Clinical proteomics in oncology : a passionate dance between science and clinic

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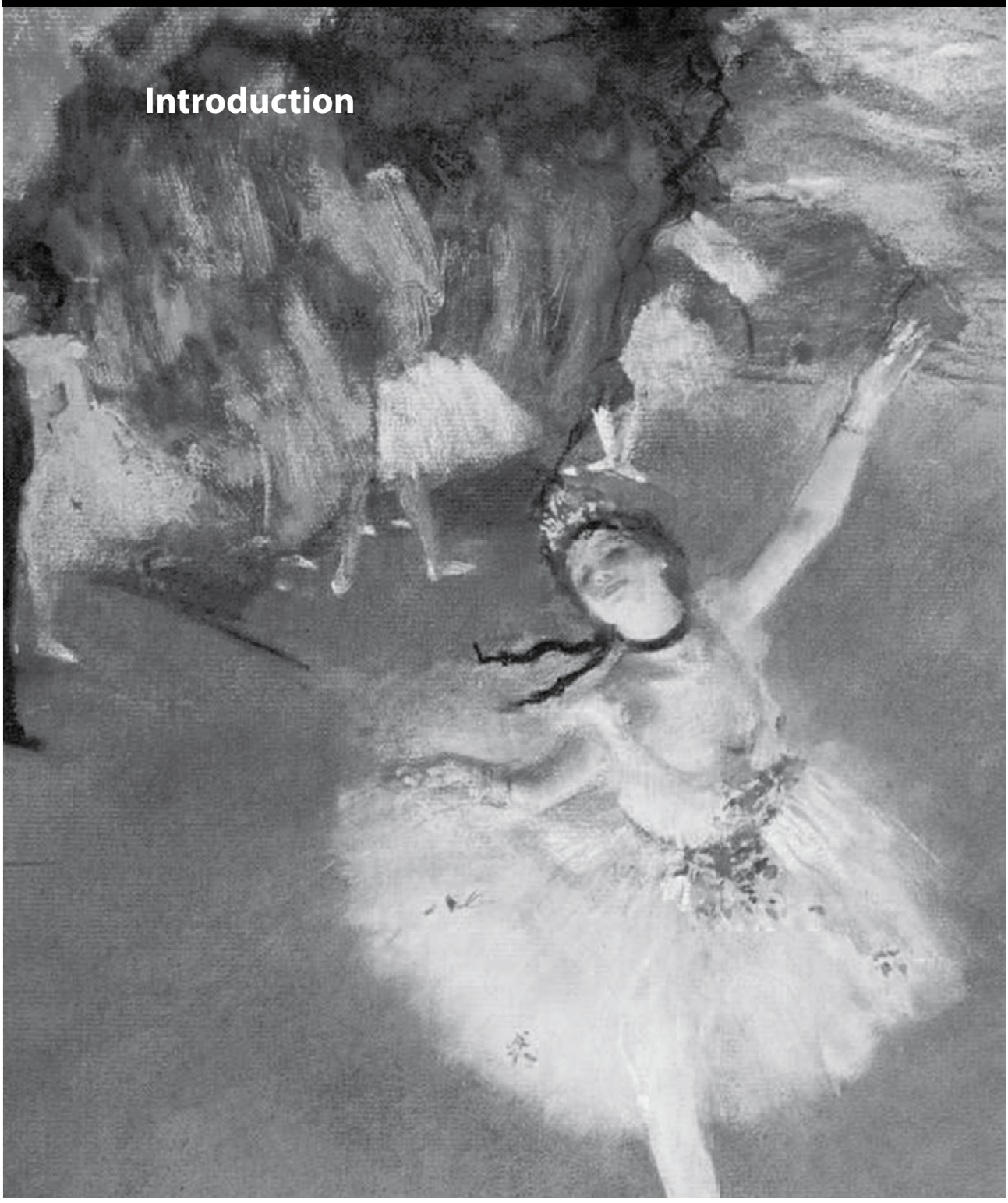
Chapter 1



