Molecular serum portraits - A step towards personalized medicine

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Molecular serum portraits –
A step towards personalized medicine

Anders Carlsson

ACADEMIC THESIS which, by due permission of the Faculty of Engineering at Lund University, will be publicly defended in Lundmarksalen, Astronomiska Institutionen, Sölvegatan 27, Lund, Friday 1st of October 2010, at 13.15 a.m.

Faculty opponent is Prof. Larry Gold, Department of Molecular, Cellular, and Developmental Biology, University of Colorado at Boulder and CEO and Chairman of the Board at SomaLogic Inc., Boulder.
"If your experiment needs statistics, then you ought to have done a better experiment"

- Ernest Rutherford
Abstract

Antibody microarray technology has the potential for playing a large roll in identifying serological biomarker panels for personalized medicine.

The aim of this thesis, based on four original papers, was to investigate if information in the serum proteome could be extracted and used for diagnostic, classificational, prognostic or treatment predictive purposes in a range of diseases.

In two studies (paper I and paper IV), the diagnosis and prognosis of breast cancer was addressed, also being the main focus of this thesis. In paper I, we identified a biomarker panel capable of stratifying serum samples from metastatic breast cancer patients from those of healthy controls. In paper IV, another panel, pre-validated in the same study, was deciphered that could be used to identify patients destined for metastatic disease in a group of newly diagnosed breast cancer patients.

Paper II and III targeted immunotherapy of glioblastoma multiforme and the diagnosis and sub-stratification of two autoimmune diseases (SLE and SSc), respectively. Also in these cases, multiple biomarker panels were identified, each capable of separating predefined cohorts of patients with relevance for applications within personalized medicine.

In conclusion, this thesis introduces the concept of personalized medicine; details the antibody microarray technology in general and the platform used for the experiments in paper I to IV; and describes the subsequent microarray data analysis.

Key words

Personalized medicine, antibody microarray, breast cancer, cancer proteomics
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My contribution to the papers in this thesis

**Paper I**
Performed the experiments and data analysis. Participated in writing the manuscript.

**Paper II**
Performed the experiments together with JI, did most of the data analysis. Participated in writing the manuscript.

**Paper III**
Performed the experiments together with JI, did the data analysis. Participated in writing the manuscript.

**Paper IV**
Performed the experiments together with MK, designed and performed the data analysis. Participated in writing the manuscript.
Original papers


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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera Toxin</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionization</td>
</tr>
<tr>
<td>FPR</td>
<td>False Positive Rate</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-Assisted Laser Desorption Ionization</td>
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<tr>
<td>MRM</td>
<td>Multiple Reaction Monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>PgR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver Operating Characteristic</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>scFv</td>
<td>single-chain Fragment variable</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
</tr>
<tr>
<td>SLEDAI</td>
<td>SLE Disease Activity</td>
</tr>
<tr>
<td>SSC</td>
<td>Systemic Sclerosis</td>
</tr>
<tr>
<td>SVM</td>
<td>Support Vector Machine</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumor-Associated Antigen</td>
</tr>
<tr>
<td>WWAS</td>
<td>Watchful Waiting with Active Surveillance</td>
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1. Introduction

During the last decades, the amount of information possible to extract from a clinical sample has increased dramatically due to the progress made in genomics and proteomics. In 2001, the Human Genome Project was completed, paving the way for the development of DNA microarrays. With this technology, genome wide expression analysis can be performed, giving detailed information about which and to what degree genes are used at a given time. In parallel with this development, technological advancements in mass spectrometry (MS) allowed for more sensitive and robust analysis on the protein level. Proteins, being the downstream products of expressed genes, constitute the most important group of molecules in biological processes, and are therefore the most interesting targets both in drug development and in the search for diagnostic or prognostic biomarkers.

Genomic and proteomic technologies both produce large amounts of data from the analysis of biological samples. The data can be mined to extract information about, for example, which genes are mutated in or being expressed and translated by the tumor cells in a breast cancer patient. The availability of this kind of information brings new opportunity in many clinical situations, including patient diagnosis and prognosis, and through this realization, the concept of personalized medicine was born.

The idea behind personalized medicine, defined as “the right treatment for the right person at the right time”, is that better clinical decisions can be made by using information about the individual patient. In other words, it is recognition of the fact that just knowing that a person has e.g. breast cancer might be inadequate in order to treat the patient. Instead, there are several factors that must be investigated in order to optimize the regimen for the individual patient.
The need for an individual-based treatment design in such complex and heterogeneous disease as cancer is very clear. The possibility of subcategorizing malignancies into more homogenous groups is perhaps the most promising way of increasing the survival of cancer patients. Already today, there are tests approved for clinical use that are indicative for alternative treatments for breast cancer, which can identify patients with tumor types susceptible for certain drugs (Herceptin) (Wolff, Hammond et al. 2007). Personalized medicine will also limit overtreatment, which will benefit both patient with less unnecessary adverse effects, and healthcare with lower costs.

The development of the highly information-generating genomic and proteomic technologies gave great hope for the rapid identification of disease-specific biomarkers, and large resources are continuously invested in biomarker research. The results have, so far, to a large degree been disappointing. Even though numerous studies with potential biomarkers have been published, only a hand-full has been approved for use in a clinical setting. Affinity proteomics has during the last years emerged as a strong contender to solve some of the issues associated with the analysis of often highly complex samples. Affinity proteomics uses the specific interaction between a binder molecule and the target molecule to catch and analyze one or several markers of interest. The binder molecule is typically an antibody or, as in the case in my studies, a fragment thereof, which has an intrinsic ability to specifically bind and capture even low levels of proteins that has been utilized in biomedical research for many years.

The antibody microarray is a rather new technology within the field of affinity proteomics. Antibody variants, each capable of binding different target molecules, are immobilized on a surface in a spatially discrete manner in the form of an array. This makes it possible to run assays in which large numbers of targets are analyzed simultaneously, while consuming minute amounts of sample.

In this thesis, based on four original papers, I have addressed the potential of using antibody microarrays in personalized medicine. I have analyzed blood-based samples (serum and plasma) from patients with cancer or autoimmune diseases, and compared them with samples from healthy individuals (paper I, II, III), individuals with benign disease (paper IV) or individuals with other, similar, disease (paper III). The common aim of these four studies has been to identify patterns in the protein composition, or molecular portraits, in the blood from different groups or subsets of patients, which later could help the
physician to make better decisions in composing the optimal regimen for a specific patient, *i.e.* personalized medicine.
2. Personalized Medicine

Personalized medicine refers to the act of tailoring the treatment for the individual patient according to the disease phenotypes he or she displays. This concept is not new – physicians have always made observations for diagnosis and to decide which treatment to use – but the tools to do it with are. After the human genome was mapped in 2001 (Lander, Linton et al. 2001; Venter, Adams et al. 2001), new technologies that can be used to quantify gene and protein expression were developed. These make it possible to use genetic, mRNA, protein and metabolic markers as guides in disease diagnostics and prognostics as well as in treatment prediction.

Today’s drugs work in an average of 50% of the patients who take them, a fact that can partly be explained by differences in the genes coding either the drug metabolizing enzymes, the drug transporting proteins or the drug target themselves (Spear, Heath-Chiozzi et al. 2001). This has two major consequences. Firstly, patients suffer from disease and adverse effects longer due to trial-and-error prescription until an effective therapy is found, adding to the fact that between 5 and 7% of all hospital admissions may be due to adverse effects (Kongkaew, Noyce et al. 2008; Ingelman-Sundberg and Sim 2010). Secondly, it leads to unnecessarily high treatment costs. Hence, getting the right drug to the right patient sooner will benefit both patient and healthcare provider.

Another important aspect of a more personalized medicine is the decreased patient noncompliance to treatment. A personalized treatment that proves more effective and has fewer side effects increases the probability that a patient will comply with it, resulting in fewer adverse health effects (Umans-Eckhausen, Defesche et al. 2003).
The basis of the research in this thesis is the application of our antibody microarray for personalized medicine of different forms of cancer (breast cancer and glioblastoma multiforme) and autoimmune diseases (systemic lupus erythematosus and systemic sclerosis). The diseases investigated, although widely different, all share the fact that an increased knowledge about the patients’ individual serum protein composition, their molecular portraits, could aid in either their diagnosis or treatment design. In the four included studies, we have identified panels of serological biomarkers indicative of several important traits and phenotypes in these diseases, ranging from diagnosis (paper I and paper III), predicting metastasizing tumors (paper IV), selecting patients eligible to specific types of therapy (paper II) to identifying patients with phenotypic disease subsets and varying disease activity (paper III).

While the main results from my studies and their potential role in personalized medicine will be presented in chapter 5 ‘Clinical Applications’, I have here exemplified the work in biomarker identification and personalized medicine by other groups, focusing on breast cancer and serum biomarkers, which have been the clinical state and sample format of main focus in my thesis work.

2.1 Breast Cancer Biomarkers

Breast cancer is the most common malignancy in women today (Jemal, Siegel et al. 2008), and is one of the diseases where personalized medicine is most developed. In two of the four studies in this thesis, I have addressed the diagnosis and prognosis of breast cancer patients (paper I and paper IV). The breast cancer mortality rate is steadily decreasing in the western world, partly due to earlier diagnosis, an effect partly attributed to mammography screening, and improvements in antihormonal and cytotoxic treatments (Sparano, Fazzari et al. 2005; Tuttle, Abbott et al. 2010). With few exceptions, the types of treatments available today are basically the same as several decades ago; no really revolutionizing new drugs have been developed. What has been revolutionized, however, is when and to whom the drugs are administered, a result of personalized medicine. The biomarkers that aid in doing this have been found by genetic analysis, gene expression analysis as well as by protein expression analysis, as depicted in figure 1.
2.1.1 BRCA1 and BRCA2

A well-known example of genetic markers indicative of disease is the test for the BRCA1 and BRCA2 gene variants indicative for hereditary breast and ovarian cancer (Nelson, Huffman et al. 2005). BRCA1 and 2 are members of the tumor suppressor gene family, and their protein products (Breast Cancer Type 1 and 2 susceptibility protein) are involved in the repair of DNA double strand breaks. Women with germline mutations in any of these genes have up to 85% risk of developing breast cancer, compared to 13% in the general population. For ovarian cancer the corresponding numbers are 60% compared to 1.7% in the general population. After positively testing for BRCA1 or BRAC2, preventive and risk reducing measures like increased frequency of mammography, chemoprevention or prophylactic surgery can be taken (Howard, Balneaves et al. 2009; Tuttle, Abbott et al. 2010). Male breast cancer is rare and accounts for less than 1% of all cancer in men. However, men with BRCA2 mutations are at 80 to 100 times greater risk than the general population (Liede, Karlan et al. 2004). Other types of cancer are also overrepresented in individuals carrying BRCA1 mutations, including prostate and pancreatic cancer (Thompson and Easton 2002).


