Chapter

Mass Spectrometry for Cancer Biomarkers

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Abstract

Cancer is considered as the second cause of morbidity and also mortality, after cardiovascular diseases. Despite the immense progress in efficacious biomarkers made in the present time, there are very few of them that can timely detect cancers or that can predict treatment outcomes or stratify patients according to their response to the treatment. Among other modern instruments involved in the research of this disease, proteomics emerged strongly, since it analyzes the "molecular effectors." Although it has some setbacks (like the lack of amplification of the signal), it is however one of the best means of investigating the presence and causes and predicting the evolution patterns of the disease. This chapter describes briefly pre-analytical (pre-MS steps), the main concepts, and the MS equipment used for such applications, followed by the presentation of several proteomic applications in melanoma, glioblastoma, pancreatic cancer, and colon cancer.

Keywords: proteomics, biomarkers, melanoma, glioblastoma, pancreatic cancer, colorectal cancer

1. Introduction

Cancer is a major challenge for health, representing the second leading cause of death worldwide. While certain progress was made in diagnostics and in some therapies for different cancers, there is still an urgent need for consistent efforts in order to set up new biomarkers for the diagnostics, prognosis, or stratification of patients. Efforts have been made to identify potential biomarkers by different approaches, like the use of techniques of genomics, transcriptomics, metabolomics, or proteomics. While the nucleic acid-based techniques present the advantage of the amplification potential, but lack the "effector" role, proteomics is considered more relevant, since proteome is directly involved in producing effective biological effects, yet it lacks the potential of amplification of nucleic acids. So, the detection of modifications, quantitative and/or qualitative, mainly affecting, for instance, low abundance proteins in biological fluids, requires increased sensitivity equipment and eventual strategies to eliminate the high-abundant and less significant ones. All these represent a major challenge of today's proteomics and will be briefly reviewed.

2. Preliminary steps

One major concern in proteomics is sample preparation, since it is generally accepted that this step is generating most of the false discoveries in the field. Therefore, we are briefly referring to this step, as well as to pre-MS steps, which are dedicated to reducing the sample complexity. Even if we do not address a total proteome, the analysis of a plasma proteome or a cellular proteome generates a large amount of data. Thus, different strategies of pre-separation are used in the proteomic analysis.

Depending on the biological materials used as start materials, it is possible that additional depletion technologies are needed, in order to reduce/eliminate major proteins (such as serum albumin or immunoglobulins).

One of the best technologies established uses an electrophoretic separation step to separate proteins; in earlier years, even the old one-dimensional sodium dodecyl sulfate (1D SDS) gel electrophoresis was used for the purpose. The next step in this approach was the use of 2D electrophoresis, which is more efficient since it uses the combination of isoelectric focusing and mass separation of proteins. The critical aspect is the time and material consumption, since for each condition one needs several runs to provide statistically significant results. Finally, the more advanced two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) was developed, which provides a significant reduction of runs (since on each gel three distinct conditions can be analyzed, such as sample, control, and sample-control mixture), and has about two orders of concentration lower. This last approach affords a good detection of low abundance proteins.

Another means of achieving the first separation of proteins is provided by capillary electrophoresis (CE). In combination with mass spectrometry (MS), this technique allows the detection of proteins [1], as well as glycoproteins [2]; besides proteomics, the technology provides in combination with MS solid platform for metabolomics studies [3, 4].

More recently, a distinct strategy was adopted, consisting in the proteolytic fragmentation of the proteins in samples (i.e., using trypsin), followed by the isoelectric focusing of the resulting peptide samples. The process usually uses single-use devices that are based on very narrow pH steps for focusing. Usually, such devices serve as the "feeder" for the mass spectrometry device, and one can obtain a complete picture of the proteins present in the sample.

3. Mass spectrometers for proteomics

Over the past decades, mass spectrometry-based proteomics was developed as a fundamental tool in identifying clinically relevant biomarkers for disease progression and assessing the organism response to treatment, with better outcome for patients.

Generally, proteomics refers to any kind of large-scale characterization of protein species in a sample given by an analytical approach, yet due to its extensive use, proteomics becomes quite synonymous with mass spectrometry analysis for protein identification, localization, interaction, abundance, and posttranslational modifications lately [5]. More specifically, in terms of biomarker discovery, MS proteomics usually indicates proteome characterization and comparison among various disease-stage models and healthy subjects.

Compared with the genomics or transcriptomics technologies, proteomics remained for years limited in terms of throughput and depth [6]. This is due to the fact that unlike nucleic acids, proteins cannot be easily amplified which limits the detection of the least abundant species in complex biological samples.

Another challenge is due to protein posttranslational modifications (PTMs). PTMs increase the complexity and dynamic range of proteome samples, compared to the genome samples, by creating a diversification that reach millions of modified protein products given the existence of protein isoforms, alternative splicing, somatic DNA rearrangements, or proteolytic events [5].

Although recent technological improvements in instrumentation also allow the analysis of intact proteins and protein complexes [6–9], there is still limited progress in adopting top-down MS strategy in a large-scale analysis of proteomes. As a consequence, the bottom-up strategy remains the main approach in MS-based proteomics [10]. In bottom-up workflows, proteins are first isolated from the biological sample, digested using single or multiple proteases to peptides (depending on the aim of the experiment), and then analyzed by mass spectrometry [11].

Due to proteome complexity, most often samples are subject to online separation in nano- or capillary C18 reversed-phase systems and even for higher dimensionality to offline or online fractionation using different chemistries at the protein or peptide level [12].

Traditionally, ion traps have been employed in discovery studies, in contrast to validation steps where triple quadrupole-type instruments have been used for targeted quantification in a larger number of samples [13].

Ion traps can store ions by applying multiple electrical fields from different directions either effectively "catching" the ions for further fragmentation or ejecting them for detection [14].

Nowadays, electrospray ionization (ESI) MS constitutes one of the most powerful analytical techniques, being capable of differentiating the pattern, mostly of proteins, between tumor and normal adjacent tissues, thus facilitating the elucidation of human tumor molecular fingerprint [15] and understanding the molecular alterations that take place during pathological processes.

ESI is a modern ionization technique that brought a big advantage in mass spectrometry since it can ionize large nonvolatile molecules without denaturation, keeping non-covalent, receptor-ligand complexes intact. As a soft ionization method, which takes place at atmospheric pressure, ESI causes little or no fragmentation of the molecules. The principle of the ESI technique is relatively simple: the sample dissolved in a polar solvent and possibly with added electrolytes up to a concentration of approximately 0.001–10 mM and passes at a slow rate (0.1–30 μl/min) through a very thin capillary (10–100 μm) held at a high potential (2–5 kV) that has an open end with a sharply pointed tip. Several parameters, among them the solvent, capillary shape, desolvation gas pressure and composition, and distance between the capillary and the counterelectrode, were found to directly influence the ESI potential value. In ESI process, a fine spray of highly charged droplets is generated and directed toward the counterelectrode. The droplets, usually smaller than 10 μm across, contain both solvent and analyte molecules. Further, by passing the droplets through a heated inert bath gas, i.e., nitrogen, the desolvation process occurs up to the moment when the surface tension can no longer sustain the charge (the Rayleigh limit). Subsequently, a Coulomb explosion takes place (**Figure 1**), generating the droplet disruption concomitant with singly or multiply charged analyte ion formation [16].

