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# Developing Novel Methods for Protein Analysis and Their Potential Implementation in Diagnosing Neurological Diseases

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## 1. Introduction

The objective of this paper is to introduce a new chapter in protein analyses and profiling in general, and to discuss on the potential practical implementation in detecting neurological diseases. A need for such a research occurs due to the arguable fact that new techniques and methods for protein analyses have been developing, and only a few have actually been implemented in routine clinical practice. This study is a part of a larger effort to develop methods in order to understand protein diversity and specificity of a human proteome in health and disease. Primarily, we will introduce and discuss some standard and already implemented techniques in clinical practice, and further we will focus on the new methods for cerebrospinal fluid (CSF) analysis as a primary biological media for study of neurological diseases.

### 1.1 Proteins in neurological diseases

Neurological conditions in patients occur as a result of a structural or biochemical abnormality in the brain, spinal cord or in the nerves leading to and from them. After the onset of symptoms, additional parameters are analyzed in order to distinguish between different, so called, patterns and contribute to the diagnosis of a disease. Protein profiling has been introduced in clinical practice 100 years ago (W. Bruno 1956; Vesterberg 1989). Cerebrospinal fluid is the media used for such investigations, due to its close proximity to the brain; therefore being a biological fluid that can potentially obtain first-hand information about the potential causes of the induced change. Changes usually occur either in the CSF physical appearance, or can be manifested through the intrinsic characteristics – mainly the protein content and composition. Protein profiling in neurological diseases, therefore,

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contributes to the overall clinical diagnosis. However, in order to acquire the complete clinical state in a patient one must include comparative protein analysis in the other biological fluids (serum, plasma or urine). Since proteins in CSF are derived from the plasma, changes in the composition or concentration in either of these media will result in changes in the other (Andersson, Alvarez-Cermeno et al. 1994). Total protein concentration reveals only little about the function of blood-brain barrier and exploits changes only after the onset of symptoms related to a neurological disorder. Also, there are neurological disorders, among others multiple sclerosis, which are characterized with normal or moderately increased total protein concentration (although changes in the individual protein concentration occurs) (Link and Muller 1971). This serious neurological disorder, and many others, mostly related to demyelization processes in the CNS; do not exhibit changes in total protein concentration (Hein Nee Maier, Kohler et al. 2008). Other neurodegenerative disorders such as Alzheimer disease (AD), Parkinson disease (PD), or amyotrophic lateral sclerosis (ALS) have different causes and affect different regions of the nervous system, and are characterized by abnormal accumulation of protein aggregates and organelles along the axon due to disruption of axonal transport. As a consequence, an early pathogenic event leading to the demise of neurons occurs (Chevalier-Larsen and Holzbaur 2006; De Vos, Grierson et al. 2008). This remark only contributes to the importance of analyzing proteins and protein profiles in such diseases.

In order to achieve our goal and to be able to correlate proteins to certain disease, we must first address to understanding the origin of the complexity of the proteome in human cells and body fluids. The importance of studying cells at the proteome level is underscored by the difficulty in predicting protein characteristics from genomic sequence data alone. These characteristics include post-translational modifications, subcellular distribution, stability, biomolecular interactions, and function. In contrast to DNA and RNA, proteins can be modified by phosphorylation, glycosylation, acetylation, nitrosation, poly(ADP ribosylation), ubiquitination, farnesylation, sulfation, linkage to glycosylphosphatidylinositol anchors, and SUMOylation. In total, there are about 300 different posttranslational modifications that have been reported. These modifications can profoundly affect protein conformation, stability, localization, binding interactions, and function (Aebersold and Goodlett 2001). Separate media further complicate the application of protein analyses. Cerebrospinal fluid is a hurdle media for protein analyses for one basic reason - limited amount of starting material that is often insufficient to carry out all the analysis required. Additional reasons for the difficult analysis of body fluid proteome is their large dynamic range reflected by the presence of very abundant proteins like albumin, and in the case of CSF, minute quantities of brain-derived proteins (Anderson and Anderson 1998). Further complicating the analysis of CSF is the possible infiltration of serum proteins that is caused by a leaky blood-brain barrier that is especially pronounced in patients with brain disorders. As a consequence, it is often impossible to know if a protein that is found in CSF is derived from the brain or serum. And not only proteins fitting in a specific family, but the separate protein fractions, and moreover, the individual proteins and even protein variants, produce useful information about the presence of all types of disorders. Considerable fact is that additional modifications in proteins occur after translation of the information written in the genes. Having this remark in mind, it is obvious that the proteome is practically more related to the phenotype of an individual, and hence, protein profiling will result in the most precise understanding of disease mechanisms as well as the

molecular effects of drugs (Turck, Maccarrone et al. 2005). The ultimate goal of proteomics in medicine, and its sub disciplines is therefore, to provide quantitative and qualitative data of sample proteins that reflect a certain phenotype, disease state or a response to disease treatment.

## 1.2 Methods for protein analysis

Analyzing proteins as a diagnostic parameter in clinical practice was introduced with the development of the traditional techniques that combined methods based on protein precipitation and colored reactions. Since the introduction of electrophoresis in routine protein profiling in patients with neurological diseases, the main goal is developing novel methods for protein analyses that will offer more information and onset into the intrinsic protein characteristics. In recent years, almost every approach used to describe a method or technique applied to study protein diversity is termed "proteomics" (Nedelkov 2005). Analyses in proteomics, especially when human plasma proteome is considered, are technically challenging because the circulating proteome is a complex mixture of diverse proteins that spans approximately 10 orders of magnitude in concentration (Anderson and Anderson 2002). Routine techniques in protein profiling include mainly electrophoretic methods and can be distinguished as: gel-based proteomics approaches, fractionation or separation with other media and support material, which are based toward the identification of abundant proteins. For these types of assays, experimental designs need to involve enrichment strategies such as immunoisolation of protein complexes of interest to reduce sample complexity and increase sensitivity of detection.

The development of non-gel-based approaches for quantitative proteomics, together with advances made to detect posttranslational modification guide progress toward delineating the mechanisms involved in nerve regeneration and degeneration dysfunctions. Such neuroproteomics approaches lay the foundation for further detailed functional studies (Sun and Cavalli 2010). Both of these approaches relay on a two stage mechanism of protein analysis: *protein separation* followed by *identification and analysis* (Turck, Maccarrone et al. 2005). Classical proteomic approaches employ fractionation on the protein level with the help of 2D-PAGE. This technique produces high resolution protein separations resulting in the display of potentially thousands of protein spots. Identification of these spots is rather difficult and requires additional informatics-based approaches in order to derive relevant conclusions. Some of the limitations of 2D-PAGE have currently been overcome with the implementation of 2D-DIGE (Difference Gel Electrophoresis) where fluorescent dyes are used to distinguish between proteins from control samples and one from individuals with certain disease (Alban, David et al. 2003). It is therefore evident that at this point in proteomic technology development the broadest proteome coverage comes from a combination of multidimensional fractionation, advanced instrumentation and additional computational techniques.

Introducing mass spectrometry in protein analysis contributes towards overcoming several of the disadvantages common for standard techniques. The most efficient and most widely used protein identification method in proteomics are MALDI-TOF-MS, SELDI-TOF-MS, ESI-MS/MS and other methods (Lahm and Langen 2000; Issaq, Veenstra et al. 2002). Using shotgun mass spectrometry approach proteins are digested by specific enzymes into small peptides and analyzed on-line by MS. This technique allows low abundant proteins to be

identified despite the presence of high abundant proteins in the samples. Further advantage was done by Aebersold and co-workers (Gygi, Rist et al. 1999). They have developed a novel isotope-coded affinity tag (ICAT) strategy that permits the stable-isotope labeling of cysteine residues in proteins, thus facilitating a quantitative global analysis of differences in protein expression. A more common approach is combining chromatography separation (usually liquid chromatography, since proteins are high molecular mass compounds) with mass spectrometry detection. Chromatographic methods reduce the complexity of protein mixtures on the basis of different binding principles, and every approach adds a unique resolving power. The proteins are usually separated on the basis of affinity, charge, hydrophobicity, or size (Fountoulakis, Takacs et al. 1999; Takacs, Rakhely et al. 2001). The choice of the chromatographic method best-suited to fulfill the experimental requirements is essential for the success of the experiment (Fountoulakis and Takacs 1998). There are several methodologies that use liquid chromatography (mainly HPLC) as a tool for protein separation. Basic LC-MS/MS techniques generally employ ion-pair reversed-phase chromatography followed by electrospray ionization and mass spectrometry analysis. Depending on the ionization technique, LC-MS/MS coupling can be done either “on-line” (where HPLC is directly coupled to the ESI source of the tandem MS) or “off-line” (where, after being resolved in the HPLC process, samples are deposited directly onto a MALDI target, mixed with corresponding matrix and analyzed via MALDI-TOF MS) (Mallick and Kuster 2010). In the last 15 years combined approaches coupling immunoaffinity separation and mass spectrometry detection have been implemented (Nedelkov 2006). Also, a new approach that is considered promising in the efforts of proteome analysis is the use of aptamers as an alternative class of reagents to use for highly multiplexed protein measurements (Brody, Gold et al. 2010).

All of the above mentioned protein composition studies can be extended to two main approaches – analyzing either individual proteins or obtaining protein profile (“group” protein analysis).

### 1.2.1 Protein profiling approach

In the “group” protein analysis, termed as protein profiling, our main focus is to detect and identify as many proteins as possible, perform qualitative analyses, and obtain protein pattern for each sample analyzed (Fig.1/a).

Electrophoresis techniques have been the hub of laboratory testing and a component of almost all diagnostic panels (McCudden, Voorhees et al. 2010). A rather intriguing fact is however, that electrophoresis has come only a short way in advancing during the years, when used for protein profiling in patients with neurological diseases. Since it was implemented as a method in routine clinical practice, electrophoresis has come only a short way in actually progressing. In routine clinical practice discontinuous polyacrylamide gel electrophoresis (DISC-PAGE) is usually applied for determining the protein profile (Monteoliva and Albar 2004). More detailed information can be obtained using denaturing sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Hu, Huang et al. 2004). In diagnosing some specific neurological diseases, isoelectric focusing (IEF) and immunofixation can be used to confirm the presence of specific oligoclonal, monoclonal or polyclonal immunoglobulin. Determination of these protein components is in many cases necessary to delineate between differential diagnoses. By combining IEF with gel electrophoresis, two dimensional gel electrophoresis is created (2D-GE), a technique, which



is probably the most frequently used methodology for protein profiling. When applied into clinical practice, this methodology is used to analyze the complete proteome present in tissue samples, biological fluids and cell lysates. The result is an electrophoregram (for 1D-GE techniques) or pattern plot (for 2D-GE) fingerprinting the specific protein composition in the analyzed sample.

