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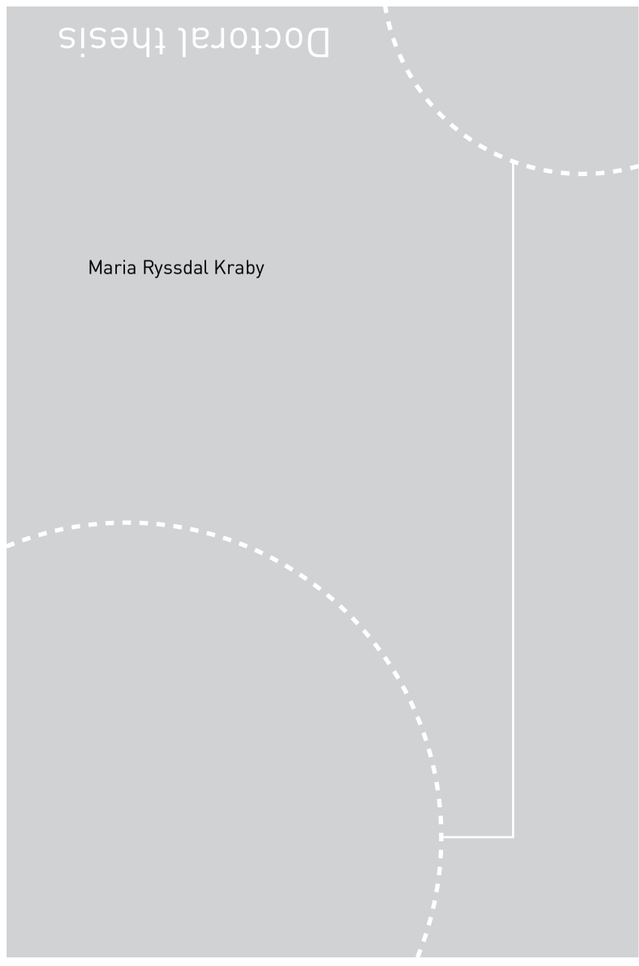
Maria Ryssdal Kraby

Tumour Vasculature in Subtypes of Breast Cancer



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Thesis for the Degree of
Philosophiae Doctor
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Department of Clinical and Molecular Medicine

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Trondheim, February 2019

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Tumorvaskulatur i subtyper av brystkreft

Brystkreft er en heterogen gruppe sykdommer. Ved hjelp av immunhistokjemi (IHK) og *in situ* hybridisering (ISH) kan brystkreftsvulster inndeles i molekylære subtyper med ulik biologi og pasientoverlevelse. Pasienter med subtypen Luminal A har best prognose. Denne subtypen har positivt uttrykk for hormonreseptorer og lite potensial for vekst. Pasienter med nonluminale subtyper har dårligst prognose. Disse svulstene er hormonreseptornegative og deles videre inn i tre grupper (HER2 type, basal fenotype (BF), og fem-negativ fenotype (5NF)) avhengig av deres uttrykk av et sett biomarkører. Ved å studere kreftsvulsten har *Breast Cancer Subtypes*-prosjektet som mål å øke kunnskapen om tumorbiologi i hver subtype, samt å identifisere faktorer som kan bidra til å bestemme pasientens prognose. Denne avhandlingen bygger på tre delarbeider som tar utgangspunkt i 909 trønderske kvinner. Kvinnene ble diagnostisert med brystkreft fra 1961 til 2008 og fulgt fra diagnosetidspunkt til 2010. Brystkreftsvulstene var på forhånd reklassifisert i molekylære subtyper med IHK og ISH.

Tilgang til blodomløpet er en forutsetning for tumorvekst og spredning. Knoppskyting av kapillærer fra eksisterende blodkar (angiogenese) er den mest kjente mekanismen tumorceller har for å få tilgang til blodkar, ved at omliggende kapillærer stimuleres til vekst (proliferasjon) og infiltrasjon i tumorvevet. Mikrokartetthet (MKT) beskriver antall blodkar i det mest karfylte området av tumoren (den vaskulære hotspoten). MKT er den mest brukte metoden for å kvantifisere blodkar i kreftsvulsten, men det er variasjon mellom studier i hvordan MKT måles. Denne avhandlingen undersøkte tre metoder for å beskrive vaskulatur i brystkreftvev; MKT, definert som gjennomsnittlig antall blodkar per kvadratmillimeter i den vaskulære hotspoten; prolifererende MKT (pMKT), definert som gjennomsnittlig antall prolifererende kar målt på samme måte som MKT; og vaskulær proliferasjonsindeks (VPI), definert som ratioen av pMKT til MKT.

I den første studien sammenliknet vi de to mest biologisk ulike subtypene, Luminal A og BF. BF-svulster hadde høyere pMKT og VPI enn Luminal A, men punktestimatet var lavt. Da vi studerte alle pasientene som én gruppe var økt MKT assosiert med dårlig prognose. Inndelt etter subtype var MKT kun en prognostisk faktor i Luminal A. Luminal A-svulstene i denne studien representerer en mer aggressiv gruppe enn Luminal A-svulster generelt, så resultatene må valideres i et mer representativt utvalg.

I den andre studien vurderte vi MKT, pMKT og VPI i de tre nonluminale subtypene. MKT var høyere i 5NF-svulster enn både HER2 type- og BF-svulster. Igjen var høy MKT assosiert med dårlig prognose da alle pasienter ble vurdert samlet. Men da vi studerte hver subtype for seg, hadde MKT kun prognostisk verdi i HER2 type og 5NF. Dette indikerer at MKT er en prognostisk faktor i noen subtyper, men ikke i andre. pMKT og VPI ga ikke informasjon om prognose i noen av studiene.

I den tredje studien studerte vi hvilket synsfeltareal brukt til å måle MKT i den vaskulære hotspoten, som ga den mest nøyaktige informasjonen om pasienters prognose. Alle kasus

fra de to tidligere arbeidene ble inkludert. Større synsfeltareal ga mindre nøyaktig prognostisk informasjon. Den mest nøyaktige informasjonen om prognose kom frem hvis vi kun inkluderte de to synsfeltene som hadde flest blodkar.

Funnene fra disse studiene bidrar med ny informasjon om tumorbiologi i de individuelle subtypene av brystkreft. De gir også informasjon om prognose i brystkreft, og resultatene indikerer at hver enkelt subtype kan ha forskjellige prognostiske faktorer. Ved å demonstrere at MKT ga mer nøyaktig prognostisk informasjon da vi brukte et mindre synsfeltareal med kun de mest karrike områdene, kan våre resultater bidra til en mer effektiv og standardisert metode for beregning av MKT i brystkreft.

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Hovedveileder: Anna M. Bofin

Biveiledere: Signe Opdahl, Lars A. Akslen, Hege G. Russnes

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i auditorium LA21, Laboratoriesenteret

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LIST OF PAPERS

This thesis is based on the following three papers:

Paper I:

Kraby MR, Krüger K, Opdahl S, Vatten LJ, Akslen LA, Bofin AM. Microvascular proliferation in luminal A and basal-like breast cancer subtypes. Journal of Clinical Pathology. 2015;68(11):891-7. doi: 10.1136/jclinpath-2015-203037. Epub 2015 Jul 14

Paper II:

Kraby MR, Opdahl S, Akslen LA, Bofin AM. Quantifying tumour vascularity in non-luminal breast cancer subtypes. Journal of Clinical Pathology. 2017;70(9): 766-774. Doi: 10.1136/jclinpath-2016-204208. Epub 2017 March 1

Paper III:

Kraby MR, Opdahl S, Russnes HG, Bofin AM. Microvessel density in breast cancer – The impact of field area on prognostic informativeness. *Manuscript submitted.*

ABBREVIATIONS

American Society of Clinical Oncology (ASCO)

Basal Phenotype (BP)

Breast Cancer Subtypes project (BCS)

Cancer-associated fibroblasts (CAF)

Cancer stem cells (CSC)

Centre for Cancer Biomarkers (CCBIO)

Cluster of differentiation 31 (CD31)

Cluster of differentiation 34 (CD34)

Cluster of differentiation 105 (CD105)

College of American Pathologists (CAP)

Confidence interval (CI)

Cumulative incidence curve (CIC)

Cytokeratin 5 (CK5)

Cytokeratin 17 (CK17)

Endothelial area (EA)

Epidermal growth factor receptor (EGFR)

Epithelial-mesenchymal transition (EMT)

Extracellular matrix (ECM)

Factor VIII procoagulant protein (VIII:C)

Fragment antigen binding (Fab)

Fragment, crystallisable (Fc)

Fibroblast growth factor (FGF)

Five-negative phenotype (5NP)

Formalin-fixed, paraffin-embedded (FFPE)

Hazard ratio (HR)

Haematoxylin-erythrosin-saffron (HES)

Human epidermal growth factor receptor 2 (HER2)

Immunohistochemistry (IHC)

In situ hybridisation (ISH)

Likelihood ratio (LR)

Matrix metalloproteinase-9 (MMP-9)

Microvessel density (MVD)

No special type (NST)

Oestrogen receptor (ER)

Prediction analysis microarray (PAM50)

Progesterone receptor (PR)

Proliferating microvessel density (pMVD)

Risk of relapse (ROR)

Tissue microarray (TMA)

Triple-negative (TN)

Tumour, node, metastasis (TNM)

Vascular proliferation index (VPI)

Vasculogenic mimicry (VM)

Vascular endothelial growth factor (VEGF)

Von Willebrand factor (vWF)

World Health Organization (WHO)

SUMMARY

Breast cancer is a group of heterogeneous diseases. Using immunohistochemistry (IHC) and *in situ* hybridisation (ISH), they can be separated into molecular subtypes with distinct biology and prognosis. The Luminal A subtype has the best prognosis. It is positive for hormone receptors and has little potential for growth and proliferation. Patients with the hormone receptor negative subtypes, the non-luminal, have the poorest prognosis. Non-luminal subtypes are further classified into three groups, depending on their expression of a set of biomarkers (HER2 type, basal phenotype (BP), five-negative phenotype (5NP)). By studying the breast tumour, the Breast Cancer Subtypes (BCS) project aims to increase our understanding of the tumour biology, and to identify prognostic factors, in each molecular subtype. This dissertation is a result of three papers (two are published, one is presented as a manuscript). The work is based on a series of 909 women from Trøndelag county, diagnosed with breast from 1961 to 2008, and followed from the time of diagnosis to 2010. Tumours from these women were previously reclassified into molecular subtypes with IHC and ISH.

Access to the vascular network is a prerequisite for tumour growth and metastasis. Angiogenesis, the sprouting of capillaries from existing vessels, is the most well-known mechanism for tumour cells to gain access to blood vessels, where nearby capillaries are stimulated to proliferation and infiltration into the tumour tissue. Microvessel density (MVD) describes the number of vessels in the most vessel-rich tumour region, called the vascular hotspot. MVD is the most commonly used method for vasculature quantification in tumour tissue. However, there are great methodological variations in MVD assessment between studies. This thesis studied three methods for quantifying the vasculature in breast cancer tumour tissue; MVD, described as the average number of vessels/mm^2 in the vascular hotspot; proliferating MVD (pMVD), which is the average number of proliferating vessels measured in the same way as MVD; and vascular proliferation index (VPI) is the ratio of pMVD to MVD.

In the first study, we compared BP and Luminal A, which are the most biologically distinct subtypes. BP tumours had higher pMVD and VPI than Luminal A, but the point estimate was low. Increased MVD was associated with poor prognosis in all cases combined. However, when separated according to subtype, MVD was a prognostic factor only in the Luminal A.

The Luminal A tumours in this study represent a more aggressive subset than Luminal A tumours as a whole, so these results should be validated in a more representative selection of Luminal A tumours.

In the second study, we considered MVD, pMVD and VPI in all the non-luminal subtypes. MVD was higher in 5NP tumours compared to both HER2 type and BP. Increasing MVD was associated with poor prognosis in all cases combined, but in analyses of each subtype separately, MVD only provided prognostic information in HER2 type and 5NP. This could imply that MVD is a prognostic factor in some subtypes, but not in others. pMVD and VPI were not associated with prognosis in either of the studies. Our results should be confirmed in a larger sample size.

In the third study, we investigated which field area used for MVD assessment that provided the most accurate prognostic information. All cases from the two previous studies were included. Prognostic information became less accurate when a larger field area was included, and the most accurate prognostic information was obtained by including the two visual field with the highest number of vessels only.

The results of these studies provide novel information about tumour biology for individual subtypes of breast cancer. They also provide information about prognostic factors in breast cancer, and imply that each subtype may have a different set of prognostic factors. By demonstrating that MVD provided the most accurate prognostic information when using a smaller field area with only the most vessel-dense visual fields, our results could contribute to a more efficient and standardised method for assessment of MVD in breast cancer.

1 INTRODUCTION

As the most common cancer in women worldwide causing over 600,000 deaths every year, breast cancer is responsible for a great part of the worldwide disease burden. Breast cancer is not one, but several diseases with diverse biology and patient outcomes. In addition to classification into histopathological type, grade and stage, breast cancers can be separated into molecular subtypes with differing biological traits, prognosis, and response to treatment. However, even within individual subtypes, prognosis varies. One-size-fits-all therapy regimens are no longer sufficient. With improved survival come new perspectives, such as overtreatment and long-term side effects in the increasing prevalence of breast cancer survival. Thus, for each subtype, there is a need for more information about its biological traits, and a need to identify additional prognostic markers.

Tumour cells need access to vasculature for growth and metastasis. There are several mechanisms by which tumour cells gain access to a blood supply, but the most well-known is when nearby blood vessels are stimulated to angiogenesis, where capillaries sprout from existing vessels. Microvessel density (MVD) is a frequently used method for quantifying tumour vasculature, and is assessed by counting microvessels in the most vascularised tumour region. Its prognostic informativeness remains controversial, and some of the discrepancies may be explained by lack of consensus in methodology. If MVD were to be assessed in a diagnostic setting, a more efficient scoring method would need to be developed. Today, there is no consensus regarding the optimal field area size that should be used in MVD assessment. Moreover, since MVD includes all tumour microvessels, it is unsuited for quantifying ongoing tumour angiogenesis. It has been suggested that vascular proliferation, which describes the proportion of proliferating vessels in a tumour, could be used to assess true angiogenesis.

This thesis is a study of MVD and vascular proliferation in several molecular subtypes of breast cancer. The aims were to describe the vascular biology of each subtype, and to assess whether these methods for measuring vasculature provide prognostic information.

2 BACKGROUND

2.1 Breast cancer

2.1.1 Breast morphology

The breast comprises 15-25 secretory lobes, which are independent glands surrounded by collagenous septa and adipose tissue (1, 2). Each lobe consists of lobules, which are separated by a collagenous connective tissue (1). Within the lobules are alveolar glands, surrounded by specialised, hormone sensitive and vascularised connective tissue (2). The functional unit of the breast is the terminal duct lobular unit (TDLU) (Figure 1). Alveolar glands secrete into ductules, which in turn lead to the terminal duct (2). Terminal ducts from one lobe unite into a larger lactiferous duct that is surrounded by circular and longitudinal smooth muscle cells (2, 3). The lactiferous ducts collect into lactiferous sinuses in the nipple, which are located right before the opening onto the surface (1).

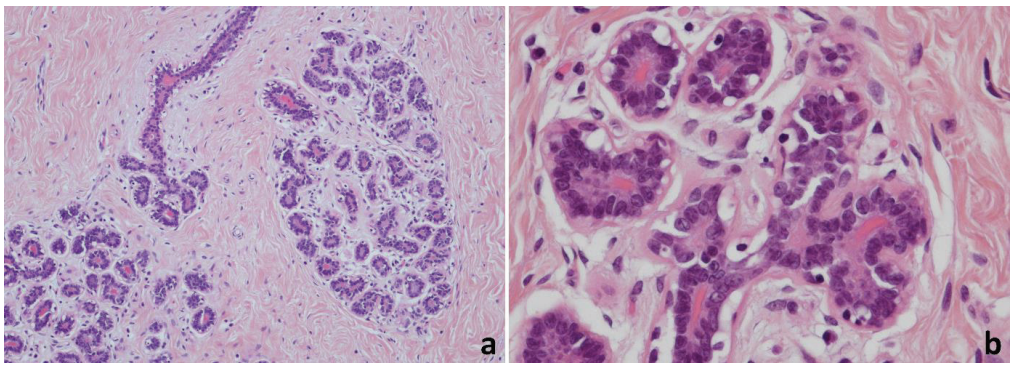

Figure 1. Normal, inactive breast tissue stained with haematoxylin-erythrosin-saffron. a: Terminal duct lobular unit (100x). b: Alveolar glands of one lobule. Epithelial cells (ductal and myoepithelial), and proteinaceous material in the lumen (400x). Photo: AM Bofin, NTNU.

There are two main cell layers in the epithelium of ducts and lobules (2), in addition to mammary stem cells and progenitor cells (4).The epithelial cells lining the lumen have columnar or cuboidal shape, and are responsible for secretion of milk in the lobules during lactation (2). Myoepithelial cells are flat and spindle-shaped, located between the epithelial cells and the basement membrane, and have the ability to contract to stimulate milk flow (1, 2). In the surrounding stroma, there are immune cells and fibroblasts, fibres of elastin and collagen, nerves and vasculature. In a normal breast, small capillaries encircle the ducts, while lobules are surrounded by fewer and larger vessels (2).

There are several individual variations in breast blood supply and drainage. The breast receives blood from the axillary, intercostal and internal thoracic arteries. The latter is usually the greatest contributor, and is also called the internal mammary artery. Venous drainage usually follows arterial distribution and is therefore mostly to the internal thoracic vein. However, some blood is also drained to axillary, intercostal and superficial neck veins. Lymphatic drainage of the breast has three dominant routes. The largest to the axilla and accounts for at least 75% of all drainage. Interstitial lymphatic regions receive less than 25%, and the final route leads to posterior intercostal lymph nodes. Additionally, some drainage passes through lymph nodes located in the supra- and infraclavicular region and in the breast tissue. Both blood and lymph vessels are potential routes for metastasis in breast cancer (2).

2.1.2 Breast cancer incidence and mortality

Breast cancer is the second most common cancer in the world, and the most common cancer among women in both developed and developing countries. With an incidence of 2.09 million new cases in 2018, breast cancer accounts for 25% of all cancer in women (5). In addition, it is likely that there is a considerable number of unrecorded cases in developing countries. More than 600,000 people died from the disease in 2018, making breast cancer the most common cause of cancer death in women worldwide (5). The St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer from 2017 emphasized the importance of developing efficient and less expensive treatment strategies for breast cancer (6).

In Norway, one in twelve women will develop breast cancer before turning 75 years of age. The Cancer Registry of Norway reported a 7% increase in breast cancer incidence rates between 2012 and 2016, compared to the previous 5-year period (Figure 2). The 5-year breast cancer survival rose from 88.6% in the previous 5-year period to 89.7% between 2012 and 2016. However breast cancer is still the second most frequent cause of cancer death in Norwegian women after lung cancer, with a 15-year survival of 76.6% (7).

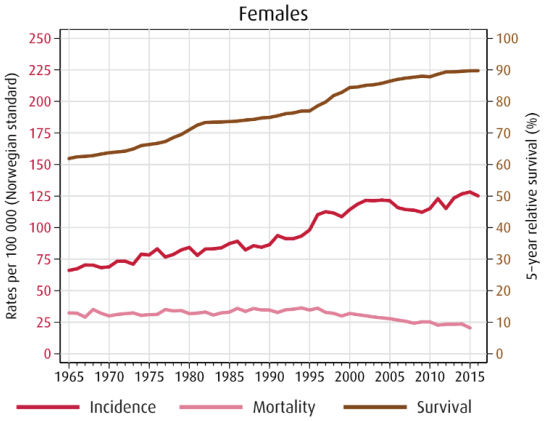

Figure 2. Trends in incidence, rates for mortality and proportions for 5-year relative survival. Reprinted from the Cancer Registry of Norway, *Cancer in Norway 2016 – Cancer incidence, mortality, survival and prevalence in Norway* (7), page no. 99, Figure 9.1-M, with permission from the Cancer Registry of Norway.

With increasing incidence and improving survival comes increasing prevalence. By the end of 2016, 45,725 breast cancer patients were still alive. Of these, 20,412 had been diagnosed with breast cancer more than ten years ago. This, and the fact that women are diagnosed at an earlier age (7), changes the perspective in breast cancer treatment. In addition to survival, other factors need to be taken into account, such as long-term side effects and quality of life. It is no longer sufficient to add years to a patients' life, but also life to the patients' years.

2.1.3. Prognostic and predictive markers

A biomarker is a feature that can be objectively measured in a biological sample, and that provides information about a normal or pathological biologic process, a clinical outcome, or about response to intervention (8). There is a distinction between a prognostic and a predictive biomarker. Prognostic biomarkers provide information on the clinical outcome in patients, regardless of the treatment they receive. Predictive biomarkers communicate how a disease can be expected to respond to a specific treatment, thereby potentially changing the prognosis (9). For example, since patients with oestrogen receptor (ER) positive breast cancers have a better prognosis than ER-negative, ER is a prognostic marker (10). Furthermore, since ER^+ breast cancers respond to tamoxifen and aromatase inhibitors, and ER^- breast cancers do not, ER is a predictive marker as well (11). However, biomarkers can also be solely predictive or prognostic.

2.1.4. Current guidelines in breast cancer diagnostics and treatment in Norway

2.1.4.1. The mammography screening programme

All Norwegian women between the age of 50 and 69 years are invited to breast mammography screening every second year. The aim is to discover breast cancers at an earlier point during disease progression thereby reducing breast cancer mortality (12).

2.1.4.2 Triple diagnostics

Triple diagnostics is offered to patients with symptoms suspicious of breast cancer, such as a lump in the breast or axilla, skin changes, inverted nipple or secretion from the nipple, or as further investigation of a pathological finding at mammography screening. Triple diagnostics comprises imaging, a clinical examination and core biopsy and/or fine-needle aspiration cytology. The aim is to establish the diagnosis of a tumour before surgery, thus enabling the tailoring of surgical treatment accordingly (12).

2.1.4.3 Pathology diagnostics after an invasive malignancy has been established

After extirpation, tumours undergo thorough examination in a department of pathology. The pathology report describes the type of specimen, the main diagnosis, histopathological type, and whether tumour cells invade vessels, skin, papilla, muscles or nerves. It reports tumour size, and whether the resected specimen has a tumour-free surface. The histopathological grade and the number of mitoses per mm^2 are given, along with the expression status of the four biomarkers ER, progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and the proliferation marker Ki67. The report also includes information about lymph node status; sentinel lymph node; total number of lymph nodes; number of positive lymph nodes, and also reports extranodal tumour tissue (12).

2.1.4.3.1 Histopathological type

The histopathological type is determined by microscopic examination of haematoxylin-erythrosin-saffron (HES) stained tissue sections, and may historically be regarded as the oldest expression of the degree of tumour differentiation and tumour morphology. Histopathological typing is carried out according to the World Health Organization (WHO) classification of tumours of the breast (13). The most common type is invasive carcinoma of no special type (NST), which is a heterogeneous group of cancers that do not fill the necessary characteristics to be classified into special subtypes (14). The second most common type is invasive lobular carcinoma, which accounts for approximately 5-15% of

breast carcinomas. Other, less common types include tubular, cribriform, medullary, metaplastic and mucinous carcinomas (Figure 3) (15, 16). Although the histopathological type can provide some additional prognostic information (16, 17), it does not influence the choice of therapy for Norwegian patients today (12).

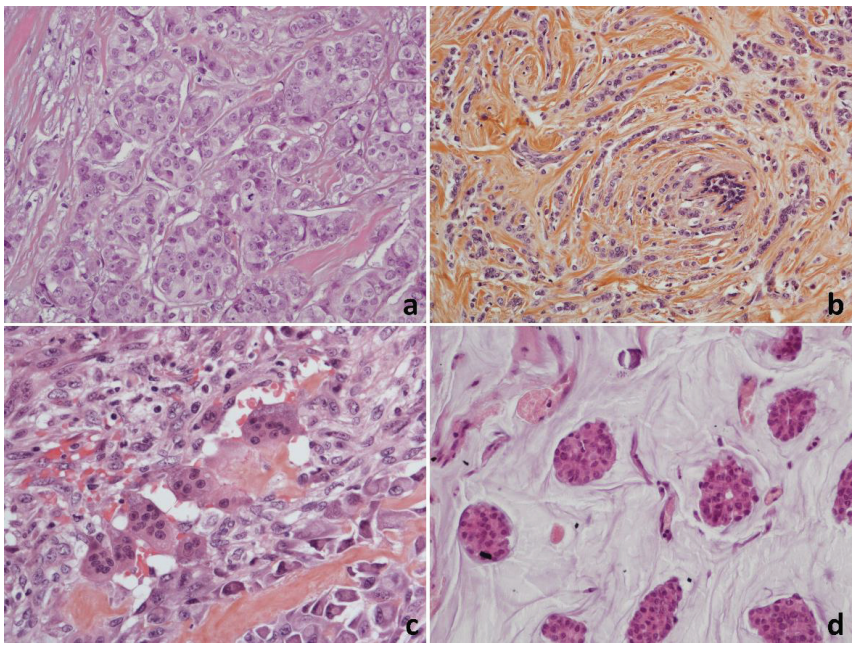

Figure 3. Histopathological types in breast cancer. Staining with haematoxylin-erythrosin-saffron. a: Invasive carcinoma of no special type (200x). b: Lobular carcinoma (200x). c: Metaplastic carcinoma (400x). d: Mucinous carcinoma (200x). Photo: AM Bofin, NTNU.

2.1.4.3.2 Histopathological grade

Histopathological grade reflects the degree of differentiation in a tumour, and is determined microscopically on HES sections. Both guidelines from Norway (12) and the Royal College of Pathology (18) recommend the Nottingham Grading System described by Elston and Ellis (19). The method was developed in 1991, and is a modification of the Bloom & Richardson method for breast cancer grading (20). The method is semi-quantitative, and three morphological features are scored 1-3 (Figure 4): 1) Tubule formation is assessed by studying the overall tumour tissue (18), and describes the 'degree of structural differentiation' (20), by expressing the proportion of tumour cells that form gland-like structures with clear lumina and polarised tumour cells as <10%, 10-75%, or>75% (18, 19). 2) Nuclear pleomorphism is assessed at higher magnification, either at the tumour edge or the least differentiated region of the tumour (18), by comparing the appearance of tumour nuclei to

that of adjacent, normal epithelial cell nuclei. The greater the variation in in chromatin pattern, nuclear size and shape; the higher the score. 3) The number of mitoses is counted in ten consecutive fields of the tumour periphery at high magnification (250x-400x) and describes the velocity of tumour cell growth. The sum of scores from each feature determines the histopathological grade. Grade 1 tumours are well-differentiated and have a score of 3-5 (Figure 5). Grade 2 tumours score 6-7 and are moderately differentiated. Poorly differentiated tumours are grade 3 and score 8-9 (19). Histopathological grading has proved to be a powerful prognostic factor in breast cancer (19-23).

Table 1. Summary of semiquantitative method for assessing histological grade in breast carcinoma

Feature	Score
Tubule formation	
Majority of tumour (>75%)	1
Moderate degree (10–75%)	2
Little or none (<10%)	3
Nuclear pleomorphism	
Small, regular uniform cells	1
Moderate increase in size and variability	2
Marked variation	3
Mitotic counts	
Dependent on microscope field area (see Table 2)	1–3

Figure 4. Method for assessing histopathological grade in breast carcinoma. Reprinted from Histopathology, 2002 (reprint version), Vol 41, Elston and Ellis, *Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up* (19), page no. 155, Table 1, with permission from John Wiley and sons (licence no. 4421370457191).

