FAMILY HISTORY AND BREAST CANCER SUSCEPTIBILITY

Clinical and Molecular studies

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Stockholm 2006
ABSTRACT

Family history and breast cancer susceptibility – clinical and molecular studies
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Apart from gender, family history is the most important risk factor for breast cancer. In 5-10 %
of the cases there is a family history pattern of an autosomal dominant disease and there is also
a familial clustering of breast cancer associated with a more modest increased risk of the
disease. Mutations in the known high risk genes BRCA1, BRCA2 and p53 account for less
than 25% of the familial risk for breast cancer, while the remainder remain genetically
unexplained despite a large effort in research. A polygenic model has been proposed to best
explain the residual familial breast cancer risk and also to contribute to sporadic breast cancer
susceptibility in interaction with environmental factors.

In order to further elucidate the impact of genetic susceptibility for familial and sporadic breast
cancer, a population-based cohort of 489 breast cancer patients from southern Stockholm was
collected. For all patients, information on family history, clinical data and 5 years follow-up
was retrieved. In addition, a risk-cohort of 350 non-BRCA1/2 familial patients from the
Stockholm region was used for these studies.

In total 32% of the patients in the population-based cohort reported a family history of breast
cancer and 10% was defined a high-risk familial group. There was no relation between family
history and age of onset, hormonal background, tumour characteristics, treatment or prognosis.

The population-based cohort was screened for mutations in BRCA1 (exon 11). Two mutations
(<1%) were detected, both in cases with a family history of both breast and ovarian cancer.

Sporadic (n=313) and familial (n=387) breast cancer cases and controls (n=760) were screened
for the rare truncating variant, CHEK2 1100delC, which has previously been shown to be
associated with familial and unselected breast cancer. Of the familial patients 2.3% carried the
variant compared to 0.7% of the controls. The prevalence was not increased in sporadic patients
(0.3%). The variant seemed to influence age at onset, with a lower mean age in carriers than in
non-carriers.

Analysis of a common single nucleotide polymorphism, C975G, in the estrogen receptor a
(ESR1) gene in 288 sporadic, 197 low risk and 191 high risk non BRCA1/2 familial breast
suggested a protective effect of the variant allele in high-risk familial breast cancer
compared to controls. No association was seen in low risk familial or sporadic cases.

Three polymorphisms in the estrogen receptor β (ESR2) gene were analysed for association
with familial and sporadic breast cancer. In total 723 breast cancer cases were genotyped, 323
sporadic cases and 400 non-BRCA1/2 familial cases. There was no overall significant
difference in genotype distribution but one common haplotype, G-A-G, was associated with an
increased risk of sporadic breast cancer indicating a role for ERβ in breast cancer susceptibility.

Keywords: breast cancer, family history, sporadic, familial, BRCA1, CHEK2, polymorphism,
association, estrogen receptor alpha, estrogen receptor beta

ISBN 91-7140-868-1
PAPERS INCLUDED IN THE THESIS

The thesis is based on the following papers, which will be referred to in the text by their Roman Numerals:

Familial Cancer 2006; in press (e-pub ahead of print)

II. Margolin S, Werelius B, Fornander T, Lindblom A. BRCA1 Mutations in a Population-based Study of Breast cancer in Stockholm County
Genetic Testing, 2004; 8, 127-132

III. Margolin S, Eiberg H, Lindblom A, Bisgaard ML. CHEK2 1100delC in Swedish familial and sporadic breast cancer
Submitted

IV. Skoglund J*, Margolin S*, Zhou XL, Maguire P, Werelius B, Lindblom A. The Estrogen receptor alpha C975G variant in familial and sporadic breast cancer - a case-control study
Anticancer research, 2006; 26, 3077-3082

V. Maguire P*, Margolin S*, Skoglund J, Sun XF, Gustafsson JÅ, Børresen-Dale AL, Lindblom A. Estrogen receptor beta (ESR2) polymorphisms in familial and sporadic breast cancer
Breast Cancer Research and Treatment, 2005; 94, 145-152

*These authors contributed equally to this work
ABBREVIATIONS

3’UTR 3’ untranslated region
aa Amino acid
AR Androgen receptor
Arg Arginine
AT(M) Ataxia teleangiectasia (mutated gene)
ATP Adenosin triphosphate
BMD Bone Mineral Density
BRCA Breast cancer gene
CGH Comparative genome hybridisation
CHEK (CHK) Check point kinase
CI Confidence Interval
COMT Catechol-O-Methyltransferase
CYP Cytochrome P450
Del Deletion
DNA Deoxyribonucleic acid
DSB Double strand break
ER, ESR Estrogen receptor
GST Glutathion S-transfases
HNPCC Hereditary non polyposis colon cancer
HR Hazard ratio
HRT Hormone replacement therapy
HWE Hardy Weinberg equilibrium
Ins Insertion
LD Linkage disequilibrium
LFS Li Fraumeini syndrome
LOH Loss of heterozygosity
LOD Logarithm of the odds
NAT N-acetyl tranferases
OCCR Ovarian cancer cluster region
OR Odds ratio
PCR Polymerase chain reaction
PgR Progesterone receptor
Ppi Inorganic pyrophosphate
PTEN Phosphatase and tensin homologue
PTT Protein truncation test
Rb1 Retinoblastoma gene
RFLP Restriction fragment length polymorphism
RNA Ribonucleic acid
RR Relative risk
SNP Single nucleotide polymorphism
STK11 Serine/threonine kinase 11
TP53 Tumour protein 53 gene
TNF Tumour necrosis factor
## CONTENTS

**CONTENTS** .......................................................................................................................................................1

**INTRODUCTION** ................................................................................................................................................3

1.1 EPIDEMIOLOGICAL AND PEDIGREE STUDIES ..........................................................................................3

1.2 HIGH-RISK SUSCEPTIBILITY GENES ........................................................................................................5

1.2.1 TP53 .........................................................................................................................................................5

1.2.2 BRCA1 .....................................................................................................................................................5

1.2.3 BRCA2 .....................................................................................................................................................6

1.2.4 Other rare high risk genes ......................................................................................................................6

1.2.4.1 PTEN ..................................................................................................................................................6

1.2.4.2 STK11 ................................................................................................................................................6

1.2.4.3 ATM ...................................................................................................................................................7

1.2.4.4 E-cadherin .......................................................................................................................................7

1.2.5 LOW RISK GENES ..................................................................................................................................8

1.3.1 DNA repair genes .......................................................................................................................................9

1.3.1.1 CHEK2 .............................................................................................................................................9

1.3.2 Steroid hormone metabolism genes .......................................................................................................10

1.3.2.1 ERα (ESR1) ......................................................................................................................................10

1.3.2.2 ERβ (ESR2) ...................................................................................................................................13

1.3.3 Carcinogen metabolism genes ..............................................................................................................14

1.3.4 Other candidate genes ..........................................................................................................................15

1.4 THE SEARCH FOR ADDITIONAL PREDISPOSING BREAST CANCER GENES – PRESENT AND FUTURE .................................................................................................................................15

1.5 CLINICAL IMPLICATIONS OF FAMILY HISTORY AND BRCA1/2 MUTATIONS ...............................................................17

1.5.1 Prevalence of family history and BRCA1 and BRCA2 mutations ...........................................................17

1.5.1.1 Family history ..................................................................................................................................17

1.5.1.2 BRCA1/2 mutations in high risk families ......................................................................................17

1.5.1.3 BRCA1/2 prevalence in Nordic countries ....................................................................................17

1.5.1.4 BRCA1/2 prevalence in unselected breast cancer and in the normal population ..............................18

1.5.2 Penetrance of BRCA1 and BRCA2 mutations .....................................................................................18

1.5.3 Phenotypic characteristics of familial breast cancer .................................................................................19

1.5.3.1 Family history of breast cancer .......................................................................................................19

1.5.3.2 BRCA1 mutation carriers .............................................................................................................19

1.5.3.3 BRCA2 mutation carriers .............................................................................................................20

1.5.3.4 Prognosis ........................................................................................................................................20

**AIMS** ...............................................................................................................................................................21

**MATERIAL AND METHODS** ..........................................................................................................................22

1.6 MATERIAL ..................................................................................................................................................22

1.6.1 Patient cohort Södersjukhuset / Huddinge (studies I-V) ........................................................................22

1.6.1.1 Classification according to family history (Paper I and II) ................................................................22

1.6.1.2 Classification according to family history (Paper III-V) ..................................................................23

1.6.1.3 Clinical parameters (Paper I-IV) ....................................................................................................23
1.6.2 Patients from Department of Clinical Genetics (Paper III-V)........................................23
1.6.3 Controls (Paper III-V)..................................................................................24
1.7 METHODS........................................................................................................24
1.7.1 PTT (Paper II).........................................................................................24
1.7.2 Multiplex PCR (Paper III)...........................................................................24
1.7.3 Pyrosequencing (Paper IV and V).................................................................25
1.7.4 Restriction fragment length polymorphism, RFLP (Paper V)......................26
1.7.5 Association analysis (Paper III-V).................................................................26

RESULTS AND DISCUSSION....................................................................................28

1.8 PAPER I..........................................................................................................29
1.9 PAPER II .......................................................................................................32
1.10 PAPER III ...................................................................................................32
1.11 PAPER IV ......................................................................................................34
1.12 PAPER V.......................................................................................................35

CONCLUSIONS.................................................................................................37

ACKNOWLEDGEMENTS...................................................................................38

REFERENCES..................................................................................................40

PAPERS...........................................................................................................58
INTRODUCTION

Apart from gender, family history is the most important risk factor for breast cancer. Other identified risk factors are mainly hormonal and life style factors and in rare cases irradiation in young age (Table 1). In most individual cases there is however no definite answer to the crucial question occupying many affected women “why me?” In the 18th century it was common, also for the medical profession, to blame the disease on damage done to the breast by blows or the accumulation of black bile after menopause (a notion going back to antiquity) which could be prevented by regular bleeding. The hereditary component, which was observed in up to one in three affected women, was explained by an inherited constitution e.g. bilious or sanguineous. In Victorian times, breast cancer was often blamed on chronic irritation by the tight habit of dressing and another common notion was that women’s inborn, but varying, emotional fragility was predisposing them to cancer, breast cancer in particular.

The modern research on hereditary breast cancer started with epidemiological studies and moved on to studies of loss of heterozygosity (LOH) and linkage studies in the nineties leading to the discovery of the breast cancer genes BRCA1 and BRCA2. Thereafter has followed an intense search for more high and low risk genes. The clinical research field of family history and BRCA1/2 include studies on prevalence and penetrance of these genes, preventive strategies and if and how family history and mutation carrier status influence clinical presentation and prognosis.

The aim of this thesis has been to study the proportion and effect of family history in breast cancer and to identify underlying inherited alleles that contribute to the genetic susceptibility to the disease. Apart from women with a strong family history of the disease, also cases with a more moderate or no family history have been the focus of these studies, groups where the cause of the disease probably is multifactorial and involves a complex interaction between genetic and environmental factors. Identification of causative biological factors may have impact on prevention and clinical management of the disease.

1.1 EPIDEMIOLOGICAL AND PEDIGREE STUDIES

In the early 1980s several systematically epidemiological studies of familial susceptibility was conducted, almost all showing an elevated risk for breast cancer in female relatives to breast cancer patients. One of the largest population-based studies was conducted in Sweden including 2660 women. Within this cohort, the relative breast cancer risk was 1.7 for women with an affected relative.