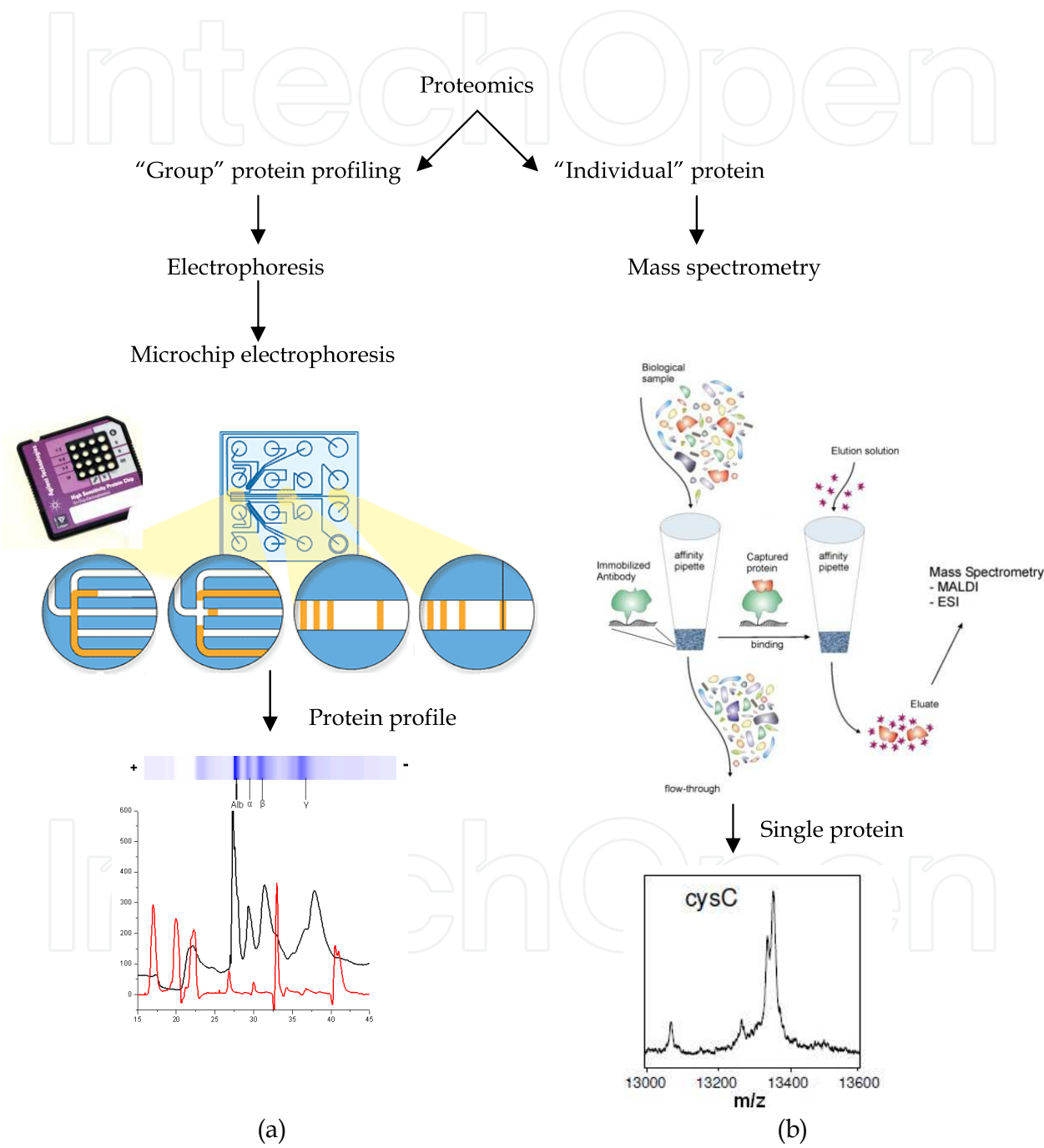


Fig. 1. Methods for protein analyses a) protein profiling using microchip electrophoresis, b) single protein analysis using mass spectrometry immunoassay (MSIA)

The general goal when using these types of qualitative group approaches is obtaining complete protein profiles both, from control samples and samples from patients with different (neurological) diseases. By comparing the obtained protein profile from the control subject (normal reference sample) to a profile originating from a patient we are able to construct a certain pattern based on the principal of similarity analysis (Trenchevska, Aleksovski et al. 2009). In our work, using this approach, we have been able to implement microchip electrophoresis method to distinguish a specific pattern for several neurological diseases (multiple sclerosis, blood-brain barrier dysfunction diseases, inflammatory conditions and autoimmune disorders) which will be discussed further in details.

### 1.2.2 Single protein assays

Although, the classical proteomics efforts are geared towards the analysis of the protein constitutes in both CSF and serum (Maccarrone, Birg et al. 2004; Maccarrone, Milfay et al. 2004), alternative strategies have been established for the identification of novel disease markers for neurological disorders. When it comes to protein analyses, often the concentration plays a significant role in contributing to diagnosis (example – determination of IgG concentration in multiple sclerosis). However, quantitation of all proteins in a sample is a Herculean task itself, due to the fact that the outcome of a proteome profiling experiment results in a content-rich and convoluted data that require powerful approaches to discover the subtle differences between the healthy and diseased samples (Figure 1a). In this regard, mass spectrometry has found its way as a sensitive technique for quantitative protein analyses. Although mass spectrometry has been a golden standard for proteomics from the very beginning, its complexity, cost, maintenance and other analytical characteristics are the underlying reason why this technique is rarely used in routine clinical practice (Rabilloud 2002). A step forward has been done with introducing immunoaffinity approaches (Engvall and Perlmann 1971; Ritchie 1999; Craig, Ledue et al. 2001; Yan, Lee et al. 2004). The pilot work has been done with introducing ELISA as a technique for quantification single proteins from biological samples. This technique is currently the most common abandoned method used in routine screening of known proteins and is commonly used as a laboratory test for detecting antibodies or rapidly screen and quantify antigens in biological samples. Several advantages have made this technique favorable; it follows simple sample preparation, it does not require specific conditions and data acquisition is relatively simple (Dufva and Christensen 2005). However, it can only be applied in analyzing known proteins, and, what is more, a suitable antibody must exist for retrieving the antigens from the sample. Also, ELISA based methods determine the total protein concentration and are lacking the ability to detect, identify or quantify protein variants, post-translational modifications or point mutations. Therefore, advances in this area are directed in developing assays that use combined techniques in order to obtain more detailed information about each protein intrinsic characteristics. Combined techniques have been conceptualized in the mid-nineties, via the SELDI (Hutchens and Yip, US patent No.5,719,060) (Engwegen, Gast et al. 2006; Poon 2007), and later by MSIA (Nelson et al., US patent No.6,974,704)(Nelson, Krone et al. 1995; Krone, Nelson et al. 1996) approach. The newly developed methodologies are the platform for biomarker discovery assays. These assays target “individual” proteins and use specific antibody towards the protein of interest. These specific proteins from the proteome are considered to be potential diagnostic markers for certain neurological diseases. MSIA methodology, which will be further discussed in details, bridges between the selectivity that can be obtained with immunoassays and the specificity of mass spectrometry detection (Fig. 1/b).

2. Electrophoresis in protein profiling

Identification and determination of different types of proteins play an important role in medical diagnosis. Conventional electrophoresis methods are well known for protein detection and analysis in several biological fluids, primarily blood serum and plasma and cerebrospinal fluid. CSF analysis, coupled with other methods, remains the cornerstone of diagnosis of various neurological diseases, including multiple sclerosis (Reiber, Otto et al. 2001; Sindic, Van Antwerpen et al. 2001). Even though electrophoresis is practically the basic technique in protein profiling, both in routine and scientific clinical practice, it provides only semi-quantitative information about the concentration of a certain protein. Regarding the protein profiling, these techniques still offer significant amount of information, especially about the abundance of specific protein classes, such as immunoglobulins. This is of great importance in demyelization neurological diseases. Additional criteria for this and other types of neurological diseases are presence of oligoclonal immunoglobulins. For detection of oligoclonal IgG in serum and unconcentrated CSF, several techniques can be used, primarily isoelectric focusing (IEF) combined with polyethylene-enhanced gel immunofixation and silver staining, CSF:serum quotient diagram and body index (Tourtellote, Povin et al. 1980; Sandic, Monteyne et al. 1994; Mitrevski, Stojanoski et al. 2001). Combining IEF and PAGE, 2D-GE methods are developed, optimizing techniques for detailed protein mapping, as mentioned previously. Introducing bioinformatics tools is further implemented in order to obtain more information from the protein profile. This approach necessitates usage of protein standards in each run. Using additional computational programs and following the advances in the field of bioinformatics, more information can be obtained from each protein profile spectra.

2.1 Conventional DISC-PAGE and IEF

Discontinuous polyacrylamide gel electrophoresis (DISC-PAGE) is one of the most widely used techniques for analytical separation of proteins and peptides. DISC-PAGE separations are based upon the intrinsic protein charge-to-mass ratio and the molecular mass of the protein (Chiou 1999). Polyacrylamide gels are thermostable, transparent, strong and chemically inert, can be prepared in different pore concentration and are non-ionic, therefore making them convenient for protein analyses (Ornstein 1964). Presented in Fig.2 are electrophoregrams obtained from normal samples (Fig.2/a) and sample from patient with blood-brain barrier dysfunction (Fig.2/b) using DISC-PAGE classical method, implemented in routine clinical practice. Each protein zone can be identified and quantified according to the peak area and when compared to standard. The obtained results for the basic parameters in protein profiling using DISC-PAGE are presented in Table 1.

Protein fraction	Optical density	Peak area	Band %	Rf	Protein fraction	Optical density	Peak area	Band %	Rf
Prealbumin	44.97	1.984	2.31	0.071	Prealbumin	52.91	4.026	4.72	0.095
Albumin	240.97	5.332	46.92	0.201	Albumin	207.6	6.138	37.97	0.299
α - globulins	45.73	1.984	3.58	0.368	α - globulins	70.89	2.640	5.93	0.442
β1 -globulins	52.44	1.736	4.03	0.461	β1 -globulins	187.8	4.686	22.01	0.620
β2 -globulins	186.52	3.534	20.27	0.594	β2 -globulins	133.2	1.650	7.45	0.694
γ -globulins	106.48	2.046	7.85	0.683	γ -globulins	158.8	4.092	21.93	0.817

a)b)

Table 1. Characteristic parameters for protein profile obtained using DISC-PAGE from a) control group (normal sample) and b) sample from patient with neurological disorder.



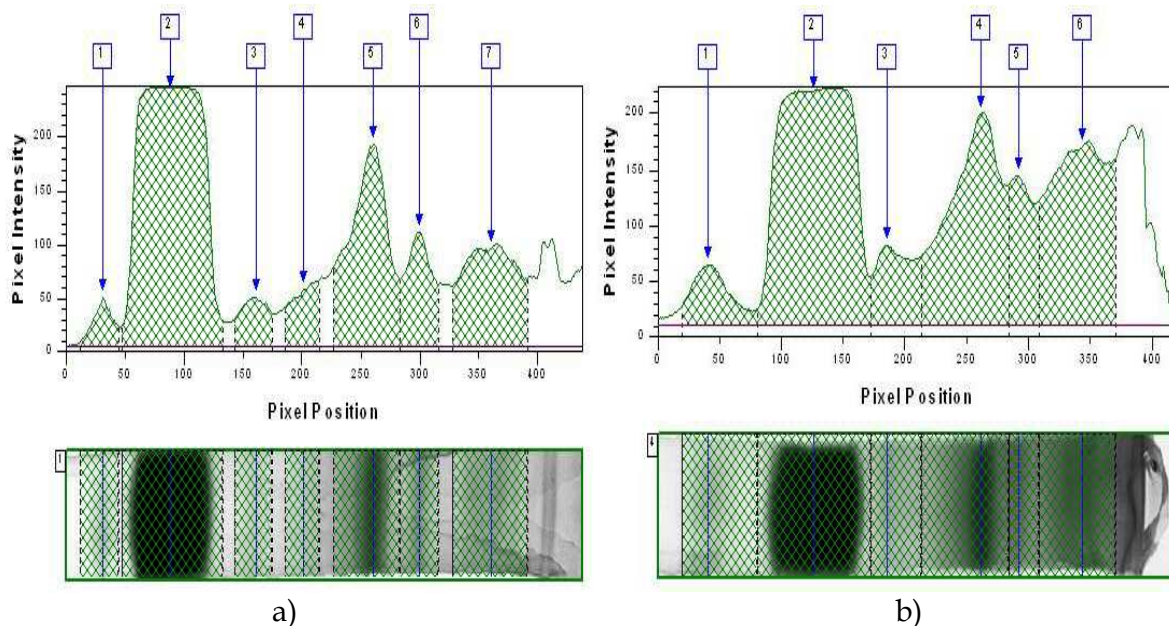


Fig. 2. Electrophoregrams presenting protein profile from CSF samples obtained from a) control group (normal sample) and b) sample from patients with dysfunction in blood-brain barrier

## 2.2 Miniaturization in electrophoresis

The miniaturization processes have been implemented in all spheres of protein research, resulting in newly developed techniques that follow the concept of microchip electrophoresis. The onset of miniaturization electrophoresis dates from 1954 when the first microchip used in the semiconductors structure was created (Manz, Graber et al. 1990). It took more than 20 years to introduce the first microchip in gas chromatography analyses in 1975. However, the great leap in applying miniaturization in biomedical sciences and research was in the beginning of 1990s, when the first commercial microchip was constructed (Woolley and Mathies 1994; Keramas, Perozziello et al. 2004; Balslev, Jorgensen et al. 2006; Geschke 2006; Geschke 2009). This pioneer technique was introduced for the first time in genomic and DNA analysis in the mid-1990s, when the first commercially available microchip for DNA and RNA analyses was produced. Protein microchips were used for the first time in 1999, when 6 fluorescent labeled proteins with molecular mass ranging between 9 and 116 kDa were successfully separated on a microchip in less than 35 s (Yao, Anex et al. 1999). Another step forward was done in 2001 when a method for fluorescent labeled protein-SDS complexes (where proteins were non-covalently bounded to the denaturing agent) separation was optimized (Jin, Giordano et al. 2001). The first commercially available microchip was known as LabChip and produced by Bousse et al. (Bousse, Mouradian et al. 2001) in Caliper Technologies, Mountain View, CA. At the same time, Agilent 2100 Bioanalyzer appears on the market, as the first commercial instrument for microchip electrophoresis separation platforms. The preliminary results using microchip technology indicated great potential, especially for implementation in routine clinical practice, which was the basic goal of developing such methods.