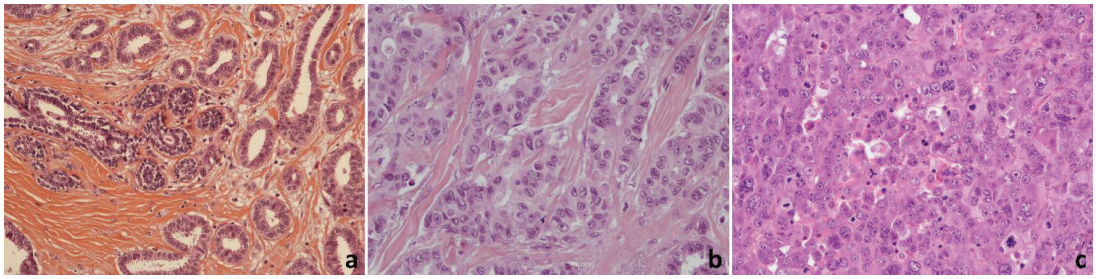

Figure 5. Histopathological grade in breast cancer. Staining with haematoxylin-erythrosin-saffron (200x)**.** a: Grade 1, tubular carcinoma. b: Grade 2 carcinoma, NST. c: Grade 3 carcinoma, NST. Photo: AM Bofin, NTNU.

2.1.4.3.3 Molecular biomarkers

In breast cancer, the four molecular biomarkers ER, PR, HER2 and Ki67 are used determine patient prognosis and predict treatment response (6, 12).

2.1.4.3.3.1 Hormone receptors

ER and PR are nuclear hormone receptors located in normal breast epithelium. ER is expressed in approximately 7% of nuclei in normal breast epithelial cells, while PR is expressed in 19% (24, 25). They are prognostic factors in breast cancer, and can be used to predict which patients that will respond to endocrine therapies such as tamoxifen and aromatase inhibitors (10, 11). ER/PR expression can be visualised using immunohistochemistry (IHC) (Figure 6), a process which is described in detail on page 37. A tumour is considered ER^+ if ≥1% of tumour nuclei display positive staining. For PR, the cut-off for positive staining is ≥1% in international guidelines (6, 10), and ≥10% in Norwegian guidelines (12). Response to treatment correlates with the degree of hormone receptor expression in the tumour (26).

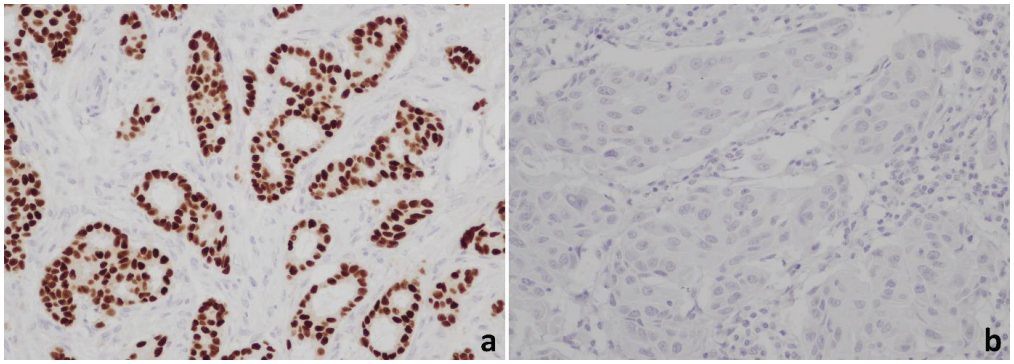

Figure 6. Immunohistochemical staining for oestrogen receptor (ER), counterstained with haematoxylin (200x). a: ER^+ staining in the nucleus of ≥1% of tumour cells. b: ER^+ staining in <1% of tumour cells. Photo: AM Bofin, NTNU.

2.1.4.3.3.2 HER2

The gene *HER2* is located on chromosome 17q12 (27). It encodes the transmembrane growth factor receptor called HER2 or Erb-B2, which participates in the regulation of normal cell growth and differentiation (28). Ten to 20% of breast tumours are amplified for HER2 and/or overexpress the HER2 receptor (29, 30), and this is associated with poorer prognosis in breast cancer (31). HER2 overexpression can be caused by amplification of the *HER2* gene, or dysregulation on transcriptional and post-transcriptional levels. The first has been reported in 92% of HER2 overexpressing tumours (28). HER2 status may be assessed by both

IHC and *in situ* hybridisation (ISH). A tumour is HER2 positive if more than 10% of the cancer cells display strong, continuous, circumferential membrane staining with IHC and/or increased copy number of *HER2* by ISH (Figure 7) (29). Therapies that target the HER2 protein, such as trastuzumab, are used in the treatment of patients with $HER2^+$ breast cancer (6), and has improved patient survival in this group (32).

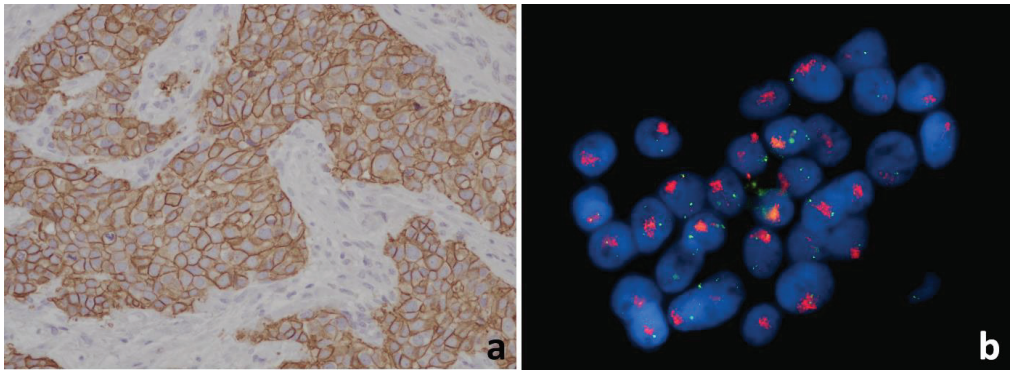

Figure 7. Expression of the human epidermal growth factor receptor 2 (HER2). a: Immunohistochemical staining displays strong, circumferential membrane staining for HER2, counterstained with haematoxylin (200x). b: In situ hybridisation displays increased copy number of the *HER2* gene (red). Chromosome 17 is green. Tumour cell nuclei are blue (DAPI) (600x). Photo: AM Bofin, NTNU.

2.1.4.3.3.3 Proliferative fraction: Ki67

Ki67 is a marker of proliferation, and is positively expressed in nuclei during all phases of the cell cycle, except G0 (33). Its reliability score for IHC in the Human Protein Atlas is classified as enhanced (34-36). In breast cancer, the Ki67 score is the percentage of tumour cells with Ki67 positively stained nuclei. A high Ki67 score is associated with aggressive tumour behaviour and poorer prognosis (37). Some found that Ki67 can be used to predict response to chemotherapy, but its predictive role is controversial (38). The Ki67 score is the most commonly used measure of proliferative fraction in breast cancer (6). However, because of the great variability between observers and institutions in Ki67 scoring, there is controversy about its clinical usefulness. The American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) guidelines do not recommend the use of Ki67 for predictive purposes (39). Nevertheless, there have been promising results regarding a common methodological standardisation through prior training on centrally stained TMA slides (40). The most recent guidelines from the St Gallen consensus acknowledged inter-laboratory discrepancies and encouraged a common scoring calibration. For now, patients with tumours that have a clearly low Ki67 proliferation index, low grade and ER^+, are not

given chemotherapy (6). According to Norwegian national guidelines, tumour cell proliferation should be measured both as a Ki67 score and as the number of mitoses per mm^2. The Ki67 score is given in percent after at least 500 tumour cells in a hotspot have been counted at 400x magnification. The cut-off for high and low Ki67 score should be decided based on the median Ki67 score in local laboratories, since laboratory routines may influence the Ki67 value. A tumour with a clearly high Ki67 score is more likely to respond to intensive chemotherapy regimens (12).

2.1.4.3.4 Staging

Breast cancers are staged according to the TNM Classification of Malignant Tumours (41), where three modalities are used for stage determination: primary tumour size and extent (T), regional lymph node involvement (N) and distant metastasis status (M). Breast cancer staging is assessed both clinically and pathologically. The clinical stage is based on physical examination and imaging. The pathological stage for T (pT) and N (pN) is assessed by gross and microscopic examination. Tumour size, lymph node status and presence of distant metastasis are important for determining treatment and predicting prognosis (26, 41).

2.1.4.4 Treatment options according to Norwegian guidelines

2.1.4.4.1 Surgery

Surgery is the pillar of breast cancer treatment. Patients either undergo a mastectomy or breast conserving therapy, and sentinel node examination is performed for assessment of nodal status. Axillary dissection is considered in cases of sentinel lymph node positivity. Patients with locally advanced breast cancer receive neoadjuvant therapy prior to reassessment of operability (12).

2.1.4.4.2 Radiation

Postoperative radiation is given to patients after breast conserving therapy; when it is uncertain if tumour removal had free margins; when primary tumours are large or locally advanced; and when patients have lymph node positive disease. The aim is to reduce the risk of locoregional disease recurrence (12).

2.1.4.4.3 Systemic adjuvant therapy

Systemic adjuvant therapy is offered to patients with established lymph node metastasis, primary tumour size >2 cm, grade 2-3, $HER2^+$ and/or hormone receptor negativity with increased proliferation status. Patients with ER^+/PR^+ tumours receive endocrine therapy anti-oestrogens (tamoxifen or aromatase inhibitors) for at least five years. Patients with $HER2^+$

positive disease receive trastuzumab in combination with taxans. Epirubicin, cyclophosphamide and taxans are all chemotherapies used in primary breast cancer treatment. Postmenopausal women treated with systemic adjuvant therapy also receive bisphosphonates (12).

2.1.5 Molecular subtypes of breast cancer

In 2000, Perou et al published *Molecular portraits of human breast tumours*, a study of the variation in gene expression patterns between different human breast tumours. The amount of mRNA in a cell provides information about which genes are expressed at that given time. With complementary DNA microarray technology, they studied the expression of a large number of genes by measuring their mRNA levels. They identified an 'intrinsic' gene subset, which comprises genes that vary little within the same individual over time, but has great variation between individuals. Through hierarchical clustering, breast tumours were classified into four main 'intrinsic' subgroups (Figure 8). The main distinction was between ER^+ and ER^- tumours. The ER^+ group overexpressed the same genes as luminal epithelial cells, and was therefore called luminal-like. In the ER^- tumours, ER and other genes commonly co-expressed with ER were down-regulated. One group of tumours had a basal epithelial cell-like gene expression pattern, positive IHC staining for cytokeratin 5/6 and 17, and was called the basal-like subtype. Another group overexpressing the *HER2* gene was called Erb-$B2^+$ (42), and later the HER2-enriched subtype (43). The last group had a 'normal breast'-like expression pattern, with high levels of genes characteristic of basal epithelial cells and adipose cells (42). The 'intrinsic' subtype classification appeared robust in subsequent validation studies, with each subtype acting as a biologically distinct disease entity (43-46), and the largest biological difference remaining between tumours that overexpressed luminal epithelial specific genes and those who did not (45, 47). The luminal-like subtype was further subdivided into Luminal A and Luminal B (44, 45), while the normal breast-like subtype was suggested to be an artefact due to contamination from normal breast tissue in the tumour sample (43). An additional subtype called the Claudin-low was also suggested (48).

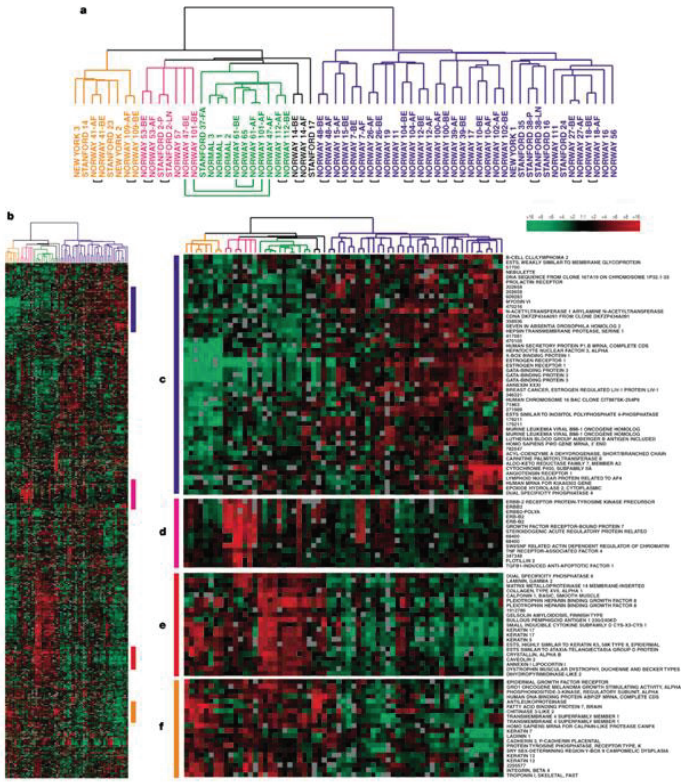

Figure 8. Cluster analysis based on the 'intrinsic' subset of 496 genes. Each column represents a breast tumour and each row represents a gene. An up-regulated gene has red colour, a down-regulated gene has green colour and the black colour means unchanged gene expression. The grey colour denotes missing data. The name of the gene is on the right side of the figure, and the colour code and letter at the left side of the figure explains which cluster the gene belongs to. The luminal-like/ER gene cluster has purple colour and the letter "c". The *HER2* gene cluster is pink and lettered "d". Both red, "e", and orange, "f", are basal-like clusters. Tumours with similar gene expression patterns are clustered together as shown in the dendrogram at the top of the figure. The group's colour describes which gene cluster each group is overexpressing. Reprinted by permission from Macmillan Publishers Ltd (licence no. 4421371112760): Nature, 2000, Vol 406, Perou et al, *Molecular portraits of human breast tumours* (42), page no. 750, Figure 3.

There are clear differences in survival between the 'intrinsic' subtypes. The Luminal A subtype has the longest survival, while the shortest is found in the HER2-enriched and basal-like subtypes (43-46, 48). However, the HER2-enriched subtype has a more favourable prognosis among patients treated with trastuzumab (49). The Luminal B subtype has an intermediate prognosis that is markedly poorer than that of the Luminal A subtype (44-46, 48, 50). Furthermore, some report that Luminal B has higher relapse-free survival than the basal-like subtype the first five years after diagnosis, but after that time point, survival is poorer for Luminal B (43).

Luminal A tumours have high expression of luminal-specific genes. p53 mutations are rare (44), and the *HER2* gene is down-regulated (44, 45). The ER signalling pathway, EGF pathway and IL6 pathway are up-regulated, and the most dominant biological processes are fatty acid metabolism and steroid hormone-mediated signalling (47). Response to chemotherapy is poor (43). Luminal B tumours have lower expression of luminal-specific genes (44), and approximately 30% overexpress the *HER2* gene (45). While Luminal A tumours are mostly grade 1 and 2, Luminal B tumours have a greater proportion of grade 3 tumours. Furthermore, the Luminal B subtype overexpresses a cluster of proliferation genes that is down-regulated in Luminal A (46).

Tumours in the *HER2*-enriched subtype overexpress the *HER2* gene, growth factor receptor-bound protein 7 and other genes in the *HER2* amplicon on chromosome 17. Mutations of p53 have been reported in 71% (5/7) (44), and a large proportion of the tumours are grade 3 (46, 48).

The Basal-like subtype is characterised by its high expression of proliferation genes and 'basal cluster genes', such as basal cytokeratins (CK5 and CK17), laminin and c-KIT (42, 44, 48, 51). They also have high expression of the epidermal growth factor receptor (EGFR) (51). Basal-like tumours do not overexpress *HER2* (44, 51), and a large proportion carries *p53* mutations (44). Women with BRCA1 mutations frequently have basal-like breast tumours (45). The tumours have several chromosomal rearrangements and genomic instability (52). The main biological processes involve many 'hallmark' genes for processes such as cell cycle and cell cycle control, and cell proliferation and differentiation (47, 53). Basal-like tumours are mostly triple-negative (TN), which means that they are negative for ER, PR and HER2, and grade 3 (46, 48). As a highly proliferative subtype, the basal-like is sensitive to chemotherapy (48, 52).

Subsequent gene expression analysis studies have demonstrated that breast cancers can be clustered into molecular subtypes in several ways (52, 54). Curtis et al identified ten integrative clusters with differing prognosis. Some clusters comprised tumours from various intrinsic subtypes, such as one cluster of ER^+, luminal tumours whose patients had the second poorest prognosis of all groups (54). Another study identified six molecular subtypes that might benefit from different therapy regimens within the TN breast cancers. Among these were the immunomedullary subtype, which had a favourable prognosis, and the

luminal androgen receptor subtype, which was less sensitive to chemotherapy and had poorer relapse-free survival (52).

There are now several multiparameter gene expression assays using 'gene signatures' to classify breast tumours into various molecular subtypes with different prognosis. Examples of such arrays are the 50-gene prediction analysis microarray (PAM50) (43), which classifies breast tumours into the original intrinsic subtypes and calculates a score for risk of recurrence (ROR) for the first ten years after diagnosis (43, 50), the MammaPrint® 70 gene prognostic signature (55), and the 21-gene assay Onco*type* DX® (56).Some of these arrays can be used to determine patient prognosis (43, 50, 55, 56), or to predict response to chemotherapy (43, 56). Clinical guidelines on diagnostics and treatment by the ASCO (39) and the St Gallen International Expert Consensus Conference (6) endorse the application of some multiparameter gene expression assays in the clinical setting, most of all in the identification of patients with hormone receptor positive, $HER2^-$ and lymph node negative tumours that might benefit from chemotherapy (6, 39).

2.1.6 Molecular subtypes defined by surrogate markers

At the time 'intrinsic' subtypes were identified, the use of gene expression analysis in everyday diagnostics and therapeutics was hindered because of its expense, complexity and the need for fresh-frozen samples. Therefore, since IHC and ISH were more accessible, feasible and affordable, they were exploited as surrogates for gene expression analysis to classify breast cancer into molecular subtypes (51, 57).

Since the greatest difference between the 'intrinsic' subtypes is the expression of luminal epithelial specific genes (42, 44, 47), IHC expression of ER and PR could be used to distinguish tumours into luminal (ER^+ and/or PR^+), and non-luminal (ER^- and PR^-) (31, 57). To distinguish Luminal A and B tumours, two features may be considered (57). Firstly, since *HER2* is down-regulated in Luminal A and up-regulated in some Luminal B tumours (45), all HER2-positive, luminal tumours were classified as Luminal B ($HER2^+$) (57). Secondly, proliferation genes are up-regulated in the Luminal B subtype, but not in the Luminal A (46). Therefore, the Ki67 score was used to distinguish the remaining Luminal B ($HER2^-$) tumours. Cheang et al identified the optimal Ki67 score cut-off for distinguishing Luminal A and B tumours as 13.25%. With this classification, the Luminal B subtype had a higher proportion of patients with younger age at diagnosis, and tumours with larger size and higher

histopathological grade than the Luminal A subtype. Compared to patients with the Luminal A tumours, patients with both $HER2^+$ and $HER2^-$ Luminal B tumours had a 1.5 times higher risk of relapse and breast cancer death (57).

The HER2-enriched subtype is non-luminal and overexpresses the *HER2* gene (42). Therefore, tumours with negative IHC staining for ER and PR, that are positive for HER2 with either IHC or ISH, can be classified as the HER2 type (31). With this classification, HER2 type tumours are frequently high-grade, lymph node positive and have a large proportion of p53 mutations (22, 23), which is similar to the HER2 enriched subtype defined by gene expression analysis (44, 46, 48).

The basal-like subtype has high expression of the basal cluster gene cytokeratin 5 (CK5) (42, 44, 45), and many basal-like tumours express the epidermal growth factor receptor (EGFR) (51). Since most basal-like subtypes are TN, some studies use the TN phenotype as an IHC surrogate for the basal-like subtype (58). However, there are potential risks of error when subtype classification is based only on absence of staining, and the TN group comprises several other subtypes in addition to the basal-like (52). Some further classified TN tumours into two groups to increase specificity for basal-like tumours (51). Tumours with positive IHC staining for either CK5 or EGFR were called the Core Basal Phenotype or the Basal Phenotype (BP), and the group negative for all markers was called either the five-negative phenotype (5NP) (30, 31) or unclassified/undetermined (22, 31, 51, 59). BP tumours were more frequently grade 3 (22, 23, 31), and had a large proportion of p53 mutations. Unlike the HER2 type, they were not more likely to be lymph node positive, and it was suggested that the subtype might have a more haematogenous way of metastasis (22, 60). Patients with BP tumours had a poorer prognosis than the whole TN group, and a clinical outcome more similar to patients with the basal-like subtype as defined by gene expression. Further classification of the TN group seemed to identify two subgroups with distinct prognosis (31), and provided more accurate prognostic information (30). While the 5NP has appeared to be a distinct subtype in some studies (23, 30), it is possible that this group comprises several biologically distinct subtypes not identified by the algorithm, or subtypes included in the algorithm that have been wrongly classified due to false negative IHC staining. Based on their expression of six biomarkers, breast carcinomas may be classified into six molecular subtypes as outlined in Figure 9 (23).

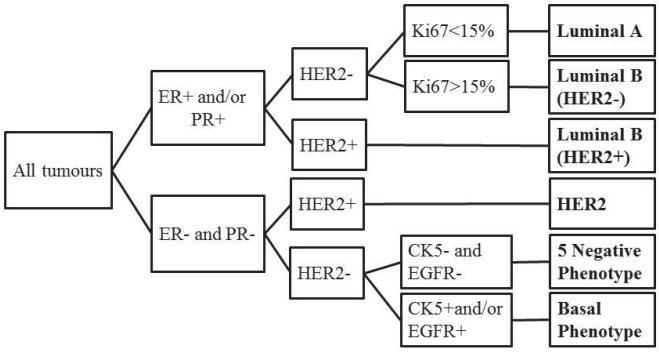

Figure 9. Classification algorithm for molecular subtyping with immunohistochemistry and *in situ* hybridisation. Oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), Cytokeratin 5 (CK5), epidermal growth factor receptor (EGFR). Reprinted from Breast Cancer Res Treat, 2013, Vol 140, Engstrøm et al, *Molecular subtypes, histopathological grade and survival in a historic cohort of breast cancer patients* (23), page 466, Figure 2. Paper distributed under a Creative Commons license, and can be used for non-commercial/educational purposes without obtaining additional permissions from Springer Nature.

With an IHC/ISH surrogate classification, the Luminal A subtype has the best survival (22, 23, 57, 61, 62) and the HER2 subtype has the poorest (22, 23, 30, 31, 61, 62). This is similar to the results from gene expression analyses. Some found the second poorest prognosis to be in the BP (22, 30, 31, 51). Others reported a worse prognosis in the 5NP compared to BP the first five years after diagnosis, even though the 5NP has a higher proportion of grade 2 tumours compared to BP (23). Most patients in the studies above were not treated with trastuzumab. Patients with Luminal B ($HER2^+$) and HER2 type tumours that did receive trastuzumab had a more favourable, intermediate prognosis (49).

Breast cancer mortality appears to follow different time-dependent patterns for the molecular subtypes (22, 23, 30, 58). Patients with Luminal $HER2^-$ tumours have an approximately constant mortality rate over time (23, 30). In contrast, the mortality rate for the other subgroups is initially high, peaks within five years after diagnosis, and then decreases rapidly (22, 30). While there are few deaths in the HER2 type and BP from ten years after diagnosis and onwards (30), it appears that patients with the Luminal A subtype continue to die from breast cancer more than a decade after diagnosis. Engstrøm et al found the time point five years after diagnosis to be of particular interest, since the molecular subtypes only provided prognostic information the first five years after diagnosis. For patients who had survived the first five years, neither grade nor subtype could be used to foresee further prognosis (23). Based on these time-dependent patterns, different subtypes

might require different follow-up regimens; while some may require frequent follow-up the first five years and after that next to none, others may need fewer controls, but for a longer time period.

There are discrepancies between the surrogate methods using IHC/ISH and the multiparameter gene expression assays using 'gene signatures' (43, 48, 50). When PAM50 was used to identify all the 'intrinsic subtypes' in a subset of ER^+ tumours, and 8.1-11% were HER2-enriched (43, 50). Furthermore, 11% of ER^- tumours were classified as Luminal (43). Among tumours classified as HER2 type with IHC, 22% were not HER2-enriched. The group of Luminal B ($HER2^+$) tumours comprised 54% HER2-enriched, 31% Luminal B and 13% Luminal A (63). Although 73-79% of TN tumours were basal-like, all 'intrinsic' subtypes were represented (43, 63). Nevertheless, the IHC/ISH surrogate markers for molecular classification of breast cancer subtypes have demonstrated important prognostic information in patients (23, 31, 51, 57) that persisted across different laboratories (30), and some even found they may perform better than the PAM50 classifier in predicting pathologic complete response (49).

However, the complexity of each tumour is still not sufficiently clarified, and the prognosis of the individual patient cannot be determined. The ultimate goal is to provide personalised therapy that is optimal for every woman’s disease. That is, sufficient to ensure survival without overtreatment, so that patients are spared unnecessary short-term and long-term side effects. However, in order to be able to provide personalised therapy, we need more information about the biology of each molecular subtype, and about how patients can be stratified into more representative and accurate risk profiles. The discovery of other molecular subtypes within current subtypes implies that it may be possible to identify even finer subgroups with differing biological traits and prognosis within the current classification. The molecular subtypes identified through IHC/ISH appear to be distinct subtypes with different prognosis (23, 30, 31, 51, 57), and can be used as the basis for further biological and prognostic studies.

2.2 Tumour biology and hallmarks of cancer

2.2.1 The tumour microenvironment

Although cancer is defined as uncontrolled cell division (64), tumour development is a multistep process of reciprocal interactions between cancer cells and the tumour microenvironment (Figure 10) (65). All cells in the tumour microenvironment impact the development of one another, and are highly plastic with time (53). The most important cells will be described briefly here, except for the endothelial cells and pericytes of the tumour-associated vasculature, which will be described in detail on page 36.

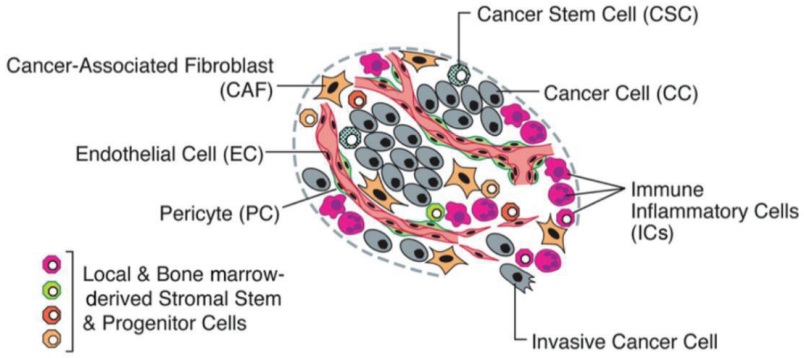

Figure 10: Cells participating in the tumour microenvironment. Reprinted from the Cell, 2011, Vol 144, Hanahan and Weinberg, *Hallmarks of Cancer: The Next Generation* (53), page no. 662, Figure 4 upper, with permission from Elsevier (licence no.4421380177674).

