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Discovery of Biomarker Signatures using Affinity Proteomics

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Molecular Portraits of Cancer

Discovery of Biomarker Signatures using Affinity Proteomics

Petter Skoog



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DOCTORAL DISSERTATION

by Petter Skoog

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To be defended at Medicon Village Hörsalen, Lund. Friday June 9th at 09:15.

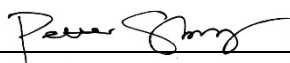
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Organization LUND UNIVERSITY Department of Immunotechnology Medicon Village (Building 406) SE-223 81 LUND Author: Petter Skoog		Document name DOCTORAL DISSERTATION Date of issue 2017-06-09 Sponsoring organization	
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Abstract <p>The use of antibodies as capture molecules in assays is a common practice. By either printing monoclonal single chain fragment variables (scFvs) on a solid support – recombinant antibody microarray – or attaching them to magnetic beads – Global Proteome Survey (GPS) – we can specifically capture and measure target proteins of interest. Using the recombinant antibody microarray, we target predominantly intact immunoregulatory proteins, and measure their expression patterns in clinical samples, such as cancer samples. With GPS, we target peptides derived from digested (cancer) samples to identify and quantify a variety of proteins. While antibody microarrays use one antibody per targeted protein, GPS targets peptides, where one antibody can bind hundreds of different proteins – i.e. one antibody per several target proteins. The latter is accomplished by designing antibodies against a short 4 to 6 amino acid long peptide motif shared among these proteins. In this thesis, both of these methods have been used to decipher cancer biomarker signatures.</p> <p>In paper I, the antibody microarray was used to examine the immunosignature of pancreatic ductal adenocarcinoma (PDAC), and how the profile of the immune response differed between cancer patients compared to both healthy and benign controls. We successfully identified an immunosignature of four to ten scFv antibodies that could classify PDAC from controls with high specificity and sensitivity.</p> <p>In paper II we investigated with the antibody microarray how the immune response evolved with, and shaped, emerging cancer cells in patients later diagnosed with breast cancer using antibody microarray. By determining protein expression profiles for cases and controls, we identified several deregulated immunological proteins that reflected evolving breast cancer, up to two years before diagnosis.</p> <p>In papers III and IV, we used both the antibody microarrays and GPS to decipher biomarker signatures to differentiate between histological grades in breast cancer tumors. With molecular signatures capable of classifying grade, we could also visualize the heterogeneity of intermediate grade 2 tumors. Grade 2 tumors have little clinical prognostic value, and a majority of samples are classified as grade 2. Results from paper IV showed that it might be possible to reclassify many of these samples as either grade 1 or 3, giving clinicians further information for optimal treatment selection.</p> <p>In conclusion, in this thesis I have demonstrated the use of profiling parts of the immune response as a tool for surveying and classifying disease, by using antibodies as specific binders to capture and measure low and high abundant proteins.</p>			
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Molecular portraits of Cancer

Discovery of Biomarker Signatures

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Your theory is crazy, but it's not crazy enough to be true.

Niels Bohr

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Original papers

This thesis is based upon the following papers, which are referred to in the text by their roman numerals (I-IV).

- I Gerdtsson A.S., Malats N., Säll A., Real F.X., Porta M., Skoog P., Persson H., Wingren C., Borrebaeck C.A.K. *A Multicenter Trial Defining a Serum Protein Signature Associated with Pancreatic Ductal Adenocarcinoma*. International Journal of Proteomics. 2015;2015:587250.
- II Skoog P.*, Borgquist S*, Pauly F*, Ohlsson M., Borrebaeck C.A.K., Wingren C. *Immunoprofiling of early breast cancer reveals disease-associated plasma protein profiles*. Manuscript submitted for publication.
- III Olsson N., Carlsson P., James P., Hansson K. Waldemarsson S., Malmström P., Fernö M., Rydén L., Wingren C., Borrebaeck C.A.K: *Grading Breast Cancer Tissues Using Molecular Portraits*. Molecular & Cellular Proteomics, 2013 23: 3612-3623
- IV Skoog P., Ohlsson M., Fernö M., Rydén L., Borrebaeck C.A.K., Wingren C. *Tumor tissue protein signatures reflect histological grade of breast cancer*. Manuscript submitted for publication.

* Shared first authors

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My contributions to the papers

- | | |
|-----|--|
| I | I co-performed the experiments, and took part in writing the manuscript |
| II | I designed the experimental work, performed the experiments, co-performed the data analysis and participated in writing the manuscript |
| III | I co-designed the experimental work, co-performed the experiments, data analysis and participated in the writing of the manuscript |
| IV | I designed the experimental work, performed the experiments, analyzed the data and was main responsible for writing the manuscript. |

Abbreviations

AIP	Autoimmune pancreatitis
ANOVA	Analysis of variance
AUC	Area under curve
BJP	Bence-Jones protein
C	Complement
CA	Carbohydrate / Carcinoma antigen
CDR	Complementary determining region
CIMS	Context-independent motif-specific
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ESI	Electrospray ionization
EUS	Endoscopic ultrasound
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FDA	Food and drug administration
FISH	Fluorescence in situ hybridization
GPS	Global proteome survey
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
LC	Liquid chromatography
MRI	Magnetic resonance imaging

MS	Mass spectrometry
NGS	Nottingham grading system
PAI-1	Plasminogen activator inhibitor-1
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
PR	Progesterone receptor
PTM	Posttranslational modification
ROC	Receiver operating characteristics
scFv	Single-chain fragment variable
SLE	Systemic Lupus Erythematosus
SVM	Support vector machine
TGF	Transforming growth factor
TNM	Tumor size, Node involvement, Metastasis status
uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor

1. Introduction

Cancer is a disease that affects everyone. Either a family member, a close friend, or yourself will most likely be diagnosed with cancer during your lifetime. One in ten Swedish women in today's society will get a breast cancer diagnosis during their lifetime. However, with increasing age and better universal health care, the occurrence of cancer will increase, and is expected to affect half of the population by 2030 (Torre, Bray et al. 2015). Hence, there is a need for better treatments, but also to better understand and subgroup different cancers. With new subpopulations, the need of additional treatment strategies of cancers rises, to further explore the concept of personalized medicine.

After the millennial shift, the human genome was fully sequenced and published (Lander, Linton et al. 2001, Venter, Adams et al. 2001). By having the complete map to all human genes at hand, technologies targeting DNA, mRNA and proteins could be further refined.

The genome contains between 19,000 – 20,500 protein-coding genes, but makes up less than 2% of the genome (Clamp, Fry et al. 2007, Ezkurdia, Juan et al. 2014). There is no accepted number of mRNAs possibly expressed yet defined (Sorek, Dror et al. 2006), but up to two thirds of all genes can produce different mRNAs (Johnson, Castle et al. 2003). To further add function and diversity, once mRNA has been translated, each protein will differ by means of post-translational modifications (PTMs) (Khoury, Baliban et al. 2011). By exploring the proteins in disease, a more precise picture of what is happening in that disease is achieved. By using antibodies as specific binders, we can capture part of the proteome that may otherwise be difficult to analyze.

The knowledge of antibodies has a long history. The first description of the antibody came from Emil von Behring and Shibasaburo Kitasato in 1890, when they showed that the transfer of sera from immunized animals to susceptible animals cured diphtheria (Bering and Kitasato 1890). The first model was proposed by Paul Erlich in 1900, where he hypothesized that a molecule on cells consisted of multiple sites for binding foreign substances, or antigens (Davies and Chacko 1993). In 1948, Astrid Fragraeus described the plasma cell as the forming

cell of antibodies (Fagraeus 1947), and in 1959, Rodney Porter and Gerald Edelman independently published the structure first of the antibody (Porter 1959, Edelman, Heremans et al. 1960).

Antibodies has long been characterized as having high binding specificity and selectivity, becoming of great use in research, diagnostics, and therapeutics. The use of antibodies as tools became easier after phage display became available, allowing the selection of binders through screening (Smith 1985).

Affinity proteomics is a field of proteomics, where using protein-specific catchers, or detection probes, the protein expression levels can be determined. Most often antibodies are used, but other choices such, as affimers (Sharma, Deacon et al. 2016) or aptamers (Brody and Gold 2000) can be used. By designing antibodies to target foremost components of the immune system, we have created an antibody microarray technology platform with tremendous versatility and strength (Borrebaeck and Wingren 2007, Borrebaeck and Wingren 2011). The microarray platform combines several hundred antibodies specific against selected target proteins, giving a relative quantification read-out. Through stringent data analysis, the difference between two states generates a candidate biomarker signature generally consisting of typically 5 to 25 antibodies (Ingvarsson, Wingren et al. 2008, Carlsson, Wingren et al. 2011, Nordstrom, Wingren et al. 2014, Delfani, Sturfelt et al. 2017).

Combining antibodies and mass spectrometry, an in-house developed method named Global Proteome Survey (GPS) (Wingren, James et al. 2009, Olsson, Wingren et al. 2011, Olsson, James et al. 2012), allows affinity enrichment of multiple peptides, combined with subsequent identification and quantification.

The aim of this thesis has been the discovery of molecular signatures that reflects prognostic or diagnostic patterns in cancer. This work is based on four original papers, targeting pancreatic cancer and breast cancer.

Using the antibody microarray platform, we have in this thesis presented three papers, generating biomarker signatures regarding breast cancer diagnostics (**paper II** and **paper IV**). In paper II we investigate the shaping of a tumor by the immune system, and paper IV focused on histological grade. The third microarray study investigated pancreatic cancer diagnosis, comparing serum samples of diseased compared to both healthy and benign controls (**paper I**). Finally, using the GPS platform, we characterized 50 primary breast cancer tumors, and found a signature of proteins reflecting histological grade (**paper III**).

2. Affinity proteomics

Affinity proteomics is most often carried out using antibodies, or engineered fragments thereof, to analyze protein expression, modifications, and distribution in health and disease (LaCava, Molloy et al. 2015).

During the last decades, the antibody microarray technology has evolved into an important tool for simultaneously measuring a large number of proteins in a specific and sensitive manner while consuming only minute amounts of the sample (Haab 2006). Many different types of samples can be analyzed in a high-throughput manner, allowing for the search of disease-specific markers in complex samples due to the high sensitivity, the resolution and reproducibility of antibody-based microarrays.

