Chapter 2

Protein profiling as a diagnostic tool in clinical chemistry, a review

Influence of pre- and post-analytical aspects

Judith A.P. Bons, Will K.W.H. Wodzig, Marja P. van Dieijen-Visser

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Abstract

Serum protein profiling by Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry appears to be an important diagnostic tool for a whole range of diseases. Sensitivities and specificities obtained with this new technology often seem superior to those obtained with current biomarkers. However, reproducibility and standardization are still problematic.
The present report shows an overview of the diagnostic value of protein profiles obtained with Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry in studies on prostate and ovarian cancer. To identify aspects important for protein profiling, we compare and discuss differences in pre- and post-analytical conditions presented in the literature supplemented with some of our own data. Further progress in protein profiling as a diagnostic tool requires a more comprehensive description of technical details in all future studies.

Introduction

Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS) is a promising new technology which was introduced by Hutchens and Yip[1]. The ProteinChip system manufactured by Ciphergen Biosystem Inc. (Fremont, CA, USA) indicated as SELDI-TOF-MS has the potential to discover useful biomarkers faster than any existing technology. The true scientific goal of serum proteomic pattern analysis is in fact biomarker discovery. However, since the study by Petricoin et al.[2] on proteomic patterns to detect ovarian cancer, the use of SELDI-TOF-MS protein profiling as a diagnostic tool, has become an important subject of investigation[3]. Until now, this approach has been suggested for diseases such as ovarian[2,4-8], prostate[9-13] and lung[14] cancer, as well as for inflammatory diseases[15,16]. For the principle of SELDI-TOF-MS, we refer to a recent overview by Wiesner et al.[17]. Figure 2.1 illustrates the serum protein profiles of two healthy controls compared to the profiles of two sarcoidosis patients.
The aim of this report is to discuss differences between the pre- and post-analytical strategies used in the various studies and to identify aspects that could be responsible for the discrepancies and might be important for future studies on protein profiling. Prostate and ovarian cancer were selected, because several studies on SELDI-TOF-MS protein profiling as a diagnostic tool for these two diseases have been published, allowing more extensive comparison with a special focus on the technical details.

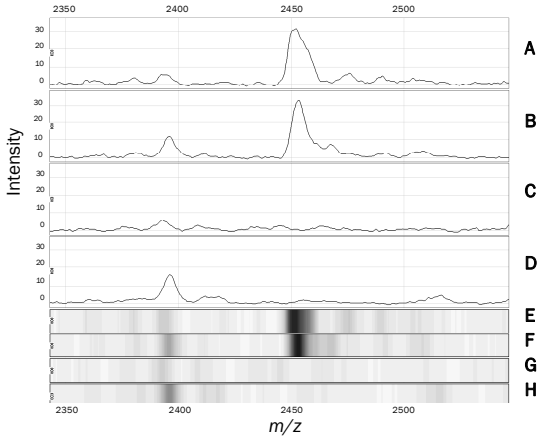

Figure 2.1 SELDI-TOF mass spectra and gel views of fractionated serum samples by centrifugation on a 30,000 Da cut-off filter on a normal phase (NP20) ProteinChip array.
Panel A and B show spectra of two healthy control samples. Panel C and D show spectra of two sarcoidosis patient samples. Panel E and F show healthy control spectra in gel view and panel G and H show sarcoidosis spectra in gel view. The intensity is displayed along the y-axis and the mass is given as mass-to-charge ratio (*m/z*) ratio on the x-axis.

Comparison of prostate and ovarian studies

Tables 2.1 and 2.2 present an overview of studies on prostate[9-13] and ovarian cancer[2,4-8] where SELDI-TOF-MS was used to discover potential biomarkers in serum by protein profiling. Many small studies have been published, but essential data on sample collection, sample preparation and post-analytical strategies are often lacking in their reports (Tables 2.1 and 2.2, open fields).
However, a comparison of different studies on protein profiling with SELDI-TOF-MS requires a clear description of all technical details. If protein profiling is to become a diagnostic tool, it is essential that the data are reproducible, which is only possible if an adequate description of the methods is included in all reports. The present review discusses aspects essential for a comparison of methods used in different studies and their influence on the final results.
Comparative studies on protein profiling have been published by Diamandis[18] and Xiao et al.[19]. Both studies clearly addressed the problems of standardization and reproducibility of protein profiling. Diamandis[18] focused on the decision algorithms obtained in three different studies on prostate cancer by Adam et al., Qu et al. and Petricoin et al.[10-12] and tried to find an explanation for the fact that they found completely different decision trees, although they used identical chip types and comparable study populations. Xiao et al.[19] compared different cancer types, ovarian, breast and prostate cancer and indicated that various statistical methods eventually constructed different classifiers with different statistical results. Pre-analytical aspects and technical details were only briefly discussed in both reviews. Therefore, our review focuses on both pre- and post-analytical aspects, supplemented with some of our own data. The description of the methods used in the different studies is summarized in the Tables 2.1 and 2.2 and discussed below.

