Evaluation of the PI3K pathway and downstream effects in Her-2 positive and negative breast carcinomas

by

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DECLARATION

I, ANNA MAGDALENA (ANINA) RADEMAN, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

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ABSTRACT

Breast cancer management continues to be a challenge due to the heterogeneity of the disease and the fact that individuals with the same stage and pathological diagnosis may respond differently to treatment as a result of differences in gene expression. Several components of the Her-2/PI3K/Akt pathway were evaluated for their relevance as potential prognostic markers or indicators for treatment. A secondary objective was to evaluate the CISH technique for its suitability for analysis of Her-2 gene amplification on archived, Papanicolaou-stained fine needle aspiration (FNA) samples.

Tissue blocks from a retrospective series of 93 primary breast carcinoma cases were selected, based on their Her-2 status. Twenty six of these cases received trastuzumab treatment while 67 did not. Expression of Her-2, ER, PI3K, PTEN, p-Akt, BCL2, NFκB, MDM2 and p53 were analysed and compared with various clinicopathological features. The CISH technique was evaluated for its suitability for analysis of Her-2 gene amplification on archived, Papanicolaou-stained FNA samples.

Her-2(+) patients showed clear benefit from trastuzumab treatment although it was not significant. Comparing Her-2 expression with downstream components in the PI3K/Akt pathway and clinicopathological parameters, significant associations were found between Her-2(+) and high NFκB expression ($p=0.035$), Her-2(+) and ductal carcinomas ($p=0.006$) and Her-2(+) and high tumour grade ($p=0.004$). There was no significant association between PI3K and PTEN expression. A significant association was found between PI3K expression and lymphovascular invasion ($p=0.012$). This study also found a strong association between both lymphovascular and lymph node involvement and a subgroup of breast cancers: Her-2(+)/ER(+)/PI3K(high expression), which was even stronger than the previously reported Her-2(+)/ER(+) subgroup. High p-Akt expression was an indicator of good prognosis. The absence of p-Akt expression was associated with a five fold increase in the risk of death compared to cases showing high p-Akt expression (HR 5.1; CI1.5-17.3). Significant associations were also found between high p-Akt and high MDM2

expression (p=0.040) and between high p-Akt and high NFκB expression (p=0.014). High MDM2 expression was associated with an absence of distant metastasis (p=0.017) and reduced risk for death. Patients with tumours showing low MDM2 expression had a significant 3.2 fold increase in risk of death compared to those with MDM2 high category tumours (HR 3.2; CI1.1 – 16.7). A strong association was also found between high MDM2 expression and ductal carcinomas (p=0.051).

This study confirmed the value of some markers already identified by other studies but also identified new markers as possible indicators of prognosis and treatment. These findings however need to be evaluated in a larger study.

In addition, a 100% concordance was found between Her-2 CISH results on corresponding cytopathology and histopathology samples which serve as evidence that the Her-2 CISH technique can be successfully performed not only on paraffin embedded tissue sections but also on stained and archived FNA samples that have been de-stained.

TABLE OF CONTENTS

Tables

Figures

Appendices

ABBREVIATIONS

AIB1	amplified in breast cancer 1
ARF	alternate reading frame
Akt	acute transforming retrovirus from AKR thymoma
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
Apaf-1	apoptotic protease-activity factor 1
ATM	ataxia-telangiectasia mutated
BAFF	B-cell activating factor
BAX	BCL2-associated X protein
BCL2	B-cell lymphoma 2
BH3	Bcl-2 homology domain 3
CAF	cyclophosphamide, doxorubicin (Adriamycin) and 5-fluorouracil
c-AMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
c-Cbl	Casitas B-lineage lymphoma
c-FLIP	(FADD-like IL-1β-converting enzyme) inhibitory protein
CDC37	cell division cycle-37
Chk2	checkpoint kinase 2
c-IAP1	cellular inhibitor of apoptosis protein 1
c-IAP2	cellular inhibitor of apoptosis protein 2
CISH	chromogenic in-situ hybridization
CDK	cyclin dependent kinase
CK2	casein kinase 2
C-RAF	RAF proto-oncogene serine/threonine-protein kinase (cellular-RAF gene)
CREB	c-AMP response element-binding protein
CTMP	carboxyl-terminal modulator protein
DAB	3,3’-diaminobenzidine-tetrahydrochloride
DFS	disease free survival
DNA-DK	DNA dependent kinase
ECD	extracellular domain

EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELAM1	endothelial-leukocyte adhesion molecule 1
ER	oestrogen receptor
ERK	extracellular signal-related kinase
FAC	fluorouracil, doxorubicin (Adriamycin) and cyclophosphamide
FADD	Fas-associated death domain
FAK	focal adhesion kinase
FDA	food and drug administration
FISH	fluorescent in-situ hybridization
FLIPL	Fas-associated death domain-like interleukin-1-ß-converting enzyme-inhibitory protein
FKHRL1	forkhead-related family of mammalian transcription factor-1
FOXO	forkhead box O
GADD45	growth arrest and DNA damage-inducible 45 proteins
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRB2	growth factor receptor-bound protein-2
GROα	growth-regulated oncogene α
GSK-3	glycogen synthase kinase 3
GT	Gallotannin
GTP	guanosine triphosphate
HR	hazard ratio
HSP90	heat shock protein-90
IGF1R	insulin-like growth factor 1 receptor
HF	hinge fragment
ICAM1	intercellular adhesion molecule 1
IHC	immunohistochemistry
IκB	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
IKK	IκB kinase
IL	interleukin

ILK	integrin-linked kinase
IRS	insulin receptor signaling
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
K-RAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LTβ	lymphotoxin β
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
MAPK	mitogen-activated protein kinase
MAPKAPK2	mitogen-activated protein kinase activated protein kinase-2
MCP-1	monocyte chemotactic protein-1
MDM2	mouse double minute 2 homolog
MEK	MAPK/ERK kinase
MMP	mitochondrial membrane permeabilization
MMP2	matrix metalloproteinase 2
MMP9	matrix metalloproteinase 9
NFκB	nuclear factor-kappa B
Nck	non-catalytic region of tyrosine kinase adaptor protein
NCoR	Nuclear hormone receptor Co-Repressor
NEMO	NF-kappa-B essential modulator
NES	nuclear export signal
NIK	NF-κB inducing kinase
NLS	nuclear localization sequence
NO	nitric oxide
NoLS	nucleolar localization signal
NPI	Nottingham prognostic index
OS	overall survival
PBS	phosphate buffered saline
PCAF	p300/CBP-associated factor
PCNA	proliferating cell nuclear antigen
PDK1	phosphoinositide dependent kinase-1
PH	plekstrin homology
PHLPP	PH domain leucine-rich repeat phosphatases

PI	phosphatidylinositol
PI3K	phosphoinositide 3 kinase
PIDD	p53-induced death domain protein
PIK	phosphoinositide kinase
PIP2	phosphatidylinositol (4,5)-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-triphosphate
PKB	protein kinase B (Akt)
PKC	protein kinase C
PLC-γ	phospholipase-C-gamma
PR	progesterone receptor
pRb	retinoblastoma protein / tumour suppressor
PRK2	protein kinase C-related kinase-2
PtdIns	phosphoinositol
PTEN	phosphatase and tensin homolog deleted on chromosome 10
PTK	protein tyrosine kinase
PUMA	p53 up-regulated modulator of apoptosis
Pxn	paxillin
RAF	rapidly accelerated fibrosarcoma
RAS	rat sarcoma
RE	response elements
RH	Rel homology
RING	really interesting new gene
RIP	receptor-interacting protein
rRNA	ribosomal RNA
RTK	receptor tyrosine kinase
5S rRNA	5S ribosomal RNA in eukaryotes binds to the L5 ribosomal protein
SH2	Src homology-2 domain
SH3	Src homology-3 domain
SHC	SH2 domain containing transforming protein
SHIP	SH2-containing inositol phosphatase
SMRT	Silencing mediator or Retinoic acid and Thyroid hormone receptors

SOS	sun of sevenless
SRC1	steroid receptor co-activator 1
STAT	signal transducer and activator of transcription
TBS	tris-HCl buffered saline
TopIIα	topoisomerase II alpha
TNF	tumor necrosis factor
TNF–R1	tumour necrosis factor-receptor1
TRADD	tumour necrosis factor receptor 1-associated death domain
TRAF2	TNF receptor-associated factor 2
TSC1	tuberous sclerosis complex 1
TSC2	tuberous sclerosis complex 2
VCAM1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
V-RAF	virus-induced rapidly accelerated fibrosarcoma
WNT	wingless-related integration site
XIAP	X-linked inhibitor of apoptosis

CHAPTER 1

INTRODUCTION

Breast carcinoma is the most common malignancy in woman in many industrialized countries but its management continues to be challenging because of the heterogeneity of the disease (Jemal *et al.* 2005). It is widely acknowledged that individuals with the same stage and pathological diagnosis may respond differently to treatment as a result of differences in gene expression. The gene expression profile predicts the tumour phenotype and the sensitivity to treatment (Pusztai 2008). Despite the substantial effort that has been invested in the identification and validation of additional prognostic markers aimed at improving risk stratification and treatment of breast cancer, very few molecular markers have proven reliable in the clinical setting. Clinically relevant molecular tests currently performed on breast carcinomas that can be used for early detection, prediction of prognosis, disease monitoring and gene-specific therapy, are limited.

One of the molecular markers that is currently available and for which a targeted monoclonal antibody therapy is available, is Her-2. Overexpression of Her-2 is usually associated with the amplification of the *Her-2* gene and a poor prognosis (Owens *et al.* 2004).

Trastuzumab, better known as Herceptin®, is a humanised targeted antibody against the extracellular domain (ECD) of the Her-2 protein. The mechanisms of action of trastuzumab include internalization and degradation of the Her-2 receptor resulting in reduced downstream signalling through the PI3K and MAPK pathways which in turn lead to the induction of the cyclin dependent kinase inhibitor, $p27^{Kip1}$, responsible for promoting cell cycle arrest and apoptosis.

Other mechanisms of action include the phosphorylation and activation of the Akt/PI3K-inhibiting molecule, PTEN, inhibition of PI3K downstream effectors such as Akt and mTOR, inhibition of Her-2 ECD proteolysis and reduced VEGF production (Sliwkowski *et al.* 1999; Cuello M *et al.* 2001; Lane *et al.* 2001; Molina *et al.* 2001; Yakes *et al.* 2002; Nagata *et al.* 2004; Owens *et al.* 2004). The theory that trastuzumab inhibits proteolysis of Her-2 ECD is controversial

though. According to a study by Liu *et al*. (2007), trastuzumab had little or no effect on the shedding of Her-2 ECD.

Trastuzumab has been shown to reduce the risk of recurrence of Her-2 positive tumours by half and the mortality rate by one third in patients with early stage breast cancer (Romond *et al.* 2005). Despite the clear benefit of trastuzumab treatment in terms of disease free survival (DFS) and overall survival (OS), about one third of patients with Her-2 overexpressing carcinomas exhibit primary resistance to this treatment and the majority of patients with metastatic breast cancer, who initially respond to trastuzumab, show disease progression within one year of commencement of treatment (Nahta and Esteva 2006).

The reported cardiac failure in 28% of patients treated with trastuzumab and the cost of trastuzumab treatment (around R400 000,00 per annum), clearly indicate the need for improved knowledge of factors that would predict treatment response (Suter *et al.* 2004). Further studies could ensure the selection of only patients that would benefit from this or any other treatment and enable oncologists to select the treatment best suited for each individual based on the genetic profile of their cancer. Based on these facts, Her-2 status alone does not seem to be sufficient to justify trastuzumab treatment. Recently, a number of biomarkers have been identified as potentially useful predictors of response to trastuzumab-based therapies. These include components of the Her-2 downstream signalling pathway, such as PTEN whose activity contributes to the efficacy of trastuzumab, while PI3K co-amplification may render trastuzumab ineffective (Nagata *et al.* 2004).

We have evaluated these as well as other components of the Her-2/PI3K/Akt pathway for their relevance as potential prognostic markers.

CHAPTER 2
LITERATURE REVIEW

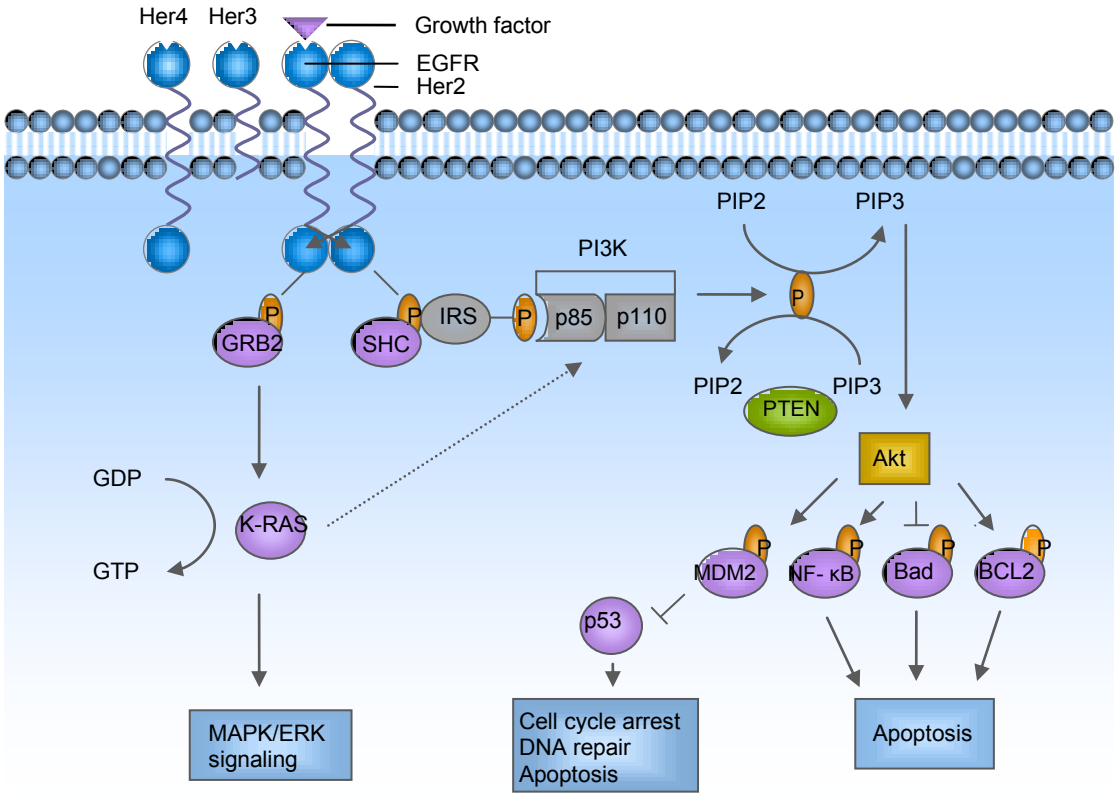


Figure 1: The Her-2/PI3K/Akt signalling pathway
Adapted from Hennessy *et al*. (2005)

2. Components of the Her-2/PI3K/Akt pathway

2.1 HER-2

2.1.1 Structure and function

The *Her-2* gene is a proto-oncogene located on the long arm of human chromosome 17(q11.2-q12). This gene encodes a 185kD transmembrane tyrosine kinase receptor protein ($p185^{HER}$,Her-2/*neu*, erbB-2) which is one of four related cell surface receptors, Her-1(erbB-1,EGFR), Her-2 (erbB-2), Her-3 (erbB-3) and Her-4 (erbB-4)(Rubin and Yarden 2001). These receptors (with the exception of Her-2 which has a closed ligand binding site) have an extracellular domain that is cysteine rich and acts as a ligand/growth factor receptor binding site, a lipophilic transmembrane domain as well as an intracellular protein tyrosine kinase (PTK) domain (van der Geer *et al.* 1994).

Binding of an epidermal growth factor (EGF) to the epidermal growth factor receptor (EGFR) binding site or high receptor density (overexpression) leads to the formation of homo- or heterodimers with other members of the Her-family. Receptor dimerization leads to the activation of intrinsic PTK activity, as well as tyrosine autophosphorylation. This in turn leads to the recruitment of a variety of signalling proteins, including the adaptor proteins, growth factor receptor-bound protein-2 (GRB2), Src homology-2 (SH2) domain containing transforming protein (SHC), nck adaptor protein (Nck), phospholipase-C-gamma (PLC-γ), signal transducer and activator of transcription (STAT), amongst other proteins (Klapper *et al.* 2000; Kuhnen and Winter 2006; Smith *et al.* 2006). Sequentially, these proteins are responsible for kinase-mediated activation of downstream signal transduction pathways such as the PI3K/Akt pathway, the mitogen-activated protein kinase (MAPK) pathway, the Janus kinase/activator and signal transducer pathway (JAK) and the PLC-γ pathway (Ross *et al.* 2003). The SH2 domain of GRB2 can either bind directly to phosphotyrosines of the activated EGFR or it can bind EGFR indirectly through the adaptor protein SHC. The SH3 domains of GRB2 are known to bind Son of Sevenless (SOS), which is an exchange factor for the GTPase, K-RAS, as well as c-Cbl, a regulator of EGFR endocytosis. Binding of the GRB2-SOS complex to the activated EGFR leads to GTP loading of K-RAS and then to the activation of K-RAS effectors, such as C-RAF kinases and PI3K. C-RAF in turn is responsible for the phosphorylation or activation of extracellular signal-related kinase (ERK) and MAPK/ERK kinase (MEK) (**Figure 1**) (Meng S *et al.* 2005; Immervoll *et al.* 2006).

The Her-2 surface receptor has no ligand binding domain of its own and therefore cannot bind growth factors. It does however, bind tightly to other ligand-bound EGFR family members to form heterodimers that stabilize ligand binding. In fact, Her-2 is found to be the preferred dimer partner for other members of the HER family. Heterodimers and especially those containing Her-2, result in stronger and prolonged signalling (Klapper *et al.* 2000). The reason for this is the relatively slow rate of ligand dissociation from Her-2 containing heterodimers as well as its slower rate of internalisation (Alroy and Yarden 1997). This effect is enhanced when Her-2 is amplified or overexpressed and leads to enhanced responsiveness to growth factors and

ultimately malignant growth (Rubin and Yarden 2001). The most potent heterodimer combination is Her2-Her3, which is also the combination most commonly found in malignancy (Alroy and Yarden 1997; Pinkas-Kramarski *et al.* 1998). In fact, Her-2 and Her-3 were described as obligate heterodimers due to their inability to signal as homodimers. This is attributed to their unique structural features. Her-2 has a closed ligand-binding domain and Her-3 an inactive kinase domain and yet when combined, they form the most potent signalling unit when it comes to cell growth and development (Garrett *et al.* 2003).

The Her-2 pathway can also be activated via proteolytic cleavage of its extracellular domain (ECD) resulting in a 95 kDa NH_2-terminally truncated membrane-bound fragment with kinase activity (Christianson *et al.* 1998).

2.1.2 Interactions with other proteins

A protein that was found to potentially play a role in Her-2 overexpression and tumourigenesis is Muc4, which is a heterodimeric transmembrane glycoprotein consisting of a mucin subunit (muc4α) and a transmembrane subunit (muc4β) (Sheng *et al.* 1990; Kozloski *et al.* 2010). Muc4 is a cell surface mucin with a protective function, protecting the epithelium from injuries and infections by keeping it lubricated (Hattrup and Gendler 2008). Abnormal expression of Muc4 has been reported in a number of malignancies where it is believed to play a role in cancer development by influencing cellular mechanisms that promote cancer development, such as cell adhesion, promotion of oncogenesis as well as cell signalling potential (Komatsu *et al.* 1997; Ponnusamy *et al.* 2008; Yonezawa *et al.* 2008; Kozloski *et al.* 2010). Muc4 is found to increase phosphorylation of Her-2 and this ultimately results in the activation of the Akt pathway (Carraway *et al.* 1999). Muc4 was also found to be responsible for suppressing the internalization of Her2-Her3 and thus enhancing the surface concentration of Her2-Her3 heterodimers (Funes *et al.* 2006).

2.1.3 Clinical implications

Her-2 is overexpressed in about 20-30% of breast carcinomas and associated with high grade breast carcinomas, high incidence of recurrence, short

remission time, distant metastases and overall poor prognosis, especially in lymph node positive disease (Slamon *et al.* 1987; Slamon *et al.* 1989; Owens *et al.* 2004; Yaziji *et al.* 2004). Her-2 overexpression is rare in benign breast disease and when present it is associated with an increased risk of subsequent invasive breast cancer (Klapper *et al.* 2000; Stark *et al.* 2000).

In addition to predicting more aggressive tumour behaviour, Her-2 overexpression may also be predictive of response to both hormonal- and chemotherapeutic agents. Her-2 overexpression has been associated with resistance to tamoxifen treatment (Dowsett 2001; Konecny *et al.* 2003; Johnston 2006). In fact, a few studies reported tamoxifen treatment to be beneficial in Her-2(-)/ER(+) tumours, but not in Her-2(+)/ER(+) tumours. One study suggested that tamoxifen treatment may actually have a detrimental effect on Her-2(+) tumours (Borg *et al.* 1994; Lohrisch and Piccart 2001). According to two preclinical studies, trastuzumab treatment is able to enhance the growth inhibitory activity of anti-oestrogens (including tamoxifen) in Her-2(+)/ER(+) breast tumours and may even reverse resistance of Her-2(+)/ER(+) tumours to anti-oestrogens (Witters *et al.* 1997; Kunisue *et al.* 2000). One study reported an improved outcome when Her-2(+)/ER(+) breast cancers in postmenopausal patients were treated with cyclophosphamide, doxorubicin, and 5-fluorouracil (CAF) regimen and tamoxifen (Albain *et al.* 2009). Her-2(+) tumours show sensitivity to dose-intensive treatment with CAF but resistance to non-anthracycline, non-taxane containing chemotherapeutic agents (Thor *et al.* 1998; Ellis *et al.* 2001; Ménard *et al.* 2001). Järvinen *et al*. (1999) suggested that the mechanism of anthracyclin sensitivity in Her-2(+) tumours may be related to the coexpression of Her-2 and TopIIα. They found TopIIα amplifications or deletions in more than 80% of Her-2(+) tumours while no TopIIα aberrations were found in Her-2(-) tumours.

Her-2 status is mainly detected by the immunohistochemistry (IHC), but the methodological and specimen variability, the variety of antibodies available, the lack of antibody sensitivity, the various scoring systems and the fact that the score is not always very objective, raises questions about the reliability of this method (Mezzelani *et al.* 1999). Fluorescent in-situ hybridization (FISH)

analysis on the other hand, is more objective, sensitive and reliable, but requires more sophisticated equipment and experience in cytogenetics (Slamon *et al.* 1987). Additionally, it is also much more expensive than the IHC method. Chromogenic in-situ hybridization (CISH) could be a more economical alternative to FISH, as it is said to be as sensitive and reliable as FISH. The only downside is that it only allows for a single colour detection of one probe at a time and in the case of polysomy of chromosome 17, it does not allow for correction of *Her-2* amplification (Vocaturo *et al.* 2006). At the time of this study, only 2 CISH kits had FDA approval, namely Invitrogen's "SPOT-light" kit for *Her-2* testing and more recently the *HER2* CISH pharmDx™ Kit from DAKO.

Staining for Her-2 receptor overexpression by IHC and testing for *Her-2/erb-B2* oncogene amplification by FISH or CISH encompass the current standard for determining patient eligibility for trastuzumab-based therapy (Jemal *et al.* 2005). Currently, local medical aid schemes will only approve trastuzumab treatment for patients with cancers shown to overexpress Her-2 by FISH analysis and may not accept an IHC (3+) result. A cost effective alternative would be to stain for Her-2 with IHC and then to confirm only the (2+) cases with CISH or FISH.

One of the most important reasons for determining the Her-2 status of a patient is that the intensity of the IHC staining or level of amplification with FISH would, to an extent, predict the response to trastuzumab treatment (Burstein *et al.* 2001; Slamon *et al.* 2001). Vogel *et al*. (2002) reported a 35% response rate for carcinomas with a Her-2 IHC score of (3+), while the (2+) cases showed only negligible benefit from trastuzumab treatment. Likewise, Burstein *et al*. (2001) reported a response rate of 34% in IHC (3+) carcinomas versus a 13% response rate in IHC (2+) carcinomas.

Lane *et al*. (2000) reported that although treatment of Her-2 overexpressing cells with monoclonal antibodies against the extracellular domain of Her-2 reduced phosphorylation and signalling of Her-2, it did not inhibit downstream signalling. As a result of these findings, this group postulated that inhibiting Her-2 receptor activity in Her-2 overexpressing tumours with the use of targeted antibodies, does not necessarily predict the cellular response to the treatment.

One possible downside of the existing Her-2 tests is that they only detect gene amplification or protein overexpression which does not necessarily reflect the functional activity of the receptor. Interestingly, in Her-2(+) invasive breast cancers, only 12% of Her-2 receptors appeared to be activated or phosphorylated while more that 58% were found to be phosphorylated in ductal carcinoma in situ (DiGiovanna *et al.* 2002). In a study involving 800 cases of Her-2 overexpressing breast cancers, adverse prognosis was limited to the cases displaying phosphorylated Her-2, suggesting that phosphorylated Her-2 may be a more powerful prognostic marker that Her-2 overexpression (Thor *et al.* 2000).

Elevated levels of Her-2 ECD (p95) have been found in the sera of patients with breast, ovarian and prostatic cancer (Leitzel *et al.* 1992; Myers *et al.* 1996; Meden *et al.* 1997). The level of p95 in the serum was found to correspond to the level of Her-2 expression and tumour load. Furthermore, this is also used as a marker for metastatic disease and predictor of recurrence, survival and response to anti-oestrogen therapy. Serum p95 has been reported to neutralize the activity of anti-Her-2 antibodies like trastuzumab that has been designed against the p95 domain of the Her-2 molecule (Leitzel *et al.* 1995; Molina *et al.* 1996; Brodowicz *et al.* 1997; Krainer *et al.* 1997). In addition, Christianson *et al*. (1998) found a strong correlation between p95 expression and nodal positivity in breast carcinomas but no significant correlation between p185 expression and nodal status or any of the other prognostic factors. In a study by Scaltriti *et al*. (2007), breast cancer cells were transfected with p95/Her-2 and Her-2 (full length) and subjected to treatment with trastuzumab and lapatinib, a Her-2 tyrosine kinase inhibitor. The cells transfected with p95/Her-2 were resistant to trastuzumab but responded well to lapatinib, indicating that breast cancers expressing p95/Her-2 require alternative or additional anti-Her-2 treatment to trastuzumab which is the current standard for Her-2-positive tumours. Finn *et al*. (2009), however did not find p95/Her-2 status to be a predictor of lapatinib benefit in Her-2(+) patients, although it correlated well with patient outcome, regardless of treatment.

Other potential mechanisms of trastuzumab resistance includes alternative or increased signalling through the PI3K/Akt pathway, downregulation of PTEN, decreased receptor-antibody interaction due to cell surface proteins such as Muc4. Muc4 was found to prevent trastuzumab binding by masking the receptor or Her-2 related receptors and non-Her-2 receptors such as insulin-like growth factor 1 receptor (IGF1R). IGF1R was found to be involved in crosstalk with Her-2 in trastuzumab resistant cells (Price-Schiavi *et al.* 2002; Nagata *et al.* 2004; Diermeier *et al.* 2005; Nahta *et al.* 2005)

Multiple studies have shown that Her-2 overexpression or amplification does play a substantial role in the prognosis of breast cancer and should be done on all breast cancers. In fact, Her-2 amplification was found to be of greater prognostic value than any other prognostic indicator, hormonal-receptor status included (Slamon *et al.* 1987; Slamon 2004).

2.2 PI3K

2.2.1 Structure and function

There are three families of phosphoinositide kinases; phosphoinositide 3-kinases (PI3Ks), PtdIns 4-kinases (PtdIns4Ks) and PtdIns-P(PIP) kinases (PIP5Ks). These phosphoinositide kinases are responsible for phosphorylating phosphoinositol (PtdIns), a phospholipid and phospholipase substrate that forms part of the cell membrane and consists of a head group and five free hydroxyl groups that can be phosphorylated.

Phosphorylated derivatives of phosphoinositol are called phosphoinositides (or inositol phosphate second messengers) and are responsible for regulating proliferation, vesicle trafficking, glucose transport, organisation of the cytoskeleton, location and activity of intracellular proteins, platelet function as well as cell survival (Fruman *et al.* 1998).

PI3K is a protein encoded by the gene, *PIK3CA,* which is a 34 kilobase gene situated on chromosome 3q26.3 and contains 20 exons. Class I_API3Ks are composed of heterodimers or subunits, a 110-120 kDa catalytic domain or kinase domain, p110, as well as an associated 61 kDa regulatory or adaptor

subunit, p85. The catalytic domain consists of an N-terminal region which binds to the regulatory subunit, p85, a RAS protein (small GTP-binding protein) binding region as well as a phosphoinositide kinase (PIK) region (Fruman *et al.* 1998). The catalytic domain possesses intrinsic protein kinase activity apart from lipid kinase activity. The protein kinase phosphorylates serine residues within the catalytic domain itself or within its associated regulatory subunit. This phosphorylation of p85α by p110α at serine 608, results in downregulation of the lipid kinase activity of p110α (Carpenter *et al.* 1993; Dhand *et al.* 1994; Stoyanova *et al.* 1997; Vanhaesebroeck *et al.* 1997). The regulatory subunit, p85, which usually associates with PI3K, lacks enzymatic activity but contains the following modular units: an N-terminal Src-homology 3 (SH3) domain, two or three proline-rich regions, a region similar to GTPase-activating proteins for Rho protein binding (Rho GAPs), as well as two Src-homology 2 (SH2) domains, one at the C-terminal (C-SH2) and one at the N-terminal (N-SH2) (Fruman *et al.* 1998). p85 is also responsible for regulating the activation and location of p110α within the cell. At the cell membrane, the p85 subunit binds to the phosphorylated receptor tyrosine kinase (RTK) which leads to conformational changes in the p110 domain and in turn, results in kinase activation (Pommery and Henichart 2005). Another way in which class I_A PI3Ks are activated or regulated is by RAS, which binds to the RAS protein binding region of p110. RAS, in turn, is activated when the bound GDP is replaced by GTP during a process of transition mediated by enzymes known as guanosine nucleotide exchange factors (GEFs). Reverse transition is modulated by GTPase-activating proteins (GAPs) which are responsible for promoting hydrolysis of the bound GTP molecules at the end of the stimulation cycle (Riuz *et al.* 2007). The RAS-GTP complex then activates downstream targets such as p110 (Rodriquez-Viciana *et al.* 1994). It is found that most of the activators of p110 via p85/pYMXM interaction, are also responsible for activating RAS (Druker *et al.* 1990; Ling *et al.* 1992). RAS and most of the YMXM- containing proteins are associated with the cell membrane, therefore recruitment of p85/p110 heterodimers not only enhances catalytic activity, but also moves the PI3K from the cytoplasm to the cell membrane where its substrates and activator RAS reside (Fruman *et al.* 1998). Activated PI3K is responsible for the phosphorylation of membrane bound PIP_2 (Phosphatidylinositol-3,4-

bisphosphate)to generate PIP_3 (Phosphatidylinositol-3,4,5-trisphosphate) (Pommery and Henichart 2005).

2.2.2 Clinical implications

Amplifications, deletions and somatic mutations of *PIK3CA* have been implicated in many cancer types, such as breast, colon, stomach, lung, liver and brain (Karakas *et al.* 2006). Campbell *et al*. (2004) discovered the highest frequency (up to 40%) of *PIK3CA* missense mutations in breast cancers. "Hot spots" for somatic mutations map to E542 and E545K in the helical (exon 9) and H1047R in the catalytic (exon 20) domain of p110α (Campbell *et al.* 2004). *PIK3CA* mutations lead to gain of function in PI3K signalling, which in turn leads to disturbances in control of cell growth and survival resulting in a competitive growth advantage, metastatic growth advantage and often therapy resistance. In fact, mutations in exon 20 are believed to inhibit the therapeutic response to EGFR-targeted therapies in patients with metastatic colorectal cancer (Kang *et al.* 2005; De Roock *et al.* 2010).

Wang *et al*. (2011) found *PIK3CA* mutations to be more frequent in elderly patients. Saal *et al*. (2005) reported a strong correlation between *PIK3CA* mutations, ER, PR and Her-2 expression and lymph node metastases. In fact, in the subgroup ER(+)/Her-2(+) and lymph node positive breast cancers, *PIK3CA* mutations were present in 58% of cases. A strong inverse relation to *PTEN* mutation was described where PTEN was functional or positive in 86% of cancers bearing *PIK3CA* mutations. Mutations in both *PIK3CA* and *PTEN* were rare. They could however not establish any significant relation between PI3K status and disease free survival or overall survival (Saal *et al.* 2005). Jensen *et al*. (2011) found that patients with *PIK3CA* mutations or high PI3K pathway activation had a significantly worse overall survival despite adequate treatment. They found no significant correlation between PTEN or pHer-2 and patient outcome (Jensen *et al.* 2011). In an independent study, Aleskandarany *et al*. (2010) found PI3K expression to be associated with poor prognostic variables such as a higher grade and size, a higher proliferation factor, nodal involvement and vascular invasion in breast cancer. Their findings concurred with other studies that found PI3K to be an independent indicator of overall

survival and disease free survival, except perhaps of tumour grade (Aleskandarany *et al.* 2010).

Johnston (2006) reported an association between the activation of the PI3K/Akt pathway and resistance to tamoxifen or conventional cytotoxic drugs and also lack of response to Her-2 targeted therapy. He also reported that in preclinical studies mTOR antagonists were able to restore sensitivity to endocrine therapy in breast cancer cells (Johnston 2006). Campbell *et al*. (2001) demonstrated that the activated PI3K/Akt pathway interacts directly and indirectly with ER, protecting cancer cells from tamoxifen-induced apoptosis and activating ER in a hormone-independent fashion. Clinical evidence suggests that PI3K and K-RAS contribute to the modulation of ER while ER in turn, activates the PI3K/Akt pathway. The activated PI3K/Akt pathway has been shown to confer anti-oestrogen resistance while inhibition of this pathway reverses the resistance (Chandarlapaty *et al.* 2011). Wang *et al*. (2011) suggested that PI3K activation may lead to resistance to trastuzumab as well as lapatinib (Wang *et al.* 2011).

In support of these findings, studies in human cancer xenografts have shown that combinations of inhibitors, targeting Her-2 and PI3K, or Her-2 and AKT are superior to single-agent treatments (Chandarlapaty *et al.* 2011; Chakrabarty *et al.* 2011).

Assessment of PI3K pathway activation may provide an additional method of identifying patients less likely to respond to Herceptin-based therapy (Berns *et al.* 2007).

2.3 PTEN

2.3.1 Structure and function

PTEN (phosphatase and tensin homologue deleted on chromosome ten) is a tumour suppressor gene (10q23.3) that is mutated at high frequency in a large number of cancers. PTEN plays a major role as regulator of signal transduction pathways that are involved in growth, adhesion, migration, invasion and apoptosis (Cully *et al.* 2006). PTEN has also been found to be of critical importance in early embryologic development (Wechsler-Reya and Scott 2001).

The protein encoded by the *PTEN* gene contains a catalytic or phosphatase domain at the N-terminal region which has both lipid phosphatase and protein tyrosine phosphatase activity. In other words, PTEN is able to dephosphorylate both tyrosine and serine/threonine residues, although its tumour suppressor function is completely reliant on its lipid phosphatase activity (Myers *et al.* 1997). An enlarged active site allows for binding phosphatidylinositol (PI) substrates, while its actin-binding and SH2 domains are responsible for binding phosphotyrosine-containing proteins. The N-terminal domain of PTEN displays a close similarity to the cytoskeletal protein tensin, which is responsible for maintaining the cellular structure and is also thought to play a role in signal transduction by binding to actin filaments at focal adhesions (Davis *et al.* 1991b; Li *et al.* 1997). As part of its protein phosphatase activity, PTEN is able to dephosphorylate and thus down-regulate the actions of focal adhesion kinase (FAK) and adaptor proteins, SHC and IRS, leading to complex dissociation between adaptor proteins and GRB2/SOS which in turn results in inactivation of the RAS/MAPK pathway (Gu *et al.* 1998; Weng *et al.* 2001). RAS is found to have the exact opposite effect to that of PTEN. While PTEN dephosphorylates PIP_3 and SHC, leading to inactivation of the Akt and MAPK pathways, RAS is able to activate both the Akt and MAPK pathways by phosphorylating MEK via RAF and PIP_3 via PI3K (Serrano *et al.* 1997). Both the FAK-$p130^{Cas}$ and SHC-ERK/MAPK pathways are implicated in cell adhesion, migration and invasion (Gates *et al.* 1994; Cary *et al.* 1996; Tamura *et al.* 1998; Gu *et al.* 1999). A C2 domain in the C-terminal region binds phospholipids and is believed to be responsible for anchoring PTEN to the plasma membrane. As the C2 domain is situated in close proximity to the catalytic domain on the PTEN protein, it is believed to position the catalytic domain in relation to its substrate, phosphatidylinositol, in the cell membrane (Lee *et al.* 1999; Yamada and Araki 2001). As part of its lipid phosphatase activity, PTEN dephosphorylates phosphoinositide substrates (at the D3 positions) and therefore negatively regulates the levels of second messengers, PIP_3 and PIP_2 to PIP_2 and PIP respectively (Maehama and Dixon 1998). Activated PI3K is responsible for phosphorylating PIP_2 and PIP to PIP_3 and PIP_2 respectively, which clearly illustrates the antagonistic functions of both PTEN and PI3K. Accumulation of

PIP_3 at the cell membrane results in recruitment of proteins containing a plekstrin homology domain from the cytoplasm to the cell membrane. One such protein is Akt/protein kinase B (PKB). Binding of Akt by PIP_3 causes conformational changes in the Akt molecule, allowing phosphorylation at both Thr-308 and Ser-473 by phosphoinositide dependent kinase-1/protein kinase C-related kinase-2 (PDK1/PRK2) complex, triggering the Akt pathway (Balendran A *et al.* 1999; Eugene S *et al.* 1999). By dephosphorylating PIP_3, PTEN acts as a tumour suppressor that negatively regulates Akt/PKB and therefore also the Akt/PI3K pathway. In the absence of PTEN, PIP_3 and PIP_2 accumulate which has the same effect as activated PI3K, triggering downstream effectors such as Akt/PKB, PDK1, protein kinase C (PKC) and GEFs amongst others (Stocker *et al.* 2002). Apart from negatively regulating Akt, PTEN also controls the transcription of the anti-apoptotic factor, NF-κB (Mayo *et al.* 2002b). PTEN is also responsible for downregulating transcription of anti-apoptotic protein BCL2, while BCL2 in turn is known to inhibit PTEN-induced chemosensitivity (Zhang *et al.* 2001). PTEN, when overexpressed, was found to play a role in recruiting $p27^{Kip1}$ to the cyclin-E/CDK2 complex, resulting in decreased cyclin-E/CDK2 activity which in turn leads to diminished pRb phosphorylation and cell cycle arrest at the S phase (Cheney *et al.* 1999). PTEN is able to autoregulate its function and expression through stabilization of another tumour suppressor, p53. PTEN and p53 are mutually dependent on each other; PTEN enhances the transactivation of p53, while p53 facilitates the transcription of PTEN (Shu *et al.* 1988; Stambolic *et al.* 2001; Mayo *et al.* 2002a; Sheng *et al.* 2002). PTEN is also responsible for protecting p53 against proteasomal degradation mediated by MDM2 (Mayo *et al.* 2002b).

