

Neurotrauma investigation through spatial omics guided by mass spectrometry imaging: Target identification and clinical applications

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Abstract

Traumatic brain injury (TBI) represents one of the major public health concerns worldwide due to the increase in TBI incidence as a result of injuries from daily life accidents such as sports and motor vehicle transportation as well as military-related practices. This type of central nervous system trauma is known to predispose patients to several neurological disorders such as Parkinson's disease, Alzheimer's disease, chronic traumatic encephalopathy, and age-related Dementia. Recently, several proteomic and lipidomic platforms have been applied on different TBI studies to investigate TBI-related mechanisms that have broadened our understanding of its distinct neuropathological complications. In this study, we provide an updated comprehensive overview of the current knowledge and novel perspectives of the spatially resolved microproteomics and micro-lipidomics approaches guided by mass spectrometry imaging used in TBI studies and its applications in the neurotrauma field. In this regard, we will discuss the use of the spatially resolved microproteomics and assess the different micro-proteomic sampling methods such as laser capture microdissection, parafilm assisted microdissection, and liquid microjunction extraction as accurate and precise techniques in the field of neuroproteomics. Additionally, we will highlight lipid profiling applications and their prospective potentials in characterizing molecular processes involved in the field of TBI. Specifically, we will discuss the phospholipid metabolism acting as a precursor for proinflammatory molecules such as eicosanoids. Finally, we will survey the current state of spatial

neuroproteomics and microproteomics applications and present the various studies highlighting their findings in these fields.

KEYWORDS

mass spectrometry imaging, spatial omics approaches, spinal cord injury, trauma brain injury

1 | INTRODUCTION

Central nervous system (CNS) injuries including injury to the brain or spinal cord and are known as traumatic brain injury (TBI) and spinal cord injury (SCI), respectively. TBI and SCI are mainly caused by factors such as motor vehicle accidents, falls, assaults/violence (resulting from gunshot injuries), penetrating objects, sport-related and most importantly military related incidents such as blast-induced injuries (Ahmed et al., 2017; Bruns & Hauser, 2003; Bryden et al., 2019; Dewan et al., 2018), and even surgical/medical complications in the case of SCI (Chen et al., 2013). Although TBI and SCI are the results of mechanical insults, their consequences are prolonged due to the diffusion of damage to other remote CNS regions in a spatio-temporal manner. The neurological outcomes appearing postinjury occur in two phases of primary and secondary injury cascades (Galgano et al., 2017; McKee & Daneshvar, 2015). Primary injury conditions of TBI are heterogeneous, occurring due to the direct mechanical insult, impacting cellular and sub-cellular structures, distorting axons and blood vessels, impairing the blood-brain barrier (BBB) functions, and damaging the underlying tissue (Galgano et al., 2017). SCI primary injury phase involves a spinal shock and/or cord compression associated with vascular compromise (Livecchi, 2011). Within minutes to hours after the primary phase, the secondary injury phase is induced involving the initiation of a neuroinflammatory and other complex responses. In TBI, this response over-activates not only recruited immune cells such as neutrophils and macrophages, but also resident cells of the brain including astrocytes and the microglia (Simon et al., 2017). In addition both TBI and SCI, plasma proteins involving the complement system are directly implicated (Alawieh et al., 2018; Hammad et al., 2018; Qiao et al., 2010). The secondary injury also entails complex mechanisms where oxidative stress, excitotoxicity, mitochondrial dysfunction, axon degeneration, apoptotic cell death are observed (Ng & Lee, 2019).

On the clinical levels, TBI and SCI survivors tend to suffer from many physical, neuropsychiatric, emotional, and other burdens that impact them as individuals as well as their families and nearby society. The financial burden including the direct and indirect costs, represents another major challenge where the global healthcare

costs are estimated to be 400 billion US dollars annually (Maas et al., 2017). Although several neurotherapeutic clinical trials have been applied rigorously to formulate approved treatment strategies, effective TBI and SCI therapies are still lacking (Donovan & Kirshblum, 2018; Mallah et al., 2020). Thus, a better understanding of the complex pathology and mechanisms involved in CNS traumas is required to establish novel therapeutic strategies. For this aim, there have been increasing efforts towards identifying TBI-specific molecular signatures utilizing mass spectrometry-based technologies, namely neuroproteomics, microproteomics and mass spectrometry imaging (MSI). In this review, we will discuss recent mass spectrometry advances and findings in the context of brain trauma and other CNS diseases using neuroproteomics, spatially resolved microproteomics, and shotgun lipidomics, all of which are part of the so-called spatial omics, guided by MSI.

2 | NEUROPROTEOMICS APPROACHES IN THE FIELD OF NEUROSCIENCE

2.1 | Introduction to neuroproteomics and microextraction approaches

The field of neuroproteomics evolved, along with its sub-disciplines such as psychoproteomics owing to the advanced approaches investigating the mechanisms and pathophysiology of several neuro-related disorders (Kobeissy, Sadasivan, Liu, et al., 2008; Ramadan et al., 2017). Neuroproteomics is the field that assesses the qualitative and quantitative changes in the proteome of the nervous system; thus, uncovering information about disease states and ensuing disease progression (Jaber et al., 2016; Kobeissy, Guingab-Cagmat, et al., 2016; Kobeissy, Sadasivan, Oli, et al., 2008; Ottens et al., 2006). Neuroproteomics approaches have contributed to a better understanding of the overall mechanisms involved in neuronal injury and neural degeneration as illustrated in other studies and reviews (Jaber et al., 2016; Kobeissy et al., 2006; Kobeissy, Guingab-Cagmat, et al., 2016; Kobeissy, Hansen, et al., 2016; Kobeissy, Sadasivan, Oli, et al., 2008; Ottens et al., 2006; Sun & Cavalli, 2010; Stevens & Kobeissy, 2012). Such an approach has led to

the implementation of robust methods for accurate in-depth protein identification, along with the possibility of sensitive measurement of relative or absolute quantitation of proteins or their modifications. These methods have overcome previous inherent challenges imposed by a high abundance of different neural cell types, with their distinct patterns of gene and protein expression, cellular heterogeneity of mixed populations within the distinct brain regions as well as the complexity associated with sample size, fractionation, and handling (Craft et al., 2013).

Recently, with the advances in neuroproteomics applications, several TBI-related pathophysiological markers have been identified through spatially resolved microproteomics, representing a new dimension to evaluate proteome changes. The application of spatially resolved microproteomic approaches in TBI would enable quantitative and comparative proteomics within a relatively small surface area (micrometers) in the lesion site and its corresponding perilesional area. In fact, one main challenge observed in previous neuroproteomics studies is the accessibility of samples or the need for relatively large-sized samples along with their scarcity (Ramadan et al., 2017). These challenges are surpassed by spatially resolved microproteomics enabling protein extraction from minute heterogeneous and/or limited

accessible tissue areas, which will also help in preserving tissue for other applications such as pathology assessment. In addition, an injured lesion in TBI is not uniform but rather varies in injury mechanisms as we move distally from the injured site; thus, the analysis of each of these regions within the same sample is crucial for understanding the role of selected regions in disease progression (Ramadan et al., 2017).