## 2.3 Use of lab-on-a-chip electrophoresis in protein profiling

Miniaturized lab-on-a-chip electrophoresis is a novel technique in protein profile analyses, introduced with the development of Agilent 2100 Bioanalyzer (Woolley and Mathies 1994). The microchip system includes system of microchannels through where gel is rushed by applying pressure. The gel contains fluorescent dye which serves as a label; therefore protein detection is done by fluorescence analysis. By running the chip, proteins can be separated according to their size, and, as a result, protein profile can be obtained. Looking into the basics of lab-on-a-chip electrophoresis it can be noted that the basis of analysis is denaturing SDS-PAGE; therefore proteins separate only according to their molecular mass and not their charge. The microchip platform consists of a system of micro-channels ranging in width from 20 to 100  $\mu\text{m}$  and height of 10-25  $\mu\text{m}$ . Protein separation is performed in a solid media (polyacrylamide gel) both in native and denaturing conditions. Gel preparation is performed in a buffered solution, therefore presenting the necessary ions for the protein separation. Created potential difference indicated protein movement through the system of channels. During their passing through the system, proteins form an affinity bond to a fluorescent dye, therefore becoming visible for detection. Using a highly sensitive fluorescent detector, signals from the proteins are noted and protein profile can be obtained from a single sample in less than one minute (Trenchevska, Aleksovski et al. 2009). LiF detector is commonly used due to its high selectivity and sensitivity – it can detect fluorescein concentration in 300 fM quantities (Ocvirk, Tang et al. 1998). This detection method is actually the critical point that allows protein analysis in such a short time.

Lab-on-a-chip protein electrophoresis employs several advantages when compared to other types of electrophoresis. It is significantly faster, shortening the time required per analysis, which is of great importance, especially for application in clinical laboratories where a large cohort of samples is analyzed on daily basis. Further, the amount of sample required for the analysis is only 5  $\mu\text{L}$ , which is convenient especially when proteins in CSF are analyzed. Also, unlike traditional electrophoresis techniques, lab-on-a-chip method uses fluorescent detector, which has higher sensitivity. The microchip is designed in a specific manner depending on the analysis required, whether it is DNA, RNA or protein. Regarding the proteins in neurological diseases, using this technique under optimized conditions, we were able to analyze both low molecular mass proteins ( $\text{MW} < 50\,000\text{ Da}$ ) and high molecular mass proteins ( $50\,000\text{ Da} < \text{MW} < 250\,000\text{ Da}$ ) (Auroux, Iossifidis et al. 2002).

### 2.3.1 Method development

In order to obtain better resolution and assay characterization, several modifications can be made. Lab-on-a-chip electrophoresis platform does not usually allow many modifications; however, several optimizations can contribute to obtaining better results. This technique so far is very well implemented in DNA and RNA assays, but its application in protein analyses is rather limited for several reasons. Lab-on-a-chip electrophoresis system uses fluorescent detector, therefore require fluorescent dyes for protein labeling. These types of dyes, however, do not form covalent bond with the labeled protein; therefore require longer incubation times in order to allow high-efficient specific binding (Floriano, Acosta et al. 2007). Having this in mind, Giordano et al. introduced a concept that significantly reduces these effects. It was noted that using SDS as denaturing agent incorporated in the gel, besides uniformly charging the proteins, favors their binding with the fluorescent dye,

further producing higher signal to the detector (Christodoulides, Floriano et al. 2007; Floriano, Acosta et al. 2007; Sloat, Roper et al. 2008). When biological samples are analyzed, further problems in developing the assay occur, due to the intrinsic complexity of the specimen itself. In order to obtain the protein profile using lab-on-a-chip electrophoresis, several conditions must be optimized.

**Sample preparation for lab-on-a-chip electrophoresis** - prior to analysis samples were stored at -20°C for two weeks. Serum and cerebrospinal fluid samples from control group (designated as normal according to their cross-reactivity toward the usual infectious agents and donor information), and from patients with different neurological diseases (primarily multiple sclerosis, than blood-brain barrier disfunction and other demyelization diseases) were used for the analyses. Samples were prepared by combining 84 µL sterile water, 4 µL sample and 2 µL denaturing agent (BME). Within each run, protein standard (ladder) was used for calibration and protein fraction identification.

**Optimization of denaturing agent concentration** - denaturation agents contribute to easier formation of protein-fluorescent dye complex therefore resulting in higher efficiency of the detector. In the sample optimization steps, we have incubated the samples with different concentration of denaturing agent beta-mercaptoethanol (BME). Sample to denaturing agent ratio used for the analysis were - 1:1; 1:1.5; 1:2; 1:2.5; 1:3; 1:3.5 and 1:5. The results have been previously published and have shown optimal protein to BME ratio of 1:3.5 (Trenchevska, Aleksovski et al. 2009).

**Optimization of incubation temperature** - Temperature is an important parameter in protein analyses due to its effect onto the protein structure (denaturation) and affinity towards the fluorescent dye, which, in turn can cause increase of the signal in the detector. When optimizing incubation temperature, samples were incubated in water bath at five different temperatures - 5°C, 22°C (room temperature), 37°C (normal body temperature), 60°C and 90°C. It was noted that optimal incubation temperature is 90°C (Trenchevska, Aleksovski et al. 2009).

**Assay procedure** - After sample preparation and following incubation under the optimized conditions, the microchip was placed in the bioanalyzer and run. In the process of electrophoretic separation in the bioanalyzer, the electric current provides ideal conditions for staining and destaining within 45 s per sample analyzed. During the separation process, the sample is initially rushed through the pores in polyacrylamide gel, and then allowed to bind with the fluorescent dye incorporated into the microchannels. When using denaturing conditions, further SDS binding causes proteins to gain net negative charge, therefore separating proteins in a sample only according to the molecular mass.

**Results processing** - After completion of the separation process, complete numerical analysis is necessary in order to obtain more information from the protein profile for each sample. Analyses regarding statistical parameters are important in clinical practice, because, in a time scale, they provide useful information about the distribution of specific protein fractions and their relation to a specific disease. Collecting such large amount of data is enabled due to the automation and development of microcomputers. Using Web-based databases, data acquisition, manipulation and computation for electrophoresis protein pattern recognition is further performed using standard statistical signal analysis (Spirovski, Stojanoski et al. 2005). In this context, the next promising area of interest is cluster analysis,

along with artificial neural networks, bioinformatics techniques that have been successfully applied to various areas in medical practice, as diagnostic systems (Vogt and Nagel 1992; Jerez-Aragones 2003), biomedical analysis (Lisboa 2002) and neuroimaging (Aizenberg, Aizenberg et al. 2001).

In our work, using the optimized lab-on-a-chip electrophoresis method, we were able to obtain the protein profile from patients with multiple sclerosis, neurodegenerative diseases and blood-brain dysfunction and further perform statistical analysis on the obtained data.

### **2.3.2 Application in clinical practice (profiling in patients with different neurological disorders)**

Lab-on-a-chip electrophoresis was used in order to confirm the “group” aspect approach in protein profiling that is of great significance in every day clinical practice. Protein profiles from patients were classified in one of the four major types according to the distribution of the five basic protein zones: prealbumin, albumin,  $\alpha$ -globulin,  $\beta$ -globulin and  $\gamma$ -globulin – normal (N), transudative (T), gamaglobuline ( $\gamma$ ) and transudative-gamaglobuline (T $\gamma$ ). When compared to protein standard, in each of the zones, different proteins can be identified. This identification, however, offers only qualitative details, and cannot be used in quantifying separate proteins with high accuracy. We have previously reported using the optimized lab-on-a-chip electrophoresis to analyze serum and cerebrospinal fluid samples from control group and patients with neurological diseases (Trenchevska, Aleksovski et al. 2009). It was noted that using this advanced technique, protein profiles can be used to obtain satisfactory qualitative analyses, therefore contributing to precise clinical diagnosis. In patients with multiple sclerosis, for example, characteristic electrophoretic patterns were noted, characterized by high IgG concentration (which is evident in 46% of all MS cases, where intrathecal IgG synthesis occurs), and normal total protein levels (Fishman 1992; Daskalovska 2000). Examples of the electrophoregrams for both normal samples and samples from patient with multiple sclerosis are presented in Figure 3.

## **3. Mass spectrometry-based protein identification protocols**

Mass spectrometry has been used in clinical practice mainly for detection of small molecules (MW<1 kDa) aiming to detect inborn errors in metabolism, or monitoring toxicity, drug and doping abuse (Ahmed 2008). Introduction to mass spectrometry in proteomics was made possible in recent years thanks to the discovery of the so called soft ionization techniques (ESI and MALDI) that were recognized by the Nobel Prize in chemistry 2002 (Tanaka, Waki et al. 1988; Fenn, Mann et al. 1989). Using either ESI or MALDI, a molecular mass with a precision and accuracy of  $\pm 0.05\%$  or better can be achieved. This depends heavily on the purity of the protein sample, the relative size of the protein, the presence or absence of post-translational modifications, the resolution of the mass spectrometer itself and so on. Mass spectrometry has been broadly used in determination of protein amino acid sequence using either tandem mass spectrometry MS/MS sequencing of enzymatically derived peptide fragments of the original protein, or sometimes direct MS/MS on the intact protein. Using these approaches, post translational modifications such as protein phosphorylation, sulfonation, oxidation and terminal amino acid cleavage can be identified. Over the past few years, the sensitivity and specificity of



mass spectrometry coupled with liquid chromatography, have improved to a degree such that protein quantification can be derived from very complex mixtures (tissues, biofluids, cell lysates etc.). Recent publications contribute to the newly developed methods for protein characterization, identification and quantification, especially regarding low abundant proteins in biological specimen (Gygi, Rist et al. 1999; Kiernan, Nedelkov et al. 2006)

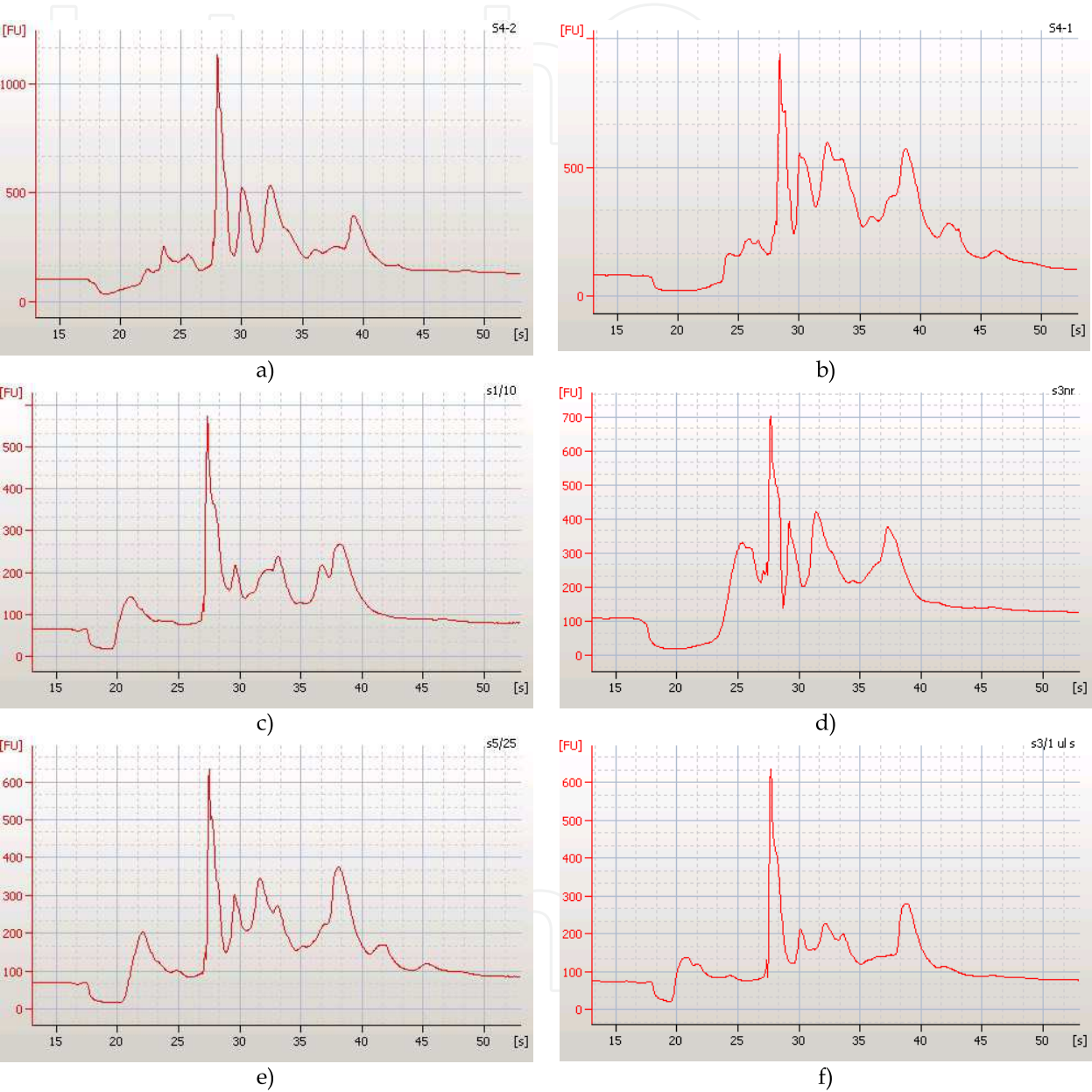


Fig. 3. Characteristic electrophoregrams obtained using lab-on-a-chip protein electrophoresis in samples from a) control group (normal sample); b) sample from patient with multiple sclerosis; c) sample from patient with polyradiculoneuropathy; d) sample from patient after ictus cerebri; e) sample from patient with blood-brain barrier disfunction and f) control sample. Noted in the electrophoregrams are differences in the protein distribution primarily in the  $\gamma$ -globulin region, which is expected in samples from patients with demyelination processes, where intrathecal IgG synthesis is dominant.