Pre-analytical aspects

Storage effects

To avoid pre-analytical errors, sample collection for proteomic analysis should be accurately described and standardized. Until now, the effects of sample storage have not been addressed systematically and the consequences of differences in sample preparation are highly underestimated. The different studies on prostate and ovarian cancer cited here generally give insufficient information on the pre-analytical conditions. In most studies, the storage-temperature of the serum samples was –80°C except in the studies by Qu et al.[12] and Zhang et al.[5] where the samples were frozen at –8°C and –70°C, respectively (see Table 2.1). Table 2.1 also illustrates that reports do not systematically describe how many freeze-thaw cycles were performed. Only the articles by Adam et al.[10] and Li et al.[13] indicate that only one freeze-thaw cycle was used. The effects of using more freeze-thaw cycles have not been investigated systematically.

Therefore we compared freshly frozen serum samples with frequently thawed serum samples. The samples were thawed at least eight times and were stored at -80°C. Freshly frozen serum and frequently thawed serum from eight sarcoidosis patients and eight healthy controls were spotted on a CM10 (weak cation exchange) and on a NP20 (normal phase) ProteinChip array. In the frequently freeze-thawed sera, three peaks were detected, allowing clear discrimination of sarcoidosis from healthy controls using the CM10 chip (*m/z* values: 3808 (up-regulation in sarcoidosis), 4277 (down-regulation in sarcoidosis), 8932 (up-regulation in sarcoidosis). However, exactly the same experiment using freshly frozen sera no longer allowed us to discriminate between sarcoidosis and controls, because the peak differences were not significant. In contrast, in the freshly frozen samples only one significant peak, with an *m/z* value of 8702 was found. This peak was different from the ones found in the frequently thawed samples. The fact that another single marker was found on the CM10 indicates that it concerns a freeze-thaw artefacts and underlines the importance standardization.
For the NP20 experiment, crude sera of 16 sarcoidosis and 16 healthy persons was fractionated by centrifugation with a 30 kDa cut-off filter. The filter was used to eliminate highly expressed proteins such as human serum albumin and immunoglobulins that interfere with the detection and identification of potentially relevant less abundant proteins. For frequently freeze-thawed filtrated sera, one peak was found to discriminate sarcoidosis from healthy controls on the NP20 (*m/z* 2454) with a mean intensity of 32.0 for healthy controls and 1.0 for sarcoidosis. However, when using freshly frozen serum samples, the peak was no longer visible, again indicating that it concerns a freeze-thaw artefact. Both experiments indicate that standardization of sample pre-treatment is essential.
However, in most proteomics evaluations archived samples are used, which are often thawed more than once. As also becomes apparent from our own data the number of freeze-thaw cycles and the freezing temperature should at least be identical for both study and control population. The problem can easily be overcome by dividing the samples in aliquots before storage.

Serum or plasma

Until now, insufficient information is available to decide whether serum or plasma should be preferred in proteomic studies. Most studies have used serum, but further research on this topic is required. The studies in this review all used human serum, except for the study of Rai et al.[8] (Table 2.1), which used human sodium ethylenediamine tetraacetic acid (EDTA) plasma for both patients and controls.