Moreover, the expression of a low expressed protein of interest may be masked and undetected due to the presence of abundant and overexpressed proteins for which conventional proteomic approaches are not able to separate (Ramadan et al., 2017). Microproteomics techniques implicate two main phases: microextraction, involving the collection of tissue and extraction of proteins from a small surface area, followed by proteomic analysis for the identification and quantification of proteins. Thus, to micro-extract proteins from a relatively small size surface area, many tools have been generated enabling high sensitivity and selectivity that warrant the success of microproteomics. For that purpose, three technologies have been developed i.e. laser capture microdissection (LCM), parafilm assisted microdissection (PAM), and liquid microjunction extraction (LMJ), illustrated below (Figure 1). A summary table comparing the

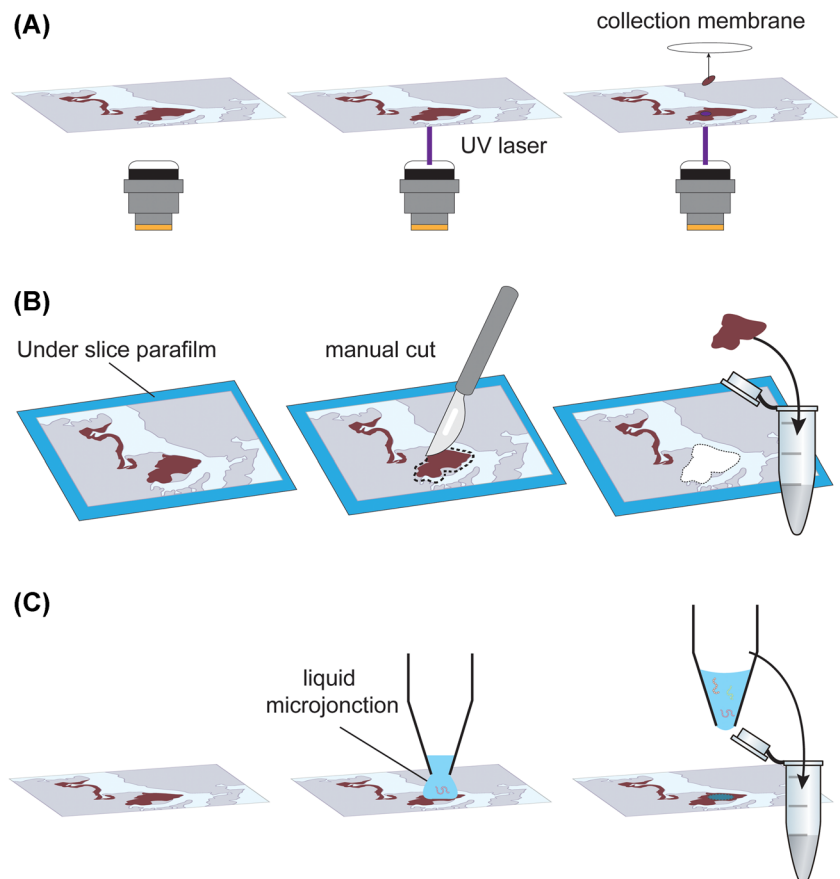


FIGURE 1 Tissue microextraction methods. The various techniques of the spatially resolved microproteomics that are used in TBI: (A) LCM, (B) PAM, and (C) LMJ. LCM, laser capture microdissection; LMJ, liquid microjunction extraction; PAM, parafilm assisted microdissection; TBI, traumatic brain injury [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/terms-and-conditions)]

differences among the three different techniques is found in Table S1.

2.1.1 | Laser capture microdissection

LCM is a method used to isolate and retrieve heterogeneous cell populations (Gutstein et al., 2008). As its name mentions, this technique relies on using a laser, either infrared (IR) or ultraviolet, under direct microscopic visualization (Chung & Shen, 2015). After the tissue is mounted on a slide, a laser pulse is fired at the desired target region within the tissue resulting in the ejection of this region from the tissue slide and its capture on a membrane/film placed just above the tissue. The precision and ability to control the laser diameter, i.e. ablation area, allows LCM to be used in single-cell isolation methods (Hu et al., 2016), as well as on subcellular levels for isolation of cell organelles, such as mitochondria, by using a laser spot size of 5 μm (Pflugradt et al., 2011). The main advantages of such a technique are the short time of tissue/cellular collection when compared to other dissection methods, along with the ability to preserve the morphology of the remaining tissue while dissecting. On the other hand, this technique has several disadvantages. Factors such as cell morphology, tissue structure, and histological visualization may result in difficulties to successfully isolate single cells or organelles from a specific tissue (Aguilar-Bravo & Sancho-Bru, 2019). If poor cellular morphology exists in ill-defined tissue, the risk for contamination will increase, especially in applications involving organelle isolation. Another limitation of LCM is its high cost. Indeed, the cost for a complete LCM apparatus, along with its corresponding microscope, may exceed 1 million dollars (Chung & Shen, 2015) along with the need for a highly skilled professional to conduct the extraction. Such special expertise is essential to obtain a dissected area that is void of any contaminated surrounding tissues.

2.1.2 | Parafilm assisted microdissection

Introduced in 2013, Franck et al. used PAM to extract tissue from small surface areas of tissue mounted on glass slides previously covered with parafilm M (Franck et al., 2013). Using a scalpel, a region of interest is manually dissected under a microscope. In their work, the authors extracted and performed proteomic identification and quantification of proteins from 3 different regions of interest with three different surface area sizes extracted from different regions of interest [ROI] (ROI1 = 3.4 mm^2 , ROI2 = 2.86 mm^2 , ROI3 = 1.77 mm^2). Quantification was achieved using label-free quantification (LFQ) based on spectral counting.

The extracted area could be as small as 600 μm in diameter depending on the desired application. More than 1000 proteins were detected from each of the dissected regions. Also, researchers showed that this approach could be applied to whole-tissue sections to obtain a protein image of the tissue, based on LFQ quantification. This technique holds the advantage of being cheaper compared to the LCM technique and can be performed without the need for state-of-the-art tools. This technique was later applied in several studies including the identification of protein biomarkers in prostate cancer (Brandão et al., 2019; Delcourt et al., 2017, 2018; Mériaux et al., 2014; Quanico et al., 2015; Quanico, Franck, Wisztorski, et al., 2017).

