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**Publisher:**  
CanAg Diagnostics AB  
Majnabbe Terminal  
SE-414 55 Gothenburg  
Sweden  
Phone: +46 31 85 70 30  
Fax: +46 31 85 70 40  
E-mail: [canag@canag.se](mailto:canag@canag.se)  
[www.canag.se](http://www.canag.se)

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Olle Nilsson

## Focus

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*Welcome to the third issue of CanAg Journal. The focus of this issue is lung cancer that is the most common cause of cancer related death in the world. It is our pleasure to present an overview on tumor markers in lung cancer written by Dr Prof Petra Stieber at Maximillian Universität in Munich.*

*There are large needs for improved therapy and early diagnosis of lung cancer and tumor markers play an important role in different areas of patient management. Reliable tumor markers with high specificity are valuable clinical tools for example diagnosis of lung cancer, differential diagnosis of the histological subtypes, monitoring of disease status and also in the future for prediction of therapy response of new designed drugs.*



### **"Tumor markers of lung cancer is a focus area for CanAg Diagnostics"**

*Currently we have five different lung tumor markers on the market and additional markers under development.*

*During 2004 and the beginning of 2005 the organization of CanAg Diagnostics has been strengthened. A Medical Marketing Team has been established to strengthen the medical marketing- and clinical documentation for our existing and novel products. Dr Sven Erickson, who joined CanAg Diagnostics in September 2004, is responsible for this group. He is also our new sales- and marketing manager for reagents and for introduction of new products. With these changes in our business organization we feel that we will improve our customer service.*

*On the next page you can read about the award we have received from EURO/DPC, "Vendor of the year". I am very thankful for this award and also proud of the competent and devoted staff we have at CanAg Diagnostics.*

**CanAg Diagnostics AB**

**Olle Nilsson**  
VP, CSO

## Vendor of the year

CanAg Diagnostics has received the award "Vendor of the year for 2004" from EURO/DPC kit division in the category "Biologicals & Chemicals".

The vendor rating system is part of Euro/DPC's Supplier Management and Quality Improvement programme and monitors vendor performance in terms of e.g. Delivery Reliability/Flexibility, Criticality, Price competitiveness and Quality Performance.

The award was presented at a supplier seminar held June 16<sup>th</sup> at Euro/DPC manufacturing facility, Llanberis in North Wales.



*"We are very proud of this award, says Christina Hall, Director for Regulatory Affairs and Quality Assurance (to the right). This is a recognition that CanAg Diagnostics has the right focus and that our monoclonal antibodies and antigens consistently show the high quality standard we always aim to fulfil. Here, together with Birgitta Grundström, responsible for monoclonal antibody purification.*

# Selection and characterisation of Monoclonal antibodies suitable for design of sandwich immunoassays



The optimal characteristics of immunological reagents differ dependent on the intended use, for instance a MAb with optimal properties for immunohistochemistry may not be optimal for serological immunoassays. Therefore the process of identification and characterisation of MAb should ideally include a functional screening based on the intended use.

In the search for optimal monoclonal antibodies (MAbs) for immunoassay development several characteristics are essential. Optimal recognition of antigen in solution is one. Recognition of independent epitopes and good kinetic properties are others. Methods for selection and characterisation of suitable MAb for design of sandwich immunoassays should include analyses of these characteristics. A systematic approach separated into three main steps for identification and characterisation of MAbs suitable for design of sandwich immunoassays is illustrated in Figure 1.

## STEP A. Functional screening

The functional screening of antibody pairs suitable for development of sandwich immunoassays includes selection of MAb's with optimal recognition of antigen in solution. The test should be made with antigen in buffer matrices and spiked into serum or plasma samples as well as with native samples containing high and low levels of the antigen.

## STEP B. Analyses of binding interactions

Knowledge of antibody-antigen interaction and the relation between antigenic

domains recognised by different MAbs is essential for selection of antibody pairs suitable for immunoassay development. Conventional immunochemical methods for analysing these parameters include cross-inhibition studies, determination of dose-response curves for all possible antibody-antigen combinations and determination of kinetic properties. The drawback of these methods is that they are time consuming and labor intensive. However, they are normally the critical step for identification of serological product leads once MAbs against a target antigen have been established. Biosensors allowing label free real time analysis of antibody-antigen binding and kinetic determinations have greatly facilitated the identification of possible sandwich pairs. The most well-known technology for label free real time analysis of antibody-antigen interactions utilise surface plasmon resonance (SPR) technology, and the most widely used system is the BiaCore instrument (BiaCore AB, Uppsala, Sweden).

However, there are other techniques and in this report we have used the Quartz Crystal Microbalance (QCM) technology to study the antigen-antibody interactions.

## QCM technology and instrumentation

The QCM technology may be used for all type of studies of antibody-antigen inter-

actions and enables real-time label free study of simultaneous binding of several antibodies directly to immobilised antigen or in a sandwich assay format.

QCM uses the piezoelectric effect. By applying an alternative potential to a piezoelectric quartz crystal, the crystal can be controlled to oscillate at its resonance frequency. Adding or removing molecules from its surface induce changes in the resonance frequency proportional to the mass of the material added or removed (figure 2). The gold sensor surface with carboxyl or biotin self assembled monolayer, is specifically coated with biomolecules. A sample flow containing the label free MAbs is passed

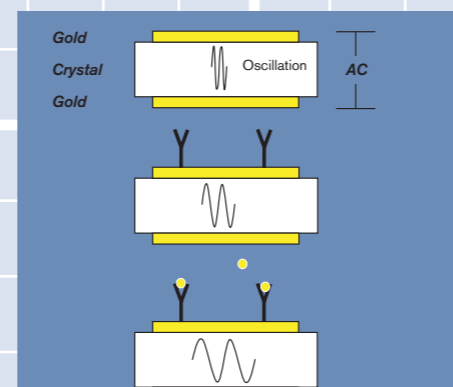


Figure 2. The piezoelectric quartz crystal can be controlled to oscillate at its resonance frequency by applying an alternating current. When molecules add mass to the gold biosensor surface the oscillation frequency decreases proportionally to the mass of the attached molecules.

above the surface, and interaction with the attached molecule is detected in real time by recording the frequency shift. The software provided with the instrument can also be used for information about kinetics, affinity ( $K_{on}/K_{off}$ ), specificity of the binding, and concentration in the sample.

## Analyses of relation between antibody binding sites

In this report the binding of PSA MAb to PSA (prostate specific antigen) was studied in order to determine the relation between the antigenic domains recognised by different CanAg PSA MAbs. The results are summarised in figure 3. The sequential addition of the different PSA MAbs resulted in a decrease in frequency, indicating that each MAb recognise an independent antigenic domain in the PSA molecule. The results show that any of the tested PSA MAbs could be used in combination for the development of PSA sandwich immunoassays.

## STEP C. Epitope mapping

Detailed knowledge about antibody binding (epitope mapping) is important to understand the antibody-antigen interaction. It also facilitates understanding of possible cross-reactivity with related and/or unrelated substances.

