1 Introduction and outline of this thesis

INTRODUCTION

1.1 DEVELOPMENT AND BURDEN OF CANCER

In this thesis the benefits and disadvantages of population screening programs for early detection of cancer will be discussed with focus on mass spectrometry (MS)-based protein and peptide profiling, introduced as a relatively new strategy.

Cancer is a major public health problem in the Western world. The global incidence of cancer will be doubled by 2050 as a result of aging and growth of the world population, and because of cancer-causing behaviours, such as smoking, diet and sun exposure.[1] Based on the GLOBOCAN 2012 estimates, about 14 million cancer cases and 8.2 million cancer deaths are estimated to have occurred in 2012. (http://globocan.iarc.fr) Various research fields come into play when aiming to improve clinical care in oncology. These can be focused on hereditary aspects and early diagnosis or on topics after clinical symptoms have revealed, such as prognosis, monitoring of disease progression and therapy efficacy or – toxicity. Most of the research programs have focused on treatment, but it is predicted that the ever-growing burden of cancer will surpass the financial capacity to treat and follow-up the millions affected.[2] Besides focusing on treatment, it might be efficient to focus on what we already know and put more effort in preventing cancer, intervening early in life and detecting cancer at an early stage.[2-4]

In the transformation from a normal cell into a malignant cancer cell, changes in multiple genes play a crucial role, such as oncogenes and tumor suppressor genes that regulate cell growth and differentiation. Most of these so-called cancer-causing mutations are acquired, and not inherited. Acquired mutations may be caused by certain exposures in the environment such as cigarette smoke, radiation and hormones. In case of hereditary cancer, a defect in one of these regulatory genes or genes responsible for maintaining the integrity of the DNA exists at birth. This makes it easier for enough mutations to build up for a cell to become cancer. Mutated genes translate into altered proteins and peptides or altered amounts of these proteins and peptides. It is beneficial to detect these proteins and peptides at an early stage of cancer development. Initially, when cancer begins to develop, symptoms are lacking and clinical signs only appear as the mass continues to grow and reaches a certain threshold. This is illustrated in figure 1. Some tumors develop gradually, others more acute. Very often the cancer is already at an advanced stage when this threshold for symptoms is reached. It seems a logical approach to use screening programs to detect a tumor at an early stage. In the next paragraphs this will be further elaborated and discussed for breast cancer and pancreatic cancer.

1.2. EARLY STAGE SCREENING

Population wide screening programs are used to detect early stage cancer to enable early intervention and reduce morbidity and mortality. Advanced cancer has a poor survival, whereas when diagnosed at an early stage, survival improves.[5] Ideally, screening tests have to be highly specific, sensitive, cost-effective and non-invasive.[5-10]
However, one of the possible downsides of screening is over-diagnosis. For all types of cancer it is known that with early detection more pre-malignant lesions will be found than that eventually will clinically manifest. Many people die with and not because of a carcinoma.[11,12] Over-diagnosis can lead to over-treatment, unnecessary anxiety, unnecessary health damage (in case of an invasive screening test) and unnecessary costs. Besides this, screening tests will miss so-called interval carcinomas, tumors which develop between two screening intervals. This will be further illustrated in following paragraphs.

