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Life Style

Molecular Pathology

and

Breast Cancer Risk

Signe Borgquist MD



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Faculty of Medicine

Malmö 2008

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Life Style Molecular Pathology and Breast Cancer Risk

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LUND
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Faculty of Medicine

Doctoral Dissertation

By due permission of the Faculty of Medicine, Lund University, Sweden,
to be defended at the Main Lecture Hall, Department of Pathology, Malmö University
Hospital, on Friday 8th of February at 9.00

Faculty opponent

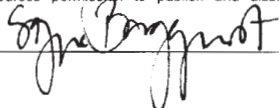
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<p>Abstract: The breast cancer diagnosis covers a wide range of tumours with different geno- and phenotypic characteristics. The diversity among studies on life-style factors and their impact on breast cancer risk might be clarified by recognizing breast cancer as a heterogeneous disease. A major aim of this thesis was to investigate the association between several life-style factors and subsequent breast cancer risk according to pathological- and tumour biological characteristics. Further, we examined two novel tumour markers with respect to their relation to established tumour characteristics, estrogen-related-life-style-factors, and/or response to endocrine breast cancer treatment. The thesis is predominantly based on breast cancer cases from the Malmö Diet and Cancer Study, a prospective cohort study including 17 035 women with baseline examinations performed 1991-1996. The study cohort was followed by record-linkage with national cancer registries and at the end of follow-up in 2004, 622 women had been diagnosed with breast cancer. Additionally, we used a consecutive cohort of 512 women diagnosed with breast cancer at the Malmö University Hospital 1988 and 1992. Immunohistochemical assessments of different tumour markers were performed using the tissue microarray (TMA) technique. Analyses of dietary habits and tumour characteristics revealed an association between low energy intakes and a more aggressive histopathological phenotype as well as over-expression of the cell cycle regulating protein, cyclin D1. A low fat intake showed similar associations to tumour characteristics. The use of combined (estrogen-progestin) hormone replacement therapy was associated with an increased risk of breast cancer, although with comparatively favourable prognostic factors, such as lobular type, low grade, low proliferation, negative/low cyclin D1 expression and maintained expression of the tumour suppressor gene, p27. In a similar way obesity was associated with an increased risk of less aggressive breast cancer characterised by lobular type, grade II, low proliferation, HER2 negativity, ERα+, PgR+, but negative ERβ expression. The enzyme HMG-CoA reductase was differentially expressed in breast cancer and high expression was associated with a smaller tumour size, low grade, low proliferation, and expression of ERα and ERβ, but not PgR. Estrogen-related factors, such as obesity and use of hormonal replacement, were associated with an increased risk of tumours with high expression of HMG-CoA reductase. The novel estrogen receptor, ERβ, was associated with expression of ERα and PgR, but not with other established prognostic factors, such as tumour size, grade, and lymph node metastases. Co-expression of both estrogen receptors indicated an improved response to endocrine therapy.</p>		
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Linjerne i et mønster løber
undertiden den modsatte vej af,
hvad man ventede. Men derfor er
det alligevel et mønster.

Karen Blixen

To 17 035 women

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LIST OF PAPERS

The thesis is based on the following papers;

- I Borgquist S, Wirfält E, Jirström K, Anagnostaki L, Gullberg B, Berglund G, Manjer J, Landberg G
Diet and body constitution in relation to sub-groups of breast cancer defined by tumour grade, proliferation and key cell cycle regulators
Breast Cancer Res. 2007; 9 (1):R11
- II Borgquist S, Anagnostaki L, Jirström K, Landberg G, Manjer J
Breast tumours following combined hormone replacement therapy express favourable prognostic factors
Int J Cancer. 2007 ;120 :2202-7
- III Borgquist S, Jirström K, Anagnostaki L, Manjer J*, Landberg G*
Anthropometric factors in relation to incidence of different tumour biological subgroups of postmenopausal breast cancer
Submitted for publication
- IV Borgquist S, Djerbi S, Pontén F, Anagnostaki L, Goldman M, Gaber A, Manjer J, Landberg G, Jirström K
HMG-CoA reductase expression in breast cancer is associated with a less aggressive phenotype and influenced by anthropometric factors
Submitted for publication
- V Borgquist S, Holm C, Stendahl M, Anagnostaki L, Landberg G, Jirström K
Estrogen Receptors α and β show different associations to clinicopathological parameters and their co-expression might predict a better response to endocrine treatment in breast cancer
In press, Journal of Clinical Pathology, Feb 2008

**These authors contributed equally as senior authors of the paper*

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ABBREVIATIONS

BCSS	breast cancer specific survival
BMI	body mass index
CI	confidence interval
CIS	carcinoma <i>in situ</i>
CDK	cyclin dependent kinase
CHRT	combined hormone replacement therapy
DFS	disease free survival
ER	estrogen receptor
ER α	estrogen receptor alfa
ER β	estrogen receptor beta
EREs	estrogen responsive elements
ERT	estrogen replacement therapy
FISH	fluorescence <i>in situ</i> hybridization
HER2	human epidermal growth factor receptor 2
HMGCoAR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
HRT	hormone replacement therapy
IHC	immunohistochemistry
MDCS	Malmö Diet and Cancer Study
MUFA	monounsaturated fatty acids
NHG	Nottingham Histological Grade
NPI	Nottingham Prognostic Index
OC	oral contraceptives
OS	overall survival
PgR	progesterone receptor
PUFA	polyunsaturated fatty acids
SFA	saturated fatty acids
TMA	tissue microarray

INTRODUCTION

Breast cancer is the most common malignancy among women worldwide and accounts for more than one million incident cases each year (Bray et al. 2004, Kamangar et al. 2006). In Swedish terms, that is a yearly incidence of 7000 women diagnosed with breast cancer and a life time risk of 10-15 % (The National Board of Health and Welfare, 2006). Over the past 20 years the breast cancer incidence has increased globally (Althuis et al. 2005) which corresponds to an increase of 1.4 % per year in Sweden (The National Board of Health and Welfare, 2006). Incidence and mortality rates generally co-varies, but vary across countries (Althuis et al. 2005). In Sweden, mortality has improved with a decrease of 14 % from the 70's to the 90's (Althuis et al. 2005). However, breast cancer still remains the leading causes of cancer death among women (Bray et al. 2004). The prognosis for Swedish breast cancer patients corresponds with a 5-year survival of 86% and a 10-year survival of 75% (The National Board of Health and Welfare, 2006). The risk of breast cancer is indeed age-related and less than 1% of breast cancer patients are younger than 30 years at diagnosis (Shannon et al. 2003). The incidence increases along with age and peaks at the age of 60-64 years (The National Board of Health and Welfare, 2006).

Several risk factors have been identified and many of them are associated with total life-time hormonal exposure, endogenous as well as exogenous hormones. Early menarche, late menopause, a low number of full-term pregnancies, and late age at first childbirth consequently lead to higher cumulative estrogen exposure. Obesity increases estrogen levels, however, the influence on breast cancer risk differs depending on menstrual status at the time of obesity (Daling et al. 2001). Furthermore, estrogen levels are altered by exposure to oral contraceptives (OC) and hormonal replacement therapy (HRT). Other life style factors such as smoking- and alcohol habits, physical exercise, and dietary intake, most likely influence breast cancer risk although the results from different studies are ambiguous. American studies on migration patterns in relation to breast cancer risk report an increase in breast cancer incidence among Asian-American women compared to Asian women suggesting a substantial impact on breast cancer risk when exposed to a Western lifestyle (Deapen et al. 2002, Ziegler et al. 1993).

The breast cancer diagnosis covers a wide range of tumours with different geno- and phenotypic characteristics. The diversity among studies on life-style factors and their impact on breast cancer risk might be clarified by recognizing breast cancer as a heterogeneous disease. In this thesis, the aim is to further study the association between life style related factors and breast cancer risk in relation to different molecular subgroups of breast cancer. This approach might improve the understanding of the impact of various risk factors and, hopefully, contribute to future prevention strategies. Furthermore, the identification of novel biological markers in breast cancer may add predictive value to existing therapeutic strategies.

TUMOUR CHARACTERISTICS

Tumour initiation and progression

Carcinogenesis is a multi-step transformation process (Hanahan et al. 2000). The initiation is most likely due to loss of DNA-damage check points leading to uncontrolled cellular proliferation. Tumour cells acquire genetic and epigenetic changes resulting in growth advantages and eventually become self-sufficient in growth signals, stimulation of angiogenesis, insensitive to inhibitory signals, and obtain capability of tissue invasion as well as shedding metastasis (Hanahan et al. 2000).

The majority of breast cancers originates from the inner layer of luminal epithelial cells in the ducts, but may develop from the lobular units as well. The conventional tumour progression model based on morphological characteristics describes an onset from normal breast tissue, via hyperplasia, atypical hyperplasia, carcinoma in situ and subsequently to invasive cancer. However, the molecular basis of disease progression in breast cancer remains poorly understood, but the introduction of high-throughput molecular profiling techniques has created unique possibilities for understanding and refining the tumour progression model (Rennstam et al. 2006). Simpson *et al* have proposed a model in which the role of de-differentiation is diminished and replaced by three rather distinctive pathways based on modern molecular profiling; one pathway for well-differentiated tumours probably evolving in a classical way, another pathway for poorly differentiated, basal-like tumours likely to use shortcuts, and, in between, intermediate grade II tumours with different pathways (Simpson et al. 2005).

Histopathology and tumour biology

All breast tumours are diagnosed and characterised according to standardised national protocols describing tumour size and type, invasiveness, histological grade, tumour stage, and expression of ER α (estrogen receptor α), PgR (progesterone receptor), and with the latest being HER2. Future diagnostic methods will probably include a variety of newly identified markers in molecular pathology.

Tumour type

The WHO-classification system (1982) used today describes mainly six different histological types with invasive ductal carcinoma being the most frequent diagnosis accounting for approximately 80% of all invasive breast carcinomas. Invasive lobular carcinomas compose another 10-15%, and the remaining proportion of invasive tumours consists of smaller groups such as mucinous, medullary, papillary, and tubular carcinomas. The prognostic value of tumour morphology is limited (Arpino et al. 2004, Jayasinghe et al. 2007, Mersin et al. 2003), despite differences in size, receptor expression, and metastatic properties (Arpino et al. 2004). Recent reports have proposed a treatment predictive value of tumour type when applying preoperative chemotherapy in favour of ductal type (Cristofanilli et al. 2005, Wenzel et al. 2007).

Histological grade (NHG)

Nottingham Histological Grading (NHG) was introduced by Elston and Ellis in 1991 (Elston et al. 1991) and is mandatory in breast cancer classification. NHG include three

parameters; (1) the percentage of open, tubular structures within the epithelial component of the invasive tumour, (2) grade of nuclear atypia, and (3) the number of mitoses. Each parameter is graded from 1-3, and the tumour grade is the sum of these three parameters. A sum of 3-5 corresponds with a grade I tumour; a sum of 6-7 with grade II tumours, and 8-9 points represents poorly differentiated grade III tumours. NHG is an established prognostic parameter (Elston et al. 1991), although the reproducibility has been questioned and recent studies suggest further refining, especially for mitotic count (Genestie et al. 1998, Mirza et al. 2002).

Table 1 Nottingham Histopathological Grading according to Elston and Ellis. (Adapted from Regional Oncologic Center, Uppsala/Örebro Region, 2007)

Nottingham Histopathological Grading (NHG)		Score
Percentage of tumour area	>75% <10% t <75%	1 2
composed of tubules (t)	<10%	3
Mitotic count in 10 high power fields	<10 10 < m < 20 >20	1 2 3
Nuclear pleomorphism	Uniform, equally sized small nuclei, no nucleoli Median size nuclei, moderate pleomorphism, nucleoli Large abnormal nuclei, pronounced pleomorphism, nucleoli	1 2 3
Sum – NHG		
Grade I	Well differentiated	3-5
Grade II	Moderately differentiated	6-7
Grade III	Poorly differentiated	8-9

Tumour stage (TNM/NPI)

The TNM classification system includes tumour size (T), axillary lymph node status (N), and distant metastasis (M). TNM is a useful system for comparison of clinical data and assessment of treatment outcomes. The value of the TNM system is continuously disputed (Benson et al. 2003), yet, in Swedish clinical praxis, TNM still plays a major part in the daily management of breast cancer and is fundamental for treatment recommendations. Each parameter in the TNM system is an independent prognostic factor (Cianfrocca et al. 2004) and the prognostic value of TNM is consequently high. Nottingham Prognostic Index (NPI) takes tumour size, NHG, and axillary lymph node status into account. NPI is estimated for all invasive breast tumours in the pathology protocol, and act as an important prognostic parameter (D'Eredita et al. 2001, Eden et al. 2004).

HER2

The human epidermal growth factor receptor 2 (HER2) belongs to the EGFR family of trans-membrane receptors (HER1, HER2, HER3, HER4). The HER2 signalling is mediated through heterodimer formation with either of the family members (Earp et al. 1995, Zaczek et al. 2005).

In today's clinical practice, breast carcinomas are classified as 0, 1+, 2+, or 3+ based on protein expression in IHC analyses (Herceptest®). HER2 gene amplification is analysed

for all 2+ and 3+ carcinomas using fluorescence in situ hybridization (FISH) (Dowsett et al. 2003). A tumour is considered HER2 positive if classified as 3+, or 2+/HER2 amplified.

In general, the frequency of HER2 positive breast cancer is estimated to be around 20% (Revillion et al. 1998). Over-expression of the HER2 protein and/or amplification of the HER2 gene are associated with aggressive tumour features and poor prognosis (Cianfrocca et al. 2004, Lohrisch et al. 2001).

Hormone receptors

The estrogen and progesterone receptors are members of the nuclear receptor superfamily of transcription factors regulating gene expression in response to endocrine signalling. To date, four different hormone receptors have been identified; estrogen receptor α , estrogen receptor β , progesterone receptor A, and progesterone receptor B.

The estrogen receptor alpha, ER α

ER α has three major functional domains; the AF-1 (ligand-independent domain), the DBD (DNA-binding domain), and the AF-2 (ligand-dependent activation domain) (Webster et al. 1988, Weigel et al. 1998). In assembly with estrogen the receptor undergoes conformational changes which allow for its binding to EREs (estrogen responsive elements) on target genes leading to gene transcription. Transcriptional activation is mediated by a number of co-regulating proteins and recruitment of co-regulators provide the receptors extensive functional flexibility (McKenna et al. 1999, McKenna et al. 2002). The ER α can also be activated in an estrogen independent manner by phosphorylation (Weigel et al. 1998).

ER α is expressed in the terminal duct lobular units in normal breast tissue, however in lower concentrations compared to breast cancer tissue (Clarke et al. 1997). ER α expression seems to alter along with breast cancer progression; in normal ductal epithelium around 10% of the cells have elevated ER α levels (Clarke et al. 1997), whereas in breast cancer the number is around 75% (Allred et al. 2004). In hyperplasia, ER α expression has been shown to correspond with increased risk of developing invasive cancer (Shaaban et al. 2002). The low ER α levels in normal breast tissue show an inter-individual variation, and additionally, women with high ER α levels have an increased breast cancer risk (Khan et al. 1994). Taken together, an elevated ER α expression might be one of the earliest events of breast cancer initiation and progression.

In vivo studies show that ER α knock-out mice do not develop mammary glands and are infertile whereas depletion of the ER β does not influence mammary development and fertility (Couse 1999, Krege 1998).