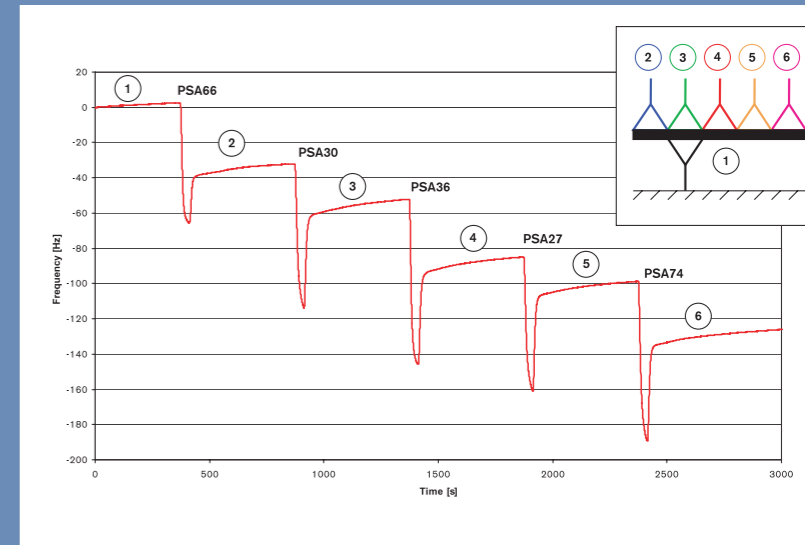


Figure 3. Sensogram for binding of different PSA MAb. The frequency shifts when different PSA antibodies bind to immobilized antigen are shown. The large negative peaks in the beginning of each injection is due to unspecific binding of proteins in the cell culture supernatant, which is washed away with buffer. The plateau represents the specific binding of PSA MAb to the PSA bound to the surface. The slight positive shift seen in the "plateau" after each injection is due to the off rate of the antibody binding.  
1) PSA has been immobilized on the surface by biotinylated PSA10 MAb. Cell supernatant containing 2) PSA66 MAb, 3) PSA30 MAb, 4) PSA36 MAb, 5) PSA27 MAb and 6) PSA74 MAb were added sequentially to the biosensor. The negative frequency shift when the bulk of protein in the cell culture supernatant has been washed away show that the antibodies have bound and added weight to the crystal, that is the PSA MAb has bound to the PSA antigen. The QCM analyses were performed using an Attana 100 QCM instrument developed by Attana AB, Stockholm, Sweden and evaluated using the Attester software provided with the instrument.

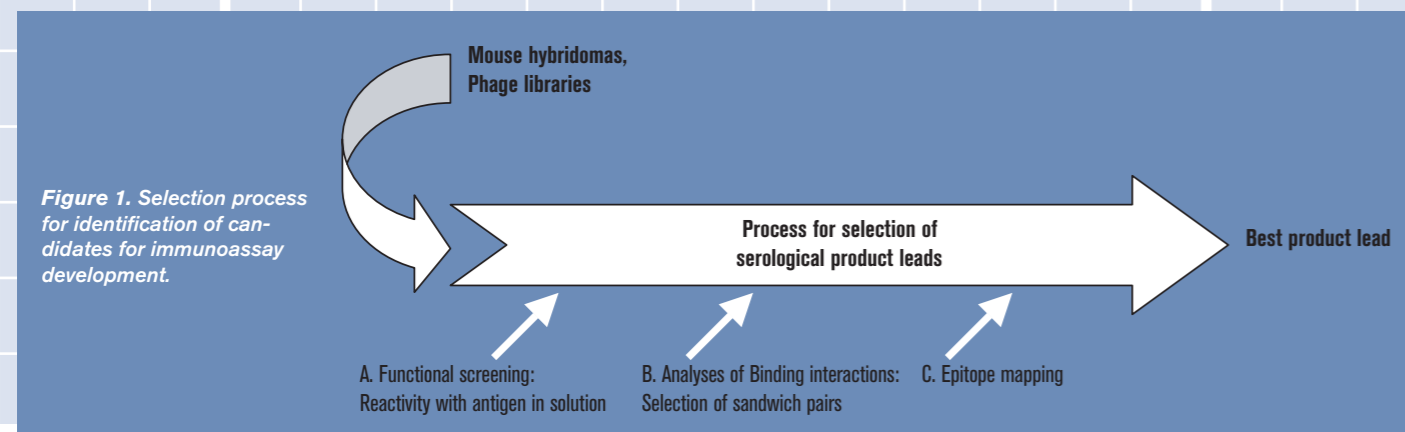


Figure 1. Selection process for identification of candidates for immunoassay development.

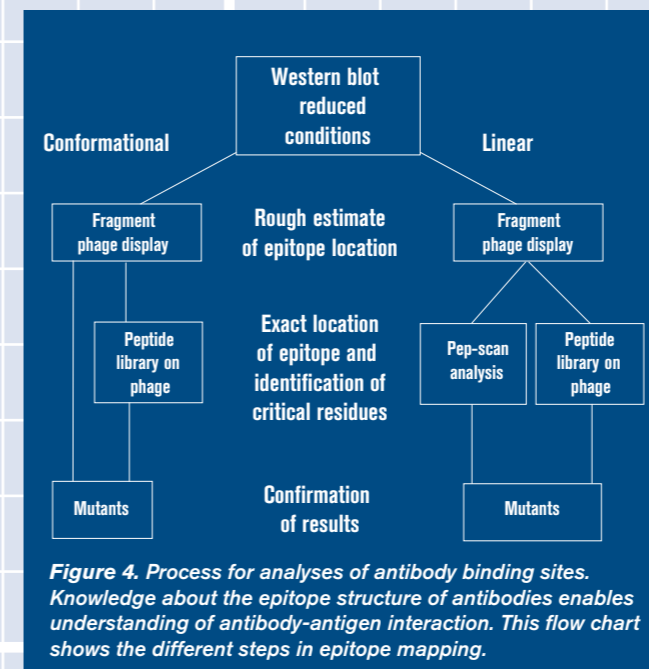


Figure 4. Process for analyses of antibody binding sites. Knowledge about the epitope structure of antibodies enables understanding of antibody-antigen interaction. This flow chart shows the different steps in epitope mapping.

Epitope mapping and analyses of possible cross-reactivity by direct binding studies or *in silico* analyses are the final step in the identification of optimal product leads. Figure 4 shows a flow chart of the different steps in epitope mapping.

## Conformational dependent versus linear epitopes

The first step in epitope mapping is to determine whether the epitope is conformation-dependent or linear. A linear epitope consists of a continuous amino acid sequence in the target protein, while a conformation-dependent epitope depends on the three dimensional structure of the protein. The type of epitope can be investigated by Western blot run under reducing conditions. By combining SDS (sodium dodecylsulphate) with a reducing agent such as dithiothreitol (DTT), that cleaves any disulphide bonds between cysteine residues, the protein gets totally unfolded and linear. Only antibodies recognizing linear epitopes, that is independent on the secondary structure for binding, will give positive signals under reducing conditions. Knowledge about the type of epitope is important to proceed with the epitope characterization and therefore the characterisation of the epitopes start with Western blot analyses in reducing and non-reducing conditions.

# Selection and characterisation of Monoclonal antibodies suitable for design of sandwich immunoassays



## Determination of detailed MAb binding site

Different methods to determine the detailed binding structure for MAb are described below (figure 5-7). The two phage display techniques are to some extent applicable for both linear and conformation dependent epitopes. However it is always difficult to obtain detailed information on binding structure for conformation dependent epitopes and mutant analyses may thus be the only useful method.