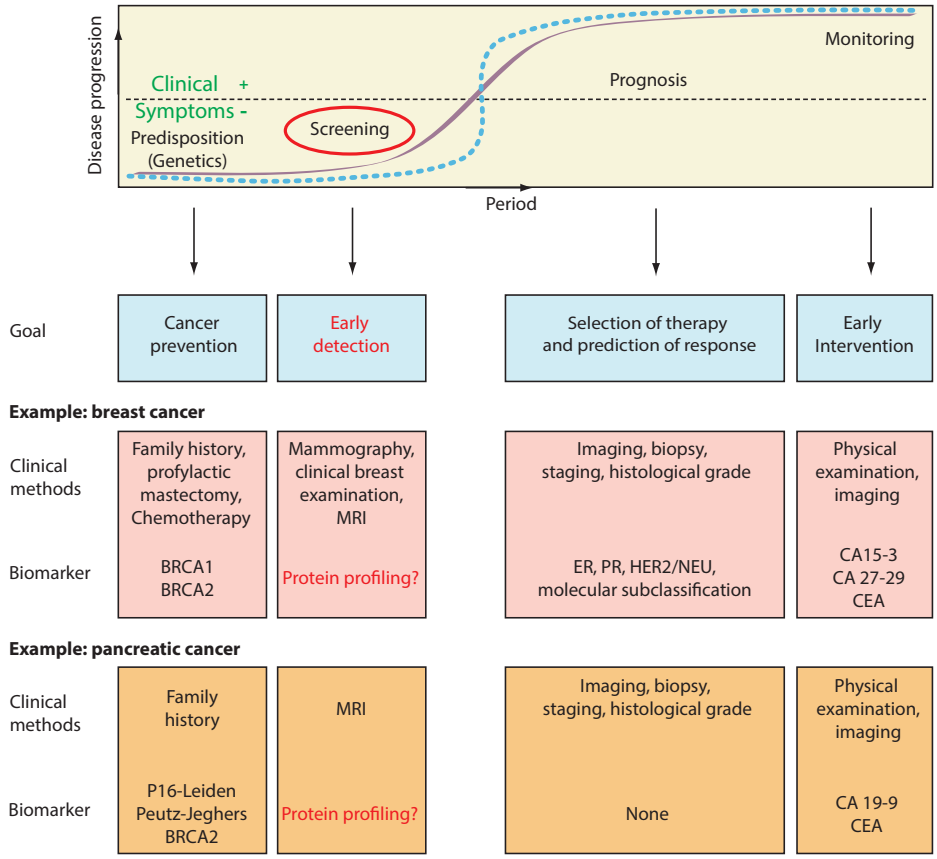

Figure 1. Stages of tumor progression with divers preventive and therapeutic options illustrated for breast and pancreatic cancer.

Screening for breast cancer

Breast cancer (BC) is the most frequently diagnosed malignancy in women with over one million new cases in the world each year and the leading cause of cancer death among females, accounting for 25% of the total cancer cases and 15% of the cancer deaths.[3,12] Lifetime risk is still increasing, currently estimated at one in 8. Approaches to decrease the risk of BC include lifestyle changes (eg, postmenopausal hormones, childbearing at a younger age, breastfeeding, weight gain, alcohol consumption, smoking and physical exercise).[12,14] Despite increasing incidence rates between 1980 and the late 1990s, annual mortality rates from BC have decreased over the last decade.[12,15] Tumor stage remains the most important determinant of the outcome. Among women with non-metastatic BC, tumor size and axillary spread are the main risk factors for recurrence of disease. This means that the ideal screening regimen for breast cancer would be one that could detect a tumor before it is large enough to be palpable.[13,16,17]

Mammography is currently the standard tool in screening and early detection of BC.[13] In the Netherlands, it is used as a population-based screening method for women from 50-75 years of age every two years. This method is non-invasive, with relatively low radiation exposure, but for patients unpleasant and sometimes very painful. Recent published results of an evaluation study for breast cancer screening in the Netherlands showed that, as in other countries, up to 20% of new incident BC are not detected by this method.[18-21] Furthermore, only for 27% the positive result of mammography was histologically confirmed as malignant.[21] Another disadvantage of screening by mammography is the prevalence of interval carcinomas. 30-40% of BC was found in between screening moments and these tumors appear more aggressive than screen detected tumors.[22] It has been debated extensively whether BC screening by mammography does more harm than good. The main questions are how large the benefit of screening is in terms of reduced BC mortality and how substantial the harm is in terms of over-diagnosis, which is defined as cancers detected at screening that otherwise would not have become clinically apparent in a woman's lifetime. Bleyer and Welch claim that there is substantial over-diagnosis, accounting for nearly a third of all newly diagnosed BCs, and that screening is having, at best, only a small effect on the rate of death from BC.[23] The independent UK panel of breast cancer screening was less pessimistic: herein it was reported that in the UK for the prevention of one mortality caused by BC three other women were unnecessary treated.[24] Recently, overdiagnosis by mammography in the Netherlands was quantified. They calculated that 11-17% of the invasive breast cancers were over-diagnosed.[25] The problem is that it cannot be predicted which women will be over-treated and which will benefit from screening and early detection. The psychological consequences are recently published by Bond et al. Having a false-positive screening mammogram can cause breast cancer-specific distress for up to 3 years.[26]

