome studies, generating large amounts of genomic data. Questions arise on how to adequately deal with these potentially sensitive data. The emergence of large (international) research projects and biobanks with increased genomic data sharing, advances in information technology, and increasingly blurred boundaries between clinical care and research make the reflection on ethical and social implications of these developments even more important [4–6]. During the past few years, multiple as well as conflicting publications have appeared on the ethical aspects of large-scale DNA sequencing studies. In the era of personalized medicine, genomics researchers are confronted with a wide variety of moral duties. In this review, we provide a comprehensive assembly of moral duties that have been attributed to genomics researchers in the literature and offer suggestions for a responsible advancement of personalized genomics.

DISTINGUISHING DIFFERENT TYPES OF MORAL DUTIES

Moral duties of genomics researchers can be grouped into four clusters: 1) Disclosure duties; 2) Consent duties; 3) Privacy duties; and 4) Social duties. Details of the review process, through which these clusters were identified, can be found in Box 1.

DISCLOSURE DUTIES

Genomics studies generate large amounts of genetic information, ranging from clinically significant genetic aberrations to variants of unknown or uncertain significance [6]. This may generate a moral obligation for researchers to disclose some of these results to participants when they are relevant for their health or that of their relatives [7]. Duties involved with individual disclosure were classified as Disclosure duties. This cluster of duties is most frequently discussed in literature and includes the duty to return results to research participants and their relatives, the duty to rescue, the duty to hunt, the duty to re-contact, and the duty to provide ancillary care.

The duty to return results to research participants

The debate in literature is mostly focused on the disclosure of individual unsolicited genetic results to research participants (also known as incidental or secondary findings). Return of aggregate research results seems consensual [6,8]. The two extreme positions of 'no disclosure at all' and 'full disclosure' of all individual genetic results are rarely advocated [6]. There is growing consensus that there is an obligation to return individual genetic results that are analytically and clinically valid, of clinical utility, and actionable, provided that the participant has consented to disclosure [9–17]. This position is based upon principles of beneficence, respect for autonomy, and reciprocity toward research participants, as reviewed previously [6]. There is however still definitional uncertainty and variable interpretation of above-mentioned terms. Genetic research findings are often uncertain, probabilistic and pleiotropic in nature [6,15,17–20]. Therefore, a qualified rather than a full-disclosure policy is often advocated as disclosure of all genetic information causes information overload, hampers decision-making and it could have potential adverse psychological, social and financial consequences for research participants [6,15,17–20]. Moreover, disclosure imposes a burden on the research enterprise. Disclosure can be time-consuming and costly, and research laboratories often lack the resources and infrastructure for adequate disclosure [6,7,10,18,20–25].

However, disclosure of unsolicited findings, even if a burdensome undertaking, may not only be beneficial to research participants but to science as well [11,15,17,20,26–28]. Firstly, when participants are contacted about individual research results, more data could be gathered (e.g. phenotypic data, family history), which can be used to interpret unclear variants [20]. Secondly, disclosure of unsolicited findings could also encourage participation in research and engage participants in science [11,15,27,28].

To conclude, there is growing consensus that unsolicited findings in genetic research should at least to some extent be disclosed to research participants applying a qualified disclosure policy, although there is still discussion on the interpretation of proposed criteria [29–32]. A balance must be sought between meeting participants' benefits and interests in obtaining genetic results, while not unjustifiably hindering the research enterprise and society's long-term interest in advancing knowledge [11,33].

The duty to return results to relatives

Individual genetic results should in the first place be returned to research participants themselves [34,35]. However, there is growing consensus that certain unsolicited findings should also be offered to relatives as they may have implications for them as well. Many authors agree that highly pathogenic and actionable variants should be disclosed to

relatives when the chances are high that they are at risk and when disclosure might prevent them from harm [34–39]. According to some, this duty may even override the privacy concerns of the research participant, when research participants refuse to disclose information to their relatives themselves or have demanded full non-disclosure [40,41]. Otherwise, obligations to notify relatives should be with the participant just as in the clinical situation, or else it would create a tremendous burden on researchers [35,37]. Obviously, a duty to disclose research results to relatives may cause a moral conflict and a balance must be found between focusing on research's central goal of producing generalizable knowledge, protecting participants' privacy, and the duty to warn participants' relatives for potential harm [35].

The duty to rescue

The obligation to return genetic results with clear health implications has been justified by some authors by a rescue paradigm [42–44]. The following criteria for the duty to rescue research participants by providing them genetic results have been proposed: 1) there is a high probability that the rescuer (i.e. researcher) will be able to avoid significant harm; 2) there is little risk of harm to the rescuer; and 3) the benefits outweigh the potential risks [42,44]. Criticism on this duty is not directed at the disclosure of clinically significant research findings. However, the rescue obligation framework is said to be inappropriate for the genomics research practice considering the nature of rescue cases, which are concrete rather than probabilistic and to a considerable extent predictable rather than unforeseeable [44]. Also the mode of dealing with clinically significant unsolicited findings should be proactive rather than reactive [44].

The duty to hunt

As stated above, there is a growing consensus to disclose clinically significant unsolicited findings. Some authors have proposed that researchers have a duty to actively search or hunt for these findings [30,45–47]. The following criteria, showing overlap with the above mentioned criteria for the duty to rescue, have been proposed that must be present for a duty to hunt: 1) high benefit for participants; 2) high need, e.g. lack of alternative resources; and 3) low burden for researchers [46,48]. Pro- and opponents of a duty to hunt vary in their opinions whether these criteria should or could be met [17,19,21,30,42,45–52]. Proponents argue that researchers currently have unique access to extensive genomic results, as there are few clinical alternatives. This carries along a duty to actively search for unsolicited findings [30,46,47]. Moreover, researchers using high-throughput sequencing methods should already for the purpose of their research itself investigate all identified variants as a standard component of their experimental setup [45,53]. Opponents focus on the differences in obligations between researchers

and clinicians. Researchers should not be obliged to actively search for genomic results that are unrelated to their research aims and beyond results yielded in their standard analysis process [21,54]. Moreover, a duty to hunt would create researchers' responsibilities and burdens that are larger than those of clinicians and increase the risk for therapeutic misconception [18,19]. Some opponents of a duty to hunt are in favor of the duty to rescue, as the latter may more appropriately balance researchers' obligations toward research participants [42–44].

The duty to re-contact

Re-contact with research participants could have several purposes, including a request for further research participation, to obtain further health information, and to return individual research results (e.g. after additional analyses or with new insights on the pathogenicity) [10]. The debate in literature is limited to the latter purpose of re-contact. Several criteria have been proposed that determine a researchers' obligation to re-contact: 1) whether the results were initially reported to research participants; 2) the level of potential benefit and risk of harm of the new genetic information; 3) the length of time since the start of the study; 4) the burden of re-contacting participants [55,56]. Objections to re-contacting for returning genetic results relate to practical aspects, such as the burden on researchers [10,57] and the increase in costs of genomics research [9]. There is currently no consensus to what extent and for which duration a duty to re-contact should exist.

The duty to provide ancillary care

The duty to provide ancillary care is one of the most recently attributed duties and relates to the theory of partial entrustment: researchers have special responsibilities toward research participants, as participants entrust important and private aspects of their health to them [13,23,31,32,43,56,58–60]. Therefore, when results are disclosed, proponents argue, all researchers, not only clinician-researchers, should provide adequate follow-up care, such as medical advice and referral [32]. This duty is also ascribed to secondary researchers who have had no direct contact with research participants [43,58]. Opponents argue that this duty is over-demanding as a researcher's aim is not to promote the health of research participants themselves [43].

CONSENT DUTIES

Several studies have discussed the typology of consent appropriate for the genomics research context [3,10,17,19,20,22,25,26,28,32,35–37,45,49,50,61–73]. The suitability of traditional informed consent given to a specific research project is challenged in genomics research due to its complexity and the risk of information overload and limited ability of participants to sufficiently understand the information given [17,68]. Questions have been raised on whether consent in genomics research can truly be 'informed' [26]. Therefore, the duty to obtain appropriate consent emerged as a topic of debate in literature.

The duty to obtain appropriate consent

Several consent alternatives have been proposed as being more suitable for genomics research. These include (the not mutually exclusive types of) broad consent [10,17,20,26,63,67,72,73], dynamic consent [69,74], tiered consent [11,20,37,50], and opt-out [11]. Broad consent has become an accepted consent model for genomics research and entails that participants give permission for a broad range of approved, but unspecified future research use with their stored samples as it is difficult to anticipate on all types of benefits and risks, considering the increasing knowledge and technological advances [10,71]. It can be combined with dynamic consent for future research applications and tiered consent for disclosure. Although broad consent is beneficial and convenient for the advancement of science as no repeated re-consent is necessary, opponents argue that broad consent cannot be regarded as adequately 'informed' and that it nullifies the concept of autonomy [17].

PRIVACY DUTIES

In genomics studies the enormous amounts of personal data generated increases the risk of individual identifiability [7,16,17,71]. Genetic data could be interesting for many third parties, such as family members, insurance companies, employers, and law courts, and may thereby lead to problems with insurance coverage, employment, stigmatization and discrimination [36]. Thus, the duty to protect participants' privacy emerged in genomics research literature [7,9,10,16,17,29,35,36,40,57,62,63,66,68,70,73,75–83].

The duty to protect participants' privacy

The duty to protect participant's privacy is important to protect the private sphere of research participants as well as to maintain public trust in the research enterprise [84].

It has been stated that the obligation to protect privacy is higher for a researcher than for a clinician, as research has no primary aim to provide benefit to patients, but it can certainly lead to harm [83]. Once genomic data is released into the public domain, there is essentially no way back [7]. Risk of re-identification and potential misuse of data should therefore be minimized [84]. Most genomic data are de-identified or anonymized, and saved and shared through controlled access databases [63]. Although the sharing of anonymized data seems favorable considering confidentiality, it is argued that no method of de-identification is perfect and the risk of identification may be greater than expected as it has been shown possible to identify individuals by linking genomic data to public information [63]. Moreover, it is not always conducive for the advancement of science, as the data's value comes most to its own if (unclear) genetic variants can be coupled to disease course, lifestyle, and social information, for which regularly updated personal information may be necessary [10,73]. Additionally, anonymization makes it difficult to re-contact participants to return relevant genetic results [29]. Currently, there is still disagreement on to what extent privacy risks actually form an imminent threat in genomics research [16,84]. Privacy measurements should be proportionate to the associated risks and some have argued that privacy risks are overstated as the examples of actual tangible harm are very limited [84]. To conclude, no consensus exists on whether and to what degree data should be anonymized. A balance needs to be found between providing adequate protection of participants' privacy and responsible use and storage of sensitive information for the advancement of science [81,82,85].

SOCIAL DUTIES

Social duties deal with the relationship between researchers on the one hand and the genomics research enterprise at large as well as society on the other hand. Social duties, like the (partially overlapping) duty to share research data and/or samples, the duty to take social responsibility and the duty to engage the community, are among the most recently emerged duties.

The duty to share research data and/or samples

The duty to share data and/or samples has been put forward as it would be beneficial to the advancement of genomic science (see: The duty to protect participants' privacy) and thereby also to the primary duty of researchers to create generalizable knowledge [10]. Sharing will enable international data use and thereby optimal utilization from public resources [7,45]. Apart from the benefit to science, data sharing is also beneficial to the public good as it may benefit future patients and it may pick up low-frequency genomic

events in specific populations [10,86]. Data sharing is increasingly being promoted and facilitated in research practice [71,87]. Open-access publications are being encouraged and several grant providers, such as the National Institutes of Health (NIH), oblige supported studies to post their data in open repositories [71]. Consent forms vary in their data sharing and privacy policies, although a trend towards data sharing can be seen [10]. However, data sharing does not seem to be the default yet, due to factors such as a competitive research environment [85,87]. Moreover, the benefits of sharing data and/or samples should be balanced with the potential privacy risks related to it.

The duty to take social responsibility

The duty to take social responsibility is put forward as a warning that injustice may occur, if genomics research studies are not performed in a wide range of populations as the research results may not be applicable to underrepresented populations [16,19,71,86]. According to some, it must be ensured that health benefits of genomics research are equally accessible to all people and researchers should be more aware about this matter. While there is a general consensus that distributive justice is a relevant consideration in genomics, the extent of obligations to promote distributive justice, such as in the form of a duty to take social responsibility remains controversial.

The duty to engage the community

The duty to engage the community is put forward by some, stating that it is important to engage participants in science, to increase their knowledge about genomics (genetic literacy) and the research enterprise [19,21,88]. This could have positive consequences for researcher-participant relationships by increasing trust and mutual understanding. This duty is however not yet widely discussed and supported.

EVOLVING TRENDS IN GENOMICS RESEARCHERS' MORAL DUTIES

Based on the literature, we provided a comprehensive assembly of moral duties that have been attributed to genomics researchers. It concerns a wide range of moral duties, showing that they should not only have technical-scientific skills but also adhere to professional moral duties. The duties can be clustered into Disclosure duties, Consent duties, Privacy duties, and Social duties, reflecting the current and most urgent ethical challenges and responsibilities in genomics research. In the course of time more duties have been attributed to genomics researchers. Duties have become more specific, detailed, and go beyond the relationship between the primary researcher and the

individual research participant [26]. For example, Disclosure duties have come up that apply or relate to secondary researchers, the research enterprise in general, participants' relatives, and society at large [12,16,19,21,29,34,36,41,49,50,54,71,73,86,88]. On the contrary, ideas concerning consent tend to become more generalized with new concepts, such as broad consent [10,17,20,26,63,67,72,73].

The principles and duties derived from literature are not unique for genomics research, as comparable duties apply to single-gene and imaging research as well [7]. However, they seem particularly pressing in the genomics context due to the unleveled amounts of data being generated, the probabilistic character of genetic information, the frequent uncertain significance of the results, the potential implications for relatives and the difficulty to guarantee privacy [7,9]. There is much at stake for research participants as well as researchers, considering for example participants' privacy and disclosure burdens for researchers. However, the contradictions between participants' and researchers' interests seem enlarged in literature. Most arguments in favor of moral duties are reasoned from the participants' perspective, based upon principles as beneficence, autonomy, and privacy. On the contrary, arguments against moral duties are mostly written from the researchers' perspective, stating that they may create an immense burden on the research enterprise and go beyond the primary purpose of research to generate generalizable knowledge.

Traditional research ethics principles focus on protecting individual participants and are reflected in consent and privacy protection duties. Gradually, these principles have become supplemented with social obligations such as data and sample sharing and community engagement duties, which are focused on the interests of society and the research enterprise at large [16,19,21,86,88]. These novel duties go partly together with the traditional duties, but they can also be in tension, for example the duty to share data and the duty to protect privacy. Also, there are potential conflicts between the duty to protect privacy and the duty to return results for which the participants should be (easily) identifiable. These conflicts all relate to the key ethical issue of how to balance the researchers' dual responsibilities to produce generalizable knowledge that is eventually also beneficial to society on the one hand, and to respect autonomy, and guarantee protection of research participants on the other hand [30,66,86]. Research participants and researchers as well as other stakeholders (e.g. participants' relatives, grant suppliers, and the public at large) have many conflicting interests, but they do also share duties and more than ever rely on each other to make sense of the enormous amounts of data generated, that may be beneficial for research participants, patients and society at large now and in the future.

THE TRANSLATIONAL GAP BETWEEN ETHICAL SCHOLARSHIP AND GENOMICS RESEARCH PRACTICE

Despite a vast amount of literature discussing and proposing moral duties for genomics researchers, their implementation in research practice is lagging behind. When looking at empirical studies investigating the opinions of researchers, they show a willingness to return certain research results and provide ancillary care [56,60]. In reality, however, practice has not matched the good will so far [18,89]. The apparent blockade between ethical debate and implementation in genomics research practice can partly be explained by the context- and time-dependence of many moral duties, no fixed hierarchy of duties, the need to weigh partially conflicting moral duties against each other, and variable interpretation of principles and criteria. How then could concrete decisions in particular circumstances be derived from these duties?

CONCLUDING REMARKS

The ultimate aim in bioethics is to reach moral justification, i.e. to establish one's case by providing sufficient and coherent reasons for it (Box 2) [90]. This requires, according to current ethical scholarship, an argumentative process of seeking equilibrium between a wide range of input, including generally accepted ethical principles and background theories as well as empirical facts, local contextual factors, and moral intuitions [91,92]. Several (conflicting) moral duties require balancing and specification in particular local contexts, and need continuous refinement [93].

We believe that ethical awareness of researchers is key to pick up the ethical debate and bridge the translational gap. Researchers are usually the first who are confronted with these moral issues and the identification and balancing of several, potentially conflicting moral duties is becoming an important aspect of a genomics researcher's profession. These skills are different from the traditional technical-scientific skills and need to be developed during education in all phases of (genomics) researchers' careers, starting during their university training.

Moreover, we believe that a social approach could complement the traditional research ethics principles to give better guidance for the moral issues at stake. Although traditional research ethics principles should not be discarded, genomics research should take place in full view of the public, having openness, veracity, and sharing of data and knowledge as important principles [9]. Other key principles should be solidarity and reciprocity,

referring to the sense of mutual dependence and the responsibility toward the health of others [66,79,94]. More awareness is needed on the idea that all who participate in or may benefit from genomics research have a shared responsibility to contribute to its ethically and socially responsible advancement [9,66,79,85,94–96]. Here we have focused on researchers' duties, but research participants for example also carry a certain responsibility to contribute to research from a solidarity principle, as they may benefit from healthcare developments made possible by human subject research in the past. Genomic science cannot progress without the participation of a sufficient number of research participants [6,11,88,94]. To be able to acquire sufficient numbers of research participants from whom more is asked, the research community should meet certain public needs (e.g. disclosure of research results, sharing data) and maintain public trust [6]. For this, researchers should become more active in the social debate. In short, the social approach asks more from researchers as well as research participants and the public in general [95].

To conclude, genomics research is besides a scientific also a social endeavor, requiring as well as thriving on many collaborations within and across societies. In other words, for the advancement of *personalized* medicine, a *collective* approach is required.

BOX 1. METHODS

A literature search was performed in PubMed and Embase databases, using a search string that combined synonyms for moral duties with synonyms for high-throughput sequencing methods (Table I). The search yielded 7.564 articles. Title and abstract of these papers were screened on inclusion and exclusion criteria, followed by full text screening using selection criteria (Fig I). Guidelines from several Genetics, Oncology, and Biomedical Ethics associations were also screened using the same criteria. In total, 149 articles were identified. We here refer to papers published from 2012 onwards, but we also included older key papers since the emergence of next-generation sequencing (85 articles in total). From these articles, moral duties, including arguments given in favor or against these duties, were collected and described (see the enumeration of moral duties). Identified moral duties were organized into clusters The discussion and conclusion that follow are based upon our analysis and interpretation of the literature, in which we aimed to point out general developments in attribution of moral duties to genomics researchers and to analyze why the implementation of these duties in research practice is difficult and what could be done to improve this. Our literature study is a modified version of the Systematic Review of Reasons approach, which is a model for systematic reviews that aims to systematically identify the reasons and arguments that have been given in literature in favor and against a normative position, claim or phenomenon [97,98].

