Proteomics-driven approach for the detection of breast cancer biomarkers

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Abbreviation list

ATP	adenosine-5'triphosphate
aa	amino acid
ANN	automated neural network
approx.	approximately
AID	autoimmune disease
AUC	area under the curve
BC	breast cancer
CAR	cancer-associated retinopathy
CEA	carcinoembryogenic antigen
CID	collision-induced dissociation
CDR	complimentary determining region
CC	craniocaudal
CTL	cytotoxic T-lymphocyte
DCIS	ductal carcinoma *in situ*
EGTA	ethylene glycol tetraacetate
ESI	electrospray ionization
ER	estrogen receptor
et al	et alia
etc.	et cetera
EDTA	ethylenediaminetetraacetate
e.g.	exempli gratia
FDA	Food and Drug Administration
HBV	hepatitis B virus
HPLC	high pressure liquid chromatography
HPV	human papilloma virus
HUPO	Human Proteome Organization
IRS	immune reactivity score
IEF	isoelectric focusing
iDC	invasive ductal carcinoma

ILC	invasive lobular carcinoma
MA	Morbus Alzheimer
major histocompatibility complex	MHC
MG	mammography
minute(s)	min(s)
MS	mass spectrometry
MRI	magnetic resonance imaging
MALDI-TOF/TOF MS	matrix-assisted laser desorption-ionization time of flight/time of flight mass spectrometry
MLO	mediolateral oblique
MLP	multilayer perceptron
OV	ovarian carcinoma
PMF	peptide mass fingerprint
PPP	Plasma Proteome Project
PSD	post source decay
PR	progesterone receptor
PRR	proline-rich region
ROC	receiver operating characteristic
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gelelectrophoresis
SD	standard deviation
SE	standard error
SELDI-TOF MS	surface-enhanced laser desorption-ionization time of flight mass spectrometry
SLE	systemic lupus erythematosus
TA	tumor antigen
TCA	trichloroacetic acid
TIL	tumor-infiltrating lymphocyte
TFA	trifluoroacetic acid
USA	United States of America

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1 Introduction

1.1 Breast cancer

Pink ribbon is the international, highly recognizable symbol of consciousness for mammary carcinoma (colloquially *breast cancer*, BC). Following chapters in introduction part point the medical background behind this disease and give a short overview of BC research with its main topic in proteomics-based approaches. The descriptions of diagnosis and treatment issues of BC are mainly based on the interdisciplinary S3 guidelines for diagnosis, therapy and aftercare of BC in Federal Republic of Germany (FRG)[1].

1.1.1 Etiology and epidemiology

Carcinogenesis in general is a multi-step event with longer latent phases, accompanied by several molecular changes, starting from mutations on the genomic level, whereas most mechanisms are not clear defined by now. Thus, mutations in genes, which have an important impact on the cell cycle (*e.g.* check-point kinases like p53 or tumor-suppressor genes like *breast cancer 1- brca1*, *etc.*) accumulate and lead to an endless replication of cancer cells. The transformed cells acquire additionally several new properties including apoptosis resistance, promoted angiogenesis, unrestricted replicative potential, invasion and metastasis potential, escaping the growth inhibitors and perpetual proliferating signaling[2]. Figure 1.1 shows the summary of tumor cells' properties with two additional complementary characteristics: escaping the obliteration through immune system and reprogramming of energy metabolism (switching to aerobic glycolysis, the Warburg effect)[3]. All these acquired properties enable growth of tumor cells and their spread over the organism leading to irreversible systemic damage.

The etiology of BC, like for most cancer entities, is unknown by now, although some risk factors are admitted from epidemiologic studies. One of the most prominent risk factors of BC is the sex affiliation as only 0.6% BC affected persons are males[4].

Likewise, aging is the another important factor: the affected woman in FRG is in average 64 years old[1]. Further risk factors are coupled with the estrogen impact on the organism like early menarche, late menopause and nulliparity. Also a continuous uptake (over 10 years) of estrogen medication during the menopause (hormone replacement therapy) may lead to a higher rate of BC[5,6]. Overall, the emergence of BC seems to be a complex, multifactorial process, whereas the (most important) components are not known up to date.

Figure 1.1: Hallmarks of cancer cells, postulated by Hanahan and Weinberg in 2011[3]. The original figure was changed by removing of therapy strategy for each hallmark.

Large population-based studies show that some of the invasive carcinoma may develop from the intraepithelial atypical hyperplasia to *in-situ* carcinoma, *e.g.* ductal carcinoma in-situ (DCIS), turning somehow into invasive malignancy[4]. The phase of developing invasive properties is latent and may last under certain circumstances for several years[7]. Only for the hereditary type of breast cancer (approximately 5% of all BC cases), it is well-determined that mutations in two tumor-suppressor genes *breast cancer 1* (BRCA1) and *breast cancer 2* (BRCA2) lead to higher rates of BC and ovarian carcinoma (OC)[8-10]. Both proteins are essential for the repair of double-strand DNA breaks[11]. The third relevant high-risk gene for the BC susceptibility is *rad51c*, which was recently identified by Meindl *et al*[12].

In 2010, 68710 women in FRG were diagnosed with primary BC[13]. In the same year, malignant diseases of the breast were the 4th leading cause of death in FRG[14] (17466 deaths)[15]. The mortality remains nearly constant over the last years. Today's medicine view of BC is of systemic nature with local component[16]. Therefore, the handling of BC requires multiple lines of actions including local and systemic adjuvant treatment.

Statistically, every 8-10th women in the Western countries is diagnosed with BC throughout life. This cancer type is therefore the most common cancer in women[17] and the second leading diagnosed cancer entity[18]. Nevertheless, regional differences in the distribution of BC are well-committed (see an overview of worldwide incidence and mortality rates in Figure 1.2). Thus, Japanese women are less frequently diagnosed with BC in Japan compared with the women in the United States of America (USA). However, women living in the USA in the second generation from the Japanese descents are indeed more often confronted with BC when compared to women living in Japan. This observation in Western countries may be

partially explained by several environmental factors, including nutrition (fat-rich diet and enhanced alcohol consumption) and lifestyle habits (*e.g.* lack of exercise). Overall in the USA, more Caucasian women are affected by BC, followed by African Americans, Japanese and Hispanics[4].

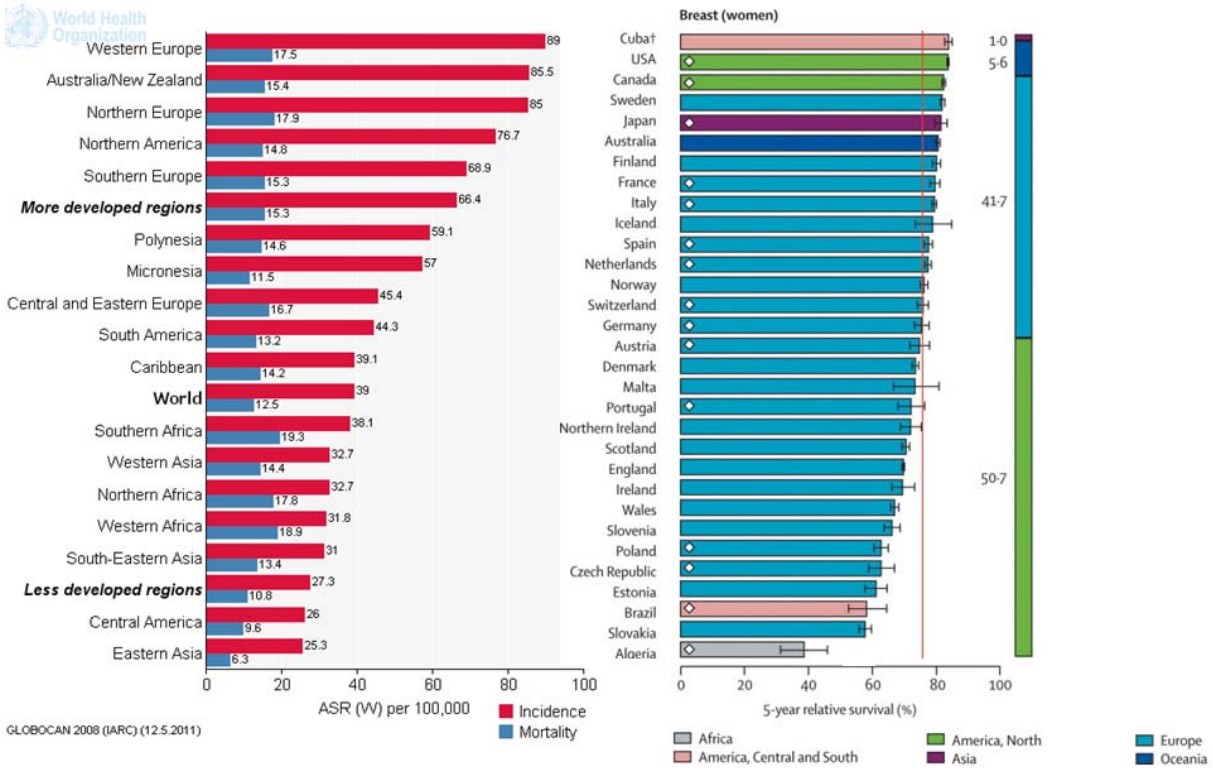


Figure 1.2: Age-standardized incidence and mortality rates of BC worldwide per 100000 in 2008 on the left side[19] and BC survival rates from different countries and continents on the right side from Coleman *et al.* 2008[20].

1.1.2 Anatomical build-up of mammae

The *mammae* are located above *major pectoralis* muscle on the rib cage's forefront and reach from the second to the sixth rib[4]. The mammary tissue consists of different layers. Briefly, the mammary gland (the glandular tissue) is surrounded by the adipose tissue. The glandular part of mamma consists of several lactiferous lumen or lobuli (*Lobulus glandulae mammariae*) secreting breast milk. The milk is transported from lobuli in *ductus lactifer*. Further breast milk transport occurs over the bigger ductus (*Ductus lactifer colligens*) which is enlarged to a milk supply unit (*Sinus lactifer*) and ends in the apical part of the breast, the papilla. In general the structure of the mammary gland is a subject of change during puberty, pregnancy and lactation period as well as at the time of the menopause[21]. The simplified build-up of mamma is depicted in Figure 1.3.

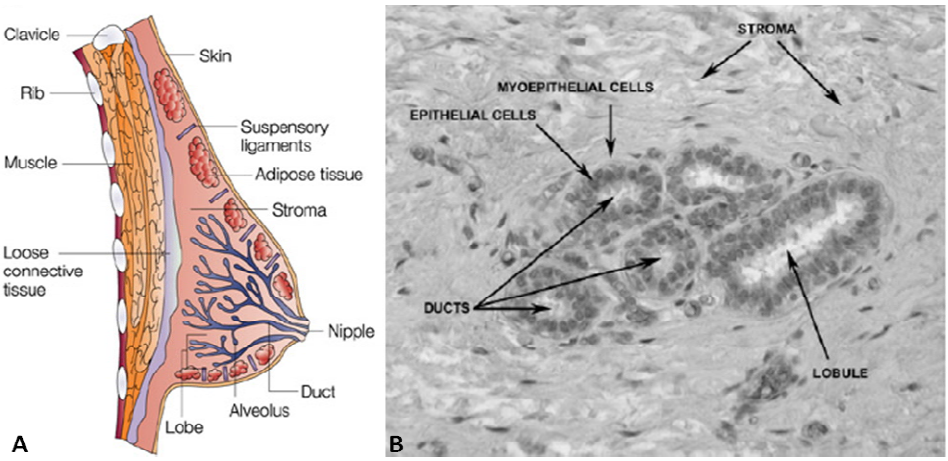


Figure 1.3: The build-up of the mammary gland. In A, the anatomical structure is shown[22], whereas on the right side in B, the histological section of breast components is depicted[23].

Besides malignant neoplasms, also several non-malignant mammary diseases: *e.g.* benign neoplasms like fibroadenoma, mammalgia (tensional mammary pain) or puerperal/non-puerperal mastitis (mammary inflammation). Due to BC as the main topic of this doctoral thesis, they are not further enlightened in the context.

1.1.3 Diagnostic options

Palpable tumors can be detected by women' themselves by a routine self-examination at home. The most efficient time point of examination is the 1st week after the beginning of menstruation. Additionally to the mandatory professional palpation, sonography (SG) of the breast and axillary region in combination with mammography (MG) are the most important diagnostic tools. Also magnetic resonance imaging (MRI) procedure may be applied for clarification of suspect findings. Currently, the gold standard for detecting BC is mammography (MG). This x-ray based technique allows detecting tumors over 0.5 cm, whereas smaller tumors are usually not detectable. During this procedure, x-ray-pictures from different angles (craniocaudal, CC and mediolateral oblique, MLO) of the compressed breast tissue are imaged and afterwards examined by two independent radiologists (see Figure 1.4 for different MG and MRI pictures).

The crucial factor of the curability of BC is its earliest possible detection. Thus, several national health-based programs with varying duration and experience exist in different countries. In FRG, every woman of 50-70 years gets every two years a tentative invitation from the citizen centre for a MG screening, a non-payment benefit from the compulsory health insurance fund. If a suspect mass in mamma is detected by MG, several additional steps are required to confirm the diagnosis. The final detection is performed via SG-guided core biopsy. Afterwards, the tissue is examined by a pathologist, who confirms the diagnosis by well-established anatomical and histological parameters.

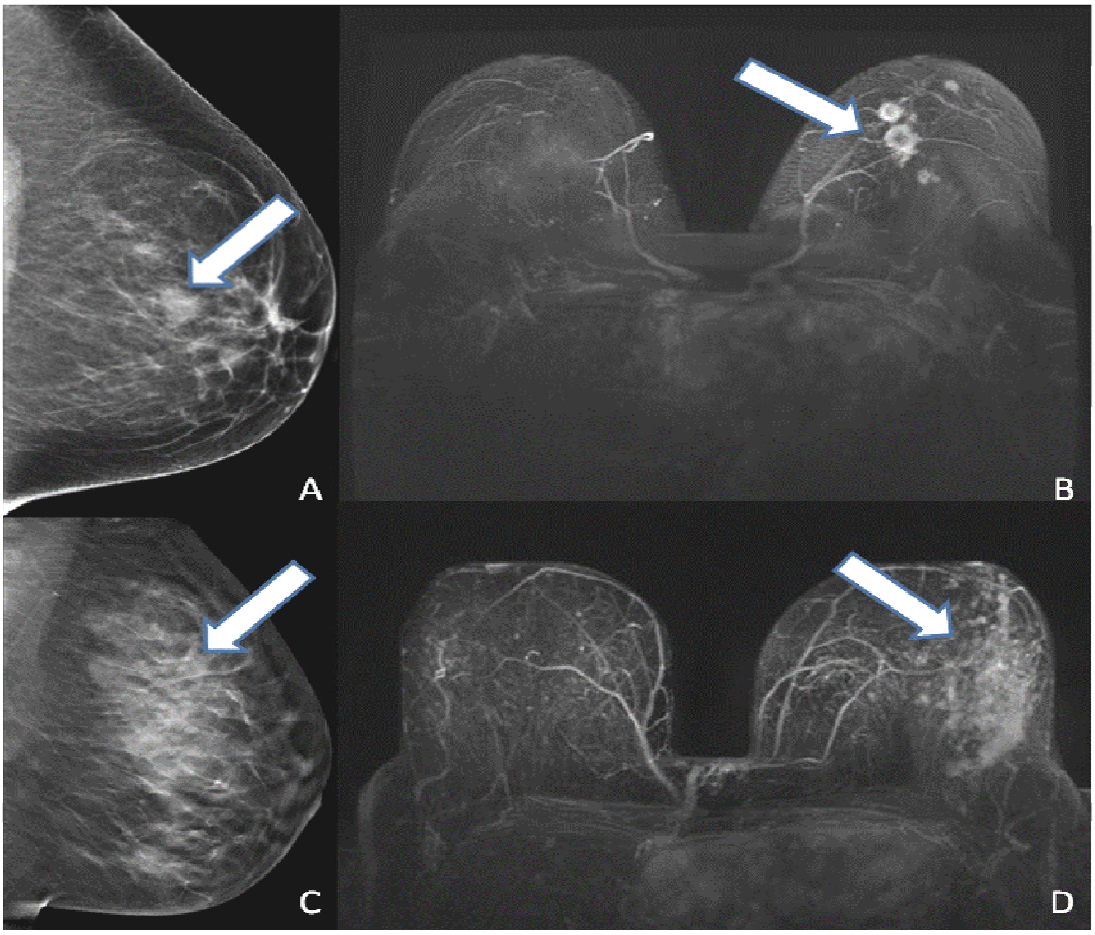


Figure 1.4: Examples of diagnosed BC are shown over MG (A+C) and MRI (B+D) images. In A: left mamma with an invasive ductal carcinoma (IDC) is shown in MLO and in B in the CC view. A multicentric invasive lobular carcinoma (ILC) in the left mamma is depicted in C in MLO and in CC view in D. Tumor locations are indicated by arrows. (Courtesy of Breast Center of the Universitaetsmedizin Mainz).

Recently, many population-based studies brought controversy into the discussion of positive effects of MG as the benefit seems not to be completely proven. Thus, women with false-positive result must undergo several unnecessary procedures, even until invasive steps including healthy tissue removal. These circumstances clearly burden healthy women mentally and physically. The potential of MG as a detection tool is in general not free of certain limitations. The overall specificity and sensitivity of MG are 92% and 75%, respectively[24,25] depending on different parameters such as the density of mammary tissue[26] or proper positioning of the device or interpretation errors[27], *etc*. The false-positive detection rate is especially high for younger women at the age of 40-49[28]. Thus, due to the high density of tissue in younger women, the sensitivity of MG for women of 40-49 years ranges from 62%-76%, indicating additional SG. Yet, the early detection of breast tumors is essential for the further treatment process making better outcome possible. All described limitations of MG as single available method for early detection of BC clearly show the need for the development of new and more specific supportive molecular tools for its diagnosis.

1.2 Proteomics-a promising branch in cancer research

The proteome is the entirety of all proteins at a certain time point, whether in an organism, a cell, or any compartment ("subproteome"). The composition and the levels of certain proteins are not constant in comparison to the genome and may significantly vary between organs and different body fluids. Therefore, studies of the proteome enlighten the impact of different factors like environment fluctuations, hormone imbalances or disease-driven changes. The proteomics branch enables more precise analyses specific for any new entered stage of disease. Its basic concepts are described in the following sections.

1.2.1 Proteomics-based tools

Proteins are macromolecules composed of amino acids (aa). Each protein is unique due to its specific multilevel structure formation. The first composition level of the protein is its primary structure or the amino acid sequence: the aa's are linked in a specific order over planar peptide bonds between the carboxyl group of one aa and the amino group of the following aa. The hydrogen bonds between these functional groups sculpt the secondary spatial structure, whether alpha-helices or beta sheet structures. The tertiary structure is determined via hydrophobic-mediated assortment of secondary structures into protein' three-dimensional shape: *e.g.* over disulfide or hydrogen bonds. The last protein structural level represents the aggregation of several single protein molecules (subunits) to the functional protein unit[29].

As already mentioned, the proteome's composition is very sensitive to different environmental fluctuations, but it is also influenced by post-translational modifications, hormone imbalances or pathological changes (intrinsic impact). This fact makes the proteomics explorations very interesting, as it provides the possibility to detect the immediate changes in the expression levels in response to a certain trigger, whether caused by environmental or pathogenic impacts. The proteomics-driven analysis approaches within the techniques are briefly described in the following.

Two different proteomics approaches are used in general: the explorative and the targeted one. The explorative analysis of complex protein samples enables the profiling of several components of the proteome and the comparison of peptide/protein levels in a steady state and after specific triggering factor: *e.g.* disease, therapeutic intervention, *etc*. The targeted approach is focused on single molecules of interest and their interaction partners. Both approaches demand in first line stable and robust protein analyzing techniques.

The analysis of native complex protein samples starts with multidimensional purification steps to target proteins of interest. One of the most investigated human (sub)proteomes,

being relevant not only in research laboratories but also in the clinical routine, is the serum or plasma proteome. The difference between both sample types is the presence of anticoagulants (citrate, heparin, ethylenediaminetetraacetate, EDTA) in plasma, which prevent the loss of fibrinogen and other aggregation components. Plasma and serum harbor a large number of proteins and serve as prior information source for the estimation of healthy or diseased condition. Despite being well investigated proteomes, the use of serum or plasma has limitations in first line due to the exceeding amount of proteins. Currently, according to the Human Proteome Organization' Plasma Proteome Project (HUPO PPP), the number of identified proteins in the plasma is around 9500, but the assumed number is much higher[30,31]. The major problem of these complex samples is the high abundance of proteins like albumin, immunoglobulins or proteins of complement system. As the result, high amount of these proteins simply covers the lower abundant proteins, making their profiling and identification very challenging. Several fractionation approaches are applied therefore prior to analysis of serum or plasma samples: *e.g.* removal of high abundant proteins via binding to the specific antibodies (depletion) or enrichment of small abundant proteins via interactions with chemically active surfaces (bead- or column-based approach)[32-34]. After these fractionation steps, further procedures for the proteome analysis can be performed. Briefly, complex samples can be separated according to the molecular weight of the components with polyacrylamide gel electrophoresis (PAGE), whether in a native or in a denatured way using the detergent sodium dodecylsulfate (SDS-PAGE). Additionally, a separation method according to the isoelectric point (isoelectric focusing, IEF) of proteins with an immobilized pH gradient may be applied prior to the described PAGE (two-dimensional, 2D-SDS-PAGE)[35,36]. Other important techniques for studies of proteins' interactions and profiling of different protein samples are the protein mass spectrometry (MS) and the highly precise protein or antibody microarray-based platform.

The MS technique enables the analysis of different molecules like peptides, carbohydrates, nucleic acids, *etc.* The protein MS, a separate branch of MS, estimates the molecular weights of proteins and their fragments by calculation of the mass/charge ratio. It is often combined with chromatography techniques for a better separation of complex samples by certain properties of proteins: *e.g.* hydrophobicity or aromatic residues of amino acids. MS can be used not only for the profiling of molecules in different samples but also for their subsequent identification. While (2D) SDS-PAGE technique is capable to resolve complex protein samples according to the distinct protein properties, the MS enables correct, graphical mapping of proteins according to the mass/charge ratio as well as subsequent protein identification. However, several pre-fractionation steps as described above are still required for the decreasing of sample complexity for reaching the proteins of interest. One of the MS techniques is the surface-enhanced laser desorption ionization time of flight (SELDI-

TOF MS), whereby even non-fractionated protein samples are transferred on the targets ("spots") with different chemical active surfaces[37,38]. Based on the interactions with the surfaces, the samples are fractionated and afterwards analyzed in the mass spectrometer. Another, but similar analyzing method which was also used in the present thesis is matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS[39,40], whereby sample fractionation is applied prior to sample transfer on a metallic target. Then, the analyte is co-cristallized with a matrix substance (*e.g.* α cyano-4-hydroxy cinnamon acid). Samples are then ionized (protonated) through matrix over the laser (ion) source and moved into gas phase (*desorption/ionization*). The bigger the molecule, the more ionization occurs. The ionized molecules are accelerated in the electrical field and moved in the vacuum tube until they reach the detector plate. The heavier molecule arrives there later than the lower weight-molecule. This effect enables the estimation of the mass/charge (m/z) ratio of each ionized molecule by the TOF analyzer. Additionally, an implemented reflector accelerates the ions in the opposite direction for better resolution. The respective molecular weights of molecules are based on the protein or peptide mixture, which is measured together with the analytes. Furthermore, for the better protein identification, the enriched proteins or the gel fraction after (2D) SDS-PAGE are digested with the endopeptidase, *e.g.* trypsin or lysine K, and the peptides are measured. Each peptide generates its unique peptide mass fingerprint (PMF)[41], due to proteinase-generated fragmentation pattern, and subsequently mapped to the according proteins over database matching (MS mode)[42]. In addition, MALDI-TOF/TOF with tandem mass analysers provides the sequence-based identification of the peptides via enhanced fragmentation mode (MS/MS analysis)[43]. The protein identification is then combined *e.g.* with the inert gas collision induction (collision-induced dissociation, CID) between both analysers. Thus, the m/z ratio of fragments is determined in the first analyser, the selected fragments are then fragmented in the collision cell and afterwards directed to the second detector[42]. Figure 1.5 shows schematically the build-up of the tandem MALDI TOF/TOF MS.

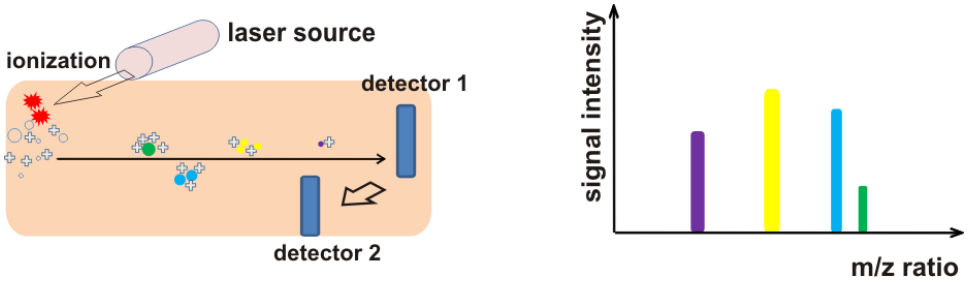


Figure 1.5: Schematically build-up of MALDI TOF/TOF MS on the left side and the m/z ratio of measured peptides on the right side. First, the samples are protonated over the ion source and moved into vacuum tube (thin black arrow) until detector 1, fragmented in the collision cell (thick black arrow) and moved to detector 2. Changed and adapted from Abramovitz and Leyland-Jones[44].

Another important strategy for detection and estimation of protein amounts in different complex samples is the antibody or protein microarray platform[45]. Here, very small amounts of analytes are fixed on an adsorbing surface, *e.g.* nitrocellulose, for capturing the corresponding molecules of interest from the complex samples. Besides, proteins or antibodies are transferred onto carrier with the help of an automatical device to prevent volume fluctuations. Additionally, several slides can be processed simultaneously in order to accelerate the procedure. The main principle is based on the common and well-established enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA) technique and offers several advantages due to its miniaturized format[46,47]. The protein microarrays are used for the detection of the respective antibodies in the samples. Thus, single proteins of complex protein samples (reversed-phase) are captured on the microarray slides. The visualization occurs via secondary antibody with coupled labeling dye or an enzyme. In case of so-called "reporter" enzymes, *e.g.* horseradish peroxidase, a chromogenic color reaction can be visualized by conversion of its substrate. By use of antibody arrays, proteins in the samples of interest may be directly labeled in the complex sample solution with a fluorescent dye and then incubated with the spotted antibodies. The general principles of protein and antibody microarrays with regard of performed experiments in the present thesis are shown in section 3.13.

Recent advances in proteomics techniques provided huge amounts of new data according to the different disease-driven changes on the proteome level. This fact enables the use of proteomics for the detection of disease-related biomarkers, described in the following section.

1.3 Biomarkers for breast cancer and other diseases

A biomarker is a possibility to highlight ("mark") a current status of a biological system. The official definition of the National Institute of Health (NIH) for biomarker is a "characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"[48]. The properties of the biomarkers should highlight the present state and discriminate it precisely from the past time point. Furthermore, biomarkers can be divided in several categories, like detection, prognostic or predictive biomarkers or even more precisely classification is possible, *e.g.* into biomarkers for certain disease stages. Thus, a prognostic cancer biomarker should provide a statement about the relapse risk and survival rates, whereas predictive biomarkers would allow a forecast about the therapeutic response. In addition, potential biomarkers offer the opportunity of targeted therapies, as certain structures of tumor cells can be selectively combated.

Several molecular biomarkers were described in the past decade, whereas the search for the biomarkers was initially oriented on the genomic and transcriptomic levels[8,49,50]. Hence, not only genomic-based investigations of certain genes, but also proteins were proposed to discriminate a certain status in a biological system. The most common purpose for measuring the levels of cancer protein biomarkers is the detection of an abnormal status or malignancy in a preinvasive stage.

The development of clinically useful biomarkers is a very challenging issue, as several investigations should be performed for the validation of selected molecules to ensure the discriminatory functions. The establishment of a population-based cancer discovery strategy was postulated by Pepe *et al.*, whereas the described guidelines were divided in five phases[51]. Thus, the first challenging phase consists of biomarkers' finding including definition of the most promising candidates. For that purpose gene or protein expression patterns of specific tumor tissues are compared with the healthy counterpart, whereas most promising down- or upregulated candidates are selected. The constructed assay is evaluated according to its operating characteristics: true-positive rate (sensitivity) and true-negative rate (specificity) resulting in the area under the receiver operator characteristic (ROC) curve (AUC: sensitivity plotted against reciprocal specificity or false-positive rate). Surely, especially the low false-positive rate of cancer diagnosis plays a very important role for the establishment of population-based screening. In the second phase the genomics- as well as proteomics-based detected biomarkers or biomarker panels (gene patterns, proteins or autoantibodies) found in different levels of phase 1 cancer patients and healthy probands are verified in serum or other body fluids of the same probands. Basically, the retrieval of these samples is minimally invasive compared with tissue samples, contributing to the realistic usage in population screenings. Also here the sensitivity and specificity with the AUC of the preliminary clinical assay are estimated. The correlations with the results from the first phase and their intra- and interinstitutional reproducibility are important parameters for the successful development of the molecular tool. Moreover, also relationships between found biomarkers and tumor' classification parameters (see next section 1.3.1 for details) or behavioral impacts, *e.g.* alcohol consumption or smoking may be discovered. The third phase of biomarker development comprises of testing, whether the selected biomarkers possess the ability to detect cancer prior to the clinical onset in the longitudinal sample testing. Thus, the conditions for the positive (=cancer) come out are simultaneously defined in this phase. The constructed assay is applied on the screening population (prospective screening) in the fourth phase. The biomarkers are therefore estimated also in subjects diagnosed with cancer to the time point of screening. Also here the respective operator characteristics are estimated. The last fifth phase evaluates the benefit of the biomarker-based screening assay for the reduce of cancer impact on the population, as it still may be

insufficient: *e.g.* due to high-costs or bad compliance of screening participants, *etc*[51]. Figure 1.6 summarizes the described main stages of cancer biomarker development and their use in the clinical routine.

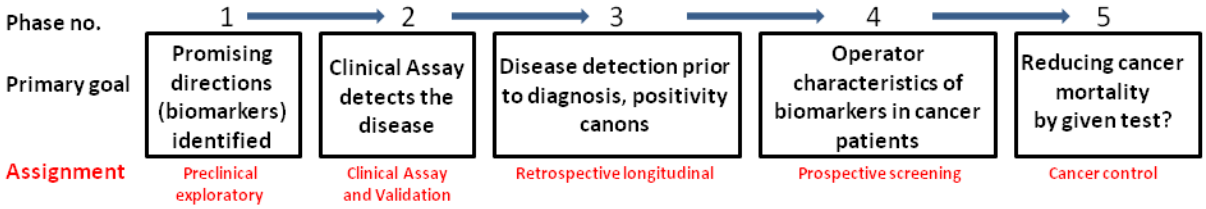


Figure 1.6: Development of population-based clinical assays based on cancer biomarkers divided in five stages with the appropriate primary goals. Changed and adapted from Pepe *et al.*[51]

The following section discusses established parameters for the characterization and treatment of BC. The described properties of tumors, together with serum biomarkers, serve as prognostic and predictive parameters (in the broader sense as biomarkers) and are designated in the respective BC guidelines[1].

1.3.1 Established parameters for classification and therapy of BC

Subsequent histological examination of tumor tissue by pathologists delivers knowledge of tumor properties over respective classification according to the international guidelines. Additional imaging examinations of the liver and the lung are required for the determination or exclusion of distant metastasis, as, together with bones, they represent most favorable targets of breast cancer metastases. Primarily, tumors are anatomically classified according to the Union internationale contre le cancer (UICC) and American Joint Committee on Cancer (AJCC) guidelines using the **T** (tumor size), **N** (node involvement) and **M** (distant metastasis) categories (**TNM** or **pTNM** staging after histological examination) and their subsequent combination to tumor stages[52-55]. The node characteristic points the affection of the axillary lymph nodes and the metastasis category marks spreading of tumor cells into other tissues and organs. Additionally, the type of carcinoma (invasive/in-situ, ductal/lobular, *etc.*) is defined. The current TNM and staging classification parameters for BC are provided in Repository figure 3.

Summing up, the histopathological investigations deliver more precise information for the therapy of the diagnosed malignancy. Besides the described tumor staging, several other parameters and properties of tumor tissue are analyzed for the post-operational term and the appropriate therapy options. The following parameters can be therefore divided into prognostic and predictive factors.

The prognostic parameters of BC help to assess the relapse and metastasis risks leading to specific *modus operandi* for affected patients. Thus, tumor size and lymph node infiltrations

are important prognostic factors as well as the age of affected women (high relapse risk for women fewer than 35 years or over 75 years)[4,56].

The histological grading of tumors is the next contribution for the prognosis. The malignancy of tissue is defined by certain criteria. For that purpose, several cellular and nucleus characteristics are evaluated for the determination of the malignancy score[57]. For example, a high rate of mitoses increases the score. Moreover, poor differentiation as a reverse event in highly aggressive tumor cells (=anaplasia) and different nucleus sizes (=anisokaryosis) increase the grade of the malignancy either. The grading criteria are summarized in Repository table 1.

For the grading of *in-situ* carcinoma[58], the Van-Nuys classification can be used[59]. The Van-Nuys-Prognostic-Index (VNPI) system is applied as a supportive tool for the therapy options of the DCIS[60]. This scoring system is based on *in-situ* carcinoma properties, including tumor and margin size as well as necrosis stage. The higher the score, the bigger is the affected area resulting in more urgent the need for surgical involvement. The updated criteria of USC(University of Southern California)/VNPI with the age of patients as additional factor are summarized and provided in Repository table 2[61].

Contemporary with the described prognostic factors, also predictive parameters are important for the following adjuvant therapy strategy as they anticipate the impact of systemic therapy options. One of the crucial factors for the successful endocrine therapy of BC patients is the estimation of the hormone receptor status of the tumor cells due to harboring of estrogen (ER) and progesterone (PR) receptors in the nuclei. These cells possess the ability for fast proliferation and tumor spreading in presence of steroids (steroid sensitivity). Therefore, these tumor cells may subsequently develop higher aggressive potential. The presence of hormone receptors in tumor cells serves as the prognostic and predictive factor simultaneously, as their presence correlates with better therapy response, whereby their lack is associated with higher relapse risk and poor survival rates[4,62]. Additionally, the PR seems to be better prognostic and the ER the better predictive factor as standalone parameters[4]. Figure 1.7 shows examples of positive staining patterns for both receptor proteins.

Another important prognostic factor is the overexpression of *human epidermal growth factor receptor* (HER2/neu) protooncogenes on the surface of breast tumor cells (approx. 20% of all BC cases)[63,64]. HER2/neu belongs to the epidermal growth factor receptor family (EGFR) with four different tyrosine kinase receptors and interacts with other receptors via dimerization.

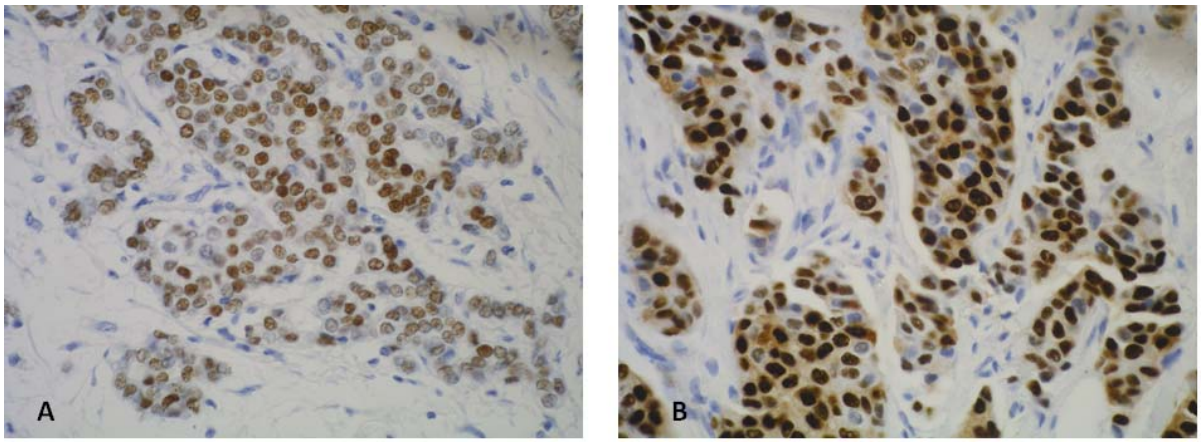


Figure 1.7: Hormone receptor protein detection in tissue sections from IDC's. The detection of both receptors is performed via immunohistochemical assay ER-ICA and PgR-ICA (Estrogen or Progesteron receptor immuno cytochemical Assay). The intensity of the staining is then calculated and summarized in an immune reactivity score (IRS, positive ≥3). In A (estrogen receptor detection) the nuclei are moderately stained (IRS=6) in a high-grade tumor, whereas in B the nuclei are strongly stained using the anti-PgR antibody (IRS=12) in the moderately differentiated tumor (Kindly provided by Institute for Pathology, Universitaetsmedizin Mainz).

The heterodimers initiate several signal cascades which trigger higher proliferation and aggressive potential of the tumor cells[65-67]. Figure 1.8 shows an example of a positive HER2/neu staining.

The presence of HER2/neu amplification or protein overexpression indicates the targeted monoclonal antibody therapy due to the worse prognosis. Additionally, coamplification of topoisomerase IIα with HER2/neu is used as a predictive marker for the anthracycline-based chemotherapy[68].

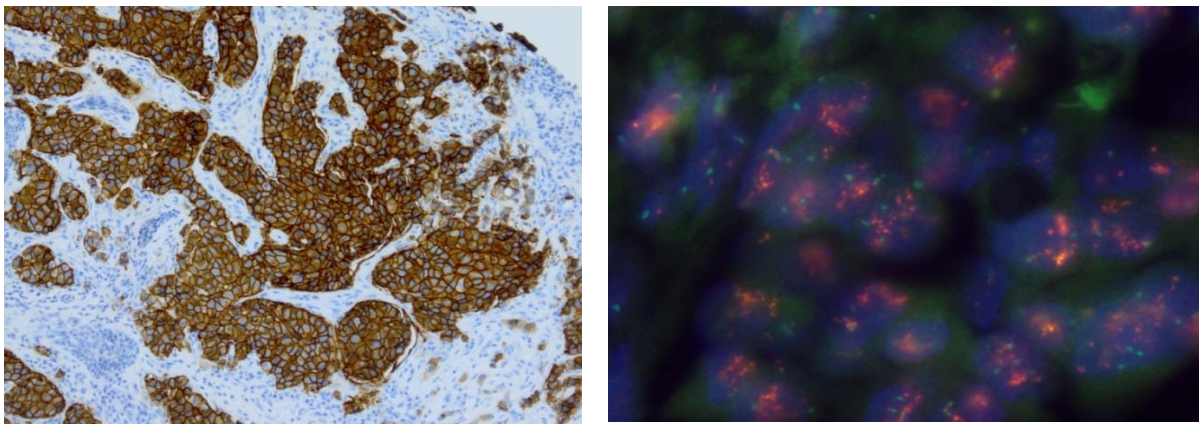


Figure 1.8: Immunohistochemical detection of HER2/neu receptor in an IDC. The HER2/neu detection in FRG is performed with *in situ* hybridization, combined with immunohistochemical detection (on the left side). The FISH-positive staining of HER2/neu genamplification is depicted on the right side (kindly provided by).

