High AURKA expression in Triple Negative Breast Cancer correlates with expression of CIP2A and PME-1

A Dissertation Presented in Partial Fulfilment of the Requirements for the Degree of Master of Science in Pathology

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To my nephews,

Logan and Arthur

L-Università ta' Malta

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Abstract

Triple negative breast cancer (TNBC) is a heterogeneous breast cancer subtype which lacks expression of receptors usually targeted in breast cancer therapy. Thus, treatment options rely only on generalised chemotherapy, which carries cytotoxic consequences. Studies have shown that the tumour suppressor, protein phosphatase 2A (PP2A) was found to be deregulated in approximately 60% of TNBCs. The aims of the study are to evaluate protein expression of AURKA and KIF2C as biomarkers of interest in relation to PP2A activity in a cohort of TNBC patients, to identify biomarkers correlating with AURKA and KIF2C at RNA level, to confirm the selected biomarkers at protein level in the TNBC cohort, and in TNBC cell models following treatment with PP2A activator FTY720.

Protein expression of AURKA and KIF2C was investigated in 160 TNBC cases and 23 normal breast epithelia by immunohistochemistry. Overexpression of these biomarkers was correlated with RNA profiles (40-gene panel, established in previous studies) assessed using a branched DNA assay to identify potential complimentary biomarkers related to AURKA and KIF2C expression. Protein expression of these biomarkers together with proliferation marker Ki67 was assessed on TNBC cell line models (N=6) treated with PP2A activator, FTY720 and on the TNBC patient cohort.

72% of TNBC patients overexpressed AURKA protein while 56% showed KIF2C protein expression. PP2A negative regulators CIP2A and PME-1 were found to be positively correlated with AURKA and KIF2C RNA and protein expression. No significant reduction was observed in TNBC cell lines following treatment FTY720.

The AURKA, CIP2A and PME-1 biomarker signature identifies a potential therapeutic group within the TNBC cohort characterised by PP2A deregulation. Furthermore, inhibitors targeting these biomarkers should also be considered for evaluation in TNBC.

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List of Abbreviations

ACTB	Actin, beta
ALK	Anaplastic lymphoma kinase
AGR2	Anterior gradient 2
ANP32A	Acidic nuclear phosphoprotein 32 family member A
APC	Anaphase promoting complex
APS	Ammonium persulfate
AURKA	Aurora kinase A
AURKB	Aurora kinase B
BCL-2	B-cell lymphoma 2
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
BTAK	Breast tumour amplified kinase
CA12	Carbonic anhydrase 12
CCN1	Cellular communication network factor 1
CDC25	Cell division cycle 25
CDH1	Cadherin 1
CDH12	Cadherin 12
CDK	Cyclin-dependent kinases
CDKN1B	Cyclin-dependent kinase inhibitor 1B
CHK1/2	Checkpoint kinases ½
CIP	CDK interacting protein
CIP2A	Cancerous inhibitor of PP2A
CTNNB1	Catenin beta 1
DAB	3,3'-Diaminobenzidine
DCIS	Ductal carcinom in-situ
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMI1	Early mitotic inhibitor 1

EN1	Engrailed Homeobox 1
ER	Estrogen receptor
ERBB3	erb-b2 receptor tyrosine kinase 3
ERBB4	erb-b2 receptor tyrosine kinase 4
ERK	Extracellular regulated kinase
ESR1	Estrogen receptor 1
FBS	Foetal bovine serum
FDA	U.S. Food and Drug Administration
FGFR2	Fibroblast growth factor receptor 2
FN1	Fibronectin 1
FOXC1	Forkhead box C1
FOXM1	Forkhead box M1
GABRP2	Gamma-aminobutyric acid type A receptor Pi subunit
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA3	GATA binding protein 3
H&E	Haematoylin and eosin
HER2	Human epidermal growth factor receptor 2
HIF1A	Hypoxia inducible factor 1 alpha subunit
HPRT1	Hypoxanthine phosphoribosyl transferase 1
ID2	Inhibitor of DNA binding 2
IC_{50}	Half maximal inhibitory concentration
IGF-1	Insulin-like growth factor 1
IHC	Immunohistochemistry
JAK	Janus associated kinase
KIAA1524	Cancerous inhibitor of protein phosphatase 2A
KIF2C	Kinesin family member 2C
KIP	Kinase inhibitory protein
LATS2	Large tumour suppressor 2
LCIS	Lobular carcinoma in-situ
LI	Labelling index

MAP	Microtubule associated protein
MCAK	Mitotic centromere associated kinase
MFI	Median fluorescence intensity
mRNA	messenger RNA
mTOR	Mammalian target of rapamycin
NASP	Nuclear autoantigenic sperm protein
NF1	Neurofibromatosis type 1
PARP	poly ADP ribose polymerase
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	PI3K catalytic subunit α
PINK1	PTEN induced putative kinase 1
PIP2	phosphatidylinositol 4,5-biphosphate
PIP3	phosphatidylinositol 3,4,5-triphosphate
PLK1	Polo-like kinase 1
PPME-1	Protein phosphatase methylesterase 1
PP2A	Protein phosphatase 2A
PPIB	Peptidyl prolyl isomerase B
PPP2CA	PP2A catalytic subunit α isoform
PPP2CB	PP2A catalytic subunit β isoform
PPP2R2A	Protein phosphatase 2 regulatory subunit B, alpha
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
RAS	Rat sarcoma
RB	Retinoblastoma 1
RPMI	Roswell Park Memorial Institute
S6K1	S6 kinase beta-1
SAPE	Streptavidin phycoerythrin
SCNN1A	Sodium channel epithelial 1 alpha subunit
SET	SET nuclear oncogene

SETBP1	SET binding protein 1
STAT	Signal transducer and activator of transcription
STK15	Serine/threonine kinase 15
SV40	Simian virus 40
TACC	Transforming acidic coiled-coil
TBP	Tata box binding protein
TBS	Tris buffered saline
TCGA	The Cancer Genome Atlas
TEMED	Tetramethylethylenediamine
TFF3	Trefoil factor 3
TMA	Tissue microarray
TNBC	Triple negative breast cancer
TNM	Tumour, Node, Metastasis
TSC1/2	Tuberous sclerosis complex 1/2
TWIST1	Twist family BHLH transcription factor 1
VEGFA	Vascular endothelial growth factor A
VEGFA	Vascular endothelial growth factor B
WEE1	Wee1-like protein kinase

Chapter I
Literature Review

1.1 Introduction

Triple negative breast cancers (TNBC) are a type of breast cancers which exhibit a high degree of heterogeneity and constitute more than 170,000 breast cancer cases around the world annually, comprising 15-20% of all breast cancer cases. The main characteristic feature of this type of cancer is that it lacks the expression of the following biomarkers: oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2/neu receptors (HER2) (Foulkes, Smith, & Reis-Filho, 2010).

To date, there are no potential therapeutic groups within this subtype, treatment relying only on chemotherapy with generalised cytotoxic effects. In a study by Baldacchino et al. (2014), the tumour suppressor protein phosphatase 2A (PP2A) was found to be deregulated in approximately 60% of basal-like class. The majority of TNBC cases fall into this class and approximately 80% of basal-like breast carcinomas are reported as triple negative breast cancer (Alluri & Newman, 2014).

PP2A is known to be reactivated via pharmacological use of FTY720. Previous studies from our research group have identified three TNBC cells lines to be sensitive to FTY720. These studies have also identified AURKA and KIF2C as biomarkers of PP2A deregulation in TNBC based on RNA expression of these biomarkers.

A subset of TNBC patients can benefit from PP2A reactivation as a therapeutic option, based on expression of biomarkers which reflect PP2A deregulation. Thus, our aims are to identify biomarkers which are complimentary to AURKA and KIF2C expression in a cohort of TNBC patients and to investigate the effect of PP2A reactivation on the expression of these biomarkers in TNBC.

1.2 Breast cancer

In 2012, there were approximately 14 million new cancer cases and 8 million deaths attributed to cancer worldwide. Breast cancer is the most commonly diagnosed cancer in females (25.2% of totals), also having the highest incidence rate (43.3 per 100,000) and the highest mortality rate in females (12.9 per 100,000) (Stewart & Wild, 2014).

There are several risk factors which can lead to breast cancer, mainly being hereditary, hormonal and reproduction related. These include nulliparity, having the first child after the age of 30, use of oral contraceptives, and use of hormone replacement therapy at menopause (Nelson et al., 2012). Etiological factors related to one's lifestyle include consumption of alcohol, inadequate nutrition and lack of physical activity (Stewart & Wild, 2014). A smaller fraction of cases is due to genetic predisposition due to the presence of mutations in Breast Cancer gene 1 (BRCA1) and Breast Cancer gene 2 (BRCA2) (Brenner et al., 2016).

1.2.1 Classification of breast cancer

Breast cancer is a complex and heterogeneous pathology, biologically, morphologically and clinically. The simplest classification of breast cancer can be based on the tumour's status, if it is in-situ (pre-invasive) or has developed invasive properties. In-situ can be further subdivided into ductal carcinoma in-situ (DCIS) or lobular carcinoma in-situ (LCIS), based on the histological and cytological features of the breast cancer. DCIS is considered to be more aggressive and carries a higher risk of becoming invasive. Ductal invasive carcinoma is frequently described, upon histology, as "not otherwise specified" or of "no special type" based on complexity and intratumour and intertumour heterogeneity, which are characteristics of breast cancer. This comprises 70% of all breast cancers (Vuong, Simpson, Green, Cummings, & Lakhani, 2014).

An important classification of breast cancer is that based on receptor status, mainly expression of hormonal receptors, namely oestrogen receptors (ER) and progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2). The presence or absence of these receptors determine prognosis and what treatment options are available for that type of breast cancer. Lack of expression of these receptors is a characteristic of the breast cancer subtype TNBC (Foulkes et al., 2010).

Furthermore, breast cancer can be subdivided into luminal A, luminal B, HER2-enriched and basal-like. The luminal subtype can be further sub-classified into type A and type B. Type A exhibits ER and/or PR positivity but is negative for HER-2 together with a low Ki67 expression, while Type B is either HER2 negative with a high Ki67 expression or HER2 positive. On the other hand, the majority of the basal-like subtype is characterised by a lack of expression of ER, PR and HER2 receptors (Goldhirsch et al., 2011).

The luminal A subtype is defined by low histological grade and good prognosis and constitutes 50 to 60% of all breast cancer cases and is also characterised by a low frequency of p53 mutations. On the other hand, luminal B breast cancers are more aggressive and present as high-grade tumours with worse prognosis and a high proliferative index. Expression of proliferation genes is high, and these tumours also exhibit a higher frequency of mutations in p53 (Cancer Genome Atlas Network, 2012). The basal-like subtype constitutes 10-20% of breast cancers and exhibit a high proliferation and high grade at histology contributing to a poor prognosis. A high frequency of p53 mutations together with loss of retinoblastoma 1 (RB1) is noted in these tumours (Perou, 2011).

1.2.2 Prognosis and therapeutic options

Target therapeutic options for breast cancer are based on drugs which arrest cancer growth by aiming to restrict tumour cell proliferation and survival via action on specific molecules.

Specific molecules are overexpressed in certain types of breast cancer which result in downstream signalling leading to gene expression for cancer cell growth, survival and proliferation (Pal, Childs, & Pegram, 2011).

Histological grade is an important element in the determination of prognosis for a patient. Three parameters of a tumour are assessed in what is known as the Nottingham histological grading system. These are how well the cellular architecture is differentiated, the nuclear grade of pleomorphism and the mitotic count/index. Each parameter is given points on a scale of one to three and then added together. The final total will determine what grade the tumour is, the lower the grade the better the prognosis for the patient. Prognosis is not solely based on histological grading. The expression of hormone receptors is also a valid contributor to patient prognosis and choice of therapeutic options (Cao & Lu, 2016).

Breast cancer staging is also an important determinant for prognosis upon which therapeutic decisions also depend. This involves tumour size (T 1-4), involvement of lymph nodes (N 1-3) and distant metastases (M 0-1), in what is known as the TNM classification. Stage 0 breast lesions are removed via lumpectomy if lesions are less than 5mm in diameter. Larger lesions are treated with adjuvant radiation therapy. Higher stage tumours are treated via surgical resections, chemotherapy and endocrine therapy following hormone receptor status evaluation (Anjum, Razvi, & Masood, 2017).

Primary breast cancers which are ER-positive present with a good prognosis with 92% of patients having a 5-year survival without undergoing chemotherapy. ER-positive breast cancer correlates strongly with low tumour grade and lower levels of p53 tumour suppressor gene loss. ER-positive patients classify for anti-oestrogen treatments, namely selective ER modulators such as tamoxifen, aromatase inhibitors and fulvestrant which is an oestrogen antagonist. Tamoxifen has been shown to reduce recurrences by 40-50%. Aromatase

inhibitors, such as anastrozole, letrozole and exemestane, act by inhibiting the aromatase enzyme thus blocking the biosynthesis of androgens leading to lower levels of oestrogen in cancer cells (Den Hollander, Savage, & Brown, 2013). On the other hand, PR positive tumours lack targeted therapeutic options but are strongly associated with ER positive tumours. Treatment of breast cancer cell lines with oestrogen led to increased levels of progesterone since it is a target gene of oestrogen activation (Cao & Lu, 2016).

HER2 overexpression is associated with a poor prognosis, yet a good predictive marker considering the current therapeutic options. HER2 is a tyrosine kinase, part of the epidermal growth factor receptor (EGFR) family, and its overexpression is associated with a high relapse rate (Cao & Lu, 2016). HER2-positive breast cancer is treated with the monoclonal antibody trastuzumab (Herceptin) (Goldhirsch et al., 2011).

The biomarker Ki67 is stained immunohistochemically in order to determine the proliferation state of the tumour since it is expressed in proliferative breast cancer cells. The Ki67 index is used to determine whether patients qualify for chemotherapy or not in ER-positive tumours. It can also be used to differentiate between Luminal A breast cancer subtype which has a low proliferative rate and a good prognosis and Luminal B breast cancer which presents with a higher proliferative rate and poorer survival (Pathmanathan & Balleine, 2013).

TNBC does not express any of the previously described receptors and therefore does not qualify for targeted therapeutic options. Therefore, treatment is based on general cytotoxic chemotherapy and prognosis is poor (Baldacchino et al., 2014). Given the lack of homogeneity, response to treatment depends on the molecular subtype of the tumour. Subtypes include basal-like 1, basal-like 2, immunomodulatory, mesenchymal, mesenchymal stem-like and luminal androgen receptor. For example, the basal-like 1 subtype has a better response to platinum agents than basal-like 2 subtype, while mesenchymal and luminal

subtypes are more sensitive to phosphatidylinositol 3-kinase (PI3K) pathway inhibitors. This heterogeneity has always proved problematic when it comes to novel therapeutic strategies. The pathologic complete response of TNBC is higher to neoadjuvant chemotherapy than that of ER-positive tumours and similar to those tumours which are HER2-positive. Nonetheless, TNBCs present with a higher risk of recurrence and a worse prognosis. This risk peaks at three years, but then exhibits a rapid decline. Recurrence of TNBC is commonly encountered in the lung and liver, with 15% of patients also having brain metastases. Recurrence is treated with cytotoxic chemotherapy with palliative care and quality of life determining dosing schedules (Ballinger, Kremer, & Miller, 2016). However, ongoing research is being conducted so as to find a more targeted treatment which also improves the quality of life of the patient. Since almost 70% of TNBCs present with 20% or more tumour infiltrating lymphocytes, immunotherapy has been considered an attractive choice to target TNBCs. Prognosis correlates with the volume of tumour infiltrating lymphocytes in the stroma. Nevertheless, results have shown that adjuvant chemotherapy was still necessary and combination treatments might be considered in the future (Szekely, Silbner & Pusztai, 2017). Clinical trials with platinum agents such as carboplatin and cisplatin are also being investigated in TNBCs. Platinum agent actions result in DNA crosslink strand breaks which lead to apoptosis in cells which are defective (Bianchini, Balko, Mayer, Sanders, & Gianni, 2016).

PI3K/AKT/mTOR and RAS/MEK/ERK pathway inhibitors are also potential therapeutic options, together with poly-ADP ribose polymerase (PARP) inhibitors. PARP inhibitors act on cancers with DNA-repair defects, such as TNBC with BRCA 1/2 mutations (Collignon, Lousberg, Schroeder, & Jerusalem, 2016). Afinitor (everolimus) is an mTOR inhibitor and acts by preventing energy to reach cancer cells. Avastin (bevacizumab) inhibits angiogenesis in various cancers including breast cancer (Masoud & Pages, 2017).

1.2 Triple negative breast cancer

As previously described, triple negative breast cancer (TNBC) is defined by the absence of hormone receptor expression, ER and PR and lack of HER2 expression. These factors are important for therapeutic decision making, absence of which leaves cytotoxic chemotherapy as the only viable option for treatment. The unavailability of these three biomarkers together with TNBC being an aggressive tumour leads to a poor prognosis for the patient (Onitilo, Engel, Greenlee, & Mukesh, 2009). TNBC also stains positive immunohistochemically for cytokeratins 5/6 and 17 and exhibits epidermal growth factor receptor (EGFR) overexpression. Genetically, there is a high P53 and MKI67 expression with low BCL-2 levels (Yadav, Chanana, & Jhamb, 2015).

TNBC accounts for approximately 15% of all breast cancers and is denoted by a short overall survival and a high risk of distant recurrence at three years post-diagnosis, with a reduction in recurrence probability afterwards and a high 5-year mortality rate. TNBC is most common in younger women (less than 40 years) with a high incidence in Afro-American women. Patient usually presents with advanced cancer staging, with a grade III tumour characterised by a higher proliferative index and lack of tubule formation (Reis-Filho & Tutt, 2008). Around 20% of patients also present with BRCA 1/2 mutations (Gonzalez-Angulo et al., 2011).

Histologically 95% of TNBCs are diagnosed as invasive ductal carcinoma of no specific type with marked nuclear pleomorphism and a high mitotic index but without any distinguishable histological characteristics. However other types have been described, such as invasive lobular carcinoma and metaplastic carcinoma with squamous and/or mesenchymal differentiation. Metaplastic carcinoma presents as high-grade lesions, with a high level of heterogeneity and have a worse outcome when compared to other TNBC types. Genetically, this type of carcinoma presents with aberrations in the Wnt and PI3K pathways (Pareja et al.,

2016). According to the Cancer Genome Atlas Research Network, TNBC and basal-like breast cancer were most commonly characterized by loss of p53, RB1, BRCA1 and PI3K catalytic subunit α (PIK3CA) (Collignon et al., 2016).

1.2.1 Molecular characteristics of TNBC

The molecular basis of TNBC has been a strong point of interest to account for the heterogeneity of this type of cancer based on common patterns of pathology and sensitivity to current and novel therapies. Some patients succeed in having an effective response to neoadjuvant chemotherapy with a good pathologic complete response at surgery, whilst other patients show no response, this further indicating the heterogeneous element of TNBC (Collignon et al., 2016).

In a study by Lehmann et al. (2011), six TNBC subtypes were identified based on gene-expression: basal-like 1, basal-like 2, mesenchymal, immunomodulatory, luminal androgen receptor and mesenchymal stem-like. In 2016, Lehmann et al. refined their TNBC subtyping into four groups, with the immunomodulatory and mesenchymal stem-like being removed from being major subtypes of TNBC (Marotti, de Abreu, Wells, & Tsongalis, 2017).

The basal-like 1 subtype has an elevated proliferation rate and is also characterized by aberrations in cell cycle checkpoint mechanisms. Ki67 mRNA expression is found to be amplified which is consistent with the high proliferation rate. On the other hand, the basal-like 2 subtype exhibits a higher expression of EGFR, TP63 and myoepithelial markers. EGFR is an essential factor for survival of the tumour cell, where it initiates a signal cascade leading to cell proliferation, angiogenesis, apoptosis inhibition and metastatic spread. EGFR expression is linked to a low response to chemotherapy (Marotti et al., 2017).

The mesenchymal subtype is characterized by epithelial-mesenchymal transition and growth factor pathways but exhibits a lower expression of genes involved in proliferation. Genes expressed in this subtype include the vascular endothelial growth factor 2 which is involved in angiogenesis and neovascularisation, and cell survival by promoting the expression of anti-apoptotic proteins. This subtype has a high response to PI3K/mTOR inhibitors (Yadav et al., 2015).

The luminal androgen receptor subtype involves androgen receptor signalling. Even though being ER-negative, pathways regulated by hormones are present in this subtype such as synthesis of steroids, metabolism of porphyrin and oestrogen. Patients in this category have a reduced disease-free survival and overall survival (Yadav et al., 2015).

A claudin-low type also exists in TNBC molecularly defined by epithelial-to-mesenchymal transition and stem-like features. However, luminal genes and genes related to proliferation are lowly expressed (Collignon et al., 2016). Spindle cell metaplastic carcinomas are classified under this category (Pareja et al., 2016).

Medullary carcinoma, a rare subtype of TNBC, has been identified via gene-expression profiling and has a better outcome than the other subtypes, being characterised by a high lymphoplasmacytic infiltration as well as well-defined borders with syncytial cellular growth and a high mitotic rate (Huober et al., 2012).

As previously described, 20% of TNBC cases present with BRCA 1/2 mutations, in what is known as “BRCAness”. The BRCA gene is important for DNA repair mechanisms and defects in these genes lead to tumour formation. BRCA genes are also involved in cell cycle control and transcription (Lips et al., 2013). Once DNA damage is detected at checkpoints,

there is arrest of the cell cycle, yet typical p53 mutations in TNBC inhibit this (Yadav et al., 2015).

