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INVITED REVIEW

Biomarkers for NeuroAIDS: The Widening Scope of Metabolomics

Gurudutt Pendyala · Elizabeth J. Want · William Webb · Gary Siuzdak · Howard S. Fox

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Abstract "Metabolomics", the measurement of metabolite concentrations and fluxes in cell systems, is an emerging science that has enormous potential and several unique characteristics. The current applications for this field are, primarily, toxicological profiling and biomarker studies. This review of metabolomics research highlights the identification of reliable biomarkers with emphasis on neuroAIDS. Such identification of candidate markers will be advantageous for tracking the progression of human immunodeficiency virus/central nervous system (HIV/CNS) disease to gain maximal benefit from antiretroviral treatment and to provide insight into the mechanism of related neuropathogenesis.

Key words neuroAIDS · biomarkers · metabolomics · CNS · HIV · SIV · mass spectrometry

Introduction

Neuropsychopathology, that is, central nervous system (CNS) dysfunction leading to cognitive and behavioral changes, continues to be the leading cause of disability and death in AIDS patients (Albright et al. 2003). Damage to the brain during human immunodeficiency virus (HIV)-induced disease was initially identified in late-stage AIDS, during which clinically diagnosed dementia and neuropathologically testable encephalitis developed in an appreciable number of individuals. However, despite the current availability of treatment, the brains of these patients remain as a target of damage and the ensuing dysfunction. In many countries, the combination of several (typically three or four) antiretroviral drugs, known as Highly Active Antiretroviral Therapy (HAART), has been successful in greatly decreasing the mortality rate and prolonging the life span of humans infected with HIV. Nevertheless, the number of individuals with HIV-related CNS disorders has increased (Dore et al. 1999; Gray et al. 2003; Masliah et al. 2000). As most HIV therapeutics do not readily cross the blood-brain barrier, the treatment of this epidemic poses a daunting task. Furthermore, worldwide, only a small fraction of those infected with HIV receive effective treatment.

Soon after entering its host, HIV migrates into the CNS, where it remains detectable throughout the course of infection. Although HIV does not infect neurons, it does attack cells of the monocytic lineage in the brain, namely, macrophages and microglia (Davis et al. 1992; Gendelman et al. 1994; Gray et al. 1993; Kaul et al. 2001). Therefore, neurotoxicity resulting from HIV infection stems from an indirect mechanism, possibly involving toxic viral proteins or inflammatory mediators produced by activated macrophages and microglia (Diesing et al. 2002; Gartner 2000; Lane et al. 1996; Nuovo and Alfieri 1996; Sharer 1992) and also from the adaptive immune response to this virus in the brain (Marcondes et al. 2001; Roberts et al. 2006). The resulting neurological, motor, and cognitive impairments in these patients have been termed "neuroAIDS".

A critical gap in the field of neuroAIDS research is the identification of reliable molecular markers that divulge the mechanisms of neuropathogenesis. Furthermore, the detec-

G. Pendyala · H. S. Fox (✉)
Molecular and Integrative Neurosciences Department,
Scripps Research Institute, 10550 N. Torrey Pines Road,
La Jolla, CA 92037, USA
e-mail: hsfox@scripps.edu

E. J. Want · W. Webb · G. Siuzdak
Department of Chemistry and Center for Mass Spectrometry,
Scripps Research Institute, 10550 N. Torrey Pines Road,
La Jolla, CA 92037, USA

tion of individuals at risk for the CNS complications of HIV infection, and those in whom CNS abnormalities may be prevented, progress, or respond to treatment, is severely hampered by the lack of reliable biomarkers for this aspect of HIV pathogenesis. Because such biomarkers are amenable to objective tracking and quantification, they indicate changes in physiological states due to pathogenic processes or therapeutic intervention. In addition to being invaluable clinically, these markers play an increasingly prominent role in drug development and medical research (Frank and Hargreaves 2003; Matsumoto and Kuhara 1996; Horning and Horning 1971).

