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Master's Thesis in Biological Chemistry

Relation Between Prognostic Factors and MicroRNAs (miR-18a/miR-18b) in Triple Negative Breast Cancer

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Sadia Hassan Raghe

Abstract

Breast cancer is the most common type of malignancy found in women. It is a very heterogenous disease and treatments vary accordingly. Triple negative breast cancer (TNBC) is the most aggressive subtype of breast cancer and is often found in younger women or women of African descent. It is recognized by its lack of expression of estrogen receptor (ER), progesterone receptor (PR) or the human epidermal growth receptor 2 (HER2). Due to the lack of these receptors, treatment options are very limited, and chemotherapy is often the only choice available for patients. However, several other ways to treat TNBC is under investigation and strong emphasis are placed to discover prognostic and predictive markers that can predict or aid in the treatment of this cancer.

MicroRNAs are short non-coding RNA molecules that form around 19-22 nucleotides in length and are tasked to up/downregulate gene expression post-transcriptionally by either binding to the messenger RNA (mRNA) targets or by cleaving them. Functionally, they maintain balance in normal cells by regulating cellular growth, proliferation, differentiation and apoptosis. In breast cancer, the expression of some of these microRNAs have been recognized to possess either oncogenic or tumor-suppressing properties. MicroRNA 18a and 18b especially, which are part of the miR-17-92 cluster, have been considered to be oncogenic, and their overexpression in tumor cells have shown to enhance cell proliferation, trigger angiogenesis, block differentiation, and promote metastasis and evasion of an apoptotic response. Due to their significant contribution to cancer growth, microRNAs in general are very valuable biomarkers to target as potential prognostic markers or novel therapeutic agents for TNBC.

The aim of this study is to count the mitotic activity index (MAI), TILs and miR-18a/b in the tumor microenvironment of TNBC to investigate whether any correlation exists between them, and the other factors and characteristics related to TNBC and its prognosis. There are two main objectives to this thesis. Firstly, to statistically analyze MAI, TILs and other characteristics of TNBC in the Norwegian cohort (n= 271) at the University Hospital of Stavanger to find good prognostic markers for TNBC, and secondly, to measure the expression of microRNAs 18a and 18b using CISH. Statistical analyses will be done to determine whether either or both of these microRNAs are significant enough to be considered as prognostic markers for TNBC.

From the larger cohort of TNBC cases of those that were alive and well, those with local reoccurrence and those dead of the disease with distant metastasis at the end of their follow-up status (n=195), the results showed that the lymph node status, when negative (p=0.000, HR=0.300, 95% CI=0.181–0.497) was the most significant factor for a good prognosis. The age at the time of diagnosis (p=0.001, HR=2.053, 95% CI=1.329–3.171) and the fibrotic focus (p=0.017, HR=1.670, 95% CI=1.092–2.55) were also significant factors that showed a trend towards greater survival and good prognosis when combined with the negative lymph node status. From the CISH group (n=42), miR-18a showed a significant correlation with the Nottingham grade (p=0.066) and tumor type (p=0.003). On the other hand, miR-18b showed correlation with MAI (p=0.056), TILs (p=0.028), lymph infiltration (p=0.001) and tumor type

(p=0.004). No difference was observed between patients with or without distant metastasis in the CISH cohort when all cases were observed together. However, when only lymph node negative cases were isolated from the rest and selected, a trend was observed in patients who didn't experience distant metastasis when they had higher numbers of positive miR-18b cells. In conclusion, the findings in this thesis suggest that the lymph node status is a good predictive prognostic marker for TNBC along with fibrotic focus. From the microRNAs, miR-18b seem to be a good prognostic marker in lymph node negative TNBC patients and should be further studied. The sample sizes used for this study were small, and these results should be interpreted with caution and further validation is recommended.

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Abbreviations

A/LR	Alive with local reoccurrence
AI	Artificial Intelligence
ALND	Axillary lymph node dissection
AP	Alkaline phosphatase
ASCO	American Society of Clinical Oncology
ATP	Adenosine triphosphate
AW	Alive and well
BC	Breast cancer
BCIP	5-bromo-4-chloro3’-indolylphosphate
BLBC	Basal-like breast cancer
BRCA1/2	BReast CAncer gene 1/2
CAMs	Cell-cell adhesion molecules
CHEK2	Checkpoint Kinase 2
CI	Confidence Interval
CISH	Chromogen In-Situ Hybridization
CTLs	Cytotoxic T lymphocytes
DCIS	Ductal carcinoma in situ
DGCR8	DiGeorge syndrome critical region 8
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DoD/DM	Dead of disease with distant metastasis
EMT	Epithelial-mesenchymal transition

ER	Estrogen Receptor
EtOH	Ethanol
FF	Fibrotic focus
FFPE	Formalin-fixed paraffin embedded
GLUT1	Glucose transporter 1
HER2	Human epidermal growth factor receptor 2
HIF-1	Hypoxia-inducible factor 1
HR	Hazard ratio
HRT	Hormone replacement therapy
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
ISH	In situ hybridization
IV	Intravenous therapy
LCIS	Lobular carcinoma in situ
LNA	Locked nucleic acid
LNP-mediated	Lipid nanoparticle-mediated
MAI	Mitotic activity index
MET	Mesenchymal to epithelial transition
miR/miRNA	MicroRNA
MQ/MilliQ water	Deionized purified water
mRNA	Messenger RNA
NBT	4-nitro-blue tetrazolium
NK cells	Natural killer cells

NST	No-special type
PARP	Poly adenosine diphosphate–ribose polymerase
PBS	Phosphate-buffered saline
PR	Progesterone receptor
PSA	Prostate-specific antigen
PTX	Paclitaxel
RB	Retinoblastoma-associated
RNA	Ribonucleic acid
RNAse	ribonuclease
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SASP	Senescence-associated secretory phenotype
SLNB	Sentinel lymph node biopsy
SREBP1	Sterol regulatory element-binding transcription protein 1
SUS	Stavanger University Hospital
TGF-β	Transforming growth factor β
TILs	Tumor infiltrating lymphocytes
Tm	Melting temperature
TME	Tumor microenvironment
TNBC	Triple negative breast cancer
TNM	Tumor-node-metastasis
TP53	Tumor protein P53

uPA/PAI-1	Urokinase plasminogen activator/plasminogen activator inhibitor-1
WHO	World Health Organization
WSI	Whole slide imaging

1 Introduction

1.1 A Brief History of Cancer

Cancer is a disease in which abnormal cells quickly divide without control and invade nearby tissues, thereby spreading to other parts of the body through the blood and lymph systems [61][67]. Due to its heterogeneous nature, a cure has yet to be found for all its various forms. Along its wake follows anxiety, fear, anger, and mourning. Countless people, among them women, men, and children have lost their lives to it and continue to do so today. It can be said surely that cancer is, if not, one of the deadliest diseases still around in the modern world. Once spoken optimistically, the U.S. President William Howard Taft said in 1910 [82]:

“Within five years, cancer will have been removed from the list of fatal maladies”.

However, it is still ongoing today. This battle is not new, nor will it be an easily won one either and strong efforts are put daily into ending this disease for good. However, the origins of cancer as a disease date far back in time, longer than what most people like to believe [50]. Animals and humans have been dying of cancer for centuries and the earliest evidence of its presence presents itself as a fossilized bone tumor in human mummies in ancient Egypt. Not known then, the cancer found in these mummies bear many similarities to osteosarcoma, a known bone cancer in today’s world [17]. The oldest description of cancer or tumors date back to 3000 BC, and this can be found in the Edwin Smith Papyrus, an ancient Egyptian book on trauma surgery. There, the removal of 8 tumors or ulcers of the breast is described by the method of cauterization with a fire drill. In the writings, the grim description reads [17]:

“There is no treatment.”

Sadly, this sentiment is not far from what is still experienced by patients today centuries later who have been diagnosed with terminal cancer. This disease is difficult to deal with, and the issue lies in how to navigate the breadth and diversity of it, its multiple genetic variations, tissue biology, histopathology, and how the cells response to therapy and treatment. No two cancers are alike, and no two patients diagnosed with the same type of cancer will experience it alike either [40]. With how complex this disease has proven itself to be over the last centuries, advancements in both medicine and technology have made it possible to develop a fighting chance against it. This is good because with an aging population, there is an expectancy for more cancer patients that demand better care and treatment. Advancements in areas like diagnostics and treatments have resulted in increased survival rates, but also an increase in costs due to high-quality testing and research for better biomarkers, which are not cheap endeavors. Cancer might be a tough opponent, but the more understood it is and its behaviors learnt, a way to counter-act it might be developed sooner [90][78].

1.2 The Hallmarks of Cancer

1.2.1 Introduction

The ability of cancer cells to grow and develop lie within their abilities to defect from the regulatory circuits that generally guide normal cell proliferation and homeostasis. As a heterogenous disease, there are several types of cancer cells that are distinct, exhibit certain characteristics only present in specific subtypes, and or take shape in specific organs [38]. However, how these cancers form, and which disruptions in their regulatory circuits cause them to disform and spread to other parts of the body is vast and difficult to grasp. For some, only one misstep in their regulatory circuit can cause cells to become cancerous, while for others, it takes a vast collection of diverse gene aberrations to come together and influence the cancer growth, thus giving each cancer its own specific genotype [61][78].

In their research (2000) to categorize how cancer cells form, Hanahan and Weinberg suggested that cancer cells need changes in six essential categories from cell physiology in order to fulfill their full potential for malignant growth [38]: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Each of these changes show how cancer cells will defend themselves against the body's efforts to eliminate them as well as establishing the multistep developments necessary for tumors to thrive [38]. In 2011, two more hallmarks of cancer emerged and were added to the list – reprogramming of energy metabolism and evading immune destruction. With the complexity of tumorigenesis, another factor that had to be considered were how normal cells contributed to the hallmarks of cancer by creating "tumor microenvironments" for cancer cells to grow, and thus two new enabling characteristics of genome instability and inflammation were also introduced. In 2022, Hanahan and Weinberg decided to incorporate four new emerging hallmarks to their list of cancer capabilities, the first being unlocking phenotypic plasticity, then non-mutational epigenetic reprogramming, polymorphic microbiomes and lastly senescent cells [40]. Recognizing these concepts still remains to be the first thing that must be done to effectively combat cancer cells in the future. The more capabilities that are discovered, the more insight is gained into how to eventually treat cancer for good [39][34].

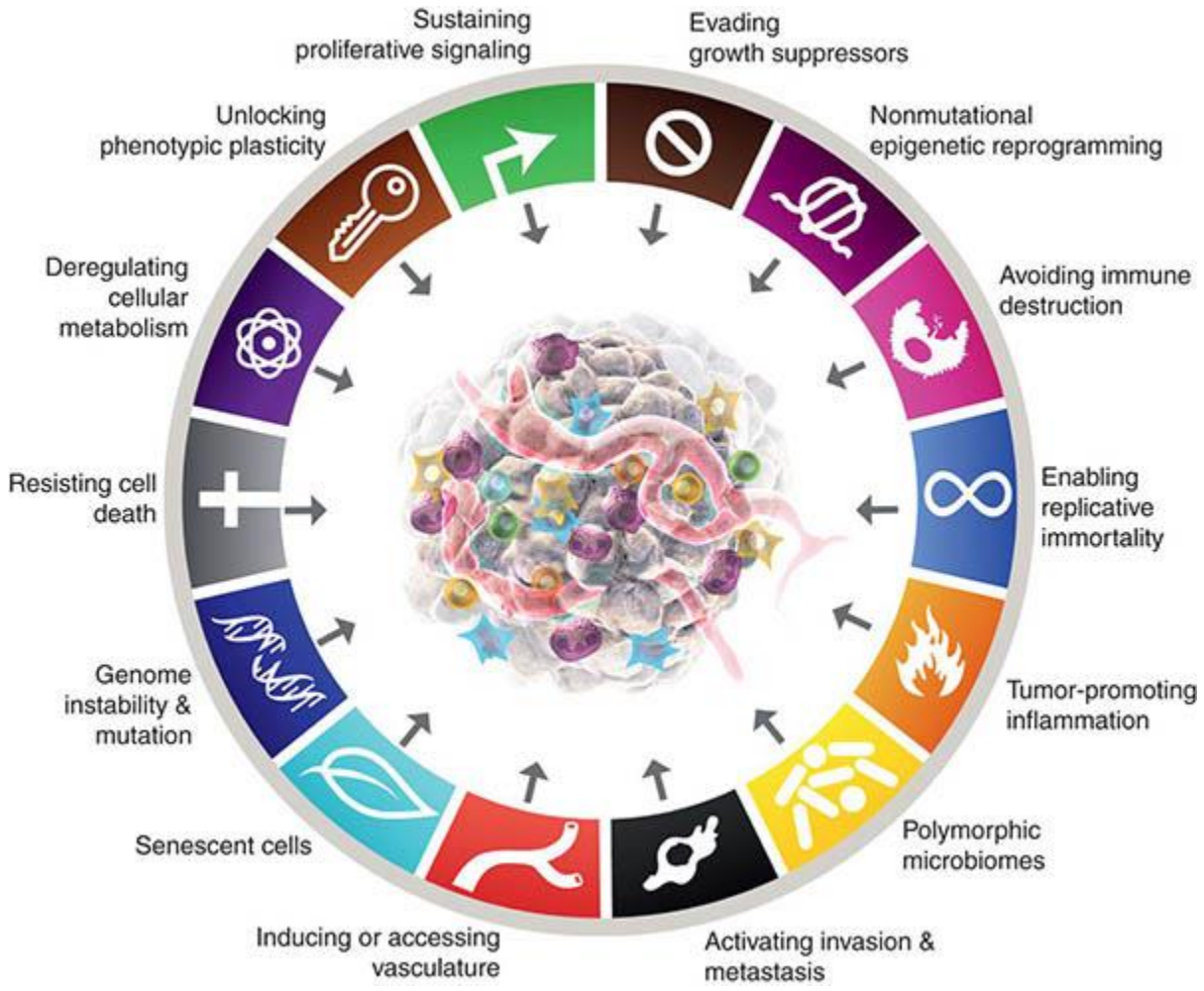

Figure 1. The New and Updated Hallmarks of Cancer (2022)[40].

1.2.2 Sustaining Proliferative Signaling and Evading Growth Suppressors

The most important trait for a cancer cell is its ability to maintain its source for division and rapid growth. Natural cell proliferation, the process of increasing the number of cells in the body, occurs normally during growth and development through life such as during childhood when the body starts to mature, or during pregnancy when a new life is growing inside the mother's womb. The general upkeep of the cells to maintain the epithelia (the colon and skin etc.) is also part of the way cell proliferation occurs naturally [39]. During normal cell division, the production and release of growth-promoting signals that begin the cell growth-and-division cycle are carefully monitored, and a state of balance is maintained. Cells are given their functions and when they reach the end of their service, they are signaled to undergo cell death (apoptosis). Every cell must undergo a quality-control system before they enter the cell cycle. The difference with cancer cells is that they do not do so, and instead act on their own to deregulate the finely tuned system in order to achieve an unstoppable growth [29] [38].

Currently, no normal cell can undergo proliferation without the presence of stimulatory signals. Cancer cells often imitate how normal growth signaling works by generating their own signals.

But in order to survive, they must also find a way to shut down tumor suppressors and anti-growth signaling pathways. Through cancer research, numerous tumor suppressors have been discovered and the various ways in which they limit cell growth and proliferation [39]. The two most known tumor suppressors encode the RB (retinoblastoma-associated) and TP53 proteins which operate as central nodes in the cellular regulatory circuits that determine whether a cell will proliferate or not, as well as activate senescence and other apoptotic pathways [39]. Once cancer cells obtain this ability to circumvent signals that do not favor their growth and mimic those that do, they can become self-sufficient and obtain the ability to avoid cell death, thus leading to the formation of cell masses that shape malignant tumors [38]. It must be noted though, that rapid formation of cancer cells does not always lead to malignant cancer, as benign tumors do exist as well [29].

1.2.3 Resisting Cell Death

The program instilled in cells to stop them from countlessly dividing and increasing in numbers is called apoptosis. It's a process where due to a series of biochemical signals the cells change, shrink, and die [38]. It was in 1972 that apoptosis, being a barrier against cancer cells, was put forth by Kerr, Willie and Currie [38]. For cancer cells, this programming is a major obstacle if they want to survive and replicate. Just evading suppressors is not enough. Resistance towards apoptosis becomes mandatory for almost every cancer cell type for the disease to take over the body and spread [38]. Observations and research over the past few years have shown that the apoptotic program is present in its latent form in all cell types [38]. This is to trigger a signal that will lead to a series of steps that will eventually kill the cell if needed. The cellular membrane is broken down, chromosomes degrade, the nucleus fragments and more in about 30 – 120 minutes. At the end of the process, the dying cell is engulfed by macrophages and disappears [38]. Whenever there are high apoptotic signaling genes/proteins activated, a corresponding low or slowly growing tumors are observed. On the opposite side, when tumor suppressing proteins that activate the apoptosis chain are deactivated, a rapid growth of tumors with low numbers of apoptotic cells are observed [38]. This is not always the case though, as other observations have been made where high apoptosis coexists with high proliferation, usually depending on the growth of the blood vessels and how much access the tumor cells have to nutrients and oxygen to keep proliferating. And the end, tumor cells must learn to adapt and interfere with the apoptotic programming in their cells to inactive the system, and effectively resist cell death and keep spreading [38].

1.2.4 Enabling Replicative Immortality

One would think that after sustaining their own growth signals, evading suppressors, and avoiding cell death that tumor cells now would have the full ability to expand and limitlessly divide. However, even with those abilities at hand, it is not enough for cancer cells to grow indefinitely. Research throughout the decades show that many, perhaps all mammalian cells, carry an intrinsic, cell-autonomous programming that limits their multiplication. This means that even though cancer cells have allowed themselves to replicate from outside signals, the limit of their multiplication is not infinite and is an intrinsic value within each cell. In order for cancer cells to divide past their limit, they must disrupt this programming as well. As cells have finite replicative potential, once they reach a certain number, they stop to grow. This is known as

senescence. However, disabling the pRB and p53 tumor suppressor proteins allows cells to replicate down further generations until they hit the stage “crisis”. In crisis, massive cell death occurs which seems counterintuitive. But occasionally, a variant might rise (1 in 10^7 chance) that somehow requires the ability to multiply without limits, hence achieving immortality [38]. The longevity of cell age is tied to the telomeres as senescence occurs from the successive shortening of the telomeres at the end of the chromosomes in cells. To combat the shortening of their telomeres, majority of cancer cells can express the enzyme telomerase, which in contrast to the normal process of cell aging, instead *adds* telomere segments to the ends of the chromosomes. With this function in place, cancer cells now have acquired the limitless potential of continuous replications in order to increase in numbers [29].

1.2.5 Inducing Angiogenesis

For a cell to sustain itself and function, it needs access to oxygen and nutrients. Angiogenesis is the process of growing new blood vessels and typically happens when new tissues are formed. This process is very carefully regulated as a network of blood vessels must be coordinated and provide nutrients as well as remove waste from all the organs interconnected [38]. With cancer cells continuously proliferating and developing new masses inside the body, one would think that with the generation of new cells, they must have the ability to trigger the growth of new blood vessels as well to sustain themselves. However, that is far from the truth here. New cells that grow away from an already established parameter of networks do not have an intrinsic ability to trigger angiogenesis. For cancer cells, this is problematic because for them to grow quickly and survive, they must develop angiogenic abilities [38].

Every cell requires to be within 100 μm of a capillary blood vessel in order to get the sufficient amount of nutrients and be able to get rid of metabolic waste and carbon dioxide. In order to sustain itself, a tumor will release soluble angiogenic factors like hypoxia-inducible factor 1 (HIF-1), and other growth factors to encourage the formation of blood vessels nearby it. This process is known as “neovascularization”. It is essential for both the development and the preservation of cancer as it allows it to metastasize too by forming routes in which the cancer cells can migrate and start anew [29]. Sometimes, cancer cells might grow too fast for angiogenesis to keep up, causing some parts of the tumor to die due to lack of nutrients and supplies. These necrotic areas of the tumor can be observed on a microscope [29].

1.2.6 Activating Invasion and Metastasis

For a tumor to keep growing and colonize other organs, it must acquire the ability to spread and go to distant areas. 90% of all cancer deaths are caused by this acquired ability to invade nearby tissues and metastasize. From its primary location, cancer cells send out pioneer cells across the body to start establishing new colonies. In a process called epithelial to mesenchymal transition (EMT), cancer cells metastasize to these new areas before reversing the process once they get there, undergoing mesenchymal to epithelial transition (MET). Initially, these new settlements will provide the cancer cells with new terrain and plenty of nutrients to replicate quickly and grow [38]. This however does not mean that the settlements will automatically turn into tumors. They can remain localized like carcinoma-in-situ for decades before starting to grow or spread malignantly. Still today, the way cancer cells invade and metastasize are not completely

understood. It is a very complex process consisting of several genetic and biochemical pathways that have yet to be fully explored [38]. Both invasion and metastasis share similar signaling processes in the way they change how the cells react to their microenvironment and their ability to trigger the activation of extracellular proteases when needed [38].

In order to invade or metastasize, cancer cells must firstly target the proteins that are involved in tethering cells to their surroundings. Thus, affected proteins to be altered include cell-cell adhesion molecules (CAMs), especially the immunoglobin and calcium-dependent cadherin families because they mediate cell-to-cell interactions. The integrins that link cells to their extracellular matrix substrates are also of importance to cancer cells [38]. The most widely known of them is E-cadherin, a homotypic cell-to-cell interaction molecule that is expressed in epithelial cells. So, it comes as no surprise when this molecule is found missing in majority of epithelial cancers as it is a major obstacle against invasion and metastasis. It is a good suppressor and the reason for why ensuring its elimination is a key step for the growth of cancer cells [38].

1.2.7 Genome Instability and Mutation

While the previous six were the original capabilities that cancer cells had to acquire in order to survive, most of them are developed directly or indirectly through two enabling characteristics, the first one being genome instability and mutations [38][39]. To start, it should not be expected that rise in mutations in the human body will automatically give rise to a higher chance to develop cancer. Quite the contrary, the genomic integrity of normal cells is kept intact by the complex systems of DNA monitoring and repair enzymes that prevent that from happening [38]. This maintenance crew ensures that DNA sequences that are put forth are correct, and that no mishap has occurred through checkpoints during critical moments like cell division [38]. For the other hallmarks of cancer to be acquired, a development of genomic instability is necessary to give rise to multiple random mutations including chromosomal rearrangements. This could potentially give cancer cells the rare genetic changes they need in order to acquire the hallmark capabilities that would otherwise be impossible to obtain due to the repair system ingrained in every cell [39].

Cancer cells technically arise due to the genetic aberrations that occur over the life span of their host. These aberrations can be somatic mutations or inherited (germline) mutations. These mutations generally slip through or directly affect the DNA repair system and once they accumulate, can provide the cancer cells with structural changes in their nucleotide sequences. This can lead to changes in their expressions and functions making them cancerous [29]. Only mutations that offer advantages to promote tumorigenesis are selected for cancer cells. Some of the changes are loss of function for tumor-suppressors, or the gain of functions for oncogenes. In either of these cases, cancer cell proliferation is favored, and these mutations are labelled as “driver mutations” [29]. For a tumor to develop, generally two to eight types of mutations must occur in one and the same cell, which usually does not happen as the DNA repair system is quick to fix any mishaps during the checkpoint processes. However, with age or environmental factors/live style, more and more cell aberrations occur, giving rise to more chances for several mutations to take form at once and combine. This is why cancer is more common in older patients. For those who carry the gene variations that store the mutations BRCA1/2, it is quite

possible to develop cancer cells earlier in life than expected. This is due to the mutations accumulating over time since birth [29].

1.2.8 Tumor-Promoting Inflammation

Every tumor contains immune cells, ranging from small densities only detectable with specific antibodies to large amounts appearing as inflammations when observed using standard histochemical staining techniques [39]. In the past, this immune response was thought to reflect the attempt of the immune system to kill the cancer cells, but now increasing evidence suggests that the inflammatory response of the immune system to the tumor can have an unexpected paradoxical effect that in actuality enhances tumorigenesis and cancer progression instead of eliminating them [39]. The immune response, especially the innate immune system, surprisingly can aid tumor progression by helping it acquire its hallmark abilities by causing an inflammation. According to Hanahan and Weinberg, it also sends "bioactive molecules to the tumor environment like growth factors, supplies with survival factors that limit cell death, proangiogenic factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis, and inductive signals that lead to activation of EMT and other hallmark-facilitating programs" [39]. Not only that, but inflammation usually occurs during the early stages of cancer progression as well, which is very helpful for general development. During inflammation, a release of reactive oxygen species also occurs, and they are actively mutagenic to nearby cells, thus aiding with the acceleration of mutations to occur too [29]. However, there is still some evidence that shows that the immune system still has antitumoral responses to many types of cancer, which is why cancer cells must develop the ability to evade termination [29].

1.2.9 Reprogramming Energy Metabolism

As proliferation and constant growth are among the main priorities of cancer cells, a new ability to utilize energy metabolism and adjust to fuel cell growth has been observed and emerged as a new hallmark in recent years. During energy metabolism, normal cells first convert glucose to pyruvate via glycolysis in the cytosol and then into carbon dioxide inside the mitochondria. Under anaerobic conditions, relatively little pyruvate is converted and sent to the mitochondria because glycolysis is favored. It has been observed that even in the presence of oxygen, cancer cells have reworked their systems to depend largely on glycolysis in what has been labelled as "aerobic glycolysis", also known as the Warburg effect [39]. This energy redirecting seems counterintuitive as cancer cells would have to compensate for 18-fold deficiency of ATP production, comparative to how normal metabolism works. They do this by upregulating glucose transporters, especially GLUT1, to increase the import of glucose into the cytoplasm as many tumors have been observed to have an increased uptake and usage of it [39]. Therefore, in a sense, they can balance out the loss with a gain that benefits them.

