Biomarkers in non-small cell lung carcinoma

To Tobias and Daniel

Örebro University

Christina Karlsson

Biomarkers in non-small cell lung carcinoma

Methodological aspects and influence of gender, histology and smoking habits on estrogen receptor and epidermal growth factor family receptor signalling

Abstract

Christina Karlsson (2011): Biomarkers in non-small cell lung carcinoma
- Methodological aspects and influence of gender, histology and smoking habits on estrogen receptor and epidermal growth factor family receptor signalling Örebro Studies in Medicine 61, 86 pp.

Non-small cell lung carcinoma is a leading cause of cancer mortality worldwide. There are gender and smoking associated differences both in tumour types and clinical outcome. Squamous cell carcinomas (SCC) are more frequent among smoking men while females develop adeno-carcinomas (ADCA). NSCLC among never smokers are mainly ADCA, and occurs mostly in females.

The present thesis elucidates the role of estrogen receptor (ER) and epidermal growth factor receptor family (EGFR/HER2-4) in NSCLC in the perspective of gender and histology as well as the influence of smoking on those biomarkers.

A recently developed technique, tissue micro array (TMA), was employed. The question of how much of a tumour tissue that needed to be included in a TMA for biomarker analysis was analyzed by a statistical approach. Data indicates a sample size of three cylinders of tumour tissue with a diameter of 0.6 mm each as being appropriate and cost-effective.

In order to optimally use the up to thousands of different tumour samples within a TMA, it would be optimal to serially cut and store slides before performing in situ detection of proteins and nucleic acids. Applying up to date methodology, and by evaluation with image analysis, data are presented that shows that such handling of TMA slides would be possible without any loss of biomarker information.

ERα is more frequently observed in ADCA and in females and a local estradiol synthesis is supported by the presence of aromatase. ERβ is identified as a positive prognostic marker in ADCA. Smoking is associated to increased levels of ERβ mRNA. EGFR over expression is associated with a ligand. Independent phosporylation of ERα. HER-4 intracellular domain may also act as a co-activator to ERα in ADCA, especially among never-smokers. The question of ER and EGFR family signalling crosstalk as a potential target for combined targeted therapy is raised.

Keywords: Non-small cell lung carcinoma, estrogen receptor, epidermal growth factor receptor, HER-4, tissue microarray, immunohistochemistry, smoking habits, in situ hybridisation

Christina Karlsson, Hälsoakademin
Örebro University, SE-701 82 Örebro, Sweden, christina.karlsson@oru.se

List of publications

This thesis is based on the following original papers, which will be referred to in the text by their roman numerals:

I   Karlsson C, Bodin L, Piehl-Aulin K, Karlsson MG. Tissue microarray Validation: A Methodological Study with Special Reference to Lung Cancer. Cancer Epidemiol Biomarkers Prev 2009;18(7):2014-21.

II   Karlsson C, Karlsson MG. Effects of long term storage on the detection of proteins, DNA and mRNA in tissue microarray slides. Accepted J Histochem Cytochem 2011-08-23, proofs included.

III   Karlsson C, Helenius G, Fernandes O, Karlsson MG. Estrogen receptor β in NSCLC – prevalence, proliferative influence, prognostic impact and smoking. *Submitted*

IV   Karlsson C, Helenius G, Karlsson MG. Nuclear HER-4 (4ICD) and estrogen receptor α in non-small cell lung carcinoma. *Submitted*

V   Karlsson C, Helenius G, Karlsson MG. Estrogen receptor α phosphorylation and EGFR in non-small cell lung carcinoma. *Manuscript*

Table of contents

Abbreviations

ABC	avidin-biotin complex
ADCA	adenocarcinoma
AF-1	activation function 1
AI	aromatase inhibitor
AR	antigen retrieval
CISH	chromogenic in situ hybridisation
DBD	DNA binding domain
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor
ERE	estrogen response element
FFPE	formalin fixed paraffin embedded
FISH	fluorescent in situ hybridisation
H&E	haematoxylin & eosin staining
HIAR	heat induced antigen retrieval
HRE	hormone response element
HRT	hormone replacement therapy
ICC	intra class correlation
ICD	intracellular domain
IHC	immunohistochemistry
ISH	in situ hybridisation
LBD	ligand binding domain
LCC	large cell carcinoma
Mab	monoclonal antibody
mMab	mouse monoclonal antibody
MW	microwave
NR	nuclear receptor
NRG	neuregulin
NSCLC	non-small cell lung carcinoma
PAP	peroxidise-anti-peroxidase
PgR	progesterone receptor
rMab	rabbit monoclonal antibody
RT	room temperature
RTK	receptor tyrosine kinase
SCC	squamous cell carcinoma
SCLC	small cell lung carcinoma
SERM	selective estrogen receptor modulator
TMA	tissue microarray
TNM	tumour, nodule, metastasis

Introduction

Lung cancer was the most commonly diagnosed cancer as well as the leading cause of cancer death in males in 2008 globally. Among females, it was the fourth most commonly diagnosed cancer and the second leading cause of cancer death. Lung cancer globally accounts for 13 % (1,6 million) of the total cancer cases and 18% (1,4 million) of the cancer deaths in 2008 (1). In Sweden the mortality for men has been steadily falling during 1987-2008, while the mortality for women has during the same period risen quite dramatically (2). In Sweden 24800 patients were diagnosed with lung cancer during 2002-2008 (53% men and 47% women) (2) of those 21.4% were squamous cell carcinomas (SCC) (26,7% men and 15.7% women) and 39.7% adenocarcinomas (ADCA) (35.2% men, 44.7% women) (2). Clearly, lung cancer is an important and widespread disease that constitutes a major public health problem. This was not always so. 150 years ago, it was an extremely rare disease. In 1878 in Dresden less than 1% of all cancers at autopsy were lung cancer. This figure rose to 10 % by 1918 (3).

Lung cancer

Lung cancer can be divided into four major histological types, ADCA, SCC, large cell carcinoma (LCC) and small cell carcinoma (SCLC). The term non-small cell lung cancer (NSCLC) is often used for ADCA, SCC and LCC together, since these types of lung cancer show similar clinical and biological characteristics. NSCLC is mostly chemo resistant and is therefore treated primarily by surgery if diagnosed at early stages. In contrast, SCLC progresses more rapidly than NSCLC, and thus is mostly far advanced at the time of diagnosis; since it is chemo- and radio- sensitive, it is treated primarily by chemotherapy and radiotherapy (4-5).

NSCLC (non-small cell lung carcinoma)

Squamous cell carcinoma

There is no squamous epithelium in the normal lung, and SCC arises from bronchial epithelia cells through squamous metaplasia and dysplasia. Over 90% of SCC occurs in cigarette smokers, arsenic as well as asbestos and various heavy metals are also strongly associated with SCC. Most SCC present as central lung tumours (4). The tumours may grow to a large mass and then cavitate. Microscopically, SCCs are highly variable. Well differentiated tumours have keratin "pearls" which are small round nests of brightly eosinophilic aggregates of keratin surrounded by concentric layers

of squamous cells. Intercellular bridges are identified in some well differentiated tumours. By contrast some tumours are so poorly differentiated that they lack keratinization and are difficult to distinguish from large cell, small cell or spindle cell carcinomas. SCC accounts for about 30% of all lung cancers (4, 6-7).

Adenocarcinoma

ADCAs are derived from alveolar or bronchioalveolar epithelial cells (in particular, type II alveolar epithelial cells and Clara cells) or mucin-producing cells (4, 6-7). ADCA is a malignant epithelial tumour with glandular differentiation or mucin production, showing acinar, papillary, bronchioloalveolar or solid with mucin growth pattern or a mixture of these patterns (4, 6-7). Most ADCAs are heterogeneous and consist of two or more of these histological subtypes. These tumours often arise at the periphery of the lungs often associated with pleural fibrosis. Although most cases are seen in smokers, it develops more frequently than other histological types of lung cancer in individuals (particularly women) who have never smoked (4, 7-8)

Large cell carcinoma

LCC is an undifferentiated non-small cell carcinoma that lacks the cytological and architectural features of SCLC and glandular or squamous differentiation. This type accounts for up to 10% of all invasive lung tumours. They have a spectrum of morphologies, and most of them consist of large cell with abundant cytoplasm and large nuclei with prominent nucleoli. Mitotic rates are high and necrosis frequent (4, 6-7).

Small cell lung cancer

SCLC originates from epithelial cells with neuro-endocrine features. This is an epithelial tumour consisting of small cells with scant cytoplasm, ill-defined cell borders, finely granular chromatin and absent or inconspicuous nucleoli. The cells are round, oval and spindle shaped. Necrosis is typically extensive and the mitotic count is high. It accounts for 20% of lung cancers and is strongly associated with smoking.

Histological sub typing of lung cancer is based on the best differentiated component.

In addition to morphological examination by light microscopy, analysing specific protein expression by immunohistochemistry (IHC) is used to further diagnose the lung tumours. The different types of NSCLC express different antigens. SCCs are positive for cytokeratin 5/6 and p63, whereas

ADCA are negative for the same markers. ADCA on the other hand expresses TTF-1, a protein which is not expressed by SCC (6, 9-10). By using IHC the group of large undifferentiated tumours will become smaller.

TNM and staging of lung cancer

TNM

The TNM system is the most widely used means for classifying the extent of cancer spread; it was developed during 1943-52 by Frenchman Pierre Denoix. Since then it has undergone revisions until the 7[Th] edition used today (11). Two classifications are described for each site; 1, clinical classification based on evidence acquired before treatment, such as physical examination, imaging, endoscopy, biopsy and surgical exploration. 2, pathological examination, based on the postsurgical histopathological classification, all cases should be confirmed microscopically (11).

The TNM system for describing the anatomical extent of disease is based on the assessment of three components: T: the extent of the primary tumour (the size and penetration into adjacent organs), N: the absence or presence and extent of regional lymph node metastasis, M: the absence or presence of distant metastasis. The addition of numbers to these three components indicates the extent of the malignant disease (11).

Stage

The stage of the disease is important for prognosis and treatment planning (4). The stage of a cancer is a description (usually numbers I to IV with IV having more progression) of the extent the cancer has spread. The basis for all staging is the TNM classification of the tumour. Staging does not change with progression of the disease as it is used to assess prognosis (4).

Lung cancer and smoking

In males, the highest lung cancer incidence rates are in Eastern and Southern Europe, North America, Micronesia and Polynesia, and Eastern Africa, while rates are low in sub-Saharan Africa. In females the highest lung cancer incidence rates are found in North America, Northern Europe and Australia/New Zeeland. The observed variations in lung cancer rates and trends across countries or between males and females within each country largely reflect differences in the stage and degree of the tobacco epidemic (12-13). Smoking accounts for 80% of the worldwide lung cancer burden in males and at least 50% of the burden in females (14-15). Male lung cancer deaths are decreasing in most Western countries where the

tobacco epidemic peaked by the middle of the last century (12, 16). In contrast lung cancer rates are increasing in countries such as China and several other countries in Asia and Africa, where the epidemic has been established more recently and smoking prevalence continues to increase or show signs of stability (1).

Generally, lung cancer trends among females lag behind males, because females started smoking in large numbers several decades later than males. Therefore lung cancer rates in females are increasing in many countries (1, 12). In addition, the relative risk of specific types of lung cancer appear to differ for men and women, and the interaction between smoking and lung cancer may not be the same for each group.

The link between smoking of cigarettes and lung cancer began to be suspected by clinicians in 1930s when they noted the increase of this "unusual" disease. A German study flatly stated in 1940 "continued use of tobacco creates a disposition to cancer at the place of provocation" (17-18). In the 1950s Doll and Hill (19-20) among others provided further evidence for a causal association between smoking and lung cancer (19-22). At that time ADCAs constituted about 5% of the cases, today the ADCAs account for about 45% of all lung cancers. A possible explanation to this shift is that design changes in cigarettes could actually have changed the location and histological distribution of lung cancers for two reasons. First the introduction of filter tip cigarettes leads to a deeper inhalation of the smoke. This inhalation transports tobacco-specific carcinogens toward the bronchioalveolar junction where ADCA often arise. Second, the composition of tobacco in the cigarettes changed during the 1950s towards stems rather than leaves, which releases higher concentrations of nitrosamines. In rodents injected with nitrosamines a higher level of ADCAs have been detected (23).

Biobanks

When tissue is removed from the body, a decision about how to preserve this tissue has to be made. This decision has to be based on what kind of information the clinician / researcher will extract from the tissue during the examinations to come. Am I interested in proteins, carbohydrates or fat, or maybe DNA or RNA? How you treat or mistreat the tissue at this time decides what questions your tissue can answer later. All tissues can be stored as unfixed (frozen) or fixed specimens.

Freezing of tissue

Freezing of tissues can damage the tissue and artefacts are produced depending on how the tissue is frozen. The rate of freezing alters the size of the ice crystals. At slow freezing rates, the ice crystals will grow quite large and the crystals themselves expand as they freeze. This expansion results in mechanical damage to the tissue. Rapid freezing results in much smaller ice crystals that are less likely to cause visible alteration to the tissue. The higher the magnification that is going to be used on the tissue, the smaller the ice crystals must be to avoid visible damage (24).

Water at atmospheric pressure is converted to ice at any temperature below 0^0C, but it is in a dynamic state and will constantly be changing shape and interacting with adjacent ice crystals. Ice-crystal damage gets worse as tissue are stored, as the ice remodels and changes its shape and size. Only when the temperature of the ice drops very low, about -130^0C for pure water, does it become stable and not recrystallize. The point at which recrystallization in tissues, which are filled with a salt solution, is inhibited is not known but is probably somewhere below -90^0C (24).

Slow freezing of tissue (containing a salt solution) results in the production of ice crystals that are pure water. It is only when the temperature is below -21^0C that the salt solution will freeze as a whole. Since water is being removed from the cellular fluid, the remaining solution becomes more concentrated. Water will be drawn out from the cells and they will shrink as a result. If the rate of freezing is high enough, then the tissue freezes as one intact block without separating into water and salt solutions (24).

Freezing will inevitably cause the morphology of the cells to be poorer than fixed tissue. However, if the desire is to extract good quality DNA or RNA from the tissue, frozen tissue is a must. Freezing preserves the nucleic acids, and does not cause the same fragmentation of DNA or RNA that fixation does. Frozen tissue is also suitable for both IHC and in situ detection of both DNA and RNA (25).

Fixation of tissue

Formalin fixed and paraffin embedded (FFPE) tissue is an invaluable resource in tumour biology research.
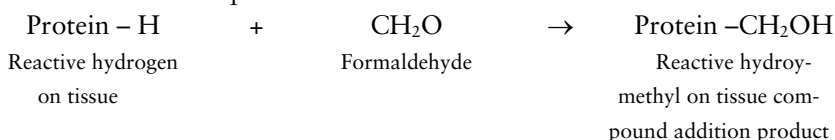
After fixation, the tissue is further processed and the end product is the tissue block. The tissue block contains a tissue sample that is resilient to long term storage. Beside tissue structure, the cellular content of proteins and nucleic acids are preserved, even after decades of storage (26-27). In the clinical setting, tissue blocks are stored, usually at ambient temperature, after initial diagnostic testing, constituting a unique biobank for retrospective studies.

Fixation is a process by which tissues are preserved from decay. A fixative usually acts to disable intrinsic bio molecules, which otherwise digest or damages the tissue sample. A fixative also protects the tissue from extrinsic damage, and may also increase the mechanical strength or stability of the treated tissue (24).
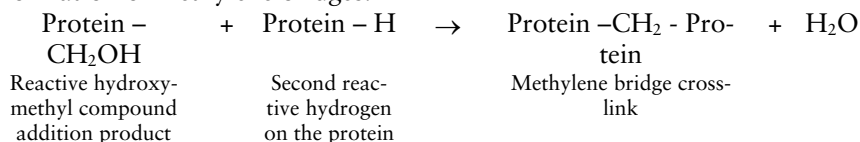
The most common fixative for light microscopy is 4% neutral buffered formaldehyde, pure formaldehyde is a vapour that when completely dissolved in water forms a solution containing 37-40% formaldehyde. The reactions of formaldehyde with macromolecules are numerous and complex. Fraenkel-Conrat and his colleagues (28-30), meticulously identified most of the reactions of formaldehyde with amino acids and proteins. In an aquous solution formaldehyde forms methylene hydrate, a methylene glycol as the first step in fixation (31).

Methylene hydrate reacts with several side chains of proteins (lysine, cysteine, histidine, arginine, tyrosine and reactive hydroxyl groups of serine and threonine) to form reactive hydroxymethyl side groups (-CH2-OH) (32).

Formation of addition products:

Protein – H	+	CH_2O	$\rightarrow$	Protein –CH_2OH
Reactive hydrogen on tissue		Formaldehyde		Reactive hydroxymethyl on tissue compound addition product

Formation of methylene bridges:

Protein – CH_2OH	+	Protein – H	$\rightarrow$	Protein –CH_2 - Protein	+	H_2O
Reactive hydroxymethyl compound addition product		Second reactive hydrogen on the protein		Methylene bridge cross-link		

With relatively short times of fixation with 4% neutral buffered formaldehyde (hours to days) the formation of hydroxymethyl side chains is probably the primary and characteristic reaction. The cross-linking tends to preserve the secondary structure of proteins and may protect significant amounts of tertiary structure as well.

Formaldehyde reacts with C=C and -SH bonds in unsaturated lipids but does not interact with carbohydrate (33-36).

While formaldehyde fixation preserves tissue morphology, it also alters the three dimensional structure of the protein. This alteration can result in a modification of the antigens epitopes and electrostatic charge. The loss of an epitope may results in an antigens ability to react with the paratope of the antibody and can only be corrected by the restoration (retrieval) of the epitope (24, 37-38).

In paraffin sections, only nucleic acids that are firmly attached to protein will be consistently retained since most fixatives act on proteins and not on nucleic acids. Additive fixatives (such as formaldehyde) combine with and alter the reactive groups, although formaldehyde is an acceptable fixative for nucleic acids. The disruption of nucleic acids into shorter fragments is a problem with all kinds of fixation of tissue. Depending on how long fragments are needed to detect within the tissue, one must take the precaution to freeze tissue as well, as in frozen tissue the detection of longer fragments of nucleic acids is more reliable (24, 37).

Immunohistochemistry

Fluorescence-based IHC was first introduced in the mid-1940s by Albert H Coons (39-40). Since then a plethora of labels have evolved, all having the disadvantage of the need for fluorescence microscopy. Many limitations were overcome with the introduction of enzymes as labels. Cells that have been labelled with an enzyme such as horseradish peroxidase, conjugated to an antibody, and visualized with an appropriate chromogen such as diaminobenzidine (24, 41) can be counterstained with traditional nuclear stains such as hematoxylin. It was only with the development of the peroxidase-anti-peroxidase (PAP) and the avidin-biotin complex (ABC) techniques that the procedure could be applied to FFPE tissues, facilitating its usefulness in tissue diagnosis (37). Since then many detection systems have been developed. Changing the labelling from the primary (direct labelling) to the secondary (indirect labelling) antibody and adding even more enzyme complexes. Today there are a number of detection systems that work well on formalin fixed and paraffin embedded tissue (39, 42).

There are numerous IHC staining techniques that may be used to localize and demonstrate tissue antigens. The selection of a suitable technique should be based on parameters such as the type of specimen under investigation (fresh frozen, FFPE, cells) and the degree of sensitivity required (24, 39).

