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DISCOVERY AND VALIDATION OF PROTEIN BIOMARKERS FOR LUNG CANCER

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We lung cancer patients cannot wait for evidence

Anders Jonasson (1955-2009).
Founder and former Chairman of the Association for the
Support of Lung Cancer Patients in Stockholm.

Not all those who wander are lost

J.R.R. Tolkien

ABSTRACT

Lung cancer is a disease characterized by high incidence and mortality rates as well as by a remarkable resistance to common treatment strategies. Reliable and validated biomarkers could represent a possible breakthrough in the management of this tumor type, by facilitating diagnosis, refining prognosis and providing guidance towards the choice of the most appropriate therapy

In this thesis we approach the field of lung cancer biomarkers using proteomics technologies on tumor and plasma samples from a large local institutional biobank.

In paper I a protocol to prepare enriched tumor cell suspensions from freshly collected surgical samples is developed. Eight cases are analyzed using a bottom-up proteomics approach. Compared with the direct lysis of fresh frozen specimens, with this sample preparation we were able to identify more than twice as many proteins. In addition, we could demonstrate an effective removal of contaminants deriving from plasma, stroma and red blood cells.

In paper II we analyze with a top-down proteomics approach tumor lysates from 39 lung cancer cases. We could observe a characteristic 3-peak profile which corresponded to the post-translational modification pattern of the calcium-binding protein S100A6. We performed an early clinical validation of the expression of this protein on 103 lung cancer samples using immunohistochemistry on specimens loaded on a tissue microarray. In cases with a negative p53 expression, the positive tumor expression of S100A6 was associated with a significant survival improvement, confirming on clinical material the pro-apoptotic role proposed for S100A6 by in vitro investigations and suggesting a functional connection with the p53 pathway.

An extensive validation of the findings obtained in paper II is performed in paper III. The expression of S100A6 and other proteins from the same protein family, namely S100A2, S100A4, S100A7, S100A8, S100A9, S100A10 and S100A11, was assessed on a large and well characterized tumor cohort consisting of 494 stage I lung cancer cases. S100A2 and S100A6 were shown to be positive prognostic factors of progression-free survival in p53-negative adenocarcinomas, whereas S100A10 was a negative prognostic factor of progression-free survival in p53-positive squamous-cell tumors.

Finally, in paper IV we evaluate the diagnostic and prognostic role of plasma level of two forms of cytokeratin 18, an indicator of cell death, in comparison with the well established biomarker Cyfra 21.1 (cytokeratin 19 fragment). The study was conducted on 179 lung cancer cases, 113 subjects with benign lung diseases and 200 healthy blood donors. Cyfra 21.1 was a more reliable diagnostic biomarker than cytokeratin 18 (diagnostic accuracy of 94% vs 56%, respectively). However, in the total lung cancer cohort and in a subset of 78 patients with stage III-IV lung cancer receiving either combination chemoradiotherapy or 1st-line palliative chemotherapy, cytokeratin 18 was the only biomarker which retained an independent prognostic potential on multivariate survival analysis.

In conclusions, the works presented in this thesis explore possibilities and limitations of biomarker research on lung cancer, highlighting the importance of the quality of the samples and of the accuracy and completeness of clinical data in order to obtain reproducible results.

LIST OF PUBLICATIONS

I. **De Petris L**, Pernemalm M, Elmberger G, Bergman P, Orre L, Lewensohn R, Lehtiö J. A novel method for sample preparation of fresh lung cancer tissue for proteomics analysis by tumor cell enrichment and removal of blood contaminants. *Proteome Science* 26;8:9, 2010.

II. **De Petris L,** Orre LM, Kanter L, Pernemalm M, Koyi H, Lewensohn R, Lehtiö J. Tumor expression of S100A6 correlates with survival of patients with stage I non-small cell lung cancer. *Lung Cancer* 63;3:410-417, 2009

III. **De Petris L**, Kanter L, Melotti F, Bergman P, Kölbeck K-G, Hellborg H, Adolfsson J, Lewensohn R, Lehtiö J. Clinical implications of the tumor expression of S100 proteins in non-small cell lung cancer. *(Manuscript)*

IV. **De Petris L,** Brandén E, Herrmann R, Chavez-Sanchez B, Koyi H, Linderholm B, Lewensohn R, Linder S, Lehtiö J. Diagnostic and prognostic role of plasma levels of two forms of cytokeratin 18 in patients with non-small cell lung cancer. *European Journal of Cancer,* in press

Related publications:

A. Pernemalm M, **De Petris L**, Eriksson H, Brandén E, Koyi H, Kanter L, Lewensohn R, Lehtiö J. Use of narrow-range peptide IEF to improve detection of lung adenocarcinoma markers in plasma and pleural effusion. *Proteomics* 9;13:3414-3424, 2009

B. Lehtiö L, **De Petris L**. Lung cancer proteomics, clinical and technological considerations. *Journal of Proteomics* E-pub June 1, 2010. Review

LIST OF ABBREVIATIONS

2DE	2-dimension electrophoresis
AUC	Area under the curve
BSC	Best supportive care
CI	Confidence interval
CK	Cytokeratin
CT	Computed tomography
ED	Extensive disease
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMEA	European MEdicines Agency
EORTC	European Organization for the Research and Treatment of Cancer
ERCC1	Excision repair cross-complementation group 1
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
FISH	Fluorescence in situ hybridization
HIV	Human immunodeficiency virus
HR	Hazard ratio
IASLC	International Association for the Study of Lung Cancer
IHC	Immunohistochemistry
iTRAQ	Isobaric tags for relative and absolute quantification
LCM	Laser capture microdissection
LD	Limited disease
MALDI	Matrix assisted laser desorption ionization
MS	Mass spectrometry
N-CAM	Neural cell adhesion molecule
NCI	National Cancer Institute
NIH	National Institute of Health
NPV	Negative predictive value
NSCLC	Non-small cell lung cancer
NSE	Neuron specific enolase
PCA	Principal component analysis
PCI	Prophylactic cranial irradiation
PET	Positron emission tomography
PLS-DA	Partial least square – discriminant analysis
PPV	Positive predictive value
PSA	Prostate specific antigens
ROC	Receiver operating characteristic
SBRT	Stereotactic body radiotherapy
SCLC	Small cell lung cancer
SEER	Surveillance Epidemiology and End Results
SELDI	Surface enhanced laser desorption ionization
SNP	Single nucleotide polymorphism
SUV	Standard uptake value
TKI	Tyrosine kinase inhibitor
TMA	Tissue microarray
TNM	Tumor Node Metastasis
TOF	Time of flight
TTF-1	Thyroid transcription factor 1
UICC	International Union Against Cancer
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

CONTENTS

NOTE TO READERS.
This thesis contains a general introduction followed by a more specific part about the objectives and results of the current study. In the general introduction section I describe the opportunities and problems related to the areas of lung cancer, biomarkers and proteomics. I tried to summarize the concepts to make them clear both to readers with a biological background as well as to those with a clinical background. For this reason I tried to avoid too specific phrasing and have approximated numerical data. Consequently, the work is far from being exhaustive and for more accurate details I refer to specific citations. I apologize with medical professionals for not being adequately evidence-based medicine, and with biology professionals for not being sufficiently molecularly consistent. However, I hope that this will facilitate the comprehension and use of this content to readers with different backgrounds.

1 INTRODUCTION

1.1 LUNG CANCER

Lung cancer is the leading cause of cancer-related mortality worldwide. The possibility that a patient with lung cancer will be alive after five years from the diagnosed is approximately 15% [1]. The 2004 WHO classification defines seven histology types of primary malignant epithelial tumors of the lung and more than 20 different variants [2]. Nevertheless, it is widely recognized that two major clinical entities exist with distinct clinical characteristics and behavior, namely small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The former represents approximately 15% of lung cancer cases. It has neuroendocrine origin and is strongly induced by smoking. SCLC cells are small in size, similar to lymphocytes and form undifferentiated patterns with large necrotic areas. SCLC is a very aggressive disease which in the majority of the cases at time of diagnosis has already spread to form distant metastases. SCLC is highly sensitive to chemotherapy and radiotherapy. However, after an initial response to treatment, resistant clones inevitably start growing and the disease rapidly progresses [3]. Epithelial lung tumors other than SCLC have been grouped into a non-further specified category named NSCLC. The major histology types comprised in this group are adenocarcinoma, squamous-cell carcinoma and undifferentiated large-cell carcinoma. Despite some epidemiological and clinical differences, NSCLC types share some common features. Approximately 30% of the cases are diagnosed at an early stage of disease, still amenable for curative surgical resection. Several CT-scan based screening studies on heavy smokers have been conducted with the aim to identify more cases at an earlier stage. However, this procedure is expensive and has not demonstrated so far to be effective in reducing lung cancer-related mortality [4]. For a long time all NSCLC types have been uniformly treated mostly with drugs and regimens towards which they show a strong primary resistance. Similarly, all NSCLC types have been studied as a unique entity in clinical trials and for clinical practice guideline purposes[5].

Nowadays a classification of lung tumors as SCLC and NSCLC is no longer sufficient and a specific definition of the correct histology is warranted in order to implement the most appropriate treatment.

The present thesis will mainly focus on NSCLC, although some aspects of SCLC will also be described. For a complete and exhaustive work about all aspects of lung cancer, from epidemiology to pathology to treatment, it is advised to refer to a special issue of the journal Chest, containing the clinical practice guidelines developed by the American College of Chest Physicians (Chest 132;3:supplement, September 2007).

1.1.1 Brief summary of epidemiology and staging

Each year, lung cancer kills approximately 3,400 people in Sweden, 340,000 in Europe and 1.4 million people in the world (Globocan 2008 www-dep.iarc.fr). Lung cancer is becoming more and more a disease of the elderly, with more than 40% of cases diagnosed in subjects aged >70 years. Smoking consumption is undoubtedly the first cause of lung

cancer and, given the poor treatment possibilities, the only real breakthrough in the fight against this disease is the control of smoking by government-mediated acts. The incidence of lung cancer is directly associated with smoking exposure, which is measured with an index known as "pack-year". One pack-year corresponds to the consumption of 1 pack of cigarettes (20 cigarettes) each day for one year, or alternatively half a pack for 2 years and so on. Given the fact that it is extremely unlikely to start smoking after the age of 20 years, the target population to educate about the damages caused by smoking are teenagers [6]. Lung tumors caused by smoking account for 75% of cases, whereas the rest is not related to this habit [7]. Lung cancer not caused by smoking is characterized by deregulation of the *EGFR* pathway, while smoking-induced tumors are based on the *RAS* pathway and the lack of p53 function caused by the selective hot-spot mutations of the *TP53* gene induced by the action of the carcinogens on the bronchial stem cell [8]. Once the policies against smoking will be more and more implemented worldwide, a definite decrease in overall lung cancer incidence and mortality will be observed, with a relative increase in the percentage of non-smoking related tumors, against which some novel and effective drugs are already available.

The most common lung cancer histology type is adenocarcinoma, followed by squamous-cell carcinoma and SCLC.

Lung cancer diagnosis and staging is mainly based on the following procedures; imaging techniques (chest X-ray, CT scan and PET scan, nowadays usually in combination with CT to merge metabolic and anatomical information), minimally invasive approaches (bronchoscopy, endobronchial ultrasound, fine-needle and middle-needle aspiration biopsies) and invasive techniques (mediastinoscopy, video assisted thoracoscopy or open thoracotomy). All these procedures aim at correctly defining the extension of the disease (stage), and to obtain a tumor sample for proper histological diagnosis.

NSCLC is staged according to the international TNM (Tumor Node Metastasis) classification [9]. The combination of the T, N and M descriptors define each stage and categorize NSCLC into three main prognostic and treatment groups; early stage (I and II), locally advanced (stage III) and metastatic (stage IV).

Recently, the International Association for the Study of Lung Cancer (IASLC) has released a proposal to update the staging system [10]. The proposal has been accepted by the International Union against Cancer (UICC) and included in the 7th edition of the TNM classification of tumors. This classification, developed on more than 130,000 cases and externally validated on >32,000 cases from the SEER database, has contributed to improve the prognostic definition of stage. The major changes concern the T and M descriptors. For example, the presence of pleural effusion has moved from T4 to M1, and consequently from stage IIIB to IV. Conversely, the presence of metastasis in the same lobe has moved from T4 to T3, and consequently to a potential resectable stage in case of N0 or N1 disease. In addition, tumor with a diameter >7 cm, previously staged as T2 if not meeting the criteria for T3 (atelectasis of the entire lung; tumor of the main bronchus at <2 cm from the carina; invasion of the thorax wall, diaphragm or pericardium), now moved definitely to the T3 category, leading to a better definition of the stage I class. These changes must be taken into account when performing biomarker research, because the prognostic definition of the 7th TNM version is much more accurate than the previous version.

SCLC is classified according to a modified system developed by the Veteran's Administration Lung Cancer study group, which recognizes only two stages. If the tumor is localized to one hemithorax and can be comprised within a unique and safe radiotherapy field it is defined as limited disease (LD), otherwise it is defined extensive disease (ED) [11].

1.1.2 Brief summary of treatment modalities

Several clinical trials on lung cancer conducted during the past decade have contributed to the acquisition of evidence-based knowledge about lung cancer treatment options, ultimately leading to an improvement of patient survival. However, it is worth noting that for both NSCLC and SCLC such improvement in survival seen in the past ten years is the result of better diagnostics, more refined staging and progress in lung medicine and supportive care rather than of significant advances in the outcome of available therapies [12, 13].

Treatment of lung cancer depends on the clinical stage at diagnosis. Table 1 summarizes the percentages of patients diagnosed at each stage, the general treatment strategy and the expected outcome in terms of 5-year survival rate.

