MD Thesis

Biomarkers to individualise adjuvant systemic therapy in early breast cancer patients

By
Maung Maung Myat Moe

Supervisors

Dr Richard Adams
Professor Robert Mansel

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1.0 Abstract

Background

Adjuvant chemotherapy, endocrine therapy, anti-HER2 therapy and radiotherapy significantly improve recurrence free and overall survivals in early breast cancers. Indications for a particular therapy have been well defined. Examples include oestrogen receptor positivity for endocrine therapy; HER2/Neu protein overexpression for anti-HER2 therapy; young age group, lymph node positivity, nuclear grade 3 and triple negativity (ie, ER/PR/HER2 negative) etc for chemotherapy; lumpectomy, > 5 cm tumour size, > 4 lymph nodes involvement etc for radiotherapy. Compared to no chemotherapy adjuvant chemotherapy can reduce the 10 years breast cancer mortality risk by one third although the absolute benefit depends on the absolute risk before the adjuvant chemotherapy as the risk reduction is proportional. The absolute risk depends on the various clinical and histopathological risk factors such as age, nuclear grade, tumour size, lymph node involvement, oestrogen hormone and HER2 receptor expressions. Various clinical guidelines, prognostic/ predictive tools and tests have been developed to calculate the absolute breast cancer specific survival risks and chemotherapy benefits to help in making the decision of "potential benefit outweighs the potential treatment toxicities" to recommend the adjuvant chemotherapy on individual basis. This principle aims to identify patients with very good prognosis for whom the toxic chemotherapy could be safely omitted and also patients with prognosis poor enough to justify offering toxic chemotherapies. However, no studies have specifically focussed on identifying patients in whom the chemotherapy could not deliver the expected benefit. Analysing molecular biomarker proteins that are functionally important in the cancer biology and chemotherapy cell killing mechanism using readily available and relatively inexpensive immunohistochemistry (IHC) method might be able to identify this

group of patients and find the targets against which novel therapy could be developed to improve their survival outcomes.

Objective

This study aims to identify potential molecular biomarkers that indicate a failure of adjuvant chemotherapy in early breast cancers in terms of cancer recurrence within five years and to create hypotheses relating to the use of effective novel targeted adjuvant therapy to improve the outcomes.

Methods

A matched case control, exploratory study was performed. Cases were patients relapsing within 5 years from the date of curative surgery and received adjuvant chemotherapy (with or without hormone/radiotherapy). Controls were patients who remained recurrence free for a minimum of 5 years from the date of surgery and had adjuvant chemotherapy (with or without endocrine/radiotherapy). Controls were matched to the cases by 10 years recurrence risk (RR) using "Adjuvant!" prognostic tool. "Matched controls" should also have positive axillary lymph nodes, Adjuvant RR at least > 50% and not more than 10% absolute points lower than their matched cases. "Low risk control" group (patients who are recurrence free for 5 years following surgery, have RR < 50%, and had adjuvant chemotherapy) was also included for the exploratory analysis purpose. Clinicopathological data was collected from the case notes. Formalin fixed paraffin embedded (FFPE) tumour blocks were retrieved from the storage and H & E slides were prepared. The most suitable part of the invasive cancer was marked on the slide guided by an experienced pathologist. Depending on the tissue availability, up to three tissue cores each measuring 0.6 mm in diameter were collected from the different parts of the block corresponding to the area marked on the H & E slides. Tissue microarray (TMA) blocks were constructed using a manual Beecham tissue arrayer®.

TMAs were cut into 4 micrometers thick slices and then mounted on super frost glass slides. IHC staining was optimised before performing final staining using Ventana automated staining machine, as per manufacturer's instruction after antigen optimisation for each proteins biomarkers namely ER, PR, EGFR, HER2, Ki-67, CK-5/6, Bcl-2, MCM-2, Bag-1, Aurora A, PDGFR alpha, CD-68, CD-71, VEGFR-2, Cathepsin L2, Plk-1 and GSTM-1. The TMA slides were scanned and digitalised images were obtained using a Mirax® scanner. The biomarker proteins expression was analysed using 20x power fields. 10% of cores were randomly examined by the second independent observer. Associations between survival outcomes and individual biomarkers or molecular subtypes (Luminal A, Luminal B, HER2 enriched, Core Basal, 5-Negative classified according to expressions of 6 biomarkers namely ER, PR, HER2, EGFR, Ki-67 and CK-5/6) were analysed by Chi-square test, independent samples Student t test, Log Rank test, Kaplan-Meier and Cox multivariate regression model using SPSS 16v software. Correlations between survival outcomes, molecular subtypes and RR according to the prognostic tools ("Adjuvant" & "OPTION") were also evaluated. The study was approved by the local ethic committee. The project was funded by the research grant from Pfizer.

Findings

The study includes a total of 178 patients (72 patients each in "cases" and "matched controls", 34 patients in "low risk control" groups). Molecular subtyping was possible for 170 patients as IHC assessment had failed in 7 patients. Luminal A (LA) is seen more commonly in the control group while Luminal B (LB), HER2 enriched (H), Core Basal (CB) and 5 markers negative (5N) are seen more commonly in the cases group.

There are no differences between the cases and the matched controls in their mean and median RR by "Adjuvant!" (10-years)/ "OPTION" (5-years). There are also no differences in

mean and median "Adjuvant!" or "OPTION" RR among different molecular subtypes. No correlation could be established between "Adjuvant!"/ "OPTION" RR and the survival outcomes in the matched controls and cases cohort.

There are statistically significant differences in RFS and OS between molecular subtypes. The median RFS and OS (months) for LA: LB: H: CB: 5N are: not reached: 58.1: 28: 15.4: 19.9 ($p = <0.001$); and not reached: 86.1: 55.9: 30.4: 26 ($p = <0.001$) respectively.

In **univariate** analysis **better** RFS and OS were observed for IHC positive Bcl-2 ($p = 0.036$ & 0.058), positive MCM-2 ($p = 0.01$ & 0.03), positive Bag-1 ($p = 0.018$ & 0.018) and negative Aurora A ($p = 0.001$ & 0.001) expressions. Trends for **better** RFS and OS were observed for negative PDGFR-α ($p = 0.07$ & 0.085) and negative CD-71 ($p = 0.097$ & 0.081) expressions.

In **multivariate** analysis, statistically significant factors (hazard ratios in the bracket) for RFS are as follows: CB (5.7) compared to LA; Bag-1+ (0.26); MCM-2+ (0.169); Aurora A+ (3.494); T3 (3.596); N1 (0.305). Significant factors for OS are: LB (2.37), CB (11.29), H (3.14), 5N (7.71); Bag-1+ (0.5); CD-68+ (0.45); MCM-2+ (0.38); Aurora A+ (2.64); N1 (0.035).

25% of Luminal A patients are cases (ie, RFS < 5 years). The 5-years recurrence risk of 25% is a lot higher than that reported for LA cancers from other studies with different risk populations. No biomarkers that could predict disease recurrence within 5-years in LA were found.

In non-luminal cancers (ie. both ER and PR negative) **positive** expressions of CK-5/6 and Aurora A were associated with worse RFS while **positive** expressions of Bag-1 and MCM-2 were associated with better RFS.

Negative Aurora A expression is associated with the better RFS and OS in non-LA patients. (No statistical significance was achieved for LA cancers.)

The IHC expressions of proteins coded for by the genes used in Oncotype Dx assays (ER, PR, HER2, Bcl-2, Bag-1, Ki-67 and Aurora A) show similar positive/ negative impact on RFS except CD-68 (as tumour associated macrophages infiltration) that is associated with the good prognosis similar to other published studies.

Conclusion

This study identified Bag-1 protein expression as a predictor for better survival, in keeping with the contribution of Bag-1 mRNA to Recurrence Score within the Oncotype-Dx tool. The finding of a trend for lower CD-71 expression (<=17) relates to better RFS (p=0.097) and OS (p=0.081) is the first observation that CD-71 (the iron transporter transferrin receptor which has been equated with proliferative capacity of tumours) may relate to its association as a poor prognostic factor, rather than a predictive factor for therapy (although a combination is probable), even in patients who had adjuvant chemotherapy. This adds to our knowledge that CD-71 relates to worsened endocrine outcome in breast cancer patients.

This study showed that 5 IHC defined molecular subtypes can predict differing survival outcomes in patients with similar "Adjuvant!" 10-years and "OPTION" 5-years RR treated with adjuvant chemotherapy. This suggests the superiority of this IHC based assessment over and above the globally utilised Adjuvant! RR score. In this patient population, LA was found to have the best survival while CB and 5N have the poorest survivals. This is in agreement with the results from previous publications. However, Luminal A cancers with OPTION 5 years RR > 40% should be offered chemotherapy due to the high 5 years recurrence rate (although this may need to be validated in an independent study) and predictive molecular markers are needed for better patient selection.

Subtypes CB & 5N, **positive** CD-71, Aurora A, PDGFR-α, and **negative** Bag-1, Bcl-2 and MCM-2 expressions were predictive of poor RFS and OS and should be used as stratification factors for novel prospective biomarker led adjuvant studies. Novel theraputic agents that modify the biological functions of these proteins should be explored in the well designed clinical trials.

2.0 Epidemiology

Over the last few decades "cancer" has become one of the most common medical illnesses in the developed world. (http://info.cancerresearchuk.org/cancerstats published in April 2011) One in 3 people in the UK will develop cancer during their lifetime and one in four people will die from it. Around 320,500 people were diagnosed with cancer in the UK in 2009, equating to a crude rate of 519 cases per every 100,000 people. The European age-standardised rate for UK is 429 per 100,000 for men and 372 per 100,000 for women. Breast, colorectal, lung and prostate cancers account for half of all cancers. (Cancer Stats Incidence 2009 - UK, CRUK, published in May 2012) In women, breast cancer is the most common type of cancer, accounting for 31% of all cancers. The age standardised rate is 124 per 100,000 women and the life-time risk is 1 in 8. In 2009, a total of 48,417 women were diagnosed with breast cancer in UK. (Cancer Stats Incidence 2009 – UK)

The risk of having breast cancer increases with age. 81% of cancers were diagnosed in 50 and over age group while 48% of cancers were diagnosed in the 50-69 year age group. (Cancer Stats Incidence 2009 – UK) Some of the other known risk factors include BRCA 1 & 2 genes mutation, early onset of menstruation, late menopause, low number of live-born children (parity), older age at first completed pregnancy, some forms of benign breast disease, exposure of developing breast tissue to radiation, use of products containing oestrogen and progestrone (either oral contraceptives or hormone replacement therapy), lifestyle parameters such as obesity, high intake of meat, saturated, monounsaturated and polyunsaturated fat.

Improvements in breast cancer management since the 1970s led to the prevention of 25-30% breast cancer deaths in the year 2000. (Peto, 2000) Estimated 10 and 20 years survival rates have improved from 54% and 44%, respectively, for women diagnosed in early 1990's to

72% and 64%, respectively, for women diagnosed in 2001 – 2003. Age standardised 5 year survival rate for 2005 – 2009 is 85.1% for England with the very similar rate for the rest of the UK. (http://info.cancerresearchuk. org/cancerstats/ types/ breast/survival/) Yet 11,556 women and 77 men died from breast cancer in year 2010 in UK. To push the current survival successes to the next level, it is of paramount importance to identify patients who are at especially high risk of recurrence and death despite adjuvant therapies, so that novel treatments can be explored for these patients. This study aims to achieve this goal by analysing biomarkers to predict failure from adjuvant chemotherapy, a treatment that can be applied to any type of cancer.

3.0 Early Breast cancer and its treatments

Breast cancer is highly curable when it is diagnosed at an early stage where the cancer is macroscopically confined to the breast and the regional lymph nodes namely ipsilateral axillary, internal mammary and the supra clavicular nodes. Surgery – either mastectomy or wide local excision - is the mainstay of the curative treatment. However, in some patients cancer would recur locally or at a distant site at a later date due to the spread of micrometastases before surgery. When micrometastases grow and become macrometastases over a variable period of time, the disease is rarely curable and most patients die from it. The Surveillance, Epidemiology and End Results (SEER) database from United States for 2003 – 2009 showed that 61% of breast cancer cases were diagnosed at an early stage while being confined to the breast; 32% were diagnosed after the regional spread (regional lymph nodes or directly beyond the primary site) and 5% were diagnosed at the metastatic stage with corresponding 5-year relative survival rates of 98.6%, 84.4% and 24.3%, respectively. (http://seer.cancer.gov/statfacts/html/breast.html#survival assessed on 08.07.13) Average survival after the identification of metastatic disease is around 22 months and the intention of treatment at this stage is to relieve symptoms and improve quality of life, although the prolongation of life is often possible. (Chia, 2007) (Gennari, 2005) To prevent such devastating outcomes, patients at risk of developing metastatic disease are offered and administered so-called adjuvant therapies with the aim of clearing "micrometastases". Currently there are chemotherapy, endocrine therapy, anti-HER2 therapy and radiotherapy available and they have been shown to improve both short and long term survivals. (EBCTCG, 2005, 2011, 2012) (Baselga, 2006)

In the past, breast cancer was treated as a single disease with mutilating extensive surgery. By the first half of the 20th century, clinicians had become aware that not all breast cancers

shared the same prognosis or required the same treatment, and attempts were made to define characteristics that could reliably distinguish those tumours that required aggressive treatment from those that did not. Clinical staging systems were introduced to classify patients for an appropriate treatment. The tumour-node-metastasis (TNM) system was developed by Pierre Denoix in the 1940s (www.uicc.org/system/files/.../History_Evolution_Milestones_0.pdf assessed on 27.11.2012) and was later updated by the International Union Against Cancer and the American Joint Committee on Cancer to divide breast cancer into different stages that have significant differences in survival. Five year survival data from US National Cancer Database for patients diagnosed in 2001 and 2002 is shown in the table below. (http://www.cancer.org/Cancer/BreastCancer/DetailedGuide/breast-cancer-survival-by-stage assessed on 08.07.13)

AJCC staging and 5 years survival rate for breast cancer	
AJCC Stage	**5-year Survival Rate**
0	93%
I	88%
IIA	81%
IIB	74%
IIIA	67%
IIIB	41%
IIIC	49%
IV	15%
(Please see appendix 1 for AJCC staging.)	

The choice of adjuvant treatments depends on the patient and tumour characteristics, potential benefits, potential toxicities and acceptability to the patient. Early Breast Cancer Treatment and Collaborative Group (EBCTCG) overviews demonstrated that some taxane-plus-anthracycline-based or higher-cumulative-dosage anthracycline-based regimens (not requiring stem cells) reduced breast cancer mortality, on average, by about one-third.

(EBCTCG, 2012) This means that the remaining two thirds of patients who really need adjuvant therapies to clear micrometastases did not achieve the expected benefit from even most effective adjuvant chemotherapy regimes. This is because although the adjuvant chemotherapy is proven to be effective, "one size fit for all" approach can't be applied. In the absence of 100% effective therapies, the best way of improving treatment outcome in the future is to give the right treatment to the right patients - a "personalised" approach. However, it remains a major challenge to identify at the individual patient level who is likely or unlikely to benefit from specific chemotherapy.

Risk can be assessed by the presence of prognostic factors. A prognostic factor is any feature of the patient or the tumour that can be used to foresee the patient's natural history in terms of cancer survival. Prognostic factors correlate with the survival in the absence of specific theraputic intervention, and are used to select patients at risk. Currently the strongest prognostic factors for the survival in the breast cancer include presence or absence of distant metastases, histologic grade, nodal status, tumour size, age, oestrogen receptor and HER2/neu status. Various prognostic tools have been developed to combine these factors for higher accuracy in risk calculation. For example, Nottingham Prognostic Index (NPI), by using tumour size, grade and lymph node status, stratifies patients into different groups that have different survival outcomes. (Rampaul, 2001)

NPI Group	NPI Score	15 years overall survival
Good	>2.4	80%
Moderate	2.4 – 5.4	42%
Poor	>5.4	23%

NPI and 15 years overall survival

Freely available internet web-based decision-making tools such as Adjuvant!, (Ravdin, 2001) PREDICT (Wishart, 2010) and OPTION (Campbell, 2010) became available to estimate 5 - 20 years disease recurrence and mortality risk as well as the benefit from various systemic adjuvant therapies based on the clinicopathological parameters such as mode of diagnosis (symptomatic or screen detected cancer), age, general health status, hormone receptor status, HER2 status, nodal status, tumour grade and size.

In contrast to the prognostic factors, the predictive factors correlate outcomes from the therapeutic intervention independent of prognosis, and have a significant impact in selected patient populations. A marker has a predictive value only if its presence or absence could foretell the outcome from a particular therapy such as response or survival; it may or may not have a prognostic value. In early breast cancer, predictive factors for systemic adjuvant therapies benefit include age, hormone receptor status and Ki-67 expression for adjuvant chemotherapy, oestrogen receptor (ER) and progesterone receptor (PR) status for endocrine therapy and HER2 expression status for anti-HER2 trastuzumab therapy.

Although a large number of molecular proteins have been extensively investigated, no tumour biological factor has any useful predictive value for response to any specific chemotherapy regime. There are some reports on predictive value of molecular markers to response to some chemotherapies such as HER2 expression for some chemotherapy regimens, (Cheang, 2012) triple negative cancers (ie. ER/ PR, HER2 expression) for classical CMF chemotherapy, (Colleoni, 2010) ER status for paclitaxel (Henderson, 2003) and ER, Ki-67, HER2 status for docetaxel (Hugh, 2009) but these are not yet fully validated to be used as a standard practice.

4. Adjuvant chemotherapy

Systemic adjuvant chemotherapy was first introduced in early 1970s, initially with single agents such as Melphalan. (Carpenter, 1983) In 1975, Bonadonna et al reported distant recurrence benefit from 12 months treatment with adjuvant polychemotherapy CMF (Cyclophosphamide, Methotrexate, 5-FU). (Bonadonna, 1976) (Bonadonna, 1995) After 27 months of study, only 5.3% of 207 women treated with CMF relapsed while 24% of 179 women without chemotherapy relapsed. The benefit was seen across all the subgroups. Since then CMF had become a standard for many years and studies have been carried out to answer various questions such as optimal number of CMF cycles (1 or 3 or 6 or 12) and its effectiveness in node negative or ER negative tumours.

After a median follow up of 28.5 years with a minimum follow up of 25.4 years, adjuvant CMF was found to significantly reduce the relapse with hazard ratio (HR) = 0.71, 95% confidence interval (95%CI) = 0.56 to 0.91; P = 0.005 and death (HR = 0.79; 95%CI = 0.63 to 0.98; P = 0.04), compared to no chemotherapy. (Bonadonna, 2005) In the node negative and oestrogen receptor negative trial, intravenous CMF significantly reduced the relapse (HR = 0.65; 95%CI = 0.47 to 0.90; P = 0.009) and death (HR = 0.65; 95%CI = 0.47 to 0.92; P = 0.01) at a median follow up of 20 years. The patients who received optimal doses of CMF (85% of the planned doses) showed a long lasting, superior benefit (RFS= 42%; 95% CI = 26% to 59%; OS = 40%; 95% CI = 26% to 55%) compared with patients who received lower doses (RFS = 26%; 95% CI = 19% to 33%; OS = 21%; 95% CI = 14% to 26%, respectively). No detrimental effect of adjuvant chemotherapy was seen for any of the subsets of patients.

After a median follow up of 25 years, 6 cycles of CMF was found equally effective as 12 cycles. (Bonadonna, 2005) 3 cycles of CMF was found to be as effective as 6 cycles for

disease free survival in ER positive women aged over 40. But it was not as effective as 6 cycles in women younger than 40 years and ER negative tumours. (Colleoni, 2002) The IBCSG Trial V also showed that a single cycle of perioperative CMF improved the outcome compared with no chemotherapy in node negative disease, but it was found to be less effective than 6 cycles of CMF in node positive disease. (Ludwig Breast Cancer Study Group, 1988) EBCTCG overview in 1998 showed that the improvement in 10-year survival from CMF chemotherapy in premenopausal women younger than 49 years of age was between 7% and 11%, depending on the nodal status (improvement from 71% to 78% in node-negative and from 42 to 53% in node-positive patients). The figures in node-positive and node-negative women aged between 50and 69 years were 3% (46 to 49%) and 2% (67 to 69%), respectively. (EBCTCG, 1998)

EBCTCG's extended 15-year follow-up meta-analysis involving more than 14,000 patients found that anthracycline-containing regimens were significantly more effective at preventing recurrence (HR = 0.89) and decrease mortality (HR = 0.84) than were CMF regimens regardless of menopausal status, age, nodal and ER status. (EBCTCG, 2005b) However only two types of anthracycline based regimens - FAC or FEC like and sequential anthracycline - Epirubicin or Doxorubicin - followed by CMF (E-CMF or D-CMF) - have shown to be superior to CMF. (De Placido, 2005) (Bonneterre, 2005) Four cycles of doxorubicin + cyclophosphamide were shown to be equally effective as 6 cycles of CMF chemotherapy. (EBCTCG, 2012) The French Adjuvant Study Group (FASG) demonstrated that the optimal epirubicin-based chemotherapy in premenopausal, node-positive breast cancer patients was six cycles of FEC50 rather than three cycles. (Fumoleau, 2003) In a planned pooled efficacy analysis of the National Epirubicin Adjuvant Trial and the Scottish Cancer Trials Breast Group BR9601 trials, where 28% of the 2,391 patients enrolled were node-negative, it showed that E-CMF gave significantly better RFS (HR = 0.70) and OS (HR = 0.64),

compared to 8 cycles of classical CMF, irrespective of the nodal status. (Poole, 2006) Cameron et al showed that D-CMF is as effective as AC-Taxol and days 1 and 8 ECF in their audit of 329 women with early breast cancer with at least 4 axillary lymph nodes involvement. (Cameron, 2002)

Newer chemotherapies that contain taxane (docetaxel or paclitaxel) are shown to further improve the survival in both node negative and positive breast cancer patients compared to chemotherapies without taxane. (EBCTCG, 2012) The anti HER2 monoclonal antibody trastuzumab added to chemotherapy improves pathological complete response rate in neoadjuvant setting (Buzdar, 2005) (Wildiers, 2011) and survival in adjuvant setting in patients with cancer that over-expresses HER2 protein. (Viani, 2007)

In premenopausal women, chemotherapy can deliver its effect via ovarian suppression in addition to the direct cell killing as shown by the high incidence of chemotherapy induced amenorrhoea in 50% to more than 65% of patients and equal effectiveness of ovarian suppression with or without tamoxifen compared to chemotherapies. (Jakesz, 2002) (Kaufmann, 2003) However, pathological complete response rate of up to 54.6% to neoadjuvant chemotherapy (docetaxel + carboplatin) in triple negative breast cancers – higher than ER/PR negative, HER2 positive and ER/PR positive patients – (Chang, 2010) and benefit of chemotherapy in postmenopausal patients confirmed the importance of direct cell killing effect of chemotherapy independent of hormonal manipulation. (EBCTCG, 2008)

Drug-induced cell death is a result of checkpoint response to DNA and other cellular damages. Apoptosis as a mechanism of chemotherapy induced cell killing was first suggested in 1975. (Searle, 1975) Conventional nonspecific cytotoxic anticancer agents cause DNA damage, genome destabilization, cell cycle arrest, and cytotoxic cell stress on rapidly dividing cancer cells, activating p53, and killing the cells by indirectly activating the intrinsic

apoptotic pathway. Thus, the efficacy of anticancer treatments depends not only on the drugs' ability to cause cellular damage but also on the cells' ability to detect and respond to such damages. Non-proliferating cells are less sensitive to chemotherapy drugs because the cells have more time to repair the damaged DNA and the converse is true for the rapidly proliferating cells.

5. Patient selection for adjuvant chemotherapy

Whether or not to recommend adjuvant chemotherapy to a patient is the balance between potential benefits and potential side effects. Benefit from adjuvant chemotherapy depends on breast cancer mortality risk before chemotherapy. The low absolute risk implies low absolute benefit. Therefore only patients with high risk features are often offered adjuvant chemotherapy as the benefit is deemed to outweigh the potential risk of unpleasant treatment toxicities. However, there is no consensus on the amount of benefit for which toxic chemotherapy is justified. Half of the patients who participated in two clinical surveys claimed that they would take chemotherapy for 0.5 - 1% absolute benefit in the recurrence risk. (Ravdin, 1998) (Jansen, 2001)

St. Gallen's consensus and NCCN guidelines provide clear treatment pathways for adjuvant therapies. Prognostic tools such as Nottingham's Prognostic Index (NPI), OPTION, PREDICT and Adjuvant are useful to estimate the risk of cancer recurrence, mortality and the benefit from adjuvant chemotherapies for an individual patient. Recently developed molecular subtyping identifies different types of cancer based on the gene or protein expressions. (Perou, 1999) (Cheang, 2009) These subtypes have different risk levels and potential benefits from chemotherapy regardless of other known risk factors such as age, size, grade and lymph node status. (Houssami, 2012) (Caudle, 2012) Commercialised multi-gene assays such as Oncotype Dx and Mammaprint utilise the expressions of various genes in cancer cells to calculate the recurrence and mortality risk and an estimate of the benefit from chemotherapy for an individual patient. (Paik, 2004) (vant' Veer, 2002) Different assays use different sets of genes and divide patients into different risk groups for recommendation of chemotherapies. These gene assays, costing in the excess of £2000 per test, claimed to be superior to the previously mentioned freely available conventional methods and tools to

select patients who could get benefit from the adjuvant chemotherapy. (www.oncotypedx.com) (www.mammaprint.com) However, none of these gene assays are designed or able to identify patients who would get metastatic disease despite adjuvant chemotherapies. The way forward to improve this is to develop and utilise various biomarkers – prognostic, diagnostic, metabolic, etc. – for better selection of patients and treatments. Cancer Biomarker Collaborative Consensus made recommendations in eight critical areas for biomarker development - bio specimens, analytic performance, standardization and harmonization, bioinformatics, collaboration and data sharing, regulations, stakeholder education and communication, and science policy - and put forward 27 recommendations with corresponding action plans to enable integration of biomarkers into development of safer, more effective and less costly drugs. (Khleif, 2010)

(A) Conventional methods

Conventionally, the risks of cancer recurrence and mortality were assessed on an individual risk factor to make decision for a particular adjuvant therapy. The St. Gallen consensus 2009 (Goldhirsch, 2009) set out the criteria for systemic adjuvant therapies as below.

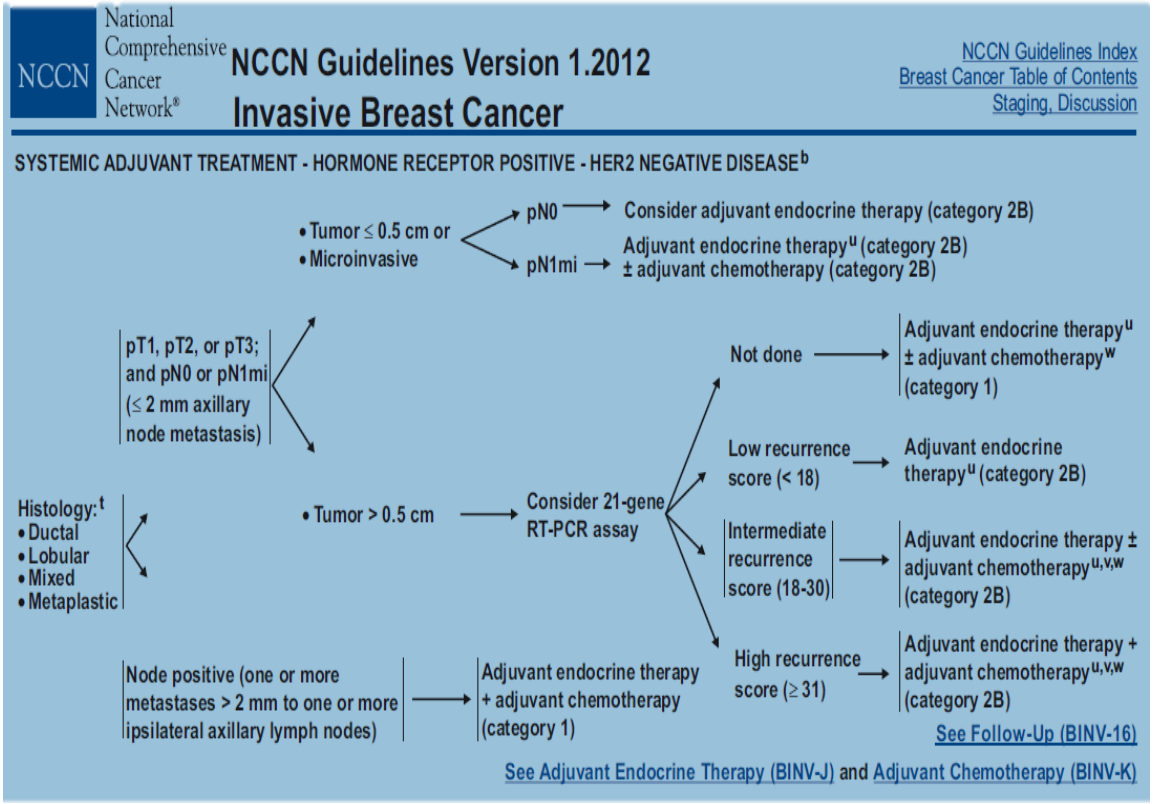
St. Gallen consensus, 2009 treatment recommendation guide table

	Relative indications for chemoendocrine therapy	Factors not useful for decision	Relative indications for endocrine therapy alone
Clinicopathological features ER and PgR	Lower ER and PgR level		Higher ER and PgR level
Histological grade	Grade 3	Grade 2	Grade 1
Proliferation	High[a]	Intermediate[a]	Low[a]
Nodes	Node positive (four or more involved nodes)	Node positive (one to three involved nodes)	Node negative
PVI (peritumoural vascular invasion)	Presence of extensive PVI		Absence of extensive PVI
pT size	>5 cm	2.1–5 cm	≤2 cm
Patient preference	Use all available treatments		Avoid chemotherapy-related side-effects
Multigene assays Gene signature	High score	Intermediate score	Low score

[a] = Conventional measures of proliferation include assessment of Ki67-labelling index (e.g. low, ≤15%; intermediate, 16%–30%; high, >30%)] and pathological description of the frequency of mitoses.

(This table was borrowed from the published article by Goldhirsch, 2009)

Well established cancer organisations such as National Cancer Institute (www.cancer.gov) and National Comprehensive Cancer Networks (www.nccn.org) published clear guidances and treatment pathways in a similar way to St. Gallens guidelines.



A slide from NCCN guideline for treatment of breast cancer (This slide was borrowed from NCCN guidelines version 1.2012 – "www.nccn.org" accessed in June 2012)

(B) Clinical Prognostic/ predictive tools

Various clinical tools have been developed to combine the different risk factors to give the best estimate of the risk and the benefits from various adjuvant treatments. The most commonly used and well-validated tools are described below.

PREDICT

The "PREDICT" tool was developed based on the outcome of 5,694 breast cancer patients from Eastern Cancer Registration and Information Centre (ECRIC), UK dataset. (Wishart, 2010) The tool is available free of charge at www.predict.nhs.uk/predict.shtml. Based on the age at diagnosis, mode of diagnosis (screening or symptomatic), tumour size, grade, nodal status, ER and HER2 status and different chemotherapies, the tool can produce 5 years and 10 years overall survival and the benefit from adjuvant endocrine therapy and chemotherapy. In ER negative disease, the 8 year actual breast cancer mortality rate was 25.0% compared to 30.6% predicted by the tool; for ER positive tumours, 8 years actual and predicted breast cancer mortality were within one percentage (8.9% vs. 9.2%). Overall model fit was good, although the fit was less good for some sub-groups. Specifically, for ER positive disease, the fit was not so good in women aged <49 years. For ER negative disease, the model fits in node negative disease, 30 to 49 mm tumours size category and high-grade tumours were not so good. The latest version incorporated the effect of HER2 status. The prediction of breast cancer specific survival was claimed to be superior to that of Adjuvant! (Wishart, 2012)

Adjuvant!

"Adjuvant!" is a web-based prognostication and treatment benefit tool for various cancers including breast cancer. (Ravdin, 2001) Based on the age, comorbidity, tumour size, grade, lymph node and ER status, it calculates 10 years risk of cancer recurrence and death, and the estimate of benefits from adjuvant therapies. The SEER (Surveillance, Epidemiology and End Results) data on 30,000 women aged 36 to 69 who were diagnosed between 1988 and 1992 and recorded in SEER registry in the United States were used to calculate the breast cancer related 5-year and 10-year mortality. Considering that on average, mortality occurs approximately 3 years after the relapse and that the annual hazard of contralateral breast cancer is approximately 0.65%, the annual hazard of relapse is estimated 1.3 times the breast cancer mortality hazard plus 0.65%. This formula is used to calculate 10 years cancer recurrence risk. The estimates of benefit from adjuvant therapies are obtained by applying proportional risk reduction (PRR) from Oxford overview or indirectly from these estimates. The PRR for combined chemotherapy and endocrine therapy is derived from the following formula.

PRR chemoendocrine therapy = 1 – [(1 – PRR chemotherapy) x (1 – Endocrine therapy)]

The tool is being updated to incorporate the impact of HER2 expression, anti-HER2 treatment and genomic assays risks. Although the "Adjuvant!" has been validated in various centres around the world, there are some uncertainties about how applicable "Adjuvant!" is to current patients diagnosed and treated in the UK. It has been shown that "Adjuvant!" overestimated the overall survival by 6% in a UK cohort of 1,065 women with early breast cancer treated in Oxford between 1986 and 1996. (Campbell, 2009)

OPTION

Using parametric regression-based survival analysis on the prognostic characteristics and outcomes of 1,844 women treated for early breast cancer at Churchill Hospital in Oxford between 1986 and 2001, "OPTION" prognostic model was developed to predict recurrence free survival. (Campbell, 2009) (Campbell, 2010) The model was able to separate patients into distinct prognostic groups, and predicted well at the patient level. It has been externally validated. When compared with the NPI, the model was able to better discriminate between women with excellent and good prognoses, and it did not overestimate the 10-year recurrence free survival to the extent observed for "Adjuvant!"

The OPTION tool can calculate estimated probability of 5, 10, 15 and 20 years RFS, before and after adjuvant treatments - radiotherapy, chemotherapy (none, CMF, Anthracycline and taxane) and endocrine therapy - using age, number of affected nodes, nuclear grade, tumour size and ER status. (www.herc.ox.ac.uk)

Nottingham Prognostic Index (NPI)

The Nottingham Prognostic Index (NPI), a prognostic scoring system developed based on a large cohort of early breast cancer (<50mm tumour size) patients treated between 1990 – 1999 in Nottingham City Hospital, UK, estimates the 10 – 15 years overall survival using tumour size, grade and lymph node status. NPI is calculated as below.

NPI = Nuclear Grade (grade 1 – 3) + Nodal status (1 = 0 nodes; 2 = 1 – 3 nodes; 3 = >4 nodes) + (tumour size in millimeter x 0.02)

The NPI index has been validated in various cancer centres. Initially, NPI index assigned patients to good (NPI score < 3.4), moderate (NPI = 3.4 – 5.4) and poor risk (NPI > 5.4) groups with 15 years survival of 80%, 42% and 13%, respectively. (Galea, 1992) Later it was extendedto six prognostic groups and the latest version divided patients into 10 different prognostic groups that have an excellent inverse correlation between median NPI value for each group and the 10 years overall survival. (Blamey, 2007a & b)

NPI range	*n*	Median NPI	10 year % Survival (BCS)	±95% CL
2.00–2.40	321	2.2	96	2
2.41–3.00	32	2.5	95	6
3.01–3.40	441	3.3	93	4
3.41–4.00	134	3.5	92	6
4.01–4.40	504	4.3	79	4
4.41–5.00	316	4.5	76	6
5.01–5.40	170	5.3	72	8
5.41–6.00	203	5.5	56	8
6.01–6.40	33	6.3	38	18
6.41–7.00	86	6.6	37	12

NPI prognostic index and breast cancer survival (This table was borrowed from the article by Blamey, 2007b)

The estimated survival for an individual NPI score is also available from the following formula (Blamey, 2007b):

10 year % survival for the individual = -3.0079 x NPI^2 + 12.30 x NPI + 83.84.

(C) Molecular subtype profiling

Intrinsic genes are genes showing significant variations in the expressions across different tumours from different patients but not between paired samples from the same tumour. Based on the intrinsic gene sets analysed by cDNA arrays, breast cancers were classified into 4 main molecular groups (Perou, 2000):

- **ER+/luminal like group** characterized by the relatively high expressions of many genes expressed by the breast luminal cells;
- **Basal like group** that expresses genes characteristic of breast basal epithelial cells such as CK-5/6, CK-14, CK-17, integrin b4 and laminin;
- **ErbB2-positive group** that overexpresses ErbB2 genes cluster that includes ErbB2 and GRB7; and
- **Normal like group** that has high expression of genes characteristic of basal epithelial cells and adipose cells, and the low expressions of luminal epithelium genes.

Luminal cancers are again divided into Luminal A and B for negative or positive, respectively, expressions of ERα gene, GATA binding protein 3, X-box binding protein 1, trefoil factor 3, hepatocyte nuclear factor 3 α, oestrogen-regulated LIV-1m MIK57 and / or HER2 (Sorlie, 2001) or Ki-67 protein IHC expression (at the cut-off point of 13.25% staining index). (Cheang, 2009)

Most commonly used platforms for gene expression profilings have been spotted complementary DNA and high density oligonucleotide microarrays. Both techniques need careful extraction of good quality RNA from the tumour. (reviewed by Cheang, 2008a) Similar molecular subtyping has been successfully performed using IMAC 30 (immobilized metal affinity capture) Protein Chip arrays. (Brozkova, 2008)

Using IHC expressions of six different molecular markers (ER, PR, HER2, EGFR, CK-5/6, Ki-67) breast cancers were classified into 5 different molecular subtypes that have different risks and survival outcomes (Cheang, 2009) (Cheang, 2008a, b):

- Luminal (ER and/or PR positive)
    - Luminal A (LA) - both Ki-67 and HER2 negative
    - Luminal B (LB) - Ki-67 and/ or HER2 positive
- HER2+/ER-PR- (H)
- Triple negatives phenotype (TNP) – ER/ PR/ HER2 negative. TNPs were again divided into:
    - Core Basal Phenotype (CB) for positive EGFR and / or CK-5/6 expression
    - 5 Markers Negative Phenotypes (5N) for negative ER/ PR/ HER2/ EGFR/ CK-5/6 expressions

Blows et al classified more than 10,000 patients from 12 studies into 6 molecular subtypes in a similar method. Luminal A was subdivided into basal marker CK-5/6 positive and negative. (Blows, 2010) Basal like breast cancers defined by IHC molecular markers were shown to have better prediction for the breast cancer survival than that defined by the gene analysis. (Cheang, 2008b) The concordances between IHC protein expressions and RT-PCR genes expressions were high for ER and HER2 but poor for PR, Ki-67 (Cobleigh, 2005) and CK-5/6. (Kordek, 2010)

There are significant differences in the incidence, survival, metastatic site specificity, and the treatment responses among molecular subtypes. LA cancers tend to have late recurrences while the basal and HER2+ groups have early recurrences. (Sorlie, 2003) The mortality rate is found to be constant over the time for LA cancers while it tends to peak within 5 years of the diagnosis and declines after that and reverses at 5–10 years in LB and non-luminal

subtypes. By 8 years after the diagnosis, there were no differences in the prognosis between LA and LB tumours; between basal marker positive and negative tumours within LA subgroup. Luminal subtypes have poor prognosis with longer follow-up time with the worst prognosis at 15 years being in the Luminal HER2 positive tumours. (Blows, 2010) In a study of molecular subtyping using gene profiling on 357 breast cancer patients, the 10-year relapse free survivals were 78% for LA cancers, 67% for LB cancers, and 64% for Luminal HER2 positive tumours. The 10-year breast cancer specific survivals (BCSS) were 92% for LA cancers, 79% for LB cancers, and 78% for Luminal HER2 positive tumours. (Cheang, 2009)

Differences in prognoses between TNP and non-TNP groups were reported to be most marked at 3 years (76.8% vs 93.5%, $p < .0001$). (Tischkowitz, 2007) CB has a slightly poorer prognosis than 5N with an absolute 10% lower 10-year BCSS. (Conforti, 2007) (Cheang, 2008b) Non-luminal HER2 positive tumours have a poorer prognosis than Luminal HER2 positive tumours, and the CB tumours have a poorer prognosis than the CK-5/6 positive LA tumours regardless of the adjuvant chemotherapy or endocrine therapy. Basal markers seem to have no prognostic significance within the HER2 positive subtypes.

In the neoadjuvant setting, LB, HER2 positive and TNP have higher pathological complete response (pCR) rate to chemotherapy than LA cancers. TNPs with higher Ki-67 expression have higher rate of pCR but the presence of residual disease is a very strong risk factor for the relapse. (Darb-Esfahani, 2009) (Carey, 2007) In the adjuvant setting the additional survival benefit from docetaxel containing chemotherapy over non-docetaxel chemotherapy was reported for LB cancers, LA cancers that had tamoxifen but not for other LA, TNP and HER2+ cancers. (Hugh, 2009) CB seemed to have less benefit from anthracycline based chemotherapy compared to 5N. (Conforti, 2007) (Cheang, 2008b)

Crabb et al used IHC expressions of eight molecular proteins (ER, PR, EGFR, HER2, CK-5/6, carbonic anhydrase IX, p53, Ki-67) to classify 4 or more lymph node positive patients treated with adjuvant chemotherapy into 3 different groups with mean 10-year RFS of 75.4%, 35.3% and 19.3%, respectively. In the validation set, differences in RFS for these subgroups remained statistically significant but were less marked. (Crabb, 2008a)

Because of the poor prognosis, basal like breast cancers were investigated extensively. The basal like cancers were characterised by the expressions of genes associated with myoepithelial basal cells: KRT5 (keratin 5), KRT17 (keratin 17), CNN1 (calponin 1), CAV1 (caveolin) and LAMB1 (laminin). (Perou, 2000) (Sorlie, 2001) (Sorlie, 2004) They are also characteristically negative for ER, PR and HER2 expressions but may express EGFR, KIT (CD117), Fascin and CD109, (Rodriguez-Pinilla, 2006) (Hasegawa, 2008) and frequently have mutated TP53 and BRCA1 genes. (Sorlie, 2003) (Laakso, 2006) The criteria of ER/PR negative, HER2 negative/low, CK-5/6 positive and/or EGFR positive IHC expressions can identify 76% of basal like cancers defined by the gene expression methods and it is 100% specific. (Nielsen, 2004) On the other hand, as much as 15-54% of basal like tumours defined on mRNA level still express at least one of ER, PR and HER2. (Reis-Filho, 2008) (Nielsen, 2004) (Calza, 2006) (Sotiriou, 2003) (Jumppanen, 2007)

The basal like cancers occur with peaks in the <35 and 51 to 65 years age groups. It is associated with many poor clinicopathologic features such as younger age, dense breast, oestrogen receptor negativity and p53 expression (Ihemelandu, 2007) (Collett, 2005) but less likely to have axillary nodal involvement (odd ratio 0.53). (Crabb, 2008b) Basal like cancers more often present as interval cancers while LA cancers present more often as screen detected cancers. (Sihto, 2008) (Collett, 2005) Basal like cancers are more often detected as an ill-defined mass (61%) while non-basal type cancers are detected as a spiculated mass

(49%). (Luck, 2008) Most of the metaplastic carcinomas and breast cancers that metastasise to the brain are more likely to to be core basal like cancers and have high nuclear grade. (Gilbert, 2008) (Hicks, 2006) Post mastectomy radiotherapy reportedly failed to improve the survival in basal like cancers although the benefit was clearly seen in ER/ PR positive patients in a study involving high risk breast cancers. (Kyndi, 2008)

BRCA1 associated cancers are more likely to be basal like subtypes compared to non-BRCA associated cancers (90% vs. 15%) (Nielsen, 2004) (Foulkes, 2003) (Lakhani, 2005) There are similarities between BRCA1 associated and sporadic basal like breast cancers such as ER negativity, high nuclear grade, high Ki-67, CK-5/6, EGFR expressions, and poor prognosis regardless of the nodal status (Foulkes, 2004) although P-cadherin and vimentin - markers associated with basal like breast cancers- are more often seen in BRCA1 associated cancers. (Arnes, 2005) (Rodriguez-Pinilla, 2007) BRCA1 mRNA expression was found to be two fold lower in sporadic basal breast cancers (as defined by CK-5/6 IHC positivity) compared to the age and grade matched non-basal ductal cancers. ID4, a negative regulator of BRCA1, was expressed at 9.1 folds higher level, suggesting a potential mechanism of BRCA1 down regulation. (Turner, 2007)

In-vitro studies of BRCA1 associated breast cancers have shown a marked sensitivity to agents such as bifunctional alkylating agent, mitomycin-C and platinum drugs that cause interstrand cross-links of DNA, etoposide and bleomycin that cause double strands breaks, but showed resistance to taxane and vinca alkaloids that target mitotic spindles and microtubules.

St. Gallen consensus 2011 recommended the use of ER, PR, HER2 and Ki-67 to subtype Luminal A, Luminal B, HER2 enriched and triple negative cancers. No adjuvant chemotherapy was recommended for Luminal A. Chemotherapies containing anthracycline

and taxane were recommended for Luminal B. For triple negative cancers, cyclophosphamide in addition to anthracycline and taxane was recommended, preferably in the dose dense form, while routine use of cisplatin or carboplatin was not recommended. Chemotherapy, anti-HER2 therapy and endocrine therapy were recommended for HER-2 positive luminal cancers and combination of chemotherapy and anti-HER2 therapy was indicated for non-luminal HER2 positive cancers. (Goldhirsch, 2011)

(D) Multigene assays

Multiple genes profilings had led to the development of various assays that offered prognosis, diagnosis and prediction of treatment outcomes in the management of breast cancer. These assays used different techniques and different combination sets of gene expressions. Although none of them has 100% sensitivity and specificity, they claimed to be superior to existing conventional methods that use clinical and histopathological features. These assays are said to give additional informations that change the direction of the treatment decisions for or against adjuvant chemotherapies in approximately 30% of the time. (Albain, 2009)

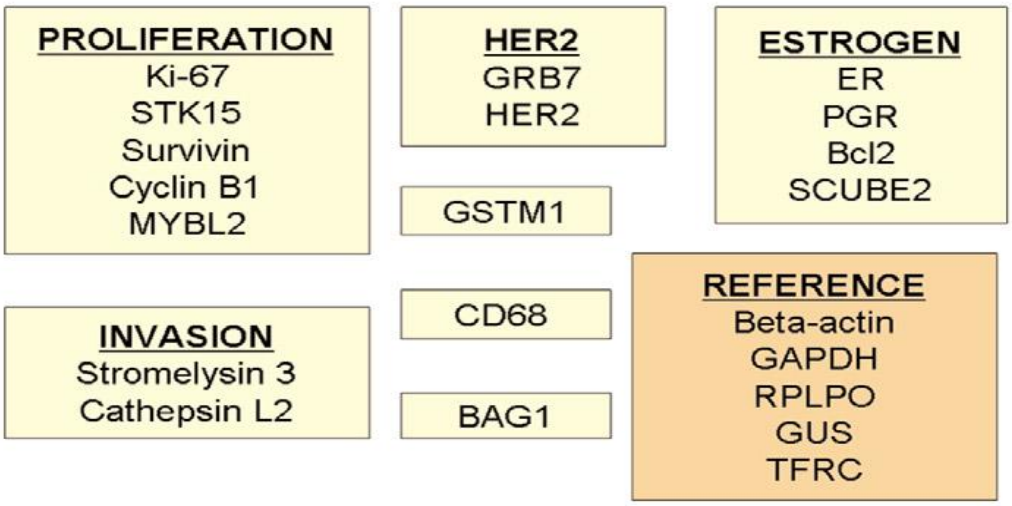
Oncotype Dx

OncotypeDx is an assay that utilises expressions of 16 cancer related genes and 5 reference genes (Table 1) in formalin fixed paraffin embedded tissues using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). (Paik, 2004) The genes are related to ‘‘ER cluster,’’ whose expressions were associated with longer distant relapse free survivals (DRFS) compared to lower or absent expressions, and ‘‘proliferation cluster’’, ‘‘macrophage cluster’’, “invasion cluster” and “HER2 cluster” whose expressions were associated with shorter DRFS. Different weights were given to different cluster genes expressions in the calculation of the recurrence score as shown in the table 1. Oncotype Dx also provides quantitative values for proliferation, luminal (ESR1, PGR) and ERBB2gene expressions.

RS is calculated as follow:

RS = 0 if RSu < 0; RS = 20 x (RSu - 6.7) if 0 ≤RSu ≤100; RS = 100 if RSu > 100.

(a)



(b)

Score	Formula
HER2 Group Score	(0.9 × GRB7) + (0.1 × HER2) If HER2 Group Score is less than 8 then the HER2 Group Score is considered equal to 8
ER Group Score	([0.8 × ER] + [1.2 × PgR] + Bcl2 + SCUBE2])/4
Proliferation Group Score	(SURV + KI-67 + MYBL2 + Cyclin B_1 + STK15)/5 If the Proliferation Group Score is under 6.5 then the Proliferation Group Score is considered equal to 6.5
Invasion Group Score	(Cathepsin L2 + Stromelysin 3)/2

(c)

RS_U =
+ 0.47 × HER2 Group Thresholded Score
− 0.34 × ER Group Score
+ 1.04 × Proliferation Group Thresholded Score
+ 0.10 × Invasion Group Score
+ 0.05 × CD68
− 0.08 × GSTM1
− 0.07 × BAG1

Table: Oncotype Dx genes list (a), individual group score calculation (b) and Recurrence score unscaled calculation (c). (This table was borrowed from the published article by Habel, 2006)

Oncotype Dx calculates the recurrence score (RS) that ranges from 0 to 100, the 10 years DRFS for an individual patient, and an estimate of adjuvant chemotherapy benefits. The assay classifies RS into 3 different risk levels: low risk for RS <18, intermediate risk for RS = 18 – 31, and high risk for RS >31. RS was associated with 10 years breast cancer death risk in patients who had adjuvant tamoxifen and also in patients who did not have any adjuvant treatment. There was also a significant association between RS and the risk for locoregional relapse. (Mamounas, 2010) (Habel, 2006)

High risk patients get most benefits from CMF chemotherapy in terms of 10 years distant disease recurrence (27.6% absolute risk reduction) while low risk patients get only minimal, if any, benefits among the participants of NSABP 20 trial. (Paik, 2006) The benefit was uncertain for the intermediate risk patients. The disease free and breast cancer specific survival benefits from CAF chemotherapy added to tamoxifen were seen in node positive patients from high risk but not low risk groups. The benefit was most significant in the first 5 years with no additional prediction beyond 5 years. Patients with a high ER expression (Allred score >6) and HER2 negative disease did not seem to gain any benefits from CAF. (Albain, 2010) RS was predictive of distance recurrence risk in tamoxifen or anastrozole treated node negative and positive postmenopausal patients. (Dowsett, 2010) The high RS was positively associated with the increased likelihood of pCR to neoadjuvant chemotherapy with doxorubicin + cyclophosphamide followed by paclitaxel in locally advanced breast cancer patients. (Gianni, 2005)

80 genes assay

Expressions of 80 different genes were analysed to predict a response to neoadjuvant chemotherapies in locally advanced breast cancers. pCR was shown to be more likely with high expressions of proliferation (including CDC20, E2F1, MYBL2, TOP2A, FBXO5, MCM2, MCM6, CDC25B) and immune related genes (including MCP1, CD68, CTSB, CD18, ILT-2, CD3z, FasL, HLA.DPB1, GBP1), and low expressions of ER related genes cluster (including PR, SCUBE2, ER, NPD009, GATA3, IGF1R, IRS1). Other genes predictive of pCR are related to apoptosis (BBC3, BAD, DR4, TP53BP1), invasion/ metastasis (FYN and MMP12), and drug resistance/metabolism (ABCC5, ALDH1A1, CYP3A4). (Gianni, 2005)

Mammaprint (70 genes assay)

Mammaprint, known as the 70 genes signature, was derived from a set of 231 genes that were differentially expressed in tumours that metastasised versus those that did not. (Vant' Veer, 2002) The proliferation is the main biological function of most of the genes. Some are associated with invasion, metastases, stromal integrity and angiogenesis. The assay divides patients into poor and good prognosis groups. It has shown that poor prognosis patients are 15 times more likely to develop distant metastases within 5 years compared to good prognosis patients among T1 – T2, N0 breast cancer patients younger than 55 years of age. (Isaacs, 2001) (Vant' Veer, 2002) 10 years distant metastases free and overall survivals for poor and good prognosis patients were 85.2% & 94.5% and 50.6% & 54.6%, respectively. (van de Vijver, 2002) The assay was also strongly predictive of survival in pT1 tumours with up to 3 positive nodes regardless of the ER status. (Mook, 2010) The test requires freshly frozen tissue or tissue collected in RNA preservative solution for the analysis. The test can't

be done on the FFPE tissue. The assay is currently marketed for invasive breast cancers T2 or smaller, any ER expressions and up to 3 positive lymph nodes.

PAM50

Prediction Analysis of Microarray (PAM) on 50 genes was developed from the original genes set that classified breast cancers into different molecular subtypes by Perou et al. (Perou, 2000) In a study that evaluated PAM50 against Oncotype Dx, 83% of Oncotype Dx low RS patients are LA cancers while 90% of high RS patients are LB cancers as classified by PAM50. 70% of LA cancers have low RS and the rests have intermediate RS. 33% and 48% of LB cancers have high and intermediate RS, respectively. There is a good agreement between the two assays for high (i.e., LB or RS > 31) and low (i.e., LB or RS < 18) prognostic risk assignments but PAM50 assigns more patients to the low risk category. About half of the intermediate RS group was reclassified as LA by PAM50. (Kelly, 2012)

PAM50 can be applied on FFPE tissues. It can calculate risk of recurrence (ROR) either based on the subtype classification alone (ROR-S) or in conjunction with the clinical features (ROR-C). The sum of the coefficients from the Cox model is the ROR score for the individual patient. The patients were categorized as low risk (ROR-S score < 23), moderate risk (score 23 – 53), and high risk (score > 53). ROR-S model has 94% sensitivity and 97% negative predictive value for identifying non-responders and pCR to taxol/FAC chemotherapy. (Parker, 2009)

ROR-S = (0.05 x basal) + (0.12 x HER2) + (-0.34 x Lum A) + (0.23 x LumB)

ROR-C = (0.05 x basal) + (0.11 x HER2) + (-0.23 x LumA) + (0.09 x LumB) + (0.17 x T)

Wound Response Signature

Chang et al. (Chang, 2004) identified a set of ‘‘core serum response’’ (CSR) genes – 512 cell cycle independent genes expressed by fibroblasts in response to serum exposure – that includes genes for entry into and progression through the cell cycle, induction of cell motility, extracellular matrix remodelling, cell–cell signalling and acquisition of a myofibroblast phenotype. Wound response signature that utilises CSR was shown to be predictive of poor overall survival and increased risk of metastases in several tumours such as breast, lung, and gastric cancers. In breast cancers with the size of <2.0 cm and any nodal status, the activated wound response signature was predictive of worse distant metastasis free and overall survival compared to the quiescent wound signature independently of any other known risk factors in the multivariate model. (Chang, 2005)

The fibroblast CSR genes set contains only 20 out of 456 genes from ‘‘intrinsic gene list’’ that was used for molecular subtyping by Perou et al. (Perou, 2000), 4 out of 128 genes that define the general metastasis signature reported by Ramaswamy et al (Ramaswamy, 2003) and only 11 out of 231 genes that van’t Veer et al analysed for Mammaprint. (van’t Veer, 2002)

Rotterdam 76 genes signature

Rotterdam 76 genes signature can predict the development of metastatic disease within 5 years in the absence of any adjuvant treatments for node negative patients with tumours 10 – 20 mm size, any ER and menopausal status. (Wang, 2005) The 5 and 10 years distant metastasis free survivals for good and poor prognosis groups were 98% & 94% and 76% & 73%, respectively. Corresponding overall survival rates were 98% & 87% and 84% & 72%, respectively. (Desmedt, 2007)

IE-IIE assay

IE-IIE assay, using 76 genes Affymetrix Human U133a Gene Chips (initially developed from supervised analyses on 822 genes), defined ER/PR positive patients into poor prognosis group (IIE) that showed high expressions of cell proliferation and anti-apoptosis genes, and the good prognosis group (IE) that showed high expressions of oestrogen and GATA3 regulated genes. IE and IIE are also associated with other known risk factors such as nuclear grades and molecular subtypes. IE tumours showed high expressions of XBP1, FOXA1, PR and many ribosomal genes. Hazard ratios for RFS and OS for group IIE compared to IE are 2.9 and 3.64, respectively. (Oh, 2006)

SET index

The SET (Sensitivity to Endocrine Therapy) index was developed from the analysis of 165 genes co-expressed with ESR1 in 437 microarray profiles from newly diagnosed breast cancers unrelated to the treatments or outcomes. (Symmans, 2010) It was significantly associated with the distant relapse or death risks in patients who had adjuvant tamoxifen or neoadjuvant chemotherapy followed by adjuvant tamoxifen (regardless of any pathologic response status), but was not prognostic in patients who did not receive any adjuvant treatments. No distant relapse or death was observed in node negative and high SET patients treated with adjuvant tamoxifen alone, and in intermediate or high SET patients treated with the neoadjuvant chemotherapy and adjuvant tamoxifen. The endocrine predictive utility of SET index was independent of pathologic response from chemotherapy. At 5 years of follow up, disease free survival rates were 100% for high or intermediate and 82.4% for low index groups using cut off SET values of 3.66 and 2.68. However, the prognosis of those with high residual disease following neoadjuvant therapies remained poor, irrespective of the SET value.

Triple Negative Breast Cancer genes assay

For triple negative breast cancers, 264 and smaller 26 genes sets can identify good and poor prognosis cancers with the hazard ratios of 4.03 and 4.08, respectively, for event free survival. The 10 years event free survival rates for good and poor risk groups are 70% and 20%, respectively. The 26-genes signature in combination with B-cell metagene can predict the response to the neoadjuvant chemotherapy. Most of the genes in the assay are related to metagenes for inflammation and angiogenesis, and are not related to other known genes signatures. (Karn, 2011)

MGH2-gene signature: 2-gene (HOXB13:IL17BR) ratio predictor

Homeobox gene B 13 (HOXB13) was shown to regulate a pathway in conjunction with the EGF signalling to promote cancer cell motility and invasion. (Ma, 2004) HOXB13 gene expression was associated with the shorter RFS while interleukin 17B receptor gene (IL17BR) expression was associated with the longer RFS in patients treated with adjuvant tamoxifen. The HOXB13:IL17BR index (cut off point of 0.06) predicted clinical outcomes in ER positive patients independently of adjuvant tamoxifen treatment in node negative but not in positive patients. (Ma, 2006) Two-gene index was a highly significant factor for predicting RFS with a hazard ratio of 3.9 in the same study. On the continuous scale, an untreated patient with a two-gene index of -2.0 has a 5-year recurrence risk of 15% (95% CI, 9.8% to 20.5%), whereas a patient with an index of +2.0 has a significantly higher 5-year recurrence risk of 36% (95% CI, 26.5% to 45.2%). (Ma, 2006) It is also predictive of an early relapse and death. (Goetz, 2006)

Other assays

Other assays include:

- eXagenBC, a prognostic (recurrence) test using 3 genes (CYP24, PDCD6IP, BIRC5) for ER positive and 3 genes (NR1D1, SMARCE1, BIRC5) for ER negative patients with any nodal status. (Davis, 2007)
- Celera metastatic score, a prognostic test that uses 14 genes multiplex RT-PCR to predict the distant metastatic disease. (Tutt, 2008)
- The Breast BioClassifier, a qRT-PCR assay that uses 55 genes for molecular subtyping. (Perrard, 2006)
- Invasive gene signature that analyses 186 genes using Affymetrix U-133 Gene Chip for any nodal and ER status. (Liu, 2007)
- Novoselect which is a combination of several pharmacogenomics gene sets (200 genes) such as the one that predicts response to neo-adjuvant chemotherapy. (Ayers, 2004) (Rouzier, 2005 a & b)
- MapQuant Dx (formerly known as the genomic grade index) that uses 97 unique genes to grade the tumour. (Sotiriou, 2006)
- Molecular grade index (MGI) that uses a set of 5 genes namely BUB1B, CENPA, NEK2, RACGAP1, and RRM2 that can differentiate histology grade 2 into MGI grade 1 and 2 (low and high risk group respectively). (Ma, 2008)
- Theros Breast Cancer Index which is a combination of MGI and 2 genes (HOXB13:IL17BR) ratio that can identify poor prognosis patients treated with adjuvant chemotherapy and endocrine therapy. (Ma, 2008)
- 7-genes immune response module, down regulation of which is associated with the high risk of distant metastases. (Teschendorff, 2007)

- Stroma derived prognostic predictor that is composed of 163 genes reflecting clusters reflecting hypoxia and angiogenesis (linked to poor outcomes) and a TH1-like immune response (linked to good outcomes). (Finak, 2008)
- Medullary like signature by Support Vector Machine (SVM) classifier that is based on 368 genes to define the low risk and high risk recurrence groups. (Sabatier, 2011)
- Cytochrome p450, 2D6 (CYP2D6) genotyping to predict a response to tamoxifen although there is still a debate on its prediction. (Fleeman, 2011) (Regan, 2012)

Phase 3 radomised trials comparing adjuvant chemotherapy against no chemotherapy based on results of genes profiling assays such as Oncotype Dx (TAILORx trial), Mammaprint (MINDACT trial), Genomic Grade (GERICO11/PACS10 trial) and PAM50 (NCI-2011-02623 trial) are currently underway to determine the best use of these assays.

6.0 Prognostic/ Predictive biomarkers

As previously mentioned, the success of chemotherapy depends not only on its cell damaging effect but also on the cell's ability to undergo cell death or repopulate through growth signalling pathways. Any functional aberration of proteins involved in these pathways will be the important factors to make chemotherapy successful or not. Correction of any aberrant proteins function by suitable drugs might be beneficial in this situation. Expression of these proteins can be therefore used as prognostic or predictive markers or both. Due to the complexity and multiplicity of pathways and the number of various proteins involved, targeting only one pathway or a few proteins had resulted in a very modest improvement in the outcome so far. In this sense, analysing and targeting functionally important proteins as many as possible from different cellular pathways seems to be the most logical way forward to improve the survival from the breast cancer. The biomarkers/ proteins included in this study are from different cellular pathways, some being involved in several pathways.

1. Cellular growth signalling pathway (ER, PR, EGFR, HER2, PDGFR-α, VEGFR-2)
2. Cell proliferation (ER, PR, EGFR, HER2, Ki-67, MCM-2, Aurora A, Plk-1)
3. Apoptosis (Bcl-2, Bag-1)
4. Cell cycle phase progression (Aurora A, MCM-2, Plk-1)
5. Angiogenesis (PDGFR-α, VEGFR-2)
6. Others (CK-5/6, CD-68, CD-71, GSTM-1, Cathepsin L2)

Oestrogen Receptor (ER)

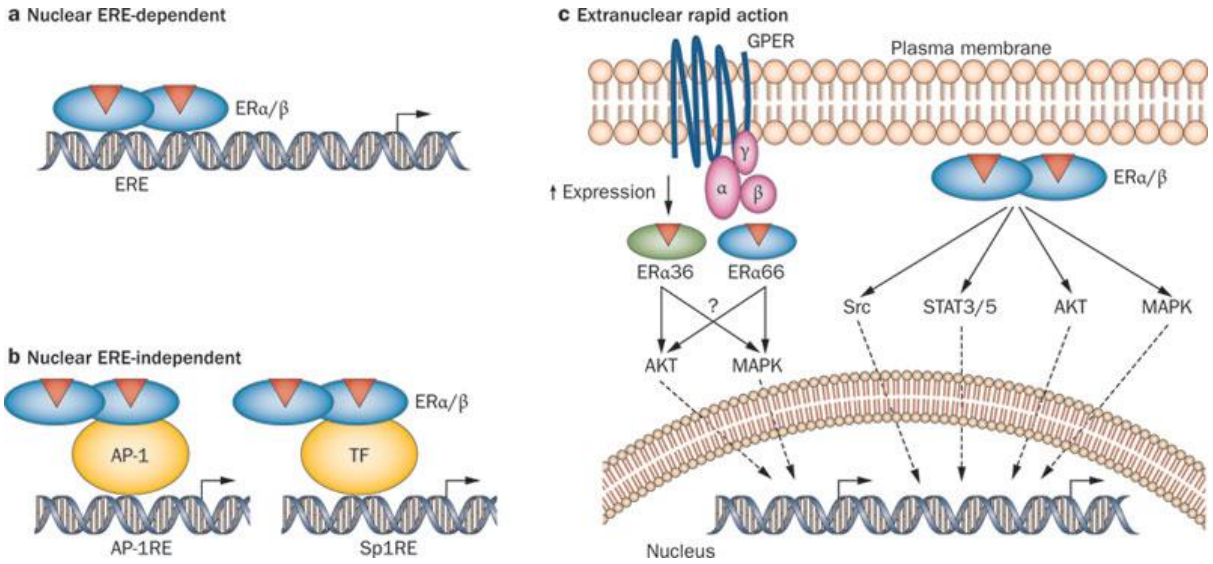
There are two isoforms of ER – α and β - coded for by 2 different genes. ERα is expressed in epithelial cells whereas ERβ is expressed in epithelial and stromal cells, including fibroblasts and endothelial cells. About 50% of breast cancer patients express both ERα and ERβ. After the oestrogen binds to the ligand binding sites, two ERs form a dimer and attach to the oestrogen response element (ERE) on the DNA leading to increased or decreased transcriptions of certain genes. The overall activity of ER is determined by not only ER levels but also by the associated co-activators and co-repressors involved in this process. ERα is involved in the oestrogen-stimulated proliferations and ERβ is involved in counteracting the ERα action leading to a decrease in the proliferation. ER reviewed in this chapter refers to ERα.

Oestrogen receptors locate in the nucleus and near the plasma membrane. The action through the nuclear ER is known as the "nuclear initiated steroid signalling (NISS)" and through the plasma membrane ER is known as the "membrane initiated steroid signalling (MISS)". NISS can be activated not only by the oestrogen but also by other factors such as insulin like growth factor-I (IGF-I), epidermal growth factor (EGF), heregulin, transforming growth factor alpha and neurotransmitters such as dopamine. NISS can also be activated, independently of ligand binding, by signalling molecules such as cyclic adenosine monophosphate and membrane permeable phosphatase inhibitors. This activation is believed to be through the phosphorylation of ER or its co-regulators at the specific sites by the growth factors and kinases such as extracellular regulated kinase (ERK 1 & 2), p38 mitogen-activated protein kinases (MAPKs), cyclin dependent kinases (CDK –2, CDK-7), c-SRC, protein kinase A, pp90rsk1 and AKT. Acquired resistance to the anti-oestrogen therapy is reported to be due to the change of ER activity from NISS to MISS. The cross talk between

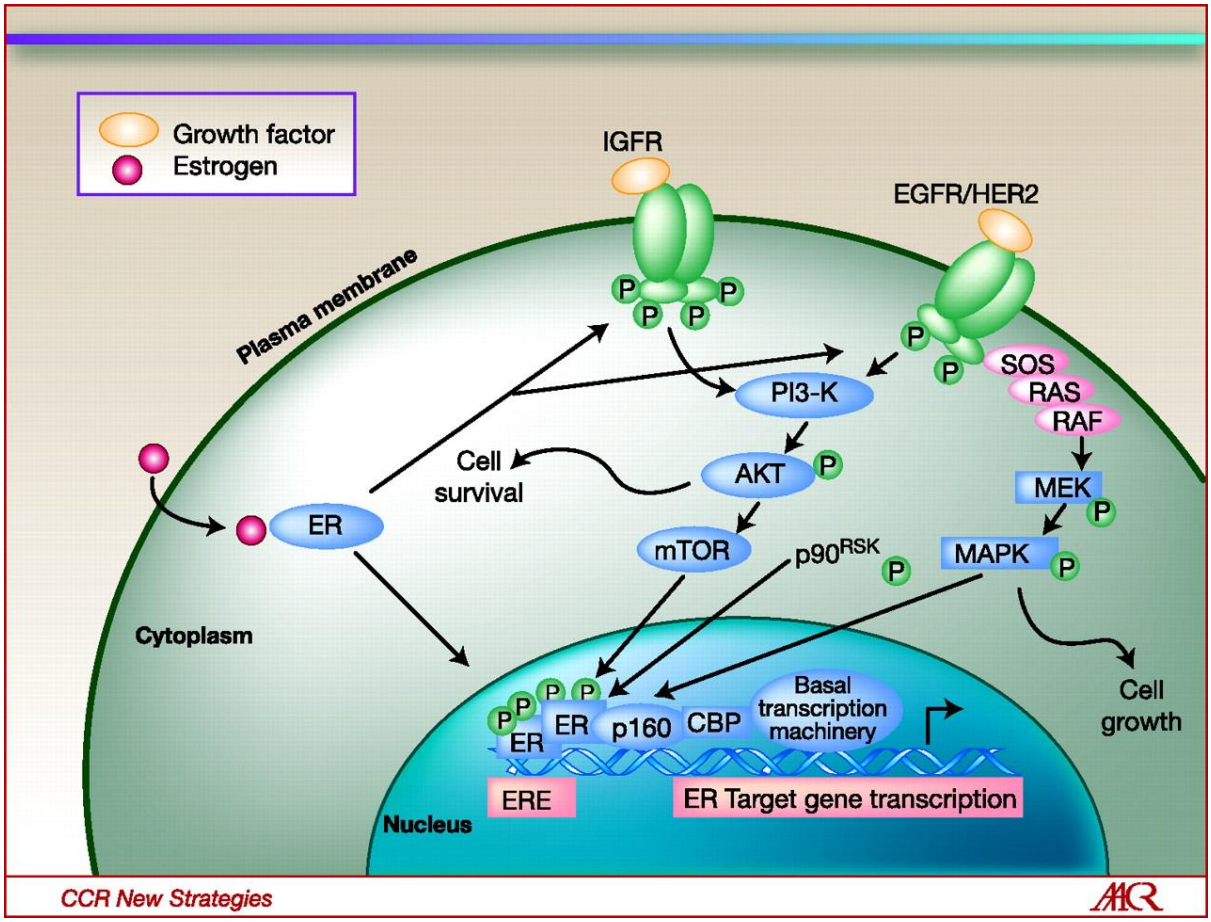
ER and the growth factors is suggested to be one of the causes for the resistance to the antioestrogen therapy. Increased HER2 activity reportedly results in more ERs outside the nucleus leading to the high levels of membrane and cytoplasmic ER with the development of the resistance to tamoxifen induced apoptosis. (Chung, 2002) Therefore a treatment with EGFR or HER2 inhibitors could overcome the resistance to the tamoxifen treatment. Loss of ER in breast cancers is in part a result of the hypermethylation and repression of the ER promoter, which prevents ER productions. The ER pathway is therefore very complex and contains co-activators, co-repressors, transcription factors that modify binding of ER to its targets and the crosstalks between ER and the growth factor receptor pathways.

ER is a prognostic factor as seen in the molecular subtypes where luminal cancers have better survival than non-luminal cancers. (Cheang, 2008a) ER positive cancers have better prognosis than ER negative cancers regardless of the PR status. ER is also a predictive factor for benefits from anti-oestrogen treatments. However, not all ER positive tumours are responsive to the anti-oestrogen therapy, and 30–40% of ER positive breast cancers will relapse or develop distant metastases despite the anti-oestrogen (tamoxifen) adjuvant treatment. (Loi, 2008) The likelihood of having benefits from the anti-oestrogen treatment depends on the degree of ER expression. No benefit was seen in cancers with poor ER expression (<10 fmol/mg cytosol protein). (EBCTCG, 2011b) "Allred score" that combines scores on the percentage of stained cells (0 – 5) and the staining intensity (0 – 3) is a useful tool to decide for or against the use of anti-oestrogen therapy. (Harvey, 1999) It has been suggested that patients with inherited nonfunctional alleles of the cytochrome P450 (CYP2D6) gene that codes for the enzyme that converts the tamoxifen into its active compounds inside the body, may not get any benefits from the tamoxifen. However this wasn't confirmed in all studies. (Fleeman, 2011) (Regan, 2012)

Low ER status is an indicator of a better response to chemotherapies compared to stronger ER positive status. In women with low ER breast cancers, the polychemotherapy significantly reduced 10 years risk of recurrence, for <50 years and 50 – 69 years age groups, by 27% & 18%, breast cancer mortality by 27% & 14%, and any cause mortality by 25% & 13% , respectively, compared to no chemotherapy. (EBCTCG, 2008)



ER signalling pathway (Borrowed from a published article by Tiano, 2012)



Cross talks between ER and EGFR/HER2 signalling pathways (Borrowed from the published article by Johnston, 2010)

Progesterone Receptor (PR)

Progesterone receptor (PR) is a member of the nuclear steroid receptor family. It is synthesized by the oestrogen through the ER pathway and the presence of PR usually indicates a functioning ER pathway. PR exists in two isoforms - PRα and PRβ - both of which are coded for by a single gene. Both PRs are found to express in the normal and malignant breast tissues. PR is made up of a central DNA binding domain and a carboxy terminal ligand binding domain. Upon ligand binding, two PRs (same or different PRs) become homo- or heterodimers that attach to the progestin response elements (PRE) in the promoters of target genes leading to the transcriptions of genes. PR has also been shown to mediate rapid activation of the Src/Ras/Raf/MAPK and also STAT signalling pathways.

Positive PR expression is an independent prognostic marker in patients treated with tamoxifen or chemotherapy or no systemic adjuvant therapy. (Liu, 2010) Fisher et al reported that the prognostic significance of PR was comparable to that of ER in NSABP B-06 study. (Fisher, 1988) PR expression, not ER expression, regardless of the HER2 expression status, was shown to be significantly and inversely associated with the stage of the breast cancer at the diagnosis which is a strong correlative of survival not affected by the intervening endocrine therapy or chemotherapy, in the multivariate analysis in a study. (Coyle, 2007) Like ER, the prognostic value of PR was lost after 10 years. (EBCTCG, 2011b) Stendahl et al reported RFS and OS benefits only for the premenopausal patients with >75% PR positive nuclei. The PR was a stronger predictor than the ER for the treatment response. (Stendahl, 2006) However, Badve et al reported that OXA1 is better than PR in predicting prognosis following an endocrine therapy. (Badve, 2007) No prognostic or predictive effect of PR was reported for a given ER status although there is a trend for benefits for ER-/PR+ patients. (EBCTCG, 2011b) (Dowsett, 2006)

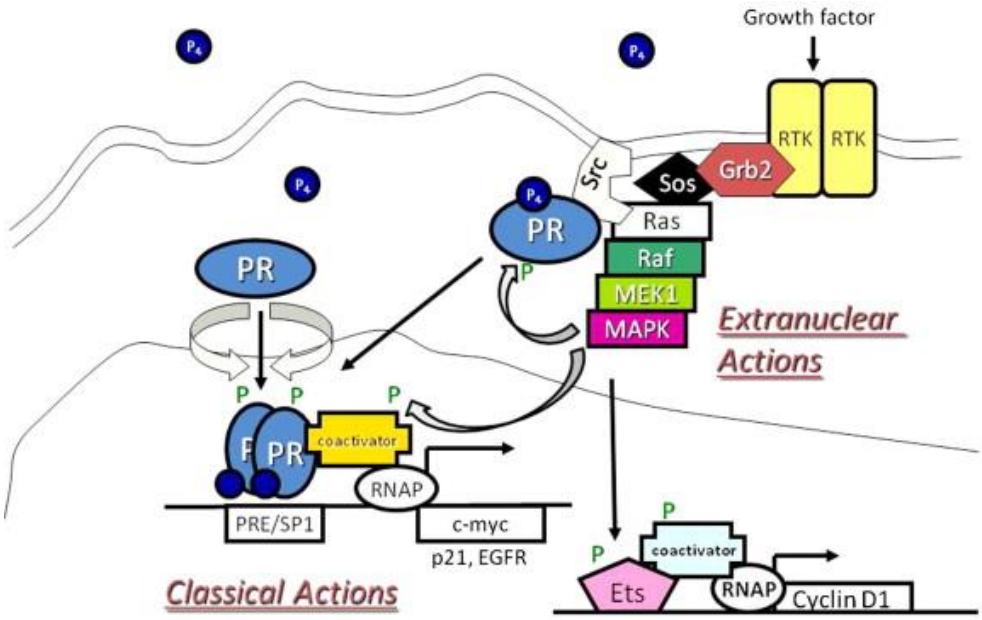
PR-α expression is believed to increase the resistance to paclitaxel by up regulating antiapoptotic gene BCL-XL in breast cancer cells. (Richer, 2002) Serum depletion induced apoptosis was shown to be inhibited by the progesterone treatment. (Ory, 2001) Radiation-induced apoptosis could be antagonised via PR in breast cancer cell lines. (Vares, 2004) Progesterone was shown to inhibit the growth of MDA-MB-231 breast cancer cells inoculated into a mice transfected with the PR cDNA and also increased the cellular differentiation of these cells probably by modifying the genome expressions. (Lin, 2001) (Leo, 2005)

Although PR expression is expected to be positive alongside ER, there are breast cancers with ER+/PR- and ER-/PR+ phenotypes. EBCTCG overview found that PR was positive in 76% of ER positive and 21% of ER negative (strictly, ER-poor) breast cancers. (EBCTCG, 2011b) The breast cancer recurrence risk was higher for the ER negative disease regardless of the PR status. ER+/PR- breast cancers may simply result from the low circulating levels of endogenous oestrogens in postmenopausal women that are insufficient to induce PR expression even though the ER pathway is intact. Therefore, a brief treatment of ER+/PR- patients with the oestrogen may restore the PR in some of the patients. Absent or low PR expression could also be due to the down regulation of PR by the overexpressed growth factor signalling, hypermethylation or genetic loss at the PR gene locus (chromosome 11q23) rather than the non-functional oestrogen receptor pathway.

ER-/PR+ expression was reported in 21% of breast cancer patients in EBCTCG's overview. (EBCTCG, 2011b) In these cancers, PR expression may be driven by the cross talks between various growth factor signalling pathways. These cancers have significantly poorer differentiation, larger tumour size and younger age compared to the ER+/PR+ cancers. ER+/PR- cancers are more commonly found in the postmenopausal and older patients. Up to 60% of ER+/PR- patients were more than 60 years old, and have worse DFS and OS

compared to ER+/PR+ cancers. (Yu, 2007) ER+/PR- status was also reported to be an independent predictor for the lymph node positivity especially in younger women. (Neven, 2004) ER+/PR- breast cancers respond less well to tamoxifen than ER+/PR+ tumours but respond better to oestrogen deprivation with aromatase inhibitors according to the ATAC trial. (Dowsett, 2005) Therefore a combination of aromatase inhibitor with growth factor inhibitors may be a better treatment option in ER+/ PR- tumours.

Statistically significant change of PR status from positive to negative was found following neoadjuvant chemotherapies. (Kasami, 2008) Distant metastases of some originally ER+/PR+ primary tumours lost PR expression and became ER+/PR-. These cancers have more aggressive course, poor survival and are resistant to the endocrine therapy. (Cui, 2005) So far, the gain of PR by metastases from originally PR negative primary tumours has not been seen yet. (Thompson, 2010) In the IHC molecular subtyping, ER+/PR-, ER-/PR+ and ER+/PR+ cancers are not sub-divided into separate groups (all were grouped together as "luminal cancers") suggesting absence of either receptor may have very little, if any, significant impact on the survival. (Perou, 2000)



PR pathway (Borrowed from the published article by Hagan, 2012)

EGFR

EGFR is a member of type one transmembrane tyrosine kinase receptors EGFR/ErbB/HER family that includes ErbB1/HER1 to ErbB4/HER4 proteins. These receptors play an essential role in the organ development and the growth by regulating differentiation, proliferation and morphology of cells and tissues. Activation of extracellular EGFR domain by its ligand causes homo- or heterodimerization of EGFRs with another EGFR molecule, or a different member of the ErbB family (e.g. HER2), which in turn induces the amplified signalling cascade.

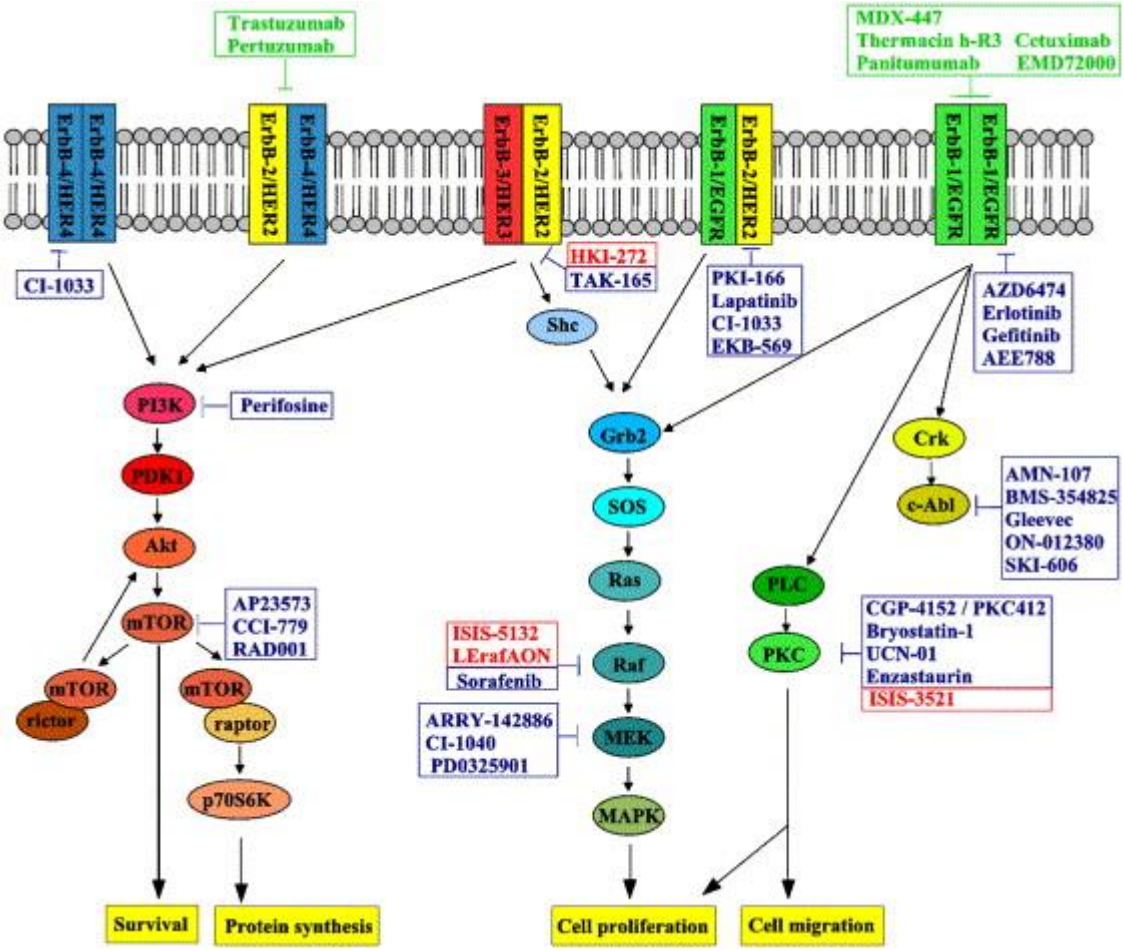
EGFR overexpression was found in a number of different cancers including lung, colorectal and breast cancers. EGFR overexpression in invasive breast cancers ranged from 14% to 91% with an average of 45% among 40 different studies included in a meta-analysis (Klijn, 1992) and up to 50% in triple negative breast cancers. (Nogi, 2009) EGFR expression was reported to be one of the poor prognostic factors and was associated with other risk factors. (Nieto, 2007) EGFR expression was correlated with the poor treatment response and the shorter survival compared to no expression. (Nieto, 2007) (Park K, 2007) (Nogi, 2009) EGFR expression was believed to be mostly limited to the basal like, HER2 and luminal B type cancers. (Meche, 2009) Arnes et al reported association between EGFR expression and CK-5 and P-cadherin positivity but not with overall survival in basal like cancers. 28% of CK-5 positive cases showed EGFR gene expression. (Arnes, 2008) Nielsen et al suggested EGFR expression as an alternative to CK-5/6 to define the basal phenotype. (Nielsen, 2004) EGFR expression by IHC was found in 54% of cancers that expressed basal cytokeratin genes and it was associated with the poor survival. (Nielsen, 2004) In a study by Park et al, IHC expression of EGFR protein was found in 20.6% of 165 cases while the gene amplification was found in only 7.9% of the cases. (Park, 2007)

Inhibition of the tyrosine kinase enzymatic activity of EGFR is a clinically relevant treatment option for breast cancer patients. Cetuximab, alone or in combination with cisplatin, was effective in vitro on breast cancer cells. (Nofech-Mozes, 2009) Small molecule TKIs such as Erlotinib, Lapatinib and Gefitinib have shown activities in breast cancers too. Gefitinib enhanced the effectiveness of tamoxifen and fulvestrant in breast cancer cell lines. (Gee, 2003) Phosphorylation of EGFR detected in triple negative tumours could be blocked by Gefitinib in vitro. (Gori, 2009) In a tamoxifen resistant, HER2 overexpressing MCF-7 breast cancer cell line (MCF-7/HER2-18) Gefitinib pre-treatment was shown to block ER-EGFR receptor cross talk, re-establish co-repressor complexes with tamoxifen-bound ER on target gene promoters, eliminate tamoxifen agonistic effects, and restore tamoxifen antitumor activity both in vitro and in vivo. (Shou, 2004) A combined treatment with EGFR inhibitor and HER2 inhibitor showed a synergistic growth inhibition in breast cancer cell lines that over express both EGFR and HER2 proteins. (Normanno, 2002)

Erlotinib and Gefitinib as monotherapy in patients with breast cancer, however, have been reported to have limited activities, with response rates of less than 5%. (Green, 2009) (Dickler, 2009) The combination of Erlotinib and Bevacizumab also had limited activities in unselected, previously treated metastatic breast cancers. (Dickler, 2008) Erlotinib with Capecitabine and Docetaxel gives an overall response rate of 67% in metastatic breast cancer patients. (Twelves, 2008) In a phase 2 study with 41 patients, a response rate of 54% (95% CI 45–75%), a stable disease 14%, and a progressive disease 32% were reported for Gefitinib plus Docetaxel. (Ciardiello, 2006) Serum HER2 and EGFR are suggested to be a predictor for early response, PFS, and OS in patients with advanced breast cancer treated with the metronomic chemotherapy. (Sandri, 2007)

EGFR expression is a potentially useful tool for prognostic and therapeutic purposes. It is not possible to say if EGFR is more commonly associated with other risk factors or EGFR

expression itself contributes to the presence of other poor prognostic factors. More research is needed to use it as a standard prognostic/ predictive factor in the daily practice for breast cancer.



