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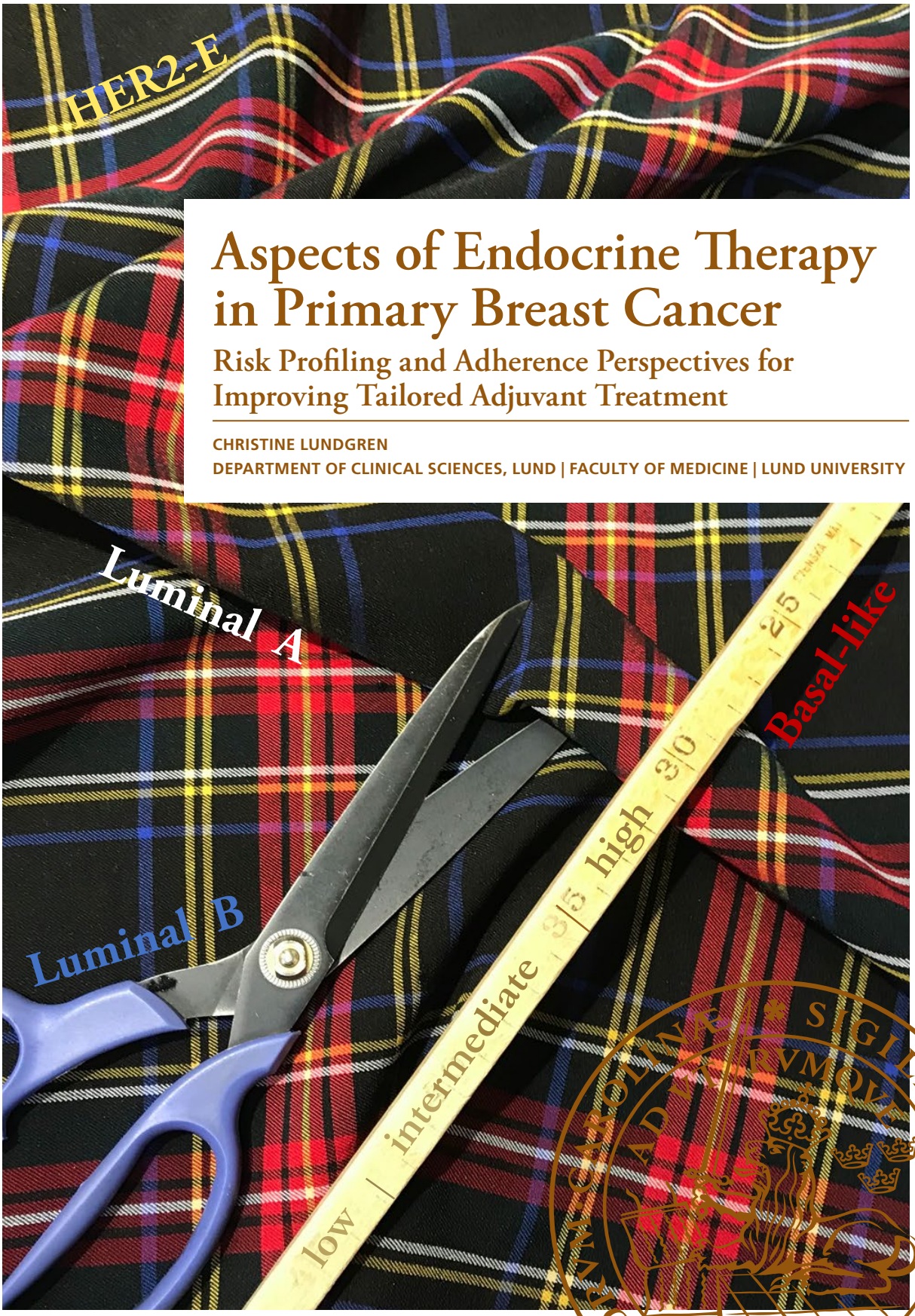

Aspects of Endocrine Therapy in Primary Breast Cancer

Risk Profiling and Adherence Perspectives for Improving Tailored Adjuvant Treatment

CHRISTINE LUNDGREN
DEPARTMENT OF CLINICAL SCIENCES, LUND | FACULTY OF MEDICINE | LUND UNIVERSITY

According to van´t Veer et al., gene expression analyses and its application for tumour signature determination is 'similar to recognition of the tartan plaids that distinguish one Scottish clan from another. In other words, the details of the individual thread colors are not as important as the overall pattern'.

Individual profiling of breast cancer patients is a combination of clinicopathological factors and the gene expression profile. These are separate details that are combined into a completeness for tailoring treatment for the individual patient.

Christine Lundgren M.D.

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Aspects of endocrine therapy in primary breast cancer

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LUND
UNIVERSITY

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Title and subtitle: Aspects of endocrine therapy in primary breast cancer - risk profiling and adherence perspectives for improving tailored adjuvant treatment

Abstract: Breast cancers are heterogeneous tumours. Prognostic markers are needed to better profile the patient´s risk, and predictive markers to indicate the expected benefit of systemic treatment. Using gene expression analyses, breast tumours can be divided into intrinsic subtypes. Immunohistochemical (IHC) markers (oestrogen and progesterone receptors (ER and PR), human epidermal growth factor receptor 2 (HER2), proliferation marker (Ki67)) and histological grade (HG) are used to classify the tumours into the corresponding surrogate subtypes. The ER-positive/HER2-negative (ER+/HER2−) tumours are divided into Luminal A and Luminal B, which are associated with different prognoses. Patients with Luminal B tumours are generally recommended adjuvant chemotherapy in addition to endocrine therapy. The agreement between the subtyping by gene expression and surrogate classification, is not perfect. Prosigna® test provides the tumour subtype and assigns the patient a relapse risk score (Risk of Recurrence (ROR) score) by gene expression analyses, predicting 10 years risk of distant metastases. This test is currently in clinical use for postmenopausal women only, and assumes treatment with endocrine adjuvant drugs. The adherence to these drugs is however known to be poor, mostly because of the side-effects. The immune system and its complex components, such as tumour-infiltrating lymphocytes (TILs) have so far proven to be important markers for certain subtypes of breast cancer, although their value in ER+/HER2− tumours is less known.

Study I: Data on the prescribed and collected endocrine drugs from the Swedish Prescribed Drug Register for patients in Region Jönköping County, was retrived. Adherence to the therapy was calculated after 3 (*n*=445) and 5 years (*n*=248), defined as collecting over or equal to 80% of the prescribed drugs during the time periods, respectively. The results showed that adherence was over 90% after both 3 and 5 years.

Study II: Patient and tumour data from over 2,000 patients included in the SCAN-B project, in which primary tumours were defined by PAM50 subtypes, was retrieved. The ER+/HER2− tumours were divided into Luminal $A_{\text{Surrogate Classification (SC)}}$ and Luminal B_{SC} according to three surrogate algorithms. The agreement between luminal subtyping by gene expression and surrogate markers showed poor results. The highest agreement was 70% for the classification mainly based on HG. By combing HG and Ki67, nine subgroups were generated, and among these, six groups (51% of the cohort) were identified having >90% Luminal A_{PAM50} tumours.

Study III: Tumour blocks from primary breast cancer tissues were collected from patients that participated in the SBII:2pre study, in which premenopausal women were randomised between 2 years of adjuvant tamoxifen or no systemic treatment. Available follow-up data was over 30 years. TILs were assessed on whole tumour sections. The results showed that a high proportion of TILs (≥50%) was associated with better prognosis in all breast cancer subtypes. Furthermore, the benefit of tamoxifen was higher in patients whose tumours had low infiltration (<50%) of lymphocytes.

Study IV: Gene expression analysis (by NanoString Breast Cancer 360™ assay) of the primary tumours from patients in the SBII:2pre study, was conducted. This assigned each tumour a PAM50 intrinsic subtype and the corresponding patient a relapse risk score (ROR score). Surrogate classification according to St. Gallen 2013 was also performed. Both PAM50 and ROR score were prognostic. After 10 years of follow-up, re-classification of Luminal B_{SC} tumours into Luminal A_{PAM50} was associated with improved prognosis as compared to those uniformly classified as Luminal B, and benefit from tamoxifen could only be demonstrated in patients with Luminal A_{PAM50} tumours.

In conclusion, the results presented in this thesis showed that risk profiling of patients with primary breast cancer can be performed using a combination of gene expression and IHC markers. These markers and tests are pieces of the puzzle to be put together for each unique patient, and the pieces should be given different weights in order to tailor adjuvant treatment. Moreover, the results indicated that good adherence to endocrine therapy is possible to achieve.

Key words: breast cancer, prognostic, predictive, gene expression, tamoxifen, premenopausal, TILs

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Aspects of Endocrine Therapy in Primary Breast Cancer

Risk Profiling and Adherence Perspectives for Improving Tailored Adjuvant Treatment

Christine Lundgren M.D.

Faculty of Medicine
Department of Clinical Sciences, Lund
Division of Oncology
Lund University, Lund
Sweden

LUND
UNIVERSITY

Author: M.D. Christine Lundgren
Department of Clinical Sciences Lund
Division of Oncology
Lund University, Sweden

Main supervisor: Professor Lisa Rydén
Department of Clinical Sciences Lund
Division of Surgery
Lund University, Sweden

Co-supervisors: Professor Olle Stål
Department of Biomedical and Clinical Sciences
Linköping University, Sweden

Associate researcher Carina Forsare
Department of Clinical Sciences Lund
Division of Oncology
Lund University, Sweden

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Faculty of Medicine
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Gene expression analyses and its application for tumour signature determination 'is similar to recognition of the tartan plaids that distinguish one Scottish clan from another. In other words, the details of the individual thread colors are not as important as the overall pattern'.

van't Veer et al.[1]

Individual profiling of breast cancer patients is a combination of clinicopathological factors and the gene expression profile. These are separate details that are combined into a completeness for tailoring treatment for the individual patient.

Christine Lundgren M.D.

Table of Contents

Abbreviations

A	adenine
AF	activation function
AI	aromatase inhibitor
AKT	protein kinase B
ALND	axillary lymph node dissection
AMP	amplification
AP1	activator protein 1
ASCO	American Society of Clinical Oncology
ATC	anatomical therapeutic chemical classification
auROC	area under the ROC curve
BC	breast cancer
BCFi	breast cancer-free interval
BCI	breast cancer index
C	cytosine
cDNA	complementary DNA, circulating DNA
CAP	College of American Pathologists
CDK	cyclin-dependent kinases
CI	confidence interval
CTC	circulating tumour cell
CTS	clinical treatment score
DCIS	ductal carcinoma *in situ*
DBD	DNA binding domain
DDD	defined daily doses
DFS	disease-free survival
DNA	deoxyribonucleic acid
E2	endogenous oestrogen (17β-oestradiol)
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
EFGR	epidermal growth factor receptor
EIA	enzyme immune assay
ER	oestrogen receptor
ERE	oestrogen response elements
ERK	extracellular signal-regulated kinase
ET	endocrine therapy
FFPE	formalin-fixed paraffin-embedded
FSH	follicle-stimulating hormone
G	guanine
GnRH	gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
HE	haematoxylin-eosin

HER2	human epidermal growth factor receptor 2
HG	histological grade
HR	hazard ratio
IF	isoelectric focusing
IHC	immunohistochemistry
IKWG	International Ki67 Working Group
ISH	*in situ* hybridization
ISPOR	International Society for Pharmacoeconomics and Outcome Research
IGF-1	insulin-like growth factor-1
iTILs	intratumoural TILs
LBA	ligand binding assay
LBD	ligand binding domain
LH	luteinizing hormone
Lum	luminal
LVI	lymphovascular invasion
LPBC	lymphocyte-predominant breast cancer
MAPK	mitogen activated protein kinase
MEMS	microelectronic monitoring system
MGI	molecular grade index
MPR	medication possession ratio
mRNA	messenger RNA
MUT	mutation
NHG	Nottingham histological grade
NK-cells	natural killer-cells
NKBC	national quality register for breast cancer
NGS	next generation sequencing
NICE	National Institute for Health and Care Excellence
NST	no special type
OFS	ovarian function suppression
OR	odds ratio
OS	overall survival
PAM50	prediction analysis of microarray 50
pCR	pathological complete response
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
PR	progesterone receptor
PRE	progesterone response element
QC	quality control
qPCR	quantitative PCR
RTK	receptor tyrosine kinases

RNA	ribonucleic acid
RNA-seq	RNA sequencing
ROC	receiver operating characteristic
ROR	Risk of Recurrence
RR	relative risk
RS	recurrence score
RTK	receptor tyrosine kinase
SCAN-B	Sweden Cancerome Analysis Network - Breast
SERM	selective oestrogen receptor modulator
SHBG	sex hormone binding globulin
SP1	specificity protein 1
SSBCG	South Swedish Breast Cancer Group
sTILs	stromal TILs
T	thymine
TCGA	The Cancer Genome Atlas Project
TGF-α	transforming growth factor-alpha
TGF-ß	transforming growth factor-beta
TMA	tissue microarrays
TME	tumour microenvironment
TMB	tumour mutational burden
TILs	tumour-infiltrating lymphocytes
TNBC	triple-negative breast cancer
U	uracil
UICC	Union for International Cancer Control
vs	versus
WHO	World Health Organization
4-OH-A	4-hydroxy-androstenedione

Populärvetenskaplig sammanfattning (summary in Swedish)

Bröstcancer är den vanligaste typen av cancer hos kvinnor. Risken för återfall och död i bröstcancer har minskat på grund av tidig upptäckt genom screeningprogrammet med mammografi samt förbättrat återfallsförebyggande, adjuvant, behandling. För yngre, premenopausala, kvinnor orsakar bröstcancer en betydande andel av förtid död. Den adjuvanta behandlingen omfattar olika typer av systemisk behandling såsom hormonell, endokrin, behandling, antikroppsbehandling och cytostatika. Att kunna undvika överbehandling med adjuvant cytostatika, som leder till negativa patient- och samhälleliga konsekvenser, utan att påverka prognosen för den enskilda patienten, är av stor betydelse. Följsamheten till den endokrina behandlingen är enligt tidigare studier otillfredsställande, främst på grund av biverkningar. När målet är att minska onödig användning av cytostatika, är kunskap om följsamheten till endokrin behandling viktig.

Bröstcancer omfattar heterogena tumörer. Genom analys av genuttryck i tumören kan bröstcancer indelas i undergrupper, så kallade subtyper, som har visat sig ha olika prognostisk och prediktiv betydelse. Immunohistokemiska (IHC) markörer såsom östrogen- och progesteronreceptorn (ER och PR), human epidermal growth factor receptor 2 (HER2), tillväxtmarkör (Ki67) och histologisk grad (HG, (Nottingham histological grade, NHG)) används för att indela tumörerna i motsvarande subtyper genom att kombinera markörerna enligt så kallade surrogatklassificeringar. De hormonellt känsliga ER-positiva/HER2-negativa (ER+/HER2–) tumörerna, indelas i två undergrupper (Luminal A och Luminal B) och representerar majoriteten av alla bröstcancertyper. Det finns dock en viktig skillnad mellan dem; Luminal B-tumörer är förknippade med sämre prognos och mycket förenklat rekommenderas patienter med Luminal A-tumörer adjuvant endokrin behandling, medan patienter med Luminal B-tumörer i allmänhet rekommenderas tillägg med cytostatika till den endokrina behandlingen. Subtypningen av luminala tumörer baserat på genuttryck har visat sig bättre kunna förutsäga prognos än motsvarande surrogatsubtyper. Flera genexpressionstester baserade på en begränsad uppsättning gener har utvecklats. Prosigna® definierar tumörens subtyp baserat en algoritm kallad PAM50, samt tilldelar patienten en riskpoäng (Risk of Recurrence (ROR) score) som anger återfallsrisken efter 10 år, förutsatt 5 år endokrin behandling. Testet är idag i kliniskt bruk för vissa postmenopausala kvinnor, men kunskapen om testets värde för premenopausala kvinnor är sparsam. Även om genexpressionstester är kommersiellt tillgängliga idag, råder osäkerhet om de ska tillämpas på alla patienter, då man även måste ta hänsyn till det hälsoekonomiska perspektivet.

För att bättre kunna skräddarsy den adjuvanta behandlingen behövs redskap för att kunna tilldela patienten en egen riskprofil gällande prognos, samt prediktiva markörer som indicerar förväntad nytta av endokrin behandling. Den premenopausala patientgruppen tenderar att erbjudas mer adjuvant behandling enbart utifrån deras yngre ålder. Tamoxifen infördes som adjuvant behandling för 30 år sedan för patienter med ER+ tumörer, där i regel 5 års behandling är standard. För patienter som vill bli gravida efter bröstcancerdiagnos rekommenderas enligt nuvarande riktlinjer 2 års tamoxifen före graviditet, vilket betonar att ytterligare kunskap om en kortare period av tamoxifen är motiverad. Då den endokrint känsliga subtypen av bröstcancer har visat sig kunna ge återfall under lång (>20 års) tid, är studier med långtidsuppföljning viktiga för att studera den långsiktiga betydelsen av markörer och genexpressionstest. Trots att genexpressionstest blivit en del av klinisk rutin, är rutinmässiga markörer med immunohistokemiska analyser fortfarande av betydelse. Immunförsvaret och dess komplexa komponenter, såsom tumörinfiltrerande lymfocyter (TILs) kan bedömas av patolog på helsnitt utan komplex teknik och denna markör har hittills visat sig vara av betydelse för att förutsäga prognosen för vissa subtyper av bröstcancer.

I den första delstudien inhämtades uppgifter om förskrivning och uttag från Läkemedelsregistret gällande endokrina läkemedel för patienter i Region Jönköpings län. Följsamheten beräknades efter 3 och 5 år genom att definiera följsamhet som uttag av 80% eller mer av de förskrivna preparaten under de respektive tidsperioderna. Resultatet visade att följsamheten var över 90% för båda tidsintervallen, vilket talar för mycket god följsamhet.

I delstudie två inhämtade vi patient- och tumördata från över 2,000 patienter som inkluderats i projektet SCAN-B, i vilken genexpressionsanalys utfördes på primära brösttumörer som tilldelade dem en subtyp. Parallellt indelade vi de ER+/HER2– tumörerna enligt tre surrogatalgoritmer, varav en var vår egen föreslagna algoritm (gradbaserad) där tumören initialt indelas genom histologisk grad. Överensstämmelsen mellan luminal subtypning enligt genexpression jämfördes med de motsvarande tre surrogatklassifikationerna. Resultatet var otillfredsställande där den gradbaserade hade bäst överensstämmelse (70%). Genom explorativa analyser påvisade vi att Ki67 och HG kan kombineras för att indela ER+/HER2– tumörer i nio undergrupper där andelen Luminal A-tumörer, enligt genexpression, i sex av dessa var så pass god att användningen av genexpressionstest i dessa grupper kan ifrågasättas.

För de tredje och fjärde delstudierna insamlades tumörklossar från patienter som deltog i SBII:2pre studien, i vilken premenopausala kvinnor randomiserades till 2 års adjuvant tamoxifen eller ingen behandling. Över 30 års uppföljningsdata fanns tillgänglig. I tredje delstudien bedömdes TILs på tumörmaterialet och syftet var att analysera dess samband med prognos och möjlig prediktion av nyttan av adjuvant tamoxifen. Resultatet visade att hög andel TILs (≥50%) var en oberoende indikator för bättre

prognos för alla subtyper av bröstcancer. Vidare fann vi att effekten av tamoxifen var större för patienter med tumörer som hade låg infiltration (<50%) av lymfocyter.

I den fjärde delstudien genomfördes genexpressionsanalys (NanoString Breast Cancer 360™ assay) av tumörer från patienter i studien SBII:2pre. Denna analys tilldelade tumören en subtyp enligt PAM50 och motsvarande patient en återfallsriskpoäng (ROR score). Vi analyserade det långsiktiga prognostiska värdet av dessa data (>30 års uppföljning relativt bröstcancerhändelser och totalöverlevnad). Dessutom indelade vi de ER+/HER2– tumörerna i surrogatsubtyper (Luminal $A/B_{\text{Surrogate Classification (SC)}}$) enligt St. Gallen 2013. Resultatet visade att subtypning enligt PAM50 var prognostiskt, där Luminal A_{PAM50} -tumörer var associerade med bättre prognos jämfört med Luminal B_{PAM50} -tumörer. Patienter med låg ROR score hade bättre prognos än de med hög ROR score. Dessutom visade vi att luminal subtypning med PAM50 verkar vara prediktivt för nyttan av adjuvant tamoxifen. Efter 10 års uppföljning hade enbart de patienter med Luminal A_{PAM50} -tumörer nytta av tamoxifen jämfört med de som hade Luminal B_{PAM50}-tumörer. I studien bekräftade vi slutligen att det skedde en betydande omklassificering (>50%) av tumörer från Luminal B_{SC} till Luminal A_{PAM50} och dessa patienter hade en bättre prognos än de som klassificerats som Luminal B genom bägge metoderna.

Sammanfattningsvis visar resultaten att riskprofilering av patienter med primär bröstcancer inte kan genomföras med ett enda test eller en markör. Kliniska testresultat baserat på genexpressionsanalys och markörer baserade på IHC, bör snarast ses som pusselbitar som ska sättas samman för varje unik patient med mål att kunna skräddarsy den adjuvanta behandlingen. Varje pusselbit måste dock ges olika vikt. En framtida utmaning är att kunna vikta dessa rätt, snarare än att hitta en pusselbit som ger hela sanningen om patientens prognos och förväntat nytta av onkologisk behandling.

List of included papers

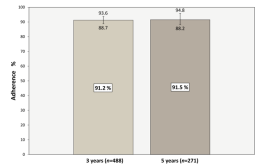
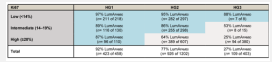
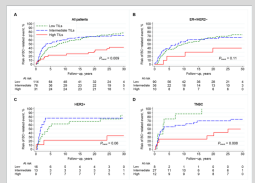
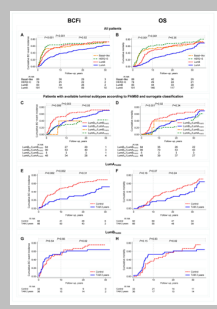
I **Good adherence to adjuvant endocrine therapy in early breast cancer - a population-based study based on the Swedish Prescribed Drug Register**
Lundgren C, Lindman H, Rolander B, Ekholm M.
Acta Oncologica. 2018 Jul;57(7):935–40

II **Agreement between molecular subtyping and surrogate subtype classification - a contemporary population-based study of ER-positive/HER2-negative primary breast cancer**
Lundgren C, Bendahl P-O, Borg Å, Ehinger A, Hegardt C, Larsson C, Loman N, Malmberg M, Olofsson H, Saal LH, Sjöblom T, Lindman H, Klintman M, Häkkinen J, Vallon-Christersson J, Fernö M, Rydén L, Ekholm M.
Breast Cancer Research and Treatment. 2019 Nov;178(2):459–67

III **Tumour-infiltrating lymphocytes as a prognostic and tamoxifen predictive marker in premenopausal breast cancer: data from a randomised trial with long-term follow-up**
Lundgren C, Bendahl P-O, Ekholm M, Fernö M, Forsare C, Krüger U, Nordenskjöld B, Stål O, Rydén L
Breast Cancer Research. 2020 Dec;22(1):140

IV **PAM50 subtyping and ROR score improve long-term prognostication for premenopausal patients included in the randomised SBII:2 trial**
Lundgren C, Bendahl P-O, Church S, Ekholm M, Fernö M, Forsare C, Krüger U, Nordenskjöld B, Stål O, Rydén L
Manuscript submitted

Thesis at a glance

Study	Aims	Patients and methods	Figure	Results and conclusions
I	To assess adherence to adjuvant endocrine therapy in a cohort with primary breast cancer, after 3 and 5 years, respectively.	Data of prescription for 488 patients from the Swedish Prescribed Drug Register was retrieved. Medication Possession Ratios (MPRs), for the time periods of 3 and 5 years were calculated. Adherence was defined as MPR≥80%.		Adherence was 91.2% after 3 years (*n*=445) and 91.5% (*n*=271) in patients who had completed 5 years of treatment. This indicated substantially higher adherence (>90%) to adjuvant endocrine therapy than previously reported.
II	To evaluate agreement between intrinsic subtyping using the PAM50 algorithm and that using surrogate classification. Identify subgroups consisting mainly of Luminal A or B subtypes using PAM50.	2,063 patients with primary ER+/HER2− breast cancer were included. These luminal tumours were analysed using RNA sequencing from the SCAN-B project providing intrinsic subtypes by PAM50. Surrogate subtyping according to three algortihms was perfomred. Agreement (%) and kappa (κ) analyses were reported.		Agreement between luminal intrinsic subtypes and surrogate subtypes was generally poor (62–70%). A combination of histological grade and Ki67 could identify patients very likely to have Luminal A$_{PAM50}$ tumours.
III	To define the prognostic effect of tumour-infiltrating lymphocytes (TILs) in premenopausal patients stratified by breast cancer subtypes. To examine the tamoxifen-predictive value of TILs.	Archival tissues from primary breast cancer (*n*=520) from the SBII:2pre trial were retrieved. Scoring of TILs was performed on whole tissue sections (*n*=447). Outcomes in relation to breast cancer free-interval (BCFi) and overall survival (OS) were analysed.		High (≥50%) infiltration of TILs was an independent favourable prognostic factor. Similar effects were observed across all subtypes. The effect of adjuvant tamoxifen was stronger in patients with ER+ tumours and TILs <50%.
IV	To evaluate the prognostic value of PAM50 subtyping and Risk of Recurrence (ROR) score, and surrogate subtyping by St. Gallen 2013 in premenopausal patients. To examine the tamoxifen-predictive effect of luminal PAM50 subtypes.	Ribonucleic acid (RNA) was extracted from the primary tumour tissues from patients in the SBII:2pre trial, and analysed by the NanoString Breast Cancer 360 ™ assay. This provided PAM50 subtypes and ROR score (*n*=437). Outcomes in realtion to BCFi and OS were analysed.		PAM50 subtyping and ROR score were prognostic in premenopausal women. A major (>50%) proportion of Luminal B$_{SC}$ tumours were classified as Luminal A$_{PAM50}$ and these patients had a better prognosis as compared to those classified as Luminal B. Luminal PAM50 subtypes had a possible predictive value of tamoxifen benefit after 10 years of follow-up.

Introduction

Breast cancers tumours are heterogeneous. The oestrogen receptor-positive/human epidermal growth factor receptor 2-negative, (ER+/HER2–) tumours are divided into two main categories; Luminal A and Luminal B, and the latter subtype is associated with worse prognosis. Therefore, these patients are treated differently regarding adjuvant therapy. Today, markers based on immunohistochemistry/*in situ* hybridization (IHC/ISH) are used in combination to classify the tumours into Luminal $A_{\text{Surrogate Classification (SC)}}$ or Luminal B_{SC}, which correspond to the intrinsic subtypes based on gene expression. International guidelines recommend multigene assays as a complement to conventional risk assessment in patient subgroups with equivocal risks. Thus far, there is more robust data for risk stratification of postmenopausal, not premenopausal, patients with ER+/HER2–, node-negative disease.

Since patients with ER+/HER2– tumours may develop recurrence decades after diagnosis, despite endocrine therapy, long-term follow-up studies are needed. Thus far, ER the only predictive marker for endocrine treatment and adjuvant tamoxifen has repeatedly shown a beneficial effect reducing recurrence and death. However, the side-effects of endocrine therapy are a major reason for low adherence, affecting the outcomes in breast cancer patients.

Immuno-oncology is an emerging research area in cancer treatment, also in breast cancer, and the infiltration of lymphocytes is thought to be associated with differences in prognosis and treatment prediction in certain breast cancer subtypes. Their value in ER+/HER2– breast tumours is however not settled.

Epidemiology

Breast cancer incidence and outcomes

Breast cancer is the most common cancer in women in Sweden. More than every 10th woman will develop breast cancer and approximately 8,300 women were diagnosed with this disease in 2019[2]. Although the incidence has increased, the relative 5-year overall survival (OS) in Sweden has steadily increased (92% in 2016)[2]. Both these

phenomena have led to an increase in the prevalence of breast cancer. The mortality is dependent on many factors such as the tumour stage and its subtype. A majority (64% in 2019) of all detected breast cancers in Sweden are diagnosed within the screening programme[3] and the improved outcomes in this disease is both due early detection in the screening programme and improved adjuvant treatment[4].

Risk factors

There are several risk factors for developing breast cancer, such as environmental factors (previous mantel irradiation for Hodgkin´s lymphoma), genomic mutations (such as *BRCA1* and *BRCA2*), family history and reproductive factors (fewer children, early menarche, late menopause)[4]. The genomic mutations are described in more detail later in the thesis.

In premenopausal women, the ovaries are the main sources of the production of endogenous oestrogen (17β-oestradiol; E2), however, the corresponding sources of this hormone in postmenopausal women are the fat tissue and adrenal cortex[5, 6]. Although the levels of E2 become reduced with age, these levels vary among women and higher levels of the endogenous oestrogen have previously been associated with increased risk of postmenopausal breast cancer[7]. Hormone replacement therapy during menopause increases the breast cancer risk as well[8], and moreover, there is an association between high breast density and breast cancer incidence[9–11].

Pathology of breast cancer

Histopathological report and tumour morphology

The pathological report of breast cancer incorporates different variables[4, 12]. For example, the relation of the tumour cells to the normal stromal area is assessed to determine whether it is an invasive or *in situ* process[4]. Furthermore, analysis of size and cell morphology, further categorisation by different IHC markers, and if needed additional methods, are performed.

Using histopathological assessment, the breast cancers are classified according to the World Health Organization (WHO) classification and the dominating subtypes are the ductal carcinoma (also defined as no special type, (NST)) and the lobular tumours[13]. The ductal carcinoma is in general recognised as a solid, grossly palpable mass[4]. The cells of the lobular carcinoma, however, are aligned in so called 'Indian files' and this tumour type generally lacks calcifications[4]. This type, in comparison to the ductal carcinoma, is more often bilateral and multicentric[14]. Furthermore, these tumours are

in general ER+ and seem to give rise to metastases diagnosed at a later time-point compared to the NST. However, if it recurs, the sites of metastases are more uncommon such as the gastrointestinal tract[15]. The medullary histological type is often poorly differentiated with marked atypia and the cells grow to form in large solid patterns and have increased lymphoplasmocytic infiltration[4]. Despite the atypia, this tumour type is associated with a better prognosis than other poorly differentiated types[16] and this might be explained by the immune response. Moreover, it is more frequently associated with younger age and germline *BRCA1* mutation[17].

Pathological features and biomarkers

ER

The ER is a ligand-activated transcription factor, belonging to the nuclear hormone receptor family[18]. Two different types of ERs are known; ERα, that was discovered in the late 1950s using radiolabelled oestradiol in rats[19] and was later cloned in 1986[20, 21], and ERβ, cloned in the 1990s by Gustafsson et al.[22]. The role of ERα in breast cancer is well documented as described below, however, the function of ERβ is less known[23] and therefore the denotation ER is restricted to ERα.

The ER contains different domains, including the deoxyribonucleic acid (DNA) binding domain (DBD), a ligand binding domain (LBD) and transactivation regions; activation function 1 (AF1, regulated by phosphorylation) and AF2 (regulated by oestrogen binding) and these latter domains recruit coregulatory proteins to the DNA-bound receptor[18, 24, 25]. The classical mechanism of ER action is based upon ligand-binding by oestrogen[25]. The most potent ligand of ER is E2 and once it binds to ER, a signalling cascade is initiated, leading to dimerization and binding of the ER directly to an oestrogen response element (ERE) that causes either increased or decreased gene transcription[24]. Moreover, ligand-bound ER can interact with other transcription factor complexes (such as specificity protein 1 (SP1) and activator protein 1 (AP1)-responsive elements) and affect transcription of genes not harbouring EREs[18]. Apart from the above-mentioned mechanism for gene activation, there is a ligand- independent mechanism of ER activation, mediated by a crosstalk among other signalling pathways[18, 24]. It is postulated that E2-mediated responses could be conducted though a G protein-coupled receptor (GPCR) and this is a possible mechanism for endocrine resistance[26] (see *Adjuvant endocrine treatment* subsection, *Introduction* chapter).