2.1.2 Oncotype DX and MammaPrint™

Onco\textsuperscript{type} DX and MammaPrint™ are two available biomarker signatures used in breast cancer prognostics. Oncotype DX is a clinically validated RT-PCR assay that predicts the likelihood of cancer recurrence and beneficial chemotherapy in early-stage, lymph node-negative, ER–positive breast cancer. It does so by analyzing the expression of 21 genes from RNA extracted from paraffin-embedded tissues, which are routinely prepared after surgical tumor removal (Cronin, Sangli \textit{et al.} 2007). A prospective study on 668 patients showed that the Recurrence Score provided by the test displays a prognostic power and reproducibility that outperforms classical clinical factors such as age and tumor size and tumor grade (Esteva, Sahin \textit{et al.} 2005). Another study using the tumors from 651 breast cancer patients determined that the assay also is able to predict how beneficial chemotherapy would be for a patient (Paik, Tang \textit{et al.} 2006), which could lead to a reduction of overtreatment.

MammaPrint™ is a 70-gene microarray-based assay capable of classifying lymph node negative, stage I or II patients with invasive breast cancer into groups according to high or low risk of distant tumor recurrence (van 't Veer, Dai \textit{et al.} 2002; Glas, Floore \textit{et al.} 2006). In a recent study, the predictive value of the classification was demonstrated as it was shown that the patients predicted with low risk did not benefit from having chemotherapy added to the endocrine therapy, while the benefit was significant for the patients classified as high risk (Knauer, Mook \textit{et al.} 2010).

Our antibody microarray, as applied in paper IV, is in a similar manner capable of extracting prognostic information about breast cancer patients, the main difference being the source of the information. While both of these RNA based require tumor tissue, our approach probes the serum proteome for similar prognostic answers. This and other differences will be discussed in some detail in chapter 5.1.2.

2.1.3 ErbB2 - Herceptin

Another example of personalized medicine in breast cancer management is the characterization of the surface marker ErbB2 (or HER2/neu) on the tumor cells. Overexpression of this receptor occurs in about 30% of breast cancer patients and is associated with poor prognosis (Kurosumi 2009). While standard treatment is ineffective against this subset of patients, an antibody drug called Herceptin® (trastuzumab) can, in combination with chemotherapy, reduce the recurrence with 52%, as compared to chemotherapy alone (Piccart-Gebhart, Procter \textit{et al.} 2005; Romond, Perez \textit{et al.} 2005). Of note, an
additional benefit of personalized medicine can be observed in this example; Herceptin® failed a phase III trial in 1997, where it was shown to be ineffective when used on the whole breast cancer population. However, careful analysis of the results revealed that the ErbB2 positive population showed significantly better results. One year later the Food and Drug Administration approved the combination of diagnosis and treatment using ErbB2 and Herceptin® (Cobleigh, Vogel et al. 1999). This individual-based observation of possible sub-populations in the patient data rescued the drug, now saving the lives of thousands of patients each year.

A schematic outline of how breast cancer is treated can be viewed in figure 2.
Figure 2. Outline of the clinical decisions in the treatment of breast cancer. *In situ ductal carcinoma* can progress to invasive cancer, and is treated with breast conserving surgery and radiotherapy for stage I or stage II tumors. Stage III patients usually require chemotherapy prior to surgery to downsize the tumor. In inflammatory stage III breast cancer, induction chemotherapy is often followed by mastectomy. For metastatic stage IV patients, the benefits of prolonged survival and reduced pain against the harm from treatment must be balanced. The choice of adjuvant therapies depends on lymph node involvement, hormone receptor status (ER/PgR) and ErbB2 overexpression (Maughan, Lutterbie et al. 2010).
2.2 Examples of Serum Biomarkers in Personalized Medicine

As established in the previous section, there are several sample sources that can be probed for information relevant in personalized medicine, including tissue, urine, blood, cerebrospinal fluid, and saliva (Radpour, Barekati et al. 2009). The human serum proteome, however, is considered to be the most complete, containing subsets all other tissue proteomes (Anderson and Anderson 2002). It is evident that the applicability of the different sample sources and technologies overlap to some degree, but also complement each other in an important way. Genomic sequencing for the identification of disease associated gene variants (i.e. BRCA1 and BRCA2) may be the most fundamental source of information, making it possible to assess the risk of developing disease. Proteomic technologies may be used in diagnostic contexts as in disease screening in body fluids, as tissue based samples are unavailable for analysis at this stage. Once the disease is diagnosed and in cases tissue thus can be accessed (e.g. tumor tissue), both gene and protein expression data may be mined for prognostic and predictive information. After primary treatment (i.e. tumor removal), the disease progression and therapy can be monitored using mainly proteomic technologies and body fluid based samples.

Serum is the predominately form of clinical specimen, routinely collected at clinics all around the world. The minimal invasiveness of blood sample collection and the abundance of information available in it, make serum an attractive source. This is appreciable by the fact that through 2008 there were 109 serum or plasma protein targets that had been FDA approved (Anderson 2010). A few of these are approved for the detection and prognosis of cancer, including prostate specific antigen for prostate cancer; CA15-3, ErbB2, CA27-29 for breast cancer; CA125 for ovarian cancer; alpha-fetoprotein for testicular cancer; and CA19-9 for pancreatic cancer (Sahab, Semaan et al. 2007).

2.2.1 Single Biomarkers

Historically, efforts in finding biomarkers have focused at one target at the time for each disease. The perhaps most commonly known single serum biomarker is Prostate Specific Antigen (PSA) that has been in clinical use since 1986 when it was FDA approved for monitoring of men diagnosed with prostate cancer. In 1994, PSA was also approved for prostate cancer detection, in combination with digital rectal examination (Reed and Parekh 2010). The use of PSA is, however, not uncontroversial – at a sensitivity level of 53% the
specificity is only 72\% (Nogueira, Corradi et al. 2010), meaning that in order to correctly diagnose 53\% of the patients that have cancer, 28\% of the healthy men taking the test will be incorrectly diagnosed with cancer, leading to overtreatment causing adverse effects like erectile dysfunction and urinary incontinence. To increase the sensitivity to 83\%, the PSA threshold level needs to be set so low that the frequency of overdiagnosis will increase dramatically, yielding a specificity of only 39\% (Nogueira, Corradi et al. 2010). Because the overtreatment rate is suspected to be so high, a strategy known as “watchful waiting with active surveillance” (WWAS) has been introduced. In a WWAS situation, a patient with elevated PSA level is closely monitored, and only if disease progression is indicated is an active treatment initiated (Corcoran, Peele et al. 2010).

Since PSA is not generally accepted to perform well enough as a prostate cancer diagnostic biomarker, large efforts are made to find new and additional ones that will increase the reliability of a blood-based prostate cancer test. For example, Steuber et al. found that increased blood concentrations of human kallikrein 2 (hK2) was indicative of locally advanced and recurrent prostate cancer in men with elevated but low PSA, and that the hK2 level on its own was indicative of aggressive tumor (Steuber, Vickers et al. 2007). Other candidate serum markers identified include uPAR (Shariat, Roehrborn et al. 2007), already known to be predictive of survival for patients with colorectal cancer (Stephens, Nielsen et al. 1999), and autoantibodies to Huntingtin-interacting protein 1 (Bradley, Oravecz-Wilson et al. 2005).

Although findings like these are encouraging, there are yet much to be learned before a prostate cancer test can be considered to have validated performance and be fully trusted in a diagnostic situation. Our antibody microarray group is currently running a large study on serum from prostate cancer patients in the hope of taking another step towards this end.

Serum biomarkers have also been identified in many other diseases than cancer (for review see Anderson 2010). Mutations in the gene for progranulin have been discovered to cause neurodegenerative disease like Alzheimer’s disease (Ahmed, Mackenzie et al. 2007), and recent studies have showed that serum levels of progranulin can be indicative of such disease, and might come to play an important role in personalizing the treatment for different forms of dementia (Sleegers, Brouwers et al. 2010).

We as many others suggest, however, that it in many cases may be impossible to find single biomarkers that alone carries sufficient diagnostic or prognostic
power to be used for applications within personalized medicine (Adib, Henderson et al. 2004; Alhamdani, Schroder et al. 2009). Instead, it is more likely that a multiplex signature is needed in order to increase the resolution. Here follows a few examples of panels of serological biomarker that have been identified with candidate use in applications for personalized medicine.

### 2.2.2 Biomarker Panels

Ovarian cancer has been the subject of several antibody microarray studies. In 2008 Visintin et al. identified a serum biomarker panel of six proteins displaying 95% sensitivity and 99% specificity for the detection between ovarian cancer and healthy controls (Visintin, Feng et al. 2008). The same year, Bertenshaw et al. assayed 204 serum molecules for dysregulation in the same disease (Bertenshaw, Yip et al. 2008). Both studies found the upregulation of CA-125 to be indicative of ovarian cancer, but the Bertenshaw study failed to repeat the results from the other five markers in the Visintin panel. This could, according to the authors, partly be explained by that the control group in the second study was composed of patients with benign gynecological conditions, rather than of healthy subjects as in the first.

In 2005, Orchekowski et al. analyzed the serum from pancreatic cancer patients using antibody microarrays, identifying a signature with sensitivity and sensitivity above 90% (Orchekowski, Hamelinck et al. 2005). The same group continued by analyzing post-translational modifications on proteins by detecting carbohydrate structures using lectins. By detecting both protein abundance and glycan variation, pancreatic cancer-associated glycan alterations could be identified on proteins including MUC1 and CEA (Chen, LaRoche et al. 2007). Using a Luminex bead array platform, Kim and colleagues were able to identify several serum markers, including soluble CD40-ligand and proapolipoprotein A1, that in different combinations could distinguish breast cancer patients from the normal population with sensitivities and specificities of around 90% (Kim, Lee et al. 2009). Several studies have also identified multi-analyte panels associated with different forms of lung cancer. In one of these, Gao et al. probed the serum from 80 patients with lung cancer, COPD or healthy controls, where seven analytes were found to significantly differ between the groups. By testing combinations of the three and five best antibodies, classifications with sensitivities and specificities above 80 and 90% could be achieved using leave-one-out cross-validation. (Gao, Kuick et al. 2005). Further, a six-analyte panel was defined for identifying a patient's pathologic nodal status, which is an important prognostic factor and is used to
guide treatment (Borgia, Basu et al. 2009). Using the panel, 88 % of the patients were correctly classified.