For older women with BC and co-morbidities it also has been assumed that diagnosis at an earlier stage through screening programs could improve the prognosis. However, a recent study showed that at this moment screening older women up to the age of 75 with mammography only led to a small decrease in incidence of advanced stage BC. Furthermore, co-morbidity and poor physical functioning of this group can lead to poor attendance to the current screening program.[27]

Mammography is also used as a yearly screening tool for young women with a high familiar risk or with a genetic predisposition, such as carriers of germline mutations in the BRCA1 and BRCA2 genes. Unfortunately, in this group the detection rate of mammography is only 40%, mainly because of the dense breast tissue.[28,29] Moreover, it was found that exposure to ionizing radiation is a relatively large risk factor for BC for this high-risk groups.[30] Especially in the young (< 35 years of age) high-risk groups the possibility of developing an interval carcinoma is considerable.[31-33] Currently, in screening programs for high-risk women magnetic resonance imaging (MRI) is added to mammography screening because it has good potential to detect mammographically occult cancers and improves metastasis free survival.[34,35] However, this relative expensive imaging technique does not distinguish benign from malignant findings and still results in a false-positive rate of 10%.[34,36-40]

To overcome the challenges such as over-diagnosis, poor attendance of older women and missing interval carcinomas, development of additional highly sensitive and specific molecular markers could be of use next to screening by mammography or MRI. (Figure 1) These molecular markers might help to pre-select women at risk of more aggressive BC. It should be noted that BC is a very heterogeneous disease with many different subtypes.[41] Currently, no single serum biomarker is available that can be used for screening of early BC. The best known carcinoma- (or carbohydrate) antigens (CA) 15-3 and CA 27-29 are the basis of well-characterized serum assays that allow the detection of circulating MUC-1 antigen in peripheral blood of patients with breast cancer. Levels correlate with disease status, although tumor marker levels cannot be used for early detection. Carcinoembryonic antigen (CEA) levels are less commonly elevated, but as with CA15-3 and CA 27-29, levels appear to correlate with disease status. Nevertheless, data up to now is insufficient to support the use of CA 15-3, CA27-29, or CEA in the diagnosis and staging of BC.[42]

Screening for pancreatic cancer

With an overall 5-year survival rate of less than 5%, patients with pancreatic cancer (PC) have an extremely poor prognosis. This explains why although the annual incidence of PC is only 8.2 cases per 100.000 males and 5.4 cases per 100.000 females, it is the fifth (male)

and fourth (female) leading cause of cancer death in developed countries.[3] The incidence of pancreatic cancer is increasing. Projections-based changes in incidence and death rates (changing demographics, diabetes and obesity) have suggested that pancreatic cancer is changing from the fourth to the second leading cause of cancer death in the U.S. by 2020. (Pancan.org) Median survival after diagnosis is only four to six months in case surgical resection is impossible (as is true for most tumors). When surgical resection is possible, 5-year survival rates increase to approximately 25% for node-negative and 10% for node-positive tumors.[43,44] Furthermore, the use of preoperative or postoperative adjuvant chemotherapy or radiotherapy has only minimally improved survival.[45,46] Again, instead of focusing on treatment, it would be much more efficient to focus on preventing cancer, intervening early in life and detecting cancer in an early stadium.