The estrogen receptor beta, ER β

During decades, ER α was recognized as the only estrogen receptor. However, in 1996 a novel estrogen receptor was identified and named ER β (Kuiper et al. 1996, Mosselman et al. 1996). The gene encoding for ER β is located on chromosome 14 (Enmark et al. 1997), whereas the ER α gene is located on chromosome 6 (Menasce et al. 1993).

The estrogen receptors differ in the aminoterminal AF-1 region (Paech et al. 1997), which is the location for interaction with other proteins in the transcriptional machinery; and to a less degree in the ligand-binding domain, AF-2 (Ogawa, Inoue, Watanabe, Hiroi et al. 1998). Despite these differences, ER β and ER α bind to estrogen with a similar affinity

(Ogawa, Inoue, Watanabe, Orimo et al. 1998), and neither does the binding affinity to DNA differ considerably (Kuiper et al. 1996). ER β expression is relatively high in the normal breast (Shaw et al. 2002) with a decreased expression along with disease progression and an altered ER β -ER α ratio (Shaaban et al. 2003, Skliris et al. 2003). ER β expression does not appear to provide significant prognostic information in contrast to ER α , whereas a treatment predictive value of ER β has been reported (Esslimani-Sahla et al. 2004, Hopp, Weiss, Parra et al. 2004).

The often contradictory results regarding the role of ER β could be explained by the presence/absence of several splice variants, in particular ER β -ex (Palmieri et al. 2002). ER beta splice variant, ER β -ex is a dominant negative repressor of ER α and has no measurable affinity for estradiol (Ogawa, Inoue, Watanabe, Orimo et al. 1998). Hence, ER β -ex represents a potential confounder in the already complex estrogen receptor story (Palmieri et al. 2002).

Recently new insights on the interaction between the two estrogen receptors reveal that ER β acts by antagonizing ER α on a very specific subset of estrogen-stimulated genes and actively prevents ER α stimulated cell growth (Lin et al. 2007) and suggestively, ER β status may be a major driver for clinical heterogeneity in ER α positive tumors.

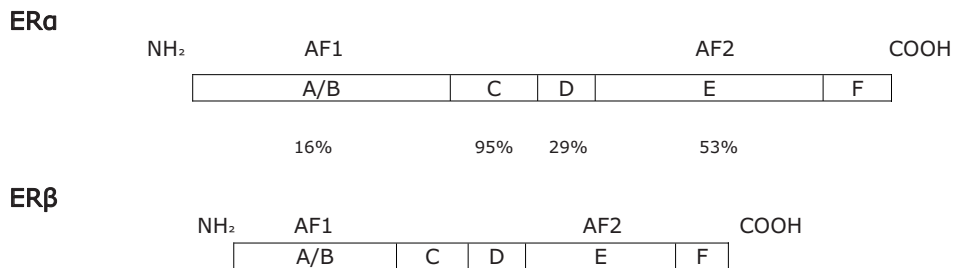


Figure 1 Schematic illustration of the estrogen receptors with the percentage of homology between protein domains listed. A/B: Transactivation domain; C:DNA binding domain; D:Dimerisation domain; E: ligand binding domain; F: Transcriptional modulation Adapted from Gruvberger-Saal, 2005.

The progesterone receptors, PgR

Another nuclear hormone receptor is the progesterone receptor which exists in two isoforms, PgR-A and PgR-B. They represent two structurally and functionally distinct nuclear receptors arising from a single gene (Conneely et al. 2003). PgR-A and PgR-B differ in their capacity as a transcription activator with PgR-B being the most powerful and further, the most predominant regulator of breast cancer cells (Soyal et al. 2002). The PgR is localized downstream of the ER α and PgR expression is dependent of estrogen stimulation (Flototto et al. 2004). Yet, recent studies indicate an estrogen independent PgR functioning pathway as well.

Most clinical studies evaluating the prognostic and predictive value of progesterone expression have not taken PgR isoforms into account. However, Hopp *et al* reported poorer disease-free survival among women with PgR-A high tumours, which was interpreted as tamoxifen resistance (Hopp, Weiss, Hilsenbeck *et al.* 2004). Both isoforms are recognized by the clinically used antibody, and generally referred to as PgR.

In ER α positive breast cancer, negative or low expression of PgR have been associated with more aggressive features like aneuploidy, high proliferation, HER1- and HER2 over-expression (Arpino *et al.* 2005). Not only is PgR expression a prognostic factor, the predictive role was identified 30 years ago (Horwitz *et al.* 1975), and several studies has confirmed the initial results (Arpino *et al.* 2005, Bardou *et al.* 2003, Stendahl *et al.* 2006).

The cell cycle

The cell cycle forms the basis for the reproduction of all cells and is fundamental for proliferation and development in all tissues of the human body.

The cell cycle consists of four active phases and two restriction points;

- G0 phase; the cell is waiting for a signal to enter the cell cycle (quiescent phase).
- G1 phase; the cell is preparing for multiplying its genome by checking for any damages.
- S phase; DNA replication takes place (synthetic phase).
- G2 phase; the cell is preparing for division.
- M phase; cell division occurs (mitotic phase).

The G1/S restriction point is regulated by cyclin D1, D2, D3, and cyclin E in cooperation with their specific cyclin-dependent kinases (CDKs). D-type cyclins and CDK4 and CDK6 form complexes, which enter the nucleus and phosphorylate the Rb protein, and eventually lead to transcription of genes required for entrance into the S-phase. In complex with CDK2, cyclin E further phosphorylates pRb, and the cell enters S-phase. The G2/M restriction point symbolizes the final check point before the cell is allowed to enter the mitotic phase and divide.

The formation of cyclin-CDK complexes stimulates the cell to "cycle", whereas the CDK inhibitors exert the opposite effect. CDK-inhibitors, which are potential tumour suppressor gene products, can be divided into two families. The CIP/KIP family includes p21, p27 and p57, which all inhibit the cyclin E/CDK2 complex. The INK4 family consists of p15, p16, p18 and p19, potential inhibitors of the cyclin D-CDK4/6 complex. Levels of cyclins vary during cell cycle, and are rapidly degraded after having completed their mission. CDK levels are, however, rather constant during the cell cycle.

In the development of the normal breast, cyclin D1 and p27, play essential roles. *In vivo* studies have shown that cyclin D1 deficient mice fail to develop normal mammary epithelium of the lobularalveolar system, and consequently impaired lactating capacity (Sicinski *et al.* 1995). Overexpression of cyclin D1 in the normal mammary glands has been shown to stimulate proliferation and eventually lead to the development of breast carcinomas (Wang *et al.* 1994). *In vivo* studies on p27 deficient mice report an observed multiorgan hyperplasi, but impaired mammary development and infertility (Deans *et al.* 2004, Fero *et al.* 1996).

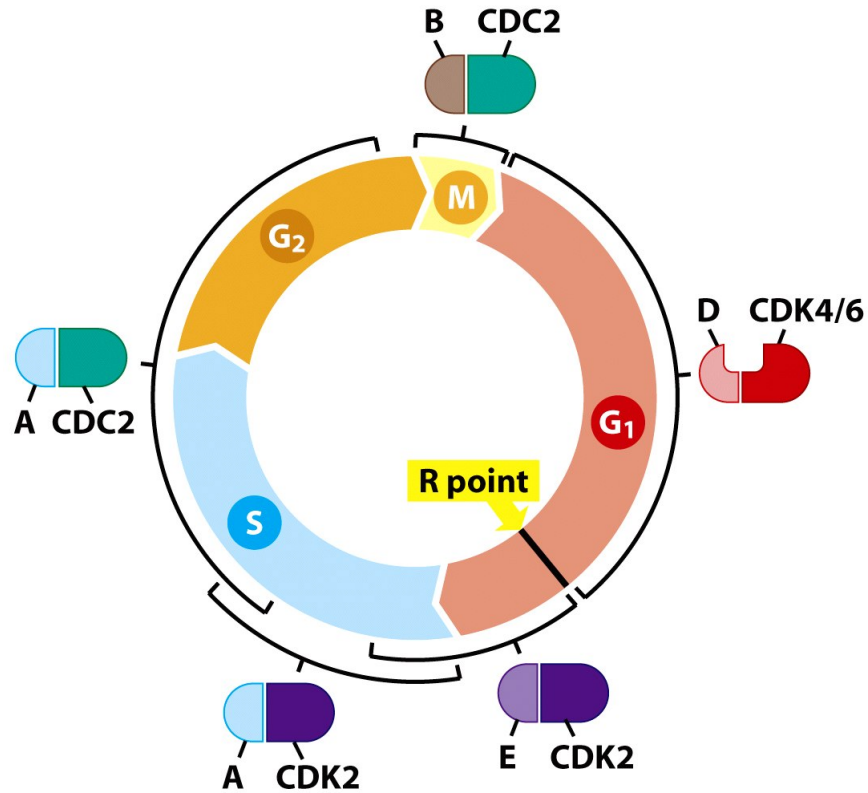


Figure 2 Schematic illustration of the cell cycle (Reprinted from "the biology of Cancer" by Robert A. Weinberg, 2007. Copyright Garland, Science, Taylor & Francis Group, LLC. With permission)

Cell cycle aberrations in breast cancer

In breast cancer cells the cell cycle is often deregulated at the G₁/S restriction point controlled by cyclin D1, cyclin E, and CDK inhibitors (Landberg 2002, Sutherland et al. 2004).

Cyclin D1 represents a downstream target for the estrogen mediated activation of the estrogen receptor α thereby providing cyclin D1 with an important role in ER α -induced proliferation in breast cancer cells (Planas-Silva et al. 1997, Resnitzky et al. 1994). Additionally, cyclin D1 can act in an estrogen-independent manner and exert its oncogenic activity by recruiting co-factors (McMahon et al. 1999).

Cyclin D1 protein overexpression is a relatively common event and has been reported in 25-50 % of all invasive breast cancers, whereas amplification of the CCND1 gene, coding for cyclin D1, is observed in around 15% (Gillett et al. 1994, Ormandy et al. 2003). Cyclin D1 overexpression increases proliferation (Lukas et al. 1994), yet the prognostic value of cyclin D1 over-expression remains to be fully clarified. Conflicting results have been published and report both reduced and improved prognosis associated with cyclin D1 overexpression (Hwang et al. 2003, Kenny et al. 1999, van Diest et al. 1997). The predictive value of cyclin D1 overexpression in tamoxifen treated women has received further attention lately, suggesting cyclin D1 overexpression to be a potential marker for tamoxifen resistance (Stendahl et al. 2004). In a study of premenopausal women, amplification of the CCND1 gene was associated with adverse effects of tamoxifen (Jirstrom et al. 2005).

Elevated levels of cyclin E may arise either indirectly through mutations in upstream mitogenic pathways, by gene amplifications or by obstructed proteolysis (Moroy et al. 2004). Several studies indicate that not only does cyclin E overexpression induce an aberrant cell cycle progression; it also leads to increased chromosomal instability. Cyclin E might therefore have multiple functions potentially involved in tumorigenesis. Cyclin E is commonly overexpressed in ER α negative breast cancer, and generally, high levels of cyclin E is correlated to a poorer outcome (Keyomarsi et al. 1994, Nielsen et al. 1996, Nielsen et al. 1999).

The tumour suppressor gene, p27, acts as an important inhibitor of the cyclin E/CDK2 complex thereby inhibiting further oncogenic activity (Slingerland et al. 2000). Decreased p27 protein expression is frequently observed in several malignancies, and the mechanism behind this appears to be caused by increased protein degradation exerted by other proteins rather than p27 gene mutations (Slingerland et al. 2000). Low p27 expression has been associated with increased proliferation and high levels of cyclin E (Cariou et al. 1998, Gillett et al. 1999). In survival studies, p27 expression seems to be an independent prognostic factor, implicating a reduced recurrence-free survival associated with low p27 levels (Cariou et al. 1998).

Proliferation

The net result of cell cycling is cellular proliferation, which is regarded as an independent prognostic factor in breast cancer. The rationale behind this is that haematogenous spread metastases is an early event, thus prognosis depends on the growth of metastases rather than presence or absence of micro-metastasis (van Diest et al. 2004). Different methods are applicable for assessment of tumour proliferation. Mitotic count represents one of the parameters on which NHG is based and is considered an established and reproducible proliferation parameter (van Diest et al. 2004). Ki67 is a proliferation associated antigen expressed in "cycling" cells, and Ki67 labelling index correlates well with mitotic count and S-phase fraction (Spyratos et al. 2002). Furthermore, topoisomerase II α and cyclin A are recently identified markers of proliferation activity displaying independent prognostic values (Bukholm et al. 2001, Rudolph et al. 1999).

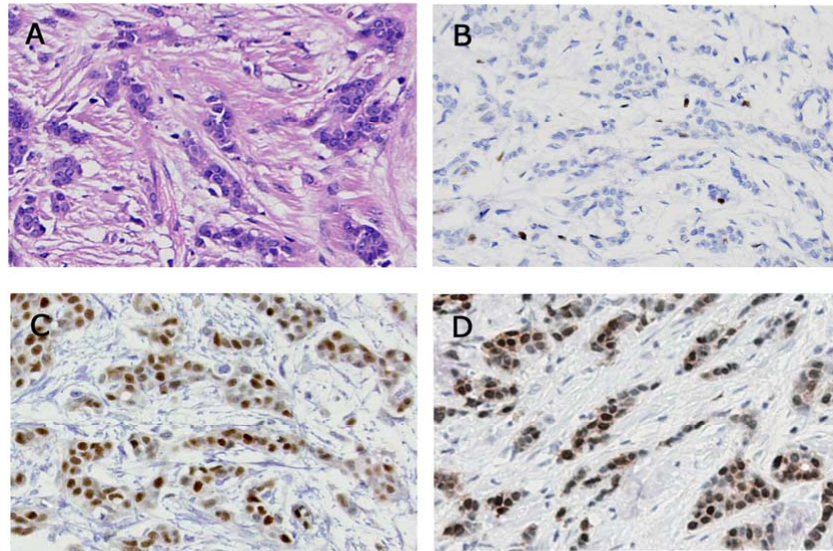


Figure 3 Example of an ERα positive grade I breast cancer with low proliferation and high p27 expression. Panel A shows routine Hematoxylin & Eosin staining. Panel B, C, and D show immunohistochemical staining for Ki67, ERα, p27, respectively.

HMG-CoA reductase

HMG-CoA reductase (HMG-CoAR) is a 90-97 kDa transmembrane glycoprotein residing in proteosomes and the endoplasmic reticulum, respectively (McGee et al. 1996). HMG-CoAR act as a rate-limiting enzyme in the mevalonate pathway (Goldstein et al. 1990) and is required for the synthesis of isoprenoids. Cholesterol represents the main product of the isoprenoid synthesis and forms the basis of all steroid hormones and is fundamental in membrane biogenesis (Di Croce et al. 1999). The other isoprenoid compounds are essential in the regulation of cell signalling by post-translational modification of proteins necessary for cellular proliferation (Kato et al. 1992). HMG-CoAR expression is regulated at different levels counting gene transcription, mRNA stability, translation, and enzyme degradation (Di Croce et al. 1996). Continuously acting feedback mechanisms assure the maintenance of adequate isoprenoid levels.

