Discovery and Validation of Biomarkers in Breast Cancer

by

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the requirements for the degree of
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I dedicate this thesis to my beloved daughters, Eirill and Åsta.

Nina Gran Egeland

In honour of my aunt Reidun

Abbreviations

AI	aromatase inhibitor
APP	analysis protocol package
BCT	breast-conserving treatment
BRCA1/2	breast cancer type 1/2 susceptibility protein
CD	cluster of differentiation
CIBERSORT	Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts
CIS	cancer *in situ*
CISH	chromogenic *in situ* hybridization
DBCG	Danish Breast Cancer Group
DIA	digital image analysis
DIG	digoxigenin
DNA	deoxyribonucleic acid
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
ERBB2	erb-B2 receptor tyrosine kinase
ER	oestrogen receptor
FFPE	formalin-fixed paraffin-embedded
HER2	human epidermal growth factor-like receptor 2
IHC	immunohistochemistry
LN	lymph node

LNA™	locked nucleic acid™
MAI	mitotic activity index
MARCKLS1	myristoylated alanine-rich C kinase substrate like-1
miRNA	microRNA
mRNA	messenger RNA
NBCG	Norwegian Breast Cancer Group
NGS	next-generation sequencing
NST	no special type
PPH3	phosphohistone H3
PR	progesterone receptor
RNA	ribonucleic acid
ROI	region of interest
RT-qPCR	reverse transcription quantitative polymerase chain reaction
TDLU	terminal ductal lobular unit
TIL	tumour-infiltrating lymphocyte
TMA	tissue microarray
TME	tumour microenvironment
TNBC	triple-negative breast cancer
TNM	tumour – node – metastasis
VEGF	vascular endothelial growth factor
WS	whole-slide

Summary

Worldwide, breast cancer is the most common malignancy among women, and although treatment and prognosis have improved substantially over the last decades, for some patients the risk of recurrence remains for several years following diagnosis. Meanwhile, many breast cancer patients receive systemic adjuvant treatment unnecessarily, since their tumours will never recur. Implicitly, these patients are being overtreated while others are being undertreated. The challenge is to identify patients with a higher risk of developing recurrences and metastasis, from those who do not need additional treatment. These women may be spared potential treatment-induced side effects. Breast cancer is a highly complex and very heterogeneous disease, displaying both inter- and intratumoural biological variation. To ensure correct diagnosis and treatment, we need more precise and improved biomarkers. Equally important as discovering new and better biomarkers is the validation of existing ones. The work described in this thesis focuses on the discovery of novel candidate biomarkers for breast cancer, but also emphasize the equally important value of validating existing ones.

The first study examined the expression of the protein MARCKSL1 by immunohistochemistry. Increased expression of MARCKSL1 was previously associated with risk for metastasis and worse prognosis in breast cancer patients, especially in those with highly proliferating tumours. In this study, we set out to validate these findings. However, in

contrast to previous findings, MARCKSL1 protein expression was not prognostic in this independent patient cohort.

In the search for novel prognostic and predictive biomarkers in breast cancer, microRNAs are now emerging as potential candidates. In previous studies, gene expression of miR-18a and miR-18b correlated with high proliferation and basal-like features of breast cancer. In the second study, we applied chromogenic *in situ* hybridization to investigate the *in situ* expression of these microRNAs in both ER^+ and ER^- tumours. Our findings revealed that miR-18a and miR-18b are specifically expressed in the stroma surrounding the tumour, especially in ER^- breast tumours that present with a high degree of tumour infiltrating lymphocytes. Additional investigations suggested that the expression of these miRNAs might be associated with macrophages.

Cell proliferation is a fundamental feature of cancer cells, and high proliferation correlates with a higher risk of recurrence and reduced survival in breast cancer. Ki-67 is a well-known marker for proliferation, but its use is controversial because of the lack of consensus regarding pre-analytical processing, optimal clinical cut-off value and a high degree of variability across laboratories. Digital pathology is becoming increasingly important in routine diagnostics and is soon to be implemented in Norway. In the third study, we employed digital image analysis to evaluate the expression of Ki-67 in tissue microarrays, in a case-control study of tamoxifen-treated patients with and without

recurrence. However, our findings do not support an increased risk of recurrence associated with Ki-67 expression.

The resulting discrepancies with previous studies discussed in this thesis, highlights the importance of performing replication and validation studies, and to critically re-evaluate previous biomarkers.

List of Publications

I *Validation study of MARCKSL1 as a prognostic factor in lymph node-negative breast cancer patients.* Nina G. Egeland*, Marie Austdal*, Bianca van Diermen-Hidle, Emma Rewcastle, Einar G. Gudlaugsson, Jan P.A. Baak, Ivar Skaland, Emilius A. M. Janssen, Kristin Jonsdottir. (2019) **PLoS One** 14(3): e0212527.

II *MiR-18a and miR-18b are expressed in the stroma of oestrogen receptor alpha negative breast cancers.* Nina G. Egeland*, Kristin Jonsdottir*, Miriam R. Aure, Kristine Sahlberg, Vessela N. Kristensen, Deirdre Cronin-Fenton, Ivar Skaland, Einar G. Gudlaugsson, Jan P. A. Baak and Emiel A. M. Janssen. (2020) **BMC Cancer** 20:377 https://doi.org/10.1186/s12885-020-06857-7.

III *Digital image analysis of Ki-67 stained tissue microarrays does not predict recurrence in tamoxifen-treated breast cancer patients.* Nina G. Egeland, Kristin Jonsdottir, Kristina L. Lauridsen, Ivar Skaland, Cathrine F. Hjorth, Einar G. Gudlaugsson, Stephen Hamilton-Dutoit, Timothy L. Lash, Deirdre Cronin-Fenton, Emiel A.M. Janssen. (2020). Accepted for publication by **Clinical Epidemiology** (07th June 2020).

* Shared first authorship.

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Errata

1) In chapter 1.6. page 83 it is stated that miRNA molecules are ~*9–24 base pairs in length.* This number is incorrect. The correct range is from 15–27 nt in humans.

2) In chapter 1.6. page 83 it is stated that *1917 mature human miRNA sequences have been identified so far.* This is not correct, as there are 1917 precursors identified, and 2654 mature miRNAs.

1 Introduction

In the course of a lifetime, cancer will affect most people in some way. If they are not themselves diagnosed with cancer, it may still affect them through the diagnosis of a spouse, close relative, friend, or colleague. Among the challenges of cancer are the complexity and variability of its manifestations. On one end of the spectrum, the disease can be mild and curable within a short time span and, following treatment, the patient may continue life largely as before. On the other end of the spectrum, cancer can be aggressive and non-responsive to therapy, spreading and quickly killing its host. Often, the reality will fall somewhere between these two extremes. Cancer patients are increasingly surviving beyond their diagnosis and many are cured. In some cases, they either live for many years with a chronic but latent disease or they complete treatment but face a lifetime of various adverse side effects. Medical science has come a long way with cancer treatments, and physicians now have an arsenal of sophisticated therapy options available for their patients with cancer. Nonetheless, cancer remains a leading cause of death, and the search continues for even better diagnostic tools, with the hopes of achieving personalized treatment and eventually eradicating both overtreatment and undertreatment in cancer therapy. To achieve these aims, we need more and better biomarkers.

This thesis focuses on the discovery and validation of (phenotypic *in situ*) biomarkers in breast cancer.

1.1 *Tumourigenesis and the Basic Principles of Cancer*

A variety of biological events and molecular changes are involved in the appearance of a tumour, a process called tumourigenesis. Some common traits of distinguish cancer cells from normal cells. In 2000, Hanahan and Weinberg published their seminal article *Hallmarks of Cancer*, describing six key biological functions or changes that cells acquire in the multistep process of becoming cancerous. In 2011, two additional hallmarks were proposed, and two enabling characteristics were added (**Figure 1**). Although these listings have been criticized [1] for simplifying the complex biology of a malignant tissue-specific disease, these hallmarks are regarded as basic principles underlying tumourigenesis. These 10 traits are briefly introduced here.

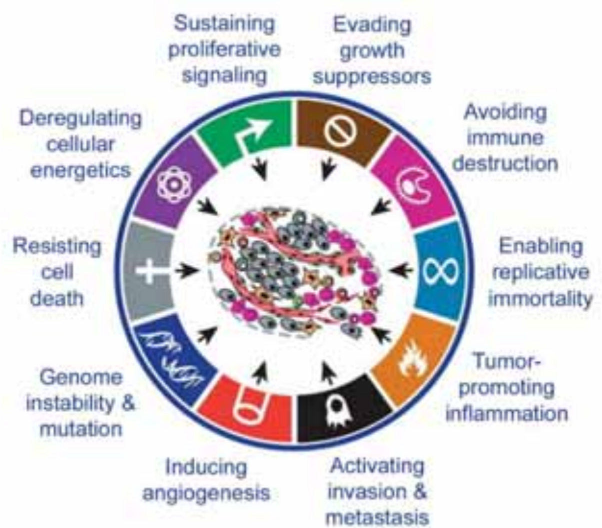

Figure 1. Hallmarks of Cancer.
Reprinted from Cell, Vol 144, Issue 5, Douglas Hanahan, Robert A. Weinberg, Hallmarks of Cancer: The Next Generation, Pages 646-674, Copyright 4721910985387 (2011), with permission from Elsevier.

1.1.1 Sustaining proliferative signalling, evading growth suppressors, and resisting cell death

In normal tissue, growth signals are carefully orchestrated to maintain a steady balance between the healthy, functioning cells that grow and divide continuously and the old or malfunctioning cells undergoing apoptosis, or controlled cell death. Every cell must pass through an intricate quality-control system to proceed through the cell cycle. This carefully controlled process ensures homeostasis in the body, fine-tuned to meet the current needs of the organism, through the release of growth factors. Cell proliferation – the process of increasing cells numbers – occurs naturally under healthy conditions, such as during childhood growth and in pregnancy. Cancer, however, results from cells dividing out of control and independently of the so-called tumour suppressors that usually inhibit cell division. Cells gaining these features become self-sufficient in growth signals, can avoid apoptosis, and are insensitive to anti-growth signals. These factors will lead to unrestrained cell proliferation and abnormal growth, and the cancerous cells proliferate and grow in number, eventually forming masses, i.e., tumours [2]. This increased cell proliferation in tissue, or neoplasms, does not always lead to cancer; sometimes, the cells will remain slow-growing and harmless, forming benign masses. When cells of the epithelium behave in this way, they are designated as “carcinomas.” When carcinomas remain in place, they are termed “carcinoma *in situ*” (**Figure 2**), and the term “cancer” is used only if the carcinoma becomes invasive and disrupts adjacent tissue.

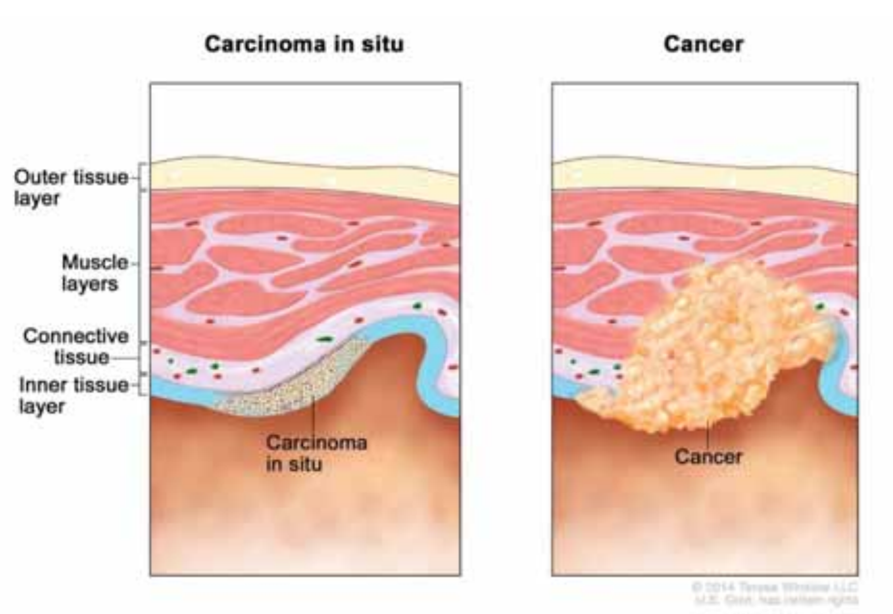

Figure 2. Carcinoma *in situ* versus Cancer.
The distinction between cancer and carcinoma *in situ* is based on the invasiveness of the tumour; a cancer disrupts its inner tissue layer and invades and harms the surrounding tissue. For the National Cancer Institute © 2011 Terese Winslow LLC, U.S. Govt. has certain rights. (Reproduced with permission).

1.1.2 Enabling replicative immortality

Normal cells have a limited ability to grow and undergo cell division and will do so only if necessary, usually being restricted to a certain number of cell cycles. In other words, normal cells “grow old”, and cell division eventually ceases, a process called “cell senescence” that results mainly from successive shortening of so-called telomeres. Telomeres are located on the end of chromosomes and consist of repetitions of the nucleotide sequence TCCCAA. This progressive erosion of telomeres is a self-protecting mechanism that occurs with every cell cycle/division, ensuring that cells do not have unlimited proliferation power [3]. However,

the vast majority of cancer cells express the enzyme telomerase, which counteracts this process by *adding* telomere repeat segments. In this way, cancer cells may acquire the potential for unlimited replication and continue to divide and give rise to even more cancer cells [2].

1.1.3 Genome instability and mutation

Genome instability references an increasing number of changes accumulating in the genome, consequently interfering with the maintenance of genome integrity and correct DNA replication. Sometimes aberrations occur in the chromosomes, leading to aneuploidy. As reviewed by Stratton et al in 2009, cancer is often said to be a disease of the genome [4]. In practical terms, all cancers arise because of genetic aberrations accumulating in one cell, either acquired over time (somatic mutations), or inherited (germline mutation). Such mutations result from errors during replication or from unrepaired or incorrectly repaired DNA damage, leading to permanent structural changes in the nucleotide sequences. These genetic mutations may accumulate in the genome and introduce error(s) into the DNA codons, causing changes in the expression or the function of the encoded proteins and ultimately leading to cells becoming cancerous [5]. Depending on the location of the deleterious modification in the genome, any of these DNA alterations can change the structure, amount, or function of a protein. For example, these pathogenic mutations could lead to loss-of-function of a tumour-suppressor or to gain-of-function of an oncogene. Uncontrolled cell proliferation will arise in each case, and cancer can be the end result.

Only mutations that confer a selective growth advantage for the cell can promote tumourigenesis and are called “driver mutations”. A typical tumour involves two to eight of these mutations, which usually develop over the course of several years [4,6]. Humans have approximately 20,500 genes, and DNA damage and spontaneous mutations occur continuously; in fact, the mutation rate in humans is estimated at roughly 0.5×10^{-9} bp^{-1} year^{-1} [7]. Fortunately, our cells have developed proficient DNA damage repair mechanisms to deal with these aberrations. Nevertheless, with time, cells acquire more and more aberrant mutations, which is why cancer becomes more common as we age [4]. Genetic variations are also a reason that some people have increased risk for developing cancer, as seen in people who are carriers of BRCA1/2 mutations.

1.1.4 Inducing and sustaining angiogenesis

As for all cells, to grow and survive, cancer cells depend on sufficient amounts of nutrients and oxygen and ways to rid of metabolic waste and carbon dioxide. Therefore, any cell requires capillary blood vessels within a distance of 100 µm [8]. As cells accumulates into a tumour of a certain size, the tumour becomes dependent on having afferent and efferent blood vessels to sustain itself [9]. Induced by a cancer-related state of hypoxia and/or inflammation, the cancer cells then release soluble angiogenic factors such as hypoxia-inducible factor 1, and various growth factors and cytokines that stimulate sprouting and ingrowth of nearby blood vessels, a process called “neovascularization”. This stimulus is referred to as the “angiogenic switch”, and vascular

endothelial growth factor (VEGF) is an important stimulator in this process, as is platelet-derived growth factor [2]. Neovascularization also involves endothelial progenitor cells (EPCs), activation of platelets, and remodelling of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs). Angiogenesis is essential for both the development and preservation of the cancer, and it contributes to enabling metastasis by providing an escape route for migrating cancer cells via its efferent blood vessels. The mechanisms of tumour-induced angiogenesis are summarized in **Figure 3**. Sometimes, the tumour grows so rapidly that angiogenesis cannot keep up, in which case some areas of the tumour without blood supply will then die, as can be seen under the microscope as necrotic areas.

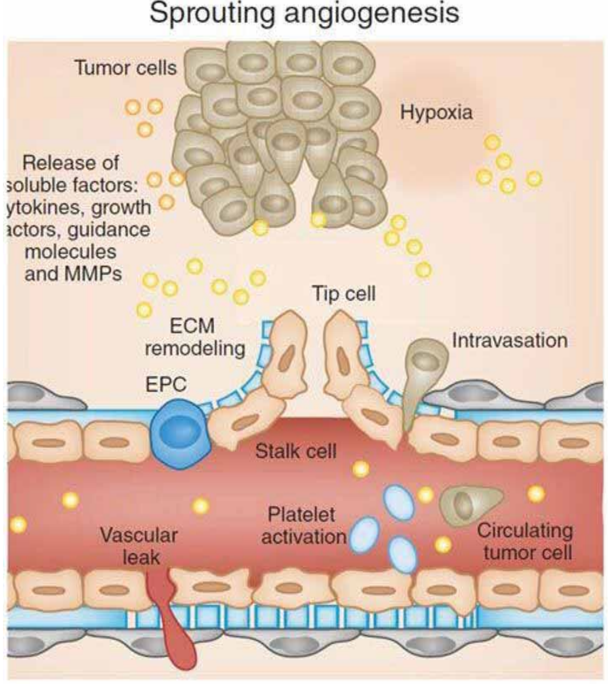

Figure 3. Mechanisms of Tumour-induced Angiogenesis.
Both hypoxia and the tumour cells cause release of several stimulatory signals such as cytokines, growth factors, and MMPs into the microenvironment. These signals stimulate angiogenic and inflammatory changes in multiple cell types. Some tumour cells may invade the surrounding vasculature and enter the circulation; whereas others cause disruption of the vascular barrier. Exposure of the basement membrane is perceived as a wound, leading to recruitment and activation of multiple cell types such as platelets, EPCs, and myeloid cells, which contribute to this process by releasing stimulatory factors into the TME. **ECM**: extracellular matrix; **EPC**: endothelial progenitor cell; **MMP**: matrix metalloproteinase; **TME**: tumour microenvironment. Adapted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Medicine. Tumor angiogenesis: molecular pathways and therapeutic targets, Sara M Weis et al, COPYRIGHT (2011).

1.1.5 Deregulating cellular energetics

Cancer cells can reprogram cellular metabolism by triggering a metabolic shift and adjusting energy metabolism to sustain themselves. The highly effective oxidative phosphorylation in mitochondria is the

default setting of energy metabolism for normal cells. This process of glycolysis takes places under aerobic conditions, in which glucose is processed into pyruvates that enter the mitochondrial tricarboxylic acid cycle (TCA), resulting in the production of energy-rich adenosine triphosphate (ATP) and carbon dioxide. Cancer cells, however, prefer a different form of energy, produced by a mechanism similar to that used under anaerobic conditions, but in which the pyruvate is converted mostly into lactate even in the presence of oxygen, depleting the amount of pyruvate available to enter the TCA (**Figure 4**). This phenomenon is also known as the Warburg effect, named after the Nobel laureate who first described it in the late 1920`s. Presumably, the rationale behind this phenomenon is that although the cancer cells generates much less energy (in the form of ATP) through glycolysis, in return, the process yields even more metabolites to fuel their growth and biosynthesis. In addition, growing tumours often result in a hypoxic environment that will benefit cells with such an aberrant metabolism independent of oxygen.

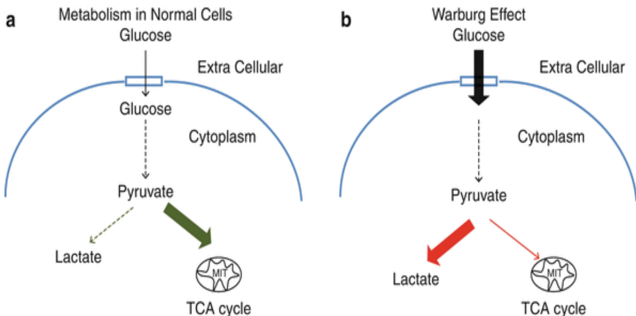

Figure 4. Metabolism in Normal vs Cancer Cells.

Cell metabolism in a) a normal cell demonstrating oxidative phosphorylation, and b) a cancer cell exhibiting the Warburg effect. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Springer eBook [10].

1.1.6 Activating tissue invasion and metastasis

Normal epithelial cells, from which most solid cancers originate, reside within the basement membrane and their tissue-specific boundaries. Gaining the ability to displace and invade the surrounding tissue is a characteristic that distinguishes malignant cells from benign masses, which “stay in place”. Tumour cells become able to invade the surrounding tissues by a multistep process, changing the phenotype and morphology of the cells. One important mechanism is the so-called epithelial-to-mesenchymal transition (EMT), in which various transcription factors transform resident epithelial cells are into a motile mesenchymal phenotype. EMT is a reversible process that is involved in physiological processes such as embryogenesis, development, stem cell behaviour, and wound healing, but this cell plasticity is also fundamental in tumourigenesis. Loss of E-cadherin, which is important for maintaining cell–cell adhesion, is another cancer cell alteration contributing to invasion.

The combination of exaggerated cell proliferation and these other hallmarks results in the formation of a malignant tumour. Some of the cancer cells may break off from the tumour and attain the ability to spread by either blood or lymph to other areas in the body and form metastases, often with detrimental consequences. Metastases are the main reason for cancer-associated deaths. The mechanisms of metastasis are highly complex and still not fully understood, although different explanatory models have been proposed, some of which were reviewed by Hunter et al in 2008 [11]. Not all cancer cells have metastatic potential;

metastatic cells must overcome many obstacles to establish a new colony of cells at a secondary location by forming viable micrometastases. Some cancers metastasize early on, whereas other cancer cells may be dormant for years before reactivating and starting to grow into a new tumour, eventually becoming clinically overt. Different types of cancers can have very different prognoses, but generally, early detection is of vital importance for treatment success and patient survival.

1.1.7 Tumour-promoting inflammation and avoiding immune destruction

A functioning immune system is critical for upholding and protecting a viable organism, and an elaborate and highly efficient immune surveillance system protects us from both exogenous and endogenous harmful events. Inflammation is a biological process that occurs in response to tissue damage, trauma, infection, or pathological events and results in a local release of numerous chemical mediators such as pro-inflammatory cytokines, histamines, and prostaglandins. This release induces growth factors and initiates the wound-healing process by stimulating new tissue growth and neovascularization. Paradoxically, cancer cells can take advantage of this protective immune response by directing these mechanisms to stimulate further tumour growth [12].

Generally, our immune system effectively identifies and destroys infections, damaged cells, or emerging (genetically) aberrant neoplastic cells. Tumour cells express specific tumour antigens on their surfaces, which the immune system recognizes either directly or indirectly via so-

called antigen-presenting cells (APCs). With recognition of these tumour-specific antigens, cytotoxic lymphocytes (T cells) of the adaptive immune system will be activated to kill these cells, whereas B cells will start to produce antibodies directed against the tumour cells. In addition, cells of the innate immune system contribute by secretion of pro-inflammatory cytokines. In most solid cancer types, immune cells, especially lymphocytes, have been found to infiltrate the tumour area. These cells are called tumour infiltrating lymphocytes (TILs) and have prognostic value in several cancer types [13,14]. The importance of the immune system in regulating cancer is evident from, for example, the elevated risks/incidences of cancer in immunosuppressed individuals [15]. It is now recognized that the adaptive and innate immune responses and their many associated immune cells play an essential but dual role in cancer, having both pro- and anti-tumour effects, depending on the type of immune cells involved [16]. Through genetic and epigenetic modifications, some tumour cells evade the natural selection pressure of immune surveillance, remaining undetected and escaping the immune system, thus enabling the clones to accumulate and continue to grow without eliciting an immune response [2,17,18]. Tumour-promoting inflammatory cells take part in both tumourigenesis and maintenance of cancer and cancer progression, and often involve and occur within the tumour microenvironment (TME). An illustration of cancer-induced inflammation and the adaptive- and innate immune responses to malignant cells is shown in **Figure 5**. Cancer immunity and the TME are discussed in further detail in section 1.6.

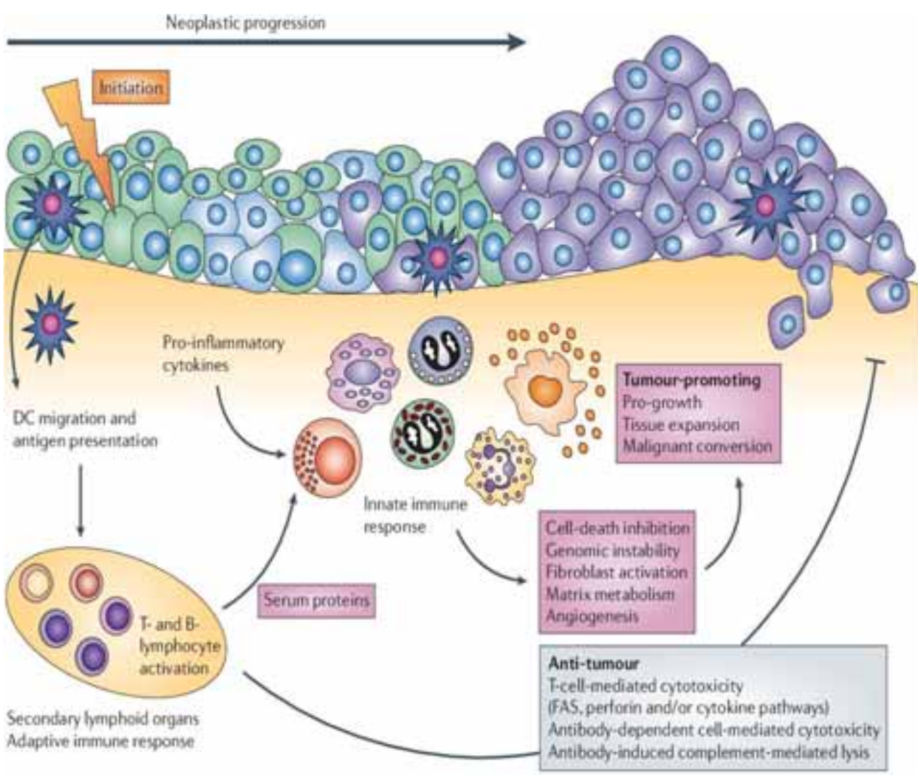

Figure 5. Cancer-induced Inflammation.
Simplified model of how adaptive and innate immune cells may respond to cancer-induced inflammation during neoplastic progression. Dendritic cells (DCs) present tumour antigens to T and B cells in lymphoid organs. Once activated, these cells elicit an adaptive immune response with both tumour-promoting and antitumour effects. B cell activation and pro-inflammatory cytokines activate innate immune cells, further promoting tumour development through mechanisms such as cell death inhibition, tissue remodelling, and induction of angiogenesis. Meanwhile, T cell-mediated and antibody-dependent cytotoxicity and tumour cell lysis have anti-tumour effects. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature. Nature Reviews Cancer. (2006). Paradoxical roles of the immune system during cancer development, Karin E. de Visser et al .Nat Rev Cancer 6, 24-37, doi:10.1038/nrc1782 [16]. [COPYRIGHT 4770741508990], 2006.

1.2 *Breast Cancer Biology and Classification*

Breast cancer is one of the oldest described cancers in human history, with the first evidence dating back to ancient Egypt [19]. Hippocrates described the different stages of breast cancer as early as 400 B.C.E [20]. Because breasts are such a visual and important part of women's physiology, they have strong symbolic associations with femininity, fertility, and motherhood. The physiological development and growth of the breasts is closely associated with the primary female steroid hormones oestrogen and progesterone. Induced by hormones during puberty, the breasts will start to develop into functional tissue consisting of mostly adipose (fatty) tissue and the functional glandular tissue lobes, or the terminal ductal lobular units (TDLU). The lobes consist of smaller sections called lobules. In the case of pregnancy and lactation, these lobules become milk-producing, branching out into a tubular network of ducts that ultimately drains into the nipple. The lobes and ducts are lined with a thin epithelial layer. In addition to the lobes, ducts, and adipose tissue, the breast also contains blood and lymph vessels, lymph nodes, nerves, and connective tissue (**Figure 6**). These components make up the stromal compartment, i.e., the part of a tissue with a primarily supporting role.

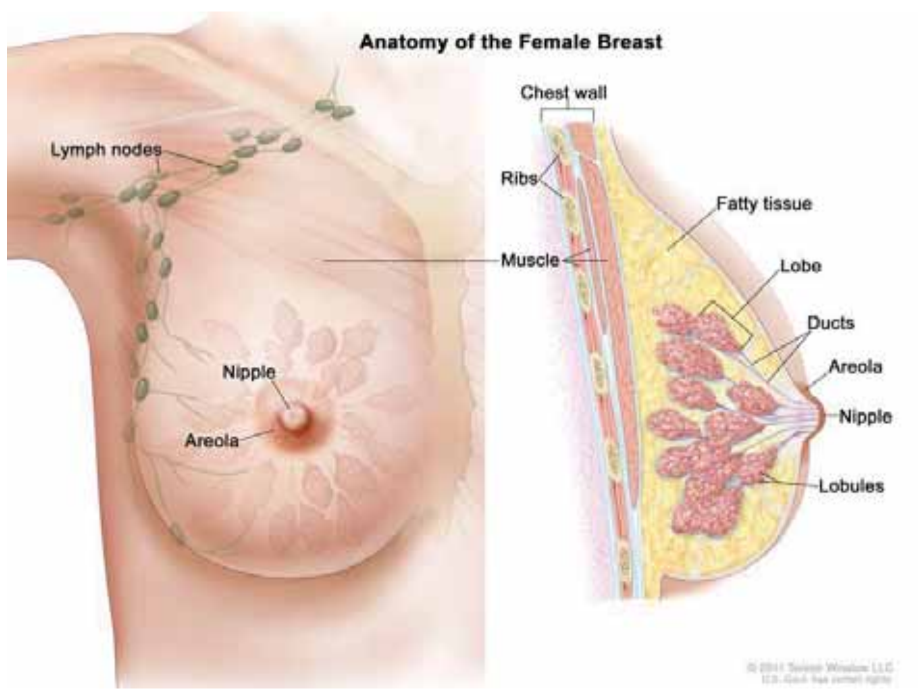

Figure 6. Anatomy of the Female Breast.
Anatomy of the female breast, illustrating the draining lymph nodes. Cross-section illustrating the organization of lobes, lobules, ducts, areola, and nipple in relation to the chest wall. For the National Cancer Institute © 2011 Terese Winslow LLC, U.S. Govt. has certain rights. Reproduced by permission.

1.2.1 Breast cancer epidemiology and aetiology

Worldwide, breast cancer is the most frequent female malignancy, with more than 2 million new cases per year. In Norway, breast cancer accounted for as many as 3568 cases of cancer and 586 deaths in 2018. Although men also may develop breast cancer (Norway 2018: 8 male breast cancers), but for the remainder of this thesis, we will focus only on breast cancer in women.

In Norway, the median age at diagnosis is 62 years, and women have an 8.89 % cumulative risk of developing breast cancer by the age of 75. Breast cancer incidence has increased over the years, likely because of earlier detection of non-symptomatic cancer through the Norwegian Breast Cancer Screening Program, which was implemented nationwide in 2005 [21]. Meanwhile, breast cancer survival has also increased (**Figure 7**), from 89.3% to 90.7% in the last 5-year period (2014–2018), most likely because of improvements in treatment [21,22]. These values translate into a substantial number of breast cancer survivors, including women who have either been fully cured, and those who knowingly or not continue living with the disease in the form of (micro-) metastases. In fact, the number of breast cancer survivors increased from 34,719 in 2008, to 49,344 by the end of 2018 [22]. Some of these women had already been diagnosed with metastases, whereas others may or may not develop them over time.

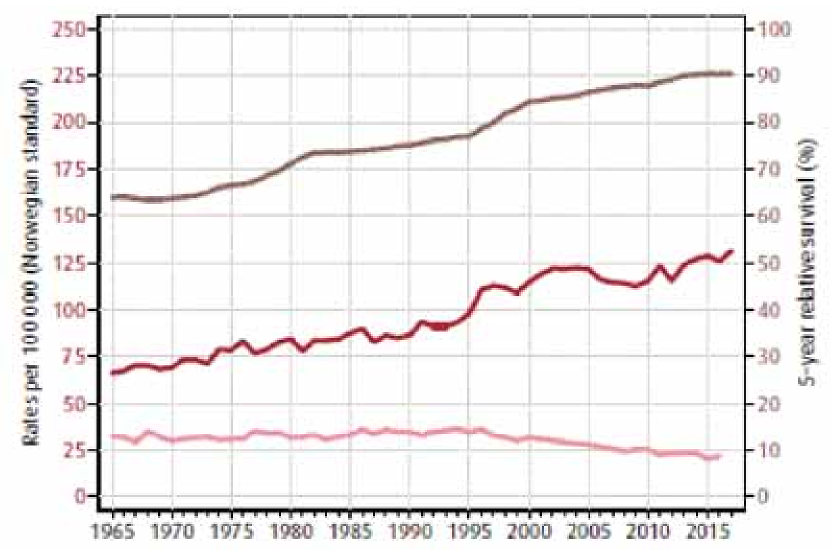

Figure 7. Trends in 5-year Relative Breast Cancer Incidence.
Trends in the 5-year relative incidence (red line), survival (brown line), and mortality rates (pink line) per 100 000 women with breast cancer in Norway, from 1965–2015. Adapted from [22].

Apart from female sex and age, known risk factors for breast cancer include excess body mass index, long-term hormone replacement therapy, and poor diet. As with most cancers, breast cancer risk may to some extent be partially preventable by reducing alcohol consumption, increasing physical activity, and maintaining a healthy diet. Pregnancy at age <25 years, multiple pregnancies, and breastfeeding also seem to play a risk-reducing role, whereas extended exposure to oestrogen seems to be disadvantageous. However, for most cases of breast cancer, generally no single direct cause is attributable. Approximately 5% to 10% of breast cancers are associated with inherited risk because of mutations in cancer-related genes, the most well-known being the BRCA1 and BRCA2 mutations, which cause approximately 2% of breast

cancers [23]. Women who inherit a mutated version of one of these tumour-suppressor genes have a high lifetime risk of developing both breast and ovarian cancer and these women are therefore offered prophylactic bilateral oophorectomy and mastectomy [24]. Other gene mutations are also associated with breast cancer, and one study found *TP53*, *PIK3CA*, *MYC*, *CCND1*, *PTEN*, *ERBB2*, *ZNF703*/*FGFR1* locus, *GATA3*, *RB1*, and *MAP3K1* to be the most frequently altered genes in primary breast cancer [25]. A recent large EU project from 2016, identified 93 genes as the main drivers of breast cancer [26].

1.2.2 Terminology and classification of breast cancer

Breast cancer is a highly complex and heterogeneous disease, displaying a multitude of both intertumoural and intratumoural biological variation. Breast cancers are therefore classified based on several clinical, histological, and molecular characteristics. The vast majority of these cancers are histologically classified as infiltrating carcinomas of no special type (NST), and usually originates in the TDLUs (**Figure 8**). According to the National Quality Registry for Breast Cancer 2018, NST carcinomas constitute around 70% of breast cancers in Norway. The remaining infiltrating carcinomas are of the special type, most frequently the infiltrating lobular carcinoma (12%) or infiltrating, other (9%). Furthermore, around 10% of suspected breast cancers turn out to be premalignant, meaning that there is a tumour but that it has not yet become invasive. These lesions are called carcinoma in situ (CIS) and may arise in the ducts (DCIS) or the lobules (LCIS) [27].

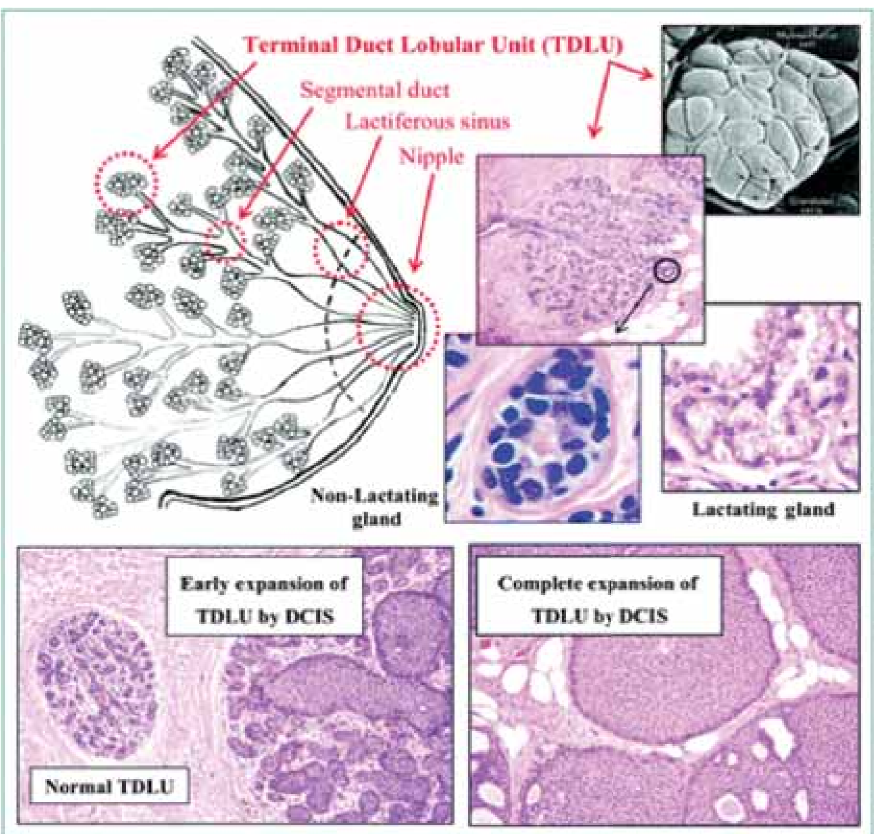

Figure 8. Ductal Carcinoma *In Situ*.
Breast cancer usually originates from epithelial cells in the TDLUs, the functional units of the breast where the milk-producing glands are located. Often, the process of a developing cancer starts with non-invasive DCIS, before it eventually becomes invasive. DCIS: ductal carcinoma in situ. TDLU: terminal ductal lobular unit. Allred, D. Craig. Ductal Carcinoma In Situ: Terminology, Classification, and Natural History. Journal of the National Cancer Institute Monographs, 2010, volume 2010, issue 41, 134-138, by permission of Oxford University Press.

Stage To be able to offer the right adjuvant treatment, clinicians must determine at the time of diagnosis the extent to which the cancer has manifested itself within the breast or the body, i.e., staging. Like most cancers, breast cancer is classified according to the TNM Staging system (see **Table 1** for summary of this system). This system relies on a set of

standardized criteria developed by the Union for International Cancer Control (UICC)[28], and is based on the size of the tumour (T), involvement of regional lymph nodes (N), and whether the cancer has metastasized (M). Each category is assigned a number to describe the extent of tumour load. Stage I describes the least advanced tumours and stage IV the most advanced, but the terms “early”, “late” and “terminal” breast cancer are also used. Generally, cancers within stage T1-2N0-1M0 are regarded as operable, whereas stages beyond T3-4N0-3M0-1 or T1-2N2-3M0-1 are considered primarily inoperable. Cancer staging is determined based first on preoperative clinical examination, imaging tests, and biopsies, i.e., clinical staging, followed by pathological staging based on findings in the surgical specimen of the primary tumour per- and postoperatively.

Most breast cancers are diagnosed before the cancer has spread to more distant sites, i.e., stages I–III, or non-metastatic disease. Furthermore, for most (>60%[29]) stage I–III breast cancers, the cancer cells have not yet spread to nearby lymph nodes, so they are lymph node negative, or LN^{-}. This status is often referred to as early stage breast cancer, which is the main focus of this thesis.

Table 1. TNM Staging System.
Simplified summary of the UICC TNM staging system for breast cancer.

CATEGORY	DESCRIPTION
TX	Primary tumour cannot be evaluated
T0	No evidence of primary tumour
Tis	Carcinoma *in situ* (DCIS/LCIS/Paget's disease).
T1–T4	Size and/or extent of the primary tumour
T1	Tumour ≤ 2 cm in greatest dimension
T2	Tumour > 2.0 ≤ 5.0 cm in greatest diameter
T3	Tumour > 5.0 in greatest diameter
T4	Tumour independent of size, but with direct extension to chest wall and/or to skin (ulceration or macroscopic nodules).
pNX	Regional lymph nodes cannot be evaluated
pN0	No regional lymph node involvement (no cancer found in the lymph nodes)
pN1-N3	Involvement of regional lymph nodes (number and/or extent of spread)
pN1	Micrometastases; or metastases in 1–3 axillary lymph nodes; and/or clinically negative mammaria interna lymph nodes with micro- or macrometastases by SLN biopsy.
pN2	Metastases in 4–9 axillary lymph nodes; or positive (by imaging) ipsilateral mammaria interna lymph nodes(s) in absence of axillary lymph node metastases.
pN3	Metastases in i. ≥10 axillary lymph nodes; or infraclavicular lymph nodes, or ii. Positive ipsilateral mammaria interna lymph nodes by imaging in presence of positive axillary lymph node (s); or iii. >3 axillary lymph nodes and micro- or macrometastases by SLN biopsy in clinically negative ipsilateral mammaria interna lymph nodes; or in ipsilateral supraclavicular lymph nodes.
MX	Distant metastasis cannot be evaluated
M0	No distant metastasis (cancer has not spread to other parts of the body)
M1	Distant metastasis (cancer has spread to distant parts of the body)

Adapted from [30]. Used with permission under the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/).

Grade In addition to stage, grade (based on the Nottingham grading system or the Elston-Ellis modification of Scarff-Bloom-Richardson grading system) is an important tumour characteristic because it has strong prognostic value [31]. Roughly speaking, the higher the grade of the tumour, the more dedifferentiated the tumour cells will be. Histological grade is based on quantification of the following morphologic features: mitotic count (0–7, 8–15, >16), degree of tubular formation (>75%, 10%–75%, <10%), and nuclear pleomorphism (uniform, moderate, high) [32,33]. Microscopic investigation of these characteristics results in an overall score of one, two, or three, correlating with increasingly worse outcome, as depicted in **Figure 9**. In Norway, the overall histological grade distribution of breast cancer is roughly 24% with grade 1, around 50 % grade 2, and 26% grade 3 [27].

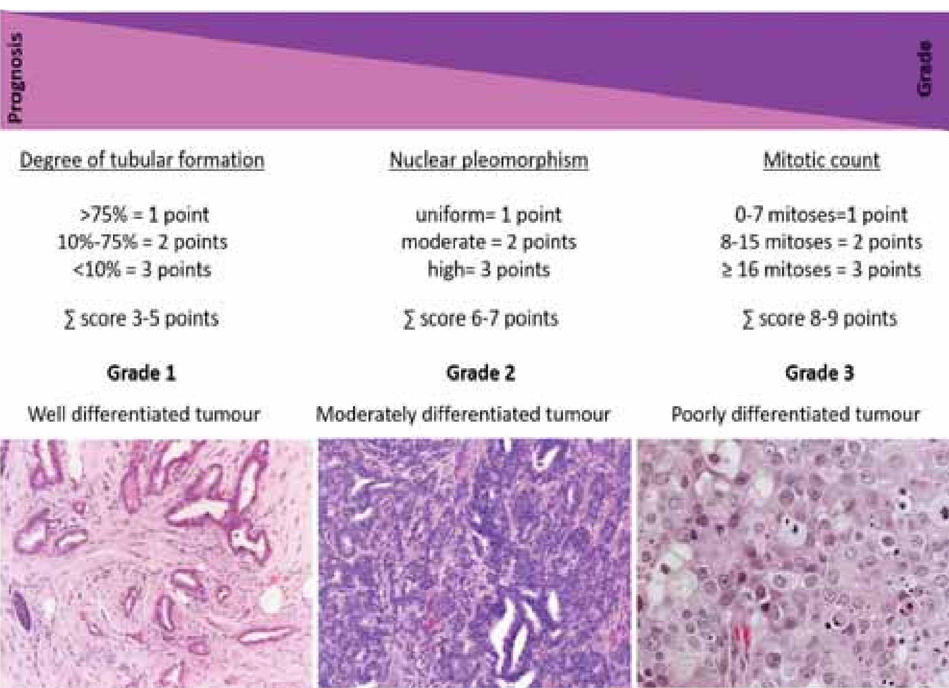

Figure 9. Histological Grade in Breast Cancer.
Histological grade is based on quantification of the morphologic features mitotic count (0–7, 8–15, >16), degree of tubular formation (>75%, 10%–75%, <10%), and nuclear pleomorphism (uniform, moderate, high). Scoring each of these characteristics results in an overall score of grade 1, 2 or 3, corresponding to well, moderately, or poorly differentiated tumours, resp. Figure based on [33,34] , images from [35].

Hormone receptors In 1896, George Beatson had already discovered that breast cancer would regress after surgical removal of the ovaries [36], demonstrating a link between growth hormones produced in the ovaries and breast cancer. However, the molecules responsible for this, i.e., the steroid hormones oestrone, oestradiol and oestriol, were not identified until 1923 [37]. Then, in 1967, Elwood Jensen discovered the oestrogen receptor (ER) in the cell nucleus, which acts as a transcription factor [38].

Hormone receptor–positive breast cancers (HR$^+$) will proliferate upon binding of their hormone ligand, so it is important to confirm a patient's hormone receptor status. The biological impact of oestrogens is reflected by the fact that most breast cancers have tumour cells that express receptors for these hormones, so that they are ER and/or progesterone receptor (PR) positive. In Norway, around 14% of all patients are ER$^-$ (<1% ER expression), whereas the rest are per definition ER positive, but to varying degrees. Around 33% of breast cancers in Norway are PR$^-$ (<10% PR expression) [27]. PR occurs in two isoforms, PRA and PRB, and the same is true for ER, which has an ERα and an ERβ isoform; however only ERα is discussed in this thesis. HR status is determined by immunohistochemistry (IHC), which will be further discussed in the Methodology section 3.4.

HR status is a feature with strong prognostic and predictive implications because HR$^+$ tumours can be treated with and respond to endocrine therapy. Such treatment has a significant impact on survival, demonstrably reducing the 5-year risk of recurrence by roughly 50% [39]. The HR$^+$ breast cancers are distinct from the HR$^-$ breast cancers, which do not express such receptors, and thus do not respond to anti-hormonal therapy. Of note, although breast cancers considered HR$^+$ tend to be positive for *both* ER and PR (ER$^+$/PR$^+$), they are not necessarily always so because cancers might have differential expression of these markers, i.e., be ER$^+$/PR$^-$ or ER$^-$/PR$^+$. These rare combinations may have clinical implications, as seen with an observed tamoxifen benefit in ER$^-$/PR$^+$ or "ER-poor"/PR$^+$ patients, suggesting that PR expression should be taken

into account when considering endocrine treatment in ER^- patients [40,41]. More recent studies also suggest that the PR status has both prognostic and predictive value in patients with luminal breast cancer patients [42].

HER2 The *ERBB2* gene encodes the oncoprotein receptor tyrosine kinase 2 erbB2, also known as HER2/*neu* (HER2), short for human epidermal growth factor-like receptor 2. As the name suggests, this receptor stimulates cell growth, and its amplification results in a more aggressive clinical behaviour of breast cancer tumours, which subsequently correlates with worse outcome in terms of relapse and survival [43]. Breast cancers that overexpress the oncogene HER2 are called HER2-positive ($HER2^+$), and in Norway these account for around 13% of breast cancers [27]. HER2 status is determined by IHC and/or *in situ* hybridization (ISH) and is an important prognostic and predictive marker. Since the late 1990s, patients with $HER2^+$ breast cancer have been treated with the anti-HER2 monoclonal antibody trastuzumab (Herceptin) or similar drugs, which has significantly improved the disease-free survival rates in this patient group [44,45].

Proliferation Cell proliferation is a fundamental feature of cancer cells, where cell growth by cell division is no longer counterbalanced by cell death, resulting in an increased mass of cells, i.e., a tumour. Not all tumours have the same growth rate, and some grow slowly whereas others are highly proliferative. The higher the cell growth rate, the more aggressive and prone to metastasis the tumour will be. Not surprisingly,

high proliferation correlates with a higher risk of recurrence and thus reduced survival cancer [46-48]. Proliferation is also an integral part of histological grade, and the degree of proliferation is of great significance for treatment decisions. Most often, proliferation of breast cancer cells is counted in haematoxylin and eosin (HE)-stained slides of tumour tissue (e.g., mitotic activity index (MAI) or mitotic count), or based on the counting of IHC markers like phosphohistone-H3 (PPH3) or Ki-67 score. The proliferation marker Ki-67 will be more thoroughly discussed in section 1.3.4.

Breast cancer subtypes Based on clinicopathological factors, the Norwegian Breast Cancer Group (NBCG) currently delineates four main groups of breast cancer: $HR^+/HER2^-$; $HR^+/HER2^+$; $HR^-/HER2^+$; and $HR^-/HER2^-$ or triple- negative breast cancer (TNBC). In addition to the clinical and histological markers described above, breast cancer is classified based on molecular subtypes derived from gene expression profiles, which for the most part correspond/overlap with the clinical subtypes. The transcript profiling of breast cancer tumours was first described in the pioneering article of Perou et al in 2000, who classified breast cancers into four main molecular, or *intrinsic*, subtypes with very different prognoses: ER^+/luminal-like (further subdivided into luminal A or luminal B), basal-like, Erb-$B2^+$, and normal breast-like [49]. This study was a breakthrough in the molecular-based classification of breast cancer, and these four intrinsic subtypes have since been confirmed in several similar studies [50,51]. Luminal A breast cancers are by far the

largest group, exhibiting the least aggressive behaviour and associated with excellent prognosis, and therefore requiring the least treatment. Luminal B tumours are characterized by higher proliferation and are often of higher grade. As such, they tend to have a prognosis that is worse than that of luminal A disease but better than that with the $HER2^+$ and TNBC subtypes (**Table 2**).

Table 2. Breast Cancer Classification.
Simplified overview of the classification of the four main clinical subtypes used by the NBCG.

Subtype	ER/PR expression	HER2 expression	Characteristics	Prognosis
$HR^+/HER2^-$				
-Luminal A-like	Positive/positive	Negative	↓proliferation/grade	Excellent
-Luminal B-like			↑proliferation/grade	Good
$HR^+/HER2^+$	Positive	Positive		Intermediate
$HR^-/HER2^+$	Negative	Positive		
$HR^-/HER2^-$	Negative/negative	Negative	TNBC, ↑↑proliferation ↑↑grade	Worse

Other studies and whole genome sequencing have unveiled an even more complex molecular portrayal of breast cancer genomics [26,52]. Today several commercially available genomic/gene expression tests are available for breast cancer recurrence risk assessment, such as Onco*type*DX®, MammaPrint/BluePrint®, and the Prosigna®. These tests separate patients into low-/medium- or high-risk groups in what is,

at least in theory, a more refined manner than traditional markers. As such, they may help identify patients who may or may not benefit from adjuvant therapy. For example, the Onco*type*DX® Breast Recurrence score is based on the activity of 21 genes, providing a recurrence risk score that may help diminish overtreatment and undertreatment [53,54]. The Prosigna® test uses the PAM50 gene signature to identify intrinsic molecular subtypes, providing a prognostic score assessing the 10-year risk of distant recurrence [55,56]. Reportedly, a combination of MammaPrint and the NGS-based BluePrint® 80-Gene Molecular Subtyping Assay could classify subtypes more precisely than traditional markers, predict treatment response to chemotherapy, and identify patients with luminal A disease who will experience no further benefit from neoadjuvant chemotherapy [57]. Whether or to what extent these tests will lead to more precise treatment selection remains to be settled, as some study findings are conflicting [58].

Since 2017, the American Joint Committee on Cancer updated their staging guidelines to include tumour grade, ER/PR status, HER2 status, and Onco*type*DX score, thereby taking into account not only the traditional biomarkers but also the molecular subtypes and prognosis [59]. **Figure 10** gives an overview of the intrinsic- and surrogate intrinsic breast cancer subtypes and how they associate with proliferation, grade, and receptor expression.

