Androgen and Estrogen Receptors in Breast Cancer. Impact on Risk, Prognosis and Treatment Prediction.

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2017

Document Version:
Publisher's PDF, also known as Version of record

Androgen and Estrogen Receptors in Breast Cancer

Impact on Risk, Prognosis and Treatment Prediction

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LUND
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DOCTORAL DISSERTATION

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To be defended at the lecture hall in Strålbehandlingshuset, 3rd floor, Skåne University Hospital, Klinikgatan 5, Lund,
Friday the 20th of January 2017, at 9.00 a.m.

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Organization LUND UNIVERSITY	Document name DOCTORAL DISSERTATION
Department of Clinical Scirences, Lund Division of Oncology an Pathology	Date of issue: January 20th 2017
Author: Karin Elebro	Sponsoring organization

Title and subtitle: Androgen and Estrogen Receptors in Breast Cancer: Impact on Risk, Prognosis and Treatment Prediction

Aim: Breast cancer research faces critical clinical challenges: to improve treatment prediction, to overcome primary and secondary resistance to treatments, to develop treatment targets for the subtypes that have few treatment options available and to optimize and personalize treatment for recurrent breast cancer. This translational thesis investigated the potential value of adding the novel hormone receptors androgen receptor (AR) and estrogen receptor-β (ERβ) to the established estrogen receptor-α (ER) as markers for improved primary and tertiary breast cancer prevention.

Methods: The observational population-based cohorts *The Malmö Diet and Cancer Study* (MDCS) and *The Breast Cancer and Blood Study* (BC-blood) were used. Immunohistochemistry of AR and ERβ isoform 1 (ERβ1), respectively, was evaluated using tissue microarrays, combined with patient and tumor characteristics and evaluated using survival analysis according to breast cancer risk or prognosis.

Results: Paper I, MDCS (n=16,459), risk for breast cancer according to AR expression (n=516): The older the woman at first childbirth, the higher the risk of AR negative (AR–) breast cancer; adjusted hazard ratios (Adj. HRs); $HR_{\leq 20yrs} = 0.35$, $HR_{>20\leq 25yrs} = 0.62$, $HR_{nulliparous} = 1.00$, $HR_{>25-\leq 30yrs} = 1.29$, $HR_{>30yrs} = 1.92$, $P_{trend} = 0.001$. No such association was seen in separate analysis for AR positive (AR+), ER positive (ER+) or ER negative (ER–) tumors, respectively, or for general breast cancer risk. Similarly, ever use of oral contraceptives increased the risk of AR– breast cancer [Adj. HR (95% confidence intervals (CI)); 2.59 (1.26-5.34)] compared to never use.

Paper II, BC blood (n=905), AR expression in relation to disease-free survival (DFS): The AR+ status was a prognostic marker for DFS (LogRank $P = 0.025$). There was an interaction between AR and ER expression with respect to prognosis ($P_{interaction} = 0.010$). ER+AR– indicated early treatment failure with aromatase inhibitors among chemo-naïve patients aged 50 or older, a finding that warrants confirmation in a randomized setting.

Paper III, MDCS (n=671), AR expression in relation to breast cancer mortality: AR expression added information compared to that of ER expression alone although to a limited extent. The previous finding of a significant interaction between AR and ER status in relation to outcome (Paper II) was not confirmed. However, the poorest prognosis was still seen among patients with AR+ER– tumors, after adjustment for confounders in short-term (up to five years) follow-up.

Paper IV, BC Blood (n=903), ERβ1 expression in relation to DFS: High ERβ1 expression defined as >75% ($ERβ1_{75}$+, 73%) was associated with lower risk of breast cancer events [Adj. HR (95% CI); 0.60 (0.41-0.89)], compared to low ERβ1 expression ($ERβ1_{75}$–). The magnitude of the association was larger in patients with ER– tumors [Adj. HR (95% CI); 0.30 (0.12–0.76)], compared with ER+ tumors [Adj. HR (95% CI); 0.66 (0.42-1.03)]. Among the 232 adjuvant chemotherapy-treated patients, $ERβ1_{75}$+ tumors were associated with lower risk of breast cancer events compared with $ERβ1_{75}$– tumors [Adj. HR (95% CI); 0.31 (0.15–0.64)]. Among the 671 chemonaïve patients, $ERβ1_{75}$ status was not associated with the outcome.

Conclusion: AR and ERβ1 added prognostic value to the clinically established ER in the adjuvant primary breast cancer setting both overall and within specific treatment groups. Future studies taking both ERβ and AR expression into consideration would be of interest as well as incorporation of genetic profiling or the IHC surrogate markers. In spite of being relatively large cohorts, future observational studies need to be even larger in order to better characterize ER– breast cancer subtypes. Validation of tumor AR and ERβ1 expression in relation to outcome and treatment type in already performed clinical trials are needed.

Key words: breast cancer, risk, prognosis, androgen receptor, estrogen receptor-β, tamoxifen, aromatase inhibitors, chemotherapy

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language: English
ISSN and key title: 1652-8220		ISBN: 978-91-7619-388-4
Recipient's notes	Number of pages 93	Price
	Security classification	

Signature Date

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Cover photo by Zora Rief

Oncology and Pathology
Department of Clinical Sciences Lund, Faculty of Medicine,
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Lund University, Faculty of Medicine Doctoral Dissertation Series 2017:7
ISBN 978-91-7619-388-4
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2017

Contents

Abbreviations

AIs – aromatase inhibitors

ALNI – axillary lymph node involvement

AR – androgen receptor

BC Blood – breast cancer and blood study

BCM – breast cancer mortality

BMI – body mass index

BRCA1 – breast cancer 1

BRCA2 – breast cancer 2

CI – confidence intervals

CMF – cyclophosphamide, methotrexate, fluorouracil

CYP450 enzymes – cytochrome P 450 enzymes

DBD – DNA-binding domain

DFS – disease-free survival

DHEA – dehydroepiandrosterone

DHEAS - dehydroepiandrosterone sulfate

DHT – dihydrotestosterone

DMFS – distant metastasis-free survival

DNA – deoxyribonucleic acid

ER – estrogen receptor-α

ERβ – estrogen receptor-β

ERβ1 – estrogen receptor β isoform 1

ERβ2/cx – estrogen receptor β isoform 2

ESR2 – estrogen receptor 2

HER2 – human epidermal growth factor receptor 2

htSNP – haplotype tagging single nucleotide polymorphism

HRT – hormone replacement therapy

IHC – immunohistochemistry

ISH – *in situ* hybridization

Ki67 –proliferation associated antigen

LAR – luminal androgen receptor

LBD – ligand-binding domain

MDCS – the Malmö diet and cancer study

MPE – molecular pathological epidemiology

mRNA – messenger ribonucleic acid

NHG – Nottingham histological grade

NTD – N-terminal domain

OC – oral contraceptive

OR – odds ratio

OS – overall survival

PR – progesterone receptor

SARM – selective androgen receptor modulator

SHBG – sex hormone binding globulin

SERD – selective estrogen receptor down-regulator

SERM – selective estrogen receptor modulator

SNP – single nucleotide polymorphism

TAM – tamoxifen

TDLU – terminal duct lobular unit

TMA – tissue microarray

TNM – tumor node metastasis

TNBC – triple negative breast cancer

List of papers

The thesis is based on the following papers:

I. Elebro K, Butt S, Dorkhan M, Jernström H, Borgquist S.

Age at first childbirth and oral contraceptive use are associated with risk of androgen receptor-negative breast cancer: the Malmo Diet and Cancer Cohort.

Cancer Causes Control 25 (8):945-957, 2014.

II. Elebro K, Borgquist S, Simonsson M, Markkula A, Jirstrom K, Ingvar C, Jernström H.

Combined Androgen and Estrogen Receptor Status in Breast Cancer: Treatment Prediction and Prognosis in a Population-Based Prospective Cohort.

Clinical cancer research 21 (16):3640-3650, 2015.

III. Elebro K, Bendahl P-O, Jernström H, Borgquist S.

Androgen Receptor Expression and Breast Cancer Mortality in a Population-based Prospective Cohort

Manuscript

IV. Elebro K, Borgquist S, Rosendahl AH, Markkula A, Simonsson M, Jirström K, Rose C, Ingvar C, Jernstrom H.

High estrogen receptor beta expression is prognostic among adjuvant chemotherapy-treated patients - results from a population-based breast cancer cohort.

Clinical cancer research [E-pub ahead of print], 2016

Reprints were made with permission from the publishers.

Related paper not included in the thesis:

Sartor H, Zackrisson S, Elebro K, Hartman L, Borgquist S.

Mammographic density in relation to tumor biomarkers, molecular subtypes, and mode of detection in breast cancer.

Cancer Causes Control 26 (6):931-939

Thesis at a glance

Paper	Questions	Methods	Results and Conclusion
I **AR**	Are hormone-related lifestyle factors associated with AR positive or AR negative breast cancer risk?	Cohort: MDCS. 16,459 women with no prior breast cancer diagnosis were followed for an average of 13 years during which 747 underwent surgery for invasive breast cancer, and their tumors were evaluated for tumor AR expression by IHC.	High age at first child birth and prior use of oral contraceptives were associated with increased risk of AR negative breast cancer. This hypothesis-generating study may contribute to improved understanding of breast carcinogenesis.
II **AR**	Does tumor AR expression add independent prognostic or treatment predictive value when added to the ER expression that is used in clinical routine?	Cohort: BC Blood. 1,026 women underwent surgery for invasive breast cancer and were followed for, on average, 5 years. The tumors were evaluated for tumor AR expression by IHC. Medical records and registry linkage provided information for analyses of DFS according to combinations of AR and ER expression in different treatment groups.	AR expression added prognostic information compared to that of ER expression alone. AR expression was differentially associated with prognosis depending on tumor ER status, and this may impact the choice of AR targeted drugs (agonistic or antagonistic) that are currently evaluated in clinical trials. A tendency towards early failure of aromatase inhibitor treatment was observed among chemonaïve patients 50 years or older who had ER+AR– tumors. This finding warrants confirmation.
III **AR**	Can the AR-related prognostic findings from Paper II be validated using breast cancer mortality as endpoint in an independent cohort of longer follow-up?	Cohort: MDCS, case-only. 910 women underwent surgery for invasive breast cancer and were followed for, on average, 10 years. The tumors were evaluated for tumor AR expression by IHC. Medical records and registry linkage provided information for analysis of BCM and all-cause mortality according to AR and ER expression.	AR expression added information compared to that of ER expression alone, although to a limited extent. The previous finding of a significant interaction between AR and ER status in relation to outcome was not confirmed. However, the poorest prognosis was still seen among patients with AR+ER– tumors, after adjustment for confounders in short-term (up to five years) follow-up.
IV **ERβ**	Does tumor ERβ1 expression add independent prognostic or treatment predictive value, when added to the ER expression that is used in clinical routine?	Cohort: BC Blood, identical patient cohort and follow-up as in Paper II. Paper II analyses were repeated for tumor ERβ1expression, assessed by IHC, and extended with secondary endpoints of DMFS and OS.	High ERβ1 expression (>75%, ERβ1$_{75}$+) was a favorable prognostic marker in this breast cancer cohort, especially among patients with ER– tumors, but also among patients with ER+ tumors. Regarding treatment, patients with ERβ1$_{75}$+ tumors who had received adjuvant chemotherapy had a superior prognosis compared to patients with ERβ1$_{75}$– tumors. Among adjuvant endocrine treated patients, no prognostic role of ERβ1 was seen.

Abbreviations: AR, androgen receptor; AR+, AR positive (>10%); AR–, AR negative(≤10%), BC Blood, Breast Cancer and Blood Study; BCM, breast cancer mortality, DFS, disease-free survival; DMFS, distant metastasis-free survival; ER, estrogen receptor-α; ER+, ER positive (>10%), ER–, ER negative (<10%); ERβ1, estrogen receptor beta isoform 1; ERβ1$_{75}$+, ERβ1$_{75}$ positive (>75%); ERβ1$_{75}$–, ERβ1$_{75}$ negative (≤75%); IHC, immunohistochemistry; MDCS, Malmö Diet and Cancer Study; OS, overall survival

Populärvetenskaplig sammanfattning (Plain Swedish Summary)

Uppskattningsvis diagnosticeras en av nio kvinnor i Sverige med bröstcancer innan 75 års ålder. År 2014 var antalet kvinnor i Sverige som lever med en bröstcancerdiagnos över 100 000. Den genomsnittliga överlevnaden vid bröstcancer är mycket god, 90% på fem års sikt och 80% på 10 års sikt.

Bröstcancer delas upp i olika grupper beroende på vilka tumörmarkörer som uttrycks/uppmäts i tumören. De olika grupperna skiljer sig åt i behandlingsval och prognos, dvs. risken för återfall och i förlängningen för död till följd av bröstcancer. Bröstcancer är alltså att betrakta som en heterogen sjukdom, dvs. en sjukdom med ”många ansikten”, och för var och en av dessa grupper finns olika aspekter som behöver förbättras: 1) att mer exakt välja ut vilka patienter som har nytta av varje specifik behandling, dvs. att minska över- och underbehandling, 2) att förstå och försöka förhindra behandlingssvikt, samt 3) att utveckla nya behandlingar. Ett exempel: en stor andel av all bröstcancer ”triggas” till tillväxt av kvinnligt könshormon, östrogen. Den klassiska östrogenreceptorn alfa (α), eller ER, kan mätas i brösttumören. När ER anses vara positiv, får kvinnan antihormonell tablettbehandling. Sådan behandling fungerar i många fall men inte i alla, och därför behövs nya markörer som kan ge en mer exakt vägledning i vilka patienter som har nytta av behandlingen, och för de patienter som sviktar på behandling behövs det nya behandlingsalternativ. Givetvis behöver vi även bättre förstå varför bröstcancer uppkommer från första början och sträva efter att förebygga sjukdomen helt och hållet. Alla dessa åtgärder sammanfattas under begreppet prevention, förebyggande arbete, som delas in i att förebygga sjukdom, att upptäcka sjukdom tidigt samt att öka chanserna till längre överlevnad eller bot, dvs. att förbättra behandling och prognos.

Kunskaperna om bröstcancer som en mångfacetterad sjukdom ökar ständigt. Bröstcancerforskningen sker prekliniskt i laboratorier, på cell- och djurförsöksnivå, samt kliniskt på patienter. Det ligger en stor utmaning idag i att forska translationellt, dvs. att koppla samman preklinisk och klinisk forskning, för att olika rön på ett effektivt sätt ska sammanstråla och komma både dagens och framtidens patienter till gagn.

Det här är en translationell avhandling i gränslandet mellan epidemiologi, dvs. läran om sjukdomars karaktär och spridning i ett samhälle, patologi, specifikt läran om tumörmarkörer, och slutligen onkologi, handläggningen av cancersjukdomar. Det övergripande målet har varit att utvärdera om en komplettering med de nyare hormonreceptorerna - androgenreceptor (AR) och östrogenreceptor beta (ERβ) - skulle kunna hjälpa till att förbättra prognosen av bröstcancer.

Avhandlingen består av fyra studier där vi studerat risk för bröstcancer samt prognos efter sjukdomen. I tre arbeten har vi studerat AR, och i ett arbete ERβ. AR och ERβ har analyserats i mikroskop i vävnadsprover som infärgats med hjälp av immunohistokemi specifik för AR respektive ERβ. Alla arbeten bygger på observationsstudier/kohortstudier, i vilka patienterna följs av forskare samtidigt som de får sin ordinarie behandling. Studierna som använts är Malmö Kost Cancer-studien (MKC, arbete I och III) och Bröstcancer och Blod-studien (BC Blod, arbete II och IV). MKC är en studie som är nästan unik i sitt slag. På 90-talet bjöd man in och undersökte noggrant 17 035 kvinnor. Av dessa hade 576 redan haft bröstcancer. Resterande 16 459 har följts via olika register, och de som fått bröstcancer, ungefär 1 000 kvinnor, har fått sina tumörer undersökta av oss forskare. På så vis har vi fått en möjlighet att studera både risk för insjuknande i specifika typer av bröstcancer samt prognos baserad på olika tumörmarkörer. BC Blod är en modernare studie, som startade 2002, och fortfarande inkluderar nya patienter. Drygt 1 100 patienter som har opererats för bröstcancer i Lund deltar idag. Kvinnorna genomgår noggranna undersökningar inför och efter att de opererats, vartefter de följs med både undersökningar, frågeformulär, journal- och registerkontroller. Studien syftar till att öka vår förståelse om prognostiska faktorer.

Frågeställningar och fynd i de olika arbetena sammanfattas nedan:

Arbete I, en utforskande studie om bröstcancerrisk: *Påverkar kända hormonella riskfaktorer för bröstcancer vilket uttryck av AR som tumören får?* Vi fann indikationer på att två sådana riskfaktorer skulle kunna öka risken för en bröstcancer som saknar uttryck av AR, AR-negativ bröstcancer: ju äldre en kvinna är när hon får sitt första barn, samt tidigare användning av p-piller. Eftersom dessa faktorer inte tycktes påverka generell risk för bröstcancer, AR-positiv, ER-positiv eller ER-negativ bröstcancer, kan fyndet på ett akademiskt plan bidra till en ökad förståelse av ARs roll i bröstcancerutvecklingen.

Arbete II, ARs roll för prognos och behandlingssvar: *Bland kvinnor med bröstcancer, skulle utvärdering av tumöruttrycket av AR förbättra informationen om prognos och behandlingssvar jämfört med den information som dagens rutinutvärdering av ER ger?* Ja, det finns hållpunkter för det. AR positivitet var generellt ett gott prognostiskt tecken. Men, om vi tittade inom ER positiva respektive inom ER-negativa bröstcancergrupperna – två grupper som har väldigt ”olika ansikten”, tycktes AR-uttrycket säga helt olika saker: AR-positivitet var gynnsamt inom ER-positiv bröstcancer, men ogynnsamt inom ER-negativ bröstcancer. Eftersom det finns målstyrda behandlingar som riktar sig mot AR, skulle ett fynd som vårt i förlängningen kunna bidra till bättre styrning av sådan behandling. Ogynnsamt uttryck borde då motverkas med motverkande antiandrogen behandling, medan gynnsamt uttryck borde ”boostas”, som med pådrivande agonistisk behandling.