Even though it is not fully known why cancer cells redirect metabolism due to the poor efficiency of ATP production that occurs during constant glycolysis, the glycolytic fueling has been linked with oncogenes and tumor suppressors that primarily aid in the hallmarks of cell proliferation, avoiding cytostatic controls and apoptosis. Another recent discovery that might link this to other hallmarks as well is that glycolysis releases intermediates formed during the

process into different pathways of biosynthesis that generate nucleosides and amino acids which further synthesize the macromolecules and organelles needed for making new cells [39]. Today, this reprogramming of energy metabolism has officially been recognized as one of the core hallmarks of cancer due to its importance of function for the disease and how it works independently, while also enhancing the other capabilities of cancer [39].

1.2.10 Evading Immune Destruction

One important factor that cancer cells must face is the immune system of the host. The immune system is there to help with the eradication of any abnormal cells or faulty DNA that causes harm to the body. A well-known theory, immune surveillance, states that the immune system is in a constant mode of surveillance, constantly monitoring the body for anything threatening, and cancer cells fall into this category perfectly. However, this would mean that cancer cells would not be able to survive the constant watching right? There is clear evidence of solid tumor cells, either in situ or malignant, located in the body while the immune system is active. This means that cancer cells have developed some way to evade as one of its emerging hallmarks. In immunocompromised individuals, an increase in certain types of cancers is given. High immunogenic cancer cells will get eliminated, but instead, they might leave behind weaker immunogenic cells. Some high immunogenic cancer cells can perform "immune editing" by disabling components of the immune system that have been sent to destroy them like paralyzing infiltrating CTLs and NK cells, or by secreting TGF-β and other immunosuppressors. As of today, the ability of cancer cells to evade the immune system has been recognized as one of the core hallmarks of cancer due to the amount of research that has been put on the importance of the interaction between them [39]. A treatment method to combat the way cancer cells evade the immune system, known as immunotherapy, has become a successful way in treating patients and allowing those diagnosed with terminal cancers to live much longer lives than originally expected. More on this treatment will be explored in a later section.

1.2.11 Unlocking Phenotypic Plasticity

What unlocking phenotypic plasticity entails lies in the process of organogenesis, which is the "development, determination and organization of cells into tissues" thus, giving them homeostatic functions [40]. During this process, the progenitor cells undergo terminal differentiation. They change their phenotypic characteristics as they fully differentiate into different cells before eventually dying off. This is not ideal for cancer cells, as organogenesis is an antiproliferative system. Cancer cells must have figured a way to circumvent this as increasing evidence has been found that indicates so. What they do is unlock the capability of phenotypic plasticity, which is usually restricted. When unlocked, progenitor cells can evade and escape their fate of terminal differentiation, which is critical for cancer cells as that eliminates the antiproliferative property of the progenitor cells [40]. This can happen in several different ways, by either reversing back to their progenitor form (dedifferentiating) after approaching full differentiation, remain partially differentiated and create neoplastic cells that way, or trans differentiation, where the cells that were committed to one pathway suddenly switch over to an entirely different development course, allowing the tissue to acquire traits it would never otherwise have acquired. Considering all this, the way cancer cells use cellular plasticity to

further advance is enough to distinguish this as a new functional hallmark capability of cancer [40].

1.2.12 Non-mutational Epigenetic Reprogramming

The second emerging hallmark is termed “non-mutational *epigenetic* reprogramming” as genome instability is already a previous established hallmark of cancer. For cancer cells, their abilities to mutate and reprogram the genome in human cells is so fundamentally important and exists in almost all types of cancer, hence why genome instability is a core hallmark. These instabilities and diversions can be found in different stages of progression, in metastatic lesions and when developing resistance to therapy. These mutations are related to defected processes of genome regulation. However, non-mutational epigenetic reprogramming has emerged as an independent way for cancer cells to reprogram their genomic expressions without relying on mutations, or evolution, instead only depending on epigenetic factors to select phenotypes that aid in their constant growth and proliferation. The use of mutation-less reprogramming can already be found in mechanisms developing the embryo, in processes like differentiation and organogenesis. Long-term memory change that occurs in adults is an example of ordinary epigenetic reprogramming in which gene, histone and chromatin structural modifications over time lose stability due to positive and negative feedback loops. By using this already established system, cancer cells can alter gene expressions epigenetically without relying on factors forcing a mutation or need to acquire the core hallmark capabilities during development and malignant spreading in the beginning [40]. One such example of this is the methylation of the BRCA1/2 gene found in breast cancer patients. Instead of a mutation giving rise to either breast or ovarian cancer in patients, the epigenic alterations occurring in BRCA1 (ex. hypermethylation) may play a key role in tumorigenesis by contributing to the initiation and malignant progression of the tumors [21].

1.2.13 Polymorphic Microbiomes

With the recent increase in technology and biomedicine, microbiota have taken a center stage of interest when it comes to medicine in cancer biology. On the surface of the epidermis, to the inner mucosa, intestinal tract, lungs, breasts and urogenital system, several microenvironments filled with a variety and diversity of microorganisms symbiotically exist. With the use of information gathered from next generation sequencing and bioinformatic technology, a strong case is building for the connection between the polymorphic microbiomes and cancer phenotypes. Stronger understanding regarding how they affect cancer development, malignant progression and response to cancer therapy can shape how cancer is dealt with in the future. This new emerging hallmark highlights the importance of these tissue microbiomes and how cancer cells are utilizing them to their advantage [40].

Among the microbiota, the gut is the most impactful and well-known microbiome related to cancer cells and how they operate. The gut is in charge of degrading and importing nutrients from the large intestine into the rest of the body to maintain metabolic homeostasis. A shift within the maintained homeostasis of the gut will often lead to diseases. One example of how influential microbiomes can be on cancer growth is seen with colon cancer. In a study done, some fecal matter was transplanted from patients with colon tumors into mice predisposed to

developing colon cancer. From this experiment, it was gathered that there exist some tumor-promoting microbiomes and some cancer-protective microenvironments that help with the pathogenesis of colon tumors, even narrowing it down to a couple bacterial species that were active in the process [40]. The questions regarding this emerging hallmark are as follows; do microbiomes, if utilized correctly, provide the ability to interfere or contribute to how cancer cells obtain the other hallmarks? Do they influence cancer progression and increase proliferation? It is undeniable that polymorphic microbiomes do possess the distinct enabling characteristics to aid hallmark capabilities of cancer in complimenting genome instability, mutation and assist in tumor-promoting inflammation [40]. Hopefully, this also means that a way to interfere with those processes will eventually emerge, easing the path to finding a way to eliminate cancer cells for good.

1.2.14 Senescent Cells

The last of the new additions to the hallmarks of cancer is senescent cells. Cellular senescence is when cells undergo an irreversible form of proliferative arrest, making them unable to continue dividing. This is a protective measure against cells potentially becoming neoplastic and a complimentary system to apoptosis as it gets rid of any abnormal, inactivated, or dysfunctional cells. When cells undergo senescence, the cell division cycle is shut off, and changes to the cell morphology begins to occur. Senescence is often induced by either DNA damage, conditions in the microenvironment prompting it to, or nutrient deprivation, and wrong cellular signaling to name a few. Even though this is a protective measure against cancer cells forming, the catch here is that senescence is formed exactly from the same processes associated with malignancy and tumor formation (aka DNA damage etc.). Thus, with further investigation into this process that halts the division of cells, it has been discovered with increasing evidence that cellular senescence in certain context can stimulate tumor growth and cancer progression instead. The main culprit behind this is believed to be SASP (senescence-associated secretory phenotype), which releases various bioactive proteins such as chemokines, cytokines, proteases etc. It can convey signaling to nearby cancer cells in paracrine fashion, promoting certain tumor phenotypes to emerge. Another way senescent cells perform these functions is to revert from their senescent state into fully functioning cells that still express SASP, becoming operable oncogenic cells. These cases are well-documented when it comes to therapy resistance as senescent cells are often induced during chemotherapy/radiotherapy [40].

1.3 Biomarkers of Cancer

1.3.1 Introduction to Biomarkers

When it comes to cancer, once a patient is diagnosed, a means to find the right treatment is necessary. Often, it is a challenge to identify and decide whether a patient should receive adjuvant treatment, chemotherapy, etc. or not [73]. Thus, biomarkers take the center stage in dealing with this issue. Under the National Cancer Institute, a biomarker is defined as “a biological molecule found in blood, other body fluids, or tissues that is a sign of normal or abnormal process, or a condition or disease” and cancer falls under these terms [42]. Therefore, the hunt for potential biomarkers that can aid in decision making when it comes to treatment or diagnosis is extremely important. Biomarkers are used to separate healthy patients from diseased

ones, and several different biomarkers are often used together to generate profiles for cancer subtypes. There are a huge variety of biomarkers out there, from using proteins such as receptors/enzymes to nucleic acids like microRNAs which will be further explored in this thesis. Biomarkers can also be alterations like gene expressions, proteomics and metabolomic signatures and can clinically be detected in circulations (blood, plasma, serum) or excretions (stool, urine etc.) by biopsies or imaging for evaluations [42]. Biomarkers can be inherited and detected in the germ line DNA when isolated from blood, or somatically detected in DNA when taken from tumor tissues [42]. The primary reason for biomarkers is to assess patients by estimating their risk for the disease, differentiate benign from malignant tumors, and even differentiate between the different subtypes of said tumor to determine a good prognosis and prediction for the health of the patient. They are also used as a tool for monitoring, determining response to treatment and to keep up with recurrence if that were to happen. Some biomarkers are used just once, while others can serve multiple different functions [42].

1.3.2 Prognostic and Predictive Biomarkers

Prognostic biomarkers are those that help in identifying the likelihood of a patient suffering from cancer progressing or reoccurring later in life. They are independent of therapy, and often provide more insight into the disease itself. The strongest prognostic factors are those that deal with proliferation [47]. Thus, prognostic biomarkers like tumor size and tumor grade have been widely used in clinical settings to determine the stage of cancer. With some limitations, tumor grading won't always line up with how different pathologists estimate the grade and the lack of reproducibility is concerning due to the high heterogeneity of the tumor itself. Therefore, recently a push towards using molecular biomarkers have been taking a precedence. With new research, these molecular biomarkers have been validated and can now help as they are multi-parameter, multi-analyte, and multi-gene tests [10]. Some of these tests that are currently in clinical practice and recommended are tests like Oncotype DX, MammaPrint and uPA/PAI-1 [10][47]. In Norway, PAM50 from Nanostring is used [71]. These are too expensive for many countries and are not widely available. Therefore, cheaper and more accessible alternatives are used to test patients such as proliferation indices like Ki67, proliferating cell nuclear antigens, estrogen receptor (ER), progesterone receptor (PR), and markers that overexpress oncogenes [12]. Predictive biomarkers help with understanding how a patient would respond to treatment, and they are more important for medicinal development to treat patients with aggressive subtypes of cancer. Currently, for breast cancer, the biomarker ER is the best predictive marker available. Even though it has prognostic abilities, the predictive value of ER is more important in clinical application, and very much so for endocrine therapy [10]. PR is associated with ER and thus also serves as a good predictive biomarker for clinical application. The evaluation of both is highly recommended by experts to be done in newly diagnosed patients along with the HER2 marker as hormonal biomarkers often provide a good indication for whether hormone treatment would be beneficial for the patient [10]. A hunt for more efficient and effective novel biomarkers is underway as well as attempts to perfect techniques to use them properly.

1.3.3 The Mitotic Activity Index (MAI)

In breast cancer, tumor proliferation is often a key in tumor grading, and one of the most important independent prognostic factors that is looked at by pathologist during grading. The

most common method to determine proliferation is simply counting cells. Currently, the subjective nature of counting is still accepted in the world as long as good reproducibility can be achieved [4]. The mitotic activity index (MAI) is one of these methods that meet the proposed requirements [4]. It is scored on a light microscope where all clear mitotic figures are counted inside an active hotspot (with lots of mitotic activity) of a tumoral area under a 40x objective magnification per unit area of 1.59mm^2. When counting MAI, a clear distinction between true mitosis from similar-looking figures must be made. Apoptotic cells, dark nuclei, necrosis, infiltrating lymphocytes, tissue artifacts and the like are discarded when counting for this reason [4].

In order to make better decisions on treatment plans for patients with low-risk early breast cancer or high-risk patients that are given dose-intensive treatments, it is important to strengthen these guidelines and protocols to achieve high accuracy and reproducibility. Even though multiple studies have researched different ways to determine the prognostic or predictive values of certain genetic profiling techniques, there are currently no published guidelines that encompass a method that can be widely used for everything at once and for all types of cancers [49]. In the 2011 St Gallen guidelines, proliferation factor Ki67 was proposed to distinguish between the "luminal A" and "luminal B"-like subtypes of breast cancer but the method was too subjective and lacking in reproducibility even when done by many experienced pathologists, though it is still included in the Norwegian guidelines due to its potential for diagnostics [49]. Currently though, MAI is still the strongest prognostic marker for breast cancer available [7].

1.3.4 Tumor-infiltrating Lymphocytes (TILs)

The immune system plays a critical role in maintaining tissue homeostasis. Through balanced activation of the innate and adaptive immune cells that respond to inflammatory reactions, and the constant "immunosurveillance" that goes on in the background to eliminate any foreign bodies that enter or trigger transformations of normal cells, the immune system is the first and last barrier of defense [83]. Cancer cells cause neoplastic transformations of normal cells that start to change and alter the orderly structure already set in place. Under normal circumstances the immune response is initiated, and the altered cells are eliminated. However, cancer cells can develop the ability to escape this elimination, in what has been described as their propensity to undergo immunoediting [83]. One of the hallmarks of cancer previously explored in this thesis expands on how cancer cells have evolved the ability to actively evade the immune system to survive and proliferate further while simultaneously taking advantage of the tumor-promoting effects that are associated with the inflammatory state of infiltrating immune cells [44]. This concept has been observed and studied in several experiments, firstly in immunodeficient mice, and later in immunocompromised patients. When tumor cells manage to escape the immunosurveillance, the immune system has effectively failed in its responsibility to eliminate the threat, allowing the tumor to develop exponentially in a new microenvironment that benefits it greatly [44]. This ability of the cancer cells to undergo malignant progression is called "immunoediting" and it falls into three stages, elimination, equilibrium, and escape [83] [28]. During elimination, cancer cells are fully eliminated following immunosurveillance. During the equilibrium stage, the cancer cells are held in place by the immune system, and neither eliminates nor escapes. Lastly, the cancer cells transform enough to bypass the immune system

and enter the escape stage where the immune system can no longer hold them back. Most patients get diagnosed during the escape stage of immunoediting [28]. However, due to the complexity and relationship between immune cells and tumor cells, the prognostic value of quantifying and using these tumors infiltrating immune cells to determine patient's response to treatments has sparked more interest for further study [44][2].

Tumor-infiltrating lymphocytes, or TILs have recently gained traction as an immunological biomarker due to their strong prognostic and predictive value, particularly for TNBCs and HER2 positive breast cancers [83]. In recent studies, methodologies on how to evaluate TILs have been explored in several studies in hopes of achieving comparability between results and establish one standardized methodology that can be followed for future trials around the world [1] [83]. In 2013, several researchers and clinical teams gathered to come to a consensus on which parameters to consider important when evaluating TILs in breast cancer. This was done to help further integrate TILs as an evaluation method in future diagnostic practices, and even establish a "immunological grade" depending on individual patient's immune response to tumor cells [83]. For TNBC which currently only has chemotherapy as a treatment option, avenues such as using TILs to determine better treatment options for patients is highly desired. Increasing evidence links patients with disease-free and overall better survival rates in TNBC with having higher levels of TILs and less chance of recurrence as the immune system plays a more active role in this subtype of breast cancer [43]. This has opened up more interest in using targeted therapy for TNBC patients and using the immune system to achieve that [93][1][2].

1.3.5 Fibrotic Focus

Fibrotic focus (FF) is concentrated at the center of invasive ductal carcinomas and consists of fibroblasts and various other collagens [41]. It is observed under the microscope as a scar-like area where strong emphasis is drawn on the reactive tumor stroma formation. High fibrosis in TNBC patients is generally marked by an increase in collagen fibers and is linked to tumor progression [41]. The fibroblasts forming FF are regarded as more proliferative when present in IDCs than when they are absent. The presence of it has also been correlated with tumor size, higher histological grade, necrosis etc. among few other characteristics of poor prognosis. Since FF is linked with tumor progression, high tumor angiogenesis ratio and hypoxia within tumors where it is present, it has been proven to facilitate metastasis, and recently a link between FF and the tumor microenvironment has been discovered. The true reasoning behind why the presence of FF gives poor prognosis still remains unknown but some studies have tried to explore the mechanisms behind it and there are still much more to discover [7] [26]. Including FF in the standard assessment routine for microscopic tissue sections would not be a difficult process as it is easy to assess, reproduce and could provide additional prognostic value as a biomarker [7].

1.3.6 MicroRNAs

MicroRNAs have become a point of study in recent years for their value as prognostic/predictive biomarkers, especially for breast cancer subtypes like TNBC that lack the hormonal receptors ER, PR and HER2. Currently, there are no effective ways to determine treatment plans for patients diagnosed with TNBC, and the 5 years survival rate for the disease in Europe is 77% while 11% of the diagnosed patients experience distant metastasis [23]. More insight into the

genomic, molecular, and biological analysis of TNBC are being done to reduce this gap of knowledge and to further understand the complexities surrounding it [27][57].

MicroRNAs, by definition, are short non-coding RNA molecules that form around 19 - 22 nucleotides in length. The main task of these non-coding RNAs is to up/downregulate gene expression post-transcriptionally by either binding to the messenger RNA (mRNA) targets or cleaving them by binding to the 3'-untranslated region (UTR) of said mRNA [86] [33]. Functionally, they maintain balance in normal cells by regulating cellular growth, proliferation, differentiation and apoptosis [33]. One microRNA can influence several signaling pathways in mRNA, whether the processes are similar to one another or different, it does not matter. It is due to their heterogenous ways of interacting with mRNA that abnormal microRNAs contribute well to cancer cells. Their interactions with their targets are dynamic and depend on multiple factors, such as their location, how much microRNAs there are comparative to target mRNAs, and their affinity towards each other. These microRNAs can be secreted into extracellular fluids, and transported via exosomes, or by binding to proteins [75]. In breast cancer, the abnormal expression of microRNAs is recognized as either oncogenes or tumor-suppressors, aiding cancer cells in proliferation and metastasis [33]. Since they significantly contribute to cancer growth, their oncogenic properties make microRNAs valuable biomarkers to target as potential prognostic markers or novel therapeutic agents [33]. Among the oncogenic microRNAs, the polycistronic miR-17-92 cluster has been found to enhance cell proliferation, trigger angiogenesis, block differentiation, and promote metastasis. In this cluster, microRNAs 18a and 18b are worth taking note of to explore further as potential biomarkers, and more on them will be discussed in the next section [86]. MicroRNAs have also been found to interact with immune cells and form cell-to-cell signaling and communication between tumor cells and their microenvironments. Thus, they play a critical role in how the tumor microenvironment influences cancer cells during the initial growth to full progression [31].

MicroRNA biogenesis occurs in several steps firstly starting with the transcription of long single stranded RNAs (ssRNAs) in the nucleus by RNA polymerase II. These are known as primary microRNAs or pri-microRNAs and they contain one or more hairpin-like structures that are only unique to them, making them recognizable targets for enzymes [47] [75]. Due to this, they can be cleaved by a complex which consists of an RNAse III enzyme (Drosha), an RNA-binding protein (DGCR8) and other accessory factors. Once the hairpin-like structure is shortened, the resulting RNA is termed the "precursor-microRNA" or "pre-miRNA" which is around 70-100 bp. Some microRNAs are theorized to be within introns, hence why they share the same regulatory elements as their targets [47]. Therefore, some can result from the splicing and debranching of introns from a pre-mRNA without having to be cleaved by Drosha and DGCR8. Pre-microRNAs are exported to the cytoplasm by the export protein Exportin 5 along with the protein guanine triphosphatase RAN, before undergoing cleavage by RNAse III enzyme complex Dicer, and fully maturing into microRNAs that are approximately 19 - 22 nucleotides in length [47][57].

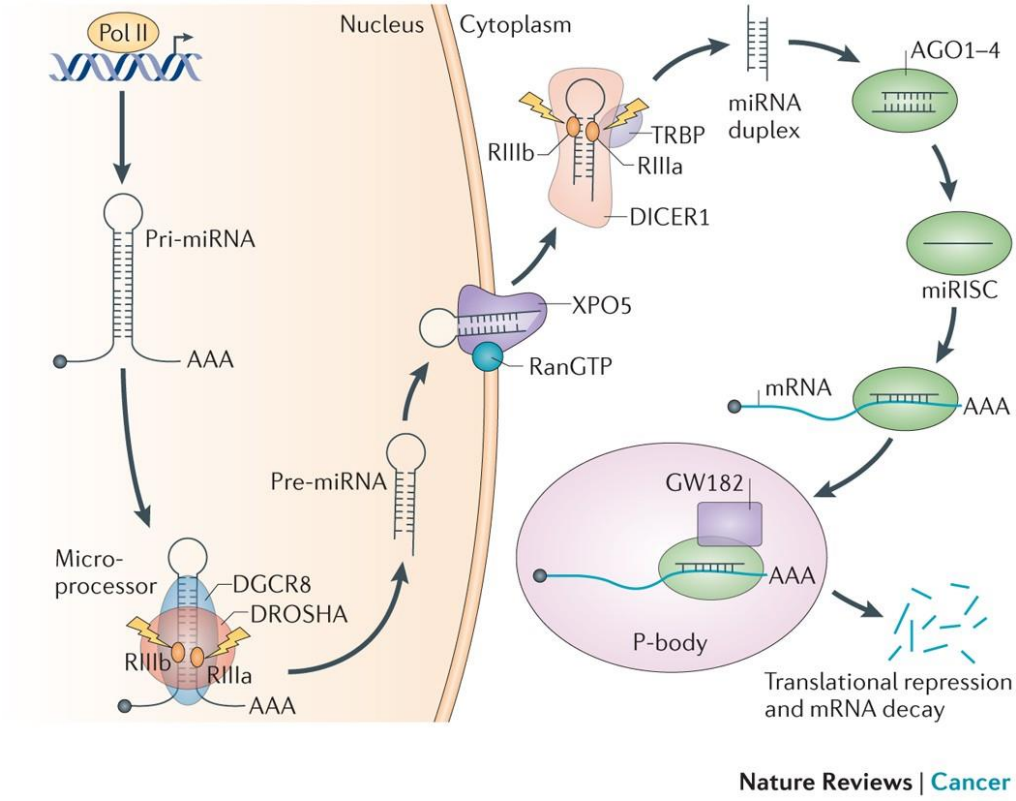

Figure 2. MicroRNA biogenesis.

1.3.7 miRNAs 18a and 18b

MicroRNAs have shown to either upregulate oncogenes or stimulate tumor suppressors [9]. In this thesis project, oncogenic microRNA's 18a and 18b were chosen for analysis as several studies have already been done on them, and further understanding is still needed [86]. The gene expressions regulated by these microRNAs' correlate strongly with high proliferation of tumor cells [29]. Located on chromosome 13, MiR-18a is found in cluster miR-17 ~ 92 while MiR-18b is found in cluster miR-106a ~ 363 on chromosome X. The microRNAs in these clusters are associated with the regulation of certain processes that ensure orderly and efficient biological activities to occur. It is when these processes become dysregulated that cancer can start to form. Especially if the microRNAs start to facilitate cell proliferation, epithelial-mesenchymal transition (EMT) and cancer metastasis due to errors in their regulated pathways. Recent discovery has shown that circulating microRNAs can be contained in secretory vesicles and be stably detected in human plasma and serum. This makes oncogenic microRNAs like 18a and 18b valuable biomarkers as they can be used to test for tumor progression or development in patients if approved [86].

1.4 Clinical Treatments for Cancer

The heterogenous nature of this disease requires correspondingly several ways to deal with. The type of treatment a patient receives depends on the cancer itself, on how far or little it has advanced in the body, and especially the patient. It is not uncommon that a patient receives multiple different types of treatment for just one cancer, as a patient can undergo chemotherapy

coupled with radiation therapy to eliminate tumor cells [62]. This is not often good, as treatments will take their toll on the body, and the more treatments a patient endures, the tougher and tougher recovery gets. So, deciding on the right treatments from the beginning is most beneficial for the patient. In order to determine the right type of treatments though, effective biomarkers must be used to figure out more information about the tumor. This method is often called tumor testing, profiling or simply tumor genetic testing as each cancer has its unique pattern that can be determined and further diagnosed/identified. Some treatments work for patients with only specific biomarkers present [62]. Some treatments will not work at all. It all depends on how much information can be withdrawn from the tumors with the available markers and technology present.

