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Review

Cancer-specific MALDI-TOF profiles of blood serum and plasma: Biological meaning and perspectives

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ABSTRACT

MALDI-TOF mass-spectrometry has become a popular tool of cancer research during the last decade. High throughput and relative simplicity of this technology have made it attractive for biomarker discovery and validation across various platforms in blood serum/plasma. Many technical approaches have been developed for plasma/serum profiling including protein-chip based SELDI-TOF mass-spectrometry, purification of serum on magnetic beads, analysis of carrier-associated fraction and mass-spectrometric immunoassays. Extensive data about the identity of differential features detected on mass-spectra up to now makes it possible to draw conclusions about potency and perspectives of MALDI-TOF mass-spectrometry in this field. A great majority of identified differentially expressed proteins are either house-keeping or inflammatory proteins as well as their modifications or fragments. Discriminating ability of mass-spectra is likely to be based on differential modification and fragmentation patterns of abundant serum proteins reflecting activity of enzymes including proteases and their inhibitors.

Contents

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1. Introduction

Cancer is a major public health problem in many parts of the world. Early detection represents one of the most promising approaches to reduce the growing cancer burden. The promise of early detection is that it will identify cancer while it is still localized and curable, preventing mortality. Cervical cancer demonstrates an example of successful reducing of disease-related deaths due to implementation of broad screening programs. However, development of effective screening tests for other cancers is still of urgent need [1].

Therefore, a lot of effort is put to the development of efficient methods of cancer diagnostics. Blood obtained by venepuncture is the most accessible human specimen, the least invasive, and feasible to monitor over long periods of time. The blood plasma may capture proteins and their fragments released from all organs and tissues in health and disease [2].

Despite a lot of attempts in searching for biomarkers using various methods no biomarker with 100% diagnostic accuracy has been found for any cancer type. Moreover, existence of such biomarkers is doubtful due to the heterogeneous nature of cancer. Most efforts are therefore concentrated on searching for panels of differentially expressed proteins/peptides instead of individual biomarkers and construction of diagnostic methods based on several features [3]. Fast development of proteomics made it possible to analyze many proteins simultaneously and facilitated numerous attempts of cancer biomarker discovery. MALDI-TOF-mass-spectrometry has become one of the most widely used proteomic technologies in cancer investigation [4–6]. Experimental pipeline commonly includes collection of mass-spectra for cancer and non-cancer sera, searching for statistically significant differences and development of diagnostic algorithm. Working with serum/plasma is a challenge due to an extremely wide concentrational range of proteins with >98% of protein mass represented by 20 high-abundant proteins [7]. MALDI-TOF profiling has limited detection sensitivity compared to other methods [8]. For example, bottom-up mass-spectrometry based on two-dimensional liquid chromatography (LC/LC)-coupled electrospray ionization (ESI)-MS/MS of trypsinized protein mixtures is capable to penetrate several orders of magnitude into the proteomic dynamic range. However, the level of sophistication and lengthy analysis time of obtained results significantly complicate probing large sample cohorts required for reliable statistical analysis. Direct mass-spectral profiling is a high-throughput method which allows rapid profiling of pre-existing peptides in hundreds of serum samples but only skims the top layer off complex proteome and peptidome, thereby limiting discovery to abundant blood proteins and their fragments. However, mass-spectral profiling is quite simple and can be in principle adopted to point-of-care analysis [9].

The present review describes different methodologies of direct MALDI-TOF profiling of blood serum/plasma and analyzes the results of their application to cancer biomarker discovery. The problems of sample handling and preprocessing and its reproducibility are also briefly discussed. Comparative proteomic profiling using liquid chromatography-tandem mass spectrometry is reviewed in [10] and, accordingly, is not in the scope of the present paper.

2. Methodology

2.1. *Sample collection and preprocessing*

Standardization of sample collection and handling is probably the main challenge of high-throughput proteomic methods for disease biomarker discovery in human serum/plasma. There is a large list of pre-analytical variables that can influence on the analysis of blood-derived samples, including time and conditions of storage, using of protease inhibitors, number of freeze/thaw cycles. A wide-scaled research carried out within the framework of the HUPO Plasma Proteome Project concerning factors affecting the reproducibility of sample preparation generated a number of recommendations for sample handling. According to their recommendations, platelet-depleted plasma is preferable to serum for certain peptidomic studies; samples should be aliquoted and stored in liquid nitrogen; the addition of protease inhibitors is recommended, but should be incorporated early and used judiciously, as some of them form non-specific protein adducts and others interfere with peptide studies. Further, the use of reference materials for quality control and quality assurance is recommended [2].

Another research group [11] examined the effect of tube type, clotting time, transport/incubation time, temperature, and storage method on protein profiles. They established less stringent standardization requirements for proteomic studies. In accordance with their data, transport of samples on ice is the most essential, whereas it does not matter if transport times are then 3 or 6 h. Based on their results, the proteomic analysis of samples from established serum banks, where samples were not collected in accordance with more stringent protocols, can be used for proteomic biomarker studies. The key factor is that all samples in the collection should have been handled in a similar manner [11].

There is some controversy about the influence of refreezing on serum/plasma composition. Many authors report minimal changes in plasma/serum profiles after several freeze/thaw cycles [12]. However, it is still generally believed that freeze/thaw cycles change the serum/plasma composition most likely due to peptide aggregation, precipitation and adsorption to surfaces, and so refreezing of plasma/serum samples is not recommended [13].

The type of anticoagulant (citrate, heparin, EDTA) has been shown to affect the mass-spectral profiles of plasma [14]. EDTA-plasma protein profiles are the most divergent from those obtained from citrate- or heparin-plasma [12].