As can be seen at both histological and molecular levels, intratumoural cancer cells can be highly heterogeneous. Different subpopulations of cancer cells can have distinct phenotypes and expression of proteins and genes. In addition to cancer cells, tumours contain cancer stem cells (CSCs), which, compared to regular cancer cells, appear to have an even greater ability to generate novel tumours when transplanted into new surroundings. They stimulate tumour progression, appear to be less sensitive to chemotherapy and may differentiate into fibroblast-like or endothelial cell-like cells if necessary. Cancer-associated fibroblasts (CAFs) stimulate tumour progression, angiogenesis, invasion and dissemination. Among the CAFs are cells that resemble regular fibroblasts that support the structural composition and maintain epithelial cells in normal tissue, and myofibroblasts that express α-smooth muscle actin and participate in wound healing and chronic inflammation. Immune inflammatory cells are present to various degrees in all tumours, and their role is somewhat paradoxical. On the one hand, immune cells are programmed to detect and exterminate potential cancer

cells in the entire organism, and they are thus to be thanked for obstructing numerous cancer diseases. On the other hand, immune cells create inflammation and stimulate wound healing, and therefore have the ability to secrete signals and enzymes needed for growth, matrix degradation, neovascularisation, and so on. Immune cells, such as macrophages, neutrophils, mast cells, T and B lymphocytes, and myeloid progenitors typically gather at the tumour margin and infiltrate the tumour microenvironment, whilst secreting free radicals and extracellular matrix (ECM) enzymes, like matrix metalloproteinase-9 (MMP-9), that enable invasion, metastasis and angiogenesis. They also synthesise signals promoting angiogenesis, cancer cell growth and proliferation. In addition, the tumour microenvironment contains bone marrow-derived stem cells and progenitor cells. These cells enhance both tumour progression and angiogenesis, and they have the potential to differentiate into various cell types. In summary, immune cells, endothelial cells, pericytes, CAFs, progenitors and stem cells can be recruited from both adjacent normal tissue and the bone marrow. Additionally, CSCs can differentiate into various cancer cell phenotypes and stromal cell-like phenotypes if needed (53).

2.2.2 Hallmarks of cancer

The hallmarks of cancer are defined as 'distinctive and complementary capabilities that enable tumour growth and metastatic dissemination' (53). In 2000, Hanahan and Weinberg presented six traits thought to be essential for tumour development in most, if not all, human tumours (Figure 11). The extent of each hallmark, the order in which they present, and whether they presented simultaneously or subsequently, could differ with each individual disease (65). An update on the hallmarks of cancer was published in 2011, where two additional hallmarks and two enabling characteristics were presented (53).

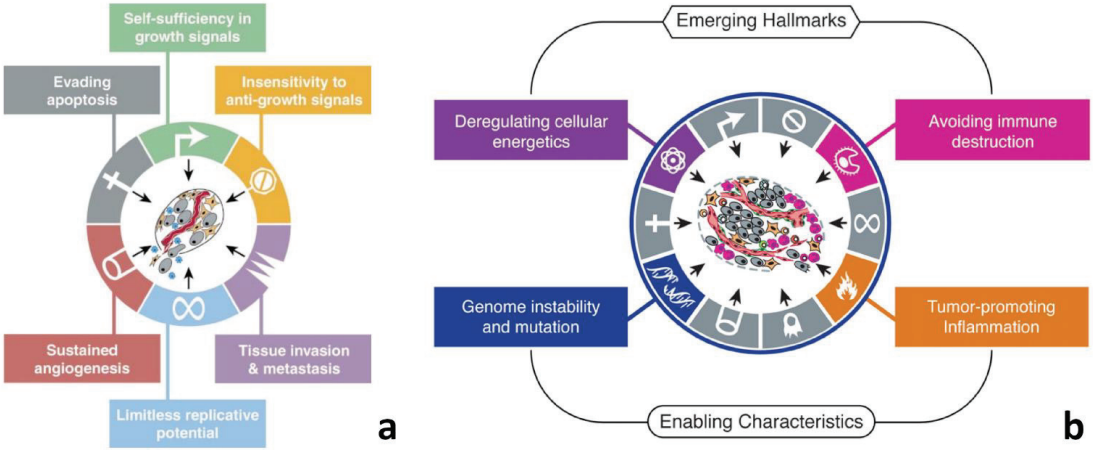

Figure 11: a: The six, original hallmarks of cancer. Reprinted from the Cell, 2000, Vol 100, Hanahan and Weinberg, *Hallmarks of Cancer* (65), page no. 58, Figure 1, with permission from Elsevier (licence no.4421380022305). **b: Emerging Hallmarks and Enabling Characteristics**. Reprinted from the Cell, 2011, Vol 144, Hanahan and Weinberg, *Hallmarks of Cancer: The Next Generation* (53), page no. 658, Figure 3, with permission from Elsevier (licence no. 4421380177674).

The hallmark of self-sufficient growth signalling was later renamed 'sustaining proliferative signalling' (53). Tumour cells can generate growth signals independent of exogenous factors through several mechanisms. Autocrine stimulation happens when tumour cells produce their own growth signals. Overexpression of receptors on the cell surface can make the cell hyperresponsive. Activation signals can be triggered even in the absence of ligand binding through structural modifications of receptors (53, 65), alterations of pathways downstream of the receptor or inactivation of negative-feedback loops. Tumour cells can also stimulate the tumour-associated stroma to paracrine, growth-promoting signalling (53).

In addition to stimulation of proliferation, tumour cells must dodge antigrowth and apoptotic signalling. Mutations or epigenetic alterations can lead to loss of function in tumour suppressor genes such as the antiproliferative Rb protein and the proapoptotic p53 protein (65). Tumour cells must also evade contact inhibition, where cell-to-cell adhesions hinder proliferation. For resisting cell death, tumour cells down-regulate molecules activating controlled mechanisms for cell death; apoptosis and autophagy. In the process of necrosis, however, tumour cells explode and release metabolites and proinflammatory signals into the tumour microenvironment. Therefore, necrosis has a tumour-promoting function through recruitment of immune cells that stimulate tumour cell proliferation, invasion, and angiogenesis (53).

Tumour cells must overcome the obstacle of limited replication in order to obtain uncontrolled mitosis. For each DNA replication, normal cells undergo telomere shortening. This limits the number of times the DNA can be copied and thus the number of mitoses a cell can undergo. Most commonly, tumour cells gain the potential for eternal DNA replication by telomere maintenance (65) through telomerase expression. The telomerase enzyme adds telomere segments to the DNA strand and thereby hinders telomere shortening (53, 65). However, since some degree of telomere shortening fosters genomic instability and tumour-promoting DNA mutations, tumour cells sometimes deliberately delay the upregulation of telomerase (53).

Induction and sustaining of angiogenesis will be described in detail on page 34. The last, original hallmark is the ability of tumour cells to invade tissue and metastasise (65). Epithelial tumour cells undergo the process of epithelial-mesenchymal transition (EMT), where they change phenotype by gaining mesenchymal cell-like traits and increased motility (53). The anchoring to other cells and to the ECM is removed by altered expression of cell-cell adhesion molecules like E-cadherin, and of integrins, which tie them to the ECM (65). Tumour cells secrete matrix-degrading enzymes, which enhances infiltration through stroma, vessels and epithelium (53, 65). The increased motility facilitates invasion and inhibits apoptosis. EMT can be constant or brief, and of varying degrees in different cancer cells. Tumour-associated stroma is an important contributor to invasion and metastasis. For instance, macrophages and other immune cells secrete matrix-degrading enzymes. After dissemination to a new location, tumour cells can reverse the EMT process to enable colonisation (53).

Since the immune system demolishes cancer cells as described above, it has been suggested that the ability of tumour cells to avoid immune cell destruction should be added as a hallmark of cancer. The second additional hallmark describes how cancer cells reprogram their energy metabolism. In normal cells under aerobic conditions, glycolysis transforms glucose to pyruvate, which is metabolised in the mitochondria through the more energy efficient and oxygen consuming citric acid cycle and oxidative phosphorylation. Under anaerobic conditions, glycolysis in preferred. The Warburg effect describes a concept where some cancer cells alter their metabolism to prefer glycolysis also under aerobic conditions. This way, they become less oxygen-dependent. To compensate for glycolysis being a less

energy effective process, tumour cells ensure that a larger amount of substrate is available by increasing the number of glucose transporters on the cell membrane. Oncogene signalling and hypoxic conditions can both stimulate tumour cells to this 'glycolytic fuelling', by increasing the levels of hypoxia-inducible factors 1-α and 2-α. These transcription factors stimulate the expression of more glucose transporters and glycolytic enzymes. Some tumours comprise synergistic cancer cell populations, where one produces lactate through 'glycolytic fuelling', which is used by the other as an energy source in the citric acid cycle (53).

In addition to the hallmarks of cancer, two particular characteristics that enable tumorigenesis have been singled out (53). The first characteristic, namely genome destabilisation and mutation, is present in near all cancer cells as an important contributor to clonal expansion, independent growth signals, resisting cell death and growth suppressors (53, 65). Elimination of DNA maintenance molecules and increased sensitivity to mutagenic agents both increase genomic instability. The second characteristic is the presence of immune cells stimulating tumorigenesis as described above (53).

2.3 Tumour vascularisation

2.3.1 The circulatory system

The circulatory system comprises vessels of the blood vascular system and the lymphatic vascular system. It has several important tasks, including transport of oxygen, carbon dioxide, nutrients, metabolic waste, hormones and cytokines, distribution of immune cells and temperature regulation. In the blood circulation, blood from the heart is pumped into the aorta and then distributed to all regions of the body. With each branching, arteries decrease in size until they become small, terminal branches called arterioles. They transport blood into the microcirculation, where the actual exchange occurs in capillaries and postcapillary venules. The greater proportion of the exchange happens in the capillaries, which form an interconnected, anastomosing web between arterioles and venules. Postcapillary venules lead blood to venules of gradually increasing diameter, which drain into veins of the systemic venous system and return the blood back to the atria of the heart. Lymph, which is redundant extracellular fluid, is transported through the lymphatic system to the blood circulation, and to lymph nodes for immunological screening (66).

Capillaries have a small diameter about as wide as one erythrocyte. They have a very thin wall, which only comprises a thin basement membrane and endothelium lining the lumen. Endothelial cells are epithelial cells with a characteristic, flattened or elliptical nucleus (66). They are only surrounded by some collagen fibrils and sporadic pericytes, which are specialised cells of the mesenchyme related to smooth muscle cells (53, 66). Exchange of gases, metabolites etc. occurs through passive diffusion, pinocytotic vesicles and intercellular spaces between endothelial cells. Endothelial cells produce material for basal lamina maintenance such, as collagen and proteoglycans, and growth factors, such as fibroblast growth factor and platelet-derived growth factor. They release factors that control blood flow and reduce formation of pathological thrombus, like nitrous oxide and prostacyclines, and interleukins that participate during acute inflammatory reactions. Lastly, they participate in the process of blood coagulation and thrombosis by synthesising, storing and secreting factors such as von Willebrand factor (vWF) (66).

2.3.2 Tumour growth depends on vascularisation

Four decades ago, Judah Folkman stated that neovascularisation is essential for tumour growth (67). Cells need oxygen and nutrients to function and survive, and they need to eliminate carbon dioxide and metabolic waste products (53). Folkman divided tumour growth into two stages; before and after vascularisation. During the avascular stage, tumour cells obtain nutrients and oxygen through diffusion. The tumour cell population is small and has a slow, linear growth rate. When the tumour reaches a size where diffusion is insufficient, it will enter a dormant state where tumour cells in the outer layer remain viable and proliferate, while other cells become degenerative and necrotic. The result is a non-growing tumour with constant size of 1-3 mm in diameter (67-69). After vascularisation, tumour growth rate quickly increases and becomes exponential (68).

In addition to increasing tumour growth, vascularisation facilitates metastasis. In the multistep process of invasion and metastasis, the presence of nearby vessels is a necessity for distant metastasis to occur. Through intravasation, tumour cells infiltrate nearby vessels and are transported through the vascular system before they extravasate into the stroma of distant tissues (53).

2.3.3 Mechanisms for tumour vascularisation

Angiogenesis is defined as the sprouting of new capillaries from existing vessels (53, 67). The process where tumour cells stimulate nearby host vessels to angiogenesis is the most well-known mechanism for tumour vascularisation (53, 67, 69, 70), and the one originally referred to by Folkman et al (67, 70). However, subsequent studies have found that tumour cells also can gain access to vasculature through other, non-angiogenic mechanisms (71-74). A vascular network may be achieved through mechanisms such as co-option (72, 75), intussusception (76, 77), vasculogenic mimicry (VM) (74, 78), vasculogenesis (53, 79), the formation of mosaic blood vessels (78, 80), and the differentiation of CSCs into endothelial cell-like cells as described above (53). Furthermore, it appears that angiogenesis and non-angiogenic mechanisms can occur in the same tumour simultaneously, or at different phases during tumour progression (71, 72).

2.3.3.1 Intussusception

Intussusception takes place when a vessel splits into two vessels (76, 77) without the occurrence of endothelial cell proliferation. There is an increase in blood vessel girth followed by endothelial cell processes protruding into the vascular lumen. After the endothelial cells have connected and formed a pillar through the lumen, the pillar is invaded by supporting cells such as fibroblasts and pericytes. It continues to increase in circumference until the vascular structure has become two, free-standing vessels (77).

2.3.3.2 Co-option

Co-option is the hijacking of existing host vessels (72, 75, 81). By migrating alongside the vessels that are already present, tumour cells get their needs for exchange met. Co-option typically occurs in highly vascularised organs, like brain, lungs and liver (72, 75).

2.3.3.3 Vasculogenic mimicry

VM occurs when highly aggressive tumour cells or CSCs gain endothelial cell-like characteristics and form *de novo* tubular networks that connect to the circulatory system (53, 74). Increased VM has been found in hypoxic tumours, and in tumours treated with antiangiogenic therapy, and some have suggested that VM might be induced as a response to chemotherapy and inhibition of angiogenesis (78).

2.3.3.4 Mosaic blood vessels

Tumour vasculature can have variants of mosaic blood vessels, where vessels comprise a combination of endothelial cells and tumour cells. These mosaic vascular structures imply that the different mechanisms described here can coexist (78).

2.3.3.5 Vasculogenesis

Endothelial cell precursors originate from the bone marrow and flow through the circulation. The tumour microenvironment can recruit endothelial cell precursors to participate in the formation of blood vessels through vasculogenesis. The endothelial cell precursors differentiate into endothelial cells and take part in the new vascular structures. This mechanism often occurs in synergy with angiogenesis (53, 79).

2.3.4 The process of angiogenesis

The process of angiogenesis is the most well-known mechanism for tumour vascularisation, and the one of particular interest in this thesis. During capillary sprouting, there are two types of endothelial cells; the tip cell and the stalk cell. The tip cell is the very first cell in the vascular sprout. It is highly polarised and stretches out long, cytoplasmic extensions called filopodia, functioning as a leader for the following endothelial cells in the vascular sprout. The tip cell does not proliferate. Stalk cells follow the tip cell while proliferating and creating the vascular lumen. While the tip cell is dependent on a gradient of angiogenic inducers to lead the direction of the sprout, stalk cells depend on their overall concentration (82).

Under normal circumstances, endothelial cells have a slow proliferation rate. Although there are some differences between tissues, the proportion of endothelial cells that are in proliferation at any given time is small (one in 10 000 in the retina, 14 in 10 000 in the myocardium) (83). The exception is physiological angiogenesis, which occurs during embryogenesis, and transiently by meticulous regulation in wound healing, in the placenta and in the female reproductive cycle (53, 84). However, in tumour tissue, angiogenesis endures so that vasculature can sprout continuously and further aid tumour expansion. The intensity of the angiogenesis varies between tumours (53, 85).

Induction and sustaining of angiogenesis is one of the six original hallmarks of cancer (53, 65). It seems to be a distinct step of tumorigenesis that takes place at an early stage in the process. The angiogenic switch determines whether endothelial cells are resting or sprouting (53, 65, 85). The balance hypothesis for the switch suggests that it is turned on or off

depending on the balance between angiogenic inducers and angiogenic inhibitors (Figure 12). If the amount of inhibitors is reduced or the amount of inducers increased, the switch is turned on (85). In tumour development, the angiogenic switch is near constantly turned on, which explains the enduring angiogenesis (53). Some tumours up-regulate angiogenic inducers, while others down-regulate angiogenic inhibitors (65).

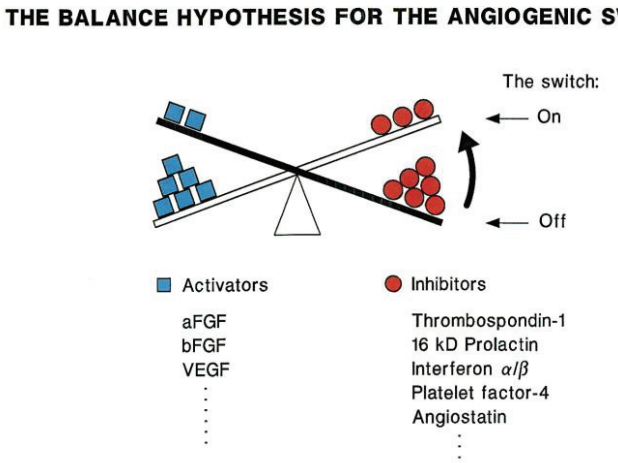

Figure 12: The angiogenic switch. Reprinted from the Cell, 1996, Vol 86, Hanahan and Folkman, *Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis* (85), page no. 359, Figure 4, with permission from Elsevier (licence no. 4421380252839).

Angiogenic inducers and inhibitors are different factors from those regulating tumour cell growth (85). Soluble factors such as the angiogenic inducers vascular endothelial growth factor A (VEGF-A) and fibroblast growth factors (FGFs), or the angiogenic inhibitor thrombospondin-1, bind to transmembrane tyrosine kinase receptors on endothelial cells (53, 65, 85). In addition to soluble factors, angiogenesis is influenced by adhesion molecules that mediate cell matrix and intercellular associations, and by integrins. Endothelial cells express different integrins when they are in a resting or an angiogenic state. Pro-angiogenic signals can be sent from tumour cells, CAFs and immune inflammatory cells (53, 86).

There is interplay between the signalling factors regulating tumour cell growth, and those regulating capillary sprouting (65). For instance, loss of p53 function can cause decreased levels of thrombospondin-1, and *ras* proto-oncogene activation can lead to VEGF expression (53, 65). The *VEGF-A* gene can be up-regulated by both hypoxic conditions and oncogene signalling, and VEGF ligands that are latent in the ECM can be cleaved and activated by extracellular matrix-degrading proteases such as MMP-9 (53, 86).

VEGF is a potent inducer of angiogenesis and stimulates vascular permeability (85). There are many anti-angiogenesis drugs, and several target the VEGF signalling, such as bevacizumab (86, 87). However, angiogenic inducers appear to be able to synergise and complement each other (53). This makes inhibition of angiogenesis more difficult. The fact that tumour cells are able to gain access to blood supply through mechanisms other than angiogenesis, further complicates the process.

2.3.4.1 Lymphangiogenesis

In addition to endothelial cells forming the blood vascular system, lymphatic endothelial cells in the tumour microenvironment are stimulated to lymphangiogenesis. Lymphangiogenesis typically occurs at the tumour periphery and in nearby normal tissue, where lymphatic vessels are functional. These lymph vessels are probable access points for the metastatic spread to regional lymph nodes and distant sites (53). The focus of the present thesis, however, is on blood vessels.

2.3.5 Tumour blood vessels

Blood vessels are heterogeneously distributed in a tumour (88). The vascular architecture is disorganised (89), where vessels have irregular shape and branching patterns, with more branching, twists, curvatures, bifurcations and large variations in lumen diameter (89-91). Tumour vessels are leaky, and their cell lining can have irregular shape and thickness, and finger-like sprouts growing into the tumour tissue. They can overlap or be loosely interconnected with intercellular openings that contribute to vessel leakiness (90). The vessels can be dysfunctional, hypoxic, have oscillating blood flow and lack control mechanisms for blood flow, which makes perfusion and transport of oxygen and nutrients inefficient (92, 93). Endothelial cells, basement membrane and pericytes are all loosely connected (94). Pericytes form protrusions surrounding endothelial cells to cover, maintain and support the function of tumour vessels (53). However, they also have several structural abnormalities, such as cytoplasmic processes invading the tumour stroma (94), and loose pericyte coverage that makes the vasculature less stable (53). Because tumour vessels tend to express both markers of blood vessels and lymphatic vessels, it has been suggested that the tumour vasculature may comprise a mix of both blood and lymphatic endothelial cells (93). Finally, tumour vessels are difficult to see using routine HES staining (Figure 13).

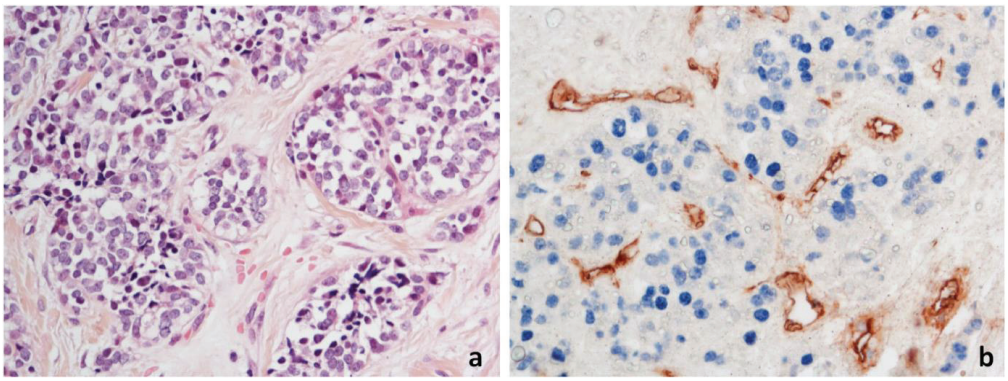

Figure 13: **Tumour blood vessels.** a: Tumour vessels are not visible with haematoxylin-erythrosin-saffron. b: Immunohistochemical staining for von Willebrand factor and Ki67. Brown staining for von Willebrand factor in the cytoplasm of endothelial cells. Proliferating nuclei display blue staining. Photo: AM Bofin/MR Kraby, NTNU.

2.3.6 Von Willebrand factor

The vWF is a large glycoprotein that has also been called the factor VIII-related protein (95). Together with the factor VIII procoagulant protein (VIII:C), it forms the Factor VIII complex through non-covalent bonds (95, 96). In this complex, vWF is considerably larger than VIII:C (95). While vWF promotes primary haemostasis by stimulating platelet adhesion and aggregation, the small VIII:C stimulates secondary haemostasis as a procoagulant cofactor in the coagulation cascade (95, 96). The second role of vWF is to stabilise, protect and increase the half-life of factor VIII in the circulation (96). vWF is named after the autosomal von Willebrand disease, where defects in vWF structure or concentration lead to prolonged bleeding-time (95). The *VWF* gene is situated on chromosome 12 (96), and its protein is synthesised by endothelial cells and megakaryocytes (97). In endothelial cells, vWF is stored in the cytoplasm, in Weibel-Palade bodies of the endoplasmic reticulum (66, 96). Therefore, the vWF antigen can be targeted in IHC/immunofluorescence studies to visualise endothelial cells of arteries, arterioles, capillaries and veins (95). In the Human Protein Atlas, the reliability score of vWF is supported, and the protein is described as being selectively expressed in the endothelial cell and megakaryocyte cytoplasm (34, 35, 98).

2.3.7 Immunohistochemistry

IHC is a procedure for detecting and visualising specific molecules in cells or tissues. Cells or tissues expressing the molecule in question are stained with a specific colour, and their morphological localisation can be identified with a bright-field microscope or a fluorescent

microscope (99). IHC was first performed by Coons et al in 1941 (100), and on formalin-fixed, paraffin-embedded (FFPE) sections in 1974 (101).

IHC is based on the immunological concept where antibodies bind to antigens (100). The antigen is the molecule we aim to detect, and the epitope is the specific region of the antigen that is recognised by the antibody. Antibodies are also called immunoglobulins. They comprise two light chains and two heavy chains with a three-dimensional, Y-shaped structure (Figure 14a). The fragment antigen binding (Fab) elements are the two ends of the forked part of the Y. Each Fab element comprises both a light and a heavy chain. The Fab elements make up the antigen-binding site, and are therefore highly variable in structure. The most commonly used immunoglobulin in IHC, IgG, is bivalent, meaning that it has two Fab regions. IgM, however, is decavalent because it comprises five forks and has a total of ten antigen-binding sites. The tail of the Y is the fragment crystallisable (Fc) element. It comprises heavy chains only, defines how the immunoglobulin functions biologically, and functions as a binding site for other antibodies. In IHC, the epitope and the Fab element attach and form a junction. Then, a detection system is used to visualise the junction (99).

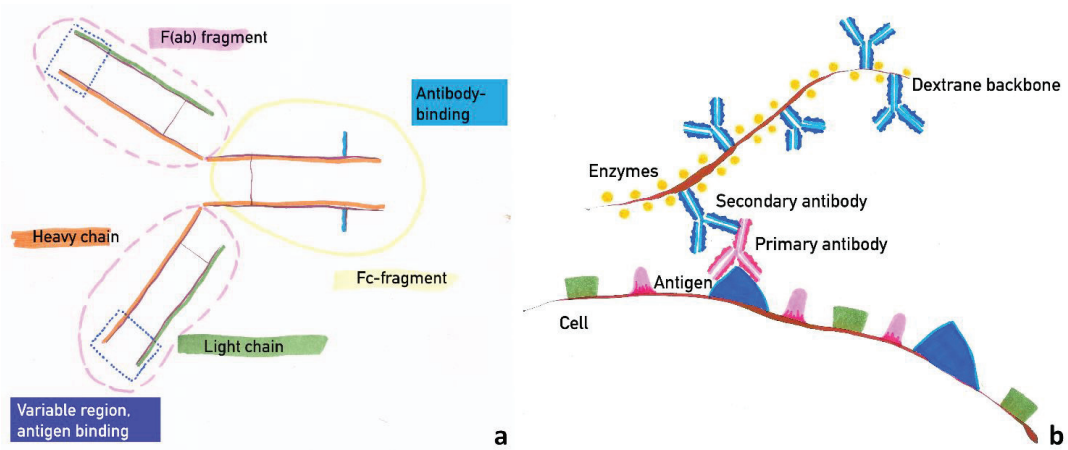

Figure 14. Immunohistochemistry. a: Structure of the antibody. Immunoglobulin IgG. **b: Indirect staining method in immunohistochemistry**. Illustration: S. Kilaas.

Immunostaining can be direct or indirect. The latter uses two antibodies rather than one, and is the most common IHC method because it is more sensitive and causes a reaction with stronger intensity. The primary antibody detects the antigen. The detection system comprises the secondary antibody that detects the primary antibody, and a label, which is either a fluorescent, an enzyme or a metal (Figure 14b). When adding the enzyme's

substrate, a chemical reaction is triggered in the cells or tissues that contain the detection system. The product of the enzyme has a specific, recognisable colour, which is displayed by all cells or tissues expressing the antigen in question (99).

