APPLICATIONS OF MASS SPECTROMETRY TO METABOLOMICS AND METABONOMICS: DETECTION OF BIOMARKERS OF AGING AND OF AGE-RELATED DISEASES

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Every 5 years or so new technologies, or new combinations of old ones, seemingly burst onto the science scene and are then sought after until they reach the point of becoming commonplace. Advances in mass spectrometry instrumentation, coupled with the establishment of standardized chemical fragmentation libraries, increased computing power, novel data-analysis algorithms, new scientific applications, and commercial prospects have made mass spectrometry-based metabolomics the latest sought-after technology. This methodology affords the ability to dynamically catalogue and quantify, in parallel, femtomole quantities of cellular metabolites. The study of aging, and the diseases that accompany it, has accelerated significantly in the last decade. Mutant genes that alter the rate of aging have been found that increase lifespan by up to 10-fold in some model organisms, and substantial progress has been made in understanding fundamental alterations that occur at both the mRNA and protein level in tissues of aging organisms. The application of metabolomics to aging research is still relatively new, but has already added significant insight into the aging process. In this review we summarize these findings. We have targeted our manuscript to two audiences: mass spectrometrists interested in applying their technical knowledge to unanswered questions in the aging field, and gerontologists interested in expanding their knowledge of both mass spectrometry and the most recent advances in aging-related metabolomics. © 2011 Wiley Periodicals, Inc., Mass Spec Rev

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I. INTRODUCTION

A. Metabonomics Versus Metabolomics

Metabo*nomics* and metabo*lomics* both involve studies of the metabolome, the collection of metabolites in a cell or organism. The distinction between these terms is tenuous, and numerous definitions have appeared in the literature. Traditionally, the term metabo*lomics* has been applied to plant studies, whereas metabo*nomics* has referred to studies involving animal models. A second way these terms have been differentiated is based on choice of analytical method, with metabo*lomics* studies typically utilizing mass spectrometry (MS) techniques, and metabo*nomics* studies usually employing nuclear magnetic resonance (NMR) spectroscopy (Cubbon et al., 2010). However, both of these definitions are now outdated, and confusion between these two terms has caused their usage to become blurred in the literature, with metabo*lomics* rapidly becoming a catchall phrase to describe both metabo*nomics* and metabo*lomics*.

A good definition of metabo*nomics* has been given by Nicholson, Lindon, and Holmes (1999), who describe metabo*nomics* as "the quantitative measurement of the time-related multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification." These authors later state that metabo*nomics* "measures the fingerprint of biochemical perturbations caused by disease, drugs, and toxins" (Lindon, Holmes, & Nicholson, 2003). In contrast to studying dynamic changes in the metabolome, one could also envision taking a sampling of as many metabolites as possible to produce a "metabolic snapshot." This process was first described by Oliver (1997), and has later come to be known as metabo*lomics*.

Among the biochemical changes which cause perturbations to the human metabolome are many diseases that typically appear with late onset in life such as diabetes and cancer, as well as changes due to aging itself. Indeed aging is being increasingly considered as a treatable condition. Since studies of aging and disease onset will almost invariably involve dynamic changes in the metabolome, they fall within the realm of metabo*nomics*, and it is the analysis of these *time-related* changes in the metabolome which will provide the focus of this review. Throughout the course of this review we will also describe several analytical techniques and statistical methods which have applications to both metabo*lomics* and metabo*nomics*, and we will use the term metabo*lomics* when referring to these techniques to indicate that they generally apply to both fields.

B. Types of Metabolomics Experiments

There are several different approaches which are typically used for analysis in metabolomics. (i) In metabolic

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fingerprinting, a subset of the intracellular metabolome is analyzed, often without identification or precise quantification of individual metabolites. Multivariate analysis techniques such as principal component analysis or independent component analysis can then be used to classify samples by characteristics such as age, gender, or disease state. (ii) Similar to metabolic fingerprinting, metabolic footprinting looks at changes in the extracellular metabolome in the nearby vicinity of cells, tissues, or organisms under study (Kell et al., 2005). (iii) Metabolic profiling involves the identification and quantification of a group of metabolites associated with a particular metabolic pathway. For example, profiling has been used to study organic acidurias (Duez, Kumps, & Mardens, 1996). (iv) A fourth approach to metabolomics is targeted metabolite analysis. This method focuses on a smaller portion of the metabolome, and involves the analysis of one or a few metabolites associated with a metabolic pathway (Dunn, Bailey, & Johnson, 2005; Ryan & Robards, 2006; Spratlin, Serkova, & Eckhardt, 2009).

C. MS Instrumentation

While the majority of our readers will likely be familiar with the standard instrumentation used in mass spectrometry, gerontological researchers unfamiliar with the technology may be quickly overwhelmed by both the variety of instruments available and the sea of acronyms associated with MS techniques. This section is not meant to be a comprehensive introduction to MS, as there are plenty of other resources available,[1] but is meant to serve as a convenient introduction to instrumentation for the non-MS specialist. Those well-versed in MS instrumentation may elect to skip this section. For expediency, a table of acronyms commonly associated with mass spectrometry is included (Table 1).

In short, mass spectrometry seeks to analyze a sample based on the mass to charge ratio (m/z) of its ionized components. MS does not discriminate between compounds in a mixture, and complex mixtures are typically separated prior to MS analysis via high-pressure liquid chromatography (HPLC) or some other analytical technique, creating the so-called ''hyphenated'' MS techniques that are discussed in Section II. A conventional mass spectrometer consists of an ionization source, mass analyzer, and detector. These components are discussed individually below.

1. Ionization Sources

Since MS analyzes samples based on the ratio of mass to charge, the first step in MS analysis is to ionize the sample to create charged species. Multiple methods have been designed for this purpose, and they fall under two broad categories of ''hard'' and ''soft'' ionization techniques. Hard ionization techniques result in a large amount of fragmentation of the analyte compounds during the ionization process. In contrast, soft ionization techniques result in little ion source fragmentation, and molecular ion peaks (peak corresponding to an ion with the same molecular mass as the unfragmented parent compound) are usually observed, and are often the only peaks observed in the mass spectrum.

Ionization methods for MS can be further classified based on the source of the ions. In gas-phase sources, the sample is converted directly to gaseous ions. This method requires high thermal stability and a low boiling point of the compounds of interest, and therefore is generally only applicable to compounds with molecular weights less than approximately 10^3 Daltons (Da). In desorption methods, a sample in either a liquid or solid state is converted to gaseous ions. Since this does not require volatilization of the analyte molecules, much higher masses can be analyzed. For example, matrix-assisted laser desorption and ionization (MALDI) can analyze samples with molecular weights $>10^6$ Da. Spray sources involve ionization of an aerosolized spray. Details of each ionization method are now presented.

a. Gas-phase sources

Two common ionization methods which employ gas-phase sources are electron impact (EI) ionization and chemical ionization (CI). Electron impact ionization is a hard ionization technique which involves the interaction of analyte molecules with high energy electrons to produce ions. Electrons are produced by running an electric current through a wire filament, and are then accelerated by a 70 eV potential applied between the filament and an anode, creating a beam of energized electrons. The sample is introduced perpendicular to the electron beam, and capture of these electrons by analyte molecules results in bond disruption and subsequent fragmentation and ionization.