2.1. Proteomics

The central dogma states that DNA gives rise to RNA, and RNA gives rise to proteins (Crick 1970). Debated for long, however it is evident that genes can produce several mRNAs due to gene alternative splicing (Johnson, Castle et al. 2003). The function of a translated protein can then vary due to, among other things, post-translational modifications (PTMs) (Khoury, Baliban et al. 2011).

Proteomics is the study of proteins in a specified compartment, at a given time point. Since the proteome will differ in different tissues, vary under stress or disease, the measurement of proteins can give more information than genetics. The correlation between up- or down-regulated mRNA levels with the corresponding protein expression levels is debated (Gygi, Rochon et al. 1999, Lu, Vogel et al. 2007, Gry, Rimini et al. 2009). However, the expressed levels of mRNA and proteins are dynamic, an mRNA can be highly expressed and have high turnover rate so that no protein is expressed at all, and vice versa (Hodgkinson, Eagle et al. 2010). While proteins in the end are the doers of the cells mechanisms and what makes a process work, mRNAs are the messengers that

the cells send to communicate and initiate the process. By measuring the protein levels, we try to decipher what has taken place in a given cell, organ, blood sample, or tumor, at a given time point.

2.2. The antibody

As a part of the adaptive and humoral immune response, the antibody has the ability to bind specifically, and with high affinity to a pathogen – the antigen. In its full format, an antibody consists two identical heavy chains, and two light chains. Together, they build a Y-shaped structure, as described in Figure 1, where the two arms bind to the antigen, while the stem has an effector function.

The antigen binding site of the antibody consist of six complementary determining regions (CDRs), three located on the light chain (blue in Figure 1) and three on the heavy chain (orange in Figure 1. By molecular cloning, the antigen-binding parts can be isolated and expressed as different constructs, containing the variable, antigen-binding domains of the full antibody. Using only the top of variable antibody arm, and connecting these with a linker, a single-chain fragment variable (scFv) is constructed, but unlike a complete antibody, only one antigen binding site is present (Huston, Levinson et al. 1988). Cloning of the whole arm creates a fragment antigen binding (Fab) construct, containing both variable, and constant domains.

The generation of antibodies against a specific protein (or compound) can be generated in several ways. Polyclonal antibodies are generated by immunizing an animal with the protein, and harvesting blood after a period of time. Polyclonal antibodies are heterogeneous, binding several epitopes of the protein, but often with lower specificity and potentially more non-specific binding (Stills, Suckow et al. 2012). In comparison, monoclonal antibodies all originates from the same B-cell, and are identical in their sequence. Production of monoclonal antibodies results in only one type of antibody, all binding the same epitope of the targeted protein. Hybridoma technology allowed for specific B-cells to be immortalized, and used for production of intact immunoglobulins (Milstein 1999).

Antibody libraries are created by either harvesting naturally occurring variable parts of the antibody repertoires from individuals, and cloned into vectors, allowing the selection of specific members that display the desired characteristics (Jirholt, Ohlin et al. 1998). Alternatively, synthetic libraries are constructed, by

mimicking the diversity found in nature with *e.g.* error-prone PCR, and cloned into a vector (Hoogenboom and Winter 1992).

Using molecular cloning allows us to use phage display for antibody libraries (Smith 1985). Through phage display, one can select and screen for antibodies with a specific and sensitive binding against most compounds of choice. This generates unique clones containing the genes encoding the variable domains, and can be transformed into different constructs, such as scFv, Fab, or a full immunoglobulin.

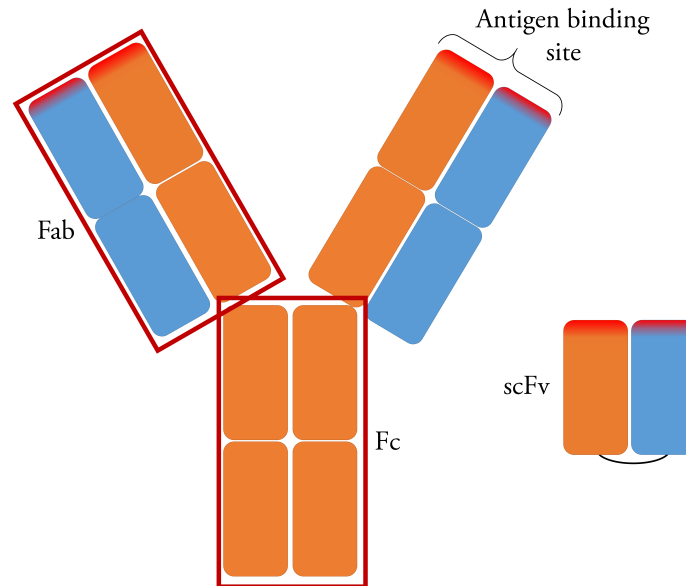


Figure 1: A schematic structure of a complete antibody, and the different components described. The heavy chain is in orange, the light chains in blue.

2.3. The microarray platform

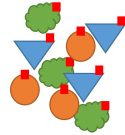
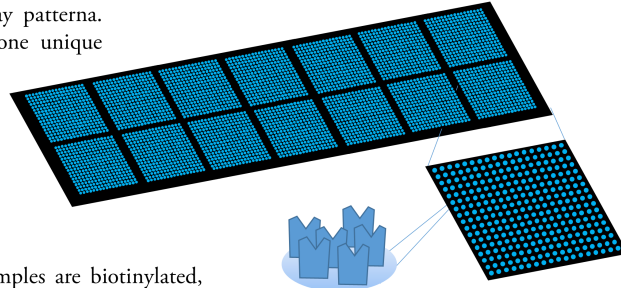
The experiments conducted in papers I-III in this thesis was based on an in-house developed and optimized recombinant antibody microarray platform, designed for protein expression profiling of complex proteomes (Ingvarsson, Larsson et al. 2007, Wingren, Ingvarsson et al. 2007, Delfani, Dexlin Mellby et al. 2016). In

the works presented in this thesis, close to 300 unique recombinant scFv has been used, targeting around 100 serum proteins. The scFv used on the microarray were selected against mainly immunoregulatory proteins, selected from three different phage display libraries (Soderlind, Strandberg et al. 2000, Sall, Walle et al. 2016). The antibody microarray platform allows the profiling of multiple proteins at the same time, in a specific and sensitive manner. The robustness of the antibody microarray platform further allows the targeting of both low- and high-abundant proteins in even crude sample formats, such as serum.

The microarrays are produced using a non-contact printer that dispenses ~300 pL sized droplets of purified scFv onto a solid slide surface. All microarray assay experiments in this thesis used ~300 different antibodies, but updated versions of the platform contain closer to 400 antibodies with an added variety of targets (Delfani, Dexlin Mellby et al. 2016, Gerdtsson, Wingren et al. 2016). These are all printed onto a surface no larger than a fingernail.

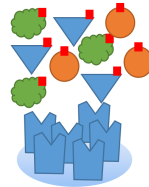
Each slide contained up to 14 sub-arrays, and 10 slides were produced per day, allowing 140 samples to be analyzed per day and workstation. After production of the slides, biotin-labelled samples were added to each sub-array, and proteins bound by the antibodies were detected by adding a streptavidin-coupled fluorophore. Using a confocal laser scanner, the intensity of the fluorophore was measured and quantified. A schematic set-up of the procedure can be seen in Figure 2.

1. Antibodies (scFvs) are printed onto a solid support surface in array pattern. Each array spot consist of one unique antibody clone.

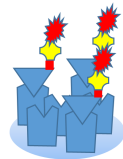


2. Serum samples are biotinylated, adding a molecule to each protein that binds strongly to streptavidin.

3. One biotin-labelled sample is added per array, allowing the antibodies to bind their respective protein.



4. Unbound protein is washed away, and addition of streptavidin coupled to a fluorophore is added, binding to the biotin on the bound proteins.



5. After unbound streptavidin is washed off, the slides are dried and scanned in a confocal laser microarray scanner. The fluorescence of each spot is quantified and used as a measure of the relative amount of protein present in the sample.

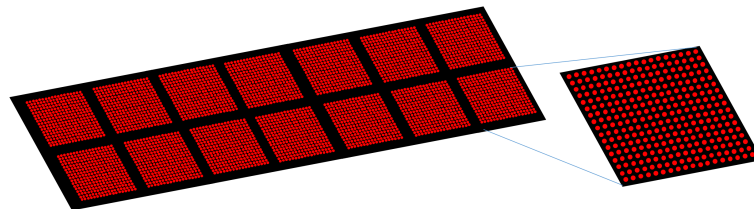


Figure 2. Overview of the experimental procedure for the antibody microarray platform.

However, by directly labelling the sample proteins with biotin, the epitope on the protein risk being masked, which could result in a reduced or even lost antibody reactivity. This problem could, however, be bypassed, or minimized, by using several antibody clones directed against the same protein, but targeting different epitopes (Borrebaeck and Wingren 2007, Borrebaeck and Wingren 2009). Quantified levels were used as a relative measurement of protein bound at each spot. Altogether, this allowed us to compare protein profiles of *e.g.* diseased samples to healthy or other controls, ultimately enabling us to decipher unique biomarker signatures for the targeted disease.

There are some limitations with the antibody microarray, *e.g.* availability of binders, and the production of both binders and arrays. Accessibility to new binders can be achieved by the availability of a phage display library, often containing up to 10^{10} members or more, thus with a wide range of specificities (Soderlind, Strandberg et al. 2000, Sall, Walle et al. 2016). However, production of binders remains a bottleneck, as each clone is produced separately. During the last years, the capacity of production of clones to the microarray has been increased by implementing a high-throughput protocol. But by scaling up the capacity from ~30 to ~300 clones per week, the production of the slides becomes another issue. But with the use of a non-contact printer, several slides can be printed each day. Frontline research aiming for novel array design, bypassing at least some of these limitations have been presented, based on engineered antibodies carrying *e.g.* unnatural amino acids (Brofelth, Stade et al. 2017). Further, by equipping the antibodies with a specific DNA-tag, self-addressing antibodies could be designed that could localize to pre-targeted sites on the slide on their own, thus eliminating the need of having to print the antibodies one by one.