2.1.3 | Liquid microjunction extraction

To achieve a more efficient microextraction, Quanico et al. (2013) developed the LMJ technique in 2013 where locally digested and extracted peptides are performed within a defined region of the tissue. Briefly, using a microdeposit system, direct on-tissue digestion is performed. Following this continuous tryptic digestion, ranging from 1 to 2 h, depending on the optimized protocol for application, peptides are microextracted using an automated liquid microjunction interface by depositing and aspirating several cycles of extraction solvents in the following order: methanol, acetonitrile, and trifluoroacetic acid. Following extraction, peptides are then subjected to LC-MS/MS analysis followed by protein identification using the bottom-up shotgun proteomics approach. This technique has the ability to extract digested peptides from an area as small as 650 μm in diameter, resulting in the identification of 1500 proteins (Quanico et al., 2013). This technique, along with the previously discussed PAM technique, have been applied to perform top-down proteomics in an effort to identify intact protein changes in several sample applications including ovarian cancer and rat brain proteomic profiling, along with their posttranslational modifications (Delcourt et al., 2017, 2018). Coupling the liquid microjunction microextraction system to a nanoscale liquid chromatography-tandem MS is also applicable. In fact, with such a setup, the authors successfully extracted and identified more than 500 proteins in an area as small as 250 μm in diameter (Quanico, Franck, Cardon, et al., 2017).

2.2 | Microproteomic applications in TBI

Spatial omics technique guided by MSI has been previously performed in the context of neurodegeneration, specifically brain injury. In a rat model of experimental

TBI, Mallah et al. (2019) used LMJ extraction followed by bottom-up shotgun proteomics to depict the underlying proteomic changes in the injury core microenvironment (approximately 1 mm² surface area of tissue) throughout the first 10 days postlesion. After subjecting the data to LFQ, protein ID's subjected to hierarchical clustering depicted several protein clusters unique to different time points within the first 10 days post insult. The authors showed that starting at day 1 postinjury and continuing until day 7, an ongoing inflammatory process is implicated with an upregulation of many blood-related factors. However, as early as day 3, the processes of DNA repair are initiated along with regeneration and astrogliosis; thus, characterizing two different opposite processes occurring within the injury site in the acute phase of injury. The use of such microproteomic approach allowed for a precise and in-depth proteomic analysis within the injury microenvironment. In an effort to investigate the proteomic link between TBI and Parkinson's disease (PD) at early stages postprimary insult, the authors then applied the same microproteomic approach on the substantia nigra region of TBI injured brains at 3 days postinjury. Several PD-related proteins were found to be significantly overexpressed in the ipsilateral substantia nigra including HMGB1, GAD1, and GPR158. This study is a clear example of the use of microsampling approaches to extract and identify proteins from small surface area or regions that are not highly accessible or easily dissected, such as the *substantia nigra*.

Many brain regions are affected at several cellular levels post-TBI. Using different microsampling techniques such as LCM, depicting genetic changes post-TBI becomes possible. In turn, those changes result in diverse effects on neurodegeneration, initiating behavioral and cognitive changes. The LCM system has been used to precisely extract and capture tissue from different hippocampal brain regions in young and aged rats before and after lateral fluid percussion TBI (Shimamura et al., 2004). In this case, the tissue was used to analyze antisense mRNA by different techniques including Northern blot and ribonuclease protection assays, which illustrated that p21, an age-associated gene, was significantly highly expressed in the hippocampal CA3 region of aged rats. Following experimental TBI, provided via the fluid-percussion model on male Sprague-Dawley rats, Boone et al. (2012) found deregulation of the circadian clock genes within the hippocampus. Furthermore, using LCM on samples from the rat hippocampus post-TBI, it was proven that stochastic gene expression is crucial for predicting whether injured neurons survive or die (Shearer et al., 2016). Accordingly, genomic engineering strategies are being implicated as novel therapeutic methods for the restoration of normal brain functions.

Following TBI, gene silencing or knockdown of injury-induced genes using RNAi could advance the functional outcome (Boone et al., 2017). In their work, Boone et al. evaluated the properties of Adeno-associated virus (AAV) siRNA vectors that target two genes with antagonistic roles in TBI pathogenesis: the nNOS, and the GPx-1.

Microarray analysis of laser-captured, virus-infected neurons in the injured hippocampus showed significantly reduced neurodegeneration process following the knockdown of nNOS, which was not the case when knocking down GPx-1 as well. These findings suggest that targeting nNOS gene could decrease the effect of TBI on memory by decreasing neurodegeneration. These examples provide further proof of the wide-field research applications of microsampling approaches such as LCM.

Microdissection and extraction offer a powerful tool for the isolation of specific cell types from their harboring tissues. Indeed, proteomic analysis of LCM enriched samples were shown to have improved peptide and protein quantification along with a more defined tumor protein expression (De Marchi et al., 2016). Therefore, such a technique can also be beneficial for studying brain tumor formation post injury, in cases such as glioblastoma, which could be linked to TBI (Tyagi et al., 2016).

Taken together, microsampling techniques studies showed a greater potential for obtaining a better understanding of the TBI pathogenesis on the the genomic and proteomic levels. Such advantages include monitoring expression levels and identification of minute changes and differences that could probably be the key to a superior characterization of the disease which can result in better optimization of treatment development. Figure 2 summarizes what we have discussed in the current section of the review. A summary of the discussed applications of microextraction is found in Table S2.

2.3 | Proteomics applications in SCI

Spatio-temporal proteomics approach on rat SCI after balloon compression has been recently performed by our group (Devaux et al., 2016). The whole spectrum of the data has allowed us to depict a complete scheme along the spinal cord axis of the cellular and molecular sequel of events occurring through the time course of the inflammatory process and abortive regeneration. Specific markers for each spinal cord segment at different time points (3, 7, and 10 days) contributing to the biochemical-pathophysiological processes were observed. Surprisingly, segments below the lesion site (caudal segments) host a robust inflammatory process accompanied by the local synthesis of neuroprotective and

regenerative molecules as depicted in Figure 3. In this study, we demonstrated that the caudal segment adjacent to the lesion site possesses, at least temporarily, harbor the intrinsic components/features that may allow axonal

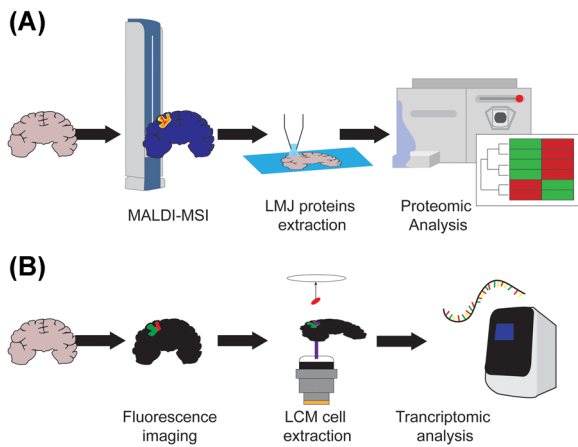


FIGURE 2 Microproteomics in TBI studies. Microproteomic techniques are applied widely in TBI studies: (A) A study using LMJ allowed to constitute a heat map of upregulated and downregulated sets of proteins. (B) LCM was used to depict genetic changes post TBI and to study the effects of AAV siRNA vectors that target two genes with antagonistic roles in TBI pathogenesis. AAV, adeno associated virus; LCM, laser capture microdissection; LMJ, liquid microjunction extraction; TBI, traumatic brain injury [Color figure can be viewed at wileyonlinelibrary.com]

regeneration. This knowledge can be used to elucidate the therapeutic window for possible therapy. However, such a caudal-to-rostral altered regenerative potential is likely hampered by inhibitory signals such as glycans that are abundantly detected or even secreted at the lesion site. Among the proteins identified from the rostral and lesion segments, some are related to chemokines, cytokines, or neurogenesis factors. In contrast, proteins from caudal segments are more related to neurocan, agrican, brevican but also to RhoA pathway and to immunoglobulins (Devaux et al., 2016, 2017).