Antibodies are mono- or polyclonal. Polyclonal antibodies have higher affinity, can interact with several epitopes in the same antigen, require less resources in production and appear more robust in situations where pre-analytical conditions are unknown. However, monoclonal antibodies are more specific and lead to less background staining (99).

Prior to IHC, tissues undergo several pre-analytical steps. After sample acquisition, tissues are immersed in a fixative for architectural and antigenic preservation (102). The most common fixative is formaldehyde. Fixation makes proteins chemically cross-linked with methylene bridges, insoluble and unreactive (99). After fixation, sections are embedded in paraffin (102). The latter further inhibits reactivity with surroundings and preserves tissue architecture. Tumours are now FFPE blocks that can be sectioned, mounted onto glass slides and dried, or they can be stored as FFPE blocks for years without loss of antigenicity (99).

Before IHC can take place, tumour sections must have their tissue reactivity restored through deparaffinisation, rehydration of biological material, and epitope retrieval. Paraffin must be removed to ensure that soluble reagents gain access to tissue (102). Epitope retrieval is fixation reversal, where the cross-linked bonds between formalin and tumour tissue are broken, and can be achieved using heat or enzymatic degradation (99).

2.3.8 Quantification of tumour vasculature

2.3.8.1 Microvessel density

In 1991, Weidner et al introduced a method for quantifying tumour vasculature called microvessel density (MVD), using IHC to visualise vWF. MVD was determined by counting microvessels in the tumour region containing the highest number of vessels (88). This region was later termed the vascular 'hotspot' (103), and reported to be found most often at the tumour margins (88, 104). Today, MVD is a frequently used method for measuring tumour-associated vasculature.

MVD reflects neither ongoing angiogenesis nor tumour growth rate. If vascular sprouting increases rapidly in a fast-growing tumour, this is not necessarily reflected by tumour vessel density (105). In fact, the MVD in a tumour does not always exceed that of corresponding

normal tissue (75, 106). In the breast, some reported that MVD was lower in the tumour tissue than the corresponding normal tissue (106). The antibodies most commonly used for MVD assessment do not differentiate between vessels created through angiogenesis and vessels from other, non-angiogenic mechanisms. Furthermore, MVD does not reflect a tumour's dependency on angiogenesis, and it can neither be used to predict which tumours will respond to antiangiogenic treatment, nor to determine the effect of antiangiogenic treatment (92, 105). Unfortunately, several studies still use the terms MVD and angiogenesis as if they were synonyms (107-109). Therefore, it is paramount to be conscious of whether conclusions drawn from a study concern MVD or angiogenesis. In this thesis, the term angiogenesis will only be used in reference to the process of capillary sprouting, and MVD to the vessel quantity in a specified tumour region.

The prognostic value of MVD is debated, and there are many studies on this topic. While some have found that MVD is associated with poor prognosis in breast cancer (88, 103, 110-114), others have not (115-123). The only meta-analysis is from 2004, included 8,936 patients from 43 studies, and found that high MVD was associated with poor prognosis with a risk ratio (RR) 1.54 (95% CI 1.29- 1.85) (124). A contributor to the discrepant results between studies may be lack of comparability, which will be described in detail on page 43.

2.3.8.2 Vascular proliferation

Quantifying the amount of vasculature in proliferation might be a better measure of ongoing tumour angiogenesis. Proliferating endothelial cells can be visualized by dual immunohistochemistry, using one antibody to visualise endothelial cell cytoplasm (for instance vWF, cluster of differentiation 31 (CD31) or cluster of differentiation 34 (CD34)), and another to target proliferating nuclei (for instance Ki67) (Figure 13b) (106, 125-127). The first studies of proliferating vasculature estimated a labelling index for the proportion of counted endothelial cells that were in proliferation (125, 126). More recent studies have assessed proliferating microvessel density (pMVD), which is the average number of proliferating vessels per square millimetre within a selected tumour area, and the vascular proliferation index (VPI), which is the proportion of proliferating vessels to the total number of vessels (MVD) (121, 127, 128).

High vascular proliferation has been associated with aggressive clinicopathological features and poor prognosis in endometrial cancer, prostate cancer and breast cancer (119, 121, 127-

129). BP breast tumours had a greater probability of having higher vascular proliferation compared to luminal tumours (129). However, vascular proliferation is breast cancer is generally low, with median VPI ranging from 1.0% to 3.4% (106, 119, 121), median pMVD ranging from 0.5 to 2.0 vessels/mm^2 (119, 121), and mean endothelial cell labelling index ranging from 2.2% to 2.7% (125, 126).

2.3.8.3 Vascular grading

In the original paper on MVD, the authors also subjectively graded the vascular hotspot at low magnification from 1+ (low MVD) to 4+ (high MVD) (88). Vascular grade was less time-consuming, correlated with MVD and other vascular measures (130), and was associated with prognosis (88, 103). However, this semi-quantitative approach at low magnification leads to loss of information. Furthermore, the low magnification makes it difficult for observers to distinguish between vessels, so there is a risk of grading antibody staining intensity rather than vessel density. This is supported by some results implying that the eye tends to detect vessel area more readily than vessel density (130). Errors in the staining process, background staining, and false positives may lead to misinterpretation. Furthermore, because the method is so subjective, there is a risk of high observer variation (131).

2.3.8.4 The Chalkley method

The Chalkley method for vasculature assessment was introduced by Fox et al in 1994 (132). After identifying three vascular hotspots at low magnification, an eyepiece graticule is used to quantify vasculature at 250x magnification in each hotspot. The Chalkley graticule covers a field area of 0.16mm^2, and comprises 25 randomly arranged points. The observer adjusts the graticule so that the maximum number of points hit a vessel. Each field is scored from zero to 25, and the average value from the three fields is the final Chalkley score of the tumour (130, 132). The Chalkley score is unit-free and provides an estimate of the relative vascular area to the total area assessed (130, 132, 133). Hence, while MVD is a density measure, Chalkley estimates area. The method has been described as simple, quick, less subjective and more reproducible than MVD (116, 123, 134). Some have found that a high Chalkley score is associated with poor prognosis in breast cancer (116, 117, 123, 130, 132), while others have not (135). Compared to MVD, the Chalkley method removes the potential source of variation that comes from having to determine whether an IHC-stained structure is one or several, distinct vessels.

2.3.8.5 Vascular pattern

It has been suggested that rather than measuring vessels purely quantitatively with methods like MVD, one should study the microvascular patterns as an expression of capillary functionality. Some found that patients whose tumours had a vascular pattern of blood-filled capillaries with increased size appeared to have a poorer prognosis compared to those with small, delicate, compressed capillaries (118). This could be supported by others reporting that tumours with low MVD and high Chalkley typically display interconnected vessels with plenty of branches, and that patients whose tumours had low MVD/high Chalkley had the poorest prognosis, followed by those with high MVD/high Chalkley (117).

2.3.8.6 Automated assessment of tumour vasculature

Several studies have used digital pathology to automatically quantify tumour vasculature (104, 112, 136-139). Software can be used to estimate the endothelial area (EA) (137-139). EA is the ratio of total endothelial area to the total analysed area, and thus resembles the Chalkley method. Other automated methods mimic MVD by estimating the number of vessels (112, 136). While some found that conventional MVD was associated with prognosis and automated MVD was not (112), others found the opposite (139). Compared to the conventional methods, automated vasculature assessment is more objective, has increased accuracy and reproducibility (104, 136). With automated methods, several vascular parameters can be studied simultaneously, such as vessel count, area, diameter, size and length. This opens for additional information about tumour biology (130, 131, 136). There is also a potential for identifying the true vascular hotspot in a section. Through slide digitisation, it is easier to re-evaluate measurements at a later time (104). However, automated methods are less able to take morphology into consideration and rely on software commands for thresholding and size setting. This puts them at greater risk of both excluding weakly stained vessels, and including false positives such as background staining and stained lymphocytes (112, 136-138). Some found automated and conventional MVD to correlate well in cases without background staining, but not when background staining was present (136). Manual adjustments by experienced personnel are needed (112, 136-138). In addition, automated methods require extra equipment and data storage solutions. A recent meta-analysis pointed out that the accuracy of automated vasculature assessment still needs to be further tested (109). Many challenges could be solved with emerging technologies such as interactive learning and texture-based recognition where software identifies cell

types without using tissue identification markers (140). Furthermore, in some fields of pathology, novel machine learning algorithms based on deep learning with deep convolutional neural networks have outperformed experienced pathologists (141).

2.3.8.7 Imaging diagnostics

There are emerging studies assessing tumour vasculature with ultrasound (91, 142), computer tomography (CT) (61, 89) and magnetic resonance imaging (MRI) (143, 144). Imaging techniques make it possible to study vasculature on a three-dimensional platform, and to identify vascular hotspots in the entire tumour (89). Several other vascular parameters may be studied, in addition to tumour haemodynamics such as blood flow, perfusion blood volume and capillary permeability. Impact of therapy may also be evaluated (91, 143). Imaging studies have mainly aimed at distinguishing between benign and malignant tumours (143, 144), or to study the differences in tumour vascularity between the tumour edge and centre (89, 142).

2.3.9 Challenges with current histopathological methods for assessment of tumour vasculature

For tumour vasculature assessment to be feasible in everyday diagnostics, it must be reliable, inexpensive, reproducible, require little training, and provide relevant information about either tumour biology, prognosis or prediction. In 1999, CAP stated that MVD was not considered to be clinically useful. An important reason for this, was the great observer variability (145), for which they quoted one study by Axelsson et al (n=220) (115). This study reported that observers were quite consistent with themselves in MVD assessment in a tumour. Between observers, however, pairwise correlation was 0.55, median variation coefficient was 23% and the kappa correlation was 0.45 (moderate agreement). When separated into high/low MVD groups, 32% of the cases had been discordantly classified between observers (115). Others considering observer variation for vessel quantification in either all tumours or a selection of tumours have found moderate (88, 110) or good (104, 111, 122, 136, 139, 146-148) reproducibility, while some reported considerable variability (134, 149). Some found a higher variation between observers (interobserver variability) than within observers (intraobserver variability) (115, 134), others did not (104), and some reported that variation was observer dependent (149). Others found that the major reason for total variation in MVD lies in the biological difference between tumours, and not in

methodological observer variation (134). Furthermore, measurement of the tumour vasculature is complicated by intratumoural vascular heterogeneity.

In response to CAP, *the Second International Consensus on the Methodology and Criteria of Evaluation of Angiogenesis Quantification in Solid Human Tumours* was published in 2002, considering the methodological issues in quantifying tumour vascularity. They identified methodological differences as important reasons for the varying results in prognostic informativeness between individual studies, and suggested that the Chalkley method with the CD34 antibody should be the standard for tumour vessel assessment (80). However, they were subsequently criticised for this choice since the Chalkley recommendation was based on only a handful of studies (123). Sixteen years later, there is still no consensus for how tumour vasculature should be assessed. New antibodies, technologies and methodologies have emerged, rendering the consensus reports outdated. MVD is the most frequently used method, (109, 124, 150-152), but there are no current guidelines for how MVD should be assessed.

An overview of the literature regarding methods for vasculature assessment in breast cancer (Table 1) reveals a lack of consistency across studies, and confirms that there is an urgent need for consensus in methodology. The following chapters systematically consider the sources of methodological variability between studies. We mainly focus on MVD in breast cancer, but most of the factors presented here are also applicable for other cancer types.

Table 1. Overview of methodology in studies of tumour vasculature in breast cancer

First author	Year	N	Anti-body	Vasculature assessment method	Magni-fication (x)	Number of fields per section	Total field area per section	For statistical analyses	
Weidner (88)	1991	49	vWF	MVD	200	1 field in 1 hotspot	0.74mm^2	Total count	Per field
	1991	49	vWF	MVD	400	1 field in 1 hotspot	0.19mm^2	Total count	Per field
	1991	49	vWF	Vascular grade	40-100	1 hotspot	NR	1+ to 4+	Per unit
Weidner (103)	1992	148	vWF	MVD	200	1 field in 1 hotspot	0.74mm^2	Total count	Per field
	1992	148	vWF	Vascular grade	40-100	1 hotspot	NR	1+ to 4+	Per unit
Horak (148)	1992	103	CD31	MVD	250	3 fields in 1 hotspot	1.14mm^2	Highest count	Per mm^2
	1992	103	CD31	MVD	400	3 fields in 1 hotspot	0.6mm^2	Highest count	Per mm^2
Bosari (113)	1992	110	vWF	MVD	200	1 field in each of 3 hotspots	NR	Mean count	Per field
	1992	110	vWF	MVD	200	1 field in each of 3 hotspots	NR	Highest count	Per field
Fox (125)	1993	20	CD31	MVD	NR	1 field in each of 3 hotspots	NR	Mean count	Per mm^2
van Hoef (122)	1993	93	vWF	MVD	100	1 field in each of 4 hotspots	1.92mm^2	Highest count	Per field
	1993	93	vWF	MVD	200	1 field in each of 4 hotspots	0.48mm^2	Highest count	Per field
Fox (132)	1994	109	CD31	Chalkley	250	1 field in each of 3 hotspots	0.48mm^2	Mean count	Chalkley score
Vartanian (126)	1994	57	CD34	MVD	200	1 field in 1 hotspot	0.72mm^2	Total count	Per field
Axelsson (115)	1995	220	vWF	MVD	250	1 field in each of 3 hotspots	0.37mm^2 /0.32mm^2	Highest count	Per mm^2
Fox (130)	1995	30	CD31	MVD	400	1 field in each of 3 hotspots	0.48mm^2	Mean count	Per mm^2
	1995	30	CD31	Chalkley	250	1 field in each of 3 hotspots	0.48mm^2	Mean count	Chalkley score
	1995	30	CD31	Vascular grade	NR	NR	NR	NR	Low/medium /high

	1995	30	CD31	Semi-automated MVD	NR	1 field	0.16mm^2	Total count	Per mm^2
	1995	30	CD31	Semi-automated MVD	NR	1 field	0.85mm^2	Total count	Per mm^2
	1995	211	CD31	Chalkley	250	1 field in each of 3 hotspots	0.48mm^2	Mean count	Chalkley score
de Jong (146)	1995	10	CD31	MVD	400	4 fields in 1 hotspot	0.64mm^2	NR	Per mm^2
	1995	10	CD31	MVD	400	10 fields in 2-3 systematically selected visual fields	1.59mm^2	NR	Per mm^2
Simpson (139)	1996	178	CD34	Automated EA	200	5 fields in 1 hotspot	0.74mm^2	Total number of pixels	Per 1000
	1996	178	CD34	MVD	200	5 fields in 1 hotspot	0.74mm^2	NR	NR
	1996	178	CD34	MVD	100	5 fields in 1 hotspot	1.06mm^2	Total count	Per field
Vermeulen (110)	1997	38	vWF	MVD	200	Whole section	NR	Highest count	Per field Per mm^2
	1997	38	vWF	MVD	400	Whole section	NR	Highest count	Per field Per mm^2
Martin (147)	1997	21	CD34	MVD	200	10 fields in 1 hotspot	6.8mm^2	Highest count	Per field
Hansen (134)	1998	40	CD34	MVD	200	1 field in 1 hotspot	0.75mm^2	Total count	Per mm^2
	1998	40	CD34	MVD	200	3 fields in 1 hotspot	2.25mm^2	Mean count Highest count	Per mm^2
	1998	40	CD34	MVD	200	Random systematic sampling of 20 fields	1.2mm^2	Mean count	Per mm^2

	1998	40	CD34	MVD	200	10 fields in each of 3 hotspots	0.67mm^2	Mean count	Per mm^2
	1998	40	CD34	Chalkley	250	1 field in each of 3 hotspots	0.2mm^2	Mean count	Chalkley score
Ingeholm (149)	1999	50	vWF	MVD	400	1 field in hotspot	0.26mm^2	Total count	Per field
	1999	50	vWF	MVD	400	1 randomly chosen field	0.26mm^2	Total count	Per field
Belien (104)	1999	10	CD31	MVD	400	10 fields in 1 hotspot	0.6mm^2	Total count Mean count	Per mm^2
	1999	10	CD31	MVD	400	Whole tumour section	NR	Total count Mean count	Per mm^2
	1999	10	CD31	Automated MVD	NR	Whole tumour section	NR	Total count Mean count	Per mm^2
de Jong (111)	2000	112	CD31	MVD	400	4 fields in 1 hotspot	0.64mm^2	Mean count	Per mm^2
	2000	112	CD31	MVD	400	10 fields in 1 hotspot	1.56mm^2	Mean count	Per mm^2
	2000	112	CD31	MVD	400	10 randomly chosen fields	1.56mm^2	Mean count	Per mm^2
Eberhard (106)	2000	24	CD34	MVD	400	At least 5 fields in hotspot	At least 0.34mm^2	NR	Per mm^2
Ahlgren (153)	2002	21	CD31	MVD	200	3 fields in 1 hotspot	2.16mm^2	Highest count Mean count	Per field
Offersen (123)	2003	455	CD34	MVD	200	1 grid field in each of 3-5 hotspots	0.75-1.25mm^2	Highest count	Per field
	2003	455	CD34	Chalkley	200	1 field (0.2mm^2) in each of 3 hotspots	0.6mm^2	Mean score	Chalkely score
Hansen (116)	2004	330	CD34	MVD	200	1 field in each of 3 hotspots	2.26mm^2	Mean count	Per mm^2
	2004	330	CD34	Chalkley	250	1 field in each of 3 hotspots	0.56mm^2	Mean score	Chalkley score
Dhakal (117)	2009	498	CD34	MVD	200	1 field in each of 3 hotspots	2.75mm^2	Highest count	Per field

	2009	498	CD34	Chalkley	200	1 field in each of 3 hotspots	0.56mm^2	Highest score	Chalkley score
Lopes (154)	2009	142	CD105	MVD	200	1 field in each of 3 hotspots	2.22mm^2	Mean count	Per mm^2
Qin Li (155)	2010	161	CD105	MVD	200	1 field in each of 5 hotspots	3.7mm^2	Mean count	NR
Safali (118)	2010	31	CD34	Automated MVD	200	1 field in each of 3 hotspots	0.24mm^2	Mean count	Per mm^2
	2010	31	CD34	Vascular pattern	NR	NR	NR	Pattern	NR
Mohammed (135)	2011	397	CD34	Chalkley	200	1 field in each of 3 hotspots	NR	Mean score	Chalkley score
Mikalsen (136)	2011	420	CD34	MVD	310	≈0.38mm^2 in one of three hotspots	NR	NR	NR
	2011	420	CD34	Automated MVD	NR	NR	NR	NR	NR
Nalwoga	2011	177	vWF	MVD	250	10 fields in 1 hotspot	4.5mm^2	Mean count	Per mm^2
(129)	2011	239	vWF	MVD	250	10 fields in 1 hotspot	4.2mm^2	Mean count	Per mm^2
Miyashita (114)	2011	105	CD31	MVD	200	One field in hotspot	NR	Total count	NR
Jafaar (107)	2012	96	CD34	MVD	200	3 fields in 1 hotspot	NR	Mean count	Per field
Arnes (121)	2012	499	vWF	MVD	250	10 fields in 1 hotspot	4.2mm^2	Mean count	Per mm^2
Keyhani (108)	2013	111	CD34	MVD	NR	1 fields in each of 4 hotspots	NR	NR	Low/mode-rate/high
Kruger (119)	2013	178	Nestin	MVD	400	10 fields in 1 hotspot	2.0mm^2	Mean count	Per mm^2
Haisan (137)	2013	50	CD34	Automated EA	NR	1-3 fields in each of 3 tumour regions	NR	Mean area	Percent (%)
Ozerdem (138)	2013	40	CD34	Automated EA	NR	1 TMA core	3.14mm^2	NR	Percent (%)
Li (156)	2013	40	CD34	MVD	200	1 field in each of 5 hotspots	3.7mm^2	Mean count	NR

Mohammed (112)	2013	33	CD34	MVD	400	3 fields in tumour	0.85mm^2	Mean count	NR
	2013	356	CD34	MVD	400	3 TMA cores	0.85mm^2	Mean count	NR
	2013	356	CD34	Automated MVD	NR	NR	NR	NR	NR
Biesaga (157)	2014	172	CD34	MVD	200	1 field in each of 5 hotspots	1.45mm^2	Mean count	Per mm^2
Li (61)	2014	255	CD34	MVD	200	5 fields in 1 hotspot	3.7mm^2	Mean count	NR
Li (143)	2015	52	CD34	MVD	100	1 field in 1 hotspot in TMA	NR	Mean count	Per mm^2
	2015	52	CD105	MVD	100	1 field in 1 hotspot in TMA	NR	Mean count	Per mm^2
Yehia (158)	2015	190	CD31	MVD	NR	4 randomly selected fields	NR	Mean count	NR
Shrivastav (159)	2016	100	CD34	MVD	400	At least 5 fields in hotspots	At least 0.95mm^2	Mean count	Per mm^2
Nasir (87)	2017	169	CD34	MVD	NR	1 TMA core	0.28mm^2	Total count	Per TMA core
Liu (160)	2017	195	CD34	MVD	200	1 field in each of 5 hotspots	NR	Mean count	NR
Bujor (161)	2018	54	CD34	MVD	400	NR	NR	NR	NR
Li (142)	2018	99	CD34	MVD	400	5 fields in 1 hotspot	NR	Mean count	NR

von Willebrand factor (vWF). Cluster of differentiation 34 (CD34). Microvessel density (MVD). Endothelial area (EA). Tissue microarray (TMA). NR: Unclear or not reported.

2.3.9.1 Study populations

Cohorts have differing nationalities, ethnicities, ages, cultures and reproductive factors, all of which can affect disease occurrence and course. Different study populations may vary with regard to distribution of histopathological type, grade, stage, subtype, and other disease characteristics. Treatment regimens may differ according to country, region, socioeconomic status and participation in clinical trials. Research groups have various access to clinical information, and distinct routines for follow-up frequency and duration. There are also profound variations in sample size. All of these factors influence results. Those who start a cohort study are usually not the ones to finish it. Therefore, research groups are usually not able to influence the original outline of the project or the pre-analytical conditions mentioned below.

2.3.9.2 Variations in pre-analytical, analytical and post-analytical phases

IHC studies comprise pre-analytical, analytical and post-analytical phases. We define the pre-analytical phase as every step from tissue sample procurement to right before the IHC staining process. All steps performed to ensure the best staining result in the IHC process are in the analytical phase. The post-analytical phase comprises steps in the interpretation and report of the IHC staining results. Every step of each phase entails possible sources of error. In addition comes the potential variability between studies due to different equipment, laboratory and interpretation routines, which may vary according to location and time period. These differences could impact the results of the individual study and the comparisons across studies. Table 2 provides an overview of potential sources of variation for each step. Some of the sources of variation specifically for tumour vasculature assessment, will be described in detail below.

Table 2: Potential sources of inter-study variability during pre-analytical, analytical and post-analytical phases in IHC studies. Based on Ramos-Vara et al (99), Hammond et al (10), Vyberg (102) and the variations observed from individual studies in the Table 1

	Step	Source of variation
Pre-analytical phase	Acquisition of sample	Warm ischemia time Cold ischemia time Size of the sample
	Fixation	Type of fixative (cross-linking vs coagulating), buffer, pH, concentration Fixative volume, temperature, time
	Decalcification	Type of decalcification solution, decalcification time
	Tissue processing	Frozen tissue vs paraffin-embedding Choice of buffer for incubation Temperature

	Tissue sectioning	Tissue section thickness Tissue adhesive Drying temperature and duration
	Storage of paraffin blocks /tissue sections	Storage time Storage conditions (light, temperature, air)
Analytical phase	Deparaffinisation	Dewaxing agent
	Epitope retrieval	Retrieval or not Heat-induced or enzymatic Detergents, buffers, pH
	Blocking endogenous enzyme activity	Protocols for blocking substrates
	Blocking nonspecific reactions	Blocking protein protocols (bovine serum albumin, casein solutions, etc.)
	Rinse	Wash buffer, number of rinses
	Incubation	Time, temperature
	Primary antibody	Mono/polyclonal, choice of clone Antigen recognition (native vs linear) Specificity Species variability (mouse, rabbit, etc.) Dilution concentration and buffer Prior validation
	Detection system	Avidin-biotin, peroxidase-antiperoxidase, polymer-based systems, etc. Prior validation
	Enzyme-substrate-chromogen	Colour detection
	Multiplex IHC	Enzyme-substrate combinations
	Counterstain	Presence/absence Contrast between chromogen and counterstain
	Coverslipping	Xylene or other mounting media
Post-analytical phase	Selection of region for IHC assessment	Number of regions Location of region in tumour Size of region (total field area for assessment)
	IHC assessment	Magnification Staining pattern (nuclear, cytoplasmic, membranous, etc.) Definition of a positively stained unit (positivity threshold, morphological context) Use of microscope or screen Conventional or automated assessment
	Scoring system	Continuous/categorical measure Staining intensity, staining density, proportion of positive cells, staining index, relative area, H-score, Allred score, etc.
	Report	Interpretation of final report Unit (Per mm^2, per field, score, grade, percentage, positive/negative, high/low) Choice of statistical analysis

2.3.9.2.1 Primary antibodies

The most commonly used primary antibodies for visualisation of vasculature, are the pan-endothelial vWF (34, 35, 98), CD31 (34, 35, 162) and CD34 (34, 35, 163). More recent studies

have attempted to stain immature vessels only as a means of identifying the vessels that are a product of recent angiogenesis. For this purpose, some use endoglin (CD105) (143), and others use nestin (119, 128).

CD31 is found on the cell membrane of platelets, monocytes, neutrophils, and some lymphocytes (34, 35, 162). CD34 has been reported to stain stromal cells, inflammatory cells and lymphatic cells in addition to endothelial cells (92, 164). vWF may also stain some lymphatic capillaries (124, 165). When compared, it has been reported that CD31 has higher sensitivity and visualises a higher number of microvessels than vWF (146, 148). However, vWF has higher specificity, with good contrast between the stained vasculature and other elements in the tissue (92, 146, 148).

2.3.9.2.2 Selection of tumour region for assessment of tumour vasculature

2.3.9.2.2.1 Why vascular hotspots?

Vessel counting is most commonly done in the vascular hotspot. However, some studies have quantified vasculature in randomly chosen fields (134, 166) or globally in the whole tumour section (104). Some report that counting in randomly chosen fields provides a smaller coefficient of variation compared to hotspots (134), while others found no relevant differences between the two (146). Nevertheless, the vascular hotspot is described as the tumour region where endothelial cells are most active, and the region with the easiest access to the circulation (80, 131). When compared, MVD assessed in the vascular hotspot provided prognostic information while MVD representing the whole tumour section did not (111).

2.3.9.2.2.2 How to identify the hotspot?

To identify the vascular hotspot, most papers use the method described in the first MVD paper, which reads: '… the areas of highest neovascularization were found by scanning the tumor sections at low power (40x and 100x) and identifying the areas of invasive carcinoma with the highest number of discrete microvessels staining for factor VIII (brown)' (88). Microvessels were subsequently defined as capillaries and small venules (103). Weidner et al counted vessels in one visual field at 200x magnification, and one at 400x magnification (88). Different interpretations and small adjustments in methodology have led to variation in subsequent original articles. Many count vessels in more than one visual field. Among these, some identify a single vascular hotspot and count vessels in several visual fields within that

hotspot (107, 119, 142, 148, 153), while others identify several hotspots and count vessels in one visual field in each (113, 117, 118, 123, 132, 135) (Table 1). Hotspot identification at low magnification makes it difficult to identify the area with the highest number of microvessels (115). This is confirmed by studies reporting that hotspot selection at low magnification failed to identify the 'true' vascular hotspot (104) and that the MVD from randomly chosen fields was higher than that from vascular hotspots in some cases (149). Furthermore, hotspot selection has been called the most subjective step in the process of vessel assessment (80, 131, 165). Exclusion of the hotspot selection step increased interobserver reproducibility for MVD in some studies (104, 110, 134), while others did not find any profound differences (104, 149).

