Chapter 1

Introduction

Introduction

Proteomics

The field of proteomics has developed rapidly in recent years. Until the mid-1990s scientists studied individual genes and proteins or a handful biologically related genes and proteins. Basis of proteomics is to characterize the behavior of the system rather than the behavior of any single component. The proteome is dynamic and in constant flux due to a combination of factors. These factors include posttranslational modifications and functional regulation of gene expression[1]. Moreover, in proteomics protein identification is not necessarily performed by complete sequence analysis, but can also be performed by partial sequence analysis with the aid of database matching tools.
Proteomic analysis requires the combination of various technologies, including biochemistry, mass spectrometry and bioinformatics. Important techniques for expression analysis of proteins are two-dimensional electrophoresis (2-DE) combined with Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS), and Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry.

2-DE

The principle of 2-DE is separation on a gel of the protein content of a sample in two dimensions according to charge and mass. Separation of protein extracts by 2-DE followed by MALDI-TOF-MS is a well-established and powerful method in the identification of differentially expressed proteins. The gels can be stained with Coomassie brilliant blue staining, radioactive or fluorescent labeling, and silver staining. The spot intensities in samples are compared among different gels. The differentially expressed proteins can be excised from the gel and after destaining, in-gel digestion with trypsin and extraction, the peptide mixtures are spotted on the target plate and analyzed with MALDI-TOF-MS. A high separation is achieved with 2-DE, but a major disadvantage of this technique, however, is its lack of real high throughput capability and the large amount of starting material required.

SELDI-TOF-MS versus MALDI-TOF-MS

There have been many reports on the application of SELDI-TOF-MS technology since its first introduction in 1993 by Hutchens and Yip[2]. SELDI-TOF-MS is an approach that tries to overcome the requirements for purification and separation of proteins prior to mass spectrometry analysis[3]. It is a novel approach to biomarker discovery that combines two powerful techniques: chromatography and mass spectrometry. One of the key features of SELDI-TOF-MS is its ability to provide a rapid protein expression profile from a variety of biological and clinical samples[4]. It consists of selective protein extraction and retention on chromatographic chip

surfaces and their subsequent analysis by a simple laser desorption/ionization mass spectrometer[5]. It differs in several aspects from conventional MALDI-TOF-MS. For MALDI-TOF-MS, analytes are directly spotted onto a plate. This is usually a metal plate. The applied samples are usually tryptic digests from proteins separated by 2-DE, although proteins purified by other separation methods are also compatible with the method. Before deposition of the analytes, the energy absorbing matrix (EAM) is placed on the plate or mixed in with the sample. The matrix will absorb energy from the laser causing the analytes to be ionized by MALDI-TOF-MS[6]. The disadvantage of the MALDI-TOF-MS technique is that for complex samples fractionation needs to take place before spotting on the gold plate. This means that there is more starting material needed for the offline sample fractionation compared to SELDI-TOF-MS analyses. The advantages of the SELDI-TOF-MS technique are the high throughput capability, the low amounts of sample material. The SELDI-TOF-MS can effectively resolve polypeptides and peptides smaller than 20 kDa[7].

ProteinChip arrays

For the SELDI-TOF-MS technique different ProteinChip arrays (Ciphergen Biosystems Inc.) are used. The chromatographic surfaces that make up the various ProteinChip arrays are uniquely designed to retain proteins from a complex sample mixture according to specific properties such as hydrophobicity, charge[4] (Figure 1.1).

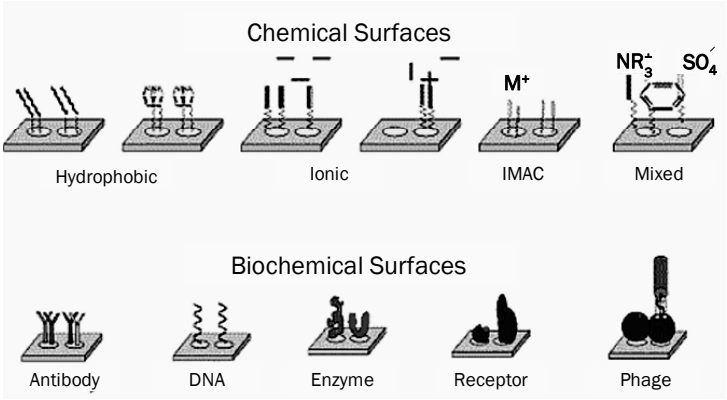

Figure 1.1 The different types of ProteinChip arrays.
The chemical surfaces are chromatographic ProteinChip arrays with hydrophobic, cationic, anionic, metal ions for immobilized metal affinity binding (IMAC) or hydrophilic spots. The biochemical surfaces are designed for coupling of biomolecules in antibody-antigen assays, DNA-protein binding experiment, coupling of enzymes, receptor-ligand interaction and for coupling of phages.