2.2. SELDI-TOF methodology for serum/plasma profiling

The most commonly used technology in cancer biomarker research is the protein-chip-based mass-spectral technology SELDI-TOF (Surface-enhanced laser desorption / ionization time-of-flight)—a modification of MALDI-mass-spectrometry developed specially for mass-spectral profiling of clinical specimen for biomedical purposes. It implies express-fractionation of biological samples on chips with various types of chromatographic surfaces prior to mass-spectral analysis. Those include normal phase, reverse-phase, metal-affinity, cation-exchange and anion-exchange arrays. Different subproteomes are predominantly purified on different chip surfaces. Besides protein fractionation, samples are also desalted on the chips. SELDI-MS is simple, high-throughput and fast. The procedure can be automated and reproducibility raises in this case manifold [15].

The common experimental pipeline contains obtaining mass-spectra from experimental cohort including control and diseased groups, peak detection and alignment and subsequent searching for *m/z* peaks which differ significantly between cancer and control. The diagnostic algorithm is commonly developed by machine-learning methods using MS data as input values. This approach means operating with mass-spectra as “black boxes” without the identification of discriminatory peaks. Many papers report diagnostic accuracy exceeding 90% [16–18]. However, the reproducibility of mass-spectral peaks selected in different research groups is commonly low, and it has caused controversy in the scientific society [19,20]. Nevertheless, development of cancer-specific ‘bar-codes’ is very attractive and attempts of ‘blind profiling’ are still proceeded [21–23]. Special attention is paid to quality control, using or adequate bioinformatical methods and correct experimental design.

A number of discriminative peaks have been identified by now. The most common methods of protein purification are 1D- and 2D-PAGE [24,25] and HPLC [26–28]. Identification is usually carried out by trypsinolysis followed by tandem mass-spectrometry. In a number of papers potential biomarkers are further validated by alternative methods, such as ELISA [19,26], Western-blot-analysis [19,29] and immunodepletion [27,30]. Although significant differences were observed in mass-spectral profiles obtained by different research groups, identified differentially expressed proteins are often similar for different laboratories. The list of identified potential cancer biomarkers is given in Table 1. Detailed discussion of their properties is provided in the next sections.

2.3. MALDI-TOF profiling of serum/plasma with chromatographic separation on beads

Besides the chips with different types of chromatographic surfaces, similar purification strategy is realized on beads, which consist of a magnetic core and chromatographic surface. The beads are thought to be more effective in protein purification from complex samples because of larger surface and greater binding capacity.

A well-known example of magnetic beads includes ClinProt beads manufactured by Bruker Daltonics. As for protein arrays, there are various types of magnetic beads: cation-exchange, anion-exchange, reverse-phase and metal-affinity. However, ClinProt magnetic beads did not become as popular as SELDI-MS and only a few papers reported their successful usage for cancer versus non-cancer discrimination [31–33].

Magnetic beads with reverse-phase surface SiMAG-C8/K were used by Villanueva et al. for peptide profiling [34]. They studied peptide profiles of patients with metastatic thyroid carcinoma [35], bladder cancer, breast cancer and prostate cancer [36]. Peaks differing in intensity between thyroid carcinoma samples and cancer-free controls were identified using tandem mass-spectrometry as complement C3f and fibrinogen alpha fragments [35].

Substantial differences were shown in peptidome profiles between patients with bladder, breast and prostate cancer and healthy donors [36]. More than 100 discriminative peaks were identified altogether including fragments of fibrinogen alpha, complement C3f, complement C4a, inter-alpha-trypsin inhibitor, apolipoproteins AI, AIV and E, transthyretin and some other proteins. The majority of discriminating peptides are fragments of high-abundant proteins most likely generated ex vivo, being the products of exoprotease activity. Thus, exoprotease activities superimposed on the ex vivo coagulation and complement-degradation pathways contribute to the generation of cancer-specific and even cancer type-specific serum peptide profiles [36].

A similar approach was used by Goldman and coworkers in their investigation of hepatocellular carcinoma [37,38]. Sample processing included desalting on C8 magnetic beads and ultrafiltration on 50 kDa Microcon membranes. Mass-spectra were obtained on an Ultraflex MALDI-TOF/TOF MS in *m/z* range of 0.9–5 kDa. Six independent biomarker peaks were found and two of them were identified as complement C3 fragment and complement C4a.

2.4. Methods used for depletion of serum/plasma from highly abundant proteins

The problem of high range of protein concentration in serum/plasma is partially overcome by removing the most abundant proteins. Many depletion approaches developed to date permitted to expand the number of lower abundance components that can be detected by proteomic techniques [39–41]. Albumin and immunoglobulins comprise about 90% of total serum protein [14] and are therefore the main targets for depletion. Depletion strategies include centrifugal ultrafiltration, organic solvent extraction, thermal treating and affinity chromatography.

Centrifugal ultrafiltration is based on removing the high-molecular-weight components through the semi-permeable

Table 1 – A list of potential cancer biomarkers found using direct mass-spectral profiling.