To use mass spectrometry for biomarker discovery in clinical proteomics is conceptually simple. What we do is compare obtained spectral peaks from specific protein or proteins in body fluids or tissue extracts in a diseased group, with those from the control group. These peaks correspond to potential biomarker molecules. MS biomarker discovery efforts have focused on four subsets of the proteome: (i) polypeptides and whole proteins in tissues or body fluids separated by electrophoresis or chromatography, with or without prefragmentation; (ii) enzymatic peptide fragments separated by HPLC and analyzed by ESI or MALDI; (iii) proteins in tissue or body fluids that are adsorbed on surface protein chips in a matrix, analyzed by SELDI and (iv) naturally occurring fragmented peptides in blood that represent low range of the plasma/serum proteome, analyzed by MALDI (van der Merwe, Oikonomopoulou et al. 2007). In our work, we mainly concentrate in analyzing intact proteins as potential biomarkers using one of the basic approaches – top down proteomic analysis.

### 3.1 Top-down proteomics analysis

As a tool for proteins and peptides separated by electrophoresis or chromatography, and with or without previous fragmentation, mass spectrometry has been applied in several different ways; the two approaches are top-down and bottom-up MS. Top-down MS is the direct analysis of proteins on the intact level. Bottom-up approach identifies proteins following enzymatic or chemical digestion of the sample resulting in the formation of much smaller peptide fragments. These smaller protein segments are much easier to analyze with low resolution MS instruments and therefore bottom-up peptide analysis is currently the most popular MS-based proteomics approach (Han, Jin et al. 2006).

The strength of top-down proteomics approach lies in the direct detection of the native molecular mass of biological protein species. Mass spectrometry detection provides information for the native protein, and also for the natively occurring small peptides, biologically generated protein cleavages, post-translational modifications or point mutations – all of which are postulated to be relevant in many diseases and other biological processes occurring in cells. Other major advantage of top-down approach is the simplified sample preparation that does not necessitate enzymatic or chemical digestion prior to analysis. Scientist using top-down approach in studying proteins are mainly oriented towards addressing clinical questions – using population screening in the complicated process of identification and validation of potential biomarkers (Whitelegge, Halgand et al. 2006).

Several techniques are favored in top-down proteomics research platform. SELDI-TOF MS is a widely used biomarker discovery method that combines the selectivity of chromatography and sensitivity of mass spectrometry detection. The major challenge for this approach is the requirement for off-line enrichment and purification of the selected biomarker candidates, followed by MS/MS identification using different MS platform (Reid and McLuckey 2002).

MALDI-TOF MS has also found its way in implementing in top-down proteomics quest for novel biomarkers. One of the disadvantages of the methods used in routine clinical biomarker discovery techniques is the complicated sample preparation which requires chromatographic or electrophoretic separation of the targeted protein. Immunoaffinity capture of a protein directly from a biological sample is a base method used in all immunoassays (such as ELISA). Therefore, by combining the selectivity of immunoaffinity capture and the specificity of mass spectrometry, a novel methodology has been developed in recent years– Mass Spectrometry Immunoassay (MSIA).

In our work we have developed several assays using MSIA approach. Using this novel platform we were able to analyze several proteins, some of which have been introduced as potential biomarkers for several neurological diseases. An introduction to the overall procedure and application of this technique will be introduced through the development of Cystatin C MSIA assay.

### 3.2 Mass spectrometry immunoassay (MSIA)

Mass Spectrometry Immunoassay (MSIA) is a novel approach that has been employed for both, qualitative and quantitative characterization of body fluid proteins. From the methodology point of view, it is a combination of today's predominant technology involved in routine clinical practice – immunoassay for targeted protein affinity extraction assessment and uses mass spectrometry detection (MALDI-TOF-MS) for achieving the specificity necessary for this type of analyses. This approach lacks the disadvantage demonstrated by the immunoassays – inability to detect protein variants, post translational modifications or mutations. On the contrary, MSIA gives onset into the detailed intrinsic protein characteristics, both for high or low abundant proteins. One great advantage of this hybrid methodology is the simplicity of protein extraction from complicated biological samples. The basic concept of the analysis includes a two-step approach; first, proteins are captured by a principle of microscale affinity by aspiration/dispense cycles on an antibody-derivatized affinity pipette, and in the second step eluted proteins are subsequently analyzed using MALDI-TOF-MS (Fig.4)

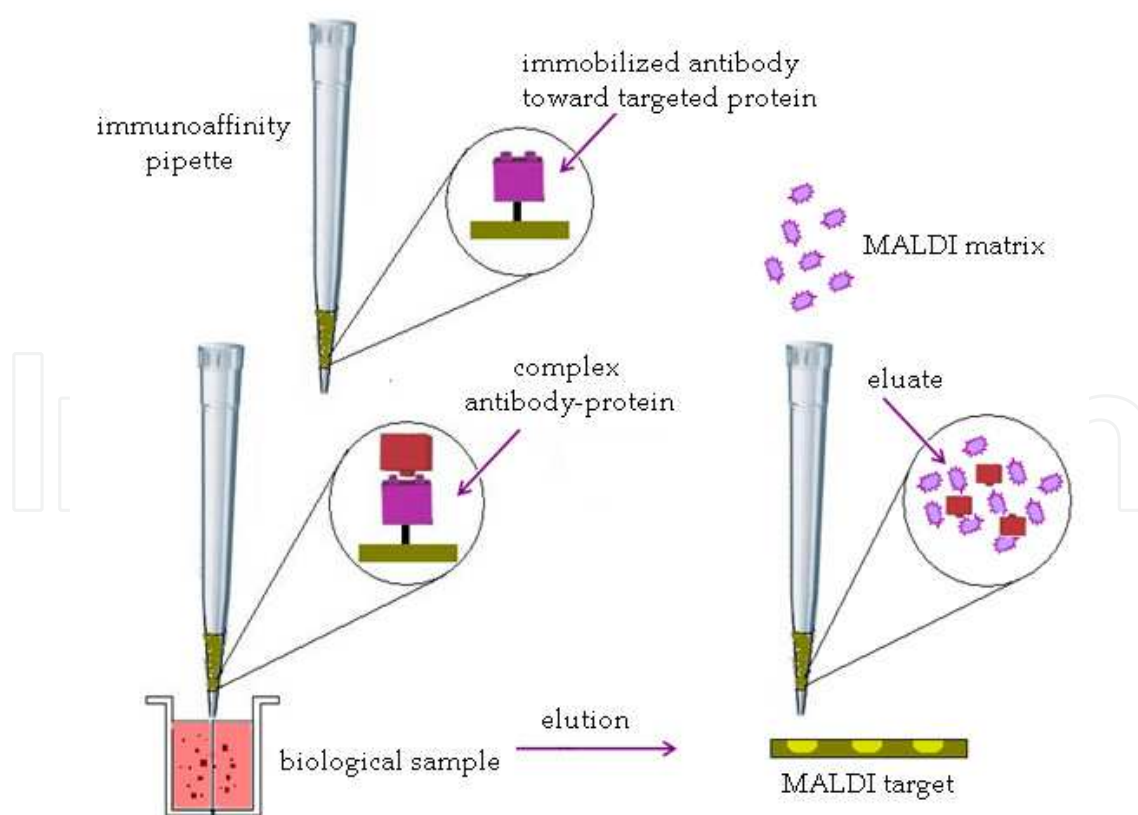


Fig. 4. Mass spectrometry immunoassay scheme

In the initial stage of this method’s development, agarose beads derivatized with an affinity ligand (e.g. polyclonal antibodies) were used to create a  $\mu\text{L}$ -scale column inside a micropipette tip (Krone, Nelson et al. 1996). These columns are further derivatized with a corresponding antibody toward the targeted protein. Additional activation of the beads is necessary prior to the derivatization process. A summary of the consecutive steps in activation and derivatization of the affinity pipettes is presented in Figure 5.

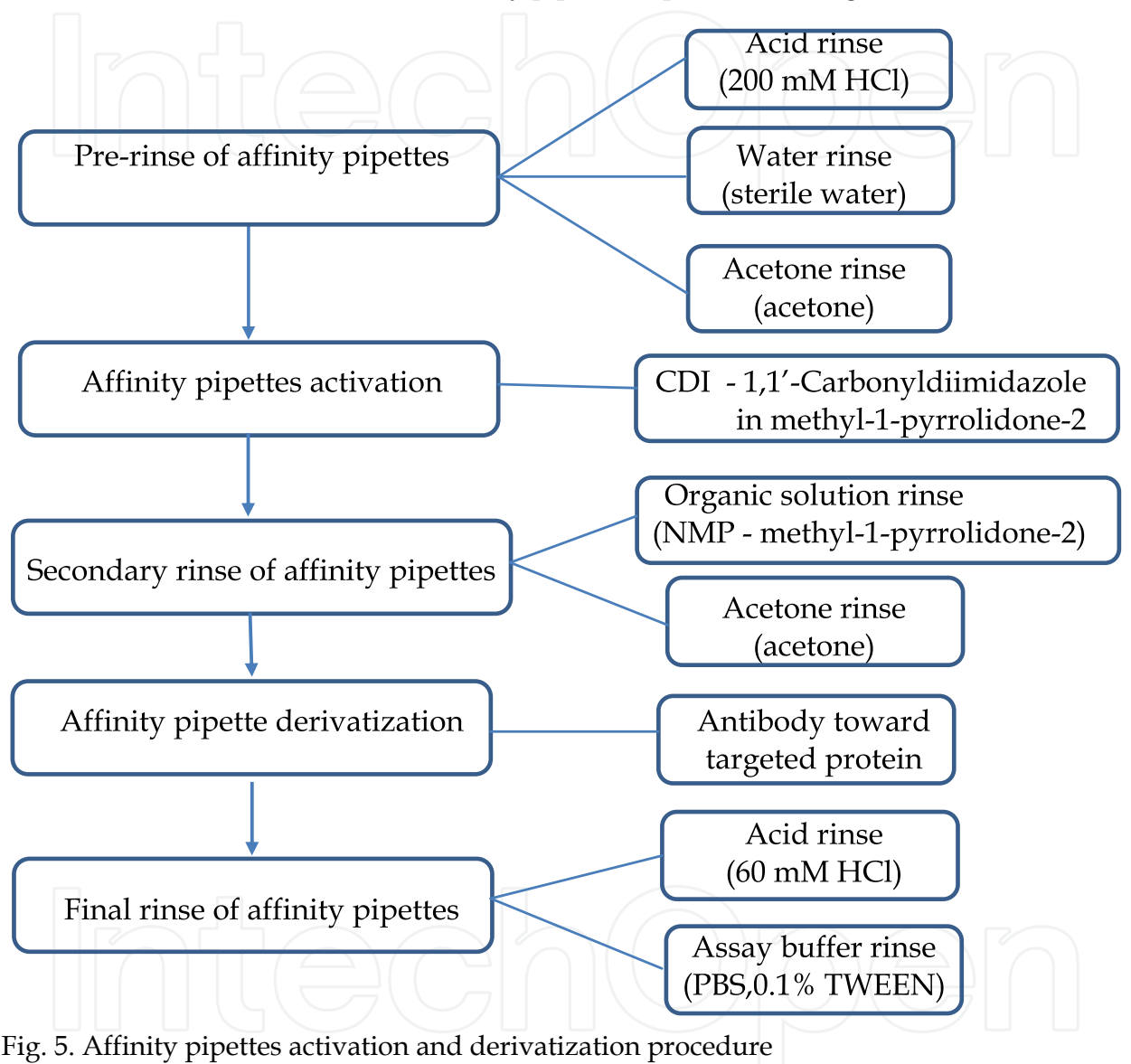


Fig. 5. Affinity pipettes activation and derivatization procedure

When the affinity pipettes are coated with the corresponding antibody, further activation of the surface is needed in order to favor protein binding. Using Multimek 96 automated 96-channel pipettor (Beckman Coulter, Brea, CA, USA), the antibody coated affinity pipettes were rinsed with buffer (PBS, with 0,1%TWEEN) in 10 aspiration/dispense cycles. Next, pipettes were immersed into a microplate containing the sample (serum, plasma, urine or CSF). Following additional buffer rinse (in order to elute the non-specific bounded proteins or other constitutes origin from the sample) and two water rinse cycles, retrieved targeted protein on the affinity pipette is ready for discharge. Figure 6 illustrates the steps in this analysis.