In ESI, the charge state of an analyte depends on the molecular structure and also on the ionizability of the molecule in a given solvent, and hence on the solvent pH, concentration, and composition [17]. In the case of small molecules (molecular weight below 2 kDa) such as metabolites, drugs, amino acids, glycans, and short peptide chains, only singly to triply charged ions are formed, while for large molecules like polypeptides and proteins (molecular weight exceeding over 2–3 kDa), multi-charged ions are mostly generated in a positive ion mode. Therefore, ESI mass spectra display multiple signals associated with species with multiple charge

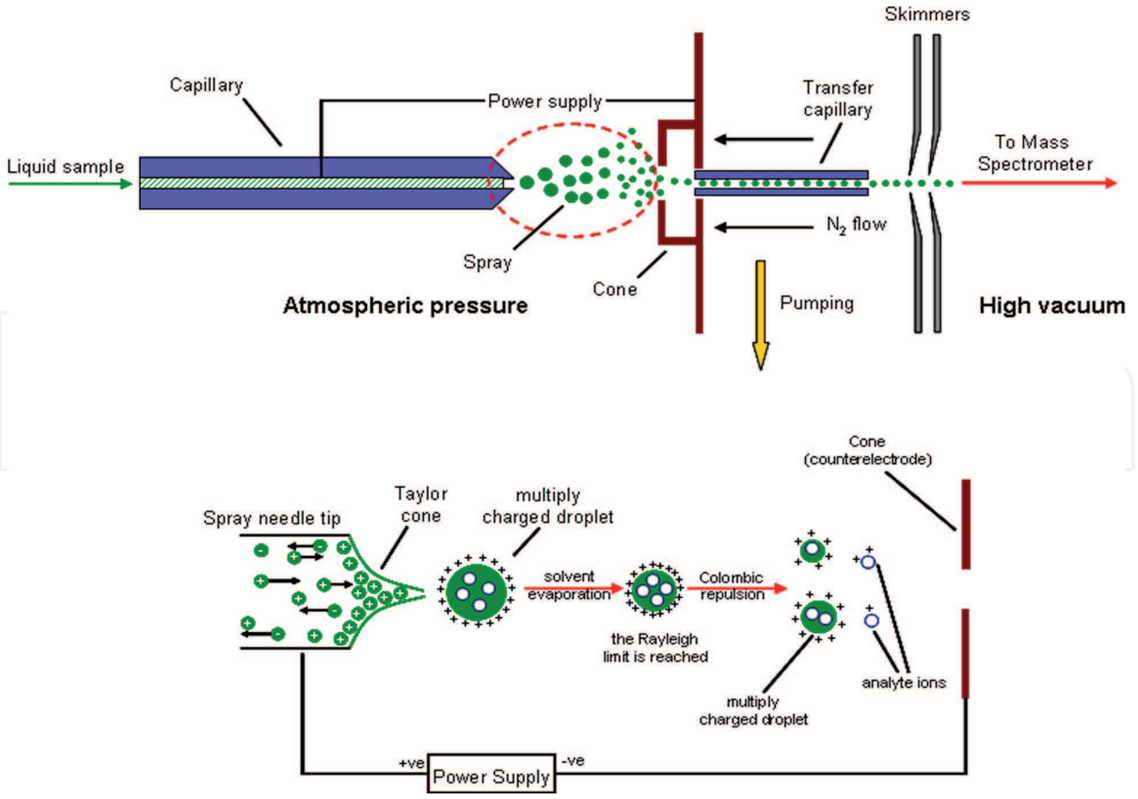

Figure 1.
The main physicochemical processes of electrospray ionization in positive ion mode.

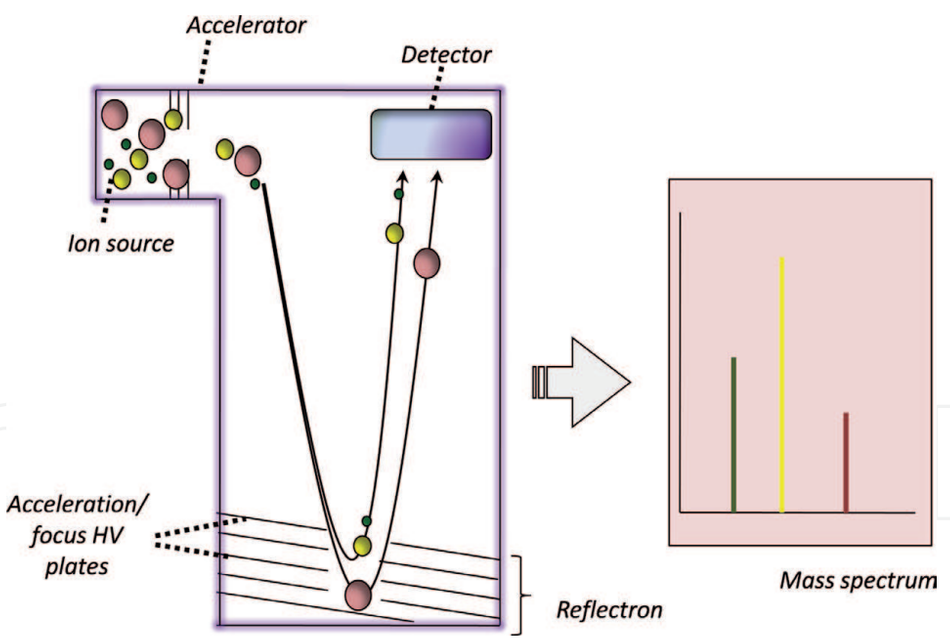

Figure 2.
Principles of ion separation in a time-of-flight analyzer.

states [17–19]. Although it brings complexity to the interpretation of the spectra, the generation of preponderantly multiply charged ions, either positive $[M + nH]^{n+}$ or negative $[M - nH]^{n-}$, represents a major advantage as it decreases the *m/z* ratios. Thus, larger molecules, i.e., biopolymers such as polypeptides, proteins, nucleic acids, anticancer drugs, etc., can be characterized by MS, using almost any type of analyzer with which ESI is compatible (**Figure 2**).

Besides ESI, one of the most frequently used ionization sources in cancer protein biomarker discovery is matrix-assisted laser desorption/ionization (MALDI).

Often considered harmonizing to its sister "soft ionization" technique ESI, MALDI is based on analyte molecule co-crystallization with an excess of matrix material (mainly small organic molecules, present in 100–100,000-fold excess compared with sample concentration). After the co-crystals were hit by pulsed laser beam (several pulses per nanoseconds), the matrix absorbs the energy of the laser; it releases the energy into the sample as heat, causing the sample to vaporize and form ions [20]. Typical MALDI devices are equipped with UV N_2 lasers with an emission wavelength of 337 nm.

The most challenging step when MALDI is used as ionization source is the selection of the optimal matrix. The matrix must fulfill a series of requirements, such as (i) to provide homogenous co-crystals with the analyte molecules, (ii) to be stable under high vacuum to prevent its sublimation, (iii) to deliver a high absorbance at the emission wavelength of the laser, (iv) to offer a high signal-to-noise ratio for the investigated sample (high sensitivity), and (v) to exhibit a low tendency of analyte-matrix ion cluster formation.

Unlike ESI, the molecules ionized by MALDI are singly charged, a feature which greatly simplifies the data analysis. Although vacuum MALDI has the capacity of a higher throughput and more tolerance for contaminants than ESI, the loss of labile moieties represents the major drawback [20–22]. However, MALDI technique is a widespread ionization technique employed in mass spectrometry imaging (MSI). MALDI MSI emerged over the last years as a key technology for label-free mapping of the spatial distribution of pharmaceuticals or biomolecules, ranging from small metabolites to large proteins, in tissue sections [23].

In MALDI MSI, a focused ionization beam is used to analyze a specific region of thin tissue slices mounted on conductive microscope slides by generating a mass spectrum that is stored along with the spatial coordination where the measurement took place. Further, by moving stepwise the sample or the ionization beam and full scan of the sample, several thousands of distribution maps, or ion images, are generated. By choosing a peak of interest from the combined spectra, the MS data maps its distribution across the sample, as a function of x and y locations, revealing therefore greater insight and aiding the understanding of molecular makeup and regional heterogeneity. Based on the high spatial resolution (>10 μm), fast acquisition speed, robustness, and relative ease handling, an entire mouse brain can be imaged successively in both positive and negative ion modes with 50 × 50 μm^2 pixels in less than 1 h by using MALDI-TOF-MSI [24].

The remarkable ability of localizing the biomolecules in tissues, even without any previous information about them, and of differentiating compounds by molecular weight, all with no need of sample cleanup or chromatography step prior to ionization, exponentially expanded the area of MALDI MSI applicability. Therefore, the method had recent major contributions in understanding the disorders by tracking proteins, lipids, and cell metabolism for correlating the biomolecular changes with diseases and improving diagnostics and drug delivery [25].

Although low-resolution instruments such as ion traps can be used for proteome characterization due to their fragmentation flexibility and fast cycle rates [26], high-resolution mass spectrometry (HRMS) remains a goal and ambition in proteomics. This is because the coeluting ions can overlap when measuring peptides in complex biological samples [27], and this can preclude the accurate charge state evaluation and quantification [28].