Potential biomarker	Protein accession number (UniProt)	Protein mass, Da	Cancer	Concentration, g/l (M) [14]	Expression
Apolipoprotein A1	P02647	28,043	Ovarian cancer [19,26,66], colorectal cancer [67], pancreatic cancer [68]	0.9–1.5 ($3.2–5.4\times10^{-5}$)	–
Apolipoprotein AII	P02652	8943 (Unknown modification [29]); 17,270 (C-term-Glu-missing homodimer [68]); 17,390 (Disulfide-linked homodimer [68])	Prostate cancer [29], pancreatic cancer [68]	0.3–0.5 ($3.4–5.7\times10^{-5}$)	±
Apolipoprotein C1	P02654	6631 (Full-size [27,67,145]); 6432 (N-threonine and proline-truncated [27])	Colorectal cancer [27,67], hepatocellular carcinoma [145]	0.04–0.07 ($6\times10^{-6}–1\times10^{-5}$)	+
Complement fragment C3a	P01024	8939	Colon cancer [27], B-cell lymphoid malignancy [28], breast cancer [75]	0.001–0.006 ($1.1\times10^{-7}–6.7\times10^{-7}$)	+
Complement fragment C4a	P0C0L4	8608	B-cell lymphoid malignancy [28]	0.0003–0.022 ($3.5\times10^{-8}–2.6\times10^{-6}$)	+
Transthyretin	P02766	13,761 (Full-size [19,28,30,56,66,68]);12,843 (10 N-terminal amino acids truncated form [26,56,66]); 13,841 (sulfonated TTR [28,66]); 13,881 (cysteinylated TTR [28,30,56,66,68]) ; 13,922 (TTR-CysGly [30,66]); 14,043 (glutathionylated TTR [56,66,68])	Ovarian cancer [19,26,56,66], B-cell lymphoid malignancy [28], colon cancer [56]; pancreatic cancer [68], mucosis fungoides [30], lung cancer [126]	0.1–0.6 ($7.1\times10^{-6}–4.3\times10^{-5}$)	–
Potential biomarker	**IPI**	**Protein mass, Da**	**Cancer**	**Concentration, g/l (M)**	**Expression**
Serum amyloid A1	P02735	11,683 (SAA1 alpha [24,89,111]) 11,527 (N-arginine truncated SAA1 [89]) 11,628 (SAA2 alpha [111])	Ovarian cancer [89], renal cancer [24], bone metastases in prostate cancer [93], pancreatic cancer [111], lung cancer [146]	0.003–2 ($2.5\times10^{-7}–1.7\times10^{-4}$)	+
Beta-hemoglobin	P68871	15,867	Ovarian cancer [19]; pancreatic cancer [111]	0.1–0.4 ($6.3\times10^{-6}–2.5\times10^{-5}$)	+
Haptoglobin-1-alpha	P00738	9200	Ovarian cancer [82], renal cancer [24]	0–0.37 ($0–4\times10^{-5}$) [147]	+
Transferrin	P02787	76,960	Ovarian cancer [19,82], colon cancer [27]	2–3 ($2.5\times10^{-5}–3.8\times10^{-5}$)	±
Alpha-1-antitrypsin	P01009	50,700 (Glycosylated [27,111])	Colon cancer [27], pancreatic cancer [111]	1.2–2 ($2.7\times10^{-5}–4.5\times10^{-5}$)	+
Immunoglobulin heavy chain	N/A	54,000	Ovarian cancer [82]	N/A	–
Cystatin-C	P01034	13,347	Hepatocellular carcinoma [23]	0.001–0.002 ($7.4\times10^{-8}–1.5\times10^{-7}$)	+

For reliably identified proteins average mass is given. For peaks identified as modifications of known proteins original *m/z* value is given.

membranes with different molecular weight cut-offs. The method is fast and inexpensive, however is not specific.

Organic solvent extraction is based on selective precipitation of high-molecular-weight proteins and extraction into organic phase of low-molecular-weight proteins and peptides. It has been shown that two volumes of acetonitrile added to serum/plasma samples efficiently precipitate high-molecular-weight abundant proteins, such as albumin, while smaller proteins and peptides remain in solution and can be subsequently analyzed by MS [12].

Another simple and inexpensive way of partial depletion of serum/plasma from abundant proteins is its thermal treatment with subsequent analysis of thermostable fraction [42]. It has been shown that albumin and immunoglobulin spots on 2D electrophoregrams are 2–3 fold less intensive in thermostable fraction compared to whole serum. The number of spots of less abundant proteins is increased in thermostable fraction.

Affinity chromatography is most widely used for depletion of high abundance proteins in serum/plasma as a first step in biomarker-related proteomic studies. A- and G-sepharose chromatographic columns are used for immunoglobulin G (IgG) removal. The most effective but expensive depletion strategy is based on affinity chromatography using polyclonal antibodies against several abundant plasma proteins [40,43,44].

Combination of different depletion strategies can be also used. For example, Fu et al. proposed using G-sepharose for removing of immunoglobulins and sodium chloride/ethanol precipitation for albumin depletion [45].

2.5. *Carrier-associated proteins and peptides*

Despite the goal of depletion methods in the removal of high-abundant proteins, it is well-known that they can act as carrier proteins and bind a vast assortment of peptides and protein fragments. Relatively long half-life of the carrier protein (19 days for albumin compared to a few hours or less for an unbound low-molecular-weight molecule) facilitates the prolonged presence of smaller peptides and proteins in the bloodstream [46,47] protecting them from renal clearance. It has been shown that under native conditions most of the low-molecular-weight molecules visualized by mass spectrometry form complexes with high-molecular-weight proteins [48].

Thus, carrier protein-bound fraction may possess high diagnostic information for many diseases.

Zhou et al. performed targeted isolation of each of highly abundant serum proteins by immunoprecipitation (albumin, immunoglobulin G (IgG), IgM, IgA, transferrin, and apolipoprotein AI) with subsequent using of microcapillary reversed-phase liquid chromatography coupled on-line with tandem mass spectrometry (MS/MS) for identification of peptides and proteins bound to high-abundant proteins. Low-molecular-weight proteins were separated from carrier proteins by ultrafiltration using 30 kDa filters. 210 proteins were identified and many of them were not found in previous studies of whole-serum proteome [49]. Twelve proteins detected in this study were clinical biomarkers, including prostate-specific antigen, pregnancy plasma protein A, meningioma-expressed antigen, and dihydropteridine reductase [49]. These data clearly demonstrate the diagnostic potential of protein/peptide fraction bound to major proteins.