The procedure for detecting protein biomarkers is very simple. A few microliters of the sample are dispensed onto the ProteinChip surface under specific binding conditions that determine which proteins will be retained by the surface. Protein specificity is achieved through the application of a series of washes with an appropriate solvent or buffer designed to elute unbound proteins and interfering substances, such as salts, detergents, lipids. Only proteins actively interacting with the spot surfaces are analyzed in the SELDI-TOF-MS (Protein Biosystem series instrument) (Ciphergen Biosystems Inc.) because all other components are washed off in advance. One of the most obvious advantages of this surface-enhanced process is that components such as salts or detergents which commonly cause problems with other analytical tools are washed away as part of the SELDI process[8].
By choosing different ProteinChip arrays with array-specific surface components, different proteins will be analyzed depending on the chip characteristics. In fact the interaction of the analyte and the chip introduces a purification step. Each combination of ProteinChip arrays together with the binding and washing buffers of choice results in a unique binding capacity for a special subset of peptides and proteins. After addition of sample and washing buffers, the EAM is applied to the ProteinChip array. The EAM will facilitate desorption and ionization in the SELDI-TOF-MS.

Desorption/Ionization process

After introducing the ProteinChip array into the SELDI-TOF-MS, a laser beam is directed onto the sample on the spot. Upon laser activation, the sample becomes irradiated and the desorption and ionization proceeds to liberate gaseous ions from the ProteinChip arrays. These gaseous ions enter the TOF-MS region of the instrument, which measures the mass-to-charge ratio (*m/z*) of molecular ions of each protein, based on its velocity through a vacuum tube[4]. The time-of-flight corresponds to the *m/z* value. As a first result, the molecules in the sample are represented in a graph with the *m/z* value on the x-axis and the corresponding signal intensity on the y-axis[8] (Figure 1.2).

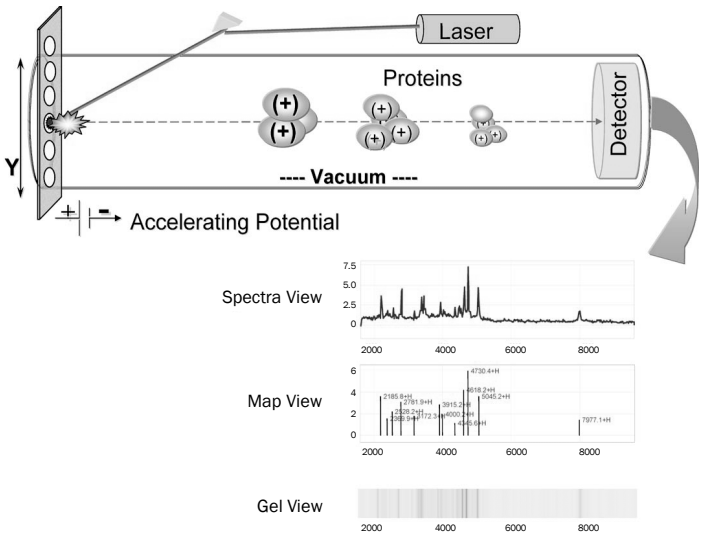

Figure 1.2 The ions of the molecules in the sample are represented in a spectra, map and gel view with the mass-to-charge ratio (*m/z*) on the x-axis and the corresponding signal intensity on the y-axis.

Biomarker Discovery

The true scientific goal of serum proteomic pattern analysis is in fact biomarker discovery. However, since the study by Petricoin et al.[9] on proteomic patterns to detect ovarian cancer, the use of SELDI-TOF-MS protein profiling as a diagnostic tool, has become an important subject of investigation too[10]. Until now, this approach has been suggested for different diseases, like ovarian[9,11-15], prostate[7,16-19] and lung[20] cancer, but also for inflammatory diseases[21,22].

Currently, the pipeline from translation of new biomarkers into tests appears to have a bottleneck at the early stages of translation of research markers into clinical tests. Research groups performing discovery and clinical studies rarely have the resources to develop prototype analyzers or test reagent sets, to manufacture them, or to proceed with other steps in commercialization. These steps usually rely on the in vitro diagnostics industry, which has had relatively low investment in the development of new markers. After development of tests, there is a need for evaluation in clinical laboratories, submission for approval by the US Food and Drug Administration[23], establishment of reimbursement rates by the Medicare system and insurers, and education of physicians about test ordering and interpretation. The process of translating new markers into clinical laboratory test entails contributions from multiple disciplines, including scientists; engineers; business, legal, and regulatory professionals; clinicians; and clinical laboratorians[24].

