

# Methodology and Applications of Disease Biomarker Identification in Human Serum

Ziad J. Sahab, Suzan M. Semaan and Qing-Xiang Amy Sang

Department of Chemistry and Biochemistry and Institute of Molecular Biophysics, Florida State University, Tallahassee, FL 32306-4390, U.S.A.

**Abstract:** Biomarkers are biomolecules that serve as indicators of biological and pathological processes, or physiological and pharmacological responses to a drug treatment. Because of the high abundance of albumin and heterogeneity of plasma lipoproteins and glycoproteins, biomarkers are difficult to identify in human serum. Due to the clinical significance the identification of disease biomarkers in serum holds great promise for personalized medicine, especially for disease diagnosis and prognosis. This review summarizes some common and emerging proteomics techniques utilized in the separation of serum samples and identification of disease signatures. The practical application of each protein separation or identification technique is analyzed using specific examples. Biomarkers of cancers of prostate, breast, ovary, and lung in human serum have been reviewed, as well as those of heart disease, arthritis, asthma, and cystic fibrosis. Despite the advancement of technology few biomarkers have been approved by the Food and Drug Administration for disease diagnosis and prognosis due to the complexity of structure and function of protein biomarkers and lack of high sensitivity, specificity, and reproducibility for those putative biomarkers. The combination of different types of technologies and statistical analysis may provide more effective methods to identify and validate new disease biomarkers in blood.

**Abbreviations:** 2-DE, two-dimensional gel electrophoresis; 2DLC-MS, two-dimensional liquid chromatography mass spectrometry; CA 15.3, cancer antigen 15.3; CA 19–9, cancer antigen 19–9, a tumor-associated antigen; CA125, cancer antigen 125, a mucin-like protein; CEA, carcinoembryonic antigen; CF, Cystic Fibrosis; CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; ESI-MS/MS, electrospray ionization tandem mass spectrometry; FDA, Food and Drug Administration; IPG, immobilized pH gradient; MALDI-TOF-MS, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry; MDLC, multidimensional liquid chromatography; MUC, mucin; MudPIT, multi-dimensional protein identification technology; OA, osteoarthritis; pI, isoelectric point; PSA, prostate-specific antigen; RA, rheumatoid arthritis; RP-HPLC, reversed-phased-high performance liquid chromatography; RPLC, reversed phase liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; SELDI, surface-enhanced laser desorption ionization; SELDI-TOF, surface-enhanced laser desorption ionization-time of flight.

**Keywords:** Serum biomarkers, proteomics, cancer signatures, disease diagnosis and prognosis, biochemical indicators, technological innovations.

## 1. Introduction

### 1.1 Proteomics

Proteomics is the science and technology of separating and identifying proteins from crude biological samples. It is mostly significant when differentially-expressed proteins between two samples that belong or that are subject to different conditions are identified. This identification will allow for the characterization of biological roles, clarification of biological mechanisms, and identification of therapeutic targets and biomarkers. The first step of a proteomic study requires almost always the separation of proteins using electrophoresis or chromatography techniques followed by the identification steps that are performed using mass spectrometry. The latter measures accurately the masses of peptides generated from the digestion of the protein by trypsin or another enzyme followed by the use of software that scan the different protein databases available to identify the protein. This identification is based on the information collected about this protein throughout the study including but not limited to isoelectric point, molecular mass, peptide masses, and specie of the biological sample. It is preferable—even required sometimes—to use immunoblotting techniques or N-terminal sequencing to validate the identity of the proteins. Proteomics studies can be applied on any protein mixture extracted from any organism including

**Correspondence:** Prof. Qing-Xiang Amy Sang, Ph.D., Department of Chemistry and Biochemistry and Institute of Molecular Biophysics, Florida State University, Tallahassee, FL 32306-4390, U.S.A. Tel: +1-850-644-8683; Fax: +1-850-644-8281; Email: qxsang@chem.fsu.edu

plant, bacterial, and animal cells. Proteomics strategies have been used to identify disease-specific protein markers called biomarkers that could provide the basis for the development of new diagnosis methodologies, treatments, and early disease detection (Hanash, 2000; Somiari et al. 2003).

## 1.2 Clinical proteomics and biomarkers

The term clinical proteomics refers to “the application of available proteomics technologies to current areas of clinical investigation” (Clarke et al. 2003). Many diseases manifest themselves through severe changes in human physiology, which forms the basis for clinical chemistry and bestows its value in diagnoses and subsequent therapeutic interventions (Bischoff and Luider, 2004). Clinical proteomics includes the global analysis of proteins expressed by the genome of an organism, with the typical aim being the evaluation of quantitative changes that occur as a function of disease, treatment, or environment (Somiari et al. 2003). Proteomics strategies have been used to recognize disease-specific protein markers called biomarkers that could provide the basis for the development of new diagnosis methodologies, treatments, and early disease detection (Hanash, 2000; Somiari et al. 2003). A biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathological processes, or pharmacological responses to a therapeutic intervention” (Atkinson et al. 2001).

The degree to which biomarkers reflect clinical outcomes judges their effectiveness. As biomarkers have different characteristics there should be statistical requirements that determine the usefulness of biomarkers as evaluations of disease progression or outcomes in clinical trials (Cummings, 2005). Those requirements should include statistical dispersion, detailed information on target populations, and specificity of the biomarker. Generally, an ideal biomarker is expected to be: able to detect a fundamental feature of a specific disease; validated in and confirmed by those specific disease cases; precise, able to detect the early stages of this specific disease and distinguish it from other similar disease cases or family members of that disease; simple to perform; reliable; non-invasive; and inexpensive if possible (Anon et al. 1998; Gao et al. 2005).

To identify new biomarkers of a certain disease *in vitro*, a three component analytical strategy was developed, which consists of (I) a cell line as the pre-clinical model, (II) a set of five well-studied drugs, three of which had been found in humans to elicit that disease, and (III) implementation of multi-dimensional protein identification technology (MudPIT) (see 3.3) to perform semi-quantitative analysis and identify protein biomarker candidates (Gao et al. 2005). MudPIT was chosen due to the demonstration of its usefulness in the identification as well as semi-quantification (relative changes/differential trends in protein abundance) of large numbers of proteins, both *in vivo* and *in vitro* (Washburn et al. 2001; Pang et al. 2002; Gao et al. 2003; Gao et al. 2004). This biomarker-identification stage generates a large list of biomarkers. Upon passing through a crucial second stage, a rate determining one, only the most appropriate subset of biomarker-candidates will be further tested by traditional immunoassays to identify and verify an ideal biomarker or the most credible biomarker for a specific disease according to aforementioned criteria (Gao et al. 2005).

## 1.3 Significance of proteomics of human samples

Mammalian samples are the most studied species in terms of protein profiling studies. *In vitro* culture of mammalian cell lines is an important resource for research, and have been used for disease-related studies (Tedeschi et al. 2005; Liu et al. 2005; Bianchi et al. 2005; An et al. 2005) as well as technology related ones (Hamler et al. 2004; Zhu et al. 2004; Buchanan et al. 2005). The protein profiles of cancerous cell lines have been compared to the profiles of normal cell lines (O'Neill et al. 2003). While tumor-derived cell lines can be useful for initial studies (Wu et al. 2002), each line displays a unique evolution that may not truly mimic real *in vivo* conditions (Ornstein et al. 2000a). A comparison between human prostate cell lines with tumor cells from prostate patients showed significant altered protein profiles (Ornstein et al. 2000b). Differentially-expressed proteins identified in human *in vivo* cancerous tissues when compared to their normal counterparts are by far more significant than *in vitro* ones. A large number of studies have been successful in identifying protein signatures of a disease or a condition from *in vivo* tissues (Wulfkühle et al. 2002; Tribl et al.

2005), as well as patient serum samples (Broeck-aert et al. 2000; Hathaway et al. 2005; Ahn et al. 2005). These differentially-expressed proteins are considered either the cause or the effect of the physiological change in the organism. Many published proteomic studies of human tumor tissue are associated with weaknesses in tumor representation, sample contamination by non-tumor cells and serum proteins. Studies often include a moderate number of tumors which may not be representative of clinical materials (Alaiya et al. 2005).

## 1.4 Human serum

Human serum is the clear portion of the human's body fluid that separates from blood upon clotting. This clear fluid provides moisture to the serous membranes in the human body. It contains 60–80 mg/mL of proteins in addition to various small molecules including amino acids, lipids, salts, and sugars (Burtis et al. 2001). Normal human serum mainly contains the following proteins: IgG, IgM (Ekdahl et al. 1994), IgA, haptoglobulin, albumin (Era et al. 1995), transferrin (Burtis et al. 2001),  $\alpha_1$ -anti-trypsin, fibrinogen,  $\alpha_2$ -Macroglobulin, and complement C<sub>3</sub>, those account for >95% of total serum proteins (Anderson and Anderson, 2002), many of which are synthesized and secreted, shed, or lost from cells and tissues throughout the body (Schrader and Schulz-Knappe, 2001; Kennedy, 2001).

Analysis of the human serum proteome, especially for biomarkers, has great potential for diagnosis and early detection of human disease. One of the difficulties to identifying a specific marker in the human serum is the low abundance of proteins secreted in the serum as a result of the disease when compared to the high concentration of albumin, resulting from daily synthesis of ~12 g in the liver and a half-life of about 21 days (McFarlane et al. 2000), that constitutes more than 50% (w/w) of the total amount of proteins in the serum. Knowing the complexity of the human proteome and the broad dynamic range in abundance of individual proteins (e.g. albumin, immunoglobulin), there is a need for sample treatment prior to biomarker identification and is feasible using different analytical techniques. A prefractionation step to eliminate albumin from the serum is therefore required prior to the proteomic study (Lee et al. 2006a).

## 1.5 Protein properties

Proteins are composed of covalently bound amino acids. There are 20 different amino acids generating an infinite number of possible proteins. Several protein physical properties should be taken into consideration for a successful proteomic project. These properties are used to separate and to identify proteins from crude biological samples. Based on the side chain properties, amino acids of a protein can form unstable interactions through electrostatic, hydrogen bonding, and hydrophobic affinities. These interactions result in the folding of proteins that hinders their separation and identification necessitating the introduction of several reagents to the protein solubilizing buffer to break these interactions. Once these interactions are neutralized, the protein becomes unfolded (denatured). After denaturing the proteins, the remaining physical properties are used to separate and purify these proteins.

### 1.5.1 Isoelectric point (pI)

Proteins are amphoteric molecules: they can carry positive, negative or a neutral charge. At a certain pH, the number of positive charges is equal to the number of negative charges: this pH is equal to the pI of the protein. The amino acids that affect the value of the protein isoelectric point the most are those with ionizable side chains: Arginine, Tyrosine, Lysine, Cysteine, Histidine, Glutamic acid, and Aspartic acid. Proteins can be separated based on their isoelectric point using ion exchange chromatography as well as immobilized pH gradient polyacrylamide gels (see 2.3.1).

### 1.5.2 Hydrophobicity

The highly hydrophobic amino acid side chains are those of: Valine, Leucine, Isoleucine, Methionine, Phenylalanine, Tryptophan, and Cysteine. Proteins can be separated based on their hydrophobicities using Reversed-Phased High performance Liquid Chromatography (RP-HPLC). The stationary phase used for RP-HPLC is composed of silica beads with carbon chains linked to it. The protein mixture is injected in the column and then an acetonitrile gradient is imposed. More hydrophobic proteins require higher concentrations of acetonitrile for their elution (see 2.2.2).

### 1.5.3 Molecular mass ( $M_r$ ) and size

Proteins can be composed of as few as tens of amino acids and as many as thousands. Most of

the time, denatured proteins having a higher number of amino acids will have a bigger size and a higher  $M_r$ . Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) (see 2.3.1) as well as size exclusion chromatography can be used to separate proteins based on their size or  $M_r$ . The approximate  $M_r$  can be measured using these techniques. To measure the exact  $M_r$  of proteins a mass spectrometer should be used.

## 2. Serum Protein Separation

### 2.1 Sample treatment using commercially-available kits

The presence of highly abundant albumin and immunoglobulins that constitute approximately 60–97% of the total serum proteins gave the urge for using special kits to eliminate those highly abundant proteins to allow the visualization of low abundant proteins, such as using Cibacron blue F3GA based-resin (Ahmed et al. 2005) and antibody-related methods.

Unfortunately, it was shown that Cibacron blue F3GA based-resin method will clear, not just the albumin, but also every protein having the same pI and those with a high affinity to Cibacron Blue dye's planar ring structure (Ahmed et al. 2003) or due to the protein's dinucleotide fold (Lollo et al. 1999). The major disadvantage of using antibody affinity methods is the loss of the low-abundance proteins bound to high-abundance carrier proteins; this loss was reported to be up to 38 proteins in one study (Yocum et al. 2005).

### 2.2 Alternative analytical techniques for serum treatments

#### 2.2.1 Ion exchange chromatography

Ion exchange chromatography is a high resolution and capacity, easily used technique for separating proteins according to their charge. As proteins are charged molecules, adding them in a column will cause an interaction with the resin displacing mobile counter ions that are bound to the resin depending on the overall charge from the amino acids in the protein, on the pH of the buffering solution, and on the distribution of charged molecules on the folded protein's surface. If the pH is above a protein's pI, the protein will bind to anion

exchangers (e.g. Diethylaminoethyl); below its pI it will bind to cation exchangers (e.g. Carboxymethyl). At its pI, a protein will not bind to either a cationic or an anionic exchanger. So by using either a cation or an anion exchanger and selecting the appropriate pH, the target protein will bind to the beads in the process of purifying this protein. The typical elution modes in Ion exchange chromatography are the salt gradient (Shan and Anderson, 2002; Winnik, 2005) or the change of the eluent pH (Shan and Anderson, 2002; Alaiya et al. 2005). Ion exchange chromatography was reported to be utilized as a fractionating technique (Qin et al. 2005; Sahab Ziad et al. 2005) and as an analysis method for serum proteins (Pappas et al. 2004).

#### 2.2.2 Reversed-phase Liquid Chromatography

High performance reversed phase liquid chromatography (RPLC) is a widely accepted easy to use technique for the separation of peptides, proteins and other biopolymers (Regnier, 1983). It has great resolving power and there is ease of concentrating collected fractions, as many RPLC separations employ volatile eluents. The mobile phase, polar aqueous-organic mixtures such as acetonitrile-water or methanol-water (solvent), in RPLC is significantly more polar than the stationary phase (Anon, 2000), non-polar hydrocarbons, waxy liquids or bonded hydrocarbons (such as  $C_4$ ,  $C_8$ ,  $C_{18}$ , and the like). The interaction of the non-polar components of the solutes (mixture of components = Experimented Sample) and the non-polar stationary phase cause the retention of the target component on the matrix, while the elution of the target molecule is achieved by utilizing a buffer (another solvent) of decreasing polarity. Most commonly used sorbents in RPLC are packed with chemically bonded octadecylsilyl coated silica, to which various functionalities such as the alkyl ( $C_{18}$  and  $C_8$ ), aromatic phenyl, and cyano and amino groups are widely bound. Other popular bonded phase columns have cyanopropyl, octasilyl, or phenylsilyl packings.