Although, initially, HRMS was available for the expensive Fourier-transform ion cyclotron resonance (FT-ICR) and magnetic instruments and only time-of-flight (TOF) instruments could be routinely used in facilities [29], developments in the late 1990s of technological ion trapping and storage resulted in the design of ion orbital trapping—*Orbitrap*—which allowed HRMS to be attained [30].

The Orbitrap consists of two outer electrodes (disposed in a special bell-like architecture) and a central spindle-like electrode between which ions are squeezed using electrical fields [31]. The *m/z* values are obtained by the Fourier transform (FT) of the image current produced by the axial oscillations of the ions injected in the mass analyzer [6].

The initial limiting factor in introducing Orbitrap in the commercial market was related to the difficulties in interfacing this system with ion sources such as electrospray ionization [32], but adding a curved ion trap, subsequently termed C-trap, for ion injection and coupling linear ion traps (LTQ) with Orbitrap, resulted in the introduction of a hybrid instrument which offered a good balance between speed, sensitivity, and resolution [33], as most of the methods in proteomics used HRMS for precursor mass measurement and the ion trap for ion fragment scanning.

Using this setup, it was possible to obtain an almost complete proteome mapping for several simpler organisms such as *E. coli* or yeasts in just a few hours [34–36].

Although it did not provide a complete proteomic map for more complex organisms, this had a significant impact in the characterization of proteomic signatures in many eukaryotic cell lines such as melanoma cells.

Mass spectrometry was found to be effective also in proteomic phenotyping of melanoma cell lines [37]. For example, Pirmoradian et al. reported the identification of more than 5000 protein groups in A375 human melanoma cell line using 4-h-long optimized gradients without any prefractionation [38]. They were able to reach this depth of the proteome using a relatively recently commercially introduced instrument setup which coupled the Orbitrap mass analyzer with a quadrupole mass filter and an enhanced FT deconvolution algorithm [39].

Besides proteomics, the hybrid platform is also instrumental in the assessment of the dynamics of protein interactions in the pathological context of melanoma [40]. This is important, since it was shown that mutations in p53 oncogene could alter its interaction partners leading to protein complexes that promote invasion and migration of tumor cells [40–42].

4. Melanoma biomarkers

The introduction of the Orbitrap mass analyzer allowed routine HRMS to be also used for biomarker discovery studies. Using an LTQ-Orbitrap, Kawahara et al. defined the phenotypic differences in the secretome of melanoma, carcinoma, and noncancerous cells [43]. It is interesting to note that the top two candidates from the carcinoma secretome were validated in the saliva of patients using pseudo-selected reaction monitoring (pseudo-SRM) experiments on the same conventional hybrid instrument, usually dedicated only to the discovery approach, which subjects the flexibility and the impact of this platform.

Counting about 55,500 deaths annually, cutaneous melanoma represents the most aggressive type of skin cancer [44]. In principle, melanoma biomarkers can provide insights into either the overall outcome of this disease or to the therapeutic response to available treatments, being classified as prognostic biomarkers, predictive biomarkers, and on-treatment biomarkers [45]. However, to date a relatively low number of melanoma biomarkers were found, making it difficult to predict the outcome in patients. Moreover, their expression levels change with the stage of the disease [46]. Importantly, melanoma seems to have the highest mutational load, and once it appeared it becomes life-threatening [47]. Several mutations are frequently linked to cutaneous malignant melanoma, and these mutations are presented in more than 85% of all new diagnosed melanoma cases.

Stable isotope labeling with amino acids mass spectrometry (SILAC-MS)—involving incorporation of labeled amino acids into all cell proteins—was recently

used in melanoma biomarker discovery. The main advantage of this method is its quantitative accuracy and reproducibility [1]. Using SILAC coupled with nano-spray tandem MS, Liu et al. highlighted several differentially expressed proteins in MA2 cells. It is the case of CUB-domain-containing protein 1 (CDCP1), a surface marker especially expressed on cells with high metastatic feature [48]. The group of Janostiak performed SILAC and tried to identify potential MELK targets. They identify MELK as a regulator of the NF-kB pathway with implications in tumor survival. MELK inhibition blocks melanoma growth; therefore, it can be viewed as a potential therapeutic target [49].

One of the most interesting approaches in cancer research is the possibility of performing proteomics on formalin-fixed paraffin-embedded (FFPE) tissues in order to denote disease-state-linked molecular fingerprints. This is significant given that clinical research units collect patient tissue and usually process them for histopathology analysis by FFPE which cross-link protein amino acids for long-term storage [50–53]. Using an LTQ-Orbitrap system and a spectral count-based quantitative strategy, [54] report the identification of a comprehensive list of 171 possible disease-state-linked melanoma biomarkers that were found as differently expressed proteins between the three analyzed stages of melanoma: benign nevi, primary, and metastatic melanoma. Quantitative MS analysis was also performed by Sengupta's group to study histone post-translational modifications in melanoma cells. Considering their findings EZH2 (histone methyltransferase) was upregulated in both FFPE samples and melanoma cell lines in concordance with increased histone H3 lysine 27 trimethylation [16]. Despite the potential to give a more appropriate insight into melanoma environment, this technique presents some limitations due to formalin-induced covalent linkage of proteins [55].

An even more exciting perspective of HRMS is the investigation of PTMs—as these can impact protein folding, traffic, degradation, signaling pathways, and ultimately cellular response [56]. This is of special interest given that these modifications cannot be captured by genomics or transcriptomics studies, while the biomarkers for cancer could be also affected by such modifications.

Using multienzyme digestion and different fragmentation techniques [57] available on the hybrid ion trap-Orbitrap platform, Chiritoiu et al. reported the occupancy of all N-glycosylation sites of tyrosinase in a melanoma cell line [58], an enzyme shown to be correlated with patient clinical evolution in melanoma [59, 60]. Moreover, recent evidence suggests that even other members of the tyrosinase-related protein family could be also linked to melanoma progression [61, 62].

An emerging field in biomarker discovery is MSI in which the analysis is performed directly on frozen tissue in order to evaluate possible disease-specific molecular fingerprints. It was already shown that MSI could be performed using both low- and high-resolution instruments [63–65] but also using the Orbitrap technology [66]. Even more, Rao et al. [67] showed that this technology can be implemented on the conventional platform of hybrid instrument LTQ-Orbitrap. MSI technology would be useful in diagnosing difficult melanocytic lesions in skin cancer [68]. Lazova and Seeley showed that this technology could differentiate Spitz nevi (an uncommon form of skin mole) from Spitzoid malignant melanoma. Even more, in a recent study, the same author reported a histopathological misdiagnosed, congenital malignant melanoma that on a more detailed look by MSI revealed evidence of a congenital melanocytic nevus with proliferative nodules [69]. These reports underline the potential impact that mass spectrometry could have in the clinic, as the next focus is on the clinical applications of this technology [70].

BRAFV600E/K mutation is a major oncogenic driver with a frequency of 40–60% between patients diagnosed with advanced melanoma cancer. This mutation is commonly associated with MAP kinase pathway activation. In this sense BRAF blockade therapy is usually combined with MEK inhibitors. In BRAF

wild-type cells, BRAF inhibitors are likely to lead to MAP kinase activation and thus are not advised for association [70, 71].

NRAS mutant melanoma is known as the second most common mutation observed in melanoma. Recent studies indicated that stage IV NRAS patients have a shorter overall survival rate than those with wild-type form [72].

New melanoma-related biomarkers were discovered using proteomic analysis of five melanoma cell lines. Two of these are represented by vimentin and nestin, intermediate filament proteins involved in cell migration, apoptosis, and cytoskeletal organization. Vimentin may play an important role in migration and invasion. Studies indicated that those cells close to blood vessels present higher levels of vimentin. Together with vimentin, the nestin level can be correlated with the aggressiveness of the tumor. Both were validated as melanoma-related proteins using samples from 40 patients with different stages of melanoma. These two proteins can be viewed as potential biomarkers to distinguish between different forms of melanoma [73].

A recent study reveals a potentially new biomarker, SerpinE2, responsible for the invasive phenotype of melanoma slow-cycling cells. Proteome analysis reports two other proteins enriched in melanoma cells, PDGFRL and BMP1 [47].