Methods for ER status determination

Initially, the ER status was mainly determined using ligand-binding assays (LBAs) in which radiolabelled ligands were employed and the oestradiol-receptor complex in the cytosol was measured[27–29]. Later on, measurement by the cytosol-based enzyme immune

assays (EIA) was performed and further on in the 1990s, the IHC assays were developed based on recognition of ER by antibodies[30, 31]. In accordance with previous Swedish guidelines, ER (and PR)-positivity was defined as >10% of positively stained tumour cells in the tissue section assessed by IHC. However, this was changed to ≥10% in the updated version in 2020[12]. According to American Society of Clinical Oncology (ASCO) guidelines and College of American Pathologists (CAP), ER status should be considered as positive if 1% or more of the tumour cells demonstrate positive staining[32]. ER+, as compared to ER–, breast cancer is associated with an early (during the first 5 years) favourable prognosis, however patients with ER+ breast cancer maintain a particular recurrence rate during long-term follow-up[33]. ER-positivity is associated with an expected benefit of endocrine therapy and more than 80% of all primary breast cancers are ER+ and these patients are in general recommended adjuvant endocrine treatment[10, 34].

Progesterone receptor (PR)

Progesterone receptor (PR) is as ER, a nuclear hormone receptor, located in the cytosol and acts as a ligand-dependent transcription factor. Once progesterone binds to the PR, there is a dimerization and translocation of the complex to the nucleus where it binds to the progesterone response element (PRE) and this in turn leads to up- or downregulation of the target genes[24]. There are two isoforms of PR (A and B) that are regulated by the ERα[35] and ER and PR are often co-expressed in breast cancer[36]. The PR-A is mainly expressed in ductal carcinoma *in situ* (DCIS) and breast malignancies[37].

Previous studies have shown that ER-positive/PR-negative (ER+/PR–) breast cancer tumours, are associated with worse outcome as compared to ER+/PR-positive (ER+/PR+) tumours[38]. This indicates that PR is a prognostic marker[39–41]. Moreover, some studies have demonstrated a possible predictive value of PR for adjuvant tamoxifen benefit[42–44]. However, according to the meta-analysis from the Early Breast Cancer Trialists' Collaborative Group (EBCTCG), adjuvant benefit of endocrine therapy in ER+ tumours did not depend on PR status[34]. ER-negative/PR+ (ER–/PR+) tumours are rare (approximately 1–4%)[45] and the existence of this subgroup is unknown[46].

HER2

The *HER2* oncogene encodes a transmembrane glycoprotein receptor with tyrosine kinase activity[47, 48]. The gene of the HER2, *Her2/neu*, located at chromosome 17q, was detected in the 1980s, providing the definition of HER2 status of breast cancer[49, 50]. The receptor consists of an extracellular, a transmembrane, and an intracellular domain. HER2 is homologous to other members of the HER (also referred to as erbB) family, including the epidermal growth factor receptor (EFGR (HER1)), HER2, HER3 and HER4[48]. The HER2 is important in triggering signalling pathways regulating epithelial

cell growth, differentiation and angiogenesis by dimerization with other HER members[48, 51–53]. HER2 status is routinely defined in all breast cancer tumours by IHC and scored as 0, 1+, 2+ or 3+ depending on the degree of staining of the membrane. The 0 and 1+ are defined as negative, however the 2+ cases are further analysed (*HER2* gene amplification) by ISH[12]. Those defined as 3+ (complete/intense membrane staining in >10% of the tumour cells) are regarded as HER2+[54]. In general, the HER2+ subtype is present in approximately 13% of breast cancer tumours in Sweden (according to the data in 2020)[3]. HER2+ breast cancer is associated with worse prognosis, but the targeted treatment with the monoclonal antibody trastuzumab, targeting the extracellular domain of the HER2 protein have improved the outcomes in these patients[55–57].

Ki67

The cell cycle is divided into different phases. The G_0 is a resting phase during which the cell grows in the interphase that comprises three steps: S phase (DNA replication), G_1 phase (gap between M and S phase) and the G_2 phase (gap between S and M phase). In the M phase, the cell division finally occurs[58]. Cyclins are the proteins that bind to cyclin-dependent kinases (CDKs), driving the cell cycle through its different phases[58]. There are different methods for assessing cell proliferation in breast cancer tumours[59]:

- DNA flowcytometry (evaluation the proportion of cells in the S phase)
- Mitoses activity (manual counting of number of mitoses using microscope observation)
- IHC staining using antibodies targeted to proteins activated during different phases of the cell cycle (Ki67, cyclin A)

Ki67 is a protein that is present in the cell nuclei at different amounts depending on cell cycle phase and could therefore be used to evaluate growth fraction of cells[60]. According to the Swedish guidelines at time of the included studies of the thesis, Ki67 was assessed by counting the percentage of positively stained nuclei in at least 200 cells in hotspots of invasive breast cancer[12]. The result is reported as the proportion (percentage) of positive stained tumour cells as a continuously value (0–100%). The Ki67 level has thus far been further divided into three categories (low, intermediate and high) based on laboratory-specific cut-off values. These guidelines will change in the updated guidelines; all cells will be counted (and not hotspots) and the lab-specific cut-offs will be replaced by ≤5% as low and ≥30% as high values as stated by the International Ki67 Working Group (IKWG)[61].

The proportion of Ki67 in breast cancer is correlated to prognosis[62, 63] and furthermore proposed as a predictive marker in the neoadjuvant setting[64]. Ki67 has also been adapted as a marker for surrogate subtyping as postulated by the St. Gallen consensus (division of luminal tumours into A and B, see *Surrogate subtyping* subsection,

Introduction chapter)[65, 66]. However, this marker is widely debated, both regarding its reproducibility, method standardization and utility, especially its intermediate values[61, 67, 68].

Histological grade

The morphologic assessment of histological grade (HG) is commonly done by the Elston Ellis grading system (Nottingham histological grade, NHG)[69]. This is a combination of the tubule formation, nuclear pleomorphism and mitotic count[12, 70]. Each of these parameters are scored as 1–3 and are finally summed up into a final score of NHG1-3 (Table 1 and Figure 1).

Table 1. Scoring of histological grade according to Nottingham histological grade (NHG)

Total score[a]	NHG	Classification of histological grade
3–5	1	Low (well differentiated)
6–7	2	Intermediate (moderately differentiated)
8–9	3	High (poorly differentiated)

[a]Based upon assessment of tubule formation, nuclear pleomorphism and mitotic activity

The HG has in previous studies shown to be of prognostic value regarding outcomes[71–73]. Based on a previous Swedish trial, HG was associated with survival, although this effect was attenuated in the later follow-up years[74]. Of notice, some tumour types, such as the medullary breast cancer, seem to have a favourable prognosis despite poorly differentiated characteristics[16]. Approximately 50% of the breast cancer tumours are HG2 and the clinical value of this intermediate group is uncertain[75].

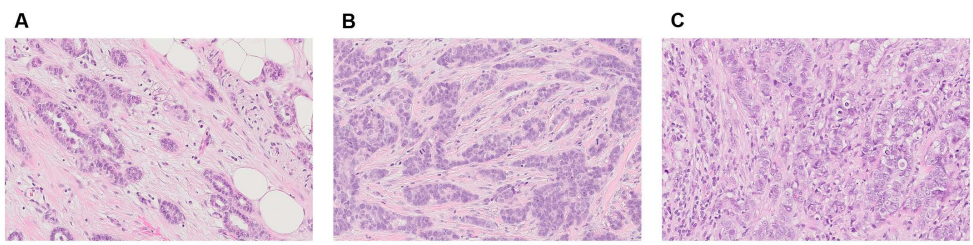


Figure 1. Histological images of breast cancer tumours
NHG (**A**) 1, (**B**) 2 and (**C**) 3. Magnification of 150×. Images are provided by courtesy of Dr. Ute Krüger.
Abbreviation: NHG, Nottingham histological grade

Lymphovascular invasion

Lymphovascular invasion (LVI) is assessed using haematoxylin-eosin (HE)-stained slides as present, absent or not possible to assess. According to the Swedish pathological guidelines, LVI is defined as present when tumour cells in cavities lined with endothelium are verified in the peritumour area[12]. If needed, IHC endothelial markers can be used as a complement. LVI is associated with younger age, positive lymph nodes, larger tumour size and poor differentiation[76, 77]. The role of LVI as a marker for

treatment decision making is not clear, however, LVI has in several publications shown to be of prognostic value both in node-negative[78] and node-positive patients[79]. Furthermore, LVI is an independent factor for outcome in the adjuvant[80] and neoadjuvant setting[81]. In a prospective population-based study, LVI was not an independent high-risk criterion, only in association with other high-risk factors[82].

Adjuvant endocrine therapy

Local and systemic treatment of breast cancer

Surgery is the primary treatment of breast cancer[4] and considered as a local treatment of this disease. In a historical perspective, the 'Halstedian' radical mastectomy was practiced as early as in the 1880s, however, in the 1960s less extensive surgery became more popular[83, 84]. Axillary lymph node dissection (ALND) was previously the staging method of the axilla, but the sentinel lymph node biopsy[85–87], as presented in the 1990s, is nowadays regarded as a standard practice in patients with a clinically node-negative axilla. However, the timing depends on whether neoadjuvant treatment is given. The addition of adjuvant radiotherapy after breast-conserving surgery, reduces the risk of recurrence by a half and the breast cancer death by rate of a sixth[88]. After mastectomy and axillary dissection, adjuvant radiotherapy reduces both recurrence and breast cancer mortality in those with positive lymph nodes, despite of whether a systemic treatment was given or not[89].

The systemic adjuvant treatment of primary breast cancer aims to eliminate microscopic cancer burden and comprises different modalities such as chemotherapy, targeted therapy and endocrine therapy. EBCTCG demonstrated in a meta-analysis in 1988, that combination therapy with cytotoxic drugs, was more effective than for single-agent therapy and that administration for 8–24 months was not more effective than for 4–6 months[90]. There are different treatment regimens in the adjuvant setting, and anthracycline- and taxane-based drugs are the current standard. In the report from EBCTCG 2012, the most efficient chemotherapy treatment regimens resulted in a relative reduction of breast cancer mortality by one third, irrespective of age and tumour characteristics, however, the absolute benefit was dependent on the absolute risk without chemotherapy (meaning the risk remaining after endocrine therapy in patients with ER+ tumours)[91]. In patients with HER2+ disease, effective results by the treatment with trastuzumab in the metastatic setting was reported in the late 1990s, and trastuzumab was proven to increase the benefit of first-line chemotherapy treatment in those patients[55, 92]. Thereafter, trastuzumab became a revolution also for patients with early HER2+ breast cancer[56, 57].

Adjuvant endocrine treatment

The adjuvant endocrine therapy of breast cancer offers a possibility to reduce recurrence and increase survival and is in general recommended for the patients with ER+ tumours[10, 93, 94]. There are several endocrine therapies in the adjuvant setting; tamoxifen, aromatase inhibitors (AIs) and gonadotropin-releasing hormone (GnRH) analogues[4] and the choice is based upon clinicopathological data, especially the menopausal status.

Historical perspective of endocrine treatment

The association between hormones and breast cancer growth was recognised in the 1896, when Beatson demonstrated that removal of the ovaries from premenopausal women with advanced breast cancer improved their prognosis[95]. After the discovery that oestrogen was produced by the ovaries[19], research focused on finding antagonists to decrease breast cancer incidence. The connection between oestrogen and breast cancer progression has been descried in epidemiological studies[96] and Lippman et al. demonstrated in 1976, the growth effect of oestrogen as well as the inhibitory effect of anti-oestrogens on cell growth in breast cancer cell lines that contained oestradiol receptors[97]. The fact that the receptors were expressed in tumour cells and could be the targeted with antihormonal drugs, led to the development of oral endocrine therapy for breast cancer.

Tamoxifen

Tamoxifen is a selective ER modifier (SERM) and was initially developed as a contraceptive drug in the 1960s[98]. Although that purpose failed, further studies resulted in tamoxifen as an effective treatment for advanced breast cancer in the 1970s, initially for postmenopausal and later on for premenopausal women[98, 99]. These results stimulated further research on the role for tamoxifen as an adjuvant treatment for breast cancer[90, 98, 100, 101]. Based on meta-analyses from EBCTCG in the 1990s, particularly after one study in 1998 showed its substantial benefit, the treatment with adjuvant tamoxifen was extended to premenopausal women[102, 103].

Since the efficacy of tamoxifen has been demonstrated, it has been used as a dominated treatment for breast cancer. The doses of tamoxifen varied in the early studies from 20–40 mg and the duration was in general 2 years, but further on, 5 years of tamoxifen was demonstrated to improve outcomes[103–106]. Based on a review from EBCTCG in 2011, after 15 years of follow-up, 5 years of adjuvant tamoxifen reduced the relative risk of recurrence by 39% (absolute gain of 13%) and the corresponding values for breast cancer mortality was 30% (absolute gain of 9%)[34]. Moreover, ER status was shown to be the only predictive marker for tamoxifen benefit.

Tamoxifen acts through its active metabolites, most importantly endoxifen, activated mainly by cytochrome P450 enzymes[107, 108]. Tamoxifen is a nonsteroidal SERM,

binding competitively to the ER forming as a receptor-ligand complex. This complex binds to the DNA and inhibits oestrogen binding to its receptor, thereby reducing breast cancer cell growth[109–111]. It is described that tamoxifen most likely modulates the ER activity by a balancing the of co-activators and co-repressors complex recruitment to the AF2 but does not prevent activation of AF1[25]. Tamoxifen has both oestrogen and anti-oestrogen actions in different target tissues and in contrast to the mammary epithelium, it mediates pro-oestrogenic effect on the uterine epithelium[100]. The ER activity in the breast epithelium is mainly because of the presence of AF2 and, whereas in tissues such as the uterus, the AF1 activity is dominating, explaining tamoxifen action as an antagonist/agonist in different tissues[25]. However, the mechanisms of action of tamoxifen are complex and tumour growth control by tamoxifen might be regulated by different mechanisms (Figure 2 and 3). Oestrogen levels are associated with an increase in the level of transforming growth factor alpha (TGF-α) which can stimulate tumour growth and angiogenesis[111]. Tamoxifen has been postulated to increase the level of oestrogen receptors but decrease tumour concentrations of TGF-α and partially inhibit tumour growth. Another suggestive mechanism of antiproliferative effect of tamoxifen is the induction of synthesis of transforming growth factor beta (TGF-ß), acting as a negative autocrine regulatory molecule[100, 112]. Tamoxifen also lowers the levels of insulin-like growth factor-1 (IGF-1), and it is known that this factor could stimulate tumour growth[113]. Moreover, tamoxifen seems to increase the sex hormone binding globulin (SHBG) in postmenopausal women, leading to a lower amount of free oestradiol in the serum[114].

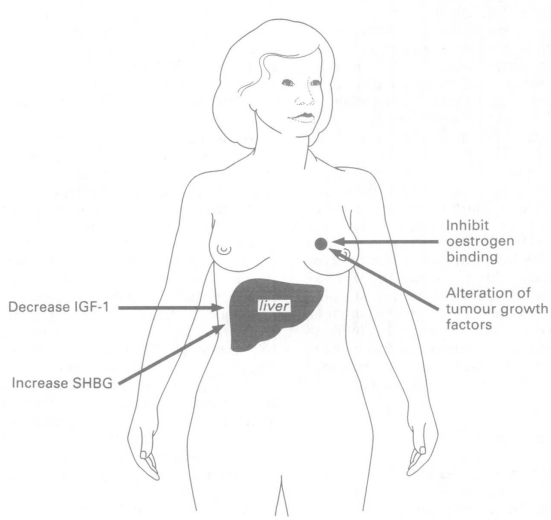


Figure 2. Tamoxifen and its mechanisms of action

Tamoxifen inhibits oestrogen binding to the oestrogen receptors and affects growth factors in the tumour. IGF-1 is reduced, and moreover, tamoxifen increases the level of ciculation SHBG in postmenopausal women. This results in lower amount of free versus SHBG-bound oestradiol in patients treated with tamoxifen. Reprinted from Jordan C,[111] with permisison from John Wiley & Sons.

Abbreviations: IGF-1, insulin-like growth factor-1; SHBG, sex hormone binding globulin

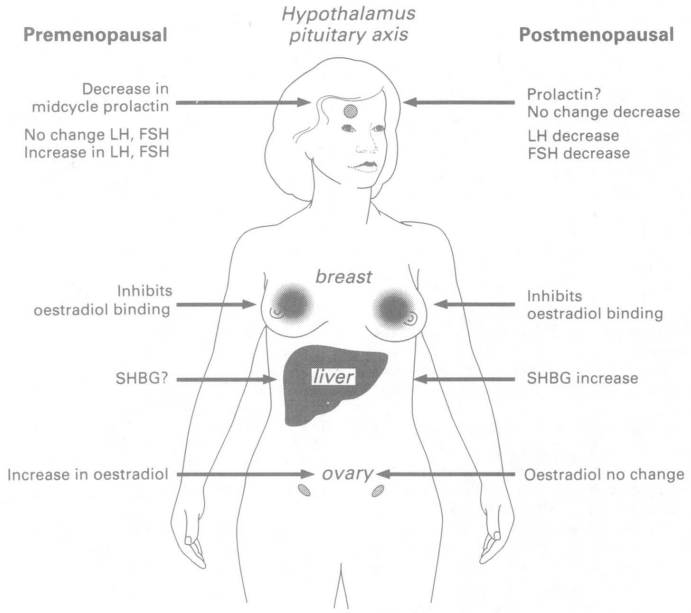


Figure 3. Endocrine effects of tamoxifen in pre- and postmenopausal women
Reprinted from Jordan C,[111] with permisison from John Wiley & Sons.
Abbreviations: FSH, follicle-stimulating hormone; IGF-1, insulin-like growth factor-1; LH, luteinizing hormone; SHBG, sex hormone binding globulin

Aromatase inhibitors

In postmenopausal women, precursors of oestrogen are mainly produced by the adrenal glands and are transformed into active oestrogen by the enzyme aromatase in different body tissues such as the fat[4]. In the 1970s it was reported that blocking of oestrogen production by enzyme inhibition could be effective and the 4-hydroxy-androstenedione (4-OH-A) was demonstrated to be a potent AI and resulted in tumour regression in rat mammary tumours[115, 116]. In the mid 1980s the first selective AI was used in clinic for breast cancer treatment and AIs (anastrozole, letrozole and exemestane) are nowadays used in both the metastatic and adjuvant settings.

There are two non-steroidal AIs; anastrozole and letrozole, and one steroidal AI; exemestane[117], but there is no difference between their clinical effects[118]. Due to their mechanism of action, they are not suitable for premenopausal women, unless a GnRH analogue is added.

Tamoxifen vs AI

The effects of tamoxifen versus (vs) AI have been studied in some meta analyses[118–120]. In the large meta analysis from EBCTCG, 32,000 postmenopausal women with ER+ disease were treated with 5 years of tamoxifen or AI administration, or as a sequenced therapy[118]. AI reduced the recurrence rates by 30% as compared with tamoxifen while

the treatment differed. In addition, 5 years of AI reduced the 10-year breast cancer mortality rates by 15% as compared with tamoxifen.

GnRH analogues

The purpose of the GnRH analogue treatment is an inhibitory effect in the ovaries, resulting a medical castration in premenopausal women (ovarian function suppression (OFS))[4]. Treatment by a GnRH analogue could be added to a backbone of an endocrine drug (tamoxifen or AI) and data from 8-years follow-up of the SOFT trial demonstrated a disease-free survival of 79% with tamoxifen monotherapy, 83% with tamoxifen and OFS, and 86% with exemestane and OFS[121]. In the HER2– cohorts receiving chemotherapy, the 8-year rate of freedom from distant recurrence was higher among those assigned to receive exemestane and OFS vs tamoxifen and OFS (7 and 5% in SOFT and TEXT trials, respectively). These results were however not replicated in the ABCSG-12 trial[122].

Extended endocrine therapy

By adding 5 years of tamoxifen after 5 years of initial tamoxifen treatment in patients with ER+ tumours, the ATLAS trial showed an absolute reduction of breast cancer recurrence and breast cancer mortality at 15 years of 3.7% and 2.8%, respectively[123]. The aTTOm trial reported similar findings[124]. Extended treatment by 5 years of AI vs placebo after 5 years of tamoxifen, was effective based on the MA.17 trial including postmenopausal patients[125, 126]. Prolonged treatment by AI after 5 years of AI (MA.17R) does seem to increase recurrence-free survival, however not OS[127].

Endocrine resistance

Although a positive ER status in the breast tumour, some of these patients do not respond to antihormonal therapy. There are many theories explaining the reasons for endocrine resistance and some might be related to the ER itself with other to signalling cellular pathways. As illustrated in Figure 4[24], different mechanisms could involve:

- Loss/mutation of the ERα
- Activation through receptor tyrosine kinases (RTK) by growth factors, hormones or cytokines that induce a signalling cascade through stimulation of signalling pathways (such as the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) and mitogen activated protein kinase (MAPK)/RAS/extracellular signal-regulated kinase (ERK) pathway)
- Overexpression of positive cell cycle regulators by activating CDK

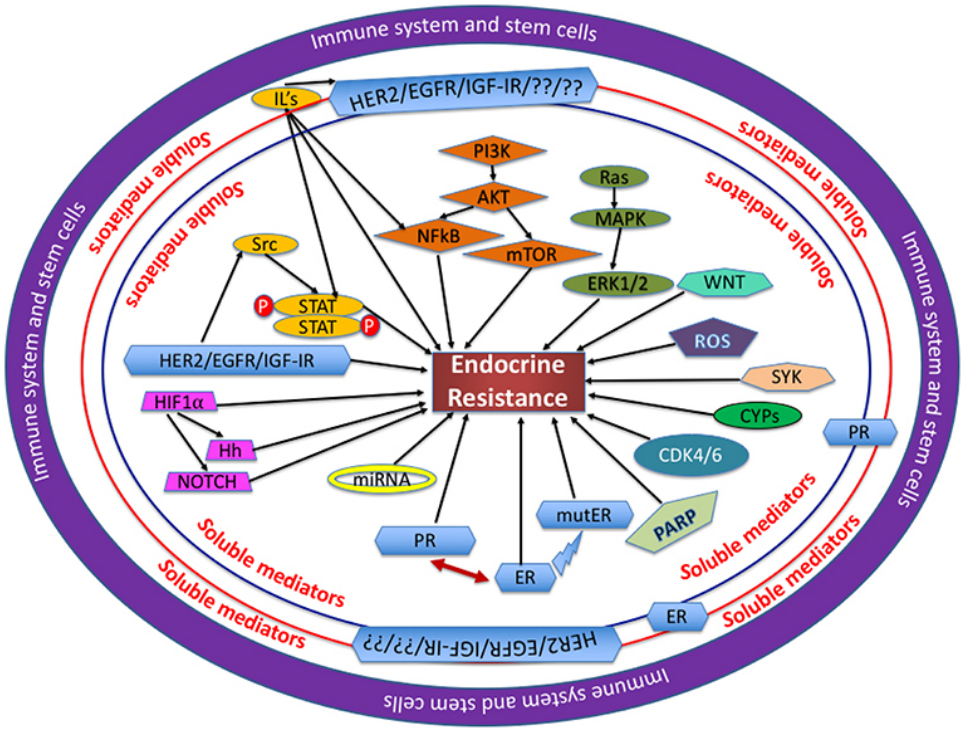


Figure 4. Mechanisms believed to be involved in endocrine resistance

Proteins, soluble mediators and transcription factors function in a complex network and these as well as other mechanisms may play a role in breast cancer cells becoming resistant to endocrine therapy.
Reprinted with permission from Rani A, et al.[24] © 2019. Note; the abbreviated genes and signalling pathways are not further explained by their complete namnes.

Side effects of endocrine therapy

Hot flushes are a common symptom in postmenopausal women and a known side-effect associated with endocrine therapy[128]. Tamoxifen is known to increase the risk of thrombo-embolic events and endometrial cancer[119, 129]. The 5-year risk of venous thrombosis/pulmonary embolus caused by tamoxifen treatment was 1.2% as compared to 0.5% when not receiving tamoxifen, and this risk was the highest during the first 2 years of treatment[130]. The risk of endometrial cancer is increasing with extended tamoxifen treatment (3.1% cumulative risk of endometrial cancer during years 5–14 vs 1.6% for controls)[123]. AIs are associated with musculoskeletal side effects such as arthralgia, joint stiffness, and/or bone pain[131, 132]. The risk of bone fracures are significantly increased in patients treated with AI than in those treated with tamoxifen (5-year risk 8.2% vs 5.5%, respectively)[118]. Side effects caused by endocrine drugs are often worse when adding OFS in premenopausal patients[133]. In a study comparing tamoxifen vs tamoxifen and OFS, the proportion of grade 3 or greater toxicity was 22% vs 12%, respectively, and the most common types of these toxicities were hot flushes, weight gain and neuropsychiatric effects (anxiety/depression)[134].

De-escalation of chemotherapy treatment

According to a meta analysis of adjuvant chemotherapy, the relative reduction of the risk of breast cancer recurrence and death are similar in all types of cancers, however, the absolute benefit is dependent on the risk at baseline[91]. The classification of ER+/HER2– tumours into Luminal A and B, is a simplified way to divide the patient into expecting 'good' and 'bad' prognosis, respectively. Both subtypes are assumed to benefit from adjuvant endocrine therapy due to ER-positivity[93, 129]. Because of the more aggressive tumour characteristics and hence worse prognosis in patients with Luminal B tumours, this is in general translated into the recommendation of adjuvant chemotherapy in a majority of these patients[10]. Overtreatment by chemotherapy drugs has patient-related and societal side-effects and causes possible long-lasting and sometimes persisting side-effects such as fatigue and neurotoxicity[135].

The economic perspective is also important such as drug costs, administration costs, hospital admission because of complications and socioeconomic effects due to loss of work and side-effects of treatment[136]. A major purpose of the commercial gene expression assays (see *Molecular subtyping of breast cancer* section, *Introduction* chapter) is to assist in prediction and prognostication, to guide treatment decision. However, one must keep in mind that this is a treatment-predictive value that ultimately states whether there is a beneficial effect of the therapy[137].

Adherence

Definition

Adherence, compliance, and persistence are terms often used in studies for the purpose of specifying how well the patients follow prescription recommendations. According to The International Society for Pharmacoeconomics and Outcome Research (ISPOR), adherence is synonymous with compliance: 'the degree or extent of conformity to the recommendations about day-to-day treatment by the provider with respect to the timing, dosage, and frequency'[138]. This is distinguished from persistence, defined as the duration of time from initiation to discontinuation of the treatment. These terms are however not easily separated in the literature. The acceptable degree of adherence is thought to range between 80-95%[139].

Methods for adherence measurement

There are different kinds of methods for adherence and these are associated with limitations. The awareness that measured adherence might affect the results, the rates

of adherence in clinical trials may be higher than in real life (named the *Hawthorne effect*)[138, 140]. Moreover, by self-reports, there is an assumed bias for overestimations[141]. Pill counting, corresponding to the return of unused pills to counter the missed doses, is also known to overestimate the adherence rate[142]. Measurement of serum and urine drugs/metabolites is another method, however, the pharmacokinetic variability may affect the accuracy of such measurements[143]. A microelectronic monitoring system (MEMS) consists of a cap on the pill bottle, counting the openings, and this method is reported to show different adherence rates as compared to self-reports or pill counting data[144]. Medication possession ratio (MPR) is a commonly used method for adherence measurement and assesses the number of doses dispensed in relation to the dispensing period[145]. The cut-off by MPR ≥80% is commonly used for adherence definition. Apart from low concordance between different types of methods used for adherence measurement, the differences in the definitions (adherence, persistence, discontinuation) in the literature complicates direct comparisons between studies in this research area[145].

Adherence to endocrine therapy in breast cancer studies

Partridge et al. performed an adherence report based on register data, that provided drug information on tamoxifen[146]. By using MPR ≥80% as adherent definition, nearly 2,400 patients met the inclusion criteria and the results showed that the adherence rate at the 4th year was only 50%. Based on other database and register studies, there is a wide range of adherence presented; 60–82% and 46–73% after 3 and 5 years, respectively[147, 148]. In Sweden, an earlier register study reported a low adherence of 69% after 3 years[149]. In another Swedish nationwide study, over 10,000 patients were included and data from the Swedish Prescribed Drug Register demonstrated adherence (defined as MPR≥80%) to be 88% after 3 years and nearly 83% after 5 years, respectively[150].

Causes and consequences of non-adherence

There are many factors associated with adherence rates including side effects, self-efficacy, perception of risks and benefits, information from the care giver at the time of prescription/communication, support, cost of the drug and subsidiaries[143]. Adherence to oral endocrine therapy is known to be a clinical challenge, mostly due to the above-mentioned side effects[151, 152].

Higher adherence has been observed among those given radiotherapy/chemotherapy and lower adherence in the youngest and oldest age groups[146, 153]. The financial aspect is also an important factor[154] and therefore, the pharmaceutical benefit of reduced costs may be an important factor for increased adherence to drug prescription. To have

regular consultations with a treating physician[146] and a perceived ability to discuss treatment options, have also been factors associated with higher adherence to tamoxifen[152]. The interventions needed to improve adherence are complex, as discussed in a Cochrane analyses regarding interventions for enhancing adherence and convenient care. These interventions among others include information, reinforcement and supportive care[155]. Both anxiety and the perception of a patient´s thoughts about the endocrine drugs, have impact on adherence[156]. A systematic review concluded that no particular difference in adherence between tamoxifen or AI was noted[157].

Low adherence to endocrine therapy is associated with worse prognosis[158, 159] and lower tamoxifen adherence has thus shown to be related to an increased risk of recurrence (hazard ratio (HR): 1.71, 95% confidence interval (CI): 1.16–2.51) and all-cause mortality (HR: 2.11, 95% CI: 1.62–2.74)[160]. According to Hershman et al. both early discontinuation and non-adherence, among those who continued, were independent predictors of mortality[161]. Moreover, low adherence to tamoxifen has shown to be associated with increased medical costs and worse quality of life[162].

Genetics

From DNA to RNA

The heredity of organisms is determined by the genes in the[58]. The chromosomes are made of tightly packed DNA helices, that are further on build up by nucleotides; A: adenine, T: thymine, G: guanine and C: cytosine. The DNA helix is double-stranded with two strands that are held together by complementary base pairs (A–T, G–C). Segments of DNA are wound up around proteins, generating nucleosomes that form the basic structural unit of the chromosome. Scattered along the DNA molecule are sequences that constitutes a gene; the region of the DNA that usually contains the coding region of a protein[58]. The genomic code in the DNA is transcribed by *transcription* into ribonucleic acid (RNA). The information in the RNA is, as for DNA, written by nucleotides sequences. However, these constitutes uracil (U) instead of thymine. In addition, the nucleotides are ribonucleotides (the sugar ribose instead if deoxyribose) and the RNA molecule is a single strand structure[58]. During transcription, where a fragment of the DNA is read, a DNA strand is liberated by unwinding of the DNA helix and this strand is used as a template for the synthesis of RNA (the coding sequence is determined by complementary base pairing). The primary RNA transcript that is copied, is modified into the protein-coding sequence by removing noncoding parts. The final product is named messenger RNA (mRNA), that is transported from the nucleus to the cytoplasm, where the code in the RNA is translated into a functional protein[58] (Figure 5).

