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## Prediction of "BRCAness" in breast cancer by array comparative genomic hybridization

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*Chapter*

**1**

## **Introduction**

*Based on the article: Prediction of BRCA status*

**SA Joesse** and J Hannemann

CML Breast Cancer. 2011 Jul; 23(2): 41-50, Leading Article.



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# Introduction

*Based on the article: Prediction of BRCA status*

**Simon A Joosse** and Juliane Hannemann

CML Breast Cancer, 2011 Jul; 23(2): 41-50, Leading Article

## General introduction about cancer

### The hallmarks of cancer

In humans, cell growth and development is a continuous process, which is strictly controlled by genes encoded by DNA of the cell nucleus. Two different types of genes regulate the mechanism of cell proliferation, the first are called proto-oncogenes that are positive regulators of cell proliferation and promote cell division and cell growth, and the second are called tumor suppressors that negatively regulate cell proliferation and suppress cell growth. Upon mutation or loss of these genetic regulators, cells may keep on dividing beyond the body's normal needs and cause harm to other tissues and body functions. This is the hallmark of cancer, a malignant growth characterized by uncontrolled, unwanted, purposeless, damaging and continuing growth of cells. These so called tumor cells differ functionally, structurally (anaplasia) and in behavior from the normal cells from which they develop; they have the potential for a limitless and uncontrolled replication independently from external stimulatory signals and uninhibited by antigrowth and apoptosis signals (1). Furthermore, not only is cancer dangerous for its direct surrounding tissue, it can be most harmful upon invasion of the body and establishment of metastasis in distant organs (2, 3).

Metastasized cancer is the leading cause of death from cancer and is considered an incurable disease (4). In 2004, around 7.4 million people died of cancer - 13% of all deaths worldwide - of which breast cancer was the most common form of cancer in women (5).

### Causes of cancer

Most cancer arises from a single cell that has accumulated DNA damage and genetic mutations to a number of key tumor suppressor genes and proto-oncogenes to escape programmed cell death and induce unlimited replication (1). These DNA mutations are sequentially acquired through time, which is why the likelihood for cancer increases with age. When a mutated predisposition gene is inherited, the risk for cancer is already present at a much younger age. Damage to genes is caused by both exogenous and endogenous factors. The exogenous factors are mainly environmental DNA damaging agents, such as ionizing radiation, ultraviolet rays, air pollution, and inhaled cigarette smoke. The endogenous factors include reactive oxygen species produced from cellular metabolism and replication errors of several cellular processes, such as DNA duplication and meiotic recombination. Although the risk for cancer by endogenous factors is difficult to control, over 30% of all cancer can be prevented by adopting a healthy life-style, *i.e.* not using tobacco, having a healthy diet, preventing infections that may cause cancer and being physically active (5, 6).

## Classification of cancer

Cancer is initially named after the site of the body from which it originates. Next, tumors are divided into groups based on the cell of origin; carcinomas are the largest group of solid tissue malignant tumors and are of epithelial origin, such as skin, colon or mammary ducts. For that reason, tumors growing in the breast are mainly classified as "breast carcinomas". Because cancer can arise from all organs and different cell types it is a complex disease consisting of many diverse entities that all have their own unique characteristics and behavior. Since the application of radiotherapy and chemotherapy in the first half of the 20th century as anti-cancer treatment, it has become clear that different tumors can respond differently to different types of therapy (7). This has motivated researchers to find tumor markers that allow for the identification of therapeutic groups to predict prognosis and adapt therapy to the clinical situation, a process which is called tumor classification. For many years now, the most important factors in tumor classification are site, degree of local and remote invasion (staging), histological type, cell

**Table 1.** Breast cancer incidence per 100,000 women in Northern America, Northern and Western Europe and Australia/New Zealand. Data source: GLOBOCAN 2008, International Agency for Research on Cancer <http://globocan.iarc.fr/>

Age (years)	Incidence
0-14	0.0
15-39	19.1
40-44	115.0
45-49	172.7
50-54	218.2
55-59	262.5
60-64	304.6
65-69	349.9
70-74	338.9
75+	341.3

structure (grading) and site dependent tumor markers (8).

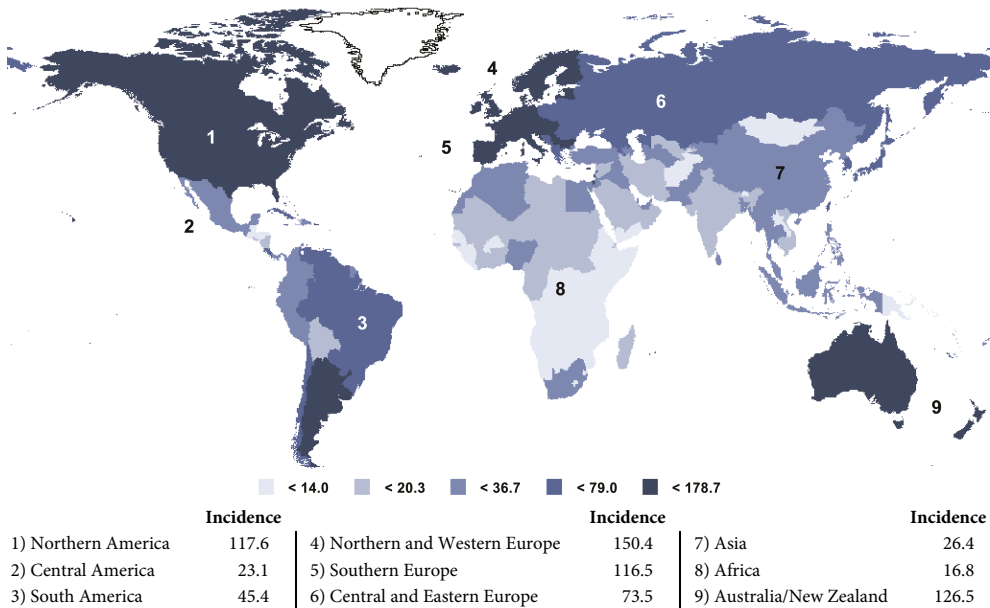
## Breast cancer

### Prevalence

Breast cancer accounts for approximately one-fourth of all cancers in women worldwide, making it the most common female malignancy. As a result of early detection and improved treatment, death rates from breast cancer have been steadily decreasing; however, breast cancer is still the leading cause of cancer-related death in women, closely followed by lung cancer. Figure 1 depicts the worldwide breast cancer prevalence; as can be seen, the incidence of breast cancer is the highest in economically developed countries, *i.e.*, in Europe, Australia and North America, where it accounts for approximately half of all the breast cancer cases worldwide. The incidence of breast cancer in Africa or Asia is about six-fold lower than that. The disease is not common until after the age of 40 and the incidence increases with age (Table 1). The average age of women to be diagnosed with breast cancer is between 60-61 years (5, 9, 10). Less than one percent of all breast cancers occur in men (11).

### Histological classification of breast cancer

A woman's breast consists of milk glands (*lobules*), tubes for transporting milk from the glands to the nipple (*ducts*), fatty and connective tissue, blood vessels, and lymph vessels (Figure 2). Pathologically, breast cancer can be divided into two main and several uncommon types. The most common type is called ductal carcinoma



**Figure 1 - Breast cancer prevalence.** Age-standardized breast cancer incidence per 100,000 women by world area in 2008 (crude rate statistic). Image and data source: GLOBOCAN 2008, International Agency for Research on Cancer <http://globocan.iarc.fr/>

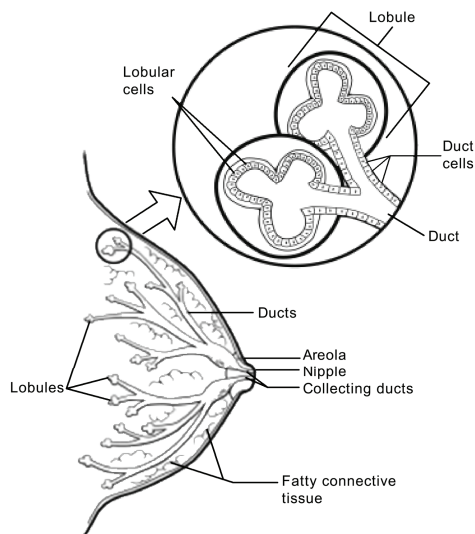
and is thought to be derived from the breast ducts; the second type is called lobular carcinoma and is deemed to have arisen from the breast lobules at the end of the ducts. However, there is no true evidence for the site of origin (duct or lobules) and classification is made on histological parameters (12). Other, less common types of breast carcinoma are tubular, invasive cribriform, medullary, mucinous, invasive papillary, invasive micropapillary, apocrine, metaplastic, glycogen-rich clear cell, lipid-rich, adenoid cystic, acinic cell, Paget's disease of the nipple, and inflammatory carcinoma. As long as a carcinoma is still growing within the ductulo-lobular system of the breast, it is called carcinoma *in situ*. Based on the histological properties, ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS) can be distinguished. If the carcinoma shows evi-

dence of breaching the basement membrane and thereby infiltrating the adjacent stroma, the tumor is classified as invasive ductal carcinoma (IDC) or invasive lobular carcinoma (ILC). To be able to estimate prognosis of breast cancer, carcinomas are generally classified by the TNM classification system and by a grading system. In the TNM system, three different parameters are assessed (Table 2). The first parameter, designated *T*, is the size or extension of the primary tumor starting from 0 (no primary tumor) to 4 (exceeding adjacent structures). The *N* parameter concerns the rate of invasion of regional nodes scored from 0 (no metastasis) to 3 (invasion beyond regional nodes). Last, the *M* component describes the presence of metastasis where M0 classifies as no remote metastasis and M1 the presence of metastasis at distant site(s). Besides size, the primary tumor is graded as a

measure for its rate of growth and cell abnormality; the most used system for this is the Bloom-Richardson-Elston grading system. By assessing tubule formation, nuclear polymorphism and mitotic rate, a carcinoma can be graded as grade 1: well differentiated; grade 2: moderately differentiated; or grade 3: poorly differentiated (13). In the past decade, new methods have been developed to classify breast cancer based on molecular characteristics; this will be discussed further on.