TNBCs carry an average of approximately 60 somatic mutations in each tumour, with 1.68 somatic mutations per Mb of coding regions. These genetic aberrations result in multiple pathway changes, which is from where tumour formation starts (Bianchini et al., 2016). In 10% or more of TNBC cases, the two genes most commonly affected by somatic mutations were TP53 and PIK3CA. Other genes which were reported to be affected by somatic mutations in TNBC include phosphatase and tensin homolog (PTEN), RB1, NF1, BRCA1, ERBB3, ERBB4 and ALK. TP53, PIK3CA and PTEN mutations are attributes to early tumour formation whereas gene mutations involved in cell motility and epithelial-to-mesenchymal transitions are present in later phases of tumour evolution (Pareja et al., 2016). MYC oncogene amplification is a common aberration in TNBC, as well as amplifications of BCL2, CCN1, EGFR, FGFR2, ID2 and NOTCH3 (Marotti et al., 2017).

1.3.2 PI3K/AKT/mTOR pathway in TNBC

The PI3K/AKT/mTOR pathway is involved in the regulation of essential cellular activities such as cell metabolism, proliferation, motility and survival (Massihnia et al., 2016). PI3K amplification is seen in 60% of TNBCs, and activation of this pathway was reported to occur more frequently in the basal subtype (Baldacchino et al., 2014).

Aberrations in signalling pathways and related control mechanisms lead to cancer growth, survival and progression. Besides the PI3K/AKT/mTOR pathway, the JAK/STAT pathway is also involved in tumorigenesis. Other pathways, which are involved in disease progression other than tumorigenesis include the Wnt/β-Catenin, Notch and Hedgehog pathways (Chalakur-Ramireddy & Pakala, 2018).

PI3Ks are heterodimers, belonging to the lipid kinase family, and consist of regulatory (p85) and catalytic (p110) subunits. There are four isoforms of PI3K namely α, β, δ, and γ. Activation of the PI3K/AKT/mTOR pathways occurs via stimulation of receptor tyrosine kinases, including Insulin-like growth factor 1 (IGF-1) receptors and HER proteins. This results in activation of PI3K which in turn phosphorylates phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) which will act as a docking site for protein kinase B (AKT) (Baldacchino et al., 2014). This leads to AKT phosphorylation resulting in protein synthesis and cell growth via activation of mechanistic target of rapamycin (mTOR) through TSC1/2 (Borg et al., 2014).

mTOR is a downstream component of the pathway and can be found in two forms, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which have different functions. mTORC1 promotes mRNA translocation to activate protein translation. It is also involved in lipid synthesis and metabolism. S6K1 is a downstream substrate of mTOR, which activation leads to oestrogen phosphorylation. mTORC2 is a regulator of AKT phosphorylation and organizes the actin cytoskeleton. Activation of the mTOR pathway is higher in TNBC than in other breast cancer subtypes and is synonymous with a poor prognosis for the patient. Furthermore, up-regulation of the PI3K/AKT/mTOR pathway leads to resistance to various forms of treatment, namely hormone-based, HER2-targeted and cytotoxic treatment (Massihnia et al., 2016).

The phosphatases PTEN and PP2A act by dephosphorylating PIP3 and inactivation of mTOR effectors, respectively, thus generating an attenuating effect on the pathway. Their function is found to be downregulated in aggressive basal-like breast carcinoma. These two phosphatases together are known to antagonise AKT phosphorylation, thus a reduction in phosphatase function results in enhanced AKT activation. Reduced BRCA1 activity is also associated

with increased AKT activity and decrease in PP2A activity, since BRCA1 is associated with activation of PP2A (Xiang, Jia, Sherris, Li, Wang, Lu, & Yang, 2011).

1.3.3 Targeting the PI3K/AKT/mTOR pathway in TNBC

ER, PR and HER2 are receptors used to predict response to therapy in breast cancer patients. As already mentioned, breast cancer patients which are positive for these receptors benefit from therapeutic options which target these receptors. On the other hand, since in TNBC these are absent, other targets are being investigated. Since the PI3K/AKT/mTOR pathway is highly activated in TNBC cases, this provides an optimal choice as a therapeutic target. Considering the high level of heterogeneity in TNBC, one should consider targeting molecular aberrations so as to provide a more personalised treatment strategy from which 60-70% of TNBC patients would benefit. To date, successful drugs targeting the PI3K/AKT/mTOR pathway in TNBC are still lacking, yet early-phase clinical trials are investigating this possibility (Bianchini et al., 2016).

In a study carried out by Borg et al. (2014), Akt activity was investigated using the triple negative breast cancer cell line, HCC1937 which was reported to have a low PP2A activity counteracted by high Akt activation, and also being BRCA-mutated. The breast cancer cells were treated with the PP2A pharmacological activator FTY720 and sensitivity was reported within an hour, resulting in increased Akt dephosphorylation. Akt rephosphorylation was stimulated using IGF-1 but was blocked by FTY720. High Akt levels are a characteristic of a subset of TNBC patients which could benefit from therapeutic options targeting the PI3K/Akt/mTOR pathway and potential activation of PP2A.

PIK3CA mutations are the most commonly reported mutations in breast cancer and TNBC is no exception. Thus, inhibitors to this molecule are being investigated as a therapeutic option. The PI3K antagonist buparlisib (BKM120) is already in a phase 3 trial and has already shown

encouraging results with improved progression-free survival, when combined with fulvestrant in HER2 negative patients. Buparlisib is in a phase 2 trial in relation to TNBC treatment (Costa, Han, & Gradishar, 2018).

Another pan-PI3K inhibitor, pictilisib, is being considered as a therapeutic option for TNBC, but reports of adverse reactions have been discouraging. On the other hand, alpelisib, which is another PI3K inhibitor, is being investigated and in hormone receptor positive, HER2 negative patients taking part in a phase 1b trial, adverse reactions have been low (Costa et al., 2018).

Ipatasertib is an AKT inhibitor and was investigated in a study involving untreated metastatic TNBC, in combination with paclitaxel. There was significant improvement in median progression-free survival when compared to the placebo group with Grades 3-4 diarrhoea and neutropenia being reported as adverse events but without treatment-related weight loss or deaths (Costa et al., 2018).

The mTOR inhibitor everolimus has been showing promising results against cancer cells in preclinical studies. In TNBC, the use of rapamycin combined with paclitaxel to treat TNBC in cell lines altered in PI3K/AKT/mTOR signalling has shown effective results. Unfortunately, clinical trials in combination with platinum agents have not given significant results yet (Massihnia et al., 2016).

Various studies have reported that PARP sensitivity increases upon PI3K/AKT inhibition. Thus, suppression of PI3K leads to an increase in sensitivity to PARP inhibitors which in turn inhibit molecules involved in DNA repair. This leads to an increase in cancer cell damage, leading to cell death (Chalakur-Ramireddy & Pakala, 2018).

1.4 PP2A deregulation in TNBC

As previously mentioned, PP2A deregulation is a common event in various cancers and breast cancer is no exception, which deregulation results in tumour aggressiveness and a poor prognosis. PP2A is also implicated in the development of therapeutic strategies for TNBC, where it was also reported that it can re-induce ER expression in ER negative breast cancer, to the benefit of TNBC patients (Zhao, Li, Zhang, Qi, Diao, Zhen, & Shu, 2017).

According to the cBioPortal for Cancer Genomics, PP2A deregulation is present in approximately 60% of basal cell breast cancer. In breast cancer patients, cancerous inhibitor of PP2A (CIP2A) is overexpressed both at mRNA and protein level, and these tumours are characterised by a high grade and aggressiveness (Baldacchino et al., 2014). In a study which involved 40 breast cancer cell lines, the highest CIP2A expression was encountered in basal-like breast cancer cell lines (Janghorban et al., 2014).

1.4.1 The PP2A tumour suppressor complex

Nearly one-third of the cellular protein functions are subject to regulations via phosphorylation and dephosphorylations by protein kinases and protein phosphatases respectively. There are two families of protein kinases, namely tyrosine kinases and serine threonine kinases. On the other hand, three classes of phosphatases exist, the tyrosine phosphatases, serine/threonine phosphatases and the dual specificity phosphatases. The number of serine/threonine kinases exceeds the number of same family phosphatases by more than 10-fold. The ability of phosphatases to form several holoenzymes, with PP2A having the capacity of forming approximately 100 heterotrimeric holoenzymes, compensates for this. Kinases and phosphatases counteract each other in order to tightly control cellular signalling pathways (Wlodarchak & Xing, 2016).

PP2A is a heterotrimer composed of three subunits, the scaffold A subunit (65kDa), the regulatory B subunit, which is present in various forms and the catalytic subunit (C subunit, 36kDa). The C subunit has two isoforms, PPP2CA aka Cα and PPP2CB aka Cβ both being ubiquitously expressed, the α isoform ten times more abundant than the β isoform. This subunit is responsible for the dephosphorylation actions. The scaffold A subunit also has two isoforms, α and β which are described as being homologous. The B subunit, on the other hand, exhibits four unrelated forms: B (B55, PPP2R2), B' (B56, PPP2R5), B" (PR48/72/130, PPP2R3) and B''' (STRN/PPP2R4). This subunit resolves the substrate specificity and localization of PP2A in the cellular environment (Grech et al., 2016).

PP2A has the major role of being a tumour suppressor complex. It regulates several signalling pathways which if left unregulated will lead to uncontrolled cancer cell growth, proliferation and inhibition of apoptosis, including the aforementioned PI3K/AKT/mTOR pathway and the c-MYC pathway. PP2A also has an essential role in cell cycle regulation, being present in various phases throughout cell cycle progression (Sablina, Hector, Colpaert, & Hahn, 2010). Reduced PP2A activity has been reported in several cancer types such as colorectal cancer and breast cancer, further emphasising its role as a tumour suppressor complex (Baldacchino et al., 2014; Cristobal et al., 2014).

PP2A activity is counteracted by various inhibitors which when overexpressed lead to carcinogenesis. The phosphoprotein SET is a strong inhibitor of PP2A activity. It is found in both the nucleus and cytoplasm and interacts with the catalytic subunit of PP2A via its C-terminal domain. SET is overexpressed in several cancers namely myelogenous leukaemia and Wilm's tumour and is also involved in Alzheimer's disease. Another oncogenic PP2A inhibitor is the microbial toxin Okadaic acid. Other inhibitors include CIP2A, calyculin A, microcystin, cantharidin, nodularm, fostriecin and tautomycin, which are reported to inhibit PP2A activity at different IC_{50} values. Another potent inhibitor is the viral protein SV40

which also has a strong oncogenic potential and acts by displacement of the PR56 subunit leading to PP2A inactivation. Both inhibition states have been reported to lead to tumour formation, highlighting the role of PP2A as a tumour suppressor (Seshacharyulu et al., 2013).

Mutations in the B regulatory subunits (mostly B55 and B56) were also reported in various cancers. A low expression of the B55 subunit was reported in acute myeloid leukaemia. Since B55α acts by dephosphorylating Akt at T308, low expression of this subunit leads to Akt activation, resulting in increased cell proliferation. In lung cancer, B56γ mutations were reported from Phe395 to Cys. Since this subunit interacts with p53 to dephosphorylate Thr55 leading to tumour suppression, mutations in B56γ lead to uncontrolled cell cancer proliferation. To date, no mutations have been reported in the catalytic subunit as an attribute to cancer formation (Seshacharyulu et al., 2013). PP2A inactivation via phosphorylation at tyrosine 307 was found to be correlated with tumour progression in HER2-positive breast cancer (Wong, Zhang, Chang, & Koay, 2010).

Furthermore, PP2A negatively regulates c-MYC, an oncoprotein which is overexpressed in various subtypes of breast cancer. On the other hand, PP2A activity is deregulated via inhibition by overexpression of SET oncoprotein and CIP2A, leading to unhindered c-MYC activity and cancer progression. In a study by Janghorban et al. (2014), PP2A deregulation was also analysed in various breast cancer cell lines, which also concluded that CIP2A was found to be enriched in TNBC cell lines and is also associated with the claudin-low subtype. This study also suggests antagonizing PP2A inhibitors so as to target c-MYC.

It has also been observed that apoptotic resistance is a common occurrence in TNBC. In a study by Li et al. (2016), it was shown that caspase-dependent apoptosis was induced following depletion of CIP2A in two TNBC cell lines, MDA-MB-231 and MDA-MB-468.

CIP2A depletion has also been associated with reduced Akt phosphorylation, also inhibiting phosphorylation of mTOR and protein S6 kinase.

1.4.2 PP2A activation as a therapeutic option

PP2A can be activated as a therapeutic option since its activation will lead to cancer suppression via attenuated signalling pathways, which are involved in carcinogenesis. Since phosphatases such as PP2A are amply expressed in normal cells, targeting cells with low PP2A activity will be restricted to cancer cells, thus avoiding cytotoxic effects on normal cells which is a major drawback of general chemotherapy (Grech et al., 2016).

FTY720 (Fingolimod) is an activator of PP2A. It is an FDA-approved therapeutic option in multiple sclerosis. In a study by Cristobal et al. (2014) on colorectal cancer patients, which were characterised by inactivation of PP2A, FTY720 proved to be a valid activator of PP2A, resulting in suppression of colorectal cell proliferation.

FTY720 acts by directly forming a bond with SET/PP2A complexes followed by disruption of the complex, thus reactivating PP2A. FTY720 sensitivity was also observed in leukemic cell lines, and also in bladder cancer cells, ovarian cancer cells and breast cancer cells. FTY720 does not seem to affect non-cancer cells since it only acts on cancer cells, reducing their proliferation and inducing apoptosis (Grech et al., 2016).

Effects on PP2A were also extended to signalling pathways, since the activation of PP2A by FTY720 has been reported to affect the PI3K/AKT pathway. Activation of PP2A leads to regulated activity of AKT resulting in antitumor effects. Cell migration and invasion were also affected (Cristobal et al., 2016).

Baldacchino et al. (2014) have also investigated a set of breast cancer cell lines for FTY720 sensitivity. It was concluded that ER-negative cell lines are the most sensitive to lower doses of FTY720. Since the ER-negative cell line BT-20 was not affected by rapamycin treatment,

which is an mTOR kinase inhibitor, it further strengthens the argument that results achieved were due to pharmacological activation of PP2A by FTY720.

1.5 PP2A's role in the cell cycle

The cell cycle is a process which is highly organized and regulated, resulting in duplication of genetic material followed by cell division. Regulation is controlled by growth-regulatory signals accompanied by the action of assigned kinases and phosphatases which conduct monitoring and yield signals to ensure that there is no genetic impairment. The cell cycle consists of four stages which are G_o/G_1, S, G_2 and M. The progression through these stages is regulated by cyclin-dependent kinases (CDKs) in collaboration with their cyclin partners. Actions from CDKs are induced by mitogenic signals in a strictly controlled process and can be inhibited when cell cycle checkpoints are activated due to the presence of DNA damage. These checkpoints are also involved in the mitotic spindle assembly and stop the cell cycle to enable repair (Figure 1.1).

Cells which are not permanently in the G_o phase due to terminal differentiation, can be re-activated to enter the cell cycle when stimulated by mitogenic factors. These induce cell proliferation via signalling pathways. On the other hand, cancer cell proliferation does not depend on these mitogenic factors to proceed (Otto & Sicinski, 2017).

PP2A's role in tumour formation and suppression can be attributed to the fact that it dephosphorylates over 300 substrates during the cell cycle, being a major regulator in signalling pathways and cell cycle checkpoints. Previous studies have emphasized the role of kinases as sole key cell cycle regulators, with phosphatases being considered nothing more than housekeeping enzymes (Wlodarchak & Xing, 2016).

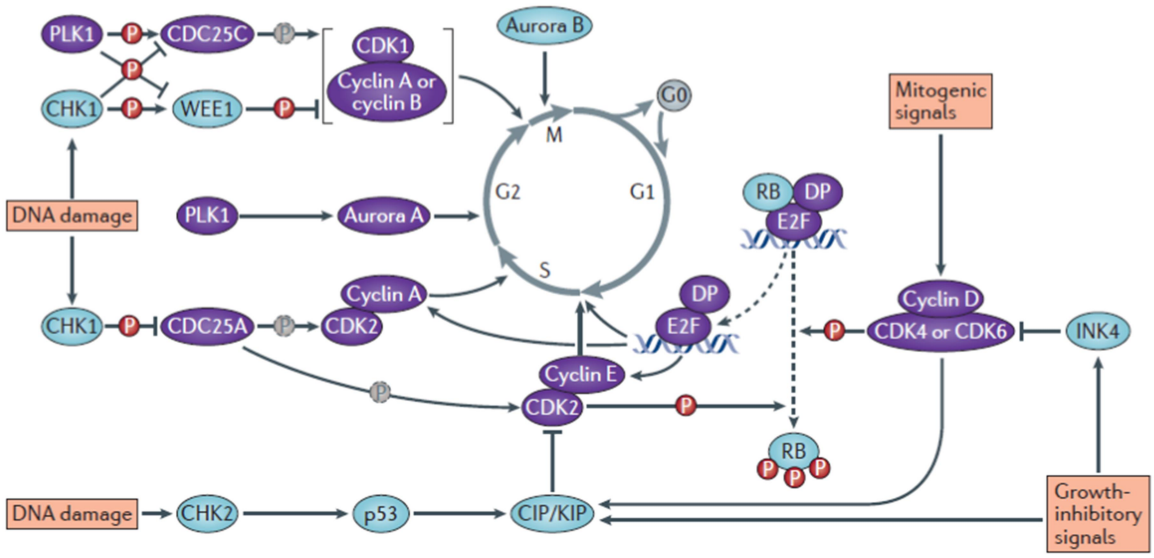

Figure 1.1 Progression of the cell cycle including regulatory proteins.

Cyclins and CDKs complexes are activated by mitogenic signals which in turn phosphorylate (P) specific targets to promote progression from the G1 phase into the S phase of the cell cycle. One of the targetted protein is RB which hyperphosphorylation leads to transcription activation by the E2 factor (E2F). Cyclin-CDK complexes also control entrance into the mitotic stage (M), together with kinases such as Polo-like kinase 1 (PLK1) and Aurora kinases (Aurora kinase A and Aurora kinase B). Checkpoint kinases such as CHK1 and CHK2 can arrest the cell cycle if DNA damage is detected during the cell cycle.

Legend: Purple ovals: positive regulators of the cell cycle. Blue ovals: negative regulators of the cell cycle. Dashed line: dephosphorylation. CDC25: cell division cycle 25. WEE1: Wee1-like protein kinase. CIP: CDK interacting protein. KIP: Kinase inhibitory protein.

In order to attain an error free cell division, CDC6 tightly regulates DNA synthesis, and is phosphorylated by cyclin E/CDK2 and cyclin A/CDK2 at a further stage. Once the desired effects have been accomplished, CDC6 is tagged for degradation, and this is accomplished via dephosphorylation by PP2A-PR-70 (Wlodarchak & Xing, 2016).

PP2A is involved in various phases of the mammalian cell cycle, acting as a major regulator. At the G2/M checkpoint, PP2A dephosphorylates CDC25 thus inhibiting the entry from the G2 phase into the mitotic phase. PP2A is also involved in the dephosphorylation of the forkhead transcription factor FOXM1, thus controlling expression of genes which act on the

cell cycle's progression during the G2 phase (Alvarez-Fernández, Halim, Aprelia, Mohammed, & Medema, 2011).

As the cell approaches the transit between the G1 phase and the synthesis (S) phase, it must go through a critical checkpoint regulated by the retinoblastoma tumour suppressor protein (RB). Kinases involved in RB phosphorylation include cyclin D/CDK4 and cyclin E/CDK2 complexes in the G1 phase whilst protein phosphatase 1 and PP2A are the key elements involved in the dephosphorylation of RB. PP2A is then present throughout the cell cycle and acts in response to various stimuli. At this point, PP2A actions can lead to cell cycle arrest following stress factor stimuli, thus discontinuing transit into the S phase and avoiding aberrant DNA synthesis (Wlodarchak & Xing, 2016).

For a cell to enter the mitotic (M) phase from the G2 phase, PP2A-B55 holoenzyme is inactivated while the activation of CDK1 occurs via phosphorylation by CDC25 kinases. If cell cycle arrest is necessary at this stage, CDC25 is phosphorylated by Chk1 which further phosphorylates PP2A-B56 so as to keep CDC25 inactive. On the other hand, the complex mechanism of positive feedback loops and regulation involving activation of CDK1 and inactivation of PP2A, aids to drive the cell into mitosis. Once activated, CDK1 partners with cyclin B to regulate almost all mitotic events, either directly or indirectly (Nasa & Kettenbach, 2018).

During mitosis, PP2A holoenzymes are essential in the reorganization of cellular structures. These include breakdown of the nuclear envelope, intracellular organelle rearrangement which include the endoplasmic reticulum and the Golgi apparatus. PP2A also regulates assembly of the mitotic chromosomes and of the mitotic spindles and is also involved in cell division via regulation of the attachment of microtubules to kinetochores (Wurzenberger & Gerlich, 2011).

Mitotic exit is initiated by a rise in activity of APC/C-CDC20. This leads to the formation and separation of the two daughter cells at interphase. APC/C activity is hindered by the spindle assembly checkpoint until there is attachment of all chromosomes to the microtubules which originate from opposite spindle poles. PP2A-B56 activates the early mitotic inhibitor (EMI1), maintaining the spindle assembly checkpoint. At mitotic exit, PP2A-B56 also mediates dephosphorylation of CDK1 substrates (Tischer, Hörmanseder, & Mayer, 2012).