Furthermore, the conditioned media (CM) from each spinal segment were used in vitro, for culturing microglial BV2 cell lines and DRGs explants, showing a lesion site-dependent impact on microglia activation and DRGs neurite outgrowth. In addition, while naive BV2 cells exhibited insignificant staining for the CX3CR1 receptor, the level of CX3CR1 was strongly enhanced in some BV2 cells after their stimulation by CM collected from SCI. The molecular data might correlate with different polarization of activated microglia and macrophages along the rostro-caudal axis following acute injury. This was partially confirmed in vivo by mapping CX3CR1 receptor distribution, revealing higher expression in the rostral segment, with potential neuroprotective action (Cizkova et al., 2014; Devaux et al., 2016). We have also established that when considering different time courses of the SCI,

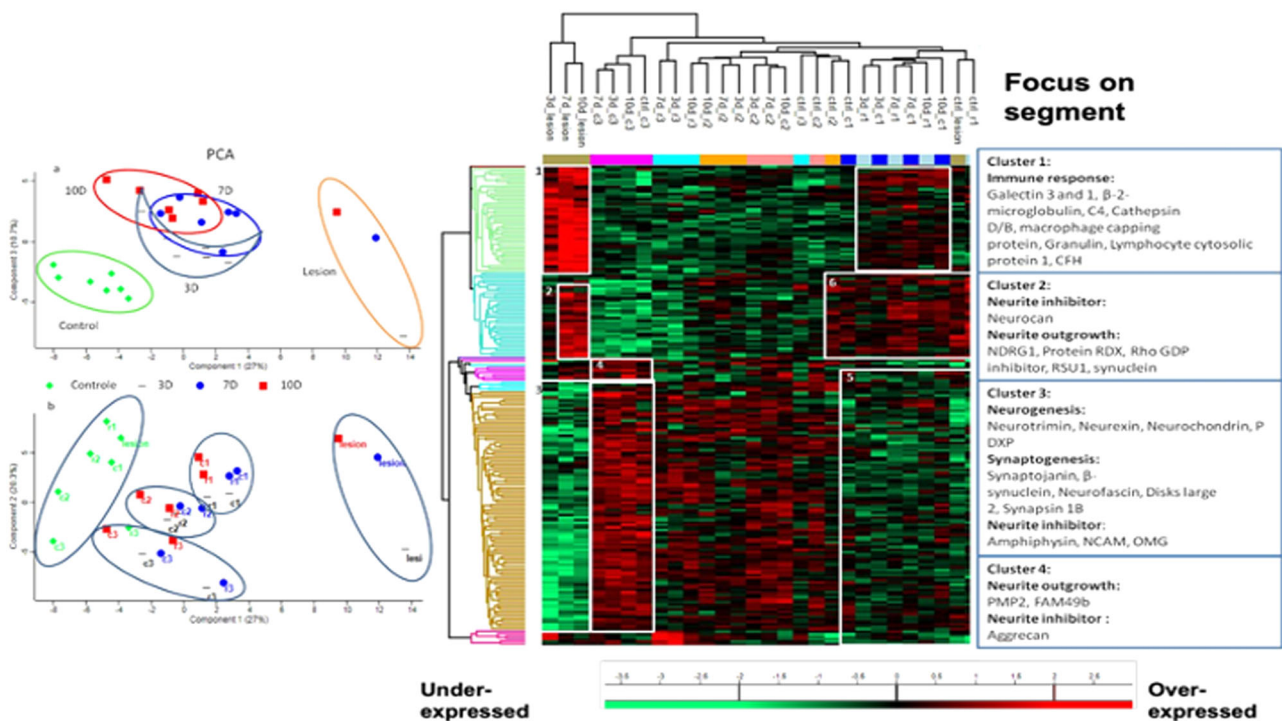


FIGURE 3 Proteomic study performed from lesion to rostral and caudal segments in time course from control, to 3, 7 and 10 days. The PCA analyses performed in time course or based on spinal cord segments is represented. A heatmap representation with clusters of proteins is also shown [Color figure can be viewed at wileyonlinelibrary.com]

the cytokine production between rostral and caudal segments appears different utilizing qualitative and quantitative assessments. At three days, cytokines allowing to attract T regulator lymphocytes have been detected in rostral but not in caudal segments which is in line with the immunocytochemical detection of these cells observed in the rostral segment.

These cells expressing CCL20 were found with a certain delay at 7 days in caudal segments. These data established a clear distinction in the nature of cells and cytokines production between rostral and caudal segments (Cizkova et al., 2014; Devaux et al., 2016). Taken together, these data suggest that polarized-regionalization in terms of inflammatory and neurotrophic responses that may occur spatially in the rostral and caudal segments. From these data, clinical applications have been investigated and bone marrow stem cells (MSCs) seemed a good way for finding neuroimmune modulators and neurites outgrowth factors (Cizkova et al., 2014). After, demonstrated success *in vitro*, alginate scaffold grafted with growth factors have been tested without stem cells (Cizkova et al., 2015) than coupled with bone MSCs which enhanced regeneration processes (Cizkova et al., 2018; Grulova et al., 2015).

Our group also used canine MSCs and demonstrated their ability to stimulate angiogenesis (Cizkova et al., 2018), antimicrobial activity, and also neuroregeneration

after SCI (Bujňáková et al., 2020; Humenik et al., 2019; Murgoci et al., 2020; Vikartovska et al., 2020) (Figure 4). Dogs with SCI treated with multiple applications of CM derived from MSCs were associated with rehabilitation strategy including under-water treadmill clearly showed high efficient recovery of motor and bladder dysfunction (Vikartovska et al., 2020) (Figure S1). These results point out the application of omics strategies from biomarkers discovery to a possible neurotherapy in spinal trauma in canine and human patients. Although the results are promising, it is necessary to validate these therapeutic approaches in a large cohorts with adequate functional analyses.