Arbete III, en valideringsstudie av fynden i arbete II: *Kan indikationerna på olika roll för AR i olika bröstcancergrupper bekräftas i vår andra studiepopulation?* Här användes MKC som har längre uppföljningstid och en annan ”endpoint”, dvs. istället för att undersöka risk för återfall (arbete II) så studerades här risk för bröstcancer-relaterad död som endpoint. Vi kunde inte fullt ut bekräfta den skillnad som AR-uttryck tycktes medföra beroende på om tumören var ER-positiv eller ER-negativ. Tendenser i samma riktning fanns dock: sämst prognos sågs hos de patienter vars tumörer var ER-negativa och AR-positiva, när vi undersökte sambanden inom de första fem åren efter diagnos och tog hänsyn till andra viktiga prognostiska faktorer.

Arbete IV, ERβs roll för prognos och behandlingssvar: *Bland kvinnor med bröstcancer, skulle utvärdering av tumöruttrycket av ERβ förbättra informationen om prognos och behandlingssvar jämfört med vad dagens rutinutvärdering av ER gör?* Vi fann att det gick betydligt bättre för de kvinnor vars tumörer hade högt uttryck av ERβ, jämfört med de kvinnor vars tumörer hade lågt ERβ uttryck. Denna skyddande effekt var extra tydlig hos de kvinnor som hade behandlats med cellgifter. Om fyndet skulle bekräftas i fler studier skulle det kunna bidra till att cellgiftsbehandling antingen kan begränsas till de kvinnor som har högst risk, eller att dessa kvinnor utöver cellgiftsbehandling får tätare kontroller och/eller annan tilläggsbehandling.

Sammanfattningsvis tyder våra studier på att både AR och ERβ skulle kunna tillföra värde till dagens kliniska utvärdering av ER. Samtliga fynd behöver först bekräftas i fler studier. Detta kan t.ex. göras genom att analysera markörerna inom ramen för redan genomförda kliniska prövningar där den medicinska behandlingen slumpats till patienterna. Sådana randomiserade prövningar ger en mer rättvisande bild av markörernas värde jämfört med de observationsstudier som vi genomfört. Våra studier var förhållandevis stora och omfattande. Ändå behöver framtida observationsstudier vara ännu större, eftersom bröstcancerns ”många ansikten” gör att tolkning av resultat försvåras när antalet patienter per grupp minskar. Ett alternativ vore studier baserade på genexpressionsanalys, en metod som kan ge en mer heltäckande biologisk bild av tumörens grupptillhörighet.

Introduction

Globally, 1.7 million women are annually diagnosed with breast cancer, and more than half a million die from the disease[1]. From a global perspective, breast cancer has been highlighted as a priority of similar importance as maternal mortality[2].

In Sweden in 2014, the number of women who were alive with a breast cancer diagnosis exceeded 100,000, and it was estimated that one in nine women would be diagnosed with breast cancer before the age of 75 years[3, 4]. The overall survival for breast cancer is high; the 5- and 10- year survival rates are 90% and 80%, respectively[3, 4]. However, depending on the breast cancer subtype, there are still critical clinical challenges to be addressed: 1) to improve treatment prediction, 2) to overcome primary and secondary resistance to treatments, 3) to develop treatment targets for the subtypes that have few treatment options available, and 4) to optimize and personalize treatment of recurrent breast cancer. All these actions are measures of prevention, particularly tertiary prevention, which may be defined as improving prognosis and providing a cure for breast cancer. Secondary prevention refers to early detection, as exemplified by the widespread use of mammography screening[5]. There is also an urgent need for primary prevention, i.e., to better predict and reduce the risk for women to develop breast cancer in the first place[6, 7].

Epidemiology is the study of the distribution of disease in a population, and it can help to better understand risk factors (i.e., exposures) for a given disease. The ultimate aim is to find indications on what exposures cause a specific disease (i.e., making causal inference). This is a difficult task and is the reason why epidemiologic findings are instead presented in terms of associations between exposures and disease as compared to statements on what exposure cause a specific disease. Risk factors may be markers for disease without effecting disease biology, or they may be determinants, which are factors that impact the development of disease. Finally, risk factors may be non-modifiable or modifiable and thus actionable in terms of preventive measures[8, 9].

In traditional epidemiology, risk factors for breast cancer have been studied while considering breast cancer as one disease, without considering the different breast cancer subtypes; thus, these studies have not identified the potentially different etiology of the various subtypes[10]. In recent years, the importance of performing risk profiling according to specific tumors and genetic markers has frequently been emphasized[11-14].

One of the most established risk factors for breast cancer is high and/or long-term hormonal exposure during a woman's lifetime, such as early menarche, late menopause or use of hormonal replacement therapy[15]. Therefore, the effect of hormones on breast cancer initiation is essential. The sex hormones androgen and estrogen, which are the focus of this thesis, are often referred to as male and female sex hormones, which is somewhat misleading since both sexes display and need both hormones[16]. The physiological effects of androgen and estrogen are mediated through their corresponding receptors, the "classical" estrogen receptor-α (hereafter referred to as ER), the more recently discovered estrogen receptor-β (ERβ) and the androgen receptor (AR), which are all commonly and highly expressed in breast cancer[17].

This thesis spans across epidemiology, pathology and oncology and addresses the potential value of adding the hormone receptors AR and ERβ to the established ER as markers for improved primary and tertiary breast cancer prevention.

Androgens and estrogens

In this thesis, hormone-related baseline exposures are investigated in relation to outcome and according to tumor AR and ERβ expression, respectively. The principle pathway from exposure to outcome consists of complex physiologic responses and endocrine signaling pathways, the basis of which is described below.

From hormone to gene expression

Androgens and estrogens are cholesterol-derived steroid hormones produced through enzymatic conversions (Figure 1) and are transported by the circulation to their target tissues to exert tissue-specific effects[16]. The hormones regulate reproductive function through an intricate feedback system emanating from the hypothalamus and pituitary gland. Production of these hormones was originally thought to come exclusively from endocrine glands such as the adrenal gland, the testis and the ovaries, but with new scientific achievement (e.g., cloning and characterization of the enzymes responsible for the steroid conversions, identification of the tissues where they are localized), non-glandular hormone production has been recognized[18]. Thus, hormones may act locally within the cell of synthesis (intracrine/autocrine) or on adjacent cells (paracrine/juxtacrine) without actually being detected in the circulating levels of hormones[18]. Adipose tissue is important for peripheral conversion of androgens to estrogens and may be regarded as an active endocrine organ secreting bioactive substances[19]. The postmenopausal

breast has been reported to have intracrine estrogen production[20, 21], and intracrine features have been described as important in the development of neoplasms[22].

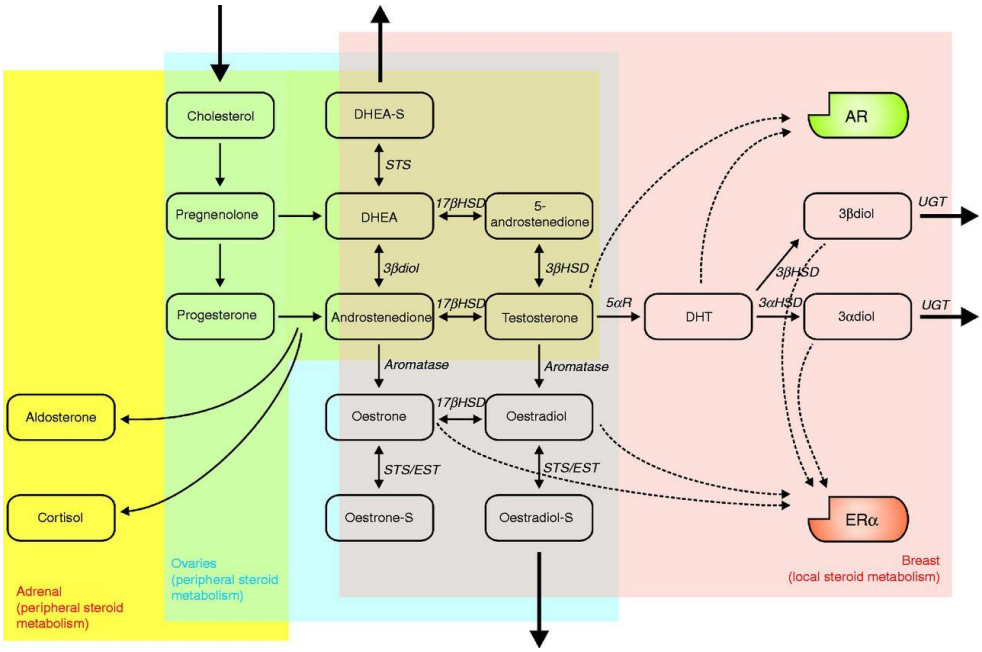

Figure 1. Predominant steroid metabolism pathways in the adrenal/ovaries and breast tissue.
Enzymes are shown in italics by the relevant reaction arrow in the figure, while the binding of selected steroids to AR or ER is shown by broken arrows. Bold arrows indicate points of intake or excretion of the steroids. Coloured boxes group the reactions occurring at peripheral or local sites.

Selected abbreviations: 5αR, 5α reductase;17β-HSDs, 17β-hydroxysteroid dehydrogenases; AR, androgen receptor; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone-sulphate, DHT, dihydrotestosterone; ERα, estrogen receptor-α; oestradiol, 17β-oestradiol; STS, steroid-deconjugating steroid sulphatase sulphatase; EST, oestrogen-specific oestrogen sulphotransferase; UGTs, steroid-conjugating UDP-glucuronosyltransferases.

Androgens and estrogens circulate either in their free active form or in their inactive form bound to sex hormone binding globulin (SHBG) or other carrier proteins such as albumin, which are produced by the liver[16]. In their target tissue, androgens and estrogens diffuse into the cell cytoplasm and bind to their specific intracellular sex hormone receptors (ER, ERβ and AR, respectively). These receptors belong to the nuclear receptor superfamily[23] and share the common structure of three functional domains: the hormone (ligand)- binding domain (LBD), the DNA-binding domain (DBD) and the transactivation N-terminal domain (NTD)[17] (Figure 2). In the classical genomic mechanism of action, the binding of hormones, or other ligands, to the receptor cause the release of chaperone heat shock proteins, followed by a conformational change of the receptor with subsequent activation and translocation

into the nucleus. The receptor complex dimerizes and binds to specific enhancer regions of target genes called response elements. This binding facilitates the recruitment of co-factors and lead to target gene transcription[17]. In addition to this classical pathway, receptor activation may also occur as a downstream event through receptors at the cell membrane or in the cytoplasm, activating intracellular signaling cascades and transcription factors. Depending on the overall conformation of the complex with its recruited co-factors, the specific gene may either be activated or repressed[24]. Since many processes/signaling pathways are ongoing at the same time, the outcome may be different in spite of the ligand being the same[23]. Activation may also occur without a ligand, for example, by posttranslational modification such as phosphorylation after which the activated receptor may interact with the responsive elements or bind to other transcription factors[25, 26].

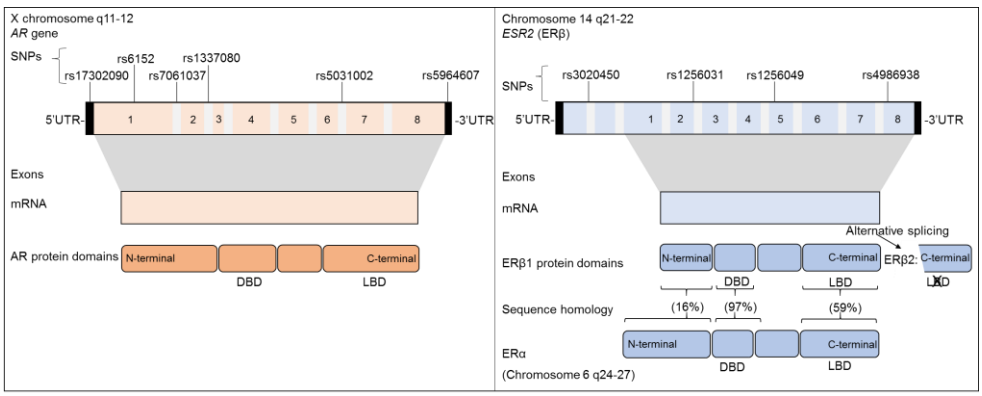

Figure 2. Schematic illustration of *AR* and *ESR2* (ERβ) genes respectively.
Location of SNPs[27, 28] (top panel). The ERβ1 protein contains an LBD whereas the protein domain of the splice variant ERβ isoform 2 (ERβ2) does not (far right). The homology sequence[29] between ERβ1 and ERα is displayed in the bottom panel (right).

Abbreviations: AR, androgen receptor; DBD, DNA-binding domain; ERα, estrogen receptor-α; ERβ1, estrogen receptor-β isoform 1; ERβ2, estrogen receptor-β isoform 2;*ESR2, estrogen receptor 2 gene*; LBD, ligand-binding domain; SNPs, single nucleotide polymorphisms.

Furthermore, the receptor gene may carry genetic aberrations that affect receptor function and efficiency. Mutations (e.g., copy number changes, insertions, deletions) may be inherited (germline) or acquired (somatic)[30]. There are also the very common point mutations where the nucleotides of the DNA-chain are exchanged with each other, called single nucleotide polymorphisms (SNPs). Polymorphisms are found in coding (exon) regions or in non-coding (intron) regions (Figure 2). The coding SNPs may cause changes in the amino acid sequence during translation, which leads to phenotypic change, or the exchanges may be neutral. Synonomous SNPs in coding regions can affect mRNA splicing and stability even though they do not affect the amoino acid sequence[31]. Non-coding SNPs may also affect phenotype, for example, through altered RNA sequences[32]. After the start of

translation, splicing events may produce different splice variants of the receptor, affecting its efficiency, such as a receptor produced that lacks the LBD, as is the case with ERβ isoforms[29].

Clinical aspects in health and disease

Disruption of any one step of the mechanisms described in the section above may influence the hormone's "net" effect on physiology and may thus cause disease, influence outcome of disease or modify the sensitivity/susceptibility for disease.

The production of androgens such as testosterone, dihydrotestosterone (DHT), androstendione, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) and estrogens such as estradiol, estrone and estrone sulfate vary between the sexes and during the course of a lifetime, and there are large inter-individual differences. The substances differ in potency and tissue-specific effects[16]. Importantly, both androgens and estrogens are essential for many organs beyond reproductive purposes. They are anabolic acting hormones in the musculoskeletal system. Both testosterone and estrogens are important for cardiovascular function, and estrogens have been explored for their potential protective cardiovascular effects. Both sexes need estrogen to produce adequate bone matrix and calcify bone[16]. Estrogens are important for cognition and may be involved in memory function, neurodegeneration and inflammation[29]. Sperm production is androgen-dependent, and a functional AR is a prerequisite for the male phenotype[33, 34]. In females, disruption of androgen metabolism may cause hyperandrogenism as in polycystic ovarian syndrome and associated metabolic disturbances such as insulin resistance and infertility[16].

There are many factors influencing hormonal exposure: The individual's hormone level is dependent on the proportion of bound versus free hormone levels, which in turn is dependent on the production of carrier protein. SHBG varies by body mass index (BMI) as well as genetic factors[35, 36]. The half-life of hormones varies with age, sex, ethnicity, concurrent disease and co-medication[16]. The different cytochrome 450 (CYP) enzymes responsible for elimination through liver, kidneys, intestine and lungs take care of all steroids, external steroids and medications. Thereby, co-medications will lead to different workload for the converting enzymes, which may influence the individual's levels[16]. The subsequently changed hormone levels may influence the susceptibility to a given disease, which may then potentially be detected in terms of risk or prognosis in relation to, for example, breast cancer.

In epidemiological studies of breast cancer risk, the circulating levels of androgens and estrogens have received substantial attention the last decades. Differences depending on assay used have been discussed[37], as well as whether testosterone is

a risk factor itself or by conversion to estrogens[38]. Regarding postmenopausal breast cancer risk, high hormone levels have been associated with an increased risk for breast cancer for all hormone types and with an inverse association to SHBG levels[39-41]. Each examined hormone, except for DHEAS, varied significantly according to tumor hormone receptor expression[41]. The most consistent finding has been the increased breast cancer risk for postmenopausal women with high estrogen levels, and especially estradiol levels[37, 42-44]. Obese postmenopausal women have a high risk due to increased aromatase activity in adipose tissues. Obese demonstrated double estradiol levels compared to lean women[44]. Smoking and alcohol have been associated both with high estrogen and high testosterone levels[35, 37, 44]. Among premenopausal women, the findings are ambiguous, in spite of the much higher premenopausal hormonal activity. There are some evidence for an increased breast cancer risk by high estrogen levels[44, 45] and moderate evidence for higher risk by higher testosterone levels[38, 43, 46].

The main source of estrogen in premenopausal women is estradiol, the most potent estrogen, which is produced by the ovaries. Due to variations during the menstrual cycle, analyses of estrogen levels need to take cycle phase into account to be reproducible[45]. Pregnant women, however, display the highest estradiol levels, which reach one-hundred fold over the levels of non-pregnant, fertile women[47]. During the post-partum period, a steep decrease in estradiol levels occurs as the placenta is expelled, and lactation is initiated. In premenopausal woman, androgens (DHEAS, DHEA, androstendione, testosterone) are produced by the adrenal glands and by the ovaries (androstendione, testosterone) in similar rates in addition to peripheral conversion of testosterone and androstenedione[48]. Bilateral oophorectomy in a fertile woman reduces serum testosterone levels to half[16].

Healthy women produce 0.5-3 nmol/l testosterone as compared to 10-40nmol/l in healthy men[16]. The postmenopausal ovary and adrenal gland retains the ability to produce androgens[22], whereas ovarian production of estradiol ceases. In postmenopausal women, the source of estrogen is mainly derived from conversion of adrenal gland derived pre-cursors taking place in the adipose tissue[29]. The less potent estrone is the postmenopausal woman's most common estrogen. Estrone and estrone sulfate are reversibly convertible to each other and to estradiol and are thus both metabolites and pre-hormones to the main estradiol. After menopause, women display lower estradiol levels than men, who instead produce similar levels throughout adult life[21]. The male estrogen levels (50-150pmol/l) are similar to the lowest level during the menstrual cycle of the fertile woman[16].