It has been recommended that a tumour section should be systematically scanned for the vascular hotspot (88), which should be in viable tumour tissue, or in adjacent stromal tissue within one 200x field diameter from the tumour tissue. It should not contain necrosis, sclerosis or normal tissue (131). Apart from these recommendations, there are no clear rules governing hotspot location in the tumour. Many have pointed out that although hotspots can be anywhere in the tumour, they are most frequently located at the tumour periphery (88, 115, 122, 126). Studies comparing MVD in the tumour periphery to the tumour centre, found the highest density at the tumour edge, although this may vary according to breast cancer subtype (61, 156). Vessels located at the invasive front, tumour centre or in adjacent tumour stroma, may have different roles in tumour biology, and thus a different impact on prognosis. This is not taken into account with the current hotspot selection method (137).

2.3.9.2.3 Microvessel counting

Vessel counts are affected by several factors, such as magnification, field area size, and the definition of what constitutes one vessel profile.

2.3.9.2.3.1 Magnification

Vessels are counted at microscope magnifications ranging from 100x to 400x (Table 1) (88, 112, 139, 142, 143, 149). Magnification can influence the vessel count. Some found good correlation between different magnifications (110, 148), but others reported that MVD was 15-32% higher per mm^2 after counting at 400x magnification than at 200x magnification (148). A 400x magnification provides better image detail and gives a more accurate vessel

identification. The higher the magnification; the easier to distinguish vessel profiles; to avoid overlooking vessels and counting vessels more than once. (110, 148, 149, 151, 165).

2.3.9.2.3.2 Field area for vessel counting

Variations in field area size for vessel counting between studies could be a contributor to the discrepant results regarding the prognostic value of MVD. Studies report large differences in field area size (Table 1), from 0.19mm^2 (88) to 6.8mm^2 (147). A recent meta-analysis of MVD in gastric cancer included 26 studies from 2000 to 2016. Some had counted vessels in two fields at 400x magnification, and others included five fields at 100x magnification (151). Even when taking different microscope lens diameters into consideration, this implies great differences in field areas between studies, and an urgent need for a common consensus regarding field area size.

Loss of information can occur both when field areas are too large and too small (131). If the field area is too small, some worry that all tumours become highly vascular so that the differences between tumours is evened out (111, 130). If the field area is too large, there is a risk of decreased MVD due to hotspot dilution (104, 111, 113, 130, 153). When comparing the highest and the mean vessel count from three visual fields at 200x, some reported more than 10% difference between the two in 61% of cases (113), and others more than 20% difference near all (19/21) tumours (153). One study compared two field areas (0.16mm^2 and 0.85mm^2), and, although they were highly correlated, mean MVD was considerably higher in the smaller field area compared to the larger (91.7vessels/mm^2 versus 42.4vessels/mm^2) (130). Some state that MVD becomes more stable, with better reproducibility, when assessed in larger field areas (115, 166). However, results of another study implied that the association between MVD and prognosis was stronger when a smaller field area had been used for vessel counting, than when a larger area had been used (111).

2.3.9.2.3.3 Definition of a vascular profile

A microvessel is defined as a clearly positively stained endothelial cell or cell cluster that is distinct from adjacent tumour cells, microvessels and other connective tissue. A discernible vessel lumen is not a necessary criterion (88). Some studies report disagreements between observers (130, 149), while the results of others implied that observers seem to agree on what constitutes a vessel (104, 110, 134).

In the identification of vessel profiles, true vessels must be distinguished from background staining, and from cells with false positive staining, like leucocytes and stromal cells. Weakly stained vessels must also be identified. Choice of primary antibody influences these considerations. However, some tumour vessels have phenotypes that make them poorly stained, despite optimal laboratory technique (149). To identify true vessels, one can to some degree rely on morphology, since endothelial cells typically have an oval shape and form a pipe-like structure or circle (66). However, vessel lumens are not always visible, and tumour vessels can be so small that they present as only one single endothelial cell that may lack its characteristic shape. Another challenge is that vasculature near the tumour edge can form maze-like patterns that are hard to distinguish from one another (149), and tumour vessels can be particularly tortuous, and will therefore be sectioned multiple times (139).

2.3.9.2.4 Number of observers and observer training

Some studies report two observers assessing sections simultaneously (61, 88, 113, 132, 153), while others report only one (103, 111, 121, 122, 134, 148). Simultaneous assessment by two observers may minimise variability, but could introduce bias since observers might unintentionally influence one another. Some endorse assessment by only one observer to ensure standardisation (121).

Observer training is recommended to increase reproducibility of vasculature assessment (131). However, the reported observer training regimens vary between original articles, and are often not reported at all. While some observers are trained in the second step of microvessel counting (119, 121), others recommend training in hotspot selection (165). Individual studies use various measures of observer variability and training effect, such as correlation coefficients the Kappa index, and difference in percentage between observers.

2.3.9.2.5 Reporting

After vessel counting, studies use the average count (107, 111, 112, 142, 159), highest count (110, 115, 122, 148) or total count (88, 126, 139, 149) to represent the tumour vasculature (Table 1). The chosen unit for vessel counts can be per field in some studies (88, 107, 117, 123, 147, 149, 153), and not reported at all in others (114, 136, 155, 156, 160). However, to make MVD studies more comparable, the number of vessels should be reported per mm^2. Similarly, it is recommended to use a continuous scale in statistical analyses instead of medians, tertiles and quartiles (131, 167). This way, the increase in one unit is universal,

enabling direct comparisons between individual studies. If cut-offs are used, the median value is usually recommended for comparable sample size in both groups (131). However, this can lead to loss of information, and information loss is smaller if more categories are used (167).

3 AIMS

The aims of this thesis were to study MVD, pMVD and VPI in molecular subtypes of breast cancer, and to study their prognostic role in patients with long-term follow-up. The specific aims of each paper were as follows:

Paper I: To compare MVD, pMVD and VPI in BP and Luminal A breast tumours, and to evaluate their impact on patient prognosis in all cases, and within each separate subtype.

Paper II: To assess MVD, pMVD and VPI in the three non-luminal subtypes of breast cancer; HER2 type, BP and 5NP. The prognostic value of each variable was studied in all tumours, and in each subtype separately.

Paper III: To critically evaluate the impact of field area size in the assessment of MVD, and to identify the field area size that provides the most accurate prognostic information in breast cancer patients.

4 MATERIALS & METHODS

4.1 The study population

The work presented in this thesis has been carried out as part of the Breast Cancer Subtypes project (BCS), which was established in 2009. One of the aims of the project is to combine population-based data and molecular pathology methods to gain a better understanding of tumour biology and prognostic markers in breast cancer. The project encompasses information about three cohorts of patients with breast cancer. This thesis is based on the first cohort, where we have information about 909 breast cancer patients and their tumours. Prior to the works of this thesis, information about the study population was linked Norwegian central health registries (168), and tumours were reclassified into histopathological type, grade and molecular subtype (23). These steps will be briefly described here. However, the present thesis had no influence on how the steps were executed.

From 1956 to 1959, all women between 20-69 years of age in four Norwegian counties were invited to participate in a survey for early breast cancer diagnosis. In the survey, women received a clinical examination from a physician and answered a questionnaire-based interview about reproductive factors. The questionnaire gathered information about age at menarche, first birth, last birth and menopause, about the number of abortions and pregnancies, the period of lactation, and occurrence of disease of the breast or genital organs. Women were followed for breast cancer occurrence if they had no previous history of breast cancer, and no breast cancer detected at the time of the survey (168, 169). This cohort is restricted to the 25,727 women from Nord-Trøndelag, born between 1886 and 1928 (62). They were followed from January 1st, 1961, to December 31st, 2008, through data linkage to the National Registry, the Cancer Registry of Norway and the Norwegian Cause of Death Registry (168).

1,379 women developed breast cancer (62). Of these, tumour tissue from patients diagnosed and treated at other hospitals were not available. For the remaining 945 cases, pathology reports and FFPE tissue from the first primary tumour was available from the Department of Pathology, St Olav's Hospital, Trondheim University Hospital (23).

Thirty-six cases were excluded due to poor quality or wrong diagnosis, leaving 909 primary breast carcinomas to be reviewed and subclassified according to histopathological type, grade and molecular subtype. After diagnosis, women had been followed until death from breast cancer, death from other causes or December 31st 2010, whichever came first (23). Eighty-five percent of the 909 women had been followed for the rest of their lives after breast cancer diagnosis, and 15% were alive at the end of follow-up.

The 909 women were an ethnically homogeneous group. Most of the patients in this cohort did not use hormonal contraception or hormone replacement therapy. None of them were included in the national mammography screening programme because of their age. Individual information about patient treatment beyond surgery is not available to the research group, but the greatest proportion of patients were likely have been treated with surgery only due to either age at diagnosis or diagnosis during periods when adjuvant treatment was limited or not available. This makes it possible to study the course of disease progression after surgery without iatrogenic systemic influences, in a group of patients with near complete follow-up time.

4.2 Norwegian national registries

All Norwegian residents and people born in Norway have a unique 11-digit identity number, which facilitates linkage of information about individuals across several national registries, including central health registries. Through Norwegian laws and regulations, there is an obligation to report to these registries (170-173), and this makes them close to complete (174). For the 909 women in the study population, information was collected from the following national registries.

4.2.1 The National Registry

The National Registry plays an important role in sustaining the rights and obligations of citizens, and comprises information including birth, names, address, death, and citizenship. Information from The National Registry is used by many institutions and organisations, including the tax authorities and other public authorities, researchers, employers, banks and insurance companies (175). Information about birth, death and emigration, which was extracted from the Cancer Registry of Norway for our study population, was based on records from the National Registry.

4.2.2 The Cancer Registry of Norway

The Cancer Registry of Norway was established in 1951, and collects data on cancer occurrence in Norway. In addition, the registry administers the national cancer screening programs, performs research and develops statistics (176). Every year, they publish a report on cancer incidence, prevalence and mortality in Norway (7). Overall completeness was 98.8% from 2001-2005 (174). Information about breast cancer diagnosis and date of diagnosis for the study population was extracted from the Cancer Registry of Norway.

4.2.3 The Norwegian Cause of Death Registry

The Norwegian Cause of Death Registry keeps a record of all deaths in Norway and of Norwegian citizens who die abroad, and comprises data from as early as 1951. Doctors are required to write a death certificate according to the WHO International Classification of Diseases, and this certificate is the basis for the official statistics on cause of death (177). Date and cause of death for the study population were extracted from the Cancer Registry of Norway because of linkage to Norwegian Cause of Death Registry.

4.3 Reclassification and subtyping

The reclassification and subtyping of the 909 breast cancers was done by other researchers in the BCS project, and has been thoroughly described by Engstrøm et al (23). New HES-stained sections were made from each tumour. Tumour size was registered and classified into three categories based on the TNM classification (≤20 mm, >20 and ≤50 mm, >50 mm) (12, 41), if measurements of the whole tumour were present in the pathology report, or if the full tumour diameter was present for measurement on the glass slide. When size was not certain, the tumour was measured on the glass slide so that the minimum tumour size was known for all cases. The 20 mm cut-off was used, and all cases with a minimum known tumour size of 20 mm were classified as 'uncertain, but ≥20mm'. However, for the cases with a smaller tumour size on the glass slide, it was not possible to exclude that they could have been larger elsewhere in the tumour. Therefore, these cases were classified as unknown (n=277, 30.4% of all tumours). Tumours were classified according to histopathologic type (13) and grade (19) by two pathologists independently. In cases of disagreement, sections were discussed and consensus was reached. Areas in the tumour periphery for tissue microarray (TMA) construction were marked by a pathologist. Before TMA construction, 15 sections were cut from the paraffin blocks and stored in the freezer.

Tissue Arrayer MiniCore® 3 with TMA Designer2 software (Alphelys, 78370 Plaisir, France) was used for TMA construction. Three 1 mm in diameter tissue cores of were extracted from each tumour and inserted into TMA recipient blocks. From the TMA blocks, sections of 4µm were cut and stained with HES, IHC and chromogenic ISH. IHC was used to stain sections for ER, PR, HER2, Ki67, CK5 and EGFR (Figure 15), and chromogenic ISH was used to assess *HER2* copy number. The HES and IHC slides were scanned using the tissue scanner Ariol™ SL-50 3.3 Scan system and analysis station (Genetix). Expression of ER, PR, HER2, Ki67, CK5 and EGFR was annotated by two researchers independently at the Ariol review station. A bright field microscope was used for determining *HER2* copy number. Cases were discussed and consensus was reached in cases of disagreement (23).

Table 1 Sources and dilutions of primary antibodies

Antibody	Clone	Manufacturer	Concentration of antibody	Dilution
ER	SP1	Cell marque	33 mg/ml	1:100
PR	16	Novocastra	360 mg/l	1:400
HER2	CB11	Novocastra	3.9 g/l	1:640
Ki67	MIB1	Dako	35 mg/l	1:100
CK5	XM26	Novocastra	50 mg/l	1:100
EGFR	2-18C9	Dako	Ready to use	No dilution

Figure 15. Sources and dilutions of primary antibodies. Oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), Cytokeratin 5 (CK5), epidermal growth factor receptor (EGFR). Reprinted from Breast Cancer Res Treat, 2013, Vol 140, Engstrøm et al, *Molecular subtypes, histopathological grade and survival in a historic cohort of breast cancer patients* (23), page 465, Table 1. Paper distributed under a Creative Commons license, and can be used for non-commercial/educational purposes without obtaining additional permissions from Springer Nature.

Cut-offs for positivity were based on guidelines at the time of subtyping (178). A section was considered positive for ER and PR if at least 1% of tumour nuclei displayed positive staining (10). For Ki67, 500 tumour nuclei were examined and the percentage of positively stained nuclei noted. If 15% or more of the tumour nuclei displayed positive staining for Ki67, the section was considered to be Ki67 high (57, 178). A tumour was HER2 positive if the *HER2* gene to chromosome 17 ratio was ≥2. In cases with unsuccessful chromogenic ISH, IHC was used (179). For CK5 and EGFR, there were no international guidelines for scoring. A staining index (SI) taking both staining intensity and the proportion of positively stained cells into

account was used. SI was calculated by multiplying intensity score with proportion score, and a value of 2 or higher was considered positive (23).

Based on their expression of the six biomarkers, breast cancers were classified into molecular subtypes according to Figure 9 (23). The reclassification complied with the REMARK recommendations for tumour marker studies (180).

4.4 Selection of cases

In Paper I, we considered MVD, pMVD and VPI in BP and Luminal A tumours. Of the 909 primary breast carcinomas in the cohort, 63 cases were classified as BP and 433 as Luminal A. To study the impact of molecular subtype, we attempted to make the groups as similar as possible with respect to other features that may also influence biology and prognosis. Therefore, one Luminal A case was selected from the cohort to match each BP case. The subtypes could not be matched for tumour size because 17 BP tumours were classified as uncertain. Instead, sections were matched for histopathological grade. Of the BP cases, there were 52 grade 3, seven grade 2 and four grade 1. There were only 42 grade 3 Luminal A tumours in the whole series, so ten grade 3 BP tumours were matched with ten grade 2 Luminal A tumours. One BP grade 1 tumour had to be excluded due to poor IHC staining. Therefore, although MVD, pMVD and VPI had been registered for four Luminal A grade 1 cases, one of the cases was randomly chosen for exclusion prior to statistical analyses. Among the 124 cases included in the analyses, average grade for BP cancers was 2.8, compared with 2.6 for Luminal A cases.

All non-luminal cases were included in the study of MVD, pMVD and VPI in Paper II. Of the 151 cases eligible for the study, 61 were BP, 60 were HER2 type and 30 were 5NP. After Paper I had been published, other projects using the same main study population discovered two misclassifications in the data set. One case classified as BP in Paper I was HER2 type, and one BP tumour that had been classified as grade 1 was in fact grade 3. This was corrected in Paper II.

Paper III is a methodological study of the field area for MVD assessment, where we aimed to include all cases with MVD data. In total, 212 tumours were included, of which 63 were Luminal A, 61 BP, 61 HER2 type and 27 5NP. We included the fourth Luminal A grade 1 case that had been excluded in Paper 1, and one HER2 type case that had been misclassified and

therefore not included in the analyses of Papers I-II even though vasculature had been quantified. Lastly, three 5NP cases were excluded because they had been counted on another microscope with a different visual field area, and therefore not eligible for the field area size study.

4.5 Immunohistochemistry

For the present study, full-face tumour sections were used. IHC was carried out in accordance with the method used by the Centre for Cancer Biomarkers (CCBIO) at the University of Bergen, Norway (127). Tissue sections were cut at 4µm from FFPE tumour tissue and mounted on Superfrost+ slides. Tumours had been sectioned prior to TMA construction, and slides had been stored in airtight boxes in a dark freezer at -20°C for one year (Luminal A and BP cases) or two years (HER2 type and 5NP cases) years. After retrieval, sections were put into a heating cabinet at 42°C overnight.

For paraffin removal, sections were immersed in Tissue Clear three times, for five minutes each time. Sections were rehydrated by being immersed in 100% ethanol twice for three minutes at a time, followed by 96% ethanol twice for two minutes at a time, then 80% ethanol once for three minutes, and lastly H_2O three times, for five minutes each time. Heat Induced Epitope Retrieval was performed in a Pre-Treatment link. Sections were immersed in Dako Target Retrieval Solution buffer pH 6, S1699 (DAKO) at 80°C. Temperature was increased to 97°C and maintained for 20 minutes before cooling.

After pre-treatment, sections were immersed in Dako Wash Buffer, S3006 10x diluted with deionized water (dH_2O) at room temperature two times, for three minutes each time. The wash buffer was used for five minutes between each step throughout the immunostaining process.

Indirect, automated immunostaining was done at room temperature, using Dako Autostainer Plus (DAKO Denmark A/S, Produksjonsvej 42 DK-2600 Glostrup, Denmark). Dual Endogenous Enzyme Block, S2003 (DAKO) was used for eight minutes for enzyme blocking. Reagents were mixed and diluted before immunostaining. Antibody diluent (DAKO S0809) and primary antibodies were blended into a cocktail. The primary antibody rabbit von Willebrand factor (Polyclonal rabbit, A0082, DAKO) was used at concentration 3.8µg/L, and mouse Ki67 (Monoclonal, clone MIB1, M7240, DAKO) at 160µg/L (Table 3). Sections were

incubated with the cocktail of primary antibodies for 60 minutes. The EnVision Detection System-Peroxidase/rabbit, K4003 contained a large sugar polymer onto which the secondary antibody for vWF and the envision molecule Horseradish Peroxidase (HRP) were hooked. Southern Biotech alkaline phosphatase/goat anti-mouse for Ki67 was added to the detection system for vWF, diluted 1:100. The detection system mixture was incubated for 30 minutes. Ferangi Blue Chromogen (BIOCARE medical, 4040 Pike Lane, Concord, CA 94520 USA) diluted with Ferangi Blue Buffer was used for Ki67 visualisation and incubated for 15 minutes. Next, sections were rinsed three times with dH_2O before they were incubated with amino-ethyl-substrate chromogen (DAKO) for 15 minutes to visualize vWF. Lastly, sections were rinsed twice with dH_2O and put into lukewarm water before coverslipping with Dako Faramount aqueous medium (DAKO). vWF positive cells displayed reddish-brown cytoplasm and Ki67 positive cells displayed blue nuclei (Figure 16). Primary antibody performance was evaluated by using tonsillar tissue, which contains both vessels and proliferating cells, as positive controls. In addition, blood vessels from adjacent normal tissue functioned as internal controls for vWF, and proliferating tumour cells worked as positive controls in a large proportion of the cases. As negative method controls, IHC was performed as described above, except the step with primary antibodies was omitted. Compared to the stained sections, there were no staining for Ki67 or vWF in the negative control.

Table 3 Sources, dilutions and detection systems for primary antibodies

Antibody	von Willebrand factor	Ki67
Host animal	Rabbit	Mouse
Clone	Polyclonal	MIB1
Manufacturer	DAKO	DAKO
Concentration	3.8µg/L	160µg/L
Dilution	1:800	1:50
Detection system	EnVision Detection System-Peroxidase/rabbit	Southern Biotech alkaline phosphatase/goat anti-mouse
Substrate for detection system	Amino-ethyl-substrate chromogen	Ferangi Blue Chromogen

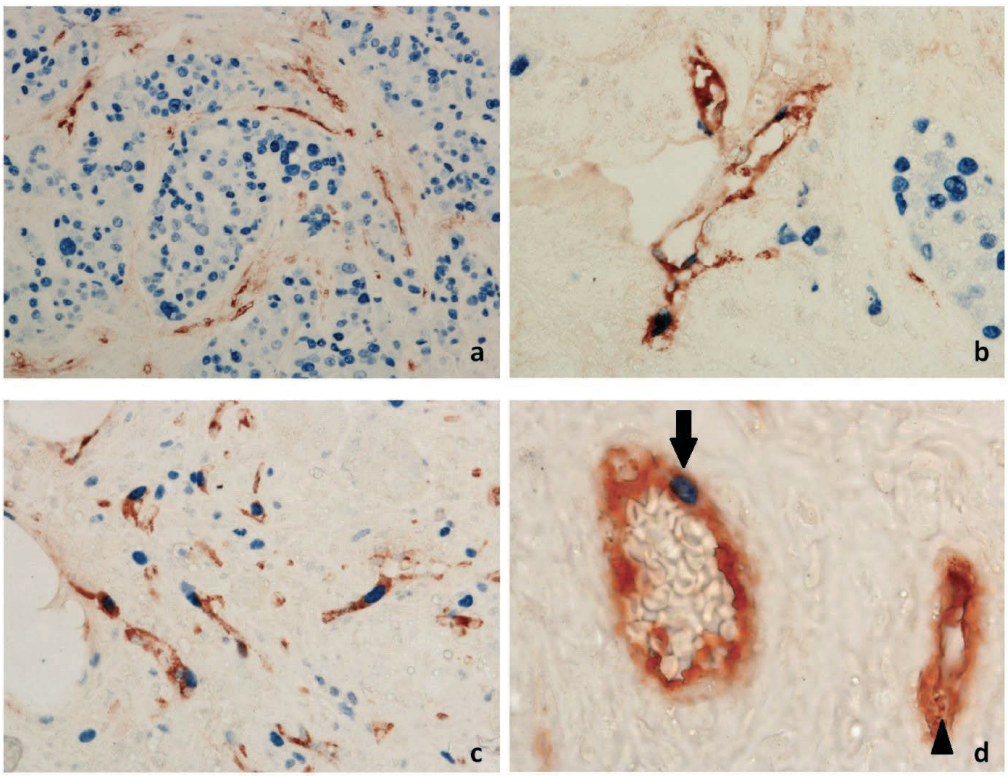

Figure 16. Breast cancer section stained with immunohistochemistry for von Willebrand factor and Ki67. Endothelial cells display brown staining for von Willebrand factor and proliferating nuclei display blue staining. a and b: Same case, HER2 type. a: 200x. b: 400x. c: HER2 type case(400x). d: HER2 type case (1000x). One proliferating vessel with a lumen filled with erythrocytes (arrow) and one non-proliferative vessel (arrowhead). Photo: AMB/MRK, NTNU.

4.6 Scoring and reporting

In this thesis, MVD, pMVD and VPI have been quantified as described by the CCBIO research group in Bergen (119, 127-129). Full-face sections were used since it is paramount to see the topography in a tumour when assessing vessel density. Furthermore, TMA cores in the material had not been taken from vascular hotspots and would not have provided the sufficient amount of tumour tissue necessary for vessel counting. For assessment to be feasible, sections had to be high quality with distinct microvessel staining and low background staining.

All cases were assessed by one observer (MRK) who had undergone prior training on a test series of 24 colon cancer sections, which had been previously scored by a co-author experienced in microvessel counting (KK) (181). First, the two observers studied and counted vessels under a conference microscope, so that the observer in training could be guided and

corrected in the identification of vessels. Then, the observer in training independently counted vessels and proliferating vessels in ten fields within preselected hotspots. To estimate intra- and interobserver agreement for MVD, pMVD and VPI, we used Kappa (κ) and Spearman rho (ρ). Spearman's rank correlation coefficient was used to rank the vessel counts of the two observers in order to estimate the linear relationship between them. Values of ρ range from -1 to +1, where +1 would indicate a perfect linear relationship between two vessel counts and 0 would indicate no linear association (182, 183). The Kappa index was used to calculate the extent of agreement between observers, beyond what could be expected due to chance. If κ=0, agreement is the same as would be expected by chance, and a positive value implies concordance greater than chance. The strength of agreement is considered good if $0.61 \leq \kappa \leq 0.80$, and very good if $0.81 \leq \kappa \leq 1.0$. A value of κ=1 signifies complete concordance (183, 184). The desired level of agreement was defined as $\rho > 0.8$ and $\kappa > 0.6$, and training was ended when this was achieved consistently over time.

Prior to vessel counting, two authors (AMB and MRK) identified hotspots by systematically examining the whole IHC section at low magnification (20x, 100x) and, since the IHC slide had no counterstain, correlating it with the corresponding HES-stained section to ensure that the chosen vascular hotspot comprised a sufficient amount of invasive breast carcinoma. In cases with scanty tumour tissue, several smaller vascular hotspots with sufficient tumour tissue were selected instead of one large hotspot.

The number of microvessels and proliferating microvessels were counted at high magnification (400x) in 10 visual fields within the hotspot. Whenever possible, vessels were counted in consecutive visual fields, and we attempted to include the visual fields with the presumed highest vessel density first. Areas with sclerosis, fibrosis, necrosis and normal breast tissue were avoided, and every counted field contained at least 50% tumour tissue (119, 129).

A vascular unit was defined as a vWF positively stained endothelial cell or cell cluster, and a vessel lumen was not necessary (88). However, in cases with microvascular bundles or long endothelial branches, each lumen or staining discontinuation was counted as a separate vascular unit. Structures with a 90° angle were defined as two vessels, also when exhibiting continuous staining (119, 129). A proliferating vessel was defined as a vascular unit containing at least one nucleus with distinct staining for Ki67, regardless of staining intensity.

Ki67-positive staining outside the endothelial cell or inside the vessel lumen was not accepted as part of a proliferating microvessel (119).

The individual vessel count for each visual field was recorded. MVD and pMVD values were given as the average number of vessels per square millimetre in the tumour hotspot. These values were estimated by dividing the average number of vessels per visual field by the visual field area of the microscope. VPI was defined as the ratio between pMVD and MVD, and given in percentages. The studies were done in compliance with the REMARK reporting recommendations for tumour marker studies (180).

4.6.1 Sorting strategies and MVD calculation according to field area

The field area of one visual field was 0.17mm^2, so MVD was assessed in a total field area of 1.73mm^2 in every tumour. In Papers I-II, we used the average number of vessels from ten visual fields only. In Paper III, however, individual vessel counts from the ten visual fields in each tumour were sorted in three different ways: 1) MVD-Consecutive, where the visual fields were organised in the original sequence in which they had been included; 2) MVD-Decreasing, where visual fields were arranged from highest to lowest vessel count; and 3) MVD-Random, where the visual field order was random. Then, for each sorting strategy, the average vessel counts per mm^2 were computed for each cumulative field area by successively including vessel counts from one additional visual field at a time.