3 | LIPID MSI IN CNS INJURY

Another advanced branch in the field of CNS biomarker discovery is the use of MSI for the detection of lipid expression changes and distribution postinital insult. Indeed, MSI is a technique used to detect the spatial distribution of different molecule species with different molecular weights defined in a region of interest on a tissue sample (Shariatgorji et al., 2014). These molecules could be drugs, metabolites, lipids, peptides, or proteins (Porta Siegel et al., 2018). Such an approach relies heavily on highly sophisticated mass spectrometry

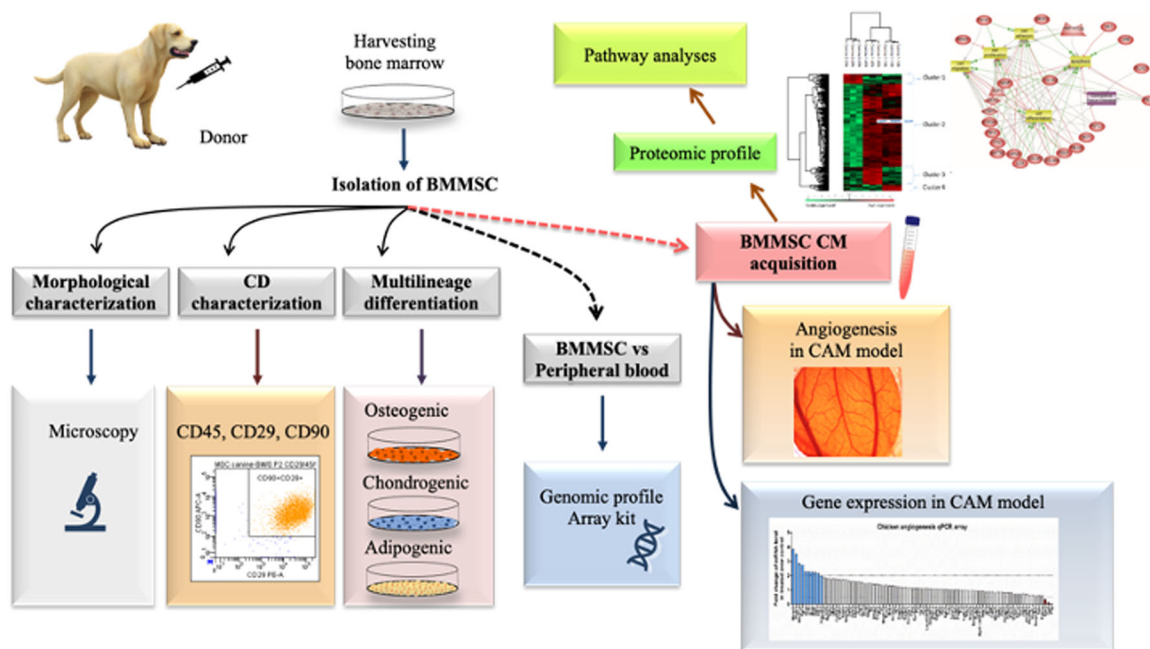


FIGURE 4 Dog bone marrow stem cells characterization. BMMSC have been characterized through their morphology, their CD present at their surface through flow cytometry analyses, multilineage differentiation through different passage. BMMSC were also analyzed at the transcript and heat map of differentially expressed transcripts of 84 selected genes in dog BMMSC has been realized. Proteomic analyses as well as system biology and biological tests like angiogenesis have been performed and compared to human bone marrow stem cells. BMMSC, bone marrow stem cells [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/terms-and-conditions)]

instrumentation and ionization techniques coupled with matrix-assisted laser desorption/ionization (MALDI) (Murayama et al., 2009). Mass spectrometry separates ionized molecules based on their respective mass/charge (m/z) ratio which can be eventually used to determine their corresponding molecular weight. In the case of MALDI MSI, a matrix is deposited on the tissue sample to extract the analytes from a specimen, and then a laser will raster throughout a region of interest and at a specified spatial resolution (Norris & Caprioli, 2013). By doing so, each laser raster point results in the ablation of molecular constituents of the tissue which will be ionized, analyzed, and detected within the instrument.

Each raster point will contain a mass spectrum of all detected ions distributed by their m/z charge and corresponding coordinates of laser ablation that will be processed into a pixel. Finally, the collection of pixels within the defined region of interest yields a molecular ion image, and each m/z value can be visualized across all mass spectra as a pseudo-colored image (Alexandrov et al., 2013). In short, MSI is a label-free approach that allows for the visualization of hundreds to thousands of spatially distributed molecules in tissue with one single experiment (Aichler & Walch, 2015). Technological advances in MSI have allowed it to become of great importance in clinical practice and research. Such advances include improvements in sample preparation, data interpretation, and instrumentation; leading to high acquisition speeds, enhanced spatial resolution improving throughput and depth, and higher spectral resolution (Buchberger et al., 2018). The development of MSI techniques as well as the different ionization sources and instrument configurations are well documented in the literature (discussed below). Therefore, we will discuss below the most recent findings of MSI techniques in the context of TBI and other CNS diseases.

3.1 | Lipid MSI applications in TBI

Lipid MSI has been employed in several preclinical rodent TBI models including controlled cortical impact (CCI), blast injury, and fluid percussion. MALDI-LTQ-XL-Orbitrap MSI depicted an increase in GM2 ganglioside and depletion of ceramide expression in the hippocampus, thalamus, and hypothalamus of mice subjected to a low-level blast TBI; thus, providing proof that a low-level nonpenetrating wave blast is sufficient to cause biochemical changes in the brain (Woods et al., 2013). In a rat CCI model, the kinetics of different membrane lipid species were monitored across the acute phase (up to 7 days) after injury using MALDI-LTQ-XL-Orbitrap MSI (Roux et al., 2016). For example, cholesterol ester

expression level did not increase until 3 days after injury and persisted till day 7. On the other hand, ceramide species increased starting from day 1 postinjury and persisted until day 7. Concerning ceramides, these findings were contrary to previously published data monitoring ceramide expression after a low-level blast wave exposure (Woods et al., 2013). Thus, these findings potentially link ceramide overexpression with two reciprocal functional processes involving direct mechanical injury and repair of contusion of neural and vascular tissue. Other studies have also confirmed the increase in ceramide after CCI in rats at 3 days after TBI using MALDI-LTQ-XL-Orbitrap MSI; however, treatment with a “decoy peptide” for dynorphin was sufficient to reduce such ceramide increase (Barbacci et al., 2017), which was shown to be mediated via reducing NMDA-dynorphin neurotoxicity.

This study shows the capabilities of lipid MSI in validating the effect of therapeutic candidates in the context of TBI. In line with these experiments, Hankin et al. (2011) also showed increased expression of ceramide, mainly Cer (d18:0/18:1) at m/z 548.5, after 72–96 h of fluid percussion injury using MALDI-TOF MSI.