Detection of potential biomarkers

The SELDI-TOF-MS technique was used to detect potential biomarkers for two chronic inflammatory diseases; sarcoidosis, ankylosing spondylitis, and to compare protein profiles of two types of lacunar stroke. These studies are described in this thesis.

Sarcoidosis

Sarcoidosis is a systemic granulomatous disorder of unknown cause characterized by its pathological hallmark, the noncaseating granuloma[25,26]. The clinical presentation of sarcoidosis is highly variable[27]. Involvement of the lungs or intrathoracic lymph nodes becomes clinically evident in 90% of the symptomatic patients during their disease and up to 30% show spontaneous remission[28]. A chronic course occurs in 10-30% of the patients, at times resulting in significant impairment of lung function[29,30]. Unfortunately, till now there is no good marker for both diagnosis and prognosis of sarcoidosis.

Ankylosing spondylitis

Ankylosing spondylitis (AS) is a chronic systemic inflammatory rheumatic disorder that primarily affects the axial skeleton, with sacroliitis as its hallmark. Spinal structural damage can be assessed on conventional radiographs as destructive and proliferative lesions ultimately leading to syndesmophyte formation. It has been estimated that at least 30% of patients do develop severe spinal restriction during the natural course of the disease. Until now there is no satisfying biomarker for diagnosis or prognosis of AS.

Lacunar stroke

Lacunar infarcts are small, deeply in the brain located infarcts, mostly caused by local pathology in a small perforating artery[31]. Lacunar stroke patients in whom cerebral imaging shows multiple additional “silent” lacunar lesions, differ clinically from those patients with only a single symptomatic lesion in that they show more extensive cerebral white matter lesions on neuro-imaging, have more often hypertension, and have worse prognosis on functional outcome, a higher stroke recurrence rate, higher short- and long-term mortality and higher rate of asymptomatic lesion progression[32-36]. Whether these two types are both ends of one pathogenetic spectrum, or represent two pathogenetically different entities, remains subject of debate. Research into the underlying pathogenetic mechanisms thus far focused on known proteins that are thought to be involved in vascular pathogenesis.

Glycomics

Glycomics or glycobiology deals with the structure and function of oligosaccharides (chains of sugars). The entire repertoire of carbohydrates in an organ is thus collectively referred to as the glycome. This area of research has to deal with an inherent level of complexity. The saccharides have a multitude of building blocks. In this thesis, we look for glycosylation defects in galactosemia. Patients with classical galactosemia, an inborn error of galactose metabolism with secondary glycosylation abnormalities, are at risk for a diminished bone mass since early age. The lack of evidence for the presence of any of the well established risk factors for a diminished bone mass such as nutritional deficiencies or other diet-related factors led us to hypothesize a glycosylation defect of proteins involved in bone metabolism in these patients. Human Insulin-like Growth Factor type I (IGF-I), a key role player in bone metabolism is over 75% bound in ternary complexes with IGF-binding protein-3 (IGFBP-3) and the acid-labile subunit (ALS). The level of IGFBP-3 glycosylation modulates the cell-binding activity of IGFBP-3. In order to elucidate if pediatric galactosemic patients have glycan abnormalities in IGFBP-3 and because IGFBP-3 levels are in the range of 10-100 nM, a specific immunoprecipitation method is needed to isolate and purify IGFBP-3. The aim was to compare 1-DE and 2-DE western blots of pediatric galactosemic patients, CDG patients and healthy controls. The discriminating isoforms need to be identified with MALDI-TOF-MS.

Scope and the aims of the thesis

The first aim of the studies presented in this thesis was to evaluate the prominent analytical aspects, which are involved in proteomics studies. The importance of the pre- and post analytical strategies in proteomics studies were outlined in **Chapter 2**.
Any new technology, particularly one being presented as a potential clinically used diagnostic tool, requires stringent quality control to evaluate analytical performance over time. In **Chapter 3** a standard protocol for calibration of the MALDI-TOF-MS part of the SELDI-TOF-MS instrument was defined and acceptance criteria for the independent certified QC samples were established.
This thesis is focused on proteomics studies in different diseases. In **Chapter 4** an overview of the different proteomics techniques to detect potential and/or common biomarkers in chronic inflammatory diseases was shown. The identified and validated proteins detected in the different studies are compared and discussed to conclude if there are some common markers which can be used in the diagnosis and prognosis of the three chronic inflammatory diseases described in this study; multiple sclerosis, rheumatic diseases and lung inflammatory diseases.
The aim of **Chapter 5** and **6** was to detect potential biomarkers, using the SELDI-TOF-MS technique, for sarcoidosis and ankylosing spondylitis, respectively.
Another SELDI-TOF-MS protein profiling study with subsequent phenotyping analysis was described in **Chapter 7** to differentiate between two types of lacunar stroke.
Another application of proteomics was to detect the glycosylation defects in galactosemia. In **Chapter 8**, a specific immunoprecipitation method to isolate IGFBP-3 from serum was described to compare 1-DE and 2-DE western blots of pediatric galactosemic patients, CDG patients and healthy controls.