A metaanalysis of 74 studies from these years quantifying the risk of breast cancer associated with a family history of the disease showed that the pooled estimate of relative risk was 1.9 with any relative and 2.1 with a first-degree relative. If both mother and sister were affected the RR was 3.6 and in general risks were increased in younger subjects and if the relative had been diagnosed before age 50. In the same period the heterogeneous nature of familiality was described by studying pedigrees. In some families, there seemed to be a strong hereditary factor leading to multiple cases of breast cancer, with cases younger than average at diagnosis and with more bilateral cases. Segregation analyses of Danish breast cancer families provided evidence for an autosomal dominant inheritance, which was later supported by American segregation studies, and a connection between premenopausal breast cancer and ovarian
Table 1. Risk factors for / associated with breast cancer (Adapted from[301]; +/-: no effect on risk, + = Relative Risk (RR) 1-2, ++ = RR 2-5, +++ RR >5). Collab. = no author listed

cancer in some families was suggested[59,102,196,302]. The lifetime risk of breast cancer in carriers was estimated to be 0.82 compared to 0.08 without the susceptibility allele[106]. In another Swedish study, a cohort of 1975 live patients in Stockholm was studied with regard to family history and 6.7% were classified as hereditary breast cancer with an autosomal dominant inheritance pattern. In this study, there was no association to bilaterality although the familial patients tended to be younger at diagnosis than sporadic patients[170]. In a cohort of 489
population-based breast cancer cases from the Stockholm region, almost 10% were defined as having high-risk family history of breast or breast-ovarian cancer (Paper I).

### 1.2 HIGH-RISK SUSCEPTIBILITY GENES

With information from segregation studies and with progress in molecular techniques an intense search for high-risk genes started in the early nineties. Linkage analysis and tumour studies (LOH-studies) were used to pinpoint regions and a candidate gene approach was sometimes used to test putative predisposing genes in selected cases.

For linkage analysis large families are needed, preferably with many affected relatives. Linkage is based on the fact that if two genetic loci lie in very close physical proximity, they are likely to segregate together during the process of meiosis. The further apart the loci are the less the chance of them being inherited together. When doing genetic mapping, linkage analysis uses known polymorphic markers scattered throughout the genome. If a certain segment of a chromosome is always shared in affected family members but not in the healthy family members, the gene involved in the disease might be localised in this area of the genome. The usual statistic measure of linkage is LOD score, which is the “logarithm of odds”. A LOD score of +3 or more is considered to be strong evidence of linkage (1000:1 odds for linkage).

Tumour studies involve screening for genetic aberrations in paired blood and tumour samples to identify genes showing LOH. LOH areas are hypothesized to harbour potential tumour suppressor genes based on Knudsons two-hit hypothesis 154.

#### 1.2.1 TP53

The rare Li Fraumeni syndrome (LFS), containing soft tissue sarcomas, brain tumours, premenopausal breast cancer and leukaemia occurring at an unusually young age, was first described in 1969 165. LFS is an autosomal dominant syndrome estimated to confer a cancer risk to carriers, up to the age of 45, of nearly 100% for females and about 75% for males, the difference because of the female breast cancer risk 57. Because of the rarity and high mortality of the syndrome, linkage analysis had been impossible and the predisposing gene, TP53, was discovered in 1990 through the candidate gene approach 178,250. The tumour suppressor gene TP53 was studied because of the growing knowledge that TP53 was inactivated in the sporadic forms of most of the cancers associated with LFS. Further studies on TP53 in young breast cancer patients and in familial breast cancer have revealed very few or no germ line mutations of the gene outside the syndrome 38,240,312.

#### 1.2.2 BRCA1

In 1990 familial breast cancer was linked, with a LOD-score of almost 6, to a marker on chromosome 17q21 111. The study was based on 23 extended families with 146 affected members and 40% of the families showed linkage, mainly early-onset families. In 1993 Narod et al. showed that also breast-ovarian cancer families were linked to the same marker 193. In 1994 BRCA1 was identified, by positional cloning, by a group in Utah 183. The function of the protein was initially unclear although in tumours from mutation carriers, loss of the wild-type allele occurred in >90% indicating a tumor-suppressing function 61,244.
1.2.3 BRCA2

Shortly after the identification of BRCA1 the second major breast cancer susceptibility gene, BRCA2, was localized to chromosome 13q12-13 by linkage in large, non-BRCA1 families by a British group. A year later, the gene was cloned and was also suggested to be a tumour suppressor gene by LOH studies in tumours of carriers. BRCA2 was found to be involved also in male breast cancer, a fact that made it possible to use linkage analysis also to find this gene.

Both BRCA1 and BRCA2 have been shown to be involved in maintaining genomic integrity. Together with several proteins including RAD 51, CHEK2, ATM and TP53 they participate in DNA repair (especially double-strand breaks) and transcriptional regulation in response to DNA damage and cell cycle control. BRCA1 has also been suggested to function as a stem-cell regulator. Almost all protein-truncating mutations (frame shift, nonsense and splice site mutations) in BRCA1 and BRCA2 known so far have shown association to disease. A missense variation, resulting in an altered but not shortened protein, is more difficult to evaluate. Large rearrangements such as deletions or insertions may be important in some populations and are not detected with routinely used screening methods.

A deletion of exon 13 in BRCA1 has been shown to have a founder effect and can be detected by using MLPA (multiplex ligation-dependent probe amplification), a method currently starting to be routine in many laboratories in addition to conventionally used screening methods. More than one thousand different mutations in each gene have been reported and 60% of these have been reported just once.

1.2.4 Other rare high risk genes

1.2.4.1 PTEN

Cowden’s disease is a rare syndrome with an autosomal dominant pattern of inheritance, named after the first described patient in 1963. Multiple hamartomas and benign tumours of the skin, mucous membranes, breast and thyroid characterize it. The susceptibility gene has been identified as the tumour suppressor gene PTEN, also known as MMAC1 and TEP1.

Women with Cowden disease are at increased risk of breast cancer but in breast cancer patients outside the syndrome mutations are rare or absent.

1.2.4.2 STK11

The Peutz-Jegher syndrome is a rare autosomal dominant disorder, caused by germ-line mutations in the STK1/LKB1 gene. The gene was mapped to 19p13.3 by linkage and is considered to be a tumour suppressor gene. Multiple gastrointestinal hamartomatous polyps, melanocytic maculae of the lips and mucous membranes and an increased risk of malignancies including breast cancer characterize the syndrome. In order to establish whether STK11/LKB1 is associated with an increased risk of breast cancer also outside this syndrome, familial breast cancer patients with LOH on 19p have been screened for germ line mutations but no mutations were identified.
1.2.4.3 ATM

Ataxia telangiectasia (AT) is an autosomal recessive disease characterized clinically by cerebellar ataxia, oculocutaneous telangiectasias, radio-sensitivity and immuno-deficiency. Homozygotes have an increased risk of malignancy particularly leukaemias and lymphomas. The ATM-gene was cloned in 1995 and appears to function as a checkpoint in response to DNA damage. ATM heterozygotes were already in the 80s reported to have an elevated risk of breast cancer in studies emanating from AT-pedigrees. In a study on 88 breast cancer families which also contained cases with other AT-related cancers such as lymphoma or leukemia, 4.3% ATM heterozygotes were found, which was more than the expected carrier frequency in the population of 1%. However, whether ATM heterozygotes have an elevated breast cancer risk or not remains controversial. A Swedish study on familial breast cancer and another study on breast cancer with young-onset, could not find an increased prevalence of protein-truncating ATM-mutations while a Dutch study found 8.5% mutations in breast cancer patients selected for young age at onset, long-term survival and bilaterality. Some recent studies indicate that non-truncating missense mutations in the ATM gene may predispose to breast cancer.

1.2.4.4 E-cadherin

E-cadherin or CDH1 encodes a protein located on the cell surface with important function in cell-cell adhesion and inactivation of E-cadherin has been suggested to contribute to a tumour’s metastatic potential. Its inactivation, which may occur through LOH, point mutations or...
methylation, is commonly a late event in cancer and associated with a worse prognosis \(^{68,311}\). Somatic mutations in E-cadherin are common in diffuse gastric cancer and germ line mutations are known to cause hereditary gastric cancer of diffuse subtype, the families often also containing multiple cases with breast cancer \(^{44,107}\). The estimated cumulative risk of breast cancer in female carriers from these families is 39% \(^{215}\). Somatic mutations are also common in invasive lobular breast cancer, the majority of mutations found in combination with LOH of the wild type E-cadherin-locus, the behaviour of a classical tumour suppressor gene \(^{36}\). In order to elucidate the possible role of E-cadherin in hereditary breast cancer, familial breast cancer cases and families with cases of breast, gastric and colon cancer, whose tumours demonstrated LOH at the E-cadherin locus, were screened for germ line mutations but no mutations were found \(^{232}\). In conclusion, E-cadherin germ line mutations are important in families with hereditary diffuse gastric cancer and do in these families confer a substantial risk also for breast cancer, but does not seem to play an important role for breast cancer susceptibility outside this rare syndrome.

### 1.3 LOW RISK GENES

In 5-10% of breast cancer cases, more often if early onset, there is a pattern in the family of a highly penetrant trait \(^{196}\). The known high-risk genes, including BRCA1 and BRCA2 and the even rarer TP53, ATM and PTEN account for less than 25% of the familial risk for breast cancer \(^{74}\). Besides this dominant inheritance of breast cancer in families, there is also the less obvious clustering of breast cancer described in epidemiological studies \(^{213}\). Familial clustering outside the high risk families could be due to shared lifestyle factors or environmental factors but recent twin studies suggest that a larger proportion of breast cancer are due to heritable factors than earlier estimates, particularly if the genetic factors are modulated by environmental risk factors \(^{167,211}\). In a study on twins from Sweden, Denmark and Finland, there was a statistically significant effect of heritable factors for breast cancer in 27% \(^{167}\). It is likely that most of the genetic susceptibility for breast cancer, as well as other common cancers, result from the combined effect of common genetic variants, each of which have a modest effect individually, the “common variant: common disease” hypothesis \(^{222}\). A polygenic model with variations in several loci, each contributing a modest independent risk has been shown to best explain the residual non-BRCA1/2 familial aggregation of breast cancer \(^{20,21}\). The effect of low-penetrant genes might also explain sporadic breast cancer \(^{212}\).

The main approach to identify common low-penetrance genes is association studies, where the frequency of a genetic variant is compared in large series of affected cases and unaffected controls (figure 2). Association studies are, in contrast to linkage studies, based on a candidate gene approach and thus limited by our current biological knowledge concerning proteins involved in carcinogenesis. A systematic review of genetic polymorphisms and breast cancer risk showed conflicting or non-conclusive results for most of the variants and few associations reported in the literature have been confirmed by subsequent studies \(^{73}\). In order to overcome problems of power, detecting variants conferring modest risks, large sample sizes are needed. In general, unselected breast cancer cases have been used for association studies, but the study size can be reduced by selecting cases enriched for genetic susceptibility such as patients with family history or with bilateral breast cancer \(^{19}\). In addition to association studies on single polymorphisms, haplotype studies are increasingly used \(^{214}\). The following list does not claim to be complete but is an example of investigated candidate genes:
Figure 2. The stages in the design of an association analysis, using cases and controls (adapted from Pharaoh et al.214).

1.3.1 DNA repair genes

During the last years numerous association studies on different genes involved in base excision repair, homologous recombination of double strand break (DSB) repair, non homologous end-joining and other aspects of maintaining DNA stability have been published, including studies of variants in BRCA1, BRCA2 and RAD51. There is, so far, no evidence of true association except for one variant in the CHEK2 gene. However some studies of other genes have shown association that need to be confirmed by larger studies.