1.4.1 Chemotherapy

The most known treatment for cancer is chemotherapy. This method of cancer treatment uses drugs to kill the fast-growing cancer cells. It is effective in stopping, slowing down the growth, or even curing cancer sometimes. Chemotherapy has the effect of decreasing the chance of reoccurrence and can also be used to shrink tumors that have grown either too big and/or are causing problems and pain to the patient [64]. There are many different cancer types that are treated by chemotherapy. In fact, for some cancers like triple-negative breast cancer, only chemotherapy is available as a standard treatment. Usually however, it is used alongside other treatments to increase their effectiveness. When it is used before surgery, it is known as neoadjuvant chemotherapy. When done after, it is referred to as adjuvant chemotherapy [64].

Even though the main point of chemotherapy is to kill cancer cells that are growing fast and dividing too quickly, it also comes with side effects including termination of healthy growing normal cells as well. Cells such as those that line the mouth, intestine, and those that help with hair growth are usually affected, which is why sores, hair loss and baldness are common side effects for those undergoing chemotherapy. It also often leads to extreme fatigue as the body is going through a tough process, essentially recovering from being poisoned [62]. The amount of chemotherapy given differs from patient to patient. How often one receives chemotherapy, which type of chemotherapy, and what dose is all related to the different cancer types, the cancer characteristics, and the patient health profiles as well where in the world they live because chemotherapy is not readily available in some countries around the world [62]. It can be received either orally by swallowing pills/capsules, get it injected directly into the body, or even applied as a cream on the skin sometimes [62].

1.4.2 Hormone Therapy

Hormone therapy is a cancer treatment that slows down the growth of cancer cells that depend on hormones to grow. This type of treatment is often used to treat cancers such as prostate cancer and various types of breast cancers. Just like chemotherapy, hormone therapy helps lessen the chance of reoccurrence or completely stops the growth of a tumor all together as the hormones it depends on are the main targets. Hormone therapy is also used to ease symptoms for patient that cannot undergo any surgery or radiation therapy [65]. There are several ways hormone therapy works; they either block the body’s ability to produce the hormones the cancer depends on, interfere with how the hormones behave in the body or bind to the hormone receptors in order to

eliminate the tumor cells. Like chemotherapy, it is often coupled with other treatments, which again depend on the patient's needs and the tumor characteristics. It can be adjuvant treatment to lower the risk of reoccurrence or be used before a surgery to reduce the size of the tumor etc. [65].

The side effects that come with hormone therapy depend on which hormones in the body are affected. Patients will respond differently to hormone therapy even if they undergo the exact same procedure. Hormone therapy side effects also differ depending on whether the patient is male or female, but the most common side effects for men with prostate cancer undergoing hormone treatment is hot flashes, loss of interest in sex, nausea and fatigue and for women with breast cancer, their side effects are almost the same with few differences such as immediately entering menopause once the treatment is started if they were previously menstruating [65]. Much like chemotherapy, the treatment duration, doses and cost depend entirely on the tumor characteristics, location and how severe the cancer is. To see if hormone therapy is working, for prostate cancer in men, often regular PSA tests are performed. If the PSA levels go down, it is a sign of the treatment working. For women with breast cancer, regular check-ups are performed, usually including examinations of the neck, underarm, chest and the breasts. Also, regular mammograms are performed along with other lab and imaging tests to monitor treatment results and record follow-ups in case of reoccurrence [65].

1.4.3 Immunotherapy

Immunotherapy is simply just assistance provided to the immune system, and helping it fight against the cancer cells more effectively. The immune system is already designed to fight against infections and diseases that enter the human body by itself and is made up of white blood cells that function as defense as well as the lymph system that is woven across the whole body partaking in its role to keep the host healthy. Cancer cells often learn to evade or manipulate the immune system to continue growing and proliferating as discussed previously in the section on the hallmarks of cancer. The main function of the immune system is to detect and destroy abnormal cells. When the immune system is responding to a tumor, certain immune cells can be detected around it or within it. These are the tumor-infiltrating lymphocytes, or TILs, which has already been explained previously in this thesis. Patients who express more TILs around their tumors often fair better than those who don not as it means that their immune system is coming to their aid [66].

Immunotherapy is an umbrella term for the multiple different types of ways cancer can be treated using the immune system. Among them are the use of immune checkpoint inhibitors, which are drugs that block the immune checkpoints. The immune system is generally kept in check to avoid strong reaction to everything that is abnormal. However, for treatment of cancer, by blocking the checkpoint inhibitors, the immune system can strongly respond to the cancer cells and hopefully prevent their spread. Other types of therapy include T-cell transfer where the natural ability of the T-cells is boosted to fight cancer. T-cells that have shown strong reactions towards the tumor are selected and grown in a lab to be put back into the body. Thus, an attack on the tumor can commence as the T-cells have an affirmed target. Monoclonal antibodies are another way to treat cancer as they are proteins created in labs designed to bind to targeted

cancer cells. Some are merely used to mark the tumor as well, so it can visually be seen easier on image analysis, while some are used for therapeutic reasons [66].

Currently, immunotherapy is not as widely available or used as the other treatments, but in recent years, its effectiveness has become more prevalent, and more research and funding is going into improving and finding better ways to implement immunotherapy as an option for cancer patients. Researchers are especially focusing on finding better ways to fight cancers that are resistant to other therapies by improving immunotherapy for those that truly benefit from it [66].

1.4.4 Radiation Therapy

Radiation therapy, or radiotherapy is another common cancer treatment alongside chemotherapy that most people have heard of. It generally uses high doses of radiation to either kill the cancer cells or shrink the tumor. Radiation therapy is not as simple as expecting the cancer cells to die after just one dose of radiation. It generally takes several high doses for the treatment to inflict enough damage to the DNA of the cancer cells so that they can stop dividing and start to die. Undergoing radiotherapy often depends on the patient and their cancer type, and there are two ways it can be done. A patient can receive an external beam of radiation which comes from a machine directly aimed at the tumor/malignant area of the body. This type of treatment is localized and targets only the spot where the tumor is located, not the entire body. On the other hand, radiotherapy can also be done as internal radiation therapy, and with this method, the patients take the radiation inside their bodies instead of receiving it as a beam. When the intake is with solids like pills, it is referred to as brachytherapy and the pill is placed near the tumor. Just like external radiation, it will remain localized and set of radiation for a while in a specific spot to eliminate or shrink the surrounding cancer cells. When the intake is with liquids, it is called systemic therapy and this type of treatment is usually either swallowed or injected through a vein using IV. The radiation then travels via the blood stream to the cancer cells and kills them [68].

Radiation therapy is both used as palliative care by easing symptoms of patients with terminal cancer, and as an active method for treating the disease as it can also cure by stopping the growth of cancer cells or slow them down, and sometimes even prevent reoccurrence later in life. Often, radiotherapy is tagged along with other treatments such as surgery, chemotherapy, and immunotherapy, but it can stand alone on its own. It is especially paired well with surgery to either shrink the tumor before removal or kill any remaining cells that stick around after surgery. However, radiation therapy is not an easy treatment and patients that choose to go through with it are on strict diets as the side effects of radiotherapy include nausea, fatigue and many more. The therapy can also kill nearby healthy cells, so constant monitoring and consistent follow-ups are extremely necessary [68].

1.4.5 Surgery

Surgery is simply the physical removal of the cancer from a patient’s body. Surgeries are not easy and will always require a period of healing afterwards. The process often requires the patients to be cut through skin, muscles and sometimes even the bone. Patients are put under anesthesia, locally numbing the area where the removal takes place as well as waiting until the patient enters deep sleep. There are several different types of surgeries that do not involve cutting into the person, among them laser therapy, photodynamic therapy etc. However, the most

familiar form of surgery is the one in which the tumor is physically removed by a surgeon. In open surgeries, a large cut is made to remove the tumor, along with some collateral healthy tissue and lymph nodes. This is to remain safe and prevent any residual cancer cells to be left behind. In minimal invasive surgery, a few small cuts are made and with the assistance of a long, thin tube with a camera attached inserted into the body, the cancer is carefully removed slowly. With this type of surgery, the healing process is easier to recover from [69].

Obviously, not all types of cancers can be treated with surgery. The cancer must be a solid tumor that is localized and not able to metastasize yet for surgery to be an effective treatment. For some people, this is all the treatment they need. For others, they undergo a surgery in which their tumor is debulked, because removing the entire thing could damage an organ. This sort of treatment is also used to ease pain and alleviate the pressure some tumors can have on certain areas of the body where they are located. With surgery, aftercare is important as pain during recovery must be tended to and infections that can potentially occur around the sore area must be properly taken care of before they worsen [69].

1.4.6 Targeted Therapy

With the new age of research, targeted therapy has presented itself as a good option for some patients. It is a type of cancer treatment that specifically targets the proteins that control cancer cell growth, proliferation and spread for the cancer it is supposed to treat. This is the beginning of precision medicine, where each treatment is tailor-fit for the patient's specific needs. The more information is uncovered for how DNA works and how cancer changes and modifies, the better designed these treatments will be [70]. Targeted therapy comes in two forms, either as small-molecule drugs, or as previously mentioned in immunotherapy, as monoclonal antibodies. The small-molecule drugs are tiny enough to enter the cells easily and can be used to directly infiltrate cancer cells. Monoclonal antibodies, however, can be produced in labs and be designed to specifically target cancer cells by attaching themselves to the receptors of specific types of cancers. Monoclonal antibodies are even used to mark cancer cells so that they can be seen and destroyed by the immune system or interfere with them so that they self-destruct as well [70].

The main issue with targeted therapy depends on whether a patient's cancer possesses the targeted signs for the drugs to take effect. Most times, patients have to get tested beforehand to see if these signs are present and if their tumor will respond correctly to the therapy provided. This is done using biomarkers by performing a biopsy starting with the removal of a piece of the tumor to directly test on it [70]. If this is successful, targeted therapy is initiated and can help the immune system defend itself by aiding in marking cancer cells, stopping them from proliferating/spreading by interfering with the proteins on the cell's surfaces and stop signals that cause angiogenesis with the use of inhibitors to stop the cancer's resource supply. Targeted therapy can directly kill the cancer cells by aiding chemotherapy/radiation drugs by guiding the medication to the location, cause apoptosis when possible, or starve the tumor of the hormones it needs to grow [70]. However, even with precise attacks, cancer cells have shown their capacity to quickly adapt and grow beyond these attempts to eliminate them, and therefore have shown resistance to targeted therapy. Another downfall to this treatment is also the cost and difficulty of developing ongoing targets that can counteract the adaptability of cancer cells perfectly for

several different patients and cancer subtypes. And just like with the other types of treatments, the side effects can be daunting to deal with too, and unfortunately, it's currently not readily available to patients [70]. Targeted therapy still holds a lot of potential to eliminate and cure cancer in the future, and research into this area is still ongoing with strong efforts today.

2 Background

2.1 Breast Cancer

2.1.1 Introduction

Breast cancer is amongst the deadliest of diseases in both developed and non-developed countries worldwide, and it is also the most common type of malignancy found in women. Globally, breast cancer cases have risen and overtaken lung cancer as the number one cause of death for the first time in 2020 and it has become the leading cause of death for both women in third-world countries and the second leading cause of death for women in America [13]. In Norway, one in ten women will develop breast cancer, and in recent years, this number has increased [80].

Breast cancer starts out in the breasts, which are made up of very fatty and dense tissues. Within these tissues lay lobes made up of thin, tube-like structures called lobules that contain the milk glands. They form a network around the tissues connecting to one another carrying the milk from the milk glands to the nipple along with the mesh of blood and lymph vessels that run alongside them [16]. Cancerous growth happens when normal cells begin to grow out of control and form a tumor mass. If this does not metastasize to further distances from the origin, it is defined as an *in-situ carcinoma* [16]. Breast cancers are characterized very differently depending on what histologic subtype they are. They can go from rapid growing tumors with potential for early metastasis which on average are 25-30% of the aggressive cases, to slow growing tumors with the same risks, just without the metastasis [80].

2.1.2 Anatomy of the Breasts

A healthy female breast consists of three types of tissues, adipose (fatty), glandular, and fibrous tissue [58]. The function of the breasts lies in their ability to lactate, in other words produce milk for breast feeding. Therefore, in between the fatty tissues that determine the size of the breasts and the fibrous connective tissues that provide support, glandular tissues that form the lobes and ducts necessary for milk production are embedded [58][60]. Each breast has approximately 12 to 20 glands, or lobes, in which each of the lobes contain smaller lobules or sacs that produce milk [60]. The milk is then carried to the nipple of the breast (the areola) through thin tubes referred to as ducts. The most common sites for cancer to start growing are in the lobes and ducts of the breasts [58][60]. Women with denser breast tissue are also at a higher risk for developing breast cancer due to the presence of more breast cells that can potentially turn cancerous. This type of development tends to happen more in younger women as the older the body gets the more glandular tissue decreases resulting in less denser breasts [72].

Aside from the tissues, the breasts are held in place by the pectoralis muscle which sits atop the ribcage for support. An important breast tissue called the "axillary tail of Spence" extends from

the breast all the way into the underarm area [76]. This tissue is particularly important because even though it does not seem to be located within the actual breast, it is still breast tissue and one in which cancer can still take root [76]. The skin that covers the breasts is the same as the rest of the body and contains the same sweat glands, hair follicles etc. [76]. Majority of the breast structure is identical to that in males, apart from the absence of the milk-producing lobules that are only present in the female breasts for breast feeding [76]. The breasts get their nutrients such as oxygen primarily from the internal mammary artery which runs underneath the main breast tissue. The breasts are also connected to the lymphatic system which flows in the opposite direction to that of the blood supply and drains the vessels into the lymph nodes [76]. The spread of breast cancer usually happens through infiltrating the lymphatic vessels to the lymph nodes, especially draining into the first node, the "sentinel lymph node". Knowledge of this drainage has led to surgeons removing the sentinel lymph node first to check for metastasis in patients [76].

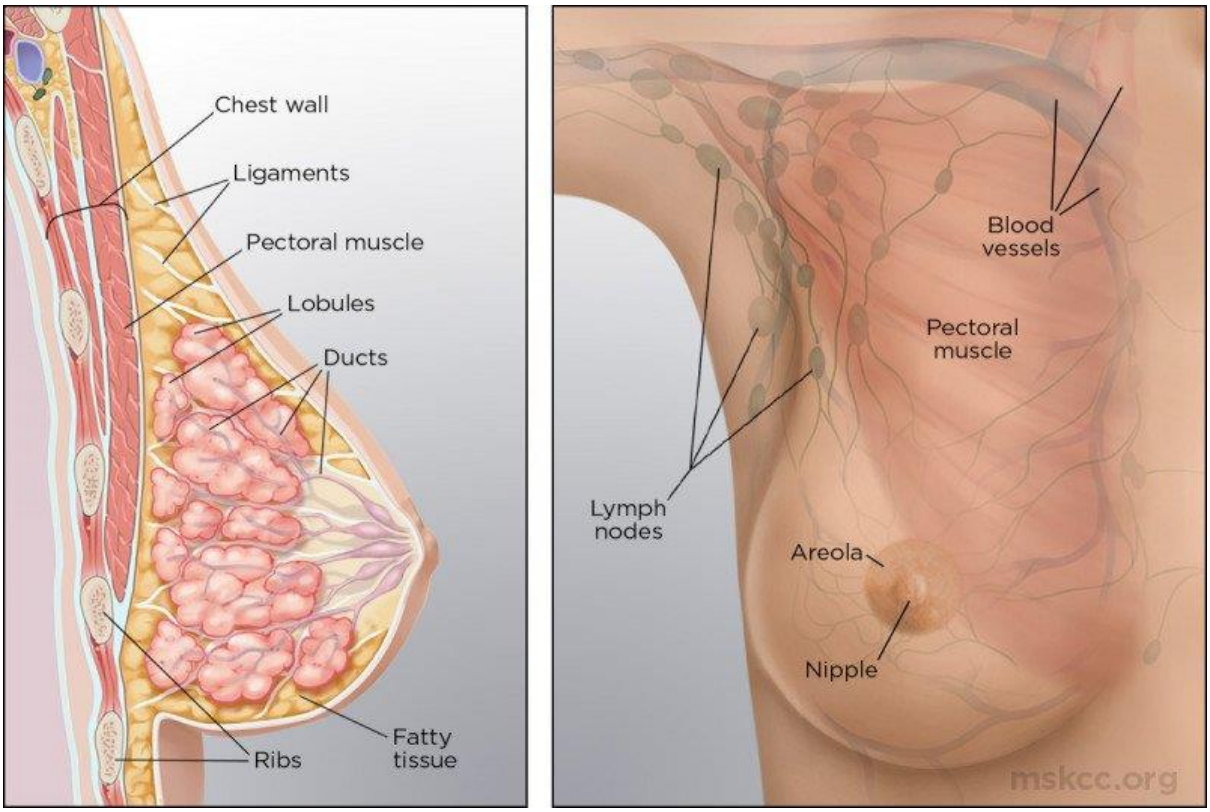

Figure 3. Anatomy of the breast [58].

2.1.3 Aetiology and Epidemiology of Breast Cancer

Breast cancer is a heterogenous disease, and multiple factors are involved for it to even occur [59]. The causes (aetiology) of breast cancer are not all known which makes it difficult to diagnose from one woman to the next. This difficulty of causation can also be seen by how the various breast cancer incidences across the world are not dependent upon the same factors to

occur or progress. Still, there are well known risk factors out there that are known to cause breast cancer that can be considered when diagnosing a patient. One major factor for causation in breast cancer is age. About 8 out of the 10 cases happen to women over the age of 50 which is the reason for why it is recommended for women between the ages of 50 to 70 to get a mammography screenings every 3 year to remain safe [72]. Family history plays a large role in whether a woman develops breast cancer in her lifetime or not. Close relatives with ovarian cancer can often indicate a higher risk for developing breast cancer in some women due to breast cancers being tied to genetics. Most cases are non-hereditary though, but the genes BRCA1 and BRCA2 greatly increase the risk of potentially developing breast cancer and are possible to be passed down from parent to child [63]. Normally, they produce proteins and are involved in DNA repair. It is the harmful variants of these genes (due to mutations or somatic alterations) that most commonly cause breast or ovarian cancer when inherited. Other genes associated with breast cancer are the TP53 and CHEK2 genes. For women with these risk factors in their family lineage, often a predictive genetics test is suggested to see if they've inherited any of these cancer-risk genes [72].

Another important causation of breast cancer are the female hormones and the frequent usage of hormonal medicine. Prolonged exposure to oestrogen can potentially cause breast cancer to form by stimulating the breast cancer cells and aid them in rapid growth. Therefore, risks for developing breast cancer may increase when overexposure to oestrogen occurs during early puberty. Interestingly, giving birth to kids decreases the chances of developing breast cancer because it interrupts the natural exposure women get to oestrogen when they have their monthly periods. So, women who decide to have children later in life increase their risk of developing breast cancer as no interruptions happen to their exposure to oestrogen during their youth. Hormone replacement therapy (HRT) increases the risk for breast cancer in woman who use them compared to women who don't, especially if used for more than 1 year. It is controversial, but some people believe pills such as contraceptives also increase risks for breast cancer, but that the risk can be decreased if the usage stops, and that within 10 years the risk levels would be back to normal [72]. Lastly, lifestyle factors can affect the risk to develop breast cancer. Obesity in women after they've entered menopause heightens their risk for breast cancer as the amount of oestrogen produced is linked with obesity. For those who drink alcohol, the risks are much higher than those who do not drink at all. [72].

Since breast cancer is a multifactorial disease, incidences, mortality rates and survival rates vary considerably across the world [55]. Therefore, narrowing down a method to determine and detect breast cancer for all is an extremely difficult task. Screening for breast cancer has been a primary way to catch the disease in its early stages, but it comes with its disadvantages in causing side-effects, over-diagnosing patients and the increased costs of improving the screenings are tough to implement for many countries [55]. Therefore, in order to combat this disease and reduce its risks and occurrences, classifying women based on their risk factors for breast cancer can be an effective way to narrow down the scope and improve risk-free methods of detection and design a more targeted breast cancer screening program in the future [55][22].

2.2 Types of Breast Cancer

2.2.1 Non-invasive Breast Cancers

The first categorial split for the differences between the types of breast cancer depends on whether the cancer is considered non-invasive or invasive. A non-invasive breast cancer stays within the milk ducts of the lobules in the breasts and do not grow outside of them into tissues within or beyond the area [14]. A tumor that remains localized in one place is often called a "carcinoma-in-situ", or pre-cancer, because it could eventually start to develop more invasive properties and start metastasis. There are only two types of cancers that fall under the non-invasive breast cancer umbrella, the most common type being ductal carcinoma-in-situ (DCIS), and the other one lobular carcinoma in situ (LCIS) [11]. DCIS starts in the milk ducts and does not spread further. The risk of it is not life-threatening, but there is a high chance for it to develop into an invasive cancer later on in the future. For LCIS, it starts in the lobules where the milk producing glands are located at the end of each breast duct and will also not spread beyond its origin. However, it carries the same risks as DCIS for the potential to develop into a more invasive version of itself later on in life for patients diagnosed with it.

2.2.2 Invasive Breast Cancers

Opposing the non-invasive breast cancers are the invasive types. As expected, this cancer is described as one that will spread outside the ducts and lobules into the surrounding breast tissue and continue onwards to other parts of the body beyond the breasts [11]. The most common type is the invasive ductal carcinoma (IDC) which around 80% of all breast cancers are diagnosed to be [11]. When detected at an early stage the cancerous cells, some of which have already started to spread to nearby lymph nodes and the armpit (axilla), they are still much present within the breasts and cannot yet be found distantly elsewhere in the body. However, in invasive lobular carcinoma (ILC), the second most common type after IDC, the cancer starts inside the milk-producing lobules and over time starts to break through the lining of the lobules into the surrounding breast tissue, spreading to the rest of the body through the lymph node system [11].

Among the rare invasive breast cancers are the Paget's disease of the nipple, where the cancer cells start to grow in the nipple or its surrounding area, the areola. In this type, the nipple becomes red, itchy, and irritated and this often becomes the first signs for the presence of breast cancer in the patient. It is not unusual that this disease also comes with the development of either DCIS or invasive breast cancer somewhere in the breast later on [11]. Another rare type of breast cancer is the inflammatory breast cancer which is a very aggressive form that attacks the blood vessels inside the skin and/or the lymphatic vessels of the breast, inflaming the breasts and changing their appearance to red. This form of breast cancer is very rare and not much information on it is available [11]. The Phyllodes tumors are also rare and although most of them are benign, they tend to grow quickly but do not spread outside the breasts, instead developing solely inside the breast's connective tissue or the stroma [11].

2.2.3 Subtypes of Breast Cancer

There are three main subtypes of breast cancer, hormone receptor positive breast cancer, HER2-positive breast cancer and triple negative breast cancer. In general, about 2/3 of all breast cancers are hormone receptor positive, meaning that for the cancer cells to grow and spread, the presence of the female hormone oestrogen and/or progesterone are needed. This dependency of the cancer cells on these hormones makes it easier to treat the disease by using hormone-blocking therapy such as tamoxifen and anastrozole instead of immediately recommending chemotherapy [11]. Hormone therapy can be recognized as targeted therapy due to the clear interference the drugs do to the growth of cancer cells that depend on solely on the blocked hormones to grow. For the HER2-positive breast cancers, they are treated with Herceptin, a drug that is designed to target these types of cancers and it has been proven to be quite an effective method. Most people are treated with this drug for 12 months if the cancer is caught early on along with other treatments such as surgery, chemotherapy or radiotherapy as follow-ups if necessary. For most hormone-receptive cancers, hormone blocking therapy is often the first treatment option [11]. The last subtype of breast cancer is triple negative breast cancer (TNBC) which will be further discussed in the next section. TNBC does not have any of the receptors usually found in the other types of breast cancers. Thus, it shows negative for oestrogen, progesterone and HER2. Unfortunately, 15% of all cases of breast cancers are TNBC and without a possibility to treat this form of the disease, only chemotherapy is recommended for patient diagnosed with TNBC [11].

2.2.4 Triple Negative Breast Cancer

Triple negative breast cancer, or TNBC, is the most aggressive subtype of breast cancer and around 10-20% of all breast cancers turn out to be TNBC for Caucasians, while the risk increases three-fold for those of African ancestry up to 40-60% [15] [87][51]. As mentioned before, it can be recognized by the lack of expression of ER, PR or HER2. It is also classified as being part of the subtype basal-like breast cancers (BLBC) due to matching the genetic expression profiling for BLBC almost to 56% [93]. To date, it remains one of the most challenging subtypes of breast cancer to deal with, and multiple therapies targeting it molecularly have rarely turned out good. Chemotherapy is the only standard option available so far to treat this disease, and more efforts are being placed in finding a better way to treat it [36]. With how fast it grows and spreads, and the limited time and treatment options available, it is without doubt that TNBC has the worst prognosis of all breast cancers [18].

The symptoms and signs of TNBC are not different from how other breast cancer symptoms usually develop. Generally, this includes finding a new lump or mass on the breast (this does not always mean it is cancer though). If the lump is hard, has an irregular shape, and remains painless, it is likely to be cancer, but for some breast cancers, malignancy can also rise from soft, round, and painful lumps. Therefore, it is important for women to be aware of how their breasts look and feel normally, and immediately go in for a checking if they discover any irregularities or changes that come unexpectedly [18]. Women who are younger than 50 are especially at risk for TNBC in contrast to how old age is considered the risk factor for the other types of breast cancers due to TNBC being linked with inheritance of cancer-risk genes. It is also more likely to occur in Black or Hispanic women, and those with the BCRA1 mutation as mentioned before [15].