Once this regulation is lost, cancer starts growing. This can result from upstream signalling pathways or by damage in genes encoding cell cycle proteins. The cell cycle is found to be deregulated in TNBC. Aberrant behaviour of Cyclin E1, which is involved in the G1-S phase transition, leads to loss of regulation during the cell cycle, and its overexpression has been reported in various cancers. In fact, overexpression is found in 30% of breast cancers, including basal-like TNBC. Cyclin E1 overexpression is also a major factor in trastuzumab resistance in HER2+ breast. Activated PP2A-B55β stabilizes Cyclin E1, thus making PP2A an attractive therapeutic target in basal-like TNBCs (Tan et al., 2014).

The PP2A B56 family regulates the dynamics of kinetochore assembly during chromosome separation at mitosis. It also regulates spatial aspects of alignment of chromosomes with microtubules. B56 deficiency has led to chromosome misalignment leading to a defective segregation of chromosomes (Zhao et al., 2017).

1.5.1 Opposing forces in cell cycle regulation, PP2A and Aurora kinase A

Serine/threonine kinases have the major role of protein phosphorylation as part of cell cycle regulation. The kinases' actions are in turn counteracted by protein phosphatases. Aurora-A kinase (AURKA) is a serine-threonine kinase with essential roles in the cell cycle. It has various aliases namely serine/threonine kinase 15 (STK15) and breast tumour amplified kinase (BTAK) (Zhang, Li, Yang, Zhang & Wang, 2015). Its roles include involvement in

mitotic entry, formation of the bipolar spindle, control of centrosome maturation and segregation during mitosis (Koh et al., 2017).

As previously described, mitotic entry is marked by a sharp increase in active kinases levels such as CDK1/cyclin B complex, PLK1, AURKA and AURKB resulting in an increase in the number of substrates being phosphorylated. Nevertheless, unless there is a decrease in phosophoprotein activity, the cell will not enter mitosis (Nasa & Kettenbach, 2018; Figure 1.2).

AURKA is responsible for the activation of CDC25B at the centrosome via phosphorylation. CDC25B then activates CDK1, partially contributing to mitotic entry. AURKA also phosphorylates LATS2 to promote centrosome maturation for nucleating microtubules. The centrosome has a major role as a microtubule-organizing centre in mitosis and mutations or loss of AURKA leads to spindles with aberrantly organized poles such as monopolar structures and short astral microtubules. AURKA interacts with proteins from the transforming acidic coiled-coil (TACC) family, targeting it to the mitotic centrosome, where it forms the TACC3-ch_TOG complex to counteract microtubule-depolymerizing kinesin KIF2C (also known as MCAK). This results in the stabilization of microtubules at centrosomes (Nikonova, Atsaturov, Serebriiskii, Dunbrack & Golemis, 2013).

AURKA is also implicated in the activation of PLK1, which occurs at the centrosome. A positive feedback mechanism is involved where PLK1 further promotes and regulates AURKA activation and functions at the centrosome. This leads to centrosome maturation and mitotic progression (Wang et al., 2014).

As the cell cycle nears the final stages of mitosis, PP2A interacts with AURKA at the spindle poles, leading to dephosphorylation of serine residue 51 and the degradation of AURKA. PP2A also stabilises pituitary tumour transforming gene 1, which in turn inhibits AURKA

(Carmena, Ruchaud & Earnshaw, 2009). Inhibition of PP2A leads to a reduction in AURKA degradation at the end of mitosis (Horn et al., 2007).

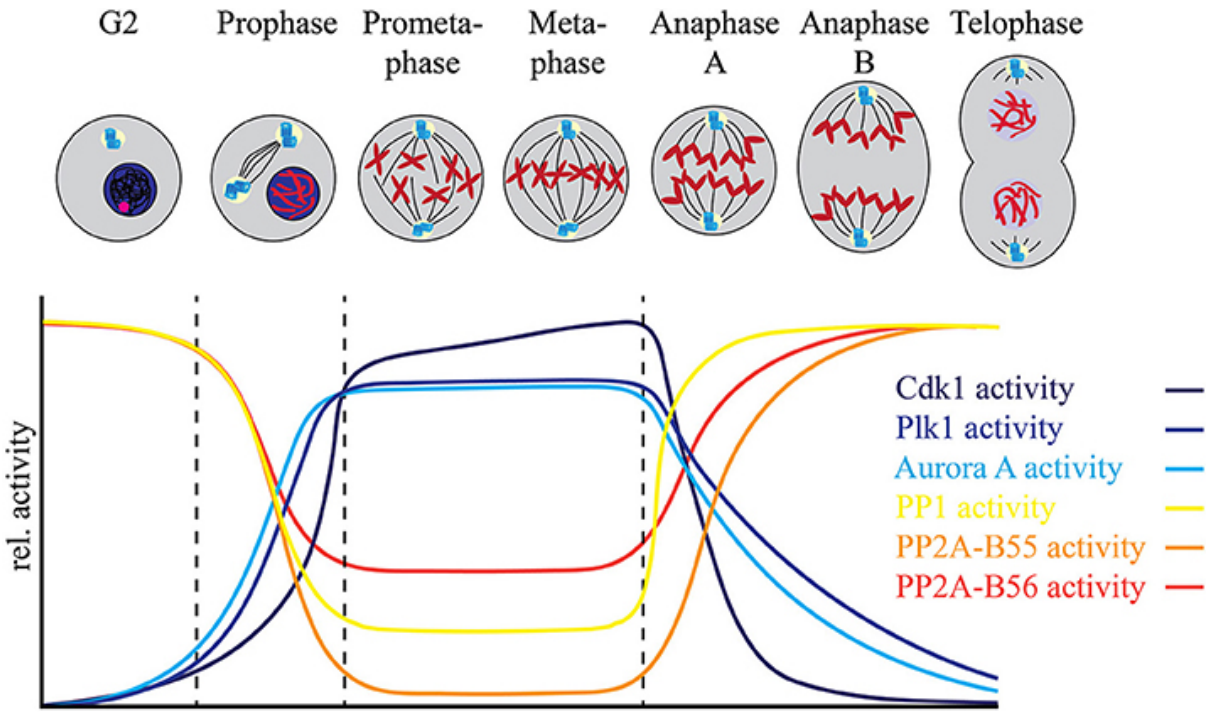

Figure 1.2 Coordinated activity between kinases and phosphatases during mitosis.

Kinases (blue colour scale) levels increase leading to entry in mitosis while phosphatase levels (red colour scale) decrease. At mitotic exit, phosphatase levels increase with subsequent lowered levels of kinases (Nasa & Kettenbach, 2018).

In the event of overexpression of AURKA, the DNA damage checkpoint at the G2 phase and the spindle assembly checkpoint during mitosis are rendered inactive. This leads to tetraploidy and centrosome amplification, which occurrence is most common where the cells exhibit defective p53-dependent DNA damage checkpoints. The sister kinase Aurora B, when overexpressed causes defective chromosome separation, resulting in aneuploidy. This strengthens the theory that Aurora kinases have oncogenic responsibilities in several types of tumour. The gene encoding AURKA is commonly found amplified in breast and prostate cancer, while AURKA as a protein is found at raised levels in various types of tumours. In studies involving mouse mammary epithelium, tumour growth was reported following

transgenic expression of AURKA after induced tetraploidy and centrosome amplification. On the other hand, cases where there was heterozygous deletion of AURKA in mice exhibited an increased incidence of cancer in different organs, thus indicating that AURKA might also have a tumour-suppressive role in specific tissues (Otto & Sicinski, 2017).

1.5.2 AURKA regulates KIF2C during mitosis

Spindle assembly and movement of chromosomes are dependent on microtubules and their dynamic properties. These involve adding and subtracting tubulin subunits which determine expansion and shrinkage of the microtubules. Some chemotherapeutic agents target microtubule dynamics and act by disturbing this process and thus disrupt spindle assembly and movement of chromosomes leading to an arrest of mitotic progression. This is due to the fact that chromosomes are dependent on microtubule dynamics via attachments to kinetochores (Manning et al., 2007).

During mitosis, proper bipolar spindle formation and correct attachment of the kinetochores of sister chromatids to the microtubules is essential to achieve an accurate segregation of chromosomes so as to avoid aberrations in genetic material in daughter cells following separation. Aberrations include chromosome instability which is a hallmark of solid tumours and a poor prognosis for the patient. Continuous incorrect attachment of chromosomes to spindle microtubules further encourages chromosomal instability (Sanhaji, Friel, Wordeman, Louwen & Yuan, 2011).

KIF2C forms part of the kinesin-13 protein class, part of the kinesin superfamily of proteins, which have an essential role in controlling microtubule dynamics. It is found localised to the spindle poles, spindle midzone and kinetochores and is also associated with the tips of growing microtubules (Manning et al., 2007).

KIF2C activity is characterised by a strong affinity for microtubule ends and catalyses the microtubule destabilisation from both ends at a considerable rate. Due to its localisation to the centrosomes, kinetochores, and spindle midzone during mitosis, it exerts an influence on various aspects of mitosis such as spindle assembly, microtubule dynamics, proper kinetochore-microtubule attachments, and positioning and segregation of chromosomes. Failure in these processes leads to chromosomal instability and aneuploidy, and consequently to tumour formation (Sanhaji et al., 2011).

In general, the microtubule-destabilizing activity of KIF2C is counteracted by microtubule-stabilizing microtubule-associated proteins (MAPs) namely XMAP2015, Tau and EB1. During interphase, activity of KIF2C is reduced due to the association of MAPs with microtubules. However, during transition into mitosis, hyperphosphorylation of MAPs dissociates them from microtubules, thus enhancing KIF2C's activity. During mitosis, KIF2C localises to the kinetochores, spindle poles and can also be found in the cytoplasmic pool. Aurora B controls the kinesin's activity at the kinetochores and ensures adequate kinetochore-microtubule attachments via phosphorylation of residue S196 (Zhang, Ems-McClung, & Walczak, 2008).

AURKA is activated by TPX2 to enable induction of bipolar spindles, further enhanced by phosphorylation of downstream targets. AURKA regulates the localization of KIF2C and its activity at the poles through two separate sites, promoting spindle polarity. The first part involving its regulation is via phosphorylation of S196, where KIF2C colocalizes with NuMA and XMAP215 at the centre of Ran asters, leading to adequate pole focusing. KIF2C localisation is also regulated via phosphorylation of S719 which is essential for bipolar spindle formation (Zhang et al., 2008).

Further regulation of KIF2C is via CDK1 phosphorylation of T537, part of the core domain of KIF2C. This leads to an attenuation in KIF2C activity and moves KIF2C from the spindle poles to other locations so as to promote correct spindle formation. On the other hand, PLK1 activates KIF2C at the kinetochores, aiding in proper microtubule attachments to the kinetochores (Sanhaji et al., 2011).

An interesting mitotic function which associates PP2A to KIF2C is the centromeric cohesion which needs to be maintained throughout the prometaphase up to the metaphase during mitosis. This is achieved by association of the shugoshin proteins with PP2A, thus preventing cohesin phosphorylation and promoting centromeric cohesion. Spindle microtubules from opposite poles capture sister kinetochores at metaphase, during which the pulling force generated is counteracted by the cohesive force at the centromeres, thus creating a stabilised environment (Tanno et al, 2010). In a study by Tanno et al. (2010) on HeLa cells, it was concluded that shugoshin proteins are phosphorylated by Aurora B, which in turn leads to the recruitment of KIF2C and PP2A which act on chromosome congression and centromeric cohesion respectively.

1.5.3 AURKA, a marker for proliferation

Proliferation can be considered a marker for breast cancer aggressiveness and a major prognostic factor for breast cancer. This is demonstrated by the fact that it is included in the Nottingham histologic tumour grading system as the mitotic index. Furthermore, prognosis based on genetic signatures in breast cancer is mostly based on genes involved in proliferation and cell cycle, further highlighting the importance of proliferation markers (Ali, Dawson, Blows, Provenzano, Pharoah & Caldas, 2012). Proliferation can also be an indicator for risk of recurrence in a patient. Treatment options in early stage ER-positive breast cancer

cases have also been based on proliferation rate status where cases characterised by high proliferation rates are more likely to benefit from chemotherapy (Baxter et al., 2015).

In a study by Ali et al. (2012), it was concluded that AURKA had a stronger prognostic value than Ki67, which is the universal marker for proliferation in various types of cancer. This study involved 3093 breast cancer patients and also indicated that AURKA can be used as an independent prognostic factor for ER+ breast cancer cases.

1.5.4 Targeting AURKA

Upregulation of AURKA leads to centrosome amplification, chromosomal instability with resulting oncogenesis. Amplification and/or overexpression of AURKA has been implicated in various solid tumours, namely ovarian and breast cancers (Zhang et al., 2015). AURKA localises to chromosome 20q13.2, a region which is found amplified in various cancer namely breast and colorectal amongst others (Koh et al., 2017).

When overexpressed, AURKA can be detected at all stages of the cell cycle, both in cytoplasm and nuclear compartments. AURKA overexpression is associated with loss of p53 as they are involved in a strictly regulated negative feedback loop which is absent in cancer cases. Studies have shown that there is a correlation between p53 expression loss and AURKA mRNA levels (Mehra, Seebriiskii, Burtness, Astsaturov, & Golemis, 2013). Blocking of AURKA activity leads to an increase in apoptosis due to upregulation of p53, whereas loss of p53 has been associated with amplification of AURKA (Mehra et al., 2013).

Targeting of AURKA is based on inhibition of the kinase, which inhibitors have been performing well on cell lines and xenograft models yet poorly in clinical trials. The highly specific AURKA inhibitor alisertib has been reported to exhibit strong efficacy in various solid tumours yet poor effects in acute myeloid leukaemia patients. A phase II study of

alisertib on breast cancer patients has shown a response in 18% of cases, 21% in small cell carcinoma of the lung and 13% in non-Hodgkin's lymphoma (Dominguez-Brauer et al., 2015).

AURKA inhibitors, combined with drugs which act by disrupting the mitotic spindle formation, target AURKA's role as a regulator in mitosis. Irreversible mitotic arrest is attained by blocking AURKA's actions together with the mechanism of microtubule assembly/disassembly via microtubule poisons, taxanes and vinca alkaloids (Mehra et al., 2013).

1.5.5 KIF2C transcriptional activation

KIF2C has been reported to be trans-activated and overexpressed in breast cancer, both in vivo and in vitro. Studies have shown that KIF2C expression levels are regulated through ectopic and endogenous p53 presence. KIF2C is not present in normal tissue except in testis. Also, studies which tackled depletion of KIF2C have shown a suppression in breast cancer growth (Shimo et al., 2008). Deregulation of KIF2C results in aberrations in spindle formaton and chromosome segregation, with consequent chromosome instability. Cancer cell proliferation proceeds regardless of these defects, and have the ability to hinder apoptosis as well, thus leading to cancer progression. Further research is required to investigate at to what level p53 regulates the transcriptional activation of KIF2C, if it affects it directly or through other transcriptional factors (Sanhaji et al., 2011).

KIF2C is one of the several genes which were reported to be trans-activated in a study involving gene expression analysis of 81 breast cancer tissues (Shimo et al., 2008). Other studies have also shown that KIF2C is also overexpressed both in vivo and in vitro with regards to breast cancer. KIF2C overexpression was also observed in gastric cancer and was also linked to tumour invasion and poor prognosis. In a study involving colorectal cancer

cases, KIF2C was also reported to be overexpressed both at mRNA and protein levels, with marked lymph node metastasis, vascular invasion and a poor prognosis as well. However, expression levels were more humble in pancreatic cancer and head and neck cancer (Sanhaji et al., 2011).

1.6 AURKA and KIF2C as potential biomarkers of oncogenic PP2A deregulation

As previously discussed, PP2A deregulation has an oncogenic effect and leads to tumorigenesis since PP2A's tumour suppressor role is attenuated. A balance is achieved during cellular processes between kinases and phosphatases, and once this equilibrium is lost, development of cancer ensues. This is the case with PP2A deregulation which is accompanied by AURKA and KIF2C overexpression in various cancers.

1.6.1 Prognostic value and predictive value

The importance of prognostic and predictive values of biomarkers is highlighted in the choice of therapeutic options which at times do not give the desired effect due to lack of distinguishing biomarkers between high-risk and low-risk patients (Zhang et al., 2015).

In a meta-analysis study by Zhang et al. (2015), it was concluded that increased AURKA expression is associated with a poor prognosis both in overall survival and disease-free progression in solid tumours. The main tumours affected were lung cancer, ovarian cancer, colorectal cancer and nasopharyngeal cancer.

In ovarian cancer, aneuploidy involving chromosome 20 is a common event and leads to an increased copy number of AURKA. Studies have shown contrasting results, some of which depend on the treatment given, yet most studies have highlighted that AURKA overexpression was related to aggressive disease progression and a poor outcome (Nikonova et al., 2013). AURKA was also found to have predictive value in a subgroup of patients

having advanced stage epithelial ovarian carcinomas, treated with Taxane-based chemotherapy (Lassmann et al., 2007).

In colon cancer, AURKA has often been associated with disease progression from a colonic polyp to invasive tumours. AURKA protein overexpression has been implicated in a large study involving colon cancer patients where there was a high frequency of recurrence in stage II and stage III cases. Another study has also shown that AURKA was commonly detected in well or moderately differentiated colon cancer cases (Nikonova et al., 2013).

AURKA overexpression has been associated with a decrease in survival in primary breast cancer cases and has been positively correlated with ER, PR and HER2 expression (Nikonova et al., 2013).

Overexpression of AURKA also contributes to a disruption in spindle checkpoint activation by therapeutic strategies involving paclitaxel and nocodazole, thus posing resistance to chemotherapeutic drugs (Goldenson & Crispino, 2015).

A reduction in AURKA expression or AURKA inhibition has been shown to be associated with a decrease in cell migration. This has been further emphasized by a study on renal tumours where overexpression of AURKA was associated with lymph node invasion (Tokes et al., 2015).

In a study by Xu et al. (2013), AURKA expression was found to be positively correlated with recurrence of TNBC in the first three years of follow-up. In the same study, it was concluded that both AURKA and Ki67 were highly expressed in TNBC and were associated with lower overall survival and disease-free survival, outlining their valuable role in prognostic determination.

In a study by Yao et al. (2015), KIF2C was found to positively correlate at RNA levels with breast cancer grades. KIF2C, together with Aurora kinase B, was also found to be valid

prognostic markers in human gliomas, with RNA expressions having a positive correlation with tumour grade. In the same study, KIF2C was also found to correlate with Ki67 expression (Bie, Zhao, Wang & Zhang, 2012). KIF2C was also found to positively correlate with Ki67 expression in a study involving colorectal cancers (Gnjatic et al., 2010), and was associated with prognosis in gastric cancer and hepatocellular carcinoma (Shen et al., 2017).

1.7 Ki67

Ki67 is a marker for cell proliferation with established prognostic and predictive value in various cancers. It is expressed in two protein isoforms and has a half-life of approximately 1 to 1.5 hours. It is present throughout the whole cell cycle with a prominent decrease in levels during the anaphase and telophase phases of mitosis. It is an important marker of tumour aggressiveness and has been relied on in various cancers as a proliferation and prognostic marker, namely breast, lung, cervical and prostate cancer (Li, Jiang, Chen, & Zheng, 2015).

Given its short half-life, the protein expression of Ki67 is the result of the balance between synthesis and degradation. It is subjected to phosphorylation and dephosphorylation during mitosis, being also subject to protease degradation. Studies have also shown that Ki67 expression correlates with that of p53, in tumours such as oral squamous cell carcinoma and breast cancer. It has been suggested that p53 inhibits Ki67 promoter activity via pathways which are p53 dependent, through a transcriptional repression of the same promoter (Li et al., 2015).

1.7.1 Ki67 as a prognostic marker for breast cancer

No cut-off value has been determined so far for Ki67 protein expression via immunohistochemistry, yet Ki67 is widely used as an additional determinant for decision

making in adjuvant therapeutic options for breast cancer (Yerushalmi, Woods, Ravdin, Hayes, & Gelmon, 2010).

In breast cancer cells, Ki67 expression rises with increasing tumour size, higher grades, ER/PR negative tumours, lymph node involvement and vascular invasion. A raised Ki67 expression is also linked to a greater risk of recurrence. Some studies have described a higher Ki67 expression in HER2-negative breast cancer whilst other have stated the contrary (Ragab et al., 2018).

Nishimura et al. (2014) have found a significant correlation between high Ki67 expression in primary breast cancers and a lower survival rate whereas Ibrahim et al. (2013) have stated lower survival rates were associated with a high K67 expression in recurrent tumours irrespective of a high or low Ki67 expression in primary breast cancers. Another study involving early breast cancer patients has shown that Ki67 expression is related to a higher risk of recurrence and poorer prognosis (Ragab et al., 2018).

In a study by Shao et al. (2011), a cohort of breast cancer patients who also had metastasis to the brain, presented with a higher Ki67 expression in comparison with a lower expression in primary breast tumours without brain metastasis. These findings were reconfirmed by studies by Tokes et al. (2015) where a higher Ki67 expression was demonstrated in primary breast cancer cases metastasising to different organs.

In normal breast tissue, Ki67 is expressed at very low levels and its levels increase according to breast cancer grade, from ductal carcinoma in situ to invasive breast cancer. As in other tumours, Ki67 correlates positively with histological grade in breast cancer, and some studies have also reported correlation with tumour size. Studies involving EGFR and HER-2 have given inconsistent results whilst p53 mutations are a characteristic in breast cancer with high Ki67 levels. Ki67 and Bcl-2 have been combined to form an index which is used as an independent predictor of survival in ER-positive breast cancer (Kontzoglou et al, 2013).

In breast cancer, Ki67 has been investigated and considered to be utilised as a prognostic and predictive marker. Nevertheless, there needs to be standardisation in different aspects of Ki67 interpretation, such as pre-analytical factors and technical issues leading to interlaboratory and interobserver variability (Kontzoglou et al, 2013).