Major risk factors for developing PC include smoking, diabetes, hereditary predisposition to PC itself or to multiple cancers and chronic pancreatitis.[47-52] Health programs to reduce smoking or obesity could help to prevent the development of PC.[4] Furthermore, screening programs may result in diagnosis of early PC. Currently, possible screening options for detection of PC are imaging techniques such as computed tomography (CT) scan, magnetic resonance imaging (MRI) and magnetic resonance cholangiopancreatography (MRCP), positron emission tomography (PET) scan and endoscopic ultrasound (EUS). These are used for diagnosis, staging and to identify patients who are eligible for resection with curative intent. CT-scan and MRI are the modalities of choice for diagnosis and to preoperatively stage the disease, when clinical presentation suspects PC. Reported sensitivities and specificities for CT-scan are 91% and 85%, for MRI 84% and 82%, respectively, and for EUS sensitivity values of up to 100% have been reported.[53,54] CT-scans and MRI perform equally for staging and detection of metastases, but a CT-scan significantly outperforms MRI in the detection of PC.[53] Dewitt and co-workers determined a pooled sensitivity of 85% and specificity of 94% for EUS, i.e. similar to CT-scan results.[54] A CT-scan is preferred in the evaluation of distant metastasis, while EUS is a more accurate device for local T (small tumors < 2cm) and N staging and for predicting vascular invasion. EUS-guided fine needle aspiration biopsy (FNA) is the best modality, with a low chance of tumor seeding, for obtaining a conclusive diagnosis when the tumor seems irresectable or when neo-adjuvant therapy is planned.[55] PET scanning with the tracer 18-fluorodeoxyglucose (FDG) relies upon functional activity to differentiate metabolically active proliferative lesions such as cancers from benign masses, most of which do not accumulate FDG with the exception of inflammatory lesions such as chronic pancreatitis. A meta-analysis described the diagnostic value of the PET-scan in case of a positive (sensitivity 92%, specificity 68%), negative (sensitivity 73%, specificity 86%) and non-conclusive (sensitivity 100%, specificity 68%) result of the CT-scan.[56] It should be noted that the more common chronic pancreatitis could imitate PC

at diagnostics and hinders patient selection for a pancreaticoduodenectomy. Although expensive and, in the case of EUS, invasive, CT-scan, MRI and EUS are candidates for a golden standard screening modality. However, screening of the normal population at this moment is not cost-effective because of the low incidence of PC.

Currently, it is recommended to screen for PC only in a selected group of patients with familiar pancreatic cancer or genetic mutations such as the p16-Leiden germline mutation. In this group the lifetime risk of PC varies between 5% and 36%.[47-49,52] It has been estimated that 5% to 10% of PC cases are associated with an inherited predisposition such as Peutz–Jeghers syndrome, familial atypical multiple mole melanoma (FAMMM), hereditary breast cancer (BRCA2 mutation carriers), and possibly Lynch syndrome. At this time, aiming for early detection in this hereditary group is carried out annually by imaging surveillance, but the optimal strategy for surveillance has yet to be determined.[57] PC in FAMMM patients often presents as a rapidly growing tumor that could originate from small precursor lesions.[58] Intensive surveillance every 6 months, for example, using alternating MRI and EUS is probably necessary to identify a tumor at a curable stage (<1 cm), however it is noted that this is very labour intensive and, in the case of EUS, invasive. Thus, the use of a low cost, highly sensitive and specific serum biomarker could aid in pre-selecting patients with early stage pancreatic cancer. (Figure 1) The mostly studied available clinical serum biomarker CA19-9 can detect higher stage tumors with good performance but misses the appropriate sensitivity and specificity for small, resectable cancers.[59] Moreover, CA19-9 is often elevated in benign cholangitis, pancreatitis and other cancers, and therefore lacks the specificity for detecting potentially curable lesions. At this moment the use of CA19-9 is only recommended for follow-up.

Clearly, an additional screening test for both malignancies is warranted. This screening test should have its own extra value next to the existing screening programs. In 2008 the World Health Organisation (WHO) reviewed and adjusted the international screening criteria of Wilson and Jungner.[10] These criteria are summed below.
The criteria of Wilson and Jungner and these of the WHO are mainly relevant to governments that consider offering a specific population based screening programme and guarantee a responsible screening.

Another very relevant issue of the 21 st Century is quality of health care. In 2006 the WHO composed 6 dimensions of quality. Any form of care should be effective, efficient, accessible, acceptable (patient-centred), equitable and safe (WHO 2006, quality of care: a process for making strategic choices in health systems).