Due to the lack of hormone expression, TNBC has fewer options, if any besides chemotherapy, than any other type of invasive breast cancer. An increasing interest in finding medications that can interfere with the processes of TNBC and how it spreads has taken root in many research labs and collaborations, and the fight to find a treatment method is underway. Still, TNBC does not respond to hormone therapy, and it is often scary for patient diagnosed with this aggressive subtype to discover that their options are limited only to chemotherapy [18][15]. If the cancer cells haven't metastasized yet, surgery can become another option, but chemotherapy might still be used to shrink the tumor before removal. Another round of chemotherapy is also often recommended to reduce the chances of reoccurrence after surgery. Depending heavily on the tumor shape, size, how the surgery went, radiation therapy might also be considered as an additional option [18].

Recently, molecular targeting therapies have been surfacing for use in the clinical setting for TNBC patients. The news is encouraging because it provides more options than just cytotoxic chemotherapy. The new therapies include PARP inhibitors olaparib and talazoparib for germline BRCA mutations "associated breast cancer (gBRCAm-BC) and most recently the checkpoint inhibitor, atezolizumab in combination with nab-paclitaxel for programmed death-ligand 1 (PD-L1+) advanced TNBC" [55][51]. More biomarkers are being looked into such as miR-18a and miR-18b from the microRNA cluster miR-17-92, which are more explored in this thesis. Antibody-drugs that were put away due to cytotoxic worries during development are being re-examined, and tumor-sequencing to identify more molecular targets for treatment options are in development. For now, TNBC patients are all put under the same umbrella with the same treatment even though each patient is different, come from different backgrounds and have vastly different health profiles. Hopefully, in the near future, TNBC might not be referred to as just TNBC, but identified more clearly by its unique characteristics and have a tailor-made treatment ready for patients diagnosed with this aggressive subtype [55].

2.3 Histological Grade, Stage and Pathology of BC

2.3.1 The Nottingham Histological Grading System

To understand how aggressive a tumor is, a "grade" is assigned to it depending on certain characteristics. The histological grading used for breast cancer is based on the Nottingham grading system, also known as the Elston-Ellis modification of the Scarff-Bloom-Richardson system. This grading system provides a way to define the aggressive potential a tumor has and is a strong prognostic factor for evaluation of breast cancer [29]. To define these grades, the Nottingham histological grading system quantifies the morphological features of the tumors by its mitotic count (0-7, 8-15, >16), degree of tubular formation (>75%, 10%-75%, <10%), and nuclear pleomorphism (uniform, moderate, high) [29]. When combined, a score of one, two or three is given to the tumor, with a higher grade being associated with a worse prognosis and a lower grade with a better prognosis. Therefore, the higher the grade of a tumor, the more dedifferentiated the cancer cells will be and the more unrecognizable the cells become [29]. The grading system is very important as it is the best way for doctors to decide on treatment options for patients [37]. Below is a figure showing how the different stages of breast cancer looks using

the Nottingham grading scale. TNBC, which is the more aggressive subtype of breast cancer is often given a score of 3.

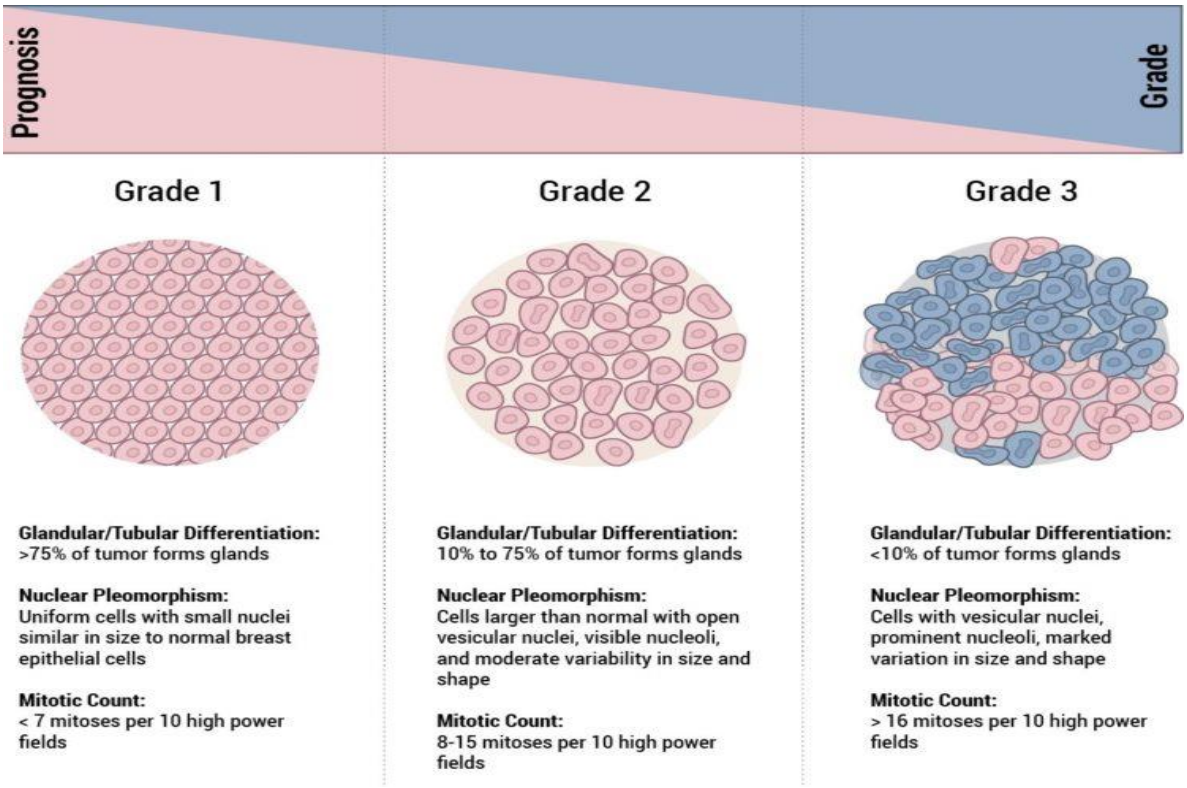

Figure 4. The Nottingham Histological Grading System for Breast Cancer [77].

2.3.2 TNM Classification

To determine how much breast cancer tumors have grown within the breasts or spread to distant parts of the body, a different kind of prognostic tool is used. Primarily for solid tumors, the pathological staging of breast cancer is done using the TNM staging system. TNM stands for Tumor, Node and Metastasis and is based on the size of the tumor, how much the cancerous cells have infiltrated the regional lymph nodes and whether the cancer cells have yet metastasized or not [29][77]. There are four recognized stages of TNM, from stage I being the least advanced tumor, to stage IV being considered terminal. Cancers found in the lower stages have a much better prognosis and survival rate than cancers who have progressed to stage IV [77][19]. Below in Figure 5 the different stages are shown. Even though this classification system is good for prognosis, a more accurate and individualized system is desired that can classify the different subtypes of breast cancer on a molecular level. Several challenges must be overcome to achieve this so until then, this system will do.

		Stage	Primary tumour (T)*	Regional lymph node status (L)	Distant metastasis (M)
T- Tumour		**0**	Tis	N0	M0
T1	Tumour ≤ 2 cm	**I**	T1	N0	M0
T2	Tumour ≥ 2 cm but ≥ 5 cm		T0	N1	M0
T3	Tumour ≥ 5 cm	**IIA**	T1	N1	M0
T4	Tumour of any size with direct extension to chest wall or skin		T2	N0	M0
N- Lymph node		**IIB**	T2	N1	M0
N0	No cancer in regional node		T3	N0	M0
N1	Regional movable metastasis	**III A**	T0	N2	M0
N2	Non-movable regional metastases		T1	N2	M0
N3	Cancer in the internal mammary lymph nodes		T2	N2	M0
M- Metastasis			T3	N1/N2	M0
M0	No distant metastases	**III B**	T4	Any N	M0
M1	Distant metastases	**III C**	Any T	N3	M0
		IV	Any T	Any N	M1

Criteria for staging breast tumours according to the UICC ICD-10 TNM classification.

*Size measurements are for the tumour's greatest dimension.

Figure 5. TNM Classification [54].

2.3.3 Histopathology of Breast Cancer

Breast cancer cells can grow in any of the mammary glands and display a wide variety of morphological, immunohistochemical and histopathological features, and often, pathologists rely on these features to divide the cancerous tissues into specific subtypes that require different clinical treatments and options [56]. Most breast cancer tissues diagnosed are carcinomas of the malignant nature. Among the malignancies, about 95% are adenocarcinomas, in which the invasive ductal carcinoma (IDC) is the most common form [56]. Primarily, breast carcinomas are determined by their histological image as that determines the growth pattern of the tumors. Pathologists have since identified several specific morphological and cytological patterns that correspond to the various subtypes of breast cancers mentioned before. These patterns are referred to as histological types, and in the World Health Organization (WHO), about 17 distinct histological special types of breast cancer are recognized that amount to 25% of all breast cancers [91]. Those with special features are called invasive carcinomas of special type, and the rest are classified as no-special type. The main benefit and clinical relevance behind these special type tumors lie in their better prognostic value compared to the no-special types [27]. Below in Figure 6, some of these special types of breast cancers that are oestrogen positive can be seen, and those that are oestrogen negative in Figure 7 [91].

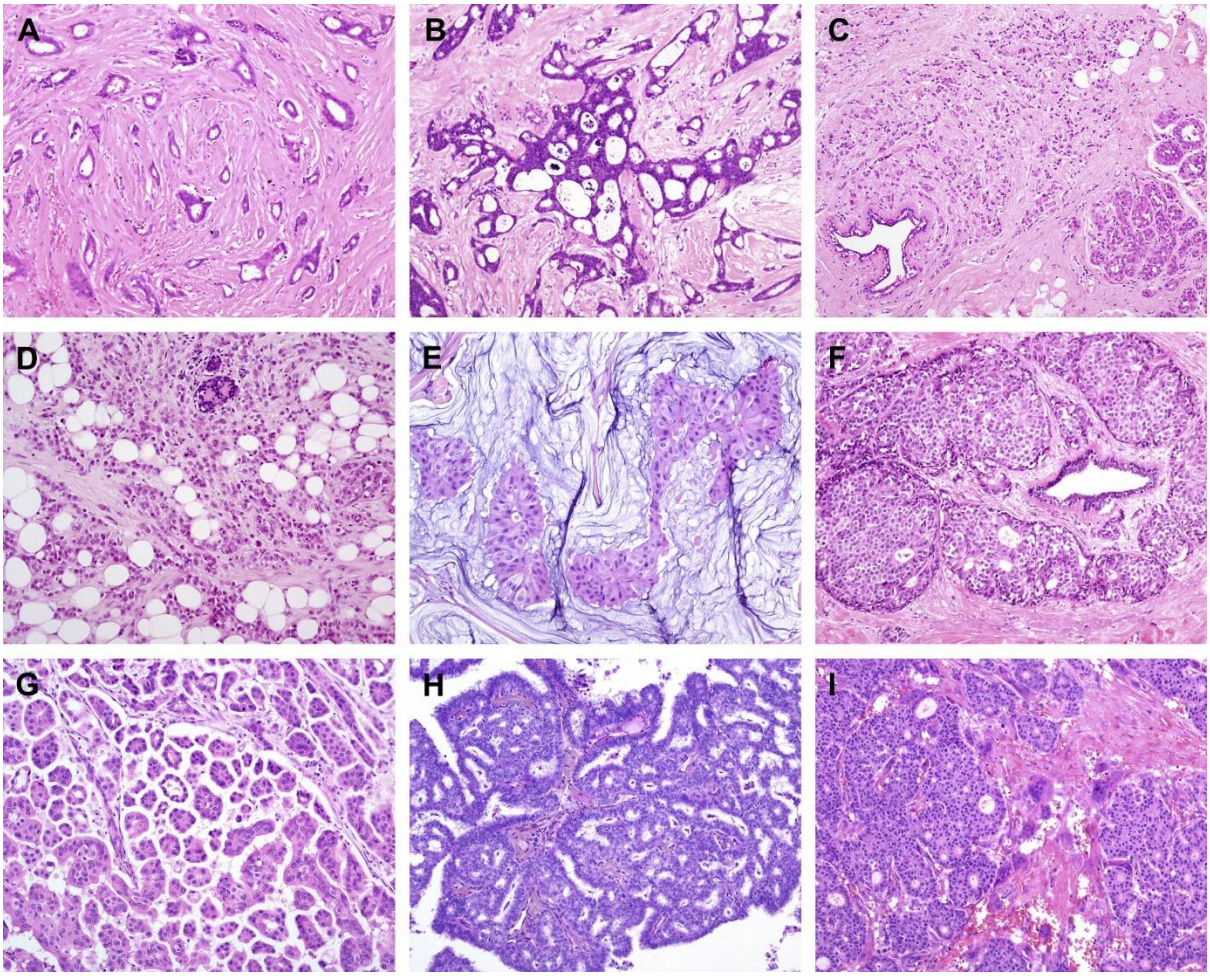

Figure 6. Histopathological images of special type breast cancers that are ER positive. (A) Tubular carcinoma, (B) cribriform carcinoma, (C) classic invasive lobular carcinoma, (D) pleomorphic invasive lobular carcinoma, (E) mucinous carcinoma, (F) neuroendocrine carcinoma, (G) micropapillary carcinoma, (H) papillary carcinoma, (I) low grade invasive ductal carcinoma with osteoclast-like giant cells [91].

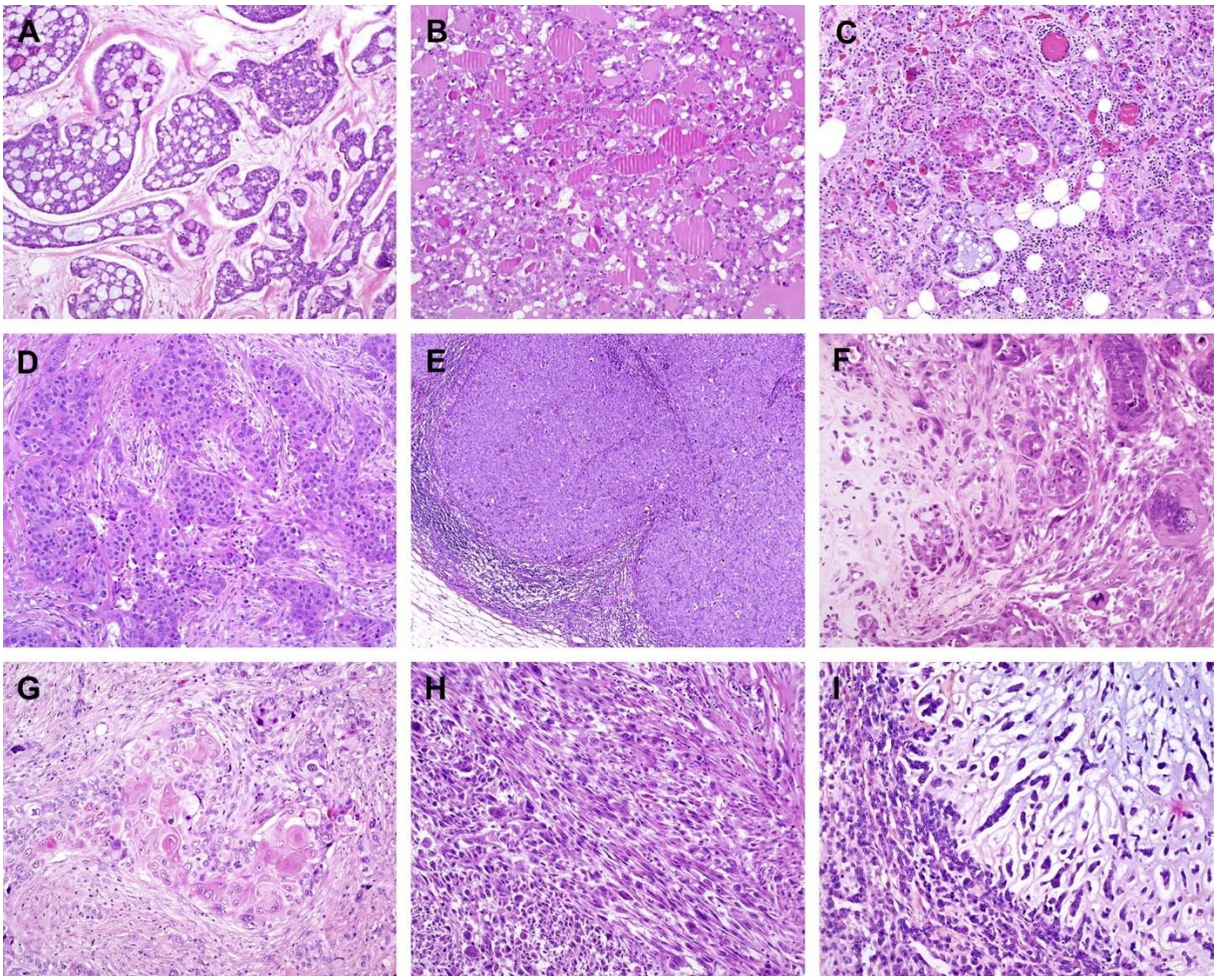

Figure 7. Histopathological images of special type breast cancer that are ER negative. (A) Adenoid cystic carcinoma, (B) secretory carcinoma, (C) acinic-cell carcinoma, (D) apocrine carcinoma, (E) medullary carcinoma, (F) metaplastic carcinoma with heterologous elements, (G) metaplastic carcinoma with squamous metaplasia, (H) metaplastic spindle cell carcinoma, (I) metaplastic matrix-producing carcinoma [91].

2.4 Immunohistochemistry of Breast Cancer

2.4.1 Hormone Receptors (ER and PR)

As breast cancer is a heterogeneous disease, various biomarkers are used to determine the molecular subtypes. Most commonly, these subtypes are divided up according to the hormone receptors ER, PR and HER2 [96]. The steroid hormone receptors ER and PR have been used as important prognostic biomarkers for BC diagnosis and critical indicators in endocrine therapy since the mid-1970s [53]. They are usually the first prognostic tools recommended clinically to be tested for when a patient is diagnosed with early BC. This is because a diagnosis with positive hormone receptors has less aggressive features and often provides a better outcome for survival for the patient due to the availability of existing hormone therapies such as endocrine therapy that can cut off the cancer's dependency on the detected hormones [53].

PR is coded by an estrogen-regulated gene, often leading to PR-positive tumors also showing positive for ER. Therefore, ER-negative tumors are often also PR-negative due to this fact. About 10% of breast cancer cases are single hormone positives, either ER+, or PR+ and these single hormone positive tumors are clinically and biologically different from the double hormone ones. How these tumors start out is yet to be found, but in some studies, a little to no difference has been observed between patients that have either type, but some other studies suggest that PR+ patients likely experience worse prognosis too [96] [53]. According to the American Society of Clinical Oncology (ASCO), more than one million women per year are diagnosed with breast cancer, and they get tested for these biomarkers specifically. This is because ER-positive breast cancers make up around 79-84% of all the cases diagnosed, and of the million cases, most of the diagnosed women are postmenopausal women. Though the detection of ER-positive breast cancer depends on factors such as age, race, screening etc., better assay protocols, newer advanced detection methods and quality assessment are needed to better the survival rate for many women [5].

2.4.2 HER2

HER2 is an oncogene that encodes for epidermal growth factor receptor with tyrosine kinase receptor [3]. The expression of this oncogene is present in 15-20% of all invasive breast cancer and its amplification is related to the protein overexpression of *HER2*. It also shows an overexpression at the mRNA level [3] [47]. This biomarker is a prognostic marker which is often associated with worse clinical outcomes for patients with an overexpression in it such as those with reoccurrence, which lead to lower survival rates. However, HER2 amplification is a good predictive factor for judging whether a patient will respond to anthracycline-based chemotherapies, and more specifically, it is also the only predictive biomarker to treat HER2 positive breast cancer with HER2-targeting agents like trastuzumab, lapatinib and pertuzumab. As these therapies are only effective in HER2 overexpressed patients, it is imperative that precise clinical assessment is done to treat these cases following the ASCO guidelines, or whatever guidelines are set in use for each country [3]. Once evaluated, a score from 0 to 3+ is given. If the samples during the analysis score above or around 2+, 3+, further immunohistochemical analysis are performed using fluorescence in situ hybridization, and it is concluded that patients with overexpression in HER2 will benefit from treatments like Herceptin [47].

2.4.3 Ki67

When it comes to what prognostic or predictive biomarkers are used to assess early breast cancer treatment options for patients, usually ER, PR and HER2 are the leading choices. For more than 30 years, these markers have been somewhat efficient in measuring cell proliferation in breast cancer and played a role in decisions regarding antineoplastic therapies etc. [74][92]. However, cell proliferation is measured in many ways, and among them is the use of immunohistochemistry assays to measure Ki67, a nuclear marker that is expressed in all of the phases of the cell cycle except the G_0 phase [74]. Even though Ki67 possesses both prognostic and predictive values, it's assessment as an actual biological marker has yet to be established as a required routine by pathologists and Ki67 is not included in the guidelines provided by the American Society of Clinical Oncology [92].

In recent years, as more investigation has been done on Ki67, a stronger case for why it should be added to the standard pathological assessment of early breast cancer is emerging. Originally identified by Gerdes and colleagues in the early 1980s, the protein was discovered to be found mainly in the nuclear cortex and the dense fibrillar components of nucleolus during interphase, and in the peripheries of condensed chromosomes during mitosis. Though its function is still unclear, the protein has an important role in cell division. As cancer cells constantly divide, Ki67 expression can be measured throughout different cell-cycle phases, especially during the G1, S, G2 and M phases. Thus, stained tumor cells are analyzed by pathologists using a normal light microscope at 40x, and a percentage is given from their count. The problem with this method is the subjectivity of how different pathologists assign scores. Estimating a percentage for Ki67 has proved to be poorly reproducible and manual counting of each slide is far too time-consuming to be done practically as part of a diagnostics routine. The argument for automated readers has been used to counter this, but unfortunately, misreading such as counting non-malignant nuclei occur and such errors cannot be accepted [92]. Even though Ki67 is not currently accepted as a clinical method of assessment for patients with breast cancer worldwide, its strong prognostic value brings interest into further researching and developing better reproducible methods and techniques for counting it so that it can be accepted as a valuable biomarker. In fact, it is already included in the Norwegian guidelines when diagnosing breast cancer [71]. There is hope that in the future, Ki67 could potentially take its place among the other biomarkers as a good prognostic tool in clinical diagnostics across the world [92].

2.6 Computational pathology

The use of machines and technology in medical research is not a foreign concept, and multiple bioengineers and professional biotechnologists apply their knowledge to the field of science every day in clinical settings and trials. However, despite all that, there has yet been any computational approaches established when it comes to cancer diagnostics. There have been amazing and remarkable advancement in technology, but still, the golden standard for diagnosis and accurate evaluation of breast cancer for patients is still through histological examinations of the tissues done by pathologist in laboratories [46]. Diagnostics is a subjective matter, and often, what one pathologist deems a grade might not line up with what another pathologist or a laboratory decides. Thus, certain guidelines are put in place for pathologists to follow, and a consensus is made if disagreements occur. These guidelines for objective grading are the Nottingham Histology Grading System, tumor size, mitotic count etc., to determine how tumors should be graded and their severity. However, due to the heterogeneity of cancers, the different stages, sizes of tumors and mitotic counting, these tasks become very time-consuming for pathologists to do [46]. Thus, if a smart and automated computational process could be developed that would objectively diagnose the cancerous tissues, the efforts of the pathologists could be focused on more interesting areas such as research and development.

Before incorporating AI into pathology, a look into digital pathology is necessary. Ever since technology advanced, medical science have gotten their hands onto mechanical systems and devices such as whole slide digital scanners that allow for pathologists to view, manage, and analyze digitized tissue on a monitor, which makes things much easier and less time-consuming. It has proven to be extremely useful when it comes to image archiving, analysis and sending the

results over to other labs for review as the images produced from these scanners are high in resolution and easily accessible once converted from the microscope to a bigger monitor [46]. This brings forth computational pathology, which is the incorporation of technologies such as WSI (whole slide imaging), multi-omics, and clinical bioinformatics in order to perform tasks that are too time-consuming and sometimes even impossible to complete manually for pathologists [46] [24][85]. Even with the growth of computational pathology, the practical effectiveness of establishing such systems in laboratories are still facing several challenges, among them how to integrate raw data from multiple different sources, the limiting hardware processing capacity of the machines, social and ethical issues pertaining to the gathering and storing of a patient's medical information digitally and more [24].

With how heterogenous cancer is, the data sources gathered from tumors come with a lot of randomness and background noise that makes it problematic for statistics and analysis done by computers to get it correct. There needs to be a thinking brain behind the operation and decision making in order to eliminate these errors. Thus, AI-based computational pathology has started to emerge, and this type of computational pathology has shown great potential and promise in increasing accuracy and establishing a standardized and hopefully, a more objective way, of diagnosing patients in the future [24] [35]. Among the biggest challenges facing AI-learning systems today are the shortage of experienced pathologist available to teach the necessary algorithms, the ever increasing heterogenous data coming in from patient care, the increased complexity in managing and sorting through data from multiple different sources, and efficiency in machine-learning to process and understand large amount of data in a short amount of time, and that is not all either [24]. For AI computational pathology to work, a direct link needs to be established between the patients and their doctors, the local laboratory and the central port for data processing and retrieval first. If this can happen, then AI technology can get advanced enough to be taught how to handle enormous amounts of data, diagnose objectively, classify, and predict correct treatment options for patients with minimum input from humans [24].