1.3.1.1 CHEK2

CHEK2 (also CHK2) is a cell cycle check-point kinas that is involved in a complicated network of proteins such as BRCA1, TP53 and ATM, regulating the cell cycle and DNA-repair 29. A protein-truncating mutation, 1100delC, previously found in rare high risk families with Li-Fraumeni syndrome, has been shown to be associated with an elevated risk for familial, non-BRCA1/BRCA2 breast cancer, however not acting as a high risk gene but rather as a modifier of so far unknown gene/s 31,180,202,281,282. A subsequent large study has also demonstrated a doubled risk in unselected cases carrying the variant 8. The frequency of the variant has been around 1% in controls, 2.0% in unselected breast cancer and 5% in familial non-BRCA1/BRCA2 breast cancer although in some populations the variant is even more rare or almost non-existent 8,141,153,180,281. In a Swedish population based breast cancer cohort, there was an increased CHEK2 1100delC prevalence in familial (2.3%) but not in sporadic breast cancer compared to controls (0.7%) and the variant seemed to influence age at onset (Paper III). The 1100delC mutation has also been shown to associate with bilateral breast cancer and was initially reported...
to increase the risk for male breast cancer by a ten-fold, however a subsequent Finnish study did not confirm this, at least not at the population level \textsuperscript{42,180,256,281}. In another study, the carriers were reported to have more receptor-positive tumours, had more often family history and had an unfavorable prognosis both regarding contra lateral breast cancer and disease-free survival \textsuperscript{67}.

Other variants in the \textit{CHEK2} gene have been reported, but they seem to confer a lower or no risk for breast cancer \textsuperscript{37,72,95,151,236}. A missense mutation, I157T, has been shown to associate to lobular breast cancer \textsuperscript{133}. Apart from breast cancer, \textit{CHEK2} also have been reported to confer a moderately increased risk of thyroid, prostate, kidney and colon cancer \textsuperscript{66}. The highest prevalence of \textit{CHEK2} 1100delC was seen in familial non- \textit{BRCA} 1/2 breast cancer also harboring colon cancer cases (18\%) \textsuperscript{181}. A subsequent Swedish study on patients with metachronous breast and colorectal cancer did not however show a statistically significant difference compared to controls (2.5\% vs. 1\%). In a Swedish case-control study on prostate cancer, the \textit{CHEK2} 1100delC variant was found in 1.2\% of the cases (sporadic: 0.7\%; familial: 1.6\%; hereditary: 1.4\%) and in 1.0\% of the controls and was concluded to be not clinically important high-risk gene for hereditary prostate cancer susceptibility in the population of southern Sweden \textsuperscript{280}.

1.3.2 Steroid hormone metabolism genes

Besides family history, the majorities of risk factors for breast cancer are related to reproductive factors and are thought to reflect longer lifetime exposures to endogenous steroid hormones \textsuperscript{122}. Both genes involved in the sex hormone biosynthesis (\textit{CYP17}, \textit{CYP19} and \textit{17 beta hydroxysteroid dehydrogenase type 2}) and the catabolism of estrogens (\textit{COMT}, \textit{CYP1A1}, \textit{CYP1B1}) as well as the steroid hormone receptors (\textit{ESR1}, \textit{ESR2}, \textit{PgR}, \textit{AR}, \textit{vitamin D receptor}) are strong candidate breast cancer susceptibility genes and have been investigated in several studies, often with inconsistent results \textsuperscript{73,185,269}. In the metaanalysis by Dunning et al in 1999, only the (TTTA)\textsuperscript{10} polymorphism in \textit{CYP19} was found to increase the risk of breast cancer with an OR of 2.3 (95\% CI 1.4-4.2) \textsuperscript{73}. The results on two restriction fragment length polymorphisms (RFLPs) in the \textit{vitamin D receptor} gene (BsmI and Apa1) are also consistent, showing an association in several studies \textsuperscript{41,65,108,109,128,172,241}. 13 SNPs in the \textit{PgR} gene was analysed in a study of >1000 breast cancer cases with no association with any variant or haplotype \textsuperscript{103}.

1.3.2.1 \textit{ERα} (\textit{ESR1})

The estrogen receptor (ER), denoted \textit{ERα} or \textit{ESR1} after the identification of \textit{ERβ}, was cloned and sequenced in 1986 although it was identified much earlier by its affinity for 17\β estradiol \textsuperscript{104,274}. It is located on chromosome 6q25.1, has eight exons and spans over 140kb. Its cDNA defines a sequence of 6322 nucleotides of which 1785 are coding while the remainder are untranslated regions at the 5’ and 3’ ends \textsuperscript{104,217}. \textit{ERα} belongs to the nuclear receptor superfamily and has six conserved domains with different function (figure 3a) \textsuperscript{217}. On estrogen binding the \textit{ER} forms homo-dimers and binds to DNA at specific sites called estrogen-responsive elements (ERE\textsubscript{s}) \textsuperscript{161}. The gene is expressed in various tissues including CNS, bone, endometrium and the mammary gland \textsuperscript{27}. \textit{ERα} is also expressed in a majority of human breast cancers and is requisite for response to endocrine treatment \textsuperscript{3,83}. Severe retardation of the mammary gland and infertility is seen in \textit{ERα} knockout mice \textsuperscript{173}.

\textit{ERα} was initially screened as a potential high-risk gene in familial breast cancer and one study indicated a possible clinical significance of the Gly160Cys variant \textsuperscript{16}. 

Figure 3a. Functional domains of ERα and ERβ. A/B domain=an amino-terminal transcription activation domain (TAF1), C domain=a central DNA binding domain (DBD) that contains two zinc-binding fingers, D-domain=a hinge region, E-domain=a hormone-binding domain (ligand binding domain, LBD) required for stable dimerization of the receptor and F region=transcriptional activating factor 2 (TAF-2).

Figure 3b. Localisation of common polymorphisms in the ERα gene, E= exon, P=promoter.

However 230 Stockholm breast cancer families were screened for this mutation and none were detected 313. More than 30 SNPs have been reported in or upstream the ERα gene, mostly rare variants (figure 3). Several positive association studies on two RFLPs in intron 1, XbaI and PvuII, have been published on both breast and endometrial cancer, however there are also negative publications 14,48,239,295,297. The most studied exonic variant is the common codon 325, CCC (pro) to CCG (pro), which has shown diverging results in association studies in both familial and sporadic breast cancer (Table 2) 64,113,147,248,294,295. In a Swedish case-control study there was an association of the codon 325 variant and high risk familial breast cancer but no effect in sporadic or low risk familial breast cancer (Paper IV).

Variants in ERα have also been studied in association with other potentially hormone related characters like bone mineral density and mammographic density. Carriers of the XbaI and PvuII polymorphisms had denser breast tissue in one study 289. Studies on bone mineral density (BMD) have also mainly focused on these two RFLPs in ERα and have demonstrated an association with BMD and the XbaI polymorphism, the results on PvuII are more contradictory 100,134,219,309. In a small study of the codon 325 polymorphism in postmenopausal women, GG homozygotes had lower femoral neck BMD compared to CC homozygotes (p=0.03).

The functional effect of the common variants in ERα is unclear. Both XbaI and PvuII are located in an apparently unfunctional area of the gene (intron I) and separated by only 50 base pairs. These polymorphisms are, not surprisingly, in strong linkage disequilibrium with each other but also with a TA repeat polymorphism upstream in the promoter region of the ER alpha, a region that might be more interesting in relation to function 30. Recently, it was noted that loss of the
PvuII site results in a potential binding site for myb transcription factors and can result in a higher ERα transcription. The polymorphism in codon 325 as well as other common SNPs in ERα (codon 10, 594) are silent since they do not cause any amino acid change. An association of these SNPs with disease might be due to linkage with other functional variant/s or a true effect on gene expression e.g on binding of transcriptional factors or splicing.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study size cases / controls</th>
<th>Population</th>
<th>Effect on risk</th>
<th>Other results</th>
<th>G allele frequency cases/controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roodi 225</td>
<td>118 ER+, 70ER- no controls</td>
<td>North-American</td>
<td>NA</td>
<td>G assoc family history (p=0.0005)</td>
<td>Spor br ca 0.14 / not done</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fam br ca 0.56 / not done</td>
</tr>
<tr>
<td>Iwase 138</td>
<td>70 / 30 British</td>
<td></td>
<td>↑ risk G vs. C (p=0.06)</td>
<td>No assoc with ER or PgR in tumor</td>
<td>0.28 / 0.13</td>
</tr>
<tr>
<td>Southey 248</td>
<td>388 / 294 (121 familial cases)</td>
<td>Australian early onset</td>
<td>None</td>
<td></td>
<td>Spor br ca 0.25 / 0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fam br ca 0.17</td>
</tr>
<tr>
<td>Schubert 235</td>
<td>133 high risk families no controls</td>
<td>North-American</td>
<td>NA</td>
<td>Did not segregate with disease</td>
<td>0.21 / not done</td>
</tr>
<tr>
<td>Curran 64</td>
<td>125 / 125 Australian</td>
<td></td>
<td>None</td>
<td></td>
<td>0.17 / 0.23</td>
</tr>
<tr>
<td>Vasconselos 294</td>
<td>70 / 69 Portuguese</td>
<td></td>
<td>↑ risk G vs. C</td>
<td>G-allele assoc N+</td>
<td>0.43 / 0.24</td>
</tr>
<tr>
<td>Kang 147</td>
<td>110 / 45 Korean</td>
<td></td>
<td>None</td>
<td>G assoc ER+, PgR+, p53- in tumor</td>
<td>0.50 / no information</td>
</tr>
<tr>
<td>Han 113</td>
<td>100 / 100 Korean</td>
<td></td>
<td>↓ risk for GG homozygotes</td>
<td>G assoc p53- in tumor</td>
<td>0.46 / 0.57</td>
</tr>
<tr>
<td>Wedrén 295</td>
<td>1296/1349 Swedish postmeno-pausal</td>
<td></td>
<td>↓ risk for GC heterozygotes for ductal but not lobular br ca</td>
<td>Assoc two haplotypes containing codon 325 pm, esp. if high BMI</td>
<td>0.20 / 0.23</td>
</tr>
<tr>
<td>Hsiao 129</td>
<td>189 / 177 Taiwanese</td>
<td></td>
<td>↓ risk w G allele</td>
<td>G assoc N+</td>
<td>0.52 / 0.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fam br ca 0.44</td>
</tr>
</tbody>
</table>

Table 2: Published studies on ERα codon 325 polymorphism (925C/G, rs1801132) and breast cancer. NA=not applicable
1.3.2.2 ERβ (ESR2)

A second estrogen receptor, ERβ or ESR2 was discovered in 1996 \(^{160,191}\). As well as ERα, ERβ belongs to the nuclear receptor super family and is activated in the nucleus by dimerization \(^{191}\). The DNA binding domains are almost homologous in ERα and ERβ (96%) and they are therefore expected to bind to Estrogen Response Elements (EREs) with similar affinity and specificity. The ligand binding (LBD) and activating function regions (TAF-1 and TAF-2) are less (60% and 30% respectively) conserved indicating a different function \(^{191}\). There are several splicing variants of ERβ, one of them ERβcx, which is identical to ERβ except for the last exon (exon 8) which is replaced by another unique sequence \(^{200}\). ERβcx lacks the ligand binding properties of ERβ and does not bind estradiol. ERβcx prefers to heterodimerise with ERα rather than ERβ, thereby inhibiting ERα DNA binding (dominant negative effect on ERα function) \(^{200}\).