Table I. Search syntaxes

Database	Search
PubMed	((((((((((ethic*[Title/Abstract]) OR moral*[Title/Abstract]) OR duty[Title/Abstract]) OR duties[Title/Abstract]) OR obligation[Title/Abstract]) OR obligations[Title/Abstract]) OR responsibility[Title/Abstract]) OR responsibilities[Title/Abstract]) OR biomedical ethics[MeSH Terms]) OR moral obligations[MeSH Terms])) AND ((((((((((genomic[Title/Abstract]) OR genomics[Title/Abstract]) OR genome[Title/Abstract]) OR genetic[Title/Abstract]) OR genetics[Title/Abstract]) OR DNA[Title/Abstract]) OR exome[Title/Abstract]) OR sequencing[Title/Abstract]) OR genomics[MeSH Terms]) OR high throughput nucleotide sequencing[MeSH Terms])
EMBASE	See PubMed

Search performed on 24 January 2016

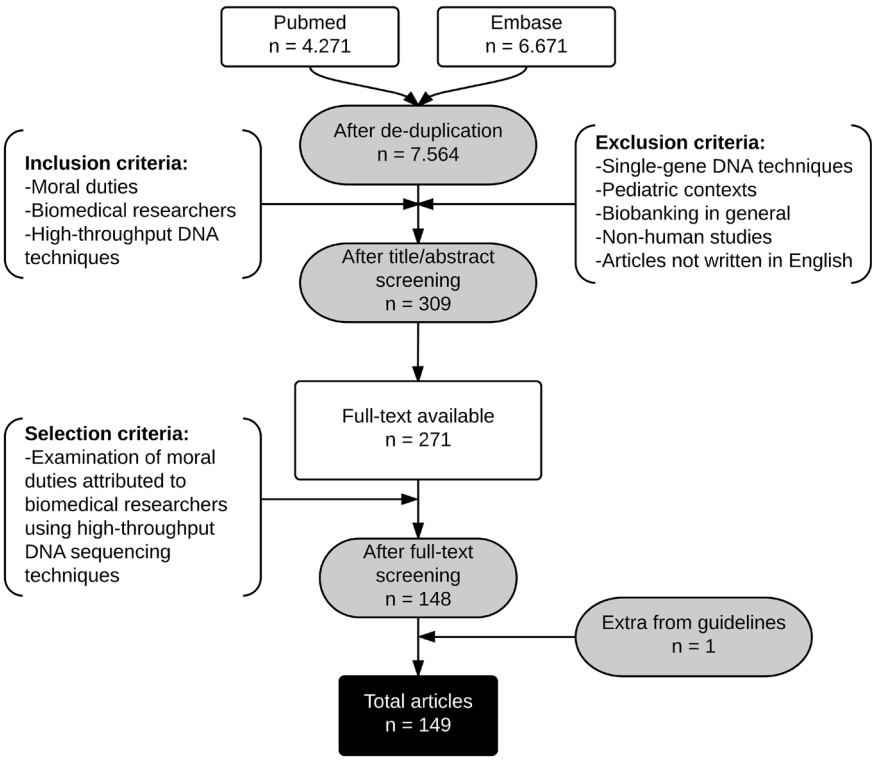

Figure I. Flow diagram of the selection and inclusion of articles

BOX 2. TRANSLATIONAL ETHICS: FROM ETHICAL THEORY TO IMPLEMENTATION OF MORAL DUTIES IN MEDICAL RESEARCH PRACTICE

How should moral duties be interpreted? What to do with conflicting moral duties, is there a hierarchy? How could concrete decisions in particular circumstances be derived from these duties? In this Box we provide a short illustration of how to make the transition from ethical theory to implementation of moral duties in medical research practice.

The ultimate aim in bioethics is to reach moral justification, i.e. to establish one's case by providing sufficient and coherent reasons for it, and is therefore related to ethical methodology [90]. Overall, it is difficult to obtain a well-developed and clear framework

or specified ethical guideline for morally right actions as the particulars of a specific case may give general concepts or considerations a special relevance [93]. Instead, one has to deal with a rather loose framework of normative deliberation [90].

Moral problems, for instance conflicting moral duties, need normative interpretation and clarification. Three ways are identified to bridge moral duties to concrete decisions or actions [93]:

1. Application: the right course of action is deduced from general rules.
2. Balancing: conflicting duties are weighed in order to determine which has priority in the specific situation.
3. Specification: the process of qualitatively tailoring our norms to cases by spelling out where, when, why, how, by what means, to whom, or by whom the action is to be done or avoided.

Of these, balancing and specification especially require extensive fine-tuning and argument [93]. For this, the method of reflective equilibrium is considered to be very potent [91,92]. To come to a coherent moral judgment or action, one has to bring a wide variety of input, such as generally accepted ethical principles (e.g. autonomy, beneficence, justice), background theories (e.g. utilitarianism, virtue ethics) as well as empirical facts, local contextual factors, and moral intuitions into a state of equilibrium or harmony [91,92]. The process can be seen as a going back and forth between beliefs originating from practice and from theory [93]. The basic criteria the outcome should meet are consistency (non-contradiction), coherence with warranted non-moral beliefs (empirical evidence), absence of bias, argumentative support, and restriction of starting premises [90]. To conclude, a moral judgment or action should be internally coherent with relevant, justified beliefs of the parties involved, requiring extensive argument and continuous refinement according to changing circumstances.

REFERENCES

1 van't Veer, L.J. and Bernards, R. (2008) Enabling personalized cancer medicine through analysis of gene-expression patterns. Nature 452, 564–70

2 Mardis, E.R. and Wilson, R.K. (2009) Cancer genome sequencing: a review. Hum. Mol. Genet. 18, R163-8

3 Garraway, L.A. (2013) Genomics-driven oncology: framework for an emerging paradigm. J. Clin. Oncol. 31, 1806–1814

4 McGuire, A.L. et al. (2008) Research ethics and the challenge of whole-genome sequencing. Nat. Rev. Genet. 9, 152–156

5 Rotimi, C.N. and Marshall, P.A. (2010) Tailoring the process of informed consent in genetic and genomic research. Genome Med. 2, 20

6 Bredenoord, A.L. et al. (2011) Disclosure of individual genetic data to research participants: the debate reconsidered. Trends Genet. 27, 41–7

7 Caulfield, T. et al. (2008) Research ethics recommendations for whole-genome research: Consensus statement. PLoS Biol. 6, 430–435

8 Beskow, L.M. et al. (2012) Offering aggregate results to participants in genomic research: Opportunities and challenges. Genet. Med. 14, 490–496

9 Conley, J.M. et al. (2010) Enabling responsible public genomics. Health Matrix Clevel. 20, 325–385

10 Allen, C. and Foulkes, W.D. (2011) Qualitative thematic analysis of consent forms used in cancer genome sequencing. BMC Med. Ethics 12, 14

11 Bredenoord, A.L. et al. (2011) Feedback of individual genetic results to research participants: in favor of a qualified disclosure policy. Hum. Mutat. 32, 861–867

12 Levesque, E. et al. (2011) Return of Research Results: General Principles and International Perspectives. J. Law Med. Ethics 39, 583–592

13 Wolf, S.M. (2013) Return of individual research results and incidental findings: Facing the challenges of translational science. Annu. Rev. Genomics Hum. Genet. 14, 557–577

14 Christenhusz, G.M. et al. (2013) Secondary variants- in defense of a more fitting term in the incidental findings debate. Eur. J. Hum. Genet. 21, 1331–1334

15 Burke, W. et al. (2014) Return of results: ethical and legal distinctions between research and clinical care. Am. J. Med. Genet. C. Semin. Med. Genet. 166C, 105–111

16 Jessri, M. and Farah, C.S. (2014) Harnessing massively parallel sequencing in personalized head and neck oncology. J. Dent. Res. 93, 437–444

17 Pinxten, W. and Howard, H.C. (2014) Ethical issues raised by whole genome sequencing. Best Pract. Res. Clin. Gastroenterol. 28, 269–279

18 McGuire, A.L. et al. (2013) Returning genetic research results: study type matters. Per. Med. 10, 27–34

19 Johnson, K.J. and Gehlert, S. (2014) Return of Results from Genomic Sequencing: A Policy Discussion of Secondary Findings for Cancer Predisposition. J. Cancer Policy 2, 75–80

20 Hallowell, N. et al. (2015) Revealing the results of whole-genome sequencing and whole-exome sequencing in research and clinical investigations: some ethical issues. J. Med. Ethics 41, 317–321

21 Weiner, C. (2014) Anticipate and communicate: Ethical management of incidental and secondary findings in the clinical, research, and direct-to-consumer contexts (December 2013 report of the Presidential Commission for the Study of Bioethical Issues). Am. J. Epidemiol. 180, 562–564

22 Johns, A.L. et al. (2014) Returning individual research results for genome sequences of pancreatic cancer. Genome Med. 6, 42

23 Pike, E.R. et al. (2014) Finding Fault? Exploring Legal Duties to Return Incidental Findings in Genomic Research. Georgetown Law J. 102, 795–843

24 Viberg, J. et al. (2014) Incidental findings: the time is not yet ripe for a policy for biobanks. Eur. J. Hum. Genet. 22, 437–441

25 Kleiderman, E. et al. (2015) Disclosure of incidental findings in cancer genomic research: Investigators' perceptions on obligations and barriers. Clin. Genet. 88, 320–326

26 Kaye, J. et al. (2010) Ethical implications of the use of whole genome methods in medical research. Eur. J. Hum. Genet. 18, 398–403

27 Bollinger, J.M. et al. (2012) Public preferences regarding the return of individual genetic research results: Findings from a qualitative focus group study. Genet. Med. 14, 451–457

28 Bookman, E.B. et al. (2013) Incidental genetic findings in randomized clinical trials: recommendations from the Genomics and Randomized Trials Network (GARNET). Genome Med. 5, 7

29 Black, L. et al. (2013) Funding considerations for the disclosure of genetic incidental findings in biobank research. Clin. Genet. 84, 397–406

30 Berkman, B.E. et al. (2014) The unintended implications of blurring the line between research and clinical care in a genomic age. Per. Med. 11, 285–295

31 Eckstein, L. et al. (2014) A framework for analyzing the ethics of disclosing genetic research findings. J. Law. Med. Ethics 42, 190–207

32 Prince, A.E.R. et al. (2015) Automatic Placement of Genomic Research Results in Medical Records: Do Researchers Have a Duty? Should Participants Have a Choice? J. Law. Med. Ethics 43, 827–842

33 Affleck, P. (2009) Is it ethical to deny genetic research participants individualised results? J. Med. Ethics 35, 209–213

34 Ayuso, C. et al. (2015) Management and return of incidental genomic findings in clinical trials. Pharmacogenomics J. 15, 1–5

35 Wolf, S.M. et al. (2015) Returning a Research Participant's Genomic Results to Relatives: Analysis and Recommendations. J. Law. Med. Ethics 43, 440–463

36 Callier, S. et al. (2014) Genomic data-sharing: what will be our legacy? Front. Genet. 5, 34

37 Fernandez, C.V. et al. (2015) Canadian Research Ethics Board Leadership Attitudes to the Return of Genetic Research Results to Individuals and Their Families. J. Law. Med. Ethics 43, 514–522

38 Boers, S.N. et al. (2015) Postmortem disclosure of genetic information to family members: active or passive? Trends Mol. Med. 21, 148–153

39 Wouters, R.H.P. et al. (2016) Am I my Family's Keeper? Disclosure Dilemmas in Next Generation Sequencing. Hum. Mutat. DOI: 10.1002/humu.23118

40 Kollek, R. and Petersen, I. (2011) Disclosure of individual research results in clinico-genomic trials: Challenges, classification and criteria for decision-making. J. Med. Ethics 37, 271–275

41 Hallowell, N. et al. (2013) The responses of research participants and their next of kin to receiving feedback of genetic test results following participation in the Australian Ovarian Cancer Study. Genet. Med. 15, 458–465

42 Ulrich, M. and M., U. (2013) The Duty to Rescue in Genomic Research. Am. J. Bioeth. 13, 50–51

43 McGuire, A.L. et al. (2014) Can I be sued for that? Liability risk and the disclosure of clinically significant genetic research findings. Genome Res. 24, 719–723

44 Garrett, J.R. (2015) Collectivizing rescue obligations in bioethics. Am. J. Bioeth. 15, 3–11

45 Tabor, H.K. et al. (2011) Genomics really gets personal: How exome and whole genome sequencing challenge the ethical framework of human genetics research. Am. J. Med. Genet. Part A 155, 2916–2924

46 Gliwa, C. et al. (2013) Do Researchers Have an Obligation to Actively Look for Genetic Incidental Findings? Am. J. Bioeth. 13, 32–42

47 Ross, K.M. and Reiff, M. (2013) A Perspective From Clinical Providers and Patients: Researchers' Duty to Actively Look for Genetic Incidental Findings. Am. J. Bioeth. 13, 56–58

48 Garrett, J.R. and J.R., G. (2013) Reframing the Ethical Debate Regarding Incidental Findings in Genetic Research. Am. J. Bioeth. 13, 44–46

49 Clayton, E.W. and McGuire, A.L. (2012) The legal risks of returning results of genomics research. Genet. Med. 14, 473–477

50 Haga, S.B. and Zhao, J.Q. (2013) Stakeholder views on returning research results. Adv. Genet. 84, 41–81

51 Anastasova, V. et al. (2013) Genomic incidental findings: reducing the burden to be fair. Am. J. Bioeth. 13, 52–54

52 Schuol, S. et al. (2015) So rare we need to hunt for them: reframing the ethical debate on incidental findings. Genome Med. 7, 83

53 Petrini, C. and Alleva, E. (2014) Incidental findings, genetic screening and the challenge of personalisation. Ann. Ist. Super. Sanita 50, 312–316

54 Jarvik, G.P. et al. (2014) Return of genomic results to research participants: the floor, the ceiling, and the choices in between. Am. J. Hum. Genet. 94, 818–826

55 Wade, C.H. et al. (2006) When do genetic researchers have a duty to recontact study participants? Am. J. Bioeth. 6, 26–27

56 Miller, F.A. et al. (2012) One thing leads to another: the cascade of obligations when researchers report genetic research results to study participants. Eur. J. Hum. Genet. 20, 837–843

57 Zeps, N. and Bledsoe, M.J. (2015) Managing the Ethical Issues of Genomic Research using Pathology Specimens. Clin. Biochem. Rev. 36, 21–27

58 Richardson, H.S. and Cho, M.K. (2012) Secondary researchers' duties to return incidental findings and individual research results: a partial-entrustment account. Genet. Med. 14, 467–472

59 Wolf, S.M. et al. (2012) Managing incidental findings and research results in genomic research involving biobanks and archived data sets. Genet. Med. 14, 361–384

60 Hayeems, R.Z. et al. (2013) Does a duty of disclosure foster special treatment of genetic research participants? J. Genet. Couns. 22, 654–661

61 Dienstmann, R. et al. (2013) Genomic medicine frontier in human solid tumors: prospects and challenges. J. Clin. Oncol. 31, 1874–1884

62 Mathaiyan, J. et al. (2013) Ethics of genomic research. Perspect. Clin. Res. 4, 100–104

63 McEwen, J.E. et al. (2013) Evolving approaches to the ethical management of genomic data. Trends Genet. 29, 375–382

64 Prucka, S.K. et al. (2015) An update to returning genetic research results to individuals: perspectives of the industry pharmacogenomics working group. Bioethics 29, 82–90

65 Garrison, N. (2015) Considerations for Returning Research Results to Culturally Diverse Participants and Families of Decedents. J. Law. Med. Ethics 43, 569–575

66 Hoedemaekers, R. et al. (2007) Solidarity and justice as guiding principles in genomic research. Bioethics 21, 342–350

67 Henderson, G.E. (2011) Is informed consent broken? Am. J. Med. Sci. 342, 267–272

68 Robinson, J.O. et al. (2013) Participants' recall and understanding of genomic research and large-scale data sharing. J. Empir. Res. Hum. Res. Ethics 8, 42–52

69 Artizzu, F. and F., A. (2008) The informed consent aftermath of the genetic revolution. An Italian example of implementation. Med. Heal. Care Philos. 11, 181–190

70 Fiore, R.N. and Goodman, K.W. (2016) Precision medicine ethics: Selected issues and developments in next-generation sequencing, clinical oncology, and ethics. Curr. Opin. Oncol. 28, 83–87

71 Peppercorn, J. et al. (2012) Ethical aspects of participation in the Database of Genotypes and Phenotypes of the National Center for Biotechnology Information: The Cancer and Leukemia Group B Experience. Cancer 118, 5060–5068

72 Chalmers, D.R.C. et al. (2014) To share or not to share is the question. Appl. Transl. Genomics 3, 116–119

73 Davey, S. (2014) Next generation sequencing: considering the ethics. Int. J. Immunogenet. 41, 457–462

74 Kaye, J. (2012) The tension between data sharing and the protection of privacy in genomics research. Annu. Rev. Genomics Hum. Genet. 13, 415–431

75 Devarakonda, S. et al. (2012) Cancer gene sequencing: ethical challenges and promises. Virtual Mentor 14, 868–872

76 Tabor, H.K. et al. (2012) Informed consent for whole genome sequencing: A qualitative analysis of participant expectations and perceptions of risks, benefits, and harms. Am. J. Med. Genet. Part A 158 A, 1310–1319

77 Edwards, K.L. et al. (2012) Genetics researchers and IRB professionals attitudes toward genetic research review: A comparative analysis. Genet. Med. 14, 236–242

78 Oliver, J.M. et al. (2012) Balancing the risks and benefits of genomic data sharing: Genome research participants' perspectives. Public Health Genomics 15, 106–114

79 Knoppers, B.M. et al. (2013) The Human Genome Organisation: Towards next-generation ethics. Genome Med. 5, 38

80 McShane, L.M. et al. (2013) Criteria for the use of omics-based predictors in clinical trials. Nature 502, 317–320

81 Rodriguez, L.L. et al. (2013) Research ethics. The complexities of genomic identifiability. Science 339, 275–276

82 Dove, E.S. et al. (2015) Genomic cloud computing: legal and ethical points to consider. Eur. J. Hum. Genet. 23, 1271–1278

83 Anwar, N. (2013) The double helix, a double edged sword: Ethical issues in genetic testing and research. J. Postgrad. Med. Inst. 27, 117–121

84 Shabani, M. and Borry, P. (2016) 'You want the right amount of oversight': interviews with data access committee members and experts on genomic data access. Genet. Med. DOI: 10.1038/gim.2015.189

85 Knoppers, B.M. et al. (2014) A human rights approach to an international code of conduct for genomic and clinical data sharing. Hum. Genet. 133, 895–903

86 Francis, L.P. (2014) Genomic knowledge sharing: A review of the ethical and legal issues. Appl. Transl. Genomics 3, 111–115

87 van Schaik, T.A. et al. (2014) The need to redefine genomic data sharing: A focus on data accessibility. Appl. Transl. Genomics 3, 100–104

88 Angrist, M. (2011) You never call, you never write: Why return of 'omic' results to research participants is both a good idea and a moral imperative. Per. Med. 8, 651–657

89 Heaney, C. et al. (2010) Researcher practices on returning genetic research results. Genet. Test. Mol. Biomarkers 14, 821–827

90 Beauchamp, T.L. and Childress, J.F. (2013) Principles of Biomedical Ethics, (7th edn) Oxford University Press.

91 Rawls, J. (1971) A Theory of Justice. Revised edition., Belknap Press of Harvard University Press.

92 Daniels, N. (1996) Justice and Justification: Reflective Equilibrium in Theory and Practice, Cambridge University Press.