Wilson and Jungner classic screening criteria

- The condition sought should be an important health problem
- There should be an accepted treatment for patients with recognized disease
- Facilities for diagnosis and treatment should be available
- There should be a recognizable latent or early symptomatic stage
- There should be a suitable test or examination
- The test should be acceptable to the population
- The natural history of the condition, including development from latent to declared disease, should be adequately understood
- There should be an agreed policy on whom to treat as patients
- The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole
- Case-finding should be continuing process and not a "once and for all" project.

Synthesis of emerging screening criteria proposed over the past 40 years

- The screening programme should respond to a recognized need
- The objectives of screening should be defined at the outset
- There should be a defined target population
- There should be scientific evidence of screening programme effectiveness
- The programme should integrate education, testing, clinical services and programme management
- There should be quality assurance, with mechanisms to minimize potential risks of screening
- The programme should ensure informed choice, confidentiality and respect for autonomy
- The programme should promote equity and access to screening for the entire target population
- Programme evaluation should be planned form the outset
- The overall benefits of screening should outweigh the harm

1.3. PROTEIN PROFILING

General introduction

The determination of the elemental composition of a protein has been boosted by the application of (two-dimensional) gel electrophoresis to separate proteins.[60] Pioneering work with regard to the identification of a protein from a gel by mass spectrometry (MS) through peptide sequencing has been performed by Matthias Mann.[61] Although this paper is by far the most cited one, several other scientists have also made crucial contributions to the field of peptide- and protein identifications by means of MS, namely Sundqvist, Heerma, Roepstorff, Hunt and Yates.[62-66] It was only in the middle 1990s, at the same time that the Human Genome Project received great attention, that mass spectrometry (MS) for protein identification grew to its current size. It was clear that the identification and characterization of all human proteins would provide biologically relevant information complementary to genomic studies, however it would take a few more years before technology was in place. This new discipline was called *proteomics* to make an analogy with the term genomics.[67] Consequently, new technologies were developed to provide innovative methods for the separation, detection and characterization of proteins, and MS has evolved from an early, laborious protein analysis tool into a high-throughput highly automated method Nowadays, MS is the method of choice in proteomics for the analysis of complex protein samples (*e.g.* human serum).[68]

Since the complete sequence map of the human genome was published in 2001, proteomics has contributed to multiple research applications in the field of medicine, clinical proteomics, biomarker discovery, and protein profiling of specific diseases.[69] However, it should be noted that the proteome is far more complex than the genome. Genes are transcribed into mRNA, but because cells can use alternative splicing, there is no one-to-one relationship between the genome and the transcriptome. The transcripts are further translated into proteins, which often undergo posttranslational modifications (PTMs), or can be aberrant in cancer cells. Therefore, one gene can result in several different protein isoforms.[70,71] Protein structure can also be influenced by environmental factors, including interaction with other proteins, degradation of compartmentalization of proteins within protein complexes. As the structure and availability of the final versions of the proteins ultimately determine the behaviour of the cell, high-throughput screening methods for changes in protein expression are considered promising targets for biomarker discovery.

In 2002 the Nobel Prize in Chemistry was awarded to John Fenn and Koichi Tanaka "for their development of soft desorption ionization methods for mass spectrometric (MS) analyses of biological macromolecules". From that moment on attention for this research field has increased enormously.[72] One of the promising applications of proteomics is to perform an MS-based proteomics experiment and map a set of (poly) peptides in a

single spectrum, i.e. a profile. In this way, possibilities are created to use profiles to discriminate cancer patients from healthy individuals. Tumors have a close interaction with their microenvironment. Tumor growth factors can be found throughout the body, and also in the serum. Matrix-assisted laser desorption/ionization (MALDI) MS and surface enhanced laser desorption/ionization (SELDI) MS have emerged as promising tools for this type of analysis.[71,73] Although profiling studies lost interest after an impressive introduction by Petricoin in the Lancet since reproducibility and validation problems, recent advances in standardisation of the sample workup, measurement, data processing and evaluation markedly improved the reliability.[74-77,79] Another drawback of the profiling approach concerns the presence of highly abundant serum proteins, such as albumin. Albumin is the most abundant serum protein (34-54 mg/ml in humans) and constitutes approximately 95% of the total serum protein content together with immunoglobulins, transferrin, macroglobulin, and apolipoproteins. This phenomenon severely hampers the analysis of the proteins present at much lower concentrations, but suitable fractionation procedures can remove these highly abundant proteins.[80-82]