Further parameters for the prognosis and therapy response in FRG are measurements of proteases' levels in tissue sections: urokinase-type plasminogen activator (uPA) and its inhibitor plasminogen activator inhibitor 1 (PAI1). Thus, high levels of these proteases (uPA

≥3 ng/ml and PAI-1 ≥14 ng/ml), together with higher grading (G2), are correlated with the high relapse risk and poorer survival rates in node-negative patients[69,70]. Likewise, another proliferation marker Ki-67 (Kiel-67) is important for the prognosis of BC. This protein is an established marker for the mitosis, as it can be detected mostly on the cells' surface during this event and not in the nucleus during the interphase of cell cycle. Ki-67 correlates with the bad prognosis in affected patients[71,72].

Recently, also gene-arrays-based parameters are considered for the prognosis of BC. Thus, commercially available assays like OncotypeDX® (Genomic Health, Inc.), Mammaprint™ (Agendia Inc., Irvine (CA), USA) or EndoPredict® (Sividon Diagnostics GmbH, Köln, Germany) provide information of putative benefit for patients from chemotherapy[73-75]. The therapy recommendation is based on the expression patterns of selected tumor genes on the arrays which are incubated with tumor tissue. The evaluated expression patterns serve for the appropriate stratification of patients into different risk groups with the respective appropriate therapy. Another meaningful feature which became important more recently is the identification of intrinsic isotypes of breast carcinoma. The differentiation is based on the data from the cDNA microarray studies, whereas the expression of several genes ("molecular patterns") stratifies tumors into:

- Basal epithelial-like,
- HER2neu$^+$ overexpressing and
- Normal Breast-like.

Later, the results of this profiling were more specified with the estimation of further ER$^+$ isotypes, namely Luminal A, Luminal B and Luminal C[76,77]. The most favorable outcome is predicted in the Luminal A-isotype molecular group, whereas the basal-like and HER2neu$^+$ (non-luminal) isotypes are associated with poor outcome[77]. The described stratification of tumors into molecular subtypes is summarized in Repository table 3[78].

Figure 1.9 summarizes different possibilities for the potential usage of cancer biomarkers with BC as example in the clinical routine. Thus, despite of the described parameters above, none of the already established factors are suitable for the early detection of BC.

Summing up, the determined BC tumor properties help the physicians to estimate the best possible individualized therapy strategy. Thus, the establishment of guidelines for nearly every treatment step, including detection, diagnosis, operational removal, following individual therapy, subsequent interdisciplinary working of professionals (certified breast centers in FRG) enables the most appropriate and standardized procedures for affected patients.

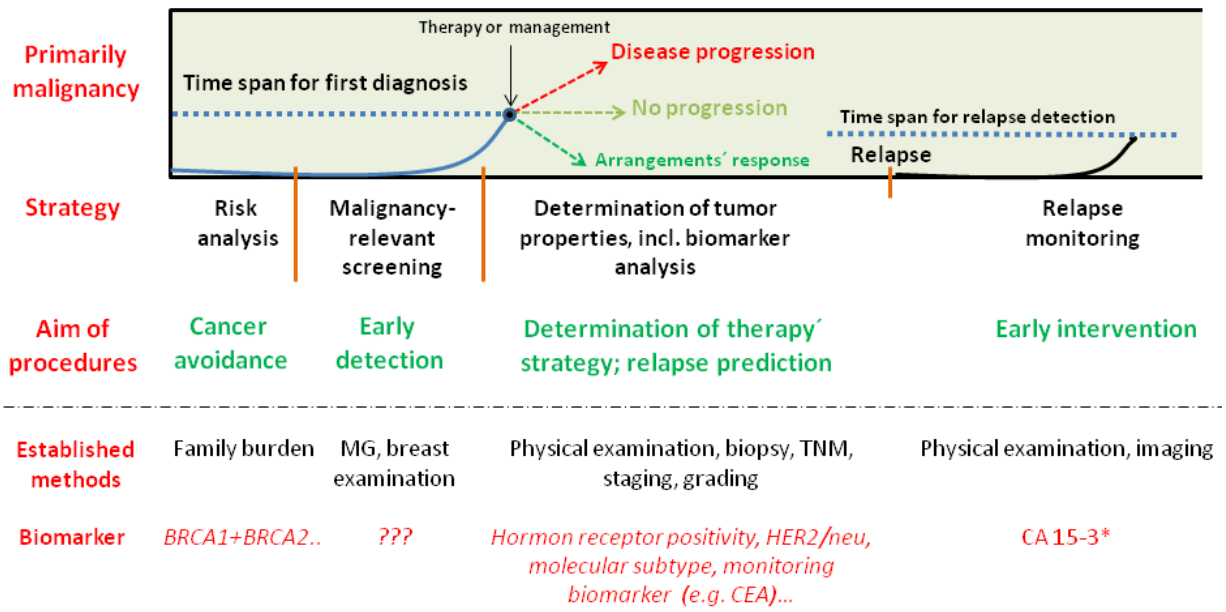


Figure 1.9: Potential usage possibilities of cancer biomarkers in the clinical routine over the dashed line and their application for BC management below. Three interrogation marks point the lack of biomarkers for its early detection. The labeled biomarker CA 15-3 points its use for the monitoring of predominantly metastasized BC in FRG. Changed and adapted from Ludwig and Weinstein[79].

As therapy options of BC is not the main point of this PhD thesis, as all samples of study participants were obtained prior to therapy, the details are not further enlightened.

1.3.2 Proteomics-based search for new breast cancer biomarkers

Since the early nineties several genomic and proteomics putative biomarkers were described for detecting BC and other cancer types[76,77,80,81]. Today, most proteomics-based studies are performed on serum samples, not least because of the standardized, well-described and easy-to use retrieval protocols. The main ideas to obtain biomarkers in serum are therefore obvious. Additionally, the development of mass spectrometry-based approaches for the comparison of peptide and protein levels enable the use of small serum amounts. Moreover, serum is easy to obtain by a minimally invasive procedure, making the sampling procedure more comfortable for the patients.

The study of Li *et al.* based on the protein profiling with SELDI-TOF MS revealed three proteins with the, which were de- or increased in the sera of BC patients[82]. In an another study, Mathelin *et al.* tried to verify these proteins in a different study population, resulting in the correct validation of two of them[83]. Also other investigations showed alterations of protein levels in serum of BC patients[84,85]. Additionally to the explorative profiling of serum proteins, also the targeted approach is widely used to examine the regulation of distinct protein with Western Blot and protein microarray platform[86-88]. Likewise, by use of MALDI–TOF/TOF MS several serum biomarkers were identified in a large cohort of studies and some of them were

validated in an independent study population[89-91]. All these investigations revealed protein alterations, but they also demonstrate the urgent need for further investigations[81,82,92,93].

One major critical point of all these investigations is the inconsistency between the investigators' results, particularly in serum or plasma. Several reports showed the alterations of protein levels and a clear impact of storage and handling procedures of serum samples. These fluctuations have led to the false assumption that the alterations are disease-specific, whereas the differences in protein levels occur only due to the different handling of samples[80,94,95]. Overall, the results convinced the investigators of identical and standardized proceeding of all patient's samples[96,97].

Different profiling studies were performed by different investigators, assuming that many of the protein deregulations would occur at the local place of disease emergence, the mamma. Therefore, several investigator groups examined the nipple aspirate fluid of BC patients and healthy probands, whereby differently regulated proteins were also detected[98-100]. Additionally, Li *et al.* found in- or decreased proteins in the ductal lavage fluid in BC patients[99].

Another interesting approach is the examination of distant body fluids for the detection of BC. Since the investigations of the serum, it is clear that BC, as a systemic disease with strong local component, is reflected by several proteomics alterations in more distant regions. Therefore, the search for the BC protein biomarkers was expanded to other body fluid like tear proteome[84,101]. The idea behind exploration of tear proteome is not new, as several studies show differences at protein level in patients with several eye diseases or diseases with an immune component[101-112] (see next section 1.4). Our recent MALDI-TOF/TOF MS investigation revealed at least 20 de- or increased proteins in tear fluid of BC patients[113].

The measurement of protein level changes is already established in the clinical routine. For example, the levels of the protein CA15-3 and CEA are measured to supervise the therapy of (metastasized) BC. However, both of them still do not provide high sensitivity and specificity to be used as an early detection tool for BC[114,115]. Also uPA and PAI levels in the sera of BC patients are used as a prognostic factor for non-metastatical BC[116] as mentioned in section 1.3.1.

Since large obtained serum profiling studies, for instance for several cancer types, many protein biomarker panels were proposed to discriminate a diseased person from the healthy group[86,117,118]. An ideal biomarker for the clinical routine should be easy to obtain, stable and reliable, meaning its absolute sensitivity and specificity. Nevertheless, the majority of the known biomarker-signatures are failed to find a way into clinical practice. Basically, the

biomarker panels could not discriminate diseased persons from healthy group with the appropriate sensitivity and specificity, making their use in the clinical routine as a universal population-based tool impossible[119]. Up to date, no protein biomarkers are available for the early detection of BC, leaving the MG as the gold standard for this purpose. Cancer in general is a multifactorial disease affecting the homeostasis of human organism on the transcriptome, proteome and metabolome levels. Therefore, different signal cascades and biological processes are changed in presence of tumor cells and it is not surprising that several molecules in affected patients are in- or decreased simultaneously. On the other side, it is crucial to separate non-specific changes on the proteomics level due to invasive procedures or non-specific concomitant symptoms.

Summing up, the search for new biomarkers as well as the validation of the already described proteins offers a promising possibility as a supportive tool for the established methods in the clinical routine and even for the earlier detection of BC prior to the clinically recognizable symptoms.

Not only altered levels of proteins in affected patients regarded much attention, but also autoantibodies which were reported in cancer patients of different entities including BC. Several investigations propose the potential of these autoantibodies to discriminate affected persons from the healthy controls. Some studies even describe the protective effect of these autoantibodies, especially of the IgM subtype. In general, antibodies are produced by important players of the human immune system: the plasma cells (differentiated B cells, see description of the plasma cells and immunoglobulins in additional chapter 8). Additionally, the immune system seems to possess enough potential to combat the cancer cells and do not let them to growth and spread over the organism, at least until certain events, whereas tumor cells escape the elimination. This supporting theory of cancer immunoediting is described in following section 1.4.1. Most of the described mechanisms are postulated for cancer in general, whereby no specific mechanisms for most cancer entities are currently committed.

1.4 Immune system and its role for cancer

1.4.1 Immunosurveillance: role of immune system in cancer

The term immunosurveillance[120] was recently broadened into *cancer immunoediting* by investigators around Gavin Dunn.[121,122]. The main point of it is the double-edge sword-role of the immune system. Thus, it seems not only to protect the host from cancer, but it can also promote tumor growth and spreading by selecting tumor cells with low immunogenicity. The tumor cells would therefore escape the attack and combating by the immune system. Generally, immunoediting can be divided into three phases: elimination, equilibrium and

escape[122,123]. The first phase includes the surveillance itself and comprises of recognition and elimination of aberrant cells by the innate and adaptive immune system. The innate system recognizes malignancies on behalf of changed composition of surrounding environment (structural aberrations and production of “alert” molecules, immunomodulatory cytokines or expression of certain surface stress signals, which activate adaptive immune response). Therefore, natural killer (NK) cells, together with T cells, dendritic cells (DC’s) and other components of adaptive immunity are alerted. The eradication of abnormal cells occurs according to the hypothesis only after all intrinsic tumor suppression mechanisms like repair or controlled apoptosis failed. The immunoediting is therefore an extrinsic tumor suppressor tool[123].

Sometimes, the aberrant cells are not eliminated by the immune system, but they are moved into dormancy by adaptive immunity, whereby the cells do not spread and metastasize[124]. On the other side, malignant cells may acquire new properties induced by genomic instability and respective mutations[125]. This circumstance impedes their recognition and subsequent elimination from the host. This second phase is called “equilibrium” and may last over several years, whereby tumor cells are occult and seem not to harm the host.

The transition into the last phase, tumor escaping, is possible via several different mechanisms, allowing tumor cells to overcome the protective function of the immune system. Presumably, these are cells with mostly new acquired genotype in the equilibrium phase. One of the strategies is the loss of tumor antigens, which make aberrant cells visible and vulnerable[126]. Additionally, tumor cells of different entities posses the molecule CD47 on the surface. This allows them to overcome the elimination by macrophages (inhibition of phagocytosis) and enables them to spread across the organism[127]. Another possibility is the induction of anti-apoptotic mechanisms by tumor cells, resulting in expression of anti-apoptotic transcription factors (*e.g. signal transducer and activator of transcription 3*, STAT3)[128]. Also the regulatory T cells or NK cells may be suppressed by tumor cells as they provide the control over the spread of tumor cells: *e.g.* via toll-like receptor 8 (TLR8) signaling or IL-13[129,130].

Summarized, tumor cells escape the combating via immune system and can therefore spread and metastasize across the organism. The major stages of cancer immunoediting are depicted in Figure 1.10.

Currently the concept of cancer editing is still highly controversial, for instance because also apparently healthy people with an intact immune system are diagnosed with cancer. Additionally, the power of the immunoediting is not well understood, *e.g.* the impact of elimination and equilibrium phases as standalone mechanisms or their combination.

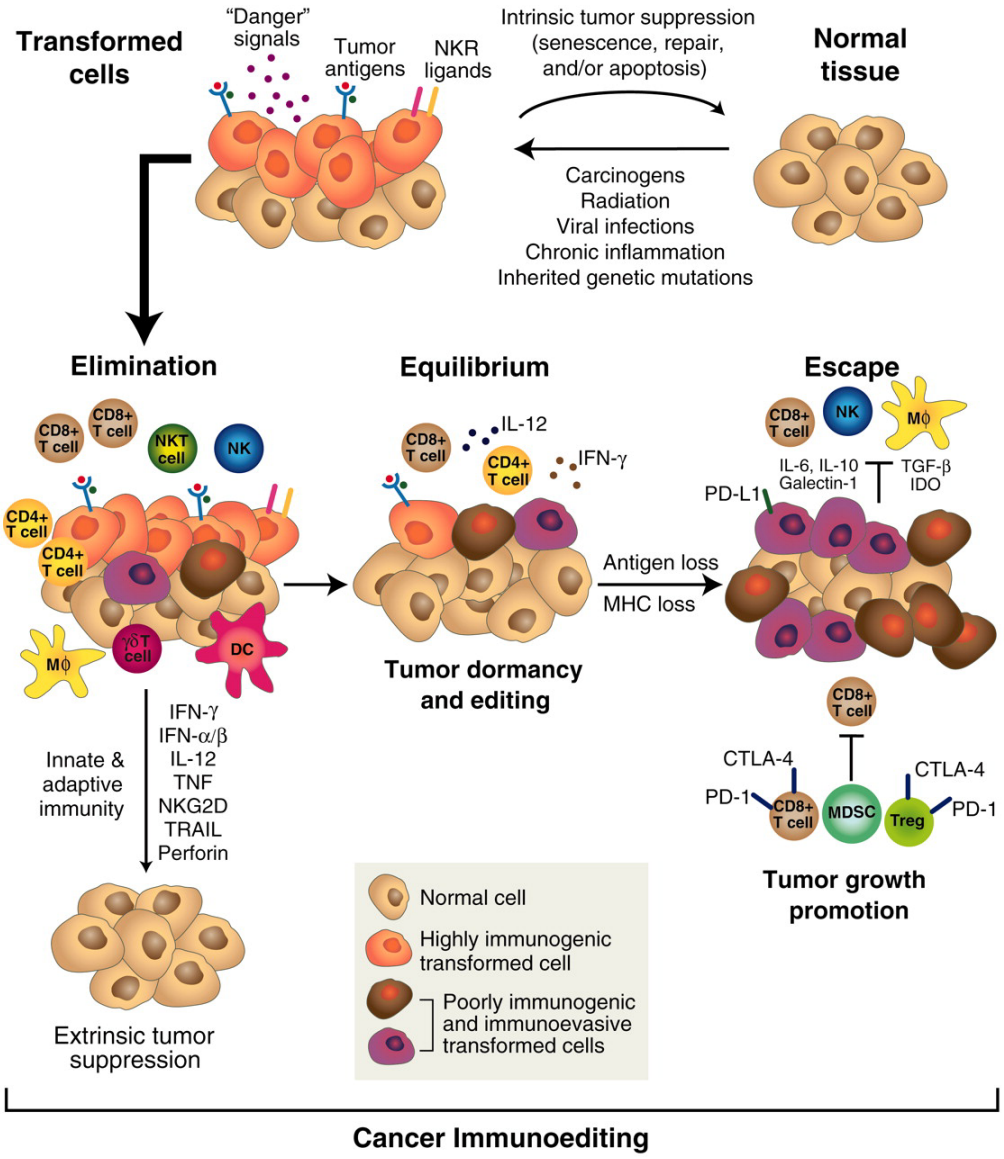


Figure 1.10: The concept of cancer immunoediting, divided in three phases[131]. Thus, it becomes active after the intrinsic tumor suppression mechanisms failed. The first phase includes the elimination of aberrant cells via innate and adaptive immunity. The survived cells may then fall into dormancy state, whereby they rest in the organism for a long period (Equilibrium). The third phase (escape) results in tumor growth due to new acquired properties of tumor cells over editing, enabling them to circumvent hindering mechanisms.

Thus, the protection by immune system seems to be insufficient in many cases and the aggressive tumor cells may somehow circumvent the safeguard mechanisms.

Nevertheless, an additional proof for cancer immunoediting hypothesis is the presence of tumor-infiltrating lymphocytes (TIL) in tumors of several entities, including breast and melanoma cancer[132-134]. Moreover, the presence of TIL's is strongly correlated with a better prognosis. A new study by Schmidt *et al.* even could show that the expression of Ig kappa C (IGKC) in node negative breast tumors could serve as a new prognostic factor. Thus, it provides nearly the same prognostic value as the previously identified B-cell metagen, which includes the expression pattern from over 60 B-cell-related genes[135,136].

1.4.2 Immunogenic tumor antigens: appearance and relevance for cancer

The first exploration of immunogenic tumor antigens (TA)'s derived from a melanoma cell line was reported in the late 70ies by Shiku *et al*. [137]. However, in the 50ies and 60ties, tumor-dependent immunity in rats was already described by RW Baldwin[138,139]. TA's are proteins expressed by the tumor cells, which may elicit an immune response in the host, promoting therefore several signal cascades and apparently also autoantibodies.

Up to date, no certain mechanisms for eliciting antibodies by the TA's are known. A big *in silico* study of Backes *et al*. in 2011 summarized several properties of immunogenic antigens in tumor diseases, normal conditions and, more known, autoimmune diseases (AID)'s, using several databases[140,141]. Thus, predominantly general properties for all immunogenic proteins were outlined: *e.g*. evolutionary conservation, coiled-coil and ELR motifs (glutamic acid (**GLU**)-leucin (**LEU**)-arginin- (**ARG**)), longer exon length, mutations, *etc.* Merely low specific features were described for immunogenic TA´s, except enrichment of different somatic mutations. Several modifications are already known to elicit the humoral autoantibody response: overexpression, posttranslational modifications, misfolding, wrong localization, *etc*[142-148]. Also the expression of fetal proteins in different cancer types in adults harbors the potential of humoral response to these antigens, as the ability for self-tolerance is developed postnatal, whereby these proteins are already absent (oncofetal proteins)[149]. Examples are cancer testis antigen NY-ESO-1 or carcinoembryogenic antigen (CEA)[144,150,151]. Some of TAA's belong to the oncogens like c-MYC or HER2/neu, tumor-suppressors like p53 or cell cycle regulating proteins like cyclin B, which is overexpressed and accumulated in cytoplasm of tumor cells instead being normally located in the nucleus and other compartments[152,153]. Again, Backes *et al.*, but also other investigators suggest that in case of tumor diseases, the immune system is stimulated by apoptosis and necrosis, whereby the release of tumor proteins provoke the emergence of autoantibodies[141,154,155]. Most of the known TA's are involved in all important signal cascades of growth, development and apoptosis, and are thought to be deeply involved in tumorigenesis[141,156]. Several tumors types in BC, melanoma, or prostate cancer express these proteins and seem to trigger the humoral response in the host, resulting in cohorts of autoantibodies detected in sera or even in distant fluids like urine as shown by Yi *et al.*[157]. One of the best analyzed proteins in connection with several cancer types is the check point kinase p53 (Tumor protein p53) and p53-autoantibodies. The protein p53 regulates the transition from G1 (cell growth) to S (mitosis) phase in the cell cycle. Indeed, if the DNA is damaged in any way, the p53 elongates the G1 phase, giving the ability to repair the defects prior to replication to prevent spreading of aberrant cells. If the DNA cannot be reconstituted, the p53 triggers the apoptosis signal cascades[158]. In many cancer types *e.g.* BC, OC, but also in other non-malignant diseases like AID's, the protein p53

undergoes severe alterations. This leads to disruption of DNA repair mechanisms followed by growth and spreading of malignant cells and subsequently to tumor formations[142,158,159]. Probably because of the *non-sense* mutations, leading in some cases to the accumulation of p53 and increased half-life of the protein, the humoral response is triggered and anti-p53 antibodies are circulating in blood[143]. The analysis of the p53 autoantibodies showed that the immunogenic epitopes belong to glycosylated N- and C-termini of the protein, whereas the mutations in the p53 are occurring in the middle sequence part[160]. Mutations in p53 and anti-p53 antibodies are additionally correlated with poor prognosis in several types of cancer, including BC[143,159].

The immunogenic impact of TA's offers a very intriguing approach for an antigen-specific tumor vaccination as some of cancer types are mediated through bacteria or viruses[161]. A summary of biological agents, which are related to the development of the annotated cancer types, is additionally provided in Repository table 6[162]. Thus, several trials with tumor vaccines are described and the investigations are promising as some cancer preventing vaccines even found the way from the bench to the clinical routine. The main idea of the preventing vaccines is to immunize the host with tumor proteins and trigger the immune system to combat them with the help of the T cells and to induce the memory cells for producing the specific antibodies against them. Examples are vaccines against human papilloma virus (HPV)-mediated cervical cancer (Gardasil®, Merck & Company) and Cervarix®, GlaxoSmithKline). Another vaccine approved for the clinical use is directed against hepatitis B virus (HBV) as the chronic infection with HBV may cause liver cancer[163]. Another immunization approach is the use of the cancer treatment vaccine. The idea behind it is the specific targeting and combating cancer cells under assumption that the immune system does not recognize aberrant cells, as they often do not express "danger signals" leading normally to the elimination. The cancer treatment vaccine should "wake up" the immune system and release danger signals to combat malignant cells. Up to date, only one cancer treatment vaccine approved by FDA is used in USA: sipuleucel-T against the metastatic prostate cancer[164].

Further cancer treatment vaccines for several types of cancer in USA are under clinical trials with currently over 30 treatment vaccines for BC[165]. The knowledge of BC-specific TA's might provide new insights into cancer preventing and also the design of treatment vaccines remains a very intriguing albeit also challenging approach.

Overall, the facts that immunogenic TA's are related to several ubiquitous functions and their malformations due to mutations, accumulations or misfolding, trigger the humoral response, let us assume that the arising antibodies are highlighting the abnormality of the signal

cascades, alternating during cancer development. The counterparts of TA's, their respective autoantibodies, especially in BC, are highlighted in the next section.

1.4.3 Autoantibodies, the humoral response of immune system in cancer

In the past decades till now, several publications reported the presence of different antibodies in serum of cancer patients. In particular, the immune system seems to react in the presence of cancer cells not only with T-cell response, which – in best case- eliminates abnormal cells with the help of cytotoxic T cells. This process occurs after presentation of antigen fragments by antigen presenting cells (APC) via uptake, fragmentation and transport to the surface and their *major histocompatibility complexes* (MHC) I or II. Nevertheless, the other players of immune response, the plasma cells, produce antibodies with support of T helper cells and belong to the humoral response of immune system (details provided in section 8.1).

The prevalence of antibodies might be more complicated for tumor diseases, as the classification of immunogenic tumor antigens is of multiple natures (as mentioned in previous section 1.4.2). This idea is supported by the findings that tumor cells may stimulate the immune system through apoptosis, inflammation, necrosis or overexpression of cell surface molecules (*e.g.* via HER2/neu overexpression)[151,152]. The function of these antibodies is however still not well understood. Overall, the immunogenicity of tumors seems to be a common phenomenon leading to the rise of cohorts of autoantibodies which are circulating in blood and other body fluids. For BC in general, different autoantibodies were also described in the past. Thus, autoantibodies against already mentioned proteins HER2/neu and further oncogenes like DJ-1, but also p53, NY-ESO-1 and heat shock proteins were detected in serum of BC patients[166-171]. Summarized, the respective, mostly intracellular tumor proteins are implicated in all important signal pathways of survival, development and apoptosis of cells and highlight aberrant processes during tumor outgrowth[141,154,171,172]. These autoantibodies are however also observed in many other cancer entities, and it makes their use as a specific tool to detect BC exclusively, questionable. Most of the studies suggest on the other side, that a combination of different autoantibody levels significantly increase the specificity and sensitivity during the discrimination of diseased cohort from the controls[173-176]. Consequently, these antibodies can eventually serve as a "hallmark" for malignancies. Even more, the detection of antibodies prior to the clinical manifestation of cancer or in the early stages makes them to a potential target of interest[154,166]. Several opportunities arise therefore with diagnosis or monitoring of the humoral immune response. Additionally, antibodies are well-studied molecules. This fact enables simple and faster handling like isolation, purifying, *etc,* and makes advanced studies more reliable. Moreover, antibodies are stable molecules, which are not degraded in normal pathways and the half-life of at least IgG and IgM isotypes

accounts for a minimum of five days[171,177]. Their appearance in serum makes them the ideal target for the screening, as serum retrieval is minimally invasive. Additionally, fast and standardized methods have been used for many years in the clinical routine. Another important point is the higher quantity of antibodies than TA's, as the immune system multiplies the humoral response to the antigens.

The quest of the autoantibody emergence and their function is not easy. Some investigators describe them as a part of natural immunity, at least the autoantibodies of the IgM isotype. As an example, by use of the human hybridoma technique[178], several IgM autoantibodies were isolated from serum of a colon, lung and pancreatic cancer patients[179]. Hereby, these IgM antibodies possessed only few or no mutations at all, which is a typical property of germ-line coded structures. Furthermore, they derived from the $CD5^+$ B cells naturally occurring in the host[180,181]. In another study, the isolation of IgM autoantibody SC-1 showed its apoptosis ability of gastric cancer cells[182]. Additionally, whole IgM's from healthy donors were isolated in the same approach, whereas the immunoglobulins also showed strong reaction with tumor cells but not with normal cells. As a conclusion, the authors stated that these antibodies, together with the innate immunity (including neutrophils, NK cells, DC's, *etc.*), are apparently involved in tumor immunosurveillance and have a protective impact, as they cross-linked the present TA's. The protective potential of autoantibodies was also reviewed by Shoenfeld and Toubi [183]. Likewise, the presence of IgG autoantibodies in serum of cancer patients is well described in the past, whereby most of investigations connect their presence with immunogenic TA's. An interesting study investigated the relationship between the presence of IgG and IgM autoantibodies and the outcome of cancer patients. Thus, the presence of anti-mucin antibodies of both isotypes in serum of breast-cancer patients were positively correlated with the disease-specific-survival (DSS) in stage 1 and 2[184].

Most of the investigative studies concentrated on the derive of so-called (auto)antibody signatures in same way like protein biomarkers, whereas a panel of putative immunogenic TA's from native tumor samples, cell culture lysates or commercially available respective single proteins is used to capture the respective autoantibodies, mostly IgG's, from sera of cancer patients and healthy probands (Immunoproteomics)[185,186].

Some analyses of cancer-driven antibodies' response showed the correlation of antibodies with the stage of cancer development[88,187-189]. Even more, likewise in AID's, autoantibodies, which are associated with the humoral response to TA's, were observed prior to the clinical diagnosis and manifestation of cancer, as shown for hepatocellular carcinoma, lung and prostate cancer as well as for BC[190-193]. These findings could enable the use of antibodies for the classification or staging, but also for the monitoring and eventually prognosis of the malignant neoplasms, *etc*. Nevertheless, it is also should be kept in mind, that antibodies to

self-antigens persist not only in the diseased patients but also in healthy subjects. Another reason for taking care and not over-interpreting the results from screening studies is the overlapping appearance of antibodies not only in different cancer types but also in AID's (*e.g.* p53)[194]. Moreover, not every patient seems to develop autoantibodies against TA's (detected in approx. 30 % of cancer patients by *e.g.* Casiano *et al.*[185]). It is therefore obligate to ensure the specificity of putative biomarkers. For that reason, the discrimination power of the antibodies should be confirmed by individual screening of studies' participants and independent validation in another study cohort (as described in chapter 1.3).

2 Objectives

BC is the most common cancer type in women worldwide. The important requirement for the successful treatment of BC is its early discovery. Despite of recent improvements in detection and treatment options, many of the female patients die from the metastatic recurrence. Up to date, the mammography remains the only available method for the early detection of BC.

The previously discovered panels of molecular biomarkers were not able to detect BC with the clinically applicable sensitivity and specificity. Furthermore, only few validation studies with the screening of individuals in an independent population were described. The identification of new biomarkers is therefore of urgent need. Taken together, the search for the most accurate biomarker signature for early detection of BC remains a great challenge but also a great opportunity to get a deeper insight in the physiological processes during cancer emergence and development.

Proteomics offers the possibility for the profiling of protein distribution in different compartments of the organism, resulting in very dynamic and constantly changing molecule patterns due to internal and external impacts. Several studies described the high number of differences in protein levels of BC patients in comparison with the CTRL subjects. Some protein tumor markers are already used in the clinical routine. *E.g.* serum titers of proteins CEA and CA15-3 are helpful prognostic biomarkers for the therapy monitoring of (metastasized) BC. Also the levels of uPA and PAI1 proteases are estimated as further prognostic biomarkers in non-metastatical BC. The aim of the current project is the proteomics-driven approach for detecting new biomarkers of primary BC in the serum of affected study population. Furthermore, the ability of alternatively regulated molecules or their combination to discriminate BC patients from the healthy cohort according to the specificity and the sensitivity should be examined by an individual profiling of age-matched individuals. Additionally, not only the detection of BC in the early stage is a very important goal of biomarker research. Indeed, the deeper proteomics-based characterization of tumor properties (BC subgroups) may lead to the development of new prognostic and predictive factors, together with the already established tumor classification possibilities.

We have shown in the previous studies decreased or increased levels of protein fragments by explorative SELDI-TOF MS and MALDI-TOF/TOF MS-driven approach not only in serum fluid but also in tear proteome of BC patients via antibody microarray platform[87,91,113]. Another study of tear protein profiling via SELDI-TOF MS revealed more differences in levels of tear proteins and their fragments in BC patients[101]. As the result, the diseased samples were

discriminated by a panel of the putative protein biomarkers from the CTRL subjects with the high specificity and sensitivity. Especially putative biomarkers from the serum and tear protein profiling via MS strategy were of primarily interest for the verification of their classification potential. The most promising candidates from these investigations should be used for the comparison of their levels in individual BC and CTRL sera via the antibody-microarray platform for estimation of their level differences and the ability to discriminate both study groups. This strategy allows simultaneous, high-throughut profiling of selected targets in individuals in a miniaturized format. Even more, as a targeted antibody-driven approach, it enables the detection of analytes' overall level in the complex biological samples.

Besides the detected protein differences in different body fluids of BC patients, many investigators reported circulating autoantibodies in serum of diseased subjects. This phenomenon is mostly known to be present in autoimmune diseases (see section 1.4.3). Thus, in- and decreased levels of autoantibodies were reported also in several cancer entities. Mostly, the IgG isotype of the autoantibodies is described, whereas some of the research groups concentrate on the IgM antibodies. This isotype seems rather to belong to the innate immunity. The origin and the function of these antibodies are currently not well understood. The autoantibodies are often detected via potentially immunogenic TA's, which are modified in different ways (see section 1.4.2 for details) and expressed on the surface of tumor cells or released from them due to secretion, apoptosis, *etc*. In the past few years, several new autoantibodies were identified in BC patients, which could discriminate diseased individuals from healthy subjects in preliminary experimental studies[171,195,196]. The new TA's should be identified in the second part of the current project via different *de novo* strategies. For the explorative approach, the isolation of potential immunogenic TA's from complex tumor protein extract with their subsequent identification was chosen. Additionally, the two-dimensional separation of tumor proteins and subsequent Western immunoblotting with autoantibody-containing serum samples should be performed for further detection of the TA's. MALDI-TOF/TOF MS with subsequent database search and protein assignment was chosen for the identification of the relevant TA's. Finally, the individual serum profiling of study participants is aspired with the highly precise protein microarray platform. This strategy represents the counterpart of antibody microarrays described above, whereby the respective autoantibodies are incubated with commercially purchased proteins (respective TA's). The most promising protein candidates from both *de novo* strategies will be selected for detection of respective autoantibodies and estimation of their level differences in individual serum samples of BC patients and CTRL probands.

3 Materials and Methods

3.1 Sample retrieval and collecting

Serum samples were collected prior to surgery and neoadjuvant treatment. Tumor tissue was retrieved during the surgery removal. All patient-related-samples and data were treated according to the Declaration of Helsinki[197].

3.1.1 Serum retrieval

Blood samples were collected using the serum tubes with clot activator (Serum-Monovette®). The samples were allowed to clot by room temperature and then centrifuged by 3220xg at 4°C. The supernatant (serum fraction) was immediately transferred into 2 ml tubes and frozen by -80°C for further analyses.

3.1.2 Tissue retrieval

Tumor samples were obtained in the Department of Gynecology and Obstetrics of the Universitaetsmedizin Mainz. The excised tissues were immediately shock-frozen in liquid nitrogen and then stored in the -80°C freezer.

3.2 Tissue processing

For the processing of whole protein extracts from breast carcinoma, the tissue was crushed and homogenized in liquid nitrogen with a pre-cooled porcelain pestle in a mortar. The tissue powder was transferred into pre-cooled 2 ml tubes (quarter-filled tube). The cells were lysed in Tris (hydroxymethyl)-aminomethan (TRIS)-borate buffer (TBS) containing 0.1 % n-Dodecyl-beta-D-maltoside and 1 µl tissue proteinase inhibitor mix. The lysates were incubated by 4°C for one hour and then centrifuged three times by 10000xg and 4°C for 20 minutes (mins) to spin down cell debris. The supernatants (containing proteins) were merged and transferred into fresh tubes.

3.3 Determination of protein concentration

Protein amounts in samples were estimated with the BCA protein Assay Kit according to the provided protocol. The measurement of the protein concentration is based on the colorimetric detection of reduced copper ions (from Cu^{2+} to Cu^{+}) by proteins in complex with bicinchoninic acid in an alkaline medium[198]. Protein amounts were calculated with help of the Multiscan Ascent photometer at 570 nm. Each protein sample was measured in duplicates and the final concentration of proteins was estimated by calculation of the mean value.

3.4 Protein precipitation

The protein precipitation was performed for additional removal of unspecific component after tissue lysis. Thus, 3x Vol of precooled acetone including 10% of trichloroacetic acid (TCA) was added to 1x Vol of samples with the desired amount of proteins. The precipitation lasted for 16 hours, mainly overnight by -80°C. The samples were then centrifuged by 10000xg for 30 mins, the supernatant was discarded and the pellets were dried by 4°C in a thermostatically controlled incubator for 30 mins. Finally, proteins were dissolved in appropriate volume of high pressure liquid chromatography (HPLC) grade water or phosphate buffer saline (PBS) in an ultrasonic water bath for 15 mins and frozen by -80°C prior to further analysis.

3.5 One-dimensional sodium dodecylsulfate-gelelectrophoresis (1D SDS-PAGE)

For the one-dimensional separation of protein extracts due to their molecular weight an Xcell SureLock™ Mini cell electrophoresis chamber and sodium-dodecylsulfate-polycrylamide gelelectrophoresis procedure (SDS-PAGE) were used. Prior to the electrophoresis procedure, protein samples were denatured and reduced with 4x NuPAGE® LDS Sample Buffer and 10x NuPAGE® Sample Reducing Agent by 90°C for 10 minutes. The samples were then chilled on ice and transferred into NuPAGE Novex 12% Bis-Tris gel lanes. Due to the molecular weight range of interest, either the NuPAGE® MOPS (3-(N-morpholino)propanesulfonic acid) SDS running buffer for medium-and large-sized proteins or the NuPAGE® MES 2-(N-morpholino)ethanesulfonic acid) SDS running buffer for small proteins was used. The SDS-PAGE was performed by 130V and 4°C. In the final step, the proteins were fixed and stained with Colloidal Blue Staining Kit according to the manufacturer's protocol and destained with H_2O_{bidest} on the next day for at least two hours under constant shaking.

3.6 Two-dimensional sodium dodecylsulfate-gelelectrophoresis (2D SDS-PAGE)

The isoelectric focusing (IEF) prior to SDS-PAGE was used for a better separation of complex protein samples. For that purpose, approximately 250 µg of the protein extract were precipitated and resuspended in 120 µl denaturation buffer (DB). Prior to IEF, the denatured sample was charged with 1 µl IPG buffer of suitable pH range and placed into 7cm strip holder with the appropriate IPG strip on the top. The sample was allowed to soak into the gel matrix of the IPG strip for 2 hours by 4°C. The steps in the IEF unit are summarized in Table 3-1.

Table 3-1: General settings of the performed IEF Additionally, 20°C for the surface temperature and amperage of 50 µA per strip were set.

Step	Voltage in V	Duration in hours
Rehydration	0	2
Step-and-Hold	8000	2
Gradient	8000	2
Step-and-Hold	4000	2
Gradient	4000	0.5
Step-and-Hold	1000	1
Gradient	1000	0.5
Step-and-Hold	500	1
Gradient	500	1
Step-and-Hold	20	12

Afterwards, the strips were briefly washed with H_2O_{bidest} and then incubated in 5 ml renaturation buffer (RB) including 12.5 mg DTT for 10 mins. The strip was then placed in 5 ml RB including 31.25 mg iodacetamid in the dark. These incubation steps denaturate and alkylate proteins to ensure the optimal separation via following 1D SDS-PAGE (see section 3.5). The strip was finally placed on the top of the NuPAGE Novex 12% Bis-Tris gel lane and covered with preheated liquid 1% agarose. After short cooling step, the SDS-PAGE was started.

3.7 SDS-PAGE-based tryptic in-gel digestion of proteins

Prior to protein digestion after 1D/2D SDS-PAGE, the lanes were divided into 16 bands (1D) or the spots directly excised (2D), and the proteins were digested with the endopeptidase trypsin according to the modified digestion protocol from Shevchenko *et al*[199]. Briefly, the gel bands were crushed with a scalpel (1D) and the 2D gel spots were directly washed with 50 mM ammoniumbicarbonate (ABC). Afterwards, the gel pieces were dehydrated with 100% acetonitrile (ACN) and then vacuum-dried. The procedure cycle was repeated twice and then the samples were coated with 75 ng trypsin solution for 45 minutes on ice for soaking into gel pieces. Then, the proteins were digested overnight by 37°C. For the additional extraction of

peptides the gel pieces were covered with 5% formic acid/50% ACN solution twice for 20 minutes. The peptides were eluted by 100% ACN under constant shaking. The supernatants of each sample were merged and vacuum-dried. Depending on the following analyses, samples were reconstituted in 0.1% trifluoroacetic acid (TFA) and then either measured directly with MALDI TOF-TOF MS or first purified and fractionated via hydrophobic interaction–based ZipTip® platform (Millipore, Billerica, USA) according to the manufacturer' protocol. Briefly, these tips contain silica particles with hydrophobic C18 alkyl chains, which bind peptides from the solubilized samples. After capturing, the tips are washed with 0.1% (TFA) and the peptides are eluted with ascending acetonitrile (ACN) concentration[200].