## Detection of breast cancer

Breast cancer can be detected by breast self-examination which, is best performed every 3 months at the end of the menstrual stage. Because breast self-examination will only detect palpable breast cancer or breast cancer in advanced stage, it does not provide early diagnosis; consequently, breast self-examination has not proven to reduce mortality in women (14). Because breast cancer has no obvious symptoms at early stage in general, the first sign of breast cancer is often an abnormality detected on a mammogram. Mammography is currently the most widely used imaging technique for early detection of breast cancer. It has been demonstrated that periodically screening by mammography can decrease breast cancer death rates; however, this technique has its limitations and does not detect all breast cancers (15). After a suspicious abnormality has been detected by mammography, additional tests such as ultrasound are performed or a needle aspiration is taken. In the presence of a highly suggestive lesion, surgery immediately follows mammography without any additional diagnostic tests. Needle biopsy is an invasive technique which can be used if needle aspiration results without a definitive diagnostic evaluation.



*Figure 2 - Anatomy of a woman's breast. Reprinted by the permission of the American Cancer Society, Inc. All rights reserved. From [www.cancer.org](http://www.cancer.org)*

It is taken using a wide-caliber needle and is followed by histological analysis of the biopsy, on which many different tumor markers can be examined, for the presence of tumor cells. Another imaging technique for breast cancer detection is magnetic resonance imaging (MRI) of which the sensitivity is much higher for infiltrating carcinomas and *in situ* lesions than mammography, but lacks high specificity. Therefore, it is suggested to use MRI in combination with mammography or ultrasound only (16). A technique that is fully under investigation is the detection of breast cancer by measurement of blood markers. Measurement of circulating tumor markers in breast cancer patients is currently most established in advanced disease, aimed at the diagnosis of metastasis and the evaluation of response to treatment (17).



**Table 2 - Cancer TNM classification table.**

Primary tumor (T)		Regional lymph node (N)		Remote metastasis (M)	
T0	None	N0	No lymph node metastasis	M0	None
Tis	Carcinoma <i>in situ</i>	N1	Metastasis in movable ipsilateral axillary lymph nodes	M1	Remote metastasis
T1	≤ 2 cm	N2	Metastasis in ipsilateral axillary lymph nodes or in internal mammary nodes		
T2	2-5 cm	N3	Metastasis in axillary lymph nodes and in ipsilateral infraclavicular, supraclavicular, or ipsilateral internal mammary lymph nodes.		
T3	>5 cm				
T4	Tumor of any size, extending through chest wall or skin				

## Treatment

The main treatment of primary breast cancer is surgery. The goals of surgery are to remove the cancerous tissue and to analyze the size, grade and other clinical factors that are important to determine sequential therapy. Removal of the tumor can be performed by lumpectomy, a partial removal of the breast which includes the tumor and some of the surrounding tissue. Lumpectomy is breast conserving surgery (BCS) and is also referred to as wide local excision. Mastectomy is the oldest known form of breast cancer treatment and means the complete removal of the breast which may include removal of skin and muscle tissue (18). After breast cancer surgery, the tumor but also the removed surrounding breast tissue is macroscopically examined and sliced for further microscopic examination. For the preservation of cellular histological markers and long term storage, tissue blocks are prepared and are either formalin-fixed and paraffin-embedded (FFPE) or frozen in liquid nitrogen. From the paraffin blocks, slices of 3-5  $\mu\text{m}$  are cut for staining with hematoxylin and eosin (H&E), and staining for estrogen receptor (ER), progesterone receptor (PR) and ERBB2 (human epidermal growth factor receptor 2). Based on the macro- and

microscopic examinations, a pathologist will determine tumor type, histological grade, invasion, tumor size, pTNM stage and hormone receptor status of the breast tumor on which further treatment is based (19). Surgery might be followed by radiotherapy to destroy any remaining tumor cells in the breast, axillary tissue or chest wall. Radiotherapy can be given externally by collimated beams of radiation or internally after lumpectomy where a small pellet of radioactive material is given directly into the tumor bed (18). Next, treatment might be followed by systemic chemotherapy. The aim of adjuvant chemotherapy is to destroy or stop any tumor cells that have invaded the body. Chemotherapy interferes with the ability of rapidly growing cells to divide, including cancer cells but also cells present in the bone marrow, hair follicles and gastrointestinal tract. Different chemotherapeutic drugs are listed in Box 1 (18). Estrogen receptor positive breast tumors are dependent on estrogen for growth and proliferation. Endocrine treatment aims at estrogen starvation of the tumor cells by blocking the production of estrogen or limiting estrogen in reaching the tumor cells (18). Other targeted therapies aim at specific proliferative cell functions. These treatment options includes the targeting of ERBB2, EGFR, tyrosine kinases (IGF), and

- ▶ Surgery: lumpectomy, mastectomy.
- ▶ Radiation: internal, external.
- ▶ Chemotherapy:
  - ▶ CMF (cyclophosphamide, methotrexate, and fluorouracil), AC (cyclophosphamide and doxorubicin), CAF (cyclophosphamide, doxorubicin, and fluorouracil), CEF (cyclophosphamide, epirubicin, and fluorouracil), TC (docetaxel and cyclophosphamide).
- ▶ Hormonal therapy: aromatase inhibitors (anastrozole, exemestane, letrozole, fadrozole), selective estrogen receptor modulators (tamoxifen, raloxifene, toremifene), oophorectomy.
- ▶ Targeted therapy: ERBB2 (trastuzumab, lapatinib), angiogenesis inhibitor (bevacizumab), EGFR inhibitor (gefitinib), tyrosine kinase inhibitor.

**Box 1** - Breast cancer treatment options (18, 20).

angiogenesis (VEGF) (18, 20). Chemotherapy can also precede surgery and is then called neoadjuvant chemotherapy. Its goal is to reduce the size of the tumor to make radical treatment intervention easier to perform and more likely to succeed (21).

## Risk factors

Each year, approximately 130 per 100 000 women are diagnosed with breast cancer in Northern America, Northern and Western Europe, Australia and New Zealand (9). Based on these data, it can be estimated that approximately one in nine women will develop breast cancer during a period of > 80 years, setting the cumulative lifetime risk of breast cancer at 11% in these regions. The risk for developing breast cancer seems to be much lower in Asia or Africa as is indicated by the incidence of the disease

(Figure 1). The international variations seem to disappear when Asian or African natives immigrate to regions with high breast cancer incidence (22-25). This has led to the conclusion that besides being a woman, significant risk of developing breast cancer lies in regional factors such as lifestyle and culture. The very first study to identify risk factors for breast cancer was performed by Janet Elizabeth Lane-Clayton and published in 1926 (26). Lane-Clayton identified that giving birth to a high number of children (>10) and giving birth to the first child at young age reduces the risk of breast cancer, women who have had no children at all, such as nuns, have a greater risk of breast cancer. These risk factors are examples of current cultural differences between the economically well and less developed countries. Besides reproductive behavior, other factors determined by lifestyle influence the risks for breast cancer; people in the western world tend to eat more animal products, eat less vegetable, have less physical activity and become older compared to people in other parts of the world. More factors that can influence the risk for breast cancer are birth weight, birth length, age at menarche, and age at menopause (27). Additional to the latter factors that are associated with the body's level of hormone exposure, one of the strongest risk factor for developing breast cancer is a family history of the disease (Box 2).

- ▶ Diet and diet-related factors
- ▶ Ionizing radiation
- ▶ Hormone and reproductive factors
- ▶ Benign breast disease
- ▶ Family history of breast cancer

**Box 2** - Factors involved in the risk of the development of breast cancer.

# Hereditary breast cancer

## History

Families with three or more close relatives with breast cancer are commonly classified as "breast cancer families" (28). In the past, segregation analyses were performed in such families, showing an autosomal dominant mode of inheritance in most cases (29-31). By linkage analysis on a large group of families with early-onset breast cancer, the locus of a high-penetrance cancer susceptibility gene was mapped on chromosome 17q12-21 in 1990 (32). Not until 1994 a candidate gene was completely characterized and truncating mutations were linked to breast cancer (33). Because it was the first gene to be associated with hereditary breast cancer, it was called "breast cancer 1, early onset" or *BRCA1*. In the same year, the second major breast cancer susceptibility gene, *BRCA2*, could be localized on 13q12-13 and was cloned just one year later by Wooster and colleagues. The identification of loss of heterozygosity (LOH) at the *BRCA2* locus and germline mutations of this gene in breast cancer demonstrated the role of *BRCA2* as tumor suppressor gene (34, 35). After the discovery of the breast cancer susceptibility genes *BRCA1* and *BRCA2*, major changes have been made in the care of women with inherited predisposition to breast cancer such as increased

screening and surveillance and risk reduction options (36). Mutations in the *BRCA1* and *BRCA2* genes are responsible for the major part of the hereditary breast cancer syndrome; however, other genes have been correlated with different forms of hereditary breast cancer syndromes. These syndromes are: Cowden disease, caused by mutation in the *PTEN* gene (37); Li-Fraumeni syndrome, caused by mutations in the *TP53* gene (38); Peutz-Jeghers syndrome, caused by mutations in the *STK11* gene (39) and Ataxia Telangiectasia, caused by mutations in the *ATM* gene (40). Furthermore, a single mutation in *CHEK2*, 1100delC, has also been reported to be associated with hereditary breast cancer (41) (Table 3). Breast cancer caused by mutations in breast cancer susceptibility genes has several distinctive clinical features such as considerably younger age at diagnosis compared to sporadic cases, the prevalence of bilateral breast cancer is higher, and associated tumors (*e.g.*, ovarian, colon, prostate, and pancreatic cancers, as well as male breast cancer) are seen in some families.