2.3.2 Clinical implications

Wang *et al*. (2011) reported PTEN loss in about one third of Her-2(+) breast cancer and did not find *PIK3CA* mutation and PTEN loss to be mutually exclusive as other studies have suggested (Saal *et al.* 2005; Wang *et al.* 2011). Perez-Tenorio e*t al*. (2007) found PTEN loss in 37% of cases which were associated with *PIK3CA* mutations, ER(+), small tumour size, low Her-2 expression and high Akt1 and cyclin D1 expressions. Saal *et al*. (2005) observed PTEN loss with nodal metastases, increased PIP_3 production and

subsequent activation of the Akt pathway, supporting findings that PTEN inhibits cell migration while PIP_3 is responsible for regulating cell motility (Depowski *et al.* 2001; Huang *et al.* 2003; Raftopoulou *et al.* 2004).

PTEN deletions or mutations have been found to generally occur late in tumour development, with a high frequency in high grade tumours while rarely present in low grade tumours (Rasheed *et al.* 1997). PTEN activity has also been found to contribute to the efficacy of trastuzumab and radiation therapy while PTEN loss was associated with increased PI3K/Akt activity and resistance to trastuzumab-based therapy in Her-2 overexpressing breast tumours, suggesting that PTEN loss could serve as a predictor of trastuzumab resistance (Nagata *et al.* 2004; Hennessy *et al.* 2005). Fabi *et al*. (2010) evaluated the protein expression of PI3K, p-Akt and PTEN in 73 metastatic breast cancer patients treated with trastuzumab and found that patients co-expressing PTEN and p-Akt had a significantly longer progression free survival (PFS) ($p = 0.01$) than those that were only PTEN positive ($p = 0.06$). They suggested that co-expression of PTEN and p-Akt may identify those patients more likely to benefit from trastuzumab-based therapy. They found co-expression of PTEN and p-Akt in 29 out of 73 Her-2(+) metastatic breast cancers (Fabi *et al.* 2010). Similar findings were reported by Esteva *et al*. (2010) who observed that PTEN loss was associated with a shorter survival time and poor response to trastuzumab therapy.

A study by Nagata *et al*. (2004) found that PI3K inhibitors were able to overcome trastuzumab resistance induced by PTEN loss, suggesting that therapies targeting PI3K could overcome this resistance.

The EGFR-selective tyrosine kinase inhibitor, gefitinib (ZD1839, “Iressa”) was found to be effective against both EGFR, Her-2 and Her-3 overexpressing tumour cells by dephosphorylating these receptors and reducing PI3K and Akt activation as a result (Moasser *et al.* 2001). It was shown that gefitinib resistance in EGFR overexpressing cells was the result of PTEN loss with subsequent hyperactivation of the Akt pathway. Reconstitution of PTEN re-

established EGFR driven Akt signalling and restored gefitinib sensitivity (She *et al.* 2003).

2.4 Akt

2.4.1 Structure and function

The Akt protein family, also known as the Protein Kinase-B (PKB) or serine/threonine-specific protein kinase family, plays an important role in regulating cell survival, growth, proliferation, metabolism, angiogenesis and migration in response to insulin, growth factors or inflammatory agents (Franke *et al.* 1995; Datta *et al.* 1999). Akt is the cellular homologue of v-Akt, a protein encoded by a gene in the acute transforming retrovirus, *Akt8*, that was isolated from a mouse T-cell lymphoma (Belacossa *et al.* 1991). The three isoforms of Akt are Akt1/PKB-α, Akt2/PKB-β and Akt3/PKB-γ. Akt1/PKB-α is involved in the cellular survival pathway, Akt2/PKB-β plays an important role in the insulin signalling pathway and Akt3/PKB-γ appears to be involved in growth and development of the brain (Yang *et al.* 2004). Akt1 is encoded by the gene, *Akt1* (14q32.32-32.33). The three isoforms of this protein show an 80% sequence correlation and consist of three regions namely, the N-terminal region that contains a PH domain, a centre region also referred to as the catalytic or kinase region and a C-terminal region which is the regulatory domain and contains a hydrophobic (HF) motif (Kannan *et al.* 2007; Yang *et al.* 2002). The PH domain shows high affinity for PI3K products, PIP_2 and PIP_3, regulates intracellular trafficking of the protein and is responsible for translocating Akt/PKB from the cytosol to the cell membrane for activation/phosphorylation (Currie *et al.* 1999; Cantley 2002). Association with PIP_2 and PIP_3 at the plasma membrane is believed to induce conformational changes in the Akt/PKB molecule, exposing two crucial amino acids, threonine (Thr308) within the activation loop of Akt/PKB and serine (Ser473) within the HF motif near the C-terminus of Akt/PKB (Stokoe *et al.* 1997; Milburn *et al.* 2003). In order to activate the kinase activity of Akt, both Thr 308 and Ser473 residues needs to be phosphorylated (Alessi and Cohen 1998; Wang *et al.* 2003; Hennessy *et al.* 2005). The kinase responsible for the phosphorylation of Thr308, is phosphoinositide-dependent protein kinase-1 (PDK1) (Walker *et al.* 1998). PDK1 contains a PH domain in its C-terminal region and is located at the cell

membrane as a result of translocation in the same manner as its substrate, Akt/PKB (Alessi *et al.* 1997). The HF motif on Akt/PKB contributes to the activating process by serving as a docking site for PDK1, allowing phosphorylation of Thr308 by PDK1 in the activation loop of the kinase domain (Balendran *et al.* 2000). For many years the identity of the kinase responsible for phosphorylating Akt/PKB on Ser473 has eluded scientists until 2005 when Sarbassov *et al*. (2005) demonstrated that it was in fact the mammalian target of rapamycin (mTOR)-rictor complex (mTOR2) that was responsible for phosphorylating Ser473 in the hydrophobic motif creating full activation of Akt1/PKB. Other factors that are reported to also phosphorylate Akt are heat shock protein-90 (HSP90), cell division cycle-37 (CDC37) and mitogen-activated protein kinase activated protein kinase-2 (MAPKAPK2) (Taniyama *et al.* 2004). Integrins are known to also activate Akt via focal adhesion kinase (FAK), integrin-linked kinase (ILK) and paxillin (Pxn). Akt can also be activated in response to cellular stress such as ultraviolet light, ischaemia, hypoxia, hypoglycaemia, heat shock and oxidative stress. There are also a variety of genetic events capable of activating Akt such as activating point mutations or amplifications of Akt, PI3K, RAS family members or growth factor receptors and loss of PTEN expression or function (Li *et al.* 1997; Yuan and Cantley 2008). The negative regulators of Akt are PTEN, SH2-containing inositol phosphatase (SHIP), carboxyl-terminal modulator protein (CTMP) and PH domain leucine-rich repeat phosphatases (PHLPP) (Basso *et al.* 2002; Fornaro *et al.* 2003; Pommery and Henichart 2005; Mendoza and Blenis 2007).

Akt promotes cellular survival by phosphorylating and inactivating the function of several pro-apoptotic proteins which include several BCL2 homology domain 3 (BH3)-only proteins. These proteins include antagonists of cell death: BAD, caspase-9, forkhead-related family of mammalian transcription factor-1 (FKHRL1) and glycogen synthase kinase 3 (GSK3) (Wang *et al.* 2002; Fornaro *et al.* 2003; Hennessy *et al.* 2005). In order to induce apoptosis, BAD, in its unphosphorylated state, associates with Bcl-xL. Phosphorylation of BAD mediated by Akt creates a binding site for 14-3-3 proteins. BAD dissociates from Bcl-xL and interacts with 14-3-3 proteins in order to prevent apoptosis (Zha *et al.* 1996; Datta *et al.* 2000). Survival factors can suppress apoptosis by

inducing phosphorylation of FKHRL1 by Akt, resulting in association with 14-3-3 proteins and subsequent cytoplasmic retention of FKHRL1. Another substrate that is inactivated by Akt phosphorylation is GSK3, which is responsible for inactivating pro-survival BCL2 member, MCL-1 (Cross *et al.* 1995; Maurer *et al.* 2006). Phosphorylation of the protease, caspase-9, by Akt decreases its protease activity and therefore also apoptosis (Cardone *et al.* 1998). Akt upregulates anti-apoptotic factors including c-AMP response element-binding protein (CREB), NF-κB, BCL2 and MDM2, leading to chromosome instability and cancer (Brunet *et al.* 1999; Carroll *et al.* 1999; Hennessy *et al.* 2005).

MDM2, an E3 ubiquitin ligase, is responsible for p53 degradation once it has been activated by Akt and translocated into the nucleus (Mayo and Donner 2001). Akt can also activate NF-κB by phosphorylating IκB kinase (IKK), resulting in transcription of pro-survival genes (Wu *et al.* 1996; Sovak *et al.* 1997). Another substrate of Akt is the forkhead box O (FOXO) family of forkhead transcription factors which are activated under stress conditions when it accumulates in the nucleus where it plays a role in the expression of pro-apoptotic genes.

Phosphorylation by Akt creates a 14-3-3 binding site, resulting in FOXO inactivation by cytoplasmic sequestration (Accili and Arden 2004). Phosphorylation by Akt down-regulates the cell-cycle inhibitory effects of both $p27^{Kip1}$ and $p21^{WAF1}$ by nuclear export and cytoplasmic localization through 14-3-3 binding (Zhou *et al.* 2001; Liang *et al.* 2002; Shin *et al.* 2002; Viglietto *et al.* 2002). Akt is able to inhibit the expression of $p27^{Kip1}$ through phosphorylation and inhibition of the FOXO transcription factors (Medema *et al.* 2000). Cell-cycle regulators c-Myc and cyclin D1 and E, and transcription factors c-jun and β-catenin all play a role in G1-to-S-phase cell-cycle transition and are all targeted by GSK3 for proteasomal degradation. Phosphorylation by Akt inhibits GSK3 function and enhances the stability of these proteins and their functions which amongst others also involve the WNT signalling pathway (Diehl *et al.* 1998; Welcker *et al.* 2003; Wei *et al.* 2005).

One of the functions of Akt is promotion of cell growth which happens primarily through the activation of mTOR-raptor complex (mTORC1) and is regulated by both nutrients and growth factor signalling. mTORC1 regulates translation initiation and plays a role in controlling cell growth. The tuberous sclerosis complex 1 (TSC1) forms a complex with tuberous sclerosis complex 2 (TSC2), thereby acting as a stabilizer of TSC2 (Chong-Kopera *et al.* 2006). By phosphorylating TSC2, Akt inactivates TSC2 and therefore indirectly activates mTORC1 resulting in cell growth (Gao and Pan 2001; Tapon *et al.* 2001; Manning *et al.* 2002). By inactivating mTORC1, activated TSC2 and its activator, AMP-activated protein kinase (AMPK), protects cells from energy deprivation-induced apoptosis (Inoki *et al.* 2003). mTORC1 not only promotes cell growth, but is also involved in regulation of cell proliferation and Akt also controls the translation of proteins essential for cell-cycle progression through phosphorylation of TSC2 and subsequent activation of mTORC1(Skeen *et al.* 2006). Akt regulates nutrient uptake and metabolism through downstream targets and stimulates glucose uptake in response to insulin. The PI3K/Akt pathway is also critical in maintaining cellular metabolism in conditions of limited growth factors such as hypoxia and glucose deprivation found in solid tumours, by regulating the expression of glycolytic enzymes (Elstrom *et al.* 2004; Hammerman *et al.* 2004). Akt is found to be involved in both normal and pathological angiogenesis stimulated by endothelial cells, cells producing angiogenic signals, such as tumour cells or in hypoxic conditions. In endothelial cells, the PI3K/Akt pathway is activated by vascular endothelial growth factor (VEGF) upon which activated Akt mediates endothelial cell survival, growth and proliferation (Fujio and Walsh 1999). One of the targets of phosphorylated Akt and more specific, Akt1, is endothelial nitric oxide synthase (eNOS) which upon activation by Akt produces nitric oxide (NO). NO is a major role player in endothelial cell migration and angiogenesis by stimulating vasodilation and vascular remodelling (Dimmeler *et al.* 1999; Ackah *et al.* 2005). Surprisingly, activated Akt1 but not Akt2 has been found to decrease mammary epithelial cell migration (Irie *et al.* 2005).

Studies done on mouse tumour models have suggested that in epithelial cells, Akt1 inhibits metastases, while Akt2 promotes metastases but that the opposite

is true in fibroblasts, where Akt1 promotes metastases while Akt2 inhibits it (Arboleda *et al.* 2003; Hutchinson *et al.* 2004; Zhou *et al.* 2006).

2.4.2 Clinical implications

Sun *et al*. (2001) found activated Akt1 to be present in tumour cells of high grade tumours that were late stage and had a poor outcome while Perez-Tenorio *et al*. (2002) linked Akt1 with poor response to endocrine therapy. Bacus *et al*. (2002) found Akt2 to be frequently associated with aggressive, Her-(2+) breast tumours and metastases. Stemke-Hale *et al*. (2008) reported the incidence of *Akt1* mutation of only 1.4% in a study involving more than 400 breast cancers. *Akt2* and *Akt3* mutations were not detected. Perez-Tenorio *et al*. (2007) found no significant association between p-Akt expression, nodal status or *PIK3CA* mutation. They did however observe elevated levels of p-Akt, total Akt and Cyclin D1 with mutated *PIK3CA* and/or Her-2 overexpression.

Davies *et al*. (2009) measured the levels of p-Akt and PTEN in melanomas and found that almost all cases with elevated levels of p-Akt had low PTEN levels. Interestingly, they also found significantly higher p-Akt and lower PTEN levels in melanomas that metastasised to the brain compared to those that metastasized to the liver or lung (Davies *et al.* 2009). Shah *et al*. (2005) found that when p-Akt expression was located primarily in the nucleus, it was associated with nodal metastases and a poor prognosis in non-small cell lung cancers.

High levels of p-Akt were found in immune-resistant tumours and associated with upregulation of anti-apoptotic molecules. This finding suggests that the PI3K/Akt pathway may represent a mechanism for immune escape and has implications in immunotherapy and presents a challenge to develop immunotherapy against immune-resistant tumour cells (Noh *et al.* 2009).

Recently the first human trial for MK-2206, a potent oral allosteric Akt inhibitor has been concluded. The results were promising, particularly in tumours where Akt activation was the result of PTEN loss, but needs further evaluation in larger studies (Yap *et al.* 2011).

2.5 NF-κB

2.5.1 Structure and function

NF-κB is a nuclear transcription factor complex that controls the expression of a large number of genes responsible for controlling normal but crucial cellular and organisational processes, such as, inflammatory and immune responses, cellular proliferation and survival. As a result, loss of NF-κB regulation has been implicated in a number of disease states, such as inflammatory, autoimmune diseases and cancer and neurodegenerative diseases, such as Parkinson and Alzheimer's disease (Gilmore 1999; Mattson and Camandola 2001; Soós *et al.* 2004; Collister and Albensi 2005). The NF-κB protein, first discovered in the laboratory of the Nobel Prize winner David Baltimore in 1986, is encoded by the gene *NF-κB* (4q24). The NF-κB protein forms part of a family of proteins that are structurally related through a conserved N-terminal DNA-binding domain called the Rel homology (RH) domain (Sen and Baltimore 1986). The RH domain serves as a dimerization interface to other NF-κB transcription factors and also binds to the inhibitory protein, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, IκB, as well as DNA. NF-κB proteins can be divided into two classes or sub-families. The class I or 'NF-κB' sub-family includes p105 and p100 and the class II or 'Rel' sub-family includes RelA, RelB, and c-Rel. The class I proteins contain a large number of ankyrin repeats that masks the nuclear localization sequence (NLS) and renders the protein inactive. The long C-terminal domain prevents nuclear localization and DNA-binding (Kieran *et al.* 1990; Davis *et al.* 1991a; Haskill *et al.* 1991; Rice *et al.* 1992; Grilli *et al.* 1993). These large precursors, p105 (NF-κB1) and p100 (NF-κB2) undergo phosphorylation followed by ubiquitination and proteasomal degradation of the C-terminus to form shorter, active DNA-binding subunits, p50 and p52 respectively (Ghosh *et al.* 1990; Naumann and Scheidereit 1994; Palombella *et al.* 1994). On their own these sub-units do not activate transcription, except when they form heterodimers with members of the class II sub-family. In addition, the p50 and p52 homodimers can also form complexes with nuclear protein Bcl-3 and as such activate transcription (Bours *et al.* 1993). In contrast, the class II proteins contain transcription activation domains in their C-terminus. All NF-κB proteins can form either homodimers or heterodimers except for RelB, which can only form heterodimers. The most

common heterodimer is p50-RelA, also commonly referred to as NF-κB and happens to be the major Rel/NF-κB complex in most cells (Grilli *et al.* 1993; Siebenlist *et al.* 1994).

In an inactive state the Rel/NF-κB heterodimer is located in the cytoplasm sequestered by the inhibitory protein IκB that is responsible for masking the NLS of NF-κB proteins by means of their ankyrin repeat domains (Davis *et al.* 1991a; Haskill *et al.* 1991; Henkel *et al.* 1992). IκB proteins are also capable of removing the Rel/NF-κB complex from its DNA-binding locus (Davis *et al.* 1991a; Ernst *et al.* 1995; Thompson *et al.* 1995; Beauparlant *et al.* 1996). A number of studies have demonstrated that IκB molecules bind specific targets therefore different IκB molecules inhibit specific Rel/NF-κB dimers (Baeuerle and Baltimore 1989; Beg *et al.* 1992; Thompson *et al.* 1995).

NF-κB is activated by 3 major pathways; the canonical or classical pathway, the non-canonical or alternative pathway and the casein kinase 2 (CK2) pathway. In the canonical pathway, activation of the Rel/NF-κB dimers occur when the enzyme IκB kinase (IKK) is activated mainly in response to bacterial and viral infections or pro-inflammatory cytokines (Baeuerle and Baltimore 1988; Rayet and Gélinas 1999). IKK, also referred to as the IKK complex, contains two kinase subunits, IKKα and IKKβ, and an associated regulatory protein IKKγ also referred to as NEMO (Zabel U and Baeuerle PA 1990). IKK, in turn phosphorylates the inhibitory protein, IκB and p105, which results in dissociation of IκB from the NF-κB complex, ubiquitination and finally proteasomal degradation of IκB and p105 resulting in the release of p50-, p65- and c-Rel-containing dimers (Baeuerle and Baltimore 1988; Hayden and Ghosh 2004). Degradation of the Rel/NF-κB inhibitor, IκB, uncovers the NLS and gives rise to an active Rel/NF-κB heterodimer. Activated Rel/NF-κB translocates into the nucleus where it binds to specific sequences of DNA called the response elements (RE). This DNA/NF-κB complex then recruits other role players such as co-activators and RNA polymerase, which are responsible for transcribing downstream DNA into mRNA. mRNA in turn, is translated into protein, which results in a change of biological function of proteins involved in immune and

inflammatory response, cell growth and proliferation to name a few (Baldwin 1996).

In the alternative pathway, IKK is activated mainly by cytokines such as B-cell activating factor (BAFF), CD40 and lymphotoxin β (LTβ) via NF-κB inducing kinase (NIK) (Coope *et al.* 2002; Dejardin *et al.* 2002; Kayagaki *et al.* 2002). IKK, in turn mediates selective phosphorylation and processing of p100 in the RelB/p100 complex to generate an active RelB/p52 complex through the process of ubiquitination and proteasomal degradation (Pomerantz and Baltimore 2002). This active RelB/p52 complex is now able to enter the nucleus to facilitate transcription.

The third pathway is IKK independent and activated in response to UV-C irradiation independent of DNA damage and is believed to activate CK2 via the p38 pathway. CK2 is responsible for phosphorylating IκB directly (Kato *et al.* 2003; Tergaonkar *et al.* 2003). Activated NF-κB turns on the genes responsible for cell proliferation and protects the cell against processes that would induce apoptosis. Activated NF-κB is responsible for the increased expression of pro-inflammatory cytokines (IL1, IL2, IL6, TNF), chemokines (IL-8, MCP-1, GROα), growth factors (VEGF, GM-CSF, MMP2, MMP9), adhesion molecules (ICAM1, VCAM1, ELAM1) and anti-apoptotic proteins (c-IAP1, c-IAP2, c-FLIP, XIAP, BCL2 family members) (Shishodia and Aggarwal 2004; Li *et al.* 2005). The external apoptotic pathway starts at the cell membrane and involves membrane death receptors. One such receptor is tumour necrosis factor-receptor1 (TNF–R1), which transduces both death and survival signals via two complexes. Complex I is situated at the cell membrane and constitutes TNF-R1, tumour necrosis factor receptor 1-associated death domain (TRADD), receptor-interacting protein (RIP), TNF receptor-associated factor 2 (TRAF2) and cellular inhibitor of apoptosis protein 1 (c-IAP1). Complex I transduces survival signals and activates NF-κB. Complex II on the other hand is located in the cytosol, constitutes Fas-associated death domain (FADD) and procaspases 9 and 10 and transduces apoptotic signals should NF-κB fail to upregulate anti-apoptotic Fas-associated death domain-like interleukin-1-ß-converting enzyme-inhibitory

protein (FLIPL) (Sheikh and Huang 2003). As a result, defective NF-κB function would render cells vulnerable to apoptosis and result in cell death.

Regulation of NF-κB transcription factors is of critical importance as deregulation has serious pathological implications. Persistent inhibition leads to inappropriate immune cell development, delayed cell growth and infertility, while persistent activation by upstream kinases (including PI3K via Akt) or genes encoding NF-κB transcription factors or mutations inactivating IκB on the other hand, lead to cancer (Micheau and Tschopp 2003; Danial and Korsmeyer 2004; Gilmore 2006; Escárcega *et al.* 2007; Perkins 2007). DNA binding by Rel/NF-κB transcription factors is regulated at multiple levels which include: dimerization, inhibition by IκB, DNA binding, interaction with other transcriptional co-activators and also with the basal transcriptional mechanism (Brasier 2006).

2.5.2 Clinical implications

There is strong evidence to support the roles of Rel/NF-κB proteins and their IκB regulators in cell proliferation and oncogenesis (Rayet and Gélinas 1999). As a matter of fact, c-Rel, RelA, p100, p105, Bcl-3 and IκB have all been associated with oncogenesis as a result of gene amplification, overexpression, re-arrangement, translocation and mutation. Constitutive activation of Rel/NF-κB proteins were found in most cancer cell lines and in many tumour types (Shishodia and Aggarwal 2004). Houldsworth *et al*. (1996) found genes encoding Rel/NF-κB proteins to be amplified or rearranged in up to 25% of certain human lymphoid tumours while Biswas *et al*. (2004) reported activated Rel/NF-κB to be present in 86% of ER- and Her2(+) breast carcinomas. Some studies have shown p100 to be overexpressed in up to 75% of breast cancer biopsies examined. In contrast to p100 rearrangements found in lymphomas, overexpression of p100 in breast cancer cells due to failure of cytokines to induce processing of p100, results in a high cytoplasmic concentration of precursor p100 as well as p52. In this setting, p100 forms tertiary complexes with p50/RelB and p50/p65 heterodimers, leading to cytoplasmic sequestration and inhibition of NF-κB-related proteins which according to Dejardin *et al*. (1995) could play a role in oncogenesis. Although NF-κB was initially linked to ER negative breast cancers, new evidence associates NF-κB with a subset of

ER(+) breast cancers that are at increased risk for relapse despite adequate hormonal treatment (Zhou *et al.* 2005).

Chronic inflammation has long been associated with carcinogenesis and tumour progression. The production of pro-inflammatory cytokines, chemokines, growth factors and anti-apoptotic proteins as a result of inflammation induced NF-κB activation were found to be involved in cancer progression and chemoresistance (Coussens and Werb 2002; Chen *et al.* 2007).

Recently the drug Gallotannin (GT) has been found to be effective in inhibiting NF-κB signalling and tumour growth in colon cancer (Al-Halabi *et al.* 2011). Phytoestrogens, genistein and quercetin have also been shown to inhibit NF-κB signalling and induce apoptosis in Her-2(+) breast cancer cells (Seo *et al.* 2011).

2.6 p53

2.6.1 Structure and function

The *p53* gene that is situated on the short arm of chromosome 17(17p13.1) was discovered in 1979. As a sequence specific transcription factor, the p53 molecule is composed of an N-terminal activation domain, a central DNA-binding domain, an MDM2 binding site and a C-terminal tetramerization domain that also includes the nuclear export signal, followed by a regulatory domain that contains the DNA-damage recognition site and nuclear localization signals (Harris 1996; Somasundaram 2000).

One of the most important functions of p53 is to guard the cell against malignant transformation. *p53* is activated by cellular stresses like DNA damage, oncogene activation, hypoxia and nutrient deprivation. p53 regulates the target genes that induce cell cycle arrest, DNA repair, apoptosis, cellular senescence or changes in metabolism (Lee *et al.* 1995; Maya *et al.* 2001; Harris and Levine 2005; Bensaad *et al.* 2006; Efeyan *et al.* 2006; Vousden and Lane 2007). Depending on the type or duration of stress, DNA damage or conditions of cellular growth, p53 would selectively activate a subset of target genes in order to achieve growth arrest, apoptosis, DNA repair or a change in differentiation

(Hupp *et al.* 1992; el-Deiry 1998). p53 regulates G1-S checkpoint in response to minimal DNA damage or other minor stress factors like heat shock, hyperoxia or hypoxia. Under these conditions, p53 would target $p21^{WAF-1}$ or 14-3-3 to induce G1 arrest to allow repair of damaged DNA (Kastan *et al.* 1991; Kuerbitz *et al.* 1992; McKay *et al.* 1999). Under conditions of extreme stress or severe DNA damage, p53 activates pro-apoptotic genes, such as, BAX, PIDD, Noxa or Puma (Miyashita and Reed 1995; Owen-Schaub *et al.* 1995; Polyak *et al.* 1997).

p53 regulates the expression of target genes by binding to the regulatory region of the target DNA (Ryan *et al.* 1993; Kaufmann 1995; Teyssier *et al.* 1999). p53 is able to regulate target genes due to its ability to selectively associate with different transcription factors, each with its own unique regulatory function. The association and regulatory function of p53 is determined by post translational modifications that are able to change the conformation of the p53 molecule and as a result its association with transcription factors and regulatory proteins (Adler *et al.* 1997; Sionov and Haupt 1999; Ashcroft *et al.* 2000). The p53 molecule has more than 18 phosphorylation sites that can be modified in response to stress or DNA damage and determines the function of the molecule. Phosphorylation of p53 was found to change during the course of a normal cell cycle and corresponds with its ability to associate with its regulatory proteins p300, MDM2 and JNK (Fuchs *et al.* 1998; Buschmann *et al.* 2000). Phosphorylation of p53 is regulated by phosphatases (Li *et al.* 2000).

p53 is kept at low levels in normal, unstressed cells to safeguard the cell against disruption of the cell cycle and untimely cell death. This is attained through the fact that p53 has a relatively short half-life and a mechanism by which unphosphorylated p53 is continuously being ubiquitinated largely by MDM2 ubiquitin ligase, a major negative regulator of p53 and degraded by the 26S proteasome. MDM2 inhibits p53 by binding its transactivation sites and by targeting p53 for proteolytic degradation. MDM2 is one of the transcriptional targets of p53. Since MDM2 is responsible for inhibiting p53 activity, this gives rise to a negative feedback loop (Ghosh and Bose 2005; Francoz *et al.* 2006). p53 positively regulates MDM2 levels while MDM2 negatively regulates p53

levels and activity. MDM2 is able to form a complex with both mutant and wild-type p53 (Barak *et al.* 1993; Wu *et al.* 1993; Freedman *et al.* 1999). MDM2 functions as an E3 ubiquitin ligase and degrades p53 by targeting it for ubiquitination. This is achieved by three mechanisms. Firstly, MDM2 recognizes, binds and blocks the N-terminal transactivation domain of p53, inhibiting its function as a transcription factor. Secondly, by binding lysine residues in the p53 C-terminus, MDM2 mediates a covalent attachment of ubiquitin to p53 and labels p53 for proteasomal degradation. Ubiquitinated p53 is then degraded by the 26S proteasome. Lastly, MDM2 exports p53 from the nucleus to the cytoplasm which is essential for p53 degradation. In the absence of MDM2-mediated degradation, p53 is activated (Barak *et al.* 1993; Wu *et al.* 1993; Kubbutat *et al.* 1997; Ghosh and Bose 2005; Francoz *et al.* 2006).

2.6.2 p53 activation following DNA damage

In the event of DNA damage, either by UV or ionising radiation or a chemical agent, multiple molecular pathways are able to activate p53, resulting in both an increase in the levels of p53 protein and its binding affinity for specific DNA sequences (Maltzman and Czyzyk 1984; Liu *et al.* 1996b). Following genotoxic stress, p53 is phosphorylated by different upstream mediators at various sites resulting in both an increased half life and transactivation activity (Meek 1994). Transcriptional activation of p53 results in both G1 and G2 arrest. Some of the upstream mediators that play a role in the activation and stabilization of p53 in response to DNA damage include ataxia-telangiectasia mutated (ATM), kinase families such as MAP kinases, cyclin dependent kinases (CDKs) and DNA dependent kinase (DNA-DK) as well as tumour suppressor proteins $p19^{ARF}$ and $p16^{INK4a}$ (Bischoff *et al.* 1990; Marshall 1994; Zhang *et al.* 1998). *ATM* is a DNA damage checkpoint gene and its product is one of the major regulators of p53 in response to ionizing-radiation induced damage. ATM kinase phosphorylates p53 on serine 15 and dephosphorylates p53 on serine 376 in order to facilitate specific binding of 14-3-3proteins to p53 and to increase sequence specific DNA binding by p53 (Matsushime *et al.* 1992; Waterman *et al.* 1998). ATM kinase phosphorylates and activates checkpoint kinase 2 (Chk2) and nuclear tyrosine kinase c-Abl, in response to DNA damage (Baskaran *et al.* 1997). Activated Chk2 not only regulates cell cycle arrest at G2 but is also able to

phosphorylate p53 at serine 20, which is known to interfere with MDM2 binding, preventing ubiquitination in response to DNA damage resulting in an increase in p53 stability. Activated c-Abl binds p53 and enhances its sequence specific transactivation ability (Goga *et al.* 1995; Hirao *et al.* 2000). Recent studies have shown that tumour suppressor $p14^{ARF}$is able to stabilize p53 by binding and promoting MDM2 for degradation. The *ARF-INK4* gene locus encodes two unrelated tumour suppressor proteins; $p16^{INK4a}$ and $p19^{ARF}$. $p16^{INK4a}$ binds and inhibits CDK4 and CDK6, while $p19^{ARF}$ binds and promotes the rapid degradation of MDM2, while modifying, stabilizing and increasing p53 levels at the same time. Ubiquitination of p53 is suppressed and p53 accumulates in the nucleus where it is stabilized and activated (Stott *et al.* 1998; Zhang *et al.* 1998).

Acetylation by p300/CBP-associated factor (PCAF) is another modification of p53 that inhibits ubiquitination by MDM2 and aids in stabilizing p53. Acetylation of p53 also aids in p53 DNA binding and stimulates p53-mediated transactivation of downstream targets through recruitment of co-activators (Dornan *et al.* 2003; Cesková *et al.* 2006).

c-Myc is a transcription factor responsible for increasing both the transcription and stabilization of p53, while c-Myc transcription in turn, is downregulated following p53 activation as part of a negative regulatory feedback loop (Hermeking and Eick 1994).

2.6.3 Cell cycle arrest and DNA repair

The first step in the process of DNA repair is to arrest the cell cycle to prevent proliferation of cells containing damaged DNA which would otherwise pose a threat of tumour development. The cell is arrested at checkpoints in the G_1/S and G_2/M phases of the cell cycle. CDK1 is a major regulator of cell cycle progression and requires phosphorylation at T161 and association with cyclin B in order to be activated. In contrast, phosphorylation of CDK1 at Y15 and T14 during G2 phase results in deactivation of CDK1, which is directly inhibited by three transcriptional targets of p53 that are $p21^{WAF1}$, Gadd45 and 14-3-3. p53 mediated transcriptional induction of $p21^{WAF1}$ plays a major role in mediating the

G1 arrest. $p21^{WAF1}$ binds to and inhibits cyclin B/CDK1 complexes by sequestering them in the nucleus. The resulting accumulation of pRb binds members of the E2F transcription family in order to constrain them. This process brings about G1 arrest. Phosphorylation of pRb by CDK4 or CDK6 in mid or late G1 phase has the effect of dissociating pRb from E2F. Free E2F is now able to activate target genes needed for the cell to enter into S phase. The next step involves the biochemical processes involved in DNA repair. Both Gadd45 and 14-3-3 play a mediating role in G2 arrest. 14-3-3 is responsible for sequestering cyclin B/CDK1 in the cytoplasm while Gadd45 prevents binding of cyclin B and CDK1 through direct interaction with CDK1. p53 is also responsible for the transcriptional repression of cyclin B. Gadd45 and $p21^{WAF1}$ are believed to play a role in DNA repair through their interaction with proliferating cell nuclear antigen (PCNA) (Serrano *et al.* 1993; Dyson 1998; Chan *et al.* 1999; Moore *et al.* 1999; Taylor and Stark 2001).

Once repair is completed successfully, the cellular functions can progress to cell division.

Should repair not be possible as a result of excessive damage, the p53-mediated apoptotic pathway comes into play (Ma *et al.* 2005).

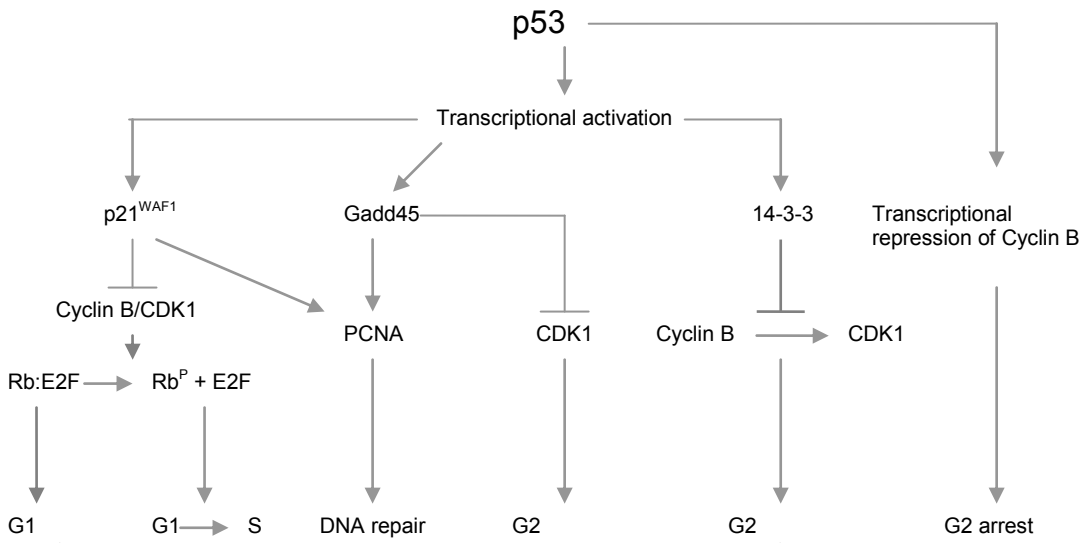


Figure 2: Cell cycle regulation by p53
Adapted from Somasundaram (2000)

2.6.4 Apoptosis

p53 upregulates the synthesis of the pro-apoptotic protein BAX, while it downregulates the synthesis of the anti-apoptotic protein, BCL2 (**Figure 3**). BAX together with other pro-apoptotic proteins aids in the release of cytochrome C proteins from the mitochondria. Cytochrome C is an apoptogenic factor, which when released into the cytosol, binds apoptotic protease activating factor-1 (Apaf-1). Apaf-1binds and hydrolyses dATP to ATP. Together, the hydrolysed dATP, Cytochrome C and Apaf-1 forms the Apaf-1/cytochrome C complex. This apoptosome complex in turn cleaves and activates procaspase-9 to yield active caspase-9 which in turn cleaves bound procaspase-3 to produce the active effector molecule caspase-3. Executioner caspase-3 cleaves key cellular proteins and dismantles the cell resulting in apoptosis. Caspases are cysteine proteases that act in a proteolytic cascade to dismantle and remove dying cells and can be divided into upstream caspases (caspases 2, 8, 9, 10 and 12) and downstream or effector caspases (caspases 3, 6 and 7). Upstream procaspases are activated by dimerization while downstream procaspases are activated by the proteolytic action of activated upstream caspases (Liu *et al.* 1996a; Zamzami *et al.* 1998; Fesik and Shi 2001; Boatright *et al.* 2003; Czerski and Nuñez 2004; Renatus *et al.* 2001). Caspase-3 is also responsible for cleaving and downregulating anti-apoptotic BCL2 while $p21^{WAF1}$, which is upregulated by p53, is in turn responsible for downregulating caspase-3. BCL2 protein suppresses the pro-apoptotic activity of BAX. BCL2 also prevents the binding of Apaf-1 to procaspase-9. The level of activation is dependent on the competition between the pro- and anti-apoptotic agents involved in the apoptotic pathway (Fussenegger *et al.* 2000; Hengartner 2000; Bose and Ghosh 2007).