References

1. Srinivas PR, Srivastava S, Hanash S, Wright GL Jr. Proteomics in Early Detection of Cancer. Clin Chem 2001;47:1901-1911.
2. Hutchens TW, Yip T. New Desorption strategies for the mass spectrometric analysis of macromolecules. Rapid Commun Mass Spectrom 1993;7:576-580.
3. Bischoff R, Luider TM. Methodological advances in the discovery of protein and peptide disease markers. J Chromatogr B Analyt Technol Biomed Life Sci 2004;803:27-40.
4. Issaq HJ, Veenstra TD, Conrads TP, Felschow D. The SELDI-TOF MS Approach to Proteomics: Protein Profiling and Biomarker Identification. Biochemical and Biophysical Research Communications 2002;292:587-592.
5. Caputo E, Moharram R, Martin BM. Methods for on-chip protein analysis. Anal Biochem 2003; 321:116-124.
6. Aebersold R, Goodlett DR. Mass spectrometry in proteomics. Chem Rev 2001;101:269-295.
7. Qu Y, Adam BL, Yasui Y, Ward MD, Cazares LH, Schellhammer PF, Feng Z, Semmes OJ, Wright GL Jr. Boosted decision tree analysis of surface-enhanced laser desorption/ionization mass spectral serum profiles discriminates prostate cancer from noncancer patients. Clin Chem 2002;48: 1835-1843.
8. Wiesner A. Detection of Tumor Markers with ProteinChip(R) Technology. Curr Pharm Biotechnol 2004;5:45-67.
9. Petricoin III EF, Mills GB, Kohn EC, Liotta LA. Proteomic patterns in serum and identification of ovarian cancer. Lancet 2002;360:170-171.
10. Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, Mills GB, Simone C, Fishman DA, Kohn EC, Liotta LA. Use of proteomic patterns in serum to identify ovarian cancer. Lancet 2002;359:572-577.
11. Kozak KR, Amneus MW, Pusey SM, Su F, Luong MN, Luong SA, Reddy ST, Farias-Eisner R. Identification of biomarkers for ovarian cancer using strong anion-exchange ProteinChips: potential use in diagnosis and prognosis. Proc Natl Acad Sci U S A 2003;100:12343-12348.
12. Zhang Z, Bast RC Jr, Yu Y, Li J, Sokoll LJ, Rai AJ, Rosenzweig JM, Cameron B, Wang YY, Meng XY, Berchuck A, Van Haaften-Day C, Hacker NF, de Bruijn HW, van der Zee AG, Jacobs IJ, Fung ET, Chan DW. Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. Cancer Res 2004;64:5882-5890.
13. Vlahou A, Schorge JO, Gregory BW, Coleman RL. Diagnosis of Ovarian Cancer Using Decision Tree Classification of Mass Spectral Data. J Biomed Biotechnol 2003;2003:308-314.
14. Ye B, Cramer DW, Skates SJ, Gygi SP, Pratomo V, Fu L, Horick NK, Licklider LJ, Schorge JO, Berkowitz RS, Mok SC. Haptoglobin-alpha subunit as potential serum biomarker in ovarian cancer: identification and characterization using proteomic profiling and mass spectrometry. Clin Cancer Res 2003;9:2904-2911.
15. Rai AJ, Zhang Z, Rosenzweig J, Shih Ie M, Pham T, Fung ET, Sokoll LJ, Chan DW. Proteomic approaches to tumor marker discovery. Arch Pathol Lab Med 2002;126:1518-1526.
16. Banez LL, Prasanna P, Sun L, Ali A, Zou Z, Adam BL, McLeod DG, Moul JW, Srivastava S. Diagnostic potential of serum proteomic patterns in prostate cancer. J Urol 2003;170:442-446.
17. Adam BL, Qu Y, Davis JW, Ward MD, Clements MA, Cazares LH, Semmes OJ, Schellhammer PF, Yasui Y, Feng Z, Wright GL Jr. Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. Cancer Res 2002;62:3609-3614.
18. Petricoin EF 3rd, Ornstein DK, Paweletz CP, Ardekani A, Hackett PS, Hitt BA, Velassco A, Trucco C, Wiegand L, Wood K, Simone CB, Levine PJ, Linehan WM, Emmert-Buck MR, Steinberg SM, Kohn EC, Liotta LA. Serum proteomic patterns for detection of prostate cancer. J Natl Cancer Inst 2002; 94:1576-1578.
19. Li J, White N, Zhang Z, Rosenzweig J, Mangold LA, Partin AW, Chan DW. Detection of prostate cancer using serum proteomics pattern in a histologically confirmed population. J Urol 2004;171: 1782-1787.

