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The role of AP-2 alpha in predicting response to chemotherapy in breast cancer patients

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Introduction

Epidemiology and pathogenesis:

Breast cancer is the commonest cancer occurring in women in Europe (1). In Switzerland, between 2003 and 2007 they were approximately 5000 new cases per year (2) and it is the primary cause of cancer related death in women, with a mortality rate of 19.2% (2). Incidence remained stable until the introduction of mammography screening in the 80s (3); an increased ensued, as mammography detected instances of carcinoma *in situ* and small invasive carcinomas that would have remained undiscovered without this method (3). Conversely, cases of advanced stage carcinoma decreased (figure 1) and currently less than 10% of cases display metastatic disease at initial presentation (3). This, combined with the availability of modern therapies, has led to the decrease in breast cancer mortality in the last decade (3,4).

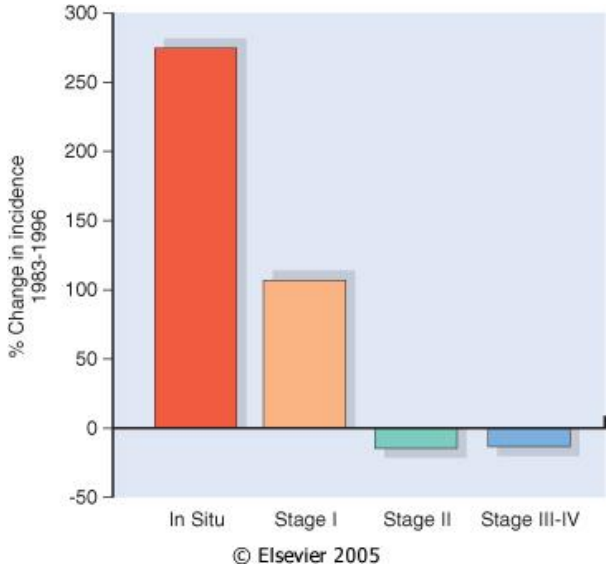


Figure 1: Change in incidence depending on stage of breast cancer from 1983 to 1996. (SEER Cancer Statistics Review, http://seer.cancer.gov/.)(3)

Breast cancer is a multifactorial disease arising from interactions between genetic, reproductive and environmental factors (5). Reproductive risk factors include the combined oral contraceptive pill, early menarche, advanced age of first pregnancy, nulliparity, hormonal replacement therapy and late menopause. Obesity, unhealthy diet, smoking, alcohol consumption and lack of exercise rank amongst the environmental factors (5). Inherited risks comprise of age, specific mutations in BRCA1/2, and familial history of cancer (5). It is believed that these interactions lead to the development of cancer via various carcinogenesis pathways (through a series of molecular alterations at the cellular level) resulting in the evasion of growth-inhibition signals, evasion of apoptosis and spread of breast epithelial cells (6). However no common genetic or functional changes can be consistently observed in every breast cancer investigated (3).

Breast cancer molecular subtypes:

The majority of breast cancers (more than 95%) are adenocarcinomas. Classification of breast cancer has been conventionally based upon histology, following the WHO 2003; Tumours of the Breast and Female Genital Organs recommendations (32). It divides breast tumours into two categories: infiltrative carcinoma and in situ (3). DCIS (ductal carcinoma in situ) and LCIS (lobular carcinoma in situ) constitute the latter category. Invasive breast malignancy is then subclassified as follows: ductal carcinoma (60-75% of all invasive carcinomas), lobular carcinoma (8%), and the remaining 15% which consist of much rarer tumours such as tubular, medullary, mucinous and papillary types (32). In total, 19 categories have been described.

Recently, by using microarray technology to define gene expression of breast cancer, four different subclasses have been described**:** luminal A, luminal B, HER2+, triple negative (8,12). This technique uses the association of gene clusters to define the subtypes (8). It has been shown that clinically these subtypes each have a distinct natural evolution and progression (figure 2); each also responds differently to adjuvant treatment (9,11,12). It is thought that these features are indicative of distinctions in specific growth rate, intracellular signal pathways and cell composition, thus explaining breast cancer heterogeneity (9). This classification is now approved and is considered integral to the efficacious treatment of breast cancer (7,10).

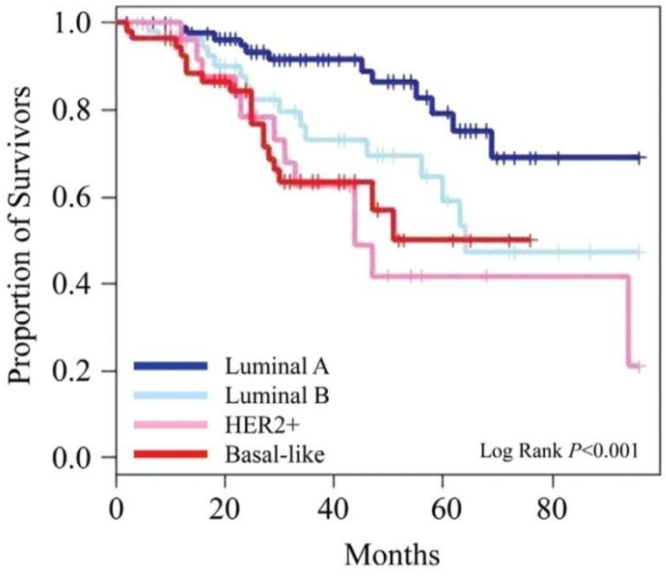


Figure 2: Survival plot of 294 breast cancer patients. A Kaplan-Meier survival plot of overall survival corresponding to 294 breast cancers from the publicly available UNC database is shown grouped by molecular subtype (11).

Microarray is not utilised in the hospital setting however, with cost concerns dictating that the subtypes are evaluated by immunohistochemistry (10). Goldhirsch and al., consider this method as a valid surrogate for microarray technology (10). Those subclasses are defined according to oestrogen and progesterone receptor positivity, HER2 status and Ki-67 (proliferation rate) (table 1) (10). The St-Gallen 2011 expert consensus created specific recommendations on how to classify breast tumours based upon the criteria mentioned above.

Table 1: breast cancer molecular subtypes: Definitions according to St-Gallen 2011 consensus

	ER and/or PR	HER2 overexpressed	Ki-67
Luminal A	Positive	Negative	Less 14%
Luminal B	Positive	Negative	High
Luminal B	Positive	Positive	Any value
HER2+	Negative	Positive	Any value
Triple negative	Negative	Negative	Any value

Adjuvant treatment:

Management of breast cancer depends on a combination of several factors: tumour biology (molecular subtype, grade, hormonal receptors, HER2 status, proliferation rate and histological type); tumour stage (tumour size and axillary lymph node involvement); peri-tumoural lymphovascular invasion; patient's age and menopausal status (13-15). Adjuvant treatment consists of chemotherapy, radiotherapy, hormonotherapy and immunotherapy. Radiotherapy follows surgery in most cases to prevent local relapse. Hormone therapy should be given if the cancer is hormone receptor positive, and should be administered for at least 5 years (14,16). Immunotherapy is used for women exhibiting HER2 overexpression. Adjuvant chemotherapy is used to destroy residual cancer cells believed to have survived elimination by surgery and radiotherapy (6). The goal is to avoid recurrence and the development of distant metastases, resulting in increased disease-free survival and overall survival (6,15). Analysis of several randomised control studies by the Early Breast Cancer Trialists' Collaborative Group has shown reductions of 12% and 6% in 10 year mortality in the cohort under 50 years old, for node positive and node negative patients respectively (4). Women aged 50 to 69 years accrued reductions of 6% and 2%, for node positive and node negative patients respectively (4).

However, over 70% of these patients would have been cured without the use of chemotherapy (7). These treatments are debilitating, with many secondary effects even leading to refractory secondary malignancies many years later. Also, some women who receive chemotherapy will still develop recurrence of the disease; 15% of early breast cancers which are classified as "low-risk" will eventually recur (7,15,20). Alighting upon the correct treatment regimen is especially problematic in early breast cancer, where differentiating those who will ultimately benefit from chemotherapy from those who will not is difficult for the clinician (7).

Clinical tools in breast cancer:

Tools have thus been developed to help the clinician make this decision, in the form of international guidelines, computer based techniques and genomic testing.