There is some evidence that $p21^{WAF1}$ has a dual role in the p53-MDM2 network. $p21^{WAF1}$ assists in the arrest of the cell cycle when it is activated by p53, but also has a second function of protecting the cell against apoptosis. MDM2 is found to be a negative regulator of $p21^{WAF1}$ independent of p53. MDM2 promotes $p21^{WAF1}$ degradation by facilitating the binding of a proteasomal subunit, which implies that MDM2 overexpression leads to lower $p21^{WAF1}$ levels.

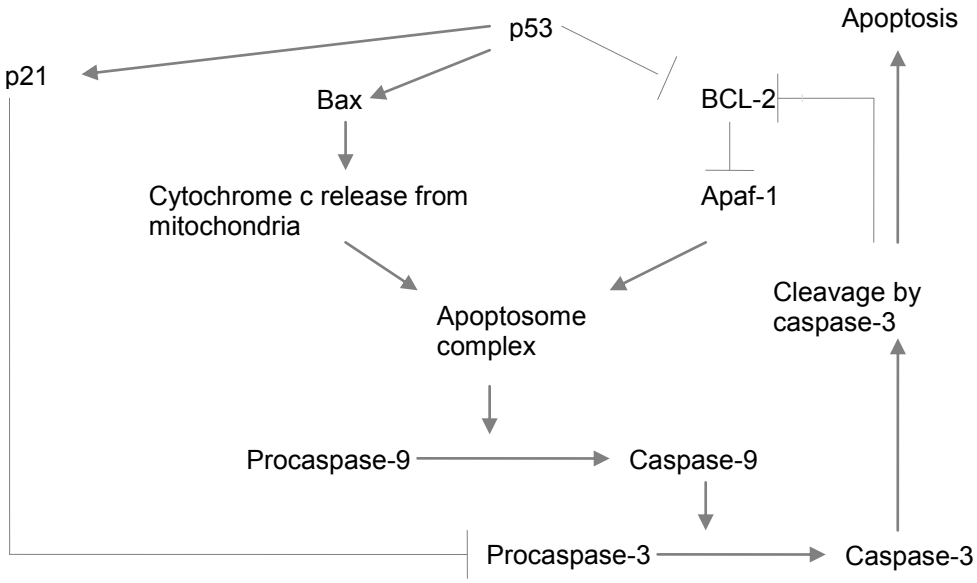


Figure 3: The p53-mediated apoptotic pathway
Adapted from Bose and Ghosh (2007)

Downregulation of $p21^{WAF1}$ activity results in enhanced $p14^{ARF}$ expression. The $p14^{ARF}$ protein in turn sequesters MDM2 in the nucleolus, which results in a decrease in p53 degradation and therefore increased levels of p53 (Javelaue and Besancon 2002; Vassilev *et al.* 2004).

2.6.5 Interactions with other proteins / cellular components

As mentioned before, PTEN and p53 are mutually dependant on each other. p53 facilitates the transcription of PTEN, while PTEN enhances transactivation of p53 and for this reason loss of p53 expression would result in a decrease in PTEN expression (Wang *et al.* 2005). PTEN also protects p53 from survival signals by opposing PI3K/Akt signalling that promotes translocation of MDM2 from the cytoplasm to the nucleus where it negatively regulates p53 (Mayo and Donner 2001). PTEN restricts MDM2 to the cytoplasm by inhibiting nuclear entry of MDM2. In the cytoplasm, MDM2 is degraded while cellular content and transactivation of p53 is increased. By protecting p53, PTEN aids in the process of sensitization of tumour cells to chemotherapeutic agents that rely on p53 activity (Mayo *et al.* 2002b).

p53 and NF-κB are regarded as functional antagonists as they are responding to different types of stress in entirely antagonistic ways. While p53 responds to intrinsic stress, like DNA damage by initiating cell cycle arrest or cell death and oxidative phosphorylation, NF-κB responds to extrinsic stress, like inflammation or cytokine activation and initiates cell division and aerobic glycolysis. It is clear that these two transcription factors cannot function in the same cell at the same time. Regulatory proteins in the activation pathway of both these transcription factors are responsible for ensuring that when the one is activated, the other is inactivated (Ak and Levine 2010).

2.6.6 Clinical implications

Mutations in the *p53* gene have been found in more than half of human cancers and more than 30% of breast cancers. Over one thousand *p53* mutation types have been identified. The majority of the mutations occur in the core domain which also contains the sequence-specific DNA binding function, resulting in loss of its transcriptional activities and hence causes the loss of tumour suppressor activity (Hollstein *et al.* 1991; Cho *et al.* 1994). In cancer cells harbouring a mutant *p53* gene, the p53 protein is unable to control cellular proliferation, resulting in inefficient DNA repair and genetically unstable cells (Levine 1997). Some mutations render p53 transcriptionally active against a different target gene, resulting in gain of function phenotypes (Blandino *et al.* 1999; Deppert *et al.* 2000). Mutant p53 expression was found to be elevated due to the fact that p53 was stabilized as a result of the inability of mutant p53 to induce expression of its major negative regulator, MDM2 (Lane 1994).

Overall phosphorylation of p53 (mutant as well as wild type) was found to be substantially increased in cancer, irrespective of the type and was believed to be due to the failure of one or more phosphatases (Minamoto *et al.* 2001). Extensive phosphorylation is expected to cause conformational change in the p53 molecule and alter its association with target molecules. This theory provides a possible mechanism by which p53 activities are altered in cancers harbouring wild type p53 (Buschmann *et al.* 2000).

Normal p53 protein is usually undetectable by IHC techniques due to its short half-life, while mutant p53 proteins accumulate in the nucleus of tumour cells due to an increased half-life and structural changes. It is noteworthy that IHC techniques may in some instances give false positive results when wild-type p53 is stabilized due to cellular stress while false negative results have been seen in some cancers harbouring nonsense mutations or deletions (Schmitt *et al.* 1998; Geisler *et al.* 2001).

p53 mutations and/or overexpression in breast carcinoma has been associated with shorter disease free survival and overall survival, low ER content and high proliferation rate. Exceptionally high p53 expression was found in ER-/PR- medullary and high grade ductal carcinomas of the breast (Borg *et al.* 1995). Cancers with mutations located in regions encoding parts of the protein involved in zinc-binding were associated with significantly shorter disease free survival and overall survival compared to mutations involving other domains (Borg A *et al.* 1995; Borresen AL 1995). Patients with tumours overexpressing both Her-2 and p53 were found to have a significantly shorter disease free and overall survival than when they were overexpressed individually (Yamashita *et al.* 2004).

p53 mutations have been associated with a good as well as poor treatment response depending on the type and mode of action of a particular treatment regimen. The fact that wild-type p53 is able to repair UV-type DNA damage is proving useful clinically in that some types of cancer carrying mutant *p53* genes may be more sensitive to adjuvant radiotherapy and certain chemotherapeutic drugs than those with wild-type p53 function. As *p53* mutation is associated with high grade malignancies and metastases, preferential killing of these cells carrying mutated *p53* would be of great benefit to cancer treatment (Sidransky *et al.* 1992; Peyrat *et al.* 1995; Stal 1995). Cytotoxic drugs like 5-fluorouracil and doxorubicin rely on functioning p53 to execute their cytotoxic effects. Breast cancers expressing high levels of wild type p53 showed an increased level of apoptosis and tumour regression after treatment with doxorubicin and radiotherapy, while p53 deficient cancers continued to grow and showed little evidence of apoptosis (Lowe *et al.* 1994). *p53* mutations especially those

involving loop domains L2 and L3 were associated with resistance to doxorubicin treatment and relapse as a result of defective apoptosis due to inactive p53. *p53* mutations were also associated with Her-2 expression, a high histological tumour grade and lack of bcl-2 expression. All of these parameters were also associated with chemoresistance, especially when present simultaneously (Geisler *et al.* 2001).

p53 mutations were also found to be present at an increased frequency in triple negative breast cancers and was shown to be a strong negative prognostic indicator for disease free survival and overall survival in this group of cancers, especially when treated with adjuvant anthracycline-based chemotherapy (Cheang *et al.* 2008; Chae *et al.* 2009). Non-inflammatory breast cancers and particularly the basal or triple negative type breast cancers with *p53* mutations showed increased sensitivity to high dose epirubicin-cyclophosphamide treatment, while those with wild type p53 showed complete resistance (Bertheau *et al.* 2002; Bertheau *et al.* 2007).

Bergh *et al*. (1995) found the mutational status of p53 of little value in predicting treatment response to radiotherapy and tamoxifen treatment in node positive breast cancers. Geisler *et al*. (2001) found no statistically significant relation between p53 immunostaining and treatment response despite its association with *p53* mutation status.
No association was found between p53 status and the clinical efficacy of trastuzumab (Köstler *et al.* 2005). Varna *et al*. (2011) found that p53 status could be useful in selecting the best cancer treatment because of its strong impact on the prognosis of breast cancer, provided that p53 status is determined by DNA sequencing and not IHC. IHC was found to be unreliable in determining p53 status. Soussi (2007) found that the prognostic significance of p53 is in fact dependent on the specific *p53* mutation present and explains why determining p53 status with IHC would not be that reliable.

2.7 MDM2

2.7.1 Structure and function

MDM2, encoded by the *MDM2* gene (12q14.3-q15), consists of 491 amino acids and has a molecular weight of 56kDa. The MDM2 molecule contains several structural domains including a N-terminal domain essential for p53 binding, a nuclear localization signal (NLS) and nuclear export signal (NES) essential for nuclear-cytoplasmic trafficking of MDM2, a central acidic region, a zinc finger (Zn) and RING finger motif as well as a nucleolar localization signal (NoLS) (Ganguli and Wasylyk 2003). The acidic region facilitates interaction between the ribosomal protein, L5 and associated 5S ribosomal RNA (rRNA) (Marechal *et al.* 1994). The function of the zinc finger domain is still poorly understood. The RING domain located near the C-terminal contains two additional zinc fingers in a ring formation enabling the molecule to bind to specific RNA structures. The RING domain contains a nucleotide binding motif, a nucleolar localization sequence and has E3 ubiquitin ligase activity that may contribute to p53 regulation. The MDM2 molecule also contains residues that when phosphorylated, play a role in MDM2 regulation (Marechal *et al.* 1994). Although the key target of MDM2 is probably p53, it also interacts with other tumour suppressor proteins, such as, pRb, p73 and $p14^{ARF}$ / $p19^{ARF}$ (Xiao *et al.* 1995; Kamijo *et al.* 1998; Pomerantz *et al.* 1998; Zhang *et al.* 1998; Dobbelstein *et al.* 1999). As discussed earlier in section 2.6.1, MDM2 is an important negative regulator of p53 in normal, unstressed cells. MDM2 inhibits p53 by binding its transactivation sites and by targeting p53 for proteolytic degradation (Leng *et al.* 1995; Haupt *et al.* 1997; Kubbutat *et al.* 1997). Three domains of the MDM2 molecule are involved in the ubiquitination process: the p53 interaction domain, the RING finger domain responsible for mono-ubiquitination and the p300 interaction domain for poly-ubiquitination (Fang *et al.* 2000; Grossman *et al.* 2003). MDM2 was found to differentially catalyze mono-ubiquitination and poly-ubiquitination of p53 in a dosage-dependent manner which implies that low levels of MDM2 would induce mono-ubiquitination and nuclear export of p53, while high levels of MDM2 would result in poly-ubiquitination and nuclear degradation of p53. The assumption was made that these two mechanisms come into play under different physiological conditions. For example, in normal unstressed cells where the MDM2 levels are kept low,

p53 is regulated by MDM2-mediated mono-ubiquitination and p53 cytoplasmic translocation. MDM2-mediated poly-ubiquitination and nuclear degradation of p53 on the other hand would be the most likely mechanism exploited when MDM2 is overexpressed in malignancy or during late stage DNA damage response (Freedman *et al.* 1999; Boyd *et al.* 2000; Shirangi *et al.* 2002; Li *et al.* 2004a; Stommel and Wahl 2004).

MDM2 not only promotes the proteolytic degradation of p53 but is also responsible for its own proteolytic degradation as well as that of other proteins (Honda and Yasuda 2000). MDM2 is regulated by phosphorylation of residues in its acidic domain which could change its function, stabilize p53 and negatively regulate p53-MDM2 interaction. As discussed earlier in section 2.6.2, $p19^{ARF}$ directly interact with MDM2 to sequester MDM2 in the nucleolus, neutralising its inhibitory effect on p53 (Pomerantz *et al.* 1998; Stott *et al.* 1998).

MDM2 serves as a substrate for Akt binding. Akt-mediated phosphorylation of MDM2 enhances MDM2-mediated ubiquitination and degradation of p53. Through this mechanism Akt indirectly reduces the levels of p53 and promotes cellular survival, proliferation and tumourigenesis. Ogawara *et al*. (2002) reported that Akt-mediated phosphorylation of MDM2 did not affect the subcellular location of MDM2. Overexpression of MDM2 can result in excessive inactivation of p53, diminishing its tumour suppressor function. MDM2 also affects the cell cycle, apoptosis and tumourigenesis through interactions with other proteins, including RAS, pRb and ribosomal protein L5 (Freedman *et al.* 1999).

There is some evidence to suggest that overexpression of MDM2 may also play a p53-independent role in G1 cell cycle arrest by regulating DNA replication provided that there is additional genetic damage (Lundgren *et al.* 1997; Jones *et al.* 1998). MDM2 overexpression may be the result of one of three mechanisms namely gene amplification, increased transcription or enhanced translation (Oliner *et al.* 1992; Watanabe *et al.* 1994; Bueso-Ramos *et al.* 1996; Landers *et al.* 1997).

2.7.2 Clinical implications

The most common mutations are found in the zinc finger domain of MDM2. It disrupts the negative regulation of MDM2 by Rb protein L11, which no longer recognizes the binding site and in doing so, creates a potential mechanism for oncogenic activation (Lindström *et al.* 2007).

Increased levels of MDM2 have been found in a wide variety of tumours including certain carcinomas, sarcomas, melanomas and some haematological malignancies. MDM2 was found amplified in breast cancers at a frequency ranging between 4% and 14% (Marchetti *et al.* 1995; McCann *et al.* 1995; Momand *et al.* 1998; Turbin *et al.* 2006).

Momand *et al*. (1998) found that *p53* mutations and *MDM2* amplifications do not generally occur within the same tumour, while Cordon-Cardo *et al*. (1994) found a significant correlation between the overexpression of both p53 and MDM2 in the same tumour and a poor survival rate ($P<0.05$) but found no correlation between MDM2 on its own and clinical outcome. A study by Al-Kuraya *et al*. (2004) based on more than 2000 cases, found no correlation between MDM2 amplification and disease free survival. Turbin *et al*. (2006) however, in a study based on the same number of cases, found a strong negative correlation between MDM2 IHC expression and disease free survival, independent of other pathological or clinical factors. The intensity of IHC staining had little or no effect on the clinical outcome (Turbin *et al.* 2006). McCann *et al*. (1995) found no correlation between p53 and MDM2 expression.

According to Sheikh *et al*. (1993) ER(+) cells express higher levels of MDM2 compared to ER- cells. They suggested that oestrogen may play an important role in growth stimulation of breast carcinoma by modulating the expression of MDM2, which in turn may inactivate the function of p53.

2.8 BCL2

2.8.1 Structure and function

The *BCL2* gene has been mapped to 18q21.3. The name is derived from a B-cell lymphoma in which it was the second protein described to be

inappropriately expressed as a result of the chromosomal translocation [t(14,18)] and associated with a poor outcome (Tsujimoto *et al.* 1984). The BCL2 protein forms part of the BCL2 family of apoptosis regulating proteins, which includes both anti-apoptotic and pro-apoptotic proteins. The anti-apoptotic proteins include BCL2 proper, Bcl-x-protein long isoforms (Bcl-$X_{L)}$, myeloid cell leukaemia sequence-1 (Mcl-1), CED-9, A1/Bfl-1 and BHRF-1. The pro-apoptotic members are divided into multidomain pro-apoptotics that include proteins like BCL2-associated X protein(BAX) and BCL2-agonist/killer (BAK) while the BCL2-homology domain 3 (BH3)-only pro-apoptotics include proteins like BCL2-agonist of cell death (BAD), BH3-interacting domain death agonist (BID), BCL2-interacting mediator of cell death (BIM) and BCL2-interacting killer (Bik). Members of the BCL2 family have one or more of four characteristic BCL2 homology (BH) domains in common. The domains are BH1, BH2, BH3 and BH4, which are crucial to the function of these members (Fesik and Shi 2001; Petros *et al.* 2004). BH1 and BH2 play a role in the function of the anion channel that regulates the release of cytochrome C from the mitochondria. BH3 is essential for heterodimerization between members and the minimum domain requirement for the pro-apoptotic function BH4 allows for dimerization with pro-apoptotic proteins (Kluck *et al.* 1997; Yang *et al.* 1997; Kelekar and Thompson 1998).

Members of the BCL2 family share a general structure that consists of a hydrophobic helix, surrounded by amphiphatic helices, which play a central role in the intrinsic pathway of apoptosis. The anti-apoptotic members of the BCL2 family form an integral part of the outer mitochondrial membrane protein and regulate the mitochondrial membrane permeabilization (MMP). MMP plays a pivotal role in the process of apoptosis and is controlled by activation or inactivation of an inner mitochondrial permeability transition pore, which regulates matrix Ca^{2+}, pH and voltage. At least two possible mechanisms have been described. The first mechanism involves a permeability transition pore complex (PTPC) formed by cyclophilin-D, peripheral-type benzodiazepine (PBR), adenine nucleotide transporter (ANT) and the voltage-dependent anion channel (VDAC). This complex then associates either with pro-apoptotic BCL2 proteins like BAX, BAK1 and BIM,

which accelerate the opening of the channel or with one of the anti-apoptotic proteins like BCL-X_L, which causes closure (Shimizu *et al.* 1999; Vyssokikh and Brdiczka 2003). The second mechanism involves the pro-apoptotic proteins and their translocation from the cytosol to the mitochondria where it forms protein pores by oligomerization and association with other proteins or by antagonizing the anti-apoptotic proteins by binding via their BH3 domains. Pro-apoptotic members like BAX, BID and BIM are inactive and must translocate from the cytosol to the mitochondria in order to induce apoptosis. Pro-apoptotic members of the BCL2 family proteins induce the release of cytochrome C from within the mitochondria into the cytosol, while the anti-apoptotic members inhibit the release of cytochrome C. Apoptosis is determined by the ratio of pro-apoptotic to anti-apoptotic proteins (Krajewski *et al.* 1995; Ogura *et al.* 1999).

The mitochondrial pathway of apoptosis is activated by various types of intra- and extra-cellular stress, including DNA damage, growth factor withdrawal, oxidative stress, stresses in the endoplasmic reticulum, death receptor stimulation, anticancer drugs and irradiation (Ron 2002; Green and Kroemer 2004). Stress, like Gamma- and UV radiation, cytotoxic drugs and removal of cytokines induce apoptosis by a mechanism that alters the MMP with subsequent cytochrome C release. Intracellular stress like oncogenes, DNA damage, hypoxia and deprivation of survival factors, activate p53 (Marshall 1994; Baskaran *et al.* 1997; Zhang *et al.* 1998).

Growth factors influence apoptosis via the PI3K/Akt pathway. Growth factors bind growth factor receptors and activate PI3K which in turn activates Akt. Activated Akt is a negative regulator of BAD, a pro-apoptotic member of the BCL2 family involved in mitochondrial apoptosis (Thompson *et al.* 1995).

p53 is responsible for transcriptional activation of the BAX, Noxa, p53-upregulated modulator of apoptosis (PUMA) and BID while repressing the anti-apoptotic proteins. Other transcriptional targets of p53 involved in the apoptotic pathway include Apaf-1 and PTEN (Oda *et al.* 2000; Thornborrow *et al.* 2002; Yu *et al.* 2003).

2.8.2 Clinical implications

As anti-apoptotic proteins, BCL2 and Bcl-X_L are able to inhibit the mitochondrial apoptotic pathway by preventing the release of cytochrome C from the mitochondrion and the subsequent activation of caspases-9 and -3. As a result both BCL2 and Bcl-X_L are able to inhibit drug and radiation induced apoptosis and therefore reduce the effectiveness of chemotherapeutic agents and even more so when they are over-expressed (Huang 2000; Kim *et al.* 2002).

In a study by Silvestrini *et al*. (1994), BCL2 expression was associated with small, slow growing, ER- and p53- tumours. The predictive value of BCL2 was found to be dependent on p53 expression. Lee *et al*. (1997) found a strong relation between hormonal status, histologic tumour and nuclear grade and BCL2 expression. Although they found an association between BCL2 status and survival, BCL2 was not a reliable indicator for overall survival. Charpin *et al*. (1998) found BCL2 expression only of limited prognostic value. They reported an association between BCL2 expression and a longer disease free survival but not with overall survival. Callagy *et al*. (2006) evaluated 930 breast tumours and found BCL2 to be an independent predictor of prognosis particularly in the first 5 years after diagnosis and a useful addition to the Nottingham prognostic index (NPI). In a study involving more than 11 000 early-stage breast cancer cases, Dawson et al. (2010) found BCL2 to be an independent indicator of favourable prognosis for all types of early breast cancer irrespective of ER or Her-2 status or type of adjuvant therapy received. Similarly, Treré *et al*. (2007) found BCL2 to be an independent predictor of clinical outcome, irrespective of nodal status. They associated BCL2(-) carcinomas with a 5x higher risk for relapse and 7x higher risk for death, compared with BCL2(+) carcinomas. Geisler *et al*. (2001) found Her-2 (+)/BCL2(-) cancers with a high histological grade to be resistant to doxorubicin.

2.9 Oestrogen Receptor

2.9.1 Structure and function

Oestrogen receptors (ER) are DNA-binding transcription factors that regulate gene expression and are activated by the hormone 17β-oestradiol to mediate the effects of oestrogen on tissue growth, development and maintenance. Two

different subtypes of oestrogen receptors, encoded by different genes, have been identified. ERα (ESR1) is encoded by a gene located on chromosome 6 (6q25.1) while ERβ (ESR2) is encoded by a gene located on chromosome14 (14q) (Moggs and Orphanides 2001; Levin 2005; Dahlman-Wright *et al.* 2006). The two subtypes show significant sequence homology and exhibit functional domains characteristic of the nuclear receptor super family. Both are composed of five domains (A-F), which include the A/B domain on the N-terminal which is an agonist-independent transcriptional activation function (AF-1) domain. The C-domain is a conserved DNA-binding domain that binds oestrogen response elements in DNA. The D-domain is a hinge region that connects the C and E domains. The E-domain is a dimerization region. The function of the F-domain on the C-terminal is unclear. (Moggs and Orphanides 2001; Nilsson *et al.* 2001)

Although both ERα and ERβ are widely expressed in different tissue types, notable differences are found in their expression patterns. ERα is expressed in the endometrium, breast cancer cells, ovarian stromal cells and the hypothalamus, while ERβ is expressed in the prostate, brain, bone, kidney, intestinal mucosa, lungs, heart and endothelial cells. These two subtypes are co-expressed in many cell types and may form homo- (αα or ββ) or heterodimers (αβ) (Li *et al.* 2004b). In their inactive state, both receptors are found in the cytosol, although a small amount of the ERs have also been found within the nucleus and cell membrane (Htun *et al.* 1999). Oestrogen is a steroidal hormone and can pass through the phospholipid of the cell membrane, therefore ERs do not need to be membrane-bound in order to bind with oestrogen. Oestrogen binding to the receptor triggers a number of events starting off with receptor phosphorylation, dimerization and migration of the receptor from the cytosol into the nucleus and subsequent binding of the receptor dimer to the promoter region of target genes, also referred to as hormone response elements. This DNA/receptor complex then recruits other proteins that are responsible for the transcription of downstream DNA into mRNA and finally protein that is able to change cellular function. This pathway is known as the genomic pathway and is also influenced by several co-repressors such as nuclear hormone receptor co-repressor (NCoR) and silencing mediator or retinoic acid and thyroid hormone receptors (SMRT) and

co-activators such as amplified in breast cancer 1 (AIB1) or steroid receptor co-activator 1 (SRC1) (Shou *et al.* 2004).

2.9.2 Clinical implications

More than half of breast cancers are found to be ER(+). Unfortunately only about half of these ER(+) breast cancers show sensitivity to hormonal treatment (e.g. tamoxifen) and a significant number of these cancers ultimately develop secondary resistance to hormonal treatment, leading to tumour progression, metastasis and death (Osborne 1998; Schiff *et al.* 2004). Richard *et al*. (1987) reported a worse relapse-free survival and overall survival in ER(-) patients compared to ER(+) patients. They also found a significantly worse relapse-free and overall survival in ER(-)/EGFR(+) patients compared to ER(-)/EGFR(-) patients. They also reported a significant association between tumour size and differentiation. Onitilo *et al*. (2009) found tumours of the subtype ER(+)/PR(+)/Her2(-) to benefit from chemotherapy while supporting the findings of other studies which have shown the ER(-)/PR(-)/Her2(-) (triple negative) subtype to be associated with large tumours, a high incidence of nodal positivity and the worst overall and disease-free survival for the first four years. After four years the mortality rate declines rapidly to be on par with other cancers.

One proposed mechanism for developing inherent resistance is the co-amplification of both human epithelial growth factors, especially Her-2 and co-activator AIB1 in ER(+) breast cancers (Osborne *et al.* 2003). In fact, tamoxifen is found to play the role of an oestrogen agonist rather than antagonist in breast carcinomas that express high levels of both Her-2 and AIB1, resulting in stimulation rather than suppression of tumour growth (Shou *et al.* 2004). Another mechanism by which oestrogen can promote growth is the fact that ER located in or near the cell membrane has the ability to activate EGFR as well as Her-2. This pathway is referred to as the non-genomic pathway. This receptor cross-talk happens in both directions and therefore ER and AIB1 in turn can also be phosphorylated by EGFR or HER-2 (Razandi *et al.* 2003). This non-genomic pathway is believed to be the reason for tamoxifen monotherapy failure and the introduction of gefitinib as an EGFR/Her-2 pathway inhibitor in order to close the so-called “loophole” and restore tamoxifen’s antagonistic

effect on ER(+) tumours. Shou *et al*. (2004) found oestrogen withdrawal in ER(+) tumour to be a valid alternative to a combination of tamoxifen and gefitinib treatment. This approach would unfortunately only be beneficial to a small fraction of ER(+) patients as less than 10% of ER(+) cancers express high levels of Her-2, suggesting the existence of another mechanism through which the majority of ER(+) cancers escape endocrine therapy.

2.10 Aims

- To determine the expression and prognostic value of selected markers in the Her-2/PI3K/Akt pathway

- To evaluate the chromogenic in-situ hybridization (CISH) technique for its suitability for analysis of Her-2 gene amplification on archived, Papanicolaou-stained fine needle aspiration (FNA) samples.

CHAPTER 3
MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Ethical Approval

Ethical approval has been obtained from the Human Research Ethics Committee at the University of Cape Town. (HREC reference number 082/2009)

3.2 Sample selection

We have selected a retrospective series of primary breast carcinoma cases based on their Her-2 immunohistochemical results. Twenty seven cases were Her-2 negative, 7 had a score of (1+), 17 had a score of (2+), while 42 had a score of (3+).

A total of 93 cases were selected for this study (referred to as cohort A). Sixty seven cases were from the archives of the NHLS Division of Anatomical Pathology at Groote Schuur hospital (cohort B), but since trastuzumab was not offered at state facilities at the time of the study, a second cohort of 26 cases from the private sector that received trastuzumab treatment was included (cohort C). These tissue blocks were obtained from two independent private pathology laboratories in Cape Town.

A limiting factor in case selection and follow up period has been the fact that routine Her-2 testing on all invasive breast cancer cases was only introduced late in 2006. For this reason, only patients who had a mastectomy after the introduction of routine Her-2 testing, unless they had Her-2 testing done on request, were included in the study. The outcome was based on the patient status on 1 July 2011. All cases were reviewed and appropriate tissue blocks selected for study.

3.3 Data Collection

The histology and cytology reports were obtained from the NHLS Division of Anatomical Pathology at Groote Schuur hospital or from private pathology laboratories.

Clinical information was obtained with the help of clinical collaborators at the oncology unit at Groote Schuur hospital and private practice oncology units. Patients that were lost to follow up or died of unrelated causes were excluded from the study.

The following clinical and pathological information was recorded:

- Clinical stage of tumour at time of mastectomy
- Histopathological grade
- Tumour type
- Age of patient at time of mastectomy
- Date of onset of treatment
- Lymph node status
- Lymphovascular invasion
- Her-2 IHC and ISH results
- Oestrogen receptor (ER) status*
- Treatment / therapy received
- Follow up of patient – minimum of 4 years:
    - Alive and disease free
    - Alive with disease
    - Dead
    - Disease free survival (DFS)
    - Overall survival (OS)

* The progesterone receptor (PR) status was not consistently tested for in the treatment work-up during this particular period. In view of this inconsistency, the PR results were not recorded in this study.

3.4 Immunohistochemistry (IHC)

IHC was performed on formalin-fixed, paraffin-embedded tissue sections.

3.4.1 Buffers and reagents used

3.4.1.1 Phosphate buffered saline (PBS) pH7.6

Used for diluting antibodies (without Tween 20) and rinsing (with Tween 20)

NaH_2PO_4 (2H20) 0.78g

Na_2HPO_4 6.4g

NaCL 42.5g

Dissolve the above in 5L distilled water.

Adjust to pH7.6 using HCL or NaOH.

Add 2.5ml Tween 20 for PBS with Tween when used for rinsing.

3.4.1.2 Tris-HCl buffered saline (TBS) pH7.6

Used for diluting antibodies and rinsing

$(HOCH_2)_3CNH_2$ 3g (TRIS hydroxymethylaminomethane)

NaCl 40.5g

1M HCl 19ml

Distilled water to 5L

3.4.1.3 0.1M Citrate buffer pH6.0

Used for antigen retrieval

Citric acid 10.5g

Dissolve in 4.0L of distilled water.

Adjust to pH6.0 with NaOH

Make up to 5L with distilled water.

3.4.1.4 1mM EDTA pH8.0

Used for antigen retrieval

EDTA 1.86g

Dissolve in 4.9L distilled water.

Adjust to pH8.0 with 1M NaOH.

Make up to 5L with distilled water.

3.4.1.5 1% Copper sulphate

Used to enhance DAB staining/colour

$CUSO_4$ 10g

Dissolve in 1L distilled water.

3.4.1.6 Scotts tap water

Used for “blueing” after counterstaining with haematoxylin

Na_2CO_3 3.5g

$MgSO_4.7H_2O$ 20g

Dissolve in 1L tap water.

3.4.2 Controls

A Study cohorts

Within the total population (93 cases – cohort A), we had a cohort that received trastuzumab treatment (26 cases – cohort C), while remaining cohort did not receive trastuzumab treatment (67 cases – cohort B) and was used as a control group.

B Test controls

Appropriate positive tissue controls were included in each test run*.
For negative controls (IHC), the primary antibody was substituted by PBS buffer.

*Please refer to **Table 1** for specific positive tissue controls that were used for each antibody.

3.4.3 Method for IHC

- Cut sections 4µm thick
- Float sections onto APES coated slides and blot
- Heat-fix the sections onto the slides by leaving slide on a hot plate (~60ºC) for at least 10 min.
- De-wax in xylene and hydrate slides in graded alcohols down to water
- Rinse slides in running tap water
- Perform the appropriate antigen retrieval by heat induced epitope retrieval using Citrate buffer at pH6.0 for 2min or EDTA buffer at pH8.0 for 90 sec, depending on specific antibody requirements (See Table1).
- Remove slides from pressure cooker and immerse in running tap water to cool down.

- Block for endogenous peroxidase activity by treating slides with 1% H_2O_2 in distilled water for 5-10 min (to block non-specific staining)
- Rinse in running tap water
- Block for non-specific binding by treating slides with normal goat serum (1:20) for 5-10 min (DAKO)
- Drain the normal serum from the slides
- Apply appropriately diluted primary antibody for required / optimised incubation time. See Table 1 for specific antibody dilutions and incubation times. Dilution of antibody p-Akt was done using TBS.
- Rinse thoroughly with PBS
- Apply Envision reagent (secondary antibody) for 25-30 min (DAKO) (NB. use the appropriate Envision reagent for each antibody i.e. goat anti-mouse OR goat anti-rabbit)
- Rinse in running tap water
- Apply the chromogenic substrate (1ml buffer + 1 drop 3,3'-diaminobenzidine-tetrahydrochloride (DAB) for 3-5 min (DAKO)
- Rinse in running tap water
- Immerse slides in 1% copper sulphate for 3-5 min (enhances the DAB staining)
- Rinse in running tap water
- Counter stain in haematoxylin
- Rinse in running tap water
- "Blue" the slides in Scott's tap water
- Rinse in running tap water
- Dehydrate in graded alcohols, rinse in xylene and mount with DPX or Entellan mounting medium.

The buffer used (either EDTA or Citrate) for antigen retrieval was determined by the suggested pH requirement specified in the datasheet provided by the supplier of the antibody.

Primary antibodies and normal goat sera were diluted in PBS without Tween unless otherwise indicated.

All rinse steps are performed with PBS with Tween unless otherwise indicated.

In the case of antibody p-Akt, PBS was substituted with TBS in all rinsing and dilution steps.

To prevent further dilution of the reagents, excess fluid was mopped up around sections before reagents were applied.

Staining was performed in a humidity chamber.

All antibodies were stored between 2-8°C.

Table 1: Summary of antibodies, antigen retrieval, clones, dilutions, controls and incubation times

Antibody	Clone	Supplier	Secondary Antibody	Ag retrieval	Positive tissue control	Dilution	Incubation time	Buffer
Her-2	Polyclonal Cat # A0485	DAKO	Goat Anti-Mouse	EDTA buffer pH8.0 90 sec	Breast carcinoma	1: 250	30 min	PBS
PI3K	3E7	Abnova	Goat Anti-Mouse	Citrate buffer pH6.0 2min	Adrenal gland	1:167	3 hours	PBS
PTEN	6H2.1	Enzo Life Sciences	Goat Anti-Mouse	Citrate buffer pH6.0 2min	Endometrium	1:300	1 hour	PBS
p-Akt	LP18	Leica Microsystems	Goat Anti-Mouse	EDTA buffer pH8.0 90 sec	Skin	1:50	14 hours	TBS
NF-κB	Polyclonal Cat # RB-9034	Thermo Scientific	Goat Anti-Rabbit	EDTA buffer pH8.0 90 sec	Prostate	1:100	40 min	PBS
MDM2	1B10	Leica Microsystems	Goat Anti-Mouse	Citrate buffer pH6.0 2min	Leiomyosarcoma	1:100	60 min	PBS
Bcl-2	124	DAKO	Goat Anti-Mouse	EDTA buffer pH8.0 90 sec	Tonsil	1:50	40 min	PBS
p53	DO-7	DAKO	Goat Anti-Mouse	d EDTA buffer pH8.0 90 sec	Colonic adenocarcinoma	1:100	40 min	PBS

Information obtained from package inserts provided by suppliers and further optimization done by researcher

3.4.4 Evaluation of slides

Evaluation of the IHC results was done simultaneously by two investigators.

Table 2: Scoring of Her-2 results

Staining pattern	**Score**	**Assessment**
No staining or membrane staining in less than 10% or tumour cells.	0	negative
Faint/barely perceptible membrane staining in more than 10% of tumour cells. The cells exhibit incomplete membrane staining.	1+	negative
Weak to moderate staining of entire membrane in more than 10% of tumour cells.	2+	equivocal
Strong staining of entire membrane in more than 10% of tumour cells.	3+	positive

Table 3: ER scoring

Based on the Allred scoring system (Allred *et al.* 2004)

Proportion					
0	1	2	3	4	5
0%	<1%	1%-10%	11%-33%	34%-66%	>66%
Intensity					
0	1	2	3		
No staining	Weak staining	Moderate	Strong		

The sum of the proportion and intensity scores was regarded as the total ER score out of a possible score of 8. According to recommended clinical practice, scores from 0 to 2 were regarded as ER negative and scores from 3 to 8 as ER positive (Lester *et al.* 2012).