Autoantibodies, antibodies directed against self-antigens, are often used in the diagnosis of autoimmune disease such as systemic lupus erythematosus (Kurien and Scofield 2006), rheumatoid arthritis (Joseph, Brasington et al. 2010) and autoimmune liver disease (Czaja 2010). They have, however, also been indicated as diagnostic factors in cancer in the form of antibodies targeting specific tumor-associated antigens (TAAs), first recognized in 1977 in patients with malignant melanoma (Shiku, Takahashi et al. 1977). It is believed that tumorigenesis induce the release of many otherwise absent proteins into the blood (Caron, Choquet-Kastylevsky et al. 2007), leading to priming of the immune system to recognize the tumor-associated epitopes as foreign (Tan 2001). As a consequence of this, the immune system’s antibody response could be regarded as an amplification of a signal indicating tumor presence. Such antigens have been identified in several forms of cancer including renal cell carcinoma (Klade, Voss et al. 2001), lung cancer (Winter, Minna et al. 1992) and breast cancer (Crawford, Pim et al. 1982).

While the use of autoantibodies as diagnostic markers is promising in many cases, the fact that they are quite rare, displaying low sensitivity, is a problem. In the cases they do exist they, however, display a high specificity of 98-99 % (Soussi 2000).

The promise of using multiplex serum biomarker signatures for increased power of classification and their use in personalized medicine is demonstrated in the above mentioned examples, as well as in paper I-IV. To detect complex and heterogenic diseases like many forms cancer, where the targets in some cases do not originate from the tumors - but rather from cells in response to them, it is very likely that the use of biomarkers panels will be necessary. The act of choosing the candidate markers, tailoring a final panel and validating it offers greater challenges, some of which will be discussed in chapter 4.

2.3 Economic Aspects of Personalized Medicine

While the tests necessary for a personalized medicine based healthcare can be expensive and entail increased costs, several calculations and simulations have nevertheless estimated that the economic benefits of finding the right treatments compensates for this in such a degree that the aggregated costs per patient will be lower. In 2005, Hornbereger et al. published an economic analysis on the use of the 21-gene Oncotype DX breast cancer assay
(Hornberger, Cosler et al. 2005), in which they concluded that if applied correctly, it will increase predictability of tumor recurrence in lymph node negative, ER positive patients, and save costs, even though the price for a single test is about $4,000 (Ross 2009). A similar simulatory analysis of the cost-effectiveness of the 70-gene MammaPrint™ assay showed that it improves quality-adjusted survival, and that it had a higher probability of being cost-effective than did tests based on classical prognostic factors such as the St. Gallen guidelines or “Adjuvant! Online” (Retel, Joore et al. 2010).

In the case of prostate cancer “WWAS situation” described in chapter 2.2, Corcoran et al. calculated that the cost for active surveillance of suspected prostate cancers lies between 43 and 79 % of the cost for up-front radical prostatectomy, even when considering that some of the WWAS patients will convert to active treatment (Corcoran, Peele et al. 2010).

The conclusion that can be drawn from this is that investing resources in personalized medicine, in spite of initial increased costs, is very likely to not only increase the survival and quality of life for the patients, but also to decrease the overall expenses for health care and society.

The development of personalized medicine will undoubtedly also have large impact on Big Pharma, and it remains to be seen if these companies will embrace the possibilities of increased drug success ratio (e.g. Herceptin) and cheaper clinical trials, or if they will see the development as a threat, as less patients will be prescribed with their drugs, limiting the revenues.
3. Antibody Microarrays

The 2001 unveiling of the human genome (Lander, Linton et al. 2001; Venter, Adams et al. 2001) generated a set of databases that can be used both when measuring mRNA expression (genomics) and for the prediction of peptides for identification in MS based technologies (proteomics). Several commercial array based platforms for the analysis of gene expression are readily available, such as the platforms from Affymetrix (www.affymetrix.com), CombiMatrix (www.combimatrix.com) and Applied Biosystems (appliedbiosystems.com). The field of MS has been revolutionized after several important developments like Matrix-Assisted Laser Desorption Ionization (MALDI), Elector Spray Ionization (ESI) and most recently Multiple Reaction Monitoring (MRM). The importance of proteomic analysis became clear after the realization of that the level of an mRNA often correlate very poorly with the level of its protein product (Maier, Guell et al. 2009). Due to alternative splicing and post-translational modifications, a single gene can be the parent of several protein products (Hoffman, Sniatynski et al. 2008), and the mRNA-based analysis does not address this fact. The dynamics of mRNA and their protein products may also differ between genes with the peak protein level lagging behind the mRNA production. The mRNA turnover rate may also differ greatly between genes, at times being so high that the protein is not expressed at all (Hodgkinson, Eagle et al. 2010). For these reasons, it is clear that to get the true picture of what is going on in the cell, the analysis in some situations needs to be performed at the protein level (see chapter 2.2).

While the MS technology has undergone an impressive development during recent years, providing many vital proteomic data (Listgarten and Emili 2005; Cravatt, Simon et al. 2007), it is still associated with certain limitations. Firstly, its use depends on a database for peptide identification, and secondly, some
proteins may not yield a sufficient number of good peptides to give significant identifications. It is also possible that specific forms of some proteins are not represented in the database. In addition, MS based proteomics often depend on a sample preparation step to reduce the complexity of the sample (Gundry, White et al. 2009). Antibody microarray-based affinity proteomics is not limited by these requirements, and has shown great promise in being a way to overcome these problems.

3.1 The Antibody Microarray Assay

3.1.1 Microarray Fabrication

To create an antibody microarray, specific and high affinity antibodies are immobilized on a surface in an organized way, typically in an array with rows and columns of antibody spots (Borrebaeck and Wingren 2007; Borrebaeck and Wingren 2009). The array printing can be done either by using a contact printer, which use dip pins to collect and dispense the probes, or a non–contact spotter which use piezoelectric technology to shoot pL scale droplets from a glass capillary (Delehanty 2004). In effect, this creates a defined spot pattern on a surface capable of binding a large number of different analytes. Our set-up is based on a non-contact spotter (paper I-IV), which generally gives better spot morphology, and is known to perform better together with proteins in solution than the metal pins of contact dispensers (Schena 2005). There is a large number of surfaces to choose as solid support, ranging from glass and polymer to nanoengineered three-dimensional polyelectrolyte films (Zhou and Zhou 2007). In an optimization study, it was concluded that a polymer slide displayed the best biocompatibility in our set-up (Ingvarsson, Larsson et al. 2007).

Before the sample can be incubated on the array, a blocking agent is added in order to avoid unspecific binding of the proteins in the sample to the surface not occupied by probes. The blocking is in our case done using fat-free milk powder dissolved in PBS buffer. After removing unbound blocking agent, the sample is added (figure 3a) and the antibody probes are allowed to interact with and bind to its target in the complex mix of molecules (figure 3b). The binding and immobilization of the sample analytes to the antibody probes locally increases the concentration of the specific analyte. By washing the array surface with buffer and a mild detergent, unbound molecules are cleared from the array, leaving only the protein analytes of interest bound to the antibodies (figure 3c).
3.1.2 Probes

There are problems associated with the use of full antibodies as probes, as shown by early antibody microarray studies. Miller et al. observed that for their set-up with 184 unique monoclonal and polyclonal antibodies, less than 50% showed acceptable activity after being arrayed (Miller, Zhou et al. 2003). Using a similar set-up, Knezevic et al. found that out of 368 antibodies, both in-house generated and from commercial sources, only 14% displayed adequate reproducibility for use in array applications (Knezevic, Leethanakul et al. 2001). Antibodies are large protein molecules, and have several domains not involved in antigen binding. Differences in these constant parts, combined with the fact that proteins tend to denature when allowed to dehydrate on hydrophobic surfaces, may account for this phenomenon (Mitchell 2002; Pavlickova, Schneider et al. 2004). Hence, only a fraction of commercially available antibodies may be possible to use in an antibody array set-up (Haab, Dunham et al. 2001; MacBeath 2002). These facts, together with the cumbersomeness of the development and production of monoclonal antibodies, and the batch-to-batch variations of polyclonal antibody production, complicate the act of scaling up the number of probes on the array. A way to overcome these problems is to use recombinant antibody probes that share a single and highly stable scaffold and are highly reproducible (Steinhauer, Wingren et al. 2002; Wingren, Ingvarsson et al. 2003). The probes used in our set-up are antibody single-chain variable fragments (scFv) selected using phage display from the n-CoDeR® antibody library (Soderlind, Strandberg et al. 2000). The single-chain construct is based on the two variable domains from the antibody heavy and light chains, tied together with a linker. The library is built around a single framework based on the VH3-23 and the VL1-47 immunoglobulin genes (Soderlind, Strandberg et al. 2000). The choice of these genes was based on their highly frequent occurrence in the human immune repertoire (Jirholt, Ohlin et al. 1998), and the excellent folding properties and high stability of their products (Worn and Pluckthun 2001).

Although antibodies are the by far most commonly used probes, there are several alternatives. Single-stranded oligonucleotides, or aptamers, selected for protein binding have been shown to be highly stable when arrayed on chip surfaces due to their reversible denaturation (Walter, Kokpinar et al. 2008). They also display high specificity, and the highly efficient and automated production process together with the possibility of simultaneously analyzing protein and nucleotide targets makes them an attractive alternative (Brody, Willis et al. 1999; Bock, Coleman et al. 2004; Anderson, Hansen et al. 2006).
Affibodies, based on a protein A domain (Renberg, Shiroyama et al. 2005), and repeat ankyrins (Binz, Amstutz et al. 2004) are examples of other scaffolds that have been evaluated for use in array applications. The antibody however, with its well-documented binding characteristics and long history in immunoassays, remains to be the by far most frequently used protein array probe.

Figure 3. Binding and detection of sample molecules. The probes are immobilized on the surface, and a sample is added (a). The sample is incubated and the probes bind to their respective target (b). The unbound molecules are washed off, leaving only the target molecules bound to the probes (c). Detection of bound molecules can be done by sandwich binder pairs where the secondary antibody is either directly labeled (d), or with a third antibody carrying an enzyme that can convert a substrate into a phosphorescent molecule (e). In our setup we directly label the antigens with biotin, which then is recognized by fluorescently labeled streptavidin.
3.1.3 Detection

There are several methods to detect how much protein has been bound the antibody spot. The most straightforward method is to directly tag the sample proteins with fluorescent dyes, typically Cy3 or Cy5 that absorb and emit light at different wavelengths (figure 3d) (Wingren and Borrebaeck 2008). This makes it possible to run two (or more) samples simultaneously on one array, each labeled with a separate color. The signals from the two samples can then be measured using different scanner wavelengths, revealing the relative concentrations of analytes between the samples (Han, Oh et al. 2009; Schroder, Jacob et al. 2010).