Metabolomics: New applications, new insights

With the advent of DNA and oligonucleotide microarray technology, one could simultaneously analyze the expression and activity of several gene transcripts, thus revolutionizing genomics and genetic research. Earlier studies from our group met with significant success in transcriptional profiling of the brain using gene microarrays (Roberts et al. 2003, 2004, 2006; Masliah et al. 2004) to address questions in neuroAIDS. But we now believe this challenge can best be met with metabolomics.

Analogous to the genome (set of all genes in an organism), the metabolome is a quantitative description of all low-molecular-weight endogenous metabolites in specified cellular, tissue or biofluid compartments. Since numerous high-profile publications on this subject have now appeared, interest in metabolomics is increasing rapidly. This branch of "omics" may even become superior to any other postgenomics technologies for pattern-recognition analyses of biological samples. Present applications for this field center around toxicological profiling and biomarker studies, and emerging data indicate that metabolomics can serve as a true window for new insights into the actual biochemical state of the cell/organism (Fig. 1). Metabolomics draws on the strengths of a century of progress in biochemistry and recent advances in technology, allowing separation and quantitative analysis, as elucidated below.

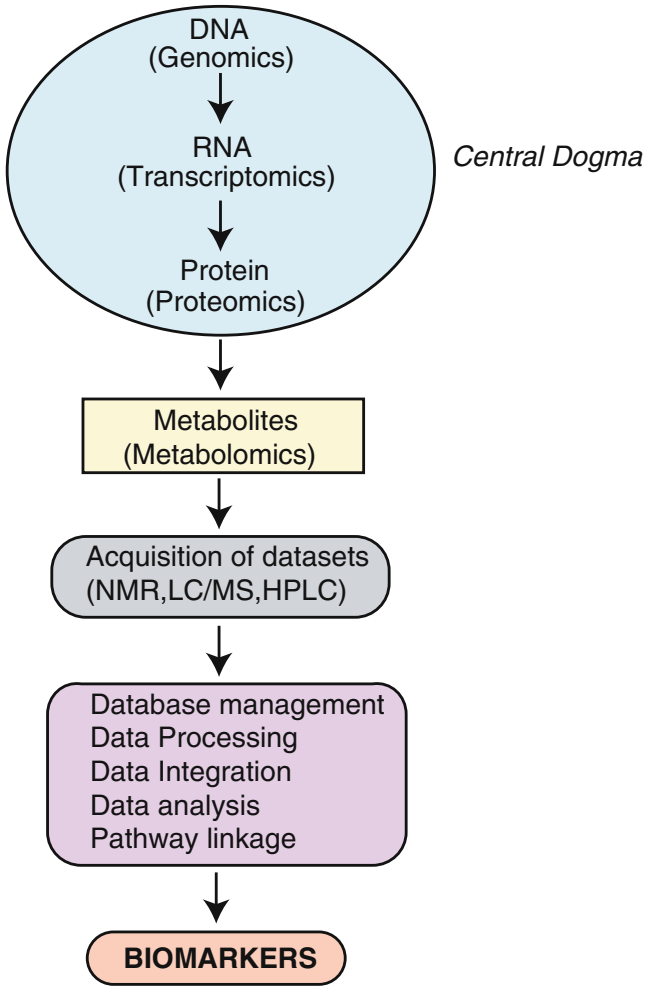

Fig. 1 Metabolomics—the new kin in the "omics" family. The position of metabolomics is shown in respect to the other "omic" approaches and to the central dogma of modern molecular biology. The initial phase involves screening of metabolites to deduce a metabolite database using a wide array of techniques. After the generation of a database from metabolic responses, this can be used in obtaining a validated biomarker (NMR: nuclear magnetic resonance; LC/MS: liquid chromatography combined with mass spectrometry; HPLC: high-performance liquid chromatography).