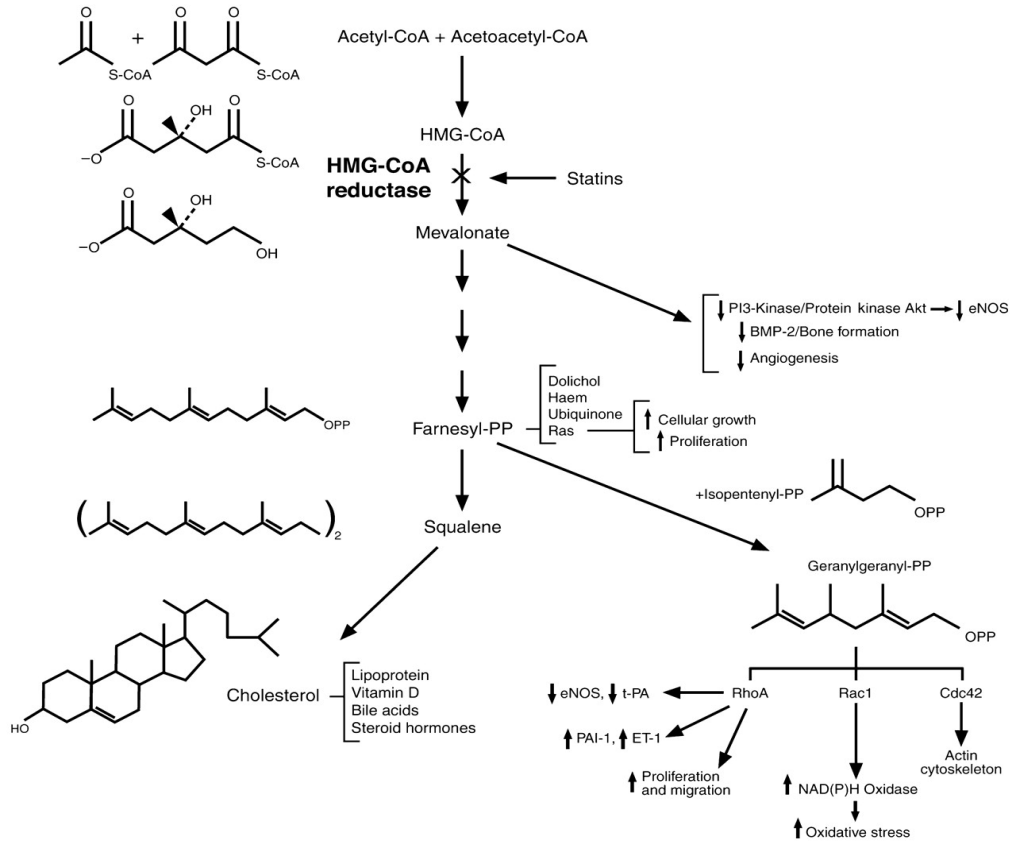


Figure 4 The mevalonate pathway. Reprinted from Liao (Liao 2002). Copyright, 2002, with permission from Journal of Clinical Investigation.

HMG-CoA reductase in cancer

In cancer cells, HMG-CoAR activity is elevated and the normal sterol feedback regulation disrupted (Mo et al. 2004). The increased HMG-CoAR levels in tumours might reflect an increased demand of non-sterol isoprenoids to maintain growth advantages (Mo et al. 2004). Both *in vitro* and *in vivo* studies on breast cancer have demonstrated how mevalonate is required for DNA-synthesis, cell proliferation, and subsequently tumour growth (Duncan et al. 2004, Wejde et al. 1992).

It has been shown that estrogen affects HMG-CoAR activity (Cypriani et al. 1988, Di Croce et al. 1996), although the molecular mechanisms are not fully understood. Recently, Croce et al identified an estrogen-responsive-element (ERE) on the HMG-CoAR gene, proposing a potential way of mediating estrogen induced effects on the HMG-CoAR gene (Di Croce et al. 1999).

The mevalonate pathway represents a potential target for cancer prevention and chemotherapy. Most epidemiological studies agree on the preventive and therapeutic effects of lipophilic HMG-CoAR inhibitors (statins). Preclinical studies support the anti-tumoural effects of statins and have reported anti-proliferative, proapoptotic, anti-invasive, as well as radiosensitizing properties of statins (Chan et al. 2003).

Several dietary components are potential inhibitors of HMG-CoAR activity. Plant isoprenoids are derived from vegetables, grains, and essential oils of fruits (Crowell 1999). Genistein is derived from soy and possess estrogenic potential. Polyunsaturated fatty acids (PUFAs), mainly from dietary fish oils, and cholesterol, represent further sources of naturally occurring HMG-CoAR inhibitors (Duncan et al. 2005). Both in vivo and in vitro studies have observed anti-carcinogenic activity following administration of naturally occurring HMG-CoAR inhibitors, and the underlying mechanism is proposed to be inhibition of HMG-CoAR activity.

CLINICAL ASPECTS

Diagnostics

During the past decade, the breast cancer diagnosis has been based on the triple diagnostic procedure including clinical examination, mammography, and fine needle aspiration. The combined triad of tests provides a sensitivity of 100% and a negative predictive value of 100% allowing triple-negative individuals surveillance instead of an open biopsy (Kaufman et al. 1994). In case of suspicious cancer in any of the diagnostic procedures, further investigation is initiated, primarily using surgical biopsy, alternatively MRI or ductography.

Treatment

Breast cancer treatment comprises both local and systemic approaches. These approaches represent a wide range of options applied in different phases of the disease, and comprise surgery, radiotherapy, chemotherapy, endocrine therapy, and antibody therapy.

Surgery

Primary surgery is applied for most patients, and performed either in a breast conserving manner or as mastectomy. The surgical treatment of choice depends on several factors, i.e. tumour size, breast size, tumour growth pattern, and finally, and most importantly, the patient's prerequisites. Following the introduction of the sentinel node (SN) technique, axillary surgery procedures have been modified. The sentinel node technique is appropriate in cases of smaller tumours and no indications of axillary metastasis. Otherwise, or in cases with positive sentinel node, axillary dissection is performed aiming at a minimum of ten examined lymph nodes. Surgery is generally not recommended in case of generalised disease at diagnosis.

Radiotherapy

In order to reduce the risk of loco-regional recurrences, women with invasive breast cancer should receive adjuvant radiotherapy to the remaining breast following breast conserving surgery (Malmstrom et al. 2003). Among women with locally advanced breast cancer, radiotherapy to the chest wall and loco-regional lymph nodes is applied following mastectomy. Postoperative radiotherapy to high-risk pre- and postmenopausal women has been shown to reduce the risk of local recurrences and improve survival (Overgaard et al. 1997, Overgaard et al. 1999).

Chemotherapy

Chemotherapy is applicable both in the neo-adjuvant, the adjuvant and in the palliative setting. Poly-chemotherapy is recommended in the neo-adjuvant and the adjuvant setting (2005). The rationale behind combining several drugs is the potential synergistic effects and different toxicity profiles which allow for more intense treatment modules. Neo-adjuvant treatment is primarily indicated in non-operable, locally advanced breast

cancer (T3-T4 tumours) and seems to generate improved local control and survival (Bergh et al. 2001). Today, adjuvant chemotherapy is indicated in case of grade III, T2, or ER α negative tumours, independent of the patient's age. For lymph node positive patients, age is taken into account in the decision of chemotherapy or not. Combination therapy is frequently used as first line therapy in the palliative setting, whereas single agent therapy is often applied in case of further recurrences.

Endocrine therapy

The aim of endocrine therapy is to diminish the stimulation of hormone sensitive breast tumour cells by estrogens.

Elimination of ovarian hormone production can be achieved by three methods; oophorectomy, radiotherapy of the ovaries, or medically induced ovarian suppression using gonadotropin-releasing-hormone (GNRH) agonists. Ovarian suppression, in either way, is predominantly applied in premenopausal women.

Two different groups of drugs with anti-estrogenic effects are applied in the adjuvant setting; selective estrogen receptor modulators (SERMs, i.e. tamoxifen) and aromatase inhibitors (AI, i.e. anastrozole, letrozole, exemestane).

Tamoxifen

Tamoxifen has been essential in breast cancer management since the early 70s and was initially used in patients with advanced disease (Cole et al. 1971), and only later on tamoxifen became an established drug in the adjuvant treatment of ER α positive postmenopausal breast cancer (1988). Since 1996, five years of adjuvant tamoxifen treatment has been recommended to postmenopausal women with breast cancer, based on the Swedish SBCG study (1996) and confirmed in several studies later on (1998). However, the recent introduction of aromatase inhibitors (AIs) has changed the management of postmenopausal breast cancer. In Sweden, tamoxifen is currently used in the adjuvant setting in N0 breast cancer with tumour size more than 10 mm independent of age. In N+M0 breast cancers, premenopausal women are recommended adjuvant tamoxifen treatment, whereas postmenopausal women receive either the tamoxifen-AI-switch model (tamoxifen 2-3 years followed by 2-3 years of AIs) or single therapy with AI depending on age and other risk factors (Regional Oncology Center, Uppsala/Örebro, 2007).

The anti-tumoural effect of tamoxifen is mediated by competitive binding to the ER α , and the tamoxifen-ER α complex probably prevents activation of co-activators resulting in deprived transcription of estrogen-regulated genes thus inhibiting cell proliferation (Shiau et al. 1998). The agonist effects of tamoxifen are well-known in bones and the uterus (Smith 2003), and a reduced cardiovascular mortality was seen among women who received two years of tamoxifen compared to five years tamoxifen therapy (Nordenskjold et al. 2005). Initially, most ER α positive patients will respond to tamoxifen (Osborne et al. 1980), but eventually the majority will develop resistance. The mechanism of resistance is not fully elucidated. Most tamoxifen resistant breast cancers are surprisingly still expressing ER α and the majority is capable of responding to alternative anti-estrogenic mechanisms exerted by fulvestrant and AIs (Buzdar et al. 2001, Howell et al. 1996).

ER β expression might influence the respond to tamoxifen and potentially predict outcome in tamoxifen-treated patients. However, conflicting results have been reported. Early and small studies have shown increased ER β expression in tamoxifen-resistant patients, whereas recent studies report the opposite, that is, high ER β expression is correlated with good prognosis for tamoxifen-treated patients but not for untreated patients

(Esslimani-Sahla et al. 2004, Hopp, Weiss, Parra et al. 2004, Mann et al. 2001). Paech *et al* have demonstrated that the ER β -tamoxifen complex can activate transcription via non-classical ER-signalling pathways through its binding to activator protein 1 transcription factors, thus indicating an agonist effect of tamoxifen in ER β high cancers (Paech et al. 1997). Murphy *et al*, conclude the contrary, that is, high expression of ER β is correlated to tamoxifen sensitivity (Murphy et al. 2002). A potential argument for the latter is that the agonist activity of tamoxifen has been located within the AF-1 domain of ER α (Berry et al. 1990) and the ER α -ER β heterodimer might diminish the agonist activity of ER α when bound to tamoxifen (Hopp, Weiss, Parra et al. 2004).

Aromatase inhibitors

The aromatase inhibitors (AIs) act by inhibiting the aromatase enzyme responsible for the conversion of peripheral circulating androgens into estrogens thereby lowering circulating estrogens to almost immeasurable levels (Geisler et al. 2005). AIs are only used in postmenopausal women with a cessation of the ovarian function. Consequently, circulating estrogens are produced in peripheral tissues by aromatization of androstendione. Results from two large studies, the ATAC-trial (Arimidex, Tamoxifen, Alone, or in Combination) and BIG 1-98 demonstrate a significant difference in disease-free-survival (DFS) in favour of AIs (Howell et al. 2005, Thurlimann et al. 2005). In a meta-analysis by Jonat *et al*, the tamoxifen-AI-switch-model was superior compared to continuously tamoxifen in terms of DFS and overall survival (Jonat et al. 2006). Given the fundamental differences in their biological function, the side-effects of AIs differ from those of tamoxifen. Joint symptoms are frequent and might limit the number of patients able to continue treatment recommendations (Crew et al. 2007). Further, AIs are associated with bone mineral density loss and increased bone turnover, thereby explaining the increased fracture risk observed among AI-users in the ATAC trial (Eastell et al. 2006). However, the risk of tamoxifen associated side-effects such as endometrial cancer, stroke, or pulmonary embolism, is decreased for AI-users (Mouridsen 2006).

Antibody therapy

Trastuzumab (Herceptin®) is a humanised monoclonal antibody recently approved for treatment of women with HER2 positive, node-positive breast cancer. Trastuzumab therapy is preferably administered in combination with other drugs, i.e. taxanes, and prescribed every 3rd week during one year. Both in primary and metastatic disease, trastuzumab has proven its efficacy (Piccart-Gebhart 2006), and recent studies on neo-adjuvant trastuzumab therapy concomitant with chemotherapy, reveal promising results (Buzdar et al. 2005). Modest side effects make trastuzumab well tolerated with the exception of cardiotoxicity which might accompany the therapy (Piccart-Gebhart et al. 2005).

Lapatinib is a dual inhibitor of HER1 and HER2, and has shown activity in advanced breast cancer, both as a single drug or in combination with trastuzumab or chemotherapy (Bilancia et al. 2007). Another targeted therapy with a specific antibody is the anti-angiogenic drug bevacizumab (Avastin®) which was recently approved in Europe for treatment of breast cancer patients with metastatic disease (Widakowich et al. 2007).

RISK FACTORS

Genetics

Breast cancer exhibits familial aggregation, and a history of breast cancer among first or second-degree relatives increases breast cancer risk. The Collaborative Group on Hormonal Factors in Breast Cancer demonstrated a lifetime excess incidence of breast cancer of 5.5% for women with one affected first-degree relative and 13.3% for women with two (2001). Relatives diagnosed at young age is another important risk marker, and recent studies indicate that survival patterns are heritable (Hartman et al. 2007, Hemminki et al. 2007). Most breast cancers are sporadic and occur without any identified breast cancer susceptibility. In contrast, around 5 % of all breast cancers are considered hereditary and develops in women with inherited breast cancer susceptibility genes, most notably *BRCA1* and *BRCA2* (Loman et al. 2003). Mutation carriers have a seriously increased breast cancer risk estimated to an average cumulative risk of 65 % for *BRCA1*-mutation carriers and 45 % for *BRCA2* (Antoniou et al. 2003). A number of breast cancer susceptibility genes have been identified, however, it is estimated that all currently known breast cancer susceptibility genes accounts for less than 25% of the familial aggregation of breast cancer. The number of identified breast cancer susceptibility genes probably represents a small fraction, and intensive research chases new candidates (Easton et al. 2007, Pharoah et al. 2007).

Diet

The etiological role of diet in breast cancer is still unclear, although migrant studies demonstrating altered breast cancer risk profiles could indicate a dietary influence (King et al. 1980). Given the complexity of dietary intake, the number of potential cancer risk-modifying agents is extensive. Furthermore, dietary assessment is complicated and several methods have been applied in different studies. Taken together, consistent associations between diet and breast cancer are difficult to prove, and might mirror a true absence of associations or may be caused by methodological difficulties (Michels, Mohllajee et al. 2007).

In vivo studies have shown inhibition of mammary tumorigenesis in energy restricted animals, independent of the amount of dietary fat (Kritchevsky 1997). The preventive role of energy restriction was supported in a human study, demonstrating a significantly reduced breast cancer incidence among women hospitalized for anorexia nervosa prior to the age of 40 years compared to the general population (Michels et al. 2004). Energy balance covers energy intake and expenditure, and the null findings might be an important parameter in breast cancer risk assessment (Silvera et al. 2006).

Fat intake has received considerable attention in breast cancer studies, although any consensus has not been reached. A meta-analysis of 12 case-control studies showed a higher breast cancer risk in the top quintile of total fat intake (Howe et al. 1990), as opposed to other case-control studies reporting negative results (Lipworth 1995). In general, prospective cohort studies have not shown any associations between total fat intake and breast cancer risk (Hunter et al. 1996). The hypothesis that a low-fat diet would reduce breast cancer risk was studied in a recent randomized controlled trial, and revealed a non-significant risk reduction in the intervention group (Prentice et al. 2006).