Table 4: Evaluation of all other IHC results

% of cells stained	**score**
<5%	0
5% - 50%	low
51% - 100%	high

3.5 Chromogenic in-situ hibridization (CISH)

CISH was performed on 10 histopathological and corresponding cytological samples. The cytological samples were archived, Papanicolaou-stained, FNA slides taken prior to the mastectomy and the histopathological samples, formalin-fixed, paraffin-embedded tissue sections taken from the mastectomy specimen.

3.5.1 Probe

The Her-2 CISH probe (SPOT-Light® HER2 CISH kit from Invitrogen) (FDA approved).

3.5.2 Sample pre-treatment for CISH

Information obtained from the SPOT-Light® HER2 CISH kit package insert (Intended for use on formalin-fixed, paraffin-embedded tissue)

3.5.3 Preparation of archived cytology slides for CISH:

- Immerse slides in xylene until coverslip and mounting medium is removed.
- De-stain in 2% acid alcohol until completely de-stained
- Soak in 100% ethanol for 5min
- Wash thoroughly in dH_2O

3.5.4 Histopathological sample pre-treatment

- Cut formalin-fixed, paraffin-embedded tissue into 4µm thick sections and float onto Histo-bond or APES treated slides
- Air dry slides or dry at 37°C, and then bake 2hours at 60°C

3.5.5 Deparaffinization

- Immerse in xylene until wax is removed
- Soak in 100% ethanol for 5min
- Wash thoroughly in dH_2O

Method for CISH for both histopathological and cytological specimens

3.5.6 Heat pre-treatment

- Place slides in slide rack. (Including supplied test control slide)
- Heat the Heat Pre-treatment solution in a beaker on a hotplate until temperature reaches ≥98°C. Cover beaker to prevent evaporation.
- Place slides in boiling solution and boil for 15 min. Ensure that the temperature remains between 98°C and 100°C.
- Transfer slides immediately to dH_2O at room temperature
- Wash in dH_2O

3.5.7 Enzyme digestion

- Remove enzyme pre-treatment solution (pepsin) from the fridge and allow to reach room temperature
- Add enough enzyme pre-treatment solution to cover the tissue sections and incubate for 5 min at room temperature
- Wash in dH_2O

3.5.8 Dehydration in graded ethanol series

- Dehydrate in graded alcohols
- Allow slides to air dry

3.5.9 Denaturation and hybridization

- Add 15µl of probe to the centre of a 22 x 22 mm coverslip
- Place coverslip, probe side down, on the appropriate area of the tissue sample on the slide
- Seal the edges of the coverslip with rubber cement in order to prevent evaporation
- Allow rubber cement to dry (10 min)
- Place slides in preheated hybridizer with humidity strips and set program to:

    A: Denature at 95°C for 5 min and

    B: Incubate overnight (±14 hrs) at 37°C

3.5.10 Stringent wash (Day 2)

- Prepare two Coplin jars containing saline sodium citrate (SSC) buffer, one at room temperature and the other heated to 70°C
- Peel off rubber cement from slides
- To remove coverslip without tearing tissue, pre-soak slides in SSC at room temperature for 2-3 min, until the coverslip slides off easily
- Rinse in SSC at room temperature
- Immerse slide in Coplin jar containing SSC at 70°C for 5 min
- Wash in dH_2O

3.5.11 Immunodetection

- Immerse slides in 30% H_2O_2 in 100% methanol (1:9) (10 min)
- Wash in PBS/Tween 20 (0.01%)
- Add CAS-Block™ - 2-3 drops per slide and incubate (10 min)
- Blot off reagent with tissue paper (do not rinse)
- Add mouse anti-digoxigenin antibody – 2-3 drops per slide and incubate
  (30 min)
- Wash in PBS/Tween 20 (0.01%)
- Add goat anti-mouse HRP polymer conjugate – 2-3 drops per slide and incubate (30 min)
- Wash in PBS/Tween 20 (0.01%)
- Prepare DAB substrate-chromogen solution: add one drop of each of the 3 solutions to 1ml of dH_2O
- Add DAB – 2-3 drops per slide and incubate (30 min)
- Place slides in slide rack
- Wash in running tap water

3.5.12 Counterstaining and mounting

- Counterstain tissue sections with haematoxylin (5 secs)
- Evaluate intensity of counterstain and stain for further (3-5secs) if needed
- Wash in running tap water

- Dehydration in graded ethanol series, immerse in xylene
- Coverslip using DPX or Entellan

2.5.13 Slide evaluation

Slides were evaluated simultaneously by two investigators using an Olympus BX43 light microscope.

An individual Her-2 gene appears as a small round single dot.
Non-amplification according to the Invitrogen Spot-light® HER2 CISH kit package insert is defined as:

- 1-5 single dots in the majority (>50%) of carcinoma cells

Amplification according to the Invitrogen Spot-light® HER2 CISH kit package insert is defined as:

- >5 dots in the majority of carcinoma cells
- Large clusters in the majority of carcinoma cells
- A mixture of multiple dots and large clusters in the majority of carcinoma cells
- A mixture of multiple dots and small clusters in the majority of carcinoma cells
- Small clusters in the majority of carcinoma cells

Unclear cases of amplification or non-amplification according to the Invitrogen Spot-light® HER2 CISH kit package insert is defined as:

- Specimens that exhibit 4-6 dots in the majority of carcinoma cells
- If the average number of dots is between 4 and 6 after 30 carcinoma cells have been counted, an additional 30 cells should be counted for a total of 60 cells.

3.6 Her-2 IHC and Her-2 CISH on corresponding histopathology and cytopathology specimens

The IHC stained slides for Her-2, a test that is routinely performed on all breast carcinomas, were re-evaluated simultaneously by two observers. We

have selected 10 cases which had both tissue blocks and archived FNA slides available, for testing. We performed Her-2 CISH analysis on the histopathology sample cut from a paraffin embedded tissue block taken from the mastectomy specimen, as well as corresponding cytology sample. The cytopathology sample was obtained by de-staining one archived, diagnostic FNA slide that was taken shortly before the mastectomy was performed.

We used the ISH results to re-classify all equivocal IHC Her-2 (2+) cases.

3.7 Determining the apoptotic index

The apoptotic index was calculated as the average of the apoptotic cell count in 50 consecutive high power fields (40X objective) in haematoxylin and eosin-stained tumour sections of all cases, using a normal light microscope (Olympus BX43). Areas of necrosis were avoided and multiple apoptotic bodies in one small area that could have originated from one cell were counted as one.

3.8 Statistical analysis

The Shapiro Wilk test was used to test whether continuous variables were normally distributed. A value of >0.05 is regarded as normal distribution.

The association between clinicopathological variables and protein expression was analyzed using analysis of variance (ANOVA) for continuous variables and the chi-square or Fishers' exact test for categorical variables. The Fisher's exact test was used if the expected cell frequency in any of the contingency tables was <5. The level of significance was set at $p < 0.05$ for all analyses.

Survival curves according to key variables was plotted using Kaplan Meier graphs and the Log Rank test for equality of survivor functions were used to assess statistical significance.

The Cox-proportional hazards model was used to estimate the hazard ratios (HR) and the 95% CI for overall and disease free survival between the

various subgroups based on their difference in protein expression, adjusting for age. A multivariate Cox regression was attempted adjusting for stage, grade and age as variables but the estimates changed dramatically from the age adjusted estimates and the model was deemed unstable.

Hazard ratio (HR) describes the relative risk based on the comparison of event rates or hazard rates between two groups over a given time period. The hazard rate is the ratio of the number of events within a group to the total number in the group over a given time period. HR = 1.0 indicates equal risk rates. HR >1.0 indicates increased risk and HR<1.0 decreased risk.

Confidence intervals (CI or 95% CI) is the range of HR's likely to contain the mean HR or in other words the mean HR has a 95% chance of being contained in the CI interval. If the CI contains 1.0, the HR is not considered statistically significant and equal risk / chance apply. If the CI is greater and excluding 1.0, it indicates increased risk while a CI less and excluding 1.0 would indicate decreased risk. For each variable, the first category was used as reference group.

Both overall and disease free survival was measured from date of first treatment to the date of related death or first relapse. Patients who died of unrelated causes where excluded from this analysis.

STATA version 12.1 (StataCorp LP, 4905 Lakeway Drive, College Station, TX77845, USA) statistical software was used for all analysis with the assistance of a biostatistician.

CHAPTER 4
RESULTS

RESULTS

4.1 Age distribution

The study included 93 invasive breast carcinomas from patients with ages ranging from 22 to 78 years. Age distribution was found to be normal according to the Shapiro Wilk test for normality (p=0.9). The mean age was 53.5 years and the standard deviation (SD) 10.76. The highest frequency of breast carcinoma cases was found in the 55-59 year old age group (**Figure 4**).

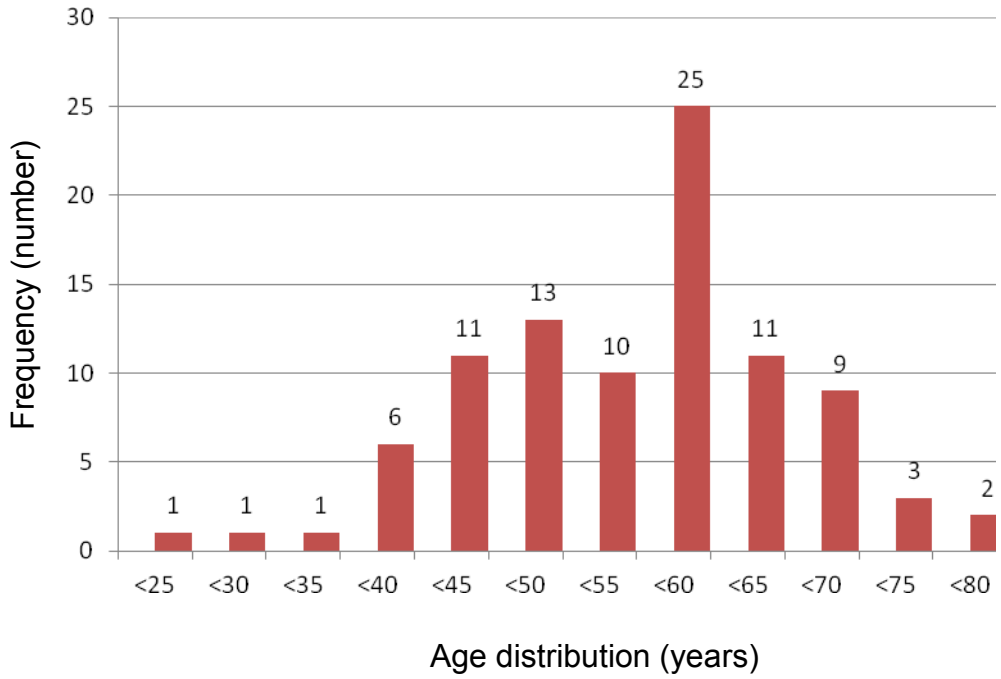


Figure 4: Histogram showing the age distribution of patients

4.2 Racial profile

The racial profile of the patients from Groote Schuur hospital was largely Coloured (42/67, 62.7%), followed by African patients (10/67, 14.9%) and White patients (3/67, 4.5%). The race was not stated in 12 (17.9%) cases. The patients from the second cohort obtained from the private laboratories were predominantly White (20/26, 76.9%), followed by Coloured patients (3/26, 11.5%). The race was not stated in 3 (11.5%) cases.

4.3 Clinicopathological characteristics

Clinicopathological characteristics such as carcinoma classification, stage, grade, lymphovascular invasion, presence of distant metastasis, presence of nodal metastasis, treatment offered and patient outcome are listed in **Table 5**.

4.4 Treatment

All patients had surgical resection. None of these patients received treatment before surgical resection. Patients who had received trastuzumab were from the private sector. Two patients were treated with a single treatment option. The majority of patients received a combination of 3 or more of the treatment options, depending on hormonal status, tumour stage and other clinical considerations (**Table 6)**.

Table 5: Clinicopathological characteristics

Characteristics	n=93	%
Carcinoma classification:		
Ductal	85	91.4%
Lobular	8	8.6%
Stage:		
I	14	15.1%
II	48	51.6%
III	30	32.3%
IV	1	1.1%
Grade:		
1	14	15.1%
2	36	38.7%
3	43	46.2%
Lymphovascular invasion:		
Y	70	75.3%
N	23	24.7%
Lymph node metastasis:		
Y	59	63.4%
N	34	36.6%
Distant metastases:		
Y	28	30.1%
N	65	69.9%
Treatment:		
Surgery - Mastectomy	81	87.1%
Surgery – Wide local excision	12	12.9%
Hormonal Therapy	61	65.6%
Chemotherapy	71	73.3%
Radiotherapy	58	62.3%
Trastuzumab	26	28.0%
Outcome:		
Disease free	59	63.4%
Alive with disease	14	15.1%
Dead	20	21.5%

Table 6: Treatment combinations

Chemotherapy only	4
Chemotherapy + hormone therapy	6
Chemotherapy + hormone therapy + trastuzumab	6
Chemotherapy + hormone therapy + radiotherapy	20
Chemotherapy + hormone therapy + radiotherapy + trastuzumab	10
Chemotherapy + radiotherapy	15
Chemotherapy + radiotherapy + trastuzumab	6
Chemotherapy + trastuzumab	4
Hormone therapy + radiotherapy	6
Hormone therapy only	13
Radiotherapy only	1
Surgery only	2

4.5 Immunohistochemistry results

4.5.1 Her-2

Twenty seven (29.0%) cases were Her-2 negative, 7 (7.5%) had a score of (1+) (considered negative), 17 (18.3%) had a score of (2+) (considered equivocal), while 42 (45.2%) had a score of (3+) (considered positive). Three Her-2 (2+) results were reclassified as Her-2 negative, while 14 were re-classified as Her-2 positive according to ISH results.

A statistically significant association was found between Her-2 results and NFκB expression (p=0.035). NFκB expression was high in 63.2% (55/87) of Her-2(+) cases compared to 36.8% (32/87) in Her-2(-) cases whereas NFκB expression was low in 16.7% (1/6) of Her-2(+) cases and 83.3% (5/6) Her-2 (-) cases (**Appendix 1**).

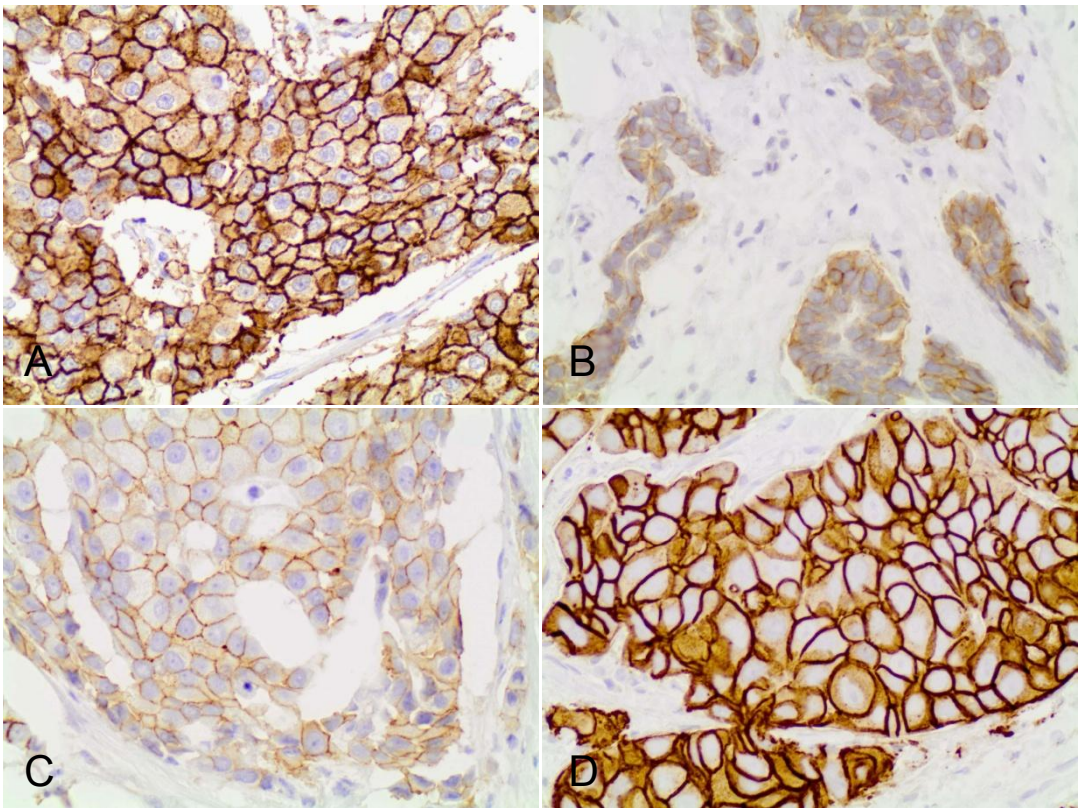


Figure 5: Representative IHC staining of Her-2 with score (**A**) Pos control. Infiltrating duct carcinoma Her-2 (3+) (400x) (**B**) Infiltrating duct carcinoma Her-2 (1+) (400x) (**C**) Infiltrating breast carcinoma Her-2 (2+) (400x) (**D**) Infiltrating duct carcinoma Her-2 (3+) (400x)

4.5.2 ER

The distribution of ER expression (proportion + intensity) were as follows: 44 cases were negative, 2 cases had a score of 2, 1 case had a score of 3, 3 cases had a score of 4, 11 cases had a score of 5, 5 cases had a scored 6, 10 cases had a score of 7 and 27 cases had a score of 8. Forty six were regarded as ER(+) and 47 as ER(-).

A statistically significant association was found between ER status and BCL2 expression (p=0.022). BCL2 expression was high in 63% (30/47) ER(+) cases and in only 36.2% (17/47) ER(-) cases, while BCL2 expression was negative in 30.8% (8/26) of ER(+) cases and in 69.2% (18/26) of ER(-) cases (**Appendix 2**).

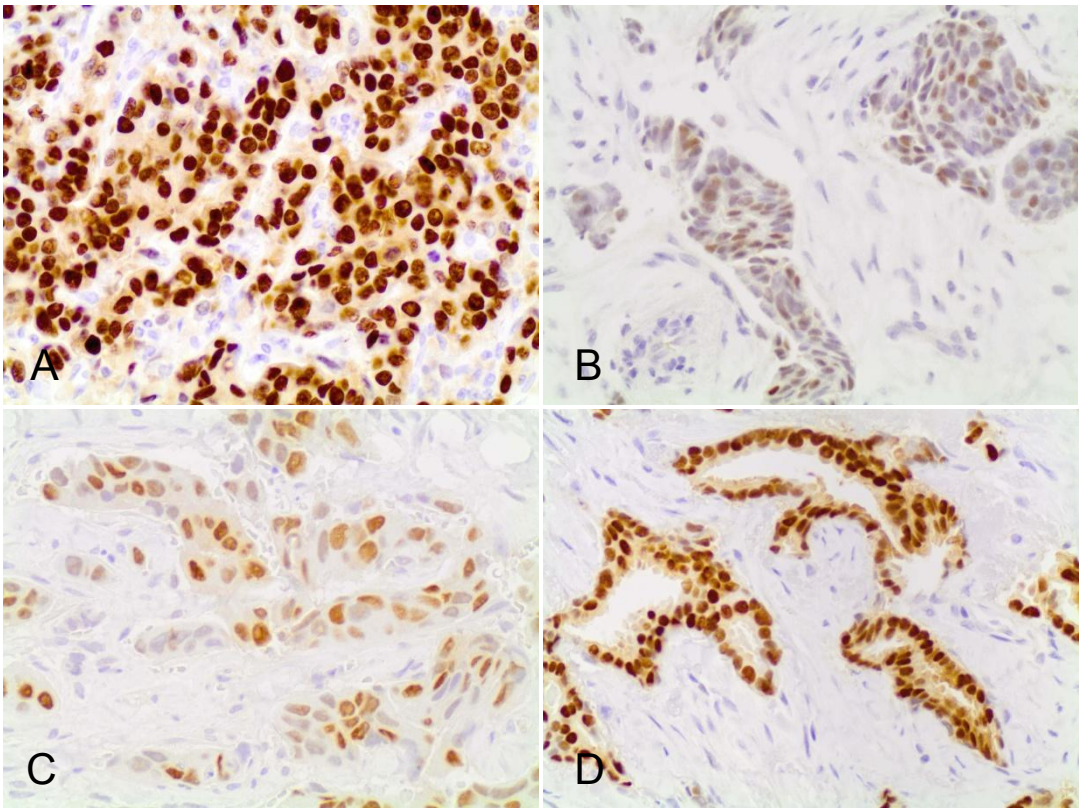


Figure 6: Representative IHC staining of ER with intensity score (**A**) Positive control ER (3+) (400x) (**B**) Infiltrating duct carcinoma ER (1+) (400x) (**C**) Infiltrating duct carcinoma ER (2+) (400x) (**D**) Infiltrating duct carcinoma ER (3+)

4.5.3 PI3K

Seventy nine (84.9%) cases showed high PI3K expression, 13 (14.0%) low and 1 (1.1%) was found to be negative for PI3K expression.

We found no significant association between PI3K and any of the other components (**Appendix 3)**.

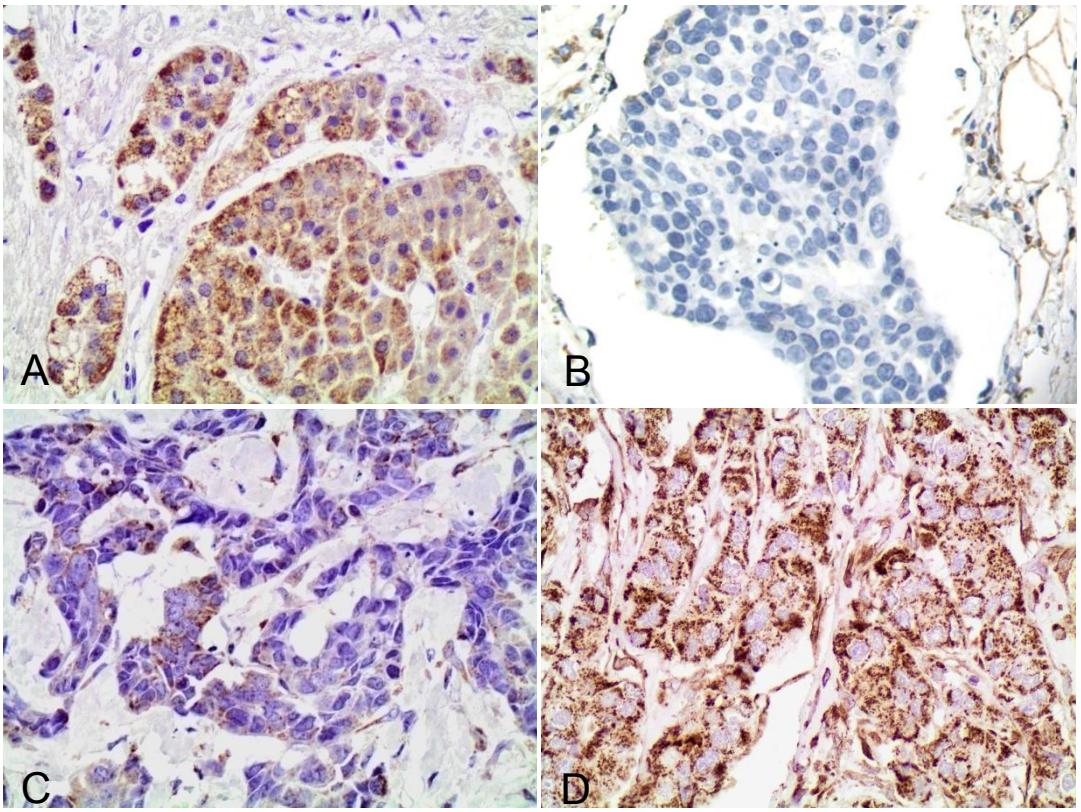


Figure 7: Representative IHC staining for PI3K (**A**) Positive control. Adrenal gland (400x) (**B**) Infiltrating breast carcinoma showing negative PI3K expression (400x) (**C**) Infiltrating breast carcinoma showing low PI3K expression (**D**) Infiltrating breast carcinoma showing high PI3K expression (400x)

4.5.4 PTEN

PTEN showed high expression in 55 (59.1%) of cases, low expression in 27 (29.0%) cases and was negative in 11 (11.8%) cases.

An association was found between PTEN and p-Akt expression (p=0.053) and between PTEN and Her-2 expression (p=0.065) although these were not statistically significant.

p-Akt expression was high in 67.8% (40/59) of cases that showed high PTEN expression, 23.7% (14/59) of cases that showed low PTEN expression and 8.5% (5/59) of cases that was negative for PTEN expression.

Her-2 was negative in 54.1% (20/37) of cases that showed high PTEN expression, 24.3% (9/37) of cases that showed low PTEN expression and in 21.6% (8/37) of cases that were negative for PTEN expression (**Appendix 4**).

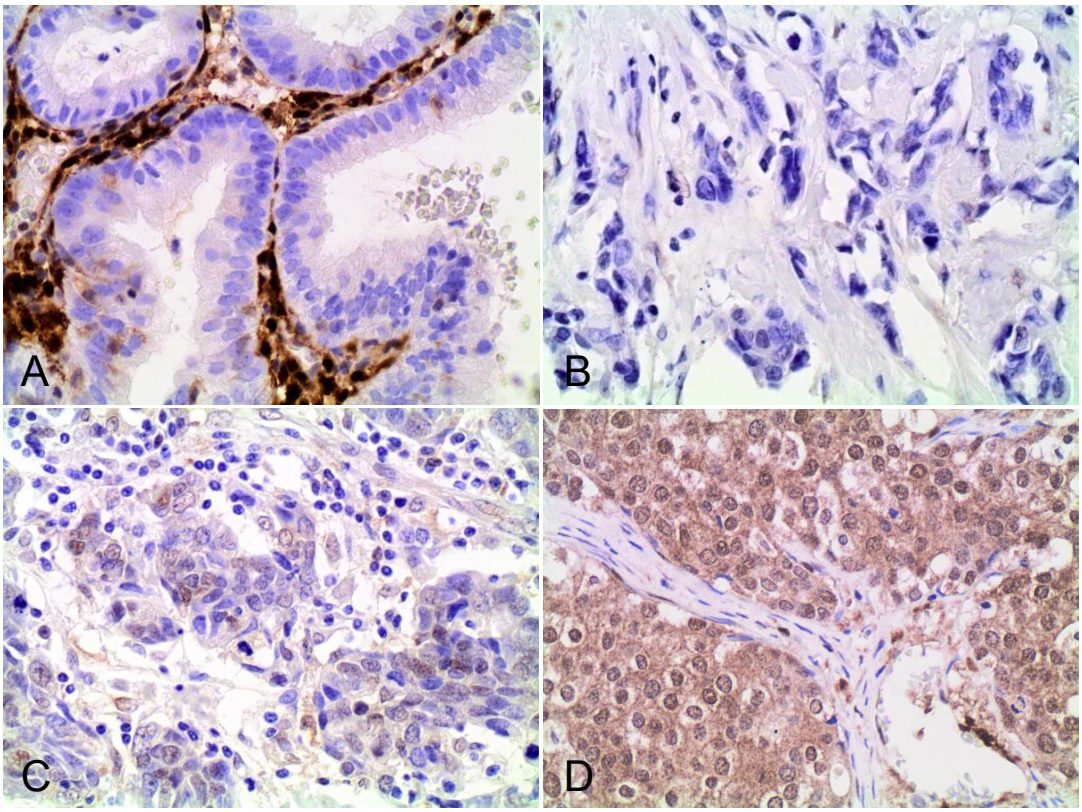


Figure 8: Representative IHC staining for PTEN (**A**) Positive and negative control. Endometrium showing non-expressing neoplastic glands and normal stroma showing high PTEN expression (400x) (**B**) Infiltrating duct carcinoma showing negative PTEN expression (400x) (**C**) Infiltrating duct carcinoma showing low PTEN expression (400x) (**D**) Infiltrating duct carcinoma showing high PTEN expression (400x)

4.5.5 p-Akt

p-Akt showed high expression in 59 (63%) of cases, low expression in 26 (28%) cases and was negative in 8 (9%) cases.

Significant associations were found between both p-Akt and NFκB expression (p=0.014) and p-Akt and MDM2 expression (p=0.040). High NFκB expression was present in 66.7% (58/87) of cases that showed high p-

Akt expression, compared to 26.4% (23/87) in cases that showed low p-Akt expression and in 6.9% (6/87) of cases that were negative for p-Akt expression. High MDM2 expression was present in 70.3% (52/74) of cases that were showing high p-Akt expression, while it was present in 26.4% (16/74) of cases showing low p-Akt expression and in only 12.2% (6/74) of p-Akt negative cases (**Appendix 5**).

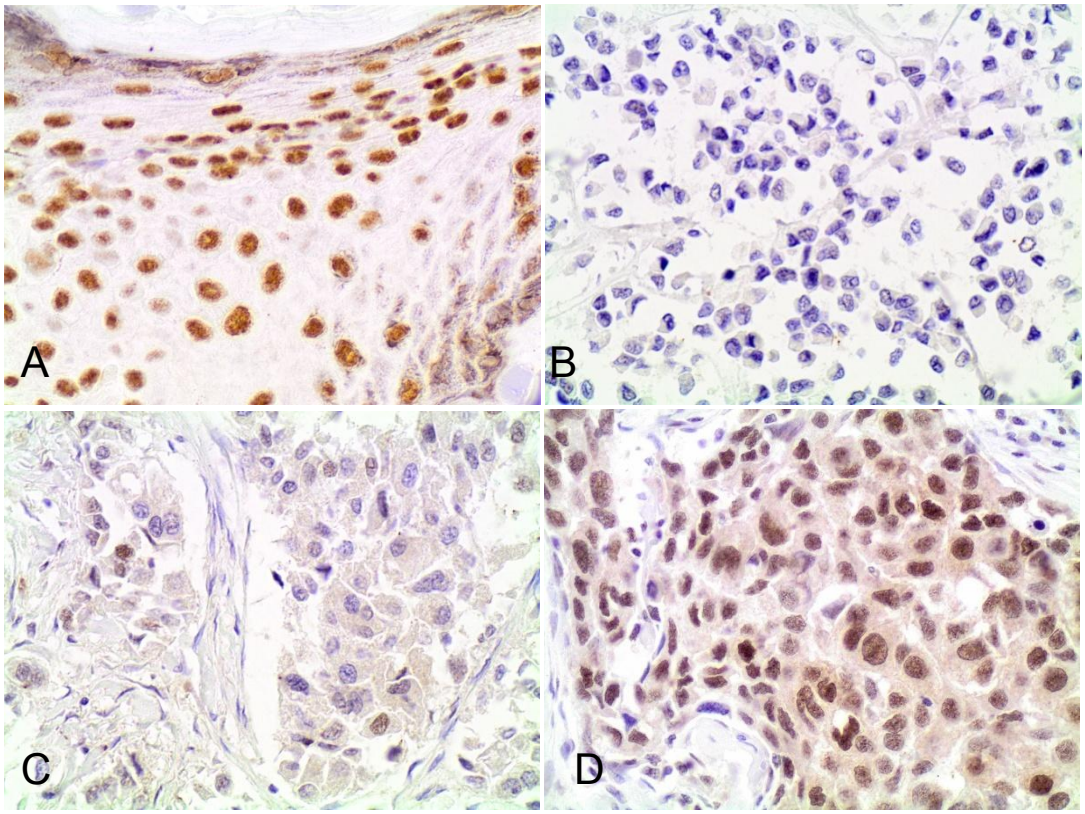


Figure 9: Representative IHC staining for p-Akt (**A**) Positive control. Skin (400x) (**B**) Infiltrating duct carcinoma showing negative p-Akt expression (400x) (**C**) Infiltrating duct carcinoma showing low p-Akt expression (**D**) Infiltrating duct carcinoma showing high p-Akt expression (400x)

4.5.6 MDM2

High expression of MDM2 was found in 74 (80%) cases, low expression in 14 (15%) cases and was negative in 5 (5%) cases.

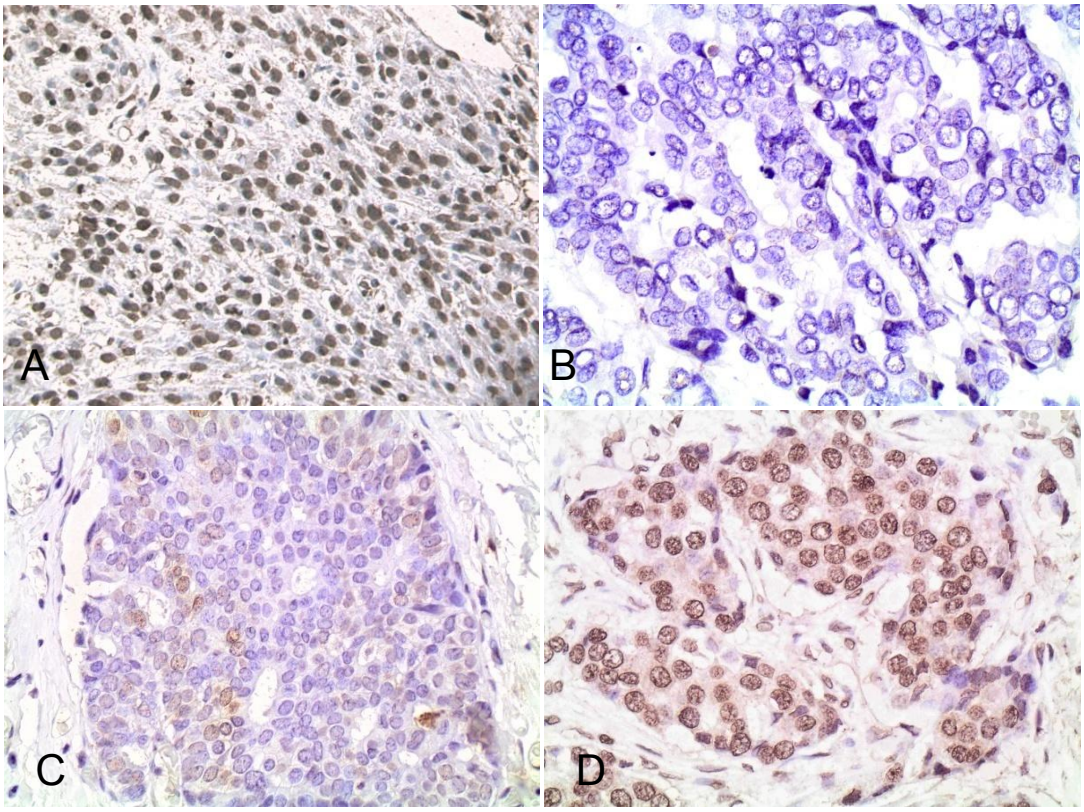


Figure 10: Representative IHC staining for MDM2 (**A**) Positive control. Leiomyosarcoma (400x) (**B**) Infiltrating duct carcinoma showing negative MDM2 expression (400x) (**C**) Infiltrating duct carcinoma showing low MDM2 expression (200x) (**D**) Infiltrating duct carcinoma showing high MDM2 expression (400x)

4.5.7 NFκB

Cytoplasmic expression of NFκB was present in all cases. High cytoplasmic expression of NFκB was seen in 87 (94%) cases while 6 (6%) cases showed low expression.

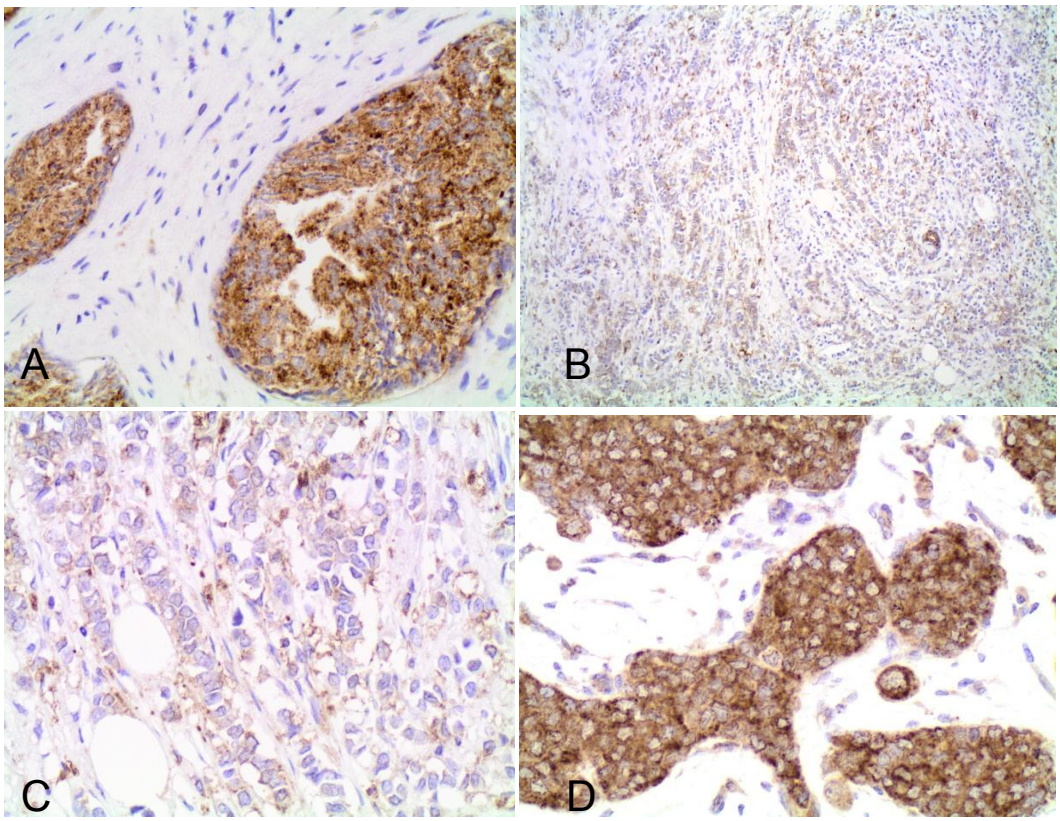


Figure 11: Representative IHC staining for NFκB (**A**) Positive control. Prostate (400x) (**B**) Infiltrating breast carcinoma showing low NFκB expression (100x) (**C**) Infiltrating breast carcinoma showing low NFκB expression (400x) (**D**) Infiltrating breast carcinoma showing high NFκB expression (400x)

4.5.8 BCL2

High BCL2 expression was seen in 47 (50%) cases, low expression in 20 (22%) cases and was negative in 26 (28%) cases.