To separate large numbers of proteins with sufficient resolution, a 1.5  $\mu\text{m}$  non porous silica-RPLC column is used. The Non-porous silica allows rapid separations of large numbers of proteins with high recovery compared to porous columns (Lubman et al. 2002). This separation is

compatible with the chromatofocusing (also known as anion exchange chromatography) separation. The samples that would be separated by non porous silica reversed phase HPLC are the fractions collected from the first chromatofocusing separation. These fractions' pH will be neutralized using HCl or NaOH, vacuum centrifuged, their precipitates will be reconstituted in water, 0.1% Trifluoroacetic acid, and 0.05% Octylglucopyranoside, and afterward will be subjected to Electrospray Mass spectrometry.

RPLC is used to fractionate serum proteins (Morris et al. 2004; Marshall et al. 2004; Qin et al. 2005; Martosella et al. 2005; Sheng et al. 2006). This method allows for a high protein recovery and is compatible with other separation techniques and can be coupled with mass spectrometry (see 2.3.2).

### 2.2.3 Size Exclusion Chromatography

Size exclusion chromatography (SEC) is a general, not a high-resolution, technique that separates mixtures based on the molecular size of the components, such as biomolecules and organic polymers and was used as one of the several means of fractionating serum proteins (Pieper et al. 2003; Kuhn et al. 2004). Adjustment of the porosity of the gel can exclude all molecules above a certain size, therefore, the solute retention time depends sensitively on the solute's size (Bloustine et al. 2003). A line of porous silica-based and polymer-based

SEC matrices is tailored for fast, high resolution of large and small biomolecules and organic polymers. The trade names for gels available commercially in a broad range of porosities are Sephadex and Sepharose (Adrados et al. 2001). For the separation of biomolecules in aqueous systems, SEC is referred to as gel filtration chromatography, while the separation of organic polymers in non-aqueous systems is known as gel permeation chromatography.

Investigation of the binding of Human Neutrophil Peptides -1, -2 and -3 to high mass plasma proteins was performed using SEC. These serum peptides may serve as blood markers for colon cancer in combination with Surface Enhanced Laser Desorption/Ionisation—Time Of Flight/Mass spectrometry (SELDI-TOF/MS) (Albrethsen et al. 2005). This strategy may also be used for other smaller markers. The advantages and disadvantages of methodologies of serum sample treatment and prefractionation are summarized in Table 1.

## 2.3 Protein separation after sample treatment

### 2.3.1 Two-dimensional Polyacrylamide Gel Electrophoresis (2-DE)

Two-dimensional polyacrylamide gel electrophoresis (2-DE) is an analytical technique that simul-

**Table 1.** Advantages and Disadvantages of Albumin Elimination *versus* Serum Fractionation.

Techniques		Advantages	Disadvantages
<b>Albumin Elimination Techniques</b>	Cibacron Blue F3GA	- Fast	- Loss of proteins bound to albumin
	Antibody-Based Albumin Elimination	- Does not require specialized instrumentation	
<b>Serum Fractionation Techniques</b>	Anion Exchange Chromatography	- High Recovery Rate	
	Reversed-Phased High Performance Liquid Chromatography	- Can be coupled with other separation techniques	- Requires specialized Instrumentation
	Size Exclusion Chromatography	- Can be optimized to fractionate the serum sample into many fractions each enriched with target proteins	- Time consuming

taneously separates thousands of proteins (Anderson and Anderson, 1977; Lilley et al. 2002) and allows comparative protein profiling in different crude biological samples. Although labor intensive, this technique is still the predominant method for protein profiling (Pandey and Mann, 2000; Westbrook et al. 2001; Huang et al. 2005).

A protein placed in a medium with a pH gradient and subjected to an electric field will move toward the electrode of opposite charge and stops when it arrives at the point in the pH gradient equal to its pI (Tribl et al. 2005; Sheng et al. 2006). Immobilized pH Gradient (IPG) strips offer the advantage of gradient stability over extended focusing runs, where its gradient's pH is related with sets of acrylamido buffers that can form almost any conceivable pH gradient (Bjellqvist et al. 1982). Proteins resolved in IPG strips in the first dimension will be applied to second-dimension gels and separated by molecular mass perpendicularly to the first dimension (O'Farrell, 1975). The pores of the second dimension gel sieve proteins according to size because dodecyl sulfate coats all proteins essentially in proportion to their mass. The net effect is that proteins migrate as ellipsoids with a uniform negative charge-to-mass ratio, with mobility related logarithmically to mass. Proteins will therefore be separated horizontally based on their isoelectric point and vertically based on their molecular mass ( $M_r$ ).

Although imperfect, 2-DE is the only analytical technique that simultaneously separates thousands of proteins and allows comparative protein profiling between different crude biological samples, for instance, proteins in hepatocellular carcinoma serum have been characterized by utilizing 2-DE separation (Lee et al. 2006b). 2-DE is incapable of detecting the majority of protein components, such as very large or small proteins, membrane-associated proteins, extremely hydrophobic, acidic or basic proteins and proteins found in low abundance within the cell (Corthals et al. 2000; Gygi et al. 2000a; Gygi et al. 2000b; Graves and Haystead, 2002; Gorg et al. 2004; Chignard and Beretta, 2004). The ones that are detected are mainly the high abundant ones (Dos Remedios et al. 2003). The limitation in protein loading on the IPG strips as well as the magnitude of protein abundance within a cell that differ by up to 10 orders of magnitude are the main cause for this discrimination.

### 2.3.2 Two-dimensional Liquid Chromatography Mass Spectrometry (2DLC-MS)

Profiling of proteins using 2-DLC can be performed (Lubman et al. 2002) using ion exchange chromatography or chromatofocusing (Whitelegge, 2005) to separate proteins based on their charge and Reversed-Phase Liquid Chromatography- Electrospray Ionization Mass spectrometer to separate proteins based on their hydrophobicities in the second dimension and measure their exact  $M_r$ . For instance, the application of 2-DLC was used: as an isolation strategy (Lam et al. 2005), to generate protein profiles (Liao et al. 2004), and for proteomic analysis (Hamler et al. 2004) for proteins in serum.

More recently in proteomics, aiming for the optimization of protein separation methods and selective depletion of the higher abundance proteins, such as immunoglobulins (e.g. IgG) and albumin, has led researchers to separate intact proteins in the liquid phase with multidimensional liquid chromatography (MDLC). MDLC is performed utilizing separation first by chromatographic focusing and second by reversed phase, and followed by mass spectrometric detection and identification (Wall et al. 2000; Gygi et al. 2002; Pieper et al. 2003; Rose et al. 2004; Fung et al. 2004; Sheng et al. 2006). Finally, database is searched by the SEQUEST algorithm. This whole procedure is referred to as multidimensional protein identification technology (MudPIT) that will be discussed later in the review (Washburn et al. 2001) (see 3.3). In proteomics studies, MDLC successfully complements 2-D electrophoresis as it overcomes difficulties encountered during analysis of samples containing proteins spreading over a large molecular weight range (Washburn et al. 2001; Han et al. 2001) or proteins with immoderate isoelectric points (pI).

### 2.3.3 Surface-enhanced Laser Desorption Ionization (SELDI)

Another versatile and convenient proteomics tool that allows semi quantitative analysis of numerous proteins in most biological samples is the surface-enhanced laser desorption ionization-time of flight (SELDI-TOF) system. Complex biological samples can be analyzed and compared to detect differentially-expressed proteins in the samples that are selectively retained by using different chromatographic surfaces (Tang et al. 2004), "chip" (a thin strip of

aluminum, which has eight small places for loading samples), such as immobilized metal affinity capture for capturing metal-binding proteins, hydrophobic for reversed-phase capture, anion and cation exchange surfaced, and pre-activated surfaces to examine receptor-ligand, antibody-antigen, DNA-protein, and the like. The number of proteins quantified in each sample is dependent on the tissue and the chip surface. Using MS, proteins retained on the array get analyzed generating a profile of the analyzed proteome. This instrument has a combination of software, Pattern Recognition Software and one for comparing groups of biological samples, which permits the detection of subtle differences between groups of samples allowing the detection and assessment of sensitivity/specificity of a very wide range of biomarkers. An example of known software used is the Ciphergen Biosystems software (Vermeulen et al. 2005). For data presentation, the two useful formats are the grey-scale and the raw spectrum (the former represents a stained one-dimensional electrophoresis gel, hence the name “gel-view”).

Currently SELDI have been used for evaluating serum finding it useful for the discovery and identification of potential biomarkers (Li et al. 2002; Petricoin et al. 2002a; Qu et al. 2002; Petricoin et al. 2002b; Petricoin et al. 2002c; Issaq et al. 2002; Miguet et al. 2006). Although limitations during large profiling experiments, where some observations noted that spectra can vary based on analytical factors (such as the time of processing, issues in the reproducibility of SELDI (Petricoin et al. 2002a; Deng et al. 2003; Diamandis, 2003), and usually unidentified individual proteins (Chignard and Beretta, 2004)), there are advantages that include prior to analysis removal of components (salts or detergents) that commonly cause problems with other analytical tools, thus leaving only those proteins that are actively interacting with the spot surfaces to be analyzed in the ProteinChip Reader (Romer et al. 2002).

### 3. Serum Protein Identification

#### 3.1 Mass spectrometry techniques

##### 3.1.1 Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS): Peptide mass measurement

Proteins are usually identified utilizing Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) (Binz et al. 2003; Aebersold and Mann, 2003; Ferguson and Smith, 2003; Heck and Krijgsveld, 2004; Feuerstein et al. 2005). After 2-DE, spots are excised and treated with TPCK-treated trypsin. While in case of using 2-DLC, the resulting fractions collected in the second dimension of the separation are vacuum dried and incubated with 0.5 µg/mL TPCK-treated trypsin. The peptides generated from the trypsin digestion are first mixed with a matrix ( $\alpha$ -cyano-hydroxycinnamic acid) (matrix application described by (Karas and Hillenkamp, 1988; Tanaka et al. 1988)), which should consist of a 100–1000-fold molar excess of the matrix over the analyte (Schiller et al. 1999). This mixture is then spotted onto a plate and allowed to evaporate forming crystals that will be analyzed by the mass spectrometer and time-of-flight analyzer. This analysis provides for measurement of the peptide fragment masses generated by trypsin digestion of the protein where the mass resolution is a measure of a spectrometer’s capability to produce separate signals of ions of similar mass (Hillenkamp et al. 1991). Finally, having obtained the molecular weight, pI, and peptide fingerprints of the protein of interest, identification of the protein should be possible using the Peptident tool that scans the Swiss-Prot protein database (UniProtKB) for possible matches.

MALDI-TOF-MS has been used to analyze serum biomarkers. For instance, it was used in combination with porous silicon nanovial arrays to achieve high-speed biomarker identification (Finnskog et al. 2006). In another study, 4 protein spots significantly down-regulated in grade 3 ovarian cancer patients were identified as isoforms of transferrin precursor using MALDI-TOF (Ahmed et al. 2005). Recently, its combination with derivatized cellulose particles represented a simple, economical, and rapid approach to generate serum protein profiles for biomarker identification (Feuerstein et al. 2005). Furthermore, one study proved that the analysis of MALDI-TOF/MS data using proteomic spectral pattern recognition software derived a distinctive molecular signature of multiple sclerosis (Avasarala et al. 2005).

##### 3.1.2 Electrospray Ionization Tandem Mass Spectrometry (ESI-MS/MS)

Electrospray ionization (Dole et al. 1968) tandem mass spectrometry (Yamashita and Fenn, 1984)

(ESI-MS/MS) is a process in which a solution containing the analyte, either from a syringe pump or as the eluent flow from liquid chromatography (in this case known as LC-ESI-MS/MS), is sprayed across a high potential difference electrospray needle. The charged droplets from the spray are desolvated by either countercurrent gas flow (present in the opposite direction to the spray) or by passage through a heated capillary, a source sampling cone; then the resulting ions after desolvation of these charged droplets are analyzed in a mass spectrometer (Dongre et al. 1997). This is a very soft method of ionization where very little residual energy is retained by the analyte. This shows the importance of ESI-MS as a technique in biological studies where the analyst often requires that non-covalent molecule-protein or protein-protein interactions are representatively transferred into the gas-phase. The fact that the production of fragmentation is very little, usually none is produced, is the major disadvantage of this technique. For structural clarification studies, the requirement for tandem mass spectrometry is a must where the analyte molecules can be fragmented. ESI-MS/MS, for instance, was coupled to microcapillary reversed-phase liquid chromatography in a study where the analysis of low molecular weight serum proteome sample resulted in the identification of over 340 human serum proteins (Tirumalai et al. 2003).

The powerfulness of mass spectrometry has been discussed above. The application of mass spectrometry for the identification of disease biomarkers in serum samples is a growing field of research holding high promise for clinical oncology. Serum biomarker proteomics continues to evolve and progress following the improvements of technology, clinical study design, and bioinformatics (Drake et al. 2006).

## 3.2 Immunological techniques

### 3.2.1 Sandwich Enzyme-Linked ImmunoSorbent Assay

A protein identification method is the “Sandwich” Enzyme-Linked ImmunoSorbent Assay (ELISA), a fast and accurate biochemical technique very useful to detect the presence and determine the concentration of low-concentrated antigen or antigen contained in high concentrations of contaminating protein in unknown samples. The

sandwich ELISA requires two antibodies that bind to epitopes (antigenic sites) that do not have common ground on the antigen. This can be accomplished with either using two monoclonal antibodies that recognize discrete sites, which allow fine detection and quantification of small differences in antigen, or using one batch of affinity-purified polyclonal antibodies to pull down as much of the antigen as possible. To quantify the assay one should use a colorimetric substrate to measure the amount of labeled detection antibody bound to the matrix. As the antigen does not need to be purified prior to use and these assays are very specific, these facts are considered major advantages of this technique. However, not all antibodies can be used. Monoclonal antibody combinations must recognize separate epitopes on the antigen so they do not hinder each other’s binding and therefore be eligible as “matched pairs.” An example of a sandwich ELISA kit that detects circulating galactomannan is the *Platelia Aspergillus*, which due to the early detection of the antigen has been a major advance for managing patients at risk for invasive *aspergillosis* (Mennink-Kersten et al. 2004). For multiplexed sandwich assays there are several labeling and detection methods that can be employed (Wodicka et al. 1997; Scorilas et al. 2000; Moody et al. 2001; Wiese et al. 2001; Huang et al. 2001; Wang et al. 2002; Woodbury et al. 2002; Tam et al. 2002; Nielsen et al. 2003).

Matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinases-2 (TIMP-2) levels in patients with systemic sclerosis were determined by means of sandwich ELISA in a study that concluded that serum TIMP-2 level can be used as a marker of the extent of skin sclerosis and disease activity added to the fact that TIMP-2 and MMP-2’s balance may play an important role in those patients (Yazawa et al. 2000). Moreover, another study used it to determine that increased pentosidine serum concentration in patients with osteoarthritis and its correlation with the cartilage destruction marker COMP (cartilage oligomeric matrix protein) in synovial fluid suggests that pentosidine may be important in osteoarthritis pathology and is a new potential osteoarthritis marker (Senolt et al. 2005).

### 3.2.2 Western blot/immunoblot

Another immunological technique, Western blot or immunoblot, (Towbin et al. 1979; Burnette, 1981) is a method in biochemistry/molecular biology/immunogenetics to detect specific protein



in a given sample of tissue homogenate or extract, while giving information about the size of that protein and how much protein has accumulated in cells. Proteins are electrophoresed into a gel (polyacrilamide gels, SDS-PAGE, are most common) to separate denatured proteins by size and charge and compared to a control, a “marker” or “ladder.” Proteins small in size migrate through the gel faster than larger ones. The separated proteins in an SDS-PAGE are then transferred out of the gel and onto a membrane, which is in general nitrocellulose or PolyVinylidene DiFluoride (a thermoplastic fluoropolymer) placed face-to-face with the gel by applying an electric current.

The detection of the membrane-blotted antigen requires: first, the blocking of the sites on the membrane that have no blotted protein from the gel, the addition and incubation of primary antibody with the membrane, which is a sensitive and specific detection tool, the addition of the secondary antibodies, which are anti-immunoglobulin antibodies coupled to a reporter group, such as to biotin, to alkaline phosphatase, e.g. goat anti-human IgG- alkaline phosphatase (Wallis et al. 1990), or to horseradish peroxidase, and directed against a species-specific portion of the primary antibody.

After excess second antibody is washed away from the blot, a substrate is added to the membrane and it will be converted by the reporter enzyme to a colored reaction product, this will cause the specific protein to be detected on the membrane to become visible, as in the case of utilizing monoclonal antibodies for SERCA 1 (clone IIIH11) and SERCA 2 (Arruda et al. 2003). In case of a horseradish peroxidase-linked secondary antibody, which is commonly used in combination with a chemiluminescent agent, the reaction product will produce fluorescence in proportion to the amount of specific protein bound. Placing a sensitive sheet of photographic film against the membrane will create an image of the antibodies bound to the blot upon its exposure to the light from the reaction. Another way of detection is by utilizing radioactive labeling (e.g. a radioactive isotope of iodine) rather than an enzyme coupled to the secondary antibody. This method is highly sensitive, but very rarely used as the other non-radioactive methods have more advantages of being cheaper, safer, and faster.

Western blot was recently used to confirm the presence of elevated serum heat shock protein 70

levels in the patients with sudden sensorineural hearing loss compared with normal controls. This study suggests that these levels of heat shock protein 70 might have a clinical role in predicting prognosis of hearing loss in the patients with sudden sensorineural hearing loss (Park et al. 2006). Also, Western blot was used to characterize Human Kallikrein 11, suggesting it as a candidate prostate and ovarian cancer biomarker, from seminal plasma (Luo et al. 2006). Moreover, it was utilized to validate that Heat-shock protein 27 is a potential biomarker for hepatocellular carcinoma and that it could help in the diagnosis of Hepatocellular carcinoma though further validation is needed (Feng et al. 2005).

### 3.3 Multi-dimensional Protein Identification Technology (MudPIT)

Multi-dimensional Protein Identification Technology (MudPIT) pioneered by Yates is an unbiased method for rapid and large-scale semi-quantitative proteome analysis and protein biomarker candidates identification (Wolters et al. 2001; Gao et al. 2005). MudPIT begins with multi-dimensional liquid chromatography, followed by tandem mass spectrometry, and finalizing by a database searching by the SEQUEST algorithm (Eng et al. 1994; Yates et al. 1995; Washburn et al. 2001), where SEQUEST is a database search program for performing protein identification and peptide sequencing by utilizing mass spectrometry fragmentation patterns to search protein and nucleotide databases (Tabb et al. 2001).

MudPIT is another approach which may help lessen many of the disadvantages associated with two-dimensional gel electrophoresis. The advantage of MudPIT using two chromatography steps interfaced back to back in a fused silica capillary is that the band broadening associated with many chromatographic steps, which can lead to resolution loss hence the running of components into one another, is avoided and also the capillary can be placed directly into the ion source of a mass spectrometer maximizing sensitivity. The mass spectrometer is a tandem electrospray, so peptides are ionized in the liquid phase, separated in a primary mass spectrometer, broken up using collision induced dissociation and analyzed again (Eng et al. 1994; Link et al. 1999; Gygi et al. 1999; Washburn et al. 2001; Han et al. 2001; Ideker et al. 2001; Tabb et al. 2002).

MudPIT was used to resolve and analyze, for example, the fractionated serum proteins which were trypsinized in an attempt to prove that several different prefractionation approaches should be used in parallel for a comprehensive identification of the serum proteins (Barnea et al. 2005). The Human Proteome Organization in another study used MudPIT to perform an extensive analysis of serum proteins in which they identified a large number of proteins (490) to further provide the foundation for future studies with clinically important disease states (Adkins et al. 2002). Analysis of the tandem mass spectra resulted in the identification of over 340 human serum proteins in a study that was trying to prove the efficacy of having Centrifugal ultrafiltration followed by MudPIT for the removal of large abundant proteins and the enrichment of the low molecular weight serum proteome (Tirumalai et al. 2003).

## 4. Biomarkers in Serum

### 4.1 Overview

Serum Prostate-Specific Antigen (PSA) and prostatic acid phosphatase are clinically established biomarkers in human serum. While PSA is used in insurance testing to assess the risk of underlying prostate cancer, other biomarkers are neither specific enough nor cost effective to use. Serum-protein biomarker approvals despite steady increases in the literature on potentially useful ones have seen a noticeable decrease. Before receiving US Food and Drug Administration (FDA) approval, biomarker candidates must undergo clinical validation. Yet that process is just beginning for most candidate markers. Moreover, among the FDA-approved biomarkers (see Table 2) only PSA is routinely used in standard clinical practice, and only two of the biomarkers have made it into the TNM staging guidelines, which is based on a combination of Tumor size or depth, lymph Node spread, and presence or absence of Metastases and provides the following information: a) a basis for prediction of survival, b) option of initial treatment, c) precise communication among healthcare providers, d) stratification of patients in clinical trials, e) and consistent reporting of the end result of cancer management. The ideal biomarker assay for staging should be specific, cost-effective, sensitive, fast, and durable against inter-operator and inter-institutional

variability. It must also display clinical value afar that of the other types of information that are already available at the time of diagnosis (Ludwig and Weinstein, 2005). The following includes a discussion of the possible serum biomarkers that were found for cancerous and other major illnesses.

### 4.2 Cancer biomarkers

Cancer or tumor markers can be defined as tools that clinicians rely upon to answer clinically related questions concerning a cancer disease (Diamandis, 2002). A more preferred specific definition of a tumor marker is: a molecule, a process, or a substance that is altered quantitatively or qualitatively in precancerous or cancerous conditions, the alteration being detectable by an assay (Hayes et al. 1996). The tumor itself or the surrounding normal tissue as a response to tumor cells can produce those alterations (Hayes et al. 1996). The tumor marker itself can be mRNA, DNA, processes (apoptosis, angiogenesis, proliferation, etc.), or protein measured qualitatively or quantitatively by a suitable assay, which can be of various formats stretching from complex animal models to immunohistochemical test kits. The immunoassay, a well-characterized methodology, is probably the most commonly used format. In spite of this, there is a rapid progress in this field, and fresh and advanced assays, like microarrays and mass spectrometry, are becoming established technologies in tumor marker research (Schrohl et al. 2003). In the following paragraphs only biomarkers in “serum” will be discussed in both cancer and other disease cases.

#### 4.2.1 Prostate cancer biomarker

To date, predictive clinical markers in prostate cancer are the prostatic acid phosphatase (Gutman et al. 1936; Yam, 1974; Vihko, 1979; Lin et al. 1980; Sakai et al. 1992; Veeramani et al. 2005) and serum PSA (Sensabaugh, 1978; Cooper et al. 1990; Grignon and Hammond, 1995; Schrohl et al. 2003; Veeramani et al. 2005). Other markers have not earned sufficient support in the literature to be recommended for routine clinical application (Grignon and Hammond, 1995). Human prostatic acid phosphatase was used prior to the availability of PSA as a valuable surrogate marker for monitoring prostate cancer (Veeramani et al. 2005). Although PSA, the tumor-associated serum protein

**Table 2.** FDA-approved and Potential Disease-serum Biomarkers.

<b>Disease</b>	<b>FDA approved Biomarkers</b>	<b>Potential Biomarkers</b>
Prostate	PSA	Prostatic acid phosphatase, CK18-Asp396, prostate secretory protein of 94 amino acids, hepatocyte growth factor activator, insulin-like growth factor binding protein-1 and -3, E-cadherin, and alpha-methylacyl-CoA racemase.
Breast	CA15.3, Her-2/neu, and CA27–29	CEA, Human kallikrein genes 5, and serum carboxyterminal telopeptide of type I collagen.
Ovarian	CA125	Isoforms of haptoglobin-1 precursor, hemoglobin- $\alpha$ , hemoglobin- $\beta$ , human kallikrein 6 & 10, Prostatein, Osteopontin, Tumor-associated trypsin inhibitor, preoperative serum Human Chorionic Gonadotropin-beta, Interleukin-8, and serum Carboxy-terminal telopeptide of type I collagen.
Lung (non-small cell lung cancer)	none	CEA and pretreatment serum macrophage colony-stimulating factor.
Lung (small cell lung cancer)	none	Pro-gastrin-releasing peptide
Liver	none	Alpha-fetoprotein
Non Seminomatous Testicular Seminoma	alpha-fetoprotein Human chorionic gonadotropin- $\beta$	
Thyroid	Thyroglobulin	MMP-2 and TIMP-2.
Medullary Thyroid	none	Calcitonin, CEA, and pro-gastrin-releasing peptide.
Melanoma	none	Melanoma-inhibiting activity
Colorectal	none	Human chronic gonadotropin-beta and D-dimer.
Pancreas	CA19–9	Human chronic gonadotropin-beta
Kidney	none	Human chronic gonadotropin-beta
Colon	none	CEA
Heart Disease	none	B-type natriuretic peptide, N-terminal pro-B-type natriuretic peptide, cardiac troponin T, and high-sensitivity CRP.
Rheumatoid Arthritis	none	CRP, erythrocyte sedimentation rate, stromelysin-1 (MMP-3), melanoma-inhibiting activity, CS846-epitope, neoepitope marker for degradation of type II collagen in cartilage, interleukin-15, cytokines, vascular endothelial growth factor, myeloid-related protein 8, CRP, S100A8/calgranulin A, S100A9/calgranulin B, S100A12/calgranulin C proteins, and serum cartilage oligomeric matrix protein.
Asthma	none	Serum urinary-trypsin-inhibitor, serum eosinophil cationic protein, and serum tryptase.
Cystic Fibrosis	none	CA 19–9, Trypsinogen, TIMP-1, collagen-IV, and prolyl hydroxylase.

marker, is useful for monitoring prostate cancer progression, it is insufficient for diagnosing primary cancers or even early prostate cancer detection mainly due to its limited specificity at the desired sensitivity (Pannek and Partin, 1998; Djavan et al. 1999; Petricoin et al. 2002b; Adam et al. 2002; Qu et al. 2002; Wang et al. 2005; Engwegen Judith et al. 2006).

Identification of serum biomarkers for prostate cancer has been the aim of many studies. In one study, caspase-cleaved cytokeratin 18 (CK18-Asp396) serum marker was used to assess tumor apoptosis *in vivo* (Kramer et al. 2006). In another study, serum prostate secretory protein of 94 amino acids is able to help identify patients with high grade prostate cancer (Nam et al. 2006). In addition, serum hepatocyte growth factor activator was elevated in patients with advanced stage prostate cancer but further studies are needed to verify its clinical value (Nagakawa et al. 2005). Serum insulin-like growth factor binding protein-1 and -3 participate in an important role in regulation of prostate cancer cell growth. Results of studying serum insulin-like growth factor binding protein-3/PSA ratio suggested that it might be a useful prognostic marker in patients with advanced prostate cancer (Miyata et al. 2003). Moreover, an 80 kDa fragment of e-cadherin was believed to be a serum biomarker in a broad spectrum of prostate cancer cases (Kuefer et al. 2005). Today assays are available to detect a humoral immune response against alpha-methylacyl-CoA racemase, which may have the potential to complement PSA screening in identifying patients with clinically significant prostate cancer (Sreekumar et al. 2004).

#### 4.2.2 Breast cancer biomarker

Serum carcinoembryonic antigen (CEA), a glycoprotein normally found in embryonic endodermal epithelium, is elevated in 30–50% of patients with symptomatic metastatic breast cancer (Mughal et al. 1983; Williams et al. 1988; Robertson et al. 1991). MUC1 mucin, a glycoprotein, is expressed on the apical surface of epithelial cells under normal conditions. Only in case of tumors, the disruption of the normal tissue architecture due to the growing tumor allows MUC1 mucin to be shed into the circulation where it can be measured via immunoassays kits to detect cancer antigen 15.3 (CA 15.3), mucin-like carcinoma associated antigen, breast

cancer mucin, and the like. Human kallikrein gene 5 was also found to be a potential biomarker in patients with ovarian and breast cancer (Yousef et al. 2003).

Currently, many biomarkers, particularly the hormonal and epidermal growth factor receptors, are utilized for breast cancer prognosis. Together with those predictive biomarkers (progesterone and estrogen receptors) (Schrohl et al. 2003), histological grade or Gleason system, axillary lymph node status, tumor size, and age, are currently used for selecting the suitable systemic therapy. Although none of the biomarkers in use have sufficient diagnostic, prognostic or predictive power across all categories and stages of breast cancer, more useful information can be generated if tumors are questioned with multiple markers with a right combination for each case, which is a challenging matter (Arciero et al. 2003; Somiari et al. 2005). In a recent study, one of the most popular serum biomarkers for the follow up of breast cancers and the early detection of breast cancer metastases is the cancer antigen Ca15.3, (Wang et al. 2005; Mathelin et al. 2006), a carbohydrate antigen commonly known as a “mucin,” which influences cell–cell interaction and cell growth (Banfi et al. 1997). In patients with early stage cancers, however, one study shows that Ca15.3 measurements are not helpful in diagnosis and the therapeutic decision making of patients with breast cancer (Lumachi et al. 2004). Her-2/neu, a cell membrane surface-bound tyrosine kinase, which is involved in the signal transduction pathways leading to cell growth and differentiation, and a glycoprotein MUC1 that can be recognized by CA27–29, a monoclonal antibody, are Food and Drug Administration (FDA) approved cancer biomarkers for monitoring breast cancer (Ludwig and Weinstein, 2005).