2.4. Immunosignaturing

When a disease occurs in the body, the immune system will react. Depending on the disease, a multitude of reactions will occur and the immune system is the tool that the body uses, with the aim to clear the disease. By surveying the immune response, and its components to detect changes in protein expression levels, we aimed to find disease-specific patterns, or signatures, that could be used to diagnose and classifies a disease. Our hypothesis was thus that by surveying the immune system, the response to a given disease would be reflected by a specific and biologically relevant clinical immunosignature (Sreekumar, Laxman et al.

2004, Ho, Hassan et al. 2005, Dunn, Koebel et al. 2006, Hughes, Cichacz et al. 2012).

The antibody microarray platform target mainly immunoregulatory proteins present in blood, giving a rapid snapshot of disease-specific alterations. Targets include cytokines, chemokines, adhesion molecules, and complement components. Recently added antibodies target among others, enzymes and signaling components. Each individual protein may not be indicative of disease, but several proteins together could reflect disease-associated changes, like a molecular fingerprint.

The interactions between cancer and the immune system are many, and during different phases of tumor development, both the cancer and the immune system adapts and evolve (Dunn, Bruce et al. 2002). The hallmarks of cancer (Hanahan and Weinberg 2011) clearly shows the importance for cancer cells to suppress and evade the immune system, to progress and survive. The concept of ‘cancer immunoediting’ describes three consecutive phases where the immune system shapes the cancer – elimination, equilibrium, and finally escape (Shankaran, Ikeda et al. 2001, Dunn, Koebel et al. 2006)

Using the antibody microarray technology platform, diagnostic and/or prognostic immunosignatures have previously been reported, for among others B cell-lymphoma (Pauly, Smedby et al. 2014), bladder cancer (Sanchez-Carbayo, Socci et al. 2006), breast cancer (Carlsson, Wingren et al. 2008, Carlsson, Wingren et al. 2011), colorectal cancer (Madoz-Gúrpide, Cañamero et al. 2007), cystic fibrosis (Srivastava, Eidelman et al. 2006), glioblastoma (Carlsson, Persson et al. 2010), *H.pylori*-induced gastric adenocarcinoma (Ellmark, Ingvarsson et al. 2006), prostate cancer (Nordstrom, Wingren et al. 2014), and systemic lupus erythematosus (SLE) (Delfani, Sturfelt et al. 2017). Together, these studies showed the robustness and applicability of the antibody microarray technology to address clinical unmet needs in disease.

2.5. Microarray data handling

After the experimental work is finished, data handling of the raw data starts. Compared to genomic arrays, non-biological variations are more platform dependent when using protein microarrays (Shi, Reid et al. 2006, Tarca, Romero et al. 2006), such as the antibody microarray. No standardized guidelines

regarding handling of data from antibody microarrays yet exists (Hamelinck, Zhou et al. 2005, Diez, Dasilva et al. 2012, Chiechi 2016). Normalization is used to eliminate any non-biological variations from the dataset, but if not carefully performed, possible biological relevant variation could be removed as well (Volkenhauer, Moller-Levet et al. 2002). In 2016, the protein microarray database was launched, to archive and analyze protein microarray data (Xu, Huang et al. 2016). The effort is of great importance, to bring a standardized work frame for data analysis. However, the difference in protein microarrays, and the potential sources of variations across different applications, will require many generalizations for a one-fit-all solution. As of today, the notion of a consensus in data handling seems far away, since a fundamental difference among platforms are present. Further, both the number of samples, analytes, but also the scientific question will differ between researchers and platforms.

2.5.1. *Pre-processing*

Pre-processing involves quantification from scanned images, adjustment of any non-specific background binding, outlier detection, and normalization. The raw-intensities from spots varies from background levels (low hundreds) to saturated (over 65,000). Depending on several factors, such as subarray position, day of analysis, or slide, intensities of the same analyte could vary. This calls for a stringent pre-processing of raw-data to minimize the effect these non-biological factors impose on the dataset.

The pre-processing compensates for non-biological sample variations, such as day-to-day or array-to-array variations. The quantified signals from scanned slides are adjusted for background noise, outliers are eliminated, and dataset is normalized. Based on our experience with the platform, two major normalization strategies have been implemented to correct for day-to-day and array-to-array variation. *Semi-global* normalization corrects for day-to-day variations (Carlsson, Wingren et al. 2008, Ingvarsson, Wingren et al. 2008), while commonly used *subtract by group mean* strategy handles array-to-array variation. A combination of these was used in paper I and paper II, while in paper IV, only *semi-global* normalization was implemented, due to that all samples were analyzed on the same day. In *semi-global* normalization, we select 15-20% of the analytes with the lowest overall variation across the entire dataset, and use these to create a scaling factor (Ingvarsson, Wingren et al. 2008). Recent research regarding the normalization of antibody microarray technology raw-data shows that application of Combat normalizing strategy was as efficient at eliminating non-biological variation, while still maintaining relevant biological variation (Delfani, Dexlin Mellby et al. 2016).

2.5.2. Data-analysis

When analyzing data from the antibody microarrays, we ask two questions, (i) which proteins are differentially expressed between our groups (often sick versus healthy), and (ii) can a protein expression profile be defined, capable of classifying the groups.

To answer the first part, standard statistical hypothesis testing, both parametric (*e.g.* t-test or ANOVA) and non-parametric (*e.g.* Wilcoxon) have been implemented. Using ANOVA, several groups can be compared (*e.g.* healthy, diseased and benign), and any variation will be statistically assessed. Wilcoxon allows comparison between related samples, such as matched controls.

For the purpose of classifying samples, we implemented a support vector machine (SVM) (Furey, Cristianini et al. 2000, Dupuy and Simon 2007). A SVM creates a hyperplane between two groups after training the machine, and depending on which side of the hyperplane a sample lands, it is predicted as either diseased or healthy. We utilized two major ways of running the SVM, depending on the number of samples analyzed.

Optimally, analyses are done by randomly dividing the dataset into two sets – a training set with two thirds of the samples, and a test set with one third of the samples. The training set is used to calibrate the model and setting up the hyperplane. Then the test set samples are tested, and the model can be evaluated. Important when running this strategy is to not have any samples in the test set if they were involved in the training, as this causes over-fitting. Several rounds of dividing the dataset into training and test sets can be done.

The second way, when the number of samples are not sufficient to adopt the training and test set model, uses all but one sample to train a model, and the remaining sample is used to test the model (Brody and Gold 2000). Depending on where in the 3D-structure, or the distance to the hyperplane, a samples receives a decision value. Once all samples have received a decision value, the model can be evaluated. Common for both models is the generation of a receiver operating characteristic (ROC) curve. One axis shows sensitivity, or true positives, the other specificity, or the false positive rate. This plot illustrates the performance of the classification, and by calculating the area under the curve (AUC), a measure of the classification can be generated. An AUC value of 1.0 is a perfect separation, and 0.5 can be seen like tossing a coin.

In addition to the SVMs, using an algorithm called backwards elimination, we condense the signature from ~300 antibodies to only contain those antibodies that

together provides the highest classification power (Carlsson, Wingren et al. 2011). This algorithm was applied to the data in papers I, II and IV.

The algorithm excludes one antibody at the time from the dataset, performs a leave-one-out cross validation of the samples, generating a ROC-curve for each dataset. This process is done for all antibodies in the dataset, until all has been excluded once. The antibody that was excluded when the smallest error in the classification was achieved will be eliminated from the dataset. The procedure is repeated until only one antibody remains. The combination of antibodies that gave the smallest error gives an approximate number of antibodies needed for optimal classification.

In paper IV, we also implemented a bootstrap strategy to increase the power of the analysis. In this approach, we randomly picked samples to create the training set, and each sample was allowed to be picked several times. Those samples that were not selected could instead be added to the test set. By reiterating this process one hundred times, all samples were left excluded from the trainingset, and could be evaluated in the model.

2.6. Mass spectrometry

Mass spectrometry (MS) has evolved since the publication of the human genome, when the blueprints for all proteins was made available (Lander, Linton et al. 2001, Venter, Adams et al. 2001). With the seemingly endless number of protein isoforms and modifications present, MS has been hailed as the method for protein discovery and quantification (Nilsson, Mann et al. 2010). The mass spectrometer is an analytical technique that measures not mass, but the mass-to-charge (m/z) ratio, of ions. The information collected is used to elucidate the composition of the analyzed sample. Early in the development of MS, only smaller compounds could be analyzed. Through the discovery of electrospray ionization (ESI) (Fenn, Mann et al. 1989), larger molecules such as proteins and peptides could be analyzed as well, though limitations still exist. The experimental procedure for analyzing a sample on a mass spectrometer consists of preparation of sample, separation of the sample, and acquisition of mass spectra.

Preparation of the sample usually involves an enzymatic cleavage of proteins to generate peptides. In this thesis, Trypsin has been the enzyme of choice, due to two factors. (i) It generally cleaves after arginine (R) or lysine (K), which are basic residues and tend to pick up a charge relatively easy, thus flying well. (ii) Due to

the occurrence of these amino acids, peptides generated from Trypsin cleavage are generally 10 to 20 amino acids in length, optimal for ionization and fragmentation in the mass spectrometer (Olsen, Ong et al. 2004).

Separation of sample, and in this case peptides, is achieved using chromatography. The most commonly used type is that of reversed-phase chromatography when analyzing peptides on a mass spectrometer (Aebersold and Mann 2003). Reversed-phase chromatography separates compounds depending on hydrophobicity, and allows the more non-polar compounds to be retained in the column, while more polar compounds are eluted at a higher pace.

The mass spectrometer basically contains three modules; an ion source (*e.g.* ESI) that transfer the sample from a liquid phase to the gas-phase, a mass analyzer to measure the mass over charge ratio (m/z) values, and an ion detector to count the ion intensities (Aebersold and Mann 2003). Ionization using ESI is based on removing all liquid of peptide-containing droplets, nebulized by a capillary at high electrical potential compared to the mass spectrometer. Each peptide is usually charged multiple times, allowing the peptide to “fly” from the ESI into the mass analyzer.

Mass analyzers measure the mass over charge ratio, and there are several types of analyzers. The orbitrap (Hardman and Makarov 2003, Hu, Noll et al. 2005) traps ions, and sends them to either an ion detector to measure the m/z , generating a MS1 spectra. These peptides can then be sent into a collision chamber for fragmentation. The peptide fragments are then transported back to the ion detector, generating a MS2 spectra. Depending on the instrument set up, up to MS10 can be generated, each additional round, smaller and smaller fragments are generated. In this manner, both the intact peptide can be measured, and then the fragments of the peptide can be measured, resulting in a mass spectrum. Using software and different databases, the different fragments together with the intact peptide m/z can be used to elucidate the amino acid sequence of the peptides in the sample.