International guidelines were created by groups of experts, interpreting data from randomized controlled trials in a form suitable for the practice of evidence based medicine (14,15). However it is not an individualized based care.
Computer-based tools, for example *Adjuvant! Online*, are easy-to-use online risk calculators tailored for clinical settings (15). They are founded on the same criteria as the guidelines, but are formulated to numerically express risk (15). These still need to be validated however, and current examples don't take histology, node status or molecular subtype into consideration (15).
The genomic tools include Oncotype Dx and Mamaprint. Oncotype Dx is a q-RT-PCR of a 21 gene signature, giving a recurrence score to predict breast cancer recurrence (specifically for women with node-negative and ER positive tumours) (15). Mamaprint uses DNA microarray to investigate the expression of 70 particular genes, and is designed to predict the risk of development of metastases in early breast cancer (specifically for node-negative, ER positive/negative cancer type) (15). In the literature, these techniques have been proven to be more effective than the present guidelines and computer based techniques (15). A panel of experts at the 12th annual St-Gallen conference in 2011 judged that Oncotype DX was helpful for decision making, while other predictive and prognostic factors didn't appropriately indicate which management to choose (10). However, Mamaprint was adjudged not to be sensitive and specific enough to use in clinic (10). Randomized control studies have yet to be carried out to prove that Oncotype Dx can in fact assist in reducing mortality (15). The limits of these techniques include cost, their restricted application to only early stage breast cancer, variability in reproducing this technic for every tumours specimen and normalization of the results (15).

The aforementioned tools may help in the direction of patient care but we believe there is a necessity for a more reliable and improved tool to assist the clinician in his judgment.

Activator-protein 2 family:

The human transcription factor activator-protein 2 alpha (AP-2 alpha) has been found to have a central role in the development of breast cancer (17,18). AP-2 alpha belongs to a family of proteins including AP-2 beta and gamma (19). AP-2 alpha is the most studied of AP-2 family subtypes in breast cancer (19). It is thought to have a tumour suppressor role in the breast, whereas the roles of the other members of this family are not yet well known (19). These 52kda proteins recognize and bind specific DNA sequences on the target genes, enhancing or suppressing their expression (19). For example, p21, ER-alpha, and HER2 number amongst AP-2 alpha's target genes, and all are essential in cell differentiation and growth (19). The general role of AP-2 in normal tissue was studied by using knock-out mouse trials, showing that mice lacking AP-2 presented with abnormal organogenesis and died at birth or soon after (19).

AP-2 alpha's role, localisation (nuclear vs cytoplasmic), regulation of ER, and its link to HER2 overexpression with regard to breast cancer are not yet well established (19). Some studies link a decrease in nuclear AP-2 alpha with more aggressive cancer types (17-19).

However, many current studies are contradictory in their findings (19). Pellikainen and Kosma suggested that one possible cause could be that the immunoshistochemical method used was not reliable, as the antibodies utilised were not specific enough (19). They also suggested that it was because AP-2 protein *in vivo* regulators could not be studied as accurately, results could be difficult to interpret (19). It is possible to study AP-2 alpha activity via a new method called protein-binding microarray (PBM) (17). Proteins, in this case AP-2 alpha, are put in contact with double stranded DNA sequences fixed on a microarray (18). This technique allows the detection of protein binding on specific DNA sequences (18). This method has been demonstrated to be reliable and highly sensitive (17). An initial study done at the University of Lausanne demonstrated that AP-2 alpha binding on DNA could have a better prognostic value than classic molecular diagnosis, and may redistribute the subtype classification (24). Now, there is a need to repeat this trial with a larger breast cancer sample size and a more robust microarray.

Objectives:

My objective is to create an anonymous database from clinical and pathologic data, to be used to correlate results from the PBM with the patient's follow-up. Then it would be to amplify by PCR some targets of AP-2 alpha to be added on a custom microarray. This custom microarray will be used to test protein extract from breast tumours samples selected from the database previously made.

In parallel, RNA will be extracted from breast tissues and quantitative RT-PCR will be done in order to correlate the AP-2 alpha binding activity on specific targets detected by PBM with the transcriptional level of those targets.

Method and materials

Patient selection:

One hundred mastectomy/tumeroctomy surgical samples were selected from the database of the pathology institute to carry out the trial. The specimens used were taken between 2000 and 2005, so as to have at least 5 years of clinical background information. Specimens were of various molecular subtypes (luminal A, luminal B, HER2+, triple negative) and also from breast tissue adjacent to the tumour. Healthy tissue obtained at mammoplasty was used as a control. All tissue samples were flash frozen and preserved at -80 C° to protect the cells' integrity (proteins in particular). Surgical samples and documentation were rendered anonymously before being processed by the lab.

Samples were selected and classified with regard to their respective predictive and prognostic factors, and added to a spreadsheet. Two different charts were formulated: the first for pathological data and the second for clinical data.

Pathological information was classified according to the following categories: date of birth, type of surgery (tumeroctomy or mastectomy), where the operation was performed, date of operation, single or multifocal tumours, tumour size, number of sentinel lymph nodes analysed, number of lymph nodes analysed, number of positive lymph nodes (metastasis, micrometastasis, isolated tumour cell), histological type of the tumour, histological type of the lymph node, grade (Elston-Ellis), lymphovascular invasion, ER%, PR%, HER2 overexpression, methodology used to detect HER2, and proliferation rate (Ki-67).

Clinical information was classified according to the following categories: date of birth, date of diagnosis, menopausal status, chemotherapy (type, number of cycles), hormonotherapy (type, dosage, time), radiotherapy (local, loco regional+/- boost), date of most recent oncology clinic appointment, date of most recent hospital admission, recurrence, distant metastasis (when, where, histological type), new tumour, and death (cause).

Frozen tissue and histological studies:

Surgically removed tumours were conserved at -80 C°. Before extraction of protein and RNA, each specimen was cut, using cryostat, into sections of 30µm and 7µm, in the following sequence: (1)x7µm section superiorly – (30)x30µm sections – (1)x7µm section medially – (30)x30µm sections – (1)x7µm section inferiorly. The 30µm sections were collected in Eppendorf tubes and saved for protein and RNA extraction. The 7µm sections were placed on histology slides and stained with HE, following the HE protocol (see appendix 1). Cancer cells were detected on these slides using microscopy, thus verifying that cancer cells were present at throughout the sample, at these three levels, superiorly, medially and inferiorly.

Preparation of target genes for custom based PBM:

Around 50 AP-2 alpha targets genes were selected for creating custom-made PBM. The following four target genes required amplification for spotting: ESR1, FABP5, WASF, TAF. Each of these genes was amplified and cloned in plasmids, pCR8®/GW/TOPO (Invitrogen), as described previously (18). For PCR amplification their respective annealing temperatures were of 62 C°, 58 C°, 58 C°, 60 C°. PCR solution was prepared following manufacturer's recommendations (Qiagen). Briefly, 250µL of solution contained 0.5 µL miniprep DNA, 2.5 µL primer 1 (100µM), 2.5 µl primer (100µM), 2.5 µL 10x tampon, 50 µL Q solution, 20 µL dNTP mix (2.5mM each), 1.25 µL high-fidelity Taq polymerase (Qiagen), 1.25 µL Taq polymerase, 147 µL water. A PCR programme was accomplished as follows : 95 C° for 10 minutes; 95 C° for 30 seconds; annealing temperature for 30 seconds; 72 C° for 1minute and 30 seconds, cycling through denaturation, annealing and extension for 35 cycles; 72 C° for 10 minutes and 10 C° for forever.

PCR products were electrophoresed in 1% agarose gel. The gel was prepared as a mix of 2 g agarose powder with 200 ml TAE and 20µl of SYBR safe DNA gel stain. The mix was then poured on an electrophoresis plate. Agarose fluorescents parts were then collected by cutting the gel under ultraviolet light source. PCR products were purified by centrifugation using Wizard SV gel and PCR Clean-Up System (Promega) (see appendix 2).

The solution was then analysed using the nanodrop 3300 (Micro-Volume Full-Spectrum Fluorospectrometer) to quantify the DNA.

Total RNA extraction and Reverse Transcription (RT):

RNA extraction was performed on frozen tissue after cryostat cut as explained above, in five samples of luminal A, five luminal B, five triple-negative and 3 HER2+ samples, using the Trizol Reagent protocol (Invitrogen, San Diego, CA) (see appendix 3) . RNA purity was analysed using the nanodrop. Reverse transcription was performed using a total of 500 ng of RNA to synthetize cDNA in a 33 µL total volume reaction (20 µL RNA + DTT 1µL, Primer 1µL, Bulk first-strand reaction mix) with the GE Healthcare first-strand cDNA synthesis kit.

Quantitative real-time PCR:

AP-2 alpha binding activity was studied using a PBM with a H-6K promoter chip Avi-HU Pro6k lot (Aviva Systems Biology, San Diego, USA) containing 6000 genes. Selected genes of interest were observed, comparing the level of binding of the transcriptor factor between normal tissue and neoplastic tissue. These genes included: ER alpha and beta, PR, HER2, MMP2, MMP9, ANXA1, WWOX, and then one gene for each molecular subtype category that is unique to that subtype (table 2). Previous published studies were consulted to choose

appropriate gene primers. Primers were inputted to the website of UCSC genome bioinformatics for validation. Applied Bio system 7900 was used to carry out the real-time PCR in triplicate for each sample using light SYBR green master mix following manufacturer instructions (Roche). Control gene's (β-actin, GAPDH, 18s) activity level was used as a reference for data normalization. Genes' reverse and forward primers are described as followed (table 3).