	Stage	% of patients	Treatment Strategy	5-year survival
NSCLC	I	17%	Surgery or SBRT with curative intent in non-operable cases	65%
	II	13%	Surgery + adjuvant chemotherapy	35%
	III	20%	Chemotherapy + radiotherapy	15%
	IV	35%	Palliative chemotherapy or biological agents	<5%
SCLC	LD	6%	Chemotherapy + radiotherapy ± PCI	25%
	ED	9%	Palliative chemotherapy	<5%

Table 1. Summary of clinical data by stage. See text for references and details (with courtesy of AIOT, Italian Association of Thoracic Oncology www.oncologiatoracica.it)

The treatment of choice for early stage NSCLC (Stages I and II) is radical surgical resection of the primary tumor, obtained with the removal of the lung lobe or sometimes of the entire lung, together with a systematical dissection of mediastinal lymph nodes [14]. When the tumor size is <5 cm and there are no nodal or distant metastases, but the patient cannot undergo surgery due to co-morbidities or reduced respiratory function, high dose hypofractionate stereotactic body radiotherapy (SBRT) can be a suitable and effective treatment option, leading to disease control and survival outcomes similar to surgery [15]. In case of stage II disease, the administration of cisplatin-based postoperative (adjuvant) chemotherapy has shown to improve patient survival by 5% at five years. On the other

hand the use of adjuvant chemotherapy is detrimental in stage IA tumors (size <3 cm) and is still a questionable approach in stage IB tumors [16].

Locally advanced NSCLC is treated with a combination of chemotherapy and radiotherapy, which can be administered sequentially or concurrently. With this treatment, objective tumor response (reduction of at least 25% of tumor volume) is obtained in approximately 65% of cases and median survival time is approximately 15 months [17]. In case or resectable IIIA disease (resectable primary tumor with the presence of ipsilateral N2 mediastinal lymph node metastases) treatment with neoadjuvant (preoperative) chemotherapy ± radiotherapy followed by surgery may improve local tumor control. However, randomized trials have not shown any advantage for this strategy as compared with chemoradiation administered with curative doses [18, 19].

The majority of clinical trials on lung cancer have been conducted in advanced/metastatic Stage IV NSCLC [20]. In brief, the current clinical practice guidelines suggest a first-line treatment consisting of a combination of a platinum compound (either cisplatin or carboplatin) with a third generation drug (taxanes, gemcitabine, vinorelbine). All combinations have different toxicity profiles, but are equivalent in terms of efficacy. For at least 10 years, the response rate to first-line chemotherapy and the median survival time have not gone beyond the disappointing levels of 35% and 7 months, respectively [5, 21]. Recently, new drugs have been developed and the attitude towards treatment choice has also changed. One of the novel agents is pemetrexed, a multitargeted antifolate. Registration trials showed that the combinations cisplatin/pemetrexed and cisplatin/gemcitabine are equally effective for the first-line treatment of NSCLC [22]. Moreover, single agent pemetrexed is equivalent to docetaxel for second-line NSCLC, namely as salvage therapy once the tumor has progressed after a first-line intervention [23]. However, subgroup analyses of those trials showed that pemetrexed is superior to the comparative regimens in NSCLC with non-squamous histology (adenocarcinoma and large-cell carcinoma), and detrimental in squamous tumors [24]. This may be ascribed to the expression levels of the enzyme thymidylate synthase, a target of pemetrexed, which are higher in squamous compared to non-squamous tumors [25]. Following this analysis, the American and European regulatory agencies (FDA and EMEA, respectively) approved the use of the drug only for the treatment of non-squamous NSCLC.

Another agent is bevacizumab, a monoclonal antibody against the vascular endothelial growth factor (VEGF). In combination with chemotherapy, bevacizumab improves survival of lung cancer patients compared to chemotherapy alone [26]. This evidence was acquired on non-squamous tumors only, because of safety issues. By inhibiting VEGF, in fact, bevacizumab exposes patients to higher risk of bleeding, and is therefore not indicated in subjects with squamous tumors.

Finally, a novel class of compounds, the inhibitors of the tyrosine kinase domain of the Epidermal Growth Factor receptor (EGFR-TKI), gefitinib and erlotinib, has also emerged as potential breakthrough in NSCLC therapy. A series of clinical trials conducted on unselected patient populations using these drugs as single agents or in combination with chemotherapy produced sobering results [27-30]. However, clinical observations suggest that EGFR-TKIs have a higher activity in adenocarcinomas, women, never smokers and East Asians. Molecular studies on tumor samples revealed

that these drugs are effective only in patients whose tumor harbor a specific mutation of the *EGFR* gene, which mainly drives the carcinogenesis profile of the tumor and confers sensitivity to these agents [31]. Again, FDA and EMEA approved the use of EGFR-TKIs for the first-line treatment of NSCLC only in tumors with confirmed *EGFR* mutations, which unfortunately accounts for approximately 15% of non-squamous tumors in Caucasians [32].

LD-SCLC is treated with a combination of chemotherapy and thoracic radiotherapy. The objective tumor response rate in LD-SCLC is approximately 80%, with a median overall survival of 20 months [33, 34]. ED-SCLC treatment is based on the combination of two old second-generation drugs, namely cisplatin and the topoisomerase II inhibitor etoposide. This regimen gives objective responses in 60% of cases and a median overall survival of 10 months [33, 35]. Although some attempts have been made in order to improve the outcome of ED-SCLC patients by introducing the topoisomerase I inhibitor irinotecan, in combination with cisplatin, evidence is still conflicting and the cisplatin-etoposide combination remains the treatment of choice in this setting [36]. In case of complete response to first-line treatment, irrespective of initial tumor stage, prophylactic cranial irradiation (PCI) is standard of care, since it has proven to reduce the incidence of brain metastasis and improve patient survival [37].

1.2 CANCER BIOMARKERS

According to the definition developed by the NIH, a biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [38]. In other words, biomarkers are measured in order to acquire relevant knowledge concerning diverse aspects of a disease, to be converted into clinically useful information that may serve to improve the disease management and, ultimately, the patient outcome. As such, it is not correct to talk about biomarkers if the findings have not undergone an evaluation at the clinical level on patient material. Conversely to what is commonly believed, biomarkers are not only, or not necessarily molecules. A biomarker is any kind of measurable change which may have clinical relevance. Biomarkers may be for example: i) certain proteins present on the tumor or released by the tumor in the blood, which indicate the recurrence of the disease after a curative surgical intervention; ii) a single nucleotide polymorphism (SNP) haplotype correlated to the risk that patients will develop a certain drug-related toxicity; iii) the expression level of mRNA or the presence of a gene mutation targeted by a drug; iv) but even the metabolic activity of the tumor, measured by the standard uptake value (SUV) of images obtained during PET examination, or the number of circulating tumor cells may as well considered biomarkers.

When performing research aiming at the discovery and validation of biomarkers, a number of issues must be taken into account.

Firstly, the method used to measure the biomarker must be standardized and reproducible. Quantification issues, as well as the determination of a cutoff value which discriminates biomarker-positive vs biomarker-negative cases may be troublesome even in commonly used methods, such as immunohistochemistry. In addition, when the sample

undergoes specific manipulation and preparation before it is analyzed, these may have unexpected effects at the molecular level, as for example mRNA rapidly degrades or phosphopeptides are very unstable. Hence the way such procedures are conducted should be taken into consideration when results are elaborated. In addition, a characteristic of high throughput technologies is the discovery of biomarkers based on the combination of different analytes instead of single molecules. In such a case, an algorithm based on all interesting variables useful to correctly define biomarker-positive vs negative samples must be developed before proceeding to clinical validation. To obtain a reliable algorithm, early during the discovery phase overfitting of data should be controlled by proper multivariate data analysis, such as PCA and PLS-DA [39, 40].

Moreover, it should be considered that the analytes selected as potential biomarkers must somehow have a relationship with the clinical question (diagnosis? prognosis? response to treatment?) and the tumor characteristics, to reduce the risk of false positive results and bias. High throughput technologies, including global genome analysis, mRNA microarray and proteomics profiling may lead to the selection of a number of genes or a number of mass spectrometry (MS) signals which may be associated with a clinical outcome in that given patient cohort, but find no plausible correlates with the clinical question. More about this aspect will be discussed below.

Finally, for an analyte to be defined as a biomarker and be introduced in the clinical practice, it is essential that it successfully undergoes validation. A high number of potential biomarkers are indeed discovered daily, which do not reach clinical practice because of insufficient validation. The clinical validation of biomarkers is a very long and expensive process, which similarly to drug development, should be conducted following three consecutive steps [41]. However, conversely to trademark drugs, which receive heavy sponsoring from pharma companies, biomarker research is mostly funded by academical institutions, and this sensibly limits the conduction of well designed prospective clinical trials.

Step 1. Retrospective analysis on biobank material. This step requires of course free access to large biobanks with accurate information about clinical variables. The selected sample size is divided into two groups, a training set and a test set. In the former, the method to quantitate the analyte is set up, cutoffs or algorithms are developed and the clinical significance of the results is corrected for all possible confounding factors. In the test set the results obtained in the training set are blindly internally validated.

Step 2. Retrospective analysis of prospective clinical trials. In this step clinical data and outcomes are prospectively monitored and collected. Samples are usually collected after the trial is completed, and biomarker analyses are associated to clinical correlates. The advantage with this step is that the intervention on patients, in terms of treatment or diagnostic procedures, is much more accurate than any retrospective biobank collection. The clinical hypothesis is prospectively stated and the sample size consequently calculated. In case of randomized trials a comparison with the diverse treatment arms can be performed and a crucial differentiation between prognostic and predictive biomarkers may be obtained (see below). The disadvantages are that it may be extremely difficult to acquire

specimens, since usually such studies are multicentric. Consequently, due to the reduction of sample size to only available cases, statistical power may not be retained to justify subgroup analyses. Regulatory agencies may however accept evidence from retrospective analysis of prospective trials to register the use of a biomarker for specific clinical purposes, as it has happened for the already mentioned case of the registration by FDA and EMEA of testing for *EGFR* mutation and EGFR-TKI treatment.

Step 3. Prospective clinical trials. The ultimate evidence to determine the real clinical role of a biomarker is validation with prospective randomized trials. There are several strategies that can be implemented to conduct such trials. It is beyond the scope of the present summary to descript such strategies in detail. However three aspects deserve further discussion. The first one is that the biomarker analysis must be performed when the patient is registered in the study, before any randomization or initiation of treatment. Secondly, the trial design must take into account the prevalence of the biomarker in the target population, in order to have two numerically comparable groups of biomarker-positive and negative cases. Finally, the expected differences in clinical outcomes by biomarker expression must be accurately retrieved from preliminary evidence and used to calculate the correct sample size. This is required in order to detect expected differences with adequate statistical power. This latter aspect may result in a considerable increase in the number of patients needed to perform the trial, *de facto* making it impossible to conduct it.

Finally, before proceeding with the description of current lung cancer biomarkers, and discussing how biomarkers are needed to improve the clinical management of this disease, the following paragraphs will describe what type of clinical functions may be accomplished by the use of biomarkers, and what the statistical requirements are that each researcher in the field must fulfill in order to evaluate whether his/her findings may become potential biomarkers.

1.2.1 Diagnostic Biomarkers

Diagnostic biomarkers are used to assist in making a specific diagnosis. In the cancer context, such biomarkers may be assessed on tumor specimens, for example to differentiate between tumors with similar morphology but different origin. The large majority of diagnostic biomarker research, however, is performed on serum or plasma. Blood is an ideal source material for biomarker analysis. It is easy to sample, relatively cheap, and multiple samplings can be performed on the same subject. In addition, in preclinical research it is not uncommon to encounter interesting molecules which can be actively released in the bloodstream by tumor cells, or leak out as a consequence of the high cell turnover rate typical of tumors. The ultimate use of diagnostic biomarkers would be to test them in a population at risk, in a screening setting, or in patients with a suspected cancer, but who could likely have either a malignant or benign disease.

The difficulties and problems of research performed directly on serum/plasma to discover diagnostic markers will be discussed later. Here it will be more specifically examined what kind of indexes must be measured when a potential diagnostic biomarker is tested on a cancer cohort and on a control group.

Firstly, a proper control group must be chosen. This should be represented by patients affected by benign diseases or by other tumor forms. Healthy donors should be avoided, partly because they do not constitute a population in which the biomarker will be tested once eventually available in the clinic, partly because of the risk to falsely consider as potential biomarkers factors which are instead related to the chronic inflammatory status that is usually associated to the tumoral disease or co-morbidities. Healthy donors may be used to determine which cutoff limit should be used when comparing the cancer and control groups. One possibility is to accept as limit the 95th percentile in blood donors, supposing that in healthy people the biomarker should be normal (or negative) in at least 95% of cases.

In addition to the control group, the cancer cohort must be carefully chosen. Usually it is composed of patients with advanced disease who have maybe already received some kind of treatment. However, results obtained in such a cohort may not be equally useful in patients with a disease at earlier stages and much smaller tumor burden, who actually also represent the target of most of diagnostic procedures involving biomarkers. The best cohort to perform diagnostic biomarker validation in cancer is represented by patients candidate to receive surgery. Usually this is a population of relatively young people, without severe co-morbidities and with a relatively small tumor burden. Besides, in such patient population, the kinetic of the biomarker may be also tested after the removal of the tumor lesion and monitored until disease recurrence. The disadvantage is that the risk to obtain negative results is extremely high, due to several issues, as for example excessive dilution of the marker, sample complexity and matrix effects due to high abundant background molecules.