Metabolomics techniques

Nuclear magnetic resonance

In the 1980s and 1990s, high-field proton nuclear magnetic resonance (NMR) became prevalent for metabolite studies (Nicholson et al. 2002) and has since been widely utilized for metabolomics research in areas as diverse as plant metabolism (Bligny and Douce 2001; Ratcliffe and Shachar-Hill 2001), Duchenne muscular dystrophy (Griffin et al. 2001), neurological disorders (Holmes et al. 2006), and hepatotoxicity and nephrotoxicity in rodents (Bollard et al. 2005; Lenz et al. 2005; Craig et al. 2006).

One key advantage of using NMR for metabolomics is the minimal sample preparation required, often involving no more than pH buffering plus the addition of a deuterated solvent such as chloroform, methanol, or acetonitrile (Defernez and Colquhoun 2003) for the study of biofluids, cell lysates, and tissues. Other advantages of NMR include both rapid and quantitative analysis (Dunn et al. 2005) and preservation of the sample, which is particularly important when amounts of material are small. By using high-throughput NMR techniques such as flow injection NMR, hundreds of samples per day can be analyzed, which makes

this technique applicable to metabolomics studies (Dunn et al. 2005). Additionally, the advent of new NMR probe techniques, particularly the introduction of cryoprobes (Spraul et al. 2003), has resulted in electronic noise reduction and up to a fourfold increase in sensitivity (Yang 2006).

However, to provide extensive coverage of biofluid and tissue metabolomes, a combination of separation and detection techniques will be needed. For example, in NMR, although analytes can be measured quickly and accurately without the need for initial sample processing, the spectra can be complex, containing overlapping resonances from components other than metabolites, including proteins, lipids, and lipoproteins (Tang et al. 2004). As a result, only a small percentage of the compounds in a sample may actually be observed, because metabolites present at low concentrations may be masked by more abundant molecules. This issue is particularly troublesome in plasma, where lipids and proteins may overwhelm the signals from small metabolites (Reo 2002).

Here, mass spectrometry (MS) can offer additional insight into the metabolome. When coupled with appropriate separation techniques, MS analysis of biofluids can offer high sensitivity and specificity. However, despite liquid chromatography (LC)-MS being the foremost technique for the analysis of known compounds (Yang 2006), as well as the determination of unknowns using tandem MS (MS/MS), one limitation is the inability of LC-MS alone to unequivocally distinguish between some coeluting stereoisomers (Dachtler et al. 2001). Additionally, some compounds do not ionize well using any of the common ionization techniques and so cannot be detected by using MS alone. Coupled NMR and MS have been combined with LC (LC-NMR-MS) and applied to metabolite studies, such as in the pharmaceutical arena (Corcoran and Spraul 2003; Yang 2006). This technique allows data from both MS and NMR to be collected in a single LC run; the complementary information thus provided makes this approach a powerful tool for the detection and identification of both known and unknown compounds (Yang 2006). Furthermore, software is being developed to cope with analysis of the complex data produced by these instruments, in particular, statistical heterospectroscopy, an approach to the integrated analysis of NMR and ultrahigh-performance liquid chromatography (UPLC)-MS data sets (Crockford et al. 2006).

Mass spectrometry in metabolomics

MS has revolutionized the biological sciences, because of its ability to monitor a vast repertoire of metabolites with high quantitative accuracy. The result is discovery of markers for disease diagnosis as well as drug metabolite analysis and pharmacokinetic studies (Maurer 2005; Ma et al. 2006). MS is already well established as tool that reproducibly quantitates small molecules and, because the metabolite profile reflects enzymatic activity, directly monitors normal biochemical events and perturbations that lead to disease. In biofluids, the dynamic range of small molecules easily exceeds 9 orders of magnitude, and the diversity of species ranges from simple amino acids to complex carbohydrates. MS offers the capability of measuring over a wide dynamic range and also of observing a diverse number of molecular species, both of which are important attributes when addressing the challenges of metabolomics (see Table 1). In this study we discuss MS in terms of varied approaches to metabolomics, with particular focus on liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS), now a commonly used method in these studies (Wilson et al. 2005a).