A softer ionization technique than EI, CI ionizes samples through collision with an ionized gas. In this technique, the ionization chamber is filled with a reagent gas such as methane, ammonia, or isobutane. Electrons entering the chamber preferentially ionize the reagent gas, which is in excess to the analyte by a factor of 10^3–10^4. This typically produces positively charged ions through proton transfer or hydride abstraction, but in some cases (for example, highly electronegative compounds) negative ions can also be produced (Dougherty, 1981). Since this is a lower energy technique than EI, less fragmentation of analytes occurs during ionization. This results in a simpler spectrum, and the molecular ion peak is usually observed. Since both EI and CI sources require the sample to be in the gas phase, they are easily coupled with gas chromatography (GC) separation (see Section II), but cannot be coupled with liquid chromatography (LC).

b. Desorption sources

Matrix-assisted laser desorption and ionization (MALDI) is one of the most commonly used ionization sources for analyzing large biomolecules such as proteins, oligonucleotides, and oligosaccharides. MALDI is a favored ionization technique due to its ability to produce high molecular weight ions combined with the fact that it is a soft technique, resulting in little or no fragmentation of analytes, which would complicate the mass spectra of large molecules. A time of flight (TOF) mass

[1]See, for example, ''Mass Spectrometry: Principles and Applications'' by de Hoffman and Stroobant (2007). A variety of electronic resources are also available, including the websites for the Scripps Center for Metabolomics and Mass Spectrometry (http://masspec.scripps.edu/mshistory/whatisms_details.php) and the American Society for Mass Spectrometry (http://www.asms.org/whatisms/index.html).

TABLE 1. Abbreviations and acronyms used in this article

Mass Spectrometry	MS	Mass Spectrometry
	CID	Collision Induced Dissociation
	m/z	Mass to Charge
	MRM	Multiple Reaction Monitoring
	SIM	Selected Ion Monitoring
	SRM	Selected Reaction Monitoring
	TIC	Total Ion Chromatogram
Ion Sources		
	APCI	Atmospheric Pressure Chemical Ionization
	CI	Chemical Ionization
	EI	Electron Impact
	ESI	Electrospray Ionization
	MALDI	Matrix Assisted Laser Desorption/Ionization
	MSLDI	Matrix Suppressed Laser Desorption/Ionization
Mass Analyzers		
	FTICR	Fourier Transform Ion Cyclotron Resonance
	IT	Ion Trap
	oa-TOF	Orthogonal Acceleration – Time of Flight
	Q-IT	Quadrupole – Ion Trap
	Q-TOF	Quadrupole – Time of Flight
	TOF	Time of Flight
Chromatography		
	GC	Gas Chromatography
	HILIC	Hydrophobic Interaction Liquid Chromatography
	HPLC	High Pressure Liquid Chromatography
	LC	Liquid Chromatography
	RPLC	Reverse-Phase Liquid Chromatography
	UFLC	Ultra-Fast Liquid Chromatography
	UPLC	Ultra Performance Liquid Chromatography
Electrophoresis		
	CE	Capillary Electrophoresis
	CZE	Capillary Zone Electrophoresis
Statistical Analysis		
	ANOVA	Analysis of Variance
	DA	Discriminate Analysis
	OPLS	Orthogonal Projections to Latent Structures
	PCA	Principal Components Analysis
	PLS	Partial Least Squares
	RFE	Recursive Feature Elimination
	SVM	Support Vector Machine
	ULDA	Uncorrelated Linear Discriminant Analysis
Nuclear Magnetic Resonance	NMR	Nuclear Magnetic Resonance
	COSY	Correlation Spectroscopy
MALDI Matrices and Derivatization Reagents		
	BSTFA	*N,*O-bis(trimethylsilyl) trifluoroacetamide
	CHCA	α-cyano-4-hydroxycinnamic acid
	DHB	2,5-dihydroxybenzoic acid
	MSTFA	*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide
	MTBSTFA	*N*-methyl-*N*-(*tert*-butyl-dimethylsilyl) trifluoroacetamide
	SA	Sinapinic Acid
Metabolomics Resources		
	HMDB	Human Metabolome Database
	KEGG	Kyoto Encyclopedia of Genes and Genomes
	NIST	National Institute of Standards and Technology
Other Abbreviations Used in this Paper		
	AMD	Age-Related Macular Degeneration
	CSF	Cerebral Spinal Fluid
	RPE	Retinal Pigment Epithelium
	T2DM	Type 2 Diabetes Mellitus

analyzer (see below) is almost universally used with MALDI due to the virtually unlimited mass range of the analyzer.

Prior to analysis by MALDI-TOF the sample of interest must first be co-crystallized with a suitable matrix compound. The matrix is usually an acid, which aids in the formation of positive ions, and is a compound which can absorb energy at the same wavelength of the laser used, typically a nitrogen laser operating at 337 nm. The sample is mixed with an excess of the matrix (typically in a water–acetonitrile solution) and allowed to dry on a specialized metal plate, forming crystals of the matrix compound which have incorporated analyte molecules into their crystal structure. A laser is then used to excite the matrix molecules, and the excited matrix molecules transfer energy to the analyte, causing ionization. Ions observed after this process typically have a m/z of $[M + H]^+$ (corresponding to addition of a proton) or $[M + Na]^+$ (corresponding to addition of a sodium ion), where M is the molecular weight of the analyte. Choice of matrix for MALDI analysis depends on the analyte of interest, with different matrices being used for proteins, peptides, oligonucleotides, and oligosaccharides. Commonly used matrices include 2,5-dihydroxybenzoic acid (DHB), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA), and α-cyano-4-hydroxycinnamic acid (CHCA).

The use of MALDI is limited when looking at compounds with molecular weights less than 1,000 Da due to interference from the sample matrix. This presents a limitation to using MALDI as an ionization source in metabolic studies, since most metabolites have molecular weights under 1,500 Da (Kell, 2004) (Fig. 1). Methods for circumventing this problem have been devised including use of high molecular weight compounds for the matrix (Ayorinde et al., 1999), derivatization of analytes (Tholey et al., 2002), and using a low matrix-to-analyte ratio to suppress matrix signals (Goheen et al., 1997; Kang, Tholey, & Heinzle, 2000). The applicability of matrix-suppressed laser desorption/ionization (MSLDI) to analyses of the metabolome is discussed in detail by Vaidyanathan, Gaskell, and Goodacre (2006) and in a recent review by van Kampen et al. (2011). Despite its limitations, several metabolic studies which employ MALDI have appeared recently in the literature including its use for diagnosis of colorectal cancer (Cristoni et al., 2009), metabolic profiling of cancer cells treated with various antitumor agents (Miura et al., 2010), and for the analysis of metabolites found in *Escherichia coli* and in extracts from islets of Langerhans (a specialized microorgan of the pancreas) of mice (Edwards & Kennedy, 2005).