Table 2: The 15 genes selected from the 50 to which AP-2 alpha binds

Genes	**Breast cancer-associated**
18s	Control genes
β-ACTIN	
GAPDH	
AP-2 alpha	Common to all
ANXA1	
WWOX	
MMP2	
MMP9	
ER alpha	Common to luminal A/B
PGR	
ERBB2	Common to HER2+/luminal B
EDG5	HER2+
RAP2B	Triple negative
TACR1	Luminal A
PON2	Luminal B

Table 3: Real-time quantitative PCR was made using the following primers:

Gene	**Gene primers (For/Rev)** **(5′→3′)**
AP2 alpha	*F: CGA TCC AGA GCT GCT TGA CC* *R: GAG CCT CAC TTT CTG TGC TTC TC*
ERBB2	*F: TCC TGT GTG GAC CTG GAT GAC* *R: CCA AAG ACC ACC CCC AAG A*
β-ACTIN	*F: TCC TTC CTG GGC ATG GAG* *R: AGG AGG AGC AAT GAT CTT GAT CTT*
GAPDH	*F: TTG TCA AGC TCA TTT CCT GG* *R: TGA TGG TAC ATG ACA AGG TGC*
18s	*F: CAA CTT TCG ATG GTA GTC G* *R: CCT TCC TTG GAT GTG GTA*
ER alpha	*F: TGG GCT TAC TGA CCA ACC TG* *R: CCT GAT CAT GGA GGG TCA AA*
PGR	*F: CGC GCT CTA CCC TGC ACT C* *R: TGA ATC CGC CCT CAG GTA GTT*
ANXA1	*F: AGG GTG ACC GAT CTG AGG AC* *R: CTG GTG GTA AGG ATG GTA TTG A*
WWOX	*F: GAG CTG CAC CGT CGC CTC TCC CCA C* *R: TCC CTG TTG CAT GGA CTT GGT GAA AGG C*
MMP2	*F: CCC TGT CAC TCC TGA GAT CTG C* *R: CAC AGT CCG CCA AAT GAA CC*
MMP9	*F: CCA CCA CAA CAT CAC CTA TTG G*

	R: ACT GGA TGA CGA TGT CTG CG
EDG5	*F: GCC TCT CTA CGC CAA GCA TTA* *R: TTG AGC GGA CCA CGC AGT A*
RAP2B	*F: AGC CAA ACG CAT CCG ACT CTC TAA* *R: CTG CAA AGA GCC ACA TTT CCA CCA*
TACR1	*F: CTC AGA CCT CTC CCC AAA CA* *R: CAC AAT GAC CGT GTA GGC AG*
PON2	*F: TGA GCT TCT TCC AAG TGT GAA TG* *R: AAA TGT GCC GGT CCA ACA G*

q-RT-PCR was performed on 16 tumour samples (5 luminal A, 3 luminal B, 3 HER2+, 5 triple negative) and 5 normal tissue samples.

Protein extraction:

Protein extraction (AP-2 alpha) was achieved as described previously (22). Tissue specimens cut in 30 µm section that had been collected in the Eppendorf tubes were thawed on ice in a solution of 1X PBS (10 mM pH 7.4 Phosphate, 137 mM NaCl, 2.7 mM KCl) containing a cocktail of protease inhibitor (Roche complete protease inhibitor cocktail tablets, Roche) followed by centrifugation of the tissue fragments at 4 C° for 5 min at 3500 rpm. Pellets were recovered into 250 µL Low Salt Lysis Buffer (10 mM Tris pH 7.8, 1.5 mM MgCl2, 10 mM KCl) and incubation allowed on ice for 15 min. Cells were lysed by addition of 0.5 µL NP-40 (0.02% masse/volume) and mixed by vortexing for 5 seconds. Nuclei were harvested by centrifugation for 5 min at 3500 rpm, 4 C° and the pellet washed with 300 µL Low Salt Lysis Buffer. After an additional centrifugation step (3500 rpm at 4C° for 5 min) the supernatant was taken off and the pelleted nuclei resuspended in 25 µL of High Salt Lysis Buffer (10 mM Tris pH 7.8, 1.5 mM MgCl2, 10 mM KCl, 400 mM NaCl, NP-40 0,033% [masse/volume]). Samples were mixed by vortexing for 5 seconds and incubated on ice for 30 minutes with vortexing every 5-10 min in order to allow extraction of nuclear proteins. The supernatant containing the nuclear proteins was transferred into new coded tubes and diluted by adding 25 µL of Low Salt Buffer to bring the final NaCl concentration to 200 mM and immediately frozen in liquid nitrogen.

Protein-binding microarray assay:

Protein-binding microarray assay was performed as described previously (18,22). Custom-made PBMs were treated prior to use as follows: slides were washed twice for 2 minutes with 1xTEN (40 mM Tris-HCL pH 7,5, 1 mM EDTA, 150 mM NaCl), then reduction of aldehydes was performed in the reduction solution (0.25% NaBH4 [masse/volume] in PBS 1X/EtOH 25%) for 10 minutes, followed by two washes of 2 minutes with 1xTEN. Slides were then blocked in the blocking solution for 1 hour (5% skimmed milk in PBS 1X/0.05% Tween-20) followed by two final washes for 2 minutes with 1xTEN. Labeling of the DNA on the arrays was carried out as describe here: slides were pre-incubate with 1xPBS containing 100 µg/mL

adenosine monophosphate (AMP), followed by application of the DNA labeling mix (1x NEB buffer 4, 100 µg/mL AMP, 0.1 mM CoCL2, 0.2 µM ddNTP-Cyanine5 [Perkin Elmer], 0.02 U/µL TdT [NEB]). After incubation for 1.5 hour at 37 C° in the absence of light, slides were washed twice for 2 minutes in 1xTEN and spun dry.

AP2-binding reaction mix (2.5 mM Tris-Hcl pH 7.9, 15 mM KCl, 1 mM MgCl2, 0.025 mM EDTA, 12.5 µg/ml BSA, 0.05 % NP40 2 % [masse/volume] skimmed milk, 0.015 µg/µL poly-dIdC, 0.15 ng/µL purified GST-AP2α or 100 to 1000 ng/µL of protein from nuclear cell extract) was applied onto each array slide and incubated at room temperature (RT) in the absence of light for 1 hour. Slides were washed 5x3 minutes with 1x PBS supplemented with 1 % (volume/volume) Tween 20, 3x5 minutes with 1x PBS containing 0.01 % (volume/volume) Triton-X-100 and spun dry. Next, the primary antibody (AP2-alpha (C-18):sc-184, Santa Cruz Biotechnologies) was applied in 1x PBS supplemented with 2% skimmed milk, and incubated for 1 hour at RT in the absence of light. Slides were washed 3x5 minutes with 1x PBS with 0.05 % (v/v) Tween 20, 3x5 minutes with 1x PBS with 0.01 % (volume/volume) Triton-X-100, before incubating with the secondary antibody (Cy3-labeled Alexa Fluor 546 goat anti-rabbit IgG, Molecular Probes) in 1x PBS supplemented with 2 % (masse/volume) skimmed milk for 1 hour at RT in the absence of light. This was followed by a final washing cycle (3x5 minutes with 1x PBS, 0.05 % [volume/volume] Tween 20, 3x5 minutes with 1x PBS, 0.01 % [volume/volume] Triton-X-100 and once for 5 minutes with 1x PBS). The Agilent G2566AA scanner was used to detect the fluorescence and this later was analyzed using the GenePix Pro6 software.

Results

Molecular subtypes:

The pathological Institute of Lausanne referred 103 tumour samples for use in this study. Of these 103, 24 tumour specimens were excluded as clinical data was not available. Of the 79 remaining, 49% were luminal A, 1% were luminal B, 9% were HER2+ and 8% were triple negative and 33% were of undetermined molecular subtype (figure 3).