Once the cutoff has been defined it is possible to proceed to determine the diagnostic performance of the biomarker. In Figure 1 the classical 2x2 table obtained in such case is depicted. Cancer and control patients correctly diagnosed by the biomarker are true positives and true negatives, respectively, whereas those who have been incorrectly diagnosed are false negatives and false positives, respectively. The table can be used to estimate the indexes of the diagnostic performance of the test, as shown in the lower panel of Figure 1. The percentage of cancer cases and of control cases that are correctly diagnosed represent the sensitivity and specificity of the test, respectively. The percentage of all cases correctly diagnosed is the diagnostic accuracy. In contrast, the percentage of cases with a positive test result who have cancer, and the percentage of cases with a negative test result who pertain to the control group are the positive and negative predictive values (PPV and NPV), respectively. Of course, a test retaining 100% specificity and 100% sensitivity has also 100% NPV and PPV. However, NPV and PPV depend on the relative quantity of cancer and control cases in the study, and ultimately on the prevalence of the disease in the population, provided that the study is properly designed [42, 43].

In the case that a cohort of blood donors is not available for testing, the biomarker may still be analyzed in the two groups (cancer vs controls), and results plotted into a receiver operating characteristic (ROC) curve, which is a function of logistic regression. A ROC curve can be used to determine the best cutoff, estimated by the index of Younden, which calculates the cut off level that retains the highest sum of sensitivity +

specificity – 1 [44]. In addition, with the ROC curve one may compare the diagnostic performance of diverse biomarkers, by comparing the area under the curve (AUC) for each biomarker (Figure 2).

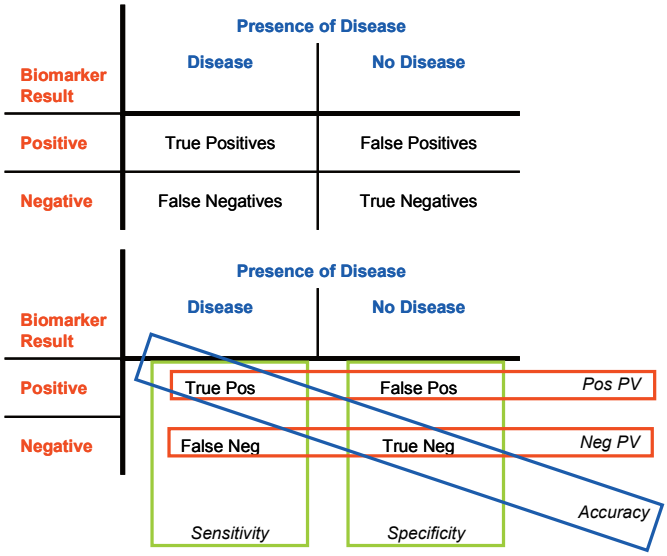

Figure 1. 2x2 table to determine the performance of diagnostic markers

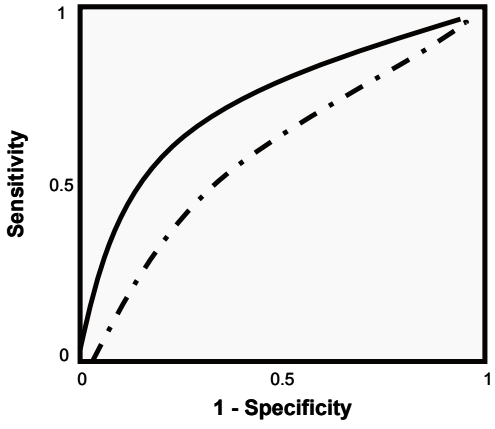

Figure 2. ROC curve. The marker depicted by the straight line has a larger AUC than the marker depicted by the dotted line, and as such it is better for diagnosis

Finally, it should be considered that the choice of the cutoff may be adjusted depending on the clinical question. In theory the aim of a diagnostic test would be to detect as many tumors as possible, and this may be obtained by decreasing the cutoff and increasing the sensitivity of the test. However, this approach is not feasible because it must be taken into consideration the number of cases that will have a false positive result and for which the use of expensive and sometime invasive procedures needed to make the diagnosis will be totally useless. The issue of the cutoff definition is actual and concrete in the field of diagnostic biomarkers. In fact, even for a widely used markers such as the prostate specific antigen (PSA) in prostate cancer there still is no consensus concerning the optimal diagnostic cutoff to use for screening purposes [45].

1.2.2 Prognostic Biomarkers

Prognostic biomarkers are used to acquire knowledge about the natural history of the disease, in terms of metastatic potential, likelihood of tumor progression and probability of patient survival, independently of treatment. There are several reasons why prognostic biomarkers are relevant in cancer research and management. First of all, when it is determined that the expression of a protein, gene or other molecules is directly correlated with an aggressive phenotype this may give important information about the biology of the disease and what pathways are activated when the phenotype is more aggressive. In addition, from the clinical point of view, the availability of validated strong and independent prognostic tumor markers may be used to stratify patients in randomized clinical trials aiming at evaluating the effect of diverse drugs. A proper patient stratification would indeed allow the composition of balanced groups, avoiding that cases with a worse prognosis may end up in only one patient group and introduce bias in the final results.

When research is performed on *in vitro* models it is not uncommon to claim that a molecule which is upregulated when the cells proliferate and express an aggressive phenotype may be a potential prognostic biomarker. However, prognosis is a function of how a disease affects patient survival, and in biomarker studies it must be considered as such. In addition, it should be taken into account that patient survival depends upon a number of known and unknown variables, and that statistical methods used to calculate this endpoint require that patients are dead (events), because living cases (censors) are not informative.

Figure 3A shows how patient prognosis is evaluated. In brief, the survival time of patients is plotted into a Kaplan-Meier curve, where the X axis represents time (days, weeks, months etc.), and the Y axis the percentage of living patients [46]. At time 0, all patients are alive, and the rate of living patients progressively diminishes with time. With such curves one can calculate some indexes that are frequently used to report patient survival. These are for example median survival (the time at which 50% of cases have died) and 1-year or 5-year survival rate (the percentage of patients still alive after 1 and 5 years, respectively). The survival of different patient groups, for example expressing high or low levels of a biomarker, may be plotted together in the same graphs, and the difference between the curves can be calculated using several methods, the most common being the log-rank test. This initial univariate analysis provides a first indication about

the potential prognostic relevance of a biomarker. However, this is not sufficient to establish that a biomarker has prognostic value, because, beside the biomarker expression, the two populations may have differential survival because of other confounding factors. At this stage, a common multivariate method, named Cox proportional hazard, is used [47]. This method is in principle a multivariate regression analysis and calculates the relative risk of death (hazard ratio, HR) for one or more groups compared to another group chosen as reference. For example, in a patient cohort, cases with low biomarker expression are used as reference, and the HR for this group (against itself) will be 1. Cases with a high biomarker expression will have a HR <1 if the biomarker is a positive prognostic factor (risk of death lower than the group with low expression) or conversely >1 if the biomarker is a negative prognostic factor (risk of death higher than the reference group). If the 95% confidence interval of the HR does not include the unit the difference between the two groups is statistically significant. After loading all covariates of interest, which may include one or more biomarkers together with other clinical variables, the model will attest what factors are still independently prognostic and, conversely, what factors are not. These methods have been largely implemented in papers II to IV of the present thesis. To avoid overfitting of the model, i.e., to avoid that the model describes noise or random errors instead of intrinsic relationships, a good rule for multivariate survival analysis is that the number of variables to be included in the model should not exceed 10% of the number of dead cases (events) present in the sample size. This may be a serious problem in multiple biomarker evaluation and in the case where the majority of patients are still alive due to inadequate follow-up.

Finally, it is worth noting that in the attempt to standardize the conduction of prognostic marker studies, in year 2005 a NCI-EORTC combined working group released a consensus document suggesting guidelines to perform studies on tumor prognostic biomarkers, known as the REporting recommendations for tumor MARKer prognostic studies (REMARK) [48]. These recommendations are presented in Table 2. This project was perceived by the scientific community as an extremely relevant initiative and the guidelines were contemporarily published in several high impact factor journals.

1.2.3 Predictive Biomarkers

Predictive biomarkers are used to determine in which subset of patients with a certain disease a drug is more effective. Predictive biomarkers are the most intriguing area of translational research in cancer. It is indeed a common clinical observation that the same treatment may induce excellent responses in some patients while in others it will not have any effect. Ideally, after testing for a panel of predictive biomarkers for different treatment regimens, one could administer the most effective drug only to those patients who, on the basis of the results from the biomarker analysis, will likely benefit most from that specific treatment. Biomarkers can also be used to predict toxicity and be particularly valuable for choosing between drugs with the same activity, but different toxicity profiles, as it is the case for regimens implemented in lung cancer treatment.

INTRODUCTION

1. State the marker examined, the study objectives, and any pre-specified hypotheses

MATERIALS AND METHODS

Patients

2. Describe the characteristics (*e.g.*, disease stage or co-morbidities) of the study patients, including their source and inclusion and exclusion criteria

3. Describe treatments received and how chosen (*e.g.*, randomized or rule-based)

Specimen characteristics

4. Describe type of biological material used (including control samples) and methods of preservation and storage

Assay methods

5. Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study endpoint

Study design

6. State the method of case selection, including whether prospective or retrospective and whether stratification or matching (*e.g.*, by stage of disease or age) was used. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time

7. Precisely define all clinical endpoints examined

8. List all candidate variables initially examined or considered for inclusion in models

9. Give rationale for sample size; if the study was designed to detect a specified effect size, give the target power and effect size

Statistical analysis methods

10. Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled

11. Clarify how marker values were handled in the analyses; if relevant, describe methods used for cutpoint determination

RESULTS

Data

12. Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the numbers of patients and the number of events

13. Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumour marker, including numbers of missing values

Analysis and presentation

14. Show the relation of the marker to standard prognostic variables

15. Present univariate analyses showing the relation between the marker and outcome, with the estimated effect (*e.g.*, hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analysed. For the effect of a tumour marker on a time-to-event outcome, a Kaplan–Meier plot is recommended

16. For key multivariable analyses, report estimated effects (*e.g.*, hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model

17. Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their statistical significance

18. If done, report results of further investigations, such as checking assumptions, sensitivity analyses, and internal validation

DISCUSSION

19. Interpret the results in the context of the pre-specified hypotheses and other relevant studies; include a discussion of limitations of the study

20. Discuss implications for future research and clinical value

Table 2. REporting recommendations for tumour MARKer prognostic studies (REMARK) (Reproduced with permission from reference 48)

As mentioned above, when working on *in vitro* or *in vivo* animal models it is not uncommon to claim to have found predictive biomarkers. For example, if two cell lines harboring a mutated or a wild type gene, or expressing high levels or low levels of a protein, are treated with a drug or radiation and only one of the two cell lines respond to treatment, it is reasonable to think that such molecular feature could be used as a predictive biomarker of treatment effect. However, a strict clinical validation is needed for a biomarker to be defined as predictive of benefit from a given treatment. Some requirements must indeed be fulfilled in order to determine if the biomarker is really predictive or rather prognostic. The main concept is that it is not enough to observe a differential effect of a drug on two groups of patients with different biomarker expression.

Let us consider the example depicted in Figure 3. In panel A, patients with a cancer disease at the same clinical setting (stage, treatment indication) are randomly allocated to receive either Treatment X (dotted line) or Treatment Y (straight line). It is essential at this stage that the biomarker is tested before randomization, in order to allow a proper stratification that will evenly distribute biomarker-positive and biomarker-negative patients in the two treatment groups. The final analysis shows that the two treatments achieve a comparable result in terms of survival. At this point (panel B) the evaluation of biomarker expression is incorporated in the analysis. By dividing patients into four groups according to treatment arm and biomarker expression, it can be observed that patients with a positive biomarker (red lines) survive longer than patients with a negative biomarker (blue lines) in both treatment arms. However, in the patient cohort with a positive biomarker expression treatment X seems to be more effective than treatment Y. The final step (panel C) is to calculate the HR of the treatment arms (X vs Y) in the two biomarker groups (positive and negative). The HRs are calculated as explained above in the paragraph on prognostic biomarkers and in the figure are depicted by the two black double-headed arrows. Finally, the differences between the two HRs obtained in biomarker-positive and negative groups, respectively must be calculated. This test is called interaction test, which must be statistically significant ($p<0.05$) in order to conclude that the biomarker is predictive [49]. In this case it is a treatment-by-biomarker expression interaction. This does not mean that the treatment X is not effective in biomarker-negative cases, or vice versa, but it only means that in the subset of patients expressing the biomarker the drug X is more effective than the drug Y. In this example the interaction test is quantitative, but it can also be qualitative, i.e. in biomarker-positive cases treatment X is more effective than Y, whereas it is the contrary in biomarker-negative cases. A couple of examples of treatment-by-biomarker interaction emerging from clinical trials conducted on lung cancer patients are described below.

Interaction between biomarker and treatment can be assessed also in retrospective data sets. However, as previously stated in section 1.2, assessment of predictive biomarkers presupposes randomization, which is ultimately achieved in prospective clinical trials. In order to calculate the correct sample size required to obtain results with adequate statistical power, the clinical study should be designed on the basis of the expected treatment-by-biomarker interaction.

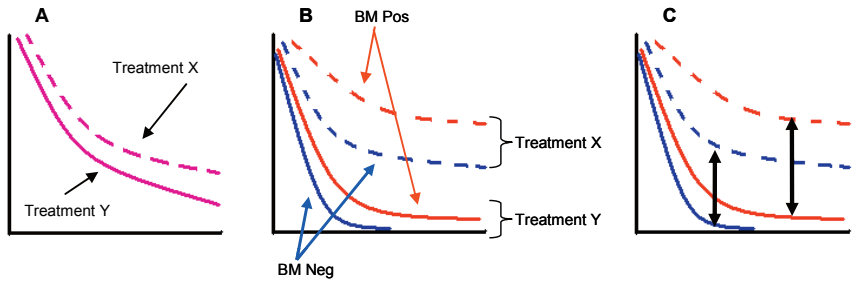

Figure 3. Interaction test for predictive biomarkers. See text for explanation. (BM Pos: biomarker-positive; BM Neg: biomarker-negative)

1.2.4 Biomarkers for disease follow-up and as surrogate end-points

The final application of biomarkers in the clinical settings is to use them as surrogate end-points in substitution of a clinical end-point. Of course, the response of the surrogate marker must reflect evidence of clinical benefit. Surrogate biomarkers are commonly used in non-cancer medicine, where death is a less common event of a disease. For instance, in HIV infection, biomarkers such as the count of CD4+ lymphocytes or the levels of HIV mRNA viral load are used to monitor the response to antiretroviral drugs and evaluate their efficacy [50]. In oncology, surrogate markers are used especially in the follow-up of ovarian and prostate cancer, which can be monitored by the serum levels of the CA125 and PSA antigens, respectively [51, 52].