Over the years, many myths surrounding the preservation and presentation of antigens in FFPE tissue have been dispelled. In the 1970´s it was thought that routine paraffin processing destroyed many epitopes and that certain antigens could never be demonstrated in paraffin sections. However, it was found that many antigens are not lost, but are masked by the process involved in formalin fixation and paraffin processing (24, 37, 39).

In situ detection of nucleic acids

In situ hybridization (ISH) is a hybridization technique that uses a labelled complementary DNA or RNA strand as a probe to localize a specific DNA or RNA sequence in a portion or section of tissue (43).

Before hybridization, sample cells and tissues are usually treated to fix the target transcripts in place and to increase access of the probe. The probe is either a labelled complementary DNA or a complementary RNA (riboprobe). The probe hybridizes to the target sequence at elevated temperature, and then the excess probe is washed away. Solution parameters such as temperature, salt and/or detergent concentration can be manipulated to remove any non-identical interactions (i.e. only exact sequence matches will remain bound). Then, the probe that was labelled with either radio-, fluorescent- or antigen-labelled bases (e.g., digoxigenin) is localized and quantified in the tissue using either autoradiography, fluorescence microscopy or immunohistochemistry, respectively. ISH can also use two or more probes, to simultaneously detect two or more transcripts (24, 43).

Fluorescent in situ hybridisation (FISH) uses fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence similarity (24, 43).

Chromogenic in situ hybridisation (CISH) utilizes conventional peroxidase or alkaline phosphatase reactions visualized under a standard bright-field microscope, and is applicable to FFPE tissues, blood or bone marrow smears, metaphase chromosome spreads, and fixed cells (43-44).

Compared to FISH, CISH offers three important advantages: a. the histological details of the paraffin section are generally better appreciated with bright field microscopy, b. the morphological details are readily apparent using low-power objectives and c. the probe signals are not subject to rapid fading (43-44).

Antigen retrieval

One of the earliest methods of antigen retrieval was proteolytic digestion employed prior to the application of the primary antibodies. A number of proteolytic enzymes served this purpose, including trypsin, proteinase K, pronase, pepsin and others (37, 39). These enzymes all differ in action and there are also variations in concentration, duration, optimal pH and temperature of digestion. Not all antigens benefit from proteolytic digestion, and some show deleterious effect with loss of staining (24, 37, 39).

Heat induced antigen retrieval (HIAR) was a major milestone, greatly enhancing the ability to demonstrate antigens in FFPE tissue (45-48). The initial technique was achieved with microwaves (MW), which has remained the most convenient. Shi et al (46) described MW heating (the studies by Fraenkel Contrat in the 1940s (30, 49-50), indicated that cross-linkage between formalin and protein could be disrupted by heating above 100^{0}C or by strong alkaline treatment) of FFPE tissue in the presence of heavy metal solutions such as lead tiocyanate, up to temperatures of 100^{0}C to "unmask" a wide variety of antigens for immunostaining. It was subsequently shown that treating the deparaffinized-rehydrated tissue section in MW irradiation in 0.01M citrate buffer pH 6.0 produced increased intensity and extent of immunostaining of a wide variety of tissue antigens (51). Since then additional techniques for this heating of the section have been employed. The use of water baths, autoclaves, hot plates and pressure cookers are described in the literature (37, 51). Also the heated solution itself has been scrutinized (52-53), and this has lead to the need to test each new antibody extensively regarding how to "unmask" the epitope. Shi and Taylor (51) suggest a "test battery" to evaluate antibodies, this test battery consists of combination of different temperatures and different pH of the retrieval solution in all to find out in what setting the staining gives the highest sensitivity and specificity.

HIAR also has an impact on restoring antigens that previously have been undetectable due to long time storing of section on slides. A number of reports stated that this loss of antigenicity, is all attributed to storage of slides (54-56), and a number of studies showed that antigen in tissues decreases over time (54, 56-57). There are however some inconsistencies in these reports, some stating antigens are lost within a week (56), while others state a couple of months (58) or even years (59). Further differences between these studies are the employment of HIAR, only a few of them use up to date antigen retrieval, and some of them none at all.

Controls

When using IHC and ISH techniques with antigen retrieval and tissue pre-treatment it is of the utmost importance to include a number of controls in the assays to avoid both false positive and false negative results.

Many factors may influence the staining result in IHC and ISH: differences in tissue fixative and fixation time, day-to-day variations due to temperature, variations due to different workers' interpretations of protocol steps or in the conditions of reagents applied on a particular day (24, 39, 60).

It is therefore important to include reagent and tissue controls for verification of IHC and ISH staining results. Positive controls are included to test a protocol or procedure and make sure it works; the ideal control is a tissue with a known positivity. Negative control is to test for the specificity of an antibody or probe involved. No staining must be shown when omitting the reagent or replacing it with normal serum or nonsense probes (24, 39, 60).

Tissue microarray

High-throughput techniques for molecular studies related to carcinogenesis, prognosis or therapy in cancer have been introduced in recent years. These methods include assays at both the genomic and proteomic levels. One technique is tissue microarray (TMA), which makes it possible to study histopathologic material from a large number of different tissue samples within a limited experimental setting. TMA analysis can thus be performed both on historical material with long follow-up and as part of ongoing clinical studies (61).

Like conventional FFPE material, TMAs are amenable to a wide range of techniques, including photochemical stains, immunological stains with either chromogenic or fluorescent visualization, ISH (including both FISH and CISH) and even tissue micro dissection techniques (61).

The TMA technology has greatly facilitated retrospective studies of FFPE tissues. Large sets of tissues can now be put together in the same block and subjected to the same laboratory treatment, thus minimizing batch to batch variability and making the analysis less time-consuming and more cost effective (62-63). However, one should keep in mind that TMA is not intended for individual clinical diagnosis, tumour classifications or grading within studies, but has been designed to facilitate biomarker studies in large tissue materials (62).

Multi tissue blocks were first introduced by Battifora (64) in 1986 with his so called "multitumour (sausage) tissue block" and modified in 1990 with its improvement "the checkerboard tissue block" (64-65). The next step in the development of TMA was described by Wan et.al. (66) who used a 16-gauge needle to bore cores from tissues blocks and array them in a multitissue straw in a recognizable pattern. This method was further modified by Kononen and (67) collaborators who developed the current technique, which uses a sampling approach to produce tissues of regular size and shape that can be more densely and precisely arrayed.

A hollow needle is used to remove tissue cylinders as small as 0.6 mm in diameter from region of interest in paraffin-embedded tissues such as clinical biopsies or tumour samples. These cylinders are then inserted in a recipient paraffin block in a precisely spaced, array pattern. Sections from this block are cut, mounted on a slide and analyzed by any method for histological analysis (61). (Figure 1)
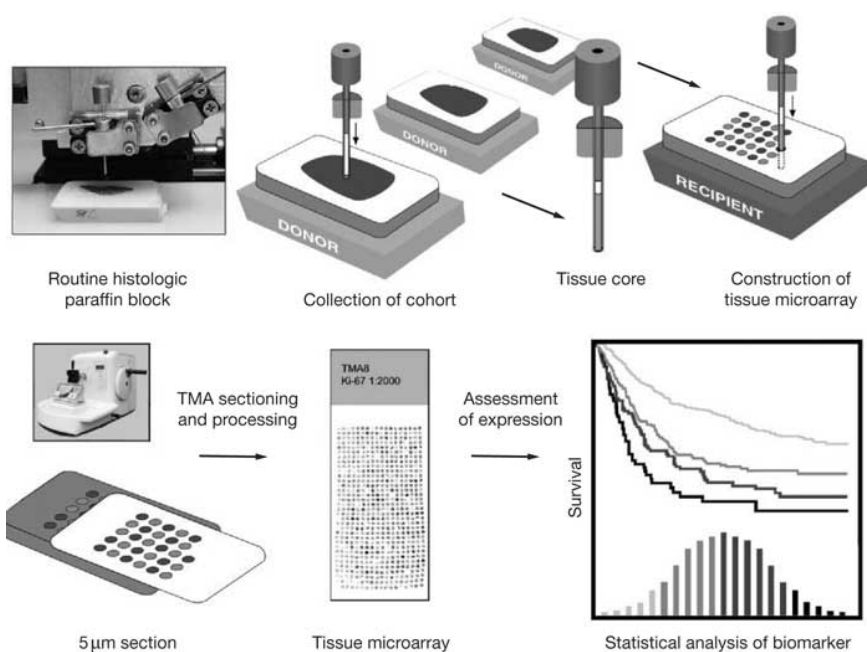
Figure 1. Archival paraffin-embedded, formalin-fixed tissues are collected. A pathologist selects representative areas from each donor tumour block, and then punches cores, 0.6–2.0 mm in diameter, from the donor block. Sections of the resultant tissue microarray are cut and transferred to glass slides for processing of biomarker status by IHC or in situ hybridization techniques (68).

When the TMA technique was first published in 1998 (67), many researchers were concerned about the small size of the tissue included in the TMA. Tissues are typically heterogeneous, with areas of different histological or even genetic features in the case of tumour tissues. In a specific tumour, heterogeneity may occur in different areas of the tumour. It is therefore of the utmost importance to select areas of the tissue that accurately represents the whole tumour (69-70).

In view of the heterogeneity of lung cancer tumours, it is vital to take extra precautions when constructing the TMA blocks in lung cancer research concerned with pathogenetic or prognostic factors and markers related to therapies (61).

Nuclear hormone receptors

Steroid or nuclear hormone receptors (NRs) constitute an important super family of transcription regulators that are involved in widely diverse physiological functions.

NRs are intracellular transcription factors that directly regulate gene expression in response to ligands. Many of the NRs act as ligand-inducible transcription factors, responding to endogenous and exogenous chemicals. A ligand binding to a nuclear receptor results in a conformational change in the receptor, which, in turn, activates the receptor, resulting in up-regulation or down-regulation of gene expression. A number of NRs, referred to as orphan receptors have no known endogenous ligands (71).

Phylogenic analysis has shown six subfamilies (NR1-6) with various groups and individual genes (72). NRs generally follow a standard blueprint, as shown in Figure 2 (73-76).
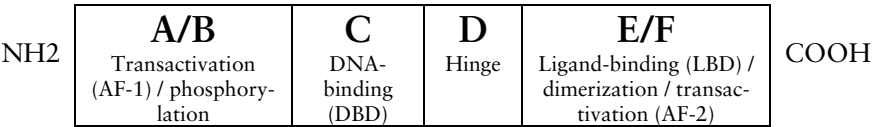
A/B	C	D	E/F	
Transactivation (AF-1) / phosphory-lation	DNA-binding (DBD)	Hinge	Ligand-binding (LBD) / dimerization / transac-tivation (AF-2)	

NH2 ... COOH

Figure 2. The general composition of a nuclear hormone receptor.

The N terminal of the NR, or A/B domain, contains the activation function 1 (AF-1) whose action is independent of the presence of ligand. The transcriptional activation of AF-1 is normally very weak, but it does synergize with AF-2 in the E-domain to produce a more robust up regulation of gene expression. The A/B domains sequence and length are highly variable between receptors and among receptor subtypes. In addition, this region is the most frequent site of alternative splicing and secondary start sites and contains a variety of kinase recognition sequences.

NRs bind to hormone response elements (HREs) in their target promoters through the DNA binding domain (DBD) or C domain. Containing two zinc fingers, the DBD is the most conserved region within the NR superfamily, that binds to specific sequences of DNA, namely HREs.

Immediately adjacent to the DBD is the D or hinge domain. This particular region has an ill-defined function. The hinge domain contains the carboxy-terminal extension (CTE) of the DBD, which may be involved in recognizing the extended 5' end of the HRE. The D-domain appears to allow for conformational changes in the protein structure following ligand

binding. Also, this region may contain nuclear localization signals and protein-protein interaction sites.

The sequence of the ligand binding domain (LBD) or E/F domain varies substantially between NRs, but they all share a common structure of 11-13 -helices organized around a hydrophobic binding pocket. The LBD also contains the AF-2 whose action is dependent on the presence of bound ligand.

NRs can also have important biological effects without ligand binding. Most NRs are phosphoproteins, and it is now known that many of these proteins are activated by crosstalk with other signal transduction pathways such as those responding to EGF and TGF-α (73-76).

Ligands for NRs are as varied as the proteins themselves. All ligands are lipophilic and can easily transverse the plasma membrane as well as the nuclear membrane, if required.

The ligand is attracted to the recptor, and a conformational change takes place, preventing the ligand's exit. Ligand binding to the NRs LBD stabilizes their structures relative to the unliganded receptor. Binding of the ligand molecule induces a conformational change in the LBD, whereby the AF-2 sequences fold back against the binding pocket, obstructing the opening and causing rearrangements in adjacent helices. In the process, a new surface is revealed that recruits specific transcriptional coactivators. This model may explain why receptor antagonists block transactivation; these compounds do not induce the proper conformational rearrangements in the LBD, interfering with the formation of the transcriptional activation complex (77-78).

Estrogen receptors

Estrogens are steroid hormones that regulate growth, differentiation and function in a broad range of target tissues in the human body. The most potent and dominant estrogen in human is 17-estradiol (E_2). The biological effects of estrogens are mediated through estrogen receptor (ER) (79).

The ER is a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding and activation of transcription. Alternative splicing results in several ESR1 mRNA transcripts, which differ primarily in their 5-prime untranslated regions. The translated receptors show less variability (80-81).

ERs belong to the large super family of NRs that bind to DNA at specific sites to control gene transcription (82). The ER gene was cloned and sequenced in 1986 (83). Recently, a second ER, referred to as ERβ, has been identified (84-85). Thus the old receptor was renamed ERα. The ERβ protein is highly homologous to ERα and specifically binds estrogens with

high affinity. While ERα is mainly found in estrogen target tissues (uterus , vagina, mammary glands), ERβ has been detected in a large variety of tissues e.g. kidney, thymus, small intestines, lung, blood leucocytes and colon (86).

The human ERα gene is located on chromosome 6 and ERβ gene on chromosome 14, demonstrating that they are in fact coded by separate genes and are distinct (86).

Human ERα and ERβ share common structural domains, which are designated A-F. The variable amino terminal A/B domain contains AF-1. This modulates transcription in a gene- and cell-specific manner. This domain is one of the least conserved domains between ERα and ERβ, exhibiting only a 30% identity (87-88). Based on functional studies, ERβ has been shown to lack AF-1 activity (89). The DBD, or C domain, is the most highly conserved region between ERα and ERβ, with 96% identity. This domain mediates receptor dimerization, and allows both receptors to bind to similar target sites. The D domain, or hinge region, is not well conserved between the receptors and it contains the nuclear localization signal. Finally, the carboxy-terminal multifunctional E/F region holds the LBD as well as sites for cofactors, a second nuclear localization signal and AF-2. In contrast to AF-1, AF-2 is a ligand-dependent activation function. The E/F domain of ERα and ERβ exhibit a sequence identity of 53%. ERβ activity is mediated by AF-2, since ERβ does not contain AF-1(Figure 3) (87-88, 90).
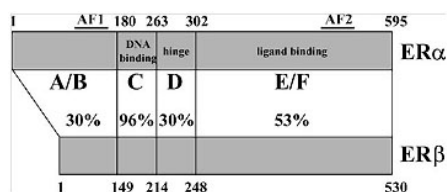
Figure 3. Structure and homology between human ERα and the long form of ERβ . Adapted from (84, 87)

The classical mechanism of ER action involves estrogen binding to receptors in the nucleus, after which the receptor dimerize and bind to specific response elements known as estrogen response elements (EREs) located in the promoter of target genes (91). Hormone binding also induces a conformational change within the LBD of receptors and this conformational change allows co activator proteins to be recruited (92). However, evidence for signalling pathways that deviate from the classical model has

emerged and it is now accepted that ERs can regulate gene expression by an number of distinct mechanisms (93). Around 1/3 of the genes in humans that are regulated by ERs do not contain ERE-like sequences. ERs can regulate gene expression without binding directly to DNA by modulating the function of other classes of transcription factors through protein-protein interaction in the nucleus (94)

In short there are four ways (Figure 4) for ER to exert its signalling mechanisms: 1) the classical mechanism of ER action, where ligand-activated receptors bind directly to EREs in the promoters of target genes. 2) non-ERE estrogen signalling pathway, ligand bound ERs interact with other transcription factors (AP-1, NF- B, SP1), forming complexes that mediate the transcription of genes whose promoters do not harbour EREs. Co-regulator molecules regulate the activity of the transcriptional com-plexes. 3) non-genomic estrogen signalling pathways, ERs and GP30 lo-cated at or near the cell membrane might elicit the rapid response by acti-vating the PI3K/Akt and/or PKC/MAPK signal transduction pathways. 4) ligand independent pathways, ERs can be stimulated by growth factors such asIGF-1, TGF-β/SMAD, EGF or Wnt/β catenin signalling pathways in the absence of ligands, either by direct interaction or by MAP and PI3K/Akt mediated phosphorylation. Since members of these signalling pathways are transcription factors, some of them, such as SMADs 3/4 , can elicit estrogen responses by interacting with ER in the non-ERE dependent genomic pathway (87-88, 93)
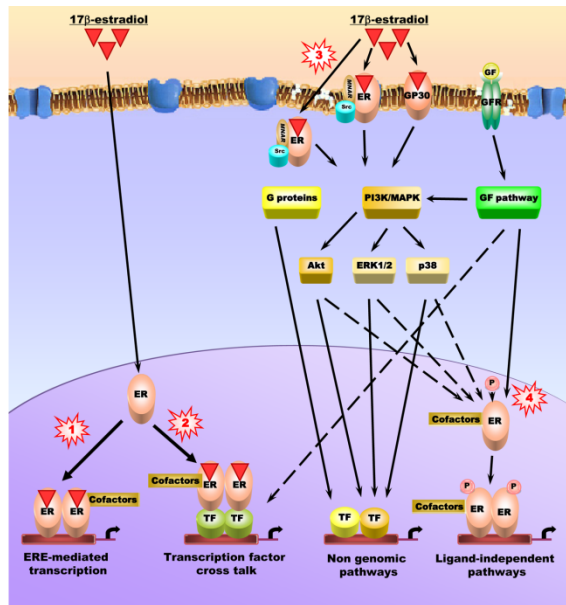
Figure 4. The four different ways for ER to signal.1: canonical estrogen signaling pathway, 2: non-ERE estrogen signaling pathway, 3: nongenomic estrogen signaling pathways and 4: ligand-independent pathways ((88)

Phosphorylation is a common covalent modification of proteins which provides an important mechanism by which the activity of transcription factors is regulated. Cell surface receptors for polypeptide hormones, cytokines etc, stimulate signal transduction pathways, leading to phosphorylation and/or dephosphorylation of substrate proteins, including transcription factors (95). The ligand-independent activity of the ER is a result of phosphorylation of the ER and creates cross-talk between the ER and other signalling pathways (96).