HER/ EGFR family signalling pathways and their inhibitors (This illustration was borrowed from the article by Kikalsen, 2006)

HER2

HER2/neu protein is a member of HER family that contains four transmembrane receptor tyrosine kinases (as mentioned in the EGFR section) that mediate cell growths, differentiations and survivals. HER2/neu gene is located on the chromosome 17q and it was first discovered in 1984. Incidence of overexpression of the HER protein or amplification of HER2 gene or both has been reported as 9.7% in node negative, <10 mm tumours (Albert, 2010), 14% in 10,000 patients participated in 12 different clinical studies (Blow, 2010), 22.7% in a very large cohort study involving 61,309 patients (Parise, 2009) and 44% among very high risk (>4 lymph nodes positive or inflammatory cancer) patients who participated in a high dose chemotherapy clinical trial. (Nieto, 2004) When a ligand binds to the extracellular domain, HER2 protein either homodimerise with another HER2 protein or hetrodimerise with other members of the family and phosphorylates the intracellular tyrosine kinase residue. Signal propagation then occurs as the enzymatic activity of one protein activates the next protein in the pathway. The main signal transduction pathways are Ras/mitogen-activated protein kinase pathway (MAPKinase), the phosphatidylinositol 3 kinase (PI3K)/Akt pathway, the Janus kinase/signal transducer and activator of transcription pathway, and the phospholipase C pathway that promotes cell proliferation, survival, motility, and adhesion.

HER2 protein over expression can be assessed by IHC using two commercially available kits, the HercepTest R (Dako, Carpinteria, CA) and Pathway™ HER-2 (Ventana, Tucson, AZ). HER2 gene overexpression can be assessed by various methods including Fluoresence in situ hybridisation (FISH), chromogenic in situ hybridisation (CISH) which has a 100% concordance with FISH and a good concordance with IHC in the 0–1+ and 3+ categories, Silver In Situ Hybridisation (SISH), Southern and Slot blotting, RT-PCR, mRNA by

microarray, dimerisation assays, phosphorylated HER2 receptor in the tissue and serum by ELISA. The concordance between IHC and FISH is nearly 98% in the laboratories that perform high volume testings. (Paik, 2002) Disadvantages of IHC method are subjective interpretations and semiquantitative results.

Patients with IHC 3+ expression (DAKO scoring), gene copy number > 6.0 per nucleus or gene ratios of HER2/CEP17 > 2.2 are considered as HER2 positive. (Wolff, 2007) HER2 protein overexpression or gene amplification was shown to have adverse prognostic effect in multivariate analysis independent of all other prognostic factors in 68 out of 107 studies with 39,730 patients in a metanalysis. The correlation between HER2 overexpression and poor prognosis could be established in 85% of studies that used FISH analysis and 100% of studies that used CISH analysis. (Ross, 2009)

HER2 overexpression is associated with other risk factors such as intermediate or high histology grade, negative ER and PR, positive lymph nodes, DNA aneuploidy, high cellular proliferation rate, p53 mutation, topoisomerase IIa amplification, alterations in a variety of other molecular biomarkers of breast cancer invasion and metastasis. HER2 positive breast cancer is also found to have a high rate of recurrence and shorter disease free and overall survival despite adjuvant chemotherapy. (Slamon, 1987) (Wright, 1989)

The coamplification of c-*myc* and HER2 was found to be correlated with the worse outcome than the amplification of either protein. Patients with c-*myc* co-amplification had a worse outcome if they were treated with chemotherapy alone, but had a 4-year recurrence free survival rate of 90% when treated with chemotherapy and trastuzumab in the NSABP-31 trial (Kim, 2005) although similar positive effect was not found in the N9831 trial. (Perez, 2011a)

The topoisomerase-II alpha (*topo-IIa*), a target protein for anthracycline, gene is located in close proximity to HER2 oncogene on chromosome 17q12-q21 and is amplified or deleted in

almost 90% of HER2 amplified primary breast cancers, making cancers to either sensitive or resistant to anthracycline chemotherapy. (Muss, 1994) (Paik, 1998) HER2 positive cancers are more sensitive to aromatase inhibitors and resistant to tamoxifen. (Houston, 1999) (Lipton, 2002) HER2 overexpressed breast cancers are also more sensitive to local radiotherapy. RFS benefit from CMF chemotherapy is smaller in HER2 positive cancers compared to HER2 negative cancers. (Stal, 1995)

Trastuzumab, monoclonal antibody that targets HER2 protein has changed the course of the HER2 overexpressed breast cancers. It can give objective response in more than one third of the HER2 positive cancers as a single agent and more than 50% in conjunction with the chemotherapy. In the adjuvant setting, one year of trastuzumab in conjunction with adjuvant chemotherapy improves both disease free and overall survivals. (Baselga, 2006) (Salmon, 2011) (Perez, 2011b) (Gianni, 2011) Lapatinib, another anti-HER2 antibody, together with chemotherapy or endocrine therapy, has also shown significant activity in metastatic breast cancers. (Cameron, 2010) New treatments targeting HER2 signalling such as HER2 vaccine, Pertuzumab, Ertumaxomab, MDX-H210, TDM-1 (Trastuzumab + fungal toxin Maytansine) and novel tyrosine kinase inhibitors are in the development stages.

Despite its own unique actions and effects, HER2 gene expression is not exclusive to the HER2 enriched molecular subtype. It is still seen in other subtypes such as luminal A and B. In the basal-like phenotype which is regarded as ER/PR/HER2 negative by IHC, HER2–positive status is found in about 10% of cases. (Harris, 2007)

Bcl-2

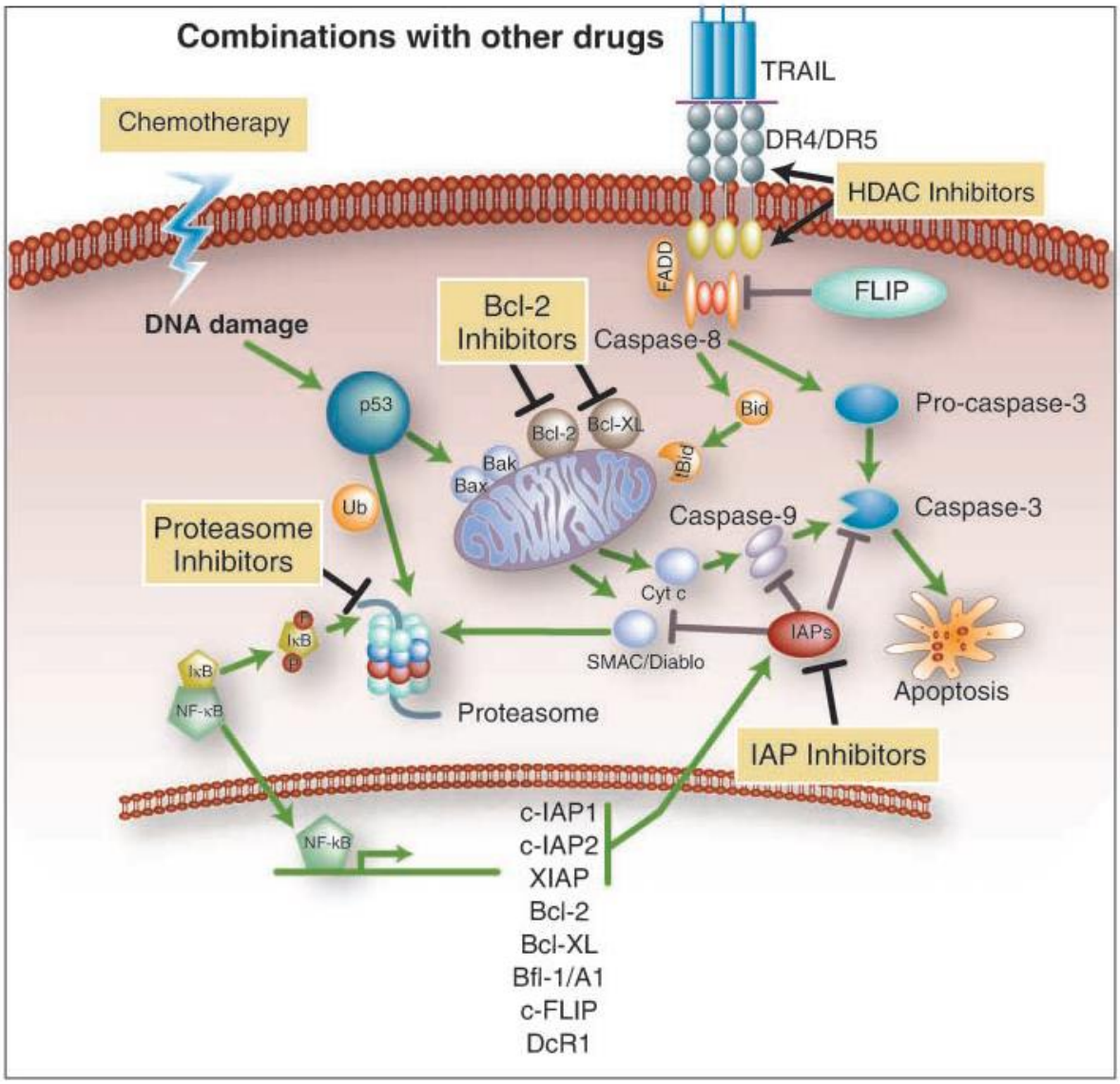
Anti-apoptic protein Bcl-2 has two isoforms - “a” (26 kDa) which is detected commonly and “b” (21 kDa) which is rarely detected. At least 17 Bcl-2 family members have been identified in mammalian cells and viruses. All members possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4). Bcl-2 family proteins can homo- or heterodimerise with each other. When anti-apoptotic Bcl-2 level is higher than pro-apoptotic Bax level, Bcl-2 is able to neutralise the ability of Bax to promote cytochrome C release and subsequent apoptosis.

Bcl-2 protein mainly localises in the nuclear membrane, outer mitochondrial membrane, nuclear pore complexes, mitochondrial junctional complexes and some parts of the endoplasmic reticulum. Bcl-2 can prevent apoptosis induced by various factors such as chemotherapeutic drugs, gamma irradiation, neurotrophic factor withdrawal from neurons, cytotoxic cytokines such as tumour necrosis factor-α, Fas-ligand, transforming growth factor-β, heat shock, calcium ionophores and chemicals that induce oxidative injury. (reviewed by Reed, 1994) It prevents cytochrome C release into the cytoplasm by forming mitochondrial pore complexes upon receiving apoptotic stimulus, preventing formation of the cytosolic apoptosome complex, and activation of the downstream caspase cascade. Bcl-2 induced resistance to apoptosis following chemotherapy can be reversed with Bcl-2 targeting therapy. Many anti-apoptotic Bcl-2 family proteins are found to be able to inhibit cell proliferation. This could be the reason for the good prognostic effect of Bcl-2 protein.

Bcl-2 expression was detected in about 80% of breast primary lesions regardless of the nodal status. (Gee, 1994) (Krajewski, 1995) Bcl-2 gene expression is found to be regulated by oestrogens in mammary epithelial cells and ER positive breast cancer cell lines. (Johnston, 1994) (Barbareschi, 1996) (Zapata, 1998) Increased Bcl-2 expression was correlated with

other good prognostic factors such as ER, PR, well differentiation, low nuclear grade, absence of positive lymph nodes, better response to endocrine therapy, and inversely correlated with p53 expression and the apoptotic index. (Won, 2010) (Lee, 2007) (Planas-Silva, 2007) Bcl-2 expression was predictive of tamoxifen sensitivity in breast cancer patients. (Elledge, 1997) (Ciocca, 2000) In a study of 13 biomarkers in 930 breast cancers on a tissue microarray, Bcl-2 was found to be predictive of better 10 years survival in univariate and multivariate analysis along with NPI and ER. Bcl-2 was also found to be a significant prognostic factor independent of NPI and the effect was maximal in the first 5 years. (Callagy, 2006)

There have been reports on association between Bcl-2 expression and better as well as worse survival outcomes following adjuvant and neoadjuvant chemotherapies. (Gasparini, 1995) (Bonetti, 1998) (Vargas-Roig, 2008) (Ogston, 2004) A lack of association between Bcl-2 expression and the survival benefit from dose dense adjuvant chemotherapy has also been reported. (Malamou-Mitsi, 2006) Correlation between high Bcl-2 expression and a better survival has been reported in high risk patients including those with over 10 positive lymph nodes. (O'Driscoll, 2003) (Kroger, 2006) (Lee, 2007) Although Bcl-2 expression did not change significantly following the chemotherapy, post neoadjuvant chemotherapy Bcl-2 expression was found to be predictive of better disease free and overall survivals. (Vargas-Roig, 2008) Bcl-2 expression is not currently used as a standard prognostic or predictive marker in breast cancers.



Apoptosis pathway and drugs targeting the pathway (Borrowed from the published article by de Vries, 2006)

Bag-1

Bcl-2-associated anthanogene 1 (Bag-1) is a pro-survival protein that was first identified as a binding partner of Bcl-2 and the activated glucocorticoid receptor. (reviewed in Cutress, 2002) It is expressed in most normal human tissues. Bag-1 gene encodes three major isoforms namely Bag-1S, Bag-1L and Bag-1M that share a common carboxy terminus. The Bag-1S is preferentially located in the cytoplasm. Bag-1L is predominantly located in the nucleus. The Bag-1M is located in both nuclear and cytoplasmic compartments, but it relocates from the cytoplasm to nucleus in response to heat shocks and hormonal stimulations. Therefore nuclear Bag-1 expression may indicate either high levels of Bag-1L or re-localisation of Bag-1M to the nucleus in response to specific signals in the tumour microenvironment.

Bag-1 is a multifunctional anti-apoptotic protein. It binds to proteins from four different subcellular compartments:

1. cytosolic domains of tyrosine kinase hepatocyte growth factor (HGF) receptor and platelet derived growth factor (PDGF) receptor on outer cell membrane to increase the protection from apoptosis by HGF and PDGF, respectively;
2. Bcl-2 on inner cell membrane to enhance the inhibition of apoptosis;
3. heat shock protein (Hsp) in the cytoplasm to inhibit the Hsp70-mediated refolding of denatured proteins, and protect cells from the heat shock induced apoptosis;
4. RAF-1 and hormone receptors such as glucocorticoid, androgen, oestrogen and thyroid receptors in the nucleus to modulate their functions.

Bag-1 promotes cellular proliferation in normal conditions but causes cell cycle arrest under a stressful condition. (Song, 2001) Overexpression of Bag-1 suppresses the activation of caspases and apoptosis induced by many factors such as Fas and TRAIL death-receptors,

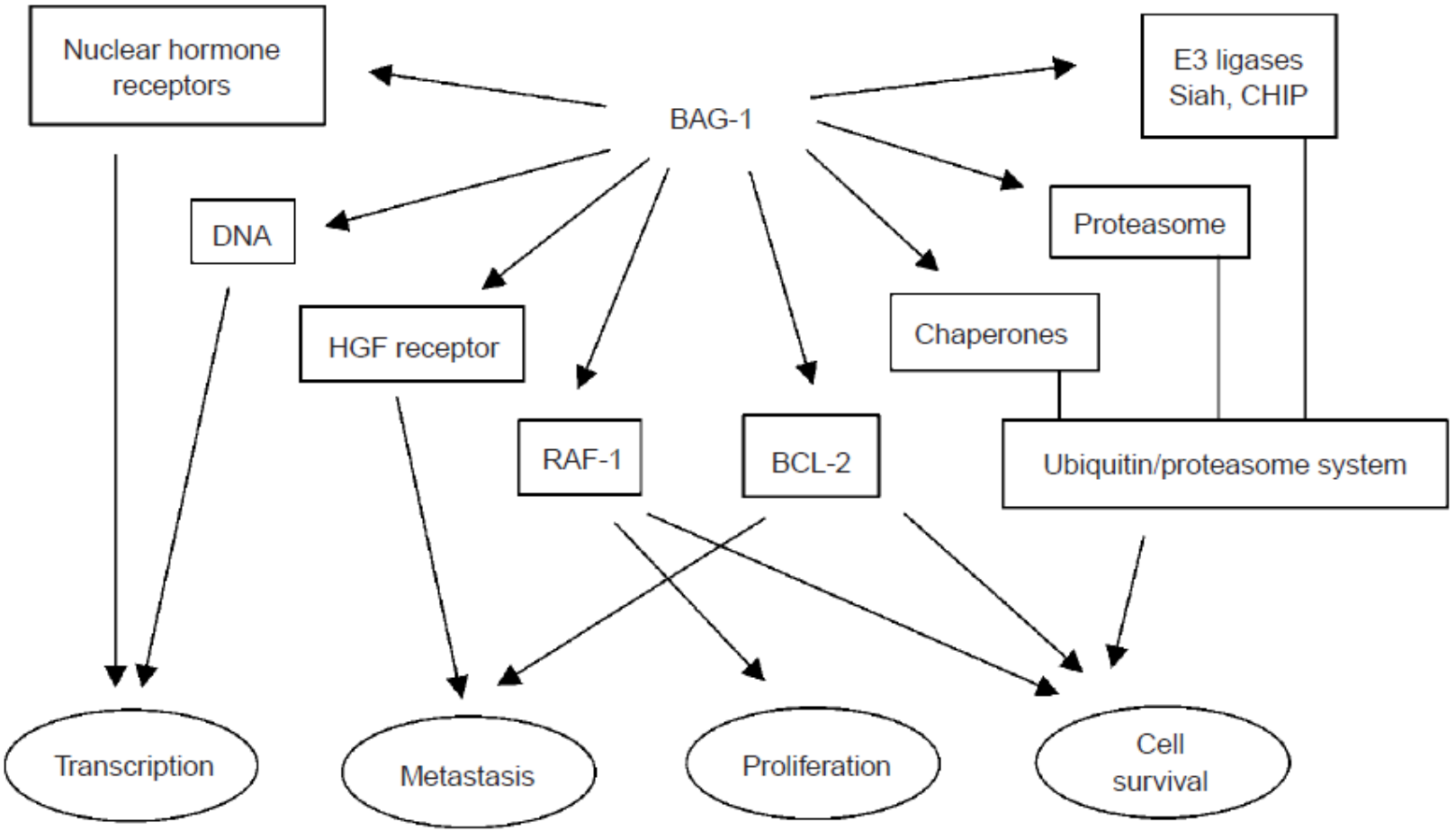
kinase inhibitors, vitamin D, retinoic acid, withdrawal of growth factors, heat shock, dexamethasone, radiation, anti-cancer drugs such as cisplatin and etoposide. Over-expression of Bag family proteins has been shown to increase the resistance to chemotherapy. Bag-1 enhances metastasis in experimental cancer models. (Shindoh, 2000) ZR-75-1 breast cancer cells transfected with Bag-1 have increased survival in the culture, and form larger tumours than non-transfected cells when injected into mammary fat pads of mice. ZR-75-1 cells with mutated Bag-1 show retarded growth in vivo and in vitro. (Kudoh, 2002) Over-expression of Bag-1 was also found to enhance cell migration and the survival in two gastric cancer cell lines. (Naishiro, 1999)

Bag-1 has been linked with the aggressiveness of breast, gastric, pancreatic, head and neck, endometrial and colorectal cancers. In breast cancer, relatively high level of Bag-1 expression by IHC was located to the cytoplasm in more than two thirds, nucleus in 0.5% to 70% and both nucleus and cytoplasm in 1% to 60% of the cases. (Tang, 1999) (Turner, 2001) (Sjostrom, 2002) (Townsend, 2002) Nuclear Bag-1 was inversely associated with the tumour grade and cytoplasmic Bag-1 was associated with the ER status. High grade tumours exhibit weak nuclear Bag-1 expression. (Tang, 2004) Bag-1 expression was more frequently found in node positive breast tumours compared to node negative tumours - 89% vs. 38% - and was predictive of good prognosis in node positive patients in the univariate analysis. There are also strong associations between Bag-1, Bcl-2, ER and PR expressions. (Nadler, 2008b) Positive, negative or no correlations between Bag-1 expression and other molecular markers such as Ki-67, Bcl-2, ER, PR and Bcl-x had also been reported. (Xie, 2004) (Sjostrom, 2002) (Tang, 2004) (Townsend, 2002) (Turner, 2001) (Brimmell, 1999) (Cutress, 2003)

The correlation between Bag-1 expression and the survival outcome was reported inconsistently. Tang et al. reported an association between high nuclear Bag-1 expression and decreased survival among 140 breast cancer patients (Tang, 2004) while Krajewski et al

reported better overall survival for Bag-1 overexpression (>20%). (Krajewski, 1999) Turner et al reported a strong association between high cytoplasmic Bag-1 and a better survival in both univariate and multivariate analysis (Turner, 2001) while Townsend et al. found no significant association between nuclear or cytoplasmic Bag-1 and survival in 160 patients. (Townsend, 2002) High nuclear Bag-1 expression was found to increase anti-oestrogen induced growth arrest in MCF-7 cells and was predictive of lower local recurrence, distant metastases and death from breast cancers in tamoxifen-treated ER+ cancer patients in univariate analysis and predictive of distant metastases in multivariate analysis (Millar, 2009). Bag-1 was found not to be predictive of a response to a chemotherapy that contains docetaxel or methotrexate and fluorouracil in advanced breast cancers. (Sjostrom, 2002)

Reduction of Bag-1 expression by antisense cDNA leads to the sensitization to apoptosis induced by many apoptotic inducers including staurosporine, paclitaxel, ATRA, and 4-HPR. (Takahashi, 2003) Only Bag-1L regulates ERα function and the expression of nuclear Bag-1L might be particularly important in determining a response to hormonal therapy in breast cancers. (Cutress, 2003) As all Bag-1 isoforms possess anti-apoptotic activity, cytoplasmic Bag-1S might be particularly important in determining responses to chemotherapy that exerts its effect via apoptosis.



Bag-1 binding partners and functions (Borrowed from the published article by Cutress, 2002)

Aurora A

Aurora A - also known as serine threonine kinase 15 (STK15), BTAK, Aurora kinase A or Aurora-2 - is a kinase protein, encoded for by a gene located at chromosome 20q13.2. It is a member of Aurora kinase family which also includes Aurora B and C. Aurora proteins need phosphorylation to become active and several different activators for Aurora A such as TPX2, Ajuba, PAK1, HEF1, hBora and ASAP have been identified. Auroras are degraded through the D (destruction) box – a sequence in the protein's structure. Aurora A is expressed in most rapidly dividing tissues like testis and thymus, and found to be low in most adult tissues likely due to the low proliferation rates.

Main function of Auroras is the regulation of cell cytokinesis. Aurora A regulates phases of mitosis that include centrosome maturation and separation, mitotic entry, bipolar spindle assembly, chromosome alignment on the metaphase plate and cytokinesis by phosphorylating different substrates. Aurora-B is a subunit of the chromosomal passenger protein complex and functions to ensure accurate chromosome segregation and cytokinesis. Aurora-C is a chromosomal passenger protein and co-localizes with Aurora-B. Aurora C is found only in the normal testicular tissue.

Auroras level is undetectable in G1 phase but increases during S phase, reaches peak at G2/M phase and then rapidly decreases at the end of mitosis. A perfect timing of Aurora activation and destruction is necessary for an effective cytokinesis. Disruption of Aurora-A function leads to G2-M arrest and severe mitotic defects such as delayed entry into mitosis, monopolar spindles, defective centrosome maturation and misalignment of chromosomes during metaphase and apoptosis. Aurora-A over expression results in a centrosome duplication, multipolar spindle, failure to complete spindle microtubule attachment, bypass of the G2-M DNA damage-activated checkpoint permitting cells to inappropriately enter anaphase despite

the presence of these abnormalities resulting in numerous chromosomal separation defects. With additional cycles this leads to an aneuploidy and progressive chromosomal instability. Aurora A over expression also leads to the increased p53 degradation, which facilitates the oncogenic transformation.

In normal cells, Aurora A is present on duplicated centrosomes and mitotic spindles from late S phase until early G1 phase during the mitosis. (Fu, 2007) (Marumoto, 2005) In malignant cells, Aurora A is detected diffusely throughout the cell as aberrant phosphorylated cystoplasmic proteins, regardless of the cell cycle position. (Gritsko, 2003) Aurora A over expression, with or without amplification, has been observed in up to 62% of breast cancers. (Miyoshi, 2001) (Tanaka, 1999) (Zhou, 1998) Potential mechanisms of Aurora A over expression include gene amplification, mRNA over expression and phosphorylation on serine 51 that prevent proteolysis. (Kitajima, 2007)

It has been shown that Aurora A over expression induces cancer cells resistant to taxane by disrupting the spindle checkpoint activated by paclitaxel or nocodazole treatment. (Anand, 2003) High Aurora A mRNA levels are associated with a lower response rate to docetaxel compared to low Aurora A mRNA levels especially in ER negative tumours (33% vs 83%). (Miyoshi, 2001) (Hata, 2005) In early breast cancer, Aurora A over expression is associated with a poor survival and also with other poor prognostic factors such as high nuclear grade and positive HER2 expression, ER/PR negativity and centromere abnormality. (Loddo, 2009) (Nadler, 2008a) (Hoque, 2003) (Royce, 2004) Cytoplasmic or nuclear localisation of Aurora A has been reported to be a critical factor for its effect on the various cancers. (Tatsuka, 2009) (Ogawa, 2008)

Aurora A inhibitors have been developed and investigated in various human cancers. JNJ-7706621 and PHA-680632 (Keen, 2004) (Plyte, 2007) are shown to decrease Aurora kinases activities by inhibiting the histone H3 phophosrylation which is a substrate for Aurora A. An

inhibitor of Aurora A and B quinazoline ZM447439 (Ditchfield, 2003) and phenylamide VX-680 are reported to target ATP-binding site of the Aurora kinases. It has been shown to be effective in leukemia, prostate, colon and pancreatic cancers. (Harrington, 2004) (Lee E, 2006) Aurora A kinase selective inhibitor MLN8054 has shown robust growth inhibition of human tumour xenografts. (Manfredi, 2007) MK615 exerts an anti-neoplastic effect on human pancreatic cancer cells in vitro by dual inhibition of Aurora A and B kinases. (Okada, 2008) Aurora A kinase inhibitor VE-465 synergizes with paclitaxel to induce 4.5-fold greater apoptosis than paclitaxel alone in 1A9 cells. Higher doses are needed to induce apoptosis in paclitaxel resistant PTX10 cell. (Scharer, 2008)

Plk-1

Polo like kinase (Plk) belongs to a family of serine/threonine kinases isoenzymes. There are 4 isoforms namely Plk-1, Plk-2, Plk-3 and Plk-4. These kinases have a conserved N-terminal catalytic domain and a C-terminal phosphopeptide-binding polo-box domain (PBD) that allows them to localize to mitotic structures. In the absence of a bound ligand, the PBD forms an intramolecular interaction with the kinase domain inhibiting its kinase activity.

Among all Plks, Plk-1 was studied most extensively in cancers. Main functions of Plk-1 are involvement in the regulation of centrosome maturation, bipolar spindle assembly, sister chromatid cohesion, activation of anaphase promoting complex, initiation of cytokinesis and regulation of the G2/M transition. Plk-1 is essential for the cell cycle to restart after a successful or failed repair of damaged DNA. The depletion of Plk-1 induces apoptosis in various cancer cell lines by affecting cell cycle profile and by damaging DNA. (Ando, 2004) (Liu, 2003) (Spankuch-Schmitt, 2002a)

Plk-1 expression is lowest during the S phase but increases in late G2, highest during mitosis, and degraded during mitotic exit. It is predominantly cytoplasmic during the S phase but is associated with condensed chromosomes during mitosis. Aurora A is responsible for the initial phosphorylation of Plk-1 at the G2/M transition. Plk-1 also regulates the localization of Aurora A to the centrosomes for proper maturation.

Plk-1 expression has been reported to be a poor prognostic factor in various carcinomas including lung, head and neck, oesophagus, stomach, endometrium, ovary, brain, skin and breast cancers. King et al. reported expression of Plk-1 in 11% of primary breast cancers and it was associated with P53 mutation and triple negativity in early breast cancers. (King, 2012) Weichert et al. reported the expression of Plk-1 and Plk-3 in 42.2% and 47.4% of breast cancers. A positive correlation was found between the Plk expression and the tumour grade,

vascular invasion, HER2 expression and proliferation markers while inverse correlation was reported between the Plk isoform expression and the estrogen receptor status. Overexpression of Plk-3 but not of Plk-1 was significantly associated with the reduced median overall and relapse free survival in multivariate analysis. (Weichert, 2005)

Because of its crucial role in the cancer pathogenesis, many agents have been investigated to target Plk-1 as a treatment. Examples are Plk-1 enzymatic inhibitors - Scytonemin, Wortmannin, Staurosporine, Morin and Quercetin; PBD inhibitors such as Thymoquinone and Purpurogallin; and agents that suppress Plk-1 expression such as Genistein, Vanillin, Silibinin, Trichostatin A and Indirubin. (reviewed by Schmit, 2010)

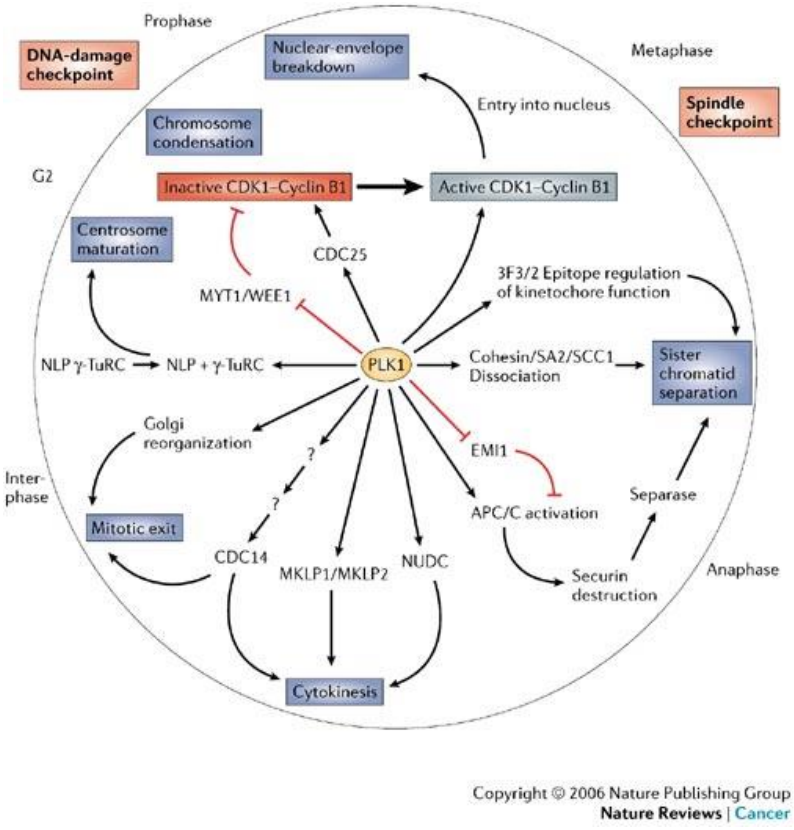
Several studies have shown that the down regulation of Plk-1 by several anti-sense and small interfering RNA (siRNA) as well as cytotoxic compounds caused cell-cycle arrest, apoptosis and decreased the growth in cancer cell lines. (Liu, 2003) (Spankuch-Schmitt, 2002a&b) (Kawata, 2008) i.v. administration of Plk-1 siRNA/atelocollagen had been shown to decrease the growth of liver metastases from lung cancer in a mouse model. (Kawata, 2008) The intravesical administration of Plk-1 siRNA inhibited the growth of bladder cancer in an orthotopic murine model. (Nogawa, 2005) Plk-1 siRNA transfection of prostate cancer cells resulted in a mitotic cell cycle arrest, failure of cytokinesis, and defects in centrosome integrity and maturation. In melanoma cells, Plk-1 inhibition resulted in a significant decrease in the viability and clonogenic survival, multiple mitotic errors, G2/M cell cycle arrest, and apoptosis. (Schmit, 2009) ON01910, a non-ATP-competitive small molecule inhibitor of Plk-1 was found to result in an induction of mitotic arrest characterized by spindle abnormalities leading to their apoptotic death in a wide variety of human tumour cells. (Gumireddy, 2005)

In HER2+ breast cancer cell lines, primary human cancer cells and orthotopic breast cancer models, intravenously injected F5-P/Plk1-siRNA complexes inhibited Plk-1 gene expression,

reduced proliferation and metastasis, induced apoptosis, and prolonged survival without evident toxicity. (Yao, 2012)

Inhibiting Plk-1 with siRNA or BI 2536 blocked the growth of triple negative breast cancer cells including the CD44high/CD24-/low TIC subpopulation and mammosphere formation.

(Hu, 2012) In a breast cancer brain metastatic xenograft model (231-BR), GSK461364A was found to inhibit the development of large brain metastases by 62% (P = 0.0001) and prolonged the survival by 17%. GSK461364A also sensitized tumour cells to radiation induced cell death in vitro. (Qian, 2011) Treatment of breast cancer cells with siRNAs targeting Plk-1 improved the sensitivity to paclitaxel and trastuzumab in a synergistic manner. (Spankuch, 2007)



Plk-1 interaction with other proteins (Borrowed from the published article by Strebhardt, 2006)

MCM-2

Minimichrosome maintenance proteins (MCM) were first found in the yeast as mutants defective in maintenance of minichrosomes. Six highly conserved members of MCM proteins (MCM 2 – 7) have been identified. They interact with each other to form a hetrohexamer complex. This complex binds to the DNA at specific sites known as replication origins in early G1 phase. Proteins namely origin recognition complex (ORC), Cdc 6 and Cdt 1 functionally interact with MCM 2-7 complex to form a pre-replication complex. MCM complex unwinds the DNA through its helicase activity that makes the binding sites on the DNA become accessible for replication and then the DNA is said to be licensed for replication. MCM complex dissociates from ORC in the S and G2 phases and they remain as a soluble nuclear pool during G2 phase and early mitosis. MCM complex makes sure the DNA replication occurs only once in each cell cycle. MCM complex is present in all phases of cell cycle and disappear when cell exits from the cell cycle, rapidly in the case of cell differentiation or more slowly in the case of quiescent G0 state.

MCM-2 expression by immunohistochemistry is localised to the nucleus. It was found in abundance in all phases of the cell cycle, but they are degraded in cells that have abandoned the cell cycle, such as quiescent or senescent state and differentiated cells. MCM-2 labelling index (LI), like Ki-67, identifies a unique licensed but non-proliferating population of tumour cells that increased significantly with tumour grade and was also of prognostic value. (Dudderidge, 2005) There was a positive correlation between the MCM-2 and Ki-67 LIs in normal breast although expression of MCM-2 was significantly higher than that of Ki-67 and showed greater variability and said to be able to identify more cells in the cell cycle than Ki-67 in a range of normal and malignant tissues. This may be because of the presence of cells in the early G1 phase of the cell cycle which may not express Ki-67. Hence, any tissue with a

high proportion of cells that are progressing slowly through G1 phase or held in G1 would be predicted to show a higher LI for MCM-2 than for Ki-67.

MCM-2 protein overexpression using different cut off values in different studies had been reported in various malignant tumours including colon, cervix, breast, oesophagus, kidney, brain, lung, thyroid, and prostate and was correlated with bad pathological features such as higher clinical stage, poor survival and NPI (in case of breast cancer). (Gonzalez, 2003) MCM-2 LI >50% was shown to be associated with significantly poor survival outcomes in breast cancers. (Kato, 2003) MCM-2 expression with a cut off point of 40% was an independent predictor of disease recurrence in the multivariate Cox regression analysis in Ta/T1 Bladder cancers. (Burger, 2007) Ki-67 LI >5% and MCM-2 LI >10% were strongly predictive of inferior disease specific survival in GISTs. (Huang, 2006) In gastric carcinoma patients, high MCM-2 LI was a poor prognostic factor in the diffuse types but not in the intestinal types. (Tokuyasu, 2008) Non-small cell lung cancer patients with less than 25% MCM-2 LI had a longer median survival compared to patients with > 25%. (Ramnath, 2001) In renal cell cancer, MCM-2 expression level is much higher than other proliferative markers such as Ki-67 and Geminin, and is correlated with the tumour grade and reduced disease free survival. MCM-2 expression is increased in prostate cancer tissues and correlated with a shorter disease free survival. (Meng, 2001)

Ki-67

Ki-67 is a large nuclear protein associated with the cellular proliferation although the exact function remains unknown. It was identified following the discovery of the prototype monoclonal antibody Ki-67, which was generated by immunising the mice with the nuclei of the Hodgkin lymphoma cell line L428. (Gerdes, 1983) The name is derived from the city of origin (Kiel) and the number of the original clone in the 96-well plate. (Scholzen, 2000) Ki-67 protein was present in the nuclei of cells in the G1, S and G2 phases of the cell division cycle as well as in mitosis. Quiescent or resting cells in the G0 phase did not express the Ki-67 proteins. (Gerdes, 1984) Therefore Ki-67 is useful to identify a cell population in the active cell cycling or tumour proliferation state. Ki-67 expression was reported to be a better marker than the mitotic count to evaluate the tumour proliferation and the prognosis. (Clahsen, 1999) However it can only give the estimate of the growth fraction but not the rate of proliferation which is also a very important factor.

Ki-67 overexpression was found to be a poor prognostic factor for the disease relapse and death in various cancers including breast cancers. Ki-67 >10% was predictive of poor survival in stage 1 and 2 breast cancer patients and also in a subset of low risk group defined by "Adjuvant!" and St. Gallen risk criteria. (Jung, 2009) Metastases in axillary nodes are likely to have higher Ki-67 expression compared to its primary breast lesions suggesting more active proliferative properties of metastasising cells. (Cabibi, 2006)

Ki-67 overexpression is predictive of a response to the chemotherapy (Penault-Llorca, 2008) (Darb-Esfahani, 2009) (Li, 2011) and endocrine therapy. (Viale, 2008) (Yamashita, 2006) (Kai, 2006) However some studies suggested that pre-treatment high expression may no longer be a poor prognostic factor after the chemotherapy or endocrine therapy. (Clarke, 1993) (MacGrogan, 1996) Instead post treatment residual Ki-67 expression may be a more

significant prognostic factor (Jones, 2009) as Ki-67 expression was found to decrease after various primary chemo, endocrine and other anticancer therapies. (Johnston, 1994) Decrease in Ki-67 expression index following neoadjuvant chemotherapy was more significant in the primary tumour than the lymph nodes metastases. (Koda, 2007a) Median reduction in Ki-67 expression following neoadjuvant FEC was 21.2%. Tumours having >75% reduction in Ki-67 expression following chemotherapy were more likely to get complete pathological response. (Burcombe, 2005) Early decrease in Ki-67 index after 10 –21 days of chemoendocrine therapy or chemotherapy alone was associated with a good clinical response, achieving either complete response or minimal residual disease. (Chang, 1999) (Assersohn, 2003) Higher Ki-67 expression after 2 weeks of endocrine therapy was statistically significantly associated with the worse RFS in a multivariate analysis in the IMPACT trial while high Ki-67 expression at baseline was not. (Dowsett, 2007) Changes in Ki-67 expression and apoptotic index at day 21 of neoadjuvant anthracycline chemotherapy did not however manage to predict the response. (Burcombe, 2006)

Post primary chemotherapy lymph node positivity and ki-67 index more than 15% are significant poor prognostic factors in breast cancer patients with hazard ratio for recurrence and death of 3.1 and 2.4 for one factor only, and 9.3 and 6.5 for both factors, compared to other patients. (Guarneri, 2009) Following neoadjuvant chemotherapy with doxorubicin + docetaxel, ER negativity and Ki-67 index above 1% are the poor prognostic factors, being the worse when both factors were present. Ki-67 index 1 or below was the only significant prognostic factor in multivariate analysis. (Lee, 2008)

ER/PR positive cancers with Ki-67 index of 13.25% was defined as luminal B subtype for which 10 years relapse free survival was 67% while that for luminal A subtype was 74% without systemic adjuvant therapy among low risk breast cancers. 10 years breast cancer specific survival was 79% and 92% respectively. (Cheang, 2009) Some studies have used

10% (Keshgegian, 1995) (Bevilacqua, 1996) or 20% (Clahsen, 1999) (Joensuu, 2003) or the mean (Goodson, 2000) or the median (Liu, 2001) as a cut off point to define the Ki-67 overexpression.

Transfection of Ki-67 cDNA resulted in 60-70% reduction in the proliferation of MDA-MB-435s cells which highly expressed Ki-67 mRNA and protein. The mobility and invasion capability were also reduced by 50 – 60% and the cell cycle analysis showed a higher proportion in G2/M and G0/G1 phases with markedly increased ratio of apoptotic cells. Therefore anti-sense Ki-67 cDNA might be a treatment option for cancers with high Ki-67 expression. (Wang, 2008)

Overall, the concordance between the RT-PCR and IHC analysis for ER, PR, and HER2 determinations was high. In contrast, the concordance between the RT-PCR measurements and IHC assay for Ki-67 was poor. (Cobleigh, 2005) (Potemski, 2006) Ki-67 expression score from TMA cores are shown to be in good agreement with that from whole tumour sections. (Giltnane, 2004) (Camp, 2000) (Nocito, 2001)

Although Ki-67 protein has been consistently shown to be a prognostic and predictive factor for treatment response and survival, there are many variations in assessment techniques and the cut off points used for dichotomisation. There is a need for an international standardization of the IHC procedures and a clinicalpathological validation by randomized, multicentre prospective studies.

CK-5/6

Intermediate-sized basic (type II) polypeptides CK-5 and 6 are members of the cytokeratin family that forms the intermediate filament cytoskeleton in epithelial cells. Their absence gives rise to a blistering skin disorder in neonatal epidermis, and haemorrhages within the embryonic liver. Mutations in CK-5 gene have been associated with epidermolysis bullosa simplex. The CK-5, CK-14 and CK-17 - high molecular weight cytokeratins - are known as basal keratins because they are expressed in the mitotically active basal layer of stratified epithelial tissues. The cells that express these keratins are also known as basal cell regardless of their position. The CK-5⁄6 is useful, especially in conjunction with the ER expression, to distinguish between the epithelial hyperplasia of usual type in a papilloma and the atypical hyperplasia or malignant epithelium. CK-5 is negative in the malignant epitheliums as they don't arise from the progenitor cells. (Otterbach, 2000) (Grin, 2009)

The genes for CK-5 and 6 are clustered in the region of chromosome 12q12-q13. In normal tissue, CK-5/6 is mainly expressed in keratinizing (epidermis) and nonkeratinizing (mucosa) squamous epithelium as well as in basal myoepithelial cell layer of the prostate, breast and salivary glands. CK-5/6 was positive in the vast majority of squamous cell carcinoma, basal cell carcinoma, thymoma, salivary gland tumour, biphasic malignant mesothelioma, transitional cell carcinoma, pancreatic , breast , ovarian and endometrial carcinomas.

CK-5 mRNA and protein are shown to be expressed in normal mammary epithelial cells culture and a few tumour cell lines (MCF-12A, MCF-10A and MCF-10F). (Subik, 2010) Therefore CK-5 expression is useful to differentiate normal from the malignant tissue. Decreased expression also correlates with the tumourgenic progression. In breast cancer, CK-5/6 expression was correlated with other poor prognostic factors although the biological function of CK-5 and 6 remained unknown.

In molecular subtype profiling, Core Basal Phenotype (CBP) was defined as tumours with overexpression of basal cytokaretins CK-5/6, CK-14, CK-17, EGFR and vimentin among ER/PR/HER2 negative patients. (Dabbs, 2006) However the definition of CBP as ER/PR/HER2 negative, CK-5/6 or EGFR positive has only 76% sensitivity to detect basal-like tumours defined by multi genes assays where CK-5/6 expression was found in only 57.6% - 62% of basal cancers. (Nielsen, 2004) (Livasy, 2006) (Lakhani, 2005) There were also discordances between mRNA and IHC methods to identify CK-5/6 positivity. Up to 48-55% cases could be CK-5/6 positive by IHC, but negative by mRNA examination. Similarly, 14% of cases with high mRNA levels were negative on IHC examination. Similar discordances were observed for CK-14 and CK-17 as well. (Kordek, 2010) CK-5/6 can be reliably analysed in the FNA specimen blocks containing at least 10 tumour cells suggesting basal like breast cancer can be identified at the time of the breast cancer diagnosis. (Delgallo, 2010)

Basal like cancers have poorer prognosis compared to other molecular subtypes. (Sorlie, 2001) Many studies found that CK-5, CK-14 and/or CK-17 are associated with higher tumour grade, poor survival and triple (ER/PR/HER2) negativity especially in node negative tumours. (Korsching, 2002) (Reis-Filho, 2008) When CK-5/6 and/or EGFR expressing tumours were analyzed without consideration of ER/PR status, the reduction in the survival increased with time, becoming more pronounced at 10 years than at 3 years. (Tischkowitz, 2007) Breast cancer patients who went on to develop brain metastases were more likely to have primary tumours that expressed CK-5/6 ($P<0.001$), EGFR ($P=0.001$) and HER2 ($P=0.001$). (Hicks, 2006)

As much as 15 - 54% of basal-like tumours defined on mRNA level still express at least one of the ER/PR/HER2 proteins. (Reis-Filho, 2008) (Nielsen, 2004) (Calza, 2006) (Sotiriou, 2003) (Jumppanen, 2007)

The hazard ratio for development of distant metastasis in bilateral breast cancer patients in whom at least one cancer was CK-5/6+ was 99.8 (P = 0.037). (Piekarski, 2006) There are also reports of correlation between basal cytokeratin expression and atypical and typical medullary carcinomas. (Gusterson, 2005) CK-5/6 expression is associated with a higher pCR rate in breast cancer patients treated with neoadjuvant anthracycline/ taxane based chemotherapy compared to others. (Darb-Esfahani, 2009) (Li, 2011)

Although CK-5 is a recognised protein to define the basal like cancer, CK-6 is not. There are also some evidence suggesting CK-6 is not expressed in normal and basal-like breast cancers. (Moll, 1998) (Bocker, 2002) Yet in some studies CK-6 and 17 expressions were used to define the basal phenotype. (Stingl, 2007) The antibody directed against CK-5 alone was said to be more sensitive than common CK-5/6 antibody - 97% vs. 59%. For positive cases, the percentage and intensity of staining was much higher with CK-5 than with CK-5/6. (Bhargava, 2008) However, CK-5/6, rather than CK-5, is commonly used in molecular subtype profilings.

CD-68

CD-68 (Cluster of Differentiation 68) is a glycoprotein with a molecular weight of 110 Kda that binds to the low density lipoprotein. It is encoded for by a gene located on the chromosome 17p13. CD-68 protein is expressed primarily as an intra-cytoplasmic molecule associated with lysosomal granules. Monoclonal mouse antibody against CD-68 labels human monocytes and macrophages. (Tran, 1998) CD-68 antibody is used to identify macrophagic cells or cells of macrophagic origin. CD-68 expression by the cancer cell was reported only in a few cancer types. In a study involving 127 breast cancers, immunohistochemistry staining for macrophage markers CD-163 and MAC387 in cancer cells was detected in 48% and 14%, respectively, of cases but CD-68 staining in cancer cells was not detected in any of the cases. (Shabo, 2008) Immunostaining for CD-68 was reported in melanoma cells in 10% of cases and was correlated with the relapse free survival. (Jensen, 2010) Malignant glioma cells showed CD-68 expression more commonly than benign glioma cells and the higher expression was associated with the poor prognosis. (Strojnik, 2009)

Tumour associated macrophages (TAMs) are one of the major components of the breast cancer stroma being seen in 90% of the cancers. (Scholl, 1994) CD-68+ macrophages infiltration is seen more commonly in invasive breast cancers compared with DCIS, benign hyperplasia or normal breast tissue. (Hussein, 2006) Decrease in CD-68+ monocytes count was seen in tumours following neoadjuvant chemotherapies. (Hornychova, 2008) However an increase in macrophages in the tumour mammary duct has been reported following an endocrine therapy. (Chen, 2009)

TAMs regulate the tumour growth in a positive or negative way through interactions between TAMs, stroma and the tumour cells. (Yoshimura, 1989) (Mantovani, 1992) Tumour cells stimulate the formation of stroma that excretes a variety of growth factors, cytokines and

proteases. Certain cytokines and chemokines promote macrophage infiltration into the tumour. Tumour cells stimulate macrophages to produce various growth factors, angiogenesis factors and matrix-degrading enzymes that in turn promote angiogenesis, tumour growth, invasion, tumour cell migration, metastasis and survival. (Bingle, 2002) (Huang, 2002) CD-68+ TAMs were shown to secrete epidermal growth factor while other cells or malignant cells did not. (O'Sullivan, 1993) Some studies suggested that the prognostic value of TAM is probably due to their positive involvement in the tumour angiogenesis and the proliferation. (Leek, 1996) (Tsutsui, 2005) (Jonjic, 1998) Macrophages express uPA that leads to the plasmin dependent release of matrix-bound heparan sulphate proteoglycan - a basic fibroblast growth factor - and transforming growth factor beta, both of which are strong angiogenic factors. A positive correlation between microvessel density, vascular invasion, uPA level, macrophage content and the proliferation rate has been reported. (Hildenbrand, 1995)

TAMs can be activated from a quiescent non-angiogenic state to an angiogenic state. (Assoian, 1987) By releasing vasoactive substances, macrophages increase vascular permeability that enables extravasation of fibrinogen. Plasmin-cleaved fibrinogen fragment E has a strong angiogenic activity. TAMs increase the synthesis of nitric oxide which is converted into active genotoxicant peroxinitrite (Maeda, 1998) (Schaffer, 2006) and cause dysregulation of production of fat derived hormones and hormone-like substances. (Lin, 2005) Macrophages content of adipose tissue is found to be higher in obese patients (Weisberg, 2003) and in patients with insulin resistance/ decreased glucose tolerance as a result of certain hormonal and non-hormonal signals. (Neels, 2006)

The active immune response, such as macrophage infiltration, to the poor tumour cell differentiation was believed to be responsible for the increase in the proliferative activity, angiogenesis and dissemination of the tumours. (Pupa, 1996) (Tsutsui, 2005) (Lin, 2007) On the other hand, inflammatory changes could be just a reflection of high grade proliferating

tumours that excrete more cytokines attracting macrophages and T lymphocytes. Higher tumour grade and proliferative activity were found to be associated with CD-68+ macrophages infiltrations. (Al Murri, 2008) Inflammatory cell infiltrates were correlated with the better cancer specific survival (Toi, 1999) although some studies failed to confirm that. (Griffith, 1990) (Wintzer, 1991) Higher density of CD-68+ macrophages and antigen presenting cells are correlated with the increasing vascularisations and decreasing differentiations in follicular, papillary and anaplastic thyroid carcinomas. (Hermann, 1994)

There are conflicting results on correlations between CD-68+ macrophages infiltration and the breast cancer survival. In a study by Lee et al (Lee, 2006) involving 679 stage 1 and 2 breast cancer patients with a median follow-up period of nearly 10 years moderate to marked intratumoral diffuse inflammation (both macrophage and lymphocytic infiltration) was seen only in 11% of the cases and associated with the better survival in grade 3 but not in other grade cancers. Some studies suggested that it was inferior to the microvessel density in predicting the disease free survival. (Tsutsui, 2005) (Shabo, 2008) (Uzzan, 2004) Murri et al reported that breast cancer survival was not associated with CD-68+ macrophages infiltration but with the positive Ki-67 labelling index, higher tumour grade, higher proliferative activity, microvessel density and negative hormonal receptor expression. (Al Murri, 2008) In colorectal cancers, 5 years cancer specific survival rate was shown to be better in patients with higher CD-68+ cells density in the lymph nodes compared to the lower CD-68+ cells – 60% vs. 38%. (Oberg, 2002) High CD-68 gene expression by RT-PCR was one of the gene expression used as a poor risk factor to calculate 10 years distant recurrence rate in Oncotype Dx prognostic gene assay. (Paik, 2004)

CD-71 (Transferrin Receptor)

CD71 - also known as Transferrin receptor (TfR) - is a type II transmembrane glycoprotein that consists of a large extracellular C-terminal domain with a binding site for the ligand transferrin, a transmembrane domain and a short intracellular N-terminal domain. (Jing, 1987) The CD-71 is an essential protein involved in the iron uptake and the regulation of cell growth and also has immunoregulatory properties.

The CD-71 expression is found at low level in cells with a low proliferation rate such as those in the vascular endothelium of brain capillaries, endocrine pancreas, seminiferous tubules of the testes, pituitary gland, luminal membranes of the breast, hepatocytes, hepatic Kupffer cells and renal tubules, but at high level in cells with a high proliferation potential such as cells in the intestinal epithelium. The CD-71 is also expressed on cells that require large amounts of iron such as placental trophoblasts and maturing erythroid cells.

There are two different TfRs - namely TfR1 and TfR2 - being produced by alternative splicing. They have some similarities in their domains except cytoplasmic domains. TfR1 has 25 folds higher affinity for the transferrin (Tf) than TfR2. (Kawabata, 1999) TfRs can form hetrodimers but commonly form homodimers. (Vogt, 2003) Expression of TfR1 was highest in the late G1 and G2/M phases. TfR1 but not TfR2 expression is regulated by intracellular iron levels. (Kawabata, 2001) (Kawabata, 2000) In normal tissues, TfR2 expression is confined to the hepatocytes and enterocytes of the small intestine. Surface expression of TfR2 was detected in a wide variety of human cancer cell lines such as HepG2 (human hepatoma), K562 HEL-R (Kawabata, 2001) (Kawabata, 1999) and selected B and myeloid cell lines. (Deaglio, 2002)

CD-71 expression is found to be much higher in malignant cells such as breast cancer, transitional cell carcinomas of the bladder, gliomas, pancreas, lung adenocarcinoma, chronic lymphocytic leukemia and non-Hodgkin's lymphoma compared to non-malignant cells. High expression of CD-71 in malignant cells is believed to be a response to meet the increased demand for iron as a cofactor of the ribonucleotide reductase enzyme involved in the DNA synthesis of rapidly dividing cells. In breast cancer cells CD-71 expression was up to 4 to 5 folds higher than that of non-malignant cells. Increased CD-71 expression was associated with poor NPI score, tumour proliferation, basal cytokeratins, p53, EGFR, HER2, steroid receptor negativity and shortened breast cancer specific survival. On multivariate analysis, CD-71 was found to be an independent prognostic factor in the ER+ cancers. Elevation of CD-71 was seen in cell models of acquired resistance to tamoxifen. Exogenous Tf was found to significantly promote the growth especially in oestrogen deprived MCF-7 cells.

Deprivation of iron by disrupting CD-71 function can be detrimental to rapidly growing tumour cells. Monoclonal antibodies of IgG, IgA, and IgM isotypes against human transferrin receptor have been successfully used to inhibit the growth of neoplastic cells. The murine monoclonal anti-human CD-71 IgA antibody 42/6 has shown some cytotoxic activities against most human malignancies by preventing Tf from binding to its receptor by non-competitive inhibition, leading to iron deprivation and subsequent growth arrest. High expression of CD-71 was found in the drug resistant cells and down regulation of these receptors by calcium channel blockers diminished the drug resistance. (Barabas, 1993)

When iron bound ligand Tf binds to TfRs (CD-71) on the cell membrane, the whole complex is internalised through the receptor mediated endocytosis. Then the iron is released inside the cytoplasm and Tf/TfR (CD-71) complex returns to the cell surface where Tf dissociates from TfR (CD-71), leaving the latter available for the next cycle of endocytosis. This mechanism of endocytosis has been exploited to deliver various substances into the cell including

cytotoxic drugs in malignancy. CD-71 conjugates with chemotherapy agents such as gemcitabine, daunorubicin, doxorubicin and cisplatin have shown to increase the chemotherapy toxicity in the cancer cells compared to the chemotherapy alone. The therapeutic window of cytotoxic drug was also increased due to the lack of cytotoxicity in normal cells. The Tf-doxorubicin conjugate can overcome the resistance to doxorubicin in human oral carcinoma cells. (Fritzer, 1996) Modified Tf-doxorubicin conjugates reversed the resistance of MCF-7 human breast cancer doxorubicin resistant cells where doxorubicin was found sequestered in cytoplasmic vesicles. (Wang, 2000) Cisplatin-Tf conjugate showed some responses in advanced breast carcinomas. Treatments with CD-71 ligand targeted toxin conjugate (Tf-CRM107) showed some complete and partial responses in malignant glioma patients. (Weaver, 2003) (Laske, 1997) Therefore the analysis of CD-71 expression in the breast cancer tissue can be of benefit for both prognostic and therapeutic purposes.

PDGFR-α

PDGFRs are dimeric molecules consisting of alpha and beta sulfate bonded chains. They have an extracellular region with five immunoglobulin-like domains, a transmembrane region, an intracellular region with a regulatory juxta membrane domain, and a catalytic tyrosine kinase domain. PDGFRα had been found to play a major role in growth factor signalling pathways in various cancers. The binding of ligand Platelet Derived Growth Factors (PDGFs) to PDGFR induces dimerization of the receptor leading to auto-phosphorylation of tyrosine residues and stimulation of kinase activity with subsequent activation of downstream intracellular cascades RAS/RAF/MAPK, PI3K/AKT and STATs, that regulate cell proliferation, differentiation, migration and survival. Vascular Endothelial Growth Factor A (VEGF-A) can activate both PDGFRs. (Ball, 2007) Activated PDGFRs interact intracellularly directly with components from different PDGFRs and their ligands and form various autocrine and paracrine loops for activation. Autocrine PDGFR stimulation has been well documented in various tumours including breast cancers. (Jechlinger, 2006)

There are 4 different PDGFs that contain one of four different polypeptide chains: PDGF-A, PDGF-B, PDGF-C and PDGF-D. The chains are linked with amino acid disulphide bonds to form homo or hetro dimers. PDGF expression has been reported in various neoplasms including glioblastomas, ovarian, prostate and up to 90% of breast cancers. High level of PDGF in breast tumours has been shown to correlate with the high invasiveness, low response to chemotherapy and decreased survival. PDGF-BB is reported to be important for the cancer cells to metastasise to the bones. (Lev, 2005) Reduction in cell migration and proliferation had been observed following the blockage of PDGF activities. (Ball, 2007)

PDGFRs are expressed on erythroid and myeloid precursors in the bone marrow, monocytes, megakaryocytes, fibroblasts, endothelial cells, osteoblasts and glial cells. In malignant

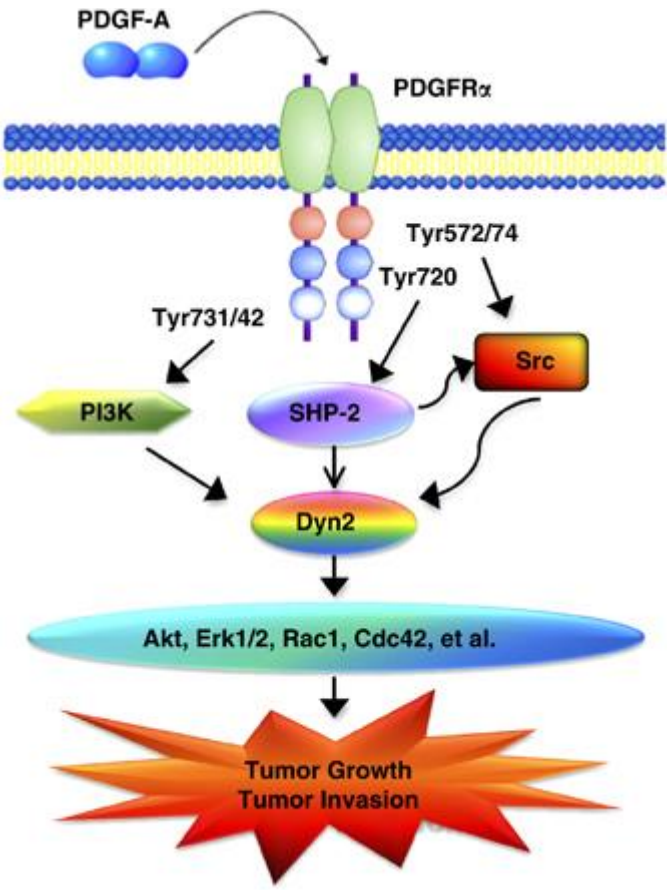
tissues, it was also found to be expressed in the stromal tissue including pericytes that support blood vessels. (Ostman, 2004) Aberrant or over-expression of PDGFRs is associated with a variety of disorders including atherosclerosis, fibrotic disease and neoplasia. Aberrant activity of PDGFR and their ligands have been reported in gastric, prostate, lung, melanomas, ovarian and colorectal cancers, (Drescher, 2007) (Ebert, 1995) (Antoniades, 1992) (Wehler, 2008) and it was associated with poor outcomes. PDGFR-α mutations had also been reported in some tumours such as GISTs (exon 12 and 18) lacking c-Kit mutation and glioblastomas (amplification of PDGFR-α genes). (Joensuu, 2005) PDGFR-α was associated with increased cell proliferation in some non-small cell lung carcinomas and rhabdomyosarcoma cell lines. (McDermott, 2009)

In human colorectal cancer, PDGFR-α, PDGFR-β and co-expression were found in 83%, 60% and 57%, respectively, of cases. PDGFR-α was mainly found in the cytoplasm of cancer cells and pericytes. PDGFR α and β expressions are significantly correlated with the lymph node metastasis and advanced UICC stages III/IV in older patients. (Wehler, 2008) PDGFRα expression was found in high frequency among metastatic prostate cancer cells taken from the bone. (Chott, 1999) (Ko, 2001) (Dolloff, 2007) More than 70% of hepatocellular carcinoma (HCC) tissues had elevated PDGFR-α. Its inhibition significantly affected HCC cell survival by modestly reducing the proliferation suggesting PDGFR-α's main function is for the survival rather than the proliferation. (Stock, 2007)

Invasive breast carcinomas expressed cytoplasmic staining of PDGFR-α in 65% and PDGFR-β in 75% of samples. (Jechlinger, 2006) Endothelial cells of breast cancer tissues express more PDGFR-β than PDGFR-α compared to the endothelial cells of normal breast tissue. (Carvalho, 2005) However, no objective responses to treatement with Imatinib were observed among the 13 metastatic breast cancer patients with PDGFR-β overexpression.

(Christofanilli, 2008) Imatinib is believed to exert its function in non-small cell lung cancer through the PDGFR-α. (Zhang, 2003)

Inhibition of PDGFR-α signalling by siRNA, small molecule inhibitor or neutralizing antibody has been shown to have anticancer effect in various cancers including GISTs, ovarian cancer, non-small cell lung cancer, rhabdomyosarcoma, mudalloblastoma, breast cancer and prostate cancer both in vitro and in vivo. But the resistance developed in one third of rhabdomyoblastomas in one study. (McDermott, 2009) (Taniguchi, 2008) (Armistead, 2007) (Schneider, 2005) Tyrosine kinase inhibitors such as Sunitinib can inhibit many proteins such as VEGFR-2, PDGFR-α, PDGFR-β, and fibroblast growth factor receptor 1 (FGFR1) tyrosine kinases involved in PDGFRs signalling cascade.



PDGFR-α signalling in glioma cell (Borrowed from the published article by Feng, 2012)

VEGFR-2

Angiogenesis is one of the major pathways involved in the pathogenesis of malignancy. One of the key components of this pathway is vascular endothelial growth factor receptors (VEGFRs), which upon binding with its ligand vascular endothelial growth factor (VEGF) produce a cascade of signals for cell proliferation and angiogenesis. VEGF is produced by many tumour cells and positively regulated by cytokines, activation of oncogenes, loss of tumour suppressor genes and hypoxia.There are mainly two VEGFRs – VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1). These tyrosine kinase receptors promote cancer cell growth indirectly through the stimulation of microvascular endothelial cells and directly by stimulating tumour cells in an autocrine fashion. Activation of VEGFR-2 alone is sufficient to elicit all proangiogenic, proliferation and survival effects associated with VEGF indicating its dominant role over VEGFR-1. (Gille, 2001)

VEGFR-1 and VEGFR-2 expressions have been widely reported in endothelial cells, bone marrow derived elements and various cancers. VEGFR-2 was found to be 3 to 5 folds higher in the endothelial cells of tumour vasculature compared to the normal tissue vasculature.

VEGFR-2 and VEGF-A are found to express together in several malignant epithelial cells such as prostate, breast, pancreas and stomach. (Takahashi, 1996) (von Marschall, 2000) (Kollermann, 2001) (Ryden, 2003) The VEGF level was found to be higher than VEGFR-2 level. (Dias, 2001) (Ferrer, 1999) High expression of VEGFR-2 was also reported in colorectal, renal and genitourinary cancers. (Giatromanolaki, 2007) (Heng, 2007) (Pouessel, 2008)

VEGFR-2 dimerises upon activation by VEGF and auto-phosphorylates in the cytoplasmic kinase domain. (Kendall, 1999) This phosphorylation triggers a cascade of events through

Raf/Mek/Erk, PI3K/Akt and PI3K/Akt/nitric oxide pathways, leading to endothelial cell proliferation, migration, apoptosis inhibition, maturation of vascular structures and vascular permeability. Blocking VEGF/VEGFR signal pathways inhibits tumour growth by decreasing the vascular density and subsequent cell death by apoptosis. (Des Guetz, 2006) The function of VEGFR-1 is not well established but it has been reported to induce protease activity in endothelial cells and stimulate the migration of macrophages into the tumour tissue. However, it has no effect on the proliferation. (Veikkola, 2000)

In breast cancers, VEGF, VEGFR-1 and VEGFR-2 all showed a predominantly membrane/cytoplasmic distribution in the tumour, and their high levels were significantly associated with poor survivals in the univariate analysis. (Ghosh, 2008) VEGF stimulates the proliferation of VEGFR-2 positive tumour cells; promotes the survival via the expression and activity of Bcl-2; and overrides the growth suppressive effects of anti-hormones. (Liang, 2006) 17β-estradiol (E2) was found to induce VEGFR-2 expression in ERα-positive ZR-75 breast cancer cells. (Higgins, 2006) Tamoxifen decreases VEGFR-2 expression more in the ER/PR negative cancers compared to ER/PR positive cancers. (Garvin, 2005) In patients with ER positive and VEGFR-2 low tumours, adjuvant tamoxifen significantly increased the recurrence free survival. But tamoxifen was found to have no effect in VEGFR-2 highly expressed tumors. In multivariate analyses, this VEGFR effect on the tamoxifen efficacy was seen in all hormone receptor positive cancers. (Ryden, 2005)

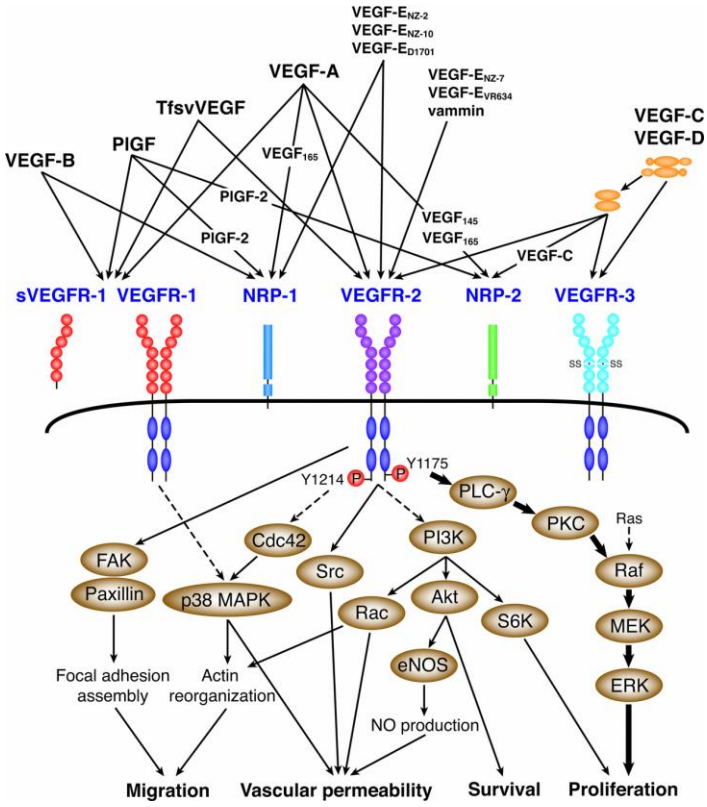
High VEGFR-2 expression was correlated with other poor risk factors such as VEGF, p38 MAPK, negative ER, triple negative cancers, larger tumours, nuclear grade 3, distant metastasis, shorter RFS and breast cancer specific survival. High intratumoral level of VEGFR-2 was suggested to be predictive of an intrinsic resistance to the adjuvant endocrine therapy. (Linderholmn, 2011) (Ryden, 2010)

In combination with anti-VEGFR-2 antibody DC 101, metronomic chemotherapy (one tenth of maximum tolerated dose) with doxorubicin, vimblastin, taxane and cisplatin showed a significant breast cancer cell death in a xenograft model, even in the absence of the tumour shrinkage, that contain high level of P-glycoprotein and multi drug resistance protein against which chemotherapy alone failed to show any effectiveness. (Klement, 2002)

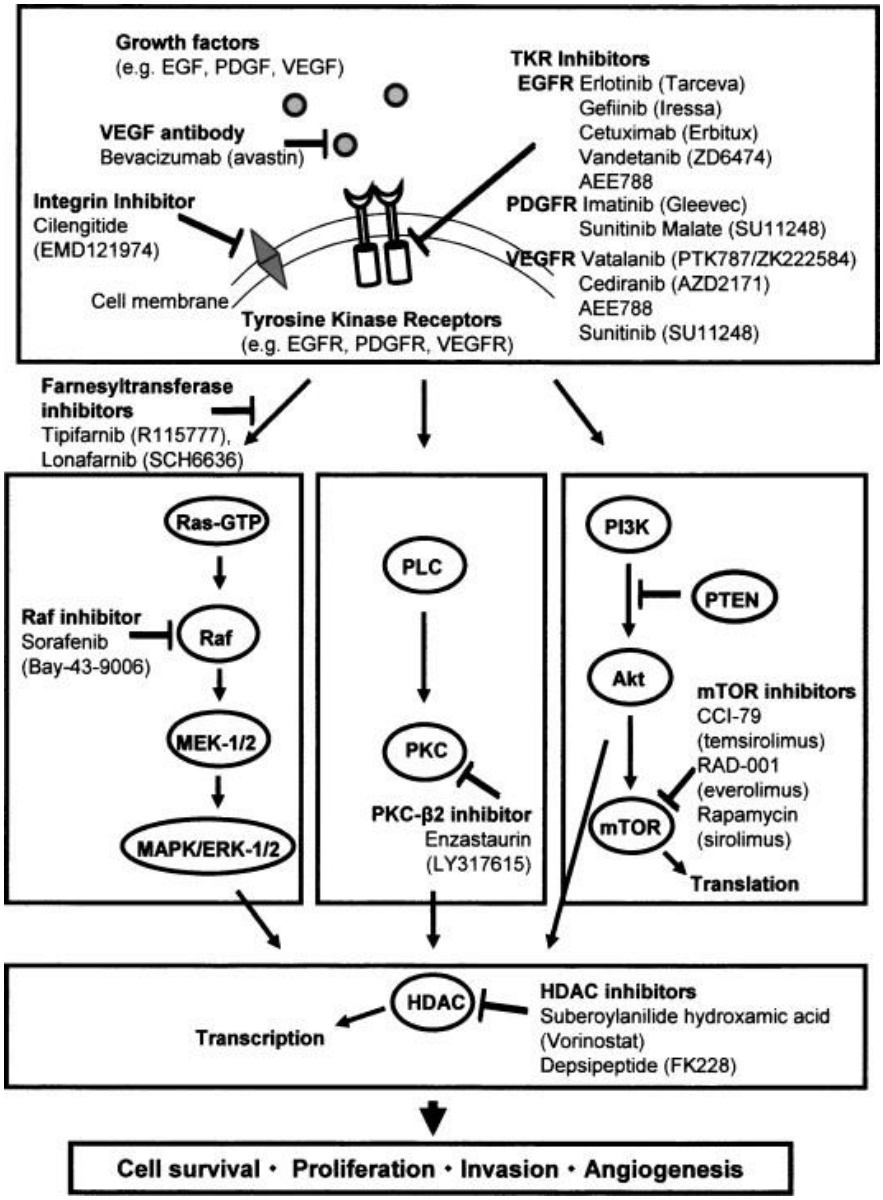
The VEGFR inhibitors such as vandetanib, sorafenib and sunitinib had been successfully tested in various cancers. Objective tumour response with oral tablet vantenatib was seen in refractory non-small lung cancer patients. (Tamura, 2006) (Holden, 2005) VEGFR-2 antagonist peptid, GU81, was found to enhance the anti-tumour activity of doxorubicin in spontaneous murine MMTV-PyMT breast tumours. (Lynn, 2010) In a phase II study, single-agent sunitinib revealed 14% response rate in breast cancers resistant to anthracycline and taxane. (Miller, 2005) More than three-fold increases in VEGF relative to the baseline, and decreases in soluble sVEGFR-2 levels by 30% in at least 88% of the patients treated with sunitinib had been reported. VEGF and VEGFR-2 levels returned to the baseline after 2 weeks off treatment. (Deprimo, 2006)

The addition of tamoxifen to epirubicin showed a significant reduction in VEGF expression in T2-4, N0-1 breast cancer patients in the neoadjuvant setting while epirubicin alone failed to do so. However, VEGFR-2 expression in the residual caner tissue was found to have increased from the base line level. The decrease in the VEGFR-2 expression was significantly associated with the response rate. This data suggests a potential synergism of these two drugs. (Mele, 2010) In a small study consisting 21 patients with the inflammatory and locally advanced breast cancers, a treatment with bevacizumab was found to be associated with a median decrease of 66.7% in phosphorylated VEGFR-2 (Y951) in tumour cells and a median increase of 128.9% in the tumour apoptosis. There were no significant changes in the microvessel density or VEGF-A expression. (Wedam, 2006)

VEGFR-2 expression by IHC was not associated with the better survival outcome in breast cancer patients treated with neoadjuvant VEGF inhibitor bevacizumab in combination with doxorubicin and docetaxel chemotherapy compared to chemotherapy alone. (Yang, 2011) Adding gefitinib to pre-operative chemotherapy paclitaxel and epirubicin in early breast cancers did not result in any different effects on the EGFR dependent pathway, proliferation, apoptosis and the VEGFR-2 expression as compared to the placebo. (Guarneri, 2008)



VEGFRs pathway (Illustration borrowed from <pubmed>16104843</pubmed> [http://www.clinsci.org/cs/rights.htm Copyright)



EGFR, PDGFR and VEGFR pathways and their inhibitors (Borrowed from the published article by Argyriou, 2009)

GSTM-1

Glutathione S-transferases (GST) are phase 2 metabolising enzymes involved in catalysing detoxification of electrophilic compounds including carcinogens, therapeutic drugs (eg. alkylating agents and platinum compounds), environmental toxins and products of oxidative stress for subsequent removal from the body. Failure to clear electrophilic carcinogenics could lead to the DNA damage and subsequent carcinogenesis. Variations within GST genes can cause a loss or reduction in the enzymatic activity and have been associated with the increased risk of several cancers. Elevated DNA adducts, sisterchromatid exchanges and somatic genetic mutations have been demonstrated in carriers of some null GST genotypes. (Rebbeck, 1999)

GSTs are divided into four classes - Alpha, Mu, Pi and Theta - based on the protein sequence similarities. At least five distinct GST-Mu (GSTM 1 - 5) isoforms have been described. GSTM-1 is the dominant isoenzyme among them and the biophysiological functions of the other isoenzymes may become conspicuous only when GSTM-1 is absent. The most reported GSTM-1 polymorphism is a gene deletion variant, known as GSTM-1- null (GSTM1-/-) with a complete absence of GSTM-1 enzyme activity.

The GSTM-1 and GSTT-1 null genotypes have been linked to the increased risk of developing cancers in lung, bladder, stomach, bowel and skin. (Setiawan, 2000) (Katoh, 1996) (Gao, 2002) (Choi, 2003) (Epplein, 2009) (Inoue, 2001) (Piao, 2009) (Carlsten, 2008) There are also studies that reported no association between the null genotype and cancers of lung, colorectal, breast, prostate and oesophagus. (Welfare, 1999) (Sivonova, 2009) (Lavender, 2009) (Zhou, 2009) Some studies suggested that GSTM-1 polymorphism alone did not increase the risk of colorectal cancer but together with GSTM-3 polymorphism it increased the risk of distal colon cancer. (Loktionov, 2001)

A meta-analysis on 59 studies involving 20,993 cases and 25,288 controls, significantly elevated breast cancer risk was found to be associated with GSTM-1 null genotype (Odd Ratio = 1.10, 95% CI = 1.04–1.16). The increased risk was noted in Caucasians, Asians and postmenopausal women. The author concluded that GSTM-1 null genotype was a low-penetrant risk factor for developing breast cancers. (Qiu, 2009) Combined effect of all three GSTT-1, GSTM-1, and GSTP-1 polymorphism have been reported to have > 3-fold increase in the breast cancer risk compared with the common genotypes. (Helzlsouer, 1998) (Steck, 2007)

However, Kadouri et al reported GSTM-1 null genotype to be associated with the low breast cancer risk although it was not statistically significant and it did not increase the risk of breast cancer in BRCA carriers. (Kadouri, 2008) (Spurdle, 2009) Null GSTM-1 gene was found not to be associated with the increased risk of breast cancer among postmenopausal women who used HRT. (The Marie-Genica consortium, 2010) When GSTM-1 gene was present, no Single Nucleotide Polymorphism (SNP) or haplotypes in the GSTMs cluster conferred conspicuous risk to the breast cancer. (Yu K-D, 2010) No increased risk of breast cancer was found among smokers with GSTM-1 null genotype although the absent GSTM-1 activities should have led to the lack of inactivation of polycyclic aromatic hydrocarbons from smoking. (McCarty, 2009) A SNP in the promoter region of GSTM-3 gene had a strong association with breast cancers when GSTM-1 was genetically deleted. Null GSTM-1 was reported to be associated with a better response to neoadjuvant chemotherapy in stage 2 and 3 breast cancers. (Oliveria, 2010)

Correlations between the risk of breast or other cancers and GSTM-1 protein expression by immunohistochemistry have never been studied.

Cathepsin L2

Cathepsins - meaning “to digest” in Greek - are lysosomal cystein proteases, a family made up of 11 different types - B, C, F, H, K, L, O, S, V ,W, and X/Z. Most cathepsins are endopeptidases whereas some are exopeptidase. Most cathepsins have a property of catalysing the cleavage of peptide bonds in the proteins in the majority of cell types. They play a role in a variety of intracellular and extracellular processes including antigen presentation, pro-hormone activation, sperm maturation and bone resorption. The activity of cathepsins is regulated by the balance between their endogenous inhibitors and the activators of their inactive precursors. Over-expression of cathepsins has been reported to be involved in glomerulonephritis and osteoarthritis. Cathepsins promote tumour invasion and metastasis by degrading components of the extracellular matrix especially the basement membrane, helping cancer cells to invade the surrounding tissues, lymphatic and blood vessels to metastasise. Cathepsins were also significantly correlated with UPA and PAI-1 enzymes that played an important role in the metastasis. (Herszenyi, 1999)

Cathepsin L2, also known as Cathepsin V, has a high sequence homology to Cathepsin L. It is encoded for by CTSL2 gene located on the chromosome 9q21-22 which is the same site as Cathepsin L. The sequences of the two enzymes are quite similar sharing 80% of the identity. Cathepsin L2 is predominantly expressed in the thymus, testis and cornea. Although Cathepsin L has been extensively studied in various cancers, Cathepsin L2 expression has not been studied for its prognosis and predictive outcomes in cancer patients.

Cathepsin L has been reported to be upregulated in a variety of malignancies including breast, lung, gastric, colon, ovary, head and neck carcinomas, melanomas and gliomas but not in normal or peri-tumoural tissues. (Santamaria, 1998) Cathepsin L is functionally active in the acidic environment. The acidification of tumour environment by the increased anaerobic

glycolysis in cancer cells is believed to promote the activities of Cathepsin L to degrade components of extracellular matrix such as collagens, fibronectins and laminins for subsequent metastasis. High expression of cathepsin L was found to be associated with deeper invasions (muscularis propria vs. mucosa) and venous invasions in gastric cancers. (Dohchin, 2000) Oral mucosal dysplasia with overexpressed cathepsin L is found more likely to progress to oral cancer than the dysplasia with no overexpression. (Macabeo-Onga, 2003) Urinary Cathepsin L level is found to be predictive of the presence and invasiveness of the bladder cancer. (Svatek, 2008) High blood level measured by ELISA is reported to be associated with a poor prognosis in colorectal cancers. (Herszenyi, 2008)

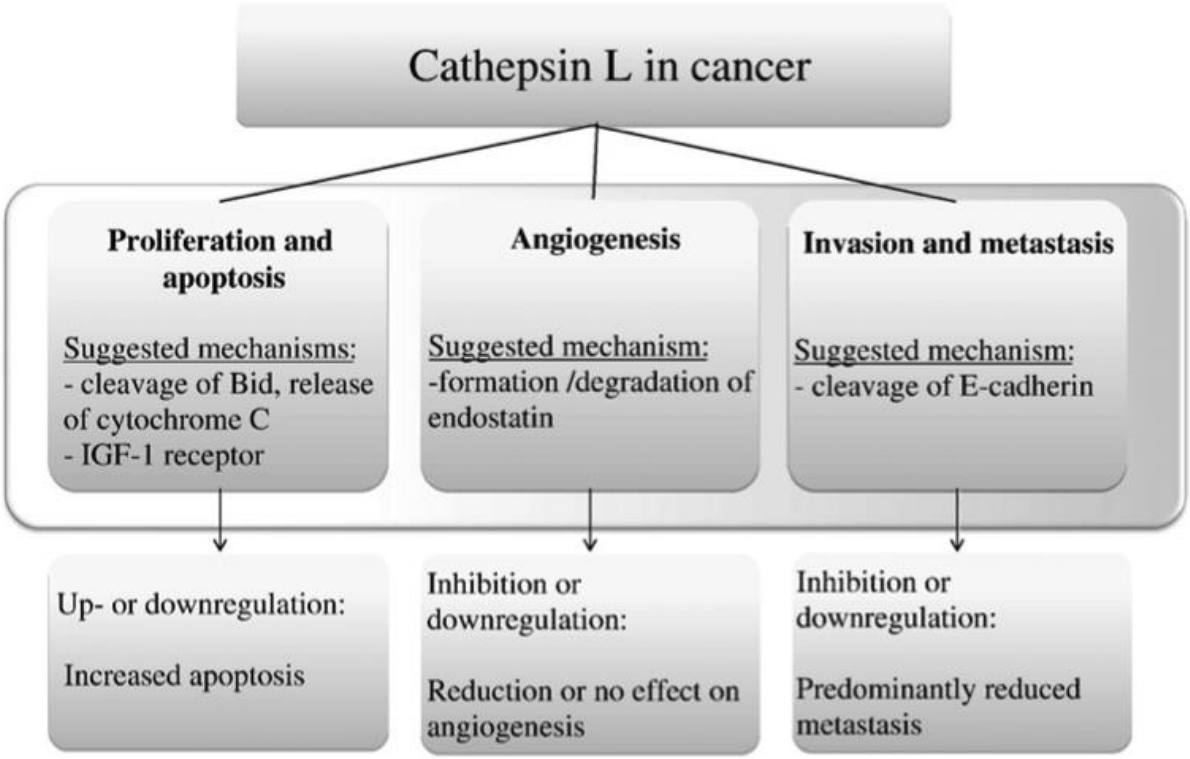
Cathepsin L was reported to be a poor prognostic factor for breast cancer recurrence that was comparable to the nodal status and the tumour grade both in node positive and negative cancers. It was inversely correlated to the hormone receptor status and the tumour necrosis. (Thomssen, 1995) (Lah, 2000) In a study of 276 breast cancer patients (52 = chemo; 95 = endocrine; 9 chemo + endocrine; 119 = no adjuvant therapy) with a median follow up of 109 months Cathepsin L was found to be prognostic of DFS and OS in all patient groups. In node positive patients, Cathepsin L was the only prognostic factor for DFS and OS. (Harbeck, 2001) This suggests that Cathepsin L is a prognostic rather than a predictive factor.