A highly stringent method of detection is to have a binder sandwich pair, where a secondary antibody binds to the antigen already immobilized by the primary antibody, in a effect creating a sandwich-like complex (figure 3e) (Huang, Huang et al. 2001). Depending on which label molecule is being used, the secondary antibody can be detected either directly (e.g. fluorescence) or by addition of an agent specific for the secondary antibody (e.g. phosphorescence or fluorescence). The use of sandwich pairs is, however, limited for two reasons. Firstly, it depends on the availability of a functioning antibody pair. The two antibodies in such a pair must not recognize overlapping antigen epitopes, or epitopes that are positioned in a way where the antibodies would sterically hinder each other. The logistics for the process of obtaining such compatible antibody pairs is complicated and a major bottleneck. Secondly, the number of sandwich pairs possible to use in a single array is limited to between 30 and 50 due to the risk of cross-reactivity (Haab 2005; Sanchez-Carbayo 2006).

Another approach with which analysis of carbohydrate post-translational modifications is possible utilizes lectins as detection probes. In this set-up, multiple differently labeled lectins can be used to multiplexly detect several forms of glycan groups, in combination with detection of the protein abundance (Chen, LaRoche et al. 2007). This method generates information that can be used to study differences in post-translational modifications of carbohydrates, and was used in the Orchekowski study of pancreatic cancer mentioned in chapter 2.2.2 (Orchekowski, Hamelinck et al. 2005).

In paper I-IV, we have used a primary labeling strategy that is based on covalently linking biotin, a small molecule with extremely high affinity for streptavidin, to the sample proteins (figure 3f), which have proved to be the most efficient method in terms of signal intensity and signal to noise ratio for
our (Wingren, Ingvarsson et al. 2007; Wingren and Borrebaeck 2008) and other’s set-ups (Kusnezow, Banzon et al. 2007). In this protocol, the proteins in the sample are tagged with by linking the biotin molecule via a reactive group that binds to primary amines on lysine and arginine residues (Wingren, Steinhauer et al. 2005). The biotin tag can then be exploited by adding fluorescently labeled streptavidin, which, after binding to biotin, can be measured with a confocal scanner. The fact that several fluorescent molecules can be attached to each streptavidin molecule also generates a sort of signal amplification in the readout step. This method was chosen in favor of a two-color approach after an optimization study that found that the different colors displayed highly different labeling efficiency, which meant that even when running the samples using criss-cross labeling, the samples are essentially analyzed as on-color experiments (Wingren, Ingvarsson et al. 2007; Wingren and Borrebaeck 2008).

An overview of the set-up for our antibody microarray platform can be found in figure 4.
Figure 4. Our current antibody microarray set-up. Spotting of scFv probes is done using a non-contact spotter on a polymer slide (a). The spotter uses piezoelectric technology to shoot pL scale droplets from a glass capillary (a1). The droplets containing highly purified scFv probes (a2) immediately dry out on the hydrophobic chip surface (a3 and a4).

After the spotting of the about 130 different scFv probes in eight replicates is completed (b), the probes are ordered one or several arrays (b1), where each spot contain one type of probe with known specificity (b2-4).

When the sample is added (c) the probes bind to their respective target (c1 and c3). A washing step clears unbound sample molecules from the surface, leaving only the target molecules bound to the probes (c2 and c4). By adding a labeled secondary reagent that binds to any molecule captured by the probes, the abundance of the target analytes can be measured using a confocal scanner and quantified using image analysis software.
4. Data Analysis

The handling of data produced by antibody microarray experiments can be challenging. In contrast to DNA microarrays, no clear guidelines yet exist. We have developed strategies for all steps in this process, spanning from chip-to-chip normalization to signature condensation and optimization in paper I-IV.

4.1 Normalization

When doing measurements on several samples over the course of an experiment, any variations observed between samples should reflect the biological differences, and not be caused by technical variations. In most cases, however, the raw data from antibody microarray experiments will display variations of both biological and technical origin (Yang, Dudoit et al. 2002). The normalization should ideally eliminate all the technical differences, i.e. labeling efficiency; array quality differences or any other technical factor that is systemic, between the arrays, leaving only the true, biologically relevant, differences in the data (Park, Yi et al. 2003).

The perhaps simplest method, developed for DNA microarrays, is global normalization, which uses the log ratios of the mean or median for all signals on the array to calculate a scaling factor for each array. All signals are then scaled accordingly. This method is, however, not very well suited for our antibody arrays since the number of probes is much smaller (hundreds as compared to tenths of thousands).

In 2005, Haab and colleagues published a study in which they undertook to determine how to optimally normalize antibody microarrays (Hamelinck,
Zhou et al. 2005). They compared seven different normalization strategies as applied to a pancreatic cancer dataset. The methods included measuring the IgM concentration on both the array and using ELISA, mean centering using all signals on the arrays, Loess normalization (regression of the two-color rations) and the use of a spike-in protein. Out of these alternatives, we chose to try a spike-in strategy when we ran the first studies, subsequently spiking all samples with the same amount of cholera toxin subunit B (CT). The anti-CT probe was titrated on the array, and a linear regression was made in order to find an equation for each array that could be used to adjust the remaining signals. Although this method worked (Ellmark, Ingvarsson et al. 2006), it was not as robust as desired. Instead we chose to use of a semi-global method, where a subset of antibodies was used to calculate a scaling factor for each array. The coefficient of variation (CV) was calculated for each probe, and the 20% of the probes with lowest CV was identified. The mean signal for each array over the selected subset of probes was calculated, and the relation between that value and the average for the same probe subset over all arrays was used as a scaling factor, and the data was adjusted accordingly. This method was used to normalize all data in all four papers included in this thesis.

4.2 Data Mining and Classification

Once the data is normalized, the process of mining it for information can begin. A simple way of doing so is to look at one protein at a time, calculating the fold change or a p-value for each protein, indicating up- or down-regulation between the sample groups. The significance of the dysregulations can then be estimated by determining a false positive rate (FPR), which takes the fact that random noise from multiple observations can correlate with the pattern you are looking for into consideration.

However, the fact that a large number of different measurements have been done adds new dimensions to what you can do with the data.

4.2.1 Unsupervised and Supervised Classification

In the studies included in this thesis, the aim has been to classify, or separate, patients into predefined groups, e.g. healthy vs. cancer or benign vs. metastatic tumor. This is done by investigating if the molecular composition of the targets analyzed in a patient look more like the ones in the patients from “group A” or “group B”.

Out of the large number of ways to do this, the perhaps simplest method is an unsupervised classification. This is typically performed using hierarchical
clustering analysis, where an algorithm arranges the samples into clusters based on which other samples they most resemble (Eisen, Spellman et al. 1998). The algorithm identifies the underlying patterns in the data, and can sort both samples and proteins accordingly. One problem with this strategy is that these pattern-recognition algorithms will find patterns in any data, thus any findings must be carefully validated both in a statistic and a scientific sense (Boutros and Okey 2005). This process is said to be unsupervised due to the fact that the algorithm has no information about which group (A or B) the samples belong to (Bowd and Goldbaum 2008), and results in a tree with branches showing the unbiased relation between the samples. If the clustering process created a tree where the members of group A and B are mostly separated onto different branches, it can be concluded that the measurements made with the array found information relevant for the state according to which the original group stratification of patients was made.

In contrast to unsupervised classification, supervised classification actively tries to identify differences between the samples in the defined groups (Bowd and Goldbaum 2008) by training it on a subset of samples, often denoted “training set”. When the model is trained, it can be tested on a new set of samples called, denoted “test set” or “validation set”. If the model was trained on data that held information relevant to the state investigated, it should be able to blindly predict the group belongings of the samples in the test set.

In paper I-IV, I have used a supervised learning method called Support Vector Machine (SVM) (Cortes and Vapnik 1995). The SVM has become a popular kernel method due to its high efficiency relative easy use, and is now considered a standard algorithm for regression and classification (Melville, Burke et al. 2009). The SVM uses the data to map the training set samples in space in a way that makes the samples from the two groups separated with a gap as large as possible (figure 5a). It then constructs a hyperplane in this gap that later can be used for classification (figure 5b). After the construction of the hyperplane, the model is tested by placing new samples from the test set in the space as defined by the model. By observing on which side of the hyperplane the test sample ends up (figure 5c and 5d), and by measuring the distance to said plane, an estimation of which group the sample belongs to can be made.
4.2.2 Evaluation of classifications

Once the group identities for all samples in the test set have been predicted by the SVM, the result can be evaluated by e.g. calculating the sensitivity and specificity of the classification. The sensitivity of a classification is determined by how many of the patients that actually are sick, were classified as sick by the SVM. Therefore, if 80 of 100 sick patients included in the study were identified as sick, the classification will have a sensitivity of 80 % (80/100). The specificity, on the other hand, measures the fraction of the patients without disease that were correctly classified, thus, if 70 of 100 healthy
individuals were classified as healthy, the specificity for the classification will be 70 % (70/100).

The sensitivity and specificity of a classification are always dependent of a threshold value. ‘Above which level of a disease marker in a sample should a patient be considered to be sick’? By modulating this threshold, the sensitivity and specificity can be changed for any given classification. The extreme situations are easy to imagine. By setting an impossibly high threshold level of the marker, every patient will be classified as healthy. This will obviously lead to that, i) every sick patient will be miss-classified as healthy, ii) all healthy patients will correctly be classified as healthy. In terms of sensitivity and specificity, this will yield a sensitivity of 0 % and a specificity of 100 %. The opposite situation is equally easy to create by assigning a threshold value that is extremely low, resulting in a sensitivity of 100 % and a specificity of 0 %. The best threshold level must therefore be found somewhere in-between. A common way of examining this is to create a Receiver Operating Characteristic (ROC) curve (Lasko, Bhagwat et al. 2005).