The same idea was illustrated by Gundry et al. [50], who investigated albumin-associated peptides and proteins of human serum using anti-albumin antibody (anti-HSA) affinity chromatography and native non-denaturing size-exclusion chromatography. Combining the results from these approaches, 35 proteins, of which 24 were full-size, were found to be associated with albumin, including both high- and low-abundant. Moreover, it was shown that albumin binds proteins reproducibly and selectively. Authors conclude that it may be necessary to probe for a particular protein both the bound and unbound fractions, to obtain a total concentration for comparison among control and affected samples [50].

Lowenthal et al. carried out another profound research of albumin-associated proteins and peptides in pooled sera samples of patients with early- and late-stage ovarian cancer and high-risk unaffected individuals [47]. They used solid-phase affinity capture for albumin binding followed by separation of bound proteins by gel-electrophoresis and identification by microcapillary reversed-phase tandem MS. In total, 1208 individual protein sequences were predicted from all 3 pools, including fragments of BRCA2, a low-abundance nuclear protein linked to cancer susceptibility. These facts highlight the method potency to detect low-abundant proteins in serum.

Lopez et al. used mass-spectral profiling of albumin-bound peptides for ovarian cancer biomarker discovery. They used Cibachron blue dye affinity chromatography-based technology on ZipPlates to bind high-abundant proteins. MALDI mass spectra were obtained. Both fragments of major proteins (transthyretin, complement, fibrinogen) and less abundant proteins potentially involved in cancerogenesis (kaseinkinase 2, transgelin) were present among discriminative peaks [51]. As a result, Lopez et al. discovered biomarker panels that could distinguish the first stage of ovarian cancer from unaffected patients with no evidence of ovarian cancer, with a sensitivity of >93% and specificity of 97%.

The same approach with isolation of carrier-associated proteins was utilized for development of diagnostic methods for uterine endometrial cancer [52], ovarian cancer [53] and cutaneous T-cell lymphoma [53], however, without identification of discriminating MS peaks.

Investigation of albumin-associated peptide fraction was also successfully used to search for biomarkers of Alzheimer disease [54].

2.6. *Exoprotease activity assay*

Results on the peptidomic profiling [17,35,36] highlighted crucial role of exoprotease activity in altering mass-spectral profiles in cancer. For a more precise evaluation of exoprotease activity Villanueva et al. designed an original ex vivo test based on quantification of degradation products of foreign labeled peptide substrates [55]. The goal of the research was to test the activity of a particular, yet molecularly undefined, subset of all aminopeptidases and carboxypeptidases within a proteome that takes part in the degradation of peptide (or peptides) with defined sequences. Labeled complement fragments and fibrinopeptide alpha were used as substrates. The substrates were chosen taking into account the results of

discovery efforts discussed above that involved comparative peptidomic analysis of sera from patients with different cancer types and control sera [35,36]. These peptides were incubated for a fixed period of time and partly digested by exoproteases present in samples. Quantitative evaluation of each degradation fragment was done by comparison of ion intensities or areas under the curve of the corresponding *m/z*-peaks with those of double-labeled reference peptides used as internal standards and spiked into the samples at known absolute concentrations simultaneously with substrates. The reference peptides were comprised by D-amino acids and were therefore fully resistant to any proteolytic degradation. After processing of mass-spectra, all peptide pairs were assigned and ratios calculated.

By simultaneous adding of the double-labeled reference peptides and substrates, equal adsorptive and processing-related losses of compared peptide fragments were guaranteed. Importantly, sequence-specific exopeptidase activity test has been shown to be much more tolerant of serum preparation variability such as clotting time, times 'left-on-the-bench' and the number of freeze/thaw cycles.

Diagnostic potential of the test was demonstrated using a sample set consisting of sera of 48 patients with metastatic carcinomas and from 48 age- and gender-matched healthy controls. Using SVM for model generation and leave-one-out cross-validation class prediction sensitivity 94% and specificity 90% were obtained [55]. Unfortunately, the sample set used in the research lacked sera of patients with benign and inflammatory conditions. Thus, diagnostic utility of this approach needs further validation.

2.7. *Mass-spectrometric immunoassay: analysis of modification patterns*

One of the most valuable properties of mass-spectral methods is their ability to distinguish between different covalent modifications of proteins, which cannot be done by immune methods, such as traditional ELISA. Systemic response to tumor invasion can be accompanied by altered activity of enzymes and therefore, altered modification patterns of target proteins, so investigation of modification patterns of proteins can be beneficial for cancer diagnostics development. Crucial role of modification pattern was demonstrated for example, by Miguet et al. in their research of B-cell lymphoma. Discriminative mass-spectral peaks with *m/z* 13,766 Da, 13,876 Da and 13,876 Da were identified as unmodified, cysteinylated and sulfite transthyretin respectively. The first two peaks in average decreased intensity in B-cell lymphoma patients and the last peak increased intensity compared to control. Despite clear difference in modification pattern, transthyretin level measured using ELISA had no significant difference between control and cancer groups [28].

For targeted analysis of modification patterns of distinct proteins immune-affinity purification can be combined with mass-spectral analysis. This methodology does not implicate cancer biomarker discovery; target proteins for immunoassay construction are chosen based on the previous experience.

This approach was utilized by Fung et al. for investigation of TTR modification pattern in sera of patients with ovarian and colon cancer. Earlier this research group showed that TTR level is reduced in both types of cancer [26]. However, mass-spectral analysis of transthyretin modifications using immunoaffinity chips showed decreased intensity of cysteinylated and glutathionylated TTR forms only in ovarian cancer sera, whereas unmodified and truncated TTR were decreased in both cancer types [56].