In a recent paper the clinical importance of serum pro-Iota collagen peptide and serum carboxyterminal telopeptide of type I collagen, CA15.3 and CEA was evaluated as compared to bone scintigraphy to detect metastatic breast cancer. It was concluded that combining serum CA15.3, carboxyterminal telopeptide of type I, and CEA would increase the sensitivity and specificity of the test, while pro-Iota collagen peptide alone or combined with carboxyterminal telopeptide of type I were not sensitive (Zissimopoulos et al. 2006).

#### 4.2.3 Ovarian cancer biomarker

Increased serum levels of CA125 (Whitehouse and Solomon, 2003), a mucin-type protein (Lloyd et al. 1997) that is often secreted into the blood by ovarian cells shed by cancer cells or also made by inflamed normal cells that line body parts, is widely used in the clinical setting to monitor ovarian cancer patients for early diagnosis of tumor recurrence (Bast et al. 1998) and follow-up of patients after treatment to calculate response (Rustin et al. 1999). However, it has low sensitivity and specificity and false-positive results can be obtained since CA125 is also produced in the ascites, a common factor present in both ovarian cancer and liver cirrhosis, and therefore the CA125 antigen alone can not be of diagnostic use in any patient with ascites or pleural effusions (Bergmann et al. 1986).

Many studies have been performed by different groups aiming to uncover serum biomarkers for ovarian cancer or even tentative ones for several stages of the cancer. One of which showed that isoforms of haptoglobin-1 precursor, a liver glycoprotein present in human serum can be used as a serum marker for ovarian cancer early detection (Ahmed et al. 2004). Determined by ELISA, two ovarian cancer biomarker proteins were discovered by another group and identified as hemoglobin- $\alpha$  and hemoglobin- $\beta$ . But additional studies are required to further validate them as biomarkers (Ahn et al. 2005). In addition to those, some studies found that serum human kallikrein 6 (a serine protease) (Diamandis, 2002; Diamandis et al. 2003; Bayes et al. 2004; Oikonomopoulou et al. 2006) and human kallikrein 10 (Luo et al. 2001) concentrations seem to be new biomarkers for ovarian carcinoma and may have value for disease diagnosis and prognosis. Prostatin, a tryptic peptidase expressed in prostate, kidney, lung and airway (Tong et al. 2004), is overexpressed in epithelial ovarian cancer and should be investigated further as a screening or tumor marker, alone and in combination with CA125 (Mok et al. 2001). Osteopontin, a N-linked glycoprotein family of calcified extracellular matrix-associated protein, has been discussed as a potential diagnostic biomarker for ovarian cancer and showed evidence of an association between levels of osteopontin in serum and ovarian cancer suggesting that it would be worthwhile for future research assessing its clinical usefulness (Kim et al. 2002; Bramwell et al. 2006). Tumor-associated trypsin inhibitor's elevated level

is another important predictor of disease stage and future prognosis in ovarian cancer (Torre et al. 1991). Moreover, a study suggested that preoperative serum Human Chorionic Gonadotropin-beta is a strong independent prognostic factor in epithelial ovarian carcinoma (Higashida et al. 2001) when measured with a sensitive and specific method (Vartiainen et al. 2001). Furthermore, another study illustrates that Interleukin-8 could possibly act as a useful monitoring marker in patients with ovarian carcinoma (Mayerhofer et al. 2001). It was also found that an increased serum Carboxyterminal telopeptide of type I collagen concentration may reflect the spreading and aggressiveness of invasively growing ovarian cancer making serum Carboxyterminal telopeptide of type I collagen a clinically useful predictor of the clinical behavior of ovarian cancer (Santala et al. 2004).

#### 4.2.4 Lung cancer biomarker

People at high risk for lung cancer have been studied in an attempt to identify new biomarkers that may be predictive of precancerous lung lesions and their possible progression to lung cancer. A study showed that serum CEA level could be a predictive factor for the efficacy of gefitinib treatment (a drug that is used to treat non-small cell lung cancer also is being studied in the treatment of other types of cancer) while also considering CEA a prognostic factor for advanced non-small cell lung cancer patients undergoing this treatment (Okamoto et al. 2005). A more recent topic-related study revealed that increased pretreatment serum macrophage colony-stimulating factor level is a significant independent predictor of poor survival in patients with non-small cell lung cancer, where CEA levels were shown but couldn't prove to be an independent prognostic factor as macrophage colony-stimulating factor did (Kaminska et al. 2006). Currently, there are no satisfactory biomarkers available to screen for lung cancer.

#### 4.2.5 Other cancer biomarkers

In order to improve the prognosis of cancer patients, it is crucial to explore serum biomarkers for its early diagnosis. One of the serum biomarkers is the alpha-fetoprotein for tumors of the liver (Johnson et al. 2000; Johnson, 2001), non seminomatous testis, and other germ cell line tumors. Human corionic gonadotropin- $\beta$ , a glycoprotein,

elevated serum level indicates the presence of metastatic testicular seminoma tumors but lactic acid dehydrogenase serum level, rather than human chorionic gonadotropin- $\beta$ , is more useful as a prognostic indicator for those patients with seminoma (Hori et al. 1997). Even though serum calcitonin and CEA are biomarkers for medullary thyroid carcinoma (Hamada et al. 1976), some recent studies report that those biomarkers are of no clinical use (Bockhorn et al. 2004), while other studies add pro-gastrin-releasing peptide, which is considered to be a specific marker for small cell lung carcinoma, as an additional marker for the diagnosis and monitoring the response to therapy in patients with medullary thyroid carcinoma (Ide et al. 2001). The evaluation of the concentration of metalloproteinase-2 and its tissue inhibitor TIMP-2 in peripheral blood serum may be useful for the differentiation between benign and malignant thyroid tumors, but their concentrations in patients with thyroid cancer did not significantly correlate with the clinical staging of thyroid cancer (Pasiaka et al. 2004). Measuring the levels of thyroglobulin, a protein produced by the thyroid gland, in the blood have been approved by the FDA as a monitoring biomarker for thyroid cancer (Ludwig and Weinstein, 2005). In systemic malignant melanoma, melanoma-inhibiting activity was found to represent a serum marker for this cancer showing a high sensitivity and specificity (Bossert et al. 1997). A studying group have discussed human chorionic gonadotropin-beta as being most commonly elevated ( $>10$  mIU/ml) in the serum of gynecological cancers especially ovarian cancer (Higashida et al. 2001), but it is also elevated in colorectal (Lundin et al. 2000), pancreas (Syrigos et al. 1998), and kidney (Torre et al. 1991), cancers and, therefore, can be referred to as a "current serum cancer marker." D-dimer's elevated levels in serum, a fibrin degradation product, has also been proposed as an important prognostic biomarker for metastatic colorectal carcinoma (Blackwell et al. 2004) but its use is limited by its low specificity. CEA is reported as a colon cancer marker having low specificity and insufficient sensitivity to be used as a screening marker, as it can be elevated by many other factors than cancer; smoking for instance raises CEA levels, but can be helpful in follow-up (Bast et al. 1996; Chatterjee and Zetter, 2005). CA19-9, a serum-derived single carbohydrate, has been approved by FDA as a cancer biomarker for monitoring pancreatic cancer

(Ludwig and Weinstein, 2005). Comparing serum lipoprotein in patients with cancer to non-cancer subjects revealed an association of cancer with low serum total cholesterol and a characteristic of low low-density lipoprotein-cholesterol, low high-density lipoprotein-cholesterol and relatively high serum triglycerides, where the abnormality is a common feature of both hematological and solid tumors and is not entirely explained by poor nutrition (Fiorenza et al. 2000). Polanski and Anderson (2006) have done comprehensive literature review and compiled a list of 1261 proteins believed to be differentially expressed in human cancer. Among them, only nine have been approved as "tumor associated antigens" by the FDA, i.e. CEA, Her-2/neu, bladder tumor antigen, thyroglobulin, alpha-fetoprotein, PSA, CA125, CA19.9, CA15.3. Only a small fraction of proteins are candidate plasma biomarkers that could be further evaluated and validated for future early cancer detection, prognosis, and monitoring efficacy of specific treatment options.

## 4.3 Biomarkers for other diseases

### 4.3.1 Heart disease

B-type natriuretic peptide, nesiritide, was reported as a prognostic marker in acute coronary syndromes (McCullough et al. 2002). Several likely uses are now considered for B-type natriuretic peptide and its terminal prohormone fragment, N-terminal pro-B-type natriuretic peptide, in adults: (I) to examine heart failure therapy's efficiency (Troughton et al. 2000; Rodeheffer, 2004); (II); to predict prognosis (Anand et al. 2003; Maisel et al. 2004); (III) to check asymptomatic persons at threat for asymptomatic ventricular dysfunction (Nakamura et al. 2002; Redfield et al. 2004); and (IV) to recognize the failure of the heart of patients having dyspnea or the like symptoms suggesting heart disease (McCullough et al. 2002; Maisel et al. 2002). Although, there are available commercial kits for B-type natriuretic peptide and N-terminal pro-B-type natriuretic peptide, which makes them candidate markers for heart disease in daily practice (Nasser et al. 2005), standardization of these assays is deficient, diverse cutoff values have been reported, and the effect of age, gender (Redfield et al. 2002), and possibly renal function, on normal values (McCullough Peter and Sandberg Keisha, 2003) may complicate the use of B-type natriuretic

peptide as a marker for heart disease. A recent study evaluating serum Levels of N-terminal pro-B-type natriuretic peptide reveal it as an early cardiac marker of carbon monoxide poisoning (Davutoglu et al. 2006) and of heart toxicity caused by amyloidogenic light chains (Palladini et al. 2003). Another study assessing serum N-terminal pro-B-type natriuretic peptide and cardiac troponin T levels and echocardiography was recently performed and concluded that their simultaneous measurement allows for precise acute pulmonary embolism prognosis (Kostrubiec et al. 2005). A scientific group hunted to describe the relationship between cholesterol and survival in patients with chronic heart failure and concluded that lower serum total cholesterol is independently associated with a worse prognosis (Rauchhaus et al. 2003).

Concentrations of C-reactive protein (CRP), serum amyloidA, and interleukin-6 were increased in patients with coronary heart disease but failed to correlate with acuteness of coronary disease. Therefore, the study concluded that these markers might reflect the diffuse atherosclerotic process in the vascular system rather than the degree of localized obstruction from coronary lesions (Rifai et al. 1999). A later study concluded that high-sensitivity CRP has a prognostic utility in patients with acute coronary syndromes, and it is also considered a strong independent predictor of future coronary events in seemingly healthy subjects (Rifai and Ridker, 2001). Recently, a study verified that the first paroxysmal episode of lone atrial fibrillation is associated with elevated high-sensitivity CRP levels, proposing that high-sensitivity CRP may be a marker for inflammatory states that may prop up the initiation of lone atrial fibrillation (Hatzinikolaou-Kotsakou et al. 2006).

#### 4.3.2 Rheumatoid Arthritis (RA)

Persistently active rheumatoid arthritis (RA), in most patients, has no early biomarker, and this leads to high possibility of joint destruction and disability (Liao et al. 2004). Although, available biomarkers, like CRP or erythrocyte sedimentation rate, offer good correspondence parallel with disease activity, yet they do not predict subsequent severity (Riel et al. 1998). The evaluation of the level of stromelysin-1 (matrix metalloproteinase-3) in serum presents a particularly useful marker of inflammatory activity in the joints of patients with

RA (Yoshihara et al. 1995; Yamanaka et al. 2000). In addition to melanoma-inhibiting activity's role in being a serum marker for progression of malignant melanoma (see 4.2.5), a study revealed that it might also be useful in the differential diagnosis of RA vs. non-destructive rheumatic diseases via checking for the presence of elevated levels of melanoma-inhibiting activity in serum, which correspondingly means a very likely joint destruction in RA (Muller-Ladner et al. 1999).

Levels of a marker for aggrecan turnover in cartilage (CS846-epitope), which is normally found in chondroitin sulfate of the cartilage proteoglycan aggrecan, are found to be elevated in serum in chronic RA, even though the levels are found to be depressed in rapid progressive RA (Mansson et al. 1995). Some studies showed that a neopeptide marker for degradation of type II collagen in cartilage, which was generated by collagenases, is increased in the serum and urine of patients with RA (Poole et al. 2004) making type II collagen in cartilage a specific marker for cleavage of type II collagen (Verstappen et al. 2006). Recent studies show that interleukin-15 may be used as a marker for the evaluation of severity of juvenile rheumatoid arthritis (Cao et al. 2006). Another study's evaluation of the degree of clinical rheumatoid arthritis activity based on the concentrations of cytokines tumor necrosis factor- $\alpha$ , interleukins -12, -15, and -18 in serum and synovial fluid concluded that cytokines concentrations could be good indicators of the degree of the general activity of RA (Petrovic-Rackov, 2006). The investigation of matrix metalloproteinases-2, -9, inhibitors of matrix metalloproteinase-1, and -2 levels in serum and synovial fluid in patients with RA and psoriatic arthritis deduced that the evaluation of synovial fluid concentrations is more reliable than that determined in serum (Giannelli et al. 2004). The vascular endothelial growth factor can also be detected in serum of patients with RA (Kikuchi et al. 1998; Harada et al. 1998), where vascular endothelial growth factor level is related to RA disease activity, therefore, suggesting that vascular endothelial growth factor may play some role in the pathogenesis of RA (Harada et al. 1998; Sone et al. 2001). Auto-antibodies to  $\alpha$ -enolase, an enzyme of the glycolytic pathway, were found to be present in the sera of patients in very early RA cases, therefore, having potential diagnostic and prognostic value for RA (Saulot et al. 2002).

In a recent paper comparing marker proteins in serum and synovial fluid in patients with advanced osteoarthritis (OA) and RA deduced that the only marker protein that revealed distinct higher levels in the serum than in the synovial fluid was matrix metalloproteinase-13, but further investigations may provide more information about the value of matrix metalloproteinase-13 as a potential marker to monitor the course of RA and OA (Andereya et al. 2006). Moreover, in another recent study myeloid-related protein 8 was confirmed by MS/MS to be present in serum of patients with erosive RA (Liao et al. 2004). Additionally, mass spectrometry identified protein biomarkers of disease severity in the synovial fluid and serum of patients with rheumatoid arthritis. Those biomarkers are high levels of CRP, S100A8/calgranulin A, S100A9/calgranulin B, and S100A12/calgranulin C proteins. Those proteins were elevated in the serum of patients with erosive disease compared to patients with nonerosive RA or healthy individuals (Liao et al. 2004).

An investigation of whether increased cartilage oligomeric matrix protein in serum is a specific marker for joint destruction done by comparing serum cartilage oligomeric matrix protein between patients with RA and patients with other inflammatory rheumatic diseases with less cartilage-destructive arthritis was able to confirm the conclusion that serum cartilage oligomeric matrix protein levels are highly specific markers for the cartilage degradation process in RA (Skoumal et al. 2004).