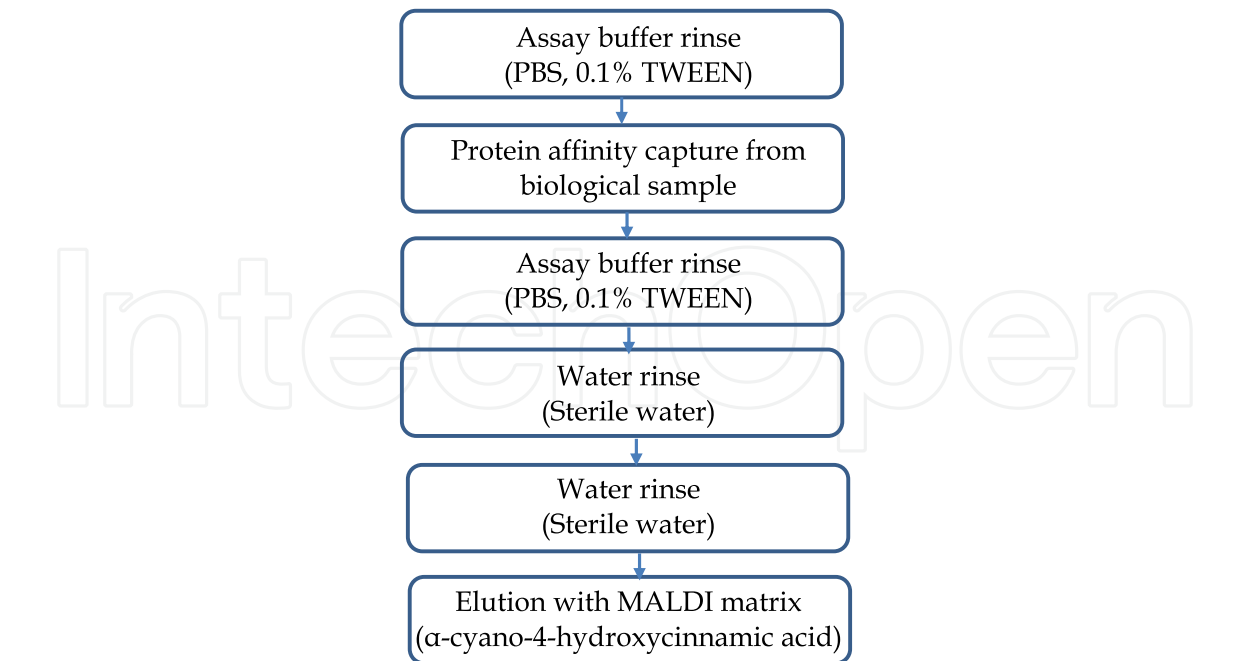


Fig. 6. Steps in MSIA protein analysis

Preparing for the second step of detection – the mass spectrometry detection, captured protein is eluted directly on the MALDI target, by 6 µL aliquots of MALDI matrix (α-cyano-4-hydroxycinnamic acid in aqueous solution containing 33% (v/v) acetonitrile and 0.4% (v/v) trifluoroacetic acid). Using this acidic matrix, captured protein are dissociated from the antibodies and eventually dispensed onto the target. Following drying and visual inspection of the sample spots, linear mass spectra were acquired using delayed extraction mode with 1.7 kV draw out pulse, 200 ns delay and a full accelerating potential of 20 kV.

	MSIA	
	Qualitative MSIA	Quantitative MSIA
Primary goal	Population proteomic studies	Biomarker discovery and validation
Samples	Large cohort of samples	Control and disease samples spiked with reference protein
Assay development	Affinity pipettes immobilized with antibody toward targeted protein	Affinity pipettes immobilized with two antibodies – toward targeted protein and IRS
Immunoaffinity separation	Retrieval of targeted protein	Retrieval of targeted protein and the IRS
Mass spectrometry detection	MALDI-TOF MS	MALDI-TOF MS
Results	Detection of protein variants, post-translational modification and point mutation	Calculating concentration of native protein and protein variants
Additional analyses	Enzymatic digestion	/

Table 2. Differences between qualitative and quantitative MSIA approaches

Although introduced in the recent years, this technique has already been implemented in developing qualitative and quantitative assays for several protein and protein variants using different biological samples as medium. There is one basic difference regarding these two approaches (Table 2). When using MSIA platform for qualitative analysis, affinity pipettes are derivatized only with antibody towards the targeted protein; additional antibody must be fitted into the affinity micocolumn in order to retrieve another protein in the quantitative assay. In quantitative MSIA, affinity pipettes are derivatized with a secondary antibody, toward a protein termed as internal reference standard (IRS). Choosing the IRS is one of the critical steps in developing of the assay, due to the high criteria required for such a protein. An important prerequisite for an IRS is that it should not be present in human plasma or serum (or other biological fluids, as well), so that its spiked concentration in the analytical samples is always constant. Also, the signal on the mass spectrometer produced by the IRS should be in close proximity to the signal of the targeted protein, in order to be able to use the same MS acquisition parameters for both proteins. The goal in developing qualitative assays is determination and identification of existing or novel protein variants and point mutations. In these type of analyses, a large cohort of samples is required for analyses in order to delineate between the “wild” isoforms (post-translational modifications, or additional derivatization) present in majority of samples, and which subsequently are termed as “normal” and the pathological variants which are only present in a small number of samples or in samples from patients with certain diseases. In quantitative assays we are able to calculate the exact concentration of each variant, which, again, by screening populations, can provide information about the range of “normal” concentration distribution of the variant and the protein in general. These correlations can further be used in discussing the potential biomarker capacity of a certain protein or variant. Presented here are results of the developed MSIA qualitative and quantitative assays for determination of cystatin C and its variants.

### 3.2.1 Qualitative CysC MSIA assay in CSF and serum

Cystatin C is a serine proteinase inhibitor belonging to the type 2 cystatin gene family (Jarvinen, Rinne et al. 1987; Mussap and Plebani 2004). It inhibits both endogenous proteases, such as liposomal cathepsins, and proteases of parasites and microorganisms. It is a non-glycosylated single chain protein with a molecular weight of 13,343. Due to the important function, cystatin C is expressed at the stable levels in most nuclear cells. Its amino acid sequence consists of 120 amino acid residues encoded by a 7.3 kb gene located in chromosome 20 (Schnittger, Rao et al. 1993). Cystatin C has been indicated in numerous pathological states (Henskens, Veerman et al. 1996; Grubb 2000; Reed 2000), most notably in renal failure (Randers, Kristensen et al. 1998; Randers and Erlandsen 1999). There are a growing number of reports demonstrating that cystatin C is more preferable than creatinine for measurement of GFR (Naruse, Ishii et al. 2009). Also, a variant of human cystatin C (L68Q) is an amyloidogenic protein deposited in the cerebral vasculature of patients with hereditary cerebral hemorrhage with amyloidosis in which patients suffer from repeated cerebral hemorrhages (Ghiso, Jensson et al. 1986; Olafsson and Grubb 2000; Calero, Pawlik et al. 2001). In the clinical practice, cysC is well-described serum marker of renal failure that is not dependent of age, sex or lean muscle mass (Seronie-Vivien, Delanaye et al. 2008; Naruse, Ishii et al. 2009). At the same time, cystatin C is becoming acknowledged as a marker for elevated risk of death from myocardial infraction and stroke (Naruse, Ishii et al.



2009). The concentration of cystatin C in healthy individuals range from 0.8 to 1.2 mg/L, depending on the measurement method (Roos, Doust et al. 2007). Increased serum levels are almost always associated with reduction in GFR. The role of cystatin C as potential biomarker for multiple sclerosis was introduced in the work of Irani et al. They have reported a correlation between the ratio of one cysC truncated form des-SSPGKPPR and native cysC peak, and the occurrence of multiple sclerosis (Irani, Anderson et al. 2006). Other research groups do not support this concept, claiming that the cleaved peptide occurs as a result of sample storage, not exclusively MS existence (Del Boccio, Pieragostino et al. 2006; Hansson, Hviid Simonsen et al. 2006; Nakashima, Fujinoki et al. 2006).

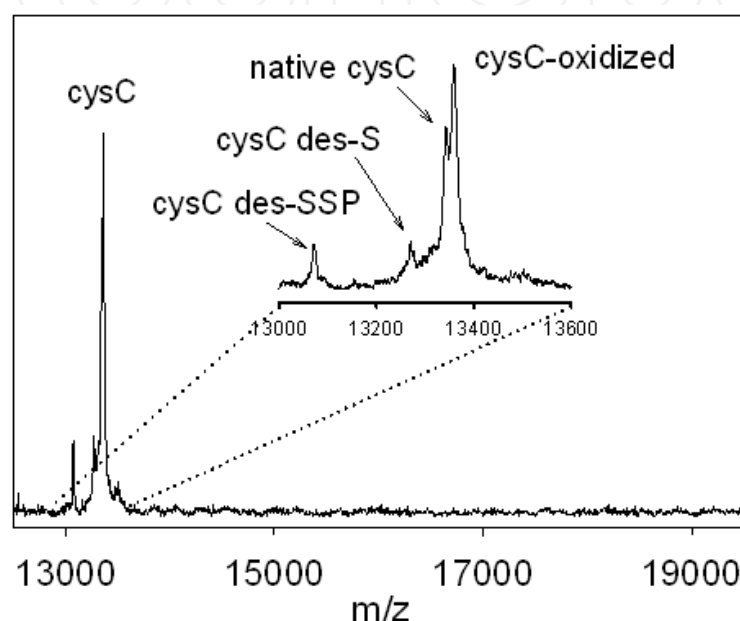


Fig. 7. Cystatin C mass spectra showing signal from native form and additional “wild type” protein variants

In previous work MSIA assays have been developed for qualitative identification of cystatin C from human plasma (Kiernan, Nedelkov et al. 2006) and urine samples (Kiernan, Tubbs et al. 2003). After several assay optimization we were able to develop MSIA assay for determination of cysC in cerebrospinal fluid both from control group and a couple of matched serum/CSF samples obtained from patients with multiple sclerosis (Trenchevska, Aleksovski et al. 2009).