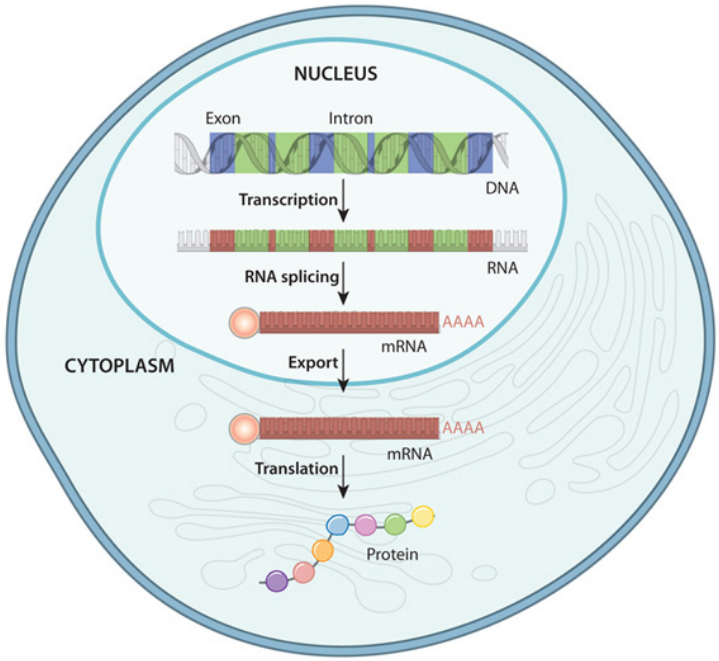


Figure 5. The transcription and translation processes in the cell
Reprinted with persmission from Nature Education[163] © 2010.
Abbreviations: DNA, dexoyribonucleic acid; mRNA, messenger RNA; RNA, ribonucleic acid

Somatic and germline mutations in breast cancer

A mutation is a heritable change in the nucleotide sequence of a chromosome[58]. Mutant proto-oncogenes correspond to overactive forms of genes named oncogenes, as compared to tumour suppressor genes in which a loss-of-function mutation results in harmful effects. Somatic mutations that occur in the somatic tissue give rise to a population of mutant cells and are not transferred to the progeny[164]. In contrast, the germline mutations are passed on to the next generation. *TP53, PIK3CA* and *GATA3* are some of the most commonly found somatic mutations in breast cancer tumours[165]. More details about the mutation framework of different subtypes are presented later in the thesis.

Approximately 5–10% of all breast cancers are caused by a germline mutations[4]. In the mid 1990s the tumour suppressor genes *BRCA1* and *BRCA2* (located on chromosomes 17q and 13q, respectively) were identified[166, 167] and mutations in these genes are characterised by an autosomal dominant pattern of inheritance with increased risk predominantly for breast and ovarian cancers. The risk of breast cancer in *BRCA1* carriers at age 70 is approximately 55-70 % and the corresponding figure for *BRCA2* is 45-70%[168, 169]. Typically, for *BRCA1* carriers, there is a particular increased risk of early

onset of the disease; about 50% are diagnosed before 50 years of age, and the triple negative breast cancer (TNBC) is the most frequent subtype[4]. The risk of contralateral breast cancer is also increased[170]. Apart from *BRCA1* and *2*, there are other known genes that increase the breast cancer risk (and also for other cancer diagnoses) such as *ATM, CHEK2, TP53* and *PTEN* and criteria for germline ontogenetic testing is stated in the Swedish national guidelines for breast cancer[10]. Moreover, risk assessment by risk calculation tools is performed based on the family history and by result from mutation analyses[171, 172].

Gene expression analysis

Abnormally functioning genes in malignant tumours appear to alter the gene expression[173]. Similarities in gene expression forms clustering of tumours that share similar sets and types of gene expressions, so called signatures, and as discussed later, gene expression analysis in breast cancer can be used for tumour profiling[1]. Several methods have been developed for gene expression analyses. The commercially available assays today are mainly based upon microarray, quantitative polymerase chain reaction (qPCR) and RNA sequencing (RNA-seq)[174].

qPCR

Polymerase chain reaction (PCR) was invented by Kary Mullis and colleagues using heat stable Taq polymerase from *Thermus Aquaticus* and is a method that is used for amplification of DNA[175]. In real-time quantitative reverse transcription PCR (qRT-PCR), RNA is first transformed into complementary DNA (cDNA) (Figure 6A) and this method makes it possible to determine the exact amounts (relative or absolute) of DNA[173, 176]. The genes of interest are amplified, and the PCR reaction is carried out through various cycles, and during the reaction, a dye or probe is incorporated, acting as a fluorescent reporter. As compared to conventional PCR, DNA quantification by qRT-PCR is based on counting the amount of reaction cycles that are needed to reach a threshold level, and this threshold is inversely proportional to the amount of the targeted gene product[173].

Microarrays

DNA microarrays are designed to analyse the expression of a large number of genes simultaneously[173]. These are slides made up by small spots in positions that contain cDNA sequences[174]. These act as probes that detect expression of genes[173, 177]. The mRNA is collected from the sample to be analysed and are, in addition to a reference sample, converted into cDNA. The samples are labelled by different sets of fluorescent probes. The samples are mixed and bound to the microarray sequences by

hybridization. Thereafter the array is scanned, and the gene expressions are measured (Figure 6B).

NanoString Technologies

The sequencing by NanoString Technologies, is performed on the nCounter© Dx Analysis System, that uses gene specific probe pairs[178]. These are hybridized directly to the mRNA sample in the solution, omitting enzymatic reactions. The DNA probe hybridises to a 70–100 base pair region of the mRNA sample. A reporter probe with a complementary sequence binds to the mRNA and a backbone DNA sequence of the probe creates a colour code by binding six segments of colour-labelled RNA, (red, yellow, blue or green). The colour code is unique to the target. A capture probe that is complementary to the mRNA target and biotin, causes immobilisation and the probe/target complexes are aligned for data collection that is performed on the nCounter© Digital Analyzer[178] (Figure 6C).

RNA sequencing

RNA sequencing (RNA-seq) directly sequences and measures gene expression at a broader range than the above-mentioned methods[173, 174]. It is based on next generation sequencing (NGS) technology, making it possible to characterise the transcriptome at single-base resolution without the need for specific probes[174]. In the procedure, different steps take place; *sample preparation, cluster generation, sequencing* and *data analysis*[179]. Both DNA and RNA can be analysed using this method. During *sample preparation,* adaptors are added to the constructed fragments. In addition, unique index sequenced 'barcodes' are added, which act as tags that makes it possible to distinguish between different libraries when analysing data (library preparation). As a result, multiple libraries might be pooled and sequenced in one run (multiplexing). C*lustering generation* is a cloning process, where the fragments are amplified in flow cells. Hybridization takes place by complementary binding and copies are created by a polymerase in the process of bridge amplification. This generates millions of copies of single stranded DNA. During *sequencing* (here described as the sequencing by synthesis method), nucleotides are added to the DNA strand by complementary binding. The nucleotide has a fluorescent tag, and this signal indicates the incorporated nucleotide, which determines the base codes. Both the forward and reverse ends are read by washing and amplification (paired end sequencing). During the *data analysis*, there is a massive reading of all nucleotides by a software program. The fragments are sorted based on the indices and the forward and reverse strands are paired and aligned as contiguous sequences and are aligned in the reference genome for identification[179, 180] (Figure 6D).

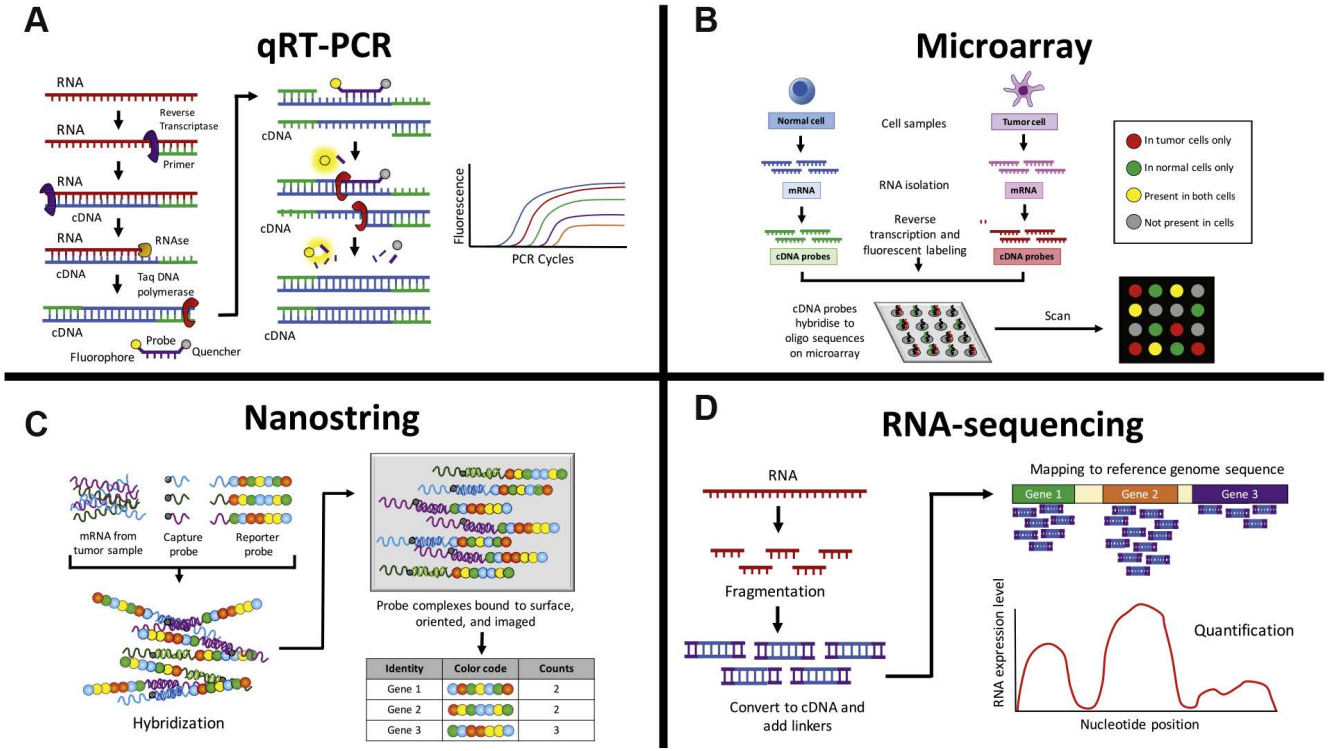


Figure 6A–D. Different techniques used for characterisation of RNA transcripts

(**A**) qRT-PCR: RNA is converted into cDNA, and amplified by PCR and bound to a flourescent probe. During synthesizing of the new DNA strand, the fluorescent probe is released. (**B**) Microarray: The extracted RNA is reverse transcribed and labelled with flourescent probes (green for normal and red for tumour DNA). The cDNA binds to complementary seqences on the microarray chip. The expression of genes corresponds to the amount of fluorescence. (**C**) NanoString: The RNA is hybridized with a capture probe and reporter probe. The reporter probe is labled by flourescent barcodes. The complex of probe/target are aligned and the barcodes are digitally read. (**D**) RNA-seq: RNA is fragmented ad reverse transcribed. Specific linkers are added and the cDNA is sequenced using NGS technology. The sequences are aligned against a reference genome to evaluate the gene expression level. Adapted and reprinted from Rogawski DS, et al.[174] with permission from Elsevier Inc. © 2017.

Abbreviations: cDNA, complementary DNA; DNA, deoxyribonucleic acid; mRNA, messenger RNA; NGS, next generation sequencing; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse-transcribed PCR; RNA, ribonucleic acid

Prognostic and predictive factors

The decision regarding the patient´s adjuvant treatment depends on prognostic and predictive factors, in addition to patient co-morbidity, medication and her/his own wishes. Prognostic factors give information on clinical outcome, regardless of the therapy planned. They reflect the biology of the tumour and the risk for metastases. Predictive factors provide, in turn, information on the probable effect of a given therapy. Both the prognostic and predictive factors help us select those patients who will derive the justified benefit, and not only the side-effects of a treatment[137, 181, 182.] Three terms are used to define the extent of the test/marker to serve as a clinical useful tool[183]:

- *Analytic validity*: does the test/biomarker perform the measurement with accuracy and reliability?
- *Clinical validity*: does the test/biomarker accurately and reliably identify a defined disorder, or is able to divide one population into groups with distinct outcome/differences?
- *Clinical utility*: is there evidence that the use of the test/biomarker to guide clinical decisions, is resulting in improved stratification than currently used methods?

Different prognostic and predictive markers in breast cancer are illustrated in Table 2. The long-term risk of recurrence after adjuvant endocrine therapy remains even 5–20 years after breast cancer diagnosis, mostly depending on the tumour size and nodal status[184]. Previously, mainly clinicopathological markers served as prognostic/predictive factors. However, as discussed in this thesis, the gene expression assays have been demonstrated to provide prognostic[185–193] and moreover also a possible chemotherapy-predictive value[194–196].

The luminal PAM50 subtype has been reported to predict the benefit of 5 years' adjuvant tamoxifen treatment in premenopausal women as compared to IHC/ISH-based luminal classification[197]. A long-term beneficial effect of 5 years' tamoxifen treatment in postmenopausal women with Luminal A_{PAM50} tumours has also been reported, however, this effect was attenuated over time in patients with Luminal B_{PAM50} tumours[198].

Staging for breast cancer

The investigation of a suspicious breast cancer tumour consists of the triple diagnostic process: mammography/ultrasound, clinical palpation and a biopsy for pathological examination[4]. Staging is important for prognostication and is performed based on the

TNM classification developed by the American Joint Committee on Cancer (AJCC) and endorsed by the Union for International Cancer Control (UICC)[199]. Tumours size (T) is defined as the largest tumour diameter. Nodal involvement (N) is the number of pathologic lymph nodes involved, and subgrouped based on their number and localization. M defines whether there are distant metastases or not. The staging system is made both clinically (physical exam with or without imaging, cTNM) and after pathologic assessment (pTNM). The updated edition (8th) of TNM, also incorporates prognostic biomarkers such as grade, HER2, ER, progesterone receptor (PR) and genomic test results[199].

Table 2. Some prognostic and predicve factors in early breast cancer

Factors	Implication	References
Prognostic		
Patient features	• Age (younger age→higher risk)	10, 200
Tumour stage	• Tumour size (increased size→higher risk) More difficult to evaluate • Nodal involvement (positive→higher risk)	184, 201, 202
Tumour morphology	• Ductal type (early recurrence as compared to lobular type) • Tubular, mucinous, medullary (more favourable prognosis)	201, 203
Pathological features and biomarkers	• Histological grade (higher grade→ higher risk) • LVI (presence→higher risk) • ER/PR (positivity→lower risk) • HER2 (positivity→in absence of therapy, higher risk) • Ki67 (higher value→higher risk) • TILs (higher level→lower risk (TNBC, HER2+ BC)	69, 78, 82, 204–213
Molecular profiling	• Gene expression assays (see *Molecular subtyping of breast cancer* section, *Introduction* chapter)	-
Predictive		
Pathological markers	• ER (positivity→effect of ET) • PR (positivity→ assumed effect of ET, however less established) • HER2 (positivity→effect of anti-HER2-blockade) • TILs (higher level→higher pCR (TNBC, HER2+ BC)	34, 129, 209, 214–216
Molecular profiling	• Gene expression assays (see *Molecular subtyping of breast cancer* section, *Introduction* chapter)	-

Abbreviations: BC, breast cancer; ER, oestrogen-receptor; ET, endocrine therapy; HER2, human epidermal growth factor receptor 2; pCR, pathological complete response; PR, progesterone receptor; TILs, tumour-infiltrating lymphocytes; TNBC, triple-negative breast cancer

Premenopausal patients and their breast cancer tumour features

Menopausal status

An important patient characteristic for prognosis and treatment decision, is the menopausal status. The median age for menopause is 51 years[10], and based on the national Swedish guidelines for breast cancer, postmenopausal status is defined as either of:

- bilateral oophorectomy
- 60 years' old
- ≤60 years' old and a menopause at least 12 months before cancer treatment and no other endocrine therapy

In the hypothalamus, the GnRH is produced and released in a pulsative manner[217]. GnRH acts by binding to its receptor in primarily the anterior pituary, which stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In the female, these hormones exert their effect in the ovaries and this in turn regulates the steroidogenesis and gametogenesis by production of oestrogen and progesterone[218]. This hormone axis is thoroughly regulated by feedback mechanisms[6].

Young age is an independent risk factor for breast cancer death[200, 219]. There are many challenges associated with the handling of breast cancer in younger patients, such as option of appropriate endocrine therapy and need for chemotherapy based on risk profiling[93, 133]. In addition, these patients are in general fertile with a possible desire to become pregnant. According to current Swedish guidelines, the recommendation is to withhold tamoxifen at least 2 months before the attempt to become pregnant[10].

There are differences in tumour biology and characteristics between younger and older women. Younger women tend to have tumours with more nodal involvement, higher grade, larger size and ER/PR-negative (ER-/PR-) status[220]. By using age 40 as a cut-off binary variable and adjusting for other variables, the outcomes in the younger patients have been shown to be significant worse[220]. Based on a gene expression classifier, the tumours in young patients (≤40 years old) are more of the Basal-like subtype and there are fewer Luminal A tumours[219, 221]. Comparing tumours from Caucasian pre- and postmenopausal women, 57% vs 67% were Luminal A and 15% and 9% were Basal-like, respectively[222]. The pattern of distribution of luminal and TNBC is somewhat similar regarding subtyping based on IHC[223, 224].

Molecular subtyping of breast cancer

Surrogate subtyping

Even though intrinsic subtyping (see below) has been known since 2000, the corresponding subtyping based on classical IHC/ISH markers has been used as standard for prognostication and treatment guidance in clinic[66, 225, 226]. At the 12th St. Gallen International Breast Cancer Conference in 2011, the expert panel endorsed surrogate classification based on ER, PR, HER2 and Ki67[65]. The cut-point for low Ki67 was set to <14%, derived from a comparison with gene expression array data, in which the optimal Ki67 value for distinguishing intrinsic the ER+/HER2– tumours as Luminal A from B was 13.25%[227]. The surrogate subtyping algorithm was further refined at the 2013 St. Gallen conference[66]. The panel voted for a threshold of ≥20% as high Ki67 and furthermore, the cut-off for high PR definition was proposed as ≥20%, based on a study by Prat et al[41]. This study showed that there were more tumours that were PR+, HG1 and had a higher gene expression of the PR gene among Luminal A_{PAM50} tumours than among Luminal B_{PAM50}. In 2014, Maisonneuve et al. proposed an updated version of the surrogate subtyping[228]. They studied more than 9,000 patients with ER+/HER2– breast cancer tumours with a median follow-up of 8.1 years and evaluated their prognosis by stratifying the tumours of these patients by Ki67 (three categories; low (<14%), intermediate (14–19%) or high (≥20%)) and PR (low/high; <20%/≥20%). Those with intermediate Ki67 and high PR, had better outcome than those with intermediate Ki67 and low PR. PR did not add additional prognostic (distant disease-free survival) information for those low (<14%) or high (≥20%) Ki67.

None of the proposed surrogate classifications have incorporated HG as a factor for distinguishing among luminal tumours. In the study by Maisonneuve et al., the authors demonstrated that patients with HG1/Luminal B_{SC} tumours, had significantly better outcomes than those with HG2 and HG3 tumours (Luminal B_{SC}). Moreover, patients with HG3/Luminal A_{SC} tumours, had worse outcomes than those with HG1 and HG2 tumours (Luminal A_{SC})[228]. Another study of HG and its importance for surrogate subtyping by St. Gallen 2013, demonstrated that mainly the HG2 subgroup, not HG1 and HG3, could further be stratified by the Ki67 and PR values regarding prognostic effect[71]. In the St. Gallen consensus statement 2017, a more non-specific classification was presented[226]. They specified a spectrum of the ER+/HER2– breast cancer tumours; Luminal A_{SC} tumours were defined as having high ER/PR and clearly low Ki67 or HG, whereas Luminal B_{SC} tumours had lower ER/PR with clearly high Ki67, high HG. Uncertainties exist about the risk for the intermediate subgroup and the possibility of multiparameter molecular markers were recommended as a tool for improved risk estimation.

According to the St. Gallen consensus from 2019, the use of molecular testing for ambiguous cases was endorsed, illustrating the progression from IHC/ISH-based surrogate subtyping to the use of genomic assays for risk stratification[93]. These previous surrogate classification algorithms described, along with the results of HG, were the rationale behind the national Swedish surrogate classification in clinical use (Figure 7)[10] (the term Luminal-like used to denote that subtype classification is based on surrogate markers).

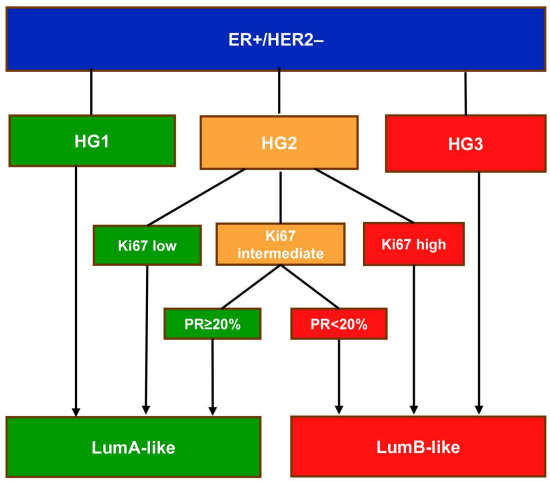


Figure 7. Pathological surrogate subtyping according to Swedish guidelines 2020[10] (adjusted version)
LumA- and B-like denotes the surrogate subtypes.
Abbreviations: ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; Lum, Luminal; HG, histological grade; PR, progesterone receptor

Intrinsic subtypes of breast cancer

In the year 2000, the intrinsic subtypes of breast cancer were presented, illustrating the different types of breast cancer based on gene expression and their distinct features[229] that have been correlated to patient outcomes[230]. As described by Perou et al., breast tumours could be categorised into different gene expression patterns based on their gene expression profile[229]. The main goal of their study was to develop a classification system for tumours based on these patterns, performed by hierarchical clustering analyses[229, 231]. A matrix displaying the ratio of abundance of transcripts of each gene to the median of the gene transcripts among all tumours are presented as red or green colours (Figure 8). The conclusions by the authors were that four clusters could be defined; *Luminal epithelial/ER+ group* (further divided into at least two groups A, B and a possible C-group, see below[230]), *basal epithelial (Basal-like) group, Erb-B2-overexpression (HER2-E) and a Normal group*. It was noted that two branches of the dendrogram separated between the tumours of ER+ and ER– and that ER+ tumours highly expressed genes that were also expressed by breast luminal cells, which was noted

by IHC analyses with antibodies for luminal cell keratins. The Basal-like tumours showed staining (by IHC) for either basal keratin 5/6 and 17. The 'normal breast genes' were associated with basal epithelial cells, adipose cells and lower luminal epithelial cells[229, 230].

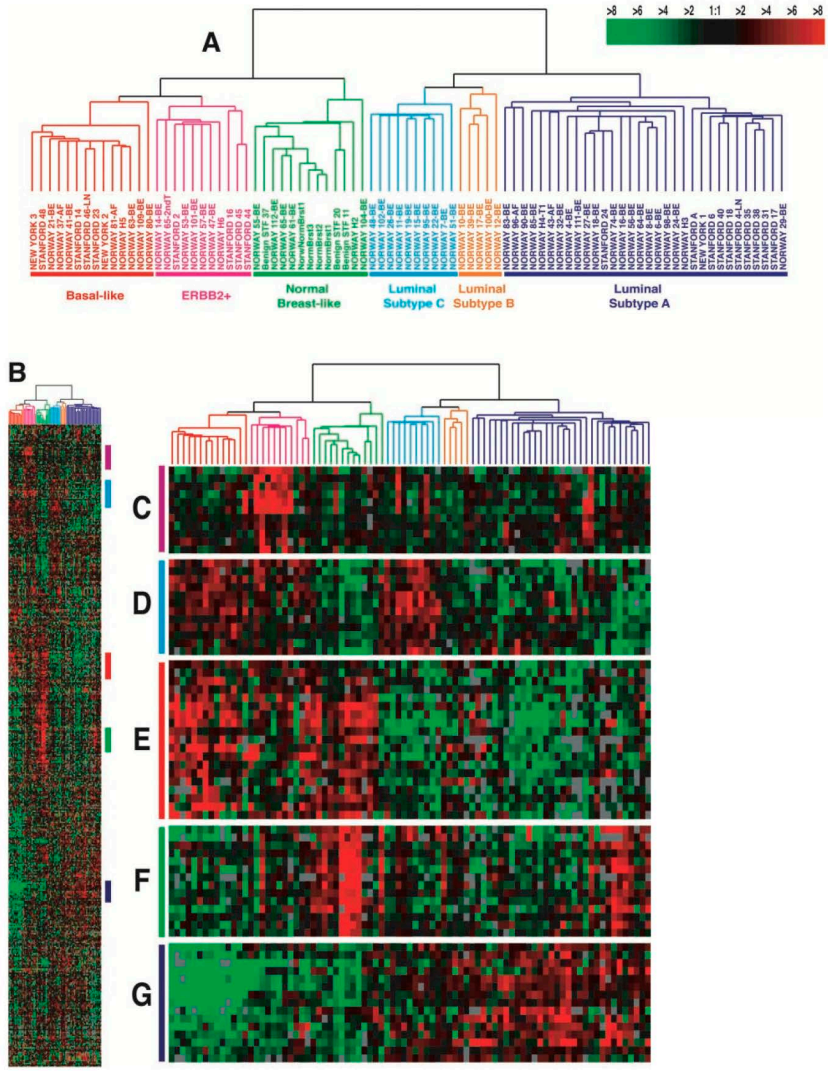


Figure 8A–G. Gene expression patterns by cluster analysis of intrinsic genes

(**A**) Cluster dendrogram illustrating the branches of intrinsic subtypes; dark blue: Luminal A, yellow: Luminal B, light blue: Luminal C, green: Normal-like, red: Basal-like, pink: ERBB2+ (HER2-E) (**B**) Gene clusters of the different intrinsic subtypes. Red and green colours represent different expression of genes (the names of the genes are omitted in the figure but available in the reference Sørlie T, et al.[230]). The coloured bars on the right represent the inserts presented in **C**–**G**. (**C**) ERBB2 amplicon cluster. (**D**) Novel unknown cluster. (**E**) Basal epithelial cell-enriched cluster. (**F**) Normal breast-like cluster. (**G**) Luminal epithelial gene cluster containing ER.

Adapted and reprinted with permission from Sørlie T, et al.[230] © 2001, The National Academy of Sciences,U.S.A.

Abbreviations: HER2, human epidermal growth factor receptor 2; HER2-E, human epidermal growth factor receptor 2–enriched; Lum, Luminal

In a study led by The Cancer Genome Atlas Project (TCGA), over 500 breast cancers were profiled[165] and by clustering, four entities of breast cancer were found, well resembling the intrinsic subtypes of Luminal A, Luminal B, HER2-E and Basal-like as defined by mRNA expression[165, 232, 233]. The specific features of the intrinsic subtypes are presented in Table 3.

Luminal A and B

Luminal tumours are the most heterogeneous in terms of gene expression and mutations[165]. A dominant feature is high mRNA expression of the luminal gene expression spectrum that predominantly contains *ESR1, GATA3, FOXA1, XBP1* and *MYB*. The luminal subgroup was proposed to be divided into two or possible three distinct subgroups, each one associated with different outcomes[230]. The Luminal B and Luminal C had lower expression of luminal-specific genes in the ER cluster. The Luminal C was furthermore defined by expression of genes of unknown function, similar to the Basal-like and HER2-E subgroups, but this subtype was never used in the clinic[230].

The upregulated genes in Luminal A tumours are more incorporated in cell differentiation and cell adhesion biological processes[41]. Luminal B tumours have a higher expression of genes related to proliferation and cell cycle such as *MKi67*, and lower expression of *PGR* and *FOXA1*[41, 233]. The Luminal A tumours incorporate lower number of mutations (less *TP53* mutations than Luminal B; 12% vs 29%, respectively) and the most frequent mutations are *PIK3CA, MAP3K1* and *GATA3*[165, 233].

HER2-E

The HER2-E subtype is characterised by expression of HER2-related genes (*Erb-B2/HER2* and *GRB7*) and has intermediate and low expression of luminal genes (*ESR1* and *PGR*) and basal genes (*keratin 5* and *FOXC1*)[233]. This subtype typically has a high mutational burden, especially regarding *PIK3CA* and *TP53*[165, 233]. Of notice, the HER2-E breast cancer subtype is not the same as HER2+ breast cancer by IHC/ISH. Prat et al. demonstrated that 45% of HER2+ tumours were presented as HER2-E[233]. There seem to exist two distinct types of the HER2+ tumour subtype; a $HER2E_{mRNA}$-subtype/HER2+ and a $luminal_{mRNA}$-subtype/HER2+, the latter incorporating a higher luminal gene expression profile[165].

Basal-like

The Basal-like tumours are often referred to as the TNBC, since they are commonly negative (according to IHC/ISH) for ER, PR and HER2[165]. However, although Basal-like tumours constitute 10–25% of all breast tumours, these make up approximately 50–75% of the TNBC subtypes[222]. The tumours of the Basal-like subtype are highly proliferative and have a high expression of genes associated with growth such as *MKI67*

Table 3. Features of intrisic subtypes of breast cancer

Subtype	LumA	LumB	HER2-E	Basal-like
TP53 pathway	*TP53* mut (12%); gain of *MDM2* (14%)	*TP53* mut (32%); gain of *MDM2* (31%)	*TP53* mut (75%); gain of *MDM2* (30%)	*TP53* mut (84%); gain of *MDM2* (14%)
PIK3CA/ PTEN pathway	*PIK3CA* mut (49%); *PTEN* mut/loss (13%); *INPP4B* loss (9%)	PIK3CA mut (32%); PTEN mut/loss (24%); INPP4B loss (16%)	PIK3CA mut (42%); PTEN mut/loss (19%); INPP4B loss (30%)	PIK3CA mut (7%); PTEN mut/loss (35%); INPP4B loss (30%)
RB1 pathway	Cyclin D1 amp (29%); *CDK4* gain (14%); low expression of *CDKN2C*; high expression of *RB1*	Cyclin D1 amp (58%); *CDK4* gain (25%)	Cyclin D1 amp (38%); *CDK4* gain (24%)	*RB1* mut/loss (20%); cyclin E1 amp (9%); high expression of *CDKN2A*; low expression of *RB1*
mRNA expression	High ER cluster; low proliferation	Lower ER cluster; high proliferation	HER2 amplification signature; high proliferation	Basal signature; high proliferation
DNA mutation	*PIK3CA* (49%); *TP53* (12%); *GATA3*(14%); *MAP3K1* (14%)	*TP53* (32%); *PIK3CA* (32%); *MAP3K1* (5%)	*TP53* (75%); *PIK3CA* (42%); *PIK3R1* (8%)	*TP53* (84%); *PIK3CA* (7%)
Protein expression	High oestrogen signalling; high MYB; RPPA reactive subtypes	Less oestrogen signalling; high FOXM1 and MYC; RPPA reactive subtypes	High protein and phospho-protein expression of EGFR and HER2	High expression of DNA repair proteins, PTEN and INPP4B loss signature (pAKT)

Adjusted and reprinted with permission from The Cancer Genome Atlas Network[165] © 2012. Published by Springer Nature.
Abbreviations: amp, amplification; DNA; deoxyribonucleic acid; HER2-E, human epidermal growth factor receptor 2-enriched; Lum, Luminal; mRNA, messenger RNA; mut, mutation; RNA, ribonucleic acid. Note: the abbreviated genes and signalling pathways are not further explained by their complete namnes.

and cytokeratins (keratins 5, 6, 14 and 17)[165, 222, 233]. *TP53* is a frequent mutation[165] and *BRCA1*-mutated hereditary breast cancer is associated with the Basal-like subtype[222]. Another special subtype of the TNBC spectrum is the Claudin-low subtype, characterised by a low expression of claudin genes[222]. These tumours, which comprise about 5–10% of tumours, are similar to the Basal-like tumours with an intense immune cell infiltration and low expression of cell-cell junction proteins.