The reactivity towards the phage displayed protein fragments is then tested in an ELISA - either by direct coating of the phage or indirectly via a catching antibody.

A prediction of the location of the epitope for linear epitopes can be done based on the reactivity with the phage displayed fragments. However for conformation-dependent epitopes, binding to the phage displayed fragments may not occur and the polypeptides have to be long enough to allow correct folding. To our experience 30-50 amino acid residues are usually sufficient to promote the right epitope conformation. By the use of overlapping peptides the location of conformation dependent epitopes can be narrowed down to a region of about 15-25 residues.

## Antigen fragments displayed on phage

The location of antibody binding can be roughly determined by analysing the reactivity of the antibody towards overlapping antigen fragments displayed on phage particles (figure 5). PCR amplified gene fragments are cloned in a phage display vector in fusion with a phage coat protein, i.e. pIII or pVIII, and the corresponding polypeptides are displayed at the N-terminus of the fusion protein on the phage surface.

## Random peptide libraries displayed on phage

The detailed amino acid sequence of the epitope and mimotope of an antibody may be further determined using random peptide libraries displayed on phage. Specific phage clones are selected for binding from large randomized

peptide libraries displayed on phage in a process called biopanning, see figure 6. The antibody is exposed to the library, non-binding clones are washed away, and binders are eluted and amplified in *E. coli*. The process is repeated 3 to 4 times with increased stringency in the washing step resulting in an enrichment of phage clones with high affinity for the antibody.

For linear epitopes sequence alignment of the peptides displayed on the phages most often indicate a consensus sequence that show the essential amino acid residues in the antibody-antigen interaction, i.e. the mimotope of the MAb. Such information is not only biologically important but also valuable for database search for proteins with the same sequence motif, proteins that might cross-react with the antibody. In the case of conformationally constrained epitopes the sequence analysis is not as straightforward and consensus sequences may not be obtained. Usually the consensus sequences show no obvious similarity with the primary protein sequence.

However, by the use of the results obtained from antigen fragment display and structural data, amino acid residues in the epitope may be identified.

## Pepscan analysis

For linear epitopes pepscan analysis is useful as a complement to the results obtained using random peptide libraries. Pepscan analysis involves synthesis of overlapping peptides (10-15 aa) covering the amino acid sequence of the antigen. Usually the peptides are linked to biotin via a short linker sequence for immobilization in streptavidin coated ELISA wells. The reactivity of an antibody towards each specific peptide can be easily determined in an ELISA assay (figure 7).

## Mutagenesis

Both for linear and conformationally constrained epitopes analysis of reactivity to mutant variants of the antigen is useful in order to confirm the results from the above mentioned techniques. For conformationally constrained epitopes in particular, mutagenesis of selected residues and/or deletion of motifs might help to pinpoint the epitope, and may for some antigens be the only method to provide detailed information about binding specificity of antibodies.

## CanChek Tumor Marker Control Serum



## SUMMARY

This report describes a systematic process for selection and characterisation of antibodies suitable for design of sandwich immunoassays. Based on the process and methods described optimal antibodies for the design of sandwich immunoassays may be selected.

Complementing the range of ELISA assays for tumor markers, CanAg Diagnostics has introduced CanChek Tumor Marker Control Serum. It is an assayed control serum to monitor the precision and accuracy of laboratory test procedures for Neuron Specific Enolase (NSE), S100B protein and Squamous Cell Carcinoma Antigen (SCCA).

The CanChek control serum is formulated using human antigens in a human serum based matrix. The controls are available in two levels in order to provide performance monitoring over the clinical range. The low level targeting the medically relevant decision points and the high level targeting clinically elevated levels. The CanChek Tumor Marker Control Serum can be used for the ELISA platform, as well as all types of random access instruments.

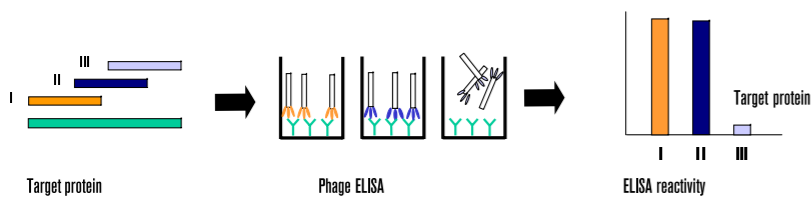


Figure 5. Phage display of protein fragment for analyses of antibody binding sites. Overlapping gene fragments are PCR amplified, cloned into phage display vector and expressed on the surface of phage particles. Each specific clone is tested for binding to antigen specific antibodies in an ELISA assay.

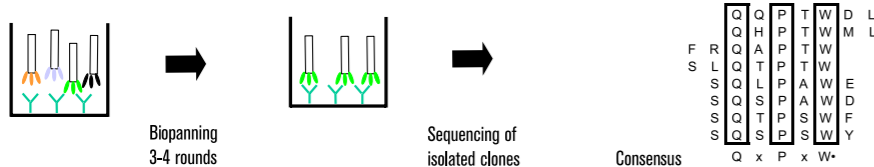


Figure 6. Biopanning of random peptide libraries. The phage displayed peptide library is panned against the antibody 3-4 times after which individual clones are amplified and sequenced. The consensus sequence indicates the mimotope of the antibody.

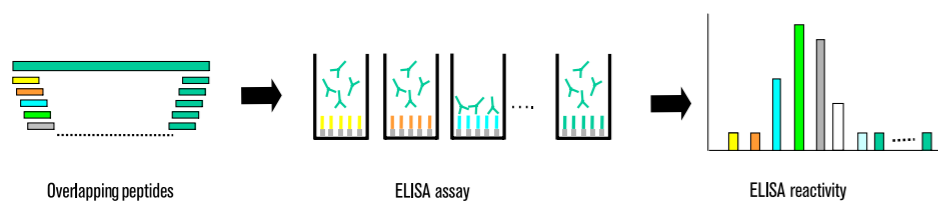


Figure 7. Principles of Pep-Scan analyses. Reactivity of overlapping peptides against the antibody tested in an ELISA assay.

# Biomarkers in Lung Cancer



In most industrialised countries, lung cancer is the most common cancer in men, and is rapidly approaching the same incidence in women. Despite worldwide efforts in improving diagnosis and therapy the five-year survival rate has not been significantly increased during the past 25 years. It is now around 13%. Lung cancer represents the prototype of a tumor induced by chemical carcinogens, and in principle its incidence could be decreased by public health measures. In industrialised countries, the association of lung cancer and cigarette smoking is strong (85%). However, in addition to exogenous physical or chemical carcinogenic influences such as from asbestos or urane, an individual genetic pre-disposition to cancer or vulnerability to the effects of carcinogens must be assumed.

