Biomarkers in non-small cell lung carcinoma
To Tobias and Daniel
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
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 adenocarcinomas (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.

ER\textsubscript{\alpha} is more frequently observed in ADCA and in females and a local estradiol synthesis is supported by the presence of aromatase. ER\textsubscript{\beta} is identified as a positive prognostic marker in ADCA. Smoking is associated to increased levels of ER\textsubscript{\beta} mRNA. EGFR over expression is associated with a ligand. Independent phosphorylation of ER\textsubscript{\alpha}. HER-4 intracellular domain may also act as a co-activator to ER\textsubscript{\alpha} 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

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Abstract


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


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 $\beta$ in NSCLC – prevalence, proliferative influence, prognostic impact and smoking. Submitted

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

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

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List of publications

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

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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 subtyping 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 7th 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 Zealand. 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°C, 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°C 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°C (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°C 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 aqueous 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:

\[
\text{Protein – H} + \text{CH}_2\text{O} \rightarrow \text{Protein –CH}_2\text{OH}
\]

Reactive hydrogen on tissue

Formation of methylene bridges:

\[
\text{Protein –CH}_2\text{OH} + \text{Protein – H} \rightarrow \text{Protein –CH}_2\text{-Protein} + \text{H}_2\text{O}
\]

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 (riboprobes). 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 Conrat in the 1940s (30, 49-50), indicated that cross-linkage between formalin and protein could be disrupted by heating above 100°C or by strong alkaline treatment) of FFPE tissue in the presence of heavy metal solutions such as lead thiocyanate, up to temperatures of 100°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).

Phylogenetic 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).

<table>
<thead>
<tr>
<th>NH2</th>
<th>A/B Transactivation (AF-1) / phosphorylation</th>
<th>C DNA-binding (DBD)</th>
<th>D Hinge</th>
<th>E/F Ligand-binding (LBD) / dimerization / transactivation (AF-2)</th>
<th>COOH</th>
</tr>
</thead>
</table>

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 receptor, 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 (E2). 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)](image)

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 complexes. 3) non-genomic estrogen signalling pathways, ERs and GP30 located at or near the cell membrane might elicit the rapid response by activating 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)
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).
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
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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).

![Aromatase diagram](image)

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.visisience.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 multicellular 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 glycoproteins 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-secretase 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 histopathologic 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 cytoplasmic 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 as re-staged according to 7ed of TNM Classification of Malignant Tumours (Sobin 2009). Survival data was obtained from the National Board of Health and
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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 system 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-2 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
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 system 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.

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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°C 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 manufacturer's 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: $2^{\Delta\text{Ct sample-Ct calibrator}}$, 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 PRA 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

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).
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.

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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°C) 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°C 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°C. (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 II A (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 phosphorylated 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 cytoplasmatic 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.

<table>
<thead>
<tr>
<th>Reference</th>
<th>clone</th>
<th>Antigen retrieval</th>
<th>Detection</th>
<th>Histology (n=)</th>
<th>Positivity</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Buffer Time (mins)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ishibashi et al 2005</td>
<td>6F11</td>
<td>-</td>
<td>ABC</td>
<td>NSCLC (228)</td>
<td>38%</td>
<td>nucl</td>
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<tr>
<td>Kawai et al 2005</td>
<td>HC-20,</td>
<td>Ci</td>
<td>Envision</td>
<td>ADCA/SCC (102)</td>
<td>72%</td>
<td>cytopl</td>
</tr>
<tr>
<td>Lau et al 2006</td>
<td>1D5</td>
<td>TE, pH 8.0</td>
<td>Envision</td>
<td>ADCA (55)</td>
<td>18%</td>
<td>nucl</td>
</tr>
<tr>
<td>Schwartz et al 2005</td>
<td>1D5</td>
<td>Ci</td>
<td>ABC</td>
<td>ADCA/SCC (45)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dabbs et al 2002</td>
<td>6F11</td>
<td>Ci</td>
<td>LSAB</td>
<td>ADCA (92)</td>
<td>7%</td>
<td>nucl</td>
</tr>
<tr>
<td>Gomez-Fernandes et al 2010</td>
<td>6F11</td>
<td>Ci</td>
<td>LSAB</td>
<td>ADCA (18)</td>
<td>5%</td>
<td>nucl</td>
</tr>
<tr>
<td>Radzi et al 2002</td>
<td>1D5</td>
<td>Ci</td>
<td>DAB</td>
<td>ADCA (14)</td>
<td>7%</td>
<td>nucl</td>
</tr>
<tr>
<td>Skov et al 2008</td>
<td>1D5</td>
<td>TE, pH 9.0</td>
<td>Envision</td>
<td>NSCLC (104)</td>
<td>3%</td>
<td>nucl</td>
</tr>
</tbody>
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rPab: rabbit polyclonal, Ci: 0.01M citrate buffer, TE: Tris-EDTA buffer, ABC: avidib-biotin complex, LSAB: labelled streptavidib-biotin, DAB, dianaminobenzidine, 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-tumoural 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 intratumoral 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 estrogen 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). Intratumoral estradiols have also been demonstrated in men and postmenopausal 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 western/European population which in general have a good nutritional state with increased average body fat with concomitant increased levels of estradiol 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 previously discussed, an ongoing ERα induced ERE dependent transcription was also indicated by the finding of PgR expression in the ERα positive tumours. 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 significantly 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 by 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 highlighted.

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 phosphorylation 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) tumors 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.
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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:

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

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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!
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