Song et al. investigated the fragmentation pattern of inter-alpha-trypsin inhibitor heavy chain (ITHC4) in sera from patients with ovarian cancer (III–IV stages), breast cancer, colon cancer, prostate and pancreatic cancer [57]. Sera were incubated with beads carrying antibodies against ITHC4, eluate was applied to SELDI chips and mass-spectra obtained. The digestion profile in proline-rich region of ITHC4 differed substantially in patients' sera with different cancer types. It was also shown that fragment ratio was not influenced by sample preparation (incubation at 4 °C and at room temperature, freezing and unfreezing).

2.8. *Mass-spectral analysis of serum digested by trypsin*

Trypsin digestion of serum with subsequent fractionation and MS/MS analysis is the classical scheme of the 'bottom-up' approach in proteomics [7,10]. This approach is very powerful because of the high sensitivity and capability of protein identification. However, this methodology in most cases is much more complicated both in sample preparation and in bioinformatical analysis of obtained data. Besides, much information about modification pattern of full-size proteins can be lost during digestion. For these reasons, there are only a few publications on biomarker searching using the bottom-up approach. Common bottom-up methods are based not on MALDI-TOF mass-spectrometry but on electrospray mass-spectrometry. Discussion of these methods is beyond the object of the present review. However, direct MALDI profiling of serum tryptic digests for discriminating cancer versus non-cancer was also carried out.

Matharoo-Ball et al. utilized a simplified approach of bottom-up analysis by direct MALDI profiling of serum tryptic digests [58]. They compared mass-spectral profiles of sera digested by trypsin obtained from patients with metastatic IV stage melanoma and unaffected persons. As a result three discriminative peaks were identified, two of which were fragments of acid glycoprotein alpha 1 and the third was a fragment of complement C3 [58]. However, it is necessary to note the incorrect experimental group design because it lacked early-stage cancer or benign conditions.

3. Full-size proteins identified as potential cancer biomarkers

All proteins identified by using MALDI-TOF mass-spectrometry (mostly, SELDI-MS) can be roughly divided into two groups: acute-phase proteins (complement fragments, acute-phase serum amyloid, alpha-1-antitrypsin) and house-keeping proteins, predominantly transport proteins (apolipoproteins, transthyretin, hemoglobin, transferrin). Alteration in concentrations of the acute-phase proteins is most likely caused by inflammation which comes along with malignization process [59–61]. Alterations in concentrations of the second group of

proteins may be the consequence of metabolic stress. Most of the biomarkers found are multifunctional proteins, and some of them influence directly or indirectly initiation and progression of malignant tumors.

There is a belief, that in inflammation the liver activity is switched to production of great amount of acute-phase proteins, and as a consequence synthesis of visceral proteins including albumin, transthyretin and transferrin is suppressed [62,63].

Below we will briefly characterize proteins identified by MALDI-TOF profiling with special emphasis to inflammation and cancer-related properties.

3.1. *Apolipoproteins*

Apolipoprotein A1 is the main component of high-density lipoproteins (HDL). It is synthesized in hepatocytes, activates plasma lecitin-cholesterol-acyltransferase facilitating etherified cholesterol incorporation into HDL [64]. Apolipoprotein A1 is well-known as a negative marker of inflammation, its concentration decreases more than 25% during inflammation [65]. Apolipoprotein A1 was shown to be a specific inhibitor of cytokine production by monocytes and macrophages in response to interaction with stimulated T-cells. According to Burger and Dayer, ApoA1 is a constitutive anti-inflammatory factor, and the decrease in HDL-associated ApoA1 level may be a signal of chronic inflammation progression [65]. It was shown that ApoA1 concentration in blood is reduced in different types of cancer. Using SELDI-MS ApoA1 was identified as a potential biomarker of ovarian cancer [19,26,65,66], colorectal cancer [67], and pancreatic cancer [68]. Surprisingly, different types of chromatographic surface were used for profiling: metalloaffinity IMAC-Cu [26,66], cation-exchange [67,68] and anion-exchange [19]. Decrease in ApoA1 level in sera of patients with breast cancer [69] and hepatocarcinoma [70] was shown using 2D-electrophoresis.

Apolipoprotein AII is the second abundant component of HDL. ApoAII exists in human blood as a homodimer with subunits bound by 6-th amino acid residues [71]. A discriminative mass-spectral peak with *m/z* 8943 Da increased in sera of patients with prostate cancer was identified as a modification of ApoAII [29]. In sera of patients with pancreatic cancer intensity of mass-spectral peak of ApoAII dimer was decreased [68].

Apolipoprotein CI is a component of very low-density lipoproteins, high-density lipoproteins and chylomicrones. It was shown to activate plasma lecitin-cholesterol-acyltransferase [71]. ApoCI contains highly conservative sequence *KVKEKLK*, having a potency to bind lipopolysaccharides, components of outer membrane of gram-negative bacteria. It was shown on mice that ApoCI binds lipopolysaccharides therefore recruiting macrophages and enhancing immune response [72].

Decreased level of ApoCI and its N-Threonine and Proline-truncated form in serum was shown using SELDI-MS profiling for patients with colon cancer [27,67].

3.2. *Complement fragments*

Complement fragments C3a and C4a, also called anaphylotoxins, are proteolytic cleavage products of complement proteins C3 and C4. Anaphylotoxin C3a posseses anti-inflammatory activities, realized by its binding with G-protein-associated protein. It causes smooth muscle contraction, increases blood vessel permeability and recruits monocytes and neutrophyls to the site of inflammation and triggers macrophages to produce active forms of oxygen [73]. Anaphylotoxin C4a possesses much weaker anti-inflammatory properties compared to anaphylotoxin C3a and its receptor remains yet unknown [74].