ERβ is expressed in many different tissues in humans, including the female reproductive system and the normal breast where ERβ is the predominant estrogen receptor \(^{27}\). There is an ERβ knockout mouse (BERKO), which develops normally, but has fewer and smaller litters than wild type mice as a result of reduced ovarian efficiency, and they lack cyclical growth in the mammary gland \(^{56,156}\). In breast cancer, ERβ is thought to counteract ERα, being anti-proliferative and pro-apoptotic \(^{231}\). About 50% of breast cancers express both ERα and ERβ whereas 10-20% don’t express any of the oestrogen receptors \(^{231}\). There are several reports demonstrating a positive correlation between ERβ expression and prognosis but also studies with neutral effect \(^{231}\). The roles of both ERβ and ERβcx as predictors of hormone treatment response in breast cancer are controversial \(^{231}\).

As well as ERα, ERβ is an obvious candidate low risk breast cancer susceptibility gene but, compared to ERα, there are fewer association studies on ERβ and so far the results are controversial \(^{91,103,117,135,315}\) (Table 3). In a Swedish population-based breast cancer, a common ERβ haplotype was associated with an increased risk of sporadic but not familial breast cancer, indicating a possible role for ERβ in breast cancer susceptibility (Paper V).

There are several studies on ERβ variants and other diseases or characteristics (e.g. androgen levels, BMD, blood pressure, Alzheimer, eating and ovulatory disorders), and recently a publication on prostate cancer showed an association with a common SNP in the promoter region \(^{90,100,197,199,226,253,263,299}\). There is also a study of a CA repeat polymorphism in ERβ that demonstrated an association of colon and rectal cancer risk in women, but not in men \(^{243}\).

---

**Figure 4.** Localisation of common polymorphisms in the ERβ gene. ERβ is located on chromosome 14q22-24 and consists of 8 exons. Exon 1-7 of ERβcx is identical to ERβ. Through alternative splicing, exon 8 is replaced by the cx sequence. P=promoter, E=exon, UTR=untranslated region
<table>
<thead>
<tr>
<th>Reference</th>
<th>Study size cases / controls</th>
<th>Population</th>
<th>Polymorphism</th>
<th>Effect on risk</th>
<th>Other results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hasegawa 117</td>
<td>93 / 91</td>
<td>Japanese</td>
<td>4 SNPs</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Forst 91</td>
<td>219 / 248</td>
<td>Finnish</td>
<td>6 SNPs including G1082A, G1730A</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Zheng 315</td>
<td>1134 / 1235</td>
<td>Chinese</td>
<td>7 SNPs including G1082A</td>
<td>Assoc with C1176G (exon 6) and intron 5 pm and postmenop. breast cancer</td>
<td>A potential synergy with C1176G and steroid sex hormones on breast cancer risk</td>
</tr>
<tr>
<td>Gold 103</td>
<td>1011 / 615</td>
<td>North-American (388 Ashkenazi Jews)</td>
<td>8 SNPs including Cx+56A/G (rs928554), G1730A (rs4986938), G1082A (rs1256049)</td>
<td>No overall assoc.</td>
<td>One haplotype (7 SNPs) assoc. with increased risk among Ashkenazi Jews.</td>
</tr>
<tr>
<td>Iobagiu 135</td>
<td>139 / 145</td>
<td>French</td>
<td>(CA)ₙ in intron 5 cut-off 22repeats</td>
<td>None</td>
<td>Association with a combination of long (CA)ₙ repeats and microsatellite markers in ESR1 and AR (gene-gene interaction)</td>
</tr>
</tbody>
</table>

Table 3. Published studies on ERβ polymorphisms and breast cancer

1.3.3 Carcinogen metabolism genes

Genes coding for enzymes involved in detoxification/metabolism of environmental carcinogens are obvious candidate genes. This group of genes includes members of the cytochrome P450 family such as CYP1A1 and CYP1B1, which are both, involved in metabolism of polycyclic aromatic hydrocarbons (PAHs) and estrogen, and CYP2D6, which is induced by cigarette smoking. In a metaanalysis of 17 association studies on the four variants that have been studied in the CYP1A1 gene, there was no consistent association between breast cancer and any of the genotypes 179.

The Glutathione S-transferases, GSTs, are a family of enzymes that detoxifies carcinogens including estrogen quinines, reactive intermediates of estrogen metabolism that can bind to DNA, by facilitating their conjugation to glutathione and subsequent excretion. There are a number of association studies of variants in members of this family, e.g. GSTM1, GSTP1 and GSTT1 and in the metaanalysis from 1999 the GSTT1 polymorphism Ile105Val was associated with a modest increase in breast cancer risk (OR 1.6, p=0.02) 73. There are a number of more recent studies of members of this family, most of them, including a study of >2000 cases and controls, however negative for association 80,186,233,287,304.
Finally, the N-acetyl-transferases, NAT1 and NAT2, are also phase II enzymes involved in the detoxification of acryl amines from tobacco smoke and also amines produced during cooking of meat. Polymorphisms in both genes result in two main phenotypes, slow acetylators (homozygous for low-activity alleles) and fast acetylators. There are several association studies on the risk of breast cancer in slow acetylators, sometimes in combination with smoking and meat consumption, mostly on NAT2. In the metaanalysis there was no association between the two NATs and breast cancer. Some recent studies have however shown an association with NAT slow acetylators, smoking and breast cancer, however not consistently, and sometimes in interaction with other gene variants e.g. GSTs.

### 1.3.4 Other candidate genes

In addition to these categories, there are also several other candidate genes, which have been studied in relation to breast cancer. The HRAS1 minisatellite has been described as a low-penetrance breast cancer susceptibility locus with a relative risk for breast cancer of 1.9 for the so-called rare alleles in a metaanalysis. Two subsequent studies, with more than 700 cases and controls each have however not shown any association. Other candidates are genes in the tumour necrosis factor (TNF) family e.g. TNF alpha, TNF beta and the TNF receptor type II. Most studied is TNF alpha where the results are inconsistent, the largest study however negative.

Somatic mutations in the TP53 gene are a common event in breast cancer as well as in other malignancies making it an obvious candidate gene. An association between the codon Arg72Pro variant has been shown in several studies on both sporadic and familial breast cancer, but like for many of the associations discussed, the results are controversial and negative studies exist.

The HER2 polymorphism 1665V has been investigated in several relatively large studies and the Val allele has been associated to an increased risk of breast cancer especially in women with early onset of and a family history of breast cancer. There are however also negative studies, the largest by Benusiglio et al on more than 2000 cases and controls. Skewed X-inactivation is also a suggested risk factor for both sporadic and non-BRCA1/BRCA2 familial breast cancer.

### 1.4 THE SEARCH FOR ADDITIONAL PREDISPOSING BREAST CANCER GENES – PRESENT AND FUTURE

After the identification of BRCA1 and BRCA2 more than a decade ago, there has been no major break-through ("BRCA3") in the search for high-risk genes leaving a substantial part of the breast cancer families genetically unexplained for. Linkage analysis was used to find both BRCA1 and BRCA2 and two factors were of importance for their successful mapping. First, the high penetrance associated with these genes made it possible to find families with many affected to use for studies. Second, a typical phenotype could be used to define families for linkage, for BRCA1 early age of onset and for BRCA2 the prevalence of male breast cancer. In fact, it has been shown that the majority of families with more than 6 breast cancer cases have mutations in one of the two known genes suggesting that other genes are likely to have a lower penetrance. This fact in addition to of the lack of an identified unique phenotypic character to be used for defining families has made it difficult to find additional genes. One genome-wide
study on non-BRCA1/2 families have been published which suggested a putative locus on chromosome 2q. No other whole genome-wide screen have been published so far, however studies have focused on separate regions such as 6q, 8p and 13q.

It has been shown that tumours of BRCA1/2 mutation carriers show different phenotypic characteristics compared to unselected tumours also with molecular techniques. Similar approaches including gene expression profiling have been used to determine phenotypic characteristics of “BRCA3” tumours, as identification of homogenous subgroups could potentially increase the power of conventional genetic analysis. In a study using microarray expression, 17 familial non-BRCA1/2 tumour clustered in two groups suggesting two predisposing loci in this cohort. Comparative genome Hybridization (CGH) was used in a study on tumours from non-BRCA1/2 breast cancer families and LOH and linkage analysis in the same families suggested a putative breast cancer locus on 13q. This region was however not confirmed in a linkage study using families from the same region or in another study on families with different ethnicity. Other studies using CGH have suggested chromosomes 6, 8, 17, 19 and 20 to be of interest.

The candidate gene approach to find new low risk breast cancer genes using single polymorphisms in association studies have also yielded limited success despite large effort. The association studies could be improved by choosing candidate genes not only from known biological function but also from linkage peaks generated from family based studies, animal models or by selecting subgroups of patients defined by a trait, (e.g. dense breast tissue) that is hereditary. Instead of studying a single or a few polymorphisms in the candidate gene, all polymorphisms in the gene could be identified, either by resequencing the gene or by using data from the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP) which now contains nearly 9 or the around 11 million SNPs with a minor allele frequency of 1% or more that are estimated to exist in the human genome. It has been shown that many SNPs are in strong linkage disequilibrium (LD) with closely located SNPs and a considerably lower number of so called tagSNPs can be chosen, either individually or in multimarker combinations, so called haplotypes, to capture most of the allelic variation in a region. These tagSNPs can then be studied in larger case-control studies thus focusing on the whole gene rather than on a single SNP.

Genome-wide association studies have until now been impossible but the technical development of high throughput SNP analytical platforms and the completion of a genome-wide high density SNP map, will make it feasible. Two main approaches to genome wide association studies have been proposed. The sequence based approach advocates screening for functional SNPs in coding regions of the genome meaning that 50 000 -100 000 SNPs would have to be genotyped. This method would be able to detect lower frequency alleles (1-20%) but misses’ functional noncoding SNPs. With the haplotype approach, 200 000-500 000 tagSNPs are needed to detect disease alleles with a frequency of >5%, but there is no prejudice about types of SNPs which might be important. For the purpose of design and analysis of genetic studies the international HapMap Consortium has created a haplotype map of the whole genome. In addition to the common variant: common disease model there is also the possibility of multiple rare variants, exemplified by the rare CHEK2 1100delC variant in breast cancer. For these variants, the genome wide approach might not work, and the candidate gene approach will still be valid.

In conclusion, the search for additional predisposing high or low-risk breast cancer genes is ongoing but is complicated by a heterogeneous disease and a complex multifactorial inheritance.
1.5 CLINICAL IMPLICATIONS OF FAMILY HISTORY AND BRCA1/2 MUTATIONS

1.5.1 Prevalence of family history and BRCA1 and BRCA2 mutations

1.5.1.1 Family history

In 5-10% of breast cancer cases there is a pattern in the family of a highly penetrant dominant trait. These families are often defined as hereditary breast cancer, however there is no international consensus on an exact criteria for this definition (cp. Amsterdam criteria for HNPCC). In general, three 1st or 2nd degree relatives with breast cancer in at least two generations, often with an age criteria, are required, but in some publications only 1st degree relatives with breast cancer are taken into account. In further 18-23% of the breast cancer patients, there is a 1st or 2nd degree relative with the disease, this group sometimes defined as familial breast cancer or two close relatives. Familial breast cancer may also be used to include all patients with a family history of the disease (sometimes also 3rd degree relatives), 10-31% in previous studies.

1.5.1.2 BRCA1/2 mutations in high risk families

It was estimated that mutations in BRCA1 and BRCA2 explained about two thirds of hereditary breast cancer. Subsequent studies have however showed lower mutation frequencies and there are large variations in prevalence of mutations due to ethnicity of the population or to selection based on family history of breast and/or ovarian cancer, male breast cancer or young age at onset.

The highest prevalence is found in populations harbouring founder mutations. These are mutations that have occurred in specific ethnic or isolated populations. Founder mutations exist in several populations and are most well characterised in the Icelandic and Ashkenazi Jewish populations. In Iceland a single mutation, BRCA2 999del5, is found in a majority of the breast cancer families. In Ashkenazim, there are three founder mutations, two in BRCA1, 185delAG and 5382insC, and one in BRCA2, 6174delT accounting for >90% of mutations in this ethnic group.