By using genetically modified antisense cDNA, Cathepsin L mRNA and protein expression level and subsequently, metastatic tumour development can be decreased. (Lah, 2006) Many drugs such as Thiosemicarbazone analouges and small molecules (peptidic compounds such as thiocarbazate, aldehyde, epoxide, nitrite, cyanopyrrolidine; and non-peptidic compounds such as azepanon, cyanamides and Cathepsin L specific CLIK-148) have been developed as cathepsin inhibitors. (Kumar, 2010) Down regulation of Cathepsin L by its antisense cloning showed decreased effects on the invasiveness of murine melanoma (Yang, 2007) and human glioblastoma cells. (Zajc, 2006) Cathepsin L specific inhibitor CLIK-148 could prevent the

cancer induced hypercalcemia and bone metastases. (Katunuma, 2002) However, some studies suggested that when Cathepsin L was inhibited by its specific inhibitors, its function may be compensated by other Cathepsins. (Hagemann, 2004)

Cathepsin L has been shown to play a major role in the sequestration of drugs. Therefore, inhibition of Cathepsin L may enhance the chemotherapeutic agents reaching the nucleus increasing the therapeutic ratio. (Lah, 2006) (Rebbaa, 2005) (Zheng, 2009) Inhibition of Cathepsin L might delay the cell cycle progression to the S phase (Goulet, 2007) or induce the senescence (Zheng, 2004) or apoptosis. Combination of chemotherapy and Cathepsin L inhibitor (Z-FF-FMK) was able to induce the senescence in various murine and human drug resistant cancer cell lines. (Zheng, 2004) (Levicar, 2003) Transfection with Cathepsin L siRNA also could reverse the drug resistance. In vivo, a combination of chemotherapeutic drugs with either broad-spectrum or Cathepsin L specific inhibitors showed favourable results. (Bell-McGuinn, 2007) (Zheng, 2009) However, the findings were not consistent and one study paradoxically reported a 50% increase in the tumour burden as compared to the chemotherapy alone. (Zhanaeva, 2005)

Some studies suggested that Cathepsin L induced the apoptosis by cleaving Bid - a Bcl-2 family member - (Stoka, 2001) resulting in the release of Cytochrome C from the mitochondria. (Green, 1998) Yet, there was no conclusive data to confirm if Cathepsin L was a pro or antiapoptotic protein. (Di Piazza, 2007) (Gocheva, 2006) (Navab, 2008)



Functional roles of Cathepsin L in cancers (Borrowed from the published article by Lankelma, 2010)

7.0 Objectives

Metastatic breast cancer is incurable. Adjuvant chemotherapy can increase the number of patients who can be cured of breast cancer by preventing or abrogating the metastasis. Breast cancer mortality has declined over the last decade due to the early diagnosis through screening programmes and better treatments. (Sant, 2006) Although it has been suggested that this improvement is partly due to a "lead time bias" from the early diagnosis of screen detected low risk cancers that would never become detectable clinically in the lifetime of an individual, there is evidence suggesting the overestimate due to the potential "lead time bias" is actually very small. (Jonsson, 2007) (Seigneurin, 2011) Despite significant advances in the adjuvant treatments for breast cancer, a large number of women still continue to relapse and die each year from breast cancer. To push the current survival successes to the next level, it is of paramount importance to identify patients who are at especially high risk of cancer recurrence and death despite adjuvant therapies, so that novel treatments can be explored for this group of patients. Because of potential toxicities, the oncology community has focused its efforts on selecting patients in whom adjuvant chemotherapy could be safely omitted or patients with a certain level of risk for which toxic chemotherapies are justified. In contrast, this study aims to identify patients in whom adjuvant chemotherapy is most likely to fail. Alternative treatments through participation in clinical trials will be most appropriate for them to improve their survival outcomes.

Current prognostic tools that based on clinicopathological features and genes/protein expression profilings aim to evaluate prognostic risks to aid making decision on adjuvant chemotherapy. Analysis of protein expression at the tissue level for this purpose will be cheaper and quicker than gene expression analysis. It is also more sensible to analyse the phenotypic profile of a cell through the proteins that are directly involved in the cellular

processes and test the benefit of targeting these proteins with novel therapeutic agents. Therefore this study evaluates the expressions of a panel of proteins that would appear to play a vital role in different cellular pathways essential to therapeutic resistance.

The following **hypotheses** are made based upon our understanding of these cellular proteins:

1. The group of patients that will relapse within 5 years of adjuvant chemotherapy (or) 5 years relapse free survival and/ or overall survival benefit could be predicted by analysis of expression of a panel of:
    a. proteins identified from the literature for molecular sub type profiling
    b. proteins coded for by genes used in Oncotype Dx gene prognostic assay
    c. proteins involved in the angiogenesis pathway
    d. proteins involved in cell cycle phase progression
2. Combined analysis of above proteins from key molecular pathways will be able to indicate the strongest candidates for future evaluation via therapeutic means, in clinical trials.
3. Integration of molecular markers and conventional clinical and pathological features might have stronger predictive power.

The different expression patterns of biomarkers in patients with different relapse free and overall survival periods may indicate the chemotherapy ineffectiveness (predictive effect) or unfavourable tumour biology (prognostic effect) or both as was the case in most biomarkers. This information is expected to be at least useful for hypothesis generation and the further research for better treatments.

8.0 Methods

Study design, study population and clinical data collection

This is a matched case controlled study. Cases are patients who had breast cancer recurrence (local or distance) within 5 years of curative surgery for early breast cancer (either wide local excision or mastectomy) followed by adjuvant chemotherapy. A period of 5 years was chosen because the main benefits of adjuvant chemotherapy for recurrence emerge during the first 5 years after the treatment. (EBCTCG, 1998) Recurrence was defined as the first reappearance of breast cancer at any site (local, contralateral or distant), as is customary in the clinical literature (for example EBCTCG, 1998). Patients in the "case" group had the breast surgery between January 1998 and October 2008. Controls were selected to be the patients who remained free of recurrence for a minimum of 5 years following curative surgery and adjuvant chemotherapy. This study started in 2008. To achieve minimum of 5 years relapse free survival, only patients who had their curative surgery before December 2003 could be selected. The patients selected for the control group had their curative surgery between August 1996 and December 2003. All patients received adjuvant radiotherapy and endocrine therapy appropriately as per standard practice at that time. All the patients were treated in a single cancer centre although the surgeries were done in 3 different hospitals and the chemotherapies were delivered on two sites within the network. The controls were matched to the cases for the 10 years recurrence risk (RR) according to "Adjuvant!" tool. (www.adjuvantonline.com) Controls have positive nodal status and RR at least 50%. Each control should have RR not more than 10% point lower than the matched "case". This is to increase the chances of finding unknown prognostic/ predictive factors. As a low risk second control, patients with recurrence risk <50% (with positive nodes) were selected in a ratio of 2 cases to 1 low risk control. The patients in this group had their curative surgery between May

1996 and October 2003. Eligible patients were identified from the department database. All patients whose paraffin embedded tissue blocks were available were screened for best matching and selection. Where there was more than one control available for a case, the control that most closely resembled the case in terms of known risk factors was selected. Type of surgery, adjuvant chemotherapy, endocrine therapy, survival data and pathological features were collected from the hospital case notes. Disease free status and survival status were confirmed by the records of patients' attendance at hospital clinics and the primary care surgeries.

During the study period, new prediction tool "OPTION" became available. The "OPTION" tool offers 5 years and 10 years recurrence risk before and after adjuvant therapies. The recurrence risks according to OPTION tool were calculated and analysed to verify that cases and controls were well matched according to the recurrence risk.

Local ethic committee reviewed and approved the study. The comittee agreed that obtaining consent from the individual patient for the use of cancer tissues was not feasible as most of the patients in the "cases" group were already deceased. Also some of the patients in the "control" group had moved to different areas.

Construction of tissue microarrays blocks

H & E slides were prepared from the archived FFPE pathology blocks. The areas that contained invasive cancers and were suitable for this study were marked on the H & E slides with the help of an experienced pathologist. 0.6 mm cores were taken from the blocks corresponding to the marked areas. A total of 3 cores, if possible, were taken from different areas of each block. The cores were transferred to the recipient paraffin blocks and tissue microarrays blocks were constructed manually using Beecham® tissue micro-arrayer.

Immunohistochemistry

Each TMA block was cut into 4µm thick sections and mounted on to the Superfrost Plus® glass slides. IHC staining was carried out using Ventana BenchMark XT automated staining machine. It is a fully automated slide preparation system. The slide kinetics have been optimised through the precisely controlled reaction environment of the BenchMark staining module with Air Vortex Mixers, Liquid coverslip, heater pad, and the E-Bar code slide label system. Air-Vortex Mixers blend the aqueous layer under the Liquid Coverslip, mixing reagents and ensuring uniform reaction kinetics across the entire surface of the slide, controlling evaporation and protecting tissue integrity. The individual slide heater pads provide highly precise heating across the entire surface of each slide. The clone and dilution of antibodies, antigen retrieval methods are as shown in the table. The procedure is as follow:

1. Formalin Fixed Paraffin Embedded TMA blocks were cut into 4 micron thick sections and mounted onto Superfrost Plus® slides (Visions Biosystems, Newcastle Upon Tyne, UK).
2. The slides were dewaxed in EZ preparation (Ventana catalogue no. 950-100) at 75º C for 4 minutes and 76º C for 4 minutes and rehydrated with buffer solution.
3. Antigen retrieval was performed by heating the slides to 100º C in buffer cell conditioner solution (CC1) for the specified duration which is shown in the table below for each antibody. The CC1 Solution is composed of Ethylenediaminetetraacetic acid 0.04%, Tris (Base) 0.12%, Boric Acid 0.05%, water and other ingredients that are below detectable level.
4. For EGFR, the slide was incubated with protease 1 enzyme for 8 minutes, and then heated to 37º C for 4 minutes.
5. I-View inhibitor (hydrogen peroxide 1.1%) was applied to block the endogenous peroxidise activity.

6. The slides were incubated with primary antibody for specified duration and temperature which is shown in the table below.
7. 0.6% gluteraldehyde was applied to fix the antibody binding to the protein.
8. Amplifier was applied to increase the signal intensity of staining. Avidin Blocker was applied to bind to endogenous excess biotin present in the tissue block to reduce non-specific staining caused by endogenous biotin present in cells and tissues. Biotin Blocker saturates the remaining binding sites of Avidin Blocker.
9. I-View Biotin Ig (Biotinlyated Ig) was applied as secondary antibody.
10. I-View Streptavidin-HRP conjugate was applied to replace the complex of avidin-biotin peroxidase.
11. The slide was incubated with I-View DAB (3,3' diaminobenzidine tetrahydrochloride) chromogen and its activator I-view H2O2 (DAB activator), which results in a dark brown precipitate at the antigen site.
12. I-View copper was applied for background colour of yellow.
13. The slides were counterstained with Haematoxylin & Eosin to visualize the nucleus.
14. The slide was then washed in Scotts tap water, dehydrated using alcohol, made clear using xylene and then the coverslip was mounted using DPX.
15. As positive controls, breast cancer tissue composites (cancer tissues with known expressions status - low, medium and high) were used for ER, PR, HER-2; Tonsil for Ki-67, MCM-2, Aurora A, CD-68 and Bcl-2; skin for CK 5/6 and EGFR; colon for PDGFR-A.
16. Negative controls were done by omitting primary antibodies.

IHC protocol for Bag-1 and CD-71

For CD71 and Bag-1 proteins, manual staining technique was used as described below.

1. Sections are collected onto Surgipath X-tra adhesive slides and dried at 37° C overnight prior to assay. Breast cancer tissues with known positive expression for Bag-1 and CD-71 were used as positive controls. The slides were dewaxed with xylene and rehydrated through graded alcohol and water as below:

    a. Xylene 1 x 20 minutes

    b. Xylene 1 x 10 minutes

    c. 100% Ethanol 2 x 2 minutes

    d. 90% Ethanol 2 x 2 minutes

    e. 70% Ethanol 2 x 2 minutes

    f. Distilled water 1 x 5 minutes

    g. PBS 1 x 5 minutes

2. Endogenous peroxidises activity was blocked by applying 3% hydrogen peroxide solution to the sections for 5 minutes. Then the slides were rinsed using distilled water for 5 minutes. To retrieve the antigen, the slides were heated in pH6 sodium citrate buffer solution in the pressure cooker for 1 minute for Bag-1 protein and microwave for 23 minutes at 560 watts for CD-71 protein. The slides were cooled under running tap water for 10 minutes.

3. To block binding interaction of antibody to non-specific sites 0.02% PBS/Tween was applied for 5 minutes. Excess block from the sections was wiped away before applying primary antibody.

4. Primary antibody was applied as below.

a. CD-71 (Mouse monoclonal IgG2: 10F11 Abcam) 1/30 in 0.1% BSA. PBS for 75 minutes at temperature 23° C

b. Bag-1 (Mouse monoclonal IgG1; SC56003 Santa Cruz) 1/150 in 0.1% BSA. PBS for temperature, 23° C duration overnight.

The slides were then washed in PBS for 1 x 3 minutes, PBS/Tween 2 x 5 minutes.

5. Dako mouse EnVision HRP-labelled polymer was applied to the sections for 45 minutes at room temperature. The slides were then washed in PBS for x 3 minutes and PBS/Tween 2 x 5 minutes.

6. Dako DAB K3468 chromogen was applied to sections for 8 minutes to give dark brown colour to the antigen antibody complex. The slides were then rinsed in distilled water 2 x 3 minutes.

7. The sections were counterstained with haematoxylin. The slides were then 'blued' under gently running tap water, air dried and covered with coverslip using DPX mountant.

8. Breast cancer tissue and cell lines with known expression status were used as positive controls.

9. Negative controls were performed by omitting primary antibodies.

Verification of specificity and sensitivity of antibodies used for IHC

Antibodies for ER, PR, EGFR, HER2, Bcl-2, CK 5/6, CD68 and Ki67 are for in vitro diagnostic use and antibodies for Aurora kinase A, MCM-2, CD-71, PDGFR alpha and Bag-1 are for research purpose only. IHC staining was carried out as per manufacturers' instructions and as per previous publications. Optimal staining protocol was achieved by staining serial tissue sections with different dilutions of antibody, different antigen retrieval conditions (heating method, temperature, duration, buffer solutions etc), different incubation periods with the antibody, use of enhancers such as AB block to minimise excess biotin and amplifier to magnify the signals, and negative controls by omitting primary antibodies. For specificity and sensitivity quality assurance, tissues or cell pellets with known negative, low, medium and high expression status were used as external controls. The staining of control sections were checked and compared with that of the previous analysis to detect minor variations in the staining intensity. When a decreased intensity was observed, the procedure was repeated. The negative tissue controls were examined after the positive tissue controls to verify the specific labelling of the target antigen by the primary antibody. The presence of an appropriately coloured reaction product within positive control cells is indicative of positive reactivity. The absence of specific staining in the negative tissue control confirms the lack of antibody cross reactivity to cells or cellular components. Also the cores on the TMA acted as internal controls.

IHC assays for MCM-2, CD-71 and Bag-1 were already developed previously for similar research projects. IHC assays for PDGFR alpha and Aurora kinase 2 were developed by using colon and tonsil tissues, respectively, as positive control tissues as per manufacturer's instructions. Optimisation was done as described above.

Monoclonal antibodies used in this study, the antigen they specifically bind to, manufacturers' recommended dilution and the references are shown in the table below.

Manufacturer	Antibody clone	Specificity	Recommended dilution	References
Leica	6F11	Nuclear ER alpha	1:40 – 1:80	Bevitt, 1997
Leica	16 (NCL-PGR-312)	Nuclear PR alpha	1:100 – 1:200	Bevitt, 1997
Ventana	4B5	c-erbB-2 protein internal domain	Pre-diluted	Akiyama, 1986
Ventana	3C6	EGFR protein external domain	Pre-diluted	Ventana data sheet, Cat no. 790-2988
Leica	MM1	Human Ki-67 nuclear antigen	1:200	Leica data sheet, product code NCL-L-Ki67-MM1
Dako	D5/16 B4	Isolated cytokeratin 5 and 6	Pre-diluted	Otterbach, 2000
Dako	124	Bcl2 oncoprotein	1:50 – 1:100	Hirakawa, 1996
Santa Cruz	3.10G3E2	Human Bag 1 protein	1:50 – 1:500	Millar, 2009
Novocastra	CRCT2.1	Human MCM2 protein	1:100	Ishimi, 1998
Leica	JLM28	Human Aurora Kinase 2	1:50	Bishoff, 1998
Abcam	ab61219	Total PDGFR alpha protein	1:50-1:100	Chan, 2010
Dako	PG-M1	Macrophage CD-68 antigen	1:50 – 1:100	Falini, 1989
Abcam	10F11	CD-71 protein	-	Habashy, 2010

Protein	Antigen retrieval Method Temperature and duration	Antibody incubation Temperature and Duration	Optimised dilution
ER	100 °C; 60 min in CC1 solution*	37°C; 36 min	1:30
PR	100 °C; 60 min in CC1 solution*	37°C; 36 min	1:100
HER2	100 °C; 60 min in CC1 solution*	37°C; 36 min	Pre-diluted
EGFR	Portease 1 at room temperature x 8 min; 37 °C for 4 min*	37°C; 36 min	Pre-diluted
Ki-67	100 °C; 60 min in CC1 solution*	40°C; 32 min	1:20
CK-5/6	100 °C; 60 min in CC1 solution*	37°C; 32 min	1:50
Bcl-2	100 °C; 60 min in CC1 solution*	37°C; 36 min	1:50
Bag-1	Pressure cooked in pH6 Na Citrate for 1 min at boiling temperature	23 °C; over night	1:150
MCM-2	100 °C; 60 min in CC1 solution	40°C; 44 min	1:30
Aurora A	100 °C; 60 min in CC1 solution	RT; 48 min	1:50
PDGFR-α	100 °C; 60 min in CC1 solution	RT; 48 min	1:100
CD-68	100 °C; 300 min in CC1 solution*	37°C; 24 min	1:50
CD-71	Microwave in pH6 Na Citrate for 23 minutes at 560 watts	23° C; 75 min	1:30
Cathepsin L2	NA	NA	NA
Plk-1	NA	NA	NA
VEGFR-2	NA	NA	NA
GSTM-1	NA	NA	NA

Antigen retrieval methods and antibody incubation conditions (*In Vitro Diagnostic assays)

Scoring biomarker expression

TMA slides were scanned and images were digitally stored using MIRAX scanner. Individual cores were viewed on the computer screen using MIRAX viewer. All invasive cancer cells in the whole core were counted and the percentage was calculated for all cancer cells in the entire core. The highest score among 3 cores was chosen as the final expression of that particular patient for the study. (82.8% - 99.2% concondence between highest staining intensity or percentage out of three cores and the whole tissue slide reading was reported for ER, PR and HER2 expressions in breast cancers by T Thompson et al, 2009) Cut off levels to dichotomise patients into positive and negative expression were as per previously published validated cut off points for ER, PR, EGFR, HER2, Ki-67, CK-5/6 and Aurora A. The level that could best differentiate cases and control was used for CD-68, PDGFR α, Bcl-2 and MCM-2. X-tile algorithm was used for Bag-1 and CD-71.

The main researcher (M Moe) scored all the TMAs. Second observer examined 10% of cores at random for each antibody to verify the result. Although there was a degree of difference between two observers on individual core results, there was a perfect match after applying the cut off point to the highest core result that dichotomised the expression as positive or negative. Therefore Kappa statistical test was not performed.

For Bag-1 and CD-71, M Moe and other two observers from Tenovus laboratory scored all the cores independently first, and then together to resolve the discordances by consensus.

Patient's characteristics and other histopatholigical data were made unknown to the observers at the time of the scoring.

IHC Scoring method

Protein	Staining	Method	Cut off point
ER	Nuclear	Allred	3+
PR	Nuclear	Allred	3+
EGFR	Membrane	Dako	2+
HER2	Membrane	Dako	2+
Ki-67	Nuclear	%, any intensity	>14%
CK-5/6	Nuclear	%, any intensity	>10%
Bcl-2	Cytoplasmic	H score	>10
Bag-1	Nuclear	H score	>70
CD-68	Tumour Associated Macrophages	Semiquantitative 0 – 4+	2+
CD-71	Nuclear, cytoplasmic	H score	>17
Aurora A	Nuclear, cytoplasmic	%, any intensity	>5%
MCM-2	Nuclear	%, any intensity	>20%
PDGFRa	Cytoplasmic	%, any intensity	>60%
VEGFR-2	NA	NA	NA
GSTM-1	NA	NA	NA
Plk-1	NA	NA	NA
Cathepsin L2	NA	NA	NA

Statistical analysis

SPSS v.16 software was used for the statistical analysis. Descriptive statistics were used for the range, mean, median and Chi square test was used for the significance tests.

Univariate and multivariate logistic regressional analyses with Chi-square test were used to determine the effect of various protein expressions and survival outcomes. Kaplan Meier survival curves and Chi-square tests were used to compare survivals between positive and negative protein expressions. Pearson correlation was used to test correlations between "Adjuvant!" recurrence risk, "OPTION" recurrence risk, survival outcomes and molecular subtypes. Box-whisker plots were used to illustrate the distribution of recurrence risk among different molecular subtypes. Statistical significance was established at a p value of < 0.05.

Molecular subtyping

Based on the expression of ER, PR, HER2, EGFR, Ki-67 and CK-5/6 proteins, the patients are classified into five different molecular subtypes as below;

	ER and/ or PR	HER2	HER2 and/ or Ki-67	CK-5/6 and/ or EGFR
Luminal A	+	-	-	-
Luminal B	+	NA	+	NA
HER2	-	+	NA	NA
Core Basal	-	-	NA	+
5-Negative	-	-	NA	-

9.0 Results

A. Patients Characteristics

Patient characteristics at the time of data collection are shown in the table below. ER, PR and HER2 status were taken from the original pathology reports on the main tumour blocks.

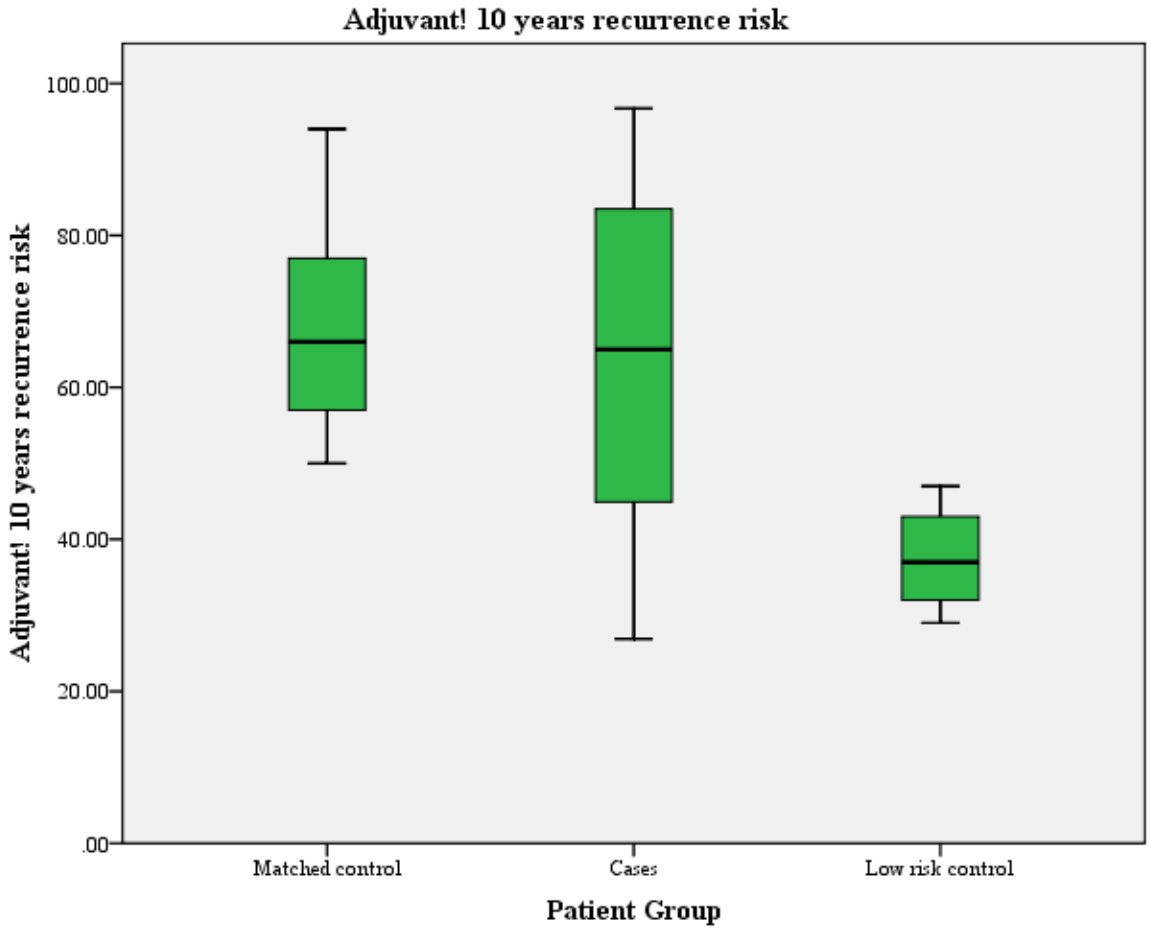
		Cases	Matched controls	Low risk control
Number		72	72	34
Median Age (p = 0.187) (Range)		57 (30 – 77)	52 (28 – 74)	51 (36 – 78)
Median DFS (months) (Range) (p = <0.001)		23.2 (4.5 – 59.9)	103. 5 (74.3 – 164.4)	105. 5 (79 – 161)
Median OS (months) (Range) (p = <0.001)		39. 7 (8.1 – 129)	104.9 (74.3 – 174.4)	106.3 (79 – 161)
Median Adjuvant! 10 years Recurrence Risk % (Range) (p = <0.001)		65 (27 – 97)	67 (50 -94)	37 (29 – 47)
Mean (Median) (Range) OPTION 5 years recurrence risk % without adjuvant chemoendocrine therapy (p = <0.001)		60.6 (60.6) (27 – 92)	60.8 (59.6) (33 – 87)	38 (38) (22 – 58)
Mean (Median) (Range) OPTION 5 years recurrence risk % after chemoendocrine therapy		36.6 (30.5) (10 – 73)	31 (33.9) (10 – 81)	21 (18) (7 – 47)
Surgery (p = 0.405)	Mastectomy	58	52	16
	WLE	24	20	18
Invasive cancer cell type (p = 0.247)	IDC	66	59	29
	ILC	6	12	2
	Others	0	1	3
Histology grade (p = 0.141)	Grade 1	1 (1.4%)	2 (2.8%)	1 (2.9%)
	Grade 2	19 (26.4%)	33 (45.8%)	11 (32.4%)
	Grade 3	52 (72.2%)	37 (51.4%)	22 (64.7%)
Tumour T stage (p = <0.001)	T1	21 (29.2%)	13 (18.1%)	24 (70.6%)
	T2	44 (61.1%)	54 (75%)	10 (29.4%)

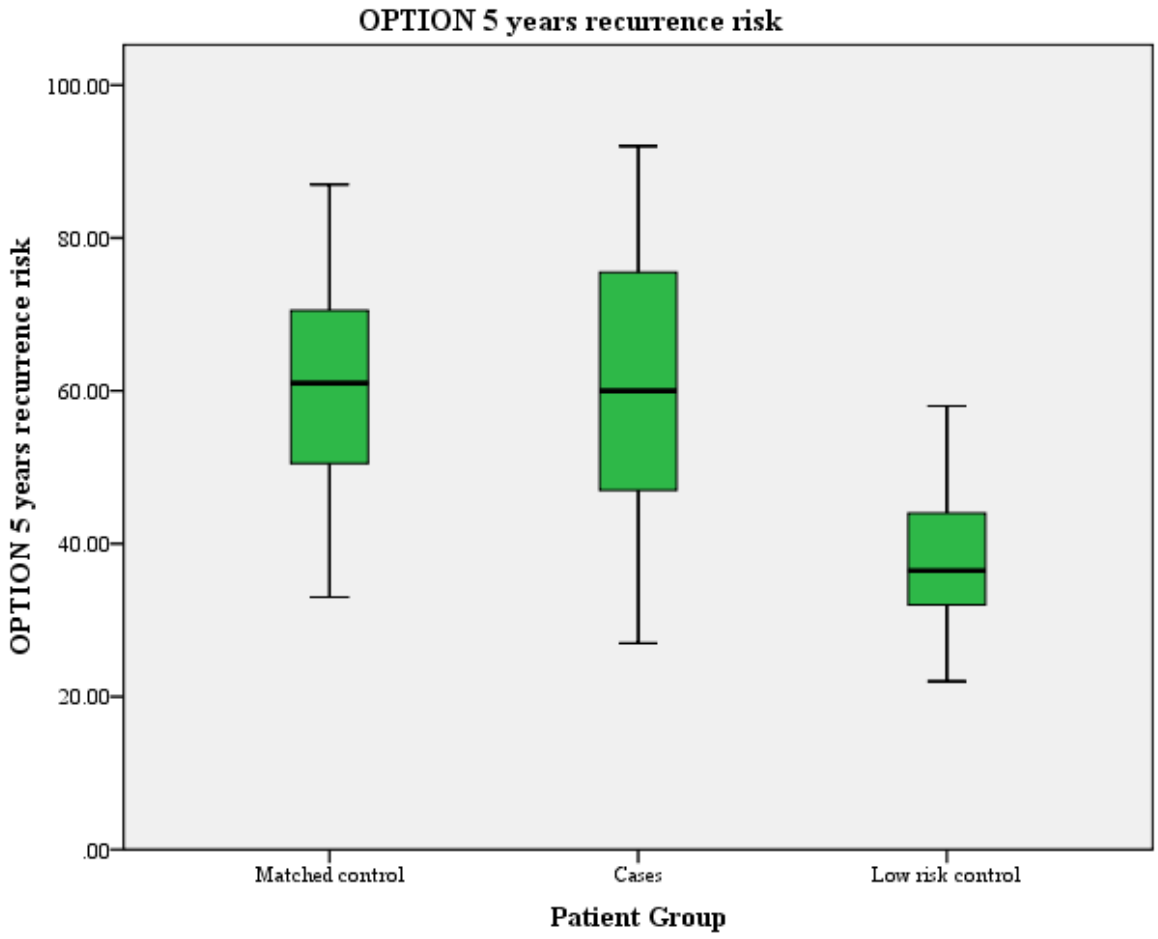
	T3	7 (9.7%)	5 (6.9%)	0
Nodal status ($p < 0.001$)	N0	14 (19.4%)	0	19 (55.9%)
	N1	29 (40.3%)	42 (58.3%)	15 (44.1%)
	N2	10 (13.9%)	18 (25%)	0
	N3	19 (26.4%)	12 (16.7%)	0
Oestrogen receptor status ($p < 0.001$)	Positive	32 (44.4%)	45 (62.5%)	13 (38.2%)
	Negative	31 (43.0%)	15 (20.8%)	17 (23.6%)
	Unknown	9 (12.5%)	12 (16.6%)	4 (5.5%)
Progesterone receptor status ($p < 0.001$)	Positive	1 (1.3%)	0	1 (1.3%)
	Negative	30 (42.8%)	12 (19.3%)	11 (17.7%)
	Unknown	41 (56.9%)	60 (83.3%)	21 (29.1%)
HER2 status ($p = 0.637$)	Positive	24 (33.3%)	6 (8.3%)	2 (2.7%)
	Negative	29 (40.2%)	14 (19.4%)	4 (5.5%)
	Unknown	19 (26.3%)	49 (68.0%)	27 (37.5%)
Chemotherapy regime ($p = 0.485$)	CMF	27 (37.5%)	33 (45.8%)	24 (70.6%)
	Anthracycline	34 (47.2%)	27 (37.5%)	9 (26.5%)
	Taxane	11 (15.3%)	12 (16.7%)	1 (2.9%)

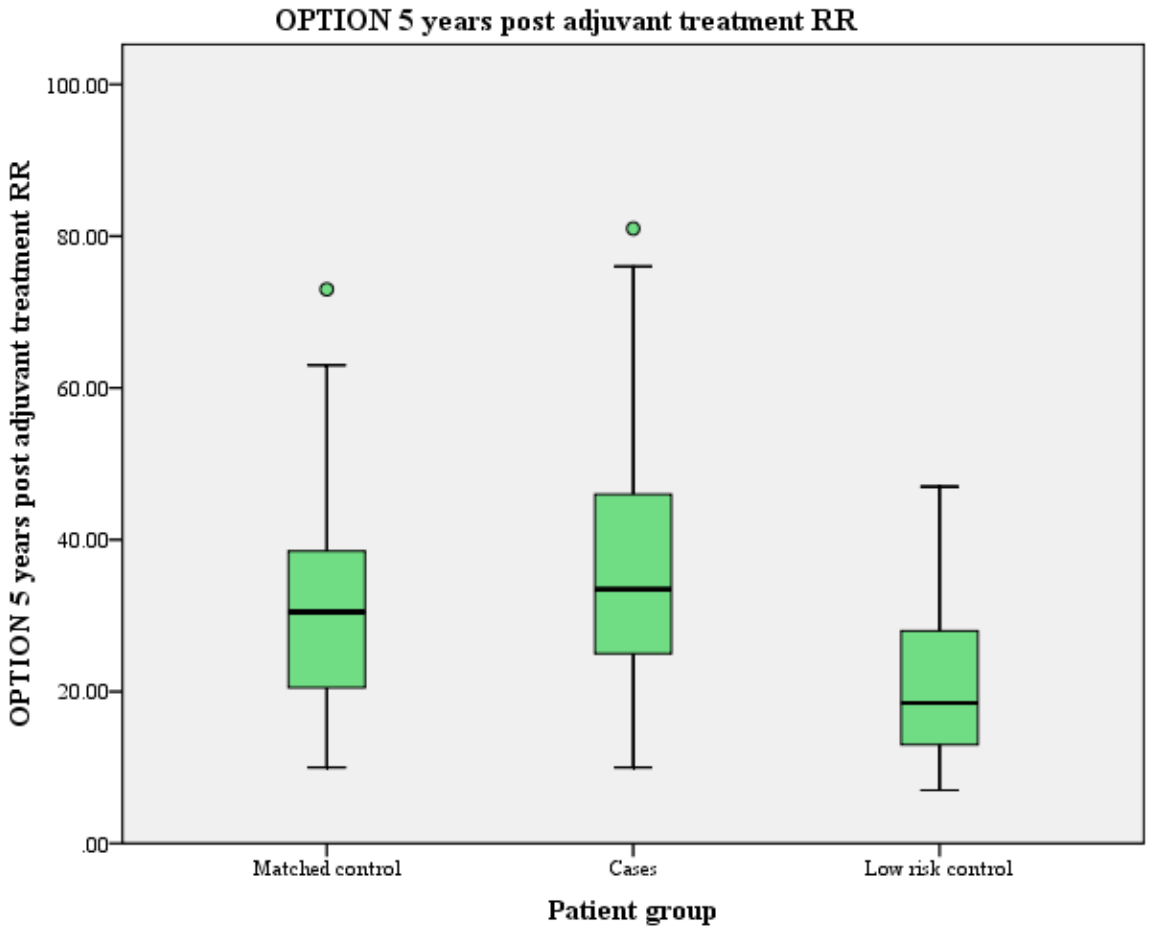
B. Comparison of Recurrence risk, RFS and overall survival between patient groups

Independent Samples Student "t" test was used to compare means of recurrence risks, recurrence free and overall survivals between different patients groups. P values shown are for the assumption of equal variance. The cases and matched controls are selected in such a way that their disease recurrence risk according to Adjuvant and OPTION tools are similar. However there are statistically significant differences in the mean recurrence risk after adjuvant treatments (chemotherapy and endocrine therapy). This is because of the endocrine treatment effect as there were more hormone receptor positive patients in the matched control group than the cases. The low risk controls have lower mean RR than the matched controls and the cases.

Patient group		Adjuvant! 10 years RR	OPTION 5 years RR	OPTION 5 years post adjuvant treatment RR
Matched control	N	72	72	72
	Mean	67.1389	60.8056	30.9722
	Median	66.0000	61.0000	30.5000
	Minimum	50.00	33.00	10.00
	Maximum	94.00	87.00	73.00
Cases	N	72	72	72
	Mean	63.5000	60.5694	36.5417
	Median	65.0000	60.0000	33.5000
	Minimum	27.00	27.00	10.00
	Maximum	97.00	92.00	81.00
Low risk control	N	34	34	34
	Mean	38.0294	37.7353	20.7941
	Median	37.0000	36.5000	18.5000
	Minimum	29.00	22.00	7.00
	Maximum	47.00	58.00	47.00
Total	N	178	178	178
	Mean	60.1067	56.3034	31.2809
	Median	58.0000	55.5000	29.5000
	Minimum	27.00	22.00	7.00
	Maximum	97.00	92.00	81.00


Adjuvant! 10 years recurrence risk
100.00
80.00
60.00
40.00
20.00
.00
Adjuvant! 10 years recurrence risk
Matched control
Cases
Low risk control
Patient Group


OPTION 5 years recurrence risk
100.00
80.00
60.00
40.00
20.00
.00
OPTION 5 years recurrence risk
Matched control
Cases
Low risk control
Patient Group


OPTION 5 years post adjuvant treatment RR
100.00
80.00
60.00
40.00
20.00
.00
OPTION 5 years post adjuvant treatment RR
Matched control
Cases
Low risk control
Patient group

Comparing recurrence risks between matched controls and cases

	Patient Group	N	Mean	Std. Deviation	Std. Error Mean	P (2-tailed test)
Adjuvant! 10 years recurrence risk	Matched control	72	67.1389	12.15198	1.43212	0.183
	Cases	72	63.4750	19.81514	2.33524	
OPTION 5 years recurrence risk	Matched control	72	60.8056	13.03909	1.53667	0.929
	Cases	72	60.5694	18.29556	2.15615	
OPTION 5 years recurrence risk after adjuvant treatments	Matched control	72	30.9722	12.67244	1.49346	**0.024**
	Cases	72	36.5417	16.44448	1.93800	
OPTION 10 years recurrence risk	Matched control	72	72.6111	10.93071	1.28820	0.888
	Cases	72	72.3056	14.80544	1.74484	
OPTION 10 years recurrence risk after adjuvant treatments	Matched control	72	39.7917	14.15302	1.66795	**0.004**
	Cases	72	47.3472	16.44467	1.93802	
Relapse free survival (months)	Matched control	72	1.0681E2	22.44024	2.64461	**<0.001**
	Cases	72	26.2097	14.71843	1.73458	
Overall survival (months)	Matched control	72	1.0690E2	22.38782	2.63843	**<0.001**
	Cases	72	44.3889	26.40500	3.11186	

Comparing recurrence risks between cases and low risk controls

	Patient Group	N	Mean	Std. Deviation	Std. Error Mean	P (2 tailed test)
Adjuvant! 10 years recurrence risk	Cases	72	63.4750	19.81514	2.33524	**<0.001**
	Low risk control	34	38.0294	5.46884	.93790	
OPTION 5 years recurrence risk	Cases	72	60.5694	18.29556	2.15615	**<0.001**
	Low risk control	34	37.7353	8.08031	1.38576	
OPTION 5 years recurrence risk after adjuvant treatments	Cases	72	36.5417	16.44448	1.93800	**<0.001**
	Low risk control	34	20.6765	9.30858	1.59641	
OPTION 10 years recurrence risk	Cases	72	72.3056	14.80544	1.74484	**<0.001**
	Low risk control	34	53.2353	7.64777	1.31158	
OPTION 10 years recurrence risk after adjuvant treatments	Cases	72	47.3472	16.44467	1.93802	**<0.001**
	Low risk control	34	31.7941	11.97669	2.05399	
Relapse free survival (months)	Cases	72	26.2097	14.71843	1.73458	**<0.001**
	Low risk control	34	1.0986E2	23.92733	4.10350	
Overall survival (months)	Cases	72	44.3889	26.40500	3.11186	**<0.001**
	Low risk control	34	1.1070E2	23.33100	4.00123	

Correlation between recurrence risk and survival outcomes

There is no correlation between the recurrence risk and the recurrence free and overall survivals for the cohort containing cases and matched controls. However when the low risk control cohort was added in the analysis, there is a significant correlation between the recurrence risk and the recurrence free and overall survivals because the cases and controls are not well matched. This means that the recurrence risk has become a predictive factor. Therefore to avoid this cofounding effect, survival analyses for molecular markers were done on the cohort after omitting low risk control patients.

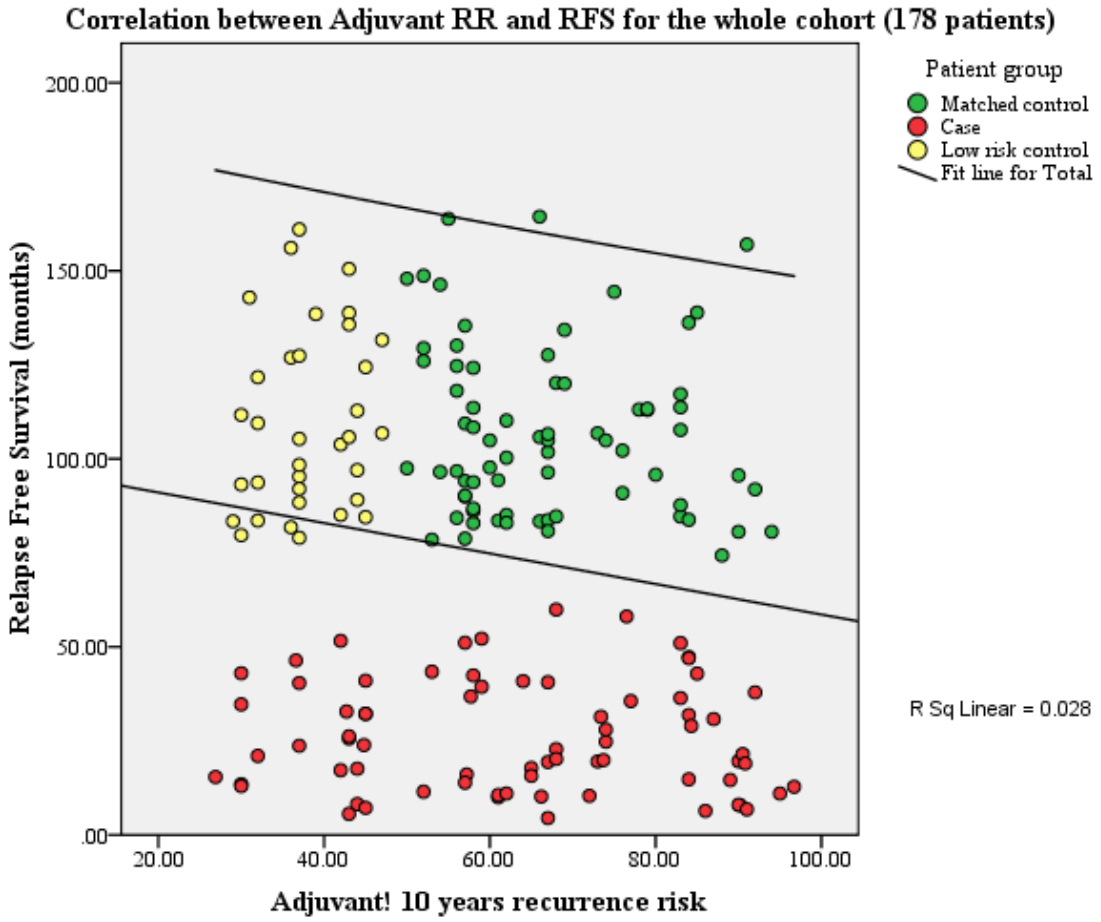
Correlation between recurrence risk and RFS & OS for the whole cohort (178 patients)

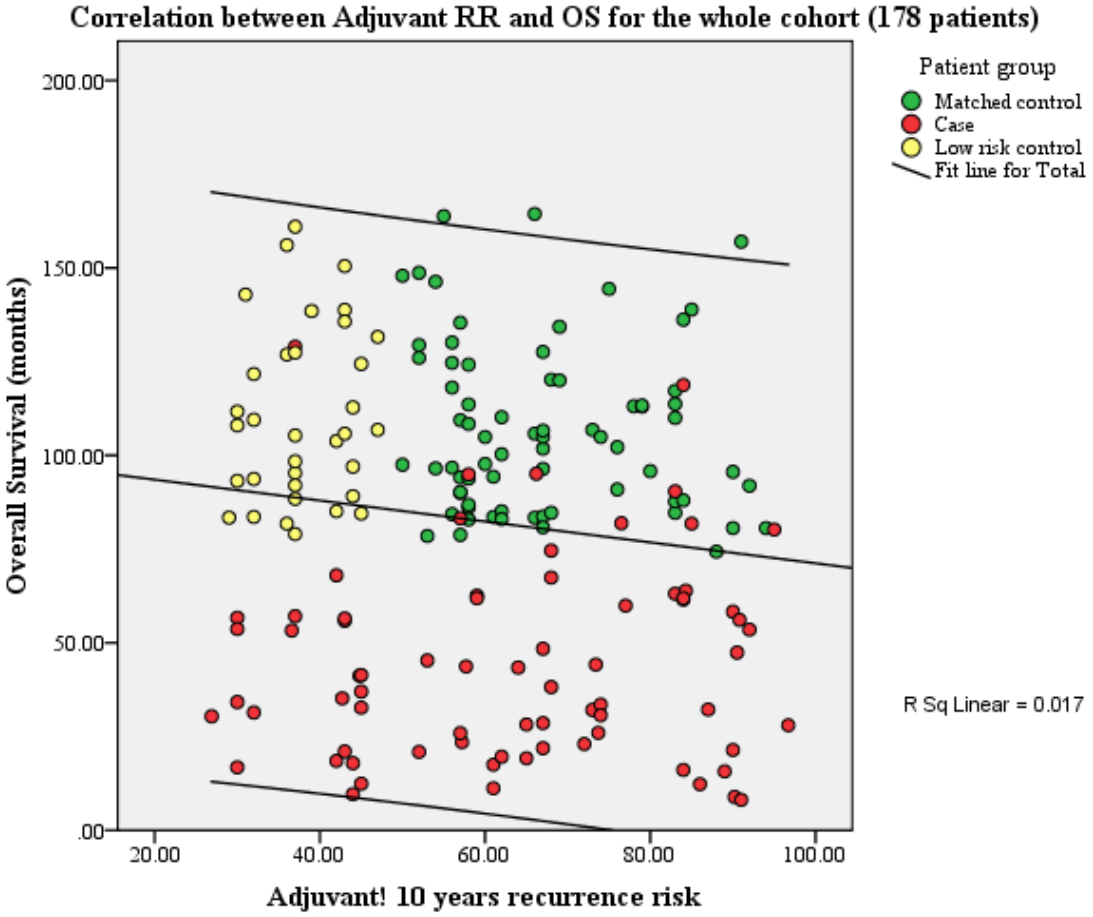
		Relapse free survival (months)	Overall survival (months)
Adjuvant! 10 years recurrence risk	Pearson Correlation	-.167	-.130
	Sig. (2-tailed)	**.026**	.084
	N	178	178
OPTION 5 years recurrence risk	Pearson Correlation	-.215	-.173*
	Sig. (2-tailed)	**.004**	**.021**
	N	178	178
OPTION 5 years recurrence risk after adjuvant treatments	Pearson Correlation	-.298**	-.286**
	Sig. (2-tailed)	**<.001**	**<.001**
	N	178	178
OPTION 10 years recurrence risk	Pearson Correlation	-.218**	-.177*
	Sig. (2-tailed)	**.003**	**.018**
	N	178	178
OPTION 10 years recurrence risk after adjuvant treatments	Pearson Correlation	-.313**	-.303**
	Sig. (2-tailed)	**<.001**	**<.001**
	N	178	178

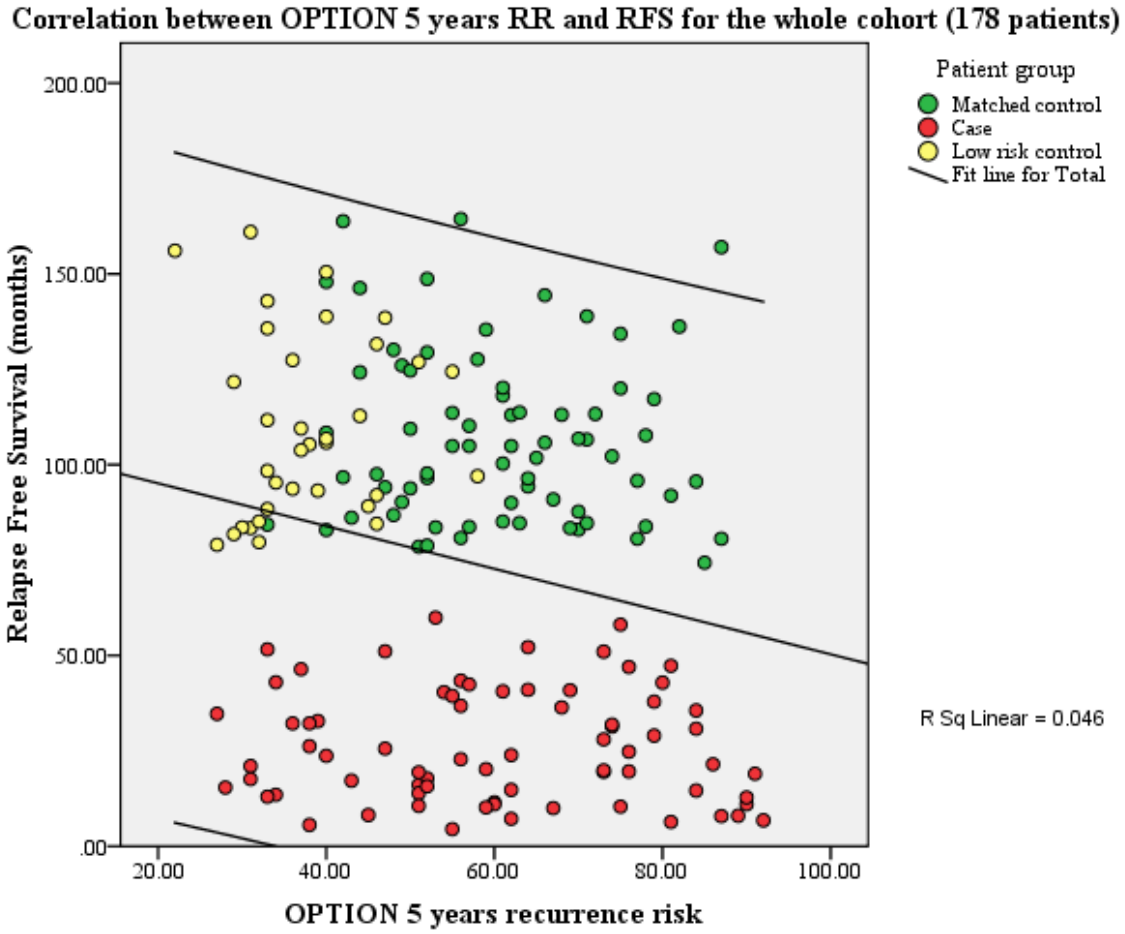
*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

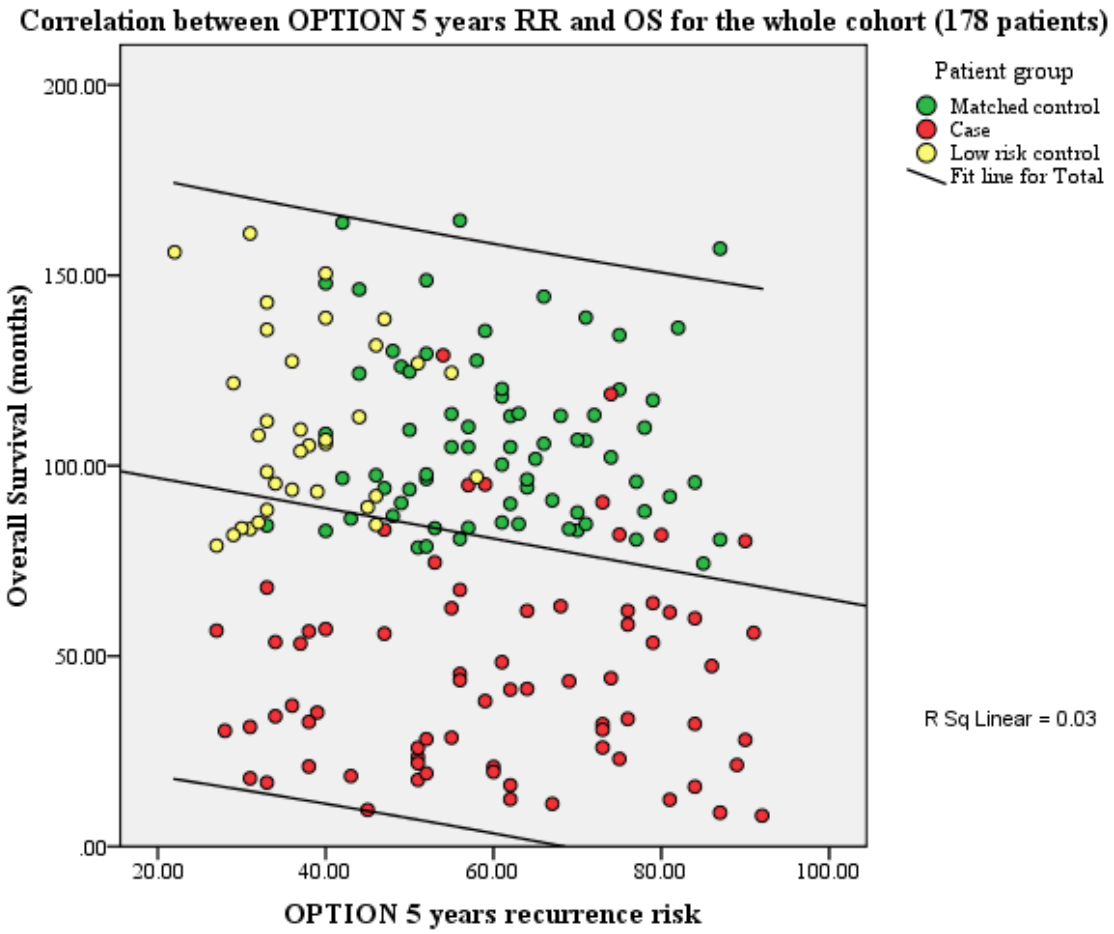
The whole cohort (178 patients)

The following graphs show that the correlations between recurrence risk and relapse free and overall survival. The best fit line and 95% confidence interval lines were also shown. The graphs show some correlation between recurrence risk and survival although the degree of correlation is small.

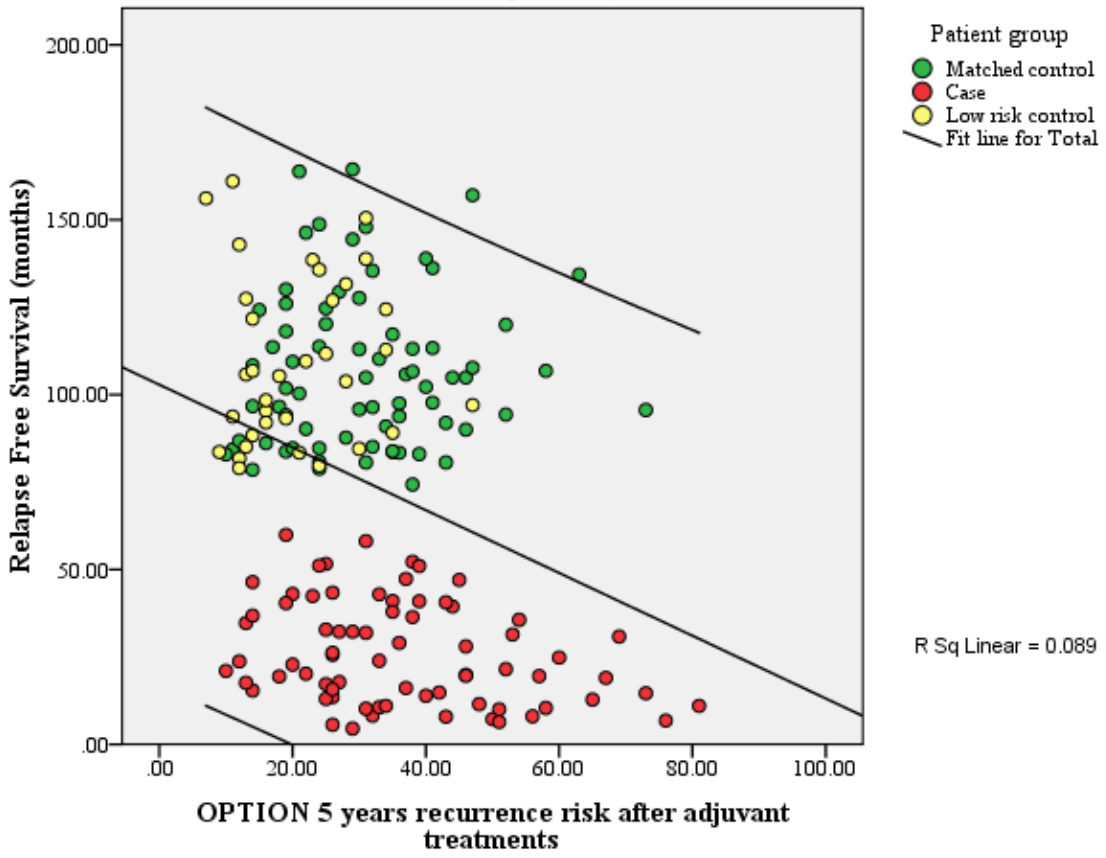



Correlation between Adjuvant RR and OS for the whole cohort (178 patients)
Overall Survival (months)
200.00
150.00
100.00
50.00
.00
20.00
40.00
60.00
80.00
100.00
Adjuvant! 10 years recurrence risk
Patient group
Matched control
Case
Low risk control
Fit line for Total
R Sq Linear = 0.017

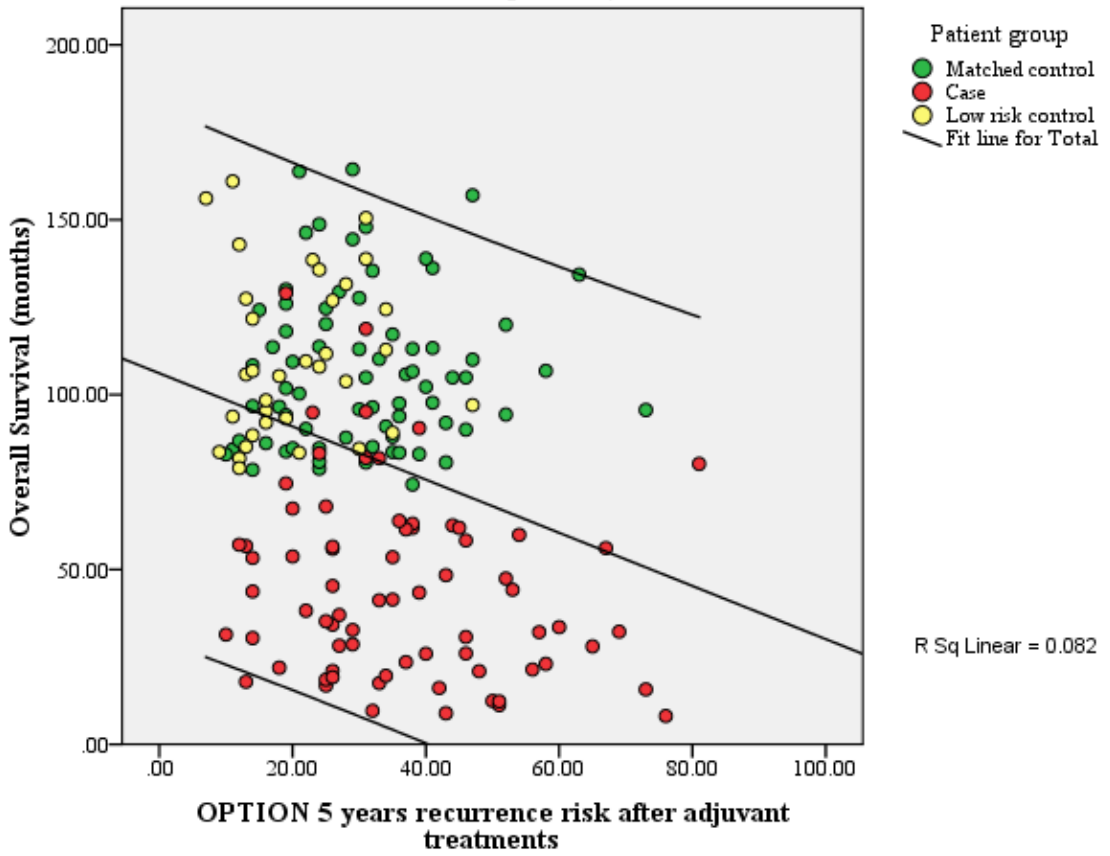

Correlation between OPTION 5 years RR and RFS for the whole cohort (178 patients)
Relapse Free Survival (months)
200.00
150.00
100.00
50.00
.00
20.00
40.00
60.00
80.00
100.00
OPTION 5 years recurrence risk
Patient group
Matched control
Case
Low risk control
Fit line for Total
R Sq Linear = 0.046


Correlation between OPTION 5 years RR and OS for the whole cohort (178 patients)
Patient group
Matched control
Case
Low risk control
Fit line for Total
R Sq Linear = 0.03
Overall Survival (months)
200.00
150.00
100.00
50.00
.00
20.00
40.00
60.00
80.00
100.00
OPTION 5 years recurrence risk

Correlation between OPTION 5 years RR post treatment and RFS for the whole cohort (178 patients)



Correlation between OPTION 5 years RR post treatment and OS for the whole cohort (178 patients)



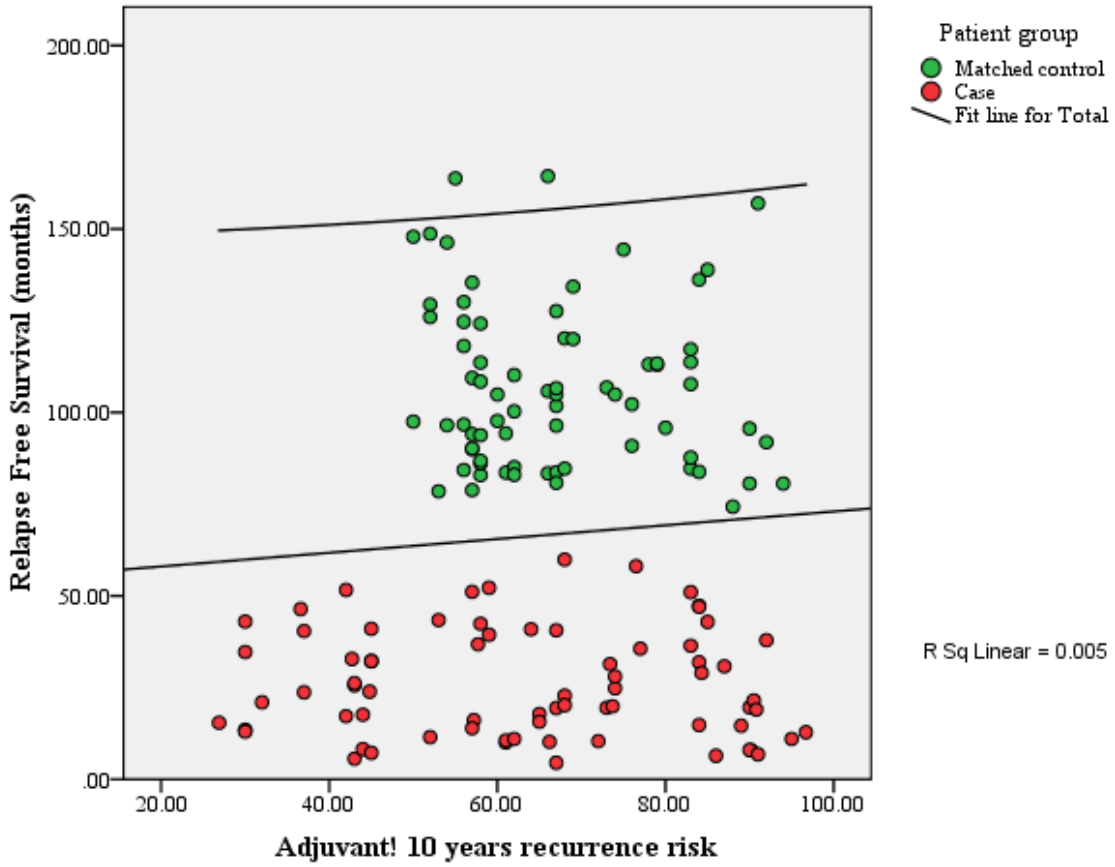
The cohort containing cases and matched controls only without low risk control (144 patients)

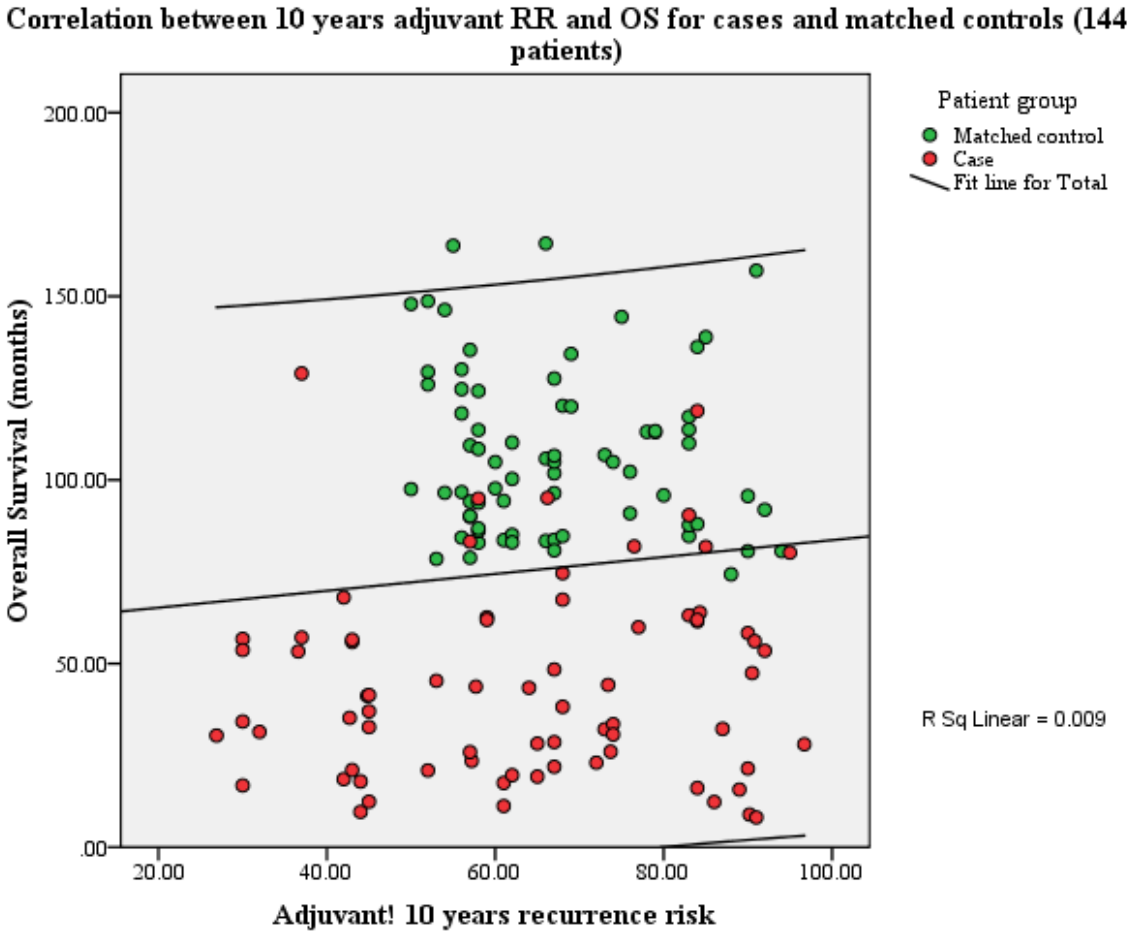
There was no correlation between recurrence risk and the survivals. However there is some correlation between OPTION post adjuvant treatment recurrence risk and the survivals.

Matched cases and controls (Total = 144 patients)		Relapse free survival (months)	Overall survival (months)
Adjuvant! 10 years recurrence risk	Pearson Correlation	.069	.095
	Sig. (2-tailed)	.410	.256
	N	144	144
OPTION 5 years recurrence risk	Pearson Correlation	-.023	.012
	Sig. (2-tailed)	.784	.884
	N	144	144
OPTION 5 years recurrence risk after adjuvant treatments	Pearson Correlation	-.210*	-.209*
	Sig. (2-tailed)	.012	.012
	N	144	144
OPTION 10 years recurrence risk	Pearson Correlation	-.028	.007
	Sig. (2-tailed)	.742	.936
	N	144	144
OPTION 10 years recurrence risk after adjuvant treatments	Pearson Correlation	-.255**	-.255**
	Sig. (2-tailed)	.002	.002
	N	144	144

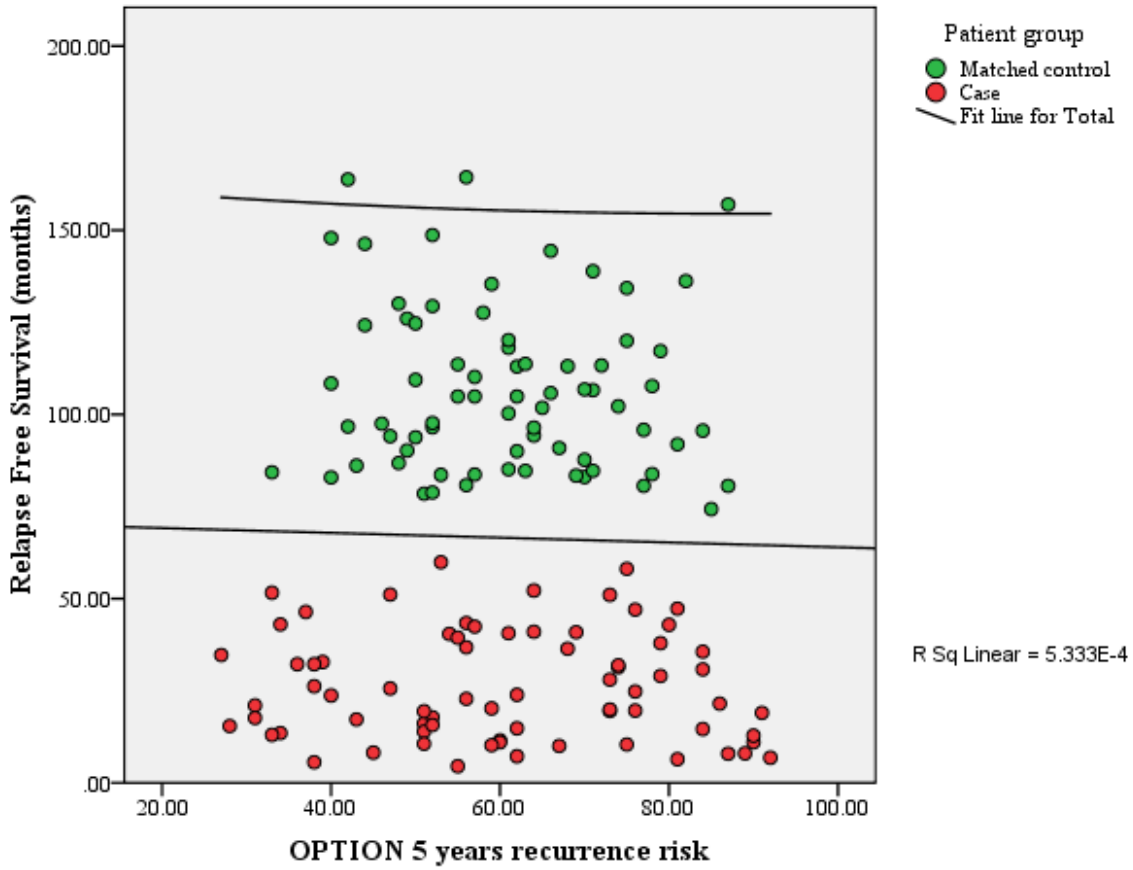
*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

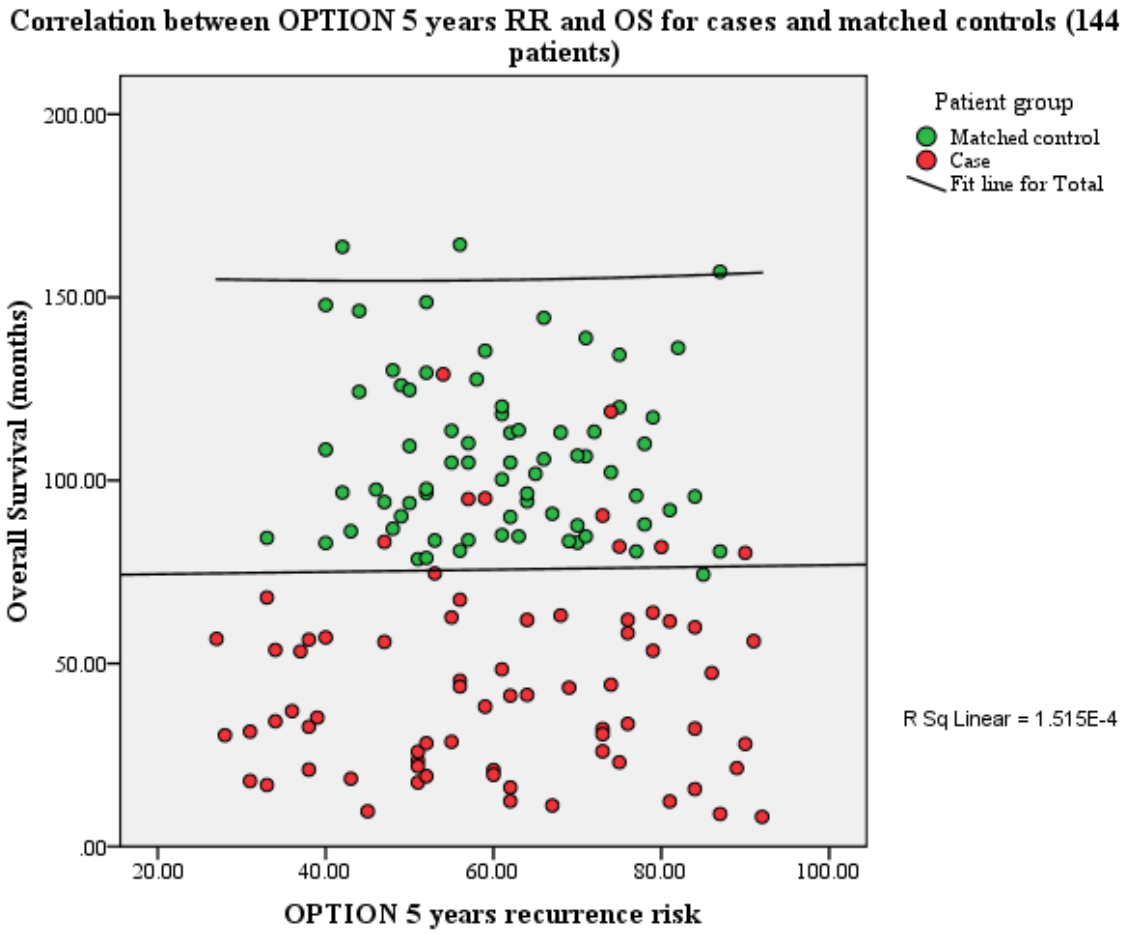
Correlation between 10 years adjuvant RR and RFS for cases and matched controls (144 patients)



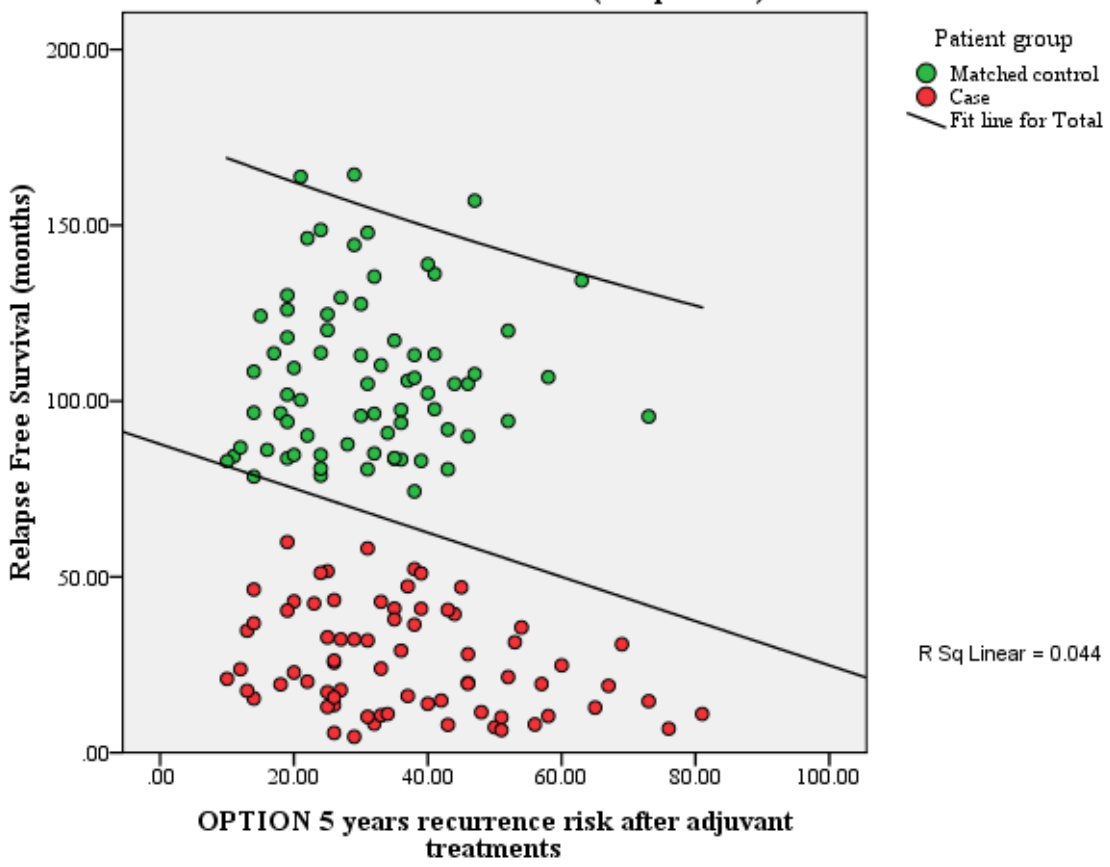

Correlation between 10 years adjuvant RR and OS for cases and matched controls (144 patients)
Overall Survival (months)
200.00
150.00
100.00
50.00
.00
20.00
40.00
60.00
80.00
100.00
Adjuvant! 10 years recurrence risk
Patient group
Matched control
Case
Fit line for Total
R Sq Linear = 0.009

Correlation between OPTION 5 years RR and RFS for cases and matched controls (144 patients)

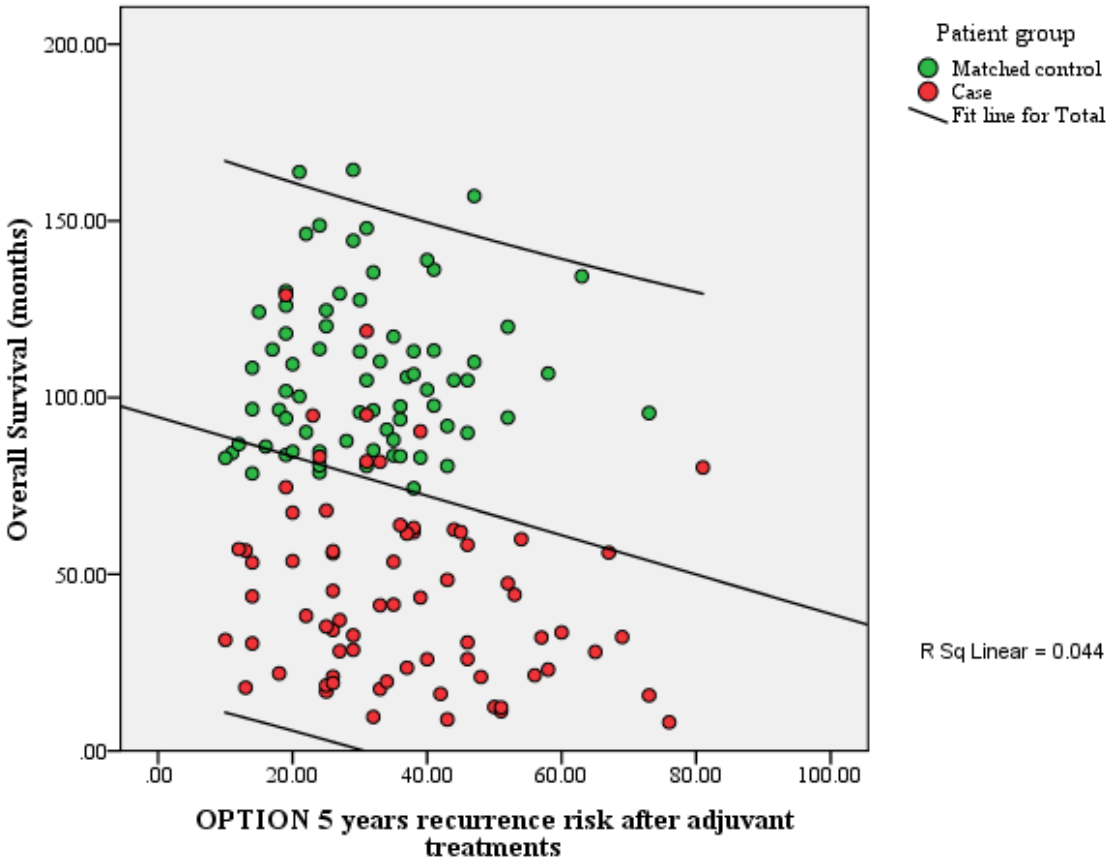



Correlation between OPTION 5 years RR and OS for cases and matched controls (144 patients)
Overall Survival (months)
200.00
150.00
100.00
50.00
.00
20.00
40.00
60.00
80.00
100.00
OPTION 5 years recurrence risk
Patient group
Matched control
Case
Fit line for Total
R Sq Linear = 1.515E-4

Correlation between OPTION 5 years RR after adjuvant treatments and RFS for cases and matched controls (144 patients)



Correlation between OPTION 5 years RR after adjuvant treatments and OS for cases and matched controls (144 patients)



C. Immunohistochemistry for individual biomarker

Immunohistochemistry was successful for ER, PR, HER2, EGFR, CK-5/6, Ki-67, Aurora A, MCM-2, Bcl-2, Bag-1, CD-68, CD-71 and PDGFRα. However it wasn't successful for Plk-1, VEGFR-2, Cathepsin L-2 and GSTM-1 despite different antigen retrieval methods and different dilutions of the reagents. Number of cores that are either lost or not good enough for analysis and the number of patients for whom no expression result is available are shown in the following table.