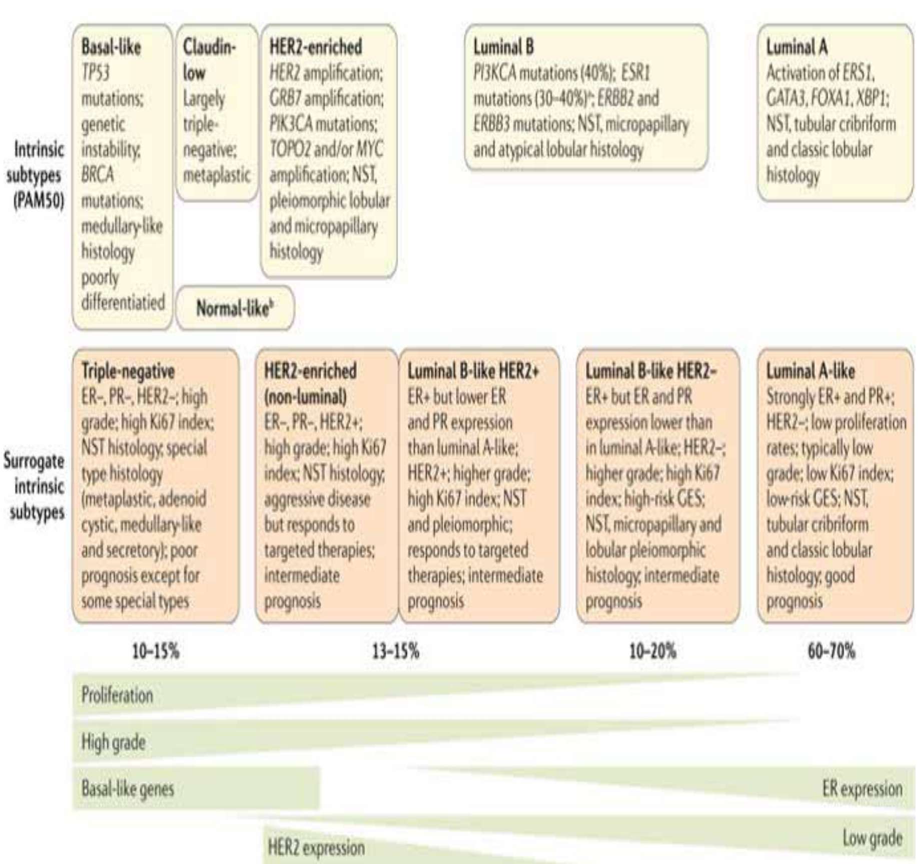

Figure 10. Intrinsic and Surrogate Intrinsic Subtypes.
Simplified overview of the intrinsic PAM50-derived and the surrogate intrinsic subtypes of breast cancer and their association with the degree of proliferation, grade, expression of basal like genes, and receptor expression. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, NATURE REVIEWS DISEASE PRIMERS (https://www.nature.com/nrdp/). Breast Cancer, [60] COPYRIGHT 4723730828679 (2019).

1.3 *Current Clinical Practice Guidelines for Treatment*

In the early days, breast cancer was treated with what was often quite radical surgery only, then chemotherapy and radiotherapy came along in the 1940s and 1950s. Since then, endocrine therapy and anti-HER2-therapy have been introduced. In recent years, the advent of gene expression assays and immune therapy has brought us to an even more tailored precision medicine. Today, an array of various treatment regimens are available, and the challenge is to decide on the best treatment for the individual patient.

1.3.1 Surgery

The primary and most useful treatment for operable breast cancer is usually surgery, with either removal of the entire breast tissue by mastectomy or the less radical breast-conserving treatment (BCT) or lumpectomy, in which the lump of cancer is removed while preserving most of the healthy breast tissue. The choice of surgery is individually assessed and depend on factors such as tumour-to-breast size relationship, extent of tumour growth, and whether free margins are assured. In times of modern surgery, BCT increasingly used and the preferred method when technically possible, usually followed by radiation therapy (RT). For patients with BRCA1/2 mutations, large/multifocal DCIS, or for whom RT is contraindicated, the breast is usually removed by a mastectomy. These patients are often candidates for primary or secondary plastic surgery breast reconstruction. In addition to the surgical removal of the primary tumour, sentinel lymph

node (SNL) biopsy is usually performed per-operatively to look for the presence of any tumour cells (**Figure 11**). If the SLN is positive for tumour cells (LN^+), axillary lymph nodes are also dissected when indicated. The rationale for this step is that any cancer cells breaking away from the primary tumour usually follow the lymph to the nearest draining lymph nodes [61].

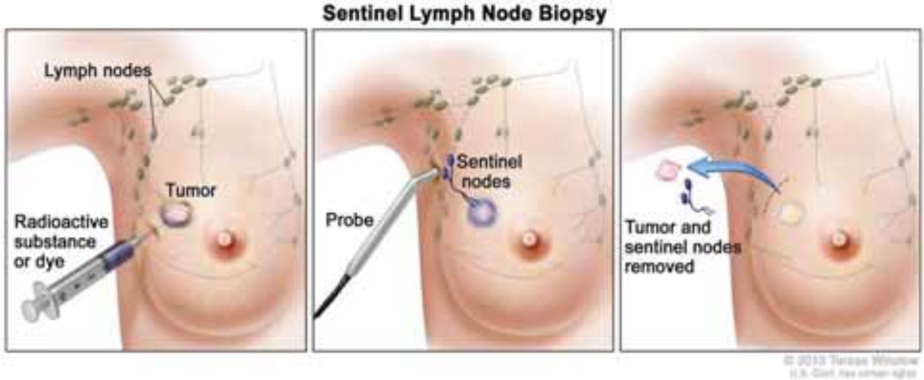

Figure 11. Sentinel Lymph Node Biopsy.
In SLN biopsy, a radioactive substance or dye is injected into the breast tissue preoperatively. This will be taken up by the draining, or sentinel, lymph nodes, which may then be detected by a probe. Both the tumour and the sentinel nodes are then removed. Printed with permission. For the National Cancer Institute © (2010) Terese Winslow LLC, U.S. Govt. has certain rights.

1.3.2 Radiation therapy

In an attempt to irradiate and kill any potential residual cancer cells after primary surgery and/or chemotherapy, RT is standard protocol for all patients who have had BCT and free margins, for tumours exceeding 50 mm (locally advanced breast cancer), and for patients with LN^+ disease. Localized beams of ionizing radiation directed at the tumour area bring substantial DNA damage to the targeted cancer cells, which subsequently

induces apoptosis [62]. RT following BCT has been shown to markedly reduce the risk of recurrence by 15% and to reduce the 15-year risk of breast cancer mortality by almost 3.8% [63,64]. In some cases, RT is used neoadjuvantly to try to shrink very large tumours preoperatively and is administered in the palliative setting to relieve suffering or at least temporarily to keep the cancer from progressing.

1.3.3 Systemic adjuvant treatment

For the least advanced tumours such as luminal A, surgical removal of the tumour by BCT might suffice. For larger and/or more aggressive tumours, more complex and intensive systemic adjuvant treatment may be necessary, including chemotherapy, radiation, targeted immune therapy, anti-HER2 therapy, or endocrine treatment. These treatments are briefly introduced here.

1.3.3.1 Chemotherapy

Systemic adjuvant therapy in the form of chemotherapy is administered to patients with breast cancer who have an estimated higher risk of recurrence, and generally, patients with low-grade LN^- luminal A disease will derive no further benefit from chemotherapy. A combination of cyclophosphamide and epirubicin (EC) is the most widely used anthracycline-based chemotherapy (previously also including fluorouracil, i.e., FEC), although taxanes (paclitaxel or docetaxel) might be added for the more aggressive subgroups such as those with luminal B, $HER2^+$, or TNBC disease [61]. Chemotherapeutic agents have different

modes of actions, but mostly they interfere with the cell cycle or induce apoptosis by impairing DNA integrity and preventing further cell proliferation. Cyclophosphamide is an alkylating agent that crosslinks DNA, leading to apoptosis; anthracyclines act by intercalating DNA strands [65]; and taxanes, originally derived from the Pacific yew tree, act by preventing the cell cycle [66]. Chemotherapy is (intravenously) administered, either before surgery as neoadjuvant treatment or shortly (within 4–6 weeks) after surgery, as adjuvant chemotherapy. Neoadjuvant chemotherapy is offered to patients with large tumours in an attempt to shrink the tumour and to assess treatment response. Chemotherapy has revolutionized cancer therapy and saved millions of lives since its implementation. Its mode of action is to attack and kill highly proliferative cells. Unfortunately, the cytotoxic effects of chemotherapeutic agents are not specific, and they also affect healthy cells that show rapid turnover, including certain immune cells (haemopoietic cells of the bone marrow), cells of the gastrointestinal tract (enterocytes), and hair follicle cells, causing sometimes severe and long-term side effects. As, such, patients often suffer immunosuppression (neutropenia), nausea, and hair loss. Chemotherapy may also induce severe allergic reactions, neuropathy, fatigue, and heart toxicity, depending on the treatment regime, dose, and frequency. Some of these side effects are addressed by simultaneously administering other, often quite potent, drugs. Furthermore, many chemotherapeutic agents have mutagenic, teratogenic, and/or carcinogenic effects and thus need to be handled with caution upon preparation, administration, and

elimination. This activity also implies that chemotherapy may in itself cause secondary cancer. Thus, chemotherapy comes with a high price, and the time-span of such treatment is therefore limited to weeks or months rather than years.

1.3.3.2 Anti-HER2 therapy

Previously, $HER2^+$ was considered to be a very aggressive form of breast cancer with a poor prognosis, as patients with $HER2^+$ tumours experience a poor response to standard chemotherapy. Today, this subtype of breast cancer is treated with a monoclonal antibody directed against the HER2 protein, thereby blocking this receptor. The anti-HER2 drugs such as trastuzumab, pertuzumab, neratinib and lapatinib have thus reduced the risk of recurrence by 45%-50% in this patient group [45,67].

1.3.3.3 Endocrine treatment

For hormone-positive breast cancers, endocrine therapy for 5–10 years is part of the adjuvant systemic treatment; endocrine therapy reduces mortality by 30% in HR^+ patients [68], and these patients thus have a more favourable diagnosis than do those with HR^- disease. The purpose of endocrine treatment in breast cancer is to prevent the observed proliferative effects that hormones have on HR^+ tumour cells. Tamoxifen, or rather its active metabolites, acts by blocking oestrogen from binding to the ER and is prescribed for premenopausal breast cancer patients. For some premenopausal patients, ovarian suppression offers additional survival benefit [69]. Such suppression can be achieved either temporarily by the effects of pharmacological treatment or

permanently by surgical removal of the ovaries. For the postmenopausal patients, the standard endocrine treatment is usually aromatase inhibitors (AIs), although tamoxifen is an important alternative treatment in these patients, too. In postmenopausal women, oestrogen is no longer produced mainly in the ovaries, but arises from the conversion of peripheral androgens by the enzyme aromatase. The most common AI's are anastrozole, letrozole, and exemestane; these drugs act by selectively inhibiting the activity of aromatase, and subsequently the oestrogen synthesis peripheral tissue [70]. Results from the comprehensive 10-year follow-up ATAC (Arimidex, Tamoxifen, Alone or in Combination) study revealed a superior effect of the AI anastrozole compared to tamoxifen, for both survival and tolerability among postmenopausal patients [71]. Tamoxifen treatment will be more extensively discussed in section 1.3.3. For the TNBCs, neither endocrine nor anti-HER2 treatment has any effect because these cancers do not express the corresponding receptors. Because of the lack of treatment options (chemotherapy only), this group unfortunately has the most unfavourable prognosis.

Of note, the prognoses for breast cancer today are based on conclusions drawn from studies that sometimes are decade old. We still do not see the potential long-term (>15 years) effects of treatments such as introducing taxanes in chemotherapy, AIs, or trastuzumab for $HER2^{+}$ patients [61]. For this reason, the prognoses for breast cancer will likely continue to improve.

1.4 *Challenges and Controversies in Breast Cancer Diagnostics and Management*

Because of continuously evolving treatment regimes, breast cancer prognosis has gotten better over the last decades, with an overall 5-year relative survival rate of 90.7% [22]. For early stage and well-differentiated HR^+ cancers with low proliferation, the prognoses are especially good; at 100% 5-year survival for stage I cancer (tumour size ≤ 2 cm, LN^-) [72]. Nevertheless, the risk of recurrence remains for as long as 20 years after the time of diagnosis, although most recur within the first 5–10 years [73]. The sooner the cancer is detected and treatment initiated, the more likely the patient is to be cured of the disease. One of the main challenges in breast cancer is to precisely identify which patients have a higher risk of developing recurrences and metastasis. If the cancer returns in the same location, the other breast, or in lymph nodes, the patient may still be cured, whereas distant recurrence is usually tantamount with dying of the disease. Despite extensive study, several challenges and controversies persist concerning breast cancer diagnostics and how best to cope with it. Major areas of discussion are the cost–benefit balance of mammography screening, genetic testing, the different types of surgery, the most optimal sequence or combinations of chemotherapy, the amounts of therapy, and additional treatments. These aspects will not be discussed further. In the following, this thesis focuses on the many different challenges in breast cancer arising from tumour heterogeneity and subsequent issues such as overtreatment and undertreatment.

1.4.1 Tumour heterogeneity

Tumour heterogeneity, which is perhaps one of the most prominent features of this disease, lies at the core of the many challenges ahead of achieving fully individualized treatment for every patient. Traditionally, breast cancer has been regarded as one disease, whereas today, it is widely recognized that breast cancer should be viewed as several distinct cancer types. Although all of these diseases arise in the breast, they may behave very differently depending on their cell biology, morphology, classification, staging, grade, and molecular subtype. As renowned surgeon and breast cancer researcher Bernard Fisher (1918-2019), stated in his retrospective commentary:

It must be appreciated that a tumor is highly likely to be composed of cells having more than one histologic type—cells that, though predominantly of one nuclear grade, might also be of other grades associated with different nuclear size, pleomorphism, prominence of nucleoli, mitosis, and DNA indices [74].

Breast cancer shows a large degree of heterogeneity, both between different patients and within each individual tumour, which ultimately plays an important part in therapy response and the course of disease. *Intertumoural* heterogeneity concerns the differences observed between different patients having the same type of cancer, as is reflected in the many different classifications of breast cancer (i.e., histological and molecular subtype, grade, receptor status, proliferation, etc.). *Intratumoural* heterogeneity references the different characteristics

within a tumour; one area of the tumour might display a typical morphological pattern, whereas in another distinct area, quite the opposite or very different characteristics could co-exist. For instance, the expression of certain receptors, or the degree of proliferation is not necessarily evenly distributed over the entire tumour region[75] (**Figure 12**), a situation known as called spatial heterogeneity.

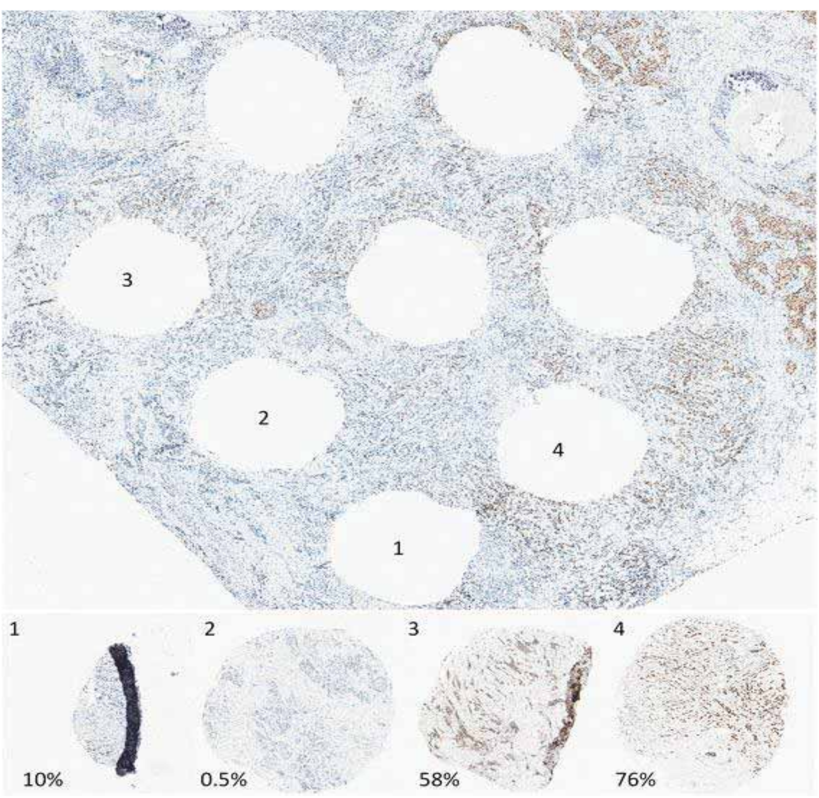

Figure 12. Heterogeneous ER expression.
Tumour heterogeneity exemplified by heterogeneous ER expression demonstrating the variability in staining across the whole tissue section, reflected in the variable expression levels in core 1-4. Copyright© Allot et al. *Intratumoral heterogeneity as a source of discordance in breast cancer biomarker classification.* Breast Cancer Res. 2016, doi: 10.1186/s13058-016-0725-1. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

How the tumour changes over time, or its temporal heterogeneity, further adds to this complexity [76]. Naturally, these variations complicate matters and are important to consider in breast cancer diagnostics. A given tissue biopsy represents a snapshot of the tumour at a fixed location and time point. How well it reflects the biology of that particular cancer will inevitably be associated with some degree of uncertainty.

Genetic heterogeneity, both within and among cancers, results from genome instability and mutations, two of the hallmarks of cancer. Practically all tumours more or less display some degree of genetic heterogeneity and thus consist of several subclones with different characteristics. These differences arise because during cell division, new mutations can occur or disappear, and over time, two cells with a common ancestor will become increasingly different from one another [6]. This divergence lies behind the concept of treatment resistance and cancer progression and is why a metastatic lesion might be quite different from the tumour mass where it originated. Selection pressure from influences such as drugs or endogenous factors from surrounding cells will create tumour subclones that have acquired treatment resistance or are more adapted to the environment, thus favouring cell survival and proliferation of those better suited clones. Thus, the cancer progresses, potentially with metastatic tumour formation of the subclone with acquired mutations [77]. During the regional evolution of cancer, two cells exposed to the same environmental stimulus might acquire different driver mutations, or one cell might develop two driver mutations. The

process of intratumoural heterogeneity and clonal evolution is depicted and summarized in **Figure 13**. The tissue and cells surrounding the tumour, i.e., the microenvironment, also play an important part in this respect and will be further described in section 1.6.

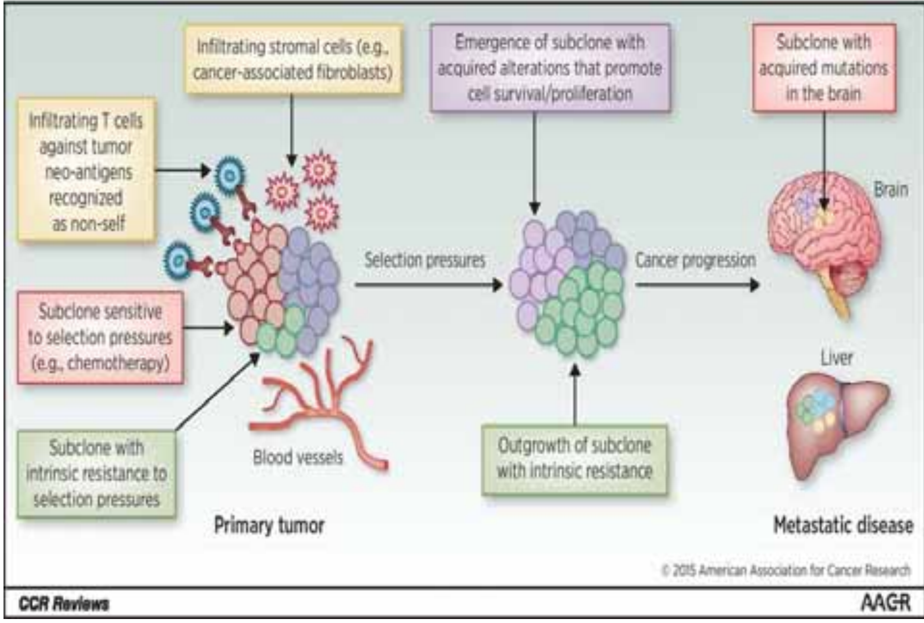

Figure 13. Intratumoural Heterogeneity and Clonal Evolution.
Microenvironmental factors such as hypoxia or infiltrating stromal or immune cells and external factors such as chemotherapy will exert selection pressure on a tumour's different subclones. Any subclones with intrinsic resistance (green) to such pressure or better suited to the environment because of acquired somatic alterations (purple) will have a survival advantage and outgrow those clones that are sensitive to a given therapy or recognized by immune cells (red). This situation will then lead to proliferation and metastatic formation of heterogenetic clones. Reprinted from Clinical Cancer Research, American Association for Cancer Research, 2015, Volume 21/Issue 6, Pages 1258-66. Mariam Jamal-Hanjani et al., Translational Implications of Tumor Heterogeneity, with permission from AACR.

1.4.2 Overtreatment and undertreatment

Breast cancer treatment has come a long way since William Halsted performed the first radical mastectomy and the early days of chemotherapy, when all women diagnosed with breast cancer were assigned almost the same treatment. A tremendous effort in breast cancer research has led to today's more personalized treatment. For every patient, the tumour specimen is carefully inspected to answer as many questions as possible and provide the most suitable therapy for that particular patient. This situation is reflected in the encouraging high survival rates for most patients breast cancer. Despite this joint effort and recent advances, many women will eventually die of their disease, either because of a higher tumour load at diagnosis or because of an early or late recurrence. Meanwhile, some patients who receive intensive chemotherapy and spend years on anti-hormonal medicines, could have managed well without them, because they would never have relapsed. These patients are in effect overtreated. For others, the treatment is inadequate or even futile, with no further benefit in survival, so that they are undertreated. Adjuvant therapy may consist of toxic drugs with potentially serious and long-term side effects, sometimes with a profound impact on a patient's quality of life. Yet the ever-present risk of recurrence, however small, leaves doctors and patients alike reluctant to opt for no additional treatment at all. Implicitly, patients are being overtreated or undertreated. Obviously, under-treatment has the most detrimental consequences for the patient, whereas overtreatment is a much more widespread problem.

So-called persistent adverse effects, or long-term side effects of cancer treatment, may substantially lessen the patient's quality of life and cause sick leave, sometimes resulting in chronic disability and an inability to work. One of the most prominent side effects is cancer-related fatigue, sometimes defined as "an overwhelming sense of tiredness, lack of energy, and a feeling of exhaustion" [78]. Although the exact biological mechanisms of treatment-induced fatigue are unknown, it is thought to be caused by the physiological strain induced on the body following systemic chemotherapy and/or radiotherapy [79]. Some studies indicate that such cancer-related fatigue may persist for several years in approximately 30 % of patients with breast cancer [80]. Breast cancer is already the second most expensive cancer in Norway in terms of treatment [81], and on top of that comes the medical costs related to persistent adverse side effects and the societal costs related to "cured" patients who are not returning to the active work force. A more targeted and cost-effective treatment of this relatively large patient group would benefit not only patients but the greater society as well.

1.4.3 Treatment resistance, focusing on tamoxifen

Luckily, most patients with early breast cancer are cured, although the threat of recurrence may remain for decades. Nevertheless, despite recent improvements and multimodal therapy options for breast cancers, regrettably, not all patients will experience a response to the current treatment regimes. Because of the aggressiveness of the cancer or genetic tumour heterogeneity, some patients respond poorly or become resistant

to treatment. This heterogeneity also explains the observation that two patients with similar tumour characteristics receiving the same treatment may experience very different treatment response and outcome.

The anti-oestrogen drug tamoxifen (tamoxifen citrate) was originally discovered in the 1960`s in the search for new contraceptive agents, but conversely it turned out to be more effective as fertility medication because it induced ovulation. In late 1977, tamoxifen was approved by the Food and Drug Administration (FDA) [82], then in 1988, the Early Breast Cancers Trialists' Collaborative Group (EBCTCG) published a large meta-analysis clearly demonstrating the positive effect adjuvant tamoxifen had on breast cancer survival [83]. Since then, tamoxifen has been used as a standard treatment regime for HR^+ premenopausal breast cancer, with (usually) 20 mg taken daily orally. Tamoxifen is on the World Health Organization's list of essential drugs, estimated to have improved the survival of millions of breast cancer patients [84]. It has also been shown to reduce the incidence of breast cancer in high-risk patients [85]. Based on the results of the international ATLAS (Adjuvant Tamoxifen Longer Against Shorter) clinical trial, the EBCTCG guidelines recommend extending the tamoxifen treatment from 5 to 10 years for some patients [86].

Tamoxifen is a selective ER modulator; it binds selectively to ER and blocks the ligand oestradiol, preventing ER activation and downstream tumour cell proliferation [87,88]. Tamoxifen is pharmacologically tissue-specific and has both agonistic (uterus) [89] and antagonistic (breast) [90]

effects on oestrogen, through recruitment of either coactivators or corepressors to the ER transcription complex [91]. Tamoxifen is metabolized in the liver by various enzymes such as cytochrome P450 2D6 (CYP2D6) into 4-hydroxy-*N*-desmethyl tamoxifen (endoxifen) and 4-hydroxytamoxifen (4-OHT), the two main active metabolites of tamoxifen. In breast cancer cells, these metabolites exert their effects by stopping oestrogen from binding to the ER, thus preventing proliferation and cell growth [92,93].

Because of genetic polymorphisms in drug-metabolizing enzymes and cancer cells being under drug therapy selection pressure (clonal expansion), treatment resistance is a well-known problem in chemotherapy, anti-HER2 therapy, and endocrine therapy. Despite the undisputable success of tamoxifen, challenges and controversies are related to it. The principal challenge with tamoxifen is drug resistance. As many as 30% of patients treated with tamoxifen are or become resistant to the drug [94,95]. For some of these patients, tamoxifen has no effect because the cancer cells are resistant to the drug from start, showing intrinsic resistance. For others, the cells might be sensitive to the drug at treatment initiation but develop resistance over time, or acquire resistance. Despite a substantial amount of research over the years, the exact mechanisms underlying resistance are not fully understood, but different mechanisms likely distinguish the two types. Results point to involvement of modification/mutations or loss of ER expression, changes in the activity of drug-metabolizing enzymes (e.g., CYP2D6), disruption of the balance of co-regulatory proteins, activation

of alternative tumour-promoting signal transduction pathways, or altered expression of certain microRNAs (miRNAs) [96-98] (**Figure 14; Appendix 2**).

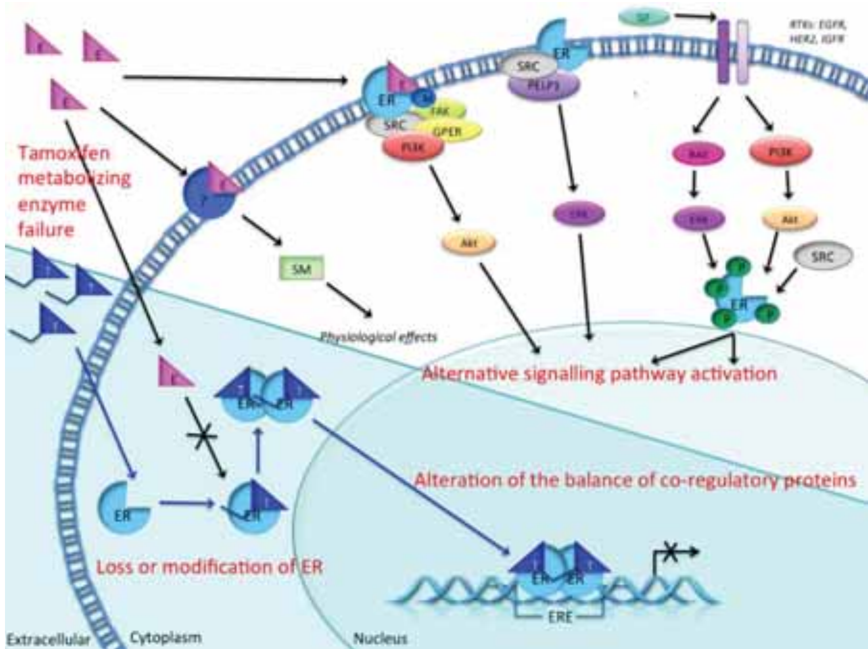

Figure 14. Possible Mechanisms of Endocrine Resistance.
The tamoxifen pathway and possible mechanisms of endocrine resistance in breast cancer cells: In the liver, tamoxifen (T) is metabolized into two active metabolites, endoxifen and 4-hydroxytamoxifen (4-OHT). Intracellularly, these metabolites can bind to oestrogen receptors (ERs), blocking the binding of its ligand, oestrogen. Upon binding with tamoxifen metabolites, ERs dimerize, resulting in an ER–tamoxifen-complex, which enters the nucleus and binds to the oestrogen response element (ERE). However, gene transcription will not be successfully activated because the ER–tamoxifen complex leads to altered balance in co-regulatory proteins. In the case of tamoxifen resistance, this blocking is compromised, likely because of a mechanism such as changes in the activity of tamoxifen-metabolizing enzymes, alternative signalling pathways for proliferation and growth, loss or modification of ER expression, alterations in the balance of co-regulatory proteins, or altered expression of microRNAs. Figure from [98] Egeland, N.G.; Lunde, S.; Jonsdottir, K.; Lende, T.H.; Cronin-Fenton, D.; Gilje, B.; Janssen, E.A.M.; Søiland, H. The Role of MicroRNAs as Predictors of Response to Tamoxifen Treatment in Breast Cancer Patients. *Int. J. Mol. Sci.* **2015**, *16*, 24243-24275 (open access article distributed under the Creative Commons Attribution License).

The most obvious predictive biomarker for tamoxifen resistance is, of course, the nuclear expression of the hormone receptors ER and PR. The exact role of PR in breast cancer is uncertain, although studies suggest that it has additional prognostic value to ER and might be independently important [99]. Some studies suggest that PR expression is in fact more important for benefit/resistance of tamoxifen than ER [41,100].

Another controversy is the distinction of ER^+ versus ER^- [101]. Although ER is measured immunohistochemically as the expression of positively stained cells on a continuous scale, the clinical cut-off is dichotomous (positive or negative). Previously, a cut-off of 10% cells positive for ER expression was regarded as ER-positive. In Norway, ER status is reported as percentage positive ER staining, to be <1%, from 1% to 10%, >10% up to 50%, or >50% [27]. Currently, any expression above 1% staining is considered ER-positive. Obviously, if one takes into account the biology and the known intratumoural heterogeneity of many breast cancers, this either/or categorization may not always reflect the biology in which ER may be expressed spatially. Intuitively, one expects a tumour with 86% ER expression to be somewhat different from a tumour with 2% positive cells and that no exact true value exists. This could be a problem both considering treatment benefit and in studies of ER^+ versus ER^- disease. However, reports suggest that any positivity of ER might make the tumour sensitive to tamoxifen, and 1% remains the current recommended cut-off [102,103]. Recently, the American Society of Clinical Oncology/College of American Pathologists recommended that ER^+ tumours within the range 1%–10% to be reported as ER low

positive, acknowledging the uncertainties associated with endocrine treatment benefit for these patients [103]. ER co-expression with PR seems to be associated with additional benefit from endocrine treatment [41,99].

Another factor reducing the effectiveness of tamoxifen treatment is that a substantial amount (>30%) of patients do not take their medication as prescribed [104-106]. This low adherence may be because of loss of motivation or adverse side effects [106-108]. Poor adherence to treatment is becoming an even bigger issue now that the Norwegian guidelines advise adjuvant tamoxifen for up to 10 years for certain patient groups.[72] If we are to treat these patients with a drug with potentially serious side effects for an entire decade, then we ought to ensure its efficacy. Doing so calls for markers hat are better able to select those patients who eventually will develop resistant disease. In this respect, a non-invasive liquid marker to monitor the effect of treatment and identify the development of resistance would be ideal. The use would be not only to monitor the effect of treatment but also to find the optimal concentration of endoxifen needed to balance the anti-tumour effect while circumventing side effects. In fact, some studies suggest that a dose as low as 5 mg of tamoxifen could maintain treatment efficacy, and this dose could be an option for those patients experiencing the most or severe side effects [85].

1.4.4 Ki-67 as a proliferation marker

Discovered more than 35 years ago, Ki-67 is a nuclear protein expressed in all cells that enter the cell cycle from late G1 to M-phase, but not in resting cells [109]. Consequently, IHC staining with antibodies against Ki-

67 protein has been widely used to assess the degree of cell proliferation, especially in cancer diagnostics. Ki-67 is encoded by the *MKI67* gene and located on chromosome 10. Although it is expressed in all phases of the cell cycle except G0, its level of expression changes through the cycle, and following mitosis, it exhibits a rapid turnover with a half-life of only one hour [110]. Although its functional role is not fully known, studies suggest that it may also play a role in rRNA synthesis [111], heterochromatin organization [112], and cell cycle regulation [113].

Ki-67 is an established yet controversial biomarker because the challenges associated with breast tumour heterogeneity also apply to Ki-67 expression. Together with MAI/mitotic count and PPH3, Ki-67 immunostaining has been widely used as a marker of proliferation in breast cancer. **Figure 15** depicts examples of low, intermediate, and high expression of Ki-67. In 2018, the national median value for Ki-67 was 17% in Norway, in accordance with the internationally reported median of 16%–27% [114].

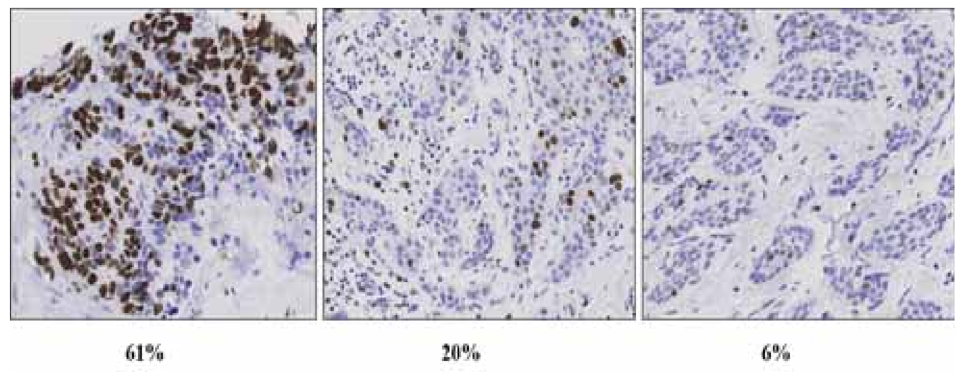

Figure 15. Representation of Ki-67 Expression.
Representation of high (61%), intermediate (20%), and low (6%) expression of Ki-67 on IHC in breast cancer tissue.

In breast cancer, early recurrence is associated with highly proliferating tumours [115], and the prognostic value of Ki-67 in breast cancer has been well documented in several studies [116-120]. In a large meta-analysis encompassing 60,000 patients in 41 studies, Ki-67 was confirmed as an independent prognostic marker for survival and recurrence in early stage breast cancer [121]. As such, proliferation can be used to guide treatment decisions for adjuvant chemotherapy [42]. In combination with histological grade, proliferation measured by Ki-67 index can be used as a surrogate marker to separate low-risk luminal A from the higher risk luminal B subtypes of breast cancer [122,123]. Ki-67 also has been used to estimate proliferation in TNBC. Furthermore, Ki-67 has predictive value for response to chemotherapy, as demonstrated in the neoadjuvant setting in which high Ki-67 scores predict complete pathological response [124].

As mentioned, a vast amount of studies underpin the prognostic value of Ki-67 in breast cancer, as well as its predictive value for the benefit of chemotherapy. Whether this evidence extends to Ki-67 having additional value in predicting treatment benefit from endocrine therapy is uncertain [125]. The few available studies are conflicting. For instance, in a review of 22 studies, Yerushalmi and colleagues examined both the prognostic and predictive potential of Ki-67 and concluded that no robust evidence supports the use of Ki-67 to identify patients who would benefit from a specific endocrine treatment [126]. Of interest, the Breast International Group (BIG)-1 98 trials demonstrated greater treatment benefit from the AI letrozole compared to tamoxifen treatment in patients with a high Ki-67 expression [120]. Furthermore, changes in Ki-67 expression (from high to low) in tumours following short-term (2 weeks) neoadjuvant endocrine therapy have also been suggested as a marker of treatment efficacy, whereas tumours with continuous high Ki-67 expression have been associated with lower-recurrence-free survival [127]. Also worth noting, after only 7 days of pre-surgical tamoxifen treatment, Cohen et al found a substantial decrease in Ki-67 expression [128]. In a randomized trial including 564 premenopausal women, those patients whose tumours expressed either high or low Ki-67 benefitted more from tamoxifen compared to those patients whose tumours expressed <u>intermediate</u> levels of Ki-67 [129]. These rather puzzling findings indicate that the relationship between Ki-67 and response to tamoxifen is highly complex. In a more recent study, Beelen et al examined the predictive value of Ki-67 in 563 post-menopausal women with ER^+ breast cancers and found that patients

with high Ki-67 did benefit from adjuvant tamoxifen [130]. These studies took place before the treatment regimens of tamoxifen were extended, and patients were only treated for 5 years or fewer.

Scoring of IHC Ki-67 is challenging, and international consensus is lacking regarding either the most optimal scoring methods or the most clinically relevant cut-off [131]. In fact, the reported optimal cut-off value for Ki-67 to separate luminal A/low risk from luminal B/higher risk patients, varies substantially among studies; and ranges from 10% to 30% [121,122,132-134]. Until recently, a cut-off value of 30% was recommended by the NBCG[135], but because of the laboratory and measurement variability, in their latest recommendations (as of 2020), the NBCG has left Ki-67-based decision making for adjuvant treatment in favour of genetic profiling (e.g. the Prosigna/PAM50 test) [61]. This decision is also in line with the American Society of Clinical Oncology Clinical Practice Guidelines [136]. Ki-67 is however still included in routine diagnostics, but is used as an alternative for genetic profiling, often in combination with histological grade and mitotic count [72].

Ki-67 is usually evaluated visually by quantifying the percentage of positively stained tumour cells on a microscope slide, which is a laborious and time-consuming effort. This observer-dependent approach makes Ki-67 quantification challenging and error-prone, leading to high variability both between observers (inter-observer variability) and among different laboratories. Interlaboratory discrepancies result from differences in pre-analytical practice and in detection and quantification

methods. Consequently, the reproducibility varies. The International Ki-67 in Breast Cancer Working Group aims to examine whether Ki-67 scoring can be adequately standardized and validated among different laboratories [131].

Digital pathology is becoming increasingly important in routine diagnostics and is scheduled to be implemented in Norway before 2022. Automated estimation of prognostic factors using digital image analysis (DIA) offers an observer-independent approach that is less prone to inaccuracy and more reproducible than standard visual assessment under a microscope. In the search for more standardized and reproducible quantification methods for Ki-67, DIA offers an objective, more reproducible, and faster method to determine the fraction of proliferating cells. Application of a high-throughput automated scoring system might eventually lead to a long-awaited standardization of quantification and perhaps to a consensus on the best clinical cut-off values for Ki-67.

1.5 *The Importance of Biomarkers*

Accurate and comprehensive pathology reports lie at the basis of any cancer diagnosis. They are the prerequisite for correct diagnosis, customized optimal treatment, and the best possible prognosis and outcome for the individual patient. Biomarkers are central in the pursuit of as much biological information as possible about a specific patient and tumour and to ensure a correct diagnosis and treatment. There are several definitions of the term "biomarker", and according to the NIH (U. S. National Institutes of Health), a biomarker has the following definition:

*A biomarker is a characteristic that is **objectively** measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention* [137].

Biomarkers can be found in tissue, blood, or other body fluids and can provide information about a variety of bodily functions and disease states. According to the FDA-NIH Biomarker Working Group, there are different categories of biomarkers, including in the categories of susceptibility/risk, diagnostics, monitoring, prognostics, prediction, pharmacodynamics/response, and safety. Two of these categories, the prognostic and predictive biomarkers, are particularly interesting when it comes to breast cancer. Prognostic biomarkers are used to *identify the likelihood of a clinical event, disease recurrence, or progression in patients who have the disease or medical condition of interest.* They are independent measures of prognosis concerning a patient's overall

clinical outcome (i.e., the risk of recurrence, survival, and mortality). Predictive biomarkers can be used to i*dentify individuals who are more likely than similar individuals without the biomarker to experience a favourable or unfavourable effect from exposure to a medical product or an environmental agent,* or in short, predict whether a patient will respond to a given therapy [138]. Biomarkers can be prognostic, predictive, or both and it is important not to confuse the two. For example, the presence of ER in a tumour indicates that it is of the luminal type and thus has a favourable prognosis; as such, ER is a prognostic marker. Meanwhile, expression of ER also can mean that this cancer will likely respond to endocrine therapy; thus, it is also a predictive marker.

1.5.1 Biomarkers in breast cancer

As of today, the biomarkers used for breast cancer are primarily the traditional clinical markers (lymph node status, age, tumour size, histological grade) and the immunohistochemically detectable markers (hormonal status (ER/PR), HER2-), and proliferation as measured by MAI, mitotic count, or Ki-67. In the last decade, the use of gene expression analyses for breast cancer has increased dramatically and may help determine risk of recurrence and response to treatment more precisely. Commercially available diagnostic or prognostic gene expression profile tests have been introduced in the clinic, such as the FDA-approved MammaPrint® 70-gene Breast Cancer Recurrence Assay, the Onco*type* DX® Breast Recurrence Score test (21 genes), and the

Prosigna® Breast Cancer Prognostic Gene Signature Assay (PAM50), discussed in the *Breast Cancer Subtypes* section above.

Another promising tool is the use of liquid biomarkers. Liquid biomarkers are derived from urine or the peripheral blood, where factors such as circulating tumour DNA (ctDNA) [139], circulating tumour cells, exosomes, or miRNAs originating from the primary tumour and/or metastatic deposits may be identified and analysed [140,141]. Tissue biopsies are cumbersome to acquire and often involve some degree of discomfort for the patient. Liquid biopsies, however, are much easier to sample and represent a rather less-invasive way to examine the state of disease and developing mutations and provide an opportunity for tumour- or therapeutic monitoring/surveillance. The challenge with circulating cancer biomarkers is finding the most suitable biofluid and detection methods that are sensitive enough to detect the small amount of biomarker present in these samples [142].

To be of clinical value, a biomarker must provide useful information and be sufficiently robust enough to be accurately measured. The measurements need to be reproducible across different platforms/observers and patients, and in addition to analytical and clinical validity, any biomarker must have clinical utility [143]. The discriminatory power of a marker means its ability to distinguish between a "positive" or "negative" test result. An ideal diagnostic marker would have a sensitivity of 100%, and at the same time be 100% specific.

This utopian scenario would mean that every person with the disease would be accurately identified, without there ever being any false negatives or false positives.

To date, no test or biomarker meets this utopian ideal. In reality, there will always be some degree of overlap where individuals without the disease will have a positive test, and vice versa. The objective must be a trade-off with the aim to maximize both the sensitivity and specificity [138]. For the prognostic and/or predictive breast cancer tests, a large portion of patients assigned to the high-risk group will never develop recurrence, and conversely, some patients defined as having low risk might still develop recurrence. For those with intermediate risk, often no clear guidelines exists. Obviously, there is a need for biomarker improvement and for even better refinement before, during, and after a breast cancer diagnosis. For instance, despite genetic risk assessment and routine mammography screening, we still cannot predict who will or will not develop invasive breast cancer. Even for the well-described BRCA mutation carriers, risk assessments are not completely clear and concise because the observed penetrance of the mutations is not at 100% [144]. Another example is that not all screening-detected lesions or CIS will develop into invasive cancer. At the time of diagnosis, most patients are eager to find out their prognosis and expect to receive the most optimal treatment. In truth, we cannot precisely predict the exact outcome for any patient and can rely only on statistics drawn from previous studies of similar events. We also cannot predict the degree to which a patient will respond to treatment, and we cannot be certain that the initial tests will

disclose all the necessary information about a particular tumour's characteristics. In the aftermath of a breast cancer diagnosis, following the initial surgery and potential adjuvant treatment, patients are offered frequent follow-up in the first couple of years, then once a year until 10 years past the diagnosis. What constitutes this follow-up is mainly a routine physical examination of the breast(s) and nearby lymph nodes, an update of the clinical history, and for women treated with BCT, a mammogram. However, these follow-ups are limited to potential detection of any sign of either local or regional recurrence, not distant recurrences. Locoregional recurrences have the potential of being curable, whereas the systemic recurrences are those that threaten a patient's life, sometimes even several years after the initial diagnosis. The reason is that we still lack sufficiently reliable methods for early detection of systemic disease, before it becomes symptomatic.

1.5.2 The quest for novel biomarkers

Collectively, the limitations of current biomarkers in breast cancer means that we need to develop novel biomarkers to improve the diagnostics and monitoring of this disease. This need has created an abundance of biomarker studies and competing research. However, the process of identifying and implementing new potential biomarkers is laborious, costly, and painstakingly time-consuming. As reviewed by Baak in 2002, biomarker development studies need to follow good laboratory practice routines and require thorough work that is well organized and standardized, adhering to strict quality control and quality assurance. A

good and standardized protocol is necessary to ensure uniformity across different observers and laboratories. Access to patient cohorts and tumour material is another requirement. Candidate biomarkers should be validated both in-house and in several independent studies. Careful optimization and standardization of pre-analytical and analytical methods is needed to ensure that results are reliable across different laboratories. All of this requires huge amounts of resources when it comes to reagents, technical equipment, time, and last but not least, funding [145]. Many new biomarkers are discovered each year, and for the most part, funding for the search for new biomarkers in cancer is still relatively obtainable. Yet, because of the lack of newsworthiness, funding for validation studies is much more difficult to attain, and many research groups are therefore less interested in embarking on such studies; hence, many of these new biomarkers will never be validated.

Another important factor is access to skilled personnel. In cancer research, there has been a dramatic development in the understanding of the molecular biology of cancer, which has led to numerous new diagnostic tests and great advancements in bioinformatics, genomics, technological improvements, and computer power. This progress necessitates a multidisciplinary approach to modern cancer research and treatment. We need trained molecular biologists and other laboratory personnel to execute the different analyses and tests, biostatisticians skilled in bioinformatics to analyse the data, and pathologists and oncologists with enough knowledge about molecular biology to interpret

the information correctly. As reviewed by Pavlou et al [146], the journey of potential new biomarkers from "bench to bedside" may take a decade and can be viewed as a pipeline consisting of four main phases: preclinical explanatory studies, assay development, retrospective validation studies, and prospective validation studies (**Figure 16**).

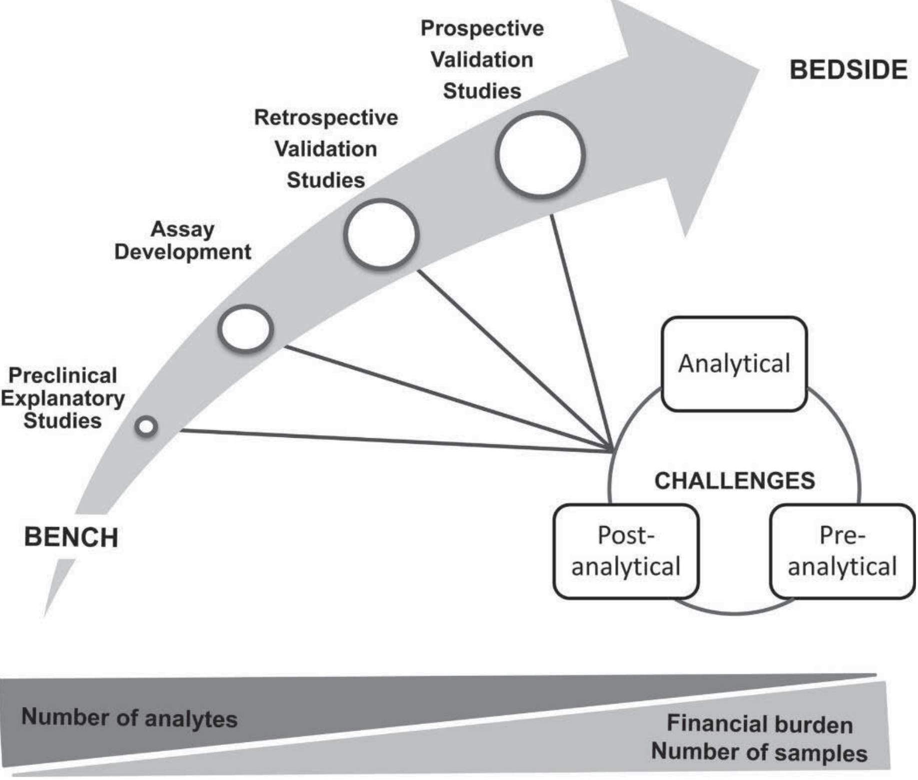

Figure 16. Pipeline of Biomarker Development.
Pipeline depicting the four main phases of biomarker development. There are several pre- and post-analytical challenges from the first phase of preclinical explanatory studies, assay development, and retrospective and prospective validation studies before a biomarker may be successfully implemented in the clinic. Pavlou, Maria P; Diamandis, Eleftherios P, The Long Journey of Cancer Biomarkers from the Bench to the Clinic, Clinical Chemistry, 2013, volume 59, issue number 1, 147-157, by permission of Oxford University Press.

Very few potential new biomarkers will succeed in reaching clinical validity; indeed, many promising candidates will fail along the way. Those biomarkers that might be considered for implementation into routine clinical practice also need be assessed for their cost benefit (the cost of the test vs the benefit for the patient/society as a whole).

1.5.3 The importance of biomarker validation and quality control

Several efforts to improve the quality of biomarker development research and implementation into the clinic have been made over the years. For instance, in 1996, Haynes et al proposed a Tumor Marker Utility Grading System, providing a utility score based on potential indicators: usefulness in risk assessment, screening, differential diagnosis, prognosis, and monitoring of clinical course. Furthermore, they introduced a scale of five different levels of evidence (LOEs) to help determine the clinical validity of a tumour marker and evaluation of marker studies [147,148]. In 2005, the European Group on Tumour Markers (EGTM) published their first guidelines on the use of tissue and serum biomarkers in breast cancer [149]. These guidelines have since been revised [150], and the latest recommendations are shown in **Table 3**.

Table 3. EGTM Recommendations on Breast Cancer Biomarkers.
Guidelines on the use of a selection of biomarkers in patients with invasive breast cancer: EGTM recommendations.

Biomarker	Recommendation	LOE	SOR
ER	For predicting the response to endocrine therapy in patients with early or advanced breast cancer. Mandatory in all patients.	IA	A
PR	In combination with ER for predicting response to endocrine therapy in patients with early or advanced breast cancer. Mandatory in all patients.	IB	A/B
HER2	For predicting response to anti-HER2 therapy in patients with early or advanced breast cancer. Mandatory in all patients.	IA	A
Ki-67	In combination with established clinical and pathological factors for determining prognosis in patients with newly diagnosed invasive breast cancer, especially if values are low or high.	IB	A/B
Oncotype DX	For determining prognosis and aiding decision making for the administration of adjuvant chemotherapy in patients with ER-positive HER2-negative lymph, node–negative and lymph node–positive (1–3 nodes) disease.	IB	A
MammaPrint	For determining prognosis and aiding decision making for the administration of adjuvant chemotherapy to patients with ER-positive, HER2-negative, lymph node–negative and lymph node–positive (1–3 nodes) disease.	IA	A
Prosigna® (PAM50)	For determining prognosis and aiding decision making for the administration of adjuvant chemotherapy to patients with ER-positive HER2-negative, lymph node–negative and lymph node–positive (1–3 nodes) disease.	IB	A

LOE: Level of Evidence, based on ref. [148]; **IA**: validation studies available=”none required”, **IB**: validation studies available=”one or more with consistent results”. **SOR**: Strength of Recommendation, based on ref.[151]. **A**: “recommendation based on consistent and good-qualitypatient-oriented evidence”. **B**: “recommendation based on inconsistent or limited quality patient-oriented evidence”. Table adapted from [150]. Published by Elsevier Ltd., https://creativecommons.org/licenses/by-ncnd/ 4.0/. Used with permission.