## Diagnosis of lung cancer

At the time of primary diagnosis, almost 50% of patients appear to have operable tumors. However, once diagnostic investigations are complete, it becomes evident that 70% of this group of patients have tumors that cannot be completely resected. The aims of diagnostic tools for lung cancer should therefore be to provide information that:

- Spare patients unnecessary operations or exploratory thoracotomies
- Encourage early surgical intervention in the 15% of patients for whom surgical intervention is likely to be effective
- Identify those patients for whom palliative resection is desirable

As many of these diagnostic procedures are invasive, they should be chosen in a stepwise manner according to the

Petra Stieber, MD and head of the research laboratory "Diagnostic Oncology" at the Institute of Clinical Chemistry, University of Munich, Germany, together with her colleagues Rudolf Hatz (Dept. of surgery), Andreas Schalhorn (Dept. of Oncology) and Stefan Holdenrieder (Clin.Chemistry) has reviewed the most important biomarkers in lung cancer and their potential clinical applications in the management of this disease, especially to support differential diagnosis in lung tumors of unknown origin using markers with a superior profile of specificity and sensitivity like ProGRP.

therapeutic consequences that can be expected;

- Basic diagnostic procedures to secure a final histological diagnosis and to identify patients with inoperable tumors
- Functional tests to determine whether patients are well enough to undergo surgery
- Diagnostic procedures for TNM (tumor, nodes, metastases) classification and staging of malignancy

## Classification of lung cancer

Most primary lung tumors can be classified into four major histological types:

- Squamous cell carcinoma
- Adenocarcinoma
- Large cell carcinoma
- Small cell lung cancer (SCLC) that accounts for 20-25% of the cases of bronchogenic carcinoma. It differs clinically and biologically from the other three histological types. However, it has become evident that many tumors have features of more than one histological type of cancer. Thus both SCLC and non-small cell lung cancer (NSCLC) represent heterogeneous groups in which there is considerable overlap among the major histological types of carcinoma of the lung.

## Tumor markers

Many different tumor associated antigens have been described and investigated in lung cancer. Depending on the clinical indication the following markers circulating in blood have proved to possess a superior profile of specificity and sensitivity: neuron specific enolase (NSE), carcinoembryonic antigen (CEA), cytokeratin 19 fragments (CYFRA 21-1) and especially Pro Gastrin Releasing Peptide (ProGRP).

NSE is a glycolytic neuronspecific isoenzyme of enolase. It consists of two almost identical polypeptide chains, each with a molecular weight of 39 kD. It is produced in central and peripheral neurons and malignant tumors of neuroectodermal origin for example SCLC, neuroblastomas and intestinal carcinoid. Furthermore, as NSE is also found in erythrocytes, plasma cells and platelets, it may be released into serum if separation from red cells does not occur within 60 minutes of venopuncture.

CEA is a glycoprotein of ~180 kD. It is one of the carcinoembryonic antigens produced during embryonal and fetal development. CEA was one of the first tumor markers to be described, and has relatively high sensitivity for many advanced adenocarcinomas, primarily colon, but also breast, stomach and lung cancer. CEA measurement is most sensitive, and serum concentrations are highest, in adenocarcinoma and large cell lung cancer.

CYFRA 21-1 detects a cytokeratin 19 fragment using 2 monoclonal antibodies (BM 21-1 and KS 19-1). Histopathological studies demonstrate that cytokeratin 19 is abundant in carcinomas of the lung. CYFRA 21-1 is especially suitable for NSCLC as it is the most sensitive tumor marker in these histologies including squamous tumors.

ProGRP (Pro Gastrin Releasing Peptide) is the more stable precursor of the gut hormone gastrin releasing peptide (GRP) originally isolated from porcine stomach. GRP is the mammalian counterpart of the amphibian bombesin - well known for the histopathological classification of lung tissues. Due to a good overall profile of specificity and sensitivity ProGRP has within a short period of time become a reliable serum marker for SCLC.

SCC is a 48 kD protein with strong homology to the serpin family of protease inhibitors. Serum measurements of SCC have been used in squamous cell carcinomas of the cervix, oesophagus, head, neck and lung. CYFRA 21-1 has replaced SCC as the marker of first choice for NSCLC due to higher sensitivity. In contrast to CYFRA 21-1, SCC has a high specificity for squamous cell carcinomas and thus represents an aid in histological diagnosis.

## INDICATIONS FOR THE DETERMINATION OF SERUM BIOMARKERS

Determination of serum tumor markers may be useful in several areas of cancer disease management, e.g. screening, primary diagnosis, differential diagnosis, prognosis, monitoring of therapy and follow-up. The following provides a review of the possible use of serum tumor marker determination in lung cancer in different areas of cancer disease management.

## SCREENING

Because of lacking sensitivity and especially lacking tumor specificity and also organ specificity neither

CYFRA 21-1, CEA nor NSE is suitable for classical screening investigations, that is single performance of a diagnostic method in asymptomatic persons or in high-risk groups like smokers. Tumor specificity could be reached for all these markers by increasing the cut off values to very high concentrations where no benign diseases are observed. These levels would mostly correspond to advanced tumor stages and not to the early stages of disease that would be of positive prognostic relevance for the patients.

This does not mean that oncological biomarkers are not released in early tumor stages and not detected in the asymptomatic screening situation. Figure 1 describes the release of CEA depending on the size of lung carcinomas as compared to healthy individuals. It can be observed that there is a clear shift of value levels for all sizes of the lung tumors and that this shift happens already within the reference range. This means that over the years when the tumor starts to grow the release of oncological biomarkers starts to increase beginning at the individual baseline values of a healthy person.

If - by consequence - kinetics or velocity of biomarkers would be observed it is highly susceptible that we could

detect early tumor stages. For CEA we know that an increase of 100% above the individual baseline values corresponds to 100% tumor specificity. Currently, we do not have the corresponding information for the other biomarkers. From a diagnostic point of view ProGRP would possess the diagnostic power and background to be suitable for screening purposes also by single investigations. ProGRP is not released by benign disorders and is only influenced by renal failure (values up to 300 pg/ml). ProGRP is not released by other cancers than neuroendocrine tumors and herewith especially by SCLC. In addition, ProGRP is released significantly already in limited disease of SCLC. By consequence a ProGRP value of > 200 pg/ml in a person without renal failure would be highly suspicious of SCLC (figure 2). However, SCLC is not the most frequent type of lung cancer and the total number of SCLC patients detected by ProGRP compared to all lung cancer patients is low. Prognosis of patients with SCLC is generally worse compared to other histologies, and the medical need of screening is thus not as evident. A combined analysis of various biomarkers with computer based interpretation of the pattern of release, is likely to increase diagnostic sensitivity as well as specificity in the near future.