The ROC curve was developed during World War II, where radar operators had to decide if a blip on the radar screen represented an enemy ship or a friendly one, a situation analogous to when classifying a patient as, e.g. being sick or healthy (Mayer 2004). The ability of the operator to do this was evaluated using a ROC curve, which is created by modulating the threshold from a value that gives a specificity of 100 % and sensitivity of 0 % (figure 6a) to a value that gives a specificity of 0 % and a sensitivity of 100 % (figure 6b). The sensitivity and specificity for each relevant threshold (one that changes the classification for at least on patient) is determined, and plotted against each other (sensitivity is plotted against 1-specificity). The plot is interpreted as a curve, and the area under the curve (AUC) is calculated. A ROC AUC of 1.0 represents prefect separation, and a value of 0.5 means that the classification is no better than a coin-toss, i.e. the prediction is correct in 50 % of the cases. The power of the ROC AUC value is that it is independent of choosing a threshold for the classification, and can therefore be used to compare completely different classifications.
The ideal setting when doing studies of the kind I have conducted is to have two (or more) independent data sets. This is, however, not always possible, especially not in an exploratory study where sample accessibility can be low (as in paper II) and experimental costs high. To handle the lack of a separate validation set in the discovery phase (paper I-III), a cross-validation strategy can be used. The simplest cross-validation strategy is leave-one-out, where instead of building a single model and testing it on new samples, the model is built with one sample left out. The left out sample is then used to test the model. The process is repeated until all samples have been left out from the

4.3 Data Dredging and Overfitting

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training and used as test sample once. This results in one classification for each sample, based on a training procedure using all other samples in the data set. Another obvious way to do validation in a single dataset this is to split it in two parts and treat them as two separate sets. This, however, requires a reasonably large dataset, and may therefore not always be applicable.

In the case of leave-one-out, there are many very similar models being built, each tested on a single sample. In the case of splitting the data into one training set and one test set, one model is trained once and later tested on several samples. One other thing, however, sets these strategies apart; the fact that you can do a leave-one-out cross-validation in only one way, while it is possible choose between any number of ways to split the data into training and test sets. The correct way to do the latter is to randomly divide the samples into training and test sets, and to do it only once. If the procedure is repeated, an average performance from all attempts should be reported. Failure to do so \textit{(i.e.} choosing to report only the value from the best performing attempt\textit{)} will result in losing the whole purpose of splitting the dataset in the first place – a leakage of information has occurred.

The most important thing to consider when handling data for the purpose of training and testing is to avoid data dredging by making sure that the flow of information is a “one-way street”. Under no circumstances, should knowledge learned from the test data be allowed to impact the training process. Training in this sense does not need to involve an advanced self-learning algorithm; the mere act of choosing a signature of genes or proteins based on their performance should in this case be considered as training. If the samples that will be used to evaluate the signature were included in the process of choosing the same signature, very little can be deduced about the signature’s performance at the test stage. This is also true when cross-validation is applied, which makes it impossible to, for example, first step select a signature based on analyte performance in the whole data set, and continue to test it in the same data. Doing so is likely to result in an overfitted model, describing random noise in the data rather than true group differences. Such a model can, without independent testing, easily be mistaken for a very good one. Overfitting can also occur if the model is excessively complex, which we avoid in all papers I to IV by only using a linear kernel in the SVM (figure 7).
Figure 7. Schematic figure of three models with various degree of overfitting. The top three panels represent three different models trained on the same dataset. The middle three panels show the trained model and the bottom three the testing in a new dataset. The leftmost model is highly overfitted in the sense that it during the training severely overinterprets the data. The center model is also overfitted, but to a lesser degree. The rightmost model can be seen as a simple linear regression, much like the linear kernel used in the SVM algorithm I have used, minimizing the noise's impact on the model training. In the bottom three panels the three models are tested on a new dataset. It is now clear that leftmost and center model are overfitted since they perform worse in the new dataset, and it is evident that their complexity is unmotivated.
4.4 Signature Optimization

After, in an unbiased way, having concluded that the data carry information about the state being investigated, it can be of interest to identify a subset of genes or proteins that together carry the information relevant for the classification. This was the case in paper IV, where we aimed to pinpoint a condensed list of markers to classify the samples in the validation dataset.

As briefly mentioned earlier in this chapter, one way of estimating if an analyte carries information is to perform a statistical test, such as t-test (if the data is normally distributed) or a Wilcoxon signed-rank test. The p-value from such a test, together with the fold change (the ratio of the average signal values between the groups) indicates with what significance and to what degree the marker is differentially expressed. While this is true on the single analyte level, combining the top ranked markers might not yield the best signature for classification. The main reason for this is correlation. If two markers, each on its own, display significant differential expression, but do so in a highly correlated way, using them both might be unnecessary since they, by definition, to a high degree contribute with the same information (figure 8a). Secondly, analytes with lower individual significance can synergize, and together be able to separate the samples (figure 8b). Thus, if designing a signature based purely on the individual performance of the markers, important information may be lost. To handle this, we, in paper IV, designed a backward elimination strategy to identify a signature that we could trust to do the classification as well as, or better than, the complete array.

Figure 8. Correlation between analytes in a dataset. (a) Two highly correlating biomarkers (x and y), each on their own separating the sample groups. Because of this correlation, it is likely unnecessary to use them both in a classifier. (b) Two analytes with low correlation and low ability to separate the sample groups. In this case, however, their ability to synergize allows for a vector to be created that perfectly separates the groups, and they might therefore both qualify for use in a classifier.
4.4.1 Backward Elimination

It is computationally impossible to test all the billions of possible combinations of biomarkers even when, as in our case, only using just over 100. As in the case of array normalization, there is no “gold standard” method that can be applied in order to render a good subset of markers. The backward elimination strategy we designed essentially estimates a relevant fraction of these combinations to test. The process starts by excluding one of \( N \) markers in the dataset, and subsequently training and testing an SVM using leave-one-out cross-validation with \( N-1 \) markers. The ROC AUC is calculated for the classification, and the marker excluded is put back in the dataset. The process is repeated until all possible \( N-1 \) marker combinations have been tested, which results in an \( N \) long list of ROC AUCs. The highest ROC AUC is identified, indicating which marker was excluded when the classifier was performing at its best. The identified marker is therefore considered to be the least important, and is henceforth eliminated from the dataset, leaving \( N-1 \) markers. The first elimination round is thereby concluded, and the procedure is reiterated; now training and testing \( N-2 \) long signatures. The iteration continues until only a single marker remains, and the order in which the markers were eliminated can now be used to build signatures of any desired length, starting from the end of the list. By observing how the ROC AUC is influenced by the removal of markers, an optimal length can also be estimated. When I in paper IV used this procedure to deduce a signature from the discovery dataset to test in the validation dataset, I added to the complexity of the above described by using an additional leave-one-out loop. By doing so I had an “unused” sample after the completion of the backward elimination procedure. The left-out sample could unbiasedly be tested using a model trained on the other samples, with a signature based on the elimination order identified. When this outer leave-one-out loop was completed, the process has generated as many elimination order lists as there are samples in the dataset. To build one final signature, I (in paper IV) scored each analyte based on its average survival during the elimination, letting only the top scoring analytes qualify for the final signature.

The details describing these processes, along with a schematic illustration, can be found in the supplementary material for paper IV.
5. Clinical Applications

In the four original papers upon which this thesis is based, we have reported disease associated serum biomarker panels that have displayed potential in a range of applications within personalized medicine. All of these studies were designed to address one or several key, unmet clinical needs, further illustrating the potential impact of personalized medicine for the direct benefit of the patients, healthcare and society in general. To this end, we employed our in-house developed recombinant antibody microarray, composed of about 130 different antibody specificities assaying mainly immunoregulatory proteins, such as cytokines, chemokines, complement factors and growth factors. The diseases investigated range from cancers (breast cancer in paper I and IV, and glioblastoma multiforme in paper II) to autoimmune disease (systemic lupus erythematosus and systemic sclerosis in paper III). This chapter will address how we in paper I-IV have applied our antibody microarrays to decipher candidate serological biomarker panels with potential use in e.g. diagnosis, classification and prognosis as well as in selecting patients eligible for therapy, taking a step towards personalized medicine in each of these diseases.

5.1 Breast Cancer

Breast cancer accounts for about 30% of the malignancies in women worldwide, making it the most common cancer in the female population (Jemal, Siegel et al. 2008; Ahmed 2010; Lorigan, Califano et al. 2010). Survival has increased over the last few decades in the Western world with the introduction of mammography for tumor detection and new adjuvant treatment alternatives (Sparano, Fazzari et al. 2005; Tuttle, Abbott et al. 2010). Approximately 30% of patients with primary breast cancer develop metastasizing disease, for which the possibility of cure is very limited
(Gonzalez-Angulo, Morales-Vasquez et al. 2007). Therefore, the need for a non-invasive test that could aid in the diagnosis of breast cancer at an early stage, the perhaps the most promising way of increasing the survival further, is greatly needed. After diagnosis and surgical removal of the primary tumor, several prognostic and predictive factors are important in designing the postoperative treatment. To date, our ability to monitor and predict the outcome in breast cancer is still very limited. In this chapter, I have addressed the results from my two breast cancer studies (paper I and IV), and how they relate and could impact the diagnosis and prognosis of breast cancer, in terms of personalized medicine.

5.1.1 Diagnosis

Breast cancer can, at the time of diagnosis, be divided into two major classes – in situ or invasive cancer (Yarnold 2009). Ductal carcinoma in situ (DCIS) is predominantly detected by mammography since it often is unpalpable (Yarnold 2009). Other imaging methods like magnetic resonance imaging and ultrasound are used in combination with mammography and clinical breast examination, and can further increase the sensitivity of tumor detection (Saslow, Boetes et al. 2007). DCIS tumors can be large, but have by definition not spread beyond the breast (Yarnold 2009). After surgical excision, the tumor is graded according to cytonuclear features into low, intermediate or high grade, with increasing risk of local recurrence (Lagios, Margolin et al. 1989). Once the tumor has been removed, the disease progress is still very difficult to predict and monitor, as high-performing biomarkers (and the required tools) are still missing.

In contrast to DCIS, invasive breast cancer infiltrates breast stroma and may spread to lymphovascular spaces and metastasize (Yarnold 2009). At the time diagnosis, invasive tumor can also be classified according to several different standards, most importantly by histological grading, separating the tumors into three classes, grade I to III, with increasing aggressiveness (Yarnold 2009).

In paper I, we set out to identify a panel of biomarkers that could distinguish between metastatic breast cancer patients hand healthy matched controls, i.e. to see if diagnostic information could be extracted from the serum proteome. Hence, crude, non-fractionated serum samples were analyzed using our antibody microarray platform, and the diagnostic power of the information gathered was tested using a leave-one-out cross-validated SVM. The cross-validated classification displayed a ROC AUC of 0.92, displaying high specificity and sensitivity. A signature composed of 11 analytes displaying
significant dysregulation was assembled, including biomarkers both previously identified as associated with breast cancer such as sialyl Lewis\(^\text{a}\) (Matsuura, Narita \textit{et al.} 1997; Kurebayashi, Nomura \textit{et al.} 2006), C3 (Li, Orlandi \textit{et al.} 2005), C4 (Lamoureux, Mandeville \textit{et al.} 1982), and IL-8 (Lin, Huang \textit{et al.} 2003; Lin, Huang \textit{et al.} 2004; Lin, Wang \textit{et al.} 2005; Nicolini, Carpi \textit{et al.} 2006), as well as previously unknown, e.g. C5, IL-5 and IL-7. Further, to investigate if the effect of different treatments and disease aggressiveness could be observed, we divided the patients into groups based on tumor stage, and whether or not they had been prescribed anti-inflammatory medication. While these subgroups were smaller, hierarchical clusterings using the top differentially expressed analytes separated the cancer patients from the healthy controls perfectly when excluding the patients on anti-inflammatory medication. The same result was seen when only using patients diagnosed with grade III or IV tumors. These results might indicate that the predictive power increases after dividing the patients into more homogenous subsets. However, the fact that the first classification, using all patients, still managed to sustain high discriminatory accuracy suggests that the information in serum was relevant and detectable, in spite of the wide array of anti-inflammatory and hormonal agents that had been prescribed.