Another important tool in this respect, to which many scientific journals now adhere, are the Reporting recommendations for tumour marker prognostic studies, or the REMARK guidelines [152].

Just as important as finding new and better biomarkers is the validation of existing ones. Even after a biomarker has been successfully implemented, it is important to continue validating its usefulness in new independent patient cohorts and different clinical settings.

1.6 *miRNAs and Their Potential as Biomarkers*

Most pathology departments have large archives of formalin-fixed paraffin-embedded (FFPE) material from years back, often containing primary tumours, lymph nodes, metastatic biopsies, and vast amounts of tissue slides with various IHC staining. This store represents an often-overlooked resource that if used wisely could support medical research. In the search for novel prognostic and predictive biomarkers in breast cancer, miRNAs (designated individually as miR), which are quite stable and detectable in FFPE material, are now emerging as potential candidates.

miRNAs are a class of small non–protein-coding RNA molecules of ~9–24 base pairs in length, which are involved in RNA silencing and post-transcriptional regulation of gene expression. Studies suggest that miRNA interactions are in fact responsible for most of human mRNA regulation [153]. In recent years, it has been recognized that miRNAs may also upregulate gene expression [154,155]. According to the latest release of the online repository miRBase (v.22.1), 1917 mature human miRNA sequences have been identified so far [156-158]. However, many of them have not yet been validated and must be regarded as candidate miRNAs. Recently, Alles et al estimated the amount of true human microRNAs to be 2300, of which 1115 are annotated in the latest miRBase version [159].

The biosynthesis of miRNAs (**Figure 17**) is initiated in the nucleus where a primary miRNA transcript (pri-miRNA) is produced by polymerase II (or III), before the pri-miRNA precursor (80–100 nt) is

cleaved by the ribonuclease Drosha, resulting in a precursor hairpin structure, the pre-miRNA (~70 nt). From the nucleus, pre-miRNA is then exported by the Exportin-5/Ran-GTP complex to the cytoplasm. The endoribonuclease Dicer, together with the double-stranded RNA-binding protein TRBP, then cleaves the pre-miRNA to a mature-length miRNA duplex. The passenger strand is degraded, and together with Argonaute (Ago2) protein, the remaining functional strand of the mature miRNA is loaded into the RNA-induced silencing complex RISC. The final step is hybridization by base pairing and subsequent silencing of target mRNAs by either mRNA degradation, translational repression, or deadenylation, depending on the degree of base-pairing complementarity [153,160].

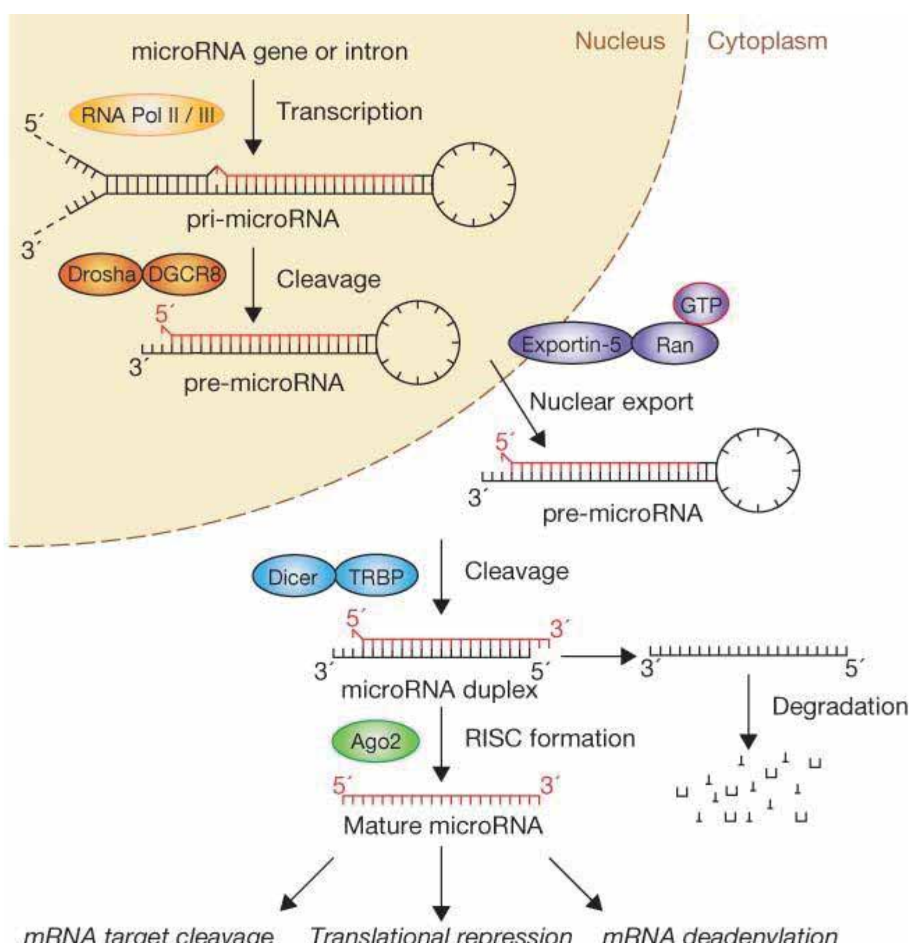

Figure 17. miRNA Biogenesis.
In the nucleus, a primary miRNA transcript (pri-miRNA) is produced by polymerase II before it is cleaved by Drosha, resulting in a precursor hairpin structure, the pre-miRNA. From the nucleus, pre-miRNA is exported by Exportin-5/Ran-GTP to the cytoplasm. The endoribonuclease Dicer, together with TRBP, then cleaves the pre-miRNA to a mature-length microRNA duplex. Together with Ago2, one functional (red) strand of mature miRNA is loaded into RISC, finally leading to silencing of target mRNAs by either mRNA cleavage, translational repression, or deadenylation. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, NATURE CELL BIOLOGY. Many roads to maturity: microRNA biogenesis pathways and their regulation, Julia Winter et al, [COPYRIGHT] (2009).

Each miRNA is (partially) complementary to one or several mRNA molecules, and some miRNAs are predicted to bind several hundred gene targets (mRNAs); meanwhile different miRNAs may also target the same gene [161]. This redundancy means that miRNAs may affect several genes or pathways simultaneously, something that could be used therapeutically as well.

miRNAs occur in all tissues and biofluids, and display temporal and spatial expression [162,163]. Since their discovery in 1993 [164], it has become increasingly clear that miRNAs are involved in a substantial number of cellular functions and play an important role in physiological and pathophysiological processes, including cancer [165-167]. In normal tissue, proper miRNA transcription, processing, and binding to complementary sequences on the target mRNA results in normal rates of cellular growth, proliferation, differentiation, and cell death. In cancer, however, miRNA expression is often dysregulated [168]. The miRNA sequences are often found within genomic regions involved in cancer or at fragile sites [169], and they can be involved in several of the hallmarks of cancer [170]. For instance, proliferation and invasion are associated with elevated expression of miR-210 [171]; miR-126, which suppress cell growth, is elevated in breast cancer cells [172], and metastatic breast cancer cells exhibit high expression of miR-10b [173]. miRNAs have also been associated with endocrine treatment resistance in breast cancer. Some candidate miRNAs involved in tamoxifen resistance include miR-10a, miR-26, miR-30c, miR-126a, miR-210, miR-342 and miR-519a [98].

Because miRNAs exert their effect at the translational level, they could provide an important connection between coding genes and various cellular processes. However, despite the increasing number of studies on miRNAs and their potential role as cancer biomarkers, as of yet, few have made it into the clinic. Mandujano-Tinoco et al recently reviewed the emerging roles for miRNAs in breast cancer, and found several of them to have potential clinical applications [174].

1.6.1 miR-18a and miR-18b in breast cancer

miR-18a and miR-18b are paralogues of the same microRNA family [175]. They are both 23 nucleotides long and their sequences differ by only one nucleotide. miR-18a belongs to the miR-17~92 cluster located on chromosome 13, whereas miR-18b belongs to the miR-106a~363 cluster located on chromosome X [176,177] (**Figure 18**).

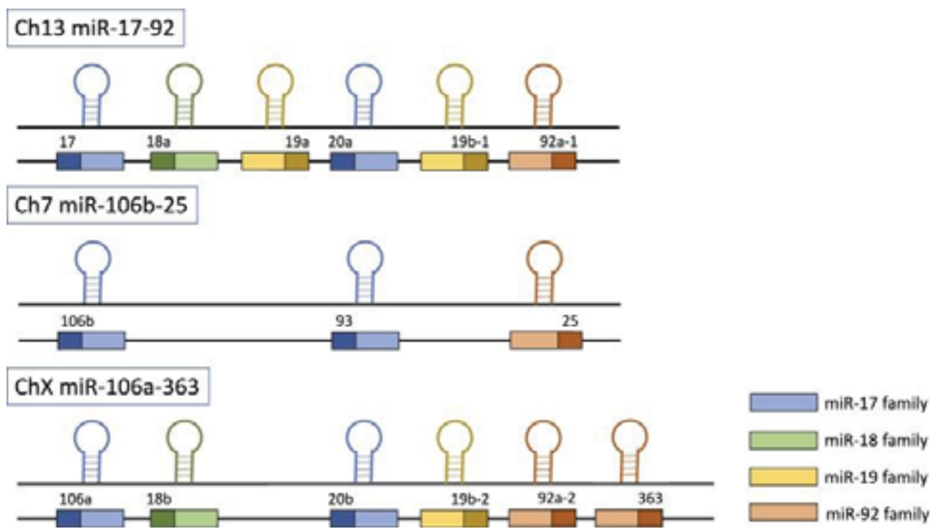

Figure 18. The miR-18-Family and its Homologs.
Illustration of the gene structure of the miR-17-92 cluster located on chromosome 13, comprising miR-18a; and its two mammalian paralogs: the miR-106b-25 cluster located on human chromosome 7, and the miR-106a-363 cluster located on the X chromosome, which comprises miR-18b. The miRNAs encoded by these three clusters are categorized into the miR-17-, miR-18-, miR-19- and miR-92 family. Figure from [175] Shen et al. Clin Trans Med (2019). The Dual Functional Role of MicroRNA-18a (miR-18a) in Cancer Development. 8:32 https://doi.org/10.1186/s40169-019-0250-9. Used with permission under the Creative Commons Attribution Non-Commercial License https://creativecommons.org/licenses/by/4.0/.

miR-18a and miR-18b show higher expression in many different tumour types, and especially in more advanced tumours, and are therefore described mostly as oncomiRNAs [176,178,179]. For instance, miR-18a is upregulated in hepatocellular carcinoma (HCC) tissue and cell lines and promotes cell migration and proliferation in HCC cell lines [180]. Similarly, miR-18b is upregulated in gastric cancer tissue and cell lines and involved in invasion and lymph node metastasis [181]. However, previous studies have described miR-18a as both a tumour suppressor [182], and an

oncomiRNA [183,184]. Likewise, miR-18b is reported to be upregulated in breast cancer cell lines and involved in cell migration [185], whereas in a melanoma cell line experiment, overexpression of miR-18b produced tumour suppressor activity [186].

Although these miRNAs have been examined previously in several studies, their exact function in breast cancer is still not fully elucidated. However, several studies have shown that the expression of miR-18a and miR-18b is associated with ER status [185,187-189], and some research suggests that ER might be a direct target of miR-18a [187,190,191]. Furthermore, microarray gene expression of miR-18a and miR-18b has previously been correlated with ER negativity, high proliferation, and cytokeratin 5/6 in breast cancer [189,192]. These findings have since been confirmed by other studies [193,194]. Of interest, circulating miR-18a has been detected in serum samples, and studies suggest it could act as a potential biomarker for early detection of cancer [195,196], whereas in TNBC, miR-18b in serum has prognostic value [193].

1.7 *The Tumour Microenvironment*

In recent years, accumulating evidence has emerged on the importance of the stromal tissue, or the tissue surrounding the tumour cells, i.e., the TME, in cancer. Indeed, the importance of TME and its recruited cells is now included and emphasized in the updated core and emerging hallmarks of cancer, as reviewed by Hanahan and Coussens [197]. A given tissue consists not only of functional cells but also of a mixture of cells with mainly supportive functions. This latter part is called the stroma and contains cells such as connective tissue, blood vessels, adipocytes, nerves, and a heterogeneous collection of immune cells. As mentioned earlier, breast cancer often starts in epithelial cells of the TDLU, the functional compartment of breast tissue, but the collection of cells surrounding the tumour is also important to consider. Stromal cells and the TME are no longer regarded as static, passive bystanders with merely supportive functions but rather are now seen as an active component that may have substantial influence on both the initiation and progression of neighbouring tumour cells, sometimes with a significant impact on the course of disease.

The TME harbours ECM, blood and lymph vessels, and a variety of different cells such as neighbouring normal cells, cancer-associated fibroblasts (CAFs), and several immune cells such as T and B lymphocytes, natural killer (NK) cells, tumour-associated macrophages (TAMs), dendritic cells, and neutrophils (reviewed in [198,199]). The TME and surrounding stroma have emerged as important contributors to the complex interplay between tumour cells and immune cells, and to the

formation and development of neoplastic cells, with the tumour and the microenvironment having reciprocal influences on each other. Hanahan and Coussens extensively reviewed the contributing role that cells of the TME have on the hallmarks of cancer and tumour development [197]. Studies demonstrate that TME-associated cells interact with and partake in bidirectional crosstalk with cancer cells [200,201]. Furthermore, small molecules such as cytokines, chemokines, and growth factors are released into the TME, and contribute to the interaction between stromal and cancer cells [202].

1.7.1 Immune cells and the TME

Especially important cells in the TME are the many cancer-associated immune cells, which may be tumour promoting or tumour inhibiting, depending on signals released in the immediate surroundings and the type of immune cells involved [203]. Immune cells of both the innate and the adaptive immune systems are diverse and numerous, each having distinct functions with different implications for tumour development and progression. The different molecular subtypes in breast cancer display various degrees of immunogenicity, with the lowest immunogenicity in the luminal subtypes and highest in the HER2+ and TNBC [204,205]. The immune microenvironment also changes during carcinogenesis, with mostly anti-tumour actions involving release of cytokines, such as transforming growth factor (TGF)β, interferon (IFN)γ, and tumour necrosis factor (TNF)α, taking place in the early phases of cancer initiation. This milieu will shift to a pro-tumour environment after

the cancer has been established, when the cytokines instead promote cancer progression, metastasis, and growth [206-208].

As mentioned in section 1.1.7, some immune cells are directed against tumour cells, whereas others act in a pro-tumour manner, with extensive crosstalk between them. Many solid cancer types exhibit aggregations of immune cells infiltrating the malignant cells. Some of the most prominent cancer-associated immune cell types are the TILs, of which the majority are T cells [209], whereas B cells may constitute up to 40% [210]. B cells are antigen presenting and secrete antibodies and cytokines. Several studies have shown that TILs may have prognostic value in breast cancer, particularly in the triple-negative and HER2$^+$ subtypes (as reviewed in [211]). For instance, in a large study of approximately 12,000 patients with breast cancer, infiltration of cluster of differentiation (CD)8$^+$ T cells in both tumour and stroma was associated with a reduced risk for breast cancer–specific mortality in patients with either ER$^-$ and ER$^+$/HER2$^+$ disease [212].

The anti-tumour response involves several cell types. Breast tumour cells express tumour antigens, which are tumour-specific peptides displayed on the surface of APCs, recognized as non-self by the immune system and thereby eliciting an immune reaction. Tumour neoantigens are presented by a major histocompatibility complex (MHC) on APCs [213]. MHC class I (MHC I) is found on the surface of most human cells, whereas the MHC class II (MHC II) is found mainly in B cells, lymphocytes, and macrophages. Immune cells express glycoproteins,

CDs, of different classes on their cell surface, of which CD4 and CD8 are expressed and central for T cells. The main effector cells in the anti-tumour immune response are the cytotoxic $CD8^+$ T cells, which have the ability to kill tumour cells directly by inducing cell lysis or apoptosis. $CD8^+$ T cells are activated by neoantigens through the T-cell receptor (TCR). This activation causes release of the cytolytic molecules granzyme B and perforin, which will lead to tumour cell lysis. The innate immune response includes NK and NK T cells that also can directly kill tumour cells. Neoantigens additionally activate $CD4^+$ (helper) T cells, which secrete cytokines such as IFNγ, interleukin (IL)-2, and TNF, further stimulating the $CD8^+$ T-cells. Once activated, $CD8^+$ T cells may also upregulate the expression of Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), subsequently inducing apoptotic pathways directed towards tumour cells [204]

The pro-tumour responses are equally complicated. Tumour cells can suppress the immune response through regulatory T cells (Tregs), induced by TAMs and tumour and/or CAF-secreted factors such as TGF-β, and inhibit activation of $CD4^+$ and $CD8^+$ T cells. Through IL-10 secretion, TAMs and Tregs may also inhibit the activity of APCs. The presence of tumour antigens and activated effector T cells causes upregulation of immune checkpoint inhibitors such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death 1 ligand 1 (PD-L1), leading to suppression of the anti-tumour immune response and creating a more pro-tumour microenvironment. This process is supplemented by recruitment of immunosuppressive cells,

myeloid-derived stromal cells (MDSCs), and Treg cells. MDSCs are recruited by tumour-secreted factors, and inhibit trafficking and activation of T cells. Secretion of VEGF and MMPs stimulates angiogenesis and invasion and contributes further to a pro-tumour microenvironment [60,204]. An overview of this extensive crosstalk between breast cancer TME and associated immune cells is shown in **Figure 19**.

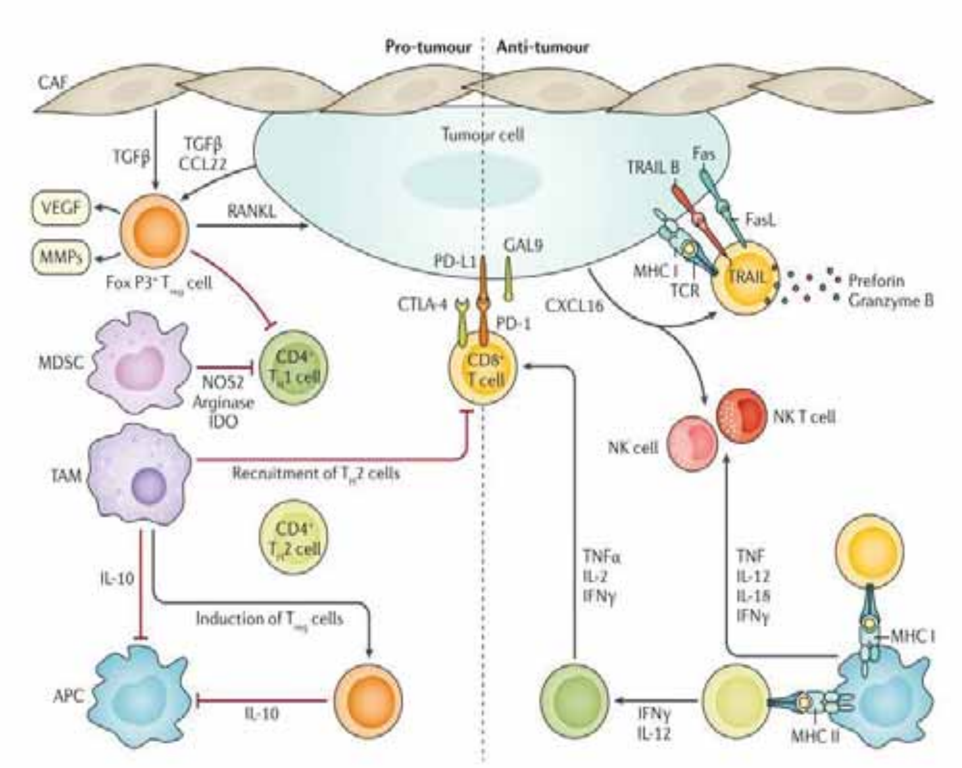

Figure 19. Immune Crosstalk in Breast Cancer TME.
Immune crosstalk in breast cancer TME involves both anti-tumour and pro-tumour activity. Anti-tumour: Breast tumour cells express neoantigens that elicit an immune reaction when being presented by MHC I or MHC II molecules on APCs. The main effector cells in the anti-tumour immune response are the cytotoxic $CD8^+$ T-cells, which are activated by neoantigens through the TCR. This activation causes release of the cytolytic molecules granzyme B and perforin, which exert their effects by tumour cell lysis. The innate immune response includes NK and NK T cells that also can directly kill tumour cells. Neoantigens also activate $CD4^+$ (helper) T cells, which secrete the cytokines IFNγ, IL-2, and TNF, further stimulating the $CD8^+$ T cells. Once activated, $CD8^+$ T cells may also upregulate the expression of FasL and TRAIL, subsequently inducing apoptotic pathways directed towards tumour cells. Pro-tumour: Tumour cells can suppress the immune response by Tregs, induced by TAMs and tumour and/or CAF-secreted factors such as TGFβ, inhibiting activation of $CD4^+$ and $CD8^+$ T cells. Through IL-10 secretion, TAMs and Tregs may also inhibit the activity of APCs. The presence of tumour antigens and activated effector T cells causes upregulation of immune checkpoint inhibitors such as CTLA-4 and PD-L1, leading to suppression of the anti-tumour immune response and creating a more pro-tumour microenvironment. This process is supplemented by recruitment of immunosuppressive cells, MDSCs, and Tregs. MDSCs are recruited by tumour-secreted factors, and inhibit trafficking and activation of T cells. Secretion of VEGF and MMPs stimulates angiogenesis and invasion, and contributes further to a pro-tumour microenvironment. **APC**: antigen-presenting cell. **CAF**: cancer-associated fibroblast. **CCL22**: CC-chemokine ligand 22. **CTLA-4:** cytotoxic T lymphocyte-associated protein 4. **CXCL16**: CXC-chemokine ligand 16. **FasL:** Fas ligand. **MDSCs:** myeloid-derived stromal cells. **MHC**: major histocompatibility complex. **MMP**: matrix metalloproteinase. **NK**: natural killer. **NOS**: nitric oxide synthase. **PD-1**: programmed cell death 1. **PD-L1**: programmed cell death 1 ligand 1. **RANKL**, receptor activator of nuclear factor-κB (RANK) ligand. **TAM**: tumour-associated macrophage. **TGFβ**: transforming growth factor-β**. TH1 cell**: type 1 T helper cell. **TRAIL**: TNF-related apoptosis-inducing ligand. **Treg**: T regulatory cell. **VEGF**: vascular endothelial growth factor. Adapted from ref. 75, CC-BY-4.0 https://creativecommons.org/licenses/by/4.0/. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, NATURE REVIEWS DISEASE PRIMERS (https://www.nature.com/nrdp/). Breast Cancer, [60] COPYRIGHT (2019).

Macrophages (meaning "big eater") are phagocytosing immune cells of the innate immune system that are tissue-resident under physiological conditions. They are recruited to the tumour site by cytokines released into the TME, where they engulf cells expressing tumour antigens. As noted, macrophages involved in cancer are called tumour-associated macrophages, or TAMs, and are the major components of immune infiltrate in cancer [214]. TAMs are involved in tumour progression and therapy resistance and are usually associated with unfavourable prognosis/worse outcome in breast cancer [215,216]. They may, however, play a dual role, depending on their phenotypic expression. For example, macrophages exhibit phenotypic plasticity, occurring as both M1 and M2 phenotypes, considered as two extremes on a functional scale. There are also the M0 cells, a non-polarized phenotype that, depending upon surrounding signals, can change to either the M1 or the M2 phenotype. Macrophages of the M1 phenotype secrete pro-inflammatory cytokines, whereas those of the M2 phenotype act mostly in an anti-inflammatory manner and are also involved in tissue remodelling and angiogenesis. TAMs are mostly of the M2 phenotype [217].

In parallel with the increasing knowledge of the key role the TME plays in cancer immunology, targeted immunotherapy has emerged as a promising new approach to cancer therapy. The immune system and immune cells of the TME can play significant roles in treatment response and are therefore interesting as candidate therapeutic targets [218]. Immunotherapy takes advantage of the body's existing intricate immune system and associated immune cells to combat cancer, by either boosting

anti-tumourigenic or inhibiting pro-tumourigenic immune cells or pathways. The objective for this approach is to achieve a more target-specific therapy directed only against cancer cells, as opposed to the more generalized range of action from traditional adjuvant systemic treatments such as chemotherapeutic agents. Several clinical studies are investigating the potential of immunosuppressive therapy for breast cancer, in particular the so-called immune checkpoint inhibitors. Immune checkpoints (e.g., CTLA-4, PD-1, PD-L1) are proteins expressed on the surfaces of T cells and/or other cells and are important regulators in the immune system, preventing T cells from attacking healthy cells. However, cancer cells may exploit these checkpoint proteins to avoid being recognized as harmful non-self. Immune checkpoint inhibitors are immune modulators that “release the brakes” on the immune system, so that T cells may again recognize checkpoint proteins [219]. Emerging immune checkpoint inhibitors for use in breast cancer are the PD-1 inhibitors pembrolizumab and nivolumab, and the PD-L1 inhibitor atezolizumab [220], as well as the CTLA-4 inhibitor ipilimumab. These drugs are indicated primarily for immunogenic TNBC, in patients harbouring a higher degree of TILS and PD-L1-expression [219]. For TAMs, breast cancer immunotherapy is mostly focused on inhibition of the pro-tumourigenic TAMs and also on repolarizing such M2-type phenotypes or directly stimulating anti-tumour M1-type macrophages [221].

1.7.2 miRNAs and the TME

miRNAs have been reported to take part in cell–cell signalling between tumour cells and the adjacent microenvironment [222,223] by means of paracrine signalling [224,225]. This communication is mediated through the release of extracellular vesicles [226], such as exosomes [218,227]. As mentioned above, miRNAs seem to act both as oncomiRNAs and tumour-suppressor miRNAs, depending on which miRNA is involved. For instance, in a breast cancer cell line, macrophages activated by IL-4 secrete exosomes containing oncogenic miRNAs, contributing to the invasiveness of breast cancer cells [228]. Meanwhile, exosomal miRNAs of breast cancer cells participate in anti-angiogenic and tumour-inhibiting signalling [229].

miRNAs are also involved in development and maturation of immune cells, and the miR-17-92 cluster is involved in regulation and expansion of $CD8^+$ T cells by targeting the PI3K-AKT-mTOR pathway [230]. Of interest, when investigating miRNAs isolated from the tumour interstitial fluid from breast cancer patients, Halvorsen et al presented a correlation between the miR17~92 cluster and $CD68^+$ cells (i.e., monocytes/macrophages) [222].

.

2 Aims of the Thesis

As is the case with any biomarker study, the overall objective of this thesis is to discover new or identify better diagnostic, prognostic, and/or predictive biomarkers for breast cancer, with the ultimate goal of avoiding unnecessary undertreatment and overtreatment and accompanying severe side effects for patients. More specifically, in light of the challenges and controversies highlighted in the Introduction, for the work described in this thesis, we have selected four phenotypic *in situ* biomarkers: three novel biomarker candidates (myristoylated alanine-rich C kinase substrate like-1 (MARCKSL1) and miR-18a and miR-18b), and one that is established yet controversial (Ki-67). By integrating different phases of biomarker development and looking at both the protein and the RNA levels, the work described in this thesis examines their potential as diagnostic, predictive, or prognostic markers in breast cancer.

Aims for Paper I Here, in an independent cohort of patients with early stage breast cancer, we aim to validate the protein expression of MARCKSL1 as a prognostic factor for survival in patients with LN^- breast cancer.

Aims for Paper II In this exploratory study, we aim to examine the cellular *in situ* expression of the miRNAs miR-18a and miR-18b in primary breast cancer FFPE tissue by the use of chromogenic ISH (CISH).

Aims for Paper III In tissue microarrays from ~1300 breast cancer tumours with long-term follow-up, we aim to use digital DIA in assessment of the IHC expression of the proliferation marker Ki-67 to examine whether DIA Ki-67 expression is associated with recurrence in tamoxifen-treated ER^+ breast cancer.

3 Materials and Methodological Considerations

3.1 *Ethical Considerations*

Applicable national ethics approvals were sought and acquired for all patient material used in this thesis. All the included studies were approved by the Regional Committees for Medical and Health Research Ethics (REC), part of the Norwegian National Research Ethics Committees. In detail, for paper **I**, the study was approved by REC Region West (REC number 210.04). For paper **II**, the Stavanger cohort was approved by REC Region West (REC number 2010/2014), whereas the Oslo2 cohort was approved by REC Region South East, approval numbers 2016/433 and 429-04148. For paper **III**, the study was approved by the REC Region West (REC number 23216), and the Regional Committee on Biomedical Research Ethics of Denmark's Central Region (Record No. 1-10-72-16-15).

3.2 *Patient Material*

For all three papers, archival FFPE primary tumour tissue from breast cancer patients was used.

The patient material for paper I consisted of FFPE tissue from 190 patients diagnosed with invasive, operable T1-2N0M0 breast cancer at the Stavanger University Hospital (SUH), between January 2002 and December 2004. Because of either contralateral breast cancer, neoadjuvant chemotherapy, being lost to follow-up, or for missing

MARCKSL1 expression, 39 patients were excluded, leaving 151 patients for further analysis.

For paper II we used different patient cohorts, as outlined in **Figure 20.**

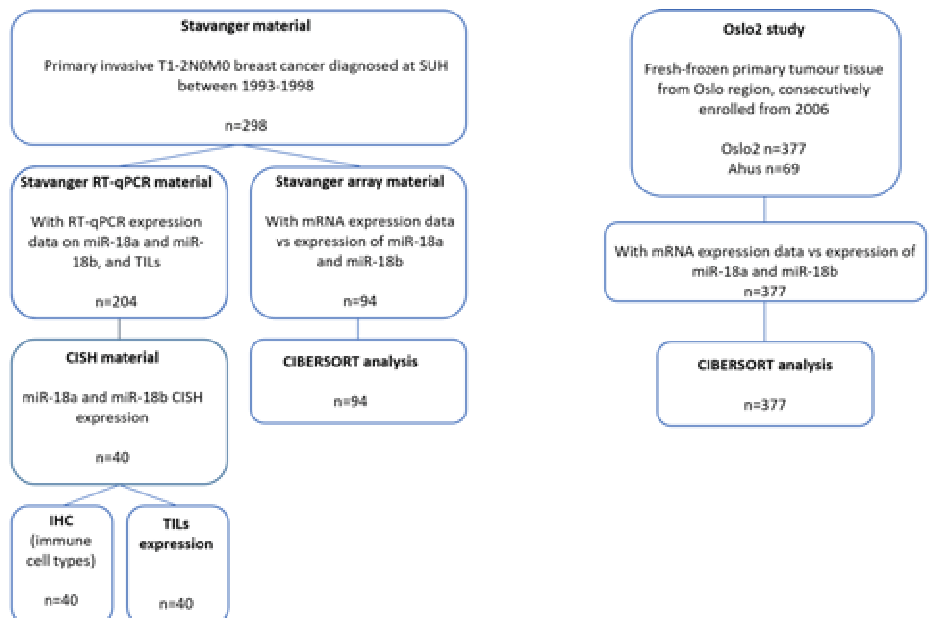

Figure 20. Study Design for Paper II.
Overview of the study design for paper II. The Stavanger RT-qPCR cohort was used for CISH experiments, as well as IHC for immune cell types and quantification of TILs, in n=40 preselected patients. The Stavanger array cohort (n=94) was used for CIBERSORT analyses, together with n=377 patients selected from the Oslo2 and the Ahus cohorts.

Stavanger material: This study included data for patients with breast cancer diagnosed with first onset invasive operable T1-2N0M0 breast cancer at the SUH between January 1, 1993, and December 31, 1998. From this Stavanger cohort, several sub-cohorts have been applied in the present study: 1) A total of 94 breast cancer cases from previous studies

[58,192] with complete mRNA and miRNA expression data, hereafter called the *Stavanger array cohort*, were included for correlation analysis between CIBERSORT output (based on mRNA expression) and miR-18a/miR-18b expression. 2) We analysed TILs in 204 samples (from our previous study [189]), and correlated these results with our previous expression data for miR-18a and miR-18b (measured by reverse-transcription quantitative PCR (RT-qPCR)), grouping the patients based on ER status and high vs low TILs on RT-qPCR. This sub-cohort is referred to here as the *Stavanger RT-qPCR-cohort*.

CISH material: A total of 40 samples from the *Stavanger RT-qPCR cohort* [189] described above were selected for CISH. The selection criteria were 20 tumours classified as ER^+ with low expression of miR-18a and miR-18b (as measured by RT-qPCR), and 20 tumours classified as ER^- with high expression of miR-18a and miR-18b (as measured by RT-qPCR).

Oslo2 material: This is a multicentre study of breast cancer patients with primary operable breast cancers, consecutively enrolled from hospitals in the Oslo region from 2006 until this writing. The cohort material consists of fresh-frozen primary tumour, lymph nodes with tumour cells, peripheral blood, and bone marrow. Patients were included at the time of primary surgery. Tumour mRNA and miRNA expression data from the Oslo2 study (n=377), including (n=69) from the Akershus University Hospital (Ahus), from 2003 to 2010 were selected for CIBERSORT analyses and correlation with miRNA expression.

For paper III, we took advantage of an existing case–control study. The source population consisted of all women (n=11,252) ages 35 to 69 living in the Jutland Peninsula in Denmark, diagnosed with non-metastatic (stages I – III) invasive breast cancer between 1985 and 2001, and registered in the clinical database of The Danish Breast Cancer Cooperative Group (DBCG) Registry [231]. The DBCG enables identification of patients and extensive clinical and treatment data. From this source population and based on the DBCG data, our collaborators from the Department of Clinical Epidemiology at Aarhus University Hospital developed a large Danish population-based case–control study called "The Jutland Breast Cancer Recurrence Biobank", consisting of FFPE breast tumour tissue, both whole-slide (WS) sections and tissue microarrays (TMAs), as well as DNA/RNA samples and clinicopathological data. "The Jutland Breast Cancer Recurrence Biobank" consisting of 541 ER^+ tamoxifen-treated (duration $\geq$1 year) breast cancer patients with recurrence and their 541 matched controls without recurrence, and 300 ER^- non–tamoxifen-treated breast cancer patients with recurrence and their 300 matched controls without recurrence (**Figure 21**). This case–control study was originally designed with the intent to evaluate multiple biomarkers in relation to tamoxifen resistance. The ER^- group was therefore included to enable a distinction of predictive from prognostic factors and has previously been used to study the potential influence of various pharmacological, genetic (e.g., CYP2D6 [232]) or molecular factors (e.g., miRNAs), on tamoxifen treatment efficacy.

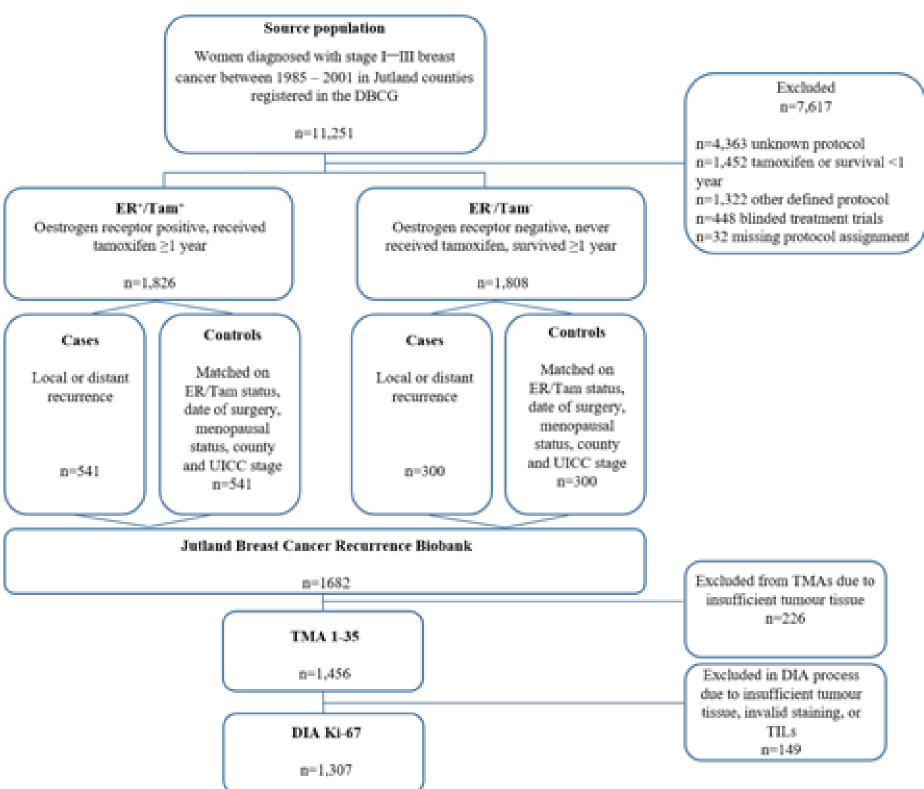

Figure 21. Study Design for Paper III.
Overview of the study design for paper III. The source population consisted of women ages 35–69 with stage I–III breast cancer, diagnosed 1985–2001 in the Jutland peninsula, registered with the Danish Breast Cancer Group (DBCG). Because of not meeting the inclusion criteria, roughly two thirds of the women were excluded. Patients were then stratified into one ER$^+$/Tam$^+$ group with/without recurrence and one ER$^-$/Tam$^-$ with/without recurrence. Patients were excluded from the TMA construction or from the DIA processing if tumour tissue was insufficient; if staining, processing, or imaging was unsatisfactory; or if Ki-67 could not be reliably quantified. Ki-67 was assessed in n=1307 patients.

The TMAs were sampled from archival primary tumour tissue donor blocks of cases and controls of the Jutland Breast Cancer Recurrence Biobank cohort, after a pathologist had identified the sampling region as invasive carcinoma. As such, all the TMAs included both ER$^+$ and ER$^-$ samples. One placental and two liver tissue cores were used as

orientation markers in each TMA. The recipient TMA blocks (n=35) were constructed using a TMAMaster (3DHistech Ltd., Budapest, Hungary), sampled from each primary breast tumour (donor block) and re-embedded into recipient TMA paraffin blocks using standard procedures [233]. For each patient sample, one to three cylindrical 1 mm diameter representative tumour cores, and one normal/tumour margin tissue core were sampled. This sampling yielded a total of roughly 5500 tumour tissue cores for subsequent Ki-67 IHC. ER status was defined as positive if ≥10% cells stained positive. Recurrent cases were defined as occurrence of any (local, regional, contralateral, or distant) breast cancer recurrence during follow-up time, as recorded in the DBCG Registry[231]. Follow-up started from one year after the date of breast cancer diagnosis until the date of the first breast cancer recurrence, or emigration, loss to follow-up, 10 years of follow-up, death from any cause, or end of study (September 1, 2006).

3.3 *Methodological Considerations*

For all three papers, the patient material used was archival FFPE tissue from primary breast tumours. The FFPE material used in these studies sometimes dates back several years. Immunohistochemical staining of ER and PR has been demonstrated to be stable in FFPE material for up to at least 40 years [234]. However, it is important to emphasize that in practice, pre-analytical variables (e.g., fixation, cutting, staining issues, region of sampling, scanning, inclusion/exclusion criteria) can have substantial effects on the outcome. When performing retrospective

studies on archival patient material, it is important to take into account the advances in methodology that have arisen over the years. We now have more sensitive and specific methods of detection, and treatment guidelines, diagnostic recommendations and methodological protocols are constantly changing.

3.3.1 Immunohistochemistry

IHC is an invaluable and extensively used technique that lies at the root of pathology. It takes advantage of the specificity of the antibody–antigen reaction of cells. First, a thin (circa 2 μm) tissue section is mounted onto a microscopy slides before being exposed to a mono- or polyclonal antibody from another species, upon which the antibodies will bind to their specific antigens expressed in the tissue sample, if present. Then, a secondary hybridization step will follow. Finally, any positive binding is visualised by treating the tissue with a chromogenic or fluorogenic substance, in order to demonstrate the presence or absence of the protein of interest. This technique enables the exact localization of the protein of interest in tissues and is widely used in cancer diagnostics. All immunostaining procedures in papers I–III were performed in accordance with recommended protocols, using a Dako Autostainer Link 48 instrument, and executed at the high-quality Department of Pathology at SUH, which participates in the NordiQC external quality programme (https://www.nordiqc.org/). IHC staining on archived FFPE breast tumour tissue was used for detection of all the proteins assessed in this

thesis: MARCKSL1, ER/PR, PPH3, HER2, CK5/6 (paper I) and for quantification of immune markers (paper II) and for Ki-67 (paper III).

3.3.2 Quantification of MARCKSL1

In 10 high-power fields of vision with a total area of 1.59 mm2, MARCKSL1 expression at the invasive front of the tumour was scored as cytoplasmic, membranous, and/or granular staining. Both intensity and number of positive tumour cells were assessed from 0–3 (0=lowest score, 3=highest score), and a total MARCKSL1 score was calculated by multiplying these two scores. A high MARCKSL1 expression was defined as a score of ≥7. The slides were scored blinded and separately by two observers.

3.3.3 Quantification of TILs

First, haematoxylin and eosin (HE) stained tissue sections were assessed according to the presence or absence of stromal tumour infiltrating lymphocytes (sTILs). Then, the relative amount of TILs in the tumour stroma area was assessed in accordance to methods described previously [209,235]. More specifically, sTILs were defined as *the percentage of tumour stroma area that contains a lymphocytic infiltrate without direct contact to tumour cells* [235]. The degree of infiltration was scored in the range of 0%–100%.

3.3.4 CISH for microRNAs miR-18a and miR-18b

CISH is based largely on the same immunogenic principles as IHC. In paper II, CISH was performed on FFPE tissue to detect miR-18a and miR-18b, using the miRCURY LNA™ microRNA ISH optimization kit (FFPE) v1.3 (Exiqon, Vedbaek, Denmark) according to the manufacturer's protocol, with some minor changes (protocol in Appendix 1). The main principles behind this protocol are shown in **Figure 22** and described in the following: First, tumour tissue slides are deparaffinized and treated with proteinase K to allow access to the miRNAs. Then, in the hybridization step, double-DIG(5`-3`)-labelled Locked Nucleic Acid™ (LNA™) probes hybridize specifically with their complementary sequences on the target miRNA in the tissue section, if present. The slide is then treated with an alkaline phosphatase (AP)-conjugated anti-DIG antibody, for a secondary enzymatic reaction binding. NBT-BCIP is added to give a blue precipitate if binding to conjugated AP. Lastly, the tissue is counterstained with Nuclear Red for background visualization. Further details on the CISH detection probes is shown in **Table 4**.

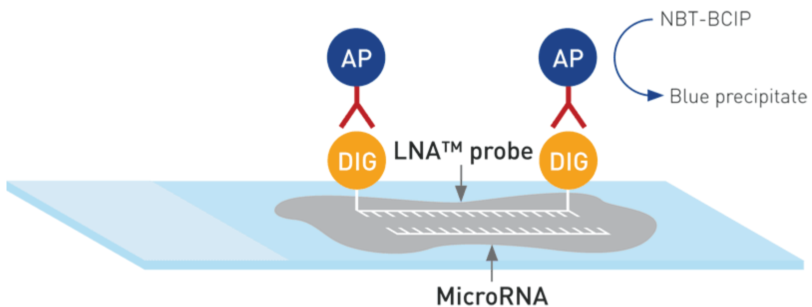

Figure 22. Principle of the CISH Experiments.
Principle of the Exiqon/Qiagen *miRCURY LNA miRNA ISH Optimization Kit* for CISH experiments. The tissue is first deparaffinized and treated with proteinase K to allow access to the miRNA. In the hybridization step, a DIG-labelled LNA probe binds specifically to its target miRNA, if present. Then the slide is treated with AP-conjugated anti-DIG antibody. Then, NBT-BCIP will give a blue precipitate if binding to conjugated AP. Lastly, the tissue is counterstained with Nuclear Red. **AP**: alkaline phosphatase. **LNA™**: locked nucleic acid™. **DIG**: digoxigenin. **NBT-BCIP**: nitro blue tetrazolium chloride-5-bromo-4-chloro-3-indolyl phosphate. Figure downloaded from [236].

Table 4. CISH Detection Probes.
Name, sequence, RNA Tm and concentration for the LNA™ 5`-3`DIG hsa detection probes (Exiqon) used in CISH experiments. Note: the sequences for miR-18a and miR-18b differ by only one nucleotide, as indicated by T and A.

LNA™ Detection / control probes	Sequence	RNA Tm calc.	Concentration
5`-3`DIG hsa miR-18a	CTA**T**CTGCACTAGATGCACCTTA	88 °C	80 nM
5`-3`DIG hsa miR-18b	CTA**A**CTGCACTAGATGCACCTTA	89 °C	80 nM
5`-DIG U6 snRNA	CACGAATTTGCGTGTCATCCTT	84 °C	2.0 nM
5`-DIG Scrambled	GTGTAACACGTCTATACGCCCA	87 °C	80 nM

Specific staining for both miRNAs was observed as a dark blue colour from the NBT/BCIP precipitation. **Figure 23** shows an example of representative staining of specific and positive CISH expression for miR-18b in the stroma of an ER^- breast tumour.

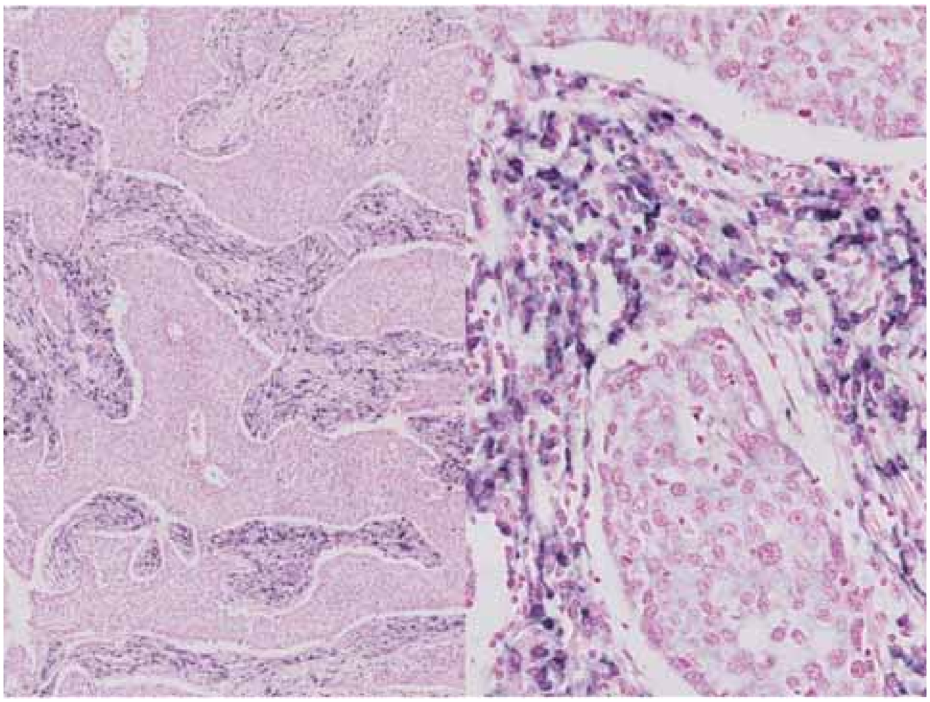

Figure 23. CISH Expression of miR-18b.
Representative positive CISH expression for miR-18b in an ER^- breast tumour, at 4× (left side) and 20× (right side) magnification.

The processed CISH sections were examined under a light microscope at 40× magnification. Cells with distinct positive staining for miR-18a and miR-18b expression were quantified by cell counting in two objectively selected hotspot areas of 1.59 mm^2. Only those slides with sufficient amounts of tumour tissue and staining intensity and with a corresponding successful U6 positive control staining were included for

quantification. Cells with nuclear miRNA staining and diffuse staining intensity were excluded. Quantifications were performed independently by two observers.

3.3.5 CIBERSORT analysis

In paper II, we employed CIBERSORT analysis to assess the amount of immune cell types in a tissue sample *in silico*. CIBERSORT (Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts) is a computational framework which, based on input from bulk tumour tissue gene expression data, compares these data with a defined signature matrix file of 22 immune cell subset (LM22), to estimate the relative abundance of these immune cells *in silico* [237,238], as described in **Figure 24**. We used mRNA expression data from the *Stavanger array* [58] (n=94) and *Oslo2* [239] (n=377) cohorts and chose the maximum number of permutations (n=1000). The output from the CIBERSORT significance analysis provided us with a quantification of the proportions of those 22 immune cell subsets.

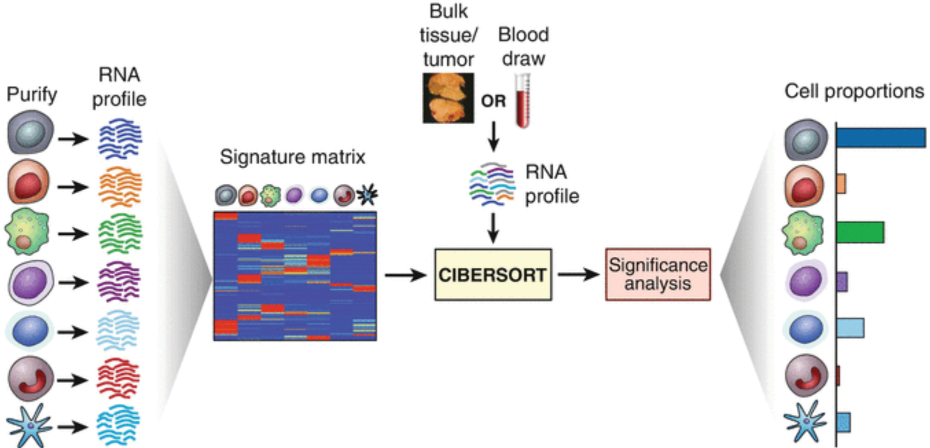

Figure 24. Principles of the CIBERSORT Workflow.
As input, CIBERSORT requires a signature matrix comprising barcode genes that are enriched in each cell type of interest. Once a suitable knowledge base is created and validated, CIBERSORT can be applied to characterize cell type proportions from bulk tissue expression RNA profiles. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature. Springer eBook. *Profiling Tumor Infiltrating Immune Cells with CIBERSORT*, Binbin Chen, Michael S. Khodadoust, Chih Long Liu et al, [COPYRIGHT] 2018.

3.3.6 Automated digital image analysis of Ki-67 score

For paper III, we applied automated scoring of the proliferation marker Ki-67 on 35 TMAs, each containing approximately 150 tissue cores, using the fully automated VIS DIA system VisioMorph (Visiopharm®, Hoersholm, Denmark), applying similar image processing principles as previously described [119].

When using automated scoring for quantification of IHC tumour markers, it is crucial to count only tumour cells to achieve representative calculations. Tumour heterogeneity makes any automated algorithm suboptimal because it to some extent unavoidably leads to overestimates and/or underestimates of some negative/positive nuclei. The algorithm

thus needs be designed so that it disregards stromal cells, artefacts, DCIS, immune cells, and other “biological noise”. Separation of these subpopulations from the tumour cells can be based on cell size, morphology, and colour. For instance, leucocytes are small, round, and dark, fibrocytes have elongated nuclei, and tumour nuclei are more closely packed and in general larger than those of the other cells. The algorithm applied in DIA in paper III was therefore specifically designed to disregard non-malignant cells and adjusted to fit the morphology of breast cancer tumour tissue.

We evaluated Ki-67 expression by employing a customized and specifically designed algorithm, or a so-called analysis protocol package (APP), for Ki-67 quantification, built on the same principles as before, although with some minor modifications and adjustments to adapt from WS to TMAs. First, the TMA slides were scanned at 40× magnification using a Leica SCN400 slide scanner (Leica Biosystems, Wetzlar, Germany) and imported into the image analysis software program Visiopharm®. A grid defining the image to be recorded was adjusted to fit each TMA, before a digital image was recorded of each tumour core.

Then, the tumour region of interest (ROI) for each core was defined semi-automatically by outlining a ROI mask of tumour cells, based on both size and morphology of the cells, which readily distinguished tumour epithelial cells from stromal cells and leucocytes (**Figure 25**). Inside the ROI mask of tumour cells, blue (negative) and brown (Ki-67–positive) nuclei were segmented using a Bayesian classifier.