Phosphorylation provides an important mechanism to regulate ERα activity (97). Activation of ERα via phosphorylation at multiple sites (S104, S106, S118, S167, S236, Y537) by multiple kinases is important because of the interaction between growth factor signalling and the ER (Figure 5). Increased growth factor signalling may account for the loss of E_2 dependence, thereby producing antiestrogen resistant tumours (87).
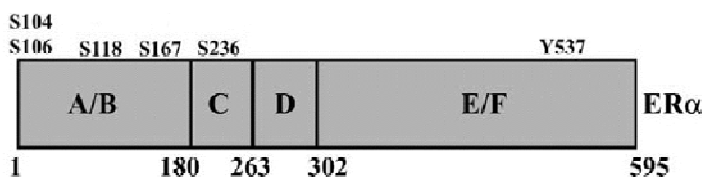
Figure 5. Kinases and ligands induce ER phosphorylation at specific sites. Adapted from (87)

Estrogens

Estrogens regulate the growth, differentiation and physiology of the reproductive process. Estrogens also influence the pathological processes of hormone-dependent cancers (79).

Estrogens are a group of steroid compounds, which are present in both men and women, though the levels are higher in women of reproductive age. Three estrogens occur naturally in the female: estradiol, which is the predominant form in non-pregnant women, estriol is the primary estrogen of pregnancy and estrone is produced during menopause (98). Estrogens are produced primarily by developing follicles in the ovaries. Some estrogens are also produced in smaller amounts by other tissues such as the liver, adrenal glands, and the breasts. These secondary sources of estrogens are especially important in postmenopausal women (79).

Estrogen, like other steroids, is derived from cholesterol. After side chain cleavage and using the delta-5 or the delta-4 pathway, androstenedione is the key intermediary. A fraction of the androstenedione is converted to testosterone, which in turn undergoes conversion to estradiol by an enzyme called aromatase. In an alternative pathway, androstenedione is aromatized to estrone, which is subsequently converted to estradiol. Estrogens can be produced de novo in some types of tumours by aromatisation of androgens (99).

Like all steroid hormones, estrogens readily diffuse across the cell membrane. Once inside the cell, they bind to and activate estrogen receptors which in turn modulate the expression of many genes. This binding of estrogen to its receptors can be classified into two pathways, genomic and non genomic as mentioned above.

Aromatase

Aromatase, also known as estrogen synthetase, catalyzes the conversion of androgens to estrogens, which is a key step in estrogen biosynthesis, and

constitutes a unique gene family (CYP19 gene) in the cytochrome P-450 superfamily (100).

The enzyme is located in the endoplasmic reticulum of the cell (100) and its activity is regulated by tissue-specific promoters that are in turn controlled by hormones, cytokines, and other factors. The principal action of the enzyme transforms androstenedione to estrone and testosterone to estradiol (Figure 6). The aromatase enzyme can be found in many tissues including gonads, brain, adipose tissue, placenta, blood vessels, skin, bone, and endometrium, as well as in tissue of endometriosis, uterine fibroids, breast cancer and endometrial cancer (101).
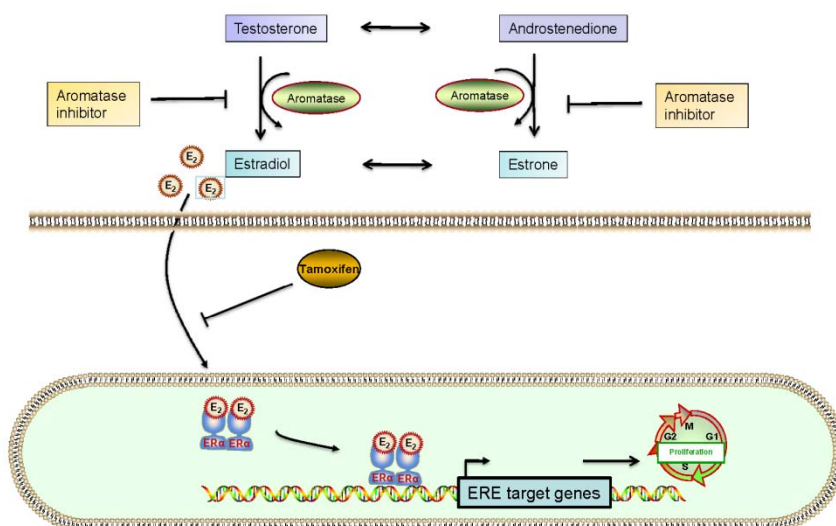
Figure 6. Aromatase is involved in two parts of the synthesis pathway: conversion of testosterone to estradiol in the ovary and conversion of androstenedione to estrone in adipose tissue (www.visiscience.com)

Progesterone receptor

The progesterone receptor (PgR) is an intracellular steroid receptor that specifically binds progesterone. In humans PgR is encoded by a single PGR gene located on chromosome 11q22, it has two main forms A and B that differ in molecular weight. PgR A and PgR B are translated from the same

gene, however their transcription is initiated at different promoters (102), PgR B contains an additional 164 amino acids at the N-terminal domain (103). The genomic organization of these PgRs are well characterized, the nuclear/genomic PgR contains three functional domains, the N-terminus, the DBD and the C-terminal LBD. Activation domains present within these functional domains are required for the DNA bound receptor to transmit a transcription activation response and these sites also serve as specific binding sites for co-activators. The N-terminal domain, the least conserved domain with respect to length and amino acid sequence, is required for the full transcriptional activity of the receptor and for other cell target gene specific responses (104). This domain contains the AF-1 and AF-3, which recruit co-activator proteins to the receptor and thereby regulate promoter specificity and also the transcriptional activity (105). The DBD is a highly conserved centrally located domain consisting of approximately 66-68 amino acids (105). Two type II zinc finger structures in this domain facilitate the binding of the receptor to specific cis-acting DNA structures (106). The DBD also contains a highly conserved AF-2 domain. The LBD is involved in binding to progesterone and heat-shock proteins. The binding of progesterone to the LBD domain induces a conformational change in the receptor, leading to its phosphorylation and dissociation from heat-shock protein and dimerisation. The ligand-receptor complex enters the nucleus and then binds and activates specific response elements in the promoter region of the target genes (103).

Receptor tyrosine kinases

One of the fundamental mechanisms by which cells in multi cellular organisms communicate is the binding of polypeptide ligands to cell surface receptor that possess tyrosine kinase activity.

Receptor tyrosine kinases (RTKs) are a diverse group of transmembrane glykoproteins that are activated by the binding of their cognate ligands. Upon ligand binding RTKs undergo dimerization and they transduce the extracellular signal to the cytoplasm by phosphorylating tyrosine residues on the receptor themselves (autophosphorylation) and on downstream signalling proteins. RTKs activate numerous signalling pathways within cells, leading to cell proliferation, differentiation, migration or metabolic changes (107-108). The RTK family includes the receptors for insulin and for many growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) (107-108).

RTKs consist of an extracellular N-terminal portion that binds polypeptide ligands, a transmembrane helix composed of 25-38 amino acids, and a cytoplasmatic portion that possesses tyrosine kinase catalytic activity. The vast majority of RTKs exist as a single polypeptide chain and are monomeric in the absence of a ligand (107).

The extracellular portion of RTKs typically contains a variety of conserved elements that are characteristic for each subfamily of RTKs; these domains contain primarily a ligand binding site (109). In contrast, the domain organization of the cytoplasmatic portion of RTKs is simpler, consisting of a juxtamembrane region (just after the transmembrane helix), followed by the tyrosine kinase catalytic domain and a carboxy-terminal region. The intracellular C-terminal region displays the highest level of conservation and comprises catalytic domains responsible for the kinase activity of these receptors, which catalyses receptor autophosphorylation and tyrosine phosphorylation of RTK substrates (109). The juxtamembrane and carboxy-terminal regions vary in length among RTKs (110).

ErbB receptor family

This is a family of four structurally related RTKs. ErbB receptors are made up of an extracellular region that contains approximately 620 amino acids, a single transmembrane-spanning region, and a cytoplasmatic tyrosine kinase domain. In general, ErbB receptors contain two cystein rich regions in their extracellular domain and a kinase domain flanked by a carboxy-terminal tail with tyrosine autophosphorylation sites (111).

Each member of the ErbB receptor family is located on different chromosomes. ErbB1 (EGFR, HER-1) are located on 7p12, HER-2 (ErbB2) on 17q11.2-q12, HER-3 (ErbB3) on 12q13 and HER-4 (ErbB4) on 2q33.3-q34.

A fundamental aspect of signalling is the interaction of two receptors. The dimerization of two ErbB family members and the transphosphorylation of their intracellular domain generate the initial signal leading to activation of numerous downstream signalling pathways. The dimerization event is regulated by extracellular ligands of the epidermal growth factor (EGF) and neuregulin (NRG) families (112-113).

The four members of the ErbB protein family are capable of forming homodimers, heterodimers and possibly higher-order oligomers upon activation by a subset of potential growth factor ligands (111).

ErbB2 and ErbB3 are functionally incomplete transmembrane receptors. They need interdependency. The ErbB2 extracellular domain is unique. It is locked in a conformation resembling the ligand-bound states of the other ErbB extracellular domains. ErbB2 is not regulated by ligands as it has no ligands. This receptor appears to be activated by transmodulations as a result of heterodimerization with other receptors (111). ErbB2 is always available for dimerization with activated ErbB family members. The ErbB3 intracellular kinase domain is also unique, it is inactive catalytically. However, ErbB3 is an efficient dimerization partner for all other ErbB family members (111).

Two out of the four ErbB proteins, namely ErbB-1 and ErbB-4, are autonomous; when bound by a ligand growth factor, they undergo dimerization and generate intracellular signals culminating in cell proliferation, migration or differentiation. The two other receptors are non-autonomous: ErbB-2 binds to no soluble ligand, but acts as a preferred partner in heterodimeric complexes with other ligand-bound ErbBs. On the other hand, ErbB-3 cannot generate signals in isolation because the kinase function of this receptor is impaired (108) (Figure 7).

Two main ligand classes have to date been identified: the splice variants of NRGs that binds exclusively to HER-3 and/or HER-4 and different EGF-related proteins (114). Binding of specific ligands to domains I and II is followed by conformational changes to the extracellular domain, exposing domain II, which facilitates receptor dimerization. Additional receptor interactions in extracellular, transmembrane and kinase domains further stabilize the dimer. Dimerization results in the activation of the kinase domain, and the induction of intracellular signalling cascades that mediate cell growth and survival. Signalling diversity depends both on the presence of specific receptors and the characteristics of individual ligands. EGFR

and HER-2 classically couple to Ras-Raf-Mek- MAPK dependent pathway, whereas HER3 is a potent activator of PI3K-Akt.
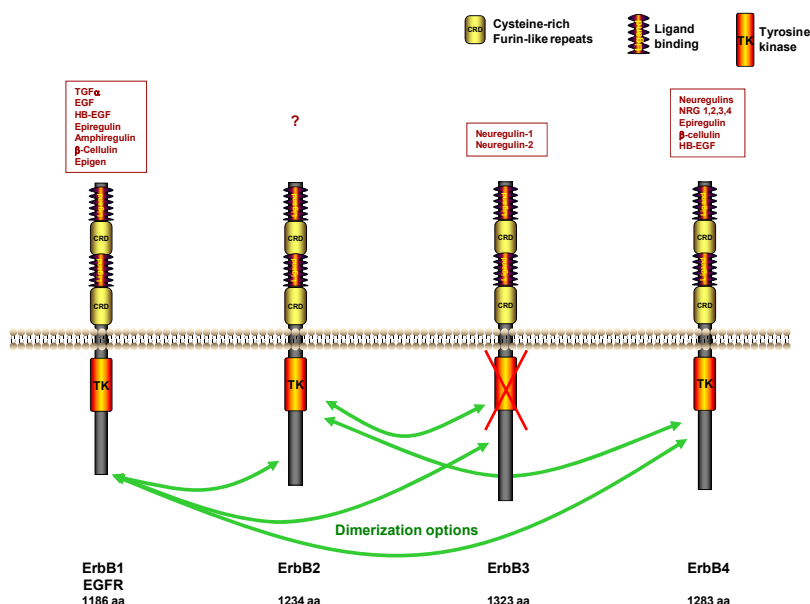

Figure 7. Members of the epidermal growth factor (EGF) family of growth factors are ligands for the ErbB receptors. Ligand binding to ErbB receptors induces the formation of receptor homo- and heterodimers and the activation of the intrinsic kinase domain, resulting in phosphorylation on specific tyrosine residues within the cytoplasmic tail. None of the ligands bind ErbB2, but ErbB2 is the preferred dimerization partner for all the other ErbB receptors. ErbB3 has impaired kinase activity and only acquires signalling potential when it is dimerized with another ErbB receptor, such as ErbB2 receptor, ErbB2 being its preferred partner. (www.visiscience.com)

The HER-4 gene is located on chromosome 2q33.3-34 and the encoded protein can be activated by both NRGs and some ligands of the EGF family (betacellulin, epiregulin, heparin binding EGF like ligand) (115).

HER-4 is a unique cellsurface receptor and mediates completely novel activities for a transmembrane tyrosine kinase. Four isoforms of HER-4 receptor have been described (JMa,JMb,Cyt1,Cyt2) resulting from alternative splicing of HER-4 mRNA (116). The JMa isoform contains an ex-

tracellular proteolytic site, which could be cleaved by the metalloproteinase tumour necrosis factor-alpha converting factor (TACE) (116). After cleavage by TACE, the remainder transmembrane cleavage product (m80) could undergo a second transmembrane γ-sectretase cleavage releasing into the cytoplasm a soluble HER-4 intracellular domain (4ICD). The 4ICD may either remain in the cytosol or translocate to the nucleus (115). These cellular responses are in many cases directly associated with a specific 4ICD subcellular context and shuttling of 4ICD between different subcellular compartments may regulate the life or death decisions in a cell (117) (Figure 8).

4ICD is characterized by multiple diverse biological activities and cellular responses including differentiation of mammary epithelial cells and lactation, activation of proapoptotic pathways, cell cycle arrest, modulation of transcription through formation of complexes with transcription factors and cell proliferation. In addition, it has been demonstrated that these diverse responses are associated with the localization of 4ICD in different cell compartments (117). Nuclear 4ICD functions as a potent ERα co-activator, directly interacting with ligand-associated ERα and promoting the proliferation of ERα positive breast tumour cells (118).

Ectodomain cleavage of the 180kDa HER-4 cell surface receptor is mediated by TACE, a member of the ADAM metalloproteinase family referred to as ADAM17. Following TACE cleavage the ca 120kDa HER-4 ectodomain, which include the ligand binding region, is shed into the extracellular milieu while the remaining 80kDa cleavage product (m80) is retained as a transmembrane peptide. The m80 harbours an active tyrosine kinase (119) and a carboxyterminus with several potential tyrosine phosphorylation sites (117).

A single transmembrane domain separates equal sized ecto- and cytoplasmatic domains. Within the ectodomain is a cleaved signal sequence and two cysteine rich regions (domains II and IV), typical or ErbB-receptors; By analogy with EGFR it seems likely that domain III mediates growth factor binding between domain IV and the transmembrane region. ErbB4 has a comparatively longer stalk region, which may make it uniquely sensitive, within this receptor family, to ectodomain cleavage. The cytoplasmatic domain contains a juxtamembrane region, a tyrosine kinase domain and a carboxyterminal domain – all typical of ErbB-receptors (115).

Ligands, which binds to ErbB4 with high affinity and specificity and which provoke receptor activation and signalling, are divided into two groups, i.e. the NRGs, also termed heregulins, and certain members of the EGF family of ErbB1 ligands (betacellulin, HB-EGF, epiregulin). There are

4 NRG genes, and the products of each are capable of recognizing ErbB4 in a biologically productive manner.
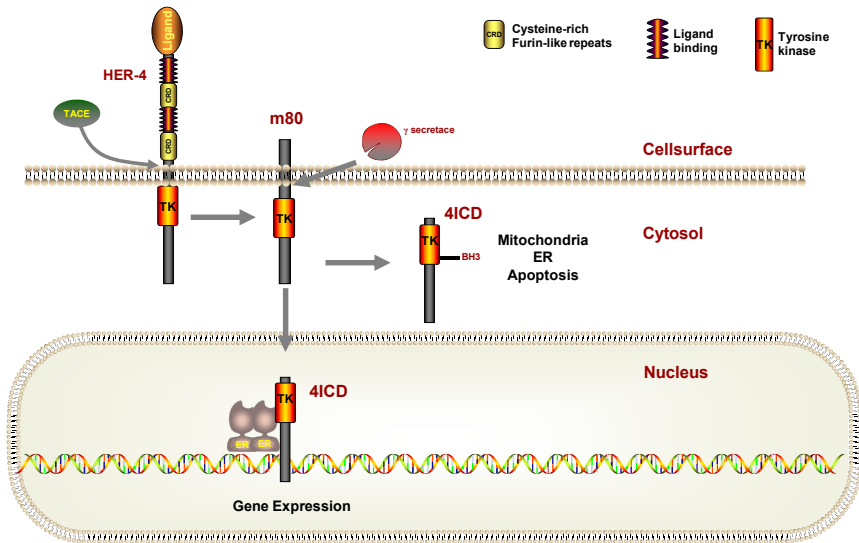
Figure 8. Ligand activated HER-4 is proteolytically processed at the cell surface through the sequential activities of TACE and γ- secretase. The HER-4 intracellular domain (4ICD) may remain in the cytosol where it accumulates within the ER and mitochondria and regulates apoptosis. Alternatively 4ICD may accumulate within the nucleus, interact with transcription factors at target promoters, and coactivate gene expression (www.visiscience.com)

Materials

Paper I

Tumour specimens

Thirty cases of lung carcinoma, (10 of SCLC, 10 of primary lung ADCA and 10 of SCC), were included in the study. One tissue block per case was selected, using the block with the largest tumour area. Ten cases of breast carcinomas were selected based on HercepTest scores in the original histo-pathologic report. Ten consecutive cases of endometrial curettages with ADCA were also included.

Before analysis the material were anonymised.

Paper II

TMA construction

A TMA block was constructed containing normal tissue from appendix, tonsil, prostate, thyroid, breast, lung colon and pancreas. In the TMA malignant tissue from colon, breast, lung, thyroid, prostate and lymph nodes were included. The tissues were chosen with the future analysis in mind; the aim was to make a TMA which could be used as control tissue for a large number of antibodies, detecting nuclear as well as cytoplasmatic and membranous antigens. All cylinders had a diameter of 1 mm and only one cylinder from each case was included in the TMA. Further details of tissue representation are presented in Paper II.

Before analysis the material were anonymised.

Paper III-V

Lung cancer specimens

262 cases of formalin fixed and paraffin embedded NSCLC (134 ADCA, 124 SCC, 3 adenosquamous carcinomas (ADSQ) and 1 large cell carcinoma (LCC) were included in the study. Among those frozen material was available in 139 cases (68 ADCA and 71 SCC). The tumour specimens were obtained during surgical procedures, mainly lobectomies and pneumonectomies between 1990 and 1995. All tumours were reclassified according to the WHO 2004 classification (Travis 2004) as well a re-staged according to 7ed of TNM Classification of Malignant Tumours (Sobin 2009). Survival data was obtained from the National Board of Health and

Welfare/causes of death database. Further details of clinical and histological features of the patiens are presented in Paper III-V.

The tumours were arranged in TMA blocks, with triplicate samples (Ø 1mm) from each tumour (120).

Methods

Immunohistochemistry (Paper I-V)

The tumours were originally FFPE and had thereafter been stored at room temperature, according to routine laboratory procedures.

Four-micrometer sections were cut from the TMA or tissue blocks, onto DAKO ChemMate Capillary gap microscope slides (Dako, Denmark), and placed at 60°C for 1 hour.