1.7.2 Ki67 as a diagnostic tool

As previously mentioned, Ki67 is an optimal indicator of cell proliferation. Ki67 protein was found to increase with tissue differentiation decrease, together with a correlation where metastasis was present. The Ki67/MIB-1 monoclonal antibody is most commonly used in immunohistochemistry and the percentage of nuclei showing positivity is known as the labelling index (LI). Studies have shown that the Ki67 LI exhibits a positive correlation between Ki67 LI and tumour grade. KI67/MIB-1 immunostaining is an essential supplementary tool aiding the pathologist in determining tumour grade and prognosis for the patient (Li et al., 2015).

In the universally adapted scoring system of Ki67 immunohistochemical staining, a cut-off value of 10% to 14% positivity has been adopted, which when surpassed indicates a high risk of poor prognosis for the patient. In 2009, the St Gallen Consensus has advised that the Ki67 (LI) is one of the key factors for treatment choice determination in hormone receptor positive breast cancer cases, determining if chemotherapy is to be included as an additional choice of treatment together with endocrine therapy (Li et al., 2015).

Ki67 expression reflects various aspects of tumour progression, from initiation to metastatic spread. In a study by Chen et al. (2012) on non-muscle invasive bladder cancer, Ki67 LI together with vascular endothelial growth factor were utilised in combination as predictive indicators to optimise personalised treatment choices. Of interest, Ki67 antibody positivity was found to be lower in poorly differentiated adenocarcinomas than in well or moderately

differentiated types. In colorectal carcinomas, Ki67 LI was lower in poorly differentiated, mucinous adenocarcinomas, Duke's stage C than in well or moderately differentiated non-mucinous carcinomas which were at early Duke's stage A or B (Nabi, Nagi, & Sami, 2008).

1.8 Aims of study

As previously described, TNBC is an aggressive breast cancer subtype lacking expression of receptors usually targeted in breast cancer. PP2A deregulation is a common event in basal-like breast cancers, the majority of which are reported as TNBC. AURKA and KIF2C are overexpressed in various tumours and RNA expression of these biomarkers was reported in previous studies from our research group to be associated with PP2A activity in TNBC.

Further evaluation of AURKA and KIF2C protein expression in a TNBC cohort and TNBC cell line models, together with the identification of correlating biomarkers can potentially offer novel therapeutic targets in TNBC based on PP2A activity.

The aims of the study are:

- To evaluate protein expression of AURKA and KIF2C as biomarkers of interest in a cohort of TNBC patients
- To identify biomarkers that correlate with AURKA and KIF2C at RNA expression
- To confirm selected biomarkers at protein level in TNBC cohort
- To investigate protein expression of AURKA together with correlating markers in TNBC cell models following treatment with FTY720

Chapter II

Materials and Methods

2.1 Design of study

In this study, the first experiment involved the evaluation of protein expression of the two novel biomarkers, AURKA and KIF2C in 160 TNBC cases and 23 normal breast epithelia. Tissues were stained by immunohistochemistry and scored using the H-score methodology based on the observations of two independent assessors.

In the second experiment, RNA expression of these biomarkers was correlated with the RNA profiles of 40 genes involved in breast cancer as established from previous studies by our research group. This was achieved using the QuantiGene® 2.0 Plex Assay from which potential complimentary biomarkers related to AURKA and KIF2C expression were identified.

In the third experiment, six breast cancer cell lines were cultured and treated with the drug of interest, FTY720. These six breast cancer cell lines were chosen based on their sensitivity to the drug, as established in a previous study by our research group.

In the fourth experiment, the protein expression of the complimentary biomarkers to AURKA and KIF2C, together with Ki67, was assessed on tissue microarrays (TMAs) of treated breast cancer cell line cell blocks and TNBC TMAs consisting of 160 cases.

2.2 Validation of the antibodies against novel biomarkers, AURKA and KIF2C using Western Blot.

The western blot technique was used for validation of the specificity of antibodies to be used in immunohistochemistry.

Acrylamide (resolving) gel was prepared with a concentration of 7.5% for KIF2C and 12.5% for AURKA. Stacking gel with 5% Acrylamide was used for both antibodies.

Table 2.1 – Reagents and volumes used to prepare gels for Western Blot technique

7.5% Resolving Gel	**12.5% Resolving Gel**	**5% Stacking Gel**
3.8ml Deionised water	3.8ml Deionised water	2.3ml Deionised water
3.72ml 1M Tris HCl (pH 8.8)	3.72ml 1M Tris HCl (pH 8.8)	0.4ml 1M Tris HCl (pH 6.8)
2.5ml 30% Acrylamide	4.16ml 30% Acrylamide	0.5ml 30% Acrylamide
0.1ml 10% Sodium dodecyl sulfate	0.1ml 10% Sodium dodecyl sulfate	15µL 10% Sodium dodecyl sulfate

Each resolving gel was then activated by the addition of 100µL Ammonium persulfate (APS) and 10µL TEMED, whilst 50µL APS and 7.5µL TEMED were added to the stacking gel. Activation was done right before use of gels. The resolving gel was then introduced into the mould just 1cm below the comb groove and topped up with water-saturated butanol. When the resolving gel set, the butanol was removed followed by a wash with deionised water. The stacking gel was then added on top of the running gel, and a plastic comb was inserted to allow formation of the wells once the stacking gel has set.

The protein lysates of breast cancer cell lines MDA-MB-231 and MCF-7 were utilised for this validation since previous studies by the same research group have shown that AURKA and KIF2C exhibit high RNA levels in these cell lines. The loading buffer was prepared by adding 100µL β-mercaptoethanol to 900µL of Dye. One-part loading buffer was added to three parts of each protein lysate and heated at 95°C for 3 minutes to achieve protein denaturation. The lysate-buffer mixtures were then pipetted into separate wells of the gel electrophoresis setup. 4µL IRDye Molecular Weight Marker 680/800 was added in an adjacent well to serve as a standard size reference.

The gel was then run at a constant of 150 volts for 55 minutes. A thick transfer paper was then soaked in transfer buffer and placed on the transfer setup followed by nitrocellulose membrane. The resolving gel was then carefully removed from the mould and transferred onto the membrane. This was then covered by a second transfer paper also soaked in transfer buffer. The protein transfer was then done at a constant of 0.3 Amps for 120 minutes.

Following the transfer, the resulting membrane was then rinsed for several times with deionised water and stained with Ponceau S which ensured that the desired transfer was achieved. After multiple washes with deionised water, the membrane was then placed in blocking buffer for two hours at room temperature. Dilutions of the antibodies were prepared in 5ml of blocking buffer. Dilution for both AURKA and KIF2C was 1:500, as established in previous studies by our research group.

Following blocking, the membrane was washed three times in 1 x Tris buffered saline with Tween-20 (TBS-T) followed by three washes of 1 x Tris buffered saline (TBS). Each wash had a duration of three minutes. This was followed by incubation in the primary antibody for two hours at room temperature on a rotary mixer. The membrane was then washed three times in 1 x Tris buffered saline with Tween-20 (TBS-T) followed by three washes of 1 x Tris buffered saline (TBS). The membrane was then incubated in the secondary antibody, complimentary to the primary, for two hours at room temperature followed by washes in TBS-T and TBS. The membranes were then scanned utilising the Odyssey (LI-COR) scanner.

2.3 Case selection

TNBC cases diagnosed between 2008 and 2016 were retrieved from the Histology database using Cognos software. 160 triple negative breast cancer cases were identified and formalin-fixed paraffin-embedded blocks were retrieved from the Histology archives at Mater Dei Hospital. Haematoxylin and eosin stained slides corresponding to these cases were examined

so as to choose the best section which is most adequate with regards to tumour consistency and cellularity.

2.4 Immunohistochemistry

Expression of AURKA and KIF2C was investigated both at protein level and RNA level, utilising formalin-fixed paraffin-embedded material.

Immunohistochemistry was the technique employed to investigate the expression of AURKA and KIF2C at protein level in triple negative breast cancer (TNBC) cases. Prior to testing AURKA and KIF2C on patient cases, the technique to be utilised was optimised for each antibody by testing various antigen retrieval procedures at different antibody dilutions. The Human Protein Atlas (https://www.proteinatlas.org/), NeXtProt (https://www.nextprot.org/) and recommendations by the manufacturer were consulted in order to identify suitable positive controls. AURKA was expected to show strong positivity on tonsil and testis controls whilst KIF2C was expected to stain strongly positive on testis and colon. The antigen retrieval techniques tested during optimisation were Citrate Buffer pH 6.0 using a pressure cooker, Citraconic Anhydride at pH 7.4 and Tris-EDTA pH 9.0 using a microwave.

Sections were cut a day beforehand on charged slides and left to dry overnight at 37°C. This was done to ensure that sections do not detach from the slides. Prior to dewax, slides were baked at 65°C for 25 minutes. Dewax involved a sequence of steps, two minutes each in the following order: xylene, xylene-alcohol, a series of degrading alcohol concentrations from 100% to 30%, and final steps in water.

Tris-EDTA was prepared by mixing 0.185g of EDTA, 500mls of distilled water, 0.61g of TRIS and 0.25mls of Tween 20. Antigen retrieval was performed using a microwave at 90°C for 25 minutes. Citrate buffer was prepared by mixing 3.15g of citric acid with 1500mls of distilled water. pH was adjusted to 6.0 with sodium hydroxide. Antigen retrieval was done

using a pressure cooker. Citraconic anhydride was prepared by mixing 250µl of citraconic with 500mls of distilled water. pH was adjusted to 7.4 with sodium hydroxide. Antigen retrieval was done using a microwave at 98°C for 30 minutes.

After being left to cool for 30 minutes, slides were rinsed in tap water and placed in 0.3% hydrogen peroxide for 20 minutes to block endogenous peroxidase activity. Slides were rinsed in tap water and mounted onto cover-plates then rinsed in phosphate buffered saline (PBS). After draining, 100µl of normal rabbit serum (NRS) (MONOSAN Cat: MONX10963) were added and incubated for ten minutes. Antibody dilutions were prepared and 100µl were then added to each respective slide.

After overnight incubation at 4°C, slides were rinsed with PBS. 100µl of secondary antibody complimentary to the primary antibody (Biotinylated Rabbit anti-mouse antibody (Dako Cat: E0354)) were then added to each slide and left for one-hour incubation at room temperature. After rinsing with PBS, 100µl of Avidin-Biotin Complex (ABC) were added and left to incubate for one hour at room temperature. Slides were then put in a trough with PBS prior to staining with 3,3'-Diaminobenzidine (DAB). Chromogen precipitation reaction was observed under the microscope to achieve optimal staining. Staining was timed on the controls then applied to the whole batch of slides.

Following staining with DAB, sections were stained with Haematoxylin as a counterstain and mounted with a coverslip.

The optimal dilutions were 1:75 with citrate buffer pH 6.0 (pressure cooker) as antigen retrieval for AURKA and 1:100 for KIF2C with citrate buffer pH 6.0 (pressure cooker) as well.

The H-score method was chosen for scoring. This involved classifying staining in four categories: 0, 1, 2, 3 with 0 being flat negative and 3 being the most intense. A percentage for

each intensity was observed and multiplied by the respective intensity to obtain the H-score ranging from 0 - 300. Three areas per tumour were selected and scored by two independent assessors.

2.5 RNA expression using QuantiGene® 2.0 Plex Assay (Thermo)

Following evaluation of H-scores of both AURKA and KIF2C in the TNBC cases, 58 TNBC cases with the highest values were used to evaluate the RNA levels of AURKA and KIF2C against a set of 40 genes involved in breast cancer, and which were investigated in a previous study. Selection of these genes was based on data analysis of Breast Cancer data retrieved from the Cancer Genome Atlas (TCGA) (Baldacchino et al., 2014) and can be classified into five groups:

- markers involved in the molecular classification of breast cancer with EN1, GABRP and FOXC1 as basal markers and AGR2, CA12, ESR1, GATA3, SCNN1A, ERBB2 and TFF3 as luminal markers
- markers representing the PP2A core complex: PP2CA, PP2CB and PPP2R2A
- negative regulators of PP2A: SET, SETBP1, CIP2A, ANP32A, PME-1 and PINK1
- Potential PP2A activity biomarkers: BCAR1, NASP, CDKN1B, AURKA and KIF2C
- breast cancer signature genes: PTEN, HIF1A, eIF4e, BRCA1, VEGFA and VEGFB
- markers involved in the epithelial to mesenchymal transition: CDH1, CDH12, CTNN1B, FN1 and TWIST1
- housekeeping genes: PPIB, HPRT1, ACTB, GAPDH and TBP

QuantiGene® 2.0 Plex Assay was used to evaluate RNA expression on material which was retrieved from the formalin-fixed paraffin-embedded material.

2.5.1 Principle of technique

QuantiGene® 2.0 Plex Assay acts by combining branched DNA signal amplification and multi-analyte profiling bead technologies (xMAP®). This leads to the concurrent detection of multiple RNA targets and enabling the quantitation of the RNA being investigated. This is based on a method which employs hybridisation leading to signal amplification.

Capture Beads, which are fluorescent microspheres, provide a surface to capture the RNA molecules. An oligonucleotide Probe Set is utilised for every RNA target, consisting of Capture Extenders, Label Extenders and Blockers. The Capture Extenders capture the target RNA via hybridisation onto the Capture Beads. A branched DNA complex (3 components – Pre-Amplifier, Amplifier and Label Probe) is bound to the captured RNA leading to signal amplification by binding to Streptavidin-conjugated R-Phycoerythrin. This results in a fluorescence signal which is read on a Luminex analyser and is reported as median fluorescence intensity (MFI) which reflects the amount of target RNA molecules present in the sample.

2.5.2 Coring of tumour

Slides stained with AURKA and KIF2C were microscopically examined to determine which area is best to core for RNA expression.

Coring involved using a 0.5mm corer to sample the formalin-fixed paraffin-embedded tissue section. The core was then released in a well on a 96 well-plate. Between each case, the corer was inserted in an empty wax block to avoid cross contamination between one case and the next. Cross-contamination was ruled out in an experiment analysing successive cores from distinct tumour samples of known profiles.

2.5.3 Lysis

A mix of homogenizing solution and proteinase K was prepared at a ratio of 60:1. 45µl of mixture was added to each well containing the core. The plate was then incubated at 65°C overnight, with shaking at 600rpm using the iEMS Shaking incubator (Thermo). The following day, the lysates were centrifuged at 1200rpm for five minutes, then transferred to a filter plate (MSBVS1210, Merck Millipore) that is placed on a fresh 96-well plate. Lysates were filtered by centrifugation at 2500rpm for 5 minutes.

2.5.4 Hybridisation

The reagents were thawed and kept on ice until the working bead mix was prepared (Table 2.2).

Table 2.2 Working bead mix for hybridisation with tissue homogenates. Volumes are per well of a 96-well plate.

Reagent	**Volume (per well)**
Nuclease-free water	4.25 µl
Lysis mixture	16.65 µl
Blocking reagent	1.00 µl
Proteinase K	0.10 µl
Capture Beads	0.50 µl
2.0 Probe Set	2.50 µl
Total	25.00 µl

The working bead mix was kept on ice and vortexed for 10 seconds before use. 25µl of bead mix were pipetted per well in the magnetic separation plate. 25µl of homogenizing solution was added to three wells to act as blanks while 25ul of patient sample lysate were added to

the respective wells. The plate was sealed using a clear plastic pressure seal and incubated for 18 to 22 hours at 54 ± 1°C at 600 rpm.

2.5.5 Washing, amplification and reading

The wash buffer was prepared by mixing deionized sterile water with Wash Buffer Component 1 and Component 2 in the ratio of 31.5:0.1:1.667. The 2.0 Pre-Amplifier QGP was centrifuged briefly and mixed with the Pre-Amplifier diluent at a ratio of 0.003:1 to prepare the Pre-Amplifier QGP Working Reagent.

Meanwhile the magnetic separation plate was mounted onto a magnetic plate washer and left to settle for one minute to effectively immobilize the magnetic beads. The plate was then inverted over a drain to empty out contents and gently blotted on a tissue paper. 100µl of wash buffer was added to each well and left for 15 seconds, followed by inversion over a sink and gentle blotting. This step was repeated for three times.

50µl of 2.0 Pre-Amplifier QGP Working Reagent were then added to each well and the plate was sealed with a foil plate seal and left on a shaker for 1 minute at 800rpm at room temperature. This was followed by incubation in the Vortemp at 50°C for one hour at 600rpm.

In the meantime, the 2.0 Amplifier QGP was thawed and centrifuged briefly and prepared at a ratio of 0.003:1 of Amplifier Diluent. Following incubation, the plate was then removed from the Vortemp and mounted onto a magnetic plate washer and left to settle for one minute. The plate was then inverted and gently blotted on a tissue paper. 100µl of wash buffer was added to each well and left for 15 seconds, followed by inversion over a sink and gentle blotting. This step was repeated for three times.

50µl of 2.0 Amplifier QGP Working Reagent were then added to each well and the plate was sealed with a foil plate seal and left on a shaker for 1 minute at 800rpm at room temperature. This was followed by incubation in the Vortemp at 50°C for one hour at 600rpm.

In the meantime, the Label Probe was thawed and centrifuged briefly and prepared at a ratio of 0.003:1 of Label Probe Diluent. Following incubation, the plate was then removed from the Vortemp and mounted onto a magnetic plate washer and left to settle for one minute. The plate was then inverted and gently blotted on a tissue paper. 100µl of wash buffer was added to each well and left for 15 seconds, followed by inversion over a sink and gentle blotting. This step was repeated for three times.

50µl of Label Probe were then added to each well and the plate was sealed with a foil plate seal and left on a shaker for 1 minute at 800rpm at room temperature. This was followed by incubation in the Vortemp at 50°C for one hour at 600rpm.

The SAPE was vortexed and centrifuged briefly and prepared at a ratio of 0.003:1 of SAPE Diluent. Following incubation, the plate was then removed from the Vortemp and mounted onto a magnetic plate washer and left to settle for one minute. The plate was then inverted and gently blotted on a tissue paper. 100µl of wash buffer was added to each well and left for 15 seconds, followed by inversion over a sink and gentle blotting. This step was repeated for three times.

50µl of SAPE Working Reagent were then added to each well and the plate was sealed with a foil plate seal and left on a shaker for 1 minute at 800rpm at room temperature. This was followed by incubation for 30 minutes at room temperature at 600rpm.

Following incubation, the plate was then removed from the Vortemp and mounted onto a magnetic plate washer and left to settle for one minute. The plate was then inverted and gently blotted on a tissue paper. 100µl of SAPE wash buffer was added to each well and left

for 15 seconds, followed by inversion over a sink and gentle blotting. This step was repeated for three times.

150µl of SAPE Wash Buffer were then added to each well and the plate was then sealed with a foil plate seal. It was then shaken at 800rpm at room temperature for 3 minutes before loading into the Luminex instrument for reading.

2.6 Cell culturing

2.6.1 Cell line thawing

Stocks of cells are kept for each cell line at -80°C or in liquid nitrogen. Dimethyl sulfoxide (DMSO) is the cryoprotective agent used to avoid the formation of ice crystals which can damage and lyse cells, but on the other hand it is used at a toxic concentration and exposure of cells to the freezing medium should be kept to a minimum.

In a laminar-flow hood and under aseptic conditions, each breast cancer cell line was transferred to a 15mL tube which was kept on ice to maintain the 4°C temperature environment. The next step involved gradual addition of cold phosphate buffered saline (PBS) (at 4°C) to the tube containing the cells in order to wash off the freezing medium. This was followed by centrifugation at 1000rpm for 4 minutes at 4°C. After discarding the supernatant, the cells were suspended in complete Roswell Park Memorial Institute (RPMI) medium, which was the medium used for cell culturing.

2.6.2 Procedure

Six triple negative breast cancer cell lines were used for this study, based on their sensitivity to FTY720, as determined in a previous study by our research group. The cell lines were MDAMB231, BT20 and Hs578t which were found to be sensitive to FTY720 at a concentration of 5µM after 24 hours and HCC1937, MDAMB468 and MDAMB436 which

were non-sensitive. A cell is considered to be sensitive if when treated with a pre-determined concentration of FTY720 reached IC_{50} (percentage viability reduced to less than 50%) after 24-hour incubation.

As previously mentioned, medium used for all triple negative breast cancer cell lines was RPMI culture medium initially supplemented with 20% Foetal Bovine Serum (FBS) and 1% Penicillin Streptomycin (PenStrep). 6mls of RPMI were used to dislodge and suspend the cell pellet in the tube following thawing and the whole volume was then transferred to a labelled T25 culturing flask. Each T25 flask was then incubated at a temperature of 37°C, 5% carbon dioxide and 95% relative humidity.

Each T25 flask was checked every three days and once the cells reached growth of 80% confluency, these were resuspended using 1x trypsin-EDTA and transferred to labelled T75 flasks. In T75 flasks used for sub culturing, RPMI was supplemented with 10% FBS. The cell cultures were maintained and sub-cultured until enough cells were available for the continuation of the study.

2.6.3 Cell seeding

The seeding density of each cell line was determined (Table 2.3). Each T75 flask was washed with its own medium before discarding the same medium. 3mLs of 1 x Trypsin-EDTA were then added to each flask. This was followed by incubation of each flask for 5 minutes; each flask being checked under the microscope to ensure that all cells were non-adherent to the flask. 2mLs of complete RPMI medium were then added to arrest the effect of trypsinisation on the cells.

Table 2.3 – Seeding densities of TNBC cell lines used for cell culturing. The seeding densities were calculated per T25 flask.