Peptide- and protein profiling workflows

In the research projects described in this thesis a high-end matrix-assisted laser desorption/ionization – time-of-flight (MALDI-TOF) mass spectrometer and a Fourier transform ion cyclotron resonance mass spectrometer (FTICR-MS) were used for sample (serum) analysis. The applied workflow is illustrated in Figure 2. First, serum samples are collected, processed and stored in a highly protocolled routine. In the LUMC serum samples have been collected since 2001 from patients with different types of cancer and from their accompanying persons. Serum is a very complex mixture and a well-defined and suitable sample clean-up procedure is required to reduce the sample complexity by removing salts and detergents to allow the acquisition of MS-profiles. Furthermore, a specific agent to capture (poly)peptides enriches the sample and thus contributes to sensitivity. In general, protein separation techniques are based on different physical properties of a protein, such as size, iso-electric point, solubility and affinity. Materials known from different chromatographic platforms are coupled to the surface of spherical magnetic beads.

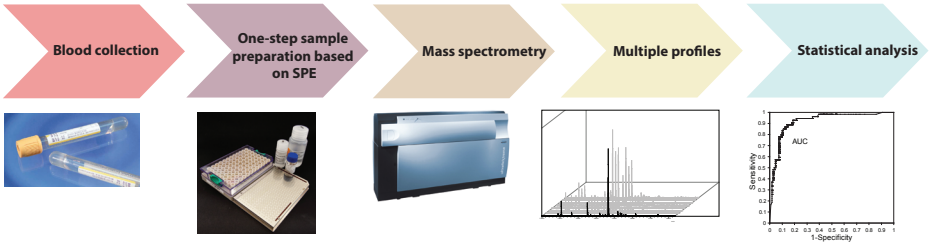

Figure 2. Applied mass spectrometry workflow, starting from serum sample to statistical analysis.

So-called functionalized magnetic beads are suited for clean-up procedures in order to obtain peptides and proteins. Solid phase extraction (SPE) methods can be carried out in a high-throughput manner by using a multichannel robot.[72,83,84]

For MALDI-TOF analysis, a small amount of sample containing various proteins and peptides is co-crystallized with light-absorbing matrix molecules on a target plate. After ionisation of the proteins, the ions are accelerated to high kinetic energy and are separated in a flight tube as a result of their different velocities. In the high-resolution mode, the ions are additionally reflected in the so-called reflectron that compensates for differences in kinetic energy. Ions are detected on a multi-channel plate detector that amplifies and thus counts arriving ions. The heavier ions travel slower than the lighter ions and will be thus detected later. Note that mass analysis is performed based on the mass-to-charge (*m/z*) ratio of the ions. Depending on the study question the MALDI-TOF can be used in linear mode that is suitable for protein analysis with (*m/z*) values of 1000 to 10.000 or in reflectron mode that allows peptide measurements from (*m/z*) values of 1000 to 4000 with higher resolution, thus showing the isotopes of the peptides.

MALDI can also be used in combination with FTICR-MS, an ultrahigh resolution instrument that traps ions in a strong magnetic field and measures ion cyclotron frequencies. As is shown by Nicolardi et al, mass measurement precision improves at least 10-fold for ultrahigh resolution data and thus simplifies spectral alignment necessary for robust and quantitatively precise comparisons of profiles in large-scale clinical studies.[85-87] From each single MALDI-FTICR spectrum an *m/z*-list can be obtained with sub-ppm precision for all different species, which is beneficial for identification purposes and interlaboratory comparisons. Furthermore, the FTICR system allows new peptide identifications from collision-induced dissociation (CID) that breaks peptides into smaller peptides to facilitate identification of the peptide.