Development of intrinsic risk profiling of breast cancer

The intrinsic subdivision of breast cancer tumours, as presented above, reflects different underlying biological phenotypes and various signalling pathways in the subsets of tumours. Using gene expression analysis, researchers have tried to find signatures associated with prognosis and prediction of response to adjuvant chemotherapy in breast cancer tumours. Parker et al. proposed in 2009 a set of genes that comprised 50 genes and additional control genes; a Prediction Analysis of Microarrays 50 (PAM50), for intrinsic subtype classification and also demonstrated the prognostic value of these different subtypes[232]. The authors also developed different variants of risk assessment scores (Risk of Recurrence (ROR) score), to estimate the patient´s risk of distant recurrence. RNA was purified from formalin-fixed paraffin-embedded (FFPE) tissues and the tumours were analysed across 1,906 intrinsic genes using hierarchical clustering. The clusters representing the Luminal A, Luminal B, HER2-E, Basal-like and Normal-like were identified. The hierarchical clustering method, by a stepwise analysis, finds the closest pair of clusters and merges them into a new parent cluster, which is illustrated in a dendrogram with several branches[231]. For each subtype, a centroid is defined. This is simply explained as a sum of vector distances of relative gene expression for all of the included genes in that specific subtype. The Pearson's correlation coefficient is used as a measure of the distance between elements and the authors used the nearest centroid-based statistic method[234]. For a new tumour sample, a comparison of its gene expression levels with regard to the distance to the centroids is performed, and the tumour subtype is assigned an intrinsic subtype based on the nearest distance to the five centroids.

Only 50 selected genes were finally used in the PAM50 algorithm. In developing the ROR score algorithm, different variants were proposed: a ROR score in correlation to subtype alone (ROR-S) or a ROR score in correlation to subtype and tumour size (ROR-T)[232]. Moreover, a ROR model with proliferation index has been developed[235]. During the last decade, several commercial multigene assays have been introduced for estimating the risk of recurrence and for selecting patients for whom adjuvant chemotherapy can be omitted (Table 4).

Risk profiling assays based on gene expression analyses

There are different strategies for the development of gene expression signatures[236, 237]. In the *top-down approach*, gene expression data is used to identify genes associated with prognosis if the outcome is known. In the *bottom-up approach*, gene expression patterns with a specified biological function are identified and then correlated to clinical outcome. In the third approach, the *candidate gene approach*, selected genes known to have biologic impact are combined into multivariate predictive models. The initial methods of fresh frozen breast cancer tissues made the logistic process in the clinical setting difficult. Progress in the analysing techniques made it possible to use archival FFPE tissue blocks[236]. Tissues could be retrieved and stored from retrospectively completed trials and also in ongoing trials for future studies.

Today, the available commercial tests are designed for risk profiling; for prognostication and to aid in treatment decision on the need of adjuvant therapy beyond endocrine therapy for patients with ER+/HER2- tumours. Among others, the National Institute for Health and Care Excellence (NICE) and ASCO propose recommendations regarding the clinical utility for the available biomarker assays[238–241]. A selection of gene expression assays is presented in Table 4 and described more in detail below.

The Prosigna® assay, providing PAM50/ROR score, is applied in one of the studies in this thesis and is thus described more thoroughly. It is worth mentioning the limitations of gene expression analyses in general, as presented by the manufacturer of the Prosigna® assay[178]:

- Prosigna® is based upon *sufficient RNA quality and quantity* for reliable results.
- The interpretation of Prosigna® results (intrinsic subtype, ROR score, risk category) should be evaluated *within the context of other clinicopathological factors, the patient's medical history and any other laboratory test results.*
- *Interfering substances* such as genomic DNA and non-tumour tissue (meaning normal tissue) affect the results and there is a need for a pathologist to determine the area of viable invasive carcinoma"

Breast Cancer Index (BCI) combines two profiles called the H:I ratio (two genes in the oestrogen signalling pathway: *HOXB13/IL17BR*) and the Molecular Grade Index (MGI) by gene expression of five genes and additional control genes[188, 242–244]. The test estimates the risk for distant recurrence (post 5 years from diagnosis and the cumulative distant recurrence risk over 10 years) after endocrine and chemo-endocrine treatment in hormone-receptor positive, lymph node-negative and -positive patients, respectively[187, 188, 245] and the validation studies have involved both pre- and postmenopausal patients. For node-positive patients, the risk is calculated by combining the BCI gene expression with tumour size and tumour grade[187]. The BCI predictive test (H:I ratio) reports the likelihood of benefit form extended adjuvant endocrine therapy after 5 years of treatment[244, 246–248]. Moreover, low H:I ratio has

previously been demonstrated to predict the effect of 5 vs 2 years of adjuvant tamoxifen[249].

Table 4. Different gene expression panels in breast cancer

Assay	Target genes[a]/method	Output	References
Breast Cancer Index (BCI) *(Biotheranostics)*	7 genes RT-PCR	Risk score Risk category Likelihood of benefit from extended ET	187, 188, 242–250
EndoPredict (EPclin) *(Myriad)*	8 genes RT-PCR	Risk score Risk category	186, 251–253
MammaPrint BluePrint *(Agendia)*	70 genes 80 genes Microarray	Risk category Subtype	190, 195, 254–260
MammaTyper *(BioTech Diagnostics)*	4 genes RT-PCR *ERBB2* (HER2), *ESR1* (ER), *PGR* (PR) and *MKI67* (marker Ki-67)	Subtype	261
OncotypeDX *(Genomic Health)*	16 genes RT-PCR	Risk score Risk category	193, 194, 262–269
Prosigna® *(Veracyte)*	50 genes NanoString Technology	Risk score Risk category Subtype	185, 192. 197, 198, 232, 270–272

[a]In addition to control genes
Abbreviations: ET, endocrine therapy; IHC, immunohistochemistry; RT-PCR, reverse transcription polymerase chain reaction

EndoPredict predicts the likelihood of distant recurrence after 10 years from diagnosis assuming 5 years of endocrine therapy[251]. This test can be used for both pre- and postmenopausal women with ER+/HER2– breast cancer in both lymph node-negative and -positive patients and measures the expression of 12 genes (3 proliferation-associated genes, 5 hormone receptor-associated genes, 3 reference (normalisation) genes and 1 control gene)[186, 251–253]. In addition to the EP score that separates the patient into low and high risk of recurrence, the EPclin score is reported by also adding data on tumour size and nodal status and these provide prognostic information beyond clinicopathological markers[186, 189, 273].

MammaPrint was the first genomic profiling tool developed for commercial use and it used microarrays that predicted recurrence in breast cancer patients irrespective of menopausal status by gene expression analyses[255]. The assay incorporates 70 genes and delivers a binary outcome: low or high risk (profile on a scale of -1.000 to + 1.000 and 0 as cut-off point)[190, 255, 274]. It has been validated in studies of retrospective, prospective non-randomised and randomised design[190, 195, 257, 260]. In a long-term follow-up study, an ultralow risk profile with a prognostic value was identified[275]. The predictive value for chemotherapy has been evaluated in the randomised, controlled prospective MINDACT study, including both node-negative and -positive patients[195]. This study showed that 46% of clinically high-risk patients were classified as genomic low risk. In patients who were defined as high clinical risk and low genomic risk, the receipt of adjuvant chemotherapy resulted in a 5-year rate of survival without distant metastasis

that was equivalent to that in patients not receiving chemotherapy. In an up-dated analysis in 2021, the beneficial effect of chemotherapy was not enhanced by nodal involvement, however, in younger patients (≤50 years), the results were not conclusive[256].

BluePrint is an intrinsic subtyper, that provides stratification of the tumour into Luminal-Type, HER2-Type and Basal-Type[258]. By combining MammaPrint and BluePrint, the Luminal-Type tumours are further stratified into Luminal-Type/MammaPrint Low Risk (Luminal A) and Luminal-Type/MammaPrint High Risk (Luminal B)[254].

MammaTyper measures ER, PR, HER2 and Ki67 at the mRNA level (*ERBB2, ESR1, PGR* and *MKI67,* respectively) in a quantitative manner based on RT-qPCR[261]. This generates the intrinsic subtypes of Luminal A_{SC} (HER2–), Luminal B_{SC} (HER2–), Luminal B_{SC} (HER2+), HER2+ (non-Luminal) and Triple negative (ductal) according to classification of negative/positive results of the gene expression analyses and subtyping (according to St. Gallen 2013[66]).

OncotypeDX measures the expression of 16 genes and additional reference genes (21 genes in total) including oestrogen receptor-, proliferation-, and invasion-related genes[191, 262, 276]. This assay estimates the 10-year risk of distant recurrence (assuming 5 years of adjuvant endocrine therapy) and reports a recurrence score (RS) (0-100) divided into categories of low, intermediate and high risk based on specific cut-offs[193, 238, 262]. This test has been validated in retrospective, prospective-retrospective, prospective non-randomised and prospective randomised studies for prognostic and chemotherapy-predictive values[194, 264, 267–269, 276]. In the randomised, controlled TAILORx study, over 10,000 node-negative women with ER+/HER2–, node-negative breast cancer, were included and 69% of the eligible participants had an intermediate RS between 11-25[194]. These patients were randomised to receive chemo-endocrine therapy or endocrine therapy only and the results indicated that at 9 years of follow-up, the rates of invasive disease-free survival were 83% and 84% in the two treatment arms, respectively. However, there has been a debate of the chemotherapy benefit in women 50 years old or younger that had a RS 16-25[194]. In the RxPONDER trial, node-positive patients with RS 0–25 were randomised to chemo-endocrine therapy or endocrine therapy alone, and the recent results indicated that postmenopausal received no benefit from chemotherapy[196]. However, this was not verified in premenopausal women as discussed in the *Future perspectives* chapter in the thesis.

PAM50 subtyping/ROR score is a prognostic gene expression analysis developed by the algorithms as presented by Parker et al.[232] (see above). In the commercial version of this test (*Prosigna Breast cancer prognostic gene signature assay (Prosigna®))* the prototypical centroids corresponding to the PAM50 algorithm by the initial study was defined using hierarchical clustering analysis and this test provided both a PAM50 subtype and a

ROR score[178]. The prognostic value of this test has been validated in two studies, the TransATAC trial and the ABCSG-8 trial, in which the prognostic value of ROR score was compared to a Clinical Treatment Score (CTS) based on an optimised combination of clinicopathological variables (age, histological grade, tumour size, nodal status and adjuvant therapy)[178, 185, 270]. TransATAC trial was an initiative in year 2002 to retrospectively evaluate patients with ER+ breast tumours included in the ATAC trial[277]. Postmenopausal patients, who did not receive adjuvant chemotherapy, but received adjuvant endocrine therapy (tamoxifen or AI), were included and the median follow-up was 10 years. Based on the prognostic results of ROR score, specific cut-offs were determined for category specification (low, intermediate and high) to correspond to a risk of <10%, 10–20%, and >20% risk of distant recurrence at 10 years after adjuvant endocrine therapy for 5 years[185]. ROR categories (for node-negative and -positive patients) were further validated in the ABCSG–8 study, in which postmenopausal women were randomised to 2 years of adjuvant tamoxifen and 3 years of additional anastrozole or a total of 5 years of tamoxifen[270]. These two randomised prospective-retrospective studies indicated that ROR score could add significant prognostic information as a continuous variable and as a categorised variable and moreover, the intrinsic luminal PAM50 subtypes (Luminal A and Luminal B) provided different prognostic information in all nodal subgroups. The risk of distant recurrence regarding PAM50 subtypes and ROR score categories for the combined validation studies in different node categories, are presented in Table 5A and B.

In developing the Prosigna® assay on the NanoString nCounter© Dx Analysis System, de novo retraining was performed on the nCounter© Analysis platform in order to develop a robust analysis, generating a gene expression assay, providing PAM50 subtypes and ROR score, which could be used in the clinic at decentralised laboratories[192]. The Prosigna® assay is intended to be used in postmenopausal women with ER+/HER2– breast cancer with node-negative or node-positive (1–3 positive nodes) status[178].

In a Danish follow-up study, it was shown that the intrinsic subtypes and ROR score delivered by Prosigna®, clearly provided information on prognostic outcomes after 10 years in postmenopausal women[271]. Sestak et al, has also demonstrated the long-term prognostic value of the ROR score[278].The validation studies did not include any premenopausal women, and thus the application of Prosigna® assay in this patient category is still unclear. However, some studies have shown the prognostic effect of this genomic test in premenopausal patients as well[197, 279]. In addition to the commercial test, assays for research intention are available such as the NanoString Breast Cancer 360™ assay run on the NanoString nCounter© SPRINT Profiler (NanoString Technologies Inc., Seattle, WA, USA)[280]. This embraces the Prosigna Breast Cancer Prognostic Gene Signature Assay in addition to other biological signatures and pathways (see *Genomic analyses* subsection, *Methods* chapter).

Table 5A. Risk of recurrence (%) according to PAM50 intrincis subtypes

Nodal status	Lum subtype	Percent without distant recurrence at 10 years (%)
N0	LumA	95
	LumB	82
N1(1–3 positive nodes)	LumA	88
	LumB	68
N2 (≥4 positive nodes)	LumA	68
	LumB	38

Data from the combined validations analyses[178, 185, 270]
Abbreviation: Lum, Luminal

Table 5B. Risk of recurrence (%) according to ROR score categories

Nodal status	ROR score categories	Percent without distant recurrence at 10 years (%)
N0	Low	96
	Intermediate	89
	High	78
N1(1–3 positive nodes)	Low	92
	Intermediate	90
	High	72
N2 (≥4 positive nodes)	High	57

Data from the combined validations analyses[178, 185, 270]
Abbreviation: ROR, Risk of Recurrence

SCAN-B

Sweden Cancerome Analysis Network-Breast (SCAN-B) is a multicentre national study that was initiated in 2010, with an overall aim to prospectively collect and analyse breast cancer tissues for research purposes with genomic analyses within a NGS platform[281, 282]. Initially this was formed by the academic group in Lund and the South Swedish Breast Cancer Group (SSBCG) in 2009 with a mission that all patients with breast cancer should at the time of diagnosis be offered new genomic and gene expression-based analyses in the clinical setting by NGS of fresh tumour tissues at the laboratory in Lund. Initially, seven hospitals participated in this study (Lund, Malmö, Helsingborg, Kristianstad, Karlskrona, Halmstad and Växjö) and Uppsala in 2013 as well as Jönköping in 2015 have joined. The intrinsic subtype of the tumour is defined according to the PAM50 algorithm (see *Genomic analyses* subsection, *Methods* chapter).

Comparison of gene expression assays

According to a study comparing between ROR score, OncotypeDx and IHC4 in the TransATAC trial, it was demonstrated the ROR score assigned fewer patients to the intermediate risk group as compared to RS of OncotypeDx, and more patients to the high-risk group[185]. Moreover, the ROR score added more prognostic information than the RS did. Similar findings were shown in another study, also demonstrating that most prognostic information was provided by ROR, BCI and EPclin as compared to CTS,

RS and the 4-marker IHC score[189]. The IHC4 is a risk predictor (low, intermediate and high categories) that combines the assessment of four IHC markers; ER, PR, HER2 and Ki67[277]. In the OPTIMA Prelim Trial, recurrence scores by Prosigna®, MammaPrint, MammaTyper, IHC4-AQUA (NexCourse Breast™) and IHC4 were compared[283]. Tumour subtyping by BluePrint, MammaTyper and Prosigna® was furthermore compared. The results showed that OncotypeDx predicted a higher proportion of tumours as low risk than those predicted as low/intermediate by Prosigna® and MammaPrint. Only 39% were classified uniformly as low/intermediate or high risk. According to tumour subtyping, only 40% had a concordance of Luminal A classification and there was a disagreement of subtyping in 41% of the tumours.

In a study based on SCAN-B, a comparison was made between 19 gene signatures (both regarding subtyping and risk scoring) in different breast cancer subgroups[284]. Risk classifier agreement (low, medium/intermediate, high risk) in ER+ tumours was 50–60%. However, by disregarding the intermediate group, the agreement between low/high was 80–95%.

Intrinsic vs surrogate subtyping

The intrinsic and surrogate subtyping are reflecting different underlying biologic features of breast cancer and therefore, a perfect agreement between them is not expected. Based on a review of Prat et el., the discordance rate between subtyping based on PAM50 and IHC/ISH was 31% across all patients and 44% within non-TNBC group[233]. The discordance rate was 38%, 49%, and 14% for the Luminal A_{SC}, Luminal B_{SC} and TNBC (to identify PAM50 Basal-like) subtypes, respectively. In another study comparing PAM50 subtypes with three surrogate classifiers, the concordance ranged from poor to moderate (kappa=0.36–0.57 and percent agreement=54–75[285]. In Table 6, the distribution of the intrinsic subtypes among different IHC-based subtypes is presented.

Table 6. Distribution of PAM50 molecular subtypes among pathology-based subtypes

IHC-based subtype	PAM50 subtype				References
	LumA	LumB	HER2-E	Basal-like	
HR+/HER2−	60%	32%	7%	1%	41, 185, 197, 235, 270, 286–289
LumA	62%	27%	10%	0.6%	41, 197, 286, 289
LumB	34%	51%	11%	4%	41, 197, 286, 289
HER2+	18%	27%	45%	11%	165, 290–293
HER2+/HR+	33%	46%	19%	2%	292, 293
HER2+/HR−	19%	4%	66%	11%	292, 293
TNBC	2%	3%	9%	86%	286, 294–296

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Abbreviations: HER2, human epidermal growth factor receptor 2; HER2-E, human epidermal growth factor receptor 2-enriched; HR, hormone-receptor; IHC, immunohistochemistry, Lum, Luminal; TNBC, triple-negative breast cancer

Immuno-oncology

The immune system and tumour microenvironment

The immune system encompasses different types of cells and signalling substances that help the host to prevent infections and is divided into the innate and adaptive immune system[297]. The innate system is responsible for the general, fast defence and is composed of barriers (skin and mucous membranes), the immune cells and proteins. In contrast, the specific defence of the adaptive immune system acts directly against specific germs. It consists mainly of T-lymphocytes (T-cells), B-lymphocytes and antibodies[297]. The T-cells are expressing complex receptor-molecules and also accessory molecules that categorise the T-cells (such as CD4 and CD8 T-cells)[298]. The response of T-cells is controlled by many checkpoints, acting as gatekeepers of immune response and is important for maintaining balance in the immune system[299].

There is a complex interaction between the host cells, signalling substances and the tumour microenvironment (TME), and the escape from the control by the immune system is regarded as one of the hallmarks of cancer[300]. The TME constitutes both innate and adaptive immune cells, and the tumour cells might cause an inhibition of the immune response by down- and upregulation of immunoreceptors[299, 301]. The tumour formation and the resulting chronic inflammation might evade the destruction by the immune system, resulting in an immunosuppressive effect[302]. Cancer *immunoediting* is a theory suggesting that the host immune system recognises the immunogenetic tumour antigens and respond to this in a three-phase action: *elimination, equilibrium* and *escape*[303]. These describe the detection and destruction of the tumour, a dormancy state (the tumour is resistant to detection), and a clinical apparent state of the tumour, respectively[303–305]. This transition might be due to different mechanisms; the tumour is no longer recognised by the immune system, insensitive to the immune mechanism or induces an immunosuppressive state. The different cells of the immune system and their functions with respect to the TME are presented in Table 7.

Tumour-infiltrating lymphocytes

Tumour-infiltrating lymphocytes (TILs) are mononuclear immune cells nested in and around the neoplastic cells and serve as a marker for tumour immunogenicity[306]. They constitute different types of cells, most commonly T-cells, but also B-cells, natural killer- (NK) cells, macrophages and dendritic cells[307].

Table 7. Different constitutions of the tumour-immune infiltrattion

Cell type	Cell subtype	Tumour effect	Function in tumour environment
Innate immune system			
Neutrophils	Tumour-associated neutrophils	Progression	Reduce T-cell proliferation
	Myeloid-derived suppressor cells	Progression	Poorly known
Macrophages	Tumour-associated macrophages M1	Suppression	Proinflammatory Produce cytokines
	Tumour-associated macrophages M2	Progression	Immunosuppressive Produce cytokines (IL-4, IL-10)
Dendritic cells	-	Suppression	Present tumour antigens to T-cells
Adaptive immune system			
Natural killer-cells	-	Suppression	Killing tumour cells
T-lymphocytes	Cytotoxic T-cells	Suppression	Release perforin and granzymes→cancer cell death
	Th1 helper T-cells	Suppression	Activate cytotoxic T-cells
	Th2 helper T-cells	Progression	Secrete cytokines
	Regulatory T-cells	Progression	Dampen immune response to tumour antigens
	Follicular helper T-cells	Suppression	Meditate B-cell activation
B lymphocytes	-	Suppression	Antibody production Antigen presentation

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The most common method for assessment of TILs is by direct quantification of histological slides stained by HE under a light microscope. However, it is possible to assess TILs in needle biopsies in the neoadjuvant setting, on tissue microarrays (TMA) or by flow cytometry and multicolour IHC staining for subsets of TILs[306]. Alternatively, gene expression-based analysis for categorisation of the immune infiltration is also a possible research method[308].

There are in general two different types of TILs; stromal TILs (sTILs); defined as the proportion of the stromal area containing infiltration of lymphocytes, with no direct contact with invasive tumour cells, and intratumoural TILs (iTILs) that are intraepithelial mononuclear cells within the nests of the tumours or in direct contact with tumours cells (Table 8)[208]. The sTILs are the most frequent TILs in breast cancer. For standardisation of the scoring, international guidelines have been implemented, as postulated by the Immuno-Oncology International TILs Working Group[208]. According to these guidelines, TILs should be assessed as average TILs in the tumour area, and not focusing on hotspots. In addition, TILs should be assessed as a continuous parameter, however, a secondary option is as a categorised variable. No formal recommendations for relevant thresholds in these categories have been made so far. The

term lymphocyte-predominant breast cancer (LPBC) is used as a descriptive term for tumours that contains more lymphocytes than tumour cells. Different studies used 50 and 60% cut-offs for this purpose[208] (Table 8).

Table 8. Definition of different tumour-infiltrating lymphocytes

Definition	Relevance
Lymphocyte-predominant breast cancer	
Defines breast cancer constituting more lymphocytes than tumour cells	• Definitions of 50–60% are used as a threshold • Can be used in predefined subgroup analyses • Description of tumours with a particularly high immune infiltrate
Stromal TILs	
Accumulation of immune cells in tumour tissue. Proportion of the stromal area containing infiltration of lymphocytes, with no direct contact with invasive tumour cells	• Predictive for increased response to neoadjuvant chemotherapy and marker for improved outcome after adjuvant chemotherapy (TNBC/HER2+ breast cancer)
Intratumoural TILs	
Intraepithelial mononuclear cells within the nests of the tumours or in direct contact with tumours cells	• More difficult to evaluate • Do not provide additional predictive/prognostic information compared to stromal TILs

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Abbreviations: HER2, human epidermal growth factor receptor 2; TILs, tumour-infiltrating lymphocytes; TNBC, triple-negative breast cancer

TILs and breast cancer subtypes

The different breast cancer subtypes have different regarding immune biology; high TILs are more commonly found in the TNBC and HER2+ than in luminal subtypes. Based on a review, the TNBC had the highest incidence of LPBC (20%), similar to HER2+ tumours (16%), but the corresponding rate for ER+/HER2– tumours was only 6%[309].

There are many studies on the prognostic value of TILs in breast cancer in the adjuvant setting[306]. Abundance of TILs has been shown to indicate good prognosis for the TNBC and HER2+ subtypes[208–213]. In the NeoALTTO study, increased level of TILs was associated with prolonged event-free survival in HER2+ tumours[310]. In the neoadjuvant setting, LPBC predicts pathological complete response (pCR) for TNBC and HER2+ breast cancer after neoadjuvant chemotherapy[209, 216]. Moreover, the FinHER study demonstrated that high level of TILs was associated with increased efficacy of adjuvant trastuzumab in HER2+ tumours[210]. An interaction between high level of stromal TILs and benefit of added neoadjuvant carboplatin has also been reported[311]. Regarding the subclasses of TILs, CD8+ TILs seem to be associate with better prognosis, especially in Basal-like and HER2+ tumours[312–314] and moreover to increased pCR in the neoadjuvant setting[315]. According to the recommendation from St. Gallen 2019, the panel argued for that TILs should be assessed and reported in TNBC[93] and it is also recommended in the updated Swedish pathological guidelines 2020[12].

For the ER+/HER2– subtype, data regarding prognostic and treatment-predictive values of TILs is sparse. Some studies have not found any conclusive results for the prognostic association of TILs in this subtype[210, 316, 317]. According to a pooled analysis of neoadjuvant therapies by Denkert et al., patients with hormone-receptor positive/HER2– tumours and low TILs levels, had an improved 10-years outcome[209]. It also seems that high TILs are linked to the increased pCR after neoadjuvant chemotherapy for this subtype (pCR rate of 6%, 11% and 28% in the low, intermediate and high TIL subgroups, respectively)[209]. However, the ability of TILs to predict the effect of neoadjuvant endocrine therapy is limited[318, 319].

Aims

Overall aims

The overall aim of this thesis was to examine different aspects of endocrine therapy in primary breast cancer; in particular, in the context of risk profiling and adherence perspectives for improving tailored adjuvant treatment. We aimed to analyse risk profiling regarding prognosis and predictive effects as defined by gene expression and IHC markers (immune infiltration as defined by TILs) in primary ER+/HER2– breast cancer and to examine the adherence to the recommended adjuvant endocrine therapy recommended. The agreement between intrinsic and surrogate subtyping and, moreover, their differences in prognosis were also analysed.

Specific aims

Study I

Examine the adherence (expressed as percentage) to adjuvant endocrine therapy in a cohort of primary breast cancer after 3 and 5 years, respectively and to investigate if there are any factors associated to adherence.

Study II

Examine the agreement (percentage and kappa statistics) between intrinsic subtyping and different surrogate subtyping classifications in ER+/HER2– breast cancer tumours. Moreover, to examine the discriminatory value of Ki67, PR and HG to identify tumour subgroups consisting mainly of Luminal A or B tumours by intrinsic subtyping.

Study III

Examine the prognostic impact of TILs in a premenopausal cohort with long-term follow-up, stratified by different breast cancer subtypes and furthermore, investigate the role of TILs as a predictive marker for tamoxifen benefit in patients with ER+ tumours.

Study IV

Examine the prognostic value of PAM50 intrinsic subtyping and ROR score in premenopausal women with early breast cancer. In patients with ER+/HER2– tumours, evaluate the prognostic differences of luminal PAM50 compared with surrogate subtyping. Furthermore, analyse tamoxifen-predictive effects of luminal PAM50 subtypes.

Methods

Study populations

Three different study populations have been used for the analyses in this thesis as presented below. The flow charts are presented separately.

Study I

The study cohort in study I included patients diagnosed with ER+ breast cancer in Region Jönköping County between 1 January 2009 and 31 December 2012 and were identified using the Swedish National Quality Register for Breast Cancer (NKBC) (*n*=802). To specify those who were recommended adjuvant therapy (*n*=634), a review of all their medical records was performed. Patients were excluded because of ER+ tumour ≤10 mm since, according to the Swedish guidelines applicable at the time, patients were generally recommended adjuvant therapy if they had an ER+ tumour >10 mm and/or lymph node positivity. Patients were also excluded for the following reasons: declined adjuvant endocrine treatment, prescription by dispensing system, relocation to another health care region, death without breast cancer during follow-up, and recurrence or contralateral breast cancer during the planned treatment period (in total *n*=142 was excluded, and *n*=4 had missing data).

Follow-up of breast cancer patients in Region Jönköping Count, was since 2016 centralised to the Department of Oncology, County Hospital Ryhov in Jönköping. Before that, the Department of Oncology, County Hospital Ryhov, Jönköping, the Department of Surgery, Highland Hospital, Eksjö, and the Department of Surgery, Värnamo Hospital, Värnamo Hospital, were responsible for the follow-up of endocrine drugs, respectively. The follow-up routines were slightly different between the hospitals. In general, all patients met a physician at the initiation of the endocrine drug and had written information about the treatment was handed out. A physician´s visit the first year and yearly control mammography were offered. In Eksjö, a nurse contacted the patient after 3 months and then yearly follow-ups were arranged. In Värnamo, the physician wrote a letter yearly to inform the patient about the mammography results and that a new prescription of the endocrine drug had been

issued. In Jönköping, a nurse initially called patients once a year for support, but this follow-up was ended in 2013.

Study II

Study II encompassed patients from the SCAN-B trial. For study II, all patients with ER+/HER2– breast cancers from SCAN-B during 2013–2017 (*n*=3,196) were initially included. We further excluded those patients who did not undergo primary surgery, had multifocal tumours, those with missing IHC data of Ki67, PR and HG and finally those whose tumours had a non-luminal intrinsic subtype. In total 1,133 patients were excluded, and 2,063 patients were included in the analyses.

Study III-IV

The study population in study III–IV included patients who participated in the SBII:2pre trial[320]. During 1984–1991, 564 premenopausal women with stage II (based upon UICC TNM, third edition (1982)) invasive breast cancer, irrespective of hormone status, were randomised between 2 years of adjuvant tamoxifen or no systemic treatment (control). In this multicentre Swedish study two centres participated: the South Eastern (Oncological Centre Lund) (*n*=137) and Southern (Oncological Centre Linköping) (*n*=427) Health Care Regions. The patients received 40 and 20 mg of tamoxifen in these two regions, respectively. The patients had radical surgery and in total eight of them received additional adjuvant chemotherapy and/or goserelin. Patients were excluded for following reasons: metastatic disease, bilateral breast cancer, or history of other malignancies. Based on the follow-up in the study by Ekholm et al., in total four patients were excluded due to protocol violation (two lacked invasive breast cancer and two had stage IV disease at time of diagnosis)[321]. Among the finally included patients, 284 were randomised to the control and 276 to the tamoxifen treatment arm, respectively.

The aims of study III were both to analyse the prognostic association to TILs and to evaluate the tamoxifen-predictive value of TILs. For these purposes we used two different subpopulations. In the prognostic analysis we selected patients allocated to no systemic therapy, with available scoring for TILs and IHC markers for subtype characterisation. Since an ER–/PR+ tumour is unlikely to represent a correct subtype[322], these were excluded from the analyses. In total 221 patients were included in the prognostic analyses. Only ER+ tumours of those successfully scored for TILs were included in the predictive analyses, (*n*=171 and *n*=150 in the control and tamoxifen arm, respectively).

In study IV, four different patient selections were used for different purposes:

- Prognostic analysis of PAM50 subtypes: all patients having tumours with available gene expression data (*n*=437)
- Comparison of prognosis between patients with luminal PAM50 and surrogate subtypes (St. Gallen 2013): all ER+/HER2– tumours with available IHC markers for surrogate subtype characterisation and luminal PAM50 subtype (*n*=207)
- Prognostic analysis of ROR score: all patients with ER+/HER2– tumours with available ROR score categories (*n*=236)
- Predictive analysis of luminal PAM50 subtype for tamoxifen benefit: all patients with ER+/HER2– tumours and available luminal PAM50 subtype (*n*=217)

Flow charts of inclusion of patients

Study I

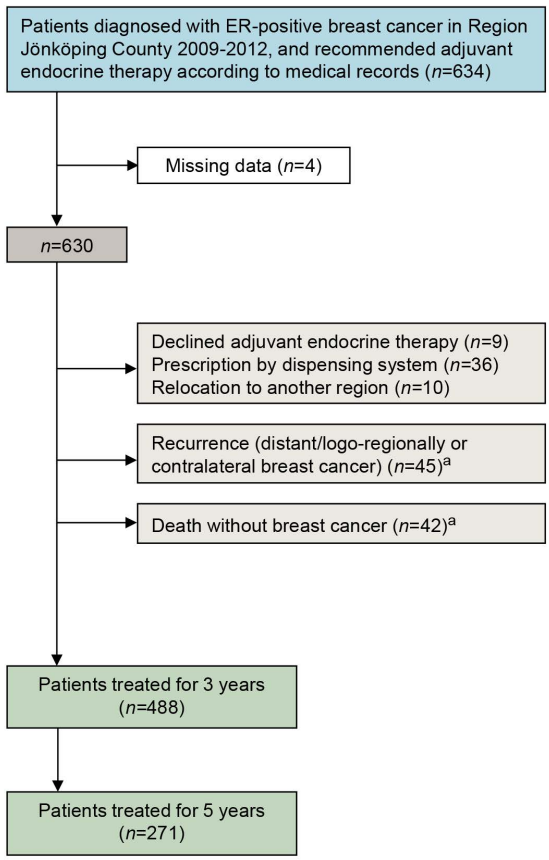

Figure 9. Flow chart in study I

[a]Twenty-three of these could have been included in the 3-year analysis. Post-hoc analysis has been done to further investigate the effect of this exclusion.