In a rat model of CCI, MALDI-TOF MSI revealed that several cardiolipin (CL) species were shown to lose their expression after initial insult in multiple brain regions including contused cortical tissue, ipsilateral hippocampus, and thalamus (Sparvero et al., 2016). Similar to CLs, phosphatidylinositol also exhibited a significant decrease in expression.

Using a developed liquid-extraction-ESI device for MSI, Guo et al. (2017) studied changes in docosahexaenoic acid (DHA)-containing lipids in rats up to 7 days post fluid percussion injury. Results showed that DHA-containing phospholipids (PLs) such as phosphatidylethanolamine (PE) (P-18:0/22:6) decreased by day 1 and then gradually increased by day 7. On the contrary, Lyso-PE (22:6) was significantly increased at 1 day and then decreased by day 7. To characterize the spatial distribution of a potential lipid biomarker, McDonald et al. monitored the expression of lysophosphatidic acid (LPA) after rat CCI using solariX FTICR-MALDI MSI (McDonald et al., 2018). Indeed, LPA levels were elevated at 1 and 3 h after injury, and surprisingly, LPA expression and spatial distribution showed significant association with β -amyloid precursor protein, demonstrating the potential link of LPA to secondary axonal injury. Following rat CCI, Mallah et al. (2018) utilized high spectral resolution MALDI-LTQ-Orbitrap-XL MSI to reconstitute a 3D volume filled visualization of lipid images from rat brains at 3 days after injury. This was achieved by the acquisition of lipid MS images of serial sections from the same brain followed by alignment and volume filling to

obtain the final 3D structure. 3D spatial segmentation depicted two injury-related clusters of lipids which corresponded to two different subsets of lipid species with higher expression levels in the injury core and its direct surrounding but not anywhere else in the brain. The same group later used a similar approach, but in a 2D manner to conduct a spatiotemporal analysis of lipid changes in rat brains post CCI up to 10 days postinjury (Mallah et al., 2019). This allowed the identification of a potential new lipid injury-related family of biomarkers, the acylcarnitines. Several lipid markers within this family, mainly palmitoylcarnitine (16:0) showed localization in the tissue directly surrounding the injury core with little to no diffusion inwards towards the epicenter of the lesion, and with direct colocalization with resident microglia of the brain. The spatial aspect of the study resulted in the detection of high expression levels of palmitoylcarnitine in the ipsilateral substantia nigra, a region involved in PD at 3 days postinjury. This study is an example of the ability of MSI to potentially bridge two different CNS neurodegenerative diseases with a potential common lipid expression phenotypes. Figure S2 represents the 3D distribution of acylcarnitines in brain post-TBI. Moreover, Table S3 summarizes the findings of the discussed results.

3.2 | Lipid MSI applications in SCI

MSI has also been employed for drug monitoring, injury characterization, and biomarker discovery in the context of SCI. Using 3D MALDI-TOF MSI in an experimental model of SCI, Quanico et al. were able to detect overexpression of long-chain acylcarnitines (LC ACs) and localize them within the lesion border (Quanico et al., 2018). These lipid species were detected as early as 3 days postinjury and persisted along the lesion margins till 7 and 10 days after SCI. LC ACs were found to colocalize with resident microglia/macrophages on the lesion border. MALDI-LTQ-Orbitrap XL MSI experiments, in a rat SCI model, were applied to assess the contribution of different adducts and their accumulation consequences on the total signal intensity of a given lipid species (Fernández et al., 2017). Results showed variations in the $[M + H]^+ / [M + Na]^+ + [M + K]^+$ relative abundance which correlated with the sample type. This abundance was strongly related to pathophysiologic changes induced by the lesion. Overall, the data was in line with the scheme of events that occurs after SCI which includes ionic homeostasis unbalance, sodium–potassium-dependent ATPase inhibition, and inflammation. MALDI-TOF MSI was used to visualize the PL distribution in the spinal cord following sciatic nerve injury

(Banno et al., 2017). Data have shown a significant increase in arachidonic acid-containing phosphatidylcholine (PC) levels in the ipsilateral ventral and dorsal horns of the spinal cord 7 days after sciatic nerve injury. This alteration was linked to the microglial activation which was shown to be diminished after treating with minocycline, a microglia suppressor.

Our group described the novel molecular and cellular processes occurring after SCI in a spatial (by focusing on the rostral, lesion, and caudal sections) and temporal (up to 10 days after injury) manner (Devaux et al., 2016). This study combined a global systems biology proteomic approach with 3D MALDI-TOF MSI and lipidomic spatial distribution profiles to study immune cell infiltration with cytokine microarrays (Figure S3 and S4). The results characterized the C1 segment as one with the presence of neurite outgrowth inhibitors; thus, the least segment to have regeneration capabilities. This was coupled with high expression of IgG in the lesion site at 3 days after injury. Finally, the lipid MSI portion of the study showed clear discrepancies between the C1 and R1 sections, with the C1 being more impacted at the level of white matter. Normal versus injured rat spinal cord tissue was assessed using desorption electrospray ionization-MSI to depict different lipid signatures of each condition. The distinction was based on the overexpression of fatty acids, diacylglycerols, and lysolipids in the injured tissue. The acquired results indicated hydrolysis of lipids during the demyelination process most probably due to the activation of the phospholipase A2 enzyme (Girod et al., 2011). Arima et al. were interested in investigating the effects of MR16-1, an anti-IL-6 receptor antibody, on the expression patterns of PLs at 7 days after injury in a mouse SCI model using MALDI-TOF MSI. Results showed that MR16-1 treatment reduced infiltration of immune cells into the spinal cord. Also, MR16-1 treatment was able to restore expression levels of DHA-containing PC to their normal levels compared to control noninjured mice (Arima et al., 2014).

3.3 | Lipid MSI application in other CNS-related injuries/disease states:

Drug distribution monitoring is one of the main applications of MSI in CNS injury and neurodegenerative diseases. To monitor the localization of a therapeutic candidate, Danegaptide (ZP1609), Wang et al. (2018) performed MSI experiments using MALDI-TOF MSI instrumentation to visually detect ZP1609 distribution in mouse brain tissue post transient middle cerebral artery occlusion. Images showed the ability of ZP1609 to penetrate the BBB and localize in the injury site. Similarly,

MALDI-LTQ-Orbitrap XL MSI was used to track the spatial distribution of Dexapramipexole, a drug able to increase mitochondrial F1Fo ATP-synthase, in brain section of a distal MCAO ischemic rodent model which subsequently resulted in a decreased infarct size (Muzzi et al., 2018). MSI has not only been investigated to monitor drug distribution in such CNS injury/disease states but it has also been employed to test the effect of therapeutic approaches on molecular species such as lipids. SIMS-TOF MSI was used to monitor the differences in the expression level of white matter lipids in neonatal rat brains after treatment with inhaled nitric oxide (iNO) up to 7 days post birth (Kadar et al., 2014). Treatment by iNO is currently used for hypoxic respiratory failure in intensive care units for preterm birth infants which showed an improved white matter maturation in the developing brain when compared to nontreated preterm born babies. The MS images showed a dramatic increase in hydroxylated sulfatides within the white matter after iNO inhalation which was previously linked to an increase in the myelination process. Koizumi et al. (2012) used MALDI-TOF mass spectrometry to measure the expression intensity of Na-adducted PC after 4 h of continuous administration of an artificial cerebrospinal fluid (Artcereb) to injured and irrigated rat brains.