4.7 Statistical analyses

4.7.1 Point estimate, p-values and confidence intervals

In statistical analyses, there are three important measures to be considered. The point estimate is the measure of effect, for example a hazard ratio (HR) or a mean difference between groups. The greater the point estimate, the stronger the association. A p-value is the probability that the observed data distribution or a more extreme distribution should arise, if there is truly no association between the variables being studied. The cut-off for statistical significance is usually defined as a p-value smaller than 0.05. The confidence interval (CI) of the test statistic assesses both statistical significance and the precision of the results. If a 95% CI, which describes the same statistical significance level as a p-value of 0.05, does not include the HR value 1, the association is statistically significant. The narrower the CI, the more precise the point estimate (185).

4.7.2 Causality versus prediction

Statistical analyses can be causal or predictive. When the aim of a study is to assess whether an exposure causes the outcome, i.e. whether the expression of a specific biomarker causes breast cancer death, a causal analysis is performed (183). However, if the attempt is to study whether the biomarker can be used to predict breast cancer death in patients, a predictive analysis is more suitable. In other words, to identify a prognostic marker, one performs a predictive analysis. This is not to be confused with the distinction between a prognostic and a predictive marker described one page 13.

When studying whether a biomarker adds prognostic information beyond that which is already known in predictive analyses, it is necessary to adjust for established prognostic markers. In studies of causal relationships, adjustments should be made for confounders, which are common causes of both exposure and outcome. Adjustments should not, however, be made for mediators, which are factors that are influenced by the exposure, and also influence the outcome.

4.7.3 Linear regression

In Papers I-II, linear regression was used to study the mean difference in MVD, pMVD and VPI (dependent variables) between molecular subtypes (independent variable). Adjustments were made for the categorical variables age (<65, 65-79, ≥80 years), grade (1, 2, 3) and stage (I, II, III-IV). Age was also adjusted for as a continuous variable, and this did not alter the results. In Paper II, adjustments were done for stage as three and four categories, and results were nearly identical.

4.7.4 Chi-squared tests

Chi-squared tests can be used to analyse associations between variables in a contingency table, usually two, by comparing the observed frequencies or proportions in each cell to the expected frequencies or proportions if there had been no relation between the variables. The greater the difference between the observed and expected data, the smaller the p-value (186). Chi-squared tests were used in Paper III to compare clinicopathological features to MVD, which was made a categorical variable by using the median value as cut-off 'high' and 'low' MVD.

4.7.5 Survival analyses

To study the amount of time to a specific event, we use survival analysis. These analyses can be used to determine treatment effect, to study time to disease, or time to death from a disease. An advantage of survival analyses, is that they can be used in situations with incomplete information on time to event, as described below (187). The aim of survival analysis is to use time data to compute, compare and interpret survival and/or hazard functions, and to study their relations to explanatory variables. There are in principle two outcomes; failure, which in this thesis is death from breast cancer, and censoring. The outcome variable in question is the time to death from breast cancer. For the censored cases, the exact time to death from breast cancer is not known, but some information about survival time is available (188). In the present study, reasons for censoring were end of follow-up (December 31st 2010) and death from other cause. Since patients only have one cause of death, the latter is defined as a competing event (189).

4.7.5.1 Kaplan-Meier methods

Kaplan-Meier survival curves with log-rank tests are a commonly used method to estimate survival functions, and were used in Paper I. The Kaplan-Meier function is a step function for cumulative survival over time, which describes the probability, from 1 to 0, of survival beyond a specified time, t (188, 190). With log-rank tests, overall comparisons of the survival curves in the same plot are performed (190). In Paper I, MVD, pMVD and VPI were classified into groups of 'high' and 'low', using the common median value for all cases as cut-off. Curves were created for all cases, and, for MVD, within each subtype separately.

4.7.5.2 Cumulative incidence

Many survival analyses assume that censoring is independent. In a situation of independent censoring, for each subgroup to be studied, the survival of cases censored at a specific time is representative of the survival of all the remaining cases in the subgroup that were not censored (188). In situations where competing risks are present, it is not possible to reliably determine whether the independence assumption is valid or not. If censoring is non-independent and we perform an analysis assuming independence, results may be biased, so that the risk of death may be overestimated. Since the Kaplan-Meier survival analysis assumes independent censoring, it may be subject to bias and less suited for survival analyses in situations with competing risks. Instead, we can use a cumulative incidence curve (CIC), which uses marginal probabilities and does not require independent censoring (189).

The cumulative risk of dying from breast cancer can be interpreted as the risk of dying from breast cancer before dying from other causes, and provides a more accurate survival estimate in situations with competing risks. (189, 191). Gray's test can be used to compare the equality of CICs in the same plot (189). In Paper II, CICs with Gray's test were used instead of Kaplan-Meier curves to account for competing risks. CICs were constructed for all cases, using the common median value as cut-off for 'high' and 'low' MVD, pMVD and VPI. For MVD, one CIC was made by separating cases into four groups, based on the 25th percentile, median value and the 75th percentile. We also made CICs for each subtype separately, using the median of each subtype as cut-off.

4.7.5.3 Cox Proportional Hazards Model

Unlike the KM plot and CIC, the hazard function does not describe the proportion that actually dies from breast cancer. Rather, it describes the momentary potential for dying from breast cancer per time unit, given survival up to that time point. If the proportion that actually dies from breast cancer were analogous to a certain distance, then the hazard function would describe the velocity at a given time during follow-up (188). By studying and comparing the hazards of groups that are distinguished by independent variables, the Cox proportional hazards model can be used to obtain a HR, which describes the quotient in hazard between the groups that are being compared (185). In Papers I-III, multiple Cox PH analysis was used to estimate the relative risk of death from breast cancer, given as HR with 95% CI. The HRs of MVD, pMVD and VPI were estimated per one unit increase, where the units were 10 vessels, 1 vessel and 1 percentage point, respectively. By using continuous estimates, we attempted to make results more comparable other studies. In Papers I-II, Cox analyses were done for all cases combined, and separately for each molecular subtype. MVD, pMVD and VPI were also categorised into two groups based on the median value in all cases. In Paper II, this was also done in the HER2 type and BP separately, but not for the 5NP since statistical power was limited by low sample size. In Paper III we focused solely on MVD, and performed independent Cox analyses for every sorting strategy and cumulative field area. After the MVD model that provided the most accurate prognostic formation had been identified, it was used to estimate the risk of death from breast cancer per 10 vessels increase within each molecular subtype.

The PH assumption in the Cox model requires that at any time point, the hazards of the groups compared are proportional, so that the HR is constant over time (185). We used log-minus-log plots and tests that are based on Schoenfield residuals, (192), which confirmed that the PH assumption was met in all analyses.

We adjusted for established prognostic factors in breast cancer. In Papers I-II, adjustments were therefore made for age (<65, 65-79, ≥80 years), grade (1, 2, 3), stage (I, II, III-IV in Paper I and I, II, III, IV in Paper II) and molecular subtype (if applicable). Stage III-IV were merged in Paper I because there were so few cases in that group (n=12). In Paper II, analyses were done adjusting for stage as both three and four categories, and results were similar. In addition to adjusting for all parameters combined, adjustments were made separately for each variable. Since there were no large differences in the point estimate or CIs, results from additional adjustments are not shown. The robustness of the findings was further evaluated by performing adjustments for age as a continuous variable and decade of diagnosis as a categorical variable, and by restricting the analyses to grade 3 tumours only. Furthermore, the 75th percentile was used as cut-off instead of the median in Kaplan-Meier plots and Cox analyses in Paper I. In Paper II, both the 75^{th} and 25^{th} percentiles were used as cut-offs. With the exception of the CIC for MVD shown in Paper II, alternative adjustments and cut-offs did not have any large impact on the results. Therefore, the median was used for optimal power.

In Paper III, adjustments were made for year of diagnosis (continuous variable), age at diagnosis (<60, 60-69, 70-79, ≥80 years), tumour diameter (≤20, >20 and ≤50, uncertain but ≥20, uncertain), lymph node status (negative, positive, unknown), grade (1-2, 3), and molecular subtype. (Luminal A, HER2 type, 5NP, BP). The groups for grade 1 and 2 were so small that we chose to merge them to increase precision. The reason we chose to adjust for tumour size and lymph node status rather than stage, was that we had data on both lymph node status and tumour size, and chose to treat each factor separately rather than together. In the early years of follow-up, stage was based on clinical examination only.

4.7.5.4 Likelihood ratio tests

In Paper III, we attempted to identify the method for MVD assessment that provided the greatest prognostic informativeness. The model with the greatest prognostic informativeness was defined as the one providing the most accurate prediction of death from breast cancer. To identify this model, we performed likelihood ratio (LR) tests, where

the likelihood functions of two Cox models were compared. The likelihood function expresses the total probability of the observed data 'as a function of the unknown parameters'. The unknown parameters are all independent covariates included in the statistical model (185, 193). In every LR-test, we compared two Cox models; one reference model that only included established predictors of breast cancer death (age at diagnosis, year of diagnosis, tumour diameter, lymph node status, grade and molecular subtype), and one model that included all covariates in the reference model plus a continuous MVD variable. An LR test statistic (χ^2) was calculated as twice the difference in log-likelihood between the two models, and described the additional prognostic information obtained by adding the MVD variable to the prediction model for breast cancer death (193). In total, 30 LR-tests were performed; one for every cumulative field area in MVD-Consecutive, MVD-Decreasing and MVD-Random. Since the MVD variable in every LR test had the same unit (increase in vessels/mm^2) and reference model, it was possible to compare the LR test statistics, and thus the prognostic informativeness, of different sorting strategies and field areas. The model that provided the highest LR test statistic, contained the MVD variable with the greatest prognostic informativeness.

STATA (13.1 and 15) was used in all statistical analyses (StataCorp LP, College Station, Texas, USA).

4.8 The Regional Committee for Medical and Health Sciences Research Ethics

To ensure ethically sound research, it is required by law that all medical and health science research projects involving the use of material or data from human beings must be approved by the regional committee for medical and health sciences research (194). There are seven regional committees, which are required to have competence on ethics, law, and relevant fields of research (195, 196).

4.9 Ethics statement

The BCS project has been approved by the Regional committee for medical and health research ethics (REK-Midt Norge, reference nr: 2009/836). A research biobank was established for the project.

The data comprise information from the approximately 22 000 women, and more than 900 cases in the pathology studies. With a data material of this size, obtaining informed consent from all participants would be impossible. The majority of the women were deceased long

before the present study started. REK granted dispensation from the general requirement of informed consent in accordance with § 35 of the Health Research Act.

Confidentiality was strictly maintained throughout the study. Patient identity has been replaced with a case number in the research data file and the linkage key is only available to the principal investigators. No direct contact occurred between researcher and participant. The participants were subject to neither risk nor gain through the research project. Nevertheless, the study may yield important findings regarding breast cancer, which may be of benefit to future generations.

5 RESULTS

5.1 Paper I

In Paper I, tumour vascularity was assessed in the two subtypes of breast cancer with the most distinct gene expression profiles; Luminal A and BP. The aims were to determine the prognostic value of MVD, pMVD and VPI in all cases and each subtype separately, and to establish whether these subtypes differed with regard to MVD, pMVD and VPI.

MVD did not differ between the two subtypes. pMVD and VPI were higher in BP compared to Luminal A. However, the point estimate was low with a 95% CI close to zero, indicating a small difference. After the study was published, it was discovered that one case had been misclassified as BP, but was in reality HER2 type. When the analyses were performed anew, there were no large differences in results for MVD, pMVD or VPI.

No association was found between vascular proliferation and prognosis in analysis of all cases combined, or in the separate subtype analyses. High MVD, however, was associated with poor prognosis in all tumours combined, with increasing risk of death from breast cancer per 10 vessels increase. When the subtypes were analysed separately, MVD was an independent prognostic factor in patients with Luminal A tumours, but not associated with prognosis in patients with BP tumours.

5.2 Paper II

In Paper II, MVD, pMVD and VPI were studied in the non-luminal breast cancer subtypes, which have the poorest prognosis in breast cancer: HER2 type, BP and 5NP. In addition to comparing tumour vascularity between subtypes, the prognostic value of MVD, pMVD and VPI was studied for all cases combined, and for each subtype separately.

MVD was higher in the 5NP compared to both BP and HER2. No differences were found in vascular proliferation between the subtypes. In the survival analyses of all cases combined, high MVD was associated with poor prognosis, regardless of cut-off. High MVD was associated with poor survival in the HER2 type and 5NP, but not in the BP. It should be noted that there were few 5NP cases, and that the statistical analyses in this group lacked power. Nevertheless, MVD remained a strong prognostic factor in all cases combined in spite of its lack of prognostic value in BP. Vascular proliferation was not associated with prognosis.

5.3 Paper III

In Paper III, the size of the field area for MVD assessment was studied with regard to hotspot dilution and prognostic informativeness. After the most informative method was identified, the prognostic value of MVD according to this method was reassessed within each molecular subtype.

For both MVD-Consecutive and MVD-Decreasing, MVD fell with increasing field area. The MVD value that provided the highest prognostic informativeness was obtained by only including the two visual fields with the greatest number of vessels (MVD-Decreasing, field are 0.35mm^2), and this method was 14% more informative than the original method of including 10 visual fields. Vessel counts sorted from highest to lowest provided more prognostic information than counts sorted consecutively or randomly. For MVD-Consecutive, inclusion of four visual fields (field area 0.69mm^2) provided the most accurate prognostic information.

When the most informative method for MVD assessment (MVD-Decreasing, field area 0.35mm^2) was used to study prognosis in each molecular subtype separately, results were in accordance with those of the two previous papers; MVD was an independent prognostic factor in the Luminal A, HER2 type and 5NP subtypes, where risk of death from breast cancer increased per 10 vessels increase. For BP, MVD was not associated with prognosis.

6 DISCUSSION

Breast cancer subtypes defined by IHC and ISH have different survival patterns and appear to be biologically distinct disease entities (22, 23, 30, 31, 57). If we can further describe the biologic characteristics and identify different prognostic markers within each subtype, breast cancer treatment could become even more individualised. Not only could it help in identifying new treatment targets and improve survival, it may also contribute to identifying women with a particularly good prognosis, so that over-treatment with possible, detrimental effects on quality of life, could be avoided.

We have studied MVD, pMVD and VPI in specific subtypes of breast cancer and their associations with prognosis. This way, we provide further knowledge of the biology and prognostic factors in each individual subtype. Furthermore, we have critically evaluated the method employed to estimate MVD.

6.1 Methodological considerations, strengths and weaknesses

6.1.1 Study population and selection of cases

The population's unique features, consists of its large sample-size and long follow-up time. It includes the majority of breast cancers occurring in women in one specific county in Norway born between 1886 and 1928. The women were ethnically homogeneous and thus representative of the majority of the Norwegian population. Reliable information on breast cancer incidence, date and cause of death, were available from high-quality national registries. There was no loss to follow-up since none of the patients emigrated after diagnosis. Eighty-five percent of the total study population were followed for the rest of their lives lives, as were 91% of the women selected for this thesis. Those who were alive at the end of follow-up were at least 82 years old, and had been followed for a median time of 13 years (IQR 9.5-21.9 years). Thus, follow-up was near complete after diagnosis. Because of different exposure before the disease, diagnosis at clinical presentation, older age at diagnosis and different treatment regimens than today, we should be aware that the study population may not be representative of patients diagnosed today. However, these apparent constraints provided us with the opportunity to study patient prognosis and the near natural course of disease progression after surgery.

We selected the Luminal A and BP subtypes in Paper I because these two groups had appeared the most biologically distinct in gene expression analyses (42, 47). Luminal A and BP tumours were matched 1:1 for histopathological grade in an effort to make the subtypes more similar with regard to other factors. As a result, the number of BP tumours determined the total sample size. Unfortunately, a complete matching for grade was not possible since the study material comprised 52 grade 3 BP tumours and 42 grade 3 Luminal A tumours. Nevertheless, the average grade was 2.8 for BP and 2.6 for Luminal A.

The results from the Luminal A cases included in Papers I and III may not be applicable to Luminal A cases of lower grade, since they were not randomly selected. We included all grade 3 Luminal A tumours, and a smaller proportion of grade 1 and 2 tumours. Luminal A tumours are mostly well-differentiated (23, 57), and some IHC subtyping algorithms classify all Luminal, grade 3 tumours as Luminal B (49, 197, 198). Thus, according to some classifications, we have studied a small subset of Luminal B ($HER2^-$) tumours. However, others using PAM50 for subtyping have reported that 23% (48) and 36% (50) of Luminal A tumours were grade 3. Nevertheless, it is likely that Luminal A cases in this thesis represent a more aggressive group of cancers than Luminal A tumours in general, and we should be aware of this when inferring from our results. When comparing the prognosis of patients with Luminal A tumours included in this thesis to those with Luminal A tumours who were not included, the latter had a better prognosis (Figure 17, Gray's test p-value=0.001). When making adjustments for age, tumour size, lymph node status, year of diagnosis and grade, the risk of breast cancer death among Luminal A cases not included was HR 1.00 (95% CI 0.48-2.06) compared to the included cases. This supports the notion that the Luminal A cases included in this thesis are not representative of the Luminal A group as a whole, but that differences in prognosis could be fully attributed to other measured and well-characterized prognostic factors. Our results should be further investigated among Luminal A cases where all grades are more equally represented.

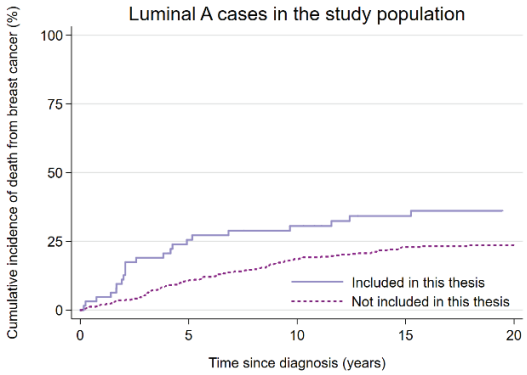

Figure 17. Luminal A cases in the study population. Gray's test p-value=0.001

Non-luminal subtypes are relatively rare (22, 30, 59, 198). The age at diagnosis for the all cases, including non-luminal, in this cohort is higher than that reported in other studies. Nevertheless, the proportion of non-luminal cases in the study population was comparable to others (30, 198). We included all HER2 type, BP and 5NP cases in this thesis.

Taken together, the selected cases in the present thesis represent a more aggressive subset of breast cancers than breast cancers in general. We should be mindful of this when interpreting the results, particularly in Paper III, which was aimed at identifying the best method of assessing MVD in breast cancer, and not in particular subtypes. Our results should therefore be validated in an independent breast cancer series where the subtype proportions are more representative of the proportions in the population.

6.1.2 Storage and fixation

In breast cancer research projects, it is not possible to have both long-term follow-up and control over the pre-analytical conditions. The present thesis is based on archival tumour tissue over a period of fifty years. Pre-analytical conditions and FFPE block storing practises may have varied during this time period. Dowsett et al have shown that antigenicity is for the most part preserved in FFPE tissue over decades (199), and Portier et al showed that cold ischemia time of up to three hours did not have a detrimental effect on IHC and ISH (200).

Antigenicity loss varies between epitopes, and is influenced by light, temperature and air. By storing unstained tissue sections, there is a risk of loss of antigenicity through aging of the section, oxidation of the slide and degradation of biomolecules (99, 201, 202). For Ki67,

which is less sensitive to variations in pre-analytical conditions than other IHC assays in breast cancer (38), there is a risk of decreased immunoreactivity when sections are stored for more than three months in room air (203). To preserve antigenicity, full-face sections that had been cut from tumour tissue before TMA construction were stored in airtight boxes in the dark at -20°C before use. The present study used two robust and well-known biomarkers and the IHC results displayed good visualisation of both. We do not have any reason to believe that antigenicity was reduced due to long-term freezing.

6.1.3 Reclassification and subtyping

Using IHC and ISH, all tumours in the present study had been previously reclassified into molecular subtypes (23). Breast cancers can be classified in multiple ways, and there is no consensus for how this should be done. The aims of this classification were to use the classification algorithm that had the closest assimilation to gene expression analyses, and also associations with prognosis. Therefore, the Cheang method was used for classifying Luminal tumours (57) and the Nielsen method for classifying basal tumours (31, 51). Laboratory work and molecular subtyping for all tumours were done at the same laboratory, using the same antibodies, IHC staining procedures, and algorithm for subtyping. Every marker used for subtyping was assessed by two researchers independently, of whom one was a pathologist, blinded for information on prognosis and other clinicopathological features. In cases of disagreement, consensus was reached after discussion.

6.1.4 Immunohistochemistry

Tumour vessels should be counted in a region with low background staining. Although CD31 and CD34 are known to be more sensitive than vWF, there is an increased risk of staining false positives, such as lymphocytes. We chose vWF because it is a more specific primary antibody with clear distinctions between stained elements and other elements in the tissue (92, 146, 148). vWF was also in use at the CCBIO research group on tumour vasculature in breast cancer, with whom we collaborated (121, 129). Some have suggested that vWF might also stain some lymphatic capillaries (124, 165). However, others point out that tumour vessels may comprise a mix of blood endothelial cells and lymphatic endothelial cells (93), so the distinction between lymphatic vessels and blood vessels may be less important in tumour tissue. For the quantification of tumour vessels contributing to growth and metastasis, vWF stands as a suitable primary antibody that also visualises vessels acquired

through some of the other, non-angiogenic mechanisms. However, since vWF stains endothelial cells regardless of vessel maturity, and some have reported that vWF stains fewer small vessels (134), the antibody may be less suitable for quantifying vascular proliferation. It might have been more optimal to use a primary antibody staining immature vessels, such as nestin or CD105, in the assessment of proliferating vessels. The use of vWF may have led to an underestimation of vascular proliferation. Median pMVD was 1.7 vessels/mm^2 in Paper I and 2.3 vessels/mm^2 in Paper II, compared to 2.0 vessels/mm^2 in a paper by Krüger et al, in which nestin and Ki67 were used (119).

All sections in this thesis were stained with the same antibodies, according to the same IHC procedure, in the same laboratory. To ensure comparability with papers on tumour vasculature from the CCBIO research group, we used the same IHC method (121, 129). The visualisation products used in this thesis (amino-ethyl-substrate and Ferangi Blue) were not soluble in water, so an aqueous medium was used for coverslipping. This medium has a risk of more air bubbles than other mounting media, but there were few air bubbles in the sections in this thesis. If present, they were easily identified and unlikely to have influenced the results. The colour on the IHC sections faded with time, and vessels were quantified shortly after IHC staining.

The IHC staining method used in this thesis did not include haematoxylin for visualisation of nuclei. The corresponding HES slide was used to ensure 50% tumour tissue in all visual fields. However, it was challenging to differentiate a lumen from a non-proliferative nucleus. With training it is possible to differentiate the two, although it is time-consuming and a potential source of error. Subsequently, we identified studies on endothelial cell proliferation in breast tumours using dual immunohistochemistry for CD31/CD34 and Ki67/Bu20a with a haematoxylin counterstain (125, 126). For further studies, we would recommend a combination of dual-colour IHC and background staining for haematoxylin.

6.1.5 Scoring and reporting

The principle challenges of vasculature assessment have been outlined in detail in the background to illustrate sources of variation between studies as a contributor to variability in results about the prognostic value of MVD. Considerations regarding the composition of the study population, variations in pre-analytical and analytical phases have been described in chapters above.

MVD, pMVD and VPI were assessed by one independent observer blinded for information about patient outcome and clinicopathological features, except molecular subtype. To ensure standardisation, the observer had undergone period of training where vessels were counted in preselected hotspots. Her vessel counts were compared to counts made by another, trained observer. When a good concordance and a linear association was achieved between observers over time for MVD, pMVD and VPI, training was ended. However, we acknowledge the possibility of personal bias being forwarded to the next observer in training. For the present thesis, we used 24 sections for observer training. A training set that is too small, or a training time period that is too long, can lead to the risk of involuntary observer image recognition that might introduce bias. A training set that is too large makes the training regimen more time consuming and less feasible in a clinical setting. There is a trade-off between observer variability and training time. BP and Luminal A cases were assessed shortly after training was ended, and all cases included in this thesis were assessed within 18 months.

The vasculature is heterogeneously organised in hotspots (88, 103), and tumour vasculature assessment in breast cancer is complicated by intratumoural vascular heterogeneity. One study compared the highest and average MVD after counting vessels in one visual field in each of three hotspots. They reported that the difference between average and highest MVD was greater than 10% in 61% of cases and greater than 20% in 15% (113). Some found greater variations in MVD within tumours than between tumours (146, 153), and the greatest difference in MVD was between tissue blocks from the same tumour (146). Others concluded that one section is representative of the tumour's vasculature (122, 147). Intratumoural vascular heterogeneity may vary according to molecular subtypes (61, 156). While HER2 type and TN tumours more frequently had higher MVD at the tumour periphery compared to the tumour edge, 86% of the Luminal A tumours had a uniform vessel distribution in the tumour section (61). Similar results were found when comparing the MVD in a group of breast cancers with aggressive features (HER2 type, grade 3, high Ki67 and $p53^{+}$) to a group with nonaggressive features (Luminal A, grade 1 and $p53^{-}$) (156).

We chose to study microvessels in vascular hotspots, since this region is most commonly used for vasculature quantification, and has the greatest potential for providing important information about biology and prognosis (80, 111, 131). Hotspots were identified by two

observers; one was an experienced pathologist, and the other had been previously trained in vessel counting. We ensured that each visual field counted contained at least 50% tumour tissue, which is more stringent than others who recommend including vessels up to the diameter of one 200-250x visual field outside the tumour periphery (131, 165). As described by Weidner et al (88), intratumoural vessel density was the only defining trait of the hotspot. Nevertheless, the vascular hotspot was located at the tumour periphery in 193 (91%) of the 212 cases in this thesis (unpublished data). This is in accordance with others who report that the hotspot is most commonly at the tumour edge (88, 115, 122, 126).

Vessels were counted at 400x magnification because this magnification provides better image detail and makes it easier to distinguish vessels (110, 148, 149, 151, 165). Vessels within the hotspot had been counted in consecutive visual fields whenever possible. However, sometimes consecutive fields could not be ensured because of insufficient tumour tissue, or because it was necessary to avoid necrosis, sclerosis or normal tissue.

A principal issue in quantifying vasculature, is how one should approach the quantification of something that essentially is one large, three-dimensional pipeline in a two-dimensional section. Where does one vessel end and another vessel begin? How can we distinguish one, long vessel from several, small vessels? Figure 18 shows some scenarios that would result in the same IHC staining pattern, although they represent different numbers or types of vessels. When we see three, distinctly stained spots in close proximity of one another, this could be three, small, independent vessels or three vessel branches from the same stem. However, it could also be one, long, tortuous vessel that looks like three vessels because of how the tumour tissue has been sectioned. In addition, it could appear as several vessels in situations with patchy IHC staining, or when the vessel wall contains non-endothelial cells, such as lymphatic endothelial cells (93) or tumour-derived cells (53, 78). There are also opposite scenarios, where vessels form maze-like patterns that make it difficult to distinguish them (149), or they may lie so close to one another that the IHC staining appears continuous. Furthermore, some tumour vessels have traits that make IHC staining weak (149). In addition comes background staining and false positively stained leucocytes or stromal cells. Endothelial cell morphology and the presence of a lumen can help distinguish true vessels from false positives to some degree, but some endothelial cells might also have been sectioned in a way that makes them lack their characteristic shape.