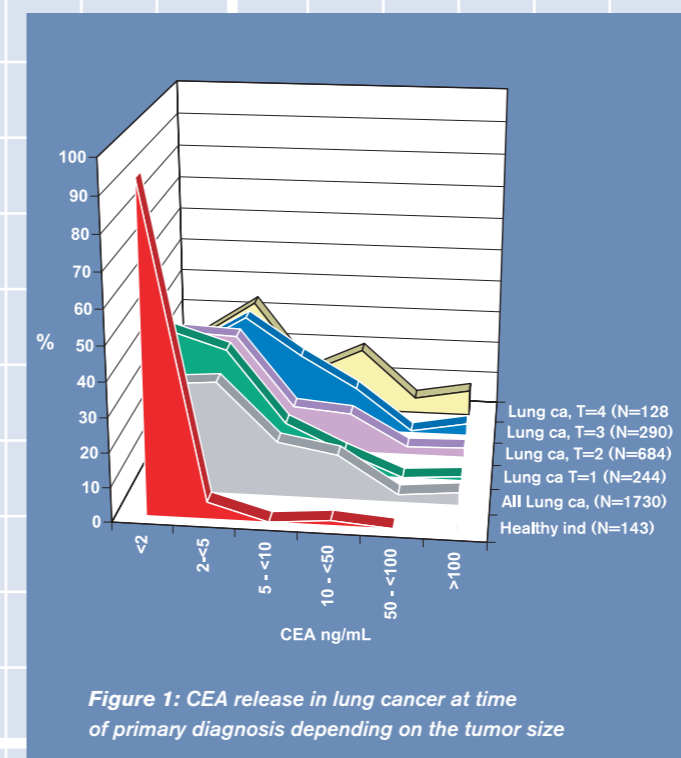


Figure 1: CEA release in lung cancer at time of primary diagnosis depending on the tumor size

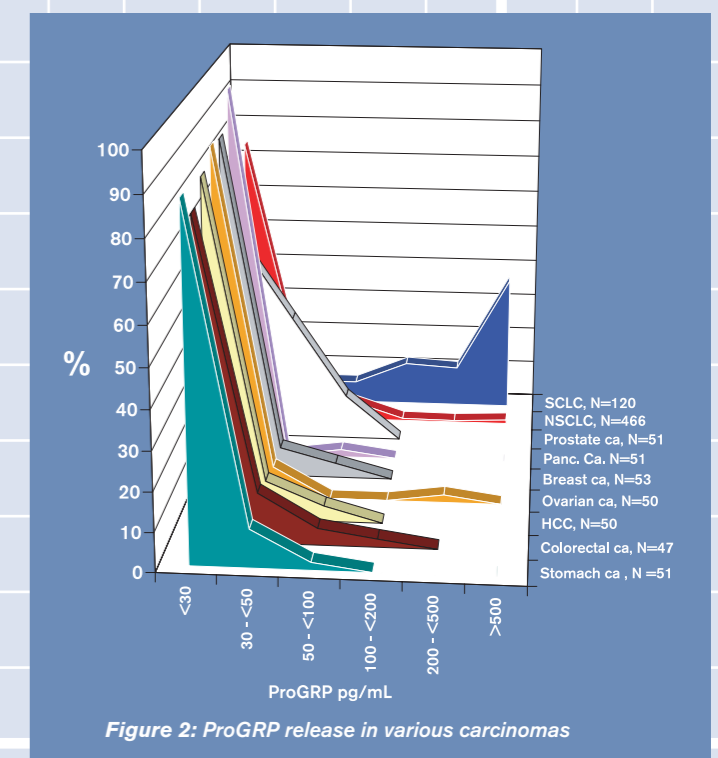


Figure 2: ProGRP release in various carcinomas

# Biomarkers in Lung Cancer



## PRIMARY DIAGNOSIS

In general, primary diagnosis and primary therapy of lung cancer is determined by clinical investigations including medical imaging, endoscopy and intra-operative findings. Although serum concentrations of CYFRA 21-1, NSE and CEA show significant correlation with tumor burden, there is no consistent relationship between production of these markers, tumor stage and tumor type. In most cases, high tumor marker concentrations reflect advanced tumor stage and therefore suggest a bad prognosis. However, low or mildly increased marker concentrations never exclude any kind of tumor disease or progression of disease. Despite these limitations, determination of tumor markers at the time of primary diagnosis is important because it allows a first estimate of tumor extent with the possible consequence of a more intense staging. It gives information about the prognosis irrespective of the tumor stage and about the histological subtype, which can lead to a different therapeutic regimen even before first surgery. Determination of CEA, CYFRA 21-1, NSE and ProGRP at the time of primary diagnosis may be performed as suggested in table 1.

In lung cancer in general (table 2) CYFRA 21-1 possesses at primary diagnosis an overall sensitivity of 40% (at a specificity of 95% for benign lung diseases, as recommended by the European Group on Tumor Markers, EGTM).

This is much better than those of CEA (25%), SCC (15%) and NSE (32%). Also in NSCLC CYFRA 21-1 was the best marker with 43% true positive test results as compared to CEA (26%) and SCC (17%). In squamous cell cancer CYFRA 21-1 is with 53% significantly superior to CEA (15%) and SCC (31%). But with respect to the histological classification SCC has a clear superior diagnostic specificity for squamous cell carcinomas, thus SCC may be useful because patients with SCC >2 ng/mL have a 80% probability of having a squamous tumor. CYFRA 21-1 in contrary is released with a high probability by all lung cancers irrespective of the histological background. In adenocarcinomas CYFRA 21-1 and CEA are with 22% and 33% sensitivity quite comparable. The combined determination increases sensitivity to 44%.

Neuron specific enolase NSE is well known since many years to be a relevant tumor marker in SCLC. Referring to benign lung diseases as relevant control group the sensitivities vary considerably depending on the extent of disease (limited disease – extensive disease) and on the composition of the control group with results ranging from 34% to 84%. It has already been reported in the literature in the 1970s that Gastrin Releasing Peptide GRP is often produced by SCLC cells and therefore could be helpful for the monitoring of these patients. But due to the instability of GRP it was not possible to measure this analyte by a

reliable method in blood. Only the development of an ELISA by Yamaguchi and coworkers in 1995 based upon the more stable precursor ProGRP led to first reliable results. The first very convincing clinical results from the same group have been confirmed by many Japanese and European colleagues.

ProGRP as single marker has a high sensitivity in SCLC as compared to benign diseases of the lung. When compared to CEA, CYFRA 21-1 and NSE, the relatively new marker ProGRP proved to be superior in tumor as well as organ specificity. The high ability of discrimination of ProGRP lies in the fact that ProGRP levels and extent of release are very low in various benign disorders and that even malignant tumors other than SCLC, with the exception of medullary thyroid carcinoma, release if at all very small amounts of ProGRP (figure 2). Depending on the extent of the disease the sensitivities for SCLC range from 47% to 80%. The diagnostic sensitivity of ProGRP is in most of the publications higher than NSE and in few publications comparable to NSE. But as expected due to the completely different pathophysiologic background of ProGRP and NSE, these two analytes have clear additive sensitivities in SCLC and play complementary roles in the diagnosis of SCLC (table 2, figure 3).