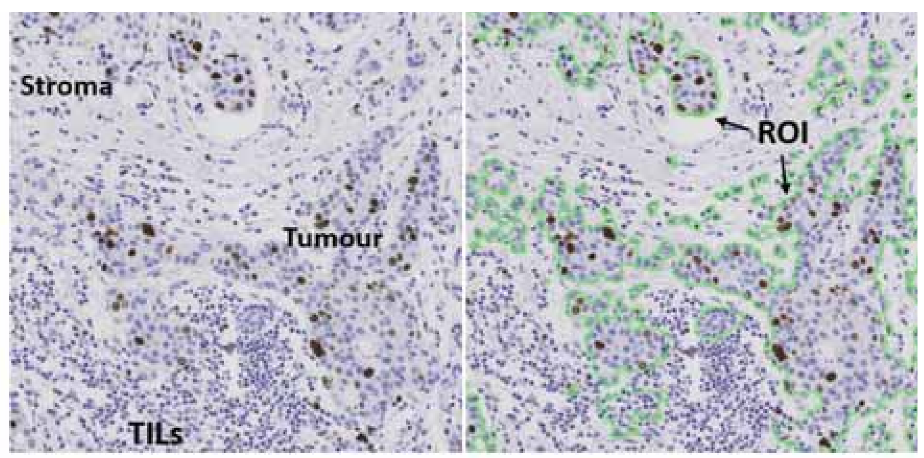

Figure 25. DIA Segmentation of Region of Interest.
Representative image of a TMA core with stromal cells, tumour cells both positive and negative for Ki-76, and TILs. The algorithm automatically segmented tumour nuclei for definition of the ROI mask. ROI (outlined in green) was defined semi-automatically in Visiopharm®, based on both size and morphology of the cells. Stroma and TILs were disregarded by the customized APP. **ROI**: region of interest. **TILs**: tumour-infiltrating lymphocytes.

Pixels of positively stained Ki-67 nuclei were identified based on their brown DAB (3,3'-diaminobenzidine) colour deconvolution, whereas pixels of the negative class (i.e., normal cells) were identified by their blue HE stain. Stromal cells were classified as background (label 003/red), and disregarded in the quantification (**Figure 26**).

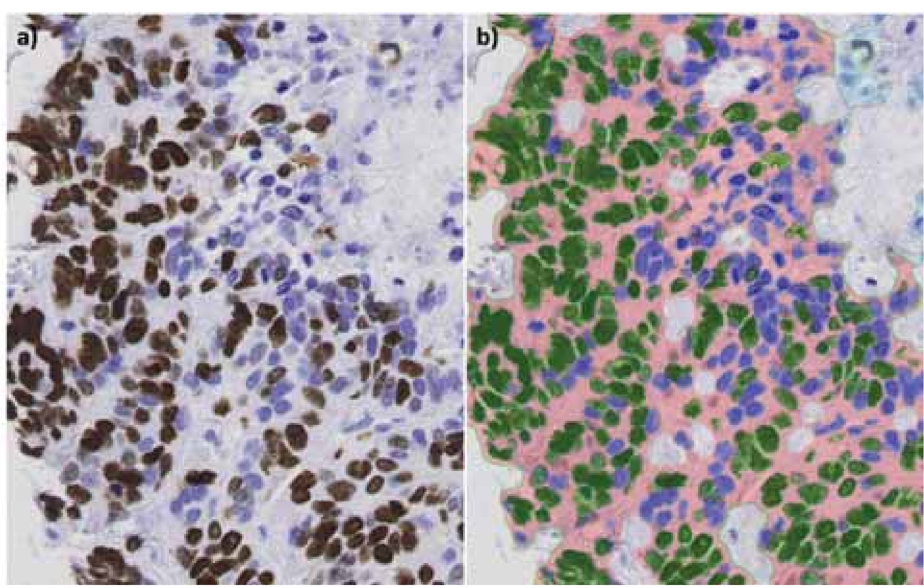

Figure 26. DIA Scoring of Ki-67.
Representation of DIA scoring of Ki-67 a) before and b) after, the customized algorithm was run in the Visiopharm® program. Ki-67–positive tumour cells were identified and scored in relation to the negative tumour cells: Ki-67 positively stained nuclei were identified based on their brown DAB staining, whereas negative cells were identified based on their blue HE stain. DIA score: In this particular tissue core, the DIA Ki-67 was calculated by the customized algorithm to be 61%.

Then, the Ki-67 score was assessed automatically by the customized APP, using the areas of classified blue and brown nuclei:

$$\%\ \text{Ki} - 67\ \text{score} = \frac{\text{area of Ki} - 67 - \text{positive tumour cells}}{\text{area positive} + \text{negative tumour cells}} \times 100$$

The proportion of tumour cells with positive Ki-67 staining was noted as a continuous metric from 0% to 100%.

To avoid any erroneously segmented areas, all cores were re-examined and either accepted, manually edited (e.g., removing any misclassifications, artefacts, DCIS, TILs, poor staining, etc.), or excluded (empty, too few tumour cells or unsuitable cores). Cores from 149 patients were excluded in their entirety, but one, two, or three tumour cores remained for most patients. Examples of cores that were excluded are shown in **Figure 27**.

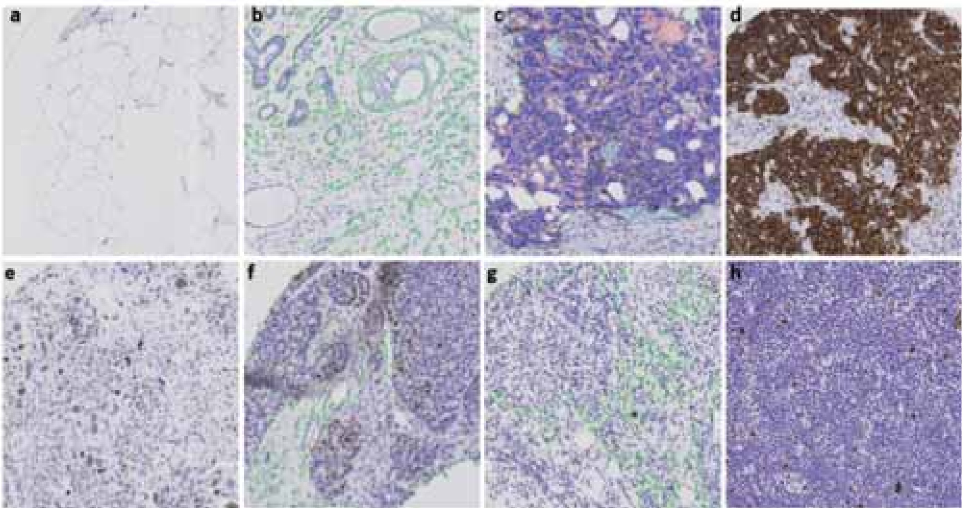

Figure 27. Cores Excluded from the DIA.
Examples of cores that were excluded and the reasons. **a)** stroma/empty core, **b)** too few tumour cells present, **c)** excessive blue HE staining, **d)** excessive membranous/cytoplasmic brown DAB staining, **e)** poor morphology/too weak staining, **f)** poor imaging/artefacts, **g)** and **h)** tumour-infiltrating lymphocytes.

For the great majority of the cores, the manual adjustments had little impact on the resulting Ki-67 score. Any considerable discrepancies between the automated APP-generated and the edited Ki-67 scores were double-checked. **Figure 28** summarizes the workflow of the digital image analysis.

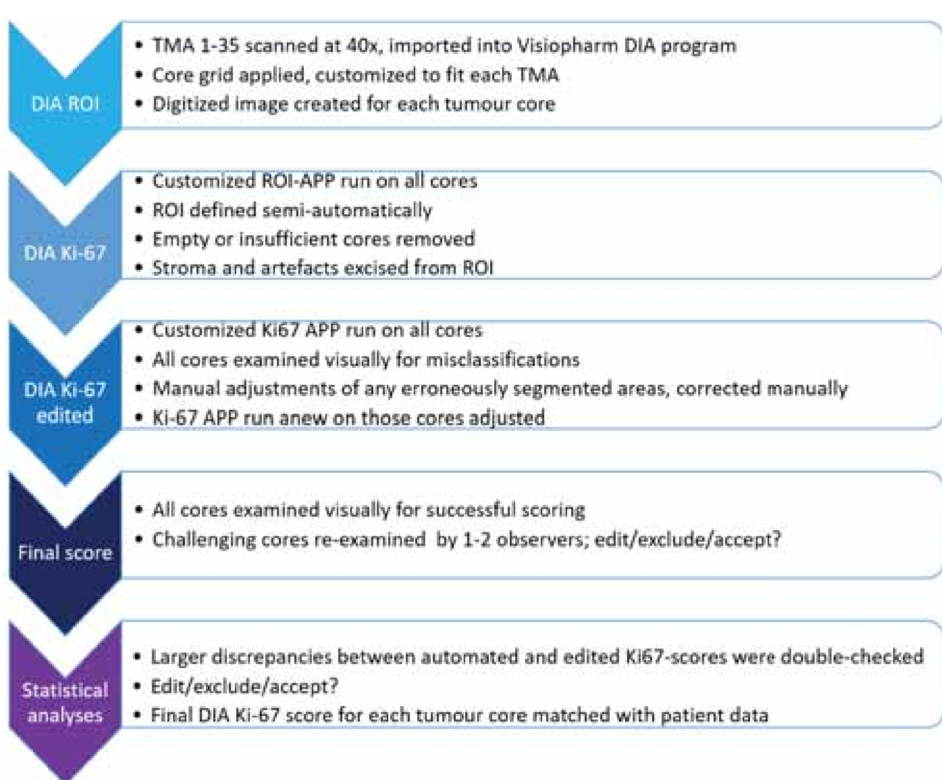

Figure 28. Summary of DIA Workflow.

3.4 Statistical Analyses

For paper **I**, statistical analysis were conducted using the software program SPSS (IBM SPSS, Version 23.0. IBM Corp. Armonk, NY, USA). The main endpoint was distant metastasis-free survival (DMFS). Differences between groups were tested using the log-rank test, and Kaplan–Meier survival curves were constructed. Cox proportional hazard analysis (method: Forward, Wald) was used to test the relative importance of potential prognostic variables, and expressed as hazard ratios (HRs) with 95% confidence intervals (CIs). Group-wise comparisons were performed using Fisher's exact test.

For paper **II**, statistical analyses were conducted using SPSS (IBM SPSS, Version 20.0. IBM Corp. Armonk, NY, USA) and R software [240]. Independent t-tests were used to test differences between patient groups. Pearson's and Spearman's correlation were used to test correlations between different expression levels. Spearman correlation coefficient was calculated to assess the association between miRNA expression and the CIBERSORT-based quantification of cell type composition.

For paper **III**, statistical analyses were conducted using SAS 9.4 (SAS Institute, Cary, NC) and within strata of the two patient groups (ER^+/Tam^+ and ER^-/Tam^-). Cases and controls were characterized using descriptive statistics. Distribution of patient clinicopathological factors was also characterized according to median Ki-67 score. The Ki-67 data were not normally distributed and were evaluated as categorical variables. A dichotomous variable of Ki-67 expression was created, in

which a Ki-67 score above, or equal to, the study sample's median Ki-67 score (6.2%) was considered positive, and a score below was considered negative for Ki-67 expression. To estimate the matched odds ratios (ORs) between Ki-67 score and breast cancer recurrence, we applied logistic regression models adjusting for the matching factors. We adjusted for potential confounding variables using unconditional logistic regression models, including the matching factors, chemotherapy, type of surgery, receipt of radiation therapy, age category, and comorbidity. We performed several sensitivity analyses and re-ran the regression models also adjusting for grade. We stratified the analyses by time to recurrence and by the receipt of chemotherapy. We also performed analyses using the median Ki-67 score separately in the two ER groups (e.g. ER^+/Tam^+ vs ER^-/Tam^-).

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4 Summary of the Papers

4.1 *Paper I Validation Study of MARCKSL1 as a Prognostic Factor in Lymph Node–Negative Breast Cancer*

MARCKSL1 is a protein associated with exocytosis, cell migration, and integrin activation. In a previous study, IHC protein expression of MARCKSL1 was identified as a prognostic factor for DMFS in 305 patients with LN^- breast cancer, especially in patients with high expression of the proliferation marker PPH3 [241]. For paper I, we wanted to further investigate the biomarker potential of MARCKSL1, and examine whether MARCKSL1 protein expression could be validated as a prognostic marker for DMSF in an independent cohort of patients with LN^- breast cancer. We evaluated MARCKSL1 expression by IHC in 151 operable T1-2N0M0 breast cancers in patients, diagnosed at SUH, January 2002–December 2004. The median follow-up time was 152 months (range, 11–189 months), during which time 13 patients (9%) developed distant metastases. Only 5/151 patients showed high (i.e., score ≥7) expression of MARCKSL1. In addition to MARCKSL1, we quantified the expression of PPH3, Ki67, CK5/6, ER/PR, HER2, and TILs. Using single (Kaplan–Meier) and multivariate (Cox model) survival analysis, we compared the results with classical prognosticators such as age, tumour diameter, grade, ER status, and proliferation. However, MARCKSL1 expression did not show any significant prognostic value for DMFS (p=0.498). Furthermore, the only classical

prognosticator assessed that had any significant prognostic value was tumour diameter (≥2 cm) (HR 9.3, 95 % CI 2.8–31.0, p <0.001).

Conclusion Contrary to previous findings, MARCKSL1 expression was not confirmed as having prognostic value in this validation study. The reason for this difference is not known, but given that both the diagnostics and treatment guidelines applied to these patients have since undergone some changes, we speculate that to some extent, this divergence might be attributable to the observed differences between the two cohorts. Nonetheless, the biological role of MARCKSL1 in breast cancer remains uncertain.

4.2 *Paper II miR-18a and miR-18b Are Expressed in the Stroma of Oestrogen Receptor Alpha–Negative Breast Cancers*

In previous studies, gene expression of miR-18a and miR-18b has correlated with high proliferation, ER^-, cytokeratin 5/6 positivity, and basal-like features of breast cancer [189,192]. For paper II, we investigated the expression level and *in situ* localization of miR-18a and miR-18b in 20 ER^+ and 20 ER^- breast tumour tissues by CISH. The resulting expression level and localization of miR-18a and miR-18b were then evaluated with respect to the presence of TILs and immunohistochemical markers for ER, CD4, CD8, CD20, CD68, CD138, PAX5, and actin. Moreover, by the use of CIBERSORT analysis, the correlation between miR-18a and miR-18b expression and the relative quantification of 22 immune cell types was assessed in two independent breast cancer cohorts (94 and 377 patients). For both miR-18a and miR-18b, CISH demonstrated distinct and specific cytoplasmic staining, principally in the intratumoural stroma and the stroma surrounding the tumour margins, and especially for the ER^- tumours. Immunostaining revealed some degree of overlap of miR-18a and miR-18b with CD68 (monocytes/macrophages), CD138 (mature plasma cells/early pre-B-cells), and the presence of high percentages of TILs, but no conclusive identification of the cell type that expressed these miRNAs could be made. However, CIBERSORT analysis showed a strong correlation between M1 macrophages and $CD4^+$ memory activated T cells with miR-18a and miR-18b gene expression.

Conclusion Our study demonstrated that miR-18a and miR-18b are specifically expressed in the stroma surrounding the tumour and that this expression is associated with ER^- breast tumours that present with a high degree of inflammation, as measured by high TILs. Immunostaining and CIBERSORT analysis suggest that the expression of these miRNAs is associated specifically with macrophages, although further analyses are needed to identify the exact subtype of immune cells. Overall, these results point to a potential role for miR-18a and miR-18b in a systemic immunological response in ER^- tumours.

4.3 *Paper III Digital Image Analysis of Ki–67–Stained Tissue Microarrays and Recurrence in Tamoxifen-treated Breast Cancer Patients*

Although somewhat less precise than gene expression signatures [242,243], the proliferation marker Ki-67 has been used as a surrogate marker to separate low-risk luminal A from higher risk luminal B breast cancer subtypes, guiding treatment decisions for adjuvant chemotherapy[122]. Whether Ki-67 expression might also be associated with response to tamoxifen therapy is less studied, however. Although the Ki-67 score has shown prognostic value in several studies, its use has been heavily debated because of the lack of consensus regarding an optimal clinical cut-off value and a high degree of variability across laboratories. Meantime, high-throughput automated scoring of Ki-67 might lead to a more objective standardization of quantification and bring us closer to a definition of appropriate cut-off values. In a case–control study (The Jutland Breast Cancer Recurrence Biobank), nested in the DBCG, we assessed the DIA Ki-67 score on TMAs in 541 ER^+ recurrent cases and their non-recurrent controls, as well as in 300 ER^- cases and controls. We hypothesized DIA of Ki-67 on TMAs could be used to objectively evaluate proliferation in breast cancer tumours and that Ki-67 may be associated with tamoxifen resistance in early stage breast cancer. We applied DIA for quantifying the expression of Ki-67, using a customized algorithm from Visiopharm. Cases and controls were matched on ER status, cancer stage, menopausal status, year of diagnosis, and county of residence. Conditional logistic regression was used to estimates ORs and

associated 95% CIs to determine the association of Ki-67 expression with recurrence risk, adjusting for matching factors, age category, comorbidity, type of surgery, receipt of chemotherapy, and radiation therapy. Ki-67 was not associated with increased risk of recurrence in tamoxifen-treated patients (adjusted OR=0.72, 95% CI 0.54–0.96), or ER-negative patients (adjusted OR=0.85, 95% CI 0.54–1.34).

Conclusion Our findings suggest that DIA Ki-67 in TMAs is not associated with increased risk of recurrence in tamoxifen-treated ER^+ breast cancer or ER^- breast cancer patients. Overall, our findings do not support an increased risk of recurrence associated with Ki-67 expression.

5 Discussion and Future Perspectives

Despite the vast amount of biomarker studies, unfortunately, few will reach clinical relevance, and many will never even be published because of neutral or negative results. To ensure correct diagnostics and optimal treatment options for patients, it is vital to validate suggested prognostic factors in new cohorts. Moreover, in the search for new biomarkers, looking at other biological entities formerly not considered to be very significant could potentially make way for unexpected discoveries.

In parallel with the ever-progressing treatment for cancer patients, patient groups are not static but continually changing. Existing biomarkers therefore need constant re-evaluation. Unfortunately, there is a tendency in some academic journals to accept only studies with significant or positive results, at the expense of studies with neutral or negative findings. Sometimes, researchers themselves are reluctant to submit studies with null results. The reason might be concerns that such studies could have little impact, fail to confirm previous studies, or be less interesting for potential readers. This situation creates a publication bias towards studies that yield significant results, at the expense of null or inconclusive results, even if the research is of equally high quality when it comes to study design and performance [244]. Nonetheless, neutral and negative studies are important to report because doing so is the only way that others may learn from previous results and experiences, avoid redundant work, further knowledge. This importance also applies to validation studies and their significance.

5.1 *MARCKSL1 and its Biomarker Potential*

In paper I we set out to validate a previously suggested, potentially new tumour marker in breast cancer, MARCKSL1 (also known as *MARCKS-Like Protein 1, MARCKS-Related Protein, MARCKS Like 1, Mac-MARCKS*, and more), in a new patient cohort. MARCKSL1 is a member of the MARCKS (myristoylated alanine-rich C kinase substrate) family of proteins, which has been implicated in a range of relevant processes, such as cell adhesion, endocytosis and exocytosis[245], macrophage spreading [246], tumour cell motility [247], and neural development[248,249]. Results of previous studies of MARCKLS1's role in cancer are conflicting. It has been associated with both tumourigenesis and metastasis[250,251], but has been proposed as a tumour suppressor[252], for instance through inhibition of VEGF-induced angiogenesis in an ovarian cancer cell line [253]. In the initial 2012 study of this marker, high IHC expression of MARCKSL1 was found to be prognostic for reduced survival in 305 patients with LN^- breast cancer with high proliferation tumours, as scored by PPH3 [241]. Since that study, few other groups have examined this protein in breast cancer. Supporting research for its potential role in tumour progression is scarce, which warrants replication studies.

Replication studies of potential biomarkers are necessary, and despite negative results, they are important because it is vital to validate both suggested and accepted prognostic factors in new independent cohorts. For instance, in the original study, very few patients had a high MARSKSL1 score, and in the search for the most prognostic cut-off,

such small numbers may result in false-positive results because of statistical overfitting or overtraining. It is important to acknowledge the possibility that this may happen in similar studies, and validating such findings in new cohorts is essential.

Because ours was a validation study, we employed the same protocol as the initial study for MARCKSL1 IHC and scoring. However, in contrast with the original study, we did not find that MARCSKL1 was prognostic. Additionally, whereas in the original study, a high MARCKSL1 total score was associated with lower age and ER^+ disease, in the validation study cohort, a higher MARCKSL1 total score was not associated with age or with ER^+ disease. There are some possible explanations for these discrepancies. The population of women diagnosed with breast cancer changes over time, with numbers of younger and earlier staged patients increasing after the introduction of the national screening program. Such changes, in addition to a more effective chemotherapy regimen[254] and increased endocrine therapy, may contribute to the observed differences in survival between the two cohorts.

Of interest, some studies have suggested that the activity of MARCKSL1 in cell migration may be depend on its phosphorylation status. Dephosphorylated MARCKSL1 compromises filopodium formation but increases actin mobility, lamellipodium formation, and migration in neurons and prostate cancer cells, whereas phosphorylated MARCKSL1 has quite the opposite effect [247]. Regrettably, in our study, the IHC performed did not distinguish the phosphorylation status of the protein.

Whether this information would have changed the overall results is uncertain. This was a small study (n=151), and future research should include more patients. In addition, it would be useful to use antibodies that could differentiate the phosphorylation statuses of MARCKSL1.

Despite the lack of significance reported in paper I, MARKSL1 may still play a role in cancer. Recent findings of MARCKSL1 in other cancers are in fact supportive of the original study by Jonsdottir et al. For instance, in both tissue and cell cultures of lung adenocarcinoma, MARCKSL1 expression was increased compared with normal lung tissue. MARCKSL1 was also found to promote cell proliferation, migration, and invasion. Upon suppression of its expression by MARCKSL1-specific small interfering RNAs, the expression of EMT-associated proteins was decreased [255]. Furthermore, as part of a 9-gene signature, upregulated MARCKSL1 was found to have prognostic value and significantly stratify patients into low- or high-risk groups in HCC [256]. In a recent paper based on microarray datasets and network analyses, MARCKSL1 was one of several gene signatures found to be differentially expressed and a potential target for detection and development in basal cell cancer [257]. Obviously, further validation studies are needed to reveal the true functions of this versatile protein, and perhaps in future studies, it will re-emerge as a potential biomarker for breast cancer.

5.2 *miR-18a and miR-18b and Their Potential as Novel Biomarkers in Breast Cancer*

In general, considering their abundance in expression throughout tissues and their implications in so many physiological and pathophysiological processes, miRNAs are promising as potential biomarkers, both in tissue and in blood. This promise is reflected in the increasing number of studies evaluating miRNAs in cancer, including breast cancer [258].

The main finding of paper II is the localization of miR-18a and miR-18b in breast cancer stromal tissue, especially in the ER^- tumours. We thus validated the findings of preceding studies demonstrating an upregulation of these miRNAs in ER^- breast cancers, albeit in stromal cells and not in the tumour itself.

Counting of TILs in the 40 samples demonstrated an association between high TILs and expression of these miRNAs. Meanwhile, when we examined the amount of TILs with the RT-qPCR data from the original *Stavanger RT-qPCR cohort* (n=204), we found that ER^- samples with high TILs had significantly ($p< 0.001$) higher expression of miR-18a and miR-18b than the ER^+ samples with high TILs. This result indicated that miR-18a and miR-18b expression is strongly associated not only with TILs but also with ER negativity.

In an attempt to come closer to a plausible identification of the specific cell types that express miR-18a and miR-18b, we performed immunostaining in serial sections of the same FFPE blocks used in CISH experiments, to compare their staining pattern. This effort did not result

in a complete match for any of the immune markers, but visually, staining for CD68 (monocytes/macrophages) and CD138 (plasma cells) seemed to be the most compatible with CISH expression, pointing to a possible association with macrophages and plasma cells. To obtain more quantifiable data on the immune infiltrate typical for this material, we performed a CIBERSORT analysis. This procedure identified M1 macrophages, memory-activated $CD4^+$ T cells, M0 macrophages, and monocytes as the top four immune cells significantly correlated with miR-18a and miR-18b expression. This result is somewhat counterintuitive considering our observation of upregulation of miR-18a and miR-18b in breast cancer. Another group recently used CIBERSORT to integrate miRNA and mRNA immune cell signatures to predict survival in ovarian and breast cancer. They reported that the most significantly prognostic immune cell type in breast cancer was the M2 macrophages, whereas M1 macrophages were the most prognostic in ovarian cancer [259], in contrast to our findings. Concurrently, our CIBERSORT results support our observed staining pattern with $CD68^+$ cells, although the M1-like phenotype of macrophages is associated with inflammation and anti-tumourigenic responses, whereas TAMs are mostly of the M2-like phenotype. However, our findings of $CD4^+$ memory T cells are consistent with those of Vahidi et al, who reported that the majority of $CD4^+$ T cells localized in draining lymph nodes were indeed memory T cells [260]. Although we have not identified the specific cells types expressing miR-18a or miR-18b, our findings suggest that

they seem to be related to immune cells, which subsequently is supported by the quantitative analysis of CIBERSORT.

Because we observed that miR-18a and miR-18b were expressed in the stroma and most likely in cells related to the lymph system, we examined patients with LN^+ breast cancer for expression of miR-18a and miR-18b using CISH. This revealed that miR-18a and miR-18b were indeed expressed in lymph nodes positive for tumour cells. However, we examined only a few lymph nodes, and this idea should be tested in larger cohorts to further examine correlations between tumours high in miR-18a and miR-18b expression and their corresponding lymph nodes, both positive and negative for malignant cells.

Unfortunately, our study has some limitations with respect to the cell type specificity and direct quantitative evidence for cell populations. It would be of great interest to further investigate which cells express these miRNAs in such a specific manner, and reveal their true function in breast cancer. Combined staining of IHC proteins together with miRNA is challenging. We have tried to apply dual staining in the CISH protocol together with IHC but regrettably, these efforts have been futile thus far, at least for these miRNAs (we did however succeed for other miRNAs). Possible solutions could involve developing a more robust working protocol for performing simultaneous ISH and IHC for selected immune cell markers. A combination of flow cytometry and cell sorting followed by RT-qPCR could also help with identifying specific subgroups of cells that express miR-18a and miR-18b, for instance by the use of CyTOF®

or the Hyperion™ Imaging System mass cytometry technology. Other options include GeoMx® digital spatial profiling technology (Nanostring) as a tool to measure both protein and RNA expression combined with morphological information, knockout studies, or other functional studies.

Ideally, this study should have been performed with more patients, but unfortunately, CISH is quite a laborious and time-consuming method, explaining the low (n=40) number of patients represented in paper II. However, solutions for automated CISH do exist (e.g., Ventana Medical System), which could facilitate examining many more patients more efficiently. Perhaps we could also have examined miR-18a and miR-18b CISH expression on corresponding lymph nodes for all patients and also in normal breast tissue.

To begin with, we plan to further examine the CISH-expression of these microRNAs in TNBC tissues. Unpublished data from the PErsonalized TREatment of high-risk MAmmary Cancer (PETREMAC) trial suggest that the response rate of patients with TNBC to a poly (ADP-ribose) polymerase inhibitor correlated with the presence of PD-L1 positivity and TILs. Considering that the expression of miR-18a and miR-18b and of TILs seems to overlap, we plan to examine whether these miRNAs might be predictive of the response to olaparib in TNBC breast tumours of the PETREMAC trial. Furthermore, the ongoing Prospective Breast Cancer Recurrence Biobank (PBCB) is an observational regional study of patients from both Haukeland University Hospital and SUH, which

consists of blood samples taken every 6 months from ~1,300 patients with breast cancer, with baseline starting at time of diagnosis, and with prospective 11 years of follow-up. That study focuses on identifying circulating tumour cells before a recurrence becomes clinically overt, and also represents an exciting opportunity to examine the expression of miR-18a and miR-18b in blood.

5.3 *Future Role of Ki-67 in Breast Cancer Diagnostics*

In paper III, our hypothesis was that higher Ki-67 expression would be associated with an increased risk of tamoxifen resistance. Our findings did not support this hypothesis; instead, somewhat surprisingly, the results suggested a 28% reduced risk of recurrence in patients with ER^+/Tam^+ disease, albeit with substantial imprecision, especially considering the large number of patients. This rather slight decrease in risk was further weakened when the ORs were calculated with adjustment for grade. This approach resulted in a 95% CI range of 0.52 to 1.04, crossing 1.0. We performed several additional statistical analyses, but none of them yielded any substantial differences from the original calculations. Based on these data, our overall conclusion remained that our findings do not support an increased risk of resistance to tamoxifen associated with Ki-67 expression in this study.

In paper III, we applied the technology from Visiopharm® using a customized and specifically designed algorithm (APP) for quantifying the expression of Ki-67. This approach was based on the experiences in a previous study in which we compared four methods for obtaining

percentages of Ki-67–positive nuclei with respect to prognosis prediction and reproducibility. These methods were 1) a ‘quick scan rapid estimate’, 2) ocular-square-guided counts by independent pathologists, 3) computerized pointgrid-sampling interactive morphometry (CIM), and 4) automated DIA. In this work, we examined 237 T1-2N0M0 breast cancer FFPE samples with these methods and concluded that both the DIA and CIM were highly reproducible and correlated well with each other, whereas the visual counts were not reproducible [119]. The same algorithm used in paper III has been successfully employed before, in a number of published studies.

Of interest, for quite a few patients (n~60), we observed distinct and excessive cytoplasmic and/or membranous Ki-67 staining across all cores. In these cases, the DIA algorithm could not distinguish individual cells, and manual editing was futile. Most of these patients were therefore excluded because the APP would overestimate the area of positively (i.e., DAB/label 01) labelled pixels. This phenomenon has been reported in a few other studies, but whether it is merely an artefactual technical issue, dependent on the Ki-67 subclone used, or might represent a true underlying biological function with diagnostic/prognostic value in breast carcinomas, is unknown [261-263]. It would therefore be interesting to take a closer look at these remarkable cases to see if there might be any associations with histopathological variables/subgroups/prognosis.

For validation purposes, we examined the reproducibility of the DIA Ki-67 scoring on TMAs in a randomly selected TMA sample by comparing the scores with visual hotspot scoring of their corresponding WS (n=20). In brief, from an area in the tumour tissue slide with the visually estimated highest amount of positive nuclei, i.e., the hotspot area, the number of positive Ki-67 tumour cells was counted in one field of vision with ≥500 tumour cells (which is in accordance with the current guidelines of our hospital). Ki-67 expression was estimated as % Ki-67 by [(#positive cells/total # of positive or negative cells)*100]. Observers were blinded to the DIA Ki-67 score, all clinical information including ER/Tam status, and case/control status. Although we did not perform any formal tests of concordance between the TMAs and corresponding WS, what we did find was similar Ki-67 scores for roughly half the patients, but varying degrees of discrepancies in the other half. All of these cores were re-examined to check the performance of the automated scoring, which turned out to be correct. The observed discrepancies were mostly caused by spatial and temporal heterogeneity between the WS and the TMA cores. Years have passed since the TMAs were first constructed, and since then, these FFPE blocks have been used in several studies, so we were unable to obtain serial sections. This obstacle makes validation studies comparing the original WSs with the TMAs difficult. Should such a validation have been informative, we ought to have ascertained the exact location of the original tumour cores and that cores were sampled from the corresponding hotspot areas of the WS, but unfortunately, we did not have this information. Still, we concluded that,

technically, the DIA of TMA set-up in itself worked well. For the majority of the tumour tissue cores, our customized APP seemed to work quite well. The APP could correctly score the Ki-67 percentage for the vast majority of cases, with no or minimal need for further editing. We excluded only around 10 % of the patients.

Results of previous studies of Ki-67 expression using DIA are conflicting. In a newly released paper, Acs et al used three different DIA platforms to score Ki-67 in a TMA of 149 breast cancers with duplicate cores. They found excellent reproducibility both between- and intra-DIA platforms, and their results are promising with regard to achieving a platform-independent, highly reproducible system for automated DIA Ki-67 scoring [132]. Of note, these scores were based on identical scanned images, whereas pre-analytical variables such as fixation, cutting, staining issues, digital image acquisition, scanning, and inclusion/exclusion criteria can have a substantial effects on the outcome of automated scoring. These issues should be considered in future studies involving automated image analyses. In a large multicentre study from the Breast Cancer Association Consortium, the researchers developed an automated protocol for large-scale Ki-67 scoring in 166 TMAs from ~9000 breast cancers. They correlated the automated Ki-67 scoring with computer-assisted visual scoring, and overall, they found good agreement and accuracy between the two methods, although the performance varied across studies. Larger discrepancies between automated and visual scores were found almost exclusively in cores of poor quality control, whereas the most optimal TMAs cores showed

higher correlation [264]. This outcome is in line with our own results, with the most heterogeneous cores having the highest variation in Ki-67 scores. Discrepancies between visual assessment and automated DIA of the Ki-67 labelling index were recently investigated and found to be caused by tumour heterogeneity in roughly half of the cases [265]. In a 2017 study of 707 breast cancers, Muftah et al examined Ki-67 expression in both whole tissue slides and TMAs and found significant concordance between Ki-67 expression in the whole tissue slides and TMA. In analysing Ki-67 score on a continuous scale and as a dichotomous value, they did find statistically significant differences, although these differences were only moderate. It is important to take into account that in their study, the TMAs cores were preselected from the tumour invasive front, in line with how visual scoring is usually done. These authors concluded that Ki-67 expression in breast cancer can be evaluated by TMAs, although with caution because of the substantial heterogeneity of Ki-67 expression. In addition, they recommended that Ki-67 expression be assessed as a continuous variable rather than by the current predefined dichotomous values [134].

Previous studies have demonstrated the importance of selectively sampling from the tumour invasive front, i.e., from hotspot areas [266]. In a 2012 study by Gudlaugsson et al, Ki-67-score was significantly higher in the periphery than in the central areas of the tumour, and these authors found a significant difference in Ki-67 score between the hotspot area and “cold“ areas [119]. The TMAs of the Jutland Breast Cancer Biobank were not constructed with scoring of Ki-67 in mind, and although the

region of sampling was within the tumour area, the cores were not preselected from the invasive tumour front or hotspot areas specifically. The random sampling of tumour tissue might fail to represent the most proliferating areas, which we know may be very different within the same sample. In essence, we cannot exclude the possibility that had the cores been collected specifically from the hotspots, the Ki-67 scores might have been higher for some of the cores. In that case, the results might underestimate the association between Ki-67 expression and risk of recurrence. However, considering the large sample size and the many cores available for each patient, this scenario is unlikely. In addition, any potential underestimation because of "non-hotspot sampling" would then apply to all sampled cores across the TMAs, and therefore to cases and controls alike. Still, careful sampling from preselected hotspot areas within the invasive front would be more in line with the accepted visual method and might facilitate a more suitable way to validate or assess the true value of Ki-67 as a prognostic marker when applying TMAs.

Previously, a 30% threshold has been used in clinical practice [135], although the optimal cut-off value for Ki-67 varied in previous reports. However, we opted to report the median Ki-67 value and hotspot based on an initial evaluation of Ki-67 expression in the study population, which showed that the median expression of Ki-67 was close to the mean and hotspot values, and few patients had Ki-67 levels above 30%. Therefore, we did not evaluate other categories and chose to report Ki-67 based on median and hotspot expression rather than a 30% threshold. According to the Norwegian Quality Pathology Report of 2016, the Ki-

67 score across national pathology laboratories was distributed as follows: 31% of breast tumours were scored below 15% (low), 39% between 15% and 30% (intermediate), and 29% above 30% (high) [267]. In comparison, the distributions in the TMAs was <15% of patients scored ≥30%, and roughly 30% were scored ≥15%, which overall is much lower than the reported clinical scores. Although the Jutland Breast Cancer Recurrence Biobank design is appropriate for robust evaluation of potential biomarkers for tamoxifen recurrence and the TMAs thus represent valuable material, this stratification of patients with equal numbers of recurrences as no recurrences does not reflect the patient population, in which the most patients will remain recurrence-free. It would therefore be interesting to perform a validation study of the DIA Ki-67 algorithm using different patient material that is representative of the patient population, in which the tissue is sampled from within confirmed hotspot areas and with the possibility of reliably comparing the Ki-67 expression outcome with visual counts.

Obviously, our findings emphasize the well-known challenges of using Ki-67 in breast cancer diagnostics, especially in terms of its prognostic or predictive value. Maybe reaching a clinically relevant cut-off value for Ki-67 is not realistic [268]. In addition, perhaps the prognostic value of Ki-67 is limited to the very high (e.g., ≥30%) or very low (≤10%) Ki-67 scores, with minimal use of reported intermediate scores. This limitation does not mean that proliferation in itself is not prognostic. Substantial evidence exists that highly proliferative tumours behave more aggressively, and are associated with disease progression. Some studies

suggest that degree of proliferation is more predictive of early recurrences [269], whereas other mechanisms, such as disseminated tumour cells dormant in bone marrow, are involved in late recurrences [270].

Furthermore, change in Ki-67 expression may be used to assess efficacy in neoadjuvant treatment by identifying low- and high responders to therapy [271]. Moreover, upregulation of different proliferation markers often coexist, and markers such as MAI and PPH3 could be used as alternatives to Ki-67 [272-274]. These markers are also more specific for the mitotic phase of the cell cycle and consequently more representative for viable proliferating cells that are in the process of mitosis. Both MAI and PPH3 are easy to measure, inexpensive, and highly reproducible[274-276]. Furthermore, in contrast to Ki-67, for MAI, a validated standardized quantification protocol has been available for many years, and many studies have shown that MAI is a better proliferation marker than mitotic count [46,272]. Previous studies have demonstrated that MAI is predictive of the effect of chemotherapy [277]. In future studies, it would therefore be of interest to compare our results with those of other available DIA platforms and with other proliferation markers such as PPH3 or MAI on the same samples. Furthermore, it will also be interesting to examine the Ki-67 index in primary tumours versus recurrent tumours, in early versus late recurrences, and in local recurrences versus systemic metastasis.

Of note, Ki-67 plays an important role in many other malignancies, including colorectal cancer[278], non–small cell lung cancer[279], and neuroendocrine tumours[280]. Despite our results, ruling out Ki-67 as a

biomarker in breast cancer would be premature because in future studies, this very interesting marker may still prove to have prognostic value in both ER^+ and ER^- disease. Certainly, the usefulness of Ki-67 as a biomarker in breast cancer remains unresolved.

5.4 Digital Pathology: Towards Standardization in Cancer Diagnostic

With the forthcoming implementation of digital pathology, cancer diagnostics in Norway is finally entering the digital era. This move represents a comprehensive change from visual to virtual microscopy and from large glass slide archives to digitized specimens. Although necessary equipment such as scanners and operational networks are costly, these expenses are believed to pay off in more economical and efficient diagnostics. By applying artificial intelligence algorithms and machine learning, nuclei segmentation and identification of tumour regions can be performed more efficiently and objectively than by using visual counts [281]. Today, around 25% of all quantitative IHC testing is performed using DIA methods, especially for ER/PR, HER2 and Ki-67 in breast cancer [282]. Digital pathology also represents an opportunity to run large-scale validation studies on potential biomarkers. In addition to Visiopharm, several different DIA platforms are available, and diagnostic artificial intelligence for quantification of, for instance, mitotic count already exists, as do commercially available APPs for both Ki-67 and PPH3. Regrettably, these have yet to be implemented into daily clinical practice, which to some extent may be because of a lack of technical requirements, trained personnel, and operating standards, but high costs are also an issue. The sheer number of different platforms and algorithms unavoidably results in a high degree of variability among them, and developing a universal protocol is challenging. Another important reason is the lack of sufficient training data for machine-

learning algorithms. Training requires a high number of digital slides, and such slides have to be thoroughly annotated by experts. Such tasks cannot be accomplished by a single pathology department. Research in the field of artificial intelligence and the development of mature systems for routine operation requires joint efforts and an interdisciplinary approach across disciplines such as pathology, image analysis, computer science, mathematics, and health economics. Such a joint effort, involving universities and all the regional pathology departments, recently was begun in the Health West region of Norway (pathology services in the Western Norwegian Health Region – a centre for applied digitization) with the objective to raise the quality of pathology services in the region.

The ongoing project *Establishment of Molecular profiling for Individual Treatment decisions in Early Breast Cancer*, i.e. the EMIT study (http://breastcancerresearch.no/studies/establishment-of-molecular-profiling-individual-treatment-decisions-in-early-breast-cancer), focuses on comparing genomics-based classification with routine pathology diagnostics. One of the work packages/study arms involves a comparison of Prosigna/PAM50 molecular profiling versus DIA of proliferation markers Ki-67, PPH3, and MAI. The hope is that this effort will lead to an answer as to which approach is better at separating patients into subgroups — a molecular based and expensive classification, or automated scoring of routine pathology proliferation markers.

This future is promising, and digital pathology will hopefully alleviate the workload on pathologists, enhance cooperation across hospitals, and possibly lead to more standardized protocols and facilitate more efficient validation studies.

6 Concluding Remarks

In the vast field of what constitutes pathology, proper tools to measure biological characteristics are essential. What is right for one patient is not necessarily right for another. Every tumour is unique, and breast cancer displays a striking diversity when it comes to tumour morphology, metabolomics, proteomics, genomics, treatment response, and clinical behaviour. This heterogeneity is something pathologists and clinicians need to take into account, and developing robust biomarkers is essential for finding the most optimal treatment, or combinations thereof. This thesis has focused on the issues of tumour heterogeneity and overtreatment and undertreatment in breast cancer and the ongoing search for better biomarkers to circumvent the many obstacles that still lie ahead. Collectively, the findings of this thesis emphasize the importance of discovering and validating potential new biomarkers, as well as critically reviewing existing ones.

Despite the vast amount of biomarker studies, studies validating previous findings are lacking. Research in itself will always bring forth new knowledge, but such knowledge is useful only if it is conveyed to the rest of the scientific community so that it can be critically re-examined in other studies. Positive or high-impact studies involving new “sexier” techniques receive a disproportionately greater share of the attention in terms of both research funding and media coverage, whereas negative or neutral studies have a tendency to be more challenging to publish. It is also challenging to receive funding for the equally important but

somewhat less desirable validation studies that might result in the implementation of biomarkers into routine clinical practice.

For those of us who are fortunate enough to live in well-developed countries, having a well-functioning health care system and access to expensive state-of-the-art treatments and care are things that we take for granted to a large extent. However, we ought to remember that these resources are unevenly distributed in a global perspective. Strikingly, low- and middle-income countries account for as many as 70% of global cancer deaths, likely because of patients not seeking help until the cancer has progressed, poorly developed medical systems, and limited available treatment options and medical personnel. In fact, it has been reported that only 26% of low-income countries have pathology services generally available [283]. By far, breast cancer research and treatment have come a long way, and individualized treatment is now becoming a reality. The next step in breast cancer should be to ensure that this knowledge would also benefit less-privileged countries, and to strive for more equal diagnostic and treatment options.

Biomarkers are now in the genomic era, and we know more than ever about the genetics of breast cancer. Still, basic pathology, IHC, and the classical and histological markers (e.g., TNM-staging, proliferation, histological subtype) are invaluable in breast cancer. Repeatedly, it has been shown that readily available classical biomarkers complement and match, or even outperform novel or more technical and sophisticated genomic tests [284]. Such tests are in practice inaccessible in large parts of

the world because of their high costs, which further urges the need to develop simpler, more low-cost biological markers that can be used as surrogates for more expensive approaches.

In recent years, we have seen a promising development in cancer treatment, in which differences in cancer biology are taken into account, acknowledging that breast cancer is a heterogenetic disease at the molecular level. This acknowledgment has resulted in the ability to de-escalate treatment, thus reducing adverse side effects. More personalized treatment will eventually yield even better health care and quality of life for patients. Meanwhile, the search for even better biomarkers continues.

Cancer, like all biology, is random, unpredictable, and does not always fit into the strict diagnostic boxes we have created in an attempt to create order in the chaos. Most likely, we may never fully understand or predict the molecular biology and behaviour of cancer cells. We are left at the mercy of hoping that the tools we develop to study nature and cancer cells will bring us a step closer to comprehending its complexity. In the meantime, we must continue to critically evaluate our existing knowledge and to be open to new ideas and perspectives. Although we may not eradicate cancer, perhaps we will be able to make it a chronic and more manageable disease.

The more I learn, the more I realize how much I don't know.

— Albert Einstein —

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Appendices

Appendix 1 – Protocol for CISH experiments: miRCURY LNA™ microRNA ISH Optimization Kit (FFPE)

Modified from the Exiqon Instruction manual v3.0. January 2016

Prior to the experiment: Cut 5-6µm FFPE sections. Let the paraffin sections dry for 1-2 hours at room temperature (RT) and store at 4°C (for up to one week). Avoid melting the paraffin until the day prior to the *in situ* hybridization analysis. Melt paraffin at 60°C for 60 minutes on the day before conducting the ISH experiment. Store slides in an RNase-free environment.

<u>Deparaffinise slides</u>

1. xylene, 5 min
2. xylene, 5 min
3. xylene, 5 min
4. 99,9 % EtOH, 5 min (immerse 2*10 times, then 5 min)
5. 96 % EtOH, 5 min (immerse 10 times, then 5 min)
6. 70 % EtOH, 5 min (immerse 10 times, then 5 min)
7. PBS, 2-5 min

<u>Incubate with Proteinase K (Prot. K).</u> Prepare the Prot. K-solution immediately before use: Prot. K 15 µg/ml: 10 ml Prot. K buffer + 7,5 µl Prot. K stock solution. Apply ~300-800 µl/slide; incubate at 37 °C for 10-30 minutes in Dako hybridizer, without humidifying strips. → 2 x PBS wash

<u>Hybridization.</u> Thaw and spin down the hybridization mixtures, apply 50 µl/slide. Apply sterile coverslips onto each section, carefully avoiding air bubbles. Place the slides in the Dako Hybridizer (with humidifying strips humidified with MQ-water) and run a program hybridizing for 55°C for 1 hour.

<u>Stringent wash</u>: Carefully remove coverslips and Fixogum. Stringent SSC-wash at hybridization temperature.

8. 5x SSC, RT
9. 5x SSC, 55 °C, 5 min
10. 1x SSC, 55 °C, 5 min
11. 1x SSC, 55 °C, 5 min
12. 0,2x SSC, 55 °C, 5 min
13. 0,2x SSC, 55 °C, 5 min
14. 0,2x SSC, RT, 5 min

Transfer to PBS.

<u>Incubate with blocking solution</u>. Apply ~500-800 µL/slide for 15 min at RT, in humidifying chamber. Protect from light. *Tissue sections not allowed to dry out during this and subsequent IHC steps.*

Blocking solution:

I. 9,9 ml MQ water
II. 1,1 ml 10x Maleic Acid buffer
III. 1,25 ml 10x Blocking Solution stock
IV. 0,25 ml sheep serum (final concentration:2%)

<u>Incubate with anti-DIG reagent</u>**.** Apply ~500 µL/slide for 60 min at 30°C, in Dako hybridizer. Protect from light.

Anti-DIG reagent:

I. 4 ml prepared blocking solution
II. 5 µl anti-DIG AP

<u>PBS-T wash, 3x 3 min.</u>

<u>Incubate with AP substrate</u>. Apply ~400µl for 120 min at 30°C, in Dako hybridizer. Protect from light.

AP substrate:

I. 10 ml MQ water
II. 1 tbl NBT/BCIP
III. 20 µl Levamisol stock solution

Incubate in KTBT buffer, 2 x 5 min.

Wash with MQ water, 2x 1 min.

Counterstain with Nuclear Fast Red, 3 min.

Rinse in tap water for 5-10 min.

Dehydrate slides.

15. 50 % EtOH, immerse 10 times, then 1 min
16. 70 % EtOH, immerse 10 times, then 1 min
17. 96 % EtOH, immerse 10 times, then 1 min
18. 99,9 % EtOH, immerse 10 times, then 1 min

Mount Slides. Mount slides with 2 drops Histokitt mounting medium. Avoid air-drying sections at this step.

Appendix 2 – Review article: The Role of MicroRNAs as Predictors of Response to Tamoxifen Treatment in Breast Cancer Patients

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Review

The Role of MicroRNAs as Predictors of Response to Tamoxifen Treatment in Breast Cancer Patients

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Abstract: Endocrine therapy is a key treatment strategy to control or eradicate hormone-responsive breast cancer. However, resistance to endocrine therapy leads to breast cancer relapse. The recent extension of adjuvant tamoxifen treatment up to 10 years actualizes the need for identifying biological markers that may be used to monitor predictors of treatment response. MicroRNAs are promising biomarkers that may fill the gap between preclinical knowledge and clinical observations regarding endocrine resistance. MicroRNAs regulate gene expression by posttranscriptional repression or degradation of mRNA, most often leading to gene silencing. MicroRNAs have been identified directly in the primary tumor, but also in the circulation of breast cancer patients. The few available

studies investigating microRNA in patients suggest that seven microRNAs (miR-10a, miR-26, miR-30c, miR-126a, miR-210, miR-342 and miR-519a) play a role in tamoxifen resistance. Ingenuity Pathway Analysis (IPA) reveals that these seven microRNAs interact more readily with estrogen receptor (ER)-independent pathways than ER-related signaling pathways. Some of these pathways are targetable (e.g., PIK3CA), suggesting that microRNAs as biomarkers of endocrine resistance may have clinical value. Validation of the role of these candidate microRNAs in large prospective studies is warranted.

Keywords: breast cancer; tamoxifen; endocrine resistance; microRNA; biomarker

1. Introduction

Breast cancer is a heterogenic disease that demands an individualized treatment plan, incorporating both patient and tumor information. The development of breast cancer is a highly complicated biological process in which the alteration of women's physiology and the hormonal status plays a significant role [1]. The biological profile of breast cancer differs between the very young (<45 years) and elderly patients (>70 years). Tumors of younger patients are more often Estrogen Receptor alpha (ER) negative (30% are ER−) with higher average proliferation (Mitotic Activity Index (MAI) = 12.8), while elderly patients more often present with ER positive tumors (90% are ER+) and a much lower average proliferation (MAI = 8.7) [2]. Currently, biomarkers such as ER, Progesterone Receptor (PgR) and the Human Epidermal growth factor-like Receptor 2 (HER2) expression level, as well as proliferation status as measured by Ki-67, roughly distinguish patients according to breast cancer subtypes and help inform treatment choice [3,4]. These biomarkers represent important biological processes in the development and progression of breast cancer. In addition to these biological factors, clinical characteristics including the extent of cancer spread, tumor size, lymph node involvement, and evidence of any metastases (TNM), are used to determine the most effective treatment course. For most breast cancer patients, surgical removal of the tumor is primary treatment. In addition, depending on the specific characteristics of the individual tumor, adjuvant therapy comprising systemic treatment with chemotherapy, endocrine therapy, anti-HER2 treatment and/or zoledronic acid, and postoperative radiation therapy are recommended to reduce the risk of relapse [5].

Despite the effectiveness of surgery and extensive adjuvant treatments in breast cancer, challenges concerning over- and under-treatment and recurrence prediction persist. Over-treatment can induce temporary or chronic side effects, significantly lowering quality of life. On the other hand, under-treatment can lead to disease recurrence and metastasis, almost always with life-threatening consequences.

Two-thirds of breast cancer patients have ER+ tumors and are candidates for endocrine therapy [6,7]. Tamoxifen is recommended for premenopausal women, in whom aromatase inhibitors (AIs) are contraindicated [8], whereas AIs are the treatment of choice for postmenopausal women. Still, tamoxifen is an alternative or sequential treatment for postmenopausal patients, depending on their risk of tamoxifen side effects [9,10]. Endocrine therapy reduces the five-year recurrence risk by about one-half [6]. However, patients with identical prognostic factors at diagnosis can vary substantially in their clinical course and treatment response. Endocrine therapy resistance can either exist from the start

of diagnosis (*de novo*/intrinsic resistance) or develops during the course of treatment (acquired resistance) [11]. Unfortunately, resistance to therapy as well as over- and under-treatment, are difficult to foresee with the current biomarkers. Thus, acquired resistance is hard to predict before a local or systemic relapse has occurred and becomes clinically overt. Therefore, improved prognostic and predictive biomarkers (measured in the primary tumor), as well as biomarkers for monitoring drug response (measured in blood) are urgently needed.