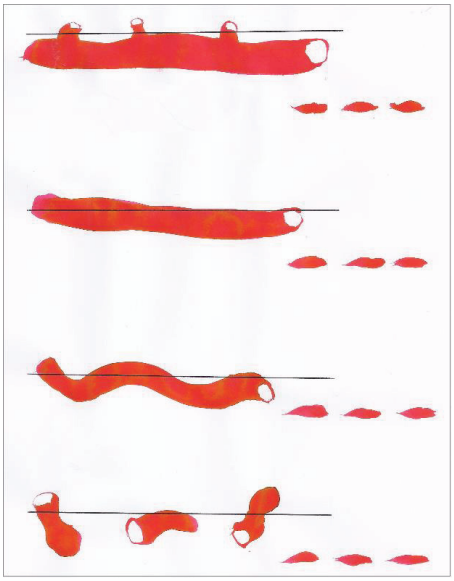

Figure 18. Example of various vasculature scenarios that provide the same immunohistochemistry (IHC) staining pattern. Upper: Three vessel branches from one larger vessel. Depending on where the section was cut, this could result in three vessels or one. Upper middle: One vessel with patchy IHC staining due to technical reasons, or the presence of non-endothelial cells in the vessel wall. Lower middle: Tortuous vessel that on sectioning gives the impression of discontinuous IHC staining. Lower: Several, small vessels. Illustration: S. Kilaas.

In the present thesis, long, vessel-like structures, and positively stained endothelial cells or cell clusters were counted as one vessel. Discontinuation in vWF staining defined a separate vascular unit. In situations with continuous IHC staining, each lumen was defined as a distinct vessel. In cases where one structure with continuous vWF staining had a 90° angle, the structure was considered as two vessels since this could mark the branching of one vessel from another. Both staining intensity and cell shape were used to distinguish endothelial cells from false positives. This set of 'rules' ensured consistency, but we are aware that there still is a possibility of having included false positives, excluded true vessels or counted vessels an incorrect number of times. Others have used different criteria for defining vessel units. For instance, some considered branching or interconnected structures with continuous staining as one vessel (112, 115, 122). In this thesis, it was considered most important that the observer was consistent, so that all vessels were considered as similarly as possible. However, as long as all observers are only consistent with themselves, different studies are not comparable. A set of guidelines would improve consistency and comparability.

There are great variations in field area size for MVD counting (88, 147), and there are no directives determining field area size. Originally, vessels had been consecutively counted in ten visual fields (total field area 1.73mm^2). During the course of our studies, we found that MVD seemed to provide prognostic information in some subtypes and not in others, but we simultaneously realised that our method for MVD assessment was time-consuming and would not be feasible in everyday diagnostics. Therefore, we wished to study whether it really was necessary to include as many as 10 visual fields, or whether we could gain the same or even more accurate prognostic information from MVD by including a smaller field area. Since we had registered every vessel count from the individual visual fields in each tumour, we compared the prognostic informativeness of the original method with ten visual fields to the prognostic informativeness if fewer visual fields had been included. We sorted vessel counts from each tumour consecutively, randomly, and in decreasing order, to compare the prognostic informativeness of 'any' fields within the hotspot to the fields with the highest vessel counts, and to fields counted consecutively.

To make our results transferable to the studies of others, MVD, pMVD and VPI were reported as standardised units (vessels/mm^2 and percent), with continuous variables in the statistical analyses.

6.1.5.1 pMVD

Since pMVD is low in breast cancer (121, 125, 126), it is prone to variation. This should be taken into consideration when interpreting the results. To ensure that the few proliferating vessels actually present in the tissue were counted, it was imperative to include a greater field area. This is an important justification for counting vessels in 10 visual fields in Papers I-II. However, in the current method for pMVD and MVD assessment, the identification of the vascular hotspot is solely based on the number of vessels, and not the number of proliferating vessels (121, 127, 129). One study reported that proliferating endothelial cells were almost exclusively located at the tumour periphery (125). In this thesis, the hotspot was located at the tumour periphery in 91% of the cases (unpublished data). Nevertheless, since the proliferating vessel density was not taken into consideration when a hotspot was chosen, we may have excluded the areas containing the highest number of proliferating vessels and thus underestimated pMVD. This may, in part, explain why vascular proliferation was not associated with prognosis in our studies, and why the differences between subtypes

were small. It is possible that the best methodology for assessing MVD is different from the best methodology for assessing pMVD.

6.1.5.2 VPI

Since pMVD is constantly low and MVD has a greater degree of variation, the values of VPI tend to be determined by MVD rather than pMVD. Therefore, it appears that VPI may not be the most appropriate method for assessing vascular proliferation in breast cancer. However, some report that VPI is a prognostic factor in breast cancer while MVD is not (119, 121), although we could not confirm this in our studies.

6.1.6 Analytical considerations

The tumours in the study population provide a snapshot in time in which we can study tumour biology. Thus, when studying the tumour, all information gained is from that same time point. It is therefore not possible within the frames of this study to know whether subtypes influence vasculature, or vice versa. We can also not know whether the factors we adjust for are common causes for, or a result from, subtypes and vasculature. Furthermore, it is possible that all of these factors may have underlying common causes of which we are unaware. Most likely, both subtype and vasculature are a result of multidirectional signalling in the tumour microenvironment, where several components influence one another in a multi-step way (53). Because of this complexity, caution should be exercised in inferences of the results. In Paper I, we may have drawn conclusions too far when we suggested that BP tumours might be more susceptible to antiangiogenic treatment, based on our finding that BP tumours had higher vascular proliferation than Luminal A. This is claim would need to be substantiated in other studies.

The aims of the survival analyses were to study whether MVD, pMVD or VPI provide additional prognostic information beyond what is already known through conventional prognostic factors. Therefore, in the adjusted Cox model, we included several established prognostic factors simultaneously. In Papers I and II, we also made adjustments for each covariate separately, but this did not alter the results. In doing so, we have made allowances for causal analyses as well, although we have not drawn any conclusions on causality from our results due to the complexity of tumour biology discussed above. Our inferences are only from the predictive analyses, i.e. whether MVD, pMVD and VPI provide additional prognostic information in breast cancer.

In Paper III, the main aim was to study the prognostic informativeness of MVD in all breast tumours. Molecular subtypes were less important. We included all cases with information about MVD to increase sample size. The greater the sample size, the more additional prognostic factors can be adjusted for without critical loss of precision. In addition to the prognostic factors adjusted for in Papers I and II, we now added year of diagnosis and adjusted for tumour size and lymph node status rather than stage.

In every step of a study, there is a risk of random misclassification of information. However, the resulting non-differential information bias would lead to a more conservative effect estimate and therefore cause type-II-errors rather than type-I-errors. In the BCS project, observers were blinded for clinical outcome during annotation, which made it more likely that misclassifications had been random if they occurred.

6.2 Discussion of the main results

6.2.1 Vasculature in the molecular subtypes of breast cancer

Folkman stated that tumours do not exceed the size of 1-3 mm without angiogenesis (67-69). However, as time has passed and non-angiogenic methods for vessel recruitment have been identified, it might have been more precise to say that tumours do not exceed 1-3 mm without tumour vasculature. It should be noted that Folkman conducted most of his experiments at earlier time points in tumorigenesis than observational studies, which mostly study tumours that are larger than 1-3 mm.

Although endothelial cell proliferation has been reported as 45x higher in breast tumour tissue compared to normal breast tissue, the absolute differences were smaller (labelling indices 2.7% and 0.06%, respectively) (126). Regardless of measuring method (LI, pMVD, VPI), vascular proliferation appears to be to be generally low in breast cancer (121, 125, 126), and this is in accordance with our results. Nevertheless, breast tumours have plenty of tumour vessels, unevenly distributed in vascular hotspots (88, 103). One study comparing six cancer types reported that vascular proliferation in breast cancer was lower than in glioblastomas and renal cell carcinomas, and higher than in lung carcinomas and prostate carcinoma. Furthermore, microvessel pericyte coverage index, which has been used as a measure of tumour vessel maturation, was highest in breast cancer (mean 67.3%) (106). High pericyte coverage index and generally low vascular proliferation could imply that breast cancers may not be among the most angiogenesis-dependent cancers. However, it is also

possible that angiogenesis may have occurred at an earlier time point during tumour development, and was therefore no longer detectable at the time of tumour extirpation. Patients in this cohort were not subjected to screening, and tumours were discovered at a later time in disease progression, when they were considerably larger than 1-3 mm. We should be mindful when we are conducting our analyses that describing the initiation of gaining access to vasculature is beyond the scope of the study. Nevertheless, it is possible that different molecular subtypes of breast cancer have distinct mechanisms for acquiring access to vasculature. The results from this thesis imply that vascular proliferation may be somewhat higher in BP tumours compared to the Luminal A tumours, and that 5NP tumours had higher MVD than both HER2 type and BP tumours. It would be interesting to study the role of other, non-angiogenic mechanisms for gaining access to vasculature across the differing molecular subtypes of breast tumours.

The BP is known to be poorly differentiated and clinically aggressive (22). It is more likely to metastasise to lung and brain tissue than non-BP subtypes (204), and it has been suggested that BP tumours have a more haematogenous way of spreading (22, 60). Yet, assessment of tumour vasculature did not provide prognostic information in this subtype. Compared to Luminal A, vascular proliferation was higher in BP tumours, although the differences were small. A possible explanation for this, and the lack of difference in MVD between the two subtypes, is that the Luminal A tumours in this thesis represent a more aggressive subset than Luminal A tumours in general. There were no differences in vascular proliferation between BP and the other, non-luminal tumours. Others have found that the BP was more likely to have high VPI compared to Luminal tumours (129) and non-BP tumours (119). One small study reported that median vascular proliferation and MVD was higher in tumours that were TN and/or basal marker positive compared to Luminal A tumours (Δ1.4 proliferating vessels and Δ6 vessels), although they reported no standard deviation or statistical test for this observation (161). Another study did not report any profound differences in vascular proliferation between subtypes. However, MVD seemed to be higher in the HER2 type and TN tumours compared to luminal tumours. They did not classify TN tumours into BP and 5NP (159). Others comparing subtypes have reported that MVD was highest in TN tumours (160) or the HER2 type (108). Several studies have failed to find any statistically significant differences in MVD between molecular subtypes (154, 155, 158), nor any association

between MVD and basal marker expression (154, 155). In this thesis, BP tumours had markedly lower MVD than 5NP tumours, but there were no differences compared to Luminal A and HER2 type tumours. Thus, our results imply that BP tumours might have higher vascular proliferation than some other subtypes, but not a higher vessel count. It would be interesting to study whether BP tumours have other mechanisms for gaining access to vasculature and the haematogenous metastasis route. VM, for instance, may facilitate tumour cell dissemination without vessel density increase, and typically occurs in highly aggressive tumours. Since tumour cells and CSCs form the vasculature (74, 78), these vessel-like structures might not have been discovered with the IHC method employed in the present study.

Although some found that the 5NP subtype appears to be a distinct breast cancer subtype with poor prognosis (23, 30), it could also be a group of misclassified tumours because of false negative IHC staining. However, that does not comply with the IHC results of the present thesis, where all 5NP cases had positive staining for vWF, and have shown positive nuclear staining for at least one primary antibody (unpublished data). The 5NP appears to be a small group of tumours, and little is known about the biology and prognostic markers for this subtype. The only systemic adjuvant treatment option today for patients with 5NP, is chemotherapy (6). Little is known about the biology and prognostic markers for this subtype. This thesis provides additional information about 5NP biology by reporting that the 5NP has higher MVD than the BP and HER2 type, both of which are described as aggressive subgroups of breast cancer (22). Our results also imply that MVD may be a prognostic factor in patients with 5NP tumours. Another study that separated TN tumours into BP (n=20) and a group negative for all markers (n=11) did not report any differences in MVD between the two (25.5 vessels and 21.9 vessels, respectively) (155). In this thesis, vascular proliferation was not increased in 5NP tumours compared to BP and HER2 type. There are several mechanisms whereby tumours gain access to vasculature without endothelial cell proliferation, such as co-option and intussusception (71, 72, 76), and their role in 5NP tumours should be further studied. However, as described above, our results may also reflect that angiogenesis occurred earlier during tumorigenesis. Furthermore, the number of 5NP tumours in this study was small, and our results should be validated in a larger sample size.

Results from previous studies have implied that the BP and 5NP subtypes are distinct groups with different survival patterns (23, 30, 31). In current recommendations for breast cancer diagnostics (6) and several studies (108, 154, 158-160), BP and 5NP tumours are clustered together as TN. In doing so, others found no difference in MVD between subtypes (154, 158), that MVD was higher in TN tumours, and/or in HER2 type tumours (108, 159, 160). In our thesis, the 5NP had the highest MVD of all non-luminal subtypes, and BP had the lowest. Furthermore, MVD was a prognostic factor among women with 5NP tumours, but not for women with BP tumours. This would not have been discovered if we had studied the TN group as a whole. Thus, our results imply that the two groups are distinct, both with regard to biology and prognostic factors.

6.2.2 Vascular proliferation and prognosis in breast cancer

Arnes et al studied the prognostic value of MVD, pMVD and VPI in three series of breast cancer patients (121). In all series, high VPI was associated with poor prognosis in univariate analyses, and VPI was an independent prognostic factor in two series. pMVD was associated with survival in univariate analyses (121). These results differ from those of the present thesis, where neither pMVD nor VPI were associated with prognosis. Our laboratory followed the same immunostaining and counting procedure as Arnes et al, and the first author underwent training under the aegis of that research group prior to vessel counting. A possible explanation for the discrepant results, is that studies had different study populations, so the cases studied by Arnes et al may have other phenotypes compared to those in the present thesis. This is supported by the differing degrees of vascular proliferation in these studies. For Arnes et al, cut-off values for high pMVD ranged from 0.48 to 1.9/mm^2, and for VPI from 0.95 to 3.3% (121). In the present thesis, median pMVD was 1.7/mm^2 and 2.3/mm^2, and median VPI was 2.3% and 3.6% in Papers I and II, respectively. Arnes et al did not classify specimens into molecular subtypes.

Krüger et al studied MVD, pMVD and VPI in 178 breast cancers, with emphasis on VPI and the BP. They classified tumours into BP or non-BP tumours, and 18/178 cases were BP when using the same classification as for the present thesis (119). High VPI (defined by the upper quartile) was associated with poor survival in adjusted and unadjusted analyses, and more likely to be found in interval breast cancers than in screening detected breast cancers. Krüger et al used nestin, and not vWF (119). Therefore, it is possible that they were able to

identify the true ongoing angiogenesis in the tumours to a greater degree than us. However, they may not have been able to identify older tumour vessels contributing to tumour growth and metastasis through other, non-angiogenic mechanisms. This may have contributed to their finding that MVD had no prognostic value.

6.2.3 MVD and prognosis in breast cancer

The only meta-analysis of the prognostic value of MVD in breast cancer is from 2004. It included 8,936 patients from 43 studies, and found that high MVD was associated with poor prognosis with a risk ratio (RR) 1.54 (95% CI 1.29- 1.85) (124). More recent meta-analyses have been performed on other cancer types. Increased MVD was, for instance, associated with poor prognosis in oesophageal squamous cell carcinoma (ESCC) (109), gastric cancer (GC) (151) and endometrial cancer (EC) (150). Meta-analyses are important because they combine the results from many small, individual studies (124). However, they are based on population-level data rather than individual patient-level data, and have a risk of publication bias. They also become less robust when considering retrospective studies and not clinical trials (124, 167). It is important to consider the results from individual papers as well. For MVD, some individual studies reported that increased MVD predicted poor prognosis in breast cancer (88, 103, 110-114) , while others did not (115-123). In this thesis, we found in all papers, that increased MVD was associated with poor prognosis in all cases combined. The results from Paper II implied that the risk of breast cancer death increases steadily with increasing vessel count, following a gradient of increasing risk rather than a specific cut-off value for high and low vessel density.

The differing methodology for MVD assessment between individual studies (Table 1) makes them less comparable and limits the conclusions that can be drawn about its prognostic role. Thus, there is a need for a standardisation in methodology. We identified one particular source of variability that we wanted to study more closely, namely the field area for vessel counting. The great variations in field area size between studies testified to a general lack of knowledge about which field area that actually provided the most accurate prognostic information. Therefore, for our third paper, we aimed to identify the optimal field area for predicting prognosis.

6.2.3.1 Field area for MVD assessment

For a method to be successfully incorporated in diagnostics, it needs to be reproducible, not too time-consuming, and require little training. But most importantly, it must provide meaningful information. The chosen method for vasculature assessment should depend on the research question (166). It is entirely possible that the best method for predicting patient prognosis may not be the best for studies of ongoing biological processes in tumour tissue, or for predicting response to therapy. For example, even though vascular proliferation was not a prognostic factor in this study, it may still provide relevant information about the biology of different subtypes. The results from Paper III are informative with regard to the prognostic potential of MVD. However, we also provide descriptive information about intratumoural vascular heterogeneity by recognising hotspot dilution, in accordance with others (104, 111, 113, 130, 153).

In the meta-analysis on MVD in breast cancer, results were not influenced when survival analyses were performed separately in studies assessing MVD in a field area greater than 0.38mm^2, and in studies with a smaller field area (124). In one study, vessels were counted in three TMA cores from tumour-rich regions (total field area 0.85mm^2), and the average MVD count was reported to have the greatest association with prognosis when compared to the maximum and minimum counts (112). However, to the best of our knowledge, the only individual study that compared the prognostic estimates from two field areas, was done in year 2000 on 112 breast tumours. Vessels had been counted in vascular hotspots at 400x magnification in four (HS-MVD4, total field area 0.64mm^2) and 10 visual fields (HS-MVD10, total area 1.56mm^2). Like us, they concluded that MVD assessed in a smaller field area within the hotspot provided the best prognostic estimate. This study had a smaller sample size than ours, and only compared two field areas (111). We compared ten areas sorted in three different ways, and our systematic approach provides novel information about the prognostic value of MVD according to field area size. Our results imply that the most important prognostic information is not acquired by studying all vessels, but rather the vessel density in a small region with the highest number of vessels. If counting consecutively, it does not seem necessary to include a field area greater than 0.69mm^2. When only including the most vessel-dense visual fields, an even smaller area (0.35mm^2) seems optimal. Furthermore, MVD-Decreasing provides more accurate prognostic information than MVD-Consecutive and MVD-Random. This implies that which visual fields observers choose to

include within the hotspot also are of importance, and not only the chosen vascular hotspot in itself. Based on our results, the aim should be to include the visual fields with the highest vessel counts only, although these findings should be validated in an independent data set with a more representative group of breast cancers.

Observer variation was not considered in this thesis, and it has been suggested that observer variability is greater when counting in a smaller field area (115, 166). However, with more standardisation and methods for automated vessel assessment (104, 112, 136-139), reproducibility may increase. Automated assessment could markedly improve both efficiency and reproducibility. Regardless of whether future methods for vessel quantification involve conventional MVD, automated assessment or imaging, our results are relevant because they tell us where to look for the information that provides most accurate prognostic information. Just because novel methods have the ability to study the entire tumour, that does not mean that they should.

Differences in prognostic accuracy between field areas were not immense. When studying each subtype separately, the best method for MVD assessment reached the same conclusion as the method used in Papers I and II. The advantage implied from our results, is that we may achieve the same or better result with a less laborious and time-consuming method. Furthermore, it is also possible to avoid hotspot dilution.

6.2.3.2 MVD and prognosis in molecular subtypes of breast cancer

In this thesis, MVD was a prognostic factor in Luminal A, HER2 type and 5NP, but not in BP. This result implies that MVD may provide prognostic information in some subtypes, but not in others.

The Luminal A cases in this study are most likely a more aggressive subset than Luminal A tumours as a whole. Nevertheless, we did identify a clear difference between the subtypes; MVD provided additional prognostic information in the Luminal A subtype, but not in the BP. However, these results should be validated in a larger and more representative subset of Luminal A cases.

In Paper II, it can be assumed that the prognostic value of MVD seen in the analyses including all cases was due to the 5NP and HER2 subtypes alone, since MVD had no prognostic value among BP patients when subtypes were studied separately. Others

reported that high MVD was associated with poor prognosis in 102 patients with TN tumours, of whom 66% were BP. They did not, however, study the prognostic value of MVD in the BP and 5NP groups separately (114). Our study is large for its kind and seems representative of the non-luminal subtypes. Nevertheless, the sample size of each molecular subtype was small, particularly for the 5NP group. The prognostic role of MVD in the non-luminal subtypes should be further investigated in studies with a larger sample size.

7 CONCLUSIONS AND FUTURE PERSPECTIVES

BP tumours had somewhat higher pMVD and VPI than Luminal A tumours, but there were no differences in MVD between the two groups. Among the non-luminal tumours, the 5NP had higher MVD than both BP and HER2 type. The largest difference in MVD was between BP and 5NP. This supports the hypothesis that these are two biologically distinct subtypes, and that important information may be lost if they are merged as one TN subtype. There were no differences in vascular proliferation between the non-luminal subtypes. The role of non-angiogenic mechanisms for tumour vascularisation in breast cancer subtypes should be further studied.

MVD was associated with prognosis in all cases, but vascular proliferation was not. Throughout the project, we identified methodological variability between studies, and potential for improvement in methodology for assessment of MVD and vascular proliferation assessment. A consistent and efficient method with low sensitivity to variation should be developed. We addressed one methodological issue for MVD, namely the size of the field area for vessel counting. Inclusion of a larger field area led to hotspot dilution. A smaller field area including only the most vessel-dense visual fields provided the most accurate prognostic information. Our results about field area size provide relevant information for further development of an improved and standardised method, regardless of it being automated or conventional.

Increased MVD was associated with poor prognosis in patients with Luminal A, HER2 type and 5NP subtypes, but not in patients with BP tumours. The prognostic role of MVD in patients with HER2 type and 5NP should be further investigated in a larger sample size.

The Luminal A subtype has the best prognosis of all subtypes. Still, patients today are treated with extensive adjuvant therapy which has several side effects that strongly affect quality of life. According to present guidelines, women should use tamoxifen or aromatase inhibitors for at least five years, and many should be offered ten years in total (205). Women in the present study did not receive systemic adjuvant therapy at all. Yet, survival in Luminal A was approximately 75% five years after diagnosis and 60% fifteen years after diagnosis, implying that a percentage of these women might not have had any survival benefit from systemic adjuvant therapy. If it were possible to identify these women, overtreatment could be avoided. There is an urgent need to discover new prognostic markers so that these so-called

super-survivors may be identified. Not only would avoidance of overtreatment lead to improved quality of life for the individual patient, but it would also benefit health economy by reducing inappropriate and expensive therapy to an increasing number of patients. In the present study, low MVD was associated with better survival in patients with Luminal A tumours. Most Luminal A tumours were grade 3, and therefore likely to represent a more aggressive subset than Luminal A tumours in general. Nevertheless, MVD was a prognostic factor in Luminal A tumours. These results should be validated and further studied in a larger, more representative subset of Luminal A tumours. If our findings persist, MVD might provide prognostic information that could aid in the identification of super-survivors.

8 ERRATA

Paper I: Table 2 states that adjustments also were made for subtype, but that is incorrect. Subtype was never adjusted for in multiple linear regression analyses, since it was the parameter to be studied.

Paper II: In Table 1, the numbers of patients who died from breast cancer and the number of patients who did not die from breast cancer have been switched. However, the manuscript text (page 768), which reads “Among BP patients, 44.3% died from breast cancer compared with 61.7% of the HER2 patients and 53.3% of the 5NP patients”, is correct.

Paper III: It is written in the article that the total field area varies from 0.14mm^2 to 6.8mm^2, but the text should have been that the total field area varies from 0.19mm^2 to 6.8mm^2.

Paper III (introduction, line 4 in first paragraph) and thesis (page 40, line 14 and page 91, line 17): The article by Lee et al (ref no. 120 in the thesis and ref no. 16 in the manuscript) does not consider prognosis. Therefore, it should not have been included as one of the references that failed to find an association between MVD and prognosis.

9 REFERENCES

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Paper I

Paper II

Paper III

Microvessel density in breast cancer – The impact of field area on prognostic informativeness

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Abstract

Aims: Tumour microvessel density (MVD) is assessed by counting vessels in the most vascularised tumour region, the vascular hotspot. Current uncertainty regarding the prognostic role of MVD in breast cancer could, in part, be explained by variations in field area size for MVD assessment. We aimed to identify the field area size that provides the most accurate prognostic information in breast carcinoma.

Methods: MVD was assessed in 212 tumours. von Willebrand factor positively stained vessels were counted in 10 consecutive visual fields in vascular hotspots. Counts from the 10 visual fields in the original counting sequence (MVD-Consecutive), visual fields sorted from highest to lowest (MVD-Decreasing), and sorted randomly (MVD-Random). After adding counts from one visual field at a time, average MVD was calculated for each cumulative field area. The prognostic informativeness of each field area and sorting strategy were compared.

Results: Median MVD decreased with increasing field size for MVD-Decreasing and MVD-Consecutive. A 0.35 mm^2 total field area comprising only the highest vessel counts provided the most accurate prognostic information (MVD-Decreasing, hazard ratio for breast cancer death 1.06 per 10 vessels/mm^2 increase, 95% CI 1.03-1.10). MVD-Decreasing gave more accurate prognostic information than MVD-Consecutive and MVD-Random, with decreasing prognostic informativeness with increasing field area.

Conclusions: Mean MVD and its prognostic informativeness decreased with increasing field area. Assessing MVD in a carefully selected small field area of 0.35 mm^2 provides the most accurate prognostic information. This could facilitate the implementation of MVD assessment in breast cancer.

Introduction

Tumour cells need access to vasculature to proliferate and metastasize (1, 2). Microvessel density (MVD) is an acknowledged and frequently used method for measuring tumour-associated vasculature (3). In breast cancer (BC), high MVD has been associated with poor prognosis in some studies (3-10), but not in others (11-17). Methodological variations may contribute to this lack of consistency across studies.

Since 1991, MVD has been used to quantify tumour vessels. Vessels are counted in "vascular hotspots", which are tumour regions with the highest number of vessels (3, 8). However, there is no consensus regarding field area or total number of visual fields in which vessels should be counted. While some report counting in a single visual field (3, 18, 19), others include up to 10 (5, 10, 13, 15, 17), and total field area varies from 0.14 mm^2 (20) to 6.8 mm^2 (21). Counting in larger field areas may reduce inter- and intraobserver variation (22). At the same time, larger areas may dilute the MVD value in a hotspot (5, 23-25). A study comparing only two field areas suggested that the prognostic value of MVD was greater when counting in a smaller field area (5). There is a need for a systematic study of the effect of field area on prognostic accuracy in breast cancer patients.

BC can be classified into molecular subtypes using immunohistochemistry (IHC) and in situ hybridisation (ISH) (26-30). We previously demonstrated that MVD is associated with prognosis in the Luminal A, HER2 type and five negative phenotype (5NP), but not in the basal phenotype (BP) (6, 7). In this study, we sought to identify the field area size for MVD assessment that provides the most accurate prognostic information in 212 breast tumours from women with long-term follow-up.