In summary, the endocrine processes described above illustrate the physiologic importance of hormones but also that the circulating levels of hormones are insufficient to understand the complexity of the effect of hormones on biology. Androgens and estrogens exert a wide variety of effects that are mediated by slow

genomic as well as by rapid non-genomic mechanisms. This is also what we use as a leverage in the treatment of disease, targeting different parts of the process, and we use this as scientific pieces to better understand the immense puzzle of breast cancer initiation and progression.

Breast cancer epidemiology

During the last decades, the incidence of breast cancer has increased across all age groups, whereas breast cancer mortality has decreased[3] (Figure 3). The increase of the incidence is considered to be a combination of a true increase and a detection effect due to mammographic screening[9]. The decreased mortality may also be attributable to earlier detection through mammography screening, but better adjuvant treatments and tumor profiling may have also added value[9].

Breast cancer is a multifactorial disease[49]. One major culprit behind the increase in breast cancer incidence is the westernized lifestyle. In the UK, lifestyle and environmental factors have been estimated to account for 27% of female breast cancers and almost 7% due to obesity alone[50]. Studies of migratory patterns have shown a substantial increased risk following migration from Asia to America, which supports a larger influence of lifestyle compared to genetic factors [51, 52]

So far, preventive measures for breast cancer have not occurred to the same extent as for cardiovascular disease[6]. The awareness of the need for preventive actions is high, and it has been postulated that half of breast cancer may be prevented if interventions would be taken among selected high risk populations[7].

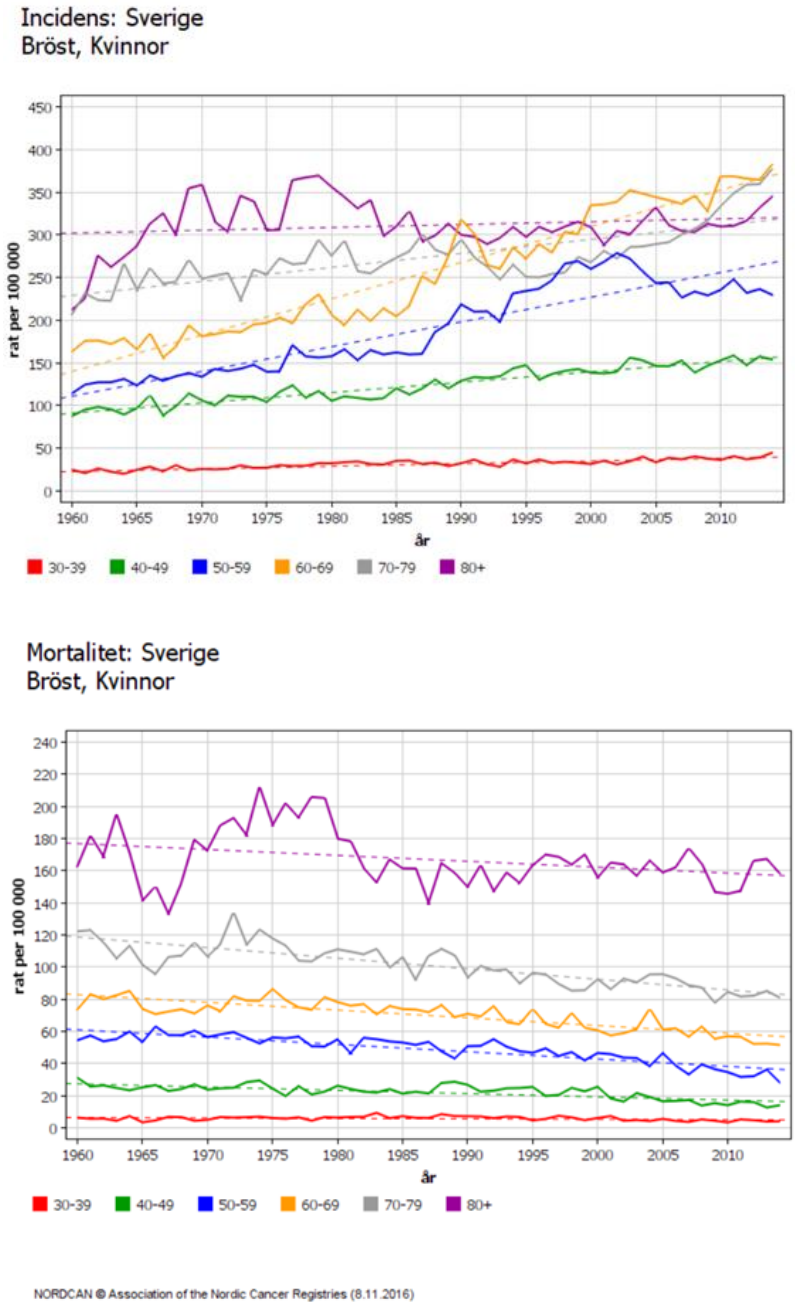

Figure 3. Breast cancer incidence and mortality over time in different age groups[3].

Risk factors

Female sex and increasing age are the most important risk factors for breast cancer[15]. The majority of breast cancers are sporadic, but around 25 % are estimated to be caused by familial factors and 5-10% by single genes[53, 54]. A family history of breast cancer confers a doubled risk compared to the general population, and this risk is higher if the first relative is of a young age[55, 56]. The uncommon highly penetrant breast cancer susceptibility genes, BRCA1 and BRCA2, are the main contributors to heriditary breast cancer[57]. Women with these mutations experience a 10-30 times increased risk of breast cancer[58] and are offered increased surveillance and preventive actions as part of clinical praxis[9]. Moderate penetrant mutations are the focus of efforts such as *The Prospective Registry Of MultiPlex Testing* (PROMPT), a prospective research platform for genetically tested high risk individuals[59]. SNPs have been shown to minimally increase risk, and there are examples of findings of SNPs increasing the risk according to triple negative breast cancer (TNBC) and in BRCA1 associated breast cancer[57], which make SNPs of high interest to include in multi-factorial preventive measures[57]. Regarding gene-environment influence, more research is needed[60].

High socioeconomic status and high educational level increase breast cancer risk[15, 61], partly due to screening attendance and differences in reproductive patterns and exogenous hormone use[62, 63]. Physical activity may reduce endogenous estrogens and has emerged as a protective factor[64-67], and high mammographic density is frequently highlighted as a risk factor [68, 69].Alcohol, previous benign breast disease and prior exposure to radiation are established risk factors, whereas smoking has not yet been established[9, 15, 35].

Reproductive factors

Reproductive factors influence breast cancer risk, are linked to lifetime hormonal exposure and may influence the hormone receptor expression of the tumor[70, 71]. The breast epithelium is considered most sensitive to hormonal stimuli during the period between menarche and first childbirth, and long duration of breast feeding has been associated with a protective effect[72, 73]. The parous breast matures during breast feeding, and it may take several pregnancies for full differentiation[72, 74].

Early menarche, late menopause, nulliparity and high age at first childbirth are considered risk factors[15, 75, 76]. Parity is a protective factor and includes the dual component of short-term increased risk after child birth and long-term protection following child birth[77-79]. Postponing first child birth to a higher age has been estimated to increase the relative risk by 3% for each delayed year[73]. When assessed by breast cancer subtype, reproductive factors have been associated with hormone-receptor positive breast cancer rather than to hormone-receptor negative breast cancer[10].

Anthropometry

Attained height is a risk factor for breast cancer that may be related to childhood energy intake[80], which is however contradicted by the association remaining in richer settings, suggesting genetic causes[81]. Obesity is a well-established risk factor for postmenopausal breast cancer, whereas it is associated with a lower risk of premenopausal breast cancer[82-84]. The increased breast cancer risk in postmenopausal women may be explained by the increased levels of circulating estrogens due to increased peripheral conversion of androgens in adipose tissue and the lower level of SHBG in obese women[35].

Exogenous hormone use

Use of oral contraceptives (OCs) has been associated with a slight risk increase in breast cancer risk, especially for women younger than 20 years using combined OC and up to 10 years afterward[85]. The impact of OC use may depend on age at initiation and timing of initiation in relation to first child birth, duration and type of OC used[86, 87]. For hormone replacement therapy (HRT) use, combined estrogen and progestin HRT was associated with an obvious risk increase, lasting up to 5 years after cessation of therapy[88].

Risk Prediction & Preventive Efforts

During the last couple decades, risk prediction models such as the Gail model have been developed, but they have not reached a wider implementation. The Gail model is based on age, family/reproductive history and previous breast biopsy[89, 90]. Another algorithm based model is the Tyrer Cuzick model[91]. There are three major current clinical guidelines to aid in clinical decision-making.

1) *The US Preventive Service Task Force* guidelines recommend prescription of medication for risk reduction of breast cancer for women aged 35 years or older without prior breast lesions and an estimated 5-year risk for breast cancer of 3% or higher estimated by a modified Gail model and taking careful individual considerations[90, 92, 93].

2) Since 2013, the guidelines from *the American Society of Clinical Oncology*[94] recommend individuals with increased risk for breast cancer to discuss tamoxifen (TAM) or aromatase inhibitors (AIs) as an option of chemoprevention based on their individual risk. Several important barriers were pointed out, and further investigations were requested.

3) *The UK National Institute of Health and Care Excellence* recommend women with a family history and a life-time risk of 30% or higher to be offered TAM or AIs and that women of 17% risk or higher should be considered for the

corresponding treatments and receive information on risk-reducing lifestyle changes[95].

The combination of SNPs with traditional risk factors have so far only had a minimal impact on risk prediction models[96] or to more accurately define risk[97]. A future development of SNPs evaluated in panels together with Gail and other risk models may take place within future prevention trials, either to include or exclude patients or to understand aberrant results[57]. In such settings, mammographic density will probably also take on an important role[68]. Currently in Sweden, the Karma intervention trial "*Karisma 2"* recently opened for inclusion of women attending mammographic screening. *Karisma 2* aims at finding the lowest threshold dose of TAM to reduce breast density while keeping the side-effects as mild as possible. This is the first attempt in optimizing a low-dose (as low as 1 mg TAM) prophylaxis regime for TAM to prevent breast cancer[98].

Clinical breast cancer

Diagnostics

The golden standard for breast cancer diagnosis is triple diagnostics including clinical examination in combination with imaging (mammography, ultrasound or magnetic resonance imaging) and cyto-pathological examination[9]. Mammography screening was introduced in Sweden in the 1980's and today around half of breast cancer patients in Sweden are diagnosed after attending screening mammography[9]. Women between 40-74 years of age are invited to screenings at 1.5-2.0 year intervals[99]. However, around 25% of the diagnosed breast cancers are detected among women outside the screening ages[100].

Prognostic and predictive markers

Clinical guidelines use prognostic and predictive markers to decide whether or not to recommend adjuvant treatment after breast surgery (prognostic markers) and which therapy to choose (predictive markers). The prognostic markers describes the natural history of the disease, whereas the predictive markers relates to the likelihood to respond to a certain treatment[101]. The factors used in the clinical guidelines today are presented below:

Patient characteristics

Age at diagnosis is foremost a risk factor for breast cancer[15] but is also used in clinical guidelines as a guide for the choice of adjuvant treatment. Very young age (<35 years) is associated with unfavorable tumor characteristics and poor clinical outcome. These patients are therefore more prone to receive heavy treatments[102]. In addition, menopausal status will impact the choice of endocrine treatment and to some extent chemotherapy[9]. In contrast, a very high age at diagnosis may confer a poor prognosis independent of tumor stage[103] and may also co-vary with co-morbidity that restrict the treatment choices[104, 105].

TNM classification

The tumor size (T), spread to regional lymph nodes (N) and absence or presence of distant metastasis (M 0/1) is collectively referred to as the TNM-classification[106] and is the most important prognostic factor[101]. Tumor size includes stepwise larger tumors with an increasing risk for recurrence—T1: 1-≤20mm, T2: 21-50mm, T3: >50mm, T4: skin or muscular involvement—irrespective of tumor size. Axillary lymph node involvement (ALNI) equals the number of involved nodes—N0: node negative, N1: 1-3 positive nodes, N2: 4-9 positive nodes, N3: ≥10 positive nodes. The tumor stage refers to the sum of T, N and M and can range from stage 1 to stage 4[107].

Histological grade

The Nottingham histological grade (NHG) includes the scoring of tumor histological parameters that gives an indication of the differentiation of the tumor[108]. The count consists of tubular formation, nuclear pleomorphisms and mitotic count, and the sum of these three parameters represents either grade 1, 2 or 3—here, grade 1 breast cancer has the best prognosis, and grade 3 has the poorest prognosis[108]. Grade 2 is an intermediate group for which additional assessment of progesterone receptor (PR), the proliferation associated antigen Ki67 or gene profiling may facilitate the estimation of patient risk for recurrence[109, 110].

ER and PR

The nuclear transcription factors ER and PR are prognostic factors that are co-expressed to a very high extent in breast cancer. In 2015, ER positivity (ER+) was reported to be 85% in Sweden nationally[111]. PR is regulated by ER, whereby the double-positive tumors (ER+PR+) may be indicative of a functional ER[112]. In the rare ER–PR+ cases, assay problems should be considered in order to avoid wrongly excluding a patient from endocrine treatment[113]. ER is a predictive marker for endocrine treatment response independent of PR according to recent findings[113]. The protective effect on prognosis by ER expression may be lost over the years, and

relapses come late, as opposed to patients with ER– tumors that rather experience early relapses[113].

Proliferation, including Ki67

Deregulation of the cell cycle check points are essential for tumor proliferation, and the net result is cellular proliferation, which is an independent prognostic factor in breast cancer. There are several known markers including the mitotic count, which is part of NHG. Ki67 is expressed in all phases of the cell cycle except for in cell cycle arrest of G0 and thus provide a measure of tumor proliferation. A high Ki67 score is an independent prognostic marker, but its predictive role in response to chemotherapy is less clear[114, 115]. The currently used cut-off for high Ki67 is 20% or a laboratory specific cut-off at two thirds of the samples[116].

HER2

HER2 is amplified in 10-30% of breast cancers and is both a prognostic factor and predictive factor for response to trastuzumab treatment. HER2-amplified tumors have poor prognosis and high risk for metastasis[9, 117, 118]. HER2 assessment is based on immunohistochemistry (IHC) score of 3+ or 2+ if confirmed amplified with *in situ* hybridization (ISH) analysis.

Molecular profiling

Tumors harbor massive amounts of genetic aberrations, and with the introduction of gene expression profiling techniques, it has become possible to study tumor-specific gene signatures more comprehensively. These improvements have rendered predominantly four molecular subtypes with significantly different prognoses: Luminal A, Luminal B, HER2-enriched and basal-like subtype[119, 120]. Luminal A and B predict endocrine treatment response. Luminal B have worse prognoses and benefit from chemo-endocrine therapy, whereas the Luminal A group probably does not[121].

In current Swedish clinical practice, the molecular subtypes are assessed through the surrogate IHC markers established at the St Gallen Consensus meeting; knowingly, they do not completely map groups by gene profiling[116, 121, 122]. In brief, Luminal A are the low-proliferative (Ki67<20%) ER+ tumors, and the Luminal B are the high proliferative (Ki67≥20-30%) ER+ tumors. Additionally, the presence of high or low histological grade in combination with PR status (≤/> 20 %) further discriminates between the two groups. HER2+ tumors belong to the HER2-enriched group irrespective of hormone receptor status. TNBC (ER–PR– HER2–) are considered the basal-like group[122]. In the Southern Region of Sweden currently, the *South Sweden Cancerome Analysis Network - Breast (SCAN-B)* are performing large-scale, real-time clinical profiling with promising results[123, 124].

Treatment

The treatment for the individual breast cancer patient is discussed and decided on at the multidisciplinary conference before and after primary surgery and is based on the available prognostic and predictive markers according to clinical guidelines[9]. In the adjuvant setting, the primary choice of surgery is breast-conserving surgery if the tumor can be radically removed with a good cosmetic result. Large and multifocal tumors are removed by modified radical mastectomy. Breast-conserving surgery followed by radiotherapy reduces local recurrences [125, 126], and these patients have similar survival rates as patients undergoing radical mastectomy[125, 127, 128]. Postoperative radiotherapy may be either local or loco-regional and serves to eliminate micro-metastatic disease. The indication is relative to the 10-year risk of local recurrence if estimated as 20% or higher[9, 128].

The absolute benefit of radiotherapy has been strongly associated with established prognostic markers and is thus interpreted to be related to an "intrinsic" risk of relapse[125, 128]. If the benefits of preventing relapses are summarized after 15 years, one breast cancer-related death is avoided for every four avoided relapses during this time[125, 126].

The sentinel node technique is used to detect micro-metastases in the axilla, and if performed with negative result (no micro- or macro-metastases), no axillary dissection is performed, which safely spares the woman the associated side effects[9, 129]. Unresectable or locally advanced breast tumors are treated with neoadjuvant chemotherapy to reduce the tumor burden prior to surgery, which is also considered for lymph-node-positive patients. In addition, patients with triple negative or HER2-amplified breast cancer are more prone to receive neo-adjuvant systemic therapy[9]. In the metastatic setting, systemic treatments serve to prolong life, and the treatment choice is based on individual risk/benefit evaluations. The selective estrogen receptor down-regulators (SERDs) such as fulvestrant are potent pure ER antagonists and are currently only prescribed in the metastatic setting[9]. Adjuvant systemic treatments are further outlined below.

Endocrine treatment

Endocrine treatment is prescribed to women with invasive and ER+ tumors; in Sweden, this is defined as >10% ER positive nuclei. Additional criteria are tumor size (>10mm) and/or lymph node positive disease. The two main endocrine treatment options are TAM and AIs that act through different mechanisms. TAM is a selective estrogen receptor modulator (SERM) that binds to ER instead of estrogen and opposes estrogenic effects in the breast, whereas it may mimic estrogen in other tissues such as the endometrium. Since it is a pro-drug, it has to be metabolized to endoxifen to become active, a process dependent on CYP enzyme activity, which varies widely between individuals. Co-medications may also affect CYP enzyme

activity. AIs create a low estrogen environment by inhibiting aromatase, which converts androgens to estradiol. This conversion occurs mainly in peripheral adipose tissue and does not affect ovarian hormone production. Thus, AIs are prescribed to postmenopausal women only or to premenopausal women after gonadotropin agonist treatment. TAM, on the other hand, may be prescribed regardless of menopausal status and is prescribed if there is uncertainty on menopausal status[9].