In particular, proteolytic proteins released in serum during the clotting phase can cause fragmentation of proteins and influence the final serum protein composition. In general, proteolytic activity in serum samples may have considerable consequences for protein profiling studies. Table 2.1 indicates that the pre-treatment of serum before spotting on the ProteinChip arrays varies considerable in the different studies making comparison of the obtained peaks/profiles difficult. However, as long as pre-treatment of serum is the same for all samples within a study, the differences observed can be considered as relevant differences between controls and the disease population.
In our own study we compared serum and EDTA plasma with and without protease inhibitors. Serum and plasma samples of eight sarcoidosis patients and eight healthy persons were spotted on CM10 and NP20 ProteinChip arrays. The mean protein peaks in serum with and without protease inhibitors were compared with the mean protein peaks in plasma with and without protease inhibitors.
Table 2.3 shows that in the *m/z* range of 2500 Da to 150,000 Da serum without protease inhibitors showed slightly more protein peaks (n=64) than serum with inhibitors (n=63). EDTA plasma with and without protease inhibitors was inferior (both n=28) on the CM10 ProteinChip array. On the NP20 ProteinChip array serum without protease inhibitors showed slightly fewer protein peaks (n=58) in the same *m/z* range than serum with inhibitors (n=63), but EDTA plasma with (n=11) or without (n=14) protease inhibitors was evidently inferior. More significant peaks that could discriminate sarcoidosis from healthy control samples were found in the serum samples with and without protease inhibitors compared to plasma samples with and without protease inhibitors on the CM10 and NP20 ProteinChip array. It is generally assumed that, more peaks can lead to more significant differences between populations as was the case in our study. Theoretically, however, plasma with protease inhibitors contains more intact proteins not attacked by proteolytic enzymes. Further examinations on the differences between serum and plasma are required.

Table 2.3 Serum and plasma samples with and without protease inhibitors were spotted on CM10 and NP20 ProteinChip arrays.

	CM10 (N peaks)	NP20 (N peaks)
Serum	64	58
Serum with protease inhibitors	63	63
Plasma	28	11
Plasma with protease inhibitors	28	14

The mean number of peaks (N) in the protein spectra (*m/z* range of 2500 Da to 150,000 Da) from eight sarcoidosis and eight healthy control samples are indicated.

Sampling time

It is known that the serum concentration of certain proteins is influenced by the sampling time, i.e., time between puncture and storage (clotting time, spinning

time and time between spinning and storage). However, the type of material also plays a role. For instance B-type natriuretic peptide (BNP) a well-known marker for heart failure, is instable in serum, as a result of the presence of proteolytic enzymes. The degradation progresses even during storage at -20°C and can only be prevented by addition of protease inhibitors or by measuring plasma BNP, instead of serum BNP[20]. Therefore, information on sampling-time should be indicated more clearly in the different studies. Table 2.1 shows that this information is lacking in most studies. This can be problematic when archived samples are used. However, in prospective proteomics studies clotting time and time between sample collection and storage should be standardized. We suggest, according to World Health Organization (WHO) recommendations on anticoagulants in diagnostic laboratory investigations (2002), the use of a clotting time of 30 min at room temperature, spinning for 15 min at a minimum speed of 1500 x *g* and storage of the samples in aliquots within one hour at –80°C after blood collection.
The consequences of differences in sample characteristics within a study population, as well as between the study and control populations, e.g., the use of fasting or non-fasting samples and age-matching of the samples should also be more rigorously standardized in future studies.

Sample preparation

Most studies used samples denatured with urea/([3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate]) (CHAPS)[4,6-10,12,13] while only one study used samples[5] fractionated with anion exchange chromatography. In the prostate and ovarian study of Petricoin et al.[2,11] sample pre-treatment is not indicated at all. Denaturing conditions allow protein-protein interaction disruption before analysis by SELDI-TOF-MS. With fractionation, the serum proteome is divided into sub-proteomes and this method markedly increases resolution and sensitivity without any loss of minor proteins. With fractionation by anion exchange chromatography the highly abundant proteins such as albumin and immunoglobulins (60-80% of total serum protein content), which can interfere with the resolution and sensitivity of the proteome profiling techniques, will be visible in specific fractions. The albumin signal will be mainly visible in fraction 3 (pH 5), fraction 4 (pH 4) and fraction 5 (pH 3). Similarly, the immunoglobulins signal should be observed in fraction 1 (pH 9) and fraction 2 (pH 7). In this procedure, the highly abundant proteins are not removed, but they are localized to one or a few particular fractions[21].
Table 2.1 shows that the denaturing steps also vary in the different studies as a result of the use of different buffer concentrations. The loss of these high abundant protein signals increased the detection of less abundant protein signals. Moreover, the total number of protein peaks with fractionated samples was larger than that seen with crude serum samples. Linke et al.[22] also illustrated that fractionation greatly increases the number of peptide and protein ion signals that can be

observed by SELDI-TOF-MS when compared to both unfractionated (only denatured) and albumin-depleted samples. By using different denaturing steps or fractionated samples, other significant peaks resulting in different biomarkers can be detected. This is one of the aspects responsible for the different results in the studies discussed here.