Introduction

COLORECTAL CANCER

Colorectal adenocarcinoma is the third most common cancer and the fourth most frequent cause of death due to cancer worldwide. Yearly almost one million new cases occur global, with 492000 related deaths.[1] In developed countries it is the second most common tumour, with a lifetime risk of 5%, but its incidence and mortality are currently decreasing.[2;3] Surgery is the cornerstone of therapy when the disease is confined to the bowel wall. This results in 70 to 80% of patients who can be resected with curative intent.[4] After curative surgery the five-year survival rate for patients with localised disease is 90%, decreasing to 65% in case of metastised disease in the lymph nodes. Adjuvant radiation therapy, chemotherapy, or both are beneficial in selected patients. For colorectal cancer the TNM staging system remains the gold standard for prognostication of the disease relying entirely on morphological and histopathological appearance of the tumour. Classification of tumours into these TNM stages with distinct clinical courses enables clinicians to define treatment. However, tumours with similar histopathological characteristics may have different clinical outcome and responsiveness to therapy.[5] Therefore, a detailed diagnosis would allow a more individualised treatment that may avoid unnecessary morbidity and increase survival. Despite these optimised treatment strategies for colorectal cancer patients, early detection of colorectal cancer will increase survival most. Colorectal cancer is optimal to employ early detection, as precancerous and early cancerous lesions are well defined in a multistep sequence of genetic alterations that result in the transformation of normal mucosa to a precursor adenoma and ultimately to carcinoma. Thus, given the natural history of the malignancy, early diagnosis appears to be the most appropriate tool to reduce disease-related mortality.[6-8]

BIOMARKERS IN COLORECTAL CANCER

Biomarkers are molecules that indicate the presence of cancer in the body. Most biomarkers are based on abnormal presence, absence or alterations in genes, RNA, proteins and metabolites. Since the molecular changes that occur during tumour development can take place over a number of years, some biomarkers may be used to detect colorectal cancer early. Furthermore, they might be used to predict prognosis, monitor disease progression and therapeutic response. Gion et al. classified different circulating biomarkers according to their clinical application.[9] These candidate biomarkers however, are frequently found in relatively low concentrations amid a sea of other biomolecules, so biomarker research and possible diagnostic tests depend critically on the ability to make high sensitive and accurate biochemical measure-

ments. Ideally, biomarkers should be specific for the disease and easy accessible, such as serum, plasma or urine, to increase their clinical applicability.

Carcinoembryonic antigen (CEA) is the best-characterised serologic tumour marker for monitoring colorectal cancer. However, its use as a population based screening tool for early detection and diagnosis of the disease is hindered by its low sensitivity and specificity. Fletcher showed that for screening purposes in a normal population, a cut-off concentration of 2.5 µg/L CEA would yield a sensitivity of 30-40%. Based on these data he calculated that there would be 250 false positive tests for every true positive test, i.e. a patient with cancer. Furthermore, 60% of the cancers would not be detected.[10-13]

Faecal occult-blood testing (FOBT) is another biomarker for which clinical trials have shown evidence of a decreased risk of death correlated with increased detection of the disease. This approach is a non-invasive option that limits the need for follow-up colonoscopy to patients with evidence of bleeding. Neoplasms bleed intermittently, however, allowing some to escape detection with faecal occult-blood testing. Annual retesting is therefore necessary but is still insufficient, detecting only 25 to 50% of colorectal cancers and 10% of adenomas. The specificity of FOBT is also limited by frequent false positive reactions to dietary compounds, medications, and gastrointestinal bleeding from causes other than colorectal cancer.[14-17] However, population screening for colorectal cancer based on FOBT is already implemented in several countries, including a trial in the Netherlands. The expectation is that even though the techniques still has its flaws, a population screening for colorectal cancer will decrease mortality with 15-20%.[14] This can be attributed to the fact that colorectal cancer develops as a multistep sequence of precancerous and early cancerous lesions over a relatively long period of time. However, these early stages of the disease can only be detected by screening. Furthermore, scientific evidence clearly shows that, in the case of CRC, early detection and treatment leads to more benefit than treatment that has started later. These reasons among other Wilson and Jungner's criteria, that also apply for colorectal cancer, are explain that a population based screening trial has started in the Netherlands, although the technique still has some limitations.[18]

BREAST CANCER

With over 1 million new cases in the world each year, breast cancer is the commonest malignancy in women and comprises 18% of all female cancers.[19] In 2005, breast cancer caused 502,000 deaths (7% of cancer deaths; almost 1% of all deaths) worldwide. The most recent data from the Surveillance, Epidemiology, and End

Results (SEER) program of the National Cancer Institute indicate that the lifetime probability of developing invasive breast cancer is one in nine.[20] Despite increasing incidence rates, annual mortality rates from breast cancer have decreased over the last decade (2.3% per year from 1990 to 2002).[21] The effect of reduction due to early diagnosis of breast cancer has been outlined with patients' data by the Surveillance, Epidemiology, and End Results program in a competing-risk analysis calculating probabilities of death from breast cancer and other causes according tot stage, race and age at diagnosis.[22] Reasons for the decline in mortality rates in western Europe, Australia and the Americas include widespread mammography screening, precise diagnosis, and increased number of women receiving tailor made treatment- including extensive use of tamoxifen and the use of chemotherapy.[23] There are many risk factors for breast cancer, including age and gender, race, lifestyle and dietary factors, reproductive and hormonal factors, family history and genetic factors, exposure to ionizing radiation and environmental factors. Although many epidemiological risk factors have been identified, the cause of any individual breast cancer is often unknown. In other words, epidemiological research informs the patterns of breast cancer incidence across certain populations, but often not in a given individual.