2.7 Objective of Thesis

This thesis is part of the Clarify Project, a project which aims to study the histological patterns and features of TNBC using image analysis, immunohistochemistry and the expression of miRNAs in the tumor cells to improve reproducibility and prognostic value of its clinical diagnosis. To do so, the project focuses on training artificial intelligence and strengthening computational pathology in order to eliminate the subjective nature of the current diagnosis for TNBC and improve upon its poor prognosis. Hopefully, the under- or overtreatment of TNBC patients will be prevented once such an automated and objective way of diagnosis is achieved [48].

There are two main objectives to this thesis. The first is to find out whether there are other factors associated with TNBC that can become good prognostic markers alongside well-established biomarkers such as MAI and TILs. Both are counted for and estimated for 271 patients diagnosed with TNBC at the Stavanger University Hospital (SUS). The results will expand the database of the Clarify project and assist in the development of image analysis tools or a machine learning system that can eventually calculate MAI, Ki-67, and PPH3 for breast

cancer tissues without the need for pathologists [48]. Statistical analysis will be performed to draw comparisons and correlations between the survival-related characteristics of the TNBC cases collected in the data base for those that were alive and well, those with local reoccurrence and those dead of the disease with distant metastasis at the end of their follow-up status (n=195). Focus will be placed on finding characteristics that display significant prognostic value within the margins set for the analysis.

The objective for the second part of this thesis is to analyze the expression of microRNA's 18a and 18b in the lymphocytes of TNBC tumors of 42 patients. The patients selected for this part are divided into two groups, those with occurrence only, and those that experienced reoccurrence of the cancer. The focus of this part will lie on whether any positive cells are found for miR-18a - and 18b, the quantity of these positive cells and whether the expression of the microRNAs show a difference between the group without reoccurrence against the group with reoccurrence [48]. The location for the expression of the microRNAs, whether they are found surrounding the tumor, on the edge of it or inside, will also be considered for the final evaluations. Independent sample T tests will be done to find correlations between the two microRNAs and the same characteristics of TNBC from the larger data base to find out if they have any significant prognostic value.

3 Materials and Methods

3.1 Background and Methodology

3.1.1 Tissue Sectioning and Staining

The breast cancer tissues used for this project were all formalin-fixed paraffin embedded (FFPE) tissue blocks dating back from 1978 to 2004, all triple negative breast cancer, diagnosed and preserved at Stavanger University Hospital. To get a thin-sliced sectioning of a breast cancer tumor that can be observed under a light microscope in high quality, a sample has to be embedded in something, and one of the most common ways to do that is through paraffin waxing [81]. For FFPE sections, formaldehyde is used to react with the nucleic acids and peptides of the tissue, forming bonds between them and cross-linking, thus preserving the general structure of the cells within. However, this can sometimes interfere with the yield of either DNA or RNA during diagnostics and with time, degradation can occur. This introduces bias to results because of the permanent changes that happen over time [47]. However, even though fresh tissue sections from frozen material are more desirable for analysis, in many cases they are also more delicate and can get easily distorted. Fortunately, for gene expression of microRNA, several recent publications have shown that results obtained from FFPE sections are quite comparable to those obtained from fresh frozen cuts, making FFPE slides sufficient for use in this experiment [47].

When it comes to staining, the most common type that has been used for the last century is the hematoxylin and eosin (H&E) staining. This staining method is so essential and often used in histology due to the ease it provides when it comes to differentiating and recognizing various tissue types/morphological changes on sample sections. It has remained unchanged due to its

ability to work well with many different types of tissues, and the broad range of what it can color in high quality. The hematoxylin in the stain reacts with the nucleic acids. This generates a deep bluish-purple color when it binds to the nucleic acids and stains it, while eosin stains proteins nonspecifically and appears pink under the microscope [20] [32]. Typically, with hematoxylin staining, cancer-type specific patterns can be recognized, which is why it is used so often in diagnostics [32]. For this project, the standard H&E staining was done on slides prepared by the Stavanger University Hospital from TNBC FFPE tissue blocks.

3.1.2 Counting the Mitotic Activity Index (MAI)

The strong prognostic value of the MAI has been studied and shown in multiple publications and is the most widely accepted form for determining proliferation. Though there have been questions regarding the reproducibility of MAI like any other methods, it has shown to be more reproducible if the aforementioned recommended guidelines in this thesis are used. It is also an easily understandable method to teach and incorporate into clinical work and it is already part of many routine histological gradings in hospitals [49].

For this thesis, MAI was evaluated for 271 BC cases of H&E-stained slides that were prepared by the University Hospital in Stavanger. The mitotic figures on the slides were clearly defined, and all necrotic or apoptotic cells were excluded from the counting to achieve maximum accuracy (see Figure 8 below to see what kind of mitotic cells were counted). At low magnification (4x on the microscope) an overview of the tissue samples was first observed. Areas where high proliferation occurred were chosen for the counting and defined as “hotspots”. These areas contained invasive tumor components that spread without the presence of necrosis or inflammation surrounding or being within it. From there, magnification was increased to 400x (objective 40x) and the selected area of $1.59mm^2$ was counted with a light microscope. Cases that were difficult to define the mitosis for, blurry, too pale or too darkly stained, too thick sectioning, had multiple necrotic areas/inflammation or the infiltrating tumor area were missing were excluded from the count. The total mitotic count was divided into two groups, those less than 10 (MAI <10) and those equal to or greater than 10 (MAI ≥ 10) for statistical analysis [49] [8]. In Figure 8, the figures counted for in MAI can be seen at 40x magnification.

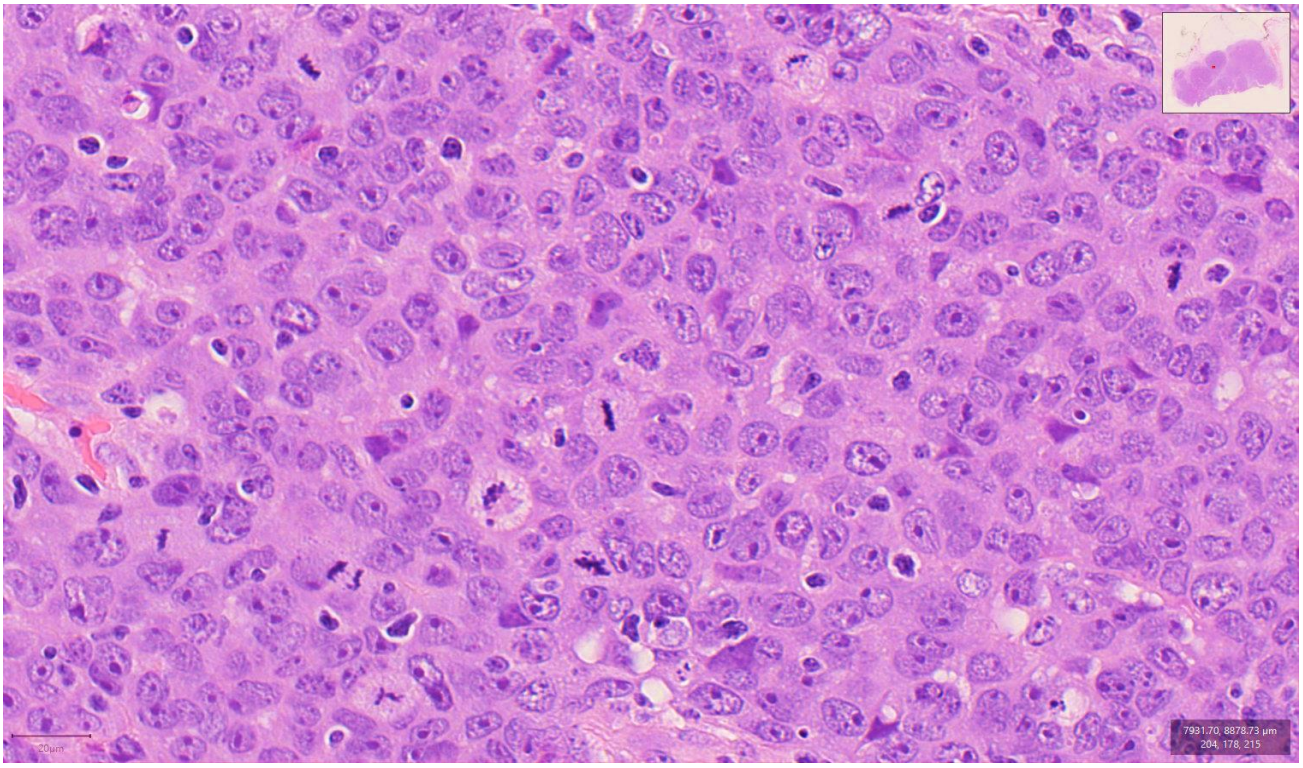

Figure 8. Shows mitotic figures counted for in a hotspot at 40x magnification following the MAI guidelines provided.

3.1.3 Counting Tumor-infiltrating Lymphocytes (TILS)

In this thesis, TILs were assessed for the same 271 cases that MAI were counted for. The H&E-stained slides were overlooked fully in low magnification. The field of view (FOV) was moved continuously until the whole slide was observed. Each slide was determined by how much of the tumor stroma was covered with the tumor-infiltrating lymphocytes. Only TILs found in the stroma around and within the invasive tumor cell areas were counted. TILs that were located within the areas of tumor cells (intratumoral TILs) were not counted. High stromal TILs generally represent a better prognosis [1]. Those found around the borders of the tissues and necrotic areas as well as polymorphonuclear cells were ignored. When determining the percentage, the criteria defined by the cooperative efforts of pathologists, oncologists and immunologists was used. Following those guidelines, the TILs were given a score between 0-100% [44]. For analysis, the TILs scores were divided into two groups, less than 15 (TILs <15) and those equal to or more than 15 (TILs ≥ 15). In Figure 9 below, a visual representation can be seen for low and high TILs in 10x and 40x magnifications.

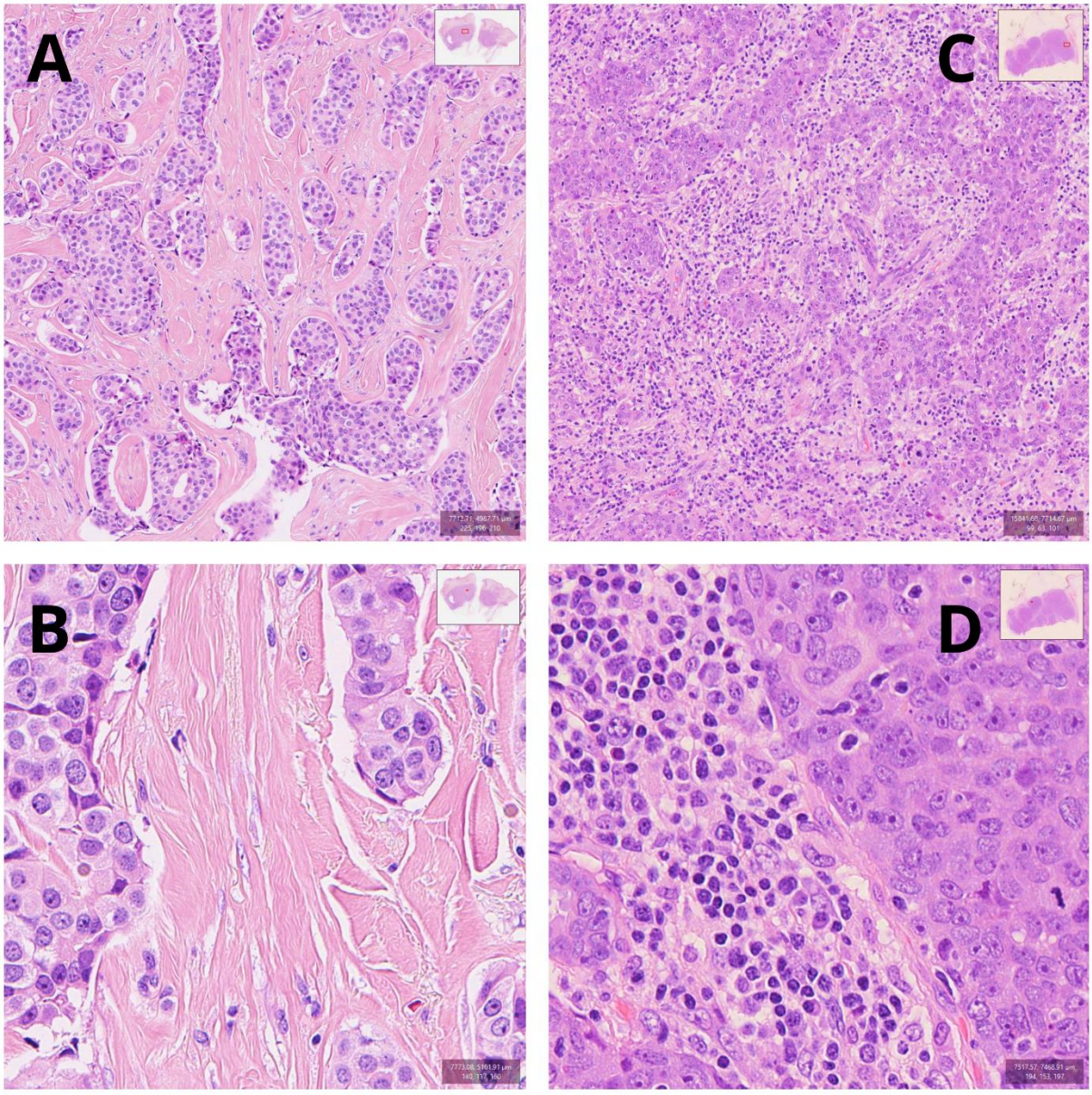

Figure 9. (A) Shows an overall view of a tissue at 10x magnification for low expression of TILs. (B) Shows 40x magnification of low expression of TILs. (C) Shows an overall view of a tissue at 10x magnification for high expression of TILs. (D) Shows 40x magnification of high expression of TILs.

3.1.4 Chromogen In-Situ Hybridization

Chromogen in-situ hybridization is a powerful technique used to visually analyze the expression of genes in specific cell types or tissue in FFPE sections. For this method to work, several steps are done precisely in order to achieve the high optimization it demands. The protocol used for this experiment was derived from Qiagen's miRCURY LNA miRNA ISH Optimization Kit, which provides a straightforward way of performing miRNA ISH analysis without having to

optimize too much and without it being too time-consuming, shortening the whole procedure to only a day to get satisfying results [79].

This kit uses locked nucleic acids (LNA) technology. LNA are a class of high-affinity RNA analogs that can be locked in the ideal Watson-Crick binding formation to stabilize the temperature of DNA or RNA stands when hybridizing to complementary strands. With this thermal stability, LNA oligonucleotides can also be made shorter while still retaining their high temperature stability. This technology of modifying LNA oligonucleotides, making different mixtures of LNA with DNA or RNA makes it possible to optimize the sensitivity and specificity for many hybridization-based projects such as the ISH protocol in use for this thesis. In general, the small sizes and GC content of microRNAs make it difficult to analyze them using traditional methods, especially through microarray profiling and high-throughput experiments. Using LNA-enhanced probes generally eliminates these challenges, making them ideal for use in a challenging procedure such as CISH [79].

Done on FFPE tissue samples from selected cases, the CISH procedure relies on the nonmammalian hapten digoxigenin (DIG) to access the double-DIG-labeled LNA probes to hybridize the desired microRNA sequences on the slide, in this case microRNAs 18a or 18b, once Proteinase K demasks them. The anti-DIG solution that is made prior to that step in the procedure contains a specific anti-DIG antibody that directly conjugates with the enzyme alkaline phosphatase (AP). The 4-nitro-bleu tetrazolium (NBT) and 5-bromo-4-chloro3'-indolylphosphate (BCIP) in the AP substrate solution releases a dark blue water- and alcohol-insoluble NBT-BCIP precipitate, which after staining makes it easier to see the probes under the light microscope [79].

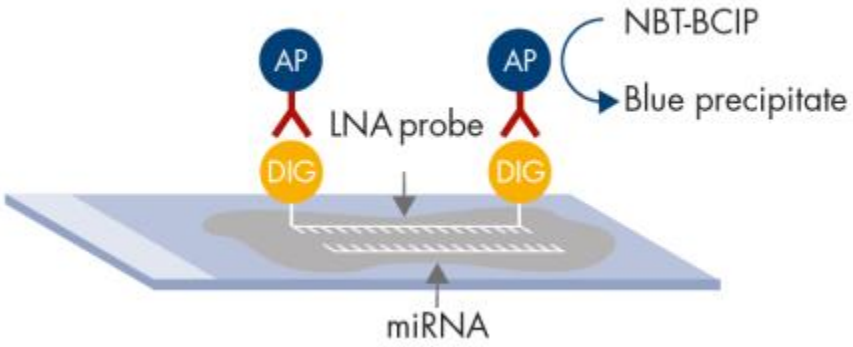

Figure 10. Principle of the colorization of CISH procedure. After the demasking with proteinase K, the LNA probes bind to their target miRNA. Then an antibody-alkaline phosphatase conjugate binds to the probes, and once the substrate is added, NBT-BCIP generates the blue precipitate [79].

3.2 Protocols and Materials

3.2.1 Probe Preparation

The hybridization probes used for this experiment were miRNA's 18a and18b, and control probes, Scramble-miR, and U6 snRNA from the miRCURY LNA miRNA ISH Optimization Kit. The powdered forms of each of these probe stocks (25 µM for 18a, 18b, Scr and 0.5 µM for U6) were spun down in a small centrifuge for a couple of seconds before they were resuspended in 40 µl of RNAse-free water, which is also referred to as MilliQ water. More details regarding the probes from the kit can be seen in Table 1 below.

Table 1: Details for the stock solutions for the probes miR-18a and miR-18b as well as the positive control (U6) and the negative control (Scr). The concentrations for each stock, their final volume and RNA degradation temperature are also displayed [79].

Probes	RNA-T_m calculated (°C)	Volume (µl)	Concentration (µM)
5'-3' DIG hsa-miR-18a-5p	83,8	40	25
5'-3' DIG hsa-miR-18b-5p	84,1	40	25
LNA U6 snRNA probe, 5' DIG-labeled	83,6	40	0.5
LNA Scramble-miR probe double-DIG labeled	87,3	40	25

The experiment requires hybridization mixes to be made with a final concentration of 80 nM each for miRNA 18a, miRNA 18b and Scramble, and 1nM for U6 [79]. To achieve this, the previously resuspended stock solutions were denatured in non-stick RNase free 1,5 mL safe-seal tubes at 90ºC for 4 minutes using a heating block. Four separate ISH buffers were made in non-stick RNase free 2 mL safe-seal tubes for each of the probes and labelled correspondingly. The ISH buffers were diluted 1:1 with MilliQ water depending on how many probes were supposed to be prepared. Immediately after the 4 minutes in the heating block, the denatured probes were mixed with the calculated amount of ISH buffer and spun down to ensure that the probes would bind to the buffer instead of each other [79]. They were kept in the dark for the duration they were not in use as the probes are light sensitive. The calculated amount needed for each probe was determined by using this following formula:

$$C_1 \times V_1 = C_2 \times V_2$$

Once the denatured probes were mixed with their respective ISH buffer thoroughly (flipping the tubes upside down and spinning), 55 µl aliquots of the individual probes were made and stored inside 0,5 mL RNase-free tubes that were put in the freezer at -20ºC until needed for the CISH procedure. The probe target sequences, their sequences and melting temperature (T_m) can be seen below in Table 2 as extracted from the kit protocol [79].

Table 2: The probes used for miR-18a and miR-18b in CISH, including target sequences, probe sequences and T_m. In addition, the positive control (U6) and the negative control (Scr) can be seen [79].

LNA™ Detection / control probes	**Target sequence**	**Probe sequence**	**RNA-T_m calculated (°C)**	**Concentration**
5'-3' DIG hsa-miR-18a-5p	MIMAT0000072: 5'UAAGGUGCAUCUAGUGCAGAUAG	CTATCTGCACTAGATGCACCTTA	83,8	80nM
5'-3' DIG hsa-miR-18b-5p	MIMAT0001412: 5'UAAGGUGCAUCUAGUGCAGUUAG	TAACTGCACTAGATGCACCTTA	84,1	80nM
U6		CACGAATTTGCGTGTCATCCTT	83,6	1nM
Scramble		GTGTAACACGTCTATACGCCCA	87,3	80nM

3.2.2 Solutions and Glassware

Before use, all glassware for the solutions were autoclaved. A thin layer of water (MilliQ/RNase-free) was poured inside the autoclave machine and each cap on the glassware were loosened and taped securely with autoclave tape that turns black when the process is complete.

Below in Table 3, the solutions prepared before the CISH protocol can be seen along with the instructions on how to make them. For each run of making the solutions, two or more PBS solutions were made as well as 0.2xSSC solution as they are more excessively used and run out quickly after a couple of days. An extra PBS is also required every time to be converted to PBS-Tween.

Table 3. Solutions prepared before the CISH protocol takes place [79].

Solutions:	**Instructions**

PBS	*Add five phosphate buffered saline tablets to be dissolved in 1000 mL of MilliQ water.*
PBS-Tween	*1000 µL of Tween-20 was added to a bottle of PBS already made.*
0.2xSSC	*Add 10 mL of 20xSSC to 990 mL of MilliQ water in a glass bottle, total volume 1 L.*
1xSSC	*Add 50 mL of 20xSSC to 950 mL of MilliQ water in a glass bottle, total volume 1 L.*
5xSSC	*Add 250 mL of 20xSSC to 750 mL of MilliQ water in a glass bottle, total volume 1 L.*
KTBT (AP-stop solution)	*7,9 g of Tris-HCl (50 mM), 8,7 g of NaCl (150mM) and 0,75 g of KCl are added to 1000 mL of MilliQ water.*
Proteinase K Buffer	*Add 1 mL of 1M Tris-HCl (pH 7.4), 400 µL of 0,5M EDTA and 40 µL of NaCl in 200 mL of MilliQ water.*

During deparaffination and the dehydration steps of the CISH protocol, various concentrations of alcohols are used. The 99% ethanol (Absolute Alcohol) and the 96% ethanol (Reduced Alcohol) are readily available for use at the hospital and are kept in a locked storage room along with another solution that does not require preparation, Xylene. The 70% alcohol concentration and the 50% alcohol concentrations are both made in the lab. Below in Table 4, the alcohols and Xylene instructions are listed.

Table 4. Alcohols prepared before the CISH protocol, and Xylene [79].

Solutions:	**Instructions**
Xylene (mixed isomers)	*Ready for use. Kept in a storage at the hospital, requires only retrieving.*
99% Ethanol (Absolute Alcohol)	*Ready for use. Kept in a storage at the hospital. Requires signature when retrieving.*
96% Ethanol (Reduced Alcohol)	*Ready for use. Kept in a storage at the hospital. Requires signature when retrieving.*
70% Ethanol	*Add 700 mL of 99% Ethanol to 300 mL of MilliQ water in marked alcohol container.*
50% Ethanol	*Add 500 mL of 99% Ethanol to 500 mL of MilliQ water in marked alcohol container.*

Lastly, for the next few solutions, they are to be made right before they are needed for each step in the CISH protocol. They are either kept in the dark or in the fridge/freezer when they are not needed. For those that are light sensitive, two/three layers of aluminum foil is wrapped around them before they were put inside the cupboard for extra protection. Below in Table 5, they are shown along with instructions.

Table 5. Solutions prepared during the CISH protocol right before use [79].

Solutions:	Instructions
Proteinase K	*Thaw, spin and mix 7,5 µL Proteinase K stock solution with 10 mL of the Proteinase K Buffer solution.* *Proteinase K Stock Solution (20 mg/mL): Aliquots of 8,5 µl/tube can be found in the freezer at -20 °C. Avoid repeated thawing and freezing.*
Blocking Solution	*Add 550 µL twice of Maleic Acid Buffer (total volume 1,1 mL), 1000 µL + 250 µL of the blocking solution (total volume 1,25 mL) and 250 µL of sheep serum to 9,9 mL of MilliQ water. Wrap it in aluminum foil and keep it in the dark until needed.*
Sheep-anti-DIG-AP (1:800)	*Add 6.5 µL to 5 mL of blocking solution. Wrap the tube in aluminum foil and keep it in the dark until needed.*
AP-Substrate	*Add 1 NBT/BCIP tablet from the fridge in 10 mL of MilliQ water and wait until the tablet is fully dissolved before adding 20 µL of Levamisole Stock Solution to the solution. Wrap it in aluminum foil and keep it in the dark until needed.*

3.2.3 The CISH Protocol

This protocol is an optimized and modified version from the one provided by Qiagen. Hybridization temperatures, duration of proteinase K treatment and heating cabinet temperatures have all been changed to yield effective results for the probes used. Equipment and surfaces used during the process such as tweezers were treated and cleaned with RNAseZap to prevent RNAse contamination [79]. The materials used can be found listed in the kit.

1 - Sectioning and melting the Paraffin Wax:

This step was completed by a supervisor in the laboratory:

Before use, the warm water bath was rinsed with MilliQ water, then 100% alcohol, then left to air dry before being filled up again with new MilliQ water. Before the slides were cut, the FFPE tissue blocks were placed on ice for 15-20 minutes to better control the outcome of the thickness of each section. From each of the tissue blocks cut, 4 sections of 5 µm were cut on an automated rotational microtome. These 4 sections are put on glass slides and are labelled 18a, 18b, U6 (positive control) and Scramble (negative control) along with the date and ID for the tissue block before they are left to air dry. Before the CISH protocol can start, the slides must be put in the heating cabinet overnight at 56°C for at least 12-19 hours. This way, it ensures that the wax melts and the tissue stick more to the slides.