Complement C3a fragment was identified by mass-spectrometry as a potential biomarker of B-cell-lymphoid malignancy [28], colon cancer [27], breast cancer [75] and hepatocellular carcinoma [25]. Complement C4a fragment was shown to be differentially expressed in B-cell-lymphoid malignancy [28].

3.3. *Haptoglobin*

Haptoglobin is a plasma sialoglycoprotein capable of binding hemoglobin with high affinity. There are several forms of haptoglobin different in structure, molecular weight and properties. Some of them are high-molecular-weight products of polymerization [76]. The main function of haptoglobins is hemoglobin binding. The Hb–Hpt complexes are recognized by receptors on hepatocytes, internalized and degraded by lysosomes. Hpt reduces the loss of iron, because the Hb–Hpt complex is not filtered through the glomeruli but is transported to the liver.

Haptoglobin is also a well-known acute-phase protein; it has antibacterial activity and plays a role in modulating many aspects of the acute-phase response. Hpt also acts as an antioxidant preventing Hb-mediated hydroxyl radical formation [76,77].

Haptoglobin is synthesized predominantly in liver in response to cytokines such as interleukin-6, interleukin-1 and tumor necrosis factor [76]. Differential expression of Hpt as well as its fragments and different glycoforms has been demonstrated for various cancer types [78–81] using 2D-electrophoresis [81], LC-MS and affinity methods [78,79,81].

Using SELDI-MS increased intensity of mass-spectral peak with *m/z* about 9200, which was identified as haptoglobin fragment, was shown in ovarian cancer [82] and renal cancer [24].

3.4. *Acute-phase serum amyloid A*

Acute-phase serum amyloid A (A-SAA) is an ancient serum protein with a wide spectrum of biological activities. In humans A-SAA is encoded by two genes with high homology: *SAA1* and *SAA2*, each of which has a number of allelic forms [83].

It is well-known that A-SAA level in blood elevates in several orders of magnitude in inflammation. Several decades ago elevated level of A-SAA in blood of patients with malignancies [84,85], including lung [84], prostate [86] and colorectal cancer [87] was found. A-SAA level in blood was shown to be correlated with disease stage [84], tumor volume [88] and metastasis [84,86]. In case of gastric cancer A-SAA was shown to be a sensitive marker of recurrence regardless of the secondary localization [88].

Association of high A-SAA concentration and disease prognosis was also studied. Biran et al. showed that for patients with late-stage cancer with A-SAA concentration more than 0.01 g/l average lifetime is shorter compared to those with A-SAA concentration less than 0.01 g/l [84]. According to the data from Chan et al., patients with A-SAA concentration in blood more than 0.097 g/l have 4-fold increased fatality risk [88]. These observations say for possible A-SAA participation in cancer progression.

Using proteomic methods A-SAA was shown to be differentially expressed in ovarian [89], lung [90,91], renal [24,67] and nasopharyngeal [92] cancer. Le et al. identified A-SAA as a potential biomarker of bone lesions in prostate adenocarcinoma [93]. For lung cancer progressive increase of A-SAA concentration with cancer stage was shown as well as A-SAA concentration decrease after tumor rejection or chemotherapy [91].

Although the main site of A-SAA synthesis in human is liver [94], A-SAA can be produced in malignant tumor as well; it was shown for colon cancer [95] and trophoblast-like choriocarcinoma [96].

A number of A-SAA properties and interactions indicate its possible role in cancer initiation and progression [97,98]. On synoviocytes it was shown that A-SAA can induce expression of transcription factor NFkB [99], which plays a key role in apoptosis suppression [100], and matrix metalloproteases MMP1, MMP3 and MMP9, contributing to intracellular matrix degradation and therefore promoting angiogenesis and metastasis [101]. All these interactions are realized by means of G-protein associated receptor FPRL1/LXA4R [99].

Recently, A-SAA has been shown to enhance the plasminogen activation (PA)-activity mediated by urokinase-type plasminogen activator (uPA) in colon cancer cell line [102]. The uPA is over-expressed in a number of cancers including colon, breast [103], gastric [104] and prostate [105] cancers and it is thought to contribute to the invasiveness of many carcinomas [105,106]. It was shown that increased concentration of uPA in breast cancer correlates with poor disease prognosis; in Germany uPA is recommended for clinical use for choosing breast cancer therapy strategy [107].

3.5. *Alpha-1-antitrypsin*

Alpha-1-antitrypsin (A1AT) is a member of the serpine family of serine protease inhibitors and is the main serine protease inhibitor in plasma. By inhibiting elastase and other leukocyte secretary serine proteases A1AT possesses anti-inflammatory properties. A1AT is known as an acute-phase protein [108], which serum concentration raises in a wide spectrum of inflammatory diseases including malignancies. Elevation of A1AT concentration in serum was shown for lung [109], prostate [109], ovarian [110], colon [27] and pancreatic [111] cancers.

Alpha-1-antitrypsin is synthesized predominantly in hepatocytes and also in macrophages and epithelial cells [112]. A1AT was shown to be synthesized in ovarian cancer cells [110] and in breast cancer cell-line MCF-7 [113].