MSIA provides excellent results for this type of single protein analysis, because it preserves the protein in its native form during the sample preparation procedure, and using the MALDI-TOF MS, allows an insight into the detailed protein structure, presence and distribution of isoforms. When completing MSIA data from analyzed cysC in human plasma sample, besides the native peak (CysC native MW=13 343), several protein variants can be noted: oxidation peak (MW=13 359), des-S variant (MW=13 260) and des-SSP variant (MW=13 076) (Fig.7)

These isoforms were noted in all samples analyzed, and also in a large population proteomics study where 1000 samples were screened using the developed MSIA cystatin C assay (Nedelkov, Tubbs et al. 2004; Nelson, Nedelkov et al. 2004). The goal of the further

research was to develop MSIA method for cysC identification in CSF. For utilization of this assay, a critical step was sample preparation in terms of optimizing the dilution. In this analysis, both control CSF samples and samples from patients diagnosed with MS were analyzed. Moreover, paired serum/CSF samples origin from same individuals was screened. As presented in the previous studies, using the optimized MSIA qualitative assay, cysC and its wild type isoforms were detected in the analyzed CSF samples (Nedelkov, Shaik et al. 2008). However, several correlations were observed when matched serum/CSF samples were analyzed. The sample that consisted most extensive truncations in the serum cysC, exploits most variants in the CSF sample as well (Fig.8/c,d). Also, additional peaks not common in serum cysC analysis were noted both in the control CSF samples and the samples from patients with MS. These are mainly truncated cysC isoforms missing 3, 4, 7, 8, 9, 10, 11, 14 and 17 N-terminal amino acids; some of which have been reported for the first time by our group (Fig. 8/a,b).

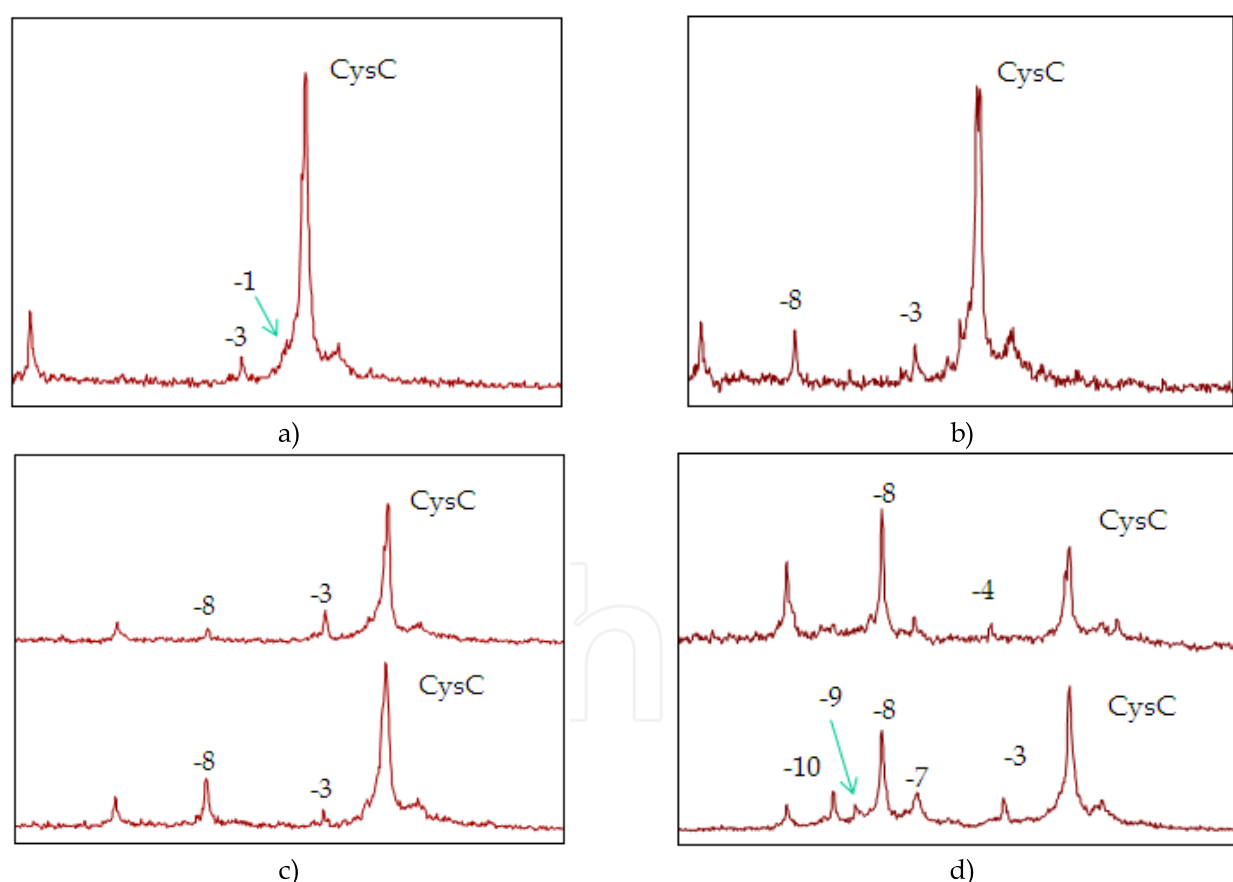


Fig. 8. MALDI-TOF MS spectra obtained after cystatin C MSIA in a) serum sample (control); b) CSF sample (control); c) paired serum/CSF sample (control) and d) paired serum/CSF sample (patients with diagnosed multiple sclerosis) (reprinted with permission from (Nedelkov, Shaik et al. 2008))

### 3.2.2 Developing quantitative MSIA assay for CysC and its variants

Developing quantitative assays for protein determination can contribute to the diagnosis and prognosis in clinical proteomics studies due to the fact that oftentimes, not only the occurrence, but also the concentration distinguishes between “normal” and “diseased” condition. Using conventional ELISA and other immunologically based quantitative assays, we can obtain information about the total protein concentration. All these techniques, however, lack the ability to quantify specific protein variants and isoforms, which in turn, are more probable biomarker candidates.

Using MSIA, we have been able to develop and validate several fully quantitative assays, both for quantitation of intact proteins, as well as protein isoforms.

There are several possible approaches regarding mass spectrometry quantitation of proteins and protein variants. One requires usage of isotope labels, whereas, second uses different protein, termed as internal reference standard for quantitation. The goal of developing platforms to be implemented in every-day clinical practice requires simplified sample preparation procedure and handling. In our work we present the developed quantitative assay for determination and quantitation of cystatin C and its variants in biological samples. Results from this quantitative assay have recently been published (Trenchevska and Nedelkov 2011). In this chapter we summarize the basic concepts and “hot spots” that need to be considered when developing this type of assay in clinical practice. The procedure includes several steps:

**Choice of internal reference standard** – There are several approaches in the process of choosing an adequate internal reference standard for a quantitative assay. An important prerequisite that an internal standard must possess is not to be present in human plasma or serum, so that its spiked concentration in the analytical sample remains constant in each analysis. Also, the signals that the IRS produces in the mass spectra should be in close proximity to the signal of the targeted protein, so that the same MS acquisition parameters can be used for analysis of both proteins. For developing quantitative assay for cysC we have used beta-lactoglobulin (BL), the major whey protein in cow’s milk, which has molecular weight of 18 281 (it is close to the MW of cysC = 13 343), and is present in many mammalian species, but not in human. In Figure 9, signals from cystatin C and the internal reference standard BL can be noted.

**Optimization of standard curve concentration range** – In this step first it is necessary to determine the ratios of immobilized cysC and BL antibodies immobilized on the affinity pipettes. In general, concentration of cysC antibody should be higher in order to accurately quantify the various amounts of cysC in the examined analytical samples. Since we can control the concentration of BL in the samples, the antibody concentration can be lower in the affinity pipettes. For the analysis we have used the following antibody ratio – cysC:BL=8.5:1 (m/m). Next, we determine the optimal concentration and volume of spiked BL in the samples, and last, we determine the concentration range for the standard curve. It is important to construct the curve in its linear range concentration and then dilute the samples if necessary, because of the greater accuracy. In this assay we have constructed a six point cysC standard curve, spanning the range from 0.0312 to 1.0 mg/L cystatin C (Fig.10).

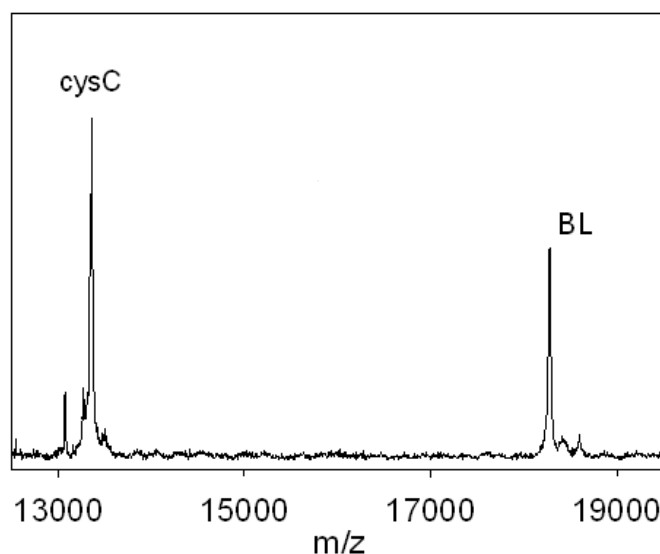


Fig. 9. Mass spectra from cystatin C and Beta lactoglobulin used as internal reference standard for quantitation

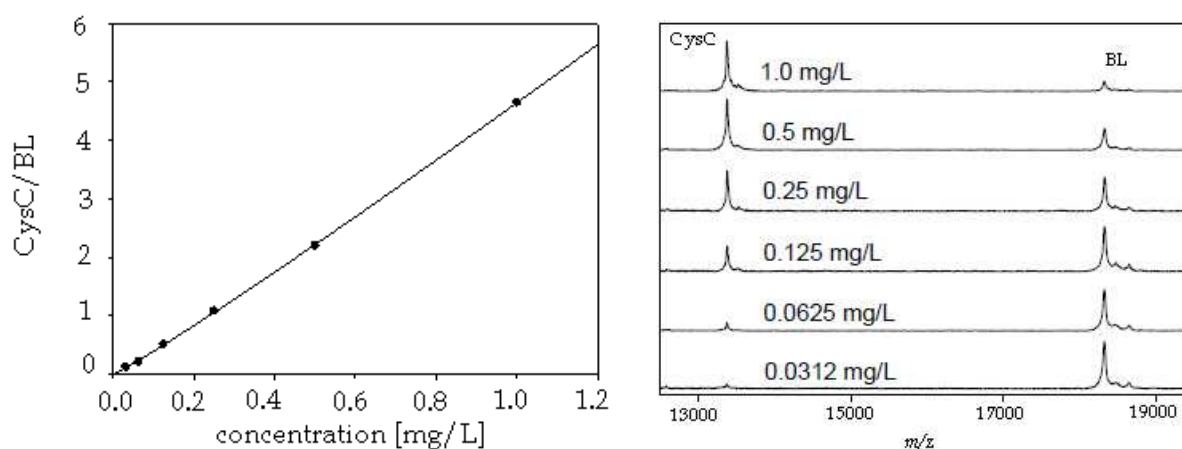


Fig. 10. Presented is a typical standard curve used for cysC quantitation (left); representative cysC mass spectra used for standard curve generation (right) (reprinted with permission from (Trenchevska and Nedelkov 2011)).

**Sample preparation** – Prior to the analysis, the analytical samples were diluted twice in the assay buffer and spiked with 5  $\mu$ L BL ( $c$  (BL) =10 mg/L).

**Assay parameters** – For this quantitative assay several characteristic parameters were analyzed in order to check the reproducibility and quality of assessment. Intra-assay precision was done by analyzing three plasma samples in replicates, each with a single standard curve. Inter-assay precision was determined by analyzing one plasma sample three times on different days with separate standard curve. Linearity of the assay was determined by analyzing serial dilutions of a sample with known cysC concentration and comparing the observed with the expected concentrations. Also, spiking-recovery experiments were performed by adding cysC standard in known concentration into the analytical sample, again with a known cysC concentration. By comparing the expected with the observed results we were able to recover the spiked cysC concentration.

**Assay validation** – In order to further validate the newly developed assay, a comparison with a well-established method was performed. Several samples were analyzed both with commercially available ELISA and with the developed MSIA method. The good correlation between both assays validated the results obtained with the new cystatin C assay (ref. statija cysC).

**Assay application** – The next phase, and practically the last step between the method and its application in the full potential is actually screening many samples and quantifying not only the native protein, but the isoforms present. Using this assay, we were able to quantify cystatin C and its variants in a total of 500 plasma samples. For all the samples, concentrations of cystatin C and its variants was determined, and averaged (average cysC concentration = 0.94 mg/L) which corresponds to the average CysC concentration previously established using ELISA (Erlandsen, Randers et al. 1998). In all the samples the “wild type” variants were noted; CysC 3Pro-OH and two truncated forms lacking one (des-S) and three (des-SSP) N-terminal amino acids respectably (Fig.11).