Once the diagnosis of breast cancer is established, the choice of initial treatment depends upon the stage or extent of disease. Although initial treatment decisions are made on the size and appearance of the primary tumour and the presence of palpable axillary nodes, the surgical and pathological findings are used to determine the pathologic disease stage, which dictates the prognosis and need for adjuvant systemic therapy. The most important are the status of the draining axillary lymph nodes, tumour size, whether the tumour expresses hormone receptors and/or the protein HER2, and a woman's age or menopausal status. Up to one-third of women with non-palpable axillary lymph nodes will be found to harbour metastases, while one-third of those with palpable nodes will be pathologically free of nodal involvement. In women with breast cancer who are younger than 50 years of age, chemotherapy increases their 15-year survival rate by 10%; in older women the increase is 3%.[24] However, chemotherapy has a wide range of acute and long-term side effects that substantially affect the patient's quality of life.[25] As it is not possible to accurately predict the risk of metastasis development in individual patients, nowadays more than 80% of them receive adjuvant chemotherapy, although only approximately 40% of the patients relapse and ultimately die of metastatic breast cancer. Therefore, many women who would be cured by local treatment alone, which includes surgery and radiotherapy, will be 'over-treated' and suffer the toxic side effects of chemotherapy needlessly.[26]

Women who have oestrogen sensitive (ER positive) tumours receive some form of hormonal therapy to block the cancer-promoting effect of oestrogen.[27] The use of tamoxifen was shown to significantly reduce the risk of recurrence and increase ten year survival in women with ER positive and ER unknown status tumours and its gradual widespread use is one of the main factors associated with the dramatic fall in mortality during the late twentieth century.[28;29] Most postmenopausal women receive tamoxifen for five years. Trials are ongoing to establish even more effective drugs and regimens for pre- and postmenopausal patients, taking into account side-effects as well as survival times. The ATAC trial recently reported its early results comparing anastrazole alone, anastrazole plus tamoxifen, and tamoxifen alone for postmenopausal women and has shown the benefits of anastrozole over tamoxifen in disease-free survival in early breast cancer.[30] In premenopausal women oestrogen production may be stopped by surgery (removing the ovaries), radiotherapy or drugs that reversibly suppress the ovaries (LHRH analogues).

In a recent meta-analysis, a mortality reduction of 38% (age <50 years) and 20% (age 50-69 years) with chemotherapy is shown, followed by a further reduction of 31% from tamoxifen. When combined together, the final mortality reductions would be 57% and 45%, respectively 57% reduction for women younger than 50 years of age and for those of age 50–69 years.[24] Moreover, breast cancer patients with the same stage of disease can have markedly different treatment responses and overall outcome.

DIAGNOSIS AND BIOMARKERS IN BREAST CANCER

The procedures most commonly used in breast-cancer diagnosis is mammography, and to a lesser extent ultrasonography, MRI, and PET. In addition, physical examination remains important because a certain proportion (11%) of breast cancers is not seen on mammography.[31] Mammography remains the most important diagnostic tool in women with breast tissue that is not dense and is used in many countries as a population based screening in woman older than 50 years. The effect of breast screening in terms of breast cancer mortality reduction persists after long-term follow-up. A recent meta-analysis of seven randomised trials – concluded that there was a 15-20% reduction in risk of death from breast cancer in women attending mammography.[32] The effect of mammography screening is age-dependent and the highest effect is seen in women aged 55-69 years. This effect was not seen in woman under the age of 50, probably because of the higher density of the breast tissue.[33] Thus, after menopause, mammography is generally the best method to discover tiny, non-palpable lesions. By contrast, ultrasonography is the most effective procedure to diagnose small tumours in women with dense breast and to differentiate

solid lesions from cystic lesions.[34] Although mammography can identify suspicious micro calcifications, it is not good at distinguishing between breast densities and has difficulty in identifying certain lobular invasive carcinomas, Paget's disease of the nipple, inflammatory carcinoma, and particularly peripheral, small carcinomas.[35] MRI is mainly used as a problem-solving method after conventional diagnostic procedures. The technique is highly sensitive and mainly used for the screening of high-risk, *BRCA*-positive patients. It is also useful for identification of primary foci in non-palpable lesions and axillary metastases with no evidence of a primary focus, and for assessment of response to neoadjuvant chemotherapy.[36] Although MRI has good diagnostic accuracy, the rate of false-positive cases is still high and MRI findings cannot be a sole indication for breast surgery.[37] PET is presently used to discover undetected metastatic foci in any distant organ and can assess the status of axillary nodes in the preoperative staging process.[38]

Currently, mammography remains the most important diagnostic tool since serum tumour markers play no role of importance in the diagnosis of breast cancer due to a lack of sensitivity and specificity. Consequently, a major focus of present research is the identification of new biomarkers and drug targets to improve (early) detection and treatment; since early detection and more individualised treatment would benefit the individual patient and avoid unnecessary morbidity.