Information about A1AT role in cancerogenesis is ambiguous. It was shown that people with reduced A1AT activity in blood due to mutations in *A1AT* gene (homozygotic Z-allele of *A1AT* gene leads to A1AT deficiency) have a susceptibility to hepatic and bladder cancer [114]. Among patients with bladder cancer Z-allele frequency is approximately 3-fold higher compared to control group [115]. A1AT was shown to inhibit cell proliferation by reducing cancer cell iron uptake in erythroleukemic cells and retyculocytes [116]. On breast cancer cell-line MCF-7 A1AT was shown to block secretion of transforming growth factor alpha therefore inhibiting cell growth on soft agar [117]. However, Petrache et al. showed on human lung cancer cell line and on mice in vivo, that A1AT can inhibit apoptosis in endothelium cells acting on caspase 3 [118].

A1AT was also shown to stimulate fibroblast proliferation and procollagen synthesis [119].

3.6. *Transthyretin*

Transthyretin (TTR) is the main transporter of thyroxin and triiodothyronine in blood and contributes to rethinol transport by interacting with rethinol-binding protein [120]. It is well-known that transthyretin concentration decreases in inflammatory conditions and insufficient nutrition [62,63,121–123].

Reduced TTR level in ovarian cancer patients was shown quite long ago, and the negative correlation between TTR blood level and tumor volume was demonstrated. TTR level in blood usually increases after chemotherapy in accordance with tumor volume reduction [124]. It was found out that lower TTR level leads to less optimistic prognosis after cytoreductive operations. Much more frequent postoperative complications up to lethal outcome were observed when TTR level was less than 0.1 g/l (7.7×10^{-6} M) [125].

Transthyretin and its modifications were identified by direct mass-spectral profiling as biomarkers of ovarian cancer [19,26,56], B-cell-lymphoma [28], colon cancer [56]; pancreatic cancer [68], mycosis fungoides [30] and lung cancer [126]. Decreased level of transthyretin in ovarian cancer sera, but not in sera of patients with breast cancer or uterus cancer was found also using 2D-electrophoresis [42].

TTR is a sensitive marker of metabolic stress, its utility as a cancer biomarker is doubtful. However, it was shown that transgenic mice lacking TTR expression have dramatically reduced retinol and retinol-binding protein levels, and it is associated with increased malignant transformation rate of ovarian epithelium [127,128].

3.7. *Transferrin*

Transferrin (Tf) is a glycoprotein whose main function is iron transport. The principal site of transferrin synthesis is liver [129]. However, it is also synthesized in some malignant tumors: small-cell lung cancer [130] and malignant T-cell lymphoma [131]. There is an assumption that Tf can act as an autocrine growth factor [130]. Neoplastic cells were shown to synthesize great amounts of TfR and therefore they have enhanced capability of iron uptake [132].

Transferrin was reported to stimulate angiogenesis by promoting endothelial cells migration [133]. Iron-bound transferrin was shown to inhibit apoptosis in ovarian cancer cells [132].

Tf was identified as a biomarker of various types of cancer including ovarian cancer, breast cancer and colon cancer. Ovarian cancer patients have reduced Tf level in blood as established by mass-spectral data [19,82] and 2D-electrophoresis

Table 2 – A list of precursor proteins of peptide potential biomarkers found by mass-spectral profiling.

Precursor protein	Protein accession number (UniProt)	*m/z* values	Cancer type
Apolipoprotein AI	P02647	1971.2; 2052.9; 3182.5	Bladder cancer [36]
Apolipoprotein AIV	P06727	1771.8; 1927.9; 2755.2	Bladder cancer [36]
		2508.2	Breast cancer [36]
Apolipoprotein E	P02649	2409.1; 2565.5	Bladder cancer [36]
Casein kinase 2, polypeptide alpha 1	P68400	1966.9	Ovarian cancer [51]
Clusterin	P10909	822.4;	Bladder cancer [36]
		1277.7	Prostate cancer and bladder cancer [36]
Coagulation factor XIII, polypeptide A1	Q9BX29	2602.2	Prostate cancer and breast cancer [36]
Complement C 3	P01024	942.4	Bladder and breast cancer [36], thyroid carcinoma [35]
		1055.6	Prostate cancer and bladder cancer [36]
		1562.8	Breast cancer [36]
		1751.9	Bladder cancer [36]
		1865.0	Prostate cancer, bladder cancer and breast cancer [36], ovarian cancer [51], thyroid carcinoma[35], hepatocellular carcinoma[37]
		1690.9; 1777.9	Prostate cancer and bladder cancer [36], ovarian cancer [51], thyroid carcinoma[35]
		2021.1	Ovarian cancer [51]
		1449.8	Prostate cancer and bladder cancer [36], thyroid carcinoma[35]
		1211.7	Bladder cancer [36], thyroid carcinoma[35]
		1739.9	Breast cancer [36], hepatocellular carcinoma[37]
		1498.9	Bladder cancer [36]
		1762.9; 1896.0	Bladder and breast cancer [36]
D-amino acid oxidase	P02671	2115.1	Ovarian cancer [51]
Fibrinogen alpha chain	Q8N3Y3	1263.6;1536.7	Prostate cancer, bladder cancer and breast cancer [36]
		758.5; 1077.5; 1206.6; 3190.4	Prostate cancer and bladder cancer [36]
		2768.3; 2816.3; 2931.2	Bladder cancer [36]
		3240.0	Prostate cancer and bladder cancer [36], ovarian cancer [51]
		2379.0	Bladder and breast cancer [36]
		2659.0	Breast cancer [36]
		1350.6	Prostate cancer, bladder cancer and breast cancer [36], thyroid carcinoma[35]
		1020.5; 1465.7	Prostate cancer and bladder cancer [36], thyroid carcinoma[35]
		905.5	Bladder and breast cancer [36], thyroid carcinoma[35]
		2771.1; 5901.7	Thyroid carcinoma[35]
Glycosyltransferase-like protein LARGE2	Q14624	1224.7	Ovarian cancer [51]
Inter-alpha-trypsin inhibitor heavy chain 4	P35908	998.5; 2115.0	Prostate cancer, bladder cancer and breast cancer [36]
		2183.9	Prostate cancer and breast cancer [36]
		3971.0	Bladder cancer [36]
		842.4; 2724.5; 3156.5	Prostate cancer and bladder cancer [36]
		1786.9;	Breast cancer [36]
		2582.4;	Ovarian cancer [51]
		2880.5;	Breast cancer, pancreatic cancer [57]
		3141.6	Breast cancer, prostate cancer, pancreatic cancer [57]
		4283.2	Breast cancer [57], pancreatic cancer [57,111]
		3956.0	Pancreatic cancer [57]
		3272.5	Bladder cancer [36], breast cancer, prostate cancer, pancreatic cancer [57], ovarian cancer [26]
		3027.6	Ovarian cancer [51], breast cancer and pancreatic cancer [57]
		2724.5	Prostate cancer and bladder cancer [36], breast, prostate and pancreatic cancer [57]
		2627.5	Bladder cancer [36]; ovarian, breast and pancreatic cancer [57]