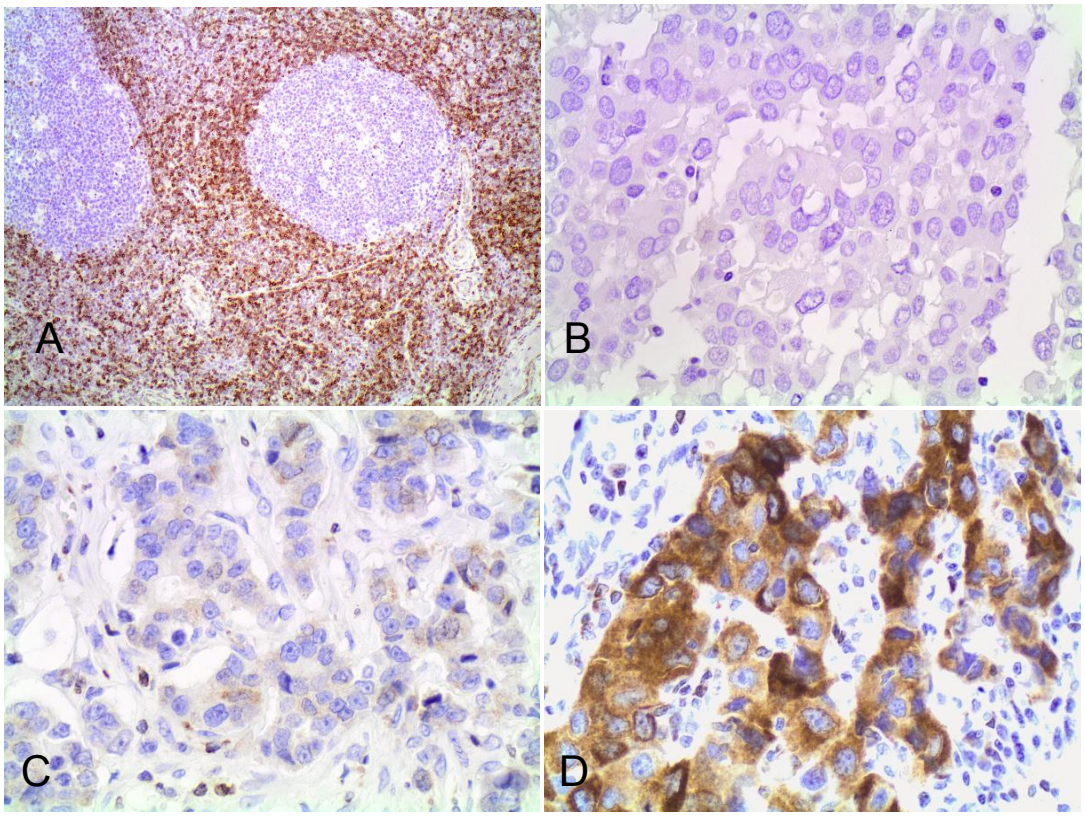


Figure 12: Representative IHC staining for BCL2 (**A**) Positive control. Tonsil (100x) (**B**) Infiltrating breast carcinoma showing negative BCL2 expression (400x) (**C**) Infiltrating breast carcinoma showing low BCL2 expression (400x) (**D**) Infiltrating breast carcinoma showing high BCL2 expression (400x)

4.5.9 p53

High expression of p53 was found in 21 (23%) cases. The same number showed low expression while 51 (55%) cases showed no p53 expression.

No statistically significant association was found between p53 and any of the other protein markers. There was however a slight association between p53 and Her-2 expression (p=0.057). Her-2 expression was negative in 10.8%

(4/37) of cases showing high p53 expression, compared to 21.6% (8/37) of cases showing low p53 expression and 67.6% (25/37) of cases where p53 expression was negative (**Appendix 9**).

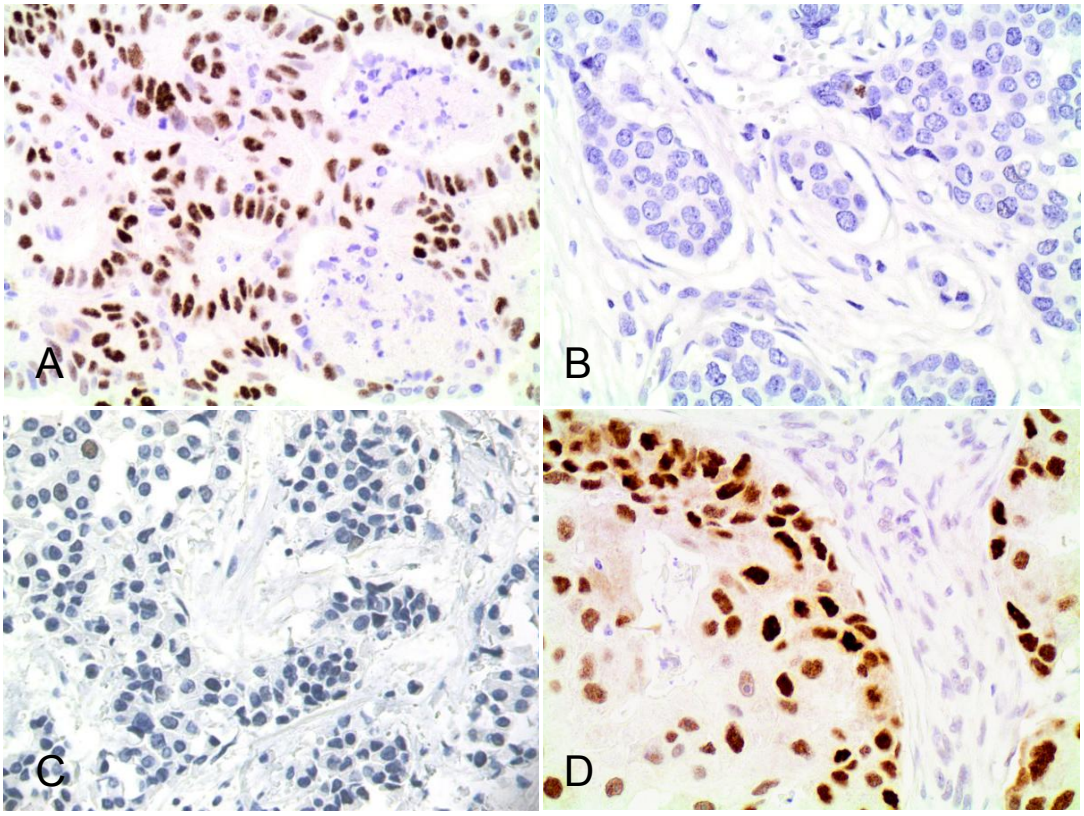


Figure 13: Representative IHC staining for p53 (**A**) Positive control. Colonic Adenocarcinoma (400x) (**B**) Infiltrating duct carcinoma showing negative p53 expression (400x) (**C**) Infiltrating duct carcinoma showing low p53 expression (400x) (**D**) Infiltrating duct carcinoma showing high p53 expression (400x)

4.6 Association between clinicopathological variables and protein expression

4.6.1 Age

A statistically significant association was found between age and Her-2 expression (p=0.044). The mean age for Her-2 (+) expression was 51.6 years (SD 8.6) compared to a slightly older 56.2 years (SD 11.7) for Her-2 (-) expression. No statistically significant association was found between age and any of the other markers (**Appendix 10**).

4.6.2 Tumour type

A statistically significant association was found between tumour type and Her-2 expression (p=0.006). Of the 56 cases that were Her-2 (+), 98.2% (55/56) were ductal carcinomas compared to 1.8% (1/56) lobular carcinomas.

A strong association was also present between tumour type and MDM2 expression (p=0.051) and a slight association between tumour type and ER expression (p=0.059). Of the 74 cases that showed high MDM2 expression, 91.9% (68/74) were ductal carcinomas and 8.1% (6/74) were lobular carcinomas.

Of the 46 cases that were ER(-), 97.8% (45/46) were ductal carcinomas and 2.2% (1/47) were lobular carcinomas. In contrast, 85.1% (40/47) of the ER(+) cases were ductal carcinomas and 6.7% (7/47) were lobular carcinomas (**Appendix 11**).

4.6.3 Tumour grade

Significant associations were found between tumour grade and p53 (p = 0.013), Her-2 (p = 0.004) and NFκB (p = 0.051) expressions (**Appendix 12**).

p53 showed a significant increase in expression with higher tumour grades (p = 0.013). High p53 expression was seen in 23.8% of grade 2 tumours and 76.2% of grade 3 tumours. High p53 expression was absent in grade 1 tumours.

Positive Her-2 expression was associated with high grade tumours and varied from 5.4% in grade 1 tumours, 39.3% grade 2 tumours to 55.4% in grade 3 tumours (p=0.004). Conversely, Her-2 expression was negative in 11 out of 14 (78.65%) grade 1 tumours.

Similarly, high NFκB expression was associated with high grade tumours and increased from 12.6% in grade 1 tumours to 39.1% in grade 2 tumours and 48.3% in grade 3 tumours (p=0.051).

4.6.4 Lymphovascular invasion

Significant associations were evident between lymphovascular invasion and both PI3K (p=0.010) and ER expression (p<0.001). High PI3K was seen in 79.7% (63/79) of cases that showed lymphovascular invasion compared to 20.3% (16/79) in cases that were negative for lymphovascular invasion.

ER was positive in 95.7% (45/47) of cases that showed lymphovascular invasion compared to only 4.3% (2/47) expression when lymphovascular invasion was absent (**Appendix 13**).

4.6.5 Lymph node metastasis

There was a significant association between ER expression and nodal involvement (p=0.002). ER expression was positive in 78.7% (37/47) of cases that showed lymph node involvement, compared to 21.3% (10.47) expression in those cases that were negative for nodal involvement (**Appendix 14**).

4.6.6 Distant metastasis

There was a statistically significant association between MDM2 and distant metastasis (p=0.017). High MDM2 expression was seen in 75.7% (56/74) of cases without distant metastases and in 24.3% (18/74) of cases with distant metastasis while MDM2 was negative in 20.0% (1/5) of cases that showed no distant metastasis and in 80.0% (4/5) of cases showed distant metastasis. There was no association between the presence of distant metastases and any of the other markers (**Appendix 15**).

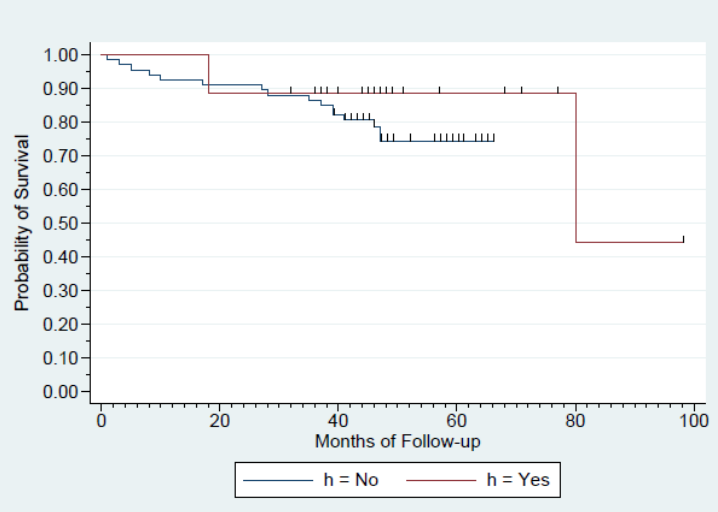
4.6.7 Apoptotic index

There was a statistically significant association between the apoptotic index and PI3K expression (p=0.014) (**Appendix 16**).

4.7 Survival analyses

4.7.1 Survival analysis according to trastuzumab treatment

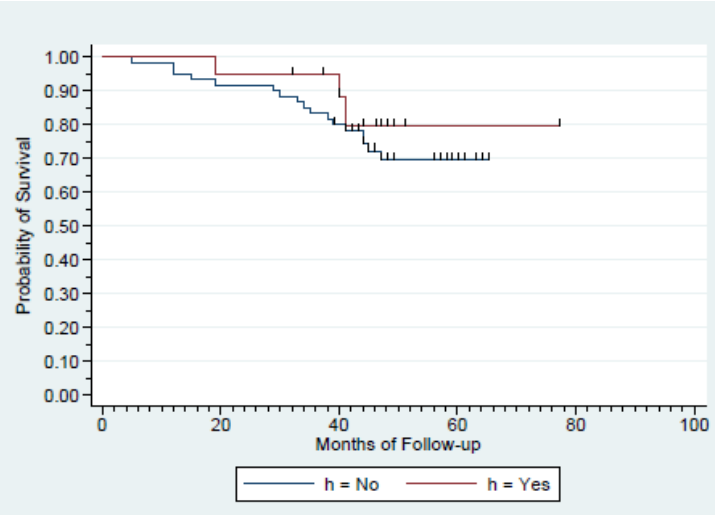
The survival graph (**Figure 14**) shows a survival advantage for cohort C, however this was not statistically significant (p=0.267). The median OS for cohort C was 80 months. Due to the relatively short follow up period and low number of regressions, the median OS for cohort B could not be determined. The 5 year OS for cohort B was 75%, in contrast to 90% for cohort C. The risk of death for cohort C was 2.5 fold less than that of cohort B (HR 0.4; CI 0.1 – 1.5) but this was not significant according to the age adjusted Cox regression analysis (**Appendix 20**).



h= trastuzumab treatment

Figure 14: The Kaplan-Meier graph comparing overall survival between patients in cohort B and cohort C

According to the log rank test there was no statistically significant difference in DFS between cohort B and cohort C (p = 0.429). The Kaplan-Meier graph shows a DFS advantage for cohort C but due to the low number of regressions the median DFS could not be calculated for either of these two groups. The 5 year DFS for cohort B was 70%, in contrast to 80% for cohort C (**Figure 15**). The risk for recurrence for cohort C was 1.7 times less than that of cohort B (HR 0.6; CI 0.2 – 2.1) (**Appendix 22**).



h= trastuzumab treatment

Figure 15: The Kaplan-Meier graph comparing disease free survival between patients in cohort B and cohort C

4.7.2 Survival analysis according to Her-2 expression

According to the log rank test, there was no statistically significant association between Her-2 expression and OS in cohort A. The median OS for Her-2 positive tumours was 80 months. The 5 year OS for Her-2 positive tumours was 79% while that of the Her-2 negative tumours was 75% (**Figure 16**).

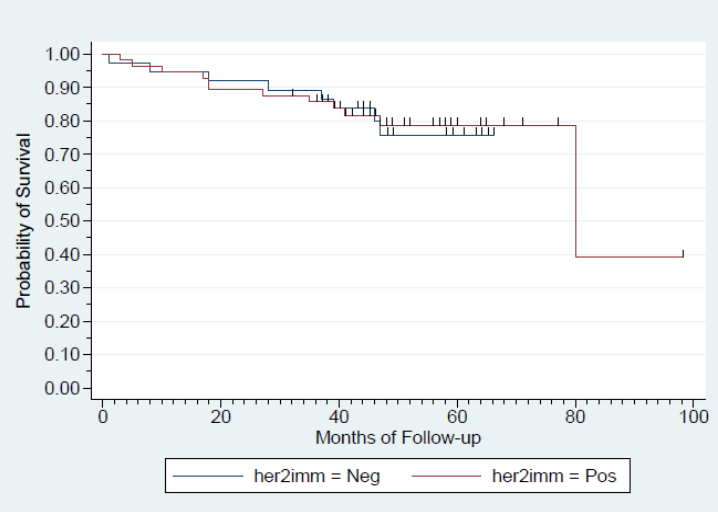


Figure 16: The Kaplan-Meier graph for OS cohort A according to Her-2 expression

The 5 year OS for the Her-2(+) cases in cohort B was 69%, while the OS for the Her-2(-) group was 78% (**Figure 17**). The risk for death in the Her-2(+)

group was 1.2 times higher than the risk in the Her-2(-) group (HR 1.2; CI 0.4 – 3.4) (**Appendix 21**).

The 5 year OS for cohort C was 91% (**Appendix 19**).

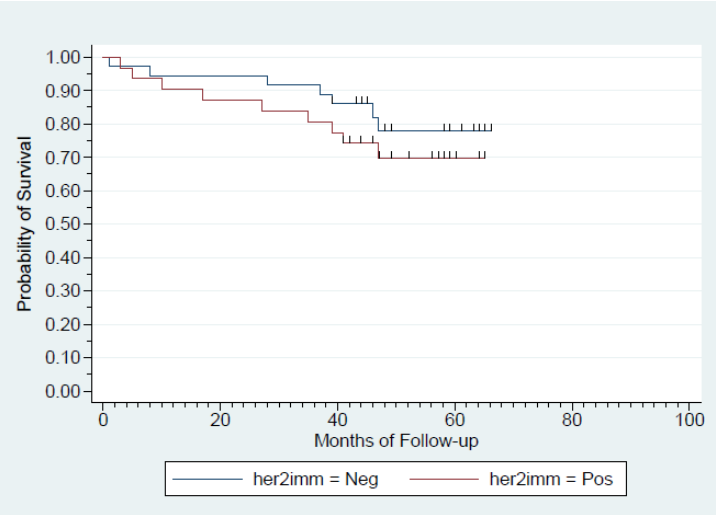


Figure 17: The Kaplan-Meier graph for OS for cohort B according to Her-2 expression

According to the log rank test there was no significant association between DFS and Her-2 expression (p = 0.473) in cohort A. The 5 year DFS for the Her-2 positive group was 77%, while that for the Her-2 negative group was 65% (**Appendix 17**). The risk for recurrence in the Her-2 positive group was 1.4 times lower than in the Her-2 negative group (HR 0.7; CI 0.3 – 1.8) (**Appendix 22**).

There was also no significant association between DFS and Her-2 expression in cohort B (p = 0.709). After 5 years, 74% of the Her-2 positive group and 65% of the Her-2 negative group remained disease free (**Appendix 18**).

The 5 year DFS for cohort C was 80% (**Appendix 19**).

4.7.3 Survival analysis according to PI3K expression

According to the Kaplan-Meier graph for OS (cohort A) (**Figure 18**), patients with tumours that showed no PI3K expression had the best OS, followed by

those with low PI3K expressing tumours. Patients with high PI3K expressing tumours had a median OS of 80 months. The 5 year OS was 75% for patients with tumours showing high PI3K expression, 92% for low PI3K expression and 100% for negative PI3K expression (**Appendix 17**). The risk for death in the PI3K low category was 3.3 fold less when compared to the PI3K high category (HR0.3; CI 0.0 – 2.3). No deaths were recorded in the PI3K negative category (**Appendix 20**).

The median DFS of patients according to PI3K expression could not be calculated due to few recorded deaths. After 5 years 68% of PI3K high category, 85% of PI3K low category and 100% of PI3K negative category patients were disease free (**Appendix 17**). The risk of recurrence for the PI3K negative category was half that of the PI3K high category (HR 0.5; CI 0.1 – 2.2) (**Appendix 22**).

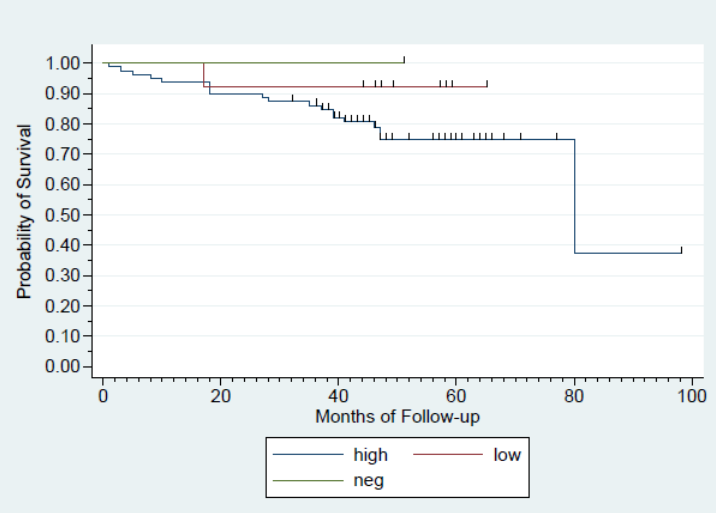


Figure 18: The Kaplan-Meier graph for overall survival for cohort A according to PI3K expression

4.7.4 Survival according to PTEN expression

In cohort A, the median OS for the PTEN high category was 80 months. The 5 year OS for the PTEN high category was 80%, PTEN low category 73% and PTEN negative category 91%. The age adjusted Cox regression analysis shows that cases that showed low PTEN expression had a 1.9 fold increased risk of death compared to cases that showed high PTEN

expression (HR 1.9; CI 0.7 – 5.2) but this was not statistically significant (**Appendix 20**). No deaths were observed in the PTEN low category.

In cohort C, there was a significant association between PTEN expression and OS (p=0.015). The median OS for the group that showed no PTEN expression was 20 months, while the median OS for the group that showed high PTEN was 80 months (**Figure 19**). The 5 year OS for the PTEN high category was 89% and the OS for the PTEN low category was 100%. The OS for the PTEN negative category was 0%, however this category only contained 1 subject (**Appendix 19**). According to the age adjusted Cox regression analysis (data not shown), the risk of death for the PTEN low category was 2 times that of the PTEN high category (HR 2.0; CI 0.1 – 34.7). Due to the limitations of small sample size of cohort C and low number of deaths recorded as a result, these results although significant, may not be reliable.

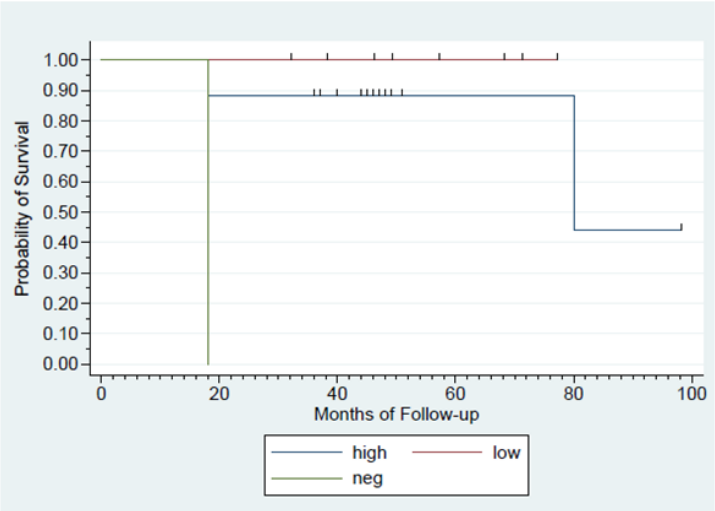


Figure 19: The Kaplan-Meier graph for overall survival in cohort C according to PTEN expression

According to the log rank test, there was no significant difference in DFS in cohort A according to the levels of PTEN expression (p = 0.367). After 5 years 70% of patients in the PTEN high category, 64% in the PTEN low category and 90% in the PTEN negative category were disease free (**Appendix 17**).

There was no significant difference in DFS according to categories of PTEN expression in the cohort B (p = 0.0955). The 5 year DFS for the PTEN high category was 68%, PTEN low category was 56% and the PTEN negative category was 100% (**Appendix 18**).

In cohort C, patients with cancers that were PTEN expression negative had a median DFS of approximately 19 months. After 5 years 80% of the PTEN high category and 100% of the PTEN low category were disease free (**Appendix 19**). The finding of 0% for the PTEN negative category was disregarded due to the presence of only a single case in this category.

4.7.5 Survival analysis according to p-Akt expression

In cohort A, there was a statistically significant association between p-Akt expression and OS (p = 0.046). The median OS for the cohort that did not express Akt was 46 months compared to 80 months for the group that showed high p-Akt expression. The 5 year OS for the p-Akt high category was 83%, the OS for the p-Akt low category was 73% and the OS for the p-Akt negative category was 46% (**Figure 20**). The p-Akt low category had a 2.1 fold increased risk of death compared to the p-Akt high category (HR 2.1; CI 0.8 – 5.9), while the p-Akt negative category had a significant 5.1 fold increased risk for death compared to the p-Akt high category (HR 5.1; CI 1.5 – 17.3) (**Appendix 20**).

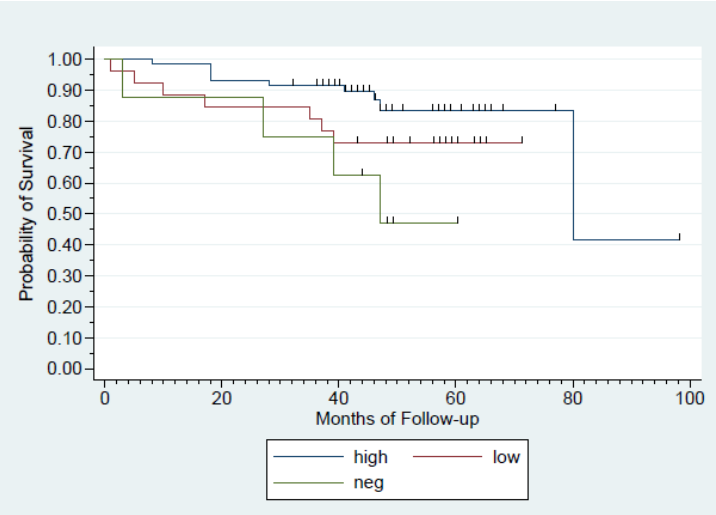


Figure 20: The Kaplan-Meier graph for overall survival for cohort A according to p-Akt expression

4.7.6 Survival analysis according to NFκB expression

Patients with high NFκB expressing cancers showed a median OS of 80 months compared to those patients with low NFκB expressing cancers which had a median OS of 48 months (cohort A). These results were not statistically significant (p=0.169). The risk of death for the group showing low NFκB expression was 3.6 times that of the group showing high NFκB expression (HR 3.6; CI 1.0 – 13.1) (**Appendix 20**).

4.7.7 Survival analysis according to MDM2 expression

In cohort A, patients with cancers that showed high MDM2 expression had a median survival of approximately 80 months. The limited number of deaths recorded in patients with cancers showing low or negative MDM2 expression prevented the determination of median survival in this group. The 5 year OS for the MDM2 negative category was 60%, for the low category 65%, and for the high category 80% (**Appendix 17**).

In cohort B, the 5 year OS for the MDM2 high category was 79%, for the low category 58% and for the negative category 60% (**Appendix 18**). The risk of death in the MDM2 low category was 3.2 fold higher than the risk in the MDM2 high category (HR 3.2; CI 1.1 – 9.7), while the risk of death in the MDM2 negative category was 2.6 fold higher than the MDM2 high category (HR 2.6; CI 0.6 – 12.2) (**Appendix 21**).

No significant association was established between MDM2 expression and DFS using the log rank analysis (p = 0.0871). The median DFS for cases showing no MDM2 expression was 34 months. After 5 years 75% of MDM2 high category, 80% of MDM2 low category and 0% of MDM2 negative category remained disease free (**Figure 21**). The risk for recurrence in the MDM2 negative category showed a 3.5 fold increase compared to the MDM2 high category (HR 3.5; CI 1.01 – 12.1) and was significant (**Appendix 22**).

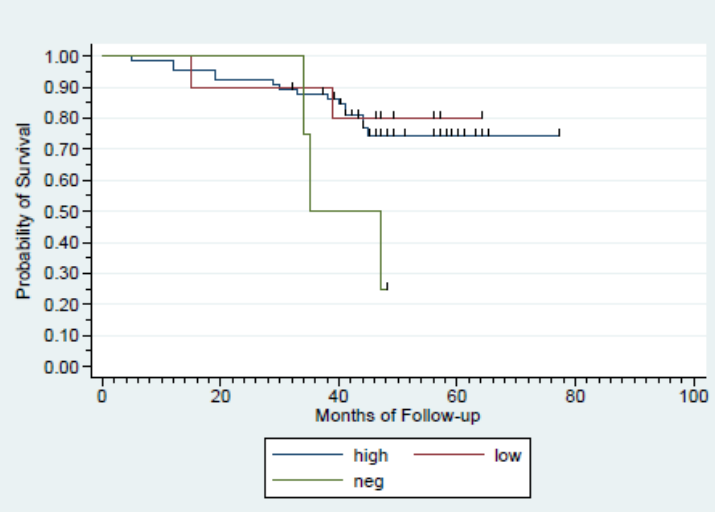


Figure 21: The Kaplan-Meier graph for DFS for cohort A according to MDM2 expression

4.7.8 Survival analysis according to BCL2 expression

There was no statistically significant association between BCL2 expression and OS (p = 0.831) in cohort A. The median OS for the group that showed high BCL2 expression was 80 months. The 5 year OS for BCL2 high category was 80%, the OS for both BCL2 low and negative categories were 75% (**Appendix 17**). There was no significant association between BCL2 expression and DFS (p = 0.952).

4.7.9 Survival analysis according to p53 expression

There was a significant association between p53 expression and OS (p=0.029) in cohort A (**Figure 22**). The median OS for patients with p53 negative cancers was 80 months. The low number of deaths recorded in the p53 low and high categories prevented the calculation of median OS in these

groups. The 5 year OS for the p53 high category was 67%, for the p53 low category 89% and for the p53 negative category 81% (**Figure 23**). Patients with tumours expressing high levels p53 had a 5.5 fold increase in the risk of death compared to those with tumours expressing low levels of p53 and this was significant (HR 0.18; CI 0.0 – 0.9). Patients with tumours negative for p53 expression had a 2.5 fold reduced risk for death, compared to those with tumours expressing high p53 levels (HR 0.4; CI 0.2 – 1.0) (**Appendix 22**).

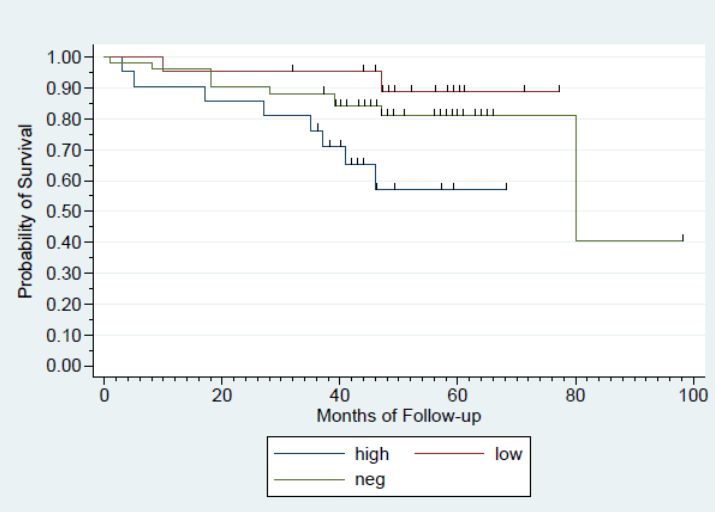


Figure 22: The Kaplan-Meier graph for overall survival according to p53 expression for cohort A

In cohort B, there was a statistically significant association between p53 expression and OS (p=0.001). The median OS for patients with tumours expressing high levels of p53 was 42 months (**Figure 23**). The 5 year OS for the p53 high category was 40%, the low category was 85% and the negative category was 83% (**Appendix 18**). According to the age adjusted Cox regression analysis (**Appendix 21**), patients with tumours expressing high levels of p53, had a 5 fold increased risk of death compared to those with tumours showing low (HR 0.2; CI 0.0 – 0.9) or negative (HR 0.2; CI 0.1 – 0.7) p53 expression. Both these findings were statistically significant.

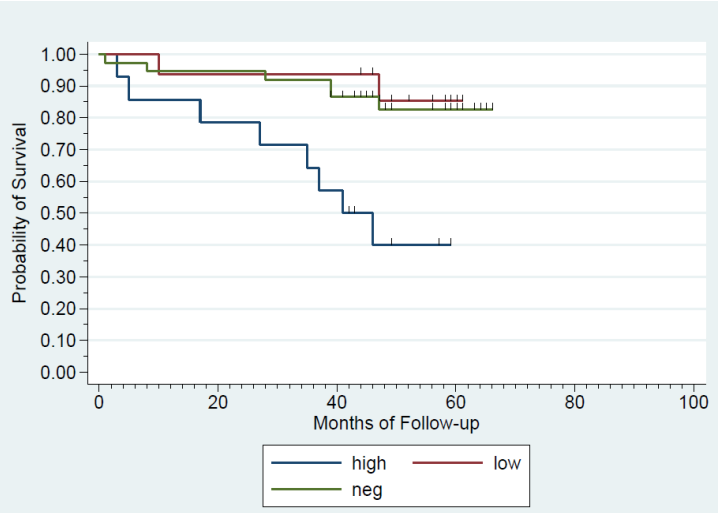


Figure 23: The Kaplan-Meier graph for overall survival according to p53 expression for cohort B

In cohort C, the median OS for the p53 negative category was 80 months. The 5 year OS for both the p53 low and high categories were 100%, while the OS for the p53 negative category was 78% (**Appendix 19**).

As a result of too few regressions in cohort A, the median DFS could not be determined in any of the categories of p53 expression (**Figure 24**). After 5 years 55% of the p53 high category, 79% of the p53 low category and 75% of the p53 negative category remained disease free (**Appendix 17**). The risk for recurrence in the p53 high category was 2.5 times more when compared to the p53 low category (HR 0.4; CI 0.1 – 1.4) and double that of the p53 negative category (HR 0.5; CI 0.2 – 1.3) (**Appendix 22**).

In cohort B, the 5 year DFS for the p53 high category was 0%, for p53 low category 75%, and for the p53 negative category 77% (**Appendix 18**). The risk for recurrence in the p53 high category was 3.3 fold higher compared to the p53 low (HR 0.3; CI 0.1 – 1.1) and negative (HR 0.3; CI 0.1 – 0.8) categories (**Appendix 23**).

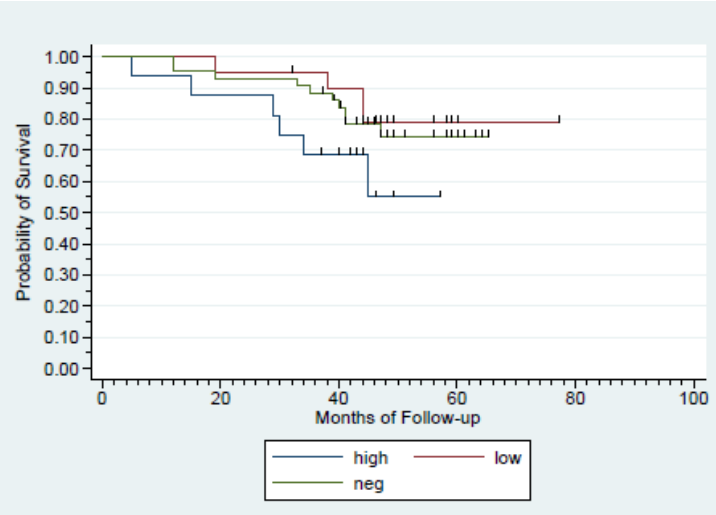


Figure 24: The Kaplan-Meier graph for disease free survival for cohort A according to p53 expression

In cohort C, the 5 year DFS for the p53 low and high categories were 100%, while that of the p53 negative category was 63% (**Appendix 19**). The Cox regression analysis for this cohort was deemed unstable due to the small sample size and too few recurrences.

4.7.10 Survival analysis according to the apoptotic index

The median OS for the apoptotic category (5 <7) in cohort A was 80 months. The 5 year OS for the (<3) category was 77%, for category (3 <5) 72%, for category (5 <7) 85%, and 87% for category (≥7) (**Figure 25**).

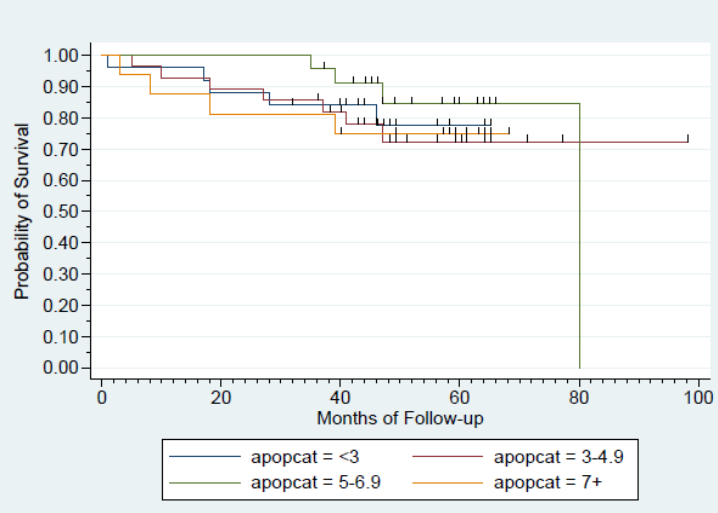


Figure 25: The Kaplan-Meier graph for overall survival for cohort A according to the apoptotic index

The overall survival according to the apoptotic index for cohort B did not reach significance in any of the 4 categories. The 5 year OS for the category (<3) was 75%, for category (3 <5) 65%, for category (5 <7) 80% and for category (≥7) 72% (**Appendix 18**).