Up until recently endocrine treatment has been prescribed for five years, either as mono-treatment AI or TAM or sequentially with switch after 2 or 3 years. AIs have been shown to be more effective than TAM, especially to reduce local recurrences and contralateral breast cancer as compared to reduction of distant metastases [9, 130].

For TAM, a carry-over effect has been described, indicating a beneficial effect after the 5-year cessation of treatment, with reductions in breast cancer mortality for up to 15 years among both pre-and postmenopausal patients[131]. Similar findings have been described in premenopausal patients in Sweden[132].

Very recently the guidelines were expanded to include up to 10 years of endocrine treatments for breast cancer patients with lymph node involvement[9]. In cases of prolonged treatment, AI is followed by TAM since AIs are not recommended above 5 years[133].

Chemotherapy

Adjuvant postoperative chemotherapy reduces the risk of poor clinical outcome from potential early microscopic disseminated tumor cells. It is given to women with lymph node positive disease or to women with other molecular subtypes than Luminal A and if other risk factors are present such as low age, TNBC or HER2 amplification in spite of small tumor size[9]. The first-choice chemotherapy is anthracycline in combination with a taxane (sequential) if it is tolerated with regard to heart disease and other co-morbidities. Poly-chemotherapy has proven superior to mono-therapy with anthracyclines alone and to CMF (cyclophosphamide, methotrexate, fluorouracil)[9, 134, 135].

Targeted therapy

Targeted treatment refers to the targeting of specific molecules with therapeutic agents such as the monoclonal antibody trastuzumab that target the extracellular receptor portion of the oncogene human epidermal growth factor receptor 2 (HER2). Trastuzumab has very successfully improved survival for patients with HER2-amplified tumors[136]. According to clinical guidelines, treatment is given concomitantly with chemotherapy, after which treatment with trastuzumab continues for a total of one year.

Breast carcinogenesis

Carcinogenesis, or tumor development, is a multi-step process including genetic and epi-genetic changes by which a normal cell is transformed to a cancer cell that is self-sufficient in growth signaling. The capacities that a cancer cell acquires were described by Hanahan *et al*. as the Hallmarks of Cancer[137, 138]: the ability of growth, invasion, survival, angiogenesis, avoiding immune destruction and deregulating of cellular energetics. These abilities are facilitated by tumor-promoting inflammation and genome instability and mutation. Another important evolutionary aspect is that different cell populations may co-exist in the same tumor, referred to as tumor intra-hetererogeneity[139, 140]. In recent years, large research efforts focus on the interaction between divergent tumor cell clones and/or tumor stem cells and their interactions with the tissues surrounding the tumor, such as stroma and extracellular matrix signaling, referred to as the tumor microenvironment[141, 142].

In the breast, most cancers originate from the terminal duct lobular unit (TDLU), which secretes the milk[143]. One model depicts cancer progression from the *in situ* component to invasive cancer. The tumor, which most often originates from the inner lining of the luminal epithelial cells, expand through the outer myoepithelial layer and through the basement membrane, enabling cell migration[141, 144].

Androgen and estrogen receptors

From normal breast to breast cancer

The main site for estrogen action in the breast is thought to be in the epithelial cells[143]. ER is expressed at low levels, around 10-25%, in the TDLU of the normal breast[143, 145-148]. Specifically, ER expression has been reported in luminal epithelial cells but not in stromal cells, however contradicted by one report of ER expression also in stromal cells[29]. Knockout mice models have shown ER to be important for normal breast development[149], whereas mouse models lacking ERβ have showed more subtle changes such as increased proliferation and decreased differentiation[150, 151]. In normal breast development, ER+ cells in the breast rarely proliferate, and when proliferation does occur, it is mainly the large amount of surrounding ER– cells that proliferate, which is thought to be driven by ER+ paracrine signalling[143, 146, 152]. In these non-malignant circumstances, the genetic landscape of the breast may be described as homogenous and the microenviroment as structured, and as a cancer develops these features turn heterogenous and unstructured[57].

After breast cancer initiation, which is still unsufficiently understood[153], ER expression increases as the cancer progresses[146, 148, 154], and ER posititivy may rise to over 80%[111]. The opposite has been described for ERβ. ERβ expression was reported to be widespread, at 70-80%, in the normal breast both in luminal and myoepithelial cells, as well as in stromal cells, endothelial cells and leukocytes[147, 152, 155]. ERβ expression then decreases in *in situ* tumors and furthermore in invasive cancers[29, 148], possibly due to inactivation by promotor methylation[156]. In patients with atypias of the breast, ERβ expression was reported associated with a lower risk to develop breast cancer[157]. ER is considered proliferative, and ERβ is considered antiproliferative. The interaction between the two estrogen receptors may occur through the forming of heterodimers[158], since ER has been shown to co-localize in the same nucleus as ERβ in human breast tumors[159]. Contrary results of no co-localization has also been reported[148]. AR has also been shown to co-localize with ER and PR in breast epithelial cells[160], which highlights that the interaction between these hormone receptors may be of importance.

AR expression was reported in luminal epithelial cells but not in myoepithelial cells or stroma[160]. However, more recent findings showed presence of AR also in stroma, fibroblasts and adipocytes[29, 152]. Normal AR signaling is an important inhibitor of ER. Mouse models have shown that impeded androgen and AR signaling have proliferative effects on the breast and may induce tumor formation[161]. AR expression is expressed at approximately 20% in the normal breast[152], and in accordance with ER, expression is higher in breast cancer cells compared to normal cells[162]. AR expression is well preserved in the metastastatic setting, and around 25% expressing AR as the sole hormone receptor[163-165]. The "homeostatic attempt" presented by Hickey *et al*. describes how AR expression successively rise during ER+ breast cancer progression in order to maintain the physiologic homeostasis normally seen between estrogens and androgens. If the upregulation of AR is insufficient, the net result will be a growth advantage and progression of disease[161].

Androgen receptor

Androgens have been used historically as treatment for breast cancer but with troublesome androgen side-effects[166-168]. Following the introduction of TAM, focus shifted away from androgens to estrogens during the last few decades. Recently, and especially since Sørlie & Perou *et al*. described the intrinsic molecular subtypes[119], there has been a revival of AR breast cancer research, particularily regarding AR's potential as a prognostic and predictive marker and as a target for treatment adopting modern agents from the treatment of prostate cancer. In prostate cancer, AR is a key oncogenic driver, and different kinds of androgen deprivation are important treatments[161, 169].

Distribution

Around 70% to 90% of all primary invasive breast cancers are AR+[170-173]. AR has often been co-expressed with ER and PR and correlated with low histological grade[170, 174-177]. In TNBC, AR+ was reported on average in one third, but the frequencies vary between studies approximately in the range of 12-55%[171, 172, 178-181]. Based on gene expression profiling, subtypes that characteristically are AR+ have emerged: Among ER– tumors, the *HER2 enriched* subtype is the most likely to express AR. The subtype *Molecular apocrine*, characteristically has high AR expression and a luminal profile inspite being ER–[182, 183]. This group is often HER2 amplified, but when it is not, it is similar to the *Luminal AR* group[38, 182, 184-186]. *Luminal AR* (LAR) is AR+ TNBC, and further subdivisions have been suggested[184, 187]. Finally, a quadruple-negative subtype has been proposed (ER–PR–HER2–AR–) and that AR should be evaluated in clinical routines[181].

Mechanisms of action

Androgens exert direct antiproliferative effects on breast cancer cells through the binding to AR and have indirect proliferative effects through its aromatization to estrogens. AR is a transcription factor regulating gene expression and has been shown to inhibit ER dependent proliferation[176]. Preclinical studies have been divergent in their results, ranging from androgens having inhibitory to stimulating effects on breast epithelium[188]. Inconsistencies may be caused by a lack of cell line models expressing AR, especially as the sole hormone receptor[189]. Other co-existing mutations and estrogen-dependent models may also interfere with the results[189].

Interestingly, AR has been found to compete with ER in the binding of estrogen response elements[176], and ERβ has been implicated in the interaction between AR and ER[190]. In one model of suggested AR mechanisms, AR acts as a tumor suppressor in ER+ breast cancer and as an oncogene in ER– breast cancer[161]. Furthermore, complex crosstalk between different signaling pathways depending on breast cancer subtype may occur, as illustrated in the model recently presented by Pietri *et al*.[191] in Figure 4.

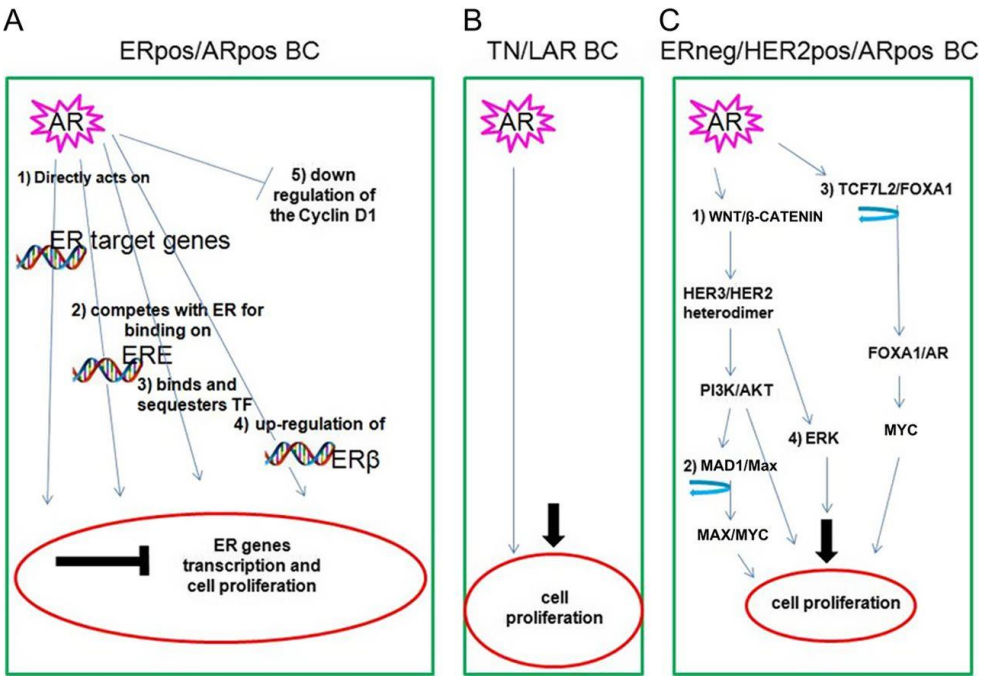

Figure 4. AR signaling effects in breast cancer subtypes.
(A) ER-positive/AR-positive breast cancer: (1) AR directly inhibits ER target genes. (2) AR competes with ER for binding on ERE. (3) AR binds and sequesters TF. (4) AR upregulates ERβ receptors. (5) AR induces direct downregulation of cyclin D1 gene expression.

(B) TN/LAR BC: AR drives tumor progression.

(C) ERneg/HER2pos/ARpos BC: (1) AR directly upregulates WNT7B, which acts on WNT/β-CATENIN, stimulating HER3 gene transcription with subsequent HER3/HER2 heterodimerization and modulation of PI3K/AKT pathway. (2) HER2/ HER3 heterodimers activate PI3K/AKT pathway, which phosphorylates MAD1, promoting its degradation and dissociation from Max with subsequent MYC-MAX heterodimerization and access to transcriptional sites. (3) AR induces dissociation of repressor transcription factor 7-like 2 (TCF7L2) from the pioneer transcription factor of AR, FOXA1, promoting AR target gene MYC transcription. (4) AR induces ErbB2 expression, which activates ERK with consequent cell proliferative effect.

Selected abbreviations: AR, androgen receptor; ER, estrogen receptor-α; ERE, estrogen response elements; HER2, human epidermal growth factor-2; LAR, luminal AR subtype; PI3K, Phosphatidylinositol 3-kinase pathway; TF, transcription factors; TN, triple negative breast cancer;

Prognosis and prediction

In relation to prognosis, AR has mainly been described as a positive prognostic factor and often within ER+ breast cancer[172, 176, 192-197]. Among ER– and TNBC breast cancers, findings have been contradictory with poor[172, 194], good[198] or no influence on outcome[176, 193, 197]. The LAR subtype has been reported to have poor pathological response following neoadjuvant chemotherapy[199]. AR has been suggested to be an oncogenic driver in Molecular apocrine subtype[38, 200] and in

LAR subtype[187]. Conversely, the poor treatment response to chemotherapy has been suggested to be related to low proliferation[181, 201].

In terms of treatment prediction, AR has been suggested to be a marker of endocrine treatment response in ER+ breast cancer[202] and there is one randomized study that reported AR to predict endocrine treatment response in ER– breast cancer[173]. However, the majority of evaluations have not been randomized for treatments. Also, preclinical findings have suggested the opposite: a potential role of AR overexpression in TAM resistance[203, 204]. Other preclinical findings have suggested that AR may interact with the cyclin-dependent kinase inhibitor p21 via an androgen responsive element in its promotor. In turn, p21 has been suggested to be affected by ER signaling and to be required for sensitivity to TAM treatment and for signaling through the MAPK pathway[189, 205-207].

AR as a therapeutic target

The largest potential for AR in future clinical breast cancer is probably as a therapeutic target. As of today there is no consensus on what agent to use in specific breast cancer subtypes[208]. Substances currently evaluated in clinical trials of different phases are listed below[191, 208-210], and several other agents are under development[169].

Agonists include selective androgen receptor modulators (SARMs), such as enobosarm/GTx024, a non-steroidal, tissue-selective AR modulator[211, 212]. Antagonists include antiandrogens of first generation, bicalutamide[213] and the more specific and potent second generation enzalutamide (formerly MDV3100)[214-218]. The CYP17A1 inhibitor abiraterone acetate, which suppresses androgen production, is another example[219].

To summarize, there are still critical gaps in the understanding of AR cellular function, especially in relation to treatment response. As of yet, there is insufficient clinical experience regarding AR's role from the different breast cancer subtypes.

Estrogen receptor β

ER was assumed to be the sole ER until the discovery of ERβ in 1996[220]. ERβ is a gene product from another chromosome than ER, but has high homology with ER except for in the NTD (Figure 2). Estradiol have similar affinity for both receptors, and ER and ERβ may bind similar DNA response elements[23]). The understanding of ERβ's role in breast cancer has been complicated by non-standardized methodology and ERβ's various isoforms[221-224], of which at least ERβ isoform 2, ERβ2/cx, has been identified in human breast tissues[222 , 225]. Also, cell line studies

of ERβ function have been hampered by a lack of models expressing endogenous ERβ[224].

This thesis investigates the first discovered, full length, wild-type isoform 1, ERβ1, which has been reported to be the only fully functional isoform[226]. The other isoforms lack an LBD (Figure 2), but may in accordance with ERβ1 act through other pathways such as non-genomic interactions[26].

Distribution

ER and ERβ has been shown to have separate functions in different organs[29]. ERβ expression has been identified across breast cancer subtypes; however, cutoffs vary markedly between studies[227]. In one report, ERβ1 expression was evenly distributed at approximately 55% across the molecular subtypes. Therein, ERβ1 was associated with smaller tumor size and low Ki67 in the overall population and among lymph node negative patients, whereas ERβ1 expression among node-positive patients were associated with poor outcome[228]. Another study reported significant differences in ERβ1 expression depending on molecular subtype: ERβ1 was more common among Luminal A and Luminal B tumors, ERβ1+ of approximately 70%, as compared to around 55% in HER2 enriched or basal-like tumors[229].

Mechanisms of action

ERβ is thought to act through the formation of homodimers, or heterodimers with ER[230]. ERβ is considered an inhibitor of ER-driven proliferation[231] and has been suggested as the main driver for clinical heterogeneity among ER+ tumors[232]. ERβ may attract different co-factors than ER does[233] and they have been suggested to impact proliferation through diverse signaling pathways[29].

In similarity with AR, a bi-faceted role of antiproliferative vs proliferative impact has been suggested for ERβ, depending on the prescence or absence of ER[26, 224, 234, 235], and, as in the case of AR, no consensus or solid conclusion may yet be drawn. Recently, when ERβ's role in TNBC subtypes has been addressed, ERβ has been suggested as a beneficial marker rather than being an aggressive feature[236-238].

Prognosis and prediction

In terms of prognosis, the lack of standardized methodology complicates a comprehensive summary of the field. Recent meta-analyses reported ERβ1 as a positive prognostic marker, either irrespective of ER expression or foremost among ER– tumors [227, 239].

In terms of treatment prediction, the main focus has been the potential of ERβ improving endocrine treatment response[240]. It has been suggested to predict

endocrine treatment response also in the ER– setting[241], but the role of ERβ in endocrine treatment response still needs further clarification[151].

ERβ as a therapeutic target

To date, clinical evaluations of ERβ as a therapeutic target are less common than AR targeting. There is a multitude of agents in early development[242-244]. The main proposed principle is selective ERβ agonistic actions, either through development of novel specific agonists or as phytoestrogens such as genistein, which is present in soybeans[245-247]. Soy isoflavones, of which genestein is one, have been indicated to reduce breast cancer risk[248, 249], but there has also been controversies regarding its protective effect [250, 251].

So far, good tolerability for selective ERβ agents have been found in trials of other diseases[252, 253]. Promising such agents have also been evaluated for menopausal symptoms [254, 255].

Background to this thesis

In the years prior to when the projects of this thesis were initiated, the common genetic variant SNPs were top research priorities in cancer research. They brought a renewed spark of interest and potential to the study of conventional risk factors for disease, since SNPs may modify the susceptibility to exposure, disease and treatment response. The Breast Cancer and Blood Study (BC Blood) in Lund, which focused on investigating genetic and environmental associations with breast cancer prognosis had been ongoing for the past nine years. Therein, *AR* genotypes, which had been high-lighted in the field of prostate cancer for quite some time, were investigated with the exciting finding of a potential predictive role for TAM response among women with primary breast cancer[27]. The Malmö Diet and Cancer Study (MDCS) was a unique local source for cancer risk analyses with a huge database of baseline characteristics of more than 17,000 women and tumor material in tissue microarrays (TMAs) from over 700 patients. MDCS had (and still has) an active breast cancer researcher network and had at that time frequently published on reproductive risk factors[256, 257], exogenous hormone use[258] and anthropometry measures[259, 260], which are all known to be related to hormone levels. The interest in obesity highlighted the modifiable risk factors and preventive medicine, and the group had an ongoing window-of-opportunity trial with statins[261, 262] as a starting point for further evaluation of statins in the preventive measure[263]. Endocrine response or resistance was evidently a clinical problem, and at large international breast cancer research meetings, both AR and ERβ were reported to be of potential interest in terms of endocrine treatment response. And so, this project came about.