The intensity expression of Na-adducted PC served as an indication of water retention in brain cells and showed that when compared to nontreated animals-Artcereb reduced the water retention in the wound area; thus, resulting in minimization of edema postimpact to the brain. The same research group previously demonstrated, using MALDI-TOF MSI on brains from a rat focal cerebral ischemia model, the upregulation and accumulation of several lyso-PC molecules within the injury site (Koizumi et al., 2010). Finally, MALDI-FT-ICR was used to visualize the lipid changes in a rat optic nerve crush model before and after treatment with inflammation-induced optic nerve regeneration (IIR) (Stark et al., 2018). This treatment increased the presence of microglia and macrophages, along with the accumulation of GM3 gangliosides and reduction in expression of lipid sulfatides, mainly sulfo-glycosphingolipids.

MSI has also been used in preclinical research studies to understand the underlying processes involved in CNS-related injuries. For example, MALDI-TOF MSI of proteins was utilized to develop a classification system in differentiating between tissue containing diffuse axonal injury within rat brain stem sections, compared to normal noninjured regions (Ren et al., 2016). In a rat model of ischemia, MALDI-TOF MSI revealed the increased expression of several PL species in the injured region, mainly *m/z* 753.7, 756.7, and 782.7, when compared to the noninjured opposite site of the same brain (Shanta

et al., 2012). In a mouse MCAO cerebral ischemia model, MALDI-TOF MSI revealed the differential spatial distribution between different ganglioside species based on the oligosaccharide structure and sphingosine chain length. While d20:1 moiety of GM1, GD1, and GT1b showed increased expression in the cortex, d14:1 moiety of GM2 and GM3 were mainly localized within the hippocampus and striatum (Whitehead et al., 2011). Nielsen et al. (2016) applied MSI on brains collected from mice subjected to permanent focal cerebral ischemia in a temporal manner starting from 2 h postinjury up till 20 days. Their study concluded that bis(monoacylglycerol) phosphate and *N*-acyl-phosphatidylethanolamines can be used as lipid biomarkers for phagocytizing macrophages/microglia and dead neurons; respectively, showing the potential lipid MSI in biomarker identification and validating the role of the neuroinflammatory processes occurring post cerebral ischemia. Table S4 summarizes all the studies discussed in this section.

4 | SPATIALLY RESOLVED LIPIDOMICS APPLICATION IN TRAUMA: SHOTGUN ANALYSIS

The need for biomarker discovery in the context of CNS injury is not restricted to the field of proteomics for the identification of protein biomarkers, but rather there is a profound need for lipid biomarkers combined with their protein counterparts to obtain a more accurate and precise diagnosis. For this, the field of lipidomics has emerged. Lipidomics is a systems-based study of all lipids and their function within a given cell, while also considering the molecules with which they interact (Watson, 2006). Lipidomics encompasses different “front end” liquid chromatography strategies utilized for lipid separation and various “back end” advances. These advances include the use of mass spectrometry for lipid discovery, identification, and categorization. Lipid-centered analysis has encountered notable interest in recent decades due to its primary involvement in health, cellular metabolism, and injury, which allowed analysis prospects for individual PL species, including structural and subtle quantitative analysis that focuses on uncovering lipid participation in cellular metabolism (Han & Gross, 2003; Tyurin et al., 2008). In regard to clinical utility, the significance of utilizing lipidomics over neuroproteomics lies in the fact that the expression of circulating lipids in the blood reflects the brain pathophysiology given the ability of lipids to cross the BBB along with other factors with a similarity of lipid content in CSF and extracellular fluids (Pardridge, 2005). Furthermore, CNS tissue has a highly concentrated lipid

content, the highest in the body, just after adipose tissue (Han, 2007). Numerous new mechanisms of lipid metabolism are becoming increasingly appreciated. These include mechanisms investigating selected gene functions, assessing drug efficiency, and recognizing innovative biomarkers. Lipids are highly saturated in the brain, where they are fundamental to CNS architecture, maintenance, and function. They are among the major constituents of cellular membranes and represent chemical energy storehouses (van Meer et al., 2008). Lipids participate in numerous cellular mechanisms, such as the physical regulation of cellular membrane properties and neurotransmitter signaling (Allen et al., 2007). Also, throughout the secondary phase of trauma, lipids are involved in the neuroinflammatory process (Lamade et al., 2020), and some lipid mediators, such as docosanoids and eicosanoids, play roles as inflammatory-response regulators in CNS injuries and disease states (Sparvero et al., 2016). The homeostasis of brain lipids is critical throughout TBI recovery. Hence, surveillance of the lipid distribution in TBI models becomes crucial, paving the way toward the characterization of the molecular processes involved in the affected zone, together with widening therapeutic horizons for future treatments. The necessity of lipids as constituents in cellular membranes, signaling molecules, and bioenergetic fuel has stimulated lipidomic-based attempts to uncover human health and disease markers.

“Shotgun Lipidomics” emerged, with its advantages and disadvantages, mainly addressing targeted lipids along with the major lipid content. Besides, advances in liquid chromatography-ion mobility-mass spectrometry offer a supplementary element for the separation of lipid species. This separation occurs due to differences in molecular size, shape, and charge, thus providing added organizational data regarding the studied lipids (Paglia et al., 2015). Shotgun lipidomics relies on the utmost usage of distinctive physical and chemical properties of lipid types, subtypes, and discrete subatomic species. This facilitates the identification and evaluation of a cell lipidome in a high-throughput style, straightforwardly from natural concentrates of biological samples. The constant advancement in MS is the major driving force of improvement in lipidomic related approaches, resulting in the understanding of the biological processes associated with lipids. The major aim behind this is to develop a reliable analysis method for lipids leading to comprehending lipids at a closer level and relating their profile to human health and diseases (Hsu, 2018).

Several studies reported an alteration in the lipid profile after TBI. One study showed PL abnormalities in the CCI mouse model up to 3 months post injury (Abdullah et al., 2014). This was evident in multiple

brain regions studied. In the cortex and cerebellum, there were decreased PC and PE levels compared to noninjured animals. Also, proportions of DHA to arachidonic acid-containing PC and PE species showed a decrease in expression values in the hippocampus, cortices, and plasma of injured mice. In a study analyzing CSF from human TBI and control patients using LC-MS/MS approach, several lipid mediators including arachidonic acid, DHA, 5- and 12-eicosatetraenoic acid all possessed increased levels in the CSF of TBI patients (Farias et al., 2011).

