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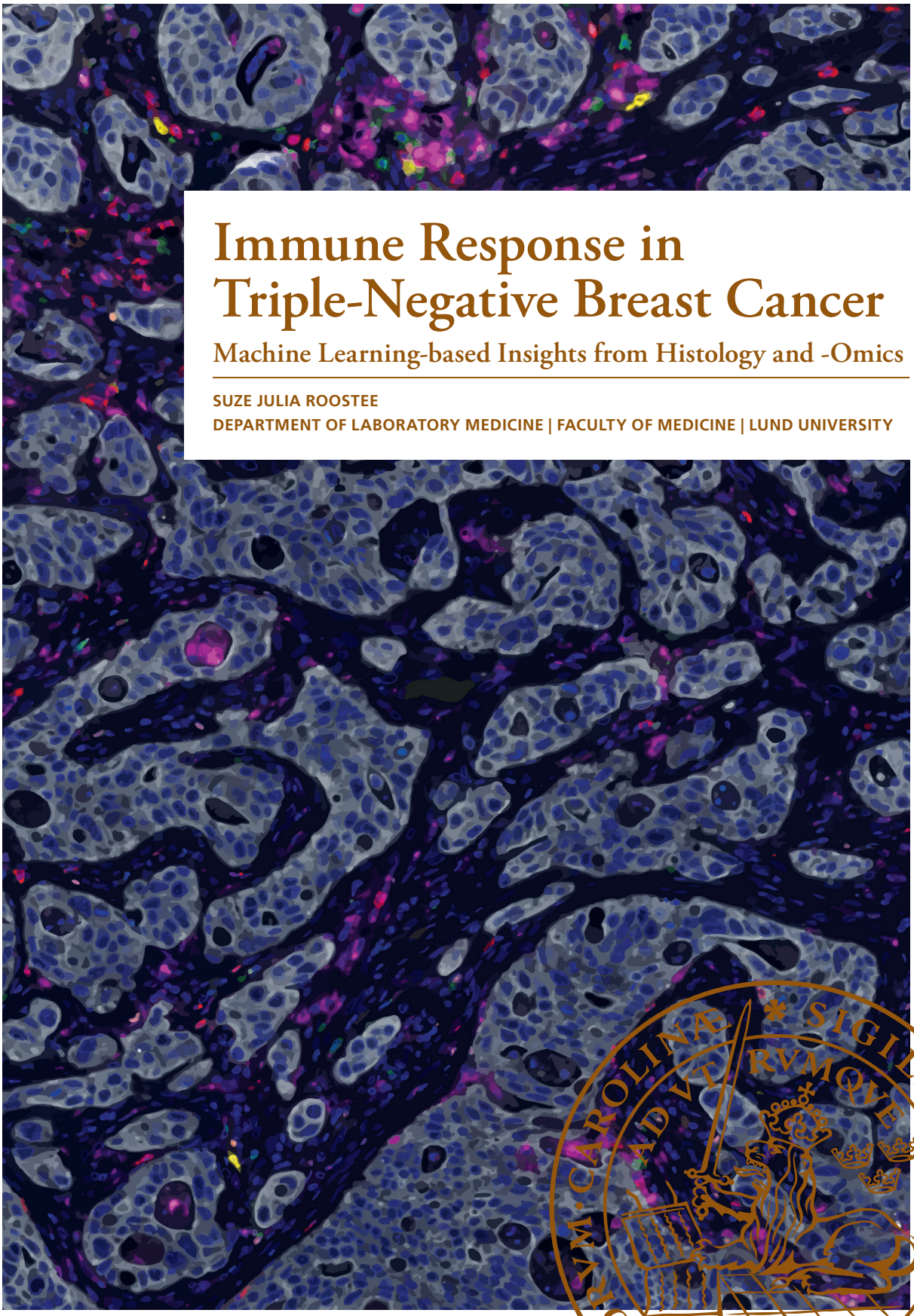

Immune Response in Triple-Negative Breast Cancer

Machine Learning-based Insights from Histology and -Omics

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by Suze Julia Roostee

LUND
UNIVERSITY

Thesis for the degree of Doctor of Philosophy

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A doctoral thesis at a university in Sweden takes either the form of a single, cohesive research study (monograph) or a summary of research papers (compilation thesis), which the doctoral student has written alone or together with one or several other author(s).

In the latter case the thesis consists of two parts. An introductory text puts the research work into context and summarizes the main points of the papers. Then, the research publications themselves are reproduced, together with a description of the individual contributions of the authors. The research papers may either have been already published or are manuscripts at various stages (in press, submitted, or in draft).

Cover illustration front High-fidelity and edited photo of multiplexed TNBC tissue

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"Wisest is she who knows she does not know."

- Jostein Gaarder *(Sophie's World)*

Contents

Scientific publications

List of papers and author contributions

This thesis is based on the following studies, referred to by their Roman numerals.

Author contributions are listed following the CRediT (Contributor Roles Taxonomy) system.

I **Tumour immune characterisation of primary triple-negative breast cancer using automated image quantification of immunohistochemistry-stained immune cells**
Suze Roostee, Daniel Ehinger, Mats Jönsson, Bengt Phung, Göran Jönsson, Gottfrid Sjödahl, Johan Staaf, Mattias Aine
Scientific Reports, 2024, 14(1):21417

Contributions: conceptualization, data curation, methodology, formal analysis, validation, visualisation, writing –original draft (lead), writing – review & editing.

II **Stand-Alone Immune Response Prediction in Triple-Negative Breast Cancer based on Gene Expression**
Suze Roostee, Fredrika Killander, Hani Saghir, Jari Häkkinen, Srinivas Veerla, Johan Vallon-Christersson, Niklas Loman, Mattias Ohlsson, Johan Staaf.
Submitted to *Breast Cancer Research and Treatment*

Contributions: conceptualization, methodology, data curation, investigation, formal analysis, validation, visualisation, writing – original draft (lead), writing – review & editing.

III **Characterization of the tumor immune microenvironment of triple-negative breast cancer through multiplex imaging and spatial transcriptomics**

Suze Roostee*, Iñaki Sasiain*, Matteo Bocci, Paulina Bolivar, Sophie Lehn, Mats Jönsson, Rosamond Nuamah, Åke Borg, Jelmar Quist, Anita Grigoriadis, Kristian Pietras, Johan Staaf. *These authors contributed equally
Manuscript

Contributions: conceptualization, data curation, methodology, data curation, investigation, formal analysis, visualisation, writing – original draft (lead).

Abstract

Breast cancer is the most common type of cancer in women worldwide. Triple-negative breast cancer (TNBC) is a subtype of breast cancer that lacks the expression of the oestrogen receptor, progesterone receptor, and amplification of HER2. This subtype is the most aggressive subtype and a heterogeneous subgroup of breast cancer, making up around 10-15% of the cases, often affecting younger patients and presenting with a higher risk of relapse. One factor that seems to positively impact patient outcomes in this heterogeneous subtype is the presence of an active immune response. This thesis focuses on improving the understanding of immune infiltration in TNBC and deriving approaches for better prognostication and treatment prediction. For this, we developed an automated image analysis pipeline and applied it to (among others) six immunohistochemical markers of immune cells. Digital cell counts extracted from five tissue microarray blocks showed how immune cells are often co-expressed in the tumour micro-environment. Furthermore, we demonstrate how a combination of immune status with DNA repair deficiency status can improve prognostication in (chemotherapy-treated) patients. In addition, we created a standalone classifier for gene expression data, based on the immunomodulatory subtype, that reflects immune response in TNBC. Our classifier was borderline non-significant in the stratification of neoadjuvant-treated chemotherapy patients and could stratify adjuvant chemotherapy-treated patients into subgroups of better or worse prognosis. Lastly, we studied the spatial heterogeneity of the tumour immune microenvironment in TNBC and its connection to molecular and genomic subtypes. We connected ecosystems in the tumour micro-environment with patterns of immune infiltration and could show TNBC specific differences. In conclusion, this thesis advances the quantitative and integrative study of the tumour (immune) micro-environment in TNBC, offering new approaches and insights to bridge the gap between immune infiltration, molecular heterogeneity, and clinical outcome.

Popular Science Summary

Breast cancer is the most common type of cancer for women all around the world, but not all breast cancers are the same. Triple-negative breast cancer (TNBC) is a less common form of breast cancer, affecting about 10–15% of women with breast cancer. What makes TNBC unique is that it lacks the (overexpression of) certain receptors or proteins that most breast cancers use to grow and spread. Unfortunately, this also means that TNBC is harder to treat because many targeted treatments are ineffective. As a result, chemotherapy is one of the few available options, making it critical to understand other factors that influence TNBC outcomes.

One important factor is the immune response. Recent research shows that the activity of the immune system can give us clues about the chances of a patient surviving TNBC, regardless of whether they receive chemotherapy. The immune system, which fights infections and diseases, has different types of cells that can work together to attack cancer. These cells can enter the tumour and the area around it, known as the 'tumour micro-environment'. Think of the tumour micro-environment as a city. Some parts of the city have many police officers (immune cells) working to keep criminals (cancer cells) under control. But not all police are equally good at their job, and some may even work against the system! Additionally, some cities (tumours) have very few or no police at all, allowing crime (cancer) to take over. Understanding how these 'police patrols' work—and why they fail in some cases—is key to finding better treatments for TNBC.

In this thesis, we focused on how the immune system interacts with TNBC and how this affects patient outcomes. First, we developed a computer tool to analyse images of TNBC tissue automatically. This allowed us to identify patterns, such as when one type of immune cell is present in high numbers, another type is often present in high numbers too. Based on these observations, we divided patients into two groups: those with 'high immune activity' and those with 'low immune activity'. Next, we combined these immune activity groups with a DNA-based classification that examines how well a tumour can repair its DNA. By combining these two pieces of information, we created four patient subgroups. Interestingly, we found that patients with high immune activity and faulty DNA repair tended to have better outcomes.

We also developed a tool that uses gene activity data to measure the immune response in TNBC. While this tool wasn't statistically perfect in predicting outcomes for patients who had chemotherapy before surgery, it was able to separate patients who had chemotherapy after surgery into better- and worse-performing groups. This shows promise for its use in identifying which patients might respond better to certain treatments.

Finally, we examined how immune cells are distributed within tumours and linked this spatial information to specific TNBC subtypes based on molecular and DNA

changes. This helped us better understand how the immune system interacts with the unique biology of different TNBC subtypes.

Taken together, this thesis added some small pieces to our understanding of the immune system's role in TNBC. By analysing the immune response in a quantitative way and linking it to patient outcomes and tumour characteristics, we have taken important steps toward improving treatments for this challenging cancer.

Populair-wetenschappelijke samenvatting

Borstkanker is wereldwijd de meest voorkomende vorm van kanker bij vrouwen, maar niet alle vormen van borstkanker zijn hetzelfde. Triple-negatieve borstkanker (TNBC) is een zeldzamere vorm van borstkanker en treft ongeveer 10–15% van de vrouwen met borstkanker. Wat TNBC uniek maakt, is dat het niet (de overexpressie van) bepaalde receptoren of eiwitten die de meeste andere vormen van borstkanker gebruiken om te groeien en zich te verspreiden, heeft. Dit betekent helaas ook dat TNBC moeilijker te behandelen is, omdat veel gerichte behandelingen niet effectief zijn. Chemotherapie is daardoor een van de weinige beschikbare opties, wat het belangrijk maakt om andere factoren te begrijpen die van invloed zijn op de uitkomsten van TNBC. Een van deze belangrijke factoren is de immuunrespons. Recentelijk onderzoek wijst uit dat de activiteit van het immuunsysteem een aanwijzing kan zijn voor de overlevingskans van een patiënt met TNBC, ongeacht of ze chemotherapie krijgt. Het immuunsysteem, dat infecties en ziekten bestrijdt, heeft verschillende soorten cellen die kunnen samenwerken om kanker aan te vallen. Deze cellen kunnen de tumor en de omgeving eromheen, wat ook wel de 'tumor micro-environment' genoemd wordt, binnendringen. Je kunt de tumor micro-environment zien als een soort stad. In sommige wijken zijn er veel politieagenten (immuuncellen) aanwezig die hard werken om criminelen (kankercellen) onder controle te houden. Maar niet alle politieagenten zijn even goed in hun werk, en sommigen zijn zelfs corrupt! Bovendien zijn er steden (tumoren) waar bijna geen politie aanwezig is, waardoor de criminaliteit (kanker) de overhand kan krijgen. Begrijpen waarom deze 'politiepatrouilles' wel of niet aanwezig zijn, is een stap in de richting van het vinden van betere behandelingen voor TNBC.

In dit proefschrift hebben we ons gericht op de wisselwerking tussen het immuunsysteem en TNBC en hoe dit de overlevingskans van patiënten beïnvloedt. Eerst hebben we een computerhulpmiddel ontwikkeld om automatisch afbeeldingen van TNBC-weefsel te kunnen analyseren. Dit stelde ons in staat patronen te identificeren, zoals dat wanneer één soort immuuncel in grote aantallen aanwezig is, een ander type vaak ook in grote aantallen aanwezig is. Op basis van deze bevindingen hebben we patiënten ingedeeld in twee groepen: degenen met 'hoge immuunactiviteit' en degenen met 'lage immuunactiviteit'. Vervolgens hebben we deze groepen gecombineerd met een classificatie gebaseerd op DNA, die aangeeft hoe goed een tumor beschadigd DNA kan repareren. Door deze twee groeperingen te combineren, hebben we vier patiëntgroepen gecreëerd. We zagen dat patiënten met hoge immuunactiviteit en een defect DNA-reparatie mechanisme vaak een betere uitkomst hadden.

We hebben ook een hulpmiddel ontwikkeld dat gebruik maakt van genactiviteitsgegevens om de immuunrespons bij TNBC te meten. Hoewel dit hulpmiddel niet statistisch perfect was in het voorspellen van uitkomsten voor patiënten die chemo-

therapie kregen vóór de operatie, kon het patiënten die chemotherapie kregen na de operatie wel onderscheiden in beter en slechter presterende groepen. Dit laat ziet dat er potentie is voor het identificeren van welke patiënten mogelijk beter reageren op bepaalde behandelingen.

Ten slotte hebben we onderzocht hoe immuuncellen binnen tumoren zijn verdeeld en deze ruimtelijke informatie gekoppeld aan specifieke TNBC-subtypen op basis van moleculaire en genetische veranderingen. Dit hielp ons om beter te begrijpen hoe het immuunsysteem samenwerkt met de unieke biologie van verschillende TNBC-subtypen.

Samenvattend heeft dit proefschrift enkele kleine puzzelstukjes toegevoegd aan ons begrip van de rol van het immuunsysteem bij TNBC. Door de immuunrespons op een kwantitatieve manier te analyseren en deze te koppelen aan patiënt-uitkomsten en tumoreigenschappen, hebben we belangrijke stappen gezet richting het verbeteren van behandelingen voor deze uitdagende vorm van kanker.

Abbreviations

ADC	Antibody Drug Conjugate
AI	Artificial Intelligence
ANN	Artificial Neural Network
APC	Antigen-Presenting Cell
AR	Androgen Receptor
BCR	B-Cell Receptor
BL1	Basal-Like 1
BL2	Basal-Like 2
BLIA	Basal-Like Immune Activated
BLIS	Basal-Like Immune Suppressed
CNN	Convolutional Neural Networks
CPS	Combined Positive Score
CTLA4	Cytotoxic T-Lymphocyte Associated Protein 4
DC	Dendritic Cell
DL	Deep Learning
ECM	Extracellular Matrix
EMT	Epithelial-to-Mesenchymal Transition
ER	Oestrogen Receptor
FDR	False Discovery Rate
FN	False Negative
FP	False Positive
FPKM	Fragments Per Kilobase of transcript per Million
FUSCC	Fundan University Shanghai Center
GO	Gene Ontology

GSEA	Gene Set Enrichment Analysis
H&E	Hematoxylin Eosin
HED	Hematoxylin Eosin DAB
HER2	Human Epidermal growth factor Receptor 2
HR	Hormone Receptor
HR	Hazard Ratio
HRD	Homologous Recombination Deficiency
HRR	Homologous Recombination Repair
IC score	Immune Cell score
IDFS	Invasive Disease-Free Survival
IHC	Immunohistochemistry
IM	Immunomodulatory
ISH	In Situ Hybridisation
ISS	In Situ Sequencing
KEGG	Kyoto Encyclopedia of Genes and Genomes
KM	Kaplan-Meier
kNN	k-Nearest Neighbours
LLM	Large Language Model
M	Mesenchymal
MDSC	Myeloid-Derived Suppressor Cell
MES	Mesenchymal
MHC	Major Histocompatibility Complex
ML	Machine Learning
mRNA	messenger Ribonucleic Acid
MSL	Mesenchymal Stem-Like

NCI	National Cancer Institute
NGS	Next-Generation Sequencing
NHGRI	National Human Genome Research Institute
NK cells	Natural Killer cells
LAR	Luminal Androgen Receptor
OS	Overall Survival
PARP	Poly(ADP-ribose) polymerase
PCA	Principal Component Analysis
pCR	pathological Complete Response
PD-L1	Programmed Death Ligand-1
PR	Progesterone receptor
RD	Residual Disease
RF	Random Forest
RFS	Recurrence-Free Survival
RGB	Red Green Blue
SCAN-B	Swedish Cancerome Analysis - Breast
scRNAseq	single-cell RNA sequencing
SMI	Spatial Molecular Imager
STEEP	Standardised Definitions for Efficacy End Points
TCGA	The Cancer Genome Atlas
TIL	Tumour Infiltrating Lymphocyte
TMArQ	Tissue microarray MArker Quantification
TMB	Tumour Mutational Burden
TNBC	Triple-negative breast cancer
TCR	T-Cell Receptor

TIME	Tumour Immune Micro-Environment
TMA	Tissue microarray
TME	Tumour Micro-Environment
TN	True Negative
TP	True Positive
TPM	Transcripts Per Million
TP53	Tumour Protein 53
UMAP	Uniform Manifold Approximation and Projection
WSI	Whole-Slide Imaging

Introduction

'Één woord verandert alles!'
- Eva Hermans-Kroot, Longeneeslijk

Cancer

Cancer - one word that changes everything. From the moment it is spoken, a large shift in an individual's life occurs. Fearing the loss of control, dependence on others and the effect of treatment are not uncommon [1]. Not only patients themselves but also their loved ones are affected by the diagnosis. They often provide the much-needed social and emotional support to the cancer patient but may feel helpless themselves as well [2].