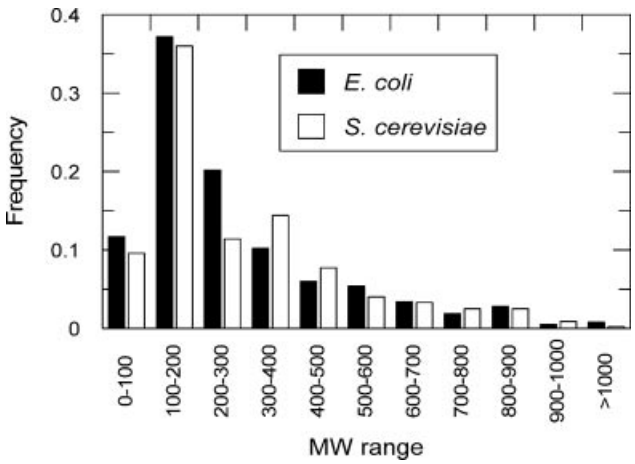

FIGURE 1. Histogram of typical molecular weights of metabolites found in *E. coli* and *S. cerevisiae*. *E. coli* data are from Nobeli et al. (2003) and was compiled based on information found in the EcoCyc database (Karp et al., 2002). It was noted that some larger molecular weight metabolites such as lipids were absent from the EcoCyc database at the time this was compiled, and are therefore absent from the analysis. Metabolite information on *S. cerevisiae* is from Forster et al. (2003), who reconstructed the metabolic network using available information. Reprinted from Kell (2004) with permission from Elsevier. Copyright 2004 Elsevier.

c. Spray sources

Also included among ionization sources are spray source techniques such as atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), which ionize analytes from a liquid spray. Since APCI and ESI generate ions directly from solution they are easily coupled with liquid chromatography separation techniques. In APCI, the liquid sample is vaporized by heating it to above 400°C and spraying it with nitrogen gas. The vaporized sample is ionized in the gas phase at atmospheric pressure by a corona discharge, an electrical discharge which is accompanied by ionization of the surrounding atmosphere. Ionized solvent molecules then transfer ionization to the analyte through a chemical ionization process. This is a relatively soft technique, and primarily generates intact molecular ions which usually contain a single charge.

Electrospray ionization (ESI) is a softer ionization technique than APCI, resulting in even less fragmentation of the analyte ions. In this technique, the aerosolized sample is sprayed into an electric field to create charged particles. A flow of warm gas is used to aid in solvent evaporation. The charge on the surface of the droplets increases as the solvent evaporates, forcing ions to be expelled from the liquid phase. ESI creates multiply charged ions, and ions of large biomolecules often have m/z ratios small enough to be analyzed by a quadrupole instrument (see below). For example, the ESI mass spectrum of apo-myoglobin (MW 16,951) shows a series of multiply charged peaks with m/z of 600–2,000 Da (Konermann et al., 1997). Both positively and negatively charged ions can be produced with this method.

2. Mass Analyzers

The mass analyzer portion of the mass spectrometer is where charged analyte molecules are separated based on m/z ratio. In addition to choice of ionization source, there are many different types of mass analyzers from which to choose. These include magnetic sector, ion trap (IT), and time of flight (TOF) analyzers, and each has its advantages and disadvantages.

a. Magnetic sector analyzers

In magnetic sector instruments ions are accelerated through an electric potential into either a permanent magnet or an electromagnet, with the kinetic energy of the accelerated ions depending on the applied potential and the charge on the ions. Ions travel in curved paths through a flight tube situated

within the magnetic field. The curved flight tube typically has a fixed arc length of 60°, 90°, or 180° (Skoog, Holler, & Nieman, 1998). The radius of each ion's flight path is determined by the initial speed of the ion after acceleration, the *m*/*z* ratio of the ion, and the strength of the magnetic field. If all ions have $z = 1$, one can scan through the magnetic field range to select for ions with a specific mass. Magnetic sector instruments typically have an upper *m*/*z* limit of 10,000 Da, but suffer from low resolution.

b. Quadrupole and ion trap analyzers

Quadrupole mass analyzers analyze samples by electrically accelerating ions through a set of four parallel metal rods (quadrupoles), arranged in a diamond pattern, via a potential of 5–10 V. Rods on opposite sides are connected electrically, with one pair of rods being connected to the positive terminal of a variable DC source, and the other pair being connected to the negative terminal. Variable radio frequency (RF) AC potentials are also applied to each pair of rods and superimposed on top of the DC potentials. While the ions are being accelerated between the sets of rods, the AC and DC potentials are simultaneously increased while kept at a constant ratio. Positively charged ions which enter the quadrupole will be drawn towards a negatively charged rod, and vice versa. When the potential is changed, the trajectory of the ion will change directions. In this way only ions with a specific *m*/*z* will have the correct trajectory to reach the detector, others will have unstable trajectories, causing them to discharge on the rods (de Hoffman & Stroobant, 2007). By varying the applied voltage, the operator can scan over the entire *m*/*z* range. Quadrupole instruments can typically resolve ions that differ by one mass unit, but are only useful for analyzing ions with *m*/*z* up to 3,000 Da.

Quadrupoles can also be operated in RF-only mode to serve as ion guides, permitting passage of ions within a transmission mass range. In this mode the RF voltage is adjusted so that all ions which have masses greater than a specified limit have stable trajectories. Ions with higher masses are poorly focused, and may crash into the rods. High molecular weight ions can be focused more efficiently by increasing the RF voltage; however, this also raises the lower mass limit (de Hoffman & Stroobant, 2007). Hexapole and octapole ion guides have also been devised, and they operate under similar principles.