A NEW DIAGNOSTIC PARADIGM: CLINICAL PROTEOMICS

Proteomics is the large-scale study of proteins, particularly their presence, structure and functions. The term 'proteomics' was coined to make an analogy with genomics, the study of the genes. Proteomics is often considered the next step in the study of biological systems, after genomics. It is more complicated than genomics, mostly because while an organism's genome is rather constant, a proteome differs from cell to cell and constantly changes through its biochemical interactions with the genome and the environment. However, this functional state of a cell is very interesting for research goals and especially in oncogenesis. Clinical proteomics is referred to as mass spectrometry based proteomics using easy accessible body fluids.

Mass spectrometry is an analytical technique used to measure the mass-to-charge ratio of ions. It is most generally used to find the composition of a physical sample by generating a mass spectrum representing the masses of sample components. The mass spectrum is measured by a mass spectrometer. Matrix-assisted laser desorption/ionisation (MALDI) is a soft ionisation technique used in mass spectrometry, allowing the analysis of biomolecules which tend to be fragile and fragment when ionised by more conventional ionisation methods. The ionisation is triggered by a laser beam

(normally a nitrogen laser). A matrix is used to protect the biomolecule from being destroyed by direct laser beam and to facilitate vaporization and ionisation. The type of a mass spectrometer most widely used with MALDI is the TOF (time-of-flight mass spectrometer), mainly due to its large mass range. These mass spectrometers use an electric field to accelerate the ions through the same potential, and then measures the time they take to reach the detector. If the particles all have the same charge, then their kinetic energies will be identical, and their velocities will depend only on their masses. Lighter ions will reach the detector first, as shown in figure 1. The TOF measurement procedure is also ideally suited to the MALDI ionisation process since the pulsed laser takes individual 'shots' rather than working in continuous operation. MALDI-TOF instruments are typically equipped with an "ion mirror", deflecting ions with an electric field, thereby doubling the ion flight path and increasing the resolution. First, a sample has to be introduced into the ionisation source of the instrument. Once inside the ionisation source, the sample molecules are ionised, because ions are easier to manipulate than neutral molecules. These ions are extracted into the analyzer region of the mass spectrometer where they are separated according to their mass-to-charge ratios (m/z). The separated ions are detected and this signal is sent to a data system where the m/z ratios are stored together with their relative abundance for presentation in the format of an m/z spectrum.

Proteomic pattern diagnostics is a recent and potentially revolutionary approach for early disease detection, prognostication, and monitoring in oncology. The use of proteomic technologies might benefit biomarker discovery and treatment modalities: serum protein profiling for early disease detection and molecular signal mapping to instigate pharmocoproteomic therapeutic interventions.[39] Thus, several authors hypothesised that proteomic patterns generated with mass spectrometry are correlated to biological events occurring in the entire organism and are likely to change in the

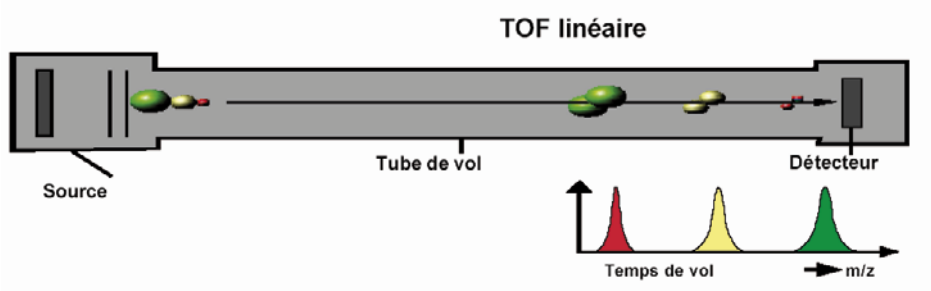


Figure 1. Schematic version of MALDI-TOF mass spectrometry principle.
Sample molecules are ionised with a laser source. Then an electric field is used to accelerate the ions in a flight tube. The detector measures their flight time. If the particles all have the same charge, then their kinetic energies will be identical, and their velocities will depend only on their masses. The smaller ones (red) will reach the detector earlier than the heavier ones (green).

presence of disease. New types of bioinformatic pattern recognition algorithms were used to identify patterns of protein changes in order to discriminate cancer patients from healthy individuals with promising results.