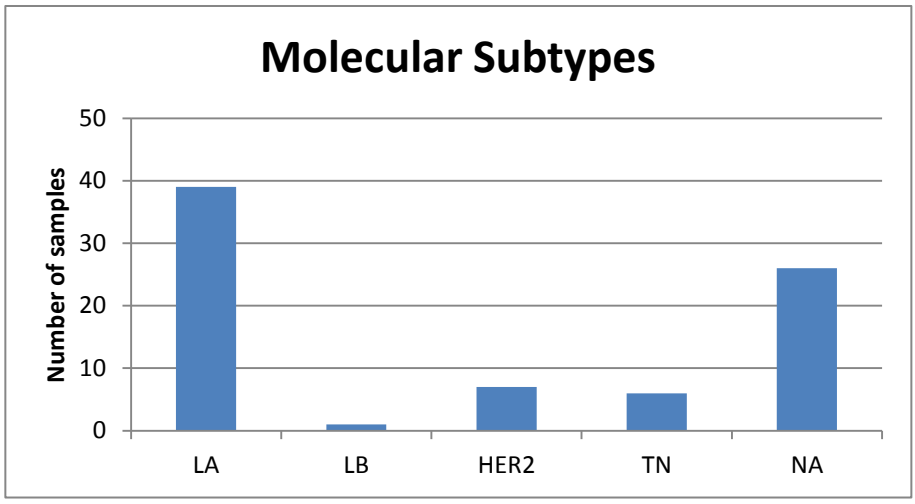


Figure 3: number of tumour samples depending on their molecular subtypes (LA= luminal A, LB= luminal B, HER2+, TN= triple negative, NA= undetermined).

Classification of the specimens was performed according to hormonal status and HER2 overexpression. This does not follow the St-Gallen recommendations (mentioned above), as it does not include the Ki-67 (MIB-1) parameter. 33% of samples remained of undetermined subtype as HER2 overexpression status was not routinely carried during the period in question (2000-2005).

The mean age of patients was 65 years old and 73% of patients had reached menopause at time of diagnosis of breast cancer.

25% of women developed metastasis, at an average time of 21.55 months post diagnosis. Incidence of metastasis in patients with different molecular subtypes was as follows: 20.5 % of luminal A, 0 % of luminal B, 50 % of triple negative, 42.9 % of HER2+, and 23.1 % of undetermined subtype (figure 4). Nearly half of the patients who developed metastasis had received chemotherapy, which was of various regime types.

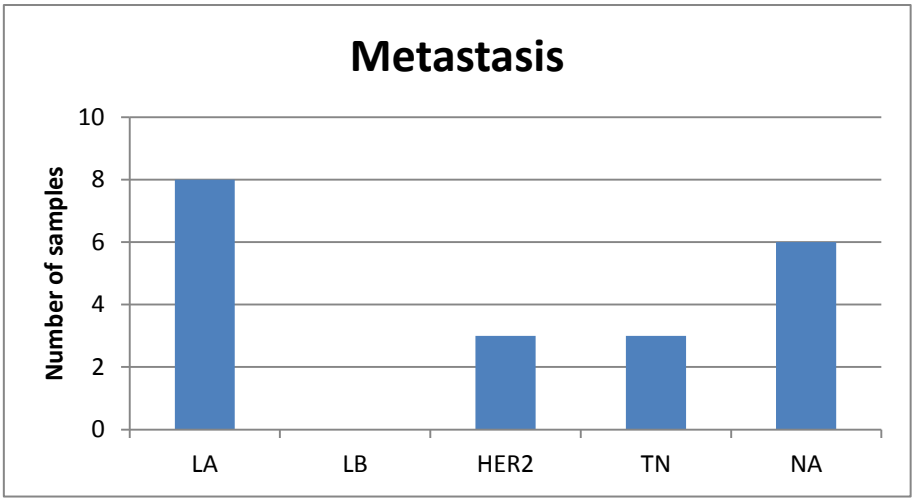


Figure 4: number of patients who developed metastasis in the 5 years follow-up depending on their respective molecular subtype (LA= luminal A, LB= luminal B, HER2+, TN= triple negative, NA= undetermined).

Chemotherapy was given to 43% of patients, with different regimes administered depending on the case. 11% of these 43% had received chemotherapy neoadjuvantly.

Relapse-free survival rates were evaluated using a Kaplan-Meier analysis curve (figure 5). Relapse is here interpreted as any evidence of recurrence or metastasis. Undetermined tumour type samples were not taken into consideration. Triple negative molecular subtype reported the lowest disease free survival rate. However, the difference between the molecular subtypes did not show statistical significance (p-value = 0.246).

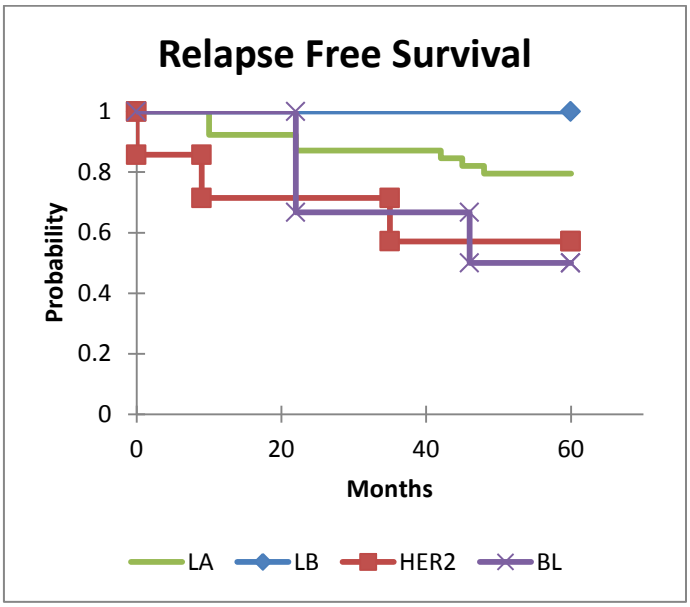


Figure 5: relapse free survival curve based on the different molecular subtypes (LA= luminal A, LB= luminal B, HER2+, BL= triple negative, NA= undetermined).

Mortality rates could not be determined as cause of death was not always reported in the clinical files.

PCR gene amplification:

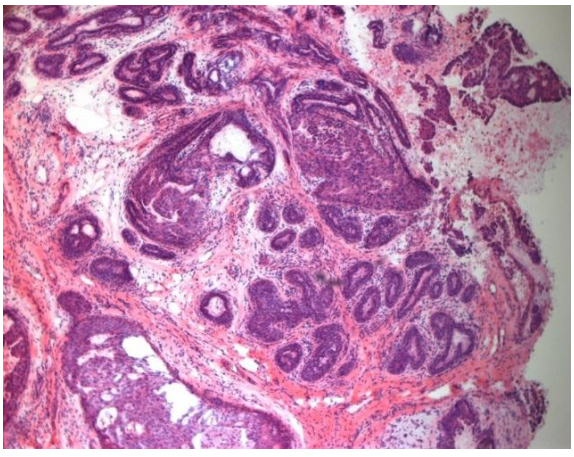
DNA quantity was analysed using the nanodrop samples of gene amplification. Results using the nanodrop are recorded in table 4.

Table 4

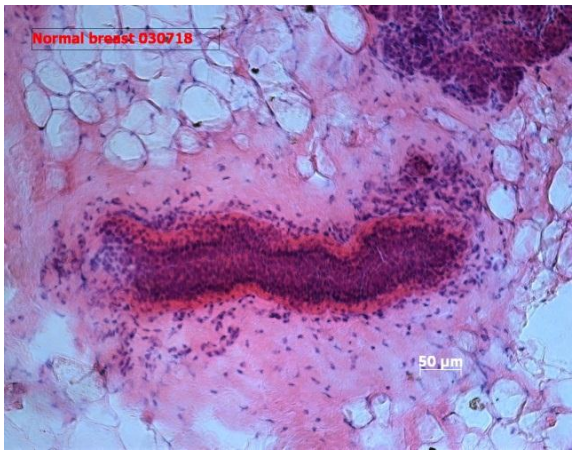
Sample ID	Ng/ul	A260	A280	260/280	260/230
ESRI	89.98	1.800	0.959	1.88	2.54
FABP5	45.55	0.911	0.504	1.81	2.26
TAF1	27.01	0.540	0.291	1.86	1.92
WASF	61.81	1.236	0.675	1.83	2.96

A280 and A260 represent the spectrum of absorbance of proteins and nucleic acids respectively (31). A230 represents the absorbance of contaminants. A260 and A280 ratio serves a control of purity of DNA. The ratio should be in the range 1.8-2.0; a decreased ratio could signify presence of contaminant (for example proteins). The A260/A230 ratio should be in the range 2.0-2.2 (31). For spotting samples were putted in a solution of 3XSSC/1.5M betaine at a final concentration of 80ng/ul.