After initial deparaffinization, antigen retrieval was carried out according to the results from using the "test battery" proposed by Shi et al (51). The IHC was performed using a ChemMate DAKO EnVision Detection Kit (Dako, Denmark), with primary antibody incubation for 25 min at room temperature. The Dako TechMate sytem was used for the IHC staining. Negative control slides were prepared by substituting the primary antibody for Dako ChemMate antibody diluent (Dako, Denmark). Positive control sections with adequate tissue recommended by the manufacturer were included.

All antibodies had previous undergone testing to obtain the highest detection sensitivity. These tests include pre-treatment, antigen retrieval testing and titration of the antibody.

Details of the antibodies analysed, including clone, pre-treatment and detection system, are given in Paper I-V.

Scoring of the positive cells were carried out at 100x – 400x magnification in an ordinary light microscope or by studying scanned slides using Aperio Image Scope. All scoring was done twice (CK), and if differences occurred the highest score was used. Scoring for EGFR and HER- 4 was done by CK and MK, if differences occurred, consensus decision was reached. Cut off limits for scoring is given in Paper I-V.

Paper I

Instead of constructing TMA blocks with different tumour material and different cylinder diameter, this study was designed to be performed on virtual cylinders. Sections of the tumours were stained for H&E and the tumour area marked (to avoid normal tissue, intra tumour haemorrhage and necrotic areas). This marking was transferred to slides stained for Ki-67, p53, ERα and HER-2. Virtual cylinders corresponding to Ø of 0.6mm and 1.0 mm were marked on the slides. 5-10 different "cylinders" were marked both at central and border parts of the tumour. Each cylinder was photographed using the Leica QWIN system (Leica, Germany) and all

counting and scoring was done using the pictures. Positive and negative cells were counted for Ki-67. Scoring was done for p53, ERα and HER-2.

Paper II

Storage of slides

The sections were divided into two batches. One for storage without any coating and one for storage with a coat of paraffin wax. The slides destined for coating were deparaffinised in xylene, air dried and then dipped in melted paraffin wax to form an even coat on the section. All slides were then stored either at room temperature (RT) or at 4°C, for 1 week, 1, 3, 6 or 12 months, thus rendering four experimental settings.

Evaluation of staining intensity

All slides were compared with the original slides stained at day 0, i.e. sectioned the same day as they were stained.

All slides were scanned by Scanscope XT (Aperio, SanDiego, CA), at 20X magnification. Intensity of positive staining was measured using Positive Pixel Count V9.0 included in the Aperio ImageScope (Aperio, SanDiego, CA), with standard settings. The greyscale is composed of values ranging from 0-255 where 0 is black and 255 is white. For these measurements positivity was counted if the values were within 0-100 in the greyscale. Details about the segmentation are given in pictures in Paper II.

Fluorescence in-situ hybridisation

Four-micrometer sections were cut from the TMA or tissue blocks, onto SuperFrost® Plus slides (Mentzel-Gläser GmbH, Germany), and placed at 60°C over night, the slides were stored as above with FISH analysis at 1, 3, 6 and 12 months.

The sections were subjected to treatment according to the manufacturer's protocol. Slides were hybridized with a probe mix (details given in Paper II) in HYBrite (Vysis, Des Plaines, IL) were denaturation was set at 6 min 73°C and hybridization for 17 h at 37°C

Signals were counted in 20 tumour cells at 60X magnification with oil immersion objective.

Chromogenic in-situ hybridisation

Four-micrometer sections were cut from the TMA or tissue blocks, onto SuperFrost® Plus slides (Mentzel-Gläser GmbH, Germany), and placed at 60°C over night. Fresh cut sections and slides stored for 12 months as above were used for CISH analysis.

Detection of kappa and lambda light chains was performed according to the manufacturer's protocol (Histosonda, Lugo, Spain). Slides were incubated with the probe (details given in Paper II) at 62^0C for 1 h. For visualization of the probe the DAKO EnVision Detection Kit (Dako, Copenhagen, Denmark) was used.

Paper III-IV

Extraction of RNA and DNA

Fresh frozen lung tumour tissue was cryosectioned and stained with haematoxylin and eosin (H&E) in order to confirm the presence of tumour cells. RNA and DNA were extracted from the same tissue using AllPrep RNA/DNA minikit (Qiagen, Germany) according to the manufactures' description.

Gene expression

Total RNA was reversed transcribed to cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, USA). Reaction plates (348 wells) were robotically prepared in the CAS 1200 (Corbett Robotics, Australia) system in 10 µl reactions containing 4 µl cDNA, 5 µl Gene Expression MasterMix (Applied Biosystems, USA) and 1 µl primers/probe (Applied Biosystems, USA). The reaction plates were analyzed in the real time PCR system 7900 HT (Applied Biosystems, USA). All samples were analyzed in duplicate and the threshold number was averaged. Relative gene expression was calculated using the comparative Ct method (Ct) with GAPDH as endogenous control. The relative quantitative gene expression were determined as follows: $2^{-(Ct\ sample-Ct\ calibrator)}$, where the Ct values of the calibrator and sample were determined by subtracting the Ct value of the target gene from the value of the GAPDH gene. Further details about probes are given in paper III-IV.

Copy number analysis

Quantitative real-time PCR was used to determine the copy number and the target gene was normalized to an endogenous reference gene, Ribonuclease P RNA component H1, known to be present in two copies in a diploid genome. As internal controls, DNA extracted from 5 breast carcinomas was used where the copy number of HER2 has been established with fluorescent *in situ* hybridization. gDNA (10 ng) was amplified in a 10 µl reaction containing 5 µl TaqMan® Genotyping Master Mix, 0.5 µl FAM™dye-labeled TaqMan® Copy Number Assay (Applied Biosystems, USA), 0.5 µl VIC™dye-labeled TaqMan® Copy Number Reference Assay

(Ribonuclease P RNA component H1 (H1RNA) gene (RPPH1) TaqMan® Copy Number Reference Assay RNase P) (Applied Biosystems, USA) and 3 µl nuclease-free water. Reaction plates (348 wells) were robotically prepared in the CAS 1200 (Corbett Robotics, Australia). The reaction plates were analyzed in the real time PCR system 7900 HT. All samples were analyzed in duplicates and the Copy Caller software v1.0 (Applied Biosystems, USA) was used to analyze the data. Further details about probes are given in paper III-IV.

Statistics

Paper I

Agreement between the percentage of positive cells found from 0.6 mm cores and 1.0 mm cores was estimated by the intra-class correlation (ICC) (121) and by 'Bland-Altman plots' (122). Both the ICC and the Bland-Altman plots were applied to all tumours together and then to each histological type. Variations in measurements between subjects and within subjects were calculated and reported as SDs.

Scoring was evaluated with weighted kappa statistics using quadratic weights (123-124) to compare scoring of different numbers of cylinders versus whole section.

Paper II

The influence of storage time for the four different storage conditions was analyzed for each antibody with one-way ANOVA statistics using STATISTIX v.8 (Analytical Software, Tallahassee, USA). Inter-assay variation using three parallel staining for CD5, CD7, ERα and PR A was analyzed in the same way.

Data for a number of antibodies with more extensive datasets (Ki-67, p53, Herceptest® and Cytokeratin 7) were analyzed using a Mixed Models Method. The analyses were performed using the procedure MIXED in SAS, version 9.2 (Cary, NC, USA).

Paper III-V

The statistical analyses were done using Statistix 8 (Analytical Software, USA).The IHC and categorized gene copy number (GCN) data was compared using chi-square test and the Wilcoxon Rank sum test was used for expression analysis. The probability of overall survival was calculated using the Kaplan-Meier method and compared using the log-rank test.

Values of $p<0.05$ were considered significant.

Ethics

Paper I-II: In accordance with the Swedish biobank act (SFS2002:297) all tissues were completely anonymised during the preparation of the TMA blocks and no clinical data were known to the investigators.

Paper III-V: Work was approved by the Örebro research ethical committee 2003-12-30, and Linkoping regional ethical review board, 2010/44-31.

Results

Paper I

Aim

The aim of this study was to investigate the diameter and number of the cylinders needed to obtain representative histopathologic material. This approach was addressed by using virtually constructed tissue cylinders from different tumour material, with emphasis on material from NSCLC as well as SCLC.

Results

Cylinder diameter

The ICC for the percentage of Ki-67 positive cells between observations for the 0.6 and 1.0 mm diameter cylinders was 0.96 for the material as a whole, which can be regarded as a very high value.

A Bland-Altman plot was constructed to analyze the possibility of a systematic difference between the different cylinder diameters. A small average difference of 0.5% between the observations from cylinders is shown, and there is no indication of a systematically changing difference. The limits of agreement are –8.1 to 9.1%, which are reasonably small as they cover approximately 95% of the differences.

Together these data show that cylinders with diameter 0.6 mm are as informative and reliable as cylinders with 1.0 mm diameter.

Number of cylinders

These data reveals that an increased number of cylinders results in a decreased SEM, the major change in SEM already occurring between observations based on one and two cylinders.

Comparison between whole sections and TMA

Data for all possible combinations of cylinders was generated and compared to whole section scoring and the agreement was evaluated by weighted kappa statistics. p53 scoring in lung carcinoma showed an almost perfect correlation (kappa 0.96-1.0). The agreement for Ki-67 in endometrial carcinomas was good (kappa 0.57-0.69) and excellent in breast carcinomas (kappa 0.85-0.91). ERα scoring between cylinders and whole sections showed excellent agreement using kappa statistics (kappa 0.94-

1.0). When using the same settings for HER-2 as for the other markers, kappa values ranged from 0.75-0.85

Paper II

Aim

The aim of this study was to evaluate, using adequately FFPE tissues and up-to date antigen retrieval techniques and sensitive detection system, factors which may render it possible to use even long term stored slides for protein, DNA and RNA in situ demonstration in order to facilitate the handling of valuable TMA materials.

Results

Baseline values were established on fresh cut sections without any previous coating or storage before staining. All different variation in storing and coating were compared to baseline values.

Inter-assay variation

In order to evaluate the influence of inter-assay variation as a co-factor in possible time-dependent effects an experiment with three parallel staining for CD5, CK7, ERα and PR A was performed. For none of the antibodies any significant difference occurred between the different experiments.

Influence of storage time – single factor analysis

Data indicates that the main factor explaining the reduced staining intensity over time is the antibody used. However, no obvious difference between different types of antibodies or cellular localisation of the antigen was found

Influence of storage time – multi factor analysis

Three factors were studied in this setting: time of storage (baseline, 1 week, 1, 3, 6 and 12 months), temperature of storage (RT or 4^0C) and paraffin coating of stored slides (Yes/No).

For p53 storage temperature seemed to be of no importance, paraffin coating of the slides significantly decreased staining intensity, especially when considering the time factor ($p<0.01$). Though statistically significant, differences were limited, 7 gray value units, taking the 256 levels in the intensity scale in account.

Sections stained with Ki-67 showed that a decreased staining intensity occurred over time regardless of storage conditions, $p<0.0001$. RT storage vs. 4^0C independently of other factors decreased staining intensity (1.5 unit, $p <0.001$), as did the presence of paraffin coating (1.5 unit, $p <0.001$). However, influence of storage conditions was limited compared to the time factor.

After storage at RT, cytokeratin 7 showed significantly decreased staining intensity compared to 4℃. (1.1 unit; p=0.027), also acting in a time dependent manner, p<0.0001. A negative impact of paraffin coating was significant, p=0.001, this also interacted with increased storage time, p<0.0001.

HER-2 IHC unexpectedly showed a time-dependent increased staining intensity, p<0.0001, due to an extreme value (10 units stronger than baseline) at 12 months for 4°C storage. However, as previously noted paraffin coating decreased staining intensity (p=0.0003) regardless of storage temperature. On average, paraffin coating decreased staining intensity with 3.3 units compared to non-coated slides.

FISH and CISH analysis

The ability to detect HER-2 amplification by the means of cluster detection was unaffected over time and regardless of storage condition. Evaluation of HER-2/CEP17 ratio in the non-amplified cases showed baseline values of 1.06 compared to of 0.99-1.12 regardless of storage condition over time and 1.04-1.07 for the different storage conditions over time.

In cases of MCL, the ability to detect a translocation 2 colour fusion signal was unchanged for all storing conditions and all time points.

Positive signals for kappa and lambda light chains visualized by CISH were detected regardless of storage conditions and time.

Paper III-V

Aim

The aim of these studies was to investigate if certain biomarkers could contribute to the known gender and smoking associated differences in pathology and prognosis in NSCLC. The biomarkers selected were ERs α and β and the EGF receptor family both at the protein level as well as mRNA and DNA levels.

Results

Firstly, the influences of a number of factors on 5-year survival rate were analysed. Among this group of NSCLC being woman (p=0.0001) and having an ADCA (p=0.0429) had a positive impact on survival. This gender associated effect was only present in the ADCA group (p=0.0003). After reclassification according to the present staging system, an increased survival among women with ADCA was found within Stage IA (tumour ≤ 3 cm Ø) and IIB (tumour 5-7 cm) no statistical differences occurred in Stage IB (tumour 3-5 cm) or Stage IIA (tumour <5 cm).

The proliferation was determined by IHC with staining for Ki-67. SCC was more proliferative than ADCA (p<0.0001), and among men the proliferation was more extensive (p=0.0265). These results shows that the SCC is a more proliferative tumour than ADCA, the SCC constitute 47% of the tumours in this study.

The results show an extensive transcription of ESR1 (ERα) in NSCLC. No differences between genders or between the two histological types, ADCA or SCC are shown.

The presence of ERα detected by IHC is more limited without any differences in histological types (ADCA 20/125, SCC 14/123) and shows a more frequent expression among females (20/97) but failed to reach significance (p=0.0568).

The expression of ERα did correlate to PgR A expression, both in the whole group (p<0.0001) in ADCA (p=0.001) as well as SCC (p=0.002) and among men (p<0.0001). However PgR A was only detected in 14 of the cases, and the distribution between genders were equal, men 9/165 and women 5/97.

The expression of ERα was furthermore associated with an improved survival, p=0.0149.

The phosphorylation site on the ERα, SER118, is possible to activate by estradiol as well as by other mechanisms such as signalling by EGFR signalling pathways. In SCC a correlation between EGFR over expression and pERαS118 was shown (p=0.0153)

n4ICD is detected both among ADCA and SCC but is most frequently expressed in ADCA (p<0.0001).

Aromatase is demonstrated in ADCA as well as SCC and more frequently among women than men (p=0.03). A significant correlation between n4ICD and aromatase was found in the overall group of NSCLC (p=0.0325). ERα also showed a significant correlation to aromatase within both women (p=0.0334) and men (p<0.0001) as well as in the two major histological types, ADCA (p=0.0216) and SCC (p<0.0001). These findings imply an intra tumour presence of aromatase.

In this material an extensive transcription of ESR2 (ERβ) was (97/137 cases expressed ESR2) found, with an almost twofold level of expression in SCC vs ADCA (p=0.0001). ERβ determined by IHC was found in 118/134 ADCA and 108/124 SCC. However no difference was found by histological type or gender.

Tumours of stage I (tumour size ≤3cm) expressed ERβ more frequently (p=0.0132) compared to the remaining cases. This effect was mainly attributed to ADCA (p=0.007) and men (p=0.0418). ERβ detected by IHC showed no influence on survival rate.

A positive prognostic influence of ESR2 expression within the ADCA group was found (p=0.0444), this effect could not be explained by gender or stage.

Information about smoking habits was available in 226 of the tumours.

In the non-smoking group (less than 100 cigarettes in a lifetime) a non significant tendency for ERα positivity (p=0.07) in the overall group, as well as the ADCAs (p=0.08) was found. The presence of the ERα coactivator n4ICD shows an obvious over expression in the non-smoking group, especially among women and ADCA morphology. This group of tumours also displays a lower fraction of proliferation than the rest of the ADCAs.

Smoking history was not associated with ERβ expression by IHC. ESR2 transcription was significantly higher among smoker as well as ex-smokers compared to never-smokers.

Discussion

What is a representative tissue sample? Already with the gross examination of the surgical specimen a selection is made, where and how to take a sample for diagnostic purposes? The tissue sample is further subject to fixation, dehydration, embedding and eventually sectioning into 4μm thin sections, followed by staining and mounting before diagnosis. This has been the basis for a successful diagnostic and classifications system since the 19th century. The microscopically examined tissue may represent as little as 1/50000 part of the original surgical specimen, but still this is the basis that treatment of patients are based on. Together with the classical H&E staining and morphological diagnosis in the microscope, the pathologist of today also has a number of analyses to rest the case on, like IHC, different ISH techniques and molecular biology assays.

Even though this is a minute sample from the whole tumour, studies show that the expression of biomarkers in the tissue is representative for the whole tumour in comparison with cytological samples (125).

TMA is an excellent technique to gather large tissue material for analysis. Representative tissue is selected from H&E stained sections and cylinders are punched from the donor tissue and placed in a reproducible pattern. The major advantage of TMAs is that they allow the performance of tissue-based assays (IHC, ISH, FISH, CISH etc) on a large number of tissue samples simultaneous in a time and cost efficient manner.

One must remember that TMAs are not intended for individual clinical diagnosis, tumours classifications or grading within studies, but has been designed to facilitate biomarker studies in large tissue materials (62).

The information that has been obtained by the original diagnosis e.g. histological type of tumour and specific morphology together with data such as gender, tumour location, tumour size and environmental factors can be included in the analysis when evaluating the biomarkers analysed in the TMA.

Multiple studies in different organ systems have demonstrated that consistent and comparable results can be obtained using TMA cylinders as with whole sections (126-128).

In order to obtain comparable results between TMAs and whole sections, two main strategies have been used. Increasing the number of cylinders from each tissue, or increasing the diameter of the cylinder taken from the tissue. It is vital to get the right sample size of cylinders to correspond to the whole section. By increasing the number of cylinders from the donor tissue included in the TMA, the number of different tumours in the TMA decreases, since the purpose of the TMA was to decrease the number of

blocks needed for the analysis. It is therefore of high priority to decide how few cylinders are needed for the specific application at hand. This also applies for increasing the diameter of the cylinders. A cylinder of larger diameter takes up more space in the TMA, thus, fewer cylinders can be arrayed. Another issue is that a small size cylinder drawn from a tissue block, does less damage than a large size cylinder from the same block. Smaller size cylinders drawn from the same block will also reflect at least some of the heterogeneity in the tissue.

A number of studies have investigated the size and number of cylinders needed for an adequate sample for their settings. Hoos et al (129) found that 3 cylinders of 0.6 mm were sufficient for human fibroblastic tumours, while Rubin et al (130) determined that prostate tumours needed 4 cylinders, and Rosen et al (63) reported that 2 cylinders of 1.0 mm were representative for ovarian tumours. Zhang and co-workers (131) used a single cylinder with 1.0 mm diameter for the detection of estrogen receptor, progesterone receptor and HER-2 in breast tissues.

The more cylinders you chose to array in a TMA, the more accurate will the comparison between TMA and whole section become, even though ours and other studies (69, 132) shows that between 2-4 cylinders from each case will provide enough information about the tissue. In our material, we chose to use 3 cylinders of 1 mm Ø, but we show that 0.6 mm would suffice.

A limiting factor with TMAs is the different length of cylinders arrayed, this could lead to a loss of material, to overcome such difficulties it may be a good decision to array extra cylinders, or to "fill the hole" with more material.