Total 178 patients Total 467 cores	Number of cores lost or not good enough for analysis (%)	Number of patients with no IHC expression result (%)
ER	66 (14.1%)	1 (0.6%)
PR	53 (11.3%)	2 (1.1%)
HER2	59 (12.6%)	4 (2.2%)
EGFR	48 (10.3%)	3 (1.8%)
CK-5/6	58 (12.4%)	3 (1.7%)
Ki-67	49 (10.5%)	3 (1.7%)
MCM-2	101 (21.6%)	10 (5.6%)
Aurora A	0 (0%)	0 (0.0%)
Bcl-2	40 (8.6%)	2 (0.1%)
Bag-1	54 (11.6%)	2 (1.1%)
CD-68	35 (7.5%)	2 (1.1%)
CD-71	50 (10.7%)	5 (2.9%)
PDGFRα	51 (10.9%)	5 (2.9%)

Immunohistochemistry results for 3 patient groups (Pearson Chi-square test)

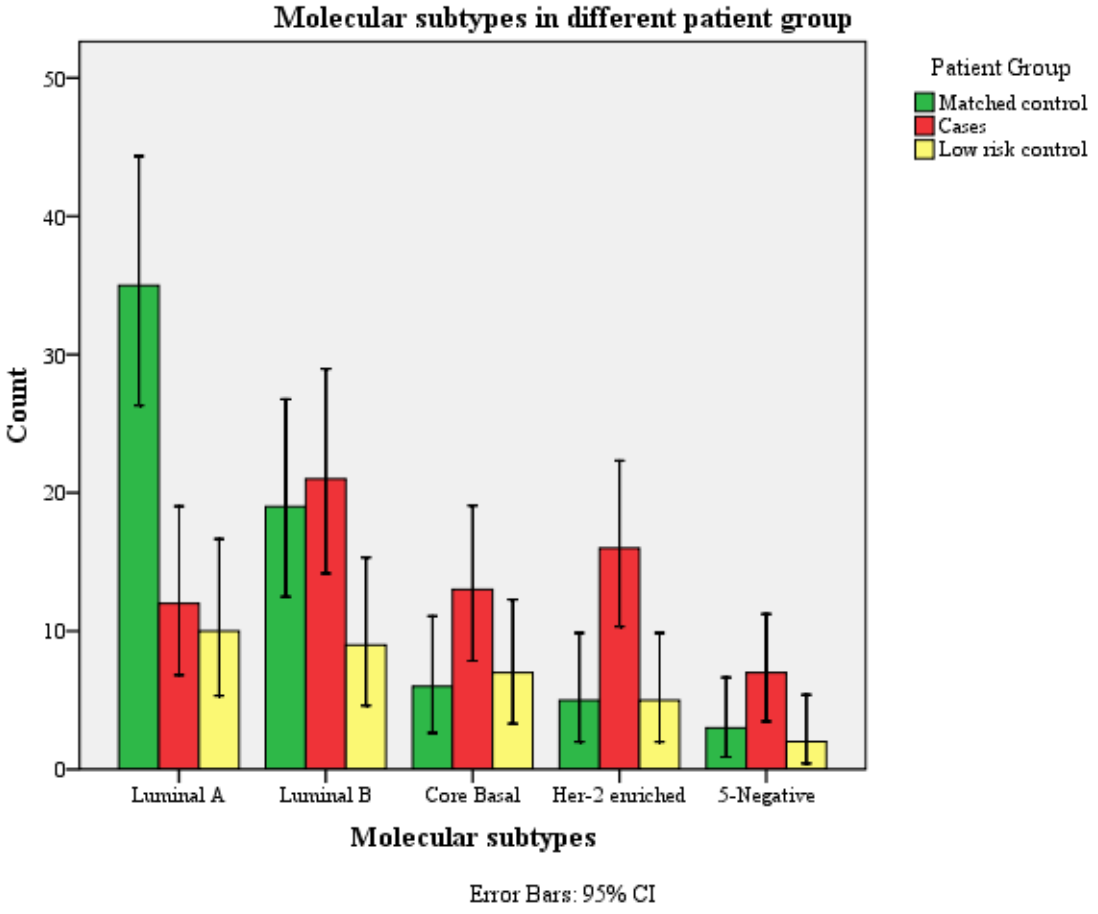
Total = 178 patients

		Matched control	Cases	Low risk control
ER	Positive	55 (76.3%)	34 (47.2%)	17 (50%)
P = **<0.001**	Negative	16 (22.2%)	38 (52.8%)	17 (50%)
PR	Positive	41 (56.9%)	14 (19.4%)	13 (38.2%)
P = **<0.001**	Negative	29 (40.3%)	56 (77.8%)	21 (61.8%)
HER2	Positive	13 (18.1%)	20 (27.8%)	9 (26.5%)
P = 0.401	Negative	55 (76.4%)	50 (69.4%)	24 (70.6%)
EGFR	Positive	4 (5.6%)	7 (9.7%)	5 (14.7%)
P = 0.304	Negative	65 (90.3%)	64 (88.9%)	28 (82.4%)
Ki-67	Positive	25 (34.7%)	52 (72.2%)	16 (47.1%)
P = **<0.001**	Negative	44 (61.1%)	19 (26.4%)	16 (47.1%)
CK-5/6	Positive	32 (44.4%)	32 (44.4%)	12 (35.3%)
P = 0.507	Negative	37 (51.4%)	37 (51.4%)	22 (64.7%)
Bcl-2	Positive	61 (84.7%)	55 (76.4%)	23 (67.6%)
P = 0.088	Negative	9 (12.5%)	17 (23.6%)	10 (39.4%)
Bag-1	Positive	37 (51.4%)	24 (33.3%)	14 (41.2%)
P = 0.074	Negative	33 (45.8%)	47 (65.3%)	18 (52.9%)
CD-68	Positive	39 (54.2%)	31 (43.1%)	20 (58.8%)
P = 0.189	Negative	31 (43.1%)	40 (55.6%)	13 (38.2%)
CD-71	Positive	24 (33.3%)	35 (48.6%)	19 (55.9%)
P = 0.062	Negative	43 (59.7%)	35 (48.6%)	13 (38.2%)
MCM-2	Positive	27 (37.5%)	13 (18.1%)	5 (14.7%)
P = **0.005**	Negative	35 (48.6%)	49 (68.1%)	25 (73.5%)
Aurora A	Positive	25 (34.7%)	43 (59.7%)	7 (20.6%)
P = **0.001**	Negative	45 (62.5%)	29 (40.3%)	22 (64.7%)
PDGFRα	Positive	58 (80.6%)	68 (94.4%)	27 (79.4%)
P = 0.099	Negative	9 (12.5%)	3 (4.2%)	5 (14.7%)

Percentage is for positive and negative expressions within each patient group.

D. Molecular Subtypes

Molecular subtyping was available for 170 patients. For remaining 8 patients one or more necessary biomarker IHC expressions were missing. Most of the matched control patients are in the Luminal A group and most of the cases are either in the Luminal B or HER2 enriched groups. Molecular subtypes are highly predictive of RFS and OS in both whole cohort as well as the cohort without low risk control patients but more significant in the latter.



Molecular subtypes and Patient Groups Cross tabulation

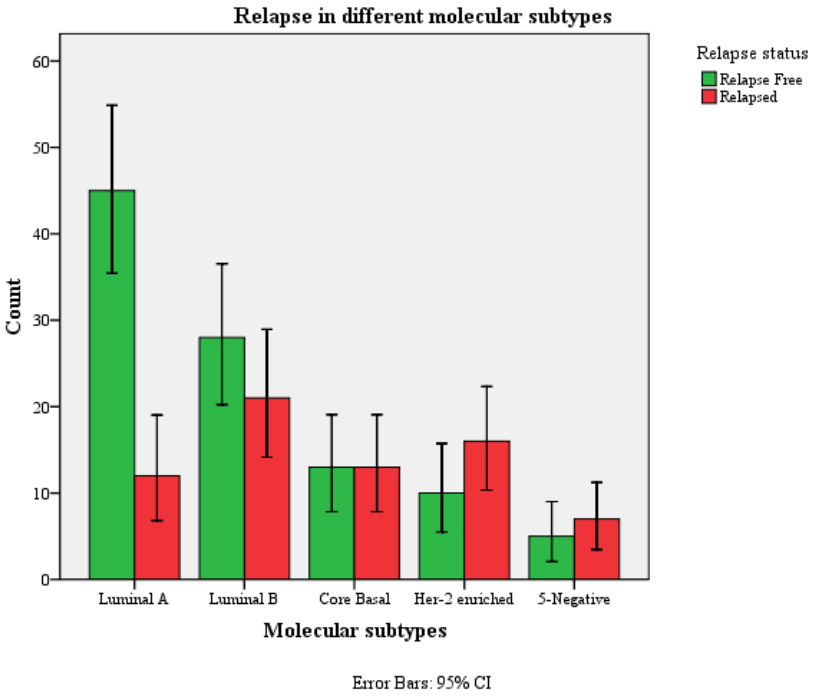
			Patient Group			
			Matched control	Cases	Low risk control	Total
Molecular subtypes	Luminal A	Count	35	12	10	57
		Expected Count	22.8	23.1	11.1	57.0
		% within Molecular subtypes	61.4%	21.1%	17.5%	100.0%
	Luminal B	Count	19	21	9	49
		Expected Count	19.6	19.9	9.5	49.0
		% within Molecular subtypes	38.8%	42.9%	18.4%	100.0%
	Core Basal	Count	6	13	7	26
		Expected Count	10.4	10.6	5.0	26.0
		% within Molecular subtypes	23.1%	50.0%	26.9%	100.0%
	HER2 enriched	Count	5	16	5	26
		Expected Count	10.4	10.6	5.0	26.0
		% within Molecular subtypes	19.2%	61.5%	19.2%	100.0%
	5-Negative	Count	3	7	2	12
		Expected Count	4.8	4.9	2.3	12.0
		% within Molecular subtypes	25.0%	58.3%	16.7%	100.0%
Total		Count	68	69	33	170
		Expected Count	68.0	69.0	33.0	170.0
		% within Molecular subtypes	40.0%	40.6%	19.4%	100.0%

Chi-Square Tests

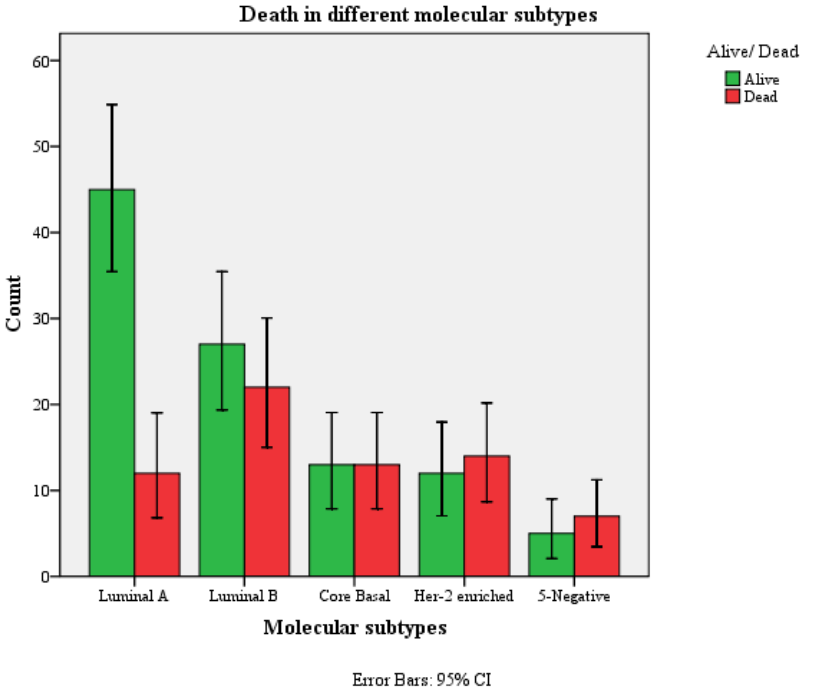
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	22.551[a]	8	**0.004**
Likelihood Ratio	23.280	8	**0.003**
Linear-by-Linear Association	7.880	1	**0.005**
N of Valid Cases	170		

a. 3 cells (20.0%) have expected count less than 5. The minimum expected count is 2.33.

Molecular subtypes and Relapse



Molecular subtypes and mortality



Molecular subtypes among relapsed and relapse free patients

			Relapse		
			Relapsed	No relapse	Total
Molecular subtype	Luminal A	Count	13	44	57
		Expected Count	23.4	33.6	57.0
		% within Molecular subtype	22.8%	77.2%	100.0%
	Luminal B	Count	22	28	50
		Expected Count	20.5	29.5	50.0
		% within Molecular subtype	44.0%	56.0%	100.0%
	Core Basal	Count	12	13	25
		Expected Count	10.3	14.7	25.0
		% within Molecular subtype	48.0%	52.0%	100.0%
	HER2 enriched	Count	16	10	26
		Expected Count	10.7	15.3	26.0
		% within Molecular subtype	61.5%	38.5%	100.0%
	5 - Negative	Count	7	5	12
		Expected Count	4.9	7.1	12.0
		% within Molecular subtype	58.3%	41.7%	100.0%
	Missing	Count	3	5	8
		Expected Count	3.3	4.7	8.0
		% within Molecular subtype	37.5%	62.5%	100.0%
Total		Count	73	105	178
		Expected Count	73.0	105.0	178.0
		% within Molecular subtype	41.0%	59.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	14.555[a]	5	**.012**
Likelihood Ratio	15.023	5	**.010**
Linear-by-Linear Association	8.460	1	**.004**
N of Valid Cases	178		

Molecular subtypes among Alive and Dead patients

			Alive/ Dead		
			Alive	Dead	Total
Molecular subtype	Luminal A	Count	46	11	57
		Expected Count	34.6	22.4	57.0
		% within Molecular subtype	80.7%	19.3%	100.0%
	Luminal B	Count	27	23	50
		Expected Count	30.3	19.7	50.0
		% within Molecular subtype	54.0%	46.0%	100.0%
	Core Basal	Count	13	12	25
		Expected Count	15.2	9.8	25.0
		% within Molecular subtype	52.0%	48.0%	100.0%
	HER2 enriched	Count	12	14	26
		Expected Count	15.8	10.2	26.0
		% within Molecular subtype	46.2%	53.8%	100.0%
	5 - Negative	Count	5	7	12
		Expected Count	7.3	4.7	12.0
		% within Molecular subtype	41.7%	58.3%	100.0%
	Missing	Count	5	3	8
		Expected Count	4.9	3.1	8.0
		% within Molecular subtype	62.5%	37.5%	100.0%
Total		Count	108	70	178
		Expected Count	108.0	70.0	178.0
		% within Molecular subtype	60.7%	39.3%	100.0%

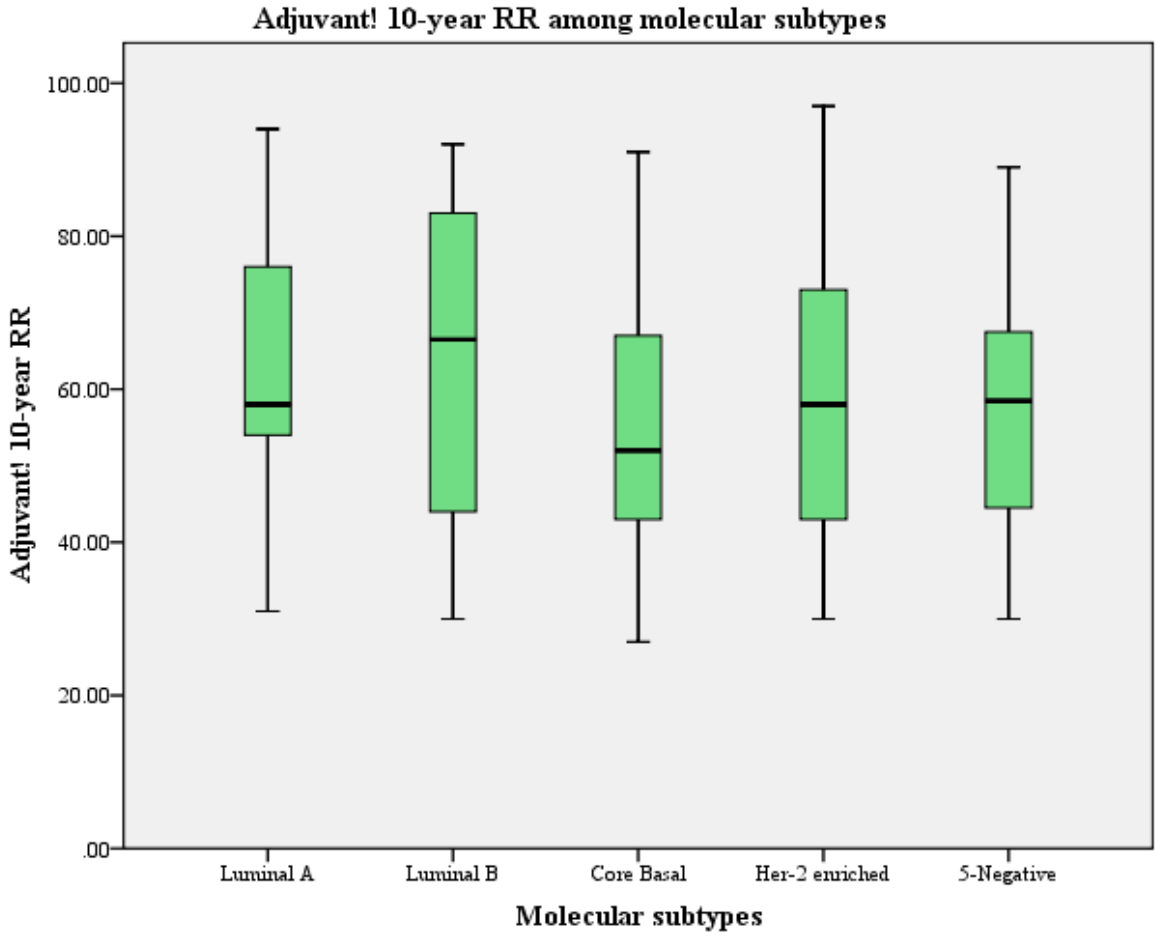
Chi-Square Tests

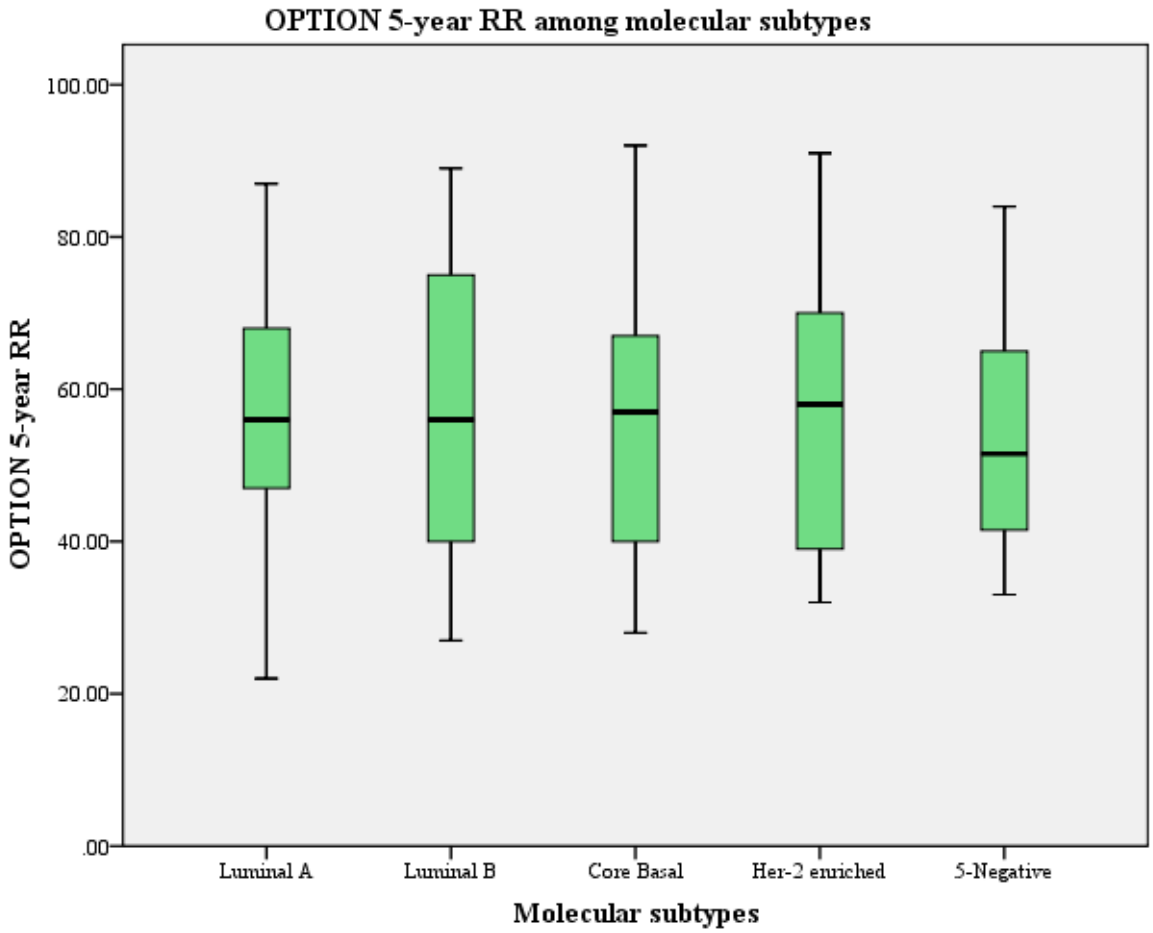
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	15.429[a]	5	**.009**
Likelihood Ratio	16.279	5	**.006**
Linear-by-Linear Association	8.262	1	**.004**
N of Valid Cases	178		

a. 3 cells (25.0%) have expected count less than 5. The minimum expected count is 3.15.

Recurrence Risk among molecular subtypes

Molecular subtypes		Adjuvant! 10-year RR	OPTION 5-year RR	OPTION 5-year post adjuvant treatment RR
Luminal A	N	57	57	57
	Mean	61.8596	56.6842	26.2632
	Median	58.0000	56.0000	24.0000
	Minimum	31.00	22.00	7.00
	Maximum	94.00	87.00	54.00
Luminal B	N	50	50	50
	Mean	62.7400	56.5600	25.3600
	Median	66.5000	56.0000	23.5000
	Minimum	30.00	27.00	9.00
	Maximum	92.00	89.00	56.00
Core Basal	N	25	25	25
	Mean	54.8400	55.8400	41.5600
	Median	52.0000	57.0000	36.0000
	Minimum	27.00	28.00	14.00
	Maximum	91.00	92.00	76.00
HER2 enriched	N	26	26	26
	Mean	58.1154	57.7692	40.8462
	Median	58.0000	58.0000	37.0000
	Minimum	30.00	32.00	19.00
	Maximum	97.00	91.00	81.00
5-Negative	N	12	12	12
	Mean	56.8333	53.5833	38.5000
	Median	58.5000	51.5000	34.0000
	Minimum	30.00	33.00	25.00
	Maximum	89.00	84.00	73.00
Total	N	170	170	170
	Mean	60.1588	56.4706	31.3412
	Median	58.0000	55.5000	29.5000
	Minimum	27.00	22.00	7.00
	Maximum	97.00	92.00	81.00


Adjuvant! 10-year RR among molecular subtypes
Adjuvant! 10-year RR
100.00
80.00
60.00
40.00
20.00
.00
Luminal A
Luminal B
Core Basal
Her-2 enriched
5-Negative
Molecular subtypes


OPTION 5-year RR among molecular subtypes
OPTION 5-year RR
100.00
80.00
60.00
40.00
20.00
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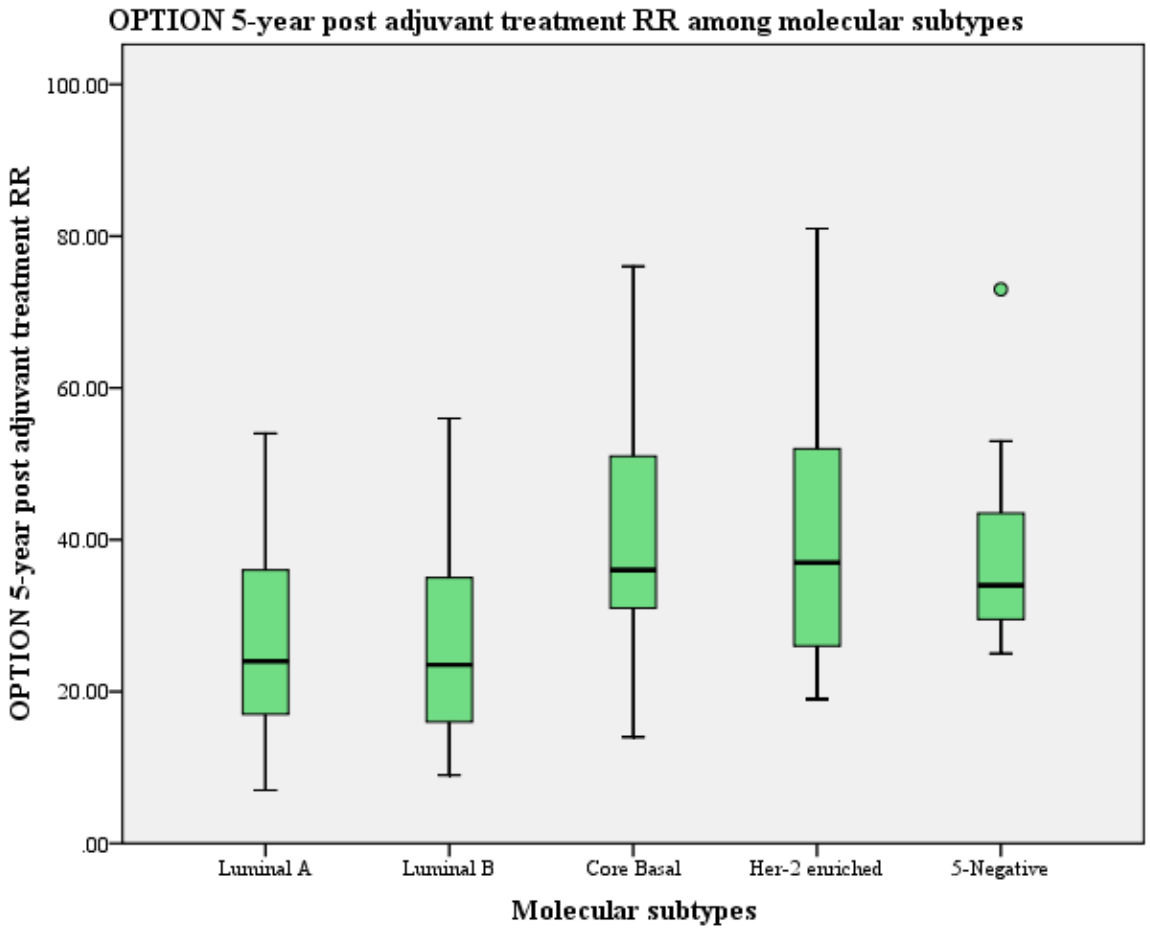
Luminal A
Luminal B
Core Basal
Her-2 enriched
5-Negative
Molecular subtypes


OPTION 5-year post adjuvant treatment RR among molecular subtypes
OPTION 5-year post adjuvant treatment RR
100.00
80.00
60.00
40.00
20.00
.00
Luminal A
Luminal B
Core Basal
Her-2 enriched
5-Negative
Molecular subtypes

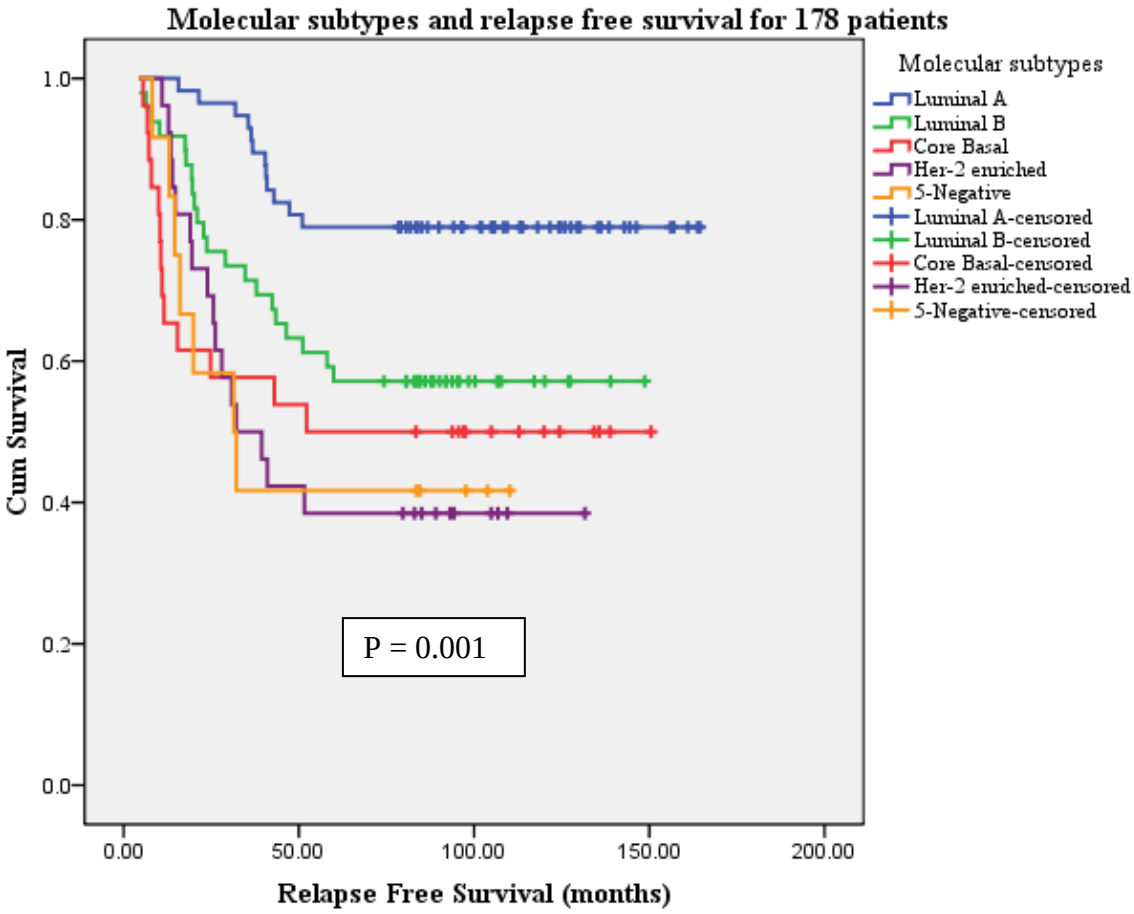
One way ANOVA comparing means of RR among molecular subtypes

There are no statistically significant differences in mean recurrence risks by Adjuvant! or OPTION (before adjuvant treatment) between molecular subtypes. But there is a statistically significant difference in mean OPTION 5-year post adjuvant treatment recurrence risks.

One way ANOVA comparing means of recurrence risk among molecular subtypes

				Sum of Squares	df	Mean Square	F	Sig.
Adjuvant! 10-year RR	Between Groups	(Combined)		1446.534	4	361.634	1.066	.375
		Linear Term	Unweighted	466.310	1	466.310	1.374	.243
			Weighted	754.263	1	754.263	2.223	.138
			Deviation	692.271	3	230.757	.680	.565
	Within Groups			5.599E4	165	339.322		
	Total			5.743E4	169			
OPTION 5-year RR	Between Groups	(Combined)		156.825	4	39.206	.128	.972
		Linear Term	Unweighted	53.954	1	53.954	.177	.675
			Weighted	15.029	1	15.029	.049	.825
			Deviation	141.796	3	47.265	.155	.927
	Within Groups			5.042E4	165	305.585		
	Total			5.058E4	169			
OPTION 5-year post adjuvant treatment RR	Between Groups	(Combined)		8833.095	4	2208.274	12.125	.000
		Linear Term	Unweighted	3456.475	1	3456.475	18.978	.000
			Weighted	6103.852	1	6103.852	33.514	.000
			Deviation	2729.243	3	909.748	4.995	.002
	Within Groups			3.005E4	165	182.128		
	Total			3.888E4	169			

Molecular subtypes and RFS by Kaplan-Meier's curve (178 patients)



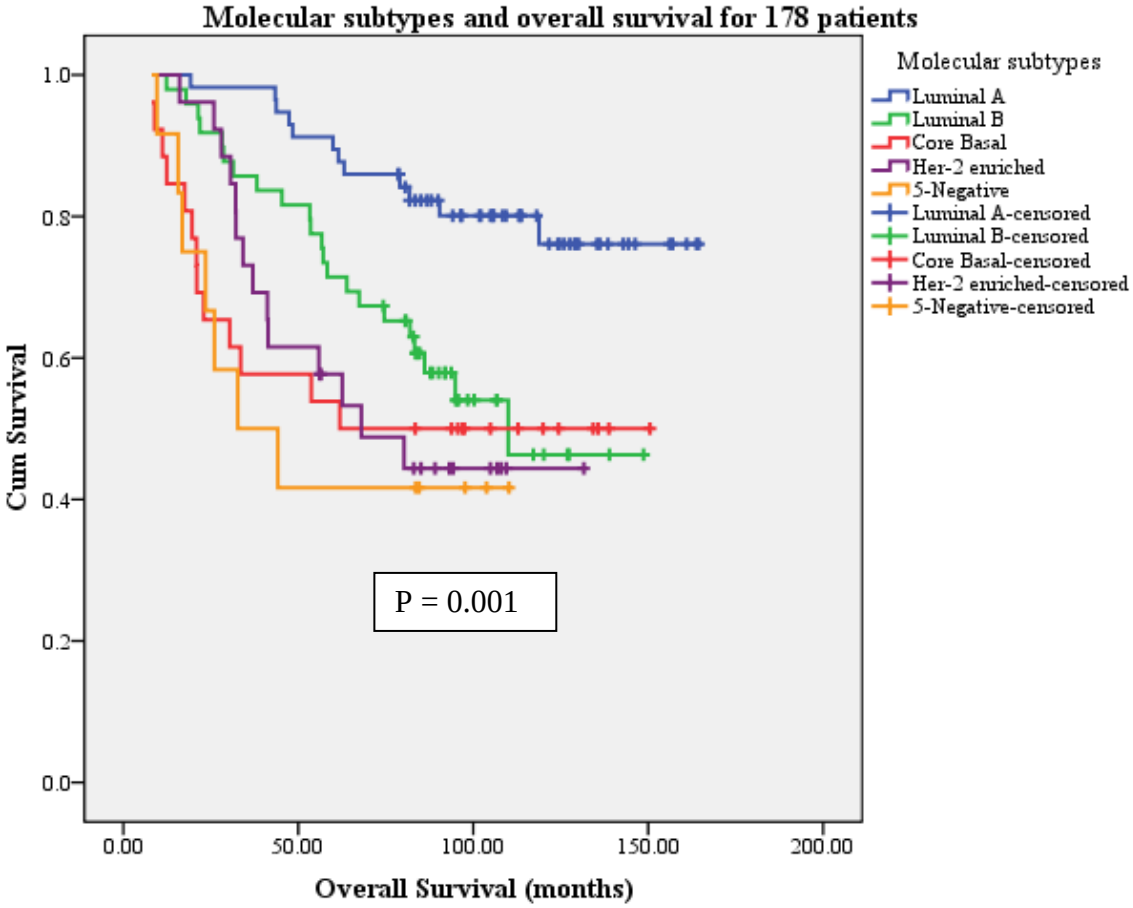
Means and Medians for RFS (months) for the whole cohort (178 patients)

Molecular subtypes	Mean[a]				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Luminal A	137.526	6.917	123.968	151.084	.	.	.	.
Luminal B	97.096	8.649	80.144	114.048	.	.	.	.
Core Basal	83.573	13.271	57.563	109.583	52.200	.	.	.
HER2 enriched	66.123	10.299	45.937	86.310	32.200	8.286	15.960	48.440
5-Negative	57.200	13.066	31.590	82.810	31.400	10.652	10.522	52.278
Overall	108.203	5.264	97.886	118.520	.	.	.	.

a. Estimation is limited to the largest survival time if it is censored.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	19.322	4	**.001**

Molecular subtypes and OS by Kaplan-Meier's curve (178 patients)



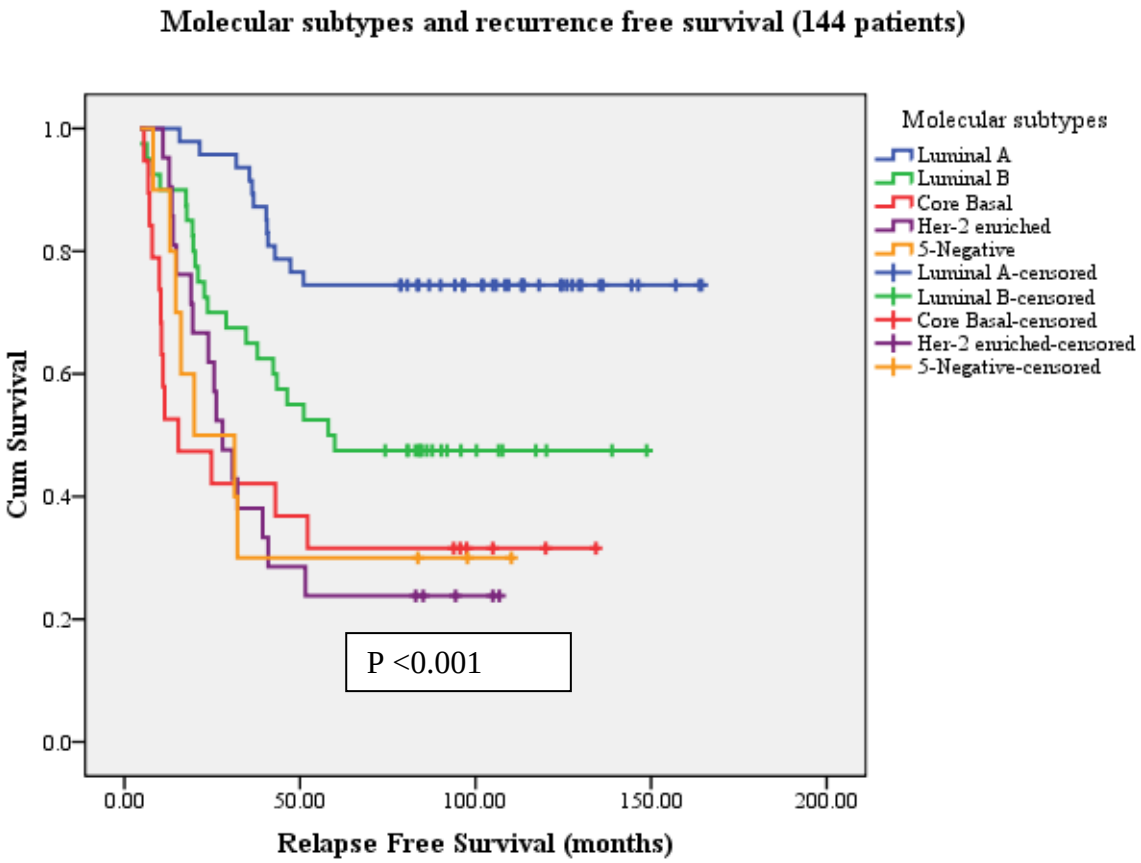
Means and Medians for OS (months) for the whole cohort (178 patients)

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
Molecular subtypes	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
Luminal A	141.574	5.919	129.973	153.175	.	.	.	.
Luminal B	102.139	7.517	87.406	116.873	110.000	.	.	.
Core Basal	87.638	12.525	63.090	112.187	61.900	.	.	.
HER2 enriched	82.169	9.177	64.182	100.156	68.000	18.302	32.129	103.871
5-Negative	59.958	12.487	35.484	84.432	32.700	15.762	1.807	63.593
Overall	114.593	4.767	105.250	123.937	.	.	.	.

a. Estimation is limited to the largest survival time if it is censored.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	18.931	4	**.001**

Molecular subtypes and RFS for cases and matched controls (<u>144 patients</u>)



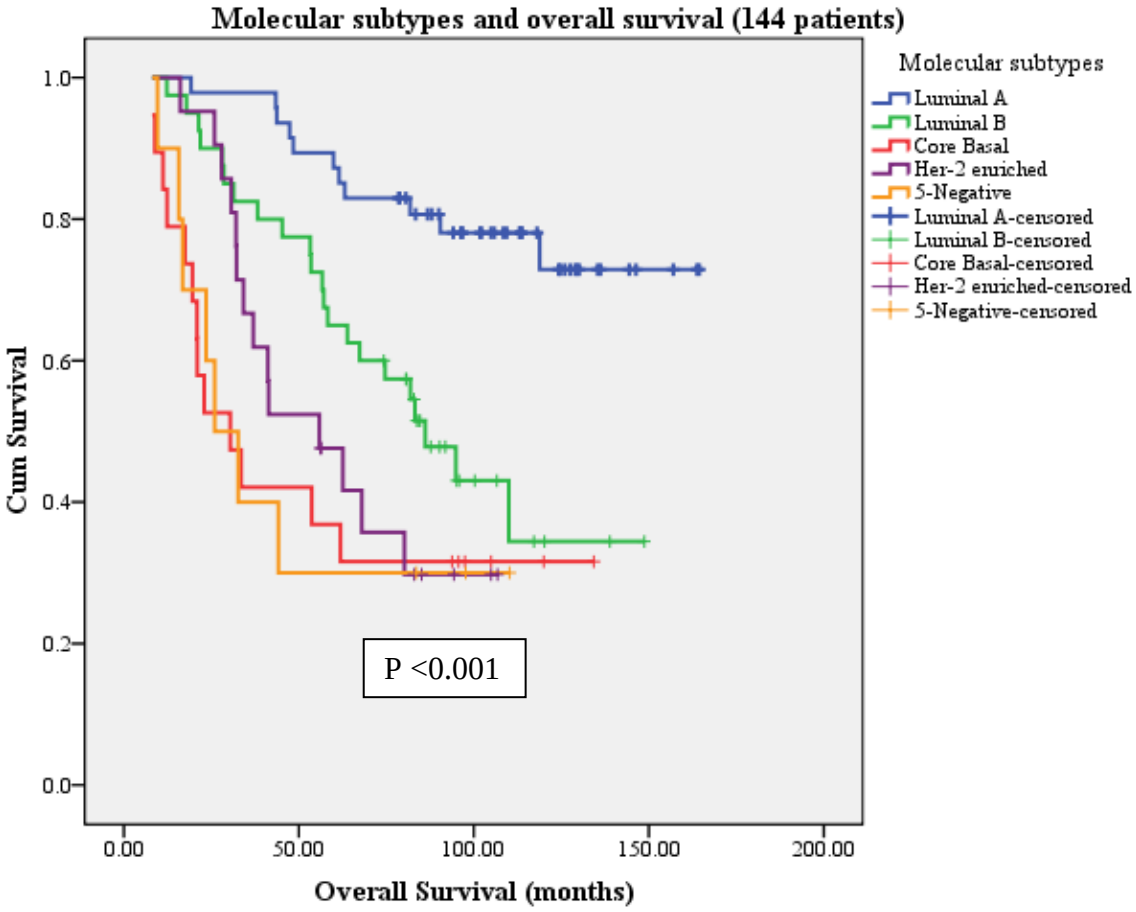
Means and Medians for Relapse Free Survival for molecular subtypes

Molecular subtypes	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
Luminal A	131.809	8.149	115.836	147.782	.	.	.	.
Luminal B	85.485	9.691	66.491	104.479	58.100	.	.	.
Core Basal	53.800	12.829	28.655	78.945	15.400	10.012	.000	35.023
HER2 enriched	44.629	7.883	29.178	60.079	28.000	3.967	20.225	35.775
5-Negative	46.600	13.358	20.418	72.782	19.900	12.096	.000	43.608
Overall	94.666	5.981	82.944	106.389	59.900	.	.	.

a. Estimation is limited to the largest survival time if it is censored.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	27.406	4	**<.001**

Molecular subtypes and OS for cases and matched controls (<u>144 patients</u>)



Means and Medians for Overall Survival for molecular subtypes

Molecular subtypes	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
Luminal A	138.440	6.908	124.899	151.980	.	.	.	.
Luminal B	91.565	8.241	75.414	107.717	86.100	11.716	63.136	109.064
Core Basal	59.363	12.065	35.716	83.010	30.400	9.068	12.626	48.174
HER2 enriched	62.176	7.293	47.882	76.470	55.900	15.070	26.363	85.437
5-Negative	49.910	12.804	24.814	75.006	26.000	7.273	11.744	40.256
Overall	103.001	5.461	92.297	113.705	110.000	.	.	.

a. Estimation is limited to the largest survival time if it is censored.

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	29.827	4	**<.001**

RFS and OS analysis for individual biomarker

Kaplan-Meier survival analyses and log rank tests were carried out to evaluate RFS and OS between the cases and the matched control groups.

RFS analysis by Kaplan Meire's curve and Log Rank test

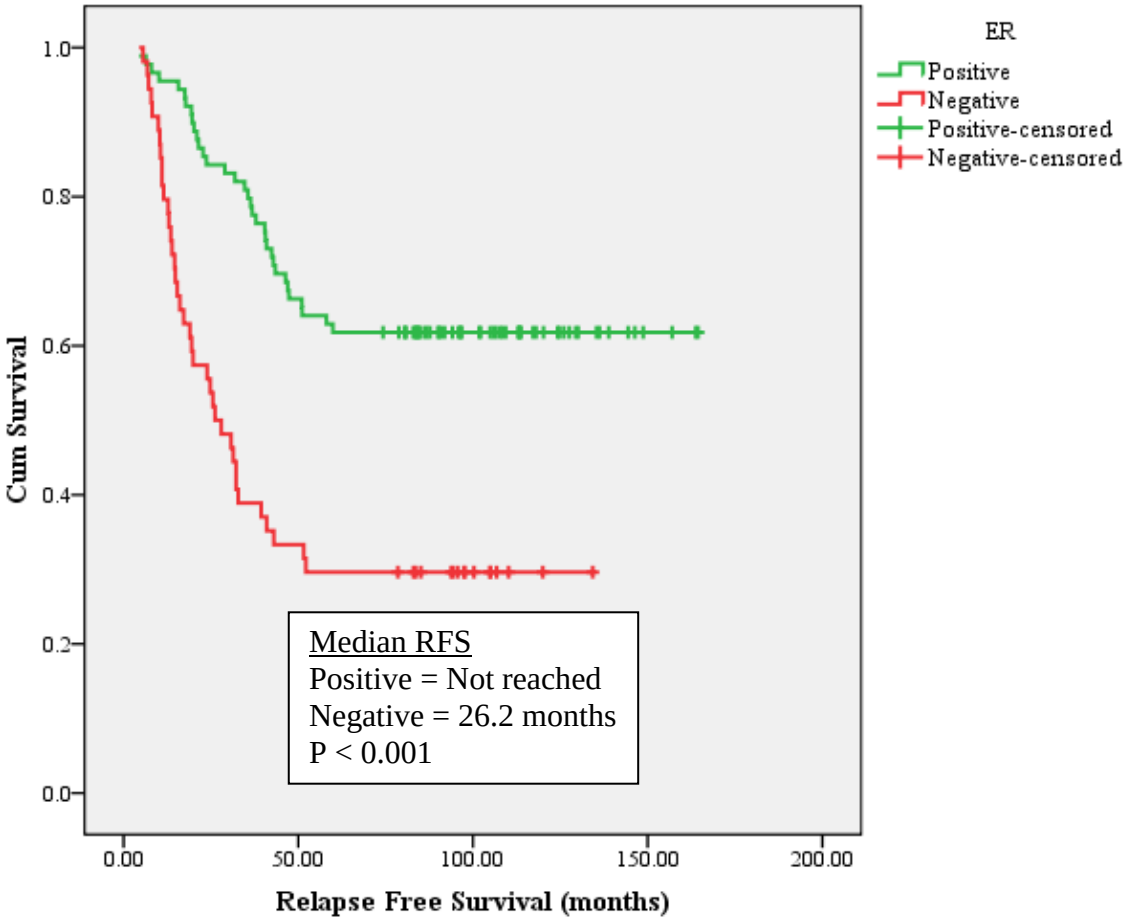
		Mean	Median
ER	Positive	113.754	Not reached
P = **<0.001**	Negative	54.7	26.2
PR	Positive	130.676	Not reached
P = **<0.001**	Negative	72.074	35.6
HER2	Positive	75.245	41
P = 0.251	Negative	98.237	Not reached
EGFR	Positive	54.482	15.4
P = 0.134	Negative	96.287	Not reached
Ki-67	Positive	63.91	30.8
P = **<0.001**	Negative	125.157	Not reached
CK-5/6	Positive	81	59.9
P = 0.955	Negative	95.322	51.1
Bcl-2	Positive	99.504	Not reached
P = **0.036**	Negative	65.819	23.9
Bag-1	Positive	106.313	Not reached
P = **0.018**	Negative	82.543	42.4
CD-68	Positive	89.366	Not reached
P = 0.424	Negative	89.327	47.3
CD-71	Positive	79.402	43
P = 0.097	Negative	102.582	Not reached
MCM-2	Positive	108.292	Not reached
P = **0.014**	Negative	83.883	42.9
Aurora A	Positive	69.591	32.2
P = **0.001**	Negative	111.762	Not reached
PDGFRα	Positive	86.319	51
P = 0.070	Negative	131.608	Not reached

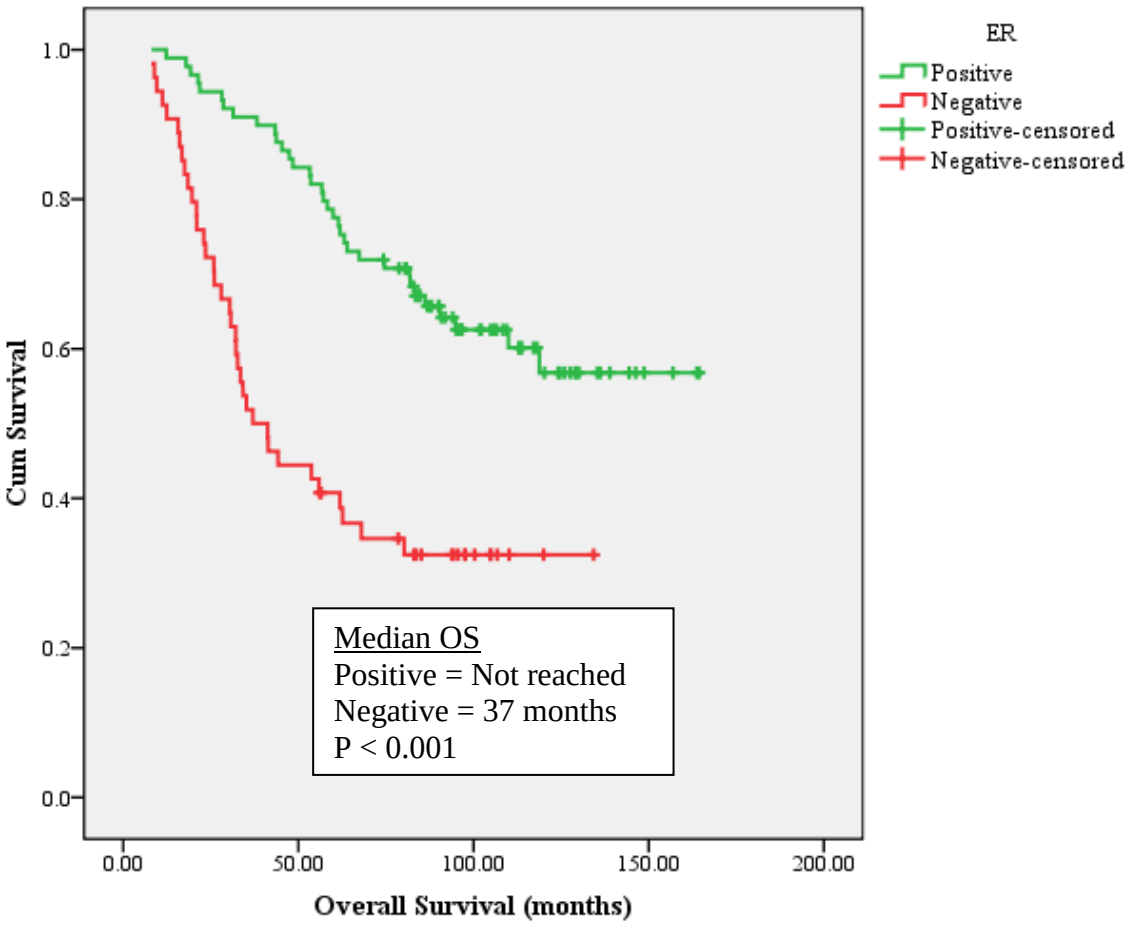
Overall survival analysis using Kaplan-Meier's curve and Log Rank test

		Mean	Median
ER	Positive	120.396	Not reached
P = **<0.001**	Negative	65.13	37
PR	Positive	130.495	Not reached
P = **<0.001**	Negative	84.905	59.9
HER2	Positive	82.076	80.2
P = 0.150	Negative	106.666	Not reached
EGFR	Positive	60.964	34.2
P = 0.123	Negative	104.753	118.8
Ki-67	Positive	77.026	57.1
P = **<0.001**	Negative	127.449	Not reached
CK-5/6	Positive	91.031	Not reached
P = 0.869	Negative	102.004	110
Bcl-2	Positive	106.726	118.8
P = 0.058	Negative	77.734	58.3
Bag-1	Positive	112.942	Not reached
P = **0.018**	Negative	92.508	63.1
CD-68	Positive	102.758	Not reached
P = 0.131	Negative	95.4	81.9
CD-71	Positive	88.586	81.8
P = 0.081	Negative	110.065	Not reached
MCM-2	Positive	109.730	Not reached
P = **0.032**	Negative	96.184	63.1
Aurora A	Positive	77.669	67.4
P = **0.001**	Negative	118.713	Not reached
PDGFRα	Positive	95.164	86.1
P = 0.085	Negative	134.725	Not reached

ER

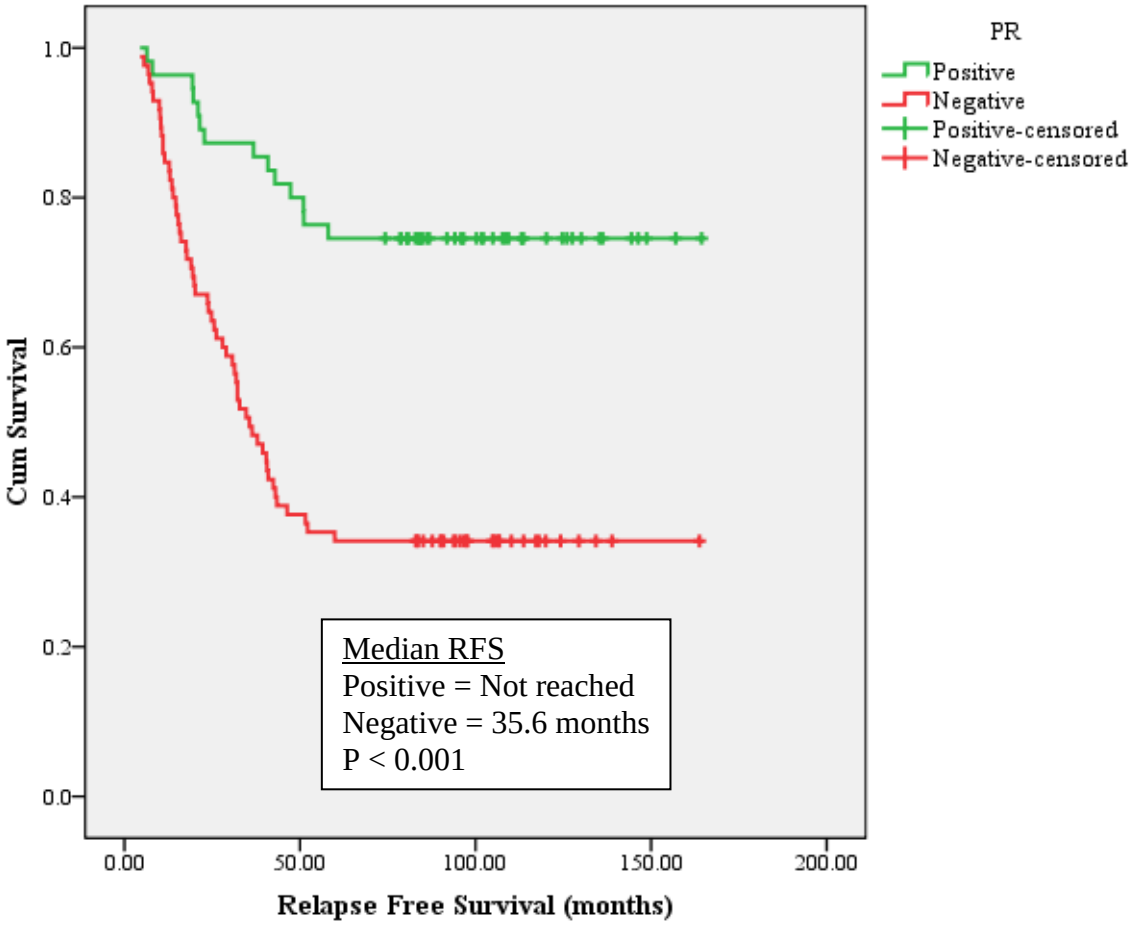
ER positive expression was associated with better RFS (p = <0.001) and OS (p = <0.001).

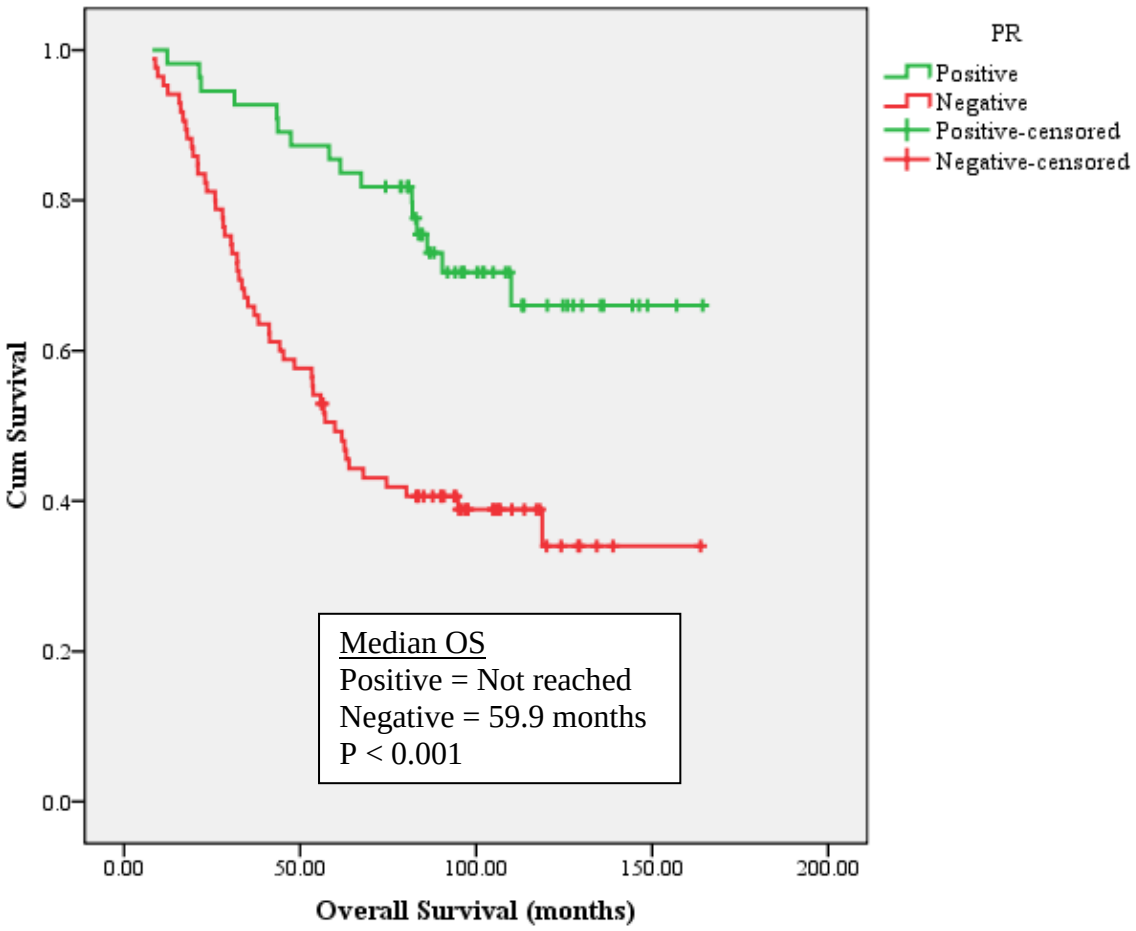



ER
Positive
Negative
Positive-censored
Negative-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
50.00
100.00
150.00
200.00
Overall Survival (months)
Median OS
Positive = Not reached
Negative = 37 months
P < 0.001

PR

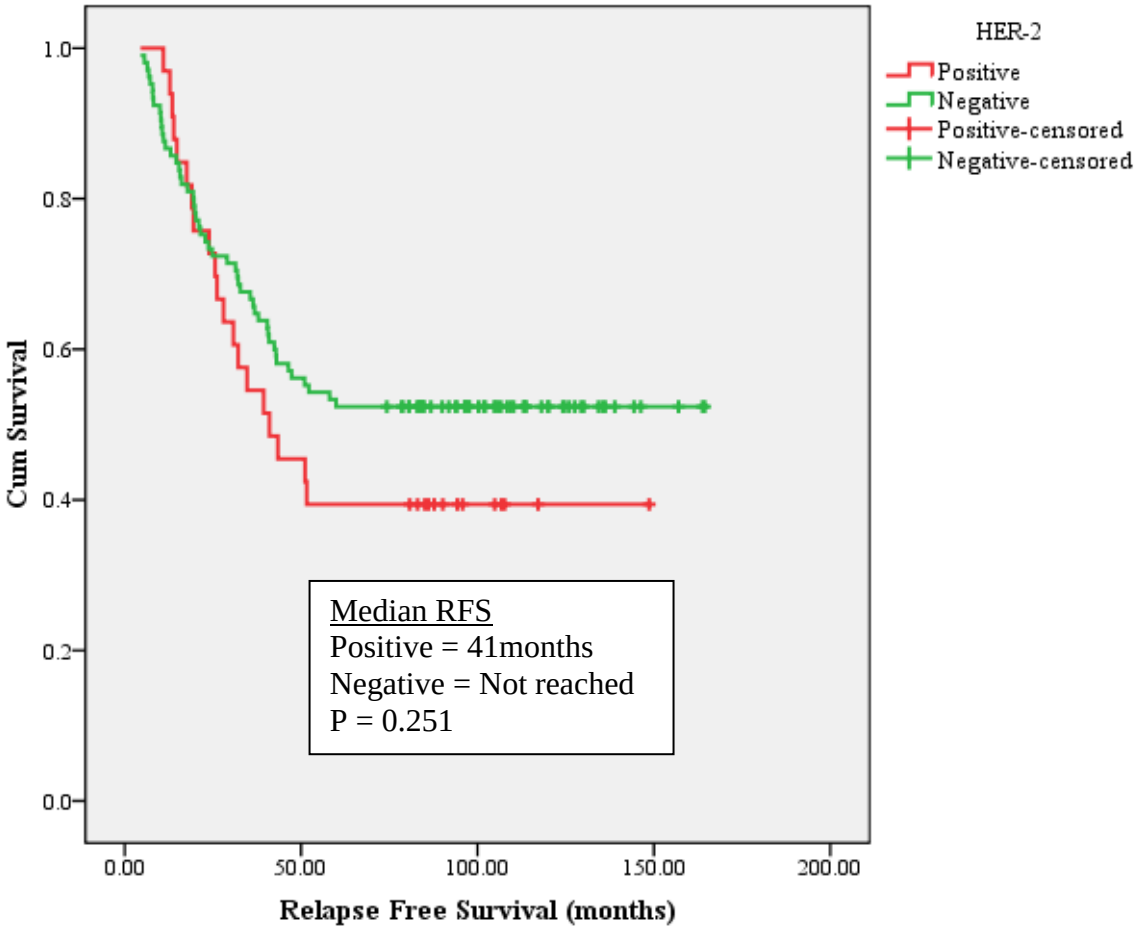
Positive PR expression was associated with better RFS (p = <0.001) and OS (p = <0.001).

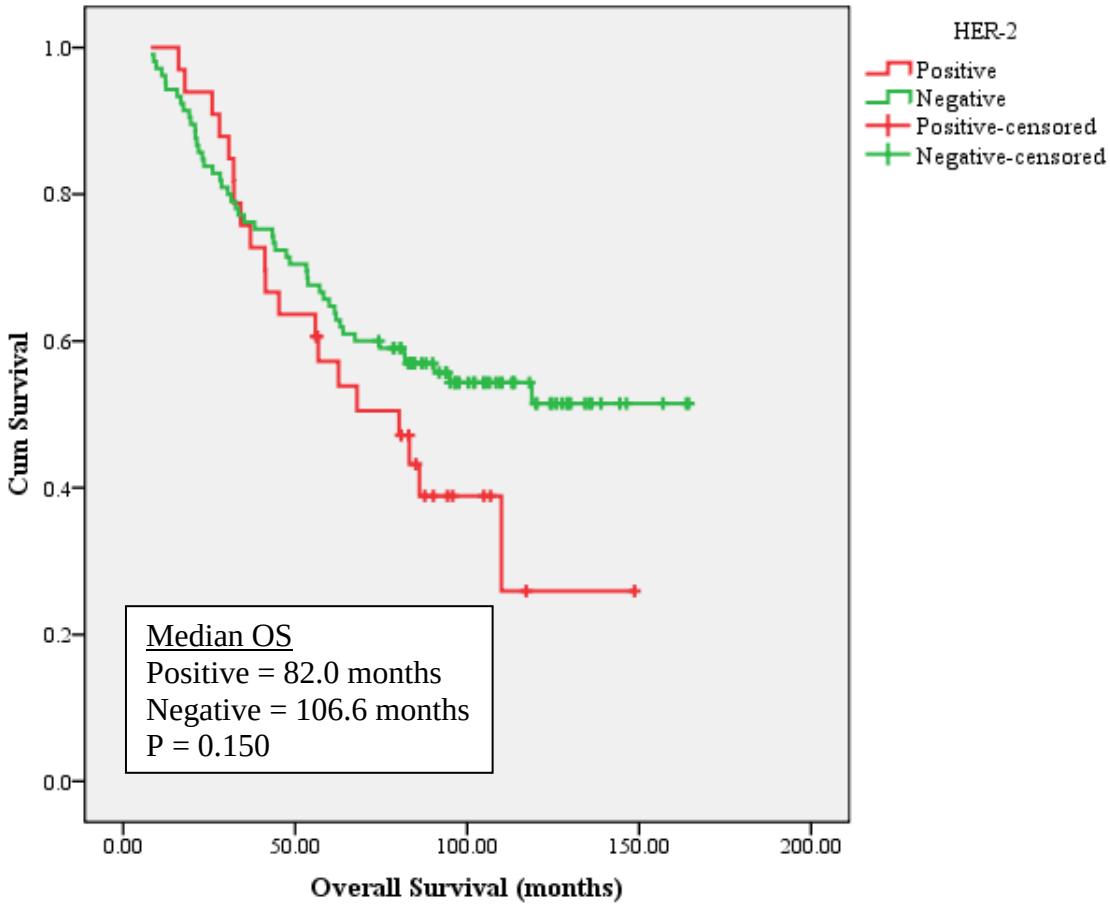



PR
Positive
Negative
Positive-censored
Negative-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
50.00
100.00
150.00
200.00
Overall Survival (months)
Median OS
Positive = Not reached
Negative = 59.9 months
P < 0.001

HER2

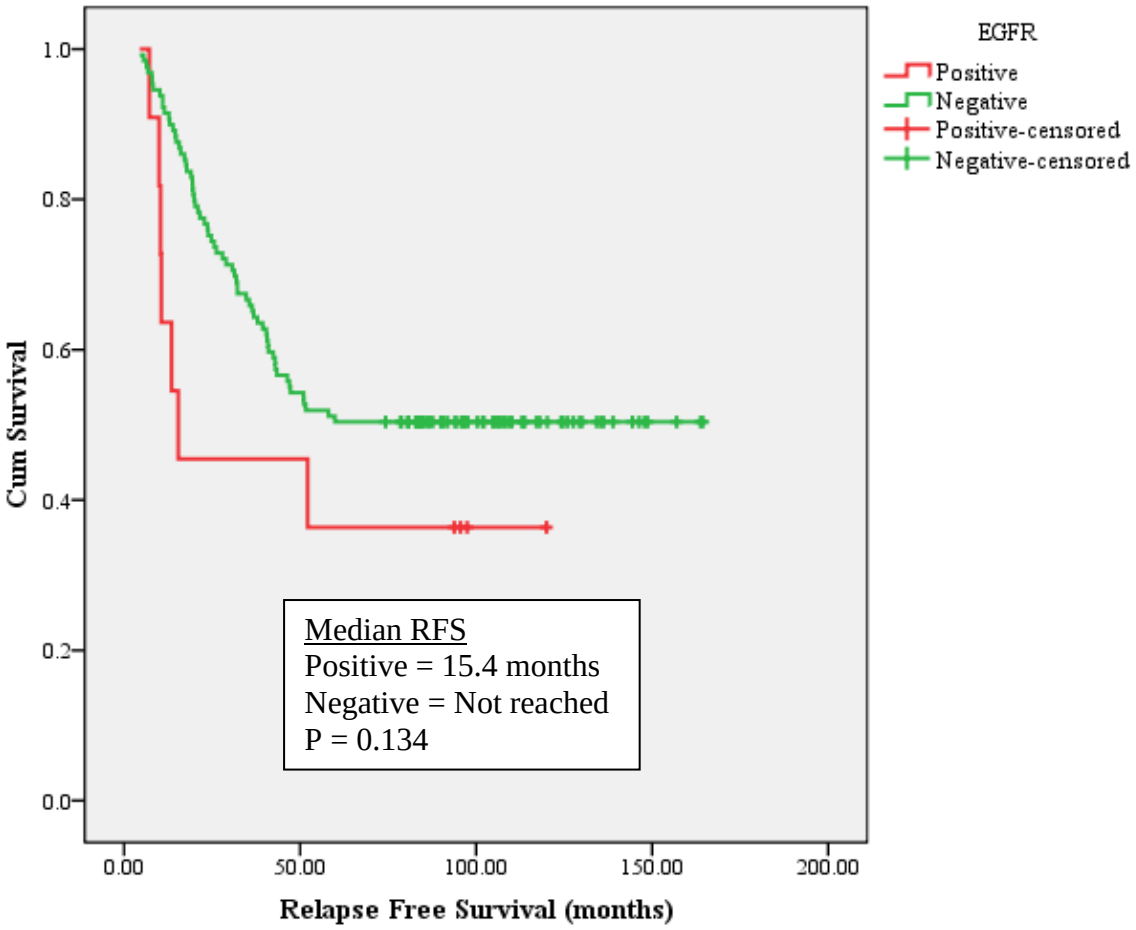
Negative HER2 expression showed a trend for better RFS (p = 0.268) and OS (p = 0.150).

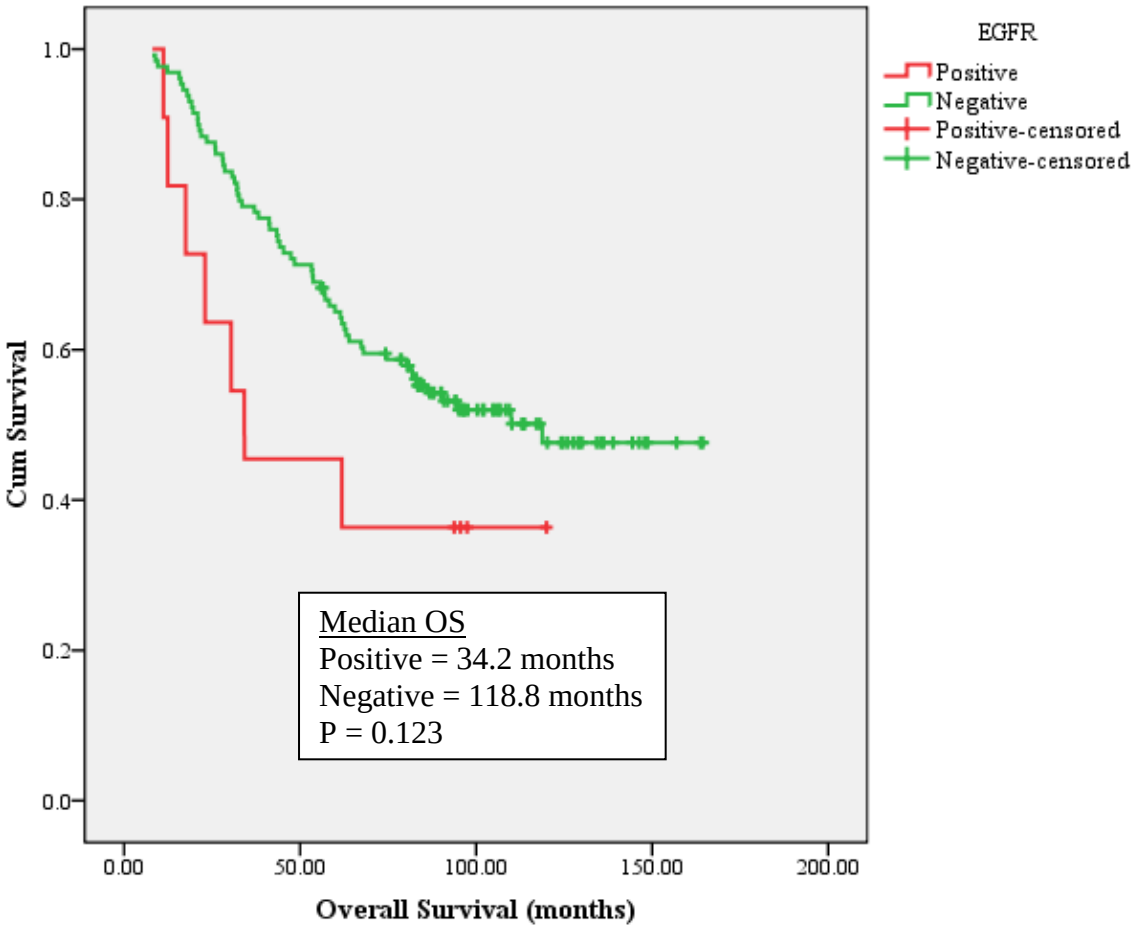



HER-2
Positive
Negative
Positive-censored
Negative-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
50.00
100.00
150.00
200.00
Overall Survival (months)
Median OS
Positive = 82.0 months
Negative = 106.6 months
P = 0.150

EGFR

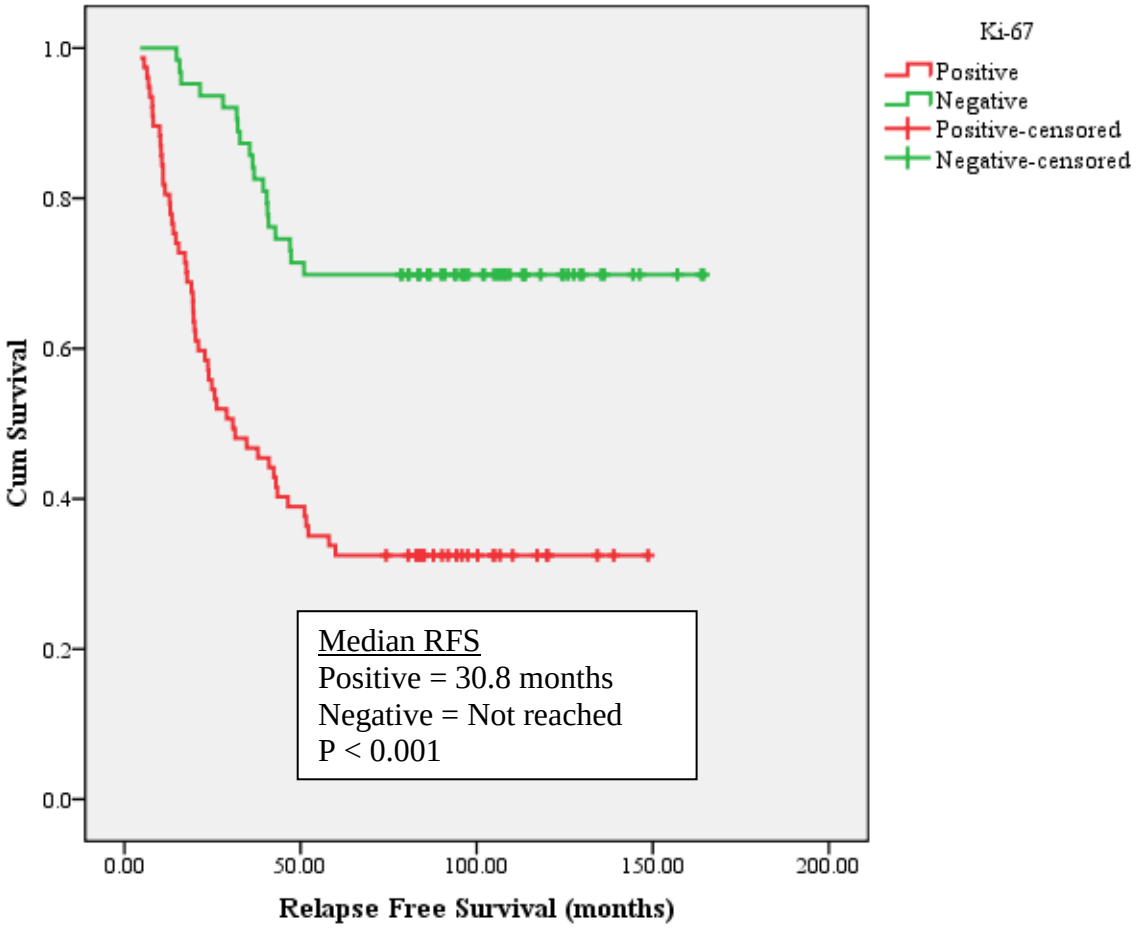
Negative EGFR expression was associated with a trend for better RFS (p = 0.144) and OS (p = 0.123)

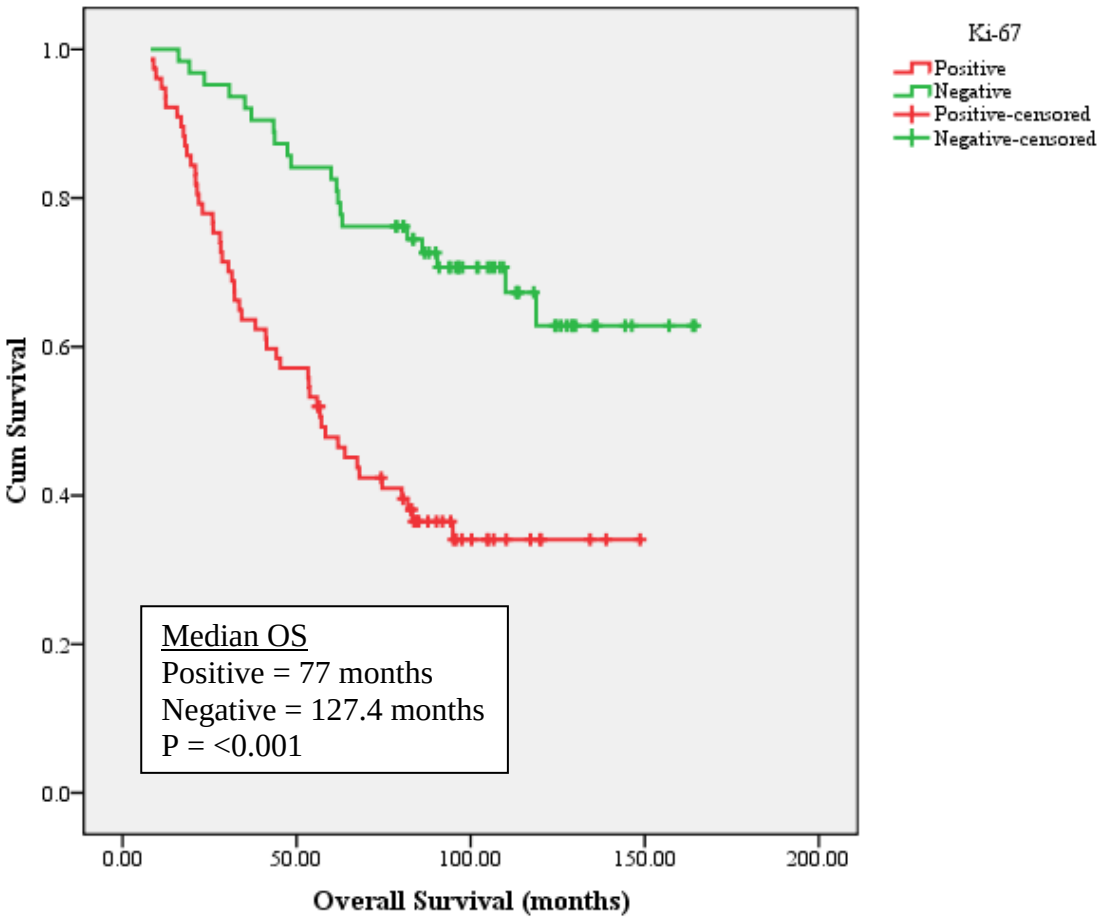



EGFR
Positive
Negative
Positive-censored
Negative-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
50.00
100.00
150.00
200.00
Overall Survival (months)
Median OS
Positive = 34.2 months
Negative = 118.8 months
P = 0.123

Ki-67

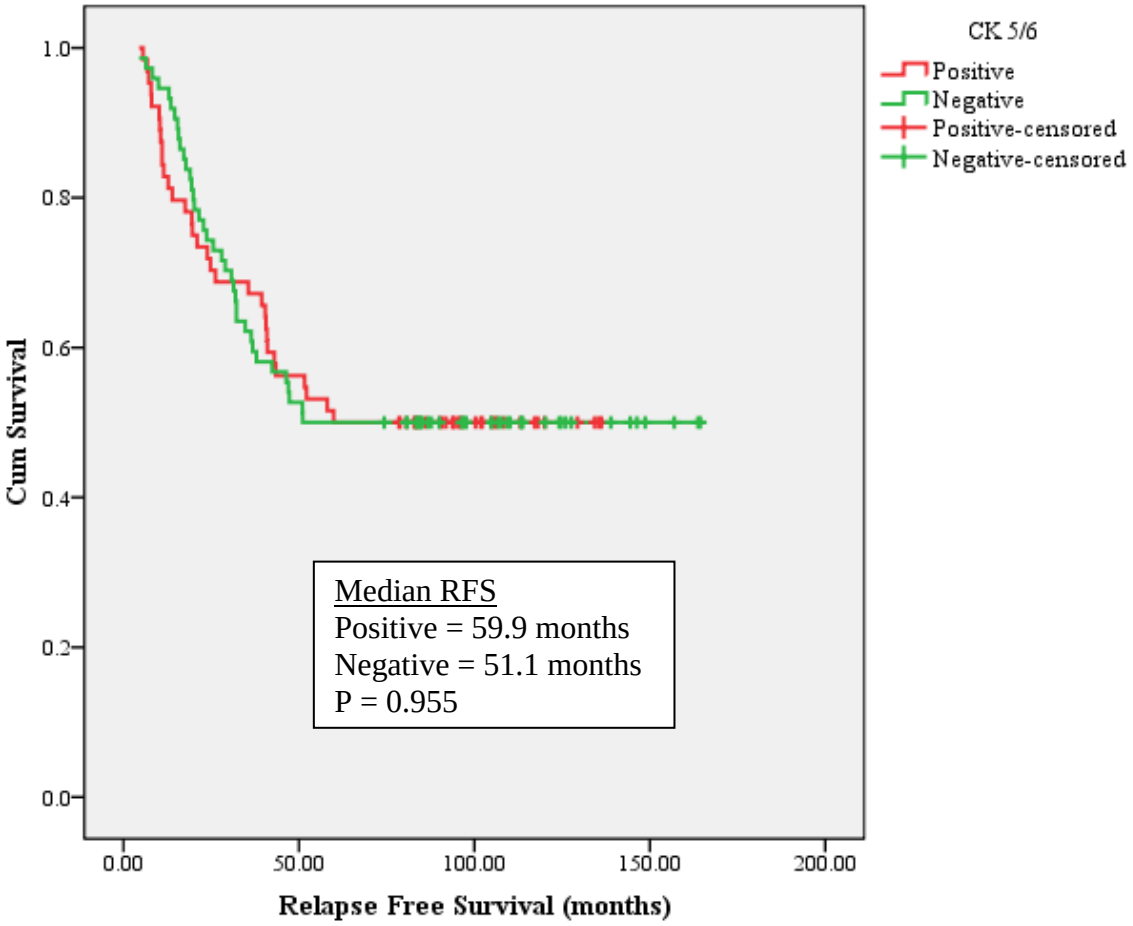
Negative Ki-67 expression was associated with better RFS (p = <0.001) and OS (p = <0.001).