Yearly cancer cases are steadily on the rise, such that every year 20 million people worldwide are newly diagnosed with cancer, and in 2022 alone, it claimed an estimated 9.7 million lives [3]. With an estimated increase to 35 million cases worldwide in 2050, cancer is becoming a more and more pressing global health issue [4].

Cancer is a complicated disease that has long eluded scientists. While first named by Hippocrates [1], cancer is as ubiquitous across life forms as it is throughout time [5]. The disease's origins can be traced back in humans all the way to Neanderthals [6] and has even been reported to have affected dinosaurs [7]. Progress and understanding were minimal until the nineteenth century, when advancements in physics, chemistry, and medicine, driven in part by war expenditure [8] and in part by 'the war on cancer', ushered in many discoveries. The development of radiation and new chemicals led to radiation therapy and chemotherapy as treatment options, in addition to the already existing treatment approach of surgery. For many cancers, these treatments still form

[1] Cancer's etymology originates from Latin, meaning crab, and was a translation by Celsus of the ancient Greek carcinoma ($\kappa\alpha\rho\kappa\acute{\iota}\omega\mu\alpha$). Hippocrates thought the large veins spreading away from a tumour (or other tissue growth) resembled the legs of a crab [5].

the cornerstone of disease management.

Although it has long been clear that cancer at its core is uncontrolled cell growth and many key discoveries -especially in the last century- have been made, the exact mechanisms driving cancer development remain partially unknown.

Hallmarks of Cancer

In 2001 Hanahan and Weinberg tried to summarise the findings of the last quarter century of oncological research and stipulated the six hallmarks of cancer [9]. These hallmarks described intrinsic characteristics of tumours and included the typical self-sufficiency of tumours in growth signals, insensitivity to anti-growth signals, tumours' ability to invade and metastasize to other tissues, their ability to keep dividing cells, induction of and access to vasculature, and evasion of apoptosis (cell death). In 2011, Hanahan and Weinberg expanded their original six hallmarks, adding two more emerging hallmarks of reprogramming cellular metabolism and avoiding immune destruction [10]. They noted that the tumour trait capturing hallmarks could not capture the full complexity of cancer and added the term "enabling characteristics", which describes the means by which malignant cells can gain the hallmarks. In 2022 and 2024 Hanahan and Hanahan and colleagues, respectively, shifted the focus from tumour traits to the complexity surrounding cancer instead [11, 12].

In this thesis, we focus on two key hallmarks of cancer: avoiding immune destruction and promoting inflammation in the context of the tumour micro-environment in triple-negative breast cancer (TNBC). We will start in the following section by providing an overview of breast cancer and its clinical context. We then move on to the biology of TNBC, where we cover more of the mutational background of TNBC and its subtypes, and continue by describing the role of inflammation and the tumour micro-environment.

Breast Cancer

Breast cancer is one of the most commonly occurring cancer types globally, with approximately one in eight women being diagnosed with the disease at some point in their life [13]. It is the most frequent cancer type among women and the second most common cancer overall after lung cancer, according to statistics from the World Health Organization [13, 14].

Globally, breast cancer represents a substantial portion of the cancer burden. Regionally, the incidence and outcomes of breast cancer show a significant variation.

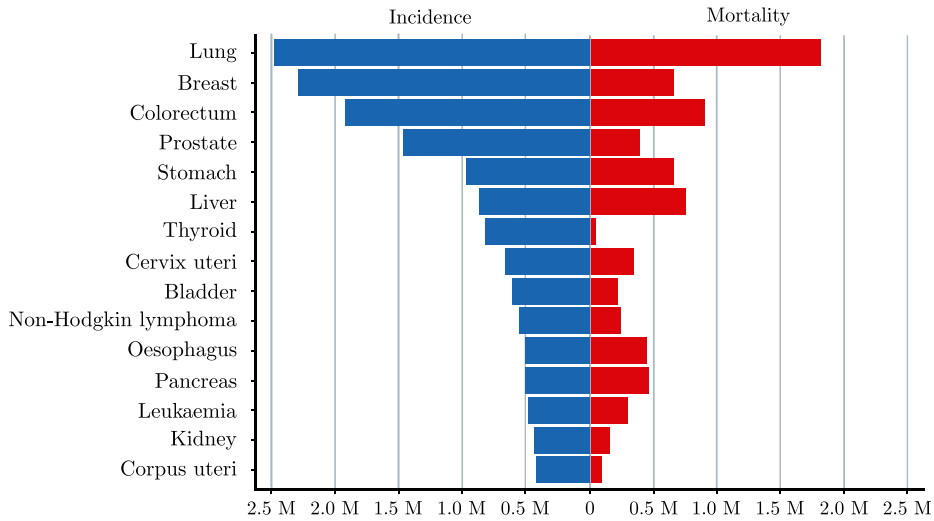

Figure 1: Incidence and mortality of breast cancer in women in absolute numbers worldwide. Data from the World Health Organization Global Cancer Observatory (https://gco.iarc.fr) [17]

The highest incidence rates are observed in high-income countries, likely due to advanced diagnostics tools and ageing populations. For example, in Europe, breast cancer accounts for about 25% of all cancer diagnoses among women [15]. In contrast, lower-income regions, such as the sub-Sahara region, often report lower incidence rates but face higher mortality due to limited access to early detection and advanced treatments [16].

Despite the significant contribution of breast cancer to cancer-related mortality, survival rates have improved over the last few decades. Important contributing factors here have been innovations in treatments, such as adjuvant chemotherapy regimes, endocrine therapies, and other targeted treatments (e.g., HER2-inhibitors). These advancements have allowed many patients to achieve long-term remission.

National screening programmes have also played a significant role in this success. For example, in Sweden, national screenings have achieved an estimated 16-25% reduction in breast cancer deaths [18]. In many countries, mammography screening is offered to women aged 40-76 years (international variations) at regular intervals (2-3 years, international variations) to detect breast cancer earlier, at a more treatable stage. Genetic screenings for high-risk mutations such as *BRCA1* and *BRCA2* have also become more widespread, enabling early detection in high-risk patients and families. Furthermore, awareness campaigns, such as during October's Breast Cancer Awareness Month, have increased public understanding and early detection [19], further improving outcomes. The impact of all these efforts is reflected in the im-

proved 5-year survival rate for breast cancer, which has reduced the number of deaths by approximately 40% compared to the 1970s [20, 21].

Although breast cancer is often treated as a single entity in statistics and public discourse, it is in reality a highly heterogeneous disease. Tumours vary widely in their genetic makeup and aggressiveness, and patient factors such as age and overall health all contribute to diverse clinical outcomes.

Risk factors

Risk factors for breast cancer include both intrinsic patient characteristics (e.g., age, sex, genetics, menopausal status, and moment of menopausal onset) and environmental/lifestyle (e.g., smoking, alcohol, lack of exercise, and obesity). By far the largest and most well-known risk factor for breast cancer is sex. Although breast cancer can occur in men, it is extremely rare and in 99% of the cases it affects women [22]. Another important risk factor, as with many cancers, is age. Especially in women over 50 years of age (postmenopausal women) the incidence of breast cancer increases significantly [23].

Hormonal factors also play an important role in the risk of developing breast cancer. Prolonged exposure to oestrogen and progesterone through factors such as late menopause, early menarche (onset of menstruation), and hormonal replacement therapies increases the risk of developing breast cancer [24]. Meanwhile, pregnancy and breastfeeding reduce the risk of developing breast cancer [25]. Genetic disposition also plays an important role. Carriers of pathogenic *BRCA1* or *BRCA2* germline mutations have a higher likelihood of developing breast cancer with up to 70% life-time risk and often have an earlier disease onset [26, 27]. Women with dense breast tissue also have a higher risk of developing breast cancer, with a combined risk of developing breast cancer being missed in early detection screenings. These women may require additional screening through, e.g., magnetic resonance imaging (MRI) or ultrasound [28]. In addition, a family history of breast cancer increases the risk of developing breast cancer even in the absence of known germline pathogenic variants in established breast cancer susceptibility genes [24].

Diagnosis

Once a tumour is suspected, further diagnostic procedures are required to confirm the malignancy of the abnormality. Additional ultrasound imaging can be implemented, especially in women with dense breasts where standard screening may not be informative enough [28]. Alternatively, magnetic resonance imaging (MRI) can be used,

but this is usually reserved for high-risk patients or to guide surgical planning [29]. If there is a suspicious finding, a biopsy, usually guided by images, is performed to obtain a tissue sample for histopathological analysis. This allows clinicians to not only confirm the presence of cancer but also to determine other critical characteristics or factors, both pathologically and potentially genomically, that can guide treatment. The following section describes (some) of these factors.

Classification

Currently, a variety of treatment strategies are available for breast cancer. To identify the most appropriate approach for each patient, oncologists often rely on key classifications such as tumour grade, stage, and histological subtype. These factors play a crucial role in tailoring treatment plans to achieve optimal outcomes.

Grade

The tumour grade informs the oncologist about the potential of the tumour to grow and spread (metastasize). Pathologists determine the grade of a tumour by investigating how closely the tissue in the histological section resembles normal tissue. The most widely used classification system is the Nottingham histological grading system, which takes into account nuclear pleomorphism, tubule formation, and mitotic activity [30, 31]. Nuclear pleomorphism describes the variability in the size and shape of the nuclei of cancer cells. The more variety in shape and size of the tumour cells, the more the tumour has evolved away from normal tissue, which is an indicator of a more aggressive tumour phenotype [32]. Tubule formation refers to how much of the tumour retains its normal glandular structures (tubules), where a higher tubule formation score is an indicator of more (normal) well-differentiated breast tissue. Mitotic activity is a measure of how quickly cells divide. Tumour cells can divide quickly, and faster-growing tumours are indicators of higher aggressiveness. These features together determine the overall grade of the tumour, which can range from grade 1: a slowly growing, normal tissue resembling tumour, to grade 3: a poorly differentiated, abnormal-looking and rapidly growing tumour.

Table 1 provides an overview of the Nottingham Grade components and how they combine into different grades. As a semi-quantitative measure of scoring, there may be some discrepancies in grading between pathologists, especially for grade 2 tumours [31]. Total scores from 3–5 indicate for well-differentiated grade 1 tumours; scores 6–7 moderately differentiated grade 2 tumours; and scores 8–9 poorly differentiated grade 3 tumours.

Table 1: Nottingham Histological Grade scoring table [31].

Histological feature	Characteristic	Score
Nuclear pleomorphism	Minimal variation in shape and size	1
Nuclear pleomorphism	Moderate variation in shape and size	2
Nuclear pleomorphism	Marked variation in shape and size	3
Tubule formation	$> 75\%$ tubule formation	1
Tubule formation	$< 75\%$ but $> 10\%$ tubule formation	2
Tubule formation	$< 10\%$ tubule formation	3
Mitotic count[a]	Mitotic count ≤ 7	1
Mitotic count[a]	Mitotic count ≥ 8 & ≤ 14	2
Mitotic count[a]	Mitotic count ≥ 15	3

[a]Based on an area of $2\,\mathrm{mm}^2$. Guidelines may vary depending on microscope field view.

Histology

Most breast cancers arise from epithelial cells that line the ducts and lobes. The most common histological subtype is invasive ductal carcinoma (IDC), which accounts for about 70–80% of breast cancer cases [33]. As the name suggests, IDC arises from the milk ducts and is characterised by its ability to spread beyond the ductal walls. It can potentially metastasize to lymph nodes and distant organs. On imaging and pathology examination, IDC often presents as a firm, irregular mass with distinct histological features such as glandular differentiation and fibrous stroma. Invasive lobular carcinoma (ILC) derives from the lobules and represents approximately all 10–15% of breast cancer cases [34]. Unlike the firm mass that IDC often presents as, ILC has a diffuse growth pattern, which makes it less visible on any imaging techniques, such as mammography screenings [35].

Stage

The most widely used staging classification is the TNM system. This system assesses the size of the primary tumour (T), the involvement of the regional lymph nodes (N), and the presence of distant metastases (M) [36]. Based on these three components, breast cancer is divided into four stages, ranging from Stage 0 to Stage IV. Stage 0 is non-invasive and remains confined within the ducts of the breast and is also referred to as ductal carcinoma in situ (DCIS). Lobular carcinoma in situ is considered benign, so it is not included at this stage [37]. Stage I and II are considered early-stage, where the tumour is still relatively small. The lymph nodes are only marginally or not at all involved at this stage. As long as the tumour is within these stages, it has not spread beyond the breast or nearby tissue and is highly treatable with surgery, radiation, or systemic therapies [38].

Table 2: Pathological stage as defined by the TNM classification (8th edition).

Stage	Tumour[a]	Node	Metastasis
0	DCIS	No lymph node involvement	No metastasis
I	Tumour size $\leq$ 20 mm	No lymph node involvement	No metastasis
I	No evidence of primary tumour	Micrometastases	No metastasis
I	Tumour size $\leq$ 20 mm	Micrometastases	No metastasis
II	No evidence of primary tumour	Metastases to axillary	No metastasis
II	Tumour size $\leq$ 20 mm	Metastases to axillary	No metastasis
II	Tumour size $>$ 20 mm but $\leq$ 50 mm	No lymph node involvement	No metastasis
II	Tumour size $>$ 20 mm but $\leq$ 50 mm	Metastases to axillary	No metastasis
II	Tumour size $>$ 50 mm	No lymph node involvement	No metastasis
III	No evidence of primary tumour	Metastases to infraclavicular	No metastasis
III	Tumour size $\leq$ 20 mm	Metastases to infraclavicular	No metastasis
III	Tumour size $>$ 20 mm but $\leq$ 50 mm	Metastases to infraclavicular	No metastasis
III	Tumour size $>$ 50 mm	Metastases to axillary	No metastasis
III	Tumour size $>$ 50 mm	Metastases to infraclavicular	No metastasis
III	Invasion of skin and/or chest	No lymph node involvement	No metastasis
III	Invasion of skin and/or chest	Metastases to axillary	No metastasis
III	Invasion of skin and/or chest	Metastases to infraclavicular	No metastasis
III	Any	Metastases to axillary	No metastasis
IV	Any	Any	Distant metastasis

[a] all tumour sizes are considered in the greatest dimension

When breast cancer progresses to Stage III, also known as locally advanced breast cancer, the tumour may be larger in size and the lymph nodes typically become more involved. Stage IV breast cancer represents metastatic breast cancer, which means that the cancer has spread to other distant organs, such as the lungs, liver, or brain [39]. When this is the case, curative treatment is no longer the main goal, but instead the focus is on controlling the cancer while maintaining quality of life [40]. Table 2 describes the TNM classification metrics. The TNM classification can be further refined by implementing the biomarkers ER, PR, and HER2, NHG, and the score for the genomic test Oncotype DX [41], which the following sections discuss in more detail. This thesis focuses on triple-negative tumours of Stage I–III.

Biomarkers

In addition to the staging, grading, and overall histology of the tumour, tumour biomarkers play a major role in the final decision about a treatment strategy for breast cancer. The most important biomarkers are typically graded by a pathologist using immunohistochemistry on a resected section of the tumour, although newer technologies based on, e.g., gene expression profiles are making their way into clinical routine.

Hormone receptors and HER2

The oestrogen receptor (ER) and the progesterone receptor (PR) are two hormone receptors (HR) that act as gene-regulating transcription factors. In healthy breast tissue, they can regulate cell proliferation and survival, but when the hormone receptors are not properly regulated, it can lead to uncontrolled cell division [42]. Clinical ER and PR staining status' are determined by a pathologist scoring the percentage of tumour cells that are positively stained for ER/PR antibodies with immunohistochemistry. Tumours are typically considered positive for the respective hormone receptors when 1% or more of the tumour cells are positively stained, but the clinical cut-off is country-dependent, with Sweden setting the cut-off at 10% [43, 44]. This cut-off is based on data showing that patients with an ER-staining status of 1–9% (ER-low) have a similar prognosis as patients with ER-zero status when treated similarly [45].

The third important biomarker is the Human Epidermal growth factor Receptor 2 (HER2, encoded by the *ERBB2* gene). This receptor is a tyrosine kinase receptor in the epidermal growth factor family (EGFR), which is involved in cell growth, proliferation and differentiation [46]. Tumours can have an amplification of the *ERBB2* gene (HER2 amplification), which can lead to overexpression of HER2. Tumours with HER2 amplification or overexpression can have uncontrolled cell proliferation, where HER2 is often the driver of tumour formation, so-called oncogene addiction. HER2 scoring is performed on a scale of 0 to 3, where a score of 3+ indicates HER2-positivity (HER2+), and 2+ tumours will be additionally tested to confirm HER2-positivity [47]. Tumours with a score of 0 and 1+ are generally considered HER2-negative (HER2-) tumours. However, more recently tumours with low HER2 expression (IHC score 1+ or 2+ without amplification) have emerged as a clinically relevant subgroup, called HER2-low, based on the possibility of treating these tumours with recently developed antibody-drug conjugates such as trastuzumab-deruxtecan.

Ki67

In addition to hormone receptors and HER2, Ki67 staining is also often performed on resected tumour tissue. This biomarker is a specific marker for cell proliferation and a good indicator of tumour aggressiveness. The higher the positivity of the Ki67 antibody staining, the more aggressive the tumour is generally [48]. Ki67 expression is also measured as a percentage of tumour cells that express Ki67.