Loss of material is also evident in the sectioning situation, due to facing of the block each time you section, material is lost. Serial sectioning is ideal with TMA, section until an empty block, and store until use for different types of analyses.

Previous results have though commented on the possibility to store sections on slides without any loss of antigenicity.

A number of studies published 1995-2005 reported about loss of antigenicity in stored sections. These studies do not show uniformity, they detect IHC with various techniques, and above all they differ in the antigen retrieval technique used. The development of HIAR showed a plethora of ways of doing this. Most common has been the overconfident belief that citrate buffer work well for all antigens. Nowadays there are studies showing that different antigens require the right pH of the retrieval solution to give a satisfactory result (52-53, 133).

The quality of a TMA experiment, such as IHC or ISH, can only be as good as the worst step in the entire process. All tissue handling starts with the removal of the tissue from the body, at that point decay starts, enzymes are released from the lysosomes into the cytoplasm, these enzymes include proteases which destroy proteins, nucleases which destroy nucleic acids and lipases which destroy lipids. These reactions taken together will result in a complete disintegration of the cells and its content (24). To avoid these processes the tissue should immediately (or at least within 30 minutes) be placed in an environment that stops these reactions. This may be done by freezing the tissue or by immersing it in a chemical fixative. The method of fixation must reflect what the tissue sample is going to be used for later on. If freezing the tissue is it just extraction of nucleic acids or is it IHC and ISH techniques that will be used, then preserved morphology is the goal. Regardless of what the tissue will be used for, the processes of fixation or freezing must be employed fast to preserve the target molecules in the right place in the tissue (24).

To preserve tissue for routine diagnostics, 4% neutral buffered formaldehyde is the most common fixative today, a gold standard. To fix tissue in formaldehyde, a process that spend the fixative, you must make sure that there are enough fixative around the tissue (x20 times at least) (24) and leave it there for 24-48 h (134-135). For IHC to give accurate results the tissue has to be properly fixed, if under fixed (i.e. not left long enough in fixative) the IHC reaction will be poor at the centre of the specimen. Another concern has also been about over fixation (leaving the specimen for too long in the fixative), here conflicting results have been shown (136-137).

Fixation changes the tissue, above all else, the structure of the proteins will change during fixation (24). This causes problem for some of the analyses that the tissue are destined for, in IHC the epitopes on the antigen can change during fixation, so that they are destroyed or masked. To overcome this problem, different techniques to restore the tissue, for detection of these specific antigens, are used. Digestion of the tissue using proteolytic enzymes is one way; here it is important to not over digest the tissue, since this leads to poor or nonexistent remaining morphology. More common today is to heat the section. A test battery of different solutions at different pH are tested along with different heating techniques and times, all to determine the best conditions for each specific biomarker. Some biomarkers require the combination of both proteolytic enzymes and heat retrieval. If that is no good, one must retort to frozen tissue instead.

IHC was developed and published for the first time in 1942 (40), using antibodies marked with fluorescents on frozen sections. The development

since then has gone via the PAP-complex, published in 1970, for the first time FFPE tissue was available for IHC analysis (138). In 1981 the ABC technique was published (139). The techniques used today are mostly based on the ABC, or rather the strong affinity between avidin/streptavidin to the vitamin biotin. The detection systems of today sometimes uses dextran backbones with added enzyme complexes, that gives distinct and clean signals (39, 140).

By using well fixed tissue samples, most tissues at our laboratory are fixed for 24-48 h, and employing the "test battery" suggested by Shi et al (51), and a very sensitive detection system for IHC, we found that even after 1 year of storage all the antigens included in the study were possible to detect and diagnose. Even the dreaded p53, which should disappear within a week.

TMA from FFPE material is thus a valuable material for cost effective analysis of biomarkers involved in for example the carcinogenetic process. By using biobanked material from previously performed diagnostic procedures large material can easily be identified. Even though FFPE material, by using modern techniques, can be used for a large range of molecular techniques, availability of prospectively biobanked frozen tissues are still an invaluable asset.

The issue of the potential roles for ERs in lung cancer has many different aspects, diagnostic, epidemiological, methodological, pathophysiological, prognostic as well as therapeutic.

In histopathology, the method of choice to determine the ER status within tumour tissues is by IHC (141). Concerning the diagnostic issue whether an ADCA within the lung is primary or not, our data support the view that nuclear ERα-protein is expressed only in a few cases of NSCLC both in primary lung ADCAs as well as SCC (142-143). Furthermore, in those cases the IHC showed only a weak and restricted staining reaction. In addition, a marker for pulmonary origin, TTF-1, may solve the question in most of the cases even though some cross reactivity have been reported, including tumours which might express ERα (144-145).

Does ERα play a significant role in NSCLC carcinogenesis?

From an epidemiological point of view, data are divergent. A role for estrogen in lung carcinogenesis is supported by a number of epidemiological studies of hormone replacement therapy (HRT) and anti-hormonal treatment (146-151). The use of estrogen replacement therapies have been associated with both increased (147, 150), questionable (152) or decreased (146, 148) risk of lung ADCAs whilst the use of oral contraceptives (146) has been shown to decrease the incidence of lung ADCA.

The methodological aspects of IHC ERs analysis are complex and, of course, crucial concerning this question. The development over the years of antibodies, antigen retrieval techniques and detection system is likely to influence the results. Compared to previous divergent reports our results are comparable with findings using the same anti-ERα primary antibody (142). The ERα antibody used here (SP1) has an excellent performance in the breast carcinoma context with well documented concordance with the effect of anti-estrogen therapy (141, 153). However, using other antibodies the reports are very divergent in NSCLC (Table 1). A high frequency of positive lung ADCAs, with a few cases with strong nuclear staining pattern (154) have been reported using 1D5 mouse monoclonal antibody (mMAb). Similar results have been reported with mMAb 6F11 (155-156). However, low or no ERα expression have been reported using 6F11 (143, 157) or 1D5 (143, 157) even on the same material reported positive using 6F11 (155). Thus, detection of nuclear ERα is dependent upon several methodological factors concerning IHC staining (158), the choice of primary antibody is of outmost importance (142).

Besides technical differences concerning e.g. fixation, antigen retrieval, IHC scoring and intracellular localization, a possible explanation to these divergent data may be that the different ERα antibodies reacts with different part of the ERα molecule (142), the presently used SP1 reacts with C-terminal. The studies reporting higher levels of ERα used antibodies either to either the full-length protein (6F11) or the A/B region located at the N-terminal (1D5). A further observation in this context, is that our study with a monoclonal antibody versus a phosphyrylated site (S-118) within the N-terminal of the ERα molecule showed a frequency of pERα in the range of 1D5 (154).

Thus, IHC detection of ERα is dependent upon IHC method. Even though our data with the SP1 antibody showed a very limited expression other data, including our pERα IHC and gene expression data indicates a more widespread protein presence in NSCLC. Since the SP1 antibody is a good biomarker for anti-ER treatment strategies in breast carcinoma (159) it may more reflect ERα acting in the ligand-dependent genomic pathway rather than the overall presence and activity of cytoplamatic and nuclear ERα. Our data where ERα detected by SP1 Mab correlates to PgR A expression indicates an ongoing ERα ligand dependent action since PgR A is widely accepted as a marker for ERα /ERE dependent transcription (160). Furthermore ERα protein expression correlated to the presence of aromatase within the tumour cells.

Table 1
Summary of earlier results concerning ERα IHC in NSCLC.

Reference	clone	Antigen retrieval		Detection	Histology (n=)	Positiv-ity	Loca-tion
		Buffer	Time (mins)				
Ishibashi et al 2005 [156]	6F11	-	-	ABC	NSCLC (228)	38%	nucl
Kawai et al 2005 [161]	HC-20, rPab	Ci	15	Envision	ADCA (102) SCC (28)	72% 75%	cytopl
Lau et al 2006 [154]	1D5	TE, pH 8.0	20	Envision	ADCA (55)	18%	nucl
Schwartz et al 2005 [157]	1D5 6F11	Ci Ci	- -	ABC ABC	ADCA/SCC ADCA/SCC	0 0	
Dabbs et al 2002 [155]	1D5 6F11	Ci Ci	- -	LSAB LSAB	ADCA (45) ADCA	0 80%	nucl
Gomez-Fernandes et al 2010 [142]	1D5 6F11 SP1	Ci Ci Ci	20 20 20	LSAB LSAB LSAB	ADCA (92) ADCA ADCA	7% 14% 27%	nucl nucl nucl
Radzi Kowska et al 2002 [143]	1D5 6F11	Ci Ci	- -	DAB DAB	ADCA (18) SCC (14)	5% 7%	nucl nucl
Skov et al 2008 [162]	1d5	TE, pH 9.0	15	Envision	NSCLC (104) NSCLC	3% 55%	nucl cytopl

rPab: rabbit polyclonal, Ci: 0.01M citrate buffer, TE: Tris-EDTA buffer, ABC: avidib-biotin complex, LSAB: labelled streptavidib-biotin, DAB, diaminobenzidine, nucl: nuclear, cytopl: cytoplasmatic

Bearing these major discrepancies in IHC staining results between studies in mind, it is not surprising that the prognostic impact of ERα in NSCLC is divergent in the literature. In our material, nuclear ERα expression had a positive prognostic impact, not only restricted to ADCAs nor to the female gender. Nuclear ERα as determined by another primary antibody, 1D5, was however without any prognostic significance (162). In contrast to these studies using yet another antibody and determining cytoplasmatic staining as positive, ERα expression was found to be a negative prognostic factor (161).

Almost all IHC determinations of ERα, as well ERβ, evaluate the nuclear ER but ERs is not only expressed in the nucleus. ERα transcripts are found by us in the majority of NSCLC cases. Earlier studies of non-tumour lung tissues of ESR1 (ERα) and ESR2 (ERβ) mRNA have revealed an extensive expression as well as the presence of transcripts in cell lines (163). A gender difference, concerning tumour tissue, has been shown (164) where tumour tissue from females more frequently expressed ERα transcripts than

males. Data supporting a functional signalling by extra nuclear ERs in lung tissues have been obtained by studying different cell fractions (165). Estrogen induced signalling could be detected both in cytosolic and plasma membrane fractions. Using confocal microscopy (165), the presence of ERα and ERβ in the cell membrane could be demonstrated. Furthermore, cytoplasmatic ERα staining have been reported on frozen sections (166) as well as in paraffin embedded material (149, 162, 166-167). It may thus be that the transcriptional analysis is a better measurement of total ERs in NSCLC.

Here we show in NSCLC, evidence for an in vivo activation by phosphorylation of ERα. IHC demonstration of pERαS118 with a nuclear expression pattern was found in the majority of our cases, biologically indicating a nuclear ERα activation (87).

A crosstalk between ERs signalling and EGFR may take place by several mechanisms (168-169). Our data indicates a role for ERα EGFR crosstalk signalling in SCC. In SCC, EGFR amplification and over expression is frequent (170). Phosphorylation at S118 is not only associated with estradiol interaction but could also be induced by the EGFR/MAPK pathways (171). In the present material, we describe a correlation between EGFR expression and phosphorylation of the ERα receptor at a site which have been associated to EGFR pathway signalling. Even though, the EGFR IHC is hampered by methodological problems (172-174), the described correlation between pERαS118 and pCREB further indicates a ligand independent ERα activation (175).

Thus, a ligand-independent ERα activation may be of importance in SCC tumourigenesis. The independence of gender in pERα detection rate further strengthens the argument that phosphorylation at S118 is mainly EGFR pathway dependent at least in SCC.

In ADCA over expression and gene amplification of EGFR are less frequent but on the other hand activating EGFR mutations can be detected in 10-30 % of ADCA, dependent on ethnical groups studied. Hitherto, EGFR mutations have not been determined in our material, but it would of course be interesting to correlate pERα status to mutational data (4, 176-177).

The presence of aromatase with a female predominance as well as the more frequent expression of ERα in ADCA may reflect that ligand dependent activation is more frequent in the ADCA group contributing a mechanism explaining the female predominance in NSCLC with ADCA morphology (178).

The biological functions for ERβ in lung carcinogenesis remain to be studied in depth (79, 179). In the normal lung, ERβ is expressed in bronchial and alveolar epithelium (180-181). We also found ERβ IHC positivity

in the majority of cases of NSCLC. Because almost all cases were found to express nuclear ERβ neither prognostical impact, nor any gender difference could be demonstrated. Others have found a less extensive expression pattern with a positive prognostic impact in the overall material (161) or among men (157, 162) and especially in combination ERα-/ERβ+ staining pattern (161). ADCAs were also associated with a more frequent ERβ staining pattern (181), especially among men (157). Once again, a number of different antibodies have been used likely contributing to the divergent results (166, 182-183).

As for ESR1, there is widespread transcriptional activity for ESR2 (ERβ). ESR2 transcripts are present in non-tumuoral lung tissues and in cell lines (163). No gender differences have been observed (164).

ERβ has in vitro, stimulatory effects on NSCLC cell lines (184). In vivo, as determined by IHC, ERβ has been correlated to proliferation in female NSCLC (185). We found a proliferation impact of ERβ expression with a positive correlation to proliferation as determined by Cyclin A expression.

ERβ may be associated with tumour differentiation in some ADCAs (186). We observed a higher frequency of ERβ positivity in Stage IA tumours which may indicate a similar mechanism in NSCLC.

ERβ protein expression may be of good prognostic impact in NSCLC (187), especially in male subjects (157, 162). To our knowledge, we report for the first time the prognostic significance of ESR2 transcripts in NSCLC. This effect was strongly associated with ADCAs but not gender specific.

ERβ activation/phosphorylation in NSCLC is not well studied (188). We made an effort to use a commercially available antibody to a phosphorylated form of ERβ on FFPE tissues without any consistent results. In the breast carcinoma situation similar mechanisms of ligand and ligand independent activation have been described and even attributed prognostic impact (189).

ERα and ERβ ligand dependent activation by estradiol may be due to systemic or local ligand synthesis. In the NSCLC perspective a local intra-tumoral synthesis would be of special interest. As discussed previously, a role for ligand dependent ER activation is implied by a number of studies. However, gender and age distribution in NSCLC highlights the question of a local estradiol synthesis.

Local estrogen synthesis may thus influence the tumour development in NSCLC, as has been shown in breast carcinomas (190). Our data support the view of lung carcinoma cells as the site for aromatase expression. We report a gender difference with a significantly more frequent expression of aromatase in female then in male tumours, with expression both in SCC as well as in ADCAs. The substrate for the aromatase driven reaction are

steroids which also may be synthesised in non-gonodal sites as the adrenal and in adipose tissue (190). Aromatase have previously been reported in NSCLC (165, 191) and associated mainly to the tumour cells (192). Intra-tumoral estradiols have also been demonstrated in men and postmeno-pausal females (193). In contrast to a previous study (194) we were not able to show any advert prognostic impact of local aromatase expression. The correlation between aromatase and ERα expression further emphasis the possibility of an active estradiol driven ERα mediated signalling in NSCLC.

These findings could be of interest as explanatory factors also for the observed increasing frequency of ADCAs among smoking men (195) and previous smokers (23). The former tendency is restricted to the west-ern/European population which in general have a good nutritional state with increased average body fat with concomitant increased levels of estra-diol precursors (4).

ER activity is also regulated by a number of co-activators / co-repressors. A further crosstalk mechanism between EGFR family signaling and ERα involves the shredded HER-4 intracellular domain, 4ICD, serving as an ERα co-activator. In vitro studies of 4ICD have described a process where 4ICD, after HER-4 ligand dependent activation, is spliced from the remaining HER-4 molecule (196) and translocates to the nucleus (196). This process is also dependent upon ER activation by estradiol [40]. n4ICD/ERα/ligand complex induces, by binding to ERE, synthesis of a number of proteins, HER-4 included (118).

We demonstrate for the first time the presence of nuclear 4ICD in SCC and ADCA with predominance in the latter group. Furthermore, n4ICD was associated with the concomitant expression of aromatase. As previ-ously discussed, an ongoing ERα induced ERE dependent transcription was also indicated by the finding of PgR expression in the ERα positive tu-mours. Our data may constitute an indication of a functioning HER-4 and ERα cross-talk in NSCLC carcinogenesis, especially in ADCA.

In breast cancer, the prognostic value of n4ICD is debated (197-198). In NSCLC, we did not find any correlation to clinical outcome concerning 4ICD expression. Even though the presence of n4ICD does not seem to be of prognostic significance, the group of n4ICD positive ADCAs has a sig-nificantly lower proliferation then the remaining ADCA group.

Does smoking influence ERs signalling pathways?

Smoking is obviously the main exogenous factor for NSCLC. Life style factors, mainly smoking, have been identified as risk-factors in a number of malignancies. Molecular pathways like K-RAS mutations associated to

smoking in NSCLC have been described. Does smoking influence ERs signalling pathways? Smoking as a risk factor for breast cancer is still debatable (199), data links ERα positive tumours to smoking among premenopausal females (200) and length of smoking (201) as well as to ERα negative tumours (202).

Concerning smoking and ERs in NSCLC data are limited. An epidemiological study (203) describes an advert effect of HRT therapy on overall survival in NSCLC patients, especially among smokers. Biological data are sparse.

We had the opportunity to investigate smoking related changes in our biomarker studies since smoking habits were known in the majority of cases included. Concerning nERα as determined by IHC, a previous study (204) found an overrepresentation of ERα expressing tumours among smokers. In our material a similar trend was observed, but did not reach statistical significance.

Concerning ERβ, we as well as others (205), have not been able to identify any differences due to smoking habits whilst others have found such a correlation (187).

An interesting observation in our material is the differences in ERβ transcriptional levels between smoker and non-smokers. Thus our and others (204) data imply that ERs mediated signalling is of more importance in the smoking induced cases, which also is of interest as a possible explanatory factor for gender differences in the histological panorama of smoking associated NSCLC (23).

In the last decades, the relative frequency of ADCA has risen compared to SCC among smoking men (195) as well as previous smokers (23). Could a role for ERβ signalling be involved in this process? Some data do support such an assumption.

The use of filter cigarettes with low-tar content was introduced some decades ago. This has been shown to change smoking habits towards more deeply inhalation, increased inhalation frequency and volume thus exposing the deeper airways to carcinogenetic agents (206). These mechanisms could be involved in the increased development of peripheral ADCA compared to SCC among men. It might also be of importance concerning the predominance of ADCAs among smoking females since filter low-tar cigarettes was earlier preferred by the female smokers (4, 206). In this context, ERβ receptor signalling is of special interest. In vitro data (184) describes ERβ activation by nicotine-derived nitrosamine. Furthermore, this activation seems to induce ERβ signalling by a non-classical genomic pathway.

n4ICD on the contrary is strongly associated with a never-smoking history in ADCA. n4ICD was also negatively correlated to proliferation.

Among smoking induced ADCA mutations of K-RAS oncogene is prevalent. Speculatively, K-RAS is a potent stimulatory oncogene and may thus contribute more to cell proliferation then mechanisms, including n4ICD, acting in K-RAS negative cases (176, 207).

This indicates a mechanism in NSCLC carcinogenesis among never-smokers that involve ERα induced signalling, including a non-classical pathway signalling where n4ICD plays a part.