20. Zhukov TA, Johanson RA, Cantor AB, Clark RA, Tockman MS. Discovery of distinct protein profiles specific for lung tumors and pre-malignant lung lesions by SELDI mass spectrometry. Lung Cancer 2003;40:267-279.
21. Poon TC, Hui AY, Chan HL, Ang IL, Chow SM, Wong N, Sung JJ. Prediction of Liver Fibrosis and Cirrhosis in Chronic Hepatitis B Infection by Serum Proteomic Fingerprinting: A Pilot Study. Clin Chem 2004;51:328-335.
22. Zhu XD, Zhang WH, Li CL, Xu Y, Liang WJ, Tien P. New serum biomarkers for detection of HBV-induced liver cirrhosis using SELDI protein chip technology. World J Gastroenterol 2004;10: 2327-2329.
23. Hackett JL, Gutman SI. Introduction to the Food and Drug Administration (FDA) regulatory process. J Proteome Res 2005;4:1110-1113.
24. Hortin GL, Jortani SA, Ritchie JC Jr, Valdes R Jr, Chan DW. Proteomics: A New Diagnostic Frontier. Clin Chem 2006;52:1218-1222.
25. Baughman RP, Lower EE, Du Bois RM. Sarcoidosis. Lancet 2003;361:1111-1118.
26. Kataria YP, Holter JF. Sarcoidosis: A Model of Granulomatous Inflammation of Unknown Etiology Associated with a Hyperactive Immune System. Methods 1996;9:268-294.
27. Judson MA, Baughman RP, Thompson BW, Teirstein AS, Terrin ML, Rossman MD, Yeager H Jr, McLennan G, Bresnitz EA, DePalo L, Hunninghake G, Iannuzzi MC, Johns CJ, Moller DR, Newman LS, Rabin DL, Rose C, Rybicki BA, Weinberger SE, Knatterud GL, Cherniak R. Two year prognosis of sarcoidosis: the ACCESS experience. Sarcoidosis Vasc Diffuse Lung Dis 2003;20:204-211.
28. Rybicki BA, Maliarik MJ, Major M, Popovich J Jr, Iannuzzi MC. Epidemiology, demographics, and genetics of sarcoidosis. Semin Respir Infect 1998;13:166-173.
29. Baughman RP, Winget DB, Bowen EH, Lower EE. Predicting respiratory failure in sarcoidosis patients. Sarcoidosis Vasc Diffuse Lung Dis 1997;14: 154-158.
30. Arcasoy SM, Christie JD, Pochettino A, Rosengard BR, Blumenthal NP, Bavaria JE, Kotloff RM. Characteristics and outcomes of patients with sarcoidosis listed for lung transplantation. Chest 2001;120:873-880.
31. Fisher CM. The arterial lesions underlying lacunes. Acta neuropath (Berl) 1969;12:1-15.
32. Boiten J, Lodder J, Kessels F. Two clinically distinct lacunar infarct entities? A hypothesis. Stroke 1993;24:652-656.
33. Mast H, Thompson JLP, Lee S-H, Mohr JP, Sacco RL. Hypertension and diabetes mellitus as determinants of multiple lacunar infarcts. Stroke 1995;26:30-33.
34. Spolveri S, Baruffi MC, Cappelletti C, Semerano F, Rossi S, Pracucci G, Inzitari D. Vascular risk factors linked to multiple lacunar infarcts. Cerebrovascular Diseases 1998;8:152-157.
35. de Jong G, Kessels F, Lodder J. Two types of lacunar infarcts, further arguments from a study on prognosis. Stroke 2002;33:2072-2076.
36. van Zagten M, Boiten J, Kessels F, Lodder J. Significant progression of white matter lesions and small deep (lacunar) infarcts in patients with stroke. Arch Neurol 1996;53:650-655.