Microphthalmia-associated transcription factor (MITF) expressed by melanoma cells is known as a driver of melanoma progression. It is involved in multiple biological processes like proliferation and differentiation. Due to its high sensitivity and specificity, this transcription factor is frequently used to differentiate highly proliferative melanoma cells from poorly invasive population [74].

Al-Ghoul et al. performed a comparative analysis of proteins expressed by either primary melanoma cells or metastatic cells. Their results indicate higher abundance of metabolic proteins upregulated in melanoma cells. About 131 proteins have a higher level than normal in these cells. Cyclophilin A, a protein linked to cancer progression and inflammatory diseases, was also identified to be upregulated in metastatic melanoma [75].

Histopathologic characteristics of 10 samples originating from patients with stage III metastatic melanoma were correlated with protein expression obtained using mass spectrometry. Authors define four proteins positively correlated to melanoma tumor, and among them are melanoma cell adhesion molecule (MUC18), melanoma chondroitin sulfate proteoglycan (CSPG4), melanoma-associated antigen D2, and melanocyte protein (melan A) [76].

S-100β serum level can reflect clinical stage. In general its concentration is less than 0.10 μg/L in healthy population. Usually S-100β level is used to monitor the response to treatment in patients with metastatic tumor. A second prognostic biomarker largely used is LDH, a well-known prognostic factor commonly used in those patients diagnosed with stage IV melanoma [46].

Based on the assumption that eIF4A1 and eIF4E are overexpressed in cancer cells, Joyce et al. tried to elucidate the possible mechanism related to invasive phenotype of cancer cells. Silencing either eIF4A1 or eIF4E reduces melanoma proliferation. Recent findings reveal that eIF4A1-depleted melanoma cells and eIF4E-depleted melanoma cells may present quite different proteomes, which may lead to hypothesis that they induce invasive phenotype through alteration of different molecular mechanisms. In order to perform this comparative proteome analysis, a MS operating in MS3 mode was used [77].

5. TOF MS applications to biomarkers of human glioblastoma

Discovery and implementation of these fragmentation techniques, together with ESI and MALDI, revolutionized the field of MS, bringing significant contributions

to the development of its analytical potential and its successes toward the topic of great interest, such as neoplastic transformations.

Accounting for 60% of all primary brain tumors, astrocytomas are already the focus of research for a few years [78]. Of these, glioblastoma multiforme (GBM), classified by World Health Organization (WHO) as having the highest malignancy degree, grade 4, is one of the most common and aggressive malignant brain tumor [79]. Treatment of GBM consists of surgical resection, radiation, and chemotherapy. Unfortunately, patients with this type of tumor have a median survival of approximately 14–15 months from the diagnosis [80, 81], with an estimated 5-year survival rate of 5%.

This poor prognosis could be allied to the richly neovascularized solid tumor profile, with many upregulated angiogenic and mitogenic factors [82], in other words, the high infiltrative and diffuse spreading of the GBM cells over long distances in the brain.

In recent years considerable efforts were invested in understanding the molecular mechanisms governing GBM tumorigenesis and also for the discovery of novel and more efficient therapeutic targets [83].

Astrocytomas and especially GBM were intensively addressed by proteomic studies, particularly by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), followed by protein digestion in a bottom-up approach. Nevertheless, the high sensitivity and reproducibility of MS, the need for significantly fewer test materials, as well as the MS relative simple working procedure over 2D-PAGE evolved MS as a powerful tool in screening cellular and tissue protein expression profiles (**Figure 3**) [84].

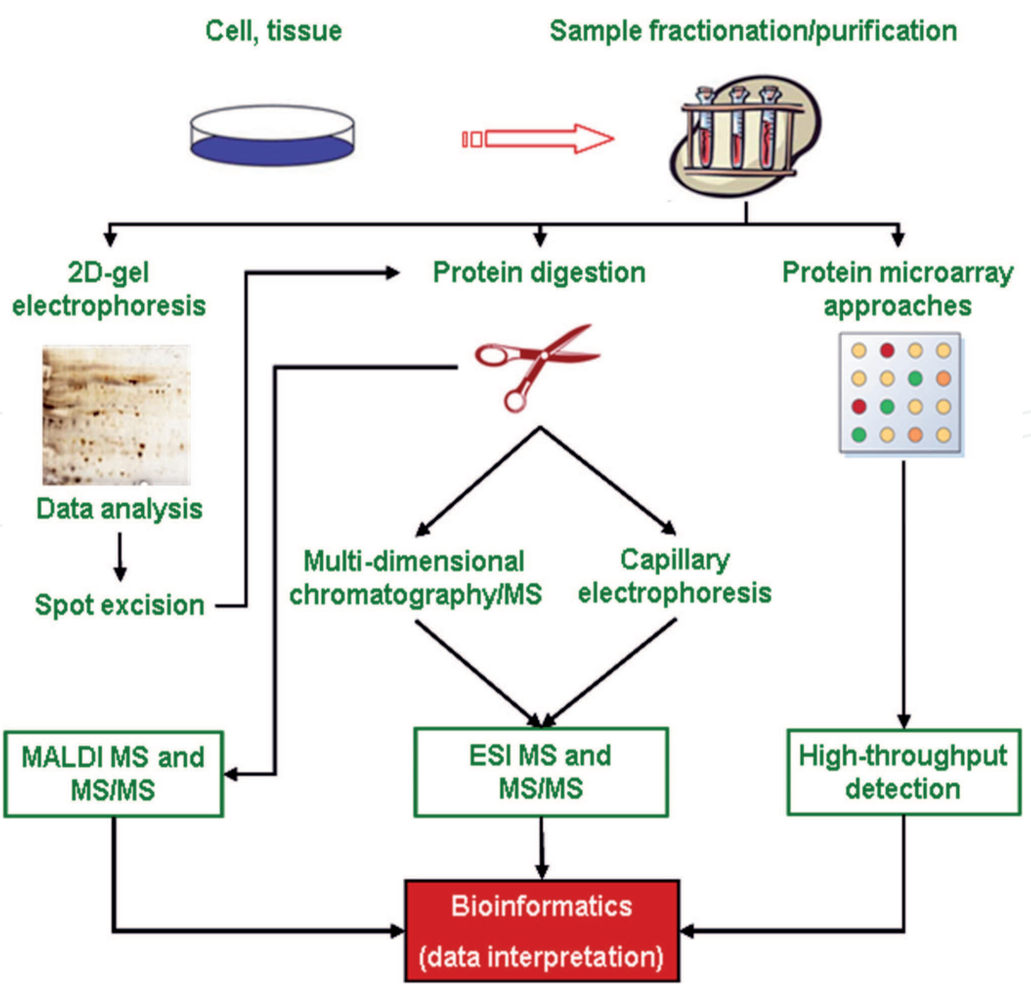

Figure 3.
Basic analytical strategies in proteomics.

In 2005, the proteome investigation in a single GBM tissue sample conducted by Wang et al. employing the combination of capillary isoelectric focusing (CIEF) and reversed-phase chromatography for multidimensional peptide separation hyphenated to QTOF MS led to the identification of a total of 6866 fully tryptic peptides, corresponding to 1820 distinct proteins [85].

MALDI-TOF-MS protein profiling has been used by Schwartz et al. to determine protein expression patterns that distinguish primary gliomas from normal brain tissue and one grade of gliomas from another, with high sensitivity and specificity. From the total of 162 tissue samples from 127 patients, including 19 patients undergoing resective surgery for nonneoplastic disease, 29 grade 2, 22 grade 3, and 57 grade 4 glioma patients that were analyzed, over 100 potential, tumor-specific biomarkers were identified. With their approach, they have distinguished glioma grades with high accuracy ranging from 76% to 97%, encountering some difficulty in distinction between the WHO grade II and III tumors [86].

A complex protein data set for GBM from direct tissue analysis including 2660 proteins with a significant number of peptide hits in total and 1401 validated protein hits with at least two unique peptide identifications was provided by the group of Huber CG [87]. Their novel methodology was based on the application of ion-pair RP chromatography to fractionate intact proteins from the tumor tissue prior to identification by MALDI-TOF/TOF-MS^2, as well as the combination of intact protein fractionation with shotgun proteomics to discover the most complex data set of proteins.