Petricoin and his co-workers were the first to state that finding a single disease-related biomarker is like searching for a needle in a haystack; each entity has to be separated and identified individually.[40;41] Moreover, they postulated that the blood proteome constantly changes as a consequence of the perfusion of the diseased organ adding, subtracting, or modifying the circulating proteome. These differences might be the result of proteins being abnormally produced or shed and added to the serum proteome, clipped or modified as a consequence of the disease process, or subtracted from the proteome owing to disease-related proteolytic degradation pathways. Therefore, protein pattern diagnostics would provide an easy and reliable tool for detection of cancer. The advantages of the proteomic pattern approach were stressed in several papers. In addition to the high sensitivity and specificity, cost-effectiveness, easy accessibility of body fluid and especially the high-throughput, ultimately allowing application in future screening studies, were mentioned.[42;43] Next to these hopeful voices, soon critical notes were made on analytical reproducibility and the use of the so-called black box approach, lacking identification of discriminating proteins.[44]

CLINICAL PROTEOMICS IN ONCOLOGY

Cancer is known to be the consequence of genetic alterations. A gene, however, is only potential information that must be put into a functional form. The DNA is transcribed into RNA before translation into protein, the functional manifestation of the genetic code. During the transformation of a healthy cell into a neoplastic cell, including alterations in expression, activity, localization and differential protein modification, changes also occur in the protein level. Identifying and understanding these changes is the underlying theme in cancer proteomics.[45]

In 2002 several studies discriminated patients with various cancers from healthy subjects on the basis of presence/absence of multiple low-molecular-weight serum proteins using SELDI-TOF mass spectrometry technologies.[42;46-48] The authors hypothesised that proteomic patterns are correlated with biological events occurring in the entire organism and are likely to change in the presence of disease. New types of bioinformatic pattern recognition algorithms were used to identify patterns of protein changes in order to discriminate cancer patients from healthy individuals with promising results. Several studies have shown that biomarkers can be identified on the basis of the presence/absence of multiple low-molecular-weight serum

proteins.[41;42;46-49] Furthermore, different profiles may be associated with varying responses to therapeutics and other clinically relevant parameters and may also serve as prediction for treatment outcome.

Although serum protein patterns showed high sensitivity and specificity as an early diagnostic tool in several studies, critical notes have been made on biological variation, pre-analytical conditions and analytical reproducibility of serum protein profiles that would make it difficult to differentiate a normal from a pathological and/or malignant status.[50] In addition, the reproducibility of serum protein profiles has been questioned, which however relates more to the bioinformatical analysis of the measured protein profiles than the capturing and measuring techniques itself.[51-53] Thus, if proteomics spectra are ultimately to be applied in a routine clinical setting, collection and processing of the data will need to be subject to stringent quality control procedures.[54]

OUTLINE OF THIS THESIS

Given the natural history of colorectal and breast cancer, early diagnosis appears to be the most appropriate tool to reduce disease-related mortality.[6;7] Currently, there is no early diagnostic test with high sensitivity, specificity and positive predictive value, which can be used as a routine screening tool. Therefore, there is a need for new biomarkers for both types of cancer that can improve early diagnosis, monitoring of disease progression and therapeutic response and detect disease recurrence. Proteomic expression profiles generated with mass spectrometry have been suggested as potential tools for the early diagnosis of cancer and other diseases. Because it is still in its infancy, many problems have to be overcome before clinical proteomics can be transferred form bench to bedside. **Chapter 2** gives an insight in the different fields of translational research in colorectal cancer by our group. In **chapter 3** reliability of human serum protein profiling using MALDI-TOF mass spectrometry is analysed. We present a pipeline for pre-processing, statistical data analysis and presentation of MALDI-TOF spectra. This novel analysis method was used to assess the effect of variable pre-analytical conditions on human serum protein profiles, and their effect on reproducibility. In line with the logistic conditions in a routine clinical setting, the effects of sample handling and storage, and also circadian rhythm factors on the serum protein profiles were analysed. In **chapter 4 and 5** the feasibility of mass spectrometry based protein profiling for the discrimination of colorectal cancer patients from healthy individuals was assessed. In addition to standardizing technical factors and biological variations, we performed blinded tests and employed a randomised block design experimentation to minimize impact of potential confounding

factors and to avoid bias. Especially, validation of our classifier, as a possible pitfall, was given much attention. Therefore, we performed a linear discriminant analysis with double cross-validation to separate cancer patients from healthy subjects. **Chapter 6** reports on results from an identical designed protein profiling study for the detection of breast cancer. In **chapter 7** a first validated study on the detection of breast cancer based on mass spectrometry generated protein profiles is described. In this study the same randomised blocked design and double cross validation is used, however the classifier was validated in an independent set of new patients and controls. Finally, the results and conclusions of all above mentioned studies and especially the current status of clinical proteomics in cancer are discussed in **chapter 8**.