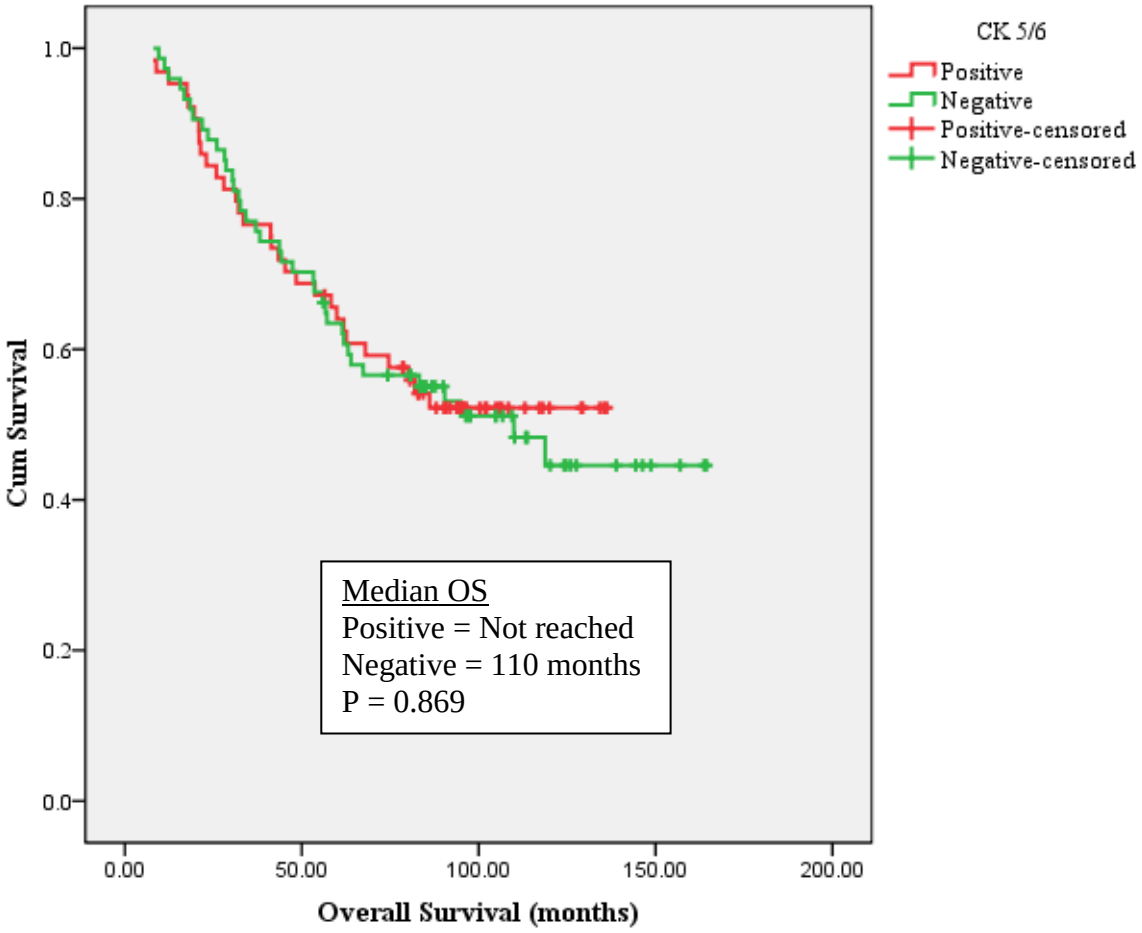



Ki-67
Positive
Negative
Positive-censored
Negative-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
50.00
100.00
150.00
200.00
Overall Survival (months)
Median OS
Positive = 77 months
Negative = 127.4 months
P = <0.001

CK-5/6

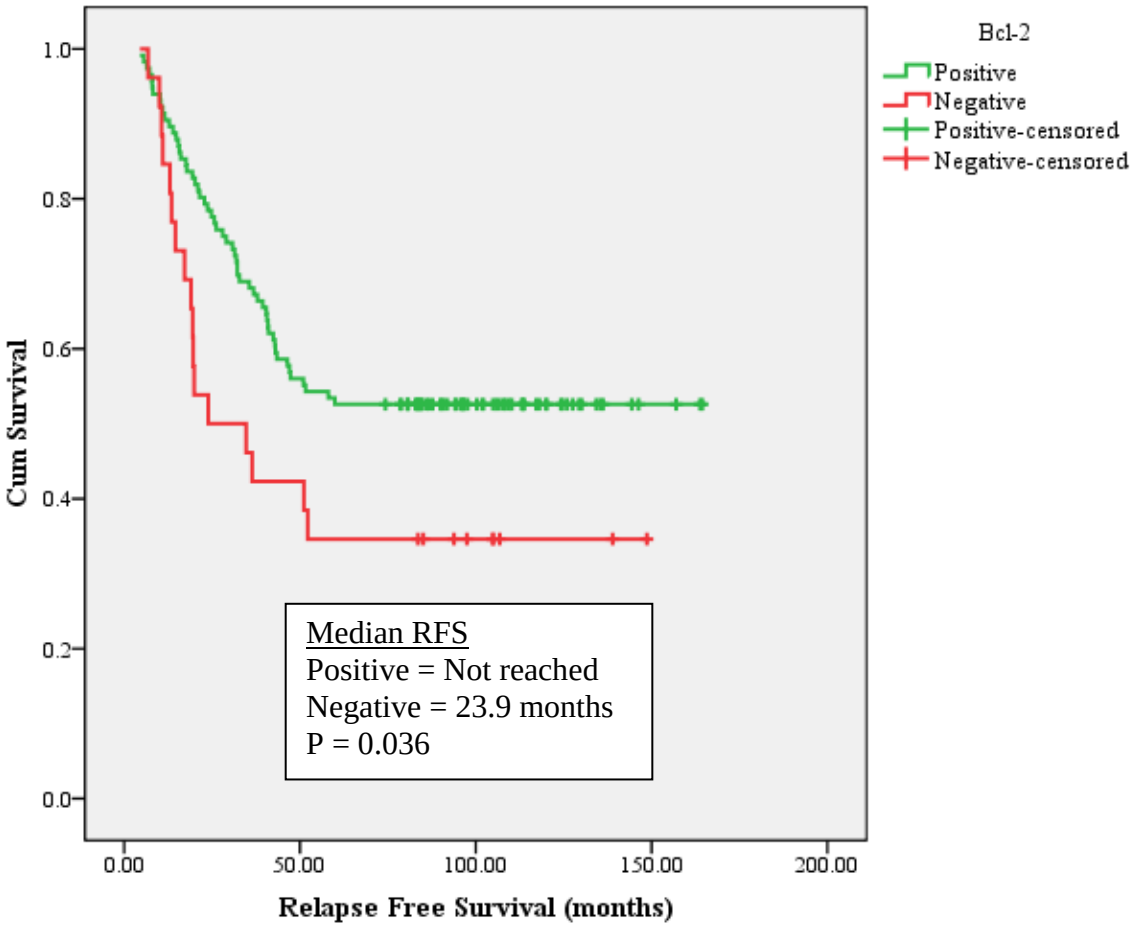
CK-5/6 expression was not associated with RFS (p = 0.971) or OS (p = 0.869).

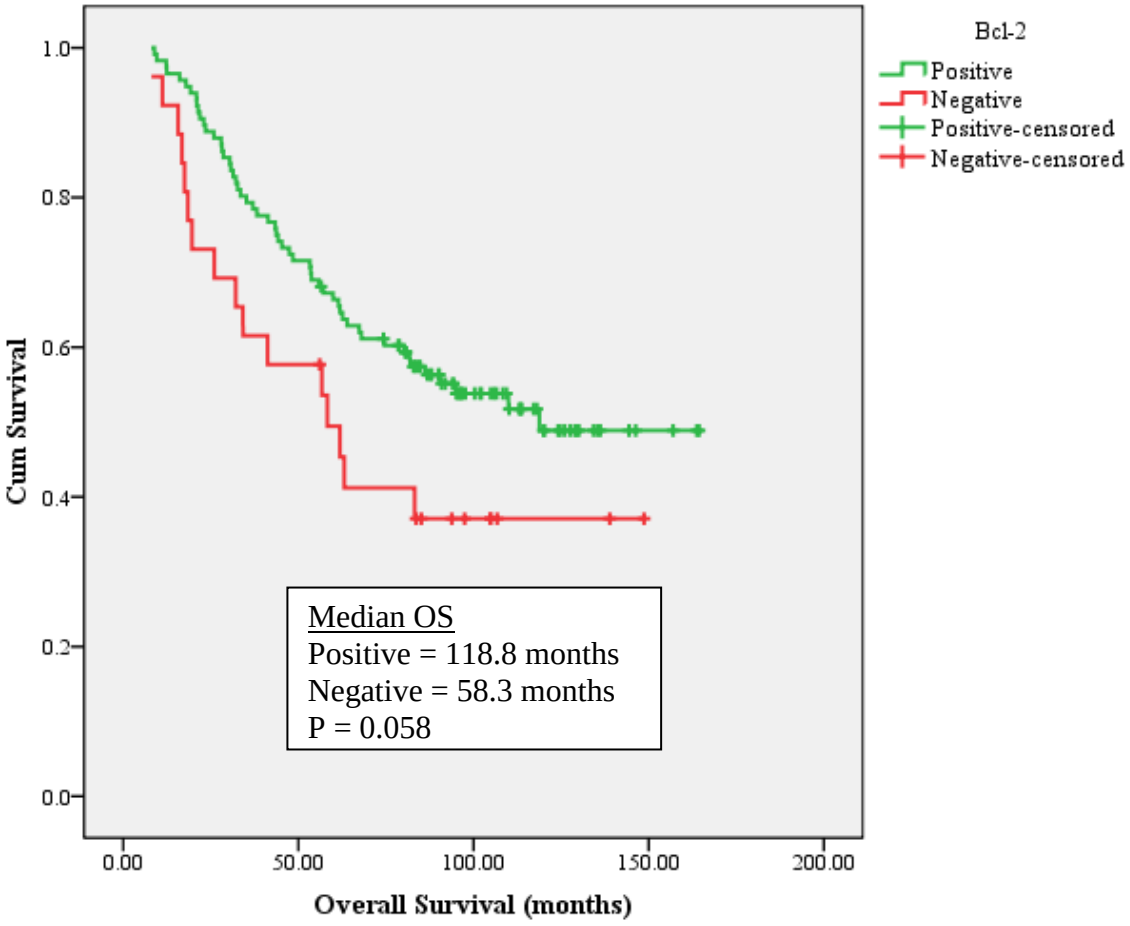



CK 5/6
Positive
Negative
Positive-censored
Negative-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
50.00
100.00
150.00
200.00
Overall Survival (months)
Median OS
Positive = Not reached
Negative = 110 months
P = 0.869

Bcl-2

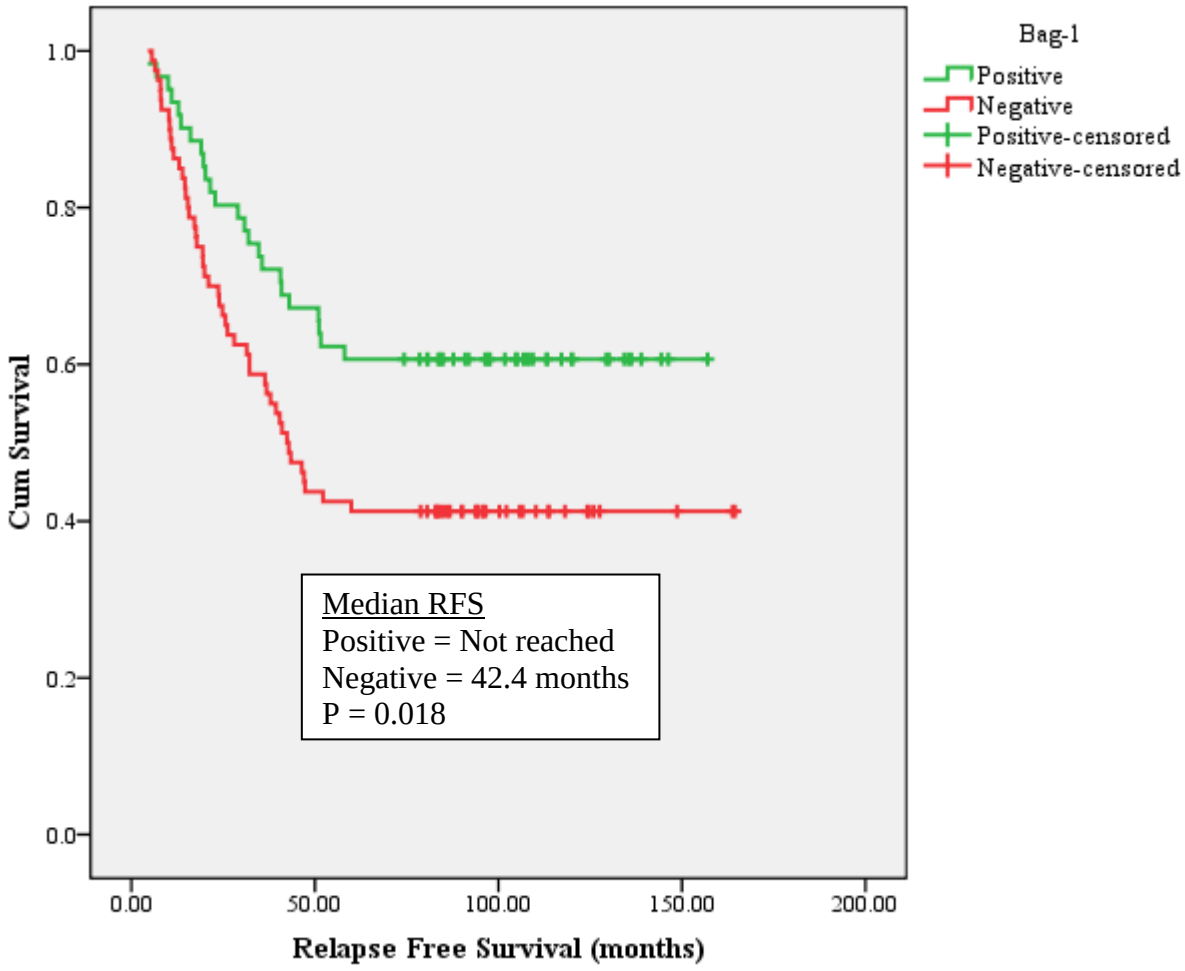
Positive Bcl-2 expression was associated with better RFS (p = 0.036) and OS (p = 0.058).

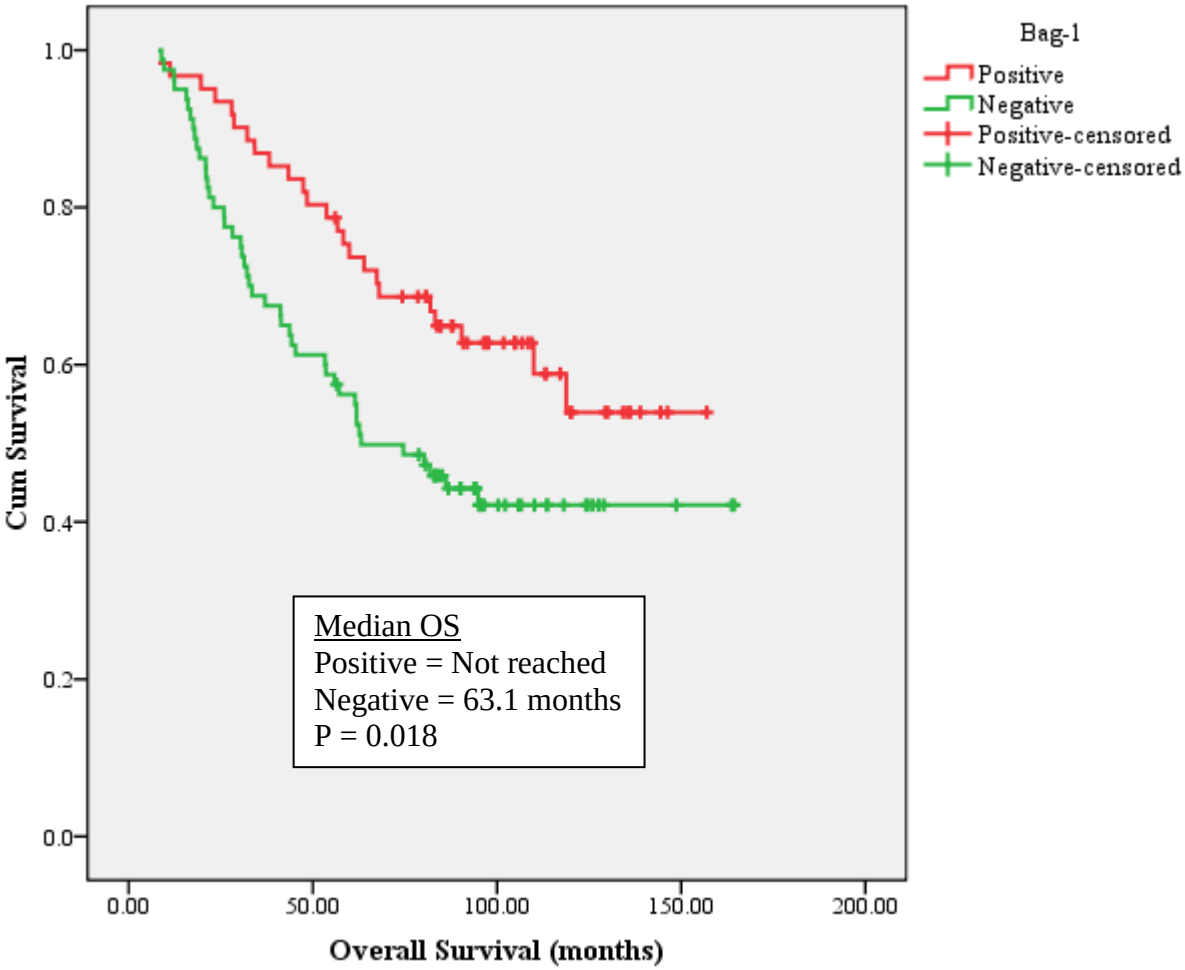



Bcl-2
Positive
Negative
Positive-censored
Negative-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
50.00
100.00
150.00
200.00
Overall Survival (months)
Median OS
Positive = 118.8 months
Negative = 58.3 months
P = 0.058

Bag-1

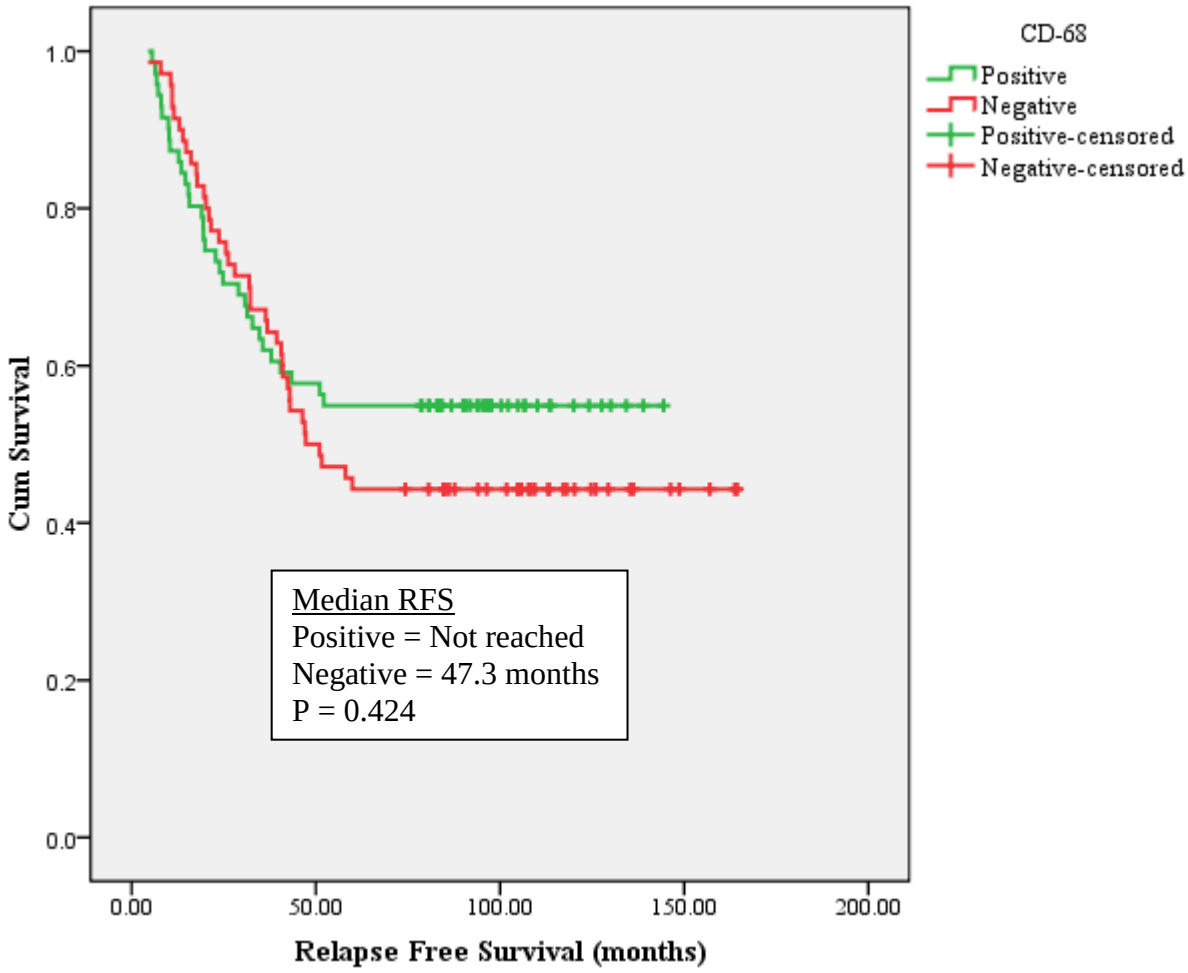
Bag-1 positive expression was associated with RFS (p = 0.018) and OS (p = 0.018).

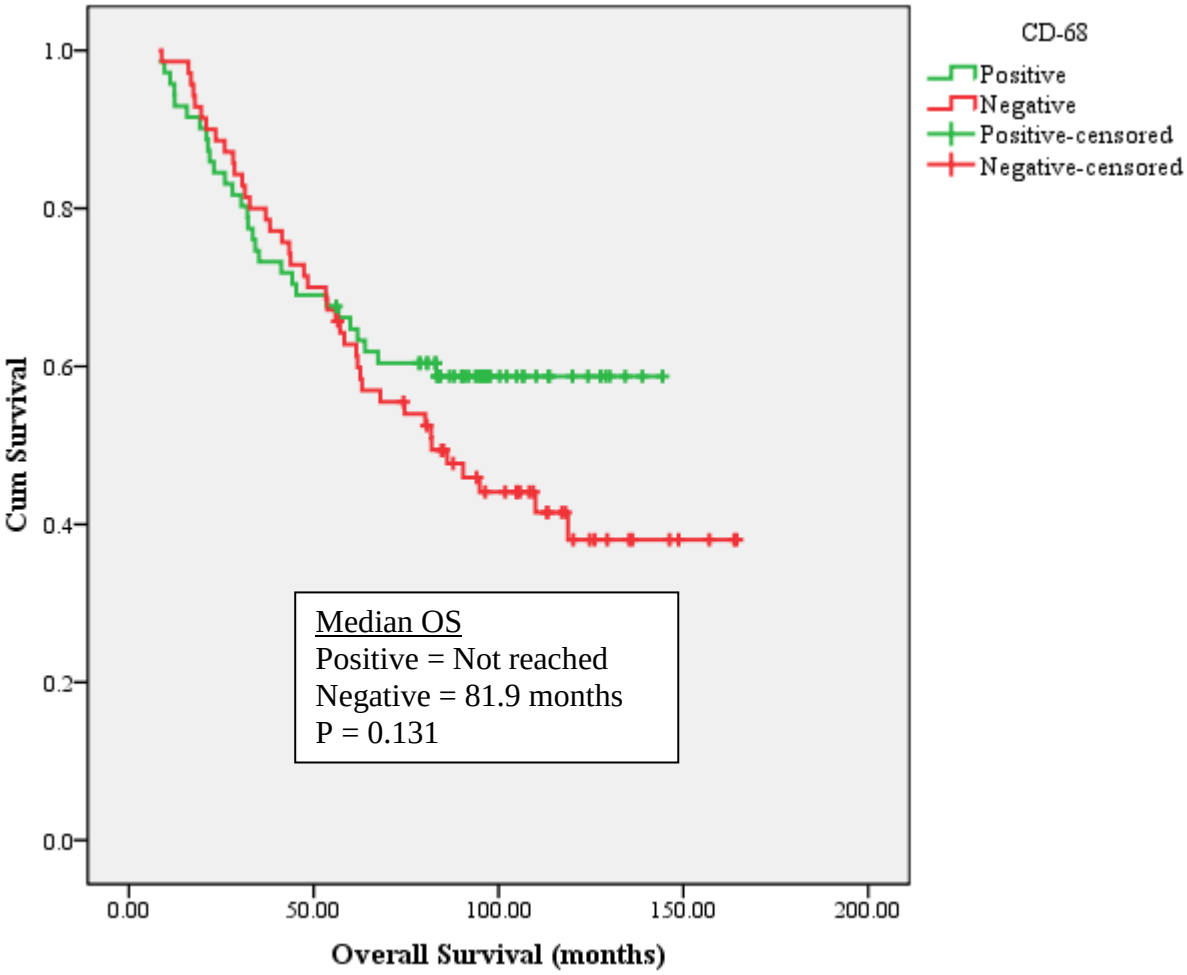



Bag-1
Positive
Negative
Positive-censored
Negative-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
50.00
100.00
150.00
200.00
Overall Survival (months)
Median OS
Positive = Not reached
Negative = 63.1 months
P = 0.018

CD-68

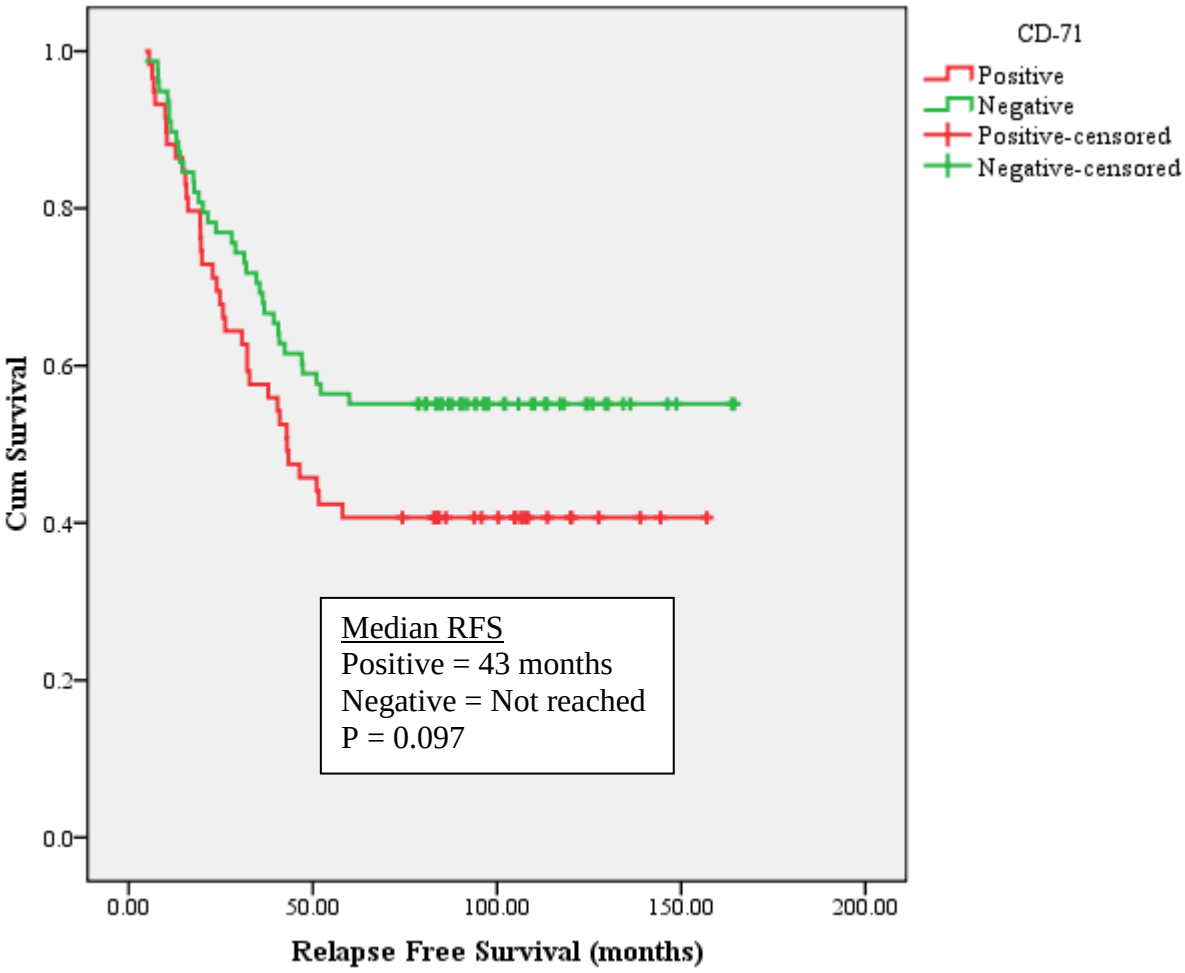
Invasive breast cancer cells did not show reaction with CD-68 antibody. Positive CD-68 expression as tumour associated Macrophages infiltration showed a trend for better RFS (p = 0.419) and OS (p = 0.131).

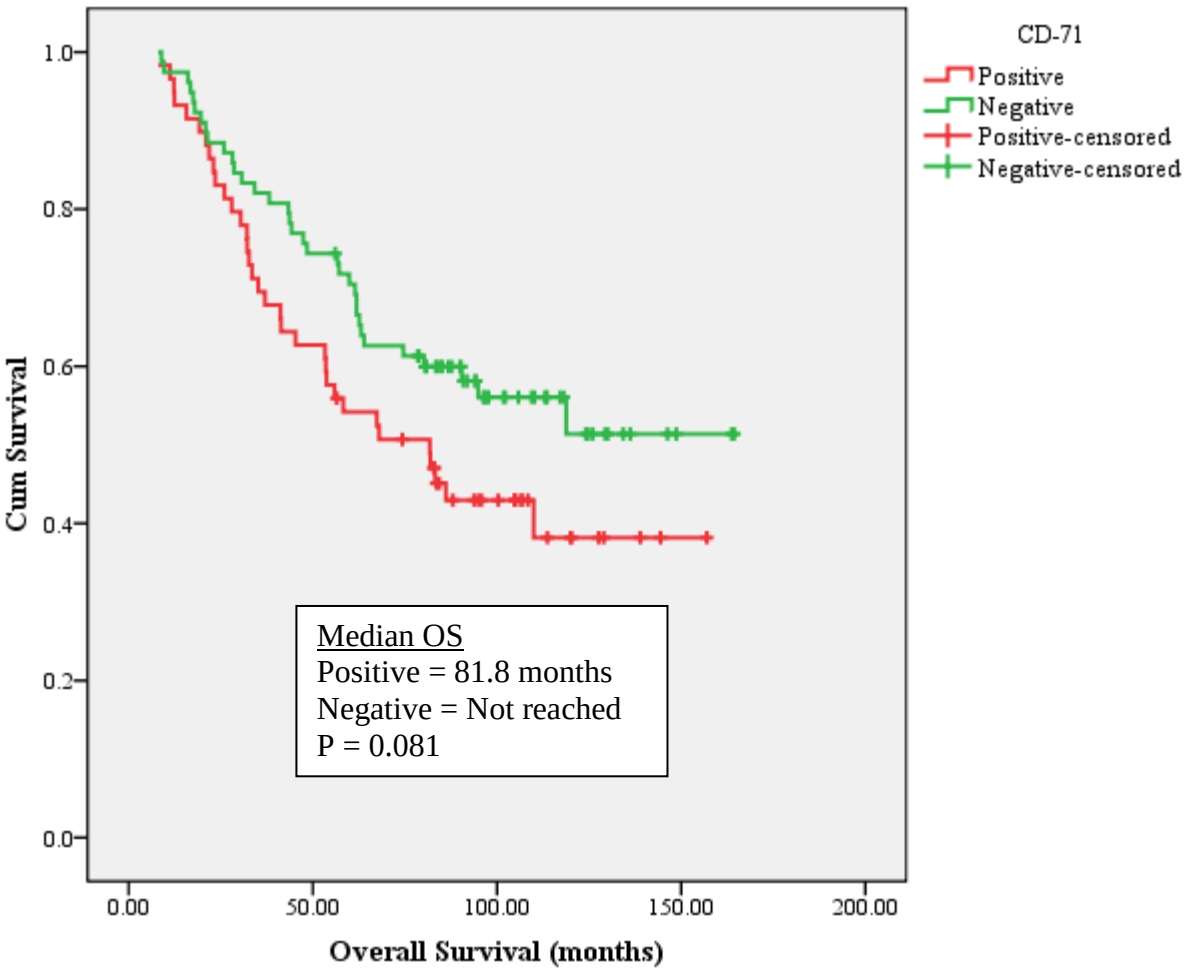



CD-68
Positive
Negative
Positive-censored
Negative-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
50.00
100.00
150.00
200.00
Overall Survival (months)
Median OS
Positive = Not reached
Negative = 81.9 months
P = 0.131

CD-71

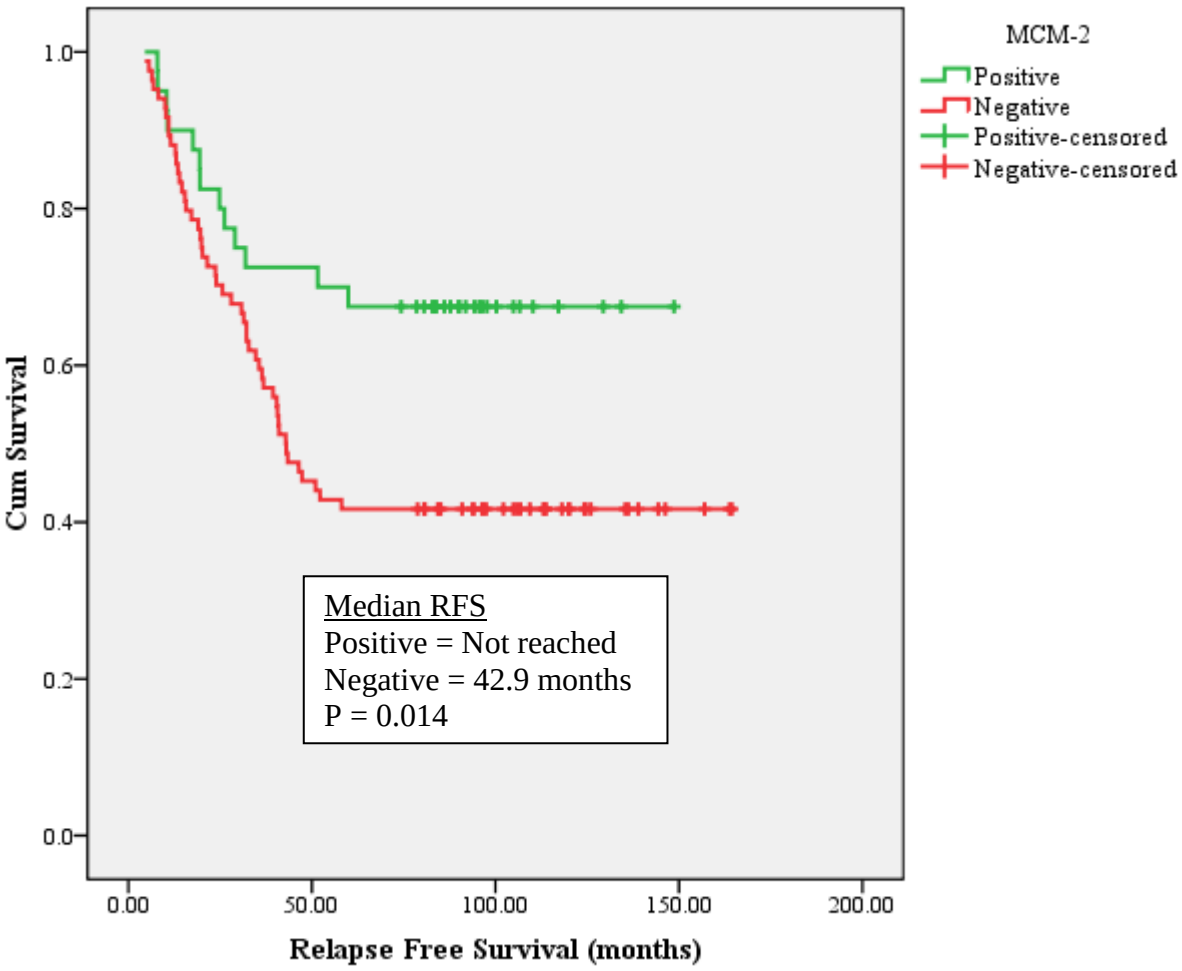
Negative CD-71 expression showed a trend for better RFS (p = 0.097) and OS (p = 0.081).

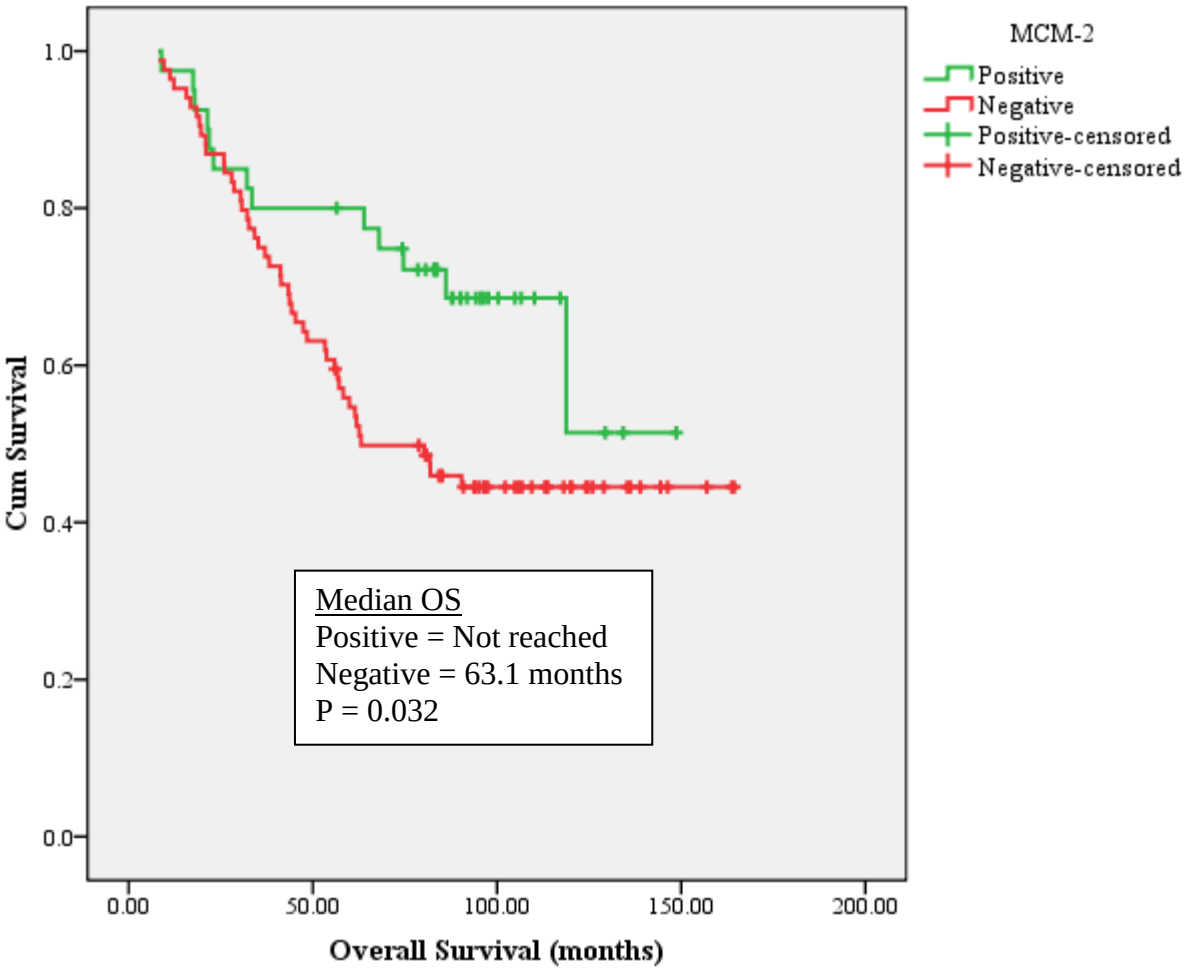



CD-71
Positive
Negative
Positive-censored
Negative-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
50.00
100.00
150.00
200.00
Overall Survival (months)
Median OS
Positive = 81.8 months
Negative = Not reached
P = 0.081

MCM-2

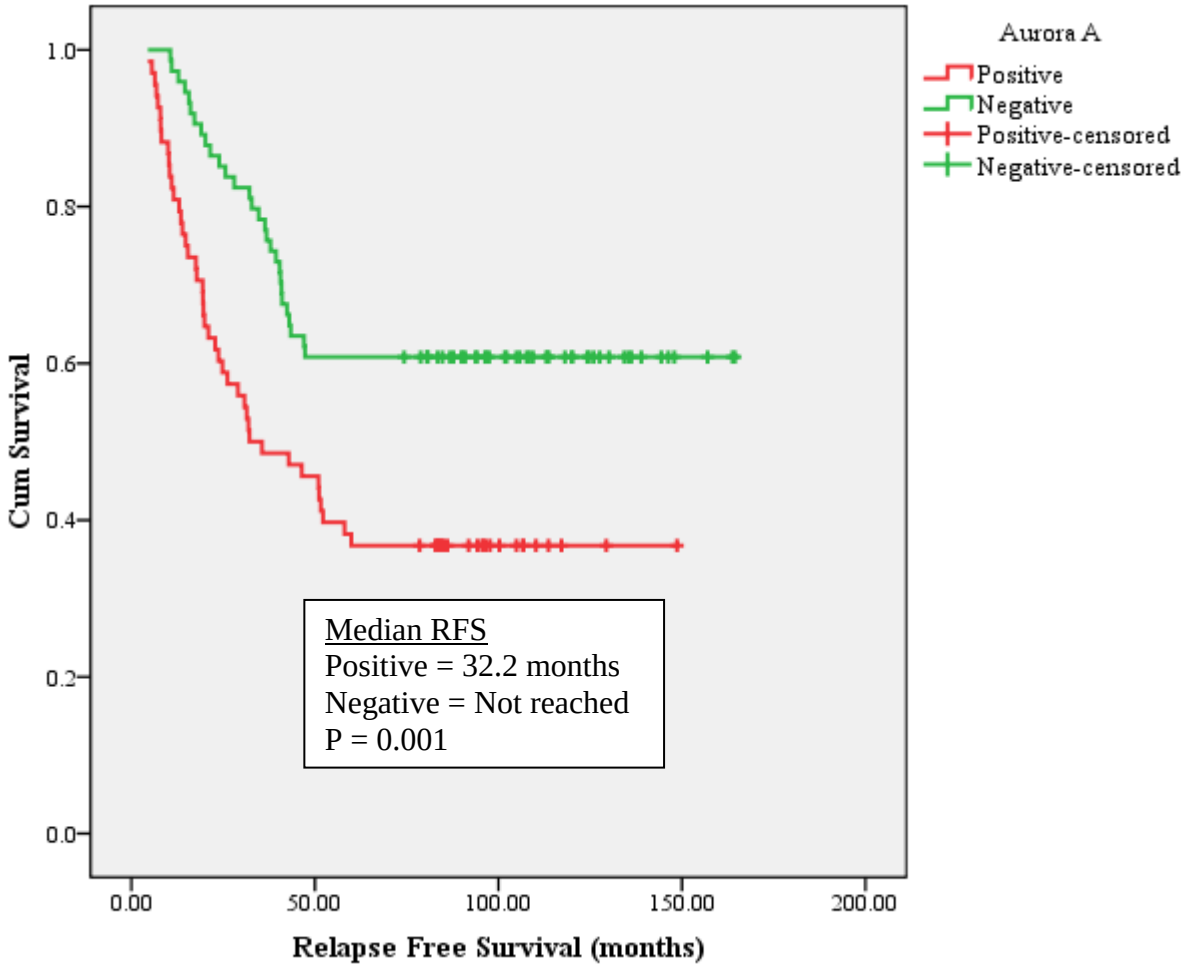
MCM-2 positive expression was associated with better RFS (p = 0.012) and OS (p = 0.032).

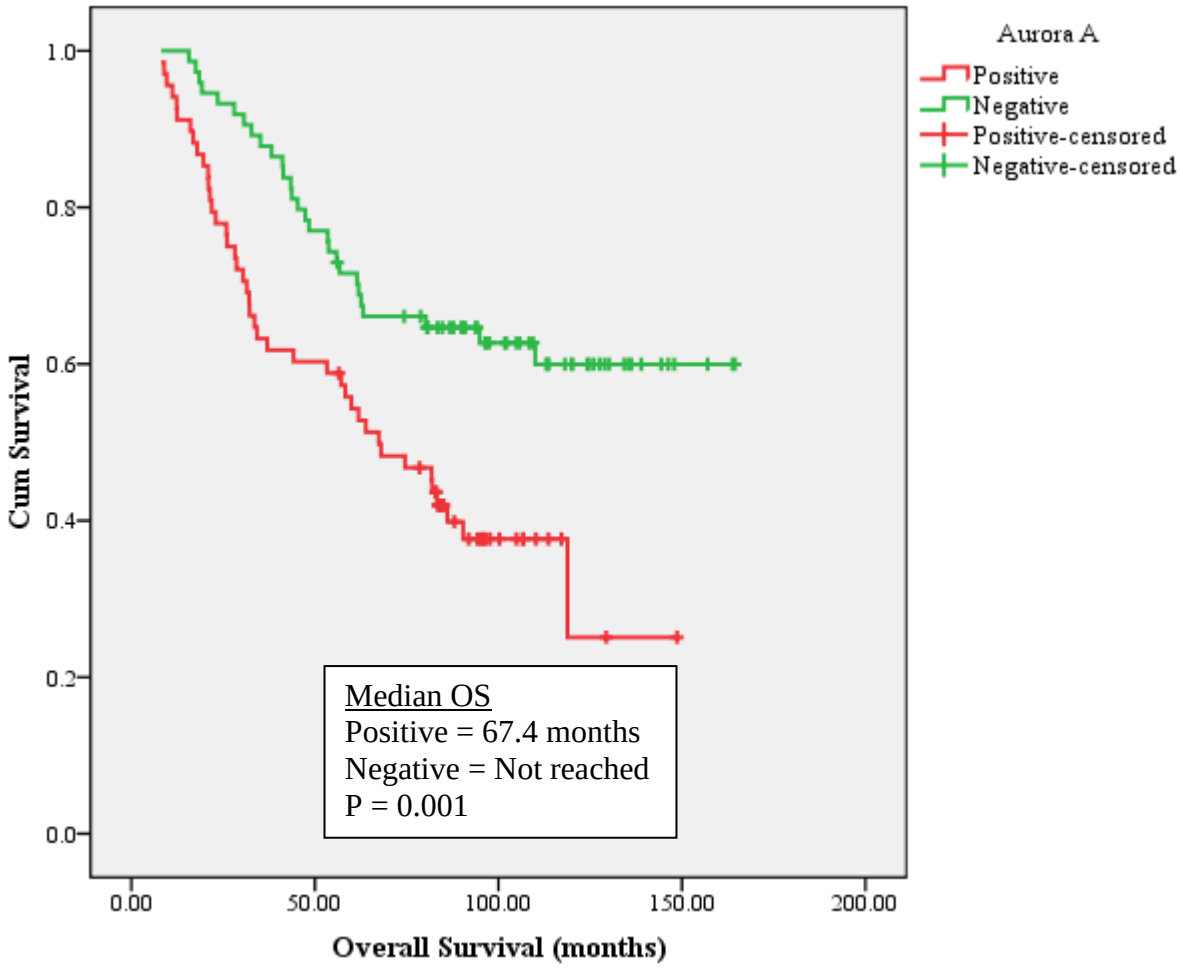



MCM-2
Positive
Negative
Positive-censored
Negative-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
50.00
100.00
150.00
200.00
Overall Survival (months)
Median OS
Positive = Not reached
Negative = 63.1 months
P = 0.032

Aurora A

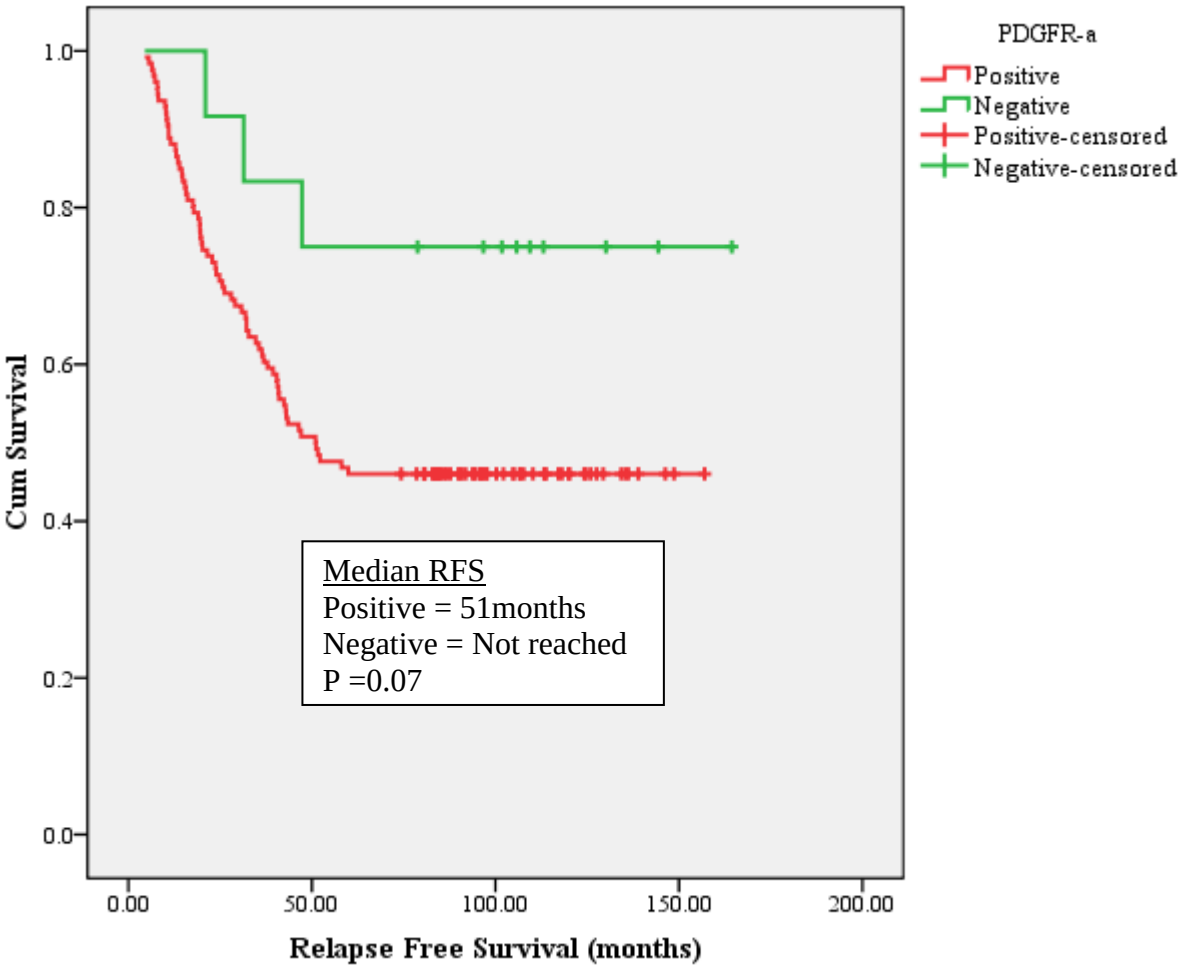
Aurora A negative expression was associated with better RFS (p = 0.001) and OS (p = 0.001).

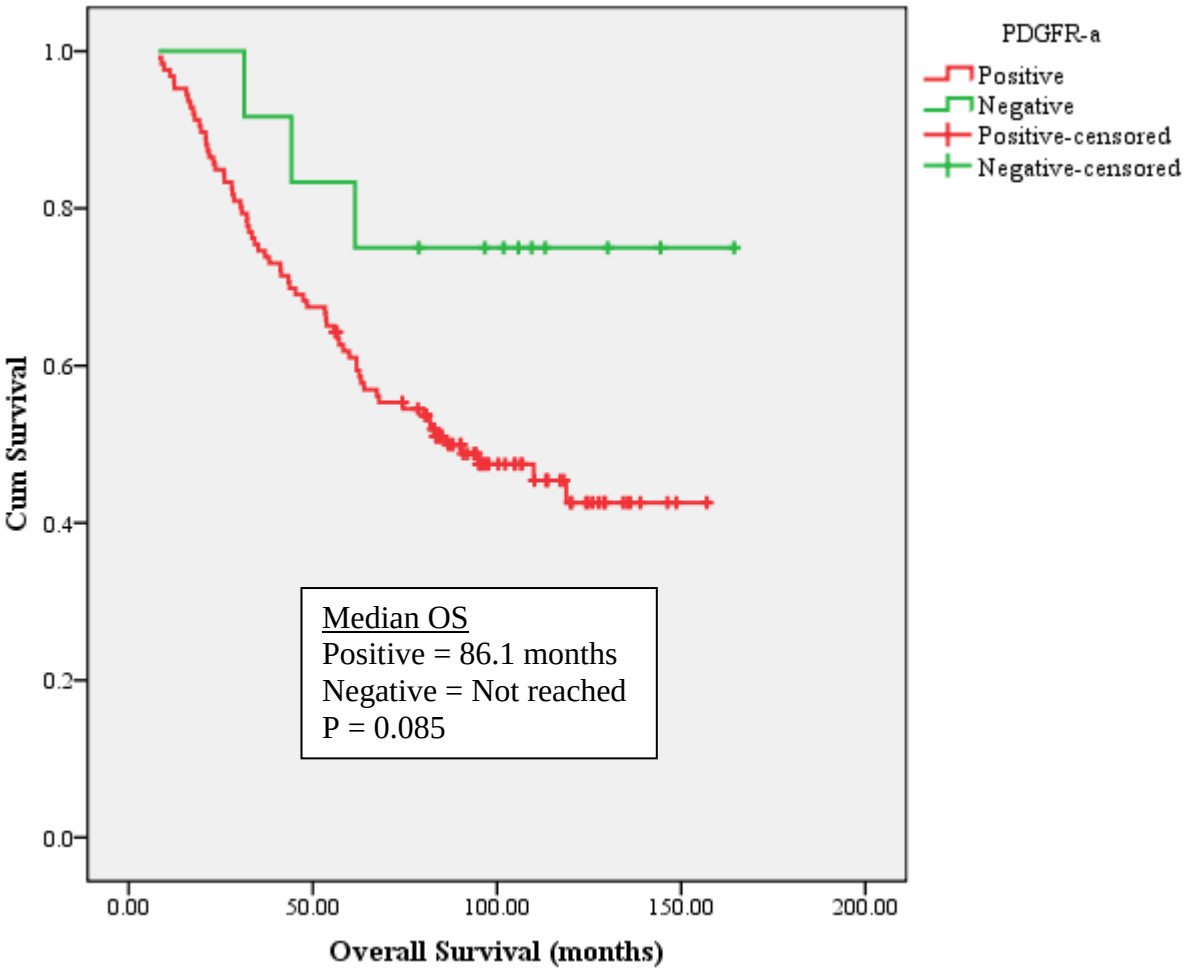



Aurora A
Positive
Negative
Positive-censored
Negative-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
50.00
100.00
150.00
200.00
Overall Survival (months)
Median OS
Positive = 67.4 months
Negative = Not reached
P = 0.001

PDGFR α

PDGFR α negative expression shows a trend for better RFS (p = 0.135) and OS (p = 0.085).




PDGFR-a
Positive
Negative
Positive-censored
Negative-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0.00
50.00
100.00
150.00
200.00
Overall Survival (months)
Median OS
Positive = 86.1 months
Negative = Not reached
P = 0.085

Binary Logistic regression analysis (Stepwise forward)

Binary logistic regression analysis was carried out for 5 years recurrence free status.

			Predicted		
			Patient group		
			Matched	Case	Percentage Correct
Step 1	Patient group	Matched control	32	25	56.1
		Case	10	48	82.8
	Overall Percentage				69.6
Step 2	Patient group	Matched control	40	17	70.2
		Case	17	41	70.7
	Overall Percentage				70.4
Step 3	Patient group	Matched control	42	15	73.7
		Case	10	48	82.8
	Overall Percentage				78.3
Step 4	Patient group	Matched control	48	9	84.2
		Case	15	43	74.1
	Overall Percentage				79.1
Step 5	Patient group	Matched control	47	10	82.5
		Case	12	46	79.3
	Overall Percentage				80.9
Step 6	Patient group	Matched control	52	5	91.2
		Case	17	41	70.7
	Overall Percentage				80.9

	B	S.E.	Wald	df	Sig.	Exp(B) (Hazard ratio)
PR +	-2.931	.728	16.205	1	**<.001**	.053
Bag-1 +	-1.685	.680	6.139	1	**.013**	.186
CD-68 +	-1.735	.665	6.810	1	**.009**	.176
MCM-2 +	-2.260	.714	10.031	1	**.002**	.104
Aurora A +	2.324	.725	10.268	1	**.001**	10.214
Nodal stage			12.928	3	**.005**	
N0	17.924	10529.041	<.0001	1	.999	60865215.432
N1	-3.014	.862	12.234	1	**<.001**	.049
N2	-2.975	.978	9.255	1	**.002**	.051
Constant	4.475	1.165	14.752	1	**<.001**	87.793

Binary logistic regression analysis showed that positive expressions of PR, Bag-1, CD-68, MCM-2, N1 and N2 were associated with lower risks with hazard ratios of 0.05, 0.18, 0.176, 0.104, 0.049 and 0.051 respectively and Aurora A positive expression was significantly associated with the increased risk (hazard ratio = 10.214) to have breast cancer recurrence in the first 5 years. The model has 91.2% specificity and 70.7% sensitivity.

Multivariate analysis (for cases and matched controls: total = 144 patients)

In Cox regression forward stepwise likelihood ratio analysis, N1 stage, positive MCM-2 and Bag-1 expressions are significantly associated with better RFS while Core Basal and positive Aurora A expression are associated with poor RFS. Similar pattern was found for the overall survival too. ER, PR, HER2, Ki-67, CK-5/6 and EGFR were not included in the model as they were used to classify molecular subtypes.

Relapse Free Survival

	B	SE	Wald	df	Sig.	Exp(B) (Hazard ratio)	95.0% CI for Exp(B)	
							Lower	Upper
Molecular subtypes			13.885	4	.008			
Luminal B	.843	.461	3.352	1	.067	2.324	.942	5.731
Core Basal	1.745	.498	12.290	1	<.001	5.726	2.159	15.191
HER2 enriched	.422	.491	.736	1	.391	1.524	.582	3.993
5-Negative	.488	.612	.636	1	.425	1.630	.491	5.411
Bag-1 +	-1.342	.386	12.075	1	.001	.261	.123	.557
MCM-2 +	-1.780	.413	18.575	1	<.001	.169	.075	.379
Aurora A +	1.251	.327	14.627	1	<.001	3.494	1.840	6.633
Grade			12.069	2	.002			
Grade 2	-1.871	1.144	2.675	1	.102	.154	.016	1.450
Grade 3	-.454	1.085	.175	1	.676	.635	.076	5.332
T stage			7.552	2	.023			
T2	-.089	.356	.063	1	.801	.914	.455	1.836
T3	1.280	.563	5.169	1	.023	3.596	1.193	10.839
Nodal stage			20.766	3	<.001			
N1	-1.188	.407	8.502	1	.004	.305	.137	.678
N2	-.958	.509	3.542	1	.060	.384	.141	1.040
N3	.396	.471	.709	1	.400	1.487	.591	3.740

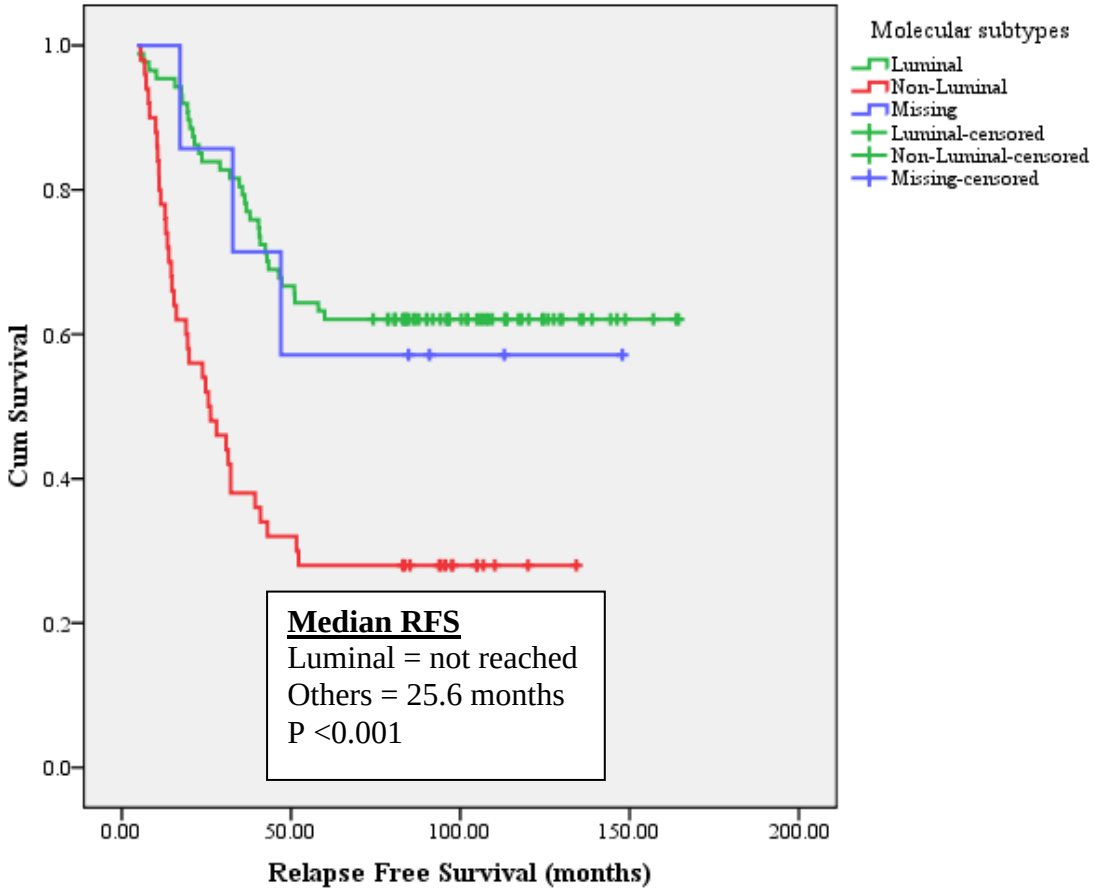
Overall Survival

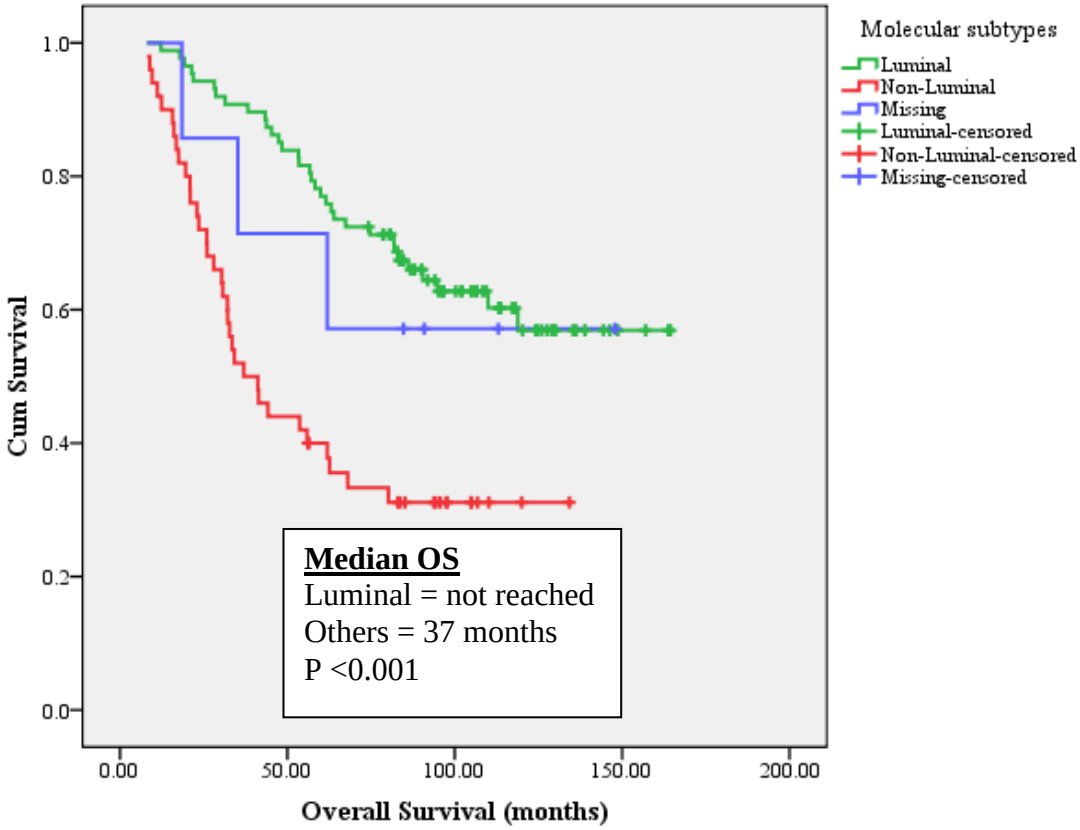
	B	SE	Wald	df	Sig.	Exp(B) (Hazard ratio)	95.0% CI for Exp(B)	
							Lower	Upper
Molecular subtypes			23.316	4	<.001			
Luminal B	.864	.438	3.889	1	.049	2.374	1.005	5.604
Core Basal	2.424	.523	21.522	1	<.001	11.293	4.055	31.449
HER2 enriched	1.144	.449	6.486	1	.011	3.140	1.302	7.576
5-Negative	2.043	.584	12.219	1	<.001	7.713	2.453	24.251
Bag-1 +	-.691	.327	4.462	1	.035	.501	.264	.951
CD-68 +	-.787	.328	5.744	1	.017	.455	.239	.866
MCM-2 +	-.960	.357	7.211	1	.007	.383	.190	.772
Aurora A +	.971	.311	9.757	1	.002	2.640	1.436	4.853
Nodal stage			13.101	3	.004			
N 1	-.894	.424	4.436	1	.035	.409	.178	.940
N 2	-.626	.532	1.384	1	.239	.535	.188	1.518
N 3	.393	.487	.649	1	.420	1.481	.570	3.849

E. Luminal (LA and LB) vs. Non-Luminal subtypes (H, CB, 5-N)

To investigate the effect of biomarkers independently of well recognised oestrogen receptor positivity, survival outcomes between luminal and non-luminal cancers were compared with Kaplan Meyer curves, and Cox regression multivariate analysis was carried out separately for both groups.

Luminal cancers have less recurrence patients compared to non-luminal cancers – 37.9% vs. 72%; $p < 0.001$. The luminal cancers have much better RFS and OS than non-luminal cancers as shown in the Kaplan Meyer curves ($p < 0.001$).





Luminal (ER/PR positive) vs. Non luminal (ER/PR negative) cancers

Pearson Chi square test p < 0.001			Molecular subtypes		Total
			Luminal	Non-Luminal	
Patient group	Matched control	Count	54	14	68
		% within Molecular subtypes	62.1%	28.0%	49.6%
	Case	Count	33	36	69
		% within Molecular subtypes	37.9%	72.0%	50.4%
Total		Count	87	50	137
		% within Molecular subtypes	100.0%	100.0%	100.0%

Cox regression analysis for Luminal cancers

Ki-67, Bag-1, MCM-2, Aurora A, grade, size and nodal stage are associated with RFS, and CK-5/6, Bag-1, CD-68, MCM-2, Aurora A, grade, size and nodal stage are associated with OS. These are statistically significant. Hazard ratios are shown in the table. ER and PR expressions are not included in the model as all the patients are ER and/or PR positive.

Cox regression analysis for RFS on Luminal cancers

	B	SE	Wald	df	Sig.	Exp(B) (Hazard ratio)	95.0% CI for Exp(B)	
							Lower	Upper
HER2 +	-1.002	1.256	.636	1	.425	.367	.031	4.309
EGFR+			.	0[a]	.			
Ki-67 +	1.745	.855	4.161	1	**.041**	5.724	1.071	30.599
CK-5/6 +	-.696	.564	1.523	1	.217	.499	.165	1.506
Bcl-2 +	.781	.856	.832	1	.362	2.184	.408	11.697
Bag-1 +	-1.988	.661	9.037	1	**.003**	.137	.037	.501
CD-68 +	-.263	.464	.322	1	.570	.768	.309	1.909
CD-71 +	-.663	.622	1.135	1	.287	.515	.152	1.745
MCM-2 +	-3.044	.907	11.255	1	**.001**	.048	.008	.282
Aurora A +	1.734	.580	8.936	1	**.003**	5.661	1.817	17.640
PDGFR α +	1.450	1.149	1.593	1	.207	4.264	.448	40.545
Grade			9.367	2	.009			
Grade 2	-3.168	1.475	4.612	1	.032	.042	.002	.758
Grade 3	-1.228	1.304	.886	1	.346	.293	.023	3.773
T stage			6.266	2	.044			
T2	.335	.623	.288	1	.591	1.398	.412	4.743
T3	2.860	1.174	5.938	1	.015	17.456	1.750	174.138
Nodal Stage			9.016	3	.029			
N1	-3.399	1.534	4.911	1	.027	.033	.002	.675
N2	-2.845	1.549	3.373	1	.066	.058	.003	1.210
N3	-1.770	1.524	1.349	1	.245	.170	.009	3.376

Cox regression analysis for OS for Luminal cancers

	B	SE	Wald	df	Sig.	Exp(B) (Hazard ratio)	95.0% CI for Exp(B)	
							Lower	Upper
HER2 +	.191	1.099	.030	1	.862	1.211	.141	10.430
EGFR+			.	0[a]	.			
Ki-67 +	1.080	.820	1.735	1	.188	2.945	.590	14.694
CK-5/6 +	-1.248	.608	4.220	1	**.040**	.287	.087	.944
Bcl-2 +	1.145	.842	1.850	1	.174	3.142	.604	16.358
Bag-1 +	-2.051	.740	7.679	1	**.006**	.129	.030	.549
CD-68 +	-1.014	.487	4.327	1	**.038**	.363	.140	.943
CD-71 +	-.354	.576	.377	1	.539	.702	.227	2.172
MCM-2 +	-1.865	.781	5.708	1	**.017**	.155	.034	.715
Aurora A +	1.826	.628	8.470	1	**.004**	6.211	1.815	21.247
PDGFR α +	1.367	1.162	1.385	1	.239	3.924	.403	38.237
Grade			5.070	2	.079			
Grade 2	-3.153	1.508	4.374	1	.036	.043	.002	.820
Grade 3	-2.144	1.376	2.427	1	.119	.117	.008	1.739
T stage			4.051	2	.132			
T2	.433	.638	.460	1	.498	1.542	.441	5.389
T3	2.173	1.080	4.050	1	.044	8.784	1.058	72.907
Nodal Stage			15.537	3	.001			
N1	-4.307	1.467	8.619	1	.003	.013	.001	.239
N2	-3.931	1.534	6.566	1	.010	.020	.001	.397
N3	-1.922	1.435	1.794	1	.180	.146	.009	2.437

Cox regression analysis for Non-Luminal cancers

Cox regression analysis was performed for Non-luminal type cancers. CK-5/6, Bg-1, MCM-2, Aurora A, grade and nodal stage are associated with RFS, and MCM-2 and Aurora A are associated with OS at statistically significant level. CK-5/6 and Aurora A are bad prognostic markers. ER and PR expressions are not included in the model as all the patients have both ER and PR negative.

Cox regression analysis for RFS for Non-luminal cancers

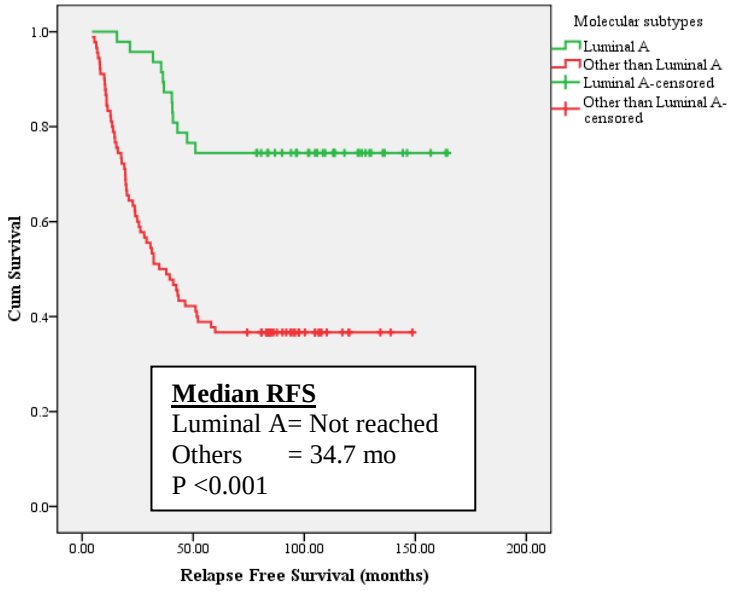
	B	SE	Wald	df	Sig.	Exp(B) (Hazard ratio)	95.0% CI for Exp(B)	
							Lower	Upper
HER2 +	-.852	.500	2.898	1	.089	.427	.160	1.138
EGFR+	.584	.935	.389	1	.533	1.792	.287	11.204
Ki-67 +	.534	.773	.477	1	.490	1.705	.375	7.759
CK-5/6 +	1.462	.685	4.560	1	.033	4.315	1.128	16.510
Bcl-2 +	-.212	.584	.131	1	.717	.809	.258	2.540
Bag-1 +	-1.244	.555	5.018	1	.025	.288	.097	.856
CD-68 +	-.184	.750	.060	1	.806	.832	.191	3.619
CD-71 +	-.181	.605	.090	1	.765	.834	.255	2.730
MCM-2 +	-1.765	.796	4.914	1	.027	.171	.036	.815
Aurora A +	1.307	.591	4.895	1	.027	3.695	1.161	11.762
PDGFR α +	.595	1.565	.145	1	.704	1.813	.084	38.930
Grade	2.146	1.364	2.475	1	.116	8.553	.590	124.000
T stage			.894	2	.639			
T2	.281	.806	.121	1	.728	1.324	.273	6.419
T3	.861	.944	.832	1	.362	2.365	.372	15.036
Nodal Stage			11.438	3	.010			
N1	-1.402	.733	3.663	1	.056	.246	.059	1.034
N2	-1.225	.968	1.603	1	.205	.294	.044	1.957
N3	1.041	1.000	1.085	1	.298	2.833	.399	20.101

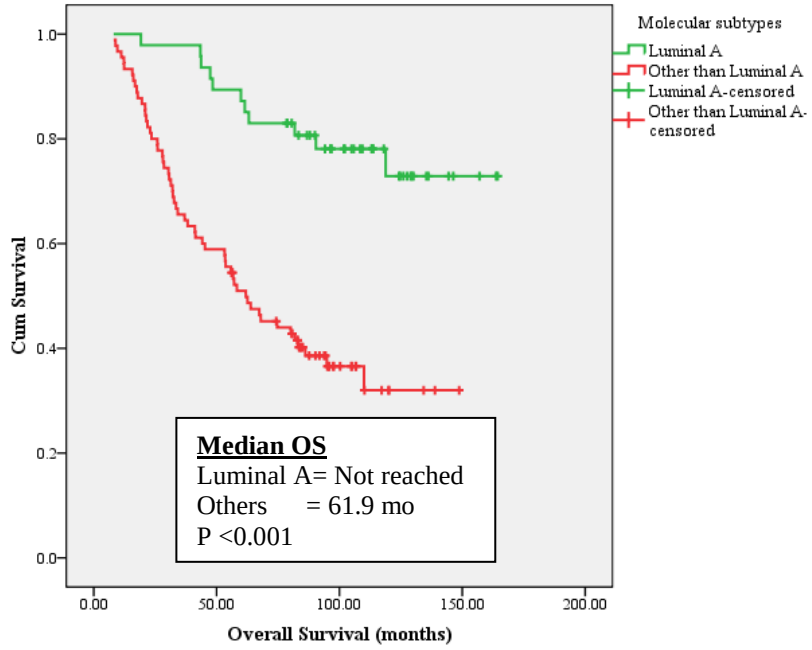
Cox regression analysis for OS for Non-luminal cancers

	B	SE	Wald	df	Sig.	Exp(B) (Hazard ratio)	95.0% CI for Exp(B)	
							Lower	Upper
HER2 +	-1.529	.560	7.457	1	.006	.217	.072	.649
EGFR+	.286	.937	.093	1	.760	1.331	.212	8.343
Ki-67 +	.715	.751	.907	1	.341	2.044	.469	8.902
CK-5/6 +	.610	.565	1.165	1	.281	1.840	.608	5.566
Bcl-2 +	-.433	.604	.515	1	.473	.649	.199	2.117
Bag-1 +	-.823	.544	2.289	1	.130	.439	.151	1.275
CD-68 +	-1.141	.706	2.613	1	.106	.320	.080	1.274
CD-71 +	1.069	.668	2.562	1	.109	2.914	.786	10.793
MCM-2 +	-1.403	.653	4.609	1	.032	.246	.068	.885
Aurora A +	1.809	.670	7.302	1	.007	6.106	1.644	22.678
PDGFR α +	-.245	1.553	.025	1	.875	.783	.037	16.433
Grade	.887	1.120	.626	1	.429	2.427	.270	21.809
T stage			.345	2	.842			
T2	-.369	.698	.279	1	.597	.691	.176	2.718
T3	-.515	1.036	.247	1	.619	.598	.079	4.551
Nodal Stage			6.867	3	.076			
N1	-.041	.655	.004	1	.951	.960	.266	3.469
N2	-.309	.878	.124	1	.724	.734	.131	4.100
N3	2.153	.969	4.938	1	.026	8.607	1.289	57.459

F. Lumina A cancers vs. other subtypes

Luminal A subtype cancers have statistically significant better RFS and OS compared to other cancers. LA group had less recurrence within 5 years compared to other cancers (25.5% vs. 63.3%; p < 0.001). None of the protein expression was associated with RFS or OS at statistically significant level among Luminal A cancers. Kaplan Meyer and Cox regression analysis were done to compare LA against others.





Distribution of Luminal A and other subtypes among cases and matched controls

Pearson's Chi square test $p < 0.001$			Molecular subtypes		Total
			Luminal A	Other than Luminal A	
Patient group	Matched control	Count	35	33	68
		Expected Count	23.3	44.7	68.0
		% within Molecular subtypes	74.5%	36.7%	49.6%
	Case	Count	12	57	69
		Expected Count	23.7	45.3	69.0
		% within Molecular subtypes	25.5%	63.3%	50.4%
Total		Count	47	90	137
		Expected Count	47.0	90.0	137.0
		% within Molecular subtypes	100.0%	100.0%	100.0%

Cox Regression analysis for Luminal A and non-luminal A cancers

ER, PR, HER2 and Ki-67 expressions are not included in the model because these expressions are used to define Luminal A and non-luminal A tumours. There are no biomarkers that can predict RFS or OS at statistically significant level for Luminal A cancers. For non-luminal A cancers, CK-5/6 (HR = 2.2), Bag-1 (HR = 0.296), MCM-2 (HR = 0.124), Aurora A (HR = 3.9), N2 (HR = 0.313) are significantly RFS, and Bag-1 (HR = 0.37), MCM-2 (HR = 0.29), Aurora A (HR = 2.7) and N2 (HR = 0.385) are significantly predictive of overall survival.

Cox regression analysis for RFS for Luminal A cancers

	B	SE	Wald	df	Sig.	Exp(B) (Hazard ratio)	95.0% CI for Exp(B)	
							Lower	Upper
EGFR +			.	0	.			
CK-5/6 +	.155	.857	.033	1	.856	1.168	.218	6.264
Bcl-2 +	-.759	1.703	.198	1	.656	.468	.017	13.193
Bag-1 +	-2.191	1.629	1.809	1	.179	.112	.005	2.724
CD-68 +	-.751	1.198	.393	1	.531	.472	.045	4.938
CD-71 +	.761	1.090	.487	1	.485	2.140	.253	18.109
MCM-2 +	-1.596	1.471	1.178	1	.278	.203	.011	3.619
Aurora A +	1.882	1.275	2.180	1	.140	6.567	.540	79.882
PDGFR α +	1.771	1.393	1.617	1	.204	5.876	.383	90.046
Grade			1.826	2	.401			
Grade 2	-2.065	1.797	1.320	1	.251	.127	.004	4.294
Grade 3	-.735	1.452	.256	1	.613	.479	.028	8.260
T stage			2.498	2	.287			
T2	.675	.964	.490	1	.484	1.964	.297	12.997
T3	3.325	2.113	2.477	1	.116	27.796	.442	1747.127
N stage			2.032	2	.362			
N2	-.135	1.340	.010	1	.920	.873	.063	12.081
N3	1.354	1.133	1.427	1	.232	3.873	.420	35.703

Cox regression analysis for OS for Luminal A cancers

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
EGFR +			.	0	.			
CK-5/6 +	-.689	.997	.477	1	.490	.502	.071	3.545
Bcl-2 +	-.680	2.295	.088	1	.767	.506	.006	45.458
Bag-1 +	-1.448	1.750	.685	1	.408	.235	.008	7.252
CD-68 +	-.808	1.382	.342	1	.559	.446	.030	6.690
CD-71 +	-.484	1.282	.142	1	.706	.617	.050	7.603
MCM-2 +	-1.958	1.878	1.087	1	.297	.141	.004	5.603
Aurora A +	1.698	1.412	1.445	1	.229	5.460	.343	86.927
PDGFR α +	1.191	1.384	.740	1	.390	3.289	.218	49.571
Grade			1.871	2	.392			
Grade 2	-3.267	2.398	1.857	1	.173	.038	.000	4.188
Grade 3	-2.110	1.880	1.260	1	.262	.121	.003	4.831
T stage			1.383	2	.501			
T2	.898	1.352	.442	1	.506	2.456	.174	34.745
T3	2.653	2.297	1.334	1	.248	14.191	.157	1280.276
Noda stage			2.599	2	.273			
N2	-.426	1.730	.061	1	.806	.653	.022	19.393
N3	1.486	1.249	1.416	1	.234	4.418	.382	51.052

Cox regression analysis for RFS for cancers other than LA

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
EGFR+	.515	.617	.695	1	.404	1.674	.499	5.614
CK-5/6+	.803	.385	4.362	1	.037	2.232	1.051	4.743
Bcl-2+	.249	.403	.382	1	.537	1.283	.582	2.828
Bag-1+	-1.216	.431	7.965	1	.005	.296	.127	.690
CD-68+	.161	.404	.160	1	.689	1.175	.533	2.592
CD-71+	-.408	.369	1.221	1	.269	.665	.323	1.371
MCM-2+	-2.090	.506	17.061	1	<.001	.124	.046	.333
Aurora A+	1.362	.399	11.673	1	.001	3.902	1.787	8.522
PDGFR α+	.658	1.299	.257	1	.612	1.932	.152	24.633
Grade	1.527	.495	9.503	1	.002	4.604	1.744	12.157
T stage			4.104	2	.128			
T2	-.438	.380	1.332	1	.248	.645	.307	1.358
T3	.714	.615	1.348	1	.246	2.041	.612	6.807
N stage			13.122	3	.004			
N2	-1.161	.468	6.148	1	.013	.313	.125	.784
N3	-1.091	.673	2.627	1	.105	.336	.090	1.257

Cox regression analysis for OS for cancers other than LA

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
EGFR+	.505	.619	.665	1	.415	1.657	.492	5.577
CK-5/6+	.209	.370	.318	1	.573	1.232	.597	2.545
Bcl-2+	.082	.413	.039	1	.843	1.085	.483	2.438
Bag-1+	-.995	.432	5.291	1	.021	.370	.158	.863
CD-68+	-.331	.375	.780	1	.377	.718	.345	1.497
CD-71+	-.109	.378	.084	1	.772	.896	.428	1.879
MCM-2+	-1.237	.419	8.712	1	.003	.290	.128	.660
Aurora A+	1.005	.396	6.448	1	.011	2.732	1.258	5.935
PDGFR α+	-.082	1.306	.004	1	.950	.921	.071	11.900
Grade	.760	.451	2.840	1	.092	2.139	.883	5.177
T stage			1.841	2	.398			
T2	-.508	.387	1.721	1	.190	.602	.282	1.285
T3	-.091	.720	.016	1	.900	.913	.222	3.748
N stage			6.056	3	.109			
N2	-.956	.476	4.028	1	.045	.385	.151	.978
N3	-.789	.676	1.363	1	.243	.454	.121	1.708

Recurrence Risk and relapse free survival among Luminal A patients

Among the LA patients the relationship between Adjuvant, OPTION RRs and relapse free survival are evaluated by scattered plot, independent 2 samples student t test and Pearson correlation test. Although the cases have higher mean Adjuvant RR than the controls, the difference was not statistically significant. The correlation between Adjuvant! RR and RFS did not show any statistical significance either. Mean OPTION 5 years RRs before and after adjuvant treatments were higher for cases than controls and the differences were statistically significant (p = 0.013 & 0.017, respectively). The correlation between RFS and OPTION 5 years RR before adjuvant treatment was significant but after adjuvant treatment was not statistically significant.

Luminal A cancers and recurrence risk analysed by independent 2 samples t test

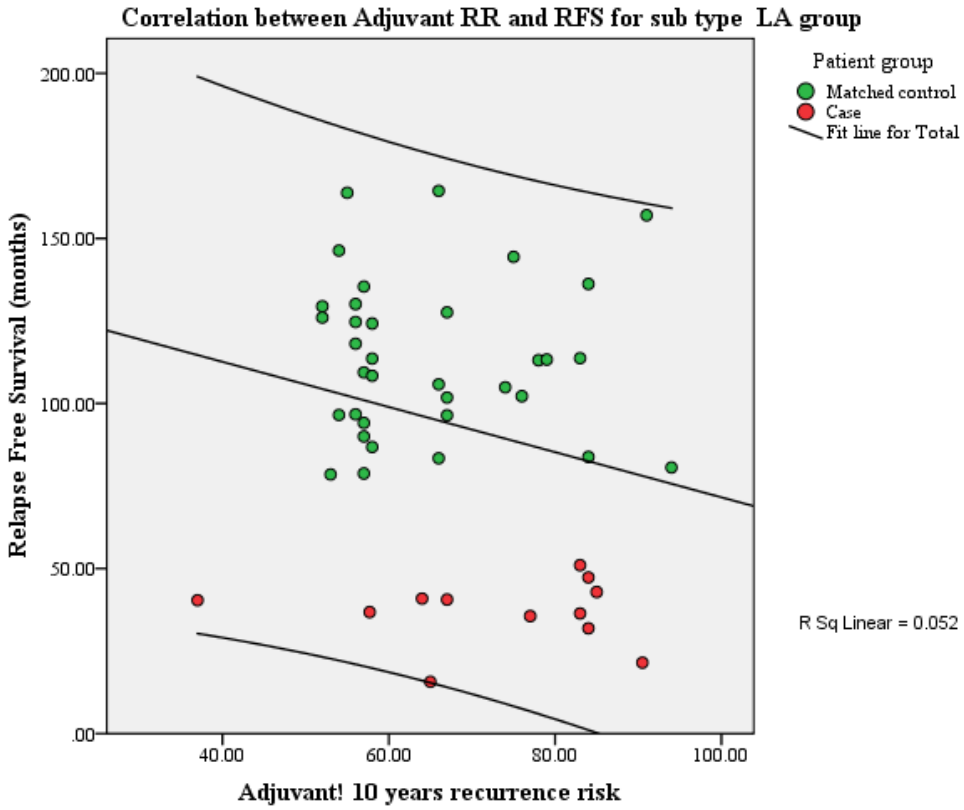
	Patient group	N	Range	Median	Mean	Std. Deviation	Std. Error Mean	P*
Adjuvant! 10 years recurrence risk	Matched control	35	52 - 94	58	65.0857	12.01561	2.03101	0.071
	Case	12	37 – 90.5	80	73.1000	15.43084	4.45450	
OPTION 5 years recurrence risk	Matched control	35	40 - 87	58	59.0000	12.72330	2.15063	**0.013**
	Case	12	52 - 86	71	69.8333	11.90747	3.43739	
OPTION 5 years recurrence risk after adjuvant treatments	Matched control	35	12 - 47	25	26.7714	9.8431	1.6637	**0.017**
	Case	12	14 - 54	37	35.4167	11.8893	3.4321	

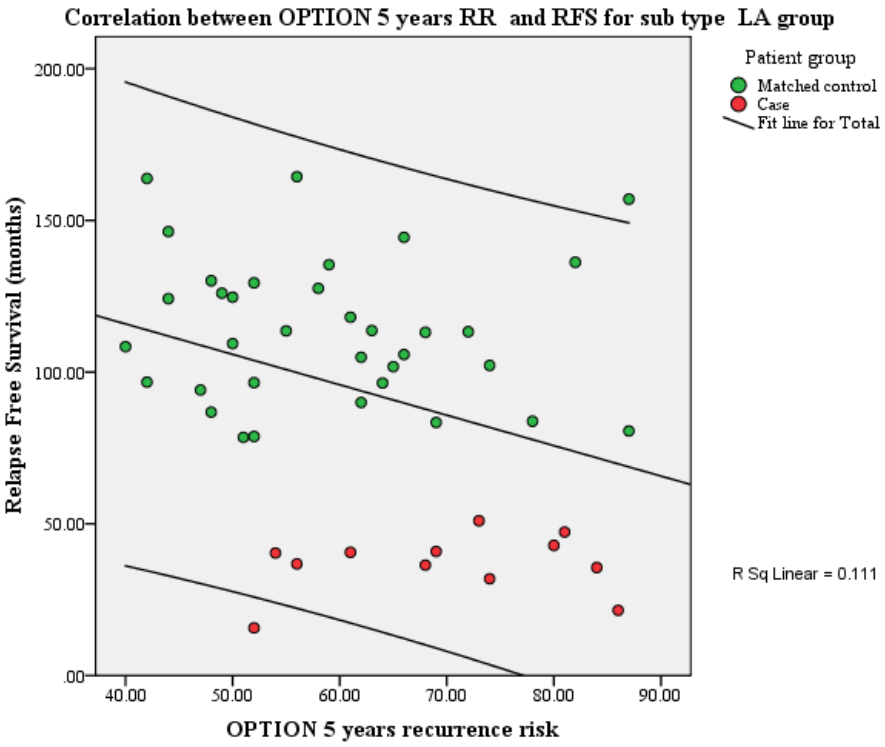
*Equal variance is assumed for calculation of p value.