Quadrupole ion trap (Q-IT) mass spectrometers typically consist of a ring-shaped electrode and two hyperbolic end cap electrodes. By varying the voltages on the electrodes, ions can either be collected within the ion trap or ejected through a hole in one of the end-cap electrodes to the detector. In addition to conventional (3D) ion traps, 2D linear ion traps (LIT) have also been devised. These ion traps have a design similar to the four rod quadrupole, with ions confined in the radial dimension by the quadrupolar field, and in the axial direction by an electric potential applied to the ends of the trap (de Hoffman & Stroobant, 2007).

c. Time of flight analyzers

When analyzing high molecular weight biomolecules, TOF mass analyzers are often employed, due to their virtually unlimited mass range. In this type of analyzer all ions with the same charge are imparted the same kinetic energy by acceleration through an electrical field and are then allowed to drift freely through a vacated drift tube, which has a length of around a meter. Since all ions of a given charge have the same kinetic energy, lighter particles will transverse the flight tube faster than heavier particles, allowing for separation of ions based on *m*/*z*. In this way ions are separated in time (as opposed to in space as in quadrupole, magnetic sector, and ion trap instruments). When used with ion sources such as MALDI, where the sample is dried onto a surface, space effects can occur causing molecules with the same *m*/*z* to have different kinetic energies and affecting resolution. Resolution in TOF instruments can be improved by use of a reflectron. A reflectron consists of a series of electrostatic plates to which a static potential is applied, and will reverse the direction of ions entering it. Higher energy ions with the same *m*/*z* penetrate deeper into the reflectron, thereby traversing a slightly longer path to the detector. This phenomenon creates a focusing effect, helping ions with the same *m*/*z*, but slightly different kinetic energies, reach the detector at the same time. This also effectively doubles the length of the flight tube, since ions must travel the length of the tube twice. Continuous ion sources such as ESI are often coupled to TOF analyzers through orthogonal acceleration (oa-TOF). This involves accelerating ions perpendicular to the initial direction of motion prior to entering the mass analyzer, and is discussed in a review by Guilhaus, Selby, and Mlynski (2000). A TOF mass analyzer can also replace the third quadrupole in a triple quadrupole mass spectrometer to create a quadrupole time-of-flight instrument (Q-TOF).

d. Fourier transform ion cyclotron resonance

Fourier transform ion cyclotron resonance (FTICR) is a form of mass analyzer which involves accelerating ions in a type of particle accelerator known as a cyclotron. The charged particles move in circular paths perpendicular to a uniform magnetic field at a characteristic frequency known as the cyclotron frequency, which is dependent on the *m*/*z* ratio. Pulsed RF radiation is used to excite ions to paths of larger radius and causes them to move in phase, which creates an image current. Detection of this current followed by Fourier transformation provides information on the *m*/*z* of the ions. This differs from other kinds of mass analyzers in that ions are not separated prior to detection. This technique offers very high resolution, and the resolution can be increased by increasing the magnetic field strength.

3. Detectors

After a sample has been ionized and sorted by mass in the mass analyzer, the information stored in that sample must be converted to an electric current for quantitation and data analysis. A variety of detectors have been devised for this purpose, two common ones are the electron multiplier and the Faraday cup. The electron multiplier tube is currently the most widely used detector in mass spectrometry. This type of detector consists of a metal conversion dynode held at a high potential opposite to the charge on the particles of interest. The charged particle strikes the conversion dynode, causing the emission of between one and three secondary particles. These particles can be either positive ions (when a negatively charged particle strikes the conversion dynode) or negative

ions and electrons (when a positively charged particle strikes the conversion dynode). The charged particles are then converted to electrons at the first dynode (de Hoffman & Stroobant, 2007). These electrons can then be directed towards a second dynode through an applied potential, causing the release of more secondary electrons. By adding several additional dynodes, a single charged particle can create a cascade and generate a very large current. Electron multipliers consisting of 20 dynodes can generate a current gain of 10^7, which results in a high sensitivity for this type of detector. Continuous-dynode electron multipliers have also been designed. These detectors consist of a trumpet-shaped piece of glass doped with high amounts of lead. Electrons skip across the inner surface of the detector, creating the cascade effect.

A Faraday cup consists of a metal cup designed to catch ions in a vacuum. The ions enter the cup and impinge on a collector electrode to create a current by either accepting or donating electrons. The collector electrode is surrounded by a metal cage to prevent the escape of reflected electrons and secondary electrons emitted from the surface when it is struck by the incident charge. Faraday cups are less sensitive than electron multiplier detectors due to the fact that there is no amplification of signal (Skoog, Holler, & Nieman, 1998). The advantage of using a Faraday cup is that they are highly accurate, as the current produced is directly proportional to the number of ions.

D. Extraction of Metabolites from Biological Samples

To carry out a metabolomics-based experiment, metabolites must be first isolated from the biological sample of interest. For animal studies, metabolites are often obtained from blood, urine, cerebral spinal fluid (CSF), or intact tissue. Generally, some form of chemical extraction is employed, with the precise method of extraction dependent upon the metabolites of interest (e.g., polar or nonpolar). Following extraction, samples are typically spiked with internal standards, and if GC-MS is to be performed, they are further treated with a derivatizing agent to assist in volatility (see Section II.A.2 for more details).

For liquid samples (blood, urine, CSF, etc.), preparation often begins with removal of suspended particles, through either centrifugation or filtration. For CSF and plasma samples, precipitation of proteins, usually by addition of acetonitrile, is performed prior to this centrifugation step (Gika et al., 2008; Li et al., 2010). Samples can then be analyzed directly by LC-MS if one is using a polar mobile phase (Williams et al., 2005b; Schnackenberg et al., 2007). Additional steps may be taken if one is interested in a specific class of metabolites. For example, following filtration of CSF samples, Myint et al. (2009) extract polar cationic metabolites by acidifying the samples and eluting them from a cation exchange cartridge.

A typical extraction procedure for tissue samples might involve grinding or sonicating tissue in an organic solvent mixture. Wu et al. (2010) and Chan et al. (2009) used a monophasic mixture of 2:5:2 chloroform/methanol/water with sonication to extract metabolites from cancer tissue samples. Alternatively, Fan et al. (2009) extracted water-soluble and polar metabolites from tissue samples using 10% trichloroacetic acid. Many investigators freeze samples in liquid nitrogen before grinding samples to minimize artifactual changes in metabolite levels during the extraction process.

E. Statistical Treatment of Metabolic Data

The metabolome is large, with almost 600 metabolites reported in *Saccharomyces cerevisiae* (Forster et al., 2003). The size of the metabolome is larger for more complex species, with more than 1,000 metabolites present in *Aridopsis thaliana*, a model plant, and up to 200,000 unique metabolites expected across the plant kingdom (Fiehn, 2001; Kell, 2004). As of the writing of this article, the Human Metabolome Database (HMDB) lists over 7,900 human metabolites, and this number is growing rapidly (Wishart et al., 2009). Consequently, metabolomics studies typically generate large sets of data. Due to the overwhelming amount of metabolites in a single analysis, univariate statistical analysis is rarely used in metabolomics. However, if the concentration of a particular metabolite is found to be significantly altered through multivariate analysis, univariate analysis can be used to test the statistical significance of the change. This typically involves the use of the Student's *t*-test or one-way analysis of variance (ANOVA).