3.8 Mass spectrometry

The identification of 2D spots was performed via stepwise elution of digested peptides (10%, 15%, 20%, 25%, 30%, 40% and 50% ACN) with the ZipTip®-based desalting system in section 3.7. The eluted peptides (3 µl) were directly spotted on the MALDI TOF/TOF polished steel target and coated by the 3 µl crystallization matrix (20 mg cinnamon acid/50% acetonitrile/2% trifluroacetic acid). All samples were measured head-to-head to avoid protein degradation and measurement's fluctuations in the MALDI mass spectrometer. For the MS spectra a laser intensity of 30% and 300 laser shots were used. For MS/MS measurement and the appropriate parent peaks, over 300 laser shots were first accumulated and 500 shots were used for enhanced peptide fragmentation. The peak detection was performed with help of the peptide standard calibrant mix. All spectra were normalized according to the total ion count[201] and visualized with BioTools™ version 3.1 as well as flexAnalysis version 2.4 software.

3.9 Protein identification and subsequent protein network analysis

After the measurement of digested proteins, all MS and MS/MS spectra in **.xml* (extensible markup language) format from one experimental run were imported into the *in-house* developed software Proteomics Pipeline Mainz (P^2M) and subsequently combined in one **mgf* file (mascot generic format). Afterwards, the file was submitted over the implemented search tool directly to the Mascot server. Following general parameters were used for the identification of the proteins: *carbamidomethyl* as a global modification due to iodide-based alkylation used in the digestion of proteins (see section 3.7 for details) and *oxidation* (M) as a variable modification with a MS tolerance of 100 ppm and MS/MS tolerance of 0.8. Additionally, the UniProtKB/Swiss-Prot database[202] for the protein identification as well as the type of mass spectrometer (MALDI TOF/TOF), the used endopeptidase (trypsin) and number of miscleavage sites (1) were chosen over the *html*-based (hypertext markup language) request. These additional parameters enable more precise matching with the virtually stored

fragment patterns in the protein databases with regard of certain variability over the experimental steps. The Mascot search tool achieves the identification of proteins over different probability based algorithms, of which the MOWSE (**Mo**lecular **We**ight **Se**arch) scoring system was used for the significance threshold[203].

Furthermore, the online software platforms were used for the estimation of protein interactions whether for submitted protein set only or for their implication in biological networks. Thus, the STRING software (Search Tool for the Retrieval of Interacting Genes/Proteins) was chosen for the simplified overview of the resulting protein relationships. The resulting interaction strength of the submitted proteins is based on the experimental genomic data and extensive literature research of more than 5 Mio. proteins (as stated at September 06, 2012)[204]. The stronger thickness of the connection lines indicates higher combined score according to the following prediction options: co-expression of genes, co-occurrence, gene fusion, database information and text mining as well as experimental junction. The identified proteins were submitted online for the analysis, whereas the appropriate gene names were identified according to the UniProtKB/Swiss-Prot[205] declaration.

The online platform IPA® (http://www.ingenuity.com) was chosen for more extensive estimation of protein involvement of the identified proteins in different pathways and their possible common interaction partners[206]. The gene names of the identified proteins were submitted in the same way as described above. In case of non-recognizing of gene names by the software the appropriate accession number from the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein) was alternatively submitted. The core analysis was performed after the successful submission of proteins. Thus, the most relevant information according to the implementation of identified peptides in canonical pathways or biological networks as well as the relevant functions were computated and obtained as output data, which were exported in **pdf* (portable device format). Furthermore, the proteins of interest were subsequently edited in the integrated tool PathFinder, whereby the links and global associations of proteins are shown more precisely.

3.10 Removal of IgG antibodies from serum or tissue samples

500 µl of individual or pooled serum samples were used for the depletion procedure. Also for the removal of IgG antibodies from tumor protein extracts the same sample volume was utilized. Thus, the serum/sera of interest were applied on the IgG depletion column and the depletion steps were performed according to the commercial' manual. The bound antibodies were then eluted in three fractions and pooled together after neutralization with the supplied buffer. The columns were reconstituted as described by the manual and stored by 4°C until

further experiments. Repository table 5 provides the composition of BC and CTRL serum pools used for the immunoprecipitation and Western immunoblotting (see next sections).

3.11 Immunoprecipitation

3.11.1 Tumor protein capturing via protein G beads-based platform

The protein G bead platform was used for the *de novo* identification of potentially immunogenic TA's. The advantage of these modified beads is the easy handling of sepharose beads with magnetic properties and subsequently reduced loss of material and achievement of high analyte recovery. All washing and eluting steps were performed at 4°C according to the supplied instructions. For the removal of buffers during all incubation steps, the magnetic rack DynaMag^{TM} -2 was used to ensure the optimal handling.

The IgG antibodies were removed from the tumor protein extract prior to capturing of autoantibodies; as even small tumors develop own blood vessels to ensure the specific reaction with immunoglobulins from the sera of study participants (see previous section 3.10). Three 5x3 cm nitrocellulose membranes were incubated overnight with 10 mg of tumor protein extract under constant rotation in 50 ml tubes. Then the isolated IgG's from mixed serum samples were transferred on the membranes. The autoantibodies should therefore bind to the appropriate antigens. Afterwards, the autoantibodies were eluted, bound to the protein G beads and cross-linked with 50 mM Dimethylpimelidate/triethanolamine for the subsequent elution of TA's only. In the next step they were incubated with fresh tumor protein extract. The bound proteins were eluted on the next day from the IgG's in 500 µl of 100 mM Glycine buffer (pH 2.7) in three fractions, subsequently neutralized by 300 µl TRIS buffer and finally vacuum-dried. The described workflow is summarized in next Figure 3.1.

The strategy of immunoprecipitation was also performed without previos enrichment of autoantibodies on the membranes with TA's. Thus, the isolated IgG antibodies from mixed serum samples were isolated and then directly captured to 500 µl of magnetical protein G sepharose beads overnight. The elution procedures as well as all washing steps were identical with the workflow described above.

Afterwards, the samples were reconstituted in 10 µl HPLC-grade water and separated per 1D-SDS-PAGE. The stained proteins bands were first excised from the gel, then digested and identified with MS (see sections 3.7 and 3.8).

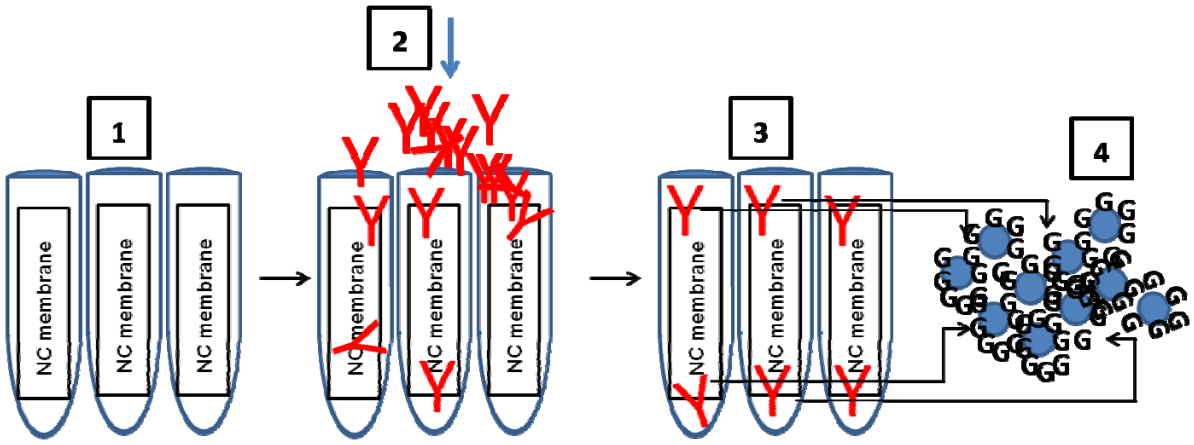


Figure 3.1: First chosen strategy for detection of immunogenic tumor antigens with previous enrichment of appropriate autoantibodies. Briefly, in the first step (1) three nitrocellulose membranes are covered with extracted liquid tumor antigens under constant shaking. The isolated IgG' from the pooled serum specimen (n=20) is added to the tumor proteins and incubated under rotation (2). After that the bound autoantibodies are eluted from the respective tumor proteins (3) and subsequently coupled to the protein G beads (4) for further incubation with fresh tumor protein extract (not shown in the sketch). Own sketch of the author.

3.12 Western immunoblotting

Protein extracts were separated per 2D SDS-PAGE (see chapter 3.6). At least two identical protein separations via IEF were performed simultaneously for the subsequent analysis of the sought tumor proteins. Likewise, the secondary SDS-PAGE separation of blotting and analytic gel was accomplished in the same electrophoretic chamber to facilitate the non-ambiguous mapping of the sought protein spot in the analytical gel. Also the immunoblotting of two gels for the incubation of tumor antigens with serum pools from BC and CTRL groups were run simultaneously in one wet blotting chamber. Afterwards, the blotting gel was rinsed with Transfer Buffer (Towbin Buffer[207], TB). A Mini Trans-Blot wet chamber was used for the subsequent protein blotting. Proteins were transferred onto nitrocellulose membrane after 1 hour blotting procedure by 100 V. The transfer was verified by Ponceau S staining. The membranes were then destained with H_2O_{bidest} for 15 minutes under constant shaking and blocked with 4% non-fat dry milk/TBS (NFDM) for 1 hour to mask the non-specific binding sites. Thereafter, the membranes were washed with 0.5% Tween20/TBS washing buffer (WB) for three times and incubated with serum samples overnight (1:20 dilution in TBS buffer). On the next day after washing with WB, the blots were incubated with the horseradish peroxidase-coupled anti-IgG antibody (1:750 dilution). Immunoreactions were visualized using 4-chloronaphthol staining/ 2%peroxide (H_2O_2)[208]. Briefly, 12 µl of H_2O_2 were dissolved in 20 ml TBS and merged with 8 ml methanol (MeOH) containing 12 mg 4-chloronaphthol. The membranes were coated with the staining solution for 20 minutes in the dark and then washed with H_2O_{bidest}. Images were scanned with a high-resolution capable scanner, and the membranes were stored at 4°C.

3.13 Microarray Platform

An *in-house* established microarray platform was used for the determination of the protein and autoantibody levels in serum samples. Antigens or antibodies of interest were spotted with a non-contact piezzo-driven microarray spotter onto nitrocellulose-coated pads on glass slides. Each analyte was spotted in triplicates to achieve the better statistical data mining. The fixed proteins on the glass slides were stored by 4°C in the dark prior to incubation steps. Each glass slide contained 16 nitrocellulose pads. It was therefore suitable for ssimultaneous creening of 16 individual samples. The slides were clamped into a slide holder with 16 well incubation chambers over them for all following incubation steps.

3.13.1 Selection of proteins for antibody microarray set

The semiquantitative comparison of several protein levels in cancer serum and healthy controls was performed via antibody-based microarray approach. Previous profiling studies according intact tear and serum protein levels in the experimental ophthalmology via SELDI-TOF and MALDI TOF/TOF MS showed decreased as well as increased proteins in the individual samples of BC patients and CTRL subjects[87,91,101,113]. The molecular weight of non-identified proteins or subunits was compared in an extensive paper research in other laboratory studies and the PubMed database, whereas this protein masses were already assigned to the corresponding proteins. The proteins inter-alpha-trypsin heavy chain inhibitor member 4 (ITIH4) and apolipoprotein CI (APOC1) were in- and decreased in serum specimens of BC cohort, respectively. proline-rich protein 4 (PROL4) was found in lower and secretoglobin family 1D member 1 (SG1D1) in higher amount In tear fluid of BC samples (unpublished data).

Regarding the results from MALDI TOF/TOF MS-based protein profiling in tear fluid of BC patients and CTRL probands, at least 20 proteins were found de- or increased in the study[113]. The most promising proteins like microtubule-associated tumor suppressor 1 (MTUS), Calgranulin A (S10A8), Calgranulin B (S10A9), Transferrin receptor protein 1 (TFR1) and Cystatin SA (CYTT) were also chosen for the verification in serum samples in the present study. MTUS1, TFR1 and CYTT were increased in the pooled BC tear fluid, whereas the calgranulin family proteins were decreased. However, the purchased anti CYTT antibody seemed also to cross-react with other cystatin family member, namely Cystatin S (CYTS) and Cystatin SN (CYTN). Therefore, it was excluded from the main analyses. Additionally to the mentioned proteins, also alpha-2-Macroglobulin (A2MG) was added to the protein list of interest for the main exploration study. The high abundant serum protease inhibitor A2MG binds several cytokines and growth factors[209] and is also involved in IL-6-mediated signaling events as shown in the Pathway Interaction Database[210]. We showed

previously in another antibody microarray study of immune system players a higher level of IL-6 in serum of BC patients[87]. For the best possible realization of the analysis platform, the appropriate, mostly polyclonal (except anti-MTUS and anti-CYTT antibodies) antibodies against these proteins, were commercially obtained.

3.13.2 Antibody-based microarray analysis

Prior to incubation steps with prespotted antibodies, serum proteins were precipitated according to chapter 3.4 and then dissolved in HPLC-grade water. 5 µg of each sample were used for the incubation of serum proteins on the antibody arrays (see section 3.3). Serum proteins were labeled with 0,3 µl DyLight649 NHS Ester prior to incubation on the microarray slides for 1 hour in the dark at room temperature. The unbound fluorescent dye was blocked with 100 µl Glycine (5 mg/ml) for one additional hour. All subarrays were blocked with 4% non-fat dry milk NFDM in PBS for 1 hour at 4°C. After blocking step, the slides were washed three times for 10 mins with 0.5% Tween20xPBS washing solution (WS) and then incubated with 100 µl labeled serum proteins under constant shaking by 4°C overnight. The workflow is additionally shown in Figure 3.2.

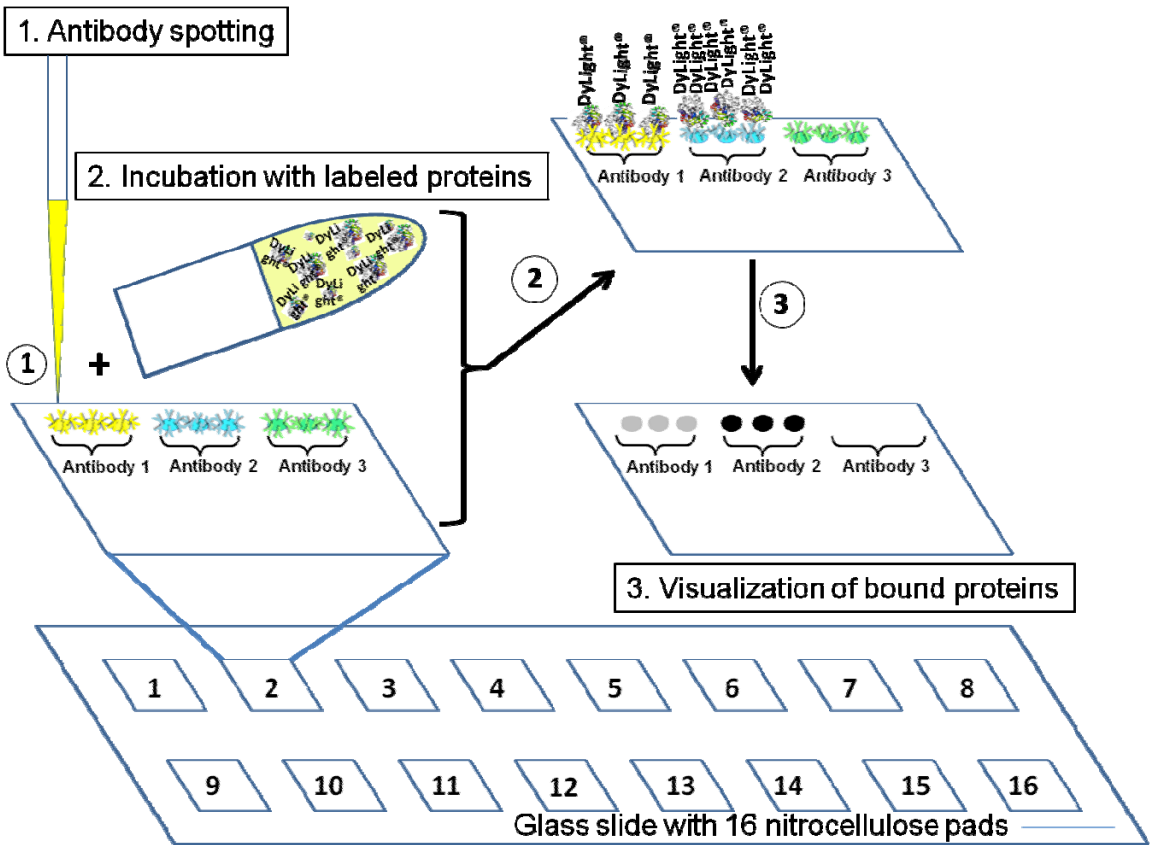


Figure 3.2: Workflow of the antibody microarray procedure. Briefly, the antibodies were spotted in triplets (1) onto nitrocellulose pads (subarrays). Each 5 µg of previously precipitated serum samples was labeled with the fluorescent dye and afterwards incubated with the antibodies (2). The slides were scanned with the microarray scanner and the bound amounts of the appropriate proteins on the respective antibodies were visualized (3). Own sketch of the author.

In parallel, negative controls were also processed, meaning the proceeding of same experimental steps without usage of serum or labeling dye. The slides were washed again on the next day once with WS, two additional times with HPLC grade H_2O and then vacuum-dried.

3.13.3 Antigen-based (reversed-phase) microarray analysis

All serum samples were dissolved 1:250 in PBS for the estimation of autoantibodies' levels in serum proteome. Identical blocking and washing steps were performed as described above in section 3.13.2. The workflow is provided in Figure 3.3.

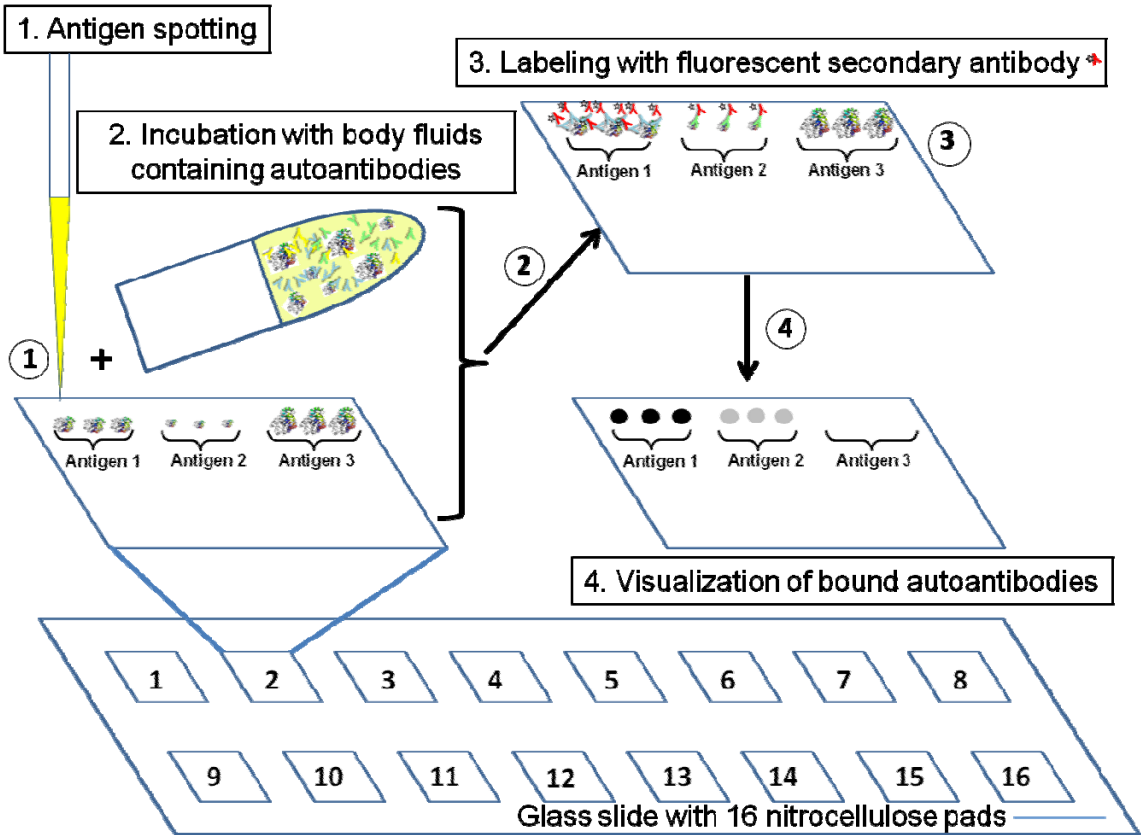


Figure 3.3: Workflow of the antigen (protein) microarray procedure. In the first step, the proteins of interest were spotted onto nitrocellulose subarrays on glass slides (1) in similar way as shown in Figure 3.2. The subarrays were then incubated with individual serum samples containing autoantibodies (2). A secondary labeled anti-IgG antibody was used for the visualization of bound molecules (3). In the last step, the slides were scanned with the microarray scanner and the protein-autoantibodies complexes were visualized (4). Own sketch of the author.

Thus, 100 µl of each diluted individual serum sample was incubated on each subarray with the proteins of interest for 16 hours at 4°C overnight under constant shaking. Additionally, after each four spotted proteins, a triplicate, containing PBS only, was spotted onto the slides to ensure proper purifying of the spotting system. On the next day, the slides were washed three times for 10 minutes with WS and then incubated with the secondary Cy™ 5-conjugated AffiniPure Goat Anti-Human IgG (H+L) antibody for one additional hour. In parallel, also negative controls were performed, meaning no addition of serum or secondary

antibody. Finally, the slides were washed again with WS and additionally two times with HPLC grade H_2O. The slides were then vacuum-dried and stored for further evaluation by 4°C.

3.13.4 Data mining I: microarray platform

The slides were scanned with Affymetrix 428™ Array Scanner. Thus, the appropriate settings of the laser power were adjusted to the signal intensities of the complete study. All slides were therefore scanned with identical settings and the scanned images in 16-bit *TIFF* (tagged information file format) were used for further evaluation of raw data. The generation of raw microarray data was performed with the TIGR Spotfinder version 3.1.1. The general background cutoff was adjusted via the visual comparison of all slides, meaning that the higher background required the higher cutoff (up to 25%). Each subarray was in parallel analyzed visually to ensure the optimal usage of the raw data. Basically, failed spotting patterns as well as inaccurate protein or antibody spots were noted and considered prior to generation of the analysis data. For the evaluation of signal intensities a grid system was generated using following settings: the appropriate number of rows and columns, Otsu segmentation method of spots[211,212] and the minimum and maximum of evaluable spot size of 15 pixels and 25 pixels, respectively. Each signal was manually checked for the generation of the raw data file, which included the mean signal intensity values. Data were exported using the **.csv* (comma separated values) format. Prior to the import of these files into STATISTICA® Software Package (see next chapter 3.13.5), additional data were subjoined: the name of the spotted proteins or antibodies, the position of them on the subarrays, the ID number of each serum sample as well as the classification of them into BC or CTRL group.

3.13.5 Data mining II: statistical evaluation of microarray data

Further evaluation of microarray data was performed with the Statistica® Software Package version 8. First, the previously noted failed spotting pattern data were removed from the final analysis table. As every molecule of interest was spotted in triplicate, only the visually good spots (at least two of each protein triplicate) were used for the estimation of the mean intensity values. Proteins or antibodies on some of the subarrays, whereas no usable values were possible to estimate due to the high background signals, were also removed from the analysis.

Z score data transformation was applied for the raw data normalization[213]. For the described experiments the difference of signal intensity of spots from one analyte on the subarray and mean intensity of all spots on this subarray was divided by standard deviation (SD) of all signal intensities of all spots on the subarray, as shown in following:

$$Z\ score_{Analyte} = \left(\frac{X_i - \bar{x}}{SD}\right)$$

$$SD = \sqrt{\frac{\sum_{i=1}^{n}(X_i - \bar{x})^2}{n-1}}$$

with number of spots on the subarray *n*, intensity of each spot X_i and mean value of the intensity of *n* spots $\bar{x}$. The mean value of each triplicate was then used for the data mining. After the normalization of raw data the appropriate statistical algorithms were applied for the estimation of significantly different levels of proteins or autoantibodies in serum ($p<0.05$). The *t*-test[214] as well as it modified version (Welch test) with separated variance calculation in each group was applied[215,216]. For more than two independent groups (subgroup analysis of BC cohort) the Kruskal-Wallis ANOVA test was used[217]. All proceeding steps of statistical analysis, as well as the generation of plots, were performed in Statistica®.

Additionally to the described algorithms, the significantly different distributed proteins or autoantibodies were analyzed for their discrimination potential using the artificial neural networks (ANN's) with the supervised training and testing pattern (back propagation)[215,218]. For the generation of the ANN's the multiple layer perceptron type of the networks (MLP) was chosen. The main principle is shown in next Figure 3.4.

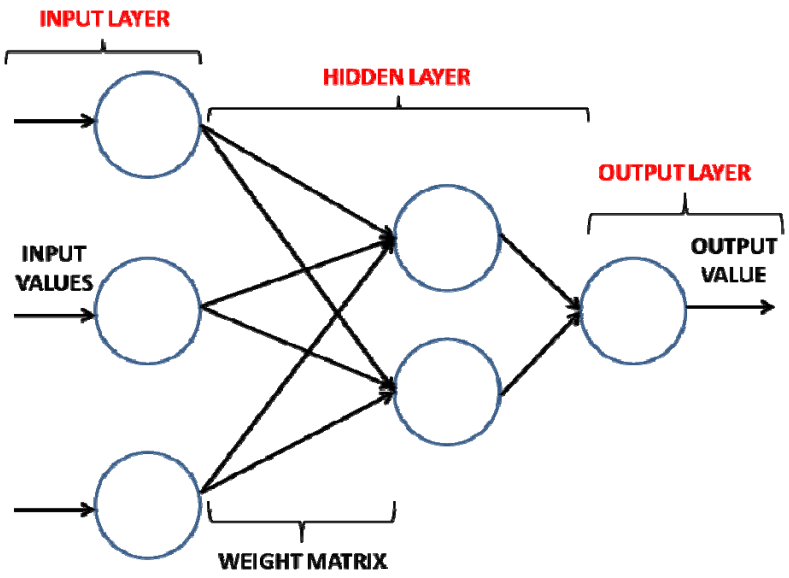


Figure 3.4: Exemplarily sketch of a multilayer perceptron consisting of three layers. The input layer shows three neurons, whereas the hidden layer posseses two hidden neurons and the output layer is made from only one neuron. Note that each neuron from the input layer is connected with all next hidden layers by a certain weight shown via the weight matrix. Own sketch of the author.

The networks were trained with normalized signal intensities of single or multiple biomarkers as inputs (input layer). Thus, training of the networks over hidden layers by changing of connection weights due to false or correct assignment of samples to the respective group

was performed. The network output in the output layer represents the mapping to BC or CTRL group. First, the networks were trained with 50% of randomly chosen data. Afterwards, the other 50% of data served as input to the previously trained networks. It is important that the test set data did not serve for the training of networks. The output of this analysis should provide therefore the ability to classify correctly new data on behalf of the training values. Following settings were performed for the generation of the MLP networks: hyperbolic tangent activation function for the hidden neurons, the identity function for the output neurons, no additional weight decay was performed. 100 networks were generated for each analysis run and the best five were retained. From these five networks, the one with the best parameters for the correct discrimination of two groups was chosen taking the achieved area under the curve (AUC) into account. The value of AUC is based on the sensitivity (true positive rate) and 1-specificity (false positive rate) of the test. These values are calculated regardless, whether the recognized probands' values belong to the diseased or healthy group. For calculation of the sensitivity and specificity of biomarkers with respect of the group affiliation (BC or CTRL), all test sets were reviewed for counting of the correct classified study participants. This additional analysis may provide the data, whether diseased or healthy subjects are better classified into respective group. Briefly, additional sensitivity was calculated by dividing the number of the correctly as diseased recognized probands by the number of all diseased patients used for the test set (calculation regarding only BC samples). The respective specificity is determined by number of all as healthy recognized probands divided by the number of all healthy subjects in the test data set (calculation regarding only CTRL samples).

3.14 List of buffers

4-Chloronaphthol Staining for HRP-coupled anti human IgG

20 ml TBS+12 µl H_2O_2, mixed with

4 ml MeOH+12 mg 4-Chloronaphtol

Blocking Buffer

4% NFDM/TBST

Denaturation Buffer (DB)

8M urea

2% CHAPS

32.4 mM dithiothreitol (DTT)

Rehydration Buffer (RB)

6M Urea

2% SDS

30% Glycerol

75 mM TRIS

pH 8.8

Transfer Buffer for Western Blotting (TB)

25 mM TRIS

192 mM Glycin

20% Methanol

TRIS Buffered Saline (TBS)

20mM TRIS

150 mM NaCl

pH 7.5

3.15 List of used chemicals

Chemical	Distributor
2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS)	Carl Roth GmbH, Karlsruhe, Germany
Acetic acid	Carl Roth GmbH, Karlsruhe, Germany
Acetone	VWR International GmbH, Darmstadt, Germany
Acetonitrile HPLC gradient grade	VWR International GmbH, Darmstadt, Germany
Alpha-cyano-4-hydroxycinnamic acid	Sigma-Aldrich Chemie GmbH, München, Germany
Ammoniumbicarbonate	Sigma-Aldrich Chemie GmbH, München, Germany
Bromophenolblue	Carl Roth GmbH, Karlsruhe, Germany
Dithiothreitol	Carl Roth GmbH, Karlsruhe, Germany
Formic acid	Merck KGaA, Darmstadt, Germany
Glycine electrophoresis grade	MP Biomedicals Inc., Santa Ana, CA, USA
Iodacetamide	Carl Roth GmbH, Karlsruhe, Germany
Methanol	Thermo Fisher Scientific Inc., Rockford (IL), USA

n-Dodecyl-beta-D-maltoside	Fluka,Chemie AG, Basel, Switzerland
Non-fat dry milk	Bio-Rad Laboratories GmbH, München, Germany
Sodium chloride	Carl Roth GmbH, Karlsruhe, Germany
Trichloroacetic acid	Sigma-Aldrich Chemie GmbH, München, Germany
Trifluoroacetic acid	Merck Darmstadt, Germany
Tris(hydroxymethyl)-aminomethan	Carl Roth GmbH, Karlsruhe, Germany
Trypsin, sequencing grade modified	Promega GmbH, Mannheim, Germany
Tween20	Carl Roth GmbH, Karlsruhe, Germany
Water HPLC-grade	Mallinckbrodt Baker, Griesheim, Germany

3.16 Commercially purchased buffers and Kits

Kits and buffers	**Distributor**
BCA protein Assay Kit	Pierce, part of Thermo Scientific, Rockford, USA
Dulbecco's Phosphate Buffered Saline (D-PBS) without Calcium, Magnesium and Phenol Red	Invitrogen, part of Life Technologies™, Darmstadt, Germany
IPG Buffer pH 3-10 and pH3-10 NL	GE Healthcare Life Science, München, Germany
NuPAGE® MOPS Buffer	Invitrogen, part of Life Technologies™, Darmstadt, Germany
NuPAGE® MES Buffer	Invitrogen, part of Life Technologies™, Darmstadt, Germany
NAb Protein G Spin Columns, 0.2mL	Pierce, part of Thermo Scientific, Rockford, USA
NuPAGE® LDS Sample Buffer	Invitrogen, part of Life Technologies™, Darmstadt, Germany
NuPAGE® Sample Reducing Agent	Invitrogen, part of Life Technologies™, Darmstadt, Germany
NuPAGE Novex 12% Bis-Tris gels	Invitrogen, part of Life Technologies™, Darmstadt, Germany
Peptide calibration standard II	Bruker Daltonik, Bremen, Germany
Ponceau S Solution	Sigma-Aldrich Chemie GmbH, München, Germany
Protease Inhibitor Cocktail for tissues	Sigma-Aldrich Chemie GmbH, München, Germany
Protein G Mag Sepharose	GE Healthcare Life Science, München, Germany

3.17 Further consumables

Consumables	Distributor
384 MTP steel polished target	Bruker Daltonik GmbH, Bremen, Germany
Centrifuge tubes	Greiner Bio-One GmbH, Frickenhausen, Germany
Cy™ 5-conjugated AffiniPure Goat Anti-Human IgG (H+L)	Jackson ImmunoResearch, Newmarkert Suffolk, UK
DyLight® 649 NHS Ester	Thermo Fisher Scientific Inc., Rockford (IL), USA
epT.I.P.S.® pipette tips	Eppendorf AG, Hamburg, Germany
FAST® slide incubation chamber	Sigma-Aldrich Chemie GmbH, München, Germany
Gloves, latex	MaiMed GmbH, Neuenkirchen, Germany
Gloves Sempercare®, nitrile	Lohmann Rauscher GmbH & Co. KG, Neuwied/Block, Germany
Microtitre plate of 384 wells with V bottom	Molecular Devices GmbH, Biberach, Germany
Nitrogen liquid	Messer Griesheim, Griesheim, Germany
ONCYTE® 16-pad Nitrocellulose Slides	Grace Bio-Labs, Inc., Bend (OR), USA
PCR 8er-SoftStrips, 0.2 ml	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Safe-Lock Tubes™	Eppendorf AG, Hamburg, Germany

3.18 Technical equipment

Equipment	Distributor
Affymetrix 428™ Array Scanner	Affymetrix Inc., Santa Clara (CA), USA
Centrifuge 5415D	Eppendorf AG, Hamburg, Germany
Electrophoresis and blotting power supply Standard Pack	Biometra GmbH, Goettingen, Germany
Magnetic rack for small (up to 2 ml) tubes DynaMag™ -2	Invitrogen, part of Life Technologies™, Darmstadt, Germany
MALDI TOF/TOF mass spectrometer ultraflex II	Bruker Daltonik GmbH, Bremen, Germany
Microplate photometer Multiskan Ascent®	Thermo Fisher Scientific Inc., Rockford (IL), USA

Mini Trans-Blot® Electrophoretic Transfer Cell	Bio-Rad Laboratories GmbH, München, Germany
Porcelain mortar and pestle	Carl Roth GmbH, Karlsruhe, Germany
Thermal mixer	Ditabis Digital Biomedical Imaging Systems AG, Pforzheim, Germany
Sample shaker and incubator Titramax 100	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
sciFLEXARRAYER S3 non-contact spotter, incl. PDC60 nozzle	Scienion AG, Berlin, Germany
Vacuum Concentrator 5305	Eppendorf AG, Hamburg, Germany
Vortex-Genie™ 2	Scientific Industries Inc., Bohemia (NY), USA
XCell SureLock™ Mini-Cell electrophoresis	Invitrogen, part of Life Technologies™, Darmstadt, Germany

3.19 Summary of Software used for data mining

Software Package	Distributor
BioTools version 3.1	Bruker Daltonik GmbH, Bremen, Germany
ImaGene version 5.5.4	BioDiscovery, Inc., Hawthome (CA), USA
IPA® integrative biomolecular analysis for complex ‘omics data	http://www.ingenuity.com, accessed at August 29, 2012
flexAnalysis version 2.4	Bruker Daltonik GmbH, Bremen, Germany
MASCOT	http://www.matrixscience.com
Microsoft Office 2007 Package	Microsoft
Proteomics Pipeline Mainz (P^2M)	University Eye Hospital, Universitaetsmedizin Mainz, Germany
Statistica® Software Package version 8.0	StatSoft, Tulsa (OK), USA
STRING 9.0 (Search Tool for the Retrieval of Interacting Genes/Proteins)	http://www.string-db.org, accessed at March 27, 2013
TIGR Spotfinder version 3.1.1	Dana-Farber Cancer Institute, Boston (MA), USA

3.20 List of purchased antibodies for microarray analysis

Gene name	Recommended protein name according to UNIPROT with abbreviation Id_HUMAN	Manufacturer/Distributor
s10a9	Anti-Protein S100-A9; S10A9	Abcam plc, Cambridge UK

cst2	Anti-Cystatin-SA; CYTT	R&D Systems, Inc., Minneapolis, USA
mtus1	Anti-Microtubule-associated tumor suppressor 1; MTUS1	Abcam plc, Cambridge UK
scgb1d1	Anti-Secretoglobin family 1D member 1; SG1D1	Santa Cruz Biotechnology, Inc., Heidelberg, Germany
itih4	Anti-Inter-alpha-trypsin inhibitor heavy chain H4; ITIH4	Abcam plc, Cambridge UK
apoc1	Anti-Apolipoprotein CI antibody; APOC1	Abcam plc, Cambridge UK
prr4	Anti-Proline-rich protein 4; PROL4	Santa Cruz Biotechnology, Inc., Heidelberg, Germany
s10a8	Anti-Protein S100-A8; S10A8	Abnova GmbH, Heidelberg, Germany
tf	Anti-Serotransferrin; TRFE	Biomol GmbH, Hamburg, Germany
a2mg	Anti-Alpha-2-Macroglobulin; A2MG	Rockland Immunochemicals Inc., Gilbertsville, USA

3.21 List of purchased antigens for microarray analysis

Gene name	Recommended protein name according to UNIPROT with abbreviation	Manufacturer/Distributor
eno1	Alpha-enolase	Abcam plc, Cambridge UK
rcvrn	Recoverin	Abcam plc, Cambridge UK
pgk1	Phosphoglycerate kinase 1	Abcam plc, Cambridge UK
hspa5	78 kDa glucose-regulated protein	Abcam plc, Cambridge UK
gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Abcam plc, Cambridge UK
prdx6	Peroxiredoxin-6	Abcam plc, Cambridge UK
capzb	CAPZB protein	Abcam plc, Cambridge UK
aldoa	Fructose-bisphosphate aldolase A	Abcam plc, Cambridge UK
stip1	Stress-induced-phosphoprotein 1	Abcam plc, Cambridge UK
h2b	Histone H2B protein	Abcam plc, Cambridge UK
ahsg	α2-hs-Glycoprotein	Sigma-Aldrich Chemie GmbH, München, Germany

4 Results

Following chapters were divided into two main parts. On the one side, the individual profiling of selected protein levels in serum samples of BC and CTRL study participants was aspired. On the other side, the immunoproteomics-based approach was performed in the second part of the thesis. It included the visualization of complex autoantibody profiles in BC and CTRL as well as the profiling of autoantibodies' levels in cancer samples only according to certain subgroup division.

4.1 Comparison of protein levels in individual BC and CTRL serum samples via antibody-microarray platform

The selection of protein candidates was described in section 3.13.1. Predominantly, the set of proteins consisted of biomarkers obtained during the previous intact protein profiling in serum and tear fluid samples of BC patients including results from the *de novo* identification of biomarkers in mixed tear proteome samples[91,101,113]. Figure 4.1 shows the aspired workflow for the selection of candidates and estimation of their level differences in serum BC and CTRL subjects.