However, the reproducibility of mass spectrometry has been an issue (Bell, Deutsch et al. 2009, Mann 2009). Together with high-profile studies that could not be confirmed (Petricoin, Ardekani et al. 2002), a skeptical view has been seen towards mass spectrometry based studies.

2.7. Global Proteome Survey

Global proteome survey (GPS) is a cross-disciplinary approach that combines affinity proteomics, using antibodies, with MS-based analysis of the sample (Wingren, James et al. 2009). The context-independent motif specific (CIMS) antibodies bind to 4 to 6 amino acid motifs at the end of the peptide. Design of each antibody allowed them to each bind several hundred different peptides (Olsson, Wallin et al. 2012). This is made possible since the motif targeted is shared among many proteins (*i.e.* peptides). Notably, the antibodies will work equally well for proteins from different species if the peptides with the desired motif(s) are present, making the technology species independent. In the end, one CIMS-antibody is thus able to bind specifically to hundreds of proteins, compared to classical antibody assays, *e.g.* ELISA, where one or two antibodies are needed to bind one protein.

The GPS procedure start with sample preparation, where the sample proteins are digested with trypsin to generate peptides. CIMS antibodies are produced, purified and covalently coupled to carboxyl-coated magnetic beads. Incubation of digested sample with the beads allow binding of those peptides containing the correct motif, while unbound peptides are washed off. Elution of any bound peptides with an acid generates a sample ready for injection into the mass spectrometer.

In comparison, the antibody microarray set-up is based on one to five antibodies per target, thus many antibodies are required for high multiplexity. It targets specific, predefined proteins (focused targets), and targeted proteins are detected and relative expression levels are determined. In contrast, the GPS platform is based on one antibody per numerous target proteins, all sharing the same motif. This allows few antibodies for multiplexicity. The GPS method targets a broad range of proteins, and the proteins are detected, identified, and quantified.

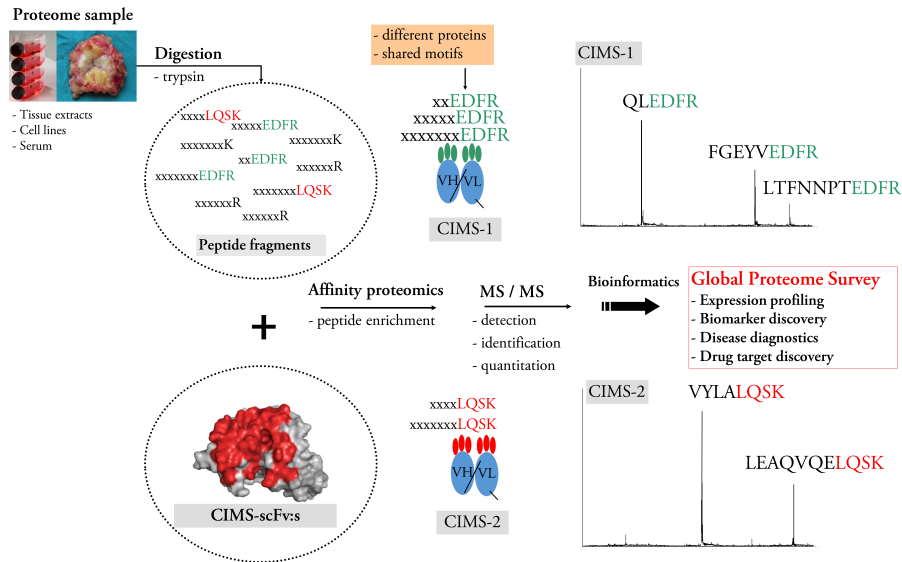


Figure 3: The GPS work flow

Recently, an updated set-up of the GPS platform was investigated, where by attachment of a biotin molecule at a specific site on the scFv, the antigen binding site was directed outwards by all scFv attached to streptavidin-coated magnetic beads (Sall, Persson et al. 2016).

Limitations of the GPS platform regards throughput, *i.e.* running time for analysis of one sample, instrument stability, and partly the inherent reproducibility with mass spectrometry.

In **paper III**, we applied the GPS platform to analyze the difference in histological grade between grade 1, 2, and 3 tumors. Of note, using only nine CIMS antibodies, we could quantify 1,388 protein groups in 52 tumors. A multivariate analysis (three-group comparison), identified 49 proteins significantly differentially expressed between the three grades. Notably, a PCA plot showed that grade 1 samples differed from grade 3 samples, but grade 2 samples were evenly spread between the grades.

3.Applications on Breast Cancer

Cancer occurs when a cell starts to proliferate and grow in an uncontrolled manner. Only 5-10% of cancers are caused by inherited mutations, while 85-90% are caused by genetic mutations that occur during one's life-time (World Cancer Report, 2014). Researchers believe that four out of ten cancers are linked to life style choices, such as tobacco, diets, alcohol, and physical inactivity (Parkin, Boyd et al. 2011). In all cancer deaths, approximately 30% are linked to smoking, 30% linked to diet, and 15% are due to infection (Anand, Kunnumakkara et al. 2008). A drastic change in life style choices can thus significantly decrease the risk for a cancer diagnosis.

In 2000, Douglas Hanahan and Robert A. Weinberg published "The Hallmarks of Cancer" that outlines six acquired capabilities that most tumors need during their development (Hanahan and Weinberg 2000), and in 2011 they added four new hallmarks (Hanahan and Weinberg 2011), all shown in Figure 4.

Breast cancer affects one in ten women during their lifetime in Sweden (www.cancerfonden.se). Nevertheless, breast cancer is not one disease, but comprises of over twenty different types, depending on origin, placement, and invasiveness (WHO classification of tumors, 2003). Genetic studies have subdivided breast cancers into five subtypes, giving physicians refined diagnostic tools for treatment plans (Sorlie, Perou et al. 2001).



Figure 4: The Hallmarks of Cancer (adapted from Hanahan and Weinberg, 2011)

Screening of women over a certain age often helps find breast cancer early, resulting in a greater chance for survival (Gotzsche and Jorgensen 2013). Screening for breast tumors is primarily done using mammography. After introduction of screening for breast cancer, the incidence-rate has stabilized in Sweden, and the mortality-rate has decreased (DeSantis, Bray et al. 2015). When an abnormal growth is detected, an ultrasound, and in rare cases an MRI, can be used to further visualize the growth, differentiating between solid mass and a mass filled of liquid. A biopsy is taken using a fine needle aspiration, and the sample will be sent for cytological evaluation. A more complete diagnosis can be set using tumor features (*e.g.* invasive or in situ), molecular (*e.g.* estrogen and progesterone receptor, and Her2 status), histological (*e.g.* grade) and genetic markers (*e.g.* *BRCA1* and *HER2* (Ludwig and Weinstein 2005)). An appropriate treatment is selected depending on a combination of these factors.

3.1. Biomarkers in Cancer

A biomarker generally refers to a measurable characteristic that can be measured objectively, and indicates a biological state or condition (Strimbu and Tavel 2010). Most biomarker tests are directed against genomics, mRNA, proteins, or metabolic markers. The perfect biomarker would be present in sick, but absent in healthy, and could easily be measured. However, the perfect biomarker seldom exists. In cancers, the biomarker is often one of our own proteins that becomes affected by the disease, and will be expressed at a higher or lower level.

The first biomarker goes back to 1847, as H.B. Jones discovered the Bence-Jones protein (BJP) in urine (Jones 1848). BJP is a free antibody light chain, often produced by multiple myeloma cells (Drayson, Tang et al. 2001, Katzmman, Abraham et al. 2005, Shaw 2006). Since then, hundreds of biomarkers have been observed in the laboratory, but the number of biomarkers in clinic are few. In 2012, there were 23 protein tumor markers approved by the food and drug administration (FDA), and currently used in clinical practice (Fuzery, Levin et al. 2013). The gap from discovery of a potential biomarker, to implementation into clinic is very large and long. The FDA has as a requirement for new biomarker assays to show that it has adequate analytical performance, *i.e.* accuracy, precision, and reproducibility, as well as clinical performance, *i.e.* sensitivity and utility (Gutman and Kessler 2006).

One of the best-known single cancer biomarker is the Prostate Specific Antigen (PSA) (Balk, Ko et al. 2003, Welch and Albertsen 2009). Although increased level of PSA acts as an indicator of prostate cancer, the use of PSA as a specific biomarker for prostate cancer has been debated (Velonas, Woo et al. 2013). In Sweden, it was decided not to screen for prostate cancer using PSA without other symptoms of prostate cancer, since the negative effects are too high, including unnecessary needle biopsy, overtreatment, and false positive diagnosis (www.socialstyrelsen.se).

Yet, instead of looking at a single biomarker, recently many tests for disease measures several genes or proteins, creating a signature to define a specific disease (Raman, Avendano et al. 2013). Since most available single biomarkers lack both sensitivity and sensitivity early in disease, a combination of low-specificity biomarkers could enhance the capability of an assay (Su 2013). It has been suggested that multiplexed biomarker signatures could increase the reliability of detection of disease, reducing many invasive and unnecessary biopsies (Arellano-Garcia, Hu et al. 2008, Wei, Patel et al. 2009).

3.2. Biomarkers in breast cancer

Breast cancer in women account for more than one in four diagnoses, and is the most common cancer diagnosis. It is also the second cause of death in female cancer patients (Siegel, Miller et al. 2016). At the same time, breast cancer research was the most NCI funded cancer area between 2010 and 2014, funding close to \$3 billion - more than double the amount of lung cancer research, placed in second (www.cancer.gov). This effort has during the last decades given physicians new access to treatment choices, and the information necessary to treat patients with the best therapy, giving a more personalized care. The efforts made in 1980 until the millennial shift gave more knowledge and insights than all the years before, in cancer prevention, early detection, and treatment. In the last two decades, a tremendous effect has been seen due to the research of protein and gene expression profiles, immunotherapy, cancer genetics, targeted therapies, and robotic surgery (Lukong 2017). The discovery of breast cancer subtypes and subsequent genetic tests, as well as new classes of drugs, has further added to the decrease in breast cancer deaths since the early 1990's (Berry, Cronin et al. 2005, Siegel, Miller et al. 2016).