Cell line	Seeding density
MDAMB231	1.6 x 10^5 cells
BT20	8.6 x 10^4 cells
Hs578t	2.5 x 10^4 cells
HCC1937	2.5 x 10^4 cells
MDAMB468	1 x 10^5 cells
MDAMB436	2 x 10^5 cells

Each flask's contents were then added to a 15mL tube labelled for each cell line and centrifuged at 1200rpm for four minutes. The supernatant was then discarded while the cell pellet was resuspended. From each tube, 100µl of cell suspension was added to 9.8mL of CASYton isotonic solution. Cells were counted using the CASY® cell counter.

Once the cell seeding amounts were determined, the determined amounts were added to 6mLs of RPMI medium in T25 flasks and left to incubate until the flasks reached confluency.

2.6.4 Treatment with FTY720

The cell lines were then treated with FTY720 at concentrations of 5µM and 10µM for 24 hours and 48 hours. Flasks with no treatment and with DMSO only (vehicle control) were incubated in parallel with the treated cell lines.

Following incubation, each T25 flask was viewed under the microscope and changes were recorded. Each flask was then washed with its own medium before discarding the same medium. 1.5mLs of 1 x Trypsin-EDTA were then added to each flask. This was followed by incubation of each flask for 5 minutes; each flask being checked under the microscope to

ensure that all cells were non-adherent to the flask. 2mLs of fresh RPMI medium were then added to arrest the effect of trypsinisation on the cells.

Each flask's contents were then added to a 15mL tube labelled for each cell line and centrifuged at 1400rpm for four minutes. Cells pellets were then trapped in a fibrin clot (cell block) and fixed in 10% neutral buffered formalin.

2.7 Cell block preparation

Cell blocks were prepared for untreated, vehicle-controlled and treated cell lines. This was done to proceed to the last part of the study which involved preparing TMAs of cell blocks in order to be stained via immunohistochemistry.

Cell blocks were prepared using the Plasma-Thrombin method where the cell pellet was re-suspended in 8 drops of plasma. This was followed by addition of 4 drops of Thrombin. Thrombin activates the fibrinogen into a fibrin clot trapping cells within. Cell blocks were fixed in 10% neutral buffered formalin for 24-48 hours prior to histology processing (Table 2.4).

Table 2.4 – Histology processing programme. List of reagents and duration of each reagent during processing of cell blocks.

Reagent	Duration
70% ethanol	60 minutes
70% ethanol	75 minutes
Absolute alcohol	60 minutes
Absolute alcohol	75 minutes
Absolute alcohol	90 minutes
Xylene	60 minutes
Xylene	60 minutes
Xylene	60 minutes
Paraffin wax	30 minutes
Paraffin wax	60 minutes
Paraffin wax	120 minutes

2.8 Tissue microarray production

Tissue microarrays (TMAs) of 1mm cores were prepared from the cell blocks and from the triple negative breast cancer cases. Blocks were cored with a 1mm diameter corer and cores were then inserted in a recipient paraffin block. Each block was cored in duplicate. A TMA map was documented to identify tumours within the TMA. Control tissues and orientation cores were included within each TMA to validate any immunohistochemical staining as well as to provide navigation points during microscopical examination (Appendix 1).

3µm sections were then cut by microtomy and immunohistochemistry (section 2.4) was performed on the TMAs for AURKA, KIF2C, Ki67 and the two biomarkers derived from correlation of RNA expressions in Section 2.5, which were PME-1 and CIP2A.

PME-1 and CIP2A immunohistochemistry technique were optimised as was done with AURKA and KIF2C prior to staining of TMAs. An H-score was obtained from two independent assessors for each biomarker for each case within the TMAs.

2.9 Statistical analysis

Microsoft Excel and IBM SPSS Statistics 23 were used for statistical analysis.

Chapter III

Results

3.1 Protein expression of AURKA and KIF2C

3.1.1 Validation of protein biomarker antibodies using Western Blot

The antibodies to the biomarkers of interest, AURKA and KIF2C, were validated using the Western Blot technique (Figure 3.1). Validation is done to ensure that an antibody is specific to the antigen being analysed and this is demonstrated by the formation of a band at the molecular weight of the target protein antigen (Bordeaux et al, 2010). A single band around the 45.8kDa mark was detected for AURKA whilst KIF2C showed the expected 81.3kDa band.

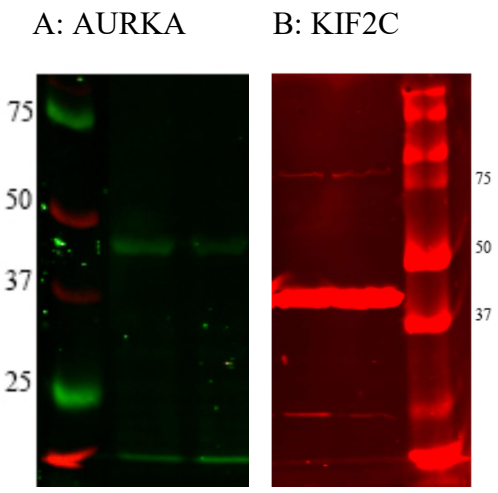

Figure 3.1: Western blot for the validation of antibodies against AURKA and KIF2C.

(A) AURKA showed a band at the expected size 45.8kDa

(B) KIF2C showed a band at the expected 81.3kDa mark. A band at 42kDa indicates the presence of β-actin used as a control

3.1.2 AURKA and KIF2C immunohistochemistry staining

160 Triple negative breast cancer (TNBC) formalin-fixed, paraffin-embedded tissue sections were stained via immunohistochemistry (IHC) for AURKA at an optimal dilution of 1:75, with citrate buffer pH6.0 at 95°C as antigen retrieval. The same antigen retrieval was utilised

for KIF2C but at a dilution of 1:100. Both biomarkers stained in a cytoplasmic pattern as illustrated in Figure 3.2 and Figure 3.3.

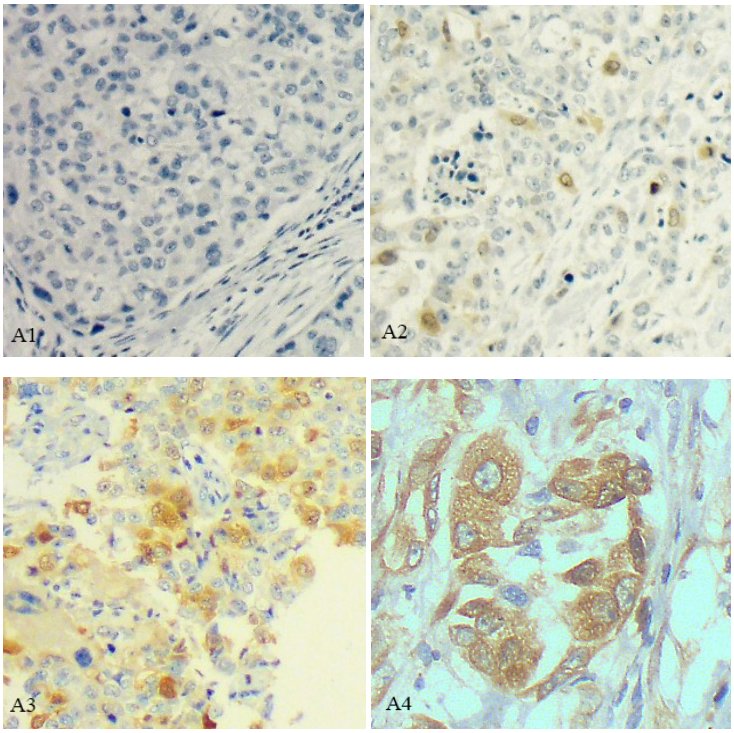

Figure 3.2: IHC staining of TNBC sections for AURKA showing different H-score intensities. Positivity is denoted by brown staining which varies in intensity whilst Harris Haematoxylin stains nuclear material as blue staining.

(A1) Intensity score: 0

(A2) Intensity score: 1

(A3) Intensity score: 2

(A4) Intensity score: 3

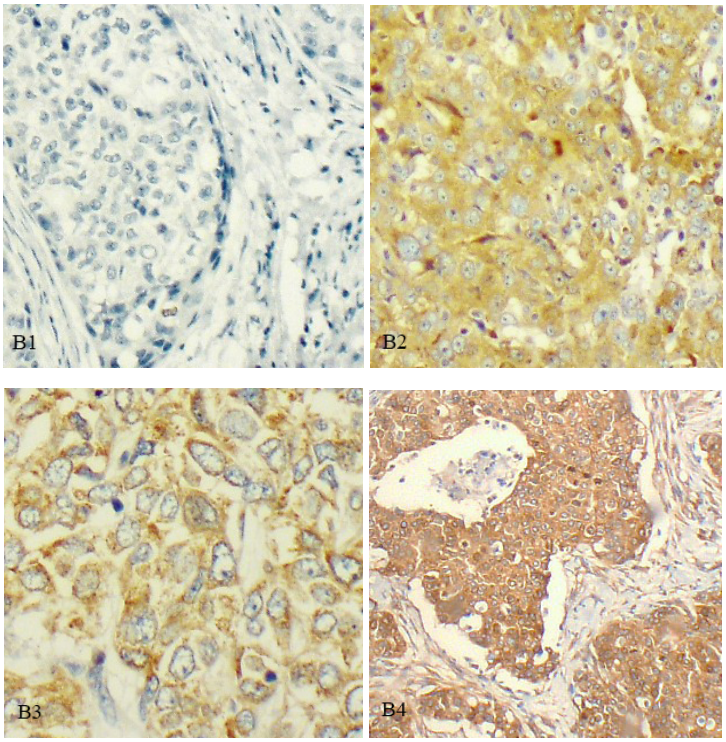

Figure 3.3: IHC staining of TNBC sections for KIF2C showing different H-score intensities. Positivity is denoted by brown staining which varies in intensity whilst Harris Haematoxylin stains nuclear material as blue staining.

(B1) Intensity score: 0

(B2) Intensity score: 1

(B3) Intensity score: 2

(B4) Intensity score: 3

3.1.3 Correlation between the two independent assessors on scoring of AURKA and KIF2C stained slides

Scoring of the slides was performed by two independent assessors for both sets of slides stained at immunohistochemistry for the biomarkers of interest AURKA and KIF2C, respectively. Intraclass correlation coefficient was performed on both sets of H-scores to measure reliability between the two independent assessors. As illustrated in Table 3.1, a strong consistency and absolute agreement was achieved between the two assessors (values between 0.75 and 0.9 indicate a good reliability (Koo & Li, 2016)).

Table 3.1 Intraclass correlation coefficient showing agreement between the two assessors for both AURKA and KIF2C H-scores at 95% Confidence Interval

Biomarker	Consistency agreement		Absolute agreement	
	Single measures	*Average measures*	*Single measures*	*Average measures*
AURKA	0.788 (0.73 – 0.84)	0.881 (0.84 - 0.91)	0.785 (0.72 – 0.84)	0.880 (0.84 – 0.91)
KIF2C	0.831 (0.78 – 0.85)	0.908 (0.88 – 0.93)	0.746 (0.33 – 0.88)	0.855 (0.50 – 0.94)

3.1.4 AURKA and KIF2C protein expression in the TNBC cohort

AURKA was found to be overexpressed in 72% of the TNBC cases (114 positive cases out of 158). Overexpression was determined by calculating the cases which scored higher than the mean AURKA expression in the normal breast tissue sections i.e. 8.17 (Figure 3.4 and 3.5). KIF2C was found to be overexpressed in 56% of the TNBC cases (87 positive cases out of 156) when threshold was calculated to be 72.56 based on the mean of normal breast tissue expression (Figure 3.6 and 3.7). Both biomarkers did not follow a normal distribution (Table 3.2).

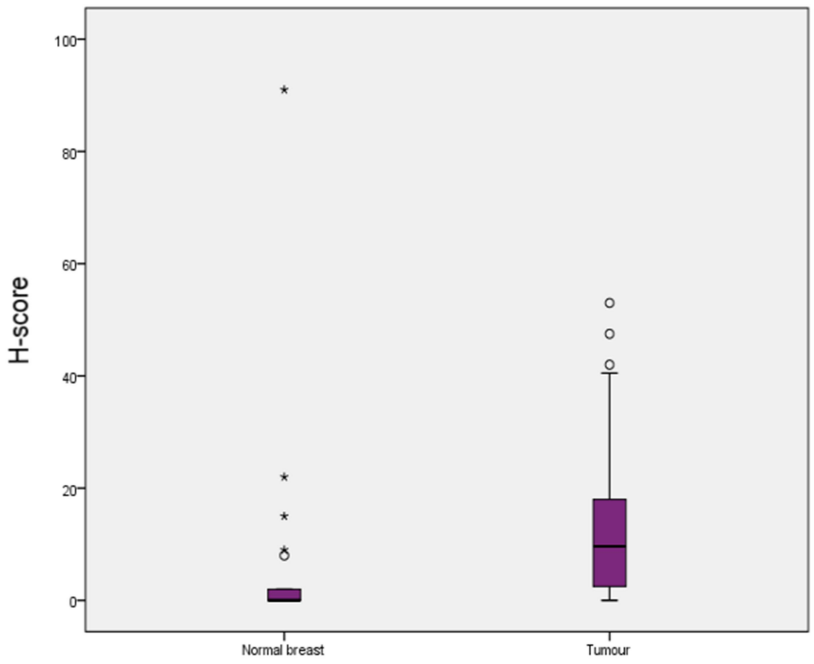

Figure 3.4 AURKA protein expression in normal breast epithelia and in TNBC cases. AURKA protein was overexpressed in 72% of the TNBC cases which scored higher than the average H-score of normal breast epithelium.

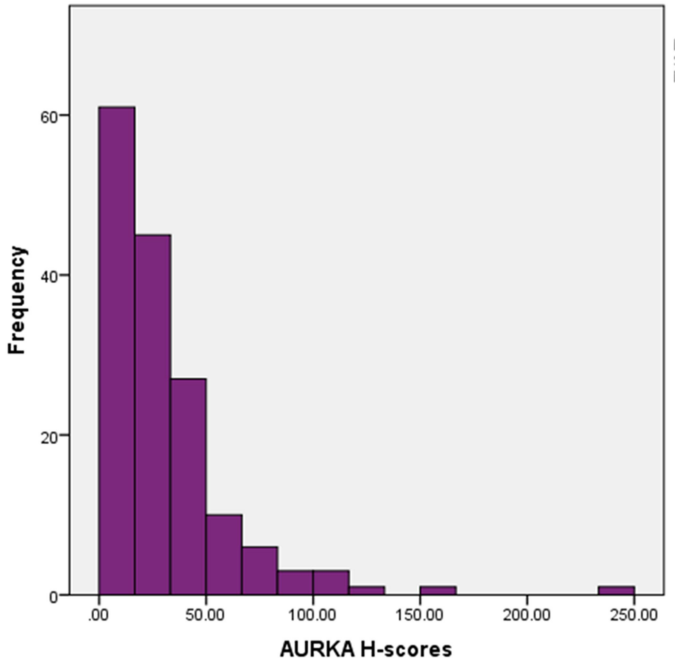

Fig 3.5 Histogram showing the distribution of AURKA H-scores. The majority of cases had an H-score of 50 or less.

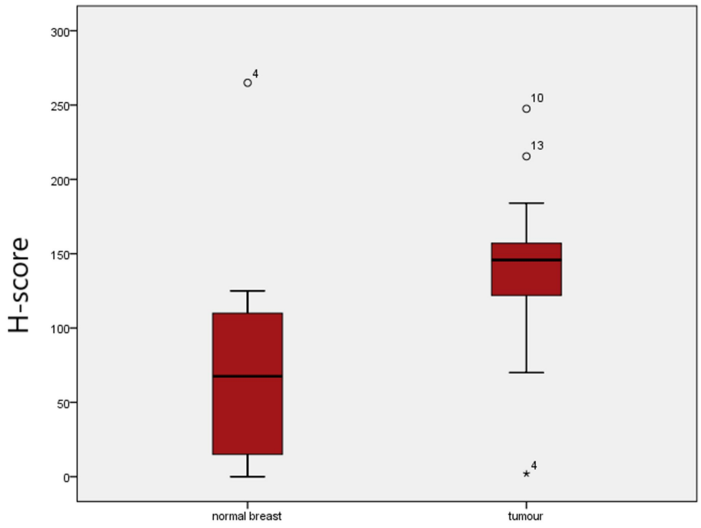

Fig 3.6 KIF2C protein expression in normal breast epithelia and in TNBC cases. KIF2C protein was overexpressed in 56% of the TNBC cases which scored higher than the average H-score of normal breast epithelium.

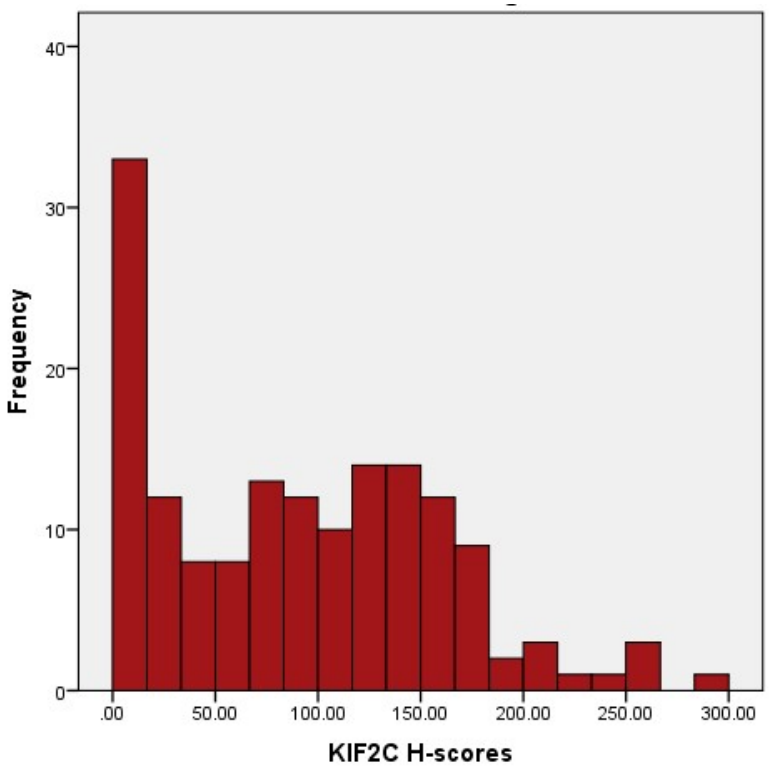

Fig 3.7 Histogram showing the distribution of KIF2C H-scores. KIF2C showed more intense staining than AURKA, as demonstrated by the distribution on this histogram.

Table 3.2 AURKA and KIF2C H-scores do not follow a normal distribution. (AURKA, D(158) = 0.182, p = 0; KIF2C, D(156) = 0.111, p = 0)

One-Sample Kolmogorov-Smirnov Test

		AURKA	KIF2C
N		158	156
Normal Parameters[a,b]	Mean	29.6519	90.3718
	Std. Deviation	32.69428	68.65298
Most Extreme Differences	Absolute	.182	.111
	Positive	.135	.111
	Negative	-.182	-.094
Test Statistic		.182	.111
Asymp. Sig. (2-tailed)		.000[c]	.000[c]

a. Test distribution is Normal.

b. Calculated from data.

c. Lilliefors Significance Correction.

3.1.5 Identification of complimentary biomarkers to AURKA and KIF2C

One of the aims of this study is to identify complimentary biomarkers to AURKA and KIF2C so that a novel biomarker signature can be established which will identify a potential therapeutic group within a subset of TNBC patients, based on PP2A deregulation. This was achieved by correlating the AURKA and KIF2C RNA expression of 58 TNBC cases with high AURKA protein expression, with the RNA expression of the 40 genes described in section 2.5.

3.1.5.1 Normalisation of data against housekeeping genes

RNA expression data was normalized to remove any variables which might affect its validity such as variations in sample consistency and those related to the assays being utilised. This is done by normalizing the expression of the gene being investigated to the expression of reference genes, which are also known as housekeeping genes. These genes are present in basic cellular functions and are known to remain constant without being affected by different cellular processes and conditions (Eisenberg & Levanon, 2013).

The three reference genes utilised in this study are Peptidylprolyl isomerase B (PPIB), Hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) and TATA-box binding protein (TBP) gene. These genes have been chosen based on their high expression and low variability and are not involved in being regulated by any of the target genes being investigated. The other two housekeeping genes Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Actin beta (ACTB) were not chosen due to a high variability, thus introducing an element of variation.

3.1.5.2 Correlation of RNA expression levels of AURKA and KIF2C with 40-plex genes

The Spearman's rank correlation coefficient was utilised for the correlation of the RNA expression levels of AURKA and KIF2C with the 40-plex genes. This was preferred over other correlation tests since protein expression data was not normally distributed and thus a non-parametric test was chosen.

AURKA and KIF2C correlated strongly between each other, whilst both biomarkers were found to be correlated with nuclear auto-antigenic sperm protein (NASP), hypoxia inducible factor 1 (HIF1A), and PP2CB.

AURKA was found to positively correlate with β-catenin (CTNNB1), BRCA1 and PP2A inhibitor CIP2A. On the other hand, there was an inverse relationship between AURKA and SETBP1. KIF2C positively correlated with PP2A deregulators SET and PME-1 whilst there was a negative correlation with TWIST, CTNNB1, PP2CA and SETBP1 (Table 3.3).

PP2A inhibitory regulators CIP2A and PME-1 were chosen as candidate biomarkers for further evaluation within the TNBC cohort and TNBC cell models (Table 3.4).

Table 3.3 Significant correlations of AURKA and KIF2C at RNA level with 40-plex assay genes. AURKA showed a positive correlation with β-catenin (CTNNB1), BRCA1 and CIP2A. KIF2C showed a positive correlation with SET and PME-1.