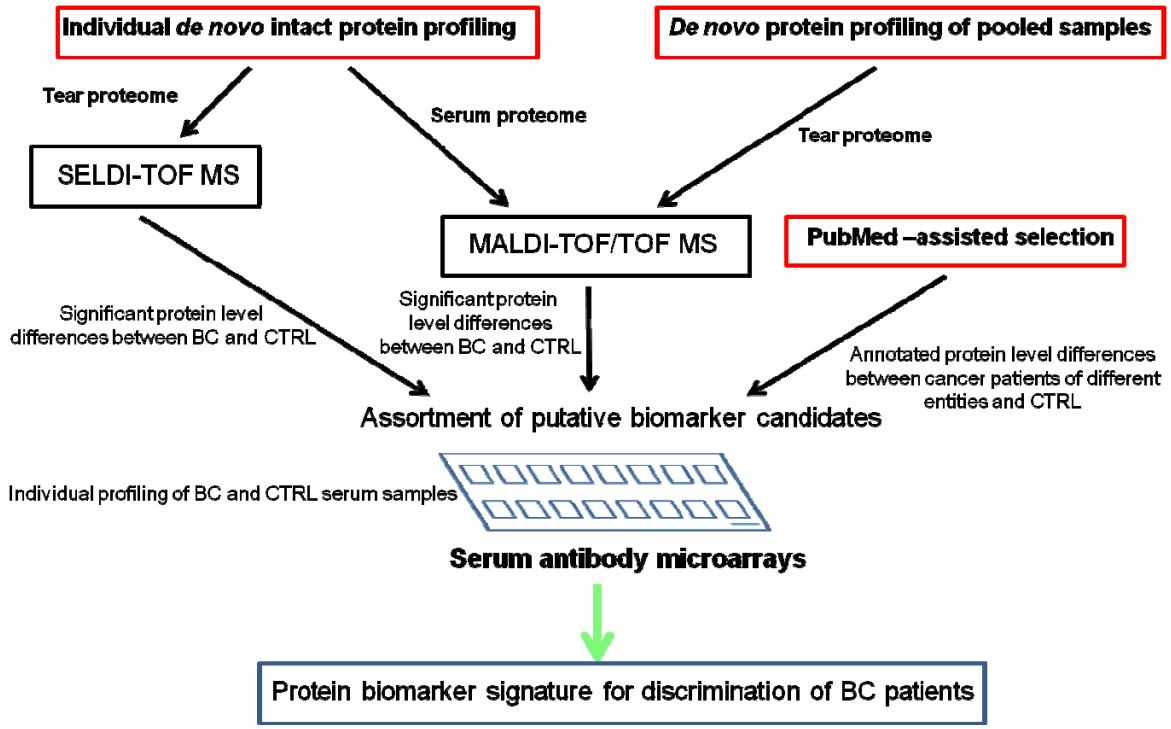


Figure 4.1: Aspired workflow of the performed antibody microarray strategy for estimation of protein level differences. The most promising candidates were taken from the preliminary studies together with the PubMed-based search of annotated level differences in other cancer entities. The levels of proteins were examined in the individual profiling of BC and CTRL subjects and their discriminatory ability was verified. Own sketch of the author.

Figure 4.2 shows the selected proteins chosen for the estimation of their levels in individual serum samples of BC and CTRL study participants. For that purpose, the respective antibodies were commercially obtained.

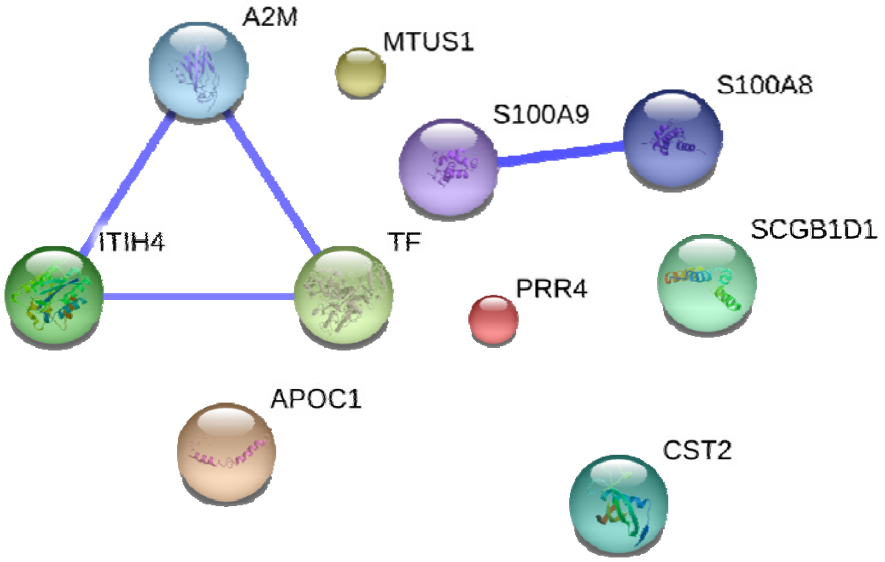


Figure 4.2: Summary of the proteins selected for their level measurement in serum of BC and CTRL probands via antibody microarray platform. The sketch was constructed with the STRING protein database[204], whereas the gene names are used instead of protein names. Deviating titles are as follows: *scgb1d1*=SG1D1, *prr4*=PROL4, *cst2*=CYTT, a2m=A2MG.

For the main study 53 BC and 39 CTRL serum specimens were used. The estimation of protein level differences between both groups was aspired as a first goal. Each subarray with all antibodies was incubated with labeled serum proteins of single study participants. Figure 4.3 shows the example of two incubated subarrays from this study with the appropriate antibodies spotted head-to-tail in triplicates on the nitrocellulose slides.

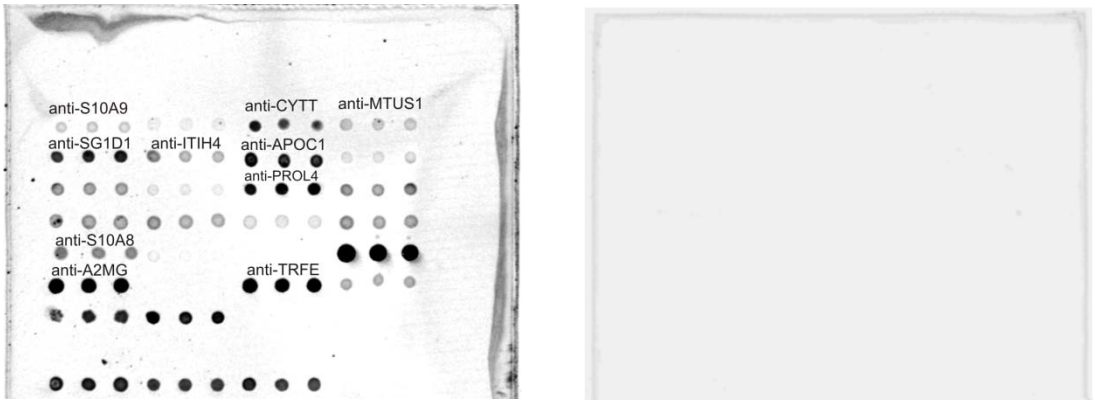


Figure 4.3: Two subarrays from the antibody microarray study. The left subarray was incubated with labeled serum. The abbreviated names of antibodies (see full list in 3.20) are placed above the appropriate positions. The right subarray was incubated with unlabeled serum (negative control).

Many more antibodies were spotted on the microarray slides for the analysis of serum samples, as 20 slides were processed simultaneously. The remaining slides were used for

further studies in the ophthalmology lab. Full list of the antibodies on these slides is provided in Repository figure 4 and Repository table 4.

Summing up, all analyzed antibodies reacted with the labeled antibodies in all serum samples of study participants, whether from the cancer or from the healthy group. Even simple visual comparison of signal from the same protein in different patients revealed variations of detected signal intensities. These interindividual differences of the proteins are shown exemplarily on two subarrays from CTRL and BC serum sample in Figure 4.4. Notably, also intensities of distinct proteins within both groups (proteins in BC or CTRL individual serum specimens) varied similarly. Additionally, signal intensities of various proteins were distinct in the serum sample itself (intraindividual differences). Thus, the signal intensity of the protein TF was considerably higher than of the protein S10A8 or S10A9 (as shown in Figure 4.3).

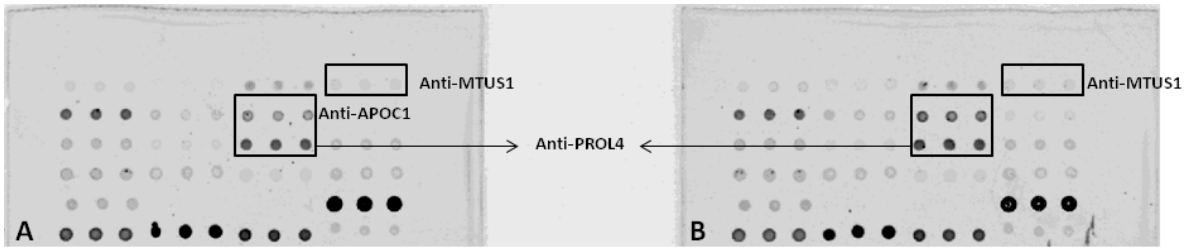


Figure 4.4: Two antibody subarrays incubated with healthy (A) and cancer (B) sample, respectively. The last two rows with antibodies are removed in comparison to Figure 4.3 as they were not used for the current study. The visual differences of the intensities between healthy and cancer subarrays (slightly higher in B) are highlighted by boxes and the respective names of the spotted antibodies.

The obtained signal intensities were evaluated with the TIGR Spot software and additional visual assessment of the individual reactions on each subarray. The signal intensities of analytes were compared between the BC and CTRL group after normalization procedures (see section 3.13.5 for details).

The analysis revealed significantly different levels of three proteins in both groups. Thus, the protein ITIH4 was decreased in the BC group (p=0.016), whereas SG1D1 and A2MG were increased (p=0.031 and p=0.034, respectively). Figure 4.5 shows the summary of distinct levels of proteins in both groups with the respective Whisker Plots.

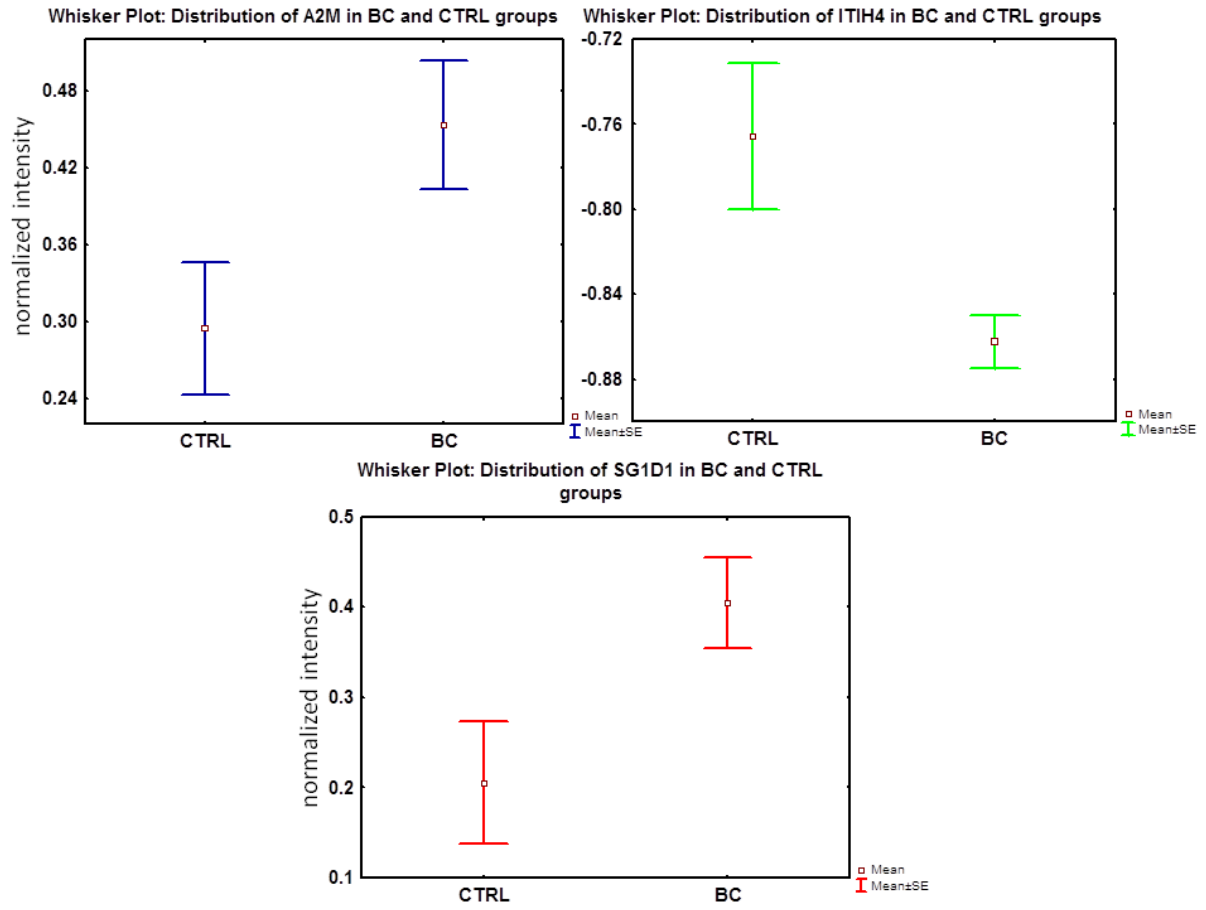


Figure 4.5: Summary of the significantly different regulated proteins in CTRL and BC groups (x-axis). The Whisker Plots were built according to the normalized signal intensity levels (y-axis) and their mean level in each group (square). The whiskers point the standard error (SE) of the signal intensities.

Another interesting approach regards the estimation of protein level differences in the cancer group only. The main idea is the establishment of biomarkers, which are specific for any of the subgroups. These findings can be used for the monitoring of the disease, if certain proteins subsequently change the level during disease progression. This effect was also shown in our previous study regarding measuring of immune system protein levels[87]. Further analyses were therefore performed for the comparison of protein levels in BC group based on TN(M)-, grading and hormone receptor status for the detection of biomarkers. Table 4-1 shows the considered characteristics of the respective removed tumors.

The distribution of protein levels in BC group was checked according to tumor size T1 and T2, node involvement rated as N_0 and N1 and subsequently differentiation of tumor cells (G1-G3). The statistical analyses revealed no significant differences between all examined subgroups.

Table 4-1: Summarized parameters from BC populationused for the estimation of defined proteins via individual serum profiling. Tumor's characteristics include TNM classification (*DCIS), grading and hormone receptor status. No patients developed metastases (M0).

Histological characteristics	BC group n =53	CTRL group n = 39
Mean age (distribution)	57.9 (34-85)	57.6(36-85)
Tumor size		
pTis	0 (%)	
pT1	30 (57.7)	
pT2	19 (36.5)	
pT3	2 (3.8)	
other*	1 (1.9)	
Node status		
Negative N0	32 (61.5)	
Positive N1	20 (38.5)	
Grading		
Well differentiated (G1)	7 (13.5)	
Moderately differentiated (G2)	30 (57.7)	
Poor/undifferentiated (G3)	15 (28.8)	
Hormone receptor		
Positive	45 (86.5)	
Negative	7 (13.5)	

Another investigation was based on the assumed differences by including only a subgroup from the BC cohort and protein level comparison with CTRL serum specimens: *e.g*. sera of only small tumors or stratified by differentiation level (G2 and G3, as only 7 G1-samples were available). Thus, only sera from moderately differentiated tumors (G2) were compared with the CTRL samples, whereas ITIH4 and A2MG were again de- and increased in BC group (p=0.025 and p=0.039, respectively). Also if only small tumors (T1) were included in the BC group the proteins SG1D1 and ITIH4 were in- and decreased in the BC group in same manner as described for the main analysis, respectively (p=0.01 for both proteins). ITIH4 was also decreased in node-positive (N1) serum samples if compared to CTRL population (p=0.02). In case of hormone receptor positive tumors, same biomarkers were found to be differently distributed in the same manner as in the main analysis (SG1D1 and A2MG were increased in BC group, p=0.02 for both biomarkers; ITIH4 was decreased, p=0.009).

The interpretation of the results in the subgroup profiling is however limited as the number of cases between the groups varied significantly. The results may be biased requiring higher as well as more similar case numbers. It is however of high interest, that the comparison of small tumors only revealed similar results with the main protein level comparison.

4.1.1 Discriminatory power of protein biomarkers

For the subsequent classification analysis, whether putative biomarkers can discriminate BC from CTRL with high specificity and sensitivity, the ROC curves were constructed with the ANN's of multilayer perceptron type (see section 3.13.5 for details). All three significantly different putative biomarkers were tested as inputs separately or as combination in order to increase the discriminatory ability. The results were obtained using 46 participants' test data (22 from BC and 24 from CTRL group).

The protein SG1D1 as a standalone input only revealed the AUC of 0.7 with the network test sensitivity of 63% and specificity of 68% (see Figure 4.6 **A** for the SG1D1 ROC curve). In case of A2MG, the same AUC of 0.7 was obtained with nearly constant sensitivity of 71% but low specificity of 55% (shown in Figure 4.6 **B**). The last tested biomarker was the protein ITIH4, which achieved an AUC of 0.63 with sensitivity of 54% and the specificity of 73% (Figure 4.6 **C**). The combination of two biomarkers for the increasing the AUC was also investigated, whereas the best discrimination was achieved by A2MG and SG1D1. Both iinputs resulted in an AUC of 0.71, whereby the sensitivity and specificity were comparable with the standalone inputs (71% and 64 %, respectively). The described ROC curve is shown in Figure 4.6 **D**.

The ROC curves were constructed by plotting the true positive rate (sensitivity) against false positive rate (1-specificity), irrespective of group belonging of analyzed samples. Additionally, the correct percentages of these operator characteristics were also calculated (see description in 3.13.5) using the formulas for the sensitivity and specificity regarding BC and CTRL groups, respectively (summarized in Table 4-2).

Table 4-2: Calculated sensitivity and specificity of putative biomarker values described above. Note that the test population (46), as well as the number of BC and CTRL values, is identical in all tests (22 and 24, respectively).

input for ANN	n BC correct prediction	n CTRL correct prediction	sensitivity in %	specificity in %
A2MG	17	14	77	58
SG1D1	22	10	100	42
ITIH4	22	6	100	25
A2MG+SG1D1	19	15	86	63
A2MG+SG1D1+ITIH4	22	8	100	33

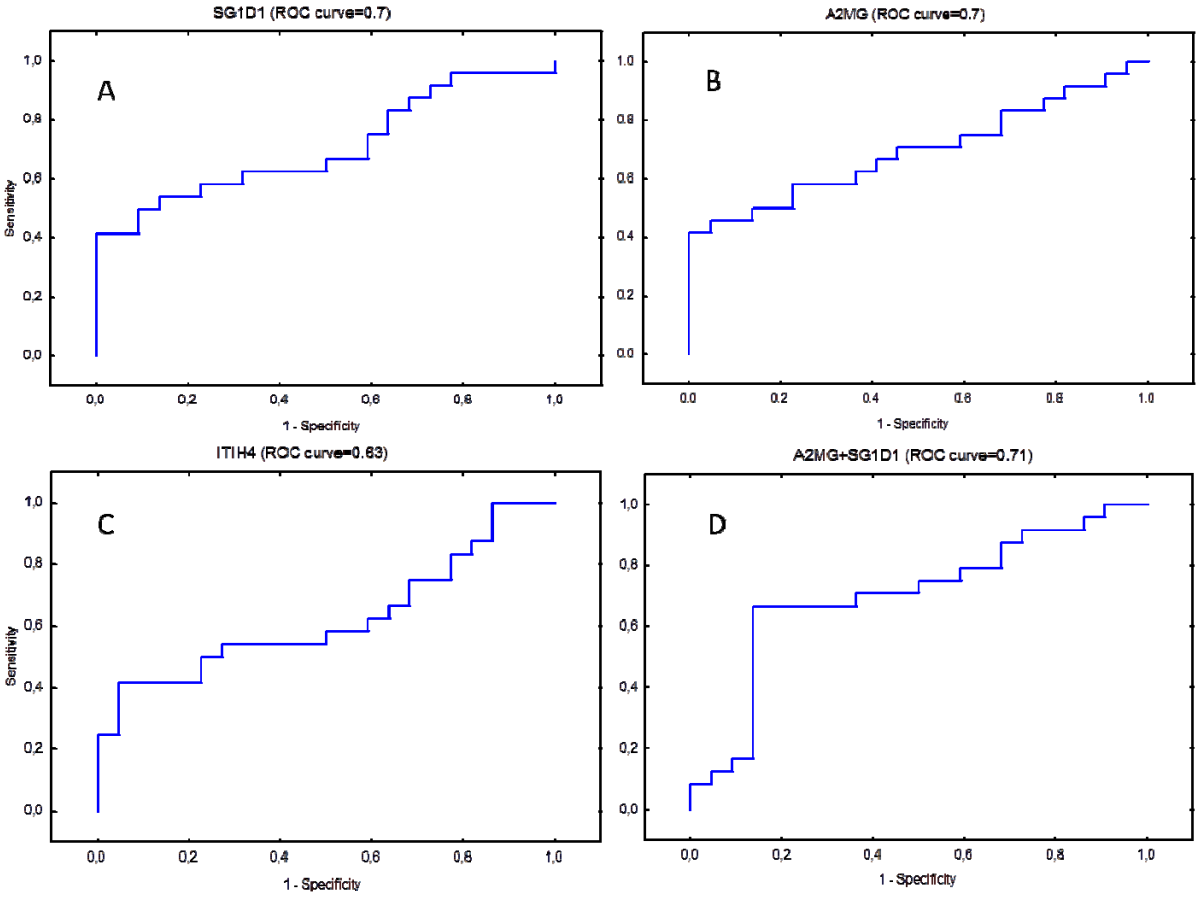


Figure 4.6: Constructed ROC curves for the discrimination of BC and CTRL groups by the different levels of: A SG1D1, B: A2MG, C: ITIH4 and D: combination of A2MG and SG1D1 as input with the sensitivity on y-axis and the reciprocal specificity (false-positive rate) on the x-axis.

Taken the values of all three proteins as inputs together, the AUC to 0.73 with the previously achieved sensitivity of 71% and specificity of 77% (as shown in Figure 4.6) was obtained. The final combined ROC curve is shown in Figure 4.7.

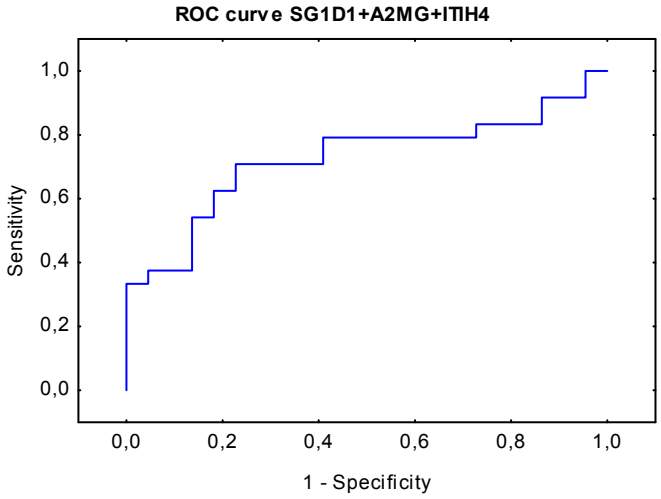


Figure 4.7: ROC curve for the discrimination of BC and CTRL groups by the protein levels of SG1D1, A2MG and ITIH4 (AUC=0.73). The reciprocal specificity is shown on the x-axis, the sensitivity of the ROC curve on the y-axis.

As it has been stated, also anti-CYTT antibody was used for the estimation of respective levels in the serum of study participants. The results were however not included in the main analyses described above, because the cross-reactions with another cystatins could not be excluded. Nevertheless, the evaluation of CYTT protein showed the significant decreased level in the BC group (p=0.007). For the sake of completeness, also ROC curves for the classification of BC and CTRL subjects with CYTT as standalone input or together with A2MG, ITIH4 and SC1D1 were constructed in the additional classification analysis.

Using the values of the protein CYTT as a standalone input, the AUC of 0.64 was achieved with network test sensitivity of 86% and specificity of 52%. From 46 participants' data of CYTT, which were used for the test set of ANN (25 belonged to the BC group and 21 to the CTRL group), 13 were correctly identified as BC and 17 as CTRL subjects. These results led to the lower clinically relevant sensitivity of 52% and the specificity of 81%. The combination of CYTT with other three putative biomarkers as inputs increased the AUC up to 0.84 with the higher sensitivity of 83% and the specificity of 82%. Additionally, from 46 test persons' data (22 from BC and 24 CTRL groups), 20 BC patients and 16 CTRL probands were correctly classified. The respective specificity and sensitivity are 67% and 91 %. Both networks with CYTT as input are shown in Figure 4.8).

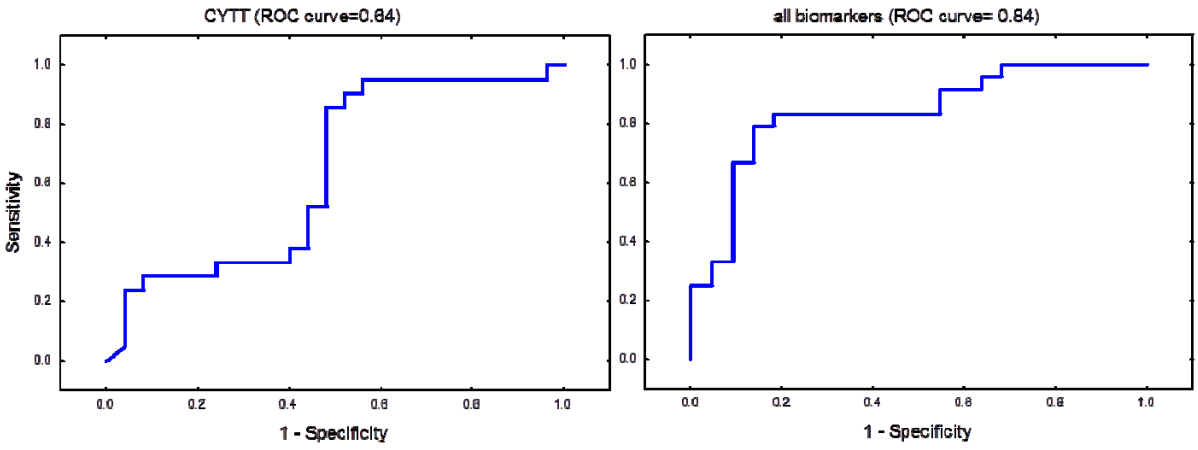


Figure 4.8: ROC curves for the discrimination of BC and CTRL groups by the protein levels of CYTT alone on the left side and the combination of CYTT, SG1D1, A2MG and ITIH4 on the right side. The reciprocal specificity is shown on the x-axis, the sensitivity of the ROC curve on the y-axis.

To sum up, the profiling of protein levels revealed significant level differences of three proteins (A2MG, ITIH4 and SG1D1) in the main analysis between the BC and CTRL groups. The discrimination aility was also tested, whereas the AUC of 0.73 was obtained. Additionally, a sensitivity regarding the BC group only of up to 100% (SG1D1, ITIH4 and the combination of all three inputs) was achieved, meaning that all BC patients were correctly recognized as diseased group.

4.2 Detection of putative immunogenic tumor antigens

TA's were identified through applying of two different strategies. On the one side, the antigens were captured via coupling to the isolated IgG antibodies, which were previously cross-linked with protein G beads (described in the section 3.11). The complimentary identification strategy of the immunogenic TA's was aspired via Western immunoblotting (see section 3.12 for details). In the second part (starting in the section 4.3), the most promising TA´s from both approaches were commercially obtained for the profiling of the respective autoantibodies and their level distribution in individual serum samples of BC and CTRL groups. The general corresponding workflow is shown in Figure 4.9.

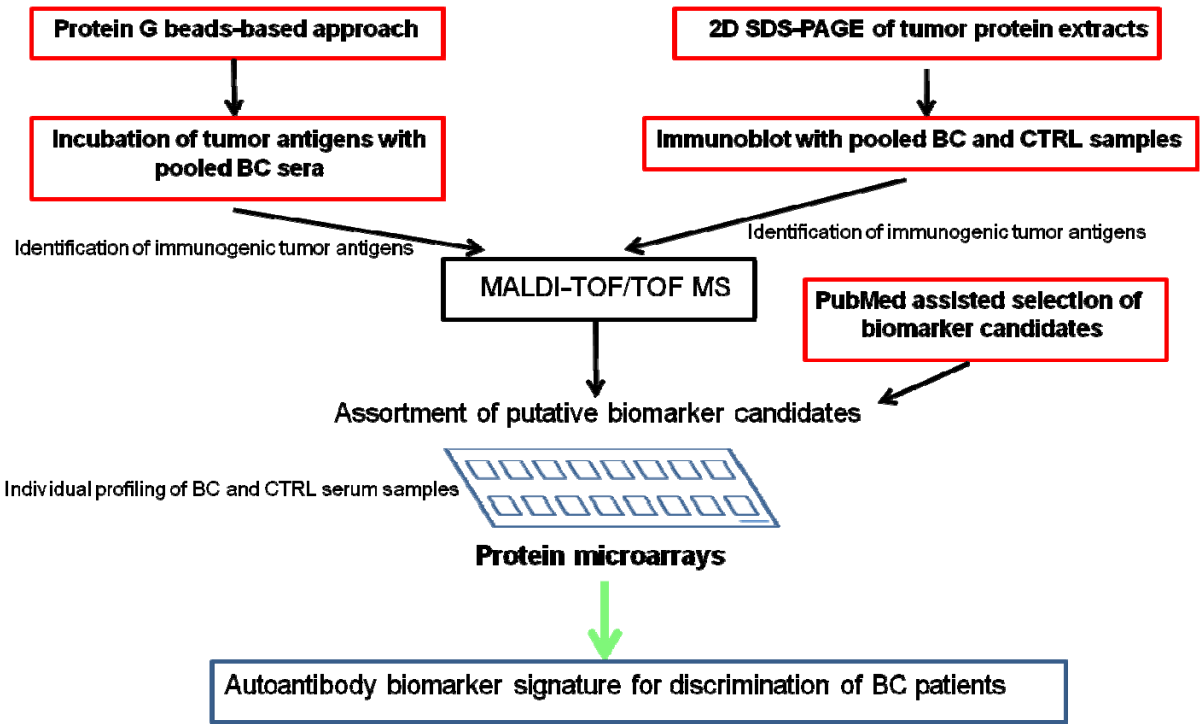


Figure 4.9: Summarizing workflow of the aspired strategy for the detection of complex autantibodies' patterns via protein G beads-based immunoprecipitation and Western immunoblotting of 2D separated TA's. The immunogenic TA's were identified via MALDI-TOF/TOF MS and the most promising candidates were commercially purchased. The levels of the respective autoantibodies in the individual profiling in serum of BC and CTRL subjects were examined and statistically different levels in both grous estimated. The discriminatory potential of the autoantibodies evaluated together as a biomarker panel were examined in the last step (bottom of the figure). Own sketch of the author.

Tumor protein extracts were obtained via the procedure described in section 3.2. Prior to all experiments 1D or 2D SDS-PAGE of every protein sample was performed for the evaluation of resulting protein patterns. Additionally, their consistence was verified after IgG depletion procedures. Figure 4.10 shows exemplarily 1D and 2D electrophoretical separations of tumor proteins.

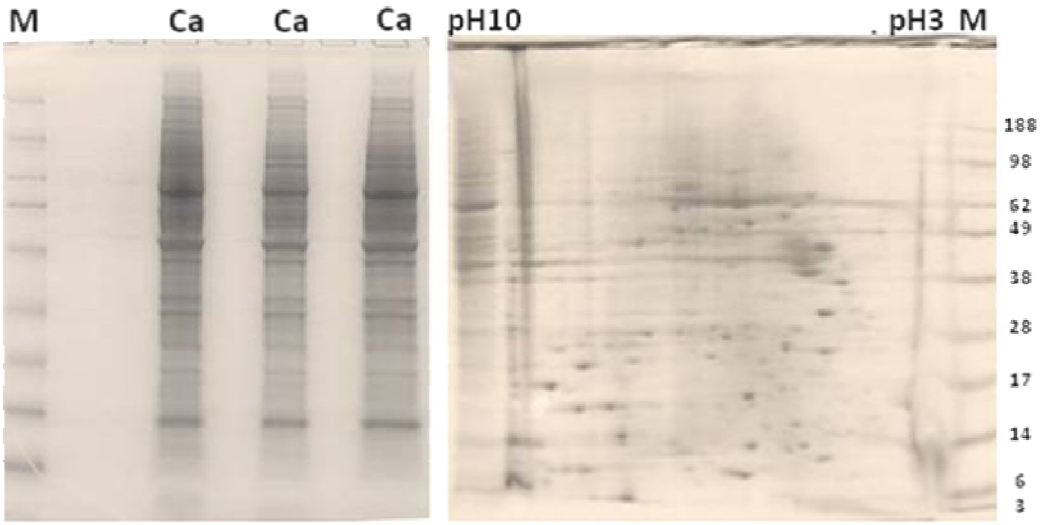


Figure 4.10: Reducing 1D and 2D SDS-PAGE of tumor protein extracts from tumor tissues of breast carcinoma (stated as Ca). On the left side, 1D SDS-PAGE of a lymph node metastasis from breast carcinoma is exemplarily shown. On the right side, the result of 2D SDS-PAGE of tumor tissue itself is depicted (for IEF the pH range of 3-10 was chosen shown above). The protein ladder (M) was simultaneously loaded onto gel for the facilitated orientation according to the given molecular weight.

4.2.1 Immunoprecipitation platform for isolation of immunogenic tumor antigens

Different strategies were performed for the isolation of relevant immunogenic TA's on behalf of the protein G-bead platform. Prior to main experiments, tumor proteins were obtained over lysis of breast carcinoma tissue samples. Predominantly, a high tumor protein amount was required for the immunoprecipitation of tumor antigens for detecting of respective autoantibodies in the sera of study participants.

The first strategy of coupling the immunogenic antigens was the primary enrichment of the putative autoantibodies (described in section 3.11.1). Afterwards, these autoantibodies were cross-linked with protein G beads and incubated with the fresh tumor protein extract. The eluted putative immunogenic TA's were separated over the 1D-SDS-PAGE, then digested and subsequently identified via MALDI-TOF/TOF MS (described in section 3.7). However, the number of obtained proteins was insufficient as most of the identified peptides belonged to immunoglobulins, which were apparently co-eluted despite the cross-linking procedure. Besides, the TA's may went missing due to several experimental steps of this strategy and the required amounts of tumor extracts were too high to repeat this procedure for several times. Thus, this handling protocol was modified to reduce the necessary amount of tumor proteins and minimize the experimental steps to prevent the loss of important molecules. Therefore, the enrichment of autoantibodies by pre-incubation with tumor proteins on the nitrocellulose membrane was left out. The isolated IgG's from the mixed serum samples were directly coupled onto protein G beads with subsequent crosslinking and then incubated

with the tumor extract. The proteins were eluted in several steps, then pooled together and dried in the vacuum centrifuge to reduce sample volume. The obtained tumor antigens were separated after reconstitution via 1 D SDS-PAGE. Figure 4.11 shows the SDS gels with separated tumor proteins using the described techniques.

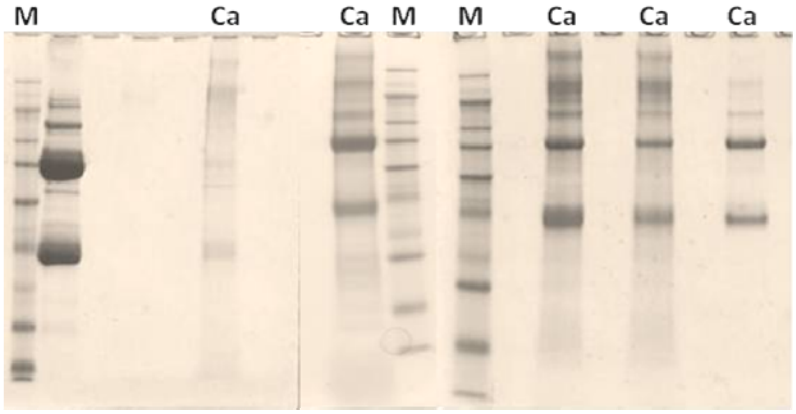


Figure 4.11: Separated tumor proteins via 1D SDS-PAGE after elution from IgG's cross-linked with protein G beads (marked with Ca-lanes). The M-lanes show the protein ladder (see Figure 4.10 for detailed molecular weights) and the lane beneath contained purified IgG's. Note the similar protein pattern in this lane and Ca-lanes. This circumstance visualized the co-elution of the immunoglobulins.

The gel lanes with eluted TA's were divided into fragments and the proteins were digested according to the method described in section 3.7. Nevertheless, high amounts of immunoglobulins were co-eluted with TA's again, even though performed checking of crosslinking sufficiency by SDS-PAGE of all washing steps after this procedure. However, the reduction of experimental steps showed a positive effect regarding the number of the identified proteins in comparison to the first chosen strategy. All acquired spectra were subsequently combined and identified via Mascot search tool (described in section 3.9). Table 4-3 summarizes the merged results of the protein identification using the MALDI-TOF/TOF MS.

Table 4-3: Summary of the identified proteins with appropriated gene names according to the merged list of all spectra deriving from the digestion of eluted TA's described above. All identified fragments of immunoglobulins were left out. The data were analyzed via the Mascot Search tool[219]. The abbreviations of protein names derived from the UniProt database[202].

ID number	UniProt entry	Abbreviation Id_HUMAN	Protein name	Gene name
1	Q9NRD9	DUOX1	Dual oxidase 1	*duox1*
2	P62805	H4	Histone H4	***
3	P60709	ACTB	Actin, cytoplasmic 1	*actb*
4	P04908	H2A1B	Histone H2A type 1-B/E	*hist1h2ab/hist1h2*

				ae
5	P02768	ALBU	Serum albumin	*alb*
6	P33778	H2B1B	Histone H2B type 1-B	*hist1h2bb*
7	Q68DK2	ZFY26	Zinc finger FYVE domain-containing protein 26	*zfyve26*
8	Q8TE59	ATS19	A disintegrin and metalloproteinase with thrombospondin motifs 19	*adamts19*
9	Q13835	PKP1	Plakophilin-1	*pkp1*
10	Q13395	TARB1	Probable methyltransferase TARBP1	*tarbp1*
11	Q9BYJ9	YTHD1	YTH domain family protein 1	*ythdf1*
12	P0C7X3	CCYL3	Putative cyclin-Y-like protein 3	*ccnyl3*
13	Q16695	H31T	Histone H3.1t	*hist3h3*
14	O94874	UFL1	E3 UFM1-protein ligase 1	*ufl1*
15	Q96N11	CG026	Uncharacterized protein C7orf26	*c7orf26*
16	Q8NHY6	ZFP28	Zinc finger protein 28 homolog	*zfp28*
17	Q8N9L9	ACOT4	Acyl-coenzyme A thioesterase 4	*acot4*
18	Q562R1	ACTBL	Beta-actin-like protein 2	*actbl2*
19	Q71U36	TBA1A	Tubulin alpha-1A chain	*tuba1a*
20	P61626	LYSC	Lysozyme C	*lyz*
21	Q96QV6	H2A1A	Histone H2A type 1-A	*hist1h2aa*
22	P07437	TBB5	Tubulin beta chain	*tubb*
23	Q13885	TBB2A	Tubulin beta-2A chain	*tubb2a*
24	Q13509	TBB3	Tubulin beta-3 chain	*tubb3*
25	Q8IZT6	ASPM	Abnormal spindle-like microcephaly-associated protein	*aspm*

26	P02747	C1QC	Complement C1q subcomponent subunit C	*c1qc*
27	Q9NY65	TBA8	Tubulin alpha-8 chain	*tuba8*
28	Q6ZRQ5	MMS22	Protein MMS22-like	*mms22l*
29	Q9H5P4	PDZD7	PDZ domain-containing protein 7	*pdzd7*
30	Q13233	M3K1	Mitogen-activated protein kinase kinase kinase 1	*map3k1*

Interestingly, different intracellular, even located in the nucleus, proteins like histones, were found inbetween of the identified proteins. Also enzymatic proteins like Acyl-coenzyme A thioesterase 4 (ACOT4) or Dual oxidase 1 (DUOX1) were found. These results can be considered as additional argument for the hypothesis of raising autoantibodies against intracellular proteins (presented in section 1.4).