93 Ghaly, M. et al. (2016) Islamic Perspectives on the Principles of Biomedical Ethics, World Scientific Publishing.

94 Meslin, E.M. et al. (2010) Research ethics in the era of personalized medicine: Updating science's contract with society. Public Health Genomics 13, 378–384

95 Virani, A.H. and Longstaff, H. (2015) Ethical considerations in biobanks: how a public health ethics perspective sheds new light on old controversies. J. Genet. Couns. 24, 428–432

96 Knoppers, B.M. and Chadwick, R. (2015) The ethics weathervane. BMC Med. Ethics 16, 58

97 Strech, D. and Sofaer, N. (2012) How to write a systematic review of reasons. J. Med. Ethics 38, 121–126

98 Sofaer, N. and Strech, D. (2012) The need for systematic reviews of reasons. Bioethics 26, 315–28

Chapter 8

Ethical considerations for modern molecular pathology

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ABSTRACT

Molecular pathology is becoming an increasingly important discipline in oncology, as molecular tumor characteristics will increasingly determine targeted clinical cancer care. In recent years, many technological advances have taken place that contributed to the development of molecular pathology. However, attention to ethical aspects has been lagging behind, as illustrated by the lack of publications or professional guidelines. Existing guidelines or publications on ethical aspects of DNA sequencing are mostly aimed at germline or tumor sequencing in clinical genetics or biomedical research settings. As a result, large differences have been shown in the process of tumor sequencing analysis between laboratories.

In this paper, we discuss the ethical issues to consider in molecular pathology by following the process of tumor DNA sequencing analysis from the pre-analytical to post-analytical phase. For a successful and responsible use of DNA sequencing techniques in clinical cancer care, several moral requirements must be met, for example those related to interpretation and returning genetic results, informed consent, and the retrospective as well as future use of genetic data for biomedical research. Many ethical issues are new to pathology or more stringent than in current practice as DNA sequencing could yield sensitive and potentially relevant data, such as clinically significant unsolicited findings. The context of molecular pathology is unique and complex, but many issues are similar to those applicable to clinical genetics. As such, existing scholarship on this discipline may be translated to molecular pathology with some adaptations and could serve as a basis for guideline development. For responsible use and further development of clinical cancer care, we recommend pathologists to take responsibility for the adequate use of molecular analyses and be fully aware and capable of dealing with the diverse, complex and challenging aspects of tumor DNA sequencing, including its ethical issues.

INTRODUCTION

Emerging ethical needs for molecular pathology

With the emergence of precision medicine, genetic analysis to molecularly characterize tumors has become a fundamental component of the diagnostic work-up of cancer patients. The development of precision medicine has been fueled by advances in DNA sequencing technologies, especially the possibility of performing massively parallel or next-generation sequencing (NGS) on formalin-fixed paraffin-embedded tissues for routine diagnostics [1–3]. Tumor (somatic) DNA sequencing is often performed at 'molecular pathology' (MP) units of pathology departments. MP has contributed to the changing role of pathologists from purely diagnostics to being actively involved in common treatment decisions and determining suitability for clinical trials based on identifying molecular targets.

However, there are several important concerns when technological possibilities outpace interpretive skills and reflection on ethical aspects [4]. With ethical aspects, we refer to those aspects that carry a normative dimension, harboring some of form of value judgment about what action or behaviour is right or wrong. Many of these issues concern how to adequately deal with the large amounts of potentially sensitive genetic data. MP publications generally only deal with technical issues, without making the normative aspects explicit [5–8]. Guidelines or publications focused on the responsible application of large-scale genetic sequencing are mostly aimed at germline sequencing in clinical genetics (CG) or primary research settings [9–21]. Also, most existing scholarship that (briefly) mention tumor DNA sequencing focus on whole exome or genome sequencing (WES, WGS), and not on targeted (cancer) gene panels generally used in MP practice [7,22,23]. As a result, large differences have been shown in the process of tumor sequencing analysis between laboratories [4,24–26].

The unique context of molecular pathology

The MP field is unique and different from CG and primary biomedical research settings. As such, ethical challenges and guidelines cannot necessarily be extrapolated from these related fields. First, the context is different. MP analysis forms an integral part of routine tumor diagnostics. As such, not all tissue can be used for genetic analysis, but should for instance be responsibly divided for morphological, immunohistochemical and molecular analyses. Second, the pathology patient population is different. In most cases, patients are either just diagnosed with a malignancy or are still undergoing diagnostics for their health complaints. Generally, they are very focused on getting a proper diagnosis and treatment, and not so much concerned or familiar with the (e.g.

hereditary) side effects of their molecular tumor workup [27–31]. Third, the analysis and input material are different. In contrast to CG, DNA analysis is performed on a wide range of (tumor) tissues in search of somatic alterations instead of using blood to look for germline mutations. The type of input material poses challenges. Reliable molecular analysis relies on an adequate amount of nucleic acids of adequate quality, but there is a trend toward increasingly smaller biopsy or cytology specimens obtained by minimally invasive techniques. Moreover, pathology specimens generally undergo formalin fixation, which causes DNA fragmentation. Although these aspects are no direct ethical issues in themselves, they may affect the reliability of MP analysis results. To determine what degree of uncertainty is still acceptable, both technical and ethical judgements are required. Hence, the technical and ethical challenges are often interwoven.

For a successful and responsible use of DNA sequencing in clinical cancer care, several moral requirements must be met [32]. In this paper, we discuss the ethical issues to consider in MP by following the process of tumor DNA sequencing from the pre-analytical to post-analytical phase. The exact practice of MP may differ between countries and clinical practice settings, but the ethical issues are to a large extent global. We make use of scholarship and ethical discussion in related fields and we analyse how they can be translated to MP.

1. PRE-ANALYTICAL POINTS TO CONSIDER: ORDERING TUMOR DNA SEQUENCING ANALYSIS

1.1. Informed consent

There has been a long-standing debate and emerging consensus in the clinical genetics and research context that obtaining informed consent is an ethical prerequisite before initiating DNA sequencing [9,19–21,33–35]. However, there is currently no guideline or consensus for informed consent in MP. It has been given little attention in the literature so far. This may be quite understandable, since genetic analysis is a logical part of tumor workup to arrive at an optimal treatment for the patient (i.e. initiated from the principle of beneficence). It may also be due to certain difficulties to implement informed consent in clinical practice. First, MP analysis is unpredictable beforehand since it heavily depends on the initial morphological diagnosis, meaning that genetic analysis may or may not be necessary, and the exact type of genetic analysis may vary from tumor to tumor. Second, pathologists usually do not have direct contact with patients and, as such, do not perform informed consent procedures themselves. Third, obtaining informed consent in the sequencing context is challenging due to its complexity,

which may lead to patients who insufficiently understand the information given and experience information overload [36]. Fourth, there is much time pressure in pathology and interrupting the usual analysis logistics to ask for informed consent for DNA analysis would inevitably lead to delays. As a consequence, consent in current practice is considered to be deficient. It can be assumed that patients are generally insufficiently informed about the ins and outs of tumor DNA sequencing and have not been given the opportunity to decide whether they would like to be informed about issues such as unsolicited findings (i.e. findings not related to the primary clinical question) (see below) [36].

However, from a moral point of view, these practical issues should not be regarded as an excuse to not at least attempt to sufficiently inform them about the potential benefits (e.g. confirming disease; determining therapeutic options), limitations (e.g. possibilities of too low tumor cell percentage or no representative tumor sample due to tumor heterogeneity), and potential side effects (e.g. discovery of variants of uncertain significance (VUS) or unsolicited (germline) findings requiring further medical investigation and causing potential psychosocial concerns for patients and their family members) [9]. The current consensus states that patients should be able to give explicit consent about whether they would like to be informed about clinically significant unsolicited findings [9,14,19–21,37]. If the patient's preference is not known and analysis yields a clinically significant unsolicited finding, it could cause moral dilemmas for pathology: should the laboratory report the finding, or not? Moreover, it would be desirable and sometimes also legally required that patients are informed about potential (anonymous) data sharing or uploading of genetic variants in online databases/repositories to improve clinical cancer sequencing and potential future use of genetic data for research purposes for which patients can give separate consent (see below).

The optimal form and timing of the informed consent procedure is still to be determined. The extensiveness of the informed consent procedure should be dependent on the extensiveness of MP analysis (i.e. number of genes involved) (Figure 1). The risk of unsolicited findings increases with increasing numbers of genes analyzed. It is not easy to define the cutoff point in molecular testing for where the risk of unexpected findings warrants informed consent. In literature, it has been shown that at least 1% of patients had unsolicited findings in large-scale NGS sequencing, rising to 16-19% when using gene panels containing 300-450 cancer-related genes [38–41]. Since many of these unsolicited findings consisted of variants of uncertain significance, a balance must be found between full informed consent with the risk of information overload on

the one hand, and too limited consent with the risk of becoming uninformed consent [37,39]. In our opinion, a separate informed consent for tumor DNA analysis is not necessary when only a limited set of genes are analyzed in which no relevant germline mutations are known to exist. In case of non-targeted sequencing of many genes and/or sequencing of genes in which relevant germline mutations exist, additional informed consent as well as a clear disclosure policy may be necessary because of a reasonable chance of unsolicited findings.

Alternative informed consent models have been proposed that may be more suitable for (large-scale) sequencing, including generic consent and a tiered disclosure system in which patients are given a set of options, for example different types of unsolicited findings they would like to be informed about [14,37,42–46]. It is generally understood that the ordering clinician (e.g. medical oncologist or pulmonologist), and not the laboratory, is responsible for obtaining informed consent [9,22]. However, although the patient is literally farther away, pathologists (or molecular biologists working in pathology) should play an active role in designing an adequate informed consent procedure for the analyses they perform. The question is whether such an informed consent procedure could still be solely executed by well-trained clinicians or that pathologists should more directly take part in such communication with patients. In any case, it is important to find a balance between providing adequate information, avoiding unnecessary fear and burden for patients (e.g. by information overload), and not taking up too much time and resources of clinicians and pathologists [46].

1.2. Who can order molecular pathology testing?

Physicians from several disciplines may be qualified to order MP analysis directly or usually indirectly via a pathologist for a tumor on which initial diagnostics has been completed. The benefit from the latter approach is that it provides an extra check, for example to check whether it concerns the right tumor type, whether the sample likely contains enough tumor cells, and whether the correct test will be performed. This prevents unnecessary testing and associated costs. A drawback may be that this takes extra time. Alternatively, pathologists can directly and independently order MP analysis, as part of a protocolled workup of certain tumor types. Time-saving approaches, such as automatic (reflex) testing, have been proposed based on diagnosis and tissue availability, for example for assessing therapeutic sensitivity for lung adenocarcinomas [7]. However, without prior consultation of the clinician, patients may not be adequately informed and may not have been able to give prior informed consent. As said before, this is especially important when performing large-scale non-targeted DNA analysis techniques. As such, the span and scope of what a pathologist is allowed to do is also an

important matter of debate. In conclusion, good communication between the pathology laboratory and clinicians is essential so that MP analyses are ordered and performed effectively and appropriately.

2. ANALYTICAL CONSIDERATIONS: PERFORMING TUMOR DNA SEQUENCING ANALYSIS

2.1. Selecting tissue for molecular pathology analysis

Pathologists have the obligation to make responsible use of tissues for morphological, immunohistochemical, and molecular analyses. The trend towards increasingly smaller patient samples makes the obligation to use tissues responsibly even more urgent. Molecular analysis relies on adequate amounts of tumor DNA, so tissue usage for morphology and immunohistochemistry should be as minimal as possible. The issue of tissue representativity is not only a technical issue (e.g. insufficient tumor material), but also an important moral issue: to what extent do the cells taken for tumor DNA analysis represent the genetic composition of the whole tumor (i.e. tumor heterogeneity), and when would this be adequate enough? When there is no uniform distribution, the tumor cells taken for analysis do not represent the composition of the whole tumor, potentially leading to false positive or false negative results and eventually, treatment failure and shorter survival [47]. In other words, tumor heterogeneity may reduce power to detect clinically relevant mutations [48]. Using smaller biopsies increases the risk for tumor heterogeneity underestimation [49,50]. Also, low tumor cell percentages make it difficult to detect clinically significant mutations due to normal tissue contamination [24]. This means decisions have to be made about a responsible cut-off or limit of detection to make the distinction between positive and negative results. For this, not only biological and technical aspects should be considered, but also moral aspects, such as what amount of risk for false positive or false negative tumor DNA alterations would be morally acceptable. As clinical cancer care will increasingly depend on the molecular tumor characteristics and targeted therapies are generally less associated with severe side effects, one could argue that it would be very important to detect as many reliable potential targetable genetic alterations as possible and determine the threshold above which a mutation is considered to be present accordingly (e.g. establishing the limit of detection). Molecular biologists or pathologists interpreting MP results should be aware of the assay's limit of detection and the risk of false negative results. Before initiating MP analysis, the presence of tumor and tumor percentage should be confirmed and determined by an experienced pathologist. In a few areas, such as lung cancer, professional organizations have created best practice recommendations and guidelines on how to adequately

use and select tumor tissue for morphological, immunohistochemical, and molecular analyses [51,52]. It would be of in depth importance to also include the perspective of patients and proceduralists who obtain the tissue samples in determining these kinds of cut-off points. It is important for proceduralists, e.g. pulmonologists and intervention radiologists, to be aware not only of the benefits (e.g. lower risk of complications, shorter recovery time), but also of the downstream risks of obtaining small biopsies or fine-needle aspirations due to limited tumor content (e.g. inadequate amount of DNA, tumor heterogeneity). Although we here focus on the pathology perspective, it is in fact a shared responsibility to successfully and responsibly obtain representative cells or tissue for MP analysis, weighing several aspects against each other and making risk-benefit calculations.

Once the decision has been made to perform MP analysis, one could decide to test only tumor tissue or also normal tissue to be able to discriminate somatic variants from germline variants (subtracting approach). In the case of WES/WGS, it is essentially impossible to interpret tumor results without a paired normal sample given the large number of genetic variants detected. For more limited genetic analysis, no consensus has been reached about which of these approaches to take, although in general tumor-only sequencing is preferred. The subtracting approach has been considered acceptable when the purpose of testing is to identify clinically significant somatic variants [9]. However, this approach masks germline variants, leading to a questionable loss of information and is also associated with increased costs. In the context of (tumor) WES or WGS in CG and research settings, there is a growing consensus that there is an obligation to actively screen for potential germline findings and to disclose clinically significant (potential) germline findings (see below) [9,10,14,15,17,18,21,53–56]. As such, it may be odd not to have such an obligation in the diagnostic MP setting when paired normal tissues are analyzed. However, when tumor-only sequencing is performed, unsolicited germline mutation may still be uncovered (see later, 2.1. Return of results) [41]. A balance should be found between meeting patients' benefits and interests in obtaining potential clinically significant unsolicited genetic results and the extra costs and other resources required for this and subsequent germline validation.

Of note, cell-free DNA (cfDNA) analyses could complement tissue and cellular analyses and thereby be useful to increase the diagnostic yield of molecular tumor work-up. For example, it is nowadays possible to detect molecular alterations in supernatants from fine-needle aspirations or cerebrospinal fluid [57–60]. The cell pellets could then be reserved for morphological, immunohistochemical, or additional molecular analyses. Interestingly, in several cases clinically significant molecular alterations were detected

only in the cfDNA in the supernatant and not in tumor cell DNA in the cell pellet [58]. Regarding tumor heterogeneity, it has been suggested that circulating cfDNA may be more representative of the whole tumor cell population than biopsy or cytology samples [61], although this needs to be further confirmed. As such, cfDNA analysis in blood may be a promising technique to monitor tumor progression and detect mechanisms of therapy resistance [61,62]. We recommend MP laboratories to actively engage in (multidisciplinary) discussions on cfDNA analysis applications and setting up analysis pipelines, as they form a promising technique for the future.

2.2. Choosing the right molecular test

Several factors can be identified that influence the choice for a MP assay, including clinical need (e.g. drug availability for actionable mutations), the type of mutations (e.g. hotspot or non-hotspot mutations), tissue availability and quality, the number of samples, the number of genes that need to be tested and workflow efficacy issues [7]. Currently, targeted NGS gene panels are commonly used in MP laboratories as they are cost- and time-effective by targeting multiple clinically actionable mutations with high coverage (i.e. sensitivity) [3,9,63,64]. It is expected that testing with large gene panels or whole exome sequencing will become more widespread in the future when this becomes technically feasible and affordable, allowing a single workflow in the MP lab [3,65].

The current discussion on which assays to use is mainly focused on the technical and financial aspects, although there are also some ethical considerations, mainly with respect to a higher chance of unsolicited findings when using larger gene panels, many consisting of VUS [39,66,67]. As written above, this risk should not be underestimated. VUS detection should usually not alter clinical management, but it has been shown that clinicians overinterpret VUS [68]. Appropriate counselling and reporting of unsolicited findings to clinicians and patients are necessary. The laboratory is not only responsible for the results being analytically valid, but should also be aware of and responsible for a workflow in which the advantages and limitations, assay options and (unsolicited) findings are explained to healthcare providers [12].

2.3. Data analysis issues

The reported genetic variants are greatly influenced by the choice of filters for variant calling parameters. Currently, there is no gold standard variant calling procedure and it is not yet clear to what extent these procedure variabilities eventually lead to reporting false positive or false negative tumor DNA sequencing results. Further research and more uniformity on this matter are needed to determine what is morally acceptable. Theoretically, bioinformatics pipelines can be set to reveal or hide potential unsolicited findings.

3. POST-ANALYTICAL CONSIDERATIONS: REPORTING, STORING AND RE-USING OF TUMOR DNA SEQUENCING ANALYSIS RESULTS

3.1. Return of results

Offering the return of clearly pathogenic genetic variants directly related to the clinical question is self-evident, but what about uncertain findings such as VUS in the genes of interest, or unsolicited findings in genes not directly related to the clinical question? In essence, the required information to disclose should be based upon the clinical indication, e.g. providing disease classification or treatment guidance [7,23]. In general, it is recommended to report only pathogenic or likely pathogenic mutations [23]. Concerning VUS, it has been proposed that they should only be reported when found in genes relevant to the testing indication [21]. With respect to unsolicited findings, there has been a long-standing debate with emerging consensus that all analytically and clinically significant unsolicited findings should be reported, provided that the patient has given consent in the context of CG and research [10,14–18,21]. Although the Association for Molecular Pathology (AMP) promotes laboratories to develop a policy for analysis and reporting of clinically significant unsolicited findings, it has not been given much attention in general pathology literature so far [22,23]. There is no clear guideline or consensus yet for the MP setting on which results to report, apart from the general recommendation to exercise extra caution with unsolicited findings [23].

The question is whether it is justifiable to limit the analysis to the diagnostic question or that there is an obligation to screen for potential clinically significant unsolicited findings [25,53]. Unsolicited findings can be considered 'foreseeable' when using larger gene panels. These gene panels often contain several genes from the set of genes of which the ACMG recommends disclosure in case unsolicited findings are detected (e.g. *TP53*, *APC,* and *PTEN*) [21]. This could be a strong argument for disclosure. However, in contrast to CG, no definite conclusion with regard to the detection of clinically significant unsolicited germline mutations can be made in MP, as sequencing takes places on tumor tissue, almost always mixed with a varying amount of normal tissue cells. However, variant frequency can, in combination with tumor percentage, suggest a germline nature. Moreover, more genetic variants could be expected in MP than in CG, as cancer is a genetic disease in itself. The majority of genetic variants may not be clinically or biologically significant, but could be considered passenger mutations [63]. It is often difficult to definitively classify a genetic variant as clinically relevant (i.e. pathogenic), which could cause unwanted psychological distress for the patients [25].

The decision on what to report to the patient has been traditionally given to the clinician. However, developments such as the rapidly evolving knowledge of cancer genetics and the visibility of genetic data in electronic health records accessible by the patients themselves, necessitates a re-evaluation of who is the appropriate provider of genetic information [25]. It becomes increasingly difficult for clinicians to decide which information is important and appropriate to report to patients [25]. A more active role for pathology in providing patients the required information has been proposed [7]. This would provide pathologists with new challenges to perhaps open up a pathology outpatient clinic or participate in a multidisciplinary outpatient setting. Pathologists should actively engage and promote discussions with other medical specialists and with patients on the handling of unsolicited findings and uncertain findings in genes of interest. They should also contribute to development and implementation of guidelines on how to manage them.