Abbrevation: ER, oestrogen receptor

Study II

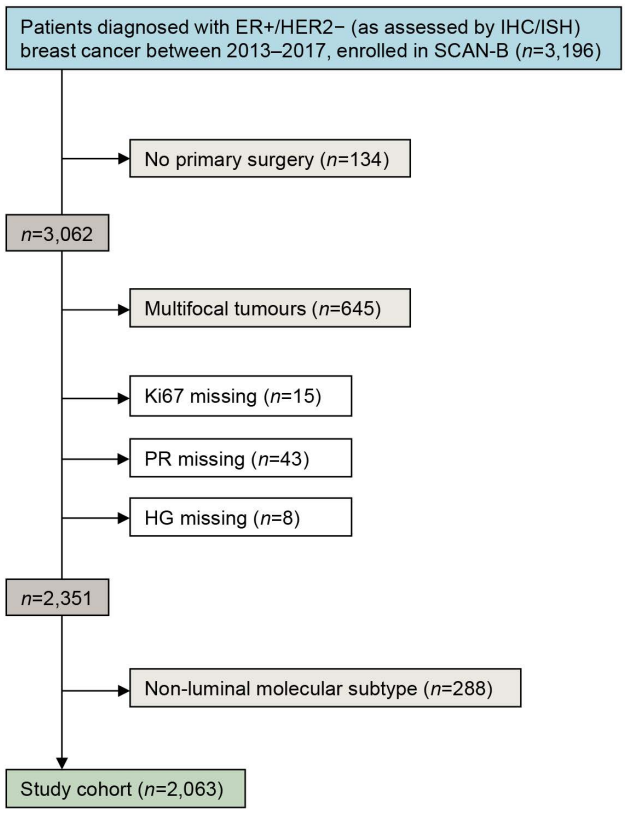

Figure 10. Flow chart in study II

Abbreviations: ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; HG, histological grade; ICH, immunohistochemical; ISH, *in situ* hybridization; PR, progesterone receptor

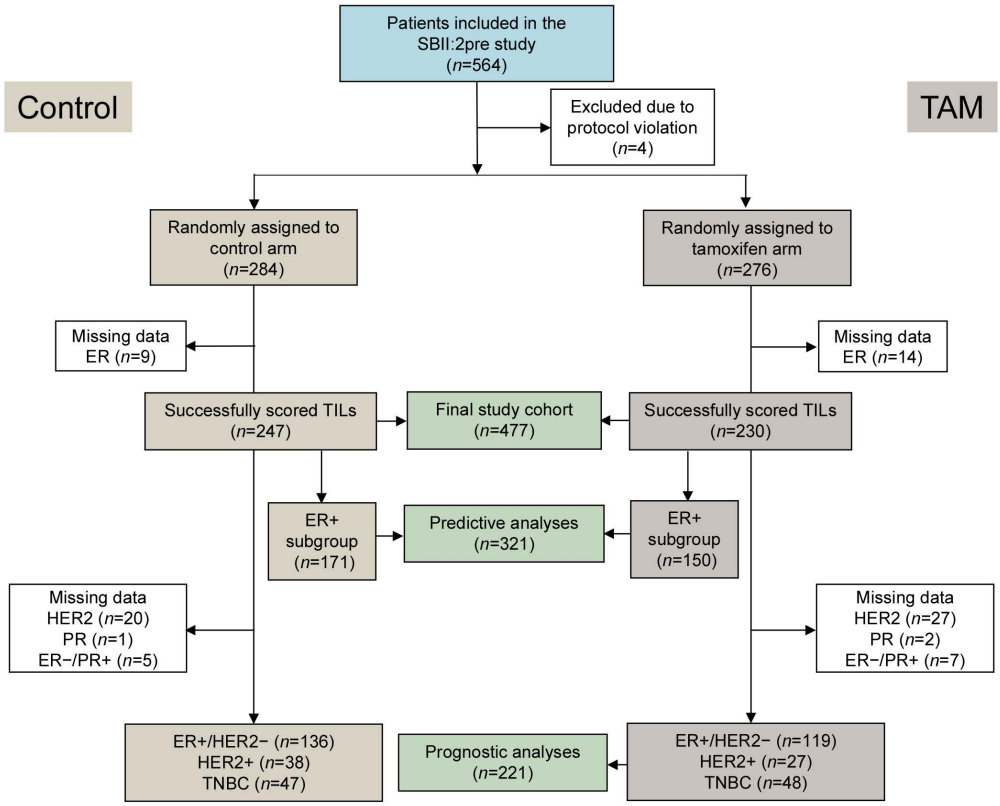

Figure 11. Flow chart in study III

Abbreviations: ER, oestrogen receptor; HER2 human epidermal growth factor receptor 2; PR, progesterone receptor; TILs, tumour infiltrating lymphocytes; TNBC, triple-negative breast cancer

Study IV

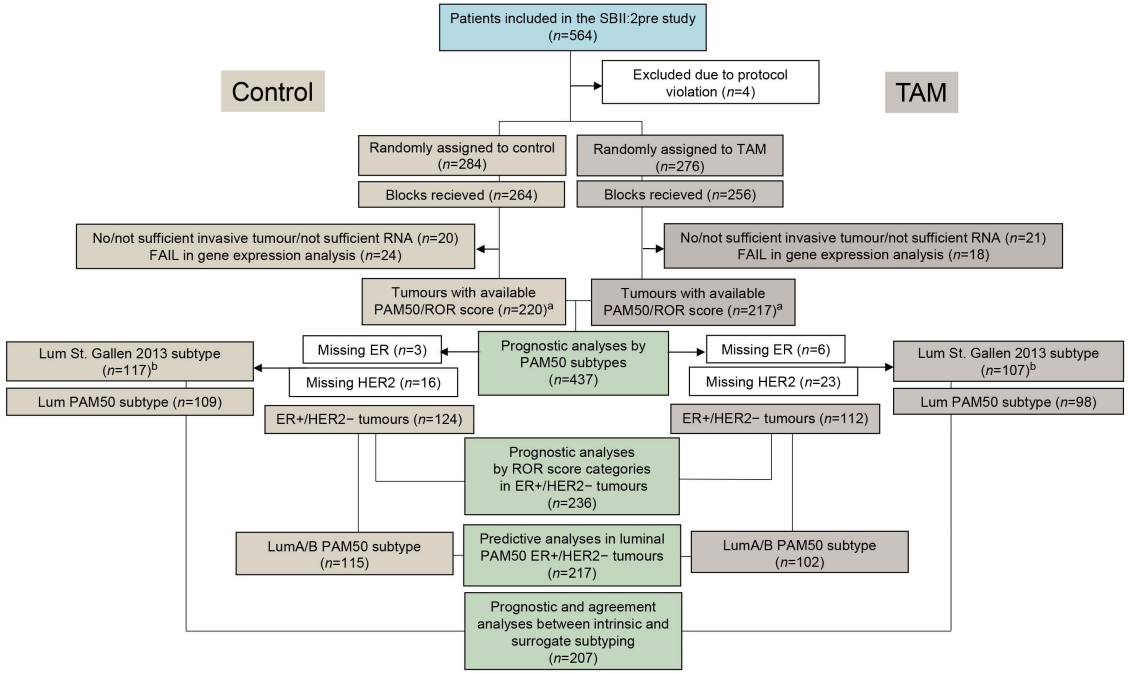

Figure 12. Flow chart in study IV

[a]Available ROR score categories in *n*=219 and *n*=216 patients in the control (no systemic treatment) and tamoxifen arm, respectively, due to *n*=1 missing tumour nodal status in each treatment arm.

[b]Defined accordingly: $LumA_{SC}$, low Ki67 (<20%) and high PR (≥20%); $LumB_{SC}$, high Ki67 (≥20%) and/or low PR (<20%). Cases with missing re-evaluated PR data, were substituted (*n*=2 in the control arm) with previously available IHC data.

Abbreviations: ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; Lum, Luminal; TAM, tamoxifen; ROR, Risk of Recurrence; SC, surrogate classification

Clinicopathological data acquisition

Study I

The clinicopathological data of the included patients were retrieved from the Swedish NKBC. Specifically, hospital (Jönköping, Eksjö, Värnamo), type of endocrine drug, age at time of diagnosis and whether radiotherapy/chemotherapy was/were given, were recorded. The medical records were studied in order to validate that the patient had been offered endocrine treatment and to verify if any of the exclusion criteria were fulfilled. To estimate patient adherence, data of the prescribed endocrine drugs was retrieved from the Swedish Prescribed Drug Register[323]. This register contains data about a patient's prescribed drugs, date of prescription, Anatomical Therapeutic Chemical Classification (ATC) codes and number of packages collected and Defined Daily Doses (DDD). The DDD is a measurement often used in combination with ATC-system and is defined as the average daily dose when the drug is used as the main indication. We retrieved data based on the ATC codes for the endocrine drugs (tamoxifen, AI, GnRH analogues). By 28 October 2016 we retrieved data from the register for all the included patients regarding date of prescription and dispersion, number of packages prescribed, the ATC code, the product name, the substance name, the DDD for each prescription, and the DDD for each package.

Study II

The genomic analyses from the SCAN-B trial provided information of the intrinsic subtype (according to the PAM50 algorithm). The tumour tissues were collected at the local pathology departments for regular IHC analyses and reported into the NKBC along with standard clinical variables. Data from this register is made available for enrolled patients and transferred to SCAN-B and retrieved simultaneously with the genomic data once requested. For the purpose of study II, genomic data and the clinicopathological data were retrospectively retrieved with permission from the SCAN-B steering committee after approval application.

Study III-IV

From the database of SBII:2pre trial, data regarding ER, PR, Ki67, NHG and HER2 were available in addition to the clinical variables age, nodal status, tumour size, histopathological type, adjuvant radiotherapy/chemotherapy/GnRH analogue

treatment and treatment arm. For the purpose of study III and IV, preserved archival FFPE tissues from breast tumours in the SBII:2pre trial were collected from seven regional biobanks (*n*=520) and stained with HE. In study III, assessments by a pathologist regarding TILs and LVI were performed according to standard methods (see below).

In study IV, reassessments were made for PR and Ki67. Information on the genomic data (PAM50 subtype and ROR score) was obtained by gene expression analyses of RNA material from the archived tumour blocks.

Immunohistochemical markers, surrogate subtyping and genomic analyses

Immunohistochemical markers

ER, PR, HER2, Ki67, NHG and LVI

In study I and II, the IHC variable ER was retrieved from the NKBC, assessed in clinical routine as defined by the Swedish guidelines. This was also true for the additional IHC variables used in study II (PR, NHG and Ki67). ER/PR-positivity was defined as >10% positively stained cells (according to the Swedish guidelines at the time of study initiation, however, in 2020 the cut-off in Swedish pathology guidelines was changed to ≥10%, but this was not changed in any study). In study II, ER/PR-positivity was defined based on the NKBC report of the variable (positive/negative) and percentage value of the receptors. In study II, HER2 status was retrieved from the NKBC and HER2+ was defined based on IHC score 3+ and *HER2* gene amplification as reported in the NKBC register.

In study III and IV, data regarding ER, PR, Ki67, NHG and HER2 was available from previous publications of the SBII:2pre trial. Hormone-receptor analyses were performed at time of primary surgery[320] according to the cytosol-based method (for the 564 patients: ER; *n*=457, PR; *n*=449). Paraffin-embedded tumour samples (*n*=500) were collected in 2003 and TMAs were constructed to assess ER/PR-status based on ICH analyses. ER/PR-positivity was defined as >10% of positively stained tumour cells. In study III and IV, a total of 560 patients were included in the analyses (see above). IHC data was used as the primary definition of ER/PR-positivity, and in tumours with missing IHC scores for ER and/or PR, the cytosol-based results were used (in total, available ER and PR status of the 560 patients was *n*=537 and *n*=535, respectively). In study IV, a re-evaluation of PR (*n*=464) based on IHC from whole tissue sections was performed in 2020 from retrieved tumour blocks according to the Swedish national

guidelines[12]. For missing data of this new PR re-evaluation, the old IHC PR-status was used, generating available PR-status (positive/negative) in 543 patients. Furthermore, for surrogate subtyping, a database variable defining PR low/high was needed (cut-off: ≥20% as high), and for this purpose, those with missing re-evaluated PR, was substituted (n=35) with previously available PR data from the IHC assessments. This generated in total 499 tumours defined as PR high/low. Histological grade was evaluated (at primary surgery and previously re-evaluated in 491 patients) as NHG on whole tissue sections in accordance with the work of Elston-Ellis[69]. Ki67 was originally assessed as a categorical variable (0–1%, 2–10%, 11–25%, 26–50%, 51–100%) and re-evaluated 2020 in 463 tumours as a continuous variable. Of these, 17 were excluded from the analyses, due to low quality and uncertainty in assessment. The assessment of Ki67 was performed by selecting hotspots and counting the percentage of positivity of 200 cells[12]. In study III and IV, the HER2-status was previously assessed on TMA, both by IHC and by *HER2* amplification using fluorescent ISH[324]. HER2 status was available for 468 tumours (out of the 560 patients) and classified as HER2+ either by HER2 3+ as assessed by IHC, and/or by *HER2* amplification by fluorescent ISH.

In project III, the presence of LVI was assessed on whole tissue sections from retrieved preserved tumour blocks from the SBII:2pre trial and defined present if tumour cells were verified in endothelial lined cavities (not by IHC endothelial markers)[12]. For stratification analyses in study III, subtyping based on IHC/ISH markers were used by the following definition: ER+/HER2–, HER2+ (irrespective of ER status) and TNBC (ER–/PR–/HER2–).

Tumour-infiltrating lymphocytes

For the purpose of study III, sections from the retrieved whole breast tumour tissues from the SBII:2pre trial, were scored for TILs. Based on the recommendation from the Immuno-Oncology International TILs Working Group[208], two classifications of TILs, sTILs and iTILs, were made based on their spatial localisation. Only sTILs were used in the statistical analysis and announced TILs in project III. The assessment was performed by a breast pathologist blinded to the patient characteristics and the outcomes (Figure 13). TILs were assessed under a light microscope with a magnification of 40× and 100× (if necessary, 200×). TILs were categorised into the following categories: 0–9%, 10–49%, 50–74% and ≥75%. In the prognostic analyses, the two latter groups were merged into one category (high), and the three groups were then denoted as low, intermediate and high, respectively. Tumours with TILs ≥50% were further defined as LPBC and those with low/intermediate TILs (<50%) were defined as non-LPBC.

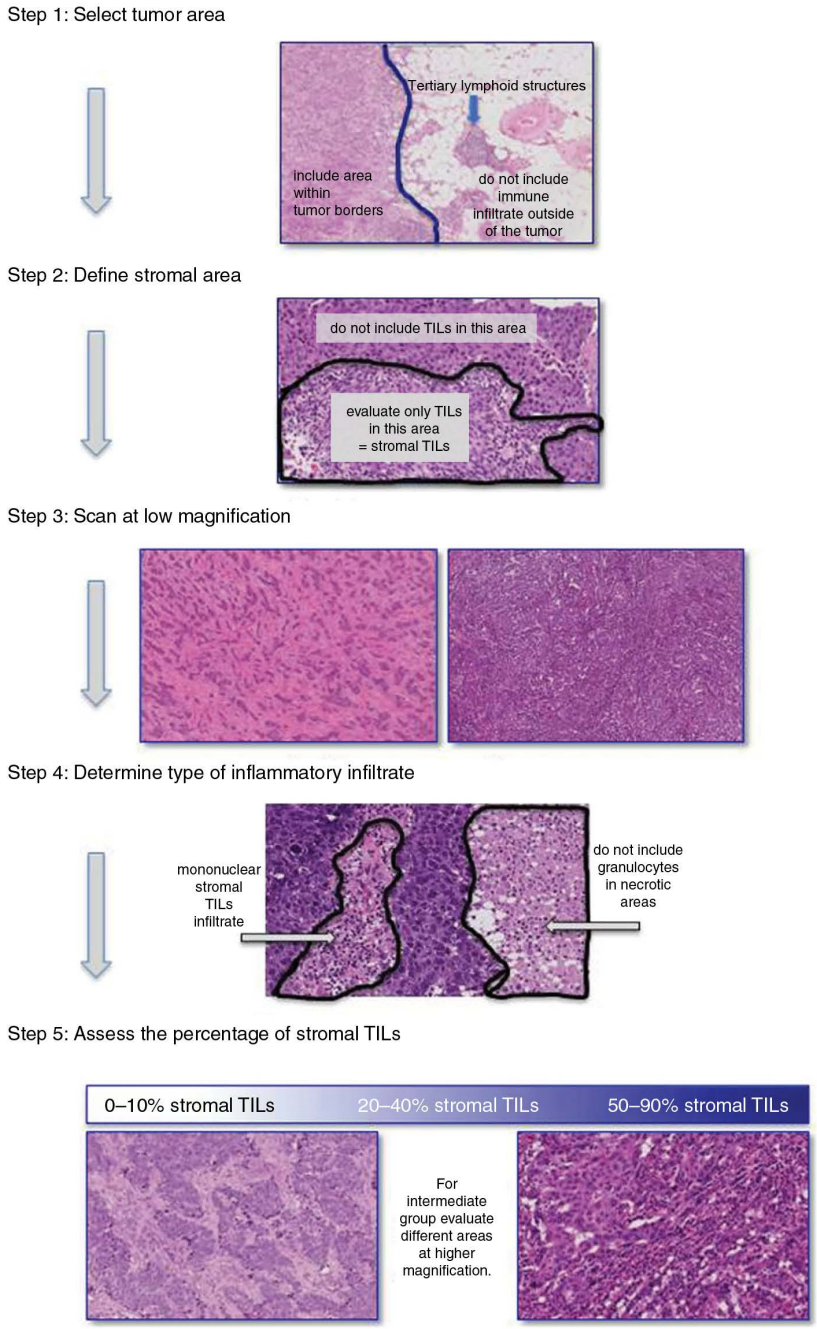

Figure 13. Assessment of stromal tumour-infiltrating lympfocytes according to the Immuno-Oncology International TILs Working Group

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Abbreviation: TILs, tumour-infiltrating lymphocytes

Genomic analyses

Gene expression analysis used in the SCAN-B trial

To determine the intrinsic subtypes in project II, gene expression as assessed by the SCAN-B algorithm was used. Fresh tissue preserved in RNAlater to preserve DNA and RNA and (as well as blood) was delivered to the SCAN-B laboratory at the Department of Oncology in Lund where the tissue was further sectioned; one part for RNA and DNA extraction and an adjacent part for FFPE to conduct a histopathological check (based on TMA)[281]. using RNA-seq, the intrinsic subtype was determined by a nearest-centroid implementation using the PAM50 genes and centroids as described by Parker et al.[232]. This method designates the tumour specimens to Luminal A, Luminal B, HER2-E, Basal-like or Normal-like subtype, according to the most frequent nearest centroid[282, 325]. Unclassified tumours were those whose correlation coefficients were below 0.2 for all the subtype centroids. To avoid the cohort dependence when assigning the PAM50 subtype, fixed reference cohorts were used for gene centring to match the original training population from Parker et al. Moreover, the subtype classifier in SCAN-B uses 100 standard populations and the subtype is determined by the most frequent subtype of these analyses. For the patients included in study II, the handling and genomic analyses of the tumour tissues were performed in accordance with the trial protocol and logistics. The luminal intrinsic subtyping by PAM50, including the SCAN-B PAM50 profile was denoted as Luminal A_{PAM50} and Luminal B_{PAM50} in this thesis.

NanoString Breast Cancer 360™ assay

In project IV, 1–5 sections (10µm thick) from FFPE tissue with invasive breast carcinoma, were used to extract total RNA (AllPrep DNA/RNA FFPE kit (Qiagen Cat: 80234, Hilden, Germany)). Genomic profiling was performed using the NanoString Breast Cancer 360™ assay on the NanoString nCounter® SPRINT Profiler (NanoString Technologies Inc., Seattle, WA, USA)[280]. The assay includes 776 genes and raw data is handled by the BC360 Data Analysis Service, provided by NanoString, Seattle (US). The assay provides a 360-degree view of the gene expressions of microenvironment and immune perspectives of breast cancer. The assay covers 23 key breast cancer pathways and processes and 48 breast cancer signatures (Figure 14). The nCounter© system uses gene-specific probe pairs and performs digital readouts of the relative abundance of mRNA transcripts by a multiplexed measurement of gene expression.

The analysis steps of the system are (also described previously in this thesis[178]:

- hybridization of the RNA to a fluorescent reporter probe and capture probe

- purification of the target/probe complexes through the nCounter© Prep plates that contains reagents for post-hybridization and immobilization on the nCounter© Cartridge
- analyses of the nCounter© Cartridge on the nCounter© Digital Analyzer

The target molecule is identified by the colour code generated by the six RNA-labelled segments of fluorescence colour dyes on the reporter probe that attaches to the target.

Breast Cancer Prognosis	**Risk of Recurrence (ROR)/Genomic Risk***							
Breast Cancer Subtyping	**PAM50 Molecular Subtypes***	Luminal A Correlation Value (PAM50)	Luminal B Correlation Value (PAM50)	HER2-Enriched Correlation Value (PAM50)	Basal-like Correlation Value (PAM50)	Claudin-Low Subtype Score	Triple Negative Subtype	
Breast Cancer Receptors	ESR1 Gene Expression	PGR Gene Expression	ERBB2 Gene Expression	AR Gene Expression				
Breast Cancer Signaling Pathways	ER Signaling	PTEN Gene Expression	CDK4 Expression	CDK6 Expression				
Tumor Mutational Response	HRD	BRCA	p53					
Tumor Regulation	Proliferation (PAM50)	Apoptosis	Differentiation	FOXA1 Gene Expression	Cell Adhesion	Mammary Stemness	RB1 Gene Expression	SOX2 Gene Expression
Tumor Immunogenicity	APM (Antigen Processing Machinery)							
Stromal Factors	Endothelial Cells	Stromal Abundance						
Inhibitory Metabolism	Hypoxia							
Inhibitory Immune Mechanisms	IDO1 Expression	PD-L1 Gene Expression	B7-H3	TGF-Beta				
Anti-Tumor Immune Activity	**Tumor Inflammation Signature (TIS)***	Interferon Gamma Signaling	MHC Class II Antigen Presentation	Cytotoxicity				
Inhibitory Immune Signaling	Inflammatory Chemokines	TIGIT Gene Expression	PD-L2 Gene Expression	PD-1 Gene Expression				
Immune Cell Abundance	Cytoxic Cell	CD-8+ T Cell	Macrophage	Mast Cell	Treg			
	***Validated Signatures**							

Figure 14. NanoString Breast Cancer 360™ assay
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Housekeeping gene geomean quality control (QC) categorised samples as PASS/BORDERLINE (≥202) or FAIL (<202). In total, 91% (437/479) of the samples in study IV passed the QC. PAM50 genes were normalised to the PAM50 housekeeper gene geomean. The correlation between the observed scaled expression for the PAM50 genes and a centroid for each of the four subtypes was then determined. The subtype with the greatest correlation value determined the intrinsic subtype. The ROR score was calculated by using the coefficients from the Cox model that includes the Pearson's coefficients to the four tumour centroids, in addition to a proliferation score and primary tumour size as an additive term. Since ROR models including 50 and 46 genes (excluding *BIRC5*, *MYBL2*, *GRB7*, and *CCNB1)*, respectively, did not differ in performance, the 46-gene expression model was used in the algorithm for ROR score calculation[192]. Tumour size was included as a binary category (cut-off ≤20 and >20 mm). A weighted sum generated a score between 0–100 and ROR categories (low, intermediate, and high) that defined according to prespecified cut-offs[178].

Surrogate subtyping

In addition to the previously established surrogate classifiers (St. Gallen 2013 and Maisonneuve) as presented in the introduction of this thesis, an experimental surrogate algorithm was proposed in study II. Histological grade has previously not been used in surrogate classifications, although it seemed to contribute to separation of tumours associated with different outcomes[71, 228] The proposed Grade-based classification emphasizes HG as the major factor for surrogate subtyping. Tumours with HG1 were classified as Luminal A_{SC} and HG3 tumours as Luminal B_{SC}. Ki67 and PR were only used for the HG2 tumours for further surrogate division (Table 9).

Table 9. Definition of different surrogate subtyping classifications for ER+/HER2− breast cancer tumours

Clinicopathological surrogate definition	Characteristics
St. Gallen 2011[225]	
$LumA_{SC}$	Low Ki67 (<14%)
$LumB_{SC}$	High Ki67 (≥14%)
St. Gallen 2013[66]	
$LumA_{SC}$	Low Ki67 (<20%) and High PR (≥20%)
$LumB_{SC}$	High Ki67 (≥20%) and/or Low PR (<20%)
Maisonneuve[228]	
$LumA_{SC}$	Low Ki67 (<14%) or Intermediate Ki67 (14–19%) and high PR (≥20%)
$LumB_{SC}$	High Ki67 (≥20%) or Intermediate Ki67 (14–19%) and low PR (<20%)
St. Gallen 2017[226]	
$LumA_{SC}$	High ER/PR and clearly low Ki67 or HG
Intermediate	Uncertainties persist about risk and degree of responsiveness to endocrine and cytotoxic therapies
$LumB_{SC}$	Lower ER/PR with clearly high Ki67, HG3
Grade-based classification	
$LumA_{SC}$	HG1 or HG2 and low Ki67 (<14%) or HG2 and intermediate Ki67 (14–19%) and high PR (≥20%)
$LumB_{SC}$	HG3 or HG2 and high Ki67 (≥20%) or HG2 and intermediate Ki67 (14–19%) and low PR (<20%)

Abbreviations: ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; HG, histological grade; Lum, Luminal; PR, progesterone receptor; SC, surrogate classification

Ethics

Study I

Study I was approved by the Regional Ethical Review Board in Linköping, registration number 2016/61-31.

Study II

Study II was covered by previous ethical approvals from the Ethics Board in Lund (2009/658, 2010/383, 2012/58, 2013/459, 2014/521, 2015/277, 2016/944). Informed consent has previously been obtained from all individual participants included in the SCAN-B study.

Study III-IV

Informed consent was obtained from all participants included in the SBII:2pre trial, and approval was provided by the ethical committees in Lund and Linköping, Sweden. The follow-up study was approved by the ethical committee of Lund (LU 2015/350) as well as a complementary approved application for tissue retrieval and genomic analysis (LU 2017/97). Biobank approval was cleared for all involved pathology departments.

Statistical considerations and follow-up data

Study I

Follow-up

Not applicable.

Calculation of adherence

For the purpose of estimating coverage of available medication of at least 80% of the days during the 3- and 5-year periods, we used the MPR method[145]. This implies the ratio of a total day´s supply to number of days in the observation period. For adherence definition, the lower limit was set at ≥80%. All prescribed endocrine drugs, as selected by the ATC-code for AI and tamoxifen, prescribed, from the first prescription until the last one before the end date in the respective time periods of 3 and 5 years, were added

and the total DDD for all was calculated. Only orally taken medication (not GnRH analogues) were considered. The sum was divided by the number of days in each time period (3 × 365 and 5 × 365 days, respectively).

To analyse the degree of non-adherence, the MPR in the non-adherent subgroup was divided into three categories; 0–30%, 31–60%, and 61–79% and among these subgroups, the percentage distribution was calculated.

Factors associated with adherence

Binary logistic regression analyses were performed to examine potential subgroups (age at time for diagnosis (<40, 40–59, 60–79, ≥80), adjuvant radiotherapy (yes/no), adjuvant chemotherapy (yes/no), hospital responsible for the follow-up (Eksjö/Jönköping/Värnamo), and treatment category (AI/tamoxifen/sequenced)) associated with adherence. This model was used to compare a binary outcome between different exposure groups, in our case defining adherence and non-adherence as the binary outcome and the subgroups mentioned above as the exposure variables. This analysis generated an odds ratio (OR) relative to a chosen reference category, the corresponding 95% CI and a *P*-value which is a measure of evidence against the null hypothesis of an OR of 1.00. If more than one explanatory factor was included in the model, adjusted ORs were calculated[326].

Study II

Follow-up data

Not applicable.

Percentage agreement (exact agreement) and kappa statistics

The main purpose of study II was to assess the agreement between two categorical variables (intrinsic and surrogate subtyping of ER+/HER2– tumours). Both percentage (also known as exact agreement) agreement (%) and Cohan´s kappa (κ) statistics for concordance analyses were used for this purpose. Percentage agreement was calculated by summing up the diagonal of the cross table and dividing by the total number of comparisons. This method, however, does not account for the agreement expected by chance. Cohan's kappa (κ) includes both the percentage agreement and agreement expected by chance. The kappa value is often interpreted by referring to the following categories: ≤0.40 poor/fair agreement; 0.41–0.60 moderate agreement; 0.61–0.80 substantial agreement; and >0.80 almost perfect agreement[327], however, other cut-offs have been suggested[326].

The kappa values are calculated as follows:

$$\kappa = \frac{A_{obs} - A_{exp}}{1 - A_{exp}}$$

The observed proportion of agreement (A_{obs}) between two readings is compared with the agreement that would be expected by chance (A_{exp}). When more categories are included, the kappa is decreasing since there are more misclassification possibilities[326].

Receiver operating characteristic analyses

Different measures which assess the ability of a classification rule to correctly classify a variable into two categories have been suggested. Sensitivity (proportion of true positives correctly identified as such) and specificity (proportion of true negatives correctly identified as such) are the most widely used measures of classification performance of a test or procedure[326].

In the case when binary classifications are derived from variables that incorporates numerical values, cut-off values and their implications can be evaluated using a receiver operating characteristic curve (ROC curve), that illustrates the sensitivity against 1–specificity simultaneously for all possible cut-off values. The area under the ROC curve (auROC) is the ability of a continuous measure to be able to discriminate between the categories. An auROC value of 1 indicates perfect discrimination (cut-off with 100% sensitivity and specificity) whereas a value of 0.5 means no capability for discrimination corresponding to a line along the diagonal[326]. In study II, ROC analysis was used to assess the performance of the IHC markers; Ki67, PR and HG in the surrogate algorithms to distinguish between Luminal A and Luminal B tumours as assesses by intrinsic subtyping. Ki67 and PR were used as continuous variables and HG as a categorical variable.

Concordance in luminal subtypes

To evaluate differences in classification of luminal tumours between intrinsic subtyping and surrogate classification, McNemar's test was used[326]. This is a test based on the chi-squared distribution, used for testing the null hypothesis of equal marginal distributions of paired nominal data, provided the number of pairs is 10 at minimum.

Study III

Follow-up

For patients included in the SBII:2pre trial, the follow-up data regarding breast cancer recurrence and death, was registered by the regional oncologic centres according to a pre-specified protocol[320]. The follow-up consisted of regular follow-up with clinical

examination, chest X-ray and mammography for 10 years. In a follow-up study by Ekholm et al. 2019[321], long-term follow-up was based on medical record reviews (predefined case report forms) from the inclusion to last health care contact/death and incorporated review of the primary diagnosis, data on recurrence (local, regional and distant), contralateral breast cancer (invasive or DCIS) and death. Events of secondary malignancy and causes of death were further obtained from the Swedish Cancer Register and the Swedish Causes of Death Register (data cut-off 30 November 2016).

Breast cancer-free interval (BCFi) was defied as first event of local, regional or distant recurrence, contralateral breast cancer (invasive or DCIS), or breast cancer-related death. The association to OS was also explored. The outcome data from the latest follow-up study database[321] regarding both BCFi and OS, was used.

Distribution of variables

Chi2 test and Chi2 test for trend were used to evaluate the evidence for differences in distributions of the clinicopathological variables between the TIL groups: low, intermediate or high. The Chi2 test is the standard test for comparison of proportions in 2 × 2 tables but can be applied to larger tables. The test is valid provided that less than all the expected numbers are >5[326]. The Chi2 test for trend is used to test whether the association between a categorical or categorised test variable and an ordered variable follows a trend, indicating a correlation between the variables. This test was used for all variables (age, nodal status, tumour size, histological grade, ER, PR, HER2, LVI), except for the non-ordinal variables histopathological type and breast cancer subtype, where conventional Chi2-test was used.