With these findings, we had for the first time identified a candidate serum biomarker panel that with high accuracy could discriminate between metastatic breast cancer patients and healthy controls. Thereby, the data demonstrated the power of both the antibody microarray technology and the panel of antibody specificities included in our platform.

There are, however, other suggested candidate serum biomarkers with demonstrated potential diagnostic value in breast cancer. In a recent publication, levels of hypermethylated gene promoter 14-3-3-sigma in DNA extracted from serum was found to differ significantly between breast cancer patients with metastatic disease and those without clinical or radiological evidence of recurring disease (Zurita, Lara \textit{et al.} 2010). In this case, the target was methylated DNA rather than a protein, but accessible in serum nonetheless. There are also other examples where serum proteins have been identified as having potential diagnostic usefulness. For example, activated leukocyte cell adhesion molecule was reported to slightly outperform both CEA and CA15-3 as a diagnostic biomarker for breast cancer (Kulasingam, Zheng \textit{et al.} 2009). The predictions in these two studies resulted in ROC AUCs of 0.80 and 0.78, respectively, but are yet to be validated in independent data.
The need for new biomarkers that could contribute to earlier detection of breast cancer, one of the most important factors in increasing survival (Duffy, Tabar et al. 2002), is very clear. The results from paper I demonstrated the feasibility of finding serological markers for this purpose, and that affinity based proteomic technologies is an attractive alternative for disease diagnostics of crude clinical samples, such as serum.

5.1.2 Prognosis

Prognostic factors in breast cancer that can identify patients destined for recurring disease is crucial in selecting patients for post operative systemic adjuvant treatment (Donegan 1997). The currently most powerful prognostic factor for breast cancer patients is axillary lymph node status, with strong negative correlation with disease-free survival (Donegan 1997; Yeatman and Cox 1999; Weigelt, Wessels et al. 2005). Other important factors for treatment selection in lymph node negative patients are age, tumor size, histologic and nuclear grade, steroid hormone receptor status, ErbB2 status, lymphatic and/or vascular invasion (Goldhirsch, Glick et al. 1998; Schnitt 2010).

In paper IV, we investigated if we could identify serological information relevant for prognostic purposes, which could generate significant added value to current methods, providing unique prognostic information in designing an optimal breast cancer regimen. By assaying the serum proteome, we set out to predict the development of metastatic disease in 45 patients diagnosed with DCIS or invasive breast cancer. A unique set of samples had been collected starting at the time of diagnosis, i.e. surgical removal of the primary tumor, and then continuously every six to twelve months for up to three years, resulting in between three and five samples per patient. This longitudinal nature of the study gave us a unique possibility to dynamically monitor the serum proteome post primary surgery.

The patients were divided into groups based on whether or not they would develop metastatic disease during seven years of follow-up data available. Our initial efforts were aimed at identifying differences in the absolute levels of the markers between the groups to be able to identify patients destined to develop metastatic tumors. While small differences were found, they were not considered statistically significant. However, the collection of longitudinal data enabled us not only to observe the absolute levels of the markers, but also monitor to how they changed over time. Thus, by comparing the levels of each marker in the samples collected at the time of primary surgery and three to six months later, the velocity with which each analyte had changed could be
calculated. This concept of analyzing the dynamics of serum markers has been explored previously – the perhaps most well known case being PSA velocity for the prognosis of prostate cancer (Taverna, Grizzi et al. 2009; Frank O’Brien, Cronin et al. 2010). Similarly, the kinetics of CA 15-3 has been associated with disease progression in breast cancer patients (Kim, Park et al. 2009).

Thirty-eight patients had samples collected at the relevant time points, and were still after at least six months undiagnosed with recurrent tumor. Consequently, any prediction made using these samples was truly a prediction. As in the earlier study, a leave-one-out cross-validated SVM was applied, but was now fed velocities from all the included markers, rather than the absolute levels. The predictive power of the cross-validated classification was estimated with a ROC AUC of 0.88, with several analyte velocities displaying highly significant dysregulation between the patient cohorts, the top three being Lewisx (p = 0.0005), IL-16 (p = 0.002) and CD40 (p = 0.0003), which all increased during the evaluated period.

To pre-validate this finding, an independent set of samples from 26 (13 + 13) new patients with samples collected the same time points (time of primary surgery and three to six months later) were analyzed. The samples were run “blindly”, i.e. without knowledge of which patients belonged to which group. Before unveiling the group identities, a panel of 21 antibodies was pinpointed using the data from the first sample set. To this end, we designed a backward elimination protocol, which is described in some detail in chapter 4.4, and to a greater extent in the supplementary appendix of paper IV. Before testing of the optimized marker panel, the datasets were normalized to each other, and a single SVM model was built using the identified markers and the samples in the first dataset. The SVM model obtained was then used to predict the outcome for the 26 new patients, which after revealing the true group identities, was found to classify the patients with a ROC AUC of 0.85. The optimized panel displayed an upregulation of several markers known to be involved in cell migration and infiltration, including CCL2, factor B, Il-5, IL-6, IL-9, IL-13, IL-18, IL-12a, Lewisx/sialyl Lewisx, TNF-β and SERPING1. The results from this pre-validation thus showed that prognostic information indeed could be extracted from the serological markers assayed, which to the best of our knowledge is demonstrated for the first time in this study. Other studies with related aims have shown known cancer serum markers like CEA, CA15-3 and NCC-ST-439 (Tampellini, Berruti et al. 2006; Yonemori, Katsumata et al. 2008; Molina, Auge et al. 2010), or serum levels of the
extracellular ligand-domains of epidermal growth factor receptor and HER-2/neu (Sandri, Johansson et al. 2007) in order to predict the response to chemotherapy treatment of metastatic breast cancer, displaying a ROC AUC of 0.72.

In today’s clinical situation, parameters such as clinical (age, menopausal status), histopathological (histological grade, lymph node status, tumor size) and hormone receptor status (ER, PgR) are used as prognostic factors. Interestingly, quantifying these parameters and including them in the SVM classifier increased the accuracy of the prediction accuracy to a ROC AUC of 0.90. Additionally, when using the conventional prognostic markers alone, the classifier produced a ROC AUC of 0.66, showing the array data was the major contributor, and that new information indeed is detected in serum. While the conventional parameters displayed poor predictive power in our sample set, it should be noted that others have with the use of similar classifiers been able to do the same prediction with a ROC AUC of 0.78 (Eden, Ritz et al. 2004).

The benefits of predictive test based on our results could be several, primarily leading to a decrease in overtreatment of those patients where breast conservative surgery is enough to clear the tumor. Another possible application is discernible in the light of this study – interestingly, a few patients that were still after six months receiving chemotherapy were predicted as belonging to the high-risk group, and were subsequently diagnosed with recurring tumor. In the clinical setting the result from this test could potentially be used as an indication of that the patient is not benefitting from the current regimen, and that a switch to an alternative chemotherapy needs be to consider. Information of this kind is essential in optimizing the treatment on an individual-based level, again illustrating the essence of personalized medicine.

In light of the increasing use of the gene expression based prognostic assays described in chapter 2.1.2, it is evident that the results from paper IV, if successfully validated, could be of great use and interest in breast cancer prognostics. This becomes even clearer when considering that our approach has the clear advantage of assaying easily obtainable blood samples, while both the OncoType DX and MammaPrint™ assays require tumor tissue. Consequently, the continuous availability of blood from the patients makes it possible to monitor the disease progression using our approach. Additionally, our results have, so far, not indicated that the application of the panel is limited to certain patient subsets, which is the case for the other two where only lymph node negative (and in the first case also only ER positive) are
eligible for the test (chapter 2.1.2). In figure 9, the candidate use of the biomarker panels identified in paper I and IV are placed in the context of where they could come to impact today’s treatment decision making.

Figure 9. The clinical decisions in breast cancer treatment, and how the results from paper I and IV are associated with them. In paper I, we discovered a biomarker signature capable of distinguishing serum from metastatic breast cancer patients from serum from healthy controls. In the second breast cancer study, paper IV, a classical problem within personalized medicine was addressed: the possibility of predicting which among newly diagnosed patients would develop metastases. A panel capable of doing this was identified and pre-validated using a new, independent set of samples.
5.2 Glioblastoma Multiforme

Glioblastoma Multiforme (GBM) is the most common and aggressive type of brain tumor, with a median survival of less than 15 months after treatment with surgery followed by radiation and concomitant chemotherapy (Ohgaki and Kleihues 2005; Quick, Patel et al. 2010). Hence, the need for new treatment alternatives is urgent. To this end, several studies have shown promising results using immunotherapy in glioma treatment, at best doubling the survival (Steiner, Bonsanto et al. 2004; Fakhrai, Mantil et al. 2006; Yamanaka 2008).

The perhaps first immunotherapy experiment dates back to 1891, where William Coley injected bacteria into an inoperable cancer patient. His idea was that the infection would have the side effect of shrinking the tumor, which was proven to be true. He continued his research, injecting over 1000 patients with bacteria or products thereof, later known as Coley’s toxins. Though he and others reported good results, the use of Coley’s toxins gradually disappeared with the introduction of radio- and chemotherapy (McCarthy 2006). With the growing understanding of immunology, Coley’s theory has been proven to be true, and since 1976 the bacteria Bacillus Calmette-Guerin has been used for the treatment in bladder cancer (Morales, Eidinger et al. 1976; Simons, O’Donnell et al. 2008). Most modern immunotherapy research, however, focus on tumor associated antigens (Copier, Dalgleish et al. 2009).

In this context, Salford et al. performed a phase I clinical study, where GBM patients where immunized with their own tumor cells, transfected with IFN-γ (Salford, Siesjö et al. 2002). The therapy resulted in a promising 60% prolongation of overall survival for the treated patients, and did not cause any serious side effects. Biomarkers for monitoring the response of immunotherapy are much sought after, primarily to be able to differentiate responding and non-responding patients as early as possible (Copier, Dalgleish et al. 2009).