Aims

Overall aim

To examine the significance of novel hormone receptors in primary invasive breast cancer in terms of risk and prognosis in general and according to treatment type. Specifically, the impact of the androgen receptor (AR) and the estrogen receptor-β isoform 1 (ERβ1) was studied.

Specific aims

AIM 1 To analyze the association between hormone-related lifestyle factors and AR-defined breast cancer risk (Paper I).

AIM 2 To evaluate tumor AR expression in relation to patient characteristics and established clinicopathological markers (Paper I-III)

AIM 3 To study tumor AR expression as a prognostic factor alone and in combination with estrogen receptor-α (ER) expression (Paper II-III).

AIM 4 To evaluate tumor AR expression as a prognostic factor according to treatment type (Paper II).

AIM 5 To examine if the previously studied germline *AR* genotypes were associated with tumor AR expression (Paper II) and if *estrogen receptor 2* (*ESR2*) genotypes were associated with tumor ERβ1 expression (Paper IV).

AIM 6 To evaluate tumor ERβ1 expression in relation to patient characteristics and established clinicopathological markers (Paper IV).

AIM 7 To study tumor ERβ1 expression as a prognostic factor alone and in combination with ER expression (Paper IV).

AIM 8 To evaluate tumor ERβ1 expression as a prognostic factor according to treatment type (Paper IV).

Patients and Methods

The papers in the thesis are based on the two cohorts described below, followed by sections on the different methods used.

The Malmö Diet and Cancer Study (Paper I and III)

MDCS is a population-based cohort initiated to better understand the relation between diet and cancer[264]. Men and women from specific birth year cohorts were invited through public advertisement in newspapers and by personal invitation to participate in the study[265]. Of 74,138 residents, a total of 68,905 eligible individuals were invited. The inclusion criteria were sufficient Swedish skills and mental abilities sufficient to understand the extensive questionnaire[264]. Of the 28,098 enrolled individuals that completed all study parts, 17,035 were women born between 1923 and 1950[266]. Baseline examinations took place between 1991 and 1996, and all enrolled individuals visited the study center twice. The first visit included baseline examinations, and participants received instructions regarding the questionnaire; at the second visit, the questionnaire was returned, and an interview on dietary habits was performed. The questionnaire included data on sociodemographic, reproductive and lifestyle factors as well as medication and health status. In addition, body measurements, and blood samples were collected. Measurements of bio-impedance were registered (BIA 103, RLJ-Systems, Detroit, MI, USA) to calculate body fat percentage. Written informed consent was obtained from the participants, and the study was approved by the Lund University Ethics Committee (Dnr 652/2005, Dnr 166/2007). The following further ethical considerations were undertaken. Following the period of participant enrollment, when additional analyses were considered, advertisements were published in local newspapers informing on the possibility to withdraw from further analyses. The analyses of the present papers were covered by the previous ethical approvals, and no new contacts were deemed necessary. A formalized application process to the MDCS directory board precedes data withdrawal from the data manager office, which distributes data by sequence numbers only.

On a yearly basis, linkage to the South Swedish Regional Tumor Registry, the Swedish Cancer Registry and the Swedish Cause of Death Registry is performed in order to identify incident breast cancer cases, vital status and cause of death. In Paper I, invasive breast cancer diagnosis according to AR status +/– was the endpoint. In Paper III, the primary endpoint was breast cancer mortality, defined as incidence of breast cancer-related death, specifically when breast cancer was the cause of death or the contributing cause of death. The secondary endpoint was all-cause mortality, defined as incidence of death from any cause. Hospital records including pathology reports were accessed to retrieve information on clinicopathological information and information on treatments.

The individuals included in each paper are outlined in Figure 5. In Paper I, the risk of developing invasive breast cancer was assessed among women without a prior breast cancer diagnosis. At the end of follow-up as of 31 December 2007, a total of 826 women were diagnosed with breast cancer, of which 747 had an invasive cancer. Due to the lack of tumor tissue to be retrieved for research purposes as well as due to prior sectioning of the TMA, the total amount of tumors for which AR expression was evaluated was 516 tumors. In Paper III, which addressed prognosis following invasive breast cancer diagnosis, a case-only analysis was performed including all individuals diagnosed with invasive cancer until 31 December 2010. In total, 1,016 women were diagnosed with breast cancer, and 948 tumors were invasive. Follow-up was extended to 31 December 2014. The following further exclusions were made: 1) Patients with bilateral cancers were excluded due to difficulties in evaluating the relation between tumor characteristics and prognosis (n=17). 2) Patients with breast-cancer related death 3) or disseminated disease ≤0.3 years to baseline (n=2 and n=14 respectively) were also excluded to ensure that the prognostic information from the tumor was not based on the metastatic setting. 4) Patients who received neoadjuvant treatment that effects the possibility to asses AR post-treatment (n=4) were also excluded. 5) Finally, one patient was diagnosed with invasive breast cancer four years prior to the operation, and medical record evaluation revealed that the patient denied all treatment. This patient was excluded as her treatment schedule deviated substantially from standard clinical care, consequently jeopardizing the clinical outcome of the breast cancer diagnosis. Thus, the maximum possible number of patients to include in survival analyses was 910 patients. Importantly, due to previous sectioning of the TMA used in Paper I, a new TMA was constructed for this study, covering incident breast cancer cases during all years from 1991 to 2010. Thus, the tumor tissues available do not completely overlap the TMA in the earlier study. Among the 910 patients, tumor tissue was available for 718 tumors, and 671 of which were successfully evaluated for AR expression.

The Breast Cancer and Blood Study (Paper II and IV)

The BC-blood study is an ongoing cohort study at Skåne University Hospital, Lund, that explores genetic and non-genetic factors in relation to primary breast cancer prognosis. The study is considered population-based since patients are not referred to other hospitals for surgery. The study was initiated in October 2002, and patients are included at their pre-operative visit. Exclusion criteria are previous breast cancer diagnosis or other cancer within the last ten years. At baseline, an extensive questionnaire on lifestyle factors, reproductive history and medications is collected, body measurements are taken and blood samples collected. Blood samples are centrifuged and frozen at -80 °C within two hours of sampling. All samples are labelled with serial codes to enable blinded analyses. Follow-up visits take place after 3-6 months, 7-9 months and after 1, 2, 3 years postoperatively, after which questionnaires are requested by mail biannually. Written informed consent is obtained from all participants, and the study was approved by the Lund University Ethics Committee (Dnr LU75-02, LU37-08, LU658-09, LU58-12, LU379-12, LU227-13, LU277-15, and LU458-15).

Hospital records including pathology reports are accessed regularly to retrieve information on clinicopathological information and information on treatments and breast cancer events. New cancers and date of death were obtained from patient charts, the South Swedish Regional Tumor Registry and the population registry, respectively.

The patients included in Papers II and IV are outlined in Figure 6. Both these studies emanated from the same cohort and had the last date of follow-up by 30 June 2014. During the study period, 2,170 patients underwent surgery for breast cancer at Skåne University Hospital in Lund. Of these, 1,116 were enrolled in the BC-blood study. Among the 1,116 women, 1,026 had invasive breast cancer that was not pre-operatively treated. TMA assessments were successful in 913 and 911 cases, respectively (Paper II, IV). Since distant metastasis screening takes place postoperatively, the final study cohort excluded patients with breast cancer events ≤0.3 years from baseline to ensure that the prognostic information based upon the primary tumor was not influenced by the primary metastatic setting. Thus, the final study cohort consisted of 905 and 903 patients, respectively (Paper II and IV).

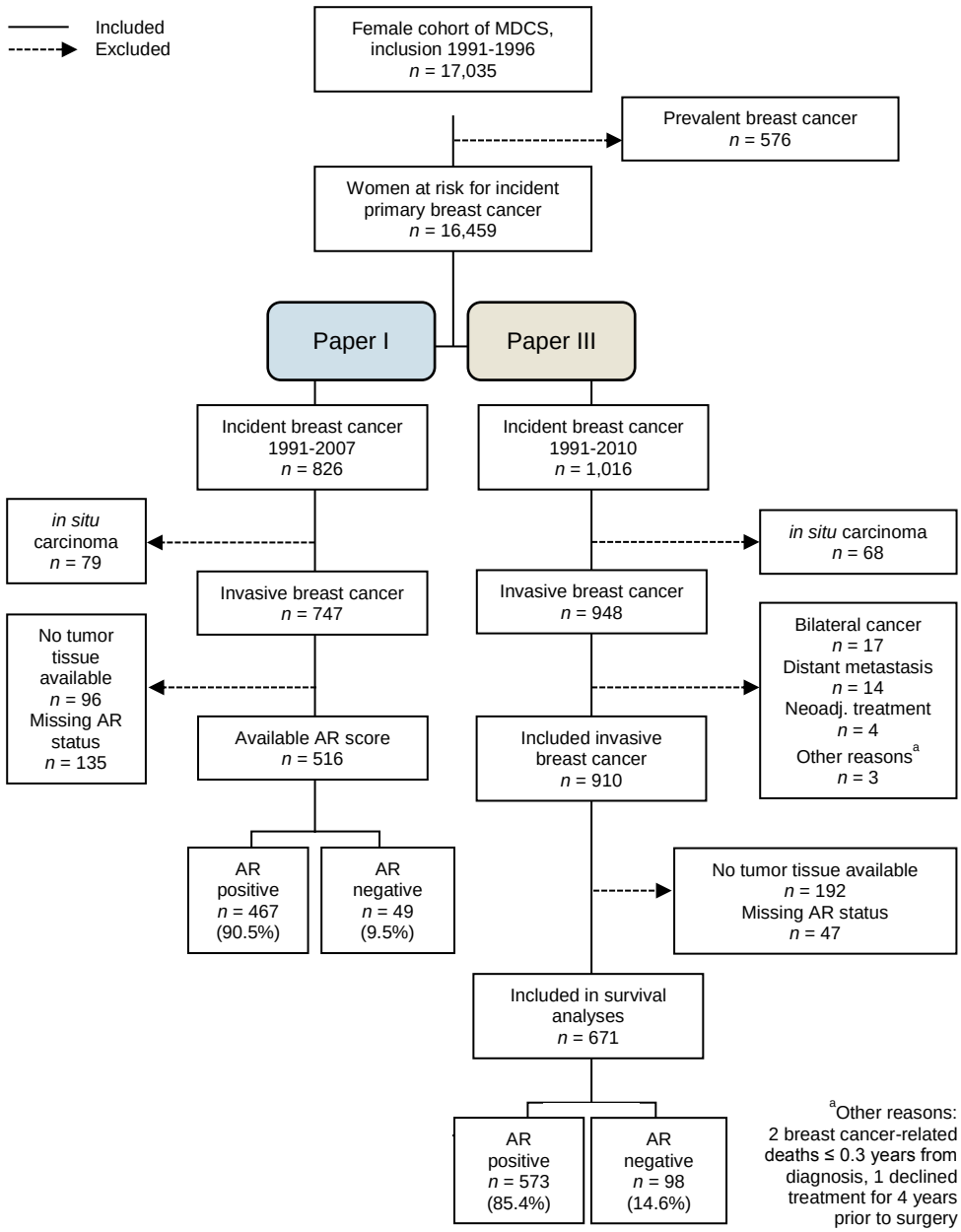

Figure 5.
The Malmö Diet and Cancer Study (MDCS). Patients included in Paper I and III.

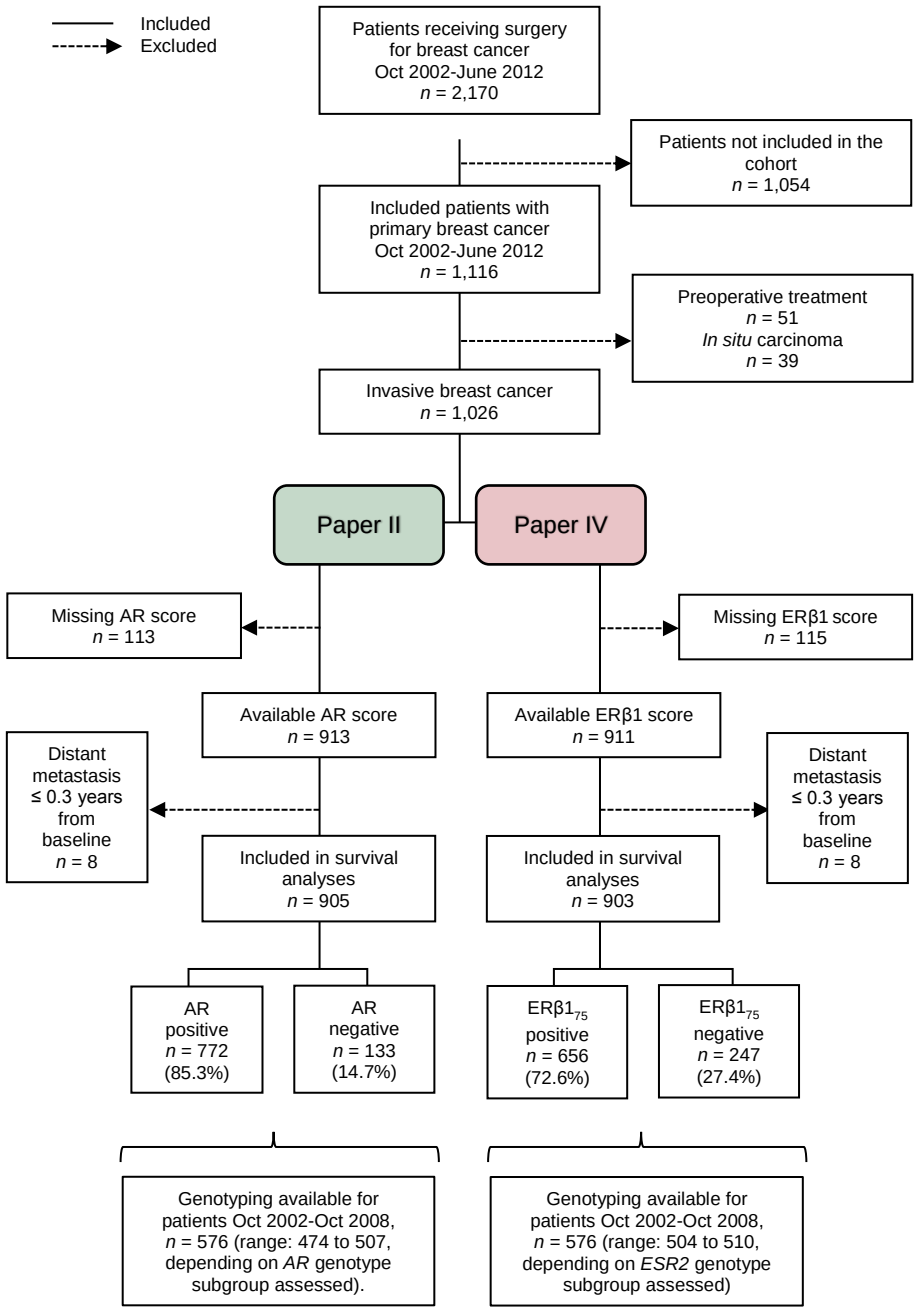

Figure 6.
The Breast Cancer and Blood Study (BC-blood study). Patients included in Paper II and IV.

Tissue microarray

TMAs were used for immunohistochemial analyses (Paper I-IV). An overview of the principle of TMA is provided in Figure 7. Cores of formalin-fixed paraffin-embedded tumor tissue from the archived tumor were inserted in a recipient paraffin block from which new slides were cut and new stainings performed. Duplicate cores were used, and the core size was 0.6 mm in diameter (Paper I, 1991-2004) or 1.0 mm (Paper I 2005-2007, and entire Paper II, III and IV). In Papers II and IV, a semi-automated TMA-arrayer (Pathology Devices, Westminister. MD, USA) was used, and in Papers I and III, a manual tissue arrayer was used (Beecher, Sun Prairie, WI, USA).

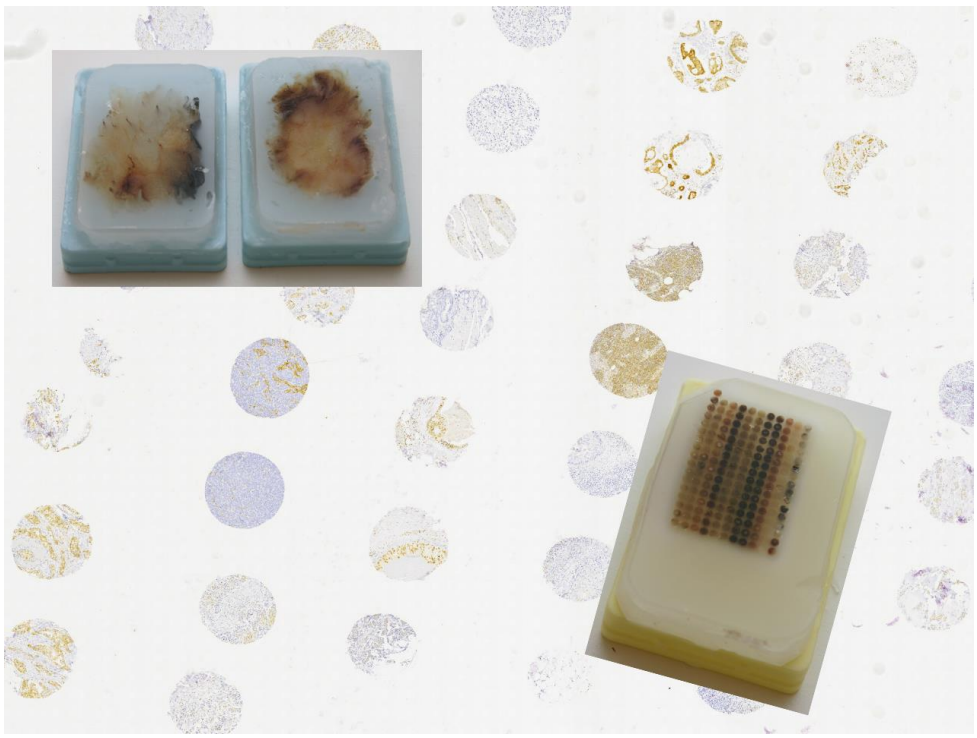

Figure 7. The tissue microarray technique.