As the matter of course, it cannot be excluded that some of the relevant TA's were not identified due to co-elution with high-abundant immunoglobulins. This fact required the improvement of the strategy for the additional identification of TA's.

4.2.2 Western immunoblotting-based strategy for the detection of autoantibodies'patterns

The use of immunoblotting enables the direct visualization of the antibodies bound to the TA's in comparison to the prior used protein G beads-based strategy. The same procedure regarding the extraction of tumor proteins was performed. The obtained tumor proteins were visually analyzed prior to the blotting procedure via SDS-PAGE (see an example of 2D SDS-PAGE in Figure 4.10).

The 2D SDS-PAGE was chosen for immunoblotting due to its more precise protein separation. Even if this procedure requires higher amounts of proteins as 1D SDS-PAGE, the used quantity of tumor antigens for blotting is definitely lower as for the protein G beads-based strategy (less than 250 µg tumor protein extract). This fact enabled multiple performances of the experiments for the better comparison of results and optimization of the used protein quantities, chemicals as well as experimental conditions to maximize the output. After 2D SDS-PAGE-based separation and adjacent immunoblotting of cancer antigens with the pooled CA and CTRL sera, different autoantibodies profiles were detected, showing the complexity of immune response. Indeed, several visualized antigen spots varied in the reaction intensity, marking different amounts of the bounded appropriate autoantibody from

the pooled sera. Repository figure 5 shows additionally similar immunoreactions obtained after multiple 2D immunoblottings confirming sustainable reaction patterns.

Interestingly, not only the immune reactions between cancer antigens and autoantibodies from breast cancer serum pool were detected but also those from the healthy serum pool. This fact confirms again the presence of autoantibodies in the healthy serum, which seem not to derive as the response to the TA's only. As described in section 1.4.3, naturally occurring antibodies persist in the serum of clinically healthy persons, whereas their role is currently not understood. The visualization of immunoreactions with BC and CTRL serum pools revealed multiple differences in the reaction intensities. Thus, the autoantibody profile patterns of healthy subjects were generally of lower intensity as also shown exemplarily in Figure 4.12. Therein, less protein spots showed immunoreactivity with CTRL serum pool.

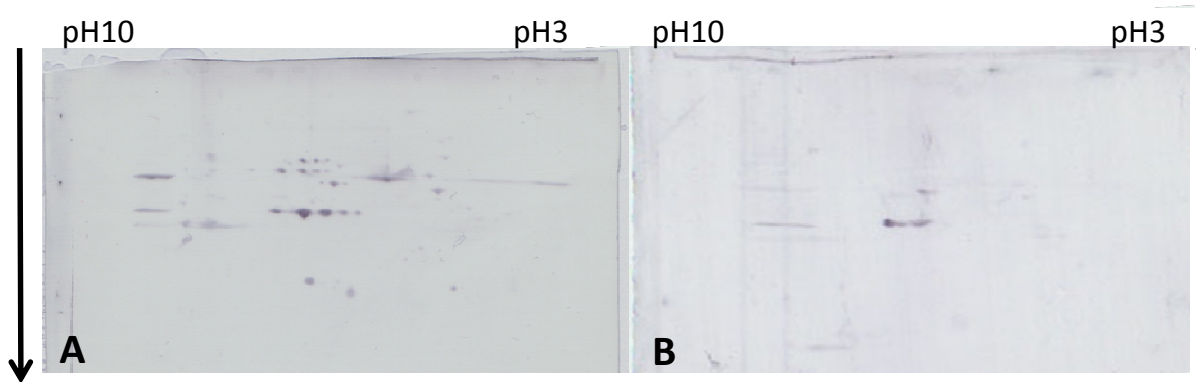


Figure 4.12: Exemplary representation of two performed immunoblots In A the visualization of immunoreactions after incubation of TA's with BC serum pool is depicted, whereas in B, the same proteins were incubated with the serum pool from the age-matched CTRL study participants. The used pH range for IEF separation is depicted above; the black arrow shows the separation direction via SDS-PAGE.

Additional confirmation of different autoantibody patterns in both groups via other experimental platform was required. Thus, the signal intensity fluctuations between immunoblots may derive from the fluctuations during the transfer onto nitrocellulose membrane, even by simultaneous blotting, or earlier due to irregular IEF. Additionally, these experiment trials were limited due to small available volume of the CTRL serum pool. On this account, all the spots of interest, even with the same reaction pattern, were analyzed and identified in the further step.

4.2.3 Identification of tumor antigens reacting with the antibodies in sera of BC and CTRL

The positions of the reaction spots (TA's and respective autoantibodies from the sera) were compared with their location on the second gel with separated tumor proteins simultaneously with the blotted gel. The identification of putative TA's was therefore facilitated by direct

picking of the respective protein spots from the analytical gel. The proteins were digested overnight and the peptides were desalted with C18 ZipTip® and eluted by ascending ACN concentration directly on the MALDI target for the identification with MALDI-TOF/TOF MS (see section 3.8). Figure 4.13 shows examples of PMF's from different sample fractions (MS spectra) and subsequently fragmented selected peaks (MS/MS spectra).

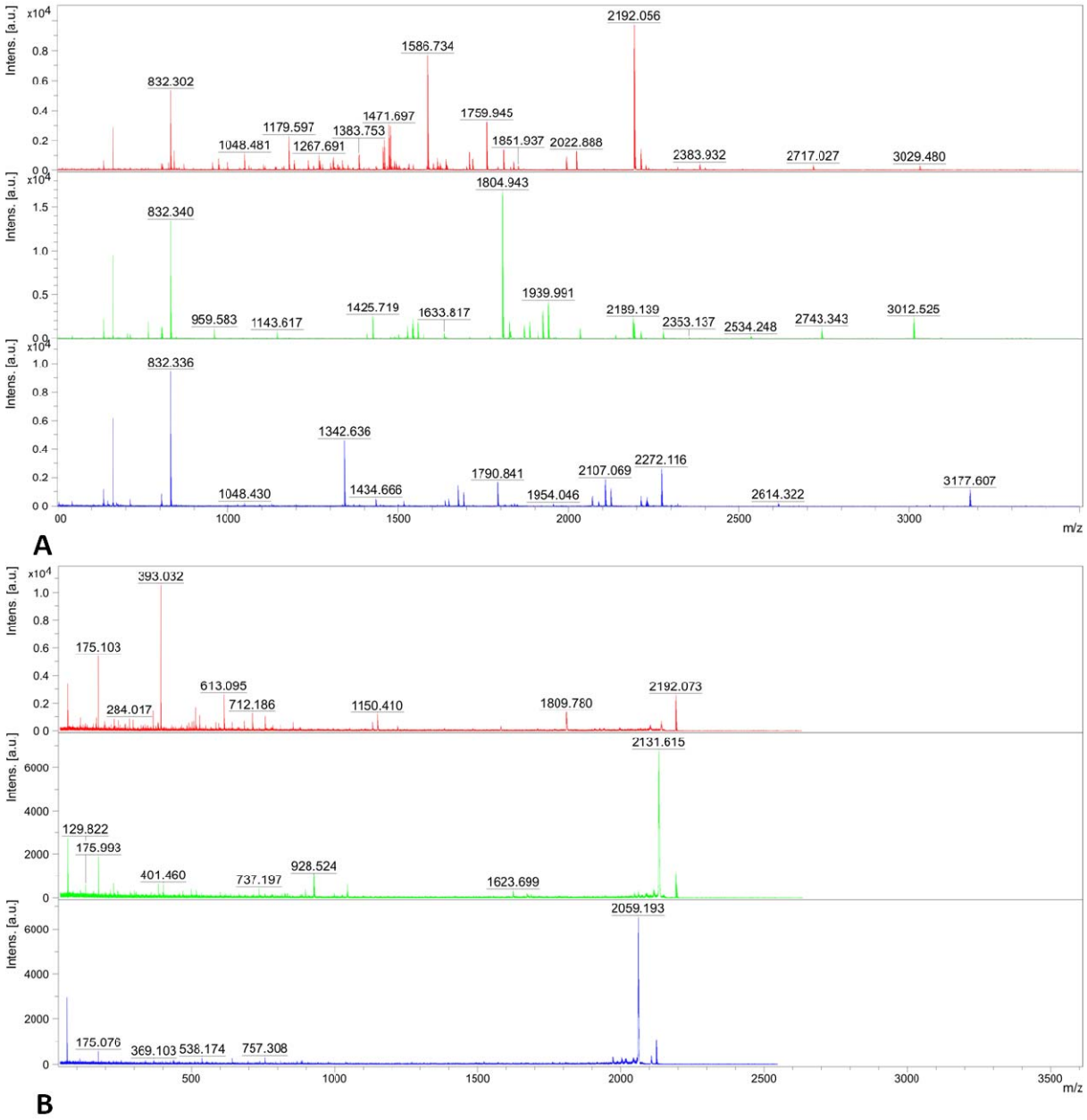


Figure 4.13: Examples of the digested TA's from the reference gel. In A, some of MS spectra are shown after proteins'digestion and desalting. The m/z ratio in Da is depicted on the x-axis, the y-axis marks the signal intensity of the detected peaks. In B, each selected fragmented peak (marked with same color as the appropriated MS spectra) is shown (MS/MS).

Figure 4.14 shows representatively one immunoblot after incubation with the BC serum pool, whereas the identified proteins are marked directly on the blot with respective protein name

in the provided Table 4-4. The encircled proteins were the most common identified proteins during several immunoblotting trials with the same tumor and sera samples.

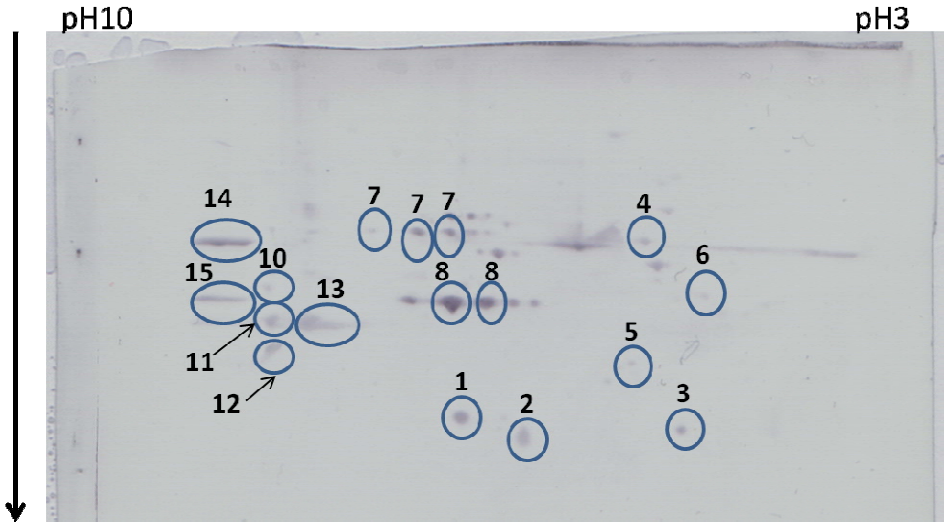


Figure 4.14: Exemplarily immunoblot, whereas the TA's were incubated with the mixed BC sera (the same immunoblot is shown in Figure 4.13). The spots of interest are marked with blue circles with appropriate numbers. The identified proteins are summarized below in the corresponding Table 4-4. The used pH range for IEF separation is depicted above; the black arrow shows the separation direction via SDS-PAGE.

Table 4-4: Identified proteins from the exemplarily immunoblot. The spot numbers are referred to the encircled protein spots in Figure 4.14 shown above.

ID number	UniProt entry	Abbreviation Id_HUMAN	Protein name	Gene name
1	P60174	TPIS	Triosephosphate isomerase	*tpi1*
2	P30041	PRDX6	Peroxiredoxin-6	prdx6
3	P52566	GDIR2	Rho GDP-dissociation inhibitor 2	*arhgdib*
4	P11021, P11142	GRP78, HSP7C	78 kDa glucose-regulated protein, Heat shock cognate 71 kDa protein	*hspa5, hspa8*
5	Q7L4N0	CAPZB	F-actin-capping protein subunit beta	*capzb*
6	P05787	K2C8	Keratin, type II cytosceletal 8	*krt8*
7, 13	P02768	ALBU	Serum albumin	*alb*
8	P06733	ENOA	Alpha-enolase	*eno1*
9	P00558	PGK1	Phosphoglycerate kinase 1	*pgk1*
10, 12	P04075, P62736	ALDOA, ACTA	Fructose-bisphosphate aldolase A, Actin, aortic smooth muscle	*aldoa, acta2*
11	P04406	G3P	Glyceraldehyde-3-phosphate dehydrogenase	*gapdh*

14	P62736, P60709	ACTA, ACTB	Actin, aortic smooth muscle; Actin, cytoplasmic 1	*acta2, actb*
15	P31948	STIP1	Stress-induced-phosphoprotein 1	*stip1*

Additionally, continually recurring spots on the immunoblots incubated with the CTRL serum pool were excised and the respective proteins were identified. As an example, the protein Alpha-enolase (ENOA) was identified on all immunoblots, non-regarding the type of the serum pool, whereas other proteins like peroxiredoxin-6 (PRDX6) or F-actin-capping protein subunit beta (CAPZB) were predominantly observed after incubation with the BC serum pool. On the contrary, protein cofilin 1 (COF1) was identified only after incubation with the CTRL serum pool (additionally provided in Repository figure 6). The main experimental steps were however predominantly realized with BC serum pools due to the limitations regarding tissue availability and amounts of the pooled CTRL serum specimens.

4.2.4 In silico analysis of identified tumor antigens

The identified TA's described in previous sections belong to the different protein types and act in several important pathways: *e.g.* signaling cascades of proliferation, energy metabolism, *etc.* Thus, some of the identified TA's were already identified in other analyses by different investigators (*e.g.* ENOA, heat shock proteins, histones, *etc.*). Summarizing networks were constructed with the help of STRING[204] software and also IPA[206] software to conduct an overview of relationships between all identified proteins and their involvement in the different biological pathways. The STRING-estimated connections between the identified proteins are shown in Figure 4.15. Notably, enzymes especially involved in glycolysis, are connected altogether.

The Ingenuity platform was used for more detailed analysis of protein relationships. This software is much complex than the STRING software and estimates the relationships between the proteins and their involvement into different pathways or their implications in the biological networks. The summary of these networks as well as the appropriate calculated probability scores of the molecular and cellular functions, canonical pathways and more additional data are provided in Repository figure 7.

Interestingly, the most significant biological network with the involvement of the submitted proteins via the Ingenuity platform as well as the most reliable disease and disorders hit, was referred as "cancer". Figure 4.16 shows this calculated cancer network with 11 implicated proteins and their interaction partners. Most of the proteins there are found in the cytoplasm. The constructed network clearly shows the involvement of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) as regulators of the pathways. This circumstance is in a good

concordance with the known relationship of BC and hormone impact on the HR^+ BC tumors predominantly used for the pooled serum experiments (details of pool composition are provided in Repository table 5)

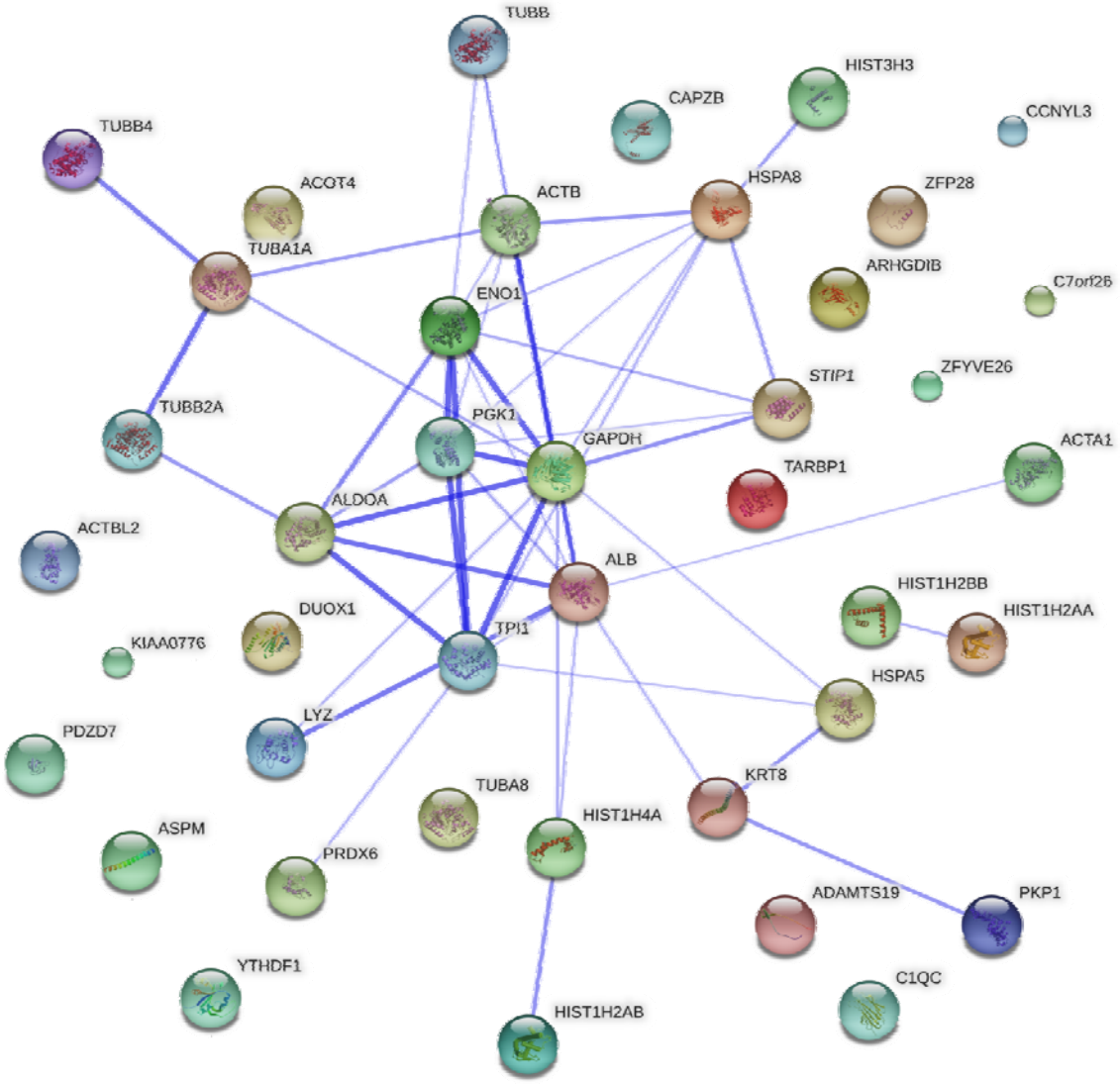


Figure 4.15: Confidence network view of the identified proteins. The respective gene names are given for every submitted protein (details are provided in Table 4-3 and Figure 4.13).

Additionally, the second network "carbohydrate metabolism, cell death and survival and gene expression" based on the significance level according to the Ingenuity scoring, is shown in Figure 4.17, whereby 10 other submitted proteins were grouped. Most of the identified cytoplasmic proteins (Figure 4.16) are in the direct relationship with the different transcription factors in the nucleus.

The results revealed additionally the submitted proteins to be significantly attenuated in over 36 canonical pathways, whereas the top canonical pathway was stated as glycolysis I (with implicated proteins ALDOA, G3P (GAPDH), ENOA (ENO1), PGK1 and TPI (TPI1)) in the

same manner as shown in the network constructed with the STRING protein software (Figure 4.15, see summarized data from the Ingenuity platform in Repository figure 8).

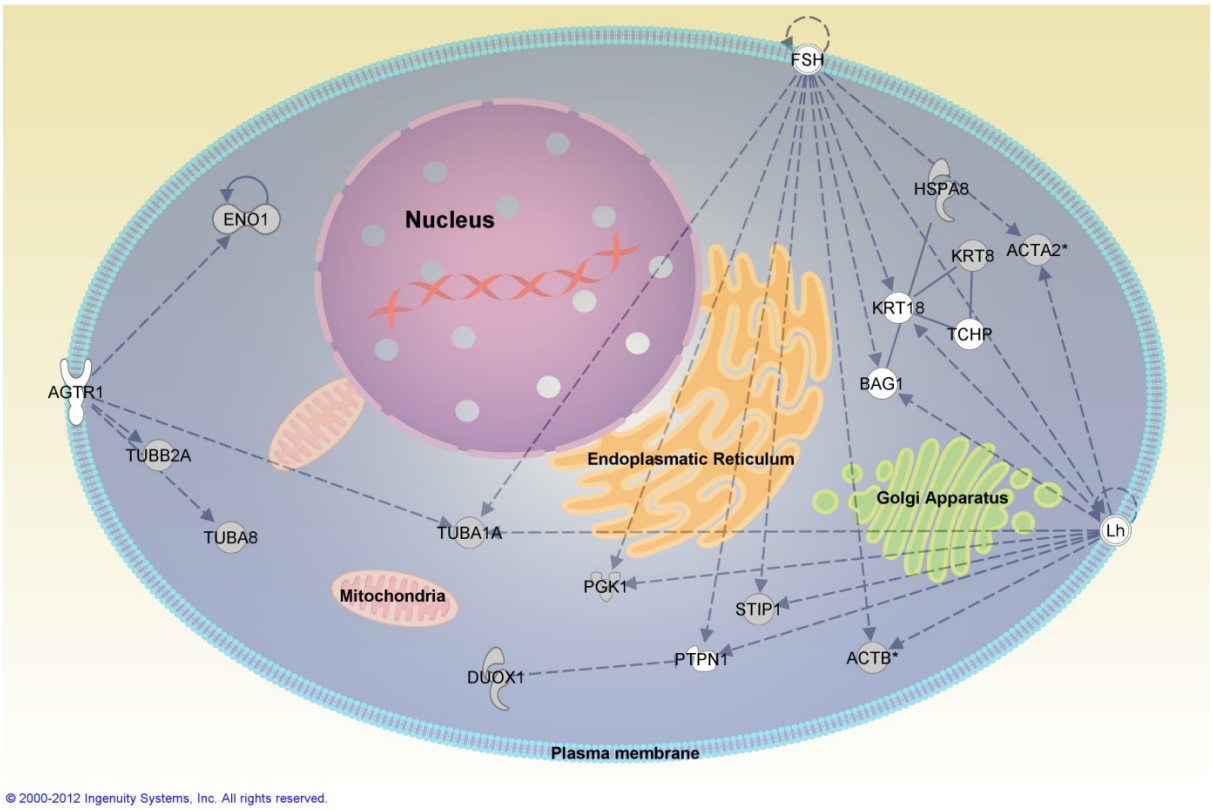


Figure 4.16: Most significant obtained network via the Ingenuity platform including 11 proteins from the submitted list (ACTB, TUBA8, TUBB2A, ENOA (ENO1), PGK1, STIP1, KRT8, HSP7C (HSPA8), DUOX1, TUBA1A). The network is stated as "Cancer, Endocrine System Disorders, Hematological Disease" and was modified with implemented Path Designer tool, The relationships are depicted with dashed arrows for indirect and solid arrows for direct relationship. The arrow direction indicates the impact direction. The circular arrow indicates the self-referential relationship. Additional details are provided in Repository figure 7.

Due to the purpose of cost-efficiency, not all identified proteins were chosen to be confirmed in the microarray-platform-based analyses. The strategy of further analysis was the combination of already known autoantibodies' responses of tumor patients with the previously unknown involvement of identified TA's and their corresponding autoantibodies. The appropriate candidates were predominantly intracellular proteins, as a large number of detected autoantibodies in cancer serum is directed against proteins from cytosol or even nucleus. Besides, a portion of the identified TA's was involved in the glycolysis. This is very intriguing as one of the tumor cells' hallmarks is the switch of the energy metabolism mechanisms. Indeed, the energy production in many tumor types, including BC is mostly restricted to the glycolysis and occurs therefore not via oxidative phosphorylation. This phenomenon is termed as the Warburg effect by its first describer Otto Heinrich Warburg and is discussed in chapter 5.

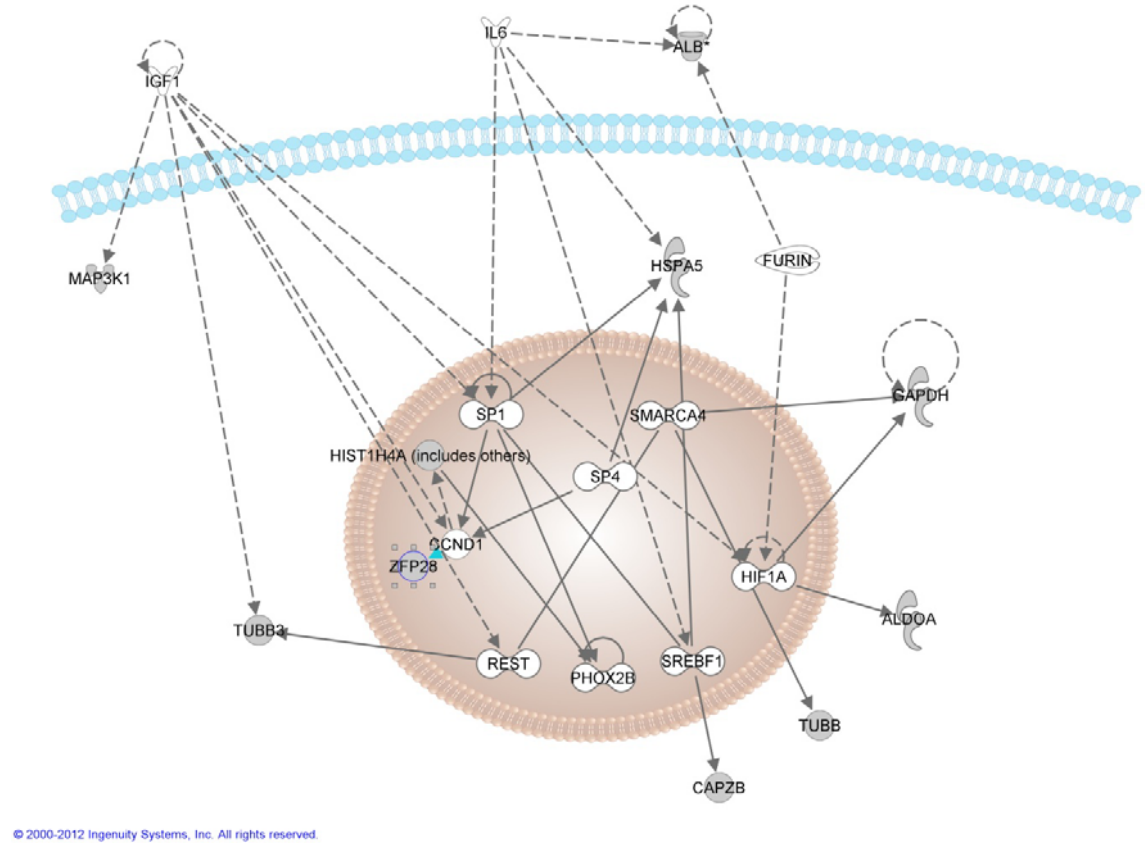


Figure 4.17: The second most significant network "Carbohydrate Metabolism, Cell Death and Survival, Gene Expression" which include following proteins: G3P (GAPDH), ALDOA, TUBB, CAPZB, ZFP28, TUBB2, M3K1 (MAP3K1), all histones, GRP78 (HSPA5) and ALBU (ALB). Note that most of the identified proteins are located in cytoplasm and are directly connected with several transcriptional factors in the nucleus (brown circle). The cell membrane is depicted with light blue color above the nucleus. Additional details are provided in Repository figure 8.

4.3 Selection of proteins for the construction of autoantibody profiling tool

The selection process of the commercially available proteins for microarray analysis was predominantly based on assumed relationships between the proteins, as estimated by *in silico* analysis. Furthermore, the selection of the already described putative TA's of other cancer entities was supported by the extensive literature research via PubMed resource (see the workflow in Figure 4.9). Glycolytic proteins ALDOA, ENOA and G3P were included in the microarray study. Additionally, STIP1 and GRP78 were purchased, as antibodies against them were reported in serum of OC patients[220,221]. The proteins COF1, PRDX6 and CAPZB demonstrated visual differences after immunoblots with BC and CTRL serum pools and were also chosen for the protein panel. Besides of the described candidates, also anti-histone antibodies were identified in cancer sera, including BC. Therefore, the protein H2B was included representatively as a nuclear protein[222,223]. Additionally, protein RECO was selected for the testing in the individual serum samples, as anti-RECO autoantibodies were described in patients with DCIS by Adamus *et al.*[224]. Additionally they were already detected in

increased amounts in subpopulation of patients with the cancer-associated retinopathy (CAR), a paraneoplastic epiphenomenon. CAR leads to retinal damage by the disturbing of photoreceptor cells. The last protein FETUA was added to the protein panel, basing on the investigations of autoantibodies in distant body fluids of cancer patients. Thus, anti-FETUA autoantibodies were reported as putative biomarker in urine of BC patients[141]. The next section describes the results from the individual serum profiling of cancer and healthy probands via constructed protein microarray-platform.

4.4 Individual screening of autoantibodies in BC and CTRL serum samples

The selected 11 proteins were applied for the individual screening of sera from 80 BC and 82 CTRL study participants from both approaches. The constructed network via the STRING software is provided in Figure 4.18. Except the proteins RECO (*rcvrn*), H2B1B (*hist1h2bb*), FETUA (*ahsg*) and CAPZB, other candidates were more or less linked together due to the co-expression, co-occurrence and other parameters described in section 3.9.

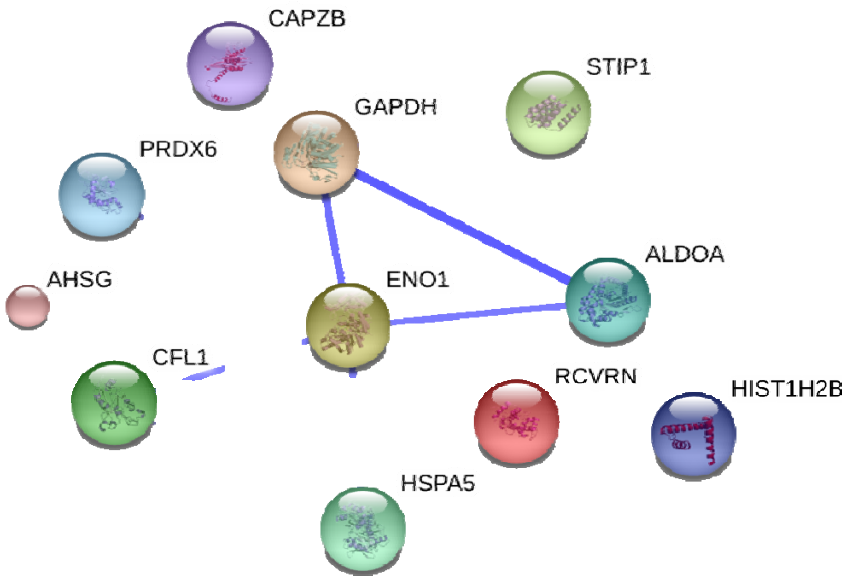


Figure 4.18: Confidence network view constructed by STRING software[204] including 11 selected proteins for the individual profiling of their respective autoantibodies in serum of BC and CTRL subjects.

The used protein microarray platform offers a high-throughput approach to profile up to 16 patient's samples simultaneously. The immunoreactions were visualized with the anti-IgG secondary antibody which enabled the capture of different isotypes of antibodies due to the presence of light chains, as they are universally distributed (see section 8.2 for details). Figure 4.19 shows an overview of two subarrays with the fixed proteins with an additional negative control subarray.

Figure 4.19: Overview of two protein subarrays for the determination of autoantibody levels in CTRL and BC probands. The positions of spotted proteins are shown on the left side. The subarray on the right side is incubated with serum sample without labeling with Cy5 dye (negative control). The proteins RECO, ALDOA and COF1 were spotted twice in different dilutions (as marked by small letters and/or numbers), whereas for the further analyze only one triplet of same dilution was used.

The complex autoantibodies' profiles in the BC and CTRL groups were visualized mostly in each patient. By visual comparison of the subarrays, several differences in the signal intensities were observed. Thus, reaction intensities with the proteins (shown in Figure 4.19) were investigated not only in one sample, but also via interindividual comparison as suggested in Figure 4.20. This circumstance enhances again the complexity of autoantibody responses and it points up the necessity of comparing not only diseased samples but also samples from the CTRL group.

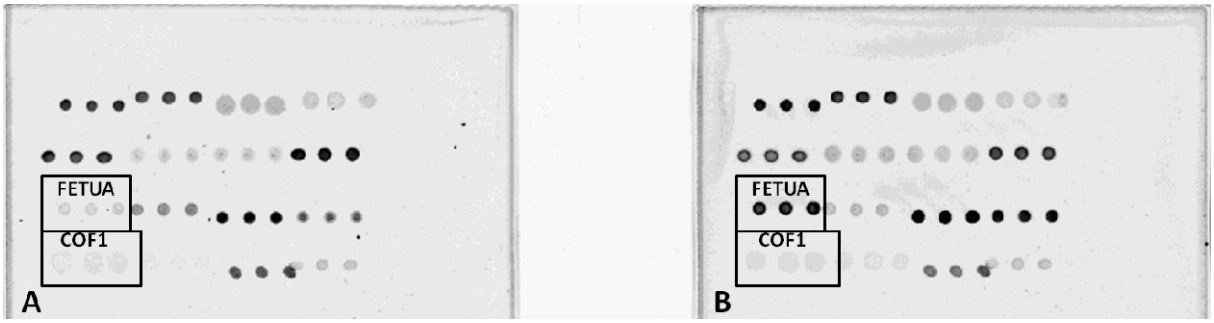


Figure 4.20: Two protein subarrays incubated with healthy (A) and with cancer serum sample (B), respectively. Some of the visible differences between signal intensities (higher in B) are marked by the boxes with the appropriate protein names over the spots.

Overall, most of the investigated proteins and their appropriate autoantibodies in the serum were visualized. In case of the protein COF1, even spotted twice in different dilutions, the antibodies against this protein were not detected on every subarray. This observation is important for the statistical data evaluation. After the analysis of the signal intensities via TIGR software (see 3.13.4 for details), the obtained data were normalized and compared between BC and CTRL groups as well as in the BC subgroups only (described in 3.13.3).

As the result, two autoautoantibodies were significantly different in BC and CTRL groups. The levels of anti-H2B autoantibodies were increased (p=0.03), whereas the anti-RECO-autoantibodies were decreased in sera of BC group (p=0.01). Figure 4.21 displays the significantly different amount of both autoantibodies in the groups.

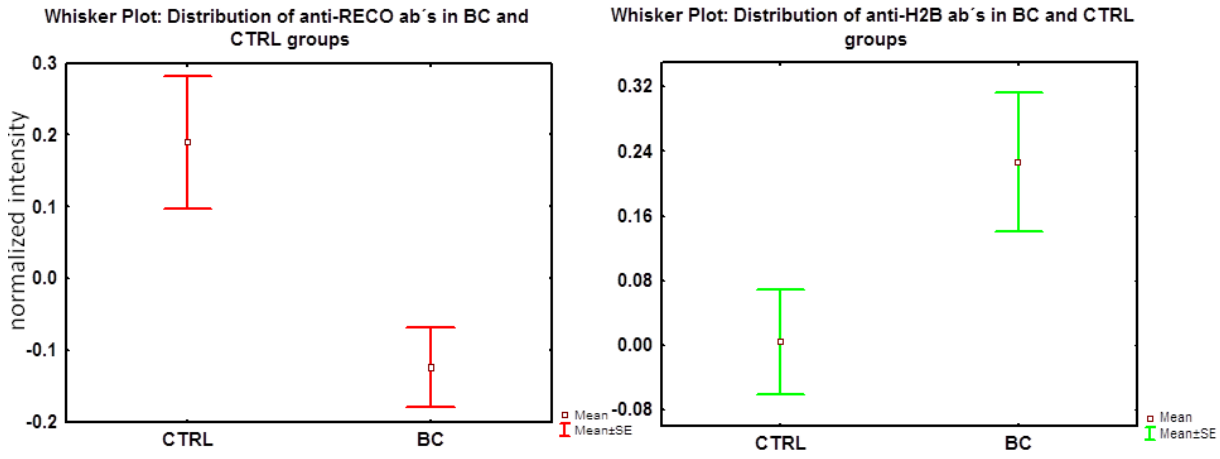


Figure 4.21: Different levels of anti-H2B- and anti-RECO-autoantibodies in BC and CTRL groups (p=0.03 and p=0.01, respectively), displayed as Whisker Plots with the mean value as a middle square and SE as whiskers. Both groups are located on the x-axis, whereas the normalized signal intensities are depicted on the y-axis.

Furthermore, it was very important not only to distinguish between the groups, but also to estimate possible differences in the levels of the autoantibodies in the BC group only, according to TNM and grading classification (see chapter 1.3.1 for details). The BC serum samples used in this study are summarized in Table 4-5 according to the respective tumors' characteristics together the hormone receptor status.

Also the profiling of the BC subgroups, according to the grading or tumor size (described in chapter 4.1) was aspired. No correlations were observed in case of moderately or high differentiated tumors or tumors with node involvement or else.

Interestingly, if only small tumors (T1, n=48) were included in the analyses for the comparison with the CTRL samples, anti-RECO autoantibodies were significantly different distributed in both groups in the same manner (p=0.002). No differences were observed by including only T2 tumors (n=26) in the BC group. Additionally, anti-RECO autoantibodies were decreased in the BC group by including moderately differentiated tumors (G2, n=40; p=0.03) or node-negative serum specimens (N0, n=48, p=0.007), and anti-H2B autoantibodies were increased in the diseased group if only well-differentiated (G1, n=14; p=0.03) samples were tested. Additionally, in the BC cohort with N1 serum specimens also anti-FETUA autoantibodies together with anti-RECO were decreased in diseased serum samples (p=0.04 p=0.02, respectively). Finally, anti-GRP78 antibodies were significantly increased in the BC cohort (p=0.026) by comparison of autoantibodies levels in poor differentiated tumors (G3, n=25) as shown in Figure 4.22.