Histology of frozen tissue slides:



A



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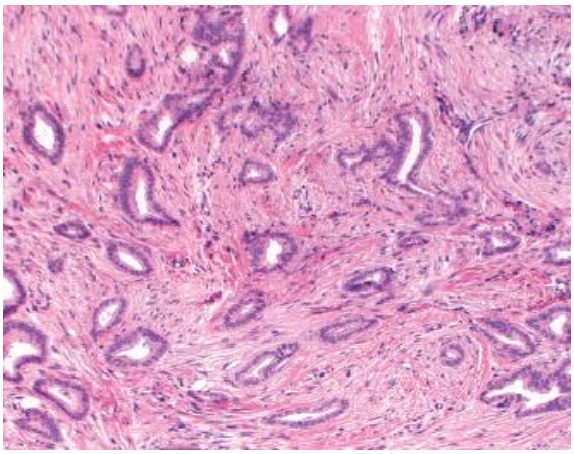


C

Figure 6: micrographs of normal breast tissue (A) (B) and tumour sample (C)

Figure 6 displays micrographs of normal breast tissue (A) and tumour tissue (B) after staining with HE. (A) Normal breast tissue shows terminal duct lobular units that is surrounded by dense fibrous tissue. (B) Normal breast tissue shows a lactiferous duct with adipose tissue visible at the periphery. (C) Well differentiated invasive carcinoma. Cords of ductal epithelial cells are seen invading into the surrounding fibrous stroma. *Image C source: Robbins Basic Pathology (3).*

Gene's expression level:

Of the three housekeeping genes (β-actin, GABDH and 18s), only β-actin was used. The amplification efficiencies of 18s were much greater than for other genes (a mean of 18 cycles compared to approximately 30 cycles for the remainder), thus rendering it unsatisfactory for normalisation. β-actin amplification efficiencies were similar to the target genes and so it was selected as our referenced gene. The delta-delta Ct method was used to assess gene expression level (30); this was calculated as fold change 2 (-Delta Delta C(T)) and normalized to β-actin gene level expression.

RAP2B, TACR, MMP9, MMP2 and PGR were excluded as their expression levels (level of mRNA) could not be accurately analysed. For example, PGR expression levels were undetermined, as levels were under the detection threshold, even in tumour samples which expressed progesterone receptors.

For each gene (AP-2 alpha, ER alpha, ERBB2, WWOX, PON2, ANXA1, EDG5) expression of mRNA was observed in normal tissue, giving us information on what occurs in non-neoplastic cells. Table 5 displays mean fold change for each genes normalized to β-actin in normal tissue. Level of expression in normal tissue was used as a reference point to compare expression of the genes in the different subtype samples.

Table 5: mean gene's fold change in normal tissue

Genes	Mean	Min-Max level
AP2alpha	1.137	0.013-1.377
ERalpha	0	0
WWOX	0.662	0.038-1.733
PON2	0.270	0.035-1.316
ANX1	0.390	0.151-1.074
ERBB2	0	0
EDG5	0.015	0-0.074

AP-2 alpha mRNA levels in the different samples exhibited decreased expression in comparison to normal tissue. However, using non-parametric statistical analysis (Mann-Whitney test), this proved non-significant with p-values as follows: triple negative 0.251, luminal A 0.175, luminal B 0.655, HER2+ 0.180 (Fig 7).

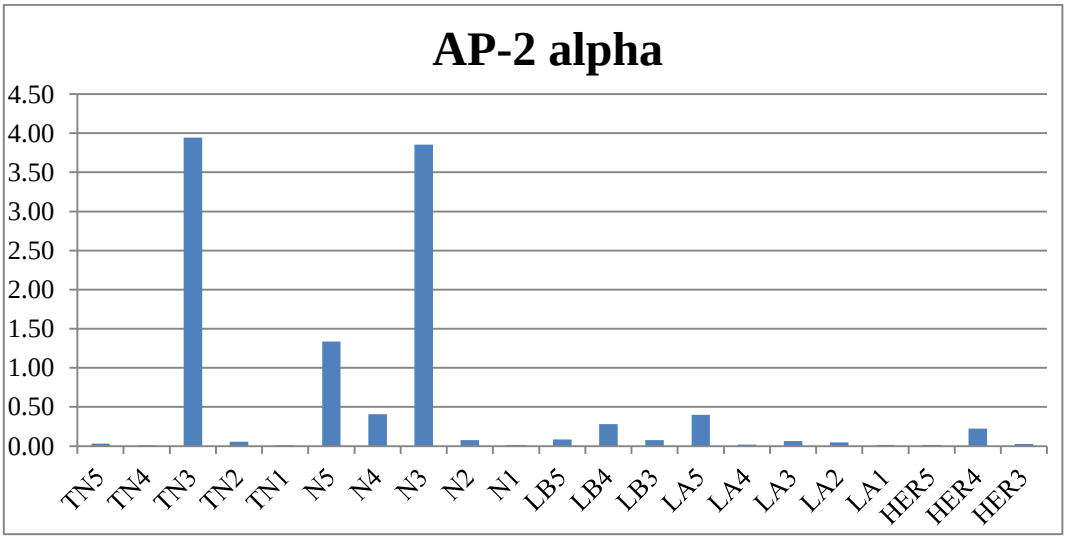


Figure 7: fold change value of Ap2 alpha normalized to β-actin in the tumours and normal samples

Using the same method with the other target genes, statistical analysis revealed non-significant correlation (p-values > 0.05) (Fig 8 A-E).

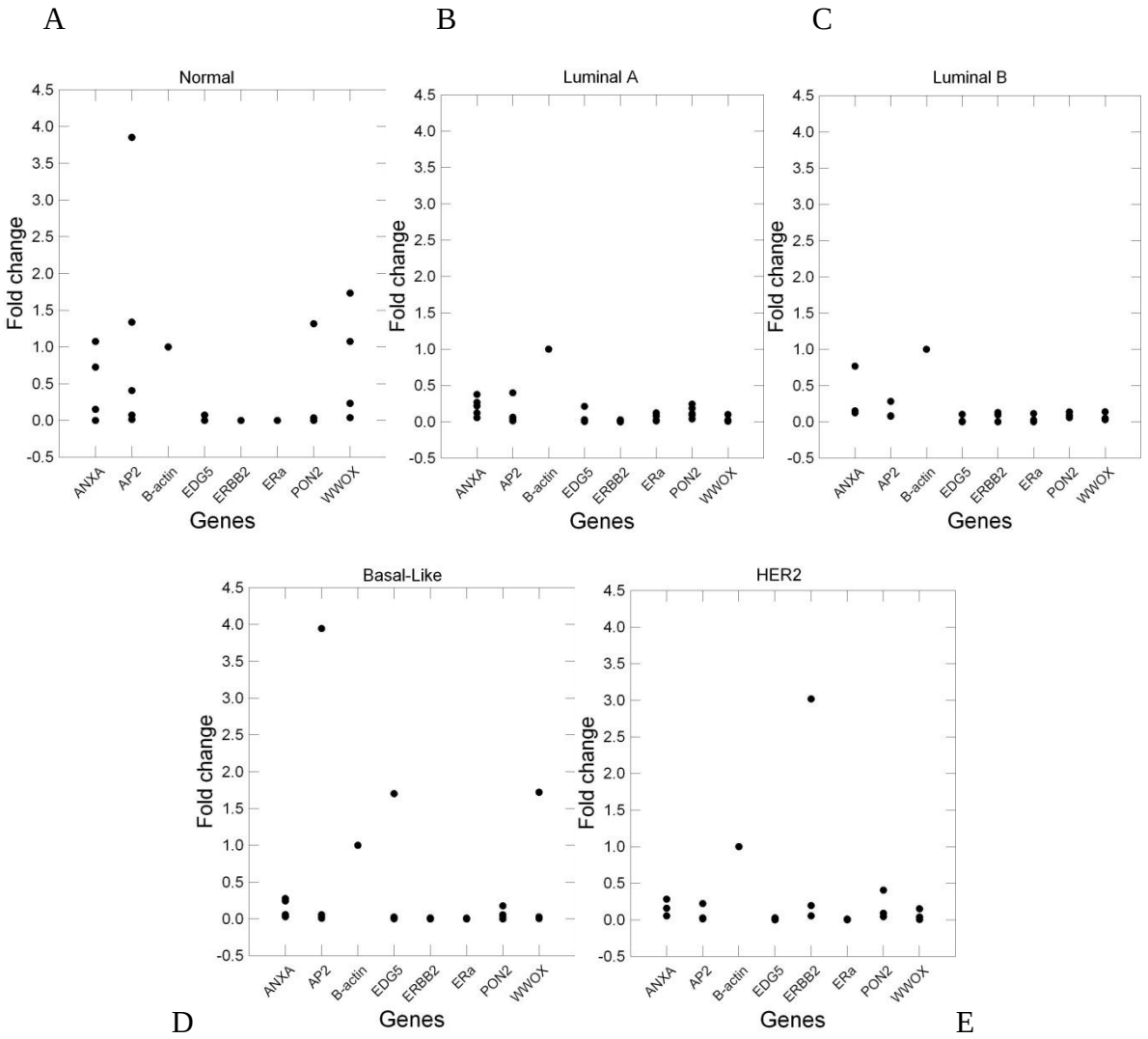


Figure 8 (A-E): level of gene's expression (EDG5, ER alpha, PON2, WWOX, AP-2 alpha, ERBB2, β-actin) as fold change normalized to β-actin in normal tissue and luminal A, luminal B, HER2+, triple negative tumour samples.