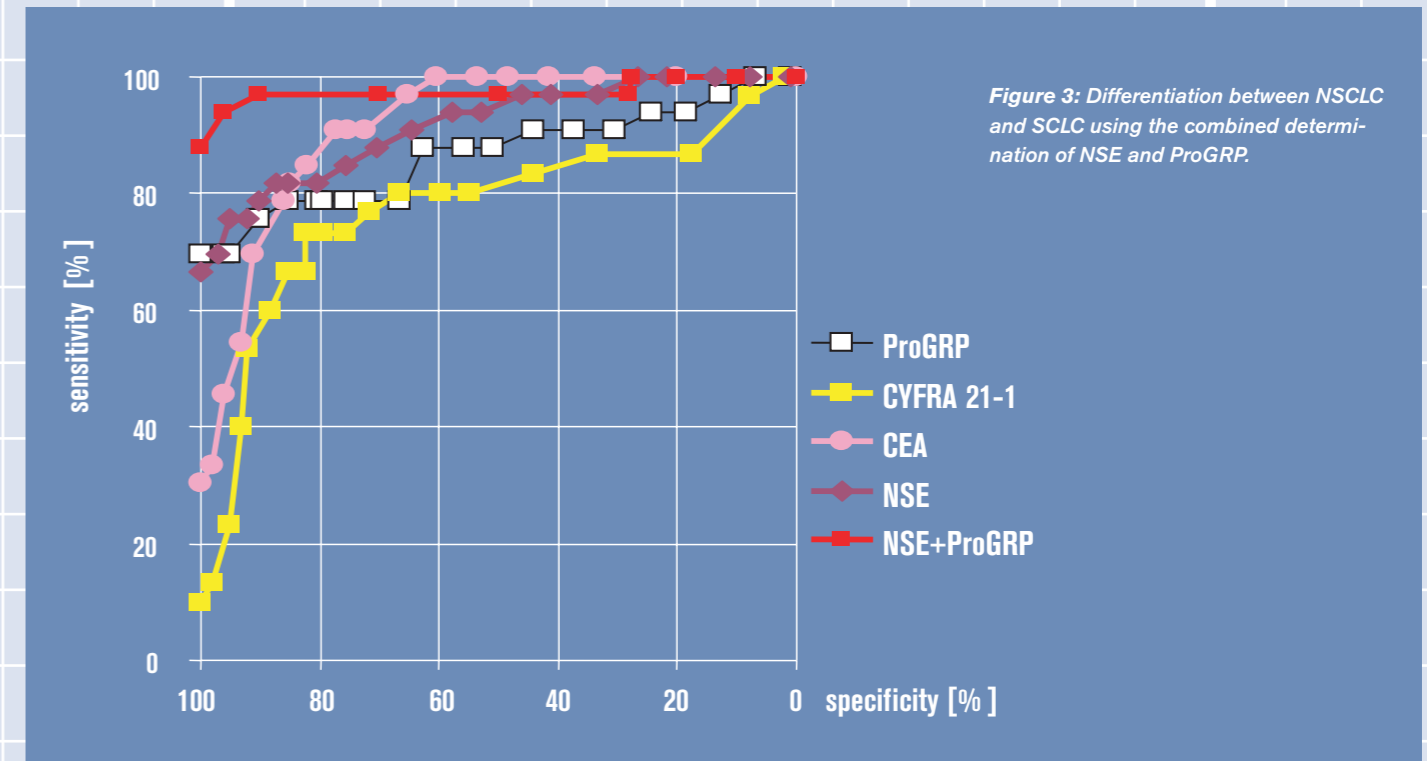


Figure 3: Differentiation between NSCLC and SCLC using the combined determination of NSE and ProGRP.

HISTORY	BEFORE THERAPY	POST-THERAPY FOLLOW-UP
unknown	CYFRA 21-1, NSE, ProGRP, CEA	after surgery: following histology without surgery: using the leading marker
adenocarcinoma	CYFRA 21-1 and CEA	CYFRA 21-1 and/or CEA
squamous cell carc.	CYFRA 21-1 and CEA	CYFRA 21-1 and/or CEA
large cell carc.	CYFRA 21-1 and CEA	CYFRA 21-1 and/or CEA
small cell carc.	NSE, ProGRP, CYFRA 21-1, CEA	NSE, ProGRP, CYFRA 21-1, CEA

Table 1: Tumor Markers in Lung Cancer

Marker	all lung cancer	NSCLC	SCLC	squamous cell	adeno	large cell
CYFRA 21-1	40	43	34	53	22	48
CEA	25	25	23	16	33	22
NSE	32	26	45	33	15	35
ProGRP	21	3	47	5	4	0
ProGRP or NSE	38	27	62	33	19	35
ProGRP or CYFRA 21-1	50	28	61	54	26	48
ProGRP or CEA	35	26	51	16	35	21
NSE or CYFRA 21-1	46	45	47	58	26	48
CEA or CYFRA 21-1	47	57	43	66	44	58
CEA or NSE	42	38	51	35	35	33

Table 2: Sensitivity (%) of CYFRA21-1, CEA, NSE and ProGRP in lung cancer (specificity 95% in benign lung diseases).

group \ marker	CYFRA 21-1 [ng/mL]	CEA [ng/mL]	NSE [ng/mL]	ProGRP [pg/mL]
colorectal	21	44	20	10
pancreas	34	28	0	0
stomach	22	23	14	0
liver	16	8	13	0
breast	26	26	10	3
ovarian	36	17	13	3
prostate	16	17	6	0
bladder	23	30	4	0

Table 3: Sensitivity [%] of CYFRA 21-1, CEA, NSE, ProGRP in other malignant diseases (Specificity 95%).

# Biomarkers in Lung Cancer

## DIFFERENTIATION BETWEEN LUNG TUMORS OF UNKNOWN ORIGIN

Again it has to be stated that all tumor associated antigens known at present like for example CYFRA 21-1, CEA, NSE and ProGRP are, or at least can be, released by benign diseases, various malignant tumors, non small cell lung carcinomas as well as small cell lung carcinomas – but the probability and extent of release into the blood circulation is different for each marker. Table 3 summarizes our findings established during the evaluation of CYFRA 21-1 and ProGRP. The specificity for these calculations is in general 95% for the corresponding benign diseases. It is obvious that CEA and CYFRA 21-1 are released by all other solid tumors, but it is also obvious that especially the ProGRP release is neglectable in other solid tumors.

For therapeutic approaches and decisions, it is extremely important to clarify the background of a lung tumor of unknown origin. In cases where no histology can be obtained due to various reasons or where histology failed, serological biomarkers can be of great relevance for differential diagnosis (figure 4, table 2).

CEA, the oldest and still the best marker for all adenocarcinomas, can be released by lung metastases of all adenocarcinomas (breast cancer, colorectal cancer, lung cancer, etc.). This means that in lung tumors of unknown origin a certain CEA-release > 20 ng/ml is highly suspicious for malignancy, but this could be either lung metastases from other primaries or any kind of primary lung cancer. Concerning the differentiation of unknown lung tumors a CYFRA 21-1-level > 15 ng/ml is indicative of a malignant lung tumor, CYFRA 21-1-values > 30 ng/ml are highly suspicious of primary lung cancer. But it is not possible to discriminate between NSCLC or SCLC by Cytokeratin 19-fragments.

According to own investigations in NSCLC, the release of ProGRP is reduced to a minimum of 3% (NSE up to 26%). In SCLC high serum levels of ProGRP are often measured, which can exceed NSE values considerably. A ProGRP-release > 300 pg/ml is indicative of primary lung cancer and is with a probability of 99% due to SCLC. A ProGRP-release of > 200 pg/ml in primary lung tumors is regardless of the pathohistological classification highly suspicious of at least a mixed histology with a small cell component.

ProGRP in combination with NSE possesses a high diagnostic ability to discriminate between NSCLC and SCLC, as can be observed in figure 3. One has to be aware of the experience that high NSE-values > 100 ng/ml can be due in few cases to other solid tumors mainly with liver metastases (stomach, colorectal), but in rare cases also due to lymphomas. One also has to be aware of that in very few cases advanced medullary carcinomas of the thyroid can lead to very high ProGRP-serum levels of several thousands pg/ml, but those cases are paralleled by a CEA- and especially significant calcitonin-release.