When testing surrogate biomarkers, as compared with diagnostic biomarkers, one can aim at maximizing marker specificity. In case of an already diagnosed disease, in fact, the cutoff for the biomarkers may be lowered in order to detect disease recurrence as early as possible.

1.3 WHY DO WE NEED BIOMARKERS FOR LUNG CANCER?

As stated above, lung cancer is characterized by high incidence and mortality rates and by a substantial lack of effective treatment strategies. Validated biomarkers may assist the disease management in various settings and aspects. This paragraph briefly describes the biomarkers currently used in the clinical setting, and what are their limitations. For more detailed reviews about this topic, please see references [53] and [54].

Diagnostic and surrogate biomarkers. A number of immunohistochemical markers are available to distinguish between the diverse lung cancer histology types in case the morphological examination alone may not be sufficient to achieve diagnosis, as it may be the case in cytological analysis. SCLC, together with the other neuroendocrine lung tumors, namely carcinoid, atypical carcinoid and large-cell neuroendocrine tumor, strongly express neuroendocrine proteins, such as NSE, Chromogranin A, CD56 (N-CAM) and CD57 (Leu7) [55]. Adenocarcinoma and undifferentiated large-cell carcinoma frequently express

TTF-1 and CK7 and are usually negative for p63 and high molecular weight CKs (CK5/6). Conversely, p63 and CK5/6 are highly expressed in squamous-cell carcinoma, which show weak or negative stainings for CK7 and TTF-1 [56]. Finally, lung tumors are usually negative for CK20, and this feature may be used to distinguish between metastases from intestinal tumors, which express CK20, and primary lung adenocarcinoma [57]. All these biomarkers are currently used in clinical practice.

As far as serum biomarkers to assist diagnosis in case of lung nodules are concerned, other strategies may be applied. For example, participants to CT-based screening programs are offered further work-up in case of detection of solid or partially solid lung nodules. Such further work-up mainly consists of minimally invasive procedures aiming at obtaining a biopsy from the nodule. Since in prospective screening trials the percentage of diagnosed lung cancer ranges from 10% till 40% of CT-scan detected lung nodule [58, 59] the availability of a specific biomarker in this setting could help to avoid futile diagnostic procedures. In the 90s many studies looked at the diagnostic role of diverse circulating biomarkers for lung cancer, such as CEA, CK18 (tissue polypeptide specific antigen, TPS) and CK19 fragment (Cyfra 21.1) [60-62]. Unfortunately these analytes have proved to be poorly diagnostic, mainly because of too low accuracy, and their use has been abandoned.

Finally, a surrogate biomarker of disease recurrence after surgical resection would be of much benefit. Today surgically-resected patients are monitored for disease recurrence by chest X-ray and clinical evaluation. Nevertheless, chest X-ray may not be enough sensitive to detect small lesions and a biomarker would much contribute to the early detection of disease recurrence.

Prognostic biomarkers. Much effort has been spent in the past years for the identification of prognostic biomarkers in lung cancer and many potential biomarkers have been discovered, none of which unfortunately achieved sufficient clinical validation. More specifically, several molecules have shown to be prognostic in a variety of clinical lung cancer settings, but none could be established as a stratification factor in prospective clinical trials, and none is used to determine the phenotypic aggressiveness of a tumor.

However, when it comes to research about prognostic factors, it is not unlikely to receive the request from journal reviewers to test the same sample cohort against "known" molecular prognostic markers. In addition, it must be taken into consideration that besides molecular features, in lung cancer several clinical characteristics are strong prognosticators of patient survival such as stage, performance status, smoking status and weight loss.

As an exemplifying summary, the number of retrospective studies assessing the prognostic role of some histological features or circulating biomarkers is so large that for some molecules it has been possible to review all results within meta-analyses. This has been the case for p53 protein expression or *TP53* gene mutation [63, 64], TTF-1 [65], EGFR [66, 67], Ki-67 [68], bcl-2 [69], microvessel density [70, 71], COX-2 [72], Her-2 [73], *K-RAS* mutation or p21 overexpression [74] and Cyfra 21.1 [75]. P53, RAS/p21, Ki-67 and Cyfra 21.1 have been associated with a worse prognosis; bcl-2 and TTF-1 tumor expression are related to a better outcome; for COX-2, EGFR, HER-2 and microvessel density no definite conclusion could be obtained. The main reasons why results have been inconclusive so far are the non reproducibility of laboratory data, often obtained with different methods, and the

inconsistency of clinical endpoints, including short follow-up time and incomplete clinical data. In addition, even in the case when it was possible to describe a clear prognostic role for a molecule by reviewing all available data, this was usually based on a general lung cancer population, from stage I till stage IV, whereas data were not clear enough to extrapolate results for a specific clinical setting, such as resectable disease only, locally advanced tumors or first-line chemotherapy.

The era of novel OMICS technologies has led to the discovery of an even higher number of potential prognostic markers. The leading role in this field has been played by genomics, and especially by mRNA microarray platforms. In a recent review by Subramanian and Simon [76], 16 studies exploring the prognostic role of gene signatures in early stage NSCLC were summarized. The authors conclude that there still is little evidence supporting the clinical use of genomic signatures to prognosticate patient survival in this setting. The most common flaws of those studies, which mainly contribute to such a statement were the following: inadequate adherence to guidelines for tumor sample handling [77]; patient selection based on the availability of tumor samples and not on the clinical question; unclear comparison between the genomic signature and known clinical prognostic factors, such as completeness of surgical resection and tumor size; and lack of external validation. As for this latter aspect, only one out of four gene signatures submitted to external validation [78] proved to have a statistically significant prognostic role in NSCLC, but it was lost after adjustment for clinical covariates [79].

Finally, it is worth noting that even in this review the authors suggest guidelines for the conduction of microarray studies for prognostic gene signatures. However, instead of highlighting the acquisition of raw data, in terms of choice of the microarray platform or conduction of the experiments, they pursue the necessity to clear delineate clinical features and outcomes, stressing how crucial this aspect is when conducting omics studies.

Predictive Biomarkers. As already stated, predictive biomarkers give information about the efficacy of a treatment. In lung cancer the majority of studies on predictive biomarker have been in the form of retrospective analysis of prospective trials. Some representative cases of lung cancer biomarkers will here be described. For comprehensive reports, two good reviews have been recently published by Rosell et al. [80] and by Aggarwal et al. [81]

EGFR status and EGFR-TKI. The presence of specific sensitizing mutations in the exons of the *EGFR* gene coding for the tyrosine kinase domain is the strongest predictive factor for successful treatment with the small molecules gefitinib and erlotinib, which are known as EGFR-TKIs. Since these drugs target that specific EGFR domain, by binding to the ATP pocket, it is obvious to assume that the efficacy of the drugs is increased if the target protein expresses higher affinity for them. However, the ultimate recognition by the scientific community of this simple linkage between target and drug had to wait for the results of a recently published prospective randomized trial. As already mentioned, a combination of clinical characteristics may be used to enrich the NSCLC patient population for the presence of somatic *EGFR* mutations. These variables are adenocarcinoma histology, East-Asian ethnicity and a status of never- or light smoker, defined as a smoking

consumption <10 pack-year. In a large prospective randomized trial (the Iressa Pan-Asia Study, IPASS) designed and coordinated by Tony Mok and colleagues from Hong Kong, patients with advanced/metastatic NSCLC and the above mentioned clinical features, who were eligible for first-line treatment (n=1217), were randomly allocated to receive gefitinib or chemotherapy, which consisted of a combination of carboplatin and paclitaxel [82]. The primary end-point of the study, progression-free survival, was met, showing an improvement for gefitinib vs chemotherapy. In addition, a subgroup analysis on 447 available tumor samples revealed a definite advantage for gefitinib in patients with *EGFR* mutation and, in contrast, a detrimental effect of this drug in cases with wild type *EGFR*. However, it must be reminded that lung tumors harboring an *EGFR* mutation represent approximately 15% of non-squamous NSCLC cases in Caucasians, limiting the marketing of the drug to a subset of cases. As a consequence, a number of other biomarkers have been tested in order to try to detect more patients who would benefit from EGFR-TKI treatment more than other available and cheaper agents.

Extensive analyses conducted on other trials explored the predictive role of EGFR protein expression or gene amplification. NSCLC expresses EGFR on the cell membrane in approximately 85% of cases, and this showed no association with anti-EGFR treatments. In addition, *EGFR* amplification, as determined by FISH, showed promising predictive potentials in retrospective cohorts [83], in single arms studies [84] and in prospective trials where EGFR-TKIs were challenged against no treatment, namely best supportive care (BSC), in the second- and third-line setting [85, 86]. However, results from a randomized trial of gefitinib vs chemotherapy failed to demonstrate any predictive effect for *EGFR* FISH [87]. The design of this trial is instructive since it shows how it is possible to merge drug efficacy and predictive biomarkers analysis in the same study. The trial was designed to demonstrate non-inferiority (i.e., equivalence of efficacy) of gefitinib vs docetaxel in second-line NSCLC. The advantage for gefitinib would have been the less harmful toxicity profile and the more convenient way of administration (oral instead of intravenous). In addition, as co-primary end-point, the investigators powered the trial in order to demonstrate superiority of gefitinib in the subset of patients with tumor expressing *EGFR* gene amplification (FISH-positive). While the primary end-point of non-inferiority was met in the overall population (n=1468), in the subset of cases with tumor sample available for analysis (n=374), *EGFR* FISH did not demonstrate any predictive effect for gefitinib.

Finally, some words should be spent about a novel biomarker for anti-EGFR treatment developed with OMICS technology. Even though the level of evidence for this biomarker is still poor, the story deserves to be told because it shows how great expectations about serum proteomics may lead to false disappointing results. In the year 2007 the Vanderbilt and Colorado Cancer centers, together with some international collaborators developed a serum proteomics classifier to identify patients who would most likely benefit from EGFR-TKI treatment [88]. The proteomics algorithm was developed on pre-treatment crude serum using a MALDI-TOF-MS technology, and consisted of a combination of 8 characteristic peak signals. The patient population, divided into training, test and validation sets, comprised a total of 460 NSCLC cases, retrospectively collected, treated with either gefitinib or erlotinib, second-line chemotherapy or with surgery alone. According to the authors, the classifier was predictive of EGFR-TKI effect because only in subjects

receiving gefitinib or erlotinib it was able to distinguish between patients with a good or bad prognosis. The advantages for this biomarker approach are the low costs, large availability of source material and good reproducibility of experimental results. As a consequence the biomarker has become a commercially available product, known as Veristrat® (Biodesix, Steamboat Springs, CO). However, TOF-MS profiling on crude serum, without any pre-fractionation or downstream resolution of a complex sample, very unlikely reveals tumor specific biomarkers, but more likely measures changes in high abundant serum proteins which reflect the chronic inflammatory condition of patients with an advanced and spread tumor disease. In fact, analysis of plasma samples from a randomized trial of erlotinib vs BSC in second- and third-line NSCLC revealed that the Veristrat® classifier could indeed distinguish between patients with good or bad prognosis, but this occurred in both the erlotinib and BSC groups, without showing any treatment-by-biomarker interaction [89].

ERCC1 expression and platinum-based chemotherapy. Platinum compounds are the corner stone of lung cancer medical treatment. Since platinum induces DNA damage, many studies have been conducted in order to determine whether the expression of proteins of the DNA repair pathway would confer resistance to platinum-based chemotherapy in lung cancer. One of these proteins is ERCC1, a component of the nuclear excision repair (NER) pathway which is activated to repair single-strand DNA breaks induced, among others, by platinum-DNA adducts. High expression of ERCC1 should in principle be associated with resistance to platinum-based treatment.

The correlation between ERCC1 tumor expression and benefit from cisplatin-based adjuvant chemotherapy was investigated in a retrospective material collected from patients participating to the first and largest randomized phase III trial of adjuvant chemotherapy, the International Adjuvant Lung Trial (IALT) [90]. In that study patients with radically resected stage I to IIIA NSCLC were randomly allocated to either observation or to receive post-operative cisplatin-based chemotherapy. The immunohistochemical expression of ERCC1 was assessed on 783 tumor samples, representing approximately 40% of the entire IALT patient population [91]. Adjuvant chemotherapy was superior to observation in the subgroup of patients with a negative ERCC1 expression, whereas it was detrimental in patients with positive ERCC1 expression. In addition, in patients allocated to the observation arm, nuclear expression of ERCC1 was associated with a significantly better prognosis. In other words, high ERCC1 levels indicate resistance of tumors to chemotherapy, while showing less aggressiveness in terms of metastatic potential. ERCC1 could be assessed for example in cases with stage I NSCLC, to tailor the use of adjuvant chemotherapy; tumors expressing ERCC1 are indeed likely not to respond to chemotherapy and in any case will likely show a better prognosis, whereas ERCC1-negative tumors have a worse prognosis while responding better to post-surgical treatment.