## Incidence and risk

Mutations in the *BRCA1* and *BRCA2* genes can be identified in approximately 80% of families with a high number of breast cancer cases (*i.e.*, four or more) diagnosed before the age of 60 years. Germline mutations in *BRCA1*

**Table 3** - Gene associated life time risk of breast cancer in female carriers (42).

Gene	Breast cancer risk	Syndrome
<i>BRCA1</i>	65%	BRCA1 syndrome
<i>BRCA2</i>	45%	BRCA2 syndrome
<i>TP53</i>	97%	Li-Fraumeni
<i>PTEN</i>	20-50%	Cowden
<i>STK11</i>	45%	Peutz-Jeghers
<i>ATM</i>	2-5 fold elevated risk	Ataxia Telangiectasia
<i>CHEK2*1100delC</i>	2-fold elevated risk	

have been detected in approximately half of familial breast cancer cases and in most cases of combined familial breast/ovarian cancers. *BRCA2* mutations are found in about thirty percent of the hereditary breast cancers. Clinically, carriers of a *BRCA* germline mutation present with a substantially higher risk of developing breast and ovarian cancer than the general population. By the age of 70 years, the breast cancer risk in *BRCA1* mutation carriers is 65% (95% CI 51-75%) and the ovarian cancer risk is 39% (95% CI 22-52%); in *BRCA2* mutation carriers, the corresponding risks are 45% (95% CI 33-54%) and 11% (95% CI 4-18%), respectively (43) (Table 3). The median age of diagnosis in mutation carriers is 42 years, approximately 20 years earlier than unselected breast cancer in the Western World and several years before mammographic screening is recommended in the general population (44). A small percentage of the hereditary breast cancer syndromes can be explained by other high- and low-penetrance breast cancer genes (45-48) (Table 3), but these will not be discussed further in this thesis. In total, it is estimated that 5-10% of all breast cancer cases are due to inherited mutations of which mutation in the *BRCA1* and *BRCA2* genes are the most frequent (49, 50). However, in an additional 15-20% of all breast cancer cases a positive family history of the disease is found; therefore, from all the families that are actually eligible and tested for *BRCA* germline mutations, only in approximately 25% a *BRCA1* or *BRCA2* mutation is diagnosed according to literature (51-54); in Dutch hospitals the current percentages are around 7-14% (55). About 10-25% of the cases tested for *BRCA* predisposition is diagnosed with an unclassified variant (UV) (55-57), whereas for the remaining breast cancer families the genetic test result is uninformative/inconclusive. In literature, the

latter families are referred to as 'non-*BRCA1/2* families' and it is likely that these people are carrier of mutations in other, still unknown, breast cancer susceptibility genes, which are collectively designated as *BRCAx* (58).

## The importance to determine *BRCA* status

As a mutation in the *BRCA1* or *BRCA2* gene is one of the greatest risk factors for developing breast and ovarian cancer, identification of such a mutation is of significant clinical value. Mutation carriers are offered special medical care to reduce the risk of cancer development and, ultimately, mortality. First of all, providing individuals general information about *BRCA1/2* mutations by genetic counseling has been shown to reduce worrying about breast cancer, reduce anxiety and depression, and increase the likelihood of participating in genetic testing (59). Second, intensified screening for early detection of cancer by both mammography and magnetic resonance imaging (MRI) has been recommended for women with *BRCA* mutations (60, 61). Furthermore, women who have been identified with a germline mutation in *BRCA1/2* can opt for prophylactic surgery which includes prophylactic bilateral mastectomy (PBM) and prophylactic bilateral salpingo-oophorectomy (PBSO), to reduce the risk of breast and ovarian cancer by 85-100% (62-67). Lastly, women with an inherited predisposition to breast cancer can be offered chemopreventive agents such as oral contraceptives (68, 69) or tamoxifen, which has been found to reduce the incidence of breast cancer in healthy *BRCA2* mutation carriers by 62% (70). Poly(ADP-ribose) polymerase 1 (PARP1) inhibitors, a novel class of drugs which is still under investigation, have shown to be highly effective against *BRCA*<sup>-/-</sup> pre-cancerous

cells and might become chemopreventive agents in the future (this will be discussed in more detail further on) (71). Although these interventions might benefit women carrying a *BRCA* mutation, they should be avoided in non-carrier relatives (true negatives).

Recently, it has been shown *in vitro* that *BRCA*-deficient cell lines display increased sensitivity to agents causing double-strand DNA breaks such as cisplatin (72, 73). These findings may open the possibility that determination of *BRCA* status may also be used to guide therapy in the near future. However, because of the lack of prospective clinical validation, *BRCA* mutation carriers are offered similar adjuvant therapy as non-hereditary breast cancer patients at the moment (74, 75).

## Eligibility for *BRCA* mutation testing

As a result of the large sizes of the *BRCA1* and *BRCA2* genes, mutation screening is expensive, complex and time-consuming. It would be inefficient to screen for inherited cancer susceptibility in all women diagnosed with breast cancer and it is therefore necessary to preselect eligible families for mutation testing. Several referral guidelines have been developed based on family characteristics that have been associated with increased risk of germline mutations in *BRCA1* or *BRCA2* for further risk evaluation (76-82). These risk factors include breast cancer onset at young age, ovarian cancer, two breast cancer primaries, a combination of breast and ovarian cancer, male breast cancer, or a known *BRCA* mutation in the family, ethnic group, and family history of breast or ovarian cancer (Box 3) (51, 54, 56, 83). In addition to the risk factors, several models have been developed to accurately evaluate the probability of a person

- ▶ Diagnosis of breast cancer at early age (<50 years)
- ▶ Triple negative breast cancer (ER, PR, ERBB2)
- ▶ Two breast cancer primaries (bilateral or ipsilateral)
- ▶ Both breast and ovarian cancer
- ▶ Ovarian/fallopian tube/primary peritoneal cancer
- ▶ One or more cases of breast and/or ovarian cancer in the family
- ▶ Clustering of breast cancer with various other cancers such as thyroid or pancreatic cancer on the same side of the family
- ▶ Presence of breast cancer in a male family member
- ▶ Known *BRCA1* or *BRCA2* mutation in the family
- ▶ Member of a population at risk (e.g., Ashkenazi Jewish)

**Box 3** - Family characteristics covering first-, second-, and third-degree relatives that have been described to be risk factors for hereditary breast cancer (51, 54, 56, 83). Affected individuals with one or more risk factors might be eligible for further risk evaluation.

carrying a *BRCA* mutation (84). Next, genetic counseling is performed with the subject and only those women with strong evidence for a germline mutation and with assumed sufficient benefit are recommended for further DNA diagnostics.

## Estimation of *BRCA* carrier probability

The most important factors that determine the individual likelihood of a deleterious *BRCA* mutation in affected or cancer-free women remain family history of breast or ovarian cancer and a known family mutation (85, 86). Several models have been developed to estimate the probability that an individual person or family is a carrier of a mutation in *BRCA1* or *BRCA2* based on their family history of cancer (*i.e.*, age of onset and type of cancer in first- and second-

degree relatives), non-hereditary risk factors (e.g., age at menarche and age at birth of first child), but also on the population prevalence of mutations, age-specific penetrance, and ethnic ancestry (87). The application of these tools is to select or exclude people from genetic counseling and genetic testing in order to provide a cost-efficient and clinically appropriate service. The currently available probability models include BRCAPRO (88-90), models from Myriad Genetic Laboratories (Salt Lake City, UT, USA) (56, 83, 91), the Couch model (also known as the

Penn model) (54, 92), IBIS (International Breast Cancer Intervention Study) (93), and BOADICEA (Breast and Ovarian Analysis of Disease Incidence and Carrier Estimation Algorithm) (94, 95). Most models can calculate a *BRCA* mutation probability for affected as well as unaffected individuals. Table 4 describes seven of these models, noting on what their estimates are based and to whom they are applicable. The results of all of these tests should be interpreted with some caution, because each model bases its calculation of risk estimate on

**Table 4** - Models to calculate the likelihood of carrying a *BRCA1* or *BRCA2* mutation. References refer to original publication and electronic tool if available.