Considering the limited accessibility to healthy brain tissue samples, especially from the same patient, achieving a normal vs. diseased brain comparison at the proteome level is challenging. Most of the time, for such investigations, control brain tissues are obtained by surgery from patients with epilepsy or autopsy. In order to identify and characterize proteins that are differentially expressed in GBM and gather information on interactions and functions that lead to this condition, ten GBM tumors (patients from 48 to 67 years) and ten epileptic brain tissue (patients from 21 to 61 years) samples were analyzed by Mirza and Shamim [88]. By SDS PAGE fractionation with internal DNA markers followed by liquid chromatography-tandem MS [87] on a LTQ-Orbitrap Velos, differential protein expression in GBM was identified. These proteins, highly specific to GBM irrespective of their location in the brain (right or left temporal region of the brain), were further characterized by Ingenuity Pathway Analysis (IPA) to assess protein interactions, functions, and upstream regulators. Such an approach provided several other upregulated proteins, such as SERPH, PDIA1, CERU, TENA, VTNC, APOE, LEG1, HRG, and FKBP5, identified to be involved in tumor progression, aggressiveness, and invasion in GBM. Besides the known GBM-associated proteins, CLIC4, NP1L1, IGKC, TAGL2, and YES, previously correlated with processes promoting cancer progression, were identified here as novel potential biomarkers of GBM [83].

Despite the significant efforts invested over the past decades in finding an appropriate treatment for GBM, almost all GBMs are ultimately resistant to existing treatment and inevitably recur [89]. Though a multimodal approach including surgery, postoperative radiotherapy, and chemotherapy is applied, the prognosis for patients with GBM remains rather poor. Hence, there is an urgent need for a better understanding of the molecular mechanisms associated with GBM, improvement of conventional treatment modalities, and/or development of new and more efficient ones. A first step in breaking the evolution and/or recurrence of this fast-growing tumor is the development and application of new, rapid, and accurate approaches to assess the treatment of biological effects, given that currently, the treatment results are radiologically evaluated several months after treatment.

Surface-enhanced laser desorption/ionization (SELDI) TOF MS demonstrated its ability in detecting the changes in protein patterns in GBM tumor tissue following radiotherapy. In the study reported by Wibom et al. [90], rat BT4C glioma cells were implanted into the brain of two groups of 12 BDIX-rats, one of the groups receiving radiotherapy. The expression of proteins in normal and tumor brain tissue, pooled at four different time points after irradiation, was investigated using SELDI TOF MS by principal component analysis (PCA) and partial least squares (PLS). The variations over time in protein expression identified by the authors, together with the tumor progression in vivo and, last but not least, the rapid and significant changes in the protein expression following irradiation, allowed the irradiated tumors to be clearly distinguished from the nontreated tumors. Therefore, the authors consider that the 77 identified signals characterized by different intensity levels in irradiated vs. nonirradiated tumors validate SELDI TOF MS as a method of choice in elucidating biological events induced by radiation which can be used as markers for monitoring the efficacy of radiation treatment in malignant glioma [90].

The pioneer study in which MRI was used to guide proteomic analysis with SELDI TOF MS in human GBM in order to demonstrate a correlation between imaging patterns and protein expression profiles belonged to Hobbs et al. [84]. Contrast-enhanced (CE) magnetic resonance imaging (MRI) represents an important diagnostic tool for correlating molecular pathophysiology with the clinical management of disease; it can supply elevated spatial resolution and molecular signatures of both healthy and diseased tissues in solid tumors. For the identification of CE and nonenhanced (NE) regions of the tumor in the four patients previously diagnosed with GBM, intraoperative stereotaxis during surgical resection in conjunction with MRI was used. Further, Hobbs et al. investigated the profile of over 100 proteins and peptide species within GBM tumors and compared the protein profiles between the CE and NE regions across four patients with MR images and confirmed diagnosis of GBM. This CE and NE comparative approach on the GBM proteomic fingerprint revealed qualitative and semiquantitative proteomic pattern differences, hinting an impaired gene expression profile that correlates with detectable tissue imaging parameters [84].

Although noninvasive imaging techniques have improved the neuroradiological diagnostic accuracy, in some cases, comprehensive specificity for discrimination of brain tumors and detection of minor differences in tumor size and behavior are difficult using imaging approaches [91]. Besides, tumor tissue extraction through biopsy or resection is not always feasible. For such reasons, identification of reliable biomarkers in the blood would facilitate the management process of GBM patients, allowing an early diagnosis, establishment of surgical intervention plans, and monitoring of the disease course and the treatment response.

Quantitative comparisons of the plasma proteomes of 14 GBM patients and 15 healthy controls using sequential window acquisition of all theoretical fragment ion spectra (SWATH) MS analyses were conducted for identifying potential biomarker candidates in plasma of GBM patients. SWATH MS, characterized by high reproducibility and reliability of quantitative information, combines a highly specific data-independent acquisition [38] method with a novel targeted data extraction strategy to mine the resulting fragment ion data sets [92].

Being a label-free analysis, SWATH MS was employed by Miyauchi et al. in conjunction with LC-MS/MS using a Triple TOF 5600 for generating a protein quantification method, called quantitative targeted absolute proteomics (QTAP) [91]. With the current approach, the authors not just estimated the origin of upregulated biomarker candidates in GBM plasma but also have examined if the upregulated biomarker candidates were elevated in GBM tissues, and if the

biomarkers were detectable in cyst fluid. Additionally, the relationships between the concentrations of biomarker candidates in plasma and the clinical presentation (tumor size, progression-free survival time (PFS), or overall survival time (OS)) of GBM patients were inspected for establishing the relationships between biomarker candidates and GBM biology. While LRG1, C9, CRP, SERPINA3, and APOB were identified by SWATH MS and QTAP analyses as upregulated biomarker candidates, compared with the healthy plasma, GSN, IGHA1, and APOA4 were identified as downregulated biomarker candidates (Miyauchi et al.). All the candidate proteins, except for CRP, were found to be highly expressed in cytosol of GBM tissues vs. noncancerous brain tissues, while GSN was identified as decreasing also in cerebrospinal fluid (CSF). Although valuable information was gained by SWATH MS and QTAP, in order to determine the feasibility of their findings in clinical application, the authors recommend further investigations using plasma from patients with postoperative GBM, glioma, and other various types of cancer [91].

Since in 2010, there was no CSF biomarker in clinical use in malignant brain tumors, neither for diagnostic nor for other purposes. The detection of recurrence or reaction to adjuvant therapy and the exploration of CSF originating from GBM patients in respect to peptide profiling were not experimented as well. Eleven CSF samples collected by lumbar puncture from four female and seven male patients with a single supratentorial GBM manifestation (median 68 years, range 43–79 years) and 13 samples from four female and nine male participants with mostly spinal canal stenosis, and used as control, were involved in a study conducted by Schuhmann and colleague [93]. The peptides, previously separated by RP HPLC in 96 fractions per sample, were resuspended in matrix solution consisting of α-cyano-4-hydroxycinnamic acid and L-fucose (co-matrix) in 0.1% acetonitrile/trifluoroacetic acid (1:1 v/v) and screened by nanoESI-QTOF-MS/MS. To detect possible marker candidates, the two peptidomes were compared using the differential peptide display approach. Additionally, patient data including age, duration of symptoms, and the morphological MRI data were correlated to the mass spectrometric signal intensity of the peptides in CSF using Spearman's rank correlation coefficient. More than 6000 peptide signals, corresponding to at least 2000 different peptides, were remarked in the peptide master displays of the two peptidomes. Of these, four CSF peptides, specific *C*-terminal fragments of α-1-antichymotrypsin, osteopontin, and transthyretin as well as a *N*-terminal residue of albumin, significantly distinguished GBM from controls in all applied statistic tests. Although these molecules are components of normal CSF, none of these peptides or their precursors was previously reported as significantly elevated in CSF of GBM patients.

Although neuronavigational guidance by MRI or positron emission tomography (PET) aids in localizing the tumor tissue, the brain shift and tissue deformation may negatively influence the accuracy of resection [94, 95]. An option to overcome these limitations is the application of intraoperative imaging; however, besides being expensive they increase the surgery time [95].