Gas chromatography/mass spectrometry

A prominent technique in the 1970s and 1980s for metabolite analyses, gas chromatography/mass spectrometry (GC/MS) is still used today for the detection of numerous metabolic disorders such as organic acidemias as well as states of steroid metabolism (Chace 2001; Kuhara 2005). GC/MS offers high resolution, efficient chromatographic separation, sensitivity, and confirmation power based on electron impact (EI) ionization mass spectra. In recent years, GC/MS has also been successfully applied to metabolomics, particularly for plant functional genomics (Fiehn et al. 2000) as a significant improvement over previously existing methods. However, this technique is not always ideal, because metabolomics studies often call for a high throughput and the detection of a wide range of metabolites, both in size and hydrophobicity. Disadvantages including convoluted sample preparation, lengthy analysis time, and limitations on the size and type of molecule that can be analyzed reduce the applicability of GC/MS to metabolomics (Table 1).

Liquid chromatography/mass spectrometry

The development of atmospheric pressure ionization techniques made the use of liquid chromatography/mass spectrometry (LC/MS) (Ma et al. 2006) advantageous for metabolomics studies. Its attributes include good sensitivity and dynamic range, simple sample preparation and analysis, as well as expanded access to metabolites of diverse chemical structure and size (Table 1). As stated above, LC/ESI-MS is a well-established tool for metabolite studies. ESI offers many benefits over other ionization techniques, such as analysis of low and high mass compounds, excellent quantitative capacity and reproducibility, high sensitivity, simple sample preparation, amenability to automation, and soft ionization (Voyksner 1997; Siuzdak

Table 1 Technical considerations of using GC/MS, NMR, ESI-LC/MS, and flow injection analysis (FIA) with ESI in metabolite profiling studies

	GC/MS	NMR	LC/MS	FIA
Sample preparation	Extraction and chemical modification needed	Typically none/minimal-pH buffering, plus addition of a deuterated solvent	Extraction often needed. Urine may simply be diluted. Plasma contains large amounts of protein that require removal	Extraction
Chromatographic separation	High-resolution separation	No separation	Medium (conventional) to high (UPLC) resolution separation	No separation
Sensitivity	millimolar to nanomolar	millimolar to high micromolar	millimolar to nanomolar	millimolar to micromolar
Speed	Slow (~30 min)	Rapid (1–5 min)	Slow to Rapid (Conventional LC up to 90min, UPLC < 2 min)	Rapid (1 to 5 min)
Quantitative accuracy	±10%	±10%	±10%	±10%
Advantages	High resolution EI-MS library available	Minimal sample preparation Nondestructive Fast Quantitative Structural information	Soft ionization. Large mass range	Data in one spectrum Fast
Disadvantages	Significant sample preparation with chemical modification, slow analysis time, harsh ionization, and limited number of molecules can be analyzed	Some chemical classes not detected. Overlapping resonances from proteins, lipids, and lipoproteins may mask signals from small metabolites	Sample destruction. Some chemical classes do not ionize and are not detected. Formation of multiple adducts may complicate data	Signal suppression from multiple components

2006). By performing chromatography to separate the thousands of small molecules present in biofluids, electrospray ion suppression can be reduced (Gangl et al. 2001) as the number of competing analytes entering the mass spectrometer ion source at any one time decreases. This combination of chromatographic separation and efficient ionization results in a selectivity that allows for both quantitation and structural information, with sensitivities in the pg/ml range achieved readily (Plumb et al. 2001). However, when combined with tandem MS/MS, ESI can be effective even without separation techniques, offering the advantage of faster analysis times and higher sample throughput. Direct ESI-MS/MS applications to metabolite screening emerged in the early 1990s, and more than 2 million newborn infants have been screened worldwide for more than 35 amino acid, organic acid, and fatty acid oxidation disorders, including phenylketonuria (Chace et al. 2003) (Fig. 2).