#### 4.3.3 Asthma biomarker

Neutrophil-mediated inflammatory events have been shown to be involved in exacerbation of childhood asthma and that the screening of urinary-trypsin-inhibitor-serum concentrations might be useful for evaluating the neutrophil-mediated inflammation in childhood asthma attack (Yasui et al. 2003). Serum eosinophil cationic protein may also be used to predict a response to corticosteroid therapy in adult patients with asthma (Sorkness et al. 2002). Serum tryptase detected with B12 monoclonal antibody-based immunofluoroassay has been suggested as a marker of allergic airway inflammation in asthma (Taira et al. 2002).

#### 4.3.4 Cystic fibrosis

In Cystic Fibrosis (CF) patients, an elevated level of serum CA 19–9 (a tumor-associated antigen)

concentration have been observed in several studies (Duffy et al. 1985; Buamah et al. 1986; Roberts et al. 1986). Trypsinogen has been reported to be an excellent screening test for CF in young infants (Cleghorn et al. 1985). As there is no straightforward correlation between CF-transmembrane conductance regulator genotype and CF lung disease severity, finding of novel therapeutic strategies that can efficiently overcome this pathology isn't easy (Kerem et al. 1990). In a recent study the elevation of tissue inhibitor of metalloproteinases-1 (TIMP-1), collagen-IV, prolyl hydroxylase serum concentrations suggest that these may be indicators of hepatic fibrogenesis in cystic fibrosis (Pereira et al. 2004).

## Conclusions

Human serum is extremely complex thus is the most difficult protein-containing sample to characterize. This review examines the various proteomic techniques utilized for each step in the process of human serum sample preparation or treatment, for its prefractionation or separation, and for the analysis to identify the corresponding serum biomarkers related to a specific disease or during different stages of a disease development. The discussed proteomic techniques are utilized for identifying protein-based serum biomarkers. The importance of biomarker identification cannot be underestimated. A fast growing area of basic, translational, and clinical research interest is the applications of proteomics techniques to preventive, diagnostic, and prognostic medicine. Protein biomarkers could facilitate the early detection of the onset of a disease at a curable stage and identification of subgroup of patients who respond well to certain types of drug treatment from those who do not. The knowledge generated may provide biochemical indicators for predicting patients' risk for cancer, heart attack, or other diseases and their different responses to the same therapy for optimal patient care and management.

Although many serum biomarkers were discussed, only a few are FDA approved for disease diagnosis and prognosis, including cancer biomarkers in human serum, CEA, Her-2/neu, bladder tumor antigen, thyroglobulin, alpha-fetoprotein, PSA, CA125, CA19.9, CA15.3, and some other cancer biomarkers, as well as cardiovascular disease biomarkers Troponin I and B-type natriuretic peptide (for more detailed review, see



Polanski and Anderson, 2006). Many biomarkers remain to be validated for accurate clinical identification of a particular disease. It is more desirable to combine different techniques and identify a panel of biomarkers to accurately measure the disease biomarker levels and establish criteria for disease diagnosis and prognosis. Moreover, many techniques still lack the proper sensitivity, specificity, and reproducibility for the identification of serum or other biomarkers. ELISA is a quantitative assay based on specific antibody-antigen binding and is commonly used to analyze biomarkers due to its sensitivity, specificity, and simplicity. Unfortunately, specific antibodies against many biomarkers are not available. In addition, due to the low abundance of some biomarkers, those biomarkers are masked by other more abundant proteins, and thus, difficult to be detected. To enhance future serum biomarker identification, techniques should be improved and combinations of different technologies and statistical analysis are required to increase the sensitivity, reproducibility, and specificity of biomarker detection.

## Acknowledgements

This work was in part supported by grants from Department of Defense Congressionally Directed Medical Research Program DAMD17-02-1-0238, Susan G. Komen Breast Cancer Foundation BCTR0504465, and Elsa U. Pardee Foundation, and a Program Enhancement grant and a Developing Scholar Award from Florida State University to Dr. Q.X. Sang.

The first two authors contributed equally to this work.

## References

- Adam, B.L., Qu, Y.S. and Davis, J.W. et al. 2002. Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. *Cancer Research*, 62:3609–14.
- Adkins, J.N., Varnum, S.M. and Auberry, K.J. et al. 2002. Toward a human blood serum proteome—Analysis by multidimensional separation coupled with mass spectrometry. *Molecular and Cellular Proteomics*, 1:947–55.
- Adrados, B.P., Galaev, I.Y. and Nilsson, K. et al. 2001. Size exclusion behavior of hydroxypropylcellulose beads with temperature-dependent porosity. *Journal of Chromatography A*, 930:73–8.
- Aebersold, R. and Mann, M. 2003. Mass spectrometry-based proteomics. *Nature*, 422:198–207.
- Ahmed, N., Barker, G., Oliva, K. et al. 2003. An approach to remove albumin for the proteomic analysis of low abundance biomarkers in human serum. *Proteomics*, 3:1980–7.
- Ahmed, N., Barker, G. and Oliva, K.T. et al. 2004. Proteomic-based identification of haptoglobin-1 precursor as a novel circulating biomarker of ovarian cancer. *British Journal of Cancer*, 91:129–40.
- Ahmed, N., Oliva, K.T. and Barker, G. et al. 2005. Proteomic tracking of serum protein isoforms as screening biomarkers of ovarian cancer. *Proteomics*, 5:4625–36.
- Ahn, W.S., Park, S.P. and Bae, S.M. et al. 2005. Identification of hemoglobin-alpha and -beta subunits as potential serum biomarkers for the diagnosis and prognosis of ovarian cancer. *Cancer Science*, 96:197–201.
- Alaiya, A., Al-Mohanna, M. and Linder, S. 2005. Clinical cancer proteomics: Promises and pitfalls. *Journal of Proteome Research*, 4:1213–22.
- Albrethsen, J., Bogebo, R. and Gammeltoft, S. et al. 2005. Upregulated expression of human neutrophil peptides 1, 2 and 3 (HNP 1–3) in colon cancer serum and tumours: a biomarker study. *Bmc Cancer*, 5:–.
- An, J., Yuan, Q. and Wang, C. et al. 2005. Differential display of proteins involved in the neural differentiation of mouse embryonic carcinoma P19 cells by comparative proteomic analysis. *Proteomics*, 5:1656–68.
- Anand, I.S., Fisher, L.D. and Chiang, Y.T. et al. 2003. Changes in brain natriuretic peptide and norepinephrine over time and mortality and morbidity in the Valsartan Heart Failure Trial (Val-HeFT). *Circulation*, 107:1278–83.
- Andereya, S., Streich, N. and Schmidt-Rohlfing, B. et al. 2006. Comparison of modern marker proteins in serum and synovial fluid in patients with advanced osteoarthritis and rheumatoid arthritis. *Rheumatology International*, 26:432–8.
- Anderson, L. and Anderson, N.G. 1977. High resolution two-dimensional electrophoresis of human plasma proteins. *Proceedings of the National Academy of Sciences of the United States of America*, 74:5421–5.
- Anderson, N.L. and Anderson, N.G. 2002. The human plasma proteome—History, character, and diagnostic prospects. *Molecular and Cellular Proteomics*, 1:845–67.
- Anon. 2000. Compendium of Chemical Terminology—IUPAC Recommendations, 2nd edn. Compiled by A.D. McNaught and A. Wilkinson. *Food Additives and Contaminants*, 17:480.
- Anon, Assoc. A. and Aging, N.I. 1998. Consensus report of the Working Group on: “Molecular and Biochemical Markers of Alzheimer’s Disease.” *Neurobiology of Aging*, 19:109–16.
- Arciero, C., Somiari, S.B. and Shriver, C.D. et al. 2003. Functional relationship and gene ontology classification of breast cancer biomarkers. *International Journal of Biological Markers*, 18:241–72.
- Arruda, A.P., Da-Silva, W.S. and Carvalho, D.P. et al. 2003. Hyperthyroidism increases the uncoupled ATPase activity and heat production by the sarcoplasmic reticulum Ca-2+-ATPase. *Biochemical Journal*, 375:753–60.
- Atkinson, A.J., Colburn, W.A. and DeGruttola, V. G. et al. 2001. Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. *Clinical Pharmacology and Therapeutics*, 69:89–95.
- Avasarala, L.R., Wall, M.R. and Wolfe, G.M. 2005. A distinctive molecular signature of multiple sclerosis derived from MALDI-TOF/MS and serum proteomic pattern analysis—Detection of three biomarkers. *Journal of Molecular Neuroscience*, 25:119–25.
- Banfi, G., Parma, P. and Pontillo, M. 1997. Stability of tumor markers CA 19.9, CA 125, and CA 15.3 in serum obtained from plain tubes and tubes containing thixotropic gel separator. *Clinical Chemistry*, 43:2430–1.
- Barnea, E., Sorkin, R. and Ziv, T. et al. 2005. Evaluation of prefractionation methods as a preparatory step for multidimensional based chromatography of serum proteins. *Proteomics*, 5:3367–75.
- Bast, R.C., Bates, S. and Bredt, A.B. et al. 1996. Clinical practice guidelines for the use of tumor markers in breast and colorectal cancer. *Journal of Clinical Oncology*, 14:2843–77.
- Bast, R.C., Xu, F.J. and Yu, Y.H. et al. 1998. CA 125: The past and the future. *International Journal of Biological Markers*, 13:179–87.
- Bayes, A., Tsetsenis, T. and Ventura, S. et al. 2004. Human kallikrein 6 activity is regulated via an autoproteolytic mechanism of activation/inactivation. *Biological Chemistry*, 385:517–24.
- Bergmann, J.F., Beaugrand, M. and Labadie, H. et al. 1986. Ca-125 (Ovarian Tumor-Associated Antigen) in Ascitic Liver-Diseases. *Clinica Chimica Acta.*, 155:163–5.

- Bianchi, L., Canton, C. and Bini, L. et al. 2005. Protein profile changes in the human breast cancer cell line MCF-7 in response to SEL1L gene induction. *Proteomics*, 5:2433–42.
- Binz, P. A., Hochstrasser, D. F. and Appel, R. D. 2003. Mass spectrometry-based proteomics: Current status and potential use in clinical chemistry. *Clinical Chemistry and Laboratory Medicine*, 41:1540–51.
- Bischoff, R. and Luidner, T.M. 2004. Methodological advances in the discovery of protein and peptide disease markers. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 803:27–40.
- Bjellqvist, B., Ek, K. and Righetti, P.G. et al. 1982. Isoelectric-Focusing in Immobilized Ph Gradients—Principle, Methodology and Some Applications. *Journal of Biochemical and Biophysical Methods*, 6:317–39.
- Blackwell, K., Hurwitz, H. and Lieberman, G. et al. 2004. Circulating D-dimer levels are better predictors of overall survival and disease progression than carcinoembryonic antigen levels in patients with metastatic colorectal carcinoma. *Cancer*, 101:77–82.
- Bloustine, J., Berejnov, V. and Fraden, S. 2003. Measurements of protein-protein interactions by size exclusion chromatography. *Biophysical Journal*, 85:2619–23.
- Bockhorn, M., Frilling, A. and Rewerk, S. et al. 2004. Lack of elevated serum carcinoembryonic antigen and calcitonin in medullary thyroid carcinoma. *Thyroid*, 14:468–70.
- Bosserhoff, A.K., Kaufmann, M. and Kaluza, B. et al. 1997. Melanoma-inhibiting activity, a novel serum marker for progression of malignant melanoma. *Cancer Research*, 57:3149–53.
- Bramwell, V.H.C., Doig, G.S. and Tuck, A.B. et al. 2006. Serial Plasma Osteopontin Levels Have Prognostic Value in Metastatic Breast Cancer. *Clinical Cancer Research*, 12:3337–43.
- Broeckaert, F., Arsalane, K. and Hermans, C. et al. 2000. Serum Clara cell protein: A sensitive biomarker of increased lung epithelium permeability caused by ambient ozone. *Environmental Health Perspectives*, 108:533–7.
- Buamah, P.K., Cornell, C. and Cassellsmith, A.J. et al. 1986. Concentrations of Trypsin, Elastase and Carbohydrate Antigen Ca 19–9 in Serum of Cystic-Fibrosis Patients. *Clinical Chemistry*, 32:913–.
- Buchanan, N.S., Hamler, R.L. and Leopold, P.E. et al. 2005. Mass mapping of cancer cell lysates using two-dimensional liquid separations, electrospray-time of flight-mass spectrometry, and automated data processing. *Electrophoresis*, 26:248–56.
- Burnette, W.N. 1981. Western Blotting—Electrophoretic Transfer of Proteins from Sodium Dodecyl Sulfate-Polyacrylamide Gels to Unmodified Nitrocellulose and Radiographic Detection with Antibody and Radioiodinated Protein-A. *Analytical Biochemistry*, 112:195–203.
- Burtis, C.A., Ashwood, E.A. and Editors, 2001. *Tietz Fundamentals of Clinical Chemistry, 5th Edition*.
- Cao, L.-F., Lu, Y.-M. and Ma, M. et al. 2006. Levels of serum interleukin-15 and the expression of T-helper lymphocyte subsets in peripheral blood of children with juvenile rheumatoid arthritis. *Zhongguo dang dai er ke za zhi = Chinese journal of contemporary pediatrics*, 8:9–12.
- Chatterjee, S.K. and Zetter, B.R. 2005. Cancer biomarkers: knowing the present and predicting the future. *Future Oncology*, 1:37–50.
- Chignard, N. and Beretta, L. 2004. Proteomics for hepatocellular carcinoma marker discovery. *Gastroenterology*, 127:S120–S5.
- Clarke, W., Zhang, Z. and Chan, D.W. 2003. The application of clinical proteomics to cancer and other diseases. *Clinical Chemistry and Laboratory Medicine*, 41:1562–70.
- Cleghorn, G., Benjamin, L. and Corey, M. et al. 1985. Age-Related Alterations in Immunoreactive Pancreatic Lipase and Cationic Trypsinogen in Young-Children with Cystic-Fibrosis. *Journal of Pediatrics*, 107:377–81.
- Cooper, E.H., Armitage, T.G. and Robinson, M.R.G. et al. 1990. Prostatic Specific Antigen and the Prediction of Prognosis in Metastatic Prostatic-Cancer. *Cancer*, 66:1025–8.
- Corthals, G.L., Wasinger, V.C. and Hochstrasser, D.F. et al. 2000. The dynamic range of protein expression: A challenge for proteomic research. *Electrophoresis*, 21:1104–15.
- Cummings, J.L. 2005. Clinical evaluation as a biomarker for Alzheimer's disease. *Journal of Alzheimer's Disease*, 8:327–37.
- Davutoglu, V., Gunay, N. and Kocoglu, H. et al. 2006. Serum levels of NT-ProBNP as an early cardiac marker of carbon monoxide poisoning. *Inhalation Toxicology*, 18:155–8.
- Deng, Y.X., Xu, Y. and Ye, B. et al. 2003. Prostate carcinoma tissue proteomics for biomarker discovery. *Cancer*, 98:2576–82.
- Diamandis, E.P., 2002. Tumor markers: past present and future. In: *Tumor Markers: Physiology, Pathobiology, Technology, and Clinical Applications*.
- Diamandis, E.P. 2003. Point—Proteomic patterns in biological fluids: Do they represent the future of cancer diagnostics? *Clinical Chemistry*, 49:1272–5.
- Diamandis, E.P., Scorilas, A. and Fracchioli, S. et al. 2003. Human kallikrein 6 (hK6): A new potential serum biomarker for diagnosis and prognosis of ovarian carcinoma. *Journal of Clinical Oncology*, 21:1035–43.
- Djavan, B., Zlotta, A. and Kratzik, C. et al. 1999. PSA, PSA density, PSA density of transition zone, free/total PSA ratio, and PSA velocity for early detection of prostate cancer in men with serum PSA 2.5 to 4.0 ng/ml. *Urology*, 54:517–22.
- Dole, M., Mack, L.L. and Hines, R.L. 1968. Molecular Beams of Macroions. *Journal of Chemical Physics*, 49:2240–&.
- Dongre, A.R., Eng, J.K. and Yates, J.R., III. 1997. Emerging tandem mass spectrometry techniques for the rapid identification of proteins. *Trends in Biotechnology*, 15:418–25.
- Dos Remedios, C.G., Liew, C.C. and Allen, P.D. et al. 2003. Genomics, proteomics and bioinformatics of human heart failure. *Journal of Muscle Research and Cell Motility*, 24:251–60.
- Drake, R.R., Schwegler, E.E. and Malik, et al. 2006. Lectin capture strategies combined with mass spectrometry for the discovery of serum glycoprotein biomarkers. *Molecular and Cellular Proteomics*, 5:1957–67.
- Duffy, M.J., Osullivan, F. and McDonnell, T.J. et al. 1985. Increased Concentrations of the Antigen Ca-19–9 in Serum of Cystic-Fibrosis Patients. *Clinical Chemistry*, 31:1245–6.
- Ekdahl, K., Roloff, J. and Oxelius, V.A. et al. 1994. Analysis of Immunoglobulin Isotype Levels in Acute Pneumococcal Bacteremia and in Convalescence. *European Journal of Clinical Microbiology and Infectious Diseases*, 13:374–8.
- Eng, J.K., McCormack, A.L. and Yates, J.R. 1994. An Approach to Correlate Tandem Mass-Spectral Data of Peptides with Amino-Acid-Sequences in a Protein Database. *Journal of the American Society for Mass Spectrometry*, 5:976–89.
- Engwegen Judith, Y.M.N., Gast Marie-Christine, W. and Schellens Jan, H.M. et al. 2006. Clinical proteomics: searching for better tumour markers with SELDI-TOF mass spectrometry. *Trends in pharmacological sciences FIELD Publication Date:2006*; 27:251–9. FIELD Reference Number: FIELD Journal Code:7906158 FIELD Call Number:.
- Era, S., Kuwata, K. and Imai, H. et al. 1995. Age-related change in redox state of human serum albumin. *Biochimica et Biophysica Acta, Protein Structure and Molecular Enzymology*, 1247:12–6.
- Feng, J.T., Liu, Y.K. and Song, H.Y. et al. 2005. Heat-shock protein 27: A potential biomarker for hepatocellular carcinoma identified by serum proteome analysis. *Proteomics*, 5:4581–8.
- Ferguson, P.L. and Smith, R.D. 2003. Proteome analysis by mass spectrometry. *Annual Review of Biophysics and Biomolecular Structure*, 32:399–424.
- Feuerstein, I., Rainer, M. and Bernardo, K. et al. 2005. Derivatized cellulose combined with MALDI-TOF MS: A new tool for serum protein profiling. *Journal of Proteome Research*, 4:2320–6.
- Finnskog, D., Jaras, K. and Ressine, A. et al. 2006. High-speed biomarker identification utilizing porous silicon nanovial arrays and MALDI-TOF mass spectrometry. *Electrophoresis*, 27:1093–103.
- Fiorenza, A.M., Branchi, A. and Sommariva, D. 2000. Serum lipoprotein profile in patients with cancer. A comparison with non-cancer subjects. *International Journal of Clinical and Laboratory Research*, 30:141–5.