A more useful statistical approach in dealing with the large amounts of data generated in metabolic studies is multivariate analysis. Multivariate analysis can be applied to reduce large volumes of data into a few dimensions for classification and prediction of outcomes. It is worth noting here that the predictive power of multivariate analysis comes from patterns in the data, and identification of specific metabolites is not necessary to discriminate different classes of data. Both unsupervised and supervised learning methods have been devised. In unsupervised strategies, no prior knowledge of the data is built into the statistical model. This is in contrast to supervised learning techniques, where a training set of data is used for prediction and classification. However, this also requires a separate set of data to test the predictions of the model which can sometimes be problematic when one is limited by a small set of samples and does not have enough independent data to build both training and validation sets. This can be circumvented by using a data-splitting method such as cross-validation, where the data are continually split into training and validation sets and the predictions of the data sets are averaged (Schlotterbeck et al., 2006).

The most commonly used unsupervised technique for identifying patterns and trends in metabolomics data is principal components analysis (PCA), and this is often used as a starting point for analysis (Schlotterbeck et al., 2006; Lu et al., 2008a; Cubbon et al., 2010). PCA attempts to reduce multi-dimensional data so that it can be plotted in a two- or three-dimensional Cartesian coordinate system, with the axes (principal components) representing the greatest variations in the data. The popularity of this technique stems from the easy graphical interpretation of the data, with clustering often observed between data points based on class-type (age, gender, etc.). Contributions of individual variables to the separation of samples can be identified by corresponding loading plots, which plot the contribution of each variable based against selected principal components (Schlotterbeck et al., 2006).

Principal component analysis (PCA) is usually followed by a supervised learning technique, such as partial least squares discriminant analysis (PLS-DA). PLS-DA attempts to maximize the covariance between the independent and dependent variables to discriminate amongst samples, and this method often discriminates between classes better than PCA. Moreover, the lack of class information when determining the principal components in PCA can lead to discrimination of samples based on factors not related to the classes of interest. A specialized form of PLS-DA is orthogonal projections to latent structures (OPLS), in which non-correlated systematic variance is removed from the model (Trygg & Wold, 2002).

Another supervised learning technique is support vector machines (SVMs). This technique was first described in 1995 by Vladimir Vapnik (Cortes & Vapnik, 1995) and functions as a binary separator to classify input data as belonging to one of two possible classes. The use of SVMs to classify LC-MS data from serum samples of ovarian cancer patients and healthy controls has been described by Guan et al. (2009), and specific details of the method as well as several SVM-based methods (for example, recursive feature elimination (RFE)) can be found there. SVMs have also been used for the prediction of breast cancer (Henneges et al., 2009) and for identifying metabolic biomarkers in prostate cancer (Osl et al., 2008).

The random forests method is an ensemble classification method developed by Breiman (2001) which classifies input data into two or more categories using decision trees. Benefits of this method are that it has good predictive performance even when most variables are noise and that it does not overfit the data (Diaz-Uriarte & Alvarez, 2006). This method is also advantageous in that it incorporates interactions among predictor variables, and returns a rank of the variables in order of importance. For example, Gall et al. (2010) used the random forest method to identify α-hydroxybutyrate as a highly ranked biomarker to separate insulin-resistant from insulin-sensitive individuals. Many other supervised methods have been used to analyze metabolomics data, including artificial neural networks (ANNs) (Zupan & Gasteiger, 1993) and classification and regression trees (CARTs) (Breiman, 1998), but their detailed discussion is beyond the scope of this article.

F. A Comparison of MS to NMR for Metabolic Studies

The two most commonly employed analytical techniques used for the analysis of complex mixtures of metabolites are mass spectrometry and NMR, and both techniques have their advantages and disadvantages. When choosing a particular technique, one must be cognizant of the type of analytes they are interested in observing. If a particular compound does not ionize well, detection by MS may be difficult. On the other hand, many compounds of biological importance (e.g., 2,8-dihydroxyadenine) are NMR invisible under normal operating conditions, due to the exchange of labile protons with water. One particular limitation of MS in metabolomics studies is the difficulty in achieving quantitative information on metabolite concentrations, due to different ionization efficiencies. Also, fragmentation may lead to multiple MS peaks derived from the same analyte. Additionally, characterization of metabolites identified by MS requires either comparison to a standard, or database searching against compiled mass spectral libraries. While several libraries have been made freely available (as discussed at the end of this article), for a particular library to be useful the mass spectra must be acquired under identical conditions. For example, spectra acquired from a quadrupole instrument will look different from spectra acquired from an ion trap. Since samples are sometimes derivatized prior to MS analysis (e.g., for GC-MS, Section II.A.2) it is also important that the same derivatizing agents have been used to compile the libraries.

Quantitative information is easily obtained by NMR, but this method requires larger sample volumes and suffers from decreased sensitivity. Metabolic fingerprinting has clinical potential in diagnosing a variety of diseases (see Section III), and while both NMR and MS have been successfully used to differentiate between healthy and diseased patients, MS has greater clinical potential as the instrumentation is less costly to purchase and maintain, and requires less space. Additionally, the greater sensitivity of MS allows for the identification of a greater number of metabolites, as many metabolites exist *in vivo* below the NMR detection limit. Another strength of MS is its ability to easily be directly coupled to a variety of separation techniques, which is less facile with NMR instrumentation. While the focus of this article is on MS technologies, it is important to recognize that NMR has also been extensively used in metabo*nomics* studies. These two techniques provide complementary information, and ideally a combination of the two methodologies should be employed for a complete study of the metabolome. A tabular comparison of the strengths and limitations of MS and NMR for metabolomics research is given in Table 2.

II. HYPHENATED AND COMBINED MS TECHNIQUES

Here we wish to provide a general overview of the various analytical techniques which have been "hyphenated" with MS for metabolomics studies. Comprehensive reviews have already been written on many of these techniques, as well as their applications to metabolomics, and we will direct the reader to those for specific details when appropriate. Hyphenated MS techniques commonly used for metabolomics have been reviewed by Bedair and Sumner (2008).