Several nutritional researchers have questioned the traditional fat debate (Taubes 2001, Willett 1994) and stated that "mainstream nutritional science has demonized dietary fat"

(Taubes 2001). Rose *et al* have proposed that the challenge is rather to balance the proportion of different fatty acids than avoiding fat (Rose 1997).

Diets are composed of several types of dietary fats, including saturated fatty acids (SFA, dairy products and meat), monounsaturated fatty acids (MUFA, i.e. olive oil), and polyunsaturated fatty acids (n-6 PUFA, vegetable oils, and n-3 PUFAs, fish).

Animal studies have shown that the tumour promoting effect of fat depends on the type and amount of fat, and not simply the caloric content (Wynder *et al.* 1997). Most likely, the ratio between two PUFAs, n-6 and n-3, is important for the tumour promoting effects of fat observed in animal studies. Further, diets rich in the eicosapentanoic acid (long-chained PUFA), present in fish oil, lack tumour promoting effects. In parallel, the low breast cancer rates in Eskimo and Japanese populations might be explained by the high content of eicopentanoic acid (Kaizer *et al.* 1989). Similar findings for oleic acid, present in olive oil, have been reported based on epidemiological studies on the relatively low breast cancer rates in Southern Europe where olive oil is a major contributor to the total amount of fat (Trichopoulou *et al.* 1993).

The association between fatty acids and breast cancer was addressed in a study from the Malmö Diet and Cancer cohort, where the authors found that a high intake of n-6 PUFAs was associated with an increased risk of breast cancer (Wirfalt *et al.* 2002). In another Swedish cohort study, a high PUFA intake was associated with an increased breast cancer risk, whereas MUFA intake and breast cancer risk were inversely associated (Wolk *et al.* 1998). SFA intake was not associated with breast cancer in neither of the Swedish studies.

In general, diet consists of three different macronutrients; fat, carbohydrates, and protein. The intake of carbohydrates has generally not been associated with an increased breast cancer risk (Holmes *et al.* 2004, Jonas *et al.* 2003, Nielsen *et al.* 2005). The number of studies on protein intake and breast cancer risk is limited.

Anthropometrics

Anthropometric measurements cover a variety of parameters; height, weight, BMI, waist – and hip circumference, waist-hip ratio, and body fat percentage are all frequently applied parameters. In analyses of anthropometrics and breast cancer, it is generally agreed that stratification for menopausal status should be performed. Body size and postmenopausal breast cancer are positively associated whereas an inverse association is seen for premenopausal breast cancer (Harvie *et al.* 2003). In the following text “breast cancer” refers to postmenopausal breast cancer.

Height

Attained height is regarded as a non-modifiable anthropometric factor. A positive association between height and breast cancer risk has been reported in several cohort studies (van den Brandt *et al.* 2000). The possible mechanisms behind this are unclear. A low stature might reflect insufficient energy intake during childhood and as energy restriction has been shown to reduce breast cancer risk (Kritchevsky 1997, Michels *et al.* 2004), this might partly explain the association. However, in relatively affluent populations, height is still considered a risk factor for breast cancer (Swanson *et al.* 1989, Trentham-Dietz *et al.* 1997). More likely, genetic and environmental factors interact in a still unsolved manner.

Weight, BMI, waist, hip, and body fat percentage

Body size is an important modifiable risk factor for breast cancer (Friedenreich 2001, Reeves et al. 2007). However, estimation of body size is not that simple and the choice of parameters often depends on the purpose and practical possibilities. Weight, BMI, waist- and hip circumference are rather inexpensive measurements and often applied in larger studies. BMI is used as an indicator of "general" body size, whereas waist- and hip circumference give information on the distribution of body fat. Assessment of body fat percentage generates an average estimate of body fat, but is a rather expensive and time-consuming method. Several anthropometric measurements have been used in breast cancer studies and it remains undecided which one of them is the most reliable predictor of breast cancer risk.

Harvie *et al*/ conducted a review on central obesity using waist circumference and waist-hip-ratio as indicators and found a positive association with breast cancer risk. Adjustment for BMI abolished the association and the authors conclude that central obesity is not a superior breast cancer predictor compared to general obesity (BMI) (Harvie et al. 2003). The increased breast cancer risk with a higher BMI is most likely due to increased concentrations of circulating sex hormones, and strong empirical evidence exists to support this underlying mechanism (Key et al. 2003). Adult weight gain has received increasing attention and is proposed as an equivalent, or even superior, risk indicator compared to BMI (Feigelson et al. 2004, Han et al. 2006). Han *et al*/ found a 4% increase in breast cancer risk for every 5 kg gained weight, however, weight gain in different periods of life might exert distinct impact on breast cancer risk and should be investigated further (Han et al. 2006).

Physical activity

Physical activity is considered a modifiable risk factor for breast cancer, and several studies have reported an inverse association (Bernstein et al. 2005, Lahmann et al. 2007, Moradi et al. 2002, Sprague et al. 2007). Some studies suggest that physical activity primarily affects postmenopausal breast cancer risk (Monninkhof et al. 2007). Both recreational, household, and occupational activities are proposed as contributors to the protective effects (Sprague et al. 2007). The biological mechanisms behind are not fully understood, however, physical activity-mediated effects such as low estrogen bioavailability, prevention of weight gain, regulated insulin sensitivity, and altered immunological functions, are plausible explanations (Hoffman-Goetz et al. 1998, Sprague et al. 2007).

Alcohol

Alcohol consumption and breast cancer risk have consistently been positively associated (Hamajima et al. 2002, Singletary et al. 2001, Zhang et al. 2007). Increased estrogen levels following a regular alcohol use is a possible mechanism, although many other factors enhance alcohol-mediated effects on breast cancer risk, i.e. low folate intake (Singletary et al. 2001, Suzuki et al. 2005). The type of alcohol beverage has not been shown to affect breast cancer risk (Tjonneland et al. 2003). The average daily alcohol intake rather than drinking frequency seems to affect breast cancer risk (Tjonneland et al. 2003), and even a moderate alcohol intake of one drink per day has been associated with increased breast cancer risk (Longnecker et al. 1995). A study on alcoholic women with a severe intake did not reveal the presumed increase in breast cancer risk (Kuper et al. 2000).

Smoking

The association between cigarette smoking and breast cancer is still controversial. A large collaborative re-analysis found no association between smoking and breast cancer (Hamajima et al. 2002). However, the association might be blurred by excluding tumour-biological factors in the risk-assessment (Russo 2002). Some studies have advocated a protective role of cigarette smoking due to potential anti-estrogenic effects (Baron et al. 1990), and dual effects of smoking has been reported and highlighted the need of separate analysis of pre- and postmenopausal women (Band et al. 2002). Cessation of cigarette smoking might be a risk indicator as higher breast cancer incidence has been reported among ex-smokers compared to current and never smokers (Manjer et al. 2000). Furthermore, ex-smokers have an increased risk of grade III and PgR negative tumours compared to never-smokers (Manjer, Malina et al. 2001).

Socioeconomic status

Women of a high socioeconomic status have an increased risk of breast cancer (Baquet et al. 2000, Faggiano et al. 1997). The different incidence between deprived and affluent women might be caused by factors like late maternal age at first childbirth, nulliparity, and use of exogenous hormones among affluent women (Brown et al. 2007). Higher attendance rates in screening programmes among affluent women, probably contribute to the different incidence rates across socioeconomic groups (Brown et al. 2007). The socioeconomic status is an important prognostic parameter in breast cancer, and most studies agree on an association between a poor socioeconomic status and poor prognosis (Baquet et al. 2000, Bouchardy et al. 2006). Especially young women of poor socioeconomic status are at high risk of dying from their breast cancer with a three-fold higher risk compared to women of high socioeconomic status (Bouchardy et al. 2006).

Endogenous hormones

An endogenous hormone is defined as a substance produced in one organ and transported to another with the purpose of exerting its metabolic effect. Steroid hormones are lipophilic molecules derived from cholesterol. When bound to albumin or serum hormone binding globulin (SHBG), they are in an inactivated state. However, the unbound and active forms diffuse through cell membranes and bind to receptors in the nucleus, mitochondria or cytoplasm. The ovarian hormones, i.e. estrogen and progesterone, are crucial in breast cancer development (Key et al. 2002) and described further below.

Estrogen

In premenopausal women, the predominant estrogen source is the ovaries. In the ovaries, cholesterol is converted to androstendione and either directly, or via testosterone, to estrone and estradiol, respectively (Clemons et al. 2001). The conversion into estrogens is regulated by aromatase enzymes which are stimulated by FSH. The lower estrogen levels in postmenopausal women are due to cessation of ovarian production of estrogens. However, compensatory estrogen production is initiated via aromatization of androgens predominantly from the adrenal glands. Liver, muscle, and

fat tissue are the predominant places for this conversion, and explain the higher levels of circulating estrogens in obese women (Key et al. 2003).

In a "paracrine" manner, local estrogen production in the breast may occur. Recent studies propose that local formation of estrogens in breast tumours may be more important than circulating estrogen in plasma for the growth and survival of estrogen-dependent breast cancer in postmenopausal women. The biosynthesis of estrogens in breast tumour tissues can follow two major and different routes, one being the aromatase pathway and another one the steroid-sulfatase (STS) pathway. There is accumulating evidence for higher estrogen concentration in breast tissue among women with breast cancer compared to those without (Chetrite et al. 2000, Nakata et al. 2003). Estrogens diffuse passively through the cell- and nuclear membranes in target cells. In complex with estrogen receptors, estrogen binds to estrogen responsive elements (EREs), whereby cell growth and differentiation is regulated.

Estrogen levels are known to affect breast density (Greendale et al. 2005), supported by the notion that hormonal replacement therapy increases breast density (Greendale et al. 2003) and tamoxifen treatment causes reduced breast density (Ursin et al. 1996).

Estrogens are essential for many normal functions in the human body (Weihua et al. 2003), i.e. breast development, bone mineral density (Clemons et al. 2001), and arterial calcification processes (Saltiki et al. 2007). Life-time exposure to endogenous estrogens varies among women and is determined by reproductive factors as age of menarche, age at first full-term pregnancy, parity, and age at menopause. Other determinants of estrogen resources are obesity, amount of exercise, and certain dietary nutrients, i.e. phytoestrogens (Clemons et al. 2001).

Estrogens are predominantly metabolized in the liver. The estrogen catabolism reveals both intra- and interpersonal variation due to gene polymorphism among catabolic enzymes (Clemons et al. 2001). Hence, further variation in the cumulative exposure to estrogen is added.

Indeed, estrogen and breast cancer are associated. Estrogens are involved in both initiation and promotion of breast cancer. The mechanisms behind are complex. Estrogen has potential genotoxic effects, and DNA-damage might occur through activation of oncogenes or other proteins that are involved in the nucleic acid synthesis. Tumour promotion is mainly facilitated by proliferative effects exerted by estrogens in complex with estrogen receptors (Russo et al. 2006).

Progesterone

Progesterone is the only endogenous progestin. The corpus luteum in the ovaries is the primary site for synthesis, and a minor amount is derived from the adrenal glands. Progesterone is a precursor of androstendione which may be converted into testosterone, and further to estrone and estradiol. The role of progesterone in breast cancer is not fully elucidated. Most epidemiological studies report no association between progesterone and breast cancer risk (Eliassen et al. 2006, Missmer et al. 2004), however, breast cell proliferation is increased in the luteal phase when progesterone levels are high (Navarrete et al. 2005). The full development of the mammary gland is not achieved until pregnancy and progesterone is proposed to play an important role in the morphological and functional changes of the breast induced during pregnancy (Soyal et al. 2002).

Exogenous hormones

Oral contraceptives

The use of oral contraceptives (OCs) is estimated to cause a slightly increased risk of breast cancer, although levelled out ten years after cessation (1996, Veronesi et al. 2005). The influence on breast cancer risk exerted by OCs depends on several different parameters. Age of OC initiation, time of initiation in relation to menarche and first child birth, duration of OC use, and type of OC (Althuis et al. 2003) are relevant parameters to take into account when estimating breast cancer risk subsequently to OC use (Ansink et al. 2007). A recent Swedish study found no increased breast cancer risk among current and ever users, while women with a history of OC use prior to the age of 20, had a markedly higher risk of early-onset breast cancer (Jernstrom et al. 2005).

Hormonal replacement therapy (HRT)

Along with menopause and hormone deprivation, menopausal symptoms arrive. The most frequent symptoms include flushes, depression, heart palpitations, fractures, variations in menstruation cycle, sweat symptoms, etc. Hormone replacement therapy (HRT), prescribed as single estrogen-therapy (ERT), was introduced in the 1960s with great expectations, yet an increased risk of endometrial cancer emerged as a severe adverse effect. The addition of progestin eliminated the increased risk (Persson 89, Voigt 91) and further on combined hormonal replacement therapy (CHRT) was recommended (Odland 2004). In CHRT, progestins are added either sequentially or continuously.

During the 1980s the use of HRT increased rapidly. In the Million Women Study (UK) recruiting participants between 1996 and 2001, half the women were current or former users of HRT (Beral 2003). However, novel consequences of the popular therapy arose. In the 1990s several meta-analyses pointed out an increased risk of breast cancer among HRT-users (1997). The Women's Health Initiative Study, a randomized controlled primary prevention trial, was stopped after a mean of 5.2 years of follow-up due to an increased risk of breast cancer in the CHRT group compared to the placebo group. Further, the risk-benefit analysis concluded that initiation and continued CHRT was not appropriate for primary prevention of coronary heart disease (Rossouw et al. 2002).

The first results from the Million Women Study were published in 2003 and showed a marked increase of breast cancer among women receiving CHRT, but not ERT. Neither the type of estrogen/progestin, nor the administration (sequential/continuous) and dose, influenced the risk of breast cancer. Further, the increased breast cancer risk seemed to vanish five years after HRT cessation (Beral 2003). The difference in breast cancer risk between ERT and CHRT has been described in several other studies (Colditz 2005, Collins et al. 2005, Greiser et al. 2005). Breast cell proliferation increases along with high endogenous progesterone levels in the luteal phase, and in a similar fashion after initiation of CHRT (Conner et al. 2003, Hofseth et al. 1999). Notably, proliferation was localized to the terminal duct-lobular unit of the breast, the site for development in most breast cancers (Hofseth et al. 1999).

Women using HRT show increased breast density on mammography (McTiernan et al. 2005) and women on continuously administrated CHRT have the most affected mammograms (Colacurci et al. 2001). A recent study reports that extensive mammographic density is associated with an increased risk of breast cancer detected by screening or during screening intervals and dense mammograms are therefore regarded as a risk factor *per se* (Boyd et al. 2007). However, dense breast tissue is more difficult to examine on mammography, and others propose that the decreased sensitivity of mammograms might influence the increased risk of interval cancer (Hofvind et al. 2006).

Thus, alternative diagnostic methods for women on HRT might be appropriate. Particularly when considering the fact that HRT-users are at high risk of lobular cancers which are often multifocal and easier detected on MRI (Biglia et al. 2007).

Anthropometric factors have been related to breast cancer risk among HRT users; lean women appear to be at higher risk compared to obese women (Lahmann et al. 2004). Lean women do have lower levels of unbound serum estradiol compared to obese women (Bezemer et al. 2005, Rinaldi et al. 2006) and the use of HRT may stimulate cell proliferation relatively more in hormone-deprived lean women than in obese women.