Often in metabolomics studies, potential biomarkers may be present in such small amounts that only the most sensitive analytic techniques can detect them. One such technology is nano-ESI-LC, which has already proved important in proteomics (Chelius et al. 2003). NanoLC/nano-ESI-MS is performed at much lower flow rates (~200 nL/min) than LC-ESI-MS (~300 μL/min) (Fig. 3), allowing for more efficient transfer of ions into the mass analyzer and a higher tolerance of complex mixtures. This improved sensitivity and dynamic range are advantageous in metabolite analysis (Griffiths et al. 1999).

Ultrahigh-performance liquid chromatography-mass spectrometry

Although the separation of components in complex mixtures makes conventional LC/MS especially useful in the initial stages of metabolomics research, traditionally, data acquisition speed is reduced and subsequent analysis time is increased. However, UPLC (Wilson et al. 2005b) utilizes columns with packing material of a much smaller particle size (1.4–1.7 μm) than traditional columns, thereby improving separation and increasing resolution. By using this approach, sample analysis times can be reduced to as little as 1 min (Wilson et al. 2005a), greatly increasing throughput. UPLC produces narrower chromatographic peaks than older methods, resulting in increased peak capacity, lower ion suppression, and improved signal-to-noise ratio, thereby heightening sensitivity. Recent studies comparing UPLC and HPLC for application to metabolomics showed that UPLC detects more components than the latter (Plumb et al. 2004). A 20% increase over the same chromatographic length was reported (Nordstrom et al. 2006), and UPLC also displayed superior retention time, reproducibility, and signal-to-noise ratios than HPLC.

Selection of potential biomarkers

Metabolomics studies generate large, complex data sets that require sophisticated software to enable interpretation. Often, two or more sample sets, such as those for individuals with disease and for healthy controls, are being compared for changes in metabolites, either increased or decreased levels. To achieve this comparison, metabolites must first be detected in all samples, matched between the samples and then evaluated for levels. Multiple adducts can be formed using LC-ESI/MS (such as sodium and potassium in positive mode, acetate in negative mode), thus complicating the data produced by increasing the number of peaks detected. It is imperative that the same metabolites are correctly identified in all samples to enable this comparison. To this end, algorithms and complete software have been written to allow peak picking and comparison. Many instrument manufacturers have produced their own software, which often works solely with data generated from a particular instrument. Some researchers desire the freedom to modify many parameters and also to compare data from different instruments and so have developed their own software, such as MZmine (Katajamaa and Oresic 2005; Katajamaa et al. 2006) and XCMS (Smith et al. 2006). These programs are freely available for download and modification.

With many of these software suites, data (e.g., peak lists) can be output in a suitable format to be analyzed using multivariate statistics, such as principal component analysis and partial least-squares discriminant analysis. These multivariate techniques can help to discern peaks with high discriminating power between the sample groups being analyzed, i.e., potential biomarkers (Lutz et al. 2006).

Biomarker characterization

Although some of the metabolites that are detected and identified in metabolomics studies may be well known and characterized, often a potential biomarker is an unknown compound, which then requires precise identification. The characterization of biomarkers is perhaps the greatest challenge facing metabolomics, given the often limited sample amount and minute quantities of some metabolites. Despite the availability of EI ionization mass spectral databases for GC/MS, one current limitation of LC/MS is the lack of complete databases with which to compare as yet unidentified compounds. However, several databases are available for such searches, such as the KEGG database (Kanehisa 2002) and METLIN (Smith et al. 2005) to find possible matches. Obtaining MS fragmentation data using a quadrupole time-of-flight (Q-TOF) and exact mass measurements by using a Fourier transform-MS (FTMS) instrument or orthogonal acceleration Q-TOF/MS (Wolff et al. 2001; Wilson et al. 2005b) for comparison can often narrow further the number of potential matches.