Materials and methods

Study population and specimen characteristics

The main study population comprised 909 primary breast carcinomas from women who were born in 1886-1928 (26), invited to participate in a survey for early BC detection in 1956-1959 (31, 32), and subsequently followed for BC occurrence from 1961 to 2008. Depending on which came first, patients were followed from diagnosis until death from BC, death from other causes or December 31st 2010. Data were linked to information from the Cancer Registry of Norway and the Cause of Death Registry of Norway (26).

Pathology reports and formalin-fixed, paraffin embedded (FFPE) tissue had been retrieved from the Department of Pathology, St Olav's Hospital, Trondheim University Hospital. As previously described (26), two pathologists independently reclassified tumours into histopathological type (33) and grade (34). After tissue microarray construction, IHC and ISH were used to reclassify BCs into molecular subtypes (Figure 1) (26).

From the main study population, the present study included all BC tumours (n=212) in which MVD had already been assessed by our group (Figure 2) (6, 7): Luminal A (n=63), BP (n=61), HER2 type (n=61) and 5NP (n=27). Vessel counting had been done on full-face sections in 10 consecutive visual fields (total field area 1.73 mm^2) at x400 magnification (6, 7).

Immunohistochemistry

Dual-stain IHC for von Willebrand factor (vWF) and the proliferation marker Ki67 had been done previously (6, 7). FFPE tumour tissue sections cut at 4µm and stored at -20°C, were heated at 42°C overnight. Heat induced epitope retrieval was done in a Pre-Treatment link with Dako Target Retrieval Solution buffer pH 6, S1699 (DAKO). Initial temperature was 80°C, increasing to 97°C for 20 minutes before refrigeration. Dako Wash Buffer, S3006 10x diluted with deionized water (dH_2O) was used twice for 3 minutes after pretreatment, and for 5 minutes between each step in the immunostaining process. Immunostaining was done at room temperature using Dako Autostainer Plus (DAKO Denmark A/S, Produksjonsvej 42 DK-2600 Glostrup, Denmark), followed by eight minutes of enzyme blocking with Dual Endogenous Enzyme Block, S2003 (DAKO). Antibody diluent and primary antibodies were mixed, using rabbit von Willebrand factor (Polyclonal rabbit, A0082, DAKO) concentrated at 3.8µg/L and mouse Ki67 (Clone MIB1, M7240, DAKO) at 160µg/L. The detection system contained EnVision Detection System-Peroxidase/rabbit, K4003 for vWF and Southern Biotech alkaline phosphatase/goat anti-mouse for Ki67, diluted 1:100. Primary antibodies were incubated for 60 minutes, and the detection system for 30 minutes. To visualise Ki67, Ferangi blue (BIOCARE medical) diluted with Ferangi Blue Buffer was incubated for 15 minutes. Sections underwent three dH_2O rinses before vWF visualisation with amino-ethyl-substrate chromogen (DAKO) for 15 minutes. Coverslipping with Dako Faramount aqueous medium (DAKO) after two dH_2O rinses and immersion in lukewarm water. Ki67-positive cells had blue nuclei and vWF-positive cells reddish-brown cytoplasm (Figure 3).

Scoring and reporting

Two observers (AMB and MRK) identified intratumoural vascular hotspots at 20x and 100x magnification. One observer (MRK) counted microvessels at 400x magnification in 10 visual fields within the hotspot in each tumour. Vessels were counted in consecutive visual fields whenever possible, aiming to commence counting in the region with the apparent highest vessel density. Regions with the highest number of vessels were always included, while areas with normal tissue, fibrosis, sclerosis and necrosis were avoided. Each visual field comprised at least 50% tumour cells. A vascular unit was defined as an endothelial cell or cell cluster with vWF-positive staining, and did not require a lumen. However, in long branches of endothelium or microvascular bundles, each lumen denoted a separate vessel. Stained structures containing a 90° angle were defined as two vessels. Vessel counts for individual visual fields were registered separately.

One visual field had an area of 0.17 mm^2, making the total area for MVD assessment 1.73 mm^2 per tumour. In previous studies, only the average vessel count from 10 visual fields were used (6, 7).

Statistical analyses

We compared the prognostic value of three different strategies for sorting vessel counts for the 10 visual fields in each case: 1) MVD-Consecutive, the original sequence in which visual fields were included; 2) MVD-Decreasing, visual fields sorted from highest to lowest vessel count; and 3) MVD-Random, visual fields sorted randomly. For each sorting strategy, we calculated the cumulative field area and average number of vessels/mm^2, by successively adding the vessel count from one visual field at a time.

Cox proportional hazards models were used to estimate hazard ratios (HRs) for BC death according to each sorting strategy, with censoring at death from other causes. For each cumulative field area in each of the three sorting strategies, HR per 10 vessels/mm^2 increase in MVD was estimated, with MVD as a continuous variable. The following factors were adjusted for by including them as covariates in all models: year of diagnosis (continuous variable); age at diagnosis (<60, 60-69, 70-79, ≥80 years); tumour diameter (≤20, >20 and ≤50, uncertain but ≥20 mm, uncertain); lymph node status (negative, positive, unknown); grade (1-2, 3); and molecular subtype. Log-minus-log plots and tests based on Schoenfeld

residuals were used to confirm that the assumption of proportionality between hazards was met.

We estimated and compared the informativeness of each strategy and cumulative field area for MVD assessment (35). In this study, informativeness reflects how well each cumulative field area of vessel counts within each sorting strategy predicts risk of death from BC. Informativeness was estimated in likelihood ratio (LR) tests and calculated as twice the difference in log-likelihood between a Cox model containing only the covariates listed above, and a model containing a continuous MVD variable in addition to the covariates. The greater the LR test statistic (χ^2); the more prognostic information the MVD value provides. Since the reference model (containing only covariates) and the units of the MVD variables (increase in 10 vessels/mm^2) were the same for all comparisons, we could compare informativeness across sorting strategies and field areas. For the most informative combination of field area and sorting strategy, we performed separate Cox analyses for each molecular subtype.

Results

Table 1 displays characteristics of the study population and tumours according to median MVD (72.9 vessels/mm^2) across all tumours, calculated for all 10 visual fields combined. Median follow-up time was 4.6 years (interquartile range, IQR, 1.9-11.2 years). MVD was below the common median in 69.8% of the Luminal A tumours, 41.0% of the HER2 type, 50.8% of the BP and 22.2% of the 5NP. Low MVD was associated with smaller tumour size, but not with stage or lymph node status.

Table 1 Descriptive characteristics and chi-square tests for the 212 breast cancer patients according to median MVD value when counted in 10 visual fields

	Total	≤72.9 vessels/mm^2	>72.9 vessels/mm^2	Chi^2
Number of cases (%)	212 (100.0)	106 (50.0)	106 (50.0)	
Median age at diagnosis, years (IQR)	72 (64-78)	73 (65-78)	69.5 (61-78)	
Median follow-up time, years (IQR)	4.6 (1.9-11.2)	6.4 (3.0-12.7)	3.2 (1.7-9.9)	
Median time to breast cancer death, years (IQR)	2.4 (1.3-4.2)	3.3 (1.3-8.1)	2.1 (1.3-3.5)	
Age at diagnosis, years (%)				0.003
<60	31	7 (22.6)	24 (77.4)	
60-69	58	29 (50.0)	29 (50.0)	
70-79	75	47 (62.7)	28 (37.3)	
≥80	48	23 (47.9)	25 (52.1)	
Type, n (%)				0.211
No special type	149	79 (53.0)	70 (47.0)	
Lobular	13	4 (30.8)	9 (69.2)	
Mucinous	1	1 (100.0)	0 (0.0)	
Medullary	15	5 (33.3)	10 (66.7)	
Papillary	9	6 (66.7)	3 (33.3)	
Metaplastic	12	7 (58.3)	5 (41.7)	
Other types	13	4 (30.8)	9 (69.2)	
Grade, n (%)				0.046
1	6	6 (100.0)	0 (0.0)	
2	49	24 (49.0)	25 (51.0)	
3	157	76 (48.4)	81 (51.6)	
Subtype, n (%)				<0.001
Luminal A	63	44 (69.8)	19 (30.2)	
Luminal B(HER2-)	0	0	0	
Luminal B(HER2+)	0	0	0	
HER2 type	61	25 (41.0)	36 (59.0)	
5NP	27	6 (22.2)	21 (77.8)	
BP	61	31 (50.8)	30 (49.2)	
Tumour diameter (mm), n (%)				0.003
≤20	79	49 (62.0)	30 (38.0)	
>20 ≤50	24	12 (50.0)	12 (50.0)	
Uncertain, but ≥20	54	17 (31.5)	37 (68.5)	
Uncertain*	55	28	27	
Lymph node status, n (%)				0.100
Negative	80	47 (58.8)	33 (41.3)	
Positive	93	43 (46.2)	50 (53.8)	
Unknown*	39	16	23	
Stage, n (%)				0.137
I	92	46 (50.0)	46 (50.0)	
II	95	53 (55.8)	42 (44.2)	
III	15	4 (26.7)	11 (73.3)	
IV	9	3 (33.3)	6 (66.7)	
Missing*	1	0	1	

*Chi^2 test does not include cases with uncertain, unknown or missing values.

Median MVD in increasing cumulative areas according to each sorting strategy are displayed in Table 2. For MVD-Consecutive, median MVD decreased with increasing field area, from 86.5 vessels/mm^2 when including one visual field to 72.9 vessels/mm^2 for 10 visual fields. In

MVD-Decreasing, median MVD was 109.6 vessels/mm^2 when only the visual field with the highest MVD was included. In MVD-Random, there were no apparent trends in median MVD with increasing field area, and the difference in median MVD was 1.1 vessels/mm^2 between the highest and lowest total areas.

Table 2 shows adjusted relative risk of death from BC per 10 vessels/mm^2 increase, LR statistic and relative informativeness of each method. The highest informativeness was found when only the two visual fields with highest MVD were included (MVD-Decreasing, total area 0.35 mm^2, HR 1.06, 95% CI 1.03-1.10, χ^2 16.08). This method was 14% more informative than the original counts in 10 visual fields (total area 1.73 mm^2, HR 1.08, 95% CI 1.04-1.12, χ^2 14.05). Furthermore, within MVD-Decreasing, informativeness fell steadily with increasing cumulative area. For MVD-Consecutive, the highest informativeness was achieved at four visual fields (total area 0.69 mm^2, HR 1.07, 95% CI 1.04-1.10, χ^2 14.36). However, it was less informative than the corresponding field area for MVD-Decreasing (HR 1.06, 95% CI 1.03-1.09, χ^2 15.69). For MVD-Random, there were no trends in informativeness.

Table 2 Prognostic informativeness according to cumulative field areas and sorting strategies

Visual fields	Cumulative field area (mm^2)	Median number of vessels/mm^2 (IQR)	HR per 10 vessels increase (95% CI)*	LR test statistic (χ^2)	Relative informativeness compared to 10 visual fields (%)
MVD-Consecutive					
1	0.17	86.5 (57.7-138.4)	1.04 (1.01-1.06)	5.69	40.5
2	0.35	86.5 (57.7-128.3)	1.05 (1.02-1.08)	10.58	75.3
3	0.52	80.7 (57.7-121.1)	1.06 (1.03-1.09)	11.42	81.3
4	0.69	79.3 (56.2-126.1)	1.07 (1.04-1.10)	14.36	102.2
5	0.87	79.6 (55.4-123.4)	1.07 (1.03-1.11)	13.28	94.5
6	1.04	77.4 (56.2-122.1)	1.07 (1.04-1.11)	13.04	92.8
7	1.21	77.0 (54.8-119.9)	1.07 (1.03-1.11)	12.74	90.7
8	1.39	76.0 (54.4-116.8)	1.07 (1.04-1.11)	12.53	89.2
9	1.56	74.3 (53.5-114.4)	1.07 (1.04-1.11)	13.46	95.8
10	1.73	72.9 (52.8-115.6)	1.08 (1.04-1.12)	14.05	
MVD-Decreasing					
1	0.17	109.6 (86.5-173.0)	1.05 (1.03-1.07)	15.38	109.5
2	0.35	103.8 (77.9-157.1)	1.06 (1.03-1.08)	16.08	114.5
3	0.52	99.0 (73.0-150.9)	1.06 (1.03-1.09)	16.01	114.0
4	0.69	94.4 (69.2-143.4)	1.06 (1.03-1.09)	15.69	111.7
5	0.87	90.5 (66.3-137.2)	1.07 (1.04-1.10)	15.39	109.5
6	1.04	86.5 (63.4-132.2)	1.07 (1.04-1.10)	14.88	105.9
7	1.21	82.4 (61.0-128.1)	1.07 (1.04-1.11)	14.65	104.3
8	1.39	78.6 (58.7-124.0)	1.07 (1.04-1.11)	14.43	102.7
9	1.56	75.3 (55.7-119.5)	1.07 (1.04-1.11)	14.18	100.9
10	1.73	72.9 (52.8-115.6)	1.08 (1.04-1.12)	14.05	
MVD-Random					
1	0.17	75.0 (51.9-115.3)	1.05 (1.01-1.08)	6.71	47.8
2	0.35	72.1 (53.3-116.8)	1.07 (1.03-1.11)	12.88	91.7
3	0.52	71.1 (51.9-118.2)	1.07 (1.03-1.11)	11.56	82.3
4	0.69	73.5 (51.9-118.9)	1.07 (1.03- 1.11)	12.81	91.2
5	0.87	71.5 (50.7-115.3)	1.08 (1.04- 1.12)	14.00	99.6
6	1.04	71.1 (51.9-114.9)	1.08 (1.04- 1.12)	14.37	102.3
7	1.21	71.3 (51.9-113.7)	1.08 (1.04- 1.12)	14.09	100.3
8	1.39	71.7 (52.6-113.5)	1.08 (1.04- 1.12)	14.39	102.4
9	1.56	72.7 (52.5-115.3)	1.08 (1.04- 1.12)	14.05	100.0
10	1.73	72.9 (52.8-115.6)	1.08 (1.04- 1.12)	14.05	

*Adjusted for year of diagnosis, age at diagnosis, tumour size, lymph node status and molecular subtype.

In the original consecutive order, the visual field with the highest vessel count was among the first four visual fields in 122 cases (57.5%), and the visual field with the second highest count was among the first four visual fields in 91 cases (42.9%). However, both were among

the first four in only 60 cases (28.3%). In 57 cases (27.7%), the two visual fields with the highest counts had been included successively.

Table 3 describes the risk of death from BC according to BC subtype for three methods; the two visual fields with highest vessel count, the first four visual fields counted consecutively and the original 10 visual fields. For the two visual fields with highest vessel count, the association with BC death was strongest for patients with Luminal A tumours (HR for breast cancer death per 10 vessels/mm^2 increase 1.16, 95% CI 1.05-1.28), compared to the HER2 type (HR 1.10, 95% CI 1.05-1.15) and 5NP (HR 1.09, 95% CI 1.01-1.18). For BP, no association was found (HR 1.00, 95% CI 0.95-1.06). A similar pattern was seen for the first four visual fields in MVD-Consecutive and for the original 10 visual fields.

Table 3 Relative risk of death from breast cancer according to MVD, stratified for molecular subtypes

MVD, microvessels/mm^2	Luminal A	HER2 type	5NP	BP
2 visual fields with the highest count				
Unadjusted HR* (95% CI)	1.09 (1.02-1.17)	1.05 (1.02-1.09)	1.09 (1.03-1.16)	1.03 (0.98-1.07)
Adjusted† HR (95% CI)	1.16 (1.05-1.28)	1.10 (1.05-1.15)	1.09 (1.01-1.18)	1.00 (0.95-1.06)
4 visual fields in consecutive order				
Unadjusted HR* (95% CI)	1.10 (1.02-1.19)	1.09 (1.04-1.14)	1.08 (1.01-1.15)	1.03 (0.98-1.08)
Adjusted HR† (95% CI)	1.18 (1.05-1.32)	1.15 (1.07-1.24)	1.11 (1.02-1.22)	1.00 (0.94-1.07)
10 visual fields				
Unadjusted HR* (95% CI)	1.12 (1.02-1.22)	1.07 (1.03-1.12)	1.10 (1.02-1.18)	1.06 (0.96-1.16)
Adjusted HR† (95% CI)	1.19 (1.05-1.35)	1.14 (1.07-1.21)	1.17 (1.04-1.32)	0.99 (0.88-1.10)

*HR per 10 vessels increase †Adjusted for age, year of diagnosis, tumour size, lymph node status, grade

Discussion

We studied the prognostic informativeness of different strategies for assessing MVD in 212 BC tumours. MVD in a total field area of 0.35 mm^2 in the most vessel-rich region of the tumour provided the most accurate prognostic information. When vessel counts were sorted in decreasing order, prognostic relative informativeness decreased with increasing cumulative area. MVD-Decreasing provided more accurate prognostic information than MVD-Consecutive and MVD-Random. When the most informative method for MVD assessment was applied to each molecular subtype separately, increasing MVD was associated with poorer prognosis in Luminal A, HER2 type and 5NP, but not in BP.

A number of factors may account for the variation across studies of the prognostic value of MVD, but the lack of consensus in methodology is an important contributor. Vessels are counted in different tumour regions (5, 36, 37), at different magnifications (4, 5, 11, 38), in

total field areas of varying size (5, 11, 12, 36, 38), and with different antibodies (3, 5, 36). The definition of what constitutes a vessel, and of how individual vessels should be distinguished, varies (3, 18, 25, 38). In statistical analyses, some use only the maximum vessel count (4, 11, 13), while others use the average (5, 12, 14) or total count (3, 39), and cut-offs for high and low MVD differ. This study analysed one source of discrepancy, the total field area for vessel counting.

The optimal method for assessment of tumour vasculature should be efficient, reproducible, require minimal training and, ultimately, provide meaningful information. Unfortunately, improving one aspect of assessment may be detrimental in others. For instance, observer reproducibility for MVD may be improved when a larger field area is assessed (11, 22), but is more time-consuming and increases the risk of observer fatigue. In the search of the optimal method, the most important factor is the information it provides, be it about tumour biology, prognosis or treatment prediction. The main focus in this study, was on the method's ability to predict patient prognosis. Prognostic informativeness as estimated here reflects how well a given model fits with the total amount of observed survival data, and thus, the most informative model need not be the one with the strongest association between MVD and prognosis. In the clinical setting, other aspects of MVD should also be considered.

When visual fields were sorted in decreasing order, median MVD fell markedly from 109.6 vessels/mm^2 for the first visual field to 72.9 vessels/mm^2 for 10 visual fields. Similarly, there was a clear declining trend when visual fields were sorted consecutively. This is in accordance with the findings of others, and supports the claim that MVD assessment in larger total field areas lead to hotspot dilution and MVD underestimation (5, 23-25).

Smaller cumulative field areas provided more accurate prognostic information compared to larger areas, and our results suggest that it is unnecessary to count vessels in an area greater than 0.69 mm^2. Furthermore, a field area of 0.35 mm^2 seems to be optimal if counting is done exclusively in visual fields with the highest vessel count. These regions may be difficult to identify prior to vessel counting. However, careful scanning of the hotspot at high magnification may be sufficient for observers to identify them through visual discrimination.

In order to count vessels in 10 visual fields consecutively, we had to construct a path within the hotspot to avoid overlapping. This sometimes required that visual fields with higher vessel counts were included later in the counting sequence. However, it is possible that the visual fields with highest MVD may have been in close topographical proximity even though they appeared distant in the consecutive counting order. An argument for consecutive counting is to prevent inclusion of the same visual fields twice. However, this is easily avoided when as few as two visual fields are assessed. Moreover, since there were no large differences in informativeness between MVD-Consecutive and MVD-Random, including fields consecutively may be more of a constraint than an aid.

When we repeated survival analyses for each subtype separately using the methods with the highest prognostic informativeness, there were no great differences in results compared to the original model with 10 consecutive visual fields. This implies that the results from our two previous studies were robust (6, 7). Although we revealed differences in prognostic informativeness between methods, they were not profound, which could imply that different studies may be comparable despite varying field areas. This favours assessment of smaller field areas, which are less time-consuming and reduces the risk of observer fatigue, while providing increased prognostic accuracy.

To the best of our knowledge, this is the first systematic study of the prognostic informativeness of different field areas for vessel counting. The 212 cases are from a well-described cohort with long-term follow-up, where the majority were only treated with surgery. This enables the study of the prognostic informativeness of MVD in the near-natural course of disease progression. The same laboratory and antibodies were used for IHC. A trained observer (MRK) counted vessels in all sections. Vessels were only counted in the consecutive field order, and visual field counts were subsequently sorted into decreasing and random order. Since we only included some molecular subtypes and only a small subset of the most frequent subtype, Luminal A, the study may not be representative for all BCs.

If MVD were to be used as a diagnostic tool, a simpler and less time-consuming method would be needed. The increasing use of digital pathology with automated MVD assessments could provide a solution to observer variability, and other methodological issues presented in this paper. Software analyses have the added advantage of assessing other vascular

qualities simultaneously, such as vessel size and shape (10). Software is used to estimate endothelial area (37, 40) or the number of vessels (10, 41). Because automated vasculature assessments are more sensitive to background staining and less sensitive to weakly stained vessels, thresholding may be challenging, with the risk of both including false positives and overlooking true vessels. Today, programs need to be adjusted manually by experienced personnel (10, 37, 40, 41), but this could be altered in the future with emerging technology such as artificial intelligence, interactive learning and deep learning algorithms (42, 43). Additionally, there is an increasing number of studies assessing tumour vasculature with ultrasound (44, 45), computer tomography (46, 47) and magnetic resonance imaging (19, 48). For both imaging and digital pathology, challenges such as the need for extra equipment and data storage, and the risk of software error must be solved. Moreover, these methods still require a methodological consensus for studies to be comparable. The findings of this study are applicable to both conventional MVD methods and novel methods for assessment of tumour vasculature.

Conclusions

The results of this study indicate that in breast cancer, careful selection of vascular hotspots combined with vessel counting in a limited field area, provide more accurate prognostic information than counting in larger field areas. MVD decreased with increasing field area and provided the most accurate prognostic information when only the two most vessel-rich visual fields were included (field area 0.35 mm^2). These results could bring us closer to a useful approach to vessel assessment.

Key messages

In breast cancer, MVD estimated in a small field area within a vascular hotspot had higher prognostic informativeness compared to estimations in larger fields.

MVD assessment in larger field areas led to hotspot dilution and may underestimate MVD.

The prognostic value of MVD varies across molecular subtypes of breast cancer.

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Author contributions

All authors actively contributed to the paper, and take responsibility and accountability for the accuracy and integrity of the work.

AMB: Study design. Selection of vascular hotspots. Interpretation of results. Writing the article.

MRK: Study design. Selection of vascular hotspots. MVD assessment. Statistical analyses. Interpretation of results. Writing the article.

SO: Study design. Guidance in statistical analyses. Interpretation of results. Writing the article.

HGR: Interpretation of results. Writing the article.

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Competing interests

The authors declare they have no competing interests.

Ethical considerations

The Regional committee for medical and health research ethics approved the study, including the dispensation from the requirement of patient consent (REK-Midt Norge, reference no.: 2009/836).

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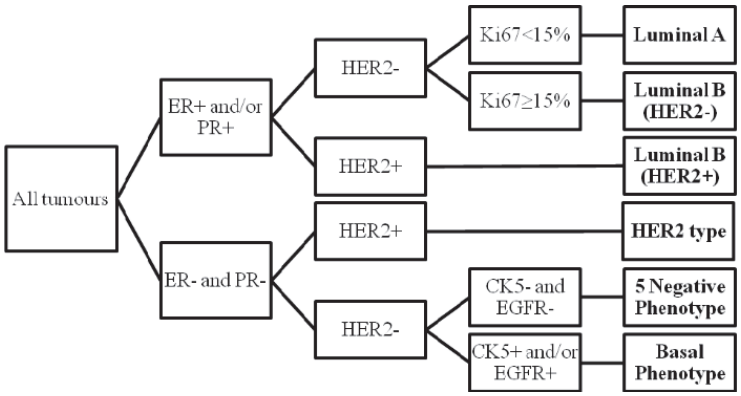

Figure 1. Molecular subtyping algorithm. Luminal A, Luminal B(HER2-), Luminal B(HER2+), HER2 type, 5 Negative Phenotype, Basal Phenotype. Oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), Cytokeratin 5 (CK5), epidermal growth factor receptor (EGFR). From Breast Cancer Res Treat, 2013, Vol 140, Engstrøm et al, *Molecular subtypes, histopathological grade and survival in a historic cohort of breast cancer patients* (26) Figure 2, page 466. Open access, permission for reprint not required.

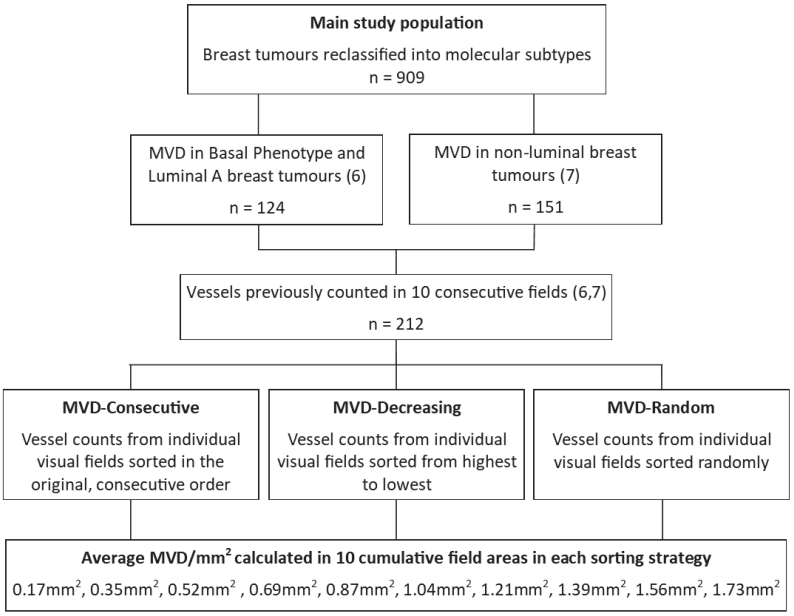

Figure 2. Flow chart of the study population. Displays included cases, sorting strategies and calculations of MVD.

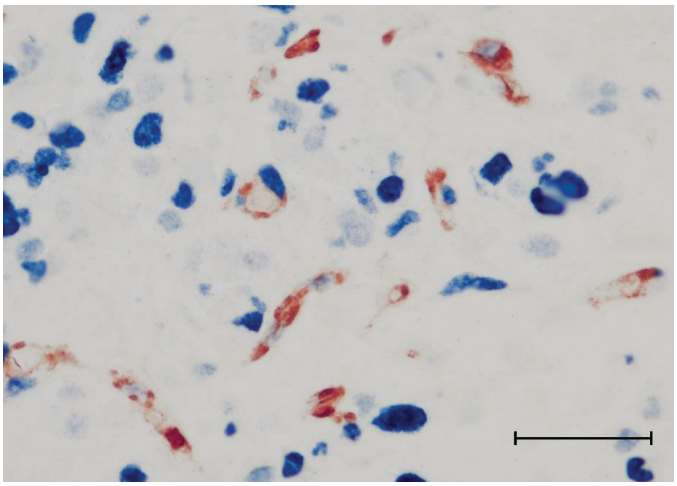

Figure 3. Immunohistochemically stained breast cancer section. Case number 255, Basal phenotype. Endothelial cells display von Willebrand factor positively stained cytoplasm as reddish-brown, and Ki67 positively stained nuclei are blue. Scale bar size: 100 µm.