Prognostic analyses of TILs

We used the cumulative incidence of events as a function of the follow-up time, which equals one minus the Kaplan-Meier estimate for OS. The log rank test was used to assess evidence for difference in effect between tamoxifen and control in the predictive analyses. The trend version of the test was used for the three ordered TIL categories (low, intermediate and high). Since two regions were included, we stratified all analyses by region, to allow for different outcomes in the two regions, but the same relative tamoxifen effect. To calculate HRs we used the Cox proportional hazards regression analysis. The effect of the cause-specific Cox regression analysis (BCFi) is discussed below (study IV).

In the prognostic analyses, only patients in the control arm were included. TILs were categorised into three groups with the following cut-offs: low: <10%, intermediate: 10–49% and high: ≥50%. In addition to analyses of all patients in the cohort, separate prognostic analyses were performed for the breast cancer subtypes (ER+/HER2–, HER2+ and TNBC). In the Cox regression analyses, we adjusted for other clinic-pathological variables (HG, age, LVI, HER2 status, nodal involvement, tumour size

and PR status). In the prognostic analysis, two potential methods could have been performed; either selection of only the control arm, or analysis of both the control and tamoxifen arms and adjust for treatment. Since the former method was used in previously published outcome papers of this study cohort, this approach was preferred also in this study.

Predictive analyses of TILs for tamoxifen benefit

Only ER+ patients were included in the predictive analyses. The rationale for this was that tamoxifen is recommended for patients with ER+ tumours. In the Cox regression multivariable analyses, PR status was omitted, due to collinearity with ER status. To further assess the predictive effect of TILs, a Cox model with a term for interaction between TIL subgroup (cut-off <50% for LPBC and ≥50% as non-LPBC) and tamoxifen was used. The cut-off of 50% was chosen post-hoc based on the estimated cumulative incidence curves, showing an effect in the low and intermediate TILs categories regarding tamoxifen benefit, but not in the high category. This cut-off value should therefore be considered data-driven.

Sensitivity test

In seven cases (in the overall study cohort), the histopathological analysis indicated that DCIS was more common in addition to microinvasion. These were finally included in the analyses, due to no difference in the main results. In addition, previous studies have demonstrated that that microinvasive tumours closely resembles more invasive tumours than DCIS[328].

Study IV

Follow-up data

See Study III. To obtain extended data regarding OS, follow-up data was retrieved from the Swedish Causes of Death Register (data cut-off for events was 10 December 2020). Results for maximum follow-up and the two time intervals 0–10 years and >10 years were reported (10 years up to maximum follow-up time, starting at year 10).

Prognostic analyses and cause-specific cumulative incidence of PAM50 and ROR score

Cumulative incidence curves, as in study III, were used to illustrate outcomes for subgroups of patients. The corresponding estimates was used for the outcome BCFi, however, with the competing event death without a preceding breast cancer-event taken into account. The log rank test was used to evaluate evidence against equality of two or more cumulative incidence curves and the one-degree-of-freedom trend version of the test was used for ordered groups (ROR score).

For comparison of cause-specific cumulative incidence curves (BCFi), a modified version of the log rank test derived by Geskus[329] was used in study IV. Competing risks occur in survival analyses when the individual is at risk of more than one type of event and each of these events prohibits the occurrence of the other[330]. The cause-specific cumulative incidence function is an estimate of the absolute or crude risk of having an event, as a function of follow-up time, accounting for the fact that it is not possible to have the event if the competing event first occurred. The method by Geskus is based on restructuring of the data and application of weights[329].

Cox regression models, stratified by region, were used to quantify relative effects on survival and cause-specific Cox-regression was used for the endpoint BCFi, censoring the follow-up at time of death for patients who died without a registered breast cancer-event. Of notice, these cause-specific HRs did not quantify exactly the effects visualised by the cause-specific cumulative incidence curves. These relative effects should formally be interpreted in a hypothetical world where patients cannot die without a preceding breast cancer-event. This could have led to contradictory results if the cumulative incidences of the competing event had varied much over groups of patients compared. However, in study IV, we performed additional analyses, illustrating there were few individuals who died without a breast cancer event and the incidences were similarly distributed among the groups analysed.

Since the proportional hazard assumption is doubtful for long-term follow-up, the HRs should be interpreted cautiously as average effects over time. Accordingly, the relative effects with follow-up restricted to 10 years were also calculated. Multivariable Cox regression analyses were adjusted for age (continuous variable), tumour size (≤20 vs >20 mm), nodal status (N0 vs N1 vs N2), NHG (1 vs 2 vs 3) and treatment arm (control vs tamoxifen). Nodal status and tumour size were omitted in the multivariable prognostic analyses of ROR score, since these variables are included in the ROR score definition.

The categorisation of the ROR score was determined based on node status according to the definition by the manufacturer: N0; low: 0–40, intermediate: 41–60, high: 61–100, N1 (1–3 positive nodes); low: 0–15, intermediate: 16–40, high: 41–100, N2 (≥4 positive nodes); high: 0–100[178]. The prognostic value of ROR score was studied in all ER+/HER2– patients and also stratified by node status (node-negative (N0) and node-positive (N+)). In the Cox regression analyses, only N1 (1–3 positive nodes) was included in the node-positive subgroup (all N2 patients are regarded as ROR score high). Moreover, since only two patients had low ROR score in the node-positive subgroup, these patients were excluded, leading to a comparison between intermediate and high ROR score.

Differences with respect to prognosis and agreement of luminal PAM50 and surrogate subtypes

For prognostic differences between luminal PAM50 and surrogate subtyping, a new variable including the four combinations of Luminal A_{PAM50}, Luminal B_{PAM50}, Luminal A_{SC} and Luminal B_{SC} tumours was created. St. Gallen 2013 surrogate subtyping was chosen, mainly since this is the most commonly known surrogate algorithm. Analyses regarding BCFi and OS were performed as described in the previous subsubsection. For percentage agreement (exact agreement) and kappa statistics, see study II.

Predictive value of luminal PAM50 for tamoxifen benefit

In the predictive analysis of differential effect of tamoxifen benefit in Luminal A_{PAM50} and Luminal B_{PAM50}, the ER+/HER2– cohort was used. The rationale for this was that ROR score is validated for this population in the clinical setting. A Cox model was fitted including an interaction variable between luminal PAM50 subgroup and treatment. As an exploratory analysis, the effect was also examined by selecting all luminal PAM50 patients, regardless of ER and/or HER2 status (n=274).

Results

The results from the four studies are presented for each study separately.

Study I – Good adherence to adjuvant endocrine therapy in early breast cancer - a population-based study based on the Swedish Prescribed Drug Register

We identified 634 patients in total who were recommended adjuvant endocrine therapy. Of these, based on prescribed drugs from the Swedish Prescribed Drug Register, 488 and 271 patients were included in the 3- and 5-year adherence analyses, respectively.

Good adherence after both 3 and 5 years

The results showed that the adherence rate was 91.2% (95% CI: 88.7–93.6; *n*=445) after 3 years, and the corresponding figure for the 271 patients who had completed 5 years of treatment was 91.5% (95% CI: 88.2–94.8; *n*=248) (Figure 15). Even though the simple regression analysis demonstrated that radiotherapy was found to be significantly associated with adherence in the 3-year analysis (OR: 1.9, 95% CI: 1.0–3.6, *P*=0.04), this could not be verified when adjusted for age at time of diagnosis, type of endocrine treatment, if radio/chemotherapy was/were given or to the hospital responsible for the follow-up (Table 10A–B).

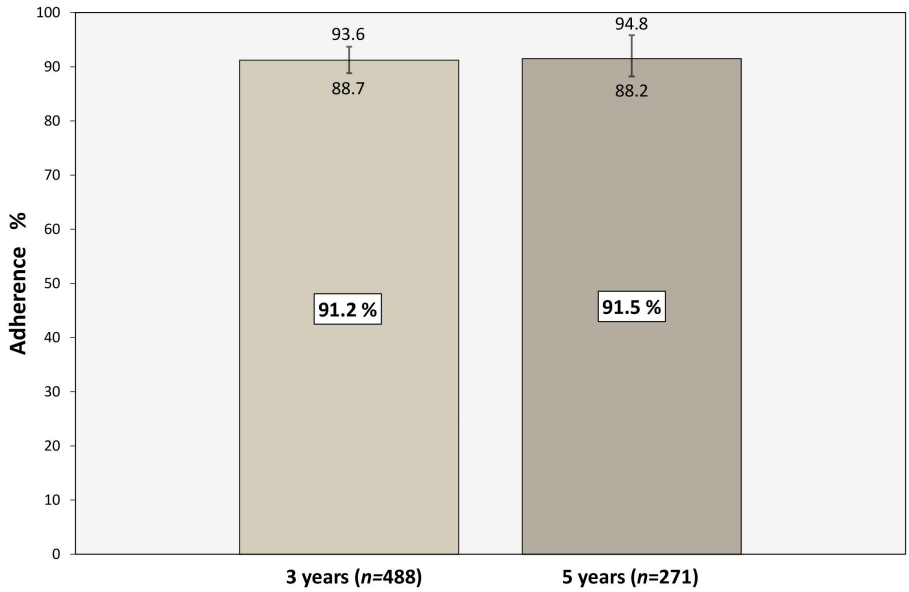

Figure 15. Percentage of patients adherent to endocrine treatment after 3 years (91.2%) and 5 years (91.5%), respectively (95% CI: 88.7–93.6 and 88.2–94.8, respectively)
Abbreviation: CI, confidence interval

Table 10A. Predictors of adherence to adjuvant endocrine treatment after 3 years (*n*=488) according to simple and multiple logistic regression analyses

Characterstic	Adherent No. (%)	Non-adherent No. (%)	OR (95 % CI) simple	*P*-value	OR (95 % CI) Multiple[b]	*P*-value
Total	445 (91.2)	43 (8.8)	(88.7–93.6)			
Hospital						
Jönköping (*n*=227)	206 (90.7)	21 (9.3)	Ref.		Ref.	
Eksjö (*n*=173)	159 (91.9)	14 (8.1)	1.2 (0.6–2.3)	0.69	1.2 (0.6–2.4)	0.70
Värnamo (*n*=88)	80 (90.9)	8 (9.1)	1.0 (0.4–2.4)	0.97	1.0 (0.4–2.4)	0.98
Type of endocrine therapy						
Tamoxifen (*n*=236)	215 (91.1)	21 (8.9)	Ref.		Ref.	
Aromatase inhibitor (*n*=96)	90 (93.8)	6 (6.3)	1.5 (0.6–3.8)	0.43	1.3 (0.5–3.8)	0.57
Sequenced (*n*=156)[a]	140 (89.7)	16 (10.3)	0.9 (0.4–1.7)	0.65	0.7 (0.3–1.5)	0.36
Age at time of diagnosis (years)						
<40 (*n*=18)	15 (83.3)	3 (16.7)	0.5 (0.1–1.8)	0.27	0.3 (0.1–1.3)	0.11
40-59 (*n*=187)	174 (93.0)	13 (7.0)	1.3 (0.6–2.6)	0.51	1.0 (0.5–2.3)	0.93
60-79 (*n*=242)	221 (91.3)	21 (8.7)	Ref.		Ref.	
≥80 (*n*=41)	35 (85.4)	6 (14.6)	0.6 (0.2–1.5)	0.24	0.7 (0.2–2.0)	0.48
Radiotherapy						
Yes (*n*=330)	307 (93.0)	23 (7.0)	1.9 (1.0-3.6)	0.041	1.6 (0.8–3.2)	0.19
No (*n*=158)	138 (87.3)	20 (12.7)	Ref.		Ref.	
Chemotherapy						
Yes (*n*=168)	159 (94.6)	9 (5.4)	2.1 (1.0–4.5)	0.056	2.0 (0.9–4.6)	0.11
No (*n*=320)	286 (89.4)	34 (10.6)	Ref.		Ref.	

[a]Sequenced treatment with tamoxifen and aromatase inhibitor
[b]Including hospital, type of endocrine therapy, age at time of diagnosis, radiotherapy and chemotherapy
Abbreviations: CI, confidence interval; OR, odds ratio

Table 10B. Predictors of adherence to adjuvant endocrine treatment after 5 years (*n*=271) according to simple and multiple logistic regression analyses

Characteristic	Adherent No. (%)	Nonadherent No. (%)	OR (95 % CI) simple	*P*-value	OR (95 % CI) multiple[b]	*P*-value
Total	248 (91.5)	23 (8.5))	(88.2–94.8)			
Hospital						
Jönköping (*n*=125)	116 (92.8)	9 (7.2)	Ref.		Ref.	
Eksjö (*n*=101)	93 (92.1)	8 (7.9)	0.9 (0.3–2.4)	0.84	0.9 (0.3–2.5)	0.81
Värnamo (*n*=45)	39 (86.7)	6 (13.3)	0.5 (0.2–1.5)	0.22	0.4 (0.1–1.3)	0.12
Type of endocrine therapy						
Tamoxifen (*n*=136)	122 (89.7)	14 (10.3)	Ref.		Ref.	
Aromatase inhibitor (*n*=44)	41 (93.2)	3 (6.8)	1.6 (0.4–5.7)	0.50	2.0 (0.5–8.7)	0.36
Sequenced[a] (*n*=91)	85 (93.4)	6 (6.6)	1.6 (0.6–4.4)	0.34	1.3 (0,4–4.0)	0.61
Age at time of diagnosis (years)						
<40 (*n*=13)	10 (76.9)	3 (23.1)	0.3 (0.1–1.1)	0.072	0.3 (0.0–1.4)	0.11
40-59 (*n*=103)	95 (92.2)	8 (7.8)	1.0 (0.4–2.5)	0.92	1.0 (0.3–2.8)	0.94
60-79 (*n*=135)	125 (92.6)	10 (7.4)	Ref.		Ref.	
≥80 (*n*=20)	18 (90.0)	2 (10.0)	0.7 (0.1–3.6)	0.69	1.0 (0.2–5.3)	0.96
Radiotherapy						
Yes (*n*=178)	166 (93.3)	12 (6.7)	1.9 (0.8–4.4)	0.16	2.1 (0.8–5.6)	0.15
No (*n*=93)	82 (88.2)	11 (11.8)	Ref.		Ref.	
Chemotherapy						
Yes (*n*=83)	76 (91.6)	7 (8.4)	1.0 (0.4–2.6)	0.98	0.9 (0.3–2.6)	0.80
No (*n*=188)	172 (91.5)	16 (8.5)	Ref.		Ref.	

[a]Sequenced treatment with tamoxifen and aromatase inhibitor
[b]Including hospital, type of endocrine therapy, age at time of diagnosis, radiotherapy and chemotherapy
Abbreviations: CI, confidence interval; OR, odds ratio

The degree of non-adherence is displayed by MPR after 3 (*n*=43) and 5 (*n*=23) years in Table 11. The results indicate that most of the non-adherent patients after 5 years of treatment had a MPR in the lowest range 0–30. There was a more even distribution among the degree of non-adherence after a shorter time of treatment.

Table 11. Descriptive data of Medical Possession Ratio (MPR) among non-adherent patients after 3 (*n*=43) and 5 years (*n*=23), respectively

MPR %	3 years *n* (%)	5 years *n* (%)
0-30	16 (37)	11 (48)
31-60	13 (30)	5 (22)
61-79	14 (33)	7 (30)

Abbreviation: MPR, medication possession ratio

Study II – Agreement between molecular subtyping and surrogate subtype classification - a contemporary population-based study of ER-positive/HER2-negative primary breast cancer

In total 2063 patients diagnosed between 2013–2017, who had an invasive ER+/HER2– breast cancer, were identified from the SCAN-B trial. All had available IHC markers for surrogate subtyping and subtyped as Luminal A_{PAM50} or Luminal B_{PAM50} by the PAM50 algorithm as used within the SCAN-B trial. The patient- and tumour characteristics are presented in Table 12.

Table 12. Tumour and patient characteristics of the included patients diagnosed with ER+/HER2− tumours by IHC/ISH with an intrinsic luminal profile (*n*=2,063)

Characteristics	Number of patients *n* (%)
Tumour size (mm)	
≤20	1,451 (71)
>20–50	557 (27)
>50	46 (2)
Missing	9
Number of positive nodes	
0	1,440 (71)
1–3	509 (25)
4–9	66 (3)
≥10	29 (1)
Missing	19
PR[a]	
Positive	1,780 (86)
Negative	283 (14)
Histological grade	
1	458 (22)
2	1,202 (58)
3	403 (20)
Ki67	
Low (<14%)	523 (25)
Intermediate (14–19%)	443 (22)
High (≥20%)	1,097 (53)
Age	
<40	14 (1)
≥40–49	106 (5)
≥50–59	356 (17)
≥60	1587 (77)
Histopathological tumour type	
Ductal/no special type	1,619 (79)
Lobular	295 (14)
Other[b]	148 (7)
Missing	1

[a]Regarded as positive if defined as positive in the Swedish National Quality Register for Breast Cancer, or a value of PR >10%
[b]Mucinous, micropapillary, papillary, neuroendocrine, clear cell carcinoma, tubular, tubulolobular, cribriform or ductal/no special type combined with other types
Abbreviations: PR, progesterone receptor; ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, *in situ* hybridization

In total 71% (*n*=1458) of the tumours were classified as Luminal A_{PAM50} and 29% (*n*=605) as Luminal B_{PAM50}. According to St. Gallen 2013, the classifications were 39% (*n*=808) and 61% (*n*=1255) as Luminal A_{SC} and Luminal B_{SC}, respectively. The corresponding figures were 43% (*n*=894) and 57% (*n*=1169) for the Maisonneuve classification, and 49% (*n*=1004) and 51% (*n*=1059) for the Grade-based classification. This indicated that a major part of the Luminal B_{SC} tumours was classified as Luminal A_{PAM50} (Table 13).

Table 13. Luminal ditributions between intrinsic subtyping (PAM50) and surrogate subtyping

	Surrogate subtyping					
PAM50 subtype	**St. Gallen 2013** *n* (%)		**Maisonneuve** *n* (%)		**Grade-based** *n* (%)	
	$LumA_{SC}$	**$LumB_{SC}$**	**$LumA_{SC}$**	**$LumB_{SC}$**	**$LumA_{SC}$**	**$LumB_{SC}$**
LumA	742 (92)	716 (57)	823 (92)	635 (54)	922 (92)	536 (51)
LumB	66 (8)	539 (43)	71 (8)	534 (46)	82 (8)	523 (49)
Total	808	1,255	894	1,169	1,004	1,059

Abbreviations: Lum, Luminal; SC, surrogate classification

Poor agreement between luminal intrinsic and surrogate subtyping

The agreement analysis indicated that the Grade-based classification had the highest rate of agreement, although moderate. The agreement between luminal PAM50 intrinsic subtypes and the surrogate classifications were as follows: St. Gallen 2013; 62% (κ=0.30, 95% CI: 0.27–0.34), Maisonneuve: 66% (κ=0.35, 95% CI: 0.32–0.38)) and Grade-based: 70% (κ=0.41, 95% CI: 0.37–0.44).

Ki67, PR and HG as surrogate markers

The proportions of low (<14%), intermediate (14–19%) and high (≥20%) Ki67 in the cohort were 25%, 22% and 53%, respectively. By using the same proportion of tumours within the three Ki67 categories as Maisonneuve et al.[228], the cut-off values were increased (low: <16%, intermediate: 16–23% and high: ≥24%) resulting in a 75% agreement between the PAM50 and the Grade-based surrogate subtyping (κ=0.46, 95% CI: 0.43–0.50). PR was used as a discriminator in the surrogate algorithms in three groups (low Ki67 (St. Gallen 2013), intermediate Ki67 (Maisonneuve) and intermediate Ki67 and HG2 (Grade-based)). According to the auROC values, PR had no value as a surrogate marker in any of these groups (auROC between 0.51–0.56). Regarding HG, the distribution of Luminal A_{PAM50} in the HG categories was HG1: 92%, HG2: 77% and HG3: 27%, showing that well-differentiated tumours were mainly Luminal A_{PAM50} (Table 14).

Table 14. Ki67 subgroup distribution and proportion of intrinsic luminal subtypes for all tumours and for HG1, HG2 and HG3

Intrinsic subtype	Ki67 <14% *n* (%)	Ki67 14–19% *n* (%)	Ki67 ≥20% *n* (%)	Total *n* (%)
All tumours (*n*=2,063)				
LumA	500 (96)	379 (86)	579 (53)	1458 (71)
LumB	23 (4)	64 (14)	518 (47)	605 (29)
Total	523 (25)	443 (22)	1,097 (53)	2,063 (100)
HG1 tumours (*n*=458)				
LumA	211 (97)	116 (89)	96 (87)	423 (92)
LumB	7 (3)	14 (11)	14 (13)	35 (8)
Total	218 (48)	130 (28)	110 (24)	458 (100)
HG2 tumours (*n*=1,202)				
LumA	282 (95)	255 (86)	389 (64)	926 (77)
LumB	15 (5)	43 (14)	218 (36)	276 (23)
Total	297 (25)	298 (25)	607 (51)	1,202 (100)
HG3 tumours (*n*=403)				
LumA	7 (88)	8 (53)	94 (25)	109 (27)
LumB	1 (13)	7 (47)	286 (75)	294 (73)
Total	8 (2)	15 (4)	380 (94)	403 (100)

Abbreviations: HG, histological grade; Lum, Luminal

Subgroups with high proportion of Luminal A_{PAM50} tumours

By classifying tumours with HG1–2 as Luminal A_{SC} and HG3 tumours as Luminal B_{SC}, the agreement with the corresponding luminal intrinsic subtypes was 80% (κ=0.46, 95% CI: 0.41–0.50). By including only HG1 and HG3 tumours (*n*=861) and defining them as Luminal A_{SC} and Luminal B_{SC} respectively, the agreement was 83% (κ=0.66, 95% CI: 0.61–0.71). When tumours were divided into nine subgroups based on HG (1–3) and Ki67 (low (<14%), intermediate (14–19%), high (≥20%)), six of these subgroups had a high proportion (91%) of Luminal A_{PAM50} tumours (Table 15).

Table 15. Proportion of Luminal A tumours, according to intrinsic subtyping by PAM50, in subgroups generated by combining histological grade (HG1–3) and Ki67 in three categories (according to Maisonneuve et al.[228])

Ki67	HG1	HG2	HG3
Low (<14%)	97% $LumA_{PAM50}$ (*n*= 211 of 218)	95% $LumA_{PAM50}$ (*n*= 282 of 297)	88% $LumA_{PAM50}$ (*n*= 7 of 8)
Intermediate (14–19%)	89% $LumA_{PAM50}$ (*n*= 116 of 130)	86% $LumA_{PAM50}$ (*n*= 255 of 298)	53% $LumA_{PAM50}$ (*n*= 8 of 15)
High (≥20%)	87% $LumA_{PAM50}$ (*n*= 96 of 110)	64% $LumA_{PAM50}$ (*n*= 389 of 607)	25% $LumA_{PAM50}$ (*n*= 94 of 380)
Total	92% $LumA_{PAM50}$ (*n*= 423 of 458)	77% $LumA_{PAM50}$ (*n*= 926 of 1,202)	27% $LumA_{PAM50}$ (*n*= 109 of 403)

The blue-coloured squares, indicate those subgroups to consist of >80% LumA tumours, as assessed by intrinsic subtyping by PAM50.
Abbreviations: HG, histological grade; Lum, Luminal

Study III – Tumour-infiltrating lymphocytes as a prognostic and tamoxifen predictive marker in premenopausal breast cancer: data from a randomised trial with long-term follow-up

In total, 447 tissue sections with available ER status were scored for TILs. The proportions of tumours in the different categories low (<10%), intermediate (10–49%) and high (≥50%) TILs were 52%, 33% and 15%, respectively. The proportions of breast cancer subtypes (*n*=415), after exclusion if missing data for HER2 and/or PR status and ER–/PR+ tumours, were ER+/HER2–: 61%, HER2+: 16% and TNBC: 23%. The distribution of TILs according to patients and tumour characteristics is presented in Table 16. The median follow-up was 28 years.

TILs and LVI as prognostic markers in premenopausal women

In patients with high TILs, the prognosis was improved as compared with that in patients with low TILs, with a relative risk reduction of 60% regarding BCFi (HR_{BCFi}: 0.40, 95% CI: 0.22–0.71, *P*=0.002) and 48% regarding OS (HR_{OS}: 0.52, 95% CI: 0.29–0.95, *P*=0.03; Table 17 and Figure 16A). The results were essentially the same in the multivariable analysis adjusting for age, nodal status, tumour size, histological grade, ER, PR, HER2 and LVI (HR_{BCFi}: 0.22, 95% CI: 0.11–0.43, *P*<0.001 and HR_{OS}: 0.23, 95% CI: 0.11–0.48, *P*<0.001). Stratified by breast cancer subtypes, the results (high vs low TILs) were essentially the same in patients with ER+/HER2– tumours (HR_{BCFi}: 0.40, 95% CI: 0.14–1.09, *P*=0.07) in HER2+ (HR_{BCFi}: 0.28, 95% CI: 0.06–0.97, *P*=0.05) and TNBC tumours (HR_{BCFi}: 0.27, 95% CI: 0.08–0.88, *P*=0.03; Table 17 and Figure 16B–D). Similar effects were seen in multivariable analyses, except for TNBC.

Presence of LVI was found to be associated with a worse prognosis (univariable analysis) in patients in the control arm (HR_{BCFi}: 1.49, 95% CI: 1.08–2.05, *P*=0.02).

TILs as a tamoxifen-predictive marker in the ER+ subgroup

The predictive analyses (*n*=321) of TILs for tamoxifen response was analysed in the ER+ cohort. The proportions of tumours with low, intermediate and high TILs were 63%, 29% and 8% in the control group (*n*=171) and 64%, 29% and 7% in the tamoxifen group (*n*=150), respectively. As shown in Figure 17A–C, tamoxifen improved outcome for those patients with low (HR_{BCFi}: 0.66, 95% CI: 0.46–0.93, *P*=0.02) and intermediate TILs (HR_{BCFi}: 0.59, 95% CI 0.35–1.00, *P*=0.05). However, the outcome did not seem to be affected by tamoxifen in the high TIL subgroup (HR_{BCFi}: 0.89, 95% CI: 0.26–3.07, *P*=0.86).

Table 16. Distribution of TILs according to patient and tumour characteristics characteristics (*n*=477)

Variable	TIL low (<10%) *n* (%)[b]	TIL intermediate (10–49%) *n* (%)[b]	TIL high (≥50%) *n* (%)[b]	*P*-value[a]
Age (years)				
<40	41 (44)	32 (34)	21 (22)	0.02
≥40	207 (54)	125 (33)	51 (13)	
Nodal status				
0	65 (47)	42 (31)	30 (22)	0.34
1–3	137 (59)	66 (28)	30 (13)	
≥4	46 (44)	47 (45)	12 (11)	
Missing	0	2	0	
Tumour size (mm)				
≤20	97 (57)	52 (31)	20 (12)	0.06
>20	151 (49)	104 (34)	52 (17)	
Missing	0	1	0	
Histological grade (NHG)				
1	44 (86)	7 (14)	0	<0.001
2	135 (70)	52 (27)	5 (3)	
3	56 (27)	91 (43)	64 (30)	
Missing	13	7	3	
ER				
Negative	45 (29)	63 (40)	48 (31)	<0.001
Positive	203 (63)	94 (29)	24 (8)	
PR				
Negative	41 (27)	63 (41)	50 (33)	<0.001
Positive	206 (64)	93 (29)	21 (7)	
Missing	1	1	1	
HER2				
Negative	205 (56)	109 (30)	50 (14)	0.001
Positive	22 (33)	28 (42)	16 (24)	
Missing	21	20	6	
LVI				
Absent	140 (54)	76 (30)	42 (16)	0.87
Present	108 (50)	81 (37)	28 (13)	
Missing	0	0	2	
Ki67 (%)				
≤10	126 (71)	40 (23)	12 (7)	<0.001
11–25	60 (56)	33 (31)	14 (13)	
≥26	22 (20)	53 (48)	36 (32)	
Missing	40	31	10	
Histopathological type				
Ductal/NST	200 (52)	134 (35)	48 (13)	<0.001
Lobular	29 (81)	7 (19)	0	
Medullary	0	3 (13)	20 (87)	
Other	9 (75)	2 (17)	1 (8)	
Missing	10	11	3	
Subtype				
ER+/HER2−	176 (69)	65 (25)	16 (6)	<0.001
HER2+	22 (33)	28 (42)	16 (24)	
TNBC	20 (21)	42 (44)	33 (35)	
Missing	30	22	7	
Total	248 (52)	157 (33)	72 (15)	

[a]Chi2 test for trend, except for the non-ordinal variables histopathological type and subtype, when conventional Chi2-test was used

[b]Percentage of patients according to the different clinicopathologic characteristics in the three TIL categories

Abbreviations: ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; LVI, lymphovascular invasion; NST, no special type; NHG, Nottingham histological grade; PR, progesterone receptor; TILs, tumour infiltrating lymphocytes; TNBC, triple-negative breast cancer

Table 17. Cox regression analyses of BCFi and OS in patients randomised to no adjuvant systemic treatment

	Univariable		Multivariable[a]	
	BCFi	**OS**	**BCFi**	**OS**
TILs, category[b]	**HR (95% CI); *P*-value**			
All subtypes	(*n*=221)		(*n*=213)	
Low (Ref.)	1.00	1.00	1.00	1.00
Intermediate	1.10 (0.78–1.54); 0.61	1.26 (0.88–1.80); 0.21	0.61 (0.40–0.93); 0.02	0.65 (0.41–1.02); 0.06
High	0.40 (0.22–0.71); 0.002	0.52 (0.29–0.95); 0.03	0.22 (0.11–0.43); <0.001	0.23 (0.11–0.48); <0.001
ER+/HER2–	(*n*=136)		(*n*=135)	
Low (Ref.)	1.00	1.00	1.00	1.00
Intermediate	1.02 (0.63–1.64); 0.94	1.02 (0.61–1.71); 0.95	0.69 (0.42–1.15); 0.16	0.65 (0.37–1.15); 0.14
High	0.40 (0.14–1.09); 0.07	0.55 (0.20–1.52); 0.25	0.20 (0.06–0.60); 0.004	0.30 (0.10–0.96); 0.04
HER2+	(*n*=38)		(*n*=35)	
Low (Ref.)	1.00	1.00	1.00	1.00
Intermediate	1.47 (0.62–3.49); 0.39	1.07 (0.45–2.56); 0.88	0.47 (0.14–1.60); 0.23	0.38 (0.11–1.31); 0.13
High	0.28 (0.08–0.97); 0.05	0.27 (0.08–0.96); 0.04	0.06 (0.01–0.56); 0.01	0.05 (0.01–0.39); 0.005
TNBC	(*n*=47)		(*n*=43)	
Low (Ref.)	1.00	1.00	1.00	1.00
Intermediate	0.76 (0.31–1.87); 0.55	1.24 (0.49–3.14); 0.65	0.59 (0.21–1.67); 0.32	1.02 (0.34–3.11); 0.97
High	0.27 (0.08–0.88); 0.03	0.44 (0.14–1.36); 0.16	0.38 (0.11–1.39); 0.15	0.59 (0.16–2.26); 0.44

All analyses are stratified by study region.
Abbreviations: BCFi, breast cancer-free interval; CI, confidence interval; ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; TILs, tumour infiltrating lymphocytes; TNBC, triple-negative breast cancer; OS, overall survival; TILs, tumour infiltrating lymphocytes

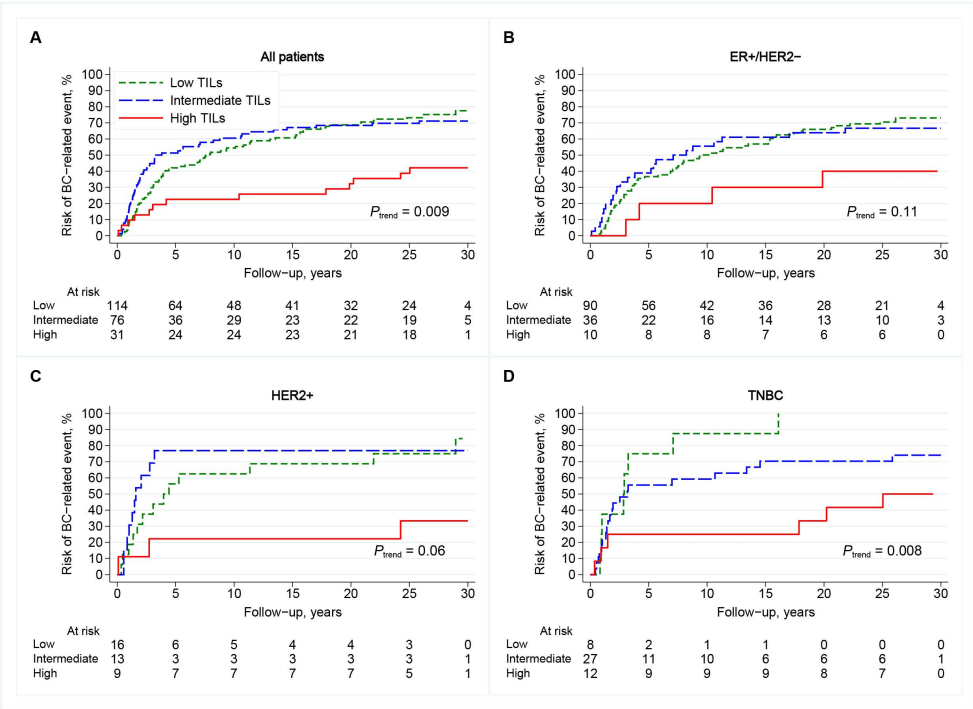

Figure 16A–D. Cumulative incidence of breast cancer-related events (in terms of BCFi) in different breast cancer subtypes.