Model	Estimates based on	Applications	References
BOADICEA ( <i>BRCA1</i> and <i>BRCA2</i> )	Extensive information on family history of cancer, male breast cancer.	Applicable for proband affected and unaffected by breast cancer.	(94-96)
BRCAPRO ( <i>BRCA1</i> and <i>BRCA2</i> )	Extensive information on family history of cancer, age at diagnosis, presence of bilateral breast cancer, male breast cancer, Ashkenazi Jewish heritage.	Applicable for individuals with or without breast or ovarian cancer.	(88-90, 97, 98)
IBIS ( <i>BRCA1</i> and <i>BRCA2</i> )	Breast/ovarian status, extensive information on family history of cancer, nonhereditary risk factors.	Applicable for proband affected and unaffected by breast cancer	(93, 99)
Myriad I ( <i>BRCA1</i> )	Bilateral breast cancer, ovarian cancer, age at diagnosis, Ashkenazi Jewish ethnicity, and family history of cancer.	Only applicable for proband affected by breast and/or ovarian cancer. Applicable to families with small numbers of affected members.	(56)
Myriad II ( <i>BRCA1</i> and <i>BRCA2</i> )	History of breast and ovarian cancer, Ashkenazi Jewish heritage, and family history of cancer.	Only applicable for proband affected by breast cancer < 50 years of age and/or ovarian cancer.	(51, 100)
Penn I/Couch ( <i>BRCA1</i> )	Age at diagnosis, family history of cancer, Ashkenazi Jewish heritage.	Applicable for proband with or without breast or ovarian cancer.	(54)
Pen II ( <i>BRCA1</i> and <i>BRCA2</i> )	Ashkenazi Jewish heritage, family history of cancer.	Applicable for proband with or without breast or ovarian cancer but with > 2 breast cancer cases in the family.	(92, 101)

different parameters, which might mean that different results are generated for the same person (102-104). It should be well understood that these models calculate the probability of a *BRCA* mutation and not the true breast cancer risk, although some of them are able to do the latter. Other models that assess the risk of developing breast cancer include the Gail, Claus, Jonker, and extended Claus models (105-108). Additional models that have been developed to assist in selecting women for referral to genetic counseling include FHAT (Family History Assessment Tool), the Manchester scoring system, and RAGs (Risk Assessment in Genetics) (109-111).

## Genetic testing

Mutations in the *BRCA1* and *BRCA2* genes are found throughout all coding regions and at splice sites, with most of these mutations being small insertions or deletions causing frameshift mutations, nonsense mutations, or splice site alterations. In order to detect these specific genetic alterations, the entire *BRCA1* and *BRCA2* genes have to be examined, which is a complex procedure. Only known founder mutations can be detected relatively easy in some high-risk families from specific ethnic groups (112). The current gold standard to determine *BRCA* mutations is direct sequencing of genomic DNA; however, since this is an expensive and time-consuming technique, many laboratories prefer the use of pre-screening techniques to detect any genetic anomalies first. Pre-screening techniques include protein truncation test (PTT), denaturing gradient gel electrophoresis (DGGE), denaturing high-performance liquid chromatography (DHPLC), single-stranded conformational polymorphism (SSCP), two-dimensional gene scanning (TDGS), fluorescent-assisted mismatch analysis (FAMA), heterodu-

plex analysis, and fluorescent conformational sensitive gel electrophoresis (F-CSGE) (113, 114). None of these techniques, including direct sequencing, is able to identify all cancer predisposing mutations in the *BRCA1* or *BRCA2* gene. Only by applying additional detection strategies for large deletions or duplications such as multiplex ligation dependent probe amplification (MLPA), can an estimated detection rate of up to 95% be achieved (115).

If a gene mutation is found, sequential clinical steps can be undertaken. If no mutation is found in a family member of a known *BRCA* mutation-carrying family (a "true negative"), the individual's risk of breast cancer is equal to that of the general population and no additional preventive measures are required. However, difficulties arise when no pathogenic mutation is found but the hereditary risk of breast cancer cannot be ruled out for this individual or family. Such test result is called uninformative or inconclusive which is absence of identification of a deleterious mutation in an entire family or identification of an unclassified variant (UV), a sequence variant of which the clinical significance is still unknown.

## *BRCA* mutations

To date of writing, 1647 and 1857 unique mutations are described in the BIC database for the *BRCA1* and *BRCA2* genes, respectively. The majority of the mutations found are frame-shift or nonsense mutations (Table 5) and about 10% of the mutations are large exonic deletions or insertions (116, 117). These mutations are described as pathogenic because they result in missing, truncated or not properly functioning protein products. Splice site alterations cause incorrect splicing and may result in unstable mRNA and thus reduced levels of protein (118).

If a mutation can be traced back to a com-

**Table 5** - Source: The Breast Cancer Information Core (BIC) Database. UV, unclassified variants.

Mutation type	BRCA1 All mutations		BRCA1 UV		BRCA2 All mutations		BRCA2 UV	
	3-prime Untranslated Region (3'UTR)	2	0.1%	1	0.1%	0		0
5-prime Untranslated Region (5'UTR)	3	0.2%	3	0.4%	10	0.5%	7	0.7%
Frameshift (F)	542	32.9%	0		544	29.3%	3	0.3%
In Frame Insertion (IFI)	1	0.1%	1	0.1%	4	0.2%	3	0.3%
In Frame Deletion (IFD)	24	1.5%	24	3.2%	23	1.2%	23	2.2%
Intervening Sequence (IVS)	264	16.0%	159	21.0%	173	9.3%	124	12.1%
Missense (M)	570	34.6%	537	71.2%	847	45.6%	812	79.2%
Nonsense (N)	194	11.8%	0		189	10.2%	1	0.1%
Splice (S)	1	0.1%	1	0.1%	2	0.1%	0	
Synonymous (Syn)	46	2.8%	29	3.8%	65	3.5%	52	5.1%
total	1647		755		1857		1025	

mon ancestor it is called a founder mutation. Such a mutation is often enriched in a certain ethnic group. The prevalence of a founder mutation can be strongly enriched among certain ethnic groups such as the Ashkenazi Jews, in which three founder mutations have been identified: the 187delAG and 5385insC in *BRCA1*, present in about 1.1 and 0.15% of the Ashkenazi Jews, and the 6174delT mutation in *BRCA2*, present in 1.5% of the Ashkenazi Jews. These three mutations account for a total *BRCA* mutation carrier frequency of 1 per 40 individuals of Ashkenazi Jewish descent, which is notably high (119-121). Another example is the founder mutation *BRCA2* 999del5 that accounts for 7-8% of female breast cancers and for 40% of male breast cancers in Iceland (122). More *BRCA* founder mutations are seen in other countries in which endogamy is a common practice among certain social or religious groups, including in the Netherlands (112, 123-125).

## Unclassified variants in *BRCA*

Besides the pathogenic mutations found in the *BRCA1* and *BRCA2* genes, more than half of the nucleotide changes in these genes occur

rarely (<1%) and their clinical significance is unknown (116). These mutations are called unclassified variants (UVs) and are generally missense (M) or intervening sequence (IVS) variants that result in substitution or loss of a single amino acid (Table 5) (118). Clinically, the identification of an UV in an individual's germline DNA is a difficult situation and to prevent unnecessary surgery, it is important to determine whether the mutation adversely affects the functions of the protein. However, determining the pathogenicity remains difficult because of the limited knowledge about the functional outcomes of such nucleotide variant; therefore, much research is currently being performed to assess the pathogenicity of each UV. Multifactorial classification models base the risk associated with a UV on combined data from variant frequency, co-segregation with cancer, and features consistent with a real pathogenic gene mutation such as family history of cancer, co-occurrence (*in trans*) of another known pathogenic mutation, tumor histopathology, loss of heterozygosity (LOH) of the wild-type allele, evolutionary conservation, and evidence from functional assays (126, 127)



(Table 6). Because multifactorial classification models are limited by the amount of families carrying the UV, complementary approaches are often required, *i.e.*, *in vitro* assays that make use of transcript and functional analyses or prediction of splicing aberrations using bioinformatics (*in silico*) (128-132). Still, determining the pathogenicity of UVs remains difficult, laborious and time consuming and new techniques are being developed to preselect for variants with high pathogenic possibility.

## Non-BRCA1/2 families

Most of the women from breast cancer families do not carry a pathogenic mutation or unclassified variant in the *BRCA1* or *BRCA2* gene (53, 55). Linkage analysis mapped a third breast cancer susceptibility gene on chromosome 13q, distinct from *BRCA2* and *Rb*, but this was opposed a few years later (133). It is now suspected that an unknown number of low penetrance genes or a combination of common

**Table 6** - Types of evidence for UV classification (126, 130).

<b>Multifactorial classification</b>	
Variant frequency	Frequency of the variant occurring in cases and controls provides a direct estimate of associated cancer risk but studies would need to be prohibitively large because of the rarity of the variants.
Co-segregation	Co-segregation with the disease in families allows for easily quantifiable and directly related risk.
Co-occurrence with deleterious mutation	UVs, co-occurring with a deleterious mutation in the same gene ( <i>in trans</i> ), can be classified as neutral if homozygotes are assumed to be embryonically lethal. However, this method exhibits less power to show causality.
Family history	Personal and family history of cancer of the carriers of the UV usually can easily be obtained; however, it is not as robust as co-segregation and the power may be low for infrequent variants.
Pathological classification	Histopathological tumor features are a potential powerful predictor for BRCA1-related tumors in which the pathological characteristics are quite distinct, however, for BRCA2-related tumors the prediction might be weak (discussed further on).
LOH	Occurrence of loss of heterozygosity in tumor DNA should be used as an adjunction to co-segregation results.
Conservation	The severity of the amino acid change and its conservation across species can be very predictive if enough evolutionary time sequence is available. Still, it is only indirectly related to disease risk.
<b><i>In vitro</i> assays</b>	
Functional analyses	Functional analyses can evaluate the effect of the variant on the protein's ability to perform (some of) its cellular functions. These assays include the determination of transcription activity, small colony phenotype, ubiquitin ligase activity, rescue of radiation resistance, embryonic stem cell-based functionality, homologous recombination, mitomycin C survival, and centrosome amplification
Transcript analyses	Transcript analysis can efficiently identify variants affecting the stability and integrity of mRNA transcripts.
<i>In silico</i> tools	Computation analysis of the UV can be used to predict the effect on mRNA splicing

variants (polygenic model) with multiplicative effects on risk may be responsible for this substantial proportion of hereditary breast cancer (114, 134-137). Researchers have come to this conclusion after investigation of a large cohort of 149 non-*BRCA1/2* breast cancer families in which linkage analysis has not been able to provide a locus on which a third major breast cancer gene might be located with statistical significance (138). Different approaches are now being examined to decrease the genetic heterogeneity and increase the statistical power of finding a breast cancer susceptibility locus, thus far without success (139). The difficulty in obtaining genetic homogeneous groups is the lack of specific familial phenotypes such as in families carrying a *BRCA1* or *BRCA2* mutation where ovarian and male breast cancer were recognized to be common. Additionally, studying families with high breast cancer incidence at early age could provide a more genetic homogeneous group but increases the likelihood of involvement of the *BRCA1* or *BRCA2* genes (140). New ways are needed to cluster families into subgroups of single-gene disorders.