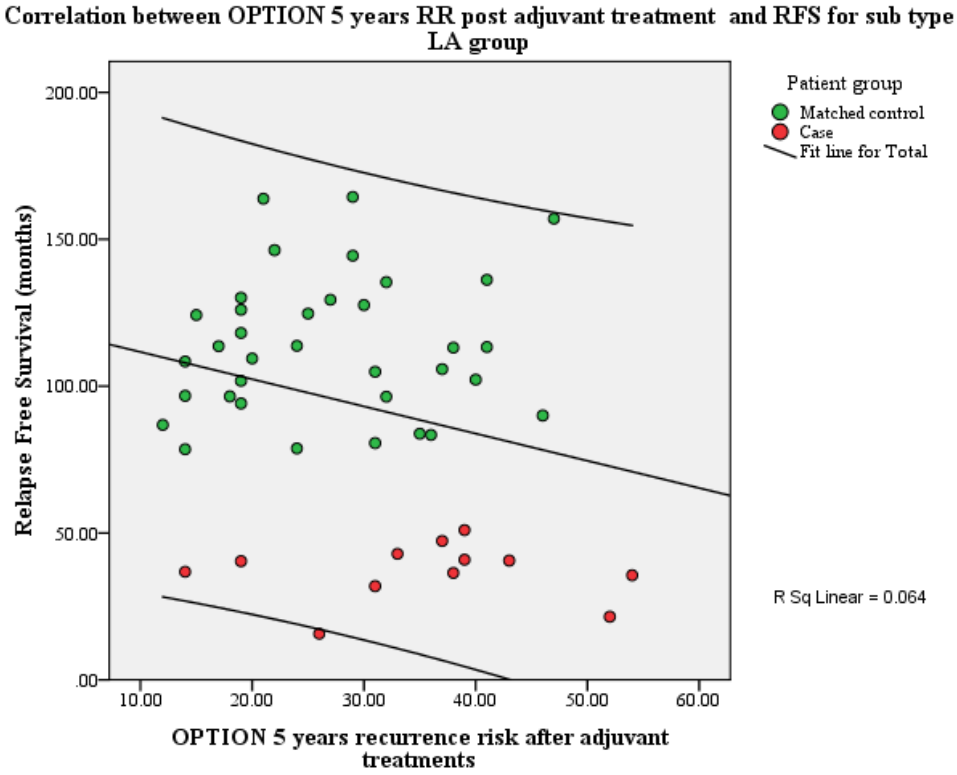
Pearson's correlation test for RFS and RR for LA cancers

	Pearson correlation	P value (2 tailed test)
Adjuvant! 10 years RR	-0.227	0.125
OPTION 5 years RR (before adjuvant treatment)	-0.333	0.022
OPTION 5 years RR (after adjuvant treatments)	-0.254	0.085

Below are the scattered dot plots showing relationships between recurrence risks and RFS for Luminal A patients. The best fit line and 95% confidence interval lines are also shown. 95% confidence interval is much narrower than that of the whole matched case control cohort that contains all molecular subgroups. This suggests the relationship between RRs and RFS is stronger for LA group than for all groups combined.




Correlation between OPTION 5 years RR and RFS for sub type LA group
Patient group
Matched control
Case
Fit line for Total
R Sq Linear = 0.111
Relapse Free Survival (months)
200.00
150.00
100.00
50.00
.00
40.00
50.00
60.00
70.00
80.00
90.00
OPTION 5 years recurrence risk


Correlation between OPTION 5 years RR post adjuvant treatment and RFS for sub type LA group
Patient group
Matched control
Case
Fit line for Total
R Sq Linear = 0.064
Relapse Free Survival (months)
200.00
150.00
100.00
50.00
.00
10.00
20.00
30.00
40.00
50.00
60.00
OPTION 5 years recurrence risk after adjuvant treatments

10. Discussion

In this study the "cases" and the "controls" are matched according to the Adjuvant recurrence risk (RR) which is calculated based on the known risk factors of tumour size, nuclear grade, nodal stage and oestrogen receptor expression. Adjuvant! RR is routinely used in today's clinical practice to make decisions on adjuvant chemotherapy and endocrine therapy. At the time of patients selection, the mean Adjuvant! RR was similar between the cases and the controls and no statistically significant correlation could be established between survivals and Adjuvant RRs. This would suggest that Adjuvant RR scoring had limited if any benefit when used alone to identify those patients for whom adjuvant therapy was ineffective within this series. The assessment of a set of specific proteins by IHC analysis has been used within this thesis to identify five different molecular subtypes (as described in previous publications) and it greatly improves the prediction of RFS and OS in a clinically valid manner. Of the 5 subtypes; Luminal A (LA) has the best RFS followed by Luminal B (LB), HER2 enriched (H), 5 negative (5-N) and Core Basal types (CB). LA again has the best OS followed by LB, H, CB and 5-N. This is in agreement with other publications. (Cheang, 2008b) However in the multivariate analysis, CB has the worst hazard ratio for RFS and OS among the five molecular subtypes but the 95% CIs for CB, H and 5-N overlap each other and therefore the statistical significance is not achieved. This may simply relate to a small sample size. The survival benefit for LA over other subtypes seen in this study is statistically highly significant.

In univariate analysis, **positive** expressions for ER, PR, Bag-1, Bcl-2, MCM-2, **negative** expressions for Ki-67 and Aurora A are correlated with better RFS. **Positive** expressions for HER2, EGFR, CK-5/6, CD-71 and PDGFR-α were associated with poor RFS as described in other publications although a statistically significant level was not achieved in this study.

Eight molecular proteins (ER, PR, HER2, Ki-67, Bcl-2, Bag-1, Aurora A, CD-68) whose genes expression s are used in the Oncotype Dx test for the calculation of recurrence scores show similar impact on the survivals (better or worse) although it is statistically significant only for ER, PR, Bcl-2 and Bag-1. Because of unsuccessful immunostainings for Cathepsin L2 and GSTM-1 proteins, IHC results were available for the proteins to represent only 5 out of 7 functional gene groups utilised in Oncotype Dx. Therefore it is not possible to comment if the IHC analysis (ie, protein level) of functional genes groups used by Oncotype Dx could produce similar informations for the disease recurrence risk.

Oestrogen receptor status was not available for some of the patients in the study groups at the start of the study (8.3% in cases, 11% in matched controls and 5.6% in low risk controls.). These were the patients treated in the late 1990's. For these patients, "ER unknown" status was used to calculate the Adjuvant RR for matching purposes. This would assign lower than actual RR for true ER negative patients and higher than actual risk for true ER positive patients. The matching for the cases and controls was done purely based on the Adjuvant RR regardless of the ER status. As a result, it was identified that there were more ER/PR positive patients in the control group and more ER/PR negative patients in the cases group. ER/PR negative patients are known to have early relapse (ie, in the first five years) and ER/PR positive patients are known to have a higher risk of late relapse after 8 years or longer period. (Blows, 2010) To avoid this bias from the higher positive ER/PR distribution in the controls group, the analysis was performed for **luminal** (LA and LB, ie. ER and/ or PR positive expressions using cut off level of Allred score 3) and **non-luminal** (CB, H and 5-N, ie, any cancers with negative ER and PR expressions) cancers.

Luminal cancers had statistically significant better RFS and OS than **non-luminal** cancers. This indicates that ER/PR expression is an important prognostic, predictive factor in this group of patients. There were 54 controls and 33 cases in the Luminal group. In multivatiate

analysis, **positive** expressions of Ki-67 and Aurora A, **negative** expressions of Bag-1, MCM-2, higher nuclear grade and nodal stage are correlated with poor RFS. This is in agreement with the already known fact that high proliferation index is a poor prognostic factor in ER/PR positive patients. (Cheang, 2009) Ki-67 (or HER2) positive tumours are classified as Luminal B tumours that have different risk level from other Luminal tumours (ie. Luminal A tumours) and have the prospect of benefiting from adjuvant chemotherapy. Surprisingly the poor prognostic effect of HER2 was not seen here. It may be due to the small number of HER2 positive patients in this group.

For **non-luminal** cancers, CK-5/6 was associated with **poor** RFS. Aurora A was associated with **poor** RFS and OS. Positive PR, MCM-2, Bag-1, lower T stage and nodal stage are associated with **better** RFS and OS. HER2 expression was associated with better overall survival. This means when ER/PR is negative, HER2 positive tumours will do better than HER2 negative tumours. Finding statistical significance for only OS but not for RFS suggests this effect may be due to the availability and use of anti-HER2 therapy for the metastatic disease within this study population or perhaps, statistical significance is not reached due to the small sample size. (Adjuvant anti-HER2 therapy was not available when the study population received its adjuvant therapies.) In the multivariate model **positive** MCM-2 and **negative** Aurora A were associated with **better** OS.

Luminal A patients are reportedly unlikely to gain benefit from adjuvant chemotherapy either because of their very good baseline prognosis or the fact that their low proliferation index will make them unresponsive to the chemotherapy. In this study, the univariate analysis with Kaplan Meyer curve showed that LA had statistically significant better RFS and OS than the other molecular subtypes (LA, H, CB, 5-N). However, within the LA group, 74.5% of patients are "controls" and 25.5% are "cases". In other words, 5 years disease recurrence rate for LA cancers in this study population is 25.5%. Theorectically, this figure could have been

higher if the adjuvant chemotherapy wasn't given. If the analysis is limited to the 35 LA patients who have OPTION RR >50%, the the recurrence rate will be 12/36 = 33.3%. Although this data is a lot higher than 5 years distant recurrence rate of 4.4% (node negative patients) – 15.5% (node positive patients) reported for LA tumours as determined by PAM50 gene expression measurement in a study involving patients treated with tamoxifen alone (53% of patiens had positive lymph nodes.) (Prat, 2012), it is similar to the 5 years recurrence rate of 29.6% among LA patients treated with the neoadjuvant chemotherapy for high risk features. (Vargo, 2011) The finding here suggests that the widely recognised notion of very good prognosis and the avoidance of adjuvant chemotherapy may not be applicable to Luminal A cancers that have OPTION or Adjuvant RR ≥ 50%. Mean and median OPTION RRs are statistically significantly higher for cases than controls. This statistically significant difference was not seen for the whole cohort with mixed subgroups as shown earlier in the results. This finding suggests that combination of molecular subgroup and RR will be more powerful than either of two alone to identify the high risk patients. Although more studies are required to define the optimal OPTION 5-year RR cut off point for better patient selections, it is reasonable to consider novel adjuvant therapies to LA cancers with OPTION or Adjuvant RR > 50% as none of the cases in the LA group had RR less than 50% except one patient who had Adjuvant RR 37%. RR >50% should also be considered as a stratification factor for LA cancers in the clinical trials. None of the proteins evaluated in this study showed any significant correlations with RFS or OS in the univariate or multivariate Cox regression analysis for Luminal A tumours. This suggests that new predictive markers are urgently needed to identify the approximately 25.5% of LA cancers with OPTION 5 years >40% or Adjuvant 10 years RR > 50% who would develop the disease recurrence within 5 years of adjuvant chemotherapy.

The predictive effect of proteins found in this study needs to be verified in a larger independent sample set – "validation set". These proteins expressions can be used for the stratification of patients in the future clinical trials. The trend for the poor prognostic effect of positive CD-71 expression is a new finding for these types of patients and was in agreement with a similar finding in patients treated with tamoxifen. (Habashy, 2010) EGFR, Ki-67, Aurora A and CD-71 had important biological functions in cancer cell growth signalling pathways and the response to the chemotherapy. The expression of these biomarkers should be considered to define a specific risk group for whom a clinical trial with novel agents could be designed. Also clinical trials for single or combination of novel agents to target these proteins should be considered. Aurora A is the protein involved in the cell cycle progression and was consistently shown to be associated with poor RFS and OS in every group analysis. Chemotherapy in combination with anti-Aurora A such as MLN 8054 (Macarulla, 2010) and Alisertib (Matulonis, 2012) that have been tested in Phase 1 and 2 trials should be evaluated in breast cancer patients with RR > 50%. Single agent Alisertib is currently in phase 2 clinical trial for metastatic solid tumours including breast cancer.

In agreement with other studies reported in the literature, the poor survival in this study was associated mainly with the expressions of proteins involved in the proliferation – Ki-67 and Aurora A. This is not surprising as proliferation has complex associations with treatment outcomes. Most of the chemotherapeutic agents such as cyclophosphamide, anthracycline, flurouracil and taxanes act as non-specific cell cycle or DNA toxins and therefore the main predictive marker would be the proliferation rate. High proliferation is an indicator for a better response to neoadjuvant chemotherapy but those who don't achieve a pCR are at the high risk of early disease recurrence and shorter survival. There were no similar parameters to predict pCR of micrometastases in the adjuvant setting although the estimate of failure rate can be worked out from the known risk factors. In patients treated with neoadjuvant

Docetaxel + Capecitabine chemotherapy, PAM-50 ROR-S scores that mainly reflect the proliferation genes expressions decreased in response to the chemotherapy and this change was correlated with the clinical response. (Korde, 2010) (Dumbier, 2011) Survival benefits from adjuvant endocrine therapy have been seen in patients only when the proliferation index falls significantly with the given treatment. "Preoperative Endocrine Prognostic Index" (PEPI), which is the Ki-67 index after the neoadjuvant endocrine treatment, is more predictive than the baseline Ki-67 for the long term survival for clinical stage 2 and 3 diseases. (Ellis, 2008) Decrease in Ki-67 expression in response to neoadjuvant anastrazole was about 75% in Luminal tumours compared to very little decrease in basal and HER2 enriched subtypes. Clinical response and surgical outcomes were similar in LA versus LB tumours; however, a PEPI of 0 (best prognostic group) was highest in the LA subset (27.1% v 10.7%; P = .004). (Ellis, 2011) In the untreated population, the prognostic impact of proliferation genes is limited to the ER+ HER2− subset since HER2+ or ER−HER2− subsets are associated with high proliferation activity. Therefore the clinical utility of most of the gene assays that largely depends on the expression of proliferative genes is mainly for the ER+ HER2− subset. There is no consensus at present on the gold standard method with a division between IHC or multigene assays, to assess proliferation status, but IHC is widely used in daily clinical practice mainly due to its lower cost.

Although, "overall survival" (OS) is regarded as the most important and meaningful end point in cancer care, "recurrence status" is the more appropriate end point to endorse the complete failure of the adjuvant chemotherapy. OS after the disease recurrence depends on the use of systemic therapies, best supportive care and patient's wishes for further active treatments. Therefore the measurement of the OS may not truly reflect the effectiveness of the adjuvant chemotherapy. However, the adjuvant chemotherapy may improve the OS by reducing the micrometastatic disease burden and delaying significant damages to vital organs.

Disease recurrence, in no doubt, indicates that the adjuvant chemotherapy did not manage to clear the micrometastases like other adjuvant therapies. However when a patient remains free of recurrence it is not possible to say if it is solely due to the adjuvant chemotherapy as there are many other possible factors such as:

1. No distant or locoregional micrometastases at the time of definitive curative surgery
2. Effect of other adjuvant therapies such as endocrine and anti-HER2 treatments while chemotherapy was actually ineffective in a particular patient
3. Combination effect of chemotherapy and other adjuvant therapies
4. Late disease recurrence that may occur after the study follow-up period.
5. Biology of individual tumour that actually dictates above mentioned factors

As routine investigations for visceral metastases are not recommended in current standard follow up practice, some patients in the "controls" group could be "patients with asymptomatic recurrent disease" at the time of the study. But 5 years breast cancer recurrence status is a very valid measure to evaluate the effectiveness of adjuvant chemotherapy because the breast cancer recurrence benefit from adjuvant chemotherapy is seen during years 0 – 4 although the mortality benefit is seen throughout the first decade. (EBCTCG, 2012) Therefore it is more reasonable to focus on identifying patients who develop a cancer recurrence rather than patients who remained recurrence free, to evaluate the effectiveness of the adjuvant chemotherapy.

More recently the focus has been made on the role of neoadjuvant chemotherapy to evaluate the effectiveness of the treatment and to find various predictive biomarkers that are associated with pCR which is a very strong indicator for long term survival. However pCR at primary tumour site doesn't mean pCR at micrometastatic site as there are patients who had disease recurrence despite achieving pCR of primary tumour. (Untch, 2011) This is also supported by the findings of pCR in the breast primary tumour but residual cancer cells in the

associated axillary lymph nodes and the negative impact of this on survival as a result (von Minckwitz, 2012). This could be due to the differing phenotype expressed between primary tumour cells population and the cells that had metastasised. This phenomenon is recognised and has been demonstrated by the finding of differing ER, PR, HER2 and other molecular proteins expression patterns between matched primary tumours cells, axillary nodes and distant metastases. Whilst these changes may occur under the influence of adjuvant therapies especially in the case of metachronous metastases, this may simply relate to the process of metastasis formation and sub clonal populations. (Sjöström-Mattson, 2009) (Bogina, 2011) (Park D, 2007) Intratumour genomic heterogeneity was recently reported in renal tumour samples with good and poor prognostic gene signatures being detected in different regions of the same tumour. (Gerlinger, 2012) In breast cancer, the discordance in expression was seen in up to 36% of patients for ER and up to 54.2% for PR, and the gain was less common than the loss at the metastatic site. (reviewed in Sari, 2010) It was also true for HER2 expression in most of the retrospective studies. (Sari, 2010) (Amir, 2008) Intratumor heterogeneity, associated with heterogeneous protein function, may foster tumour adaptation and therapeutic failure through Darwinian selection. (Gerlinger, 2012)

An increase of Ki-67 immunoreactive cells in matching axillary lymph nodes (ALN) compared with that of primary tumours (PT) was observed in 84% of cases (mean 17%; vs. 8%; $p<0.001$), whereas in 16% of the cases Ki-67 index was two to six times lower in the ALNs than in the corresponding PTs (mean 3.2% vs. 12.5%; $p<0.005$) according to a study involving 160 node positive breast cancer patients. The discordance between ALN and PT Ki 67 expressions was independent of the histology and the grade. (Cabibi, 2006) Destructive effect of neoadjuvant chemotherapy on neoplastic cells was seen to a lesser degree in the lymph nodes metastases compared to the primary tumour cells. (Koda, 2007b) Reduction in the expression of the Ki-67 and Bcl-2 by neoadjuvant chemotherapy was also relatively

smaller in ALN than the PTs indicating the different biology between the primary tumour and metastasised tumour cells that respond differently to the same chemotherapy. (Koda, 2007a)

"Matched case control" design was used here because it requires smaller sample size, lower cost, readily available follow up data and is less time consuming. It is however a challenge to have an exact matching between cases and controls because of the many known risk factors, which create a large number of stratification groups. To overcome this problem Adjuvant tool was used to estimate the overall risk based on a number of well recognised risk factors for the individual patient. Although the cases are defined as patients with breast cancer recurrence within 5 years, Adjuvant 10 year recurrence risk (RR) was used for matchingbecause Adjuvant! 5 years recurrence risk was not available. Each "control" was selected to have RR either higher than or equal to or not more than 10% points below that of the matched "case" to increase the probability of different survival outcome being due to the factors in question other than known risk factors. When OPTION tool from Oxford became available recently for 5 years RR, credibility of the matching was examined by OPTION 5 years RR using final ER/PR status from the study and this demonstrated that mean risks between the cases and controls were not significantly different. There was also no difference in OPTION 10 years RR. However, there were statistically significant differences in OPTION 5 and 10 years post adjuvant therapies RR between cases and controls, the latter having lower risk. This is mainly due to the risk reduction from the endocrine therapy as there were more patients with ER or PR positive tumours among controls. At the time of the patient selection, ER/PR status was not available for some of the the patients treated before year 2000 and "ER unknown" status was used to calculate RR. This would have assigned lower than the actual risk for patients that eventually turned out to be ER/PR negative but higher than the actual RR for the patients eventually turned out to be ER/PR positive. The post adjuvant therapy RR for some ER/ PR positive cancers could be lower than the actual risk because OPTION tool allocates same

amount of benefit for adjuvant endocrine therapy regardless of Allred score and either single ER or PR or both expression. Another reason is that OPTION gives higher benefit for aromatase inhibitors than tamoxifen and as a result controls who switch to AI in year 3 or 4 and who had extended endocrine therapy with AI were assigned much lower RR than the other patients who had tamoxifen only. The cases did not get this advantage because their cancers relapsed before they had an opportunity to switch to AIs. According to the published data, nearly half of postmenopausal women actually do not complete the recommended five years course of tamoxifen therapy and therefore the endocrine therapy benefit given here for full 5 years treatment could be an overestimate. (Owusu, 2008) To avoid this potential confounding effect, the analysis was done for ER/PR positive and negative patients separately. By analysing that way, however, the sample size gets smaller as a result and this could have reduced the power of the study and the chance of finding significant results. By assigning more than one control for each case could have overcome this problem but from the statistical point of view there is very little to be gained by including more than two controls per case. (Lewallen 1998) However, it proved difficult to find enough suitable controls because the chemotherapy was not very commonly used in patients whose recurrence free status was long enough. There was also an obstacle in identifying suitable tumour blocks to retrieve from the storage.

Tissue Micro Arrays analysis has revolutionised the exploratory translational research. This technique saves the labour, cost, time, invaluable limited cancer tissues and ensures optimised IHC standardized processing between different samples. The down side, however, is the loss of tissue cores during the processing. Loss of up to 5 – 10% cores has been reported for 0.6 mm cores. The loss can be minimised by using the bigger size of 1 – 2 mm cores or multiple cores for each patient. . The validity of TMA analysis in breast cancer was confirmed by the comparison with whole section analysis and two 0.6 mm cores yielding

comparable results for ER expression in more than 95% of cases, the figure rising to 99.5% when the core number was increased to 5. (Camp, 2000) Even one 0.6 mm core was reported to be sufficient to give results equal or even superior to the whole section for ER, PR and p53 in breast cancer. (Torhorst, 2001) Very good concordance between 3 TMA cores and whole slides analysis for ER, PR, HER 2 by both IHC and FISH had been reported (Thomson T, 2009, 2010) and large scale translational TMA studies had adopted 3 cores protocol. (Ali, 2013) (Bartlett, 2010) (van der Hage, 2011) (Cuzick, 2011) In the present study loss of cores ranged from 0% (Aurora A) to 21.6% (MCM-2) with an average of 10.9% for all antibodies. However the final expression result was not available only for maximum 5.6% of patients in the case of MCM-2 protein with an average of 1.8% for all proteins.

Immunohistochemistry was chosen as the method to evaluate this range of biomarkers because of a number of advantages such as its wide availability, relatively low cost, easy preservation of stained slides and preservation of morphology. However the disadvantages include different expression pattern from potential variability in technical issues such as tissue fixation in formaldehyde, tissue processing and embedding procedure in heated paraffin wax, storage condition and duration, intensity of antigen retrieval, type of antibody (polyclonal versus monoclonal), lack of a positive internal control signal and system control samples etc. As a standard for tissue fixation, neutral buffered formalin 10%, which contains 4% formaldehyde, is used to induce the formation of crosslinks between proteins or between proteins and nucleic acids involving hydroxymethylene bridges, masking the antigen-binding sites by altering the 3-dimensional structure of proteins. Such masking of epitopes needs to be reversed by several antigen retrieval methods before antigen antibody reaction can occur. Delay to fixation can cause proteolytic degradation and loss of immunoreactivity. Insufficient fixation time can cause incomplete process as the crosslinking is a slow process requiring 24 to 48 hours to complete. This could lead to coagulation fixation during the tissue dehydration

by alcohol resulting in a variable admixture of crosslinking and coagulation fixations that accounts for many of the observed variations in IHC. Therefore it has been recommended that the tissue is fixed within one hour from collection point in 10% neutral pH, phosphate-buffered formalin for a minimum of 6 hours to improve standardization and reliability of IHC. In invasive breast cancer, tissue fixation for a period just beyond 72 hours does not result in diminished sensitivity of ER, PR, or HER-2 IHC assays when compared with tissue fixed for a shorter period. (Tong, 2011) American Society for Clinical Oncology (ASCO) and College of American Pathologists (CAP) implemented guidelines for IHC processing for ER and PR (between 6 – 72 hours fixation time) and HER2 (at least for 6 hours but not more than 48 hours). (Wolff, 2007) (Yaziji, 2008) (Hanna, 2007) (Hammond, 2010) (Walker 2008) There was no similar guidance for other proteins included in this study. By using the samples stored in the pathology departments in the same cancer network and automated highly accurate IHC machine to process TMA slides reduce these confounding factors.

Another issue with IHC is the measurement of staining index and applying a semi-quantitative subjective slide scoring system to dichotomise the results for positive and negative expression. There is no consensus on which part of the whole tissue slide, which usually contains very heterogenous cancer tissue, should be selected for the scoring although this is not an issue for the 0.6mm core tissues on the TMA. There are well established scoring methods for ER, PR and HER2 but not for the other molecular proteins that have been studied extensively to date. Many published studies used different cut-off value to define positive/ negative or over/ under expression of a particular molecular protein. “X-tile” statistics, the median or mean value, “no or any staining” or “previously reported cut off value” have been used to define the cut-off point in various studies. Different cut off levels make it difficult to compile the results from different studies and make a valid conclusion.

On the other hand multi-gene assays are said to be more accurate and reproducible compared to IHC methods that do not use internal reference proteins for normalization. These assays are based on RNA analysis and the credibility of the assays depends on the good quality and quantity of RNA. Several factors, including prolonged time from excision to freezing or fixation and prolonged storage in formalin fixed paraffin blocks can produce wide variability in mRNA quality. Therefore high standard processing, storage, and preparation techniques are essential for the success of multigene assays. Dutch multi-institutional pilot study suggested that good quality RNA can be harvested from the material maintained in high salt fixative solutions (RNAlater; Ambion, Austin, TX) in over 95% of cases.

There are discordances between gene expression assays and IHC expression analysis. Of the 626 ER positive tumours analysed in a microarray test set, 73% were luminal (A or B), 11% were HER2 enriched, 5% were basal-like, and 12% were normal-like by gene analysis. The ER negative tumours comprised 11% luminal, 32% HER2-enriched, 50% basal like, and 7% normal-like. 64% and 6% of clinically HER2 positive cases are classified as HER2 enriched and basal subtypes, respectively, by gene analysis. 56%, 24% and 9% of clinically HER2 negative cases are reclassified as luminal, basal like and HER2 enriched cancers, respectively, by the gene analysis. (Parker, 2009) The similar discordance was found for dichotomised IHC expressions and RT-PCR gene analysis method for CK-5/6, CK-14 and CK-17. 14% of cases dichotomised based on quartiles and ROC as negative on IHC examination for CK-5/6 are found to have high CK-5 mRNA levels. (Kordek, 2010) Ki-67, whose expression contributes significantly to the positive recurrence score of Oncotype Dx, was found to overexpress in some of the patients in low risk group of the Oncotype Dx. (Gwin, 2009)

There are also discordances between risk assessments by multigene assays and clinicopathologic prognostic tools such as St. Gallen, National Institute of Health (US)

guidelines, OPTION and Adjuvant tool. “High risk” as determined by gene assay could be defined as “low risk” by clinicaophathologic tools/ guidelines and vice versa. (van de Vijver, 2002) (Campbell 2010) Analysis of 97 genes classifies breast cancers into 2 distinct molecular grades – Genomic Grade Index 1 and 2 which are strongly associated with histologic grade 1 and 3 respectively. (Sotiriou, 2003 & 2006) Histologic grade 2 tumours were thought to be a mixture of GGI 1 and 2. Classifying histologic grade 3 into 2 by IHC could be because of the loss of mitotic figures as mitosis managed to complete before the tumour specimen was fixed in the formalin.

Performances of 6 different multigene assays - Oncotype Dx recurrence score (GHI), PAM-50 ROR-S/P, Mammaprint (NKI70), Rotterdam 76 (ROT76), Genomic index of Sensitivity to endocrine therapy (SET) and oestrogen induced gene set (IE-IIE) - were tested in ER+ patients who had only adjuvant tamoxifen. All the assays mainly differentiate Luminal A subtypes from the other subtypes. The high hormonal sensitivity groups (SET-high and IE-like) and low risk of recurrence groups (PAM50-RORS-low, PAM50-RORP-low, GHI-low, ROT76-good and NKI-good) were largely composed of luminal A tumours (>71%–100%). All predictors identified groups of node-negative patients with 93.7%–97.9% and 88.4%–96.2% distant recurrence free survival at 5.0 and 8.5 years, respectively, although the number of patients in each group differed. (Prat, 2012) Multivariate analyses including two predictors at a time revealed that, in most cases, many of these correlated predictors, in particular the PAM50-RORP, GHI, NKI70 and SET, remained statistically independent of each other. The risk group assignment concordance among these predictors was found to be 36% for PAM50-RORP versus GHI, 54% for PAM50-RORP (low/medium versus high) versus NKI70 and 74% for GHI (low/intermediate versus high) versus NKI70. (Prat, 2012) Less than 25% of the genes were shared between signatures, except for 9 and 11 genes of the GHI signature (n = 21) that were present in the IE-IIE and PAM50, respectively, and 15 genes of the IE-IIE

signature that were present in PAM50. In spite of relatively little gene overlap, all predictors were significantly correlated with PAM50-RORS, IE-IIE and GHI showing the highest correlation between them. (Prat, 2012)

Intrinsic subtypes, 70-gene signature, wound response signature, and Recurrence Score were found to be highly concordant in classifying patients into low and high-risk groups. But combining these signatures did not yield a significant improvement in the predictive accuracy, suggesting that the prognostic information provided by these signatures is largely overlapping. (Fan, 2006) However, in a study with 295 breast cancer patients, wound response signature was detected in tumours of patients who died of breast cancer regardless of 70-genes poor prognosis signature status. Almost all of the basal-like tumours were found to express both 70-gene poor prognosis signature and the activated wound-response signature. (Chang, 2005) Patients with both the activated wound-response signature and the 70-gene poor prognosis signature had a risk of metastatic disease 6.4-fold higher than patients with 70-gene good prognosis signature with 10 years distant metastases free survival rate of only 47%, a good indication for most aggressive adjuvant chemotherapy. (Chang, 2005)

There have been reports on correlations between different molecular subtypes according to IHC or multi-genes based expression assays and the chemotherapy effectiveness. More than 20% improvement in 5 year OS and RFS from CEF over CMF was found in HER2 enriched tumours while the benefit was only 2% in other molecular subtypes. Within clinically defined HER2 + tumours, 79% were classified as the HER2 Enriched subtype by the gene expression and this subset was strongly associated with better response to CEF versus CMF (62% vs. 22%, $P < 0.001$). There was no significant difference in survival benefits between CEF and CMF in basal-like tumours. (Cheang, 2012)

In a study involving 62 breast cancer patients treated with neoadjuvant chemotherapy (paclitaxel followed by CAF) the basal-like and HER2 enriched subgroups were associated with the highest rates of pCR, 45% for both, while the luminal tumours had a pCR rate of only 6 %. No pCR was observed among the normal-like cancers. The molecular class was not independent of the conventional cliniocopathologic predictors of response such as ER status and the nuclear grade. None of the 61genes associated with pCR in the basal-like group were associated with pCR in the HER2 enriched group, suggesting that the molecular mechanisms of chemotherapy sensitivity may vary between these two ER negative subtypes. (Rouzier, 2005a) There were no studies to evaluate the effect of drug pharmacokinetics or cellular transmembrane transport system in individual patient for each molecular subtype. pCR (no residual disease in breast or lymph nodes) is suggested as a suitable surrogate end point for patients with luminal B/HER2-negative, HER2-positive (non-luminal), and triple-negative disease but not for those with luminal B/HER2-positive or luminal A tumours by a study that retrospectively evaluated 6,337 patients treated with the neoadjuvant chemotherapy. (von Minckwitz, 2012)

Using inexpensive IHC method for molecular protein expressions many studies have been carried out to reproduce the different risk levels defined by expensive multigenes assays such as Oncotype Dx test. A mitotic count score greater than 1 combined with a negative PR expression by IHC was shown to be predictive of an intermediate or high Oncotype DX recurrence risk group. (Auerbach, 2010) IHC4+C score (ER, PR, HER2, Ki-67 IHC expressions and clinicopathological features) was shown to give recurrence risk very similar to Oncotype Dx recurrence risk score in ER+ patients participated in an adjuvant endocrine trial that compared anastrozole against tamoxifen. (Cuzick, 2011) By applying IHC4+C score to intermediate risk group defined by Adjuvant! RR, 15 of the 26 patients was reclassified as low risk and no patient was reclassified as high-risk group. Of the 59 patients classified as

intermediate risk group by the NPI, 24 were reallocated to a low risk group and 13 to a high risk group.

In Oncotype Dx test, ER, PR, Bag-1 and Bcl-2 expressions contribute to the minus score (lower recurrence risk) while HER2, Ki-67, Aurora A and CD-68 expressions contribute to the plus score (higher recurrence risk). Findings in this study are in agreement with that pattern except CD-68 IHC expression (as tumour associated macrophage infiltration) which was correlated with good prognosis in this study, similar to better prognostic effect reported in the colorectal cancer. (Oberg, 2002) However the contribution by CD-68 expression to the Oncotype Dx recurrence score (+0.05 x CD-68 expression) was the smallest among all the other genes in the assay. (Paik, 2004) Paradoxically N0 stage was significantly associated with higher risk of disease recurrence in this study. This was probably because only node positive patients were selected for the control group while there were some node negative patients in the "case" group.

IHC was not successful for Cathepsin L-2, GSTM-1, Plk-1 and VEGFR-2 proteins. This was due to a number of factors including limited TMA tissues with core losses, time, financial constraints for consumables, techniques which needed further optimisation or the antibodies that are not considered specific enough. The other available methods such as heat induced epitope retrieval in Sodium citrate buffer or 1 mM EDTA buffer adjusted to pH 8 or Tris-EDTA buffer using either pressure cooker or microwave, enzymatic retrieval methods such as pipetting and immersion and different antibody dilution are worth trying for the successful IHC staining for these potential biomarkers, if circumstances had allowed.

Agendia has marketed many gene assays for clinical use. TargetPrint® and MammaPrint® can report ER, PR and HER2 expression status by single gene expression. BluePrint is an 80-gene expression signature that classifies breast cancer into **B**asal-type, **Lu**minal-type and **E**RBB2-type cancers. The BluePrint Molecular Subtyping Profile in combination with MammaPrint

test result provides a greater level of clinical information to assist in therapeutic decision-making. TheraPrint is a microarray-based gene expression panel of 56 genes that have been identified as potential targets for prognosis and predictor for response to therapeutic treatments. DiscoverPrint® is a tool for the development of companion diagnostics in clinical trials of oncology therapeutics for the biotechnology and pharmaceutical industry. (www.agendia.com)

Recently a new web based tool "www.recurrenceonline.com" has been launched. It is accessible online and available free of charge. It is based on analysing standard genome-wide microarrays and is able to compute varied prognostic parameters simultaneously. It supports only Affymetrix HGU133A and HGU133plus2 microarrays in raw CEL files format. This tool automatically evaluates uploaded microarray data to provide Oncotype Dx recurrence risk for node negative, ER positive patients, independent recurrence risk classification by using the 6 strongest genes for any nodal and ER status, 4 strongest genes for node negative ER positive patients, 3 strongest genes for node positive patients, and also expression of ER and HER2 receptors. The tool has been validated using data from 2,472 publicly available microarrays. (Gyorffy, 2012)

Samples of web pages for one step online interface for input (A) and result (B) of "Recurrence online" (This illustration was borrowed from the published article by Gyorffy, 2012)

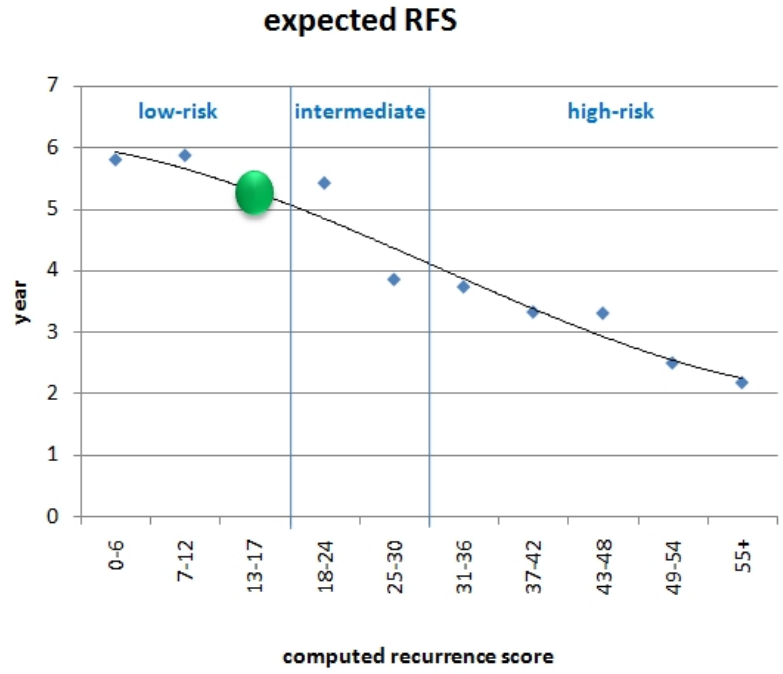
Recurrence Online

Determining the odds of breast cancer recurrence

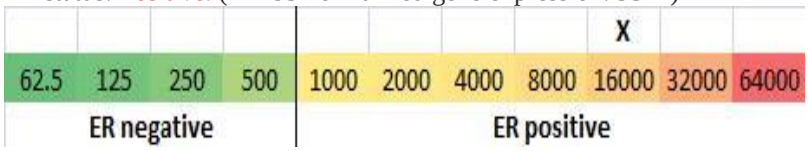
Patient ID: GSM177892.CEL
Lymph node status: Negative
Date: 2012-07-29
Microarray platform: HGU133A
Array Quality Test: passed

Risk category using strongest genes: low risk (mean expression: 540)
Category: lymph node negative ER positive
Genes used in the analysis: MELK,CDC2,TOP2A and PRC1

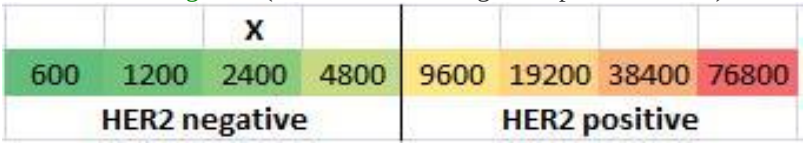
Computed Recurrence Score: 14 (low risk)
Eligibility Test: passed.
The recurrence score can be used to predict the probability of distant recurrence in patients with breast cancer who have estrogen-receptor positive tumors with no lymph nodes involved:



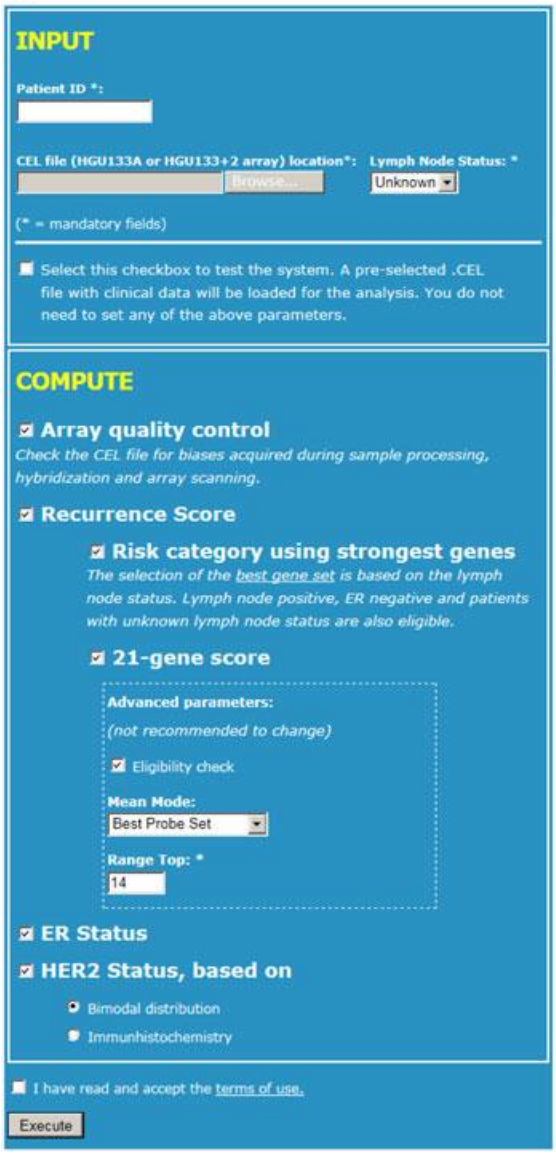
ER status: Positive. (MAS5 normalized gene expression: 9541)



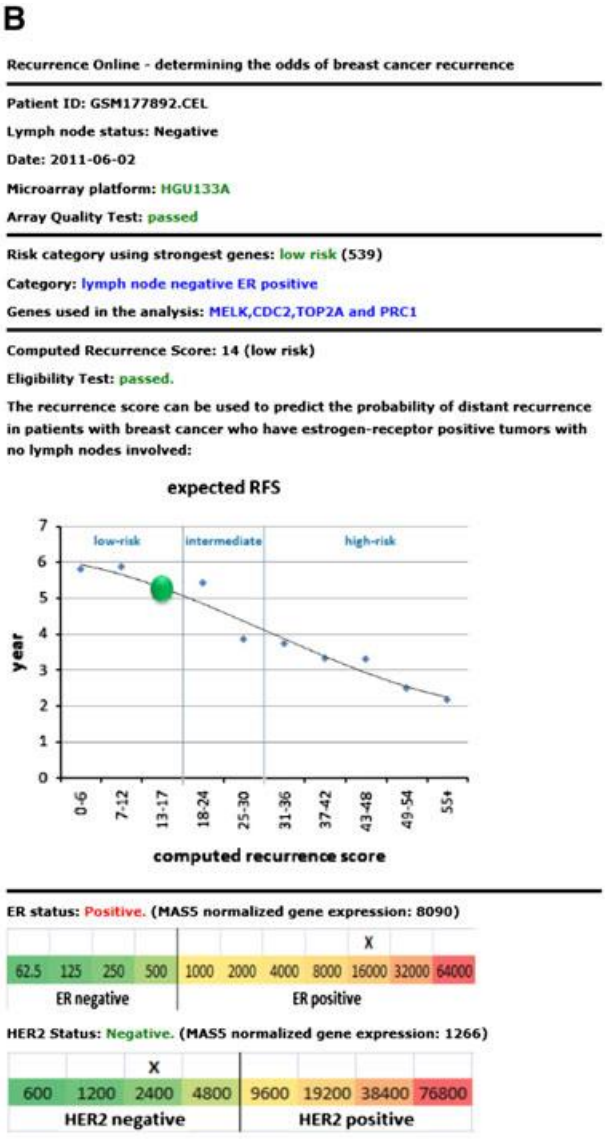
HER2 Status: Negative. (MAS5 normalized gene expression: 1493)



A



B



11. Conclusion

In conclusion this matched case control study has shown that molecular subtypes have predictive effect for survival and are superior to the clinical prognostic tools such as Adjuvant and OPTION in breast cancer patients with RR >50 who had adjuvant chemotherapy. However, in this population, Luminal A cancers have significantly higher recurrence risk despite adjuvant chemotherapy, which is a completely different finding to Luminal A patients found in other breast cancer population. Therefore, this would suggest that Luminal A patients with OPTION 5-year recurrence risk >50 should be offered adjuvant chemotherapy. No predictive markers could be found to predict the recurrence in this Luminal A group. New predictive markers/ factors are urgently needed to identify such patients. IHC expressions of proteins coded for by the genes used in the Oncotype Dx show similar positive/negative effect on the survival outcomes although statistically significance level is reached only for a few proteins. There was an opposite finding for CD-68 when it was analysed as an expression of macrophages. Aurora A expression is predictive of poor outcome regardless of ER/PR status and may be useful to identify patients suitable for evaluation of combined chemotherapy and Aurora A kinase inhibitors. As the gene assay based prognostic tools will, almost inevitably, become more affordable in the future, the integration of genes assay analysis, IHC analysis and clinicopathological parameters should help create better risk stratification and prediction of treatment outcome. In this way breast cancer adjuvant trials could be better informed grouping appropriate patients into appropriate therapies, leading to a significant improvement in breast cancer survival for the future.

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13. References

Akiyama T, Sudo C, Ogawara H, et al. The product of the human c-erbB-2 Gene: A 185-kilodalton glycoprotein with tyrosine kinase activity. Science: 232: 1644-1646. (1986)

Al Murri A, Hilmy M, Bell J, et al. The relationship between the systemic inflammatory response, tumour proliferative activity, T-lymphocytic and macrophage infiltration, microvessel density and survival in patients with primary operable breast cancer. Br J Cancer: 99: 1013 – 1019. (2008)

Albain K, Barlow W, Shak S, et al. Prognostic and predictive value of the 21-gene recurrence score assay in postmenopausal women with node-positive, oestrogen-receptor-positive breast cancer on chemotherapy: a retrospective analysis of a randomised trial. Lancet Oncol: 11: 1: 55-65. (2010)

Albain K, Paik S, van't Veer L. Prediction of adjuvant chemotherapy benefit in endocrine responsive, early breast cancer using multigene assays. Breast: 18: Suppl 3: S141-5. (2009)

Albert J, Gonzalez-Angulo A, Guray M, et al. Estrogen/progestrone receptor negativity and HER2 positivity predict locoregional recurrence in patients with T1a,b N0 breast cancer. Int J. Rad Onco Biol Phys: 77: 5: 1296 – 1302. (2010)

Ali A, Provenzano E, Bartlett J, et al. Prognosis of early breast cancer by immunohistochemistry defined intrinsic sub-types in patients treated with adjuvant chemotherapy in the NEAT/BR9601 trial. Int J Cancer: 133: 1470–1479. (2013)

Amir E, Ooi W, Simmons C, et al. Discordance between receptor status in primary and metastatic breast cancer: an exploratory study of bone and bone marrow biopsies. Clin Oncol (R Coll Radiol): 20: 10: 763-8. (2008)

Anand S, Penrhyn-Lowe S, Venkitaraman A. Aurora-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. Cancer Cell: 3: 1:51-62. (2003)

Ando K, Ozaki T, Yamamoto H, et al. Polo-like kinase 1 (Plk1) inhibits p53 function by physical interaction and phosphorylation. J Bio Chem: 279: 25549–25561. (2004)

Antoniades H, Galanopoulos T, Neville-Golden J, O'Hara C. Malignant epithelial cells in primary human lung carcinomas coexpress in vivo platelet-derived growth factor (PDGF) and PDGF receptor mRNAs and their protein products. Proc Natl Acad Sci USA: 89: 9: 3942-6. (1992)

Argyriou A, Antonacopoulou A, Iconomou G. Treatment options for malignant gliomas, emphasizing towards new molecularly targeted therapies. Crit Rev in Onco/Hemato. 69: 3: 199-210. (2009)

Armistead P, Salganick J, Roh J, et al. Expression of receptor tyrosine kinases and apoptotic molecules in rhabdomyosarcoma: correlation with overall survival in 105 patients. Cancer: 110: 2293–303. (2007)

Arnes J, Brunet J, Stefansson I, et al. Placental Cadherin and the Basal Epithelial Phenotype of BRCA1-Related Breast Cancer. Human Cancer Biology. Clin Cancer Res: 11:11:4003- 11. (2005)

Arnes J, Collett K, Akslen L. Independent prognostic value of the basal-like phenotype of breast cancer and associations with EGFR and candidate stem cell marker BMI-1. Histopathology: 52:3:370-80. (2008)

Assersohn L, Salter J, Powles T, et al. Studies of the potential utility of Ki67 as a predictive molecular marker of clinical response in primary breast cancer. Br Cancer Res Treat: 82: 113–123. (2003)

Assoian R, Fleurdelys B, Stevenson H. Expression and secretion of type beta transforming growth factor by activated human macrophages. Proc Natl Acad Aci USA: 84: 6020-6024. (1987)

Auerbach J, Kim M, Fineberg S, et al. Can Features Evaluated in the Routine Pathologic Assessment of Lymph Node–Negative Estrogen Receptor Positive Stage I or II Invasive Breast Cancer Be Used to Predict the Oncotype DX Recurrence Score? Arch Pathol Lab Med: 134:1697–1701. (2010)

Ayers M, Symmans W, Stec J, et al. Gene expression profiles predict complete pathologic response to neoadjuvant paclitaxel and fluorouracil, doxorubicin, and cyclophosphamide chemotherapy in breast cancer. J Clin Oncol: 22: 2284-93 (2004)

Badve S, Turbin D, Thorat M, et al. FOXA1 expression in breast cancer correlation with luminal subtype A and survival. Clin Cancer Res: 13: 4415 – 21. (2007)

Ball S, Shuttleworth C, Kielty C. Vascular endothelial growth factor can signal through platelet-derived growth factor receptors. J Cell Biol: 177: 3: 489-500. (2007)

Barabas K, Faulk W. Transferrin receptors associate with drug resistance in cancer cells. Biochem and Biophys Res Commu: 197: 2: 702 – 8. (1993)

Barbareschi M, Caffo O, Veronese S, et al. Bcl-2 and p53 expression in node-negative breast carcinoma: a study with long-term follow-up. Human Pathology: 27: 1149-1155. (1996)

Bartlett J, Thomas J, Ross D, et al.Mammostrat® as a tool to stratify breast cancer patients at risk of recurrence during endocrine therapy. Breast Cancer Research: 12: R47. (2010)

Baselga J, Perez E, Pienkowski T, Belld R. Adjuvant Trastuzumab: A Milestone in the Treatment of HER-2-Positive Early Breast Cancer. The Oncologist: 11(suppl 1): 4–12 (2006)

Bell-McGuinn K, Garfall A, Bogyo M, et al. Inhibition of cysteine cathepsin protease activity enhances chemotherapy regimens by decreasing tumour growth and invasiveness in a mouse model of multistage cancer. Cancer Res: 67:15: 7378–7385. (2007)

Bevilacqua P, Verderio P, Barbareschi M, et al. Lack of prognostic significance of the monoclonal antibody Ki-S1, a novel marker of proliferative activity, in node-negative breast carcinoma . Br Cancer Res Treat: 37: 2: 123 – 133. (1996)

Bevitt D, Milton I, Piggot N, et al. New monoclonal antibodies to oestrogen and progesterone receptors effective for paraffin section immunohistochemistry. J Pathol:183:2:228-32. (1997)

Bhargava R, Beriwal S, McManus K, Dabbs D. CK5 Is More Sensitive Than CK5/6 in Identifying the "Basal-like" Phenotype of Breast Carcinoma. Am J Clin Pathol: 130: 724-730. (2008)

Bingle L, Brown N, Lewis C. The role of tumourassociatedmacrophages in tumour progression: implications for new anticancer therapies. J Pathol: 196: 254 - 65. (2002)

Bischoff J, Anderson L, Zhu Y, et al. A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers. EMBO: 17: 11: 3052–3065. (1998)

Blamey R, Ellis I, Pinder S, et al. Survival of invasive breast cancer according to the Nottingham Prognostic Index in cases diagnosed in 1990–1999. E J Cancer: 43: 10: 1548-55. (2007a)

Blamey R, Pinder S, Ball G, et al. Reading the prognosis of the individual with breast cancer. E J Cancer: 43: 10: 1545-1547. (2007b)

Blows M, Driver E, Schmidt K, et al. Subtyping of Breast Cancer by Immunohistochemistry to Investigate a Relationship between Subtype and Short and Long Term Survival: A Collaborative Analysis of Data for 10,159 Cases from 12 Studies. PLoS Med: 7:5: e1000279. doi:10.1371/journal.pmed.1000279. (2010)

Bocker W, Moll R, Poremba C, et al. Common adult stem cells in the human breast give rise to glandular and myoepithelial cell lineages: a new cell biological concept. Lab Invest: 82: 737-746. (2002)

Bogina G, Bortesi L, Marconi M, et al. Comparison of hormonal receptor and HER-2 status between breast primary tumours and relapsing tumours: clinical implications of progesterone receptor loss. Virchows Arch: 459:1:1-10. (2011)

Bonadonna G, Brusamolino E, Valagussa P, et al. Combination chemotherapy as an adjuvant treatment in operable breast cancer. N Engl J Med: 294: 8: 405-10. (1976)

Bonadonna G, Moliterni A, Zambetti M, et al. 30 years' follow up of randomised studies of adjuvant CMF in operable breast cancer: cohort study. BMJ: 330: 7485:217. (2005)

Bonadonna G, Zambetti M, Valagussa P. Sequential or alternating doxorubicin and CMF regimens in breast cancer with more than three positive axillary nodes: Ten-year results. JAMA: 273: 542-547. (1995)

Bonetti A, Zaninelli M, Leone R, et al. Bcl-2 but not p53 expression is associated with resistance to chemotherapy in advanced breast cancer. Clin. Cancer Res: 4: 2331–2336. (1998)

Bonneterre J, Roché H, Kerbrat P, et al. Epirubicin increases long-term survival in adjuvant chemotherapy of patients with poor-prognosis, node-positive, early breast cancer: 10-year follow-up results of the French Adjuvant Study Group 05 randomized trial. J Clin Oncol: 23: 12: 2686-93. (2005)

Brimmell M, Burns JS, Munson P, et al. High level expression of differentially localized BAG-1 isoforms in some oestrogen receptor-positive human breast cancers. Br J Cancer: 81: 1042–1051. (1999)

Brozkova K, Budinska E, Bouchal P, et al. Surface-enhanced laser desorption/ionization time-of-flight proteomic profiling of breast carcinomas identifies clinicopathologically relevant groups of patients similar to previously defined clusters from cDNA expression. Br Cancer Res: 10:R48 (doi:10.1186/bcr2101) (2008)

Burcombe R, Makris A, Richman P, et al. Evaluation of ER, PgR, HER-2 and Ki-67 as predictors of response to neoadjuvant anthracycline chemotherapy for operable breast cancer. Br J Cancer: 92: 147 – 155. (2005)

Burcombe R, Wilson G, Dowsett M, et al. Evaluation of Ki-67 proliferation and apoptotic index before, during and after neoadjuvant chemotherapy for primary breast cancer. Br Cancer Res: 8: 3: R31. (2006)

Burger M, Denzinger S, Hartmann A. Mcm2 predicts recurrence hazard in stage Ta/T1 bladder cancer more accurately than CK20, Ki67 and histological grade. Br J Cancer: 96: 1711 – 1715. (2007)

Buzdar A, Ibrahim N, Francis D, et al. Significantly higher pathologic complete remission rate after neoadjuvant therapy with trastuzumab, paclitaxel, and epirubicin chemotherapy: results of a randomized trial in human epidermal growth factor receptor 2-positive operable breast cancer. J Clin Oncol: 23: 16: 3676-85. (2005)

Cabibi D, V. Mustacchio V, A. Martorana A, et al. Lymph Node Metastases Displaying Lower Ki-67 Immunostaining Activity than the Primary Breast Cancer. Anticancer Res: 26: 4357-4360 (2006)

Callagy G, Pharoah P, Pinder S, et al. Bcl-2 is a prognostic marker in breast cancer independently of the Nottingham Prognostic Index. Clin Cancer Res: 12: 8: 2468–2475. (2006)

Calza S, Hall P, Auer G, et al. Intrinsic molecular signature of breast cancer in a population-based cohort of 412 patients. Br Cancer Res: 8: 4: R34. (2006)

Cameron D, Anderson A, Toy E, et al. Block sequential adriamycin CMF--optimal non-myeloablative chemotherapy for high risk adjuvant breast cancer? Br J Cancer: 87:12:1365-9. (2002)

Cameron D, Casey M, Oliva C, et al. Lapatinib plus capecitabine in women with HER-2-positive advanced breast cancer: final survival analysis of a phase III randomized trial. Oncologist: 15: 9: 924-34. (2010)

Camp R, Charette L, Rimm D. Validation of tissue microarray technology in breast carcinoma. Lab Invest: 80: 12: 1943 – 1949. (2000)

Campbell H, Gray A, Harris A, Briggs A, Taylor M. Estimation and external validation of a new prognostic model for predicting recurrence-free survival for early breast cancer patients in the UK. Br J Cancer: 103: 776-786. (2010)

Campbell H, Taylor M, Harris A, Gray A. An investigation into the performance of the Adjuvant! Online prognostic programme in early breast cancer for a cohort of patients in the United Kingdom. Br J Cancer: 101: 1074 – 1084. (2009)

Cancer Stats Incidence 2009 - UK, Cancer Research UK, published in May 2012

Carey L, Dees E, Sawyer L et al. The triple negative paradox: primary tumour chemosensitivity of breast cancer subtypes. Clin Cancer Res: 13:8:2329-34. (2007)

Carlsten C, Sagoo G, Frodsham A. Glutathione S-Transferase M1 (GSTM1) polymorphisms and lung Cancer: A literature-based systematic HuGE review and meta-Analysis. Am J Epidemiol: 167: 759 –774. (2008)

Carpenter J, Maddox W. Melphalan adjuvant therapy in breast cancer. Lancet: 2: 8347: 450-1. (1983)

Carvalho I, Milanezi F, Martins A, et al. Overexpression of platelet-derived growth factor receptor alpha in breast cancer is associated with tumour progression. Breast Cancer Res: 7: R788-R795. (2005)

Caudle A, Yu T, Tucker S, et al. Local-regional control according to surrogate markers of breast cancer subtypes and response to neoadjuvant chemotherapy in breast cancer patients undergoing breast conserving therapy. Br Cancer Res: 14: 3: R83. (2012)

Chan F, Liu Y, Sun H, et al. Distribution and possible role of PDGF-AA and PDGFR-alpha in the gastrointestinal tract of adult guinea pigs. Virchows Arch: 457: 381-8. (2010)

Chang H, Glaspy J, Allison M, et al. Differential Response of Triple-Negative Breast Cancer to a Docetaxel and Carboplatin-Based Neoadjuvant Treatment. Cancer: 116: 4227–37. (2010)

Chang H, Nuyten D, Sneddon J, et al. Robustness, scalability, and integration of a wound-response gene expression signature in predicting breast cancer survival. Proc Natl Acad Sci USA: 102: 3738–43. (2005)

Chang H, Sneddon J, Alizadeh A, et al. Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds. PLoS Biol: 2: 2: E7. (2004)

Cheang M, Chia S, Voduc D et al. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J Nat Cancer Inst: 101: 736–750. (2009)

Cheang M, van de Rijn M, Nielsen T. Gene expression profiling of breast cancer. Annu Rev Pathol: 3: 67 – 97. (2008a)

Cheang M, Voduc D, Bajdik C, et al. Basal-Like Breast Cancer Defined by Five Biomarkers Has Superior Prognostic Value than Triple-Negative Phenotype. Clin Cancer Res: 14 : 5: 1368-76 (2008b)

Cheang M, Voduc K, Tu D, et al. Responsiveness of Intrinsic Subtypes to Adjuvant Anthracycline Substitution in the NCIC.CTG MA.5 Randomized Trial. Clin Cancer Res: 18: 8: 2402–12 (2012)

Chen Y, DeVries S, Anderson J. Pathologic and biologic response to preoperative endocrine therapy in patients with ER-positive ductal carcinoma in situ. BMC Cancer: 9:285: 1-9. (2009)

Chia S, Speers C, D'yachkova Y, et al. The impact of new chemotherapeutic and hormone agents on survival in a population-based cohort of women with metastatic breast cancer. Cancer: 110: 973–979. (2007)

Choi S, Yun K, Kim T, et al. Prognostic potential of glutathione S-transferase M1 and T1 null genotypes for gastric cancer progression. Cancer Lett: 195: 169-175. (2003)

Chott A, Sun Z, Morganstern D, et al. Tyrosine kinases expressed in vivo by human prostate cancer bonemarrow metastases and loss of the type 1 insulin like growth factor receptor. Am J Pathol: 155: 1271 - 9. (1999)

Chung Y, Sheu M, Yang S, et al. Resistance to tamoxifen-induced apoptosis is associated with direct interaction between Her2/neu and cell membrane estrogen receptor in breast cancer. Int J Cancer: 97: 3: 306-12. (2002)

Ciardiello F, Troiani T, Caputo F, et al. Phase II study of gefitinib in combination with docetaxel as first-line therapy in metastatic breast cancer. Br J Cancer: 94: 11: 1604–1609. (2006)

Ciocca D, Elledge R. Molecular markers for predicting the response to tamoxifen in breast cancer patients. Endocrine: 13: 1–10. (2000)

Clahsen P, van de Velde C, Duval C, et al. The utility of mitotic index, oestrogen receptor and Ki-67 measurements in the creation of novel prognostic indices for node-negative breast cancer. E J Surg Oncol: 25: 4: 356 – 363. (1999)

Clarke R, Laidlaw I, Jones L, et al. Effect of tamoxifen on Ki67 labelling index in human breast tumours and its relationship to oestrogen and progesterone receptor status. Br J Cancer: 67: 606–611. (1993)

Cobleigh M, Tabesh B, Bitterman P, et al. Tumor gene expression and prognosis in breast cancer patients with 10 or more positive lymph nodes. Clin Cancer Res: 11:24:8623-31. (2005)

Colleoni M, Cole B, Viale G, et al. Classical cyclophosphamide, methotrexate, and fluorouracil chemotherapy is more effective in triple-negative, node-negative breast cancer: results from two randomized trials of adjuvant chemoendocrine therapy for node-negative breast cancer. J Clin Oncol: 28:18: 2966-73. (2010)

Colleoni M, Litman H, Castiglione-Gertsch M, et al. Duration of adjuvant chemotherapy for breast cancer: a joint analysis of two randomised trials investigating three versus six courses of CMF. Br J Cancer: 86: 1705 – 1714 (2002)

Collett K, Stefansson I, Eide J, et al. A Basal Epithelial Phenotype Is More Frequent in Interval Breast Cancers Compared with Screen Detected Tumours. Cancer Epi Biomarkers Prev: 14: 5: 1108–12. (2005)

Conforti R, Boulet T, Tomasic G, et al. Breast cancer molecular subclassification and estrogen receptor expression to predict efficacy of adjuvant anthracyclines-based chemotherapy: a biomarker study from two randomized trials. Ann Onco: 18: 9:1477-83. (2007)

Coyle Y, Xie X-J, Hardy D, et al. Progesterone receptor expression is a marker for early stage breast cancer: Implications for progesterone receptor as a therapeutic tool and target. Cancer Letters: 258: 253–261. (2007)

Crabb S, Bajdik C, Leung S, et al. Can clinically relevant prognostic subsets of breast cancer patients with four or more involved axillary lymph nodes be identified through immunohistochemical biomarkers? A tissue microarray feasibility study. Br Cancer Res: 10: R6 (doi:10.1186/bcr1847) (2008a)

Crabb S, Cheang M, Leung S, et al. Basal breast cancer molecular subtype predicts for lower incidence of axillary lymph node metastases in primary breast cancer. Clin Br Cancer: 8: 3: 249-256. (2008b)

Cristofanilli M, Morandi P, Krishnamurthy S et al. Imatinib mesylate (Gleevec_) in advanced breast cancer-expressing C-Kit or PDGFR-*b*: clinical activity and biological correlations. Ann Onco: 19: 1713–1719. (2008)

Cristofanilli M, Morandi P, Krishnamurthy S, et al. Imatinib mesylate (Gleevec) in advanced breast cancer-expressing C-Kit or PDGFR-b: clinical activity and biological correlations. Ann Onco: 19: 1713–1719. (2008)

Cui X, Schiff R, Arpino G, et al. Biology of progesterone receptor loss in breast cancer and its implications for endocrine therapy. J Clin Oncol: 23: 7721–35. (2005)

Cutress R, Townsend P, Brimmell M, et al. BAG-1 expression and function in human cancer. Br J Cancer: 87: 834-9. (2002)

Cutress R, Townsend P, Sharp A, et al. The nuclear BAG-1 isoform, BAG-1L, enhances oestrogen-dependent transcription. Oncogene: 22: 32: 4973-82. (2003)

Cuzick J, Dowsett M, Pineda S, et al. Prognostic value of a combined estrogen receptor, progesterone receptor, Ki-67, and human epidermal growth factor receptor 2 immunohistochemical score and comparison with the Genomic Health recurrence score in early breast cancer. J Clin Oncol: 29: 32: 4273-8. (2011)

Dabbs D, Chivukula M, Carter G, et al. Basal phenotype of ductal carcinoma in situ: recognition and immunohistologic profile. Mod Pathol: 19:1506-1511. (2006)

Darb-Esfahani S, Loibl S, Müller B, et al. Identification of biology-based breast cancer types with distinct predictive and prognostic features: role of steroid hormone and HER2 receptor expression in patients treated with neoadjuvant anthracycline/taxane-based chemotherapy. Br Cancer Res: 11: R69 (doi:10.1186/bcr2363) (2009)

Davis L, Harris C, Tang L, et al. Amplification patterns of three genomic regions predict distant recurrence in breast carcinoma. J Mol Diagn: 9: 327–336. (2007)

De Placido S, De Laurentiis M, De Lena M, et al. GOCSI Cooperative Group. A randomised factorial trial of sequential doxorubicin and CMF vs CMF and chemotherapy alone vs chemotherapy followed by goserelin plus tamoxifen as adjuvant treatment of node-positive breast cancer. Br J Cancer: 92: 3: 467-74. (2005)

Deaglio S, Capobianco A, Cali A, et al. Structural, functional, and tissue distribution analysis of human transferring receptor-2 by murine monoclonal antibodies and a polyclonal antiserum. Blood: 100: 3782– 3789. (2002)

Delgallo W, Rodrigues J, Bueno S, et al. Cell blocks allow reliable evaluation of expression of basal (CK5/6) and luminal (CK8/18) cytokeratins and smooth muscle actin (SMA) in breast carcinoma. Cytopathology: 21: 4: 259-66. (2010)

Deprimo S, Friece C, Huang X, et al.: Effect of treatment with sunitinib malate, a multitargeted tyrosine kinase inhibitor, on circulating plasma levels of VEGF, soluble VEGF receptors 2 and 3, and soluble KIT in patients with metastatic breast cancer. J Clin Oncol: 24: 22s. (2006) (suppl; abstr 578)

Des Guetz G, Uzzan B, Nicolas P, et al. Microvessel density and VEGF expression are prognostic factors in colorectal cancer. Meta-analysis of the literature. Br J Cancer: 94: 1823-1832. (2006)

Desmedt C, Piette F, Loi S, et al. Strong Time Dependence of the 76-Gene Prognostic Signature for Node-Negative Breast Cancer Patients in the TRANSBIG Multicenter IndependentValidation Series. Clin Cancer Res: 13:3207-3214. (2007)

deVries E, Gietema J, deJong S. Tumor Necrosis Factor ^Related Apoptosis-Inducing Ligand Pathway and Its Therapeutic Implications. Clin Cancer Res: 12: 2390-2393. (2006)

Di Piazza M, Mader C, Geletneky K, et al. Cytosolic activation of cathepsins mediates parvovirus H-1-induced killing of cisplatin and TRAIL-resistant glioma cells. J Virology: 81: 8: 4186–4198. (2007)

Dias S, Hattori K, Heissig B, et al. Inhibition of both paracrine and autocrine VEGF/VEGFR-2 signaling pathways is essential to induce long term remission of xenotransplanted human leukemias. Proc Natl Acad Sci USA: 98: 10857–10862. (2001)

Dickler M, Cobleigh M, Miller K, et al. Efficacy and safety of erlotinib in patients with locally advanced or metastatic breast cancer. Br Cancer Res Treat: 115: 1:115-21. (2009)

Dickler M, Rugo H, Eberle C, et al. A Phase II trial of Erlotinib in combination with Bevacizumab in patients with metastatic breast cancer. Clin Cancer Res: 14: 23: 7878–7883. (2008)

Ditchfield C, Johnson V, Tighe A, et al. Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. J Cell Biol: 161: 267-80. (2003)

Dohchin A, Suzuki J, Seki H, et al. Immunostained cathepsins B and L correlate with depth of invasion and different metastatic pathways in early stage gastric carcinoma. Cancer: 89: 3: 482-7. (2000)

Dolloff N, Russell M, Loizos N, Fatatis A. Human Bone Marrow Activates the Akt Pathway in Metastatic Prostate Cells through Transactivation of the A-Platelet–Derived Growth Factor Receptor. Cancer Res: 67: 2: 555–62. (2007)

Dowsett M, Cuzick J, Wale C, et al. Prediction of risk of distant recurrence using the 21-gene recurrence score in node-negative and node-positive postmenopausal patients with breast cancer treated with anastrozole or tamoxifen: a TransATAC study. J Clin Oncol: 28:11:1829-34. (2010)

Dowsett M, Cuzick J, Wale C, et al. Retrospective analysis of time to recurrence in the ATAC trial according to hormone receptor status: a hypothesis generating study. J Clin Oncol: 23: 7512-7. (2005)

Dowsett M, Houghton J, Iden C, et al. Benefit from adjuvant tamoxifen therapy in primary breast cancer patients according oestrogen receptor, progesterone receptor, EGF receptor and HER2 status. Ann Onco: 17: 818–826. (2006)

Dowsett M, Smith I, Ebbs S et al. Prognostic Value of Ki67 Expression After Short-Term Presurgical Endocrine Therapy for Primary Breast Cancer. J Nat Cancer Inst: 99: 167–70. (2007)

Drescher D, Moehler M, Gockel I, et al.Coexpression of receptor-tyrosine-kinases in gastric adenocarcinoma-a rationale for a molecular targeting strategy? World J Gastroenterol: 13: 26: 3605-3609. (2007)

Dudderidge T, Stoeber K, Loddo M, et al. MCM2, Geminin, and ki-67 define proliferative state and are prognostic markers in renal cell carcinoma. Clin Cancer Res: 11: 2510-2517. (2005)

Dunbier A, Anderson H, Ghazoui Z, et al. Association between breast cancer subtypes and response to neoadjuvant anastrozole. Steroids: 76:8:736-40. (2011)

Early Breast Cancer Trialists' Collaborative Group, Writing committee, Darby S, McGale P, Correa C, et al. Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer mortality: meta analysis of individual patient data for 10,801 women in 17 randomised trials. Lancet: 378:9804: 1707-16. (2011a)

Early Breast Cancer Trialists' Collaborative Group, Writing committee, Davies C, Peto R, Gray R, et al. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet: 378:9793: 771-84. (2011b)

Early Breast Cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet: 365: 9472: 1687 – 1717. (2005b)

Early Breast Cancer Trialists' Collaborative Group. Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. Lancet: 366: 9503: 2087–2106 (2005a)

Early Breast Cancer Trialists' Collaborative Group. Polychemotherapy for early breast cancer: an overview of the randomised trials. Lancet: 352: 9132: 930-942 (1998)

Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Adjuvant chemotherapy in oestrogen-receptor-poor breast cancer: patient-level meta-analysis of randomised trials. Lancet: 371: 29–40 (2008)

Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Comparisons between different polychemotherapy regimens for early breast cancer: meta-analyses of long-term outcome among 100 000 women in 123 randomised trials. Lancet: 379: 432–44 (2012)

Ebert M, Yokoyama M, Friess H, et al. Induction of platelet-derived growth factor A and B chains and over-expression of their receptors in human pancreatic cancer. Int J Cancer: 62: 5: 529-35. (1995)

Elledge R, Green S, Howes L, et al. Bcl-2, p53, and response to tamoxifen inER-positive metastatic breast cancer: a Southwest Oncology Group study. J Clin Oncol: 15: 1916–1922. (1997)

Ellis M, Suman V, Hoog, J et al. Randomized phase II neoadjuvant comparison between letrozole, anastrozole, and exemestane for postmenopausal women with estrogen receptor-rich stage 2 to 3 breast cancer: clinical and biomarker outcomes and predictive value of the baseline PAM50-based intrinsic subtype--ACOSOG Z1031. J Clin Oncol: 29: 17: 2342-9. (2011)

Ellis M, Tao Y, Luo J, et al. Outcome prediction for estrogen receptor-positive breast cancer based on postneoadjuvant endocrine therapy tumor characteristics. J Nat Cancer Inst: 100: 1380–8. (2008)

Epplein M, Wilkens L, Tiirikainen M, et al. Urinary isothiocyanates; glutathione S-transferase M1, T1, and P1 polymorphisms; and risk of colorectal cancer: the Multiethnic Cohort Study. Cancer Epi Biomarkers Prev: 18: 314-320. (2009)

Falini B, Flenghi L, Pileri S, et al. M15.1. PG-M1 a new mAb directed against a fixative-resistant, macrophage-restricted epitope of the CD68 molecule. In: Knapp W, Dörken B, Gilks W, et al., editors. Leukocyte typing IV. White cell differentiation antigens. Proceedings of the 4th International Workshop and Conference; 1989 Feb 21-25; Vienna, Austria. Oxford, New York, Tokyo: Oxford University Press. p. 928-30. (1989)

Fan C, Oh D, Wessels L, et al. Concordance among gene-expression-based predictors for breast cancer. N Eng J Med: 355:560-9. (2006)

Feng H, Liu K, Guo P, et al. Dynamin 2 Mediates PDGFRα-SHP-2-Promoted Glioblastoma Growth and Invasion. Oncogene: 31:21: 2691–2702. (2012)

Ferrer F, Miller L, Lindquist R, et al. Expression of vascular endothelial growth factor receptors in human prostate cancer. Urology: 54: 567–572. (1999)

Finak G, Bertos N, Pepin F, et al. Stromal gene expression predicts clinical outcome in breast cancer. Nat Med: 14:5: 518–27. (2008)

Fisher B, Redmond C, Fisher E, et al. Relative worth of estrogen or progesterone receptor and pathologic characteristics of differentiation as indicators of prognosis in node negative breast cancer patients: findings from national surgical adjuvant breast and bowel project protocol B-06. J Clin Onco: 6: 1076–1087. (1988)

Fleeman N, Saborido C, Payne K, et al. The clinical effectiveness and cost-effectiveness of genotyping for CYP2D6 for the management of women with breast cancer treated with tamoxifen: a systematic review. Health Technol Assess: 15: 33: 1-102. doi: 10.3310/hta15330. 2011 (2011)

Foulkes W, Brunet J-S, Stefansson I, et al. The Prognostic Implication of the Basal-Like (Cyclin Ehigh/p27low/p53_/Glomeruloid-Microvascular-Proliferation) Phenotype of BRCA1-Related Breast Cancer. Cancer Res: 64: 830–835. (2004)

Fritzer M, Szekeres T, Szuts V, et al. Cytotoxic effects of a doxorubicin—transferrin conjugate in multidrug-resistant KB cells. Biochem Pharmacol: 51: 489– 493. (1996)

Fu J, Bian M, Jiang Q, Zhang C. Roles of aurora kinases in mitosis and tumorigenesis. Mol Cancer Res: 5: 1-10. (2007)

Fumoleau P, Kerbrat P, Romestaing P, et al. Randomized trial comparing six versus three cycles of epirubicin-based adjuvant chemotherapy in premenopausal, node-positive breast cancer patients: 10-year follow-up results of the French Adjuvant Study Group 01 trial. J Clin Oncol: 21: 2: 298-305. (2003)

Galea M, Blamey R, Elston C, Ellis I. The Nottingham Prognostic Index in primary breast cancer. Breast Cancer Res Treat: 22: 3: 207-19. (1992)

Gao C, Takezaki T, Wu J, et al. Sugimura H, Tajima K. Glutathione-S- transferases M1 (GSTM1) and GSTT1 genotype, smoking, consumption of alcohol and tea and risk of esophageal and stomach cancers: a case-control study of a high-incidence area in Jiangsu Province, China. Cancer Lett: 188: 95-102. (2002)

Garvin S, Nilsson U, Dabrosin C. Effects of oestradiol and tamoxifen on VEGF, soluble VEGFR-1, and VEGFR-2 in breast cancer and endothelial cells. Br J Cancer: 93: 1005 – 1010. (2005)

Gasparini G, Barbareschi M, Doglioni C, et al. Expression of bcl-2 protein predictsefficacy of adjuvant treatments in operable node-positive breast cancer. Clin Cancer Res: 1: 189–198. (1995)

Gee J, Harper M, Hutcheson I, et al. The Antiepidermal Growth Factor Receptor Agent Gefitinib (ZD1839/Iressa) Improves Antihormone Response and Prevents Development of Resistance in Breast Cancer in Vitro. Endocrinology: 144: 11: 5105–5117. (2003)

Gee J, Robertson J, Ellis I, et al. Immunocytochemical localization of Bcl-2 protein in human breast cancers and its relationship to a series of prognostic markers and response to endocrine therapy. Int J Cancer: 59: 619-628. (1994)

Gennari A, Conte P, Rosso R, Orlandini C, Bruzzi P. Survival of metastatic breast carcinoma patients over a 20-year period: a retrospective analysis based on individual patient data from six consecutive studies. Cancer: 104: 1742–1750. (2005)

Gerdes J, Lemke H, Baisch H, et al. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol: 133: 1710– 1715. (1984)

Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer: 31:13–20. (1983)

Gerlinger M, Rowan A, Horswell S, et al. Intratumor Heterogeneity and Branched Evolution Revealed by Multiregion Sequencing. N Eng J Med: 366: 883-92. (2012)

Ghosh S, Sullivan C, Zerkowski M, et al. High levels of vascular endothelial growth factor and its receptors (VEGFR-1, VEGFR-2, neuropilin-1) are associated with worse outcome in breast cancer. Hu Path: 39: 1835–1843. (2008)

Gianni L, Dafni U, Gelber R, et al. Treatment with trastuzumab for 1 year after adjuvant chemotherapy in patients with HER2-positive early breast cancer: a 4-year follow-up of a randomised controlled trial. Lancet Onco: 12: 3: 236-44. (2011)

Gianni L, Zambetti M, Clark K, et al. Gene expression profiles in paraffin-embedded core biopsy tissue predict response to chemotherapy in women with locally advanced breast cancer. J Clin Onco: 23: 29: 7265-77. Epub 2005 Sep 6. (2005)

Giatromanolaki A, Koukourakis M, Sivridis E, et al. Activated VEGFR2/KDR pathway in tumour cells and tumour associated vessels of colorectal cancer. E J Clin Invest: 37: 11: 878–886. (2007)

Gilbert J, Goetz M, Reynolds C, et al. Molecular analysis of metaplastic breast carcinoma: high EGFR copy number via aneusomy. Mol Cancer Ther: 7:4: 944–951. (2008)

Gille H, Kowalski J, Li B, et al. Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). J Biol Chem: 276: 3222–3230. (2001)

Giltnane J, Rimm D. Technology insight: identifi cation of biomarkers with tissue microarray technology. Nat Clin Pract Oncol: 1: 2: 104 – 111. (2004)

Gocheva V, Joyce J. Cysteine cathepsins and the cutting edge of cancer invasion. Cell Cycle: 6:1: 60–64. (2007)

Goetz M, Suman V, Ingle J, et al: A twogene expression ratio of HOXB13 and IL-17BR for prediction of recurrence and survival in women receiving adjuvant tamoxifen. Clin Cancer Res: 12:2080-2087. (2006)

Goldhirsch A, Ingle J, Gelber R, et al. Thresholds for therapies: highlights of the St Gallen international Expert Consensus on the Primary Therapy of Early Breast Cancer 2009. Ann Onco: 20: 1319–1329 (2009)

Goldhirsch A, Wood W, Coates A, et al. Strategies for subtypes—dealing with the diversity of breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Onco: 22: 1736–1747. (2011)

Gonzalez M, Pinder S, Callagy G, et al. Minichromosome Maintenance Protein 2 Is a Strong independent prognostic marker in breast cancer. J Clin Onco: 21:23:4306-13. (2003)

Goodson W, Moore D, Ljung B, et al. The prognostic value of proliferation indices: a study with in vivo bromodeoxyuridine and Ki-67. Br Cancer Res Treat: 59: 2: 113 – 123. (2000)

Gori S, Sidoni A, Colozza M, et al. EGFR, pMAPK, pAkt and PTEN status by immunohistochemistry: correlation with clinical outcome of HER2-positive metastatic breast cancer patients treated with trastuzumab: Ann Onco: 20: 4:648–654. (2009)

Goulet B, Sansregret L, Leduy L, et al. Increased expression and activity of nuclear cathepsin L in cancer cells suggests a novel mechanism of cell transformation. Mol Cancer Res: 5:9: 899–907. (2007)

Green D, Reed J. Mitochondria and apoptosis. Science: 281 : 5381: 1309–1312. (1998)

Green M, Francis P, Gebski V, et al. Gefitinib treatment in hormone-resistant and hormone receptor-negative advanced breast cancer. Ann Onco: 20: 11: 1813 - 7. (2009)

Griffith C, Ellis I, Bell J, et al. Density of lymphocytic infiltration of primary breast cancer does not affect short term disease-free interval or survival. J R Coll Surg Edinb: 35: 289–292. (1990)

Grin A, O'Malley F, Mulligan A. Cytokeratin 5 and estrogen receptor immunohistochemistry as a useful adjunct in identifying atypical papillary lesions on breast needle core biopsy. Am J Surg Pathol: 33:1615–1623. (2009)

Gritsko T, Coppola D, Paciga J, et al. Activation and overexpression of centrosome kinase BTAK/Aurora A, in human ovarian cancer. Clin Cancer Res: 9: 1420–1426. (2003)

Guarneri V, Frassoldati A, Ficarra G, et al. Phase II, randomized trial of preoperative epirubicin-paclitaxel +/- gefitinib with biomarker evaluation in operable breast cancer. Br Cancer Res Treat: 110: 1:127 - 34 (2008)

Guarneri V, Piacentini F, Ficarra G, et al. A prognostic model based on nodal status and Ki-67 predicts the risk of recurrence and death in breast cancer patients with residual disease after preoperative chemotherapy. Ann Onco: 20: 1193–1198. (2009)

Gumireddy K, Reddy V, Cosenza C, et al. ON01910, a non-ATP-competitive small molecule inhibitor of Plk1, is a potent anticancer agent. Cancer Cell: 7: 275 – 286. (2005)

Gusterson B, Ross D, Heath V, Stein T. Basal cytokeratins and their relationship to the cellular origin and functional classification of breast cancer. Br Cancer Res: 7: 143-148 (2005)

Gwin K, Pinto M, Tavassoli F. Complementary value of the Ki-67 proliferation index to the oncotype DX recurrence score. Int J Surg Pathol: 17:4:303-10. (2009)

Gyorffy B, Benke Zs, Lanczky A, et al. RecurrenceOnline: an online analysis tool to determine breast cancer recurrence and hormone receptor status using microarray data. Br Cancer Res Treat: 132:3:1025-34. (2012)

Habashy H, Powe D, Staka C, et al. Transferrin receptor (CD71) is a marker of poor prognosis in breast cancer and can predict response to tamoxifen. Br Cancer Res Treat: 119: 2: 283-93. (2010)

Habashy H, Powe D, Staka C, et al. Transferrin receptor (CD71) is a marker of poor prognosis in breast cancer and can predict response to tamoxifen. Breast Cancer Res Treat: 119: 283-93. (2010)

Habel L, Shak S, Jacobs M, et al. A population-based study of tumour gene expression and risk of breast cancer death among lymph node-negative patients. Br Cancer Res: 8: 3: R25. (2006)

Hagan C, Daniel A, Dressing G. Role of phosphorylation in progesterone receptor signaling and specificity. Mol and Cell Endo: 357: 43–49. (2012)

Hagemann S, Günther T, Dennemärker J, et al. The human cysteine protease cathepsin V can compensate for murine cathepsin L in mouse epidermis and hair follicles. E J Cell Biol: 83: 11–12: 775–780. (2004)

Hammond M, Hayes D, Dowsett M, et al. American Society of Clinical Oncology/ College Of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. J Clin Onco: 28: 2784–2795. (2010)

Hanna W, O'Malley F, Barnes P, et al. Updated recommendations from the Canadian National Consensus Meeting on HER2/neu testing in breast cancer. Curr Onco: 14: 149–153. (2007)

Haralambieva E, Boerma E-J, van Imhoff G, et al. Clinical, immunophenotypic, and genetic analysis of adult lymphomas with morphologic features of Burkitt lymphoma. Am J Surg Pathol: 29: 1086-94. (2005)

Harbeck N, Alt U, Berger U, et al. Prognostic impact of proteolytic factors (urokinase-type plasminogen activator, plasminogen activator inhibitor 1, and cathepsins B, D, and L) in primary breast cancer reflects effects of adjuvant systemic therapy. Clin Cancer Res: 7: 9: 2757-64. (2001)

Harrington E, Bebbington T, Moore J, et al. VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth in vivo. Nat Med: 10: 262–267. (2004)

Harris L, You F, Schnitt S, et al. Predictors of resistance to preoperative trastuzumab and vinorelbine for HER2-positive early breast cancer. Clin Cancer Res: 13:,1198 –1207. (2007)

Harvey J, Clark G, Osborne C, Allred D. Estrogen Receptor Status by Immunohistochemistry Is Superior to the Ligand-Binding Assay for Predicting Response to Adjuvant Endocrine Therapy in Breast Cancer. J Clin Onco: 17: 5: 1474-1481. (1999)

Hasegawa M, Moritani S, Murakumo Y, et al. CD109 expression in basal-like breast carcinoma. Path International: 58: 288–294. (2008)

Hata T, Furukawa T, Sunamura M, et al. RNA interference targeting Aurora kinase A suppresses tumor growth and enhances the taxane chemosensitivity in human pancreatic cancer cells. Cancer Res: 65: 2899–905. (2005)

Helzlsouer K, Selmin O, Huang H, et al. Association between glutathione S-transferase M1, P1, and T1 genetic polymorphisms and development of breast cancer. J Natl Cancer Inst: 90: 7: 512–518. (1998)

Henderson I, Berry D, Demetri G, et al. Improved outcomes from adding sequential Paclitaxel but not from escalating Doxorubicin dose in an adjuvant chemotherapy regimen for patients with node-positive primary breast cancer. J Clin Oncol: 21: 6: 976-83. (2003)

Heng D, Rini B, Garcia J, et al. Prolonged complete responses and near-complete responses to sunitinib in metastatic renal cell carcinoma. Clin Genitourin Cancer: 5: 7: 446-451. (2007)

Herrmann G, Schumm-Draeger P-M, Muller C, et al. T Lymphocytes, CD-68 positive cells and vascularisation in thyroid carcinomas. J Cancer Res Clin Onco: 120: 651 – 6. (1994)

Herszényi L, Farinati F, Cardin R, et al. Tumour marker utility and prognostic relevance of cathepsin B,cathepsin L, urokinase-type plasminogen activator, plasminogen activator inhibitor type-1, CEA and CA 19-9 in colorectal cancer. BMC Cancer: 8: 194:1186 -1471. (2008)

Herszenyi L, Plebani M, Carraro P, et al. The Role of Cysteine and Serine Proteases in colorectal carcinoma. Cancer: 86: 7: 1135-42. (1999)

Hicks D, Short S, Prescott N. Breast cancers with brain metastases are more likely to be estrogen receptor negative, express the basal cytokeratin CK5/6, and overexpress HER2 or EGFR. Am J Surg Pathol: 30:1097–1104. (2006)

Higgins K, Liu S, Abdelrahim M, et al. Vascular endothelial growth factor receptor-2 expression is induced by 17beta-estradiol in ZR-75 breast cancer cells by estrogen receptor alpha/Sp proteins. Endocrinology: 147: 7: 3285-95. (2006)

Hildenbrand R, Dilger I, Horlin A, Stutte H. Urokinase and macrophages in tumour angiogenesis. Br J Cancer: 72: 818-823. (1995)

Hirakawa N, Naka T, Yamamoto I, Fukuda T, Tsuneyoshi M. Overexpression of bcl-2 protein in synovial sarcoma: A comparative study of other soft tissue spindle cell sarcomas and an additional analysis by fluorescence in situ hybridization. Hum Pathol: 27:1060-5. (1996)

Holden S, Eckhardt S, Basser R, et al: Clinical evaluation of ZD6474, an orally active inhibitor of VEGF and EGF receptor signaling, in patients with solid, malignant tumors. Ann Onco: 16: 1391-1397. (2005)

Hoque A, Carter J, Xia W, et al. Loss of aurora A/STK15/BTAK overexpression correlates with transition of in situ to invasive ductal carcinoma of the breast. Cancer Epidemiol Biomarkers Prev: 12: 1518- 22. (2003)

Hornychova H, Melichar B, Tomsova M. Tumor-infiltrating lymphocytes predict response to neoadjuvant chemotherapy in patients with breast carcinoma. Cancer Invest: 26: 10: 1024-31. (2008)

Houssami N, Macaskill P, von Minckwitz G, et al. Meta-analysis of the association of breast cancer subtype and pathologic complete response to neoadjuvant chemotherapy. E J Cancer. [Epub ahead of print] (2012 Jul 3).