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Histopathological analyses

Information on tumor size, ALNI, histological grade, IHC markers (ER, PR, Ki67, HER2) and HER2 amplification data were collected previously, as described in more detail in each paper. Of note, the MDCS cohort included tumors diagnosed 1991-2010, and all MDCS tumor specimens 1991-2004 were re-evaluated by one pathologist. Also, IHC markers were from TMA assessments up to 2007 (Paper I and part of Paper III). The BC-Blood Study, which was initiated in 2002, was designed to collect clinical data from medical records and pathology reports from the beginning. Certain factors such as HER2 status and Ki67 were not implemented in clinical routine until later and are therefore only available for part of the cohort.

Immunohistochemistry

IHC assesses protein expression in tissue. Specific antibodies are tagged with a visible label and interact with target antigens, resulting in a visible brown stain within the histological tissue context. Some pros of IHC are its use on formalin-fixed tissue (i.e., readily accessible) and on TMAs (low amount of tissue needed). The suggested cons are antibody selection, risk for poor fixation (variation of staining quality) and lack of standardized methods of evaluation.

For IHC analysis, 4-μm sections were automatically pretreated using the PT Link system and stained (Autostainer Plus, Dako, DK) for the monoclonal AR antibody Ab-1 (clone AR441, dilution 1:200, Thermo Scientific) and the ERβ1-specific monoclonal antibody M7292 (clone PPG5/10, dilution 1:20, Dako), respectively.

Microscopy assessments

Similar principles were applied for the IHC assessments in all papers. Semi-quantitative scoring of tumor receptor fractions (0, 1-10%, 11-50%, 51-75% and 76-100%) of positively stained nuclei were performed and dichotomization to positive/negative (high/low) was performed at either >10 or >75%, as outlined in each paper. If the duplicate cores were discordant, the fraction of positively stained nuclei was estimated by visual inspection across both sampled cores. In Papers I, II and IV, intensities (none, weak, moderate, strong) were documented but not used in analyses. In these papers, a light microscope was used, and analyses were repeated two times independently by KE. In case of discrepancies, a third evaluation was made together with a co-worker to reach consensus. For Paper III, which was performed last in the sequence of studies and as the third paper on AR expression,

assessments were performed by KE one time only and using the digital pathology platform PathXL (http://www.pathxl.com, PathXL Ltd., UK).

Genetic analyses

In Paper II and IV, respectively, the previously studied *AR* and *ESR2* genotypes[27, 28] were assessed for associations with the corresponding protein expression in the tumors. The genetic analyses were performed previously (Oct 2002 - Oct 2008), which at baseline included 634 individuals or 576 after the exclusion of in situ tumors and patients that received pre-treatment prior to surgery.

In brief, genomic DNA was extracted from the leukocyte portion of whole blood using a Wizard genomic DNA purification kit (Promega, Madison, WI, USA). Genotyping was performed by two methods for the different SNPs, sequencing and TaqMan, via the manufacturer's protocol in laboratories at the Region Skåne Competence Center in Skåne University Hospital Malmö. For quality control, duplicate samples were run in over 10% with 100% concordance[27, 28].

The *AR* gene is located on the X chromosome (q11-12), meaning that women have two copies. The *ESR2* gene is located on chromosome 14 (q21-22). The locations of the SNPs are illustrated in Figure 2. The assessed SNPs were the following: In Paper II, the six haplotype tagging single nucleotide polymorphisms (htSNPs) in the *AR* (rs1337080, rs17302090, rs6152, rs7061037, rs5031002 and rs5964607) had been selected since they were shown to capture 95 % of the *AR* haplotypes among men[267]. Five common associated diplotypes were identified. Diplotypes present in <1% of the patients were clustered together into a composite group of rare diplotypes. Seven patients were missing due to failed SNP analysis[27]. In Paper II, with analyses of *AR* genotypes in relation to tumor AR expression, the number individuals ranged from 474 to 507 depending on the *AR* genotype subgroup assessed.

In Paper IV, the selected htSNPS (rs4986938, rs1256031, rs1256049 and rs3020450) of *ESR2* were chosen since the National Cancer Institute's Breast and Prostate Cancer Cohort Consortium had identified these to tag the six major haplotypes of the *ESR2* gene[268]. The four htSNPs were associated with seven haplotypes and eight diplotypes. Due to a lack of information on genotypes, haplotype construction was solved by imputation in two cases, leaving 8 cases without a haplotype. Diplotype construction could not be performed for 10 patients. The diplotype variants present in <5 % of patients were classified as rare. In Paper IV's analyses of *ESR2* genotypes in relation to tumor ERβ expression, the number of individuals ranged from 504 to 510 depending on the genotype subgroup assessed.

Statistical analyses

The main statistical analyses in all four papers are survival analyses, which were performed either to study risk of developing breast cancer (breast cancer according to tumor-specific AR expression as the event, Paper I) or prognosis (specified breast cancer-related events, Papers II-IV) and stratified by tumor receptor expression. Subsequently, the baseline characteristics (continuous or categorized variables) were derived from either healthy individuals (Paper I) or breast cancer patients at diagnosis (Paper II-IV), as described in details in each paper. Distributional differences between groups were calculated as appropriate depending on variable type (Chi-2 test, Fishers exact test, Mann Whitney U test, or logistic regression) and are presented with the associated *P*-values and/or odds ratios (OR) and 95% confidence intervals (CI). Two-way interaction terms were included in the logistic regression models to address whether the association between two factors was modified by the presence or absence of a third factor.

Univariable survival analyses were performed by log-rank test (Paper I-IV) and presented by Kaplan Meier estimates (Paper I, II and IV) or cause-specific cumulative mortality graphs (Paper III). These methods can, for each factor studied, be used to describe the relationship between time to event studied and factor level and to test the null hypothesis of equality of the survival or cumulative incidence functions. To obtain effect measures (i.e. hazard ratios) univariable and multivariable Cox regression analyses were used. Any skewed continuous variables were ln-transformed or categorized prior to use in multivariable analyses.

In Paper I, adjustments were made for potential confounders for breast cancer risk. In Papers II-IV, the potential confounders were mainly established prognostic factors. Interaction terms were included in multivariable Cox regression models to evaluate the evidence for differential effect on survival for one factor depending on the level of another factor. Therein, the adjustments for potential confounders were performed stepwise using several models in order to better understand the contribution or impact of potential confounders in relation to the impact of the studied receptor on outcome (Papers II-IV). In Paper III, the assumption of proportional hazards was formally tested by Schoenfeld's test and by visual inspection of log minus log survival curves. Since the criteria of proportional hazards was not met in the overall follow-up, further analyses were performed for subdivided time frames in which the assumption was better fulfilled.

In Paper III, *P*-values were interpreted as level of evidence against each null hypothesis, whereas in Papers I, II and IV, *P*-values less than 0.05 were considered significant. All tests were two-sided, and nominal *P*-values without adjustments for multiple testing are presented.

All statistical analyses were performed in SPSS 19.0 and 22.0 (IBM), with the following exceptions: cause-specific cumulative mortality (Paper III) was estimated using the user contributed program stcompet.ado for the statistics package Stata version 14.1 (StataCorp LP, College Stataion, TX, USA). Stata was also used to draw the cumulative mortality graphs and to test proportional hazards assumptions (Paper III).

Methodological considerations

The findings (i.e. statistical associations) described in this thesis have, in addition to true associations, two main alternative explanations: chance or bias. Chance, or random errors, is related to study precision. By increasing the sample size, the impact of chance will be reduced, but this does not reduce the effect of bias, which is a systematic error. One common source of bias in observational studies is residual confounding of the effect of main interest. A confounder is a factor associated with both exposure and outcome that is not caused by either of them, and residual refers to a factor that is not included in the statistical modelling procedure. Bias of this kind, as well as other forms of bias, will affect the external validity a study, i.e., its generalizability. Another type of bias is information bias, which may either bias the results towards the null or away from the null hypothesis. The accuracy of the study depends on the total error, i.e., both the validity and the precision.

Below, the study methodology is discussed from the viewpoints of 1) study design, 2) precision and 3) validity. Table 1 at the end of this chapter summarizes the strengths and limitations for each paper.

Study design

In the population-based prospective MDCS (Paper I and III), 40% of the invited women were enrolled in the study[264]. The educational level was higher and the percentage of foreign-born women were lower compared to the background population of women living in the city of Malmö during this period[266], which may limit the generalizability of the study. However, one also has to consider that breast cancer incidence varies nationally in Sweden, i.e., there is a higher incidence of breast cancer in southern Sweden[269]. Following the years of MDCS inclusion (1991-1996), the breast cancer incidence was higher among the participants compared to the background population, whereas breast cancer mortality was lower. This may reflect a higher amount of screening-detected tumors and better health among MDCS-participants compared to non-participants[266]. Since the distribution of the clinicopathological characteristics were distributed in accordance with previous studies, we consider it reasonable to make internal comparisons with

relative risks, which are less sensitive to the potential selection bias of higher health-concern than, for example, the study of incidence rates and prevalence would have been. However, women entering the study at age 60 have a lower risk to develop incident primary breast cancers compared to younger women while included in the study. Since any cancer diagnosis prior to inclusion led to exclusion from the study (Paper I), either because of death prior to inclusion or because we excluded all prevalent cancers the risk was different depending on age at inclusion. Therefore, all analyses were adjusted for age at inclusion.

The population-based prospective BC Blood Study (Paper II and IV) enrolled 51% of the breast cancer patients who underwent breast surgery at Skåne University Hospital in Lund. The follow-up rates for enrolled patients were high: the percentage of patients alive and breast cancer-free at 3, 5, 7 and 9 year follow-ups were 94%, 93%, 86% and 80%, respectively[270]. The major reason for non-participation was a lack of research nurses, rather than patients declining participation[27]. A comparison between participants (2002-2012) and all women operated on in Lund during the same period showed similar characteristics in terms of tumor ER/PR expression and median age at diagnosis[271]. Thus, the study cohort is considered to reflect the underlying population, and the results are therefore considered to be generalizable.

Precision

We perform statistical tests on our clinical data sets in order to evaluate differences/effects between groups. What we actually want to know is whether our finding is representative for the underlying population so that we may claim that our results are generalizable. A statistical test assumes that there are no differences between groups (the null hypothesis) and is reported by the point estimate (effect) of the analysis supplemented with the *P*-value and the 95% CI of the effect. The *P*-value is the probability of getting an effect as large as the one we found, or even larger, if there is no difference in the background population to which we want to generalize our results. Hence, the *P*-value does not inform us about the strength of the association, and it does not separate biased findings from "true" causal findings. The 95% CI on the other hand, may provide narrow or broad intervals, which do tell us something about the precision of our estimate. The interval presented covers the "true" effect in the population with 95% certainty.

There is a biological background and hypothesis that underlies the aim of each study in this thesis. However, some of the analyses are also carried out without strictly following the pre-specified hypotheses and are thus of a more exploratory nature (Paper I, II and IV) as compared to only testing a pre-specified hypothesis (Paper

III). When a large number of analyses/tests are performed, there is always an increased risk of chance findings (false rejection of the null hypothesis, type I or α error), which should be taken into account in the interpretation of the results. Likewise, the different subgroup analyses performed in the studies in this thesis, markedly reduced the number of individuals and events in the analyses, which reduced power and increased the risk of missing associations that may have been detected in larger datasets (false non-rejection of the null hypothesis, type II or β error). In the hypothesis-generating analyses, the aim is to report plausible biological associations/hypotheses for future studies to further validate. Therefore, one may say that type I error to a certain extent is acceptable and that the type II error (i.e. the overlooked true associations) are left to be discovered. Adjustments for multiple testing may be left out for such reasons. The risk is, however, that findings will be interpreted as definitive findings, without being further validated. Caution in interpretation and presentation is thus important, and confirmatory studies should be undertaken.

Validity

Validity comprise two parts: internal (i.e. target or source population) and external (outside target population). The validity of the studies is affected by the quality of the registry data. In Sweden, the civil registration number provide 100% coverage for residents in Sweden. All deaths are also reported to the Swedish Cause of Death Registry. The Swedish Cause of Death Registry has been reported to have complete and valid data from an international perspective, with the highest accuracy for cancer diagnoses[272]. The completeness of the Cancer Registry is considered to be high with low underreporting of breast cancer[273]. The following sections outline other aspects of validity and measures taken to ensure validity.

Misclassification

The method used to collect the information may have limitations that can impact data validity. Self-reported baseline patient characteristics may be over- or underreported, and certain groups or exposures may be more affected than others with the risk of introducing systematic errors.

To ensure validity of anthropometry, trained research nurses performed the measurements and the measurements were standardized (Paper I-IV). The plastic cups used for breast size measurements (Paper II and IV) have been reported to be reliable estimates of breast size[274, 275], and breast size was assessed among patients without prior breast surgery. Self-reported alcohol habits are prone to bias[276]; thus,

an underestimation was likely present in both cohorts. In the BC Blood study, the questionnaire on alcohol consumption used validated questions from the AUDIT questionnaire which is a strength[277] (Paper II and IV). Self-reported reproductive history has previously been reported to be of high validity[278], and the reported age at first childbirth was thus considered valid (Paper I). Current HRT as assessed in Paper I may underestimate former use, which consequently will be included among non-users, and may cause an underestimation of the risk of HRT use compared to non-use in relation to breast cancer risk (Paper I). Since information on OC was assessed as ever using OC, the risk of recall bias may be higher for OC use as compared to current use of HRT (Paper I).

Both studies collected exposure data prior to any type of event analyzed in the four papers. Therefore there is minimal risk of recall bias. Recall bias is also related to the interest for the event in question and is thus less likely in the BC-blood study in which all participants are enrolled prior to surgery [279] (Paper II and IV).

Confounding

Confounding is a central issue in observational studies since potential confounders may not be equally distributed across groups as they are in randomized studies. Confounders are accounted for in the analyses by performing adjustments and stratifications. In spite of such efforts, there is a risk for unmeasured confounding, which should be taken into consideration in the interpretations.

The breast cancer risk analyses in Paper I were adjusted for age, height and weight as a measure of body constitution as well as for occupation type. High socioeconomic status is an established risk factor for breast cancer[15], and the MDCS included both education level (O-level college, ≤ 9 years; A-level college, ≤ 12 years, University) and occupation type (manual worker, non-manual worker, employer- self- employed), which may both be regarded as measures of socioeconomic status. To support the selection of which co-variables to include in our multivariable analyses, we performed a backward conditional test, which rendered the variable "occupation type" of larger importance compared to "educational level"; hence, this factor was selected. Another option would have been to use directed acyclic graphs (DAGs)[280]. Since MDCS participants were born 1923-1950 and the majority was retired at the time of inclusion, an interpretation is that occupational type better reflects socioeconomic status as compared to educational level in this historic perspective. Further discussion on methodological aspects in relation to Paper I is found in the Results and Discussion section, Aim 1. Paper II-IV adjusted mainly for established prognostic factors, as outlined in the individual papers.

Tumor classification, tissue microarray and immunohistochemistry

The breast cancer diagnostic period for participants in the MDCS (Paper I and III) stretches over many years: 1991-2010. In the early years (1991-2004), re-evaluation of the tumor classification was made on whole section slides, which reduced misclassification bias. One pathologist blinded for clinical outcome performed the re-evaluation, thus reducing inter-observer variability. In more recent years (2005-2010), the standardization of pathology reports enabled the use of tumor information from pathology reports directly. The BC Blood Study used clinical information from the start (2002-2012) (Paper II and IV).

The use of TMAs (Papers I-IV) enabled additional markers to be evaluated for research purposes with limited use of tissue and less time-consuming evaluations. The TMA technology has been questioned in relation to the intra-tumor heterogeneity, and one study of tumor AR IHC compared TMA with whole sections and recommended whole section use[281]. However, in the present studies, the absolute majority of the duplicate cores were similar in their biomarker expression. In case of discrepancies, the two cores total tumor area were evaluated together. Generally, the use of duplicate TMA cores have been shown to reasonably reduce the impact of tumor heterogeneity, and the majority of studies have quite consistently argued that duplicate 0.6 mm cores are sufficient[282-284]. Also, a review wisely summarized TMAs to be as good as the cohorts they were taken from and pointed out that use of whole sections may also lead to post-hoc decisions causing bias, whereas TMAs rather offer a prospective sampling of the tumor which reduces such problems[282].

Up to 2007, the TMAs in the MDCS (Paper I and III) were used for evaluation of clinically established markers (i.e. ER, PR, HER2, Ki67) in order to reach high completeness of the database. There was more than one observer who performed these evaluations, so there exists a risk of inter-observer variability. In 2008-2010, pathology reports were used for all clinically used tumor variables. No skewness was observed depending on diagnostic period, except for PR status for which a rise in the amount of PR+ positive cases were noted when leaving TMA assessments in 2007. This was pointed out in Paper III as a limitation, which restrained us from exploring PR further.

The IHC (ER, PR and HER2) and HER2 ISH used in clinical routines undergo yearly controls for reproducibility with very good results (kappa-value 0.80 and 0.90, respectively)[9]. The internationally used ER and PR expression cut-offs at 1% could not be used due to lack of such data in our databases. Since Swedish clinical guidelines use 10% as a cut-off, it was considered relevant to apply the 10% cut-off to our cohorts in which patients were treated according to Swedish guidelines[9]. The number of ER and PR 1-9% tumors are few, and patients with these phenotypes

have a prognosis similar to those with ER– tumors[285, 286]; these patients are often recommended treatments in addition to endocrine treatment[121].

The Ki67 cut-off at 10% used in the MDCS (Paper I and III) was set during a time-period when no consensus had been reached regarding Ki67 cut-off. This might be regarded as controversial, but it is from before international consensus of 14% or 20% or laboratory-specific cut-offs[115, 116, 121]. Despite recent years´ intensive efforts to reach a consensus for Ki67 evaluation, it is still debated. Thus, we consider it appropriate to include Ki67 with 10% cut-off in the descriptive analyses and that it may add information, especially for very low proliferative tumors.