Table 4-5: Summarized parameters from BC population used for the estimation of autoantibodies' levelsin individual profiling via microarray platform. Tumor's characteristics include TN(M) classification (*DCIS), grading and hormone receptor status. No patients developed distant metastases (M0).

Histological characteristics	BC group n =80	CTRL group n = 82
Mean age (distribution)	59.8 (34-85)	59 (36-84)
Tumor size	(%)	
pTis	0 (0)	
pT1	48 (61)	
pT2	26 (33)	
pT3	4 (5)	
other*	1 (1)	
Node status		
Negative	48 (61)	
Positive	31 (39)	
Grading		
Well differentiated (G1)	14 (17.7)	
Moderately differentiated (G2)	40 (50.6)	
Poor/undifferentiated (G3)	25 (31.6)	
Hormone receptor		
Positive	65 (82.3)	
Negative	14 (17.7)	

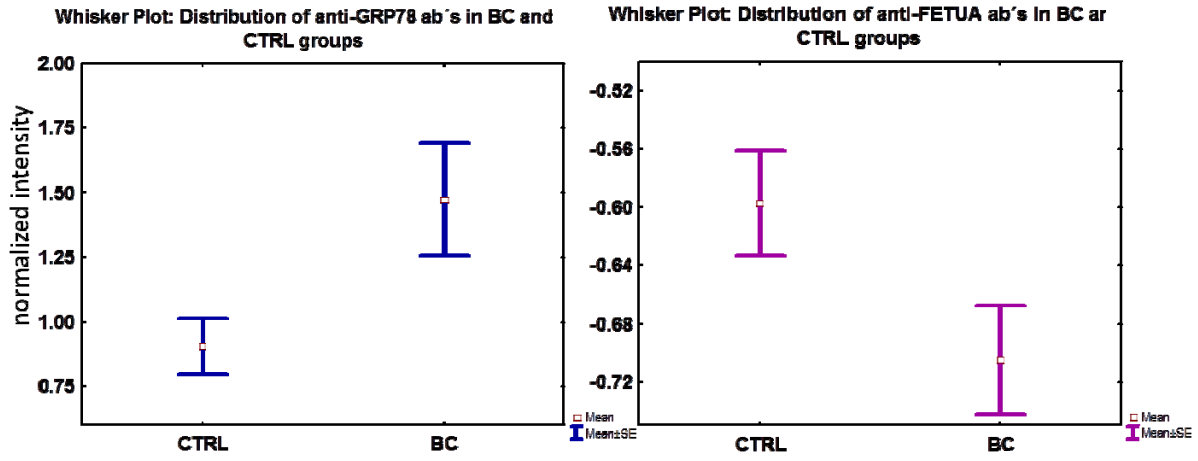


Figure 4.22: Different levels of anti-GRP78 autoantibodies in poor differentiated BC subgroup and CTRL probands

It was already described in section 4.1 by the evaluation of subgroups' data that the statistical analysis should not be overinterpreted due to the highly unequal sample sizes between the groups. Further analyses are needed with higher number of cases in the investigated subgroups.

4.5 Discrimination power of differently distributed autoantibodies

The ANN's were constructed using the signal intensities of immunoreactions between the proteins H2B and RECO and the appropriate autoantibody cohorts as inputs for the determination of discrimination power. They were used as standalone inputs or as a combination of both variables.

Summarized, the standalone inputs H2B reached an AUC of 0.61 with sensitivity of 63% and specificity of 64%, and RECO an AUC of 0.66 with 61% and 63%, respectively. The combination of both putative classification inputs was aspired for the possible enhancement of correct group recognition. Thereby, the combined AUC of 0.66 was achieved with the sensitivity of 63% and specificity of 60% by use of 81 values for the train set (43 from BC and 38 from the CTRL group). All calculated ROC curves are summarized in Figure 4.23.

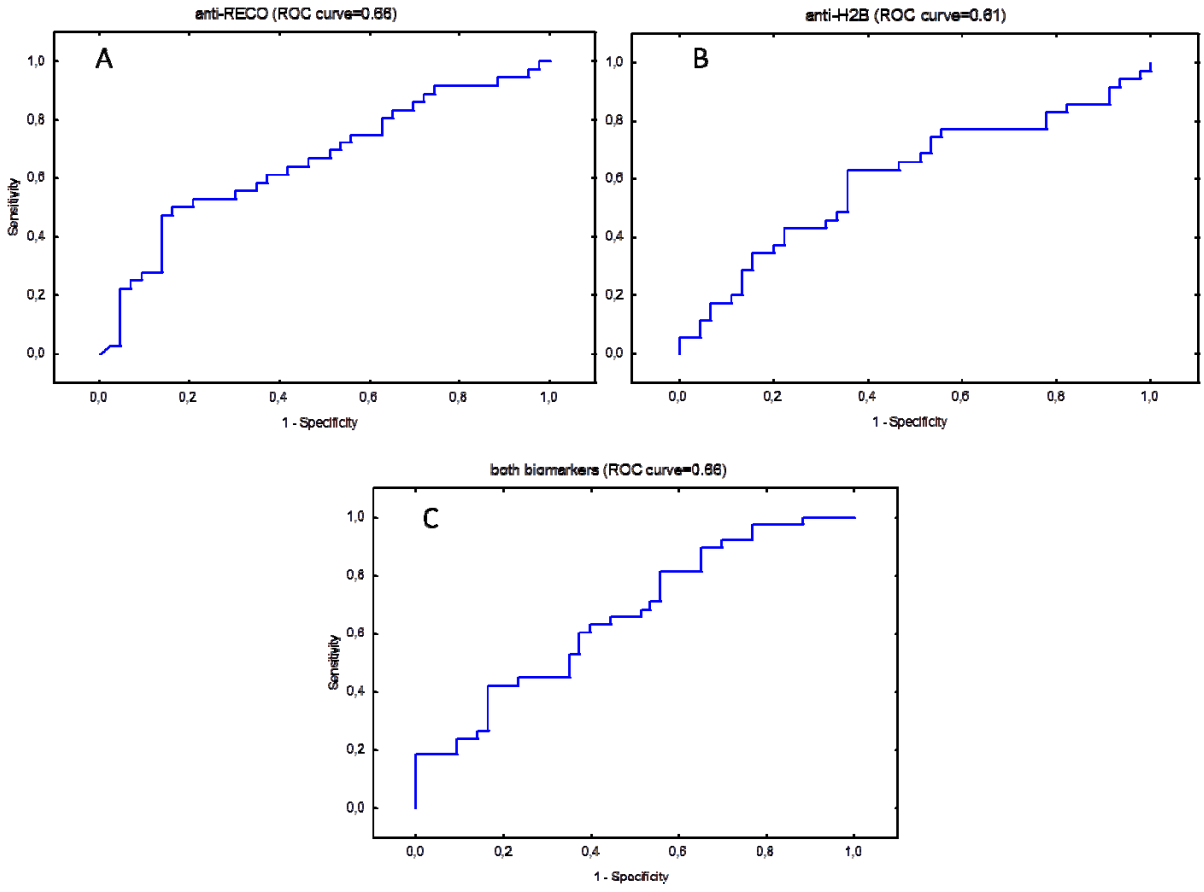


Figure 4.23: Summary of the constructed neural networks using A: values of the anti-RECO autoantibodies, B: anti-H2B autoantibodies and C: combination of both. In each graph, the sensitivity on the y-axis is plotted against the reciprocal specificity of the constructed networks on the x-axis.

The values of sensitivity and specificity regarding BC or CTRL group, together with number of used cases for the test data sets were additionally calculated and summarized in Table 4-6 as provided in section 3.13.5. The use of the combination of both values slightly increased the sensitivity and enhanced the specificity of the test. It means that healthy

subjects were more often recognized as CTRL. However, the sensitivity of only 40-42 % was not sufficient for the correct classification of diseased patients.

Table 4-6: Summary of the calculated clinically relevant sensitivity and specificity for each input value discussed above.

input for ANN	n BC test data	n CTRL test data	n BC correct prediction	n CTRL correct prediction	sensitivity in %	specificity in %
anti-RECO	43	36	17	21	40	58
anti-H2B	45	35	18	27	40	77
anti-RECO+anti-H2B	43	38	18	31	42	82

4.6 Recognition of DCIS by use of neural networks

In addition to the BC cohort, also serum samples from a patient with DCIS were included in all protein and autoantibody level measurements. DCIS is a specific non-invasive case of BC, which may turn into invasive carcinoma (see chapter 1.1.1 for details). Prior to DCIS diagnosis, the woman was not suffering from any other malignancies. These samples were measured with all other serum specimens simultaneously. The aim of this approach is to determine, whether DCIS samples are classified as BC group with the help of artificial neural networks. This circumstance may be useful for the detection of malignant affections in addition to the routine MG. The analyses were based on the previously identified protein and autoantibody biomarkers (see sections 4.1. and 4.4).

First, the ANN' were constructed based on the data set of measured protein levels in sera (see chapter 4.1). The training set consisted of random 50 % of data. As inputs, the normalized signal intensities of the putative biomarkers CYTT, A2MG, SG1D1 and ITIH4, whether as standalone inputs or in combination, were used.

100 neural networks were subsequently trained. The best five ANN's were chosen for the prediction of DCIS patient affiliation. In the next step, the signal intensities of all measured proteins from this sample were used as input and predictions based on the best five networks were made. All retained networks recognized DCIS sample as BC group, when the combination of CYTT+A2MG or of all four biomarkers (+SG1D1+ITIH4) was used as inputs.

The same procedure was performed, using significantly different levels of autoantibodies in the sera of BC and CTRL samples (described in 4.4). As inputs, the signal intensities of anti-

RECO and anti-H2B as a combination were used. Also using this combination, the DCIS serum sample was affiliated to the BC group.

The general principle of the recognition strategy is shown in Figure 4.24.

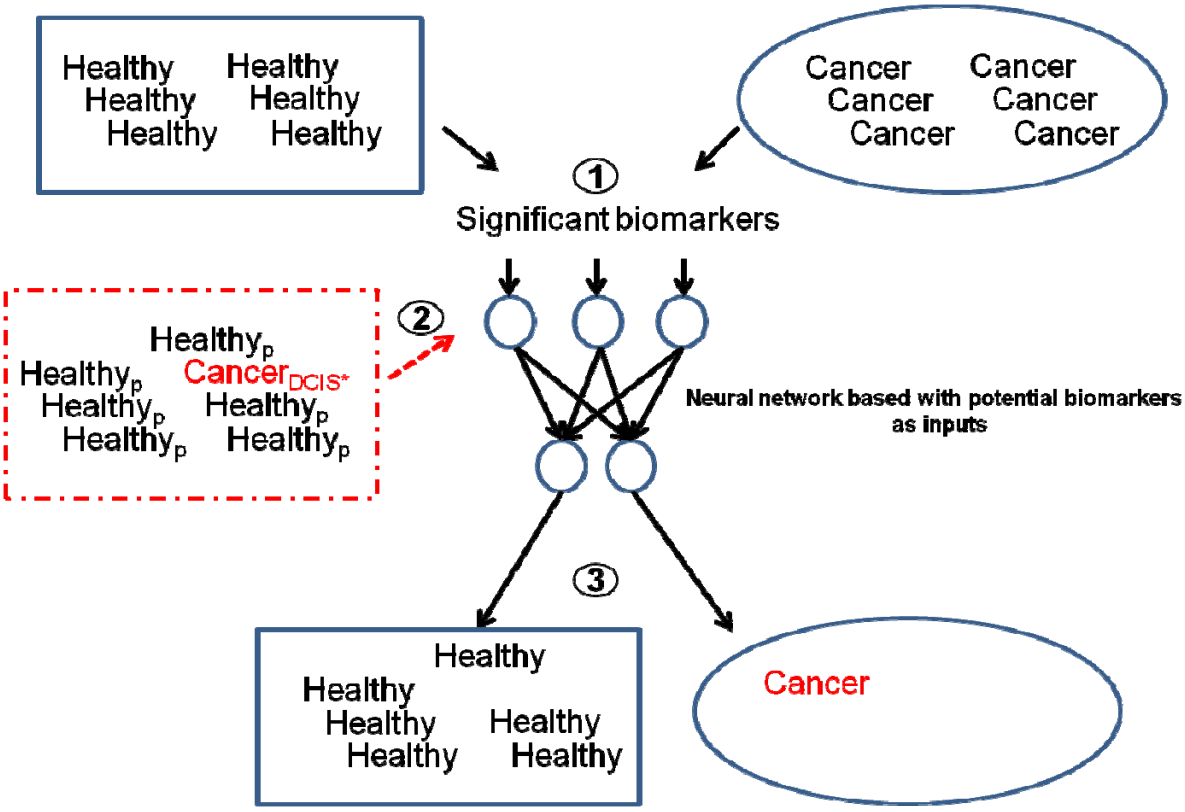


Figure 4.24: Schematically build-up of the used strategy for the recognition of DCIS patient with identified protein or autoantibody biomarkers. In the first step (1), individual serum samples of both groups (marked as Healthy and Cancer, respectively) were compared and in- or decreased biomarkers were estimated. A neural network with the MLP principle (see chapter 3.13.5 for details) was trained with their normalized intensity as inputs variables. The values of the same biomarkers from other population, which were not used for the training of the network, were tested in the second step (2) (the new population is highlighted with the dashed red line and the classification is shown in same way as above, whereas "p" for "putative" status is subscripted. The network could successfully discriminate the DCIS sample cancer serum specimen, as shown in the bottom of the figure (3).

4.7 Summary

Overall, the results of the PhD thesis point the complexity of changes on the proteomics level in presence of breast tumors. These changes were observed not only in the levels of selected proteins. Moreover, a complex pattern of circulating autoantibodies was visualized in BC and CTRL sera and their levels were successfully compared in individual samples, whereas significant differences were detected. The results are discussed in details in the next chapter.

5 Discussion

5.1 Estimation of protein levels in serum of BC and CTRL subjects

Recent developments of the proteomics-based platforms offer several opportunities for the direct level estimation of peptides and proteins in a high number of individual samples. Especially the microarray platform enables the simultaneous high-throughput sample comparison. The analytes of interest (commercially available antibodies) were transferred on the nitrocellulose-coated glass slides and incubated with individual serum samples of BC and CTRL subjects. Thus, it was possible to compare 16 individual samples on one slide with appropriate 16 nitrocellulose pads.

Construction of the antibody microarray set

The choice of antibodies, purchased for the level measurement in BC and CTRL of the appropriate proteins in sera of study participants, was based on the previous studies of the BC proteomics-based project. Partly, the proteins ITIH4 and APOC1 were chosen to improve the results of the SELDI-TOF- and MALDI-TOF/TOF MS-based protein profiling study in serum of BC and CTRL participants[91]. Briefly, the molecular weights of 14 significantly different distributed proteins were compared with fragments already identified by other investigators via PubMed-based literature search. Protein fragments of ITIH4 with molecular weight of 3972 Da and 4283 Da were increased in the sera of BC patients in our profiling, whereas putative fragments of APOC1 of 6629 Da and 6630 Da were decreased. The described fragment of ITIH4 (3970.97 Da by Villanueva *et al.*) was identified by estimation of protein levels in bladder, prostate, breast cancer and control serum samples[90]. It was observed in a higher level in bladder cancer samples, than in CTRL. The ITIH4- 4283 Da fragment was also increased in BC and pancreatic cancer samples. Lower level of APOC1 fragment (6631 Da) was found in serum of pappilary thyroid carcinoma samples compared to the CTRL population in another proteomics-based study of Fan *et al*[225]. Therefore, the current antibody-driven study should examine the general protein levels of ITIH4 and APOC1 in serum of BC and CTRL cohorts.

Our another antibody microarray-based investigation of protein levels in serum of BC and CTRL subjects revealed higher level of the cytokine inteleukin-6 (IL6) in the diseased patients[87]. The protein A2MG was added to the protein candidates due to its involvement into IL6-mediated signaling pathway[210].

The next biomarker candidates (MTUS1, S10A8, S10A9, TF and CYTT) derived from another previous comparative study of tear protein levels in BC and CTRL groups[113]. The

implementation of the protein Proline-rich protein 4 (PROL4) and SG1D1 was based on the previous SELDI-TOF MS-based intact tear protein profiling of BC and CTRL cohort[101]. The proteins were not identified simultaneously in the study, but the spectra of the proteins with significantly different levels in both groups were compared with previous studies in the experimental ophthalmology. Thus, the protein PROL4 showed decreased level in the BC group, and SG1D1 was increased. Up to date, no investigations were made according to the levels of PROL4 in the serum. Its expression in serum was however assumed based on the parallel approaches regarding expression of proteins in the serum. The function of PROL4 is currently unclear. Eventually, it might play a protection role by modulating the microflora of tear film[107,226]. The aim of including potential biomarkers from the tear proteome-based studies was the verification of biomarker potential in the serum.

The antibody microarray study shows different levels of proteins

The main protein array study was based on the data of 39 CTRL and 52 BC patients. 51 from 52 cancer patients were diagnosed with invasive BC; one patient was diagnosed with DCIS. The obtained data were used for the comparison of the appropriate protein levels not only in both groups, but also for the subgroup analysis in BC cohort according to different tumor properties. The statistical analysis revealed differences in protein amounts in the diseased and healthy groups. Thus, the protein ITIH4 was significantly decreased in BC group, whereas SC1G1 and A2MG were increased.

ITIH4 is decreased in BC sera compared to the CTRL samples

According to previous studies, fragments of ITIH4 were reported to be alternatively regulated in various diseases[90]. ITIH4 is an acute phase glycoprotein with the molecular weight of 120 kDa. It is cleaved by different endoproteases and exoproteases, especially in the proline-rich region of the COOH terminus (Gly^{611}-Gln^{730} aa positions). Two fragments (3972 Da and 4283 Da) were assigned to ITIH4 in our previous intact profiling study of serum proteins in BC and CTRL. It confirmed the results of several investigators, as they were also increased in BC sera in other proteomics-based studies[90,91,227]. Also other fragments of the cleaved ITIH4 were related to several diseases. Table 5-1 shows the summary of amino acid sequences of these peptides[90,227,228]. All fragments are cleavage products of ITIH4' PRR and of a kallikrein-released fragment (Pro^{662}-Arg^{688}, biologically active peptide, propeptide).

The main idea behind identification of these different fragments of ITIH4 and their relation to the several diseases is the assumption that the cleavage products are results of different exo- and endoprotease activity of tumor cells[90]. In the current study, the ITIH4 level in serum of BC was decreased (p=0.04), which is not necessarily a contradictory result.

Table 5-1: Cleavage fragments of the PRR region of ITIH4 (first eight fragments taken and changed from van den Broek *et al.*[227]). The number behind the abbreviation depicts the peptide length and the three-digit numbers points the respective aa position. The last both fragments were assigned to significantly different amounts of fragments in our previous proteomics-based intact profiling serum study of BC and CTRL samples[91].

ITIH4 peptide length	aa position	aa sequence
ITIH4-21	ITIH4 [667-687]	SRQLGLPGPPDVPDHAAYHPF
ITIH4-22	ITIH4 [666-687]	SSRQLGLPGPPDVPDHAAYHPF
ITIH4-25	ITIH4 [663-687]	GVLSSRQLGLPGPPDVPDHAAYHPF
ITIH4-26	ITIH4 [662-687]	PGVLSSRQLGLPGPPDVPDHAAYHPF
ITIH4-27	ITIH4 [661-687]	RPGVLSSRQLGLPGPPDVPDHAAYHPF
ITIH4-28	ITIH4 [660-687]	FRPGVLSSRQLGLPGPPDVPDHAAYHPF
ITIH4-29	ITIH4 [659-687]	NFRPGVLSSRQLGLPGPPDVPDHAAYHPF
ITIH4-30	ITIH4 [658-687]	MNFRPGVLSSRQLGLPGPPDVPDHAAYHPF
ITIH4-38	ITIH4 [650-687]	QAGAAGSRMNFRPGVLSSRQLGLPGPPDVPDHAAYHPF
ITIH4-40	ITIH4 [650-689]	QAGAAGSRMNFRPGVLSSRQLGLPGPPDVPDHAAYHPFRR

The main difference between current study and previous investigations is the use of an anti-ITIH4 antibody, which may react with different fragments of ITIH4 protein. Basically, the used sequence for the immunization of the host for raising the anti-ITIH4 antibodies corresponds with the 744-758 *aa* of the human ITIH4 (not located in the PRR region). Given the assumption that a special, disease-related activity of proteases, leads to an increase of some small cleavage fragments which may not bind to the anti-ITIH4 antibody at all, the antibody-protein- complex amount would decrease in the diseased cohort. However, further investigations regarding measurements of ITIH4 cleavage fragments are necessary for result confirmation. The amount estimation of the fragments can be performed using a semiquantitative approach or alternatively by absolute quantification strategy. As example, the exploration of ITIH4 fragments in serum by van den Broek *et al.* was performed by absolute quantification of fragments using high sensitive liquied chromatography LC/MS/MS

technique. Briefly, the calibration and working solutions for the estimation of the analyte amounts were achieved by the synthesis of ITIH4 cleavage fragments (see first eight synthesized peptides in Table 5-1). The appropriate peptides were measured in serum samples. Then, the amount of ITIH4 fragments of interest was estimated according to the amounts of diluted synthesized standard peptides. Afterwards, the technique was applied to six samples of BC and CTRL groups, whereas all described fragments of ITIH4 were increased in BC samples[227].

Despite the detection of ITIH4 level changes in cancer serum samples of different entities, no exact explanation is given to the present date. Nevertheless, during the intact proteomics-based individual profiling of BC and CTRL serum samples as well as by the current antibody-microarray approach, ITIH4 or its cleaved fragments played an important role by distinguishing BC samples from CTRL group. As is has been stated above, changes of levels may be due to different protease activities in presence of cancer cells[90]. The proteases may eventually increase their activity for combating cancer cells to prevent tumor cells' spread. This circumstance may explain our previous findings of increasing ITIH4 fragments in serum of BC patients[91] and also the decrease of ITIH4 by measuring its overall level via capturing the protein itself, as it may be more often cleaved. Both examinations confirm the alternative levels of this protein in connection with BC and may contribute to the insights of deregulated pathways in cancer. As another hypothesis, lower levels of protease inhibitor could point deregulated chemical pathways and signal cascades leading to changes of concentrations of cascade members and substrates resulting in the obtained observations. This idea is supported *e.g.* by Zore *et al.* by their investigations of complex Cathepsin B (cysteine proteinase) and Cystatin C (its inhibitor) and comparison of their single levels in diseased and normal serum samples. As a result, the amount of the complex form was decreased in sera of patients with malignant lung tumors and colorectal cancer in advanced stages[229]. Often the proteases, at least lysosomal proteases like stefins, are released through leakage of cells during apoptotic or necrotic processes as observed in cancer patients or individuals with inflammatory diseases[230-232]. These observations led to the assumption that an efficient protease inhibition is disturbed by different sophisticated mechanisms during cancer development and progression[229].

SG1D1 is increased in cancer serum specimens

Secretoglobins are small proteins including lipophilins and mammaglobins secreted by different cell types and tissues. Lipophilins are putative mammalian counterparts of the rat prostatein, a steroid binding protein with extensive homology, whereby the functional similarity is not well understood[233,234]. Mammaglobins belong to the uteroglobin family[235]. These proteins can form homo- or heterodimers or multimers over disulfide bonds and bind

progesterone and other aromatics[236,237]. Additionally, they are involved in different pathways for immune regulation[238,239]. The alternative name for SC1D1 is lipophilin A (UniProt/SwissProt database entry O95968). An investigational study by Lehrer *et al*. identified lipophilin A in non-denatured tear fluid sample existing as a heterodimer form of lipophilin A and lipophilin C[240]. Previously, no measurements of lipophilin A in serum were reported, at least the extensive search of scientific publication in the PubMed database did not provide any hits. Therefore the anti-lipophilin A antibody was used for the testing of the sera samples of BC and CTRL individuals. In the current study this protein was increased in serum samples of BC patients. Previously, two another secretoglobin family members, lipophilin B and mammaglobin A were reported to form a complex in breast cancer tissue, whereby only the second single protein was proposed as a highly specific biomarker for BC due to its overexpression[241-245]. Moreover, another member of secretoglobin family, lipophilin C (or lacryglobin) acting as heterodimer lipophilin AC in tear fluid, was correlated with BC as higher levels of lacryglobin in tear fluid of BC were detected by a proteomics-based investigation of Evans *et al*.[246] Additionally, mammaglobin A was also found in higher amounts in serum of BC patients by Bernstein et *al.*[247]. All these proteins can form homo- and heterodimers, bringing rather more difficulties into interpretation of results, whether the antibodies on the antibody microarrays or in other proteomics-based approaches detect single lipophilin A or multimers. No data regarding putative homo- or heterodimer forms of lipophilin A in other body fluids besides tear proteome are currently available. Nevertheless, increased levels of lipophilin A in BC serum samples are in good concordance with previously reported higher levels of secreted proteins in BC tissues[230-232]. Additionally, as SG1D1 is homologous to steroid-binding prostatein in rats, it may also be implicated in estrogen binding, and its different level in BC serum samples may refer to the hormone impact on breast tumors (see section 1.3.1 for details).

Also protein A2MG is increased in cancer serum compared to the healthy serum

A2MG belongs to the family of alpha-macroglobulins, which act like ITIH4 as protease inhibitor. Normally, A2MG is produced in liver by hepatocytes and also by microglia cells, and then it is secreted into blood stream[248,249]. A2MG exist in a homotetrameric form (also other forms were more recently detected) and is a very potent inhibitor, due to the so-called *bait* region, which traps proteases[250,251]. Thus, after their docking to this region and subsequent cleavage, the built complex is attacked and eliminated by macrophages. Structural polymorphisms of A2MG, especially in the *bait* region, are linked to the risk of Morbus Alzheimer (MA), as a part of beta-amyloid deposit cleavage. Normally, A2MG binds the beta amyloid peptide, whereas the potentially affected inhibitor function may lead to the fibril formations (plaques), a common event of MA[252-255]. A2MG is involved into blood coagulations

processes by inhibiting fibrinolysis and thrombin[256-258]. Additionally, A2MG may also bind different cytokines and acts therefore as a protein carrier[259]. A proteomics-based study with salivary proteins in edentulous patients with diabetes type II reported decreased levels of A2MG in comparison to diabetes-free healthy controls[260]. In another study by Smorenburg *et al.*, A2MG was shown to be secreted by colon cancer metastases cells in liver of the rats, although higher levels of A2MG were only detected in one study animal (n=8)[261]. We reported in our previous study higher levels of the IL-6 in serum of BC patients[87]. As A2MG is involved in IL-6-linked pathway[210], also its increase is plausible due to the cancer-specific changes in signaling pathways. Higher levels of A2MG in serum of pancreas cancer patients were further reported by Hanas *et al*[262]. Also measurements of protein levels in $HER2^+$-BC patients prior to chemotherapy and thereafter were performed by Mazouni *et al.*, whereby A2MG level was decreased in serum samples of therapy responder group[263]. According to this LC-MALDI-TOF/MS-based study, A2MG may serve as a potential prognostic biomarker in $HER2^+$ BC patients during chemotherapy. Additionally, a report by Ikari *et al*. suggest cell spreading function of A2MG, at least for the vascular smooth muscle cells which may eventually lead to tumor dissemination[264]. Relying on the performed extensive literature research, no significant changes of A2MG serum levels in BC serum and healthy controls in general were previously reported. As described for other increased or decreased proteins in this study several investigations of these protein levels in serum of diseased patients were performed with contradictory results. Higher levels of A2MG, a potential inhibitor of a large amount of proteases, may potentially be linked to the increased amounts of different proteases released from the tumor cells[230-232]. Further investigations are urgently required for the estimation, whether the increase of the proteins is caused by the enhanced response of a host, as a result of secretion by BC cells or their specific exo- and endoproteases' activity, which may also explain lowever levels in the present study.

Additional findings according the serum level of cystatin family proteins

The last protein found in decreased amounts in BC samples is the cysteine protease inhibitor Cystatin-SA (CYTT, *cst2*). It was excluded from the main analysis results because of the cross-reactions with other cystatins. Cystatin family includes intracellular stefins (type 1), secreted cystatins with protective functions (type 2) and kininogen-like family cystatins (type 3)[230,265,266]. Cystatin SA (CYTT) belongs to the secreted extracellular proteins. Other type 2 cystatins, revealing over 90% identity with CYTT, are cystatin SN (CYTN) and Cystatin C (CYTC)[265-267]. CYTT is normally expressed in submandibular and sublingual saliva. Previously, we reported increased levels of CYTT in mixed tear samples of BC patients[113]. CYTN is expressed like CYTT in saliva and also in urine, serum and malignant colorectal tissue[267,268]. CYTC is expressed in many body fluids and is a potential marker for Creutzfeldt-

Jakob disease[269]. No significant differences in levels of CYTN in the serum of BC and CTRL subjects were obtained in the same groups by our previous microarray-based investigations (unpublished data). Nevertheless, it is one of the possible cross-reacting partners of CYTT, together with CYTS. Different levels revealed in this study may therefore be due to the alternate levels of CYTT, CYTS or CYTN. From that reason the results were excluded from the main study. The comparison on microarray data of BC and CTRL cohort in the present experiment showed significantly reduced CYTT levels (p=0.01) in BC serum samples. Another study by Streckfus *et al.* showed lower levels of CYTT in mixed saliva samples from IIb stage (see Repository figure 3 for BC staging classification) of IDC patients in comparison to healthy saliva sample[270]. Lower levels of CYTT were found in mixed saliva samples of IDC IIa by examination of saliva proteins between IDC IIa and IIb stages. Additionally, decreased levels of CYTT in mixed saliva samples of $HER2^+$ BC patients in comparison to $HER2^-$ BC patients were also reported by the same author[271]. The possible explanation for any alterations in CYTT levels in saliva is the presence of HER2 receptors on the ductal cells of the salivary gland.

Due to the possible cross-reactions, this circumstance may potentially affect protein binding to the anti-CYTT antibody by allosteric competition and non-specific binding. Therefore, the estimated lower CYTT levels in BC group cannot be precisely explained. One possible reason is a modulation of signal cascades for arresting the secretion of this protease inhibitor and therefore a higher level of different proteases. Interestingly, some investigations clearly showed higher serum levels of CYTC in cancer patients and also in individuals with inflammatory diseases. For example, higher amounts of CYTC were found in sera of colorectal cancer[232] or in pleural fluid of patients with neoplastic and inflammatory lung diseases[272,273]. Even more, the high level of CYTC in serum was correlated with poor survival of colorectal cancer patients[232]. Also in case of CYTN, higher levels of this protein were measured in sera and urine fluid of colorectal cancer patients[268]. Additional studies with anti-CYTC and anti-CYTN antibodies as well as epitope screening studies for the detection of protein binding sites should be performed additionally. These investigations could help to estimate of binding specificity of used antibodies, as extensive identification of the important binding sites helps to ensure the validity of acquired results.

Discriminatory potential of putative protein biomarkers

In the present study with three significantly in- or decreased proteins, BC serum samples were discriminated from CTRL cohort with an overall sensitivity of up to 71 % in case of using all three biomarkers as inputs. The overall specificity of 77% was achieved. This circumstance does not enable the appropriate discrimination of CTRL persons, meaning that the use of this putative biomarker signature would lead to a high number of false positive

results. Healthy women would therefore receive false suspicious finding, which is a harmful emotional and physical experience. Even if the estimated values were lower in comparison to the MG (75% sensitivity and 92% specificity)[24,25], the results are nevertheless promising for the further development of the test. Additionally, the estimation of the sensitivity regarding the recognition of BC samples from the diseased group revealed a value of up to 100% in case of using SG1D1 or ITIH4 as standalone inputs or together with A2MG. These findings point the ability of detected biomarkers to highlight the aberrant processes in BC patients.

Additionally, false-positive results may in fact highlight early aberrant processes prior to clinical establishment point the possibility of recognizing malignancies more timely. We could also use the potential of the artificial networks, based on the protein levels examined with the same antibody microarray approach[87], for the recognizing of later diseased patients (IDC and DCIS respectively) ca. 3 years prior to the clinical diagnosis (unpublished data). Also the number of the so-called converters is too small to generalize the statement about the potential of the found putative biomarkers, it gives at least an outlook for deepening the current investigations.

It was however of main interest to combine the putative biomarker signature together with the MG, which is still the only available method for the early detection of BC. In fact, the use of CYTT together with other three biomarkers as input for the classification of patients with ANN's increased the discrimination power of the biomarkers and enabled the achievement of an AUC of 0.84 with much higher sensitivity of 83% and specificity of 82%. Summarized, the obtained high sensitivity of the derived signature resulting in even perfect discrimination of the diseased patients according to the BC subgroup only enables further development and improvement of the protein blood test. Eventually, the blood screening with the putative protein biomarkers would point the negative result based on the protein values, whereas MG examination may still indicate further tests.

Limitations of the present antibody microarray study

Several reports according to the measurements of protein levels in serum of cancer patients and healthy controls were described[81,92,93]. Additional investigations dealt with different phases and stages of diseases, comparison of protein levels prior to therapy or afterwards, monitoring the response to medication, *etc*[87,91,274]. Serum or plasma is a very potent source of protein biomarkers for discriminating multiple conditions in response to the environmental, medical or malignant impact. Moreover, investigations on the proteomics level offer several advantages in comparison to genomics-based strategies as changes of the intern or extern condition directly lead to measurable differences in the proteome. However, this circumstance may not always have a supportive effect. In contrast, by analyzing protein

levels it is important to minimize non-specific protein alterations: *e.g.* by simultaneous or head-to-tail processing steps of protein samples and identical handling of all analytes to prevent fluctuations and distortions of measurements. The advantage of proteomics as a mirror for any new or changing impact, whether of endogenous or of environmental origin, turns into a challenging factor, as proteomics fluctuations may not only derive from the malignancy alone. At the one side, the relevant biomarkers, specific for the examined condition, may be masked by *e.g.* degradation or cleavage products. On the other side, some effects may just be caused by different handling of samples and are not even remotely linked to the specific changes due to an external impact. Engwegen *et al.* reported in their study of SELDI-TOF- MS-based profiling of colon carcinoma serum samples the effects on results caused by different storage and clotting temperature, type of storage tubes and number of freeze-thaw cycles[275]. Thus, some of the proposed biomarkers for colon carcinoma were only based on the variances in processing steps. Interestingly, the authors stated the occurrence of several peptides deriving from the protein degradation and leading to the accumulation of artificially emerged peptides, even by the storage temperature of -20°C. However, these putative biomarkers still enabled the discrimination of the diseased samples from healthy control. Another investigator groups did not confirmed differences in protein profiling patterns of serum proteins stored by -80°C or -20°C.[96,276,277]

Many studies compare protein levels in diseased and healthy group without further investigations of the results. Meanwhile, investigations according biomarker specificity could stratify them *e.g.* into staging-related subgroup labels. The subgroup analyses were also performed in our previous studies of antibody-based serum profiling via microarray platform[87] or intact protein profiling of serum proteins by SELDI-TOF and MALDI-TOF/TOF MS[91], whereby different proteins were found to be in- or decreased in distinct subgroups of the used BC population. On the one side, discriminatory biomarkers, which stratify BC from CTRL group and do not vary in any cancer subgroup, may be further evaluated in an independent study, as they may present cancer-specific events. On the other side, the discovery of further biomarkers for BC subgroup distinction: *e.g.* lymph node involvement or metastasis presence/absence provides a possibility for the monitoring of the disease, progression or invasion status, *etc*. Additionally, better knowledge of cancer emergence and development may be obtained, as increased or decreased levels of biomarkers reflect deregulated pathways and signaling cascade in different BC stages considered in the subgroups. The examination of present BC group according to the lymph node involvement, tumor size and grading revealed no differences of protein levels in distinct cohorts.

Different distribution of proteins was checked based on the including of BC sera from only small tumors (T1, associated with better prognosis[56]), whereas ITIH4 was again decreased

and SG1D1 increased in the diseased group in the same way as in the main analysis. Both proteins may be therefore used for detection of at least small tumors, which may have a promising approach for further development of early detection of BC. Additionally, ITIH4 was differently distributed in the same way in moderately differentiated or node-positive tumors. Thus, analyses regarding levels of ITIH4 are needed to evaluate the clinically relevant benefit as a standalone biomarker. However, it should be kept in mind that unequal sample sizes were used for the estimation of differences in the subgroups. Despite of using different statistical algorithms, statistical bias due to this circumstance may not be excluded. More additional investigations with (more) equal sample sizes should be performed for the estimation of the results' validity.

Nevertheless, another additional challenge of comparing human cancer samples, which affects predominantly females is the correct interpretation of conducted results. As example, further investigations regarding hormonal status are needed. One of the critical points is the appropriate selection of patients and their age-matching in both cohorts. In this study the mean age distribution of the BC patients was 57.9, for CTRL subjects 57.6. The mean age of a BC female patient is 64 years[1]. This study included female subjects of age range 34/36-85 (BC/CTRL, respectively). As no clinical characteristics regarding the postmenopausal status were available, no further analysis was performed considering this stratification. Regarding however the hormone sensitivity of many breast tumors, it remains a crucial step to validate the obtained biomarkers in next studies, whereas the postmenopausal status of the study participants is documented. According to these findings, it is difficult to interpret, whether the changes of levels of described proteins derives from cancer presence only as alterations of the hormonal status may additionally play an important role.

Summarized, the discriminatory ability of these proteins, at least the high clinical sensitivity, offers the potential for further development and modification to improve the correct classification of both cohorts.

5.2 Detection of autoantibodies's patterns in sera of BC and CTRL groups

The autoantibody-based immune response in presence of tumor cells in humans was already described in several studies[146,185,278,279]. Additionally, autoantibodies of the IgM isotype may additionally persist as a component of the innate immunity[180,181,280].

The aim of this project was the discovery of new autoantibodies in serum fluid with subsequent identification of the appropriate TA's. This was realized through two different approaches. The first performed strategy was the protein G beads-based method of

capturing the TA's and subsequent incubation with the pooled serum samples containing autoantibodies as well as protein identification via MALDI-TOF/TOF MS. Another complimentary proceeded strategy was the separation of TA's by 2D SDS-PAGE and immunoblotting with pooled serum samples with the same protein identification procedure. The second extended part of the autoantibody-driven biomarker approach is the exploration of the autoantibody levels in individual serum samles using the most promising potential immunogenic TA's from both *de novo* identification experiments.

Protein G bead strategy for isolation of immunogenic tumor antigens

Using human samples in proteomics-based biomarker approach for detection of putatively specific autoantibodies in serum or other body fluids allowed several advantages. Thus, human native proteins and individual human serum samples from BC patients and healthy controls enabled the detection of putative *in vivo* immune reactions. Additionally, autoantibodies derive in early cancer stages or even prior to diagnosis and are stable molecules as they are not degraded by proteolytic processes[171,177].

Two different approaches were used to achieve best possible results for the identification of putatively immunogenic TA's: with or without prior enrichment of TA-specific autoantibodies. The IgG's from the native tumor protein sample were removed for both strategies prior to incubation steps with serum samples to achieve better specificity of results due to decrease of cross-reactions. This step is important, as also very small mammary tumors develop own blood vessel system. Overall, the method of the prior enrichment of potentially relevant autoantibodies of IgG isotype with their further coupling to the protein G beads and incubation with new tumor proteins could theoretically enhance the specificity of the identified TA's. In fact, some proteins like actin family members were identified after the subsequent MS analysis. Nevertheless, most of identified peptides were fragments of the immunoglobulins. Therefore, the output was not sufficient to support the chosen strategy. Besides, also high required amounts of tumor protein extracts did not allowed multiple repeats of the experiments.