## Treatment

Patients with hereditary breast cancer are offered bilateral mastectomy as treatment and to simultaneously decrease the risk for local recurrences or secondary primaries. As such, breast conserving surgery (BCS) is not the best therapy in *BRCA*-mutation carriers as these patients still have a substantial increased risk for local recurrences compared to sporadic breast cancer patients or *BRCA*-mutation carriers undergoing mastectomy; nevertheless, survival after mastectomy or BCS has not been shown to be significantly different (141). Chemotherapy after BCS has been shown to decrease the risk for local recurrence from 23.5% to 11.9% in *BRCA*-

mutation carriers, however, adjuvant chemotherapy by itself does not have any additional effect (positive or negative) on survival compared to patients with sporadic breast cancer (141, 142). It is thought that *BRCA* deficient tumors might be more sensitive to poly(ADP-ribose) polymerase inhibitors, but limited clinical data are available (this will be discussed in more detail further on) (143). *In vivo* and *in vitro*, homozygote *BRCA*-mutated cells have been found to be more radiosensitive due to the lack of proper DNA repair by homologous recombination and, additionally in *BRCA1* deficient cells, due to the lack of the cell-cycle G2-M checkpoint to stop cells before mitosis upon DNA damage (144-146). It would be very interesting to exploit this in cancer treatment; on the other hand, radiotherapy could then also increase the risk for secondary cancers. However, the currently available data from clinical studies do not provide evidence of hypersensitivity for radiotherapy in breast cancer patients carrying a *BRCA*-mutation or increased cancer sensitivity, cancer recurrences are reported to be similar compared to patients with sporadic breast cancers (147). Taken together, so far the clinical treatment of hereditary breast cancer patients does not differ from the treatment given to patients with sporadic breast cancer although the roles of *BRCA1* and *BRCA2* in DNA repair might be a future target in cancer treatment for these patients.

## Histopathological features

Breast cancer type, histological grade, invasion, estrogen receptor (ER) status, progesterone receptor (PR) status, and ERBB2 (human epidermal growth factor receptor 2) status are routinely determined histological features to guide therapy.

**Table 7 - Common histopathological features of hereditary and sporadic breast tumors. Negative status for ER, PR and ERBB2 is also referred to as "triple-negative". Data summarized from (148-151).**

	BRCA1 (%)	BRCA2 (%)	non-BRCA1/2 (%)	Sporadic (%)
Invasive lobular carcinoma	7	13	14	12
Invasive ductal carcinoma	74	71	73	69
Medullary carcinoma	18	3	2	3
Grade I	2	20	24	22
Grade II	24	42	44	42
Grade III	73	38	32	36
ER				
positive	21	65	72	66
negative	79	35	28	34
PR				
positive	20	49	60	56
negative	80	51	40	44
ERBB2				
positive	7	6	3	18
negative	93	94	97	82
Triple-negative	57	23	14	11
p53				
positive	45	27	12	27
negative	55	73	88	73
KRT5/6				
positive	65	7	13	8
negative	35	93	87	92

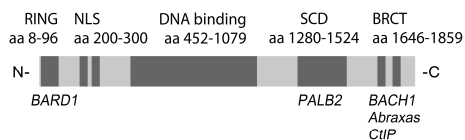
Hereditary breast cancers have several apparent features that separate them from sporadic breast cancers. Compared to sporadic breast tumors, *BRCA1*-associated breast tumors generally are grade III, hormone (ER and PR) and ERBB2 receptor negative (also referred to as triple-negative), often show p53 protein accumulation, much lymphocyte infiltration and a high expression of K5/6 (149-151). Most *BRCA2*-associated breast tumors are grade 2/3 and are often ER and ERBB2 positive (149). In contrast to *BRCA1*-related breast tumors, *BRCA2*-related breast tumors show much less distinctive features as compared to age-matched sporadic breast tumors (Table 7). Similar to *BRCA2*-

associated breast tumors, non-*BRCA1/2* breast cancer is a heterogeneous group that is comparable to sporadic breast cancer. Significant differences have been found in keratin expression, as K14 and K5/6 are higher expressed in *BRCA2*-associated and non-*BRCA1/2* breast cancer (148, 149). Although several studies have been performed to classify hereditary breast cancer based on histopathological features (152), it should be noted that none of these features in itself or in combination is unique to any of the hereditary breast cancers; therefore, histopathological features are not being used to identify hereditary breast cancer cases.

# Molecular biology of hereditary breast cancer

## The *BRCA1* gene

The human *BRCA1* gene contains 24 exons, encoding for a protein of 1863 amino acids (33). The protein contains multiple functional domains, including a highly conserved RING finger domain in its N-terminal region, two nuclear localization signals (NLS) that are located in the *BRCA1* gene in exon 11, a DNA binding domain between amino acids 452-1079, an SQ-cluster domain (SCD) between amino acids 1280-1524, and tandem BRCT repeats in its C-terminal region (Figure 3) (153). The *BRCA1* protein interacts directly or indirectly with many other molecules, including tumor suppressors, proto-oncogenes, DNA damage repair proteins, cell cycle regulators, as well as with transcriptional activators and repressors (114, 154). Normally, *BRCA1* is part of a heterodimer together with *BARD1* of which the interaction is mediated by alpha-helical units adjacent to the RING domain (155). Without *BARD1*, *BRCA1* is unstable and is rapidly degraded; it would be unable to perform its tumor suppressor functions (156). The *BRCA1*-*BARD1* complex serves as an ubiquitin ligase *in vitro* (157), however, *in vivo* it is largely unknown what its substrates are. *BRCA1* is localized to the site of the double-strand break by binding Abraxas at the BRCT repeats, followed by interaction with *RAP80* (158, 159). Not only is the *BRCA1*-Abraxas-*RAP80* complex involved in DNA repair, it also regulates phosphorylation of *CHK1* kinase through a yet still unknown mechanism (160). *CHK1* kinase is involved in DNA damage-driven cell cycle checkpoint control and is important to arrest cells to allow

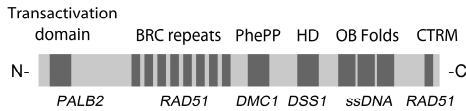


**Figure 3** - Functional domains in the *BRCA1* protein (dark gray) and interacting proteins (bottom).

them time for DNA repair before mitotic entry. Furthermore, *BRCA1* plays a role in replication checkpoints that are activated in response to replicative stress such as collapsed or stalled replication forks (146, 161), but also in mitosis control where the *BRCA1*/*BARD1* heterodimer is required for mitotic spindle pole assembly (162). Finally, the ubiquitination of topoisomerase IIa, which is involved in the decatenation of replicated DNA, is regulated by *BRCA1* (163). Loss-of-function mutations of *BRCA1* would therefore result in pleiotropic phenotypes, including defective DNA damage repair, a defective G2/M cell cycle checkpoint, abnormal centrosome duplication, chromosome damage, aneuploidy, and impairment of the spindle checkpoint (164, 165).

## The *BRCA2* gene

The human *BRCA2* gene covers 70kb of genomic DNA and has 27 exons, encoding for a protein of 3418 amino acids (35). While *BRCA1* has a wide range of functions in many different cellular processes, the primary function of *BRCA2* is limited to homologous recombination, both in meiosis and repair of double-strand breaks (154, 166). Through the interaction with *PALB2*, *BRCA2* is located to the site of damage together with *BRCA1*. *BRCA2* is able to bind to single strand DNA through interaction with *DSS1* to the helix-rich domain (HD) (Figure 4). A tower domain emerges from the second OB (oligonucleotide binding) fold, which is topped



**Figure 4** - BRCA2 protein organization, domains indicated in dark gray and interacting proteins below (154, 166). HB: helical domain, OB: oligonucleotide-binding, CTRM: c-terminal RAD51 binding motive.