Calibration

The calibration step is very important to calculate the exact mass accurately. Table 2.2 illustrates that different calibrants have been used in the prostate and ovarian studies. In the prostate studies Adam et al.[10] and Qu et al.[12] used the All-in-1 peptide molecular weight standard of Ciphergen, while Li et al.[13] used the All-in-1 protein molecular weight standard of Ciphergen. Banez et al.[9] used two calibrants from Ciphergen (insulin and ubiquitin standards). The article by Petricoin et al.[11] provided no information about the calibration. In the ovarian studies, also different calibrants were used (see Table 2.2). Vlahou et al.[6] used All-in-1 peptide molecular weight standard of Ciphergen, while Kozak et al.[4] used eight calibrants ranging from 5734-77,490 Da from Ciphergen. Ye et al.[7] used two calibrants of 5734 and 12,231 Da. Zhang et al.[5] described that the spectra were externally calibrated, but did not mention the calibrants. The articles by Petricoin et al.[2] and Rai et al.[8] gave no information at all about the calibration process. Because not all groups used the same calibrants, their studies may not be directly comparable. For comparison of data between laboratories, it is necessary to use the same calibrants.

The consequence of using different calibrants is that different *m/z* values are detected. If potential biomarkers of small molecular weight are found, it is important to calibrate with calibrants of low molecular weight; similarly, for larger proteins, calibrants with higher molecular weight have to be used.

For instance, the *m/z* values of albumin (*m/z* 66,433) and IgG (*m/z* 147,300) are approximately 500 (0.8%) and 700 Da (0.5%) lower with the All-in-protein standard than with the All-in-1 peptide standard. Dynorphin (*m/z* 2148) and human insulin (*m/z* 5808) are approximately 900 (42%) and 700 Da (12%) higher with the All-in-1 protein standard than with the All-in-1 peptide standard (own data). In fact, the best way to calculate mass accuracy is to calibrate internally because of the spot-to-spot variability. For the identification step, it is even more important to calibrate internally, because exact mass accuracy is needed for the peptide mapping. It should be noted that the Protein Biosystem II (PBSII) is a low resolution instrument and peptide mass fingerprinting is better performed on high resolution mass spectrometers. Although the PBSII can be used for peptide mass fingerprinting, the mass ranges of the measured fragments need a very large window for database searching.

Matrix

After addition of the sample and washing buffers, the EAM is applied on the ProteinChip array. The EAM facilitates desorption and ionization in the ProteinChip Reader. The molecular weight of the proteins and peptides dictates which matrix should be used. In theory, sinapinic acid (SPA) is used for proteins larger than 15,000 Da and α-cyano-4-hydroxy-cinnamic acid (CHCA) is used for the proteins and peptides smaller than 15,000 Da. In practice, SPA is used with a low and high laser intensity. The laser intensity needs to be increased by increasing molecular weights. Table 2.2 shows that all prostate studies used SPA, except the study by Petricoin et al.[11] for which no matrix information was given. The ovarian studies used SPA, except in the study by Petricoin et al.[2] in which CHCA was used. The *m/z* values found in the study by Petricoin et al. are also much smaller than the *m/z* values found in the other ovarian studies. This might explain the choice for CHCA as a matrix.
Baggerly et al.[23] found that the first two biomarkers (*m/z* 435 and 466) in the ovarian study by Petricoin et al.[2] are below 600 Da and thus questionable in terms of matrix contamination.

Post-analytical aspects

Patient population

Table 2.1 indicates that the number of patients and healthy controls in the training and validation sets of the different studies varied enormously. The reliability of the results improves with increasing numbers of patients and healthy controls in the training and test sets. For different studies a clear description of the training and validation population is essential, such as the severity of disease. Because SELDI-TOF-MS fingerprinting probably measures peptides present in high abundance in serum (e.g. mg/l to g/l range) the molecules detected probably originate from common disease mechanisms or general protection mechanisms, i.e. epiphenomena of the diseases, such as acute phase response, cachexia etc. It is clear that the robustness of the technology should be validated by comparing patient groups with comparable disease mechanisms. Method validation should therefore be extended not only to healthy controls, but also to diseases with comparable generalized disease conditions (infection, cachexia etc).