PD-L1

More recently, Programmed Death Ligand-1 (PD-L1) has been added as a biomarker for (metastatic) TNBC. PD-L1 is a protein that is present across the cell membrane (transmembrane) of both tumour cells and non-tumour cells (e.g. immune cells) and acts as a kind of brake on the immune response. PD-L1 staining can be evaluated according to different metrics, depending on the drug under consideration [49, 50]. For instance, for atezolizumab an Immune Cell (IC) score of immune cells positively stained for PD-L1 that is $\geq 1\%$ would indicate treatment, while for pembrolizumab (combined with chemotherapy) a Combined Positive Score (CPS) of tumour cells, lymphocytes, and macrophages for PD-L1 $\geq 10\%$ would indicate treatment. A problem with PD-L1 assessment is that different antibodies are recommended for specific drugs, so-called companion diagnostics.

$$\text{IC} = \frac{\text{PD-L1 positively stained immune cells}}{\text{tumour area}}$$

$$\text{CPS} = \frac{\text{PD-L1 positively stained tumour cells, lymphocytes and macrophages}}{\text{all vital tumour cells}}$$

Clinical subgroups

The exact proposed treatment strategy for a specific patient is discussed during a multidisciplinary panel with different experts, including an oncologist, but above the factors are important considerations. Patients can be divided into clinical subgroups based on the above classification parameters, with the clinical subgroups guiding the treatment decisions.

ER+/HER2-, Ki67 low

These tumours often have a low grade and a lower risk of relapse over the longer term. They are generally the breast tumours with the best prognosis and may receive only endocrine therapy [51].

ER+/HER2-, Ki67 high

While still positive for ER-expression, the higher proliferation is a marker of increased tumour aggressiveness and these tumours are also characterised by often higher

grades and an increased risk of relapse over the long term as opposed to Ki67 low tumours [51].

HER2+

HER2+ tumours are also often of higher grades and show a higher risk of relapse with an increasing risk of relapse already in the shorter term. ER-positivity in HER2+ tumours is usually a sign of less aggressive disease and is therefore a positive compendium biomarker. Of key relevance for patients with HER2+ disease is the possibility to have targeted anti-HER2 therapy, e.g., trastuzumab.

Triple-negative

Tumours that are negative for ER, PR, and HER2 are called triple-negative tumours. They are typically the most aggressive tumours that occur in younger patients and are tumours with a higher risk of relapse. Historically, the lack of ER, PR, and HER2 expression has left these patients without any targeted treatment options [51]. These tumours often have high Ki67 levels, relapse already at an earlier time point than the other subtypes, and have a higher risk of metastasizing to other sites in the body [51].

Intrinsic subtypes

As mentioned earlier, gene expression profiling is increasingly recommended for breast cancer patients, which means that genomic profiles are increasingly available in the clinic. In 2000, Perou and Sørlie et al. developed the intrinsic subtypes based on DNA microarray data of 42 patients and 17 cell lines using over 8000 genes [52]. The subtypes found in this study were called intrinsic subtypes and were able to stratify patient groups, showing that distinct underlying biological processes can predict the aggressiveness of a tumour and the type of treatment it will respond to. Later, in 2009, Perou and colleagues created a classifier that would only require 50 genes and named it PAM50[2] [53]. The 50-gene panel made clinical implementation more practical and is currently in use in clinics worldwide as a standardised test under the name Prosigna [54].

The four distinct intrinsic subtypes are Luminal A, Luminal B, HER2-enriched, and Basal-like.

[2]PAM after the method Prediction Analysis of Microarray

Luminal A

Luminal A tumours are characterised by high expression levels of ER-related genes and show a lower proliferation level. These tumours are often of a lower grade with a lower risk of relapse. These tumours often fall in the ER+ clinical subgroup with low Ki67 proliferation marker levels.

Luminal B

Like Luminal A tumours, Luminal B tumours have high expression levels of ER-related genes. These tumours also have higher proliferation levels and are more aggressive than Luminal A tumours. They often present with a higher grade than Luminal A tumours and a higher risk of relapse. These tumours often fall in the ER+ subgroup but have a higher Ki67 marker level.

HER2-enriched

HER2-enriched tumours express high levels of HER2-related genes, such as *ERBB2* and *GRB7*, and often show high expression levels for proliferative genes as well [55]. Fibroblast Growth Factor Receptor 4 (FGFR4) is one of the tyrosine kinase receptor genes that often show high expression levels in HER2-enriched tumours and is included in the PAM50 gene panel [53]. A majority of HER2-enriched tumours display clinical HER2-amplification, although a subset of ER+ tumours with clinical HER2-amplification is subtyped as Luminal B [56].

Basal-like

Basal-like tumours lack the expression of oestrogen-related genes, nor have they any HER2-related expression, but show high expression levels of Ki67 and basal cytokeratins. While a large proportion of TNBCs are PAM50 subtyped as basal-like, the clinical subgroup of TNBC is not equivalent to basal-like disease.

Clinical subgroups and intrinsic subtypes

Clinical subgroups based on immunohistochemistry and gene-level derived intrinsic subtypes show significant overlap but are not exactly a one-to-one grouping. Figure 2 shows how tumours from patients enrolled in the Sweden Cancerome Analysis -Breast (SCAN-B) observational study are classified across clinical subgroups and PAM50.

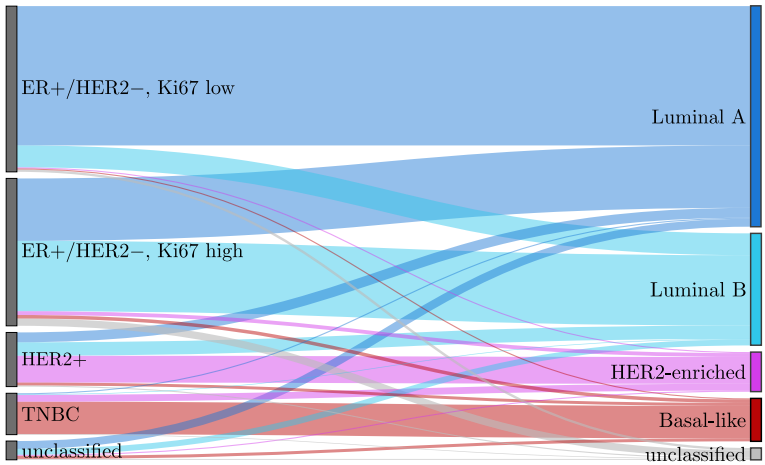

Figure 2: Link between biomarker-based clinical subgroups and PAM50 subtypes, based on supplementary data from [57].

Current treatment strategies

Historically, breast cancer was treated through surgery and until the late 1970's radical mastectomy, where the entire breast and surrounding muscles were removed, was still a popular treatment choice. Over time research has fortunately shown that equivalent effectiveness can be reached through less radical procedures, including breast-conserving surgery. Over time, more and more targeted approaches have emerged for specific breast patient subgroups, which has helped in improving the overall survival of breast cancer patients. If breast cancer is diagnosed in an earlier stage, less radical treatment (with reduced associated patient risks and societal costs) is often required, and patient survival is better, highlighting again the importance of nationwide screening programs.

Surgery

Surgery is one of the cornerstones of breast cancer treatment, especially in the early stages of the disease, when the entire tumour can still be removed by surgery. During surgery, the entire tumour is removed with a margin of normal tissue around it. A pathologist judges whether the edges of the resected section are 'clean', i.e. no tumour is present. Whether just the tumour is removed, or an entire breast i.e. mastectomy, depends on the the risk of recurrence, stage, and location of the tumour, but also importantly the patient preference. Patients with TNBC are high risk of recurrence patients, who often undergo mastectomy. If tumour cells are present in lymph nodes, these can also be surgically removed.

Radiotherapy

Another important treatment approach is radiotherapy. Here, high-energy radiation rays can be directed to target the tumour, including also the lymph nodes if the tumour has spread there. Radiation therapy is typically used to reduce local recurrences in the breast or lymph nodes but has side effects for patients.

Chemotherapy

Treatment with cytotoxic agents is also a common treatment approach in breast cancer, especially for patients with a more aggressive disease., such as triple-negative patients [38]. While chemotherapy can be given as a stand-alone treatment option, it is usually combined with surgery (adjuvant chemotherapy). When given after surgery the goal is to remove any potential leftover tumour cells and reduce the risk of recurrence. Chemotherapy can also be given before surgery (neoadjuvant). This has as benefit that the treatment gives an early indication of chemotherapy response, and (if effective) a smaller tumour can be removed during surgery, potentially facilitating breast-conserving surgery. Chemotherapy is an important treatment option for patients with TNBC and neoadjuvant chemotherapy is commonly used in Sweden for these patients. Since TNBCs are often highly proliferative tumours, cytotoxic agents that target cell division can be very effective in reducing tumour size.

Endocrine

A key treatment regime for hormone-positive breast cancers is the use of endocrine therapy. Through reduction of ER activity, the tumour cells that are 'addicted' to oestrogen can be targeted and the main driver of the tumour reduced/blocked. Different endocrine options exist for ER disruption, including aromatase inhibitors and selective ER modulators. As this treatment option only exists for hormone-positive tumours, triple-negative patients and HER2+ HR- patients do not benefit from endocrine therapies.

HER2-targeted immunotherapy

In the late 1990s, HER2-targeted treatment became available for HER2+ patients, drastically changing their fate [58]. Passive immunotherapy with monoclonal antibodies like trastuzumab (brandname Herceptin) bind to the HER2 receptor and through their binding disrupt several downstream processes, including the inhibition

of the PI3K pathway, eventually leading to cell death [59]. As triple-negative patients do not overexpress HER2 at the same level as HER2+ tumours, this therapy option is not suitable for them. While conventional HER2-targeted treatment is not recommended to patients with TNBC, a substantial proportion of these patients have tumours that are classified as HER2-low, making them potentially treatable with recent antibody-drug conjugates targeting HER2-low tumours [60].

Immune Checkpoint Blockade Therapies

A more recent treatment strategy that has emerged for patients with TNBC is the use of immune checkpoint blockade therapies. These drugs have as goal to remove inhibitory factors from T-cell functioning, thereby increasing the body's natural immune response against tumour cells. Specific targets for the drugs include PD-1 or PD-L1 inhibitors, such as atezolizumab and currently pembrolizumab. Other targets include cytotoxic T-lymphocyte associated protein (CTLA-4), a protein expressed on the cell surface of T-cells, in particular regulatory T-cells. This protein plays an important role in the regulation of immune activation/immune response and by blocking the protein through antibody binding this brake is removed. The increased T-cell activity can help in the anti-tumoural immune response.

Immunotherapy is not without side effects and immune-related adverse advents are common. Symptoms can be relatively mild, such as fatigue, diarrhoea, and rash, to occasionally severe and potentially life-threatening, including pneumonitis and hepatotoxicity [61].

PARP inhibitors

Poly(ADP-ribose) polymerase (PARP) inhibitors have also more recently become available as therapeutic option for breast cancer patients with *BRCA* mutations. The *BRCA* mutations impair the tumour suppressor genes *BRCA1* and/or *BRCA2* (upon loss of the second copy of these genes). The functional loss of these tumour suppressor genes leads to an inability for homologous recombination repair (HRR) also called homologous recombination deficiency (HRD). Some tumours who do not have mutations in the *BRCA* genes, but in other genes related to the HRR pathway, still exhibit the same HRD phenotype. The PARP inhibitors block the PARP enzymes, which are critical for repairing single-strand DNA breaks. Accumulation of the single-strand breaks without repair will eventually lead to double-strand breaks in the DNA. HR deficient tumour cells are unable to repair the double strand breaks, thereby resulting incancer cell death. Patients with *BRCA* mutations or BRCAness can both benefit from the PARP inhibitors. Recent whole genome sequencing studies in TNBC have

demonstrated that close to 60% of patients have tumours with an HRD phenotype, of which approximately 70% could be explained by alterations in four HRR genes *BRCA1*, *BRCA2*, *PALB2*, and *RAD51C* [62].

Anti-Trop2 Antibody Drug Conjugates

In more severe cases where the breast cancer cannot be surgically removed or has spread outside of the breast or fully metastasized to distant organs, antibody drug conjugates (ADCs) specific to Trop2 can be used for TNBC or HR-positive HER2- breast cancers [40]. Upon binding of the monoclonal antibody in the conjugate to Trop2 on the cell surface, the cell takes up the small cytotoxic molecule (SN-38), which inhibits topoisomerase I. Inhibition of topoisomerase I DNA blocks cell division and tumour cells are no longer able to proliferate. Side effects of Anti-Trop2 ADCs are typical of monoclonal antibody adverse effects, including fatigue, diarrhoea and nausea, but also neutropenia.

Other targeted treatments

Other targeted treatment options in breast cancer include PI3K pathway inhibitors, which are currently only clinically approved for hormone receptor positive, HER2- patients, and, CDK4/6 inhibitors, which are also only approved for hormone receptor positive, HER2- patients. However, they are typically not included for use in patients TNBC [38, 40].

Future treatment strategies for TNBC

Improving treatment strategies for patients with TNBC can take several approaches. First, the development of new treatments that can effectively target (specific subgroups of) TNBC patients, potentially in combination with immune checkpoint inhibitors and chemotherapy, is an obvious, but difficult and unpredictable road. Separately, the identification of better predictive biomarkers can help to determine who and when to treat and may thereby also minimise the adverse events often associated with antibody-drug conjugates and immune checkpoint inhibitors. Alternatively, treatment de-escalation, such as reducing the extent of surgical interventions or omitting adjuvant chemotherapy in patients with an excellent prognosis is another approach [38]. For this, retrospective studies based on treatment-naïve (no adjuvant therapy) patient cohorts have shown that patients with very high counts of tumour infiltrating lymphocytes (TILs) have an excellent prognosis after surgery alone [63]. As such, TILs have emerged as an additional important histological feature, especially for HER2+

and TNBC, representing immune response in the tumour micro-environment. TIL scores that have been validated for diagnostics purposes are quantified as a percentage of TILs in the stroma surrounding the tumour environment [64].

Biology of TNBC

TNBC as a subtype

TNBC is a disease that is characterised by and named for the lack of hormonal receptors and HER2. In reality, TNBC is a highly heterogeneous subgroup, with diverse biology underlying the disease. Both phenotypically and genomically/intrinsically there is a large variety between TNBC tumours from different patients. Several classification strategies for TNBC have been suggested, ranging from immune characterisation to RNA-expression profiles. Patients with triple-negative disease are often younger, have a more aggressive disease, and are at a higher risk for relapse.

Molecular landscape of TNBC

TNBC are tumours that often have highly rearranged genomes and contain defects in their DNA repair pathways, which can lead to an accumulation of a high total number of mutations and structural rearrangements in the tumour genomes, especially when compared to ER+ breast cancers [65]. This tumour mutational burden (TMB) is in TNBC often associated with a better clinical outcome, although reports vary [66, 67]. Mutations in high-risk genes such as *BRCA1* and *BRCA2* are relatively common in TNBC [62]. These alterations can result in homologous recombination deficiency (HRD), where DNA double-strand breaks are not effectively repaired anymore. In a recent study it was reported that close to 60% of tumours in a population representative triple negative breast cancer cohort showed an HRD phenotype, indicating that HRD is a dominant mutational process that shape a large proportion of tumour genomes [62]. The most common molecular alterations in TNBC are *TP53* mutations, occurring in $60 - 70\%$ of the cases according to [68, 69], with a higher frequency among basal TNBCs. Other key mutations include *PIK3CA*, which is the second most mutation gene in TNBC [68].

To better understand the genetic diversity in TNBC and potentially aid clinical decisions several subtyping schemes have been suggested. The following sections describe some of the proposed classification systems based on transcriptomic data.

Lehmann

The Lehmann classifications were created using k-means clustering on gene expression (microarray) profiles from 587 TNBC samples from 21 breast cancer data sets [70]. TNBC samples total were used to perform the clustering and initially six clusters were identified as the optimal number of clusters. These classes were Basal-like 1 (BL1), Basal-like 2 (BL2), Mesenchymal (M), Mesenchymal stem-like (MSL), luminal androgen receptor (LAR), and immunomodulatory (IM). Later, this was refined to four clusters and an 'overlaying' classification for IM, because the MSL and IM class were based on transcripts from tumour-associated stromal cells and TILs respectively, so not considered tumour-intrinsic [71]. The next section describes the four classes as Lehmann et al defined them in 2016 and the immunomodulatory class.

Basal-like 1 (BL1) Basal-like 1 (BL1) tumours comprise a large portion of TNBC, accounting for approximately 35% of TNBCs. This subtype is characterised by a favourable clinical response with 41% of patients achieving a pathological complete response (pCR) following neoadjuvant therapy [71]. Histopathologically, BL1 tumours are enriched for medullary carcinomas, a pathology common in patients with the *BRCA1* mutations, especially in younger patients. On a molecular level, BL1 tumours exhibit a relatively high mutational burden with an average mutation rate of 2.1 mutations per megabase pair (mut/Mbp) and show significant enrichment for homologous recombinant deficiency (HRD). Clinically, these tumours demonstrate the lowest risk for progression among Lehmann subtypes [72].

Basal-like 2 (BL2) The Basal-like 2 (BL2) subtype represents around 22% of TNBCs and is distinguished by a lower pCR rate with only 18% of patients achieving pCR in clinical studies [71]. Histologically, BL2 tumours are enriched for metaplastic carcinomas, a rare and aggressive form of breast cancer. These tumours often display a mix of histological features, suggesting they arrive from highly plastic, young cells, capable of differentiating into different types of mature cells. BL2 tumours are hypothesized to originate from myoepithelial cells and are at the highest risk for progression. Genomically, BL2 tumours have a lower mutational load, with about 1.2 mut/Mbp.

Mesenchymal (M) The Mesenchymal (M) subtype were originally classified alongside the mesenchymal-like (MSL) subtype, but upon revision of the subtypes the MSL subtype was dropped, with the difference between types mainly attributed to a higher immune infiltration in MSL tumours [71]. Representing 25% of TNBC tumours, M tumours are often relatively "immune cold", lacking prominent lymphocyte and monocyte signatures and are notably deficient in antigen-presenting and effector im-

mune cell populations, contributing to a colder immune micro-environment. Despite the lower immune infiltration about 40% of the tumours achieved a pCR, ranking M tumours just behind BL1 tumours in that aspect [71]. Histopathologically, M tumours are enriched for rare malignant phyllodes tumours, which exhibit a distinctive leaf-like growth pattern. Genomically, this subtypes is marked by high mutational burden, averaging 2.3 mut/Mbp. However, the high mutational load does not translate well to an improved progression-free interval, which suggests other factors drive these tumours. M tumours have a relatively high mutational rate of epigenetic modifiers and exhibit loss of chromatin remodelling genes. Furthermore, these tumours are enriched for homologous recombination deficiency (HRD) and show a higher prevalence of DNA repair gene deletions, including *BRCA1*, *BRCA2*, and *ATM*, providing potential therapeutics target for DNA-damaging agents.