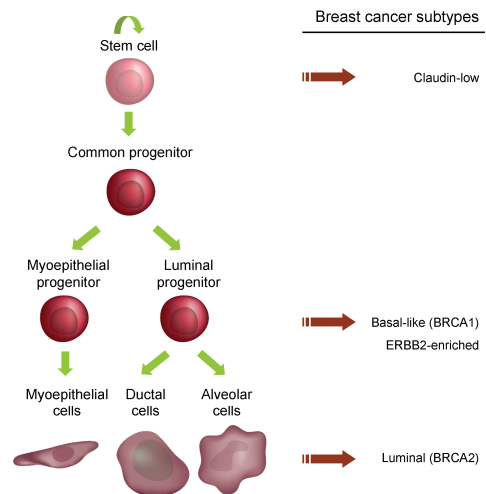
by a three-helix bundle for DNA binding. Although ssDNA is preferred by BRCA2, the structure of the tower domain suggests the possibility of binding to duplex DNA too (167). Next, RAD51 is loaded onto the 3'-strand overhang, which is bound to the BRC repeats of BRCA2 to facilitate DNA repair (Figure 6). Through the CTRM domain, BRCA2 stabilizes the resulting nucleoprotein filament. Because RAD51 is required for DNA-repair by homologous recombination, it is not surprising that BRCA2-deficient cells exhibit genetic instability (168, 169).

## BRCA1 and mammary stem cell differentiation

Normal stem cells are primitive undifferentiated cells that are capable of self-renewal while maintaining the undifferentiated state but have the potency to differentiate into specialized cell types. Stem cells maintain the growth of normal proliferative tissue such as intestinal epithelium, skin, or bone marrow but also guarantee tissue regeneration after injury. Stem cells are the top of the cellular hierarchy and give rise to progenitors with more restricted lineage potential (Figure 5) (170). It is postulated that similar to normal proliferative tissue, the growth of a tumor is driven by a limited number of so called cancer stem cells (CSC) (171). Cancer stem cells maintain the growth of the neoplastic clone and give rise to rapidly proliferating and more

differentiated cells that form the bulk of the tumor. One of the CSC concepts is that the tumor-initiating cell was originally an adult stem cell or a progenitor cell that has accumulated (epi)genetic damage resulting in tumorigenesis (172, 173).

The cyclical nature of mammary gland growth and involution during each pregnancy suggests the presence of stem cells in breast tissue (176, 177), but a consensus on the phenotypic definition of normal human mammary stem cells is still lacking at this point (178). Mammary adult stem cells can differentiate into two distinct cell types: luminal and myoepithelial (Figure 5). The luminal cell layer in mammary ducts is composed of progenitor luminal cells

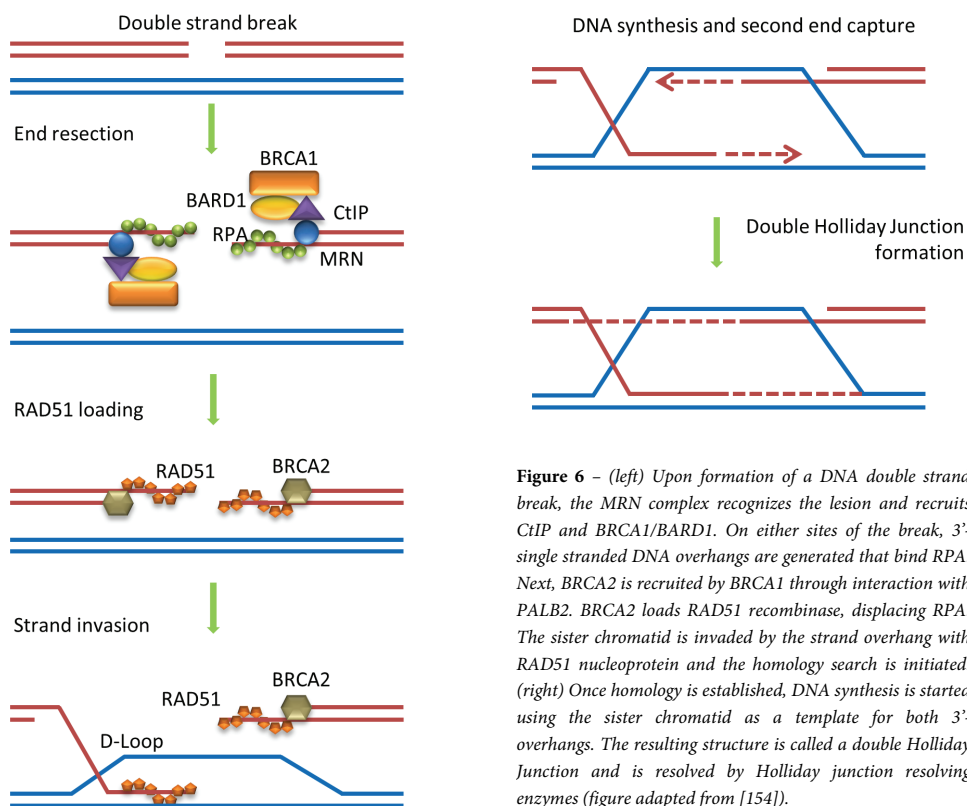


**Figure 5** - Stem cells have the ability to self-renew and give rise to more differentiated progenitor cells. The progenitor cells will further differentiate into myoepithelial, ductal, and alveolar cells and form the lobules and ducts in the breast. Depending on the cell of origin, different subtypes of breast cancer can arise. Source: (174, 175).

lacking expression of estrogen receptor (ER) and differentiated luminal cells that express ER and progesterone receptor (PR) (179, 180). BRCA1 plays an important role in the differentiation from ER negative progenitor cells to mature ER positive luminal cells. Women with a *BRCA1*-mutation often show entire lobules in the breast tissue to be ER negative and ALDH1 (a stem cell marker) positive, although histological normal, whereas this is not seen in non-mutation carriers (181). Loss of BRCA1 function results in blocked epithelial differentiation which leads to growth of undifferentiated luminal progenitor cells. Because BRCA1 also functions in DNA repair, these progenitor cells are prime targets for further carcinogenic events (182).

## The roles of BRCA1 and BRCA2 in DNA repair

DNA damage occurs continuously throughout a person's whole life, and is caused by both exogenous and endogenous stresses. Different DNA damaging sources cause different types of DNA damage and of these, double strand breaks are the severest because it affects both strands of the duplex, thus no intact complementary strand is available as a template for repair (73). Inappropriate repair of such DNA damage in a cell can lead to either loss of viability or to chromosomal alterations that increase the likelihood of cancer development (183). Fortunately, all mammalian cells possess two enzymatic path-



**Figure 6** – (left) Upon formation of a DNA double strand break, the MRN complex recognizes the lesion and recruits CtIP and BRCA1/BARD1. On either sites of the break, 3'-single stranded DNA overhangs are generated that bind RPA. Next, BRCA2 is recruited by BRCA1 through interaction with PALB2. BRCA2 loads RAD51 recombinase, displacing RPA. The sister chromatid is invaded by the strand overhang with RAD51 nucleoprotein and the homology search is initiated. (right) Once homology is established, DNA synthesis is started using the sister chromatid as a template for both 3'-overhangs. The resulting structure is called a double Holliday Junction and is resolved by Holliday junction resolving enzymes (figure adapted from [154]).

ways that mediate the repair of DNA double-strand breaks (DSB): homologous recombination (HR) and non-homologous end-joining (NHEJ). The HR pathway is a very accurate repair mechanism in which a homologous stretch of DNA on a sister chromatid serves as a template to guide repair of the broken strand. It is most active in the late S-G2 phase of the cell cycle and accounts for the repair of ~10% of DSBs in mammalian cells. The role of BRCA1 is to remodel the chromatin to make the DNA damaged site become accessible to the DNA repair machinery; next BRCA2 directly translocates the DNA repair protein RAD51 to facilitate the repair (Figure 6). As both BRCA1 and BRCA2 are involved in DSB repair by homologous recombination, lack of one of these genes will result in HR defects and leaves a cell with only NHEJ to repair double strand breaks (184). In NHEJ, which can take place during the whole cell cycle, the two ends of the broken DNA molecule are processed to form compatible ends that are directly ligated. Because this repair mechanism lacks a homologous sequence control system, deletion, inversion, or any other type of abnormality in the genome could occur as a consequence (185). NHEJ is therefore recognized as a potentially less accurate form of DSB repair. The functions of BRCA1 and BRCA2 can become completely inactivated only, when both maternal and paternal genes have been silenced. The chances of losing both gene copies during a lifetime have been shown to be relatively small (186, 187); however, women carrying a germline mutation in BRCA1 or BRCA2 already have one silenced copy and the chance of losing the second copy is relatively high. The loss-of-function of the second allele, often referred to as the 'second-hit' (188) and in most cases caused by loss of heterozygosity (LOH), can lead to cancer formation (189).

## Synthetic lethality in *BRCA* mutated tumors

PARP1 and PARP2 are proteins involved in the repair mechanism called base excision repair (BER), which is a key pathway for the repair of DNA single-strand breaks (SSB) (190). When a single-strand break is not repaired and encountered by a DNA replication fork, the fork will stall and eventually collapse, which will lead to DNA double-strand break formation (191). Usually, such breaks are repaired by homologous recombination (HR) or non-homologous end joining (NHEJ); however, in BRCA1- or BRCA2-deficient cells, the homologous recombination repair pathway is impaired and the cell has to resort to NHEJ only, which is error-prone. This has led to the hypothesis that homologous recombination deficient (HRD) cells, such as tumor cells in *BRCA1*-mutated breast cancer, might be hypersensitive to the inhibition of PARP and crippling the BER mechanism. In contrast, normal cells with at least one functional copy of *BRCA1/2* should be able to repair the breaks, resulting in chromosomal stability and cell viability (192). Indeed, preclinical studies have shown that PARP inhibitors are synthetic lethal in *BRCA* mutated cells (193, 194).