AR and ERβ antibodies

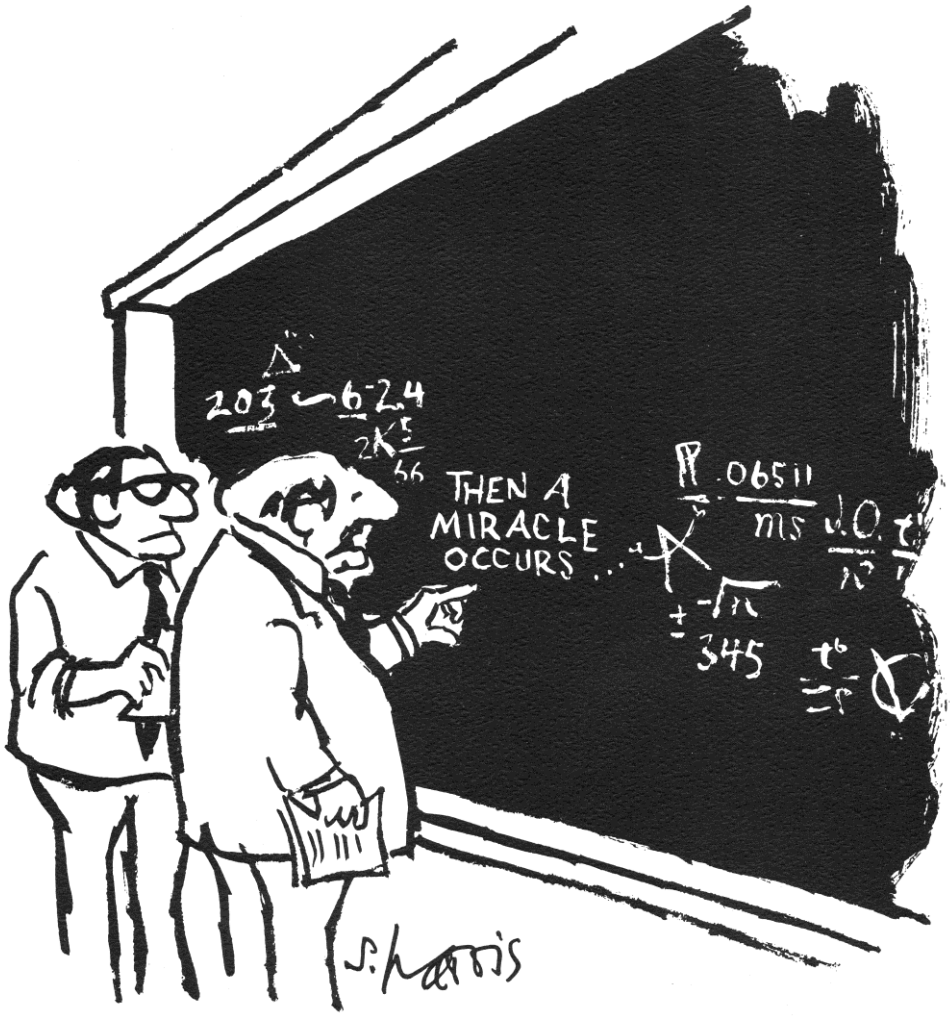
Prior to staining tumor tissues from the study cohorts, we performed a test staining using a TMA breast cancer test panel that our lab has used in previous studies to test ER, PR and HER2 stains (Papers I-IV). For both AR and ERβ1, the test staining worked to our satisfaction, with positively and negatively stained tumor cores that were easy to assess, using a protocol similar to the recommendations from the manufacturer. This test panel was co-stained with the samples from the cohort in order to ensure coherent quality (Papers I-IV).

Early use of AR antibodies was challenging due to the lack of a monoclonal antibody that was specific in identifying AR only. In recent years, the use of the commercial antibody clone AR441 that we applied have become commonly used in the majority of published studies[181, 287, 288]. AR441 specifically identifies AR, as validated in Western Blot[289] . We did not consider an antibody validation procedure in our lab necessary prior to the use of the AR antibody for research purposes.

Similarly, the ERβ1 antibody clone PPG5/10[290] has been thoroughly validated[291-296] and does not stain ER or the other ERβ isoforms. PPG5/10 that identifies ERβ1 is directed towards the C-terminal part of ERβ1, which is unique since ERβ splice variants do not have an LBD (Figure 2)[224]. We thus consider M7292 as a well-validated antibody.

Table 1. Strengths and limitations in each Paper.

Paper	Strengths	Limitations
I **AR**	Large, prospective, well-characterized population-based study with long-time follow-up. Detailed participant, patient and tumor information. Associations of AR and tumor characteristics similar to previous studies. The most widely recognized AR antibody and cut-off was used. This was the first study of AR-defined breast cancer risk. Analyses repeated for ER+/– revealed no association for ER-defined risk, strengthening the findings. Multivariable analyses adjusted for other risk factors. Missing data were described, and sensitvity analyses were performed.	Higher education and better health among participants compared to non-participants may introduce selection bias. Patient characteristics from baseline only; exposure after baseline not accounted for. Histopathological analyses mainly based on TMA, not whole sections. Low percentage of AR negative tumors. Significant number of tumors with missing AR status. Significant amount of patients not included in survival analyses due to missing data. Exploratory study with multiple analyses carries the risk of chance findings. Competing risks not accounted for in analyses.
II **AR**	Large, prospective, population-based study with detailed follow-up. Information on *AR* genotypes available. Similar age and ER status as background population indicated a representative study population. The most widely recognized AR antibody and cut-off was used. Multivariable analyses adjusted for other prognostic factors.	Relatively short follow-up. Not a randomized trial. Treatment prediction may only be addressed as prognostic according to receptor expression within the specific treatment groups. Tissue microarray analysis of AR, not whole sections. Subgroup-analyses with few patients and events reduced statistical power and precision.
III **AR**	Large, prospective, well-characterized population-based study of long follow-up. The most widely recognized AR antibody and cut-off was used. The study adhered to a prespecified hypothesis and followed a statistical plan with the aim to validate findings in Study II. Multivariable analyses adjusted for other prognostic factors. Formal test for non-proportional hazards were performed.	Higher education and better health among participants compared to non-participants. No information on relapse and distant metastasis available. Register data used carries a risk of misclassification. Histopathological analyses mainly based on TMA, not whole sections. Significant amout of patients not included in survival analyses due to missing data. Larger cohort required to validate AR findings in ER– subgroup.
IV **ERβ**	Large, prospective, population-based study with detailed follow-up. Information on *ESR2* genotypes available. Similar age and ER status as background population indicated a representative study population. A previously validated and recognized ERβ antibody was used. The ERβ cut-off used was associated with tumor characteristics similarly to previous studies. Multivariable analyses adjusted for other prognostic factors.	Relatively short follow-up. Not a randomized trial. Treatment prediction may only be addressed as prognosis by receptor status within treatment groups. The ERβ cut-off used was higher than many other studies. Tissue microarray analysis of ERβ, not whole sections. Subgroup-analyses with few patients and events reduced statistical power and precision.



"I THINK YOU SHOULD BE MORE EXPLICIT HERE IN STEP TWO."

Results and discussion

The results of each specific aim are now presented and discussed.

Aim 1. Lifestyle factors in relation to AR-defined breast cancer risk (Paper I)

The risk to develop breast cancer defined as either AR+ or AR– was investigated according to different risk factors in comparison with the rest of the cohort. Separate analyses were performed and compared for AR+ breast cancer, AR– breast cancer and generally for invasive breast cancer. Any finding related to AR expression was separately assessed according to ER expression in order to distinguish AR-driven effects from ER-driven effects.

Older age at first childbirth and ever use of OC were significantly associated with a higher risk for AR– breast cancer in both univariable and multivariable analyses. For age at first childbirth, there was a five-fold increase in risk for AR– breast cancer from the youngest to the oldest age category. Ever users of OC had a more than two-fold increase in risk for AR– breast cancer compared to never users. These factors were not associated with increased risk for AR+ or general invasive breast cancer.

There are still huge gaps in our knowledge regarding the impact that hormone related factors exert on the path from exposure through the multistep process of cancer initiation and progression to the clinical outcome, i.e., the clinically detectable tumor. For example, it is probably not sufficient to consider a woman's exposure to circulating hormone levels since the hormone content within the breast is unknown and may be of considerable importance for tumor growth[21, 22, 48]. The integrated effect of several hormones on the breast over time may be important and is difficult to assess[13]. Efforts to perfom risk profiling according to specific marker may add important information. This task is however difficult and requires rigorous study designs and large sample sizes[13]. The results generated may be difficult to interpret, and to implement novel preventive strategies based on such risk profiling may be even more difficult. In spite of all these difficulties, it is important to modernize risk profiling in order to improve the understanding of breast carcinogenesis and to develop novel preventive strategies.

The present study may be limited by the sample size and must be interpreted with caution. Many tests were performed, and the risk factors identified were associated with AR– breast cancer, the rarest AR-defined subtype. In addition, the amount of missing data may impact the conclusions drawn from the study. Thus, we have to consider the possibility of chance findings, low precision in the detected risk estimates and also the risk of unmeasured confounding as an explanation to the associations seen. Our method also did not take competing risks into account, which would have been preferable. The largest risk for competing events in analyses of AR– risk, lie in the censoring of AR+ breast cancer, followed by the missing AR subgroup, and finally the *in situ* tumors of which there were relatively few in comparison to the 16,459 women at baseline. Since analyses were performed also for risk of AR+ breast cancer and did not reveal any associations, the AR missing subgroup was considered the main competing event in this study. Another competing event was women that died prior to event.

There were no large differences in the distribution between the AR+, AR– and AR missing subgroups, and the AR missing subgroup was not considered to exert a risk of selection bias in this study. The small AR– sample size restrained us from further formal subgroup assessments in relation to ER and/or PR expression. Such analyses would have been of particular interest, since there may be a differential prognostic role of AR expression in relation to ER status; however, this issue could not be addressed in this study. In order to overcome parts of these issues, the analyses were repeated separately for ER expression (+/–) and did not indicate ER as a driver of the reported associations. Also, sensitivity analyses were performed in which analyses were repeated with the missing AR categorized as either AR+ or AR–.

After our study was published, the large American Nurses' Health Study reported on risk factors according to AR expression[297]. Having sufficiently large samples and very long follow-ups, some interesting observations were reported. Zhang *et al*. hypothesized that postmenopausal obesity may be associated with AR regulated pathways and to be reflected in AR+ breast cancer risk rather than in AR– risk. The risk was, however, elevated in both groups, thus not indicating a particular importance of AR expression. This is in line with our study findings on anthropometry where no increased breast cancer risk by AR expression was seen. In analyses of combined AR/ER/PR expression, Zhang *et al*. found the strongest effect in ER+/PR+/AR+ as well as ER+/PR+/AR–, indicating another driver to the association than AR expression. This assumption is in line with our hypothesis of a differential prognostic role of AR (Paper II and III), and Zhang *et al*. base their argument on the laboratory findings that demonstrate that androgen acts as estrogen agonists in low-estrogen environments and as antagonists in high- estrogen environments[38, 161]. The authors conclude that it must rather be PR expression acting as an ER activator[298] and that PR expression may be of higher significance for reproductive risk factors than was previously assumed. Since several risk factors

demonstrated slightly stronger associations for AR– tumors than for AR+ tumors, and Zhang *et al*. also found an association between BMI and ER–PR–AR– tumors, the authors postulate that yet other pathways such as the insulin pathway may be involved in the pathway from exposure to cancer in relation to obesity[297].

In this study, we did not take PR expression into account, but this would be of interest in future studies. There are more interesting findings on progestins and PR expression in relation to both OC use and age at first childbirth. Early first pregnancy in women may protect from breast cancer by a reduction of PR+ cells, down-regulation of paracrine mediators and a beneficial reduction of stem/progenitor cells[299]. Based on such findings, modulation of endogenous progesterone levels by anti-progestin therapy may be considered as a novel measure for breast cancer prevention[6, 300].

In relation to OC use, our findings were in line with previous findings that have mainly reported associations with ER– breast cancer risk[13] and may be explained by OCs influencing the testosterone/estrogen ratio by reducing free androgens, which in turn would allow estrogen to exert proliferative effects on the breast[162]. OC users have been reported to have lower androstenedione and testosterone levels compared to non-users[35, 301].

To conclude, the results of this study should be considered as contributions to hypothesis generation and may improve our understanding of hormonal breast carcinogenesis. In future studies of breast cancer risk according to AR, an approach using molecular subtypes may be more suitable with larger opportunities to take the differential role of AR depending on breast cancer subtype into account.

Aim 2. Tumor AR expression in relation to patient characteristics and established clinicopathological markers (Paper I-III)

Patient characteristics at diagnosis in relation to AR expression (>10%) were foremost examined in the BC Blood Study (Paper II). Therein, there was slight evidence that patients with smaller breast volumes were more likely to have AR+ tumors compared to patients with larger breasts. Analyses stratified by age ≥/< 50 years used as a proxy marker for menopausal status revealed that the finding emanated from the patients aged less than 50 years and was thus interpreted as driven by younger age (Paper II). Other anthropometric measurements, reproductive factors and use of exogenous hormones were not associated with AR expression, irrespective of age ≥/< 50 years (Paper II). Patients who were older at diagnosis were more likely to have AR+ tumors compared to younger patients. In analyses stratified by age ≥/< 50 years, this association was seen among the younger patients only (Paper II). In MDCS, where most patients were postmenopausal at diagnosis, age at diagnosis was not associated with AR expression (Paper III).

AR expression was associated with favorable tumor characteristics such as low histological grade, ER and PR co-expression (Paper I-III) as well as low proliferation index (Paper I+III). Regarding tumor size, there was slight evidence of an association between AR+ and smaller tumor size (Paper II). However, when results from the three studies (Paper I-III) were compared, there was no convincing association between AR expression and tumor size; AR+ tumors were consistently of smaller size (71-73% ≤20mm, Paper I-III). However, the size distribution among AR– tumors varied markedly between the three studies. AR expression was not associated with lymph nodal status.

In subgroup analyses among ER–PR– tumors with regard to HER2 status and AR expression, TNBC expressed AR+ in up to half of the tumors (30-50% AR+, Paper I-III), and in ER–PR–HER2+, the majority of tumors was AR+ (70-100% AR+, Paper I-III). However, these subgroup analyses should be interpreted with caution due to large missing numbers.

The finding of higher age at diagnosis among patients with AR+ tumors is in line with severeal previous findings[193, 194, 302-304]. The association between established favorable tumor characteristics has been repeatedly shown[170-172, 174-177, 194, 302, 304-307].

We anticipated that anthropometric measures were related to tumor AR expression, which was not confirmed in our study. Similarly, the Nurses' Health Study did not find supporting evidence for an AR dependency among obese women in relation to breast cancer risk[297], as discussed above (aim 1). The association between obesity and postmenopausal breast cancer is well established, and this association may be impacted by inflammatory responses that contribute to tumurogenesis rather than androgen dependent pathways[308, 309].

We did, however, find an association between AR and with breast size, which is closely associated with BMI, which in turn is associated with breast density. We have previously assessed mammographic density in relation to detection mode and AR expression in the MDCS (corresponding to breast cancer cases in Paper I)[310]. Therein, clinically detected tumors were rather AR– than AR+, and BMI was inversely associated with density irrespective of detection mode[310].

Aim 3. Tumor AR expression as a prognostic factor alone and in combination with ER expression (Paper II-III)

The prognostic role of AR expression was assessed by different endpoints in the two cohorts, and univariable and multivariable analyses according to AR expression were performed in three steps: for the overall cohort, divided by ER status +/– and for AR and ER status combined.

In univariable analyses for disease-free survival (DFS) as well as for breast cancer mortality (BCM), AR expression was significantly associated with a favorable prognosis. In multivariable analyses with adjustments for age, tumor characteristics and treatments, AR expression was not associated with prognosis, irrespective of endpoint.

In analyses stratified by ER status, there were indications of a differential prognostic role of AR expression depending on tumor ER status. In terms of DFS, AR expression in patients with ER+ tumors had a better prognosis compared to patients with ER+AR- tumors, whereas patients with AR+ER– tumors had worse prognoses. This interaction between AR and ER expression in relation to outcome was significant (Paper II). In terms of BCM, no significant interaction between AR and ER expression was observed, irrespective of follow-up period (Paper III).

When prognosis was assessed in a model where patients were grouped by the combinations of AR and ER expression, and analyses were adjusted for potential confounders, there was slight evidence for a worse prognosis for patients with AR+ER– tumors, and this finding was stronger in short-term follow-up analyses (BCM, Paper III). In terms of DFS, the corresponding results were more pronounced. Patients with AR+ER– tumors had the worst prognosis compared to all other combinations, irrespective of adjustment model used. This finding remained in subgroup analyses among patients with HER2 status available and for which analyses were adjusted for trastuzumab treatment (Paper II).

There is mechanistic support for the differential prognostic role of AR depending on ER expression. AR may interact with ER in a competitive manner[176] and bind to estrogen response elements on the ER with an anti-proliferative effect by affecting downstream target genes[161, 202]. In a low-estrogen environment, AR would rather act as an oncogene stimulating tumor growth[161]. Such preclinical findings gave rise to a recent meta-analysis which did not, however, support the hypothesis or AR being a negative prognosticator in ER– tumors[287]. This may have several explanations, e.g., the heterogeneity regarding both study populations and treatments received as well as the low frequency of ER– tumors. ER– tumors are not to be considered as one subgroup, it is rather heterogenous and the low frequency carries significant limitations as to the power of the analyses. There is also a lack of standardized methods concerning antibodies and cut-offs in the assessment of AR, which significantly impede consistent results. To date, the largest cohort in the field of AR-studies[172] found a significant interaction between AR and ER in relation to outcome, which is similar to our results. To the best of our knowledge, this is the only other study that performed formal interaction analyses.

The interest in AR is steadily increasing, and since there are many ongoing trials with AR targeted treatment[213, 216, 311, 312], (ClinicalTrials.gov identifier: NCT01842321, NCT01889238, NCT02689427, NCT02676986, NCT02457910,

NCT02463032, NCT02368691), a better understanding of AR's prognostic role is highly desirable.