## Computer assisted differential diagnosis

Keller and coworkers tried to improve the diagnostic efficacy of tumor markers by mathematical evaluation of a tumor marker profile using fuzzy logic modeling. As compared to single tumor marker determinations the combined use of several markers and the additional interpretation by fuzzy logic improved the diagnostic accuracy significantly (additional 20%). This approach of computer assisted interpretation will certainly lead to a significant improvement in diagnostic oncology in the near future.

## PROGNOSIS

### Non-small cell lung cancer

The 5-year survival rate of NSCLC-patients after resection treatment depends clearly on the tumor stage at the time of the operation. The existence of resectable early stage disease (stage I, II and resectable IIIA) is the most powerful prognostic factor in NSCLC. Despite this, within 5 years after surgery, approximately half of the patients with apparent complete resection develop recurrence. This observation is certainly related to specific biological characteristics of tumors influencing their malignant potential. It is also related to the problems of efficient staging and of detecting occult disease from the beginning of tumor disease.

### Small cell lung cancer

In SCLC clinical stage at initial presentation is one of the most powerful prognostic factors identified in most studies. The TNM classification system was found to be inadequate for the staging of SCLC, as the extent of nodal involvement did not correlate with prognosis. This led to the development of the simple two-stage system of limited disease (LD) and extensive disease (ED). The median survival time without specific therapy would be 14 weeks for "limited disease" and 7 weeks for "extensive disease". After chemotherapy the remission rate varies between 70 and 95%. The median survival rate is about 10 to 15 months for limited disease and 7 to 10 months for extensive disease. 10 to 30% of the patients with limited disease live longer than 2 years compared to < 5% of patients with extended disease. In addition to clinical stage poor performance status, age > 70 years, male gender and paraneoplastic Cushing's syndrome were found to be significant clinical factors in determining prognosis in SCLC.

## Contribution by biomarkers

It has been proven by many, mostly retrospective investigations that tumor markers as well as "classical" analytes of laboratory medicine like LDH, albumin and urea can predict outcome in lung cancer patients. But the main problem in comparing data about prognostic evaluations is the heterogeneity of the

study populations (mixture of early and advanced stages, mixture of various histological types), univariate and multivariate analyses, observation of single and often new parameters instead of observation of new variables in comparison to already established ones especially clinical variables, not defined procedure how "optimized" cut off values have been chosen and even different logistics for the choice of cut off values within the same investigation and many other pitfalls. By consequence the results are heterogeneous and often even confusing. At present, large prospective trials are ongoing evaluating all clinical and laboratory variables in parallel.

In addition to this difficult background, there has to our knowledge, not been done any prospective randomized intervention trial based on tumor markers or other laboratory findings at the time of primary intervention in lung cancer. By consequence even the high independent prognostic significance of several analytes like LDH, NSE or CYFRA 21-1 is at the moment just of informal character but does not yet influence therapeutic decisions in a defined way.

## MONITORING OF THERAPY AND FOLLOW-UP

One main indication for tumor marker determinations in lung cancer is the control of efficacy of therapy and the postoperative follow-up care. An absolute prerequisite for any kind of monitoring investigations is the use of the same test kits for tumor marker determinations. Changing methods in this situation without performing one to two determinations for both methods - old and new - in parallel means not only wasting money but also most certainly many false negative and false positive test results with harmful consequences for the patient.

### Post-surgery

As for most of the other tumor markers in various carcinomas the speed of postoperative decrease of the corresponding tumor marker serves as a first sign of prognosis. It is important to state that the postoperative control investigation must be performed irrespective of the level before first treatment, also levels within the reference range can be patho-

logical for the individual patient (Figure 1). After curative surgery, all markers can display an initial short-term increase followed by a rapid decline to individual baseline levels. The decline curve of tumor markers appears to depend on both biological half-life of markers and residual tumor cells. The biological half-life time of cytokeatin markers as well as SCC-Ag were found to be very short: 1.5- 3 hours. Therefore, after curative i.e. R0 resection the levels of these markers decrease rapidly and fall into the reference range within 1-2 days after surgery. The half-life of CEA is longer, ranging from 1 to 4 days. The time it takes for CEA to reach normal levels depends on its initial level.

It is important to note that renal or liver dysfunction may affect clearance of tumor markers and by consequence prolong their half-time. It can be assumed that, in the absence of renal or liver impairment, prolonged marker clearance and/or an elevated plateau suggest the presence of residual tumor cells. Tumor marker values which are decreasing slowly and perhaps even not moving down to the range < the 95% percentile of healthy individuals can be a sign of non-curative surgery or insufficient staging at primary diagnosis (occult metastases). Determination of postoperative individual baseline values is necessary for follow-up and especially for detection of recurrent disease. Even if no follow-up care is performed and the patient is coming back after a certain period of time with symptoms, the interpretation of tumor marker findings at this time will only be possible on the basis of the postoperative individual baseline values.

## Controlling systemic therapy

Several studies on NSCLC have been performed assuming a 40% marker increase as disease progression and a 65% decrease as partial response. Most studies have reported that CYFRA 21-1 had the best concordance with clinical evaluations, the concordance in response to treatment varied between 59 and 75%. In the detection of progressive disease specificity was 100% and sensitivity 52% and 69% respectively, the concordance with remission much lower, only 42%. CYFRA 21-1 changes alone after one course of chemotherapy seem not to be sufficient to predict the future response after three courses

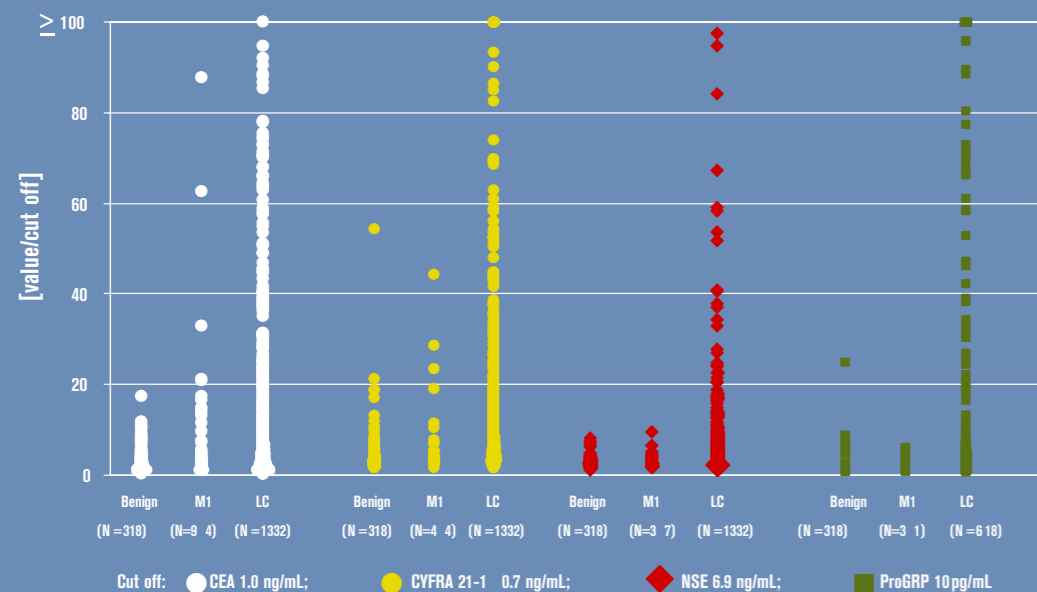


Figure 4: DOT Plots CEA CYFRA 21-1, NSE, ProGRP Benign Lung Diseases, Lung Metastases (M1) and Primary Lung Cancer (LC).