Luminal Androgen Receptor (LAR) The Luminal Androgene Receptor (LAR) subtype accounts for approximately 17% of TNBCs. Patients with LAR tumours are typically older, and about 29% of them have a pCR, which is modest compared to BL1 and M tumours. Histopathologically, LAR tumours are more frequently invasive lobular carcinomas, originating in glandular tissues, and are thought to derive from luminal progenitor cells. The LAR subtype stands out for its significant enrichment in hormone signalling pathways, steroid synthesis, androgen/oestrogen metabolism, and overexpression of androgen receptors, together reflecting its likrly reliance on hormone signalling for growth [73]. Genomic features of LAR include a relatively low mutational burden, averaging 1.8 mut/Mbp, and the relative absence of DNA repair and cell cycle alterations.

Immunomodulatory (IM) The immunomodulatory (IM) subtype was initially proposed as one of the Lehmann subtypes, but was not retained as a distinct subtype upon the revision in 2016 [71]. This decision reflected the underlying thought that immune-enriched features are not exclusive to a single TNBC subtype, but are reflective of variations in the immune micro-environment and can occur across subtypes. Binary IM classifications for tumours are still possible, for instance by using a correlation cut-off to the IM centroid correlation value for a tumour. This allows the categorisation of tumours into IM-positive (immune hot) and IM-negative (immune cold), alongside the four other subtypes classifications. IM tumours are characterised by significant enrichment in genes involved in immune signalling and immune cell pathways, including those related to for instance antigen presentation and cytokine signalling.

Burstein

In 2015 Burstein et al. also classified TNBC further, this time using mRNA expression (Affymetrix) and DNA profiling of 198 TNBC samples [74]. Burstein et al. clustered tumours into four groups using the top median-centred genes using non-negative matrix factorisation. Similarly to Lehmann they found a LAR subtype, a mesenchymal subtype (MES) and two basal like subtypes, of which one was immune infiltrated (BLIA) and one immune cold (BLIS).

Basal Like Immune Activated (BLIA) Basal Like Immune Activated (BLIA) tumours are characterised by significant immune activation. BLIA tumours frequently exhibit mutations in Tumour Protein T53 (TP53), a hallmark of many aggressive tumours, and are associated with high chromosomal instability and elevated HRD scores. Cyclin Dependent Kinase 1 (CDK1) amplification are more common in this subtype. Despite these markers of genetic disruption, BLIA tumours maintain lower aberrant cell fractions and exhibit increased immune cell infiltration into the tumour micro-environment. Immune infiltration is a defining feature of this subtype, marked by e.g. CTLA4 overexpression. BLIA tumours demonstrate lower levels of angiogenesis and metastasis-related markers, and have been proposed as good targets for immunotherapy.

Basal Like Immune Suppressed (BLIS) The Basal Like Immune Suppressed Subtype (BLIS) is the most prevalent subtype among the Burstein subtypes. Like the BLIA subtype, BLIS tumours exhibit high chromosomal instability and elevated HRD scores, along with frequent loss of *TP53*. The main contrast to BLIA tumours is the markedly reduced immune infiltration and activity. This suppression may be linked to the overexpression of V-Set Domain Containing T-Cell Activation Inhibitor 1 (*VTCN1*) , a negative regulator of T-cell mediated immune response, and BLIS tumours are then also associated with higher reoccurrence rates [74].

Mesenchymal (MES) The Mesenchymal (MES) subtype as defined by Burstein et al. shows significant enrichment in pathways related to the extracellular matrix (ECM), epithelial-to-mesenchymal transition (EMT), and angiogenesis. This subtype also exhibits particular involvement of the cell cycle, mismatch repair, and DNA damage networks (HRD), as well as hereditary breast signalling pathways. MES tumours are notable for expressing genes typically to other non-epithelial cell types, such as *OGN* (typically associated with osteocytes) and *ADIPOQ* and *PLIN1* (adipocytes), and important growth factors, such as IGF-1. MES tumours typically have an increased level of normal immune cell infiltration and a lower fraction of aberrant cells.

Luminal Androgen Receptor (LAR) The Luminal Androgen Receptor (LAR) subtype according to the Burstein classification stands out due its classification as ER- tumours (according to international/US standards), but having high expression of oestrogen-related genes, such as *FOXA1*, *GATA3*, *PGR*, and *XBP1*, indicating some ER related activation. ERBBs mutations are also commonly found in LAR tumours, which may be contributing to their resistance against trastuzumab. As the name suggests, the LAR subtype is marked by androgen receptor (AR) and MUC1 overexpression, highlighting the importance of androgenic signalling. LAR is frequently characterised by mutations in *PIK3CA* (present in about half of the cases), leading to hyperactivation of the PI3K/AKT signalling pathway.

FUSCC

In 2019 another TNBC study conducted by researcher at the Fudan University Shanghai Center (FUSCC) added an additional perspective on TNBC subtyping. They analysed 465 primary TNBC samples, mainly with patients from the East-Asian population [75]. Using consensus clustering by resampling, the study identified (again) four TNBC subtypes and associated gene targets for each. Especially the identification of gene targets for each subtype added critical groundwork for subtype specific treatment strategies in TNBC [76].

Basal Like Immune Suppressed Subtype (BLIS) The (BLIS) is the most prevalent among the FUSCC subtypes, encompassing around 39% of the tumours. BLIS tumours are characterised by their immune-suppressed profile. Additional hallmarks of this subtype are the upregulation of genes involved in cell cycle regulation, reflecting their heightened proliferative activity, and activation of DNA repair pathways, suggesting a reliance on mechanisms to mitigate genomic instability.

Immunomodulatory (IM) The IM subtype accounts for 24% of the TNBC tumours and is distinguished by its pronounced immune-related gene expression profile. This subtype is characterised by high expression of genes involved in immune cell signalling and cytokine signalling pathways, indicating the presence of an active immune response in these tumours.

Mesenchymal (MES) The MES subtype is the least common FUSCC subtype, with approximately 15% of the tumours being classified as such. MES tumours are characterised by a mesenchymal-like phenotype, and show enrichment for genes involved

in mammary stem cell pathways, such as the JAK/STAT3 signalling pathway, which is responsible for the maintance of breast cancer stem cells [76].

Luminal Androgen Receptor (LAR) The Luminal Androgen Receptor (LAR) subtype constitutes 23% of the TNBC tumours in the FUSCC classification and is primarily defined by the activation of AR signalling pathways.

TNBC subtype consensus The three different subtyping approaches underscore a recurring set of biological themes that reflect the heterogeneity of TNBC. Across the Lehmann, Burstein, and FUSCC schemes, a consistent presence of a basal-like phenotype emerges (also consistent with the high fraction of basal tumours in TNBC according to the breast cancer wide PAM50 classification). The basal-like tumours are characterised by their high proliferative capacity and expression of basal cytokeratins. Another recurrent theme is the immune landscape, with either basal subtypes classified according to their immune infiltration (Burstein, FUSCC) or immune infiltration as a separate additional class (Lehmann-revised). Additionally the LAR subtype stands out distinctly and is present as its subtype in all three classification schemes, with high levels of hormonally influenced androgen receptor signalling, despite TNBCs lack of oestrogen or progesterone on IHC stainings. Finally, the mesenchymal subtype, also present in all three classification systems, reflects a theme of a subtype with features of epithelial-mesenchymal transition and is enriched in stromal and developmental pathways.

The intersection of immunity and the tumour micro-environment

To fully understand the role of immune cells in cancer, and specifically TNBC for this thesis, it is essential to have a general understanding of the immune response to tumour cells.

Immune invasion

The immune system has two arms of immune response: the innate and adaptive immune response. The innate immune response serves as the body's first line of defence, providing a rapid but non-specific response to pathogens. Meanwhile, the adaptive immune response is a more specialised arm of immune response. It responds more slowly but has the ability to recognise specific antigens and generate immunological memory. This is also the arm of immune response that often clears up malignant cells,

before they can grow out to become full tumours. The interplay between the immune response and a tumour is called the cancer-immunity cycle [77].

The cancer-immunity cycle involves the following steps:

1. Release of cancer cell antigens
2. Cancer cell antigen presentation (innate) (dendritic cells (innate) & APCs (adaptive))
3. Priming and activation (APCs (innate &adaptive) & T-cells (adaptive))
4. Trafficking of T-cells to tumours (cytotoxic T-cells (adaptive))
5. Infiltration of T-cells into tumours (cytotoxic T-cells (adaptive), endothelial cells (conditional innate))
6. Recognition of cancer cells by T-cells (cytotoxic T-cells (adaptive), cancer cells)
7. Killing of cancer cells (immune and cancer cells)

While the cancer-immunity cycle seems straightforward, the ability of tumours to escape this response and/or turn inflammation into a tumour-progressing factor make the dynamics between tumours and the immune system highly complex. Additionally, these dynamics are tumour-type dependent. Breast cancers are generally considered less immunogenic compared to some other cancers , but TNBC stands out for its relatively high immunogenicity [78].

Tumour micro-environment

As mentioned earlier in the hallmarks of cancer, a tumour is not simply a collection of malignant cells. To survive, grow and metastasize, a tumour recruits and manipulates the cells in its surroundings. This environment, known as the tumour micro-environment (TME) [79], is a complex ecosystem that includes tumour cells, immune cells, the vasculature and lymphatics supporting the tumour, and stromal components, such as fibroblasts. At its core, a tumour resembles a complex and dysfunctional organ.

In recent years, significant attention has been directed towards the immune cells within the TME, often referred to as the tumour immune micro-environment (TIME) [80–82]. While the immune system is supposed to recognise and eliminate malignant cells before they can develop into tumours, cancer cells can evade and subvert these

defences, and even use the influx of immune cells to their own benefit, where cancer cells can use this influx for a supply of nutrients, growth-factors, and other resources to grow and expand [10].

When it comes to the TIME, different types of immune cells from the innate and adaptive immunity play various roles [83]. The following sections summarise the roles of various immune cells and other cells important in the TIME of TNBC.

Lymphocytes

Lymphocytes are immune cells that are produced by the bone marrow and circulate in the blood and lymphatic system [84]. Their name reflects their close association with lymphatic tissues, including the lymph nodes and the spleen. There are three main categories of lymphocytes: B-lymphocytes (B-cells), T-lymphocytes (T-cells), and natural killer (NK) cells. B-cell, and T-cells are part of the adaptive immune response, while NK cells are typically considered part of the innate immunity, although they show some overlap with the adaptive immune response.

Infiltration of lymphocytes (tumour infiltrating lymphocytes (TILs)) is on average higher in TNBC tumour tissue than in other breast cancer subtypes, with a median of 20% in TNBC compared to HER2+ breast cancer (15%) and ER+, HER2- (10%) breast cancer according to the breast international group 2-98 trial [85].

B-lymphocytes B-lymphocytes, or B-cells, can bind antigens and present antigens to T-cells. Upon binding of antigens to the B-cell receptor (BCR), B-cells proliferate. Some B-cells can differentiate into antibody-producing plasma cells, that can target specific tumour cells, while others will become memory B-cells, maintaining the immune response against this specific type of tumour cell. All activated types of B-cells can be found in the TME [86]. Increased B-cell infiltration in the tumour is associated with a more favourable prognosis in patients with TNBC [87–89], but B-cells seem to play contradictory roles, depending on the subtype of B-cell [90]. Some subtypes, including some plasma cells, can act immunosuppressively [91].

T-lymphocytes T-lymphocytes, or T-cells form a significant part of the TILs in TNBC. They can be broadly classified into two categories, effector T-cells and memory T-cells. Effector T-cells include the T-helper cells, cytotoxic T-cells, and regulatory T-cells. As the name suggests, T-helper cells 'help' the overall immune response by activating other immune cells. Mature T-helper cells express the glycoprotein CD4 on the cell surface, which is also their defining marker for e.g., immunohistochemical stains. Cytotoxic T-cells, also called killer T-cells or cytotoxic T-lymphocytes (CTLs),

can directly kill malignant cells through recognition of antigens presented by the malignant cell via class I major histocompatibility complex (MHC) on the cell surface. The binding of the T-cell receptor (TCR) to class I MHC is supported by the glycoprotein CD8, which is also a defining marker for cytotoxic T-cells. Regulatory T-cells are a crucial controlling part of the immune system and important to avoid, e.g., autoimmunity. However, in the context of cancer, they can dampen immune response too strongly, thereby supporting a tumour-promoting micro-environment. Regulatory T-cells are marked by the expression of CD4 and FOXP3 on their cell surface. Memory T-cells are able to 'remember' previously encountered antigens, such as presented by e..g. malignant cells. Stem cell memory T-cells can differentiate into different types of T-cells, including effector cells described above, and tissue-resident memory T-cells. Of all T-cell types, especially cytotoxic T-cells and activated or tissue-resident memory T-cells have been associated with a better prognosis in TNBC [92, 93].

Although the classification described above seems rather clear cut, single cell data from various studies have indicated that T-cells have a more continuous phenotype than appears in the above classification system, and cannot always be that easily differentiated [94–96]. More and more research is also focused on the spatial distribution of different types of T-cells, including spatial predictors of prognosis or response to therapy [97]. Together with B-cells, T-cells can form a tertiary lymphoid structure (TLS). This structure can arise in the TME and closely resembles a germinal centre, where lymphocytes can mature and proliferate [98]. TLSs are a good prognostic marker for cancer patients, including breast cancer and TNBC patients [99–101].

Natural Killer cells Unlike B- and T-cells, natural killer (NK) cells are part of the innate immune system, although they share some functional overlap with the adaptive immune system [84]. NK cells do not have an antigen receptor and therefore cannot be sensitised for specific antigens but can detect and kill malignant cells without. This makes them an important part of the early anti-tumour response, where they target MHC-1 deficient cells through lysis. NK cells can also further enhance the immune response by releasing antigens, which can be recognised by other immune cells. However, their role in TNBC is not entirely clear cut, as immature NK cells have also been reported to promote TNBC progression [102].

Monocytes

Monocytes are the largest immune cells and can destroy other cells through phagocytosis, a process in which one cell 'eats' another. While monocytes are part of the immune system and can contribute to cytotoxic activity, they can also contribute

to tumour progression [103]. Depending on the type of monocyte, they can be involved in several angiogenic activities, such as remodelling the ECM, activating angiogenesis, and suppressing anti-tumour immunity. Monocytes can differentiate into macrophages and monocyte-derived dendritic cells (moDCs) [83].

Macrophages Macrophages in the TME are often referred to as tumour-associated macrophages (TAMs) and display functional diversity. They can be broadly classified into two subtypes: M1-like and M2-like macrophages, but similar to T-cells, the classification does not appear binary in single-cell studies, but more of a continuous spectrum. M1-like macrophages are generally associated with anti-tumour immunity, actively suppressing tumour progression through activation of immune responses. In contrast, M2-like macrophages contribute to tumour progression and metastasis. They downregulate anti-tumour immunity while promoting angiogenesis, remodelling of the ECM, invasion of tumour cells, and metastasis [104].

Myeloid-derived suppressor cells Myeloid-derived suppressor cells (MDSCs) are a diverse group of immature myeloid cells that suppress the activity of other immune cells, specifically T-cells and NK cells. Their immunosuppressive behaviour leads to a favourable environment for tumour progression and metastasis and is also suspected to contribute to drug resistance.

Dendritic cells Dendritic cells (DCs) are a type of antigen sensing and antigen-presenting cells (APCs) [105], that can present their antigens on MHC-class I or II. They are part of the innate immune response with several types of DCs existing, including the monocyte-derived DCs, and are critical in linking the innate and adaptive immunity response. Mature DCs move to the lymph nodes to activate cytotoxic or helper T-cells (see also step 2 and 3 in the cancer-immunity cycle). Increased presence of a specific subset of DCs, the conventional DC1-enriched subset, was a good prognostic marker for TNBC patients in the study by Michea et al [106].

Aims

Overall aims

The aim of this thesis was to improve the understanding of immune infiltration in triple-negative breast cancer and derive approaches for better prognostication and treatment prediction.

Specific aims

Study I

In Study I, we aimed to develop an image processing workflow for immunohistochemistry-stained tissue micro-array cores and to analyse a set of immune markers to characterise the tumour immune micro-environment in triple-negative breast cancer.

Study II

In Study II, we aimed to create stable a immune classifier for triple-negative breast cancer based on transcriptomic data that could provide prognostic (and treatment predictive) information.

Study III

In Study III, we aimed to characterise the tumour immune micro-environment in triple-negative breast cancer in more depth compared to Study 1, and specifically study the use of ecosystems and spatial dispersion patterns in relation to other multi-omics annotations.

Material and Methods

Patient cohorts

Swedish Cancerome Analysis Network - Breast

The Swedish Cancerome Analysis Network - Breast (SCAN-B) is a southern Swedish initiative started in 2010 [107, 108]. This observational cohort study gives all women diagnosed with breast cancer the opportunity to participate in the study with the goal of reducing breast cancer mortality and using more personalised treatment. Researchers can access collected patient material - tissue and data in the medical charts, such as survival, metastasis, etc. - for their research projects (when the right ethical permits are in place). Since 2010, more than 17,000 patients have been enrolled in SCAN-B with a total of 7,868 samples with RNA-sequencing [57] publicly available at the time of writing.

Fudan University Shanghai Cancer Center

The triple-negative breast cancer cohort from the Fudan University Shanghai Cancer Center (FUSCC) is a cohort of 465 primary tumours with dominantly East-Asian origin [75]. The cohort contains clinical, genomic and RNA-sequencing data. The patients included were diagnosed with malignant breast cancer and retrospectively selected for inclusion in the study. The patients underwent treatment at the FUSCC between 2007 and 2014. Of the 465 patients included, RNA-sequencing data on primary tumour tissue was available for 360 patients, which we used in Study II.