To date, clinical data on the use of PARP inhibitors as anticancer drugs are limited and only Phase I and II studies have been performed; however, these studies have shown promising results in terms of antitumor activity (143, 195-197). This new therapy has the potential to improve current therapy options for *BRCA*-mutation carriers, but patients with sporadic breast cancer with HRD might also benefit from this synthetic lethality. It should, however, be noted that there is currently no clinical test available to routinely investigate *BRCA* or homologous recombination status in sporadic

breast cancer. As BRCA-deficient breast tumors have not shown to be a histopathological unique entity, genetic markers found by microarray experiments might form the basis for a tool that is able to indicate HRD or BRCA-deficiency in the future.

## Microarray technology

A microarray is a solid surface, generally a glass slide, on which multiple known nucleotide sequences, called probes, have been immobilized in gridded formation. The probes function as target on which fluorescently labeled DNA or cDNA can be hybridized. A quantitative measurement of hybridized sample to each probe can be made by comparison to a reference sample. In this thesis, two different microarray technologies are described, the first technique can be used to measure gene expression levels and the second to measure DNA copy number levels. The advantage of using microarrays as compared to other techniques that are able to quantitatively measure gene expression or copy number levels is its ability to perform thousands to even millions of measurements in parallel. Gene

expression (GE) microarrays can be used to measure the amount of mRNA expression of basically every known gene in the human genome compared to a standard (198). The technique to study copy number levels is called comparative genomic hybridization (CGH) and as a microarray application array-CGH (aCGH). This technique can be used to measure the amount of DNA copies of most part of the genome (199). Both microarray techniques have been employed to search for markers that can be used as a target for anticancer drugs, but also to identify profiles on which breast cancer can be separated into molecular subtypes. This thesis will concentrate on the latter methodology, the identification of profiles specific for tumor subclasses.

## Breast cancer subtypes

Clinically, breast cancer can be classified into two main groups: the estrogen receptor (ER) negative and ER positive breast tumors. However, an important hallmark in breast cancer classification was the identification of multiple subtypes within the ER negative and positive

**Table 8** - Data from 214 breast tumors, metastasis and normal tissue were excluded from the original study (200).

	Luminal A (%)	Luminal B (%)	ERBB2-enriched (%)	Basal-like (%)	Claudin-low (%)	Normal-like (%)
Prevalence	31	18	13	24	11	3
Grade I	23	3	4	2	0	17
Grade II	49	26	39	10	24	17
Grade III	28	71	57	86	76	66
ER						
positive	87	86	22	13	10	67
negative	13	14	78	87	90	33
PR						
positive	73	56	21	5	30	40
negative	27	44	79	95	70	60
ERBB2						
positive	10	26	70	9	31	60
negative	90	74	30	91	69	40

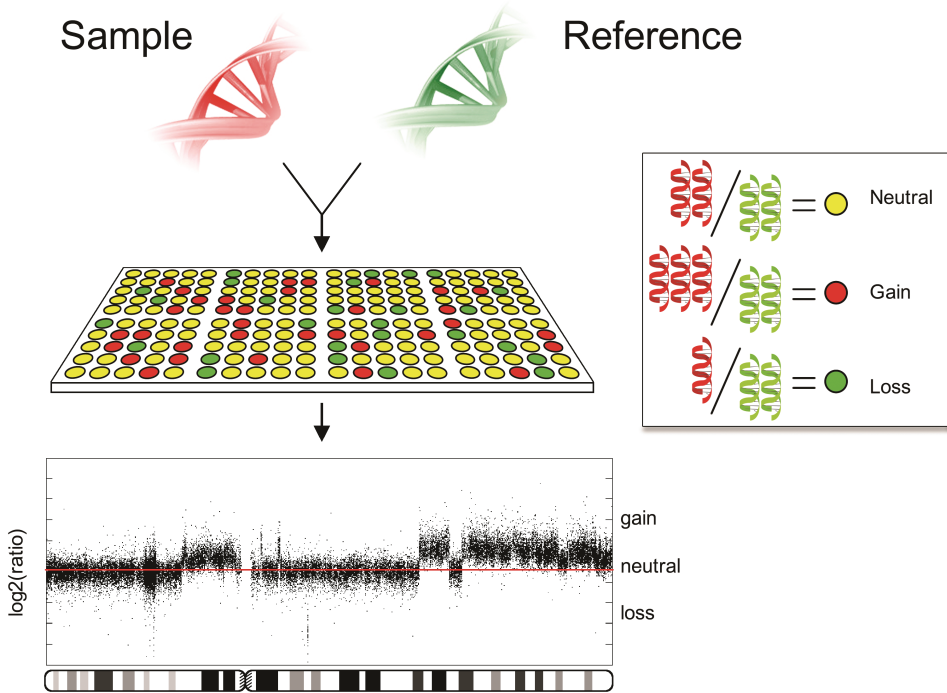


tumors based on gene expression profiles (201). To date, six different molecular subtypes have been described and are known as luminal A, luminal B, ERBB2-enriched, basal-like, normal-like and claudin-low (202); the latter group was formerly classified as a subgroup within the basal-like subtype and was called basal-like B (203). The molecular subtypes are associated with differences in histopathological feature, clinical outcome and response to chemotherapy. Patients with luminal A tumors have the longest survival times, patients with basal-like or ERBB2-enriched subtypes have the shortest survival times, and patients with luminal B or claudin-low tumors have an intermediate survival (200, 204). Basal-like and claudin-low breast tumors are in general ER, PR and ERBB2 negative (triple-negative) while ERBB2-enriched generally show overexpression of ERBB2 (Table 8). It is postulated that the basal-like and ERBB2-enriched breast tumors originate from ER negative luminal progenitor cells, while claudin-low tumors originate directly from mammary stem cells (Figure 5) (174, 175, 205). Luminal breast tumors are often ER positive and originate from differentiated luminal cells. Studies investigating hereditary breast cancer have shown that the majority of breast tumors from *BRCA1* mutation carriers are of the basal-like subtype (74-90%), while breast tumors from *BRCA2* mutation carriers are more heterogeneous but predominantly of luminal B type (204, 206, 207).

## Genomic instability and CGH

Genomic instability is one of the main characteristics of cancer and includes aneuploidy, polyploidy, translocations and amplification (1). Genomic changes are the causative factors in the initiation, development, and progression in breast neoplasms (208). These

aberrations can be studied and characterized to better understand the evolutionary pathways a cell undergoes to ultimately grow out to cancer. Errors in chromosome duplication, segregation and telomere dysfunction in the absence of caretaker genes are examples from which chromosomal aberrations can arise (209). During tumorigenesis, DNA regions that include oncogenes are frequently amplified causing overexpression of the gene and giving the cell growth advantage; tumor suppressor genes are often lost during the evolutionary process of cancer so cells can escape cell death. It has been noticed that several aberrations are recurrent in breast cancer such as amplifications of the genes *MYC* on chromosome 8q24 and *ERBB2* on chromosome 17q12 (210). Investigating DNA copy number alterations across a tumor's entire genome was a challenging task until the introduction of comparative genomic hybridization (CGH) technology (211). Improvements in the conventional or metaphase CGH, together with the development of microarray technology, led to the introduction of array CGH. Compared to metaphase CGH, array CGH has the advantages of being a high-throughput technology and providing a better resolution (199). The array CGH platform used in this thesis consisted of large-insert clones called BAC (bacterial artificial chromosome) clones, providing a genome wide resolution of 1 Mb on average. The procedure of performing an array CGH experiment is devised from several steps: DNA extracted from tumor material and reference DNA are differentially labeled with fluorescent dyes Cy5 and Cy3, respectively, and mixed in a 1:1 ratio. To block repetitive sequences,  $C_{ot}$ -1 DNA is added. Subsequently, the mixture is co-hybridized on a glass slide spotted with DNA probe sets under controlled temperature and humidity conditions. Lastly, the fluorescence of the hybridized



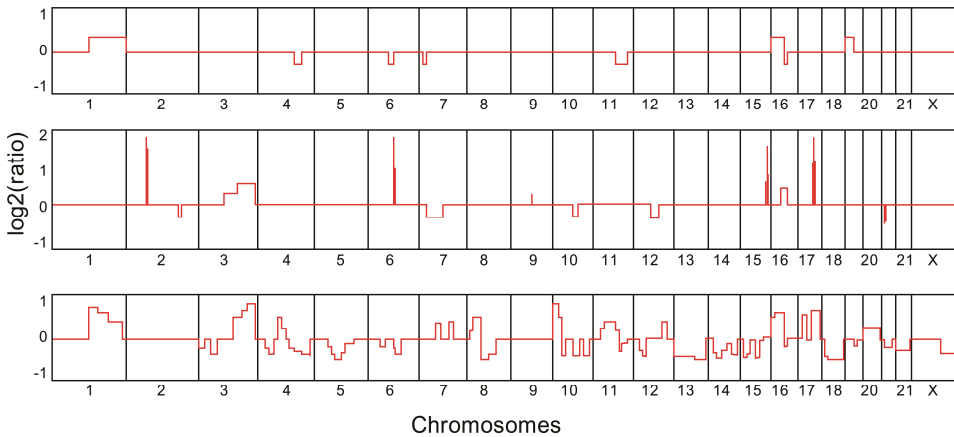
**Figure 7** - Sample DNA labeled with red fluorescent dye and reference DNA labeled with green fluorescent dye are co-hybridized to a microarray. A red-to-green ratio profile is generated from the intensity measurements. A higher red signal compared to green signal means gain/amplification of sample DNA, whereas less red signal means loss/deletion of sample DNA.