Comparison of the unknown metabolite with the chromatographic retention time, tandem MS data, and exact mass measurements of commercially obtained standards or

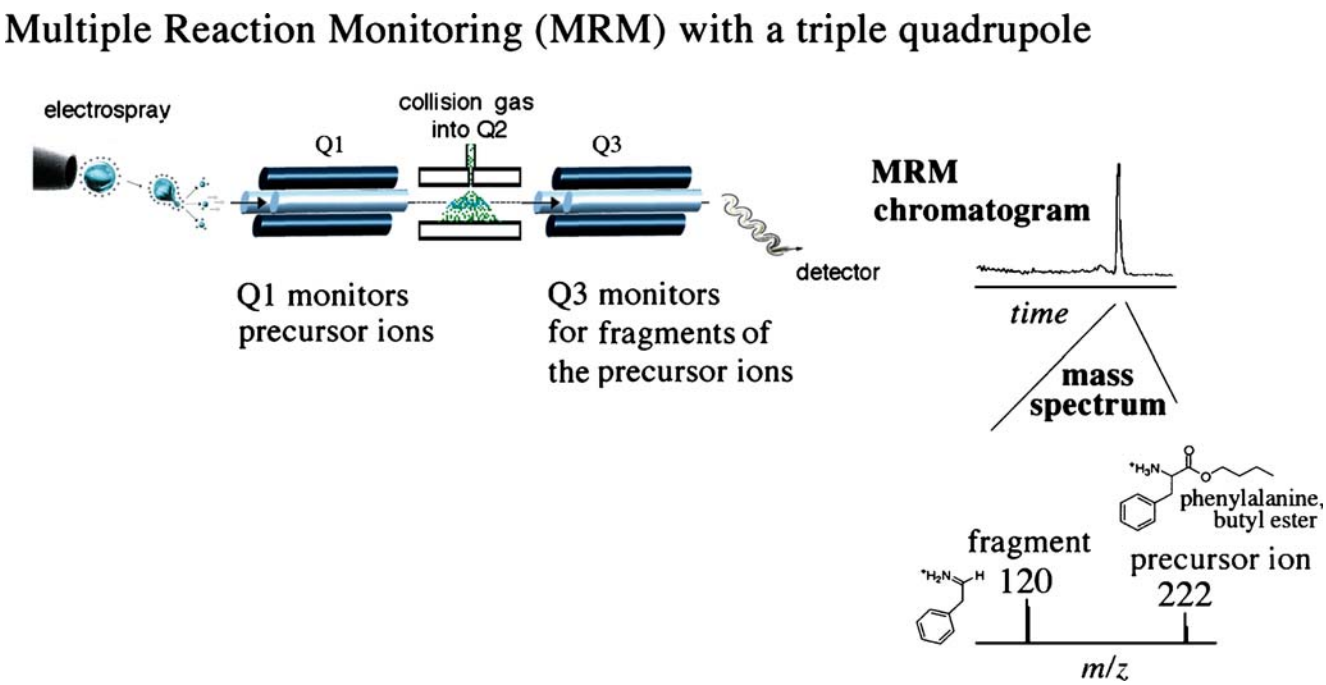

Fig. 2 Direct ESI-MS/MS experiments are now standard for monitoring over 35 different diseases in neonates, including disorders of amino acid, organic acid, and fatty acid oxidation. Here, an example of monitoring for phenlyketonuria (PKU) in a dried blood spot from an infant is shown using a triple quadrupole mass analyzer.