In families with both breast and ovarian cancer the mutation frequency is higher than in families with breast cancer only. In a large collaboration study of 237 families with at least four cases of breast cancer, 81% of the breast-ovarian cancer families were due to BRCA1 and 14% to BRCA2. In families with breast cancer only 33% mutations in both genes were detected.

1.5.1.3 BRCA1/2 prevalence in Nordic countries

Scandinavian studies on BRCA1 and BRCA2 frequency in selected high-risk families vary between countries and also within Sweden probably due to different founder mutations and fairly stable populations with little migration. In Finnish breast and breast-ovarian cancer families 21% mutations in the high risk genes were detected, 10% in BRCA1 and 11% in BRCA2. In Norway four local mutations account for 68% of BRCA1 mutation carriers and BRCA2 mutations are very rare.

In a study of breast and ovarian cancer families in southern Sweden there was a BRCA1 mutation frequency of 23%, and 11% mutations in BRCA2. On the west coast of Sweden, there is a strong effect of a single founder, 3171ins5 in BRCA1, and 40% of high-risk families...
are BRCA1 carriers while only 3% are detected in BRCA2 \(^{81}\). In 106 high-risk breast cancer families from the Stockholm region only one BRCA1 mutation was found. In the same study, 20 breast-ovarian cancer families were screened and 7 were BRCA1 mutation carriers (35%) \(^{314}\). In BRCA2, only 2 mutations in 162 Stockholm families (1%) were detected \(^{54}\). In another study from Stockholm, where more strict criteria were used for screening, there were 7% BRCA1 mutations in familial breast cancer and 34% in families with breast and ovarian cancer. Again, only 1% of the families showed BRCA2 mutations \(^{22}\). In previous studies on BRCA1 in patients from the Stockholm region, 70% of the mutations, including the main founders, have been detected in exon 11 \(^{22,23,314}\).

1.5.1.4 BRCA1/2 prevalence in unselected breast cancer and in the normal population

The mutation frequency in breast cancer patients, not selected for family history or age varies. Several of these studies focus on founder mutations previously detected in high risk families in their own population, while a few have screened the whole gene/s. In the ethnically selected populations of Ashkenazi Jews and Icelanders, founder mutations are found in 7-12% \(^{87,271,293}\). In other studies, the reported mutation frequencies vary between 2% and 6% \(^{17,51,207,257,288}\). The lowest prevalence in unselected breast cancer, <1% BRCA1 mutations, was found in a population-based breast cancer cohort from the Stockholm region (Paper II).

In the general population the prevalence of BRCA1 mutations is estimated to be less than 0.1% \(^{6}\). In the Icelandic population 0.6% are carriers of the BRCA2 founder mutation and in slightly more than 2% of the non-cancer Ashkenazim one of the three founders are detected \(^{87,271}\).

1.5.2 Penetrance of BRCA1 and BRCA2 mutations

The early estimates of the penetrance of BRCA1 and 2 were based on families showing linkage to either of the genes \(^{75,88}\). Families suitable for linkage studies are extreme high-risk families with multiple cases of breast and ovarian cancer and the estimates are therefore relevant to that type of families but might overestimate the risk for carriers with more modest family history. Another approach has been to use breast cancer patients, not selected for family history, in order to get less biased data. The most used group for these studies have been Ashkenazim as they have a higher prevalence of mutations \(^{87,152,293}\). In the largest of these studies, based on 1008 index cases, the lifetime risk of breast cancer among female mutation carriers was 82%, similar to risks in families with many cases. Lifetime risks for ovarian cancer were 54% and 23% for BRCA1 and BRCA2 mutation carriers respectively. The penetrance seemed to be higher in later birth cohorts \(^{152}\).

In 1997, Struewing et al. presented a penetrance study, not based on cases but on >5000 Jewish non-cancer individuals, and in those identified 120 mutation carriers. The estimated risk of breast cancer, by the age of 70, was 56% and for ovarian and prostate cancer 16% each \(^{252}\). All the penetrance studies on Ashkenazim are based on the three founder mutations in this population. Another study of the penetrance of a founder mutation is from Iceland and population based. The estimated risk of breast cancer for female carriers of the 999del5 mutation in BRCA2 was 37% by the age of 70 \(^{272}\). Interestingly, a recent publication on the Icelandic founder mutation showed a four-fold increase in penetrance over time (1920-2000) \(^{277}\).

A metaanalysis of 22 population-based studies including 500 mutation carriers estimated a risk of breast cancer, by age 70 of 65% in BRCA1 mutation carriers. The corresponding figure for
BRCA2 was 45%. The risk of ovarian cancer was 39% and 11% respectively. Also this study showed a reduction in risk in women from earlier birth cohorts.

These penetrance studies assume that all truncating mutations confer the same cancer risk. However, there is some evidence that the mutation position in the gene influences the risk. For BRCA1, mutations in the central region of the gene seem to lead to a lower risk for breast cancer than other mutations, whereas mutations toward the 3’ end have a lower proportion of ovarian cancer. In BRCA2, there is an “Ovarian Cancer Cluster region” (OCCR) in the central region, where mutations are associated with a higher ovarian cancer risk and lower risk for breast cancer than other mutations in the gene.

The interindividual variability in cancer risk among BRCA1/2 carriers may also be explained by risk modifying genes either in the DNA repair pathway (figure 1) or other low risk candidate genes, individual characteristics (e.g. reproductive history) and exogenous exposure (oral contraceptives, HRT, smoking). There are only a few association studies on potential genetic modifiers (e.g. RAD51, HRAS1, AR) but no validated data as yet. Studies on reproductive factors suggest an influence on breast cancer risk also in BRCA1/2 carriers. Early age at menarche has been associated with an increased risk of breast cancer among women with BRCA1 (but not BRCA2) mutations and parity seems to be have an opposed effect on risk in BRCA1 vs. BRCA2 carriers. Long term breast feeding has been shown to decrease breast cancer risk in BRCA1 carriers while the effect of oral contraceptives on breast cancer risk in BRCA1/2 carriers is still unclear.

1.5.3 Phenotypic characteristics of familial breast cancer

1.5.3.1 Family history of breast cancer

Most studies on family history and phenotypic characteristics of breast cancer have focused on frequency of bilateral tumours and age at diagnosis. Bilaterality seems to be slightly more frequent in patients with family history, the difference in several studies however often not large enough to reach statistical significance. There is no convincing data that a family history of breast cancer is more prevalent in cases with young age of onset, even though this might be a common clinical apprehension.

1.5.3.2 BRCA1 mutation carriers

Mutations in BRCA1 mainly confer a considerably elevated risk for female breast and ovarian cancer but the risk for cancer of the cervix and corpus uteri, the fallopian tubes and the peritoneum is also higher than in non-carriers. The risk of cancer in male carriers does not seem to be increased.

The breast tumours from BRCA1 carriers differ from those in non-carriers. The majority of the tumours are histologically ductal and of grade III with mainly a higher mitotic count reflecting in a high S-phase. Medullar or atypical medullar tumours are more often found in BRCA1 carriers and the tumours often have a heavy lymphocyte infiltration or so called pushing margin. Additionally, the BRCA1 positive patients generally have hormone-receptor negative as well as HER-2 negative tumours. There are also more somatic p53 mutations in tumours from BRCA1 patients. Recently, it was also shown that BRCA1 related tumours, much more often are of a basal epithelial type than other breast cancers, a feature that can be recognised with immunohistochemistry, and might be useful in the clinic in recognising potential mutation carriers.
1.5.3.3  BRCA2 mutation carriers

Germ line mutations in BRCA2 are associated with breast cancer in both female and male carriers and ovarian cancer. In addition, there is a two-fold relative risk for other cancers in both men and women, the risk most pronounced for cancer of the prostate, pancreas and the gallbladder/bile duct. In contrast to BRCA1 induced tumours, breast tumours of BRCA2 carriers show no significant differences compared to non-carriers regarding histological features and hormone-receptor expression.

1.5.3.4 Prognosis

Just as for phenotypic characteristics and familial breast cancer it is hard to compare prognostic data on familial breast cancer in studies with a different classification of family history. However, in most studies there is no statistically significant prognostic difference related to a family history of the disease, even though in several studies familial patients tend to do slightly better than sporadic patients.

Tumours of BRCA1 carriers are usually characterized by several negative prognostic factors (high grade, lack of hormone receptor expression). In a review of 10 studies on mutation carriers and breast cancer prognosis published in 1999, there was no statistically significant difference in survival in 8 studies, and in two small studies carriers did worse than non-carriers. Other, more recent studies have shown a higher risk for contra lateral recurrence but other outcomes were not significantly different in carriers compare to patients without family history. In a study on Ashkenazi women undergoing breast-conserving treatment for invasive breast cancer, BRCA1 mutations, but not BRCA2 mutations, were associated with reduced survival, but the poor prognosis was mitigated by adjuvant chemotherapy. The risk for metachronous ipsilateral disease did not appear to be increased for either BRCA1 or BRCA2 mutation carriers, at least up to 10 years of follow up.
**AIMS**

**Paper I:** Define the proportion of different levels of family history in a cohort of population-based breast cancer patients, and to determine whether familial breast cancer have phenotypic characteristics, including prognosis, different from those of sporadic patients.

**Paper II:** Investigate the prevalence of *BRCA1* mutations in a population-based material of breast cancer patients from the Stockholm region, and determine whether there is a reason to screen breast cancer patients, not selected for family history, for *BRCA1* mutations.

**Paper III:** Evaluate the prevalence of the rare truncating variant *CHEK2* 1100delC in Swedish sporadic and familial breast cancer and if possible, clarify its role as a modifier or a low risk gene.

**Paper IV:** Clarify the role of the C975G variant in *ER alpha (ESR1)* as a low penetrance susceptibility allele in sporadic and familial breast cancer.

**Paper V:** Investigate the role of three polymorphisms in *ER beta (ESR2)* as low penetrance susceptibility alleles in sporadic and familial breast cancer.
MATERIAL AND METHODS

1.6 MATERIAL

1.6.1 Patient cohort Södersjukhuset / Huddinge (studies I-V)

From October 1998 to May 2000 all new patients with a surgically treated primary invasive breast cancer admitted to the Department of Oncology at Huddinge University Hospital and Söder Hospital (Södersjukhuset/SÖS) (covering the population of 850 000 people in southern Stockholm) were asked to take part in a research study on BRCA1/2 and other genetic risk factors in unselected breast cancer. The inclusion of the patient was done after oral and written consent and took place at the postoperative visit or, if more appropriate, at a subsequent visit. A blood sample was then obtained.

A questionnaire was given to all patients to be completed on their first visit to the clinic. Information was obtained regarding breast- and ovarian cancers and other cancers including age of diagnosis in other family members (parents, siblings, maternal/paternal grandparents, maternal/paternal aunts and uncles and others). There was no confirmation of the diagnoses of the relatives from pathology reports. Patient data on hormonal factors (parity, age at first delivery, age at menarche and menopause, use of contraceptives or HRT) and former cancer diagnoses was also obtained, and sometimes supplemented with information from the patients’ records.

If the patient had a previous breast cancer, the patient was considered eligible if the present cancer was contralateral, or ipsilateral with a different histopathological and/or receptor pattern and localized in another sector of the breast. If the two cancers had similar properties, the latter was regarded as a local recurrence and the patient was excluded from the study.

All patients included in the study were offered the possibility of later knowing the result of the BRCA1 screening and if appropriate at the time were referred to genetic counselling.