In recent years, the interest for different molecular profiles within especially the ADCA group has been raised. EGFR mutations have been identified to correlate to a never-smoking history of NSCLC patients as well as with ADCA morphology and female gender (177). We found n4ICD to correlate to ADCA morphology. In ADCA, n4ICD was also more frequently found among never-smokers. The same group of never-smokers may also more frequently express ERα (204).

Can ERs and/or 4ICD serve as therapeutic targets and biomarkers in NSCLC?

Some of our findings may also be of interest in the clinical context of personalized medicine. The role for EGFR intervention in NSCLC is well established (6). Epidemiological as well as experimental studies support the presence and function of ERs in NSCLC. From epidemiological studies some data indicate ERs as a potential therapeutic target. The selective estrogen receptor modulator (SERM) tamoxifen reduces the mortality, but not the incidence, of NSCLC in a recent large epidemiological study (159). Adding tamoxifen to conventional pharmacological treatment (208) did not contribute to treatment outcome; however, no correlation to ER status was performed.

In the breast carcinoma situation ERα (141, 153) serves as biomarkers for the sensitivity of anti-estrogen substances, either SERM or aromatase inhibitor (AI). Furthermore, ERβ is a marker for sensitivity tamoxifen in ERα negative breast carcinomas (209). Also in the breast carcinoma situation, n4ICD is a better marker then ERα IHC for sensitivity to Tamoxifen (210).

Reports describing an in vitro effect of both Tamoxifen (211) and of anti-aromatase substances (212-213) in NSCLC are of course of interest in this context. Another interesting observation is that inhibition of aromatase, also have an in vitro anti-tumoral effect in NSCLC (214). Using an animal model, an inhibitory effect of tumour progression was observed when supplying the animals with an AI (212-213).

Since there may be EGFR-ERs crosstalk in NSCLC in vitro report of SERM/AI and EGFR inhibitors are of special interest. An additative effect

of EGFR and ER inhibition of the proliferation of NSCLC cell lines have been demonstrated (211) which in cell lines studies were limited to ADCA cell lines, at least one of which bearing a EGFR mutation. A case report describing excellent results of combined AI and EGFR intervention has been published (215).

Thus, no firm evidence has yet been established for the potential role for ERs associated intervention in NSCLC.

Conclusion

In the present thesis methodological aspects of NSCLC studies have been discussed. TMA studies can serve as a convenient model for biomarker studies using a limited sampling size of a few TMA cylinders. TMA material can cost-effectively be used and optimally handled be serial cutting and long term storage of slides, both for protein detection by up to date IHC methods and for in situ detection of both mRNA and DNA.

Using material from a clinical biobank, FFPE and fresh frozen tissues were used to elucidate factors contributing to the gender associated differences in NSCLC morbidity. Once again, methodological questions concerning ERs determination are high-lighted.

The present data support a role for ERs in NSCLC and describes some aspects of ERs signalling that may contribute to differences in NSCLC between gender and smokers and never-smokers.

ERα nuclear expression as determined by IHC correlates to PgR A and aromatase expression indicating a ligand/ERE dependent mechanism, associated to female gender and ADCA morphology. ERα is also a biomarker for favourable prognosis in NSCLC. A local estradiol synthesis is supported by the presence of aromatase. Aromatase is more frequently expressed among females and in ADCA.

ERα activity as determined by phosporylation is widespread in NSCLC and pERα is associated with EGFR over expression in SCC.

ERβ nuclear expression by IHC is associated with smaller (Stage IA) tumours of ADCA morphology and of male gender. ESR2 (ERβ) transcripts are present at higher levels among smokers then never-smokers. An ESR2 (ERβ) transcript is a favourable prognostic indicator in ADCA. The ERβ data may indicate a role for ERβ signalling contributing to the trend of increased ADCA morphology among male smokers.

A new mechanism for EGFR family and ERα crosstalk in NSCLC is described. n4ICD which can act as a co activator to ERα recruiting and translocating 4ICD/ERα complex to the nucleus is detected in NSCLC, especially in ADCA. Furthermore, this is observed most frequently among never-smokers, a group in which where ADCA morphology prevails.

Thus, a number of observations indicate the potential role ERs signalling in NSCLC. Accumulating evidence support a role for intervention in ERs signalling by SERM or AI, especially in combination with EGFR inhibitors in NSCLC treatment. If and when such clinical studies will be performed it is of outmost importance to evaluate which biomarkers within the ERs-EGFR network that can serve as potential predictors of treatment outcome and to establish solid and reproducible methods for biomarker analysis.

Acknowledgements

This study was performed at the School of Health and Medical Sciences at Örebro University. All support by the Deptartment of Biomedicine at Örebro University and the Department of Laboratory Medicine / Pathology is gratefully acknowledged.

I wish to express my sincere gratitude to all friends and colleagues who have made this thesis possible by supporting me in different ways, especially:

Karin Piehl-Aulin, my first supervisor, who supported me even though my interests was way out of line from your own research.

Ulf Tidefelt , my second supervisor, for continuous support.

Gisela Helenius, my co-supervisor for introducing me into the world of molecular genetics, and making me take up knitting again.

Lennart Bodin, my co-author, for valuable statistic advice.

IngBritt Ryding, who once upon a time employed me at the Dept of Pathology (it meant a lot in my life).

Björn Risberg, for introducing me into the world of research.

Siw Lunander, my "boss" for making it possible for me to make this journey.

Aleksandra Kolaric, for friendship and FISH, we had a great time in Münster, also learning some things about microscopy.

Gabriella Lillsunde-Larsson, for always being there listening to everything (mostly moaning), I will do the same for you.

All my colleagues, past and present, at the School of Health and Medical Sciences

Friends and relatives for support

My family, for always being there. Including my last supervisor, **Mats.** I love you!

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011 Mar-Apr;61(2):69-90.

2. . The National Board of Helath and Welfare. 2010.

3. Witschi H. A short history of lung cancer. Toxicol Sci. 2001 Nov;64(1):4-6.

4. Travis WD BE, Müller-Hermelink HK, Harris CC, editor. Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart. Lyon: IARC Press; 2004.

5. ROC. Vårdprogram för lungcancer och mesoteliom. 2010.

6. Hirsch F, Harper P, editors. Lung Cancer. London: Remedica; 2009.

7. Rubin R, Strayer D. Rubin´s Pathology Clinocopathologic Foundations of Medicine. 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2011.

8. Subramanian J, Govindan R. Lung cancer in never smokers: a review. J Clin Oncol. 2007 Feb 10;25(5):561-70.

9. Pardo J, Martinez-Penuela AM, Sola JJ, Panizo A, Gurpide A, Martinez-Penuela JM, et al. Large cell carcinoma of the lung: an endangered species? Appl Immunohistochem Mol Morphol. 2009 Oct;17(5):383-92.

10. Kargi A, Gurel D, Tuna B. The diagnostic value of TTF-1, CK 5/6, and p63 immunostaining in classification of lung carcinomas. Appl Immunohistochem Mol Morphol. 2007 Dec;15(4):415-20.

11. Sobin L, Gospodarowicz M, Wittekind, editors. TMN Classification of Malignant Tumours, 7th edition. NewYork: Wiley-Liss; 2009.

12. Bray FI, Weiderpass E. Lung cancer mortality trends in 36 European countries: secular trends and birth cohort patterns by sex and region 1970-2007. Int J Cancer. 2010 Mar 15;126(6):1454-66.

13. Youlden DR, Cramb SM, Baade PD. The International Epidemiology of Lung Cancer: geographical distribution and secular trends. J Thorac Oncol. 2008 Aug;3(8):819-31.

14. Ezzati M, Henley SJ, Lopez AD, Thun MJ. Role of smoking in global and regional cancer epidemiology: current patterns and data needs. Int J Cancer. 2005 Oct 10;116(6):963-71.

15. Ezzati M, Lopez AD. Estimates of global mortality attributable to smoking in 2000. Lancet. 2003 Sep 13;362(9387):847-52.

16. Weir HK, Thun MJ, Hankey BF, Ries LA, Howe HL, Wingo PA, et al. Annual report to the nation on the status of cancer, 1975-2000, featuring the uses of surveillance data for cancer prevention and control. J Natl Cancer Inst. 2003 Sep 3;95(17):1276-99.

17. Muller F. Tabakmissbrauch und Lungencarcinom [Tobacco abuse and lung cancer]. . Krebsforsch Z. 1939;49:57-85.

18. Muller F. Abuse of Tobacco and Carcinoma of Lungs. The journal of the american medical association. 1939;113(14):1372.

19. Doll R, Hill AB. The mortality of doctors in relation to their smoking habits; a preliminary report. Br Med J. 1954 Jun 26;1(4877):1451-5.

20. Doll R, Hill AB. Lung cancer and other causes of death in relation to smoking; a second report on the mortality of British doctors. Br Med J. 1956 Nov 10;2(5001):1071-81.

21. Doll R, Hill AB. Smoking and carcinoma of the lung; preliminary report. Br Med J. 1950 Sep 30;2(4682):739-48.

22. Wynder EL, Graham EA. Tobacco smoking as a possible etiologic factor in bronchiogenic carcinoma; a study of 684 proved cases. J Am Med Assoc. 1950 May 27;143(4):329-36.

23. Thun MJ, Lally CA, Flannery JT, Calle EE, Flanders WD, Heath CW, Jr. Cigarette smoking and changes in the histopathology of lung cancer. J Natl Cancer Inst. 1997 Nov 5;89(21):1580-6.

24. Bancroft J, Gamble M, editors. Theory and Practice of Histological Techniques. 6th ed. Philadelphia: Churchill Livinstone Elsevier; 2008.

25. Dillner J, editor. Methods in biobanking. newYork: Humana Press; 2011.

26. Litlekalsoy J, Vatne V, Hostmark JG, Laerum OD. Immunohistochemical markers in urinary bladder carcinomas from paraffin-embedded archival tissue after storage for 5-70 years. BJU International. 2007 May;99(5):1013-9.

27. Shibata D, Martin WJ, Arnheim N. Analysis of DNA sequences in forty-year-old paraffin-embedded thin-tissue sections: a bridge between molecular biology and classical histology. Cancer Research. 1988 Aug 15;48(16):4564-6.

28. Fraenkel-Conrat H, Mecham DK. The reaction of formaldehyde with proteins; demonstration of intermolecular cross-linking by means of osmotic pressure measurements. J Biol Chem. 1949 Jan;177(1):477-86.

29. Fraenkel-Conrat H, Olcott HS. Reaction of formaldehyde with proteins; cross-linking of amino groups with phenol, imidazole, or indole groups. J Biol Chem. 1948 Jul;174(3):827-43.

30. Fraenkel-Conrat H, Olcott HS. The reaction of formaldehyde with proteins; cross-linking between amino and primary amide or guanidyl groups. J Am Chem Soc. 1948 Aug;70(8):2673-84.

31. Singer SJ. The properties of proteins in nonaqueous solvents. Advances in protein chemistry. 1963;17:1-68.

32. Means GE, Feeney RE. Reductive alkylation of proteins. Anal Biochem. 1995 Jan 1;224(1):1-16.

33. McGhee JD, von Hippel PH. Formaldehyde as a probe of DNA structure. II. Reaction with endocyclic imino groups of DNA bases. Biochemistry. 1975 Mar 25;14(6):1297-303.

34. McGhee JD, von Hippel PH. Formaldehyde as a probe of DNA structure. I. Reaction with exocyclic amino groups of DNA bases. Biochemistry. 1975 Mar 25;14(6):1281-96.

35. McGhee JD, von Hippel PH. Formaldehyde as a probe of DNA structure. 3. Equilibrium denaturation of DNA and synthetic polynucleotides. Biochemistry. 1977 Jul 26;16(15):3267-76.

36. McGhee JD, von Hippel PH. Formaldehyde as a probe of DNA structure. r. Mechanism of the initial reaction of Formaldehyde with DNA. Biochemistry. 1977 Jul 26;16(15):3276-93.

37. Al-Mulla F, editor. Formalin-Fixed and Paraffin-Embedded Tissues Methods and protocols. London: Humana Press; 2011.

38. Fox CH, Johnson FB, Whiting J, Roller PP. Formaldehyde fixation. Journal of Histochemistry and Cytochemistry. 1985 Aug;33(8):845-53.

39. Kumar G, Rudbeck L, editors. Immunohistochemical (IHC) Staining Methods Education Guide. Carpinteria: Dako North America; 2009.

40. Coons A, Creech H, Jones N, Berliner E. The Demonstration og Pneumococcal Antigen in Tissues by the Use of Fluorescent Antibody. The Journal of Immunology. 1942;45(3):159-70.

41. Nakane PK, Pierce GB, Jr. Enzyme-labeled antibodies: preparation and application for the localization of antigens. J Histochem Cytochem. 1966 Dec;14(12):929-31.

42. Skaland I, Nordhus M, Gudlaugsson E, Klos J, Kjellevold KH, Janssen EA, et al. Evaluation of 5 different labeled polymer immunohistochemical detection systems. Appl Immunohistochem Mol Morphol. 2010 Jan;18(1):90-6.

43. Gruver AM, Peerwani Z, Tubbs RR. Out of the darkness and into the light: bright field in situ hybridisation for delineation of ERBB2 (HER2) status in breast carcinoma. J Clin Pathol. 2010 Mar;63(3):210-9.

44. Tanner M, Gancberg D, Di Leo A, Larsimont D, Rouas G, Piccart MJ, et al. Chromogenic in situ hybridization: a practical alternative for fluorescence in situ hybridization to detect HER-2/neu oncogene amplification in archival breast cancer samples. Am J Pathol. 2000 Nov;157(5):1467-72.

45. Leong AS, Milios J, Duncis CG. Antigen preservation in microwave-irradiated tissues: a comparison with formaldehyde fixation. J Pathol. 1988 Dec;156(4):275-82.

46. Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem. 1991 Jun;39(6):741-8.

47. Leong AS-Y, Milios J. Comparison of antibodies to oestrogen and progesterone receptors and the influence on microwave-antigen retrieval. Appl Immunohistochem. 1993;1:2-88.

48. Gown A, de Wever N, Beattifora H. Microwave-based antigenic unmasking: a revolutionary new technique eor routine immunohistochemistry. Appl Immunohistochem. 1993;1:259-66.

49. Fraenkel-Conrat H, Brandon BA, Olcott HS. The reaction of formaldehyde with proteins; participation of indole groups; gramicidin. J Biol Chem. 1947 Apr;168(1):99-118.

50. Fraenkel-Conrat H, Brandon BA, Olcott HS. The reaction of formaldehyde with proteins. IV. Participation of indole groups. Gramicidin. J Biol Chem. 1947;168:99-118.

51. Shi S-R, Taylor C, editors. Antigen Retrieval Immunohistochemistry Based Research and Diagnostics. Hoboken: John Wiley & Sons Inc; 2010.

52. Yamashita S. Heat-induced antigen retrieval: mechanisms and application to histochemistry. Progress in Histochemistry and Cytochemistry. 2007;41(3):141-200.

53. D'Amico F, Skarmoutsou E, Stivala F. State of the art in antigen retrieval for immunohistochemistry. Journal of Immunological Methods. 2009 Feb 28;341(1-2):1-18.

54. DiVito KA, Charette LA, Rimm DL, Camp RL. Long-term preservation of antigenicity on tissue microarrays. Lab Invest. 2004 Aug;84(8):1071-8.

55. Fergenbaum JH, Garcia-Closas M, Hewitt SM, Lissowska J, Sakoda LC, Sherman ME. Loss of antigenicity in stored sections of breast cancer tissue microarrays. Cancer Epidemiol Biomarkers Prev. 2004 Apr;13(4):667-72.

56. Jacobs TW, Prioleau JE, Stillman IE, Schnitt SJ. Loss of tumor marker-immunostaining intensity on stored paraffin slides of breast cancer. J Natl Cancer Inst. 1996 Aug 7;88(15):1054-9.

57. Prioleau J, Schnitt SJ. p53 antigen loss in stored paraffin slides. N Engl J Med. 1995 Jun 1;332(22):1521-2.

58. Bertheau P, Cazals-Hatem D, Meignin V, de Roquancourt A, Verola O, Lesourd A, et al. Variability of immunohistochemical reactivity on stored paraffin slides. J Clin Pathol. 1998 May;51(5):370-4.

59. Shin HJ, Kalapurakal SK, Lee JJ, Ro JY, Hong WK, Lee JS. Comparison of p53 immunoreactivity in fresh-cut versus stored slides with and without microwave heating. Mod Pathol. 1997 Mar;10(3):224-30.

60. Burry RW. Controls for immunocytochemistry: an update. J Histochem Cytochem. 2011 Jan;59(1):6-12.

61. Simon R, editor. Tissue Microarrays Methods and Protocols. London: Humana Press; 2010.

62. Eguiluz C, Viguera E, Millan L, Perez J. Multitissue array review: a chronological description of tissue array techniques, applications and procedures. Pathology, Research and Practice. 2006;202(8):561-8.

63. Rosen DG, Huang X, Deavers MT, Malpica A, Silva EG, Liu J. Validation of tissue microarray technology in ovarian carcinoma. Mod Pathol. 2004 Jul;17(7):790-7.

64. Battifora H, Kopinski M. The influence of protease digestion and duration of fixation on the immunostaining of keratins. A comparison of formalin and ethanol fixation. J Histochem Cytochem. 1986 Aug;34(8):1095-100.

65. Kraaz W, Risberg B, Hussein A. Multiblock: an aid in diagnostic immunohistochemistry. J Clin Pathol. 1988 Dec;41(12):1337.

66. Wan WH, Fortuna MB, Furmanski P. A rapid and efficient method for testing immunohistochemical reactivity of monoclonal antibodies against multiple tissue samples simultaneously. J Immunol Methods. 1987 Oct 23;103(1):121-9.

67. Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nature Medicine. 1998 Jul;4(7):844-7.

68. Giltnane JM, Rimm DL. Technology insight: Identification of biomarkers with tissue microarray technology. Nat Clin Pract Oncol. 2004 Dec;1(2):104-11.

69. Camp RL, Neumeister V, Rimm DL. A decade of tissue microarrays: progress in the discovery and validation of cancer biomarkers. Journal of Clinical Oncology. 2008 Dec 1;26(34):5630-7.

70. Torhorst J, Bucher C, Kononen J, Haas P, Zuber M, Kochli OR, et al. Tissue microarrays for rapid linking of molecular changes to clinical endpoints. American Journal of Pathology. 2001 Dec;159(6):2249-56.

71. Nagy L, Schule R, Gronemeyer H. Twenty years of nuclear receptors: Conference on Nuclear Receptors: from Chromatin to Disease. EMBO Rep. 2006 Jun;7(6):579-84.

72. A unified nomenclature system for the nuclear receptor superfamily. Cell. 1999 Apr 16;97(2):161-3.

73. Kumar R, Thompson EB. The structure of the nuclear hormone receptors. Steroids. 1999 May;64(5):310-9.

74. Bourguet W, Germain P, Gronemeyer H. Nuclear receptor ligand-binding domains: three-dimensional structures, molecular interactions and pharmacological implications. Trends Pharmacol Sci. 2000 Oct;21(10):381-8.

75. Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ. Nuclear receptors and lipid physiology: opening the X-files. Science. 2001 Nov 30;294(5548):1866-70.

76. Wahli W, Martinez E. Superfamily of steroid nuclear receptors: positive and negative regulators of gene expression. FASEB J. 1991 Jun;5(9):2243-9.