2- Deparaffinization and rehydration of slides (approx. 40 min):

This step takes place under the fume hood. Place three glass jars labelled Xylene I, Xylene II and Xylene III together in a row. Then place another three glass jars labelled Abs (absolute alcohol) I, Abs II, and Abs III after them. Fill up the Xylene jars and the 99% Absolute Alcohol jars with enough solution to cover the slides. Grab 96% alcohol, 70% alcohol and 50% alcohol and put them under the fume hood. Place one glass jar labelled "Deparaffinization" at the end. This will be used for the 96% and 70% alcohol step. Put the timer on 5 minutes and start. For the absolute alcohol, just dip the slides 10 times in the first two jars before leaving it in in the last one for the rest of the 5 minutes [79]. The instructions for this step can be read on Table 6 below.

Table 6. Deparaffinization and rehydration [79].

Deparaffinization Steps	Solution	Time
1	Xylene I	5 minutes
2	Xylene II	5 minutes
3	Xylene III	5 minutes
4	99,9% Abs I	Dip in 10 times
5	99,9% Abs II	Dip in 10 times
6	99,9% Abs III	5 minutes
7	96% Ethanol	5 minutes (change once around the 2:30 mark)
8	70% Ethanol	5 minutes (change once around the 2:30 mark)
9	PBS	2-5 minutes in total (Change once and use a separate jar)

3 - Proteinase K incubation and PBS wash:

During the deparaffinization step, grab the proteinase K stock solution (20 mg/mL). It is in the freezer and keep it in the dark. When it thaws, flick and spin the stock solution before taking 7,5 µL and adding that to 10 mL of proteinase K buffer and mixing the solution well.

Dry the back of the slides and put them on the hybridizer. Use approximately 700 µL of the proteinase K solution and add that quickly to each slide to avoid drying them out. The slides are left to be incubated for 30 minutes at 37°C in a hybridizer (Vysis Hybrite™ Slide Stainer, Vysis, Inc., Downers Grove, Illinois, USA) without humidity strips. After the 30 minutes are up, the slides are washed with PBS solution twice to rinse off the proteinase K solution for approximately 2-4 minutes.

4 – Dehydration of slides:

A glass jar labelled “Dehydration I” was used for this step. Follow the instructions in Table 7.

Table 7. First dehydration of slides [79].

Dehydration of slides	Steps	Time
1	70% Ethanol	1 minutes (change ethanol once around the 30 second mark)
2	96% Ethanol	1 minutes (change ethanol once around the 30 second mark)
3	99,9% Ethanol	1 minutes (change ethanol once around the 30 second mark)
4	Air dry on clean paper towels.	15 minutes

5 – Hybridization:

While the slides are air-drying on the paper towel, prepared aliquots of each probe are grabbed from the freezer and put in the drawer to thaw and avoid light as they are very sensitive. During the 15 minutes of air drying, each slide is labelled with the name of the probe, date and case number for the tissue. The coverslips that will be used are grouped together and wrapped twice with aluminum foil, previously baked in a normal household oven for 8 hours at 180°C. They are clean and sterilized and are stored in a box until further needed. Coverslips that are not used from the group are rewrapped and placed in another clean box for use for another time. Once the probes are thawed, they are flicked to mix and spun down before placed back into the drawer until they are needed as well. Humidifying strips soaked in 50% alcohol are placed inside the hybridizer, and the program is set to 50°C.

After the 15 minutes are up, it is essential to work quickly in order to avoid light exposure and drying out the slides. The order they are arranged in is as follows, Scr, 18a, 18b, and finally U6. This is done so in order to minimize possible contamination occurring between the probes and the controls. 50 µL of the hybridization mix is added to each slide before the coverslip is gently put on. If tilted slightly from the side when putting the coverslip on, minimum amount of bubbles form. However, if a lot of air bubbles form, gently tapping on the glass is recommended to get rid of the bigger bubbles before the edges are sealed with Fixogum (Marabu, Bietigheim-Bissingen, Germany). The slides are put in the hybridizer with the negative and positive controls on each end and the samples in the middle. The program is set to run for 1 hour and 30 minutes. From this point on, great care is taken to never let the slides dry out until the procedure is complete [79].

6 - Stringent wash:

After hybridization is done, the Fixogum is removed gently using a pair of tweezers. The coverslips are then pushed off from one side and dragged carefully off without tearing the tissue.

The slides are immediately placed in a room temperature 5xSSC solution. After approximately 5 minutes, the stringent wash starts by following these steps.

Table 8. Stringent Wash and PBS rinse [79].

Stringent Wash	Solution	Time	Temperature
1	5xSSC	5 minutes	50 °C (Hybridization Temperature)
2	1xSSC	5 minutes	50 °C (Hybridization Temperature)
3	1xSSC	5 minutes	50 °C (Hybridization Temperature)
4	0.2xSSC	5 minutes	50 °C (Hybridization Temperature)
5	0.2xSSC	5 minutes	50 °C (Hybridization Temperature)
6	0.2xSSC	5 minutes	Room Temperature
7	PBS	2-5 minutes	Room Temperature

7 - Blocking:

During the stringent wash, start making the blocking solution according to the instructions in Table 5. The solution is light sensitive, so the tube is wrapped in aluminum foil and stored in the cupboard until needed. A humidifying chamber was prepared while the slides remained in the PBS solution at the end of the stringent wash and 750 µL of the blocking solution was applied to each slide, before the chamber was covered to protect them from the light. The incubation was left at room temperature for 15 minutes [79].

8 - Anti-DIG incubation and PBS-T wash:

The anti-DIG reagent was made during the 15 minutes it takes to incubate the slides with the blocking solution. The instructions in Table 5 were followed and the tube was covered in aluminum foil to protect it from the light and stored in the cupboard until needed.

Once the time was up, the slides were drained from the blocking solution by tilting them on a clean paper towel before drying off the edges. This is to prevent the anti-DIG solution from running off the sides while they are inside the hybridizer. Once the slides were placed inside the hybridizer, approximately 450 µL of the solution was applied quickly in order to avoid drying them out. The negative and positive controls were placed on the edges again, while the case slides were kept in the middle. The slides were left to incubate at 30°C for 1 hour and 15 minutes.

After the incubation, they were washed with PBS-T at room temperature for 9 minutes, changing the solution three times (3 x 3) [79].

9 - AP substrate incubation:

The AP substrate solution was made during the stringent wash step as 1 tablet of NBT/BCIP was added to 10 mL of MilliQ water to have it fully dissolve before 20 µL of Levamisole stock

solution could be added to it during the PBS-Tween wash step. The tube was then protected from light until needed [79].

Once the PBS-T wash was done, the slides were thoroughly dried off around the edges to prevent the AP-substrate solution to run off and the slides were then placed in the hybridizer in the same order they were for the anti-DIG step. Approximately 400 µL of AP substrate was added to each slide and they were left to incubate for 1 hour and 50 minutes at 30°C [79].

10 - KTBT buffer (AP-stop solution) incubation:
The slides were incubated in KTBT buffer twice for 10 minutes total (changing once around the 5-minute mark) to stop the reaction [79].

11 - Wash and counter stain:
The slides were washed with MilliQ water at room temperature twice for 1 minute (2 minutes total), before they were submerged in Nuclear Fast Red for 2 minutes for counterstaining. Then the slides were washed in running tap water for 5 minutes [79].

12 – Dehydration of slides and mounting:
One glass jar was used for the following dehydration of the slides:

Table 9. Last dehydration of slides [79].

Dehydration of slides	Steps	Time
1	50% Ethanol	1 minutes
2	70% Ethanol	1 minutes
3	96% Ethanol	1 minutes
4	99,9% Ethanol	1 minutes

After dehydration, the slides were placed on a clean paper towel before quickly applying 1 drop of Histokitt mounting medium (Glaswarenfabrik Karl Hecht, Sondheim/Rhön, Germany) and mounting with the coverslips (Menzel, Braunschweig, Germany). Air bubbles were gently pressed until they were removed and residual glue on the sides were wiped off clean [79].

13 - Counting positive cells
The tissue sections were observed under a light microscope the same day, or the next. An area of 1,59mm^2 containing invasive tumor cells and tumor infiltrating lymphocytes (TILs) was selected, and hot spots with the most positive cells were counted. For each case, both their U6 and Scr slides were checked to make sure the process worked. Along with the positive and negative controls, another control slide was included in the process with known positivity for either 18a or 18b to make sure that everything in the procedure fell within the parameters and requirements for successfully staining microRNAs 18a and 18b [79].

4 Results

4.1 Statistical Analysis

For the statistical analysis of the datasets presented in this thesis, the software IBM SPSS Statistics (version 26) was used. From the program, Kaplan-Meier survival curves were drawn for relevant data along with calculations of their log rank p values. Using Cox regression, the relative significance of the variables was assessed and the hazard ratio (HR) along with the 95% confidence interval (CI) range were reported. The TNBC database contained 271 patients (195 of which were assessed for prognostic characteristics) while the CISH database contained 42 cases. Due to the small sample sizes, values that fall under 0.1 for log rank p value were considered significant while those greater than 0.1 were not further analyzed. Both univariate and multivariate analyses were done for the TNBC database, while only a univariate analysis was done for the CISH data base along with generating box plots for significant variables [30].

4.2 Triple Negative Breast Cancer Database

4.2.1 Univariate data analysis

For the 271 patients in the TNBC database, the median age at diagnosis was 55 years old and the median for the follow-up time was 128 months. In this thesis, the survival-related characteristics for those that were alive and well (AW, n=113), those with local reoccurrence (A/LR, n=5) and those dead of the disease with distant metastasis (DoD/DM, n=77) at the end of their follow-up status (eofus) (n=195 in total) were reported below in Table 10.

From the analyzed characteristics, only those with a log rank p value < 0.1 were considered significant due to the small sample size. As can be seen in Table 10., the age at the time of diagnosis had a p-value of 0.001 (HR=2.053, 95% CI=1.329–3.171). This was divided into two categories in the program, those of age ≥55 and those of age<55 (annotated as Age_55 in SPSS). TILs had a p-value of 0.044 (HR=0.567, 95% CI=0.323–0.993), the fibrotic focus (FF) a p-value of 0.017 (HR=1.670, 95% CI=1.092–2.55), the lymph node status (l_status) a p-value of 0.000 (HR=0.300, 95% CI=0.181–0.497), and finally the tumor type had a p-value of 0.045 (HR=0.462, %95 CI =0.213–1.001). These characteristics were taken further for a multivariate analysis. The rest of the variables can be seen displayed in Table 10 below.

Table 10. Univariate data analysis for triple-negative breast cancer patients. Events included were for all recurrences (AW, A/LR, and DoD/DM).

Characteristics				
Norway	**Events / At Risk %**	**Log Rank p Value**	**HR**	**95% CI**
Age				
< 55	34/135 (75%)	0.001	2.053	1.329 – 3.171
≥ 55	52/133 (61%)			
Tumor Size (cm)				
< 2	31/106 (71%)	0.471	1.181	0.751 – 1.859
≥2	47/152 (69%)			

Nottingham Grade				
1	1/ 2 (50%)	0.652	0.436	0.056 – 3.370
2	12/44 (73%)			
3	51/169 (70%)		0.535	0.073 – 3.884
MAI				
< 10	22/85 (74%)	0.201	1.375	0.842 – 2.247
≥ 10	58/174 (67%)			
TILs				
< 15	65/188 (65%)	0.044	0.567	0.323 – 0.993
≥15	15/70 (79%)			
Fibrotic Focus				
Absent	39/149 (74%)	0.017	1.670	1.092 – 2.554
Present	47/119 (61%)			
Fibrotic Focus Size				
< 1/3 of the tumor	39/98 (60%)	0.587	1.235	0.576 – 2.646
> 1/3 of the tumor	8/21 (62%)			
Lymphocyte Infiltration				
Yes	71/225 (68%)	0.582	0.855	0.490 – 1.493
No	15/43 (65%)			
Necrosis				
Absent	50/154 (68%)	0.941	0.984	0.641 – 1.510
Present	36/113 (68%)			
Lymph Node Status				
Positive	43/104 (59%)	0.000	0.300	0.181 – 0.497
Negative	24/131 (82%)			
Tumor Type				
Ductal	79/230 (66%)	0.045	0.462	0.213 – 1.001
Other	7/38 (82%)			

4.2.2 Multivariate data analysis

Presented below in Table 11, is the multivariate data analysis for the characteristics selected from the univariate data analysis of the previous section. Using cox regression and applying the method “forward wald” in IBM SPSS, the characteristics with the strongest prognostic values for survival were the lymph node status of the patients, followed by the age at the time of diagnosis (Age_55) and lastly the fibrotic focus (FF). The tumor type and TILs were not significant enough and thus exempt from showing up in the final results.

Table 11. Multivariate data analysis for the characteristics: lymph node status, fibrotic focus, and age_55 in patients with recurrence. The tumor type and TILs were included in the run but were not significant enough to appear.

Omnibus Tests of Model Coefficients			
Step	Overall (score)	Change From Previous Step	Change From Previous Block

	-2 Log Likelihood	Chi-square	df	Sig.	Chi-square	df	Sig.	Chi-square	df	Sig.
1[a]	785.070	34.597	2	.000	33.151	2	.000	33.151	2	.000
2[b]	776.843	42.679	3	.000	8.226	1	.004	41.378	3	.000
3[c]	770.263	48.990	4	.000	6.581	1	.010	47.958	4	.000

a. Variable(s) Entered at Step Number 1: Lymph node status

b. Variable(s) Entered at Step Number 2: Age divided into two groups 55

c. Variable(s) Entered at Step Number 3: Fibrotic focus

d. Beginning Block Number 1. Method = Forward Stepwise (Wald)

Kaplan-Meier recurrence-free survival curves were created from the significant factors determined by the multivariate analysis for those that were AW, A/LR and DoD/DM. Since the lymph node status had the highest prognostic value, it was set up as the strata, while the other characteristics were compared to it as factors.

Lymph node status vs Age of diagnosis (Age_55):

Table 12 shows the comparison between the lymph node status and the age at the time of diagnosis when the group is split into two. From the table, it is clear that age is not a strong prognostic factor for patients diagnosed with lymph node positive BC with p=0.314. On the other hand, the values for patients diagnosed with lymph node negative BC are p=0.049, thus significant enough to be considered for this thesis.

Table 12. Overall comparison between the lymph node status of patients and the age at the time of diagnosis when split into two groups (Age_55) for all recurrences.

Overall Comparisons

Lymph node status		Chi-Square	df	Sig.
positive	Log Rank (Mantel-Cox)	1.013	1	.314
	Breslow (Generalized Wilcoxon)	1.051	1	.305
negative	Log Rank (Mantel-Cox)	3.873	1	.049
	Breslow (Generalized Wilcoxon)	3.447	1	.063

Test of equality of survival distributions for the different levels of Age divided into two groups 55.

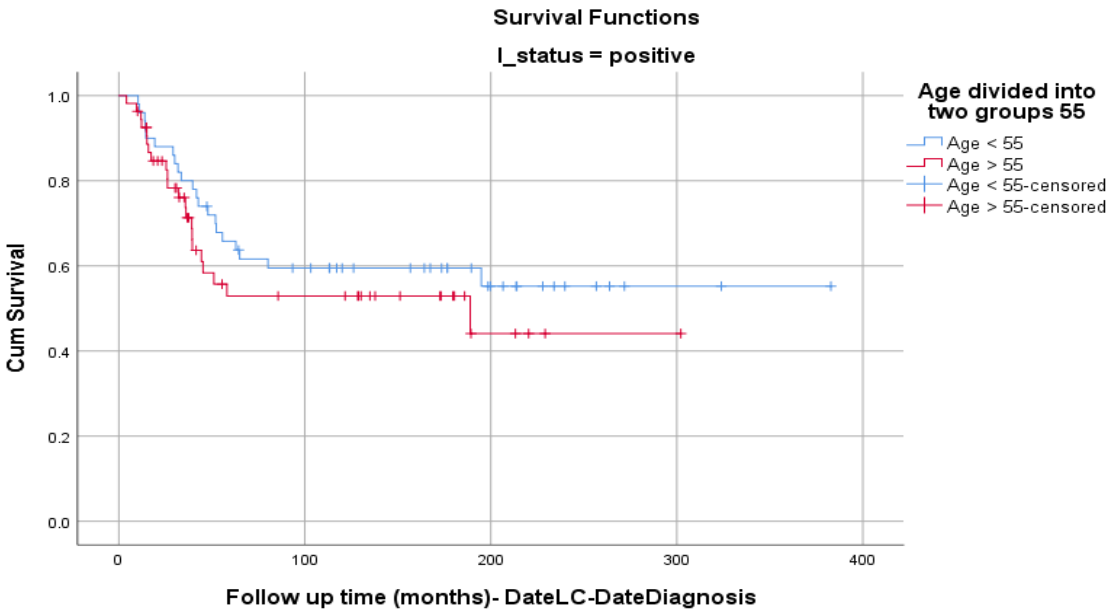

Figure 11. Kaplan-Meier recurrence-free survival curve for the correlation of age at the time of diagnosis (Age_55) for lymph node positive breast cancer patients.

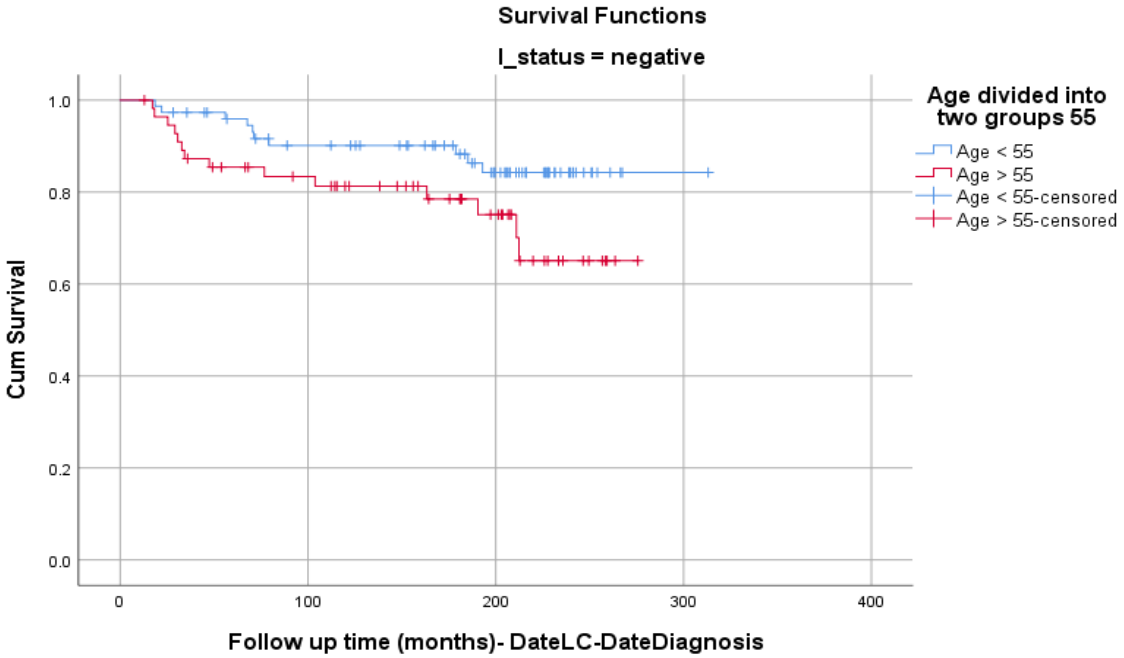

Figure 12. Kaplan-Meier recurrence-free survival curve for the correlation of age at the time of diagnosis (Age_55) for lymph node negative breast cancer patients.

There is a greater separation between the two groups (Age_55) for lymph node negative BC patients than for those with lymph node positive BC when Figures 11 and 12 are observed.

Lymph node status vs Fibrotic focus:

Table 13 shows the correlation between the absence or presence of fibrotic focus when it comes to the lymph node status of the patients diagnosed. From the table, it can be seen that lymph node negative patients when correlated with fibrotic focus show the most significance (p=0.008), and quite the opposite case is seen for lymph node positive patients (p=0.236). This trend is similar to how the age at the time of diagnoses correlated with the lymph node status of the patients.

In Figures 13 and 14, the Kaplan-Meier recurrence-free survival curves can be seen for both scenarios. In Figure 13, an overlap is observed between the groups with both fibrotic focus present and absent, rendering the results insignificant. A greater separation is observed visually in Figure 14 for the lymph node negative patients as those who are lymph node negative but also do not have fibrotic focus present display a better survival rate.

Table 13. Overall comparison between the lymph node status of patients and the presence/absence of fibrotic focus in the tumor tissue.

Overall Comparisons

Lymph node status		Chi-Square	df	Sig.
positive	Log Rank (Mantel-Cox)	1.402	1	.236
	Breslow (Generalized Wilcoxon)	1.200	1	.273
negative	Log Rank (Mantel-Cox)	7.085	1	.008
	Breslow (Generalized Wilcoxon)	9.004	1	.003

Test of equality of survival distributions for the different levels of Fibrotic focus.

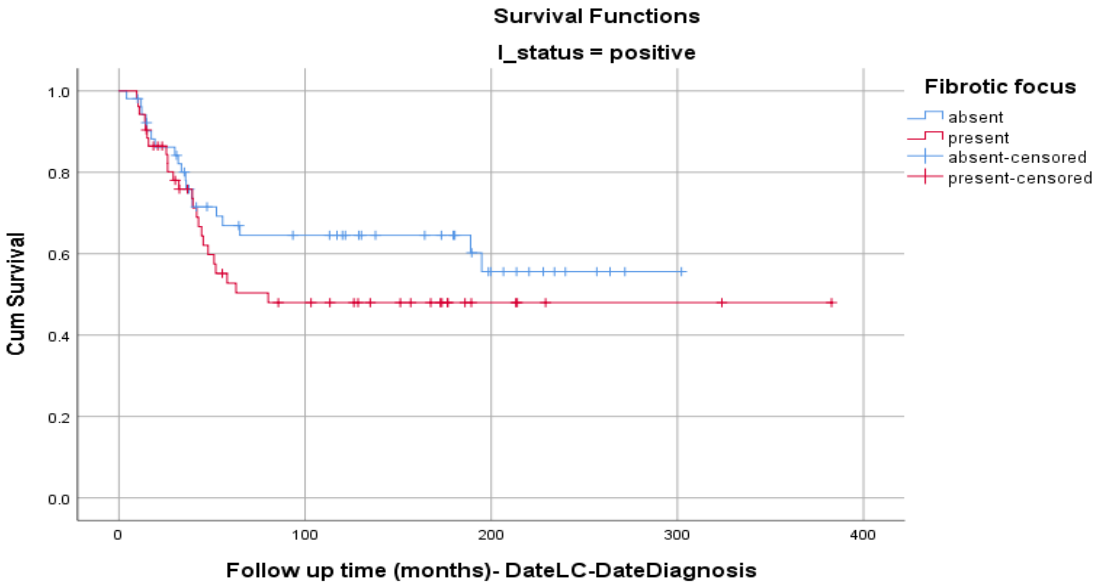

Figure 13. Kaplan-Meier recurrence-free survival curve for the correlation of the absence or presence of fibrotic focus for lymph node positive breast cancer.

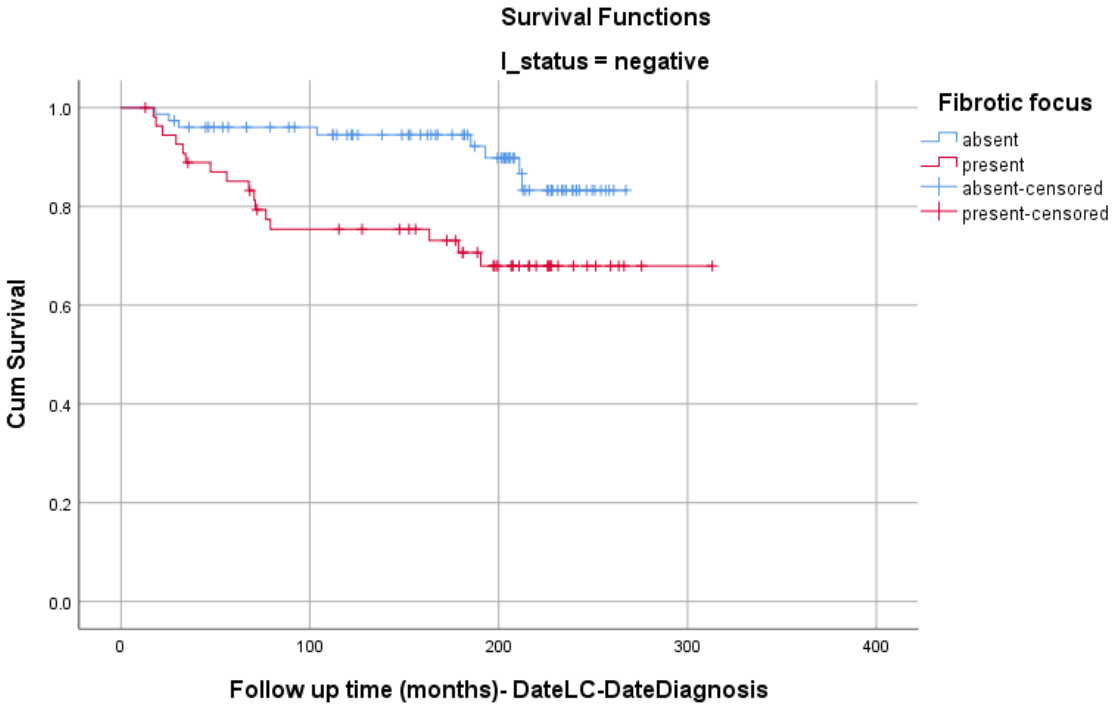

Figure 14. Kaplan-Meier recurrence-free survival curve for the correlation of the absence or presence of fibrotic focus for lymph node negative breast cancer.