Although there are several types of breast cancers (Lakhani, Ellis. I.O. et al. 2012), the most important aspect to become cancer-free, is an early detection (Etzioni, Urban et al. 2003, Wolf, Wender et al. 2010, McPhail, Johnson et al. 2015). With early detection, the tumor is often smaller and easier to remove completely by surgery, making post-surgical treatment both less harsh and less necessary (Blanks, Moss et al. 2000). The importance of early detection is thus very significant, representing a key unmet clinical need.

A detailed diagnose of breast cancer takes several factors into account (Carlson, Allred et al. 2009). A full classification of the tumor would contain histopathology type, grade, stage, receptor status, and the presence or absence of genes as determined by DNA testing. Stage is the process of determining how much cancer, and where in the body it is located (Edge and Compton 2010), and is based on the TNM system (Edge, Byrd et al. 2010). TNM evaluation regards the size of the primary tumor (T), possible cancer cells in local lymph nodes (N), and if the tumor has metastasized (M). Presence or absence of hormonal receptors give treatment options, and if applicable, increases life expectancy of the patient (Fisher, Jeong et al. 2004). Genetic tests for single genes (*e.g.* *BRCA1*) or multiple genes (*e.g.* MammaPrint™) further help stratify the tumors for selecting the optimal treatment plan.

3.2.1. *BRCA1 and BRCA2*

Women in high-risk groups are screened for two breast cancer predictive genes, *BRCA1* and *BRCA2* (Miki, Swensen et al. 1994, Wooster, Neuhausen et al. 1994). Both genes act as tumor suppressors, but mutations in one or two of these genes renders the proteins non-functional, and increases the risk of cancer. By testing the genetics, prevention of disease can be managed by an increase in mammography, preventive chemotherapy or prophylactic surgery. The ‘Angelina Jolie-effect’ started in 2013, when the film actress tested for mutations in *BRCA1*, and had subsequent risk-reducing mastectomy. The rate of referrals to genetic centers that test for *BRCA1* mutations in UK rose with 50% in the following months after Jolie went public with advice to get tested (Evans, Barwell et al. 2014).

3.2.2. *Hormonal status and proliferation*

Two hormonal receptors are tested for – the Estrogen receptor (ER) and Progesterone receptor (PR) (Ciocca and Elledge 2000). Both receptors act for growth and proliferation of the cancer cells. About two out of three breast cancers are positive for hormonal receptors (Anderson, Katki et al. 2011). These tumors are often treated with hormonal therapy, usually using Tamoxifen, which inhibits estrogen to bind the receptor on the tumor cell, thus decreasing the growth of the tumor.

The oncogene *HER2* is overexpressed in about 20% of breast cancers, and gives treating physicians both prognostic and predictive information (Hondermarck, Tastet et al. 2008). The presence of the protein Her2 on tumors can be determined through genetic testing using FISH, or protein detection using IHC of a cross section of the tumor. Recommended assays are a combination of both. Her2 positive tumors can be targeted by monoclonal antibodies, like Trastuzumab (Herceptin) (Tan and Swain 2003).

However, between 10-17% of all breast cancer tumors are triple-negative, *i.e.* does not express ER, PR or Her2, and these tumors cannot be treated with hormone therapy or antibodies targeting Her2 (Rakha, El-Sayed et al. 2007, Reis-Filho and Tutt 2008). The scientific interest in triple negative tumors is increasing, looking for therapeutic alternatives to treat these tumors since only conventional chemotherapy becomes available (Bartlett, Thomas et al. 2010).

For prognosis, Ki-67 is a protein that increases in level as a cell prepares to divide (Dowsett, Nielsen et al. 2011). By detecting the ratio of cancer cells that are positive for Ki-67, it can determine how quickly the tumor cells are dividing. The more Ki-67 present, the faster the tumor is growing, rendering it more aggressive.

Several other factors are included in determining the subtype of a breast cancer, including tumor size, stage, lymph node involvement, and histological grade. Together, these factors help determine the therapy to maximize the chance for a cure.

3.2.3. Monitoring disease response

Two biomarkers, CA 15-3 and CA 27-29, are used to monitor the disease response to therapy (Harris, Fritsche et al. 2007), as increased levels are associated with poorer diagnosis and recurrence. CA 15-3 is the most widely used biomarker in serum in breast cancer (Duffy, Shering et al. 2000). In malignant cells, the *MUC1*-gene overexpress the heavily glycosylated protein Mucin-1, coating the cell surface, and increasing levels are released into the blood stream (Klee and Schreiber 2004).

3.2.4. Prognosis

Urokinase plasminogen activator (uPA) is an extracellular matrix-degrading protease, who interacts with plasminogen activator inhibitor-1 (PAI-1) (Duffy, McGowan et al. 2014). The increased levels of both proteins in lymph node negative breast cancer indicates higher risk for tumor progression and metastasis. One prospective study showed that for early breast cancer patients with high levels of uPA/PAI-1, additional adjuvant chemotherapy was beneficial, while patients with low levels could be spared chemotherapy after surgery (Harbeck, Schmitt et al. 2013). Several expert panels recommend measurement of uPA/PAI-1 in the US and Europe (Duffy, McGowan et al. 2014). Nonetheless, they are seldom tested since the assay for measurement requires large amounts of tumor tissue, which is hard to spare in the lymph node negative tumors.

3.2.5. Genetic subtyping

In 2001, Therese Sörlie and colleagues presented a genetic classification of breast cancer tumors based on RNA (Sorlie, Perou et al. 2001). The sub-classification of tumors into five subtypes (listed in order of prognosis) consisting of Luminal A (ER positive and slow growing), Normal-like (ER positive, slow growing), Luminal B (ER-positive but fast growing), Her2-rich (Her2 positive), and Basal-like (ER/PR/Her2 negative). Although the subtyping can generate important recurrence and prognostic value for patients, the use of genetic subtyping is not common in Europe unless protein biomarker tests cannot classify the tumors.

Classification of tumors into hormone, or Her2, positive tumors is mainly useful for treatment selection. However, since recurrence prognostics is not binary, *i.e.*

no test can predict if recurrence will occur or not, assays where end-results predict an *e.g.* 40% chance for recurrence is based on population, not individuals, and can be hard to apply on a specific patient.

3.2.6. *Multiplexed biomarker panels*

Multiplexed biomarker assays are used in determining different cancers, and most are based on genes, and mRNA is usually used.

van't Veer and colleagues developed the *Oncotype DX* Test in 2002 (Cronin, Sangli et al. 2007). It targets 21 transcripts using RT-PCR, assessing the risk of cancer recurrence in early-stage breast cancer, and can determine how beneficial chemotherapy would be for the patient. It divides patients into three groups depending on score, where a low score indicates low risk for cancer recurrence if hormone therapy was administered. A large ongoing study, TAILORx, could assess the benefits for the patients with low score from the test (Sparano, Gray et al. 2015). The study confirmed the use of *Oncotype DX* test to allow low-score patients to not undergo chemotherapy, when classical diagnosis would suggest otherwise. However, since only 16% of patients fall in the low-scoring category, while 67% fall in the intermediate category where therapy is randomized.

MammaPrint™ targets 70 gene transcripts with a microarray-based assay (van 't Veer, Dai et al. 2002). It is capable of classifying early-stage, lymph node negative patients into low and high-risk groups of distant tumor recurrence. It was the first assay to be cleared by the FDA targeting multiple variables to yield a single, patient-specific result in 2007 (Terry 2010).

Prosigna™ is a genetic test of mRNA, indicated to use on early-stage, post-menopausal women, with or without lymph node involvement, and who are hormone receptor positive (Nielsen, Wallden et al. 2014). The test is based on a 50 gene subtype predictor, PAM50 (Parker, Mullins et al. 2009). The test shows an underlying intrinsic subtype, Luminal A or B, Her2, or Basal-like. Depending on subtype, the use of adjuvant endocrine therapy can be selected.

EndoPredict combines a genetic test, combined with tumor size and lymph node status. The test is eligible for early-stage, hormone receptor-positive, Her2-negative patients with maximum of 3 involved lymph nodes (Buus, Sestak et al. 2016). Results give treating doctors information if only hormone therapy decrease the risk for distant metastasis after 10 years.

Four other assays currently under evaluation are Breast Cancer IndexSM (BCI), Mammostrat™, Blueprint® and TargetPrint™ (Raman, Avendano et al. 2013). BCI analyzes the activity of seven genes in lymph node negative, hormone receptor

positive tumors to predict the risk of recurrence in 5-10 years (Zhang, Schnabel et al. 2013). It has shown greater prognostic accuracy compared to the *Oncotype DX* test (Sestak, Zhang et al. 2016). The study could also re-stratify low and intermediate risk group patients in regards to distant recurrence. Mammostrat™ is a tissue-microarray assay that targets the expression of five immunohistochemical markers to assess patients undergoing hormone treatment and their risk of future relapse (Bartlett, Thomas et al. 2010). Results give insight in prognosis, recurrence, predictive, and therapy selection. Both Mammostrat and *Oncotype DX* assess the risk for distant recurrence, but differ in what they measure. While *Oncotype* measures RNA from tumors, Mammostrat™ examines cancer cells only through IHC (Acs, Kiluk et al. 2013). BluePrint™ targets 80 genes to establish newly diagnosed, untreated tumors into three subtypes – basal, luminal or Her2 (van de Vijver, He et al. 2002, Fan, Oh et al. 2006). TargetPrint™ analyzes the ER, PR and Her2 expression levels, to determine if the patient is a candidate for hormone therapy. However, in a prospective study, only the results for ER showed that mRNA microarray analysis was in concordance with IHC, while both PR and Her2 showed lower concordance rates (Viale, Slaets et al. 2016). However, at least one study (Cardoso, van't Veer et al. 2016) showed the issue of over-diagnosing, when calibration of the test was incorrect. A brief summary of the assays above is shown in Table 1.