Bioinformatics and biostatistics

Peak detection, laser settings and data analysis software affect the ultimate *m/z* values found. Table 2.2 shows that the laser intensity and laser sensitivity and the number of averaged laser shots varied or were not indicated in the different

studies. Peak detection was described in minimal detail. Since different software was used in the prostate cancer studies as well as in the ovarian cancer studies it is very difficult to compare the different *m/z* values and sensitivities and specificities found in studies using the same ProteinChip array. Decision trees can be based on Area Under the ROC curves, but also on intensities making it hard to compare the results of studies using different kind of software for the final classification.
Recently Diamandis[18] compared the decision algorithms obtained in three different studies on prostate cancer and tried to explain the fact that completely different decision trees were found even when using identical chip types and comparable study populations[2,10,12]. According to Diamandis the most likely explanation for these differences is that the methods for extracting potential molecules are very sensitive to the experimental details or to serum storage conditions, even if the same extraction devices are used. We now include a comparison with other prostate studies and have included ovarian studies. The *m/z* values and the sensitivity and specificity results are completely different for all studies. Different software was used in all studies, which makes it very hard to compare these results. As for the pre-analytical strategy, the post-analytical strategy has an enormous impact on the final results. It should be noted that careful and precise selection of the peak labeling settings and normalization of peak intensities are considered critical for biomarker identification and for the efficient and reliable performance of any learning algorithm used in conjunction with the SELDI-TOF-MS system[6].

Identification

Recently Malik et al.[24] identified an isoform of apolipoprotein AII (ApoA-II) giving rise to a *m/z* 8946 SELDI-TOF-MS “peak” that is specifically over expressed in prostate disease. Immunochemistry revealed that ApoA-II is indeed over-expressed in prostate tumors. The fact that this peak was detected only in the study by Qu et al.[12] and not in the other prostate studies discussed here (Table 2.2) again shows the limited agreement between the different studies, obviously caused by differences in the experimental set-up.
Ye et al.[7] identified the α-chain of haptoglobin giving rise to a peak at *m/z* 11,700. The peak intensity was significantly higher in ovarian cancer. The candidate biomarker was purified by affinity chromatography, and its sequence was determined by liquid chromatography-tandem mass spectrometry. An antibody was generated from the synthesized peptide for quantitative validation of the patients and controls. This peak was not detected in the other ovarian studies, which used serum samples. However, Rai et al.[8] identified another haptoglobin peak in plasma samples at 9.2 kDa as a putative biomarker for ovarian cancer.

Immunoassay using SELDI-TOF MS

Wright et al.[25] described a novel SELDI-TOF-MS immunoassay using a ProteinChip platform to capture and detect prostate cancer-associated biomarkers by either binding single or two different antibodies to pre-activated chips. Four well-characterized prostate cancer-associated biomarkers, prostate specific antigen (PSA)(free and complexed forms), prostate specific peptide (PSP), prostate acid phophatase (PAP) and prostate specific membrane antigen (PSMA), were identified in cell lysates, serum and seminal plasma. This study successfully demonstrated the direct capture and detection of the four known prostate cancer biomarkers on both chemical and biologically defined chip surfaces. Xiao et al.[26] used the same SELDI-TOF-MS immunoassay and patient population as described by Wright et al.[25] to capture PSMA, followed by mass spectrometry to detect and quantify the antigen. This SELDI immunoassay format was successful in measuring PSMA in serum from normal healthy men and men diagnosed with either benign or malignant prostate disease. PSMA was captured from serum by anti-PMSA antibody bound to ProteinChip arrays. The captured PSMA was detected by SELDI-TOF-MS and quantified by comparing the mass signal integrals to a standard curve established using purified recombinant PSMA. The average serum PSMA value for prostate cancer was significantly different from that of benign prostate hyperplasia (BPH) and the control group. These results suggest that serum PSMA may be a more effective biomarker than prostate-specific antigen for differentiating benign from malignant prostate disease and warrants additional evaluation of the SELDI-TOF-MS PSMA immunoassay to determine its diagnostic utility. These two studies indicate that standardization using the same serum samples and performing the same specific immunoassay, leads to the same results with respect to PSMA.