While many new biomarkers have been described over the last decades very few have made it from the laboratory into the clinic. Pultz *et al.* recently reviewed several biomarkers from relevant literature and sorted them according to their potential clinical relevance. They suggested that 15 of these markers should be validated in the clinic; amongst which microRNA was mentioned [12]. MicroRNAs are a short form of non-coding single-stranded RNA about 22 nucleotides in length. MicroRNAs regulate gene expression by posttranscriptional repression or degradation of mRNA, most often leading to gene silencing [13].

Although recently discovered, a great body of evidence is accumulating implying that miRNAs might provide both predictive and prognostic potential as biomarkers. In this review, we discuss the potential clinical utility of microRNAs as determinants of tamoxifen resistance in breast cancer patients, and how and where they interact with biological pathways in order to mediate such tamoxifen resistance.

1.1. Estradiol and the Estrogen Receptor

As illustrated in Figure 1, the biological activities of estrogens are mediated by ERs, which upon activation by cognate ligands form homodimers, or heterodimers with other ER-ligand complexes [14,15], and activate transcription of specific genes containing the estrogen response element (ERE) [16,17].

1.2. Endocrine Treatment Regimens

Endocrine treatment regimens for breast cancer patients comprise a dual strategy by either blocking the estrogen action at the ER-level (tamoxifen), or by inhibition of the *in vivo* estrogen synthesis in the whole body. In postmenopausal patients the latter is achieved by AIs alone, while pre-menopausal women need ovarian function suppression (OFS) and AIs in concert. The current treatment regimens for pre-, peri- and postmenopausal ER+ breast cancer women in Norway are illustrated in Figure 2. These national guidelines are based on international recommendations [3], and are similar to the guidelines of the National Comprehensive Cancer Network [5]. Note that endocrine treatment has been extended to 10 years of treatment as a result of recent publications [7,18].

Tamoxifen is a selective ER modulator (SERM) and the most frequently used anti-estrogen adjuvant treatment for ER+ pre-menopausal women. Tamoxifen is also a standard endocrine therapy for treatment of postmenopausal women with breast cancer, although AIs are more frequently used (see Figure 2 above). Depending upon the tissue, tamoxifen may function as an agonist or antagonist, recruiting either coactivators or corepressors to the ER transcription complex [19]. Tamoxifen exhibits antagonistic effects in breast tissue, thus has preventive effects on breast cancer development [20] and cytotoxic effects on breast cancer cells [21]. Tamoxifen also exerts agonistic effects in the uterus, increasing the risk of endometrial hyperplasia and malignancy [22].

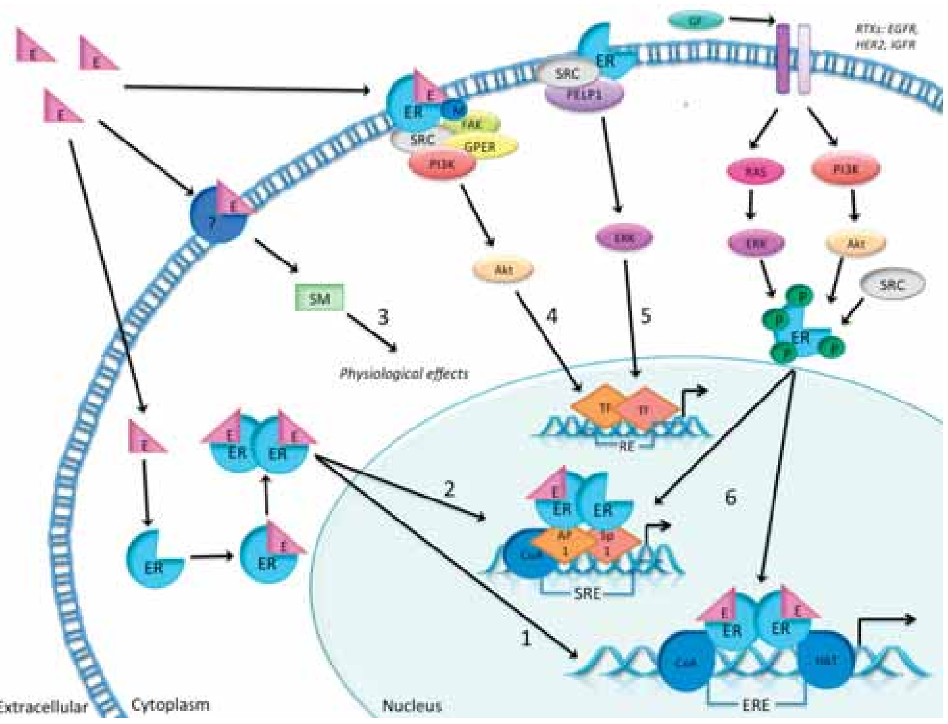


Figure 1. Simplified possible molecular signaling pathways (**1** to **6**) of estrogen (E) and estrogen receptors (ER). (**1**) Classical and direct pathway: ligand activation is followed by binding to the estrogen response element (ERE), including coactivators (CoA) and histone acetyl transferases (HATs) before gene regulation is modified; (**2**) tethered pathway: ligand dependent pathway which includes protein-protein interaction with other transcription factors, e.g., activator protein 1 (Ap1) and specificity protein 1 (Sp1), after ligand activation, thereby regulating genes by indirect DNA binding following serum response element (SRE) activation of transcription; (**3**) non-genomic ligand dependent reaction: the receptor (e.g., classical ER, ER isoform or other receptors) is activated by a ligand, which may be associated with the membrane. This is then followed by signaling cascades initiated by second messengers (SM), initiating a rapid physiological response, which does not involve gene regulation; (**4**) ligand-dependent reaction: ER is methylated by ligand induction and ER–phosphoinositide 3-kinase (PI3K)–steroid receptor coactivator (SRC)-focal adhesion kinase (FAK) forms a complex that further activates the serine/threonine–protein kinase Akt, which then activates transcription without ER binding to DNA; (**5**) ligand independent reaction: ER–SRC–proline-, glutamic acid and leucine-rich protein 1 (PELP1) forms a complex which then activates transcription, also without ER binding to DNA; (**6**) another ligand independent reaction activates through other signaling pathways, like growth factor signaling by downstream events of receptor tyrosine kinase (RTKs), such as epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2) and the insulin-like growth factor receptor (IGFR) [11,23].

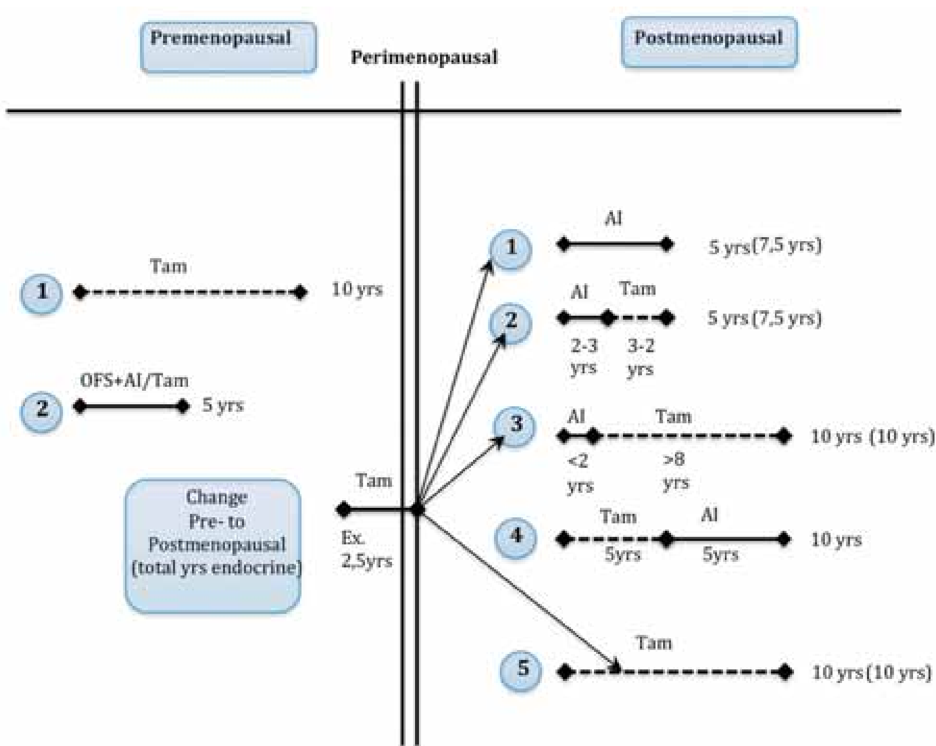


Figure 2. Overview of the adjuvant endocrine treatment guidelines for ER+ breast cancer patients according to the Norwegian Breast Cancer Group (NBCG) 2015 [24], and based on international recommendations (St. Gallen, 2013). There are two options for premenopausal patients (1 and 2 on the left side) and five options for postmenopausal patients (1–5 on the right side) comprising aromatase inhibitor (AI), tamoxifen and ovarian function suppression (OFS) alone or in combination. Total duration of endocrine treatment for a premenopausal patient that becomes postmenopausal after two or five years on tamoxifen (example) is illustrated in brackets. The choice between alternatives 1–5 is made individually based upon tumor biology, side effects and preferences among clinicians and patients. Peri: perimenopausal; TAM: tamoxifen; Yrs: years; Dotted line: years on tamoxifen; Solid line: years on AI.

The tamoxifen metabolic pathway is complex. In general, tamoxifen is oxidized in the liver by phase I metabolism involving various enzymes encoded by polymorphic genes including cytochrome P450 2D6 (CYP2D6) into two active metabolites: 4-hydroxy-*N*-desmethyl tamoxifen (endoxifen) and 4-hydroxytamoxifen (4-OHT). Endoxifen and 4-OHT then undergo phase II conjugation reactions and further find their way into the cancer cells. In breast cancer cells, 4-OHT acts as an antagonist preventing estrogen from binding to the ER, thus preventing proliferation and cell growth [25,26]. Levels of estrogen have been correlated with tamoxifen metabolite concentration in serum [27,28]. Due to polymorphic metabolic enzymes in the tamoxifen pathway, there are inter-individual differences

in the concentration of the active metabolites in serum and therefore a potential for variation in drug effectiveness [29]. The serum concentration of tamoxifen and its metabolites increases with age, which could explain part of the inter-patient variation of active metabolite concentration in serum [30].

In postmenopausal patients, peripheral conversion of androgens into estrogens takes place in various tissues [31]. Third generation AIs cause more than 98% inhibition of this extra-ovarian aromatase activity and create extremely low serum and tissue levels of estrogens [32,33]. The systemic hypo-estrogenic state produced by AIs may explain their superiority to tamoxifen when administered upfront adjuvantly in postmenopausal patients [34,35]. However, this beneficial difference in survival disappears after two years of AI treatment [35], and therefore tamoxifen treatment for at least three years might also follow in postmenopausal patients [24] (Figure 2).

1.3. Resistance to Tamoxifen

In approximately 30% of ER+ breast cancer patients, endocrine treatment fails due to tamoxifen resistance [36]. As illustrated in Figure 3, mechanisms of tamoxifen resistance may involve changes in the activity of enzymes that metabolize tamoxifen, loss or modification of ER expression, alterations in the balance of co-regulatory proteins, altered expression of specific microRNAs, or the activation of alternative signal transduction pathways that can further promote tumor growth [37,38]. Regardless, it is likely that the pathways involved in *de novo versus* acquired resistance are different [39]. Therefore, monitoring the development of resistance to tamoxifen and the exploration of new therapeutic targets is pivotal.

1.4. MicroRNAs

MicroRNAs are defined as short non-protein-coding RNA molecules, of which the mature form is about 22 nucleotides in length. Each microRNA is complementary or partially complementary to one or more mRNA molecules, and its main function is to post-transcriptionally down-regulate gene expression by either binding directly to its mRNA target, or by cleaving target mRNA by binding to its 3′-UTR region. According to the microRNA database miRBase v.21, more than 2603 human microRNAs have been identified so far [40]. A single microRNA can potentially target up to 200 mRNAs; and the same mRNA molecule may also be targeted by different microRNAs [41,42], underlining the wide range and complexity of their functions. MicroRNAs have been shown to play a pivotal role in numerous biological processes, cellular pathways and networks. Many major cellular functions such as development, differentiation, growth, metabolism, survival, motility and proliferation are, in part, regulated by microRNAs. Since the link between cancer and microRNAs was first demonstrated in 2002 [43], microRNAs have also been shown to be involved in multiple cancer types, and microRNA-encoding genes are often located at genomic regions known to be associated with cancer [44]. More specifically, microRNAs are often involved in mechanisms underlying tumorigenesis and tumor progression, where they act as either tumor suppressor microRNAs or as tumor-promoting microRNAs [45].

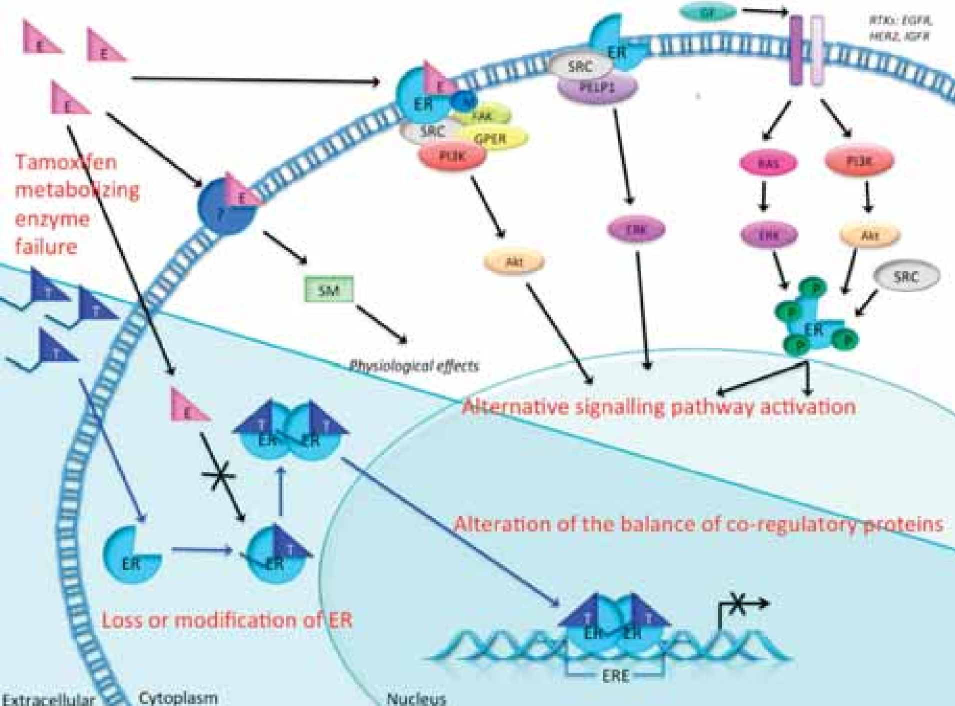


Figure 3. The tamoxifen pathway and possible mechanisms of endocrine resistance in breast cancer cells. Prior to entering the breast cancer cell, tamoxifen (T) is metabolized in the liver into the two active metabolites, endoxifen and 4-hydroxytamoxifen (4-OHT). When these metabolites enter the cell (blue background) they can bind to estrogen receptors (ERs), thereby blocking the binding of estrogen. ERs bound to tamoxifen then dimerize, enter the nucleus and bind to estrogen response element (ERE). However, the necessary coactivators will not be recruited by the ER–tamoxifen complex. Only corepressors are recruited, therefore gene transcription is not activated. In tamoxifen resistance, this blocking is compromised due to several possible mechanisms: e.g., changes in activity of the metabolizing enzymes of tamoxifen, loss or modification of ER expression, alternative signaling pathways for proliferation and growth, and alterations in the balance of co-regulatory proteins and altered expression of microRNAs [37,39]. Black arrow: normal estrogen pathway. Blue arrow: tamoxifen pathway. Crossed arrow: disrupted pathway.

2. Methods

2.1. Literature Search

To find relevant original articles for tamoxifen related microRNA, we performed a search in PubMed on the first of July 2015 using the words "microRNA" and "tamoxifen" (89 articles), filtering for human species (53 articles). Further selection, excluding review articles and focusing on studies that included patient material only, resulted in six studies [46–51] (see Table 1).

Table 1. Candidate microRNAs involved in tamoxifen response.

MicroRNA	Material and Patients	Clinical Outcome	Reference	Predicted Targets or Pathways
miR-342-5p	FFPE of tamoxifen-treated primary tumor, $n = 16$	Ten patients responded to tamoxifen and had non-recurrent disease: two-fold the level of miR-342 expression; Six patients developed recurrences and metastasis during tamoxifen treatment and had low levels of miR-342	[46]	Target genes: *GEMIN4* and *BMP7* Predicted pathways: cell death, apoptosis of breast cancer cells, mitotic roles of polo-like kinase
miR-30c-5p	Primary tumors, tamoxifen-treated following advanced disease, $n = 246$	Increasing levels of miR-30c was associated with clinical benefit of tamoxifen treatment, as measured by longer PFS	[49]	Target genes: *PPARGC1B*, *Makorin-3*, *UBAC1*, *PTPDC1* Predicted pathways: HER2, signal ransduction, and oncology pathway, RAC1 cell motility signaling pathway
miR-210-3p	Fresh-frozen tamoxifen-treated primary tumors, $n = 89$	High level of miR-210 expression was associated with a higher risk of recurrence than a lower level of miR-210	[50]	Target genes:*ACVR1B, CBFA2T1* Predicted pathways: cell cycle, cell adhesion and immune response
miR-26a	Frozen tamoxifen-treated tumors of metastatic patients, $n = 235$	Higher levels of miR-26a were significantly associated with clinical benefit (*i.e.*, complete or partial response, or stable disease), and with favorable TTP (*i.e.*, first detection of disease progression)	[51]	Target genes: *CDC2*, *CCNE1* Predicted pathways: cell cycle regulation pathway
miR-126-5p and miR-10a-5p	FFPE from postmenopausal tamoxifen-treated patients, Validation set: $n = 34$ with recurrence; $n = 47$ without recurrence	Low expression of miR-10a and miR-126 correlated significantly with reduced relapse-free time	[47]	Target genes: n/a Predicted pathways: n/a
miR-519a-3p	GEO datasets of breast cancer patients, Discovery set: $n = 632$, Validation set: $n = 586$	High expression of miRNA-519a correlated significantly with poorer disease-free survival in ER^+ breast cancer patients	[48]	Target genes: *PTEN*, *RB1*, *CDKN1A/p21* Predicted pathways: PI3K/Akt pathway

FFPE: Formalin-fixed and paraffin-embedded; GEO: Gene Expression Omnibus; PFS: progression-free survival; TTP: time to progression.

2.2. Ingenuity Pathway Analysis

To investigate the biological interactions of the seven tamoxifen-related microRNAs from our literature search with other molecules, we used *in silico* analysis to find predicted targets and identify their corresponding networks. The predicted targets and networks were generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN, Redwood City, CA, USA). This software collects information about molecule-to-molecule interactions, biological networks and canonical pathways in the Ingenuity Knowledge database. This information is also reviewed by experts to ensure good quality information. Additionally, the software calculates a *p* value (right-tailed Fisher's exact test) to determine the probability that the input genes are connected to a verified network or pathway by chance alone.

First, we used IPA to find the experimentally observed and highly predicted targets for each of the seven microRNAs, to be used further in an IPA core analysis (see Table 2). Then, for each of the resulting target lists, we used IPA to perform a core analysis considering only direct relationships between molecules, in humans, resulting in biological networks (see Figures 4–6).

Table 2. Number of experimentally observed and highly predicted gene targets for the candidate microRNAs listed in Table 1.

MicroRNA	No. of Target Genes
miR-342-5p	337
miR-30c-5p	1420
miR-210-3p	78
miR-26a	892
miR-126a-5p	37
miR-10a-5p	338
miR-519a-3p	86

3. MicroRNAs in Breast Cancer

3.1. MicroRNAs in Breast Cancer Tumor Tissue

In breast cancer, several microRNAs are aberrantly expressed in tumor tissue compared to normal tissue. In a recent review, van Schooneveld lists some of the best described microRNAs in breast cancer including the oncogenic microRNAs miR-10b, -21, -155, -520c, -373, and the tumor suppressor microRNAs miR-31, -125b, -126, -200, -206, and -335 [52].

A well-known oncogenic microRNA is miR-21, which is overexpressed in breast cancer [53–55] and has been correlated with advanced stage, lymph node metastasis and poor prognosis [55]. Correspondingly, cell growth, migration and proliferation were inhibited when miR-21 was knocked down in MCF-7 and MDA-231 cells [56].

Among other well-studied microRNA clusters, the miR-17-92 cluster (comprising miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1) has been implicated in breast cancer by several studies. For example, in breast cancer cell lines, miR-17 has been shown to play an important role in promoting tumor cell migration and invasion [57]. In a study of ER+ breast cancer patients, ER has been shown to be a direct target of miR-18a and miR-18b [58], and miR-18a, together with miR-18b,

has been associated with features of basal-like breast cancer [59]. Moreover, miR-17, miR-18a and miR-20a showed enhanced expression in triple-negative tumors compared to luminal A tumors [60]. In addition, miR-92a has been associated with tumor grade, cell migration and macrophage infiltration in breast cancer [61].

Studies report distinct functions of individual microRNAs, demonstrating that microRNAs have cell-, tissue- and organ-specific functions. As microRNAs appear to play important roles in breast cancer development, it seems likely that they have potential utility as prognostic and predictive biomarkers.

3.2. Circulating MicroRNAs in Breast Cancer

MicroRNAs have been detected in the circulation, either bound to lipids or proteins, inside apoptotic bodies from dead cells, or as part of circulating exosomes [62]. The presence of circulating microRNAs has also been shown in breast cancer patients. For instance, differential concentrations of miR-16, miR-107, miR-130a and miR-146a microRNAs were shown in plasma from 111 patients with different cancer subtypes [63]. In a cohort of 89 breast cancer patients (range 31–82 years), Roth *et al.* (2010) found elevated levels of circulating miR-10b, miR-155 and miR-34 in cell-free serum from breast cancer patients, compared with 29 healthy controls. The differences in relative concentrations of these microRNAs could be used to distinguish healthy controls from breast cancer patients, as well as metastatic (n = 30) from non-metastatic (n = 59) disease. Furthermore, in the 59 patients without distant metastases, higher levels of serum miR-34a correlated with advanced tumor stages [64]. In 2012, Madhavan *et al.* demonstrated a significant correlation between higher levels of eight circulating microRNAs and circulating tumor cells (CTC), in metastatic breast cancer patients (n = 133) compared with healthy controls (n = 76), thus showing the potential of circulating microRNAs as surrogate markers for CTCs. In addition, they found that miR-200b was a promising prognostic marker of both overall- and progression-free survival (PFS) [65].

Compared to intracellular microRNA or microRNA in cell-free blood (plasma or serum), exosomes have proved to be an enriched and protective source of circulating microRNAs [66,67]. Tumor cells secrete exosomes, so-called tumor-derived exosomes [68], in higher amounts than normal cells. In fact, the cargo of the tumor-derived exosomes has been shown to reflect the cell and tissue it originates from. This opens up the possibility to use tumor-derived exosomes detected in blood to gain information on the remaining tumor cells, providing a minimally invasive biomarker to detect tumor cells, which might also reveal some of the oncogenic features of the tumor.

The recent finding of microRNA in tumor-specific exosomes increases the potential for using microRNAs in blood as biomarkers for monitoring breast cancer characteristics and maybe even therapy response. Several studies have demonstrated the presence of tumor-derived exosomes containing microRNA, suggesting their potential as diagnostic, prognostic and predictive biomarkers.

Furthermore, exosomes originating from drug resistant breast cancer cells have been shown to mediate drug efflux and resistance through so-called exosomal shuttle-microRNAs [69]. In a recent study of chemo-resistant breast cancer cells (resistant to Adriamycin and Docetaxel), exosomes were shown to mediate such chemo-resistance to cells that were still sensitive to these drugs. This transfer of resistance was likely due to intercellular transfer of specific exosomal microRNAs, potentially miR-100, miR-222 and miR-30a [70]. Moreover, in another recent study of tamoxifen-sensitive and

tamoxifen-resistant MCF-7 cells, exosomes released from resistant cells, were able to enter into tamoxifen-sensitive cells and release miR-221 and miR-222. These microRNAs then reduced the expression of p27 and ER in the recipient cells, thus decreasing their sensitivity to tamoxifen [71].

3.3. Tamoxifen-Related MicroRNAs Found in Breast Cancer Tissue

In 2010, Cittelly *et al.* showed that miR-342-5p was differentially expressed in tamoxifen-sensitive *versus* tamoxifen-resistant cell lines. MicroRNA-342 expression was shown to be suppressed in the tamoxifen-resistant breast cancer cells, while a miR-342 inhibitor could promote resistance in the tamoxifen-sensitive cells. In addition, 16 tamoxifen-treated primary breast tumors ($n = 6$ with recurrence, $n = 10$ without recurrence) were analyzed by *in situ* hybridization (ISH) for miR-342 expression. Due to the low sample number estimates were imprecise, but the ISH results indicated that the level of miR-342 expression was about two-fold higher in tumors from the tamoxifen responders (*i.e.*, those without recurrence or metastasis), compared to the non-responders. Furthermore, they performed a search for potential gene targets by microarray analysis of tamoxifen resistant breast cancer cell lines with restored level of miR-342 and control cells. This microanalysis showed that 13 genes were differentially expressed, of which GEMIN4 and BMP7 were validated as direct targets of miR-342. By using Ingenuity Pathway Analysis (IPA) for identification of functional pathways enriched with miR-342 regulated genes, they identified the Cell Death and Apoptosis of Breast Cancer Cells pathways as being the most significant. By canonical pathway analysis, IPA identified Mitotic Roles of Polo-Like Kinase as the pathway in which miR-342 genes were most significantly enriched [46].

In another study of 246 ER+ advanced breast cancers, higher expression of miR-30a-3p, miR-30c and miR-182 was associated with better response to tamoxifen treatment as measured by longer progression-free survival time; however, only miR-30c was shown to be an independent predictor. These patients were initially hormone-naïve, and received tamoxifen treatment following metastases or recurrence. For some of the samples included in this study both microRNA and mRNA expression data was available and used to analyze the potential underlying biological pathways associating these microRNAs with tamoxifen resistance. Accordingly, by using Global Test/Biocarta, miR-30c was found to be significantly correlated to HER2, signal transduction, and oncology pathway, whereas genes related to miR-30a-3p expression were significantly associated with Ceramide signaling pathway. Furthermore, both miR-30c and miR-30a-3p were negatively associated with the RAC1 cell motility signaling pathway. By searching publicly available databases, they also reported PPARGC1B, Makorin-3, UBAC1 and PTPDC1 as target genes for both miR-30c and miR-30a [49].

In 89 ER+ tamoxifen-treated breast cancers, a higher risk of recurrence and poorer clinical outcome was associated with a high level of miR-210 expression, compared with low miR-210 expression. Overexpression of miR-210 in ER+ MCF7 cells, and repression in ER− MDA-MB-231 cells induced the altered expression of several genes (data not shown). Gene set enrichment analysis of these differentially expressed genes showed their involvement in biological pathways involved such as cell cycle, cell adhesion and immune response [50].

Low levels of Enhancer of Zeste homolog 2 (EZH2), a target of miR-26a, have been associated with favorable outcome in tamoxifen-treated patients [72]. In a retrospective study by Jansen *et al*. of 235 tamoxifen-treated patients with metastatic disease, high levels of miR-26a and decreased (EZH2)

expression was associated with clinical benefit and favorable time to progression. Furthermore, pathway analysis on microarray data from 65 of these tumors using the Global Test Approach (GTA) indicated the cell cycle regulatory pathway and the gene CDC2 to be correlated with miR-26a expression [51]. In cell line models CDC2 has been linked to tamoxifen response [73]. Patients with lower mRNA levels of CDC2 also showed a delay in disease progression compared to those with higher levels [51].

In a retrospective study, miR-126 and miR-10a were reported as being independent predictors for tumor relapse in a study restricted to post-menopausal women with breast cancer following tamoxifen treatment. By microarray profiling, they screened 12 patients (matched on age at diagnosis, tumor size, grade, nodal status, PgR-and Her2/neu-status, ER immune reactive (IR) score and radiotherapy) with ($n = 6$) and without ($n = 6$) relapse following five years of tamoxifen treatment for 1105 microRNAs. Of the 20 resulting microRNAs, miR-126 and mir-10a were confirmed by qRT-PCR in a set of 81 patients with and without relapse [47].

More recently, Ward *et al*. described that the microRNA cluster C19MC (comprising 50 microRNAs) is upregulated in tamoxifen-resistant *versus* tamoxifen-sensitive breast cancer cells; miR-519a was the microRNA most highly correlated with tamoxifen resistance. Oncogenic miR-519a was also demonstrated to increase resistance to tamoxifen-induced apoptosis as well as cell viability and cell cycle progression. In addition, the oncogenic properties of miR-519a were confirmed in gene expression datasets (Gene Expression Omnibus; GEO) of breast cancer patients. Among patients who received tamoxifen, higher expression of miR-519a was correlated with poorer disease-free survival in patients with ER+ tumors. Furthermore, by using algorithms for microRNA predictive targets, they validated the tumor suppressor genes PTEN of the PI3K/Akt pathway, and retinoblastoma protein (RB1) and CDKN1A/p21 as direct targets of miR-519a [48].

3.4. Candidate MicroRNAs in Signaling Pathways and Their Relevant Target Genes in Tamoxifen Resistance

For each of the seven candidate microRNAs involved in patient tamoxifen response, our IPA analyses generated a list of predicted target genes (Table 2).

Based on these results, IPA generated several networks depicting various direct and indirect targets for each of the selected microRNAs (Figures 4–6). The networks are graphically represented as explained in the legend in the figure.

3.4.1. Targets of miR-26a

The IPA-analyses demonstrate that six of the seven microRNAs are directly or indirectly associated with estrogen receptor; *i.e.* miR-126, miR-210, miR-26a, miR-519a, miR-30c and miR-342. As seen in Figure 4A, only miR-26a is highly predicted to directly target the *ER gene* (synonymous to *ESR1* shown in Figure 4), while the other microRNAs are only indirectly linked to ESR1.

In the top network of miR-26a seen in Figure 6D, the well-known tumor suppressor protein retinoblastoma 1 (RB1) is predicted to be a direct target of miR-26a. As mentioned, Ward *et al*. identified RB1 to be targeted by miR-519a, but this is not mapped as a target in our IPA network for miR-519a. In 2007, deregulation of the RB1 pathway was shown to be associated with early recurrence

following tamoxifen monotherapy [74]. Also, cyclin-dependent kinase 6 (CDK6) is a direct target of miR-26a.

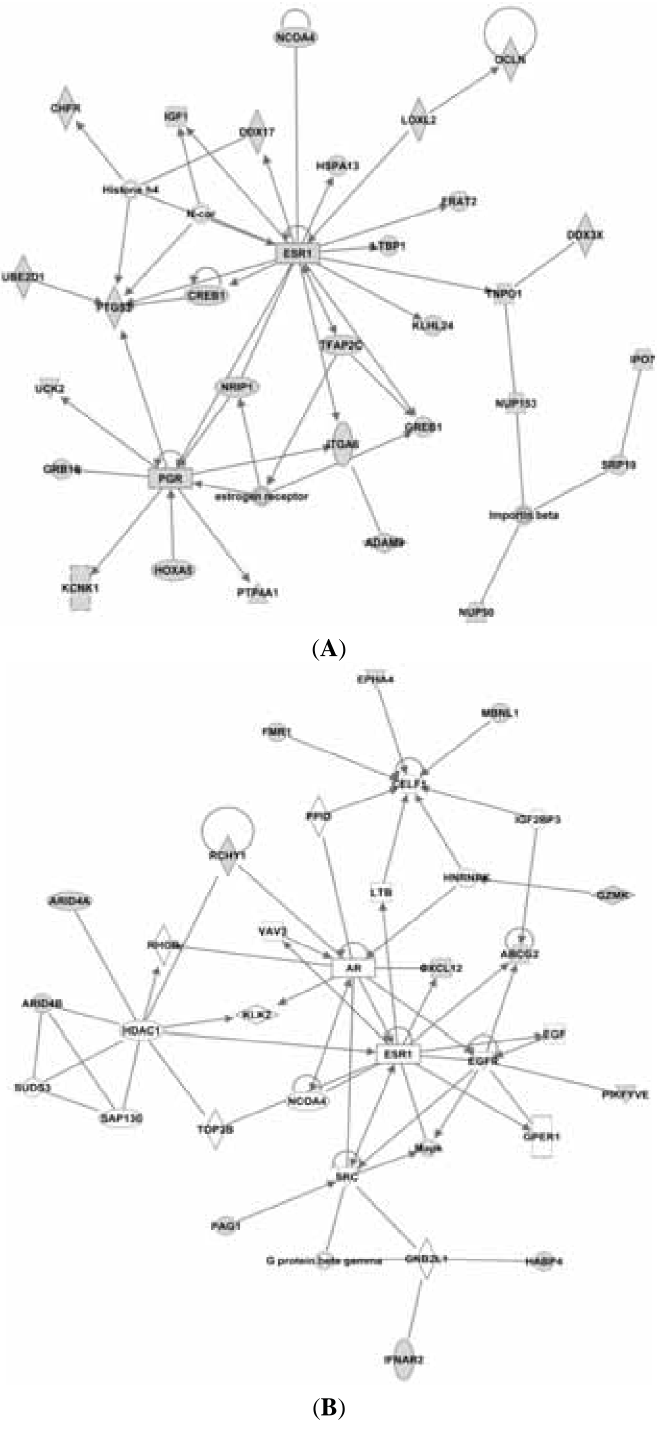
As seen in Figure 4A, insulin-like growth factor 1 (IGF1) is also predicted as a direct target for miR-26a, this is very interesting as IGF1 is highly expressed in the presence of estradiol [75]. IGF1 binds to the IGF1 receptor (IGF1R) and activates downstream pathways such as mitogen-activated protein kinase (MAPK) and PI3K pathways [76]. IGF1R and ER are strongly connected [77], and because of their crosstalk, the combination of IGF1R and ER antagonists has been clinically tested; although without convincing results [78].

3.4.2. Targets of miR-519

In Figure 4B for miR-519a, Phosphoprotein membrane anchor with glycosphingolipid microdomains 1 (PAG1) is seen as a direct target, while the proto-oncogene tyrosine-protein kinase Src is seen as an indirect target for miR-519a. Interestingly, by inhibiting the activity of Src in MCF7-cells, estrogen-stimulated proliferation was blocked [79]. Similarly, when constitutively active Src was expressed in endocrine-sensitive MCF-7 cells, the cells response to tamoxifen was attenuated, whereas tamoxifen-resistant MCF7-cells were re-sensitized when Src was suppressed. Additionally, elevated Src activity in tumor tissue was associated with clinically poor prognosis [80]. Furthermore, Src expression and Src-phosphorylation has been found to be increased in tamoxifen resistant T47D-cells. In the same study, membrane expression of Src on tamoxifen-treated breast tumor cells was associated with reduced disease-free and overall survival [81]. EGFR, MAPK and GPER1 are also indirect targets of miR-519a. GPER (G protein-coupled estrogen receptor) is inversely associated with tamoxifen resistance as confirmed in a cohort study of 103 patients [82]. Moreover, in a study by Yuan *et al.*, GPER was shown to be important in the initiation/induction of tamoxifen resistance, and is thought to contribute to tamoxifen resistance by interaction with EGFR during long-term treatment with tamoxifen in breast cancer cells. This crosstalk leads to phosphorylation of MAPK and AKT thus stimulating ER-independent gene transcription and development of tamoxifen resistance [83].

3.4.3. Targets of miR-210

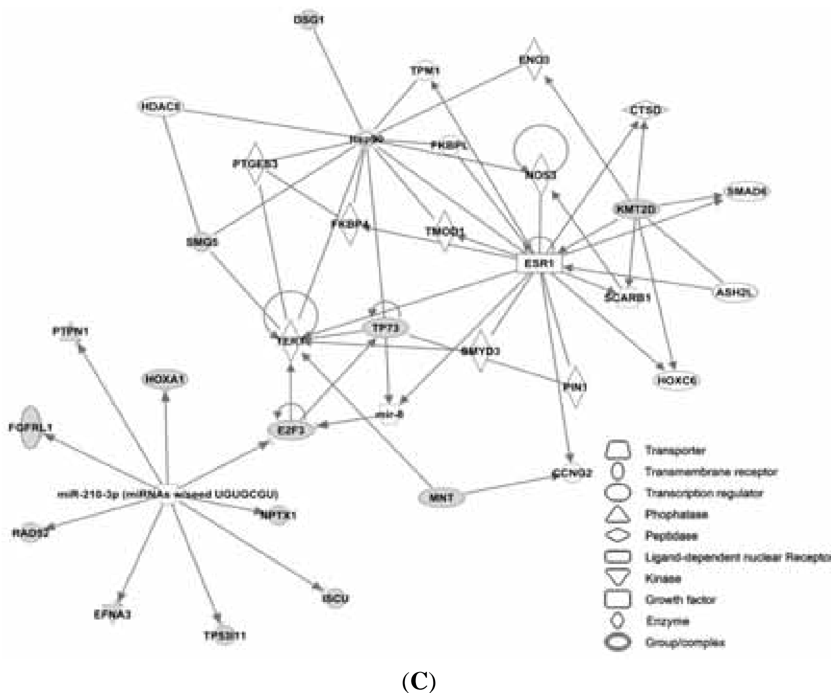
Homeobox A1 (HOXA1) is predicted to act as a direct target for miR-210 (Figure 4C). The HOXA1 is an ER-regulated gene and the HOXA1 locus is believed to be involved in promoting growth of tamoxifen resistant breast cancer cells. ER forms a complex with lysine (K)-specific demethylase 3A (KDM3A), which indirectly regulates the transcriptional outcome of the HOXA1 locus. This results in increased activation of ER in the presence of tamoxifen [84,85]. Heat shock protein 90 (Hsp90) is another direct target of miR-210. Chaperone molecules, of which Hsp90 is one of the most common, are involved in many important cellular pathways, especially in regulating the folding and sorting of proteins, as well as in the cells response to stress, cellular homeostasis, and cell cycle control [86]. It has been shown that tamoxifen and its metabolite 4-OHT may enhance the ATPase activity of Hsp90. The active metabolite was identified as a putative ligand for Hsp90 [87]. More recently, inhibition of Hsp90 has been shown to dramatically impair the emergence of resistance to hormone antagonists (tamoxifen and fulvestrant) in both cell culture and mice [88].



(**A**)

(**B**)

Figure 4. *Cont.*



(**C**)

Figure 4. IPA networks for miR-26a, network 2 (**A**), miR-519a, network 1 (**B**) and miR-210, network 1 (**C**), centered on the estrogen receptor (ESR1). These networks created by IPA comprise networks with ER as a direct (miR-26a) or indirect (miR-519a and miR-210) target. Shaded boxes refer to direct targets whilst clear boxes refer to indirect targets of the specific miRNA.

3.4.4. Targets of miR-30c

In network 3 of miR-30c in Figure 5A, an estrogen receptor complex was found as an indirect target of miR-30c. In addition, forkhead box A1 (FOXA1) is a predicted target of miR-30c in this network, and is associated with ER. FOXA1 is important in ER-binding to chromatin, and is shown to be important for ER functioning as well as endocrine response in breast cancer cells [89,90].

miR-30c had the highest number of predicted target genes (1420), and as presented in Figure 6A, all were direct targets. Among them are cytochrome P450, family 24, subfamily A, polypeptide 1 (CYP24A1), phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta (PIK3CD), phosphoinositide-3-kinase regulatory subunit 2 (PIK3R2) and TIMP metallopeptidase inhibitor 3 (TIMP3). CYP24A1 is a member of the cytochrome P450 superfamily. This enzymatic family plays important roles in drug metabolism and the synthesis of steroids and cholesterol. CYP24A1 is involved in regulation of vitamin D3 level, calcium homeostasis and the vitamin D endocrine system [91]. In both tamoxifen-sensitive and -resistant breast cancer cells, 1α,25-dihydroxyvitamin D3 has an antiproliferative effect [92]. TIMP3 inhibits matrix metalloproteinases, and is seen as a direct target of

miR-30c, but also a direct target for miR-221 and miR-222. Suppression of these microRNAs leads to an increased sensitivity to tamoxifen, mediated by TIMP3, in ER+ MCF-7 cells [93].

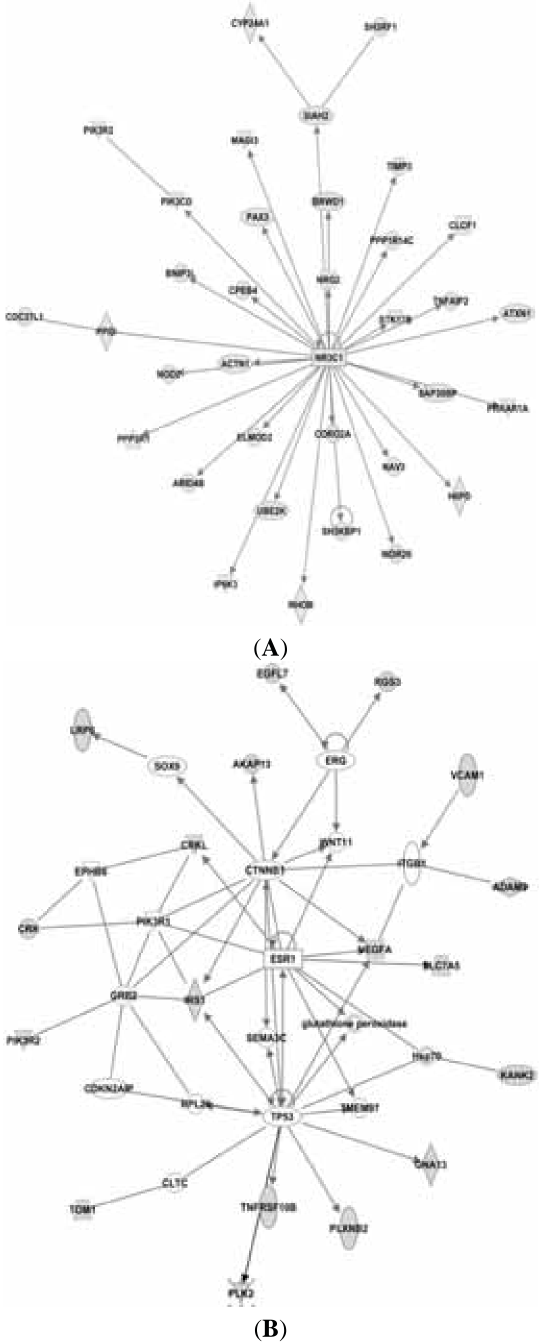
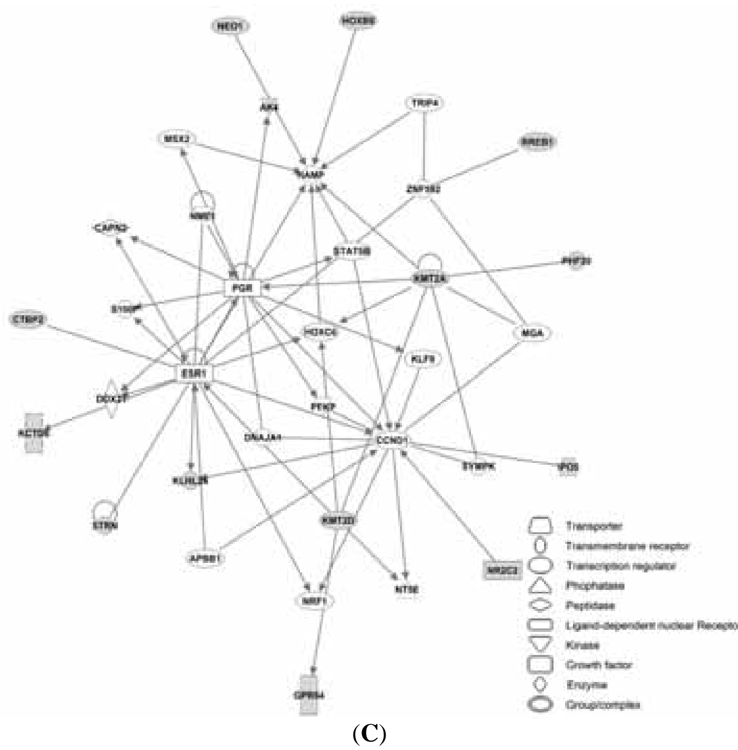


Figure 5. *Cont.*



(**C**)

Figure 5. IPA networks for miR-30c, network (**A**), miR-126, network 1 (**B**) and miR-342, network 2 (**C**), centered on the estrogen receptor (ESR1). These networks created by IPA, comprise networks with ER as an indirect target (miR-126 and miR-342), whereas miR-30c has an ER-complex as an indirect target. Shaded boxes refer to direct targets whilst clear boxes refer to indirect targets of the specific miRNA.

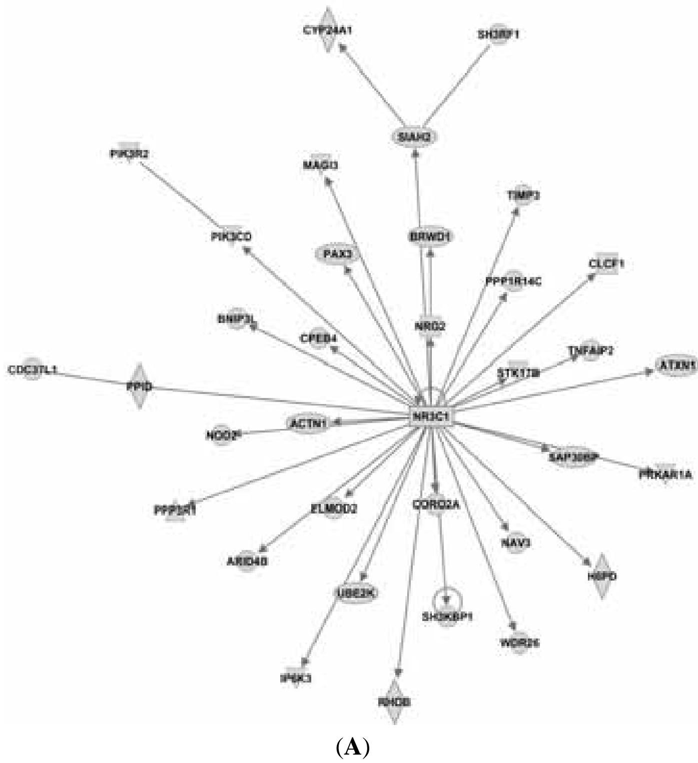
In addition, PIK3CD and PIK3R2 are also two direct targets of miR-30c. PIK3R2 is also seen as a direct target of miR-126 (see Figure 5A). Another such kinase, the phosphatidylinositol-4,5-bisphosphate 3-kinase (PIK3CA), is a predicted direct target gene of miR-10a. PIK3CD, PIK3R2 and PIK3CA are all members of the Class I phosphoinositide 3-kinase (PI3K) enzymes, which have been shown to be involved in several types of cancer and involved in the Akt/mTOR pathway [94,95]. PI3KCA mutations are frequent in breast cancer, especially in ER+ breast cancer. In fact, 40% of luminal breast cancers have PI3KCA mutations [96], making this the most common mutation in breast cancer. Furthermore, the overall mutation rate in the whole PI3K pathway in breast cancer is >70% [97]. Activation of the PI3K pathway can lead to activation of proliferation, or growth and inhibition of apoptosis. Even though PIK3CA mutations have a high mutation frequency in breast cancer patients, it does not seem to be a good independent predictor in the context of endocrine therapy [98].

3.4.5. Targets of miR-126

As seen in Table 2, miR-126 had the fewest predicted targets genes (37) in our IPA analysis. In network 1 of miR-126 (Figure 5B), PI3KR2 is shown as a direct target, whereas Hsp 70, CLTC and TP53 are shown as indirect targets. Hsp70 is a component of the molecular chaperone machinery, which aids in assembly and trafficking of steroid receptors. Clathrin heavy chain (CLTC) is involved in intracellular trafficking as well as endocytosis, and was recently identified as a target of miR-574-3p, which again was shown to modulate tamoxifen-resistance in MCF-7 cells [99].

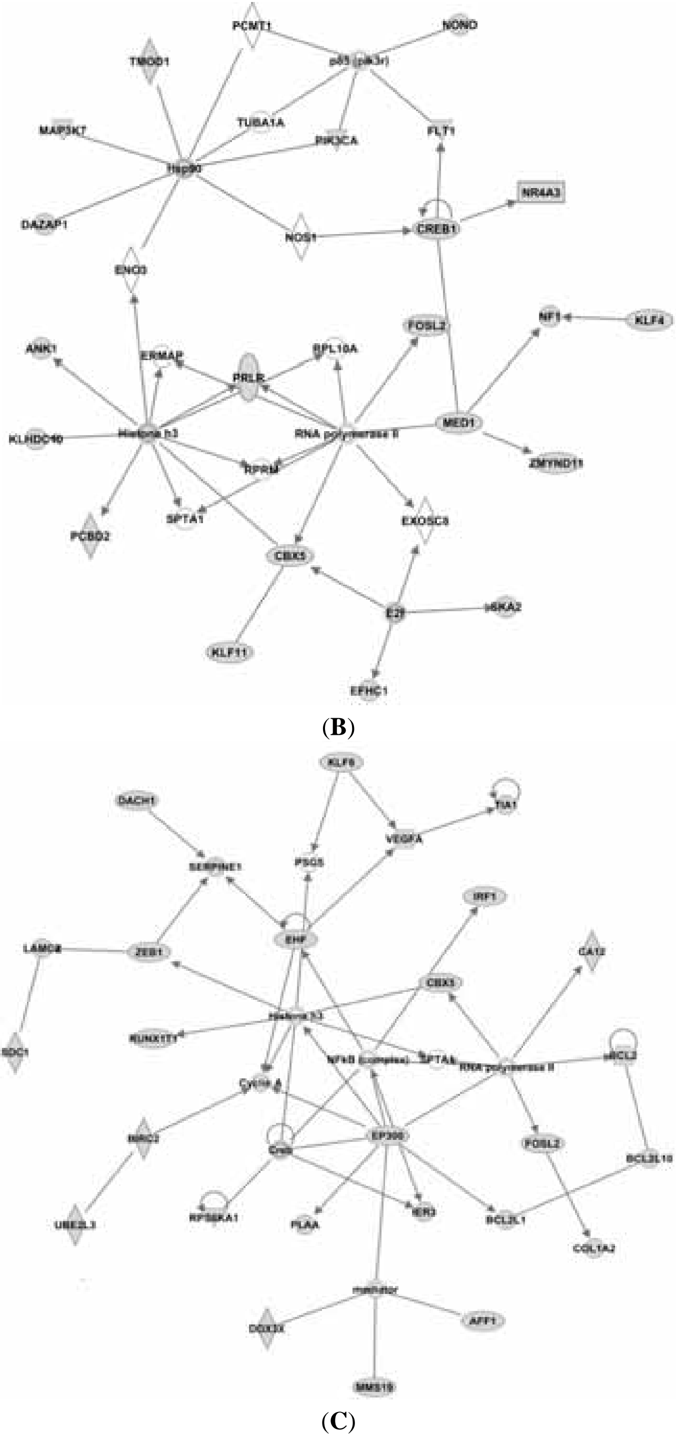
3.4.6. Targets of miR-342

As seen in Figure 6C, the transcription factor Zinc Finger E-box Binding Homeobox (ZEB1) is a direct target of miR-342. ZEB1 has previously been associated with increased tamoxifen resistance and reduced expression of miR-200 in LY2 endocrine resistant breast cancer cells [100]. As illustrated in the network, another direct target of miR-342 is B-cell CLL/Lymphoma 2 (BCL2), an oncogene that is involved in regulation of apoptosis. In tamoxifen resistant cell line studies miR-15a and miR-16 have also been shown to activate BCL2 expression, and thereby promote resistance in HER2/ER^{+} breast cancer cells [101]. In Figure 5C both ESR1 and PGR are shown as indirect targets of miR-342. Homeobox B1 (HOXB1) is seen as a direct target.