Table 1: Genetic assays and intended use

	Prognostic	Predictive	Diagnostic	Recurrence	Therapy selection	Stages	Hormone receptor status
Oncotype DX	Yes	No	No	Yes	Yes	I and II	Positive
MammaPrint	Yes	No	No	Yes	Yes	I and II	Positive
Prosigna	Yes	No	No	Yes	Yes	I and II	Positive and negative
EndoPredict	No	No	No	No	Yes	I and II	Positive
Breast Cancer Index	Yes	No	No	No	Yes	I and II	Positive
Mammostrat	Yes	No	No	Yes	Yes	I and II	Positive
BluePrint	No	No	Yes	No	Yes	I and II	Positive and negative
TargetPrint	No	No	No	No	Yes	I and II	Positive and negative

As can be seen from Table 1, all test targets therapy selection, while none are predictive, and only one is diagnostic. This set the market up for diagnostic, and most importantly, for predictive tests to find breast cancer early.

3.3. Cancer and the immune response

The idea that the immune response is involved in the emergence of cancer was first mentioned in 1957 (Burnet 1957) and has been refined and extended into the concept of immunoediting (Ikeda, Old et al. 2002, Dunn, Koebel et al. 2006). The theory includes three consecutive stages – Elimination, Equilibration, and Escape – where both the innate and adaptive immune responses shape these stages. The first stage, elimination, is the normal process where the immune system encounters and removes cells that have gained some cancer characteristics, initially through effector cells such as NK cells (Kim, Emi et al. 2007, Koebel, Vermi et al. 2007). During tumor progression into the equilibrium state, a balance between the immune system and the cancer cells is maintained, the cancer cells not being destroyed, nor being able to form a clinically apparent tumor (Mantovani, Allavena et al. 2008). The selection process often produces tumors cells lacking in production of major histocompatibility complexes class I and II, leading to both a decrease in presented tumor antigens, as well as the mechanism for cytotoxicity through CD8+ T-cells (Kim, Emi et al. 2007). A tumor, shaped and sculpted by the immune system, can then enter the escape phase, where growth and proliferation is no longer blocked by the immune system, having created a tumor microenvironment with an immunosuppressive ability (Teng, Swann et al. 2008, Kraman, Bambrough et al. 2010).

Diagnostic tools used today looks at clinically apparent tumors, those already in the escape phase, at the time of diagnosis (Kim, Emi et al. 2007). These tumors have established an environment around them to grow, resulting in a tumor that can be difficult to remove completely and have a good prognosis. Any assay to find cancer earlier than those available today could help with overall survival in all types of cancer. With the introduction of mammography, a decrease of mortality in women over 50 with 16-32% (Schopper and de Wolf 2009). However, up to 25% of all new breast cancer cases affect women under 50, not yet eligible for the screening program. A recent study, using mass spectrometry, focused on finding potential biomarkers indicating early detection of breast cancer. They saw that

there was an alteration in serum protein profiles up to three years prior to diagnosis (Opstal-van Winden, Krop et al. 2011).

In **paper II**, the aim of the study was to identify an immunosignature to diagnose early breast cancer with the antibody microarray. To this end, samples from 255 women were analyzed, of whom 85 up to two years later received a cancer diagnosis. However, even though the time before diagnosis was given, the actual time from sampling to a clinical tumor was harder to determine, highlighted by the fact that the largest tumor was 120 mm in size, which suggests that the breast cancer has been present for a long time.

The samples were sub-grouped based on both time between the sampling and diagnosis, as well as tumor size at the time of diagnosis. Tumors above the size of 20 mm were excluded, to remove any uncertainty of due to uncertainty of where in the timeline they belonged. From the analysis, we could with moderate performance classify early breast cancer samples compared to matched controls.

Data instead indicated that we could detect features that could be related to immunoediting. Even though they were not in accordance with an early diagnosis, the biological relevance was of interest, and importance.

After dividing the early breast cancer samples into four groups, each in about six month intervals from diagnosis, a pattern of mainly upregulated proteins was seen in three of the groups, the fourth showing mainly downregulation, compared to healthy controls. Samples from 6 to 12 months before diagnosis showed the highest number of differentially expressed proteins, indicating significant changes in the immune response and cancer-associated processes.

In the study by Opstal-van Winden and colleagues, C3a was identified as a potential biomarker for early detection. In our study, the complement protein C3 was identified, and C3 is proteolytically cleaved into C3a and C3b upon activation of the complement pathways. Further, we identified several deregulated anti-tumor and immunosuppressive proteins, previously identified in immunoediting (Dunn, Koebel et al. 2006). Highlighting immunoediting, we looked at the stages before clinically apparent tumors emerged. The immunoprofiles of early breast cancer patients showed that key cytokines, previously described as markers for tumor progression, were deregulated, like cytokines with anti-tumor properties, *e.g.* IL-1 α , IL-1 β , IL-12, and IFN- γ , and tumor-promoting proteins such as IL-10, TGF- β , and VEGF (Dunn, Bruce et al. 2002, Irshad, Grigoriadis et al. 2012). Even though large changes in protein expression profiles were seen to change over time, the ratio of IL-10 to IL-12 or IFN- γ did not change significantly, and the balance between these proteins is

essential when determining in which phase the tumor is regarding immunoediting (Irshad, Grigoriadis et al. 2012, Mittal, Gubin et al. 2014). The first phase of immunoediting, elimination, generates higher expression of IL-12 and IFN- γ , promoting tumor immunity. Subsequently, the equilibrium phase instills a balance between tumor immunity cytokines and immunosuppressive cytokines. Finally, the balance shift towards IL-10 production, increasing the immunosuppressive nature surrounding the manifested tumor.

3.4. Histological grade

Histological grade is one of the oldest and most used prognostic factors in breast cancer patients (Bloom and Richardson 1957). Histological grade can be seen as the aggressive potential of the tumor, and is subdivided into three grades, 1 to 3. Most often, the grading follows the Nottingham Grading System (NGS), an Elston-Ellis modified protocol by Scraff, Bloom, and Richardson (Elston and Ellis 1991). It is determined by microscopic evaluation of a cross-section of tumor. Three categories are evaluated; (i) tubule formation, (ii) mitotic count, and (iii) nuclear pleomorphism (Elston and Ellis 1991, Christgen, Langer et al. 2016). Tubule formation evaluates how much of the tumor that has normal milk duct structures. Mitotic count determines how many dividing cells are present, indicating the rate of growth. Nuclear polymorphism evaluates the size and shape of the nucleus in the tumor cells. Each category is scored 1-3, where a score of 1 indicates that the cell looks like a normal cell or tissue, and a score of 3 means that the cells look most abnormal. A combined score of 3-5 is classified as a grade 1 tumor, 6-7 a grade 2 tumor, and 8-9 a grade 3 tumor.

While grade 3 tumors are more proliferative and poorly differentiated, grade 1 tumors are slow-growing and well differentiated. Grade 2 tumors are moderately differentiated, but has less prognostic information for physicians to determine a proper treatment plan (Sotiriou, Wirapati et al. 2006). Since about half of all breast cancers are classified as grade 2 tumors, the need for improved classification, and further sub-classification, of grade 2 tumors can be seen. Both genetic and proteomic efforts have been made to subgroup tumors, and evident from foremost genetic studies are that grade 2 tumors can be divided into two groups – one more like grade 1 tumors, the other more like grade 3 tumors (Ivshina, George et al. 2006).

One study suggests that a grade 1 tumor does not progress into grade 3, but that grade 1 and grade 3 tumors are of different types (Roylance, Gorman et al. 1999). They showed that 65% of all grade 1 tumors lack the long arm of chromosome 16, while 84% of grade 3 tumors has an intact chromosome 16. This entails that a potentially binary classification regarding grade, those characterized as grade 2 become either over or undertreated.

In **paper III**, the GPS platform, based on nine antibodies against five peptide motifs, was applied to characterize the molecular expression profiles of histological grade of breast cancer tumors. Analyzing 52 breast cancer tumors, we were able to identify over 2,100 proteins, and quantify close to 1,400 proteins.

Using ANOVA, we could differentiate grade 1, 2, and 3, using a 49-plex signature, with high specificity and sensitivity. Training a model with two thirds of the samples, and testing on the remaining test samples gave a ROC AUC of 0.86 for differentiating between grade 1 and grade 3 samples. This confirmed that grade 1 and grade 3 were two separate groups. However, grade 2 was confirmed as a heterogeneous grade, but data indicated that it could be divided using a molecular protein expression profile into two groups, one more related to grade 1, the other more like grade 3. Applying a SVM, using leave-one-out cross validation, classification of grade 1 *versus* grade 3 was achieved using a 50-plex signature, resulted in a AUC of 0.92.

From the biological viewpoint, differentially expressed proteins upregulated in grade 1 were associated with ECM and stroma, *e.g.* Keratocan and Spondin-1. Proteins significantly upregulated in grade 3 were associated with mitosis and cell proliferation. Together, this indicates that low grade tumors have a more structurally intact structure, both inside the cell as well as surrounding the tumor. High grade tumors have lost the normal expression of these structural proteins, and levels of proteins involved in proliferation, *e.g.* CDK1, MCM2, and MCM7, has increased. These findings concur with the fundament that on which histological grade is based – mitosis, nuclear pleomorphism, and differentiation.

As mentioned, the GPS surveys the global proteome, detecting proteins regardless of pathway or process. By using both the antibody microarray, that surveys the immune response, as well as a global proteome survey, we could complement both studies, searching in different aspects of the cancer proteome. To add more insight into the histological grade in breast cancer, in **paper IV** we performed an antibody microarray analysis on 50 solid tumor tissue extracts. With this technology, we could look at a specific aspect of the biology of breast cancer tumors, and find protein expression profiles to classify histological grade. Using 297 recombinant scFv, we targeted 98 immunoregulatory proteins and 30 short peptide motifs.

Initial data analysis, using SVM with leave-one-out cross-validation showed that grade 1 and grade 3 could be separated, with an ROC AUC of 0.83, while grade 2 showed to be heterogeneous, overlapping both grade 1 and grade 3. To condense the number of analytes that impact the classification, backwards elimination was performed to separate grade 1 from 3, and the resulting signature was used to analyze grade 2 samples. Results showed that most grade 2 samples could potentially be reclassified as grade 1 or grade 3 tumors.

Through bootstrapping, we were able to train a model using grades 1 and 3, and testing on grade 2. However, when building the training sets, some samples were picked more than once, leaving approximately 30% of the samples out of the training set, and could instead be added to the test set. Followed by backwards elimination, the twenty most informative antibodies were used as a signature, and the test set was applied to the model. One hundred rounds of training and testing gave a result from minimum 25% to 45% of grade 1 and 3 samples, as well as grade 2.