- Fung, K.Y.C., Askovic, S. and Basile, F. et al. 2004. A simple and inexpensive approach to interfacing high-performance liquid chromatography and matrix-assisted laser desorption/ionization-time of flight-mass spectrometry. *Proteomics*, 4:3121–7.
- Gao, J., Garulacan, L. A. and Storm, S. M. et al. 2004. Identification of in vitro protein biomarkers of idiosyncratic liver toxicity. *Toxicology in Vitro*, 18:533–41.
- Gao, J., Garulacan, L.A. and Storm, S.M. et al. 2005. Biomarker discovery in biological fluids. *Methods*, 35:291–302.
- Gao, J., Opiteck, G.J. and Friedrichs, M.S. et al. 2003. Changes in the protein expression of yeast as a function of carbon source. *Journal of Proteome Research*, 2:643–9.
- Giannelli, G., Erriquez, R. and Iannone, F. et al. 2004. MMP-2, MMP-9, TIMP-1 and TIMP-2 levels in patients with rheumatoid arthritis and psoriatic arthritis. *Clinical and Experimental Rheumatology*, 22:335–8.
- Gorg, A., Weiss, W. and Dunn, M.J. 2004. Current two-dimensional electrophoresis technology for proteomics. *Proteomics*, 4:3665–85.
- Graves, P.R. and Haystead, T.A.J. 2002. Molecular biologist's guide to proteomics. *Microbiology and Molecular Biology Reviews*, 66:39–+.
- Grignon, D.J. and Hammond, E.H. 1995. College of American Pathologists Conference XXVI on clinical relevance of prognostic markers in solid tumors. Report of the Prostate Cancer Working Group. *Archives of pathology and laboratory medicine FIELD Publication Date:1995*, 119:1122–6. FIELD Reference Number: 79 FIELD Journal Code: 7607091 FIELD Call Number:.
- Gutman, E.B., Sproul, E.E. and Gutman, A.B. 1936. Significance of increased phosphatase activity of bone at the site of osteoplastic metastases secondary to carcinoma of the prostate gland. *Am. J. Cancer*, 28:485–95.
- Gygi, S.P., Corthals, G.L. and Zhang, Y. et al. 2000a. Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proceedings of the National Academy of Sciences of the United States of America*, 97:9390–5.
- Gygi, S.P., Rist, B. and Aebersold, R. 2000b. Measuring gene expression by quantitative proteome analysis. *Current Opinion in Biotechnology*, 11:396–401.
- Gygi, S.P., Rist, B. and Gerber, S.A. et al. 1999. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nature Biotechnology*, 17:994–9.
- Gygi, S.P., Rist, B. and Griffin, T.J. et al. 2002. Proteome analysis of low-abundance proteins using multidimensional chromatography and isotope-coded affinity tags. *Journal of Proteome Research*, 1:47–54.
- Hamada, S., Ishikawa, N. and Yoshii, M. et al. 1976. Roles of Circulating Carcinoembryonic Antigen and Calcitonin in Diagnosis of Medullary-Thyroid Carcinoma—Comparative-Study. *Endocrinologia Japonica*, 23:505–10.
- Hamlar, R.L., Zhu, K. and Buchanani, N.S. et al. 2004. A two-dimensional liquid-phase separation method coupled with mass spectrometry for proteomic studies of breast cancer and biomarker identification. *Proteomics*, 4:562–77.
- Han, D.K., Eng, J. and Zhou, H.L. et al. 2001. Quantitative profiling of differentiation-induced microsomal proteins using isotope-coded affinity tags and mass spectrometry. *Nature Biotechnology*, 19:946–51.
- Hanash, S.M. 2000. Biomedical applications of two-dimensional electrophoresis using immobilized pH gradients: Current status. *Electrophoresis*, 21:1202–9.
- Harada, M., Mitsuyama, K. and Yoshida, H. et al. 1998. Vascular endothelial growth factor in patients with rheumatoid arthritis. *Scandinavian Journal of Rheumatology*, 27:377–80.
- Hathaway, B., Landsittel, D.P. and Gooding, W. et al. 2005. Multiplexed analysis of serum cytokines as biomarkers in squamous cell carcinoma of the head and neck patients. *Laryngoscope*, 115:522–7.
- Hatzinikolaou-Kotsakou, E., Tziakas, D. and Hotidis, A. et al. 2006. Relation of C-reactive protein to the first onset and the recurrence rate in lone atrial fibrillation. *American Journal of Cardiology*, 97:659–61.
- Hayes, D.F., Bast, R.C. and Desch, C.E. et al. 1996. Tumor marker utility grading system: A framework to evaluate clinical utility of tumor markers. *Journal of the National Cancer Institute*, 88:1456–66.
- Heck, A.J.R. and Krijgsveld, J. 2004. Mass spectrometry-based quantitative proteomics. *Expert Review of Proteomics*, 1:317–26.
- Higashida, T., Koizumi, T. and Yamaguchi, S. et al. 2001. Ovarian malignant mixed mesodermal tumor producing the free form of the b-subunit of human chorionic gonadotropin. *International Journal of Clinical Oncology*, 6:97–100.
- Hillenkamp, F., Karas, M. and Beavis, R.C. et al. 1991. Matrix-Assisted Laser Desorption Ionization Mass-Spectrometry of Biopolymers. *Analytical Chemistry*, 63:A1193–A202.
- Hori, K., Uematsu, K. and Yasoshima, H. et al. 1997. Testicular seminoma with human chorionic gonadotropin production. *Pathology International*, 47:592–9.
- Huang, C.M., Elmetts, C.A. and van Kampen, K.R. et al. 2005. Prospective highlights of functional skin proteomics. *Mass Spectrometry Reviews*, 24:647–60.
- Huang, J.X., Mehrens, D. and Wiese, R. et al. 2001. High-throughput genomic and proteomic analysis using microarray technology. *Clinical Chemistry*, 47:1912–6.
- Ide, A., Ashizawa, K. and Ishikawa, N. et al. 2001. Elevation of serum pro-gastrin-releasing peptide in patients with medullary thyroid carcinoma and small cell lung carcinoma. *Thyroid*, 11:1055–61.
- Ideker, T., Thorsson, V. and Ranish, J.A. et al. 2001. Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. *Science*, 292:929–34.
- Issaq, H.J., Veenstra, T.D. and Conrads, T.P. et al. 2002. The SELDI-TOF MS approach to proteomics: Protein profiling and biomarker identification. *Biochemical and Biophysical Research Communications*, 292:587–92.
- Johnson, P.J. 2001. The role of serum alpha-fetoprotein estimation in the diagnosis and management of hepatocellular carcinoma. *Clinics in liver disease*, 5:145–59.
- Johnson, P.J., Poon, T.C.W. and Hjelm, N.M. et al. 2000. Structures of disease-specific serum alpha-fetoprotein isoforms. *British Journal of Cancer*, 83:1330–7.
- Kaminska, J., Kowalska, M. and Kotowicz, B. et al. 2006. Pretreatment serum levels of cytokines and cytokine receptors in patients with non-small cell lung cancer, and correlations with clinicopathological features and prognosis—M-CSF—An independent prognostic factor. *Oncology*, 70:115–25.
- Karas, M. and Hillenkamp, F. 1988. Laser Desorption Ionization of Proteins with Molecular Masses Exceeding 10000 Daltons. *Analytical Chemistry*, 60:2299–301.
- Kennedy, S. 2001. Proteomic profiling from human samples: the body fluid alternative. *Toxicology Letters*, 120:379–84.
- Kerem, E., Corey, M. and Kerem, B.S. et al. 1990. The Relation between Genotype and Phenotype in Cystic-Fibrosis—Analysis of the Most Common Mutation (Delta-F508). *New England Journal of Medicine*, 323:1517–22.
- Kikuchi, K., Kubo, M. and Kadono, T. et al. 1998. Serum concentrations of vascular endothelial growth factor in collagen diseases. *British Journal of Dermatology*, 139:1049–51.
- Kim, J.H., Skates, S.J. and Uede, T. et al. 2002. Osteopontin as a potential diagnostic biomarker for ovarian cancer. *Jama-Journal of the American Medical Association*, 287:1671–9.
- Kostrubiec, M., Pruszczyk, P. and Bochowicz, A. et al. 2005. Biomarker-based risk assessment model in acute pulmonary embolism. *European Heart Journal*, 26:2166–72.
- Kramer, G., Schwarz, S. and Hagg, M. et al. 2006. Docetaxel induces apoptosis in hormone refractory prostate carcinomas during multiple treatment cycles. *British Journal of Cancer*, 94:1592–8.
- Kuefer, R., Hofer, M.D. and Zorn, C.S.M. et al. 2005. Assessment of a fragment of e-cadherin as a serum biomarker with predictive value for prostate cancer. *British Journal of Cancer*, 92:2018–23.
- Kuhn, E., Wu, J. and Karl, J. et al. 2004. Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and C-13-labeled peptide standards. *Proteomics*, 4:1175–86.