			AURKA_3HK	KIF2C_3HK	NASP_3HK	HIF1A_3HK	PPP2CB_3HK	GAPDH_3HK	CTNNB1_3HK	BRCA1_3HK	KIAA1524_3HK	SETBP1_3HK	SET_3HK	PPME1_3HK	PPP2CA_3HK	TWIST1_3HK
Spearman's rho	AURKA_3HK	Correlation Coefficient	1.000	.656**	.351**	.466**	.367**	.452**	-.398**	.270*	.341**	-.302*	.113	.235	-.221	-.147
		Sig. (2-tailed)	.	.000	.007	.000	.005	.000	.002	.046	.010	.021	.396	.075	.095	.271
		N	58	57	58	58	58	58	58	55	57	58	58	58	58	58
	KIF2C_3HK	Correlation Coefficient	.656**	1.000	.594**	.385**	.322*	.416**	-.283*	.145	.257	-.342**	.261*	.289*	-.282*	-.343**
		Sig. (2-tailed)	.000	.	.000	.003	.015	.001	.033	.292	.056	.009	.050	.029	.033	.009
		N	57	57	57	57	57	57	57	55	56	57	57	57	57	57
	NASP_3HK	Correlation Coefficient	.351**	.594**	1.000	.430**	.542**	.500**	.072	.425**	.358**	-.040	.352**	.456**	-.223	-.102
		Sig. (2-tailed)	.007	.000	.	.001	.000	.000	.593	.001	.006	.765	.007	.000	.093	.447
		N	58	57	58	58	58	58	58	55	57	58	58	58	58	58
	HIF1A_3HK	Correlation Coefficient	.466**	.385**	.430**	1.000	.455**	.501**	-.085	.186	.121	-.127	-.018	.024	-.096	.067
		Sig. (2-tailed)	.000	.003	.001	.	.000	.000	.527	.174	.369	.343	.896	.860	.472	.619
		N	58	57	58	58	58	58	58	55	57	58	58	58	58	58
	PPP2CB_3HK	Correlation Coefficient	.367**	.322*	.542**	.455**	1.000	.439**	.026	.442**	.490**	.028	.050	.289*	-.281*	.133
		Sig. (2-tailed)	.005	.015	.000	.000	.	.001	.845	.001	.000	.835	.707	.028	.033	.320
		N	58	57	58	58	58	58	58	55	57	58	58	58	58	58
	GAPDH_3HK	Correlation Coefficient	.452**	.416**	.500**	.501**	.439**	1.000	-.277*	.171	-.026	-.434**	.070	.123	-.351**	-.293*
		Sig. (2-tailed)	.000	.001	.000	.000	.001	.	.036	.213	.850	.001	.604	.357	.007	.026
		N	58	57	58	58	58	58	58	55	57	58	58	58	58	58
	CTNNB1_3HK	Correlation Coefficient	-.398**	-.283*	.072	-.085	.026	-.277*	1.000	.003	.026	.457**	.250	.229	.402**	.251
		Sig. (2-tailed)	.002	.033	.593	.527	.845	.036	.	.983	.848	.000	.059	.084	.002	.058
		N	58	57	58	58	58	58	58	55	57	58	58	58	58	58
	BRCA1_3HK	Correlation Coefficient	.270*	.145	.425**	.186	.442**	.171	.003	1.000	.449**	.294*	.212	.272*	-.048	.031
		Sig. (2-tailed)	.046	.292	.001	.174	.001	.213	.983	.	.001	.029	.120	.044	.728	.820
		N	55	55	55	55	55	55	55	55	54	55	55	55	55	55
	KIAA1524_3HK	Correlation Coefficient	.341**	.257	.358**	.121	.490**	-.026	.026	.449**	1.000	.193	.261*	.235	.024	.211
		Sig. (2-tailed)	.010	.056	.006	.369	.000	.850	.848	.001	.	.151	.050	.079	.857	.115
		N	57	56	57	57	57	57	57	54	57	57	57	57	57	57
	SETBP1_3HK	Correlation Coefficient	-.302*	-.342**	-.040	-.127	.028	-.434**	.457**	.294*	.193	1.000	.098	.096	.476**	.511**
		Sig. (2-tailed)	.021	.009	.765	.343	.835	.001	.000	.029	.151	.	.463	.474	.000	.000
		N	58	57	58	58	58	58	58	55	57	58	58	58	58	58
	SET_3HK	Correlation Coefficient	.113	.261*	.352**	-.018	.050	.070	.250	.212	.261*	.098	1.000	.343**	.051	-.038
		Sig. (2-tailed)	.396	.050	.007	.896	.707	.604	.059	.120	.050	.463	.	.008	.705	.778
		N	58	57	58	58	58	58	58	55	57	58	58	58	58	58
	PPME1_3HK	Correlation Coefficient	.235	.289*	.456**	.024	.289*	.123	.229	.272*	.235	.096	.343**	1.000	-.001	-.171
		Sig. (2-tailed)	.075	.029	.000	.860	.028	.357	.084	.044	.079	.474	.008	.	.993	.199
		N	58	57	58	58	58	58	58	55	57	58	58	58	58	58
	PPP2CA_3HK	Correlation Coefficient	-.221	-.282*	-.223	-.096	-.281*	-.351**	.402**	-.048	.024	.476**	.051	-.001	1.000	.310*
		Sig. (2-tailed)	.095	.033	.093	.472	.033	.007	.002	.728	.857	.000	.705	.993	.	.018
		N	58	57	58	58	58	58	58	55	57	58	58	58	58	58
	TWIST1_3HK	Correlation Coefficient	-.147	-.343**	-.102	.067	.133	-.293*	.251	.031	.211	.511**	-.038	-.171	.310*	1.000
		Sig. (2-tailed)	.271	.009	.447	.619	.320	.026	.058	.820	.115	.000	.778	.199	.018	.
		N	58	57	58	58	58	58	58	55	57	58	58	58	58	58

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 3.4 Spearman correlation of RNA expression of AURKA and KIF2C with selected biomarkers CIP2A and PME-1.

			AURKA	KIF2C	CIP2A	PME-1
Spearman's rho	AURKA	Correlation Coefficient	1.000	0.656**	0.341**	0.235
		Sig. (2-tailed)	.	.000	.010	.075
		N	58	57	57	58
	KIF2C	Correlation Coefficient	0.656**	1.000	0.257	0.289*
		Sig. (2-tailed)	.000	.	.056	.029
		N	57	57	56	57
	CIP2A	Correlation Coefficient	0.341**	0.257	1.000	0.235
		Sig. (2-tailed)	.010	.056	.	.079
		N	57	56	57	57
	PME-1	Correlation Coefficient	0.235	0.289*	0.235	1.000
		Sig. (2-tailed)	.075	.029	.079	.
		N	58	57	57	58

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

3.1.6 Protein expression of AURKA, CIP2A, PME-1 and Ki67 on TMAs

TMAs consisting of the entire TNBC cohort were stained immunohistochemically with AURKA, CIP2A, PME-1 and Ki67. Protein expression of AURKA showed a significant correlation with protein expression of PP2A inhibitors CIP2A and PME-1 and with the proliferation marker Ki67. CIP2A failed to correlate with Ki67 whilst PME-1 showed a strong correlation with all three biomarkers, i.e. AURKA, CIP2A and Ki67 (Table 3.5; Appendix B).

CIP2A stained mainly in a cytoplasmic pattern but membranous staining was also noted. PME-1 stained exclusively in a cytoplasmic pattern on the TNBC TMA sections (Figure 3.8).

Table 3.5 Spearman correlations of AURKA, CIP2A, PME-1 and Ki67 expressed as protein in TNBC cohort TMAs. KIF2C protein expression was not evaluated on the TMAs.

			AURKA	CIP2A	PME-1	Ki67
Spearman's rho	AURKA	Correlation Coefficient	1.000	0.170*	0.342**	0.342**
		Sig. (2-tailed)	.	.037	.000	.000
		N	155	152	152	149
	CIP2A	Correlation Coefficient	0.170*	1.000	0.382**	0.074
		Sig. (2-tailed)	.037	.	.000	.364
		N	152	154	154	151
	PME-1	Correlation Coefficient	0.342**	0.382**	1.000	0.243**
		Sig. (2-tailed)	.000	.000	.	.003
		N	152	154	154	151
	Ki67	Correlation Coefficient	0.342**	0.074	0.243**	1.000
		Sig. (2-tailed)	.000	.364	.003	.
		N	149	151	151	151

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

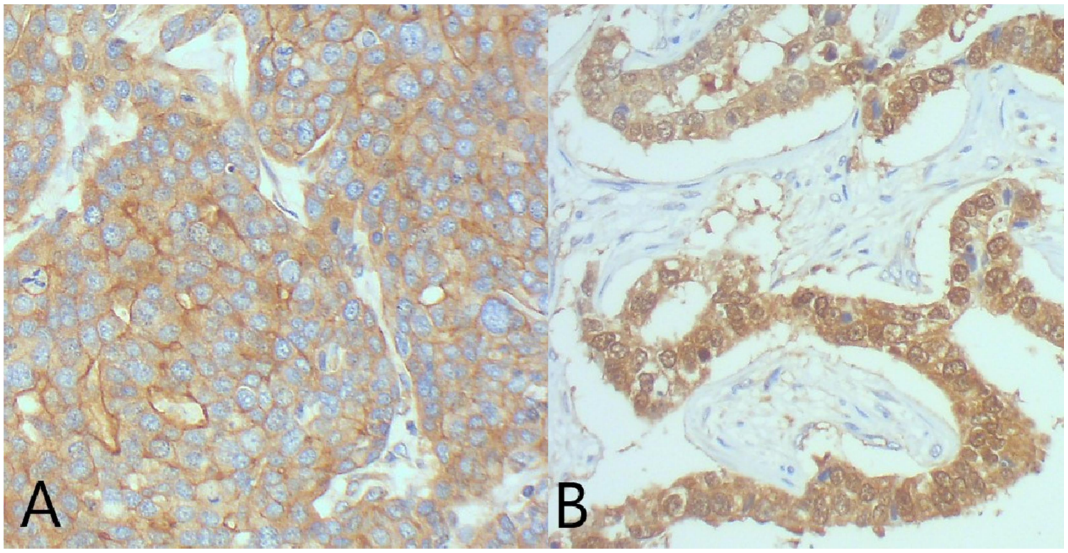

Figure 3.8 IHC staining of CIP2A and PME-1 on TNBC sections. CIP2A (A) staining was predominantly cytoplasmic but membranous staining was also noted. PME-1 (B) stained exclusively in a cytoplasmic pattern.

3.1.7 Effect on protein expression of biomarkers in treated TNBC cell lines with FTY720

Protein expression of AURKA, PME-1 and CIP2A was evaluated in six TNBC cell line models. The sensitivity of these cell lines to FTY720 was established in previous studies by our research group, thus sub-dividing the cell lines into sensitive and non-sensitive. The three sensitive cell lines were MDAMB231, Hs578t and BT20 whilst MDAMB468, MDAMB436 and HCC1937 were non-sensitive cell lines to FTY720.

At steady state, there was no significant difference in AURKA, CIP2A and PME-1 protein expression when evaluating sensitive cell lines versus non-sensitive cell lines (Table 3.6). Cell lines were treated with FTY720 at two concentrations, 5μM and 10μM. No statistically significant difference was noted for the two concentrations across the TNBC cell lines for the

three biomarkers (Table 3.7 and Table 3.8). No significant difference was noted between sensitive cell lines at steady state and after treatment with 5μM FTY720 (Table 3.9).

Table 3.6 Comparison of protein expression of AURKA, CIP2A and PME-1 at steady-state. There was no significant difference in protein expression of AURKA, CIP2A and PME-1 between sensitive and non-sensitive cell lines at steady state.

	AURKA	CIP2A	PME-1
Mann-Whitney U	8.000	12.000	7.000
Wilcoxon W	29.000	33.000	28.000
Z	-1.604	-.962	-1.764
Asymp. Sig. (2-tailed)	.109	.336	.078
Exact Sig. [2*(1-tailed Sig.)]	.132[b]	.394[b]	.093[b]

a. Grouping Variable: Sensitivity

b. Not corrected for ties.

Table 3.7 Comparison of AURKA, CIP2A and PME-1 between TNBC cell lines at steady state and TNBC cell lines treated with 5μM FTY720. No significant difference in protein expression of AURKA, CIP2A and PME-1 between steady state TNBC cell lines and treated cell lines with 5μM FTY720.

	AURKA	CIP2A	PME-1
Mann-Whitney U	35.000	29.000	18.500
Wilcoxon W	113.000	107.000	96.500
Z	-.094	-.656	-1.641
Asymp. Sig. (2-tailed)	.925	.512	.101
Exact Sig. [2*(1-tailed Sig.)]	.964[b]	.553[b]	.102[b]

a. Grouping Variable: Dose2

b. Not corrected for ties.

Table 3.8 Comparison of AURKA, CIP2A and PME-1 between TNBC cell lines at steady state and TNBC cell lines treated with 10µM FTY720. No significant difference in protein expression of AURKA, CIP2A and PME-1 between steady state TNBC cell lines and treated cell lines with 10µM FTY720.

	AURKA	CIP2A	PME-1
Mann-Whitney U	2.000	11.000	14.000
Wilcoxon W	8.000	89.000	20.000
Z	-2.311	-.183	-.578
Asymp. Sig. (2-tailed)	.021	.855	.563
Exact Sig. [2*(1-tailed Sig.)]	.018[b]	.923[b]	.633[b]

a. Grouping Variable: Dose2

b. Not corrected for ties.

Table 3.9 Comparison of AURKA, CIP2A and PME-1 between sensitive TNBC cell lines at steady state and sensitive TNBC cell lines treated with 5µM FTY720. No significant difference in protein expression of AURKA, CIP2A and PME-1 between steady state sensitive TNBC cell lines and treated cell lines with 5µM FTY720.

	AURKA	CIP2A	PME-1
Z	-.816[b]	-1.069[c]	-1.069[c]
Asymp. Sig. (2-tailed)	.414	.285	.285

a. Wilcoxon Signed Ranks Test

b. Based on positive ranks.

c. Based on negative ranks.

3.1.8 Correlation with clinicopathological parameters

Clinicopathological significance of the biomarkers expressions in our TNBC cohort was investigated via descriptive analysis of the data retrieved from the histology database. Significance of the biomarkers' expression in relation to available data reflecting aggressiveness of the tumours was also evaluated (Appendix C).

The mean age in the TNBC cohort was 61 years of age, with 48 percent of the patients falling under the mean age. 79% (124/157) of the TNBC cases were diagnosed as Grade 3 tumours, indicating an element of aggressiveness in our TNBC cohort.

Nevertheless, no significantly higher biomarker expression was noted in the TNBC cohort with vascular invasion, with Ki67 showing a trend towards an association with vascular invasion (Table 3.10). Of note, data on vascular invasion was only available for 120 cases out of the 157 cases in our TNBC cohort. No significantly higher biomarker expression was noted in the TNBC cohort when involvement of lymph nodes against the biomarkers' expression was evaluated (Table 3.11), with AURKA and Ki67 trending towards an association with lymph node involvement. Data was available for 138 out of 157 cases. Of note, data was considerably limited on mitotic activity, thus limiting the possibility of analysis on this parameter.

Table 3.10 Analysis of biomarker protein expression between TNBC cases positive and negative for vascular invasion.

	AURKA	CIP2A	PME-1	Ki67
Mann-Whitney U	1633.500	1637.500	1808.000	1245.000
Wilcoxon W	3778.500	3782.500	3404.000	3390.000
Z	-1.022	-.950	-.063	-2.590
Asymp. Sig. (2-tailed)	0.307	0.342	0.950	0.010

a. Grouping Variable: VASCINV

Table 3.11 Analysis of biomarker protein expression between TNBC cases positive and negative for lymph node involvement.

	AURKA	CIP2A	PME-1	Ki67
Mann-Whitney U	1786.500	1781.000	2086.500	1513.500
Wilcoxon W	2914.500	2862.000	6457.500	2594.500
Z	-1.847	-1.604	-.238	-2.565
Asymp. Sig. (2-tailed)	0.065	0.109	0.812	0.010

a. Grouping Variable: LNPOSNEG

Chapter IV

Discussion

4.1 AURKA and KIF2C protein levels are overexpressed in the TNBC cohort

AURKA and KIF2C protein expression was evaluated in a cohort of TNBC patients. The results indicated that AURKA and KIF2C protein levels are overexpressed in the TNBC tissue sections, when compared to normal breast tissue epithelia.

Previous studies in our research group have identified AURKA and KIF2C as potential biomarkers of PP2A activity based on their high RNA expression in TNBC cell lines known to have low PP2A activity. This was further affirmed by a downregulation in RNA expression of AURKA and KIF2C upon PP2A activation with the PP2A activator, FTY720, in the same cell lines.

These results are also in agreement with the study by Baldacchino et al. (2014) where it was reported that PP2A deregulation is present in approximately 60% of basal-like breast tumours, based on in silico analysis of the cBioPortal for Cancer Genomics. Other studies also report that AURKA and KIF2C protein was found to be overexpressed in various tumours (Gnjatic et al., 2010; Goldenson & Crispino, 2015; Katsha et al., 2015; Nikonova et al., 2013; Shen et al., 2017) contributing to tumour cell proliferation and tumour progression.

Of interest, AURKA protein overexpression was higher than protein expression of KIF2C in the TNBC cohort. AURKA protein was overexpressed in 114 out of 158 of the TNBC cases (72%) whilst KIF2C protein was overexpressed in 87 out of 156 cases (56%). AURKA is known to be involved in the localization and regulation of KIF2C, yet the difference between AURKA overexpression when compared to KIF2C overexpression in this study can be attributed to KIF2C's regulation not being solely dependent on AURKA. Zhang et al. (2008) report that AURKB is also a regulator of KIF2C, and phosphorylates KIF2C at S-196, which is the same protein location targeted by AURKA in the regulation of KIF2C.

Both AURKA and KIF2C have stained in a cytoplasmic pattern. Subcellular localization of a protein gives an indication of its function on a cellular level. Localization in the cytoplasm is a common event in tumours and according to Das et al. (2010) the presence of AURKA in the cytoplasm leads to centrosome amplification, with resulting tumorigenesis.

These results demonstrate that a subset of TNBC patients can be classified on protein expression of both biomarkers with special emphasis on AURKA due to its direct interaction with PP2A. This subset of patients can then benefit from targeted therapeutic options via PP2A activation, or via direct targeting by AURKA inhibitors.

4.2 Correlation of AURKA and KIF2C with PP2A negative regulators CIP2A and PME-1

AURKA and KIF2C RNA expression was correlated against a 40-gene plex consisting of breast cancer molecular classifiers, PP2A subunits and inhibitors, epithelial to mesenchymal transition markers and other genes related to breast cancer, across a cohort of TNBC cases with high AURKA protein expression. This was done to identify biomarkers which would support the use of a panel defining a signature which would classify a subset of TNBC patients in relation to PP2A deregulation.

Results demonstrate a correlation between AURKA and KIF2C RNA expression and PP2A negative regulators CIP2A and PME-1. AURKA protein expression was then correlated with CIP2A, PME-1 and Ki67 across the entire TNBC cohort, where AURKA showed a strong correlation with all three biomarkers.

These results further contribute to previous studies where PP2A deregulation is a major factor in TNBC, which deregulation was attributed to PP2A inhibition by negative regulators (Baldacchino et al., 2014). As previously described, AURKA is known to be regulated via dephosphorylation by PP2A shifting the cell cycle to mitotic exit (Horn et al, 2007). This

suggests that inhibition of PP2A leads to AURKA overexpression with resulting tumour cell proliferation.

Furthermore, correlation of AURKA with CIP2A at both RNA and protein level suggests a relationship at both transcriptional and translational level between the two biomarkers. CIP2A is known to inhibit the negative regulation of the oncoprotein Myc by PP2A (Come et al., 2009) whereas Myc has been reported to be involved in the upregulation of AURKA (den Hollander et al, 2010 & Nikonova et al., 2013). This proposes another mechanism which links and explains the correlation between AURKA expression and CIP2A expression in our study, other than PP2A deregulation.

Results also show that AURKA correlates with PME-1 at protein level but not at RNA level. PME-1 is known to regulate PP2A post-translationally via demethylation of the catalytic subunit of the phosphatase (Kaur & Westermarck, 2016). This implies that PME-1 post-translational regulation of PP2A affects AURKA at protein level but not at RNA level.

Nevertheless, PME-1 showed a very strong correlation with AURKA and Ki67 at protein level, suggesting a valuable oncogenic role in the proliferation status of TNBC. This role has already been investigated and confirmed in glioma and endometrial adenocarcinoma, implicating PME-1 as a positive regulator of the ERK and Akt signalling pathways, both of which are negatively regulated by PP2A (Puustinen et al., 2009 & Wandzioch et al, 2014). Of interest, KIF2C is upregulated by the ERK signalling pathway, offering a possible explanation for the correlation between KIF2C and PME-1 at RNA level in this study (Zaganjor et al., 2014).

4.3 Biomarkers' protein expression not affected by FTY720 in treated TNBC cell lines

Protein expression of AURKA, CIP2A and PME-1 was evaluated in six TNBC cell lines, three of which were established in previous studies from our research group to be sensitive to

PP2A activator FTY720 treatment, based on a reduction in cell viability after treatment with FTY720. These studies have also shown that AURKA, KIF2C and CIP2A RNA levels were downregulated after treatment with FTY720. This was mostly pronounced in the TNBC cell line MDAMB231.

In this study, results indicate that there was no difference in protein expression of AURKA, CIP2A and PME-1 between sensitive and non-sensitive cell lines at steady-state. In line with results from previous studies, it would be expected that at steady state, the three biomarkers would be overexpressed in cell lines which are then characterised by PP2A reactivation upon treatment with FTY720. Furthermore, no significant changes were observed after treatment with FTY720, in both sensitive and non-sensitive cell lines. Nevertheless, it must be noted that all three biomarkers were highly expressed as protein across all cell lines.