The model gives a binary result, and grade 2 samples were classified as either grade 1 or grade 3 in each round. Depending on the total number of times classified as either grade, a consensus grade was determined. Two samples were reclassified as grade 1, eleven as grade 3, and four samples were still intermediate.

However, this model generated one hundred different signatures, and was dependent on the composition of the test set. By using the twenty most occurring antibodies as a consensus signature, the bootstrap and testing was performed again. Two grade 2 samples were reclassified as grade 1, while thirteen were reclassified as grade 3, and two remained as grade 2.

A shift in focus compared to paper III, where classification of grades using molecular multiplexed signatures was the major focus, in paper IV we applied the antibody microarray to analyze grade 2 in more detail. Since there is evidence that tumor grade is not a progressing factor (Roylance, Gorman et al. 1999), but rather a type, correct classification is paramount. Further, 30-60% of tumors are graded in the intermediate grade 2 (Sotiriou, Wirapati et al. 2006), with little clinical information, the need for a more robust and easily made classification system is needed. The consensus signature of classification of histological grade consisted of several proteins important in structure, and a decrease in levels in more aggressive tumors. Most proteins were found with increased levels in higher grade, and correlated with decreased structural integrity, increased proliferation and higher metastatic capability.

Conclusions of **paper III** and **paper IV** were that higher histological grade clearly shows a downregulation of proteins involved in structural integrity of both tumor cells and surrounding tissue. Further, the proliferative ability, and the number of mitotic cells, are increased, as well as proteins that deregulate the ECM surrounding the tumor to potentially pave the way for future metastatic cells. Grade 2 tumors were seen as a heterogeneous group, and could potentially be subdivided into two separate groups, one more closely related to grade 1, and one more related to grade 3. Together, the GPS platform and the antibody microarray technology complemented each other, where GPS found motif-containing peptides from different part of the proteome, while the antibody microarray targeted the immune response against breast cancer tumors.

Histological grade is a measure of the aggressive potential of the tumor, and by defining the grade using a molecular signature, less inter-observer discrepancy could be obtained. Further, since most tumors are graded as intermediate, the clinical relevance for these tumors are low. With a more unbiased grading system, most tumors today graded as intermediate, could be redefined as low- or high-grade, thus giving clinicians a better base for treatment decision.

4. Applications on Pancreatic Cancer

Around 900 people are diagnosed with pancreatic cancer each year in Sweden (www.cancerfonden.se), and over 50,000 new cases are diagnosed in the US each year (Siegel, Miller et al. 2016). It is the fourth leading cause of cancer deaths worldwide (Rossi, Rehman et al. 2014), and it has been estimated that there will be more deaths from pancreatic cancer than breast cancer in Europe by 2017 (Ferlay, Partensky et al. 2016). Fifty per cent of patients diagnosed with pancreatic cancer has distant metastases, 30% have local and/or regional spread, and only 3% have tumors confined to the pancreas (Pannala, Basu et al. 2009). Given the late diagnosis of most pancreatic cancer patients, the overall five-year survival is less than 5% and the median survival is only 6 months (Chang, Wong et al. 2014). Overall, cancer deaths in the US has decreased by 23% over the last two decades, but for pancreatic cancer patients, the number of deaths has increased (Siegel, Miller et al. 2016).

Patients diagnosed with pancreatic cancer at stage I, a tumor with local spread, surgery to remove the tumor is preferred. Chemotherapy is usually given as a prophylactic treatment, and overall 5-year survival for this group is 20% (Ahrendt and Pitt 2002, Wang and Kumar 2011). This shows that early detection is crucial for a longer survival of patients with pancreatic cancer.

The symptoms of pancreatic cancer are vague, and contribute to the late detection of pancreatic cancer (Hidalgo 2010). Around 80% of pancreatic tumors are located in the body of the pancreas, causing a compression of the surrounding structures, the bile and pancreatic ducts, duodenum, and mesenteric and coeliac nerves (Evans, Abbruzzese et al. 1997). The effect of the tumor on the pancreas cause diffuse symptoms, such as jaundice, abdominal pain, type II diabetes, and weight loss.

At the moment, there is no screening program for pancreatic cancer. This is mainly due to that there is no available test that is reliable and accurate enough to find large numbers of pancreatic cancers at an early stage, and does not indicate

cancer when no disease is present, a false positive. However, recent studies have shown a great opportunity to screen high risk groups, and that the cost to society is relevant (Larghi, Verna et al. 2009, Ghatnekar, Andersson et al. 2013, Lu, Xu et al. 2015). Most screenings are accomplished using endoscopic ultrasound (EUS), as it can detect small lesions with a low risk of complications. MRI screening complement EUS, and though it cannot detect solid pancreatic lesions caused by tumors as well as EUS, it has a greater sensitivity regarding cystic lesions (Harinck, Konings et al. 2016). Conclusions from screening reports is that there is a great need for an assay that can help detect pancreatic cancer early.

4.1. Pancreatic Ductal Adenocarcinoma

Pancreatic ductal adenocarcinoma is the most common type of pancreatic cancer, comprising about 85% of all diagnosed cases (Ryan, Hong et al. 2014). Surrounding the pancreatic tumor, a larger than normal stroma, or tumor microenvironment, lies as a protective barrier, protecting against drug delivery and leading to a higher drug resistance than other tumors (Feig, Gopinathan et al. 2012).

Close to all patients with pancreatic cancer has one or more out of four genetic defects (Maitra and Hruban 2008, Hidalgo 2010), but averaging 63 mutations of consequence per tumor (Jones, Zhang et al. 2008). *KRAS2* is mutated in over 90% of patients, leading to an activated form of Ras, and causing increased proliferative and survival signaling (Biankin, Waddell et al. 2012). In nearly 95% of tumors, *p16/CDKN2A* is inactivated (Schutte, Hruban et al. 1997), normally functioning as a regulator in the G1-S transition phase in the cell cycle. Loss of function further increase the proliferative ability of the cancer cells. *TP53* is abnormal in 50-75% of tumors, leading to increased genomic instability, along with loss of cell ability to control DNA damage in control checkpoints (Olivier, Hollstein et al. 2010). *SMAD/DPC4* is lost in half of pancreatic cancers, causing an abnormal signaling pathway through transforming growth factor β (TGF- β) (Jones, Zhang et al. 2008).

Pancreatitis is the inflammation of the pancreas. There are three types of pancreatitis, acute, chronic, and autoimmune pancreatitis (AIP). Acute pancreatitis is usually caused by gallstones and/or heavy alcohol consumption, or in cases, such as infections of mumps, trauma, or certain medications. Chronic

pancreatitis can follow as a result of acute pancreatitis, but also from heavy alcohol use, high blood levels of calcium or blood fats. Common for both types is that the inflammation causes the enzymes produced by the pancreas to be activated in the pancreas. Autoimmune pancreatitis have two subgroups, type 1 and type 2 (Pearson, Longnecker et al. 2003). Type 1 AIP is distinctive since high levels of IgG4- positive cells has infiltrated small ducts in the pancreas, together with fibrosis around ducts and veins (Chari, Smyrk et al. 2006). Type 2 AIP is confined to the pancreas, and exhibits granulocytic endothelial lesions, and can cause pancreatic damage (Sah, Chari et al. 2010).

The symptoms for pancreatitis are very similar to those of pancreatic cancer, further diffusing the line between correct diagnosis of pancreatic cancer. There is no assay available as of today that can reliably differentiate between pancreatic cancer, pancreatitis, and healthy.

4.2. Biomarkers in Pancreatic Cancer

The only FDA approved biomarker for pancreatic cancer is the carbohydrate antigen 19-9 (CA19-9), or *sialyl Lewis a* (Koprowski, Herlyn et al. 1981). Discovered in a study of patients with colon and pancreatic cancer, CA19-9 was revealed as an elevated serum marker. However, CA19-9 also has an elevated level in later stages of other pancreatic diseases (Ong, Sachdeva et al. 2008), making it hard to discriminate between benign disease and malignant pancreatic cancer. Further, not all patients express CA19-9 (Hamanaka, Hamanaka et al. 1996), leading to false negative diagnosis unless sialyl Lewis status is determined.

A more adopted strategy to discover pancreatic cancer early is to scan high-risk groups for early signs of cancer (Canto, Harinck et al. 2013). For pancreatic cancer, people with high incidence of pancreatic cancer in the family history are included, as well as people with hereditary pancreatitis, or if you have one family member with pancreatic cancer and linked cancer syndrome, e.g. *BRCA1* gene mutation. These patients take a blood test, and every three years they undergo a CT scan or ultrasound of the pancreas. In addition, sampling of the pancreatic juice can be performed to determine the changes in three particular genes, p53, K-Ras, and p16.

Recently, the interest of micro-RNAs (miRNAs) in pancreatic cancer has given many candidates for pancreatic cancer specific biomarkers. (Li and Sarkar 2016). Together with CA19-9, miR-16 and miR-196a has been shown to be more

effective in classifying pancreatic cancer from both pancreatitis and healthy samples than CA19-9 alone (Liu, Gao et al. 2012). These test are however not FDA approved, and even though micro-RNAs boost the diagnostic performance of pancreatic cancer versus healthy, the aspect of differentiating from pancreatitis must not be excluded.

The in-house developed antibody microarray platform has previously been used for classification of pancreatic cancer, regarding diagnosis and prognosis (Ingvarsson, Wingren et al. 2008), classification of pancreatic cancer against benign (pancreatitis) and healthy controls (Wingren, Sandstrom et al. 2012), and profiling different stages of disease against healthy controls (Gerdtsson, Wingren et al. 2016).

4.3. Immunosignaturing of pancreatic ductal adenocarcinoma

In **paper I**, we performed protein expression profiling of pancreatic cancer samples using the antibody microarray. We compared pancreatic cancer serum samples with healthy controls and benign controls. The benign controls consisted of patients diagnosed with acute or chronic pancreatitis, Langerhan neoplasm or pancreatic neoplasm. By randomly dividing the analyzed samples into ten training and test sets, we identified the 25 top predicative antibodies in each training set, and they were evaluated in the corresponding test set. The average AUC from ROC curves was 0.98 for PDAC versus healthy controls, and 0.67 for PDAC versus benign controls. Implementing a backward elimination strategy to identify the number of antibodies sufficient to discriminate between PDAC and healthy resulted in a panel of four to ten antibodies. However, given the difficulty of separating PDAC from benign controls, a larger number of antibodies was required to separate these groups (average 67 antibodies), indicating that a smaller immunosignature of 10 or less antibodies might not be specific enough for pancreatic cancer.