The biological association between CHRT use and breast cancer is not clear. Most studies agree on a tumour promoting effects of hormones (Anderson et al. 1989), whereas hormonally induced tumour initiation appears more controversial (Dietel et al. 2005).

Reproductive factors

The breast undergoes continuous changes during the reproductive phase in a woman's life. The reproductive history includes several factors such as age at menarche, age at first full-term pregnancy, parity, breast feeding, and age at menopause. A relatively high cumulative estrogen exposure obtained through early menarche, late menopause, and nulliparity increases the risk of breast cancer (Veronesi et al. 2005). Oppositely, high parity, young age at first full-term pregnancy, and breast feeding have been associated with a protective effect on breast cancer risk (Clavel-Chapelon 2002, Veronesi et al. 2005). Giving birth has shown dual effects on breast cancer risk with a temporary increased breast cancer risk subsequent to birth in the first five to ten years (Lambe et al. 1994, Liu et al. 2002). Neither spontaneous nor induced abortions seem to influence breast cancer risk (Clavel-Chapelon 2002, Michels, Xue et al. 2007, Paoletti et al. 2003).

AIMS OF THE THESIS

The general aim of this thesis was to study the association between life style factors and breast cancer risk by sub-grouping breast cancer according to molecular pathology parameters with the anticipation of contributing to novel prevention strategies.

The specific aims of each paper are listed below:

- Explore the association between sub-groups of breast cancer and several dietary and anthropometric factors (Paper I)
- Evaluate the association between hormonal replacement therapy and the risk of specific breast cancer sub-groups (Paper II)
- Analyse the association between different anthropometric measurements and the risk of breast cancer defined by histological and tumour biological characteristics (Paper III)
- Study the intracellular distribution and various expression of HMG-CoA reductase in breast cancer and analyse its relationship with estrogen-related factors (Paper IV)
- Investigate the relationship between ER β expression and established pathological parameters in breast cancer, and study the predictive potential of ER β expression among tamoxifen treated patients (Paper V)

SUBJECTS AND METHODS

Study cohorts

The Malmö Diet and Cancer Study (Paper I-IV)

The Malmö Diet and Cancer Study (MDCS) is a prospective cohort study. Participants were recruited from a source population defined as all persons living in Malmö and born between 1926 and 1945. In 1994, the source population was extended to include women born between 1923 and 1950, and men born between 1923 and 1945. The only exclusion criteria was mental incapacity or inadequate language skills in Swedish (Berglund et al. 1993). Recruitment was performed by public advertisement (posters and pamphlets) and personal invitations (letters and telephone calls) (Manjer et al. 2002). Participation was voluntary and without any financial compensation. Baseline examinations were initiated in March 1991 and conducted until September 1996. Participants visited the MDC screening centre twice. At the first visit, they received instructions on how to register meals in the menu-book and how to fill in questionnaires concerning demographic, socioeconomic, and various life style factors, including dietary habits. Furthermore, anthropometric measures and blood samples were taken. The purpose of the second visit was to ensure completion of the questionnaires and it also included a dietary interview. At the end of baseline examinations, 28 098 participants had completed all study parts, of whom 17 035 were women (Manjer, Carlsson et al. 2001).

Breast cancer cases were ascertained by record linkage with the Swedish Cancer Registry and the Southern Swedish Regional Tumour Registry. Vital status was retrieved from the Swedish Cause of Death Registry.

In Paper I, end of follow-up was 31 Dec 2001. Following baseline examinations, a total of 440 women were diagnosed with incident breast cancer. Fifty cases were diagnosed as in situ breast cancer, and in 44 cases adequate tumour samples were not available at the Department of Pathology, either due to that surgery had been performed at another hospital or that the amount of tumour material left for histopathological evaluation was insufficient. The remaining cohort of 346 women with invasive breast cancer forms the study population in Paper I.

In Paper II, the peri- and postmenopausal cohort was extracted and included 12 583 women among which 512 had been diagnosed with breast cancer previously and classified as prevalent breast cancer. By the end of follow-up (31 Dec 2001), 332 women had been diagnosed with incident breast cancer. In situ carcinoma accounted for 30 cases, in 36 cases there was no available or inadequate amount of tumour tissue, and the remaining 266 women diagnosed with invasive breast cancer form the cohort of cases in this paper. The cohort of 11 739 peri- and postmenopausal women without prevalent or incident breast cancer, represents the healthy control subjects.

In Paper III, the follow-up period was extended to 31 Dec 2004 and by then; a total of 622 women within the MDCS had been diagnosed with incident breast cancer. However, this study on anthropometric factors was restricted to analyses of peri- and postmenopausal women (n=12 583) with no prevalent breast cancer or HRT use at baseline which reveals a study population of 9685 women. In the study population, 305 women were diagnosed with incident breast cancer and in 248 cases invasive and sufficient tumour material was provided. The remaining breast cancer population consisted of 31 in situ carcinomas and 26 cases with unavailable tumour samples.

In Paper IV, end of follow-up was the same as in Paper III revealing 622 incident breast cancers. Within the MDCS, three different study populations were defined. For the

correlation analyses, the cohort of all invasive breast cancer cases with available tumour material, was applied and counted 511 cases. The remaining cases included 72 in situ carcinomas and 39 cases with unavailable tumour material. The analyses of HRT use included the peri- and postmenopausal cohort with no prevalent breast cancer (n=12 071) and included 464 incident breast cancer cases. Invasive breast cancer accounted for 382, another 45 were carcinoma in situ, and in 37 cases tumour material was not available. For analyses of anthropometric factors, the study cohort of Paper III was used.

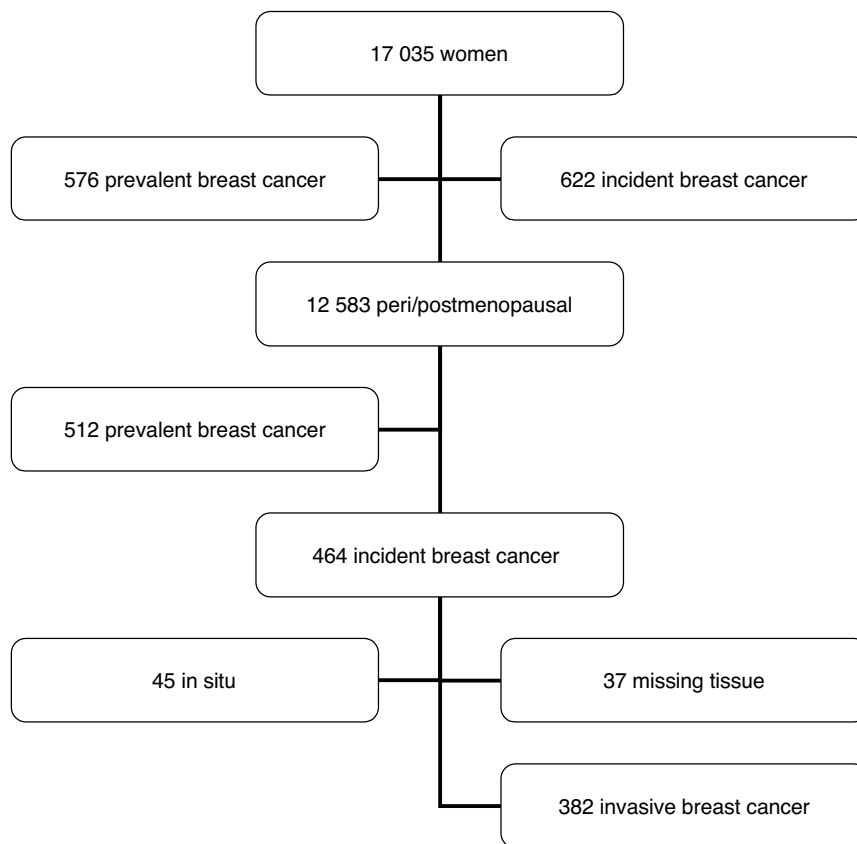


Figure 5 Flow-chart of the Malmö Diet and Cancer cohort

The Consecutive Breast Cancer Cohort (Paper V)

The Consecutive Breast Cancer Cohort (CBCC) is a consecutive series of unselected breast cancer patients diagnosed with invasive breast cancer at the Department of Pathology, Malmö University Hospital, between 1988 and 1992. Breast cancer samples were collected, and data on treatment and disease outcome were retrieved from medical records. The CBCC includes 512 women with invasive breast cancer and adequate data on treatment and disease outcome was retrieved in 389 cases. Data on the remaining proportion was either insufficiently described in medical records or lost due to follow-up outside the Malmö University Hospital.

Pathological re-evaluation

Invasive breast tumours from MDCS and CBCC were collected and re-evaluated by a senior breast pathologist according to tumour type (WHO) (1982), NHG (Elston et al. 1991), tumour size, and lymph node involvement. Furthermore, areas with invasive breast cancer were marked for the construction of the tissue microarray (TMA).

Tissue microarray

The tissue microarray (TMA) technology was developed in the late 1990s (Kononen et al. 1998) and is now a commonly used method for high-throughput analyses of protein expression in tumour samples. The construction of the TMAs can be done either manually or using an automated arrayer. In paper I-II and V the manual arrayer (MTA-1) was used, and in paper III-IV an automated arrayer was used as well (ATA-27), both devices were provided by Beecher Inc., Sun Prairie, Wisconsin, USA. Generally, two tissue cores were retrieved from each donating paraffin block and arranged in a recipient block. Each recipient block contained approximately 200 cores corresponding to 100 patients. In order to consume a minimum of tumour tissue, small tissue cores are desired, and cores of 0.6 mm were preferred.

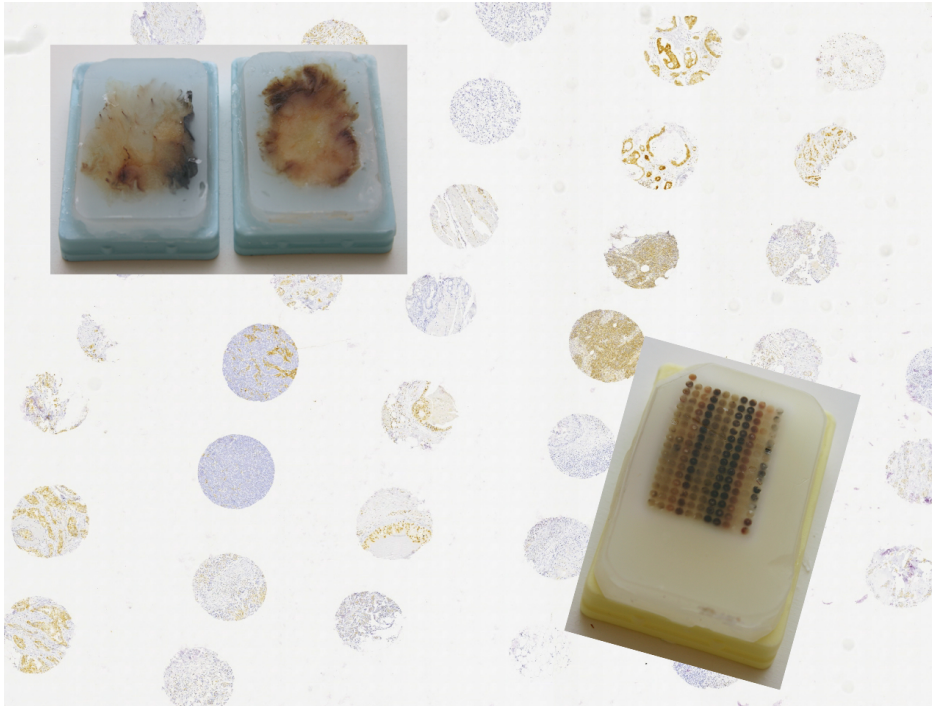


Figure 6 Schematic presentation of the tissue microarray technique.

Immunohistochemistry

For immunohistochemical (IHC) analyses, sections of four μm were cut from the recipient block, and dried, deparaffinised, rehydrated and microwave treated in a citrate buffer (pH6.0) for antigen retrieval. All automated IHC processing was performed using the DAKO Techmate 500 system (DAKO, Copenhagen, DK), except for ER α and PgR, where the Ventana Benchmark system was used (Ventana medical Systems Inc., AZ, USA). The scoring system of the immunostained slides is described in details in the relevant article. Verification of the two relatively new antibodies against ER β and HMG-CoAR was performed in cell lines by comparison of Western Blot analyses and IHC staining on TMAs from cell pellets of the corresponding cells. Further description of the antibody verification is provided in paper IV-V.

Table 2 Summary of the antibodies used.

Marker	Manufacturer	Clone	Dilution	Processing system	Paper
ER α	Ventana	6F11	Prediluted	Ventana Benchmark	I-V
ER β	Novocastra	EMR02	1/25	Dako Techmate 500	II-V
PgR	Ventana	16	Prediluted	Ventana Benchmark	II-V
Cyclin D1	Dako	DSC-6	1/100	Dako Techmate 500	I-III
Cyclin E	S:ta Cruz	HE12	1/100	Dako Techmate 500	I-II
P27	Dako	SX53G8	1/200	Dako Techmate 500	I-IV
Ki67	Dako	M7240	1/200	Dako Techmate 500	I-V
HER2	Zymed	Z4881	1/100	Dako Techmate 500	II-IV
HMG-CoAR	Upstate	07-457	1/250	Dako Techmate 500	IV

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) was used for detection of HER2 amplification in paper II. In brief, DNA in the formalin-fixed paraffin-embedded tissue specimens was denaturated and allowed to hybridise with two fluorescent signals. Evaluation of HER2 gene copy number (hybridization) was evaluated using a fluorescence microscope. In each tumour core at least 30 cells were evaluated, and the HER2 gene was considered amplified in tumour cells with a fluorescence signal number greater than 5.

Statistical methods

All statistical analyses were performed using SPSS version 11.0, 12.0, and 13.0 (SPSS Institute, Chicago; IL, USA). All statistical tests were two-sided and p-values less than 0.05 were considered significant.

In Paper I, continuous variables (age, dietary and anthropometric variables) were ln-transformed prior to analysis in order to normalize data distribution and reduce skewness. Prior to the transformation, a small number (0.01) was added to continuous variables in order to handle zero intakes. Age-adjusted analysis of variance (ANOVA) was applied for comparison of means. Analyses of dietary intake were further adjusted for diet assistant, seasonal period of dietary data collection, method version, and recent changes in dietary habits. All analyses of dietary intake were repeated in energy-adjusted models. Stratification for menopausal status was performed for analyses of anthropometric factors, and separate analyses for pre- and post menopausal women were repeated.

In Paper II-IV, breast cancer incidence in different exposure groups was calculated per 100 000 person-years. Cox's proportional hazards analysis was used for estimation of relative risks in different exposure groups. The multivariate analyses included potential confounders which are described in details in each paper. Using an unconditional logistic regression model in the case-to-case-analysis, the heterogeneity between relative risks

was examined in paper II and IV. In Paper IV, correlation analyses were performed using the Chi-square test.

In paper V, the Chi-square test was used for analyses of correlation between categorical variables, Spearman's test was applied for linear variables, and the Mann Whitney's U-test was applied for comparison of medians. The end point in survival analyses was disease-free survival (DFS), equivalent with recurrence-free survival. A univariate Cox's proportional hazard analysis was used for calculation of disease-free survival in different sub-groups. Survival curves were generated using the Kaplan-Meier method and statistical significance determined by the log-rank test. Survival analyses were repeated for breast cancer specific survival (BCSS) and overall survival (OS).