These results suggest that the effect of FTY720 on sensitive cell lines is present at a pre-translational stage, resulting in the lack of reduction effect in protein expression. Similar results were observed in the study by Tseng et al. (2012) where the effect of the drug bortezomib was limited to a downregulation in CIP2A RNA levels, whilst protein levels were unaffected.

Of interest, in this study, PP2A negative regulator SET did not correlate with AURKA and KIF2C at RNA level in the TNBC cohort with high AURKA expression. FTY720 acts on SET-PP2A complexes, re-establishing PP2A activity by disrupting the complex (Westermarck & Hahn, 2008). A low SET expression in our cohort might provide a plausible explanation as to why no significant reduction was achieved in protein expression of our biomarkers.

Studies involving FTY720 reactivation of PP2A in tumours have mostly focused on PP2A negative regulators SET and CIP2A, omitting the relatively unexplored field of PME-1 in

breast cancer. PP2A inhibition via enzymatic demethylation of the catalytic subunit by PME-1 results in denaturation of the affected site (Sangodkar et al, 2016), and this might give an insight into the post-translational mechanism by which PME-1 hinders the effect of PP2A reactivation by FTY720.

These results also indicate that protein expression of our panel of biomarkers is not ideally applied to classify a subset of TNBC patients for treatment via PP2A activation by FTY720. Nevertheless, one should consider treatment of TNBC patients expressing these biomarkers via inhibitors which directly target the biomarkers, either individually or as a combined therapeutic regime of inhibitors against these biomarkers.

Combined therapeutic options can also include the application of these biomarker inhibitors together with chemotherapeutic options since the expression of these biomarkers might be linked to chemoresistance by the tumour cells. Case in point is the study by Wang et al. (2017) where tumour cells expressing AURKA were resistant to platinum therapeutic agent cisplatin, yet promising results were achieved when AURKA was inhibited by alisertib leading to sensitization of the tumour cells to cisplatin.

4.4 CIP2A offers a potential therapeutic target in TNBC

CIP2A is reported to be overexpressed in various malignancies including breast cancer (Come et al, 2009). In a study by Niemela et al. (2012), CIP2A clustered mainly with the basal subtype of breast cancer, and the highest CIP2A overexpression was reported in basal-like breast cancer cell lines, out of the 40 cell lines being investigated. CIP2A is not reported to be expressed in normal breast tissue, thus targeting of CIP2A would limit the effect of therapy to tumour cells expressing CIP2A only.

Various studies involving TNBC have shown promising results when targeting CIP2A. Liu et al. (2016) report that pharmacological intervention on CIP2A via inhibitors led to an increase

in PP2A expression with subsequent decrease in p-Akt signalling. This led to a decrease in tumour cell proliferation and an increase in the apoptotic rate of breast cancer cells.

Similar results were achieved through the use of the proteasome inhibitor bortezomib, where tumour cell apoptosis was once again induced in TNBC cell lines. This was once again achieved by the downregulation of CIP2A expression with associated downregulation of p-Akt. Thus, CIP2A can also act as a predictive biomarker classifying TNBC patients to targeted therapy by bortezomib (Tseng et al., 2012).

Furthermore, CIP2A was downregulated in TNBC after treatment with TD52 which is a derivative of erlotinib (Liu et al., 2017). Furthermore, in a study by Huang et al. (2017), TNBC cell lines were treated with Arctigenin, a drug derived from the seeds of *Arctium lappa*, where once again CIP2A was downregulated at both RNA and protein levels. This also involved the downregulation of p-Akt, leading to apoptosis of the TNBC cells.

Considering the promising results from these studies, CIP2A has a valuable oncogenic role in TNBC and is a targetable option in TNBC characterised by CIP2A overexpression. The correlation of CIP2A with AURKA at both RNA and protein level in our cohort suggests that this subset of patients might benefit also from a combined therapeutic regime consisting of both CIP2A and AURKA inhibitors.

4.5 PME-1 – a promising novel oncogenic marker

PME-1 is a relatively novel biomarker in the cancer field yet recent studies on various tumours have shown that PME-1 overexpression leads to malignant transformation via activation of the Akt signalling pathway (Jackson and Pallas, 2012). In line with the strong correlation of PME-1 protein expression with AURKA, CIP2A and Ki67 in this study, PME-1 merits further investigation as a targetable biomarker which would in theory lead to attenuation of the Akt signalling pathway in the TNBC cohort.

In a study by Wandzioch et al. (2014), PME-1 was reported to be overexpressed in endometrial adenocarcinoma cells, also correlating with tumour cell proliferation and invasive properties via activation of the ERK and Akt pathway and related PP2A inhibition. PME-1 was also implicated in human malignant glioma by protecting the ERK pathway from PP2A regulation. Moreover, PME-1 expression was reported to be correlated with tumour cell proliferation and a shift from a low to a high grade of the tumour (Puustinen et al., 2009).

The aza-β-lactam, ABL127, and the sulfonyl acrylonitrile, AMZ-30, were recognised as potential PME-1 inhibitors in a study by Bachovchin et al. (2011). This was substantiated in a study by Pusey et al. (2016), where a decrease in tumour cell proliferation was once again observed in a study involving endometrial carcinoma.

These studies together with our results offer a new insight in PME-1 being a targetable therapeutic option. PME-1 inhibition leads to attenuation of the ERK and Akt pathways, which are reported to be highly activated in TNBC (Corkery et al., 2009), leading to a reduction in tumour cell proliferation.

4.6 Association of biomarker signature with clinicopathological parameters

Overexpression of AURKA, CIP2A, PME-1 and Ki67 has been implicated as being associated with tumour cell proliferation together with tumour progression and aggressiveness (Kaur et al., 2016; Ali et al., 2012; Soofiyani et al., 2017; Zhang et al., 2015). Nevertheless, protein expression of the biomarkers in this study was not found to be correlated with neither vascular invasion nor lymph node involvement when evaluated across the TNBC cohort.

One major limitation which might have contributed to absence of association with regards to these clinicopathological parameters is that data was retrieved from reports which were not well annotated with regards to relevant information on this aspect. A substantial number of

reports had missing data regarding the presence or absence of vascular invasion, lymph node involvement and overall staging of the tumour.

The neoadjuvant therapeutic approach to TNBC might also be implicated in the lack of association with these parameters. Neoadjuvant therapy has a relatively good pathological response in TNBC, with regards to lymph nodes generally being clear of tumour following post-treatment surgery. Data from histology reports was not always well annotated with regards to therapy received prior to surgery. One can recommend that the biomarker signature is investigated on breast cores rather than breast resections, since these would have been sampled prior to therapy. However, this would result in a limitation if the biomarker panel was evaluated on TMAs rather than whole sections, due to lack of patient material.

Furthermore, with the introduction of breast screening, a number of TNBC cases which were included in this study might have been diagnosed at an early stage. Data available on this factor was also limited and could not be associated with the expression of the biomarkers.

In order to properly assess the prognostic power of the biomarker signature, one can further review the H&E slides so as to gather as much data as possible regarding each case. Nonetheless, this would result in a very laborious and time-consuming process.

4.7 Further work and limitations

The identification of PME-1 as an important biomarker in this study merits further investigation by evaluating the expression at RNA and protein level in a larger number of breast cancer cell lines, also investigating the effect of FTY720 treatment in these cell lines on PME-1 expression.

The effect of inhibitors to AURKA, CIP2A and PME-1 on TNBC cell line models should also be further investigated. This will give more light to the possibility of these biomarkers becoming a targetable therapeutic group within a subset of TNBC patients.

One major limitation of this study was the laborious and time-consuming process of evaluating protein expression of the TNBC sections using the H-score system. TMAs have facilitated the process yet given the heterogeneity of TNBC, whole tissue section microscopic examination would give a more specific picture.

With the advent of digital pathology, scoring of immunohistochemistry slides would be made easier using proper software to evaluate the slides with regards to protein expression. The use of the software would also introduce an element of standardisation since H-scoring is a subjective procedure.

4.8 Conclusion

The identification of PP2A negative regulators, CIP2A and PME-1 as correlating biomarkers with high AURKA expression in TNBC further substantiates PP2A deregulation in TNBC. As already described, PP2A deregulation was mainly attributed to inhibitory mechanisms, leading to hinderance of PP2A's role as a tumour suppressor with oncogenic consequences.

Since no targeted therapeutic options are available for treatment of TNBC, this biomarker signature can potentially classify a subset of TNBC patients into a specific therapeutic group. Treatment options can be based on PP2A activation via pharmacological intervention by FTY720 or directly targeting the expressed biomarkers via inhibitors to these biomarkers. This study favours the latter scenario which would offer novel therapeutic options in TNBC since to date, treatment options are mainly based on generalised chemotherapy.

References

Ali, H. R., Dawson, S. J., Blows, F. M., Provenzano, E., Pharoah, P. D., & Caldas, C. (2012). Aurora kinase A outperforms Ki67 as a prognostic marker in ER-positive breast cancer. *British journal of cancer*, *106*(11), 1798.

Alluri, P., & Newman, L. A. (2014). Basal-like and triple-negative breast cancers: searching for positives among many negatives. *Surgical Oncology Clinics*, *23*(3), 567-577.

Alvarez-Fernández, M., Halim, V. A., Aprelia, M., Mohammed, S., & Medema, R. H. (2011). Protein phosphatase 2A (B55α) prevents premature activation of forkhead transcription factor FoxM1 by antagonizing cyclin A/cyclin-dependent kinase-mediated phosphorylation. *Journal of Biological Chemistry*, *286*(38), 33029-33036.

Anjum, F., Razvi, N., & Masood, M. A. (2017). Breast Cancer Therapy: A Mini Review. *MOJ Drug Des Develop Ther*, *1*(2), 00006.

Bachovchin, D. A., Mohr, J. T., Speers, A. E., Wang, C., Berlin, J. M., Spicer, T. P., ... & Nomura, D. K. (2011). Academic cross-fertilization by public screening yields a remarkable class of protein phosphatase methylesterase-1 inhibitors. *Proceedings of the National Academy of Sciences*, *108*(17), 6811-6816.

Baldacchino, S., Saliba, C., Petroni, V., Fenech, A. G., Borg, N., & Grech, G. (2014). Deregulation of the phosphatase, PP2A is a common event in breast cancer, predicting sensitivity to FTY720. *EPMA Journal*, *5*(1), 3.

Ballinger, T., Kremer, J., & Miller, K. (2016). Triple negative breast cancer-review of current and emerging therapeutic strategies. *European Oncology and Haematology*, 12(2), 112-117.

Baxter, E., Gondara, L., Lohrisch, C., Chia, S., Gelmon, K., Hayes, M., ... & Tyldesley, S. (2015). Using proliferative markers and Oncotype DX in therapeutic decision-making for breast cancer: the BC experience. *Current Oncology*, *22*(3), 192.

Bianchini, G., Balko, J. M., Mayer, I. A., Sanders, M. E., & Gianni, L. (2016). Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. *Nature reviews Clinical oncology*, *13*(11), 674.

Bie, L., Zhao, G., Wang, Y. P., & Zhang, B. (2012). Kinesin family member 2C (KIF2C/MCAK) is a novel marker for prognosis in human gliomas. *Clinical neurology and neurosurgery*, *114*(4), 356-360.

Borg, N., Baldacchino, S., Saliba, C., Falzon, S., DeGaetano, J., Scerri, C., & Grech, G. (2014). Phospho-Akt expression is high in a subset of Triple Negative Breast Cancer Patients. *Editorial Board*, 52.

Brenner, D. R., Brockton, N. T., Kotsopoulos, J., Cotterchio, M., Boucher, B. A., Courneya, K. S., ... & Friedenreich, C. M. (2016). Breast cancer survival among young women: a review of the role of modifiable lifestyle factors. *Cancer Causes & Control*, *27*(4), 459-472.

Cancer Genome Atlas Network. (2012). Comprehensive molecular portraits of human breast tumours. *Nature*, *490*(7418), 61.

Cao, S. S., & Lu, C. T. (2016). Recent perspectives of breast cancer prognosis and predictive factors. *Oncology letters*, *12*(5), 3674-3678.

Carmena, M., Ruchaud, S., & Earnshaw, W. C. (2009). Making the Auroras glow: regulation of Aurora A and B kinase function by interacting proteins. *Current opinion in cell biology*, *21*(6), 796-805.

Chalakur-Ramireddy, N. K., & Pakala, S. B. (2018). Combined drug therapeutic strategies for the effective treatment of Triple Negative Breast Cancer. *Bioscience reports*, *38*(1), BSR20171357.

Chen, J. X., Deng, N., Chen, X., Chen, L. W., Qiu, S. P., Li, X. F., & Li, J. P. (2012). A novel molecular grading model: combination of Ki67 and VEGF in predicting tumor recurrence and progression in non-invasive urothelial bladder cancer. *Asian Pacific Journal of Cancer Prevention*, *13*(5), 2229-2234.

Chen, J., Li, S., Zhou, S., Cao, S., Lou, Y., Shen, H., Yin, G. & Li, G. (2017). Kinesin superfamily protein expression and its association with progression and prognosis in hepatocellular carcinoma. *Journal of cancer research and therapeutics*, *13*(4), 651.

Collignon, J., Lousberg, L., Schroeder, H., & Jerusalem, G. (2016). Triple-negative breast cancer: treatment challenges and solutions. *Breast Cancer: Targets and Therapy*, *8*, 93.

Costa, R. L., Han, H. S., & Gradishar, W. J. (2018). Targeting the PI3K/AKT/mTOR pathway in triple-negative breast cancer: a review. *Breast cancer research and treatment*, 1-10.

Cristóbal, I., Madoz-Gúrpide, J., Manso, R., González-Alonso, P., Rojo, F., & García-Foncillas, J. (2016). Potential anti-tumor effects of FTY720 associated with PP2A activation: a brief review. *Current medical research and opinion*, *32*(6), 1137-1141.

Cristóbal, I., Manso, R., Rincón, R., Caramés, C., Senin, C., Borrero, A., Martinez-Useros, J., Rodriguez, M., Zazo, S., Aguilera, O. & Madoz-Gúrpide, J. (2014). PP2A inhibition is a common event in colorectal cancer and its restoration using FTY720 shows promising therapeutic potential. *Molecular cancer therapeutics*, *13*(4), 938-947.

Das, K., Lorena, P. D. N., Kuan Ng, L., Shen, L., Lim, D., Yun Siow, W., ... & Salto-Tellez, M. (2010). Aurora-A expression, hormone receptor status and clinical outcome in hormone related cancers. *Pathology*, *42*(6), 540-546.

Den Hollander, J., Rimpi, S., Doherty, J. R., Rudelius, M., Buck, A., Hoellein, A., ... & Goga, A. (2010). Aurora kinases A and B are up-regulated by Myc and are essential for maintenance of the malignant state. *Blood*, *116*(9), 1498-1505.

Den Hollander, P., Savage, M. I., & Brown, P. H. (2013). Targeted therapy for breast cancer prevention. *Frontiers in oncology*, *3*, 250.

Dominguez-Brauer, C., Thu, K. L., Mason, J. M., Blaser, H., Bray, M. R., & Mak, T. W. (2015). Targeting mitosis in cancer: emerging strategies. *Molecular cell*, *60*(4), 524-536.

Foulkes, W. D., Smith, I. E., & Reis-Filho, J. S. (2010). Triple-negative breast cancer. *New England journal of medicine*, *363*(20), 1938-1948.

Gnjatic, S., Cao, Y., Reichelt, U., Yekebas, E. F., Nölker, C., Marx, A. H., ... & Horn, C. (2010). NY-CO-58/KIF2C is overexpressed in a variety of solid tumors and induces frequent T cell responses in patients with colorectal cancer. *International journal of cancer*, *127*(2), 381-393.

Goldenson, B., & Crispino, J. D. (2015). The aurora kinases in cell cycle and leukemia. *Oncogene*, *34*(5), 537.

Goldhirsch, A., Wood, W. C., Coates, A. S., Gelber, R. D., Thürlimann, B., Senn, H. J., & Panel Members. (2011). Strategies for subtypes—dealing with the diversity of breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. *Annals of oncology*, *22*(8), 1736-1747.

Gonzalez-Angulo, A. M., Timms, K. M., Liu, S., Chen, H., Litton, J. K., Potter, J., ... & Hortobagyi, G. N. (2011). Incidence and outcome of BRCA mutations in unselected patients with triple receptor-negative breast cancer. *Clinical Cancer Research*, *17*(5), 1082-1089.

Grech, G., Baldacchino, S., Saliba, C., Grixti, M. P., Gauci, R., Petroni, V., Fenech, A. G. & Scerri, C. (2016). Deregulation of the protein phosphatase 2A, PP2A in cancer: complexity and therapeutic options. *Tumor Biology*, *37*(9), 11691-11700.

Horn, V., Thélu, J., Garcia, A., Albiges-Rizo, C., Block, M. R., & Viallet, J. (2007). Functional interaction of Aurora-A and PP2A during mitosis. *Molecular biology of the cell*, *18*(4), 1233-1241.

Huober, J., Gelber, S., Goldhirsch, A., Coates, A. S., Viale, G., Öhlschlegel, C., ... & Thürlimann, B. (2012). Prognosis of medullary breast cancer: analysis of 13 International Breast Cancer Study Group (IBCSG) trials. *Annals of oncology*, *23*(11), 2843-2851.

Iacono, M. L., Monica, V., Saviozzi, S., Ceppi, P., Bracco, E., Papotti, M., & Scagliotti, G. V. (2011). Aurora Kinase A expression is associated with lung cancer histological-subtypes and with tumor de-differentiation. Journal of translational medicine, 9(1), 100.

Ibrahim, T., Farolfi, A., Scarpi, E., Mercatali, L., Medri, L., Ricci, M., ... & Amadori, D. (2013). Hormonal receptor, human epidermal growth factor receptor-2, and Ki67 discordance between primary breast cancer and paired metastases: clinical impact. *Oncology*, *84*(3), 150-157.

Janghorban, M., Farrell, A. S., Allen-Petersen, B. L., Pelz, C., Daniel, C. J., Oddo, J., ... & Sears, R. C. (2014). Targeting c-MYC by antagonizing PP2A inhibitors in breast cancer. *Proceedings of the National Academy of Sciences*, *111*(25), 9157-9162.

Katsha, A., Belkhiri, A., Goff, L., & El-Rifai, W. (2015). Aurora kinase A in gastrointestinal cancers: time to target. *Molecular cancer*, *14*(1), 106.

Kaur, A., & Westermarck, J. (2016). Regulation of protein phosphatase 2A (PP2A) tumor suppressor function by PME-1. *Biochemical Society transactions*, *44*(6), 1683-1693.

Koh, H. M., Jang, B. G., Hyun, C. L., Kim, Y. S., Hyun, J. W., Chang, W. Y., & Maeng, Y. H. (2017). Aurora Kinase A Is a Prognostic Marker in Colorectal Adenocarcinoma. *Journal of pathology and translational medicine*, *51*(1), 32.

Kontzoglou, K., Palla, V., Karaolanis, G., Karaiskos, I., Alexiou, I., Pateras, I., Konstantoudakis, K. & Stamatakos, M. (2013). Correlation between Ki67 and breast cancer prognosis. *Oncology*, *84*(4), 219-225.

Lam, A. K. Y., Ong, K., & Ho, Y. H. (2008). Aurora kinase expression in colorectal adenocarcinoma: correlations with clinicopathological features, p16 expression, and telomerase activity. Human pathology, 39(4), 599-604.

Lassmann, S., Shen, Y., Jütting, U., Whiele, P., Walch, A., Gitsch, G., ... & Werner, M. (2007). Aurora-a is a predictive marker for stage III epithelial ovarian cancers. *Verhandlungen der Deutschen Gesellschaft fur Pathologie*, *91*, 225-232.

Lehmann, B. D., Bauer, J. A., Chen, X., Sanders, M. E., Chakravarthy, A. B., Shyr, Y., & Pietenpol, J. A. (2011). Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *The Journal of clinical investigation*, *121*(7), 2750-2767.

Li, S., Feng, T. T., Guo, Y., Yu, X., Huang, Q., Zhang, L., Tang, W. & Liu, Y. (2016). Expression of cancerous inhibitor of protein phosphatase 2A in human triple negative breast cancer correlates with tumor survival, invasion and autophagy. *Oncology letters*, *12*(6), 5370-5376.

Li, L. T., Jiang, G., Chen, Q., & Zheng, J. N. (2015). Ki67 is a promising molecular target in the diagnosis of cancer. *Molecular medicine reports*, *11*(3), 1566-1572.

Lips, E. H., Mulder, L., Oonk, A., Van Der Kolk, L. E., Hogervorst, F. B. L., Imholz, A. L. T., ... & Nederlof, P. M. (2013). Triple-negative breast cancer: BRCAness and concordance of clinical features with BRCA1-mutation carriers. *British journal of cancer*, *108*(10), 2172.

Liu, C. Y., Huang, T. T., Chen, Y. T., Chen, J. L., Chu, P. Y., Huang, C. T., ... & Tseng, L. M. (2019). Targeting SET to restore PP2A activity disrupts an oncogenic CIP2A-feedforward loop and impairs triple negative breast cancer progression. *EBioMedicine*, *40*, 263-275.

Liu, H., Qiu, H., Song, Y., Liu, Y., Wang, H., Lu, M., ... & Zhang, Z. (2017). Cip2a promotes cell cycle progression in triple-negative breast cancer cells by regulating the expression and nuclear export of p27Kip1. *Oncogene*, *36*(14), 1952.