Since the introduction of fluorescence-guided surgery (FGS) in 1998 by Stummer et al. subsequent to proagent 5-aminolevulinic acid (5-ALA) treatment, contrast enhancement by a fluorescent dye has progressed and represents today an important and powerful technique in GBM resection [94, 95]. After oral application of 5-ALA approximately 3 h before anesthesia, in a dose of 20 mg/kg body weight, the small molecule crosses the abnormal blood-brain barrier into the peritumoral tissue and accumulates within the tumor. As a precursor of heme B, in an intermediate step of the heme biosynthesis cascade, 5-ALA is converted to protoporphyrin IX, a red fluorescent at 635 nm upon blue light excitation. Since high-grade gliomas are known to be characterized by augmented cell proliferation, tumor cell density and

microvessel density, the greater fluorescence intensities generated by accumulation of high cellular PpIX concentrations, improve differentiation of tumor from healthy brain tissue during intraoperative fluorescence microscopy [95–97]. Nevertheless, in some cases, false-positive and false-negative fluorescence results cannot be excluded. Hence, molecular and elemental MSI was introduced as a complementary technique to examine the potential chances and boundaries of fluorescence diagnosis. Within the molecular MSI domain, MALDI MS was demonstrated to have a high applicability, even of FGS for meningioma, since it accomplishes the investigation of intact molecules by soft ionization [98].

A comprehensive study on the fluorescent drug PpIX and the biosynthesis end product heme B, based on MALDI MSI followed by quantitative Fe analysis via laser ablation inductively coupled plasma (LA-ICP) MS, was performed by Kröger et al. in the group of Karst [95]. Three GBMs after and two GBMs without 5-ALA administration, one gliosarcoma (GSM), one low-grade glioma, and two reactive brains were characterized using an ion trap-TOF mass analyzer and α-cyano-4-hydroxycinnamic acid as matrix for MALDI.

With their approach, Kröger et al. enabled the detection of PpIX distribution in GBS, where high tumor density exhibited intense PpIX signal during surgery. Heme B and enhanced Fe accumulation was detected only in regions of blood vessels and hemorrhage, corroborating the hampered transformation from PpIX to heme B in GBM tissue. In the case of GSM, the absence of PpIX accumulation determined by the nonfluorescent appears during FGS, generated a false-negative fluorescence diagnosis [95].

Although initially reported in 1895, GSM, a rare primary neoplasm of the CNS, did not gain a broad acceptance until 1955, when three cases of patients with this malignancy were described in detail [99]. Thus, hitherto, only a limited number of case reports and researches were published, and from those, just a few were targeting the identification of potential biomarker species in GSM.

Defined by the WHO as a variant of GBM, accounting for about 2% of all the GBM, GSM affects preponderantly the adult population in the fourth to the sixth decade of life, more frequently males than females (male/female, 1.8:1) [79]. Consisting of alternating areas displaying glial and mesenchymal (sarcomatous) differentiation, GSMs are typically located in the cerebral cortex, involving the temporal, frontal, parietal, and occipital lobe in decreasing frequency [100]. The epidemiology and natural history of this rare malignancy appears similar to GBM, with a slightly greater predisposition for temporal lobe involvement. As for GBS, the prognosis for GSM after surgical resection and chemotherapy is limited, even inferior to those observed in GBM patients, with a median survival of 11–12 months, with less than 10% survival after 2 years following diagnosis [101]. Such a reduced life expectancy may be caused by the widespread penetration of the GSM cells into the brain tissue that makes the tumor cells slightly inaccessible to treatment methods and so the treatment fails [102].

It is broadly accepted that abnormalities in glycoconjugate glycosylation pathways are responsible for atypical cell surface—glycocalyx—molecular structure, and the latter one accompanies malignant alteration of cells. Since the cell surface carbohydrates are involved in a variety of interactions of the cell with its extracellular environment, any change in their expression cause tumor cell migration, metastasis, and invasiveness [103].

In view of the range of glycosyl epitopes that designate tumor-associated antigens [104, 105], some of them endorsing proliferation and metastases and some suppressing the tumor progression [106], surveying the changes in the profile of the glycoconjugate molecules liable for such effect, represents an alternative to the proteomic studies. Atypical glycosylation associated with oncogenic transformation

was first confirmed with gangliosides [107]. Gangliosides (GGs) are sialic acid-containing glycosphingolipids integrated into the outer leaflet of the cellular membrane bilayer and predominantly enriched in microdomains.

Within the intermolecular interactions and through glycosynapses, GGs are known to regulate the cell-cell recognition and intercellular adhesion and to modulate signal transduction pathways. The stimulation of cell growth inhibition and cell differentiation and/or apoptosis of certain GGs is well demonstrated for nearly two decades [108]. For instance, the cell number in primary cultures of high-grade human GBM, ependymomas, mixed gliomas, astrocytomas, oligodendrogliomas, and gangliogliomas, as well as of the rat 9 L cell GSM cell line, was shown to decrease following treatment with GM3. In the same time, it had a slight effect on the cell number in cultures of a normal human brain [100]. Given the correlation of different malignancy grade and median survival time with GG composition and content in different types of glioma tumors, GGs can be considered as tumor markers and used as therapeutic targets or vaccine development [109].

A diminished amount of complex GGs in tumors is connected to the dedifferentiation processes, reflecting the GG pattern alteration in ontogenesis during differentiation of nervous tissue: an increased content of complex GGs and a reduced volume of simple GGs [110, 111].

Together with the immunochemical and immunohistochemical methods, several other biophysical techniques were introduced over the years for mapping and structural analysis of GGs in different gliomas, such as HPLC, infrared spectroscopy, and confocal microscopy via anti-GG monoclonal antibodies [112–114]. Additionally, the implementation of miniaturized, integrated devices for sample infusion into MS simplified the laborious chemical and biochemical MS strategies by reducing sample handling and sample loss, eliminating potential cross-contamination, and increasing ionization efficiency and quality of spectra as well as the possibility to perform unattended, high-throughput experiments [115]. The world's first fully automated nanoelectrospray system and perhaps the most popular and widely used device is the NanoMate robot produced by Advion BioSciences (**Figure 4**), in which 100 or 400 nanospray emitters are integrated onto a single silicon substrate, from where electrospray is established perpendicular to the substrate. Considering the (i) automated infusion into MS at low flow rates in the nanoliter range (only 50–100 nL/min), (ii) minute amount of sample, (iii) technical quality of the nanosprayers, (iv) reproducibility of the experiment, (v) formation of preponderantly multiply charged ions, and (vi) minimized in-source fragmentation of labile groups attached to the main structural backbone, in some cases, chip-based devices not just eliminated the need for separation prior to MS analysis but also provided more robust and quantitative analyses than LC- or CE-MS [116].

The first investigation of GG pattern in a human GSM specimen by sensitive and accurate MS methods, complemented by HPTLC analysis was conducted in 2006 [100]. The current approach was developed and optimized in order to detect and structurally characterize the tumor-associated species, which might serve as potential diagnostic markers or specific target molecules for the production of antitumor therapeutic agents. Primarily, the densitometric analysis conducted in two complex GG mixtures, one originating from a 47-year-old male presenting GSM tumor and one from adult frontal cortex used as control, indicated a severalfold decrease (7.4×) of the total GG content in GSM vs. normal human brain (**Figure 5**), which was in agreement with previous reports on other glioma types. Further, by employing chip-based nanoESI QTOF MS and ultrahigh-resolution nanoESI FTICR MS, Vukelić et al. demonstrated for the first time the reduction in the total GG content and the altered pattern in GSM vs. control tissue [100]. While GM1a, GD1a, and GD1b abundances were very low in GSM, GD3 species were found to account

Figure 4.
NanoMate robot with fully automated chip-based ESI (courtesy of Advion BioSciences).