A Dutch summary of this thesis is written in **chapter 9**.

REFERENCES

1. Weitz,J., Koch,M., Debus,J., Hohler,T., Galle,P.R., and Buchler,M.W. (2005) Colorectal cancer. *Lancet*, 365, 153-165.
2. Russo,M.W., Wei,J.T., Thiny,M.T., Gangarosa,L.M., Brown,A., Ringel,Y., Shaheen,N.J., and Sandler,R.S. (2004) Digestive and liver diseases statistics, 2004. *Gastroenterology*, 126, 1448-1453.
3. Jemal,A., Tiwari,R.C., Murray,T., Ghafoor,A., Samuels,A., Ward,E., Feuer,E.J., and Thun,M.J. (2004) Cancer statistics, 2004. *CA Cancer J Clin*, 54, 8-29.
4. Pfister,D.G., Benson,A.B., III, and Somerfield,M.R. (2004) Clinical practice. Surveillance strategies after curative treatment of colorectal cancer. *N.Engl.J Med.*, 350, 2375-2382.
5. Liefers,G.J. and Tollenaar,R.A. (2002) Cancer genetics and their application to individualised medicine. *Eur.J.Cancer*, 38, 872-879.
6. Ruo,L., Gougoutas,C., Paty,P.B., Guillem,J.G., Cohen,A.M., and Wong,W.D. (2003) Elective bowel resection for incurable stage IV colorectal cancer: prognostic variables for asymptomatic patients. *J.Am.Coll.Surg.*, 196, 722-728.
7. Gill,S. and Sinicrope,F.A. (2005) Colorectal cancer prevention: is an ounce of prevention worth a pound of cure? *Semin.Oncol.*, 32, 24-34.
8. Hawk,E.T. and Levin,B. (2005) Colorectal cancer prevention. *J Clin Oncol*, 23, 378-391.
9. Gion,M. and Daidone,M.G. (2004) Circulating biomarkers from tumour bulk to tumour machinery: promises and pitfalls. *Eur.J Cancer*, 40, 2613-2622.
10. Duffy,M.J., van Dalen,A., Haglund,C., Hansson,L., Klapdor,R., Lamerz,R., Nilsson,O., Sturgeon,C., and Topolcan,O. (2003) Clinical utility of biochemical markers in colorectal cancer: European Group on Tumour Markers (EGTM) guidelines. *Eur.J.Cancer*, 39, 718-727.
11. Fletcher,R.H. (2002) Rationale for combining different screening strategies. *Gastrointest.Endosc.Clin.N.Am.*, 12, 53-63.
12. Winawer,S., Fletcher,R., Rex,D., Bond,J., Burt,R., Ferrucci,J., Ganiats,T., Levin,T., Woolf,S., Johnson,D., Kirk,L., Litin,S., and Simmang,C. (2003) Colorectal cancer screening and surveillance: clinical guidelines and rationale-Update based on new evidence. *Gastroenterology*, 124, 544-560.
13. Ouyang,D.L., Chen,J.J., Getzenberg,R.H., and Schoen,R.E. (2005) Noninvasive testing for colorectal cancer: a review. *Am.J.Gastroenterol.*, 100, 1393-1403.
14. Pignone,M., Rich,M., Teutsch,S.M., Berg,A.O., and Lohr,K.N. (2002) Screening for colorectal cancer in adults at average risk: a summary of the evidence for the U.S. Preventive Services Task Force. *Ann.Intern.Med.*, 137, 132-141.
15. Ransohoff,D.F. and Lang,C.A. (1997) Screening for colorectal cancer with the fecal occult blood test: a background paper. American College of Physicians. *Ann.Intern.Med.*, 126, 811-822.
16. Ransohoff,D.F. (2005) Colon cancer screening in 2005: status and challenges. *Gastroenterology*, 128, 1685-1695.
17. Mandel,J.S., Bond,J.H., Church,T.R., Snover,D.C., Bradley,G.M., Schuman,L.M., and Ederer,F. (1993) Reducing mortality from colorectal cancer by screening for fecal occult blood. Minnesota Colon Cancer Control Study. *N.Engl.J.Med.*, 328, 1365-1371.
18. de Visser,M., van Ballegooijen,M., Bloemers,S.M., van Deventer,S.J., Jansen,J.B., Jespersen,J., Kluft,C., Meijer,G.A., Stoker,J., de Valk,G.A., Verweij,M.F., and Vlems,F.A. (2005) Report on the Dutch consensus development meeting for implementation and further development of population screening for colorectal cancer based on FOBT. *Cell Oncol.*, 27, 17-29.
19. Cancer Facts and Figures 2007. American Cancer Society. 2007.
20. Jemal,A., Siegel,R., Ward,E., Murray,T., Xu,J., Smigal,C., and Thun,M.J. (2006) Cancer statistics, 2006. *CA Cancer J.Clin.*, 56, 106-130.