The panel illustrates the result of different levels of TILs in (**A**) all patients, and patients with the following breast cancer subtypes: (**B**) ER+/HER2−, (**C**) HER2+, and (**D**) TNBC. The patients were allocated to no adjuvant systemic treatment and TILs were categorised as low: <10%, intermediate: 10–49% and high: ≥ 50%.

Abbreviations: BCFi, breast cancer-free interval; ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; TILs, tumour infiltrating lymphocytes; TNBC, triple-negative breast cancer

In the interaction analysis, including tamoxifen treatment and TILs as an interaction variable, no evidence of tamoxifen treatment and level of TIL infiltration on BCFi was found ($P_{interaction}$=0.65). In this analysis, the TIL variable was divided into two categories at the exploratory value of 50% (based on the predictive results above). The definition was non-LPBC as <50% vs LPBC as ≥50%. In total (n=321) 93% were categorised as non-LPBC and 7% as LPBC (Table 18).

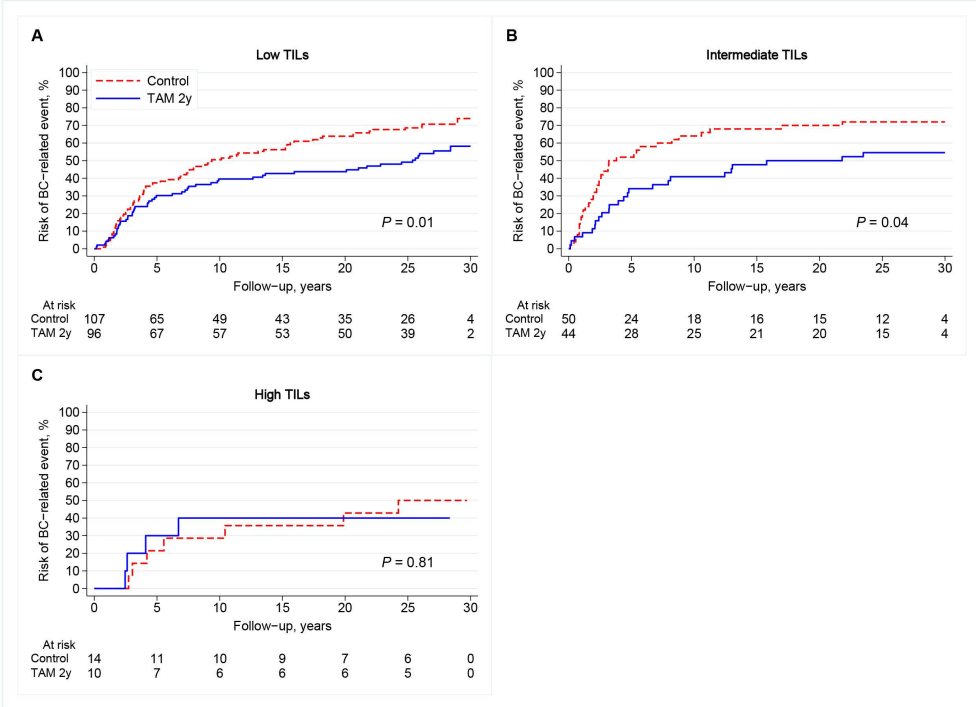

Figure 17A–C. Cumulative incidence of breast cancer-related events (in terms of BCFi) stratified by treatment allocation (control vs TAM)

The results are shown for patients whose tumours were ER-positive and had (**A**) low TILs (<10%), (**B**) intermediate TILs (10–49%), and (**C**) high TILs (≥50%).

Abbreviations: BCFi, breast cancer-free interval; ER, oestrogen receptor; TAM, tamoxifen; TILs, tumour infiltrating lymphocytes

Table 18. Predictive value of TILs for TAM response with respect to breast cancer-free interval (ER+ cohort)

	Univariable (*n*=321)		Multivariable[a] (*n*=277)	
Variable	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**
TAM vs control in TILs <50%	0.63 (0.47–0.84)	0.002	0.60 (0.43–0.83)	0.002
TAM vs control in TILs ≥50%	0.84 (0.24–2.86)	0.77	0.90 (0.22–3.64)	0.88
Interaction TILs x TAM (HR ratio)	0.75 (0.21–2.65)	0.65	0.67 (0.16–2.83)	0.59

Separate effects of tamoxifen in the two TIL groups were estimated by changing the reference group for TILs in the Cox model with main effects for treatment and TILs and an interaction effect. The HR for interaction (0.75) is the ratio between the tamoxifen effects in low and high TILs, i.e. 0.63/0.84. All analyses were stratified by study region.

[a]The following variables were included in the multivariable analysis: age (≥40 vs <40 years), nodal status (0 vs1–3 vs ≥4), tumour size (>20 mm vs ≤20), histological grade (1 vs 2 vs 3), ER (positive vs negative), PR (positive vs negative), HER2 (positive vs negative) and LVI (present vs absent)

Abbreviations: ER, oestrogen receptor; CI, confidence interval; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; TAM, tamoxifen; TILs, tumour infiltrating lymphocytes

Study IV – PAM50 subtyping and ROR score improve long-term prognostication for premenopausal patients included in the randomised SBII:2 trial

In total 437 tumours (and corresponding number of patients) had available PAM50 subtypes and ROR score (220 and 217 tumours in the tamoxifen and control arm, respectively). The proportions of Luminal A, Luminal B, HER2-E, and Basal-like intrinsic subtypes identified by PAM50 (n=437) were 44%, 19%, 17%, and 20%, respectively (Figure 18). The median ROR score was 56 and the proportions among patients with available nodal size status classified into the low, intermediate, and high ROR categories (n= 435) were 10%, 24%, and 66%, respectively. See Table 19 for patient- and tumour characteristics. The median follow-up time was 28 and 33 years in terms of BCFi and OS, respectively.

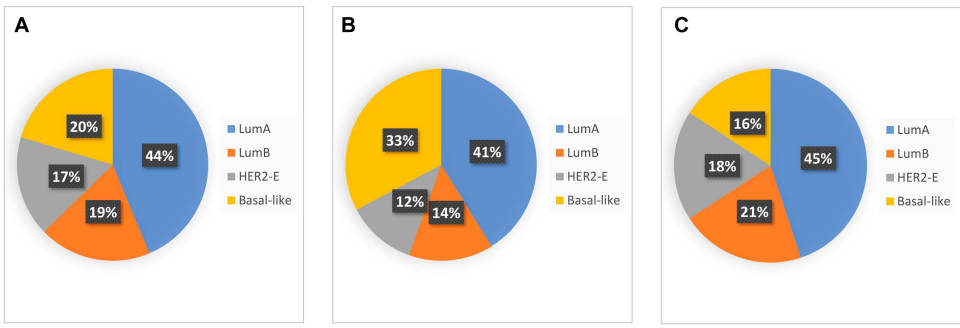

Figure 18. Distribution of PAM50 subtypes in (**A**) all (n=437), (**B**) node-negative (n=119), and (**C**) node-positive (n=316) patients

In total, n=2 missing cases with nodal status.
Abbreviations: HER2-E, human epidermal growth factor receptor 2-enriched; Lum, Luminal

Prognostic value of PAM50 subtypes

Patients with Luminal B_{PAM50} as compared to Luminal A_{PAM50} tumours, had a higher incidence of breast cancer events (0–10 years: HR_{BCFi}: 1.88, 95% CI: 1.31–2.71, P=0.001) and this was true also in the maximum long-term follow-up analysis (HR_{BCFi}: 1.60, 95% CI: 1.17–2.18, P=0.004; Figure 19A). The results also indicated increased overall mortality in patients whose tumours were Luminal B_{PAM50} as compared to Luminal A_{PAM50} (0–10 years: HR_{OS}: 2.33, 95% CI: 1.52–3.58, P<0.001; maximum follow-up: HR_{OS}: 1.41, 95% CI: 1.03–1.93, P=0.03; Figure 19B). The results were similar after adjusting for other clinicopathological variables (Table 20).

Table 19. Patient and tumour characteristics (*n*=560) in control and tamoxifen treatment arms, respectively

Characteristics	Control group *n* (%)	TAM-treated group *n* (%)
Follow-up BCFi/OS, years		
Median	28/33	28/33
Range (10th–90th percentiles)	(25–31)/(30–35)	(25–30)/(31–35)
Age (years)		
Median	45	45
Range	27–58	26–57
<40	59 (21)	51 (19)
≥40	225 (79)	225 (82)
Tumour size (mm)		
Median	22	25
Range	2–50	5–75
≤20	121 (43)	86 (31)
>20	163 (57)	189 (69)
Missing	0	1
Nodal status		
Median number of positive nodes	1	1
Range	0–22	0–21
Node-negative	75 (27)	83 (30)
Node-positive	208 (74)	192 (70)
Missing	1	1
Histological grade (NHG)		
1	32 (12)	27 (11)
2	115 (44)	105 (42)
3	116 (44)	117 (47)
Missing	21	27
ER		
Positive	191 (70)	171 (65)
Negative	84 (31)	91 (35)
Missing	9	14
PR		
Positive	185 (67)	163 (61)
Negative	92 (33)	103 (39)
Missing	7	10
HER2		
Negative	203 (84)	197 (87)
Positive	38 (16)	30 (13)
Missing	43	49
LVI		
Absent	138 (56)	124 (52)
Present	109 (44)	113 (48)
Missing	37	39
Ki67 (%)		
<14	18 (8)	25 (11)
14–19	25 (11)	27 (12)
≥20	184 (81)	167 (76)
Missing	57	57
TILs (%)		
<10	129 (52)	123 (52)
10–49	86 (35)	75 (32)
50–74	27 (11)	31 (13)
≥75	7 (3)	8 (3)
Missing	35	39

Histopathological type		
Ductal/NST	209 (84)	200 (83)
Lobular	22 (9)	21 (9)
Medullary	14 (7)	11 (5)
Other	5 (2)	10 (4)
Missing	34	34
Subtype (IHC/ISH)		
Luminal	148 (64)	132 (61)
HER2+	38 (16)	30 (14)
TNBC	46 (20)	54 (25)
Missing	52	60
PAM50 intrinsic subtype		
LumA	101 (46)	90 (42)
LumB	41 (19)	42 (19)
HER2-E	39 (18)	35 (16)
Basal-like	39 (18)	50 (23)
Missing	64	59
ROR score[a]		
Median	56	56
Range	0–94	1–94
Low	22 (10)	23 (11)
Intermediate	48 (22)	55 (26)
High	149 (68)	138 (64)
Missing	65	60
N0[b]		
Low (0–40)	19 (33)	20 (32)
Intermediate (41–60)	11 (19)	21 (34)
High (61–100)	27 (47)	21 (34)
Missing	18	21
N1 (1–3 positive nodes)[b]		
Low (0–15)	3 (3)	3 (3)
Intermediate (16–40)	37 (35)	34 (32)
High (41–100)	65 (62)	71 (66)
Missing	34	28
N1 (1–3 positive nodes)[b]		
High (0–100)	57 (100)	46 (100)
Missing	12	10
Adjuvant chemotherapy and/or goserelin		
No	275 (99)	269 (99)
Yes	4 (1)	4 (2)
Missing	5	3
Adjuvant radiotherapy		
No	37 (16)	46 (20)
Yes	199 (84)	186 (80)
Missing	48	44

[a]The ROR score categories were defined by the following cut-offs based on N-status; N0; low: 0–40, intermediate: 41–60, high: 61–100, N1; low: 0–15, intermediate: 15–40, high: 41–100,N2; high: 0–100
[b]ROR score stratified by nodal status
Abbreviations: BCFi, breast cancer free-interval; ER, oestrogen receptor; HER2–E, human epidermal growth factor receptor 2–enriched; ISH, *in situ* hybridization; Lum, Luminal; LVI, lymphovascular invasion; NHG, Nottingham histological grade; NST, no special type; PR, progesterone receptor; ROR, Risk of Recurrence; TAM, tamoxifen; TILs, tumour infiltrating lymphocytes; TNBC, triple-negative breast cancer

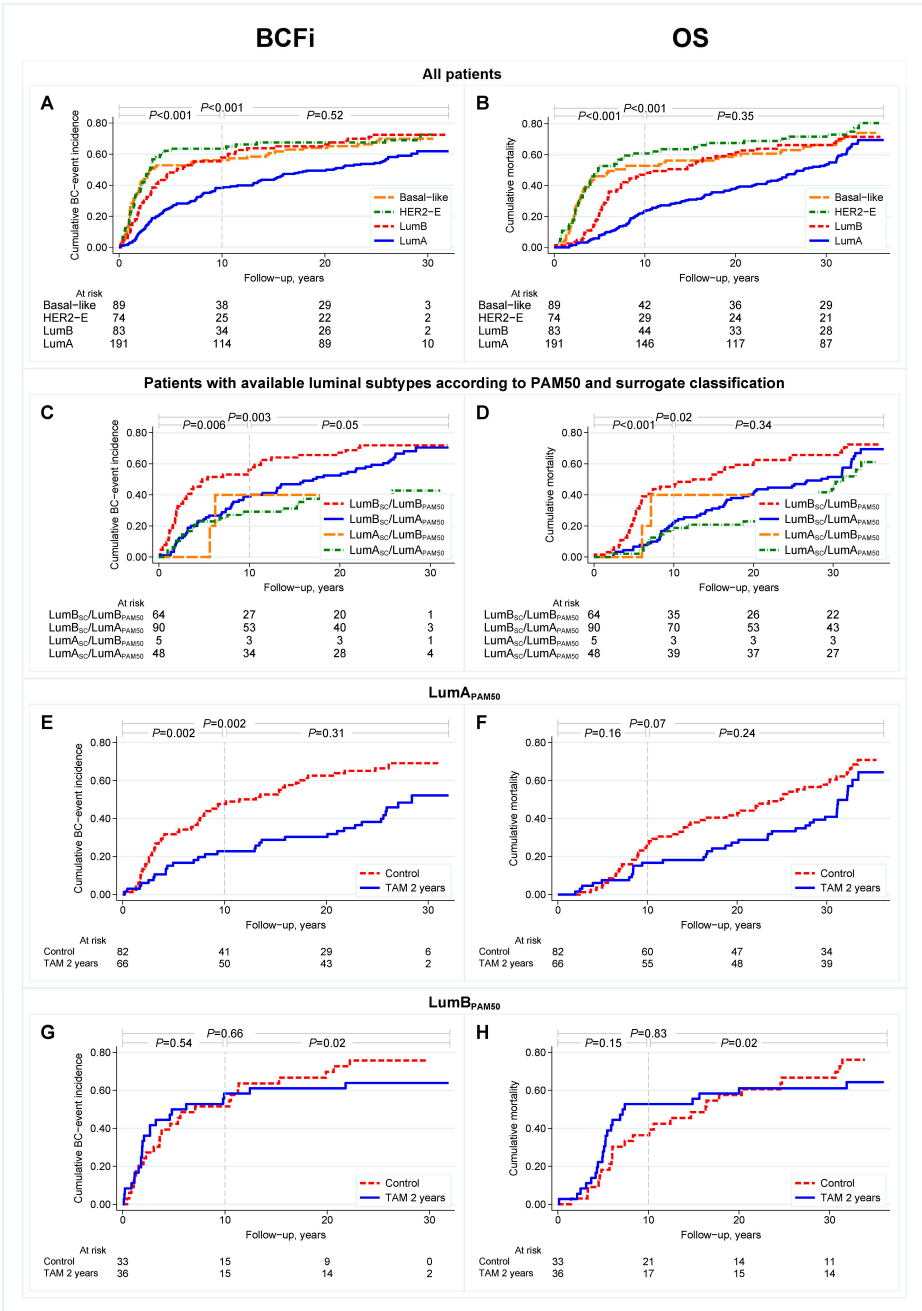

Figure 19A–H. Prognostic analyses of PAM50 subtypes, surrogate subtyping and tamoxifen-predicitve effect of luminal PAM50

Cumulative incidence in patients in terms of BCFi and OS for different time intervals (**A–B**) in all PAM50 subtypes, (**C–D**) by combining luminal PAM50 and surrogate subtyping by St. Gallen 2013, (**E–H**) of luminal PAM50 subtypes by stratification of treatment allocation (TAM vs control) in patients whose tumours were (**E–F**) LumA and (**G–H**) LumB by PAM50.
Abbreviations: BCFi, breast cancer free-interval; HER2-E, human epidermal growth factor receptor 2-enriched; Lum, Luminal; OS, overall survival; SC, surrogate classification

Agreement and prognostic effect of luminal PAM50 and St. Gallen 2013 surrogate subtypes

In total 207 patients (ER+/HER2–) were included in the agreement analyses between luminal PAM50 and St. Gallen 2013 surrogate subtypes. The proportions of Luminal A_{PAM50} and Luminal B_{PAM50} were 66% and 34%, respectively. The corresponding values for St. Gallen 2013 surrogate subtypes were 26% and 74%, respectively. The agreement between luminal PAM50 subtypes and the corresponding surrogate subtypes by St. Gallen 2013 were 54% (κ=0.22, 95% CI: 0.12–0.30). In total, 58% (90/154) of patients classified as Luminal B_{SC}, were classified as Luminal A_{PAM50}.

The outcome in patients classified into the four different combinations of St. Gallen 2013 and PAM50 subtypes (Luminal B_{SC}/Luminal B_{PAM50} (*n*=64), Luminal B_{SC}/Luminal A_{PAM50} (*n*=90), Luminal A_{SC}/Luminal B_{PAM50} (*n*=5), and Luminal A_{SC}/Luminal A_{PAM50} (*n*=48)) is illustrated in Figure 19C–D. After 10 years of follow-up, patients with tumours classified as Luminal B_{SC}/Luminal A_{PAM50}, had better prognosis as compared to those with tumours classified uniformly as Luminal B_{SC}/Luminal B_{PAM50} (HR_{BCFi}: 0.52, 95% CI: 0.33–0.83, *P*=0.006; HR_{OS}: 0.37, 95% CI: 0.21–0.66, *P*=0.001). This is illustrated in Table 20, also presenting results from the multivariable analyses. The prognostic effects became weaker with long-term (>30 years) follow-up.

Predictive value of luminal PAM50 subtyping for tamoxifen benefit

The predictive value of luminal PAM50 subtypes was analysed in the ER+/HER2– cohort (*n*=217). After 10 years of follow-up, a beneficial effect of adjuvant tamoxifen was observed in patients with Luminal A_{PAM50} tumours (HR_{BCFi}: 0.41, 95% CI: 0.23–0.74, *P*=0.003; Figure 19E). However, patients with Luminal B_{PAM50} tumours had no effect of adjuvant tamoxifen ($HR_{BCFi:}$ 1.19; 95% CI: 0.63–2.27, *P*=0.59; Figure 19G). The interaction term analysis demonstrated that tamoxifen effect that was threefold better in patients with Luminal A_{PAM50} tumours as compared with those with Luminal B_{PAM50} tumours (HR_{BCFi}: 0.34, 95% CI: 0.14–0.83, *P*=0.02). The results were almost similar regarding OS (Table 21 and Figure 19F and H). We also performed an exploratory analysis by selecting all patients with luminal PAM50 subtypes, regardless of ER and/or HER2 status (*n*=274) and this resulted in similar findings (data not shown).

Table 20. Prognostic effect of PAM50 subtypes and St. Gallen 2013 surrogate subtypes regarding BCFi and OS for different time intervals (uni- and multivariable analyses)

	Univariable		Multivariable[a]	
	BCFi	OS	BCFi	OS
	HR (95% CI); *P*-value			
PAM50 intrinsic subtype[b]	**0–10 years**			
	(*n*=437, *n*=218 events)	(*n*=437, *n*=176 events)	(*n*=411, *n*=203 events)	(*n*=411, *n*=166 events)
LumA (Ref.)[b]	1.00	1.00	1.00	1.00
LumB	1.88 (1.31–2.71); 0.001	2.33 (1.52–3.58); <0.001	1.78 (1.18–2.68); 0.006	2.08 (1.28–3.38); 0.003
HER2-E	2.55 (1.77–3.69); <0.001	4.08 (2.70–6.18); <0.001	1.99 (1.23–3.24); 0.005	2.94 (1.71–5.03); <0.001
Basal-like	2.09 (1.45–2.99); <0.001	3.36 (2.23–5.06); <0.001	1.95 (1.19–3.22); 0.008	3.00 (1.73–5.22); <0.001
	>10 years[b]			
	(*n*=210, *n*=68 events)	(*n*=261, *n*=124 events)	(*n*=199, *n*=66 events)	(*n*=245, *n*=119 events)
LumA (Ref.)	1.00	1.00	1.00	1.00
LumB	1.05 (0.55–2.00); 0.89	0.85 (0.52–1.39), 0.50	1.92 (0.92–4.01); 0.08	1.16 (0.66–2.04); 0.62
HER2-E	0.51 (0.20–1.29); 0.15	0.70 (0.38–1.29); 0.25	1.27 (0.33–4.90): 0.73	1.77 (0.76–4.14); 0.19
Basal-like	0.95 (0.50–1.82); 0.88	0.69 (0.40–1.17); 0.17	2.58 (0.94–7.08); 0.07	1.90 (0.86–4.18); 0.11
	Maximum follow-up time[c]			
	(*n*=437, *n*=286 events)	(*n*=437, *n*=300 events)	(*n*=411, *n*=269 events)	(*n*=411, *n*=285 events)
LumA (Ref.)	1.00	1.00	1.00	1.00
LumB	1.60 (1.17–2.18); 0.004	1.41 (1.03–1.93); 0.03	1.66 (1.17–2.37), 0.005	1.48 (1.04–2.10); 0.03
HER2-E	1.84 (1.32–2.56); <0.001	1.99 (1.45–2.73); <0.001	1.81 (1.16–2.81); 0.009	2.19 (1.43–3.36); <0.001
Basal-like	1.69 (1.24–2-31), 0.001	1.68 (1.24–2.28); 0.001	1.92 (1.24–2.99); 0.004	2.24 (1.45–3.45); <0.001
St. Gallen 2013/PAM50 subtype	**0–10 years**			
	(*n*=207, *n*=87 events)	(*n*=207, *n*=60 events)	(*n*=205, *n*=87 events)	(*n*=205, *n*=60 events)
$LumB_{SC}/LumB_{PAM50}$ (Ref.)	1.00	1.00	1.00	1.00
$LumB_{SC}/LumA_{PAM50}$	0.52 (0.33–0.83); 0.006	0.37 (0.21–0.66); 0.001	0.50 (0.29–0.84), 0.009	0.38 (0.20–0.74); 0.004
$LumA_{SC}/LumB_{PAM50}$	0.49 (0.12–2.05), 0.33	0.76 (0.18–3.20); 0.71	0.77 (0.18–3.32); 0.72	1.12 (0.25–5.07); 0.88
$LumA_{SC}/LumA_{PAM50}$	0.39 (0.21–0.73), 0.003	0.32 (0.15–0.68); 0.003	0.45 (0.23–0.91); 0.03	0.44 (0.19–1.02); 0.05
	>10 years[b]			
	(*n*=117, *n*=42 events)	(*n*=147, *n*=70 events)	(*n*=115, *n*=41 events)	(*n*=145, *n*=70 events)
$LumB_{SC}/LumB_{PAM50}$ (Ref.)	1.00	1.00	1.00	1.00
$LumB_{SC}/LumA_{PAM50}$	1.30 (0.62–2.69); 0.49	1.11 (0.62–1.98); 0.72	0.64 (0.26–1.60); 0.34	0.59 (0.30–1.15); 0.12
$LumA_{SC}/LumB_{PAM50}$	-	-	-	-
$LumA_{SC}/LumA_{PAM50}$	0.45 (0.16–1.23); 0.12	0.81 (0.41–1.59); 0.54	0.26 (0.08–0.80); 0.02	0.39 (0.18–0.84); 0.02

	Maximum follow-up time[c]			
	(*n*=207, *n*=129 events)	(*n*=207, *n*=130 events)	(*n*=205, *n*=128 events)	(*n*=205, *n*=130 events)
$LumB_{SC}/LumB_{PAM50}$ (Ref.)	1.00	1.00	1.00	1.00
$LumB_{SC}/LumA_{PAM50}$	0.70 (0.47–1.02); 0.06	0.65 (0.44–0.96); 0.03	0.58 (0.37–0.90); 0.02	0.50 (0.32–0.79); 0.003
$LumA_{SC}/LumB_{PAM50}$	0.34 (0.08–1.41); 0.14	0.37 (0.09–1.52); 0.17	0.53 (0.12–2.24); 0.38	0.48 (0.11–2.05): 0.32
$LumA_{SC}/LumA_{PAM50}$	0.39 (0.23–0.67); 0.001	0.51 (0.32–0.83); 0.007	0.39 (0.24–0.70); 0.002	0.43 (0.25–0.74); 0.003

[a]All analyses were stratified by study region and adjusted for age (continuous), tumour size (>20 mm vs ≤20), NHG (1 vs 2 vs 3), nodal status (N0 vs N1 vs N2) and treatment arm in addition to surrogate/molecular subtype

[b]10 years up to maximum follow-up time, starting at year 10

[c]32 and 36 years regarding BCFi and OS, respectively

Abbreviations: BCFi, breast cancer free-interval; CI, confidence interval; HER2-E, human epidermal growth factor receptor 2-enriched; HR, hazard ratio; Lum, Luminal; NHG, Nottingham histological grade; OS, overall survival; SC, surrogate classification; TAM, tamoxifen

Table 21. Predictive value (univariable analyses) of luminal PAM50 subtype for TAM response with respect to BCFi and OS (ER-positive/HER2-negative cohort)

	BCFi	OS
	HR (95% CI); P value	
	0–10 years	
	(*n*=217, *n*=92 events)	(*n*=217, *n*=64 events)
TAM vs control in $LumA_{PAM50}$	0.41 (0.23–0.74); 0.003	0.61 (0.30–1.26); 0.18
TAM vs control in $LumB_{PAM50}$	1.19 (0.63–2.27); 0.59	1.76 (0.85–3.63); 0.13
Interaction luminal PAM50 subtype x TAM (HR ratio)	0.34 (0.14–0.83); 0.02	0.35 (0.13–0.97); 0.04
	>10 years[a]	
	(*n*=121, *n*=43 events)	(*n*=153, *n*=74 events)
TAM vs control in $LumA_{PAM50}$	0.69 (0.35–1.37); 0.29	0.74 (0.44–1.25); 0.26
TAM vs control in $LumB_{PAM50}$	0.17 (0.04–0.80); 0.03	0.25 (0.08–0.77); 0.02
Interaction luminal PAM50 subtype x TAM (HR ratio)	4.05 (0.74–22.1); 0.11	2.95 (0.85–10.2); 0.09
	Maximum follow-up time[b]	
	(*n*=217, *n*=135 events)	(*n*=217, *n*=138 events)
TAM vs control in $LumA_{PAM50}$	0.52 (0.34–0.81); 0.004	0.71 (0.46–1.08); 0.11
TAM vs control in $LumB_{PAM50}$	0.80 (0.45–1.41); 0.44	0.87 (0.49–1.54); 0.63
Interaction luminal PAM50 subtype x TAM (HR ratio)	0.65 (0.32–1.34); 0.24	0.82 (0.40–1.65); 0.57

All analyses were stratified by study region

[a]10 years up to maximum follow-up time, starting at year 10

[b]32 and 36 years regarding BCFi and OS, respectively

Abbreviations: BCFi, breast cancer-free interval; CI, confidence interval; ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; Lum, Luminal; OS, overall survival; TAM, tamoxifen

Prognostic value of ROR score in the ER+/HER2- subgroup

Among all patients (ER+/HER2– subgroup, *n*=236), the distributions of low, intermediate, and high ROR score categories were 13%, 27%, and 60%, respectively. For all patients, high vs. low ROR score was associated with worse outcome (0–10 years: HR_{BCFi}: 2.37, 95% CI: 1.16–4.72, *P*=0.02) and the effect in the maximum long-term follow-up analysis was less pronounced (HR_{BCFi}: 1.70, 95% CI: 1.01–2.85, *P*=0.04; Figure 20A). The corresponding results regarding OS are presented in Table 22 and Figure 20B.

Stratified by nodal status, the distributions of ROR score categories were N0 (*n*=60): 48%, 22% and 30%; N+ (1–3 positive nodes, *n*=123): 2%, 42% and 57%, respectively. The outcomes are illustrated in Figure 20C–F. The prognostic results were essentially the same for node-negative patients (0–10 years: HR_{BCFi}: 2.53, 95% CI :1.04–6.12, *P*=0.04; maximum long-term follow-up: HR_{BCFi}: 1.69, 95% CI: 0.79–3.58, *P*=0.17). For node-positive (1–3 positive nodes) patients, the low ROR category was omitted, due to small sample size (*n*=2) and the results indicated that high vs intermediate ROR score was associated with worse outcome (0–10 years: HR_{BCFi}: 1.99, 95% CI: 1.08–3.66, *P*=0.03).