The effect of immunotherapy often vary greatly between patients, as observed in earlier studies (Steiner, Bonsanto et al. 2004) and paper II, where the average survival time for patients experiencing high treatment efficiency was almost the double that for while the patients where the therapy appeared to have lesser effect. Hence, in paper II, we targeted this discrepancy in order to find serum biomarkers that could guide in identifying patients that would benefit from the therapy. Consequently, we analyzed serum samples from the 18 grade IV GBM patients included in the immunotherapy protocol. The tumor
cells from eight of the patients had showed sufficient cell growth after isolation, and was subsequently transfected and used for immunization in the respective patients between eight and 14 times. The remaining 10 patients were used as a control group in parallel with 17 healthy control subjects. The samples were collected preoperatively, and then again after the fourth and eighth immunization.

A leave-one-out cross-validated SVM was employed in an initial attempt to classify the patients as GBM or healthy, using the preoperatively collected sample for the GBM patients and serum from healthy controls. The model’s predictive power was found to be poor, displaying a ROC AUC of only 0.60. We continued to try to classify subsets of the GBM patients vs. Healthy controls, by dividing them into groups based on if they were included in the immunization protocol or not. The classification accuracy of the immunized patient cohort was increased to some extent, displaying a slightly larger ROC AUC of 0.69.

The average survival in the immunized group was significantly higher (524 days) than that of the non-immunized group (298 days), yet large differences could, as mentioned earlier, be found within the two groups. Therefore, the patients could be further subdivided into long and short survival in both the immunized and non-immunized groups. The immunized long survivors lived for an average of 692 days after diagnosis; the immunized short survivors for 357 days. For the non-immunized group, the corresponding numbers were 470 and 184 days. Again, SVM models were constructed and leave-one-out cross-validated to classify the patients in the smaller subgroups and the healthy controls. The predictions of the patients in the refined groups versus the healthy controls were generally found to be more accurate; all except the short surviving, non-immunized group displayed ROC AUCs between 0.81 and 0.91.

As one of the key areas within immunotherapy research is the identification of biomarkers for monitoring and prediction of patients eligible for treatment (Copier, Dalgleish et al. 2009), we proceeded to see if the samples collected from the immunized GBM patients after the fourth and eighth immunization could be used to monitor the effects of the treatment. By again dividing the immunized patients into the survival-based groups described above, hypothesizing that the immunization treatment worked more efficiently in the former than the latter, indeed, larger differences in the plasma protein profile could be seen between the operation and after four immunizations for the patients with long survival than for the patients with short survival (ROC
AUC of 0.81 vs. 0.50). We also observed that two key T-helper-1 direction cytokines, IL-8 and IFN-γ, were upregulated in the immunized patients, particularly those with long survival, indicating an activated immunoresponse.

GBM tumors display a high degree of molecular heterogeneity, and the need for a personalized medicine approach is evident. It is known that GBM feature genetic variations, which make the tumors differently susceptible to different treatments, and research is ongoing on how to exploit these in order to personalize regimens (Kumar, Zhong et al. 2008). There are also efforts being made in proteomic profiling using MS technology, including the identification of profiles that indicate tumor grade and patient survival (Schwartz, Weil et al. 2005), and capable of distinguishing of high-grade GBM from other tumors of glial origin (Schwartz, Weil et al. 2005). In this study (paper II) we for the first time outlined the potential of antibody microarrays for protein expression profiling for improved GBM classification. Although the number of samples was limited, and confounding factors may exist in some of the subset stratifications, these results indicate that the array of assayed targets may be very well suited for applications within immunotherapy.

5.3 Systemic Lupus Erythematosus and Systemic Sclerosis

Autoimmune diseases are a major cause of death in the western world, affecting 3-8 % of the population (Kunz and Ibrahim 2009). Autoimmunity is a result of the adaptive immune system breaking self-tolerance, and attacking self-tissue (Ermann and Fathman 2001), causing a wide spectrum of diseases including rheumatoid arthritis, psoriasis, Crohn's disease, multiple sclerosis, and type-1 diabetes. This class of disorders manifests a wide array of clinical features, and requires early detection and constant monitoring. Thus, the characterization of biomarkers for these purposes is greatly needed. In this context, we in paper III for the first time addressed autoimmune diseases using our antibody microarray platform. The patients included in the study suffered from either systemic lupus erythematosus (SLE) or systemic sclerosis (SSc), which are two severe, chronic autoimmune diseases.

SLE is an incurable disease, and has so many different symptoms that it often is mistaken for other indications (D'Cruz, Khamashta et al. 2007). As a result of this, many patients may suffer from the disease symptoms for years before they are correctly diagnosed. The pathogenesis of SLE is unclear, but defects in the clearance of apoptotic cells leading to macrophage uptake and presentation of intracellular components to T- and B-cells has been suggested to initiate the autoimmune process (Munoz, Gaipl et al. 2005). Symptoms
typically involve the skin and joints, but can in more severe cases include cardiovascular events and damage to virtually any organ system, particularly the kidneys. In the latter cases the disease is often mortal. Today, SLE is diagnosed when at least 4 of 11 complex clinical criteria are met (Tan, Cohen et al. 1982; Hochberg 1997). Hence, patients diagnosed with the same disease may display completely different symptoms, making the need for improved diagnostics obvious.

SSc, the other disease included in this study, is a chronic connective tissue disorder known for its unpredictable course, therapy resistance and high mortality (Matucci-Cerinic, Steen et al. 2009). The diagnosis of SSc is, like SLE, based upon the evaluation of several clinical parameters (LeRoy, Black et al. 1988). SSc is further subcategorized into limited cutaneous, dominated by vascular manifestations, and diffuse cutaneous SSc, dominated by progressive fibrosis of the skin, lungs, and other internal organs (Varga and Abraham 2007). The situation is further complicated by the fact that SLE patients’ disease can transform into SSc, and vice versa (Lorber, Gershwin et al. 1994).

The need for biomarkers to diagnose, stratify clinical phenotypes, and monitor the activity of these diseases is significant, why we in paper III analyzed the serum of 30 SLE patients, 20 SSc patients and 15 healthy controls, in order to identify the first generation of candidate serum biomarkers.

To this end, a leave-one-out cross-validated SVM was employed in order to separate the SLE and SSc patients from each other and from healthy control subjects. These attempts resulted in classifications with ROC AUCs of 0.76 for SLE vs. Healthy, 0.59 for SLE vs. SSc and 0.49 for SSc vs. Healthy. This could be explained by the previously mentioned heterogeneity of both SLE and SSc, why treating them as single diseases might not be optimal. Subsequently, we subdivided the patients according to a priori determined phenotypes. The SLE patients were divided into three groups with increasing symptom severity, with the third and most seriously afflicted group being composed of patients with renal involvement, or glomerulonephritis, which is very common and one of the most severe manifestations of SLE (Mok, Tang et al. 2005).

This subdivision indeed increased the resolution of the classification, with a maximum ROC AUC of 0.99 for the SLE patients with glomerulonephritis vs. the healthy controls. The SLE patients with intermediate and the least serious symptoms were harder to classify with ROC AUCs of 0.67 and 0.55, respectively. When the same subgroups of were compared to the SSc patients,
similar results were obtained. Additionally, patients belonging to several of the different phenotypic subgroups were accurately identified, which is of outmost importance in designing optimal treatment regimens for the individual patients. Today, this grading is based solely on clinical parameters, and our findings may be a first step in finding serological biomarkers for this purpose.

SLE is also characterized by periods of remission and flare, for which there currently are no validated predictive biomarkers (Rovin, Birmingham et al. 2007; Liu and Ahearn 2009). It has been demonstrated that the long-term accumulation of damage due to these periods of active disease correlate with death (Stoll, Sutcliffe et al. 2004). While SLE is incurable, the flares are treatable, making the discovery of biomarkers to predict flares and monitor treatment essential. For this reason, we continued by investigating if the degree of SLE activity could be detected in the serum proteome. A commonly used measure of disease activity is the SLE Disease Activity Index, SLEDAI, which is derived from 24 different clinical factors (Gladman, Goldsmith et al. 2000). Several serum proteins including C1q \( (p = 9.6\times10^{-6}) \), known to be associated with the disease (Dillon, D'Souza et al. 2009), and other complement factors displayed high correlation with the patient SLEDAI. Other studies have correlated disease activity with serum levels of soluble ST2 (Mok, Huang et al. 2010) and autoantibodies against C-reactive protein (Sjowall, Zickert et al. 2009). Further, by dividing the patients into three groups based on their disease activity, several markers were found to display significantly altered levels between the cohorts. An SVM could also classify the patients into any of these groups with high accuracy, suggesting that serological information in the future might be used to predict disease flares.

In this study we demonstrated the feasibility of antibody microarrays as a tool for SLE diagnostics and personalized disease activity monitoring using blood-based protein profiles. More detailed diagnosis and closer monitoring of SLE patients are important instruments in increasing their quality of life and survival.

### 5.4 Gastric Adenocarcinoma and Pancreatic Cancer

The platform developed by our group has been successfully applied in several other diseases, such as gastric adenocarcinoma (Ellmark, Ingvarsson et al. 2006), pancreatic adenocarcinoma (Ingvarsson, Wingren et al. 2008), preeclampsia (Dexlin et al. in press; Wingren et al. manuscript in preparation), pancreatitis (Sandström et al. manuscript in preparation), mantle cell lymphoma (Dexlin et al. manuscript in preparation), CLL (Wingren et al,
manuscript in preparation) and using other sample formats than serum, e.g. plasma (refs), urine (Kristensson et al, manuscript in preparation), cell lysate and tissue extracts (Dexlin et al manuscript in preparation), intact cells (Dexlin, Ingvarsson et al. 2008).

In addition to the four papers included in this thesis, I have been involved in two of these studies, namely gastric adenocarcinoma (Ellmark, Ingvarsson et al. 2006) and pancreatic cancer (Ingvarsson, Wingren et al. 2008).

In the first study Helicobacter pylori-positive and -negative stomach tissue from and gastric adenocarcinoma patients and normal controls were analyzed for differential protein expression (Ellmark, Ingvarsson et al. 2006). Adenocarcinoma is the second leading cause of cancer-related mortality worldwide (Alberts, Cervantes et al. 2003; Parkin, Bray et al. 2005), and is highly interesting as the risk of being affected is influenced by the chronic inflammation caused by H. pylori infection (Peek and Blaser 2002). Here, we for the first time showed that it was possible to identify candidate expression signatures associated with either gastric cancer or H. pylori infection, and thus that the identified cancer signature was not detecting a general inflammatory disease state.