1.6.1.1 Classification according to family history (Paper I and II)

Patients were classified according to their level of family history on either maternal or paternal side in the following groups. If affected relatives were reported on both maternal and paternal side, the patient was classified according to the most prominent family history.

- Breast-ovarian cancer family: Patients with both breast and ovarian cancer or ovarian cancer in any 1\textsuperscript{st} – 3\textsuperscript{rd} degree relative
- Familial breast cancer: Patients with two or more 1\textsuperscript{st} or 2\textsuperscript{nd} degree relatives with breast cancer.
- Two close relatives: Patients with one 1\textsuperscript{st} or 2\textsuperscript{nd} degree relative with breast cancer.
- Cancer family: Patients with three or more 1\textsuperscript{st} or 2\textsuperscript{nd} degree relatives with cancer, other than breast or ovarian cancer, or two cancer cases in 1\textsuperscript{st} degree relatives.
- Sporadic: If the patient did not fit into any of the groups mentioned, the patient was classified as sporadic and then sub-grouped according to age at onset: 35 years or younger, 36 to 50 years and over 50 years, with the exception of those not knowing their family cancer history.
For the comparison of different levels of family history and clinical parameters in paper I the breast-ovarian cancer families and familial breast cancers were put together in a "high risk familial breast cancer" group. The group of two close relatives and cancer families, who hypothetically can harbor low risk genes formed a "low risk familial breast cancer" group and the sporadic cases were presented as 50 years or younger, or older than 50 years. As a comparable group to the "young sporadic cases", the familial high and low risk patients, 50 years or younger were united in a "young familial group".

1.6.1.2 Classification according to family history (Paper III-V)

In association studies of candidate low risk genes there is less evidence to use ovarian cancer to define genetic susceptibility for breast cancer. The breast-ovarian cancer families were therefore reclassified according to breast cancer family history only. The cancer families were classified as sporadic and those with unknown family history are also included in the sporadic cases. Three groups were formed:

- High-risk familial breast cancer: Three 1st or 2nd degree relatives with breast cancer.
- Low risk familial breast cancer: Two 1st or 2nd degree relatives with breast cancer
- Sporadic: No family history of breast cancer

1.6.1.3 Clinical parameters (Paper I-IV)

Information on clinical parameters (age at onset, age at menarche and menopause, parity, age at first delivery, oral contraceptives, HRT, tumour characteristics including bilaterality, stage, grade (Elston-Ellis) and hormone receptors (ER, PgR), method of surgery, adjuvant therapy, recurrences and deaths from breast cancer and new breast primaries) was obtained from the patient’s questionnaire, medical record and pathology reports.

1.6.2 Patients from Department of Clinical Genetics (Paper III-V)

In paper III-V, the population-based cohort collected at SÖS/HS was supplemented with breast cancer patients collected at the Department of Clinical Genetics at Karolinska University Hospital. These patients were selected because of their family history and had been collected either as part of a previous research project or in the clinic (after informed consent). Altogether 350 cases were used, 248 cases in paper III, 265 cases in paper IV and 259 cases in paper V.

These patients were defined according to their family history as described for the SÖS/Huddinge cohort (Paper III-V) and 224 had high risk and 126 low risk family history. All cases had proceeded through genetic counseling and those fulfilling the criteria for BRCA1/2 screening (Table 4) had been screened negative for mutations. For these cases family history was known and for most cases age at diagnosis (missing in 9 patients). The mean age at diagnosis was 54 years (24-92 years).
Table 4: The Stockholm criteria for BRCA1/2 screening

1. Two or more 1st or 2nd degree relatives with at least one case of breast cancer and one case of ovarian cancer or a single individual with both breast and ovarian cancer.
2. Three or more 1st or 2nd degree relatives with breast cancer, at least one with onset before 51 years of age.
3. Families with only two 1st or 2nd degree relatives with breast cancer, at least one with onset before 41 years of age.
4. One single individual with breast cancer before 36 years of age.
5. Two or more 1st or 2nd degree relatives with ovarian cancer.
6. One single woman with ovarian cancer before 51 years of age.

1.6.3 Controls (Paper III-V)

As controls for paper III-V, we used DNA from blood-donors collected as control material for association studies at Karolinska University Hospital, Stockholm, Sweden. 760 controls were used in paper III, 665 in paper IV and 480 in paper V. The material is anonymised, but information on gender of the individuals is available.

1.7 METHODS

DNA was obtained from the collected blood samples, using standard phenol/chloroform extraction.

1.7.1 PTT (Paper II)

The Protein Truncation Test (PTT) is an in vitro coupled transcription and translation method for detecting protein truncating mutations (Figure 5). Advantages of PTT are that it is a relatively quick, robust and simple method to screen large exons for disease causing mutations. Mutations close to the start or the end of a transcript might however escape detection (can be avoided by using overlapping DNA fragments) and large deletions, rearrangements or insertions are not identified. Mutations detected by PTT are confirmed, and the exact position identified, by direct sequencing. PTT is used on DNA and is suitable for mutation screening of the large exon 11 in BRCA1 and exon 10 and 11 in BRCA2. If smaller exons are screened with PTT, cDNA is required.

1.7.2 Multiplex PCR (Paper III)

Multiplex Polymerase Chain Reaction (PCR) is a variant of PCR, which enables simultaneous amplification of several targets in one reaction by using more than one pair of primers. Several CHEK2 exons, including exon 10 where 1100delC is located, are duplicated across the genome. These homologous duplications or amplicons result in similar, non-functional copies, which have to be avoided when examining the functional CHEK2 gene. In paper III, two separate pairs of primers were used, one pair specific for the mutation 1100delC and one pair specific for the wild-type. The two reactions were run separately, each in multiplex with a control PCR. The products were separated on Agarose gels.
Figure 5. **PTT**: Sequences for T7 RNA polymerase and a translation initiation site are incorporated in the PCR product in a first PCR reaction (amplification of the DNA segment of interest) and in a second step the PCR product is incubated with T7 polymerase, ribonucleotides, rabbit ribosomes and a mixture of amino acids, one of which is radio-labelled. Under suitable conditions, transcription of the PCR product to mRNA and a subsequent translation to a protein occur. The synthesized peptides are then separated by gel electrophoresis and the bands visualized on an X-ray film. If the protein product is shortened by nonsense or a frame shift mutation, an extra band will be detected on the film.

1.7.3 Pyrosequencing (Paper IV and V)

Pyrosequencing is an approach for real-time DNA sequencing and is widely used for SNP detection. The pyrosequencing enzymatic cascade starts with the release of pyrophosphate (PPI) as a result of nucleotide incorporation by DNA polymerase. The PPI formed in the DNA polymerase reaction is converted to ATP by ATP sulfurylase and the ATP production is used to generate light by the firefly luciferase, seen as a peak in a pyrogram (Figure 6). Unincorporated nucleotides are degraded by apyrase before adding the next nucleotide, allowing a repeated addition of nucleotides. The sequence can be determined since the added nucleotide in each step is known.

Figure 6: Pyrogram readouts from genotyping of polymorphism rs 928554 in ERβ.
1.7.4 Restriction fragment length polymorphism, RFLP (Paper V)

Inherited polymorphisms might be located in a cleavage site for restriction endonucleases. If a single base change (SNP or mutation) is present in a particular cleavage site in one of the DNA molecules the site will no longer be recognized by the restriction endonuclease and the different sized DNA fragments can be detected by Southern blot. RFLPs are used as genetic markers in whole genome linkage studies but also in association studies of SNPs (Figure 7).

![Restriction enzyme binding site](image)

**Figure 7.** In paper V, RFLP was used to evaluate the rs928554 SNP instead of pyrosequencing, due to the suboptimal sequence surrounding this variant. Samples homozygous for the G allele demonstrated 2 bands upon digestion, one 187 bp band and a second of 78bp. Heterozygote samples produced 4 bands of size 187, 149, 78 and 38 bp each. The A allele homozygous variant samples produced 3 bands of size 149 bp, 78 bp and 38 bp each.

1.7.5 Association analysis (Paper III-V)

Genotypic and allelic data was compared between cases and controls using the chi-square test or if more appropriate the Fisher’s exact test. Odds ratios were calculated with 95% confidence intervals with wt/wt as reference genotype. The genotype frequencies in controls were found to be in Hardy-Weinberg equilibrium (HWE).

In the ER beta (Paper V) we evaluated three polymorphisms both separately in cases and controls and as haplotypes. A haplotype is the physical arrangement of loci along a chromosome or in a single gene. Linkage disequilibrium (LD) refers to the fact that alleles at neighboring sites can co-occur on the same haplotype more often than is expected by chance and can be measured by $|D'|$ ranging from 0 (no disequilibrium) to 1 (complete disequilibrium) $^{283}$. Haplotype testing, instead of testing separate SNPs, may increase the power in association studies $^{25}$. In a candidate gene, there may be tens or more SNPs, and a negative association does not rule out another important variant in the gene. With the haplotype approach, it is possible to investigate the whole gene, using only a few markers so called tagging SNPs, since closely located SNPs often are in LD with each other $^{214}$. It may also be that a specific haplotype harbor two or more variants that are functionally important only if they occur together, and the effect is not detected if the single polymorphisms are analyzed separately $^{214}$. 


There are several algorithms used to define haplotypes blocks from unphased genotype data on single SNPs, one of them developed by Gabriel et al. This method is based on pairwise estimates of $|D'|$ and each comparison is called strong LD, inconclusive or strong recombination. A block is constructed if 95% of informative comparisons are in strong LD. In paper V we used the computer programs Haploview v 3.1.1, which is based on this algorithm and the UNPHASED program (including COCAPHASE) to estimate LD in the region and association of inferred haplotypes.
RESULTS AND DISCUSSION

A total of 785 patients underwent surgery for breast cancer and received postoperative treatment / follow-up at the Department of Oncology at Huddinge University Hospital and Södersjukhuset (SÖS) during the period. 89 of these were identified through the registration at the Oncologic Center (OC) in Stockholm after the study was completed and were never registered in the study due to logistic problems. Of the remaining 696 patients, 489 patients, all women, were willing to take part in the study (70%). In total 207 patients, including 1 man, were registered in the study but did not participate, mainly because they were not invited but also because of co-morbidity like dementia or in some cases declined participation. In total, 62 % of the eligible patients were included in the study (489/785) (Figure 8).

![Diagram](image)

**Figure 8. Patients in the population-based cohort from SÖS/Huddinge.**

The non-participating cases (n=207) were slightly older than the participating group (p<0.01), but there was no significant difference in stage (p=0.36) or in the rate of self-reported family history between groups (p=0.70) (Table 4).

In the southern part of Stockholm there is a relatively high rate of immigrants and information on ethnicity was obtained from the patient’s record. In 46 cases there was a non-Swedish background (in 3 cases only assessed from the name only) and 30 of these had European descent (12 from Nordic countries), 7 were from South America, 4 from the Middle East, 1 from the East Asia and one from Africa.
Family history is an important risk factor for breast cancer and a dominant inheritance pattern is seen in 5-10% of the cases \cite{196}. This high-risk group has been the focus of an intense research for high-risk susceptibility genes during the past two decades. However, the possibility that low-penetrance genes may play an important part in breast cancer susceptibility and the result of recent twin studies suggesting that a more substantial share of the breast cancer susceptibility is due to genetic factors than only the high risk families, has created a renewed interest in the more modest familiality described by earlier epidemiological studies \cite{11,167,211-213}.

In this study we wanted to define the proportion of different levels of family history in a population based cohort of breast cancer and to identify possible differences between familial and sporadic patients concerning age at onset, hormonal background, tumor characteristics and prognosis.