(continued on next page)

Table 2 (*continued*)

Precursor protein	Protein accession number (UniProt)	*m/z* values	Cancer type
Inter-alpha-trypsin inhibitor heavy chain 4			
		2358.1	Breast cancer [36,57]; prostate and pancreatic cancer[57]
		2271.1	Prostate cancer and bladder cancer [36], breast and pancreatic cancer [57]
		3156.6	Prostate and pancreatic cancer [57]
Keratin 2A	P01042	1041.7	Ovarian cancer [51]
Kininogen 1	P02766	904.5; 1060.6;	Bladder and breast cancer [36]
		2209.1	Bladder cancer [36]
		1943.9	Prostate cancer and bladder cancer [36]
Transthyretin	Q01995	2451.1;	Breast cancer [36]
		2898.5	Ovarian cancer [51]
Transgelin	P02763	2345.2	Ovarian cancer [51]
Alpha-1 acid glycoprotein	P02763	1160.8; 1753.2	Melanoma [58]

[134], but patients with breast [69] and colon [27] cancer had enhanced level of transferrin.

3.8. *Cystatin C*

Cystatin C is a secreted inhibitor of cysteine proteases [135]. It is known to be a marker of glomerular fitration [136]. Increased level of cystatin C in serum was shown by ELISA for patients with melanoma and colon cancer [137]. Cystatin C was also shown to be a potential marker for relapse in patients with non-Hodgkin B-cell lymphoma [138]. Cystatin C was shown by SELDI-MS profiling to be increased in sera of patients with hepatocellular carcinoma [23].

The data concerning identified differentially expressed proteins permit us to draw some conclusions. First, all described proteins are abundant serum proteins rather than products of cancer cells. All found biomarkers are present in blood in rather high, commonly micromole concentrations. Second, most biomarkers are not specific for a distinct cancer type, and their concentration differs in the same direction in different cancer types. In this regard transferrin is an exception because its concentration is increased in colon cancer [27] and breast cancer [69], whereas decreased in ovarian cancer [19,82,134]. And finally, many potential biomarkers found using mass-spectral methods represent modified proteins or protein fragments (fragment of inter-alpha-trypsin inhibitor heavy chain, apolipoprotein CI fragment, cysteinylated, glutathionylated and truncated forms of transthyretin, haptoglobin fragment), demonstrating the perspective of targeted investigation of modification patterns.

4. Peptide fragments and how they arise

It is well-known that cancer is characterized by an altered activity of many proteases, and this property can be used for diagnostic purposes [139–141]. Most of the enzymes themselves are low-abundant proteins, especially during early stages of cancer, and therefore cannot be detected in traditional MS-based profiling schemes. However, low-molecular-weight spectral region can reflect protease activity and is thought to be a rich source of potential biomarkers.

Accumulation of selected peptides may depend on pathways of proteinase activation that are affected by pathophysiologic processes in cancer. As demonstrated by Villanueva et al., many discriminative *m/z* peaks correspond to products of ex vivo exoprotease activity [36].

The list of precursor proteins of peptide biomarkers discovered by MALDI-TOF profiling of serum/plasma with different sample preprocessing is given in Table 2. Although the MS detection sensitivity is higher for low-molecular-weight molecules, most of the peptides identified as potential biomarkers correspond to digestion products of highly abundant proteins, such as fibrinogen, complement components and apolipoproteins.

5. Concluding remarks

MALDI-TOF profiling is no longer thought to be helpful in searching in blood serum/plasma for cancer-specific proteins produced by cancer tissue. Differentially expressed proteins should rather be searched directly in cancer tissue with subsequent validation in serum/plasma by quantitative methods—either traditional immunoassays or mass-spectrometric-based assays such as multiple reaction monitoring [4,142]. Nevertheless, the idea of utilizing sets of discriminative *m/z* values as multiplex biomarker still exists. Diagnostic potential of mass-spectral profiles has been demonstrated in numerous researches.

MALDI-MS is fast and can be in principle adapted to point-of-care analysis. The main problem to be solved is reproducibility. Composition of truncated forms of proteins and peptide fragments in blood is highly dependent on sample collection. Therefore, standardization of sample collection and handling is strictly required [143]. Validation groups should be carefully designed [144] and involve thousands of individuals. They should include samples from patients with different pathologies such as benign tumors, acute conditions and cancers.

The future of proteomic profiling of serum/plasma for medicine is most likely associated with the increasing of sensitivity and overall diagnostic accuracy along with increasing throughput of mass-spectral platforms and deepening in the proteome by using advanced methods of depletion and affinity enrichment for targeted analysis of modification patterns.

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