Houston S, Plunkett T, Barnes D, et al. Overexpression of c-erbB2 is an independent marker of resistance to endocrine therapy in advanced breast cancer. Br J Cancer: 79: 1220 -1226. (1999)

http://info.cancerresearchuk.org/cancerstats published in April 2011

http://info.cancerresearchuk.org/cancerstats/types/breast/survival/

http://www.cancer.org/Cancer/BreastCancer/DetailedGuide/breast-cancer-survival-by-stage

Hu K, Law J, Fotovati A, Dunn S. Small interfering RNA library screen identified polo-like kinase-1 (PLK1) as a potential therapeutic target for breast cancer that uniquely eliminates tumour-initiating cells. Br Cancer Res:14:R22 doi:10.1186/bcr3107 (2012)

Huang H, Huang W, Lin C, et al. Immunohistochemical expression of p16INK4A, Ki-67, and Mcm2 proteins in gastrointestinal stromal tumors: prognostic implications and correlations with risk stratification of NIH consensus criteria. Ann Surg Onco: 13: 12: 1633-44. (2006)

Huang S, Van Arsdall M,Tedjarati S, et al. Contributions of stromal metalloproteinase-9 to angiogenesis and growth of human ovarian carcinoma in mice. J Nat Cancer Inst: 94: 1134-42. (2002)

Hugh J, Hanson J, Cheang M, et al. Breast cancer subtypes and response to docetaxel in node-positive breast cancer: Use of an immunohistochemical definition in the BCIRG 001 Trial. J Clin Oncol: 27:1168-1176. (2009)

Hussein M, Hassan H. Analysis of the mononuclear inflammatory cell infiltrate in the normal breast, benign proliferative breast disease, in situ and infiltrating ductal breast carcinomas: preliminary observations. J Clin Pathol: 59: 972–977. (2006)

Ihemelandu C, Leffall L, Dewitty R, et al. Molecular Breast Cancer Subtypes in Premenopausal and Postmenopausal African-American Women: Age-Specific Prevalence and Survival. J Surg Res: 143: 109–118. (2007)

Inoue H, Kiyohara C, Shinomiya S et al. Glutathione S-transferase polymorphisms and risk of colorectal adenomas. Cancer Lett: 163: 201-206. (2001)

Isaacs C, Stearns V, Hayes D. New prognostic factors for breast cancer recurrence. Semin Onco: 28: 53-67. (2001)

Ishimi Y, Komamura Y, You Z, et al. Biochemical function of mouse Minichrosome Maintenance protein 2. J Biological Chemistry: 273: 14: 8369–8375. (1998)

Jakesz R, Hausmaninger H, Kubista E, et al. Randomized adjuvant trial of tamoxifen and goserelin versus cyclophosphamide, methotrexate, and fluorouracil: evidence for the superiority of treatment with endocrine blockade in premenopausal patients with hormone-responsive breast cancer--Austrian Breast and Colorectal Cancer Study Group Trial 5. J Clin Onco: 20: 24: 4621-7. (2002)

Jansen S, Kievit J, Nooij M, et al. Patients' preferences for adjuvant chemotherapy in early-stage breast cancer: is treatment worthwhile? Br J Cancer: 84: 12: 1577–1585. (2001)

Jechlinger M, Sommer A, Moriggl R, et al. Autocrine PDGFR signaling promotes mammary cancer metastasis. J Clin Inves: 116: 6: 1561-70. (2006)

Jensen T, Schmidt H, Steiniche T, et al. Melanoma cell expression of macrophage marker in AJCC stage 1/2 malignant melanoma. J Clin Onco: 28: suppl; abstr e19034. (2010)

Jing S, Trowbridge S. Identification of the intermolecular disulfide bonds of the human transferrin receptor and its lipid-attachment site. EMBO J: 6: 327– 331. (1987)

Joensuu H, Isola J, Lundin M, et al. Amplifi cation of erbB2 and erbB2 expression are superior to estrogen receptor status as risk factors for distant recurrence in pT1N0M0 breast cancer: a nationwide population based study. Clin Cancer Res: 9: 3: 923 – 930. (2003)

Joensuu H, Puputti M, Sihto H, et al. Amplification of genes encoding KIT, PDGFRalpha and VEGFR2 receptor tyrosine kinases is frequent in glioblastoma multiforme. J Pathol: 207: 224–231. (2005)

Johnston D, MacLennan A, Sacks M, et al. Modulation of Bcl-2 and Ki-67 expression in oestrogen receptor-positive human breast cancer by tamoxifen. E J Cancer: 30: 1663-1669. (1994)

Johnston S. New strategies in estrogen receptor-positive breast cancer. Clin Cancer Res: 16:1979-1987. (2010)

Jones R, Salter J, A'Hern R. The prognostic significance of Ki67 before and after neoadjuvant chemotherapy in breast cancer. Br Cancer Res Treat: 116:53–68. (2009)

Jonjic N, Valkovic T, Lucin K, et al. Comparison of microvessel density with tumour associated macrophages in invasive breast carcinoma. Anticancer Res: 18: 3767–3770. (1998)

Jonsson H, Bordás P, Wallin H, et al. Service screening with mammography in Northern Sweden: effects on breast cancer mortality - an update. J Med Screen: 14: 2:87-93. (2007)

Jumppanen M, Gruvberger-Saal S, Kauraniemi P et al. Basal-like phenotype is not associated with patients survival in estrogen-receptor-negative breast cancers. Br Cancer Res: 9: 1: R16. (2007)

Jung S-Y, Han W, Lee J, et al. Ki-67 expression gives additional prognostic information on St. Gallen 2007 and Adjuvant! online risk categories in early breast cancer. Ann Surg Onco: 16:1112–1121. (2009)

Kadouri L, Kote-Jarai Z, Hubert A. Glutathione-S-transferase M1, T1 and P1 polymorphisms, and breast cancer risk, in BRCA1/2 mutation carriers. Br J Cancer: 98: 2006 – 2010. (2008)

Kai K, Nishimura R, Arima N, Miyayama H, Iwase H. P53 expression status is a significant molecular marker in predicting the time to endocrine therapy failure in recurrent breast cancer: a cohort study. Int J Clin Onco: 11: 426–33. (2006)

Karn T, Pusztai L, Holtrich U, et al. Homogeneous Datasets of Triple Negative Breast Cancers Enable the Identification of Novel Prognostic and Predictive Signatures. PLoS ONE: 6:12: e28403. (2011)

Kasami M, Uematsu T, Honda M, et al. Comparison of estrogen receptor, progesterone receptor and Her-2 status in breast cancer pre- and post-neoadjuvant chemotherapy. The Breast: 17: 523-527. (2008)

Kato H, Miyazaki T, Fukai Y, et al. A New Proliferation Marker, Minichromosome Maintenance Protein 2, Is Associated With Tumor Aggressiveness in Esophageal Squamous Cell Carcinoma. J Surg Onco: 84: 24–30. (2003)

Katoh T, Nagata N, Kuroda Y, et al. Glutathione S-transferase M1 (GSTM1) and T1 (GSTT1) genetic polymorphism and susceptibility to gastric and colorectal adenocarcinoma. Carcinogenesis: 17: 1855-1859. (1996)

Katunuma N, Tsuge H, Nukatsuka M, Fukushima M. Structure-based development of cathepsin L inhibitors and therapeutic applications for prevention of cancer metastasis and cancer-induced osteoporosis. Adv in Enzy Regul: 42: 159–172. (2002)

Kaufmann M, Jonat W, Blamey R, et al. Survival analyses from the ZEBRA study. goserelin (Zoladex) versus CMF in premenopausal women with node-positive breast cancer. E J Cancer: 39 :12:1711-7. (2003)

Kawabata H, Germain R, Vuong P et al. Transferrin receptor 2-alpha supports cell growth both in iron-chelated cultured cells and in vivo. J Biol Chem: 275: 16618 – 16625. (2000)

Kawabata H, Nakamaki T, Ikonomi P, et al. Expression of transferrin receptor 2 in normal and neoplastic hematopoietic cells. Blood: 98: 2714– 2719. (2001)

Kawabata H, Yang R, Hirama T, et al. Molecular cloning of transferring receptor 2. A new member of the transferrin receptor-like family. J Biol Chem: 274: 20826 – 20832. (1999)

Kawata E, Ashihara E, Kimura S, et al. Administration of PLK-1 small interfering RNA with atelocollagen prevents the growth of liver metastases of lung cancer. Mol Cancer Ther: 7: 9: 2904–12. (2008)

Keen N, Taylor S. Aurora-kinase inhibitors as anticancer agents. Nat Rev Cancer: 4: 927–936. (2004)

Kelly C, Bernard P, Krishnamurthy S, et al. Agreement in risk prediction between the 21-gene recurrence score assay (Oncotype DX®) and the PAM50 breast cancer intrinsic Classifier™ in early-stage estrogen receptor-positive breast cancer. Oncologist: 17: 4: 492-8. (2012)

Kendall R, Rutledge R, Mao X et al. Vascular endothelial growth factor receptor KDR tyrosine kinase activity is increased by autophosphorylation of two activation loop tyrosine residues. J Biol Chem: 274: 10: 6453–6460. (1999)

Keshgegian A, Cnaan A. Proliferation markers in breast carcinoma. Mitotic figure count, S-phase fraction, proliferating cell nuclear antigen, Ki-67 and MIB-1. Am J Clin Pathol: 104: 1: 42 – 49. (1995)

Khleif S, Doroshow J, Hait W. AACR-FDA-NCI cancer biomarkers collaborative consensus report: advancing the use of biomarkers in cancer drug development. Clin Cancer Res: 16:13: 3299–318. (2010)

Kim C, Bryant J, Horne Z et al. Trastuzumab sensitivity of breast cancer with co-amplification of HER2 and cMYC suggests pro-apoptotic function of dysregulated cMYC in vivo. Br Cancer Res Treat: 94:S6 (2005) (abstr 46)

King S, Purdie C, Bray S ,et al. Immunohistochemical detection of Polo-like Kinase-1 (PLK1) in primary breast cancer is associated with TP53 mutation and poor clinical outcome. Br Cancer Res: 14:R40. (2012)

Kitajima S, Kudo Y, Ogawa I, et al. Constitutive Phosphorylation of Aurora-A on Ser51 Induces Its Stabilization and Consequent Overexpression in Cancer. PLoS ONE: 2: 9: e944. (2007)

Klement G, Huang P, Mayer B, et al. Differences in therapeutic indexes of combination metronomic chemotherapy and an anti-VEGFR-2 antibody in multidrugresistant human breast cancer xenografts. Clin Cancer Res: 8: 221–232. (2002)

Klijn J, Berns P, Schmitz P, et al. The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. Endo Rev: 13: 3 -17. (1992)

Ko Y-J, Small E, Kabbinavar F, et al. A multi-institutional phase II study of SU101, a platelet-derived growth factor receptor inhibitor, for patients with hormone-refractory prostate cancer. Clin Cancer Res: 7: 800 - 5. (2001)

Koda M, Lenczewski A, Sulkowska M, et al. The effect of chemotherapy on status of estrogen receptors in primary tumors and lymph node metastases of human ductal breast cancer. Oncol Rep:17:2:385-91. (2007b)

Koda M, Sulkowska M, Kanczuga-Koda L, et al. The effect of chemotherapy on Ki-67, Bcl-2 and Bak expression in primary tumors and lymph node metastases of breast cancer. Oncol Rep: 18:1:113-9. (2007a)

Kollermann J, Helpap B. Expression of vascular endothelial growth factor (VEGF) and VEGF receptor Flk-1 in benign, premalignant, and malignant prostate tissue. Am J Clin Patho: 116: 115–121. (2001)

Korde L, Lusa L, McShane L, et al. Gene expression pathway analysis to predict response to neoadjuvant docetaxel and capecitabine for breast cancer. Br Cancer Res Treat: 119:3:685-99. (2010)

Kordek R, Potemski P, Kusinska R, et al. Basal keratin expression in breast cancer by quantification of mRNA and by immunohistochemistry. J Exp Clin Cancer Res: 29:39. (2010)

Korsching E, Packeisen J, Agelopoulos K, et al. Cytogenetic alterations and cytokeratin expression patterns in breast cancer: integrating a new model of breast differentiation into cytogenetic pathways of breast carcinogenesis. Lab Invest: 82: 11: 1525 -33. (2002)

Krajewski S, Blomvqvist C, Franssila K, et al. Reduced expression of pro-apoptotic gene Bax is associated with poor response rates to combination chemotherapy and shorter survival in women with metastatic breast adenocarcinoma. Cancer Res: 55: 4471-4478. (1995)

Kroger N, Milde-Langosch K, Riethdorf S, et al. Prognostic and predictive effects of immunohistochemical factors in high-risk primary breast cancer patients. Clin Cancer Res: 12: 159-168. (2006)

Kudoh M, Knee D, Takayama S, Reed C. Bag1 proteins regulate growth and survival of ZR-75-1 human breast cancer cells. Cancer Res: 62: 1904-1909. (2002)

Kumar G, Chavarria G, Charlton-Sevcik A, et al. Design, synthesis, and biological evaluation of potent thiosemicarbazone based cathepsin L inhibitors. Bioorganic & Medicinal Chemistry Letters: 20: 1415–1419. (2010)

Kyndi M, Sørensen F, Knudsen H, et al. Estrogen receptor, progesterone receptor, HER-2, and response to postmastectomy radiotherapy in high-risk breast cancer: The Danish Breast Cancer Cooperative Group. Clin Onco: 26: 1419-1426. (2008)

Laakso M, Tanner M, Nilsson J, et al. Basoluminal carcinoma: a new biologically and prognostically distinct entity between basal and luminal breast cancer. Clin Cancer Res: 12: 4185-91. (2006)

Lah T, Cercek M, Blejec A, et al. Cathepsin B, a prognostic indicator in lymph node-negative breast carcinoma patients: comparison with Cathepsin D, Cathepsin L, and other clinical indicators. Clin Cancer Res: 6: 578–584. (2000)

Lah T, Durán Alonso M, Van Noorden C. Antiprotease therapy in cancer: hot or not? Expert Opinion on Biol Therapy: 6: 3: 257–279. (2006)

Lakhani S, Reis-Filho J, Fulford L, et al. Prediction of BRCA1 status in patients with breast cancer using estrogen receptor and basal phenotype. Clin Cancer Res: 11: 14: 5175-80. (2005)

Lankelma J, Voorend D, Barwari T, et al. Cathepsin L, target in cancer treatment? Review Article. Life Sciences: 86: 7-8: 225 – 233. (2010)

Laske D, Youle R, Oldfield E. Tumor regression with regional distribution of the targeted toxin TF-CRM107 in patients with malignant brain tumours. Nat Med: 3: 12: 1362-8. (1997)

Lavender N, Benford M, VanCleave T. Examination of polymorphic glutathione S-transferase (GST) genes, tobacco smoking and prostate cancer risk among Men of African Descent: A case-control study. BMC Cancer: 9:397;doi:10.1186/1471-2407-9-397. (2009)

Lee A, Gillett C, Ryder K, et al. Different patterns of inflammation and prognosis in invasive carcinoma of the breast. Histopathology: 48: 692–701. (2006)

Lee E, Frolov A, Li R, et al. Targeting Aurora kinases for the treatment of prostate cancer. Cancer Res: 66: 4996–5002. (2006)

Lee J, Im Y, Lee S, et al. Evaluation of ER and Ki-67 proliferation index as prognostic factors for survival following neoadjuvant chemotherapy with doxorubicin/docetaxel for locally advanced breast cancer. Cancer Chemother Phar: 6:4:569-77. (2008)

Lee K, Im S, Oh D, et al. Prognostic significance of bcl-2 expression in stage III breast cancer patients who had received doxorubicin and cyclophosphamide followed by paclitaxel as adjuvant chemotherapy. BMC Cancer: 7:63. (2007)

Leek R, Lewis C, Whitehouse R, et al. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res: 56: 4625– 4629. (1996)

Leica data sheet, product code NCL-L-Ki67-MM1. www.Leica.com accessed on 02.08.13

Leo J, Wang S, Guo C, et al. Gene regulation profile reveals consistent anticancer properties of progesterone in hormone-independent breast cancer cells transfected with progesterone receptor. Int J Cancer: 117: 561–568. (2005)

Lev D, Kim S, Onn A, et al. Inhibition of platelet-derived growth factor receptor signaling restricts the growth of human breast cancer in the bone of nude mice. Clin Cancer Res: 11:1:306-14. (2005)

Levicar N, Dewey R, Daley E, et al. Selective suppression of cathepsin L by antisense cDNA impairs human brain tumor cell invasion in vitro and promotes apoptosis. Cancer Gene Therapy: 10: 2: 141–151. (2003)

Lewallen S, Courtright P. Epidemiology in Practice: Case-control studies. Community Eye Health: 11:28: 57–58. (1998)

Li X, Liu M, Zhang Y, et al. CK5/6, EGFR, Ki-67, cyclin D1, and nm23-H1 protein expressions as predictors of pathological complete response to neoadjuvant chemotherapy in triple-negative breast cancer patients. Med Onco: Sppl 1: 129-34. (2011)

Liang Y, Brekken R, Hyder S. Vascular endothelial growth factor induces proliferation of breast cancer cells and inhibits the anti-proliferative activity of anti-hormones. Endocrine-Related Cancer: 13: 905 – 919. (2006)

Lin E, Pollard J. Tumor-associated macrophages press the angiogenic switch in breast cancer. Cancer Res: 67: 5064–5066. (2007)

Lin V, Eng A, Hen N, et al. Effect of progesterone on the invasive properties and tumour growth of progesterone receptor-transfected breast cancer cells MDA-MB-231. Clin Cancer Res: 7: 2880–2886. (2001)

Lin Y, Berg A, Iyengar P, et al. The hyperglycemia-induced inflammatory response in adipocytes: the role of reactive oxygen species. J Biol Chem: 280: 4617–26. (2005)

Linderholm B, Hellborg H, Johansson, et al. Vascular endothelial growth factor receptor 2 and downstream p38 mitogen-activated protein kinase are possible candidate markers of intrinsic resistance to adjuvant endocrine treatment in steroid receptor positive breast cancer. Br Cancer Res Treat: 125: 2: 457-65. (2011)

Lipton A, Ali S, Leitzel K, et al. Elevated serum Her-2/neu level predicts decreased response to hormone therapy in metastatic breast cancer. J Clin Onco: 20: 1467-1472. (2002)

Liu R, Wang X, Chen G, et al. The prognostic role of a gene signature from tumorigenic breast cancer cells. N Eng J Med: 356: 217-226. (2007)

Liu S, Chia S, Mehl E, et al. Progesterone receptor is a significant factor associated with clinical outcomes and effect of adjuvant tamoxifen therapy in breast cancer patients. Br Cancer Res Treat: 119: 53–61. (2010)

Liu S, Edgerton S, Moore D, Thor A. Measures of cell turnover (proliferation and apoptosis) and their association with survival in breast cancer. Clin Cancer Res: 7: 6: 1716 – 1723. (2001)

Liu X, Erikson R. Polo-like kinase (Plk) 1 depletion induces apoptosis in cancer cells. Proc Natl Acad Sci USA: 100: 5789–5794. (2003)

Livasy C, Karaca G, Nanda R, et al. Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. Mod Pathol: 19: 264–71. (2006)

Loddo M, KingsburyS, Rashid M, et al. Cell-cycle-phase progression analysis identifies unique phenotypes of major prognostic and predictive significance in breast cancer. Br J Cancer: 100: 959 – 970. (2009)

Loi S, Haibe-Kains B, Desmedt C, et al. Predicting prognosis using molecular profiling in estrogen receptor-positive breast cancer treated with tamoxifen. BMC Genomics: 9:239. (2008)

Loktionov A, Watson M, Gunter M, et al. Glutathione-S-transferase gene polymorphisms in colorectal cancer patients: interaction between GSTM1 and GSTM3 allele variants as a risk modulating factor. Carcinogenesis: 22: 7: 1053-1060. (2001)

Luck A, Evans A, James J, et al. Breast Carcinoma with Basal Phenotype: Mammographic Findings. AJR: 191: 346–351. (2008)

Ludwig Breast Cancer Study Group. Combination adjuvant chemotherapy for node-positive breast cancer. Inadequacy of a single perioperative cycle. N Eng J Med: 319:677-683. (Trial V) (1988)

Lynn K, Udugamasooriya G, Roland C, et al. GU81, a VEGFR2 antagonist peptoid, enhances the anti-tumour activity of doxorubicin in the murine MMTV-PyMT transgenic model of breast cancer. BMC Cancer: 10: 397. (2010)

Ma X, Hilsenbeck S, Wang W, et al. The HOXB13:IL17BR Expression Index Is a Prognostic Factor in Early-Stage Breast Cancer. J Clin Onco: 24:4611-4619. (2006)

Ma X, Wang Z, Ryan P, et al. A two-gene expression ratio predicts clinical outcome in breast cancer patients treated with tamoxifen. Cancer Cell: 5 :6:607-616. (2004)

Ma X-J, Salunga R, Dahiya S, et al. A Five-Gene Molecular Grade Index and HOXB13:IL17BR are complementary prognostic factors in early stage breast cancer. Clin Cancer Res: 14: 2601-2608. (2008)

Macabeo-Onga M, Shiboskia C, Silverman S, et al. Quantitative analysis of cathepsin L mRNA and protein expression during oral cancer progression. Oral Onco: 39: 638–647. (2003)

Macarulla T, Cervantes A, Elez E, et al. Phase I study of the selective Aurora A kinase inhibitor MLN8054 in patients with advanced solid tumors: safety, pharmacokinetics, and pharmacodynamics. Mol Cancer Ther: 9:10:2844-52.

MacGrogan G, Mauriac L, Durand M, et al. Primary chemotherapy in breast invasive carcinoma: predictive value of the immunohistochemical detection of hormonal receptors, p53, c-erbB-2, MiB1, pS2 and GST pi. Br J Cancer: 74: 1458–1465. (1996)

Maeda H, Akaike T. Nitric oxide and oxygen radicals in infection, inflammation, and cancer. Biochemistry (Moscow): 63: 854–65. (1998)

Malamou-Mitsi V, Gogas H, Dafni U, et al. Evaluation of the prognostic and predictive value of p53 and Bcl-2 in breast cancer patients participating in a randomized study with dose-dense sequential adjuvant chemotherapy. Ann Onco: 17: 1504–1511. (2006)

Mamounas E, Tang G, Fisher B, et al. Association between the 21-gene recurrence score assay and risk of locoregional recurrence in node-negative, estrogen receptor-positive breast cancer: results from NSABP B-14 and NSABP B-20. J Clin Onco: 28:10:1677-83. (2010)

Manfredi M, Ecsedy J, Meetze K, et al. Antitumor activity of MLN8054, an orally active small-molecule inhibitor of Aurora A kinase. Proc. Nat Acad Sci USA: 104: 4106 – 4111. (2007)

Mantovani A, Bottazzi B, Colotta F, et al. The origin and function of tumour associated macrophages. Immuno Today: 13: 265–270. (1992)

Marumoto T, Zhang D, Saya H. Aurora-A—a guardian of poles. Nat Rev Cancer: 5: 42–50. (2005)

Matulonis U, Sharma S, Ghamande S, et al. Phase II study of MLN8237 (alisertib), an investigational Aurora A kinase inhibitor, in patients with platinum-resistant or -refractory epithelial ovarian, fallopian tube, or primary peritoneal carcinoma. Gynecol Onco: 127:1:63-95. (2012)

McCarty K, Santella R, Steck S, et al. PAH–DNA Adducts, Cigarette Smoking, GST Polymorphisms, and Breast Cancer Risk. Envi Health Perspec: 117: 4: 552-8. (2009)

McDermott U, Ames R, Iafrate J, et al. Ligand-dependent PDGF receptor-alpha activation sensitizes rare lung cancer and sarcoma cells to PDGF receptor kinase inhibitors. Cancer Res: 69: 9: 3937–3946. (2009)

Meche A, Cîmpean A, Raica M. Immunohistochemical expression and significance of epidermal growth factor receptor (EGFR) in breast cancer. Romanian J Morph Embry: 50: 2: 217–221. (2009)

Mele T, Generali D, Fox S, et al. Anti-angiogenic effect of tamoxifen combined with epirubicin in breast cancer patients. Br Cancer Res Treat: 123: 3: 795 – 804. (2010)

Meng M, Grossfeld G, Williams G, et al. Minichromosome Maintenance Protein 2 Expression in Prostate: Characterization and Association with Outcome after Therapy for Cancer. Clin Cancer Res: 7: 2712–2718. (2001)

Mikalsen T, Gerits N, Moens U. Inhibitors of signal transduction protein kinases as targets for cancer therapy. Biotechnol Annu Rev:12:153-223. Review (2006)

Millar E, Anderson L, McNeil C, et al. BAG-1 predicts patient outcome and tamoxifen responsiveness in ER-positive invasive ductal carcinoma of the breast. Br J Cancer: 100: 123 – 133. (2009)

Millar K, Anderson L, McNeil C, et al.BAG-1 predicts patient outcome and tamoxifen responsiveness in ER-positive invasive ductal carcinoma of the breast. Br J Cancer:100:123-133. (2009)

Miller K, Burstein H, Elias A, et al. Phase II study of SU11248, a multitargeted receptor tyrosine kinase inhibitor (TKI), in patients (pts) with previously treated metastatic breast cancer (MBC). J Clin Onco: 23:19s. (2005) (suppl; abstr 563)

Miyoshi Y, Iwao K, Egawa C, Noguchi S. Association of centrosomal kinase STK15/BTAK mRNA expression with chromosomal instability in human breast cancers. Int J Cancer: 92: 370 – 3. (2001)

Moll R. Cytokeratins as markers of differentiation in the diagnosis of epithelial tumors. Subcell Biochem: 31: 205-262. (1998)

Mook S, Knauer M, Bueno-de-Mesquita J, et al. Metastatic potential of T1 breast cancer can be predicted by the 70-gene MAmmaPrint Signature. Ann Sur Onco: 17: 5: 1406 – 1413. (2010)

Muss H, Thor A, Berry D, et al. c-erbB-2 expression and response to adjuvant therapy in women with node-positive early breast cancer. N Eng J Med: 330: 1260-1266. (1994)

Nadler Y, Camp R, Giltnane J, et al. Expression patterns and prognostic value of Bag-1 and Bcl-2 in breast cancer. Br Cancer Res: 10: R35. (doi:10.1186/bcr1998) (2008b)

Nadler Y, Camp R, Schwartz C et al. Expression of Aurora A (but Not Aurora B) Is Predictive of Survival in Breast Cancer. Clin Cancer Res: 14: 14: 4455- 4462. (2008a)

Naishiro Y, Adachi M, Okuda H, et al. BAG-1 accelerates cell motility of human gastric cells. Oncogene: 18: 3244–3251. (1999)

Navab R, Pedraza C, Fallavollita L, et al. Loss of responsiveness to IGF-I in cellswith reduced cathepsin L expression levels. Oncogene: 27: 37: 4973–4985. (2008)

Neels J, Olefsky J. Inflamed fat: what starts the fire? J Clin Invest: 116: 33–5. (2006)

Neven P, Huang H, Vanspauwen R, et al: The prognostic and predictive value of the progesterone receptor in women with an oestrogen receptor positive breast cancer. E J Cancer: 2: 46 – 48. (2004)

Nielsen T, Hsu F, Jensen K, et al. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res: 10:5367-5374. (2004)

Nieto Y, Nawaz F, Jones R, et al. Prognostic Significance of Overexpression and Phosphorylation of Epidermal Growth Factor Receptor (EGFR) and the Presence of Truncated EGFRvIII in Locoregionally Advanced Breast Cancer. J Clin Onco: 25: 4405-4413. (2007)

Nieto Y, Nawaz S, Shpall E, et al.Long-Term Analysis and Prospective Validation of a Prognostic Model for Patients with High-Risk Primary Breast Cancer Receiving High-Dose Chemotherapy: Clin Cancer Res:10:2609–2617. 2609- 2617. (2004)

Nocito A, Bubendorf L, Tinner E, et al. Microarrays of bladder cancer tissue are highly representative of proliferation index and histological grade. J Patho: 194: 3: 349 – 357. (2001)

Nofech-Mozes S, Trudeau M, Kahn H, et al. Patterns of recurrence in the basal and non-basal subtypes of triplenegative breast cancers, Br Cancer Res Treat: doi 10.1007/s10549–008–0295–8. (2009)

Nogawa M, Yuasa T, Kimura S, et al. Intravesical administration of small interfering RNA targeting PLK-1 successfully prevents the growth of bladder cancer. J Clin Invest: 115: 978–985. (2005)

Nogi H, Kobayashi T, Suzuki M. EGFR as paradoxical predictor of chemosensitivity and outcome among triple-negative breast cancer. Onco Rep: 21: 2: 413-7. (2009)

Normanno N, Campiglio M, De Luca A, et al. Cooperative inhibitory effect of ZD1839 (Irresa) in combination with trastuzumab (Herceptin) in human breast cancer cell growth. Ann Onco: 13: 65-72. (2002)

O'Driscoll L, Linehan R, Kennedy S. Lack of prognostic significance of survivin, survivin-DEx3, survivin-2B, galectin-3, bag-1, bax-a and MRP-1 mRNAs in breast cancer. Cancer Letters: 201: 225–236. (2003)

Öberg A, Samii S, Stenling R, Lindmark G. Different occurrence of CD8+, CD45R0+, and CD68+ immune cells in regional lymph node metastases from colorectal cancer as potential prognostic predictors. Int J Colorectal Dis: 17: 25–29. (2002)

Ogawa E, Takenaka K, Katakura H, et al. Perimembrane Aurora-A Expression is a Significant Prognostic Factor in Correlation with Proliferative Activity in Non-Small-Cell Lung Cancer (NSCLC). Ann Surg Onco: 15: 2: 547–554. (2008)

Ogston K, Miller I, Schofield C, et al. Can patients' likelihood of benefiting from primary chemotherapy for breast cancer be predicted before commencement of treatment? Br Cancer Res Treat: 86: 181–189. (2004)

Oh D, Troester M, Usary J et al. Estrogen-Regulated Genes Predict Survival in Hormone Receptor–Positive Breast Cancers. J Clin Onco: 24:1656-1664. (2006)

Okada T, Sawada T, Osawa T, et al. MK615 inhibits pancreatic cancer cell growth by dual inhibition of Aurora A and B kinases. W J Gastroenterol: 14: 9: 1378-1382. (2008)

Oliveira A, Rodrigues F, Santos R, et al. GSTT1, GSTM1, and GSTP1 polymorphisms and chemotherapy response in locally advanced breast cancer. Genetics and Mol Res: 9:2: 1045-1053. (2010)

Ory K, Lebeau J, Levalois C, et al. Apoptosis inhibition mediated by medroxyprogesterone acetate treatment of breast cancer cell lines. Br Cancer Res Treat: 68: 187– 198. (2001)

Ostman A. PDGF receptors-mediators of autocrine tumour growth and regulators of tumour vasculature and stroma. Cytokine Growth Factor Rev: 15: 275–286. (2004)

O'Sullivan C, Lewis C, Harris A, McGee J. Secretion of epidermal growth factor by macrophages associated with breast carcinoma. Lancet: 342:8864:148-9. (1993)

Otterbach F, Bankfalvi A, Bergner S, et al. Cytokeratin 5 ⁄ 6 immunohistochemistry assists the differential diagnosis of atypical proliferations of the breast. Histopath: 37: 232–240. (2000)

Otterbach F, Bankfalvi A, Bergner S, et al. Cytokeratin 5/6 immunohistochemistry assists the differential diagnosis of atypical proliferations of the breast. Histopathology:37:232-40. (2000)

Owusu C, Buist D, Field T, et al. Predictors of tamoxifen discontinuation among older women with estrogen receptor-positive breast cancer. J Clin Onco: 26:4:549–55. (2008)

Paik S, Shak S, Tang G, et al. A multigene assay to predict recurrence of tamoxifen treated, node-negative breast cancer. N Eng J Med: 351:27: 2817–2826. (2004)

Paik S, Tan-Chui E, Bryan J, et al. Successful quality assurance program for HER-2 testing in the NSABP trial for Herceptin. Br Cancer Res Treat: 76: suppl 1: S31. (2002)

Paik S, Tang G, Shak S. Gene expression and benefit of chemotherapy in women with node-negative, estrogen receptor-positive breast cancer. J Clin Onco: 24: 3726– 3734. (2006)

Parise C, Bauer K, Brown M, Caggiano V. Breast Cancer Subtypes as Defined by the Estrogen Receptor (ER), Progesterone Receptor (PR), and the Human Epidermal Growth Factor Receptor 2 (HER2) among Women with Invasive Breast Cancer in California, 1999–2004. The Breast J:15:6:593–602 (2009)

Park D, Kåresen R, Noren T, Sauer T. Ki-67 expression in primary breast carcinomas and their axillary lymph node metastases: clinical implications. Virchows Arch: 451:1:11-8. (2007)

Park K, Han S, Shin E, et al. EGFR gene and protein expression in breast cancers. E J Surg Onco: 33: 956-960. (2007)

Parker J, Mullins M, Cheang M, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Onco: 27:1160–7. (2009)

Penault-Llorca F, Abrial C, Raoelfils I, et al. Changes and predictive and prognostic value of the mitotic index, Ki67, cyclin D1, and cyclo-oxygenase-2 in 710 operable breast cancer patients treated with neoadjuvant chemotherapy. Oncologist: 13: 1235–45. (2008)

Perez E, Jenkins R, Duec A, et al. C-MYC Alterations and Association With Patient Outcome in Early-Stage HER2-Positive Breast Cancer From the North Central Cancer Treatment Group N9831 Adjuvant Trastuzumab Trial. Clin Onco: 29:651-659. (2011a)

Perez E, Romond E, Suman V, et al. Four-year follow-up of trastuzumab plus adjuvant chemotherapy for operable human epidermal growth factor receptor 2-positive breast cancer: joint analysis of data from NCCTG N9831 and NSABP B-31. J Clin Onco: 29: 25:3366 -73. (2011b)

Perou C, Jeffrey S, van de Rijn M, et al. Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. Proc Nat Acad Sci USA: 96: 16: 9212 – 9217. (1999)

Perou C, Sorlie T, Eisen M, et al. Molecular portraits of human breast tumours. Nature: 406: 747–752. (2000)

Perreard L, Fan C, Quackenbush J, et al. Classification and risk stratification of invasive breast carcinomas usng a real time quantitatitive RT-PCR assay. Br Cancer Res: 8: R23. (2006)

Peto R, Boreham J, Clarke M, et al. UK and USA breast cancer deaths down 25% in year 2000 at ages 20—69 years. Lancet: 355: 9217: 1822. (2000)

Piao J-M, Shin M-H, Kweon S-S, et al. Glutathione-S-transferase (GSTM1, GSTT1) and the risk of gastrointestinal cancer in a Korean population. W J Gastroenterol: 15: 45: 5716-5721. (2009)

Piekarski J, Biernat W. Clinical significance of CK5⁄6 and PTEN protein expression in patients with bilateral breast carcinoma. Histopathology: 49: 248–255. (2006)

Planas-Silva M, Bruggeman R, Grenko R, Smith J. Overexpression of c-Myc and Bcl-2 during progression and distant metastasis of hormone-treated breast cancer. Exp Molecular Path: 82: 85–90. (2007)

Plyte S, Musacchio A. PLK1 inhibitors: setting the mitotic death trap. Curr Biol: 17: R280–R283. (2007)

Poole C, Earl H, Hiller L, et al. Epirubicin and cyclophosphamide, methotrexate, and fluorouracil as adjuvant therapy for early breast cancer. N Eng J Med. 355: 18: 1851-62. (2006)

Potemski P, Pluciennik E, Bednarek A, et al. Ki-67 expression in operable breast cancer: a comparative study of immunostaining and a real-time RT-PCR assay. Pathol Res Pract. 202: 7: 491-5. (2006)

Pouessel D, Culine S, Verhoest G, et al. Renal cell carcinoma and antiangiogenic therapies. Presse Med: 37: 4: 2: 628-633. (2008).

Prat A, Parker J, Fan C, et al. Concordance among gene expression-based predictors for ER-positive breast cancer treated with adjuvant tamoxifen. Ann Onco: doi:10.1093/annonc/mds080. (2012)

Pupa S, Bufalino R, Invernizzi A, et al. Macrophage infiltrate and prognosis in c-erbB-2-overexpressing breast carcinomas. J Clin Onco: 14: 85–94. (1996)

Qian Y, Hua E, Bisht K, et al. Inhibition of Polo-like kinase 1 prevents the growth of metastatic breast cancer cells in the brain. Clin Exp Metastasis: 28: 8: 899-908. (2011)

Qiu L-X, Yuan H, Yu K-D, et al. Glutathione S-transferase M1 polymorphism and breast cancer susceptibility: a meta-analysis involving 46,281 subjects. Br Cancer Res Treat: DOI 10.1007/s10549-009-0636-2. (2009)

Ramaswamy S, Ross K, Lander E, Golub T. A molecular signature of metastasis in primary solid tumours. Nat Genet: 33:1:49-54. (2002)

Ramnath N, Hernandez F, Tan et D-F, et al. MCM2 is an independent predictor of survival in patients with non–small-cell lung cancer. J Clin Onco: 19:4259 -4266. (2001)

Rampaul R, Pinder S, Elston C, Ellis I; Nottingham Breast Team. Prognostic and predictive factors in primary breast cancer and their role in patient management: The Nottingham Breast Team. E J Surg Onco: 27:3:229-38. (2001)

Ravdin P, Siminoff I, Harvey J. Survey of breast cancer patients concerning their knowledge and expectations of adjuvant therapy. J Clin Onco: 16:2:515-21. (1998)

Ravdin P, Siminoff L, Davis G, et al. Computer program to assist in making decisions about adjuvant therapy for women with early breast cancer. J Clin Onco: 19:4:980-991. (2001)

Rebbaa A. Targeting senescence pathways to reverse drug resistance in cancer. Cancer Letters: 219: 1: 1–13. (2005)

Rebbeck T, Walker A, Jaffe J, et al. Glutathione S-transferase-mu (GSTM1) and -theta (GSTT1) genotypes in the etiology of prostate cancer. Cancer Epidemiol Biomarkers Prev: 8:4 Pt 1:283-7. (1999)

Reed J. Bcl-2 and the regulation of programmed cell death. J Cell Biology: 124: 1-6. (1994)

Regan M, Leyland-Jones B, Bouzyk M, et al. CYP2D6 genotype and tamoxifen response in postmenopausal women with endocrine-responsive breast cancer: the breast international group 1-98 trial. J Nat Cancer Inst: 104: 6: 441-51. (2012)

Reis-Filho J, Tutt A: Triple negative tumours: a critical review. Histopath: 52: 108-118. (2008)

Richer J, Jacobsen B, Manning N, et al. Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. J Biol Chem: 277: 5209 – 5218. (2002)

Rodriguez-Pinilla S, D Sarrio, E Honrado, et al. Prognostic Significance of Basal-Like Phenotype and Fascin Expression in Node-Negative Invasive Breast Carcinomas. Clin Cancer Res: 12: 5: 1533-39. (2006)

Rodríguez-Pinilla S, Sarrió D, Honrado E, et al. Vimentin and laminin expression is associated with basal-like phenotype in both sporadic and **BRCA1**-associated breast carcinomas. J Clin Patho: 60: 9: 1006-12. (2007)

Ross J, Slodkowska E, Symmans W, et al. The HER-2 receptor and breast cancer: ten years of targeted anti–HER-2 therapy and personalized medicine. Oncologist: 14: 320–368. (2009)

Rouzier R, Perou C, Symmans W, et al. Breast cancer molecular subtypes respond differently to preoperative chemotherapy. Clin Cancer Res:11: 5678–85. (2005a)

Rouzier R, Pusztai L, Delaloge S, et al. Normograms to predict pathologic complete response and metastases free survival after preoperative chemotherapy for breast cancer. J Clin Onco: 23: 8331-9. (2005b)

Royce M, Xia W, Sahin A, et al. STK15/aurora-A expression in primary breast tumors is correlated with nuclear grade but not with prognosis. Cancer: 100: 12 - 9. (2004)

Rydén L, Jirström K, Bendahl P, et al. Tumor-specific expression of vascular endothelial growth factor receptor 2 but not vascular endothelial growth factor or human epidermal growth factor receptor 2 is associated with impaired response to adjuvant tamoxifen in premenopausal breast cancer. J Clin Onco: 23: 4695 - 4704. (2005)

Ryden L, Jirstrom K, Haglund M, et al. Epidermal growth factor receptor and vascular endothelial growth factor receptor 2 are specific biomarkers in triple-negative breast cancer. Results from a controlled randomized trial with long-term follow-up. Br Cancer Res Treat: 120: 2: 491-8. (2010)

Ryden L, Linderholm B, Nielsen N, et al. Tumor specific VEGF-A and VEGFR2/KDR protein are co-expressed in breast cancer. Br Cancer Res Treat: 82:147-154. (2003)

Sabatier R, Finetti P, Cervera N et al. Gene expression profiling and its utility in prediction of local relapse after breast-conserving therapy in early breast cancer. Cancer Genomics Proteomics: 8:4:199-209. (2011)

Sandri M, Johansson H, Zorzino L, et al. Serum EGFR and serum HER-2/neu are useful predictive and prognostic markers in metastatic breast cancer patients treated with metronomic chemotherapy. Cancer: 110: 509 – 17. (2007)

Sant M, Francisci S, Capocaccia R, et al. Time trends of breast cancer survival in Europe in relation to incidence and mortality. Int J Cancer: 119:10:2417-22. (2006)

Santa Crutz data sheet - SC560003. http://datasheets.scbt.com/sc-56003.pdf, accessed on 02.08.13

Santamaria I, Velasco G, Cazorla M, et al. Cathepsin L2, a novel human cysteine proteinase produced by breast and colorectal carcinomas. Cancer Res: 58: 1624–30. (1998)

Sari E, Guler G, Hayran M, et al. Comparative study of the immunohistochemical detection of hormone receptor status and HER-2 expression in primary and paired recurrent/metastatic lesions of patients with breast cancer. Med Oncol: 28: 1: 57-63. (2011)

Schaffer A, Muller-Landner U, Scholmerich J, Buchler C. Role of adipose tissue as an inflammatory organ in human diseases. Endo Rev: 27: 449–67. (2006)

Scharer C, Laycock N, Osunkoya A, et al. Aurora kinase inhibitors synergize with paclitaxel to induce apoptosis in ovarian cancer cells. J Trans Med: 6: 79. (2008)

Schmit T, Ledesma M, Ahmad N. Modulating Polo-Like Kinase 1 as a Means for Cancer Chemoprevention. Pharm Res: 27: 6: 989–998. (2010)

Schmit T, Zhong W, Setaluri V, et al. Targeted depletion of Polo-like kinase (Plk) 1 via lentiviral shRNA or a small-molecule inhibitor causes mitotic catastrophe and induction of apoptosis in human melanoma cells. J Invest Dermatol: 129: 12: 2843 – 2853. (2009)

Schneider L, Clement C, Teilmann S, et al. PDGFRalpha signalling is regulated through the primary cilium in fibroblasts. Curr Biol: 15: 1861– 6. (2005)

Scholl S, Pallud C, Beuvon F, et al. Anti-Colony-Stimulating Factor-1 Antibody Staining in Primary Breast Adenocarcinomas Correlates With Marked Inflammatory Cell Infiltrates and Prognosis. Nat Cancer Inst: 86:120 -126. (1994)

Scholzen T, Gerdes J. The Ki-67 Protein: From the Known and the Unknown. J Cell Physio: 182:311–322. (2000)

Searle J, Krishan A, Frei E. Correspondence. Cancer Res : 35:2900-2901 (1975)

Seigneurin A, François O, Labarère J, et al. Overdiagnosis from non-progressive cancer detected by screening mammography: stochastic simulation study with calibration to population based registry data. Br Med J: 343:d7017. doi:10.1136/bmj.d7017. (2011)

Setiawan V, Zhang Z, Yu G, et al. GSTT1 and GSTM1 null genotypes and the risk of gastric cancer: a case-control study in a Chinese population. Cancer Epi Biomarkers Prev: 9: 73-80. (2000)

Shabo I, Sta O, Olsson H, et al. Breast cancer expression of CD163, a macrophage scavenger receptor, is related to early distant recurrence and reduced patient survival. Int J Cancer: 123: 780–786. (2008)

Shindoh M, Adachi M, Higashino F, et al. BAG-1 expression correlates highly with the malignant potential in early lesions (T1 and T2) of oral squamous cell carcinoma. Oral Oncology: 36: 444-449. (2000)

Shou J, Massarweh S, Osborne C, et al. "Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer," J Nat Cancer Inst: 96: 12: 926–935. (2004)

Sihto H, Lundin J, Lehtimäki T, et al. Molecular subtypes of breast cancers detected in mammography screening and outside of screening. Clin Cancer Res.14:13: 4103-10. (2008)

Sivoňová M, Waczulíková I, Dobrota D, et al. Polymorphisms of glutathione-S-transferase M1, T1, P1 and the risk of prostate cancer: a case-control study. J Exp Clin Cancer Res: 28: 32. (2009)

Sjostrom J, Blomqvist C, von Boguslawski K, et al. The predictive value of bcl-2, bax, bcl-xL, bag-1, fas, and fasL for chemotherapy response in advanced breast cancer. Clin Cancer Res: 8: 811-816. (2002)

Sjöström-Mattson J, Von Boguslawski K, Bengtsson N, et al. The expression of p53, bcl-2, bax, fas and fasL in the primary tumour and lymph node metastases of breast cancer. Acta Onco: 48: 8: 1137-43. (2009)

Slamon D, Clark G, Wong S, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science: 235: 177-182. (1987)

Slamon D, Eiermann W, Robert N, et al. Adjuvant trastuzumab in HER2-positive breast cancer. N Eng J Med: 365: 14: 1273-83. (2011)

Song J, Takeda M, Morimoto R. Bag1-Hsp70 mediates a physiological stress signalling pathway that regulates Raf-1/ERK and cell growth. Nat Cell Biol: 3: 276–282. (2001)

Sorlie T, Perou C, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumour subclasses with clinical implications. Proc Nat Acad Sci USA: 98: 10869–10874. (2001)

Sorlie T, Tibshirani R, Parker J, et al. Repeated observation of breast tumour subtypes in independent gene expression data sets. Proc Natl Acad Sci USA: 100: 8418–8423. (2003)

Sorlie T. Molecular portraits of breast cancer: tumour subtypes as distinct disease entities. E J Cancer: 40: 2667 - 75. (2004)

Sotiriou C, Neo S, McShane L, et al. Breast cancer classification and prognosis based on gene expression profiles from a population-based study. Proc Nat Acad Sci USA:100: 10393-10398. (2003)

Sotiriou C, Wirapati P, Loi S, et al. Gene expression profiling in breast cancer: understanding the molecular basis of histologic grade to improve prognosis. J Nat Cancer Inst: 98: 4: 262-72. (2006)

Spankuch B, Kurunci-Csacsko E, Kaufmann M, Strebhardt K. Rational combinations of siRNAs targeting Plk1 with breast cancer drugs. Oncogene: 26: 5793–5807. (2007)

Spankuch-Schmitt B, Bereiter-Hahn J, Kaufmann M, et al. Effect of RNA silencing of polo-like kinase-1 (PLK1) on apoptosis and spindle formation in human cancer cells. J Nat Cancer Inst: 94: 1863–1877. (2002a)

Spankuch-Schmitt B, Wolf G, Solbach C, et al. Downregulation of human polo-like kinase activity by antisense oligonucleotides induces growth inhibition in cancer cells. Oncogene: 21: 3162 – 3171. (2002b)

Spurdle A, Fahey P, Chen X, et al. Pooled analysis indicates that the GSTT1 deletion, GSTM1 deletion, and GSTP1 Ile105Val polymorphisms do not modify breast cancer risk in BRCA1 and BRCA2 mutation carriers. Br Cancer Res Treat: DOI 10.1007/s10549-009-0601-0. (2009)

Stal O, Sullivan S, Wingren S, et al. c-erbB-2 expression and benefit from adjuvant chemotherapy and radiotherapy of breast cancer. E J Cancer: 31A: 2185-2190. (1995)

Steck S, Gaudet M, Britton J, et al. Interactions among GSTM1, GSTT1 and GSTP1 polymorphisms, cruciferous vegetable intake and breast cancer risk. Carcinogenesis: 28: 9: 1954–1959. (2007)

Stendahl M, Rydén L, Nordenskjöld B, et al. High progesterone receptor expression correlates to the effect of adjuvanttamoxifen in premenopausal breast cancer patients. Clin Cancer Res: 12: 4614-4618. (2006)

Stingl J, Caldas C. Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis. Nat Review: 7: 791 – 799 (2007)

Stock P, Monga D, Tan X, et al. Platelet-derived growth factor receptor-A: a novel therapeutic target in human hepatocellular cancer. Mol Cancer Ther: 6: 7. (2007)

Stoka V, Turk B, Schendel S, et al. Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. J Biol Chem: 276:5:3149-57. (2001)

Strebhardt K & Ullrich A. Targeting polo-like kinase 1 for cancer therapy. Nat Reviews Cancer: 6: 321-330. (2006)

Strojnik T, Kavalar R, Zaje I, et al. Prognostic impact of CD-68 and kallikrein 6 in human glioma. Anticancer Res: 29: 3269 – 3279. (2009)

Svatek R, Karam J, Karakiewicz P, et al. Role of urinary cathepsin b and l in the detection of bladder urothelial cell carcinoma. J Uro: 179: 478 – 484. (2008)

Symmans W, Hatzis C, Sotiriou C, et al. Genomic Index of Sensitivity to Endocrine Therapy for Breast Cancer. J Clin Onco: 28:27: 4111-4119. (2010)

Takahashi N, Yanagihara M, Ogawa Y, et al. Down-regulation of Bcl-2-interacting protein BAG-1 confers resistance to anti-cancer drugs. Biochem Biophys Res Commun: 301: 798–803. (2003)

Takahashi Y, Cleary K, Mai M, et al. Significance of vessel count and vascular endothelial growth factor and its receptor (KDR) in intestinal-type gastric cancer. Clin Cancer Res: 2: 1679–1684. (1996)

Tamura T, Minami H, Yamada Y, et al. A phase I dose escalation study of ZD6474 in Japanese patients with solid, malignant tumors. J Thorac Onco: 1:1002-1009. (2006)

Tanaka T, Kimura M, Matsunaga K, et al. Centrosomal kinase AIK1 is overexpressed in invasive ductal carcinoma of the breast. Cancer Res: 59: 9:2041- 4. (1999)

Tang S, Beck J, Murphy S, et al. BAG-1 expression correlates with Bcl-2, p53, differentiation, estrogen and progesterone receptors in invasive breast carcinoma. Br Cancer Res Treat: 84: 203–213. (2004).

Tang S, Shehata N, Chernenko G, et al. Expression of BAG-1 in invasive breast carcinomas. J Clin Onco: 17: 1710-1719. (1999)

Taniguchi E, Nishijo K, McCleish A. PDGFR-A is a Therapeutic Target in Alveolar Rhabdomyosarcoma. Oncogene: 27: 51: 6550 - 60. (2008)

Tatsuka M, Sato S, Kanda A, et al. Oncogenic Role of Nuclear Accumulated Aurora-A. Mol Carcinogenesis: 48: 810–820 (2009)

Teschendorff A, Miremadi A, Pinder S, et al. An immune response gene expression module identifies a good prognosis subtype in estrogen receptor negative breast cancer. Genome Biology: 8:R157. (2007)

The MARIE-GENICA Consortium on Genetic Susceptibility for Menopausal Hormone Therapy Related Breast Cancer Risk. Genetic polymorphisms in phase I and phase II enzymes and breast cancer risk associated with menopausal hormone therapy in postmenopausal women. Br Cancer Res Treat: 119: 463–474. (2010)

Thompson A, Lee B, Jordan L, Quinlan P. Prospective comparison of switches in biomarker status between primary and recurrent breast cancer: the Breast Recurrence In Tissues Study (BRITS). Br Cancer Res: 12: R92. (2010)

Thomson T, Zhou C, Ceballos K, Knight B.Tissue Microarray for Routine Clinical Breast Biomarker Analysis. The British Columbia Cancer Agency 2008 Experience. Am J Clin Pathol: 133: 909-914. (2010)

Thomson T, Zhou C, Ceballos K, Knight B.Tissue Microarray for Routine Analysis of Breast Biomarkers in the Clinical Laboratory. Am J Clin Pathol: 132:899-905 (2009**)**

Thomssen C, Oppelt P, Jaenicke F, Graeff H. Prognostic value of the cysteine proteases cathepsin B and cathepsin L in human breast cancer. Clin Cancer Res: 1: 741–746. (1995)

Tiano J & Mauvais-Jarvis F. Importance of oestrogen receptors to preserve functional β-cell mass in diabetes. Nat Rev Endo: 8 : 342-351. (2012)

Tischkowitz M, Brunet J-S, Bégin L, et al. Use of immunohistochemical markers can refine prognosis in triple negative breast cancer. BMC Cancer: 7: 134. (2007)

Toi M, Ueno T, Matsumoto H, et al. Significance of thymidine phosphorylase as a marker of protumor monocytes in breast cancer. Clin Cancer Res: 5: 1131–1137. (1999)

Tokuyasu N, Shomori K, Nishihara K, et al. Minichromosome maintenance 2 (MCM2) immunoreactivity in stage III human gastric carcinoma: clinicopathological significance. Gastric Cancer: 11: 1: 37- 46. (2008)

Tong L, Nelson N, Tsourigiannis J, et al. The Effect of Prolonged Fixation on the Immunohistochemical Evaluation of Estrogen Receptor, Progesterone Receptor, and HER2 Expression in Invasive Breast Cancer: A Prospective Study. Am J Surg Patho: 35: 545–552. (2011)

Torhorst J, Bucher C, Kononen J, et al. Tissue microarrays for rapid linking of molecular changes to clinical endpoints. Am J Patho: 159: 2259-2256. (2001)

Townsend P, Dublin E, Hart I, et al. BAG-i expression in human breast cancer: interrelationship between BAG-1 RNA, protein, HSC70 expression and clinico-pathological data. J Patho: 197: 51 – 59. (2002)

Tran C, Wolz P, Egensperger R, et al. Differential expression of MHC class II molecules by microglia and neoplastic astroglia: Relevance for the escape of astrocytoma cells from immune surveillance. Neuropath Appl Neurobio: 24: 293–301. (1998)

Tsutsui S, Yasuda K, Suzuki K, et al. Macrophage infiltration and its prognostic implications in breast cancer: the relationship with VEGF expression and microvessel density. Onco Rep: 14: 425–431. (2005)

Turner B, Krajewski S, Krajewska M, et al. BAG-1: a novel biomarker predicting long-term survival in early-stage breast cancer. J Clin Onco: 19: 992-1000. (2001)

Turner N, Reis-Filho J, Russell A, et al. BRCA1 dysfunction in sporadic basal-like breast cancer. Oncogene: 26: 2126–2132. (2007)

Tutt A, Wang A, Rowland C, et al. Risk estimation of distant metastasis in node-negative, estrogen receptor-positive breast cancer patients using an RT-PCR based prognostic expression signature. BMC Cancer: 8:339. (2008)

Twelves C, Trigo J, Jones R, et al. Erlotinib in combination with capecitabine and docetaxel in patients with metastatic breast cancer: a dose-escalation study. E J Cancer: 44: 3: 419–426. (2008)

Untch M, Fasching PA, Konecny G, et al. Pathologic complete response after neoadjuvant chemotherapy plus trastuzumab predicts favorable survival in human epidermal growth factor receptor 2-overexpressing breast cancer: results from the TECHNO trial of the AGO and GBG study groups. J Clin Onco: 29:25:3351-7. (2011)

Uzzan B, Nicolas P, Cucherat M, Perret G. Microvessel density as a prognostic factor in women with breast cancer: a systematic review of the literature and meta-analysis. Cancer Res: 64: 2941–2955. (2004)

van de Vijver, He Y, van 't Veer L, et al. A gene-expression signature as a predictor of survival in breast cancer. N Eng J Med: 347: 25: 1999 – 2009. (2002)

van der Hage J, Mieog J, van de Vijver M. Impact of established prognostic factors and molecular subtype in very young breast cancer patients: pooled analysis of four EORTC randomized controlled trials. Breast cancer res. 13:3:R68. (2011)

Van't Veer L, Dai H, van de Vijver M, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature: 415:530 – 536. (2002)

Vares G, Ory K, Lectard B, et al. Progesterone prevents radiation-induced apoptosis in breast cancer cells. Oncogene: 23: 4603–4613. (2004)

Vargas-Roig L, Cuello-Carrio´ F, Ferna´ndez-Escobar N, et al. Prognostic value of Bcl-2 in breast cancer patients treated with neoadjuvant anthracycline based chemotherapy. Mol Onco: 2:102-111(2008)

Vargo J, Beriwal S, Ahrendt G, et al. Molecular class as a predictor of locoregional and distant recurrence in the neoadjuvant setting for breast cancer. Oncology: 80: 5-6:341-9. (2011)

Veikkola T, Karkkainen M, Claesson-Welsh L, et al. Regulation of angiogenesis via vascular endothelial growth factor receptors. Cancer Res: 60: 203-212. (2000)

Ventana Data sheet, Cat no. 790-2988. www.ventana.com accessed on 02.08.13

Viale G, Regan M, Mastropasqua M, et al. Predictive Value of Tumor Ki-67 Expression in Two Randomized Trials of Adjuvant Chemoendocrine Therapy for Node-Negative Breast Cancer. J Nat Cancer Inst: 100: 207 – 212. (2008)

Viani G, Afonso S, Stefano E, et al. Adjuvant trastuzumab in the treatment of her-2-positive early breast cancer: a meta-analysis of published randomized trials. BMC Cancer: 7:153 doi:10.1186/1471-2407-7-153 (2007)

Vogt T, Blackwell A, Giannetti A, et al. Heterotypic interactions between transferrin receptor and transferrin receptor 2. Blood: 101: 5: 2008-14. (2003)

von Marschall Z, Cramer T, Hocker M et al. De novo expression of vascular endothelial growth factor in human pancreatic cancer: evidence for an autocrine mitogenic loop. Gastroentero: 119: 5: 1358–1372. (2000)

von Minckwitz G, Untch M, Blohmer J, et al. Definition and impact of pathologic complete response on prognosis after neoadjuvant chemotherapy in various intrinsic breast cancer subtypes. J Clin Onco: 30:15:1796-804. (2012)

Walker R, Bartlett J, Dowsett M, et al. HER2 testing in the UK: further update to recommendations. J Clin Patho: 61: 818–824. (2008)

Wang F, Jiang X, Yang D, et al. Doxorubicin—gallium—transferrin conjugate overcomes multidrug resistance: evidence for drug accumulation in the nucleus of drug resistant MCF-7/ADR cells. Anticancer Res: 20: 799– 808. (2000)

Wang R, Luo D, Ma X, et al. Antisense Ki-67 cDNA transfection reverses the tumorigenicity and induces apoptosis in human breast cancer cells. Cancer Invest: 26:8:830-5. (2008)

Wang Y, Klijn J, Zhang Y, et al. Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. Lancet: 365: 671–679. (2005)

Weaver M, Laske D. Transferrin receptor ligand-targeted toxin conjugate (Tf-CRM107) for therapy of malignant gliomas. J Neurooncol: 65: 1: 3-13. (2003)

Wedam S, Low J, Yang S, et al. Antiangiogenic and antitumor effects of bevacizumab in patients with inflammatory and locally advanced breast cancer. J Clin Oncol: 24: 5: 769-777. (2006)

Wehler T, Frerichs K, Graf C, et al. PDGFRα/ß expression correlates with the metastatic behavior of human colorectal cancer: A possible rationale for a molecular targeting strategy. Onco Reports: 19: 697-704. (2008)

Weichert W, Kristiansen G, Winzer K, et al. Polo-like kinase isoforms in breast cancer: expression patterns and prognostic implications. Virchows Arch: 446: 4: 442-50. (2005)

Weisberg S, McCann D, Desai M, et al. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest: 112: 1796–808. (2003)

Welfare M, Adeokun A, Bassendine M, Daly A. Polymorphism in GSTP1, GSTM1 and GSTT1 and susceptibility to colorectal cancer. Cancer Epi Biomark Prev: 8: 289-292 (1999)

Wildiers H, Neven P, Christiaens M, et al. Neoadjuvant capecitabine and docetaxel (plus trastuzumab): an effective non-anthracycline-based chemotherapy regimen for patients with locally advanced breast cancer. Ann Onco: 22:3:588-94. (2011)

Wintzer H, Bohle W, von Kleist S. Study of the relationship between immuno-histologically demonstrated lymphocytes infiltrating human breast carcinomas and patients' survival. J Cancer Res Clin Onco: 117: 163–167. (1991)

Wishart G, Azzato E, Greenberg D, et al. PREDICT: a new UK prognostic model that predicts survival following surgery for invasive breast cancer. Br Cancer Res. 12:1:R1. Epub 2010 Jan 6. (2010)

Wishart G, Bajdik C, Dicks E, et al. PREDICT Plus: development and validation of a prognostic model for early breast cancer that includes HER2. Br J Cancer: 107:5:800-7. (2012)

Wolff A, Hammond M, Schwartz J et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Onco: 25: 118–145. (2007)

Won K, Kim G, Kim Y, et al. Clinicopathologic correlation of beclin-1 and bcl-2 expression in human breast cancer. Human Path: 41: 107–112. (2010)

Wright C, Angus B, Nicholson S, et al. Expression of c-erbB-2 oncoprotein: a prognostic indicator in human breast cancer. Cancer Res: 49: 2087-2090. (1989)

www.seer.cancer.gov

Xie X, Clausen O, Boysen M. Bag-1 expression as a prognostic factor in tongue squamous cell carcinomas. Laryngoscope: 144:1785–90. (2004)

Yamashita H, Toyama T, Nishio M, et al. P53 protein accumulation predicts resistance to endocrine therapy and decreased post-relapse survival in metastatic breast cancer. Br Cancer Res: 8: R48. (2006)

Yang S, Steinberg S, Nguyen D, Swain S. p53, HER2 and tumor cell apoptosis correlate with clinical outcome after neoadjuvant bevacizumab plus chemotherapy in breast cancer. Int J Onco: 38: 5: 1445 - 52. (2011)

Yang S, Zhou X, Zhu H, et al. Amplification and overexpression of Aurora-A in esophageal squamous cell carcinoma. Onco Reports: 17: 1083-1088. (2007)

Yao D, Sun M, Huang Y. Targeted Delivery of PLK1-siRNA by ScFv Suppresses Her2+ Breast Cancer Growth and Metastasis. Sci Transl Med: 4: 130: 130 - 48. (2012)

Yaziji H, Taylor C, Goldstein N, et al. Consensus recommendations on estrogen receptor testing in breast cancer by immunohistochemistry. Appl Immunohistochem Mol Morphol: 16: 513–520. (2008)

Yoshimura T, Robinson E, Tanaka S, et al. Purification and amino acid analysis of two human glioma-derived monocyte chemoattractants. J Exp Med: 169: 1449–1459. (1989)

Yu K, Liu G, Di G, et al. Progesterone receptor status provides predictive value for adjuvant endocrine therapy in older estrogen receptor-positive breast cancer patients. The Breast: 16: 307–315. (2007)

Yu K-D, Fan L, Di G, et al. Genetic variants in GSTM3 gene within GSTM4-GSTM2-GSTM1-GSTM5-GSTM3 cluster influence breast cancer susceptibility depending on GSTM1. Br Cancer Res Treat: 121: 2: 485-96. (2010)

Zajc I, Hreljac I, Lah T. Cathepsin L affects apoptosis of glioblastoma cells: a potential implication in the design of cancer therapeutics. Anticancer Res: 26: 5A: 3357-64. (2006)

Zapata J, Krajewski M, Krajewski S, et al. Expression of multiple apoptosis-regulatory genes in human breast cancer cell lines and primary tumours. Br Cancer Res Treat: 47: 129-140. (1998)

Zhanaeva S, Korolenko T, Khoshchenko O, et al. Effect of cysteine protease inhibitor Ep-475 on TNF-alpha-independent cyclophosphamide-induced apoptosis in mouse lymphosarcoma LS cells. Bull Exp Biol Med: 139: 21: 86-9. (2005)

Zhang P, Gao Y, Turner S, Ducatman S. Gleevec (STI-571) inhibits lung cancer cell growth (A549) and potentiates the cisplatin effect in vitro. Mol Cancer: 2: 1. (2003)

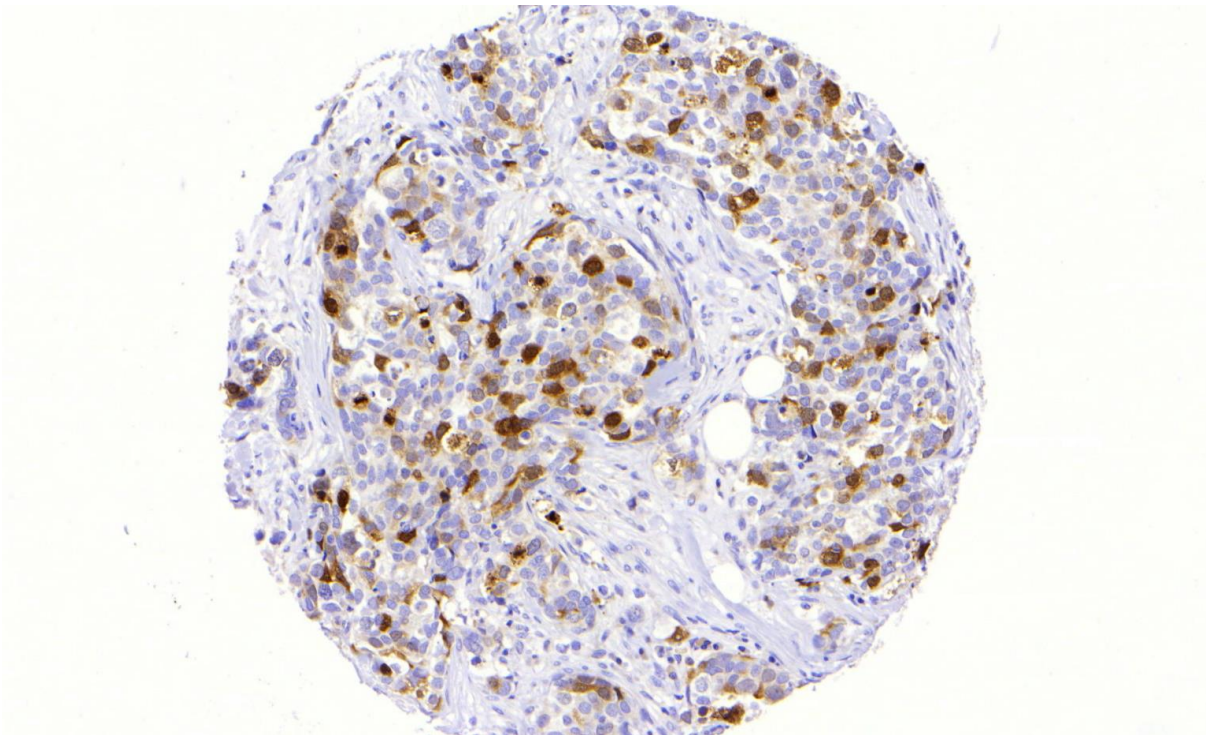
Zheng X, Chou P, Mirkin B, et al.Senescence-initiated Reversal of Drug Resistance: Specific Role of Cathepsin L. Cancer Res: 64:1773-1780. (2004)

Zheng X, F Chu, P Chou, et al. Cathepsin L inhibition suppress drug resistance in vitro and in vivo: a putative mechanism. Am J Physiol cell physiol: 296: 65 - 74. (2009)

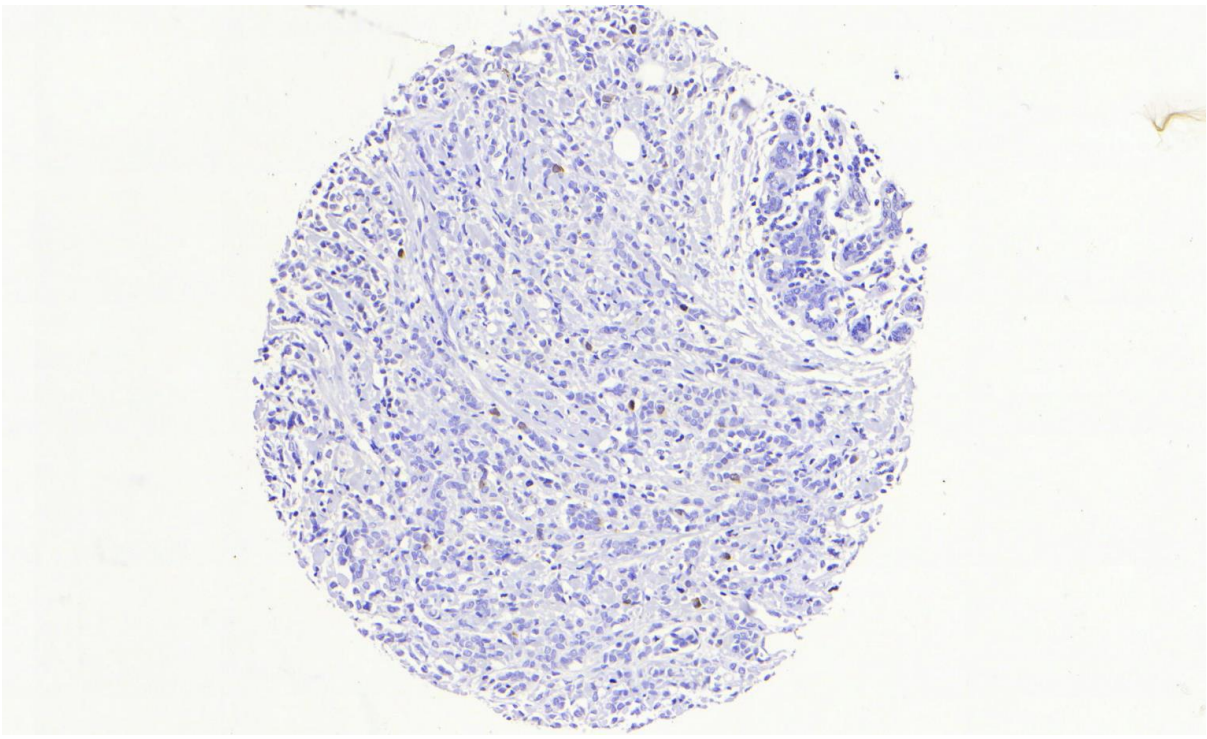
Zhou H, Kuang J, Zhong L, et al. Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nat Genet: 20:189–193. (1998)

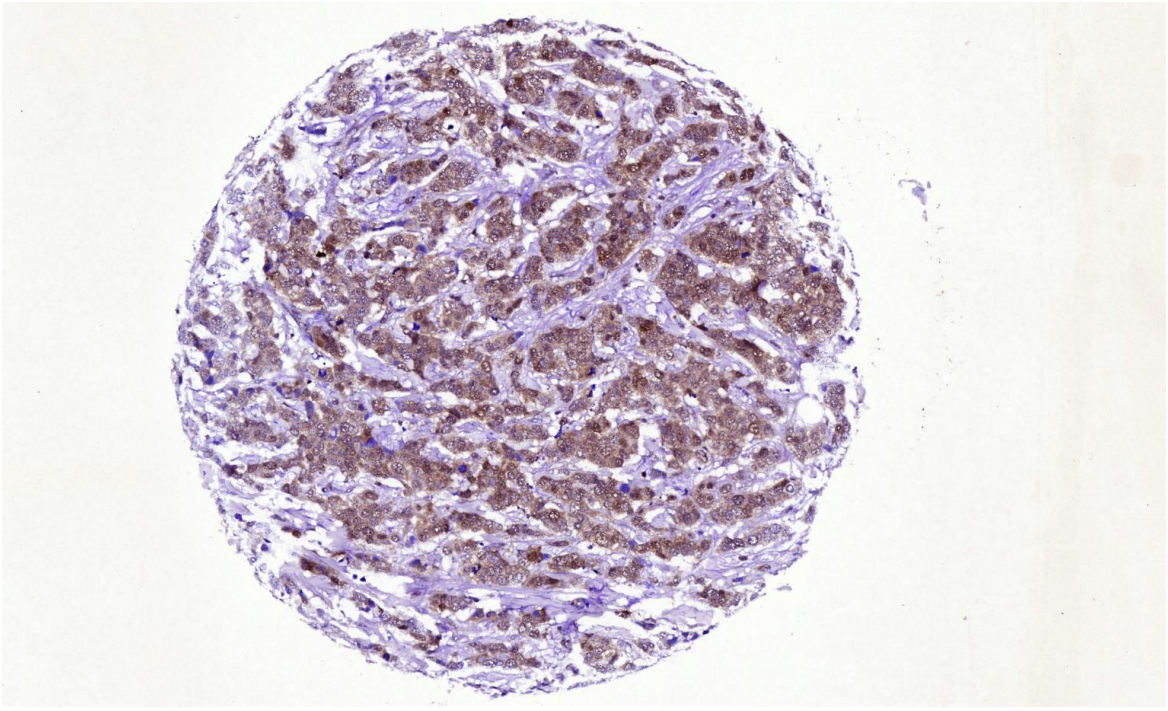
Zhuo W-L, Zhang Y-S, Wang Y, et al. Association Studies of CYP1A1 and GSTM1 polymorphisms with esophageal cancer risk: Evidence-based meta-analyses. Arch Medm Res: 40: 169-179 (2009)

14. IHC images



Aurora A positive (above) and negative (below)





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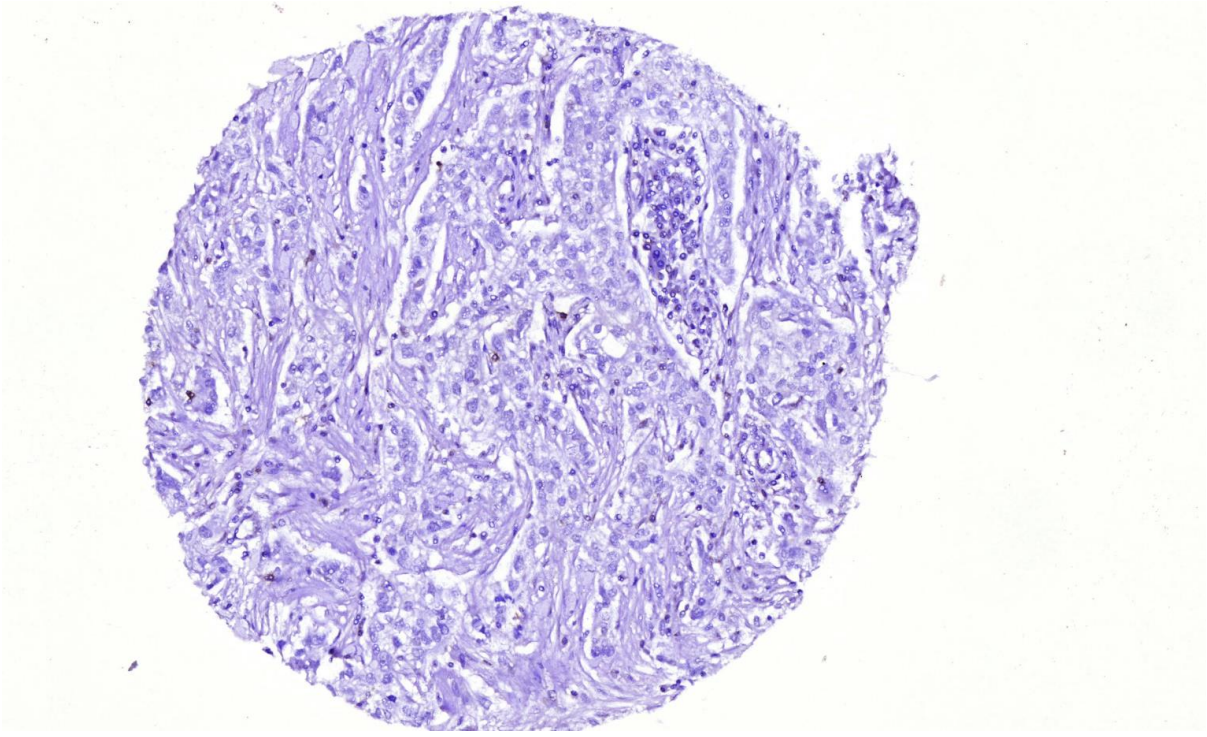

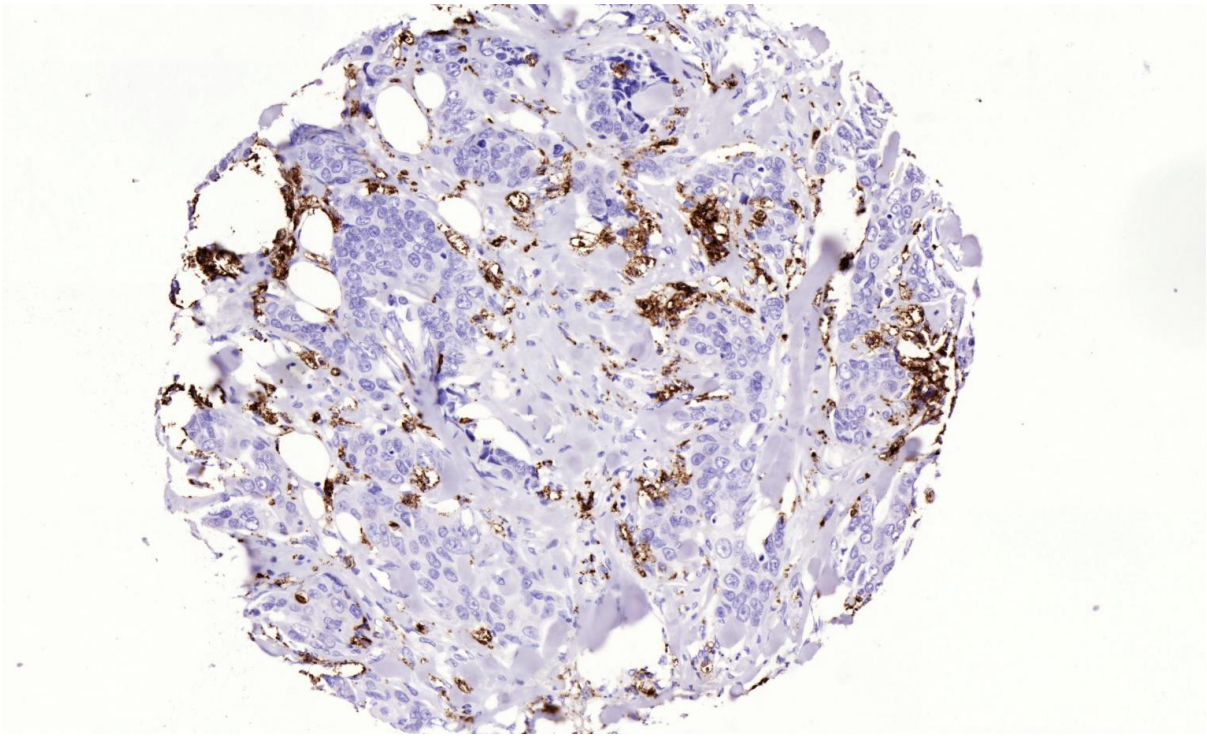

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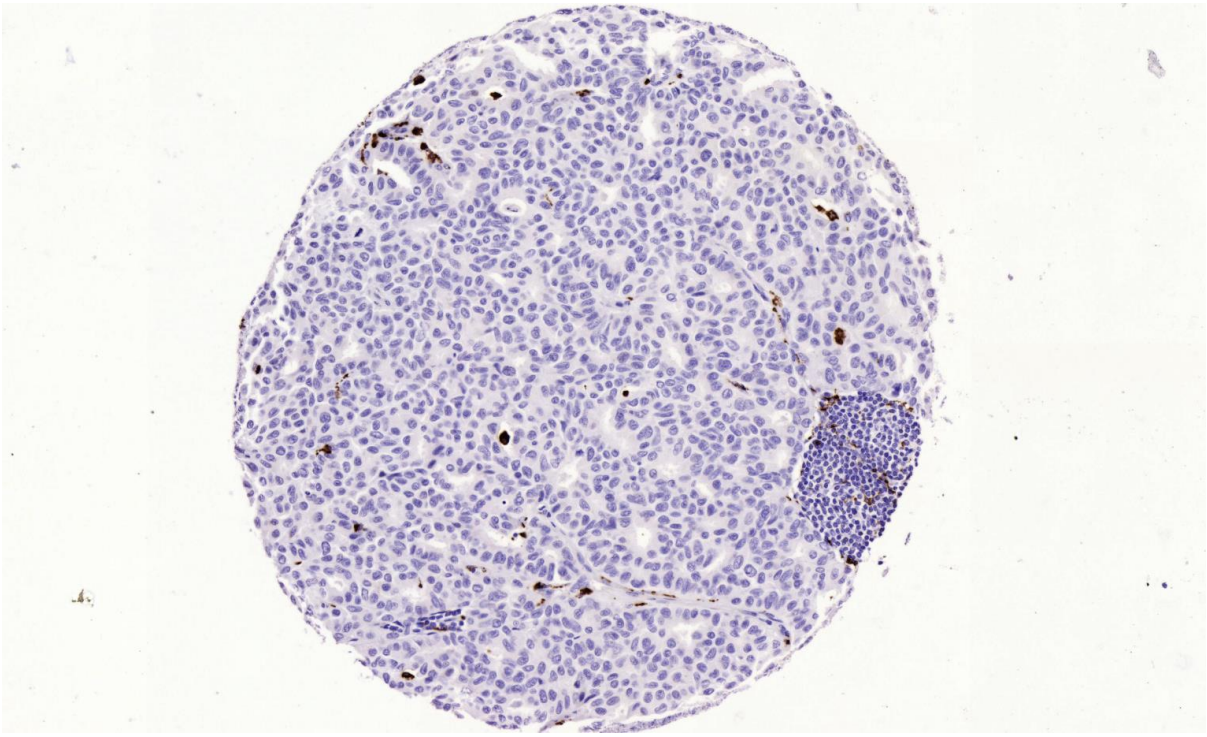