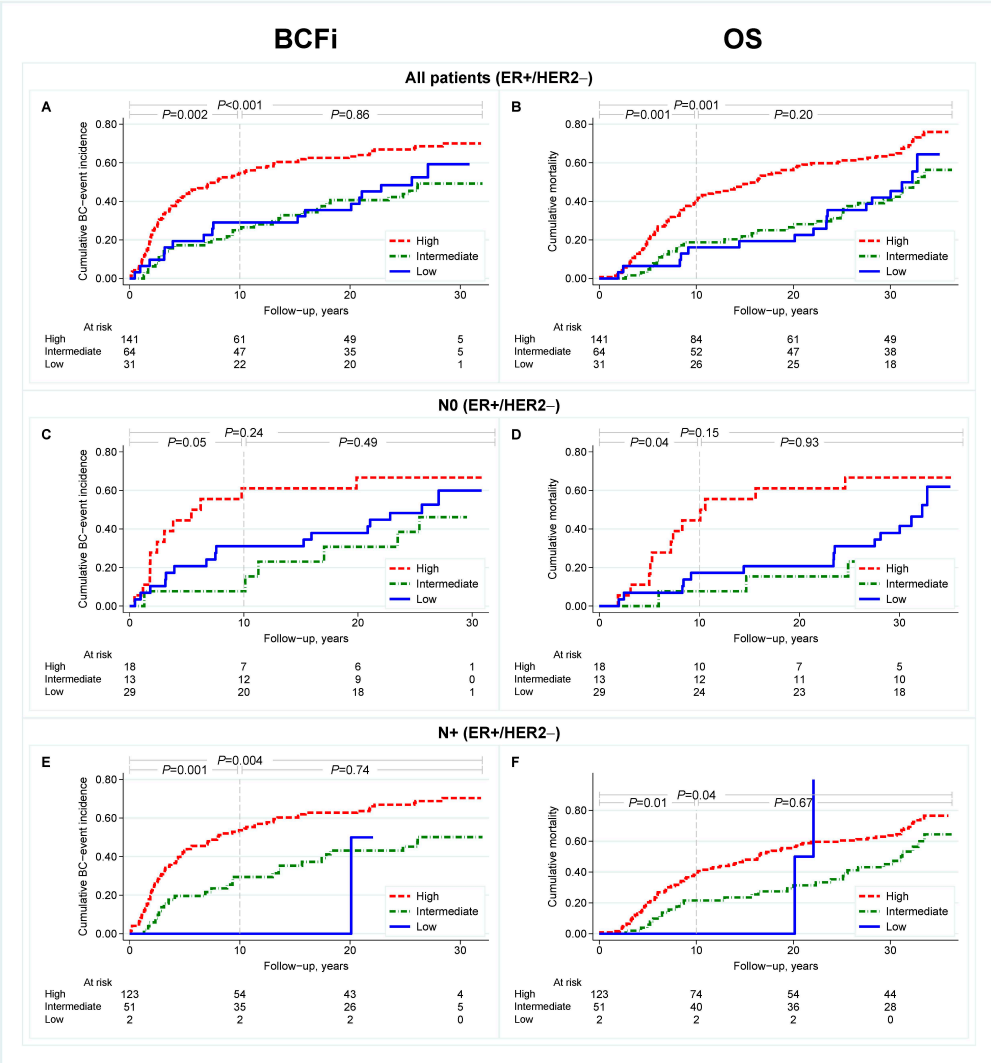

Figure 20A–F. Prognostic analsyes of ROR score in different nodal cateories

Cumulative incidence with respect to BCFi and OS for different time intervals in (**A–B**) all ER-positive/HER2-negative (ER+/HER2−) patients, patients whose tumours were ER+/ HER2− and were (**C–D**) node-negative and (**E–F**) node-positive.
Abbreviations: BCFi, breast cancer free-interval; ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; OS, overall survival; ROR, Risk of Recurrence; TAM, tamoxifen

Table 22. Prognostic effect of ROR score regarding BCFi and OS in different time intervals in all, node-negative, and node-positive (1–3 positive nodes) subgroups of patients with ER-positive/HER2-negative tumours (univariable analyses)

	All patients		Node-negative		Node-positive[a]	
	BCFi	**OS**	**BCFi**	**OS**	**BCFi**	**OS**
	HR (95% CI); *P*-value					
	0-10 years					
ROR score	(*n*=236, *n*=102 events)	(*n*=236, *n*=74 events)	(*n*=60, *n*=21 events)	(*n*=60, *n*=14 events)	(*n*=121, *n*=49 events)	(*n*=121, *n*=36 events)
Low (Ref.)	1.00	1.00	1.00	1.00	-	-
Intermediate	0.83 (0.37–1.88); 0.66	1.19 (0.42–3.37); 0.75	0.20 (0.03–1.59); 0.13	0.40 (0.05–3.43); 0.40	1.00	1.00
High	2.37 (1.18–4.72), 0.02	2.99 (1.20–7.46); 0.02	2.53 (1.04–6.12); 0.04	3.17 (1.03–9.75); 0.04	1.99 (1.08–3.66); 0.03	1.84 (0.91–3.74); 0.09
	>10 years[b]					
ROR score	(*n*=130, *n*=43 events)	(*n*=162, *n*=77 events)	(*n*=39, *n*=13 events)	(*n*=46, *n*=16 events)	(*n*=69, *n*=21 events)	(*n*=84, *n*=38 events)
Low (Ref.)	1.00	1.00	1.00	1.00	-	-
Intermediate	0.85 (0.36–2.01); 0.71	0.76 (0.37–1.56); 0.45	1.22 (0.38–3.92); 0.74	0.38 (0.08–1.75); 0.21	1.00	1.00
High	0.88 (0.39–2.01); 0.77	1.24 (0.65–2.35); 0.51	0.37 (0.05–3.03); 0.36	1.18 (0.37–3.79); 0.79	1.19 (0.50–2.80); 0.70	1.02 (0.54–1.93); 0.96
	Maximum follow-up[c]					
ROR score	(*n*=236, *n*=145 events)	(*n*=236, *n*=151 events)	(*n*=60, *n*=34 events)	(*n*=60, *n*=30 events)	(*n*=121, *n*=70 events)	(*n*=121, *n*=74 events)
Low (Ref.)	1.00	1.00	1.00	1.00	-	-
Intermediate	0.84 (0.47–1.53); 0.58	0.89 (0.49–1.61); 0.70	0.66 (0.26–1.69); 0.39	0.39 (0.11–1.35); 0.14	1.00	1.00
High	1.70 (1.01–2.85); 0.04	1.77 (1.06–2.97); 0.03	1.69 (0.79–3.58); 0.17	1.96 (0.91–4.22); 0.09	1.68 (1.03–2.75); 0.04	1.34 (0.84–2.14); 0.22

All analyses are stratified by study region. The ROR score categories are defined by the following cut-offs based on *N*-status; N0; low: 0–40, intermediate: 41–60, high: 61–100, N1; low: 0–15, intermediate: 16–40, high: 41–100, N2: high: 0–100.

[a]Only N1 (1–3 positive nodes) are included in the node-positive definition. Since only *n*=2 patients are defined as ROR low in the N1 category, these are omitted from the analyses

[b]10 years up to maximum follow-up time, starting at year 10

[c]32 and 36 years regarding BCFi and OS, respectively

Abbreviations: BCFi, breast cancer free-interval; CI, confidence interval; ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; OS, overall survival; ROR, Risk of Recurrence

Strengths and limitations

Study	Strengths	Limitations
I	• Population-based • Data from the Swedish Prescribed Drug Register • Review of medical records → many patients excluded due to recurrence/death were still on drugs at time for event	• Small sample size • Assumption that prescribed pills were actually taken by the patient • Definition of MPR → source for overestimation • Patients excluded due to recurrence or death without breast cancer
II	• Population-based, multicentre study • Large cohort of more than 2,000 patients • All age groups included • Based on the PAM50 algorithm (Parker et al.[232])	• Intrinsic subtyping in SCAN-B not clinically validated • SCAN-B has limited data on small, lower grade tumours • No prognostic effect of results because of short follow-up • Ki67 and HG have a limited reproducibility
III	• Randomised controlled study including a control (no systemic treatment) arm • Pure premenopausal cohort • Long-term follow-up based on journal reviews and register data • Tamoxifen is still a recommended adjuvant endocrine drugs for premenopausal women with ER+/HER2− disease and therefore, the results could be applicated to contemporary patients • TILs assessment in line with international consensus • Assessments of TILs based on whole tissue sections • Prognostic data on LVI	• Old preserved tumours → poorer quality of the tissues • Mixture of microinvasion and *in situ* in some tissue sections • Small sample sizes of subgroup analyses • TILs not assessed as a continuously parameter • Data-driven cut-off in the predictive interaction analysis • No distinction between Luminal A/B tumours
IV	• Randomised controlled study including a control (no systemic treatment) arm • Pure premenopausal cohort • Long-term follow-up based on journal reviews and register data • Tamoxifen as a currently recommended adjuvant drug (see Study III above) • Prolonged follow-up regarding overall survival • Reassessments of Ki67 and PR based on whole tissue sections • Validated gene expression assay	• Ki67 assessment of old preserved tissue blocks is associated with uncertainty and Ki67 is sentistive to antigen decay with long storage in paraffin • Small sample sizes of subgroup analysis→low statistical power, especially for ROR score in different nodal categories • PAM50/ROR score validated for 5 years of endocrine therapy as compared to 2 years in the SBII:2pre trial

Discussion

Discussions of the study results of the different studies included in the thesis is presented below. The strengths and limitations are presented previously.

Study I

The effect of adjuvant endocrine therapy in primary breast cancer is well established and is the backbone treatment for patient diagnosed with ER+ tumours[34]. Even though 5 years of adjuvant therapy is the standard of care, only 2 years of therapy has shown to provide a long-term carry-over effect[331]. Decreased adherence to endocrine drugs is known to be associated with increased risk of death[159, 161]. Importantly, risk estimation for 10 years' distant recurrence by gene expression assays assume that the patient is treated for 5 years with adjuvant endocrine therapy[178, 251, 262]. Taken together, to encourage and ensure a patient's adherence to adjuvant endocrine therapy is important. Many previous adherence studies have so far shown disappointing results in the adjuvant setting and differences in methods used for adherence definitions and calculations, which makes the comparison among study results difficult. The results of study I showed very good adherence among a Swedish population diagnosed with early breast cancer; over 90% after 3 and 5 years.

The reasons for the good adherence shown in study I were not further investigated, but the lower limit of the CIs is higher than those in many other studies. One could speculate whether the follow-up routines and health care support were the major contributing factors. However, even though there were some differences in follow-up routines among the three hospitals, no difference in adherence was observed between them. This could be associated with the small number of patients included, which is a limitation in this study. No specific factors were predictive to adherence in study I. According to previous data, patients seeing a physician seemed to have higher adherence[146] and adherence might also be higher in patients who had an improved ability to discuss treatment options[152]. Socioeconomic and welfare factors can also contribute to adherence rates. Since all residents in Sweden receive pharmaceutical benefits meaning reduced out-of-pocket costs for prescribed medication, this may affect the level of adherence seen in our study as compared to those in other international

studies. A major reason for ending endocrine therapy is side-effects, and since the quality of life must be taken into consideration in the discussion with the patient, 100% adherence may not be achievable. For some patients, stopping the treatment before 5 years could be reasonable and encouraged[331]. It is necessary that the organisation monitors the adherence at their practices and should have a structure for patient support and adverse events management for those treated with endocrine drugs. To provide the patient with relevant and supporting information, the understanding, the need, its purpose and side effects, management of the side effects and supportive health care, are all important factors which ideally should be included in the surveillance of breast cancer patients.

Study II

Although the intrinsic breast cancer subtypes were defined based on gene expression in year 2000, surrogate subtyping based on routine IHC/ISH markers has been used routinely in the clinic to categorise the ER+/HER2– tumours into the Luminal A_{SC} and Luminal B_{SC} subtypes. It is important to note that surrogate IHC markers is an assessment at the protein level. The gene expression analyses in intrinsic subtyping, is however based on the measurement of mRNA transcript levels of genes depicting the underlying biological pathways. Furthermore, RNA is undergoing modifications and the reason for the outperformance of genomic signatures as compared to clinicopathological factors reported in the literature, might be due to the fact that genomic signatures also incorporate other biological processes, not provided in the finally expressed protein.

Study II was a population-based study, including over 2,000 tumours. The agreement between luminal intrinsic subtyping as assessed by PAM50 and different algorithms for surrogate subtyping was poor. There were considerably more Luminal A_{PAM50} tumours than those defined by the surrogate classifications. A major finding was that HG, as an initial stratifying tool in the surrogate algorithm, seemed to contribute to a more precise agreement. When defining HG1-2 as Luminal A_{SC} and HG3 as Luminal B_{SC}, the agreement with PAM50 was higher than those in any of the other surrogate classifications in this study. Interestingly, PR was found not to add any value in the surrogate classifications. This analysis was however only restricted to agreement and not to any prognostic outcome. Similar results have previously been presented, and the authors concluded that patients with ER+/HER2–/HG1 are more Luminal A_{SC} and ER+/HER2–/HG3 are more Luminal B_{SC}, independent of PR and Ki67[71].

Selection of IHC markers to be used in the surrogate classifications and their specific cut-offs for luminal division, have changed since the algorithm by St. Gallen 2011 was presented[225]. In 2017 the use of HG was incorporated and moreover, gene expression

signatures were recommended for patients determined to be in the "in between" category of uncertain prognosis[226]. According to the results of the exploratory analysis, approximately 50% of the tumours, by combining Ki67 and HG, would be considered to include most of Luminal A_{PAM50} tumours, and that genomic assays can be questioned to apply in this subgroup. Nevertheless, these nine groups constructed partly by Ki67 (and HG), were a result of strict cut-off values. The specific cut-off values for biomarkers are often based on statistical considerations and expert opinions. In study II, we changed the cut-off values for Ki67 in an exploratory analysis and adjusted into the same percentiles as in Maisonneuve et al. study[228]. This resulted in a higher agreement between surrogate subtyping and PAM50 intrinsic subtyping. The issue regarding cut-off values of Ki67 is often debated and pathological assessment of Ki67 percentage and the specific cut-offs are associated with uncertainties and lack of reproducibility[61, 332, 333]. The proposed upcoming national Swedish guidelines regarding breast cancer treatment, has changed the cut-offs for Ki67, with the intermediate range defined as in-between 5–30% (no reference). Even though Ki67 has a clinical validity for prognostication, only very low (≤5%) and very high (≥30%) values can be regarded as reliable as stated by the IKWG[61]. The reproducibility is also questioned for HG determination[334]. The HG2 subgroup represents an intermediate risk group, not informative to the clinicians and comprise a majority of the tumours. In the Grade-based classification proposed in study II, only HG2 tumours were further classified by PR, and Ki67, however, the agreement was still not perfect.

Nonetheless, standard clinicopathological criteria are although still important for prognostication[335]. The fact that a large proportions of Luminal B_{SC} tumours are Luminal A by intrinsic subtyping, is associated with a risk of wrong risk assessment. This is of clinical importance, since the risk for breast cancer events in patients with Luminal B_{SC} tumours might be overestimated, possibly affecting treatment decisions for those with ER+/HER2– tumours. In the future, surrogate subtyping might be replaced by gene expression profiling in a large proportion of breast cancer patients. Gene expression assays are likely clinically attractive, but there is a need for more prospective studies and evidence to determine the optimal and most cost-effective use of the assays[274, 336]. The application of genomic profiling in all patients does not seem to be necessary, only in patients with an ambiguous risk. The definition of this group is a future issue.

Study III

The prognostic and treatment-predictive value of TILs in ER+/HER2– tumours is not clearly defined. In study III it was shown that high infiltration of TILs was associated with a relative reduction of the incidence of invasive breast cancer-related events by

60% after approximately 30 years of follow-up. The results were similar in all breast cancer subtypes, including the ER+/HER2– tumours. The multivariable analyses also emphasized high TILs as an important independent long-term favourable prognostic factor.

Previous studies on the prognostic effect of TILs in the ER+/HER2– breast cancer tumours as well as neoadjuvant studies did not provide consistent results and were based on mixed adjuvant chemo-endocrine treatment[209, 210, 316]. In the meta analysis of six neoadjuvant chemotherapy studies, patients with ER+/HER2– tumours and low level of TILs had an improved OS after 10 years[209]. Based on other adjuvant studies on TILs and their association with prognosis, our results have yet not been confirmed yet[210, 316, 317]. A possible predictive value of TILs on tamoxifen benefit (BCFi) was seen in patients with ER+ tumours and non-LPBC tumours (TILs <50%), while no effect was shown in patients with LPBC tumours (TILs ≥50%). No significant interaction was however seen, probably due to a low number of included patients. No previous study including premenopausal patients only and randomised to adjuvant tamoxifen vs not, has evaluated the treatment benefit in association with TILs. When no long-term outcome data is available, a neoadjuvant study design makes it possible to assess treatment effect, such as change in Ki67 expression or pCR-rate. Based on a neoadjuvant endocrine therapy study, a higher Ki67 suppression in tumours with high (≥10%) TILs was reported[319]. The underlying mechanisms related to the lack of improved outcome by tamoxifen treatment in patients with ER+/LPBC tumours was not further analysed in study III. There might be a complex interaction between tamoxifen, immune environment and signalling pathways. Based on a previous study, antioestrogens might induce a suppression of the immune system through a TGF-β-dependent mechanism[337]. According to *in vitro* and *in vivo* studies, tamoxifen is thought to modulate the immune system via the P-glycoprotein and induces a shift from cellular to humoral immunity that would rather limit its antitumoural effect[338]. One could also hypothesize that the tumour mutational burden (TMB) explains a possible endocrine resistance noted in the high TILs subgroup, since increased immune infiltration is related to a higher TMB. Tumours with high TMB and favourable immune infiltrates has been proven to be associated with a prolonged survival[339]. Because the Luminal B tumours are associated with higher expression of genes associated with proliferation, immune response and gene mutations as compared to Luminal A subtypes[41, 165, 233], this suggests that there might be a difference in the immunogenicity also between the luminal tumours. No stratification by luminal subtypes was performed, but a distinction between Luminal A and Luminal B tumours and the association to TILs would be interesting to evaluate in future studies.

The diverse results of prognostic and predictive effects of TILs in ER+/HER2– breast cancer studies, might be influenced by factors such as selection of study participants, shorter follow-up and the low proportion of LPBC in these tumours. In study III,

whole tissue sections were used, but previous studies have also assessed TILs on TMA[208]. A more refined way of defining TILs is at the subset level, dividing them into for example CD8+ and T regulatory cells. Moreover, even if TILs should be defined as a continuous variable, the application of TILs as a categorised variable is in line with other studies[209]. As a supplement to the previous IHC markers in this study, assessment of LVI was performed and it was also shown to be a prognostic factor. This finding is in line with previous results in both node- negative and positive patients[78, 79]. LVI should be highlighted in risk assessment in premenopausal women in combination with other prognostic factors.

Study IV

The results of study IV demonstrated that PAM50 subtypes and ROR score could provide long-term (>30 years) prognostic information in premenopausal patients and indicated a possible tamoxifen-predictive effect by luminal intrinsic subtyping after 10 years. The prognostic effect of Prosigna® in premenopausal women have been demonstrated in previous studies, but none with >30 years' follow-up data[197, 279]. According to a review of published articles and abstracts that evaluated the use of signatures in young women, genomic testing resulted in a higher proportion of intermediate- to high-risk categorisation (as classified by EndoPredict, Mammaprint and OncotypeDx)[340]. Young women with low risk had a 6-year distant recurrence-free survival of 94% and a 5-year OS of nearly 100%. However, they seemed to receive more chemotherapy than the older women. The possible predictive value of chemotherapy based on PAM50/ROR score in premenopausal patients could not be analysed in our study. Results from gene expression signatures used in randomised, controlled trials seem to indicate that the predictive effect is depending on age and menopausal status. Data from the TAILORx trial indicated a beneficial effect of adjuvant chemotherapy in patients ≤50 years and a recurrence score of 16–25[194]. Importantly, in the follow-up analysis of TAILORx, the addition of clinical risk stratification (based on tumour size and HG) to intermediate (for those treated with endocrine only or chemo-endocrine therapy) and high (for those treated with chemo-endocrine therapy) RS by OncotypeDX, provided prognostic information regarding distant recurrence in premenopausal women[265]. The results from the RxPONDER demonstrated that node-positive premenopausal women with a RS≤25 did have an absolute benefit of 5.2% from additional chemotherapy at 5-years follow-up[196]. An updated exploratory analysis of the MINDACT trial, also demonstrated a possible age-dependent (≤50 years) benefit of chemotherapy in those with low genomic/high clinical risk[256].

The Luminal B tumours are known to have a higher expression of genes associated with proliferation[41]. Therefore, our result, indicating that patients with Luminal B_{PAM50} tumours had worse prognosis than those with Luminal A_{PAM50} tumours, was expected. The finding of a possible tamoxifen-predictive effect of luminal PAM50 was however a novel result. Among patients with ER+/HER2– tumours, only those with Luminal A_{PAM50} tumours had a beneficial effect of tamoxifen after 10 years of follow-up. This predictive effect remained after selecting all luminal PAM50 patients, regardless of ER/HER2 status by IHC/ISH. This indicated that PAM50 could be used up-front in clinical practice for defining who would be recommended endocrine therapy. However, these results should be further verified in larger cohorts. Our results demonstrated however, that the luminal PAM50 subtypes seemed to be a predictive marker at the gene expression level in addition to ER status.

Previous data has confirmed the poor concordance between intrinsic and surrogate subtyping[285, 341]. Our results of study IV verified the results regarding agreement as reported in study II. Over 50% of the Luminal B_{SC} tumours were re-classified as Luminal A_{PAM50}. In Study IV it was, as compared to study II, also possible to evaluate the prognostic effect of this disagreement and we showed that these patients had an improved prognosis than those classified as Luminal B_{SC}/Luminal B_{PAM50}. Viale et al. demonstrated similar findings, however, they used BluePrint and MammaPrint for subtyping[341].

In the validation studies of Prosigna®, postmenopausal women were treated with 5 years of endocrine therapy and demonstrated that the ROR score was significantly related to probability of distant recurrence after 10 years of follow-up[178, 185, 270]. This was also true for the time interval of 5–10 years after 5 years of endocrine therapy treatment[278]. In the prognostic evaluation of ROR score, we selected the ER+/HER2– subgroup and we showed better long-term prognosis in patients with low vs high ROR score. The effects were similar in node-negative patients, however, in the node-positive cohort, the number of patients with low ROR score was too low (n=2) to be meaningful for inclusion in the statistical analysis. Therefore, we only analysed intermediate vs. high ROR score in this subgroup. The premenopausal patients in the SBII:2pre cohort had in general more aggressive tumour characteristics and about 70% of them were node-positive; median ROR score was as high as 56. The cumulative-incidence curves clearly showed that the outcomes in the respective ROR categories seemed to be worse as compared to the validation studies. However, a separation of prognostic effect for these two ROR categories was demonstrated. The study was under-powered to define the prognostic value of ROR score by nodal status.

Conclusions

The specific conclusion of the studies included in this thesis are listed below.

- Adherence to adjuvant endocrine treatment was satisfactory high in a subset of Swedish breast cancer patients.
- No specific factors (age, endocrine therapy, radiotherapy/chemotherapy or hospital) associated with non-adherence could be found.
- Good adherence to endocrine therapy could be affected by many factors such as organisation, patient support during treatment, thorough patient information, however, these hypotheses were not confirmed.
- Agreement, regarding the division of ER+/HER2– tumours into Luminal A and B subtypes, between intrinsic subtyping (according to the PAM50 algorithm) and different surrogate subtyping classifications was in general poor (62–70%).
- Among three different surrogate subtyping classifications, the Grade-based, in which HG was the major factor, had the highest agreement to intrinsic subtyping, implicating that HG had an important role in dividing ER+/HER2– tumours into Luminal A_{SC} or Luminal B_{SC} subtypes.
- By combing categories of HG and Ki67 (1, 2, 3 and low, intermediate, high, respectively) nine subgroups were generated and among these, six groups (51% of the cohort) were identified having >90% Luminal A_{PAM50} tumours. These patients may not benefit from gene expression assays, especially if other clinicopathological factors indicate a low risk of recurrence.
- A substantial proportion (>50%) of Luminal B tumours according to St. Gallen 2013 surrogate subtyping, was re-classified as Luminal A_{PAM50} with evidence of improved prognosis in these patients as compared to those whose tumours were classified uniformly as Luminal B.
- High infiltration of TILs was associated with better outcomes regarding breast cancer events in premenopausal women who received no adjuvant therapy. This effect was similar regardless of the breast cancer IHC subtype and after almost 30 years of follow-up. The positive prognostic value of high TIL

infiltration was retained in multivariable analysis, emphasizing TILs as an important independent long-term favourable prognostic factor.

- Adjuvant tamoxifen was associated with improved prognosis in patients with ER+/non-LPBC (TILs<50%), tumours, indicating its possible predictive value.
- PAM50 subtypes were of prognostic importance in premenopausal patients; those with Luminal B_{PAM50} vs. Luminal A_{PAM50} tumours had worse long-term prognosis.
- After 10 years of follow-up, the incidence of breast cancer events was reduced by two thirds by tamoxifen in patients with Luminal A_{PAM50} tumours, while no effect was seen in those with Luminal B_{PAM50}.
- ROR score was prognostic in premenopausal women with ER+/HER2–; high vs low ROR score was associated with worse long-term prognosis, with a similar trend in the node-negative subgroup. In node-positive patients, high vs. intermediate ROR score was associated with worse outcome. Due to low power (small cohort), the effect of the ROR score in different node categories needs further investigation.

Future perspectives

Despite the steady evolution of clinical tools and improved knowledge in oncology, a single blood sample that can predict the optimal treatment for a breast cancer patient is still sought after. Yet, is it possible to achieve this goal in reality? The search for breast tumour markers that assist in the clinic for prognostication and treatment prediction will continue in the future.

A treatment decision for the individual patient will not be based upon one tool/test or marker. This thesis emphasizes the complexity of risk profiling and treatment decision in early breast cancer. The clinical assessment of the patient´s characteristics, hereditary background, specific tumour characteristics including IHC markers, gene expression signature and tumour mutations in the tumour tissue, are to be combined to tailor the most appropriate treatment for the individual patient. The challenge will be to interpret the different tests and analyses, combine and weigh them and finally to communicate them to the patient. How to combine all this information is a future challenge. The patient´s preferences must also be considered and will play a larger role in the future.

Optimising adherence to assure study treatment effectiveness

Non-adherence to endocrine therapy is a clinical issue in early breast cancer. There are many studies confirming the effectiveness of these drugs and lack of adherence to adjuvant therapy is associated with worse prognosis in these patients[161]. In the future, specific side effect receptions for support could perhaps help increase adherence and improve patient education[342]. Moreover, consultations with on-site pharmacists could also facilitate adherence[343]. Since new technology is becoming an integrated part of health care, user-friendly supporting applications for patients might also play an important role in the future[344].

Health care providers treating patients with adjuvant endocrine treatment do have different structures. It is encouraging to perform future adherence studies to more carefully study subgroups and identify predictors of adherence in a specific organisation, rather than nationwide register studies. By increasing this knowledge at

the specific treatment site, regional health care providers may more precisely direct the right resources and develop the most appropriate structure for breast cancer treatment organisation and follow-up.

Genomic risk profiling – what about change of therapy, premenopausal and node-positive patients?

It is necessary to perform studies that aim to observe to what extent a genomic test changes the actual decision-making on the choice of adjuvant therapy in clinical routine, and what effect the change of therapy has on outcome. The estimate of 'how many of the changes in therapy are correct and how many are wrong' has to be settled in terms of beneficial effect of the chemotherapy withdrawal. As stated by Mushlin: 'In order to decide whether 'to genetic test or not to test' we need to pursue a robust agenda on their comparative effectiveness. This must include their clinical accuracy compared to current or alternative ways of providing information to guide clinical decision-making'[345]. A broad range of results regarding the impact of multigene assays on decision impact has been reported. The net change in the percentage of patients with a chemotherapy recommendation before and after the test ranged from an increase by 1% to a decrease by 23% among United Kingdom studies and a decrease by 0–64% across European studies[274].

A future perspective of multigene assays will certainly focus on patients with ER+, node-positive breast cancer. Node-positive patients have an increased long-term risk of recurrence[184]. However, this patient group is not homogenous[346]. Gene expression analyses may reveal specific subsets among the node-positive patients with improved prognosis and no need of adjuvant chemotherapy. Studies based on multigene assays including node-positive patients with low-risk scores have demonstrated that they have a favourable outcome[195, 196, 253, 268, 271, 272, 347]. The ongoing OPTIMA-trial will probably answer whether Prosigna® could be predictive for chemotherapy requirement in node-positive patients[348]. The added value of adjuvant chemotherapy for node-positive patients with Luminal A tumours is thus still an open question. A meta-analysis of six studies did not demonstrate a beneficial effect of chemotherapy in these patients[349], and it is recommended only for patients with Luminal A tumours and ≥4 positive nodes[94, 350]. The management of patients with node-positive status is under debate[351] and the results of ongoing studies as well as prolonged follow-up of completed studies are necessary before a general recommendation on abstaining from adjuvant chemotherapy in all node-positive patients can be made.

Importantly, randomised controlled studies of the chemotherapy predictive effect of multigene assays in premenopausal women are warranted. No firm results are available

so far, as presented in the discussion of study IV. In the premenopausal cohort of the RxPONDER trial, OFS was given in 15.9% vs 3.7% (endocrine therapy vs chemo-endocrine therapy) of the patients, respectively, and 47.9% vs 26.4% reported menstruation after 6 months of treatment, respectively[196]. Studies so far underscore that premenopausal patients constitute a specific entity and de-escalating strategies for them will be more challenging than in postmenopausal women. In the OPTIMA trial (ISRCTN:42400492), OFS is mandatory for all premenopausal women in order to eliminate the risk of confounding from different rates of chemotherapy-induced menopause between the arms[348]. Further studies of multigene assays including premenopausal patients are warranted, especially concerning the effect of ovarian suppression in addition to endocrine therapy as compared to chemotherapy.

The effect of multigene tests for guiding treatment decision in the neoadjuvant setting is another perspective which is highlighted in ongoing trials (ClinicalTrials.gov Identifier: NCT03749421). There are also ongoing studies such as the TAILOR RT (ClinicalTrials.gov Identifier: NCT03488693), that aim to analyse the effect of adding regional radiotherapy based on Oncotype DX. In addition, a retrospective trial has shown that RS could be used to assess the risk of locoregional recurrence in node-positive patients and thus used in decision regarding radiotherapy[352].

Immune-oncology and ER+/HER2- breast cancer

The future role of TILs in ER+/HER2– breast cancer is still not settled, and more studies are needed. The development of immune-related treatment by checkpoint inhibitors is of interest and has been effectful in solid tumours associated with high TMB[353, 354]. Regarding breast cancer, immune-related treatment has been promising particularly in TNBC[355–357]. Since the ER+/HER2– breast cancer is 'immune cold' with lower immune infiltration, future studies may focus on how to best increase the immunogenicity by immunomodulation of these tumours[358, 359].

A future interesting study would be by to combine the results of TILs from study III with data from the NanoString's Breast Cancer 360™ assay in study IV. This assay also delivers the pathways of inhibitory immune mechanism, (PD-L1 gene expression), immune cell abundance at the gene expression level and the Tumour Inflammation Signature[280]. The latter is a gene signature known to be associated with response to PD-1/PD-L1 inhibitors pathway blockade and identifies 'cold' or 'hot' tumours[360].

Further profiling at different levels

It is important to support logistics in clinic to collect both blood samples and primary and recurrent tissues in currently treated patients as a platform to make future translational studies possible. Circulating tumour cells (CTCs) are cancer cells that can be detected by capturing them in peripheral blood[361], are most frequently found in the metastatic setting[362]. In the early-stage breast cancer, enumeration of CTCs could be used as a prognostic marker[361, 363], but its clinical utility must be further investigated. Moreover, circulating DNA (cDNA), assessed in plasma or serum, is a promising marker providing information on genetic and epigenetic profiles, and might aid in tailoring therapies[364].

The predictive value of tumour mutations identified in cDNA samples and its importance for guiding treatments is another a future aspect of tumour profiling. The presence of PIK3CA mutation in hormone-receptor positive/HER2– metastatic breast cancer has shown to be predictive to the efficacy of the alpha-specific PI3K inhibitor alpelisib[365]. Although the value of this mutation in early breast cancer is unclear, mutation panels will probably help selecting specific therapies in the future also in this setting. The ongoing studies on how to overcome endocrine resistance, will also certainly continue[24, 366]. In study IV, extraction of DNA was additionally performed from the preserved primary tissues in the SBII:2pre trial, and mutation status and TMB and their correlation to prognosis in premenopausal patients is a forthcoming study. Different areas of future approaches to further personalise treatment and profiling in early breast cancer is outlined in Figure 20.

Metabolomics (sometimes denoted metabonomics) is an emerging technique that measures the metabolic response to biological stimuli or genetic manipulations[367]. In the future, the measurement of these molecules produced by the tumour cells could serve as markers for personalised medicine in early breast cancer[368] by creating a metabolic profile of the tumour.

The profiles of future primary breast cancers will certainly be complex but more informative and helpful to making decisions on optimal treatment regimens.

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