Similar results were obtained on the same patient cohort with the cell cycle regulator p27, for which a higher expression correlated to resistance to chemotherapy [92]. The molecular rationale behind this observation is that p27, a tumor suppressor gene, blocks the cell in the G1 phase of the cell cycle, preventing the phosphorylation of pRB, and thus

protecting the tumor cell from the cytotoxic effects of drugs, which preferably act when the cells are in the late G1 or in the G2 phases of the cell cycle.

Tumor expression of ERCC1 has also been investigated in correlation with response to first-line chemotherapy for advanced disease. In this setting a prospective randomized phase III trial designed to determine the predictive effect of *ERCC1* mRNA expression on cisplatin-docetaxel chemotherapy showed an association with response to treatment, but no effects on survival [93]. Further studies revealed that a more appropriate biomarker in this field could be BRCA1, a component of the mismatch repair pathway. High *BRCA1* mRNA levels, in fact, sensitize the cells to apoptosis induced by antimicrotubule agents, such as vinorelbine and taxanes, and at the same time confer resistance to platinum [94, 95]. All these therapeutic agents are currently implemented in lung cancer treatment, and their use could be customized according to BRCA1 expression. Prospective trials are ongoing.

1.4 SOME ASPECTS OF PROTEOMICS TECHNOLOGIES

1.4.1 General considerations about MS-based and gel-based proteomics

Principles of mass spectrometry. Mass spectrometry (MS) technology is based on the following three components: a ion source, a mass analyzer and a detector. For the sake of simplicity, and to better clarify the principles of MS proteomics, the matrix assisted laser desorption ionization (MALDI) time of flight (TOF) MS instrument will be described (Figure 4A) [96]. The protein or peptide mixture is placed on a support, a metal plate called MALDI target, and mixed with a matrix. A laser hits the sample and with the aid of the matrix it ionizes proteins which are subjected to a voltage and guided to free flight through a vacuum tube. The proteins/peptides will receive the same initial kinetic energy and hence fly in the charge-free tube with a speed which is inversely proportional to the mass of the protein. The detector at the end of the tube detects the proteins as they reach it and, by calculating the time of flight (TOF), will display the results of the profile. This is a mass spectrum were the X axis represents the mass-to-charge ratio (*m/z*) of the proteins, and the Y axis the intensity of each peak. The latter is based on the number of ions that reach the detector and will be used to determine the relative quantity of each protein/peptide in that specific sample and to compare different samples. When analyzing undigested protein samples, MALDI-TOF-MS is a good technology to explore the low molecular weight proteome, but it is not effective to study proteins with a molecular weight >50 kDa. The advantages of this technology are that only small amount of source material is required and that it is possible to analyze at the same time a large number of samples at relatively low cost. A major drawback, on the other hand, is that the high dynamic range of protein concentration typical of complex mixtures, such as serum/plasma or tumor cell lysates, will lead to the detection of high abundant proteins only, which will eventually mask the presence of low abundant and potentially more interesting components with biomarker potential. In addition, after the peaks of interest have been selected, in most of the cases

proteins must be purified and identified through laborious and time-consuming in liquid chromatographic procedures.

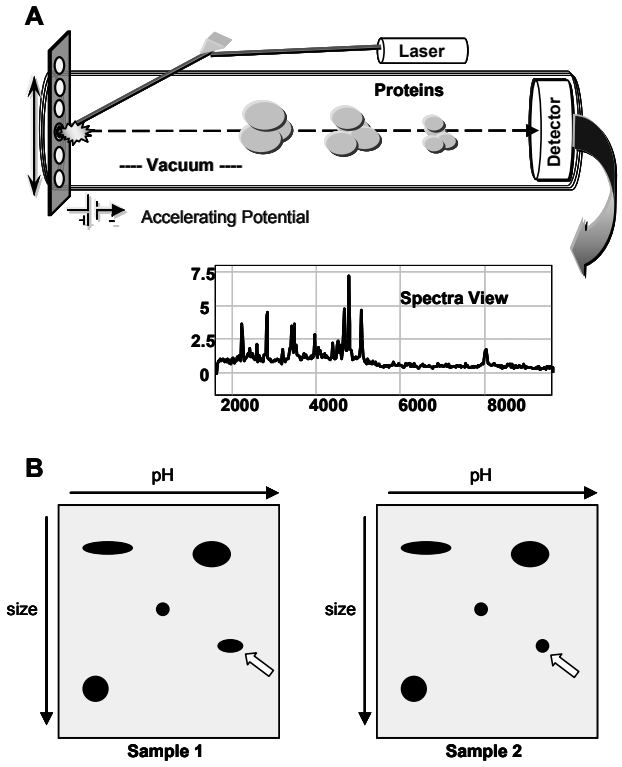

Figure 4. A: Schematic representation of MALDI-TOF-MS. B: Schematic representation of 2DE (white arrows represent potential biomarker showing different intensity in the two samples).

A modified MALDI technology, known as surface enhanced laser desorption ionization (SELDI) MS was developed in order to increase the proteome coverage of MALDI by creating an enrichment of subsets of proteins based on specific chemical or biological properties [97]. In brief, the MALDI target in the SELDI method is chemically active, with anionic, cationic or hydrophobic properties. After proper preparation of the protein mixture, the SELDI target, called chip, retains only those proteins which show affinity with the surface, and discard the others, which can be analyzed by using another chip with different properties. A useful application of SELDI is the possibility to immobilize a monoclonal antibody on the chip surface, and analyze the specific protein that binds to the antibody. With this method it is possible to confirm if the identified protein corresponds to the peak of interest selected from the initial profiling spectra. SELDI technology has been implemented in paper II of the present work.

Another interesting application of MALDI-MS is called MALDI imaging [98]. A tissue section is directly placed on the MALDI target and analyzed in order to retrieve protein profiles characteristic for each tissue area, including tumor islands, areas of normal parenchyma and stroma. One of the problems associated with this application is that protein ionization may be hampered by competition with other biomolecules, such as lipids. In addition, even though it has been reported that with this method it was possible to distinguish tumors from normal cases or from pre-neoplastic lesions [99], results confirming the capacity to identify biomarkers by comparing samples morphologically similar but with different clinical behavior are still preliminary [100].

In order to increase the potentialities of proteomics technologies in terms of proteome coverage, a number of novelties have been introduced, such as diverse ion sources and mass analyzer. One important innovation known as tandem mass spectrometry consists in the introduction of another mass analyzer after the first one (for a recent review on mass spectrometry see reference [101]). This technology is primarily applied to smaller molecules, such as peptides, lipids and metabolites. In tandem MS analysis intact molecules are analyzed and based on this result automatically selected to fragmentation to gain structural information. In this operation mode of a tandem MS instrument, molecules such as peptides are selected for fragmentation in the first mass analyzer and then directed to a collision chamber where peptides are fragmented into single amino acids or groups of few amino acids. The second serial mass analyzer measures the mass of each amino acid or peptide fragments and merges this latter information to give the amino acid sequence for that peptide. This is obtained by matching measured spectra with theoretical spectra [102]. The theoretical spectral data are available on databases which have been derived using the DNA sequence data from the human genome project. This data is translated into protein sequence data and converted to peptide data by theoretical enzyme digestion. The enzymes used for the digestion of proteins in peptides cut only at specific points. In case of trypsin, this occurs after arginine and lysine except when either is followed by proline [103]. Protein identification is then acquired by matching this information with web based resources of human proteome/genome. A tandem mass spectrometer (MALDI-TOF/TOF) instrument has been used in paper I of the present thesis.

Principles of gel electrophoresis. The most common gel-based technology implemented today in proteomics is 2-dimension gel electrophoresis (2DE) [104]. In brief, the protein mixture extracted from a sample is separated in two dimensions. The first one is the pH at which the protein is neutral charged (isoelectric point, pI), and the second dimension, which is orthogonal to the first one, is the protein size (Figure 4B). After electrophoresis the gel is stained with diverse dyes, revealing spots which contain the proteins that have migrated and separated. In a variant of the method proteins can be labeled prior to the separation. After scanning, the acquired images are used to determine the relative quantity of each protein spot in the different samples. The quantification is performed by comparing the optical density of the same spot present in several gels. In order to proceed to protein identification, the spots of interest are cut, the protein content is digested into peptides and commonly analyzed with a MALDI-TOF-MS machine, which reveals the characteristic molecular weight of each peptide, and produce a so-called peptide mass fingerprint (PMF).

In alternative, each peptide can be further analyzed using MS/MS analysis revealing its amino acid sequence. Advantages of 2DE are: i) the technology is well developed and established; ii) analysis of intact proteins allows detection of differential proteolysis; iii) it can resolve highly related proteins. Proteins harboring different post-translational modifications, for example, might have a very similar mass but different pIs; and iv) the method is quantitative, giving clinically relevant information about the relative quantity of each protein according to the clinical question. However, a number of disadvantages of 2DE must be acknowledged [105]: i) the throughput is "slow". Each sample must be run in a separated gel, or in alternative very few samples may be labeled with diverse fluorescent dyes and run together in the same gel [106]; ii) in order to select the spots of interest extensive gel matching is required and this may be challenging when many samples are compared; iii) it has a limited dynamic range, revealing mainly high abundant proteins; iv) although the method is good to study large proteins (molecular weight >20 kDa), the resolution is very low for the low molecular weight proteome (mw <20 kDa). As a consequence, for example, 2DE is not optimal to study serum or plasma samples, which will show only highly abundant circulating proteins and not reveal potential tumor biomarkers, which could be smaller and low abundant; and v) some classes of proteins are difficult to resolve, such as for example highly hydrophobic proteins, like membrane proteins, which migrate very poorly into the second dimension as well as proteins with extreme pI or molecular weight [107, 108].

1.4.2 Top-down and bottom-up proteomics

Top-down proteomics. MALDI- or SELDI-TOF-MS profiling and 2DE profiling are all examples of top-down proteomics. Intact proteins are analyzed generating a protein expression profile based on MS spectra or on gel spot patterns which retain clinically relevant information. As already stated, the high complexity of the tissue or serum proteomes may hamper the resolution and proteome coverage of these approaches. A number of pre-fractionation strategies have hence been developed. In terms of analysis on serum/plasma or other biological fluids, such as pleural effusion, affinity-based high abundant protein depletion is a feasible strategy to enrich the samples for potential biomarkers [109]. In terms of tissue, the major strategies have been to enrich the sample for tumor cells, as performed on paper I of this thesis, or to fractionate subcellular components into a nuclear, a membrane and a soluble proteins fractions [110].

Bottom-up proteomics. Not to be confounded with "Bottoms up!" proteomics, which occurs when publications are celebrated, bottom-up proteomics is maybe the most common experimental workflow in MS-proteomics. Proteins from a given sample are upfront digested to peptides, which may be separated firstly by pI, using an iso-electric focusing strip (IEF), and then further subjected to chromatographic separation. Each resulting fraction is then analyzed with tandem MS. A number of data analysis tools are subsequently used in order to determine protein identity and expression level in that sample. Protein quantification is one of the challenges of this approach. In order to keep the possibility to quantify protein expression, it is crucial that peptides subjected to all pre-

fractionation steps still reflect the protein content when they are analyzed. An effective application in this field has been based on isotopic labeling, known as isobaric tags for relative and absolute quantification (iTRAQ) [111]. In brief, to each sample a different isobaric tag is added (maximum of eight samples in each experiment). The tags bind covalently to all the peptides. At this point all the eight samples are pooled together, because all isobaric tags have the same mass and chromatographic properties. However, after fragmentation in the MS/MS instrument each tag gives rise to a reporter ion with diverse mass, which can be quantified in the MS/MS spectrum and ultimately reveal the relative quantity of that peptide and corresponding protein in each given sample. This pooling strategy reduces significantly the costs and time of each run because all the samples are processed together. Additionally the analytical variation is reduced since the pre-fractionation is performed at the same time for each of the eight samples.

Compared to the top-down approach, advantages of bottom-up proteomics are: i) the upfront digestion of proteins to peptides allows in principal a broader proteome coverage, since even the extreme proteins such as very hydrophobic or charged proteins will yield some peptides suitable for MS-analysis. However, the sample complexity is increased; and ii) the immediate protein identification permits to tackle the biomarker-related clinical question using global approaches such as the analysis of pathways that are involved in that specific process and facilitates validation with other targeted methods, such as ELISA, western blot and IHC.

1.4.3 Clues for sample preparation and some indications about biobanking of clinical material

When performing biomarker research using proteomics, or other discovery-based high throughput technologies, it is crucial that the analyzed samples are of high quality, well characterized and collected according to strict standard operating procedure. In addition, even clinical information should be prospectively collected and registered in proper case report forms (CFR). Outcomes should be clearly stated and pursued. In lung cancer, for example, it is relevant to specify what TNM system is to be used, if either the 6th or the 7th TNM versions, how to score smoking consumption and how to define tumor progression or response to treatments.

Biobanking of clinical material has progressively become standard practice in single Institutions and in cooperative groups worldwide [112]. At Karolinska University Hospital the group for Thoracic malignancies in a joint effort between the department of Oncology and Pathology and the Department of Respiratory medicine, has collected from year 2005 approximately 1900 plasma samples, 100 samples of pleural effusion and 150 tumor samples. Patient cases consist of subjects with either lung cancer, lung metastases from other malignancies or benign diseases.

Guidelines on how to biobank biological fluids, tumor tissue and ethical issues can be found in references [113], [114] and [115], respectively.

Here all indications and operating procedures needed for correct biobanking of clinical material will not be discussed. However, some important issues are worth mentioning.

In terms of plasma and biological fluids, it is crucial to collect and register for each sample also laboratory data of hematology and clinical chemistry. Even though this information might seem irrelevant, it must be considered that potential biomarkers in plasma may derive from blood components and not from tumors. For example, when comparing serum samples from patients with or without cancer, it can occur that markers found at higher concentration in cancer cases rather than in the other non-malignant condition is a product of leukocytes, which besides circulating in the blood and likely being the source of a number of protein products are also usually higher in tumor-bearing patients compared to individuals with other disease conditions [116].