- Lam, Y.W., Mobley, J.A. and Evans, J.E. et al. 2005. Mass profiling-directed isolation and identification of a stage-specific serologic protein biomarker of advanced prostate cancer. *Proteomics*, 5:2927–38.
- Lee, H.J., Lee, E.Y. and Kwon, M.S. et al. 2006a. Biomarker discovery from the plasma proteome using multidimensional fractionation proteomics. *Current Opinion in Chemical Biology*, 10:42–9.
- Lee, I.N., Chen, C.H. and Sheu, J.C. et al. 2006b. Identification of complement C3a as a candidate biomarker in human chronic hepatitis C and HCV-related hepatocellular carcinoma using a proteomics approach. *Proteomics*, 6:2865–73.
- Li, J.N., Zhang, Z. and Rosenzweig, J. et al. 2002. Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. *Clinical Chemistry*, 48:1296–304.
- Liao, H., Wu, J. and Kuhn, E. et al. 2004. Use of mass spectrometry to identify protein biomarkers of disease severity in the synovial fluid and serum of patients with rheumatoid arthritis. *Arthritis and Rheumatism*, 50:3792–803.
- Lilley, K.S., Razzaq, A. and Dupree, P. 2002. Two-dimensional gel electrophoresis: recent advances in sample preparation, detection and quantitation. *Current Opinion in Chemical Biology*, 6:46–50.
- Lin, M.F., Lee, C.L. and Wojcieszyn, J.W. et al. 1980. Fundamental biochemical and immunological aspects of prostatic acid phosphatase. *Prostate (New York, NY, United States)*, 1:415–25.
- Link, A.J., Eng, J. and Schieltz, D.M. et al. 1999. Direct analysis of protein complexes using mass spectrometry. *Nature Biotechnology*, 17:676–82.
- Liu, C.C., Huang, J.S. and Tyrrell, D.L.J. et al. 2005. Capillary electrophoresis-electrospray-mass spectrometry of nucleosides and nucleotides: Application to phosphorylation studies of anti-human immunodeficiency virus nucleosides in a human hepatoma cell line. *Electrophoresis*, 26:1424–31.
- Lloyd, K.O., Yin, B.W.T. and Kudryashov, V. 1997. Isolation and characterization of ovarian cancer antigen CA 125 using a new monoclonal antibody (VK-8): Identification as a mucin-type molecule. *International Journal of Cancer*, 71:842–50.
- Lollo, B.A., Harvey, S. and Liao, J. et al. 1999. Improved two-dimensional gel electrophoresis representation of serum proteins by using ProtoClear (TM). *Electrophoresis*, 20:854–9.
- Lubman, D.M., Kachman, M.T. and Wang, H.X. et al. 2002. Two-dimensional liquid separations-mass mapping of proteins from human cancer cell lysates. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 782:183–96.
- Ludwig, J.A. and Weinstein, J.N. 2005. Biomarkers in cancer staging, prognosis and treatment selection. *Nature Reviews Cancer*, 5:845–56.
- Lumachi, F., Basso, S.M.M. and Brandes, A.A. et al. 2004. Relationship between tumor markers CEA and CA 15–3, TNM staging, estrogen receptor rate and MIB-1 index in patients with pT1–2 breast cancer. *Anticancer Research*, 24:3221–4.
- Lundin, M., Nordling, S. and Carpelan-Holmstrom, M. et al. 2000. A comparison of serum and tissue hCG beta as prognostic markers in colorectal cancer. *Anticancer Research*, 20:4949–51.
- Luo, L.Y., Bunting, P. and Scorilas, A. et al. 2001. Human kallikrein 10: a novel tumor marker for ovarian carcinoma? *Clinica Chimica Acta*, 306:111–8.
- Luo, L.Y., Shan, S.J.C. and Elliott, M.B. et al. 2006. Purification and characterization of human kallikrein 11, a candidate prostate and ovarian cancer biomarker, from seminal plasma. *Clinical Cancer Research*, 12:742–50.
- Maisel, A., Hollander, J.E. and Guss, D. et al. 2004. Primary results of the rapid emergency department heart failure outpatient trial (REDHOT). *Journal of the American College of Cardiology*, 44:1328–33.
- Maisel, A.S., Krishnaswamy, P. and Nowak, R.M. et al. 2002. Rapid measurement of B-type natriuretic peptide in the emergency diagnosis of heart failure. *New England Journal of Medicine*, 347:161–7.
- Mansson, B., Carey, D. and Alini, M. et al. 1995. Cartilage and Bone Metabolism in Rheumatoid-Arthritis—Differences between Rapid and Slow Progression of Disease Identified by Serum Markers of Cartilage Metabolism. *Journal of Clinical Investigation*, 95:1071–7.
- Marshall, J., Jankowski, A. and Furesz, S. et al. 2004. Human serum proteins pre-separated by electrophoresis or chromatography followed by tandem mass spectrometry. *Journal of Proteome Research*, 3:364–82.
- Martosella, J., Zolotarjova, N. and Liu, H.B. et al. 2005. Reversed-phase high-performance liquid chromatographic prefractionation of immunodepleted human serum proteins to enhance mass spectrometry identification of lower-abundant proteins. *Journal of Proteome Research*, 4:1522–37.
- Mathelin, C., Cromer, A. and Wendling, C. et al. 2006. Serum biomarkers for detection of breast cancers: A prospective study. *Breast Cancer Research and Treatment*, 96:83–90.
- Mayerhofer, K., Bodner, K. and Bodner-Adler, B. et al. 2001. Interleukin-8 serum level shift in patients with ovarian carcinoma undergoing paclitaxel-containing chemotherapy. *Cancer*, 91:388–93.
- McCullough, P.A., Nowak, R.M. and McCord, J. et al. 2002. B-type natriuretic peptide and clinical judgment in emergency diagnosis of heart failure—Analysis from breathing not properly (BNP) multinational study. *Circulation*, 106:416–22.
- McCullough Peter, A. and Sandberg Keisha, R. 2003. Sorting out the evidence on natriuretic peptides. *Reviews in cardiovascular medicine FIELD Publication Date:2003*, 4 Suppl 4:S13–9. FIELD Reference Number: 38 FIELD Journal Code: 100960007 FIELD Call Number:.
- McFarlane, I., Bomford, A. and Sherwood, R. 2000. *Liver Disease and Laboratory Medicine*, ACB Venture Publications, London.
- Mennink-Kersten, M.A.S.H., Donnelly, J. P. and Verweij, P.E. 2004. Detection of circulating galactomannan for the diagnosis and management of invasive aspergillosis. *Lancet Infectious Diseases*, 4:349–57.
- Miguet, L., Bogumil, R. and Decloquement, P. et al. 2006. Discovery and Identification of Potential Biomarkers in a Prospective Study of Chronic Lymphoid Malignancies Using SELDI-TOF-MS. *Journal of Proteome Research*, 5:2258–69.
- Miyata, Y., Sakai, H. and Hayashi, T. 2003. Serum insulin-like growth factor binding protein-3/prostate-specific antigen ratio is a useful predictive marker in patients with advanced prostate cancer. *Prostate*, 54:125–32.
- Mok, S.C., Chao, J. and Skates, S. et al. 2001. Prostatein, a potential serum marker for ovarian cancer: Identification through microarray technology. *Journal of the National Cancer Institute*, 93:1458–64.
- Moody, M.D., Van Arsdell, S.W. and Murphy, K.P. et al. 2001. Array-based ELISAs for high-throughput analysis of human cytokines. *Biotechniques*, 31:186–+.
- Morris, D.L., Sutton, J.N. and Harper, R.G. et al. 2004. Reversed-phase HPLC separation of human serum employing a novel saw-tooth gradient: Toward multidimensional proteome analysis. *Journal of Proteome Research*, 3:1149–54.
- Mughal, A.W., Hortobagyi, G.N. and Fritsche, H.A. et al. 1983. Serial Plasma Carcinoembryonic Antigen Measurements During Treatment of Metastatic Breast-Cancer. *Jama-Journal of the American Medical Association*, 249:1881–6.
- Muller-Ladner, U., Bosserhoff, A.K. and Dreher, K. et al. 1999. MIA (melanoma inhibitory activity): a potential serum marker for rheumatoid arthritis. *Rheumatology*, 38:148–54.
- Nagakawa, O., Yamagishi, T. and Fujiuchi, Y. et al. 2005. Serum hepatocyte growth factor activator (HGFA) in benign prostatic hyperplasia and prostate cancer. *European Urology*, 48:686–90.
- Nakamura, M., Endo, H. and Nasu, M. et al. 2002. Value of plasma B type natriuretic peptide measurement for heart disease screening in a Japanese population. *Heart*, 87:131–5.
- Nam, R.K., Reeves, J.R. and Toi, A. et al. 2006. A novel serum marker, total prostate secretory protein of 94 amino acids, improves prostate cancer detection and helps identify high grade cancers at diagnosis. *Journal of Urology*, 175:1291–7.
- Nasser, N., Bar-Oz, B. and Nir, A. 2005. Natriuretic peptides and heart disease in infants and children. *Journal of Pediatrics*, 147:248–53.
- Nielsen, U.B., Cardone, M.H. and Sinskey, A.J. et al. 2003. Profiling receptor tyrosine kinase activation by using Ab microarrays. *Proceedings of the National Academy of Sciences of the United States of America*, 100:9330–5.

- O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry*, 250:4007–21.
- Oikonomopoulou, K., Scorilas, A. and Michael, I.P. et al. 2006. Kallikreins as markers of disseminated tumour cells in ovarian cancer—A pilot study. *Tumor Biology*, 27:104–14.
- Okamoto, T., Nakamura, T. and Ikeda, J. et al. 2005. Serum carcinoembryonic antigen as a predictive marker for sensitivity to gefitinib in advanced non-small cell lung cancer. *European Journal of Cancer*, 41:1286–90.
- O'Neill, K.A., Miller, F.R. and Barder, T.J. et al. 2003. Profiling the progression of cancer: Separation of microsomal proteins in MCF10 breast epithelial cell lines using nonporous chromatophoresis. *Proteomics*, 3:1256–69.
- Ornstein, D.K., Englert, C. and Gillespie, J.W. et al. 2000a. Characterization of intracellular prostate-specific antigen from laser capture microdissected benign and malignant prostatic epithelium. *Clinical Cancer Research*, 6:353–6.
- Ornstein, D.K., Gillespie, J.W. and Pawletz, C.P. et al. 2000b. Proteomic analysis of laser capture microdissected human prostate cancer and in vitro prostate cell lines. *Electrophoresis*, 21:2235–42.
- Palladini, G., Campana, C. and Klersy, C. et al. 2003. Serum N-terminal pro-brain natriuretic peptide is a sensitive marker of myocardial dysfunction in AL amyloidosis. *Circulation*, 107:2440–5.
- Pandey, A. and Mann, M. 2000. Proteomics to study genes and genomes. *Nature*, 405:837–46.
- Pang, J.X., Ginanni, N. and Dongre, A.R. et al. 2002. Biomarker discovery in urine by proteomics. *Journal of Proteome Research*, 1:161–9.
- Pannek, J. and Partin, A.W. 1998. The role of PSA and percent free PSA for staging and prognosis prediction in clinically localized prostate cancer. *Seminars in urologic oncology*, 16:100–5. FIELD Reference Number: 40 FIELD Journal Code: 9514993 FIELD Call Number:.
- Pappas, P.A., Tzakis, A.G. and Saudubray, J.M. et al. 2004. Trends in serum citrulline and acute rejection among recipients of small bowel transplants. *Transplantation Proceedings*, 36:345–7.
- Park, S.N., Yeo, S.W. and Park, K.H. 2006. Serum heat shock protein 70 and its correlation with clinical characteristics in patients with sudden sensorineural hearing loss. *Laryngoscope*, 116:121–5.
- Pasiaka, Z., Stepien, H. and Czyz, W. et al. 2004. Concentration of metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 in the serum of patients with benign and malignant thyroid tumours treated surgically. *Endocrine regulations*, 38:57–63.
- Pereira, T.N., Lewinson, P.J. and Smith, J.L. et al. 2004. Serum markers of hepatic fibrogenesis in cystic fibrosis liver disease. *Journal of Hepatology*, 41:576–83.
- Petricoin, E.F., Ardekani, A.M. and Hitt, B.A. et al. 2002a. Use of proteomic patterns in serum to identify ovarian cancer. *Lancet*, 359:572–7.
- Petricoin, E.F., Ornstein, D.K. and Pawletz, C.P. et al. 2002b. Serum proteomic patterns for detection of prostate cancer. *Journal of the National Cancer Institute*, 94:1576–8.
- Petricoin, E.F., Zoon, K.C. and Kohn, E.C. et al. 2002c. Clinical proteomics: Translating benchside promise into bedside reality. *Nature Reviews Drug Discovery*, 1:683–95.
- Petrovic-Rackov, L. 2006. Evaluation of the degree of clinical rheumatoid arthritis activity based on the concentrations of cytokines TNF-alpha, IL-12, IL-15, and IL-18 in serum and synovial fluid. *Vojnosanitetski pregled. Military-medical and pharmaceutical review*, 63:21–6. FIELD Reference Number: FIELD Journal Code: 21530700R FIELD Call Number:.
- Pieper, R., Gatlin, C.L. and Makusky, A.J. et al. 2003. The human serum proteome: Display of nearly 3700 chromatographically separated protein spots on two-dimensional electrophoresis gels and identification of 325 distinct proteins. *Proteomics*, 3:1345–64.
- Polanski, M. and Anderson, N.L. 2006. A list of candidate cancer biomarkers for targeted proteomics. *Biomarker Insights* 2:1–48.
- Poole, A.R., Ionescu, M. and Fitzcharles, M.A. et al. 2004. The assessment of cartilage degradation in vivo: development of an immunoassay for the measurement in body fluids of type II collagen cleaved by collagenases. *Journal of Immunological Methods*, 294:145–53.
- Qin, S.Z., Ferdinand, A.S. and Richie, J.P. et al. 2005. Chromatofocusing fractionation and two-dimensional difference gel electrophoresis for low abundance serum proteins. *Proteomics*, 5:3183–92.
- Qu, Y.S., Adam, B.L. and Yasui, T. et al. 2002. Boosted decision tree analysis of surface-enhanced laser desorption/ionization mass spectral serum profiles discriminates prostate cancer from noncancer patients. *Clinical Chemistry*, 48:1835–43.
- Rauchhaus, M., Clark, A.L. and Doehner, W. et al. 2003. The relationship between cholesterol and survival in patients with chronic heart failure. *Journal of the American College of Cardiology*, 42:1933–40.
- Redfield, M.M., Rodeheffer, R.J. and Jacobsen, S.J. et al. 2002. Plasma brain natriuretic peptide concentration: Impact of age and gender. *Journal of the American College of Cardiology*, 40:976–82.
- Redfield, M.M., Rodeheffer, R.J. and Jacobsen, S.J. et al. 2004. Plasma brain natriuretic peptide to detect preclinical ventricular systolic or diastolic dysfunction—A community-based study. *Circulation*, 109:3176–81.
- Regnier, F.E. 1983. High-performance liquid chromatography of biopolymers. *Science (Washington, DC, United States)*, 222:245–52.
- Riel, P.L.V., Wiinands, M.J. and Putte, L.B.V.D. 1998. Evaluation and management of active inflammatory disease, London: Mobsy.
- Rifai, N., Joubran, R. and Yu, H. et al. 1999. Inflammatory markers in men with angiographically documented coronary heart disease. *Clinical Chemistry*, 45:1967–73.
- Rifai, N. and Ridker, P.M. 2001. High-sensitivity C-reactive protein: A novel and promising marker of coronary heart disease. *Clinical Chemistry*, 47:403–11.
- Roberts, D.D., Monsein, D.L. and Frates, R.C. et al. 1986. A Serum Test for Cystic-Fibrosis Using Monoclonal-Antibody 19–9. *Archives of Biochemistry and Biophysics*, 245:292–4.
- Robertson, J.F.R., Pearson, D. and Price, M.R. et al. 1991. Prospective Assessment of the Role of 5 Tumor-Markers in Breast-Cancer. *Cancer Immunology Immunotherapy*, 33:403–10.
- Rodeheffer, R.J. 2004. Measuring plasma B-type natriuretic peptide in heart failure—Good to go in 2004? *Journal of the American College of Cardiology*, 44:740–9.
- Romer, T.G., Rezcallah, M.S. and Boyle, M.D.P. 2002. Use of surface enhanced laser desorption ionization time-of-flight [SELDI-TOF] mass spectrometry to analyze post-translational modification of bacterial virulence factors. *Recent Research Developments in Molecular Microbiology*, 1:1–18.
- Rose, K., Bougueleret, L. and Baussant, T. et al. 2004. Industrial-scale proteomics: From liters of plasma to chemically synthesized proteins. *Proteomics*, 4:2125–50.
- Rustin, G.J.S., Nelstrop, A.E. and Bentzen, S.M. et al. 1999. Use of tumour markers in monitoring the course of ovarian cancer. *Annals of Oncology*, 10:21–7.
- Sahab Ziad, J., Suh, Y. and Sang Qing-Xiang, A. 2005. Isoelectric point-based prefractionation of proteins from crude biological samples prior to two-dimensional gel electrophoresis. *Journal of proteome research*, 4:2266–72.
- Sakai, H., Igawa, T. and Saha, P.K. et al. 1992. Department of Urology, Nagasaki University School of Medicine, Japan, pp. 77–80.
- Santala, M., Ristel, J. and Kauppila, A. 2004. Comparison of carboxy-terminal telopeptide of type I collagen (ICTP) and CA 125 as predictors of prognosis in ovarian cancer. *Anticancer Research*, 24:1057–62.
- Saulot, V., Vittecoq, O. and Charlionet, R. et al. 2002. Presence of autoantibodies to the glycolytic enzyme alpha-enolase in sera from patients with early rheumatoid arthritis. *Arthritis and Rheumatism*, 46:1196–201.
- Schiller, J., Arnhold, J. and Benard, S. et al. 1999. Lipid analysis by matrix-assisted laser desorption and ionization mass spectrometry: A methodological approach. *Analytical Biochemistry*, 267:46–56.
- Schrader, M. and Schulz-Knappe, P. 2001. Peptidomics technologies for human body fluids. *Trends in Biotechnology*, 19:S55–S60.
- Schrohl, A. S., Holten-Andersen, M. and Sweep, F. et al. 2003. Tumor markers—From laboratory to clinical utility. *Molecular and Cellular Proteomics*, 2:378–87.