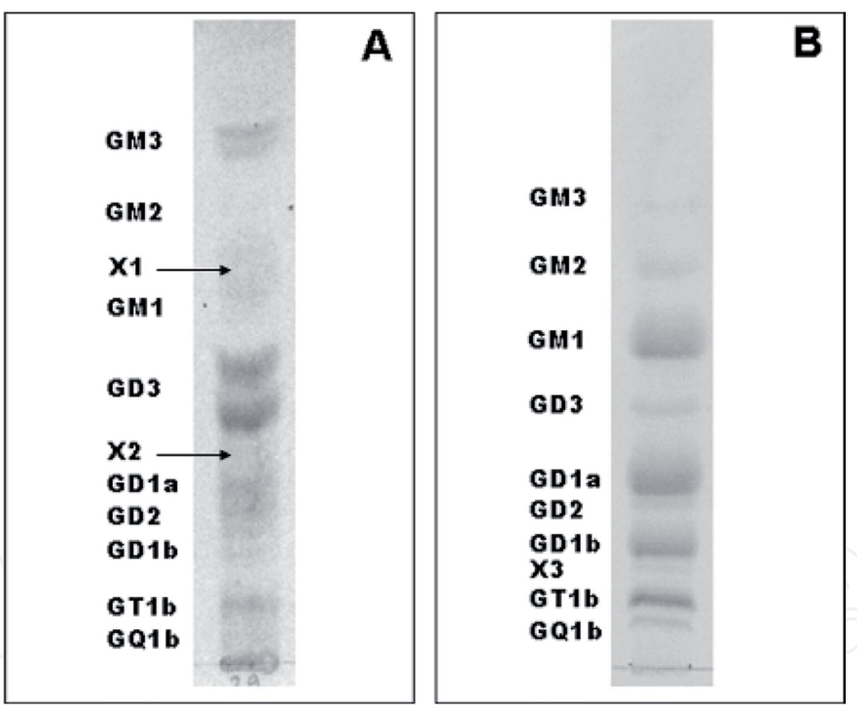

Figure 5.
Compositions of GGs isolated from human gliosarcoma specimen (A) and normal human frontal cortex (B), as revealed by HPTLC followed by densitometric scanning. Reprint with permission from Vukelić et al. [100].

for nearly 50% of the total GG content, being the most emphasized fraction and the only one expressed in higher absolute concentration than in normal cortex (approximately 128%). Therefore, the authors hypothesized that the combination of both lower overall biosynthetic rate, due to change in expression of certain glycosyltransferases, and higher turnover rate is responsible for such a pattern. At the same time, the considerably higher amount of potentially proapoptotic GD3 than of the *O*-acetyl GD3 species observed by the authors supported the assumption of Kniep et al. [117], according to which, *O*-Ac-GD3 could by itself be responsible

for the protection of tumor cells from apoptosis. Moreover, the high sensitivity and mass accuracy of QTOF and FTICR MS and MS/MS permitted them the assignment of unusual GG minor species. Among these species, GM4, Hex-HexNAc-nLM1, Gal-GD1, Fuc-GT1, GalNAc-GT1, *O*-Ac-GM3, di- *O*-Ac-GD3, *O*-Ac-GD3, and *O*-Ac-GT3, considered as brain developmental antigens [118], were not reported previously as glioma-associated structures [100]. The fully CID sequencing at low energies of *O*-Ac-GD3(d18:1/20:0), exhibiting higher abundance in GSM vs. normal human brain, provided structural evidence to postulate a novel *O*-Ac-GD3 isomer, *O*-acetylated at the inner Neu5Ac-residue, previously not structurally confirmed.

In order to reveal the role of GG as tumor-associated antigens, a detailed depiction of native GG mixtures from normal human brain (NB), GBM and corresponding peritumoral tissue [119], was conducted recently by HPTLC and MS [110]. Particular attention was paid to the GG expression in PT, since the biochemical processes occurring within this area are believed to be responsible for aggressive infiltration of tumor cells into the surrounding brain tissue and therefore for high incidence of GBM recidivism and poor prognosis of GBM [110, 120].

While the frontal cortex of a 54-year-old who died in a traffic accident was used as control, the GBM and the corresponding PT were collected from a 70-year-old female patient, whose brain MRI showed an infiltrative tumor lesion measuring 47 × 43 × 40 mm in the right frontal and insular region. This approach revealed distinctive changes in GG expression in GBS. Total GG content in GBS was approximately five times lower than in the NB, while approximately two times lower than in the PT. For MS analyses, the extracted GG mixture dissolved in methanol up to 0.8 mM concentration was infused into a Bruker amaZon ETD ion trap system. Whereas predominantly C18 chains were found to characterize the NB and PT, shorter (C16) and longer (C22, C24), unsaturated (C24:1) and with odd number carbon atom (C17, C19) fatty acids residues were detected in GBS [110]. The most abundant GG in GBS (accounting for 53% of the total GG content) was GD3(d18:1/18:0), followed by GD3(d18:1/24:0) that was exclusively detected in GBS tissue. Likewise, proportions of GM3, GM2, GD2, and O-Ac-GD3/nLM1 were much higher in GBS than in the PT and NB tissue. In contrast, GM1, GD1a, GD1b, and GT1b constituted the most abundant GG structures in NB, their amount being considerably lower in GBS compared to NB and PT. The content of GGs modified by *O*-acetylation, most often of the sialic acid, was also found altered in GBS. The previous reports, according to which *O*-acetylation exhibits antagonistic effect on GD3 function, thus modulating its activities [121] and protecting the cell from its proapoptotic activity [122], herein confirmed (i) the high intensity of *O*-Ac-GGs in healthy brain tissue, (ii) the low concentrations in PT, and (iii) the detection of only one ion corresponding to *O*-Ac-GT1(d18:1/18:0) in GBM sample. *O*-Ac-GD1 was detected in NB and PT, but not in GBS, while *O*-Ac-GD3 species were found exclusively in GBS.

6. Applications of MS in pancreatic cancer (PDAC)

Although it has a lower incidence and prevalence compared to other cancers, PDAC is one of the deadliest, patients affected by PDAC having a very low life expectancy. This is due to the low success in the detection of this disease, as well as to a high resistance to therapies. Therefore, it is of major interest to set up biomarkers or biomarker panels dedicated to the earlier detection of the disease, as well as biomarker panels for the prediction of therapy outcomes. Many works have been dedicated to these aspects, including approaches based on genomics, proteomics, metabolomics, etc., or based on combination of such techniques.

Liu et al. used a combination of MS-intensive methods such as isobaric tags for relative and absolute quantitation with two-dimensional liquid chromatography-tandem mass spectrometry (iTRAQ-2DLC-MS/MS) and 1D-targeted LC-MS/MS, on serum samples from healthy people (normal control, NC), patients with benign diseases (BD), and PC patients to identify novel biomarkers of PC [123]. From more than 1000 identified proteins, 142 were identified as differentially expressed. For the diagnosis of PC, a novel biomarker panel consisting of apolipoprotein E (APOE), inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3), apolipoprotein A-I (APOA1), and apolipoprotein L1 (APOL1), in combination with CA19-9, statistically improved the sensitivity (95%) and specificity (94.1%), outperforming CA19-9.

Radon et al. used the LC-MS/MS analysis on urine samples from patients and controls and identified LYVE-1, REG1A, and TFF1 as candidate biomarkers [119]. Further the selected biomarkers were investigated by ELISA over a larger cohort (192 PDAC patients and 87 normal urine samples) and provided an AUC for the panel of 0.90, with the detection of PDAC in stages I–II.

Nigjeh et al. [124] applied a methodology of data-independent acquisition (DIA) [38] that included sample preparation with high reproducibility and separation by LC-MS/MS; they estimated the applicability in the detection of pancreatic cancer, based on a plasma spectral library consisting of over 14,000 identified peptides obtained from more than 2300 plasma proteins. The reliability of quantification was examined for the identified peptides, in constant retention times and signal intensity. The linear dynamic range and the lower limit of quantification were evaluated and pointed toward a critical role of the sample complexity for the optimization of DIA settings. Validation of the assay based on cohort of clinical plasma demonstrated the robustness and a unique advantage for targeted analysis of plasma samples for biomarker development.

In the study of Yoneyama et al. [125], LC-MS/MS proteomics was applied for the quantitation of plasma proteins, in combination with specific antibodies. The results showed that insulin-like growth factor-binding proteins (IGFBP)2 and IGFBP3 display the potential to distinguish early-stage PDAC patients from normal controls and that IGFBP2 was increased in intraductal papillary mucinous neoplasms (IPMNs). Moreover, PDAC diagnosis based on a combination of IGFBP2 and IGFBP3 and carbohydrate antigen 19-9 (CA19-9) is significantly more efficient compared to only CA19-9. These prove that IGFBP2 and IGFBP3 can represent supplemental or compensatory biomarkers to CA19-9 and by using this biomarker combination may improve the prognosis of PDAC patients.