In total 174 (35%) of the 489 patients in the study reported a family history. Almost 10% had either high-risk familial breast cancer or familial breast-ovarian cancer, while the remainder was classified as low risk familial breast cancer (Figure 9). Among the patients 50 years and younger at onset (n=100), 40% reported a family history compared to 35% of those, older than 50 years at onset (n=389). 25% had solely paternal family history, a group that often is missing in studies on family history and breast cancer because only 1st degree relatives are included. Many of the high-risk families were small and most were defined as late-onset according to age at onset of the major part of affected family members (Figure 10).

Age at onset was similar in the familial and sporadic cases (59 vs. 61 years, p=0.14). Regarding hormonal factors, there was no major difference either, and a family history of breast cancer did not influence women in a deterrent way on parity, use of oral contraceptives or HRT.

<table>
<thead>
<tr>
<th></th>
<th>Participating (n=489)</th>
<th>Non-participating (n=207)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean age at diagnosis (years)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>64</td>
</tr>
<tr>
<td>Minimum-maximum age</td>
<td>27-88</td>
<td>25-95</td>
</tr>
<tr>
<td><strong>Family history</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovarian cancer in patient or close relative</td>
<td>3%</td>
<td>2%</td>
</tr>
<tr>
<td>Familial breast cancer</td>
<td>6%</td>
<td>5%</td>
</tr>
<tr>
<td>One 1st or 2nd degree relative w. breast ca.</td>
<td>22%</td>
<td>22%</td>
</tr>
<tr>
<td>Sporadic (no breast or ovarian cancer)</td>
<td>66%</td>
<td>66%</td>
</tr>
<tr>
<td>Not known (e.g. adopted)</td>
<td>2%</td>
<td>5%</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>56%</td>
<td>49%</td>
</tr>
<tr>
<td>II</td>
<td>37%</td>
<td>43%</td>
</tr>
<tr>
<td>III</td>
<td>6%</td>
<td>7%</td>
</tr>
<tr>
<td>IV</td>
<td>0%</td>
<td>1%</td>
</tr>
</tbody>
</table>

*Table 4: Comparison participating vs. not participating patients*

1.8 PAPER I

Family history is an important risk factor for breast cancer and a dominant inheritance pattern is seen in 5-10% of the cases \cite{196}. This high-risk group has been the focus of an intense research for high-risk susceptibility genes during the past two decades. However, the possibility that low-penetrance genes may play an important part in breast cancer susceptibility and the result of recent twin studies suggesting that a more substantial share of the breast cancer susceptibility is due to genetic factors than only the high risk families, has created a renewed interest in the more modest familiality described by earlier epidemiological studies \cite{11,167,211-213}.

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Age at onset was similar in the familial and sporadic cases (59 vs. 61 years, p=0.14). Regarding hormonal factors, there was no major difference either, and a family history of breast cancer did not influence women in a deterrent way on parity, use of oral contraceptives or HRT.
Tumor size did not differ according to family history but younger women (≤ 50) tended to have larger tumors, at least in part due to the mammography screening program in Stockholm starting at the age of 50 at the time for the study. There were more node-positive patients in the young sporadic group compared to the familial patients of the same age (52% vs. 32%, p=0.06), without obvious explanation in other tumor characteristics like grade and hormone receptor positivity.

As anticipated because of the regional and national guide-lines concerning breast cancer treatment, family history had no major impact on the method of surgery or adjuvant treatment although interestingly, more young node-negative patients with a family history received chemotherapy, compared to sporadic patients (44% vs. 31%).

Regarding prognosis, there was no statistically significant difference in recurrence free survival between patients with a family history of breast cancer and sporadic patients after a median follow-up of 4.7 years. If the high-risk familial group was analyzed separately, there was a non-significant advantage in the multivariate analysis (controlling for age at diagnosis, tumor size and nodal status) with a HR of 0.73 (95% CI 0.3-1.8) compared to the sporadic group. Due to few events (n=76), we chose to analyze all recurrences together (loco regional recurrence, distant recurrence and contra-lateral breast cancer). Recently there was a Swedish publication on the risk of contra lateral breast cancer in patients with a family history of breast cancer\textsuperscript{238}. In this study, there was a 27% risk of developing a contralateral breast cancer after 20 years follow up compared to the expected risk of 5% among breast cancer patients in general. In our study cohort, where we currently have a follow up of 5.5 years, 5% (8/164) of the familial patients have been diagnosed with a new contralateral breast cancer compared with 3% (10/307) of the sporadic patients. Altogether18 new malignancies other than breast cancer, 8 in sporadic patients (3%) and 10 in familial patients (6%) have been diagnosed during follow-up (among them 6 endometrial tumors, 4 lung cancers and 2 ovarian cancers).
Figure 10: Pedigrees of patients classified as breast-ovarian cancer families in paper I and II. In paper III-V these families were reclassified to * high familial breast cancer, ** low risk familial breast cancer and *** sporadic breast cancer. Black symbols are breast or ovarian cancer cases, grey symbol indicates another malignancy, number under the symbol is age at diagnosis, if known. BR=breast cancer, OV=ovarian cancer, ENDO= endometrial cancer, PR=prostate cancer, HEM=hematological malignancy, MEL=malignant melanoma, URO= urological tumour other than prostate, PULM=lung cancer, GI=gastrointestinal malignancy, GYN=gynecological malignancy.
**1.9 PAPER II**

The mutation frequency of BRCA1 and BRCA2 in women with breast cancer varies according to family history, age at diagnosis and ethnicity. Previous studies on the contribution of mutations in these genes in breast cancer unselected for age and family history has reported mutation frequencies between 1% and 12%, however screening methods, ethnicity, study size and to what extent the gene/s have been screened have differed. In familial breast cancer from the Stockholm region, previous studies had shown a comparatively low frequency of BRCA1 and almost none in BRCA2 but less was known of the mutation frequency in patients without family history or very young age. We therefore wanted to clarify the proportion of BRCA1 mutations in unselected breast cancer cases from the Stockholm region.

Two BRCA1 mutations were identified in the population-based study cohort of 489 individuals with a new diagnosis of breast cancer (0.4%). Both mutations were found in families with both breast and ovarian cancer.

Since the screening was limited to exon 11, 0.4% is an underestimation, however in previous studies from Stockholm more than 70% of the mutations in BRCA1 were detected in exon 11, including the Stockholm founders 22,23,314. There is a possibility that the mutation spectrum might be different in unselected patients but this is not supported by the literature 18. Large genomic rearrangements are not detected with PTT or other common screening methods including sequencing, but PTT has otherwise proven a reliable method for exon 11 screening with high sensitivity and specificity of disease causing mutations 125. Our estimate of the mutation frequency in unselected patients from the Stockholm region is ≤1%, which internationally is a low figure, probably reflecting the relatively low rates detected also in high risk families in our region.

In conclusion, BRCA1 mutations are rare in unselected breast cancer cases in the Stockholm region and are likely to be found only in high-risk patients, especially in families with both breast and ovarian cancer. There is no need to screen breast cancer patients in general for BRCA1 mutations. Family history should be used to define those new breast cancer patients who would benefit from genetic counseling.

All patients included in the study were offered the possibility of knowing the result of the BRCA1 screening. 105 patients have shown interest, and 38 were informed and could discuss their family history and risk on a medical visit, free of charge, while the remainder chose a telephone call from the principal investigator. 14 patients have been referred for proper genetic counseling. Two more mutation carriers have been detected in this cohort through clinical mutation screening, one patient from a breast-ovarian cancer family had a mutation in BRCA1 and one patient from a premenopausal breast cancer family had a mutation in BRCA2.

**1.10 PAPER III**

The protein truncating variant 1100delC in CHEK2 is almost the only low risk variant which has, with certainty, been shown to associate with breast cancer. The variant was first shown to associate with familial non-BRCA1/2 breast cancer and a large pooled study has also reported an effect in unselected breast cancer with a RR of 2.8. However, if the variant acts a modifier of still unknown high-risk gene/s or is a low risk variant on its own is unclear. The variant is rare, and its frequency in the normal population varies between countries.
In our control material consisting of 760 blood-donors from the Stockholm region, 0.7% carried the variant which is a lower prevalence than seen in the “high prevalence” countries Finland and the Netherlands but higher than in Central or Southern Europe, where the variant is non-existing. There are two other recent Swedish studies on CHEK2 1100delC, one in prostate cancer and one in breast-colon cancer families, and their control group carrier frequencies were 1%, both studies however smaller than the present study\textsuperscript{136,280}.

Among the familial patients, 2.3% carried the variant, similar in high and low risk cases but only one of the sporadic patients was a variant carrier (0.3%). In this study, as well as in paper IV and V, the study material consisted of patients recruited from two sources, the population based material from the Department of Oncology at SÖS/Huddinge and the patients from the Department of Clinical genetics, selected on family history. If separately analyzing the population based cohort, the variant frequency was 1.1% (5/452) which is slightly higher than in the controls (p=0.41)(Table 5).

Interestingly, the mean age was 10 years lower in variant carriers than in non-carriers and this was found in both materials constituting the study population indicating a true difference despite small sample size (p<0.01 in the combined material). As anticipated, the variant did not segregate with disease in the families where DNA was available from more family members. Our data, with an association of the variant only in patients with a family history of breast cancer and a possible influence of the variant on age at onset, rather support the role of the CHEK2 1100delC as a modifier than being a low risk gene of its own.

Although there is an association of CHEK2 with breast cancer, the effect is modest and the variant rare. In the clinical setting, there is no need for CHEK2 1100delC screening at present but the variant might prove interesting in combination with other genetic/non-genetic factors in the future.

<table>
<thead>
<tr>
<th></th>
<th>CHEK2 1100delC+ /total tested</th>
<th>p-value\textsuperscript{1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Familiar Risk cohort</td>
<td>5/247 (2.0%)</td>
<td>0.07</td>
</tr>
<tr>
<td>Population-based cohort</td>
<td>5/452 (1.1%)</td>
<td>0.51</td>
</tr>
<tr>
<td>Sporadic breast cancer</td>
<td>1/313 (0.3%)</td>
<td>0.68</td>
</tr>
<tr>
<td>Familial breast cancer</td>
<td>4/139 (2.9%)</td>
<td>0.04</td>
</tr>
<tr>
<td>Controls</td>
<td>5/760 (0.7%)</td>
<td></td>
</tr>
</tbody>
</table>

\textit{Table 6}. Prevalence of CHEK2 1100delC in familial risk cohort from Department of Clinical Genetics and in Population based cohort from SÖS/Huddinge compared to controls. \textsuperscript{1} p-values were calculated with Fisher’s test for association.
1.11 PAPER IV

Estrogen plays a central role in both normal mammary development and breast carcinogenesis and the estrogen receptors ERα and ERβ are obvious candidate breast cancer susceptibility genes. We chose to study a common synonymous SNP (pro/pro) in exon 4 of ERα (C975G, rs18011132) a variant previously studied with non-consistent results in breast cancer, the studies however small and the material selected in different ways.

In our material of 388 familial cases subdivided in high and low risk, 288 sporadic cases and 653 controls, the frequency of the C975G variant in ERα was lower in high risk familial cases compared to controls (18 versus 22%, p=0.046). The odds ratio for GG homozygotes compared to CC homozygotes was 0.2 (95% CI 0.06-0.8). There was no association of the variant with sporadic or low risk familial breast cancer. If the population based material from SÖS/Huddinge was analyzed separately, there was no difference in variant frequency in breast cancer cases (22%) compared to controls. Interestingly, in the group of bilateral cases (n=39), a group hypothetically enriched for genetic susceptibility, none were GG homozygotes and the G allele frequency was 19%, thus similar to the high-risk familial group.