21. Edwards,B.K., Brown,M.L., Wingo,P.A., Howe,H.L., Ward,E., Ries,L.A., Schrag,D., Jamison,P.M., Jemal,A., Wu,X.C., Friedman,C., Harlan,L., Warren,J., Anderson,R.N., and Pickle,L.W. (2005) Annual report to the nation on the status of cancer, 1975-2002, featuring population-based trends in cancer treatment. *J.Natl.Cancer Inst.*, 97, 1407-1427.
22. Schairer,C., Mink,P.J., Carroll,L., and Devesa,S.S. (2004) Probabilities of death from breast cancer and other causes among female breast cancer patients. *J.Natl.Cancer Inst.*, 96, 1311-1321.
23. Peto,R., Boreham,J., Clarke,M., Davies,C., and Beral,V. (2000) UK and USA breast cancer deaths down 25% in year 2000 at ages 20-69 years. *Lancet*, 355, 1822.
24. (2005) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet*, 365, 1687-1717.
25. Eifel,P., Axelson,J.A., Costa,J., Crowley,J., Curran,W.J., Jr., Deshler,A., Fulton,S., Hendricks,C.B., Kemeny,M., Kornblith,A.B., Louis,T.A., Markman,M., Mayer,R., and Roter,D. (2001) National Institutes of Health Consensus Development Conference Statement: adjuvant therapy for breast cancer, November 1-3, 2000. *J.Natl.Cancer Inst.*, 93, 979-989.
26. Weigelt,B., Peterse,J.L., and 't Veer,L.J. (2005) Breast cancer metastasis: markers and models. *Nat.Rev.Cancer*, 5, 591-602.
27. Wishart,G.C., Gaston,M., Poultsidis,A.A., and Purushotham,A.D. (2002) Hormone receptor status in primary breast cancer--time for a consensus? *Eur.J.Cancer*, 38, 1201-1203.
28. Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. (1992) 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Early Breast Cancer Trialists' Collaborative Group. *Lancet*, 339, 1-15.
29. Tamoxifen for early breast cancer: an overview of the randomised trials. (1998) Early Breast Cancer Trialists' Collaborative Group. *Lancet*, 351, 1451-1467.
30. Baum,M., Budzar,A.U., Cuzick,J., Forbes,J., Houghton,J.H., Klijn,J.G., and Sahmoud,T. (2002) Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomised trial. *Lancet*, 359, 2131-2139.
31. Benson,S.R., Blue,J., Judd,K., and Harman,J.E. (2004) Ultrasound is now better than mammography for the detection of invasive breast cancer. *Am.J.Surg.*, 188, 381-385.
32. Gotzsche,P.C. and Nielsen,M. (2006) Screening for breast cancer with mammography. *Cochrane.Database.Syst.Rev.*,CD001877.
33. Nystrom,L., Andersson,I., Bjurstam,N., Frisell,J., Nordenskjold,B., and Rutqvist,L.E. (2002) Long-term effects of mammography screening: updated overview of the Swedish randomised trials. *Lancet*, 359, 909-919.
34. Helvie,M.A., Chan,H.P., Adler,D.D., and Boyd,P.G. (1994) Breast thickness in routine mammograms: effect on image quality and radiation dose. *AJR Am.J.Roentgenol.*, 163, 1371-1374.
35. Gordon,P.B. and Goldenberg,S.L. (1995) Malignant breast masses detected only by ultrasound. A retrospective review. *Cancer*, 76, 626-630.
36. Kneeshaw,P.J., Turnbull,L.W., and Drew,P.J. (2003) Current applications and future direction of MR mammography. *Br.J.Cancer*, 88, 4-10.
37. Szabo,B.K., Aspelin,P., Wiberg,M.K., and Bone,B. (2003) Dynamic MR imaging of the breast. Analysis of kinetic and morphologic diagnostic criteria. *Acta Radiol.*, 44, 379-386.
38. Wahl,R.L., Siegel,B.A., Coleman,R.E., and Gatsonis,C.G. (2004) Prospective multicenter study of axillary nodal staging by positron emission tomography in breast cancer: a report of the staging breast cancer with PET Study Group. *J.Clin.Oncol.*, 22, 277-285.
39. Posadas,E.M., Simpkins,F., Liotta,L.A., Macdonald,C., and Kohn,E.C. (2005) Proteomic analysis for the early detection and rational treatment of cancer--realistic hope? *Ann.Oncol.*, 16, 16-22.