3.2. Data storage and re-analysis

Cancer genetics is an evolving field. Genetic alterations of which the clinical significance is still unclear today may be classified in the future as definitely pathogenic or benign. The possibility of reanalysis is a big advantage of DNA sequencing, maximizing its benefit for clinical care [69,70] Current literature on (whether and to what extent there is an obligation of) genetic reanalysis is focused on the CG context using WES/WGS. In this context, it has been shown that in 10-16% of previously undiagnosed cases explanatory variants were discovered by re-analysis 1-3 years later and that multiple cases with previously detected VUS could be downgraded [69–73]. The question is then whether laboratories should (systematically) re-analyse stored genetic data. For the CG context, periodic reanalysis has been recommended in non-diagnostic cases, although a recent interdisciplinary working group came to the consensus that laboratories have no obligation to routinely analyse data [69,71,74,75]. In the MP context, laboratories are encouraged to set up clear and appropriate policies, but discussion in literature let alone consensus in the MP context are lacking on what would be an appropriate re-analysis and re-contact policy [10,23,76–79]. In contrast to CG, MP analysis has more time pressure as patients are currently suffering from (often metastastic) cancer. As such, re-analysis a couple of years later may be of less value as in the CG context as part of the patients may already be in a condition too poor for targeted therapy or have already died from their disease. However, there may be some indications in which reanalysis in MP is of value, for example for patients with VUS in genes of interest. Another question is whether it should be on the clinician's initiative or that it should be a standard process initiated by the pathology laboratory, for which type of results it should be performed and for how long after the initial analysis [25]. Apart from the

desirability, the practical feasibility should also be considered, as re-analysis would require significant resources, including adequate storage, regular updating of analysis tools and genetic variant databases, reporting as well as re-contacting patients, for which there may be no reimbursement [25,71,73,75]. When laboratories do not offer (standard) re-analysis, it would be advisable to state that the classification of genetic variants is based on knowledge at the time the sequencing analysis took place [74]. It also important to keep in mind that when re-analysis is not based on the laboratory's initiative, there is chance of inequity as well-informed, higher-educated patients may request re-analysis more often than lower-educated patients [74]. This will be even more the case in countries with no universal health coverage.

3.3. Data sharing

To be able to build knowledge more rapidly about specific genetic aberrations and their association with disease types and therapeutic response and to improve clinical care, the ACMG and AMP state that laboratories should be encouraged to share their genomic/genetic data in public databases or resources, such as Clinvar, My Cancer Genome and COSMIC [11–13,23]. MP laboratories frequently make use of these resources to interpret genetic results. We believe that MP laboratories should also be encouraged to share their genetic data with these resources, based upon the principle of reciprocity, i.e. the idea that all those involved in cancer genetics and clinical cancer care have a shared responsibility for its further development. However, data sharing can be in tension with confidentiality and privacy [74]. Even when data are anonymized or de-identified, genetic information may be potentially re-identifiable, although it still unclear on to what extent privacy risks form an actual threat to patients [80]. Currently, no consensus exists on to what degree genetic data should and can be anonymized. A balance must be found between maximal use of genetic data for the advancement of clinical care and future patients, and protecting patient's privacy [80].

3.4. Future scientific use

With the emergence of MP a new type of archived 'material' beside *tissues*, namely genetic *data*, has been introduced. They form a valuable source for future scientific purposes as large quantities of genetic data are needed to obtain adequate statistical power and discover new genetic associations with disease (outcome) [81–84]. Laws and regulations on the use of left-over patient *tissues* vary between countries. In some countries patients should give explicit permission for scientific use of their tissues (opt-in), whereas in other countries these materials could be used unless patients actively oppose (opt-out) [85–91]. Considering *data*, the new EU General Data Protection Regulation sets conditions for protecting citizens' personal data. This includes sensitive

personal data such as genetic data, for countries in the European Union [92]. Processing of genetic data will in principle be forbidden without explicit consent, but could under certain conditions exceptionally be admitted by (national) law for scientific purposes [92,93]. To conclude, MP laboratories should ensure that archived tissues and genetic data are responsibly taken care of, but could also be shared with researchers as long as patients' rights and respect for their autonomy and privacy are guaranteed.

DISCUSSION

In this paper, we showed that ethical issues are present in every stage of the MP analysis procedure. Many of these issues are new to pathology or more stringent than before as DNA sequencing could yield potentially sensitive data. Several ethical principles are at the basis of these ethical issues, including beneficence, non-maleficence, confidentiality, reciprocity, and equity. These ethical principles are sometimes in conflict with each other. In these cases, the pathologist, the clinician, the patient, and other relevant stakeholders should together take these aspects into consideration in order to come to a decision. Moreover, we showed the ethical pre-requisites of performing MP analysis adequately are dependent on the extensiveness of the analysis method (Figure 1).

Although the context of MP is unique, many issues are similar to those applicable to CG, in which much more scholarship on ethical aspects exists. This is the case, for example, for issues like informed consent and return of results. In both contexts, it is important that patients are aware of the DNA analysis being performed with its associated benefits and risks, and that clinically significant findings are returned.

However, one cannot just directly apply scholarship in CG to pathology practice. In essence, there are two main differences that make the MP context more complex and that should be taken into account when managing ethical issues. First, a pathologist usually does not have direct contact with patients, in contrast to a clinical geneticist. This makes the practical execution of issues like informed consent and return of results challenging. Second, MP analysis is complex as it is not a stand-alone analysis but forms a part of tissue diagnostics in general for patients who usually are hardly aware of the implications of tumor DNA sequencing. Also, the tumor tissues are always mixed with normal tissue cells making results more difficult to interpret.

As said before, scholarship on ethical aspects of MP and tumor DNA sequencing is limited. The AMP has published guidelines in which there is some attention for ethical

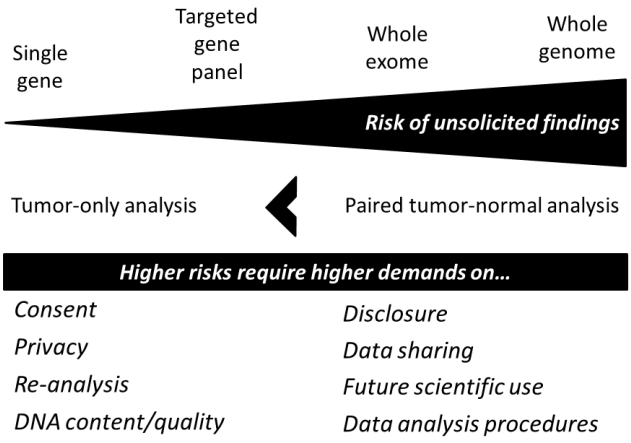

Figure 1. High-complexity assays demand higher ethical requirements.
The ethical pre-requisites of performing MP analysis adequately are dependent on the extensiveness of the analysis method. When larger gene panels are used and/or when normal tissue is also sequenced beside tumor tissue, the chance of detecting unsolicited findings (e.g. potential germline mutations) increases. This poses extra demands on the consent and disclosure procedure as well as privacy measurements. Moreover, as usually more genetic variants (of uncertain significance) are detected when using whole exome or whole genome sequencing, data sharing and re-analysis as well as a clear protocol for future scientific use (with appropriate consent) are more important compared to when single gene or limited gene panels are used. Whole exome and whole genome sequencing also pose higher requirements on DNA content and quality as well as analysis procedures, in which not only biological and technical aspects, but also value judgments are involved.

issues. These guidelines are however quite open-ended, for example encouraging laboratories to set up adequate disclosure and re-analysis procedures. A genuine discussion among pathologists is lacking about what an appropriate disclosure or re-analysis procedure would be, illustrating that there is little awareness on ethical issues amongst pathologists. This is, for instance, also reflected in the lack of ethical committees in pathology associations. However, the complexity of the MP context and the fact that pathology in general has traditionally been functioning behind the scenes, having no direct relations with patients, should not be an excuse to inhibit discussion and reflection on ethical aspects. Instead, it should be seen as an interesting new dimension of this rapidly evolving and important medical specialty that concerns increasing numbers of patients, in fact many more than those referred to CG.

Thus, pathology should take the lead in this era of tumor DNA sequencing, not only with respect to the technical aspects, but also to these ethical challenges. Pathologists should take responsibility for the adequate use of molecular analyses, for example by promoting the development of adequate informed consent and disclosure procedures.

This would require more interaction and collaboration with stakeholders, including patients, clinicians, clinical geneticists, bio-informaticians, bio-molecular scientists, clinical scientists, lawyers and ethicists, so that clinical cancer care can be truly tailored for well-informed patients and clinicians [9,25,94]. Tighter cooperation and discussions with clinical geneticists are also desirable. The disciplines of pathology and CG have perhaps developed too independently with respect to the technical as well as ethical aspects and might even be viewed as competitors in providing clinical DNA sequencing services. As they encounter similar technical and ethical issues, it would be very valuable to make use of each other's expertise and integrate (ethical) debates, while being aware of the differences. Moreover, pathologists could act as educators of clinicians and patients about the benefits, risks, and limitations of tumor DNA sequencing and personalized (genetic) medicine, so that molecular testing is properly used and interpreted [95]. These competencies are rather new for this discipline that has been traditionally acting 'behind the scenes' of clinical care, but are exciting and should be developed during pathology training [63]. In the end, biological, technological as well as ethical aspects need to be combined and shaped into a responsible use and further advancement of MP within medicine.

CONCLUSION

The era of personalized cancer care calls for an active role of pathology as a specialty. Pathology should get prepared to be fully aware of and capable of dealing with the diverse, complex but challenging aspects of tumor DNA sequencing, including its ethical aspects. These are a pre-requisite for good clinical practice. To make clinical cancer treatment truly personalized, pathology should get the patient behind the tissue and genetic data more into sight.

REFERENCES

1 van't Veer LJ, Bernards R. Enabling personalized cancer medicine through analysis of gene-expression patterns. *Nature* 2008; **452**: 564-570

2 Garraway LA. Genomics-driven oncology: framework for an emerging paradigm. *J Clin Oncol* 2013; **31**: 1806-1814

3 Dietel M, Johrens K, Laffert MV, *et al.* A 2015 update on predictive molecular pathology and its role in targeted cancer therapy: A review focussing on clinical relevance. *Cancer Gene Ther* 2015; **22**: 417-430

4 Vis DJ, Lewin J, Liao RG, *et al.* Towards a global cancer knowledge network: dissecting the current international cancer genomic sequencing landscape. *Ann Oncol* 2017; **28**: 1145-1151

5 Deans ZC, Costa JL, Cree I, *et al.* Integration of next-generation sequencing in clinical diagnostic molecular pathology laboratories for analysis of solid tumours; an expert opinion on behalf of IQN Path ASBL. *Virchows Arch* 2017; **470**: 5-20

6 Li MM, Datto M, Duncavage EJ, *et al.* Standards and Guidelines for the Interpretation and Reporting of Sequence Variants in Cancer. *J Mol Diagnostics* 2017; **19**: 4-23

7 Cree IA, Deans Z, Ligtenberg MJL, *et al.* Guidance for laboratories performing molecular pathology for cancer patients. *J Clin Pathol* 2014; **67**: 923-931

8 Lee RE, Henricks WH, Sirintrapun SJ. Laboratory Information Systems in Molecular Diagnostics. *Adv Anat Pathol* 2016; **23**: 125-133

9 Robson ME, Bradbury AR, Arun B, *et al.* American Society of Clinical Oncology Policy Statement Update: Genetic and Genomic Testing for Cancer Susceptibility. *J Clin Oncol* 2015; **33**: 3660-3667

10 Allen C, Foulkes WD. Qualitative thematic analysis of consent forms used in cancer genome sequencing. *BMC Med Ethics* 2011; **12**: 14

11 ACMG Board of Directors. Points to consider in the clinical application of genomic sequencing. *Genet Med* 2012; **14**: 759-761

12 Rehm HL, Bale SJ, Bayrak-Toydemir P, *et al.* ACMG clinical laboratory standards for next-generation sequencing. *Genet Med* 2013; **15**: 733-747

13 ACMG Board Of Directors. Laboratory and clinical genomic data sharing is crucial to improving genetic health care: a position statement of the American College of Medical Genetics and Genomics. *Genet Med* 2017; **19**: 721-722

14 Bredenoord AL, Onland-Moret NC, Van Delden JJM. Feedback of individual genetic results to research participants: in favor of a qualified disclosure policy. *Hum Mutat* 2011; **32**: 861-867

15 Levesque E, Joly Y, Simard J, *et al.* Return of Research Results: General Principles and International Perspectives. *J Law, Med Ethics* 2011; **39**: 583-592

16 Wolf SM. Return of individual research results and incidental findings: facing the challenges of translational science. *Annu Rev Genomics Hum Genet* 2013; **14**: 557-577

17 Christenhusz GM, Devriendt K, Dierickx K. To tell or not to tell? A systematic review of ethical reflections on incidental findings arising in genetics contexts. *Eur J Hum Genet* 2013; **21**: 248-255

18 Burke W, Evans BJ, Jarvik GP. Return of results: ethical and legal distinctions between research and clinical care. *Am J Med Genet C Semin Med Genet* 2014; **166C**: 105-111

19 ACMG Board of Directors. Points to consider for informed consent for genome/exome sequencing. *Genet Med* 2013; **15**: 748-749

20 ACMG Board of Directors. ACMG policy statement: updated recommendations regarding analysis and reporting of secondary findings in clinical genome-scale sequencing. *Genet Med* 2015; **17**: 68-69

21 Kalia SS, Adelman K, Bale SJ, *et al.* Recommendations for reporting of secondary findings in clinical exome and genome sequencing, 2016 update (ACMG SF v2.0): a policy statement of the American College of Medical Genetics and Genomics. *Genet Med* 2017; **19**: 249-255

22 Hegde M, Bale S, Bayrak-Toydemir P, *et al.* Reporting Incidental Findings in Genomic Scale Clinical Sequencing—A Clinical Laboratory Perspective. *J Mol Diagnostics* 2015; **17**: 107-117

23 Richards S, Aziz N, Bale S, *et al.* Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015; **17**: 405-423

24 Samuels S, Balint B, von der Leyen H, *et al.* Precision medicine in cancer: challenges and recommendations from an EU-funded cervical cancer biobanking study. *Br J Cancer* 2016; **115**: 1575-1583

25 Hehir-Kwa JY, Claustres M, Hastings RJ, *et al.* Towards a European consensus for reporting incidental findings during clinical NGS testing. *Eur J Hum Genet* 2015; **23**: 1601-1606

26 Amendola LM, Jarvik GP, Leo MC, *et al.* Performance of ACMG-AMP Variant-Interpretation Guidelines among Nine Laboratories in the Clinical Sequencing Exploratory Research Consortium. *Am J Hum Genet* 2016; **98**: 1067-1076

27 Gray SW, Park ER, Najita J, *et al.* Oncologists' and cancer patients' views on whole-exome sequencing and incidental findings: results from the CanSeq study. *Genet Med* 2016; **18**: 1011-1019

28 Vadaparampil ST, Quinn G, Malo TL, *et al.* Knowledge About Hereditary Colorectal Cancer Among Colorectal Cancer Survivors. *Genet Test Mol Biomarkers* 2010; **14**: 603-609

29 Donovan KA, Tucker DC. Knowledge about genetic risk for breast cancer and perceptions of genetic testing in a sociodemographically diverse sample. *J Behav Med* 2000; **23**: 15-36

30 Kinney AY, Croyle RT, Dudley WN, *et al.* Knowledge, attitudes, and interest in breast-ovarian cancer gene testing: a survey of a large African-American kindred with a BRCA1 mutation. *Prev Med (Baltim)* 2001; **33**: 543-551

31 Weinrich S, Vijayakumar S, Powell IJ, *et al.* Knowledge of hereditary prostate cancer among high-risk African American men. *Oncol Nurs Forum* 2007; **34**: 854-860

32 Dry S, Grody WW, Papagni P. Stuck between a scalpel and a rock, or molecular pathology and legal-ethical issues in use of tissues for clinical care and research: what must a pathologist know? *Am J Clin Pathol* 2012; **137**: 346-355

33 Scollon S, Bergstrom K, Kerstein RA, *et al.* Obtaining informed consent for clinical tumor and germline exome sequencing of newly diagnosed childhood cancer patients. *Genome Med* 2014; **6**: 69

34 Tafe LJ. Targeted Next-Generation Sequencing for Hereditary Cancer Syndromes: A Focus on Lynch Syndrome and Associated Endometrial Cancer. *J Mol Diagn* 2015; **17**: 472-482

35 Kuhlen M, Borkhardt A. Cancer susceptibility syndromes in children in the area of broad clinical use of massive parallel sequencing. *Eur J Pediatr* 2015; **174**: 987-997

36 Bijlsma RM, Wessels H, Wouters RHP, *et al.* Cancer patients' intentions towards receiving unsolicited genetic information obtained using next-generation sequencing. *Fam Cancer* 2018; **17**: 309-316

37 Bredenoord AL, Bijlsma RM, van Delden H. Next Generation DNA Sequencing: Always Allow an Opt Out. *Am J Bioeth* 2015; **15**: 28-30

38 Bijlsma RM, Bredenoord AL, Gadellaa-Hooijdonk CG, *et al.* Unsolicited findings of next-generation sequencing for tumor analysis within a Dutch consortium: clinical daily practice reconsidered. *Eur J Hum Genet* 2016; **24**: 1496-1500

39 Schrader KA, Cheng DT, Joseph V, *et al.* Germline Variants in Targeted Tumor Sequencing Using Matched Normal DNA. *JAMA Oncol* 2016; **2**: 104-111

40 Mandelker D, Zhang L, Kemel Y, *et al.* Mutation Detection in Patients With Advanced Cancer by Universal Sequencing of Cancer-Related Genes in Tumor and Normal DNA vs Guideline-Based Germline Testing. *JAMA* 2017; **318**: 825

41 Catenacci DVT, Amico AL, Nielsen SM, *et al.* Tumor genome analysis includes germline genome: are we ready for surprises? *Int J cancer* 2015; **136**: 1559-1567

42 Lolkema MP, Gadellaa-van Hooijdonk CG, Bredenoord AL, *et al.* Ethical, legal, and counseling challenges surrounding the return of genetic results in oncology. *J Clin Oncol* 2013; **31**: 1842-1848

43 Platt J, Cox R, Enns GM. Points to consider in the clinical use of NGS panels for mitochondrial disease: an analysis of gene inclusion and consent forms. *J Genet Couns* 2014; **23**: 594-603

44 McGuire AL, Hamilton JA, Lunstroth R, *et al.* DNA data sharing: research participants' perspectives. *Genet Med* 2008; **10**: 46-53

45 Dondorp W, Sikkema-Raddatz B, de Die-Smulders C, *et al.* Arrays in postnatal and prenatal diagnosis: An exploration of the ethics of consent. *Hum Mutat* 2012; **33**: 916-922

46 Rigter T, Henneman L, Kristoffersson U, *et al.* Reflecting on earlier experiences with unsolicited findings: points to consider for next-generation sequencing and informed consent in diagnostics. *Hum Mutat* 2013; **34**: 1322-1328

47 Bredenoord A, Dondorp W, Pennings G, *et al.* Preimplantation genetic diagnosis for mitochondrial DNA disorders: Ethical guidance for clinical practice. *Eur J Hum Genet* 2009; **17**: 1550-1559