Several studies, all however small, contradict our result (Table 2)\textsuperscript{138,225,294}. There is some support in studies presented as negative\textsuperscript{64,248,295}. In a recent large Swedish study on postmenopausal breast cancer, there was a slightly lower G allele frequency in cases compared to controls (20% vs. 23%, NS)\textsuperscript{295}. In this study there was an association with the C975 variant and ductal, but not lobular cancer. Two studies have shown a lower G allele frequency in familial cases only and one small study suggested a protective effect of the GG genotype in the Korean population where the G allele is more common than in Caucasians\textsuperscript{113,129,249}.

In the last few years there has been a rapid technical development of SNP analysis and molecular knowledge including the dbSNP database and the completion of the HapMap project\textsuperscript{13,159}. Two recent large studies have focused on several polymorphisms in the ERα gene, one Swedish study on postmenopausal breast cancer and an American study made by Gold et al.\textsuperscript{103,295}. The Swedish study focused on 5 variants including the two RFLPs in intron 1, the C975G variant in our study, the promoter (TA)\textsubscript{n} repeat and a rare synonymous SNP in exon 3. Two haplotypes, both including the C975G SNP and either of the RFLPs in intron 1 were shown to associate with ductal, but not lobular cancer. Unfortunately the potentially interesting promoter variant (TA)\textsubscript{n} was not possible to evaluate due to problems with HWE in the controls. The American study is the most thorough and focused on 17 variants in the ER alpha gene, and observed three common haplotypes, composed of 8 SNPs including the C975G variant, that were associated with a decreased risk of breast cancer, and another haplotype that was associated with an increased risk\textsuperscript{103}. When testing the SNPs separately there was no overall statistically significant association, however in a subgroup of Ashkenazi Jewish cases, three SNPs were associated with disease, one in the promoter and the other two in exon 1 and intron 1 respectively. There was no information on family history in this study.

In the Huddinge/SÖS patient cohort there was a possible association with the C975G variant and hormone receptor status of the breast tumour. In patients with ER negative tumours, 20% were CG heterozygotes compared to 35% of ER positive patients (p=0.022). If this was a true association, a lower frequency of GG homozygotes, among ER negative patients, would be expected, which was not seen. The group of GG homozygotes with information on ER in the tumour was however small (n=15) and a previous Korean study support a correlation with the G-allele and hormone receptor expression, although there are also negative studies\textsuperscript{138,147,225}. There
was no difference in genotype distribution in the SÖS/Huddinge cohort of patients regarding histology (ductal vs. lobular), PgR status of the tumour or age at diagnosis (≤50 years vs. > 50 years).

In conclusion, our data indicate an association of the common exonic synonymous (Pro/Pro) variant C975G in ERα with a protective effect on familial high-risk non-BRCA1/2 breast cancer susceptibility. The high-risk families are believed to segregate high-penetrance alleles, which may be modified by this variant or another variant in linkage disequilibrium with this. Further studies should use strategies in which a comprehensive set of SNPs, in and upstream ERα, are identified either through resequencing or using data from the dbSNP database. These should be validated and a smaller set used to tag common haplotypes in the gene in a larger independent case/control material with thorough knowledge of family history and clinical information.

1.12 PAPER V

The second estrogen receptor, ERβ, was identified 1996 and there are only a few reports on its possible association with breast cancer susceptibility (none when our study was initiated). We chose to study three common polymorphisms in the ERβ gene; G1082A in exon 5 (rs1256049), G1730A in the 3´ untranslated region (3´UTR) of exon 8 (rs4986938) and a G>A polymorphism located 56 bases 3´ of the ERβ alternative transcript cx (exon 9) for association with breast cancer (Figure 4). These SNPs were identified through publications where the whole gene had been screened for mutations and were found to have a minor allele frequency above 1%.

Our material consisted of 723 breast cancer cases, divided accorded to family history in sporadic (n=323) and familial breast cancer (n=400), and 480 controls. The familial patients were further subdivided into 212 familial high-risk cases and 188 familial low risk cases.

The SNPs were genotyped by pyrosequencing (G1082A and G1730A) or RFLP (cx+56A/G). The genotype and allele frequencies of each variant was tested in each of the three breast cancer groups (high risk familial, low risk familial and sporadic) and compared to controls. There was no overall statistically significant difference in genotype distribution in cases and controls. In the low risk group, there was a suggestively protective effect for heterozygotes of the G1730A variant (p=0.09, OR 0.73, 95% CI 0.50-1.05) and a similar non-significant elevated risk was seen with the G allele of the G1082A SNP in sporadic cases (p=0.08, OR 1.6, 95%CI 0.98-2.97). If the population-based cohort from SÖS/Huddinge (n=474) was tested separately compared to controls there was no overall difference for any of the genotypes either.

There is a large amount of linkage disequilibrium in the ERβ gene and all three SNPs were shown to be in strong LD, with |D'| values ranging from 0.86-1.00. We then included our SNPs in haplotype construction and these inferred haplotypes were tested for association in each of the breast cancer groups and the controls. We identified seven different haplotypes in our material, two of which we considered rare (frequency<0.005%). There was an increased risk of breast cancer for one haplotype 1-2-2 (G-A-G) in sporadic breast cancer compared to controls (p=0.03) and the contrary haplotype 2-1-1 (A-G-A) was associated with a decreased risk of breast cancer (p=0.03). In the high-risk familial group the haplotype frequencies were similar as in the controls.

Our results indicate possible effect of inherited ERβ haplotypes and breast cancer susceptibility in patients without an extensive family history. Only one other study has used the haplotype approach when examining ERβ for association with breast cancer. Gold et al identified a haplotype consisting of 7 SNPs including our three, which was associated with an increased risk
of breast cancer in an Ashkenazi Jewish population and also could narrow the region further to identify a related haplotype (5 SNPs including ours) which was also associated with breast cancer risk (p=0.001). Three other studies on individual SNPs in ER beta have been negative in relation to breast cancer, these however smaller in study size (Table 3)\textsuperscript{91,117,135}. Even though the SNPs in our study are probably not causative (synonymous or untranslated) they may be in LD with an as yet unidentified causative variant/s within or close to the ER\(\beta\) gene. Further larger studies, preferably in materials like ours with a well-defined family history, of the ER\(\beta\) locus are required to fully understand its role in breast cancer susceptibility.
CONCLUSIONS

Around 35% of breast cancer patients reported a family history of some degree, and 10% constituted a high-risk group. There was no relation between family history and age at onset, hormonal background, tumor characteristics, treatment or prognosis in our material, which was population-based, and prospectively collected and also included paternal inheritance.

Screening for BRCA1 mutations in a population-based cohort of breast cancer patients from our region revealed only two mutation carriers, both in cases with a family history of breast and ovarian cancer. There is no need for general screening of BRCA1 in breast cancer patients, however family history should be used to select patients who would benefit from genetic counseling.

The rare truncating variant, 1100delC, in checkpoint kinase CHEK2 exists in the Swedish population. In our material the prevalence was increased in both high and low risk familial breast cancer compared to controls. The variant seems to influence age at onset and this together with lack of association in sporadic patients may indicate a role as a modifier of still unknown high-risk gene/s rather than being a true low penetrance gene.

Analysis of a common single nucleotide polymorphism, C975G, in the ERα gene in sporadic and familial breast cancer suggested a protective effect of the variant allele in high-risk familial breast cancer compared to controls. No association was seen in low risk familial or sporadic cases. There was also a possible association between genotype and ER expression in the breast tumours.

Three polymorphisms in the ERβ gene were analyzed for association with familial and sporadic breast cancer, both separately and as haplotype constructions. There was no overall significant difference in genotype distribution but one common haplotype was associated with an increased risk of sporadic breast cancer indicating a role for ERβ in breast cancer susceptibility.

This thesis aimed to illuminate the contribution of low risk alleles in breast cancer susceptibility. The design used selected candidate gene variants to be studied in cohorts of breast cancer patients selected by family history into different risk groups. The compiled results support an effect of low risk alleles and suggest that the effect may vary from acting as low risk factors on their own to acting with an additive or modifying effect with other genetic or non-genetic risk-factors. Further studies of breast cancer as a complex disease need larger breast cancer cohorts to elucidate the genetic susceptibility to breast cancer.
ACKNOWLEDGEMENTS

I would like to express my gratitude to those who contributed to this study, in particular:

**Annika Lindblom**, my main supervisor, for your constant and never failing support and encouragement. You have a deep knowledge and unique devotion in the cancer genetics research field, which you have generously shared with me. Thank you also for mentorship in other areas of life.

**Tommy Fornander**, my supervisor and mentor in the clinic for many years, for sincere support and for your unique ability to elucidate complicated issues both in research and in the clinic.

**Lars-Erik Rutqvist**, former head of the Department of Oncology at Huddinge University Hospital / Södersjukhuset, for support and for sharing your great experience in breast cancer research.

**Jonas Bergh**, present head of the Breast and Sarcoma section at the Department of Oncology at Karolinska University Hospital, for your profound interest in breast cancer and support of my present and future research projects.

**Barbro Werelius**, technician at Annika Lindblom´s lab, for skilful and kind support.

**Johanna Skoglund**, fellow PhD student at the lab, for excellent collaboration and good spirits.

**Hemming Johansson** for invaluable statistical assistance.

Former and present members of Annikas lab and the Department of Clinical genetics: **Paula Maguire** for fruitful collaboration, **Norma Lundberg** for assistance with patient administration, **Xiao-Lei Zhou, Tatjana Djuricinovic, Jana Vandrovcova, Heléne Fischer, Tao Liu, Liping Luo, Quan Du, Johanna Rantala** and **Berith Wejderot** for contribution to the scientific atmosphere.

**Marie Luise Bisgaard** for pleasant and rewarding collaboration and support.

The dedicated colleagues, working with hereditary cancer in the clinic and also present or former PhD students of Annika´s: **Anna von Wachenfeldt** at Södersjukhuset and **Brita Arver** and **Annelie Liljegren** at Radiumhemmet, for interesting discussions and good company.

**Christoffer Lageroos**, for valuable computer assistance

**Inga-Britt Pettersson**, former research secretary for your professionalism, support and concern in great as in little things.

**Ulla Glas**, former head of the Department of Oncology at Södersjukhuset, for generous support during the first years of my career in Oncology.

**Asgerdur Sverrisdottir**, former colleague, fellow PhD student and roommate at Södersjukhuset for many years, for support and friendship.

All colleagues belonging to the breast-section at the Department of Oncology at Karolinska University Hospital, Södersjukhuset for continuous encouragement and for creating a wonderful atmosphere in spite of heavy work load: **Mariann liristo** for generous support in administrative issues, **Ann-Marie Billgren, Ingyveldur Björnsdottir, Ulla Blom-Goldman, Tomas Jansson, Reza Khoshnoud, Else Svensson** and **Gerard Winblad**.
All other present and former colleagues at the Department of Oncology, Karolinska University Hospital at Södersjukhuset/Huddinge, especially all those who have assisted in informing the patients and collecting the blood samples for these studies.

All present and previous nurses at the Oncologic Out-patient Department, breast section and Oncologic Day-Care Unit at Södersjukhuset/Huddinge for help with research blood samples and support in the daily work, especially Tina Bondesson, Lotta Bodell and Lena Ljungkvist Brodin

All breast cancer patients who have participated in the trials.

Tova Hannegård-Hamrin for long-time friendship and linguistic advice.

Family and friends, especially Gregory, Dan and Rebecka, for encouragement, love and perspective in life.

These studies were supported by grants from the the Nilsson-Ehle foundation, the Swedish Cancer Society, the Gustav V Jubileé Foundation, the FoU Foundation, the Swedish Doctoral Society, the Swedish Society of Medicine, Huddinge University Hospital, Karolinska University Hospital, Bert von Kantzows foundation and the Swedish EU-R&D Foundation.
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