The most promising results were obtained without prior enrichment of autoantibodies, whereby IgG's were first isolated from pooled serum samples of 20 BC patients and directly bound to the protein G beads. After that, tumor proteins were incubated with the captured IgG's and eluted in the last step of the considerably simplified experiment. During this experiment, over 30 different proteins were identified after incubating TA's with the isolated IgG's which were captured on the G beads. The protein identification list included again several parts of immunoglobulins. However, different additionaly proteins were also identified, *e.g.* many histone proteins. All of them belong to the core histones (H2A, H2B, H3

and H4), whose octamer is wrapped by chromatin and sealed by the linker histone H1. Also other identified proteins are of particular interest: *e.g.* cytoplasmic actin (ACTB), tumor suppressor E1 UFM1-protein ligase 1 (UFL1) or metabolic enzymes dual oxidase 1 (DUOX1) and acyl-coenzyme A thioesterase 4 (ACOT4). Notably, many autoantibodies against further enzymes were discovered (anti-TPIS and anti-ENOA, *etc.*).

Limitations of the performed strategy

Obviously, artificial reaction patterns due to the influence of experimental setup cannot be excluded. Basically, it is difficult to avoid that the relevant immunogenic TA's are removed together with the IgG's during the depletion procedure prior to incubation with the serum samples (see section 3.10). Furthermore, whole amounts of IgG, including potential autoantibodies against tumor antigens, were isolated from the pooled BC sera and captured on the protein G beads. This step is also error-prone under certain circumstances, as immunoglobulins may carry proteins or peptides and the binding of the relevant TA's may be complicated. Therefore, some relevant immune response players may not be detected. Moreover, this approach requires very high amounts of tumor proteins, so it cannot be performed several times because of the cost- efficiency and lack of the material. The first step of the enrichment experiment required capturing of tumor proteins on three nitrocellulose membranes, whereas the protein amounts of over 10 mg were used for each membrane. Besides, after incubation with serum pools and subsequent elution of TA's, the high protein volume required following drying in a vacuum centrifuge. Also this step may be critical for later investigations as some of the relevant peptides may stick on the surface of the tubes during the time-consuming procedure.

Additionally, the strategy of binding immunoglobulins to protein G beads followed by incubation with tumor proteins did not visualized autoantibody patterns, because only TA's are eluted in liquids from the bound IgG antibodies. Also several steps of this approach are error-prone and each additional processing step may lead to loss of potentially important antigens. One of the disrupting facts of the G bead- directed detection of TA's is the missing knowledge about sterically properties of TA's in the used buffers, whereby high amount of proteins may interact between each other and hinder the proper binding of respective autoantibodies. This circumstance certainly has an additional disturbing impact on the binding to the IgG's on the protein G beads in the prior step. The disadvantage of binding of tumor proteins *in solutio* is fixed by the prior separation and immobilization of proteins by 2D SDS-PAGE, a common proteomics strategy which was used in addition to the described protein G beads coupling strategy.

Immunoblotting – the second strategy for the identification of immunogenic TA's

The idea of immobilizing TA's via electrophoresis, whether one- or two dimensional with subsequent immunoblotting, was often described for the *de novo* detection of immunogenic antigens and is still widely used by different research groups[146,171]. The pursuit of this strategy in comparison to another carried out experiments should improve the identification of the TA's with immunogenic potential because of their spatial distance. Additionally, this strategy allows simultaneous visualization of antigen-antibody immune reactions by chromogenic protein detection. Even the use of the inexpensive horseradish peroxidase-coupled secondary anti-IgG antibody with low sensitive 4-chloronaphthol-based visualization method was sufficient to detect immunoreactions on all Western Blots. The cost factor and much smaller required tumor protein quantity (500 µg of each tumor protein extract were precipitated and then separated per 2D SDS-PAGE) made this approach more user-friendly and allowed several repeated measurements and experimental steps for the improvement of results. Thus, Western immunoblotting was performed for several times and the immune reaction patterns were compared from attempt to attempt. The visualization of signals revealed complex profiles on each blot. In parallel to the incubation with pooled BC serum samples, also an additional CTRL serum pool was used for the incubation with the separated TA's (see Figure 4.12). The immunoreactions were also observed as a confirmation of the natural occurrence of autoantibodies. Additionally, some of the immunoreactive spots varied in their intensities between BC or CTRL serum pools. *E.g.* the protein COF1 was only identified after incubation of tumor proteins with CTRL serum pool (see Repository figure 6). Overall, more spots were detected on the Western Blot membranes incubated with BC sera. Several steps like reducing and protein alkylation were additionally left out from the common digestion protocol described in 3.7 due to the specific treatment of IEF-separated proteins. This advantage may also contribute to more robust results, as every additional processing step could lead to loss of yielded proteins.

The identification of TA's revealed intriguing results. First, many of them were located in cytoplasm of cells similar to the proteins already identified with the protein G bead platform described above. Additionally to the detected enzymes via immunoprecipitation, five enzymes were involved in the glycolysis (ALDOA, TPIS, PGK1, G3P and ENOA). These results show consistently a specific pattern of autoantibodies' arise, at least of the IgG isotype, as they are directed against intracellular enzymes. Besides these results, also heat-shock proteins were identified: 78 kDa glucose-regulated protein (Heat shock 70 kDa protein 5, GRP78), and 60 kDa mitochondrial heat shock protein (CH60). In addition, some of the identified proteins were ubiquitously distributed (*e.g.* actins, tubulins, *etc.*).

As the identification of several potentially immunogenic TA's delivered promising results, the next step was the estimation of their respective autoantibodies' levels in the individual serum samples of BC and CTRL. Thus, more precise results after the analysis regarding the relevancy of the findings for the clinical utility were expected.

Further limitations of the used immunoblotting strategy

The correct and reproducible 2D SDS-PAGE separation of the proteins is susceptible to buffer composition, protein solubilization, reduction and alkylation *etc*[281-283]. It is also important that all IEF strips and SDS gels should run simultaneously to reach the most possible convergence in the protein separation. All performed standardizing procedures were already described in the section 3.6.

Additionally, for further examinations of autoantibody patterns in serum samples of healthy controls also native breast tissue samples should be examined instead of tumor protein separation. This possibility is however limited, as breast tissue is not homogenous and consists of different layers (as described in 1.1.2). For this reason, different reaction patterns may be visualized, depending on the composition of the tissue samples.

Only one tumor sample was used for the 2D SDS-PAGE separations and Western Blotting (IDC, T4 N1 M0 G3). Some reports suggest that the tumor antigen-mediated autoantibody response may vary in different cancer entities[284] or correlate with tumor' staging[195,285]. Thus, for a more detailed detection and interpretation of autoantibody patterns, also other tumor samples should be used for the enhanced detection of potentially immunogenic TA's. However, recent attempts with another tumor samples did not showed many differences after the visualization of the immune reactions. Also a lot of tumor proteins were identified again like ENOA and TPI. These observations lead to the assumption that at least some of the raised autoantibodies may persist in the serum independently from tumor staging.

The use of 4-chloronaphthol as a visualizing agent for horseradish peroxidase-coupled antibody had however an insufficient detection power, when individual serum samples were incubated with immobilized tumor proteins on the nitrocellulose membrane in first trials. This circumstance clearly shows on the one side, that another, more sensitive detection technique is necessary to obtain optimal results. On the contrary, the visualized autoantibody profiles with pooled serum samples showed that the visualized patterns may be visualized due to additive effects and did not represent individual reaction patterns. Additionally, the relatively high required amounts of serum samples (at least 500 µl) did not allow subsequent use of the individual profiling. For this reason only pooled serum samples were used for the Western Blot-based incubations with tumor samples.

In silico examination of putative immunogenic antigens

The submitted proteins belonged to the biological networks connected to cancer or carbohydrate metabolism (conducted via Ingenuity software in Figure 4.16 and Figure 4.17), whereby the submitted enzymes were involved in the glycolysis canonical pathway. Besides, the protein IL6 was also implicated in the cancer network. It was previously found in our investigation to be increased in the serum samples of BC patients[87]. Addtionally, IL6 increases the level of the protein GRP78 (*syn*. HSPA5), as shown by the investigators Vollmer *et al.*[286]. GRP78 was also identified as the immunogenic TA. These observations may support the hypothesis that autoantibodies hallmark the deregulated pathways in presence of cancer. For example, the protein M3K1 is involved in the activation of extracellular signal-regulated kinases (ERK), c Jun N-terminal kinases (JNK) and nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) signal transduction pathways, leading to activation of gene transcription and cell proliferation. M3K1 is additionally differently expressed in breast tumor tissues of different isotypes and has been also associated with the susceptibility for BC[287,288]. As the MAPK-pathway is also associated with the HER2/neu receptor activity, its activating mutations, at least in HER2/neu-positive breast tumors, were already described[289]. Therefore, anti-M3K1 autoantibodies may also label deregulated pathways in BC.

Consistently with the protein G beads-based approach, also cytoplasmic actin was identified as an immunogenic tumor protein, together with the muscle-specific actin ACTA and the cytoplasmic actin 1 ACTB. More interesting is however that a lot of autoantibodies against glycolysis-involved enzymes were persisting in the serum. As already described, tumor cells seem to prefer glycolysis for the energy metabolism. This effect was described by Otto Warburg stating that fast proliferating cells, including cancer cells, use the glycolysis and subsequently lactic acid fermentation for the energy metabolism on the contrary to common and more energy-efficient oxidative phosphorylation of adenosine-5'triphosphate (ATP)[290]. As the oxidative phosphorylation may be turned off in the tumor cells due to mutations in mitochondria' structures, increased rates of glycolysis and lactic acid fermentation were reported in several tumor entities. Thus, some of the tumors are located in the hypoxic area and their metabolism is therefore adapted to the environment, enabling fast cell proliferation[291]. The reasons for this phenomenon in tumor cells are not definitely understood to the present date. One possible explanation of the rise of autoantibodies against glycolytic enzymes may derive from hypoxia together with higher acidosis leading to the leakage of tumor proteins via apoptosis and/or necrosis[292-294]. Glycolytic enzymes are therefore released into the extracellular matrix or may be found eventually in aggregations on the surface of the tumor cells due to higher glycolysis rates. The immune system may subsequently react with

the rise of the autoantibodies against these normally intracellular, predominantly cytoplasmic proteins. Indeed, also most of the identified immunogenic TA's derived preferably from the cytoplasm of the cells leading to the assumption that autoantibodies may veritably originate as a response to the unusually high amounts of proteins, released or presented on the aberrant cells. Obviously, even if the immune system produce protective or regulating autoantibodies in the presence of malignant cells, the response cannot hold back, at least at the certain progression level, the emergence and spread of cancer (see section 1.4.1).

In conclusion, the chosen Western immunoblotting strategy for detecting the relevant immunogenic TA's was sufficient to detect complex autoantibody profiles after incubation of separated and blotted TA's with pooled BC and age-matched CTRL serum samples. Different detected immunoreactions varied in their intensities and also the incubation of TA's with the CTRL serum pool revealed some alternative reactions (see Figure 4.12).

The visualization of complex autoantibody patterns provides only limited information about the levels of autoantibodies and their relevance for the possible discrimination of BC patients. The main idea of this approach is the application of a protein signature based on the autoantibody response for the correct and specific separation of BC patients from CTRL subjects. In the best case, this signature would facilitate with the sufficient sensitivity and specificity the diagnosis of BC, together with MG as an additional detection tool in the clinical routine. As an option, some biomarkers could potentially serve for the estimation of tumor' staging, which can be a supportive strategy for the disease monitoring.

5.3 Individual serum profiling of autoantibodies in BC and CTRL groups

The highly precise technique of protein microarray was chosen for this part of the project. The counterpart of the used microarrays is the antibody-based microarray platform, which revealed interesting results after the subsequent individual profiling of proteins in serum samples of BC and CTRL subjects (see section 4.1 for details). The detection of different isotypes of autoantibodies was achieved using the secondary anti-IgG antibody containing the light chains, which are universally distributed in different isotypes of immunoglobulins (see section 8.2 for details).

Selection of the biomarker candidates for the protein microarray slides

The selection of commercially available proteins was based on the identified TA's after explorative experiments with pooled serum samples. Although the identified proteins from the protein G beads-based platform and immunoblotting did not show much overlapping, except

actin family proteins, some similarities were nevertheless noticeable. The main goal of the protein selection was the inclusion of identified intracellular and nuclear proteins as well as the combination with already described appearance of autoantibodies in BC and another cancer entities serum or in other sample sources. Thus, a portion of proteins possessed enzymatic properties and was involved in the energy metabolism over glycolysis. The enzymes ENOA, G3P and ALDOA were therefore selected as first-line immunogenic TA's. ENOA was found to be overexpressed in different tumors and also anti-ENOA autoantibodies in serum of different cancer types were described[195,295]. Anti-ENOA antibodies were also observed in sera of patients with CAR, which are however proposed to be directed against retinal ENOA[296,297]. Also the protein RECO was purchased due the reported presence of anti-RECO-autoantibodies in CAR-patients with DCIS[224]. *E.g.* in case of STIP1, its higher autoantibody titer was already described in connection with another gynecological malignancy, the ovarian carcinoma[220]. Also anti-GRP78 autoantibodies were detected in OC serum samples, whereby they seem to play a protective role, preventing the spread of tumor cells[221]. Antibodies against proteins from peroxiredoxin family were already found to be increased in serum esophageal carcinoma patients[298]. PRDX6 was therefore chosen for the protein test set. Anti-FETUA autoantibodies were found in urine of BC patients[157]. It belongs to the fetuin family and is also present in fetal blood. Therefore, as also predicted by investigators, this protein might be useful in connection with putative autoantibody signature, as autoantibodies against fetal structure and appropriate proteins were previously reported in cancer sera[150]. H2B was used to determine its autoantibody levels in BC group due to their detection in BC serum samples and other cancer entities[222,223].

As already described, only very small amount of serum was sufficient for this type of investigations, which is a very important cost-efficiency factor. In this case, only 1 µl of each serum samples (diluted 1:250) was used for each experiment. Also the amounts of proteins spotted on the glass slides were low (normally 1µg per antigen triplet or lower). This is also a very important issue, as the commercially available proteins are usually expensive (ranging from few hundred € up to several thousands) and their small required amounts can help to save the consumption costs. These advantages allowed repeating of several measurements in comparison to Western Blot and protein G beads-based strategy, whereby 500 µl of limited pooled serum samples were used for each prior experiment. The additive effects of these samples can be avoided with the individual profiling of samples leading to more precise results and potentially relevant proteins for the discrimination of BC from CTRL subjects. The relevant tumor characteristics were used for the expanded serum profiling regarding autoantibodies' levels in the BC subgroups (see Table 4-5).

Different autoantibody levels in sera of BC and CTRL groups

The main analysis of autoantibody levels in BC and CTRL samples revealed statistically significant differences in levels of antibodies against the proteins RECO and H2B. Interestingly, anti-RECO antibody levels were lower in BC patients (p=0.01), whereas anti-H2B antibodies were increased in the diseased cohort (p=0.03). The expression of recoverin is usually restricted to retina and pineal gland[299-302]. It belongs to the calcium-binding proteins and regulates normally the light/dark adaptation in the retina[303] via recoverin-mediated blocking of rhodopsin kinase, which phosphorylates the photoactivated rhodopsin molecules[304,305]. Primarily, anti-RECO antibodies were found in patients with different malignancies with the subsequent vision lost. CAR was first described by *Sawyer et al.* as a remote tumor effect causing progression of visual lost[306]. CAR is however not a common paraneoplasy and occurs in round 10% of cancer cases of different entities with the prevalence in women of 2:1[224,307]. Apparently, anti-RECO autoantibodies are somehow able to maintain the ocular-blood barrier and affect the retinal protein recoverin following by caspase-3- and caspase-6-mediated apoptosis and subsequent loss of sight[308,309]. Additionally, also healthy individuals showed the presence of anti-RECO antibodies, and in the present analysis the levels in the BC group were even decreased. Despite of many investigations of RECO and anti-RECO autoantibodies, most of the analyses are based however only on the cancer serum samples or with a small control group. The fact that also healthy individuals clearly demonstrate the presence of these autoantibodies may raise additional questions according the harmful potential of them in the host. One hypothesis may be the generation of the specific autoantibodies against RECO by the tumor protein RECO which is expressed on the tumor surface or within the cells. Also in cancer cell line the expression of RECO was observed in several investigations including breast cancer cell lines[310-314]. The investigators around Maeda *et al.* showed the expression of RECO predominantly in the perinuclear region of cells with endomembrane system association[314]. This circumstance may indirectly point the possibility to secrete RECO into the tumor environment or express the molecules on the surface of tumor cells, leading to arise of anti-RECO autoantibodies. Different immunogenic epitopes of RECO were also described. For example, in serum of a small cell lung cancer patient, as shown by Adamus *et al*., the anti-RECO autoantibodies reacted with the synthesized peptides, which were identical with the RECO sequence (62-68 *aa*) and different other *aa* positions[315,316]. The investigators around Senin *et al*. reported experiments with autoantibodies against RECO with two different immune dominant epitopes: anti-RECO-60-72 and anti-RECO-80-92. In the presence of these antibodies, RECO lost its ability to inhibit the phosphorylation of rhodopsin, pointing clearly the active inhibitory function of antibodies[317]. Additional analysis showed a Ca^{2+}-dependent manner of autoantibody binding to recoverin in the competitive ELISA study with

$CaCl_2$ or calcium chelator, ethylene glycol tetraacetate acid (EGTA), due to conformational changes in RECO molecule. As the result, RECO is recognized by different anti-RECO antibodies, however, not in the same strength. That means that RECO possess so-called calcium-sensitive conformational epitopes, which are recognized by a special cohort of anti-RECO autoantibodies[316]. RECO seems to be a potential immunogenic antigen with subsequent activation of T cells and anti-RECO autoantibodies[318].

Not only increased levels of autoantibodies were observed in disease conditions but also the opposite: *e.g.* decreased anti-ENOA autoantibodies in later stage of BC[195]. One of possible explanations in case of decreased autoantibody levels may be the loss of natural immunity. The potential role of autoantibodies has not been specified so far. The investigators Brandlein or Toubi assume that autoantibodies, at least of the IgM isotype, may have several regulatory functions. Decreased levels of autoantibodies may therefore be due to aberrant processes in connection with cancer emergence and development, leading to disturbing of the natural immunity[181,280]. Thus, tumor cells can silence the immune response by producing immunosuppressive cytokines and lead to the tumor immune escape[319]. As the result, tumor cells are not recognized and eliminated by the immune system and further development and growth of tumor cells, including metastasis, occur. As shown *e.g.* by Shih *et al*. the levels of anti ENOA-antibodies are decreased in late stages of breast and lung cancer patients in comparison to the healthy controls[195]. Therefore, decreased levels could be interpreted as the suppression of the immune response by arresting plasma cells through cytokine production. It is also possible that changes in TA's like. mutation or overexpression, contribute to epitope changing for antibody deriving leading to the production of other specific immunoglobulins with the new complimentary determining region (CDR). The relevant autoantibodies may therefore not be detected in the BC group, as the immunogenic epitopes are subjects for changes, due to aberrant protein structure. However, as the first step, the differences in levels of autoantibodies in BC and CTRL should provide in best case statistically significant alterations with the ability to correctly discriminate both groups

Interestingly, as described by Adamus, different autoantibodies, but not anti-RECO, were detected even prior to the clinical diagnosis of breast cancer[307] patients with the diagnosed retinopathy. *E.g*. autoantibodies directed against carbonic anhydrase II and transducin were measured in serum of future cancer patients[307]. This finding points the potential of autoantibodies for early detection of malignancies, although the described autoantibodies seem not to be specific for any entity of cancer. Figure 5.1 shows original sketch of the presence of autoantibodies against retinal structures in serum of tested study participants prior to cancer diagnosis.

Although primarily autoantibodies against retinal structures were described, they should be somehow able to maintain the ocular barrier and damage the respective targets in the retina. Adamus suggests that autoantibodies overcome it over unusually high concentrations and unspecifically intermalized by retina cells via endocytosis, whereas no exact explanations were provided[308,315].

On the other side, these proteins may be just expressed on the tumor cells, as already described for recoverin[314] causing the humoral immune response.

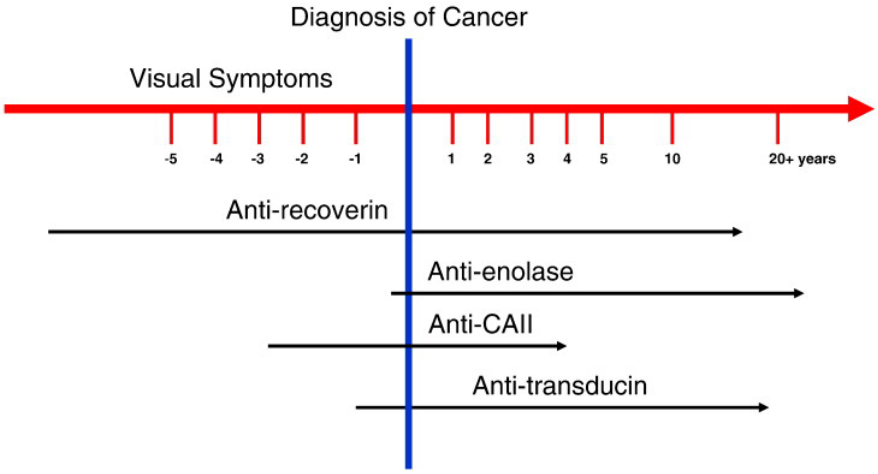


Figure 5.1: Presence of autoantibodies against retinal antigens in serum of cancer patients with visual disorders prior or simultaneously with the clinical diagnosis[307]. Thus, anti-transducin autoantibodies were detected in serum of later diagnosed colon cancer patients, anti-enolase autoantibodies by later prostate cancer, anti-carbonic anhydrase II (CAII) autoantibodies in lung, colon and prostate cancer. Also anti-recoverin autoantibodies were found in later lung cancer patients.

The level of autoantibodies against the protein H2B was significantly increased in the BC group on the contrary to the anti-RECO autoantibodies. Histone H2B belongs to the core histones, which build the so-called nucleosome with the DNA wound on it. Each histone can be post-translationally modified in case of transcription activation or repression. The combination of the modifications, basically on the N-Terminus of the histones may deliver the epigenetic code, a potential possibility for transcription regulation[320]. The modifications result in more or less strong affinity to DNA, enabling or inhibiting gene transcription. Figure 5.2 shows general modification possibilities on the N-Terminus of the histones.

The H2B protein was chosen for the verification of the respective autoantibodies' levels representatively. Thus, the identification list of the potentially immunogenic TA's included also other histone family members (see Table 4-3). As histones are highly conserved proteins, it may be difficult to recognize the identified proteins precisely.The findings should be therefore validated with other histone proteins.

Additionally, several isotypes of histone H2B exist, varying in some *aa* positions. No special isoform of the purchased protein was selected for capturing the appropriate antibodies in the serum of study participants. Therefore, several types of the autoantibodies were bound and visualized.

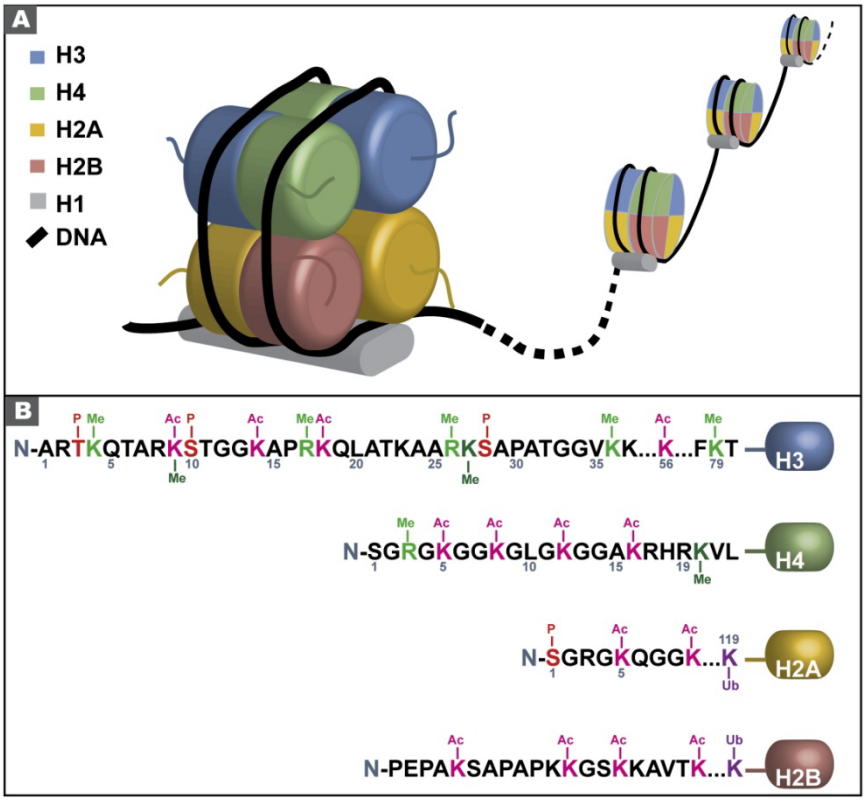


Figure 5.2: Structure of a histone octamer. The octamer is schematically shown in A, consisting of linker histone H1, core histones H2A and H2B dimers and H3 and H4 histones. In B, possible histone modifications leading to the transcription activation are shown above the sequence, inhibitory modifications are depicted below. The figure is taken from Hamon *et al*[321].

The presence of antibodies against different histones is not a rare event and has been predominantly observed in the AID's as described for SLE[322,323], whereby the diagnosis setting requires the routine blood test for the detection of anti-histone antibodies[324]. Autoantibodies to the histone dimers, at least in SLE-patients (*e.g.* anti-H2A-H2B), were described by Portanova *et al*[322]. Additionally, these autoantibodies are present in patients with rheumatoid arthritis[222] and systemic sclerosis (scleroderma), whereas they were correlated with a decreased survival[325]. Interestingly, the presence of anti-H2B antibodies in cancer serum were already reported by Kamei *et al.*, whereby cancer patients, at least with cervical cancer, were discriminated from healthy control subjects. The classification of patients with other cancer entities was less successful (*e.g.* 37% detection rate by lung cancer, 42% of colon cancer, *etc.*)[223]. Again, also in case of anti-histone autoantibodies no exact explanation of the rising is currently provided. One of the hypothetic explanations is the mentioned release of nuclear fragments from tumor cells as a result of apoptotic and necrotic

processes, which occur during tumor development and metastasis[292-294,326]. Additionally, high amounts of lactic acid due to the Warburg effect (see section 5.1) may cause apoptosis of the surrounding cells leading to release of intracellular proteins[291]. Additional studies with different modifications of histones are required for the confirmation of obtained results.

Another interesting approach included in the investigation is the alternative composition of the diseased group. The idea behind this is the question regarding changing levels of autoantibodies and a putative correlation of autoantibodies with tumor characteristics. These findings may facilitate monitoring of therapy success or disease progression. Besides, the presence of the specific autoantibodies for certain stage of BC may be a supportive tool for the histological examination and precise tumor characteristics. Otherwise, it may specify the conclusions from the findings, if the detected and identified biomarkers also vary significantly inbetween stages. The BC subgroups were compared in the present study not only within the BC cohort, but also with CTRL samples. The statistical significance of biomarkers was verified according to the grading (G) and tumor size (T). Most interesting results were achieved, by including only small or well- and moderately differentiated tumors in the BC group. The statistical analysis revealed that anti-RECO and anti-H2B autoantibodies were significantly de- or increased in the sera of BC patients, respectively, likewise in the whole BC population in the general analysis. The finding of alternatively regulated autoantibodies in small tumors supports the idea of an early immune response of the host and may be a promising tool for the detection of small tumors, which are hardly recognizable with the MG, and may lead to a development of a supportive clinically useful blood screening test for the early detection of BC. This circumstance shows again the promising potential of autoantibodies' level comparison, especially in small tumors with better prognosis. In node negative patients also anti-FETUA-antibodies were significantly decreased. Previously, the investigators around Yi et al. showed higher levels of anti-FETUA in serum and urine of BC patients[157]. However, the study population was much younger than in the present project, whereby the mean age of BC cohort (59.8) of the present investigation is closer to the average age of BC-affected women (64 years)[1]. Additionally, no TNM classification of BC patients was provided in the respective publication. In the cohort of poor differentiated tumors G3 the anti-GRP78 autoantibody was significantly increased (p=0.026) in the BC group (25 samples total). Also in patients with OC, anti-GRP78 autoantibodies were already reported, whereas they even revealed a possible protective effect[221]. The findings should be however specified with the appropriate number of sample cases as partly very low case numbers in the distinct BC subgroups were used for these additional analyses. Thus, the statistical difference in the autoantibodies' levels may be biased. However, although the results should not be overinterpreted, they still may point the trend of early antibody arise in small tumors.

Discriminatory potential of the putative autoantibody biomarkers

As revealed by the construction of the ANN's with both biomarkers as inputs, the sensitivity of the test was very low (63%, see section 4.5 for details). The specificity of the test, if both biomarkers were tested together as inputs, was only 60%. These values were too low for the correct discrimination of both groups. On the other side, by estimation of the specificity regarding the recognition of CTRL subjects in the healthy group, a value of 82% was achieved if both biomarkers were used as inputs. The autoantibodies were therefore more able to recognize at least the healthy subjects. This circumstance may also contribute to further development of BC detection tool.

In the current study no women from the control group was diagnosed with BC until the completion of the PhD thesis. Therefore, no predictions are possible regarding the time of the autoantibodies' arise. This fact relies not only on the described literature and findings of their detection prior to clinical onset of certain diseases (as shown above by Adams[307] for cancer but also for AID's[327]). With the protein biomarker signature from the previous antibody microarray profiling of serum protein levels, two later diseased patients were correctly correctly recognized as BC with the help of the constructed ANN's[87] (described in section 5.1). The achieved results may increase the potential of detected biomarkers for the early detection of BC, whereas they were regarded as the "false-positive" results at the time of the profiling.

Summarized, the discrimination ability of autoantibody biomarkers revealed quite the opposite results as the use of protein marker levels, whereby the achieved sensitivity was much higher in comparison to the specificity of the test (see section 5.1 for details). Thus, more healthy participants were correctly classified. This result offers nevertheless an equally perspective for the further development of the biomarker signatures for the detection of BC.

Effective recognition of the DCIS serum sample using ANN's

This additional project investigated the ability of the constructed neural networks to recognize a DCIS serum sample as diseased. It was indeed possible to classify this non-invasive breast malignancy as BC. The networks correctly identified DCIS specimen as cancer sample, whereby the values of the biomarkers from this patient was not used whether for the train nor for the test set. Even more, as DCIS is an often preliminary stage of invasive breast cancer, the discrimination of this serum sample from the healthy study participants showed the enhanced potential of the derived autoantibody signature. However it should be also taken into account that only one serum sample was used for this analysis, which is definitely an insufficient fact for the general postulation of this finding.

General facts regarding different levels of autoantibodies in BC and CTRL individual serum samples

Summarized, the new findings clearly show the ability of proteomics technique to differentiate between BC patients and CTRL and even the subgroups of BC according to the histological characteristics. Although the findings are not free of limitations, they may be improved in further explorations. Overall, the first results provide the fundament for an establishment of a biomarker assay, which can discriminate BC patients from the CTRL group. Nevertheless, it should be kept in mind that also healthy patients, at least cancer-free, also possess the autoantibodies against all used proteins in the constructed screening microarray platform. The investigations showed a sophisticated autoantibody pattern in probands. Generally, the most promising identified tumor proteins via Western Blot and G-bead-platform, which are putative counterparts of autoantibodies, were used in the individual screening with the result that not all of them are present in serum samples of both groups in significantly different levels. As already discussed above, it can be attributed to the undesired additive effects of pooled serum samples, whereby signal differences are artificially enhanced in both groups. Moreover, the autoantibodies against these proteins seem to be widely distributed in most samples, even of the CTRL group. Also the question about raising mechanisms of autoantibodies should be further discussed. Perhaps the most plausible explanation is the presenting of different tumor antigens by cells themselves or the secretion or leakage of intracellular structures into tumor microenvironment[141,154,155]. Whether these antibodies possess some regulatory mechanisms, remains unclear. Additionally, increased levels of antibodies in cancer may contribute to the protective autoimmunity as described by Toubi and Shoenfeld and others, as they were correlated with the improved survival of cancer patients[280]. Most of the described protective autoantibodies are of the IgM isotype coded in the germ line (see chapter 8.1 for short overview of antibodies). They possess the ability to bind different structures of tumor antigens leading to their apoptosis[179,280]. Due to usage of only unmodified proteins for detecting autoantibodies, not all cancer-related differences in autoantibody levels were observed: according to several investigations, a lot of tumor antigens possess aberrant structure because of occurring mutations or post-translational modifications. Therefore, additional investigations with modified proteins for capturing more autoantibodies are needed.

Despite of the future potential for the BC detection applying the found biomarkers, many difficulties in the interpretation of the results may occur. Therefore the conclusions should be interpreted with caution. On the one side, the presence of autoantibodies is clearly shown not only in the diseased cohort but also in the healthy group. The relevancy of these findings should be further specified in larger population-based studies. *E.g.* Adams reported the

presence of autoantibodies against retinal structures in the long period prior to the clinical diagnosis of cancer[307]. However, no additional information about the patients: *e.g.* hereditary predisposition, age, environmental impacts were provided. More analyzed data are therefore necessary for appropriate conclusions.

Another important issue is the use of the biomarker signature for the discrimination of diseased patients from the healthy controls versus usage of single biomarkers. Also in the present studies, the combination of many biomarkers increased the specificity and the sensitivity of the classification possibility, especially in the study of protein levels in the BC and CTRL groups. This finding correlates with many other investigations, whenever by measuring the levels of serum proteins or autoantibodies. Basically, the results underlined the affection of whole organism by breast cancer, whereby several signal cascades and the upcoming processes on different levels are modified in many ways. As still no other BC detection possibilities besides imaging techniques are available, the additional tools like molecular biomarkers remain as a promising approach with still ongoing search for the appropriate molecules. In best case, the use of a biomarker panel would allow a highly sensitive and specific detection of early stage BC leading to treatment' facilitation and making full recovery more reliable.

6 Perspectives

The results of this PhD project show alternative levels of proteins and autoantibodies in serum samples of BC patients and CTRL subjects. These biomarkers may help to developa clinically usable molecular tool for the earliest possible detection of BC, as only MG is currently available for that purpose. As the first goal, the results should be verified in several manners in additional studies which are further briefly described with the respective usable strategies. In general, the next aspired step is foremost the validation of results in an independent study population and subsequent multicentre studies with population-based analysis. Additional requirements of these tools should be their high intra- and inter-institutional reproducibility and cost-efficiency. In best case, a biomarker signature is then available, which can be used in future as supportive method to detect BC.

Verification and validation of de- and increased proteins in serum of BC patients

The antibody-microarray-driven approach revealed de- and increased proteins in BC patients. Further investigations are necessary to confirm the specificity of these findings. Especially the effect of CYTT measuring and the significant improvement of sensitivity and specificity of patient' discrimination from CTRL subjects should be verified. For that purpose, the used protein panel should be again examined and validated in an independent population, including serum samples from primary BC and age-matched CTRL probands. Also the subgroup analysis should be simultaneously performed. Thus, preliminary findings of the current protein study suggested that biomarkers from the main analysis were in- and decreased *e.g.* also in small tumors (T1) in comparison to the CTRL subjects. Due to the very unequal sample sizes the results should be verified with appropriate number of samles. The same approach may be applied for the examination of protein level differences between different subgroups of the BC cohort. The obtained results may be furthermore investigated in an enhanced study with different types of BC, as almost all tumors belonged to the invasive ductal type in the present investigations. Thus, the inclusion of serum samples from DCIS and LCIS patients may provide an enhanced insight into protein levels changes. Additionally, the effective recognition of these patients as cancer group should be examined again, as only one serum sample from one DCIS patient was used in present investigations.

The results of studies may deepen the understanding of mechanisms which cause the observed effects. Thus, the emergence and development of the tumors seem to change the common biological pathways and interrupt the signal cascades, leading instead to aberrant pathways. As a hypothesis, different exoprotease activity may be reflected in different levels

of protein fragments[90]. Otherwise, some of intracellular proteins may in released into body fluids via apoptosis and necrosis of cells during cancer progression.

Verification of alternative autoantibodies' levels in serum of BC patients

Most of the identified immunogenic TA's in both exploration aapproaches were not included into the protein microarray study of individual autoantibody levels in serum samples. Thus, more candidates may be purchased for further profiling: *e.g.* other histones and histones with different modifications, as well as other nuclear and intracellular proteins.

The present study offered not only increased but also decreased levels of autoantibodies in serum of BC patients. The findings should be also verified in an independent population with the used protein-microarray platform as described above. Additionally, also other tumor types should be used for experiments as only one tumor sample from an advanced invasive ductal tumor was applied in the present experiments. Further analyses with tumor samples with different anatomical and histological properties may deepen the understanding of autoantibody arise and lead to establishment of early biomarkers for the detection of BC. One of the main requirements for a BC detection tool is the early and constant presence of in- or decreased autoantibodies in all examined BC serum samples, derived from tumors of different characteristics. On the other hand, more specific biomarker for each BC staging may also be postulated in these comparative investigations, if they precisely discriminate the ongoing changes according to advanced BC stages.

The same approach regarding subgroup analyses of BC group (*e.g.* according to grading, or TNM classification parameters, *etc.*) as described for the different protein levels ahould also be applied. Additionally, anti-RECO autoantibodies were decreased in small tumors (T1) in the same way like in the main analysis including the overall BC study population. This finding should be verified with the appropriate number of BC samples for more robust statistical analysis.

Apparently, a large number of the autoantibodies circulate as a humoral response to the immunogenic TA's. After verification and validation of the autoantibodies, the epitope screening of TA's should be performed for the more precise findings. Consequently, additional information of autoantibodies' deriving would be available, as the mechanism is not completely understood. Thus, different investigations already described the modified epitopes[141,171]. As the result, also a new panel of immunogenic structures of tumor fragments (short immunogenic peptide sequences) may be used instead of whole proteins enabling in best case the more sensitive and specific detection of BC.

Exploration of autoantibodies of the IgM isotype in serum of BC patients

The detection of de- and increased autoantibodies in individual serum of study participants was performed with the secondary anti-IgG antibody which also enabled capturing of other isotypes. Some reports (Toubi *et al.,* Braendlein *et al.*)[181,280] indicated the presence of IgM autoantibodies, which may also have a potential in discrimination of BC patients from CTRL controls. The next investigation projects concerns the detection and identification of TA's after the incubation with serum samples of BC and CTRL study participants, whereas only IgM autoantibodies are visualized. The possible correlations with previously identified TA's and the appropriate IgG autoantibodies should also be investigated.

Primarily, Western immunoblotting and protein G beads-based platform could be used for the visualization of new IgM autoantibodies' patterns and subsequent identification of putatively immunogenic TA's via MS. Subsequently, the individual profiling of serum samples regarding levels of IgM and IgG autoantibodies, respectively, is aspired. Eventually, autoantibodies of both isotypes, specific for BC, could be correlated according to the time point of appearance and tumor characteristics.