48 Daniels M, Goh F, Wright CM, *et al.* Whole genome sequencing for lung cancer. *J Thorac Dis* 2012; **4**: 155-163

49 Gerlinger M, Rowan AJ, Horswell S, *et al.* Intratumor Heterogeneity and Branched Evolution Revealed by Multiregion Sequencing. *N Engl J Med* 2012; **366**: 883-892

50 Swanton C. Intratumor Heterogeneity: Evolution through Space and Time. *Cancer Res* 2012; **72**: 4875-4882

51 Travis WD, Brambilla E, Noguchi M, *et al.* Diagnosis of Lung Cancer in Small Biopsies and Cytology: Implications of the 2011 International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society Classification. *Arch Pathol Lab Med* 2013; **137**: 668-684

52 Lindeman NI, Cagle PT, Aisner DL, *et al.* Updated Molecular Testing Guideline for the Selection of Lung Cancer Patients for Treatment With Targeted Tyrosine Kinase Inhibitors. *J Mol Diagnostics* 2018; **20**: 129-159

53 Wouters RHP, Cornelis C, Newson AJ, *et al.* Scanning the body, sequencing the genome: Dealing with unsolicited findings. *Bioethics* 2017; **31**: 648-656

54 Wolf SM. Return of individual research results and incidental findings: Facing the challenges of translational science. *Annu Rev Genomics Hum Genet* 2013; **14**: 557-577

55 Green RC, Berg JS, Grody WW, *et al.* ACMG recommendations for reporting of incidental findings in clinical exome and genome sequencing. *Genet Med* 2013; **15**: 565-574

56 Wolf SM, Annas GJ, Elias S. Point-counterpoint. Patient autonomy and incidental findings in clinical genomics. *Science* 2013; **340**: 1049-1050

57 Rolfo C, Mack PC, Scagliotti G V., *et al.* Liquid Biopsy for Advanced Non-Small Cell Lung Cancer (NSCLC): A Statement Paper from the IASLC. *J Thorac Oncol* June 2018

58 Asaka S, Yoshizawa A, Saito K, *et al.* Rapid point-of-care testing for epidermal growth factor receptor gene mutations in patients with lung cancer using cell-free DNA from cytology specimen supernatants. *Int J Oncol* 2018; **52**: 2110-2118

59 Guibert N, Tsukada H, Hwang DH, *et al.* Liquid biopsy of fine-needle aspiration supernatant for lung cancer genotyping. *Lung Cancer* 2018; **122**: 72-75

60 Hiemcke-Jiwa LS, ten Dam-van Loon NH, Leguit RJ, *et al.* Potential Diagnosis of Vitreoretinal Lymphoma by Detection of *MYD88* Mutation in Aqueous Humor With Ultrasensitive Droplet Digital Polymerase Chain Reaction. *JAMA Ophthalmol* July 2018

61 Cheung AH-K, Chow C, To K-F. Latest development of liquid biopsy. *J Thorac Dis* 2018; **10**: S1645-S1651

62 Volik S, Alcaide M, Morin RD, *et al.* Cell-free DNA (cfDNA): Clinical Significance and Utility in Cancer Shaped By Emerging Technologies. *Mol Cancer Res* 2016; **14**: 898-908

63 Roychowdhury S, Chinnaiyan AM. Translating genomics for precision cancer medicine. *Annu Rev Genomics Hum Genet* 2014; **15**: 395-415

64 Giardina T, Robinson C, Grieu-Iacopetta F, *et al.* Implementation of next generation sequencing technology for somatic mutation detection in routine laboratory practice. *Pathology* 2018; **50**: 389-401

65 Li T, Kung H-J, Mack PC, *et al.* Genotyping and genomic profiling of non-small-cell lung cancer: implications for current and future therapies. *J Clin Oncol* 2013; **31**: 1039-1049

66 Kurian AW, Hare EE, Mills MA, *et al.* Clinical evaluation of a multiple-gene sequencing panel for hereditary cancer risk assessment. *J Clin Oncol* 2014; **32**: 2001-2009

67 Tung N, Battelli C, Allen B, *et al.* Frequency of mutations in individuals with breast cancer referred for *BRCA 1* and *BRCA 2* testing using next-generation sequencing with a 25-gene panel. *Cancer* 2015; **121**: 25-33

68 Plon SE, Cooper HP, Parks B, *et al.* Genetic testing and cancer risk management recommendations by physicians for at-risk relatives. *Genet Med* 2011; **13**: 148-154

69 Costain G, Jobling R, Walker S, *et al.* Periodic reanalysis of whole-genome sequencing data enhances the diagnostic advantage over standard clinical genetic testing. *Eur J Hum Genet* 2018; **26**: 740-744

70 Hiatt SM, Amaral MD, Bowling KM, *et al.* Systematic reanalysis of genomic data improves quality of variant interpretation. *Clin Genet* 2018; **94**: 174-178

71 Wenger AM, Guturu H, Bernstein JA, *et al.* Systematic reanalysis of clinical exome data yields additional diagnoses: implications for providers. *Genet Med* 2017; **19**: 209-214

72 Nambot S, Thevenon J, Kuentz P, *et al.* Clinical whole-exome sequencing for the diagnosis of rare disorders with congenital anomalies and/or intellectual disability: substantial interest of prospective annual reanalysis. *Genet Med* 2018; **20**: 645-654

73 Wright CF, McRae JF, Clayton S, *et al.* Making new genetic diagnoses with old data: iterative reanalysis and reporting from genome-wide data in 1,133 families with developmental disorders. *Genet Med* January 2018

74 Vears DF, Sénécal K, Clarke AJ, *et al.* Points to consider for laboratories reporting results from diagnostic genomic sequencing. *Eur J Hum Genet* 2018; **26**: 36-43

75 Steward CA, Parker APJ, Minassian BA, *et al.* Genome annotation for clinical genomic diagnostics: strengths and weaknesses. *Genome Med* 2017; **9**: 49

76 Conley JM, Doerr AK, Vorhaus DB. Enabling responsible public genomics. *Health Matrix Clevel* 2010; **20**: 325-385

77 Wade CH, Kalfoglou AL. When do genetic researchers have a duty to recontact study participants? *Am J Bioeth* 2006; **6**: 22-26

78 Miller FA, Hayeems RZ, Li L, *et al.* One thing leads to another: the cascade of obligations when researchers report genetic research results to study participants. *Eur J Hum Genet* 2012; **20**: 837-843

79 Zeps N, Bledsoe MJ. Managing the Ethical Issues of Genomic Research using Pathology Specimens. *Clin Biochem Rev* 2015; **36**: 21-27

80 Vos S, van Delden JJM, van Diest PJ, *et al.* Moral Duties of Genomics Researchers: Why Personalized Medicine Requires a Collective Approach. *Trends Genet* 2017; **33**

81 Foekens JA, Wang Y, Martens JWM, *et al.* The use of genomic tools for the molecular understanding of breast cancer and to guide personalized medicine. *Drug Discov Today* 2008; **13**: 481-487

82 Ioannidis JPA. Population-wide generalizability of genome-wide discovered associations. *J Natl Cancer Inst* 2009; **101**: 1297-1299

83 Francis LP. Genomic knowledge sharing: A review of the ethical and legal issues. *Appl Transl Genomics* 2014; **3**: 111-115

84 Oosterhuis JW, Coebergh JW, van Veen E-B. Tumour banks: well-guarded treasures in the interest of patients. *Nat Rev Cancer* 2003; **3**: 73-77

85 International Ethical Guidelines for Health-related Research Involving Humans Prepared by the Council for International Organizations of Medical Sciences (CIOMS) in collaboration with the World Health Organization (WHO). Available from: https://cioms.ch/wp-content/uploads/2017/01/WEB-CIOMS-EthicalGuidelines.pdf.

86 Human Tissue Act. Available from: https://www.legislation.gov.uk/ukpga/2004/30/contents

87 Giesbertz NAA, Bredenoord AL, van Delden JJM. Inclusion of residual tissue in biobanks: opt-in or opt-out? Caplan A, ed. *PLoS Biol* 2012; **10**: e1001373

88 Gefenas E, Dranseika V, Serepkaite J, *et al.* Turning residual human biological materials into research collections: playing with consent. *J Med Ethics* 2012; **38**: 351-355

89 Riegman PHJ, van Veen E-B. Biobanking residual tissues. *Hum Genet* 2011; **130**: 357-368

90 Federal Policy for the Protection of Human Subjects. Available from: https://www.gpo.gov/fdsys/pkg/FR-2017-01-19/pdf/2017-01058.pdf.

91 Medical Research Involving Human Subjects Act. Available from: http://wetten.overheid.nl/BWBR0009408/2017-03-01.

92 General Data Protection Regulation. Available from: http://ec.europa.eu/justice/data-protection/reform/files/regulation_oj_en.pdf.

93 Chassang G. The impact of the EU general data protection regulation on scientific research. *Ecancermedicalscience* 2017; **11**: 709

94 Stoeklé H-C, Mamzer-Bruneel M-F, Frouart C-H, *et al.* Molecular Tumor Boards: Ethical Issues in the New Era of Data Medicine. *Sci Eng Ethics* 2018; **24**: 307-322

95 Schrijver I, Aziz N, Farkas DH, *et al.* Opportunities and challenges associated with clinical diagnostic genome sequencing: a report of the Association for Molecular Pathology. *J Mol Diagn* 2012; **14**: 525-540

Chapter 9

Summarizing discussion and future perspectives

SUMMARIZING DISCUSSION

In the first part of this thesis, we investigated several types of tissue biomarkers for *BRCA1/2*-related breast cancer that may be used to identify potential *BRCA1/2* germline mutation carriers and stratify patients for subsequent germline mutation testing. In the second part of this thesis, we investigated 'gen-ethical' aspects of translational (cancer) research and clinical cancer care with respect to the emergence of molecular pathology.

In **Chapter 2** an overview of the literature was given concerning hereditary breast cancer syndromes in general, including *BRCA1/2* germline mutation-related breast carcinomas. Several studies have investigated the tumor characteristics of *BRCA1*- and *BRCA2*-related breast carcinomas, including protein expression by immunohistochemistry. Few studies have however taken the next step to develop a prediction model based upon these differences between *BRCA*-related and sporadic breast carcinomas, especially with regard to *BRCA2*-related breast carcinomas [1–5].

In **Chapter 3** we performed the largest comprehensive immunoprofiling study in *BRCA1/2*-related breast carcinomas published so far. From this, a promising, accurate immuno-based prediction model was developed to identify *BRCA1/2* germline mutation-related breast carcinomas. The prediction model is based upon 14 predictors (age, mitotic activity index, cyclinD1, ERα, ERβ, FGFR2, FGFR3, FGFR4, GLUT1, IGFR, Ki67, MLH1, p120, and TOP2A) with excellent discriminative performance (AUC = 0.943 (95%CI: 0.909-0.978)) and reasonable calibration. Also, the performance seems to be better than current known prediction models based on clinical, morphological, and/or immunohistochemical characteristics, and similar to that of molecular models. This tool could help in predicting which breast cancer patients may have an underlying *BRCA1/2* germline mutation, largely independent of clinical characteristics. As such, the model could be used as a pre-selection tool for germline mutation testing. Before the model could be implemented in clinical practice, the reproducibility among pathologists of scoring the selected immunostains should be evaluated. Moreover, external validation is needed to test generalizability of this model. Further research is also required to find out for which group of breast cancer patients this prediction model will be (most) useful and cost-effective.

In **Chapter 4**, we assessed the diagnostic value of *BRCA1/2* methylation in distinguishing *BRCA1/2* germline mutation-related from sporadic breast carcinomas. *BRCA* methylation analysis may be used as a potential pre-screening test for *BRCA1/2* germline mutation analysis as it has been proposed in literature that *BRCA* promoter

methylation and germline mutations are mutually exclusive [6–16]. For routine testing, more confirmation was needed, especially with regard to the best CpG sites to target, and a robust assay needs to be available that works well on FFPE tumor material. We analysed *BRCA1/2* methylation in *BRCA1/2* germline mutation-related and sporadic breast carcinomas using a recently developed *BRCA* MS-MLPA assay, a technique well-suited for daily pathology practice. We discovered that the diagnostic value of *BRCA* promoter methylation analysis in distinguishing *BRCA1/2*-related from sporadic breast carcinomas seems to be considerably dependent on the targeted CpG sites. Some CpG sites were methylated more frequently in *BRCA1/2*-related compared to sporadic carcinomas (those located at Chr17:41277395, Chr13:32889621, Chr13:32889836, Chr13:32889672, Chr13:32889683, and Chr13:2889608) (genomic locations based upon GRCh37/hg19). Other CpG sites were methylated more frequently in sporadic carcinomas (those located at Chr17:41277429 and Chr17:41277323). Daniels *et al.* (2016) also recently discovered that *BRCA1* methylation frequencies vary between CpG sites [17]. As a consequence, the diagnostic value of *BRCA* methylation analysis in distinguishing *BRCA1/2*-related from sporadic breast carcinomas is considerably dependent on the targeted CpG site(s). Sensitivity and specificity were most balanced when using all four *BRCA2* probes to rule in *BRCA1/2* germline mutations when methylation is detected in at least one the *BRCA2* probes targeting CpG sites located at Chr13:32889621, Chr13:32889836, Chr13:32889672, Chr13:32889683, and Chr13:2889608 (sensitivity 69.4%, specificity 87.5%). We observed frequent *BRCA* methylation in *BRCA*-related breast carcinomas: at least 63.8% of *BRCA1/2*-related carcinomas showed methylation of at least one of the targeted CpG sites in the *BRCA1* or *BRCA2* promoter. As such, our findings did not support the general assumption in literature that *BRCA* promoter methylation and *BRCA* germline mutations are mutually exclusive. To our knowledge, this was the largest study investigating *BRCA1* as well *BRCA2* methylation in both *BRCA1* and *BRCA2* germline mutation-related breast carcinomas, and sporadic breast carcinomas. Our findings are important for adequate use of *BRCA* methylation analysis as a pre-screening tool for *BRCA* germline genetic testing, or to test eligibility for certain therapeutic strategies (see Future perspectives). Further research is required to assess which other CpG sites are important in distinguishing *BRCA1/2* germline mutation-related and sporadic breast carcinomas, especially with respect to *BRCA2* methylation as evidence is still limited.

As our methylation results were different from several previously published studies, we performed the first systematic review that investigated to which extent *BRCA* promoter methylation has exactly been reported in breast as well as ovarian carcinomas of *BRCA* germline mutation carriers (**Chapter 5**). We also investigated which CpG sites have been investigated by the studies and what the diagnostic value would be if *BRCA*

methylation analysis were to be used to differentiate *BRCA* germline mutation-related from sporadic carcinomas (**Chapter 5**). The 21 articles that met the inclusion criteria showed that *BRCA* methylation occurs rarely in *BRCA1/2*-related breast and especially ovarian carcinomas, although methylation frequencies varied between individual CpG sites. *BRCA1* methylation was found in at least 3.6 and 1.1% of *BRCA1/2*-related breast and ovarian carcinomas, respectively. *BRCA2* methylation was observed in at least 5.3% breast and 0.0% ovarian carcinomas of *BRCA1/2* germline mutation carriers. In general, if *BRCA* methylation were used to exclude *BRCA* germline mutations when methylation is found, it would have an excellent sensitivity of at most 100%. The specificity would be poor, at most 23.1%, when compared with available data on sporadic carcinomas. However, it should be taken into account that the selected studies show large differences in methodology, quality and risk of bias. They generally performed a limited methylation and incomplete mutation analysis. Not more than 40% of the CpG sites in respectively the *BRCA1* and *BRCA2* promoter region were studied in at least one of the studies. The largest and qualitatively best studies show that *BRCA* methylation is exceedingly rare in especially *BRCA*-related ovarian carcinomas. However, the occurrence of *BRCA* methylation in breast or ovarian carcinomas from *BRCA* carriers may be underestimated. This would have major implications for clinical practice, including referral for genetic testing and *BRCA*ness analysis for treatment decision-making (see Future perspectives). Currently, it is unclear which specific CpG sites have the highest diagnostic value in distinguishing *BRCA*-related from sporadic breast and ovarian carcinomas.

In **Chapter 6**, we performed a miRNA profiling study to investigate which miRNAs are deregulated in *BRCA1/2* germline mutation-related breast carcinomas. This could give more insight into *BRCA1/2*-associated breast carcinogenesis and yield new diagnostic biomarkers, or therapeutic targets. It has been shown previously that miRNAs play a role in sporadic breast carcinogenesis [18–21], but little was known about miRNA expression in *BRCA1/2*-related breast cancers. As miRNAs are relatively resistant to degradation caused by the formalin fixation process, miRNA biomarkers could be implemented relatively easily in pathology practice [22]. We observed that many more miRNAs were differentially expressed between breast carcinomas and normal breast tissues from *BRCA* carriers, compared to available data in literature on sporadic breast carcinomas and normal breast tissues from non-*BRCA*-mutation carriers. This might be related to the chromosomal instability characteristic of *BRCA1/2*-associated breast carcinomas, leading to loss of chromosomal regions and consequently, miRNA genes. Multiple deregulated miRNAs may be potentially specific to *BRCA1/2*-related breast carcinogenesis. Especially, *BRCA2*-related breast carcinomas did not show any similarities in up-regulated miRNAs with sporadic breast carcinomas.

This is remarkable as *BRCA2*-related carcinomas otherwise show many similarities with sporadic carcinomas. However, we also discovered several miRNAs that were deregulated in *BRCA1/2*-related as well as sporadic breast carcinomas. This suggests the existence of common miRNA-regulated pathways involved in breast carcinogenesis in general. Further research is needed to validate our results and to investigate whether these deregulated miRNAs may also be used as a potential tool for diagnosis or target for therapy.

Leftover human bio-specimens are a rich source for biomedical (cancer) research. More and more analyses can be performed on formalin-fixed paraffin-embedded tissues, including DNA sequencing analyses. The scope has changed from single-gene testing to large-scale genomics studies. For example, next-generation sequencing has elucidated the genomic landscape of breast cancer [23]. Moreover, large (international) research projects and biobanks have emerged that share and store samples and/or data, for example The Cancer Genome Atlas studies (https://cancergenome.nih.gov/) and the Genomic Data Commons Data portal that researchers can use to access cancer genomic data (https://portal.gdc.cancer.gov/). These developments create ethical challenges and moral duties for researchers performing these studies, as large amounts of potentially sensitive data are being generated and shared. In hereditary breast cancer research, for example, there has been discussion whether *BRCA1/2* gene sequencing can be performed in research settings and under which conditions [24–26]. We were confronted ourselves by a moral dilemma while performing the immunoprofiling study described in **Chapter 3**. Interestingly, a small number of clinically sporadic breast carcinomas was identified by this model to have a high probability of being *BRCA1/2*-related. Further investigation is needed on the possible underlying mechanisms. Are they 'just' false positives, do they bear subclinical *BRCA1/2* germline or somatic mutations, or may *BRCA1/2* methylation play a role here? However, before analyzing this further, specific consent from the patients or their relatives is highly desirable as it may uncover potential *BRCA1/2* germline mutations.

In **Chapter 7**, we reviewed which moral duties genomics researchers in the era of personalized medicine are confronted with, based on literature, and we analysed how researchers may deal with them in a morally responsible way. A wide range of moral duties has been attributed to genomics researchers, which we have grouped in duties related to disclosure, consent, privacy, and social responsibilities. We conclude that awareness and concrete implementation in research practice is lagging behind. Several (conflicting) moral duties require balancing and specification in particular contexts, and need continuous refinement [27]. We believe that ethical awareness of researchers

is key to identify and to some extent also balance several, potentially conflicting moral duties. These skills are different from the traditional technical-scientific skills and need to be developed during education of (genomics) researchers' careers. Also, to be able to acquire sufficient numbers of research participants from whom more is asked with respect to privacy, the research community should to some extent meet their needs (e.g. disclosure of research results), and maintain public trust [28]. In short, genomics research asks more from researchers as well as research participants [29].