If an inferior prognosis of patients with ER–AR+ is confirmed, it would be possible both to arrange for closer surveillance of these patients and to target with anti-androgens. For patients with ER+AR+ tumors, agonistic actions may be evaluated, such as SARMs. One complicating factor is the possibility of a different role of AR in the adjuvant and the metastatic setting, as the latter can be challenged by resistance to treatments. For example, it has been suggested that AR have an oncogenic role in endocrine resistant settings similar to the ER– setting, which would then require/motivate anti-androgen treatment in addition to endocrine treatment. Clinical trials today display a variety of strategies, including enzalutamide as a window-of-opportunity trial for early AI treated ER+ breast cancer patients[313], as well as SARMs for both ER+ and ER– breast cancer, as exemplified by a recently registed trial of a SARM in metastatic TNBC[210, 314].

Far larger and strictly stratified observational studies using standardized AR assessment are needed. Another option to better understand the role of AR in relation to treatment resistance and the potential use of AR as a treatment target would be assessment in already performed clinical trials. Inclusion of AR evaluations in clinical routines has also been suggested[208].

Aim 4. Tumor AR expression as a prognostic factor by treatment type (Paper II)

In order to explore the potential treatment predictive value of AR expression, analyses of DFS were stratified by treatment type and ER status.

AR expression did not impact prognosis among chemotherapy-treated patients, irrespective of whether they had ER– tumors and received chemotherapy only or if they had ER+ tumors and received chemotherapy followed by endocrine therapy.

AR expression in relation to endocrine treatment was further addressed in patients with ER+ tumors, aged 50 or older, who did not receive chemotherapy. In comparison to patients with AR+ tumors, patients with AR– tumors who received sequential TAM/AI treatment experienced breast cancer events earlier during follow-up. This finding was stronger in patients who ever received AIs and was not evident among patients who ever received TAM or who received TAM only. Thus, the finding was interpreted as an AI-driven effect rather than a TAM-driven effect. The subgroup of patients who received AI only did not include enough patients with AR– tumors for meaningful analyses.

Since preclinical data have indicated a role of AR overexpression in resistance to endocrine treatment[203, 315], the above analyses were repeated using the alternative

AR cut-off at 75%. The association of AR negativity and early AI failure remained, and no associations in relation to TAM emerged.

AR expression has previously been associated with response to endocrine treatment among patients with ER+ tumors[194] and for chemo-endocrine treatment in a AR + luminal B like subgroup[192]. Park *et al*. showed that high expression of AR (>50%) is prognostic in terms of DFS and OS and is a predictive factor for endocrine treatment. Low or intermediate AR-levels may indicate whether or not there is a need for the addition of chemotherapy [177]. Since TAM and AI impede breast cancer development by different mechanisms, our aim was to differentiate the predictive role of AR between the two. Our results were to some extent hampered by our study design of an observational study. Due to the high degree of co-expression of AR and ER, the number of AR– tumors in the analyses was low, and a larger cohort would be needed to draw solid conclusions. The finding should thus be interpreted with caution bearing in mind that there were no randomization of treatments.

Mechanistically, an explanation for the finding of early failure to AI treatments among patients with AR– tumors may be found in the altered androgen levels following AI treatment since androgens may reduce tumor cell proliferation through their action via the AR[316, 317]. However, the finding may not be attributed to a total lack of AR but rather a modification of effect, since our repeated analyses using the higher alternative cut-off for AR showed similar results. Therein, we had several events in the AR_{75}– group, and since AR expression existed in both groups, the finding may not be contributed to an absolute lack of AR expression.

One possible next step would be to address AR expression rather as the ratio to ER, which has been done by others[217]. This may enhance the understanding of AR actions, due to the suggested competitive interactions between these two receptors[176]. In conclusion, the result is considered hypothesis-generating, and there is still a need confirmation in a randomized setting.

Aim 5. Specific genotypes in relation to the corresponding tumor receptor expressions (Paper II, IV)

The previously studied germline *AR* diplotypes were not associated with tumor AR expression. No association was seen between the previously studied *ESR2* genotypes and tumor ERβ1 expression.

These genotypes have mainly been addressed in relation to risk or prognosis earlier rather than in relation to the corresponding tumor expression[318, 319]

, as we did in this study. We could not demonstrate any associations, although we consider the choice of genotypes investigated to be relevant choices in both cases.

The *AR* diplotypes were selected by the SNPs that capture 95 % of the *AR* haplotypes among men and that had been associated with risk of prostate cancer[267]. The selected AR diplotypes did not tag for CAG or GGC repeat length polymorphisms[27, 320].

Changes in the AR gene easily bring about phenotypic changes in men and are associated with different degrees of androgen insensitivity[33]. In women, there has been one previous study in which an association between the diplotypes were weakly associated with androgen levels in women and modified by exogenous hormone use[321], and this prompted us to explore these diplotypes in relation to AR in the present study[321]. It may be that the phenotypic impact is different in women or that the tumor harbors too many genetic changes that the association between a germline feature and the corresponding receptor expression is not so relevant. Regarding *ESR2*, the interpretation may be similar. Possibly, the genotypes affect tumerogenesis on a systemic level that will not be mirrored by the tumor protein expression of a specific receptor.

Aim 6. Tumor ERβ1 expression in relation to patient characteristics and established clinicopathological markers (Paper IV)

Patients who had tumors with high ERβ1 expression (>75%, ERβ1$_{75}$+) were on average older at diagnosis and had smaller breast volumes compared to patients with ERβ1$_{75}$ negative (ERβ1$_{75}$–) tumors. Regarding tumor characteristics, ERβ1$_{75}$+ was associated with small tumor size, lymph node negativity, low histological grade and ER, PR, and AR co-expression. There was an interaction between ERβ1$_{75}$ and AR expression depending on the ER status of the tumor. HER2-amplified tumors were more often ERβ1$_{75}$– than ERβ1$_{75}$+, and this finding appeared driven by the ER+ subgroup.

Hence, ERβ1$_{75}$+ was associated with favorable tumor characteristics, in line with previous findings[229, 322-324]. Other studies reported a lack of associations[228, 241, 325, 326], whereas inverse associations between ERβ1$_{75}$+ and favorable tumor characteristics have not been described, which strengthens our finding. Comparison between ERβ studies is difficult, since the study populations have been heterogeneous, and a wide range of different antibodies and cut-offs have been applied[327, 328]. When assessed, patient characteristics such as age at diagnosis[228, 324, 329] or menopausal status[241, 323, 325, 326] have not been associated with ERβ1 expression. The association with breast size has not been explored previously, and smaller breast volumes could possibly be a favorable "host" factor to address together with other anthropometric measures.

The significant interaction between ERβ1$_{75}$ and AR in relation to ER has not been described previously and merits further attention, possibly by future studies using a

triple-signature[17]. There are preclinical findings in which ERβ has been suggested as a mediator between AR and ER interactions[190, 330], and as illustrated in Figure 4[191]. Our prognostic findings suggested that ERβ1_{75} and AR (Paper IV and Papers II and III, respectively) may have distinctly different impact on clinical outcome depending on tumor ER expression; ERβ1_{75} displays a protective effect irrespective of ER status, whereas AR may signal poor prognosis in the ER– setting. Findings such as these may impact the choice of agonists or antagonists when aiming to target ERβ1_{75} and AR in clinical trials. Thus, it would be of interest to better characterize ERβ1 and AR with regard to their cellular function, the distribution of ERβ1 and AR between patients and their prognostic roles in relation to each other and to ER. Interestingly, the transdermal CR1447 (4-OH-testosterone) currently in clinical Phase 2 trial of postmenopausal advanced AR+ TNBC will evaluate treatment effects by biopsis non only on AR levels but also ERβ[331].

Aim 7. Tumor ERβ1 expression as a prognostic factor alone and in combination with ER expression (Paper IV)

The prognostic role of ERβ1 expression was assessed according to high or low ERβ1 expression (ERβ1_{75}+/–). Analyses were performed according to different clinical endpoints and in three steps: for the overall cohort, stratified by ER status +/– and for ERβ1 and ER status combined.

The median follow-up was 5 years. In terms of DFS, patients with ERβ1_{75}+ tumors had approximately two thirds the risk of a breast cancer event compared to patients with ERβ1_{75}– tumors. This protective effect of high ERβ1 expression remained in all multivariable models. In the ER– subgroup, patients with ERβ1_{75}+ tumors had one third the risk of an event compared to patients with ERβ1_{75}– tumors. In the ER+ subgroup, the magnitude of the protective effect by ERβ1_{75}+ was smaller and not statistically significant. When the prognosis was assessed in a model where patients were grouped by the combinations of ERβ1_{75} and ER expression, ERβ1_{75} status seemed to distinguish between good and poor prognosis regardless of ER status.

Similar findings were found for the endpoint distant metastasis-free survival (DMFS), with the exception of the ER+ subgroup in which ERβ1_{75} expression was not associated with outcome. In terms of overall survival (OS), there was a protective effect of ERβ1_{75}+ irrespective of subgroup.

Our findings were in line with a recent meta-analysis that summarized ERβ1 as an independent prognostic marker for DFS (irrespective of ER status), while the prognostic role observed in relation to OS was dependent on ER expression[239]. Defining the clinical value of ERβ is, however, complex, given the different isoforms and their potentially different biological effects[148, 224]. Especially isoform ERβ2/cx have been reported to play a potential role in breast cancer by forming

heterodimers with ER[222, 332]. Related clinical studies have, however, been inconsistent, displaying positive, negative or neutral effects on outcome[225, 323, 326]. We chose to study ERβ1, which has been reported as the only fully functional ERβ and for which there is a validated antibody[224, 226]. Using a high ERβ1 cut-off similar to ours, a TNBC cohort of more than 500 patients have reported findings similar to ours[324].

In our study, the prognostic benefit of ERβ1$_{75}$+ was mainly attributable to the ER– group; this is a novel finding that suggests a role of ERβ1 in hormone-independent settings and for which there are plausible mechanistic explanations: ERβ ligand-independent actions and basal activity has been reported to be more pronounced for ERβ than for ER[333]. A shift of ERβ transcriptional binding sites may be seen in the absence of ER expression[334]. Furthermore, in vitro studies have suggested that ERβ may be related to suppression of EGFR expression[335, 336], and in an obesity-model the otherwise beneficial effect of ERβ on EGFR signaling was inhibited and resulted in tumor growth[337]. In TNBC cell lines, ERβ has been suggested to reduce invasiveness by influencing expression of mutant p53 target genes[338], and an ERβ agonist was reported to reduce tumor invasiveness[339].

If the beneficial prognostic role of ERβ1$_{75}$+ is confirmed to be irrespective of ER status, this may have implications for the development of ERβ-targeted drugs, where the finding would suggest ERβ agonists rather than antagonistic targeting ERβ[324]. At least one ongoing trial is evaluating an ERβ agonist at the moment, a Phase 0 study in the pre-surgical TNBC setting (ClinicalTrials.gov identifier: NCT0235202). Also, a recent Phase 2 trial suggested that estradiol treatment may be beneficial in selected ERβ+ TNBC populations[340]. Treatment alternative for ER– subtypes are scarse, and since ERβ may have potential as a treatment target[330], ERβ assessments in clinical trials have been sought[237].

Aim 8. Tumor ERβ1 expression as a prognostic factor by treatment type (Paper IV)

In order to explore the potential treatment predictive value of ERβ1 expression, analyses of DFS, DMFS and OS were stratified by treatment type and ER status.

Given results from previous studies that indicated a predictive role of ERβ for endocrine treatment response[241, 323, 341], we hypothesized that ERβ1 would impact clinical outcomes, especially among endocrine-treated patients. However, we could not confirm this hypothesis in this study. The minor prognostic impact by ERβ1$_{75}$+ observed among endocrine-treated patients was restricted to subgroups who also received chemotherapy and was interpreted as an affect driven by chemotherapy. Instead, our main finding was that patients with ERβ1$_{75}$+ tumors who received adjuvant chemotherapy had one-third the risk for a breast cancer event compared to

patients with ERβ1$_{75}$– tumors. This protective effect was seen irrespective of ER status, and ERβ1$_{75}$ expression did not affect prognosis among chemonaïve patients. In terms of DMFS, patients treated with chemotherapy were also protected by high tumor ERβ1$_{75}$ expression, and no effect was seen for chemonaïve patients or as a prognostic factor by endocrine treatment and type. In terms of OS, there was an overall lower risk of death among patients with ERβ1$_{75}$+ tumors in the general study population.

The potential importance of ERβ in the absence of ER expression has mostly been studied to better understand whether or not endocrine treatments may add value to patients with ER–ERβ+ tumors[241, 323, 342]. A recent randomized trial reported ERβ1 to be prognostic for patients with ER+ERβ1+ tumors who switched from TAM to AI, while no gain was observed among patients with ER+ERβ1– tumors. Therein, ERβ1 did not impact survival in their overall cohort of endocrine-treated patients, which highlight the complexity of endocrine treatment response[343].

In previous ERβ studies, chemotherapy has not been addressed, although study populations may have received chemo-endocrine therapy[344], such as in a cohort that displayed prognostic results similar to ours[325]. Also, in accordance with our study, most studies have been observational studies[229] with substantial limitations in addressing treatment prediction. Correspondingly, Wang *et al*. reported clinical benefits of high ERβ1 expression in a large retrospective series of TNBC patients who underwent curative surgery and received adjuvant chemotherapy only[324].

There is preclinical support for a protective effect of ERβ expression regarding chemotherapy response, derived from chemotherapy treated cell models, in which a chemo-sensitizing effect has been seen[253, 345, 346]. As previously pointed out, potential interactions between ERβ and AR may be of interest for future studies, especially in ER– tumors. McNamara *et al*. recently stressed that intracrine estrogen features of the breast motivate further assessments of ERβ in relation to TNBC. The authors also suggested that ERβ may be a modulator in the molecular LAR subtype, which is a highly androgen-enriched molecular subtype[183, 184]. Finally, in a recent review, McNamara *et al*. also hypothesized that AR+ in the LAR subgroup carry low proliferation signatures and that AR thus would be responsible for the poor chemotherapy response observed in this subgroup[199, 208, 218, 347]. Taken together, it may be relevant to address not only AR in the LAR subtype but also to perform stratifications by ERβ expression.

Conclusions

This thesis indicates that assessments of the novel hormone receptors AR and ERβ1, respectively, may add prognostic value to the clinically established ER in the adjuvant primary breast cancer setting. The conclusions according to the specific aims were as follows:

- Older age at first childbirth and ever use of OCs were associated with a higher risk for AR-negative breast cancer. These risk factors were not associated with invasive breast cancer in general or with AR+ or ER+/– breast cancer, respectively.
- AR expression was associated with favorable established tumor characteristics.
- AR expression was associated with a favorable breast cancer prognosis in terms of DFS and BCM. Prediction of breast cancer prognosis was improved by combining AR and ER status. In terms of DFS, the prognostic value of AR expression was significantly different depending on the ER status of the tumor, a finding which may influence the treatment choices (antagonist/agonist) when targeting AR. Larger cohorts are needed in future studies in order to better understand the role of AR, especially in ER– tumors. The inclusion of gene expression analyses may add valuable information.
- AR negativity may be an indicator of early AI treatment failure in patients with ER+ tumors, aged 50 or older, who did not receive chemotherapy.
- The previously studied germline *AR* diplotypes and *ESR2* genotypes were not associated with tumor expression of their corresponding receptors, AR and ERβ1.
- High ERβ1 expression was associated with higher age at diagnosis, smaller breast volumes and favorable tumor characteristics. There was an interaction between $ERβ1_{75}$ and AR expression depending on the ER status of the tumor.
- Patients with high tumor ERβ1 expression had a better prognosis compared to patients with low ERβ1 expression. The association remained significant in analyses adjusted for ER expression. The magnitude of the association was larger among patients with ER– tumors. This novel finding suggests a role of ERβ1 in hormone-independent settings.
- High ERβ1 expression was a favorable prognostic marker in chemotherapy-treated patients but not chemonaïve or in endocrine-therapy-treated patients.

Clinical implications and future perspectives

In relation to AR, the potential differential prognostic role may have implications for the choice and development of clinical AR targeted trials. There is yet no consensus on whether or not selecting AR antagonists or agonists as treatment targets is preferable in any breast cancer subtype. The forthcoming reports of ongoing clinical trials—combined with observational studies like ours and mechanistic studies on treatment resistance—will impact the future direction. Most likely the value of a wider clinical implementation will be for patients with TNBC where the heavily hormonally enriched LAR subgroup may benefit AR-targeted treatments largest and in the metastatic setting for patients that have suffered from treatment resistance. In the long run, there may be development of resistance to AR treatments as well, as seen in the field of prostate cancer. In prostate cancer, the induction of a constitutively active ARs that does not bind ligand is seen[348]. The corresponding splice variant has also been identified in human breast tissues and the antiandrogen enzalutamide has been shown to upregulate splice variant expression[349, 350]. Future splice variant targeting may have future therapeutic potential[100].

In relation to ERβ, the different isoforms and their potentially different biological effects complicate comparison between studies, and a consensus or standardization needs to be reached on what antibodies and cut-offs to apply. Our finding on high ERβ expression as a potential marker for chemotherapy response is well worth pursuing. The first step may be to evaluate how high ERβ1 expression affects outcome in already performed clinical trials. Since intracrine estrogen metabolism in TNBC was recently highlighted [330], and AR is of high interest in the molecular LAR subtype, future studies taking both ERβ and AR expression into consideration would be of interest[208].

In spite of being based on relatively large cohorts, the findings from this thesis strongly indicate the need for future large studies in order to better distinguish the etiologic and prognostic differences of AR and ERβ depending on breast cancer subtype. In particular, the combined effect of different hormone receptors is of high interest as well as the variations within the ER– setting. The era of gene expression profiling is close to clinical implementation, and future risk and prognostic studies need incorporation of either genetic profiling or the IHC surrogate markers to generate a more comprehensive understanding of hormonal carcinogenesis and treatment response. There will always be parts of clinical reality that cannot afford expensive analyses, and therefore research may well be continued on two frontiers to provide a basis for both clinical realities.

Furthermore, we will probably see more on integrative approaches such as integrating polygenic scores to the established risk factors of traditional

epidemiology as well as the combining of different modalities, such as the use of density in mammographic imaging. More complex groups require more complex study designs, and importantly, the information load created will require stringent interpretions and close collaborations with biostatisticians and bioinformaticians. Molecular pathological epidemiology (MPE), in which molecular markers are assessed to gain a deeper epidemiological understanding, may be a comprehensive way forward. The MPE approach is derived from a modern understanding of cancer development and takes the diversity within tumors into consideration when investigating epidemiological associations[351, 352].

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