ER-alpha expression:

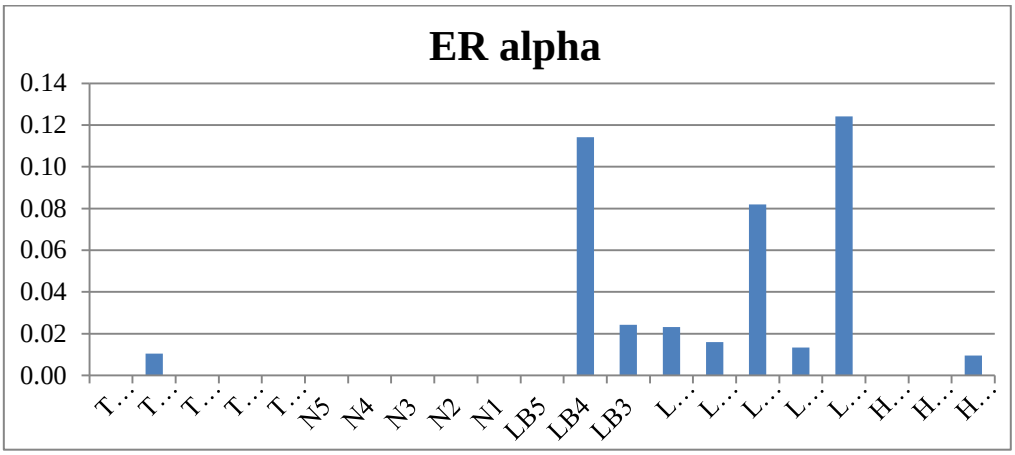


Figure 9 : fold change value of ER alpha normalized to β-actin in the tumours and normal samples

ER alpha expression results were in line with that expected (figure 9). HER3 and TN4 samples were both classified molecularly as HER2 overexpression and triple negative, even though ER receptor expression was under 20%. It is uncertain if we can classify them as such, from a molecular standpoint.

WWOX and AP-2 alpha expression:

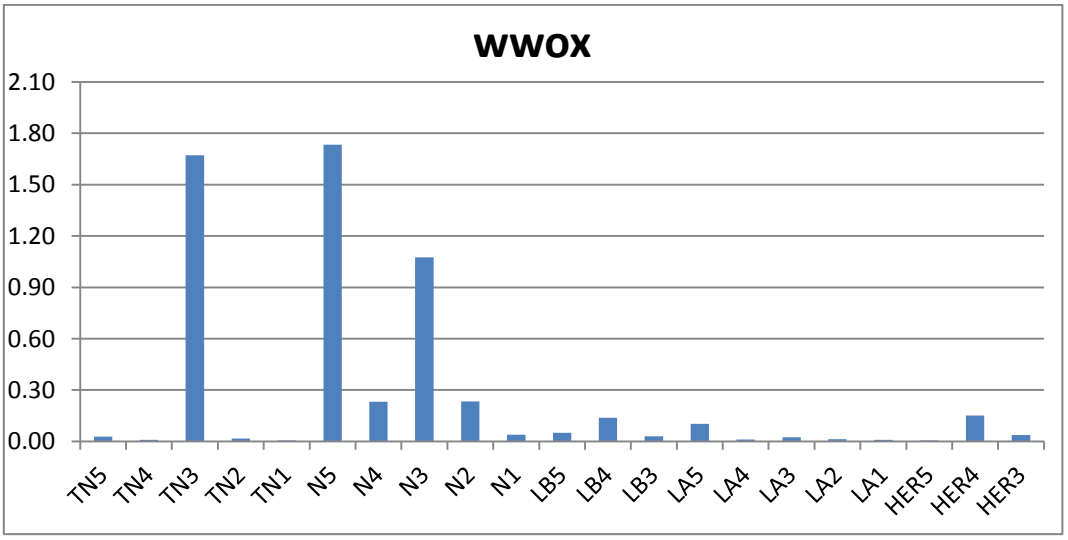


Figure 10: fold change value of WWOX normalized to β-actin in the tumours and normal samples

Analysis of the graph of fold change value correlation to gene expression of WWOX (figure 10) reveals a surprisingly similar pattern to that with AP-2 alpha. Using a zero correlation test, the correlation was proven with a p-value < 0.05 and correlation of 0.846. This could mean there is a direct link between these two genes.

Association between tumour characteristics and AP-2 alpha mRNA expression:

The cut-off point to establish underexpression of AP-2 alpha in tumour samples compared to normal tissue was determined as the mean value of fold change of all the samples: 0.108. Two levels of underexpression were created. Of the 11 samples, 3 were slightly underexpressed (Ap2-) and 8 were more underexpressed (Ap2--). These two groups can be separated statistically using a paired-test, with a P-value of 0.036 making the difference significant. Using the same principle separation between groups were significant except for EDG5 and ERRB2 with p-values of 0.026 for ER alpha, p-value of 0.039 for WWOX, p-value of 0.0.44 for PON2, p-value of 0.002 for ANXA1 and EDG5 p-value of 0.216.

We correlated the mRNA expression level of each sample with the tumours' pathological characteristics and clinical data (menopausal status, tumour size, lymphovascular invasion, lymph nodes metastasis, histological grade, mastectomy, metastasis, chemotherapy and radiotherapy). LB5, HER5, LA3, TN3, TN4 were excluded as insufficient clinical data was available for each of these samples. No significant statistical difference (using Fisher's exact test) was found between AP-2 alpha level of expression and pathological characteristics or clinical data. Results are shown in table 6. Correlation with for other genes was also neither significant. WWOX gene results were the same as AP-2 alpha.

A	Ap2-	Ap2--	P-value
Menopaused			
Yes	3	6	0.509
No	0	2	
Tumour size			
>2cm	1	4	0.455
<2cm	2	4	
LVI			
Yes	0	4	0.212
No	3	4	
Grade			
1 or 2	2	4	0.455
3	1	4	
Mastectomy			
Yes	2	7	0.436
No	1	1	
Lymph Nodes Metastasis			
Yes	0	4	0.212
No	3	4	
Metastasis			
Yes	0	2	0.509
No	3	6	
Chemotherapy			
Yes	0	4	0.212
No	3	4	
Radiotherapy			
Yes	2	2	0.255
No	1	6	

B	Wwox-	Wwox--	P-value
Menopaused			
Yes	3	6	0.509
No	0	2	
Tumour size			
>2cm	1	4	0.455
<2cm	2	4	
LVI			
Yes	0	4	0.212
No	3	4	
Grade			
1 or 2	2	4	0.455
3	1	4	
Mastectomy			
Yes	2	7	0.436
No	1	1	
Lymph Nodes Metastasis			
Yes	0	4	0.212
No	3	4	
Metastasis			
Yes	0	2	0.509
No	3	6	
Chemotherapy			
Yes	0	4	0.212
No	3	4	
Radiotherapy			
Yes	2	2	0.255
No	1	6	

C	Era+	Era++	P-value
Menopaused			
Yes	2	7	0.655
No	0	2	
Tumour size			
>2cm	0	5	0.273
<2cm	2	4	
LVI			
Yes	1	6	0.509
No	1	3	
Grade			
1 or 2	2	5	0.382
3	0	4	
Mastectomy			
Yes	2	2	0.655
No	0	7	
Lymph Nodes Metastasis			
Yes	0	4	0.382
No	2	5	
Metastasis			
Yes	0	2	0.655
No	2	7	
Chemotherapy			
Yes	0	4	0.382
No	2	5	
Radiotherapy			
Yes	1	3	0.509
No	1	6	

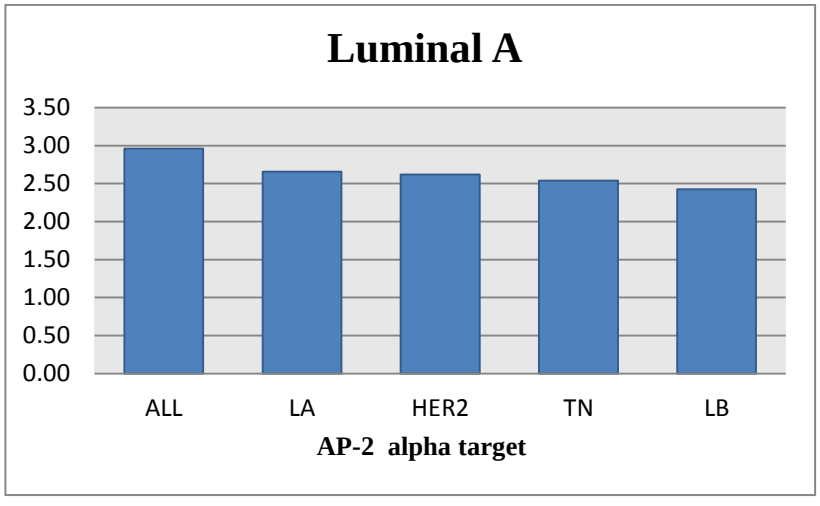
D	Anxa-	Anxa--	P-value
Menopaused			
Yes	3	6	0.509
No	1	1	
Tumour size			
>2cm	2	3	0.455
<2cm	2	4	
LVI			
Yes	0	4	0.106
No	4	3	
Grade			
1 or 2	2	4	0.455
3	2	3	
Mastectomy			
Yes	2	7	0.109
No	2	0	
Lymph Nodes Metastasis			
Yes	2	2	0.212
No	2	5	
Metastasis			
Yes	0	2	0.382
No	4	5	
Chemotherapy			
Yes	1	3	0.424
No	3	4	
Radiotherapy			
Yes	2	2	0.212
No	2	5	