In **Chapter 8**, we outlined the ethical issues that need to be considered with the emergence of molecular pathology. Molecular pathology is becoming a more important discipline in oncology, as molecular tumor characteristics will increasingly determine targeted clinical cancer care. This is changing the traditional role of pathologists from purely diagnostics to being actively involved in common treatment decisions based on identifying molecular targets. The advances in technological sequencing possibilities have outpaced interpretive skills, and attention to the ethical aspects has been lagging behind. Most existing guidelines or publications on ethical aspects of DNA sequencing are aimed at germline or tumor sequencing in clinical genetics or biomedical research settings. The molecular pathology context is however unique and complex as pathologists do not have direct patient contact, causing for example specific challenges to the implementation of adequate informed consent and return of results including unsolicited findings (e.g. *BRCA* mutations). We showed that ethical issues are present in every stage of the molecular pathology analysis procedure, starting at the order for tumor DNA sequencing and continuing after the results are disclosed with ethical issues related to data storage, re-analysis and future scientific use. For responsible further development of clinical cancer care, we recommend pathologists to take responsibility for the adequate use of molecular analyses and be fully aware and capable of dealing with the diverse, complex and challenging aspects of tumor DNA sequencing, including its ethical aspects.

To summarize, we identified several potential tissue biomarkers that could be used to identify *BRCA1/2* germline mutation carriers amongst breast cancer patients. We developed the most accurate and comprehensive immuno-based prediction model so far that could be used for detecting *BRCA1* as well as *BRCA2* germline mutation carriers, although it would require further external validation. We gained more insight into *BRCA1/2* methylation in breast and ovarian cancer. At the moment *BRCA1/2* methylation is not yet usable as a biomarker because of its complexity (CpG site dependency). We discovered many deregulated miRNAs, which may be potentially specific to *BRCA1/2*-related breast carcinogenesis and which, surprisingly, may be able to

distinguish *BRCA2*-related from sporadic breast carcinomas. Furthermore, we outlined that a wide range of moral duties has been attributed to genomics researchers, related to disclosure, consent, privacy, and social responsibilities. However, awareness and concrete implementation of these duties in research practice is lagging behind. Lastly, we showed that ethical issues are present in every stage of the molecular pathology analysis procedure. These include issues related to consent, disclosure as well as storage and future use of genetic data. Current molecular pathology guidelines are focused on technical issues and attention let alone consensus on ethical issues has been lagging behind. Pathologists should be encouraged to take responsibility for the adequate and responsible use of the molecular analyses their laboratories perform.

PERSONAL REFLECTION

Since I started my pathology residency and started doing biomedical research for this thesis, I came across several ethical issues related to my work as a biomedical researcher and pathologist in training. For example: what should I do with sporadic breast cancer cases that according to my prediction model would be at high risk of having an underlying *BRCA1/2* germline mutation? Are patients sufficiently informed about the advanced genetic analyses molecular pathology is able to perform on their tumor tissues? What should we do with unsolicited findings, which could uncover a potential hereditary cancer syndrome? Are we obliged to report these findings as we focus on tumor rather than germline DNA sequencing? For me, it has been a truly fulfilling and informative experience that I was able to reflect on my profession(s) and explore its moral dimensions in this thesis as well. I realized that I do not only want to perform translational research from a biomedical perspective, but at least as much I am driven to bridge the translational gap between ethical scholarship and pathology (research) practice. Ethics is not and should not be limited to the working field of ethicists alone as I learned about the value of ethical parallel research. I am driven to raise awareness on ethical challenges among my fellow biomedical researchers and pathologists, not to complicate matters, but to strive for responsible research and clinical (cancer) care, including development and implementation of exciting new technologies. I am happy I came to the realization that in this era of increasing subspecialization there is, in fact, still a need for bridge builders with a broad interest and a broad palet of skills to look and work beyond disciplines.

FUTURE DIRECTIONS

The research presented in this thesis has focused on 1) the potential role of several tissue biomarkers in predicting whether a breast tumor may be *BRCA1/2* germline mutation-related, and 2) which ethical requirements need to be met for responsible advancement of cancer research and clinical cancer care.

With respect to the first focus, further validation of biomarkers in general to check their robustness and reproducibility as well as identifying for which breast cancer patients the usage of these biomarkers would be most valuable and (cost-)effective, are desirable. Also, investigating combinations of tissue biomarkers, analysed serially or simultaneously, would be valuable before implementation in clinical practice. In the future, it will be more important to determine *BRCA1/2* mutations in breast cancer, as it may also have therapeutic consequences (e.g. indication for chemotherapy and treatment with PARP inhibitors) apart from the recognition of hereditary breast cancer [30, 31]. This is especially true for ovarian carcinomas, but it may be the case for breast carcinomas in the future as well. PARP inhibitors, already registered for ovarian carcinomas with either germline or somatic *BRCA* mutations, have also been shown to be effective in *BRCA1/2*-related breast cancers in phase II trials [32, 33]. Also, several studies are or have been investigating different chemotherapy regimens with promising results [30, 31]. Thus, it might be interesting to investigate the (cost-) effectiveness of our immuno-based prediction model in predicting *BRCA*ness in view of potential therapeutic consequences.

BRCA methylation analysis may also in the future be performed to determine eligibility for PARP inhibitor treatment as it has been shown that breast carcinomas with *BRCA1* methylation respond well to PARP inhibitor therapy [33–35]. However, it is still unknown which CpG sites are most important in predicting response to PARP inhibitors. These may be different from the CpG sites that may distinguish *BRCA1/2* germline mutation-related from sporadic breast carcinomas. Thus, further research is needed on which CpG sites to target for these two applications.

Research in oncology, including breast cancer, is increasingly characterized by large-scale genetic studies. Next-generation sequencing panels have been developed that can reliably detect *BRCA* mutations in formalin-fixed paraffin-embedded tumor tissues [36, 37]. Also, research techniques may be developed that can integrate clinical, radiological, pathological, genomic, and epigenetic (e.g. methylation, miRNA) data. With the rapid advances in sequencing technology, making it easier, faster, and less expensive, it may

be the case that many breast cancer patients may undergo (standard) *BRCA* sequencing in the future [38]. For ovarian cancer, it has recently be proposed that all patients with invasive epithelial ovarian, Fallopian tube, or peritoneal cancer, should be referred for *BRCA* genetic testing, irrespective of age or family history [39–42], as 20% harbours a *BRCA1/2* mutation (about 15% germline and 5% somatic mutations) [37, 39]. In breast cancer, the baseline risk of having a *BRCA1/2* mutation is lower with 11% of breast cancer patients having a *BRCA1/2* mutation (7% germline, 3% somatic) [43, 44]. Currently, *BRCA1/2* population-based genetic screening is not cost-effective in the general population or high-risk groups based upon family history, except among Ashkenazi Jews [45]. This leaves room for pre-screening tools such as immunohistochemistry. Moreover, the proportion of variants of unknown significance will likely increase with standard *BRCA* sequencing, yielding ethical challenges with respect to counselling and decision-making for clinicians and patients [46–48].

With respect to the second ethical focus of this thesis, it will be likely that (large-scale) DNA sequencing will increasingly take place in (breast) cancer research and clinical care. In general, careful evaluation of the benefits and harms of *BRCA1/2* mutation analysis is necessary for its consequences and influence on the well-being of breast cancer patients [24, 49–52]. Previous research has shown that breast cancer patients are willing to undergo genetic counseling and testing before surgery [49]. Moreover, patients have a low threshold in undergoing prophylactic surgeries to avoid potential future cancer [51]. However, it is important to realize that there are some breast cancer patients who over-estimate their cancer risk, experience increased distress, and may undergo unnecessary surgeries. Differences in risk perception and levels of distress have been shown between women affected and unaffected with breast cancer and women with and without a positive family history [50, 51]. Genetic counseling should take these differences into account to be able to adequately support and guide women.

As shown in **Chapter 7**, genomics researchers not only need to have technical skills, but also have moral duties toward their research and colleagues, research participants, and society in general. Further research is needed on the best ways to educate today's and future scientists and create awareness for identifying and balancing moral duties. In **Chapter 8**, we described the emergence of molecular pathology and the ethical issues associated with it. Further research is needed on how pathologists could learn to identify and deal with ethical issues and how to adequately act as educators of clinicians and patients about the benefits, risks, and limitations of DNA sequencing, so that clinicians and patients are able to make properly informed decisions concerning individual cancer therapy. For instance, interdisciplinary courses and collaborations

that bring together (bio)medical and ethics students and professionals should be encouraged to stimulate interdisciplinarity and ethical parallel research. The humanities should become a standard part of (bio)medical training. Also, it would be desirable that scientific (bio)medical journals have attention for ethical studies and that (bio)medical funding systems are aware of the value of ethical parallel research and provide support. Moreover, pathology should actively engage in interdisciplinary collaborations. For example, interdisciplinary symposiums and other meetings would be useful, where health care professionals, scientists, ethicists, policy makers, but also patients could meet to discuss issues like consent, disclosure, and 'hunting' (i.e. actively searching) for potential hereditary diseases in molecular pathology. Traditionally, pathologists have no direct patient contact. However, in the era of molecular pathology, patient interaction is desirable to become aware of patients' ideas and preferences on above-mentioned matters to come to a broadly accepted policy, so perhaps pathologists should start to participate in multidisciplinary outpatient clinics, or even open up their own outpatient clinic. There are exhilarating times ahead for molecular pathology as it is expected that tumor DNA analysis will become important for more and more tumor types and will involve an increasing number of genes. The risk of detecting potential clinically significant unsolicited findings will thereby increase. Also, techniques like liquid biopsies and organoid technology, which are currently only available in research settings, may enter clinical cancer care in the future and bring along unique challenges and ethical issues [53, 54]. This makes the awareness of ethical issues by pathologists and being capable of dealing with them adequately even more important in the future.

To conclude, this thesis shows that there are indications that pathology could play a role in the early detection of *BRCA1/2* germline mutation carriers among breast cancer patients. Several tissue biomarkers (immunohistochemistry, *BRCA* methylation, miRNAs) may be useful in distinguishing *BRCA1/2* germline mutation-related from sporadic breast carcinomas, independent from clinical factors. However, further validation and determination of the best (combination of) technique(s) as well as the exact target population are needed. Technical advances alone are however not enough for the (responsible) advancement of personalized cancer care. It calls for an active role of researchers and pathologists, who should be aware of their moral duties and responsibilities with respect to participants, patients and society.

REFERENCES

1. Hassanein M, Huiart L, Bourdon V, Rabayrol L, Geneix J, Nogues C, et al. Prediction of BRCA1 Germ-Line Mutation Status in Patients with Breast Cancer Using Histoprognosis Grade, MS110, Lys27H3, Vimentin, and KI67. Pathobiology. 2013;80:219–227.
2. Lakhani SR, Van De Vijver MJ, Jacquemier J, Anderson TJ, Osin PP, McGuffog L, et al. The pathology of familial breast cancer: predictive value of immunohistochemical markers estrogen receptor, progesterone receptor, HER-2, and p53 in patients with mutations in BRCA1 and BRCA2. J Clin Oncol. 2002;20:2310–8.
3. Lakhani SR, Reis-Filho JS, Fulford L, Penault-Llorca F, van der Vijver M, Parry S, et al. Prediction of BRCA1 status in patients with breast cancer using estrogen receptor and basal phenotype. Clin Cancer Res. 2005;11:5175–80.
4. Spurdle AB, Couch FJ, Parsons MT, McGuffog L, Barrowdale D, Bolla MK, et al. Refined histopathological predictors of BRCA1 and BRCA2mutation status: a large-scale analysis of breast cancer characteristics from the BCAC, CIMBA, and ENIGMA consortia. Breast Cancer Res. 2014;16:3419.
5. Honrado E, Osorio A, Palacios J, Milne RL, Sánchez L, Díez O, et al. Immunohistochemical expression of DNA repair proteins in familial breast cancer differentiate BRCA2-associated tumors. J Clin Oncol. 2005;23:7503–11.
6. Esteller M, Fraga MF, Guo M, Garcia-Foncillas J, Hedenfalk I, Godwin AK, et al. DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet. 2001;10:3001–3007.
7. Tapia T, Smalley S V, Kohen P, Muñoz A, Solis LM, Corvalan A, et al. Promoter hypermethylation of BRCA1 correlates with absence of expression in hereditary breast cancer tumors. Epigenetics. 2008;3:157–63.
8. Dworkin AM, Spearman AD, Tseng SY, Sweet K, Toland AE. Methylation not a frequent "second hit" in tumors with germline BRCA mutations. Fam Cancer. 2009;8:339–346.
9. Goodheart MJ, Rose SL, Hattermann-Zogg M, Smith BJ, De Young BR, Buller RE. BRCA2 alteration is important in clear cell carcinoma of the ovary. Clin Genet. 2009;76:161–167.
10. Kontorovich T, Cohen Y, Nir U, Friedman E. Promoter methylation patterns of ATM, ATR, BRCA1, BRCA2 and P53 as putative cancer risk modifiers in Jewish BRCA1/BRCA2 mutation carriers. Breast Cancer Res Treat. 2009;116:195–200.
11. Tung N, Miron A, Schnitt SJ, Gautam S, Fetten K, Kaplan J, et al. Prevalence and predictors of loss of wild type BRCA1 in estrogen receptor positive and negative BRCA1-associated breast cancers. Breast Cancer Res. 2010;12:R95.
12. Rennstam K, Ringberg A, Cunliffe HE, Olsson H, Landberg G, Hedenfalk I. Genomic alterations in histopathologically normal breast tissue from BRCA1 mutation carriers may be caused by BRCA1 haploinsufficiency. Genes Chromosom Cancer. 2010;49:78–90.
13. Lisowska KM, Dudaladava V, Jarzab M, Huzarski T, Chmielik E, Stobiecka E, et al. BRCA1-related gene signature in breast cancer: the role of ER status and molecular type. Front Biosci (Elite Ed). 2011;3:125–36.
14. Lips EH, Mulder L, Oonk A, van der Kolk LE, Hogervorst FBL, Imholz ALT, et al. Triple-negative breast cancer: BRCAness and concordance of clinical features with BRCA1-mutation carriers. Br J Cancer. 2013;108:2172–2177.
15. Toffoli S, Bar I, Abdel-Sater F, Delree P, Hilbert P, Cavallin F, et al. Identification by array comparative genomic hybridization of a new amplicon on chromosome 17q highly recurrent in BRCA1 mutated triple negative breast cancer. Breast Cancer Res. 2014;16:466.
16. Severson TM, Peeters J, Majewski I, Michaut M, Bosma A, Schouten PC, et al. BRCA1-like signature in triple negative breast cancer: Molecular and clinical characterization reveals subgroups with therapeutic potential. Mol Oncol. 2015;9:1528–1538.
17. Daniels SL, Burghel GJ, Chambers P, Al-Baba S, Connley DD, Brock IW, et al. Levels of DNA Methylation Vary at CpG Sites across the BRCA1 Promoter, and Differ According to Triple Negative and "BRCA-Like" Status, in Both Blood and Tumour DNA. PLoS One. 2016;11:e0160174.

18. Volinia S, Calin G a, Liu C-G, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A. 2006;103:2257–61.

19. Iorio M V, Ferracin M, Liu C-G, Veronese A, Spizzo R, Sabbioni S, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 2005;65:7065–70.

20. Farazi TA, Spitzer JI, Morozov P, Tuschl T. miRNAs in human cancer. J Pathol. 2011;223:102–15.

21. Lowery AJ, Miller N, Devaney A, McNeill RE, Davoren P a, Lemetre C, et al. MicroRNA signatures predict oestrogen receptor, progesterone receptor and HER2/neu receptor status in breast cancer. Breast Cancer Res. 2009;11:R27.

22. Xi Y, Nakajima GO, Gavin E, Morris CG, Kudo K, Hayashi K, et al. Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. RNA. 2007;13:1668–1674.

23. Kalita-de Croft P, Al-Ejeh F, McCart Reed AE, Saunus JM, Lakhani SR. 'Omics Approaches in Breast Cancer Research and Clinical Practice. Adv Anat Pathol. 2016;23:356–367.

24. Schmidt MK, Vermeulen E, Tollenaar RAEM, Veer LJV, Leeuwen FE van. Regulatory aspects of genetic research with residual human tissue: Effective and efficient data coding. Eur J Cancer. 2009;45:2376–2382.

25. Vermeulen E, Schmidt MK, Aaronson NK, Kuenen M, van Leeuwen FE. Obtaining "fresh" consent for genetic research with biological samples archived 10 years ago. Eur J Cancer. 2009;45:1168–1174.

26. Pulford DJ, Harter P, Floquet A, Barrett C, Suh DH, Friedlander M, et al. Communicating BRCA research results to patients enrolled in international clinical trials: lessons learnt from the AGO-OVAR 16 study. BMC Med Ethics. 2016;17:63.

27. Ghaly M, Ali M, Auda J, Beauchamp TL, Al-Qaradaghi A, Bredenoord AL, et al. Islamic Perspectives on the Principles of Biomedical Ethics. London: World Scientific Publishing; 2016.

28. Bredenoord AL, Kroes HY, Cuppen E, Parker M, van Delden JJM. Disclosure of individual genetic data to research participants: the debate reconsidered. Trends Genet. 2011;27:41–7.

29. Virani AH, Longstaff H. Ethical Considerations in Biobanks: How a Public Health Ethics Perspective Sheds New Light on Old Controversies. J Genet Couns. 2015;24:428–432.

30. Bayraktar S, Glück S. Systemic therapy options in BRCA mutation-associated breast cancer. Breast Cancer Res Treat. 2012;135:355–66.

31. Smith KL, Isaacs C. BRCA Mutation Testing in Determining Breast Cancer Therapy. Cancer J. 2011;17:492–499.

32. Tutt A, Robson M, Garber JE, Domchek SM, Audeh MW, Weitzel JN, et al. Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial. Lancet. 2010;376:235–44.

33. Dizdar O, Arslan C, Altundag K. Advances in PARP inhibitors for the treatment of breast cancer. Expert Opin Pharmacother. 2015;16:2751–8.

34. Lee J-M, Ledermann JA, Kohn EC. PARP Inhibitors for BRCA1/2 mutation-associated and BRCA-like malignancies. Ann Oncol. 2014;25:32–40.

35. Livraghi L, Garber JE. PARP inhibitors in the management of breast cancer: current data and future prospects. BMC Med. 2015;13:188.

36. Badoer C, Garrec C, Goossens D, Ellison G, Mills J, Dzial M, et al. Performance of multiplicom's BRCA MASTR Dx kit on the detection of BRCA1 and BRCA2 mutations in fresh frozen ovarian and breast tumor samples. Oncotarget. 2016;7:81357-81366.

37. Weren RDA, Mensenkamp AR, Simons M, Eijkelenboom A, Sie AS, Ouchene H, et al. Novel BRCA1 and BRCA2 Tumor Test as Basis for Treatment Decisions and Referral for Genetic Counselling of Patients with Ovarian Carcinomas. Hum Mutat. 2017;38:226-235.

38. Stoppa-Lyonnet D. The biological effects and clinical implications of BRCA mutations: where do we go from here? Eur J Hum Genet. 2016;24 Suppl 1:S3-9.