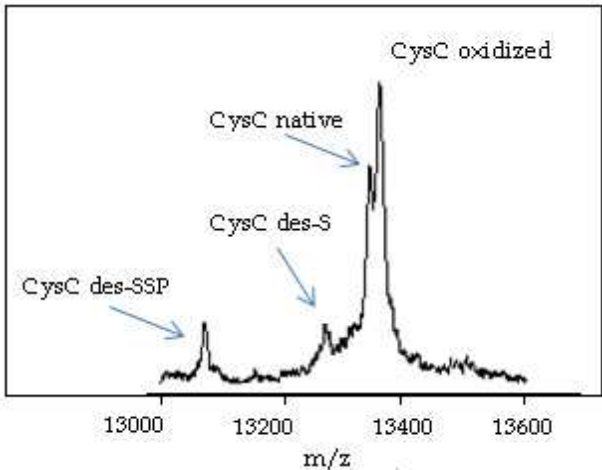


Fig. 11. Typical MALDI-TOF spectra from cystatin C showing the detected variants

Presented in Table 3 are partial results from the quantitative analysis of cystatin C, where we were able to calculate the concentration of each variant present in the analytical sample.

sample	Native cysC [mg/L]	Hydroxyl cysC [mg/L]	cysC des-S [mg/L]	cysC des-SSP [mg/L]	Total cysC [mg/L]
1	0.25	0.33	0.07	0.07	0.71
2	0.32	0.41	0.08	0.03	0.84
3	0.39	0.48	0.09	0.08	1.04
4	0.42	0.54	0.08	0.08	1.11
5	0.35	0.46	0.11	0.11	1.02
6	0.27	0.35	0.07	0.09	0.79
7	0.24	0.34	0.06	0.04	0.68
8	0.69	0.83	0.15	0.18	1.85
9	0.45	0.58	0.12	0.11	1.27
10	0.29	0.38	0.08	0.09	0.85

Table 3. Example of quantitative data obtained for cystatin C protein variants



#### 4. Further strategies in clinical proteomics

From a diagnostic point of view, it is of great advantage to analyze and assess biological markers of disease from several biological fluids from the same individual. Albeit on a small scale, the results shown here indicate that such studies are possible if the right assays are utilized. The developed MSIA method provides a unique way of delineating protein isoforms and their abundance in serum and CSF. This way, additional population proteomics studies can be done, that will provide with further insight into the physiology of biological processes and diseases. Group protein profiling still remains an irreplaceable method in clinical practice, due to the amount of information that can be provided in a single analysis. When combined with the advanced methods for statistical classification and analysis and using the tools of bioinformatics, analyses at a large scale can be performed. Obtained data can further be used in creating software programs and data bases for recognition and identification of specific protein profiles.

Further analyses regarding our group will progress in several directions: (1) develop quantitative assays for other proteins that are considered to be potential diagnostic markers for specific neurological diseases; (2) implement these assays to fully quantify proteins and protein isoforms in serum and cerebrospinal fluid samples; (3) introduce the concept of immunoaffinity separation techniques into clinical practice (4) develop algorithms for protein profile data base creation and pattern recognition and (5) investigate the possibility to implement the novel methods to analyze proteins using other media such as tears and saliva.

#### 5. Conclusions

Analyzing proteins is a complicated task since many approaches can be followed (Andersson, Alvarez-Cermenio et al. 1994; Anderson and Anderson 1998; Bakhtiar and Nelson 2001; Diamandis 2003; Conrads, Hood et al. 2004; Bons, Wodzig et al. 2005; Anderson 2010). There are two majorly differentiated approaches that can be applied in routine clinical practice, analyzing protein profile (protein profiling) and choosing single protein (biomarker discovery) and optimizing methods for identification and determination. Regarding these remarks, there is a quite divergence between the “group” protein screening and “individual” protein determination. However, by combining different novel techniques we are able to bridge the gap in between. In addition, we can discuss the necessary development both in the methodology and techniques in order to have the highest impact on the study of the molecular composition of CSF proteins.

The application of these novel techniques to the study of neurological disorders is providing an insight into the pathogenesis of neurodegeneration, and is fueling major efforts in biomarker discovery. In the protein profiling concept we are able to analyze patterns obtained from the proteome of a patient with a certain disease, and compare it with a sample from a control group. This approach is still widely used in screening patients with neurological diseases, using electrophoresis techniques as a basic technique. By comparing these profiles, a typical prototype pattern is created for separate conditions, and by simply comparing these patterns (nowadays with the use of informatics tools and statistics programs) differential diagnosis is possible.

This approach, however, only presents the big picture. It is the single protein biomarker assays that are more and more abundant in clinical laboratories. The quest for novel biomarkers results in developing techniques and mechanisms of investigating protein intrinsic characteristics at a molecular level.

From the obtained results we can conclude that even though in routine clinical practice enzymatic immunoassays are still dominant, especially for quantitative protein analysis, they have one crucial disadvantage. These assays are oblivious to protein modifications, unless there is an antibody toward that exact modification. The mass spectrometry immunoassay, on the other hand, is designed to detect protein modifications and intact proteins in a single assay. This qualitative determination is already a way to delineate between control subjects and diseased samples. With the added quantitative feature, this and similar assays are poised to change the way we look proteins and protein modifications and their role in health and disease.

An integrated evaluation of the data that are obtained by all the holistic approaches, in combination with clinical and epidemiological data will eventually not only increase our understanding of disease mechanisms, but subsequently also enable us to develop more specific and individualized medicines and therapies.

All our efforts are oriented toward the main goal – to aid in the process of revealing the protein complexity of neurological disorders and contribute to its better understanding.

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## 7. References

- Aebersold, R. and D. R. Goodlett (2001). "Mass spectrometry in proteomics." *Chem Rev* 101(2): 269-95.
- Ahmed, F. E. (2008). "Mining the oncoproteome and studying molecular interactions for biomarker development by 2DE, ChIP and SPR technologies." *Expert Rev Proteomics* 5(3): 469-96.
- Aizenberg, I., N. Aizenberg, et al. (2001). "Cellular neural networks and computational intelligence in medical image processing." *Image Vision Comp.* 19: 177-183.
- Alban, A., S. O. David, et al. (2003). "A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard." *Proteomics* 3(1): 36-44.
- Anderson, N. L. (2010). "The clinical plasma proteome: a survey of clinical assays for proteins in plasma and serum." *Clin Chem* 56(2): 177-85.

- Anderson, N. L. and N. G. Anderson (1998). "Proteome and proteomics: new technologies, new concepts, and new words." *Electrophoresis* 19(11): 1853-61.
- Anderson, N. L. and N. G. Anderson (2002). "The human plasma proteome: history, character, and diagnostic prospects." *Mol. Cell. Proteomics* 1(11): 845-67.
- Andersson, M., J. Alvarez-Cermenio, et al. (1994). "Cerebrospinal fluid in the diagnosis of multiple sclerosis: a consensus report." *J Neurol Neurosurg Psychiatry* 57(8): 897-902.
- Auroux, P. A., D. Iossifidis, et al. (2002). "Micro total analysis systems. 2. Analytical standard operations and applications." *Anal Chem* 74(12): 2637-52.
- Bakhtiar, R. and R. W. Nelson (2001). "Mass spectrometry of the proteome." *Mol Pharmacol* 60(3): 405-15.
- Balslev, S., A. M. Jorgensen, et al. (2006). "Lab-on-a-chip with integrated optical transducers." *Lab Chip* 6(2): 213-7.
- Bons, J. A., W. K. Wodzig, et al. (2005). "Protein profiling as a diagnostic tool in clinical chemistry: a review." *Clin Chem Lab Med* 43(12): 1281-90.
- Bousse, L., S. Mouradian, et al. (2001). "Protein sizing on a microchip." *Anal Chem* 73(6): 1207-12.
- Brody, E. N., L. Gold, et al. (2010). "High-content affinity-based proteomics: unlocking protein biomarker discovery." *Expert Rev Mol Diagn* 10(8): 1013-22.
- Calero, M., M. Pawlik, et al. (2001). "Distinct properties of wild-type and the amyloidogenic human cystatin C variant of hereditary cerebral hemorrhage with amyloidosis, Icelandic type." *J Neurochem* 77(2): 628-37.
- Chevalier-Larsen, E. and E. L. Holzbaur (2006). "Axonal transport and neurodegenerative disease." *Biochim Biophys Acta* 1762(11-12): 1094-108.
- Chiou, S.-H., Wu, S.-H. (1999). "Evaluation of commonly used electrophoretic methods for the analysis of proteins and peptides and their application to biotechnology." *Analytica Chimica Acta* 383: 47-60.
- Christodoulides, N., P. N. Floriano, et al. (2007). "Lab-on-a-chip methods for point-of-care measurements of salivary biomarkers of periodontitis." *Ann N Y Acad Sci* 1098: 411-28.
- Conrads, T. P., B. L. Hood, et al. (2004). "Proteomic patterns as a diagnostic tool for early-stage cancer: a review of its progress to a clinically relevant tool." *Mol Diagn* 8(2): 77-85.
- Craig, W. Y., T. B. Ledue, et al. (2001). *Plasma proteins: Clinical utility and interpretation*. Scarborough, ME, Foundation for Blood Research.
- Daskalovska, V. (2000). *Multipla skleroza*. Skopje.
- De Vos, K. J., A. J. Grierson, et al. (2008). "Role of axonal transport in neurodegenerative diseases." *Annu Rev Neurosci* 31: 151-73.
- Del Boccio, P., D. Pieragostino, et al. (2006). "Cleavage of cystatin C is not associated with multiple sclerosis." *Ann Neurol*.
- Diamandis, E. P. (2003). "Point: Proteomic patterns in biological fluids: do they represent the future of cancer diagnostics?" *Clin. Chem.* 49(8): 1272-5.
- Dufva, M. and C. B. Christensen (2005). "Diagnostic and analytical applications of protein microarrays." *Expert Rev Proteomics* 2(1): 41-8.

- Engvall, E. and P. Perlmann (1971). "Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G." *Immunochemistry* 8(9): 871-4.
- Engwegen, J. Y., M. C. Gast, et al. (2006). "Clinical proteomics: searching for better tumour markers with SELDI-TOF mass spectrometry." *Trends Pharmacol Sci* 27(5): 251-9.
- Erlandsen, E. J., E. Randers, et al. (1998). "Reference intervals for serum cystatin C and serum creatinine in adults." *Clin Chem Lab Med* 36(6): 393-7.
- Fenn, J. B., M. Mann, et al. (1989). "Electrospray ionization for mass spectrometry of large biomolecules." *Science* 246(4926): 64-71.
- Fishman, R. A. (1992). *Cerebrospinal fluid in diseases of the nervous system*. Philadelphia, W.B.Saunders Corp.
- Floriano, P. N., S. Acosta, et al. (2007). "Microchip-based enumeration of human white blood cells." *Methods Mol Biol* 385: 53-64.
- Fountoulakis, M. and B. Takacs (1998). "Design of protein purification pathways: application to the proteome of *Haemophilus influenzae* using heparin chromatography." *Protein Expr Purif* 14(1): 113-9.
- Fountoulakis, M., M. F. Takacs, et al. (1999). "Enrichment of low abundance proteins of *Escherichia coli* by hydroxyapatite chromatography." *Electrophoresis* 20(11): 2181-95.
- Geschke, O. (2006). "Miniaturization--not just a matter of size." *Anal Bioanal Chem* 385(8): 1350.
- Geschke, O. (2009). "Microfluidics for bioapplications." *Anal Bioanal Chem* 395(3): 619.
- Ghiso, J., O. Jensson, et al. (1986). "Amyloid fibrils in hereditary cerebral hemorrhage with amyloidosis of Icelandic type is a variant of gamma-trace basic protein (cystatin C)." *Proc Natl Acad Sci U S A* 83(9): 2974-8.
- Grubb, A. O. (2000). "Cystatin C--properties and use as diagnostic marker." *Adv Clin Chem* 35: 63-99.
- Gygi, S. P., B. Rist, et al. (1999). "Quantitative analysis of complex protein mixtures using isotope-coded affinity tags." *Nat Biotechnol* 17(10): 994-999.
- Han, X., M. Jin, et al. (2006). "Extending top-down mass spectrometry to proteins with masses greater than 200 kilodaltons." *Science* 314(5796): 109-12.
- Hansson, S. F., A. Hviid Simonsen, et al. (2006). "Cystatin C in cerebrospinal fluid and multiple sclerosis." *Ann Neurol*.
- Hein Nee Maier, K., A. Kohler, et al. (2008). "Biological markers for axonal degeneration in CSF and blood of patients with the first event indicative for multiple sclerosis." *Neurosci Lett* 436(1): 72-6.
- Henskens, Y. M., E. C. Veerman, et al. (1996). "Cystatins in health and disease." *Biol. Chem. Hoppe Seyler* 377(2): 71-86.
- Hu, Y., X. Huang, et al. (2004). "Recent advances in gel-based proteome profiling techniques." *Mol Biotechnol* 28(1): 63-76.
- Irani, D. N., C. Anderson, et al. (2006). "Cleavage of cystatin C in the cerebrospinal fluid of patients with multiple sclerosis." *Ann Neurol* 59(2): 237-47.
- Issaq, H. J., T. D. Veenstra, et al. (2002). "The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification." *Biochem Biophys Res Commun* 292(3): 587-92.