E	Pon2-	Pon2--	P-value
Menopaused			
Yes	4	6	0.636
No	0	1	
Tumour size			
>2cm	2	3	0.455
<2cm	2	4	
LVI			
Yes	0	4	0.106
No	4	3	
Grade			
1 or 2	2	4	0.455
3	2	3	
Mastectomy			
Yes	2	7	0.109
No	2	0	
Lymph Nodes Metastasis			
Yes	2	2	0.212
No	2	5	
Metastasis			
Yes	0	2	0.382
No	4	5	
Chemotherapy			
Yes	1	3	0.424
No	3	4	
Radiotherapy			
Yes	2	2	0.212
No	2	5	

Table 6 (A-E): correlation of genes' expression level with pathological characteristic and clinical data

Protein-binding microarray:

During my internship at the laboratory, two tumour samples were tested with the PBM method. These were samples TN5 and LA3. The results for these two samples exhibited the molecular characteristics that we expected: Genes that represent the TN category exhibited the highest AP2 binding in PBM using TN5 protein extract, and genes that represent the LA category exhibited the highest AP2 binding using LA3 protein extract (figure 12).



A

B

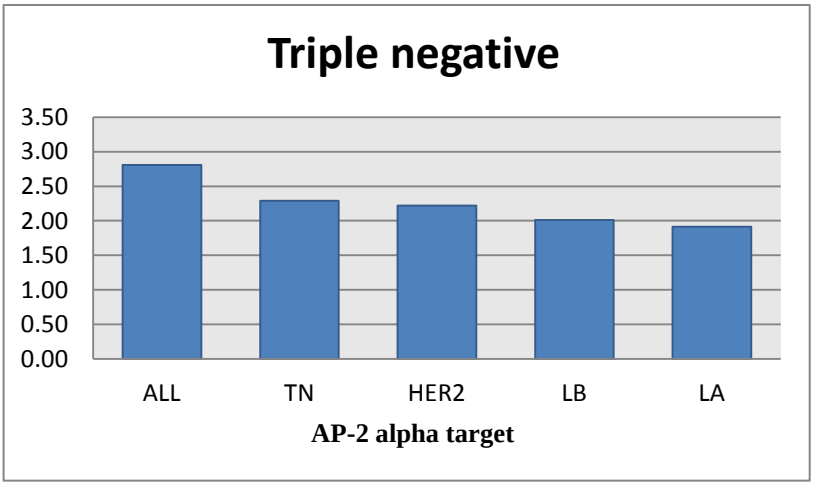


Figure 12: PBM results from luminal A (A) and triple negative (B) samples. Results are expressed as the mean of the mean of the log (protein/DNA) of each target gene to which AP-2 alpha was bound. AP-2 alpha target genes were classified depending on their association with the four molecular subtypes (e.g. LA represents the mean of the log (protein/DNA) of target genes found in luminal A type tumours. ALL represents genes that are common to the four subtypes.

Discussion

Maximal data extraction from this study necessitates a more complete classification for the molecular breast subtypes; this requires determination of HER2 status and Ki-67. The 26 samples which remained undetermined could significantly alter the results. In the literature 42% of invasive carcinomas were found to be of luminal A subtype, 28% were luminal B, 6-15% were triple negative, and 8-20% were HER2+ (25). In this study however, results are different due to both the undetermined samples and the small numbers involved.

Luminal type cancers (luminal A>luminal B) have been previously shown to exhibit the best relapse free survival rates, with triple negative and HER2+ displaying shorter times to relapse (12). Our results do not display a significant difference regarding time to relapse between the different molecular subtypes. Also, the luminal B subgroup showed the best results for relapse free survival time as no recurrence or metastasis was found in the only patient in this subgroup. This anomaly is again likely to be a product of the limited sample size, the distribution of the undetermined samples, and above all, the fact that a fraction of the luminal A tumour samples will likely be classified as luminal B once HER2/Ki-67 status is determined. Indeed, incidence of metastasis in the luminal A cohort seems rather elevated (12). Overall survival rates could not be established here as it was not possible to obtain mortality data, and thus comparison with the literature cannot be achieved.

The selection of genes to study in the q-RT-PCR was limited to those 50 genes to which AP-2 alpha can bind. The literature did not provide specific primers for all of these genes, while some others did not correlate with the data available on the website. This forced a default selection rather than a specific selection.

Recommendations in the literature on an appropriate threshold for determining oestrogen and progesterone receptor positivity vary between approximately 1% and 45% (23), depending on the protocol of the laboratory involved In the United States, the American Society of Clinical Oncology/College of American Pathologists recommend ER or PR threshold levels of positivity should be decreased from 10% to 1% and that ER/PR testing be standardized internationally (24). In the University Hospital of Lausanne this cut off point is 20 %. Some samples may not be classified as luminal, as exemplified by the two tumour samples tested by q-RT-PCR (HER3 and TN4). These displayed expression of the ER alpha gene, but at a low level of ER/PR positivity (ranging from 1% to 19%), terming them ER/PR negative under present designation. In clinical practice however, following the St-Gallen guidelines of 2009, these patients are still treated with hormonotherapy. In the follow-up to this study, decisions should be taken regarding which molecular subtype classification these samples are assigned.

No significant correlation was found between the genes' expression level and the pathological/clinical data. This could be due to the small sample size, troubleshooting during the total RNA extract experiment (e.g. presence of Rnase or Dnase, inadequate tumour mass

in the specimen, inadequate quantity of RNA taken from the sample and also the disparity in the quantity taken between the different specimens).

Although there appeared to be some inverse proportionality between AP-2 alpha expression level and menopausal status, tumour size, lymphovascular invasion, lymph node metastasis, histological grade, mastectomy, metastasis, chemotherapy and radiotherapy, the limited size of the sample precluded a statistically significant correlation.

Result of WWOX and AP-2 alpha of the q-RT-PCR showed a strong correlation, suggesting WWOX is directly regulated by AP-2 alpha. In fact, WWOX physically binds to AP2-alpha and gamma (27). WWOX is a tumour suppressor whose role has been proven in a couple of carcinoma types (lung, ovarian, stomach, oesophageal). Ekizoglu and al., demonstrated by PCR and direct sequencing that mutations in WWOX have a role in breast cancer pathogenesis (28). Studies have shown there was reduction of expression of WWOX in breast cancer, and even poor expression could be associated with triple negative molecular subtype (28). There remains a paucity of data on the exact frequency of WWOX expression in breast cancer (28). It was also suggested that WWOX could have a regulatory role in oestrogen receptor metabolism (29). Its interaction with AP-2 alpha needs to be determined, creating new molecular pathways to be explored.

In my one month placement at the laboratory, I was able to test two samples (TN5 and LA3) with the PBM method. This showed that AP-2 alpha bound to a group of genes reflecting each of the molecular subtypes. Not enough tumour samples could be tested during my placement, and so there was inadequate information on Ap2 binding activity to allow correlation with clinical data. Limitation in time was also an issue, as learning basic laboratory skills proved difficult in just one month. This study is ongoing at the laboratory of Professor Mermod at EPFL.

Conclusion

Although the q-RT-PCR results were not significant for AP-2 alpha and for the other genes, this could be a product of an insufficient sample size. In that case, repeating the experiment with more tumour samples would increase the power of the study and in the future it may possible to definitively correlate the level of expression with the pattern of binding activity found on PBM. Interaction between AP-2 alpha and WWOX has been described here, however this warrants further study.

The preliminary data from the two tumour samples analysed by protein binding microarray displayed the results we expected. Follow-up to the current PBM study should be performed with special regard for the current guidelines recognizing the need for more accurate classification of the samples i.e. agreement on the threshold level for oestrogen and progesterone receptors. Currently, work is in progress to determine HER2 and Ki-67 status.