Castillo et al. [126] examined analyzed exosomal surface proteins, using a combination of liquid chromatography and mass spectrometry. Simultaneously, PCR was applied on samples from 74 patients (a total of 136 exosome samples) to detect baseline KRAS mutation rates in patients subjected to therapy. The exosome proteomic analysis identified a set of biomarker candidates for PDAC: CLDN4, EPCAM, CD151, LGALS3BP, HIST2H2BE, and HIST2H2BF. In 44.1% of the patients under active therapy, KRAS mutations were detected in total (unsorted) exosomes; the yield increased to 73.0% when exosome capture was conducted based on selected biomarkers.

7. Applications of MS in colorectal cancer

Kirana et al. used a combination of 2D-DIGE and MALDI-TOF mass spectrometry for 125 tumor tissue samples in different TMA stages of CRC. Out of 55 proteins with differential expression, a group of ten protein biomarkers was

selected, comprising HLAB, protein 14-3-3β, LTBP3, ADAMTS2, JAG2, and NME2; they were significantly associated with clinical parameters relevant for tumor progression, invasion, and metastasis. Kaplan-Meier survival curve demonstrated the marked expression of six proteins that could be associated with improved survival from CRC. The level of expression of HLAB, ADAMTS2, LTBP3, JAG2, and NME2 on tumor cells was associated with progression and invasion, metastasis, and CRC-specific survival; all these may be useful biomarkers for stratification of CRC patients regarding low and high risk of metastasis. A combination of microdissection with 2D-DIGE and MALDI-TOF MS was demonstrated to be useful in the identification of biomarkers for the prediction of the risk of liver metastases [127].

Suwakulsiri et al. applied quantitative mass spectrometry to examine the proteomes of two classes of extracellular vesicles—exosomes and shed microvesicles (sMVs) [133]. Two populations of sMVs were investigated—released from primary (SW480) and metastatic (SW620) human isogenic-colorectal cell lines. One thousand two hundred ninety-five and one thousand three hundred proteins were identified in SW480-sMVs and SW620-sMVs, respectively, using quantitative mass spectrometry. Analysis of Gene Ontology identified processes of "cell adhesion" (CDH1, OCLN, CTN families), "signaling pathway" (KRAS, NRAS, MAPK1, MAP2K1), and "translation/RNA related" (EIF, RPL, HNRNP families) in both types of sMVs. Remarkably, SW480- and SW620-sMVs displayed distinct signatures; SW480-sMVs was enriched in ITGA/B, ANXA1, CLDN7, CD44, and EGFR/NOTCH signaling networks, while SW620-sMVs are higher in PRKCA, MACC1, FGFR4, and MTOR/MARCKS signaling networks. Jimenez et al. evaluated the proteins in small and large extracellular vesicles (SEVs and LEVs, respectively) [128], applying isobaric tag for relative and absolute quantitation liquid chromatography (iTRAQ-LC) [87] tandem mass spectrometry (MS) on EVs from colon cancer cell line (SW-480) and the lymph node metastatic line (SW-620) and a CRC patient primary culture. Bioinformatic analyses showed that SEVs contain higher levels of proteins involved in cell-cell junctions, cell-matrix adhesion, exosome biogenesis, and diverse signaling pathways and that LEVs contain higher levels of proteins associated with the biogenesis of ribosomes and RNA biogenesis and processing and with metabolism.

Kit et al. [129] analyzed the patterns of expression for proteins implicated in cell signaling, using paired samples tumor/nontumor colon cancer patients, with the purpose of finding protein clusters capable of differentiation between patients having non-metastatic and metastatic colon cancer. They found in tumoral mucosa nine proteins upregulated significantly: among these are protein kinase C gamma, c-Myc, MDM2, and pan-cytokeratin, while GAP1 was significantly downregulated. Pan-cytokeratin and APP appeared upregulated in tumor compared to nontumor tissue and were thus included in the predictive cluster for discrimination of cancer type. All applied methods of regression/clustering confirmed the presence of increased concentrations of S-100b and phospho-Tau-pSer199/202, and their predictor value for non-metastatic colon cancer. Further investigations are required for the validation of potential protein markers for colon cancer development and metastatic progression.

Lee et al. [130] used liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis for identification of potential biomarkers in colon cancer. They identified tetraspanin 1 (TSPAN 1) as a potential noninvasive biomarker.

Liu et al. [123] applied MADLI-TOF-MS for the analysis of the N-glycome of IgG in patients with colorectal benign tumors, colorectal cancer, and normal individuals. The results identified nine IgG N-glycans that were differentially expressed in patient groups. Moreover, five of them were significantly modified in CRC patients in all TNM stages. Principal component analysis (PCA) indicated evident differentiation between benign, cancer patients and normal individuals. The ROC analysis suggests that these five IgG N-glycans were correlated with the progression of

CRC. Analysis of IgG N-glycosylation suggests that core-fucosylation, sialylation, and sialo core-fucosylation were probably correlated with development of CRC.

Hu et al. [131] used stable isotope labeling by amino acids in cell culture (SILAC) technology. Thus, HCT116-I8 and HCT 116 cells were cultivated with either "light media" (Arg0, Lys0) or heavy media (Arg10, Lys8), for 7 days, followed by lysis of cultures, protein digestion, and MS analysis on an LTQ-Orbitrap mass spectrometer. The outcome of the study was Cdc42-Cdc42BPA signaling as prognostic biomarker and therapeutic target for colon cancer invasion. Also, the authors discovered a decreased expression of E-cadherin and increased expression of vimentin in highly invasive cell lines.

Alvarez-Chaver et al. [132] investigated the proteomic profile of tumor and mucosa in CRC, using a combination of 2D electrophoresis and mass spectrometry, revealing nucleotide-diphosphate kinase as a candidate biomarker. They finally used an ELISA kit for the validation of the biomarker.

8. Conclusions

Mass spectrometry provides powerful instruments for the analysis of biological systems, with dedicated instruments for different classes of analytes, such as proteomes, metabolomes, genomes, transcriptomes, etc. The power of such systems is usually backed by powerful bioinformatic tools, eventually playing at different levels (the "built-in" instrument that controls effectively the instrument), and the post-analytical bioinformatics tools that help in in-depth analysis and interpretation of findings.

We have tried to illustrate the state of the art concerning the available technology in MS for proteomics, and we have illustrated these with application in biomarker discovery in melanoma, glioblastoma, pancreatic cancer, and colorectal cancer.

Similar approaches are developed also for other solid cancers—such as gastric, hepatic, pulmonary, ovarian, or prostate—but, unfortunately, there is not enough space and time to approach them in this very chapter.

The core of the proteomic approaches is still based on two sample types: tissue (tumoral, peritumoral, and nontumoral—generally collected surgically or biopsies collected presurgically) and blood (most often, serum/plasma, more recently circulating tumor cells (CTCs) or circulating tumor stem cells (CTCS), and, in the last years, exosomes). Exosomes may represent an interesting option, since they bear significant cytosolic contents of the original cells (like nucleic acids and proteins) and also have a specific molecular surface signature of the originating cells. The major question on ECVs is represented by the amounts of such entities that can be recovered from small volumes of blood. Several other sample types were considered in the recent literature as sources for discovery of biomarkers and further as biological samples for diagnostics. One first example is represented by tears; it is considered that they have the overall load of plasma proteins, but devoid of the major ones (such as serum albumin), and could serve as biological material for diagnostics, prognostics, and patient stratification. Other such samples are saliva and urine; as in the case of tears, their collection is completely noninvasive.

Once one or several biomarkers are identified, next stages of pre-validation and validation are required, usually these will be achieved using non-MS techniques, such as ELISA, Western blot, or the use of multiplex platforms (Luminex, Meso Scale, etc.). One major problem in the progress toward clinical application is represented by the existence of equipment and detection kits validated for such applications, and this might represent a major problem for the future.

Finally, we want to encourage all who can implicate in cancer proteomics, thus accelerating the progress in knowledge.

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