39. Vergote I, Banerjee S, Gerdes A-M, van Asperen C, Marth C, Vaz F, et al. Current perspectives on recommendations for BRCA genetic testing in ovarian cancer patients. Eur J Cancer. 2016;69:127–134.
40. Arts-de Jong M, de Bock GH, van Asperen CJ, Mourits MJE, de Hullu JA, Kets CM. Germline BRCA1/2 mutation testing is indicated in every patient with epithelial ovarian cancer: A systematic review. Eur J Cancer. 2016;61:137–45.
41. Daly MB, Pilarski R, Axilbund JE, Berry M, Buys SS, Crawford B, et al. Genetic/Familial High-Risk Assessment: Breast and Ovarian, Version 2.2015. J Natl Compr Canc Netw. 2016;14:153–62.
42. Lancaster JM, Powell CB, Chen L, Richardson DL, SGO Clinical Practice Committee. Society of Gynecologic Oncology statement on risk assessment for inherited gynecologic cancer predispositions. Gynecol Oncol. 2015;136:3–7.
43. Winter C, Nilsson MP, Olsson E, George AM, Chen Y, Kvist A, et al. Targeted sequencing of *BRCA1* and *BRCA2* across a large unselected breast cancer cohort suggests that one-third of mutations are somatic. Ann Oncol. 2016;27:1532–1538.
44. Koboldt DC, Fulton RS, McLellan MD, Schmidt H, Kalicki-Veizer J, McMichael JF, et al. Comprehensive molecular portraits of human breast tumours. Nature. 2012;490:61–70.
45. D'Andrea E, Marzuillo C, De Vito C, Di Marco M, Pitini E, Vacchio MR, et al. Which BRCA genetic testing programs are ready for implementation in health care? A systematic review of economic evaluations. Genet Med. 2016;18:1171–1180.
46. Varesco L, Viassolo V, Viel A, Gismondi V, Radice P, Montagna M, et al. Performance of BOADICEA and BRCAPRO genetic models and of empirical criteria based on cancer family history for predicting BRCA mutation carrier probabilities: a retrospective study in a sample of Italian cancer genetics clinics. Breast. 2013;22:1130–5.
47. Narod SA. BRCA mutations in the management of breast cancer: the state of the art. Nat Rev Clin Oncol. 2010;7:702–7.
48. Eccles DM, Mitchell G, Monteiro ANA, Schmutzler R, Couch FJ, Spurdle AB, et al. BRCA1 and BRCA2 genetic testing-pitfalls and recommendations for managing variants of uncertain clinical significance. Ann Oncol Off J Eur Soc Med Oncol. 2015;26:2057–65.
49. Schwartz MD, Lerman C, Brogan B, Peshkin BN, Halbert CH, DeMarco T, et al. Impact of BRCA1/BRCA2 Counseling and Testing on Newly Diagnosed Breast Cancer Patients. J Clin Oncol. 2004;22:1823–1829.
50. van Roosmalen MS, Stalmeier PFM, Verhoef LCG, Hoekstra-Weebers JEHM, Oosterwijk JC, Hoogerbrugge N, et al. Impact ofBRCA1/2 testing and disclosure of a positive test result on women affected and unaffected with breast or ovarian cancer. Am J Med Genet. 2004;124A:346–355.
51. Shkedi-Rafid S, Gabai-Kapara E, Grinshpun-Cohen J, Levy-Lahad E. BRCA genetic testing of individuals from families with low prevalence of cancer: experiences of carriers and implications for population screening. Genet Med. 2012;14:688–94.
52. Brédart A, Kop J-L, De Pauw A, Caron O, Fajac A, Noguès C, et al. Effect on perceived control and psychological distress of genetic knowledge in women with breast cancer receiving a BRCA1/2 test result. The Breast. 2017;31:121–127.
53. Bredenoord AL, Clevers H, Knoblich JA. Human tissues in a dish: The research and ethical implications of organoid technology. Science. 2017;355.
54. Friedrich MJ. Going With the Flow: The Promise and Challenge of Liquid Biopsies. JAMA. 2017;318:1095–1097.

Chapter 10

Dutch summary / Nederlandse samenvatting

INTRODUCTIE

Borstkanker is de meest voorkomende vorm van kanker bij vrouwen. Ongeveer één op de acht vrouwen in de Westerse wereld krijgt in haar leven borstkanker. Een familiaire belasting is één van de grootste risicofactoren. In 5-10% van de gevallen betreft het erfelijke borstkanker, meestal als gevolg van een kiembaanmutatie in de genen *BRCA1* of *BRCA2*. Het opsporen van potentiële *BRCA1/2* kiembaanmutatiedraagsters is belangrijk, omdat deze vrouwen een kans hebben van 50-60% om in hun leven borstkanker te ontwikkelen en 6-39% om eierstokkanker te ontwikkelen. Het hebben van een *BRCA1/2* kiembaanmutatie heeft belangrijke preventieve en ook steeds meer therapeutische consequenties. Wat betreft preventie kan gedacht worden aan chirurgie waarbij borsten en eierstokken preventief worden verwijderd of surveillance door middel van mammografie of MRI. Wat betreft behandeling wordt er vaker gekozen voor een borstamputatie dan voor een borstsparende behandeling. Daarnaast heeft een *BRCA1/2* kiembaanmutatie steeds meer consequenties voor aanvullende systeemtherapie (andere chemotherapieschema's of gebruik van PARP remmers).

Op dit moment komen borstkankerpatiënten in aanmerking voor genetisch onderzoek op basis van met name klinische criteria, bijvoorbeeld wanneer er sprake is van een jonge patiënt, aanwezigheid van dubbelzijdige borstkanker, of het familiair voorkomen van borst- en ovariumkanker of andere *BRCA1/2*-gerelateerde tumoren (**Hoofdstuk 1**). Deze criteria zijn echter niet optimaal, aangezien is voorspeld dat 5% tot 25% van de *BRCA1/2*-mutatiedraagsters wordt gemist. Dit kan onder andere worden verklaard uit het feit dat families kleiner worden, dat sommige patiënten al wat ouder zijn bij diagnose en dat de mutaties ook kunnen worden overgeërfd via mannen die minder vaak borstkanker ontwikkelen.

DIT PROEFSCHRIFT: EEN ZOEKTOCHT NAAR WEEFSELBIOMARKERS VOOR *BRCA1/2*-GERELATEERDE BORSTKANKER

De centrale vraag van dit proefschrift was: in hoeverre kan de klinische pathologie een rol spelen bij de vroegopsporing van *BRCA1/2*-kiembaanmutatiedraagsters onder borstkankerpatiënten? Meer specifiek: welke weefselbiomarkers zouden gebruikt kunnen worden bij het identificeren van potentiële *BRCA1/2*-kiembaanmutatiedraagsters? Het doel van dit proefschrift was dan ook om specifieke *BRCA1/2*-gerelateerde tumorkarakteristieken met betrekking tot eiwitexpressie of moleculaire afwijkingen in

het DNA te identificeren die gebruikt zouden kunnen worden voor het opsporen van *BRCA1/2*-gerelateerde borstkanker op het moment van reguliere pathologiediagnostiek (Deel 1 van dit proefschrift). Op basis van die tumorkarakteristieken zou dan een inschatting gemaakt kunnen worden of een patiënt zou moeten worden doorverwezen naar de klinische genetica voor aanvullend genetisch onderzoek. Onze hypothese was dat specifieke *BRCA1/2*-gerelateerde tumorkarakteristieken geïdentificeerd kunnen worden, omdat deze tumoren gekenmerkt worden door een defect herstelmechanisme van DNA-dubbelstrengsbreuken (homologe recombinatie) met genomische instabiliteit tot gevolg. Eerdere studies hebben al morfologische, immuunhistochemische en moleculaire verschillen aangetoond tussen *BRCA1/2*-gerelateerde en sporadische borstkanker (**Hoofdstuk 2**). Weinig studies hebben echter geprobeerd deze te vertalen naar een klinisch toepasbare test, met name als het gaat om *BRCA2*-gerelateerde borstkanker. Overigens moet opgemerkt worden dat sporadische borsttumoren ook tekenen van een *BRCA*-deficiëntie kunnen tonen, als gevolg van andere mechanismen dan een *BRCA* kiembaanmutatie, bijvoorbeeld door *BRCA* somatische mutaties ontstaan in de tumor of epigenetische *BRCA* veranderingen, zoals methylering.

De technologische mogelijkheden voor het analyseren van tumorweefsel zijn enorm uitgebreid in de afgelopen jaren. Een van de grootste ontwikkelingen betreft het gebruik van grootschalige DNA sequentieanalyse in het kankeronderzoek als ook in de reguliere pathologiediagnostiek. Dit heeft zelfs geleid tot een nieuw subspecialisme: de moleculaire pathologie. Deze technologische ontwikkelingen brengen ook (nieuwe) morele verantwoordelijkheden met zich mee voor zowel biomedische onderzoekers als pathologen, omdat ze grote hoeveelheden potentieel privacygevoelige data opleveren. Ethische reflectie en discussie hierover lopen echter achter. Tijdens het werken aan dit proefschrift realiseerde ik me dat voor een verantwoorde vooruitgang van het kankeronderzoek en de zorg voor kankerpatiënten aan bepaalde ethische vereisten moet worden voldaan, zodat onder andere de autonomie van proefpersonen en patiënten en transparantie worden gewaarborgd. Derhalve besloot ik om ook een aantal 'gen-ethische' aspecten van het doen van biomedisch (kanker)onderzoek en van de moderne (moleculaire) pathologiepraktijk in dit proefschrift mee te nemen (Deel 2 van dit proefschrift). Hierbij waren de onderzoeksvragen als volgt: met welke (nieuwe) morele verantwoordelijkheden worden kankeronderzoekers geconfronteerd als zij grootschalige DNA sequentieanalysetechnieken gebruiken? En hoe kunnen ze daar adequaat mee omgaan? Welke ethische aspecten moeten worden overwogen bij de opkomst van de moleculaire pathologie? En hoe moet daarmee worden omgaan voor een verantwoorde ontwikkeling van 'personalized cancer care'?

DEEL 1: IMMUUNHISTOCHEMISCHE EN MOLECULAIRE ASPECTEN

In **Hoofdstuk 3** hebben we de grootste immunoprofiling studie in *BRCA1/2*-geassocierde borstkanker verricht tot nu toe. Hierbij werd gekeken naar verschillen in morfologische kenmerken en met name eiwitexpressie tussen *BRCA1/2*-gerelateerde en sporadische borsttumoren. Eiwitexpressie werd onderzocht door middel van immuunhistochemie, een techniek die in principe beschikbaar is in alle pathologielaboratoria. Hieruit hebben we een veelbelovend, accuraat predictiemodel kunnen ontwikkelen om *BRCA1/2*-gerelateerde borstkanker te kunnen identificeren. Dit predictiemodel bestaat uit 14 predictoren (leeftijd, mitotische activiteitsindex, en expressie van de eiwitten cyclineD1, ERα, ERβ, FGFR2, FGFR3, FGFR4, GLUT1, IGFR, Ki67, MLH1, p120 en TOP2A), dat zeer goed onderscheid kan maken tussen *BRCA1/2*-gerelateerde en sporadische borsttumoren (AUC = 0.943 (95%CI: 0.909-0.978)), waarbij de voorspelde en daadwerkelijke kansen redelijk goed overeenkomen. Van enkele eiwitten in het model is bekend van andere publicaties dat deze verschillend tot expressie komen tussen *BRCA1/2*-gerelateerde en sporadische borstkanker. De nauwkeurigheid van ons model lijkt beter dan de huidige gepubliceerde predictiemodellen gebaseerd op klinische, morfologische en/of immuunhistochemische kenmerken en vergelijkbaar met moleculaire predictiemodellen. Ons predictiemodel, dat nagenoeg onafhankelijk is van klinische variabelen, zou derhalve kunnen bijdragen aan het identificeren van borstkankerpatiënten met een hoog risico op een onderliggende *BRCA1/2* kiembaanmutatie. Dit model zou bijvoorbeeld kunnen dienen als screeningstest voor kiembaanmutatieanalyse. Voordat het model in de klinische praktijk geïmplementeerd kan worden, dient echter nog aanvullend onderzoek verricht te worden. Zo moet de reproduceerbaarheid van het beoordelen van de immuunhistochemische kleuringen tussen verschillende pathologen worden onderzocht en is nog externe validatie nodig om de generaliseerbaarheid van het model te testen. Ook zal verder onderzoek gewenst zijn om te kijken voor welke groep patiënten het gebruik van dit predictiemodel het meest waardevol is, gezien het feit dat er extra kosten mee gemoeid zijn.

In **Hoofdstuk 4** hebben we gekeken naar de diagnostische waarde van *BRCA1/2* promoter methylering voor het onderscheiden van *BRCA1/2*-gerelateerde en sporadische borsttumoren. DNA-methylering is een epigenetisch proces waarbij een methylgroep aan een histoneiwit van het DNA wordt toegevoegd. Dit gebeurt met name in zogenaamde CpG-gebieden. DNA-methylering speelt een belangrijke rol bij de ontwikkeling van meerdere vormen van kanker, waaronder borstkanker, omdat het een remmend effect heeft op genexpressie van o.a. tumorsuppressorgenen. In de literatuur

is gesuggereerd dat *BRCA1/2* methylering nagenoeg alleen voorkomt in sporadische borsttumoren, waardoor *BRCA1/2* methyleringsanalyse potentieel gebruikt zou kunnen als screeningstest voor *BRCA1/2*-kiembaanmutatieanalyse. Dit is echter nog niet uitgebreid onderzocht. Wij hebben *BRCA1/2* methylering bepaald in *BRCA1/2*-gerelateerde en sporadische borsttumoren middels een nieuw ontwikkelde MS-MLPA (Methylation-Specific Multiplex Ligation-dependent Probe Amplification) assay. Dit is een snelle en relatief goedkope techniek die goed werkt op formaline-gefixeerd weefselmateriaal en is derhalve goed toepasbaar in de dagelijkse pathologiepraktijk. Wij ontdekten dat *BRCA* methyleringsfrequenties sterk verschillen tussen CpG gebieden. De diagnostische waarde van *BRCA1/2* methyleringsanalyse is daardoor sterk afhankelijk van de keuze van CpG gebieden. Sommige CgP gebieden waren zelden gemethyleerd in sporadische tumoren terwijl andere gebieden juist zelden gemethyleerd waren in erfelijke tumoren. De sensitiviteit en specificiteit waren het meest met elkaar in balans wanneer alle *BRCA2* CpG gebieden in deze assay werden gebruikt en wanneer er sprake was van methylering van tenminste één van deze CpG gebieden (sensitiviteit 69.4%, specificiteit 87.5%). In totaal toonde tenminste 63.8% van de *BRCA1/2*-gerelateerde tumoren methylering van tenminste één van de onderzochte CpG gebieden in de *BRCA1* of *BRCA2* promoter. Derhalve ondersteunen onze resultaten niet de eerdere aanname dat *BRCA* promoter methylering zelden voorkomt in *BRCA*-gerelateerde borsttumoren. Voor zover wij weten is dit de grootste studie die zowel *BRCA1* als *BRCA2* methylering heeft onderzocht in zowel *BRCA1*- als *BRCA2*-gerelateerde en sporadische borsttumoren. Onze resultaten zijn belangrijk voor een adequate toepassing van *BRCA* methyleringsanalyse als screening voor *BRCA* mutatieanalyse. Nader onderzoek is nodig ter verificatie en om te bepalen welke eventuele andere CpG gebieden in de *BRCA1* en *BRCA2* promoter belangrijk kunnen zijn in het onderscheiden van *BRCA1/2*-gerelateerde en sporadische borsttumoren.

Omdat onze methyleringsfrequenties verschilden van eerder gepubliceerde studies, hebben we in **Hoofdstuk 5** de eerste systematische review uitgevoerd waarbij is gekeken naar hoe vaak *BRCA* promoter methylering nu precies is aangetoond in borst- en ovariumtumoren van *BRCA1/2* kiembaanmutatiedraagsters en wat de diagnostische waarde zou zijn als *BRCA* methyleringsanalyse gebruikt zou worden om onderscheid te maken tussen *BRCA1/2*-gerelateerde en sporadische tumoren. We hebben daarbij ook gekeken welke CpG gebieden zijn onderzocht in de verschillende studies. In totaal voldeden er 21 studies aan de inclusiecriteria voor onze review. Deze studies toonden aan dat *BRCA* methylering in het algemeen zeer weinig voorkomt in *BRCA1/2*-gerelateerde borst- en ovariumtumoren. *BRCA1* methylering werd aangetoond in tenminste 3.6% van de borst- en 1.1% van de ovariumtumoren. *BRCA2* methylering werd aangetoond

in tenminste 5.3% van de borst- en 0.0% van de ovariumtumoren. In het algemeen zou, als methyleringsanalyse werd uitgevoerd om een *BRCA* kiembaanmutatie uit te sluiten wanneer methylering wordt aangetroffen, de sensitiviteit maximaal 100% zijn, maar de specificiteit hooguit 23.1%. Hierbij is er vergeleken met beschikbare data in sporadische tumoren. Echter, er moet worden opgemerkt dat de studies grote verschillen toonden in methodologie, kwaliteit en risico op bias. Verder werd er in het algemeen een beperkte methylering- en incomplete mutatieanalyse verricht. Derhalve kan het zo zijn dat de frequentie van *BRCA1/2* methylering in *BRCA1/2*-gerelateerde tumoren onderschat wordt met potentieel grote klinische consequenties. Daarnaast is gebleken dat de methyleringsfrequentie varieert tussen CpG gebieden. Op dit moment is nog onduidelijk welke CpG gebieden de hoogste diagnostische waarde hebben in het onderscheiden van *BRCA1/2*-gerelateerde en sporadische tumoren.

In **Hoofdstuk 6** hebben we een miRNA profiling studie verricht om te onderzoeken welke miRNAs gedereguleerd zijn in *BRCA1/2*-gerelateerde borstkanker om meer inzicht te krijgen in *BRCA1/2*-gerelateerde carcinogenese en mogelijke diagnostische biomarkers of therapeutische targets te kunnen identificeren. miRNAs zijn korte RNA moleculen die invloed hebben op genexpressie. Als miRNAs binden aan mRNA moleculen wordt het aflezen van het mRNA tegengehouden en daarmee wordt genexpressie geremd. Hoewel er meerdere studies zijn gedaan naar miRNA deregulatie in sporadische borstkanker, was er nog weinig bekend over miRNA deregulatie in erfelijke borstkanker. miRNA biomarkers zouden vrij gemakkelijk in de pathologiepraktijk geïmplementeerd kunnen worden, omdat ze vrij resistent zijn tegen het degradatieproces veroorzaakt door formalinefixatie. We ontdekten dat er veel meer miRNAs verschillend tot expressie komen tussen borstkanker- en normale borstweefsels van *BRCA1/2* mutatiedraagsters, vergeleken met beschikbare data over sporadische tumoren. Dit zou mogelijk te maken kunnen hebben met de genomische instabiliteit in *BRCA1/2*-gerelateerde borsttumoren. We vonden echter ook meerdere miRNAs die zowel gedereguleerd zijn in *BRCA1/2*-gerelateerde als sporadische borstkanker. Meer onderzoek is nodig om onze resultaten te verifiëren en om te kijken of de dereguleerde miRNAs ook gebruikt kunnen worden als nieuwe aangrijpingspunten voor diagnostiek of therapie.