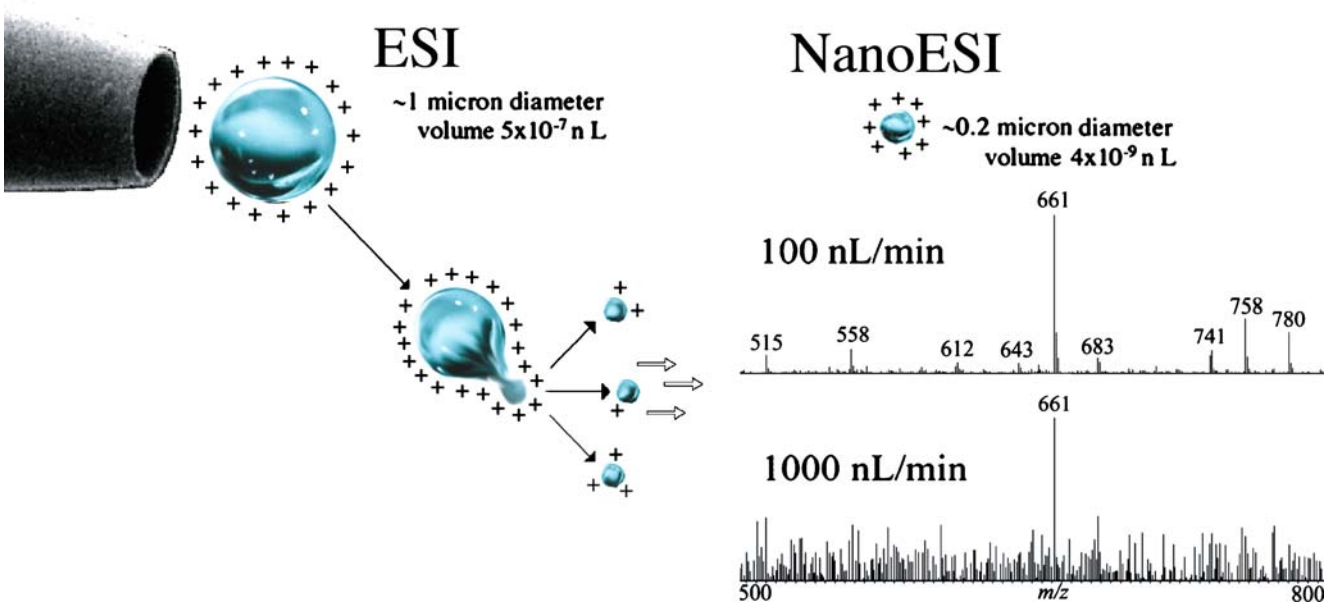

Fig. 3 NanoESI creates a smaller droplet than conventional ESI, which provides for less signal suppression and greater coverage of metabolites. Comparison of LC/MS spectra of methanol-extracted serum samples analyzed at flow rates of 100 and 1000 nl/min show a significant improvement in the signal-to-noise ratio at the lower flow rate, resulting in enhanced detection of metabolites.

synthesized compounds can aid the identification of biomarkers. Ultimately, upon identification of a biomarker, extraction combined with flow injection analysis can be used to quantify the compound with the aid of authentic standards (Chace 2001). Here, samples are introduced directly into the mass spectrometer, without prior separation. This method is rapid, with an analysis time of 2 min or less per sample and can include automated procedures for peak assignment (Castrillo et al. 2003).

However, despite the important information gleaned from MS, ultimately the combination of many technologies, including high-sensitivity capillary NMR experiments to provide metabolite structure characterization down to a low microgram level (Olson et al. 2004), chemical modification experiments will often be required to identify unknown metabolites.