In terms of tissue samples, there are two aspects that deserve some considerations. The first one is the tumor heterogeneity and the second is the use of corresponding normal tissue as control group. Lung cancer is an example of how both these aspects may hinder biomarker discovery with omics technologies.

As shown in Figure 5 tumor samples not only consist of cancer cells, but also inflammatory cells, stromal components and necrosis. Strategies aiming at enriching for tumor cells only, by using for example laser capture microdissection (LCM) or positive selection of epithelial cells with antibody-coated beads, should be implemented before extracting proteins for analysis. This aspect has been approached in paper I in this thesis.

As far as normal tissue is concerned, it should be noted that this is in general an inadequate source of "control" samples. Firstly, all normal tissues are composed of highly differentiated cells and, consequently, proteins and genes will be easily found to be down-regulated in tumors as compared to normal samples, making it very difficult to translate such findings into clinically useful biomarkers. Secondly, even in normal tissues cell heterogeneity is of concern. Normal lung is an emblematic example of this. Normal lung cells, namely alveolar and bronchial cells, are only a minority component of the lung parenchyma, which contains mainly vascular and support structures and, last but not least, air. Finally, lung cancer does not generate from normal cells, but from stem cells which are more sensitive to carcinogenesis [117]. Comparing cancer with a highly differentiated normal tissue therefore definitely cannot serve to unravel indicators of neoplastic transformation.

1.4.4 Clues for tissue microarray

Tissue microarray (TMA) technology has revolutionized biomarker research by drastically decreasing the costs and increasing the throughput of biomarker evaluation on tissue samples. The basis of TMA consists in the array of several diverse samples into a unique tissue block (Figure 6 [118]). In brief, representative areas from formalin-fixed paraffin embedded (FFPE) tumor blocks are selected by a skilled pathologist, according to the clinical question. This may not necessary mean only solid tumor area, but may include metastatic areas, invading fonts, stroma or vascular structure, or even normal tissue beside the tumor. A bioptic core, with a diameter of either 0.6 or 1 mm, is taken from the donor block and arrayed into the host paraffin block, where recipient holes were previously created. A recipient block can comfortably host from 80 till 200 cores, depending on the core diameter. It is crucial to take at least two or three cores from different areas of each tumor case, in order to guarantee a fair coverage of tumor

heterogeneity. From the resulting TMA block, tissue sections are cut and mounted on microscope glasses for analysis with mainly IHC or FISH.

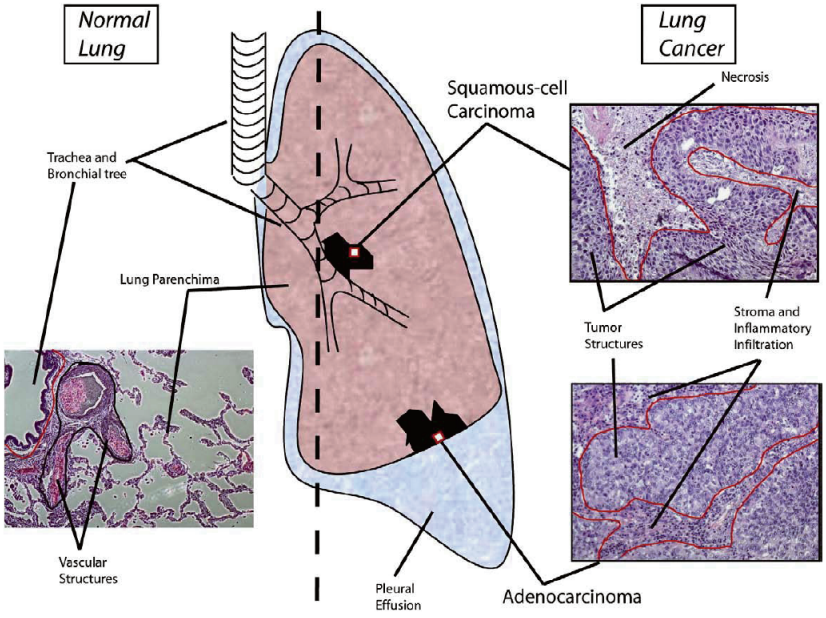

Figure 5. Schematic representation of the anatomical and histological structures of normal lung and lung cancer (reproduced with permission from reference 53)

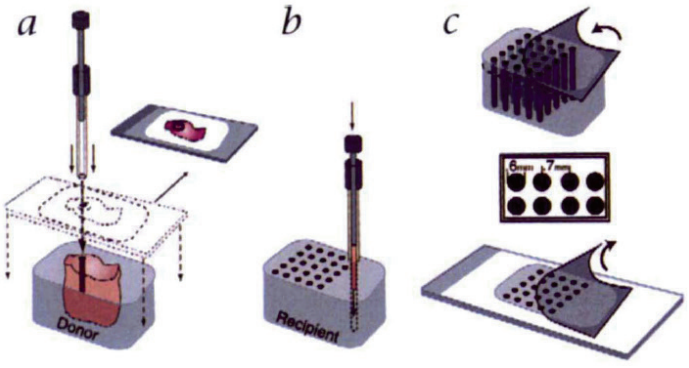

Figure 6. Tissue microarray construction (reproduced with permission from reference 118)

Advantages of TMA are the following [119]: i) the source material, namely FFPE blocks, is largely available; ii) staining variability is reduced since several samples are processed under the same experimental condition; iii) the costs of supplies, including antibodies and reagents, are drastically reduced; iv) the original donor block is substantially preserved, and several core biopsies can be obtained without altering the architecture of the original samples; and v) multi-tissue TMA can be constructed, permitting the evaluation of antibodies in a number of diverse tissues. This feature is particularly relevant in proteomics, where for example antibodies for newly discovered potential biomarkers may not have already undergone extensive validation.

TMA technology has also a number of limitations, which only partially hinder the possible applications of this technology, but must be taken into consideration when planning TMA-based research in order to avoid unexpected tangles. Firstly, at least 10% of cases will result non assessable, partly because the cores do not represent the original sample, partly because for low differentiated tumors with a pattern of scattered cells, cancer cells in the cores may not be enough for evaluation, partly because during staining procedures some cores can get lost. It is impossible to extract RNA or DNA from TMA cores for genomics analysis, whereas it is indeed possible to obtain cells through LCM, for assessment of specific gene mutations. In addition, the applicability of TMA in clinical routine is limited since patient samples must be analyzed sequentially and can rarely be merged into a single block. For each case it may not be available in the selected cores all structures, such as peritumoral stroma or normal tissue, limiting the possibility to obtain a neat contrast between tumor and non tumor areas. Another problem is representation of tumor heterogeneity, which may not be adequately ensured even in case of multiple cores. As a consequence, the real expression of a protein may be underestimated. From the technical point of view, usually from a FFPE block it is possible to cut up to 250 slices. This is not the case for TMA, first of all because the area of the donor block which is biopsied may not be thick enough. Secondly because after 50-60 slices multiple core losses may disrupt the architecture of the TMA, making it not possible to orientate under the microscope with enough confidence to permit an optimal evaluation of the stainings. For this reason, it is risky to construct TMA from small bioptic material obtained with middle-needle biopsies instead of surgical material.

1.4.5 Clues for immunohistochemistry

Immunohistochemistry (IHC) is an established method to determine the expression of a protein on a tissue. With this method, information about the protein level is merged with relevant information about the localization of the antigen in the cells, if nuclear, cytoplasmatic or membrane, and on which cells, including stromal and normal cells. IHC is quite a simple method with which good results can be obtained if it is performed by skilled hands. In brief, after proper preparation tissue sections are incubated with a primary antibody directed against the antigen of interest, and subsequently with a secondary antibody directed against the primary antibody. The secondary antibody is conjugated to an enzyme (indirect IHC) which is then exposed to an appropriate chromogen that will be optically visualized. In case the primary antibody is directly

conjugated to the enzyme, IHC is called direct, which is more simple than indirect IHC, but may be less sensitive since in indirect IHC the signal is amplified by the addition of the secondary antibody. Explicative protocols on how to effectively perform IHC can be found in the Abcam® website (www.abcam.com/technical) and in the Atlas Antibodies AB website (www.atlasantibodies.com).

Besides this simplistic description, there are at least three aspects of IHC that deserve some further discussion.

Antigen retrieval. This procedure is performed on the tissue slice after deparaffination, in order to optimally expose antigens for the binding with the primary antibody. Formalin fixation may indeed cause cross-links between the antigen and other proteins, which need to be disrupted. There are basically two methods for antigen retrieval. One is based on heating, usually with microwave and the aid of a buffer, such as citrate (pH 6) or EDTA (pH 9). The second method is enzymatic, based on proteases such as pronase E or proteinase K. Although the most appropriate antigen retrieval method to use rather depends on the antibody than on the antigen, it is worth noting that enzymatic antigen retrieval may cause the disruption of small tissue areas on microscope slides. If this will not affect entire tissue sections, it may create severe problems when performing IHC on TMA slides. Its use for this latter application is hence strongly discouraged.

Primary antibody. The availability of an antibody for a specific antigen is a mandatory step in order to validate early proteomics findings on large patient cohorts. For some antigens a number of different antibodies may be available. The first selection criterion to choose an antibody is based on the fact that manufacturers advise its use for IHC. Antibodies which have not been tested for this purpose, but have for example been developed for western blot or immunoprecipitation, will not necessarily function with IHC, and will likely generate significant signals in the stroma making the evaluation of the stainings almost impossible. In addition, it may be reasonable to suppose that a monoclonal antibody is better than a polyclonal one. In fact, compared to the latter, monoclonal antibodies recognize only a specific epitope of a given antigen and have low batch to batch variability. However, sometimes polyclonal antibodies may work excellently even on TMA section, especially if the expression of the target protein is at very low levels.

Moreover, although a primary antibody may have been extensively tested it is always advisable to control its binding specificity using both positive and negative controls. As external positive control it is good to have within the same staining session a separate section of a tissue known to express that protein. An important reference in this field is being built by the Human Proteome Resource (www.proteinatlas.org). A web-based protein atlas containing IHC stainings with a large number of primary antibodies in an atlas of normal and cancer tissues is publicly available and constantly updated with new antibodies. This source can be used to choose the proper positive controls. Internal positive controls are structures that positive stains even if the tumor cells are negative. Internal controls may consist for example of macrophages, which

usually uptake the majority of antibodies. This may be a drawback with TMA, since the cores may be too small to contain representative cells outside the tumor islands. As far as negative controls are concerned, some common practices should be avoided. For example, omitting the primary antibody is only a negative control for the secondary antibody. Since the latter is broadly used, and is produced on a species which is not human neither the species of the primary antibody, a negative control for the secondary antibody is not necessary and may be avoided, saving time and precious tissue sections. The most appropriate negative control for the primary antibody consists of immunoglobulins of the same species from which the antibody is produced and that share with it the same Ig isotype. With this tool it is possible to distinguish if there is an unspecific binding on tissue, depending on the Fc domain of the immunoglobulin and not on the idiotope.

Scoring of staining results. IHC is a qualitative method and as such it is virtually unsuitable for biomarker evaluation. For some antigens, such as nuclear p53, p21 and ERCC1 it is possible to count the number of cells and set up a cutoff for positive expression, for example at 1%, 5% or 10% of cells. However, for a great number of cytoplasmatic or membrane antigens the evaluation based only on number of positive cells may not be adequate. Some proteins may be expressed in the majority of cells, with a staining intensity that varies between the diverse tumor samples. Moreover, even within the same sample protein levels may be higher in certain cells compared to others. Pathologists prefer therefore to consider only the number of cell stained, and not staining intensity because the latter is dependent upon experimental conditions and exposure to the chromogen.

In order to find a compromise solution, a scoring system based on the number of cells and the staining intensity may be developed. Usually, to the percentage of positive cells a numerical score is assigned, with 0 if negative, 1 if <25%, 2 if between 25% and 50%, 3 if between 50% and 75% and 4 if >75%. Similarly, a numerical score from 0 to 3 is progressively assigned to the staining intensity, if negative, weak, moderate or strong, respectively. The final result is obtained by multiplying the scores of the % of cells by intensity, and ranges from 0 to 12. This system converts IHC into a semi-quantitative method, and the sample cohort can be categorized changing the cutoff of the score and challenging it to the clinical correlates. The risk of this system is to overestimate the relevance of staining intensity. A weak staining in >75% of cells or a moderate intensity in 25% to 50% of cells will have indeed the same score of 4. It is relevant to interpret the results in view of the protein function. For example, it is reasonable to speculate that if a protein must be strongly expressed in >75% of cells in order to be associated with a clinical outcome, this will likely be a minor finding and by itself not sufficient to clarify the clinical role of that biomarker.

2 AIMS OF THE THESIS

The general aim of this thesis was to explore the field of lung cancer biomarkers using mainly proteomics technology. The large biobank material prospectively collected at our Institution was therefore studied with the following specific aims:

Paper I: To optimize a protocol to obtain tumor cell enrichment from surgical samples for mass spectrometry based analysis

Paper II: To perform top-down proteomics on tumor cell lysates and proceed to early validation of the findings

Paper III: To expand the validation of the findings from Paper II on a large and well defined patient cohort in order to acquire information concerning the prognostic role of the expression of tissue biomarkers

Paper IV: To evaluate the diagnostic and prognostic significance of circulating biomakers in lung cancer and control cases.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Sample preparation to increase proteome coverage and improve biomarker discovery potentials of mass spectrometry based proteomics

In the year 2005, when establishing the set-up for a prospective biobanking of lung tumors, we aimed at elaborating a protocol for the collection of enriched tumor cell suspensions from surgical specimens. We intended to develop a method which would be cheap, easy to perform without requiring specific equipment, reproducible and allowing the removal of stromal and blood/plasma contaminants, which can constitute a major source of confounding factors in tissue proteomics.