- Jarvinen, M., A. Rinne, et al. (1987). "Human cystatins in normal and diseased tissues--a review." *Acta Histochem* 82(1): 5-18.
- Jerez-Aragones, J. M. (2003). "A combined neural network and decision trees model for prognosis of breast cancer relapse." *Artf.Intell.Med.* 27: 45-63.
- Jin, L. J., B. C. Giordano, et al. (2001). "Dynamic labeling during capillary or microchip electrophoresis for laser-induced fluorescence detection of protein-SDS complexes without pre- or postcolumn labeling." *Anal Chem* 73(20): 4994-9.
- Keramas, G., G. Perozziello, et al. (2004). "Development of a multiplex microarray microsystem." *Lab Chip* 4(2): 152-8.
- Kiernan, U. A., D. Nedelkov, et al. (2006). "Multiplexed mass spectrometric immunoassay in biomarker research: a novel approach to the determination of a myocardial infarct." *J Proteome Res* 5(11): 2928-34.
- Kiernan, U. A., D. Nedelkov, et al. (2006). "High-throughput affinity mass spectrometry." *Methods Mol Biol* 328: 141-50.
- Kiernan, U. A., K. A. Tubbs, et al. (2003). "Comparative urine protein phenotyping using mass spectrometric immunoassay." *J. Proteome Res.* 2(2): 191-7.
- Krone, J. R., R. W. Nelson, et al. (1996). "Mass spectrometric immunoassay." *SPIE* 2680: 415-421.
- Lahm, H. W. and H. Langen (2000). "Mass spectrometry: a tool for the identification of proteins separated by gels." *Electrophoresis* 21(11): 2105-14.
- Link, H. and R. Muller (1971). "Immunoglobulins in multiple sclerosis and infections of the nervous system." *Arch Neurol* 25(4): 326-44.
- Lisboa, P. J. G. (2002). "A review of evidence of health benefit from artificial neural networks in medical intervention." *Neural.Net.* 15: 11-39.
- Maccarrone, G., I. Birg, et al. (2004). "In-depth analysis of the human CSF proteome using protien prefractionaltion." *Clin.Proteomics* 1: 333-364.
- Maccarrone, G., D. Milfay, et al. (2004). "Mining the human cerebrospinal fluid proteome by immunodepletion and shotgun mass spectrometry." *Electrophoresis* 25(14): 2402-12.
- Mallick, P. and B. Kuster (2010). "Proteomics: a pragmatic perspective." *Nat Biotechnol* 28(7): 695-709.
- Manz, A., N. Graber, et al. (1990). "Miniaturized total Chemical Analysis systems: A Novel Concept for Chemical Sensing, Sensors and Actuators." 244-248.
- McCudden, C. R., P. M. Voorhees, et al. (2010). "Interference of monoclonal antibody therapies with serum protein electrophoresis tests." *Clin Chem* 56(12): 1897-9.
- Mitreviski, A., K. Stojanoski, et al. (2001). "Detection of oligoclonal IgG bands in cerebrospinal fluid on polyacrylamide support media: comparisson of isoelectric focusing and disc electrophoresis." *Acta Pharm.* 51(3): 163-171.
- Monteoliva, L. and J. P. Albar (2004). "Differential proteomics: an overview of gel and non-gel based approaches." *Brief Funct Genomic Proteomic* 3(3): 220-39.
- Mussap, M. and M. Plebani (2004). "Biochemistry and clinical role of human cystatin C." *Crit Rev Clin Lab Sci* 41(5-6): 467-550.
- Nakashima, I., M. Fujinoki, et al. (2006). "Alteration of cystatin C in the cerebrospinal fluid of multiple sclerosis." *Ann Neurol.*



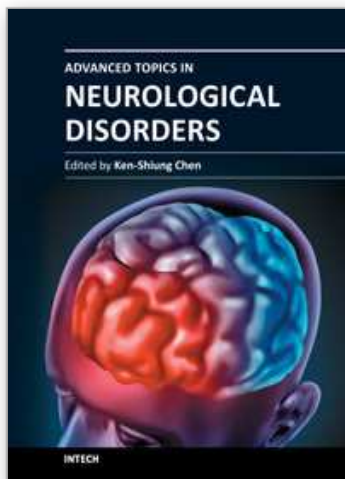
- Naruse, H., J. Ishii, et al. (2009). "Cystatin C in acute heart failure without advanced renal impairment." *Am J Med* 122(6): 566-73.
- Nedelkov, D. (2005). "Population proteomics: addressing protein diversity in humans." *Expert Rev Proteomics* 2(3): 315-24.
- Nedelkov, D. (2006). "Mass spectrometry-based immunoassays for the next phase of clinical applications." *Expert Rev Proteomics* 3(6): 631-40.
- Nedelkov, D., S. Shaik, et al. (2008). "Targeted Mass Spectrometric Immunoassay for DETECTION of Cystatin C Isoforms in Cerebrospinal Fluid." *The Open Proteomics Journal* 1: 54-58.
- Nedelkov, D., K. A. Tubbs, et al. (2004). "High-Throughput Comprehensive Analysis of Human Plasma Proteins: A Step toward Population Proteomics." *Anal. Chem.* 76(6): 1733-7.
- Nelson, R. W., J. R. Krone, et al. (1995). "Mass-Spectrometric Immunoassay." *Anal. Chem.* 67(7): 1153-1158.
- Nelson, R. W., D. Nedelkov, et al. (2004). "Quantitative mass spectrometric immunoassay of insulin like growth factor 1." *J. Proteome Res.* 3(4): 851-855.
- Ocvirk, G., T. Tang, et al. (1998). *Analyst* 123: 1429-1434.
- Olafsson, I. and A. Grubb (2000). "Hereditary cystatin C amyloid angiopathy." *Amyloid* 7(1): 70-9.
- Ornstein, L. (1964). "Disc Electrophoresis. I. Background and Theory." *Ann N Y Acad Sci* 121: 321-49.
- Poon, T. C. W. (2007). "Opportunities and limitations of SELDI-TOF-MS in biomedical research: practical advices." *Expert rev. Proteomics* 4(1): 51-65.
- Rabilloud, T. (2002). "Two-dimensional gel electrophoresis in proteomics: old, old fashioned, but it still climbs up the mountains." *Proteomics* 2(1): 3-10.
- Randers, E. and E. J. Erlandsen (1999). "Serum cystatin C as an endogenous marker of the renal function--a review." *Clin. Chem. Lab. Med.* 37(4): 389-95.
- Randers, E., J. H. Kristensen, et al. (1998). "Serum cystatin C as a marker of the renal function." *Scand J Clin Lab Invest* 58(7): 585-92.
- Reed, C. H. (2000). "Diagnostic applications of cystatin C." *Br J Biomed Sci* 57(4): 323-9.
- Reiber, H., M. Otto, et al. (2001). "Reporting cerebrospinal fluid data: knowledge base and interpretation software." *Clin Chem Lab Med* 39(4): 324-32.
- Reid, G. E. and S. A. McLuckey (2002). "'Top down' protein characterization via tandem mass spectrometry." *J Mass Spectrom* 37(7): 663-75.
- Ritchie, R. F., Ed. (1999). *Serum Proteins in Clinical Medicine*. Scarborough, ME, Foundation for Blood Research.
- Roos, J. F., J. Doust, et al. (2007). "Diagnostic accuracy of cystatin C compared to serum creatinine for the estimation of renal dysfunction in adults and children--a meta-analysis." *Clin Biochem* 40(5-6): 383-91.
- Sandic, C. J. M., P. Monteyne, et al. (1994). "Polyclonal and oligoclonal IgA synthesis in the cerebrospinal fluid of neurological patients. An immunoaffinity-mediated capillary blot study." *J. Neuroimmunol.* 94: 103-111.

- Schnittger, S., V. V. Rao, et al. (1993). "Cystatin C (CST3), the candidate gene for hereditary cystatin C amyloid angiopathy (HCCAA), and other members of the cystatin gene family are clustered on chromosome 20p11.2." *Genomics* 16(1): 50-5.
- Seronie-Vivien, S., P. Delanaye, et al. (2008). "Cystatin C: current position and future prospects." *Clin Chem Lab Med* 46(12): 1664-86.
- Sindic, C. J., M. P. Van Antwerpen, et al. (2001). "The intrathecal humoral immune response: laboratory analysis and clinical relevance." *Clin Chem Lab Med* 39(4): 333-40.
- Sloat, A. L., M. G. Roper, et al. (2008). "Protein determination by microchip capillary electrophoresis using an asymmetric squarylium dye: noncovalent labeling and nonequilibrium measurement of association constants." *Electrophoresis* 29(16): 3446-55.
- Spirovski, F., K. Stojanoski, et al. (2005). "Comparison of statistical cluster methods in electrophoretic protein pattern analysis." *Acta Pharm* 55(2): 215-22.
- Sun, F. and V. Cavalli (2010). "Neuroproteomics approaches to decipher neuronal regeneration and degeneration." *Mol Cell Proteomics* 9(5): 963-75.
- Takacs, M., G. Rakhely, et al. (2001). "Molecular characterization and heterologous expression of hypCD, the first two [NiFe] hydrogenase accessory genes of *Thermococcus litoralis*." *Arch Microbiol* 176(3): 231-5.
- Tanaka, K., H. Waki, et al. (1988). "Protein and polymer analyses of up to m/z 100,000 by laser ionization time-of-flight mass spectrometry." *Rapid Commun. Mass Spectrom.* 2: 151-153.
- Tourtellote, W. W., A. R. Povin, et al. (1980). "Multiple sclerosis: measurement and validation of central nervous system IgG synthesis rate." *Neurology* 30: 240-244.
- Trenchevska, O., Aleksovski, V., Stojanoski, K., (2009) "Advanced techniques in clinical practice: Use of lab-on-a-chip electrophoresis and other methods in protein profiling." *Journal of Medical Biochemistry* 29: 1-5.
- Trenchevska, O., Aleksovski, V., Stojanoski, K., (2009) (2009). "Temperature and denaturing substances influence on lab-on-a-chip electrophoresis." *Journal of Medical Biochemistry* 28: 1-5.
- Trenchevska, O. and D. Nedelkov (2011). "Targeted quantitative mass spectrometric immunoassay for human protein variants." *Proteome Sci* 9(1): 19.
- Turck, C. W., G. Maccarrone, et al. (2005). "The quest for brain disorder biomarkers." *J Med Invest* 52 Suppl: 231-5.
- van der Merwe, D. E., K. Oikonomopoulou, et al. (2007). "Mass spectrometry: uncovering the cancer proteome for diagnostics." *Adv Cancer Res* 96: 23-50.
- Vesterberg, O. (1989). "History of electrophoretic methods." *J Chromatogr* 480: 3-19.
- Vogt, W. and D. Nagel (1992). "Cluster analysis in diagnosis." *Clin Chem* 38(2): 182-98.
- W. Bruno, M. D. V., M.S. Abraham Saifer, M. Abraham, M.D. Rabiner, (1956). A.M.A. *Arch. Neurol. Psychiatry*, 75(5): 472-487.
- Whitelegge, J., F. Halgand, et al. (2006). "Top-down mass spectrometry of integral membrane proteins." *Expert Rev Proteomics* 3(6): 585-96.
- Woolley, A. T. and R. A. Mathies (1994). "Ultra-high-speed DNA fragment separations using microfabricated capillary array electrophoresis chips." *Proc Natl Acad Sci U S A* 91(24): 11348-52.

- Yan, W., H. Lee, et al. (2004). "A dataset of human liver proteins identified by protein profiling via isotope-coded affinity tag (ICAT) and tandem mass spectrometry." *Mol. Cell. Proteomics* 3(10): 1039-41.
- Yao, S., D. S. Anex, et al. (1999). "SDS capillary gel electrophoresis of proteins in microfabricated channels." *Proc Natl Acad Sci U S A* 96(10): 5372-7.

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