In the second study, we analyzed the serum proteome of patients with pancreatic cancer (Ingvarsson, Wingren et al. 2008). Pancreatic cancer is one of the most aggressive cancers with a five-year survival rate of less than 5 %, and a death to incidence ratio of 99 % (Laheru and Jaffee 2005). It rapidly metastasizes to liver, lung and lymph nodes, and is highly resistant to therapy (Garcea, Dennison et al. 2005). Pancreatic cancer is extremely difficult to diagnose, a fact contributing to the high mortality rate. Therefore, a sensitive and specific blood based test would be of great value.

The array analysis revealed a set of proteins with substantially altered expression between the pancreatic cancer patients and the healthy controls. By employing an SVM, we could separate the patients from the healthy controls with 100 % sensitivity and specificity. Further, the patients were split into two groups based on short (< 12 months) or long (> 24 months) survival, and could be classified with a specificity of 83 % at a sensitivity of 100 %.

In conclusion, the findings from these and other studies, both published and ongoing, all point to that i) the panel of assayed markers is highly relevant for many diseases and disease states, ii) the signals detected are not merely caused by general inflammation, iii) the identified signatures display a high degree of
disease specificity, since although some overlap is found between the disease signatures, they all include large unique components. While we have yet to run a large study where several forms of cancer are compared, the fact that the different identified cancer-associated signatures have unique compositions suggests that stratification of different malignancies, even when compared to each other, is likely to be possible.
6. Concluding Remarks

This thesis is based upon four original papers, in which I have applied our recombinant antibody microarray platform to survey the serum proteome for biomarker signatures with diagnostic, prognostic or treatment predictive value. This kind of information is important in personalized medicine, where the molecular phenotype of the individual patient can contribute in the clinical decision-making, regarding e.g. treatment.

In two of the studies, I have analyzed a total of three independent sets of breast cancer patients. In the first (paper I), we were able use the microarray data to discriminate between serum from metastatic breast cancer patients and healthy, matched controls. Here, we for the first time showed that breast cancer could be detected using only the information available in blood. In the second breast cancer study (paper IV) we set out to see if information of prognostic value could be discovered in the serum proteome, by analyzing two independent sample sets. To this end, we analyzed the velocity with which the targeted biomarkers changed in the blood of patients during the first three to six months after primary surgery. Indeed, using these velocities rather than the absolute levels, we could correctly predict the outcome in regard to distant tumor recurrence for most patients. These results could have great impact on how decisions for the treatment of breast cancer are made, resulting in reduced overtreatment as well as increased survival due to a more personalized medicine.

While my research has had breast cancer diagnostics and prognostics as a main focus, I have also addressed other aspects of cancer and autoimmune disease. In paper II we analyzed the serum from GBM patients undergoing immunotherapy. The patients included in the protocol were immunized with their own tumor cells after transfection with IFN-γ, displaying a 60 % increase
in survival time. The main finding in this study was that we could stratify GBM patients into groups based on the beneficial response of the therapy, indicating that information that could aid in selecting eligible patients could be extracted, again demonstrating the great potential of antibody microarrays in personalized medicine.

In paper III we concluded that the biomarker panel assayed on our arrays also was applicable in autoimmune disease research, as several key observations were made while analyzing the serum from patients with SLE and SSc. Here, we found that we could identify a number of candidate biomarker signatures for SLE diagnosis; stratification of predefined phenotypic subgroups patients; and for identification of active as defined by SLEDAI. Findings of these sorts are of great importance since diagnosing, stratifying and monitoring this heterogeneous disease could lead to a more personalized treatment. Being able to predict and treat disease flares is the perhaps main factor in increasing the survival and quality of life for SLE patients.

From the experiences made in these studies, we could conclude that a significant amount of information about many diseases and disease states was available in the relatively limited part of the serum proteome that we assayed. The platform will in the near future be expanded with a number of new antibody specificities, in some cases directed against targets already identified as interesting in certain diseases, thus raising the potential of our assay even further.

The results from these exploratory studies certainly need to be corroborated in larger validation studies. In the case of successful validation, it may also be necessary to transfer the assay to different platform more directly compatible with the clinical laboratories around the world. I hope to be able to continue to work against these goals, and to in the future be a part of helping and saving patients with these and other diseases.
Om man vill likna en cell vid något ligger en enorm fabrik nära till hands. Fortsätter man liknelsen kan cellens kärna, som innehåller arvsmassan i form av DNA, jämföras med ett bibliotek placerat inuti fabriken. I detta centralt belägna bibliotek står långa rader av bokhyllor fyldiga till bredden med böcker, som i detta exempel får motsvara generna i vårt DNA. Böckerna innehåller ritningar som beskriver alla de verktyg, maskiner och faciliteter som fabriken rymmer och använder. Böckernas ryggar, alla märkta med en unik streckkod, scannas kontinuerligt av ett stort antal bibliotekarier. När en streckkod stämmer överens med en order som bibliotekarien fått från fabrikschefen, öppnar denne försiktigt boken och kopierar den. Kopian motsvarar ett så kallat mRNA, som är genens arbetskopia.

Kopian skickas ut ur biblioteket och in i fabriken där robotar, i cellen motsvarade av ribosomer, står redo att läsa viken ritning som helst och tillverka vad den än beskriver. I cellen använder ribosomer mRNA som mall när de tillverkar proteiner som är nästan alla geners slutprodukt, och är de molekyler som är cellens verktyg och utför cellens funktioner. Blixtnabbt står ett antal exemplar av det beställda verktyget (proteinet) redo, och ritningskopian (mRNA) förstörs. Det nya verktyget plockas upp av ett transportband som läser av vart det ska och levererar det till rätt adress i fabriken.

Fabriken, i sig komplex som en liten värld, är bara en liten del av ett enormt industrikomplex, liksom celler som tillsammans bygger upp en vävnad eller ett organ. Vissa celler är specialiserade på att ta order om och utföra rörelser (muskelceller), andra på att observera färg eller styrka på ljus från omvärlden (celler i ögat), ytterligare andra på att känna igen och ha ihjäl inkräktare
(immunförsvarscells). Alla har de samma bibliotek av gener att tillgå – dess specialisering sker i valet av vilka gener som används, och i vilken grad dessa uttrycks.

År 2001 stod ett av de mest ambitiösa forskningsprojektens inom biologi och medicin färdigt – man hade kartlagt hela den mänskliga genuppsättningen, dvs., man hade läst igenom bibliotekets alla böcker (även om man fortfarande inte vet vad alla beskriver). Man insåg snabbt att genom att mäta vilka och hur många färdiga ritningar som skickas för produktion, d.v.s. mängden av mRNA för varje gen det finns i cellen vid ett specifikt tillfälle, kan man få en god bild av vad den håller på med. Tekniker för att snabbt och samtidigt analysera uttrycket av tiotusentals olika gener togs fram, bl.a. s.k. DNA arrayer. Med dessa tekniker kan man analysera cellerna i en vävnad och få fram en lista över alla gener som översätts till mRNA, och hur aktivt de uttrycks. Genom att analysera vävnad från sjuka och friska personer kan man jämföra dessa listor, och hitta mönster gemensamma för de sjuka som de inte delar med de friska. Detta har man gjort bl.a. för olika former av cancer, och i många fall hittat gener som är starkt överuttryckta i tumörvävnad.

Den teknik jag använt mig av i de studier som ingår i denna avhandling är på många sätt analog med den ovan beskrivna. Vad vi gjort, på ett sätt ingen annan gjort tidigare, är att istället för att mäta hur generna uttrycks på mRNA-nivå, ge oss på genernas slutprodukt – proteinerna. Det har nämligen visat sig att även om mRNA är helt nödvändigt för att skapa proteiner, så är korrelationen mellan mRNA- och proteinnivån ofta väldigt dålig. D.v.s. den bild man får av cellens aktivitet genom att titta på mRNA är något skev.

Varför väljer då inte alla att mäta proteinnivåer istället för mRNA nivåer? Jo, tekniker för att mäta biomolekyler kräver nästan alltid en s.k. bindare. En bindare är en molekyl som har en förmåga att binda till en målmolekyl. Vid analys av genuttryck är målmolekylen mRNA, vars uppbyggnad gör det väldigt lätt att designa och tillverka en bindare som kan binda upp målmolekylen. Bara genom att veta sekvensen för genen man vill mäta kan man med ett ”musklick” designa en komplementär DNA sekvens som med hög tillförlitlighet kan användas att binda till och mäta denna.

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1 I själva verket är det inte mRNA som används utan istället s.k. cDNA som skapas genom att man översätter RNA tillbaka till DNA, eftersom DNA har bättre bindningsegenskaper. Detta görs på ett sätt så att mängdförhållandet mellan olika mRNA i provet fortfarande återfinns i cDNA.
För analys på proteinnivå är skapandet av motsvarande bindare mycket svårare. Interaktionen mellan proteiner är mycket mer komplex än den mellan DNA och RNA molekyler, vilket får till följd att man (än så länge) inte kan designa en proteinbindare med ”papper och pennan”. För att få fram sådana har vi därför fått vända oss till kroppens geniala immunförsvar som är specialiserat på just detta.


Vår teknikplattform består nu, rent fysiskt, av en panel av ca 130 olika antikroppar av det här slaget, ”framavlade” att binda till ett specifikt mänskligt protein som kan hittas i blod. Genom att med en robot spotta ut små droppar (0,000.000.000.3 liter) innehållande de olika antikropparna på ett litet plastchip, kan vi nu genom att sätta ett blodprov på chippet, mäta förekomsten av dessa proteiner i blodet. Antikropparna som sitter fast på ytan av chippet binder till de proteiner i blodet som de är framavlade för, och genom att sedan tvätta bort alla blodproteiner som ej bundit till någon antikropp, har vi sedan bara kvar de proteiner vi är intresserade av att mäta.

Vi har under ett antal år gjort sådana mätningar på blodprov från patienter med olika tillstånd, allt från cancer till autoimmuna sjukdomar, och jämfört nivåerna av proteinererna med dem hos friska människor eller patienter med andra, liknande, sjukdomar. Vi har sedan undersökt om dessa nivåer på något vis korrelerar med sjukdomen – d.v.s. kan vi hitta mönster i blodet som ”skvallrar” om sjukdomen?
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Information framtagen med analyser både på gen- och proteinnivå som kan användas i prognostiska eller behandlingsprediktiva syften som beskrivet ovan, ingår i begreppet ”personalized medicine” som återfinns i titeln av denna avhandling. Att skräddarsy behandlingen för varje individuell patient baserat på sådana data kommer sannolikt vara en av de viktigaste utvecklingsfronterna inom sjukvården under de kommande decennierna.
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_Chip, chip – Array!_
References