We therefore took samples from macroscopically representative tumor directly taken after surgical resection and developed a protocol based on mechanical mincing, cell filtration, erythrocyte lysis and sequential sample-wash to remove plasma proteins. The cell filtration step was required to remove macroscopic stromal or normal structures, such as bronchiole surrounded by infiltrating tumoral lesions. From the final cell suspension, cytospin glasses were obtained to evaluate the cell content to compare with the histological composition of the original specimen.

Eight samples (two adenocarcinomas, two squamous-cell carcinomas, two large-cell carcinomas and two samples of normal lung parenchyma) were included in this analysis. Each specimen was cut in two. One piece was prepared according to the mentioned protocol to obtain enriched tumor cell suspensions (ETS). The other piece was fresh frozen (FF) and a direct tissue lysis was performed at time of analysis.

The eight ETS and the eight corresponding FF samples were analyzed using quantitative iTRAQ-liquid chromatography-tandem MS (iTRAQ-LC-MS/MS) in order to determine protein content and proteome coverage. An additional adenocarcinoma sample was prepared in five replicates to determine reproducibility of the method. The latter resulted to be very good, with coefficient of variations below 15% for the entire preparation workflow and below 10% for the analytical LC-MS/MS workflow.

In terms of protein content, the number of proteins identified from the ETS and FF samples was 244 and 109, respectively. These proteins showed only a 16.7% of overlapping. Analysis of Gene Ontology (GO) terms revealed a trend towards overrepresentation of extracellular and tissue functions, such as cell communication and organization, defense response, extracellular and membrane proteins, in the FF samples compared to the ETS samples.

Interestingly, the analysis of protein content based on the quantification of each protein revealed that hemoglobin and albumin were among the top five most abundant proteins in the FF samples. Conversely, in the ETS samples albumin ranked 33rd and hemoglobin was not identified at all, indicating a very effective removal of plasma and erythrocytes. Moreover, among the 10 most abundant proteins we identified cytokeratin 19, ubiquitin and ATP5A1, all proteins known to be expressed in cancer cells [120, 121, 122].

Finally, the cytological analyses on cytospin glasses from the ETS samples revealed that the percentage of epithelial cells in the cancer samples ranged between 30% and 90%, with the remaining component being mainly inflammatory cells. Although this may seem a poor

enrichment of tumor cells, it should be noted that these evaluations corresponded to the histological structure of the original specimens. Moreover, less than 10% of epithelial normal lung cells (bronchial or alveolar) could be isolated from the samples of normal lung parenchyma.

The quality of tissue samples to analyze is a matter of concern in proteomics-based biomarker discovery. Researchers dealing with this issue implement different strategies, such as: i) extract proteins with direct tissue lysis [123-127]; ii) macrodissection of frozen samples with sequential control of section till tumor cell content is at least 70% [128, 129]; iii) laser-capture microdissection [130, 131]; and iv) MALDI imaging [99, 121, 132].

In our study we show a feasible and reproducible method to prepare fresh lung cancer samples for proteomics analysis, with the major advantage of an efficient removal of contaminants from stroma, red blood cells and plasma proteins. However, our method may be hampered by the presence of inflammatory cells. To further develop this protocol, positive selection for epithelial cells with the aid of antibody-coated beads could be added after ETS have been prepared [133]. This latter application could also yield a fair enrichment of normal lung cells, which were poorly collected with our method.

3.2 PAPER II

Biomarker discovery on tissue and initial validation of findings

In a previous top-down SELDI-TOF-MS proteomics study on a panel of lung cancer and colorectal cancer cell lines we observed that after irradiation a 3-peak proteomic profile between 10.1 kDa and 10.4 kDa was altered. The protein responsible for this profile was purified and identified as the calcium-binding proteins S100A6. The peak at the lower *m/z* value corresponded to the native protein, whereas the peaks at 10.2 kDa and 10.4 kDa corresponded to post-translational modifications, namely cysteinylation and glutathionylation, respectively. At baseline only the unmodified and cysteinylated forms were present, whereas after irradiation a shift from the cystein to the glutathione peaks could be observed. A similar pattern was observed for another member of the S100 protein family, i.e. S100A4. Moreover, S100A6 showed to translocate from the nucleus to the cytoplasm in response to irradiation. These changes were dependent upon the presence of wild type *TP53* [134].

With the aim to evaluate the clinical significance of these *in vitro* findings we used cell lysates from 39 NSCLC cases collected during the 1980s. Tumors were of either the adenocarcinoma or squamous-cell carcinoma histology and at stage I in 80% of cases. The samples were collected and prepared directly after surgery using a method similar to the one developed in Paper I [135]. However, here an enriched tumor cell suspension was obtained with fractionation based on Percoll gradients.

Tumor lysates were analyzed with the same top-down SELDI-TOF-MS proteomics approach using a reverse phase hydrophobic surface (H50) and similar instrument settings as the cited *in vitro* study. Analysis of spectra revealed the characteristic 3-peak pattern in the 10 to 10.5 kDa area. The identification of those 3 peaks as S100A6 was confirmed with antibody-capture experiments using the affinity SELDI chip surface performed on tumor lysates from two adenocarcinoma specimens (prepared using the protocol developed in paper I), one sample of malignant pleural effusion and plasma samples from two advanced stage NSCLC and two healthy donors.

The most prominent of the three peaks, corresponding to the cysteinylated form of S100A6 at 10.2 kDa, showed a variable intensity among the 39 tumor samples. Dichotomized at the median intensity level, patients with higher expression of S100A6 had an improved survival compared with those with low levels (median survival of 35 months vs 18 months, respectively; p value non significant).

We then performed an early validation of these findings on a larger patient cohort, using IHC on TMA. This TMA was automatically constructed and contained 0.6-mm duplicate cores. We evaluated the expression of S100A6, S100A4 and p53 on 103 stage I NSCLC cases treated with curative surgical resection. Expression of the three proteins was positive in 26, 22 and 31 cases, respectively. The expression of S100A6 and S100A4 was associated with a higher tumor differentiation and adenocarcinoma histology, whereas the opposite was observed for p53 (higher in squamous tumors and in those with a lower differentiation). As a consequence, the two S100 proteins co-expressed on the same tumor cases, whereas a negative association was observed with p53 expression.

The presence of S100A6 in the entire patient cohort was associated with improved survival. Median overall survival time was 92.5 months vs 61.5 months in cases with high vs low/negative S100A6 expression (p=0.07). This difference became statistically significant when only 72 cases with negative p53 staining were considered (p=0.02). On multivariate analysis, the expression of S100A6 was confirmed as being an independent prognostic factor (HR 0.47, 95% CI 0.27-0.81; p=0.007). This result supported the *in vitro* evidence about the dependence of S100A6 kinetics and functional p53, and suggested a similar interaction in terms of clinical correlates. In fact, a positive IHC staining for p53 reflects the presence of a missense mutation that leads to the nuclear accumulation of the protein [136]. Conversely, the protein product of wild type *TP53* is below the detection limit of IHC.

The expression of S100A6 has been evaluated in other tumor forms, including melanoma, osteosarcoma, pancreas, thyroid, colorectal, prostate and gastric cancer [137-148]. However, in terms of the prognostic role of this protein, results vary depending on tumor form and histology.

From the functional point of view, S100A6 has shown to bind p53 and increase its transcriptional activity [149]. In addition, S100A6 binds to MDM2 inhibiting its interaction with p53[150]. These molecular observations may explain the upregulation of S100A6 in response to genotoxic stress caused by irradiation observed *in vitro*, and its translocation to the cytoplasm, maybe to bind to MDM2 in addition to effects on cytoskeleton. It is therefore coherent to speculate that tumors expressing high levels of S100A6 and wt *TP53* would show an increased activity of the apoptotic machinery. However, the prognostic implications of our results will be discussed later in the light of the findings of paper III, where a validation of S100A6 and other S100 proteins on a larger and better characterized patient cohort was performed.

In terms of the characteristic post-translational modification pattern of S100A6, this was also later described in another study in correlation with telomerase dysfunction [151]. A shifting to the glutathionylated form as a consequence of the radiation-induced oxidative stress, a common mechanisms to regulate protein function [152], could increase the affinity of S100A6 for calcium with a consequent increase of its biological activity, similar to what has been reported for S100A1 [153]. In the present paper, we could confirm the presence of this PTM pattern on clinical material, with a neat preponderance of the cysteinylated form. The clinical significance of such finding is unclear, and at the present time further investigation is hampered by the lack of a panel of specific antibodies for the separate detection of each modification.

Finally, besides its intracellular functions, including calcium homeostasis and effects on cytoskeleton, S100A6 exerts also extracellular activities, such as the regulation of secretory processes [154]. As we detected the presence of S100A6 in plasma and pleural effusion, this aspect would deserve further studies in the light of possible applications of S100A6 as circulating biomarker. Among others, the kinetic of S100A6 in response to irradiation in a clinical setting would be worth investigating, even though such analyses should be performed on multiple tumor samplings before and after radiotherapy, whereas a circulating marker would be much easier to monitor.

3.3 PAPER III

Validation of tissue biomarkers

In Paper II we obtained promising results concerning the prognostic role of the tumor expression of S100A6 in NSCLC in correlation with p53 status. However, a sample size of approximately 100 cases is definitely not sufficient to perform multiple biomarker analysis and to evaluate survival outcomes, especially if the patient population consists of stage I tumors, a subset of cases potentially cured with surgical resection. We hence collected a larger patient material in order to validate and expand those findings.

The patients included in this study comprised 494 subjects who received curative surgical resection for stage I NSCLC, including adenocarcinoma (54%), squamous-cell carcinoma (32%) and large-cell carcinoma (8%). Compared to the patient cohort of Paper II, we here pursued an extensive collection of clinical characteristics, including tumor localization, smoking history, time to tumor recurrence (progression-free survival), localization of the first metastatic site and overall survival. In this cohort the follow up time exceeded 10 years in the majority of patients.

The choice to perform a validation study on node-negative cases was based on several considerations. Firstly, since even this cohort was collected retrospectively, we aimed at obtaining samples that would be as homogenous as possible. The inclusion of tumors at later stages would have probably introduced additional confounding factors, such as possible administration of perioperative treatments, in the form of chemotherapy and radiotherapy. Secondly, it is reasonable to suppose that in case of node-negative tumors, the clones responsible for progression would derive from the primary lesion and depend on its phenotype. Conversely, in the case of lymph node spread, the molecular phenotype of the metastasis may well be different from the primary lesion, would likely be the source of further cancer progression and should be evaluated together with the primary tumor. In addition, since the study is based on TMA samples, small lymph node metastases are not an optimal source material for this technology.

The tumor expression of diverse S100 proteins was assessed with IHC on TMA slides. TMAs were manually constructed and contained 1-mm duplicate cores.

The following proteins were assessed: S100A2, S100A4, S1006, S100A7, S100A8, S100A9, S100A10, S100A11 and p53. S100A6 and p53 were scored according to the same systems used in Paper II, whereas for the other proteins a semi-quantitative method based on staining intensity and percentage of positive cells was implemented. The selection of which S100 proteins to evaluate was based on previous studies showing a role for each protein in cancer [155-161] and on the availability of validated antibodies for IHC.

The expression of S100 proteins was mainly cytoplasmatic. A characteristic granular pattern could be observed for S100A2 and S100A6, in 45% and 15% of positive cases, respectively. This

may suggest a role in intracellular vesicle trafficking or possible implications in extracellular functions. The percentage of cases resulting positive for the expression of p53 was 45%, 37% for S100A2, 33% for S100A4, 65% for S100A6, 63% for S100A7, 34% for S100A8, 49% for S100A9, 33% for S100A10 and 75% for S100A11. An association between the expression of some S100 proteins and histology could be observed, with squamous tumors showing a stronger immunoreactivity for S100A8, S100A9 and S100A2, and non-squamous tumors for S100A4 and S100A6. No clear histology-specific clustering emerged for the other markers. Furthermore, S100A8, S100A9 and S100A11, as well as p53, were more expressed in tumors with a lower grade of differentiation, whereas the opposite was the case for S100A2, S100A4 and S100A6. Finally, only S100A4 and S100A10 showed a weak but significant association with the occurrence of central nervous system metastasis as the first site of tumor recurrence.

It is worth noting that, compared to Paper II, we here report a much higher expression of S100A6 (26% vs 65% of cases, respectively) despite using the same antibody and the same scoring system on similar patient populations, i.e., stage I NSCLC. These discordant observations can be explained by several factors. Firstly, here we include not only adenocarcinomas and squamous tumors, but also large-cell and undifferentiated carcinomas. Secondly, we here used TMA of 1-mm duplicate cores, which compared to the 0.6-mm duplicate core TMA used in Paper II increases the evaluable tumor areas of approximately 150%. As a consequence, in Paper II we may have underestimated the real expression of S100A6, given the fact that the pattern of this protein may be patchy in a number of cases. Finally, if we consider the S100A6 expression by histology in the two studies, in Paper II S100A6 was present in 50% of adenocarcinomas and in 2% of squamous tumors. In Paper III, 45% of squamous and 78% of adenocarcinomas were S100A6-positive. In addition, the positive association between S100A6 expression and grading could be observed in adenocarcininomas but not in squamous-cell carcinomas, where for example half of G3 cases were scored as S100A6 positive.

Interesting results emerged from survival analysis, which were conducted with enough confidence due to the high accuracy of the clinical information and the relatively large sample size.