The Cancer Genome Atlas

The Cancer Genome Atlas (TCGA) was a program initiated in the US in 2006 as a joint effort between the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI). TCGA has collected in total about 20,000 samples from 33 different tumour types. It includes $> 1{,}000$ breast cancer samples, depending on the release version. While the TCGA program has ended, the data stays publicly available for researchers and has been the source of a large number of research studies worldwide. For Study II, we used publicly available RNA-sequencing data for 86 triple-negative breast cancers from the TCGA program.

Histological slides

Following tumour removal at surgery (surgical resection) and clinical procedures, tumour tissue can be leftover, which can then become available for research (with ethical permits in place, placed in a biobank). Tumours are usually embedded in a paraffin block and have been fixated prior (often with 10% formalin) to minimise tissue degradation, which can lead to among others, non-specific staining. When tissue is requested for research purposes a, or multiple, slice(s) of this block can be taken, which is called sectioning. Usually sectioned slices are between $3 - 6$ mm thick. The thicker the slice, the darker any staining will be, as more antigen will be retained as more tissue is available. Thinner slices will lead to lighter staining and have a better resolution of the tissue with morphology being clearer. Artefacts that can arise during sectioning include tissue folding, tearing, or wrinkling, which can affect stain quality and subsequent tissue evaluation. Following sectioning, the tissue undergoes staining. Usually, the first staining is a hematoxylin and eosin staining (H&E staining), or an exclusively hematoxylin staining. Hematoxylin highlights nuclei in cells, while eosin stains cytoplasmic and extracellular components. The H&E stain allows for a general overview and understanding of the tissue morphology. For more specific insights, additional staining techniques can be performed, such as immunohistochemistry (IHC), immunofluorescence, and in situ hybridisation (ISH). IHC uses antibodies to detect specific proteins and is also the technique used for hormone or HER2 receptor staining in clinical diagnostics.

So far, we have been speaking of tumour tissue as full sections, or so-called 'whole (section) slides'. However, the use of tissue in research is often done on a smaller scale, minimising the cost of expensive staining and maximising the use of the little amount of patient tissue available.

Figure 3: Histological slides a) Whole-slide section with H&E staining b) TMA with IHC staining.

Tissue Microarrays

The development of a tissue microarray (TMA) is a technique that allows the analysis of multiple tissue sections on one block. Relevant tumours in paraffin blocks, called donor blocks, are selected. Following H&E staining on a whole section from each block, one or multiple areas of interest on the whole section are chosen for the TMA. The selected area on the donor block is punctured with a special type of hollow needle, leading to the extraction of a cylindrical piece of tissue from the donor block, usually about 0.6 mm to 2 mm in diameter. This piece of tissue is placed on the block that will become the TMA, the recipient block. The process continues in the same manner with the other donor blocks and some controls, placing the extracted tissues in a grid-like structure on the recipient block. Once all tissue has been placed, additional paraffin is added to stabilise the block. Next, the TMA block can be sectioned and single TMA slides, placed on glass slides, stained for markers of interest. Aside from the previously mentioned benefits, TMAs increase the tissue throughput and standardisation of tissue staining of different tumours, making it a valuable tool in research.

In Studies I and III we used TMA slides analysed either by single-plex IHC (Study I), multiplexed IHC (Study III) or spatial transcriptomics (Study III).

Digital Pathology

Traditionally, tissue slides are evaluated under microscopes and this is still a common practice. However, a significant shift in the practice of pathology is taking place, moving from microscope workflows to digitised workflows instead. In 1994 the first

commercial slide scanner became available, which allowed for the digitisation of the glass slides., or whole slide imaging (WSI) [109]. Whole slide images are usually created at different resolutions, conceptually resembling a pyramid of images. An overview scan is made at a low resolution, capturing the full tissue (or TMA) in one shot. To create a 'zoom' effect, additional scans are made at higher resolutions, and the scans are stitched together, creating a full higher resolution image. Additional information, such as the patient ID, can be stored alongside the image(s) in the same file.

A large benefit of digital slides, other than the flexibility and efficiency they provide pathologists for examining the slides, is the computational image analysis their digitisation allows. Especially since the rise of artificial intelligence (AI) and particularly since AI's success in image analysis through implementations of deep learning (DL), has pathology moved towards a more digital and computational approach. Additionally, the lack of pathologists world-wide has led to an increase in collaboration between pathologists and AI, called human-in-the-loop systems. Current research is moving beyond the detection of clear morphological features (for the human eye) and aims to integrate genomics and transcriptomics with whole slide images, towards an even more sophisticated form of computational pathology.

For the analysis of IHC stained TMAs in Studies I and III we implemented a digital/computational pathology approach.

Statistics

Statistical analysis is required to interpret the collected data, such as cell counts. A key concept for statistics is probability, which quantifies the likelihood of an event occurring, and hypothesis testing, which is used to test whether sufficient evidence exists in sample data (e.g., all women enrolled in SCAN-B) to support a conclusion on the population (e.g., all women with breast cancer). Here, a null hypothesis typically states that there is no significant difference between the tested variables. The p-value is used to quantify the probability of obtaining the observed data if the null hypothesis is true. Typically a p-value lower than 0.05 is used to reject the null hypothesis and indicate significance in medical science. However, the likelihood of rejecting the null hypothesis increases with an increasing number of hypothesis tests being carried out. This is why in areas with a high rate of multiple testing, such as transcriptomics, correction methods are in place for the p-values to control the False Discovery Rate (FDR), such as Benjamini–Hochberg and Bonferroni correction. Statistical tests are susceptible to two types of errors, type I and type II errors. A type I error is a rejection of the null hypothesis, when this should not have been rejected and is also called a false

positive. A type II error is the opposite, where the null hypothesis should have been rejected, and is called a false negative. Controlling the FDR is a form of controlling the type I errors.

Parametric and non-parametric tests

Based on the underlying assumptions statistical analysis can be broadly categorised into two categories, parametric and non-parametric tests. Parametric tests assume a distribution that approximates the normal distribution, while non-parametric tests do not assume a normal distribution of the data. In study I, II, and III we implement the non-parametric Spearman correlation for continuous variables, and the Wilcoxon signed-rank and Kruskal-Wallis test for continuous and categorical variables.

Survival analysis

An important aspect of analysis in medical research is analysis that gives more insight into which factors contribute to e.g. patient survival, tumour reoccurrence, or tumour metastasis. A vital aspect of this analysis is the time component, we are not just interested in 'if' something happens, but also 'when'. Time-to-event analysis, often also called survival analysis, analyses the time to a specific 'event' or endpoint. Standardisation guidelines for specific clinical endpoints are described by for instance the Definition for the Assessment of Time-to-event- Endpoints in CANcer (DATECAN) [110] and the Standardised Definitions for Efficacy End Points (STEEP) [111]. A key feature of survival analysis is the ability to handle censored data. When the event of interest has not occurred before the end of the recorded follow-up, or a patient is otherwise lost to follow-up, the data points are right censored. These patients are still included in the analysis until the last recorded moment.

Survival analysis is often performed using the Kaplan-Meier (KM) estimator, and KM curve, with the latter visualising for which fraction of patients the event of interest has not yet occurred [112].

To gain even more insight into the effect of a variable on the survival measure, Cox Proportional Hazard models can be implemented [113]. While KM models require a categorical or binary variable, Cox models can handle both categorical and continuous variables. Additionally, Cox models can handle more than one variable at a time (multivariate) as opposed to KM models, which are univariate only. Multivariate analysis can give a better insight into potentially confounding factors. Hazard ratios (HRs) are the primary output of Cox models, representing the relative risk of the event occurring for different groups. An HR > 1 indicates an increased risk, while

HR < 1 indicates a decreased risk.

For the survival analyses in Study I, we used invasive disease-free survival (IDFS) as the clinical endpoint. In Study II, we used, based on available clinical data for included cohorts, IDFS for the SCAN-B cohorts, recurrence-free survival (RFS) for the FUSCC cohort, and overall survival (OS) for TCGA patients. In Study III, we used IDFS as the clinical endpoint for SCAN-B patients.

Machine Learning

Machine Learning (ML) is a subset of AI that focuses on developing algorithms and statistical models enabling computers to 'learn' and make decisions or predictions without the specific task being hard coded[3]. AI encompasses the broad concept of machines performing tasks that typically require human intelligence, such as robotics, image recognition, and natural language processing. A subset of ML called Deep Learning (DL) focuses on algorithms inspired by the structure and function of the human brain. DL uses Artificial Neural Networks (ANNs) with multiple layers, often referred to as 'deep' networks, which can learn from complex data. DL has transformed image processing, enabling computers to extract detailed insights from visual data, such as face recognition in photos. Another prominent application of ML and DL today is in Large Language Models (LLMs), like ChatGPT and other chatbots, which use DL to process and generate human-like text.

ML has made big impacts in medical research, including cancer research. Examples include the implementation of ML in the detection of subtle tissue alterations in mammography screenings that are difficult to spot with the human eye [114, 115], create new breast cancer classifications as in PAM50 and the TNBC subtypes, and classify tumour grade on histological sections [116, 117].

Supervised learning

When working with labelled data with categories (e.g., 'cancer' and 'no cancer'), numerical values (e.g. correlation scores), or pixel-based image (segmentation) masks, we can apply machine learning approaches that fall under the category of supervised learning. In supervised learning, the algorithm is trained using a dataset (called training set) containing both input data (e.g., images) and corresponding labels (e.g., cancer/no cancer). This allows the algorithm to learn patterns that associate the inputs with their labels, effectively enabling it to classify new, unseen data. If the perfor-

[3]Code based on human-crafted logic, often a manually set of rules.

mance is suboptimal, or if we think further improvement is possible, the algorithm can be fine-tuned by adjusting some of its settings, called hyperparameters.

Once the algorithm has learned on the training set, a separate part of the data set is used. The algorithm has not yet seen this 'test' set, which means it can be used to test whether the algorithm has learned sufficiently from the training data and can make accurate predictions on the test set. Once an optimised model has been achieved, the final step takes place. As the model has at this point seen the test data several times, the performance on the test set is not a good indicator of the model's generalisation anymore. Therefore, the model is validated on a third data set, called the validation set. This validation provides a more realistic impression of the model's performance on new applications.

Performance metrics

As we saw in the section above, when training a supervised algorithm the performance of the algorithm on the specific task matters. Calculation of performance metrics is vital in understanding and improving model performance. This section introduces the most important performance metrics for supervised learning. The choice of metric depends on the type of problem - classification, regression, or segmentation - and the specific goal. For classification tasks, commonly used metrics include accuracy, precision, recall, sensitivity, and specificity.

When calculating classification performance metrics, several additional metrics are important. True Positives (TP) describe data 'positive' (e.g. cancer) points that are classified correctly. True Negative (TN) would in this case be the 'negative' (no cancer) data points that are classified correctly. Additionally, False Positive (FP) are the cases that are classified as 'positive' (cancer) by the model, but which are in truth negative (no cancer). Lastly, False Negatives (FN) are the cases that are classified as 'negative' (no cancer) by the model, but which are in truth positive (cancer). Depending on the intended use of a model, different metrics -derived from the true and false positives/negatives- can take more priority in the model evaluation.

Accuracy describes the model's overall performance and is calculated as:

$$\text{Accuracy} = \frac{\text{TP} + \text{TN}}{\text{TP} + \text{TN} + \text{FP} + \text{FN}}$$

Precision, a measure of the accuracy of positive predictions, is calculated as:

$$\text{Precision} = \frac{\text{TP}}{\text{TP} + \text{FP}}$$

Recall, also known as sensitivity, is a measure of the model's ability to retrieve (recall) the positive cases, and is calculated as:

$$\text{Recall} = \frac{\text{TP}}{\text{TP} + \text{FN}}$$

In Study II we implemented accuracy, precision, and recall as model evaluators as we wanted to strike a balance between the model's overall performance (accuracy) and the performance on 'positive' (immune hot) samples.

Random Forest

Random Forests (RFs) are a popular machine learning method that can be used for both regression and classification of tabular data [118]. At the base, RFs are built on the concept of decision trees, which form the building blocks of a RF. A decision tree is a hierarchical model that partitions data into subsets based on feature values, ultimately leading to a prediction. At each node of the tree, the model selects a feature and a threshold value that best split the data into more homogeneous subsets. The homogeneity of the subsets can be calculated using different metrics, such as the Gini impurity measure. The data splitting continues until a certain stop condition has been satisfied. This can be the total number of splits, the number of data points left in a subgroup, or the arrival at a fully homogenous subgroup. The resulting structure bears resemblances to a tree, with branches that represent decision rules and leaf-like nodes that represent the tree's predictions. The drawback with decision trees is that they can suffer from overfitting, especially when a single tree 'grows' many deep branches that are supposed to fit a complex dataset with non-linear relationships.

To overcome this limitation of a single decision tree, a RF is an ensemble of decision trees. Each tree in the forest is trained on a random subset of the training set, a process called bootstrapping, and a random subset of features. Both measures help to reduce overfitting and allow the forest to generalise better. When it comes to predictions, each tree gets to vote on the class label for a data point. The collective decision of the forest is determined by aggregating the votes, with the majority vote serving as the final prediction of the forest.

In Study II, the final implemented model was a RF, as implemented in scikit-learn [119], which showed a slightly better performance than the other tested supervised models.

Cell segmentation

Image segmentation is the process by which an image is partitioned into meaningful segments, which can correspond to objects, shapes, boundaries, or other features. The segmentation mask for an image is, in effect, a pixel-by-pixel labelling of the image. When we want to predict segmentation masks for new images, for example, because we want to know which part of the image visualises a tumour area, a type of DL model called convolutional neural networks (CNNs) [120, 121] has proven very successful, and in particular, the U-Net architecture [122]. Cell segmentation is a form of image segmentation where the focus is on identifying individual cells in an image, and is useful, e.g., in quantifying cell populations and analysing spatial relationships within a (tumour) tissue. However, cell segmentation has its own challenges, such as correct segmentation of cells in crowded cellular environments, irregular cell shapes and sizes, and the variety of colours between stains and scanners. Additionally, creating training labels for segmentation is labour intensive. In recent years, several cell segmentation approaches built on CNN have emerged. The following section describes one of these models, called StarDist, that we have implemented in Study I and Study III.

StarDist StarDist is a cell segmentation algorithm that applies a star-convex polygon representation [123, 124]. It assumes that each cell in the image can be represented by a shape where each line drawn from a central point inside the shape to any point on the shape's boundary stays within the object. A CNN takes the input image and predicts a probability map where cells are likely located, and a distance map with radial distances for each pixel. The probability map is used to identify cell centres, while the radial distance predictions are converted into polygonal boundaries. A non-maximum suppression step resolves potential overlaps to ensure each cell is individually segmented. The final output is a segmentation label map, where each cell has a unique label ID.

Another cell segmentation method that has gained a lot of popularity is Cellpose [125, 126]. While Cellpose does not make the assumption of cells being star-convex polygons, it has been reported to be computationally more intensive than StarDist.

Unsupervised learning

As opposed to supervised learning, unsupervised machine learning handles datasets that lack pre-defined labels or target outcomes. Instead, the goal is to uncover hidden structures or patterns within the data. This is achieved by employing algorithms that can infer relationships and groupings directly from the input data. A common task in unsupervised learning is clustering, where the algorithm identifies groups of similar

data points, enabling the understanding of underlying distributions or segmentation of data into meaningful categories. Another key application of unsupervised learning is dimensionality reduction. Here, the algorithm transforms high-dimensional data into a lower-dimensional representation, often by identifying more informative or interpretable features (components). These new variables help simplify the data while retaining essential information, facilitating visualisation, analysis, or other downstream tasks. Unsupervised learning is particularly valuable in scenarios where labelled data is unavailable, such as exploratory data analysis, like in single-cell analysis, or preprocessing for supervised models.

Clustering

Clustering is an unsupervised machine learning technique that groups data points based on their similarities, with similar points being grouped closer together in a multi-dimensional space. The concept of similarity varies depending on the context and is typically quantified using mathematical metrics. An often used metric is the Euclidean distance, which calculates the straight-line distance between two points in (a Euclidean) space [4]. Other metrics can also be used, but are outside the scope of this thesis. The following sections introduce hierarchical clustering and k-nearest neighbour clustering.

Hierarchical clustering Hierarchical clustering is a cluster analysis method that constructs a hierarchy of clusters based on the similarity between data points. This process relies on distance metrics such as Euclidean distance, Manhattan distance, cosine similarity, or Pearson correlation to quantify how close or similar data points are. An important aspect of hierarchical clustering is linkage, which determines how the distance between clusters is measured. Various linkage methods include single linkage, complete linkage, mean linkage, and centroid linkage, each influencing the clustering outcome differently. The results are often visualized using a dendrogram, a tree-like diagram that illustrates the similarity between data points and the order in which clusters are formed, providing a clear view of the hierarchical relationships in the dataset. In Studies I and III, we implemented hierarchical clustering.

k-Nearest Neighbour clustering k-Nearest Neighbour (kNN) clustering is a method that forms clusters based on the proximity of data points, represented as a directed graph where connections are determined by closeness. Closeness is defined using various distance metrics, such as Euclidean or Manhattan distances, which measure how

[4] basically, a flat surface

similar or near data points are to each other. An important parameter in this approach is the choice of the number of nearest neighbours to consider, which directly influences the clustering outcome. In Study III, we implemented kNN as part of the single-cell analysis (cluster annotation).

Dimensionality reduction

High-dimensional data, such as bulk or single-cell gene expression data, often presents challenges for analysis and interpretation due to the so-called 'the curse of dimensionality' [127, 128]. This curse of dimensionality describes the difficulties related to high-dimensional data, such as the sparsity of the data points in high dimensions, and the reduced effectiveness of distance measures, such as Euclidean distance. Dimensionality reduction is the process of transforming high-dimensional data into a lower-dimensional space, while retaining its essential information. Various dimensionality reduction techniques exist, but the most common ones in analysis of gene expression data are Principal Component Analysis (PCA) and Uniform Manifold Approximation and Projection (UMAP). Figure 4 visualises the different effects that PCA and UMAP can have on the same data.