In conclusion, in recent years, management of breast cancer has been primarily based on the biology of the tumour itself, rather than the stage of the cancer. The trend toward a more biologic model can be noted annually in the findings of the St-Gallen consensus. This has an important impact in research, patient care and treatment, and this PBM study aims to further this development of a biologic based model of management. Indeed, by using the PBM method there is a hope to better describe and understand breast cancer. The role of AP-2 alpha as a negative prognostic indicator in cancers thought of as low risk under current classifications warrants further investigation. The use of PBM in this regard as a clinical tool may lead to better prognosis of the disease and even to new treatment development.

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Dr Maryse Fiche, médecin adjointe, Institut Universitaire de Pathologie, CHUV

Dr Khalil Zaman, médecin associé, Centre Coordonné d'Oncologie, CHUV

Appendix

Appendix 1:

Put the sections fixation in acetone at -20C° for 10min. Air dry and then put in a hematoxillin for 3 min. Rinse in tap water for 3 min while regularly changing the water. Put in an eosin 0.25% for 30 seconds. Rinse in tap water and dH2O. Put successively for a couple of seconds EtOH95%, EtOH 100% and xylene. Finally mount with Eukitt on the slides.

Appendix 2:

Wizard SV Gel and PCR Clean-Up System: Gel Slice and PCR Product Preparation

A. Dissolving the Gel Slice

1. Following electrophoresis, excise DNA band from gel and place gel slice in a 1.5ml microcentrifuge tube.

2. Add 10µl Membrane Binding Solution per 10mg of gel slice. Vortex and incubate at 50–65 C° until gel slice is completely dissolved.

B. Processing PCR Amplifications

1. Add an equal volume of Membrane Binding Solution to the PCR amplification.

Binding of DNA

1. Insert SV Minicolumn into Collection Tube.

2. Transfer dissolved gel mixture or prepared PCR product to the Minicolumn assembly. Incubate at room temperature for 1 minute.

3. Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

Washing

4. Add 700µl Membrane Wash Solution (ethanol added). Centrifuge at 16,000 × g for 1 minute. Discard flowthrough and reinsert Minicolumn into Collection Tube.

5. Repeat Step 4 with 500µl Membrane Wash Solution. Centrifuge at 16,000 × g for 5 minutes.

6. Empty the Collection Tube and recentrifuge the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

Elution

7. Carefully transfer Minicolumn to a clean 1.5ml microcentrifuge tube.

8. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × g for 1 minute.

9. Discard Minicolumn and store DNA at 4 C° or –20 C°.

Appendix 3:

Add 1ml per tube of Trizol on frozen cells and vortex vigorously. Leave for 5 min in ambient temperature. Add 200ul of chloroform. Vortex for 20s and then leave for 5min in ambient temperature. Centrifuge for 15min at ambient temperature. In a new tube, place 500ul of aqueous solution and 500ul of isopropanol. Leave to rest for 10min in room temperature and centrifuge for 15min 4 C° 12000g. Eliminate the supernatant and add 1ml d'EtOH 70% -20 C° to the sediment . Centrifuge for 5min at 4C 10000g. Repeat the procedure. Eliminate the maximum alcohol possible with a pipette and let the sediment dry at air temperature. Reprendre le culot dans 50ul d'eau nano-pure Rnase free+ 1ul de Rnasine. Conserve at -80 C°.

References

Articles:

1 Ferlay J, Parkin DM, Steliarova-Foucher E. Estimates of cancer incidence and mortality in Europe in 2008. Eur J Cancer 2010; 46:765-81

2 Institut national pour l'épidémiologie et l'enregistrement du cancer NICER. Le cancer en Suisse: les chiffres. 2009. Available from: http://assets.krebsliga.ch/downloads/krebszahlen_11_2009_f.pdf

3 Kumar V, Cotran R, Robbins S. Robbins Basic Pathology. 7th Edition. Saunders; 2003

4 Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 2005; 365:1687-717.

5 Song M, Lee KM, Kang D. Breast cancer prevention based on gene-environment interaction. Mol Carcinog 2011; 50:280-90.

6 Swart R, Downey L, Lang J, Thompson P, Livingston R, Stopeck A. Breast cancer. eMedecine Oncology, updated: May 2010. Available from: http://emedicine.medscape.com/article/283561-overview

7 Hodgkinson VC, Eagle GL, Drew PJ, Lind MJ, Cawkwell L. Biomarkers of chemotherapy resistance in breast cancer identified by proteomics: current status. Cancer Lett 2010 Aug; 294:13-24.

8 Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature 2000; 406:747-52.

9 Cheang MC, van de Rijn M, Nielsen TO. Gene Expression Profiling of Breast Cancer. Annu Rev Pathol 2008; 3:67-97.

10 Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thürlimann B, Senn HJ, Panel members. 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 Oncol 2011; 22:1736-47.

11 Sandhu R, Parker J, Jones W, Livasy C, Coleman W. Microarray-Based Gene Expression Profiling for Molecular Classification of Breast Cancer and Identification of New Targets for Therapy. LabMed 2010; 41:364-372.

12 Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 2001; 98:10869– 10874.

13 NIH Consens Statement. Adjuvant therapy for breast cancer. 2000; 17:1-35.

14 National Institute for Health and Clinical Excellence 2009. Early and locally advanced breast cancer: full guideline CG80. London: National Institute for Health and Clinical Excellence.

15 Muñoz M, Estévez LG, Alvarez I, Fernández Y, Margelí M, Tusquets I, and al. Evaluation of international treatment guidelines and prognostic tests for the treatment of early breast cancer. Cancer Treat Rev 2008; 34:701-9.

16 Aapro MS. Adjuvant therapy of primary breast cancer: a review of key findings from the 7th international conference, St. Gallen, February 2001. Oncologist 2001; 6:376-85.

17 Turner BC, Zhang J, Gumbs AA, Maher MG, Kaplan L, Carter D, et al. Expression of AP-2 transcription factors in human breast cancer correlates with the regulation of multiple growth factor signalling pathways. Cancer Res 1998; 58:5466-72.

18 Egener T, Roulet E, Zehnder M, Bucher P, Mermod N. Proof of concept for microarray-based detection of DNA-binding oncogenes in cell extracts. Nucleic Acids Res 2005; 33:e79.

19 Pellikainen JM, Kosma VM. Activator protein-2 in carcinogenesis with a special reference to breast cancer--a mini review. Int J Cancer 2007; 120:2061-7.

20 Ravdin PM, Siminoff LA, Davis GJ, Mercer MB, Hewlett J, Gerson N, et al. Computer program about adjuvant therapy for woman, J Clin Oncol 2001; 19:980-91.

21 Lim LH, Pervaiz S. Annexin 1: the new face of an old molecule. FASEB J 2007; 21:968-75.

22 Kerschgens J, Renaud S, Schütz F, Grasso L, Egener-Kuhn T, Delaloye JF, et al. Protein-binding microarray analysis of tumor suppressor AP2α target gene specificity. PLoS One 2011; 6:22895.

23 Monoclonal Mouse Anti-Human Estrogen Receptor αααα Clone 1D5 Code M7047. Available from: www.dako.com/download.pdf?objectid=104482007.

24 Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. Arch Pathol Lab Med 2010; 134:48-72.

25 Cheang MCU, Chia SK, Voduc D et al. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J Natl Cancer Inst 2009; 101: 736–750.

26 Layfield LJ, Gupta D, Mooney EE. Assessment of Tissue Estrogen and Progesterone Receptor Levels. A survey of current practice, techniques, and quantitation methods. Breast J 2000; 6:189-96.

27 Aqeilan RI, Palamarchuk A, Weigel RJ, Herrero JJ, Pekarsky Y, Croce CM. Physical and functional interactions between the Wwox tumor suppressor protein and the AP-2gamma transcription factor. Cancer Res 2004; 64:8256-61.

28 Ekizoglu S, Muslumanoglu M, Dalay N, Buyru N. Genetic alterations of the WWOX gene in breast cancer. Med Oncol 2011; [Epub ahead of print].

29 Nunez MI, Ludes-Meyers J, Abba MC, Kil H, Abbey NW, Page RE, et al. Frequent loss of WWOX expression in breast cancer: correlation with estrogen receptor status. Breast Cancer Res Treat 2005; 89:99-105.

30 Livak K, Schmittgen T. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)) method. Methods 2001; 25:402–408.

31 Thermo Fisher Scientific, NanoDrop products. T009-Technical Bulletin; Nanodrop 1000 and 8000. Available from: http://www.nanodrop.com/Library/T009-NanoDrop%201000-&-NanoDrop%208000-Nucleic-Acid-Purity-Ratios.pdf

32 WHO Classification of tumours. Tumours of the Breast and Female Genital Organs. F. Tavassoli and P. Devilee Editors; IARC Press 2003.