In the entire patient population, only the nuclear expression of p53 was a significant prognostic factor of worse overall survival ($p=0.02$). No other marker showed any prognostic potential with regard to either overall or progression-free survival. We hence performed subgroup analysis by histology and p53 expression. In adenocarcinoma, positive expression of S100A2 was associated with improved overall survival (log-rank $p=0.03$). In p53-negative cases S100A2 was a prognostic factor of improved progression-free survival ($p=0.007$), whereas it was the opposite in p53-positive cases ($p=0.3$). A similar behavior could be observed for S100A6. Positive S100A6 tumor expression was associated with a slightly worse outcome in squamous tumors ($p=0.2$) and with an improved progression-free survival in adenocarcinomas ($p=0.05$). This latter association became statistically significant when considering only p53-negative adenocarcinomas ($p=0.03$). These results were similar to those obtained in paper II, although with regard to a different clinical end-point, namely progression-free vs overall survival. However, in early stage radically resected node-negative NSCLC progression-free survival can be used as a surrogate end-point of overall survival, since those cases that progress will die of cancer, whereas those that do not progress will likely be cured by surgery. Moreover, it is reasonable to speculate that the possible prognostic effects of a marker expression on the primary tumor will reflect tumor recurrence more than the later behavior of the malignancy in terms of treatment response. Finally, in squamous tumors, S100A10 was a predictor of better progression-free survival in p53-negative cases ($p=0.4$) and the opposite in p53-positive samples ($p=0.009$).

Multivariate analysis on progression-free survival, adjusted by tumor stage and grading, showed that independent prognostic biomarkers were S100A2, but not S100A6, in p53-negative adenocarcinomas (HR 1.38, 95% CI 1.00-1.96; p=0.04), and S100A10 in p53-positive squamous tumors (HR 0.66, 95% CI 0.48-0.91; p=0.01).

Finally, we could not demonstrate any improvement in prognostic definition when combining the evaluation of diverse S100 protein pairs, selected by connections in functional activities. Tested pairs were S100A8/S100A9, S100A2/S100A4, S100A4/S100A6 and S100A7/S100A11. This suggests that the mutual interaction of these proteins has poor clinical significance.

The S100-protein family is composed by approximately 20 different members that share a common calcium-binding motif and are partly regulated by intracellular calcium levels [162]. The lack of an enzymatic domain suggests that the main functions of these proteins are exerted via protein-protein interaction. The activity of S100 proteins has been implicated in a variety of disease, from cardiovascular to inflammatory diseases to cancer. In oncology, the expression of a number of S100 proteins has been associated to tumor phenotype and patient outcome, even though results have not always been consistent [163].

In this study we show prognostic implications of tumor expression of S100A2, S100A6 and S100A10 in early stage NSCLC. The introduction of S100A2 and S100A10 in prognostic models based on histology and p53 status may be useful to identify NSCLC patients at higher probability of recurrence despite the early node-negative stage and, as such, possibly amenable of treatment with adjuvant chemotherapy.

No other relevant clinical correlations could be observed, neither when exploring functional pairs of diverse S100 proteins. This suggests that to understand more about the function of these important regulators, the evaluation on clinical material should be expanded to include the specific and direct interaction partners for each S100 protein, to connect clinical outcome to particular molecular functions.

3.4 PAPER IV

Validation of plasma biomarkers.

In Papers I, II and III we performed biomarker studies on the tissue level. In this last paper we intended to explore the diagnostic and prognostic potentials of circulating biomarkers. To this aim we selected cytokeratin 18 (CK18). This molecule is indeed released in the bloodstream as a consequence of cell death. Since lung cancer is a disease characterized by various grades of necrosis, a marker of cell death may be of particular relevance to assess this feature which has shown prognostic implications at the histologic level [164]. In addition, antibody-based assays are available to measure not only total CK18, but also a caspase-cleaved CK18 product (ccCK18) which results from apoptosis [165]. The ratio between total CK18 and ccCK18 may provide information about the amount of apoptosis relative to total cell death in each given sample.

Using commercially available ELISA assays, we determined the plasma levels of CK18 and ccCK18 of three separate and well characterized patient cohorts: 179 subjects with stage I-IV NSCLC, 113 cases with benign lung diseases and 200 healthy blood donors. In the lung cancer cohort samples were obtained at baseline, prior to any anticancer intervention. In order to better define the diagnostic and prognostic role of CK18, we measured also circulating levels of

cytokeratin 19 fragment (Cyfra 21.1), which has been largely studied in lung cancer and showed to be of diagnostic [166] and mainly prognostic [75] relevance in this disease.

Plasma levels of total CK18, ccCK18 and Cyfra 21.1 were all significantly higher in the lung cancer group compared to the control groups ($p<0.0001$ on non-parametric tests). In the specific of the NSCLC cohort, both total CK18 and ccCK18 were higher in advanced disease stage and in patients with a worse performance status. In the NSCLC and benign diseases groups the three analytes showed variable grades of co-expression, with the strongest association observed between total CK18 and Cyfra 21.1 (Spearman's Rho = 0.72). A weaker association was seen between ccCK18 and total CK18 or Cyfra 21.1 (Rho = 0.54 and 0.49, respectively).

Diagnostic role. The diagnostic role of the biomarkers was determined by comparing the NSCLC and benign diseases groups. For the two CK18 forms, the cutoff value was chosen at the 95% specificity level in the healthy donor group. These values resulted to be 104 U/L and 302 U/L for ccCK18 and total CK18, respectively. For Cyfra 21.1 we applied the established value of 3.6 ng/ml.

The performance of the three biomarkers is summarized in the table below, revealing an impressive diagnostic capability for Cyfra 21.1 and low potentialities for the two CK18 forms.

	Total CK18	**ccCK18**	**Cyfra 21.1**
Sensitivity	34%	39%	97%
Specificity	90%	83%	90%
Negative Predictive Value	47%	46%	94%
Positive Predictive Value	85%	85%	94%
Accuracy	56%	56%	94%
AUC-ROC	0.70	0.67	0.98

Prognostic role. To determine whether baseline CK18 levels would have any prognostic relevance in NSCLC we dichotomized lung cancer patients using the same cutoff values implemented in the diagnostic test. This was not feasible for Cyfra 21.1, since 97% of NSCLC cases had plasma levels above the cutoff. Hence, the median Cyfra 21.1 value in this cohort, corresponding to 6 ng/ml, was used.

On univariate analysis, all three markers were significantly associated with worse outcome. In terms of CK18, median survival was 11.5 months vs 28 months in patients with ccCK18 below or above the cutoff, respectively (log rank $p=0.0005$), and 7.5 months vs 36 in patients with total CK18 levels below and above the cutoff, respectively (log rank $p<0.0001$). On multivariate analysis, adjusted by clinical variables including gender, age, performance status, stage, smoking history, histology and whether or not patients received chemotherapy, total CK18 was the only biomarker which retained an independent prognostic role. HR (95% CI) was 0.64 (0.50-1.01) for low vs high total CK18 ($p=0.0004$), it was 1.16 (0.92-1.47) for low vs high ccCK18 ($p=0.19$) and 0.80 (0.63-1.01) for low vs high Cyfra 21.1 ($p=0.061$).

In addition, we sought to explore the prognostic role of baseline levels of the three biomarkers in a selected NSCLC cohort of patients with locally advanced or metastatic disease receiving a combination of chemotherapy and radiotherapy or 1st-line palliative chemotherapy (n=78). Even in this group the cutoffs used for total CK18 and ccCK18 were 302 U/L and 104 U/L, respectively, while for Cyfra 21.1 again the median value in the cohort (8 ng/ml) was chosen. Both Cyfra 21.1 and total CK18 were statistically significant prognostic markers on univariate Kaplan-Meier analysis, whereas no differences in patient survival were observed for ccCK18. For this reason the

latter analyte was not included in further multivariate models. Multivariate analysis, adjusted by age, gender, performance status, smoking, histology and treatment setting, confirmed total CK18 as the only independent prognostic biomarker (HR 0.70, 95% CI 0.52-0.94, p=0.018 for total CK18 vs HR 0.98, 95% CI 0.73-1.32, p=0.9 for Cyfra 21.1).

Finally, as previously stated, a further application of the concomitant measurement of both total CK18 and ccCK18 may be used in order to determine the amount of apoptosis relative to total cell death. In the present study, the ccCK18/total CK18 ratio did not reveal any additional information as compared to each single biomarker.

The potentialities of cytokeratin 18 as circulating tumor biomarker have been largely explored in a number of tumor forms, including colorectal, head and neck, pancreas, breast, gastrointestinal and testis cancer [167-172]. All reports discarded the possibility to implement this analyte in diagnostic test, basically for too low sensitivity in detecting the presence of the tumors. However, some reports associated elevated serum levels of CK18 with a worse outcome.

In lung cancer, an example where the clinical evaluation of plasma CK18 levels could be of great value is in the SCLC histology, which is characterized by high necrosis rates, leading to elevated CK18 baseline levels. In correspondence with response to treatment, which occurs in the majority of cases, both ccCK18 and total CK18 decrease, while they increase again at tumor progression [173].

An interesting application of CK18, and especially of the caspase-cleaved form, is its testing early after anticancer treatment in order to determine whether there has been an apoptotic response in the tumor, and investigate this kinetic in terms of predictive potentials. The results from our paper suggest that in NSCLC such study could lead to interesting findings. In patients with advanced disease, in fact, baseline levels of ccCK18 (apoptosis) are not correlated with survival, but may well show kinetics reflecting the early occurrence of treatment-induced apoptosis. In addition, in this setting the use of the caspase-cleaved/total CK18 ratio (apoptosis/total cell death) could represent a more suitable application than when calculated prior to treatment.

The planning for the conduction of such a prospective study is presently under consideration at the Oncology clinic in Stockholm.

4 GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The overall aim of this thesis was to explore the possibilities and analyze the limitations of biomarker studies in lung cancer using proteomics technologies. Despite the fact that the analyses were conducted on biobank material, our studies were characterized by high accuracy and completeness of clinical information. This aspect is crucial when investigating the clinical role of biomarker expression in order to reduce the probability to have false positive results.

The general conclusions of this thesis are here summarized.

- Preparing fresh lung cancer tissue from surgical specimens using a protocol that aims at tumor cell enrichment and removal of stromal, erythrocyte and plasma contaminants increases the outcome of proteomics analysis, both in terms of quantitative accuracy of tissue proteomics and proteome coverage.

- The post-translational modification pattern of the calcium-binding protein S100A6, showed in *in vitro* models, is conserved in clinical material, including tissue, plasma and pleural effusion.

- Selected S100 proteins may be used to refine the prognosis of early stage node-negative radically resected NSCLC. Specifically, S100A2 and S100A6 are positive prognostic factors of progression-free survival in p53-negative adenocarcinomas and S100A10 is a negative prognostic factor in p53-positive squamous-cell tumors. However, only S100A2 and S100A10 were deemed as independent prognostic factors on multivariate analysis.

- Circulating levels of cytokeratin 18, a marker of cell death, is an inaccurate diagnostic biomarker in NSCLC, but nonetheless it is a stronger prognostic factor than Cyfra 21.1, independently of therapeutic intervention.

The field of lung cancer biomarker research using proteomics technologies is broad and in continuous evolution and as such it is premature to make conclusive statements. Nevertheless, there are some aspects which need some further considerations.

First of all, biomarker research needs to be standardized in its structural aspects. The clinical question, outcomes and criteria for patient selections must be clearly defined. Sufficient information on experimental methodology should be provided, as well as an accurate description of statistical methods, which should include proper adjustments and corrections for multiple testing. Finally, the results should be confirmed on independent test

sets [174]. Though this might seem an obvious consideration, the majority of studies in the field do not completely adhere to these basic requirements.

Secondly, as far as methodology is concerned, in this thesis we implemented bottom-up proteomics to determine the quality of tissue samples prepared with an in-house developed method and top-down proteomics for biomarker discovery. In the validation step we used two well established and reproducible antibody-based methods, namely IHC on TMA and ELISA on plasma samples. However, proteomics technologies are rapidly evolving towards robust and better standardized methods and a number of innovations are worth mentioning in anticipation that these methods will boost the output in terms of novel clinically useful markers.

The interpretation of IHC stainings on TMA is time-consuming and dependent upon the expertise of the observer. Automatic evaluation of the stainings, currently implemented in large Institutions, increases the throughput of the method and reduces the variability related to the judgment of the pathologist [175]. Moreover, IHC has serious limitations when it comes to quantification of protein expression. A modified IHC method based on fluorescent dyes could improve this aspect both in terms of dynamic range of quantification and sensitivity of detection [176]. Moreover, protein extracts as well as circulating proteins can be accurately quantified with antibody-free methods, such as absolute quantification (AQUA) and other targeted mass spectrometry detection methods.

Finally, the strongest limitation of current OMICS research is the lack of integration between the diverse methods of molecular profiling. Genomics aims at assessing mutations or single nucleotide polymorphisms (SNP), gene expression (transcriptomics by arrays or deep sequencing), miRNA regulation (miRNA-microarrays), global regulation of gene transcription (epigenomics) and global gene copy number changes (CHG arrays). With proteomics it is possible to determine protein levels (as seen in this thesis), to dramatically increase the throughput by antibody-based profiling methods currently under development (assays such as Luminex), to study protein-protein interaction (with proximity ligation assays or other types of interaction scans) and the activity state of proteins (phosphoproteomics and other post-translational modification studies). Largely available web-based resources for elaboration of data and pathway analysis should be constantly implemented in order to merge all these data sets in a systems biology approach [177].

In anticipation of the drastic reduction of lung cancer incidence and mortality as a natural consequence of global smoking control, the path to pursue in future research can be envisioned as a combination of integrated knowledge and technological innovations. The conduction of prospective clinical trials which incorporate biomarker evaluation and interpretation of the outcomes in their design will definitely much contribute to the achievement of significant results in this field.

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