DEEL 2: 'GEN-ETHISCHE' ASPECTEN

In deel 2 van dit proefschrift hebben we de 'gen-ethische' aspecten van het doen van biomedisch (kanker)onderzoek en van de moderne (moleculaire) pathologiepraktijk onderzocht, als een reflectieve aanvulling op het biomedische onderzoek uit het eerste deel. Zoals eerder beschreven wordt het huidige kankeronderzoek en de huidige kankerdiagnostiek steeds meer gekenmerkt door het gebruik van grootschalig genetisch onderzoek met behulp van next-generation sequencing. Deze geavanceerde technieken kunnen ook steeds meer worden toegepast op formaline-gefixeerd restmateriaal. Restmateriaal vormt een rijke bron van samples voor biomedisch (kanker)onderzoek. De ontwikkelingen in DNA sequentieanalysetechnieken, bioinformatica en het opslaan en delen van weefsels en data hebben geleid tot een beter begrip van het ontstaan van kanker en dragen in toenemende mate bij aan de ontwikkeling van personalized cancer care en precision medicine. Hierbij wordt de behandeling van kanker gebaseerd op specifieke genetische afwijkingen in de tumor van de patiënt. Aan de andere kant zorgen deze ontwikkelingen ook voor (nieuwe) morele verantwoordelijkheden en dilemma's voor onderzoekers en pathologen. In het erfelijke borstkankeronderzoek is er bijvoorbeeld discussie in hoeverre en onder welke voorwaarden er *BRCA* mutatieanalyse mag worden verricht op restmateriaal. Hoe kunnen de nieuwe, krachtige onderzoeksmethoden zodanig worden ingezet dat ze bijdragen aan een verantwoorde ontwikkeling van personalized cancer care?

In **Hoofdstuk 7** hebben we geanalyseerd welke morele verantwoordelijkheden de afgelopen jaren in de internationale literatuur zijn toegeschreven aan onderzoekers die gebruik van maken grootschalige DNA analysetechnieken (genomisch onderzoekers). We hebben in kaart gebracht dat een steeds breder palet aan morele verantwoordelijkheden aan onderzoekers wordt toegeschreven, gerelateerd aan terugkoppeling van resultaten, toestemming, privacy en maatschappelijke verantwoordelijkheden. De implementatie van deze morele verantwoordelijkheden in de onderzoekspraktijk loopt echter achter. Dit heeft deels te maken met de context- en tijdsafhankelijkheid van morele verantwoordelijkheden. (Conflicterende) morele verantwoordelijkheden vereisen afweging en specificatie in een specifieke context. We menen dat ethisch bewustzijn bij onderzoekers de sleutel is tot implementatie in de onderzoekspraktijk, om morele verantwoordelijkheden te identificeren en ze ook tot op zekere hoogte te kunnen afwegen tegen elkaar. Deze vaardigheden zijn anders dan de traditionele technisch-wetenschappelijke vaardigheden en zullen tijdens de opleiding van onderzoekers moeten worden ontwikkeld. Genomisch onderzoek vraagt meer van zowel onderzoekers als proefpersonen. Om een voldoende aantal proefpersonen te

verkrijgen voor een wetenschappelijk onderzoek van wie meer wordt gevraagd onder andere wat betreft privacy, moet het biomedische onderzoek tot op zekere hoogte aan hun behoeftes tegemoetkomen (bijvoorbeeld het terugkoppelen van onderzoeksresultaten) en maatschappelijk vertrouwen in de wetenschap waarborgen.

In **Hoofdstuk 8** hebben we geanalyseerd welke ethische aspecten overwogen moeten worden bij de opkomst van de moleculaire pathologie. Moleculaire pathologie wordt een steeds belangrijker specialisme in de oncologie, omdat moleculaire tumorkarakteristieken toenemend bepalend zijn voor de behandeling. Hierdoor verandert ook de rol van de patholoog in de zorg, welke niet meer alleen de diagnostiek omvat, maar ook steeds meer betrekking heeft op het behandelbeleid. De opkomst van de moleculaire pathologie is mogelijk gemaakt door grote technologische ontwikkelingen in de DNA sequentieanalyse. Aandacht voor de ethische aspecten van de moleculaire pathologie blijven echter achter. Zo zijn de meeste richtlijnen of publicaties over ethische aspecten van DNA sequentieanalyse gericht op kiembaanmutatieanalyse in de klinische genetica of tumor DNA sequentieanalyse in de context van biomedisch onderzoek. Wij laten zien dat ethische aspecten aanwezig zijn in elke fase van het moleculaire pathologieproces. Dit begint bij de aanvraag en loopt door nadat de uitslagen zijn teruggekoppeld, met ethische aandachtspunten gerelateerd aan data-opslag en nader gebruik van genetische data voor onderzoek. Richtlijnen uit aanverwante vakgebieden kunnen als uitgangspunt dienen voor de omgang met deze ethische aspecten. Ze kunnen echter niet zo maar direct worden overgenomen, omdat de moleculaire pathologiecontext uniek en complex is, onder andere omdat pathologen niet direct patiëntencontact hebben. Dit geeft specifieke uitdagingen voor bijvoorbeeld de implementatie van informed consent en de terugkoppeling van nevenbevindingen (zoals mogelijke *BRCA* kiembaanmutaties). Voor een goede en verantwoorde ontwikkeling van de kankerzorg, willen wij pathologen aanmoedigen om verantwoordelijkheid te nemen voor het adequaat gebruik van moleculaire analyses en zich bewust te zijn van en om te kunnen gaan met de diverse, complexe en uitdagende aspecten van tumor DNA sequentieanalyse, inclusief de ethische aspecten ervan.

TOEKOMSTPERSPECTIEF

Het onderzoek beschreven in dit proefschrift was gericht op 1) de potentiële rol van weefselbiomarkers in het voorspellen of een borsttumor gerelateerd kan zijn aan een *BRCA1/2*-kiembaanmutatie en 2) de ethische aandachtspunten waarmee rekening gehouden moet worden voor een verantwoorde ontwikkeling van het kankeronderzoek en de zorg voor patiënten met kanker in de moleculaire pathologiepraktijk.

Met betrekking tot het onderzoek in het eerste deel van het proefschrift is zoals hierboven ook al beschreven verdere validatie van de biomarkers in het algemeen wenselijk om onder andere hun reproduceerbaarheid te controleren en om te bepalen voor welke borstkankerpatiënten het gebruik van deze biomarkers het meest waardevol en (kosten)effectief is. Ook is het interessant om hierbij de waarde van combinaties van weefselbiomarkers te onderzoeken. De verwachting is dat het in de toekomst steeds belangrijker wordt om *BRCA1/2* mutaties te analyseren, niet alleen vanwege het erfelijke aspect, maar omdat het in toenemende mate ook therapeutische consequenties heeft voor bijvoorbeeld chemotherapie en behandeling met PARP remmers. Ook wordt het voor de behandeling steeds belangrijker om *BRCA1/2* afwijkingen anders dan kiembaanmutaties te detecteren, zoals *BRCA1/2* methylering. Er is aangetoond dat borsttumoren met *BRCA1* methylering goed reageren op PARP inhibitietherapie. Het is echter nog onduidelijk welke CpG gebieden belangrijk zijn bij het voorspellen van de respons op PARP inhibitietherapie. Dit zouden andere CpG gebieden kunnen zijn dan degene die *BRCA1/2* kiembaanmutatie-gerelateerde van sporadische tumoren kunnen onderscheiden. Vervolgonderzoek is nodig welke CpG gebieden het belangrijkst zijn voor deze twee verschillende toepassingen.

Waarschijnlijk gaat er nog meer gebruik worden gemaakt van (grootschalig) DNA-onderzoek in het (borstkanker)onderzoek en de zorg. Het wordt steeds gemakkelijker en goedkoper om DNA sequentieanalyseonderzoek te verrichten, waardoor patiënten met borstkanker wellicht in de toekomst standaard (*BRCA1/2*) mutatieanalyse zouden kunnen ondergaan. Op dit moment is dit nog niet kosteneffectief, waardoor er nog ruimte is voor pre-screeningstechnieken als immuunhistochemie of methylering, zoals beschreven in het eerste deel van het proefschrift. Hoe dan ook is zorgvuldige evaluatie van de voordelen en nadelen van *BRCA1/2* mutatieanalyse noodzakelijk voor de potentiële consequenties op het welzijn van borstkankerpatiënten. Eerder onderzoek heeft aangetoond dat borstkankerpatiënten erg bereid zijn om genetische counseling en onderzoek te ondergaan en dat zij laagdrempelig kiezen voor prophylactische chirurgie. Het is echter belangrijk te realiseren dat sommige patiënten hun risico op borstkanker overschatten met veel psychologische gevolgen. Het is belangrijk om hiermee rekening te houden bij de counseling om patiënten zo goed mogelijk te kunnen begeleiden.

Uit ons onderzoek in het tweede deel van het proefschrift blijkt dat genomisch onderzoekers niet alleen technische vaardigheden moeten hebben, maar ook morele verantwoordelijkheden hebben jegens hun onderzoek, collega's, proefpersonen en de maatschappij in het algemeen. Verder onderzoek is nodig om te bepalen wat de beste manieren zijn om de wetenschappers van vandaag en de toekomst hiervan bewust

te maken en hen te leren hoe zij morele verantwoordelijkheden kunnen balanceren. Daarnaast blijkt uit ons onderzoek dat de opkomst van de moleculaire pathologie ook gepaard gaat met meerdere ethische kwesties. Verder onderzoek is nodig hoe pathologen het beste opgeleid kunnen worden zodat ze dergelijke ethische aspecten kunnen herkennen en ermee om kunnen gaan. Ook moet gekeken worden hoe pathologen andere clinici en patiënten het beste kunnen voorlichten over voordelen, risico's en beperkingen van tumor DNA analyse, zodat clinici en patiënten goede, geïnformeerde beslissingen kunnen nemen over tumor DNA diagnostiek. Hierbij is het belangrijk een overvloed aan informatie en onnodige onrust over de mogelijke risico's te voorkomen. Voor het bovenstaande zouden interdisciplinaire cursussen en andere initiatieven die (bio) medische en ethische studenten en professionals samenbrengen gestimuleerd moeten worden. Dergelijke initiatieven dragen bij aan interdisciplinariteit, wederzijds begrip en kunnen ethisch parallel onderzoek stimuleren. Biomedische ethiek zou een standaard onderdeel moeten worden de opleiding van (bio)medici (hetgeen in de Nederlanse curricula inmiddels vaak het geval is). Ook zou het wenselijk zijn als (bio)medische wetenschappelijke tijdschriften aandacht hebben voor ethische studies en dat fondsen de waarde inzien van ethisch onderzoek en dit ook financieel ondersteunen. Daarnaast zou de pathologie actief moeten deelnemen aan interdisciplinaire samenwerkingsverbanden en initiatieven, zoals symposia waar artsen, wetenschappers, ethici, beleidsmakers en patiënten samenkomen om over zaken als consent en terugkoppeling te discussiëren. Van oudsher hebben pathologen geen direct patiëntencontact. Echter, in het tijdperk van de moleculaire pathologie is interactie met patiënten wenselijk om kennis te kunnen nemen van hun ideeën en voorkeuren over bovenstaande kwesties. Zeker met de verwachting dat genetische analyses steeds grootschaliger worden met een grotere kans op nevenbevindingen is het belangrijk om ook van het patiëntenperspectief bewust te zijn. Er zijn spannende tijden in het vooruitzicht voor de (moleculaire) pathologie met al deze nieuwe te verwachten technologische mogelijkheden. Dit maakt het bewust zijn van ethische aspecten door pathologen en met ethische kwesties kunnen omgaan nog belangrijker.

CONCLUSIE

Samenvattend toont dit proefschrift aan dat de pathologie een rol kan spelen in de opsporing van *BRCA1/2* kiembaanmutatiedraagsters onder borstkankerpatiënten. Meerdere weefselbiomarkers (op basis van eiwitexpressie, *BRCA* methylering en miRNA expressie) kunnen daarbij nuttig zijn, die tot op zekere hoogte onderscheid kunnen maken tussen *BRCA1/2*-gerelateerde en sporadische borstkanker, onafhankelijk

van klinische variabelen. Echter, verdere (externe) validatie en bepaling van de beste (combinatie van) techniek(en) zijn noodzakelijk voor implementatie in de medische praktijk. Daarnaast zijn technologische ontwikkelingen alleen niet voldoende voor een verantwoorde ontwikkeling van personalized cancer care. Tegelijkertijd moeten onderzoekers en pathologen zich bewust zijn van hun morele verantwoordelijkheden richting de proefsersonen/patiënten en de maatschappij als geheel.

Addendum

GLOSSARY

Actionability	Refers to the ability that the genetic variant can be acted upon.
Analytic validity	Refers to the accuracy of the research finding.
Autonomy	An individual's right to self-determination.
Basal-like breast cancer	Breast cancer with high expression of basal cytokeratins and associated genes, and low or no expression of ER, PR and HER2.
Beneficence	Performing an action for the benefit of others. Beneficence performing an action for the benefit of others.
BRCAX	Familial breast cancer with a still unknown genetic aberration.
Clinical/reproductive significance	Refers to what extent the genetic variant has clinical or reproductive implications; for example, with respect to treatment or screening.
Clinical utility	Indicates to what extent the genetic variant has clinical implications (e.g. interventions) to change health outcome.
Clinical validity	Refers to the existence of a causal relation between the genetic variant with pathology/clinical outcome.
Confidentiality	The right of an individual to have personal, identifiable health information kept private.
Equity	The concept of social justice or fairness.
Ethics	The scholarly discipline that studies and examines morality; for example, by analyzing what to do when certain moral principles are in conflict with each other.
Familial cancer	Family history of one or more first or second-degree relatives with breast cancer that does not fit the more stringent definition of hereditary breast cancer.
Hereditary cancer	Families with multiple cancers that fit Mendelian patterns of inheritance (i.e. dominant, recessive, X-linked).

High-throughput sequencing	Sequencing technology that allows sequencing massive amounts of DNA or RNA at once by multiple parallel sequencing reactions (i.e. massively parallel sequencing).
Luminal type breast cancer	Breast cancer with high expression of hormone receptors and associated genes, of which some overexpress HER2.
Molecular pathology	The subdiscipline within pathology that analyzes molecular alterations in (diseased) tissues; for example, by performing DNA sequencing to analyze somatic mutations in tumors.
Morality	Refers to norms about right and wrong human conduct.
Next-generation sequencing	Synonym of high-throughput sequencing.
Non-maleficence	The ethical principle of to 'do no harm'.
Penetrance	he probability that a particular phenotype/disease is expressed in an individual with a particular genotype.
Personal utility	Indicates to what extent the genetic variant has implications for someone's personal life, irrespective of medical significance.
Personalized or precision medicine	Individualized approach to disease treatment and prevention. In the case of cancer, the patient is treated according to specific genetic alterations which have been detected in the tumor. For this, predictive biomarkers that can predict therapeutic sensitivity are required.
Reciprocity	Performing actions with others for mutual benefit or giving back to others for some benefit received.
Triple-negative breast cancer	Breast cancer negative for ER, PR and HER2.
Unsolicited findings	Findings not related to the primary clinical or research question, also known as incidental or secondary findings.
Whole-exome sequencing	Technology which sequences all coding regions of the genome (i.e., the exome).
Whole-genome sequencing	Technology which sequences the entire genetic code (i.e., the genome).

LIST OF ABBREVIATIONS

BRCA1/2	*BRCA1* and *BRCA2*
BRCA1/2-N	Normal breast tissues from *BRCA1* and *BRCA2* germline mutation carriers
BRCA1/2-related	Related to a *BRCA1* or *BRCA2* germline mutation
BRCA1-C	Breast carcinomas from *BRCA1* germline mutation carriers
BRCA1-N	Normal breast tissues from *BRCA1* germline mutation carriers
BRCA2-C	Breast carcinomas from *BRCA2* germline mutation carriers
BRCA2-N	Normal breast tissues from *BRCA2* germline mutation carriers
CMI	Cumulative methylation index
CpG	Cytosine phosphate guanine
ECM	Extracellular matrix
ER	Estrogen receptor
FC	Fold change
FDR	False Discovery Rate
FFPE	Formalin-fixed paraffin-embedded
Healthy-N	Normal breast tissues from non-mutation carriers
HER2	Human Epidermal Growth Factor Receptor 2
IPA	Ingenuity Pathway Analysis
MS-MLPA	Methylation-Specific Multiplex Ligation-dependent Probe Amplification
Non-*BRCA*-related-N	Normal breast tissues from patients not known with a *BRCA1* or *BRCA2* germline mutation
PARP	Poly (ADP ribose) polymerase
PR	Progesterone receptor
Sporadic-C	Sporadic breast carcinomas

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CURRICULUM VITAE

Shoko Vos werd geboren op 4 oktober 1988 in Amersfoort als dochter van Hans Vos en Yuko Vos-Aoki. Van 2000 tot 2006 doorliep zij de middelbare school aan het Stedelijk Gymnasium Johan van Oldenbarnevelt te Amersfoort (summa cum laude). Daarna volgde de studie Geneeskunde aan de Universiteit Utrecht. Zowel haar bachelor- (2009) als masterdiploma (2013) behaalde ze met het judicium cum laude. Tijdens haar geneeskundestudie heeft ze verbreding en verdieping gezocht op het gebied van het humanities. Zo nam zij deel aan een universiteitsbreed Honours programma gericht op wetenschapsfilosofie en volgde zij een minor filosofie van de kunsten. Ook deed zij onder leiding van prof.dr. F.G. Huisman (Geschiedenis van de geneeskunde) onderzoek naar de translatie van wetenschappelijke kennis naar toepassingen in de klinische praktijk in de medische genetica.

Tijdens haar master geneeskunde kwam zij in aanraking met de pathologie. Tevens begon zij toen met haar onderzoek voor dit proefschrift. Gedurende 8 maanden verbleef zij in Baltimore, Verenigde Staten, voor haar afstudeeronderzoek aan de Johns Hopkins School of Medicine, onder begeleiding van assistant prof.dr. V. Raman en prof.dr. P.J. van Diest (Pathologie). Haar onderzoek werd bekroond met de Professor Chris Gips studentenprijs voor jong wetenschappelijk talent in de geneeskunde (2014). Na terugkeer uit de Verenigde Staten studeerde ze af en begon ze met haar opleiding tot patholoog aan het UMC Utrecht, welke ze tot op heden combineerde met het doen van onderzoek voor dit proefschrift.

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LIST OF PUBLICATIONS

Vos S, van Diest PJ, Ausems MGEM, van Dijk MR, de Leng WWJ, Bredenoord AL. Ethical considerations for modern molecular pathology. *In press. The Journal of Pathology.*

Vos S, Elias SG, van der Groep P, Smolders YH, van Gils CH, van Diest PJ. Comprehensive proteomic profiling-derived immunohistochemistry-based prediction models for *BRCA1* and *BRCA2* germline mutation-related breast carcinomas. *American Journal of Surgical Pathology. 2018;42:1262-1272.*

Vos S, van Diest PJ, Moelans CB. A systematic review on the frequency of *BRCA* methylation in breast and ovarian carcinomas of *BRCA* germline mutation carriers: mutually exclusive, or not? *Critical Reviews in Oncology/Hematology.* 2018;127:29-41.

Vos S, Moelans CB, van Diest PJ. *BRCA* methylation in sporadic versus *BRCA* germline mutation-related breast cancers. *Breast Cancer Research.* 2017;19:64.

Vos S, van Delden JJM, van Diest PJ, Bredenoord AL. Moral duties of cancer genomics researchers: why *personalized* medicine requires a *collective* approach. *Trends in Genetics.* 2017;33:118-128.

Vos S, Vesuna F, Raman V, van Diest PJ, van der Groep P. miRNA expression patterns in normal breast tissue and invasive breast cancers of *BRCA1* and *BRCA2* germline mutation carriers. *Oncotarget.* 2015;6:32115-32137.

Vos S, van der Groep P, van der Wall E, van Diest PJ. Hereditary breast cancer: molecular pathogenesis and diagnostics. In: *eLS.* John Wiley & Sons, Ltd: Chichester, 2015.

"I am made and remade continually.
Different people draw different words from me."

Virginia Woolf, The Waves, 1931