(**A**)

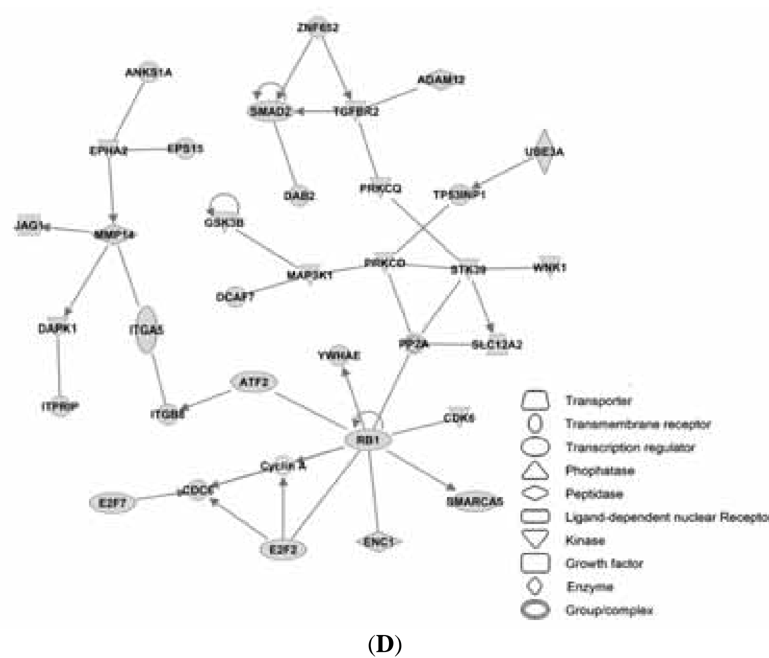
Figure 6. *Cont.*



(**B**)

(**C**)

Figure 6. *Cont.*



(**D**)

Figure 6. Top networks of miR-30c, network 1 (**A**), miR-10a, network 1 (**B**), miR-342, network 1 (**C**) and miR-26a, network 1 (**D**). Shown here are networks with the highest scores calculated by IPA, showing direct targets of the miRNAs. Shaded boxes refer to direct targets whilst clear boxes refer to indirect targets of the specific miRNA.

3.4.7. Targets of miR-10a

As seen in Figure 6A, apart from PIK3CA, miR-10a also targets heat-shock protein 90 (Hsp90) and Histone H3. Hsp90 is discussed previously, see Figure 4C. Histone H3 is part of the nucleosome and involved in transcription regulation and proliferation. Interestingly, miR-10a and miR-342 share five targets in their top rated networks; CBX5, FOSL2, Histone H3, RNA polymerase II and spectrin, alpha, erythrocytic 1 (SPTA1). Chromobox homolog 5 (CBX5) is a heterochromatin protein associated with centromeres. Spectrin alpha 1 (SPTA1) is a scaffold protein. FOS-Like antigen 2 (FOSL2) is a member of the Fos gene family that encodes leucine zipper proteins and have been implicated in cell proliferation, transformation and differentiation [91]. However, no correlations between these genes and tamoxifen resistance have been reported at this moment.

4. Discussion

In the present paper, we review the potential of miRNAs as biomarkers for predicting the response to tamoxifen in breast cancer patients (Figure 7). Surprisingly, among more than 2400 known microRNAs, only seven have been associated with tamoxifen resistance according to our literature search, where we focused only on studies that included patient material. These seven microRNAs

could well be part of the bigger picture, as many more microRNAs have been correlated with tamoxifen resistance from *in vitro* analyses.

Our IPA analysis suggests that signaling involved in endocrine resistance can be divided in two main signaling networks: One related to ER-signaling (Figure 4) and the second to membrane-related receptors (*i.e.*, EGFR, HER-2, IGF) (Figure 5). As observed in these predicted target networks and discussed before, tamoxifen resistance does not seem to be directly related to changes in ER itself, and, as Figure 5 implies, changes in various molecules in the network surrounding ER could contribute to tamoxifen resistance. These networks and pathways may have an important role in tamoxifen resistance, and therefore the role of these microRNAs should be further investigated. Notably, no link between microRNAs and tamoxifen metabolic pathways (e.g., CYP2D6) was found in our IPA analysis. This may reflect the negative results in studies examining the relation between genetic polymorphisms and relapses on tamoxifen treatment [102].

As an alternative explanation of endocrine resistance, the switch to other ER signaling independent pathways (e.g., the EGFR pathway) that drive the cellular survival processes are reported [36,103]. The IPA analyses also suggest that microRNAs related to signal networks independent of ER signaling might have a direct and strong connection to these pathways, indicating that these mechanisms are more important predictors of endocrine resistance than ER-related signaling networks.

The underlying heterogeneity of breast tumors is one important explanation of this observation [104]. In ER+ tumors (*i.e.*, ≥1% positively stained tumor cells by immunohistochemistry) most clones may respond to endocrine treatment, while others are non-responders. In the former type, up-regulation of various receptor tyrosine kinases (RTKs), *i.e.*, HER-2 may take place [105,106]. This phenomenon is thought to be an escape mechanism from endocrine control of cancer cells.

Examples of microRNAs involved in the switch to alternative ER-independent pathways are miR-10a, miR-126 and miR-30c relation to PI3K signaling. This pathway is a central node in mediating growth factor receptor signaling, and is known to characterize the more aggressive and less endocrine sensitive luminal B-subtype of breast cancers [107]. Studies indicate that only a slight loss in inhibition of this potent signaling pathway (*i.e.*, loss of PTEN) is enough to induce endocrine resistance. Interestingly, this escape from endocrine control can be restored by targeting mTOR, Protein Kinase B (Akt) or Mitogen Activated Kinase that are located downstream in the same pathway [108]. Moreover, lessons from the treatment of metastatic ER+ breast cancer patients support the importance of targeting RTKs to restore the endocrine sensitivity in breast cancer tissue. Reliable predictive markers for the PI3K/Akt/mTOR axis are necessary to indicate the need for co-targeting PI3K and ER pathways to restore endocrine sensibility. The importance of these microRNAs is demonstrated since they may help identify the timing of change in treatment strategy.

Interestingly, microRNAs are involved in various hallmarks of cancer and can interact with several characteristics at the same time [109]. From our list of microRNAs involved in tamoxifen resistance (Table 2), cell proliferation and invasion is enhanced by elevation of miR-210 [50,110], cell survival is promoted by a decrease in miR-26a [111], and angiogenesis is stimulated by elevation of miR-126 [112]. Multigene assays show that proliferation related genes dominate the basis of the predictive effect of chemotherapy in endocrine responsive early breast cancer [113,114]. However, molecular subtyping is suitable for short-term evaluation only (*i.e.*, the first five years of follow, as they seem to lose their power in long-term perspectives [115]).

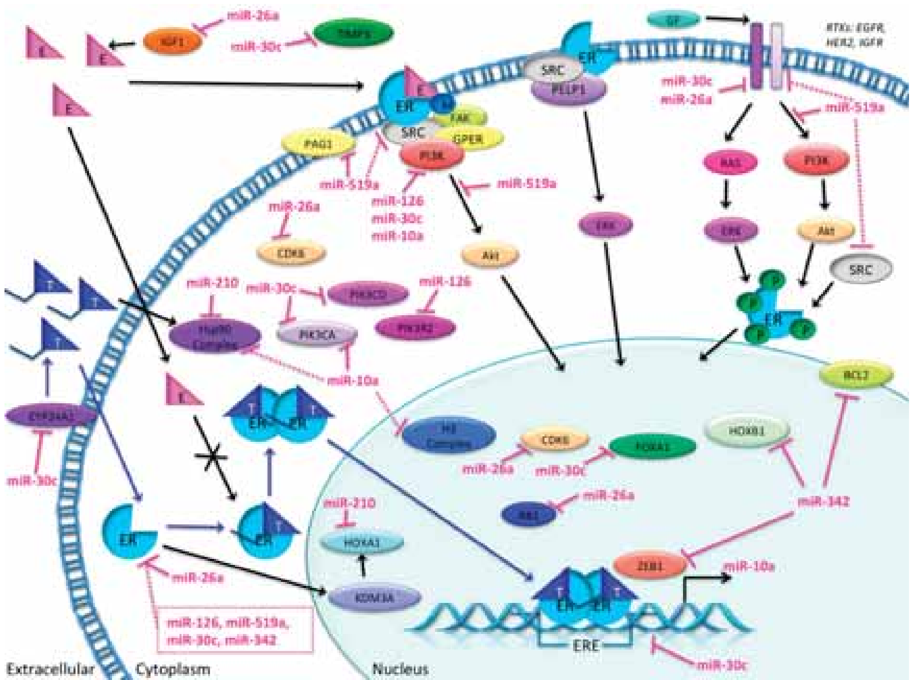


Figure 7. Overview of the possible involvement of candidate microRNAs in tamoxifen resistance pathways, based on the present literature search and IPA analysis. MicroRNAs miR-10a, -26a, -30c, -126, -210, -342-5p and -519a and their direct (pink lines) or indirect (dotted lines) targets. BCL2: B-cell CLL/lymphoma 2; CDK6: cyclin-dependent kinase 6; CYP24A1: cytochrome P450, family 24, subfamily A, polypeptide 1; IGF1: insulin-like growth factor 1; FOXA1: forkhead box A1; HOXA1: homeobox A1; HOXB1: homeobox B1; Hsp90: heat-shock protein 90. KDM3A: lysine (K)-specific demethylase 3A. PAG1: phosphoprotein membrane anchor with glycosphingolipid microdomains 1; PI3K: phosphoinositide 3-kinase; PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; PIK3CD: phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta; PIK3R2: phosphoinositide-3-kinase, regulatory subunit 2 (beta); RB1: retinoblastoma 1; TIMP3: TIMP metallopeptidase inhibitor 3; ZEB1: zinc finger E-box binding homeobox 1. Black arrow: normal pathway. Blue arrow: tamoxifen pathway. Crossed arrow: disrupted pathway. Pink inhibition arrow: direct inhibition by miRNA. Dotted pink inhibition arrow: indirect inhibition by miRNA.

Thus, since microRNAs interact with several hallmarks of cancer simultaneously, detailed characterization of these microRNAs may provide an improved understanding of the underlying resistance mechanism as compared to the currently established biomarkers in breast cancer. Promising predictors for late recurrences are involvement of micro-environmental stromal factors and the EMT processes [116]. Factors that can predict long-term cancer cell survival will be of particular interest since endocrine therapy is given over a long time span (Figure 2).

Clinicians face various challenges when deciding the most effective endocrine therapy for each patient. The following four clinical scenarios all have the need for reliable biomarkers that can point out which patients can be assigned tamoxifen treatment from those who cannot. First, in younger patients, tamoxifen is increasingly given as adjuvant therapy to patients operated for ductal carcinoma *in situ*. It increases event-free survival, even at the population level [117]; Second, in the younger pre-menopausal patients with invasive ER+ breast cancer, it is recommended to administer either 10 years with tamoxifen, or five years with OFS in addition to tamoxifen or AI (Figure 2). The latter regime is shown to be superior to tamoxifen monotherapy, but is not very well tolerated [118]; Third, in postmenopausal women AIs for five years upfront or for at least two years followed by tamoxifen for three years is recommended (Figure 2); Fourth, the elderly co-morbid patients are often treated with endocrine treatment only, while tumor size is under surveillance. Elderly women are especially vulnerable to AIs, e.g., due to high fracture rates [119]. Since the side effects of AIs are quite substantial and probably highly underestimated [120], biomarkers that can identify tamoxifen-responsive tumors, so that AIs can be avoided, are clinically relevant. Selection of the tamoxifen-sensitive tumors by means of reliable biomarkers would be clinically very helpful in managing these patients, also to indicate development of endocrine resistance so other treatment options can be considered (*i.e.*, radiation therapy or surgery). All these scenarios share the need to distinguish between tamoxifen and alternative regimens that comprise either OFS or an AI. Today, clinicians are not able to take into account the heterogeneity of the tumors; as a consequence, we treat breast cancer with a wide specter of ER positivity (*i.e.*, ranging from ≥1% up to 100%) with the same endocrine strategy.

The tumor heterogeneity calls for a double strategy to monitor endocrine effectiveness. During the early phase of the tamoxifen treatment, identification of possible endocrine sensitive/responsive tumors might provide important information whether or not endocrine therapy will work. Later, during long-term follow-up, markers that can identify emerging endocrine resistant clones at an early stage might allow change of therapy before the endocrine resistance becomes clinically evident. This "dual-approach" is more in line with the intrinsic tumor biology of breast cancer. In the large adjuvant ATAC [34] and BIG-1 98 trials [35], tamoxifen was compared with anastrozole and letrozole, respectively. These studies provide valuable long-term follow-up data on relapse and survival in four different treatment arms. In order to get an indication of which microRNA might depict endocrine sensitivity in the adjuvant setting, microRNA profiling of patients from these two studies could promptly provide valuable information on this issue. Such studies would also provide microRNA profiles from treatment arms that switch from tamoxifen to AI and vice versa. Moreover, microRNA analyses in population-based epidemiological studies (e.g., our ongoing work) will strengthen the findings from clinical trials. Candidate microRNAs identified should be validated in other prospective trials. Such prospective studies should comprise a combinatory analysis of microRNA expression profiles in the primary tumor together with concurrent free microRNAs in serum or plasma. Moreover, patients enrolled in neo-adjuvant treatment trials and endocrine-controlled comorbid elderly patients, are especially suitable for repeated tumor biopsies during treatment. Repeated microRNA profiling of both primary tumors and plasma microRNAs during treatment will elucidate which microRNAs might be promising marker candidates; both for endocrine sensitivity (if the tumor shrinks) as well as endocrine resistance (if the tumor stops responding or increases in size).

5. Conclusions

In conclusion, endocrine treatment remains one of the most important strategies for eradicating and/or controlling ER positive breast cancer. Biomarkers of endocrine responsiveness and resistance are urgently needed to help clinicians in making treatment decisions. As microRNAs are able to exert control at the translational level, they are an important link between coding genes and the various cellular processes. Hence, microRNAs are certainly promising candidate biomarkers that could be used in the clinic to guide tamoxifen treatment. One immediate aim must be to include a combined microRNA profiling in tumor tissues and in plasma in on-going and future clinical studies with long-term follow-up. This approach seems like a small step for the role of microRNA as a biomarker, but will undoubtedly bring the clinical knowledge of microRNA in endocrine treatment in breast cancer a giant leap forward.

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Author Contributions

Nina G. Egeland and Siri Lunde contributed equally to this work. Nina G. Egeland and Siri Lunde performed the literature research, prepared figures and tables and co-wrote the manuscript. Kristin Jonsdottir performed the IPA analyses and contributed with writing sections of the manuscript. Emiel A. M. Janssen and Håvard Søiland participated in the conception and coordination of the manuscript, supervised the work and contributed with writing sections of the manuscript. Tone H. Lende, Deirdre Cronin-Fenton and Bjørnar Gilje contributed with editing of the manuscript and made critical revisions to the manuscript. All authors contributed to, and have given approval to, the final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Paper I

RESEARCH ARTICLE

Validation study of MARCKSL1 as a prognostic factor in lymph node-negative breast cancer patients

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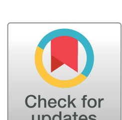

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Abstract

Protein expression of Myristoylated alanine-rich C kinase substrate like-1 (MARCKSL1) has been identified as a prognostic factor in lymph-node negative (LN^-) breast cancer patients. We aim to validate MARCKSL1 protein expression as a prognostic marker for distant metastasis-free survival (DMFS) in a new cohort of LN^- breast cancer patients. MARCKSL1 expression was evaluated in 151 operable $T_{1,2}N_0M_0$ LN^- breast cancer patients by immunohistochemistry. Median follow-up time was 152 months, range 11–189 months. Results were compared with classical prognosticators (age, tumor diameter, grade, estrogen receptor, and proliferation) using single (Kaplan-Meier) and multivariate (Cox model) survival analysis. Thirteen patients (9%) developed distant metastases. With both single and multiple analysis of all features, MARCKSL1 did not show a significant prognostic value for DMFS (p = 0.498). Of the assessed classical prognosticators, only tumor diameter showed prognostic value (hazard ratio 9.3, 95% confidence interval 2.8–31.0, p <0.001). MARCK SL1 expression could not be confirmed as a prognostic factor in this cohort. Possible reasons include changes in diagnostic and treatment guidelines between the discovery and validation cohorts. Further studies are needed to reveal the potential biological role of this protein in breast cancer.

Introduction

Breast cancer is a leading cause of death for women in the western world. In Norway women have a cumulative risk of 8.6% for developing the disease before the age of 75 [1]. About 50% of these women will present with stage I-II lymph-node negative (LN^-) cancer [2]. Stage I-II patients generally have a good prognosis, with a five-year survival rate of 89–99% [1], and not

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all may benefit from additional chemotherapy [3]. Still, approximately 12% of LN⁻ patients will experience recurrence, even up to 20 years after the initial diagnosis [4–6]. This, in combination with few recurrences among many patients with a good prognosis, leads to both over- and undertreatment of patients. Consequently, there is a need for new prognostic factors, as unnecessary chemotherapy and radiation may cause late-onset adverse effects, which reduce the quality of life of cancer survivors [7, 8].

Previous research has identified several proliferation markers such as phosphohistone H3 (PPH3), Ki-67 and mitotic activity index (MAI) as prognostic factors for LN⁻ breast cancer [9–13]. PPH3 demarks mitotic chromatin condensation, a late stage in mitosis, while Ki-67 is a nuclear protein that is expressed in all active phases of the cell cycle [10]. MAI, which is the number of cells undergoing mitosis, has been shown to be the best prognostic factor in LN⁻ patients younger than 55 years [13, 14]. Proliferation markers make up an important part of prognostic gene signature markers [12], and according to the latest guidelines of the Norwegian Breast Cancer group (NBCG), high proliferation is an indicator for chemotherapy [15]. However, even though proliferation markers are statistically very prognostic they are still not specific enough, as only 30–40% of patients with highly proliferating tumors will develop distant metastases [13]. As such, there is a need for markers highlighting additional aspects of tumor cell aggressiveness.

The metastatic potential of tumor cells depends on invasion through the extracellular matrix (ECM). This invasion is a multistep process involving cellular deformation and degradation of the ECM [16]. The myristoylated alanine-rich C-kinase substrate (MARCKS) family of proteins function in cytoskeletal regulation, protein kinase C signaling, and calmodulin signaling, and are implicated in cell motility, adhesion, and mitogenesis [17–19]. The MARCKS family of proteins differ in subcellular location and membrane binding affinity, and includes the myristoylated alanine-rich C-kinase substrate-like 1 (MARCKSL1), also known as MARCKS-related protein (MRP) and MARCKS-like protein (MLP) [20]. MARCKSL1 is a membrane-bound actin cytoskeleton regulator [18, 21], associated with tumorigenesis in several cancer types [22, 23]. In breast cancer cell lines, MARCKSL1 knockdown results in decreased migration [24]. Furthermore, MARCKSL1 was the strongest upregulated gene in response to estradiol in estrogen receptor alpha (ERα) positive cells co-cultured with bone cells; suggesting a more aggressive tumor phenotype associated with bone metastasis [25]. In contrast, MARCKSL1 also exhibits anti-angiogenic effects in ovarian tumors by suppressing VEGFR2-dependent AKT/PDK-1/mTOR phosphorylation [26]. In addition, in another breast cancer cell line experiment, MARCKSL1 was shown to suppresses LOXL2 induced oncogenesis and stimulating apoptosis [27], thereby acting as a tumor suppressor. The role of MARCKSL1 in tumor progression thus remains to be elucidated.

In a previous study by the authors, MARCKSL1 protein expression was found to be the strongest prognosticator for metastasis-free survival in node-negative breast cancer patients, with additional value in those with high proliferation [28]. Patients with high MARCKSL1 protein expression showed a 44% survival at 15 years follow up, versus 92% survival in those with low expression, yielding a hazard ratio (HR) of 5.1, confidence interval (CI) 2.7–9.8. Since then, few other studies on the MARCKS family of proteins in breast cancer have been published, and the results are conflicting. This calls for further studies on MARKSL1, examining its validity as a clinical biomarker.

Therefore, in the current study we aim to validate the prognostic value of MARCKSL1 protein expression in a new cohort of LN⁻ breast cancer patients. The MARCKSL1 expression was compared with classical prognosticators such as age, tumor diameter, grade, hormone receptor status, presence of tumor-infiltrating lymphocytes (TILs) and proliferation, with distant metastases free survival as the endpoint.

Materials and methods

Patients and pathology

Prior to commencement, the study was approved by the Norwegian National Research Ethics Committees/Regional Committees for Medical and Health Research Ethics (REC) West (REC number 210.04). The REMARK guidelines for reporting tumor marker studies were followed [29]. All 190 patients, <71 years of age at diagnosis, were diagnosed with invasive, operable ($T_{1,2}N_0M_0$) breast cancer at the Stavanger University Hospital (SUS), between January 15, 2002 and December 22, 2004. Thirteen patients could not be assessed for MARCKSL1 expression, and 26 patients were lost to follow up, had contralateral breast cancer either prior to inclusion or at follow up, or had received neoadjuvant chemotherapy, leaving 151 patients for analysis (Fig 1). There were no significant differences between the original 190 and final 151 cases in any of the features analyzed. The patients were treated according to the national guidelines of the Norwegian Breast Cancer Group. The tumor size was measured in the fresh specimens following excision and cut in slices of 0.5 cm. The axillary fat was examined macroscopically and all detectable lymph nodes were prepared for histology. The median number of identified lymph nodes was two (range 1–21). All tissues were fixed in buffered 4% formaldehyde and embedded in paraffin. Histological sections (4 μm) were made and stained with hematoxylin–erythrosine–saffron (HES). Histological type and grade were assessed by two pathologists (EG and JPAB) according to the World Health Organization criteria [30]. MAI was assessed as described elsewhere [31].

Immunohistochemistry

ER and progesterone receptor (PR), PPH3, Ki-67, cytokeratin 5/6 (CK5/6), human epidermal growth factor receptor 2 (HER2), and MARCKSL1 expression were determined by immunohistochemistry (IHC) in whole sections. Antigen retrieval and IHC techniques were based on DAKO technology [9]. In brief, formalin fixed paraffin-embedded (FFPE) sections, 4 μm thick, serially sectioned following HES sections, were mounted onto silanized slides (#S3002, DAKO, Glostrup, Denmark). Antigen retrieval was performed with a highly stabilized retrieval system (ImmunoPrep; Instrumec, Oslo, Norway) using 10 mM Tris/1 mM EDTA (pH 9.0) as the retrieval buffer. Sections were heated for 3 min at 110˚C followed by 10 min at 95˚C then

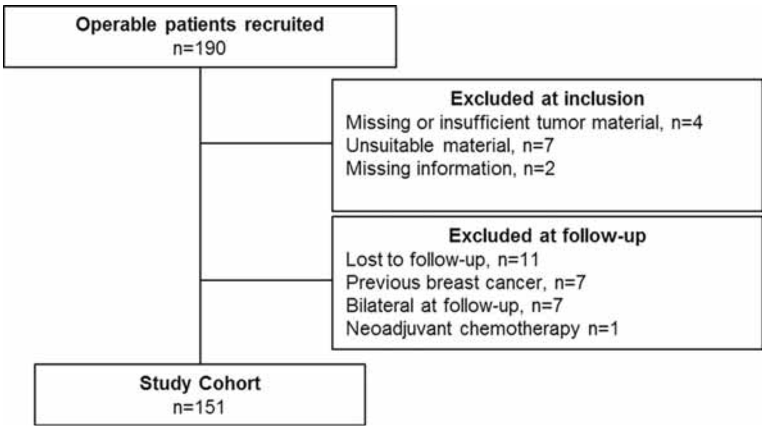

Fig 1. REMARK diagram illustrating patient flow in the study.

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cooled to 20˚C. ER (clone SP1, Neomarkers/LabVision, Fremont, CA, USA) was used at a dilution 1:400. PR (clone SP2, Neomarkers/LabVision) was used at a dilution of 1:1000. Rabbit polyclonal anti-PPH3 (ser 10) (Upstate #06–570; Lake Placid, NY) was used at a dilution of 1:1500. Ki-67 (clone MIB-1, DAKO, Glostrup, Denmark) was used at dilution 1:100. CK 5/6 (Clone D5/16 B4, Dako, Glostrup, Denmark) was used at a dilution of 1:100. Mouse monoclonal MARCKSL1 (Clone K53, sc-130471, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1:300. All antibodies were incubated for 30 min at 22˚C. The EnVision™ FLEX detection system (Dako, K8000) was used for visualization. Sections were incubated for 5 min with peroxidase-blocking reagent (SM801), 30 min with the primary antibody, 20 min with the EnVision™ FLEX/HRP Detection Reagent (SM802), 10 min with EnVision™ FLEX DAB+ Chromogen (DM827)/EnVision™ FLEX Substrate Buffer (SM803) mix and 5 min with EnVision™ FLEX Hematoxylin (K8008). The slides were dehydrated and mounted. All immunohistochemical stainings were performed using a Dako Autostainer Link 48 instrument and EnVision™ FLEX Wash Buffer (DM831). For HER2 assessments, DAKO HercepTest™ was used according to the procedures of the manufacturers.

Quantification of PPH3, Ki67, CK5/6, ER, PR, HER2, TILs and MARCKSL1

The PPH3 expression was evaluated by subjective count by counting the number of PPH3-positive objects at 40× by two independent pathologists in 10 adjacent fields of vision (FOV), or a total of 1.59 mm2, in the most PPH3-positive areas. For measuring percentage of Ki-67 positive cells, the semi-automatic interactive computerized QPRODIT system (Leica, Cambridge) was used [32]. For each measurement 250–350 fields of vision were selected, the Ki-67 percentage was defined as [(Ki-67 positive)/ (Ki-67 positive + Ki-67 negative)] x 100. The percentage of CK5/6 positive tumor cells in each tumor was scored using a continuous scale of 0–100%. In the final analysis, all tumors with any CK5/6 staining in tumor cells were grouped as being positive as described before [9]. ER was scored as positive when nuclear staining was present in $>$1% and scored negative when $<$1%. PR was scored as positive when nuclear staining was present in $>$10%, borderline 1–10% and negative when $<$1%. HER2 was scored according to the DAKO HercepTest scoring protocol. All 2+ and 3+ cases were regarded as positive. All sections were independently scored by two of the authors (BH and EJ). Tumor infiltrating lymphocytes (TILs) were scored semi-quantitatively in HE-stained tissue sections according to the presence or absence of stromal TILs. The relative amount of TILs in the tumor stroma area was then assessed according to the recommendations described by Salgado et al [33]. The degree of infiltration was scored in the range of 0–100%. Positive TILs were defined as $\geq$1%.

MARCKSL1 was scored in the same way as in our previous study [28] using the following criteria: overall diffuse cytoplasmic (referred to as cytoplasmic hereafter) staining, membrane staining, and granular staining in 10 high power fields (1.59 mm^2), usually the invasive front of the tumor (S1 Fig). For each of the criteria scoring from 0 to 3+ (0 = lowest score, 3+ = highest score) was given by assessing both intensity and number of positive tumor cells. For the membrane staining, the Dako HER2 scoring guideline was used. A total MARCKSL1 score was calculated by adding all the scores from the different criteria, resulting in a minimum score of 0 and a maximum score of 9 (S2 Fig). As in Jonsdottir et al, a high MARCKSL1 expression was defined as a score of $\geq$7 [28]. The slides were scored blinded and separately by two of the authors ER and EJ.

Survival endpoints

For survival analysis, the main endpoint was distant metastasis-free survival (DMFS). To determine the probability that patients would remain free from distant metastasis, we defined

recurrence as any recurrence at a distant site. Patients were censored from the date of their last hospital visit for death from other causes than breast cancer, or local or regional recurrences. If a patient's status during follow-up indicated a confirmed metastasis without a recurrence date, the last follow-up visit date was used. Age, time to first recurrence and survival time were calculated relative to the primary diagnosis date.

Statistical analyses

Statistical analysis was performed in SPSS (SPSS, Chicago, IL, USA), version 23. Kaplan-Meier survival curves were constructed and differences between groups were tested by the log-rank test. The relative importance of potential prognostic variables was tested using Cox-proportional hazard analysis (method: Forward, Wald) and expressed as HR with 95% CI. Group wise comparisons were performed using Fisher's exact test.

Results

Median age at diagnosis for the included patients was 55 (range 28–70) years with a median follow-up time of 152 months (range 11–189 months). In total, 13/151 patients (8.6%) developed distant metastasis and 11/151 patients (7.3%) died of breast cancer related disease. Survival and tumor related characteristics for distant metastasis-free survival (DMFS) are shown in Table 1.

Of the analyzed factors, only tumor diameter showed prognostic value (HR = 9.3, $p<0.001$, 95% CI = 2.8–31.0). MARCKSL1 protein expression was not a significant prognostic factor ($p = 0.498$). MARCKSL1 membrane score ($p = 0.263$), cytoplasmic score ($p = 0.221$) and granular score ($p = 0.307$) were not independently prognostic for DMFS either. Of the 151 patients, five (3%) had high MARCKSL1 protein expression and four (3%) had no MARCKSL1 expression in the cytoplasm, membrane or granules. MARCKSL1 protein expression did not have additional prognostic information in the group of patients with PPH3 $\geq$13 (Fig 2). No patients with low proliferation (MAI<10) showed high MARCKSL1 protein expression, therefore this subgroup could not be assessed for additional prognostic information by MARCKSL1, as suggested previously [28].

In the multivariate analyses, we included all variables showing $p<0.1$ with regards to DMFS in univariate analysis (tumor size, triple negative receptor status, TILs $\geq$1%). Tumor size was the strongest prognostic factor for DMFS (n = 140, 93%), and the only significant factor.

MARCKSL1 protein expression was higher in ERα and PR negative tumors ($p = 0.029$ and $p = 0.012$, respectively), and tumors with high proliferation (MAI $\geq$10 ($p = 0.004$) or PPH3 $\geq$13 ($p = 0.005$)), but did not differ between categories of age $\geq$55, Nottingham grade, tumor size ($\geq$2 cm), HER2, triple negative receptor status, Ki-67 $\geq$15 or $\geq$30. Tumor size was not a significant prognostic factor in ERα+ patients <55 years (Kaplan Meier $p = 0.286$), and no other factors were significant in this group. There was no difference in survival between Luminal A (ERα+, Ki-67 <15%) and Luminal B (ERα+, Ki-67 $\geq$15%) (Kaplan Meier $p = 0.362$) patients.

MARCKSL1 protein expression was significantly correlated to tumor size (Spearman's rho 0.220, $p = 0.007$), Nottingham grade (rho 0.263, $p = 0.002$), MAI (rho 0.316, $p<0.001$), PPH3 (rho 0.317, $p<0.001$) (Fig 3) and Ki-67 (rho = 0.269, $p = 0.001$).

Finally, we compared our validation cohort to the previous cohort to assess possible causes of the lack of prognostic value of MARCKSL1 (S1 Table). The validation cohort had fewer tumors larger than 2 cm (20% vs. 30%, $p = 0.018$), younger patients at diagnosis (51% versus 40% <55 years, $p = 0.035$), higher frequency of chemotherapy (56% vs. 15%, $p<0.001$) and

Table 1. DMFS in lymph node-negative breast cancer patients.

Characteristic	Distant metastasis			
	Event / at risk (%)	**Log rank p value**	**HR**	**95% CI**
Age				
<55	6/77 (92)	0.466	1.5	0.5–4.5
≥ 55	7/74 (91)			
Tumor diameter				
<2	5/121 (96)	<0.001	9.3	2.8–31.0
≥2	8/30 (73)			
Nottingham grade				
1	2/24 (92)	0.451		
2	4/60 (93)		1.0	0.2–5.2
3	5/34 (85)		2.3	0.4–12.3
Estrogen receptor				
Negative	3/24 (88)	0.413	1.7	0.5–6.2
Positive (≥ 1%)	10/126 (92)			
Progesterone receptor				
Negative	3/38 (92)	0.221	1.1	0.3–4.3
Positive (≥ 10%)	8/108 (93)		5.3	0.7–42.6
Borderline (1–9%)	1/3 (67%)			
Her2				
Negative	6/27 (78)	1.000	1.0	0.3–3.6
Positive	4/19 (79)			
Triple negative				
Any receptor positive	11/137 (92)	0.062	3.8	0.8–17.2
Triple negative	2/7 (71)			
MAI				
<10	8/108 (93)	0.296	1.8	0.6–5.5
≥ 10	5/40 (88)			
MAI				
0–2	4/66 (94)	0.473		
3–9	4/42 (91)		1.6	0.4–6.5
≥10	5/40 (88)		2.2	0.6–8.3
Ki-67				
0–9%	5/69 (93)	0.477	1.5	0.5–4.6
10–100%	8/78 (90)			
Ki-67			1.7	
<15%	5/81 (94)	0.324		
15–30%	4/39 (90)			0.5–6.5
>30%	4/27 (85)		2.5	0.7–9.9
PPH3				
<13	7/94 (93)	0.427	1.6	0.5–4.6
≥13	6/54 (89)			
TILS				
0%	3/72 (96)	0.063	3.2	0.9–11.6
≥1%	10/77 (87)			
CK5/6				
Negative	0/11 (100)	0.324	-	-
Positive (≥1%)	13/138 (91)			

(*Continued*)

Table 1. (Continued)

Characteristic	Distant metastasis			
	Event / at risk (%)	**Log rank p value**	**HR**	**95% CI**
MARCKSL1 total score				
Low (0–6)	13/146 (91)	0.498	-	-
High (7–9)	0/5 (100)			
MARCKSL1 total score in patients with PPH3>13				
Low (0–6)	6/49 (87)	0.417	-	-
High (7–9)	0/5 (100)			

CI, confidence interval; HR, hazard ratio; MAI, mitotic activity index

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endocrine therapy (24% vs 6%, p <0.001). At follow-up in the new cohort, there were fewer distant metastases (9% vs 15%, Fisher's exact test p = 0.055) and fewer deaths from breast cancer (7% vs 11%, Fisher's exact test p = 0.312) although not a significant reduction in either. There were more PR positive and fewer HER2 positive tumors in the validation cohort. In contrast with the validation study, high MARCKSL1 total score was associated with ER positivity

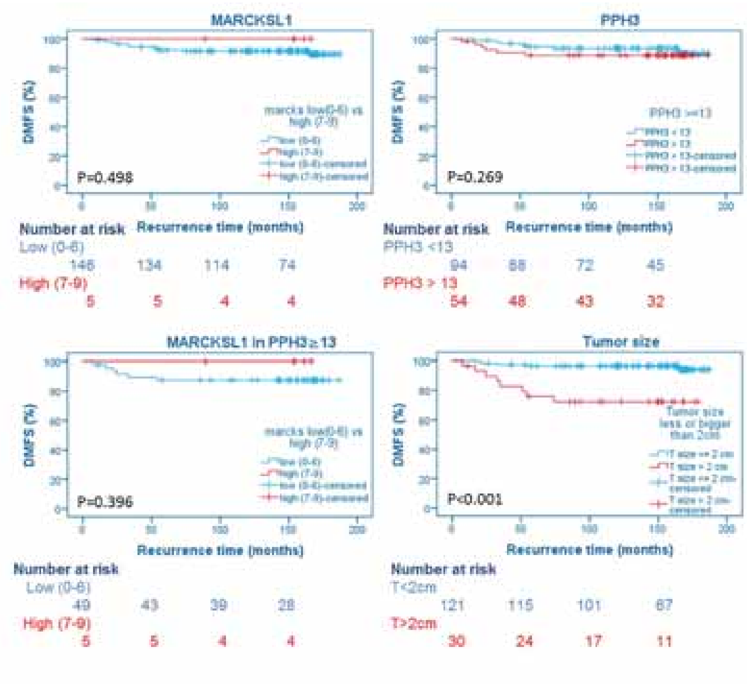

Fig 2. Long-term recurrence-free survival curves according to PPH3 status, MARCKSL1 protein expression score, MARCKSL1 protein expression score in patients with PPH3 ≥ 13, and tumor size. (DMFS, distant metastasis-free survival).

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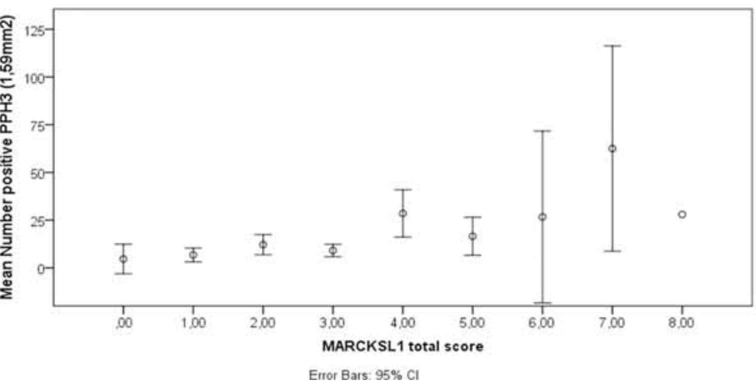

Fig 3. Correlation between MARCKSL1 total score and PPH3 in breast tumors.

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and lower age in the previous cohort, whereas in the new cohort it was associated with ER negativity and not with age.

Discussion

This study was performed as an independent validation of the prognostic value of MARCKSL1 protein expression in LN⁻ breast cancer patients under 71 years of age. To ensure correct diagnostics and optimal treatment options for patients, it is vital to validate suggested prognostic factors in new cohorts. Our study represents an awaited validation study for the use of MARCKSL1 as a prognostic marker in node-negative breast cancer. In the study by Jonsdottir et al, all LN⁻ patients diagnosed between 1993 and 1998 at the Stavanger University Hospital (SUH) were included [28]. For our validation study, we have collected samples from the same population as the exploratory cohort, and all available consecutive LN⁻ breast cancer patients <71 years at SUH in the study period (2002–2004) were included.

The current study showed that MARCKSL1 protein expression was not a significant predictor for recurrence in the validation cohort (p = 0.498). Additionally, previously validated prognostic factors (Ki-67, PPH3, and MAI) were not prognostic either, leaving only tumor size (with a cut-off at 2 cm) as a significant predictor for recurrence. Only five tumors (3%) were scored as high MARCKSL1 protein expression in our validation cohort, compared to 8% in the discovery cohort. None of the five experienced any metastasis. MARCKSL1 correlated significantly to tumor proliferation as measured by Ki-67, MAI and PPH3. High tumor proliferation, measured by, for example Ki-67, is an indicator for chemotherapy according to the NBCG guidelines [15]. As a result, chemotherapy reduces these patients' risk of recurrence but also negates the prognostic appearance of Ki-67. We speculate that while MARCKSL1 was prognostic in an earlier population, changes in chemotherapy guidelines have altered the overall survival and the specific need for prognostic factors in Norwegian breast cancer patients [1, 34].

Few other studies have investigated the role of the MARCKS family of proteins in breast cancer. In a previous study by the co-authors [28], increased MARCKSL1 gene expression was not found to be predictive; rather, low MARCKSL1 mRNA levels were predictive of recurrence. Although we did not perform gene expression analysis in the validation cohort, this discrepancy may be due to different activity of MARCKSL1 dependent on its phosphorylation

status. Phosphomimetic MARCKSL1 has been shown to inhibit migration, whereas dephosphorylated MARCKSL1 induces migration in neurons [19]. Differential phosphorylation could explain the apparent oncogenic and antitumor effects of MARCKSL1 reported in different studies. The IHC analyses performed here do not discriminate between protein phosphorylation statuses.

Alternatively, MARCKSL1 activity may be suppressed by microRNA suppression of protein translation. In fact, in a human breast carcinoma cell line, knockdown of MARCKSL1 by 5'isomiR-140-3p overexpression led to a decrease in the migratory potential of cells [24], in line with the findings of Jonsdottir *et al*. [28].

Reasons for the lower MARCKSL1 scores overall in the current compared to previous cohort could be that the patients in the validation cohort are diagnosed at an earlier stage, with younger patients and smaller tumors (S1 Table). Additionally, the change in chemotherapy type and frequency may contribute to fewer recurrences and deaths (9% vs 15% distant metastases in the current vs previous cohort, respectively). A key explanation is the introduction of mammography screening and the change in chemotherapy indications and type in recent years, resulting in earlier stages and younger age at diagnosis and fewer recurrences and cancer deaths [34]. The population of women diagnosed with breast cancer changes over time, with younger and earlier staged patients increasing after the gradual introduction of the national screening program in Norway from 1996 [2, 15]. Concurrently, the chemotherapy regimen was changed from cyclophosphamide, methotrexate and fluorouracil (CMF) to the more effective anthracycline-based fluorouracil, epirubicin and cyclophosphamide (FEC) [35]. In addition, the indication for endocrine therapy (in addition to HR+) changed from tumor size > 20mm in 2000, to a tumor size $>$11mm in 2003 [2, 3, 35]. Increased use of endocrine therapy may also contribute to the increased metastasis-free and overall survival in the validation cohort compared to the exploratory cohort [2]. Furthermore, breast cancer recurrence is known to occur up to 20 years following diagnosis [4], which extends beyond the time span of both this and the previous study.

Possible limitations to the current study include the relatively small study size, lack of mRNA measurements, and the change in treatment regimes. Due to increased survival in recent years, a greater study size may be needed to obtain sufficient numbers of recurrences. Additionally, had mRNA measurements been performed, these could explain whether reduced MARCKSL1 expression was due to reduced gene expression or other factors. Finally, as mentioned, other studies have observed opposite effects of MARCKSL1 depending on its phosphorylation status [19]. This is also the case with the much more studied MARCKS [36].

In conclusion, in this second cohort MARCKSL1 protein expression could not be confirmed as a prognostic factor. Thus, with changes both in the diagnosed population and how they are treated, the search for prognostic biomarkers must continue in new directions. Further studies are needed to reveal the potential biological role of this protein in breast cancer.

Supporting information

S1 Fig. Example of MARCKSL1 (myristoylated alanine-rich C kinase substrate like-1) staining (brown staining). A) Strong membrane staining. B) Strong granular staining. C) Strong cytoplasmic staining. D) Negative/Weak staining.
(PDF)

S2 Fig. MARCKSL1 expression scores in a) validation cohort and b) discovery cohort (Jonsdottir et al. 2012).
(PDF)

S1 Table. Comparison of the discovery cohort and the validation cohort.
(PDF)

S2 Table. Patient characteristics data.
(XLSX)

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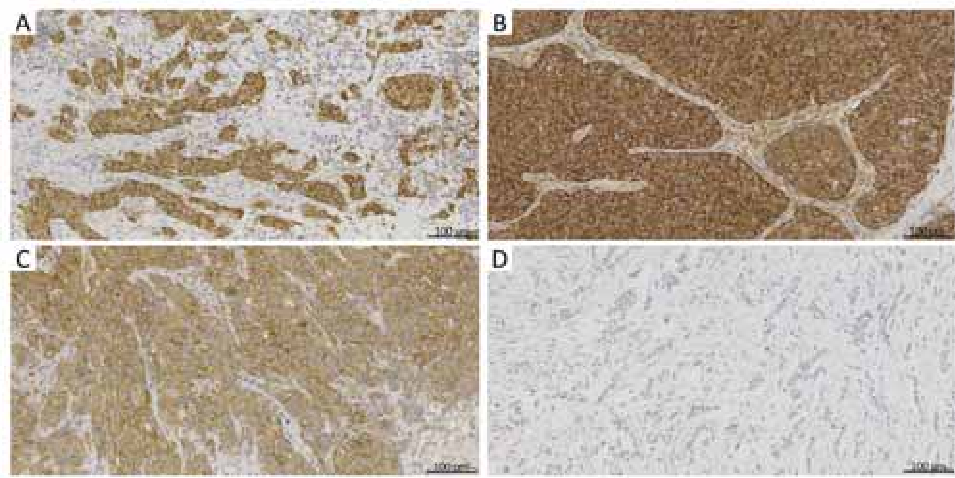
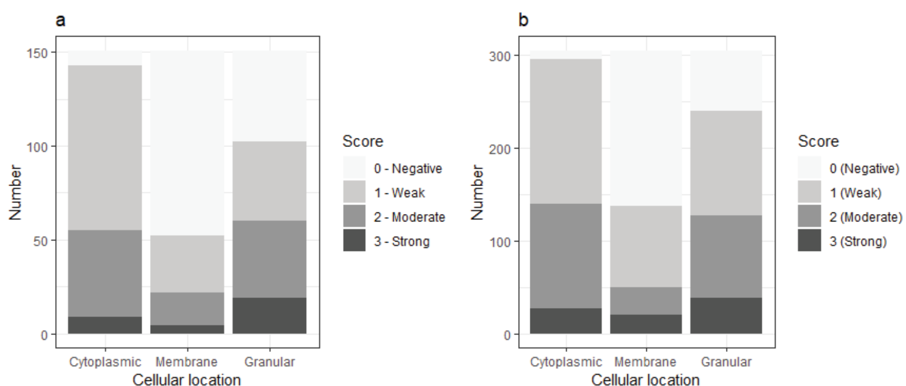
Supplementary material to Paper I

Validation study of MARCKSL1 as a prognostic factor in lymph node-negative breast cancer patients. Egeland NG, Austdal M, van Diermen-Hidle B, Rewcastle E, Gudlaugsson EG, Baak JPA, et al. (2019) Validation study of MARCKSL1 as a prognostic factor in lymph node-negative breast cancer patients. PLoS ONE 14(3): e0212527. https://doi.org/10.1371/journal.pone.0212527.



S1 Fig. Example of MARCKSL1 (myristoylated alanine-rich C kinase substrate like-1) staining (brown staining). A Strong membrane staining. **B** Strong granular staining. **C** Strong cytoplasmic staining. **D** Negative/Weak staining.



S2 Fig. MARCKSL1 expression scores in a) validation cohort and b) discovery cohort (Jonsdottir et al. 2012)

S1 Table. Comparison of the discovery cohort and the validation cohort.

Characteristic			
	Jonsdottir et al. 2012 (n=305)	Validation (n=151)	P (Fisher's exact test)
Cohort	1993-1998	2002-2004	
Age			0.035
<55	40%	51%	
≥ 55	60%	49%	
Tumor diameter			0.018
<2	70%	80%	
≥2	30%	20%	
Nottingham grade			0.158
1	30%	20%	
2	44%	51%	
3	26%	29%	
Estrogen receptor			0.327
Negative	17%	16%	
Positive (≥ 10%)	81%	84%	
Borderline (1-9%)	2%	0%	
Progesterone receptor			<0.001
Negative	22%	26%	
Positive (≥ 10%)	62%	72%	
Borderline (1-9%)	16%	2%	
Her2+[a]			<0.001
Negative	87%	60%[a]	
Positive	13%	40%[a]	
Triple negative			0.648
Negative	87%	84%[a]	
Positive	13%	16%[a]	
MAI			0.363
<10	71%	66%	
≥ 10	29%	34%	
Ki-67			0.167
0-9%	52%	44%	
10-100%	48%	56%	
PPH3			0.676
<13	61%	64%	
≥13	39%	36%	
CK5/6			0.845
<10	93%	93%	
≥10	7%	7%	
TILs			<0.001
<1%	13%	48%	
≥1%	87%	52%	
MARCKSL1 total score			0.097
Low (0-6)	92%	97%	
High (7-9)	8%	3%	
Chemotherapy			<0.001
Yes	15%	55%	
No	85%	45%	
Endocrine therapy			
Yes	6%	23%	<0.001
No	94%	77%	
Distant metastases	15%	9%	0.055
Deaths from breast cancer	11%	7%	0.312

[a]HER2 status missing for 105 patients.

Paper II

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BMC Cancer

RESEARCH ARTICLE Open Access

MiR-18a and miR-18b are expressed in the stroma of oestrogen receptor alpha negative breast cancers

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Abstract

Background: Previously, we have shown that miR-18a and miR-18b gene expression strongly correlates with high proliferation, oestrogen receptor -negativity (ER$^-$), cytokeratin 5/6 positivity and basal-like features of breast cancer.

Methods: We investigated the expression and localization of miR-18a and -18b in formalin fixed paraffin embedded (FFPE) tissue from lymph node negative breast cancers ($n = 40$), by chromogenic in situ hybridization (CISH). The expression level and in situ localization of miR-18a and -18b was assessed with respect to the presence of tumour infiltrating lymphocytes (TILs) and immunohistochemical markers for ER, CD4, CD8, CD20, CD68, CD138, PAX5 and actin. Furthermore, in two independent breast cancer cohorts (94 and 377 patients) the correlation between miR-18a and -18b expression and the relative quantification of 22 immune cell types obtained from the CIBERSORT tool was assessed.

Results: CISH demonstrated distinct and specific cytoplasmic staining for both miR-18a and miR-18b, particularly in the intratumoural stroma and the stroma surrounding the tumour margin. Staining by immunohistochemistry revealed some degree of overlap of miR-18a and -18b with CD68 (monocytes/macrophages), CD138 (plasma cells) and the presence of high percentages of TILs. CIBERSORT analysis showed a strong correlation between M1-macrophages and CD4+ memory activated T-cells with mir-18a and -18b.

Conclusions: Our study demonstrates that miR-18a and miR-18b expression is associated with ER- breast tumours that display a high degree of inflammation. This expression is potentially associated specifically with macrophages. These results suggest that miR-18a and miR-18b may play a role in the systemic immunological response in ER$^-$ tumours.

Keywords: Breast cancer, microRNA, In situ hybridization, Tumour microenvironment, Macrophages, Tumour associated macrophages (TAM)

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Background

Oestrogen receptor alpha (ER) expression is the most widely used predictive biomarker for breast cancer. Most patients with ER positive (ER^+) tumours receive adjuvant endocrine therapy and have a good prognosis. In contrast, ER negativity (ER^-) is found in roughly 15% [1, 2] of all breast cancers, and these tumours are often associated with high proliferation and a relatively poor prognosis. Additionally, there are few effective adjuvant therapy options for this group and for the so-called triple-negative (TNP) breast cancers that lack expression of ER, progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Therefore, there is a need for identifying new prognostic biomarkers and more specific, novel targets for therapy. Also, more knowledge of the biology of these tumours is necessary to improve the prognosis of patients with ER^- breast cancer.

MicroRNAs are defined as short non-coding RNA molecules, of which the mature form is about 22 nucleotides in length. Each microRNA is complementary or partially complementary to several mRNA molecules, and its main function is to post-transcriptionally downregulate gene expression by either binding directly to its mRNA target, or by cleaving target mRNA by binding to its 3′-untranslated region (UTR) [3]. Some microRNAs are predicted to bind several hundred gene targets (mRNAs), and different microRNAs can also target the same gene [4]. Studies of mammalian cells have shown that microRNAs are one of the largest groups of translational regulators in human cells [3], and they are known to play a significant role in many cellular functions [5] and in a number of diseases, including cancer [6, 7].

Previously, we have shown that gene expression of miR-18a and miR-18b is strongly correlated with high proliferation, ER^- and cytokeratin 5 and – 6 positivity (CK5/6+) [8, 9]. MiR-18a belongs to the miR-17 ~ 92 cluster located on chromosome 13, while miR-18b belongs to the miR-106a ~ 363 cluster located on chromosome X [10, 11]. MiR-18a and miR-18b, and their cluster members, are mostly described as onco-microRNAs because they show higher expression in many different tumour types, and especially in more advanced tumours [10, 12]. Several studies have shown that the expression of miR-18a and miR-18b is associated with ER- status [8, 13–15], and research suggests that ER can be a direct target of miR-18a [13, 16, 17].

Besides cancer cells, tumour tissue is made up of stromal cells such as fibroblasts, adipocytes, endothelial cells and various immune cells. The tumour microenvironment (TME) contains a heterogeneous collection of immune cell types, such as T-cells and B-cells, natural killer cells, macrophages, dendritic cells and neutrophils (reviewed in [18, 19]). MicroRNAs are also involved in the interplay between cancer and immune cells [20]. It has been reported that microRNAs take part in cell-cell signalling and communication between tumour cells and the surrounding microenvironment [21], by means of paracrine signalling [22, 23] and release of extracellular vesicles [24], especially exosomes [25, 26]. It is now recognized that the TME plays a critical role in both initiation and progression of cancer, and thus has prognostic potential. The cells within the TME take part in bidirectional cross-talk and interactions with the malignant cells, and they can have pro- or anti-tumour functions, depending on the type of immune cells involved [27, 28]. Cancer-associated immune cells also play a role in treatment response [26], and may have therapeutic potential. Several studies have shown the prognostic relevance of tumour-infiltrating lymphocytes (TILs) in breast cancer, especially in the HER2-positive and triple-negative subtypes (reviewed in [29]).