Also for these investigations, the epitope mapping described above is a useful tool to obtain more details above the rising mechanism of autoantibodies. A possible additional project is the identification of possible post-translational modifications of the TA's, which may also provide more insights into tumor-specific autoantibody response. Also the determination of immunogenic epitopes and their subsequent use on protein microarrays may lead to a more precised tool for the classification of cancer patients and increase sensitivity and specificity of the biomarker panel.

Further search for putative biomarkers of other body fluids

Additionally, the established protein or antibody microarray platform can be used for the profiling of other body fluids like urine or saliva samples, as several reports indicated altered amounts of distinct proteins or autoantibodies in these body fluids, distant from the tumor location. The retrieval of samples is non-invasive and could serve as an alternative to serum. Also an enhanced tear protein profiling is possible, as previous studies revealed several alterations of at least protein levels in BC patients.

7 Summary

BC is the most often diagnosed cancer entity of women worldwide. Even with the improved detection rate through the single available detection tool, the mammography, as well as developing of therapy strategies, many patients die because of the high recurrence rate. Especially small tumors under 0.5 cm are barely detectable. Additionally the benefit of mammography in younger women is decreased because of higher tissue density. The curability of mammary carcinoma is correlated with the early time point of the diagnosis which enables better prognosis for the affected patients.

Proteomics is one of the dynamic tools for the successful examination of changes on the protein level. It can be used for the comparison of the peptide and protein levels in cancer patients and healthy controls (CTRL). Several investigations of protein biomarkers showed the potential of some molecules to discriminate BC patients from CTRL subjects with the rising specificity and sensitivity when multiple biomarkers are combined to the biomarker panel. Up to date, no molecular biomarkers are usable in the clinical routine for the early detection of BC.

In this PhD thesis different proteomics-based investigations were performed for the detection of protein and autoantibody biomarkers in serum samples of the BC and CTRL subjects. In the first part, protein levels of candidates mostly identified in previous profiling studies were investigated via the antibody-microarray platform. Using the commercially available antibodies and serum samples of BC (n=52) and CTRL (n=39) study participants, three proteins were found in statistically significant different levels in the BC group: secretoglobin family 1D member 1 (SG1D1, p=0.031, increased), alpha-2 macroglobulin (A2MG, p=0.034, increased) and inter-alpha-trypsin inhibitor heavy chain family, member 4 (ITIH4, p=0.016, decreased). The use of the combined biomarker signature allowed the discrimination of cancer patients from healthy control with a sensitivity of 71% and specificity of 77%.

The second part of this PhD thesis was dedicated to the exploration of autoantibody profiles in patients with BC and in CTRL probands. The *de novo* profiling of potentially immunogenic tumor antigens (TA's) was performed with protein G beads-based platform (immunoprecipitation) and 2D SDS-PAGE in combination with subsequent Western immunoblotting. The TA's were identified with the help of MALDI TOF/TOF MS. Afterwards, the autoantibody levels were verified based on the individual serum profiling via the protein microarray platform. The commercially available proteins were incubated with serum samples of BC patients (n=80) and controls (n=82). As the result, antibodies against the protein histone 2B (H2B) were found to be increased (p=0.03) and antibodies against

recoverin (RECO) were decreased in cancer patients (p=0.01). The use of their combination allowed the detection of cancer patients with the sensitivity of 63% and specificity of 60 %.

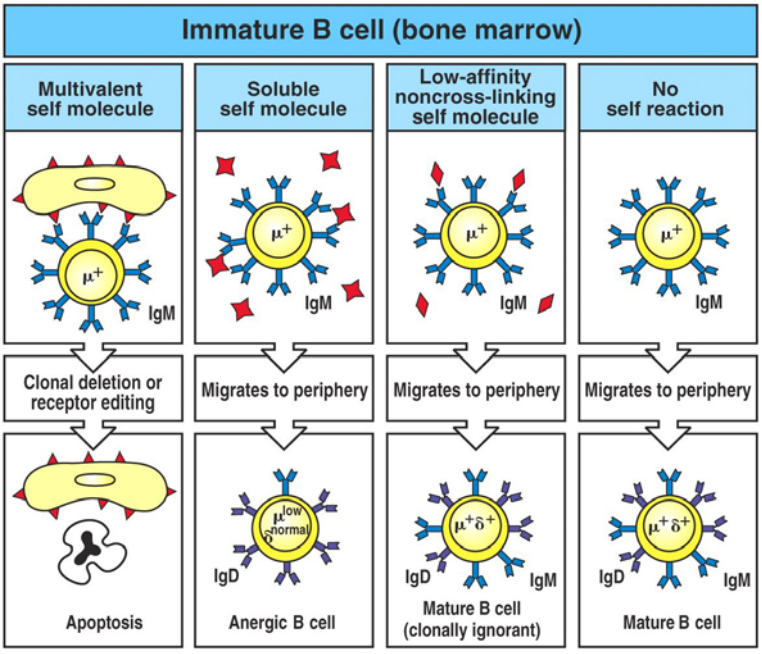
The findings of this PhD thesis underline deregulated serum protein and autoantibody levels in the presence of BC. Further investigations are needed to confirm the results in an independent study population. Additional studies with different tumor samples of different BC staging parameters should improve the findings and give more information about the specificity of the potential biomarkers. The approval and validation of examined molecules could lead to the development of a supportive molecular diagnostic tool together with mammography for the earliest possible detection of BC as a crucial requirement for its optimal healing.

8 Addendum

8.1 Plasma cells produce immunoglobulins after differentiation from B cells

An immature B cell expresses IgM molecules (bound immunoglobulin=B cell receptor) on the surface. It migrates from the bone marrow into the peripheral lymphatic organs, whereas the crucial steps are preceded prior to the development of the mature B cells (summarized in Repository figure 1). Thus, the cells are continuously exposed to host-own antigens. B cell cohort with the strong binding capacity these antigens are eliminated prior to migration by the clonal deletion. Otherwise, further rearrangement of the receptor gene segment (receptor editing) is also possible, whereby the B cells may then loose autoreactive properties[328].

In some cases, if the potentially not autoagressive B cells still recognize self antigens (soluble crosslinking or low-affinity noncrosslinking own molecules), they are freed into the peripheric lymphatic organs and remaining unresponsive or ignorant, respectively. The unresponsive B cells persist in the lymphatic organs and express only low levels of IgM. The ignorant B cells may be become self-reactive, *e.g.* due to higher amounts of antigens as observed in different AID´s. The B cell cohort without self reactivity migrates into the lymphatic organs and undergo maturing processes in the lymphoid follicles after repeated positive selection procedure[328].



Repository figure 1: Possible fates of an immature B cell. Original picture from Janeway *et al.*[328].

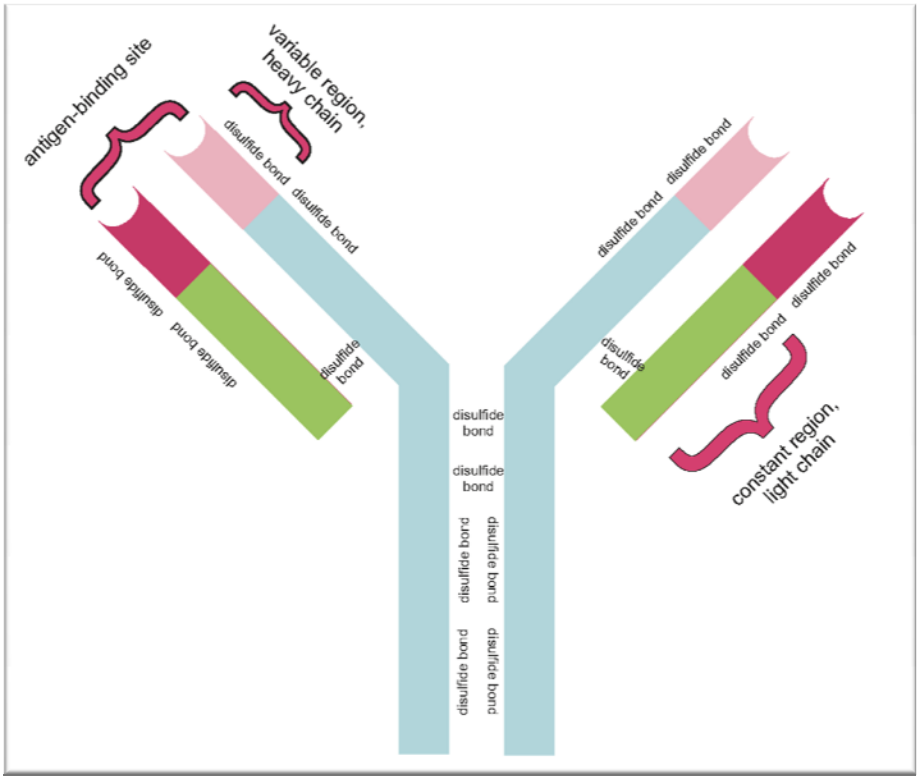
Mature B cells expressing high amounts of IgM are then able to bind foreign antigens persisting in the host. The antigens are internalized and presented over special molecules: major histocompatibility complex, (MHC) to another class of lymphocytes, the helper T cells, whereby B cells becomes activated. The interaction wth the helper T cell results in the proliferation and differentiation into the *plasma cells* (humoral immune response), now able to generate circulating antibodies. These antibodies possess the same specificity as the respective surface receptor on the B cell. Additionally, B cells perform an isotype switching, resulting predominantly in the IgG isotype. The specific isotype and subclass are provided by released cytokins of the helper T cells (see next section 8.2 for details). Antibodies may neutralize the foreign antigens or opsonize them for their subsequent elimination by killer cells. Additionally, antibodies can activate the complement system for the same purpose. The B cells may also become activated without interaction with helper T cells, *e.g.* over bacterial polysaccharides. Besides the arise of the antibody-secreting plasma cells, B cells differentiate into long-lived memory cells, which play an important role for the neutralization of recurring antigens of the same specificity[328].

8.2 Structure of immunoglobulins

Immunoglobulins can be divided into isotypes with distinct functions coded in the structure of the heavy chain: immunoglobulin **A**, **M**, **G**, **E** and **D** (IgA, IgM, IgG, IgE and IgD, respectively), whereas the isotypes are further divided into subclasses (*e.g.* IgG_1 or IgG_2).

The IgG represents the most abundant isotype of immunoglobulins. One IgG molecule consists of two light (L) and two heavy (H) glycan chains which are linked over the disulfide bridges (two H and H+L chains). The chains are composed of variable (V_H and V_L, antigen binding) and constant (C_H and C_L, activation of effector functions) regions. The constant region determines the isotype of the immunoglobulins: *e.g.* α (IgA) and γ (IgG) for the heavy chains. The light chains are universally distributed in all isotypes of immunoglobulins. Each antibody recognizes only one specific epitope of an antigen. Due to required high efficiency regarding elimination of different antigens, the immunoglobulins possess an incredible number of specific antigen binding sites. This circumstance is enabled over: *e.g.* somatic recombination or different combination of gene segments and H+L chains of the V region, as well as further hypermutations. The specificity of secreted antibodies by plasma cells is the same with the respective B cell membrane-bound immunoglobulin. Only the C-Terminus of the constant region is hydrophilic in comparison to the hydrophobic membrane-bound counterpart, enabling the secretion of the molecule[328].

Repository figure 2 shows the build-up of one IgG molecule.



Repository figure 2: Schematically build up of IgG. One molecule consists of two heavy (blue-pink) and light chains (green-red). The upper tops of one heavy and one light chain bind the antigens on the antigen-binding site. This amino-terminal site is termed as the variable region (V_H and V_L for the heavy and light chain, respectively); the downstream fragment is termed as constant region (C_H and C_L)[328]. Own sketch of the author

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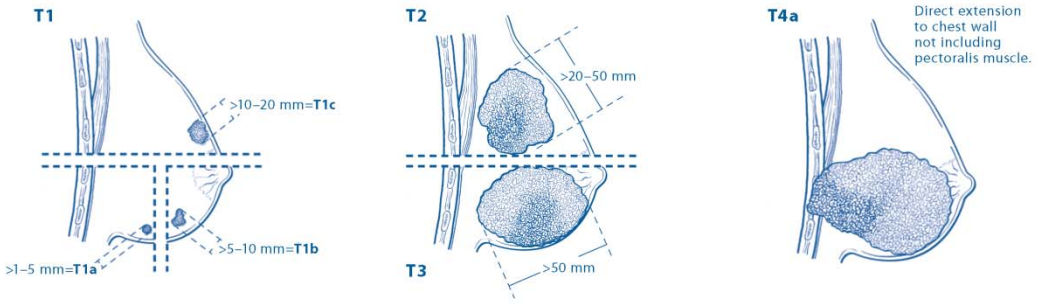

Primary Tumor (T)

- TX Primary tumor cannot be assessed
- T0 No evidence of primary tumor
- Tis Carcinoma in situ
- Tis (DCIS) Ductal carcinoma in situ
- Tis (LCIS) Lobular carcinoma in situ
- Tis (Paget's) Paget's disease of the nipple NOT associated with invasive carcinoma and/or carcinoma in situ (DCIS and/or LCIS) in the underlying breast parenchyma. Carcinomas in the breast parenchyma associated with Paget's disease are categorized based on the size and characteristics of the parenchymal disease, although the presence of Paget's disease should still be noted

- T1 Tumor ≤ 20 mm in greatest dimension
- T1mi Tumor ≤ 1 mm in greatest dimension
- T1a Tumor > 1 mm but ≤ 5 mm in greatest dimension
- T1b Tumor > 5 mm but ≤ 10 mm in greatest dimension
- T1c Tumor > 10 mm but ≤ 20 mm in greatest dimension
- T2 Tumor > 20 mm but ≤ 50 mm in greatest dimension
- T3 Tumor > 50 mm in greatest dimension

- T4 Tumor of any size with direct extension to the chest wall and/or to the skin (ulceration or skin nodules)
  Note: Invasion of the dermis alone does not qualify as T4
- T4a Extension to the chest wall, not including only pectoralis muscle adherence/invasion
- T4b Ulceration and/or ipsilateral satellite nodules and/or edema (including peau d'orange) of the skin, which do not meet the criteria for inflammatory carcinoma
- T4c Both T4a and T4b
- T4d Inflammatory carcinoma (see "Rules for Classification")

Distant Metastases (M)

- M0 No clinical or radiographic evidence of distant metastases
- cM0(i+) No clinical or radiographic evidence of distant metastases, but deposits of molecularly or microscopically detected tumor cells in circulating blood, bone marrow, or other nonregional nodal tissue that are no larger than 0.2 mm in a patient without symptoms or signs of metastases
- M1 Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven larger than 0.2 mm

ANATOMIC STAGE/PROGNOSTIC GROUPS			
Stage 0	Tis	N0	M0
Stage IA	T1*	N0	M0
Stage IB	T0	N1mi	M0
	T1*	N1mi	M0
Stage IIA	T0	N1**	M0
	T1*	N1**	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1*	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Notes

* T1 includes T1mi.

** T0 and T1 tumors with nodal micrometastases only are excluded from Stage IIA and are classified Stage IB.

- M0 includes M0(i+).
- The designation pM0 is not valid; any M0 should be clinical.
- If a patient presents with M1 prior to neoadjuvant systemic therapy, the stage is considered Stage IV and remains Stage IV regardless of response to neoadjuvant therapy.
- Stage designation may be changed if postsurgical imaging studies reveal the presence of distant metastases, provided that the studies are carried out within 4 months of diagnosis in the absence of disease progression and provided that the patient has not received neoadjuvant therapy.
- Postneoadjuvant therapy is designated with "yc" or "yp" prefix. Of note, no stage group is assigned if there is a complete pathologic response (CR) to neoadjuvant therapy, for example, ypT0ypN0cM0.

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Repository figure 3: The TNM-based staging system for breast carcinoma downloaded from the site http://www.cancerstaging.com/staging (at April 11, 2013 under "Breast small poster 8.5x11 inches"). The classification is based on[53].

Repository table 1: Summary of the criteria for the estimation of histological grading in breast carcinoma. Adapted from Elston and Ellis[57].

Feature	Score
Tubule formation:	
Majority of tumor (>75%)	1
Moderate degree (10-75%)	2
Little or none (<10%)	3
Nuclear pleiomorphism:	
Small, regular uniform cells	1
Moderate increase in size and variability	2
Marked variation	3
Mitotic counts	3
Dependent on microscope field area	1-3
3-5 points: grade I – well-differentiated 6-7 points: grade II – moderately differentiated 8-9 ponts: grade III – poorly differentiated	

Repository table 2: Summary of the updated Van Nuys Prognostic Index (VNPI) criteria for the treatment option of DCIS. The single criteria are summarized to the certain score. Original tables from Silverstein 2003[61].

The USC/Van Nuys Prognostic Index scoring system. One to three points are awarded for each of four different predictors of local breast recurrence (size, margin width, pathologic classification, and age). Scores for each of the predictors are totaled to yield a VNPI score ranging from a low of 4 to a high of 12

Score	1	2	3
Size (mm)	≤15	16–40	≥41
Margin width (mm)	≥10	1–9	<1
Pathologic classification	Nonhigh grade without necrosis (nuclear grades 1 or 2)	Nonhigh grade with necrosis (nuclear grades 1 or 2)	High grade with or without necrosis (nuclear grade 3)
Age (yr)	>60	40–60	<40

Tumor characteristics, recurrences, and breast cancer deaths by USC/Van Nuys Prognostic Index Groups. Patients treated with mastectomy are not included in this table since they are at limited risk for local recurrence

	VNPI 4, 5 or 6	VNPI 7, 8, or 9	VNPI 10, 11, or 12	TOTAL
No. breast conservation pts	230	400	76	706
Average age (yr)	57	53	48	54
Average size (mm)	8.3	18.0	38.2	17.0
Average nuclear grade	1.65	2.45	2.89	2.24
No. of recurrences	3 (1%)	78 (20%)	38 (50%)	119 (17%)
No. invasive recurrences	0 (0%)	34 (44%)	15 (39%)	49 (41%)
5 & 10-yr local recurrence-free survival	99%/97%	84%/73%	51%/34%	85%/76%
Breast cancer deaths	0	5	1	6
5 & 10-Yr breast cancer specific survival	100%/100%	100%/98.1%	97.9%/979%	99.7%/98.6%

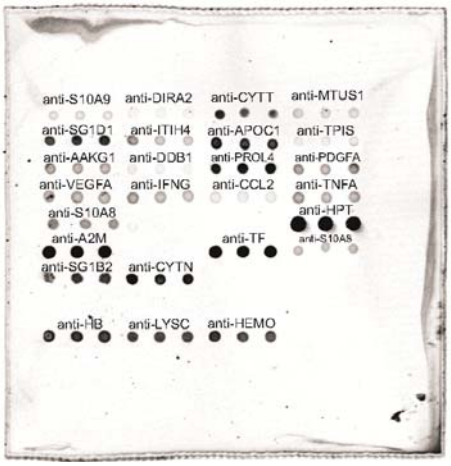
Treatment guidelines by USC/Van Nuys Prognostic Index Score

4 to 6	Excision alone
7 to 9	Excision + radiation
10 to 12	Mastectomy

Repository table 3: St. Gallen' conference-based definition of intrinsic isotypes of breast carcinoma as well as systemic treatment recommendations for each group. Original table from Goldhirsch *et al.* 2011.[78] Additional references from the table: (7): Cheang *et al.*[329]; (76): Hammond *et al.*[330]; (77): Wolff *et al.*[331]; (78): Wirapati et al.[332]; (79): Cheang *et al.*[333].

Intrinsic Subtype (1)	Clinico-pathologic definition	Notes
Luminal A	**'Luminal A'** ER and/or PgR positive(76) HER2 negative (77) Ki-67 low (<14%)*	This cut-point for Ki-67 labelling index was established by comparison with PAM50 intrinsic subtyping (7). Local quality control of Ki-67 staining is important.
Luminal B**	**'Luminal B (HER2 negative)'** ER and/or PgR positive HER2 negative Ki-67 high	Genes indicative of higher proliferation are markers of poor prognosis in multiple genetic assays (78). If reliable Ki-67 measurement is not available, some alternative assessment of tumor proliferation such as grade may be used to distinguish between 'Luminal A' and 'Luminal B (HER2 negative)'.
	'Luminal B (HER2 positive)' ER and/or PgR positive Any Ki-67 HER2 over-expressed or amplified	Both endocrine and anti-HER2 therapy may be indicated.
Erb-B2 overexpression	**'HER2 positive (non luminal)'** HER2 over-expressed or amplified ER and PgR absent	
'Basal-like'	**'Triple negative (ductal)'** ER and PgR absent HER2 negative	Approximately 80% overlap between 'triple negative' and intrinsic 'basal-like' subtype but 'triple negative' also includes some special histological types such as (typical) medullary and adenoid cystic carcinoma with low risks of distant recurrence. Staining for basal keratins (79) although shown to aid selection of true basal-like tumors, is considered insufficiently reproducible for general use.

*This cut-point is derived from comparison with gene array data as a prognostic factor [7]. Optimal cut-points in Ki-67 labelling index for prediction of efficacy of endocrine or cytotoxic therapy may vary.
**Some cases over-express both luminal and HER2 genes.



Repository figure 4: Overview of all spotted antibodies on antibody microarrays with the UniProt/SwissProt protein abbreviation above the respective triplicates.

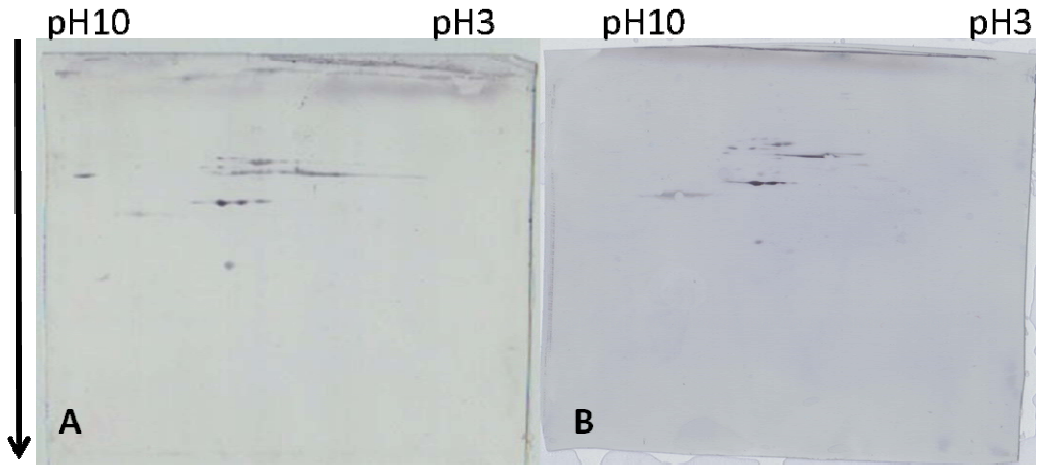
Repository table 4: List of the antibodies spotted on the protein microarrays with the respective manufacturers. These antibodies were not used for the estimation of protein levels in the current project (see section 4.1).

Recommended protein name according to UniProt with respective abbreviations: X_HUMAN	**Manufacturer/Distributor**
Anti-GTP-binding protein Di-Ras2; DIRA2	Abcam plc, Cambridge UK
Anti-Triosephosphate isomerase; TPIS	Sigma-Aldrich Chemie GmbH, München, Germany
Anti-5'-AMP-activated protein kinase subunit gamma-3; AAKG1	Sigma-Aldrich Chemie GmbH, München, Germany

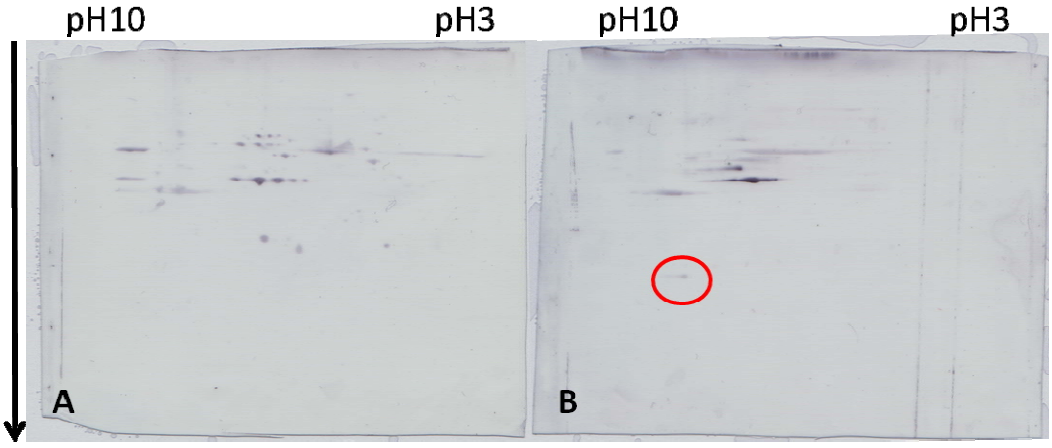
Anti-DNA damage-binding protein 1; DDB1	Sigma-Aldrich Chemie GmbH, München, Germany
Anti-Platelet-derived growth factor subunit A; PDGFA	Epitomics, an Abcam Company; Abcam plc, Cambridge UK
Anti-Vascular endothelial growth factor receptor A; VEGFA	Acris Antibodies GmbH, Herford, Germany
Anti-Interferone gamma; IFNG	BD Pharmingen™Biosciences, Franklin Lakes (NJ), USA
Anti-C-C motif chemokine 2; CCL2	Acris Antibodies GmbH, Herford, Germany
Anti-Tumor necrosis factor-alpha; TNFA	Acris Antibodies GmbH, Herford, Germany
Anti-Haptoglobin; HPT	Rockland Immunochemicals Inc., Gilbertsville (PA), USA
Anti-Hemoglobin; HB	Lifespan Biosciences Inc., Seattle (WA), USA
Anti-Lysozyme C; LYSC	Santa Cruz Biotechnology, Inc., Heidelberg, Germany
Anti-Hemopexin; HEMO	US Biological; http://www.usbio.net

Repository table 5: Summarized parameters from BC and CTRL population used for immunoprecipitation and Western immunoblotting. Serum samples were pooled accrding to group belonging. Tumor's characteristics include TN(M) classification (*DCIS), grading and hormone receptor status. No patients developed distant metastases (M0).

Histological characteristics	BC group n =20	CTRL group n = 20
Mean age (distribution)	54 (36-84)	53.7 (36-81)
Tumor size	(%)	
pTis	0 (0)	
pT1	10 (50)	
pT2	8 (40)	
pT3	1 (5)	
other*	1 (5)	
Node status		
Negative	11 (55)	
Positive	8 (40)	
Grading		
Well differentiated (G1)	3 (15)	
Moderately differentiated (G2)	10 (50)	
Poor/undifferentiated (G3)	6 (30)	
Hormone receptor		
Positive	17 (85)	
Negative	2 (10)	

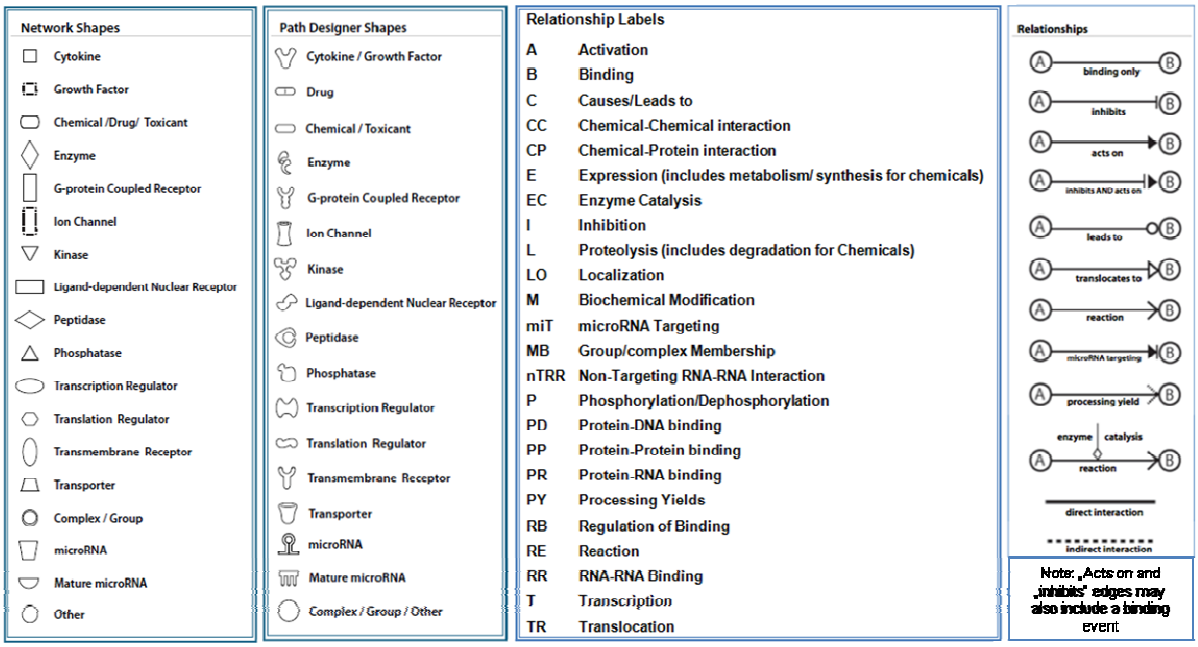


Repository figure 5: Exemplarily display of two immunoblots (A and B) of the transferred tumor proteins onto nitrocellulose membrane and incubated with same BC serum pool. The blots were performed at different time points, whereas similar reaction pattern was obtained. The used pH range for IEF separation is depicted above; the black arrow shows the separation direction via SDS-PAGE.



Repository figure 6:Exemplarily image of differently incubated immunoblots with BC (A) and CTRL serum pool (B), whereas the identified protein (mixture of cofilin 1 and destrin) was identified after incubation with healthy serum pool only (encircled red in B). The used pH range for IEF separation is depicted above; the black arrow shows the separation direction via SDS-PAGE.

IPA Legend



Repository figure 7: Legend summary for the protein interaction analysis via the online software Ingenuity (http://www.ingenuity.com), downloaded from the site at 09/17/2012

Additional notes to the figure above (taken from the online IPA legend):

Relationships: A relationship with an X over it indicates that the interaction does not occur. These relationshis are only used in Disease pathway to indicate an interaction that would normally happen in the absence of the disease, but does not happen in the disease context. An arrow pointing from A to B signifies different actions for different circumstances, as described below.

For signaling pathways:

An arrow pointing from A to B signifies that A causes B to be activated (includes any direct interaction: *e.g.* binding, phosphorylation, dephosphorylation, *etc.*)

For metabolic pathways:

An arrow pointing from A to B signifies that B is produced from A.

For ligands/receptors:

An arrow pointing from a ligand to a receptor signifies that the ligand binds the receptor and subsequently leads to activation of the receptor. This binding event does not necessarily directly activate the receptor, activation of the receptor could be caused by events secondary to the ligand/receptor binding event.

Repository table 6: Biological agents defined as human carcinogens according to the WHO International Agency for Research on Cancer Monograph Working Group IARC[162].

Group 1 agent	Cancers for which there is sufficient evidence in humans	Other sites with limited evidence in humans	Established mechanistic events
Epstein–Barr virus (EBV)	Nasopharyngeal carcinoma, Burkitt's lymphoma, immune-suppression-related non-Hodgkin lymphoma, extranode NK/T-cell lymphoma (nasal type), Hodgkin's lymphoma	Gastric carcinoma,* lympho-epithelioma-like carcinoma*	Cell proliferation, inhibition of apoptosis, genomic instability, cell migration
Hepatitis B virus (HBV)	Hepatocellular carcinoma	Cholangiocarcinoma,* non-Hodgkin lymphoma*	Inflammation, liver cirrhosis, chronic hepatitis
Hepatitis C virus (HCV)	Hepatocellular carcinoma, non-Hodgkin lymphoma*	Cholangiocarcinoma*	Inflammation, liver cirrhosis, liver fi brosis
Kaposi's sarcoma herpes virus (KSHV)	Kaposi's sarcoma,* primary effusion lymphoma*	multicentric Castleman's disease*	Cell proliferation, inhibition of apoptosis, genomic instability, cell migration
Human immunodefi ciency virus, type 1 (HIV-1)	Kaposi's sarcoma, non-Hodgkin lymphoma, Hodgkin's lymphoma,* cancer of the cervix,* anus,* conjunctiva*	Cancer of the vulva,* vagina,* penis,* non-melanoma skin cancer,* hepatocellular carcinoma*	Immunosuppression (indirect action)
Human papillomavirus type 16 (HPV-16)†	Carcinoma of the cervix, vulva, vagina, penis, anus, oral cavity, and oropharynx and tonsil	Cancer of the larynx	Immortalisation, genomic instability, inhibition of DNA damage response, anti-apoptotic activity
Human T-cell lymphotrophic virus, type-1 (HTLV-1)	Adult T-cell leukaemia and lymphoma	..	Immortalisation and transformation of T cells
Helicobacter pylori	Non-cardia gastric carcinoma, low-grade B-cell mucosa-associated lymphoid tissue (MALT) gastric lymphoma*	..	Inflammation, oxidative stress, altered cellular turn -over and gene expression, methylation, mutation
Clonorchis sinensis	Cholangiocarcinoma*	..	..
Opisthorchis viverrini	Cholangiocarcinoma	..	Inflammation, oxidative stress, cell proliferation
Schistosoma haematobium	Urinary bladder cancer	..	Infl ammation, oxidative stress

IPA® Summary of Analysis

INGENUITY® SYSTEMS

Analysis Name: SwissprotID_Ing_Final - 2012-08-29 01:35 PM
Analysis Creation Date: 2012-08-29
Build version: 172788
Content version: 14197757 (Release Date: 2012-08-11)

Analysis settings

View
Reference set: Ingenuity Knowledge Base (Genes Only)
Relationship to include: Direct and Indirect
Includes Endogenous Chemicals
Optional Analyses: My Pathways My List

Filter Summary:
Consider only molecules and/or relationships where
(species = Human) AND
(confidence = Experimentally Observed) AND
(tissues = Other Dendritic cells OR Other NK cells OR Lung OR Monocyte-derived macrophage OR Neutrophils OR Mammary Gland OR Ovary OR Vd2 Gamma-delta T cells OR Vd1 Gamma-delta T cells OR Placenta OR Cytotoxic T cells OR Tissues and Primary Cells not otherwise specified OR Organ Systems not otherwise specified OR Other Immune cells OR Nervous System not otherwise specified OR Salivary Gland OR Other Nervous System OR T lymphocytes not otherwise specified OR Other B lymphocytes OR Hypothalamus OR CD56bright NK cells OR Pancreas OR Central memory helper T cells OR Cerebral Cortex OR Retina OR Other T lymphocytes OR Thymus OR Th1 cells OR Effector memory helper T cells OR Dendritic cells not otherwise specified OR Large Intestine OR Small Intestine OR Other Cells OR Spleen OR Bladder OR Olfactory Bulb OR Pituitary Gland OR Substantia Nigra OR Dorsal Root Ganglion OR BDCA-1+ dendritic cells OR Prostate Gland OR Immature monocyte-derived dendritic cells OR Activated helper T cells OR Epidermis OR Testis OR Murine NKT cells OR Other Tissues and Primary Cells OR Macrophages OR Natural T-regulatory cells OR Activated Vd1 Gamma-delta T cells OR BDCA-3+ dendritic cells OR Liver OR Naive B cells OR Memory B cells OR Th2 cells OR Central memory cytotoxic T cells OR Effector T cells OR Activated Vd2 Gamma-delta T cells OR Effector memory cytotoxic T cells OR Other Organ Systems OR Skeletal Muscle OR NK cells not otherwise

specified OR Cells not otherwise specified OR Effector memory RA+ cytotoxic T cells OR Naive helper T cells OR CD56dim NK cells OR Immune cells not otherwise specified OR Hippocampus OR Activated CD56bright NK cells OR Kidney OR Uterus OR Monocytes OR Heart OR B lymphocytes not otherwise specified OR Cerebellum OR Plasmacytoid dendritic cells OR Amygdala OR Adipose OR Stomach OR Mature monocyte-derived dendritic cells OR Activated CD56dim NK cells)

Top Networks

ID	Associated Network Functions	Score
1	Cancer, Endocrine System Disorders, Hematological Disease	19
2	Carbohydrate Metabolism, Cell Death and Survival, Gene Expression	17
3	Cellular Assembly and Organization, DNA Replication, Recombination, and Repair, Cell Cycle	2
4	Cellular Development, Reproductive System Development and Function, Hereditary Disorder	2
5	Cellular Development, Cellular Growth and Proliferation, Developmental Disorder	2

Summary of Analysis - SwissprotID_Ing_Final - 2012-08-29 01:35 PM

Top Bio Functions

Diseases and Disorders

Name	p-value	# Molecules
Cancer	2.32E-08 - 3.87E-02	22
Dermatological Diseases and Conditions	2.32E-08 - 3.79E-02	14
Hematological Disease	2.32E-08 - 3.08E-02	10
Inflammatory Disease	2.32E-08 - 4.58E-02	17
Cardiovascular Disease	3.75E-08 - 4.72E-02	6

Molecular and Cellular Functions

Name	p-value	# Molecules
Cell Death and Survival	9.77E-05 - 4.58E-02	8
Amino Acid Metabolism	2.60E-03 - 1.04E-02	1
Cellular Compromise	2.60E-03 - 1.55E-02	2
Cellular Function and Maintenance	2.60E-03 - 4.83E-02	3
Drug Metabolism	2.60E-03 - 1.55E-02	4

Physiological System Development and Function

Name	p-value	# Molecules
Cardiovascular System Development and Function	9.77E-05 - 4.08E-02	3
Hair and Skin Development and Function	5.19E-03 - 2.57E-02	2
Hematological System Development and Function	5.19E-03 - 4.83E-02	2
Organ Morphology	1.04E-02 - 1.04E-02	1
Reproductive System Development and Function	1.04E-02 - 2.32E-02	1

Summary of Analysis - SwissprotID_Ing_Final - 2012-08-29 01:35 PM

Top Canonical Pathways

Name	p-value	Ratio
Glycolysis I	2.24E-09	5/23 (0.217)
Germ Cell-Sertoli Cell Junction Signaling	4.18E-09	8/157 (0.051)
Sertoli Cell-Sertoli Cell Junction Signaling	7.24E-09	8/184 (0.043)
Gap Junction Signaling	1.05E-07	7/158 (0.044)
Gluconeogenesis I	2.7E-07	4/24 (0.167)

Top Molecules

This analysis has no expression values.

Top Upstream Regulators

Upstream Regulator	p-value of overlap	Predicted Activation State
NEIL2	7.25E-05	
HNRNPU	7.25E-05	
Lh	8.35E-05	
FSH	2.39E-04	
POLR2A	3.73E-04	

Repository figure 8: Final analysis report of the submitted proteins to Ingenuity platform which is depicted and discussed in this doctoral thesis (see sections 4.2 and 5.2 for details)

9 Acknowledgements

Curriculum vitae

10 References

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11 Statutory declaration (Eidesstattliche Erklärung)

I hereby confirm that I composed this doctoral thesis on my own and only with the declared resources.

Hiermit versichere ich, dass ich die vorliegende Dissertation selbstständig und nur mit den angegebenen Hilfsmitteln angefertigt habe.

Mainz, den

_____ Ksenia Keller