Lymph node status vs Age_55 with/without Fibrotic focus:

Four different groups were tested for by combining the age at the time of diagnosis and the absence or presence of fibrotic focus by constructing Kaplan-Meier survival curves. To differentiate between them and find the most significant one regarding recurrence-free survival, these four new variables were created in IBM SPSS to separate them as such; age <55 without fibrotic focus (annotated as 1), age <55 with fibrotic focus (annotated as 2), age ≥55 without fibrotic focus (annotated as 3) and age ≥55 with fibrotic focus (annotated as 4). From Table 14, lymph node negative patients have the highest significance (p=0.013) over lymph node positive patients (p=0.401). In Table 15, the percentage for survival is higher in groups 1 and 3 where fibrotic focus is absent compared to 2 and 4 where it is present, and this is true for both lymph node positive and negative patients. However, the percentages for survival rates are much higher in lymph node negative patients than in the lymph node positives.

Table 14. Overall comparison of the lymph node status when correlated with the four new variables created; age <55 without fibrotic focus (annotated as 1), age <55 with fibrotic focus (annotated as 2), age ≥55 without fibrotic focus (annotated as 3) and age ≥55 with fibrotic focus (annotated as 4).

Overall Comparisons

Lymph node status		Chi-Square	df	Sig.
positive	Log Rank (Mantel-Cox)	2.937	3	.401
	Breslow (Generalized Wilcoxon)	3.065	3	.382
negative	Log Rank (Mantel-Cox)	10.771	3	.013
	Breslow (Generalized Wilcoxon)	12.333	3	.006

Test of equality of survival distributions for the different levels of Age_FF.

Table 15. Case processing summary for the lymph node status against the four variables: age <55 without fibrotic focus (annotated as 1), age <55 with fibrotic focus (annotated as 2), age ≥55 without fibrotic focus (annotated as 3) and age ≥55 with fibrotic focus (annotated as 4).

Case Processing Summary

				Censored	
Lymph node status	Age_FF	Total N	N of Events	N	Percent
Positive	1	27	9	18	66.7%
	2	23	12	11	47.8%
	3	25	10	15	60.0%
	4	29	12	17	58.6%
	Overall	104	43	61	58.7%
Negative	1	44	2	42	95.5%
	2	31	8	23	74.2%
	3	32	6	26	81.3%

	4	24	8	16	66.7%
	Overall	131	24	107	81.7%

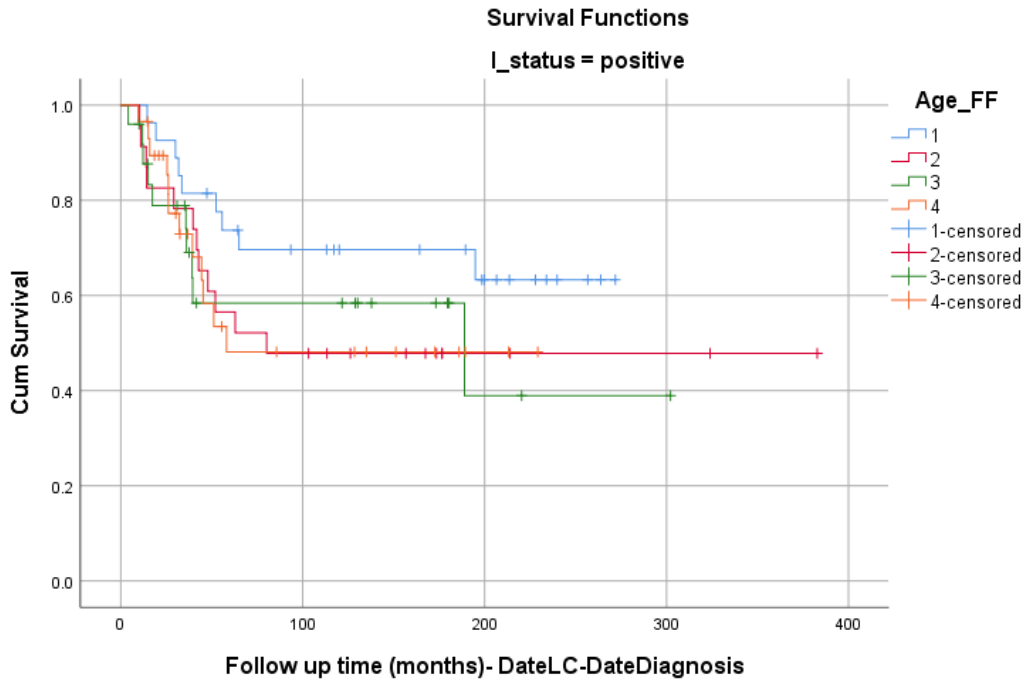

Figure 15. Kaplan-Meier recurrence-free survival curve for the correlation between the four new variables (1, 2, 3, 4) for selected patients with lymph node positive status.

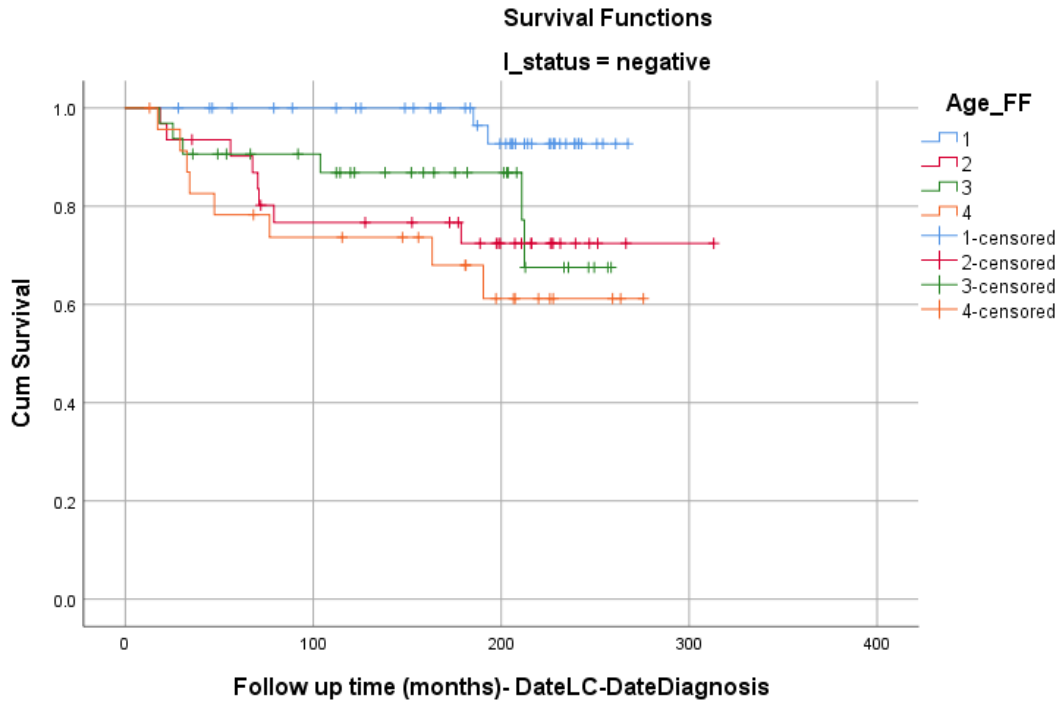

Figure 16. Kaplan-Meier recurrence-free survival curve for the correlation between the four new variables (1, 2, 3, 4) for selected patients with lymph node negative status.

Above in Figures 15 and 16, the Kaplan-Meier recurrence-free survival curves show that a clear separation exists between group 1 and the other three in lymph node negative patients as opposed to how it follows a similar path to the others in lymph node positive patients despite having a higher survival rate. To further analyze and compare this, a new variable was created to isolate group 1 as one factor while combining the other three into another one group annotated as "others". In Table 16, this new correlation and comparison between the lymph node status of the patients and the two groups can be seen. It is clear that those with lymph node negative status have a higher significance (p=0.003) than those with lymph node positive status (p=0.087). The significant value for the lymph node positive group is potentially due to the inclusion of patients with fibrotic focus absent in the calculation (from group 3).

Table 16. Overall comparison of the lymph node status when correlated with group age <55 without fibrotic focus (annotated as 1), and the other variables (2, 3 and 4) grouped into another variable annotated as "others".

Overall Comparisons

Lymph node status		Chi-Square	df	Sig.
positive	Log Rank (Mantel-Cox)	2.931	1	.087
	Breslow (Generalized Wilcoxon)	3.056	1	.080
negative	Log Rank (Mantel-Cox)	8.624	1	.003
	Breslow (Generalized Wilcoxon)	9.452	1	.002

Test of equality of survival distributions for the different levels of Age_FF_Two_groups.

In Table 17 below, the case summary can be seen. For lymph node positive patients, the survival rate for those younger than 55 without fibrotic focus is around 67%, while the other cumulative group has a survival rate of 56%. The difference of survival between the two is approximately 10%. Regarding the lymph node negative patients, the rate of survival for patients younger than 55 without fibrotic focus increases to approximately 96%, a significant increase in survival rate compared to lymph node positive patients, while the cumulative group displays an overall survival rate of 75%. The difference between the two is approximately 20%, a twofold increase

in survival for those that are lymph node negative compared to lymph node positive. In Figures 17 and 18, these correlations can be observed on the recurrence-free survival curves where a higher degree of survival and separation can be seen in the lymph node negative patients over the lymph node positives.

Table 17. Case processing summary of the lymph node status when correlated against group 1 (age <55 without fibrotic focus), and group 2 (2, 3 and 4) annotated as "others".

Case Processing Summary

Lymph node status	Age_FF_Two_groups	Total N	N of Events	Censored N	Censored Percent
positive	Age < 55, FF_absent	27	9	18	66.7%
	Others	77	34	43	55.8%
	Overall	104	43	61	58.7%
negative	Age < 55, FF_absent	44	2	42	95.5%
	Others	87	22	65	74.7%
	Overall	131	24	107	81.7%

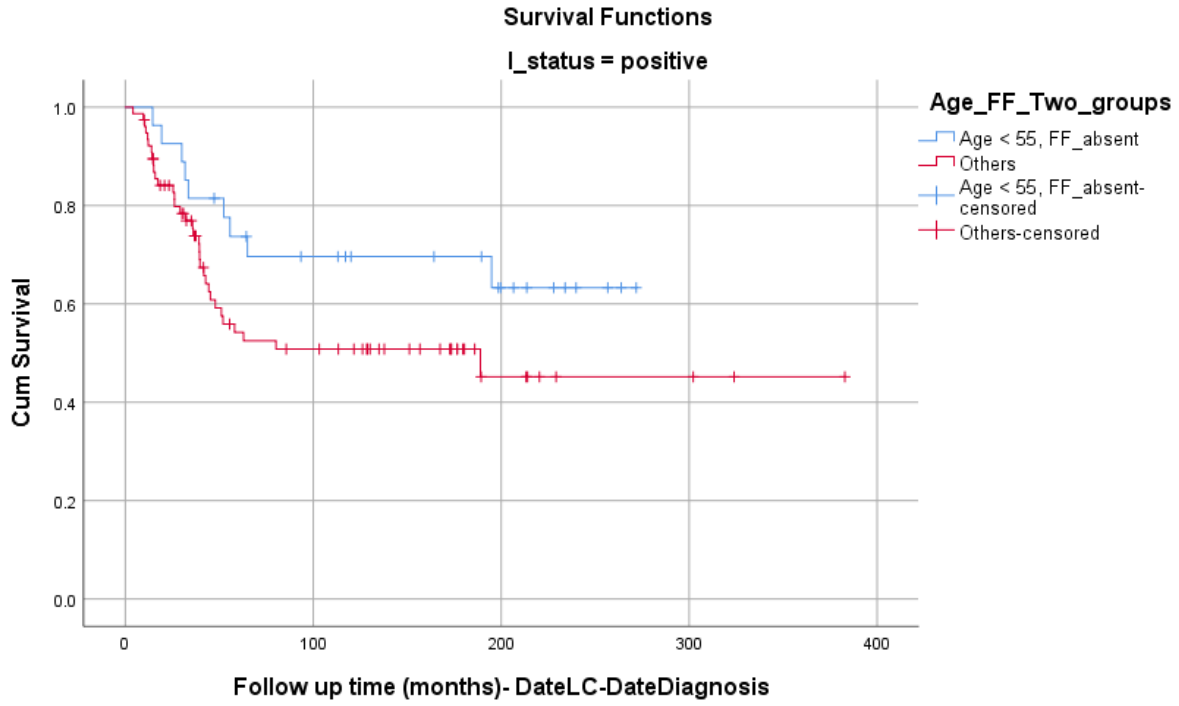

Figure 17. Kaplan-Meier recurrence-free survival curve for the correlation between group 1 and the cumulative group annotated as "others" containing factors 2,3 and 4 against patients with lymph node positive status.

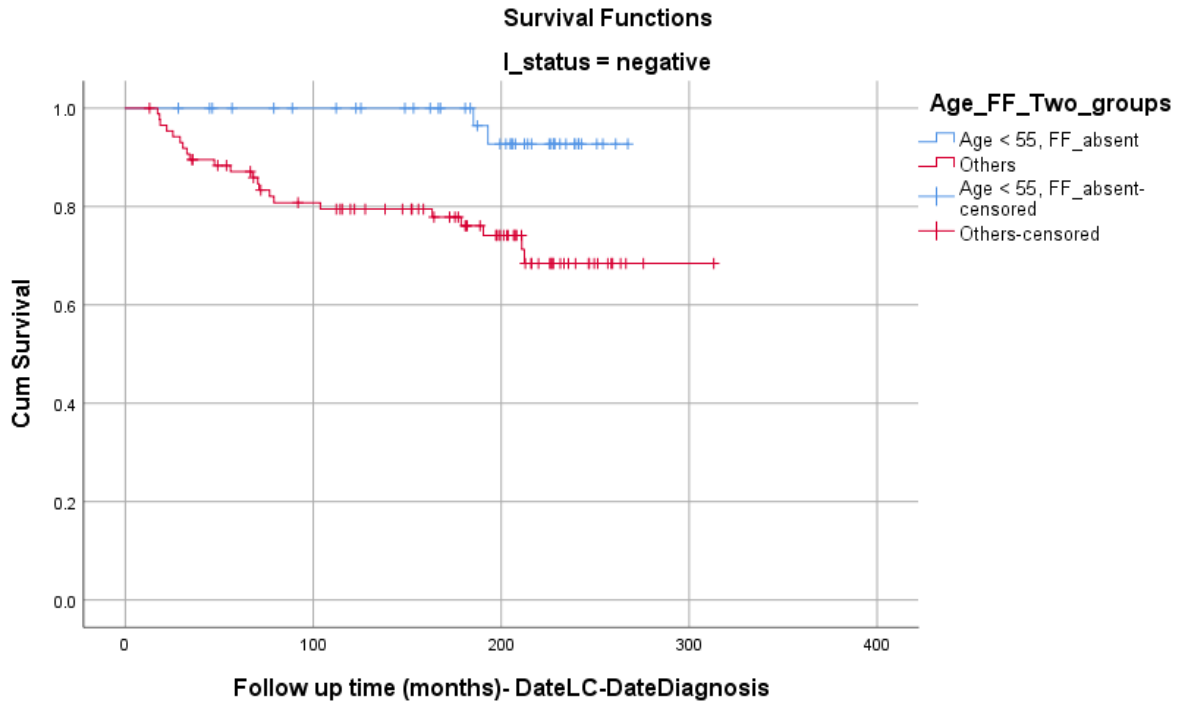

Figure 18. Kaplan-Meier recurrence-free survival curve for the correlation between group 1 and the cumulative group annotated as "others" containing factors 2,3 and 4 against patients with lymph node negative status.

4.3 CISH Database

4.3.1 Univariate data analysis

Forty-two cases (diagnosed between 1974 – 2004) were selected to undergo the CISH procedure to evaluate the expression of microRNAs 18a and 18b in the tumor tissue. Approximately half of the patients experienced recurrence in general (n=19) while the others did not (n=23). Below in Table 18., the tumor-related characteristics of TNBC can be seen when an individual sample T test is performed against microRNAs 18a and 18b. Due to the small sample size (n=42), characteristics with a significance (sig. (2-tailed)) less than 0.1 were considered.

For microRNA 18a, only two characteristics were considered significant, the Nottingham grade with a p-value of 0.066 and the tumor type with a p-value of 0.003. For microRNA 18b, four characteristics were significant, MAI with a p-value of 0.056, TILs with p-value of 0.028, lymphocyte infiltration with a p-value of 0.001 and the tumor type with a p-value of 0.004. From the significant factors, microRNA 18b had a higher count of stained cells when MAI was greater than or equal to 10 (median n=113), compared to when MAI was less than 10 (median n=39). For TILs, a percentage of 15% or higher correlated with a median count of 165 cells when compared to low TILs (<15%) having a median of 37 cells. A greater difference is seen for patients that have lymphocyte infiltration present with a median of 104 cells stained while only a median of 4 cells were counted for when lymphocyte infiltration was absent. Lastly, majority of

the cases for TNBC fell under “ductal” typing (for both microRNA 18a *and* 18b) with a median count of 103 cells for miR-18b, while those with other typing had a median count of 14 (again for miR-18b).

Table 18. Univariate data analysis for microRNAs 18a and 18b.

Characteristics								
Norway	**MicroRNA 18a**	**Mean**	**Range**	**Sig. (2-tailed)**	**MicroRNA 18b**	**Mean**	**Range**	**Sig. (2-tailed)**
Age								
< 55	18	2.06	0 - 8	0.351	18	52.72	0 - 351	0.146
≥55	24	3.71	0 - 38		24	121.5	0 - 872	
Tumor Size (cm)								
< 2	10	2.20	0 – 6	0.471	10	106.3	9 – 351	0.691
≥2	32	3.25	0 - 38		32	87.56	0 - 872	
Nottingham Grade								
1	0	-	-	0.066	0	-	-	0.286
2	4	1	0-2		4	49.25	1 – 153	
3	36	3.39	0 -38		36	101.6	0 - 872	
MAI								
< 10	12	2.75	0 – 8	0.827	12	38.67	1 – 153	0.056
≥ 10	30	3.10	0 - 38		30	113.4	0 - 872	
TILs								
< 15	24	1.79	0 – 7	0.210	24	37.17	0 – 351	0.028
≥15	18	4.61	0 - 38		18	165.2	15 – 872	
Fibrotic Focus								
Absent	20	2.5	0 – 10	0.615	20	111.2	0 – 872	0.493
Present	22	3.45	0 - 38		22	74.59	0 – 556	
Fibrotic Focus Size								
< 1/3 of the tumor	21	3.52	0 – 38	1.524	21	74.62	0 – 556	0.619
> 1/3 of the tumor	1	2	2		1	74	74	
Lymphocyte								

Infiltration								
Yes	37	3.27	0 – 38	0.142	37	103.9	0 - 872	0.001
No	5	1	0 - 5		5	3.80	0 – 10	
Necrosis								
Absent	21	2.86	0 – 10	0.885	21	54.52	0 – 351	0.149
Present	21	3.14	0 - 38		21	129.5	0 – 872	
Recurrence								
No recurrence	23	3.74	0 – 38	0.370	23	116.9	0 – 872	0.260
Recurrence	19	2.11	0 - 7		19	61.95	0 – 351	
Lymph Node Status								
Positive	24	1.87	0 – 8	0.288	24	52.87	0 – 351	0.142
Negative	12	5.42	0 - 38		12	177.17	0 - 872	
Tumor Type								
Ductal	37	3.41	0 – 38	0.003	37	102.5	0 – 872	0.004
Other	5	0	0		5	14.4	0 - 34	

A box plot was constructed to visually see if there was any difference between general recurrence and non-recurrence cases for the expression of microRNA 18b in the tumor cells, but as can be seen in Figure 19, not much difference was observed. Between the two categories, the box plot shows an almost equal distribution. However, a clear distinction can be seen when only the lymph node status is selected for the miR-18b expression. In Figure 20, lymph node negative patients have a higher separation compared to lymph node positives.

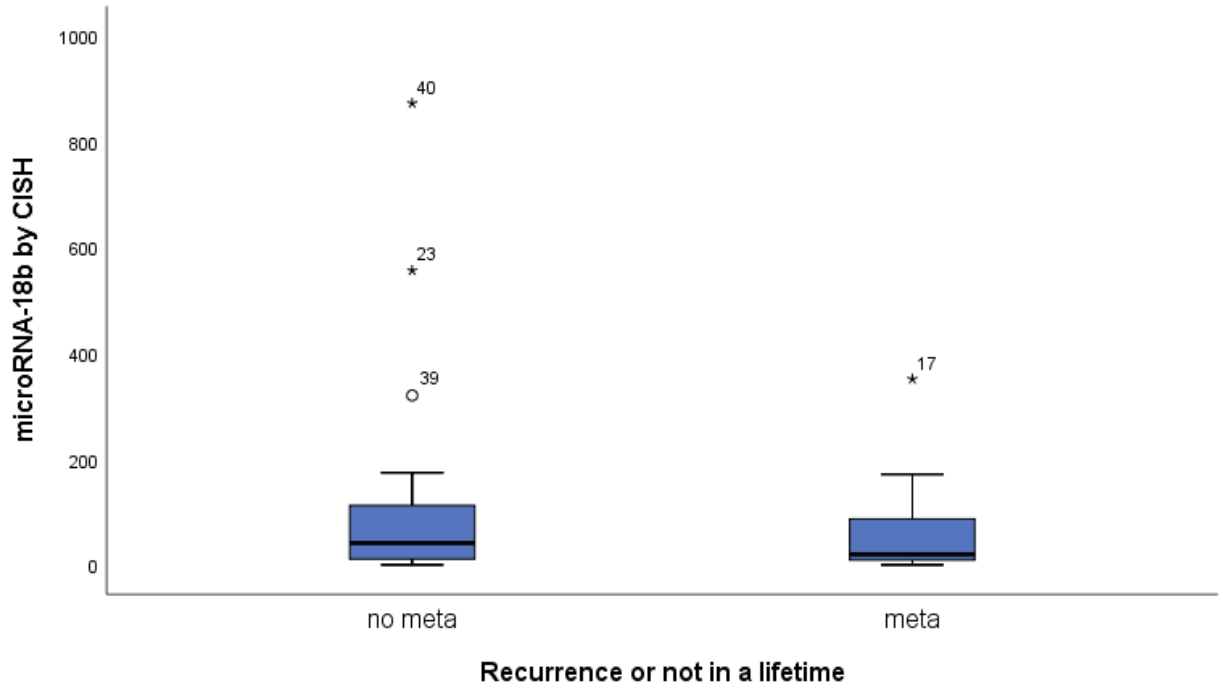

Figure 19. Correlation between microRNA 18b expression and general recurrence in TNBC patients.

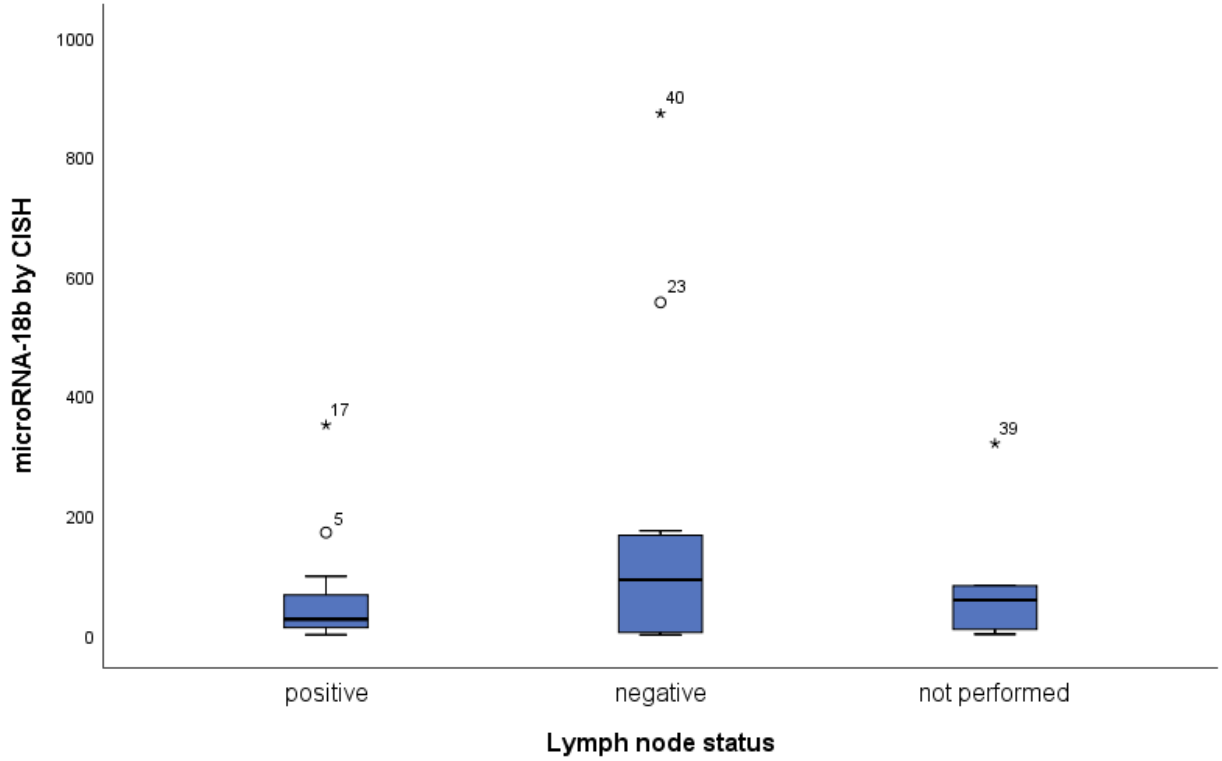

Figure 20. Correlation between microRNA 18b expression in lymph node positive and negative patients, including not performed cases.