Marotti, J. D., de Abreu, F. B., Wells, W. A., & Tsongalis, G. J. (2017). Triple-Negative Breast Cancer: Next-Generation Sequencing for Target Identification. *The American journal of pathology*, *187*(10), 2133-2138.

Masoud, V., & Pagès, G. (2017). Targeted therapies in breast cancer: New challenges to fight against resistance. *World journal of clinical oncology*, *8*(2), 120.

Massihnia, D., Galvano, A., Fanale, D., Perez, A., Castiglia, M., Incorvaia, L., ... & Castorina, S. (2016). Triple negative breast cancer: shedding light onto the role of pi3k/akt/mtor pathway. *Oncotarget*, *7*(37), 60712.

Manning, A. L., Ganem, N. J., Bakhoum, S. F., Wagenbach, M., Wordeman, L., & Compton, D. A. (2007). The kinesin-13 proteins Kif2a, Kif2b, and Kif2c/MCAK have distinct roles during mitosis in human cells. *Molecular biology of the cell*, *18*(8), 2970-2979.

Mehra, R., Serebriiskii, I. G., Burtness, B., Astsaturov, I., & Golemis, E. A. (2013). Aurora kinases in head and neck cancer. *The lancet oncology*, *14*(10), e425-e435.

Nabi, U., Nagi, A. H., & Sami, W. (2008). Ki-67 proliferating index and histological grade, type and stage of colorectal carcinoma. *J Ayub Med Coll Abbottabad*, *20*(4), 44-8.

Nasa, I., & Kettenbach, A. N. (2018). Coordination of Protein Kinase and Phosphoprotein Phosphatase Activities in Mitosis. *Frontiers in cell and developmental biology*, *6*, 30.

Nelson, H. D., Zakher, B., Cantor, A., Fu, R., Griffin, J., O'meara, E. S., ... & Mandelblatt, J. S. (2012). Risk factors for breast cancer for women aged 40 to 49 years: a systematic review and meta-analysis. *Annals of internal medicine*, *156*(9), 635-648.

Neophytou, C., Boutsikos, P., & Papageorgis, P. (2018). Molecular Mechanisms and Emerging Therapeutic Targets of Triple-Negative Breast Cancer Metastasis. *Frontiers in Oncology*, *8*, 31.

Niemelä, M., Kauko, O., Sihto, H., Mpindi, J. P., Nicorici, D., Pernilä, P., ... & Westermarck, J. (2012). CIP2A signature reveals the MYC dependency of CIP2A-regulated phenotypes and its clinical association with breast cancer subtypes. *Oncogene*, *31*(39), 4266.

Nikonova, A. S., Astsaturov, I., Serebriiskii, I. G., Dunbrack, R. L., & Golemis, E. A. (2013). Aurora A kinase (AURKA) in normal and pathological cell division. *Cellular and Molecular Life Sciences*, *70*(4), 661-687.

Nishimura, R., Osako, T., Nishiyama, Y., Tashima, R., Nakano, M., Fujisue, M., ... & Arima, N. (2014). Prognostic significance of Ki-67 index value at the primary breast tumor in recurrent breast cancer. *Molecular and clinical oncology*, *2*(6), 1062-1068.

Onitilo, A. A., Engel, J. M., Greenlee, R. T., & Mukesh, B. N. (2009). Breast cancer subtypes based on ER/PR and Her2 expression: comparison of clinicopathologic features and survival. *Clinical medicine & research*, *7*(1-2), 4-13.

Otto, T., & Sicinski, P. (2017). Cell cycle proteins as promising targets in cancer therapy. *Nature Reviews Cancer*, *17*(2), 93-115.

Pal, S. K., Childs, B. H., & Pegram, M. (2011). Triple negative breast cancer: unmet medical needs. *Breast cancer research and treatment*, *125*(3), 627-636.

Pareja, F., Geyer, F. C., Marchiò, C., Burke, K. A., Weigelt, B., & Reis-Filho, J. S. (2016). Triple-negative breast cancer: the importance of molecular and histologic subtyping, and recognition of low-grade variants. *NPJ breast cancer*, *2*, 16036.

Pathmanathan, N., & Balleine, R. L. (2013). Ki67 and proliferation in breast cancer. *Journal of clinical pathology*, *66*(6), 512-516.

Perou, C. M. (2011). Molecular stratification of triple-negative breast cancers. *The oncologist*, *16*(Supplement 1), 61-70.

Ragab, H. M., Samy, N., Afify, M., El Maksoud, N. A., & Shaaban, H. M. (2018). Assessment of Ki-67 as a potential biomarker in patients with breast cancer. *Journal of Genetic Engineering and Biotechnology*.

Reis-Filho, J. S., & Tutt, A. N. J. (2008). Triple negative tumours: a critical review. *Histopathology*, *52*(1), 108-118.

Sablina, A. A., Hector, M., Colpaert, N., & Hahn, W. C. (2010). Identification of PP2A complexes and pathways involved in cell transformation. *Cancer research*, *70*(24), 10474-10484.

Sangodkar, J., Farrington, C. C., McClinch, K., Galsky, M. D., Kastrinsky, D. B., & Narla, G. (2016). All roads lead to PP2A: exploiting the therapeutic potential of this phosphatase. *The FEBS journal*, *283*(6), 1004-1024.

Sanhaji, M., Friel, C. T., Wordeman, L., Louwen, F., & Yuan, J. (2011). Mitotic centromere-associated kinesin (MCAK): a potential cancer drug target. *Oncotarget*, *2*(12), 935.

Seshacharyulu, P., Pandey, P., Datta, K., & Batra, S. K. (2013). Phosphatase: PP2A structural importance, regulation and its aberrant expression in cancer. *Cancer letters*, *335*(1), 9-18.

Shao, M. M., Liu, J., Vong, J. S., Niu, Y., Germin, B., Tang, P., ... & Gary, M. T. (2011). A subset of breast cancer predisposes to brain metastasis. *Medical molecular morphology*, *44*(1), 15-20.

Shimo, A., Tanikawa, C., Nishidate, T., Lin, M. L., Matsuda, K., Park, J. H., ... & Nakamura, Y. (2008). Involvement of kinesin family member 2C/mitotic centromere-associated kinesin overexpression in mammary carcinogenesis. *Cancer science*, *99*(1), 62-70.

Soofiyani, S. R., Hejazi, M. S., & Baradaran, B. (2017). The role of CIP2A in cancer: a review and update. *Biomedicine & Pharmacotherapy*, *96*, 626-633.

Székely, B., Silber, A. L., & Pusztai, L. (2017). New therapeutic strategies for triple-negative breast cancer. *Oncology*, *31*(2).

Stewart, B. W. K. P., & Wild, C. P. (2014). World cancer report 2014. *Health*.

Tan, Y., Sun, D., Jiang, W., Klotz-Noack, K., Vashisht, A. A., Wohlschlegel, J., Widschwendter, M. & Spruck, C. (2014). PP2A-B55β antagonizes cyclin E1 proteolysis and promotes its dysregulation in cancer. *Cancer research*, *74*(7), 2006-2014.

Tanno, Y., Kitajima, T. S., Honda, T., Ando, Y., Ishiguro, K. I., & Watanabe, Y. (2010). Phosphorylation of mammalian Sgo2 by Aurora B recruits PP2A and MCAK to centromeres. *Genes & development*, *24*(19), 2169-2179.

Tischer, T., Hörmanseder, E., & Mayer, T. U. (2012). The APC/C inhibitor XErp1/Emi2 is essential for Xenopus early embryonic divisions. *Science*, *338*(6106), 520-524.

Tökés, A. M., Szász, A. M., Geszti, F., Lukács, L. V., Kenessey, I., Turányi, E., ... & Bálint, K. (2015). Expression of proliferation markers Ki67, cyclin A, geminin and aurora-kinase A in primary breast carcinomas and corresponding distant metastases. *Journal of clinical pathology*, *68*(4), 274-282.

Tseng, L. M., Liu, C. Y., Chang, K. C., Chu, P. Y., Shiau, C. W., & Chen, K. F. (2012). CIP2A is a target of bortezomib in human triple negative breast cancer cells. *Breast cancer research*, *14*(2), R68.

Umene, K., Yanokura, M., Banno, K., Irie, H., Adachi, M., Iida, M., ... & Tominaga, E. (2015). Aurora kinase A has a significant role as a therapeutic target and clinical biomarker in endometrial cancer. *International journal of oncology*, *46*(4), 1498-1506.

Vermeulen, K., Van Bockstaele, D. R., & Berneman, Z. N. (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell proliferation*, *36*(3), 131-149.

Vuong, D., Simpson, P. T., Green, B., Cummings, M. C., & Lakhani, S. R. (2014). Molecular classification of breast cancer. *Virchows Archiv*, *465*(1), 1-14.

Wang, R. X., Chen, S., Jin, X., & Shao, Z. M. (2016). Value of Ki-67 expression in triple-negative breast cancer before and after neoadjuvant chemotherapy with weekly paclitaxel plus carboplatin. *Scientific reports*, *6*, 30091.

Wang, G., Jiang, Q., & Zhang, C. (2014). The role of mitotic kinases in coupling the centrosome cycle with the assembly of the mitotic spindle. *J Cell Sci*, *127*(19), 4111-4122.

Wlodarchak, N., & Xing, Y. (2016). PP2A as a master regulator of the cell cycle. *Critical reviews in biochemistry and molecular biology*, *51*(3), 162-184.

Wong, L. L., Zhang, D., Chang, C. F., & Koay, E. S. (2010). Silencing of the PP2A catalytic subunit causes HER-2/neu positive breast cancer cells to undergo apoptosis. *Experimental cell research*, *316*(20), 3387-3396.

Wurzenberger, C., & Gerlich, D. W. (2011). Phosphatases: providing safe passage through mitotic exit. *Nature reviews Molecular cell biology*, *12*(8), 469.

Xiang, T., Jia, Y., Sherris, D., Li, S., Wang, H., Lu, D., & Yang, Q. (2011). Targeting the Akt/mTOR pathway in Brca1-deficient cancers. *Oncogene*, *30*(21), 2443.

Xu, S., Li, S., Guo, Z., Luo, J., Ellis, M. J., & Ma, C. X. (2013). Combined targeting of mTOR and AKT is an effective strategy for basal-like breast cancer in patient-derived xenograft models. *Molecular cancer therapeutics*.

Xu, J., Wu, X., Zhou, W. H., Liu, A. W., Wu, J. B., Deng, J. Y., ... & Liu, Q. (2013). Aurora-A identifies early recurrence and poor prognosis and promises a potential therapeutic target in triple negative breast cancer. *PloS one*, *8*(2), e56919.

Yadav, B. S., Chanana, P., & Jhamb, S. (2015). Biomarkers in triple negative breast cancer: A review. *World journal of clinical oncology*, *6*(6), 252.

Yao, F., Zhang, C., Du, W., Liu, C., & Xu, Y. (2015). Identification of gene-expression signatures and protein markers for breast cancer grading and staging. *PloS one*, *10*(9), e0138213.

Yerushalmi, R., Woods, R., Ravdin, P. M., Hayes, M. M., & Gelmon, K. A. (2010). Ki67 in breast cancer: prognostic and predictive potential. *The lancet oncology*, *11*(2), 174-183.

Zhang, X., Ems-McClung, S. C., & Walczak, C. E. (2008). Aurora A phosphorylates MCAK to control ran-dependent spindle bipolarity. *Molecular biology of the cell*, *19*(7), 2752-2765.

Zhang, J., Li, B., Yang, Q., Zhang, P., & Wang, H. (2015). Prognostic value of Aurora kinase A (AURKA) expression among solid tumor patients: a systematic review and meta-analysis. *Japanese journal of clinical oncology*, *45*(7), 629-636.

Zhao, H., Li, D., Zhang, B., Qi, Y., Diao, Y., Zhen, Y., & Shu, X. (2017). PP2A as the Main Node of Therapeutic Strategies and Resistance Reversal in Triple-Negative Breast Cancer. *Molecules*, *22*(12), 2277.

Appendix A – TMA map

Protein expression of AURKA, CIP2A and PME-1 was evaluated on tissue microarrays consisting of duplicate cores from each TNBC case. Cores from various types of specimens and breast cancer cell lines were used to provide orientation and navigational points during microscopic examination (Figure A1).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Placenta	Placenta	Placenta	Placenta	Placenta	Placenta	Placenta	Placenta	Placenta	Placenta	Placenta	Placenta
B	Endo											Kidney
C	Endo											Kidney
D	Endo											Kidney
E	SmallINT											Kidney
F	SmallINT	Colon	Colon	Appx	Appx	Myometrium	SKBR3	ERPR pos	MDAMB231	BT474	MDAMB453	Kidney
G	SmallINT						SKBR3		MDAMB231	BT474	MDAMB453	Skin
H	Kidney											Skin
I	Kidney											Skin
J	Liver	Liver	Liver	Liver	Liver	Liver	Liver	Liver	Liver	Liver	Liver	Liver

Figure A1: Map of TMA used for the evaluation of biomarker protein expression. Fields highlighted in yellow are orientation cores used as reference points for navigation during microscopic examination. White fields hosted cores from the TNBC cases.

Appendix B – Raw data TMAs

Table B1 outlining the H-scores for AURKA, CIP2A, PME-1 and Ki67 on TMAs. NC: no core was present on the slide.

Case number	AURKA	CIP2A	PME-1	Ki67
1	1	35.5	87.5	22.5
2	9.5	22.75	47.5	11.5
3	6.5	135	9	1.75
4	0.5	36.25	55	7.75
5	1	280	87.5	0
6	0.5	72.5	0	0
7	0	16.5	0	32.5
8	0	102.5	11	6.25
9	2.5	25	79	NC
10	6.5	85	82.5	91.5
11	0.5	54	25	1.5
12	17	57.5	189	6.5
13	1.75	8.75	21.25	15.5
14	0	95.75	69.5	7
15	0	0	0	NC
16	0.25	6.25	57.5	0
17	0	100	67.5	7
18	0	25	42.5	22
19	0	58.75	0	24
20	1.25	36.75	22.5	7.5
21	49	13.5	32.5	49
22	31.75	74.5	46.25	21
23	0	112.5	35	0
24	2.5	0	0	80
25	5	72.5	0	1.5
26	18.5	0.25	0	1.5
27	0	165	100	0.5
28	0.5	62.5	25	3
29	0	15.5	3.75	3
30	0	12.5	0	0
31	0.5	NC	NC	NC
32	12.5	0.25	0	48
33	1.25	0	0	0
34	0	45	0	2
35	0.75	91	70	8
36	0	0	0	0

Case number	AURKA	CIP2A	PME-1	Ki67
37	6	0	1	0.5
38	0	0	24	25
39	0	72.5	217.5	43
40	1.5	32.5	270	6.5
41	0	9	16.25	26
42	13	100	72.5	80
43	12.75	26	85	6
44	55	52.5	12	1
45	0.75	38.75	38.75	5
46	0	47.5	0	2
47	0.5	10	5	11
48	73.25	9	17.5	63
49	12.5	51.25	189	1.5
50	3	87.5	7	2.5
51	0	0	0	1
52	0	16	2.5	48
53	0	43	39	2
54	0	15	13.75	2.5
55	0.5	91	7	0
56	0	18.75	0	24
57	1	157.5	13	0
58	1	54.5	2.5	0
59	111.25	60	52.5	88
60	0	0	17.5	0
61	0	2.5	16	0
62	6	72.5	131.25	14.5
63	0	62.5	0	12
64	0	0	0	5
65	0	20.5	0	10.5
66	0	0	0	0
67	6.25	85	188	29
68	0	12	14	16
69	0	50	45	14
70	0.75	70	75	0
71	0	112.5	52.5	7.5
72	0	42.5	1.25	4.5
73	0.5	9.5	57	0.5
74	22.5	80	107.5	55
75	4	0	62.5	21.5
76	0.25	51.25	28.75	2.5
77	0	50	70	0.5
78	0	14	0	6
79	0	33	13	3.5
80	0	20	11	19

Case number	AURKA	CIP2A	PME-1	Ki67
81	0	65	52	6.5
82	0	5	3.5	30
83	0	5	5	NC
84	0	20	0	0
85	5.75	0	0.75	0.5
86	18.25	42.5	0	45
87	0.5	36.25	65	0.5
88	0	92	48	3
89	0	NC	NC	NC
90	33	42	71.5	24.5
91	4.25	25	105	98
92	NC	8	16	7
93	11	43	125	22
94	67.5	230	75	50
95	60	227.5	107.5	85
96	7	55	84	46
97	2.75	0	0	64
98	5	34	40	49
99	8	30	0	9.5
100	7.25	99	0	14
101	0	24.5	26.25	6.5
102	0	3.25	0	26
103	21	NC	NC	NC
104	72.5	37	34.5	20
105	0	19	0	1
106	0	0	0	0
107	5.75	22.5	6.25	14.5
108	7	97.5	80	4.5
109	16.5	102.5	2	15
110	0	30	32	4.5
111	73.75	101	16.25	81
112	0	72.5	3.25	35
113	0.25	76.25	77.5	11
114	1.5	0	107.5	46.5
115	1.5	7.5	17	52
116	0	87.5	0	3
117	8	8.5	20	51
118	1.5	80	75	85
119	0	11	47.5	3
120	1	32	12.5	1.5
121	73	52	116	72
122	0	21	11	33
123	1.25	22	0	2.5
124	2	5	72.5	0

Case number	AURKA	CIP2A	PME-1	Ki67
125	17.5	87.5	102.5	54
126	2.5	22.5	167.5	75
127	3.17	119	76	21
128	0.5	36	22	21
129	0	82.5	42.5	18.5
130	0	28	0	0
131	0.25	2.25	0	0
132	0	81.25	1	26
133	8	37.5	22.5	42.5
134	2	31.5	67.5	32
135	0	26	36	11.5
136	0.5	0	60	91
137	4	72.5	0	75.5
138	2.5	25	0	84
139	0	18.75	0	30
140	0	7	0	1
141	0	20	0	76
142	0	97.5	52	22
143	NC	0	0	0
144	0	59	52.5	21.5
145	0	0	0	4
146	0	90	5	37
147	0	110	58.75	16
148	0	25	0	7
149	0	100	140	3.5
150	0	0	0	8.5
151	0	89	23	1
152	30.75	80	49	32
153	0	30	0	9
154	0	72.5	12.5	23
155	0.75	126.25	0	0
156	0	100	0	0
157	0	0	44	0

Appendix C – Clinicopathological parameters

Table C1 outlining clinicopathological data

Y – present

N - absent

Case number	Age	Vascular invasion	Lymph node invasion
1	65	Y	N
2	32	N	N
3	69		
4	30	Y	Y
5	51	N	N
6	38	Y	Y
7	38	Y	Y
8	50	Y	N
9	70	N	N
10	79	N	N
11	50	Y	N
12	66	Y	Y
13	61	Y	Y
14	43	Y	N
15	71	N	N
16	58	Y	
17	58	Y	
18	76	N	N
19	74	Y	N
20	65	N	N
21	55	Y	Y
22	77	N	N
23	84	N	N
24	68		N
25	63	Y	Y
26	60	Y	N
27	44	Y	Y
28	44	Y	Y
29	44	Y	Y
30	54	Y	N
31	65		N
32	65		N
33	65		
34	79		N
35	49	N	N
36	60	N	N

37	60	N	N
38	62		
39	62		
40	62		
41	62	Y	Y
42	43	N	N
43	74	Y	Y
44	70	N	Y
45	69		
46	58	N	N
47	45	N	N
48	33	N	N
49	59		N
50	64	N	N
51	64	N	N
52	81	N	N
53	77		Y
54	68	Y	Y
55	76	Y	N
56	61	N	N
57	72	Y	N
58	58	Y	N
59	81	Y	N
60	51	Y	N
61	83		N
62	70	Y	Y
63	55		Y
64	73	Y	
65	73	Y	
66	58	Y	Y
67	68		Y
68	65	Y	Y
69	85	Y	
70	52		Y
71	55	N	N
72	78	Y	N
73	45	Y	Y
74	45	Y	N
75	71	Y	
76	75		N
77	65	Y	Y
78	55	N	N
79	46	Y	N
80	46	Y	N
81	39	N	N
82	73	Y	Y

83	59	N	N
84	57	Y	N
85	70	Y	N
86	65	N	N
87	54		N
88	44	N	N
89	56	N	Y
90	72	N	N
91	86	N	N
92	50	Y	Y
93	79	Y	N
94	79	Y	N
95	79	Y	N
96	44	N	Y
97	68	N	N
98	64	Y	N
99	37		N
100	86	Y	N
101	38	Y	Y
102	51	Y	N
103	46	Y	Y
104	46	Y	Y
105	46	Y	Y
106	46	Y	Y
107	44	N	N
108	40		
109	76	N	N
110	59		N
111	56	N	N
112			
113	47	N	N
114	41	N	N
115	37		N
116	78	Y	
117	78	Y	N
118	85	N	N
119	56	Y	Y
120	62	N	Y
121	59		Y
122	62	Y	N
123	71		Y
124	56	Y	Y
125	71	N	N
126	51		Y
127	55	Y	N
128	79	N	N

129	77		Y
130	65	Y	Y
131	65	Y	Y
132	79		N
133	78		N
134	64	N	N
135	78	N	Y
136	51	N	Y
137	51	N	Y
138	51	N	N
139	32		N
140	50		Y
141	26		Y
142	55	N	N
143	84	N	N
144	84	N	N
145	55	N	N
146	67	Y	Y
147	61	N	N
148	76		Y
149	46	Y	Y
150	61	N	N
151	38		N
152	49	N	N
153	70	N	Y
154	69	N	N
155	68	N	N
156	53	N	N
157	44	Y	Y