Here, we applied chromogenic in situ hybridisation (CISH) and immunohistochemistry (IHC), to locate and identify which cells express miR-18a and miR-18b in breast cancer. To further investigate the origin of these cells, we applied the analytical tool CIBERSORT [30] that uses gene expression data from bulk tumour to deconvolute expression and derive relative quantification of hematopoietic cell populations, to assess which cell types miR-18a and miR-18b are associated with.

Methods

Patients

This study was approved by the Norwegian Regional Committees for Medical and Health Research Ethics (REC). All patients were treated according to the national guidelines of the Norwegian Breast Cancer Group (NBCG) at the time of diagnosis.

Stavanger cohort: Breast cancer patients diagnosed with first onset invasive operable ($T_{1,2}N_0M_0$) lymph node negative breast cancer at the Stavanger University Hospital between January 1, 1993 and December 31, 1998. From this Stavanger cohort, several sub-cohorts have been used in the present study: 1) A total of 94 lymph node negative breast cancer patients from previous studies [9, 31] with complete mRNA- and microRNA expression data, hereafter called the Stavanger array-cohort, were included for correlation analysis between CIBERSORT output (based on mRNA expression) and miR-18a/miR-18b expression. 2) We analysed TILs in 204 samples (from our previous study [8]), and correlated this with our previous expression data of miR-18a and -18b (measured by quantitative real-time PCR (qPCR)), and grouped the patients based on ER status and high vs low TILs. This sub-cohort will here onwards be referred to as the Stavanger qPCR-cohort.

CISH cohort: Based on our previous findings of higher amounts of miR-18a and -18b in ER^- breast cancer, a total of 40 samples from the *Stavanger qPCR-cohort* [8] described above were selected for CISH as follows: 20 tumours classified as ER^+ with low expression of miR-18a and miR-18b (as measured by qPCR), and 20 tumours classified as ER^- with high expression of miR-18a and miR-18b (as measured by qPCR). We analysed TILs in the 40 tumours and correlated this with the CISH expression of miR-18a and -18b. For the patients' clinical characteristics, please see additional files (Additional file 1, S1 Table). Furthermore, CISH was performed also on lymph nodes histologically negative for tumour cells from two ER^- patients, as well as on lymph nodes histologically positive for metastasis from four ER^+ or ER^- patients. Also, CISH was performed on a test block consisting of several tumour types from different patients, and on a lymph node diagnosed as reactive lymphadenitis from the neck (this patient had no history of breast cancer and had no other clinical symptoms).

Oslo2 cohort: a multicentre study of breast cancer patients with primary operable breast cancers consecutively enrolled from hospitals in the Oslo region from 2006 until today. Patients were included at the time of primary surgery. Tumours from the Oslo2 study ($n = 308$) and from a similar study conducted at the Akershus University Hospital (Ahus), Norway, from 2003 to 2010 ($n = 69$) were selected for CIBERSORT analyses and correlation to miRNA expression. Total RNA was isolated from fresh-frozen (Oslo2) or RNA*later*® (Ahus) material using TRIzol™, and microRNA expression data for altogether 377 tumours were correlated with matching CIBERSORT output based on mRNA expression [32, 33] using Agilent microarrays (Agilent Technologies, Santa Clara, CA, USA). For CIBERSORT analyses, both lymph node positive and -negative patients were included ($n = 377$).

Histopathology and immunohistochemistry

The tumour tissue was fixed in buffered 4% formaldehyde and then embedded in paraffin. Sections were cut at a thickness of four μm and stained with haematoxylin, erythrosine and saffron. The histological type was assessed according to the World Health Organization criteria [34] and the tumour grade was assessed according to the Nottingham grading system [35]. ER was scored positive if ≥1% of tumour cells exhibited nuclear staining, while all others were scored negative. All sections were scored independently by two experts.

IHC was used to detect ER, CD4, CD8, CD20, CD68, CD138, PAX5 and actin. These methods were based on DAKO technology as described previously [36]. In brief, FFPE-sections of 2 μm thickness were mounted onto Superfrost Plus slides (Menzel, Braunschweig, Germany). Antigens were retrieved with a highly stabilized retrieval system (ImmunoPrep; Instrumec, Oslo, Norway) using 10 mM TRIS/1 mM EDTA (pH 9.0) as the retrieval buffer. Slides for actin staining were not treated with retrieval buffer. Sections were heated for 3 min at 110 °C followed by 10 min at 95 °C then cooled to 20 °C. The sections were incubated with monoclonal antibody at the dilutions stated in Table 1.

MicroRNA and mRNA expression profiling

The microRNA and mRNA expression profiling data from fresh-frozen tumour tissue used in this analysis have been published previously [9, 31–33].

Chromogenic in situ hybridization

CISH was performed on FFPE tissue using miRCURY LNA™ microRNA ISH optimization kit (FFPE) v1.3 (Exiqon, Vedbaek, Denmark). The manufacturer's protocol was followed with some minor changes. Briefly, 5 μm thick paraffin sections were mounted on Superfrost™ Plus glass slides and incubated overnight at 55 °C. The slides were deparaffinised with xylene and alcohol dilutions. The slides were then washed in PBS for 4 min, digested with 15 μg/ml of Proteinase K at 37 °C for 30 min, and washed in PBS before dehydration through a series of graded alcohol. The slides were hybridized with double DIG labelled Locked Nucleic Acid™ (LNA™) (Exiqon) probes at 55 °C for 1 h (see Additional file 2, S2

Table 1 Monoclonal antibodies used in IHC staining

Antibody	Clone	Concentration	Target cells	Company
ER	SP1	1:400	Epithelial cells	Thermo Scientific, Waltham, USA
CD4	4B12	1:100	CD4+ T-cells	Novocastra, Newcastle Upon Tyne, UK
CD8	C8/144B	1:50	CD8+ T-cells	DAKO, Agilent Technologies, Santa Clara, CA, USA
CD20	L26	1:1000	B-cells, neoplasms of B-cells	DAKO
CD68	PG-M1	1:400	B-cells, macrophages, histiocytes, dendritic cells, NK cells	DAKO
CD138	B-A38	1:50	Plasma cells and some epithelial cells	AbD Serotec (BioRad), Kidlington, UK
PAX5	24	1:100	B-cells	BD Biosciences, San Diego, USA
Actin	1A4	1:300	Fibroblasts	DAKO

Antibody, clone, concentrations used, Target cells and company of the antibodies used in IHC staining

Table). The probe concentrations were 80 nM for the hsa-miR-18a-5p, hsa-miR-18b-5p and scramble probe, and 2.0 nM for the positive control probe U6. After hybridization the slides were washed consecutively with 5x SSC, 1x SSC and 0.2x SSC (Sigma Aldrich, St. Louis, MO, USA) at 50 °C for a total of 30 min. Then the slides were incubated with blocking solution containing 1x Maleic acid buffer (Roche, Mannheim, Germany), 10x Blocking Solution (Roche) and 2% sheep serum (Jackson Immunoresearch, Suffolk, UK) for 15 min, before application of 1:800 dilution of sheep anti-DIG alkaline phosphatase (Roche) at 30 °C for 30 min. The slides were then washed with 1% Tween-PBS for 3 × 3 min, before they were incubated with AP substrate containing NBT/BCIP (Roche) at 30 °C for 110 min. This allowed for visualization of antibody signals by NBT-BCIP. Sections were then washed twice for 5 min in KTBT buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM KCl), before being rinsed in ultrapure water 2 × 1 min. For nuclear counterstaining, the sections were immersed in Nuclear Fast Red (Sigma Aldrich, St. Louis, MI, USA) for 3 min, and then rinsed in running tap water for 5 min. Finally, the sections were dehydrated through a series of graded alcohol, and subsequently mounted by Histokitt mounting medium (VWR, Oslo, Norway).

Quantification of miR-18a and miR-18b

Specific staining for both microRNAs was observed as a dark blue colour from the NBT/BCIP precipitation. The sections were examined by light microscopy, and positive miR-18a and miR-18b staining was quantified by cell counting in two selected areas with the highest number of positive cells within the tumour area. In these hotspot areas all positive cells were counted at 40x in an area of 1.59 mm^2. The two areas were scored separately, and the sum of both made up the final score for each slide. Dark blue cells without a visible nucleus or distinct cell membrane were excluded, as were light purple cells. Sections with negative U6 staining or slides in which a substantial amount of material was lost during the experimental treatment, were also excluded from the study ($n = 4$).

Scoring of lymphocyte infiltration

The variable degree of lymphocytic infiltration in HE-stained tissue sections was evaluated semi-quantitatively. First, the sections were assessed according to the presence or absence of stromal tumour infiltrating lymphocytes (sTILs). Second, the relative amount of TILs in the tumour stroma area was assessed according to the method described by Denkert et al. [37]. The degree of infiltration was scored in the range of 0–100%.

CIBERSORT analysis

CIBERSORT (Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts) is a computational framework, that on the background of gene expression data and a signature gene file quantifies the relative or absolute levels of member cell types in a mixed cell population [30]. We ran CIBERSORT using the LM22 signature gene file which provides the relative abundance of 22 distinct mature human hematopoietic populations. The mRNA expression data from the Stavanger array [31] ($n = 94$) and Oslo2 [32] ($n = 377$) cohorts were used and the maximum number of permutations ($n = 1000$) were chosen. The output from CIBERSORT was a matrix with quantification of the 22 cell types for each tumour sample.

Statistical analyses

Statistical analyses were conducted using the software program SPSS (version 20.0, SPSS, Chicago, IL, USA) and R [38]. Differences between patient groups were tested using an independent T-test and correlations between different expression levels were done using both Pearson's and Spearman's correlation. To assess the association of cell type composition with miR-18a and -18b, we calculated the Spearman correlation coefficient between miR-18a and -18b expression and the CIBERSORT-based quantification of 22 hematopoietic cell types. Finally, the correlations were ranked in decreasing order.

Results

Expression of miR-18a and miR-18b in FFPE tissue

Detection of miR-18a and miR-18b by CISH in FFPE tissue from breast cancer patients resulted in strong and specific cytoplasmic staining in cells in the intratumoural stroma (Fig. 1) or stroma surrounding the tumour margin (Fig. 2). Both microRNAs were typically found in round shaped cells located within the tumour stroma, although some stained cells were more elongated and outstretched (Fig. 2). Little or no expression was found within the epithelial tumour cells or in cells further (> 0.5 mm) away from the tumour area (Figs. 2 and 3). As expected based on our selection criteria, miR-18a and miR-18b had a significantly lower expression level in ER^+ tumours in comparison to ER^- tumours (Independent T-test, $P < 0.001$ for miR-18a and $P = 0.002$ for miR-18b) (Figs. 3, 4, and Table 2). The expression levels of miR-18a and miR-18b, as measured by CISH-expression levels, showed a strong correlation (Pearson's correlation coefficient of 0.85 $P < 0.001$). Furthermore, these CISH-expression levels correlated well with those measured by qPCR in our previous study [8], for both miR-18a ($r = 0.75$ Spearman's rho test $P < 0.001$) and for miR-18b ($r = 0.64$, $P < 0.001$).

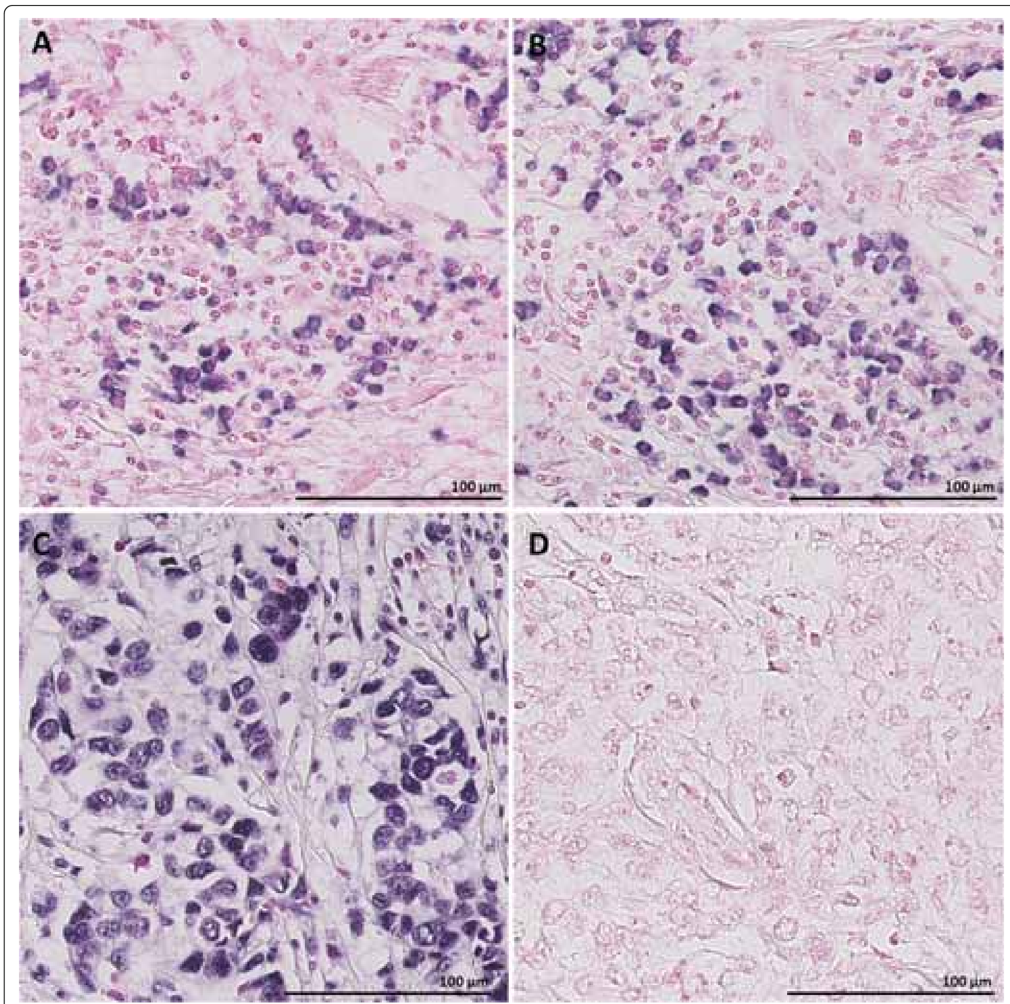

Fig. 1 40x magnification illustrating representative CISH staining with: **a**) LNA 5`-3'DIG hsa (80 nM) miR-18a probe showing strong and specific staining in stroma, **b**) LNA 5`-3'DIG hsa (80 nM) miR18-b probe showing strong and specific staining in stroma, **c**) U6 snRNA positive control probe showing overall nuclear staining, and **d**) negative probe (scrambled) probe with no hybridization signal. Scale bar 100 μm

MiR-18a and miR-18b expression pattern in relation to immunohistochemical markers

Although a strong and specific staining method was established, CISH has its limitations. Assessing the CISH slides only, pathologists were in doubt of the cell type that showed positive miR-18a and miR-18b expression. In an attempt to identify the cell type, we performed IHC on serial sections of the same breast tumours, and the corresponding lymph node samples. The following IHC-markers were used: CD4, CD8, CD20, CD68, CD138, PAX5 and actin (Table 1).

A comparison between the different IHC stains and the CISH results for miR-18a and miR-18b showed some overlap with the expression of CD68 (monocytes/macrophages), CD138 (plasma cells) and actin (smooth muscle), although a complete match was not observed (Fig. 5). Actin staining identified fibroblast cells which were mostly oblong and outstretched, and since miR-18a and miR-18b were mainly expressed in round shaped

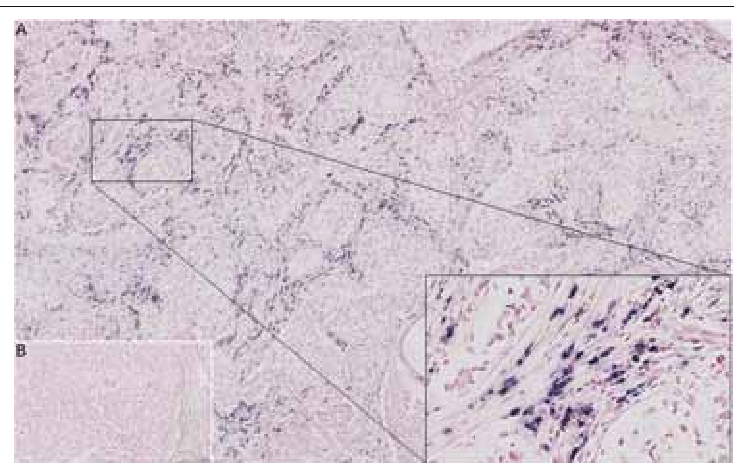

Fig. 2 Representative miR-18b CISH expression in an ER$^-$ breast tumour (sample id: 20 ER$^-$ in Table 2). **a**) Specific blue (NBT/BCIP) staining for LNA 5`-3'DIG hsa miR-18b (80 nM) probe. **b**) Negative control, i.e. staining with an LNA 5`-DIG scrambled probe (80 nM)

cells, we hypothesized that the miR-18a and miR-18b positive cells are more likely to be associated with cells of lymphoid or myeloid origin.

Based on this we also stained lymph nodes from breast cancer patients both positive and negative for cancer cells (based upon histology from HE-slides), for the same IHC-markers as mentioned above, as well as for miR-18a and miR-18b. In the lymph nodes containing tumour cells, all the miR-18a- and miR-18b-positive cells were localized close to or in between the tumour cells (Fig. 6), thus following the pattern we observed in the primary tumours (for comparison with corresponding primary tumour, see Additional file 3, S1 Fig). This seemed especially true in patients with ER$^-$ tumours (ER$^-$ tumour in Fig. 6, an ER$^+$ tumour in Additional file 4, S2 Fig). For the IHC markers, only the expression patterns for CD68 and CD138 showed some similarity with miR-18a and miR-18b expression, both in location and the shape and size of the positively stained cells (Fig. 6). Lymph nodes from breast cancer patients without tumour cells had a much more scattered staining pattern for miR-18a and miR-18b, with few positive cells and more stained cells in germinal centres. These results suggest that the miR-18a and miR-18b-expressing cells could be part of an immune response directed towards the tumour, and more specifically towards ER$^-$ tumour cells.

To investigate whether this reaction was cancer specific, we also analysed lymph nodes from patients with non-malignant disease, here in a case of reactive lymphadenitis (Fig. 7). In these non-malignant reactive lymph nodes miR-18a and -18b staining was mostly observed in the germinal centres. This, was in contrast with our observation in lymph nodes containing cancer cells where no staining was observed in the germinal centres. Again, comparison with the staining patterns for the IHC markers showed only partial overlap with CD68 and CD138 (Fig. 7).

MiR-18a and miR-18b expression pattern in relation to tumour infiltrating lymphocytes

Measurement of TILs in the 40 ER$^+$ and ER$^-$ tumour tissues showed that the ER$^-$ tumours had a significantly higher number of TILs (Independent T-test, $P = 0.0001$, boxplot in Fig. 8). As such we analysed TILs in 204 samples from our previous study [8], and compared it with the expression of miR-18a and -18b (measured by qPCR). Patients were also grouped based on ER status and high vs low TILs (Additional file 5, S3 Fig). Although not significant, a difference was observed between ER$^-$/high TILs versus ER$^-$/low TILs for both microRNAs. Additionally, the ER$^-$ with high TILs had significantly ($P < 0.001$) higher expression of miR-18a and miR-18b, than the ER$^+$ patients with high TILs (see boxplot in Additional file 5, S3 Fig). Again, this suggests that miR-18a and miR-18b expression is related to TILs and ER$^-$ cancers. Furthermore, miR-18a and -18b expression was also found in the stroma of both pancreatic cancer, and lung cancer tissue (Additional file 6, S4 Fig).

MiR-18a and miR-18b expression and CIBERSORT

To address the heterogeneity of immune cells in bulk tumour tissue, and to further investigate which type of cells express miR-18a and/or miR-18b, we used

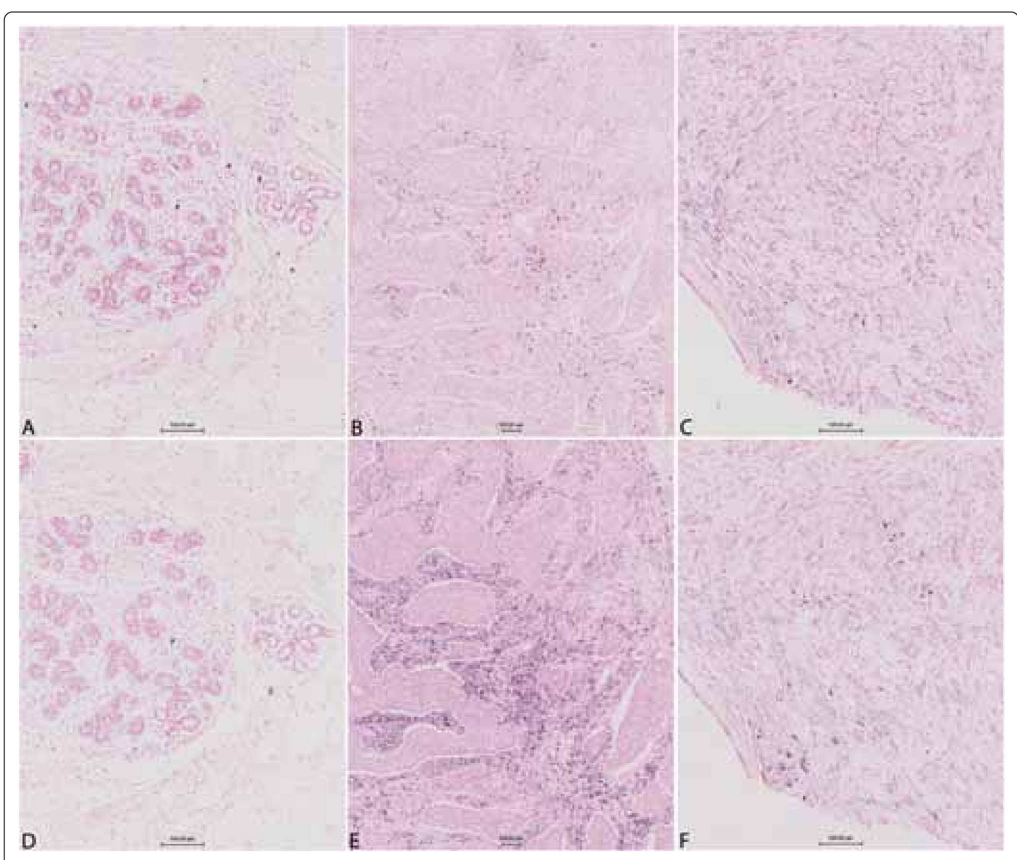

Fig. 3 Differential CISH expression levels of LNA 5`-3'DIG hsa (80 nM) probes for miR-18a and miR-18b in ER^+ vs ER^- breast cancers. Top row: miR-18a expression. Bottom row: miR-18b expression. **a**) and **d**) normal epithelial cells in an ER^- tumour (sample id: 14 ER^- in Table 2). **b**) and **e**) shows an ER^- tumour (sample id: 13 ER^- in Table 2) with higher expression of miR-18b in comparison with miR-18a. **c**) and **f**) shows an ER^+ tumour (sample id: 04 ER^+ in Table 2)

CIBERSORT [30] to characterize the cell composition of the bulk tumour tissue from which mRNA and miRNA was isolated. The output from CIBERSORT is a matrix of quantification levels of each of 22 immune cell types for each tumour. For each cell type, we took the vector of relative or absolute levels for all tumours and then compared this with the expression of miR-18a and miR-18b for the same tumours. The resulting Spearman's Rho correlation coefficients are shown in the attached Table 3. For both relative and absolute assessment, the "M1 macrophages" cell type had the highest and most significant correlation in the Stavanger data. The cell type with the second highest correlation was "memory activated CD4+ T-cells". For the Oslo2 cohort, the latter cell type showed the highest positive correlation and the "M1 macrophages" showed the second highest correlation (Table 3).

Discussion

High expression of miR-18a and miR-18b is known to be associated with ER^- breast cancer, high proliferation and worse prognosis [8, 39]. However, the role and function of these two microRNAs is not well understood. Our current in situ localisation shows that miR-18a and miR-18b are specifically expressed in the intratumoural stroma and in the stroma directly surrounding ER^- tumours with a high degree of TILs. Additionally, the current study demonstrates the specificity of our CISH protocol, and subsequently confirms our previous qPCR

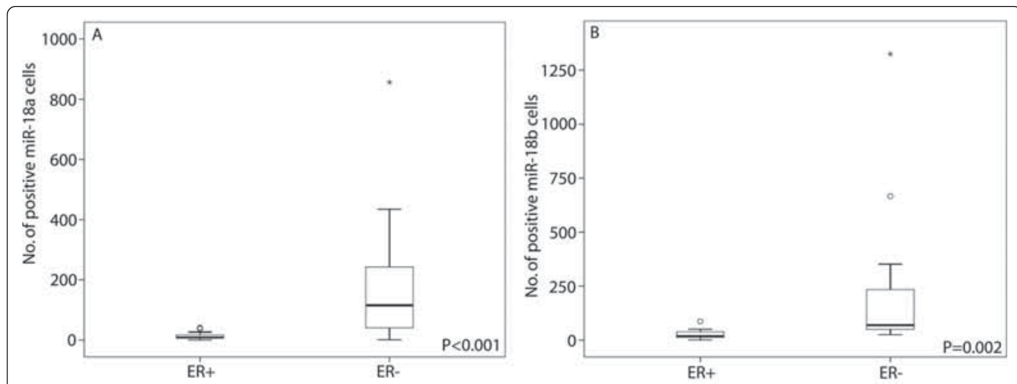

Fig. 4 Boxplot of CISH expression quantification, as measured by counting the number of positively stained cells in the $n = 20$ ER$^+$ vs the n = 20 ER$^-$ breast cancers of **a**) miR-18a and **b**) miR-18b. Central line in boxes represent the median value, boundaries of boxes represent the interquartile range and ends of whiskers represent the minimum and maximum values, excluding outliers. *P*-values were obtained using independent T-test

results that miR-18a and miR-18b are highly expressed in ER$^-$, and low in ER$^+$ breast cancers [8, 9].

Few studies have evaluated miR-18a or miR-18b expression patterns in cancer tissue using CISH, and only Guo et al. [40] showed that miR-18a is expressed in the tumour cells of ER$^+$ breast cancer tissue. These authors also describe that miR-18a is significantly under-expressed in ER$^-$ breast cancers, this is in contrast to most studies that report higher expression in ER$^-$ breast cancers. There are some technical differences in the CISH protocol between Guo et al. and the current study, and this might explain the differences in level of expression and location. The localisation of miR-18a and miR-18b expression is important in order to understand the role of these microRNAs in ER$^-$ breast tumours and in breast cancer progression.

Table 2 Quantification of cells positive for CISH expression of miR-18a and miR-18b

# / ER-status	miR-18a	miR-18b	# / ER-status	miR-18a	miR-18b
01 ER-	13	26	01 ER+	0	1
02 ER-	87	35	02 ER+	11	6
03 ER-	309	41	03 ER+	4	7
04 ER-	11	46	04 ER+	9	10
05 ER-	42	48	05 ER+	9	12
06 ER-	29	54	06 ER+	7	14
07 ER-	82	57	07 ER+	27	15
08 ER-	150	57	08 ER+	8	16
09 ER-	99	60	09 ER+	14	16
10 ER-	211	63	10 ER+	1	18
11 ER-	76	75	11 ER+	9	20
12 ER-	1	83	12 ER+	8	26
13 ER-	39	131	13 ER+	13	31
14 ER-	257	182	14 ER+	19	31
15 ER-	227	211	15 ER+	5	34
16 ER-	435	257	16 ER+	7	43
17 ER-	262	309	17 ER+	14	44
18 ER-	131	352	18 ER+	21	48
19 ER-	209	666	19 ER+	38	51
20 ER-	856	1325	20 ER+	41	86

Quantification of miR-18a and miR-18b expression, visualised by CISH, in 20 ER-positive and 20 ER-negative breast cancer tumours, sorted by miR-18b-expression

The use of CISH in the current study clearly shows that the expression of miR-18a and miR-18b is located in the stroma of ER$^-$ breast cancer with a high number of TILs. Furthermore, in breast cancer patients these microRNAs are also observed in lymph nodes both with and without macroscopically confirmed tumour cells. In lymph nodes from patients without cancer, the miR-18a and -18b positive cells were found only in the germinal centres. Meanwhile, in metastatic lymph nodes of breast cancer patients, miR-18a and -18b positive cells were found close to the tumour cells, and absent in the germinal centres. These observational results should be considered preliminary, nonetheless they do suggest a potential migration or activation of specific immune cells, related to ER$^-$ breast tumour cells.

In accordance with the stromal localization of miR-18a and -18b positive cells demonstrated by CISH and the partial overlap with CD68 staining, CIBERSORT analyses in two different cohorts showed a significant positive correlation between M1 / M0 macrophages and the expression of miR-18a and miR-18b. Similarly, Halvorsen et al. showed a correlation between the miR17 ~ 92 cluster and CD68 positive cells (i.e. monocytes/macrophages) when investigating

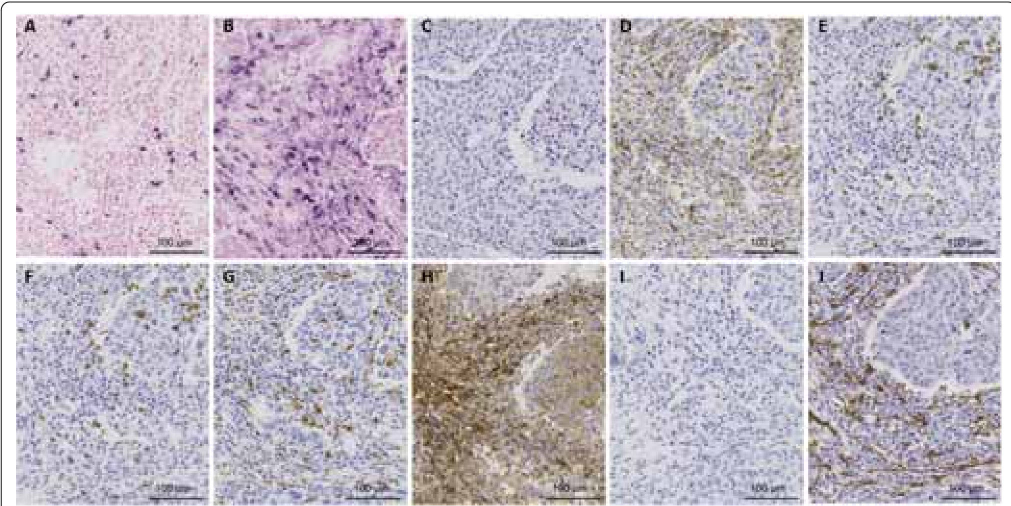

Fig. 5 Expression pattern in an ER⁻ breast tumour (sample id: 13 ER⁻ in Table 2) of **a**) CISH LNA 5`-3'DIG hsa (80 nM) miR-18a probe and **b**) CISH LNA 5`-3'DIG hsa miR-18b (80 nM) probe in comparison to IHC staining for **c**) ER, **d**) CD4, **e**) CD8, **f**) CD20, **g**) CD68, **h**) CD138, **i**) PAX5 and **j**) actin. Scale bar 100 μm

microRNAs isolated from the tumour interstitial fluid from breast cancer patients [21].

Interestingly, together with several other microRNAs, miR-18b has been suggested to play an important part in macrophage lineage development, through regulation of important macrophage transcription factors such as PU.1, RUNX1, CSFR1, PPARα and PPARγ [41]. As such, one might stipulate that overexpression of miR-18b might lead to increased expression of cytokines such as IL1β, IL-6 and TNFα, thereby increasing a pro-

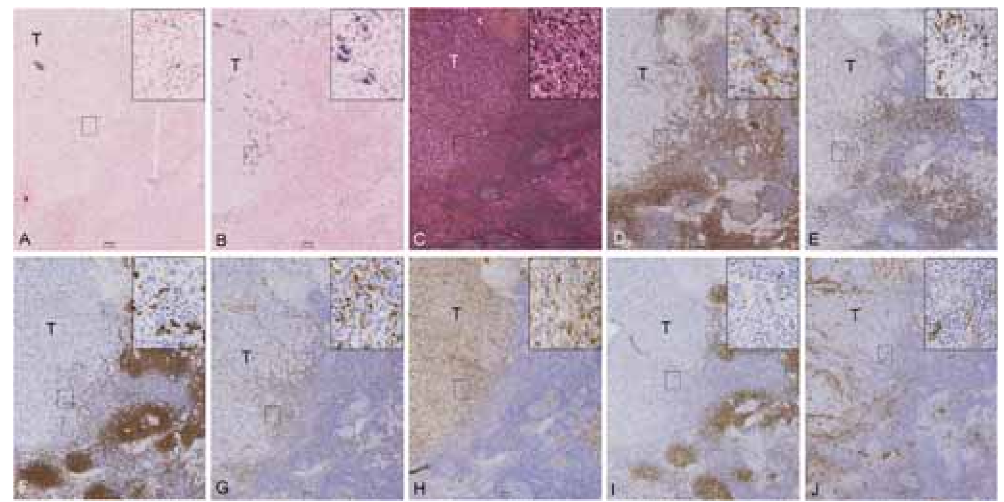

Fig. 6 Expression pattern comparison in a lymph node with tumour infiltration from a patient with an ER⁻ breast tumour. **a**) CISH LNA 5`-3'DIG hsa (80 nM) miR-18a probe and **b**) CISH LNA 5`-3'DIG hsa (80 nM) miR-18b probe, in comparison to IHC staining for **c**) HE, and IHC-staining for **d**) CD4, **e**) CD8, **f**) CD20, **g**) CD68, **h**) CD138, **i**) PAX5, and **j**) actin. **T** indicates tumour area. Scale bar 100 μm

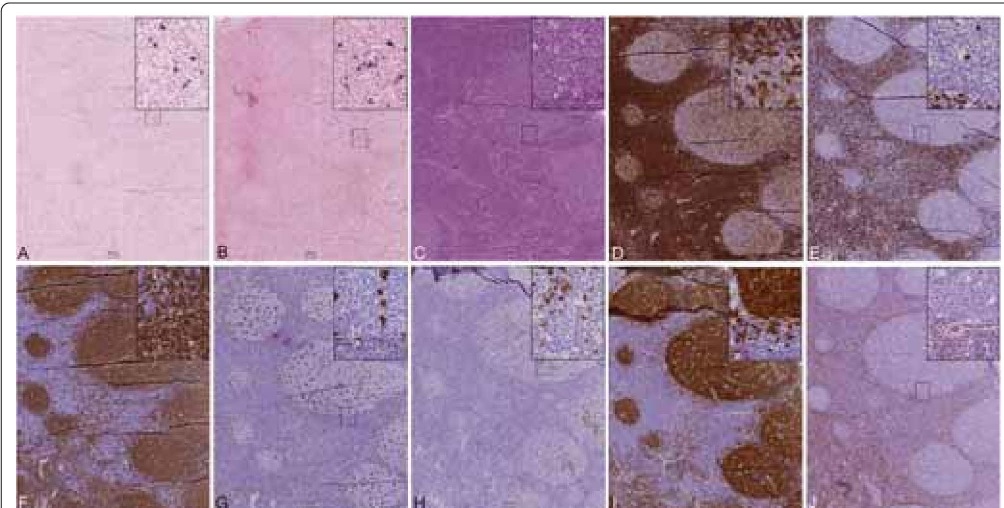

Fig. 7 Expression pattern comparison in a benign lymph node diagnosed as reactive lymphadenitis from the neck. **a**) CISH LNA 5`-3'DIG hsa (80 nM) miR-18a probe and **b**) CISH LNA 5`-3'DIG hsa (80 nM) miR-18b probe, in comparison to IHC staining for **c**) HE, and IHC-staining for **d**) CD4, **e**) CD8, **f**) CD20, **g**) CD68, **h**) CD138, **i**) PAX5, and **j**) actin. Scale bar 100 μm

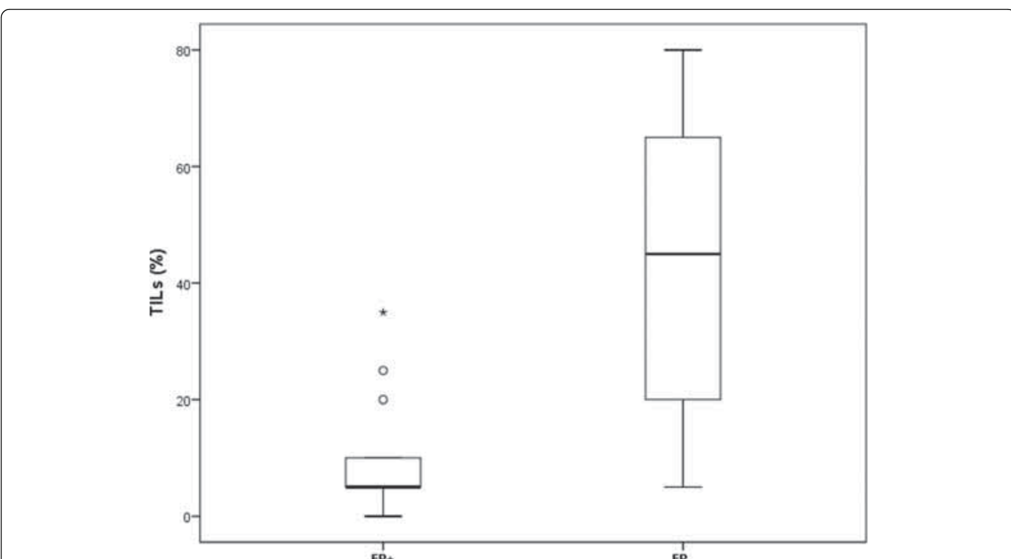

Fig. 8 Boxplot of TILs quantification, as measured by scoring the % of tumor-infiltrating lymphocytes in the n = 20 ER^+ vs the n = 20 ER^- breast cancers in the CISH-cohort. Central line in boxes represent the median value, boundaries of boxes represent the interquartile range and ends of whiskers represent the minimum and maximum values, excluding outliers

Table 3 CIBERSORT analyses, Stavanger array cohort and Oslo2 cohort

Stavanger array cohort (n = 94)					Oslo2 cohort (n = 377)				
	hsa-miR-18a		hsa-miR-18b			hsa-miR-18a		hsa-miR-18b	
	Corr.[a]	*P*	Corr.[a]	*P*		Corr.[a]	*P*	Corr.[a]	*P*
M1 Macrophages	0.415	0.001	0.387	0.001	Memory activated CD4+ T-cells	0.265	0.001	0.303	0.001
Memory activated CD4+ T-cells	0.328	0.001	0.308	0.002	Activated Dendritic cells	0.245	0.001	0.242	0.001
M0 Macrophages	0.299	0.003	0.279	0.006	M1 Macrophages	0.241	0.001	0.286	0.001
Monocytes	0.159	0.125	0.157	0.132	Neutrophils	0.177	0.001	0.151	0.003

List of the top four immune cells that correlate with miR-18a and miR-18b expression. [a] indicates Absolute Spearman's Rho correlation

inflammatory condition in the TME. Additionally, miR-18a-5p can promote carcinogenesis by directly targeting interferon regulatory factor 2 (IRF2) [42]. IRF2 is a member of the IRF family, which has the ability to exert anti-oncogenic activities; others showed that IRF2 is an important regulator of the pro-inflammatory response in macrophages by controlling HIF-1α–dependent glycolytic gene expression and glycolysis [43]. Interestingly, miR-18a has also been identified as an upstream regulator of hypoxia-inducible factor 1α (HIF1A) [44, 45]. HIF1A is associated with macrophage function, whereby its overexpression induces macrophage M1 polarization [46], and it also plays a role in centrosome aberrations and tumour progression in TNP breast cancer [47].

CIBERSORT analyses in two independent cohorts resulted in a positive miR-18a and -18b correlation with CD4+ T-cell memory cells; a subset of T-cells that can recognize foreign invaders such as bacteria or viruses, as well as cancer cells. Vahidi et al. [48] recently studied different subtypes of memory T-cells in the CD4+ population in tumour draining lymph nodes of 52 untreated breast cancer patients. Among all the CD4+ memory T-cells, more than 70% of the cells exhibited a memory phenotype, and in the tumour positive lymph nodes the frequency of T stem cell memory cells was higher than in tumour negative lymph nodes [48]. Jiang et al. studied the role of the miR-17 ~ 92 cluster during the T-cell antigen response and showed that miR-18a counteracts other microRNAs (e.g. miR-17 and miR-19 display a pro-Th1 function) by inhibition of proliferation and an increase in activation-induced cell death of CD4+ T-cells [49].

Both we and others have shown that miR-18a and miR-18b are related to ER^- tumours, and several studies have shown that both miR-18a and miR-18b directly repress ER activity [13–15, 17, 50, 51] and thus direct the location of these microRNA to the cancer cells.

It has also been demonstrated that microRNAs have the ability to take part in crosstalk between tumour cells and the microenvironment, by exosomal delivery [25, 26]. From different cancer studies circulating miR-18a has been detected as a potential microRNA biomarker for early detection of cancer in serum samples [52, 53]. Meanwhile, analysis of serum samples from 60 breast cancer patients with triple-negative tumours showed that miR-18b has prognostic value for distant metastases and overall survival [39]. These results show that miR-18a and -18b could be detected in liquid biopsies, and might therefore be potential biomarkers for tumour progression and worse prognosis in breast cancer.

The IHC staining performed and presented in the current study does not show a complete overlap with miR-18a and miR-18b (Table 1). Still, the finding of expression of these microRNAs in both elongated and smaller round cells could fit with expression in both macrophages and T-cells. A combination of both IHC and CISH on the same slide, or staining with several antibodies simultaneously, might be an appropriate way to improve the identification of the proper cell type(s) expressing these microRNAs.

Based on existing literature (cited above) and our studies, we can only conclude that miR-18a and miR-18b appears to be highly expressed among TILs in ER^- breast cancer, and that the expression of these microRNAs is correlated with a worse prognosis in these patients. While miR-18a and -18b might be linked to macrophages and memory T-cells, we speculate that these cells are not effective enough to stop the tumours from forming metastases.

There are some limitations to this study; first of all, we have only evaluated 40 patients with the CISH method. Second, the in situ expression of miR-18a and -18b did not show a complete overlap with any of the IHC markers. Third, although the CIBERSORT results were significant in two independent cohorts, this data shows only an association between the miR-18a and miR-18b expression and the different immune cells. These results should therefore be interpreted with caution. The exact function of these microRNAs in breast cancer stromal tissue, what type of cells express them, and how they relate to the infiltration of immune cells such as macrophages, needs further investigation.

Conclusions

In conclusion, our results show that miR-18a and miR-18b are highly expressed in the stromal compartment

adjacent to ER$^-$ tumour cells, especially in areas containing a high degree of infiltrating lymphocytes. The expression of miR-18a and miR-18b is positively correlated with the presence of macrophages and CD4 memory T-cells. We hypothesize that the expression of these microRNAs is related to a systemic immunological response, possibly produced by monocytes/macrophages that are activated in lymph nodes, and thereafter homed towards specific tumours. Further investigation in larger patient cohorts is needed to validate these miR-18a and miR-18b-expressing stromal cells as macrophages.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12885-020-06857-7.

Additional file 1: S1 Table. Patient characteristics in the CISH cohort.

Additional File 2: S2 Table. Name, sequence, RNA Tm and concentration for the LNA™ 5`-3'DIG hsa detection probes (Exiqon) used in CISH experiments.

Additional file 3: S1 Fig. miR-18b expression in primary tumour corresponding to Fig. 6. Positive and specific CISH expression of LNA 5`-3'DIG miR-18b (80 nM) in the stroma of a representative lymph node-positive primary breast cancer tumour.

Additional file 4: S2 Fig. Expression pattern comparison in a lymph node with tumour infiltration from a patient with an ER$^+$ breast tumour. A) CISH LNA 5`-3'DIG hsa (80 nM) miR-18a probe and B) CISH LNA 5`-3'DIG hsa (80 nM) miR-18b probe, in comparison to IHC staining for C) HE, and IHC-staining for D) CD4, E) CD8, F) CD20, G) CD68, H) CD138, I) PAX5, and J) actin.

Additional file 5: S3 Fig. Expression measured with qPCR in ER$^+$ and ER$^-$ breast cancers with high and low TILs of A) miR-18a and B) miR-18b. Central line in boxes represent the median value, boundaries of boxes represent the interquartile range and ends of whiskers represent the minimum and maximum values, excluding outlies. *P*-values were obtained using independent T-test.

Additional file 6: S4 Fig. CISH expression demonstrating strong and specific positive staining with LNA 5`-3'DIG hsa (80 nM) miR-18b probe expression in A) pancreatic cancer, and B) lung cancer.

Abbreviations

ER: Oestrogen receptor alpha; TME: Tumour microenvironment; FFPE: Formalin fixed paraffin embedded; CISH: Chromogenic in situ hybridization; TILs: Tumour infiltrating lymphocytes; TAM: Tumour associated macrophages; IHC: Immunohistochemistry; qPCR: Quantitative real-time PCR; PR: Progesterone receptor; HER2: Human epidermal growth factor receptor 2; TNP: Triple-negative phenotype

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Authors' contributions

EAMJ and KJ conceived and designed the study. KJ and IS contributed to the experimental design of CISH. NGE optimized the CISH protocol and performed the CISH experiments. EAMJ and NGE quantified the microRNAs and scored the lymphocyte infiltration. EAMJ, KJ, IS, EG and NGE analysed and interpreted the CISH/TILs/IHC results and images. EG provided clinical input. MRA, KS and VNK provided the Oslo2 data. KJ performed the statistical analyses in SPSS. MRA performed and analysed the CIBERSORT analyses. DCF, JPAB, MRA, KS and VNK provided intellectual and critical input. MRA, KS, VNK, DCF and EAMJ contributed to interpretation of the results and critically revised the manuscript. EAMJ and KJ overviewed and coordinated the study. NGE and KJ made the figures and tables and wrote the manuscript. All authors reviewed and approved the final version of the manuscript. KJ and NGE contributed equally to this work.

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Availability of data and materials

The mRNA dataset for the Stavanger array cohort is publicly available at the online Gene Expression Omnibus (GEO) repository: accession number GSE46563. The Oslo2 microRNA expression data are available from the GEO repository with accession number GSE58210, while the mRNA expression data has accession number GSE58212. Additional datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Regional Committees for Medical and Health Research Ethics (REC), part of the Norwegian National Research Ethics Committees. The Stavanger cohort was approved by REC Region West, approval number 2010/2014. As this is a retrospective study, approval was given without written consent from the patients. All insights in a patient's journal were monitored electronically, and all except the treating physician were required to state the reason why they needed to read that patient's journal. This log was always open for the patient to view. The Oslo2 cohort was approved by REC Region South East, approval number 2016/433 and 429–04148. These patients were included at the time of primary surgery after giving written informed consent.

Consent for publication

Not applicable.

Competing interests

None of the authors have any financial or other relationships with entities that have investment, licensing, or other commercial interests in the subject matter under consideration in this article.

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Supplementary material to Paper II

MiR-18a and miR-18b are expressed in the stroma of oestrogen receptor alpha negative breast cancers. Egeland, N.G., Jonsdottir, K., Aure, M.R. et al. MiR-18a and miR-18b are expressed in the stroma of oestrogen receptor alpha negative breast cancers. BMC Cancer 20, 377 (2020). https://doi.org/10.1186/s12885-020-06857-7.

S1 Table. Patient characteristics in the CISH cohort.

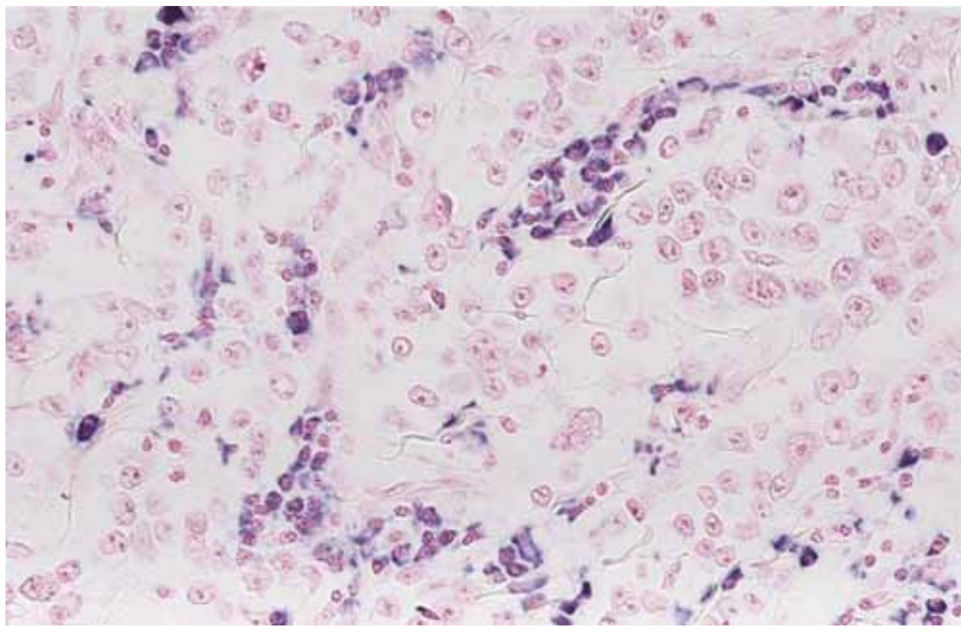
Patient characteristic	Frequencies n=40
Age	
<55 years	23
≥55 years	17
Nottingham grade	
1	13
2	9
3	18
Tumour size	
<2 cm	25
≥2 cm	15
ER	
Positive	20
Negative	20
PR	
Positive	15
Negative	25
HER2*	
Positive	2
negative	37
TNP	
Positive	23
Negative	17

*HER2 missing for 1 patient

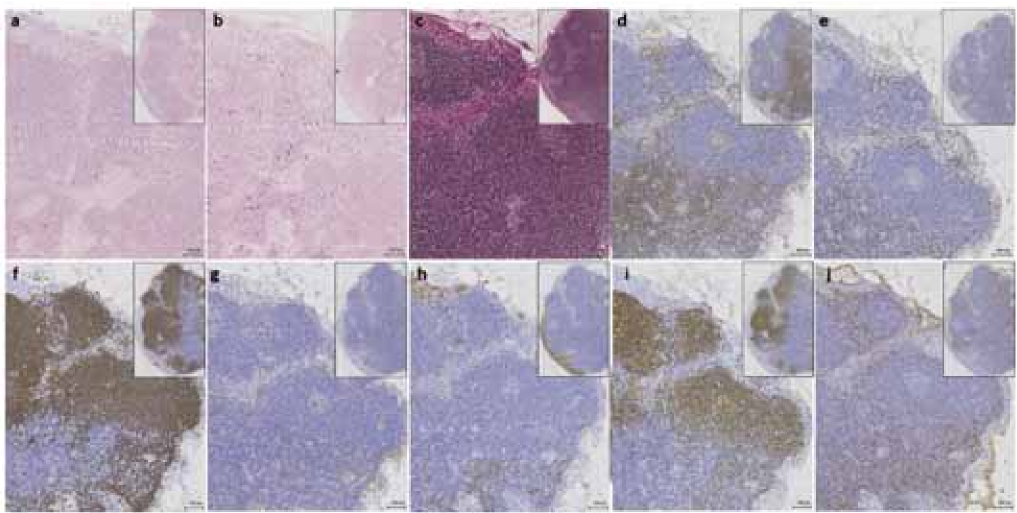
S2 Table. Name, sequence, RNA Tm and concentration for the LNA™ 5`-3`DIG hsa detection probes (Exiqon) used in CISH experiments.