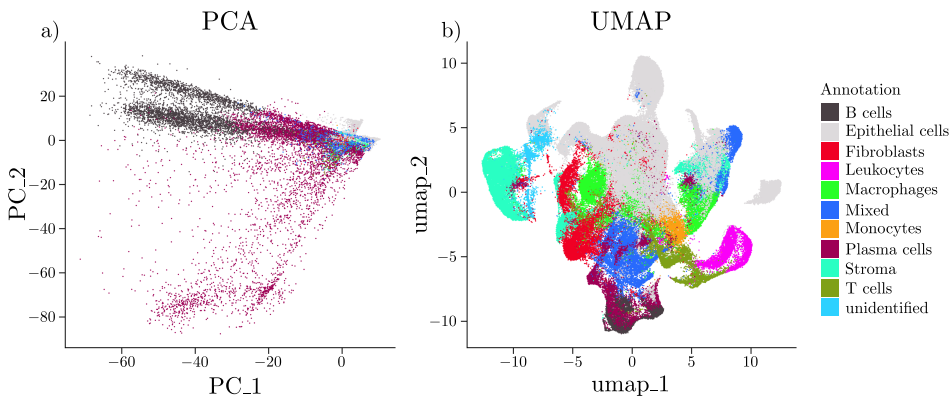

Figure 4: Dimensionality reduction with a) PCA and b) UMAP applied to the spatial single-cell data used in Study I.

Principal Component Analysis Principal Component Analysis (PCA) is a commonly used linear dimensionality reduction technique that transforms the data so that the principal components (the features after transformation) capture the largest variation in the data [129]. The first principal component is the one that captures the largest variation in the data, with the second capturing the next most variance, and so on. As PCA is a linear method, it is useful for datasets with linear relationships, but is less powerful in complex data with non-linear structures.

Uniform Manifold Approximation and Projection Uniform Manifold Approximation and Projection (UMAP) is a non-linear dimensionality reduction technique that can maintain both local and global structures within the data [130]. It takes several hyperparameters, including the number of components and the number of nearest neighbours. In contrast, PCA does not take any hyperparameters. UMAP builds a weighted k-nearest neighbour graph, which is then optimised to approximate the underlying manifold (the geometric space) of the data. The high-dimensional space is then projected into a lower-dimensional space -often for visualisation purposes- while preserving local relationships between data points. As UMAP is a non-linear technique, it is very useful on complex data sets, and a popular method (currently golden standard) in single-cell analysis [131].

Community detection Community detection is a clustering approach that identifies groups of densely connected nodes, or communities, within a network. Here nodes represent data points, such as cells, and edges denote similarities or connections between them. The process begins by constructing a kNN graph, which links data points to their nearest neighbours based on proximity. The graph is then partitioned into clusters of densely connected nodes, revealing the community structure within the dataset. Two popular methods for community detection in single-cell analysis are the Louvain and Leiden algorithms, which detect similar cell communities for annotation. While Louvain remains commonly used Leiden is the current gold standard for cluster stability. However, Leiden is slower and more computationally intensive to run, leaving Louvain to stay popular. In Study III we implement the Louvain algorithm as part of the single-cell analysis cluster (cluster annotation).

Image processing

In image processing, relevant information is enhanced in (digital) images through the use of signal processing. It is closely tied to computer vision, where the goal is to extract a specific meaning (for example, the number of cell objects) from an image. In Studies I and III, we have used a mix of image processing and computer vision techniques to extract information from the IHC and multiplexed IHC TMA cores.

Colour deconvolution Colour deconvolution is a technique in image processing that can transform images from one colour space to another. A common colour space for images is the RGB colour space, where each channel in the 3-channel image corresponds to Red, Green, and Blue respectively. To detect stains in images of IHC-stained tissue it can be useful to convert the scanned RGB image into a colour space

that aligns better with the stains, such as the Hematoxylin (nuclear stain), Eosin (cytoplasmic stain), DAB (marker stain) (HED) space [132]. By applying a deconvolution matrix tailored to the spectral properties of the dyes, the deconvolution effectively isolates the contribution of Hematoxylin, Eosin, and DAB.

Automated thresholding Automated thresholding is a technique in image analysis used to divide (segment) images into different components (e.g., object and background) based on pixel intensity. This approach eliminates the need for manual threshold selection. Various methods exist for automated thresholding, such as Otsu thresholding and triangle thresholding. Triangle thresholding is particularly effective for images with a clear peak and long tail in their intensity histograms. The triangle method works by constructing a straight line between the peak of the histogram and the end of the tail, and finding the point along the histogram that maximises the perpendicular distance to this line. In Studies I and III, we implemented thresholding in the image analysis pipeline for IHC and multiplexed IHC.

Edge detection / Hough circle detection Edge detection is a technique in image analysis to identify boundaries or contours within an image by detecting sharp changes in pixel intensity. It serves as a precursor to many higher-level tasks, such as object detection and shape recognition. Among various edge detection methods, the Hough transform is particularly useful for its ability to detect specific shapes like ellipses or circles [133]. Hough circle detection works by transforming points in the edge-detected image space into a parameter space where potential circle candidates are represented as intersections of curves. By accumulating votes in this parameter space, the algorithm can identify circles. In Study I, we implemented Hough circle detection in the image analysis pipeline.

Transcriptomics

Transcriptomics is the study of the transcriptome (the expressed genes) and its functions. By profiling the gene expression, transcriptomics can help to give a detailed view of tumour biology. Transcriptomics analysis of breast cancer has been widely used for more than two decades and gained significant importance with the first report of the 'intrinsic' mRNA subtypes [52]. Today, mRNA profiling has reached clinical implementation based on commercial assays like Oncotype DX, MammaPrint, and Prosigna, which can provide treatment decision support for specific patient subgroups. Transcriptomics has also been extensively used to subdivide triple-negative breast cancer into molecular subsets with proposed treatment importance [71, 74,

75]. While microarrays were the main technology used for gene expression analysis for many years, next-generation sequencing-based RNA-sequencing has now become the golden standard.

Next Generation Sequencing

Next-generation sequencing (NGS) is a high-throughput sequencing method. It relies on a reference genome and can be used to sequence both DNA (DNA-sequencing) and RNA (RNA-sequencing) [134]. RNA needs to be reverse-transcribed (to cDNA) before it can be sequenced. Once sequencing is completed the raw output is typically a FASTQ file that contains the sequence reads. The sequence reads can be mapped and aligned to a reference genome (resulting in a BAM file) after which gene counts can be generated. Raw gene counts can be normalised into alternative metrics. RNA-sequencing of a group of cells (e.g. a macrodissected tumour piece) is often referred to as bulk RNA-sequencing to distinguish it from RNA-sequencing of single cells (often referred to as scRNAseq). More recently, RNA-sequencing has also been included in certain spatial transcriptomic workflows to allow mRNA profiling of single cells in situ. In Studies I-III, we used the available SCAN-B bulk RNA-sequencing data of triple-negative breast cancers.

bulk RNAseq The analysis of bulk RNA-sequencing data often involves normalising raw read counts to minimise experimental bias. Several normalisation approaches exist, with different benefits [135]. In Study I, we used expression estimates in the form of Transcripts Per Million (TPM), involving first normalisation for gene length followed by normalisation for sequencing depth. Together, this leads to the sum of the reads of each sample being the same. In Studies II and III, we used expression estimates in the form of Fragments Per Kilobase of transcript per Million mapped reads (FPKM), which is the standard output from the SCAN-B sequencing analysis pipeline [57, 107]. FPKM is a simple expression level normalisation method that normalizes read counts based on gene length and the total number of mapped reads.

Differentially expressed genes can be analysed by for instance functional enrichment analysis. Examples include gene set enrichment analysis (GSEA), as well as enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways or Gene Ontology (GO) gene functions to identify altered processes between groups (performed in for example Study II).

A limitation of bulk RNA-sequencing is that the derived expression profile for a tumour is a mixture of expression patterns of the different cell types (malignant and nonmalignant) and their proportion in the analyzed tumour tissue piece. Different

cell deconvolution approaches, like the Cell-type Identification by Estimating Relative Subsets of RNA Transcripts (CIBERSORT) [136], try to address this limitation by attempting to characterise the cell composition of complex tissues based on their gene expression profiles. In Study I, we implemented CIBERSORT and compared it to in situ cell marker counts, illustrating that some caution may be warranted for the former.

Spatial Transcriptomics

In spatial (single-cell) transcriptomics, the transcriptome of individual cells and their spatial position in the tissue are measured. It is a rapidly evolving field where techniques are constantly updated to be more spatially precise and capture a larger transcriptome. In general, the techniques for spatial transcriptomics can be divided into two main categories: sequencing-based, and imaging-based, but some overlap in methods can exist [137].

Next-generation sequencing (sequencing based)

In sequencing-based spatial transcriptomics, a spatial barcode is added before library preparation and sequencing. The barcode is used to map back to the position on the slide, while the rest is mapped to the genome to identify the transcript. While sequencing-based approaches were initially more spot-based (so semi-single-cell), the spot resolution has improved leading to even subcellular resolutions in newer methods.

Imaging based spatial omics

Imaging-based spatial transcriptomics can be divided into two subgroups; in situ sequencing (ISS) and in situ hybridisation (ISH). In Study III, we implemented spatial transcriptomics by using the Nanostring CosMx Spatial Molecular Imager (SMI), which is an example of in situ hybridisation spatial transcriptomics.

In situ sequencing In situ sequencing (ISS) spatial transcriptomics methods amplify and sequence the transcripts in the tissue. First, padlock probes are hybridised to the RNA in the tissue. Next, the probes are ligated, thereby forming a circle and going into rolling circle amplification. Fluorescent probes then bind to the amplified product, are imaged, and removed, over several cycles. Sequenced transcripts are assigned to specific cells based on cell segmentation of fluorescent tissue stains.

In situ hybridisation For spatial transcriptomics with in situ hybridisation (ISH) a variation on fluorescence in situ hybridisation (FISH) takes place. As in FISH, the RNA in the tissue is first exposed to allow for probe hybridisation. This is followed by the hybridisation step, where in fluorescent reporter sets bind cyclically, are imaged, cleaved, and removed. Additionally, tissue stains are also created to allow for morphology-based cell segmentation, with for instance DAPI (nuclei), a membrane stain (CD298), and PanCK. The transcripts, derived from the fluorescent reporters, are assigned to cells using image-based cell segmentation. Transcripts that fall within the cell boundaries are assigned to that cell. This means that high-quality cell segmentation is required to avoid hybrid cells in downstream annotation. This can occur when cell segmentation captures two (or more) cells, but assigns it as one, and transcripts from different cells get assigned to this one cell. Similarly, transcripts can be not assigned to any cell.

Analysis of spatial transcriptomics

Alongside the development of spatial transcriptomic techniques, there has also been a development of the analysis of spatial transcriptomics, often based on single-cell analysis methods [138–141]. New methods are still being developed that try to account for challenges specific to spatial transcriptomics. Current spatial transcriptomics analysis is very similar to single-cell analysis. Once transcripts have been assembled, they are filtered based on quality and normalised. Following PCA and the construction of a nearest-neighbour graph, the data are usually visualised with UMAP. The nearest-neighbour graph and community detection (Louvain or Leiden) can identify clusters in the data. For biological interpretation, the clusters need to be annotated. While automated approaches exist, they don't always extend well to new data sets or are only developed for a specific tissue type [142–144]. Often manual annotation of the clusters is required, for instance, based on marker genes or top differentially expressed genes. Two popular tools are Seurat [139] and Squidpy [140]. In Study III, we have implemented Seurat for cell type annotation and Squidpy for further spatial analysis.

Results and Discussion

Overview and relationship of included studies

This thesis comprises three studies that all focus on the characterisation and analysis of the tumour immune micro-environment in early-stage, surgically treated TNB and the associations of the immune micro-environment with somatic alterations, molecular subtypes, and patient prognosis. As a basis for all studies, we used a multi-omic, population-representative, early stage, TNBC cohort consisting of SCAN-B patients enrolled between 2010-2015 in the Skåne healthcare region. This cohort had whole genome sequencing, RNA-sequencing, DNA-methylation, patient treatment and outcome, and tumour tissue suitable for in situ analyses available.

In Study I, we focussed on the tumour immune micro-environment, assessed through IHC for commonly used cell markers for T-cells, B-cells, and macrophages by developing an automated image analysis pipeline that can quantify stained cells. This pipeline allowed us to perform an analysis of multiple markers that is more quantitative compared to for example analysis based on human grading. Following the implementation of the pipeline, we proceeded to demonstrate covariance between immune cell markers and investigated individual markers' associations with mRNA expression patterns and patient outcome after adjuvant chemotherapy, and whether different somatic alterations could explain a more immune-hot or immune-cold micro-environment in our tumours.

In Study II, we turned from IHC to mRNA expression, based on RNA-sequencing to investigate the tumour immune micro-environment in TNBC. We focussed on an in literature repeatedly reported immune phenotype (the IM subtype) of TNBC and developed a stand-alone classifier for this phenotype for optimised patient prognosis using machine learning. We showed that this classifier captured a more immune-hot phenotype in situ in TNBC by integrating data also used in Study I. We validated the developed classifier in independent cohorts with RNA-sequencing for prognostic association and tested its ability to predict response to standard-of-care neoadju-

vant chemotherapy. Lastly, we investigated on a more general level how the tumour immune micro-environment changed during neoadjuvant chemotherapy treatment pressure in TNBC by analysing RNA-sequencing data from paired tumour biopsies taken before and after treatment from patients that did not achieve a complete pathological response.

Finally, in Study III we aimed to connect the in situ-based profiling aspect from Study I with high-dimensional mRNA profiling (as exemplified in Study II) by applying multiplexed IHC, where multiple markers are analysed on the same tissue section, and state-of-the-art spatial transcriptomics to the same cohort used in Study I-II. To facilitate multiplexed IHC analyses we first, similar to Study I, developed an automated image analysis pipeline that assigned cell phenotypes of B-cells, T-cells, macrophages, tumour cells, and other cells, to detected cell objects. Next, we used the phenotyped cell objects to form tumour ecosystems by cell neighbourhood analysis that accounted for the phenotype of a cell and its spatial relation to other cells in the tissue. Based on the identified tumour ecosystems we investigated their associations with molecular phenotypes, mRNA expression patterns, and association with patient outcome after adjuvant chemotherapy. We also applied spatial transcriptomics to tissue microarray sections to further delineate the tumour immune micro-environment in our triple-negative tumours. This analysis allowed for a more detailed cell phenotyping that we used in a pilot analysis to characterise the same molecular subtypes as analysed by the multiplexed IHC.

Study I
Tumour immune characterisation of primary triple-negative breast cancer using automated image quantification of immunohistochemistry-stained immune cells

Background

Tissue micro-arrays (TMAs) are a standardised and high-throughput method to analyse single or multiple antibodies on subsequent tumour sections. The interpretation of the antibody stains requires a scoring or quantification of the positive stains, for which pathologists remain the gold standard. However, despite standardisation efforts in pathologists' scoring a variation can exist between individual pathologists, and sometimes even for the same pathologist, also called inter- and intra-observer variability. Additionally, it requires a substantial amount of manual work to score large TMAs with many cores (tumours) for several markers, which leads to a time and cost bottleneck. Even at smaller scales, but especially for full cohort studies a full

quantitative analysis of individual cells becomes unfeasible, leaving manually annotated samples to be (at best) semi-quantitative. An alternative to manual scoring and an increasingly more used approach is the use of digital image analysis, often referred to as digital or computational pathology, where pipelines and models, also involving deep learning models, of varying complexity are implemented.

To allow for high throughput analysis of TMAs with multiple single-plex IHC staining[5], we aimed in Study I to develop an automated high throughput quantitative (and ideally spatial) analysis that would facilitate the data analysis of multiple IHC stainings targeting different immune cell markers. Based on this pipeline, we proceeded to characterise a multi-omic TNBC cohort stained by multiple immune markers to gain a better understanding of the interactions between somatic tumour alterations and the tumour immune micro-environment in TNBC.

Results and Discussion

We developed an image processing pipeline called Tissue microarray MArker Quantification (TMArQ), that can quantify and localise DAB staining based on commonly used image processing techniques and starDist cell segmentation and implemented this to analyse a TMA cohort of 218 SCAN-B patients with TNBC (drawn from a population-representative cohort originally described by Staaf et al [62]. The used TMA comprised of 5 blocks, had two 1 mm cores per patient and was stained for 7 immune markers (CD3, CD4, CD8, FOXP3, CD20, CD68, PD-L1 (SP142 antibody, Roche Diagnostics) as well as P53 through single-plex IHC. To validate the TMArQ pipeline we first compared our digital cell counts to pathology annotated scores for CD20 (TMA-based), PD-L1 (TMA-based), TILs (whole-slide based) available for the cohort. In addition, we also validated the pipeline versus human estimated CD3 scores in two additional TMA cohorts representing other malignancies, malignant melanoma, and bladder cancer. Overall, we found a good concordance (correlation) of the digital cell counts with human-derived scores, supporting the usefulness of the automated pipeline for research-based analysis of tumour TMA cohorts.

Scoring of TILs is becoming more clinically relevant in the routine management of TNBC based on repeated reports that they function as a prognostic biomarker, as well as influence the function of chemotherapy and immunotherapy, making them relevant for the treatment of TNBC [145]. For TILs, we observed a clear correlation between our automated cell counts for the CD3 pan-lymphocyte marker and pathology-based TIL scores. This indicates that an estimate of a general immune response may be obtained even from an automated leukocyte count on dual 1 mm TMA

[5] i.e. each section is stained by only one antibody

cores. An important limitation to this conclusion is however that our IHC staining was performed on cores from tumour-rich areas that may not capture the full range of the immune response in certain cases. In comparison, for TIL-scores, full sections are typically recommended over biopsies in guidelines [64] and TIL-estimates from whole slide evaluations have been reported to be higher than in matched TMA core analysis [146]. As such, TMA analyses performed similarly as in this study will likely be able to provide accurate correlative estimates of, for example, TIL levels on a group level, while at the patient-specific level whole slide evaluations are still needed, especially in a clinical setting.

Notably, the increased relevance of the tumour immune micro-environment in many malignancies also stresses important practical aspects in TMA production, in particular where to take the core punches, as well as how many cores should be taken per tumour. Here, in silico sampling of TMA equivalent cores from whole slide images for Ki67 estimation in breast cancer has shown that optimal tissue sampling for IHC biomarker evaluation is dependent on the heterogeneity of the studied tissue, which may require a substantial number of TMA cores to be sampled to achieve a low error in heterogenous tumours [147].