Statistics

MS is an attractive analytical method in clinical proteomics research for its ability to simultaneously profile hundreds of across a wide range of molecular weights. MS produces a sequence of intensity readings for each sample on a pre-defined, fixed and ordered set of contiguous bins within a given mass/charge range, which discretizes the signal. The recorded intensity for any bin thus corresponds to the total number of particles detected within the *m/z* range spanned by that bin. The profile can be thought of as an extremely high-dimensional histogram, as the number of bins will typically be in the thousands, recording the distribution of ionized particles within a serum sample. Bins are usually chosen to be of equal length at the time scale, which implies bin widths will be exponentially increasing with *m/z* value at the transformed scale. Given the above description, there are obvious analogies between profiles and the data provided

by other so-called high-dimensional bioinformatic methodologies, like microarrays or SNP data, for example. It is however important to realize that in contrast to these other related bioinformatic technologies, MS data have a key distinguishing feature as spectra are functions on a *m/z* interval. The visual impression of these spectra is as a dense mixture of narrow but possibly overlaying peaks of varying intensity. This makes for a considerably richer and more complex data structure in comparison to microarray or SNP data and there is thus considerable interest in the development of appropriate statistical methodology for the analysis of such data, as well as evaluation of application of existing statistical methodology for this new data type.[88]

In 2008, our group organized a competition on clinical mass spectrometry based proteomic diagnosis. Eleven international statistical groups participated and constructed a diagnostic classification rule for allocation of future patients on a blinded calibration set. This classification rule was then tested on a blinded validation set. A variety of statistical methods was used to create a classification rule. This competition showed that a discriminating profile could be created independently of the chosen statistics with consistent results of 80% accuracy.[89]

The approach used in this thesis is based on a full implementation and application of double cross-validatory calibrated linear discriminant analysis. Each sample was assigned to the group for which the probability was highest. A key feature of this methodology is that the classifiers for prediction can jointly be optimized while simultaneously validated error rates are calculated. This methodology leaves the size of the training data nearly intact.[90]

Furthermore, a technique is used for combining the distinct spectral expressions from BC patients for the calibration of a diagnostic discriminant rule. This is achieved by first calibrating two distinct prediction rules separately, each on only one of the two available spectral data sources. A double cross-validatory approach is used to summarize the available spectral data using the two classifiers for posterior class probabilities, on which a combined predictor can be calibrated.[91]

1.4. OUTLINE OF THIS THESIS

Currently, no early diagnostic biomarkers are available that detect breast cancer or pancreatic cancer with high sensitivity and specificity and therefore can be applied as a routine screening tool. Mass spectrometry-based peptide and protein profiles have been suggested as a strategy for the (early) diagnosis of cancer and other diseases. In this thesis, we have investigated serum protein and peptide profiles and evaluated whether these could be used as a sensitive and specific test for the early detection of breast cancer and pancreatic cancer.

In Chapter 2, the current status of the research programs for protein profiling as a screening tool for breast (and colorectal) cancer is discussed. An overview is given of the different profiling methods and MS-derived candidate biomarkers that have the potential for implementation in a clinical setting. In our study on serum samples from women with breast cancer promising results were obtained when compared with the sensitivity and specificity of the current diagnostic methods. To this end, high-resolution MS peptide profiles of BC patients were compared to those from healthy controls. Moreover, the combination of data from two profiling methods improved the predictive performance. These results are described **Chapter 3.**

In **Chapters 4 and 5** the results of MS based protein and peptide profiling methods for the discrimination of pancreatic cancer were further validated. **In Chapter 4** a SPE method with WCX magnetic beads was used and a discriminating profile was identified with high sensitivity and specificity and could be validated in an independent patient-control set. In **Chapter 5** the results of peptide profiling using an RPC-18 magnetic bead SPE method are reported. The promising results of this discriminating profile were validated and compared with currently used diagnostic methods for PC. Furthermore, the most important discriminating peaks were evaluated and identified. **Chapter 6** reports the results of a fully automated one-step SPE serum sample cleanup in combination with fast MALDI acquisition and ultrahigh precision 15 tesla FTICR read-out. This approach was used on a calibration and validation set of PC patients and healthy controls. The benefits of this approach are the possibility to resolve peaks in more detail and identify the peptides due to the low parts per million (ppm) mass accuracy of the species in the FTICR profiles.

Finally, the results and conclusions of the above mentioned studies and especially the current status of clinical proteomics in cancer care are discussed in **Chapter 7**. A Dutch summary of this thesis is written in **Chapter 8.**

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