LNA™ Detection/control probes	Sequence	RNA Tm calc.	Concentration
5`-3`DIG hsa miR-18a	CTA**T**CTGCACTAGATGCACCTTA	88 °C	80 nM
5`-3`DIG hsa miR-18b	CTA**A**CTGCACTAGATGCACCTTA	89 °C	80 nM
5`-DIG U6 snRNA	CACGAATTTGCGTGTCATCCTT	84 °C	2.0 nM
5`-DIG Scrambled	GTGTAACACGTCTATACGCCCA	87 °C	80 nM

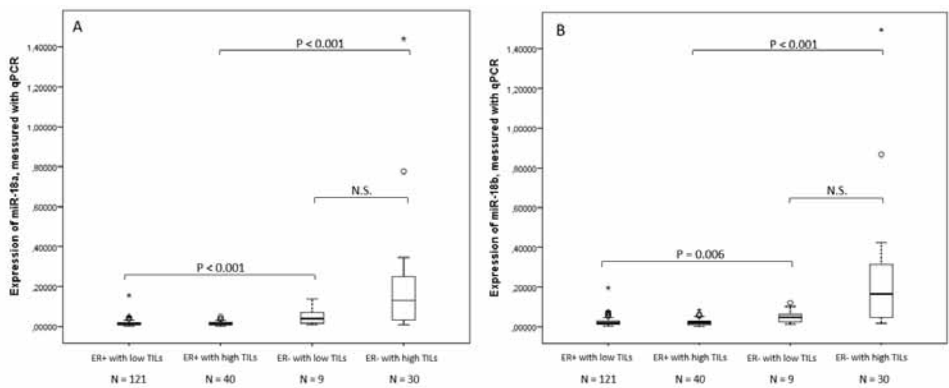
Note: the sequences for miR-18a and miR-18b differ by only one nucleotide, as indicated by **T** and **A**.



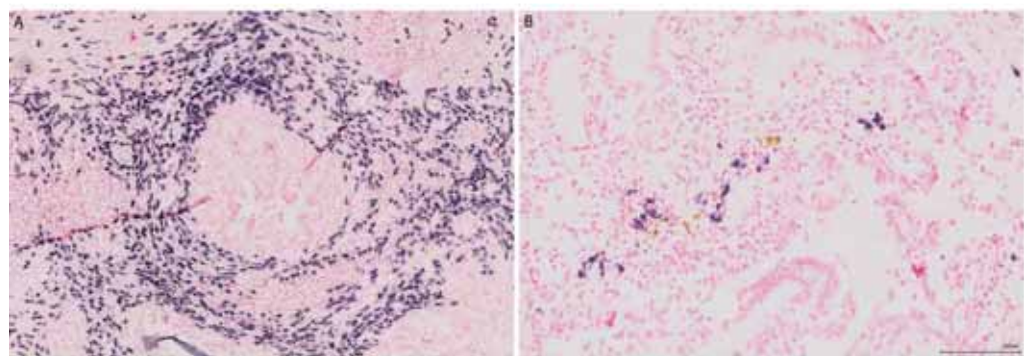
S1 Fig. miR-18b expression in primary tumor corresponding to Fig. 6. Positive and specific CISH expression of LNA 5`-3`DIG miR-18b (80nM) in the stroma of a representative lymph node-positive primary breast cancer tumor.



S2 Fig. Expression pattern comparison in a lymph node with tumour infiltration from a patient with an ER$^+$ breast tumour. a) CISH LNA 5`-3`DIG hsa (80nM) miR-18a probe and **b**) CISH LNA 5`-3`DIG hsa (80nM) miR-18b probe, in comparison to IHC staining for **c**) HE, and IHC-staining for **d**) CD4, **e**) CD8, **f**) CD20, **g**) CD68, **h**) CD138, **i**) PAX5, and **j**) actin.



S3 Fig. Expression measured with qPCR in ER^+ and ER^- breast cancers with high and low TILs of A) miR-18a and B) miR-18b. Central line in boxes represent the median value, boundaries of boxes represent the interquartile range and ends of whiskers represent the minimum and maximum values, excluding outlies. P-values were obtained using independent T-test.



S4 Fig. CISH expression demonstrating strong and specific positive staining with LNA 5`-3`DIG hsa (80nM) miR-18b probe expression in A) pancreatic cancer, and **B)** lung cancer.

Paper III

Clinical Epidemiology

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ORIGINAL RESEARCH

Digital Image Analysis of Ki-67 Stained Tissue Microarrays and Recurrence in Tamoxifen-Treated Breast Cancer Patients

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Purpose: The proliferation marker Ki-67 has been used as a prognostic marker to separate low- and high-risk breast cancer subtypes and guide treatment decisions for adjuvant chemotherapy. The association of Ki-67 with response to tamoxifen therapy is unclear. High-throughput automated scoring of Ki-67 might enable standardization of quantification and definition of clinical cut-off values. We hypothesized that digital image analysis (DIA) of Ki-67 can be used to evaluate proliferation in breast cancer tumors, and that Ki-67 may be associated with tamoxifen resistance in early-stage breast cancer.

Patients and Methods: Here, we apply DIA technology from Visiopharm using a custom designed algorithm for quantifying the expression of Ki-67, in a case–control study nested in the Danish Breast Cancer Group clinical database, consisting of stages I, II, or III breast cancer patients of 35–69 years of age, diagnosed during 1985–2001, in the Jutland peninsula, Denmark. We assessed DIA-Ki-67 score on tissue microarrays (TMAs) from breast cancer patients in a case–control study including 541 ER-positive and 300 ER-negative recurrent cases and their non-recurrent controls, matched on ER-status, cancer stage, menopausal status, year of diagnosis, and county of residence. We used logistic regression to estimate odds ratios and associated 95% confidence intervals to determine the association of Ki-67 expression with recurrence risk, adjusting for matching factors, chemotherapy, type of surgery, receipt of radiation therapy, age category, and comorbidity.

Results: Ki-67 was not associated with increased risk of recurrence in tamoxifen-treated patients (ORadj =0.72, 95% CI 0.54, 0.96) or ER-negative patients (ORadj =0.85, 95% CI 0.54, 1.34).

Conclusion: Our findings suggest that Ki-67 digital image analysis in TMAs is not associated with increased risk of recurrence among tamoxifen-treated ER-positive breast cancer or ER-negative breast cancer patients. Overall, our findings do not support an increased risk of recurrence associated with Ki-67 expression.

Keywords: breast cancer, tamoxifen, proliferation, Ki-67, recurrence risk, tissue microarray, TMA, digital image analysis, DIA

Introduction

In spite of the relatively good prognosis for early-stage breast cancer, studies with as long as 20 years of follow-up suggest that the risk of recurrence remains consistently elevated after diagnosis.[1,2] Around 70% of breast cancers are estrogen receptor (ER) positive ($^{+}$). These patients are candidates for receiving endocrine therapy, which reduces the five-year risk of recurrence by about one-half.[3] Tamoxifen is an ER modulator that selectively binds to the ER and blocks its

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ligand estradiol, thereby preventing ER activation and downstream tumor cell proliferation.[4,5] Guidelines recommend tamoxifen as treatment for premenopausal ER$^+$ patients, and is an important alternative and/or sequential treatment in postmenopausal patients. The tamoxifen treatment regimen has recently been extended from five to 10 years.[6] Unfortunately, as many as 30% of breast cancer patients are, or become, resistant to the drug, either de novo or by acquired resistance,[7,9] resulting in recurrence of disease. Furthermore, up to one-half of all patients stop taking their medication because of adverse side effects or for other reasons.[10,12]

Ki-67 immunostaining is used as a marker of proliferation and has a well-documented prognostic value in breast cancer.[13,16] Together with histological grade, Ki-67 proliferation index can be used as a surrogate marker to separate low-risk Luminal A subtype (i.e. low proliferation, low grade, hormone receptor (HR)$^+$, human epidermal growth factor-receptor 2 (HER2) negative ($^-$) from the higher risk Luminal B subtype (i.e. high proliferation, high grade, HR^{+-}/HER2$^+$).[17] In this way, proliferation can be used to guide treatment decisions regarding the use of adjuvant chemotherapy.[18] Changes in Ki-67 expression in tumors following short-term neoadjuvant endocrine therapy have also been suggested as a marker of treatment efficacy; thus, breast cancer patients with tumors with high Ki-67 expression after treatment showed lower recurrence-free survival.[19] The association of pre-treatment Ki-67 proliferation index with recurrence risk among women treated with tamoxifen therapy is, however, unclear. In the NSABP B-14 trial, among 16 cancer-related genes examined, *MKI-67* gene expression of Ki-67 was not associated with the effectiveness of adjuvant tamoxifen treatment.[20] A second randomized trial including 564 premenopausal women reported a more complex relationship between Ki-67 index and tamoxifen response; patients whose tumors showed either high or low Ki-67 levels benefitted more from tamoxifen compared with patients whose tumors had intermediate levels of Ki-67 expression.[21] As such, further evidence is needed on the potential association between Ki-67 value and tamoxifen therapy.

Scoring Ki-67 on tissue sections is challenging, not least because of a lack of standardized methods for performing, scoring and interpreting Ki-67 immunohistochemistry (IHC).[22] Ki-67 is traditionally evaluated visually with a standard microscope rather than by using digital image analysis (DIA). As a result, the reproducibility varies. There is no international consensus regarding scoring methods or the most clinically relevant cut-off, although until recently, a cut-off value of 30% was recommended by the Norwegian Breast Cancer Group.[23] Compared with standard visual scoring of Ki-67, DIA offers a more objective, rapid and more reproducible method to determine the fraction of proliferating cells.[16] We hypothesized that DIA of Ki-67 stained sections can be used to efficiently evaluate proliferation in breast cancer tumor specimens; we applied this methodology to investigate the potential association of the Ki-67 index with a response to tamoxifen therapy.

Patients and Methods

Study Population

The source and study populations have been previously described.[24] In brief, the source population consisted of all women (n=11,252) aged 35 to 69 living in the Jutland Peninsula in Denmark, diagnosed with non-metastatic (stages I–III) invasive breast cancer between 1985 and 2001, and registered in the clinical database of The Danish Breast Cancer Group (DBCG) Registry.[25] The Jutland Breast Cancer Recurrence Biobank contains tumor sections, DNA/RNA, tissue microarrays (TMAs) and clinicopathological data. Derived from this biobank, the study population consisted of 541 ER$^+$ breast cancer patients treated ≥1 year with Tamoxifen® (grouped ER$^+$/Tam$^+$) with recurrence and their 541 matched controls without recurrence, together with 300 ER-negative (ER$^-$) non-tamoxifen-treated (grouped ER$^-$/Tam$^-$) breast cancer patients with recurrence and their 300 matched controls without recurrence. An overview of the study design is shown in Figure 1. We sought to evaluate any association between Ki-67 score, as measured using the DIA-Ki-67 score, and breast cancer recurrence among women with estrogen receptor-positive and -negative breast cancer, treated with and without tamoxifen, respectively. Patients not meeting the inclusion criteria were excluded. Controls were matched to cases according to group (ER$^+$/Tam$^+$ or ER$^-$/Tam$^-$), year of diagnosis, derived UICC (Union for International Cancer Control) I–III cancer stage, menopausal status, and county of residence at the time of diagnosis. Controls were sampled using incidence density sampling whereby controls had to be alive and at risk of breast cancer recurrence on the date their corresponding case recurred.[26] Without replacement, controls were selected from members of the source population, who were not diagnosed with a breast cancer recurrence or contralateral

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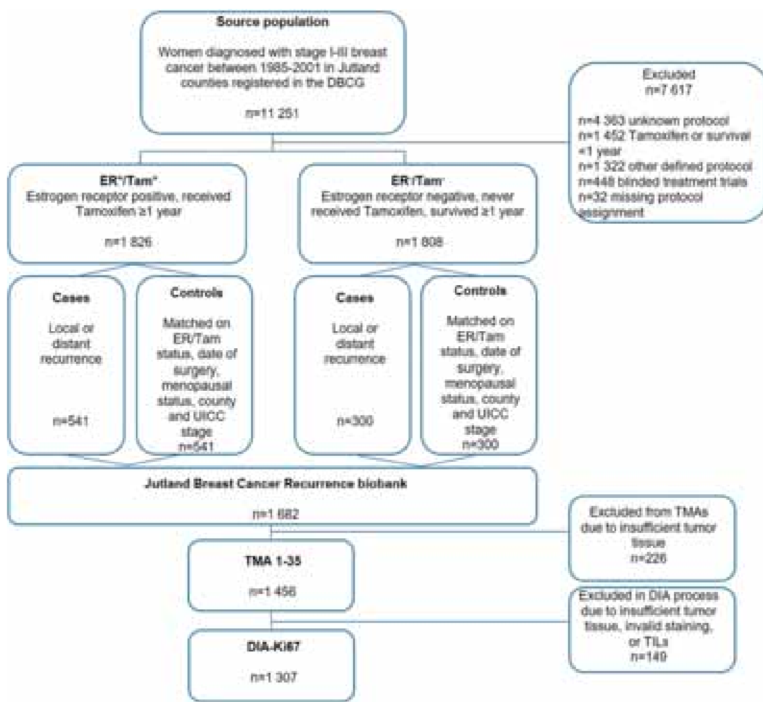

Figure 1 Study design.
Notes: The source population consisted of all female residents aged 35–69 of Denmark's Jutland Peninsula between 1985 and 2001, who were diagnosed with non-metastatic breast cancer. Two-thirds of the women (n = 7617) were excluded because of an unknown treatment protocol or because they did not meet the inclusion criteria. Ki-67 results were missing if tissue was unavailable or if the tumor core was unsatisfactory after processing, staining, and imaging.

breast cancer by the time of the matched case's recurrence. ER status was defined as positive if ≥10% cells in tumor sections stained positive. Depending on the guidelines in Denmark at the time of diagnosis, ER^+/Tam^+ women were assigned to tamoxifen therapy protocols of >1 year. Recurrent cases were defined as the occurrence of any (local, regional, contralateral or distant) breast cancer recurrence during follow-up time, as recorded in the DBCG Registry. Follow-up time started from 1 year after the primary surgery date until the date of the first breast cancer recurrence, death from any cause or emigration (assessed by DBCG registry), loss to follow-up, 10 years of follow-up or September 1, 2006 (i.e. end of study).

Data Collection from Danish Registries

Patient data were collected from the DBCG registry (date of diagnosis, UICC stage, tumor size, node status, histological grade, ER/progesterone receptor (PR) status, surgery type, chemotherapy, radiotherapy and hormonal therapy), and the Danish National Registry of Patients (comorbid diseases prevalent up to 10 years before breast cancer diagnosis).

Tumor Tissue Microarray Construction

Archived formalin-fixed, paraffin-embedded (FFPE) primary tumor tissue blocks from the cases and controls were collected from pathology departments of treating hospitals. A pathologist reviewed hematoxylin- and eosin (HE)-stained tumor whole sections and identified regions of invasive carcinoma for sampling into TMAs. Using a TMA Master (3DHistech Ltd., Budapest, Hungary), cylindrical 1 mm diameter cores were sampled from each primary breast tumor (donor block) and re-embedded into recipient TMA paraffin blocks (n=35) using standard procedures.[27] One placental and two liver tissue cores were used as orientation markers in each TMA. From each patient sample, one to three representative tumor cores and one core with normal or tumor margin tissue were sampled, yielding a total of some

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5500 tumor tissue cores available for IHC staining. Patients' samples were not included in the TMAs if their tumor tissues could not be analyzed because of inadequate material (n=226) (Figure 1).

Immunohistochemistry for Ki-67

We assessed Ki-67 expression in all 35 TMAs using IHC. Methods for tissue processing, antigen retrieval, antibody dilution and signal detection have been described,[28,29] and are outlined in the Supplementary Material. Laboratory personnel were blinded to all clinical information, including ER/Tam status and case/control status.

Automated Digital Image Assessment of Ki-67 Scores

Ki-67 expression was evaluated using the fully automated VIS DIA VisioMorph system (Visiopharm®, Hoersholm, Denmark), using similar image processing principles as described previously.[16] In brief, all TMA-slides were scanned at 40x magnification using a Leica SCN400 slide scanner (Leica Biosystems, Wetzlar, Germany) and imported into the image analysis software program Visiopharm®, and a digital image was recorded of each core. We employed a customized analysis protocol package (APP) for Ki-67 quantification based on the same principles as before, with minor modifications and adjustments. Detection was based on both size and morphology of the nuclei; the tumor region of interest (ROI) was defined by outlining an ROI mask of tumor cells (Figure 2). Inside this mask of tumor cells, blue (negative) and brown (Ki-67-positive) nuclei were segmented using a Bayesian classifier. Pixels that contributed to Ki-67 positively stained nuclei were identified based on their brown DAB (3,3′-diaminobenzidine) color deconvolution, whereas pixels of the negative class were identified by their blue HE stain. All cores were examined after the ROI had been defined, to be either accepted or edited (by manually removing any DCIS, TILs, artefacts, misclassifications, or empty cores and excluding unsuitable cores). All pixels of an image were then assigned a label for being either tumor cells expressing the Ki-67 (label 001/green), or negative tumor/normal cells (label 002/blue). Stromal cells were classified as background (label 003/red), and disregarded in the quantification (Table S1). Labelling of image pixels and subsequent classification of cells are shown in Table S1.

The Ki-67 score was then calculated automatically by the customized APP (Ki-67 score= [(area of Ki-67-positive tumor cells)/(area positive + negative tumor cells) x 100]), using the areas of classified negative cells (i.e. blue nuclei) and classified positive cells (i.e. brown nuclei) (Figure 3). Again, any erroneously segmented areas were corrected manually. For some of the cores, the material was missing (n~350), tumor tissue was absent or less than 100 tumor cells were present (n~260), the Ki-67-staining was either too weak (n~45) or too excessive (n~60), or poor quality imaging or resolution (n~25), or displayed a combination of reasons; these were all excluded and the cores marked as missing. In total, n=149 patients had insufficient or invalid tissue material on the tissue microarrays (TMAs) to be appropriately

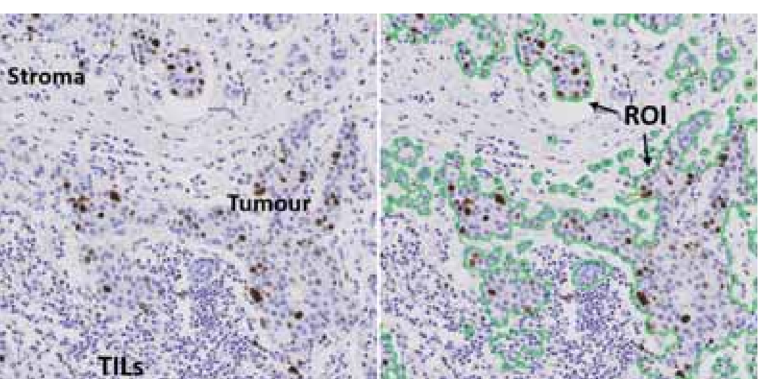

Figure 2 Tumor region of interest (ROI).

Notes: ROI (outlined in green) was defined semi-automatically in Visiopharm®, based on both size and morphology of the cells. Stroma and TILs were disregarded by the customized APP.

Abbreviations: APP, analysis protocol package; TILs, tumor-infiltrating lymphocytes.

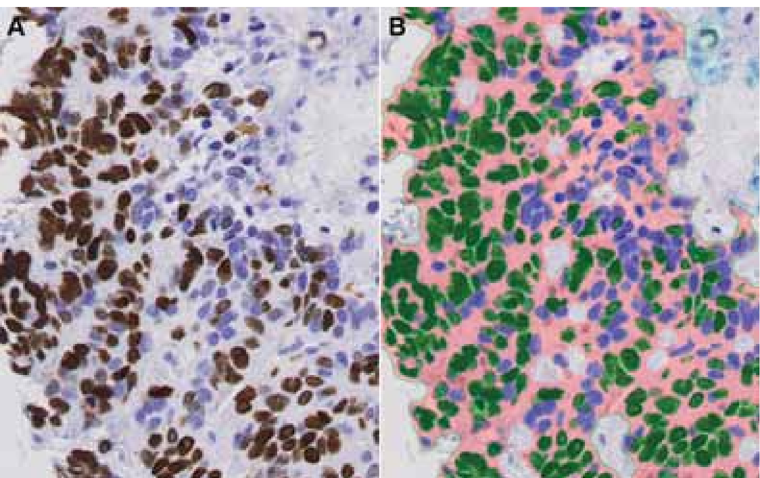

Figure 3 Representation of DIA scoring of Ki-67.
Notes: (**A**) before, and (**B**) after, the customized algorithm was run in the Visiopharm® program. Ki-67-positive tumor cells were identified and scored in relation to the negative tumor cells: Ki-67 positively stained nuclei were identified based on their brown DAB staining, whereas negative cells were identified based on their blue H&E stain. Ki-67 score= [(area of Ki-67-positive tumor cells)/(area positive + negative tumor cells) x 100]. DIA score in this particular core was calculated by the customized algorithm to be 61%.
Abbreviation: DIA, digital image analysis.

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scored, and were therefore excluded (examples of the exclusion criteria are shown in Figure S1). Finally, all cores were re-examined by two observers, adjusting the ROI or the labelling when appropriate, as well as excluding unsuitable cores. We set 15% as the limit of acceptance of intra-patient variability in the Ki-67 score (between the different cores from the same patient). In those cases in which this was exceeded, the cores were re-examined and accepted, edited further or discarded. For the great majority of the cores, the manual adjustments performed had little impact on the Ki-67 score. Larger discrepancies between the automated APP-generated and the edited Ki-67 scores were double-checked. During the DIA-scoring, observers were blind to all clinical information, including ER/Tam, and case/control status.

Ki-67 Score

Out of 1456 individual patients with samples distributed in the 35 TMAs, 149 patients (~10%) were excluded during the DIA process (Figure 1), leaving a total of 1307 patient samples with one or more cores. More specifically, all three cores remained for 830 of the patients, two cores remained for 302 patients, and one core remained for 174 patients. Table S2 summarizes the TMA-DIA set-up. The proportion of tumor cells with a positive Ki-67 staining was noted as a continuous metric from 0% - 100%. For each patient between one and three cores were available, the final index being calculated as mean of the scores for the individual cores. The hotspot core was defined as the single core for each patient with the highest Ki-67 score. DIA-Ki-67 scores ranged from 0% - 92%. We created a dichotomous variable of Ki-67 expression in the primary breast tumors. A Ki-67 score above, or equal to, the study sample's median Ki-67 score (6.2%) was considered positive and a score below the study sample's median Ki-67 score was considered to indicate no Ki-67 expression. In line with previous recommendations,[23] we initially examined the distributions of Ki-67 with a 30% cut-off. This was, however, abandoned due to low numbers of patients above 30%.

Statistical Analyses

All statistical analyses were conducted using SAS 9.4 (SAS Institute, Cary, NC), and within strata of the two patient groups (ER^+/Tam^+ and ER^-/Tam^-). Cases and controls were characterized using descriptive statistics. Distribution of patient clinicopathological factors was also characterized according to median Ki-67 score. The data on Ki-67 were not normally distributed and were therefore evaluated as a categorical variable. To estimate the matched odds ratios

(ORs) between Ki-67 score and breast cancer recurrence, we applied logistic regression models adjusting for the matching factors. We adjusted for potential confounding variables using unconditional logistic regression models, including the matching factors, chemotherapy, type of surgery, receipt of radiation therapy, age category and comorbidity. We performed several sensitivity analyses: We reran the regression models also adjusting for grade. We stratified the analyses by time to recurrence, and by the receipt of chemotherapy. We also performed analyses using the median Ki-67 score in ER^+/T^+ patients, and the median Ki-67 score in ER^-/Tam^- patients.

Results

Descriptive and clinical characteristics of the study population differed little between the cases and their controls (Table 1). These characteristics were evenly distributed across Ki-67 score according to below (Table S3) or above (Table S4) the median Ki-67 score. DIA-Ki-67 scores were equally distributed across strata, both for the dichotomous value and the hotspot median. DIA-Ki-67 score was missing for 213 patients in the ER^+/Tam^+ group and for 118 patients in the ER^-/Tam^-. In the ER^+/Tam^+ group, the majority (~80%) of patients had a histological grade of either I or II. Conversely, for the ER^-/Tam^- group, the majority (~80%) had a histological grade of II or III. At the time of diagnosis, most patients had tumor stage II (46% ER^+ and 51% ER^-) or III (52% ER^+ and 41% ER^-). More women were older than 55 years in the ER^+/Tam^+ group compared with the ER^- group, and accordingly, more women were postmenopausal in the ER^+/Tam^+ group, compared with the ER^-/Tam^- group (94% vs 60%). For quite a high number of patients, information was missing on grade, especially for the controls (around 25%). These patterns are consistent with the selection of patients into tamoxifen treatment according to Danish guidelines in place at the time of the diagnoses.

For each ER/Tam group, estimates of the association between breast cancer recurrence and DIA-Ki-67 score are displayed in Table 2. DIA-Ki-67 score was not associated with increased risk of breast cancer recurrence, neither in the ER^+/Tam^+ (ORadj =0.72, 95% CI 0.54, 0.96), nor the ER^-/Tam^- groups (ORadj =0.85, 95% CI 0.54, 1.34). This was evident, both when assessing all available cores in each of the individual patients, or just the hotspot core (Table 2), using median DIA-Ki-67 score (6.2%) as a cut-off. We also examined the distributions of the mean and hotspot DIA-Ki-67 scores across ER/Tam strata with a 30% cut-off, but this cut-off was abandoned due to very few patients ≥30% (Table S5), and since the median expression of Ki-67 was close to the mean and hotspot values (Table S6). Furthermore, analyses using the median Ki-67 expression for each ER-stratum did not change the overall estimates, although for the ER^- group with fewer patients, the adjusted ORs were higher but still with wide 95% intervals (Table S7). The sensitivity analyses additionally adjusting for grade did not materially change the effect estimates (ORadj2 = 0.74, 95% CI 0.52, 1.04) (Table S8); nor did the analysis stratifying by time to recurrence (Table S9), or by receipt of chemotherapy, although the ER^- patients with chemo did have higher ORs (Tables S10 and S11).

Discussion

Our findings suggest that a high Ki-67-score is not associated with a greater risk of breast cancer recurrence, either in tamoxifen-treated patients, or in patients with ER^- negative tumors. In fact, somewhat puzzling, our findings point to the opposite association, adding further complexity to the existing discussion concerning the association of Ki-67 proliferation score with recurrence in tamoxifen-treated breast cancer patients.[30]

Our study has several strengths including the large number of patients (n=1307), each with up to three representative tumor cores on the TMAs and the use of high-quality IHC assays. We had complete follow-up data from the DBCG clinical database, comprehensive data on patient, tumor and treatment characteristics.[25,31] Additional strengths include the application of a technically advanced digital scoring system, for precisely assessing proliferation scores, specifically in tumor cells in the tissue cores.

Our study has some limitations. Although all patients were assigned tamoxifen for 1, 2 or 5 years, most patients who were assigned tamoxifen for only 1 or 2 years at diagnosis, took tamoxifen for a longer duration because of the emerging evidence of a survival benefit.[24] In accordance with the guidelines at the time, the threshold for ER positivity was ≥10% positively stained cells, whereas nowadays, with more sensitive detection methods, a 1% threshold is used. In addition, the TMAs of the Jutland Breast Cancer Biobank were not constructed specifically for assessing Ki-67; therefore, although the region of sampling was within the tumor area, and up to three large cores (diameter 1 mm) were taken from each tumor, the cores were not selected from the invasive tumor front only. Ki-67 staining can be heterogeneous in

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Table 1 Patient and Clinical Characteristics for Cases and Controls of the Jutland Breast Cancer Recurrence Biobank

Patient Characteristics	ER^+/Tam^+ No. (%)				ER^-/Tam^- No. (%)			
	Recurrent Cases n=541		Controls n=541		Recurrent Cases n=300		Controls n=300	
DIA Ki-67 score								
< Median	245	(57)	276	(63)	76	(31)	79	(33)
Median or above	188	(43)	160	(37)	170	(69)	157	(67)
Missing	108		105		54		64	
Hotspot								
< Median	243	(56)	272	(62)	80	(33)	80	(34)
Median or above	190	(44)	164	(38)	166	(67)	156	(66)
Missing	108		105		54		64	
Year of diagnosis								
1985–1993	235	(43)	234	(43)	107	(36)	100	(33)
1994–1996	113	(21)	112	(21)	81	(27)	83	(28)
1997–2001	193	(36)	195	(36)	112	(37)	117	(39)
Age at diagnosis								
35–44	16	(3.0)	13	(2.4)	68	(23)	58	(19)
45–54	116	(21)	111	(21)	120	(40)	113	(38)
55–64	286	(53)	281	(52)	82	(27)	86	(29)
65–69	123	(23)	136	(25)	30	(10)	43	(14)
Menopausal status								
Premenopausal	34	(6.3)	34	(6.3)	121	(40)	121	(40)
Postmenopausal	507	(94)	507	(94)	179	(60)	179	(60)
UICC tumour stage								
I	9	(1.7)	9	(1.7)	25	(8.3)	25	(8.3)
II	250	(46)	250	(46)	153	(51)	153	(51)
III	282	(52)	282	(52)	122	(41)	122	(41)
Histological grade								
I	108	(25)	144	(35)	27	(11)	23	(10)
II	234	(54)	215	(52)	125	(49)	98	(43)
III	92	(21)	57	(14)	103	(40)	106	(47)
Missing	107		125		45		73	
Surgery type								
Breast-conserving	58	(11)	71	(13)	47	(16)	56	(19)
Mastectomy	483	(89)	470	(87)	252	(84)	244	(81)
Missing	0			0	1		0	
Systemic adjuvant chemotherapy								
Yes	70	(13)	65	(12)	248	(83)	188	(63)
No	471	(87)	476	(88)	52	(17)	112	(37)
Radiation therapy								
Yes	183	(34)	191	(35)	128	(47)	123	(47)
No	358	(66)	350	(65)	166	(56)	137	(53)
Missing	0		0		6		40	
Tamoxifen protocol, years								
1	257	(48)	261	(48)	-		-	
2	98	(18)	92	(17)	-		-	
5	186	(34)	188	(35)	-		-	

Abbreviations: ER, oestrogen receptor; Tam, tamoxifen; UICC, Union for International Cancer Control.

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Table 2 Associations Between Ki-67 Expression and Breast Cancer Recurrence Within ER/Tam Groups

Ki-67 Expression	ER^+/Tam^+			ER^-/Tam^-		
	Cases/ Controls (n)	Matched OR (95% CI)	Adjusted OR[a] (95% CI)	Cases/ Controls	Matched OR (95% CI)	Adjusted OR[a] (95% CI)
All cores						
<median	245/276	1	1	76/79	1	1
≥median	188/160	0.74 (0.56,0.98)	0.72 (0.54,0.96)	170/157	0.87 (0.59,1.30)	0.85 (0.54,1.34)
Hotspot						
<median	243/272	1	1	80/80		
≥median	190/164	0.75 (0.57,1.00)	0.73 (0.55,0.98)	166/156	0.92 (0.62,1.3)	0.86 (0.55,1.35)

Notes: [a]Adjusted for year of diagnosis, menopausal status, county of residence, UICC stage, chemotherapy, type of surgery, age category, receipt of radiotherapy and comorbidity.
Abbreviations: OR, odds ratio; CI, confidence interval.

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breast cancers, and correct sampling is therefore important. The periphery of the tumor is most often the area with the highest percentage of Ki-67-positive cells i.e. the so-called hotspot areas.[16,32] As a consequence, our analysis might misclassify Ki-67 scores compared with scores obtained from the invasive front of the tumor alone, as is the currently recommended protocol for performing Ki-67 assays in breast cancer.[23] The International Ki67 in Breast Cancer Working Group recommends Ki-67 scoring should be counted in at least 500 tumor cells, a higher number than the cell-limit of 100 we have applied herein.[33] In addition, our reported median Ki-67 of 6.5% is low compared to other studies.[34] However, in a previous report by co-authors, the reported DIA-Ki-67 threshold was 6.5%, this being the most robust and strongest prognosticator; which is in concordance with the present study.[16] Follow-up started 1 year after the time of diagnosis, therefore any recurrences within the first year are not recorded. Early recurrences are often associated with highly proliferating tumors,[35] which would bias towards the null. The DBCG follow-up program continues up to 10 years after diagnosis. Consequently, recurrences that occur later are not recorded in this study. We did not have access to tumor biopsies of recurrences, and were therefore unable to evaluate any change in Ki-67 levels over time.

Previous studies on the association of Ki-67 score and response to tamoxifen therapy are conflicting.[36] Yerushalmi and colleagues examined the prognostic and predictive potential of Ki-67 scores in breast cancer in a review of 22 studies. They concluded that, based on the existing literature, no robust evidence could be found recommending Ki-67 as a tool to identify patients who would benefit from a specific endocrine treatment.[36] The Breast International Group (BIG)-1 98 trials showed that the aromatase inhibitor letrozole resulted in greater treatment benefit compared with tamoxifen treatment for patients with a high Ki-67 labelling index.[37] However, in a small study of 70 post-menopausal tamoxifen-treated breast cancer patients, high (cut-off 30%) Ki-67 was associated with tamoxifen resistance and poor prognosis, in terms of recurrence and survival.[38] Furthermore, Ki-67 score was higher among patients who developed early relapse (within the first 24 months) after starting tamoxifen, yet there was no difference in relapse risk for those with tumors expressing high versus low Ki-67.[38] In a more recent study, Beelen et al tested the expression of Ki-67 score in a cohort of 563 post-menopausal women with ER^+ breast cancers, and found that patients with high Ki-67 counts did benefit from adjuvant tamoxifen.[39] However, in their study, high Ki-67 was defined as ≥5% expression. Moreover, tamoxifen efficacy was reduced in patients whose tumors had a high mitotic count, but in patients with low mitotic count, tamoxifen was of benefit. At the same time, they observed that patients with tumors with a high mitotic count could still have low tumor Ki-67 scores, and that mitotic count outperformed Ki-67 with regard to prediction of the benefit of endocrine treatment.[39] Of note, both these studies included postmenopausal patients. Our study population consisted of mostly postmenopausal, but also some premenopausal patients. Others have examined the potential effect of pre-surgical short-term endocrine treatment on Ki-67 score. Dowsett et al studied 158 patients with HR^+ primary disease, and correlated the change in Ki-67 score in tumor biopsies taken before and 2 weeks after, treatment with anastrozole and/or tamoxifen. They reported that only the change in Ki-67 level was associated with treatment benefit, whereas the absolute level of Ki-67

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expression at baseline was not significantly associated with recurrence-free survival.[19] Similarly, Cohen et al found a 40% mean decrease in Ki-67 scores after only 7 days of pre-surgical treatment and suggested using change in the Ki-67 index in future endocrine treatment trials.[40]

Taken together, these studies demonstrate the complexity that exists when trying to assess tumor cell proliferation and its potential effects on tamoxifen treatment. The prognostic information associated with the Ki-67 analysis may be limited to very high or very low index scores.[21] There is no consensus regarding the definition of high/low Ki-67 proliferation indices. For instance, the reported optimal cut-off value of Ki-67 to separate Luminal A and Luminal B breast cancers, or low-risk from high-risk patients, varies substantially across studies (i.e. from 10% to 30%).[17,38,41,42]

Muftah and co-workers analyzed Ki-67 scores in breast cancer, comparing whole tissue sections with TMAs constructed with a single 0.6 mm diameter core from each patient sample. They conclude that Ki-67 expression in breast cancer can be evaluated in TMAs, as long as the allowance is made for the substantial heterogeneity of Ki-67 expression.[42] In comparison with Muftah and co-workers' study, and other published studies, our TMAs were constructed using up to three tumor cores, each 1mm in diameter, thus including in the TMAs considerably larger areas of tumor tissue for assessment. This was done in order to improve the representativeness of the TMAs, and we believe it enhanced the precision of our study. The amount of tumor tissue included in our TMAs was considerably greater than that recommended as a minimum by Khoury et al, who conclude that either three 0.6-mm cores or a single 1.0-mm core was adequate to be representative of whole tissue sections.[43]

We hypothesized that DIA of Ki-67 score could be used to efficiently evaluate proliferation in breast cancer tumors, and that high DIA-Ki-67 scores might be associated with response to tamoxifen. As we have shown, the DIA set-up and the automated DIA-Ki-67 scoring were successful. However, it is important to emphasize that in practice, pre-analytical variables (e.g. fixation, cutting, staining issues, region of sampling, scanning, inclusion/exclusion criteria) can have substantial effects on the outcome of automated scoring.[44]

In our study, we also examined the Ki-67-score in ER^- tumors, and did not observe any substantial differences in this when comparing recurrent cases and controls. Our results are in line with the variability seen in other studies,[45] and underline the complexity and well-known challenges of using the Ki-67 index as a biomarker in clinical decision-making.[46,47]

Conclusion

In summary, we found that the Ki-67 index (as measured digitally by image analysis in TMAs) was not associated with increased risk of recurrence among tamoxifen-treated ER^+ breast cancer or ER^- breast cancer patients. Overall, our findings do not support an increased risk of recurrence associated with Ki-67 expression. Future work should aim to standardize and define a clinically relevant Ki-67 threshold before it is used for clinical decision-making in tamoxifen-treated breast cancer patients.

Abbreviations

APP, analysis protocol package, DIA, digital image analysis; ER^+, estrogen receptor positive; ER^-, estrogen receptor negative; IHC, immunohistochemistry; ROI, region of interest; Tam, tamoxifen; TMA, tissue microarray.

Ethics Approval and Informed Consent

This study was approved by the Norwegian National Research Ethics Committees/Regional Committees for Medical and Health Research Ethics (REC) West (REC number 23216), and the Regional Committee on Biomedical Research Ethics of Denmark's Central Region (Record No. 1-10-72-16-15). According to Danish law, registry-based research is exempt from informed consent requirements.

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Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it

critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

Timothy L Lash reports receiving personal fees from Amgen, outside the submitted work. The authors report no other conflicts of interest in this work.

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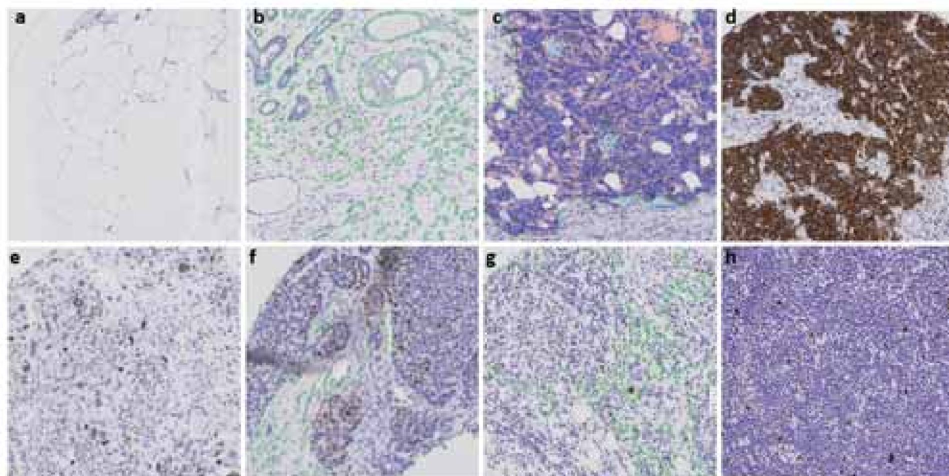
Supplementary material to Paper III

Digital Image Analysis of Ki-67 stained Tissue Microarrays does not predict Recurrence in Tamoxifen-treated Breast Cancer Patients. Egeland NG, Jonsdottir K, Lauridsen KL, Skaland I, Hjorth CF, Gudlaugsson EG, Hamilton-Dutoit S, Lash TL, Cronin-Fenton D, Janssen EAM. Digital Image Analysis of Ki-67 Stained Tissue Microarrays and Recurrence in Tamoxifen-Treated Breast Cancer Patients. Clin Epidemiol. 2020;12:771-781 https://doi.org/10.2147/CLEP.S248167.

Immunohistochemistry for Ki-67

Histology sections of 4 µm were cut from each TMA block and mounted onto silanized slides (#S3003; Dako, Glostrup, Denmark) and dried overnight at 37°C followed by 1 hour at 60°C. Sections were deparaffinised in xylene and rehydrated in decreasing concentrations of alcohol. Antigen was retrieved with a highly stabilized retrieval system (ImmunoPrep, Instrumec, Oslo, Norway) using 10 mM TRIS/1 mM EDTA (pH 9.0) as the retrieval buffer. Sections were heated for 3 min at 110°C followed by 10 min at 95°C, then cooled to 20°C. Ki-67 was detected using clone MIB-1 (DAKO, Glostrup, Denmark) at 1:100 dilution (Dako antibody diluent S0809), before the sections were incubated for 30 min at 22°C. For visualization, the EnVisionTM Flex detection system (Dako, K8000) was used. Sections were incubated for 5 min with peroxidase-blocking reagent (SM801), 20 min with the EnVisionTM FLEX/HRPDetection Reagent (SM802), 10 min with EnVisionTM FLEX DAB+ Chromogen (DM827)/EnVisionTM FLEX Substrate Buffer (SM803) mix and 5 min with EnVisionTM FLEX Hematoxylin (K8008). The slides were then dehydrated and mounted. All immunohistochemical stainings were performed using a Dako Autostainer Link 48 instrument and EnVisionTM FLEX Wash Buffer (DM831) and were controlled in the NordiQC external quality programme.

Supplemental Figures



S1 Figure. Examples of cores that were excluded due to the following reasons: a) stroma/empty core, b) too few tumor cells present, c) excessive blue HE-staining, d) excessive membranous/cytoplasmic brown DAB-staining , e) poor morphology/too weak staining f) poor imaging/artefacts, g) and h) tumor-infiltrating lymphocytes.

Supplemental Tables

S1 Table. Labelling of image pixels and subsequent classification of cells in Visiopharm®.

Assigned label	Color	Detection of pixels	Classification
Label 001	Green	Brown/DAB	Ki-67 positive
Label 002	Blue	Cells/HE	Ki-67 negative
Label 003	Red	Stroma	Background

DAB: 3,3'diaminobenzidine. HE: hematoxylin- and eosin.

S2 Table. Summary of the TMA-DIA set-up.

Patient IDs on the 35 TMAs	Excluded (%)	Ki-67 hotspot Range (%)	Patients with Ki-67 score ≥15%	Patients with Ki-67 score ≥30%	Cores remaining after DIA		
					3	2	1
1456	149 (10)	0 - 92	451	196	830 (57%)	302 (22%)	174 (12%)

S3 Table. Patient characteristics with Ki-67 median=low.

Patient characteristics Ki-67 median=low	ER/TAM type							
	ER+/TAM+				ER-/TAM-			
	Case status				Case status			
	Cases		Controls		Cases		Controls	
	N	%	N	%	N	%	N	%
Total	245	100.0	276	100.0	76	100.0	79	100.0
Year of diagnosis								
85-93	100	40.8	106	38.4	19	25.0	20	25.3
94-96	55	22.4	62	22.5	25	32.9	21	26.6
97-01	90	36.7	108	39.1	32	42.1	38	48.1
Age at diagnosis								
35-44	10	4.1	<= 5		12	15.8	12	15.2
45-54	50	20.4	62	22.5	32	42.1	28	35.4
55-64	134	54.7	<= 142		21	27.6	26	32.9
65-70	51	20.8	71	25.7	11	14.5	13	16.5
Menopausal status								
Premenopausal	17	6.9	15	5.4	29	38.2	34	43.0
Postmenopausal	228	93.1	261	94.6	47	61.8	45	57.0

Patient characteristics Ki-67 median=low	ER/TAM type							
	ER+/TAM+				ER-/TAM-			
	Case status				Case status			
	Cases		Controls		Cases		Controls	
	N	%	N	%	N	%	N	%
UICC tumor stage								
I	<=5		<= 5		7	9.2	<= 5	
II	109	44.5	117	42.4	38	50.0	36	45.6
III	<= 135		<= 158		31	40.8	<= 42	
Histological grade								
I	61	24.9	95	34.4	11	14.5	13	16.5
II	95	38.8	101	36.6	36	47.4	28	35.4
III	33	13.5	17	6.2	22	28.9	19	24.1
Missing	56	22.9	63	22.8	7	9.2	19	24.1
Surgery type								
Breast conserving	20	8.2	42	15.2	11	14.5	12	15.2
Mastectomy	225	91.8	234	84.8	65	85.5	67	84.8
Systemic adjuvant chemotherapy								
No	211	86.1	251	90.9	<= 5		26	32.9
Yes	34	13.9	25	9.1	<= 75		53	67.1
Radiation therapy								
No	171	69.8	179	64.9	43	56.6	36	45.6
Yes	74	30.2	97	35.1	32	42.1	34	43.0
Missing	0	0	0	0	1	1.3	9	11.4
Tamoxifen protocol, years								
N/A	0	0	0	0	76	100.0	79	100.0
1	116	47.3	126	45.7	0	0	0	0
2	40	16.3	46	16.7	0	0	0	0
5	89	36.3	104	37.7	0	0	0	0

S4 Table. Patient characteristics with Ki-67 median=high.

Patient characteristics Ki-67 median=high	ER/TAM type							
	ER+/TAM+				ER-/TAM-			
	Case status				Case status			
	Cases		Controls		Cases		Controls	
	N	%	N	%	N	%	N	%
Total	188	100.0	160	100.0	170	100.0	157	100.0
Year of diagnosis								
85-93	78	41.5	72	45.0	64	37.6	55	35.0
94-96	33	17.6	29	18.1	43	25.3	46	29.3
97-01	77	41.0	59	36.9	63	37.1	56	35.7

Patient characteristics Ki-67 median=high	ER/TAM type							
	ER+/TAM+				ER-/TAM-			
	Case status				Case status			
	Cases		Controls		Cases		Controls	
	N	%	N	%	N	%	N	%
Age at diagnosis								
35-44	<= 5		9	5.6	38	22.4	32	20.4
45-54	44	23.4	30	18.8	66	38.8	59	37.6
55-64	<= 97		83	51.9	50	29.4	46	29.3
65-70	46	24.5	38	23.8	16	9.4	20	12.7
Menopausal status								
Premenopausal	13	6.9	15	9.4	65	38.2	59	37.6
Postmenopausal	175	93.1	145	90.6	105	61.8	98	62.4
UICC tumor stage								
I	<= 5		<= 5		8	4.7	13	8.3
II	85	45.2	<= 84		95	55.9	85	54.1
III	<= 102		75	46.9	67	39.4	59	37.6
Histological grade								
I	23	12.2	23	14.4	7	4.1	<= 5	
II	93	49.5	73	45.6	72	42.4	50	31.8
III	44	23.4	32	20.0	66	38.8	<= 70	
Missing	28	14.9	32	20.0	25	14.7	36	22.9
Surgery type								
Breast conserving	23	12.2	14	8.8	24	14.1	35	22.3
Mastectomy	165	87.8	146	91.3	145	85.3	122	77.7
Missing	0	0	0	0	1	0.6	0	0
Systemic adjuvant chemotherapy								
No	166	88.3	138	86.3	33	19.4	68	43.3
Yes	22	11.7	22	13.8	137	80.6	89	56.7
Radiation therapy								
No	112	59.6	102	63.8	96	56.5	70	44.6
Yes	76	40.4	58	36.3	70	41.2	62	39.5
Missing	0	0	0	0	4	2.4	25	15.9
Tamoxifen protocol, years								
N/A	0	0	0	0	170	100.0	157	100.0
1	86	45.7	75	46.9	0	0	0	0
2	29	15.4	27	16.9	0	0	0	0
5	73	38.8	58	36.3	0	0	0	0

S5 Table. Distributions of the mean and hotspot Ki-67 score across ER/TAM-strata.

	ER+/TAM+ No. (%)		ER−/TAM− No. (%)	
	Cases	Controls	Cases	Controls
Mean Ki-67 (3 cores)				
<30%	420	424	198	188
30% or above	13	13	45	47
Mean Ki-67 (3 cores)				
< median*	245	277	74	79
Median or above	188	160	169	156
Hotspot				
<30%	408	412	181	172
30% or above	25	25	62	63
Hotspot				
<median*	242	273	79	80
Median or above	191	164	164	155

*medians are full sample medians

S6 Table. Descriptives; age at surgery, Ki-67-mean and hotspot by ER/TAM group.

	ER^{+}/TAM^{+}				ER^{-}/TAM^{-}				OVERALL MEAN
	N	Mean (Std Deviation)	Median	Range	N	Mean (Std Deviation)	Median	Range	
AGE AT SURGERY	962	59.1 (6.7)		34 (35-69)	539	52.7 (8.9)		34 (35-69)	56.8
KI-67 MEAN	870	7.5 (9.1)		79 (0-79)	478	17.2 (17.3)		90 (0.01-90	10.9
HOTSPOT	870	9.7 (10.8)	6.7	79 (0-79)	478	21.4 (20.1)	15.2	92 (0.03-92)	13.8

S7 Table. Adjusted ORs by ER-specific medians.

ER+/Tam+	ER-/Tam-
Adjusted OR (95%)	Adjusted OR (95%)
0.79 (0.60,1.06)	1.18 (0.78,1.80)

S8 Table. Associations between Ki-67-expression score and breast cancer recurrence within strata, adjusted also for grade.

DIA Ki-67 expression	ER+/Tam+				ER-/Tam-			
	Recurrent cases/controls (n)	Matched OR (95% CI)	Adjusted OR (95%)[a]	Adjusted OR2 (95%)[b]	Recurrent cases/controls or means	Matched OR (95% CI)	Adjusted OR (95%)[a]	Adjusted OR2 (95%)[b]
All cores								
<median	245/276	1	1	1	76/79	1	1	1
≥median	188/160	0.75 (0.55,1.03)	0.72 (0.54,0.96)	**0.74 (0.52,1.04)**	170/157	1.00 (0.66,1.53)	0.85 (0.54,1.34)	**0.89 (0.53,1.50)**
Hotspot core								
<median	243/272	1	1	1	80/80	1	1	1
≥median	190/164	0.78 (0.57,1.07)	0.73 (0.55,0.98)	**0.80 (0.57,1.13)**	166/156	1.05 (0.69,1.59)	0.86 (0.55,1.35)	**0.87 (0.52,1.49)**

[a] Adjusted for year of diagnosis, menopausal status, county of residence, UICC stage, receipt of chemotherapy, type of surgery, age category, receipt of radiotherapy and comorbidity. Line number two includes grade (high number of missing reduced sample size).
[b] Adjusted also for grade.

S9 Table. Association between DIA-Ki-67 score and breast cancer recurrence, by median time to recurrence.

	Time to recurrence (yrs post diagnosis)	Median time to recurrence (yrs)[ζ]	Cases/ controls	Matched OR (95% CI)[a]	Adjusted OR (95% CI)[b]
ER+/Tam+					
	1-5	2.8	329/125	0.75 (0.48,1.17)	0.74 (0.47,1.18)
	6-10	6.7	104/311	0.86 (0.53,1.40)	0.83 (0.50,1.37)
ER-/Tam-					
	1-5	2.1	223/70	0.69 (0.37,1.27)	0.68 (0.34,1.39)
	6-10	6.9	23/166	1.85 (0.68,5.04)	1.40 (0.40,4.90)

[ζ] Median time to recurrence based on recurrent cases.
[a] Estimated using conditional logistic regression with conditioning on the matched factors (diagnosis year, county of residence, menopausal status and UICC stage).
[b] Estimated using logistic regression with additional adjustment for chemotherapy, receipt of radiotherapy, type of surgery, age category at diagnosis and comorbidity. Due to missing values, adjusted analyses were calculated in lower number of cases/controls than the matched analysis (For ER+/Tam+: 2.8 yrs=320/121, 6.6 yrs=100/304, and for ER-/Tam-: 2.1 yrs=210/60, 6.9 yrs=22/136).

S10 Table. Adjusted ORs by chemo - overall median.

	ER^+/Tam^+	ER^-/Tam^-
	Adjusted OR (95%)	Adjusted OR (95%)
No Chemotherapy	0.64 (0.47,0.88)	0.19 (0.04,0.94)
Chemotherapy	1.38 (0.55,3.47)	1.02 (0.62,1.69)

S11 Table. Adjusted ORs by chemo and ER-specific medians.

	ER^+/Tam^+	ER^-/Tam^-
	Adjusted OR (95%)	Adjusted OR (95%)
No Chemotherapy	0.73 (0.54,0.99)	0.75 (0.24,2.31)
Chemotherapy	1.43 (0.52,3.92)	1.40 (0.87,2.26)