The median OS for cohort C according to the apoptotic index was 80 months. The 5 year OS for the category (<3) was 85%, for category (3 <5) 90%, for category (5 <7)100%, and for category (≥7) 80%. This was not statistically significant (**Appendix 19**).

The median DFS for cohort C could not be established for any of the apoptotic categories. The 5 year DFS for apoptotic category (<3) was 85%, for category (3 <5) 67%, for category (5 <7) 64% and for category (≥7) 69% (**Figure 26**) (**Appendix 17**).

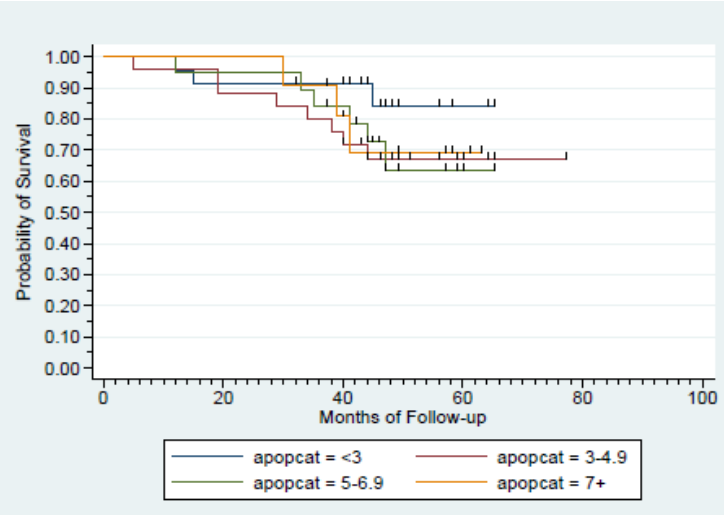


Figure 26: The Kaplan-Meier graph for DFS for cohort A according to the apoptotic index

A median DFS was not reached in any of the categories of apoptosis. The 5 year DFS for apoptotic category (<3) was 80%, for category (3 <5) 63%, for category (5 <7) 65% and for category (≥7) 62% (**Appendix 18**).

In cohort C, the median DFS for apoptotic category (5 <7) was around 41 months. The 5 year DFS for apoptotic category (3 <5) was 75% and 0% for both categories (5 <7) and (≥7). No cases were recorded for the category (<3) (**Appendix 19**).

4.7.11 Survival analysis according to age group

Overall survival for cohort A showed a significant association with age (p=0.028). The median OS for patients in the 45 to 65 year category was 80 months. The 5 year OS for the <45 year group was 65%, for the 45 to 65 year group 76%, and for the ≥65 year group 100% (**Figure 27**). When an age adjusted Cox regression analysis was performed (**Appendix 20**), patients in the 45 to 65 year category had half the risk of death compared to the <45 year group (HR 0.5; CI 0.2 – 1.3). No deaths were recorded in the ≥65 year age group.

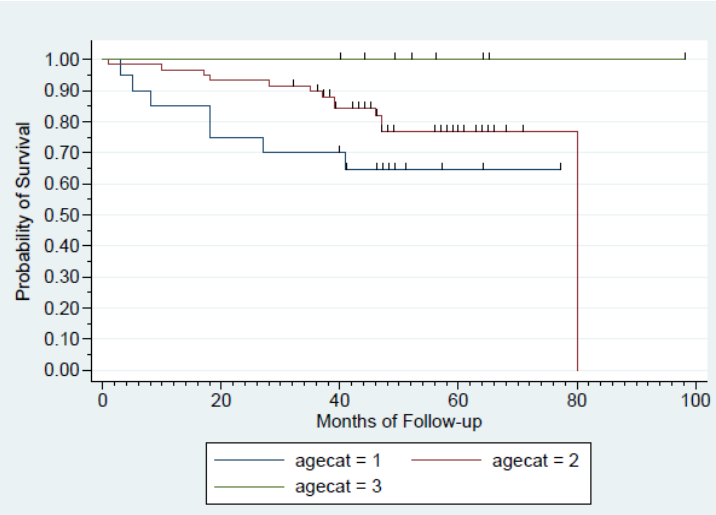


Figure 27: The Kaplan-Meier graph for overall survival for cohort A according to age category*

*agecat 1 : <45 years (n=20), agecat 2 : 45 to 65 years (n=59) and agecat 3 : ≥65 years (n=14).

Log Rank test: (2 degrees of freedom), Chi2 = 7.16, Pr>Chi2 = **0.028,** N=93

The Kaplan-Meier graph for OS according to age category for the cohort of patients who did not receive trastuzumab treatment (**Figure 28**) shows that the overall survival in the age group ≥65 years was better than any other group. Again the <45 year age group showed the worst overall survival of the three groups. The 5 year OS for the <45 year age group was 57%, for the 45 to 65 year old group 74% and for the ≥65 year group 100%. After 5 years 75% of the <45 year age group, 65% of the 45 to 65 year old group and 88% of the ≥65 year group were disease free (**Appendix 18**).

The 5 year OS in cohort C for the age category <45 was 75%, for age category 45 to 65 years 92% and for ≥65 year age group 100%. The 5 year OS in cohort B for the <45 year age group was 57%, for the 45 to 65 year age group 74% and for the ≥65 year old group 100%.

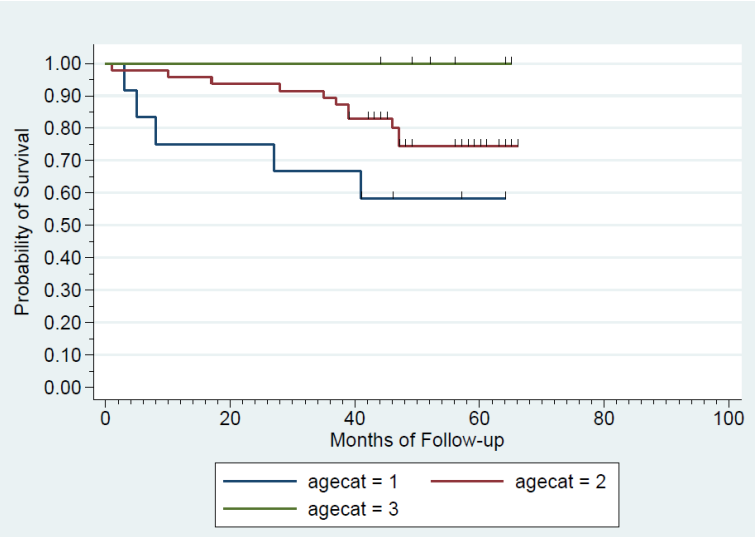


Figure 28: The Kaplan-Meier graph for overall survival according to age category for cohort B

In cohort B, the 5 year DFS for the age group <45 was 75%, for the age group 45 to 65 years 65% and for the ≥65 age group 88%. Due to the low number of recurrences in cohort C, a median DFS could not be determined in any of the age categories. After 5 years 85% the of the <45 year age group was disease free, while no subject was disease free in either the 45 to 65 year old group or the ≥65 year age group (**Appendix 19**).

4.8 Comparing Her-2 evaluation by IHC and CISH on corresponding histopathological and cytopathological specimens

We performed Her-2 CISH analysis on the histology as well as the corresponding FNA (cytology) sample. An IHC score of <2 was regarded as negative, a score of 2 was equivocal and a score of 3 was positive. CISH on the other hand needs a score of >5 to be considered positive for amplification. Comparing Her-2 CISH results performed on cytopathological and histopathological specimens, there was a 100% correlation between Her-2 CISH done on a de-stained, archived FNA (cytopathological) samples and the corresponding histopathological samples cut from paraffin embedded tissue blocks. In addition, there was a 100% correlation between the positive and negative IHC and CISH results. Out of the 6 Her-2 (2+) IHC scores, we found the Her-2 CISH to be amplified in 1 case and not amplified in 5 cases on both histopathological and cytopathological samples.

Table 7: Comparison between Her-2 IHC and Her-2 CISH on corresponding histopathological and cytopathological specimens

Case no.	Her-2 IHC	Her-2 CISH on Histo	Her-2 CISH on Cyto
54	2	6.2	>10
56	3	10	>10
60	0	1.6	1.6
65	2	2.1	3.6
73	2	3.8	3.9
79	2	2.3	2.7
83	3	7.5	10
87	3	6.2	7
103	2	2.1	3.9
231	2	1.5	2.3

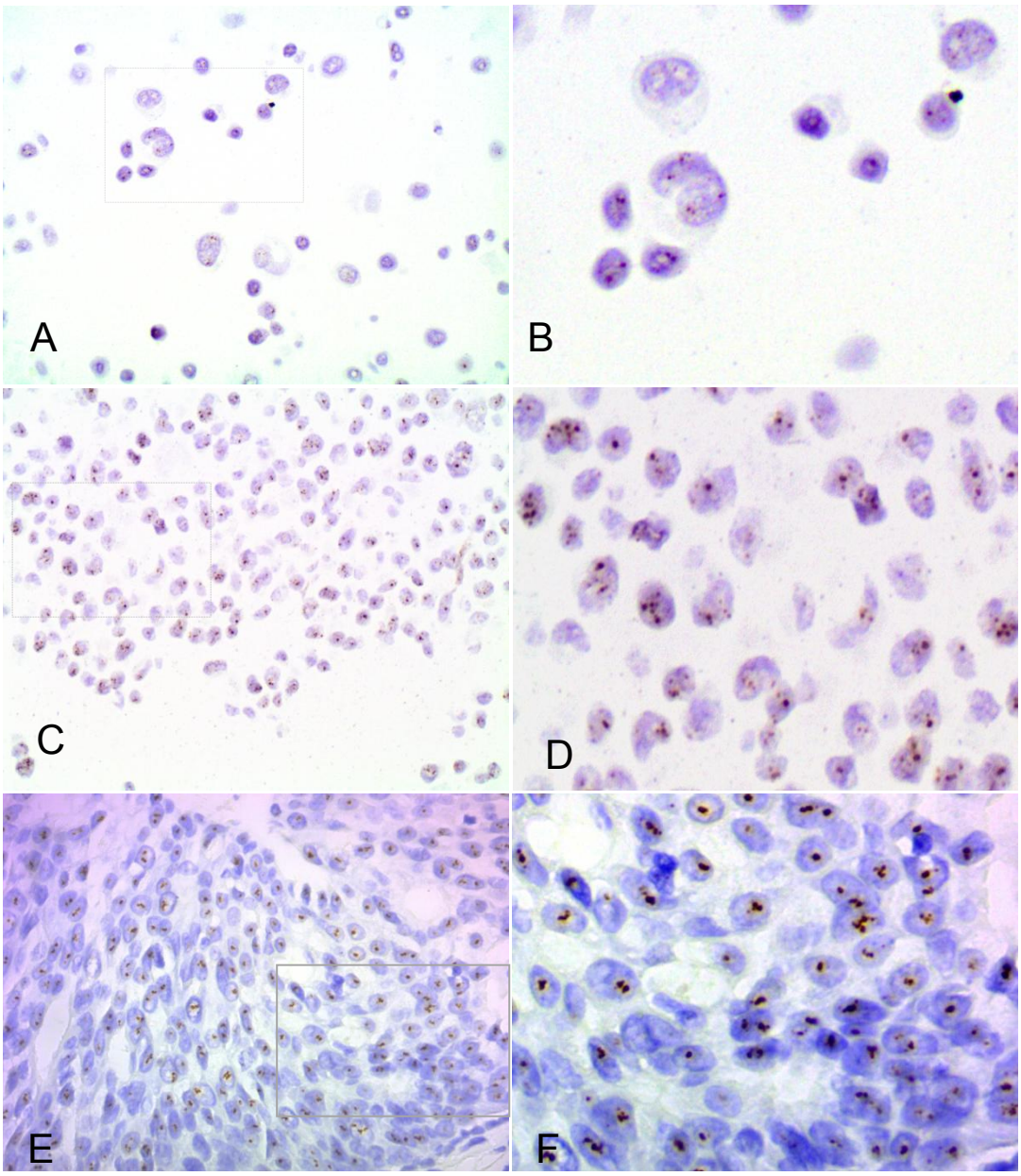


Figure 29: Her-2 CISH control results (**A**) Pos control (cell line) 5-10 copies (original magnification 400x) (**B**) Pos control 5-10 copies (±900x) (**C**) Pos control (cell line) 10+ copies (original magnification 400x) (**D**) Pos control 10+ copies (±900x) (**E**) Positive control histology 10+ copies (original magnification 400x) (**F**) Positive control histology 10+ copies (±900x)

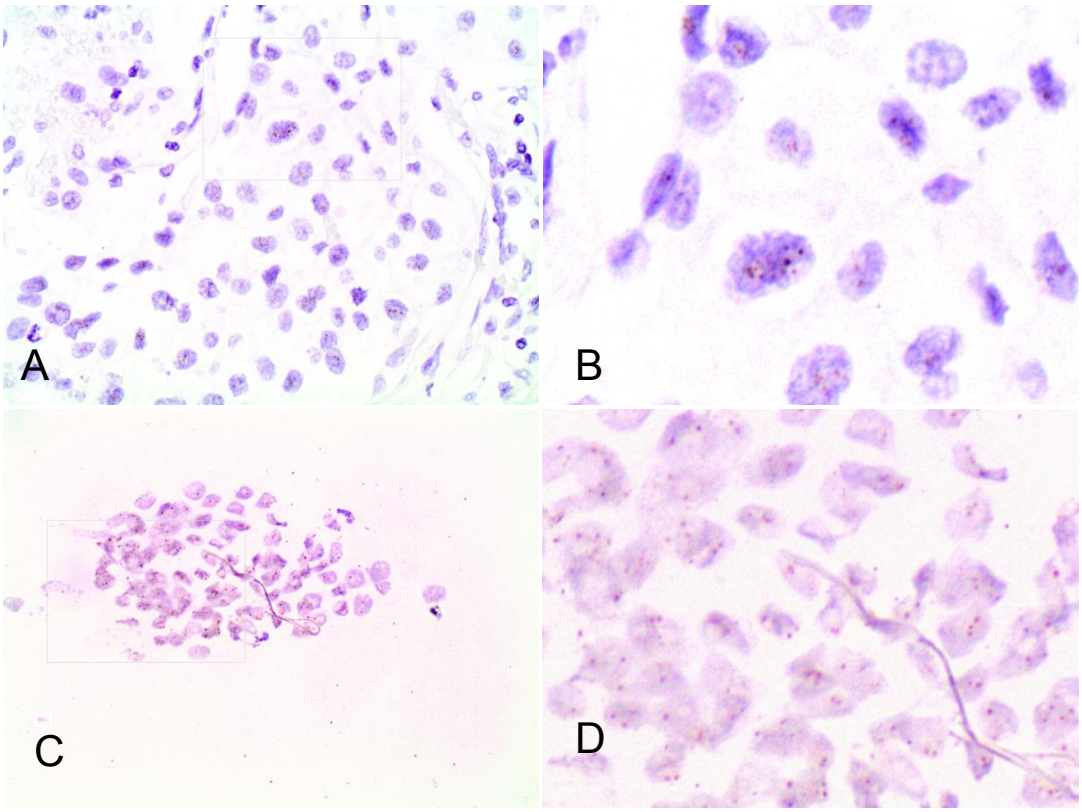


Figure 30: Her-2 CISH results (**A**) 5-10 copies histology (original magnification 200x) (case 87) (**B**) 5-10 copies histology (±900x) (case 87) (**C**) 3-5 copies cytology (original magnification 200x) (case 103) (**D**) 3-5 copies cytology (±900x) (case 103)

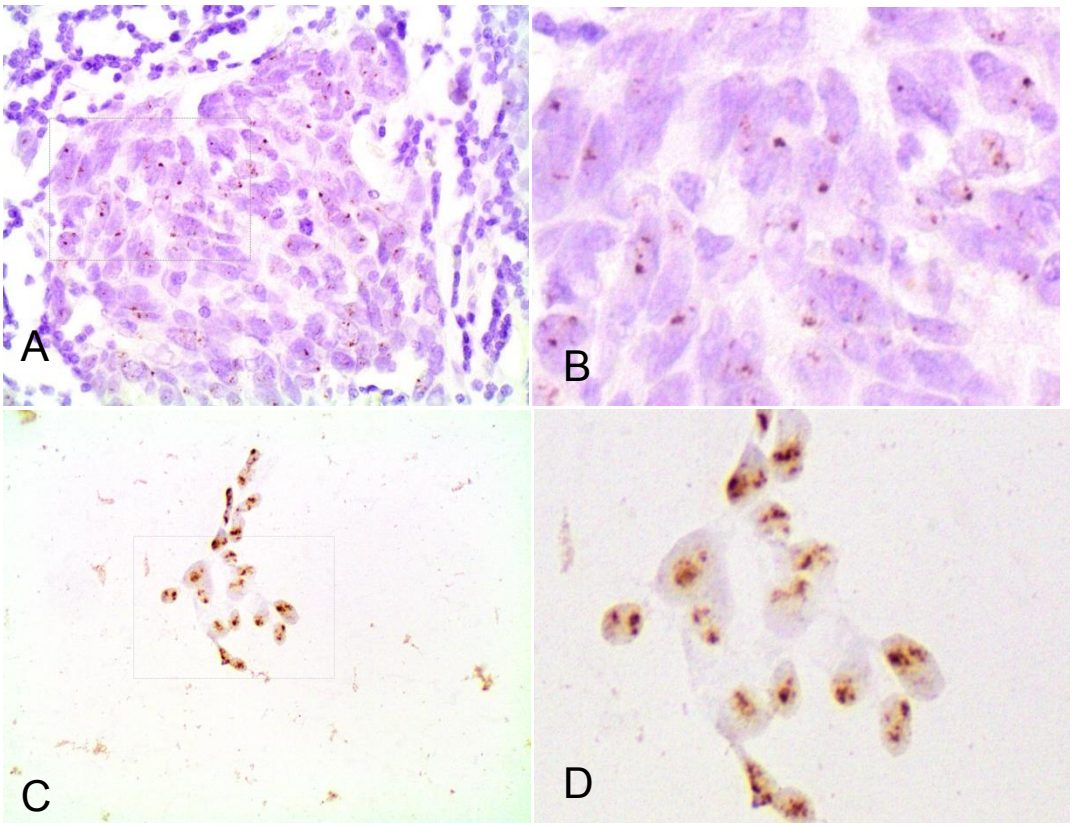


Figure 31: Her-2 CISH results (**A**) 5-10 copies histology (original magnification 200x) (case 83) (**B**) 5-10 copies histology (±900x) (case 83) (**C**) 10+ copies cytology (original magnification 200x) (case 83) (**D**) 10+ copies cytology (±900x) (case 83)

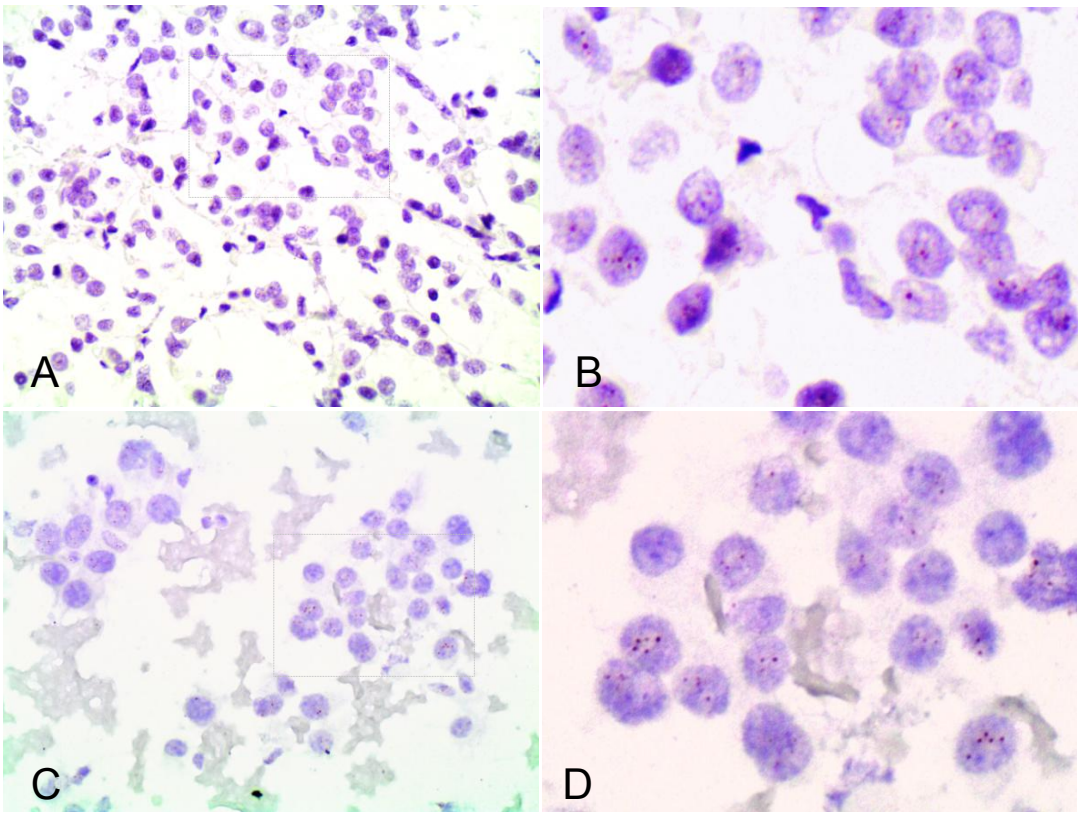


Figure 32: Her-2 CISH results (**A**) 3-5 copies histology (original magnification 200x) (case 73) (**B**) 3-5 copies histology (±900x) (case 73) (**C**) 3-5 copies cytology (original magnification 200x) (case 73) (**D**) 3-5 copies cytology (±900x) (case 73)

CHAPTER 5
DISCUSSION

DISCUSSION

5.1 Introduction

Breast cancer is a multifaceted disease with almost unique protein signature, prognosis and treatment response for each patient even for the same tumour type and grade. These findings underline the importance of being able to tailor cancer treatment based on the genetic profile for each individual. This approach ensures that every patient gets the treatment that would benefit her most, while at the same time minimizing the side effects of the treatment.

Our results reveal some statistically significant associations between clinicopathological features, treatment response and protein expression. Some of the proteins tested show promise as markers for prognostic profiling and clinical management.

5.2 Trastuzumab benefit

In order to assess the benefit derived from trastuzumab treatment, we compared the OS and DFS in cohort C which was given trastuzumab treatment to cohort B that was not given trastuzumab treatment. Although none of the results were statistically significant, it showed that trastuzumab did in fact lower the risk for both death and recurrence in cohort C and improved both the 5 year DFS and OS.

5.3 Her-2

Her-2 was positive in 60.8% of the breast cancers in our study. However this most likely is an overestimation of the frequency of Her-2 positivity in our population as our cases were selected based on their Her-2 status in order to have representation of both Her-2 positive and negative cancers. Slamon *et al.* (1987) reported Her-2 overexpression in 20 – 30% of breast cancers.

In an attempt to try and understand what effect Her-2 signalling has on downstream components of the PI3K/Akt pathway, we compared the expression of Her-2 to that of other components in the pathway. There was no significant association between Her-2 expression and PI3K (p=0. 730), p-

Akt (p=0.803), MDM2 (p=0.197), BCL2 (p=0.109) or ER (p=0.581) expression. The lack of an association between Her-2 and PI3K expression suggests that Her-2 may not be responsible for the increase in PI3K expression. The increase in PI3K expression may be due to the loss of PTEN function or due to an activating mutation within the *PIK3CA* gene itself. Since there was no association between PTEN and PI3K (p=0.812), the likely reason for PI3K activation is an activating mutation in the *PIK3CA* gene. We found no association between Her-2 and p-Akt expression which is in contrast to the findings of Park and Kim (2007) who found significant associations between both nuclear and cytoplasmic p-Akt expression and Her-2 overexpression in a study involving 127 breast cancer cases. Similarly Wu *et al.* (2008) reported a statistically significant association between p-Akt and Her-2 expression in a study involving 141 breast cancer samples. Vestey *et al.* (2005) on the other hand in a study involving 97 breast cancer cases, found no association between p-Akt and Her-2 expression (p=0.545). In the analysis it became evident that different immunohistochemical scoring methods were employed in the various studies, which may explain the discrepant findings. What the abovementioned studies did have in common was the fact that they have used both the staining intensity and proportion in scoring p-Akt expression, while our study used proportion of staining only due to the subjectivity of intensity assessment and the variation in staining batches. Another factor that may have influenced the results was the number of categories and how the scores were re-categorized. There was however a significant and direct association between Her-2 and NFκB expression (p=0.035). NFκB expression was predominantly high in Her-2 positive cases and predominantly negative in Her-2 negative cases. Similarly, Shapochka *et al*. (2013) reported a significant association between Her-2 and NFκB expression. The positive association between Her-2 and NFκB expression and the absence of any significant association between Her-2 and PI3K or p-Akt, suggests that Her-2, in the majority of cases, may be a factor responsible for the activation of NFκB. This finding confirms the findings of Merkhofer *et al*. (2010) who showed that Her-2 activates NFκB through the canonical pathway by inactivating IKKα.

A slight association was present between Her-2 and p53 (p=0.0566). Yamashita *et al*. (2004) found that patients with tumours that were positive for both Her-2 and p53 expression, had a significantly decreased DFS when compared to patients with a larger tumour size and positive lymph node status. It was also reported that patients with both Her-2 and p53 positive tumours showed benefit when treated with high dose FAC (fluorouracil, doxorubicin, cyclophosphamide) (Thor *et al.* 1992).

There was a slight association between Her-2 and PTEN expression (p=0.065) where PTEN negative expression was predominantly observed in Her-2(-) cases and PTEN low and high expression in Her-2(+) cases. The influence of PTEN expression on the prognosis of Her-2 positive breast cancer patients treated with trastuzumab or radiation is somewhat controversial. A few studies have suggested that PTEN contributes towards the efficacy of trastuzumab and radiation therapy and that PTEN loss is associated with resistance to trastuzumab treatment (Nagata *et al.* 2004; Hennessy *et al.* 2005; Esteva *et al.* 2010). Yet another study concluded that PTEN status had no effect on trastuzumab benefit in Her-2 positive patients (Perez *et al.* 2013).

A statistically significant association was found between Her-2 expression and tumour type (p = 0.006). Of the 56 Her-2 positive cases the majority were ductal carcinomas (55) and only one case was a lobular carcinoma. Our observations are supported by the findings of both Porter *et al.* (1991) and Hoff *et al.* (2002), who found Her-2 expression to be diminished or absent in classic invasive lobular carcinoma. Hoff *et al.* (2002) reported statistically significant associations between Her-2 gene amplification and tumour type (p<0.005). They found absence of Her-2 amplification in 99% of invasive lobular carcinomas and low grade ductal carcinomas and suggested that should the Her-2 gene be amplified in any of the two abovementioned conditions, those cases be re-evaluated to exclude a misclassification. Similar results were reported by Porter *et al.* (1991) who found a single case of Her-2 amplification amongst 62 cases of lobular carcinoma. Upon review it was found that it might not have been a true amplification. A larger study

by Ariga *et al.* (2005) involving 390 invasive ductal carcinomas and 128 invasive lobular carcinomas, reported Her-2 amplification in 17% of ductal carcinomas and 6% of lobular carcinomas. Their finding of 6% of lobular carcinomas showing Her-2 amplification was higher than our findings as well as the findings of Hoff *et al.* (2002) and Porter *et al.* (1991).

There was a statistically significant association between Her-2 expression and tumour grade (p=0.004). Her-2 expression was found to be mostly negative in the lower grade tumours but showed a significant increase with increasing tumour grades. Her-2 expression increased from 5.4% in grade 1 tumours to 39.3% in grade 2 tumours and 55.4% in grade 3 tumours. Similar findings were reported by Hoff *et al.* (2002) who found statistically significant associations between *Her-2* gene amplification and tumour grade. Ariga *et al.* (2005) have also reported statistically significant associations between *Her-2* amplification and tumour grade. Hoff *et al.* (2002) found no *Her-2* amplification in 72 out of 73 (99%) low grade ductal carcinomas, confirming a significant association between tumour grade and Her-2 status. Although we found no Her-2 expression in 11 of the 14 (78.6%) grade1 carcinomas, we found Her-2 negative expression more or less evenly distributed between the different tumour grades. It was suggested that the reason for the strong association between Her-2 expression and tumour grade may in part be due to the fact that both the rate of internalization and ligand dissociation from Her-2-containing dimers are very slow, with longer and more intense signalling as a result (Alroy and Yarden 1997; Klapper *et al.* 2000). The effect is more pronounced when Her-2 is overexpressed and leads to enhanced growth responsiveness and ultimately, malignant growth (Rubin and Yarden 2001).

We did not find any association between Her-2 expression and lymphovascular invasion, lymph node involvement or distant metastasis. Widodo *et al.* (2013) showed significant associations between lymphovascular invasion and p53 expression and between lymphovascular invasion and proliferation rate, but also did not find any association between lymphovascular invasion and Her-2 status.

According to the age adjusted Cox regression analysis, Her-2(+) expression was associated with an increased risk of death, when compared to Her-2(-) expression (HR1.2; CI 0.4 – 3.4) in cohort B. The 5 year OS for the Her-2(+) cases in cohort B was 69%, compared to 91% in cohort C. The 5 year OS for the Her-2(-) cases in cohort B was 78%. Although these results were not statistically significant, it shows that trastuzumab treatment is effective in reducing the risk of death in Her-2(+) tumours.

This study was not able to test the effectiveness of trastuzumab treatment on Her-2(-) tumours but results of a recent retrospective study at the University of Michigan showed that patients with Her-2(-) breast cancer that were incorrectly categorized as Her-2(+) and were treated with trastuzumab benefitted just as much as women who were actually Her-2(+). They found Her-2 selectively expressed in cancer stem cells but because of the small numbers it did not reach the Her-2(+) threshold. These tumour stem cells, although present in small numbers stimulate tumour growth and spread and are resistant to current chemo- and radiation treatments. Researchers also found that primary Her-2(-) tumours showed higher Her-2 expression in bone metastases compared to the primary tumour. Trastuzumab was able to selectively target these metastatic cells leading to survival benefit (Boltz 2013).

5.4 PI3K

PI3K showed high expression in 85.4% of our total study population, low expression in 12.7% and was negative in 1.8% of cases. *PIK3CA* mutations or PI3K pathway activation has been associated with a poor OS irrespective of treatment (Jensen *et al.* 2011). PI3K pathway activation has also been associated with resistance to tamoxifen and conventional chemotherapeutic drugs and lack of response to anti-Her-2 drugs. In an attempt to try and understand the role of PI3K in the Her-2/PI3K/Akt pathway we compared PI3K expression to other components in the pathway but did not find any association between PI3K expression and any of the other components in the pathway.

The relationship between PI3K and PTEN is somewhat controversial and many different findings have been reported. In this study, PI3K was positive in 79 cases (85%) of which PTEN was positive in 47 (59.5%), showed low expression in 22 (27.8%) and negative in 10 (12.7%) cases. No case was found to be negative for both PTEN and PI3K expression. Saal *et al.* (2005) found an inverse relationship between PI3K mutations and PTEN loss, where PTEN was positive or functional in 86% of cancers bearing *PIK3CA* mutations. Our findings were not as striking as the findings of Saal *et al.* (2005) although we looked at PI3K immunoexpression and not mutations as they did. Wang *et al.* (2011), on the other hand, did not find *PIK3CA* mutations and PTEN loss to be mutually exclusive as 3 out of 4 patients with PI3K mutations were also found to have no PTEN expression.

No significant association between PI3K and tumour type was observed. A significant association was present between PI3K expression and lymphovascular invasion ($p = 0.012$) and a slight association between PI3K expression and lymph node involvement ($p=0.062$). Of the cases with high PI3K expression, 82.9% had lymphovascular invasion and 17.1% did not have lymphovascular invasion. It could be argued that the PI3K/Akt pathway would most likely be activated in high grade tumours and that lymphovascular invasion would be more frequent in these tumours, but no association was found between PI3K and tumour grade. There is no literature on the association between lymphovascular invasion and PI3K expression, although Saal *et al.* (2005) described a strong relationship between *PIK3CA* mutations, Her-2 expression and ER/PR status in lymph node positive breast cancers. They found *PIK3CA* mutations in 58% of Her-2(+)/ER(+) breast cancers. We found a stronger association between PI3K expression and Her-2(+)/ER(+) cancers. PI3K was overexpressed in 25 of the 27 (92.6%) Her-2(+)/ER(+) cases in this study. Lymphovascular invasion was present in 23 of these 25 (92.0%) Her-2(+)/ER(+)/PI3K high expression cases. The lymphovascular status was not available in the remaining 2 cases. Lymph node involvement was present in 18 (72.0%) of these Her-2(+)/ER(+)/PI3K high expression cases. Our findings not only confirms a

strong association between PI3K expression and the Her-2(+)/ER(+) subtype but also shows a strong association between a subtype Her-2(+)/ER(+)/PI3K high expression cancers and both lymphovascular and lymph node involvement.

5.5 PTEN

We found PTEN loss in only 11.8% of breast cancer cases in our study. Pérez-Tenorio *et al.* (2007) found PTEN loss in 37% of breast cancer cases, while Panigrahi *et al.* (2004) reported PTEN loss in 8% of their study population. Other studies have also reported a lack of consistency in PTEN expression and suggested that it may be due to the use of different antibodies, tissue processing methods, scoring methods and cut-off criteria. Loss of PTEN expression reported in Her-2 positive cancers ranged between 15% and 65% (Nagata *et al.* 2004; Esteva *et al.* 2010; Jensen *et al.* 2011). We observed loss of PTEN expression in 5.3% of Her-2(+) cancers which is lower than the reported findings and suggests that loss of PTEN function in our cohort population played a smaller role in tumour development than reported by other studies.

According to our findings high PI3K expression was found in 90.9% of PTEN negative tumours. Barbareschi *et al.* (2012) on the other hand reported PTEN loss and *PIK3CA* mutations to be present in the same cancer in 13% of their cases.

No association was found between PTEN and Her-2 expression (p=0.065) and only a slight association between PTEN and p-Akt expression (p=0.0534). Contrary to findings by Stemke-Hale *et al.* (2008) who found p-Akt to be present in higher levels in PTEN low than PTEN high cancers, we found high expression of p-Akt in 67.8% of cases that showed high PTEN expression and only in 8.5% of cases that were negative for PTEN expression. These results are surprising since Akt is supposedly activated by loss of PTEN and/or PI3K activation. A study by Panigrahi *et al.* (2004) assessing the role of PTEN in signalling pathways also reported findings of a positive correlation rather than an inverse relationship between PTEN and

Akt. They suggested that ER, rather than PTEN, is the most common regulator of Akt. When comparing ER(+) expression with p-Akt expression, high p-Akt expression was present in (31/47) 66.0% of ER(+) cases, low expression in (12/47) 25.5% and no expression in (4/47) 8.5%, similar to the findings of Panigrahi *et al.* (2004). Esteva *et al*. (2010) found no correlation between PTEN loss/*PI3K* mutations and p-Akt in a study involving 137 Her-2 overexpressing breast cancer cases. Interestingly, Fabi *et al.* (2010) found co-expression of PTEN and p-Akt to be associated with a lower risk for progression and an enhanced treatment response when treated with trastuzumab.

No association was found between PTEN and PI3K, MDM2, BCL2, NFκB or p53 expression. Nor was there any significant association between tumour grade and PTEN expression (p=0.592). By contrast, Rasheed *et al.* (1997) found PTEN expression to be associated with high grade tumours while PTEN was rarely present in low grade tumours. The distribution of PTEN expression was found to be similar across all tumour grades.

Loss of PTEN expression correlated with an increased risk of death in our total study population. Low PTEN expression was associated with a 1.6 fold increased risk of death compared to high PTEN expression. The number of PTEN negative cases and recorded deaths were unfortunately too small to produce a reliable result. A statistically significant association was present between PTEN expression and OS in cohort C, which is consistent with previous studies that found PTEN activity to contribute to the efficacy of trastuzumab (Nagata *et al.* 2004; Hennessy *et al.* 2005; Esteva *et al.* 2010).

High PTEN expression was associated with a decreased risk for progression in cohort C. Our findings support the findings of those studies who found PTEN to be a valuable prognostic marker and powerful predictor of trastuzumab resistance (Nagata *et al.* 2004; Esteva *et al.* 2010). A recent study looking at activating mutations in *PIK3CA* as well as Her-2 and PTEN status in patients with Her-2 amplified breast cancer that had recurrent disease after adjuvant trastuzumab treatment or showed disease progression

while on trastuzumab treatment, found PTEN to be absent or significantly diminished in 59% of cases and activating mutations in the *PIK3CA* gene in 29% of cases. Her-2 expression was lost in less than 5% of cases (Chandarlapaty *et al.* 2011). Nagata *et al.* (2004) found that PI3K inhibitors were able to overcome trastuzumab resistance caused by PTEN-loss and subsequent PI3K/Akt pathway activation.

5.6 p-Akt

Since Akt seems to be a key player in the PI3K/Akt pathway, we compared p-Akt expression with the expression of both upstream and downstream components in this pathway. Phosphorylated Akt (p-Akt) was found to be overexpressed in (59/93) 63.4% of breast cancers in our study.