A. MS Coupled with Chromatography

1. LC-MS

Liquid chromatography is rapidly replacing GC as the chromatographic method of choice for metabolomics applications, as the two methods have similar sensitivity but LC requires less sample preparation time (Griffin & Shockcor, 2004). This is due to the need to derivatize samples prior to separation by GC (see below). A commonly used form of LC is high performance liquid chromatography (Williams et al., 2005b; Wilson et al., 2005; Yang et al., 2005), which traditionally involves partitioning analytes between a nonpolar organic mobile phase and a polar stationary phase at high pressures. For metabolomics studies, however, reverse-phase liquid chromatography (RPLC) is more commonly used. In this method the polarities of the two phases are reversed, which is useful for the separation of non-polar metabolites. Two specialized forms of LC which have been hyphenated

TABLE 2. A comparison of NMR and MS for use in metabolomics

	NMR	MS
Sensitivity	The sensitivity for a particular analyte in NMR depends on how many equivalent protons are contributing to a signal, as well as whether that signal is split. For a singlet methyl resonance, the detection limit is 5 μM. This is doubled in the case of a doublet resonance. When only a single proton contributes to the signal the limit is 15 μM for a singlet and 30 μM for a doublet (Wevers et al., 1999). Sensitivity in NMR also depends on magnetic field strength, with higher field magnets providing greater signal to noise. The signal to noise for a particular sample can be increased by increasing acquisition time, and it is directly proportional to $\sqrt{n}$, where n is the number of scans.	Sensitivity in MS is affected by choice of both ionization technique and detector (Section I.C), but in general MS is much more sensitive than NMR. ESI ionization sources have sensitivities in the range of high femtomole (10^{-15} mole) to low picomole (10^{-12} mole), with some nanoESI techniques having sensitivities as low as high zeptomole (10^{-21} mole). MALDI and APCI sources have sensitivities in the femtomole range. CI and EI sources are less sensitive, with picomole sensitivities. Electron multiplier detectors are inherently more sensitive than Faraday cups, achieving electronic gains on the order of 10^6.
Sample Preparation	No sample preparation is required	MALDI MS techniques require the sample to be mixed with a suitable matrix prior to analysis. In liquid spray ionization sources, choice of solvent is important: ESI requires use of volatile organic solvents to aid in evaporation. GC-MS requires samples to be derivatized prior to GC to increase volatility and stability.
Quantification	Quantification is easily achieved by integration of signals	Determining quantitative information from peak integration in MS is difficult since different metabolites ionize with different efficiencies. Quantitative information on a metabolite peak can be obtained in one of three ways (i) by integrating it against a reference sample of the same compound, but this requires that the identity of the metabolite be known and that peak information be compared between two separate runs, which can introduce error, (ii) comparing the relative ratios of a set of peaks across a series of spectra, and (iii) by addition of a stable-isotope version of the metabolite of interest to the sample. This method is limited to one metabolite at a time, potentially costly, and requires dedicated instrumentation.
Structural Information	NMR is far superior to MS for structural elucidation of unknown metabolites, and additional information can be obtained through the use of 2D NMR techniques such as correlation spectroscopy (COSY)	Tandem MS fragmentation patterns can give clues as to the connectivity and the presence of specific functional groups in an unknown metabolite. Identification of metabolites is often expedited by comparison to internal standards and searching against mass spectral libraries
Non-Destructive?	Yes	No

with MS for metabolomics studies are ultra-performance liquid chromatography (UPLC) and hydrophobic interaction liquid chromatography (HILIC). General reviews on the use of LC-MS in metabolomics are given by Lu et al. (2008a) and by Theodoridis, Gika, and Wilson (2008).

a. UPLC-MS

Ultra-performance liquid chromatography (UPLC), also known as ultra-fast liquid chromatography (UFLC), is a relatively new method of chromatography. It operates under the same principles as RPLC but uses a stationary phase with a much smaller particle size (<2 μm vs. 3.5–5 μm), as well as higher pressures and flow rates. The use of the smaller particle size, combined with operating pressures in the range of 6,000–15,000 psi (compared to the 5,800 psi maximum for HPLC), results in a 3- to 5-fold increase in sensitivity, a doubling of peak capacity, 5- to 10-fold faster analysis times, and better resolution (Plumb et al., 2005; Wilson et al., 2005; Lu et al., 2008a; Ma et al., 2009). Like RPLC, UPLC uses a polar mobile phase, typically consisting of water and acetonitrile. Recently, UPLC-MS has been used for metabolic analysis of liver samples (Masson et al., 2010), plasma (Plumb et al., 2006; Williams et al., 2006; Zhang et al., 2009b), urine (Plumb et al., 2005; Ma et al., 2009; Wang et al., 2009; Want et al., 2010), and hair samples (Kawanishi et al., 2007).

b. HILIC-MS

A novel LC technique which can be coupled to MS for metabolomics studies is hydrophobic interaction liquid chromatography (HILIC). HILIC is similar to normal phase LC, in that the stationary phase is polar; however, in contrast to normal phase LC, the mobile phase in HILIC uses water miscible solvents, such as acetonitrile or methanol, and also contains a substantial amount of water (Dettmer, Aronov, & Hammock, 2007). Typical stationary phases in HILIC consist of underivatized silica or silica which has been covalently modified with various polar functional groups such as aminopropyl- and

cyanopropyl groups, or zwitterionic functional groups (Godejohann, 2007). The water in the mobile phase is retained by the stationary phase to form an immobile layer into which polar and charged solutes can be partitioned and retained. This method has an advantage over normal and reverse-phase LC in that RPLC poorly retains polar analytes, and normal phase LC uses solvents that are incompatible with MS. An overview of HILIC-MS and its applications to metabolomics is given by Cubbon et al. (2010).

2. GC-MS

An alternative to using LC for separation prior to MS is to use gas chromatography (GC). Samples must be derivatized prior to separation by GC, which increases the volatility and stability of the analytes. Frequently this is accomplished by oximation of carbonyl groups to prevent formation of derivatives and to prevent cyclization of reducing sugars. This is then followed by derivatization of hydroxyl, amine, and thiol groups by a silylating reagent such as *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA), *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), or *N*-methyl-*N*-(*tert*-butyl-dimethylsilyl) trifluoroacetamide (MTBSTFA) to increase volatility (Fiehn et al., 2000).[2] For this reason sample preparation can sometimes be time consuming; however, better reproducibility is obtained with GC than with LC methods, and better compound separation is achieved in the gas phase than the liquid phase. GC-MS is the analytical method of choice for plant metabolomics (Griffin & Shockcor, 2004), but has also been applied to studies of kidney cancer (Kind et al., 2007), atherosclerosis (Teul et al., 2009; Chen et al., 2010b), diabetes (Yuan et al., 2007), and acute coronary syndrome (Vallejo et al., 2009).

Sources of bias when using GC-MS for metabolomics are discussed in detail by Kanani, Chrysanthopoulos, and Klapa (2008), and are briefly summarized here. Bias in metabolomics occurs in two forms. Type A biases are universal biases which affect all metabolites in a sample equally and can be corrected for by addition of an internal standard. Bias which affects individual metabolites differently is known as Type B bias. The primary source of bias in GC-MS is the sample derivatization step, which introduces both Type A and, to a greater extent, Type B biases. The concern with this step is that, when dealing with a biological sample consisting of hundreds to thousands of organic compounds with varying functional groups all competing for the same reagent, one must be cautious to use enough derivatizing agent to ensure that it is adequately in excess. One also needs to identify the correct duration of the derivatization step for a particular sample, to adequately allow enough time for complete derivatization of all metabolites present in that sample. Incomplete derivatization of compounds with multiple functional groups may also result in multiple peaks eluting for the same metabolite (Xu, Zou, & Ong, 2010). Additionally, Type B biases can be introduced during the data acquisition process. To reduce these biases one must ensure that they are acquiring data within the linear range of the instrument, and that operating conditions are constant across samples from which data are to be compared. For example, variations in the operating conditions of the gas chromatograph can introduce drift in the retention times of metabolites, and changes in mass spectrometer conditions can introduce bias as well.