METHODOLOGICAL CONSIDERATIONS

Tumour classification and tissue microarray

Analyses of different breast cancer subgroups may be influenced by misclassification of breast tumours, and, in order to minimize the risk of misclassification bias, all tumours were re-evaluated on whole section slides by one pathologist, thus the problem of inter-observer variability was consequently reduced. The pathologist was blinded to all data on life style parameters and tumour assessments made as objective as possible and information on pathology data is considered valid.

Advances in proteomics have increased the need to evaluate large numbers of molecular targets for their diagnostic, predictive or prognostic value in breast cancer. The conventional molecular pathology techniques are often time-consuming, and require larger amounts of tissue, thereby limiting the number of tissues that can be evaluated. Thus, the introduction of tissue microarray (TMA) was an appealing alternative method consuming both less tissue and less time. Today, TMA is a well-established and frequently used method for high-throughput analyses. However, some aspects are still debated, i.e. the size of the core and the number of cores.

The preferred sizes of the core biopsies differ among studies and vary from 0.6 mm and up to 2.0 mm. In order to consume a minimum of tissue and still maintain a reasonable amount of tissue for evaluation, we preferred a size of 0.6 mm. Compared to analyses of whole sections, a TMA core of 0.6 mm has been shown to provide equal and adequate results from immunohistochemical (IHC) analyses on tumour markers (Abd El-Rehim et al. 2005, Camp et al. 2000, Zhang et al. 2003). One could question whether archival tissues are suitable for renewed IHC evaluation many years after surgery. However, in a TMA study, Camp *et al* have demonstrated that the antigenicity of the proteins is preserved (Camp et al. 2000).

Few studies have proposed a single core to be sufficient (Zhang et al. 2003), whereas most others favour two cores, bearing in mind that a minor fraction of cores is commonly lost during IHC processing (Abd El-Rehim et al. 2005, Camp et al. 2000). In this study, we used two cores per patient and in cases with only one interpretable core left; IHC scoring was based on the remaining core.

In our studies, marker expression did not differ between the majority of duplicate cores and in the rare cases with a heterogeneous expression; evaluation was based on the core with highest expression. In paper I-IV all tumour cores were individually scored twice by the same investigator and in case of discrepancy a third re-evaluation was performed by the same investigator and followed by a final decision. TMA scoring was performed by the same investigator in all studies from the Malmö Diet and Cancer cohort and thereby the inter-observer variation was reduced. In paper V, the evaluation of ER β expression was performed by one investigator in similarity with paper I-IV, whereas another investigator evaluated ER α , PgR, Ki67, and HER2, which might cause inter-observer variation. However, as each tumour marker was evaluated by the same investigator, we considered the problem with inter-observer variation to be of less importance.

Antibody validation

The number of available antibodies *per* antigen is plentiful and the choice of a favoured antibody should be a critical decision and preferably followed by internal validation. Especially, novel antibodies need further validation (Pozner-Moulis et al. 2007).

In our studies, we have used two less established, yet commercial, antibodies against HMG-CoAR and ER β , which are further discussed below.

For validation of the HMG-CoAR antibody, four different human tissue lysates (normal breast tissue, breast tumour tissue, normal prostate tissue, prostate tumour tissue) and two cell lysates (skin, epidermoid carcinoma and liver, hepatocellular carcinoma) were analyzed. The Western Blot (WB) analysis showed a single band corresponding to a molecular weight of 90 kDa most likely representing HMG-CoAR. Neither the intra-tumoural, nor the sub-cellular distribution of HMG-CoAR in breast cancer has been described previously, thus staining on whole slides was performed for further characterisation. We found that HMG-CoAR expression was homogenously distributed in breast tumours. Thus, evaluation of HMG-CoAR on TMAs was less likely to show various expressions in between cores belonging to the same patient.

Prior to IHC analyses on the TMAs, the ER β antibody was validated on WB and by IHC using five different breast cancer cell lines. The WB analyses revealed a single band at the molecular weight corresponding to full length ER β , and, furthermore, we used an antibody not able to detect ER β cx. There was a good correspondence between results from WB analyses and IHC; the cell line displaying the strongest band on WB also revealed the strongest staining intensity. Further validation with an ER β negative cell line was not feasible despite several attempts to retrieve such a cell line. We did, however, have a rather high fraction of ER β negative tumours compared to other studies (Fuqua et al. 2003, Gruvberger-Saal et al. 2007) indicating specific antibody recognition. Taken together, we regard the ER β antibody used as valid and specific for ER β (Fuqua et al. 1999, Pavao et al. 2001).

Internal validity

Detection bias

Mammography attendance is necessarily associated with the detection of a breast cancer. The question to be asked is how, or if, different exposures are related to the attendance rates and whether these exposures affect radiological imaging, which could bias the results.

Several reports indicate that women with poor socio-economic circumstances are less prone to attend mammography screening programmes (Lagerlund et al. 2002, Zackrisson et al. 2004). Further, the mammography attendance rate may be associated with BMI as obese women (BMI 35-40 kg/m²) seem to be less likely to attend mammography compared to women with a normal BMI (Wee et al. 2004). Mammography specificity may be influenced by obesity, and a 20% increased risk of false-positive results among obese women has been reported (Elmore et al. 2004). Moreover, tumour palpability is most likely influenced by body constitution with obese women tending to have non-palpable tumours (Chagpar et al. 2007). To our knowledge, no reports on a potential association between dietary habits and mammography attendance have been found. HRT-users do attend mammography screening programmes more frequently compared to non-users (Banks et al. 2002, Kavanagh et al. 2000) leading to differences in detecting early breast cancers depending on HRT-use. Meanwhile, HRT-use influence mammography imaging with denser breast tissue among HRT-users and consequently, an increased rate of false negative as well as false positive mammography results (Kavanagh et al. 2000, Patella et al. 2005).

Taken together, mammography attendance and radiological imaging may be related to several factors that are known breast cancer risk factors. In paper II, we addressed this problem and hypothesised that HRT-users would attend mammography more frequent and probably be diagnosed with smaller tumours. However, we found that tumours among HRT-users were larger and the risk of detection bias probably limited. Similar findings were obtained in paper III with larger tumours in obese women and obesity being a risk factor ought not to have caused a detection bias.

Misclassification

Over- and underreporting is central in nutritional epidemiology based on self-reported dietary data and might cause misclassification. Different motives for misreporting have been recognized; social desirability (reporting dietary habits reflecting social norms), attention (influenced reporting due to observation of the subject), and unacceptability (misreporting of sensitive information). Subsequently, it is essential whether misreporting occurs more frequently in certain groups resulting in systematic errors. Most likely, personality traits influence misreporting (Novotny et al. 2003, Tooze et al. 2004), however, this would probably not cause differential misclassification related to exposure- or disease status. Under-reporting of energy intake is common, and probably more frequent among subjects with a high BMI (Novotny et al. 2003, Prentice 1996). In paper I, analyses on dietary variables were not adjusted for BMI; however, we adjusted for the variable "recent food habit changes". This variable was recently analysed in a study from the MDCS, demonstrating that "recent food habit changers" reported lower energy intake compared with "non-changers" (Sonestedt et al. 2007), which parallels a low energy intake reported by obese subjects. Another source of misclassification of reported dietary habits could be instrumental weaknesses in the applied method. However, the validity of dietary assessment in MDCS has been examined in a previous study, which indicated a high validity (Callmer et al. 1993).

Misclassification of self-reported data on HRT-use is less likely as misreporting is rare (Banks et al. 2001). Further, in the MDCS, HRT-use was assessed in two ways, using both a diary and a questionnaire, thereby minimising the risk of misclassification (Merlo et al. 2000). In this study, we had access to data on current and non-users and consequently, recall bias on HRT was considered less important. A minor fraction of former users might be concealed within the group of non-users, and the database did not reveal possibilities of extracting such information. Yet, a classification of former users as non-users would lead to an underestimation of the HRT associated risks.

Confounding

A true confounder is associated with both exposure- and outcome variables without being caused by either of them. In paper I analyses were adjusted for age and dietary related factors (dietary interviewer, season, method version, recent changes of dietary habits, and energy); however, other potential confounders such as established factors for breast cancer were not included, which could have influenced the results. The choice of not including breast cancer risk factors in paper I was decided on as it was a descriptive study in an unexplored field combining life style data with tumour biological markers, whereas factors related to the assessment of diet were included in order to obtain the best possible descriptive information on diet in different sub-groups. In paper II-IV, potential confounders associated with breast cancer risk were included in multivariate analyses, which merely changed the results slightly compared to crude analyses. A family history of breast cancer was not included as co-variate, which might be a limitation in our study. Data on family history of breast cancer were not available in the database and we were consequently unable to adjust for it. However, we consider it reasonable to perform analyses without adjusting for family history of breast cancer as non-sporadic breast

cancer cases constitute a minor proportion of all breast cancer (Loman et al. 2003) and is less likely to influence the results. Another co-variate which might have been appropriate to include in this study, is physical activity. However, in the MDCS, the validity of physical activity has been questioned (Mattisson et al. 2005), hence we decided not to include physical activity. In paper V, all survival analyses were adjusted for age. One may question the lack of further co-variables such as other tumour characteristics. However, due to the strong interaction among tumour characteristics which might have lead to over-adjustment; we decided to restrict adjustment to age. Furthermore, the main scope of paper V was to study the expression of ER β and we found an even distribution of ER β negative and positive tumours in different sub-groups of important prognostic parameters, such as size, grade, and lymph node status.

Statistical considerations

In some of the papers a relatively large number of comparisons have been performed, which may be questioned. A type 1 error, or α -error, occurs when the null hypothesis is rejected when it is actually true, meaning that a difference is observed although there is none. Type 1 errors are often referred to as false positive. A 95% confidence interval implies a 5% risk of observing false positive results due to coincidence of findings. An important concern in our studies is mass significance and some might recommend modifying results using Bonferroni's correction. However, most modern statisticians regard Bonferroni as rather conservative (Perneger 1998) and increasing the risk of type 2 errors. Type 2 error, or β -error, implies failing to reject the null hypothesis when it is not true, leading to false negative results. Hence, we decided not to make Bonferroni's correction, although interpretation should be done with caution. Another statistical issue to be addressed is the rather small sub-groups emerging in the studies and subsequently limited statistical power. Risk estimates in small groups often result in wide confidence intervals and consequently poor precision. Consequently, such risk estimates will also need careful interpretation.

External validity

The background population in Malmö might be a selected cohort compared to the average Swedish population as the breast cancer incidence in Skåne (146.4/100 000) exceeds the general breast cancer incidence in Sweden (141.3/100 000) (The National Board of Health and Welfare, 2005). Furthermore, studies from the MDCS have shown that participants probably exhibit a selected part of the background population, displaying a higher breast cancer incidence compared to the source population. Additionally, the study found that a higher proportion of the participants reported "good health" compared to the mailed health survey. (Manjer, Carlsson et al. 2001). We do, however, regard it as possible to make internal comparisons within the study. Moreover, comparing with the existing literature, our results on associations between life style factors and breast cancer risk are generally in agreement with others, and we believe it is reasonable to apply the relative risks obtained in our studies to the general population.

RESULTS AND DISCUSSION

Paper I

In this study, we explored various breast cancer subgroups in relation to body constitution and dietary factors in a subset of 346 invasive breast cancers from the MDCS.

Pathological re-evaluation according to tumour type and grade revealed an almost similar distribution of different histological types, but slightly fewer grade III tumours compared to an unselected set of patients (CBCC, paper V). Using the TMA method, we assessed the expression of ER α , cyclin D1, cyclin E, p27, and Ki67, and internal associations between all evaluated tumour characteristics, demonstrated expected associations thus making it reasonable to perform further internal comparisons to potential risk factors, i.e. dietary- and anthropometric factors.

Five different anthropometric measures were analysed for their association with the above mentioned tumour characteristics by comparison of mean values. The results indicated an association between tall stature and grade I tumours. BMI and hip circumference were positively associated with tumour grade, although displaying a discontinuous trend over mean values with the lowest mean among women with grade I tumours and the highest mean for women with grade II tumours. However, given the heterogeneity within the grade II carcinomas, no firm conclusion can be drawn. Few comparable studies have investigated the relationship between obesity and grade in breast cancer, and a recent study could not detect any association (Chagpar et al. 2007). Thus, analysis including further tumour characteristics is motivated. However, this study, using a more descriptive approach, was not able to detect any further associations.

Dietary habits were characterised by total energy intake, macronutrient intake (fat, carbohydrate, and protein), alcohol, and three different groups of fatty acids (saturated fatty acid (SFA), mono-unsaturated fatty acid (MUFA), and poly-unsaturated fatty acid (PUFA)).

Analyses indicated an association between reported low energy intake and tumours of nuclear grade III, high proliferation (Ki67), and overexpression of cyclin D1. Animal studies have shown a reduced risk of mammary tumours among energy-restricted rodents (Thompson et al. 1999) and, additionally, reduced tumour cell proliferation via G1 cell cycle arrest (Zhu et al. 1999). The latter finding is not concordant with our results; however, it is unknown whether the beneficial effect of energy restriction is applicable for humans. Body constitution can be an indirect measure of energy restriction, and some studies propose an increased breast cancer risk in adult life following low childhood BMI (Hilakivi-Clarke et al. 2001, Magnusson et al. 1998), as opposed to other studies reporting a decreased risk of breast cancer and cancer in general following severe anorexia nervosa (Mellekjaer et al. 2001, Michels et al. 2004). These studies did not evaluate the risk of different breast cancer sub-groups and breast cancer risk assessments were not carried out in this pilot study, hence further comparisons are not possible.

Total fat intake was inversely associated with proliferation and cyclin D1 expression with the strongest associations seen in analyses using dichotomized variables of Ki67 and cyclin D1, and weakened results following energy-adjustment. In general, human studies on fat-mediated effects on tumour characteristics have been limited to studies on the impact on fatty acids and not total fat intake. In the literature, many attempts to link a particular dietary factor to breast cancer risk has been found, although the results are contradictory (Willett 2001). We could not demonstrate significant associations between carbohydrate, protein, or alcohol intakes and tumour characteristics.

Low intakes of SFAs, MUFAs, and PUFAs, were associated with tumours with high proliferation, and results were most evident in the dichotomized model, and corresponded with the results on total fat intake. Following energy-adjustment, PUFA and proliferation sustained a significant association, contrary to SFA and MUFA. Low SFA- and MUFA intake was further associated with cyclin D1 over-expression, whereas only low SFA-intake was associated with a high nuclear grade. Breast cancer protective effects have been demonstrated for diets high in n-3 PUFAs, and animal studies report reduced tumour growth and metastasis subsequent to n-3 PUFA high diets (Hilakivi-Clarke et al. 2004). Other studies report an increased breast cancer incidence associated with higher intake of n-6 PUFAs (Wirfalt et al. 2002). Different effects on expression of cell cycle proteins has been shown, with increased cyclin D1 mRNA levels associated with n-6 PUFAs and, oppositely, a n-3 PUFA-mediated reduction in cyclin D1 levels (Hilakivi-Clarke et al. 2004). However, due to excessively many variables included in this study, further sub-analyses on n-6 and n-3 PUFAs were not carried out, but would be of great interest in future and larger studies.

In conclusion, this study indicates that a lower energy intake, especially fat intake, is associated with breast tumours characterised by more malignant features. Further studies are needed to achieve firm conclusions in this field.

Paper II

We analysed the risk of different breast cancer subgroups according to HRT-use in the cohort of 12 583 peri- or postmenopausal women in the MDCS with 332 incident breast cancers until end of follow-up, 31 Dec 2001.