Novel biomarkers for SIV-associated CNS dysfunction

\Any discourse on the benefit of metabolomics for enlarging knowledge in the medical sciences would be incomplete without presenting a vivid example of its application. One such example is the study of simian immunodeficiency virus (SIV), nonhuman primate lentiviruses that can cause AIDS-like disease in certain monkeys. In this model system, AIDS develops and progresses in far less time than in humans yet recapitulates most aspects of HIV-induced neuropathology (Sharer et al. 1988; Rausch et al. 1999; Fox et al. 1997). Choosing an optimal parameter that enables one to monitor the metabolic state of the brain under varying conditions is a prerequisite to a successful quest for measurable biomarkers. Two major areas that are being extensively pursued in biomarker discovery are neuroimaging and biochemical markers in body fluids such as cerebrospinal fluid (CSF), plasma, and urine. The role of neuroimaging is beyond the scope of this review and is not discussed further. However, CSF is close to the site of neuropathology in the brain and serves as an ideal source of the biochemical markers of interest here. An added advantage is that multiple samples can be obtained from the same individuals over time, thus making it feasible to conduct longitudinal molecular analyses of changes during the course of neurological diseases. For these reasons, there is a growing consensus that the CSF is the most ideal material for biomarker discovery with respect to neurodegenerative diseases.

Before the advent of treatment for AIDS, examination of the CSF for the presence and amount of virus as well as signs of immune activation was helpful in the diagnosis of HIV-associated dementia (Brew 2001; McArthur et al. 2003). Increases in other markers, such as cytokines and chemokines in the CSF, have also been found in patients with HIV-associated dementia (Conant et al. 1998; Kelder et al. 1998; Mastroianni et al. 1992; Kolb et al. 1999). However, these correlations may not hold in the current era of HAART. Furthermore, only one study found a marker (CSF levels of HIV-1 RNA) that predicts the development of neurocognitive abnormalities (Ellis et al. 2002); however, antiviral therapy may weaken this correlation (Sevigny et al. 2004; Cysique et al. 2005).

Although metabolomic studies are absolutely feasible in humans, the SIV-monkey model allows controlled, readily

manipulatable experiments in which to discover potential biomarkers for CNS disease in the setting of HIV. Serial CSF samples of either historically archived or prospectively obtained materials can be examined by using metabolomic technology and, through bioinformatics-assisted analysis, mined for candidate markers that can then be assessed in samples derived from humans. We believe that the reduced "noise" in the laboratory-controlled SIV-monkey system offers a high likelihood of success in identifying the biomarkers of HIV infection, making the subsequent studies in samples from humans much more efficient.

Several experimental SIV infections result in neurological disease. The two most potent of these systems involve either combining an immune suppressing SIV stock and a neurovirulent SIV molecular clone for infection of pigtailed macaque monkeys (Mankowski et al. 2002), or the use of a pathogenic SIVmac strain to infect rhesus macaques whose CD8 cells are depleted during the acute stage of infection (Williams et al. 2001; Roberts et al. 2003). However, the vast majority of these animals die within 3 months from related CNS disease, making discovery of predictive biomarkers problematic, as comparison with the much longer-lived controls that did not develop CNS disease was quite limited.

Although requiring a longer time period of experimentation, we believe that examination of a more typical disease course in the SIV-monkey model would be preferable for the identification of biomarkers applicable to human neuroAIDS. An ideal model would include the following stages: (1) acute infection, during which viruses enter the brain; (2) chronic infection, during which a low-level viral/immune interaction occurs in the brain; (3) treatment, using regimens similar to HAART; and (4) development (or not) of CNS disease. Because identifying molecular markers for neuroAIDS is so valuable for both diagnostic and etiopathogenic purposes, we believe the recent developments in metabolomics utilizing LC/MS give this technique a distinct advantage in achieving that goal. Furthermore, our recent studies indicate that high-quality metabolomic profiling by LC/MS can be obtained from frozen (−80°C) archived samples, which greatly enhances the ability to find candidate biomarkers.

Conclusion

Although the science of metabolomics has a longer history than some technologies of the present postgenomics era, recognition of its potential has lagged behind that of other high-throughput methods. Yet, this conduit promises to yield new insights into human physiology and profound discoveries that will strengthen and support medical science.

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