40. Petricoin,E.F. and Liotta,L.A. (2002) Proteomic analysis at the bedside: early detection of cancer. *Trends Biotechnol.*, 20, S30-S34.
41. Wulfkuhle,J.D., Liotta,L.A., and Petricoin,E.F. (2003) Proteomic applications for the early detection of cancer. *Nat.Rev.Cancer*, 3, 267-275.
42. Petricoin,E.F., Ardekani,A.M., Hitt,B.A., Levine,P.J., Fusaro,V.A., Steinberg,S.M., Mills,G.B., Simone,C., Fishman,D.A., Kohn,E.C., and Liotta,L.A. (2002) Use of proteomic patterns in serum to identify ovarian cancer. *Lancet*, 359, 572-577.
43. Petricoin,E.E., Paweletz,C.P., and Liotta,L.A. (2002) Clinical applications of proteomics: proteomic pattern diagnostics. *J.Mammary.Gland.Biol.Neoplasia.*, 7, 433-440.
44. Diamandis,E.P. (2004) Analysis of serum proteomic patterns for early cancer diagnosis: drawing attention to potential problems. *J.Natl.Cancer Inst.*, 96, 353-356.
45. Srinivas,P.R., Verma,M., Zhao,Y., and Srivastava,S. (2002) Proteomics for cancer biomarker discovery. *Clin.Chem.*, 48, 1160-1169.
46. Adam,B.L., Qu,Y., Davis,J.W., Ward,M.D., Clements,M.A., Cazares,L.H., Semmes,O.J., Schellhammer,P.F., Yasui,Y., Feng,Z., and Wright,G.L., Jr. (2002) Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. *Cancer Res.*, 62, 3609-3614.
47. Rai,A.J., Zhang,Z., Rosenzweig,J., Shih,I., Pham,T., Fung,E.T., Sokoll,L.J., and Chan,D.W. (2002) Proteomic approaches to tumor marker discovery. *Arch.Pathol.Lab Med.*, 126, 1518-1526.
48. Yanagisawa,K., Shyr,Y., Xu,B.J., Massion,P.P., Larsen,P.H., White,B.C., Roberts,J.R., Edgerton,M., Gonzalez,A., Nadaf,S., Moore,J.H., Caprioli,R.M., and Carbone,D.P. (2003) Proteomic patterns of tumour subsets in non-small-cell lung cancer. *Lancet*, 362, 433-439.
49. Petricoin,E.F., III, Ornstein,D.K., Paweletz,C.P., Ardekani,A., Hackett,P.S., Hitt,B.A., Velassco,A., Trucco,C., Wiegand,L., Wood,K., Simone,C.B., Levine,P.J., Linehan,W.M., Emmert-Buck,M.R., Steinberg,S.M., Kohn,E.C., and Liotta,L.A. (2002) Serum proteomic patterns for detection of prostate cancer. *J.Natl.Cancer Inst.*, 94, 1576-1578.
50. Boguski,M.S. and McIntosh,M.W. (2003) Biomedical informatics for proteomics. *Nature*, 422, 233-237.
51. Somorjai,R.L., Dolenko,B., and Baumgartner,R. (2003) Class prediction and discovery using gene microarray and proteomics mass spectroscopy data: curses, caveats, cautions. *Bioinformatics.*, 19, 1484-1491.
52. Yasui,Y., Pepe,M., Thompson,M.L., Adam,B.L., Wright,G.L., Jr., Qu,Y., Potter,J.D., Winget,M., Thornquist,M., and Feng,Z. (2003) A data-analytic strategy for protein biomarker discovery: profiling of high-dimensional proteomic data for cancer detection. *Biostatistics.*, 4, 449-463.
53. Baggerly,K.A., Morris,J.S., and Coombes,K.R. (2004) Reproducibility of SELDI-TOF protein patterns in serum: comparing datasets from different experiments. *Bioinformatics.*, 20, 777-785.
54. Coombes,K.R., Fritsche,H.A., Jr., Clarke,C., Chen,J.N., Baggerly,K.A., Morris,J.S., Xiao,L.C., Hung,M.C., and Kuerer,H.M. (2003) Quality control and peak finding for proteomics data collected from nipple aspirate fluid by surface-enhanced laser desorption and ionization. *Clin. Chem.*, 49, 1615-1623.