77. Francis GA, Fayard E, Picard F, Auwerx J. Nuclear receptors and the control of metabolism. Annu Rev Physiol. 2003;65:261-311.

78. Smirnov AN. Nuclear receptors: nomenclature, ligands, mechanisms of their effects on gene expression. Biochemistry (Mosc). 2002 Sep;67(9):957-77.

79. Chen GG, Zeng Q, Tse GM. Estrogen and its receptors in cancer. Med Res Rev. 2008 Nov;28(6):954-74.

80. Flouriot G, Brand H, Denger S, Metivier R, Kos M, Reid G, et al. Identification of a new isoform of the human estrogen receptor-alpha (hER-alpha) that is encoded by distinct transcripts and that is able to repress hER-alpha activation function 1. EMBO J. 2000 Sep 1;19(17):4688-700.

81. Kos M, Reid G, Denger S, Gannon F. Minireview: genomic organization of the human ERalpha gene promoter region. Mol Endocrinol. 2001 Dec;15(12):2057-63.

82. Evans RM. The steroid and thyroid hormone receptor superfamily. Science. 1988 May 13;240(4854):889-95.

83. Green S, Walter P, Greene G, Krust A, Goffin C, Jensen E, et al. Cloning of the human oestrogen receptor cDNA. J Steroid Biochem. 1986 Jan;24(1):77-83.

84. Ogawa S, Inoue S, Watanabe T, Hiroi H, Orimo A, Hosoi T, et al. The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha in vivo and in vitro. Biochem Biophys Res Commun. 1998 Feb 4;243(1):122-6.

85. Mosselman S, Polman J, Dijkema R. ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett. 1996 Aug 19;392(1):49-53.

86. Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, et al. Human estrogen receptor beta-gene structure,

chromosomal localization, and expression pattern. J Clin Endocrinol Metab. 1997 Dec;82(12):4258-65.

87. Pearce ST, Jordan VC. The biological role of estrogen receptors alpha and beta in cancer. Crit Rev Oncol Hematol. 2004 Apr;50(1):3-22.

88. Roman-Blas JA, Castaneda S, Largo R, Herrero-Beaumont G. Osteoarthritis associated with estrogen deficiency. Arthritis Res Ther. 2009;11(5):241.

89. Hall JM, McDonnell DP. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology. 1999 Dec;140(12):5566-78.

90. Ellmann S, Sticht H, Thiel F, Beckmann MW, Strick R, Strissel PL. Estrogen and progesterone receptors: from molecular structures to clinical targets. Cell Mol Life Sci. 2009 Aug;66(15):2405-26.

91. Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, et al. Mechanisms of estrogen action. Physiol Rev. 2001 Oct;81(4):1535-65.

92. Rosenfeld MG, Glass CK. Coregulator codes of transcriptional regulation by nuclear receptors. J Biol Chem. 2001 Oct 5;276(40):36865-8.

93. Bjornstrom L, Sjoberg M. Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Mol Endocrinol. 2005 Apr;19(4):833-42.

94. Gottlicher M, Heck S, Herrlich P. Transcriptional cross-talk, the second mode of steroid hormone receptor action. J Mol Med. 1998 Jun;76(7):480-9.

95. Chen D, Pace PE, Coombes RC, Ali S. Phosphorylation of Human Estrogen Receptor α by Protein Kinase A Regulates Dimerization. Mol Cell Biol. 1999 Feb;19(2):1002-15.

96. Lannigan DA. Estrogen receptor phosphorylation. Steroids. 2003 Jan;68(1):1-9.

97. Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S, Nakshatri H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. J Biol Chem. 2001 Mar 30;276(13):9817-24.

98. Ascenzi P, Bocedi A, Marino M. Structure-function relationship of estrogen receptor alpha and beta: impact on human health. Mol Aspects Med. 2006 Aug;27(4):299-402.

99. Russo J, Russo IH. The role of estrogen in the initiation of breast cancer. J Steroid Biochem Mol Biol. 2006 Dec;102(1-5):89-96.

100. Sebastian S, Bulun SE. A highly complex organization of the regulatory region of the human CYP19 (aromatase) gene revealed by the Human Genome Project. J Clin Endocrinol Metab. 2001 Oct;86(10):4600-2.

101. Simpson ER, Clyne C, Rubin G, Boon WC, Robertson K, Britt K, et al. Aromatase--a brief overview. Annu Rev Physiol. 2002;64:93-127.

102. Hanekamp EE, Gielen SC, Smid-Koopman E, Kuhne LC, de Ruiter PE, Chadha-Ajwani S, et al. Consequences of loss of progesterone receptor expression in development of invasive endometrial cancer. Clin Cancer Res. 2003 Sep 15;9(11):4190-9.

103. Gadkar-Sable S, Shah C, Rosario G, Sachdeva G, Puri C. Progesterone receptors: various forms and functions in reproductive tissues. Front Biosci. 2005;10:2118-30.

104. Leonhardt SA, Boonyaratanakornkit V, Edwards DP. Progesterone receptor transcription and non-transcription signaling mechanisms. Steroids. 2003 Nov;68(10-13):761-70.

105. Conneely OM, Mulac-Jericevic B, Lydon JP. Progesterone-dependent regulation of female reproductive activity by two distinct progesterone receptor isoforms. Steroids. 2003 Nov;68(10-13):771-8.

106. Freedman LP. Anatomy of the steroid receptor zinc finger region. Endocr Rev. 1992 May;13(2):129-45.

107. Hubbard SR, Till JH. Protein tyrosine kinase structure and function. Annu Rev Biochem. 2000;69:373-98.

108. Bublil EM, Yarden Y. The EGF receptor family: spearheading a merger of signaling and therapeutics. Curr Opin Cell Biol. 2007 Apr;19(2):124-34.

109. Zwick E, Bange J, Ullrich A. Receptor tyrosine kinase signalling as a target for cancer intervention strategies. Endocr Relat Cancer. 2001 Sep;8(3):161-73.

110. Fischer EH. Cell signaling by protein tyrosine phosphorylation. Adv Enzyme Regul. 1999;39:359-69.

111. Grandis JR, Sok JC. Signaling through the epidermal growth factor receptor during the development of malignancy. Pharmacol Ther. 2004 Apr;102(1):37-46.

112. Baiocchi G, Lopes A, Coudry RA, Rossi BM, Soares FA, Aguiar S, et al. ErbB family immunohistochemical expression in colorectal cancer patients with higher risk of recurrence after radical surgery. Int J Colorectal Dis. 2009 Sep;24(9):1059-68.

113. Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. Nat Rev Mol Cell Biol. 2006 Jul;7(7):505-16.

114. Harris RC, Chung E, Coffey RJ. EGF receptor ligands. Exp Cell Res. 2003 Mar 10;284(1):2-13.

115. Carpenter G. ErbB-4: mechanism of action and biology. Exp Cell Res. 2003 Mar 10;284(1):66-77.

116. Elenius K, Corfas G, Paul S, Choi CJ, Rio C, Plowman GD, et al. A novel juxtamembrane domain isoform of HER4/ErbB4. Isoform-specific tissue distribution and differential processing in response to phorbol ester. J Biol Chem. 1997 Oct 17;272(42):26761-8.

117. Jones FE. HER4 intracellular domain (4ICD) activity in the developing mammary gland and breast cancer. J Mammary Gland Biol Neoplasia. 2008 Jun;13(2):247-58.

118. Zhu Y, Sullivan LL, Nair SS, Williams CC, Pandey AK, Marrero L, et al. Coregulation of estrogen receptor by ERBB4/HER4 establishes a growth-promoting autocrine signal in breast tumor cells. Cancer Res. 2006 Aug 15;66(16):7991-8.

119. Linggi B, Cheng QC, Rao AR, Carpenter G. The ErbB-4 s80 intracellular domain is a constitutively active tyrosine kinase. Oncogene. 2006 Jan 5;25(1):160-3.

120. Karlsson C, Bodin L, Piehl-Aulin K, Karlsson MG. Tissue microarray validation: a methodologic study with special reference to lung cancer. Cancer Epidemiol Biomarkers Prev. 2009 Jul;18(7):2014-21.

121. Dunn G. Design and analysis of reliable studies. New York: Oxford University Press; 1989.

122. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet. 1986 Feb 8;1(8476):307-10.

123. Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics. 1977 Mar;33(1):159-74.

124. Fleiss J, Levin B, Paik M. Statistical Methods for Rates and Proportions. Third ed. New York: Wiley-Interscience; 2003.

125. Bozzetti C, Franciosi V, Crafa P, Carbognani P, Rusca M, Nizzoli R, et al. Biological variables in non-small cell lung cancer: comparison between immunocytochemical determination on fine needle aspirates from surgical specimens and immunohistochemical determination on tissue sections. Lung Cancer. 2000 Jul;29(1):33-41.

126. Badve SS, Baehner FL, Gray RP, Childs BH, Maddala T, Liu ML, et al. Estrogen- and progesterone-receptor status in ECOG 2197: comparison of immunohistochemistry by local and central laboratories and quantitative reverse transcription polymerase chain reaction by central laboratory. J Clin Oncol. 2008 May 20;26(15):2473-81.

127. Choi PJ, Yang DK, Son CH, Lee KE, Lee JI, Roh MS. Fascin immunoreactivity for preoperatively predicting lymph node metastases in peripheral adenocarcinoma of the lung 3 cm or less in diameter. Eur J Cardiothorac Surg. 2006 Sep;30(3):538-42.

128. Donati V, Faviana P, Dell'omodarme M, Prati MC, Camacci T, De Ieso K, et al. Applications of tissue microarray technology in immunohistochemistry: a study on c-kit expression in small cell lung cancer. Hum Pathol. 2004 Nov;35(11):1347-52.

129. Hoos A, Urist MJ, Stojadinovic A, Mastorides S, Dudas ME, Leung DH, et al. Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors. Am J Pathol. 2001 Apr;158(4):1245-51.

130. Rubin MA, Dunn R, Strawderman M, Pienta KJ. Tissue microarray sampling strategy for prostate cancer biomarker analysis. Am J Surg Pathol. 2002 Mar;26(3):312-9.

131. Zhang D, Salto-Tellez M, Putti TC, Do E, Koay ES. Reliability of tissue microarrays in detecting protein expression and gene amplification in breast cancer. Mod Pathol. 2003 Jan;16(1):79-84.

132. Fons G, Hasibuan SM, van der Velden J, ten Kate FJ. Validation of tissue microarray technology in endometrioid cancer of the endometrium. J Clin Pathol. 2007 May;60(5):500-3.

133. Shi SR, Liu C, Taylor CR. Standardization of immunohistochemistry for formalin-fixed, paraffin-embedded tissue sections based on the antigen-retrieval technique: from experiments to hypothesis. Journal of Histochemistry and Cytochemistry. 2007 Feb;55(2):105-9.

134. Buesa RJ. Histology without formalin? Ann Diagn Pathol. 2008 Dec;12(6):387-96.

135. Werner M, Chott A, Fabiano A, Battifora H. Effect of formalin tissue fixation and processing on immunohistochemistry. Am J Surg Pathol. 2000 Jul;24(7):1016-9.

136. De Marzo AM, Fedor HH, Gage WR, Rubin MA. Inadequate formalin fixation decreases reliability of p27 immunohistochemical staining: probing optimal fixation time using high-density tissue microarrays. Hum Pathol. 2002 Jul;33(7):756-60.

137. Wester K, Wahlund E, Sundstrom C, Ranefall P, Bengtsson E, Russell PJ, et al. Paraffin section storage and immunohistochemistry. Effects of time, temperature, fixation, and retrieval protocol with emphasis on p53 protein and MIB1 antigen. Appl Immunohistochem Mol Morphol. 2000 Mar;8(1):61-70.

138. Sternberger LA, Hardy PH, Jr., Cuculis JJ, Meyer HG. The unlabeled antibody enzyme method of immunohistochemistry: preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem. 1970 May;18(5):315-33.

139. Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem. 1981 Apr;29(4):577-80.

140. Skaland I, Nordhus M, Gudlaugsson E, Klos J, Kjellevold KH, Janssen EA, et al. Evaluation of 5 different labeled polymer immunohistochemical detection systems. 2010 Jan.

141. Yaziji H, Taylor CR, Goldstein NS, Dabbs DJ, Hammond EH, Hewlett B, et al. Consensus recommendations on estrogen receptor testing in breast cancer by immunohistochemistry. Appl Immunohistochem Mol Morphol. 2008 Dec;16(6):513-20.

142. Gomez-Fernandez C, Mejias A, Walker G, Nadji M. Immunohistochemical expression of estrogen receptor in

adenocarcinomas of the lung: the antibody factor. Appl Immunohistochem Mol Morphol. 2010 Mar;18(2):137-41.

143. Radzikowska E, Langfort R, Giedronowicz D. Estrogen and progesterone receptors in non small cell lung cancer patients. Ann Thorac Cardiovasc Surg. 2002 Apr;8(2):69-73.

144. Cameselle-Teijeiro J, Abdulkader I, Piso-Neira M, Reyes-Santias R, Alfonsin-Barreiro N. Thyroid transcription factor-1 expression in endometrioid tumors: a note of caution. Hum Pathol. 2011 Jul;42(7):1053-5; author reply 5-6.

145. Matoso A, Singh K, Jacob R, Greaves WO, Tavares R, Noble L, et al. Comparison of thyroid transcription factor-1 expression by 2 monoclonal antibodies in pulmonary and nonpulmonary primary tumors. Appl Immunohistochem Mol Morphol. 2010 Mar;18(2):142-9.

146. Kreuzer M, Gerken M, Heinrich J, Kreienbrock L, Wichmann HE. Hormonal factors and risk of lung cancer among women? Int J Epidemiol. 2003 Apr;32(2):263-71.

147. Liu Y, Inoue M, Sobue T, Tsugane S. Reproductive factors, hormone use and the risk of lung cancer among middle-aged never-smoking Japanese women: a large-scale population-based cohort study. Int J Cancer. 2005 Nov 20;117(4):662-6.

148. Schabath MB, Wu X, Vassilopoulou-Sellin R, Vaporciyan AA, Spitz MR. Hormone replacement therapy and lung cancer risk: a case-control analysis. Clin Cancer Res. 2004 Jan 1;10(1 Pt 1):113-23.

149. Schwartz AG, Wenzlaff AS, Prysak GM, Murphy V, Cote ML, Brooks SC, et al. Reproductive factors, hormone use, estrogen receptor expression and risk of non small-cell lung cancer in women. J Clin Oncol. 2007 Dec 20;25(36):5785-92.

150. Taioli E, Wynder EL. Re: Endocrine factors and adenocarcinoma of the lung in women. J Natl Cancer Inst. 1994 Jun 1;86(11):869-70.

151. Wu AH, Yu MC, Thomas DC, Pike MC, Henderson BE. Personal and family history of lung disease as risk factors for adenocarcinoma of the lung. Cancer Res. 1988 Dec 15;48(24 Pt 1):7279-84.

152. Adami HO, Persson I, Hoover R, Schairer C, Bergkvist L. Risk of cancer in women receiving hormone replacement therapy. Int J Cancer. 1989 Nov 15;44(5):833-9.

153. Cheang MC, Treaba DO, Speers CH, Olivotto IA, Bajdik CD, Chia SK, et al. Immunohistochemical detection using the new rabbit monoclonal antibody SP1 of estrogen receptor in breast cancer is superior to mouse monoclonal antibody 1D5 in predicting survival. J Clin Oncol. 2006 Dec 20;24(36):5637-44.

154. Lau SK, Chu PG, Weiss LM. Immunohistochemical expression of estrogen receptor in pulmonary adenocarcinoma. Appl Immunohistochem Mol Morphol. 2006 Mar;14(1):83-7.

155. Dabbs DJ, Landreneau RJ, Liu Y, Raab SS, Maley RH, Tung MY, et al. Detection of estrogen receptor by immunohistochemistry in pulmonary adenocarcinoma. Ann Thorac Surg. 2002 Feb;73(2):403-5; discussion 6.

156. Ishibashi H, Suzuki T, Suzuki S, Niikawa H, Lu L, Miki Y, et al. Progesterone receptor in non-small cell lung cancer--a potent prognostic factor and possible target for endocrine therapy. Cancer Res. 2005 Jul 15;65(14):6450-8.

157. Schwartz AG, Prysak GM, Murphy V, Lonardo F, Pass H, Schwartz J, et al. Nuclear estrogen receptor beta in lung cancer: expression and survival differences by sex. Clin Cancer Res. 2005 Oct 15;11(20):7280-7.

158. Goldstein NS, Hunter S, Forbes S, Odish E, Tehrani M. Estrogen receptor antibody incubation time and extent of immunoreactivity in invasive carcinoma of the breast: the importance of optimizing antibody avidity. Appl Immunohistochem Mol Morphol. 2007 Jun;15(2):203-7.

159. Bouchardy C, Benhamou S, Schaffar R, Verkooijen HM, Fioretta G, Schubert H, et al. Lung cancer mortality risk among breast cancer patients treated with anti-estrogens. Cancer. 2011 Mar 15;117(6):1288-95.

160. Boney-Montoya J, Ziegler YS, Curtis CD, Montoya JA, Nardulli AM. Long-range transcriptional control of progesterone receptor gene expression. Mol Endocrinol. 2010 Feb;24(2):346-58.

161. Kawai H, Ishii A, Washiya K, Konno T, Kon H, Yamaya C, et al. Estrogen receptor alpha and beta are prognostic factors in non-small cell lung cancer. Clin Cancer Res. 2005 Jul 15;11(14):5084-9.

162. Skov BG, Fischer BM, Pappot H. Oestrogen receptor beta over expression in males with non-small cell lung cancer is associated with better survival. Lung Cancer. 2008 Jan;59(1):88-94.

163. Mollerup S, Jorgensen K, Berge G, Haugen A. Expression of estrogen receptors alpha and beta in human lung tissue and cell lines. Lung Cancer. 2002 Aug;37(2):153-9.

164. Fasco MJ, Hurteau GJ, Spivack SD. Gender-dependent expression of alpha and beta estrogen receptors in human nontumor and tumor lung tissue. Mol Cell Endocrinol. 2002 Feb 25;188(1-2):125-40.

165. Pietras RJ, Marquez DC, Chen HW, Tsai E, Weinberg O, Fishbein M. Estrogen and growth factor receptor interactions in human breast and non-small cell lung cancer cells. Steroids. 2005 May-Jun;70(5-7):372-81.

166. Skliris GP, Parkes AT, Limer JL, Burdall SE, Carder PJ, Speirs V. Evaluation of seven oestrogen receptor beta antibodies for immunohistochemistry, western blotting, and flow cytometry in human breast tissue. J Pathol. 2002 Jun;197(2):155-62.

167. Stabile LP, Davis AL, Gubish CT, Hopkins TM, Luketich JD, Christie N, et al. Human non-small cell lung tumors and cells derived from normal lung express both estrogen receptor alpha and beta and show biological responses to estrogen. Cancer Res. 2002 Apr 1;62(7):2141-50.

168. Levin ER. Bidirectional signaling between the estrogen receptor and the epidermal growth factor receptor. Mol Endocrinol. 2003 Mar;17(3):309-17.

169. Arpino G, Wiechmann L, Osborne CK, Schiff R. Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. Endocr Rev. 2008 Apr;29(2):217-33.