Additionally, different PLs can hydrolyze non-oxidized or oxidized fatty acyl chains in the brain after TBI, showing an accumulation of the resulting products after both experimental and clinical TBI (Anthonymuthu et al., 2017, 2018). Other studies reported a delay in PC peroxidation, observed a day after the increase in CL oxidation (Bayir et al., 2007; Ji et al., 2012). This indicates that PL oxidation could be involved in the occurrence of secondary measures after trauma. Due to the fact that long-chain PUFAs help maintain membrane fluidity, configuration, and function (Erdman et al., 2011), antioxidant remedies could prove to be beneficial in conserving neuronal membrane integrity following TBI (Abdullah et al., 2014). Using phospholipidomics analysis, CL expression profile in pediatric TBI revealed a time-dependent increase in plasma CL expression level coupled with a decrease in brain expression level (Anthonymuthu et al., 2019).

Noteworthy, the CL levels which had the most variance between naïve and injured plasma were the ones most saturated within the brain. CLs possess several functions including being essential components of membranes, bioenergetic fuel production, and serving as signaling molecules (Horikoshi et al., 1989). In the context of cerebral ischemia, it has also been shown that brain type-CL accumulates in the plasma after injury, and in expression levels proportional to injury severity (Anthonymuthu et al., 2019). As CL is normally positioned within mitochondria exclusively, the identification of brain-type CLs in systematic circulation indicates mitochondrial dysfunction and thus can be used as potential biomarkers for TBI patients. Moreover, plasma-accumulated CLs were shown to contribute to inflammation and immunity (Balasubramanian et al., 2015; Chakraborty et al., 2017). Taking into consideration the participation of extracellular mitochondrial components in regulating immune responses, we can say that CLs are not only biomarkers but are also major contributors in inflammation regulation in response to injury. Since the brain contains these unique lipids, they represent a valuable target for biological physical alterations as well as prospective couriers after TBI.

The major task in lipidomics became the determination of mechanisms responsible for alterations in lipid homeostasis. Lipidomics may also benefit the drug-production industry, aiding in the expansion of therapeutic intervention capacities. Lipidomics may also employ the available models to test the effectiveness of any established drug. Finally, as a member of the “Omics” community, the field of lipidomics is still in its infancy, allowing scientists to exploit it for biomarker detection and analysis in different organelle, cells, organs, and entire body systems (Han et al., 2007).

5 | CONCLUSION AND CLINICAL PERSPECTIVE

CNS injury is a major leading cause of death and disability and is gaining much attention in recent years. Biomarkers associated with the injury are much needed, which also helps in developing a promising treatment. With the recent advances in neuroproteomics analysis and the ability of sensitive measurements, scientists are using this approach to reveal the protein expression within the CNS in injury and disease states. This detection of altered proteins is of great importance to comprehend injury severity and outcome. Along with proteins, lipids and their metabolites can be considered as candidate biomarkers. Advanced imaging techniques identified a new lipid injury-related family that has never been discussed before in the context of TBI such as the acylcarnitines. Since lipids make up a great part of the CNS and are major players in essential processes of the brain and inflammatory mechanisms, shotgun lipidomics studies identified an alteration in PL profiles and CLs distribution in the brain. Moreover, increased plasma levels of CLs were shown to be associated with mitochondrial dysfunction which can be used as a potential therapeutic target for TBI patients.

The detection of biomarkers is achievable by the use of preclinical animal models and studying tissue from human biopsies (from live patients) and postmortem tissues. For the latter two, there are pros and cons for their respective use. In the case of brain injury, the amount of tissue obtained while collecting a biopsy during surgical intervention is much less than harvesting a complete postmortem brain. This is especially important when studying key brain regions, in which a biopsy is not accessible or cannot be removed. Thus, for studies that focus on multiple interconnected brain regions, obtaining biopsies during the surgical intervention will not be achievable and data will not be comprehensive. Another main factor is the time for sample collection. The time to collect and store or experiment with a

biopsy collected at a surgical site is much faster than collecting a postmortem brain. In fact, the time interval to collect a postmortem brain can range anywhere from 1 h to over 24 h while the body is refrigerated awaiting autopsy (Blair et al., 2016). MSI can be applied to both biopsies from human live patients and postmortem tissue. However, the long waiting times to collect postmortem brains affect the expression level of several classes of biological molecules in the brain including lipids and proteins as well as contribute to their degradation. For example, neuropeptides are rapidly modified postmortem, and the longer the wait time, the more postmortem degradation occurs (Gemperline et al., 2014). Metabolite MSI is highly affected by postmortem autolysis and it is suggested that investigators establish the stability of their studied compounds of interest to confirm that the data they obtain are not a result of postmortem autolysis (Dienel, 2020). This is not the case when obtaining a fresh biopsy and directly processing it with specific procedures that halt the degradation process. Thus, the use of one over the other is mainly a result of the study aims and questions to be addressed.

Finally, optimization of microproteomics techniques, starting with microextraction techniques including LCM, PAM, and LMJ and followed by proteomics approaches (shotgun proteomics, top-down proteomics, etc.) would potentially improve the characterization and

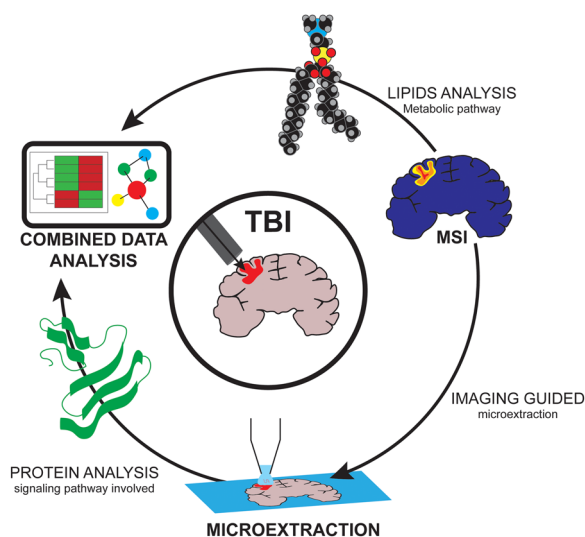


FIGURE 5 After MSI from the molecular images obtained, spatially resolved microproteomic is performed and from the collected data systemic biology analyses are realized. In parallel, spatially resolved lipidomic is done and the metabolomic pathways constructed. After combined analyses are undertaken and integration of pathways (proteomic and metabolomic) are carried out in regards with the physiopathology of TBI. MSI, mass spectrometry imaging; TBI, traumatic brain injury [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/terms-and-conditions)]

identification of proteins associated with TBI and other CNS injuries/disease states. Similarly, progress in lipid MSI studies coupled with lipidomics will help further identify lipid biomarkers. Taken together, these developments and advances present a promising approach to develop treatments for TBI and thus, warrant more investigation and analysis (Figure 5).

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

All authors have contributed to the paper writing.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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