Only lymph node negative patients were selected in SPSS, and another box plot was drawn to see if a difference would occur between the metastasis and no metastasis cases when lymph node positive patients were excluded from the selection. In Figure 21, an unequal distribution is observed between the two groups in the box plot. A greater expression of microRNA 18b exists in lymph node negative cells with no recurrence (n=8) compared to those who got recurrence in general (n=4). Below in Table 19, a summary of only the lymph node negative cases for microRNA 18b expression can be seen.

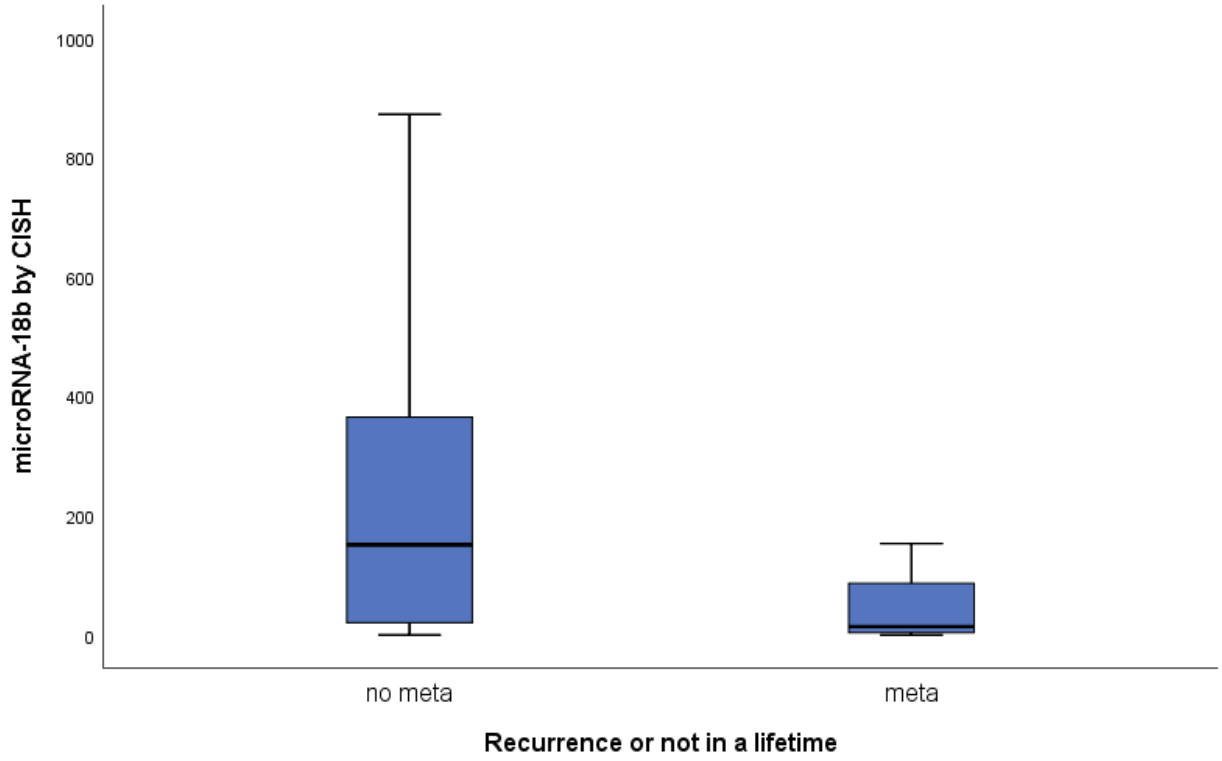

Figure 21. Correlation between microRNA 18b expression in lymph node negative TNBC patients.

Table 19. Summary of microRNA 18b expression in selected lymph node negative TNBC patients regarding the recurrence and non-recurrence of the cancer.

Lymph Node Negative Selected Cases			
Norway Recurrence	Micro RNA 18b	Mean	Range

No Recurrence	8	243.13	0 – 872
Recurrence	4	72.30	0 - 153

4.3.2 Expression and Patterns of MicroRNA's 18a and 18b in Tumor Tissue

The results obtained from the CISH procedure for microRNAs 18a and 18b can be seen in Figure 22 below. The digital imaging shows a 40x magnification of a specific area on the tumor tissue. For both microRNAs 18a and 18b (80 nM), strongly stained cells can be observed in the stromal area. For the control probe U6 (1nM), overall staining of the cells can be seen, while the negative control Scramble (80 nM) shows no signs of any stained cells. From Table 18., the count for microRNA 18a is very low for this selected group of 42 TNBC patients. For the cells that were counted for microRNA 18a, the majority of them displayed no distinct pattern. A few were found scattered randomly around in the stroma or inside the tumor, and if more than 3 were counted, they were found either at the edge of the tissue or surrounding a tumor mass in no particular manner.

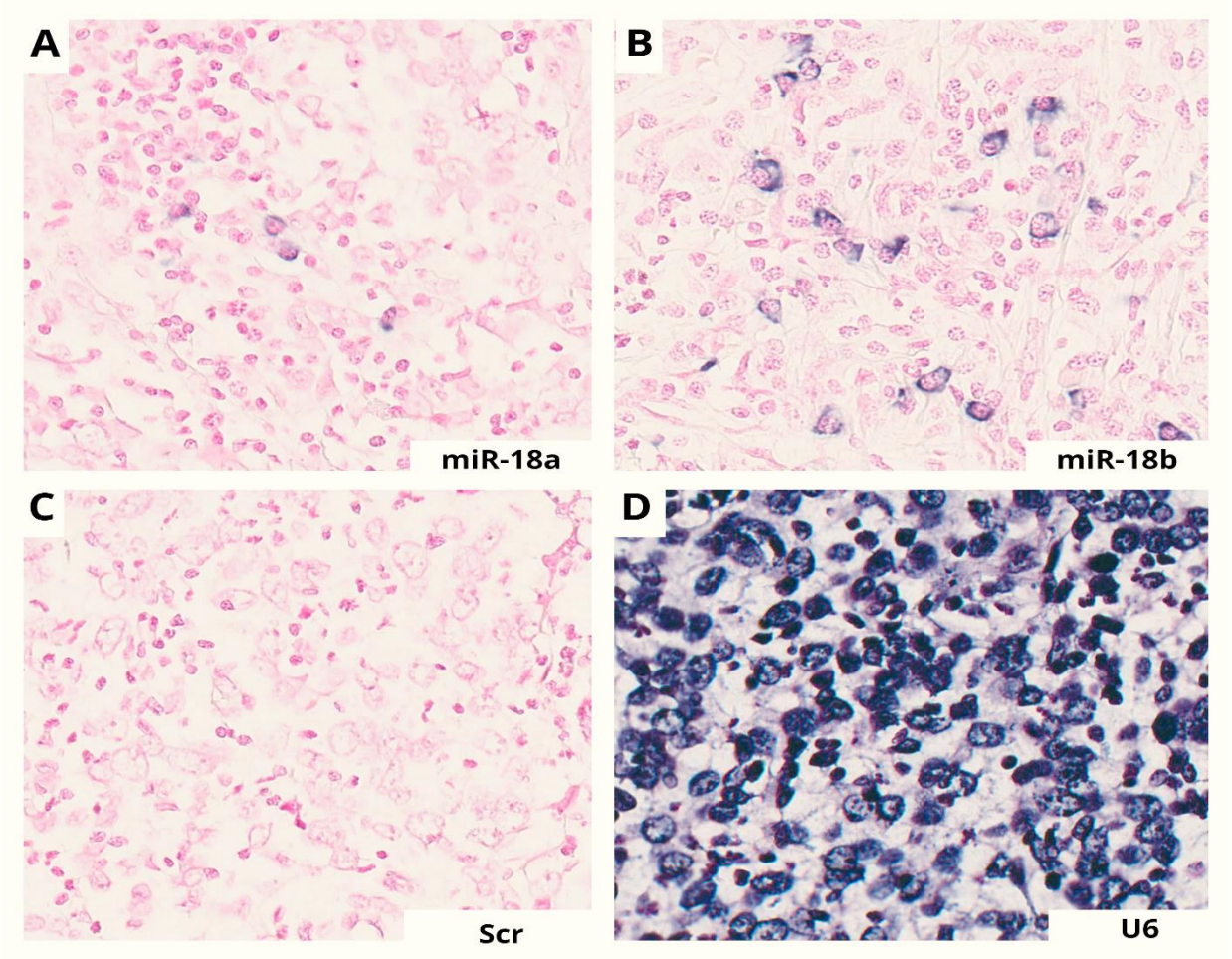

Figure 22. Expression of microRNAs 18a and 18b in TNBC along with U6 and Scramble as controls.

For microRNA 18b, an opposite observation is made. From the results listed in Table 18, the range of microRNA 18b-stained cells counted for, reached 872 at maximum. Thus, distinct patterns could be observed compared to miR-18a. Tissues with higher counts of stained cells were found surrounding the tumor mass in the stroma or concentrated heavily at the edges of the tissue. Whenever intratumoral staining was observed, the count inside the tumor was very low. However, when the stained cells surrounded the tumor mass, the count was very high. These patterns repeated themselves for the several the cases counted for in this thesis.

5 Discussion

5.1 Prognostic Markers for TNBC

5.1.1 Introduction

The first objective of this thesis was to find out whether there were any other prognostic factors aside from MAI and TILs for TNBC, and whether those factors held any merit in predicting treatment options for patients in the future. From the univariate data analysis pertaining to the 195 TNBC patients, the lymph node status, fibrotic focus, age, tumor type and TILs were the most significant characteristics with p-values lower than 0.1 (Table 10). The survival rates were the highest for those under the age of 55 (75%), without fibrotic focus (absentees had a percentage of 74% survival), with TILs more than 15% (79%), lymph node negative status (82%) and tumor types other than ductal (82%). When correlated in a multivariate analysis, the TILs and tumor type disappeared, and only the age at the time of diagnosis, the lymph node status and fibrotic focus remained as the most significant prognostic markers from the data set. These three factors will be further discussed in the next sections.

5.1.2 Age at the time of diagnosis

There is a clear relationship between cancer development in women, and the age at which they are diagnosed. Previously mentioned in the aetiology and epidemiology section of breast cancer, about 8 in 10 women develop this disease around the age of 50 [72]. More so, for TNBC patients, it is quite common for women to be diagnosed before even reaching the age of 50. This is most likely due to the inheritance of a faulty BRCA1/2 gene from either parent. When a harmful variant of either one of these genes is inherited, the risk of breast cancer appearing in these younger women increases markedly [63]. In Norway, an estimation of 15-20% of cases diagnosed are due to women inheriting a BRCA1 mutation gene from their family lineages. Here in Rogaland southwest Norway, the most incidences of BRCA1 gene mutation are found to be approximately 3-4% more than anywhere else in the country [88].

Still, age seems to be a good indicator for determining whether a patient is more likely to develop basal-like BC like TNBC or other types of BC like luminal A or B (which are less common in younger women). The differentiation provides an insight into further treatment options [45]. With the median age of diagnosis being 55 from Table 10, it can be seen that those above the age of 55 or equal to have a lower survival rate of 61% compared to those younger than 55 which have a survival rate of 75%.

With most research regarding TNBC focusing on younger patients diagnosed with the disease, older patients are often left unrepresented in clinical trials. With an aging population, there is a significant rise of TNBC being diagnosed in the elderly, which cannot be ignored anymore [89]. A study done in Sweden by Tzikas et al. compares whether the disease is the same or worse when diagnosed in the elderly, compared to when it is diagnosed in young women. The experiment was done to determine whether any differences could be observed between the two groups regarding the biology, recurrence rate, survival etc., for the primary diagnosis of TNBC. The cutoff age to separate young patients from older patients were set at 40 and 74 years respectively. Though they could not demonstrate that older patients were diagnosed with a more advanced TNBC from the beginning, the study showed that the survival rates were markedly worse for the elderly regardless. This is likely due to adjuvant chemotherapy not being given to majority of elderly patients in the study compared to younger patients [89]. The availability of chemotherapy to younger patients seems to be the differentiating factor between their survival rates when put against those of the elderly and this is probably similar for the Norwegian cohort as well. At the end of their follow-up status, treatments given to older patients might stop permanently. In the likelihood of a recurrence happening, the chances for survival will be low due to the general decline of their health. Even though exceptions should be made for fit elderly people that can still undergo treatments, this proposal is often met with hesitance as older patients are more sensitive to adverse events occurring, as these treatments can be very taxing to the body. However, the age at the time of diagnoses still gives a window into what options might be available for certain patients and holds a predictive prognostic value that is worth looking more into.

5.1.3 Lymph Node Status

The results between patients diagnosed with lymph node negative TNBC compared to those with lymph node positive TNBC show a vast difference in their survival rates, with the survival rate for those with lymph node negative status increasing 23% (Table 10). This lines up with what is already expected of the lymph node status. If the status is positive, then cancer cells have already been found in at least one of the axillary lymph nodes if not spreading more, increasing the staging and grade at which the disease is diagnosed. If the status is negative, none of the axillary lymph nodes contain any cancer cells, therefore increasing survival rates. Several studies have already been performed to confirm these prognostic and predictive values of the lymph node status for determining treatment options for affected TNBC patients [25].

When a patient first comes for a diagnosis, methods such as the sentinel lymph node biopsy (SLNB), axillary lymph node dissection (ALND) and other pathological methods are commonly used to evaluate the status in TNBCs [25]. To prevent lymph node metastasis, sometimes ALND can sufficiently be done to reduce the risk, but patients suffer from side effects such as numbness, stiffness in the upper body and also lymphedema with a chance of extra-axillary lymph node metastasis still happening [25]. From the data used for this thesis, the lymph node status was the most significant factor of the three characteristics of TNBC from the multivariate analysis (Table 11). In a study performed by Yin et al., 849 women with TNBC from China (the Shanghai Cancer Center/Hospital of Fudan University) where analyzed to better understand this relationship between the lymph node status and its prognostic value in a large cohort of patients

[94]. From among the results discovered for the lymph node status, the survival rate was 89% for those with negative status. As the lymph node numbers increased, the survival rate began to drop to 81%, 66% and 58% respectively for N1, N2 and N3 patients [94]. They observed a large drop in survival between N1 patients and N2, potentially suggesting more intensive chemotherapy is needed for patients that have N2-N3 grade [94]. This confirms that the more cancer cells start to migrate in the lymph nodes, the worse the prognosis gets for TNBC patients. The negative lymph node status provides a positive trend towards survival and should be explored further due to its great prognostic value.

5.1.4 Fibrotic Focus

The fibrotic focus is the last of the significant factors from the multivariate data analysis done on the TNBC database. The absence of it indicated a higher survival rate for patients with 74% while its presence showed a 61% survival rate from the univariate analysis. Previous studies have already reported on the fact that fibrotic focus tends to be more present in invasive ductal carcinomas that display aggressive characteristics, much like TNBC [41]. As majority of this data base contains tumors of ductal typing, the results obtained in this thesis project line up with what of what previous researchers have found. One study done by Hasebe et al. was to investigate whether the presence of FF changed the outcome of survival for patients with IDCs. The purpose was to determine if FF really was a good prognostic marker. Four hundred thirty-nine cases of IDC of the breasts between the years 1993 to 1999 were used for the base of their study at the National Cancer Center Hospital East in Japan. The study concluded with the presence of FF being a useful parameter in predicting tumor recurrence and distant metastasis, especially in lymph node negative IDCs and IDCs positive for either ER or PR, or both in some cases. They also concluded that the absence or presence of FF was an independent prognostic marker for lymph node negative IDC patients [41]. Those results point in the same direction as the Norwegian patients examined in this thesis project. In both figures 4 and 6, the absence of FF shows a significantly higher survival rate than when it is present in lymph node negative patients. Looking at another study done on FF, Baak et al. (2005) explored the prognostic value of MAI and FF in 448 patients with lymph node negative BC, with patients younger than 55 that did not undergo adjuvant systemic treatment. From that cohort, FF was determined to show strong prognostic value and inter-observable reproducibility, as well as being an additional prognostic value to the MAI in invasive ductal or mixed-ductal BC patients when the score was less than 10 [7].

The reason behind why the presence of FF in TNBC patients predicts a poor prognosis is yet to be understood. A study done by Ding et al. explored this question in their study where they defined the fibrotic grades in TNBC patients based on evaluating their FF. They concluded that high fibrosis, marked by increased collagen fibers, was an independent adverse prognostic factor in TNBC, and that it also showed interactions with TILs. Tumors that had high fibrosis were characterized by having an immunosuppressive TME with minimal immune cell infiltration and an increase in fibroblasts. The main focus of their study, however, was to explore the molecular features of fibrosis in TNBC at the genomic and transcriptomic levels to further understand the reasoning behind its poor prognosis. A relation was drawn between high fibrosis and the potential formation of an immunosuppressive tumor microenvironment through the activation of

the TGF-β pathway [26]. This could explain why the presence of FF leads to poor prognosis in TNBC patients. They mentioned in their study that this might be the first systematic analysis done on fibrosis in TNBC patients that is comprised of pathological evaluation and multiomics data. Unfortunately, not a lot of work has been done in the past on the absence or presence of FF in BC and currently not enough is available to draw full conclusions and understandings on FF. However, with more studies exploring the significance of FF in BC, new perspectives around the mechanisms behind FF can hopefully be achieved in the near future [26].

5.2 Prognostic Value of MicroRNAs 18a and 18b in TNBC

5.2.1 MicroRNA 18a

From the univariate analysis for microRNA 18a, only the tumor grade and tumor type showed any significance as independent factors correlating to miR-18a with p-levels of 0.066 and 0.003 respectively. Between the two microRNAs, miR-18a is the one most studied individually across the board in relation to its ability to offer ways to facilitate cancer screenings, diagnoses, and treatments for multiple different types of cancers. As a microRNA that exhibits a dual function in cancer progression, miR-18a has the ability to overexpress factors involved in signaling pathways that either promote or inhibit oncogenesis with most research focused on signaling pathway targets that are involved with cell proliferation, EMT, and TME. It is unique due to its conserved sequence that is responsible for the multistep biogenesis at the Drosha-mediated levels in essential biological and pathological processes [86]. In breast cancer (especially TNBC), miR-18a was found to contribute to tumor progression. In a study done by Zhang et al., miR-18a showed significant decrease of expression in highly lung-metastatic sublines from parental BC cells after an in vivo selection of the breast cancer cells with highest metastatic potential were selected, followed by further validation of the results by qPCR. Within the study, they found that the 3′-UTR of sterol regulatory element-binding transcription protein 1 (SREBP1) was a target for miR-18a. High expression of this protein correlated with worse survival outcomes for patients than those with lower SREBP1, potentially opening up the door for SREBP1 to be further explored as its own prognostic biomarker in the future [95].

The fact that miR-18a is linked to tumor progression makes sense with how the tumor grade and typing were significant factors relating to it according to the independent T-tests from the univariate data analysis. The number of cells expressed in the tissues were very low for most of the cases in this thesis project, which suggests that miR-18a was very downregulated. This lines up with the results obtained in Zhang et al.'s study as well. However, there are other studies that point to the overexpression of microRNA 18a in TNBC patients contributing to the ability of cancer cells to survive under stressful conditions, such as developing a resistance to chemotherapy by utilizing autophagy and maintaining homeostasis [6]. Enforced miR-18a overexpression directly leads to increased autophagy levels, similar to what happens when the mTOR signaling pathway is inhibited (the rapamycin effect), contributing to the development of paclitaxel (PTX) resistance for chemotherapies containing it [6][84]. In another study, the overexpression of miR-18a also directly led to Dicer repression at the mRNA and protein levels while also reducing PTX induced apoptosis in TNBC patients undergoing chemotherapy [84]. With PXT being a widely used mitotic inhibitor-based chemotherapeutic reagent for treating

TNBC, it is common for resistance to develop in patients as it is administered in both neoadjuvant and adjuvant treatments. The resistance to PTX in TNBC cells is a complex matter tied to mechanisms like the upregulation of the P-glycoprotein, a mutation in the drug target, changes in the cell cycle and more. For analyzing the results, qRT-PCR and Western blot analysis were used [84]. It is clear that the methods of measuring miR-18a used in both of those studies were different to the CISH protocol employed in this thesis, so there is unfortunately no direct comparison to be drawn from them. Still, insight into how the upregulation or downregulation of miR-18a operates in TNBC patients is valuable information that should be further explored using the same or different methodologies to conclude whether it's a good prognostic biomarker for this disease or not.

5.2.2 MicroRNA 18b

Just like miR-18a, miR-18b has been studied for its potential as a biomarker for breast cancer for a while, though not as much has been explored for it in comparison. From the data analyses, MAI ($p=0.056$), TILs ($p=0.028$), lymphocyte infiltration ($p=0.001$) and tumor type ($p=0.004$) were the most significant factors correlating to it independently. Comparing the maximum positive cells counted for miR-18a to miR-18b, there is a vast difference between the two with the maximum number of cells counted for miR-18a being 38 while 872 cells were counted for miR-18b. In this small sample of 42 patients, miR-18b is clearly very upregulated and expressed in the cells around the tumor tissues. From the bigger TNBC data base analysis, the lymph node status of the patients was the most significant factor, and for the CISH data base, the lymphocyte infiltration correlated strongly with miR-18b. In an attempt to see whether any distinction would occur between the expression of miR-18b in the different lymph node statuses of the patients, box plots were made. A clear upregulation of miR-18b can be observed in lymph node negative patients compared to lymph node positive patients and those not performed cases (Figure 20). Interestingly though, there really is no distinction between recurrence and non-recurrence cases in expression of miR-18b unless only lymph node negative TNBC patients are selected (Figures 19, 21). Only then can a clear distinction be observed in the box plot.

In breast cancer, miR-18b has shown to negatively regulate the estrogen receptor (ER)-α signaling while also showing high expression in ER-α negative samples [33]. In another study, low expressions of miR-18b led to improved survival rates for patients with the breast cancer subtype HER2 negative breast cancer. Though not clear as to what the role of miR-18b is regarding its functions and targeting sites for cancer cells, a study done by Fonseca-Sanchéz et al. have potentially identified receptors and genes linked to miR-18b that play key roles in cell migration and metastasis [33]. It has been shown that miR-18b is overexpressed, especially in TNBC (17-fold). An inverse relationship exists between the expression of miR-18b and hormonal receptors HER2/neu in breast cell lines and tumors, which supports the other studies done previously on the inhibition of ER-α (a target of both miR-18a and miR-18b) being due to miR-18b [33]. Much like miR-18a, further research is needed before miR-18b can be declared a good prognostic marker for TNBC.

5.3 Limitations

This thesis has its limitations. Firstly, the sample sizes of the two databases analyzed statistically were smaller than desired. Both the TNBC group (n=195) and the CISH group (n=42) had a cutoff point for the log rank p-value of <0.1, when the recommended value is usually p<0.05. This means that the results obtained from this thesis should be approached with caution and further validated to confirm whether or not the conclusions drawn are sound.

Secondly, most of the patients from both databases were recorded to have undergone adjuvant chemotherapy for at least a week (either FEC or CMF according to the guidelines at the time of their diagnosis) though some patients were also listed under other types of treatment [71]. Without all the patients having undergone the same treatment, there is no way of knowing whether or not the expression of microRNAs 18a or 18b affected the survival rate differently due to the differing treatments some patients underwent at the time and the durations they were treated for after their tumors were removed. The same is true for the other factors in the bigger TNBC data base [71].

Thirdly, some studies tested for the regulation of the microRNAs in vitro cells, which are not comparable to the FFPE tissue blocks used for CISH in this thesis [33]. The CISH procedure itself is a very sensitive method where slight changes in temperature or fixation times can result in very low numbers of microRNA 18a and 18b being expressed. Studies that utilized other methods for detecting the microRNA, like qRT-PCR can not be compared to the results obtained from CISH as both methodologies differ greatly in how they are performed and could provide conflicting results.

6 Conclusion

The findings of this thesis point to the lymph node status being the most productive prognostic marker for TNBC, especially if the patients are confirmed to be lymph node negative as that carries a predictive value towards their survival rate. When coupled with the age at the time of diagnosis (<55) and an absence of fibrotic focus, the chances of their survival increase drastically. For the microRNAs tested in this thesis, no significant correlation could be drawn between microRNA 18a, and the factors relating to TNBC to establish any prognostic value. Even though opposing results have been found for microRNA 18a in TNBC patients and reported in several studies, it is undeniable that its upregulation or downregulation still possesses oncogenic effects that can be utilized to further understand how it interacts with signaling pathways that assist in suppressing cancer cells. For microRNA 18b, a trend towards its expression being a good prognostic marker in lymph node negative TNBC patients has been observed. Though no therapeutic targets have yet been found for it, a correlation is drawn between miR-18b with MAI, TILs, the tumor type and lymphocyte infiltration, so further research into that should be looked into. This thesis has showcased the importance of the lymph node status as a prognostic marker in TNBC patients, and the potential for microRNA 18b to act as a prognostic marker in lymph node negative TNBC patients.

7 Bibliography

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