170. Hirsch FR, Varella-Garcia M, Bunn PA, Jr., Di Maria MV, Veve R, Bremmes RM, et al. Epidermal growth factor receptor in non-small-cell lung carcinomas: correlation between gene copy number and protein expression and impact on prognosis. J Clin Oncol. 2003 Oct 15;21(20):3798-807.

171. Chen D, Washbrook E, Sarwar N, Bates GJ, Pace PE, Thirunuvakkarasu V, et al. Phosphorylation of human estrogen receptor alpha at serine 118 by two distinct signal transduction

pathways revealed by phosphorylation-specific antisera. Oncogene. 2002 Jul 25;21(32):4921-31.

172. Anagnostou VK, Welsh AW, Giltnane JM, Siddiqui S, Liceaga C, Gustavson M, et al. Analytic variability in immunohistochemistry biomarker studies. Cancer Epidemiol Biomarkers Prev. 2010 Apr;19(4):982-91.

173. Lee HJ, Xu X, Choe G, Chung DH, Seo JW, Lee JH, et al. Protein overexpression and gene amplification of epidermal growth factor receptor in nonsmall cell lung carcinomas: Comparison of four commercially available antibodies by immunohistochemistry and fluorescence in situ hybridization study. Lung Cancer. 2010 Jun;68(3):375-82.

174. Mathieu A, Weynand B, Verbeken E, Da Silva S, Decaestecker C, Salmon I, et al. Comparison of four antibodies for immunohistochemical evaluation of epidermal growth factor receptor expression in non-small cell lung cancer. Lung Cancer. 2010 Jul;69(1):46-50.

175. Dutertre M, Smith CL. Ligand-independent interactions of p160/steroid receptor coactivators and CREB-binding protein (CBP) with estrogen receptor-alpha: regulation by phosphorylation sites in the A/B region depends on other receptor domains. Mol Endocrinol. 2003 Jul;17(7):1296-314.

176. Minna JD, Roth JA, Gazdar AF. Focus on lung cancer. Cancer Cell. 2002 Feb;1(1):49-52.

177. Shigematsu H, Lin L, Takahashi T, Nomura M, Suzuki M, Wistuba, II, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. J Natl Cancer Inst. 2005 Mar 2;97(5):339-46.

178. Ivanova MM, Mazhawidza W, Dougherty SM, Klinge CM. Sex differences in estrogen receptor subcellular location and activity in lung adenocarcinoma cells. Am J Respir Cell Mol Biol. 2010 Mar;42(3):320-30.

179. Siegfried JM, Hershberger PA, Stabile LP. Estrogen receptor signaling in lung cancer. Semin Oncol. 2009 Dec;36(6):524-31.

180. Patrone C, Cassel TN, Pettersson K, Piao YS, Cheng G, Ciana P, et al. Regulation of postnatal lung development and homeostasis by estrogen receptor beta. Mol Cell Biol. 2003 Dec;23(23):8542-52.

181. Omoto Y, Kobayashi Y, Nishida K, Tsuchiya E, Eguchi H, Nakagawa K, et al. Expression, function, and clinical implications of the estrogen receptor beta in human lung cancers. Biochem Biophys Res Commun. 2001 Jul 13;285(2):340-7.

182. Carder PJ, Murphy CE, Dervan P, Kennedy M, McCann A, Saunders PT, et al. A multi-centre investigation towards reaching a consensus on the immunohistochemical detection of ERbeta in archival formalin-fixed paraffin embedded human breast tissue. Breast Cancer Res Treat. 2005 Aug;92(3):287-93.

183. Speirs V, Green CA, Shaaban AM. Oestrogen receptor beta immunohistochemistry: time to get it right? J Clin Pathol. 2008 Oct;61(10):1150-1; author reply 1-2.

184. Majidi M, Al-Wadei HA, Takahashi T, Schuller HM. Nongenomic beta estrogen receptors enhance beta1 adrenergic signaling induced by the nicotine-derived carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in human small airway epithelial cells. Cancer Res. 2007 Jul 15;67(14):6863-71.

185. Abe K, Miki Y, Ono K, Mori M, Kakinuma H, Kou Y, et al. Highly concordant coexpression of aromatase and estrogen receptor beta in non-small cell lung cancer. Hum Pathol. 2010 Feb;41(2):190-8.

186. Konstantinopoulos PA, Kominea A, Vandoros G, Sykiotis GP, Andricopoulos P, Varakis I, et al. Oestrogen receptor beta (ERbeta) is abundantly expressed in normal colonic mucosa, but declines in colon adenocarcinoma paralleling the tumour's dedifferentiation. Eur J Cancer. 2003 Jun;39(9):1251-8.

187. Wu CT, Chang YL, Shih JY, Lee YC. The significance of estrogen receptor beta in 301 surgically treated non-small cell lung cancers. J Thorac Cardiovasc Surg. 2005 Oct;130(4):979-86.

188. Sanchez M, Picard N, Sauve K, Tremblay A. Challenging estrogen receptor beta with phosphorylation. Trends Endocrinol Metab. 2010 Feb;21(2):104-10.

189. Hamilton-Burke W, Coleman L, Cummings M, Green CA, Holliday DL, Horgan K, et al. Phosphorylation of estrogen receptor beta at serine 105 is associated with good prognosis in breast cancer. Am J Pathol. 2010 Sep;177(3):1079-86.

190. Bulun SE, Lin Z, Zhao H, Lu M, Amin S, Reierstad S, et al. Regulation of aromatase expression in breast cancer tissue. Ann N Y Acad Sci. 2009 Feb;1155:121-31.

191. Verma MK, Miki Y, Sasano H. Aromatase in human lung carcinoma. Steroids. 2011 Jul;76(8):759-64.

192. Miki Y, Suzuki T, Abe K, Suzuki S, Niikawa H, Iida S, et al. Intratumoral localization of aromatase and interaction between stromal and parenchymal cells in the non-small cell lung carcinoma microenvironment. Cancer Res. 2010 Aug 15;70(16):6659-69.

193. Niikawa H, Suzuki T, Miki Y, Suzuki S, Nagasaki S, Akahira J, et al. Intratumoral estrogens and estrogen receptors in human non-small cell lung carcinoma. Clin Cancer Res. 2008 Jul 15;14(14):4417-26.

194. Mah V, Seligson DB, Li A, Marquez DC, Wistuba, II, Elshimali Y, et al. Aromatase expression predicts survival in women with early-stage non small cell lung cancer. Cancer Res. 2007 Nov 1;67(21):10484-90.

195. Khuder SA, Mutgi AB. Effect of smoking cessation on major histologic types of lung cancer. Chest. 2001 Nov;120(5):1577-83.

196. Junttila TT, Sundvall M, Lundin M, Lundin J, Tanner M, Harkonen P, et al. Cleavable ErbB4 isoform in estrogen receptor-regulated growth of breast cancer cells. Cancer Res. 2005 Feb 15;65(4):1384-93.

197. Srinivasan R, Gillett CE, Barnes DM, Gullick WJ. Nuclear expression of the c-erbB-4/HER-4 growth factor receptor in invasive breast cancers. Cancer Res. 2000 Mar 15;60(6):1483-7.

198. Thor AD, Edgerton SM, Jones FE. Subcellular localization of the HER4 intracellular domain, 4ICD, identifies distinct prognostic outcomes for breast cancer patients. Am J Pathol. 2009 Nov;175(5):1802-9.

199. Li CI, Malone KE, Daling JR. The relationship between various measures of cigarette smoking and risk of breast cancer among older women 65-79 years of age (United States). Cancer Causes Control. 2005 Oct;16(8):975-85.

200. Lissowska J, Brinton LA, Zatonski W, Blair A, Bardin-Mikolajczak A, Peplonska B, et al. Tobacco smoking, NAT2 acetylation genotype and breast cancer risk. Int J Cancer. 2006 Oct 15;119(8):1961-9.

201. Al-Delaimy WK, Cho E, Chen WY, Colditz G, Willet WC. A prospective study of smoking and risk of breast cancer in young adult women. Cancer Epidemiol Biomarkers Prev. 2004 Mar;13(3):398-404.

202. Manjer J, Malina J, Berglund G, Bondeson L, Garne JP, Janzon L. Smoking associated with hormone receptor negative breast cancer. Int J Cancer. 2001 Feb 15;91(4):580-4.

203. Ganti AK, Sahmoun AE, Panwalkar AW, Tendulkar KK, Potti A. Hormone replacement therapy is associated with decreased survival in women with lung cancer. J Clin Oncol. 2006 Jan 1;24(1):59-63.

204. Raso MG, Behrens C, Herynk MH, Liu S, Prudkin L, Ozburn NC, et al. Immunohistochemical expression of estrogen and progesterone receptors identifies a subset of NSCLCs and correlates with EGFR mutation. Clin Cancer Res. 2009 Sep 1;15(17):5359-68.

205. Ali G, Donati V, Loggini B, Servadio A, Dell'Omodarme M, Prati MC, et al. Different estrogen receptor beta expression in distinct histologic subtypes of lung adenocarcinoma. Hum Pathol. 2008 Oct;39(10):1465-73.

206. Wynder EL, Muscat JE. The changing epidemiology of smoking and lung cancer histology. Environ Health Perspect. 1995 Nov;103 Suppl 8:143-8.

207. Rodenhuis S, Slebos RJ. Clinical significance of ras oncogene activation in human lung cancer. Cancer Res. 1992 May 1;52(9 Suppl):2665s-9s.

208. McClay EF, Bogart J, Herndon JE, 2nd, Watson D, Evans L, Seagren SL, et al. A phase III trial evaluating the combination of cisplatin, etoposide, and radiation therapy with or without tamoxifen in patients with limited-stage small cell lung cancer: Cancer and Leukemia Group B Study (9235). Am J Clin Oncol. 2005 Feb;28(1):81-90.

209. Gruvberger-Saal SK, Bendahl PO, Saal LH, Laakso M, Hegardt C, Eden P, et al. Estrogen receptor beta expression is associated with tamoxifen response in ERalpha-negative breast carcinoma. Clin Cancer Res. 2007 Apr 1;13(7):1987-94.

210. Naresh A, Thor AD, Edgerton SM, Torkko KC, Kumar R, Jones FE. The HER4/4ICD estrogen receptor coactivator and BH3-only protein

is an effector of tamoxifen-induced apoptosis. Cancer Res. 2008 Aug 1;68(15):6387-95.

211. Shen H, Yuan Y, Sun J, Gao W, Shu YQ. Combined tamoxifen and gefitinib in non-small cell lung cancer shows antiproliferative effects. Biomed Pharmacother. 2010 Feb;64(2):88-92.

212. Marquez-Garban DC, Chen HW, Goodglick L, Fishbein MC, Pietras RJ. Targeting aromatase and estrogen signaling in human non-small cell lung cancer. Ann N Y Acad Sci. 2009 Feb;1155:194-205.

213. Weinberg OK, Marquez-Garban DC, Fishbein MC, Goodglick L, Garban HJ, Dubinett SM, et al. Aromatase inhibitors in human lung cancer therapy. Cancer Res. 2005 Dec 15;65(24):11287-91.

214. Verma MK, Miki Y, Sasano H. Aromatase in human lung carcinoma. Steroids. 2011 Mar 30.

215. Giovannini M, Belli C, Villa E, Gregorc V. Estrogen receptor (ER) and epidermal growth factor receptor (EGFR) as targets for dual lung cancer therapy: not just a case? 2008 Jun.

Publications *in the series*
Örebro Studies in Medicine

1. Bergemalm, Per-Olof (2004). *Audiologic and cognitive long-term sequelae from closed head injury.*

2. Jansson, Kjell (2004). *Intraperitoneal Microdialysis. Technique and Results.*

3. Windahl, Torgny (2004). *Clinical aspects of laser treatment of lichen sclerosus and squamous cell carcinoma of the penis.*

4. Carlsson, Per-Inge (2004). *Hearing impairment and deafness. Genetic and environmental factors – interactions – consequences. A clinical audiological approach.*

5. Wågsäter, Dick (2005). *CXCL16 and CD137 in Atherosclerosis.*

6. Jatta, Ken (2006). *Inflammation in Atherosclerosis.*

7. Dreifaldt, Ann Charlotte (2006). *Epidemiological Aspects on Malignant Diseases in Childhood.*

8. Jurstrand, Margaretha (2006). *Detection of Chlamydia trachomatis and Mycoplasma genitalium by genetic and serological methods.*

9. Norén, Torbjörn (2006). *Clostridium difficile, epidemiology and antibiotic resistance.*

10. Anderzén Carlsson, Agneta (2007). *Children with Cancer – Focusing on their Fear and on how their Fear is Handled.*

11. Ocaya, Pauline (2007). *Retinoid metabolism and signalling in vascular smooth muscle cells.*

12. Nilsson, Andreas (2008). *Physical activity assessed by accelerometry in children.*

13. Eliasson, Henrik (2008). *Tularemia – epidemiological, clinical and diagnostic aspects.*

14. Walldén, Jakob (2008). *The influence of opioids on gastric function: experimental and clinical studies.*

15. Andrén, Ove (2008). *Natural history and prognostic factors in localized prostate cancer.*

16. Svantesson, Mia (2008). *Postpone death? Nurse-physician perspectives and ethics rounds.*

17. Björk, Tabita (2008). *Measuring Eating Disorder Outcome – Definitions, dropouts and patients' perspectives.*

18. Ahlsson, Anders (2008). *Atrial Fibrillation in Cardiac Surgery.*

19. Parihar, Vishal Singh (2008). *Human Listeriosis – Sources and Routes.*

20. Berglund, Carolina (2008). *Molecular Epidemiology of Methicillin-Resistant Staphylococcus aureus. Epidemiological aspects of MRSA and the dissemination in the community and in hospitals.*

21. Nilsagård, Ylva (2008). *Walking ability, balance and accidental falls in persons with Multiple Sclerosis.*

22. Johansson, Ann-Christin (2008). *Psychosocial factors in patients with lumbar disc herniation: Enhancing postoperative outcome by the identification of predictive factors and optimised physiotherapy.*

23. Larsson, Matz (2008). *Secondary exposure to inhaled tobacco products.*

24. Hahn-Strömberg, Victoria (2008). *Cell adhesion proteins in different invasive patterns of colon carcinoma: A morphometric and molecular genetic study.*

25. Böttiger, Anna (2008). *Genetic Variation in the Folate Receptor-α and Methylenetetrahydrofolate Reductase Genes as Determinants of Plasma Homocysteine Concentrations.*

26. Andersson, Gunnel (2009). *Urinary incontinence. Prevalence, treatment seeking behaviour, experiences and perceptions among persons with and without urinary leakage.*

27. Elfström, Peter (2009). *Associated disorders in celiac disease.*

28. Skårberg, Kurt (2009). *Anabolic-androgenic steroid users in treatment: Social background, drug use patterns and criminality.*

29. de Man Lapidoth, Joakim (2009). *Binge Eating and Obesity Treatment – Prevalence, Measurement and Long-term Outcome.*

30. Vumma, Ravi (2009). *Functional Characterization of Tyrosine and Tryptophan Transport in Fibroblasts from Healthy Controls, Patients with Schizophrenia and Bipolar Disorder.*

31. Jacobsson, Susanne (2009). *Characterisation of Neisseria meningitidis from a virulence and immunogenic perspective that includes variations in novel vaccine antigens.*

32. Allvin, Renée (2009). *Postoperative Recovery. Development of a Multi-Dimensional Questionnaire for Assessment of Recovery.*

33. Hagnelius, Nils-Olof (2009). *Vascular Mechanisms in Dementia with Special Reference to Folate and Fibrinolysis.*

34. Duberg, Ann-Sofi (2009). *Hepatitis C virus infection. A nationwide study of assiciated morbidity and mortality.*

35. Söderqvist, Fredrik (2009). *Health symptoms and potential effects on the blood-brain and blood-cerebrospinal fluid barriers associated with use of wireless telephones.*

36. Neander, Kerstin (2009). *Indispensable Interaction. Parents' perspectives on parent–child interaction interventions and beneficial meetings.*

37. Ekwall, Eva (2009). *Women's Experiences of Gynecological Cancer and Interaction with the Health Care System through Different Phases of the Disease.*

38. Thulin Hedberg, Sara (2009). *Antibiotic susceptibility and resistance in Neisseria meningitidis – phenotypic and genotypic characteristics.*

39. Hammer, Ann (2010). *Forced use on arm function after stroke. Clinically rated and self-reported outcome and measurement during the sub-acute phase.*

40. Westman, Anders (2010). *Musculoskeletal pain in primary health care: A biopsychosocial perspective for assessment and treatment.*

41. Gustafsson, Sanna Aila (2010). *The importance of being thin – Perceived expectations from self and others and the effect on self-evaluation in girls with disordered eating.*

42. Johansson, Bengt (2010). *Long-term outcome research on PDR brachytherapy with focus on breast, base of tongue and lip cancer.*

43. Tina, Elisabet (2010). *Biological markers in breast cancer and acute leukaemia with focus on drug resistance.*

44. Overmeer, Thomas (2010). *Implementing psychosocial factors in physical therapy treatment for patients with musculoskeletal pain in primary care.*

45. Prenkert, Malin (2010). *On mechanisms of drug resistance in acute myloid leukemia.*

46. de Leon, Alex (2010). *Effects of Anesthesia on Esophageal Sphincters in Obese Patients.*

47. Josefson, Anna (2010). *Nickel allergy and hand eczema – epidemiological aspects.*

48. Almon, Ricardo (2010). *Lactase Persistence and Lactase Non-Persistence. Prevalence, influence on body fat, body height, and relation to the metabolic syndrome.*

49. Ohlin, Andreas (2010). *Aspects on early diagnosis of neonatal sepsis.*

50. Oliynyk, Igor (2010). *Advances in Pharmacological Treatment of Cystic Fibrosis.*

51. Franzén, Karin (2011). *Interventions for Urinary Incontinence in Women. Survey and effects on population and patient level.*

52. Loiske, Karin (2011). *Echocardiographic measurements of the heart. With focus on the right ventricle.*

53. Hellmark, Bengt (2011). *Genotypic and phenotypic characterisation of* **Staphylococcus epidermidis** *isolated from prosthetic joint infections.*

54. Eriksson Crommert, Martin (2011). *On the role of transversus abdominis in trunk motor control.*

55. Ahlstrand, Rebecca (2011). *Effects of Anesthesia on Esophageal Sphincters.*

56. Holländare, Fredrik (2011). *Managing Depression via the Internet – self-report measures, treatment & relapse prevention.*

57. Johansson, Jessica (2011). *Amino Acid Transport and Receptor Binding Properties in Neuropsychiatric Disorders using the Fibroblast Cell Model.*

58. Vidlund, Mårten (2011). *Glutamate for Metabolic Intervention in Coronary Surgery with special reference to the GLUTAMICS-trial.*

59. Zakrisson, Ann-Britt (2011). *Management of patients with Chronic Obstructive Pulmonary Disease in Primary Health Care. A study of a nurse-led multidisciplinary programme of pulmonary rehabilitation.*

60. Lindgren, Rickard (2011). *Aspects of anastomotic leakage, anorectal function and defunctioning stoma in Low Anterior Resection of the rectum for cancer.*

61. Karlsson, Christina (2011). *Biomarkers in non-small cell lung carcinoma. Methodological aspects and influence of gender, histology and smoking habits on estrogen receptor and epidermal growth factor family receptor signalling.*