Reproducibility

Recently, Semmes et al.[27] published the first study on reproducibility of serum protein profiling by SELDI-TOF-MS. Across-laboratory measurement of three *m/z* peaks in a standard pooled serum revealed 0.1% coefficient of variation (CV) for mass accuracy. The CV's for signal-to-noise ratio's were 34-40% and the variations in the intensities of the three peaks for all laboratories were 15-36%[27]. Using an algorithm developed in a single laboratory, all six laboratory sites achieved perfect blinded classification for all samples when boosted alignment of raw intensities was used. Although these results look promising and show that under strict operating procedures good across-laboratory reproducibility can be achieved, further examination of the reproducibility of peak finding and algorithm assessment is required. The fact that Semmes et al. demonstrated that good across-laboratory reproducibility can be achieved after instrument calibration and

output standardization, again underlines that sample collection and handling are of extreme importance in proteomic studies. Invalid sample collection seems an important source of error.

Lee et al.[28] also indicated that it is hard to reproduce experiments. They investigated renal cell carcinoma and included samples from patients with renal cell carcinoma, patients with benign urological diseases and healthy controls in the training set. An initial blind group of samples was used to test the models. Sensitivities and specificities of 81.3-83.3% were achieved. However, subsequent testing 10 months later with a different blind group of samples resulted in much lower sensitivities and specificities (41.0-76.6%). Factors such as changing laser performance and a different batch of ProteinChips might be responsible for the different results.

Baggerly et al.[23] indicated that artifacts associated with the technology could be responsible for the discrimination between cancer and healthy samples. Changes that could introduce such artifacts include differential handling and/or processing of the samples, changes in the type of ProteinChip array and mechanical adjustments to the mass spectrometer itself. Whenever possible, standard protocols should be drawn up to minimize the effect of irrelevant sources of variation to prevent major technological differences from overwhelming the biology associated with the outcome of interest. Careful experimental design can help. Randomizing the samples, can ensure that changes in the machine calibration, differences in chip quality and variations in the reagents, are not accidentally detected as biological differences. Keeping the operators blinded to the nature of the samples can also help ensure that systematic differences in processing do not occur inadvertently. Results must also be carefully calibrated and revalidated after every shift in the protocol. The same samples must be processed using both versions of the protocol, and the classification results confirmed. On the analytical side, standardization of for instance baseline correction is also important. With the current technology this is essential, since matrix distortions are often severe. After baseline correction, normalization is necessary. Normalizing for total ion current can ensure that the same amount of ion current has reached every spot.

Conclusions

The aim of this review was to discuss differences and to identify aspects important for future studies on protein profiling. The most important aspects appear to be differences in sample storage and pre-treatment, as well as the data analysis strategy. Pre-analytical strategies, such as storage conditions and sample pre-treatment, varied enormously between the different studies and the effects were highly underestimated, as was illustrated by our own data.

It is essential that sample collection from both the patient and control populations should be completely identical and accurately standardized in future studies. Because of the enormous variation between the different studies in both pre- and post-analytical aspects and the poor description of technical details and software, it is hard to find a clear explanation for the fact that completely different *m/z* values, sensitivities and specificities were found, even in studies using identical chip types and comparable study populations[9,10,12].

We conclude that protein profiling seems promising regarding the reported diagnostic values. However, it can only become a reliable diagnostic tool if in the end, it fulfils the criteria for reproducibility and standardization that are generally accepted for diagnostic tests in clinical chemistry.

The present overview clearly underlines the need for better standardization and careful description of the methods including technical details in all future studies, in order to allow comparison between studies. Moreover, the effect of pre-and post-analytical variables on protein profiling needs further and more systematic investigation.

Since different studies have shown the importance of standardization, standard protocols for proteomic studies using the SELDI-TOF-MS would be useful. A standard protocol for the collection of serum samples according to WHO (2002) is suggested. Other international organizations, such as the International Federation of Clinical Chemistry (IFCC) and Human Proteome Organization (HUPO) that are looking into some standardization issues. The HUPO Proteomics Standards Initiative (PSI) defines community standards for data representation in proteomics to facilitate data comparison, exchange and verification. PSI currently develops standards for two key areas of proteomics, mass spectrometry and protein-protein interaction data, as well as a standardized general proteomics format. Although, the IFCC and HUPO focus on standardization in proteomics studies, further recommendations for protein profiling studies with the SELDI-TOF-MS analysis are not available yet and could be very useful[29,30].

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