In Study I, we also compared the digital cell counts to gene expression profiles (TPM data) for CD3, CD4, CD8, CD20, CD68, and FOXP3 in the TNBC cohort and for CD3 in the malignant melanoma and bladder cancer cohorts (microarray-based) as well. The validation showed good agreements to manual annotations and Spearman correlations ranging from 0.33 (CD68) to 0.59 (CD8) to gene expression values. The highest correlations were observed for the T-cell marker CD8 and the CD3 pan-lymphocyte marker, in line with literature [148].

We also compared the digital cell counts to bulk RNA-based deconvolution (CIBERSORT) and RNAseq-derived immune metagene scores based on the immune response metagene originally reported by Fredlund et al [149]. Correlation of the Fredlund mRNA immune metagene with digital cell counts showed correlation estimates ranging from 0.43 to 0.58, with the highest agreement observed for the cytotoxic T-cell marker CD8 (Figure 5a).

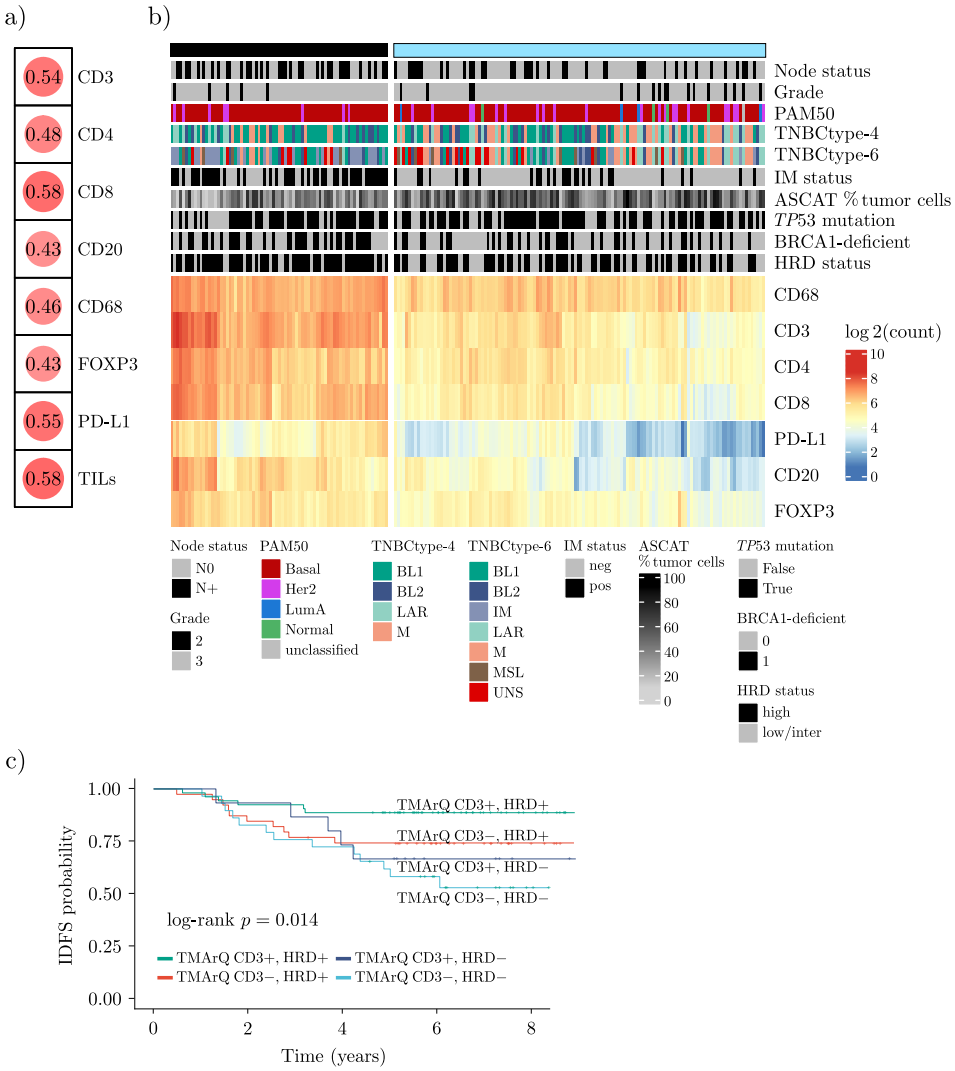

Figure 5: Digital cell count clustering and correlation RNA-sequencing based immune response estimation. a) Spearman correlation of rank scores for the Fredlund et al. gene expression immune metagene versus TMArQ mean cell counts (mean counts of both cores/sample) and whole slide TIL estimates. **b)** Clustered co-expression matrix of average TMArQ cell counts (mean of cores/sample) for all markers and samples. Clustering was performed using Euclidean distance and complete linkage and samples were divided into two main clusters. Sample annotation track include tumour status by tumour grade, lymph node status (N0/N+), PAM50 molecular subtypes, proposed TNBC gene expression subtypes (TNBCtype-4 and TNBCtype-6), mRNA derived immunomodulatory subtype (IM) status, and whole genome sequencing determined tumour cell content percentage by ASCAT, TP53 mutation status, BRCA1-deficiency status (mutation or promoter hypermethylation inactivation), and HRD-status. Log transformation of TMArQ counts was performed before analyses. **c)** Kaplan-Meier plot of invasive disease-free survival (IDFS) as clinical endpoint for patients treated with adjuvant chemotherapy stratified by TMArQ CD3-low/high and whole genome sequencing based HRD-status (positive/negative). The p-value was calculated using the log-rank test. Figure adapted from Study I.

Interestingly, we also observed a high correlation between the mRNA immune meta-

gene score and whole-slide TIL estimates (Figure 5a). While this observation is outside the original scope for Study I, it is relevant for mRNA-based profiling studies lacking matched in situ tissue resources (often the typical case), as it can be viewed as support for in silico-based conclusions about immune status on at least a group level basis (given a similar tumour biology setting, for example, TNBC).

Our findings also highlighted that higher CD3 counts were connected to the mRNA-based Lehmann classification for the immunomodulatory (IM) subtype, both as an independent label and when overlaid on the BL2 and LAR subtypes. Although the matched digital CD3 cell counts from Study I were not included for tumours in Study II due to the timing of the manuscripts, the observation stated above supports the IM subtype classification as one, of many, representative mRNA-based immune status classifications in TNBC. By analysing digital cell counts between cores from the same tumour sample and the same marker we were able to capture some of the spatial heterogeneity inherent to TNBC, but also stressing the importance of an appropriate core selection strategy in TMA production.

Another interesting possibility with digital cell counts, due to their more quantitative nature, is the application of clustering algorithms. Unsupervised hierarchical clustering of the digital cell counts from the seven markers demonstrated a general trend towards higher co-occurrence of all immune cell types within a given tumour (Figure 5b). This general trend may not be surprising considering that many of the analysed immune cell types may act together in mounting a tumour immune response. However, it should be noted that for some cell types, like macrophages, we cannot discriminate between pro- and anti-inflammatory subtypes based on the used CD68 marker alone.

Finally, we analysed the association of digital cell counts for our markers to patient outcome after adjuvant chemotherapy. Survival analysis using univariate Cox regression revealed that digital quantifications of CD3, PD-L1, CD8, and CD4 positive cells were all associated with a better invasive disease-free survival after standard-of-care adjuvant chemotherapy. This finding is consistent with the established association of an increased immune response with better outcome in TNBC. Interestingly, this suggests that simple estimates such as automated leukocyte counts on dual 1 mm TMA cores can be used to probe relevant clinical correlations in a high-throughput fashion. In addition to the established role of the immune response as a prognostic variable in TNBC, DNA repair deficiency typically manifested as homologous recombination deficiency (HRD) has also been suggested as a prognostic and potentially predictive marker in chemotherapy-treated patients [62, 150, 151]. Given the comprehensively profiled SCAN-B cohort, we combined a binarised TMArQ CD3 digital cell count classification with HRD status determined from whole genome sequencing to investigate synergistic prognostic effects.

As seen in Figure 5c this analysis identified that patients with TMArQ CD3+ and HRD-positive tumours showed the best prognosis compared to the other subgroups. Importantly, Figure 5c demonstrates the importance of considering multiple biomarkers for optimal patient management, for instance, patients with CD3+ but HRD-negative tumours appear to have a worse outcome compared to CD3- but HRD-positive patients. Moreover, while statistical tests showed that HRD-positive TNBCs in general had a more immune-warm phenotype it should be stressed that considerable heterogeneity in cell marker counts was observed between HRD-positive and HRD-negative tumours. This indicates that a highly rearranged tumour genome (or tumour mutational burden) is not equivalent to an immune-warm phenotype in situ per se in TNBC.

Limitations and Future Perspective

While this study adds some valuable insight into the immune micro-environment in TNBC, some limitations apply. First, the staining order for TMA cores was not recorded, which left us unable to properly account for the vertical distance between cores from the some stack, including any positional shifts that may have occurred during tissue slicing. Still, we observed the highest marker-to-marker correlations within cores as opposed to between cores, indicating that stained sections used in the study were likely taken in close proximity to each other.

For the assessment of marker stainings, we did not differentiate between staining locations within the cell (cell surface, nucleus, or cytoplasm) for different markers, which could have improved staining assessment on the individual marker level. Additionally, in our current approach cells have a binary classification of the staining being present or absent, which ignores any biological meaning that may be tied to varying staining intensities.

For cell-specific markers like CD8, CD20 etc. this may be less important, but for other tumour markers (like Ki67) it would be a highly relevant limitation. For most analyses in this study, we used the mean digital counts for cores from the same tumour sample. However, as we also showed in the comparison of cores from the same tumour sample, spatial heterogeneity exists in TNBC. The use of average digital counts, while useful for most analyses, ignores this spatial heterogeneity.

Despite these limitations, Study I demonstrates how the application of an open-source, automated pipeline for IHC-based cell detection and quantification can be used to target the tumour immune micro-environment in TNBC, and how results from in situ analysis can be combined with genomic data to investigate the correlation of somatic alterations with general immune status. Digital image analysis tools

will likely facilitate the next step in tumour immune micro-environment characterisation of breast cancer by allowing more quantitative profiling that can also incorporate spatial positions of detected cell objects in the tumour tissue. Interestingly, based on a more quantitative type of data we can start to apply methods, such as clustering, typically used for high-dimensional omics studies to multi-layered IHC data. Moreover, a more quantitative and spatial approach may potentially allow for better integration with omics data such as DNA methylation, gene expression profiling, and whole genome sequencing. In the end, such improvements to conventional IHC data can further facilitate the next step in phenotypic and integrative cancer studies.

Study II
Stand-Alone Immune Response Prediction in Triple-Negative Breast Cancer based on Gene Expression

Background

TNBC remains one of the most aggressive breast cancer subtypes, with early relapses and poor patient outcomes. Immune response has gained significant attention as a prognostic factor, especially for treatment naïve patients in the early-stage setting. Existing approaches to determine immune response include TIL-scoring on whole-section H&E stains (by a pathologist) and mRNA expression profiling. For mRNA expression multiple immune signatures, including immune phenotypes, have been reported in the literature. Examples include immune response metagenes that typically comprise a set of immune response pathway-associated genes (e.g., the immune response metagene reported by Fredlund et al. [152] used in Study I) but also immune phenotype subtypes like the immunomodulatory (IM) subtypes reported by Lehmann et al. [70] and Jiang et al. [75].

An immune response measurement by TILs or gene expression is typically not a bimodal distribution of values from distinct immune-hot or immune-cold tumours, which we also demonstrated in Study I and which has been reported in the literature. This means that classification into categorical groups becomes inherently dependent on arbitrary cut-offs (e.g. 30% TILs or the median level of a group) and on the cohort composition in the training cohort. Both the biological composition (number of immune hotcold tumours and their level of infiltration guided by tissue sampling etc) and the association with patient outcome (e.g., if cohorts are population-representative or not) are influential factors in cohort composition. For gene expression data, arbitrary cut-offs and differences in cohort composition between training and test sets may lead to inconsistencies and suboptimal classification when predictors

are applied to independent cohorts, illustrated for example for nearest centroid-based classifiers like PAM50 [153].

Despite progress in the field, there lies a challenge in the stability of mRNA-based classifiers on the level of individual samples, as well as the ability to truly classify a single sample without the need for calibration or normalization. Recent examples of reported single-sample predictors based on RNA-sequencing that have been validated versus clinical and commercial alternatives include predictors of Risk-of-Recurrence (ROR), PAM50 subtypes, and clinical ER, PR, and HER2 status [57]. While such predictors may help to move RNA-sequencing closer to clinical practice, neither ROR, PAM50 nor the most used commercially available gene expression-based risk predictors appear to have an immediate value for prognostication and treatment prediction in TNBC.

In Study II, we aimed to develop a stable stand-alone classifier that can predict immune response in individual patients using RNA-sequencing data and machine learning. As a basis for the training, we used the population-representative SCAN-B TNBC cohort from Study I ($n = 235$ patients with RNA-sequencing data), and the IM subtype definition by Lehmann et al. [70]. The rationales for selecting the latter as a basis for supervised training were twofold. Firstly, we observed a strong association with prognosis after adjuvant chemotherapy in the cohort from Study I. Secondly, the IM subtype classification demonstrated an association with in situ estimates of immune cell markers and TILs in Study I.

The availability of stand-alone classifiers for mRNA-based prediction (for e.g. immune status) may further enhance the usability of RNA-sequencing-based analysis in both a more routine clinical context but also for translational endpoints in clinical trials. Especially for clinical trials the controlled collection of tissue appropriate for high-dimensional profiling is becoming more frequent to maximise the scientific output of these costly studies.

Results and Discussion

We developed a stand-alone predictor using a RF classifier trained on bulk RNA-sequencing data as expressed in FPKM values and a consensus immunomodulatory 'hot' (positive) or 'cold' (negative) classification derived from the Lehmann subtypes. Before deciding on the RF classifier, we tested several machine learning algorithms in the SCAN-B training cohort to assess the suitability of different models.

Two challenges in the training process were to derive a predictor capable of predicting a single sample without calibration or normalization, represented only by an array

of gene expression values, and to define suitable IM training labels. The latter is a challenge as the only publicly available predictor of the Lehmann et al. subtypes is a web-based tool implementing the proposed centroid-based prediction by Lehmann et al. [71]. To derive the IM training labels we therefore first created a consensus IM classification by repeated classification of different training cohort subsets (randomly selected) by using the web tool, combined with survival analysis to derive a prognostically optimal cut-off. The final consensus labels were then used as the training labels.

We also performed a stability analysis similar to the one outlined by Paquet et al. [153] for PAM50 to investigate the stability of the online implemented Lehmann et al. subtyping to non-randomized perturbations by specifically excluding large numbers of samples of a single subtype followed by reclassification. As expected, the online centroid-based subtype predictor behaved similarly as demonstrated for PAM50 centroid-based classification [153] when performing non-randomized perturbations involving selective exclusion of previously classified IM-positive or IM-negative tumours. Of the original IM-negative samples 38.3% were reclassified to be IM-positive when only presented with other IM-negative samples. Of the IM-positive samples 73.8% were reclassified to be IM-negative when presented only with other IM-positive samples. These results highlight that cohort composition has a significant effect on the classification of individual samples and will importantly not only affect an IM classification but also the classification of the other TNBCtype subtypes (BL1, BL2, LAR, M) using the online tool. Importantly, this finding supports the need for a stand-alone gene expression predictor to increase the usefulness of an IM-phenotype classification in early-stage TNBC.

When comparing our RF classifier predictions of IM-positivity or IM-negativity to TIL-scores we found that the predicted IM-positive tumours exhibited significantly higher TIL-estimates. Consistently, the genes with a classification importance score > 0 in the model were strongly enriched for different immune response-associated gene ontology processes, and several of the highest-ranked genes have previously been associated with immune response in cancer. Together, these results support that the derived prediction model captures an immune-hot phenotype in TNBC.

We validated the derived RF classifier for prognosis in three independent cohorts: TCGA, FUSCC, and SCAN-B, encompassing a total of 651 patients. Patients who were classified as having a high immune infiltration according to our predictor, had higher diversity in T-cell receptor genes, indicating that this feature correlates with an immune-hot phenotype in treatment-naïve TNBC (a similar correlation was also found in the training cohort). Additionally, there was a strong statistical association between the predicted IM-positive subtype and a gene-expression-derived immune metagene rank score (Fredlund et al.) in all validation cohorts. Together, this again

supports that the derived IM-predictor identifies a generally immune-hot phenotype.

For prognostic associations, we found mixed results depending on the validation cohort. In the TCGA validation cohort, the predicted IM status was statistically significant using overall survival as the clinical endpoint but the predicted IM status was not significant in the chemotherapy-treated subset of the Chinese FUSCC cohort using relapse-free survival as the clinical endpoint. Interestingly, the original FUSCC-reported IM subtype [75] was also not statistically significant in the FUSCC chemotherapy-treated patient subset. Moreover, the proportion of FUSCC-reported IM-positive tumours was notably lower compared to the proportion of our predicted Lehmann IM-positive tumours in FUSCC. Together, this may suggest an impact of ethnicity, treatment regimes, or cohort selection behind the conflicting results compared to the TCGA and SCAN-B validation cohorts. In the SCAN-B validation cohort, the predicted IM status was borderline non-significant in the full chemotherapy-treated cohort, while statistically significant in the adjuvant-treated cohort (i.e., excluding neoadjuvant-treated patients). As SCAN-B mirrors current treatment guidelines during patient inclusion years, unlike a randomised clinical trial, the patients in the SCAN-B validation cohort (2015-2018) typically received neoadjuvant chemotherapy when presenting more aggressive tumour characteristics at diagnosis and thus likely had a poorer prognosis.