[2]A detailed guide to derivatization techniques and reagents is provided by Sigma-Aldrich and can be found online at: http://www.sigmaaldrich.com/etc/medialib/docs/Supelco/Application_Notes/4537.Par.0001.File.tmp/4537.pdf.

B. MS Coupled with Capillary Electrophoresis

Capillary electrophoresis (CE) is a technique which involves separating charged species based on their differential movement through a buffer to which a DC potential has been applied, and is useful for separating a wide range of compounds including inorganic anions, amino acids, organic acids, nucleotides, and carbohydrates (Soga & Imaizumi, 2001). CE is an ideal technique for separation of biological samples pre-MS, due to the small sample size required, and can be routinely performed on samples with volumes as small as 0.1–10 nL. This is extremely advantageous when dealing with biological samples as, for example, often only a few microliters of urine are available from small animals such as mice. In fact, CE-ESI-MS has been used for metabolomics studies of single cells (Lapainis, Rubakhin, & Sweedler, 2009), and has even been used to study sub-cellular components (Chiu et al., 1998). Due to the small injection volumes often used in CE, however, this technique sometimes suffers from lack of sensitivity, and on- or off-line sample preconcentration methods may need to be used (Ptolemy & Britz-McKibbin, 2006; Chalcraft & Britz-McKibbin, 2009).

Typically, when coupling CE to MS, the simplest form of CE, capillary zone electrophoresis (CZE) is used (Monton & Soga, 2007; Bedair & Sumner, 2008). In this mode the buffer composition is kept constant throughout the capillary and ionic species separate into distinct zones based on their mobilities, which may be either completely or partially resolved. The coupling is commonly done with a sheath-flow interface, in which a coaxial sheath liquid transfers solutes eluting from the CE capillary to an ESI ion source (Liu, Zhang, & Dovichi, 2005; Bedair & Sumner, 2008). This is necessary due to the fact that the composition of the buffer used for CZE (typically 10^{-5}–10^{-3} M in aqueous solution) is substantially different from that needed for ESI (10^{-7}–10^{-4} M buffer in an organic solvent) (Moseley et al., 1992). Lee et al. have used CE-ESI-MS to analyze glutathione metabolites in human plasma samples (Lee & Britz-McKibbin, 2009) and, as will be discussed later, CE-TOF-MS has been applied to the metabolic profiling of urine samples from accelerated aging mice (Nevedomskaya et al., 2010). Comprehensive reviews of CE-MS for metabolomics research have been written by Ramautar, Somsen, and de Jong (2009) and Monton and Soga (2007).

C. MS Coupled with NMR

The advent of flow-through technology for NMR probe heads has allowed NMR to be coupled with LC-MS, with the eluent being split post-LC into both an NMR spectrometer and mass spectrometer for simultaneous analysis of peaks as they come off the chromatography column—a general schematic for this setup is given in Figure 2. This technique is extremely

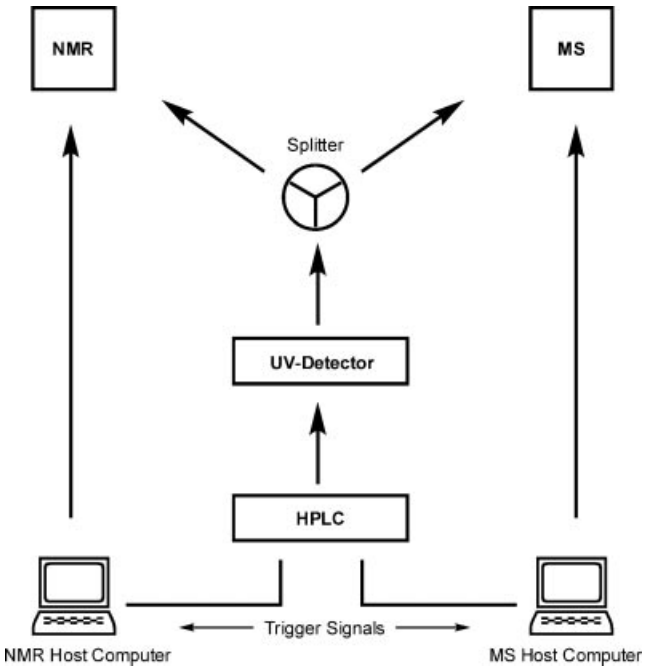

FIGURE 2. Schematic representation of HPLC-NMR-MS instrument setup.

powerful for metabolomics research in that it is able to combine data obtained from both instruments for metabolite structure elucidation (Burton et al., 1997; Shockcor et al., 2000; Bajad et al., 2003; Godejohann, 2007). There are multiple modes in which the LC-NMR can be operated, including on-flow and stopped-flow methods. In the on-flow method, sensitivity is limited due to the amount of time the sample spends in the NMR probe. This can be circumvented using stopped-flow measurements, however, peak broadening can occur on the column while the NMR data are being acquired (Godejohann, 2007). A third operational mode involves storing the peaks individually into capillaries as they come off the column, and then transferring them later to the NMR flow probe for data acquisition. General details of the LC-NMR-MS technique can be found in reviews by Lindon, Nicholson, and Wilson (2000) and Silva (2003).

D. MS Coupled with MS

Mass spectrometry (MS) can also be coupled with itself in a procedure known as tandem mass spectrometry, sometimes employing just a single mass analyzer. This is often abbreviated as MS/MS or MS^2. There are several types of experiments which can be performed using MS/MS analysis:

(i) A *product ion scan* involves selection of a precursor ion by the first mass analyzer, followed by fragmentation into product ions. This fragmentation is often accomplished by collision induced dissociation (CID), in which analyte molecules are accelerated in an electric potential and allowed to collide with neutral molecules of an inert gas (often He, Ne, or Ar). For magnetic sector, quadrupole, and TOF instruments, the resulting fragment ions are analyzed in a second mass analyzer. A commonly used instrument for this type of analysis is the triple quadrupole mass spectrometer, which consists of three quadrupoles connected in tandem. The first and third quadrupoles act as mass filters, while the second quadrupole (non-mass filtering) serves as a collision chamber. In other MS instruments, such as QIT and FTICR instruments, the analyses of the precursor and product ions are conducted sequentially in time within the same mass analyzer. For example, all ions other than a selected precursor ion can be expelled from the mass analyzer, followed by fragmentation by CID within the analyzer.