DNA is measured and specialized software converts the fluorescent intensity data to a linear red-to-green ratio profile (called CGH profile) that correlates with the hybridization intensity, which mainly depends on the extent and size of the tumor's DNA copy number changes (Figure 7).

## CGH profiles

Since the development of comparative genomic hybridization (CGH), chromosomal aberrations (*i.e.*, aneuploidy, polyploidy and amplifications) of genomic DNA in breast cancer, have been under extensive study to identify novel candidate cancer genes (212). Currently, three different types of profiles of

genetic alterations detected by array-CGH are described (Figure 8). The first profile exhibits only few gains or losses of whole chromosome arms with most characteristically gain of chromosome 1q and 16p and loss of 16q. This profile is mainly associated with ER positive breast cancers and those of the Luminal A subtype. The second type of profiles contains high-level DNA amplifications with a moderate complex pattern of other gains and losses seen along the whole genome, typically for Luminal B and ERBB2-enriched subtype breast tumors. The last type of profiles is characterized by a complex pattern of numerous small aberrations including gains, losses and amplifications, and is associated with *TP53* mutated, basal-like and claudin-low



**Figure 8** - Three different types of array-CGH profiles are identified in breast cancer research. A simple profile with only a few aberrations (upper), a moderate complex profile with high-level amplifications and several other aberrations (middle), and a complex profile with many gains, losses and amplifications (bottom).

subtype breast tumors (209). Because both *BRCA1* and *BRCA2* genes are part of DNA repair pathways, lack of function of one of these genes causes impaired DNA repair by homologous recombination, resulting in an accumulation of genetic errors and chromosomal instability (184). This thesis concentrates on characterizing the CGH profiles of *BRCA1*- and *BRCA2*-mutated breast tumors, and to extract the aberrations specific for *BRCA1*- or *BRCA2*-deficiency.

## This thesis

### Tumor based prediction of *BRCA* status

Although the inclusion criteria for *BRCA* mutation screening mentioned earlier cover many of the characteristics of typical *BRCA* families, mutation carriers might be missed because of lack of a family history of cancer or due to late age at breast cancer onset. In addition to pedigree-based risk assessment for *BRCA*

mutations, a number of strategies exist to determine tumor-specific characteristics on which association with *BRCA* defects might be deduced. A recent study showed that on mammography and MRI, *BRCA*-associated lesions were more often described as rounded and with sharp margins compared with an age- and tumor type-matched control group; however, the prognostic value of this has yet to be evaluated (213). Morphological investigations show clear differences between *BRCA1*-mutated and age-matched sporadic breast tumors: the lesions are mainly of higher grade, have more pleomorphisms, a higher mitotic count, and less tubule formation. In addition, *BRCA1*-associated carcinomas are more often of the medullary type. *BRCA2*-mutated breast tumors, on the other hand, are generally more similar to sporadic breast tumors, but they show less tubule formation and the occurrence of tubular carcinoma is less common (214-218).

Histological, *BRCA1*-mutated breast tumors are in general estrogen receptor (ER), progesterone receptor (PR) and ERBB2 negative; whereas

this "triple-negative" phenotype is present in only 11% of sporadic breast tumors (152, 219-221). Other characteristics can be found at the molecular level; these include loss of heterozygosity (LOH) of the *BRCA* genes (222), frequent *TP53* mutations in *BRCA1*-mutated tumors (223), hypermethylation of *BRCA1* gene promoter CpG islands (224), and chromosomal aberrations (225). Although these characteristics are not unique to *BRCA*-mutated breast tumors and can therefore not be solely used to identify association with mutated *BRCA*, they might be of indicative value in cases of naïve inherited breast cancer susceptibility. Tumor characteristics can be informative not only for untested but also for *BRCA*-tested individuals. Members of breast cancer families may misinterpret uninformative mutation screening test results (57, 226, 227); hence, for such families, deciding for the right prevention and treatment strategies can be difficult. The reasons for an uninformative *BRCA* status test result might be defects in other genes that have thus far not been correlated with breast cancer predisposition (*i.e.*, *BRCAX*) or (epi)genetic defects located in the *BRCA1* or *BRCA2* genes missed by genetic testing (115). Besides the pedigree based risk assessment for *BRCA* mutations, so far no other strategies exist that identify association with *BRCA* defects after routine diagnostics has not been able to identify a mutation. The work described in this thesis explored the possibility to predict the likeliness of *BRCA* association in breast cancer based on the tumor's chromosomal aberrations using array CGH.

## Array CGH — When and How

After a tumor has been surgically removed, the tissue will usually be fixed in formalin and subsequently embedded in paraffin for histopathological diagnosis and long-term storage.

Quality of the tissue's DNA is mostly affected by variability in sample fixation time and duration of storage (228). To improve efficiency of array CGH experiments, we investigated whether we could assess the quality of the tumor's DNA prior to array CGH assays. **Chapter 2** of this thesis describes a relatively easy to perform multiplex PCR test to determine whether the DNA quality is sufficient for a successful array CGH hybridization (229). After DNA of sufficient quality has been collected, array CGH experiments can be carried out. Hybridization and slide washing was formerly performed by hand, due to technical advances this process can now also be performed in an automated fashion. Automated hybridization will reduce handling time and, most importantly, improve the reproducibility compared to manual array CGH hybridization. **Chapter 3** describes the optimization of automated array CGH for formalin-fixed, paraffin-embedded breast cancer tissues (230).

## Predicting BRCAness by array CGH

The current strategy to identify *BRCA1* and *BRCA2* mutation carriers is direct sequencing preceded by pre-screening for abnormalities; however, it still remains unclear to what extent *BRCA* mutation carriers are missed by this approach. Additionally, the detection of variants of unknown clinical significance is emotionally and clinically challenging. Therefore, an additional tool that would indicate the involvement of *BRCA* in the development of the individual breast tumor would be an asset to current clinical diagnostics. Since both *BRCA1* and *BRCA2* genes are involved in DNA repair, *BRCA*-associated tumors are characterized by intensive genomic instability (231-233). This thesis describes the studies of these chromoso-

mal aberrations by array CGH, which has led to the identification of the specific aberrations of *BRCA1*- and *BRCA2*-associated breast tumors separately (234, 235). **Chapter 4** describes the usage of *BRCA1* specific chromosomal aberrations to identify *BRCA1*-associated breast tumors from a cohort in which no *BRCA1/2* mutations had been found by routine diagnostics. **Chapter 5** describes a similar process, but for *BRCA2*-association.

## **BRCAness in sporadic breast cancer**

In contrast to other cancer predisposition genes, neither *BRCA1* nor *BRCA2* has been found to be mutated in sporadic breast cancer (186, 187). It has however, come to the attention of investigators that a small subset of sporadic breast cancers is remarkably similar in many aspects to *BRCA1*-mutated tumors, this cancer group is known as the basal-like breast cancer subtype (236, 237). Tumors of the basal phenotype are seen in 2-18% of sporadic breast tumors. They show IHC positivity for basal intermediate filaments (e.g., K5, K14), are usually of high grade with large central acellular zones comprising necrosis, tissue infarction, collagen, and hyaline material, and are generally estrogen receptor (ER), progesterone receptor (PR), and ERBB2 negative (152, 220). Not only phenotypically but also genetically, sporadic basal-like tumors are similar to hereditary *BRCA1*-mutated breast tumors, as has been shown by genome wide gene expression profiling (238). **Chapter 6** in this thesis shows that also a set of characteristic cytogenetic changes in *BRCA1*-associated breast cancers can be found in sporadic basal-like breast tumors. These microarray studies suggest that similar cancer promoting pathways may lead to the development of these two tumor

groups (239). It is therefore hypothesized that sporadic breast cancer displaying genomic similarities with hereditary *BRCA*-mutated tumors, exhibit dysfunctional *BRCA* pathways and therefore deregulated homologous recombination. Tumors with homologous recombination deficiency (HRD) are highly sensitive to agents inducing DNA double strand breaks. **Chapter 8** of this thesis will, among other subjects, discuss how prediction of *BRCA*-association can assist in clinical care of sporadic breast cancer patients by demonstrating its predictive value for therapy response and survival.

## **BRCAX**

In many of the breast cancer families, no mutation is found in any of the known breast cancer susceptibility genes and so far, identification of a third *BRCA* gene has been unsuccessful. It is therefore likely that the non-*BRCA1/2* breast tumors are a heterogeneous group consisting of a collection of low penetrance genes or a combination of common variants with multiplicative effects on breast cancer risk (114, 134-137). To be able to locate potential loci on which breast cancer susceptibility genes might be located, homogeneous groups have to be identified first; however, non-*BRCA1/2* breast cancer families do not show any typical phenotypes such as the *BRCA1* or *BRCA2* families do. Because it has been shown that *BRCA1*- and *BRCA2*-mutated breast tumors display distinctive chromosomal aberrations (**Chapter 4 & 5**), it might be possible that tumors caused by other breast cancer susceptibility genes (i.e., *BRCAX*) could also display such characteristic profiles. **Chapter 7** discusses the use of array CGH with the aim to describe more homogeneous groups in non-*BRCA1/2* families, which could be applied for linkage studies in the future.

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