(sensitivity 52%, specificity 56%, PPV 45%). In SCLC NSE is described since more than 10 years to be a relevant parameter in reflecting the clinical course and the response to therapy. It has to be noted that during the course of chemotherapy, temporary NSE increases occur, in the case of response, 24 to 72 hours after the first cycle as a result of tumor cytolysis. Elevated pretreatment serum levels decrease rapidly within a week or by the end of the first treatment cycle. In contrast, failure of therapy is associated with persistently elevated, or only intermittently decreased concentrations, which do not return to the individual baseline values.

In general clinical practice NSE has since many years been the only useful activity marker for monitoring of disease course and treatment of patients with SCLC, based on a high diagnostic profile (sensitivity 93%, PPV 92%) being superior to LDH. Up to now there exist no evaluations on the combined use of ProGRP and NSE under systemic therapy. Our own experiences demonstrate that ProGRP is a very reliable parameter for therapy response in those patients with primarily elevated ProGRP.

It must be stressed, however, that observation of tumor marker kinetics without reacting on the results cannot be a helpful procedure. In our opinion, this means that – outside of clinical studies – imaging techniques like X-ray and CT scans can be reduced significantly as long as the relevant tumor markers are decreasing and the patient's clinical condition is improving or at least stable. If therapeutic consequences cannot be drawn tumor marker determinations are not indicated. Increasing tumor markers point to progressive disease very early, often weeks (or months) before imaging techniques prove the progression of the disease. Whether an immediate change of treatment will lead to prolongation of survival is unknown especially in the situation of lung cancer where the number of systemic therapies available is limited.

## Detection of recurrent disease

After radical surgery or in complete remission, all patients should be post-examined every 3 months within the first 2 years, later on every 6 months as the risk of recurrence is remarkably high (70 to 90%). The investigations should include chest radiography, computed

tomography of the chest, clinical chemistry (renal and liver function, LDH, blood sedimentation rate), tumor markers according to Table 1, bronchoscopy and sceleron scintigraphy.

During follow-up an increase in tumor marker starting from the individual baseline values can be the first sign of recurrent tumor disease. Only few studies have reported on the use of serum tumor markers in monitoring the course of disease after curative surgery. This is certainly due to the fact that in the past there were no really efficient therapeutic approaches available at time of recurrent disease which led to a defensive and partly negative attitude in the follow-up situation. In NSCLC CYFRA 21-1 is known for its high efficacy in the early detection of recurrent disease. At a specificity of 100% CYFRA 21-1 detects recurrent disease in 79% of patients with no increased CYFRA 21-1-release by the primary tumor and in 100% of patients with an increased release by the primary tumor. The lead-time varies between 2–14 months. In SCLC the combination of ProGRP and NSE detects recurrent disease in 79% of the patients. The median lead-time is around 35 days for ProGRP, no lead time could be found for NSE.



Dr Sven Erickson is our new Marketing Manager. Since August 2004 he is responsible for marketing and sales strategies for reagents, as well as launch strategies for new products.

Dr Sven Erickson received his PhD in experimental oncology at the Karolinska Institute in 2000. In his thesis he investigated how the anti-tumor drug Interferon-alpha affects cell cycle regulatory proteins and cellular proliferation in malignant cells. During a post-doc at AstraZeneca he continued his research with studying transgenic cancer models.

Prior to joining us at CanAg Diagnostics, Dr Sven Erickson held several positions, both within marketing and medical support, at Roche Pharmaceuticals, Sweden.

## 96<sup>th</sup> American Association for Cancer Research (AACR) Annual Meeting, Anaheim, California, April 16-20, 2005

## SUMMARY

Among many tumor-associated antigens and substances described and evaluated NSE, ProGRP, CYFRA 21-1 and CEA proved to be the most relevant tumor markers in lung cancer with high diagnostic capacity. The relatively bad prognosis for lung cancer patients in general, and the lack of satisfactory treatment modalities for recurrent disease at least in the past, have led to a restrictive and even destructive attitude for the application of tumor marker determinations, especially in follow-up care. Generally, follow-up investigations of any kind are of limited use in the absence of alternative therapy. But besides these limitations tumor markers as non-invasive, reproducible, fast and inexpensive method provide very helpful diagnostic tools in differential diagnosis and in monitoring the efficacy of therapy.



AACR is an international meeting on cancer research. It was held at the Anaheim Convention Center, with 10 000 participants from all over the world. Together with IBL CanAg Diagnostics had a booth that was well-attended. A scientific program with more than 6 200 posters, 30 symposia and more than 50 “morning sessions” featuring experts in different fields. In addition, a number of oral presentations in minisymposia and educational session.

This was the 96th AACR-meeting in short. The focus of the programme was integration of science and clinic. In plenary lectures basic science aiming at improving diagnosis and treatment of cancer was presented. It is obvious that knowledge in areas of signal pathways and molecular biology is constantly increasing. However, it takes time for scientific achievements to reach the clinic and to result in more specific therapies and improved cancer diagnosis.

# Distributor in Argentina – Bioars S.A.



Bioars S.A. is an IVD Company for diagnostic and integrated equipment and has its head office in Buenos Aires, Argentina. Bioars has totally 11 employees and 99% of their product list is CE marked or FDA approved. In 2004 the strategy was focused on increasing the business in the ELISA, IFA and Hematology segments and also on the development of new market segments. To achieve this Bioars started new collaborations with leading companies worldwide such as: CanAg Diagnostics, Sweden (Tumor Markers), Immuno Concepts, USA (Autoimmunity) and Helena Laboratories, USA (Electrophoresis).

Through the distributor agreement Bioars S.A. has the exclusive right to distribute CanAg Diagnostics' EIA kits in all Argentine territory. This agreement allows all Argentine laboratories access to a complete line of products for tumor marker diagnosis.

**“ Quality and Innovation are the main characteristics of CanAg Diagnostics' EIA kits.”**

## Conferences

### **CANAG DIAGNOSTICS AB WILL BE ATTENDING THE FOLLOWING MAJOR CONFERENCES THIS YEAR:**

#### **6th World Congress on Melanoma**

September 6-9  
*Vancouver, Canada*

#### **4th International Conference on Biochemical Markers for Brain Damage**

September 8-10  
*Maine, USA*

#### **ISOBM 2005**

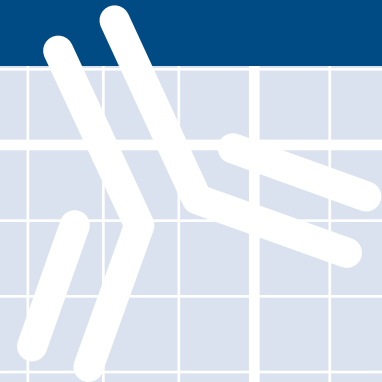
September 24-28  
*Rhodes, Greece*

#### **Medica 2005**

November 16-19  
*Düsseldorf, Germany*

#### **Hamburger Symposium über Tumormarker**

November 27-29  
*Hamburg, Germany*



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