- Scorilas, A., Bjartell, A. and Lilja, H. et al. 2000. Streptavidin-polyvinylamine conjugates labeled with a europium chelate: Applications in immunoassay, immunohistochemistry, and microarrays. *Clinical Chemistry*, 46:1450–5.
- Senolt, L., Braun, M. and Olejarova, M. et al. 2005. Increased pentosidine, an advanced glycation end product, in serum and synovial fluid from patients with knee osteoarthritis and its relation with cartilage oligomeric matrix protein. *Annals of the Rheumatic Diseases*, 64:886–90.
- Sensabaugh, G.F. 1978. Isolation and Characterization of a Semen-Specific Protein from Human Seminal Plasma—Potential New Marker for Semen Identification. *Journal of Forensic Sciences*, 23:106–15.
- Shan, L. and Anderson, D.J. 2002. Gradient chromatofocusing. Versatile pH gradient separation of proteins in ion-exchange HPLC: Characterization studies. *Analytical Chemistry*, 74:5641–9.
- Sheng, S., Chen, D. and Van Eyk, J.E. 2006. Multidimensional liquid chromatography separation of intact proteins by chromatographic focusing and reversed phase of the human serum proteome—Optimization and protein database. *Molecular and Cellular Proteomics*, 5:26–34.
- Skoumal, M., Haberhauer, G. and Feyertag, J. et al. 2004. Serum levels of cartilage oligomeric matrix protein are elevated in rheumatoid arthritis, but not in inflammatory rheumatic diseases such as psoriatic arthritis, reactive arthritis, Raynaud's syndrome, scleroderma, systemic lupus erythematosus, vasculitis and Sjogren's syndrome. *Arthritis Research and Therapy*, 6:73–4.
- Somiari, R.I., Somiari, S. and Russell, S. et al. 2005. Proteomics of breast carcinoma. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 815:215–25.
- Somiari, R.I., Sullivan, A. and Russell, S. et al. 2003. High-throughput proteomic analysis of human infiltrating ductal carcinoma of the breast. *Proteomics*, 3:1863–73.
- Sone, H., Sakauchi, M. and Takahashi, A. et al. 2001. Elevated levels of vascular endothelial growth factor in the sera of patients with rheumatoid arthritis—Correlation with disease activity. *Life Sciences*, 69:1861–9.
- Sorkness, C., McGill, K. and Busse, W.W. 2002. Evaluation of serum eosinophil cationic protein as a predictive marker for asthma exacerbation in patients with persistent disease. *Clinical and Experimental Allergy*, 32:1355–9.
- Sreekumar, A., Laxman, B. and Rhodes, D.R. et al. 2004. Humoral immune response to alpha-methylacyl-CoA racemase and prostate cancer. *Journal of the National Cancer Institute*, 96:834–43.
- Syrgos, K.N., Fyssa, I. and Konstandoulakis, M.M. et al. 1998. Beta human chorionic gonadotropin concentrations in serum of patients with pancreatic adenocarcinoma. *Gut*, 42:88–91.
- Tabb, D.L., Eng, J.K. and Yates, J.R., III. 2001. Protein identification by SEQUEST. *Proteome Research: Mass Spectrometry*, 125–42.
- Tabb, D.L., McDonald, W. H. and Yates, J.R. 2002. DTASelect and contrast: Tools for assembling and comparing protein identifications from shotgun proteomics. *Journal of Proteome Research*, 1:21–6.
- Taira, M., Tamaoki, J. and Kondo, M. et al. 2002. Serum B12 tryptase level as a marker of allergic airway inflammation in asthma. *Journal of Asthma*, 39:315–22.
- Tam, S.W., Wiese, R. and Lee, S. et al. 2002. Simultaneous analysis of eight human Th1/Th2 cytokines using microarrays. *Journal of Immunological Methods*, 261:157–65.
- Tanaka, K., Waki, H. and Ido, Y. et al. 1988. Protein and polymer analyses up to m/z 100,000 by laser ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry*, 2:151–3.
- Tang, N., Tornatore, P. and Weinberger, S.R. 2004. Current developments in SELDI affinity technology. *Mass Spectrometry Reviews*, 23:34–44.
- Tedeschi, G., Cappelletti, G. and Negri, A. et al. 2005. Characterization of nitroproteome in neuron-like PC12 cells differentiated with nerve growth factor: Identification of two nitration sites in alpha-tubulin. *Proteomics*, 5:2422–32.
- Tirumalai, R.S., Chan, K.C. and Prieto, D.A. et al. 2003. Characterization of the low molecular weight human serum proteome. *Molecular and Cellular Proteomics*, 2:1096–103.
- Tong, Z.Y., Illek, B. and Bhagwandin, V.J. et al. 2004. Prostin, a membrane-anchored serine peptidase, regulates sodium currents in JME/CF15 cells, a cystic fibrosis airway epithelial cell line. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 287: L928–L35.
- Torre, G.C., Rembado, R. and Barbetti, V. et al. 1991. Serum Levels of Tumor-Associated Trypsin-Inhibitor (Tati) in Benign and Malignant Gynecological Diseases. *Scandinavian Journal of Clinical and Laboratory Investigation*, 51:15–8.
- Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets—Procedure and Some Applications. *Proceedings of the National Academy of Sciences of the United States of America*, 76:4350–4.
- Tribl, F., Gerlach, M. and Marcus, K. et al. 2005. “Subcellular proteomics” of neuromelanin granules isolated from the human brain. *Molecular and Cellular Proteomics*, 4:945–57.
- Troughton, R.W., Frampton, C.M. and Yandle, T.G. et al. 2000. Treatment of heart failure guided by plasma aminoterminal brain natriuretic peptide (N-BNP) concentrations. *Lancet*, 355:1126–30.
- Vartiainen, J., Lehtovirta, P. and Finne, P. et al. 2001. Preoperative serum concentration of hCG beta as a prognostic factor in ovarian cancer. *International Journal of Cancer*, 95:313–6.
- Veeramani, S., Yuan, T.C. and Chen, S.J. et al. 2005. Cellular prostatic acid phosphatase: a protein tyrosine phosphatase involved in androgen-independent proliferation of prostate cancer. *Endocrine-Related Cancer*, 12:805–22.
- Vermeulen, R., Lan, Q. and Zhang, L.P. et al. 2005. Decreased levels of CXC-chemokines in serum of benzene-exposed workers identified by array-based proteomics. *Proceedings of the National Academy of Sciences of the United States of America*, 102:17041–6.
- Verstappen, S.M.M., Poole, A.R. and Ionescu, M. et al. 2006. Radiographic joint damage in rheumatoid arthritis is associated with differences in cartilage turnover and can be predicted by serum biomarkers: an evaluation from 1 to 4 years after diagnosis. *Arthritis Research and Therapy*, 8:–.
- Vihko, P. 1979. Human Prostatic Acid-Phosphatases—Purification of a Minor Enzyme and Comparisons of the Enzymes. *Investigative Urology*, 16:349–52.
- Wall, D.B., Kachman, M.T. and Gong, S.Y. et al. 2000. Isoelectric focusing nonporous RP HPLC: A two-dimensional liquid-phase separation method for mapping of cellular proteins with identification using MALDI-TOF mass spectrometry. *Analytical Chemistry*, 72:1099–111.
- Wallis, R.S., Amirtahmasseb, M. and Ellner, J.J. 1990. Induction of Interleukin 1 and Tumor-Necrosis-Factor by Mycobacterial Proteins—the Monocyte Western-Blot. *Proceedings of the National Academy of Sciences of the United States of America*, 87:3348–52.
- Wang, C.C., Huang, R.P. and Sommer, M. et al. 2002. Array-based multiplexed screening and quantitation of human cytokines and chemokines. *Journal of Proteome Research*, 1:337–43.
- Wang, X.J., Yu, J.J. and Sreekumar, A. et al. 2005. Autoantibody signatures in prostate cancer. *New England Journal of Medicine*, 353:1224–35.
- Washburn, M.P., Wolters, D. and Yates, J.R. 2001. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nature Biotechnology*, 19:242–7.
- Westbrook, J.A., Yan, J.X. and Wait, R. et al. 2001. Zooming-in on the proteome: Very narrow-range immobilised pH gradients reveal more protein species and isoforms. *Electrophoresis*, 22:2865–71.
- Whitehouse, C. and Solomon, E. 2003. Current status of the molecular characterization of the ovarian cancer antigen CA125 and implications for its use in clinical screening. *Gynecologic Oncology*, 88:S152–S7.
- Whitelegge, J. 2005. Tandem mass spectrometry of integral membrane proteins for top-down proteomics. *Trac-Trends in Analytical Chemistry*, 24:576–82.
- Wiese, R., Belosludtsev, Y. and Powdrill, T. et al. 2001. Simultaneous multianalyte ELISA performed on a microarray platform. *Clinical Chemistry*, 47:1451–7.

- Williams, M.R., Turkes, A. and Pearson, D. et al. 1988. The Use of Serum Carcinoembryonic Antigen to Assess Therapeutic Response in Locally Advanced and Metastatic Breast-Cancer—a Prospective-Study with External Review. *European Journal of Surgical Oncology*, 14:417–22.
- Winnik, W.M. 2005. Continuous pH/salt gradient and peptide score for strong cation exchange chromatography in 2D-nano-LC/MS/MS peptide identification for proteomics. *Analytical Chemistry*, 77:4991–8.
- Wodicka, L., Dong, H.L. and Mittmann, M. et al. 1997. Genome-wide expression monitoring in *Saccharomyces cerevisiae*. *Nature Biotechnology*, 15:1359–67.
- Wolters, D.A., Washburn, M. P. and Yates, J.R. 2001. An automated multi-dimensional protein identification technology for shotgun proteomics. *Analytical Chemistry*, 73:5683–90.
- Woodbury, R.L., Varnum, S.M. and Zangar, R.C. 2002. Elevated HGF levels in sera from breast cancer patients detected using a protein microarray ELISA. *Journal of Proteome Research*, 1:233–7.
- Wu, W.G., Tang, X.M. and Hu, W. et al. 2002. Identification and validation of metastasis-associated proteins in head and neck cancer cell lines by two-dimensional electrophoresis and mass spectrometry. *Clinical and Experimental Metastasis*, 19:319–26.
- Wulfkuhle, J.D., Sgroi, D.C. and Krutzsch, H. et al. 2002. Proteomics of human breast ductal carcinoma in situ. *Cancer Research*, 62:6740–9.
- Yam, L.T. 1974. Clinical significance of the human acid phosphatases. Review. *American Journal of Medicine*, 56:604–16.
- Yamanaka, H., Matsuda, Y. and Tanaka, M. et al. 2000. Serum matrix metalloproteinase 3 as a predictor of the degree of joint destruction during the six months after measurement, in patients with early rheumatoid arthritis. *Arthritis and Rheumatism*, 43:852–8.
- Yamashita, M. and Fenn, J.B. 1984. Electrospray Ion-Source—Another Variation on the Free-Jet Theme. *Journal of Physical Chemistry*, 88:4451–9.
- Yasui, K., Kanda, H. and Iwanami, T. et al. 2003. Increased serum concentration of urinary trypsin inhibitor with asthma exacerbation. *European Respiratory Journal*, 22:739–42.
- Yates, J.R., Eng, J.K. and McCormack, A.L. et al. 1995. Method to Correlate Tandem Mass-Spectra of Modified Peptides to Amino-Acid-Sequences in the Protein Database. *Analytical Chemistry*, 67:1426–36.
- Yazawa, N., Kikuchi, K. and Ihn, H. et al. 2000. Serum levels of tissue inhibitor of metalloproteinases 2 in patients with systemic sclerosis. *Journal of the American Academy of Dermatology*, 42:70–5.
- Yocum, A.K., Yu, K. and Oe, T. et al. 2005. Effect of immunoaffinity depletion of human serum during proteomic investigations. *Journal of Proteome Research*, 4:1722–31.
- Yoshihara, Y., Obata, K.I. and Fujimoto, N. et al. 1995. Increased Levels of Stromelysin-1 and Tissue Inhibitor of Metalloproteinases-1 in Sera from Patients with Rheumatoid-Arthritis. *Arthritis and Rheumatism*, 38:969–75.
- Yousef, G.M., Polymeris, M.E. and Grass, L. et al. 2003. Human kallikrein 5: A potential novel serum biomarker for breast and ovarian cancer. *Cancer Research*, 63:3958–65.
- Zhu, K., Kachman, M.T. and Miller, F.R. et al. 2004. Use of two-dimensional liquid fractionation for separation of proteins from cell lysates without the presence of methionine oxidation. *Journal of Chromatography A*, 1053:133–42.
- Zissimopoulos, A., Petrakis, G. and Stellos, K. et al. 2006. Procollagen-I, collagen telopeptide I, CEA, CA 15–3 as compared to bone scintigraphy in patients with breast cancer. *Hellenic journal of nuclear medicine*, 9:60–4.