Further, for the first time – to our knowledge – we could with a proteomic approach show that the position of the tumor within the pancreas could be determined using serum samples. The location of the tumor has shown prognostic relevance, those located in body or tail having a poorer survival compared to neck

tumors (Artinyan, Soriano et al. 2008). Together, these findings showed that we could classify pancreatic cancer patients from healthy controls with excellent separation, from benign controls with fair separation. Using the antibody microarray, we could determine the location of the tumor, further adding to the prognostic information that could influence treatment selection in resectable tumors.

5. Concluding remarks

With the emergence of new and improved therapeutic drugs for treating different cancers in the last decades, the importance of an early, correct, and easy diagnose of cancer has increased. Defining subtypes becomes essential to be able to select the optimal treatment, where biomarkers play a key role. However, the large discrepancy of the number of biomarkers potentially found, and the number of biomarkers approved by the *e.g.* the FDA, shows that several techniques still have development to be had. The need for improved therapies, as well as diagnostic tools, initiated the Cancer MoonshotSM initiative to accelerate cancer research aims of therapy, prevention, and early diagnosis.

The aim of this thesis was to decipher molecular portraits of cancer. To this end, a recombinant antibody microarray platform technology was used, containing close to 300 recombinant antibodies, targeting the immune response. In addition, an in-house developed technique, GPS, combining the specificity of affinity proteomics and power of mass spectrometry, allowed for a specific survey of the proteome. Compared to other proteomic approaches, the sensitivity and reproducibility of the antibody microarray is a great advantage. Using antibodies, targeting low-abundant proteins in otherwise difficult sample formats such as serum, becomes possible, and allows for a great opportunity in biomarker research.

A big opportunity to find key answers and mechanisms in how different cancers develop, behave, and respond to different treatments, lies within the field of proteomics. In the last decades, proteomics has evolved both in technical advancement, but also with the understanding on how to process samples, and what to look for. Through the possibility to use blood samples instead of tissue, the accessibility to actual samples increases.

A general consensus lies in the fact that early detection of cancer improves outcome for patients. To this end, in paper II, we analyzed samples collected up to two years before diagnosis. Surveying the immune system, using the antibody microarray platform, we could see that patients up to two years before diagnosis showed altered levels of serum proteins. And some proteins, *e.g.* C3, had

previously been marked as a candidate for early detection. Foremost, in this study, data indicated that we could identify several candidates that are involved in immunoediting, and the different stages thereof.

Further, in the area of personalized medicine, we aimed to develop a molecular signature that reflects histological grade in breast cancer tumors. To this end, in **paper III** and **paper IV**, we used two different proteomic technologies – the antibody microarray, and the GPS platforms, to find and identify biomarker signatures. Histological grade is of importance in the selection of therapy, since it reflects the aggressiveness of the tumor. However, the intermediate grade 2 bears little clinical prognostic importance, and a deeper understanding of these tumors could be of great importance. In **paper III**, we used the GPS platform to identify a 49-plex signature that could differentiate the different grades from each other. Further, the protein levels indicated that the more differentiated grade 1 tumors were in fact more conserved regading structure within and surrounding the tumor. Grade 3 tumors had lower stromal and ECM-associated protein levels, indicative for metastatic behavior. Grade 2 samples, were as expected a heterogeneous grade, overlapping both. This showed that several of the grade 2 tumors should be able to be subdivided into those more, and less aggressive.

In **paper IV**, we further looked at the potential of subdividing grade 2 tumors into grade 1 or grade 3. The antibody microarray platform was used to target another window of the proteome, compared to the GPS platform. Using the immune system as a sensor of disease, we could see that grade 2 samples could be classified as more like grade 1 or grade 3 tumors. In addition, we could see some samples from both grade 1 and grade 3 were transferred as well. Together with paper III, these studies analyzed two different parts of the proteome, to give complementary views regarding the same scientific question.

While my main focus has been on breast cancer, I also had the opportunity to address pancreatic cancer. Implementing the microarray platform, we aimed to find a biomarker signature targeting pancreatic cancer, a research area in desperate need of new actionable results. In 2013, the US signed into law, Recalcitrant Cancer Research Act, calling on the NCI to develop frameworks for improving the survival of pancreatic cancer. With this in mind, in paper I, we identified an immunosignature, classifying pancreatic cancer samples from healthy controls, as well as benign controls, such as pancreatitis. Classifying pancreatic cancer against normal controls, AUC values of 0.95-0.98 were obtained, using as little as four antibodies. Classification against benign controls gave AUC values of up to 0.88, showing both the similarities of mainly pancreatitis to pancreatic cancer, but also the possibilities to create a test for further subdivision.

In summary, this thesis has focused on the diagnostic capabilities gained from using antibodies as affinity probes, by searching complex samples and differentiating protein levels. Although further validation will be needed, the studies hopefully give a small piece of a larger puzzle, regarding diagnosing pancreatic cancer, early detection of cancer, and personalizing medicine for patients inflicted with breast cancer. In the future, these studies will hopefully add to help complete the understanding of cancer, making a step towards an increased standard of care.

Populärvetenskaplig sammanfattning

I modern tid har en förbättrad sjukvård gett oss längre liv. När man ser hur förekomsten av cancer ökat de senaste årtionden, räknar man med att varannan person kommer drabbas av cancer efter år 2030. En förbättrad sjukvård baseras på en ökad förståelse av sjukdomar, varför de uppstår, och därmed hur man ska kunna behandla, och förhoppningsvis bota dessa.

För att man ska få cancer, måste de sjuka cellerna kringgå en hel del kontrollpunkter som varje unik cell har. Hur de kringgår alla kontrollpunkter är olika för olika typer, men stort sett finns det tio egenskaper som en cancercell måste förvärva för att kunna överleva. Dessa tio egenskaper kallas för "Hallmarks of Cancer". Ett av dessa Hallmarks är att undvika immunförsvaret, och följaktligen kan vi förstå att immunförsvaret formas av cancer, och tvärt om.

I min avhandling har jag tittat på hur kroppen har reagerat på cancer med hjälp av immunsvaret. Vid sjukdom försvarar sig kroppen mot det främmande med hjälp av immunförsvaret, och det immunsvaret som uppstår.

Jag har försökt hitta signaturer, bestående av vilka protein, eller byggstenar, i kroppen som ändras, och hur de ändras. Genom att mäta de svar kroppen har mot till exempel cancer, kan vi ge en sjukdom en specifik signatur, och jämföra denna med en frisk individ. För när en person blir sjuk, kommer immunförsvaret aktiveras och olika delar av det ökar i kroppen, medan andra minskar. Är sjukdomen uppkommen av yttre faktorer, som bakterier eller virus, eller inre som cancer eller autoimmunitet, ser dessa nivåer olika ut.

Immunförsvaret är komplext och består av många olika delar. En viktig del av immunförsvaret är antikroppen, en molekyl som binder till olika patogener, som bakterier eller virus. Genom att binda till patogen, kan kroppen bli av med hotet som orsakar sjukdom. Genom att använda konstgjorda antikroppar och bestämma vad dom binder, kan vi med vår teknologi se efter hur mycket av de andra delarna av immunförsvaret ändras i olika sjukdomar.

För att kunna mäta hur mycket antikroppen har bundit använder vi oss av två olika metoder. Microarray, som består av en yta med olika antikroppar fästa på. Dessa antikroppar binder sin motpart, eller målprotein, som i vårt fall är olika delar av immunförsvaret, och sen mäter vi hur mycket varje antikropp bundit. GPS använder antikroppar som binder delar av protein, peptider, som man sedan kan båda mäta, och även bestämma vilka aminosyror de består av i en masspektrometer. Den största skillnaden mellan dessa sätt är att i microarray undersöker vi en mindre, men väl definierad del av immunsvaret, medan med GPS mäter vi protein som härstammar från överallt i kroppen.

Genom att använda oss av de olika egenskaperna som immunförsvaret har kan vi därmed mäta immunförsvarets respons till sjukdomar, och sen jämföra skillnader i sjuka mot friska. Alternativt använder vi oss av antikroppen som "magnet" för att fånga upp en del av många olika peptider, och därefter mäter vi dessa för att kunna göra jämförelser.

I min avhandling har vi undersökt bröstcancer och bukspottskörtelscancer. I två av artiklarna vill vi se om vi kan hitta hur skillnader i proteinnivåer beroende på hur aggressiv en bröstcancertumör är. Målet är att i framtiden kunna ersätta dagens metod, där man tittar på en bit av tumören och ser hur den ser ut, för att uttala sig om detta. Den största vinsten med detta kan bli att hälften av alla patienter som idag hamnar i en mittenkategori, istället kan fördelas in i antingen en mindre, eller mer aggressiv kategori, där behandlingarna är väldigt olika.

En tredje artikel tittar på hur immunförsvaret ändrar karaktär innan man får bröstcancer. Idag finns en teori om att en cancercell och immunförsvaret går igenom tre faser, och om en sjuk cell går från den första vidare till den tredje så får man cancer. Dessa är eliminering, där immunförsvaret tar död på den sjuka cellen, den andra är jämvikt, där immunförsvaret har koll på canceren men inte kan ha ihjäl den ännu, och till sist flykt, där cellerna ändrats så mycket att immunförsvaret inte kan rå på dem, och blir cancer. För att undersöka dessa förändringar använde vi oss av blodprov tagna upp till två år innan patienterna blev diagnostiserade med bröstcancer.

Det sista arbetet handlar om bukspottskörtelscancer. Denna cancer drabbas ca 900 svenskar av varje år, men mindre än 10% överlever mer än ett halvår. En stor anledning till detta är att man allt försent hittar sjukdomen, och att den då redan är för långt gången för att kunna behandlas effektivt. I studien hittar vi signaturer som ska kunna särskilja patienter med cancer från friska, vilket kan leda till en tidigare diagnos, med ökad överlevnad.

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