Baseline characteristics differed among groups defined by HRT-use. CHRT-users were younger, higher educated, more often previous users of oral contraceptives (OC), had a lower BMI, had a more frequent alcohol intake, and were more often current smokers as compared to ERT-users and non-users. The characteristics of CHRT-users in the MDCS correspond well with other recent European reports (Bakken et al. 2001, Nagel et al. 2007, van Duijnhoven et al. 2006). HRT-use is associated with several factors, i.e. societal circumstances, the attitude of the physician, potential contradictions to HRT, and the extent of menopausal symptoms (Heitmann et al. 2005). Deficiency symptoms are caused by low available serum-levels of estrogen, which may be due to a lower BMI or current smoking (Nagel et al. 2005). Alcohol consumption, however, lead to higher levels of estrogen (Onland-Moret et al. 2005), and the more frequent alcohol intake among CHRT-users might reflect societal factors rather than hormonal circumstances. The observed association between OC-use and HRT-use can hardly be explained by altered estrogen levels during menopausal years, and might mirror a general acceptance of exogenous hormones. Further gynaecological history did not differ remarkably among HRT-defined groups, apart from an expected higher fraction of peri-menopausal women among CHRT-users.

Use of HRT regimens lacking progestins, estrogen replacement therapy (ERT), did not influence breast cancer risk. Few women used single-progestin regimens (PRT), among whom only one was diagnosed with breast cancer and further risk analyses for PRT were inappropriate. Current use of estrogen and progestin combined HRT (CHRT), increased the overall risk of breast cancer. The observed differentiated risk estimates depending on the favoured HRT regimen is concordant with most other studies (Colditz 2005). The biological role of progestins in breast cancer development is still unclear; some have proposed a synergistic effect of estrogen and progestin in tumour promoting processes (Bigsby 2002), and novel studies point out non-progesterone-like effects of the synthetic progestins used in HRT (Campagnoli et al. 2005) and might be avoided by using natural progesterone instead (Fournier et al. 2005). In the present study, data on continuous or sequential regimens of CHRT were not included and further sub-grouping was avoided. In similarity, was information on administration (transdermal or oral) not taken into account, whereas vaginal administrated hormonal replacement was considered less relevant in this study and therefore not included. The risk of breast cancer is most likely not affected by the preferred CHRT regimen or administration route (Collins et al. 2005).

All incident invasive breast tumours were re-evaluated concerning histological type, grade, tumour size, and lymph node metastasis. Following CHRT use, we found a high risk of lobular cancer (ILC) in the multivariate analysis, with a relative risk estimate exceeding the general risk of breast cancer among CHRT-users. The association between CHRT and ILC has been reported previously (Biglia et al. 2007), indicating that the raising incidence of ILC might be caused by CHRT. Although ILC is associated with presumably favourable prognostic characteristics, i.e. low grade, low proliferation, and HER2 negativity, women with ILC do not display improved clinical outcome compared to other histological types (Arpino et al. 2004). Delayed diagnosis due to the multifocal growth pattern of ILC failing to form distinctive masses, may influence survival. Furthermore, histological sub-typing is probably of limited value in investigation of the effects of exogenous hormones, and with modern tumour analysis techniques further insight can be achieved.

An increased risk of grade I tumours was seen among CHRT-users, and further confirmed in heterogeneity analyses. Similar results was obtained in another Malmö study (Manjer,

Malina et al. 2001). We further analysed each grade-component separately and found that nuclear atypia was associated with CHRT-use, but tubular formation was not. Tumours with a low mitotic index (NHG based) and low proliferative activity estimated by Ki67 nuclear fraction, were more frequent among current users of CHRT compared to ERT- or non-users, tallying other studies (Biglia et al. 2005, Manjer, Malina et al. 2001, Sacchini et al. 2002).

Risk-analyses according to hormone receptor expression revealed almost identical results in sub-group analysis indicating that CHRT did not influence the expression of neither ER α , nor ER β and PgR. The ER α negative group was rather small with a correspondingly wide confidence interval and solid conclusions are difficult to draw. Our results concerning ER α and PgR expression do, however, correspond with results from large studies as The Women's Health Initiative Trial (Chlebowski et al. 2003). Regrettably, no other studies have addressed ER β expression following HRT use.

The evaluation of HER2 status was performed by IHC followed by FISH analysis. Unfortunately, many cases were lost in the preparation for FISH-analysis, hence making the corresponding results rather weak. We decided to dichotomize IHC obtained HER2 results to achieve an adequate number of cases in each sub-group, and found a higher risk of HER2 (2+,3+) tumours compared to HER2 (0,1+) among CHRT-users. However, we are well aware of the risk of type 1 error and the lack of related studies to verify our preliminary findings.

Finally, we analysed the potential impact of CHRT on tumour expression of two cell cycle regulating proteins, the oncogene cyclin D1 and the suppressor gene p27. We found that CHRT-use was associated with an increased risk of tumours with no or low cyclin D1 expression, whereas over-expression of cyclin D1 was not significantly associated with CHRT in the multivariate analysis. However, there is a risk of type 2 error in this group due to the rather small sample size. The biological explanation for a lower content of the protein cyclin D1 among CHRT-users is uncertain and has, until now, not been addressed in the literature. Although cyclin D1 is a downstream target of the estrogen mediated activation of the estrogen receptor, cyclin D1 has ER independent functions as well, and the role of both ERT and CHRT in cyclin D1 defined breast cancer will need further studies with improved statistical power. Cases were more equally distributed in p27 defined sub-groups, and we observed a higher risk of tumours with a normally high expression of p27, compared to more malignant phenotypes with no or low expression of p27. A lower p27 expression might be an estrogen-mediated effect of CHRT, whereas progesterin is likely to play the opposite role (Gompel et al. 2004).

In conclusion, ERT was not a risk factor for breast cancer in general and neither for any of the examined sub-groups. Current CHRT-use increases the general risk of breast cancer, however, mostly affecting the risk of less malignant phenotypes, which might indicate that CHRT-induced breast tumours may originate from dormant, low-malignant tumour cells rather than inducing breast cancer *de novo*.

Paper III

Body constitution, height, weight, BMI, waist- and hip circumference, and body-fat percentage, was related to the risk of postmenopausal breast cancer defined by various pathological and tumour-biological parameters.

Among 9685 peri- and postmenopausal women, with no prevalent breast cancer, or HRT-use at baseline, 305 women were diagnosed with incident breast cancer until end of follow-up, 31 Dec, 2004. The association between anthropometrics and breast cancer differs according to menopausal status making stratification necessary, hence, the present study focused on peri- and postmenopausal breast cancer. In anthropometric studies, exclusion of HRT-users is now well-established (Feigelson et al. 2004, Huang et al. 1999, Morimoto et al. 2002). HRT is a strong risk factor for breast cancer by which associations between body constitution and breast cancer are blurred. Furthermore, BMI and HRT are often reported negatively associated, probably due to low endogenous hormone levels in lean women resulting in more frequent HRT-use (Nagel et al. 2007). Further, exclusion of all prevalent breast cancer cases was decided on as it might have influenced body constitution. Some might argue that exclusion of all subjects with prevalent cancer would be appropriate in this study, but this will also introduce selection of an improperly healthy population. We did, however, repeat analyses with exclusion of all prevalent cancers and found similar results.

We compared baseline characteristics among women with incident breast cancer and the rest of the study cohort and modest differences were observed. Breast cancer cases were slightly older, had a higher age at menarche, at first child birth, and at menopause, and finally, had more frequent alcohol consumption habits and were more often ex-smokers. These observations are in line with similar comparisons performed in paper II, and the minor differences between the two studies, i.e. age at baseline and educational level is probably explained by the exclusion of HRT-users in paper III.

There are numerous anthropometric measurements, all with different advantages depending on the study aim, resulting in an individual choice of measurement in each study. To date, it is still unclear which anthropometric measure represents the best predictor of breast cancer risk. We used six well-established measurements in this study where we focused on breast cancer risk defined by rather novel sub-groups. Examples of other anthropometric measures in the literature are waist-hip-ratio, skin fold measurements, breast size, and weight change in particular time periods (Friedenreich 2001). Especially, weight change has received much attention in epidemiological studies with many attempts to define the most vulnerable time of weight gain according to breast cancer risk (Ballard-Barbash 1994, Friedenreich 2001) and would probably be of interest to evaluate in further similar studies.

All anthropometric measures were categorized into quartiles. The overall risk of invasive breast cancer was positively associated with height, corresponding with the findings in paper I, as well as weight, BMI, waist – and hip circumference, and body fat percentage. The highest point estimate was observed for waist circumference measuring abdominal obesity, which is reasonable bearing in mind that many hormonal and metabolic changes associated with abdominal adiposity are associated with increased breast cancer risk as well (Ballard-Barbash 1994).

Anthropometric factors are often regarded as modifiable risk factors, with the exception of height, which displays different associations with breast cancer risk in sub-group analysis. Height is mainly determined by genetic factors and, in particular populations, further influenced by nutritional conditions in childhood (van den Brandt et al. 2000). Hence, height may be described on its own opposite to the remaining five anthropometric measures with rather identical associations to breast cancer risks.

In analyses of histological type, we found a higher risk of invasive ductal carcinoma (IDC) in the fourth quartile for all anthropometric measures, although displaying a risk estimate rather similar to the general risk of invasive breast cancer. A high risk of ILC was only seen in the top quartiles of waist- and hip circumference and might mirror increased levels of bio-available estrogen in similarity with the increased risk for ILC among CHRT-users. Anthropometrics and risk of certain histological subtypes of breast cancer has been addressed by Li *et al*, although not demonstrating a significant risk of ILC in obese women (Li *et al*. 2006, Li *et al*. 2003). There are several methodological differences between the present study and the studies performed by Li *et al* and further studies are needed to clarify true associations.

Women in the top quartiles of anthropometric measures had an increased risk of grade II tumours. In comparison with the results obtained in paper I, both studies indicate an association between obesity and grade II breast cancer. Further conclusions are difficult to draw as grade II tumours are a heterogeneous group with a variety of biological disparities, and comparable studies have not been found. Proliferation was estimated by expression of Ki67, and risk analysis revealed that women in the fourth quartile had a higher risk of tumours with low proliferation.

ER α , ER β , and PgR expression were dichotomized according to the clinically used cut-off at 10%. Results demonstrated an unambiguous pattern with a high risk of ER α +, ER β -, and PgR+ breast tumours among obese women, true for all anthropometric factors except height. We hypothesised that associations might be strengthened in repeated analysis within the ER α positive group. The results for modifiable anthropometric factors were similar, but a higher risk for ER β positive tumours among tall women emerged. Our results on ER α - and PgR positive tumours in obese women are in accordance with previous studies (Rosenberg *et al*. 2006, Suzuki *et al*. 2006). On the contrary, ER β status has only been included in one previous study on anthropometrics and breast cancer (Sherman *et al*. 2007). In the study performed by Sherman *et al*, ER β status was not associated to the analysed variable, BMI, but comparison with this study is difficult due to several differences in methodological conditions.

Obesity was further associated with a higher risk of tumours not over-expressing cyclin D1 and tumours with a maintained high level of p27. However, the results on cell cycle regulating proteins were complicated to interpret with less consistent trends. The risks of cyclin D1 low tumours was, to some extent, differentially associated with anthropometrics, however displaying a significant "p for trend" in all measures, except for height. In analyses of p27 defined breast cancer, p27 low tumours were associated with waist and p27 high tumours with hip circumference. Distribution of fat might be relevant for tumour expression of p27, and women with abdominal obesity should then be at a higher risk of more malignant p27 low tumours. However, abdominal obesity did not differ from other obesity measurements in risk analyses using other tumour characteristics and further studies are needed for clarification of the impact on p27 defined breast cancer risk by various obesity markers. Taken together, obesity may be associated with the expression of cell cycle proteins, but further investigations are needed.

In conclusion, obesity, estimated in various ways, is associated with an increased risk of breast cancer of a less aggressive phenotype displaying low ER β expression.

Paper IV

The expression of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR) in invasive breast cancer was mapped and correlated to other tumour characteristics and estrogen-related life style factors, such as HRT and obesity.

HMG-CoAR was expressed in the cytoplasm displaying diversity in fraction and intensity among tumours. Membranous staining was observed in 15 % of the evaluated tumours, whereas nucleus staining was absent in all cases in accordance with the reported sub-cellular localisation of HMG-CoAR in endoplasmatic reticulum and peroxisomes (Aboushadi et al. 2000). As opposed to the normal glandular epithelium, HMG-CoAR expression was stronger in apocrine epithelium as reported by others (Celis et al. 2006).

In correlation analyses, expression of HMG-CoAR (fraction and intensity) was negatively associated with tumour size, grade, and proliferation. Up-regulation of HMG-CoAR activity is a well-established event in cancer cells, probably reflecting an increased demand for isoprenoids (Mo et al. 2004). The observed absent or low HMG-CoAR expression in more malignant tumours might be explained by an inability to preserve the mevalonate pathway in poorly differentiated cancer cells combined with a resistant sterol feed-back mechanism (Mo et al. 2004).

HMG-CoAR (fraction and intensity) correlated positively to both estrogen receptors, but not to the progesterone receptor. The association between HMG-CoAR and ER α support the notion of high HMG-CoAR expression in less malignant breast tumours. The clinical relevance of ER β expression in breast cancer is not yet evident and the impact of the association to HMG-CoAR is difficult to elucidate from this study. A strong positive association between HMG-CoAR and the tumour suppressor gene p27 was found, further indicating a positive prognostic value of HMG-CoAR in breast cancer. We could not demonstrate a significant association with cyclin D1 expression, however, tumours with strong HMG-CoAR intensity had a non-significantly lower fraction of cyclin D1 negative tumours compared to tumours with absent or low HMG-CoAR expression.

Membranous and cytoplasmic HMG-CoAR expression were differentially associated to established tumour characteristics, especially HER2 status. The HMG-CoAR and HER2 staining pattern are comparable, both presenting with membranous patterns and might contribute to the observed association. However, the statistical power is limited given the relatively low number of tumours with HMG-CoAR membranous staining, and further biological clarification is beyond this first, descriptive study on HMG-CoAR expression.

Tumour specific expression of HMG-CoAR in breast cancer has not been reported earlier, hence, knowledge concerning valid antibodies is sparse. For IHC detection of HMG-CoAR, we used a commercial antibody, but given the few studies in this area, we performed an internal antibody validation. The Western blot analysis revealed a single band corresponding to the molecular weight of HMG-CoAR, and the antibody was verified in both negative and positive cell lines. HMG-CoAR has been described in two variants with slightly different molecular weights, probably corresponding to two different sub-cellular localizations (Aboushadi et al. 2000), and it might be questioned whether this antibody recognized both variants. We cannot rule out the possibility that only one HMG-CoAR variant was recognized by the antibody used and additional studies are needed to determine the sub-cellular localisation of the HMG-CoAR recognised by the antibody used in this study.

Several studies have demonstrated HMG-CoAR regulating properties exerted by estrogens (Di Croce et al. 1996, Messa et al. 2005, Ness et al. 2000), which encouraged to further investigation of the association between HMG-CoAR expression and estrogen-related factors as HRT-use and obesity (Yasui et al. 2005).

In comparison with paper II, this study included another 182 breast cancer cases (follow-up extended to year 2004), and showed similar results for CHRT-use as a strong predictor for incident breast cancer. Further analyses revealed a significant risk for all breast cancer sub-groups defined by HMG-CoAR expression among CHRT-users. The risk-estimates were higher for tumours with high expression of HMG-CoAR, although the association did not reach statistical significance in the heterogeneity analysis. However, in paper II, CHRT-use was associated with favourable tumour characteristics and supports the present findings given the previously described associations between HMG-CoAR and other tumour parameters.