Considering that the usage of neoadjuvant therapy is increasing in TNBC, we also investigated the treatment-predictive association of the IM classifier by applying it to RNA-sequencing data of pre-treatment core needle biopsies from a cohort of 116 neoadjuvant treated SCAN-B patients. In this cohort, we found that the predicted IM status was borderline non-significantly associated with treatment response ($p = 0.054$, Fisher's exact test). To further explore the IM predictor in neoadjuvant-treated patients we focused on patients with residual disease at surgery (i.e., after completed treatment) that had matched pre-treatment core needle biopsy tissue and post-treatment surgical tumour tissue. Analysing these patients allowed us to investigate how neoadjuvant chemotherapy treatment pressure affects the tumour immune micro-environment on a general level based on changes in predicted IM status. In these patients, we observed that the predicted IM status changed in 22.2% of the cases, involving in all but one case a shift from a pre-treatment predicted IM-positive status to an IM-negative status post-treatment. This suggests a reshaping of the tumour immune micro-environment during treatment to a more immune cold environment in most tumours. Consistently, previous studies have also reported indications of immune depletion in post-treatment specimens as one component of the tumour reshaping during neoadjuvant treatment pressure [154, 155].

Limitations and Future Perspective

Several limitations apply to this study. Our predictor was exclusively developed with and intended for FPKM gene expression data, meaning that any gene expression in other formats would first have to be converted to FPKM values. The best validation results were observed in the SCAN-B validation cohort, which has the most similarity to the training cohort when it comes to patient demographics and ethnicity, population representativeness, sequencing facility, protocols, and initial data processing. Additionally, the current RF model relies on a large gene set, which could be reduced to a smaller gene set to expand the usability of the predictor to datasets where not the entire gene set is available. Another limitation of this study is the difference in treatment approaches between training and validation cohorts, and the current treatment guidelines. Very few patients in the training cohort received neoadjuvant chemotherapy. In the SCAN-B validation cohort, this fraction was higher due to changes in the national treatment guidelines and today, the fraction of patients receiving neoadjuvant treatment is even higher. Another change to pre-operative treatment between our used cohorts and the current clinical context is the recent introduction of immune checkpoint inhibitors into clinical practice. As our analyses are all based on retrospective cohorts, no patient in the training or validation cohorts received immune checkpoint inhibitors, leaving that perspective out of this study. Finally, our analyses also point towards the complexity of the classification problem itself, i.e., what defines an immune-hot, clinically relevant, TNBC (e.g. $> 10\%$ TILs or $> 30\%$ TILs, etc).

While the current study focused on the repeatedly reported IM mRNA subtype, other training labels (e.g., quantiles of immune gene rank scores, expression of individual immune-associated genes, or even categorical TIL-based training labels) could have been used instead, most likely generating highly similar results (based on the correlation of the features). For future studies, an alternative approach could be to instead train directly on clinical outcomes as these typically occur close to diagnosis for patients with TNBC. Additionally, including other clinicopathological variables, such as lymph node status, or other transcriptomic-derived data into mixed models could potentially enhance the prognostic/predictive power.

Finally, placing the focus on patients not achieving a pathological complete response after neoadjuvant therapy (meaning that there is tumour tissue left in the breast at surgery) could be another interesting approach, as these patients have a higher risk of metastatic relapse [156]. Here, we might need to focus more on the post-treatment tumour tissue as opposed to the pre-operative biopsy when developing predictors, as neoadjuvant treatment pressure may substantially remodel the tumour ecosystem, something for which we saw preliminary evidence in this study.

Study III
Characterization of the tumour micro-environment of triple-negative breast cancer through multiplex imaging and spatial transcriptomics

Background

The tumour micro-environment and in particular the tumour immune micro-environment have emerged as important factors influencing progression, therapeutics response and overall patient outcome in TNBC. The tumour immune micro-environment in TNBC is known to exhibit considerable variability ranging from tumours with high immune infiltration ('hot' tumours) to tumours with little to no immune infiltration ('cold' tumours). Different spatial patterns, such as immune infiltration that is restricted to the tumour periphery (immune-excluded) as opposed to immune infiltration into the tumour environment (immune-enriched) have also been observed. While the degree of immune infiltration as measured by for instance TILs has been established as a prognostic factor in TNBC, new studies suggest that the spatial distribution of immune cells also plays an important role in predicting clinical outcome [97].

Aside from the variability in immune infiltration, TNBC is also a disease with a high diversity in molecular alterations, including a high proportion of tumours with DNA repair deficiency (typically manifested as HRD based on *BRCA1*/*BRCA2*-deficiency), different mRNA subtypes, and potentially also epigenetic subtypes that together shape the tumour genomes and likely also the tumour micro-environment. To increase our understanding of TNBC we need to even better connect somatic alterations (the genotype) with the tissue phenotype (the tumour micro-environment composed of both malignant and non-malignant cell types).

Recent advancements in multiplex and spatial single-cell transcriptomics are providing powerful new tools for high-resolution mapping of cellular composition and spatial relationships in tumour micro-environments and between somatic alterations. By leveraging these methods it is now becoming possible to dissect the tumour micro-environment and tumour immune micro-environment, at a single-cell resolution while preserving the spatial context, thereby bridging a gap in comprehensive tumour characterisation.

In Study III, we aimed to connect the in situ-based profiling aspect from Study I with high-dimensional mRNA profiling (as exemplified in Study II) by applying multiplexed IHC and spatial transcriptomics to the TNBC cohort used in Studies I and

II. The overall ambition was to address important limitations of both Study I and II, including primarily to better explore the spatial localisation of different cell types and their cell-specific expression patterns in situ, generating new knowledge that could be contrasted versus somatic alterations, molecular subtypes, and patient outcome.

Results and Discussion

To facilitate the characterisation of the TNBC micro-environment by multiplexed IHC analysis, we began by establishing an automated cell segmentation and phenotyping pipeline, similar to TMArQ in Study I. To this end, we implemented starDist cell segmentation, pixel intensity-based automatic threshold, Gaussian Mixture Modelling, and a Genetic Algorithm. We applied the automated cell phenotyping pipeline to 6-plex multiplexed IHC data (Akoya PhenoImager), with the immune cell markers CD8, CD4, FOXP3, CD20, CD68, as well as the epithelial panCK marker, generated on the same TMA used in Study I (encompassing 343 analysable cores that represented 194 unique tumours).

As a first validation step, we compared our automatic pipeline to output from standard software (Inform, Akoya Biosciences), which requires human input. Our developed cell phenotyping approach correlated well to cell phenotype counts (Figure 6a).

To move beyond cell phenotype counts (the focus of Study I), we assigned individual cells to spatial ecosystems by a cell neighbourhood analysis including a consensus-based clustering approach based on k-means and hierarchical methods (Figure 6b). Based on the enrichment of specific cell types, we labelled the ecosystems as; tumour, stroma: undefined, stroma: macrophage enriched, immune: B-cell enriched, immune: cytotoxic T-cell enriched, and immune: T-helper enriched (Figure 6c). Similar to Study I, we found high covariance of specific immune cell ecosystems: B-cell enriched, immune: cytotoxic T-cell enriched, and immune: T-helper enriched (Figure 6d), suggesting that high cell counts typically imply a spatial aggregation.

We used survival analysis to investigate the presence of immune ecosystems with patient outcome after adjuvant chemotherapy. Based on univariate Cox regression with invasive disease-free survival as the clinical endpoint, we found that the B-cell enriched immune ecosystem was associated with a better outcome ($\mathrm{HR} = 0.83, 95\%\,\mathrm{CI} = 0.70\text{–}0.98, \mathrm{p} = 0.03$) followed by the cytotoxic T-cell enriched ecosystem ($\mathrm{HR} = 0.86, 95\%\,\mathrm{CI} = 0.75\text{–}0.98, \mathrm{p} = 0.03$).

To investigate how the derived tumour ecosystems were associated with different proposed molecular subtypes in TNBC, we compared ecosystem cell counts to different mRNA subtypes (PAM50 and the Lehmann et al. subtypes), novel DNA methylation

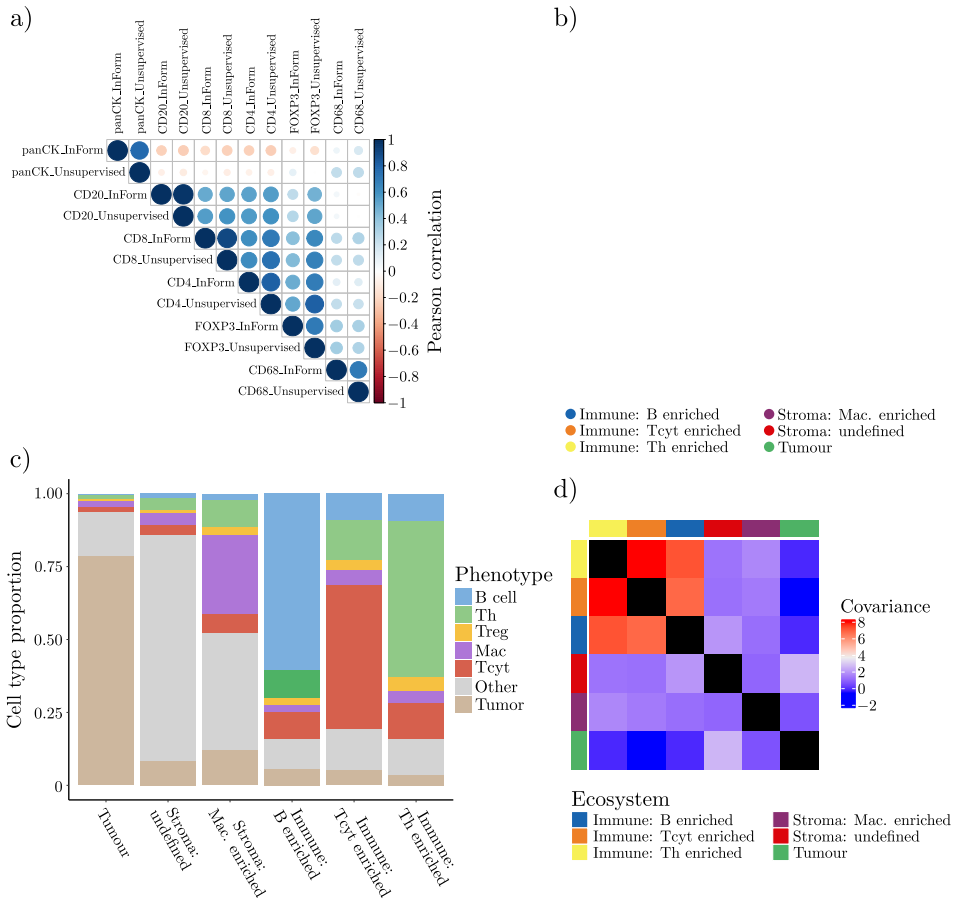

Figure 6: Multiplexed IHC pipeline validation and cellular ecosystem detection a) The marker positivity of the automated cell phenotype (_Unsupervised) counts aligned with those identified by Akoya InForm analysis, showing Pearson correlation values ranging from 0.7 to 0.97. **b)** Six cell ecosystems were identified based on cell density, each with unique cell type compositions, as illustrated for 9 selected TMA cores. **c)** Differences in cellular composition across ecosystems were used to guide annotation of the ecosystems. **d)** Covariance analysis of the ecosystem counts showed strong positive covariance among the immune ecosystem cubic root normalized counts, indicating high collinearity of immune ecosystem profiles within patients. Figure adapted from Study III.

subtypes (based on a study currently under review), and HRD status.

These analyses corroborated previous reports in the literature and the results in Study I regarding immune infiltration levels of HRD-positive versus HRD-negative tumours, a generally higher immune infiltration in the PAM50 basal vs non-basal subtype, and the difference in immune infiltration levels between the different Lehmann subtypes.

We also investigated the immune patterns in proposed novel DNA methylation phenotypes derived in the same TNBC cohort (manuscript currently under review), for which a similar analysis has not yet been conducted. The epigenetic subtypes com-

prise three basal subtypes and two non-basal subtypes. We found that one of the basal epitypes showed an immune-hot phenotype with higher values of all three immune ecosystems and TILs. Regarding the non-basal epigenetic subtypes, both subtypes showed a similar composition of the B-cell and T-helper enriched ecosystems. Nevertheless, the cytotoxic T-cell enriched ecosystem showed a trend towards a different composition, with borderline nonsignificant higher counts detected in one of the non-basal subtypes.

To investigate the observational differences in tumour immune ecosystems between the various TNBC classifications more deeply, we used spatial transcriptomic data based on Nanostring CosMx analysis of one TMA block representing 25 unique tumours across 41 cores. The aim of this analysis was to investigate the occurrence and spatial distribution of cell phenotypes on a more detailed level compared to the multiplexed IHC. We performed cell phenotyping of the spatial transcriptomic data and compared it to the corresponding tumour ecosystem data on a core-by-core level, finding the highest agreement for the B-cell enriched immune ecosystem. Similar to findings in both Study I and by the multiplexed IHC, we observed high variability in cell phenotype proportions across tumours, while less variation was observed between cores from the same patient. Again, this demonstrates the heterogeneity of the tumour immune micro-environment in TNBC. An important limitation of the spatial transcriptomic analysis was a limited expression depth for many genes in our pilot analysis, which unfortunately made cell annotation less fine-grained. When comparing the cellular composition defined by spatial transcriptomics versus the PAM50 basal/non-basal classification and the Lehmann subtypes we found consistent results with the multiplexed IHC analyses and what is known from the literature.

Limitations and Future Perspective

Limitations of this study included the reliance on TMAs as opposed to whole-slide data for spatial profiling. TMA cores may, due to their small size and selection (i.e., where the punch for the core was made), not fully represent the heterogeneity of the complete tumour. However, creating a similar dataset based on whole-slide multiplexed IHC tissue would be costly and time-consuming to generate, and more challenging to analyse due to the large file size of the raw image data. The 6-plex immune panel used for the multiplexed IHC allowed us to perform the initial spatially directed analyses more consistently and robustly compared to the repeated singleplex IHC in Study I. Still, the 6-plex immune panel did not give us the ability to derive more specific immune cell phenotypes (e.g., M1-like/M2-like macrophage classification) that would allow us to move substantially beyond the main observations in Study I. Next to the spatial transcriptomics that we implemented with this in mind, a more targeted

multiplexed IHC that can delineate cell subtypes (like M1-like/M2-like macrophages) could be used in an extension of this or future studies.

An additional limitation of this study was that our estimates of cell phenotypes and tumour ecosystems were based on TMA cores, similar to the digital cell counts in Study I, whereas the TIL-scores were based on whole-slide tissue sections. Still, the immune infiltration scores derived from multiplexed IHC showed good agreement to the TIL-scores, just as in Study I.

For the spatial transcriptomic analyses, the two main limitations were the limited sample numbers analysed in this pilot and the limited depth of the gene expression that could be mapped to segmented cells. The small sample set made subgroup analyses challenging and may hamper the generalisability of the results. Expanding the sample set for spatial transcriptomics to a larger part of the available cohort would allow for validation and potential extension of the existing results. Moreover, spatial transcriptomic analysis is a rapidly evolving field with respect to both technology and data analysis. Improving the basic processing, such as cell segmentation, of the existing Nanostring CosMx data could also be an important step in improving the output of our existing analyses. Alternatively, other spatial transcriptomics methods, like the 10x Genomics Xenium platform can potentially allow for gene expression profiling with more depth in TNBC tissue, allowing for better cell delineation.

Taken together, Study III provides an additional characterisation of the tumour immune microenvironment in TNBC alongside Studies I and II. It provides in situ confirmation of conclusions about general patterns linked to commonly used or novel molecular stratifications of TNBC (transcriptional, genetic, and epigenetic). Study III also provides multiplexed in situ support for conclusions about the collinearity of the studied immune cell types reported in Study I. However, despite all of these analyses, there is still a need to further and better delineate the heterogeneity of the tumour immune micro-environment within molecular subsets of TNBC to fully understand what drives tumours to be immune hot or immune cold.

Conclusion

Conclusion

This thesis aimed to enhance our understanding of immune infiltration in triple-negative breast cancer and to develop approaches for better prognostication and treatment predictions with a particular focus on the tumour micro-environment. Collectively, the findings contribute insight into the tumour immune micro-environment and its interplay with molecular and genomic profiles in triple-negative breast cancer.

First, our development and application of an open-source pipeline for automated immunohistochemistry-based cell detection and positive marker staining in Study I demonstrated how digital analysis of single-plex immunohistochemistry stained tissue can facilitate the characterisation of the tumour micro-environment in triple-negative breast cancer. Our approach highlights the prognostic value of immune infiltration makers even from tissue microarrays, and their potential, as shown with the general T-cell marker CD3, to refine genomic-derived classifications, such as the genetic homologous recombination deficiency (HRD) phenotype. Moreover, the spatial data generated in Study I can provide a basis for sophisticated analyses of cell-cell interactions and exemplify how quantitative profiling of the tumour micro-environment can complement -omics-driven cancer research.

Second, our development of an RNA-sequencing-based stand-alone classifier for immune response in triple-negative breast cancer in Study II further highlights the prognostic value of immune infiltration in these tumours. The predictor, based on the IM-subtype classification proposed by Lehmann et al., demonstrated strong associations with increased tumour-infiltrating lymphocytes, expression of immune-related genes in transcriptomic data, and higher diversity of T-cell receptor genes. The classifier also showed a borderline significant correlation with a better response to neoadjuvant chemotherapy treatment and improved patient outcomes in independent cohorts.

Finally, in Study III, we studied the spatial heterogeneity of the tumour immune

micro-environment in triple-negative breast cancer and its connection to molecular and genomic subtypes. By integrating multiplexed immunohistochemistry stained tissue analysis with bulk tumour multiomic analyses and spatial single-cell transcriptomics we were able to connect ecosystems in the tumour micro-environment to known patterns of immune infiltrations and could show subtype-specific differences.

In conclusion, this thesis advances the quantitative and integrative study of the tumour (immune) micro-environment in triple-negative breast cancer, offering new approaches and insights to bridge the gap between immune infiltration, molecular heterogeneity, and clinical outcome. This thesis also illustrates the complexity of the tumour immune micro-environment in triple-negative breast cancer, and the heterogeneity of it in currently proposed molecular subtypes of the disease. These contributions provide a pathway for the refinement of prognostic and predictive strategies with potential implications for personalised medicine, especially with respect to immune checkpoint inhibitors.

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