No association was found between p-Akt and Her-2 or ER expression. Our findings differ from that of Park and Kim (2007) who reported a significant association between p-Akt and Her-2 overexpression. They also reported a significant association between nuclear p-Akt expression and ER expression. Stemke-Hale *et al.* (2008) found *Akt* mutations to be restricted to ER positive cases and found no *Akt* mutations in Her-2 positive breast cancer. Admittedly, they looked at *Akt* mutations and not protein expression.

There was no significant association between p-Akt and PI3K expression and only a slight association between p-Akt and PTEN expression. Fabi *et al*. (2010) reported significant associations between both p-Akt and PI3K and between p-Akt and PTEN expression. Other studies found PTEN expression to be lower when the levels of p-Akt were increased and the PI3K/Akt pathway activated (Kang *et al.* 2005; Stemke-Hale *et al.* 2008). Since there was no association between Her-2 and p-Akt expression or PI3K and p-Akt expression, it is possible that p-Akt was activated by an activating mutation within the *Akt* gene or by a mechanism other than the Her-2 pathway. *Akt* mutations appear to be rare having been found in only 1.4% of cases in a large breast cancer study (Stemke-Hale *et al.* 2008). A further possibility perhaps could be that PTEN, in an attempt to overcome or compensate for PI3K activity, was up-regulated by a negative control mechanism in response

to the increase in PIP3 concentration, which is the product of PI3K activity. PTEN response was not effective enough to completely block PI3K signalling.

We did find significant associations between p-Akt and NFκB expression (p=0.014) and between p-Akt and MDM2 expression (p=0.040). Similarly, deGraffenreid et al. (2004) reported a positive association between p-Akt and NFκB expression. They suggested that NFκB may be the reason for tamoxifen resistance in p-Akt positive breast cancers due to its role in resistance to apoptosis and induction of angiogenesis and invasion. They have suggested that inhibition of NFκB may be an effective strategy in reversing tamoxifen resistance in these cases. A study by Schmitz et al. (2006) involving 121 breast cancer cases reported a significant association between p-Akt and its downstream effector, MDM2 as well. Breast cancers expressing both p-Akt and MDM2 had a significantly worse prognosis compared to cancers expressing p-Akt alone. The prognosis was even worse when p53 in addition, was negative.

We found no association between p-Akt and BCL2 or p53 expression, nor did we find any significant association between p-Akt expression and tumour type, lymphovascular invasion, lymph node involvement, distant metastasis or the apoptotic index. There was however a slight association between p-Akt expression and tumour grade (p=0.066).

Patients with tumours showing low p-Akt expression had a 2.1 fold increased risk of death compared to those that showed high p-Akt expression (HR 2.1; CI 0.8 – 5.9), while the group that showed no p-Akt expression had a significant 5.1 fold increased risk of death compared to those showing high p-Akt expression (HR 5.1; CI 1.5 – 17.3). These results shows p-Akt expression to be an independent prognostic factor but were unexpected as p-Akt is supposedly associated with high grade tumours, treatment resistance and poor outcome (Sun *et al.* 2001; Cicenas *et al.* 2005; Schmitz *et al.* 2006). Looking at the findings of more recent studies it appears that the earlier perceptions of the role of p-Akt in survival has somewhat changed as

reflected in recent findings. According to Yang *et al.* (2010), p-Akt significantly improves DFS in node positive breast cancers treated with paclitaxel. Aleskandarany *et al.* (2011), on the other hand did not find p-Akt to be a prognostic marker but a study by Fohlin *et al.* (2013) found Akt2 expression to be associated with a good long term prognosis in ER(+) breast cancer. We found p-Akt to be a prognostic indicator irrespective of ER status. p-Akt expression is associated with a better outcome.

5.7 BCL2

In our study population, BCL2 was positive in 50.5% of cases which is somewhat lower than the findings of Dawson *et al.* (2010), who reported positive BCL2 expression of 73% of breast cancers. They found ER(+) expression in 86% and Her-2(+) expression in 8% of these cases, while we found ER(+) expression in 63.8% and Her-2(+) expression in 55.3% of BCL2 positive cases. We saw a significant association between BCL2 and ER expression (p=0.022) but did not find any significant association between any of the other upstream components and BCL2. Our findings are supported by the findings of Silvestrini *et al.* (1994) who reported an association between BCL2 expression and low grade, slowly proliferating, ER(+) tumours.

Since there was no significant association between BCL2 and p-Akt, it is possible that BCL2may be activated in a p-Akt independent manner, including an activating mutation within the *BCL2* gene or another independent mechanism.

In addition, we found no significant association between BCL2 expression and any of the clinicopathological variables. We did not find a significant association between OS or DFS and BCL2 expression. Dawson *et al.* (2010) found BCL2 to be a favourable, independent, prognostic marker in early stage breast cancer. They have also reported a strong association between the intensity of BCL2 IHC staining and OS. Women, whose cancers showed the most intense BCL2 staining, had the best OS. We have opted not to use staining intensity but rather extent of staining to score protein expression

because staining intensity is subjective and is influenced by many factors such as tissue fixation, detection method and batch variation.

5.8 MDM2

MDM2 showed high expression in 79.6% of cases, low expression in 15.1% and no expression in 5.4% of cases. We saw a significant association between MDM2 and p-Akt expression (p=0.040) but there was no association between MDM2 and any of the other upstream components. These results confirms the role of Akt in MDM2 activation and are consistent with those previous studies that found associations between Akt and MDM2 activation and is in keeping with the Akt signalling hypothesis (Wang *et al.* 2011).

There was no association between MDM2 and p53 expression. MDM2 showed high expression in 78.4% of cases in which p53 was negative and is in keeping with normal p53 expression and negative control exerted by MDM2. In addition, MDM2 showed high expression in 71.4% of cases in which p53 was highly expressed. This may be explained by the presence of dysfunctional MDM2 molecules that are unable to bind and label p53 for degradation or alternatively, p53 has undergone post-translational modification and has been stabilized as a result of oncogenic stress, leading to an increase in its half life and making it resistant to MDM2-mediated ubiquitination and degradation as MDM2 is unable to ubiquitinate p53 in its phosphorylated state (Meek 1994; Goga *et al.* 1995; Hirao *et al.* 2000; Ghosh and Bose 2005; Francoz *et al.* 2006).

There was also a slight association between MDM2 expression and tumour type (p=0.051). MDM2 expression was associated with ductal rather than lobular carcinomas.

A statistically significant association was found between MDM2 expression and absence of distant metastasis (p=0.017). The highest expression of MDM2 was found in cases that showed no distant metastasis. This may be an indication of a protective role for MDM2 against distant metastasis and is

also in keeping with the findings of Hori et al. (2002) who associated MDM2 expression with favourable prognostic indicators. The role for MDM2 as prognostic indicator is controversial as it has been associated with both favourable and poor prognoses (Onel and Cordon-Cardo 2004).

A positive association was present between MDM2 expression and OS. According to the age adjusted Cox regression analysis, low MDM2 expression was associated with a 2.4 fold increase in risk of dying compared to high MDM2 expression, while no MDM2 expression, was associated with a 2.8 fold increased risk of dying compared to high MDM2 expression. Cases that showed high MDM2 expression showed a noticeable survival advantage to those cases that showed only low or no MDM2 expression. These findings are in contrast with the findings from other studies which found MDM2 expression to be associated with a poor clinical outcome (Jiang *et al.* 1997; Wang *et al.* 2005; Schmitz *et al.* 2006; Turbin *et al.* 2006). A few studies have reported a positive relationship between MDM2 expression and clinical outcome in both breast cancers and melanomas (Hori *et al.* 2002; Polsky *et al.* 2002). Polsky*et al.* (2002) found MDM2 to be an independent predictor of survival in primary malignant melanoma and reported statistically significant associations between MDM2 overexpression and both DFS and. In support of our findings, a study by Hori *et al.* (2002) reported a positive association between MDM2 overexpression and favourable prognostic parameters such as low grade tumours and the absence of lymph node involvement.

5.9 NFκB

NFκB expression was found to be linked to high grade carcinomas, with high NFκB expression present in 12.6% of grade 1 tumours, 39.1% in grade 2 tumours and 48.3% in grade 3 tumours ($p = 0.051$). Our findings are supported by the findings of Ozbek *et al.* (2012) who reported an association between NFκB expression and tumour grade in renal cell carcinomas. Tannahill *et al.* (2009) on the other hand, did not find any association between cytoplasmic or nuclear NFκB expression (both total and phosphorylated) and tumour grade. We found strong cytoplasmic staining for NFκB but very little evidence of nuclear staining in our study. Where nuclear

staining was observed, it was present in less than 3% of the tumour cells. The antibody datasheet provided by the manufacturer, indicated that cytoplasmic staining should be expected. We contacted the manufacturer and they indicated that the data sheet should in fact state that both nuclear and cytoplasmic NFκB staining is expected. We subsequently found a paper by Miyamoto *et al.* (1994), with very similar findings. They metabolically labelled mature B cells and fragmented them into nuclear and cytoplasmic pools before immuno-precipitating it with antibodies specific for NFκB and IκB. They detected around 10% of the total NFκB protein in the nucleus of unstimulated, mature B cells, while the rest of the NFκB proteins (around 90%) was found sequestered by IκB's in the cytoplasm. It was the findings of Cogswell *et al.* (2000) however that provided an explanation for our findings. They demonstrated the presence of p52, p50 and c-Rel subunits in the nucleus of almost all breast tumour cases, while the p65 subunit, which happens to be the subunit our NFκB antibody was directed against, was virtually undetected. Their findings explain why we could not demonstrate significant nuclear staining for NFκB which in our case was directed specifically at the p65 subunit of the classic p50/p65, NFκB heterodimer. They suggested that a mechanism other than the typical mechanism of NFκB activation may be involved. They also demonstrated the presence of p65 in the nucleus of cell lines but found that it differs from p65 expression in breast tumours. They suggested that difference in p65 behaviour may be due to the extracellular environment which is the culture medium in cell lines or even the number of genetic replications of the immortal cell line (Cogswell *et al.* 2000). This fact would, to an extent, explain the discrepancy in the findings between different studies. If the extracellular environment did in fact have an influence on p65 activation, one could postulate that p65 is activated directly or indirectly by an extracellular stimulus that is also present in the culture medium. The reason for the increase in cytoplasmic NFκB expression with increase in tumour grade remains unclear. We found no significant association between NFκB expression and lymphovascular invasion.

We found statistically significant associations between NFκB and Her-2 expression ($p = 0.035$) and between p-Akt and NFκB expression ($p=0.014$).

5.10 p53

A statistically significant association was found between p53 expression and tumour grade (p = 0.013). High p53 expression increased from 0% in grade1 tumours to 13.9% in grade 2 tumours and 37.2% in grade 3 tumours. This confirms the findings of other studies who reported significant associations between p53 expression and tumour grade (Ostrowski *et al.* 1991; Sirvent *et al.* 1995). Since wild type p53 is usually not detected by immunohistochemistry at normal levels of expression due to its short half life and negative regulation by MDM2, an increase in p53 expression towards the higher tumour grades suggests an increase in the frequency of *p53* mutations or alternatively, an increase in p53 activity due to wild type p53 being stabilized as a result of an increase in oncogenic stress signals. Similarly, in a large study, Olivier *et al.* (2006) found *p53* mutations to be more frequent in aggressive phenotypes such as high grade, large size, lymph node positive and low hormone content cancers. These results confirm our findings.

A slight association was present between p53 and Her-2 expression (p=0.057). Camerini *et al*. (2011) found no association between p53 and Her-2 expression but reported an association between p53 and tumour stage.

Overexpression of nuclear p53 has been associated with a poor prognosis. Sirvent *et al.* (2001) reported a statistically significant association between p53 expression and shorter DFS and OS. Similarly, we found a statistically significant association between p53 expression and OS (p = 0.029). Patients with tumours expressing low levels p53 had a 5.5 fold reduction in risk for dying compared to those with tumours expressing high levels of p53. Patients with tumours negative for p53 expression had a 2.5 fold reduced risk of dying, compared to those with tumours expressing high p53 levels (HR 0.4; CI 0.2 – 1.0). These findings confirm the findings of Sirvent *et al.* (2001) who reported a significant association between nuclear p53 expression and poor prognosis.

5.11 Oestrogen receptor (ER)

A statistically significant association was evident between ER and BCL2 expression (p=0.022) and ER expression and lymphovascular invasion (p< 0.001). ER expression was negative in all cases without lymphovascular invasion and positive in 64.3% of cases with lymphovascular invasion.

In addition, a statistically significant association was found between ER expression and lymph node involvement (p=0.002). These findings are in contrast to those of Sofi *et al*. (2012) who did not find any correlation between ER/PR expression and lymph node involvement in breast carcinomas.

ER expression was more frequent in lobular compared to ductal carcinomas.

5.12 Age

A statistically significant association was present between patient age and Her-2 expression (p=0,044). This is consistent with the findings of at least one other study linking Her-2(+) tumours with a younger age and a possible contributing factor to the more aggressive tumour behaviour and prognosis in the younger population (Anders *et al.* 2011).

There was no other association found between age and protein expression.

p53, although not statistically significant, showed a decrease in expression with increase in age, a finding that is supported by the findings of another study (Stark *et al.* 2003). To try and find an explanation for this observation, we looked at the various reasons for p53 immunoexpression. p53 expression could be linked to gain of function mutations in the *p53* gene which could be further enhanced by the inability of mutant p53 to induce translation of MDM2, the negative regulator of p53. Another possible reason for the accumulation of p53 could be that wild-type p53 is activated (phosphorylated) and stabilized under conditions of stress which include oncogenic stress. The half life of p53 is increased under these conditions of stress. Negative regulation by MDM2 is inhibited due to the fact that MDM2

is degraded under these conditions. In addition, MDM2 is only able to degrade unphosphorylated p53, which further enhances the level of wild type p53 (Meek 1994; Stott *et al.* 1998; Zhang *et al.* 1998). Since it is impossible to distinguish between mutated and wild type p53 looking at protein expression, it is possible that some of the p53 expression observed was actually wild-type p53 and not the product of mutated *p53*. It has been shown that the efficiency of the immune system as well as DNA repair diminish with age (Vyjayanti and Rao 2006; Woodland and Blackman 2006; Yamada *et al.* 2006). One could postulate that the accumulation of DNA mutations over a life time in the *p53* gene itself or the proteins involved in its activation in response to cellular stress or DNA damage, results in a decline in p53 activity and/or efficiency with increasing age and could possibly be a reason why most cancers arise in the last quarter of life and why we see diminished p53 expression with age. Feng *et al.* (2007) tested this theory in a mouse model where mice were irradiated with gamma rays at ages of 6 months, 20 months, 24 months and 28 months. Both the levels of p53 response to radiation as well as the level of p53 phosphorylation were lower in the older mice. They also found lower levels of p53 mediated apoptosis in the spleens of older mice compared to the younger mice. The decline in p53 activity correlated progressively with an increase in age. They also found a decline in total RNA as well as protein levels of ATM kinase in older mice. ATM kinase is responsible for stabilization and activation of p53 following DNA damage or cellular stress and could be a possible mechanism by which p53 activity is diminished in old age. This decline in activation was only seen in the p53 pathway. Activation of the Akt and mTOR pathways did not show a decline with an increase in age (Feng Z *et al.* 2007).

Olivier *et al.* (2006) in a comprehensive study reported a significant association between *p53* mutations and age with a noticeable decline in mutations in the cancers of women older than 60 years of age (Olivier M *et al.* 2006). Although this could be a reason for the decrease in p53 expression in older patients and confirms an association between *p53* mutation and expression, the reason for the decline in *p53* mutations with increasing age is not clear.

A strong association was found between age and overall survival (OS) across all three cohorts, which was statistically significant in cohort A, but not in the two treatment dependent cohorts due to the limitation of sample size. In all three cohorts, the <45 year group had the worst median overall survival followed by the 45 <65 year group. The ≥65 year age group showed the best median OS across all cohorts.

These findings suggest an association between age and survival and also prove age to be a strong prognostic indicator in the premenopausal population. This however is not a new finding but confirms that although the incidence of cancer is lower in the premenopausal population, carcinomas in this population tend to be more aggressive possibly as a result of oestrogen stimulation. In keeping with this fact our study found that postmenopausal women and in particular those who had an oophorectomy, also had the best overall and disease free survival rates in the group that received trastuzumab therapy. Looking at these findings, the use of anti-oestrogens or oestrogen-deprivation therapy to either block the effect of oestrogen on these cancers or diminish the level of circulating oestrogen, seems like an effective treatment option. Unfortunately, as mentioned before, Her-2 overexpression has been associated with resistance to anti-oestrogens, and in particular to tamoxifen, one of the most popular anti-oestrogens (Dowsett 2001; Lipton *et al.* 2002; Johnston 2006). More than one study has suggested that anti-oestrogen therapy may actually have a detrimental effect on Her-2(+) cancers (Borg A *et al.* 1994; Lohrisch and Piccart 2001). Results of a few *in vitro* studies have shown that trastuzumab may actually be able to enhance the growth inhibitory effect of anti-oestrogens and may even reverse the resistance of Her-2(+) cancers to anti-oestrogens like tamoxifen and suggested that the combination of trastuzumab and an anti-oestrogen may actually be a powerful treatment approach (Witters *et al.* 1997; Kunisue *et al.* 2000; Argiris *et al.* 2004). The unfortunate reality is that unless trastuzumab becomes more affordable, only a few of Her-2(+) patients will be able to afford this benefit. One can only hope that with the original patent for trastuzumab expiring in 2014, similar drugs will become more affordable and available to patients with breast cancer and not just a privileged few.

5.13 Her-2 ICH and CISH on matched cytopathology and histopathology samples

We assessed the newly available brightfield chromogenic in-situ hybridization (CISH) method for effectiveness and suitability as a testing method for Her-2 status on both formalin fixed paraffin embedded tissue and archived, Pap-stained, FNA samples. There was 80% concordance between Her-2 IHC and Her-2 CISH results. Inaccuracy with both IHC and ISH remains a major issue worldwide. The accuracy of Her-2 testing is crucial as a false positive result will lead to unnecessary and wasteful expenditure. A false negative result would deprive a patient of trastuzumab therapy. In an attempt to improve the accuracy of Her-2 testing in routine practice, the American Society of Clinical Oncology together with the College of American Pathologists (2007) published guidelines for Her-2 testing and analysis. They however could not clearly demonstrate the superiority of either the IHC or ISH method for Her-2 testing, but found the IHC method to be more dependent on pre-analytical variables than the ISH method, while the IHC method proved more reliable in fresh frozen tissue than the ISH method (Wolff AC *et al.* 2007). They identified a list of variables as possible sources of Her-2 testing variation.

There was 100% concordance of Her-2 CISH results on corresponding cytopathology and histopathology samples. These findings serve as evidence that Her-2 CISH can be performed not only on paraffin embedded tissue but also on stained and archived FNA samples that have been de-stained for CISH. The advantage of being able to use stained FNA samples over unstained FNA samples is that it allows you to assess the material beforehand to make sure that you do in fact have adequate material and mark areas of interest before de-staining it. We have also noticed a slightly higher score in the cytology samples which we have attributed to the possibility of antigen masking in formalin fixed paraffin embedded tissues. The fact that some nuclei are sliced when tissue is sectioned as part of the histology preparation may also explain the slightly lower average score. We also observed that in cases where the nuclear detail on the FNA sample was partly obscured mainly due to drying artefact, the gene amplification could

still be assessed but it was more difficult to identify nuclear borders. Liquid based cytology preparations should be an ideal method for CISH analysis as the material is normally very well preserved and concentrated in a small area on the slide. The smaller area allows the use of a smaller amount of probe which makes it more cost effective. The advantages of this method are that there is no need for a special dark room or fluorescent microscope and the signals will not fade as is the case with a fluorescent signal.

CHAPTER 6
CONCLUSION AND FUTURE RESEARCH

In spite of the limitations of this study on breast cancer, there are interesting conclusions with potential clinical significance.

Her-2(+) immunoexpression was associated with ductal carcinomas and high tumour grade.

High PI3K expression was associated with lymphovascular invasion and lymph node metastasis.

This study also found a strong association between a specific subgroup of breast cancer [Her-2(+)/ER(+)/PI3K (high expression)] and both lymphovascular involvement and lymph node metastasis.

High p-Akt expression was an indicator for good prognosis. The absence of p-Akt expression was associated with five fold increase in the risk of death compared to cases showing high p-Akt expression.

High MDM2 expression was associated with an absence of distant metastasis and reduced risk of death (3.2 fold decrease in risk of death compared to low MDM2 expression).

One of the shortcomings of this project was the small sample size, especially the cohort that received trastuzumab treatment, which in some instances proved too small to produce a reliable or significant result.

Since this study focused on the immunohistochemical detection of proteins, it would be important to confirm and correlate these results with molecular genetic analyses.

In addition, the chromogenic in-situ hybridization (CISH) technique for determining Her-2 amplification can be done successfully on archived, Pap-stained, FNA samples.

Based on these findings we propose that liquid based cytological preparations would be ideal for CISH analysis as the material is normally very well preserved and concentrated in a small area on the slide. The smaller area allows the use of a small amount of probe which makes it more cost effective.

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APPENDICES

Appendix 1: Correlation or Her-2 results with protein expression

Variable	N	Her-2 pos	Her-2 neg	p-value
PI3K high	79	48 (60.8%)	31 (39.2%)	0.730
PI3K low	13	7 (53.8%)	6 (46.2%)	
PI3K neg	1	1 (100%)	0 (0%)	
PTEN high	55	35 (63.6%)	20 (36.4%)	0.065
PTEN low	27	18 (66.7%)	9 (33.3%)	
PTEN neg	11	3 (27.3%)	8 (72.7%)	
p-Akt high	59	37 (62.7%)	22 (37.3%)	0.803
p-Akt low	26	14 (53.8%)	12 (46.2%)	
p-Akt neg	8	5 (62.5%)	3 (37.5%)	
NFκB high	87	55 (63.2%)	32 (36.8%)	**0.035**
NFκB low	6	1 (16.7%)	5 (83.3%)	
MDM2 high	74	46 (62.2%)	28 (37.8%)	0.197
MDM2 low	14	9 (64.3%)	5 (35.7%)	
MDM2 neg	5	1 (20%)	4 (80%)	
BCL2 high	47	26 (55.3%)	21 (44.7%)	0.109
BCL2 low	20	10 (50%)	10 (50%)	
BCL2 neg	26	20 (76.9%)	6 (23.1%)	
p53 high	21	17 (81%)	4 (9%)	0.057
p53 low	21	13 (61.9%)	8 (38.1%)	
p53 neg	51	26 (51%)	25 (49%)	
ER pos	47	27 (57.4%)	20 (42.6%)	0.581
ER neg	46	29 (63.0%)	17 (37.0%)	

Analysis was done using Fishers exact test and/or Chi square test as appropriate

Appendix 2: Correlation of ER status with protein expression

Variable	N	ER pos	ER neg	p-value
PI3K high	79	43 (54.4%)	36 (45.6%)	0.105
PI3K low	13	4 (52.0%)	9 (69.2%)	
PI3K neg	1	0 (0%)	1 (100%)	
PTEN high	55	31 (56.4%)	24 (43.6%)	0.370
PTEN low	27	12 (44.4%)	15 (55.6%)	
PTEN neg	11	4 (36.4%)	7 (63.6%)	
p-Akt high	59	31 (52.5%)	28 (47.5%)	0.898
p-Akt low	26	12 (46.2%)	14 (53.8%)	
p-Akt neg	8	4 (50.0%)	4 (50.0%)	
NFκB high	87	45 (51.7%)	42 (48.3%)	0.435
NFκB low	6	2 (33.3%)	4 (66.7%)	
MDM2 high	74	37 (50.0%)	37 (50.0%)	0.384
MDM2 low	14	6 (42.9%)	8 (57.1%)	
MDM2 neg	5	4 (80.0%)	1 (20.0%)	
BCL2 high	47	30 (63.8%)	17 (36.2%)	**0.022**
BCL2 low	20	9 (45.0%)	11 (55%)	
BCL2 neg	26	8 (30.8%)	18 (69.2%)	
p53 high	21	9 (42.9%)	12 (57.1%)	0.367
p53 low	21	9 (42.9%)	12 (57.1%)	
p53 neg	51	29 (56.9%)	22 (43.1%)	
Her-2 pos*	56	27 (48.2%)	29 (51.8%)	0.581
Her-2 neg*	37	20 (54.1%)	17 (45.9%)	

Analysis was done using Fishers exact test and/or Chi square test as appropriate
* Her-2 IHC 2+ results were reclassified according to subsequent ISH results.

Appendix 3: Correlation of PI3K results with protein expression

Variable	N	PI3K high	PI3K low	PI3K neg	p-value
PTEN high	55	47 (85.4%)	7 (12.7%)	1 (1.8%)	0.812
PTEN low	27	22 (81.5%)	5 (18.5%)	0 (0%)	
PTEN neg	11	10 (90.9%)	1 (9.1%)	0 (0%)	
p-Akt high	59	49 (83.1%)	9 (25.3%)	1 (1.7%)	0.827
p-Akt low	26	22 (84.6%)	4 (15.4%)	0 (0.0%)	
p-Akt neg	8	8 (100%)	0 (0%)	0 (0%)	
NFκB high	87	74 (85.1%)	12 (13.8%)	1 (1.1%)	0.616
NFκB low	6	5 (83.3%)	1 (16.7%)	0 (0%)	
MDM2 high	74	64 (86.5%)	9 (14.1%)	1 (1.4%)	0.425
MDM2 low	14	10 (71.4%)	4 (28.6%)	0 (0%)	
MDM2 neg	5	5 (100%)	0 (0%)	0 (0%)	
BCL2 high	47	40 (85.1%)	7 (14.9%)	0 (0%)	0.666
BCL2 low	20	18 (90.0%)	2 (0.1%)	0 (0%)	
BCL2 neg	26	21 (80.8%)	4 (15.4%)	1 (3.8%)	
p53 high	21	16 (76.2%)	5 (23.8%)	0 (0%)	0.514
p53 low	21	18 (85.7%)	3 (14.3%)	0 (0%)	
p53 neg	51	45 (88.2%)	5 (9.8%)	1 (2.0%)	
ER pos	47	43 (91.5%)	4 (8.5%)	0 (0%)	0.105
ER neg	46	36 (78.3%)	9 (19.6%)	1 (2.2%)	
Her-2 pos*	56	48 (85.7%)	7 (12.5%)	1 (1.8%)	0.730
Her-2 neg*	37	31 (83.8%)	6 (16.2%)	0 (0%)	

Analysis was done using Fishers exact test and/or Chi square test as appropriate
* Her-2 IHC 2+ results were reclassified according to subsequent ISH results.

Appendix 4: Correlation of PTEN results with protein expression

Variable	N	PTEN high	PTEN low	PTEN neg	p-value
PI3K high	79	47 (67.1%)	22 (27.8%)	10 (12.7%)	0.812
PI3K low	13	7 (53.8%)	5 (38.5%)	1 (7.7%)	
PI3K neg	1	1 (100%)	0 (0%)	0 (0%)	
p-Akt high	59	40 (67.8%)	14 (23.7%)	5 (8.5%)	0.053
p-Akt low	26	13 (50.0%)	10 (38.5%)	3 (11.5%)	
p-Akt neg	8	2 (25.0%)	3 (37.5%)	3 (37.5%)	
NFκB high	87	53 (60.9%)	23 (26.4%)	11 (12.6%)	0.114
NFκB low	6	2 (33.3%)	4 (66.7%)	0 (0%)	
MDM2 high	74	44 (59.5%)	20 (27.0%)	10 (13.5%)	0.213
MDM2 low	14	6 (42.9%)	7 (50.0%)	1 (7.1%)	
MDM2 neg	5	5 (100%)	0 (0%)	0 (0)%	
BCL2 high	47	32 (68.1%)	13 (27.7%)	2 (4.3%)	0.163
BCL2 low	20	10 (50.0%)	6 (30.0%)	4 (20.0%)	
BCL2 neg	26	13 (50.0%)	8 (30.8%)	5 (19.2%)	
p53 high	21	12 (57.1%)	8 (38.1%)	1 (4.8%)	0.236
p53 low	21	9 (42.9%)	8 (38.1%)	4 (19.0%)	
p53 neg	51	34 (66.7%)	11 (21.6%)	6 (11.8%)	
ER pos	47	31 (66.0%)	12 (25.5%)	4 (8.5%)	0.370
ER neg	46	24 (52.2%)	15 (32.6%)	7 (15.2%)	
Her-2 pos*	56	35 (62.5%)	18 (32.1%)	3 (53.6%)	0.065
Her-2 neg*	37	20 (54.1%)	9 (24.3%)	8 (21.6%)	

Analysis was done using Fishers exact test and/or Chi square test as appropriate
* Her-2 IHC 2+ results were reclassified according to subsequent ISH results.

Appendix 5: Correlation of p-Akt results with protein expression

Variable	N	p-Akt high	p-Akt low	p-Akt neg	p-value
PI3K high	79	49 (62.0%)	22 (27.8%)	8 (10.1%)	0.827
PI3K low	13	9 (69.2%)	4 (30.8%)	0 (0%)	
PI3K neg	1	1 (100%)	0 (0%)	0 (0%)	
PTEN high	55	40 (72.7%)	13 (23.6%)	2 (3.6%)	0.053
PTEN low	27	14 (51.9%)	10 (37.0%)	3 (11.1%)	
PTEN neg	11	5 (45.4%)	3 (27.3%)	3 (27.3%)	
NFκB high	87	58 (66.7%)	23 (26.4%)	6 (6.9%)	**0.014**
NFκB low	6	1 (16.7%)	3 (50.0%)	2 (33.3%)	
MDM2 high	74	52 (70.3%)	16 (21.6%)	6 (12.2%)	**0.040**
MDM2 low	14	5 (35.7%)	7 (50.0%)	2 (14.3%)	
MDM2 neg	5	2 (40.0%)	3 (60.0%)	0 (0%)	
BCL2 high	47	33 (70.2%)	11 (23.4%)	3 (6.4%)	0.538
BCL2 low	20	10 (50.0%)	8 (40.0%)	2 (10.0%)	
BCL2 neg	26	16 (61.5%)	7 (26.9%)	3 (11.5%)	
p53 high	21	13 (61.9%)	5 (23.8%)	3 (14.3%)	0.754
p53 low	21	14 (66.7%)	5 (23.8%)	2 (9.5%)	
p53 neg	51	32 (62.7%)	16 (31.4%)	3 (5.9%)	
ER pos	47	31 (66.0%)	12 (25.5%)	4 (8.5%)	0.898
ER neg	46	28 (60.9%)	14 (30.4%)	4 (8.7%)	
Her-2 pos*	56	37 (66.1%)	14 (25.0%)	5 (8.9%)	0.803
Her-2 neg*	37	22 (59.4%)	12 (32.4%)	3 (8.1%)	

Analysis was done using Fishers exact test and/or Chi square test as appropriate
* Her-2 IHC (2+) results were reclassified according to subsequent ISH results.

Appendix 6: Correlation of MDM2 results with protein expression

Variable	N	MDM2 high	MDM2 low	MDM2 neg	p-value
PI3K high	79	64 (81.0%)	10 (12.7%)	5 (6.3%)	0.425
PI3K low	13	9 (69.2%)	4 (30.8%)	0 (0.0%)	
PI3K neg	1	1 (100%)	0 (0%)	0 (0%)	
PTEN high	55	44 (80.0%)	6 (10.9%)	5 (9.1%)	0.214
PTEN low	27	20 (74.1%)	7 (25.9%)	0 (0%)	
PTEN neg	11	10 (90.9%)	1 (9.1%)	0 (0%)	
p-Akt high	59	52 (88.1%)	5 (8.5%)	2 (3.4%)	**0.040**
p-Akt low	26	16 (61.5%)	7 (26.9%)	3 (11.5%)	
p-Akt neg	8	6 (75.0%)	2 (25.0%)	0 (0%)	
BCL2 high	47	34 (72.3%)	11 (23.4%)	2 (4.3%)	0.085
BCL2 low	20	18 (90.0%)	0 (0%)	2 (10.0%)	
BCL2 neg	26	22 (84.6%)	3 (11.5%)	1 (3.8%)	
p53 high	21	15 (71.4%)	4 (19.0%)	2 (13.3%)	0.603
p53 low	21	19 (90.5%)	2 (9.5%)	0 (0%)	
p53 neg	51	40 (78.4%)	8 (15.7%)	3 (5.9%)	
ER pos	47	37 (78.7%)	6 (12.8%)	4 (8.5%)	0.384
ER neg	46	37 (80.4%)	8 (17.4%)	1 (2.2%)	
Her-2 pos*	56	46 (82.1%)	9 (16.1%)	1 (1.8%)	0.197
Her-2 neg*	37	28 (75.7%)	5 (13.5%)	4 (10.8%)	
NFκB high	87	69 (79.3%)	13 (14.9%)	5 (5.7%)	>0.999
NFκB low	6	5 (83.3%)	1 (16.7%)	0 (0%)	

Analysis was done using Fishers exact test and/or Chi square test as appropriate
* Her-2 IHC 2+ results were reclassified according to subsequent ISH results.

Appendix 7: Correlation of NFκB results with protein expression

Variable	N	NFκB high	NFκB low	p-value
PI3K high	79	74 (93.7%)	5 (6.3%)	0.616
PI3K low	13	12 (92.3%)	1 (7.7%)	
PI3K neg	1	1 (100%)	0 (0%)	
PTEN high	55	53 (96.4%)	2 (3.6%)	0.114
PTEN low	27	23 (85.2%)	4 (14.8%)	
PTEN neg	11	11 (100%)	0 (0%)	
MDM2 high	74	69 (93.2%)	5 (6.8%)	>0.999
MDM2 low	14	13 (92.9%)	1 (7.1%)	
MDM2 neg	5	5 (100%)	0 (0%)	
p-Akt high	59	58 (98.3%)	1 (1.7%)	**0.014**
p-Akt low	26	23 (88.5%)	3 (11.5%)	
p-Akt neg	8	6 (75.0%)	2 (25.0%)	
BCL2 high	47	44 (93.6%)	3 (6.4%)	0.74
BCL2 low	20	18 (90.0%)	2 (10.0%)	
BCL2 neg	26	25 (96.2%)	1 (3.8%)	
p53 high	21	21 (100%)	0 (0%)	0.180
p53 low	21	18 (85.7%)	3 (14.3%)	
p53 neg	51	48 (94.1%)	3 (5.9%)	
ER pos	47	45 (95.7%)	2 (4.3%)	0.435
ER neg	46	42 (91.3%)	4 (8.7%)	
Her-2 pos*	56	55 (98.2%)	1 (1.8%)	**0.035**
Her-2 neg*	37	32 (86.5%)	5 (13.5%)	

Analysis was done using Fishers exact test and/or Chi square test as appropriate

* Her-2 IHC 2+ results were reclassified according to subsequent ISH results.

Appendix 8: Correlation of BCL2 results with protein expression

Variable	N	BCL2 high	BCL2 low	BCL2 neg	p-value
PI3K high	79	40 (50.6%)	18 (22.8%)	21 (26.6%)	0.666
PI3K low	13	7 (53.8%)	2 (15.4%)	4 (30.8%)	
PI3K neg	1	0 (0%)	0 (0%)	1 (100%)	
PTEN high	55	32 (58.2%)	10 (18.2%)	13 (23.6%)	0.163
PTEN low	27	13 (48.1%)	6 (22.2%)	8 (29.6%)	
PTEN neg	11	2 (18.2%)	4 (36.4%)	5 (45.4%)	
MDM2 high	74	34 (45.9%)	18 (24.3%)	22 (29.7%)	0.085
MDM2 low	14	11 (78.6%)	0 (0%)	3 (21.4%)	
MDM2 neg	5	2 (40.0%)	2 (40.0%)	1 (20.0%)	
p-Akt high	59	33 (55.9%)	10 (16.9%)	16 (27.1%)	0.538
p-Akt low	26	11 (42.3%)	8 (30.8%)	7 (26.9%)	
p-Akt neg	8	3 (37.5%)	2 (25.0%)	3 (37.5%)	
NFκB high	87	44 (50.6%)	18 (20.7%)	25 (28.7%)	0.740
NFκB low	6	3 (50.0%)	2 (33.3%)	1 (16.7%)	
p53 high	21	8 (38.1%)	5 (23.8%)	8 38.1%)	0.387
p53 low	21	13 (61.9%)	2 (9.5%)	6 (28.6%)	
p53 neg	51	26 (51.0%)	13 (25.5%)	12 (23.5%)	
ER pos	47	30 (63.8%)	9 (19.2%)	8 (17.0%)	**0.022**
ER neg	46	17 (37.0%)	11 (23.9%)	18 (39.1%)	
Her-2 pos*	56	26 (46.4%)	10 (17.9%)	20 (35.7%)	0.109
Her-2 neg*	37	21 (56.8%)	10 (27.0%)	6 (16.2%)	

Analysis was done using Fishers exact test and/or Chi square test as appropriate
* Her-2 IHC 2+ results were reclassified according to subsequent ISH results.