In general, obese women have higher levels of endogenous estrogen (Mahabir et al. 2006, Yasui et al. 2005), and we hypothesised that obesity could influence the risk of HMG-CoAR defined breast cancer. We found an increased risk of HMG-CoAR high (fraction and intensity) tumours in obese women belonging to the top quartile of waist and BMI corresponding with results achieved from analyses on HRT. Studies on life style factors and HMG-CoAR expression in breast cancer are sparse. However, one study has investigated the effects of dietary factors on HMG-CoAR activity and found that the weak estrogen genistein (soy) mediated inhibition of breast cancer cells, and the authors concluded that it was partially caused by HMG-CoAR inhibition (Duncan et al. 2005).

In conclusion, HMG-CoAR is differentially expressed in invasive breast cancer and is associated with favourable tumour characteristics. This study further indicated that estrogen-related life style factors, such as HRT-use and obesity, might influence HMG-CoAR expression in breast cancer.

Paper V

A consecutive breast cancer cohort (CBCC) of 512 invasive breast cancers diagnosed at the Malmö University Hospital between 1988 and 1992 forms the basis of this study. We analysed the association between the recently identified estrogen receptor, ER β and established clinicopathological parameters. Furthermore, the response to endocrine therapy in relation to ER β status was analysed, the endpoint being disease-free survival (DFS) defined as time to first recurrence.

The expression of ER β was strongly associated with ER α - and PgR expression. Co-expression of the estrogen receptors has commonly been reported, whereas ER β -PgR co-expression is ambiguously reported (Fuqua et al. 2003). Despite interrelation of all three hormone receptors, they were differently associated with other tumour parameters. Contrary to ER β , the expression of ER α revealed expected associations with age at diagnosis, tumour size, grade, HER2 status, and proliferation (Ki67). Similar results have been reported others (Fuqua et al. 2003) whereas others report associations between ER β and less aggressive tumour characteristics (Speirs et al. 2004). We hypothesised that combinations of ER α -ER β sub-groups might clarify associations to other pathology parameters however; no further information was achieved by doing so.

The prognostic significance of ER β was analysed using DFS as end-point, yet the results revealed no significant association between ER β expression and DFS, irrespectively of co-expression with ER α . Repeated analyses for overall survival (OS) provided similar results. The strongest determinants for DFS in this study was tumour size, grade, and lymph node status, however, subgroups of these parameters were equally distributed among ER β subgroups. Currently, the prognostic value of ER β status is still debated (Speirs et al. 2004) and further studies are needed to clarify its role as a prognostic marker.

ER α is generally considered a favourable prognostic marker in breast cancer during the first years after diagnosis, although only showing a modest effect on recurrence rate over years (Osborne 1998). We analysed the prognostic value of ER α in the CBCC with a median follow-up time of 106 months and neither DFS, nor OS, were associated with ER α status. In contrast to the estrogen receptors, PgR expression was associated with significantly improved survival, an observation supported by other studies (Mohsin et al. 2004).

The role of ER β as a predictor of response to endocrine therapy has received remarkable attention since its identification in 1996. In a recent review, Murphy et al, report that co-expression with ER α (ER α +ER β +) has almost consistently been associated with an increased likelihood of response to endocrine therapy (Murphy et al. 2006). Additionally, ER α negative patients might benefit from endocrine therapy if tumours express ER β (Gruvberger-Saal et al. 2007). Therefore, we wanted to investigate the predictive significance of ER β expression in endocrine treated patients. First, DFS was estimated for endocrine treated patients with ER α +ER β +, ER α +ER β -, ER α -ER β +, or ER α -ER β - tumours and revealed an adverse outcome for patients with ER β negative tumours, although this was not statistically significant. In patients who had not received endocrine therapy, DFS did not differ according to ER β expression. Furthermore, we investigated outcome according to endocrine therapy in separate analyses for patients with ER β + and ER β - tumours. Endocrine therapy did not influence DFS among patients with ER β + tumours whereas ER β - patients did worse following endocrine therapy compared to ER β + patients.

In the interpretation of the results it must be remembered that this study was not a randomised trial. Moreover, when we investigated potential differences among endocrine treated patients and others, we observed associations between endocrine therapy and low age at diagnosis, large tumour size, high tumour grade, and positive node status. However, we believe that this study indicates a role of ER β in the response to endocrine

therapy and our results are generally in line with those of others (Hopp, Weiss, Parra et al. 2004, Iwase et al. 2003), supporting our findings.

In conclusion, ER β is strongly related to other hormone receptors, however not displaying a significant prognostic role in breast cancer. Co-expression of both estrogen receptors may predict an improved response to endocrine therapy.

CONCLUSIONS

In this thesis we found that molecular characteristics of breast cancers vary by different life style factors. By gaining further insight into the relationship between etiological exposures and aberrant molecular pathways in breast cancer, we might eventually be able to identify molecular targets for risk assessment, prevention, and novel therapeutic targets.

Specifically we conclude that:

- A low energy intake prior to breast cancer diagnosis is associated with a more malignant phenotype characterised by nuclear grade III, a high proliferation index, and cyclin D1 over-expression.
- A lower total fat intake is associated with breast cancer displaying high proliferation and especially, a low intake of polyunsaturated fatty acids was associated with high proliferation.
- Combined hormonal replacement therapy is associated with an increased risk of less malignant breast cancer, characterised by lobular type, low grade, low proliferation, negative/low cyclin D1 expression, and maintained p27 expression.
- Obesity is associated with a high risk of breast cancer characterised by lobular type, grade II, low proliferation, HER2 negativity, ER α +, PgR+, but negative ER β expression.
- The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR) is differentially expressed in the cytoplasm in invasive breast cancer and associated with small tumour size, low grade, low proliferation, and expression of ER α and ER β , but not PgR.
- High HMG-CoAR expression in breast cancer is associated with estrogen-related factors such as hormonal replacement therapy and obesity.
- ER β expression is associated with ER α - and PgR expression in breast cancer, but not with tumour size, grade, lymph node metastasis, HER2, or proliferation.
- The expression of ER β in breast cancer is not a prognostic factor, but co-expression of both estrogen receptors is associated with an improved response to endocrine therapy.

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

(Summary in Swedish)

Bröstcancer är globalt sett den vanligaste tumörsjukdomen bland kvinnor med över en miljon nya fall per år. I den industrialiserade delen av världen kan en utav nio kvinnor förvänta sig att insjukna i bröstcancer under sin livstid. Antalet kvinnor som insjuknar i bröstcancer ökar, vilket sannolikt har många bidragande faktorer. En viktig faktor är introduktionen av mammografiscreening som resulterar i att brösttumörer diagnostiseras mycket tidigare och i vissa fall handlar det om att man identifierar tumörer som kanske aldrig hade upptäckts under kvinnans livstid. Andra möjliga orsaker till det ökande antalet fall kan vara ändrade livsstilmönstrar vad gäller sent barnafödande, antal barn, användning av hormoner, fetma, rökning och ändrade kost- och alkoholvanor.

Under många år har man studerat kopplingar mellan livsstilsfaktorer och bröstcancer, men resultaten skiljer sig åt mellan olika studier och det har således varit svårt att fastställa tydliga samband. En förklaring till detta kan vara att bröstcancer ofta har studerats som en och samma sjukdom. Experimentella studier har dock visat att det finns en stor biologisk variation mellan olika brösttumörer, varför det är lämpligt att studera bröstcancer som en heterogen sjukdom för att bättre kunna förstå verkningsmekanismen mellan yttre livsstilsfaktorer och uppkomsten av en tumör.

Målsättningen med våra studier har varit att undersöka sambandet mellan ett antal livsstilsfaktorer och risken för att insjukna i specifika bröstcancertyper definierade utifrån histopatologiska och tumörbiologiska karakteristika. Målsättningen har varit att identifiera faktorer som dels påverkar risken för speciellt aggressiva brösttumörer, dels hänger samman med hur den enskilda kvinnan svarar på behandlingen, och slutligen hur detta kan påverka prognosen på lång sikt. Detta kan förhoppningsvis öka vår kunskap om hur vi kan minska insjuknande och död relaterat till bröstcancer.

I Malmö finns ett unikt material för att just studera olika livsstilsfaktorer och deras betydelse för uppkomsten av tumörsjukdomar: Under 1990-talet etablerades Malmö Kost Cancer studien där 17 035 frivilliga kvinnor deltog. Deltagarna lämnade detaljerade upplysningar om bland annat matvanor, livsstilsvanor, medicin, utbildning, yrke, tidigare sjukdomar och mycket mer. Vid studiestart bedömdes även kroppsmått och det togs ett antal blodprover. Studien stängdes 1996 och sedan dess har man årligen kopplat Malmö Kost Cancer databasen mot cancerregistret och på så sätt identifierat eventuella nya cancerfall. Denna avhandling är baserad på de kvinnor som deltog i Malmö Kost Cancer studien och som insjuknade i bröstcancer fram till och med 2004.

I våra studier analyserade vi vävnad från bortopererade bröstcancer som hade arrangerats i så kallade vävnadsarrayer för att möjliggöra analys av hundratals patientprover samtidigt. Med denna metod kunde vi på ett såväl reagens- som vävnadsbesparande sätt analysera uttrycket av olika proteiner i relation till olika biologiska undergrupper av tumörer, livsstilsrelaterade faktorer och/eller sjukdomsutfall.

I den kliniska vardagen analyseras bröstcancer för ett flertal patologiska karakteristika som i sin tur används för vägledning i valet av rätt behandling samt prognostisk information till patienten. Modern bröstcancerforskning har bidragit till att det ständigt identifieras nya lovande tumörmarkörer som dels ökar förståelsen för tumörers biologi, dels möjliggör en långt mera individuell behandlingsplan för den enskilda patienten. Vi ville därför karakterisera brösttumörer baserat på såväl etablerade patologiparametrar som uttryck av proteiner vilka på olika sätt upp- eller nedreglerar cellens förmåga att

dela sig. Därutöver undersökte vi förekomsten av en nyligen identifierad hormonreceptor i bröstcancer, östrogenreceptor β samt ett enzym, HMG-CoA reductas, som har en betydande roll i kolesterolsyntesen, men sannolikt även i tumörutveckling.

Denna avhandling består av fyra arbeten baserade på 622 bröstcancerfall från Malmö Kost Cancer studien samt ett arbete baserat på 512 bröstcancerfall från en konsekutiv kohort från Malmö.

I det första arbetet undersökte vi relationen mellan matvanor och ett antal tumörkaraktäristika. Våra resultat visade att ett lågt rapporterat samlat energiintag var relaterat till mera aggressiva tumörkaraktäristika samt ett högt uttryck av ett tumörstimulerande protein, cyclin D1. Det samlade energiintag består generellt av fett, kolhydrater och protein. Våra resultat visade en koppling mellan lågt fettintag och hög celldelningsfrekvens, medan intaget av kolhydrater och protein inte visade något samband med tumörkaraktäristika. Många epidemiologiska studier har visat en ökad risk för bröstcancer vid högt fettintag, och våra resultat indikerar att lågt fettintag är relaterat till mer aggressiva brösttumörer och *vice versa*.

I det andra arbetet undersökte vi risken för att insjukna i olika typer av postmenopausal bröstcancer efter hormonanvändning i klimakteriet. Vi fann, i likhet med många andra studier, en väsentlig ökad risk för bröstcancer vid användning av kombinerade hormonpreparat innehållande både östrogen och progesteron, men inte för preparat enbart bestående av östrogen. Hormonanvändning ökade risken specifikt för tumörer som vi hade karakteriserat som "snälla", dvs. med bevarad cellstruktur, låg celldelningsfrekvens, lågt uttryck av cyclin D1 och ett normalt uttryck av ett tumörhämmande protein, p27. Däremot fann vi ingen skillnad i uttrycket av hormonreceptorer bland kvinnor som hade använt hormoner jämfört med icke-användare.

I det tredje arbetet undersökte vi sambandet mellan kroppsbyggnad och risken för olika typer av postmenopausal bröstcancer. Vi använde sex olika mått på kroppsbyggnad – längd, vikt, body mass index (BMI), höft- och midjeomfång samt kroppsfett i procent. Alla sex mått visade ett positivt samband med en generellt ökad risk för bröstcancer. Vi fann vidare att långa kvinnor hade en ökad risk för mindre aggressiv bröstcancer. Oavsett vilket av de övriga fem mått vi analyserade visade våra resultat att överviktiga kvinnor hade en ökad risk för "snällare" tumörer med låg cell delningsfrekvens, negativ HER2 status, och ett högt uttryck av östrogen- och progesteronreceptorer. Däremot var övervikt inte kopplat till uttrycket av östrogenreceptor β .

I det fjärde arbetet undersökte vi tumöruttrycket av enzymet 3-hydroxy-3-methylglutaryl-coenzym A reductas (HMG-CoAR) och dess relation till mera etablerade tumörkaraktäristika samt till östrogenrelaterade livsstilsfaktorer som hormonanvändning och övervikt. Vi fann att HMG-CoAR var uttryckt i tumörcellers cytoplasma, men inte i kärnorna, och att uttrycket varierade starkt mellan olika brösttumörer. Resultaten visade vidare ett samband mellan högt uttryck av HMG-CoAR och gynnsamma tumörkaraktäristika såsom liten tumörstorlek, välbevarad cellstruktur, låg celldelningsfrekvens, och ett högt uttryck av båda östrogenreceptorerna, men dock inte av progesteronreceptorn. I likhet med det andra och tredje arbetet fann vi att hormonanvändande och överviktiga kvinnor hade en ökad risk för sannolikt mindre aggressiva tumörer, i detta fall tumörer med högt HMG-CoAR uttryck.

Det femte arbetet baserades på en kohort av kvinnor som mellan 1988 och 1992 opererades för bröstcancer i Malmö, sammanlagt 512 kvinnor. I detta arbete undersökte vi förekomsten av östrogenreceptor β i brösttumörer och hur ett högt uttryck var kopplat till övriga tumörkaraktäristika, samt till överlevnad efter anti-hormonell behandling. Vi fann att högt uttryck av östrogenreceptor β var relaterat till högt östrogenreceptor α och progesteronreceptor uttryck, men inte till övriga etablerade tumörkaraktäristika. I

överlevnadsanalyser var östrogenreceptor β inte en prognostisk faktor och således inte relaterad till sjukdomsåterfall, men däremot fann vi att bland de kvinnor som hade fått antihormonell behandling var prognosen bäst för de kvinnor vars tumörer uttryckte båda östrogenreceptorerna.

Med denna avhandling kan vi konkludera att ett flertal livsstilsfaktorer är kopplade till specifika typer av bröstcancer; lågt fettintag och aggressiva tumörkaraktistika var relaterade till varandra, medan övervikt och hormonanvändning för klimakteriebesvär ökade risken för snällare brösttumörer. HMG-CoA reductas är en ny tumörmarkör i bröstcancer, som kan vara kopplad till östrogennivåer i kroppen. HMG-CoA reductas kan utgöra ett potentiellt mål för cancerbehandling med kolesterolsänkande läkemedel (statiner) och det återstår att undersöka om det skulle kunna ha betydelse i behandlingen av bröstcancer. En nylig identifierad östrogenreceptor är relaterad till den traditionella östrogenreceptorn i bröstcancer och kan eventuellt bidra med ökad kunskap om vilka kvinnor som har störst nytta av endokrin behandling.

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Original papers (I-V)