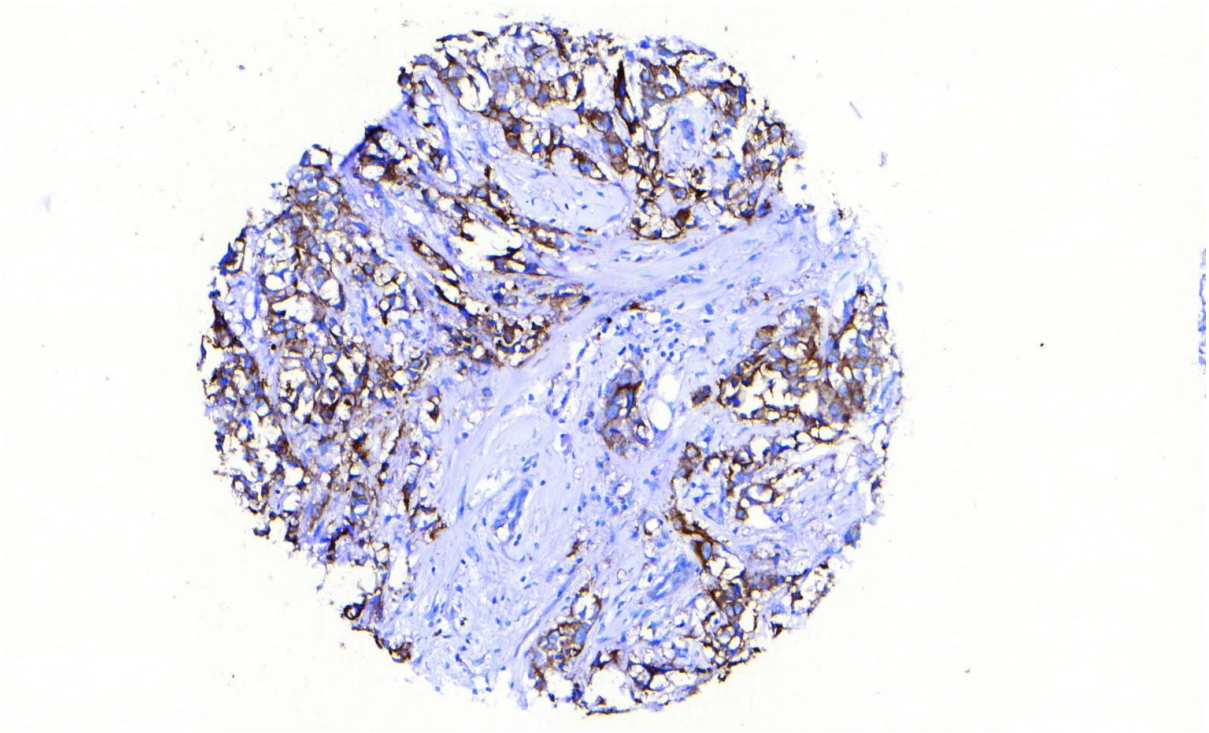

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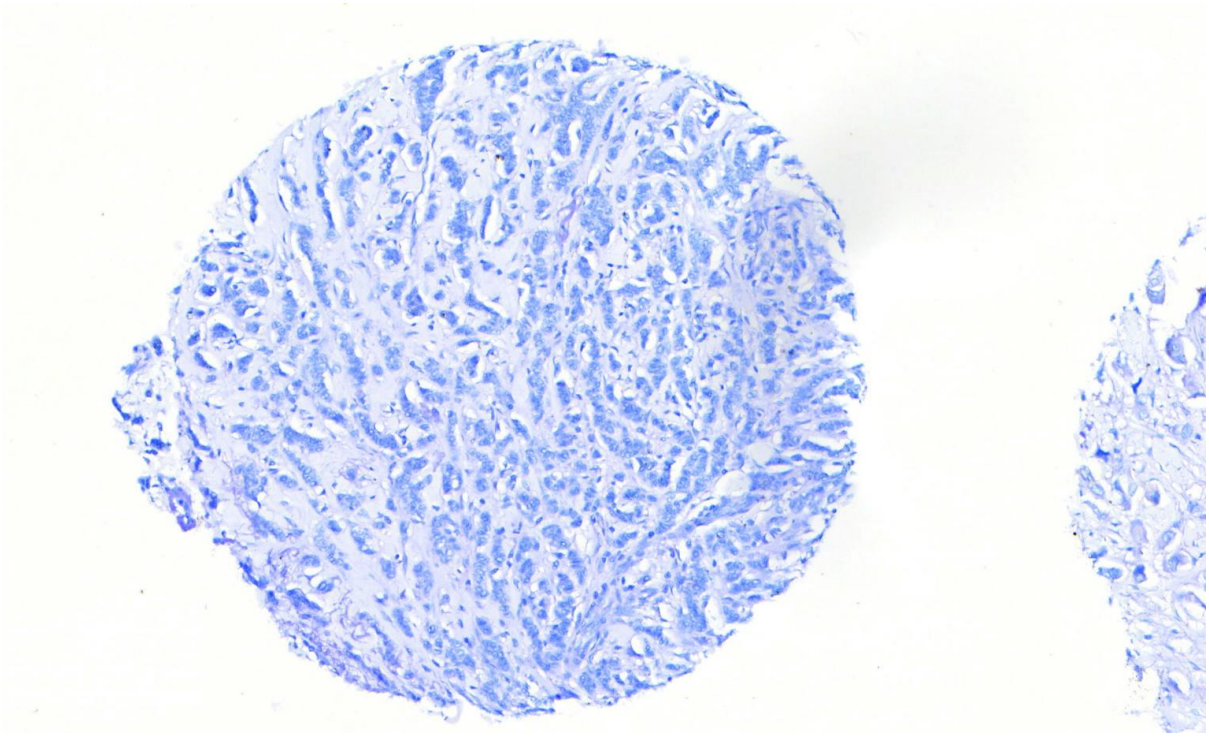

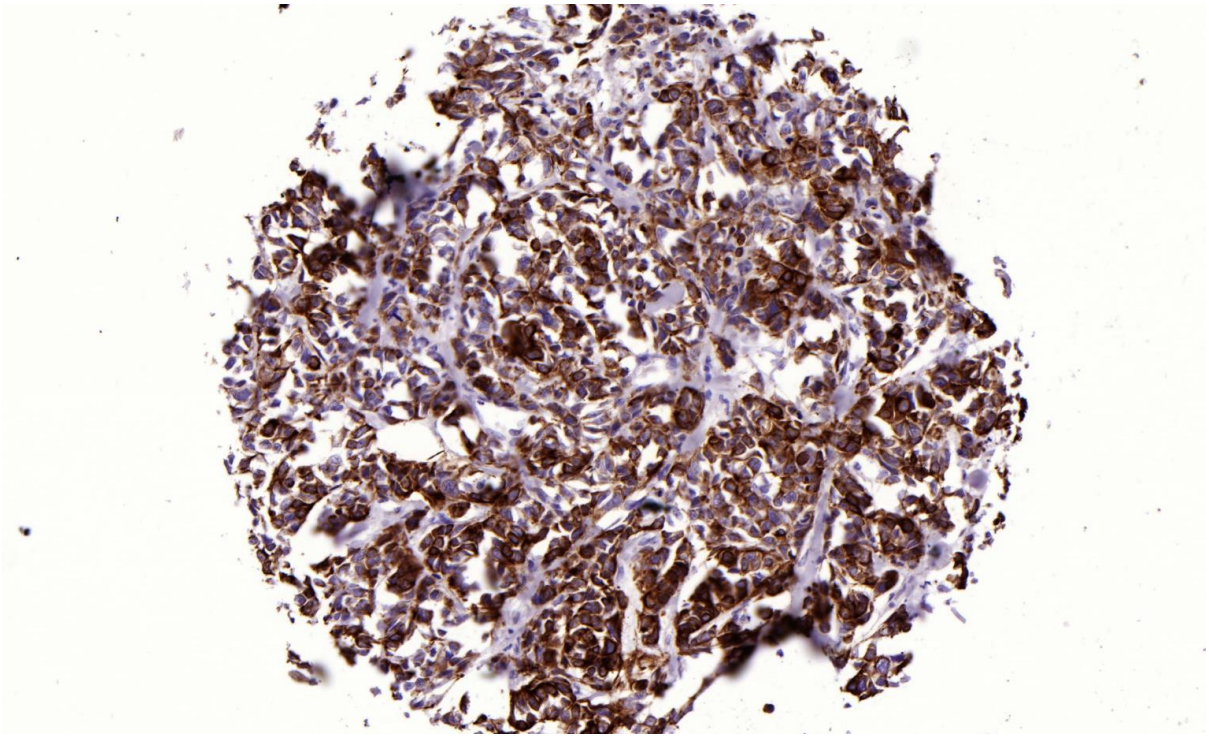

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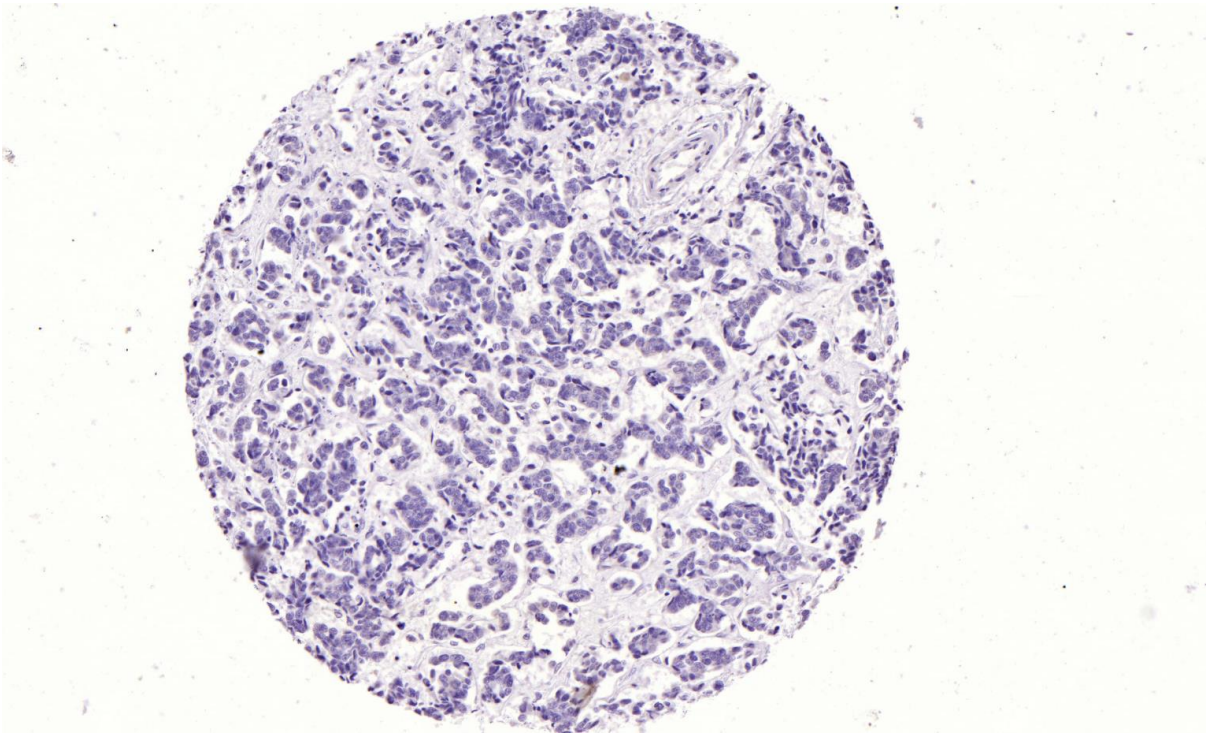

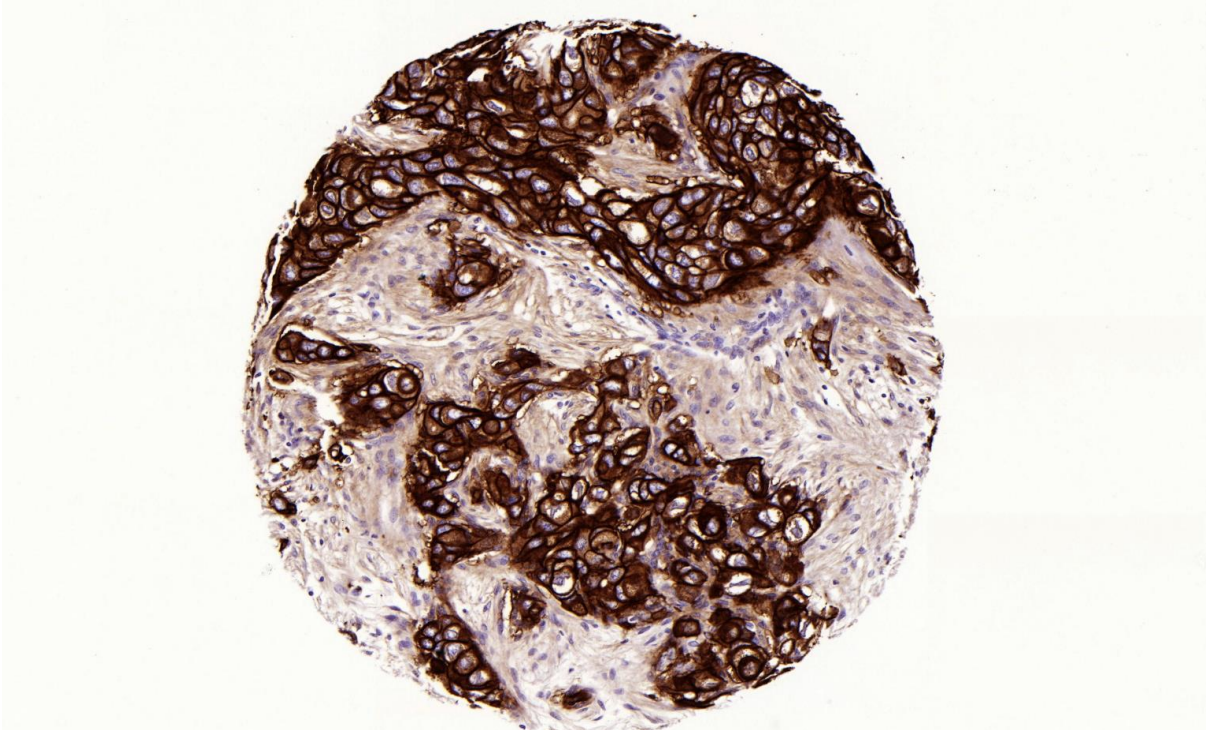

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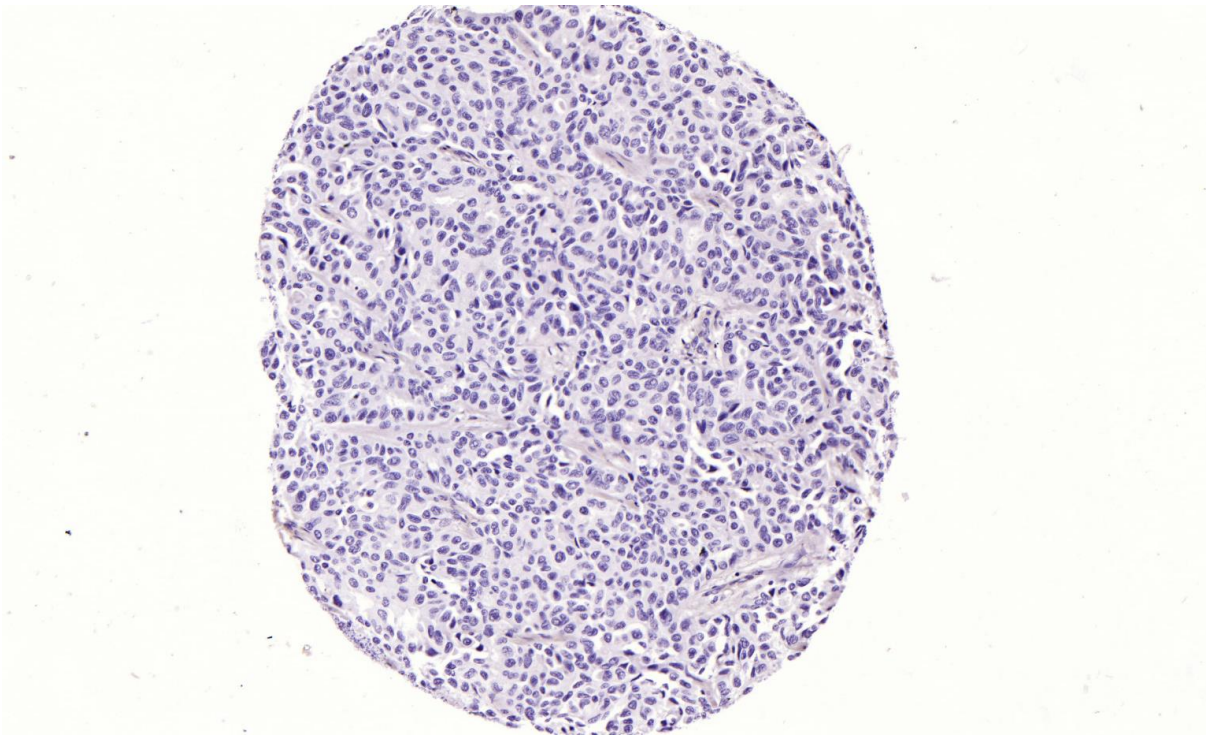

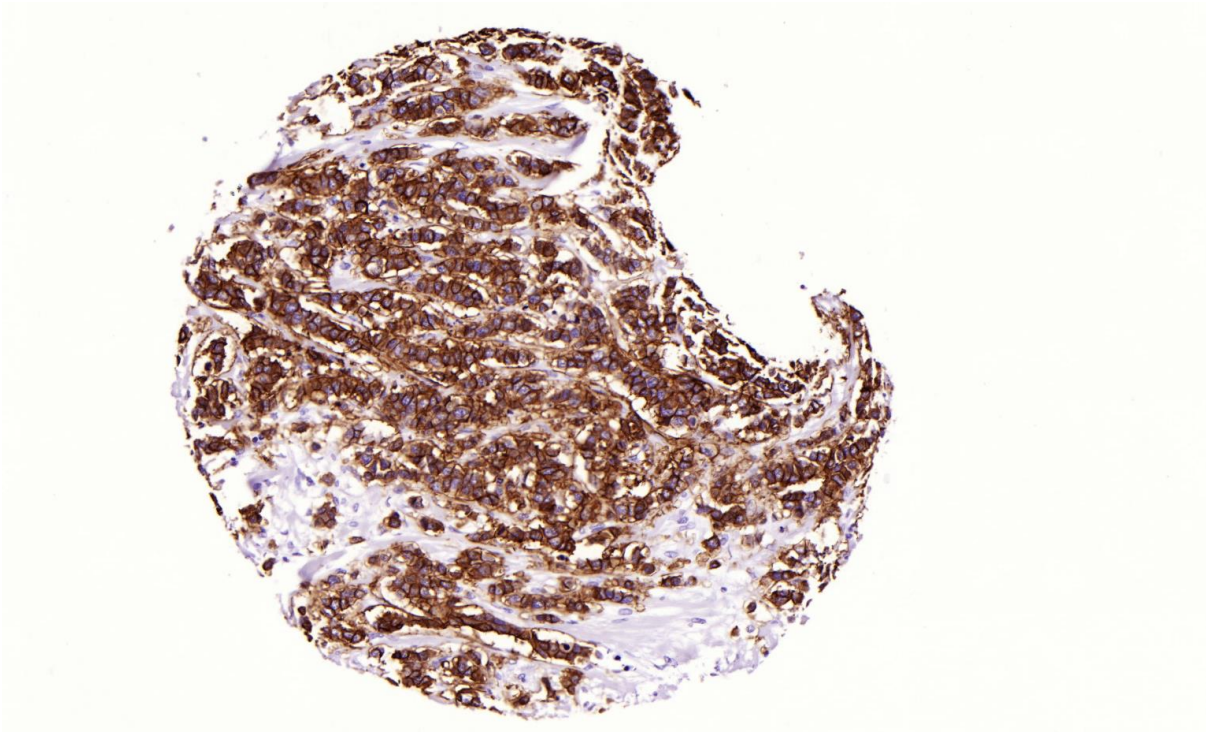

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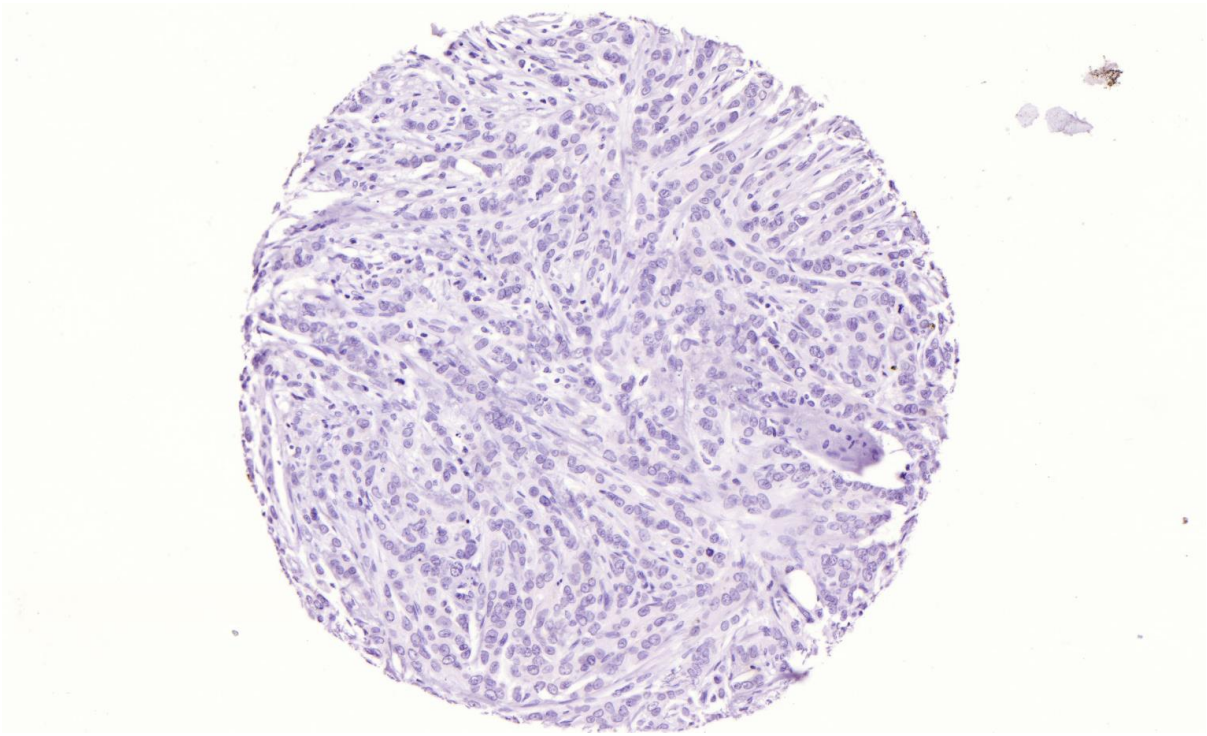

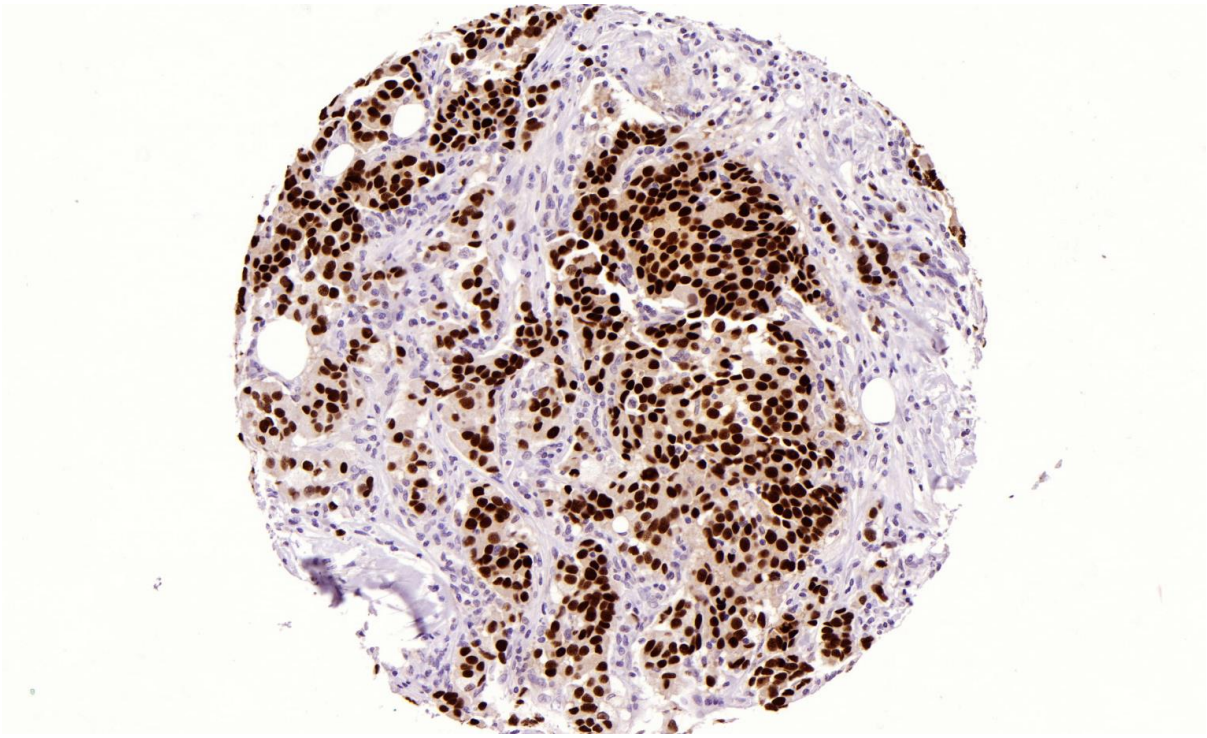

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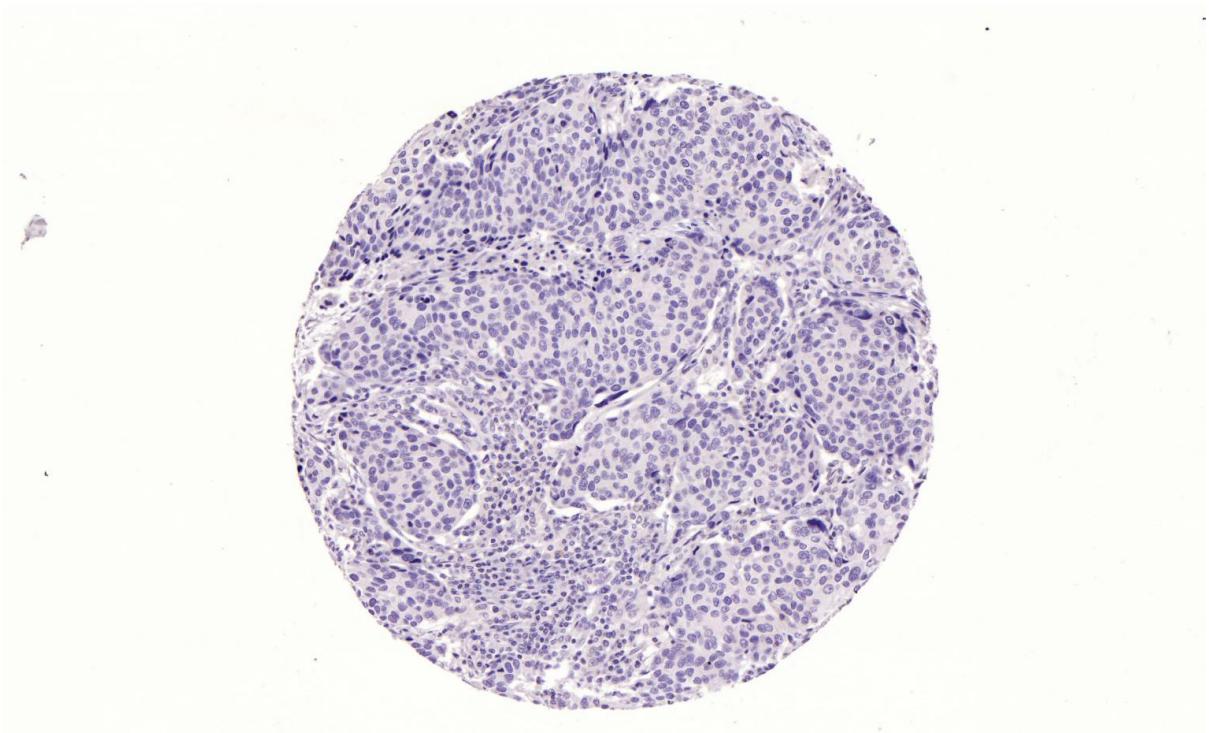

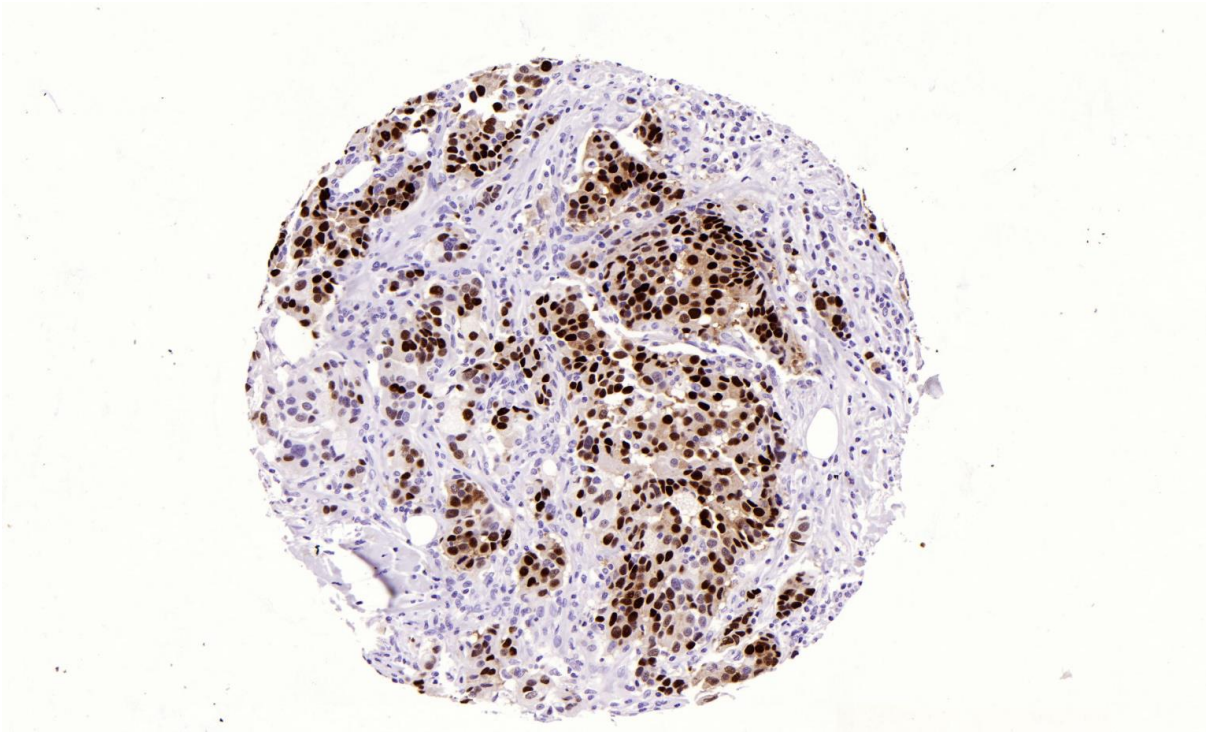

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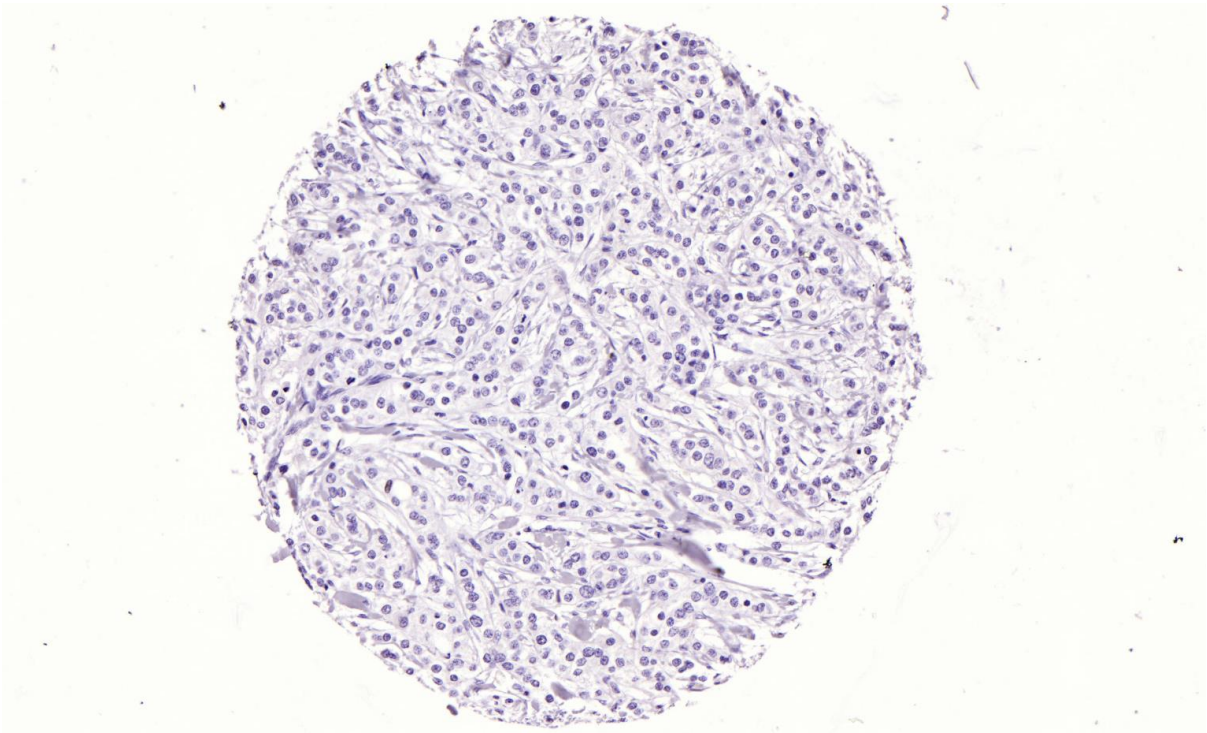

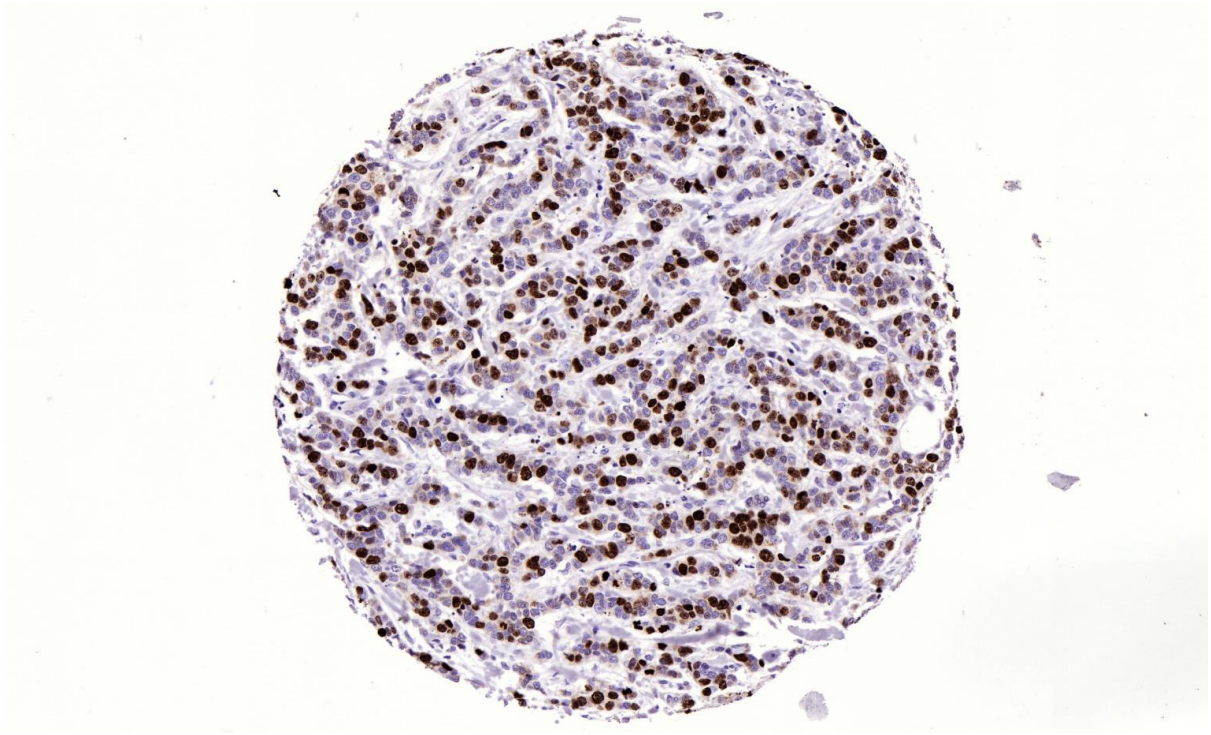

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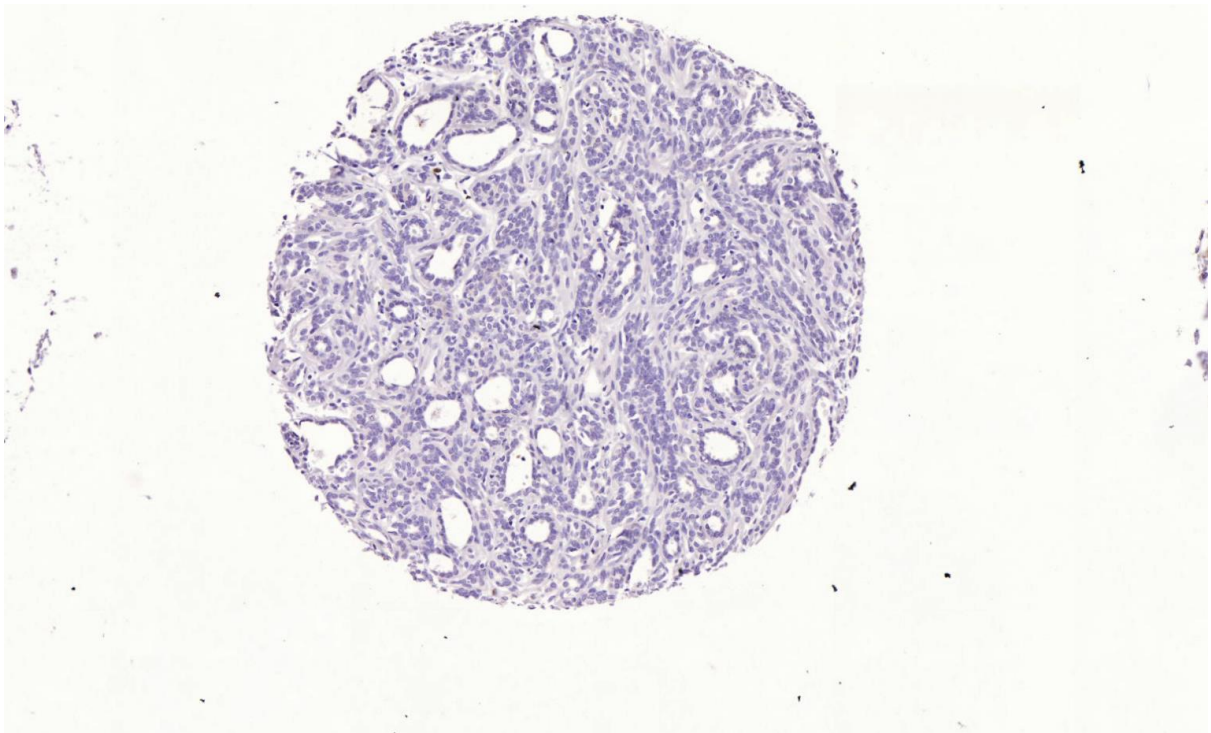
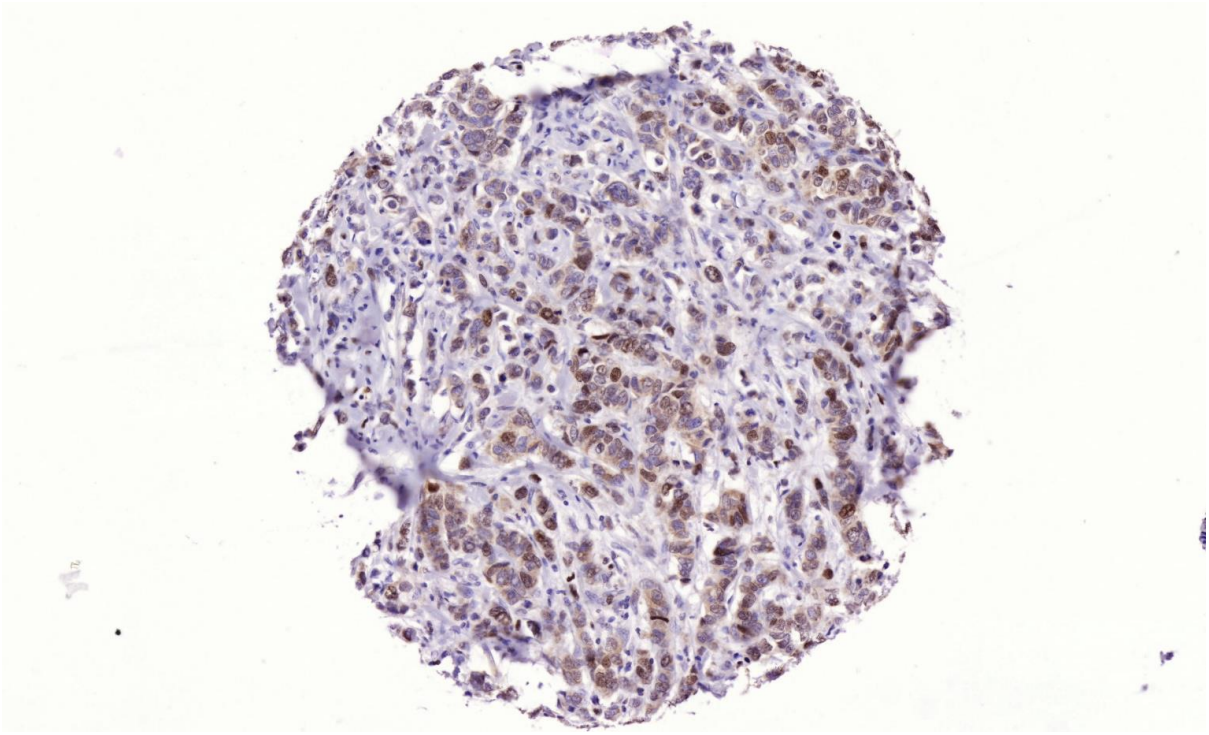
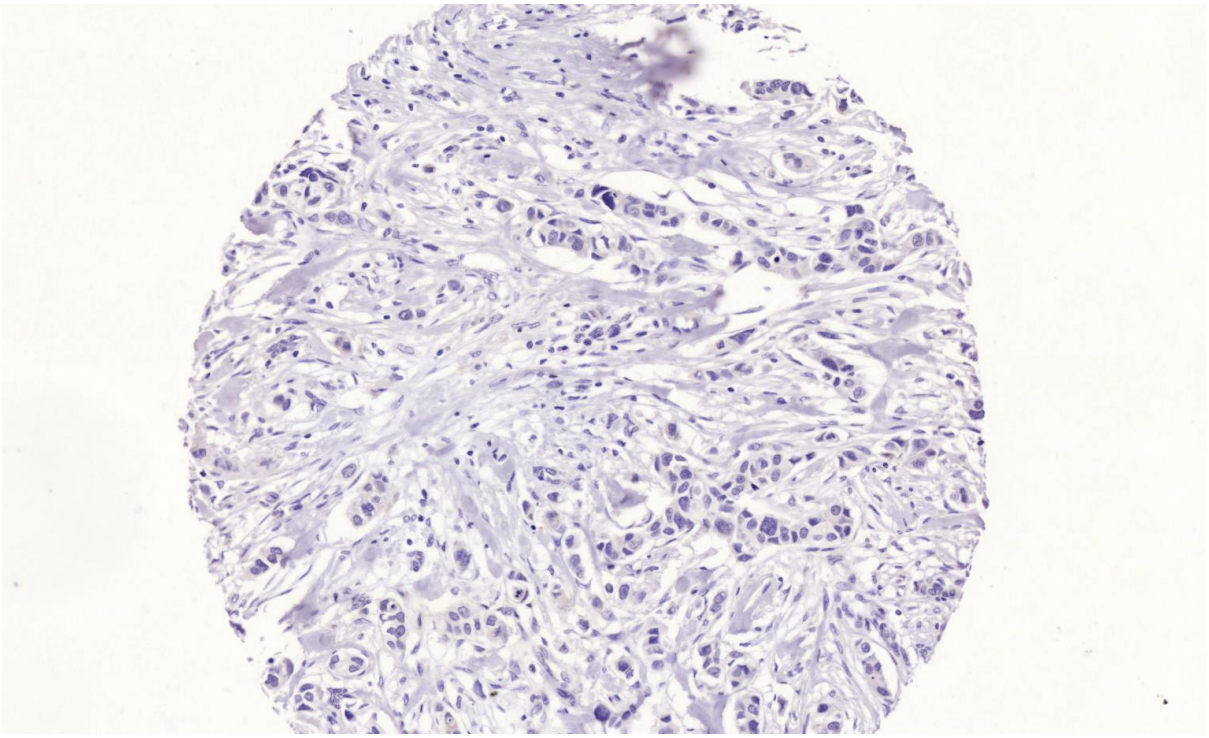
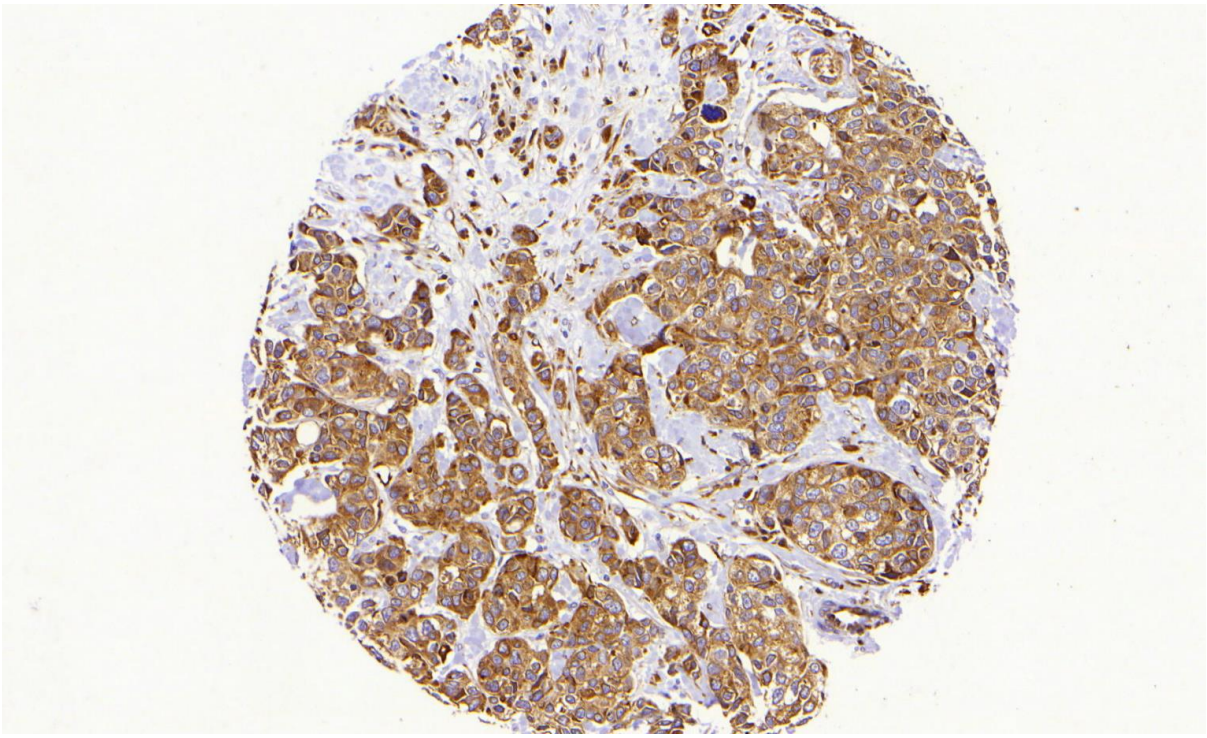


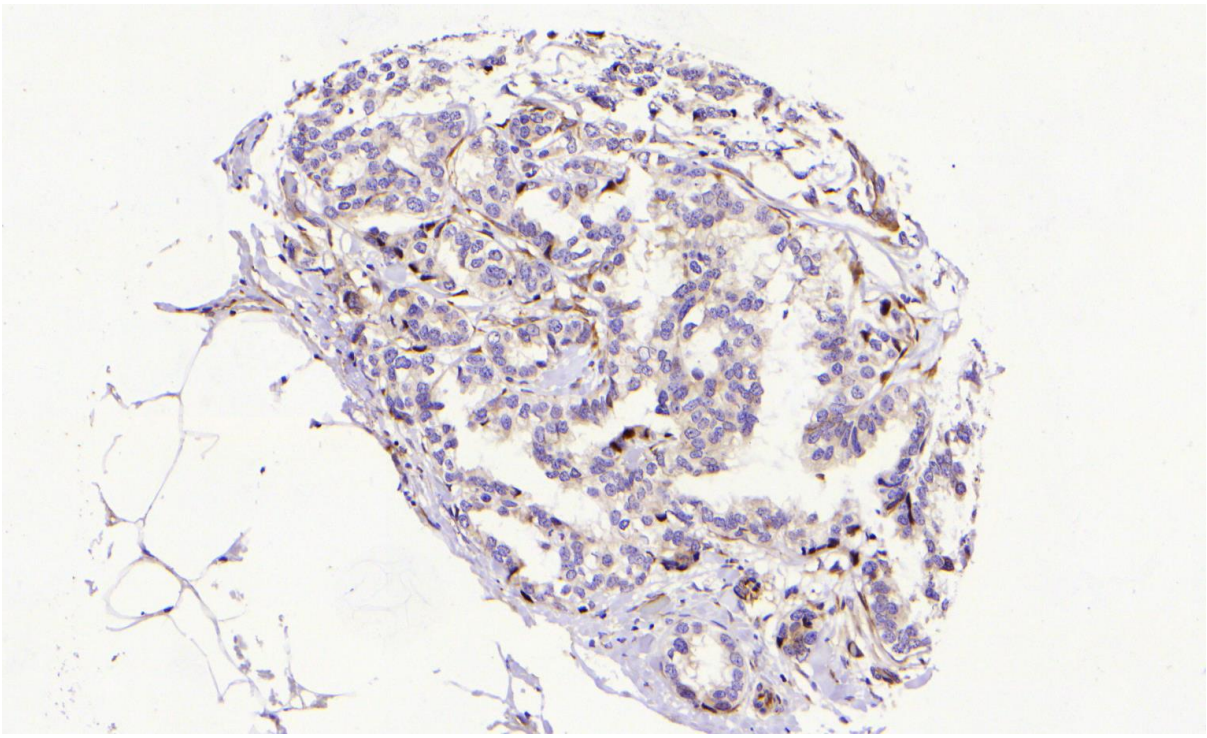


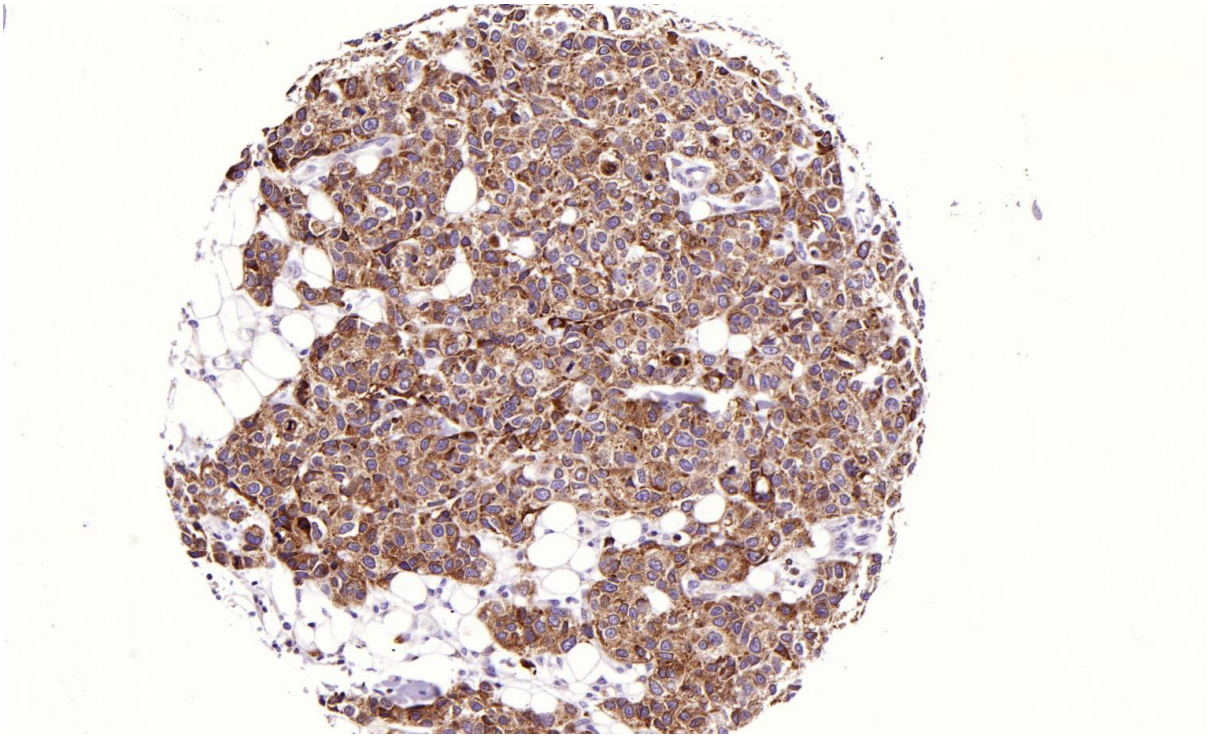
MCM-2 positive (above) and negative (below)



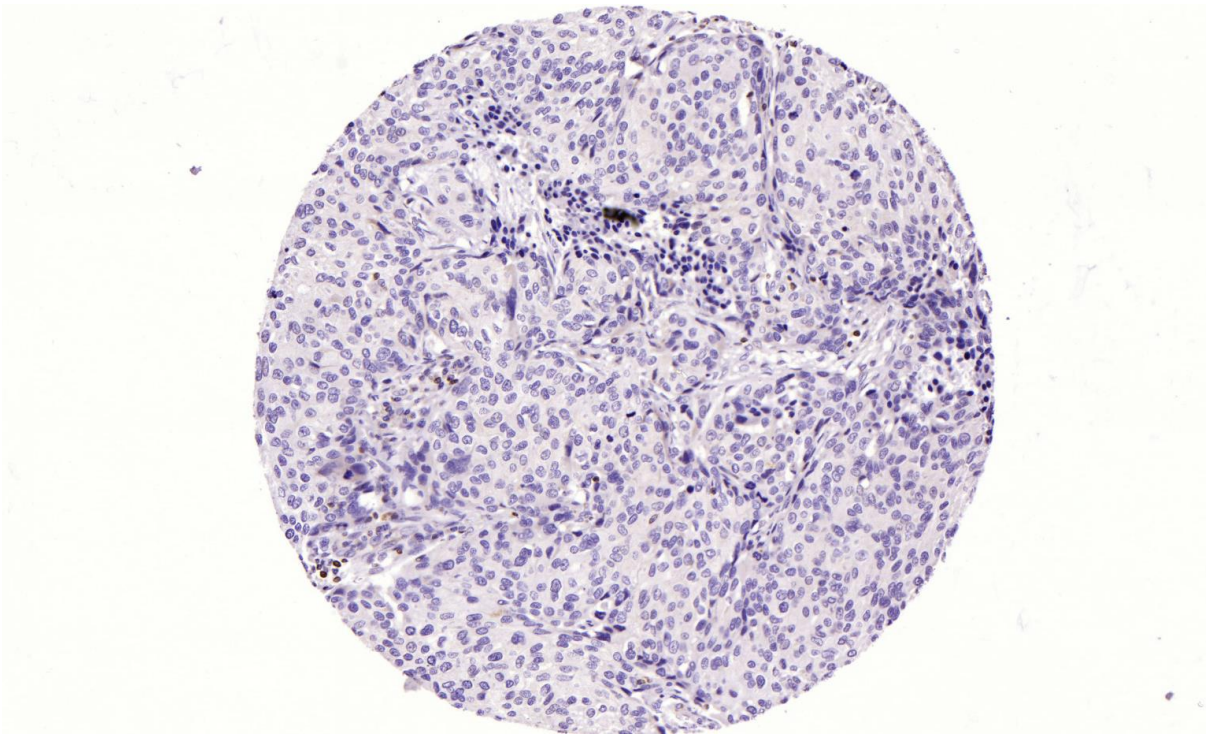


PDGFR-α positive (above) and negative (below)





Bcl-2 positive (above) and negative (below)



15. Appendix

1. Contribution list to this research work

M Moe developed the hypothesis after a thorough literature review, designed the study, secured the funding from Pfizer, reviewed the patients' clinicopathologic data in the hospital database, selected the study population, retrieved the FFPE blocks, actively involved in marking the area on H & E stained slides for TMA cores with the help of consultant pathologists, construction of the TMA blocks with the help of the technician, automatic (XT Benchmark machine) and manual IHC staining with the help of senior technicians from Singleton hospital pathology laboratory and senior scientists from Tenovus Centre for Cancer Research laboratory in Cardiff, respectively. M Moe analysed all the TMA cores for expression of individual protein and the supervisor Dr Richard Adams independently scored 10% of random TMA cores to verify the results. Bag-1 and CD-71 expressions were scored by M Moe together with Dr J Gee and Dr P Finlay from Tenovus centre for cancer research, Cardiff. M Moe analysed the data using SPSS v.16 and a senior statistician from Tenovus laboratory in Cardiff examined the results. M Moe wrote the thesis. The whole research project was carried out under the supervision of Dr Richard Adams (Oncology, Velindre Hospital, Cardiff) and Professor Robert Mansel (Surgical department, University hospital of Wales, Cardiff).

2. Abbreviations list

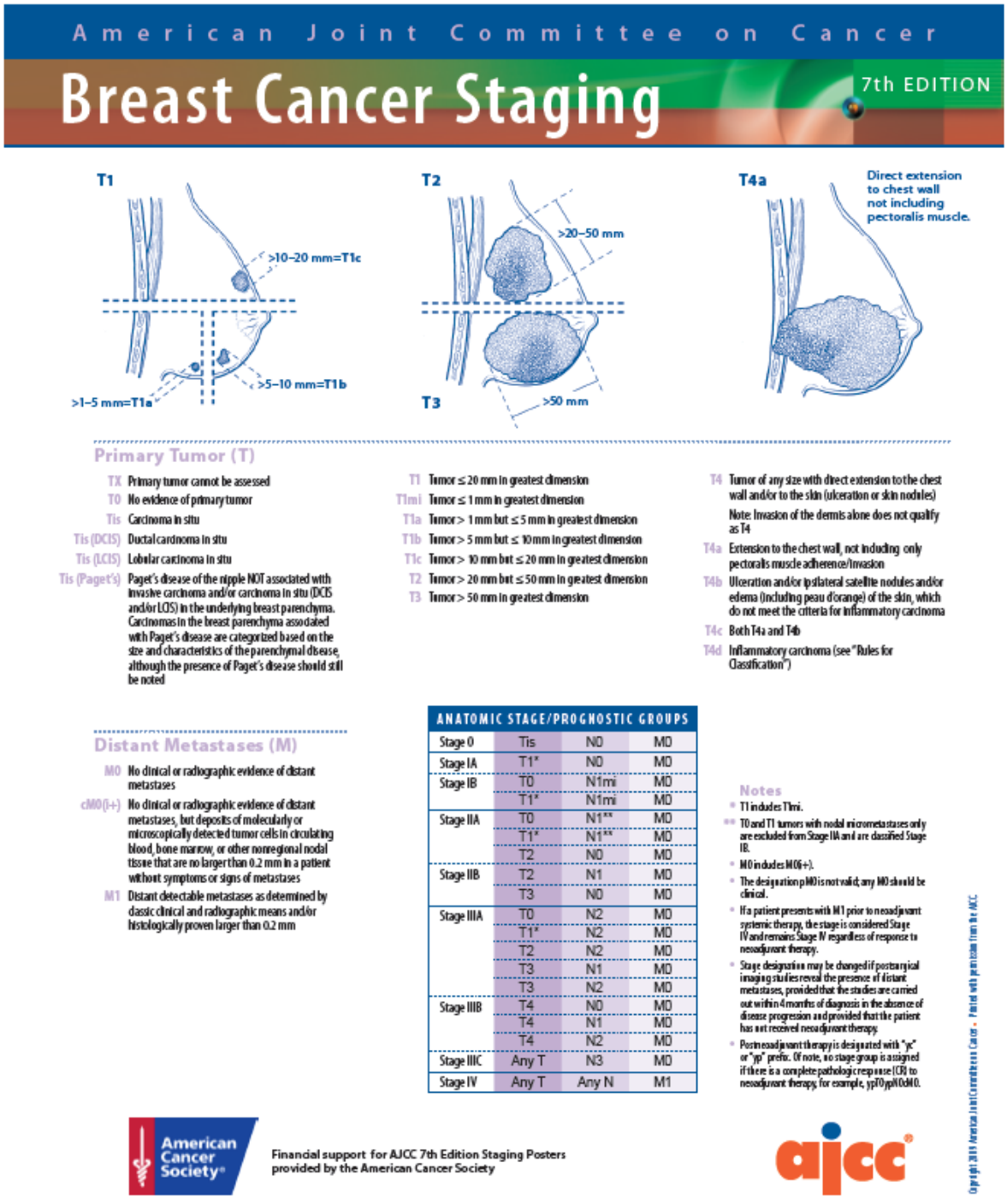
5N: 5 markers negative
AC-Taxol: Adriamycin, cyclophosphamide - taxol
AJCC: American Joint Committee on Cancer
ALN: Axillary lymph nodes
ASCO: American Society for Clinical Oncology
ATAC: Adjuvant Tamoxifen, Anastrozole combination trial.
ATP: Adenosine triphosphate
Bag-1: Bcl-2 associated anthanogene 1
BCSS: Breast cancer specific survivals
BRCA: Breast Cancer gene
CAP: College of American Pathologists
CB: Core Basal
CBP: Core basal phenotype
CC1 solution: cell conditioner 1 solution
CD-68: Cluster of differentiation 68
CD-71: Cluster of differentiation 71
cDNA: complimentery DNA
CEL: CIMFast Event Language (file format)
CI: Confidence interval
CISH: Chromogenic in situ hybridisation
CK-5/6: Cytokeratin 5/6
CMF: Cyclophosphamide, Methotrexate, 5-Fluorouracil
CRUK: Cancer research, UK
CSR: Core serum response
CYP2D6: Cytochrome p450 2D6
DCIS: Ductal carcinoma in-situ
D-CMF: Doxorubicin – CMF
df: degree of freedom
DNA: Deoxyribonucleic Acid
DPX: Di-N-Butyle Phthalate in Xylene
DRFS: Distant relapse free survivals
EBCTCG: Early Breast Cancer Treatment and Collaborative Group
E-CMF: Epirubicin - CMF
ECRIC: Eastern Cancer Registration and Information Centre
EDTA: Ethylenediaminetetraacetic acid
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
ELISA: Enzyme linked immunosorbent assay
ER: Oestrogen receptor
ERE: Oestrogen response element
FAC: 5-Flurouracil, Adriamycin, Cyclophosphamide
FASG: The French Adjuvant Study Group
FEC: 5-Flurouracil, Epirubicin, Cyclophosphamide
FFPE: Formalin fixed paraffin embedded
FGFR1: Fibroblast growth factor receptor 1
FISH: Fluoresence in situ hybridisation
FNA: Fine needle aspiration
GGI: Genomic Grade Index

GHI: Genomic Health Index
GIST: Gastrointestinal stromal tumour
GSTM-1: Glutathione S transferase Mu 1
H & E: Haematoxyline & Eosin
H score: Histochemical score
H: HER2 enriched
HCC: Hepatocellular carcinoma
HER2: Human epidermal growth factor receptor 2
HGF: Hepatocyte growth factor
HOXB13: Homeobox gene B 13
HR: hazard ratio
HRP: Horseradish peroxidase
HRT: Hormone replacement therapy
Hsp: Heat shock protein
IBCSG: International breast cancer study group
IGF-I: Insulin like growth factor-I
IHC: Immunohistochemistry
IMAC 30: Immobilized metal affinity capture 30
IMPACT: Immediate Preoperative Anastrozole, Tamoxifen, or Combined With Tamoxifen (trial)
IUC: International Union Against Cancer
LA: Luminal A
LB: Luminal B
LI: Labelling index
MAPKinase: Ras/mitogen-activated protein kinase
MCM-2: Minichrosome maintenance protein 2
MGI: Molecular grade index
MISS: Membrane initiated steroid signalling
N: Nodal stage
NCCN: National Colleborative Cancer Network
NISS: Nuclear initiated steroid signalling
NKI: Netherlands Cancer Institute (in Amsterdam)
NPI: Nottingham Prognostic Index
NSABP: National surgical adjuvant breast and bowel project
ORC: Origin recognition complex
OS: Overall survival
PAI-1: Plasminogen activator inhibitor 1
PAM: Prediction Analysis of Microarray
PAM50-RORP: PAM50 risk of recurrence (based on) proliferation
PAM50-RORS: PAM50 risk of recurrence (based on) subtype
PBD: Polo-box domain
PBS: Phosphate Buffered Saline
pCR: Pathological complete response
PDGF: Platelet derived growth factor
PDGFRα: Platelet derived growth factor receptor alpha
PEPI: Preoperative endocrine prognostic index
PI3K/Akt: Phosphatidylinositol 3 kinase
Plk-1: Polo like kinase 1
PR: Progesterone receptor
PRE: Progestin response elements

PRR: Proportional risk reduction
pT: Pathological tumour stage
PT: Primary tumours
PVI: Peritumoural vascular invasion
qRT-PCR: Quantitative reverse transcriptase polymerase chain reaction
RFS: Relapse free survival
RNA: Ribonucleic acid
ROR: Risk of recurrence
ROR-C: ROR in conjunction with the clinical features
ROR-S: ROR based on the subtype classification alone
ROT76: Rotterdam 76
RR: Recurrence risk
RS: Recurrence score
RT: Room temperature
RT-PCR: Reverse transcriptase polymerase chain reaction
S.E: Standard Error
SEER: Surveillance, Epidemiology and End Results
SET: Sensitivity to Endocrine Therapy
Sig.: Significance
siRNA: Small interfering RNA
SISH: Silver In-Situ Hybridisation
SNP: Single Nucleotide Polymorphism
SPSS v.16: Statistical Product and Service Solutions (software) version 16
T: Tumour size stage
TAM: Tumour associated macrophages
Tf: Transferrin
TfR: Transferrin receptor
TKI: Tyrosine kinase inhibitor
TMA: Tissue microarray
TNM: The tumour-node-metastasis system
TNP: Triple negatives phenotype
UICC: Union for International Cancer Control
UK: United Kingdom
uPA: urinary Plasminogen Activator
US: United States
VEGFR-2: Vascular endothelial growth factor receptor 2

3. AJCC staging

This AJCC staging poster was downloaded on 08.07.2013 from the website http://www.cancerstaging.org/staging/index.html.



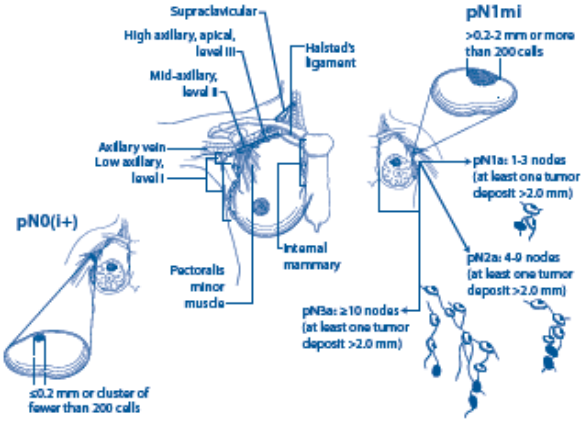
Regional Lymph Nodes (N)

CLINICAL

NX	Regional lymph nodes cannot be assessed (for example, previously removed)
N0	No regional lymph node metastases
N1	Metastases to movable ipsilateral level I, II axillary lymph node(s)
N2	Metastases in ipsilateral level I, II axillary lymph nodes that are clinically fixed or matted; or in clinically detected* ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastases
N2a	Metastases in ipsilateral level I, II axillary lymph nodes fixed to one another (matted) or to other structures
N2b	Metastases only in clinically detected* ipsilateral internal mammary nodes and in the absence of clinically evident level I, II axillary lymph node metastases
N3	Metastases in ipsilateral infraclavicular (level III axillary) lymph node(s) with or without level I, II axillary lymph node involvement; or in clinically detected* ipsilateral internal mammary lymph node(s) with clinically evident level I, II axillary lymph node metastases; or metastases in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement
N3a	Metastases in ipsilateral infraclavicular lymph node(s)
N3b	Metastases in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)
N3c	Metastases in ipsilateral supraclavicular lymph node(s)

Notes

* "Clinically detected" is defined as detected by imaging studies (excluding lymphoscintigraphy) or by clinical examination and having characteristics highly suspicious for malignancy of a presumed pathologic macrometastasis based on fine needle aspiration biopsy with cytologic examination. Confirmation of clinically detected metastatic disease by fine needle aspiration without excision biopsy is designated with an (f) suffix, for example, cN3a(f). Excisional biopsy of a lymph node or biopsy of a sentinel node, in the absence of assignment of a pT, is classified as a clinical N, for example, cN1. Information regarding the confirmation of the nodal status will be designated in site-specific factors as clinical, fine needle aspiration, core biopsy, or sentinel lymph node biopsy. Pathologic classification (pN) is used for excision of sentinel lymph node biopsy only in conjunction with a pathologic T assignment.



PATHOLOGIC (PN)*

pNX	Regional lymph nodes cannot be assessed (for example, previously removed, or not removed for pathologic study)
pN0	No regional lymph node metastasis identified histologically Note: Isolated tumor cell clusters (ITC) are defined as small clusters of cells not greater than 0.2 mm, or single tumor cells, or a cluster of fewer than 200 cells in a single histologic cross-section. ITCs may be detected by routine histology or by immunohistochemical (IHC) methods. Nodes containing only ITCs are excluded from the total positive node count for purposes of N classification but should be included in the total number of nodes evaluated.
pN0(i–)	No regional lymph node metastases histologically, negative IHC
pN0(i+)	Malignant cells in regional lymph node(s) no greater than 0.2 mm (detected by H&E or IHC including ITC)
pN0(mol–)	No regional lymph node metastases histologically, negative molecular findings (RT-PCR)
pN0(mol+)	Positive molecular findings (RT-PCR)**, but no regional lymph node metastases detected by histology or IHC
pN1	Micrometastases; or metastases in 1–3 axillary lymph nodes; and/or in internal mammary nodes with metastases detected by sentinel lymph node biopsy but not clinically detected***
pN1mi	Micrometastases (greater than 0.2 mm and/or more than 200 cells, but none greater than 2.0 mm)
pN1a	Metastases in 1–3 axillary lymph nodes, at least one metastasis greater than 2.0 mm
pN1b	Metastases in internal mammary nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected***
pN1c	Metastases in 1–3 axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected
pN2	Metastases in 4–9 axillary lymph nodes; or in clinically detected**** internal mammary lymph nodes in the absence of axillary lymph node metastases
pN2a	Metastases in 4–9 axillary lymph nodes (at least one tumor deposit greater than 2.0 mm)
pN2b	Metastases in clinically detected**** internal mammary lymph nodes in the absence of axillary lymph node metastases
pN3	Metastases in 10 or more axillary lymph nodes; or in infraclavicular (level III axillary) lymph nodes; or in clinically detected**** ipsilateral internal mammary lymph nodes in the presence of one or more positive level I, II axillary lymph nodes; or in more than three axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected***; or in ipsilateral supraclavicular lymph nodes
pN3a	Metastases in 10 or more axillary lymph nodes (at least one tumor deposit greater than 2.0 mm); or metastases to the infraclavicular (level III axillary lymph) nodes
pN3b	Metastases in clinically detected**** ipsilateral internal mammary lymph nodes in the presence of one or more positive axillary lymph nodes; or in more than three axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected***
pN3c	Metastases in ipsilateral supraclavicular lymph nodes

Notes

* Classification is based on axillary lymph node dissection with or without sentinel lymph node biopsy. Classification based solely on sentinel lymph node biopsy without subsequent axillary lymph node dissection is designated (sn) for "sentinel node," for example, pN0(sn).
** RT-PCR: reverse transcriptase/polymerase chain reaction.
*** "Not clinically detected" is defined as not detected by imaging studies (excluding lymphoscintigraphy) or not detected by clinical examination.
**** "Clinically detected" is defined as detected by imaging studies (excluding lymphoscintigraphy) or by clinical examination and having characteristics highly suspicious for malignancy of a presumed pathologic macrometastasis based on fine needle aspiration biopsy with cytologic examination.

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4. Intended publications from this research work

Intended publications from this research are:

1. Molecular markers by IHC to predict 5 years recurrence in early breast cancer patients who received adjuvant chemotherapy
2. Could IHC expressions of molecular proteins coded for by the cancer related genes used in Oncotype Dx be predictive of 5 years recurrence in early breast cancer patients who received adjuvant chemotherapy?

5. Presentations of this research work to cancer conferences

A. The abstract of the poster presented to 2nd IMPAKT breast cancer conference, 2011 (Poster number: 89p). (Published as an abstract in Annals of Oncology supplement:22:S2:ii46. (2011)

89P. A STUDY SUGGESTING SUPERIORITY OF IMMUNOHISTOCHEMICAL (IHC) MOLECULAR SUBTYPES BASED UPON ARCHIVED FORMALIN FIXED PARAFFIN EMBEDDED (FFPE) BLOCKS, PREDICTING 5 YEARS RELAPSE FREE SURVIVAL (RFS) AND OVERALL SURVIVAL (OS) IN EARLY BREAST CANCER PATIENTS WHO RECEIVED ADJUVANT CHEMOTHERAPY, COMPARED TO ADJUVANT! RECURRENCE RISK (RR)

M. Moe1, R. Adam2 1. Oncology, Singleton Hospital, Swansea/UNITED KINGDOM, 2. Oncology, Velindre Hospital, Cardiff/UNITED KINGDOM

Aim: To predict 5 years RFS and OS (months) by analysing molecular subtypes based on ER, PR, HER2, EGFR, Ki67, CK 5/6, MCM2, Aurora A, Bcl-2, PDGFRa protein expression by IHC in a matched case control study.
Method: 72 cases (R) (relapsed within 5 years of curative surgery), 72 (C) controls (matched to cases by Adjuvant! RR and node+), 34 low risk control (LC) (Adjuvant! RR <50%) were identified. (Control = no relapse > 5 years). Tissue Micro Arrays were constructed with cores from invasive cancer tissue. IHC staining was performed for each antibody. Protein expression was evaluated on digitised images (Mirax _) (by first author) and independently validated. 5 molecular subtypes were analysed based upon IHC [Luminal A (LA = ER/PR+, HER2-,Ki67-) and B (LB = ER/PR+, HER2/Ki67+), HER2 enriched (H = ER-,PR-, HER2+), core basal (CB = ER-, PR-, HER2-, CK5/6/ EGFR+), 5 negative (5N = ER-,PR-,HER2-,EGFR-,CK5/6-)] together with MCM2, Aurora A, Bcl2 and PDGFRa. SPSS v.16. was used for statistical analysis.
Findings: For R:C:LC groups, age (median) = 30-77 (57): 28 – 74 (52): 36 – 78 (51); RFS (median) = 4.5 – 59.9 (23.7): 74.3 – 164.4 (103.5): 79 – 161 (104.5); OS (median) = 8.1 – 139.4 (41.2): 74.3 – 164.4 (104.9): 79 – 161 (105); Adjuvant! RR (median) = 26.9 – 96.7 (65): 50 – 94 (66): 29 – 47 (37). All but 4 patients of R group had died from breast cancer. 3 patients from C & LC died from non-breast cancer causes. Subtypes are: LA = 57 (32%), LB = 49(27.5%), H = 26 (14.6%), CB = 26 (14.6%), 5N = 12 (6.7%), missing = 8 (4.5%). For subtypes LA: LB: H: CB: 5N, mean Adjuvant! RR = 62: 62: 56: 58: 56 (p = 0.54), median RFS = (has not reached): (has not reached):32.2: 52.2: 31.4 (p = 0.001), and OS = (has not reached): 110: 68: 61.9: 32.7 (p = 0.001), (Kaplan-Meier analysis, log-rank test) respectively.
Conclusions: The data suggests five molecular subtypes are more predictive of 5 years RFS and OS than Adjuvant! RR. N has lowest survival followed by H (RFS) or CB (OS). Data on overall analysis including MCM2, Aurora A, Bcl2 and PDGFRa will be presented.
Disclosure: M. Moe: I received research grant from Pfizer. All other authors have declared no conflicts of interest.

B. The abstract of the poster presented to 7th NCRI conference, 2011 (poster number: B71)

Defining and optimising risk stratification in early breast cancer using a focused panel of Immunohistochemical (IHC) molecular markers: a single institution study.

Maung Moe[1], Robert Mansel[4,3], Richard Adams[2]
[1]Singleton Hospital, Swansea, UK, [2]Velindre Hospital, Cardiff, UK, [3]Cardiff University, Cardiff, UK, [4]UHW, Cardiff, UK

Background

An inexpensive, readily available technique that utilises IHC expressions of proteins involved in breast cancer cell molecular pathways, seems a logical way to stratify patients for risk assessment and choice of successful treatments. Here we explore a panel of markers in a case control study.

Method

72 cases (relapse within 5yrs of curative surgery), 72 controls, (recurrence free > 5yrs), matched to cases by Adjuvant! recurrence risk. Tissue microarrays constructed with cores from formalin fixed paraffin embedded tissue. Optimised protein IHC expression was evaluated on digitalised images (Mirax). 5 molecular subtypes [Luminal A (LA = ER/PR+, HER2-,Ki67-) and B (LB = ER/PR+, HER2/Ki67+), HER2 enriched (H = ER-,PR-, HER2+), core basal (CB = ER-, PR-, HER2-, CK5/6/EGFR+), 5-negative (5N = negative for ER,PR,HER2,EGFR,CK5/6)] together with MCM2, Aurora A, Bcl2, PDGFRa and CD68 expressions were analysed. SPSS 16v. used for statistical analysis.

Results

All had adjuvant chemotherapy. All but 3 cases had died from recurrent disease. Median (m) RFS and mOS = 23.2mo & 39.7mo. All but 3 controls remain free of recurrence: median follow-up = 103.5mo (74.3 – 164.4). mRFS and mOS for subtype LA = not yet & not yet; LB = 58.1 & 86.1; CB = 15.4 & 30.4; H = 28 & 55.9; 5N = 19.9 & 26 ($p = <0.0001$ & <0.0001 by Log rank test). Better mRFS and mOS were found for positive Bcl2 ($p = 0.036$ & 0.058) and MCM2 ($p = 0.022$ & 0.048), negative Aurora A ($p = 0.01$ & 0.001) and PDGFRa ($p = 0.07$ & 0.086) expressions. Results of multivariate analysis and CD68 expression will be presented.

Conclusion

Subtypes CB & 5N, negative Bcl-2 & MCM2, positive Aurora A and PDGFRa expressions were predictive of poor RFS and OS and should be used as stratification factors for novel prospective biomarker led adjuvant studies.

C. The Abstract of the poster presented in 34th Annual San Antonio Breast Cancer Symposium (poster number: P1-07-21); published in Supplement to Cancer Research:71:24:p-196s.

Analysis of Molecular Markers by Immunohistochemistry (IHC) Method on Formalin Fixed Paraffin Embedded (FFPE) Tissues Could Predict Shorter Recurrence Free Survival (RFS) and Overall Survival (OS) among Patients Who Have Received Adjuvant Chemotherapy for Early Breast Cancer.

Moe M, Gee J, Finlay P, Mansel R, Adams R. Singleton Hospital, Swansea, United Kingdom; Velindre Hospital and Cardiff University, Cardiff, United Kingdom; Cardiff University, Cardiff, United Kingdom

Background: Various molecular markers assessed by IHC (ER, PR, HER2) and gene expression profiling (e.g. Oncotype Dx) have been developed as prognostic and predictive tools for breast cancer. Gene profiling is said to be superior to IHC but at a considerable cost with limited availability. IHC is relatively inexpensive and more readily available. If early breast cancer patients who are going to relapse within 5 years of curative surgery despite adjuvant chemotherapy could be identified by IHC on FFPE tissue alternative adjuvant therapies could be explored. In this context, here we evaluate IHC for expression of a panel of molecular markers implicated in: growth signalling pathways (ER, PR, HER2, EGFR, CD71, Ki67, MCM2), cell survival (Bcl-2, Bag 1), angiogenesis (PDGFRa) and cell cycle progression (Aurora A, MCM2). Of note, this study includes markers of breast cancer molecular subtype (ER, PR, HER2, Ki67, EGFR, also CK5/6) and several proteins encoded by genes in the Oncotype Dx test (ER, PR, HER2, Ki 67, Bcl2, Bag1 and CD68).

Materials and Method: 72 cases (R) relapsing within 5 years of curative surgery, 72 controls (C), relapse free > 5 years were identified from the hospital records. All patients had adjuvant chemotherapy. Controls were matched to cases by Adjuvant! recurrence risk (ARR). Optimised IHC was performed on FFPE TMA slides using a Ventana autostainer. Protein expression was evaluated on digitalised images (Mirax scanner). Survival analysis by molecular markers expression and also 5 molecular subtypes, Luminal A (LA = ER/PR+, HER2-, Ki67-), Luminal B (LB = ER/PR+, HER2/Ki67+), HER2 enriched (H = ER-, PR-, HER2+), Core Basal (CB = ER-, PR-, HER2-, CK5/6/EGFR+) and 5-negative (5N = negative for ER,PR,HER2,EGFR,CK5/6)], were performed. SPSS 16v. was used for statistical analysis.

Findings: All but four cases had died at the time of analysis. Four controls developed relapse at 83.8, 90.6, 107.7, 127.6 months respectively. Two controls died from non-breast cancer causes. Median (m) follow-up for the controls group (ie. mOS)was 104.9 mo (72.8 - 164.4). For cases, mRFS and mOS were 23.2 (4.5 - 59.9) and 39.7(8.1 - 129). mRFS and mOS for IHC molecular subtypes were: Subtype LA = not yet & not yet; LB = 58.1 & 86.1; CB = 15.4 & 30.4; H = 28 & 55.9; 5N = 19.9 & 26 ($p < 0.0001$ & <0.0001 by Log rank test). Better RFS and OS were found for positive Bcl2 ($p = 0.036$ & 0.058) and MCM2 ($p = 0.022$ & 0.048), negative Aurora A ($p = 0.01$ & 0.001) and PDGFRa ($p = 0.07$ & 0.086) expressions. For this study cohort there was no correlation between ARR and survival outcome or molecular subtypes. Result of ongoing multivariate analysis and correlation between survival and CD68, CD71 and Bag 1 expressions will be presented in the conference.

Discussion: Subtypes CB & 5N, negative Bcl-2 & MCM2, positive Aurora A & PDGFRa expression as measured by IHC were predictive of poor RFS and OS. While these findings need to be verified in an independent cohort, IHC profiles nevertheless have potential to stratify different risk groups for clinical trials and effective adjuvant treatments.