Appendix 9: Correlation of p53 results with protein expression

Variable	N	p53 high	p53 low	p53 neg	p-value
PI3K high	79	16 (20.2%)	18 (22.8%)	45 (57.0%)	0.514
PI3K low	13	5 (38.5%)	3 (23.1%)	5 (38.5%)	
PI3K neg	1	0 (0%)	0 (0%)	1 (100%)	
PTEN high	55	12 (21.8%)	9 (16.4%)	34 (61.8%)	0.236
PTEN low	27	8 (29.6%)	8 (29.6%)	11 (40.7%)	
PTEN neg	11	1 (9.1%)	4 (36.4%)	6 (54.5%)	
MDM2 high	74	15 (20.3%)	19 (25.7%)	40 (54.1%)	0.603
MDM2 low	14	4 (28.6%)	2 (14.3%)	8 (57.1%)	
MDM2 neg	5	2 (40.0%)	0 (0%)	3 (60.0%)	
p-Akt high	59	13 (2.0%)	14 (23.7%)	32 (54.2%)	0.754
p-Akt low	26	5 (19.2%)	5 (19.2%)	16 (61.5%)	
p-Akt neg	8	3 (37.5%)	2 (25.0%)	3 (37.5%)	
NFκB high	87	21 (24.1%)	18 (20.7%)	48 (55.2%)	0.180
NFκB low	6	0 (0%)	3 (50.0%)	3 (50.0%)	
BCL2 high	47	8 (17.0%)	13 (27.7%)	26 (55.3%)	0.387
BCL2 low	20	5 (25.0%)	2 (10.0%)	13 (65.0%)	
BCL2 neg	26	8 (30.8%)	6 (23.1%)	12 (46.1%)	
ER pos	47	9 (19.1%)	9 (19.1%)	29 (61.7%)	0.367
ER neg	46	12 (26.1%)	12 (26.1%)	22 (47.8%)	
Her-2 pos*	56	17 (30.4%)	13 (23.2%)	26 (46.4%)	0.057
Her-2 neg*	37	4 (10.8%)	8 (21.6%)	25 (67.6%)	

Analysis was done using Fishers exact test and/or Chi square test as appropriate
* Her-2 IHC 2+ results were reclassified according to subsequent ISH results.

Appendix 10: Association between age and level of protein expression

Variable	N	Mean age	SD	p-value
PTEN neg	11	52.1	8.2	0.154
PTEN low	27	54.7	9.8	
PTEN high	55	53.1	11.7	
NFκB low	6	60.2	10.5	0.595
NFκB high	87	53.1	10.7	
MDM2 neg	5	53.8	5.8	0.850
MDM2 low	14	51.1	13.6	
MDM2 high	74	53.9	10.5	
p53 neg	51	54.2	10.3	0.148
p53 low	21	53.5	9.9	
p53 high	21	51.6	12.8	
BCL2 neg	26	55.2	3.7	0.813
BCL2 low	20	50.6	10.9	
BCL2 high	47	53.7	10.4	
p-Akt neg	8	51.4	3.7	0.874
p-Akt low	26	53.6	10.9	
p-Akt high	59	53.7	10.4	
PI3K neg	1	40.0	-	0.392
PI3K low	13	55.3	11.3	
PI3K high	17	53.3	10.7	
ER neg	46	52.8	10.8	0.476
ER pos	47	54.1	10.8	
Her-2 Neg	37	56.2	8.6	**0.044**
Her-2 Pos	56	51.6	11.7	

Analysis of variance (ANOVA) was used to analyze level of protein expression and age (continues variable).

Appendix 11: Association between tumour type and protein expression

Variable	N	Ductal	Lobular	p-value
PTEN high	55	50 (90.9%)	5 (9.1%)	>0.999
PTEN low	27	25 (92.6%)	2 (7.4%)	
PTEN neg	11	10 (90.9%)	1 (9.1%)	
NFκB high	87	81 (93.1%)	6 (6.9%)	0.082
NFκB low	6	4 (66.7%)	2 (33.3%)	
MDM2 high	74	68 (91.9%)	6 (8.1%)	0.051
MDM2 low	14	14 (100%)	0 (0%)	
MDM2 neg	5	3 (60.0%)	2 (40.0%)	
p53 high	21	21 (100%)	0 (0%)	0.172
p53 low	21	20 (95.2%)	1 (4.8%)	
p53 neg	51	44 (86.3%)	7 (13.7%)	
BCL2 high	47	42 (89.4%)	5 (10.6%)	0.135
BCL2 low	20	17 (85.0%)	3 15.0%)	
BCL2 neg	26	26 (100%)	0 (0%)	
p-Akt high	59	54 (91.5%)	5 (8.5%)	0.183
p-Akt low	26	25 (96.2%)	1 (3.8%)	
p-Akt neg	8	6 (75.0%)	2 (25.0%)	
PI3k high	79	72 (91.1%)	7 (8.9%)	0.630
PI3k low	13	12 (92.3%)	1 (7.7%)	
PI3k neg	1	1 (100%)	0 (0%)	
ER pos	47	40 (85.1%)	7 (6.7%)	0.059
ER neg	46	45 (97.8%)	1 (2.2%)	
Her-2 pos*	56	55 (98.2%)	1 (1.8%)	**0.006**
Her-2 neg*	37	30 (81.1%)	7 (18.9%)	

Analysis was done using Fishers exact test and/or Chi square test as appropriate
* Her-2 IHC 2+ results were reclassified according to subsequent ISH results.

Appendix 12: Association between tumour grade and protein expression

Variable	N	grade1	grade2	grade3	p-value
PTEN high	55	8 (14.5%)	24 (43.6%)	23 (41.8%)	0.592
PTEN low	27	5 (18.5%)	7 (25.9%)	15 (55.6%)	
PTEN neg	11	1 (9.1%)	5 (45.5%)	5 (45.5%)	
NFκB high	87	11 (12.6%)	34 (39.1%)	42 (48.3%)	0.051
NFκB low	6	3 (50.0%)	2 (33.3%)	1 (16.7%)	
MDM2 high	74	10 (13.5%)	31 (41.9%)	33 (44.6%)	0.590
MDM2 low	14	3 (21.4%)	3 (21.4%)	8 (57.1%)	
MDM2 neg	5	1 (20.0%)	2 (40.0%)	2 (40.0%)	
p53 high	21	0 (0.0%)	5 (23.8%)	16 (76.2%)	**0.013**
p53 low	21	4 (19.0%)	11 (52.4%)	6 (28.6%)	
p53 neg	51	10 (19.6%)	20 (39.2%)	21 (41.2%)	
BCL2 high	47	11 (23.4%)	17 (36.2%)	19 (40.4%)	0.215
BCL2 low	20	2 (10.0%)	9 (45.0%)	9 (45.0%)	
BCL2 neg	26	1 (3.8%)	10 (38.5%)	15 (57.7%)	
p-Akt high	59	8 (13.6%)	23 (39.0%)	28 (47.4%)	0.928
p-Akt low	26	5 (19.2%)	9 (34.6%)	12 (46.2%)	
p-Akt neg	8	1 (12.5%)	4 (50.0%)	3 (37.5%)	
PI3k high	79	10 (12.7%)	33 (41.8%)	36 (45.6%)	0.275
PI3k low	13	4 (30.8%)	3 (23.1%)	6 (46.2%)	
PI3k neg	1	0 (0.00%)	0 (0.00%)	1 (100%)	
ER pos	47	6 (12.8%)	23 (48.9%)	18 (38.3%)	0.123
ER neg	46	8 (17.4%)	13 (28.3%)	25 (54.3%)	
Her-2 pos*	56	3 (5.4%)	22 (39.3%)	31 (55.4%)	**0.004**
Her-2 neg*	37	11 (29.7%)	14 (37.8%)	12 (32.4%)	

Analysis was done using Fishers exact test and/or Chi square test as appropriate

* Her-2 IHC 2+ results were reclassified according to subsequent ISH results.

Appendix 13: Association between lymphovascular invasion and level of protein expression

Variable	N	No Invasion	Invasion	p- value
PTEN high	55	13 (23.6%)	42 (76.4%)	0.777
PTEN low	27	8 (29.6%)	19 (70.4%)	
PTEN neg	11	2 (18.2%)	9 (81.8%)	
NFκB high	87	22 (25.3%)	65 (74.7%)	>0.999
NFκB low	6	1 (16.7%)	5 (83.3%)	
MDM2 high	74	17 (23.0%)	57 (77.0%)	0.656
MDM2 low	14	5 (35.7%)	9 (64.3%)	
MDM2 neg	5	1 (20.0%)	4 (80.0%)	
p53 high	21	6 (28.6%)	15 (71.4%)	0.403
p53 low	21	7 (33.3%)	14 (66.6%)	
p53 neg	51	10 (19.6%)	41 (80.4%)	
BCL2 high	47	13 (27.7%)	34 (72.3%)	0.858
BCL2 low	20	4 (20.0%)	16 (80.0%)	
BCL2 neg	26	6 (23.1%)	20 (76.9%)	
p-Akt2 high	59	12 (20.3%)	47 (79.7%)	0.175
p-Akt2 low	26	10 (38.5%)	16 (61.5%)	
p-Akt2 neg	8	1 (12.5%)	7 (87.5%)	
PI3K high	79	16 (20.3%)	63 (79.7%)	**0.010**
PI3K low	13	7 (53.8%)	6 (46.1%)	
PI3K neg	1	0 (0.0%)	1 (100%)	
ER pos	47	2 (4.3%)	45 (95.7%)	**<0.001**
ER neg	46	21 (45.7%)	25 (54.3%)	
Her-2 pos*	56	16 (28.6%)	40 (71.4%)	0.337
Her-2 neg*	37	7 (18.9%)	30 (81.1%)	

Analysis was done using Fishers exact test and/or Chi square test as appropriate
* Her-2 IHC 2+ results were reclassified according to subsequent ISH results.

Appendix 14: Association between lymph node metastasis and protein expression

Variable	N	Yes	No	p-value
PI3K high	79	53 (67.1%)	26 (32.9%)	0.062
PI3K low	13	5 (38.5%)	8 (61.5%)	
PI3K neg	1	1 (100%)	0 (0%)	
PTEN high	55	37 (67.3%)	18 (32.7%)	0.574
PTEN low	27	15 (55.6%)	12 (44.4%)	
PTEN neg	11	7 (63.6%)	4 (36.4%)	
p-Akt high	59	40 (67.8%)	19 (32.2%)	0.444
p-Akt low	26	15 (57.7%)	11(42.3%)	
p-Akt neg	8	4 (50.0%)	4 (50.0%)	
NFκB high	87	55 (63.2%)	32 (36.8%)	0.999
NFκB low	6	4 (66.7%)	2 (33.3%)	
MDM2 high	74	47 (63.5%)	27 (36.5%)	0.999
MDM2 low	14	9 (64.3%)	5 (35.7%)	
MDM2 neg	5	3 (60.0%)	2 (40.0%)	
BCL2 high	47	27 (57.4%)	20 (42.6%)	0.558
BCL2 low	20	14 (70.0%)	6 (30.0%)	
BCL2 neg	26	18 (69.2%)	8 (30.1%)	
P53 high	21	14 (66.6%)	7 (33.3%)	0.228
P53 low	21	10 (47.6%)	11 (52.4%)	
P53 neg	51	35 (68.6%)	16 (31.4%)	
ER pos	47	37 (78.7%)	10 (21.3%)	**0.002**
ER neg	46	22 (47.8%)	24 (52.2%)	
Her-2 pos	56	34 (60.7%)	22 (39.3)	0.502
Her-2 neg	37	25 (67.6%)	12 (32.4%)	

Analysis was done using Fishers exact test and/or Chi square test as appropriate

Appendix 15: Association between distant metastasis (other than regional lymph nodes) and level of protein expression

Variable	N	No	Yes	p-value
PTEN high	52	39 (75.0%)	16 (25.0%)	0.165
PTEN low	27	16 (59.3%)	11 (40.7%)	
PTEN neg	11	10 (90.9%)	1 (9.1%)	
NFκB high	87	62 (71.3%)	25 (28.7%)	0.361
NFκB low	6	3 (50.0%)	3 (50.0%)	
MDM2 high	74	56 (75.7%)	18 (24.3%)	**0.017**
MDM2 low	14	8 (57.1%)	6 (42.9%)	
MDM2 neg	5	1 (20.0%)	4 (80.0%)	
p53 high	21	12 (57.1%)	9 (42.9%)	0.122
p53 low	21	18 (85.7%)	3 (14.3%)	
p53 neg	51	35 (68.6%)	16 (31.4%)	
BCL2 high	47	33 (70.2%)	14 (29.8%)	0.837
BCL2 low	20	13 (65.0%)	7 (35.0%)	
BCL2 neg	26	19 (73.1%)	7 (26.9%)	
p-Akt high	59	42 (71.2%)	17 (28.8%)	0.882
p-Akt low	26	18 (69.2%)	8 (30.8%)	
p-Akt neg	8	5 (62.5%)	3 (37.5%)	
PI3K high	79	53 (67.1%)	26 (32.9%)	0.532
PI3K low	13	11 (84.6%)	2 (15.4%)	
PI3K neg	1	1 (100%)	0 (0%)	
ER pos	47	30 (63.8%)	17 (36.2%)	0.198
ER neg	46	35 (76.1%)	11 (23.9%)	
Her-2 pos	56	39 (69.6%)	17 (30.4%)	>0.999
Her-2 neg	37	26 (70.3%)	11 (29.7%)	

Analysis was done using Fishers exact test and/or Chi square test as appropriate

Appendix 16: Association between apoptotic index and protein expression

Variable	N	< 3	3 <5	5 <7	≥7	p- value
PTEN high	55	19 (34.5%)	16 (29.1%)	12 (21.8%)	7 (12.7%)	0.479
PTEN low	27	4 (14.8%)	9 (33.3%)	8 (29.6%)	6 (22.2%)	
PTEN neg	11	2 (18.2%)	3 (27.3%)	3 (27.3%)	3 (27.3%)	
NFκB high	87	23 (26.4%)	26 (29.9%)	21 (24.1%)	18 (20.7%)	0.798
NFκB low	6	2 (33.3%)	2 (33.3%)	2 (33.3%)	0 (0.0%)	
MDM2 high	74	21 (28.4%)	22 (29.7%)	18 (24.3%)	12 (16.2%)	0.439
MDM2 low	14	4 (28.6%)	4 (28.6%)	2 (14.3%)	4 (28.6%)	
MDM2 neg	5	0 (0.0%)	2 (40.0%)	3 (60.0%)	0 (0.0%)	
p53 high	21	4 (19.0%)	8 (38.0%)	3 (14.3%)	6 (28.6%)	0.490
p53 low	21	5 (23.8%)	7 (33.3%)	7 (33.3%)	2 (9.5%)	
p53 neg	51	16 (31.4%)	13 (25.5%)	13 (25.5%)	8 (15.7%)	
BCL2 high	47	11 (23.4%)	13 (27.7%)	14 (29.8%)	8 (17.0%)	0.887
BCL2 low	20	7 (35.0%)	6 (30.0%)	3 (15.0%)	4 (20.0%)	
BCL2 neg	26	7 (26.9%)	9 (34.6%)	6 (23.1%)	4 (15.4%)	
p-Akt high	59	16 (27.1%)	16 (27.1%)	15 (25.4%)	11 (18.6%)	0.643
p-Akt low	26	8 (30.8%)	10 (38.5%)	4 (15.4%)	4 (15.4%)	
p-Akt neg	8	1 (12.5%)	2 (25.0%)	4 (50.0%)	1 (12.5%)	
PI3K high	79	17 (21.5%)	26 (32.9%)	21 (26.6%)	13 (16.5%)	**0.014**
PI3K low	13	8 (61.5%)	1 (7.7%)	1 (7.7%)	3 (23.1%)	
PI3K neg	1	0 (0.0%)	1 (100%)	0 (0.0%)	0 (0.0%)	
ER pos	47	12 (25.5%)	15 (31.9%)	13 (27.7%)	7 (14.9%)	0.854
ER neg	46	13 (28.3%)	13 (28.3%)	10 (21.7%)	9 (19.6%)	
Her-2 pos	56	15 (26.8%)	19 (33.9%)	13 (23.2%)	9 (16.1%)	0.838
Her-2 neg	37	10 (27.0%)	9 (24.3%)	10 (27.0%)	7 (18.9%)	

Analysis was done using Fishers exact test and/or Chi square test as appropriate

Appendix 17: Median and 5 year OS and DFS for the total population of 93 subjects according to the Kaplan-Meier graph

Variable	Category	N	Median OS	5 yr OS	Median DFS	5 year DFS
Trastuzumab	No	67	80 months	90%	-	80%
	Yes	26	-	75%	-	70%
Stage	1 (T1)	14	-	86%	-	80%
	2 (T2)	48	80 months	85%	-	75%
	3 (T3)	30	-	62%	-	61%
	4 (T4)	1	-	0%	-	0%
Grade	1	14	-	94%	-	74%
	2	36	-	78%	-	82%
	3	43	80 months	72%	-	64%
Age	1(<45)	20	-	65%	-	80%
	2 (45-65)	59	80 months	76%	-	65%
	3 (>65)	14	-	100%	-	85%
PTEN	High	55	80 months	80%	-	70%
	Low	27	-	73%	-	64%
	Neg	11	-	91%	-	90%
NFκB	High	87	80 months	80%	-	73%
	Low	6	48 months	50%	-	60%
MDM2	High	74	80 months	80%	-	75%
	Low	14	-	65%	-	80%
	Neg	5	-	60%	34 months	0%
Akt	High	59	80 months	83%	-	71%
	Low	26	-	73%	-	68%
	Neg	8	46 months	46%	-	80%
PI3K	High	79	80 months	75%	-	68%
	Low	13	-	92%	-	85%
	Neg	1	-	100%	-	100%
P53	High	21	-	57%	-	0%
	Low	21	-	89%	-	79%
	Neg	51	80 months	81%	-	75%
BCL2	High	47	80 months	80%	-	68%
	Low	20	-	75%	-	76%
	Neg	26	-	75%	-	79%
Her2	Pos	33	80 months	79%	-	77%
	Neg	46	-	76%	-	65%
ER	MH (pos)	47	80 months	82%	-	70%
	NL (neg)	46	-	73%	-	75%
Apop index	< 3	25	-	77%	-	85%
	3 <5	28	-	72%	-	67%
	5 <7	23	80 months	85%	-	63%
	≥ 7	16	-	87%	-	69%

Appendix 18: Median and 5 year OS and DFS for the cohort of 67 patients that did not receive trastuzumab treatment according to the Kaplan-Meier graph

Variable	Category	N	Median OS	5 yr OS	Median DFS	5 year DFS
Stage	1 (T1)	8	-	80%	-	86%
	2 (T2)	37	-	84%	-	73%
	3 (T3)	21	-	56%	-	56%
	4 (T4)	1	-	0%	-	0%
Grade	1	12	-	92%	-	71%
	2	27	-	79%	-	78%
	3	28	-	63%	-	60%
Age	1(<45)	12	-	57%	-	75%
	2 (45-65)	47	-	74%	-	65%
	3 (>65)	8	-	100%	-	88%
PTEN	High	38	-	76%	-	68%
	Low	19	-	62%	-	56%
	Neg	10	-	100%	-	100%
NFκB	High	61	-	77%	-	70%
	Low	6	47 months	50%	-	60%
MDM2	High	50	-	79%	-	74%
	Low	12	-	58%	-	78%
	Neg	5	-	60%	36 months	0%
Akt	High	34	-	81%	-	67%
	Low	25	-	72%	-	68%
	Neg	8	47 months	47%	-	80%
PI3K	High	57	-	72%	-	67%
	Low	10	-	90%	-	80%
	Neg	0	-	-	-	-
P53	High	14	42 months	40%	45 months	41%
	Low	16	-	85%	-	75%
	Neg	37	-	83%	-	77%
BCL2	High	35	-	77%	-	76%
	Low	15	-	77%	-	76%
	Neg	17	-	70%	-	70%
Her2	Pos	36	-	70%	-	74%
	Neg	31		78%		65%
ER	MH (pos)	32	-	76%	-	69%
	NL (neg)	35	-	72%	-	70%
Apop index	< 3	18	-	75%	-	80%
	3 <5	18	-	65%	-	64%
	5 <7	19	-	80%	-	66%
	≥ 7	11	-	72%	-	63%

Appendix 19: Median and 5 year OS and DFS for the cohort of 26 patients that did receive trastuzumab treatment according to the Kaplan-Meier graph

Variable	Category	N	Median OS	5 yr OS	Median DFS	5 year DFS
Grade	1 (T1)	2	-	100%	80 months	94%
	2 (T2)	9	80 months	91%	-	-
	3 (T3)	15	-	78%	-	78%
	4 (T4)	0	-	-	-	-
Stage	1	6	-	100%	-	100%
	2	11	-	78%	-	-
	3	9	80 months	94%	80 months	94%
Age	1 (<45)	8	-	75%	-	85%
	2 (45-65)	12	80 months	92%	-	0%
	3 (>65)	6	-	100%	-	0%
PTEN	High	17	80 months	89%	-	80%
	Low	8	-	100%	-	100%
	Neg	1	18 months	0%	18 months	0%
NFκB	High	26	80 months	88%	-	80%
	Low	0	-	-	-	-
MDM2	High	24	80 months	87%	-	87%
	Low	2	-	100%	-	100%
	Neg	0	-	-	-	-
Akt	High	25	80 months	87%	-	80%
	Low	1	-	100%	-	-
	Neg	0	-	-	-	-
PI3K	High	22	80 months	86%	-	71%
	Low	3	-	-	-	-
	Neg	1	-	-	-	-
P53	High	7	-	100%	-	100%
	Low	5	-	100%	-	100%
	Neg	14	80 months	78%	-	63%
BCL2	High	12	-	92%	-	68%
	Low	5	-	0%	-	100%
	Neg	9	80 months	0%	-	87%
Her2	Pos	26	80 months	92%	-	80%
ER	MH (pos)	15	80 months	94%	-	66%
	NL (neg)	11	-	0%	-	89%
Apop index	< 3	25	-	85%	-	-
	3 <5	28	-	90%	-	75%
	5 <7	23	80	100%	41 months	0%
	≥ 7	16	-	80%	-	0%

Appendix 20: Analysis of overall survival (OS) of complete sample of 93 cases, using Cox regression analysis adjusted for age

Variable	Category	N	Person time	Deaths	Death rate	95% CI	HR	95%CI
Trastuzumab	No	67	3177	16	5.0	(3.1 – 8.2)	1.0	(ref)
	Yes	26	1237	4	3.2	(1.2 - 8.6)	0.4	(0.1 – 1.5)
Stage	1 (T1)	14	747	1	1.3	(0.2 - 9.5)	1.0	(ref)
	2 (T2)	48	2338	8	3.4	(1.7 – 6.8)	2.1	(0.2 – 17.1)
	3 (T3)	30	1287	11	8.5	(4.7 – 15.4)	5.0	(0.6 – 42.4)
	4 (T4)	1	42	0	0.0	-	-	-
Grade	1	14	731	1	1.4	(0.2 – 9.7)	1.0	(ref)
	2	36	1758	7	4.0	(1.9 – 8.4)	3.1	(0.4 – 25.6)
	3	43	1925	12	6.2	(3.5 – 11.0)	3.8	(0.5 – 29.6)
Age	1(<45)	20	796	7	8.8	(4.2 – 18.4)	1.0	(ref)
	2 (45-65)	59	2873	13	4.5	(2.6 – 7.8)	0.5	(0.2 – 1.3)
	3 (>65)	14	745	0	0.0	-	-	-
PTEN	High	55	2606	12	4.6	(2.6 – 8.1)	1.0	(ref)
	Low	27	1273	7	5.5	(2.6 – 11.5)	1.6	(0.6 – 4.3)
	Neg	11	535	1	1.9	(0.3 – 13.3)	0.4	(0.1 – 3.5)
NFκB	High	87	4110	17	4.1	(2.6 – 6.7)	1.0	(ref)
	Low	6	304	3	9.9	(3.2 – 30.6)	3.6	(1.0 – 13.1)
MDM2	High	74	3614	13	3.6	(2.1 – 6.2)	1.0	(ref)
	Low	14	550	5	9.1	(3.8 – 21.8)	2.4	(0.8 – 6.9)
	Neg	5	250	2	8.0	(2.0 – 32.0)	2.8	(0.6 – 13.0)
Akt	High	59	2836	9	3.2	(1.7 – 6.1)	1.0	(ref)
	Low	26	1261	7	5.6	(2.6 – 11.6)	2.1	(0.8 – 5.9)
	Neg	8	317	4	12.6	(4.7 – 33.6)	**5.1**	**(1.5 – 17.3)**
PI3K	High	79	3738	19	5.1	(3.2 – 8.0)	1.0	(ref)
	Low	13	625	1	1.6	(0.2 – 11.4)	0.3	0.0 – 2.3)
	Neg	1	51	0	0.0	-	-	-
P53	High	21	839	8	9.5	(4.8 – 19.1)	1.0	(ref)
	Low	21	1073	2	1.9	(0.5 – 7.4)	**0.18**	**(0.0 – 0.9)**
	Neg	51	2502	10	4.0	(2.1 – 7.4)	0.4	(0.2 – 1.0)
BCL2	High	47	2338	10	4.3	(2.3 – 7.9)	1.0	(ref)
	Low	20	976	4	4.1	(1.5 – 11.0)	0.9	(0.3 – 2.8)
	Neg	26	1100	6	5.5	(2.5 – 12.1)	1.6	(0.5 – 4.4)
Her2	Neg	33	1780	8	4.5	(2.2 – 9.0)	1.0	(ref)
	Pos	46	2634	12	4.6	(2.6 – 8.0)	0.7	(0.2 -1.7)
ER	MH (pos)	47	2303	9	3.9	(2.0 – 7.5)	1.0	(ref)
	NL (neg)	46	2111	11	5.2	(2.9 – 9.4)	1.2	(0.5 – 3.1)
Apop index	< 3	25	1079	5	4.6	(1.9 – 11.1)	1.0	(ref)
	3 <5	28	1335	7	5.2	(2.5 – 11.0)	1.1	(0.4 – 4.6)
	5 <7	23	1202	4	3.3	(1.2 – 8.9)	0.7	(0.2 – 3.4)
	≥ 7	16	759	4	5.3	(2.0 – 14.1)	1.2	(0.4 – 5.3)

Appendix 21: Analysis of overall survival for the cohort of 67 cases that has not received trastuzumab treatment, using Cox regression analysis adjusted for age

Variable	Category	N	Person time	Deaths	Death rate	95% CI	HR	95% CI
Stage	1 (T1)	14	425	1	2.4	(0.3 – 16.7)	-	(ref)
	2 (T2)	48	1850	6	3.2	(1.5 – 7.2)	1.4	(0.2 – 11.5)
	3 (T3)	30	860	9	10.5	(5.4 – 20.1)	4.3	(0.5 – 34.3)
	4 (T4)	1	42	0	0.0	-	-	-
Grade	1	14	644	1	1.6	(0.22 – 11.0)	-	(ref)
	2	36	1346	50	3.7	(1.5 – 8.9)	2.5	(0.3 – 21.2)
	3	43	1187	10	8.4	(4.5 – 15.7)	4.5	(0.6 – 36.0)
Age	1(<45)	12	448	5	11.2	(4.65 – 26.8)	1.0	(ref)
	2 (45-65)	47	2299	11	4.8	(2.6 – 8.6)	0.4	(0.1 – 1.2)
	3 (>65)	8	430	0	-	-	-	-
PTEN	High	38	1825	9	4.0	(2.6 – 9.5)	1.0	(ref)
	Low	19	835	7	8.4	(4.0 – 17.6)	1.9	(0.7 - 5.2)
	Neg	10	517	0	-	-	-	-
NFκB	High	61	2873	13	4.5	(2.6 – 7.8)	1.0	(ref)
	Low	6	304	3	9.9	(3.2 – 30.6)	2.8	(0.8 – 10.2)
MDM2	High	50	2495	9	3.6	(1.9 – 6.9)	1.0	(ref)
	Low	12	432	5	4.8	(4.8 – 27.8)	**3.2**	**(1.1 – 9.7)**
	Neg	5	250	2	2.0	(2.0 – 32.0)	2.6	(0.6 – 12.2)
Akt	High	34	1670	5	3.0	(1.2 – 7.2)	1.0	(ref)
	Low	25	1190	7	5.9	(2.8 – 12.3)	2.0	(0.6 – 6.3)
	Neg	8	317	4	12.6	(4.7 – 33.6)	**4.4**	**(1.2 – 16.7)**
PI3K	High	57	2689	15	5.6	(3.3 – 9.3)	1.0	(ref)
	Low	10	488	1	2.1	(0.3 – 14.5)	0.4	(0.1 – 2.9)
	Neg	0	-	-	-	-	-	-
P53	High	14	518	8	15.4	(7.7 – 30.9)	1.0	(ref)
	Low	16	796	2	2.5	(0.6 – 10.0)	**0.2**	**(0.0 – 0.9)**
	Neg	37	1863	6	3.2	(1.4 – 7.2)	**0.2**	**(0.1 – 0.7)**
BCL2	High	35	1678	8	4.8	(2.4 – 9.5)	1.0	(ref)
	Low	15	758	3	4.0	(1.3 – 12.3)	0.6	(0.2 – 2.3)
	Neg	17	741	5	6.7	(2.8 – 16.2)	1.8	(0.6 – 5.9)
Her2	Neg	36	1762	7	4.0	(1.9 – 8.3)	1.0	(ref)
	Pos	31	1415	9	6.4	(3.3 – 12.2)	1.2	(0.4 – 3.4)
ER	MH (pos)	32	1504	7	4.7	(2.2 – 9.8)	1.0	(ref)
	NL (neg)	35	1673	9	5.4	(2.8 – 10.3)	1.1	(0.4 – 2.9)
Apop index	< 3	25	804	4	5.0	(1.9 – 13.3)	1.0	(ref)
	3 <5	28	816	6	7.4	(3.3 – 16.4)	1.8	(0.5 – 6.5)
	5 <7	23	983	3	3.1	(1.0 – 9.5)	0.7	(0.2 – 3.1)
	≥ 7	16	535	3	5.6	(1.8 – 17.4)	0.9	(0.89 – 0.99)

Appendix 22: Analysis for disease free survival (DFS) of complete sample, using Cox regression analysis adjusted for age (N=79)

Variable	Category	N	Person time	Regressions	Regr. rate	95% CI	HR	95%CI
Trastuzumab	No	19	2775	17	6.1	(3.8 – 9.9)	1.0	(ref)
	Yes	60	818	3	3.7	(1.2 – 11.4)	0.6	(0.2 – 2.1)
Stage	1 (T1)	14	577	2	3.5	(0.9 – 14.0)	1.0	(ref)
	2 (T2)	29	2063	10	4.8	(2.6 – 9.0)	1.5	(0.3 – 7.1)
	3 (T3)	36	911	8	8.8	(4.4 – 17.6)	2.9	(0.6 – 14.9)
	4 (T4)	1	42	0	-	-	-	-
Grade	1	14	689	3	4.3	(1.4 – 13.5)	1.0	(ref)
	2	29	1356	5	3.7	(1.5 – 8.9)	0.8	(0.2 – 3.5)
	3	36	1548	12	7.8	(4.4 – 13.6)	1.9	(0.5 – 6.9)
Age	1(<45)	15	665	3	4.5	(1.5 – 14.0)	1.0	(ref)
	2 (45-65)	51	2298	15	6.5	(3.9 – 10.8)	1.5	(0.4 – 5.2)
	3 (>65)	13	630	2	3.2	(0.8 – 12.7)	0.7	(0.1 – 4.2)
PTEN	High	48	2181	12	5.5	(3.1 – 9.7)	1.0	(ref)
	Low	21	940	7	7.4	(3.6 – 15.6)	1.4	(0.6 – 3.7)
	Neg	10	472	1	2.1	(0.3 – 15.0)	0.4	(0.05 – 2.8)
NFκB	High	74	3351	18	5.4	(3.4 – 8.5)	1.0	(ref)
	Low	5	242	2	8.3	(2.1 – 33.0)	1.6	(0.4 – 7.3)
MDM2	High	65	2964	15	5.1	(3.1 – 8.4)	1.0	(ref)
	Low	10	465	2	4.3	(1.1 – 17.2)	0.8	(0.2 – 3.5)
	Neg	4	164	3	18.3	(5.9 – 56.7)	**3.5**	**(1.01 – 12.1)**
Akt	High	52	2297	12	5.2	(3.0 – 9.2)	1.0	(ref)
	Low	22	1062	7	6.6	(3.1 – 13.8)	1.3	(0.5 – 3.3)
	Neg	5	234	1	4.3	(0.6 – 30.3)	0.8	(0.1 – 6.0)
PI3K	High	65	2948	18	6.1	(3.8 – 9.7)	1.0	(ref)
	Low	13	594	2	3.4	(0.8 – 13.5)	0.5	(0.1 – 2.2)
	Neg	1	51	0	-	-	-	-
P53	High	16	631	6	9.8	(4.4 – 21.8)	1.0	(ref)
	Low	20	977	4	4.1	(1.5 – 11.0)	0.4	(0.1 – 1.4)
	Neg	43	2003	10	5.0	(5.0 – 2.7)	0.5	(0.2 – 1.3)
BCL2	High	38	1836	11	6.0	(3.3 – 10.8)	1.0	(ref)
	Low	17	749	4	5.3	(2.0 – 14.2)	0.9	(0.3 – 2.9)
	Neg	24	1008	5	5.0	(2.1 – 11.9)	0.9	(0.3 – 2.5)
Her2	Neg	33	1780	8	4.5	(2.2 – 9.0)	1.0	(ref)
	Pos	46	2634	12	4.6	2.6 – 8.0)	0.7	(0.3 – 1.8)
ER	MH (pos)	39	1765	10	5.7	(3.0 – 10.5)	1.0	(ref)
	NL (neg)	40	1828	10	5.5	(2.9 – 10.2)	1.0	(0.4 – 2.3)
Apop index	< 3	23	1041	3	2.9	(0.9 – 8.9)	1.0	(ref)
	3 <5	25	1114	8	7.1	(3.6 – 14.4)	2.7	(0.7 – 10.4)
	5 <7	19	866	6	6.9	(3.1 – 15.4)	2.5	(0.6 – 10.0)
	≥ 7	11	533	3	5.6	(1.8 – 17.5)	2.1	(0.4 – 10.7)

Appendix 23: Analysis of DFS of cohort of cases that has not trastuzumab treatment, using Cox regression analysis adjusted for age (N=60)

Variable	Category	N	Person time	Regressions	Regr. rate	95% CI	HR	95%CI
Stage	1 (T1)	7	370	1	2.7	(0.4 – 19.2)	1.0	(ref)
	2 (T2)	36	1679	9	5.4	(2.8 – 10.3)	2.0	(0.2 – 15.5)
	3 (T3)	16	684	7	10.2	(10.2 - 21.5)	3.9	(0.5 – 13.8)
	4 (T4)	1	42	0	-	-	-	-
Grade	1	12	602	3	5.0	(1.6 – 15.5)	1.0	(ref)
	2	25	1184	5	4.2	(1.8 – 10.1)	0.8	(0.2 – 3.5)
	3	23	989	9	9.1	(4.7 – 17.5)	2.0	(0.5 – 7.3)
Age	1(<45)	8	334	2	6.0	(1.5 – 24.0)	1.0	(ref)
	2 (45-65)	44	2012	14	6.9	(4.1 – 11.7)	1.2	(0.3 – 5.1)
	3 (>65)	8	422	1	2.4	(0.3 – 16.8)	0.4	(0.03 – 4.2)
PTEN	High	35	1614	10	6.2	(3.3 – 11.5)	1.0	(ref)
	Low	16	708	7	9.9	(4.7 – 20.7)	1.6	(0.6 – 4.2)
	Neg	9	453	0	-	-	-	-
NFκB	High	55	2533	15	5.9	(3.6 – 9.8)	1.0	(ref)
	Low	5	242	2	8.3	(2.1 – 33.0)	1.5	(0.3 – 6.8)
MDM2	High	47	2193	12	5.5	(3.1 – 9.6)	1.0	(ref)
	Low	9	418	2	4.8	(1.2 – 19.1)	0.8	(0.2 – 3.8)
	Neg	4	164	3	18.3	(5.9 – 56.7)	3.2	(0.9 – 11.3)
Akt	High	33	1479	9	6.1	(3.2 – 11.7)	1.0	(ref)
	Low	22	1062	7	6.6	(3.1 – 13.8)	1.1	(0.4 – 3.1)
	Neg	5	234	1	4.3	(0.6 – 30.3)	0.7	(0.1 – 5.4)
PI3K	High	50	2318	15	6.5	(3.9 – 10.7)	1.0	(ref)
	Low	10	457	2	4.4	(1.1 – 17.5)	0.7	(0.1 – 2.9)
	Neg	0	-	0	-	-	-	-
P53	High	11	406	6	14.8	(6.6 – 32.9)	1.0	(ref)
	Low	16	771	4	5.2	(1.9 – 13.8)	0.3	(0.1 – 1.1)
	Neg	33	1598	7	4.4	(2.1 – 9.2)	**0.3**	**(0.1 – 0.8)**
BCL2	High	31	1498	9	6.0	(3.1 – 11.5)	1.0	(ref)
	Low	13	580	4	6.9	(2.6 – 18.4)	1.2	(0.3 – 4.1)
	Neg	16	697	4	5.7	(2.2 – 15.3)	1.0	(0.3 – 3.1)
Her2	Neg	33	1509	10	6.6	(3.6 – 12.3)	1.0	(ref)
	Pos	27	1266	7	5.5	(2.6 – 11.6)	0.8	(0.3 – 2.2)
ER	MH (pos)	29	1319	8	6.1	(3.0 – 12.1)	1.0	(ref)
	NL (neg)	31	1456	9	6.2	(3.2 – 11.9)	1.0	(0.4 – 2.7)
Apop index	< 3	17	784	4	3.8	(1.2 – 11.9)	1.0	(ref)
	3 <5	17	757	6	7.9	(3.6 – 17.6)	2.2	(0.5 – 8.8)
	5 <7	17	788	3	6.3	(2.6 – 15.2)	1.6	(0.4 – 6.9)
	≥ 7	8	407	3	7.4	(2.4 – 22.9)	2.1	(0.4 – 10.2)