(ii) In a *precursor ion scan*, one examines all of the precursor ions capable of fragmenting to produce a particular product ion.

(iii) *Neutral-loss scanning* involves looking at pairs of precursor and product ions that differ by a defined constant neutral loss. This is useful when analyzing a class of compounds that is known to have a specific fragmentation pattern.

(iv) In *selected reaction monitoring* (SRM) a particular product ion of a selected precursor ion is monitored. Multiple precursor-product ion pairs can be monitored simultaneously, which is known as *multiple reaction monitoring* (MRM).

Tandem MS has recently been used for studies of age-related macular degeneration (Wang et al., 2006; Murdaugh, Dillon, & Gaillard, 2009; Murdaugh et al., 2010a,b) and for the metabonomic analysis of urine from breast (Chen et al., 2009b) and lung cancer patients (An et al., 2010; Yang et al., 2010).

III. APPLICATIONS OF MASS SPECTROMETRY TO AGING STUDIES

In this section and the next we discuss the use of mass spectrometry-based metabonomics to investigate the process of aging in humans, as well as in several model organisms. We have restricted our discussion to MS-based studies, but in select instances we have also elected to mention significant findings from studies undertaken using NMR. In the current section we focus on MS investigations measuring metabolic alterations related to the aging process itself. In Section IV we discuss changes associated with various late-life disease states. A large number of MS-based aging studies have been undertaken which focus exclusively on the proteome. Since the focus of this review is only on the metabolome, readers interested in proteomic studies are directed to an excellent review by Schoneich (2005).

At this point it is worth digressing for the non-gerontologist to consider what initially seems like a trivial question—What is aging? Robert M. Pirsig in his autobiographical book "Zen and the Art of Motorcycle Maintenance" discusses the term quality and the trouble one runs into when trying to define it. Aging is similar to quality in that it is also very difficult to define. Aging is not a disease state that we recover from. Aging is distinguishable from age-associated diseases.

Aging is recognizable by us all yet occurs neither at the same rate in any two individuals, nor in the exact same manner. For the purpose of this review we will define aging as a narrowing over time of life's homeostenosis window (see next paragraph). Within this framework we can at least separate the *process of aging* from *processes that result in aging* even if we do not exactly understand aging. The process of aging can then be descriptively defined in terms of a state- or time-dependent function. With much greater scientific effort, processes that facilitate the appearance of aging can also be elucidated.

Metabolites are, in essence, the business end of life. One can argue that life is defined by the ability to maintain concentrations of the small metabolites of intermediary metabolism within discrete ranges that effectively represent stable states of dynamic equilibria. For reasons unknown, this range of allowed equilibria seems to narrow with time—so-called homeostenosis. This is the phenomenon we call aging. Metabonomics therefore provides a wonderful tool to study aging because it provides a powerful means of cataloguing changes in the process over time, at the molecular level. Through the mining of such descriptive data, metabonomics can potentially aid in the identification of processes that lead to aging (Kristal & Shurubor, 2005; Kristal et al., 2007).

A. Aging Studies in C. elegans

The nematode *Caenorhabditis elegans* has long been a stalwart of genomics research (Brenner, 1974). Over the last decade this model organism has provided some of the most significant advances in our understanding of eukaryotic aging (Fontana, Partridge, & Longo, 2010; Kenyon, 2010; Rea et al., 2010). Only recently, however, has research on *C. elegans* entered the realm of metabonomics (Blaise et al., 2007; Butler et al., 2010; Falk et al., 2008; Fuchs et al., 2010; Reinke et al., 2010). Interestingly, almost all studies have employed NMR-based methods, and to our knowledge there exists only one article in the literature which uses MS to study aging in *C. elegans* (Butler et al., 2010). Further, this study used an HPLC method for generation of data, and only utilized MS for metabolite identification. Given that MS-based strategies stand to provide a rich trove of metabolite data, the use of such metabonomics approaches to look at the effects of age on *C. elegans* is an area waiting to be explored. Here we will digress momentarily to present the most recent findings regarding aging in *C. elegans*; however, the reader should be aware that these are mainly NMR-based studies.

Regarding the most recent metabonomics studies in *C. elegans*, Fuchs et al. (2010) used proton NMR to study three classes of long-lived *C. elegans*: (i) dauer larvae—which arise naturally when food or environmental conditions become limiting and which, as an alternate developmental stage specialized for survival, can live up to eight times longer than normal-developing worms, (ii) adult worms containing one of several mutations in the insulin/insulin-like signaling pathway, and (iii) a translation-defective mutant. Intriguingly, despite the varied nature of these three classes of long-lived worms, the authors were able to distinguish a common metabolic signature that was distinct from that found in wild-type worms.

In another NMR-based study, Reinke et al. (2010) examined the effect of altered diet on both lifespan and metabolic profile of *C. elegans*. Two strains of *E. coli* are commonly employed as food source to maintain and culture *C. elegans*—OP50 and HT115. The latter strain has been engineered for feeding-RNAi purposes, which is an easy and powerful means of inhibiting the expression of any gene of interest in *C. elegans* (Timmons, Court, & Fire, 2001). Wild-type animals cultured on HT115 live significantly shorter than animals cultured on OP50. Reinke et al. observed significant dietary effects on metabolic profile, mitochondrial DNA copy number and larval development. Indeed, the reported dietary effects were similar in magnitude to metabolic changes induced by various genetic modifications, or RNA interference-mediated gene suppression, and were thus far from minor.

Finally, within *C. elegans* two classes of mutants are known that disrupt mitochondrial electron transport chain function: relative to wild-type animals, one is long-lived (Mit mutants) and the other is short-lived (ByBy mutants). Butler et al. (2010) used a metabolic footprinting approach to look at the differences in metabolites excreted by both classes of mutants relative to wild-type worms. By using HPLC and PCA, the authors were able to segregate long-lived Mit mutants from wild type worms and, separately again, short-lived ByBy mutants. Several LC peaks which contributed the greatest to the PCA clustering were selected for further analysis. These peaks were analyzed via LC-ESI-MS/MS and identified based on fragmentation patterns. In this way it was found that the concentration of pyruvate was increased in the long-lived Mit mutants. Also noted was a LC peak which was enriched in the short-lived ByBy mutants. This peak was found to contain a mixture of metabolites including lactate, α-ketoglutarate, and the dipeptide Gly-Pro. This is supportive of the concept that metabolic pathways are altered in long-lived *C. elegans*, and that the metabolic pathways available to an organism may play a role in longevity.

B. Aging Studies in Rodents

Several studies have appeared in the last 5 years that have employed metabonomics to study aging in rodents. Typically, such studies have limited their sample collection to plasma or urine—samples that are relatively easy to obtain in a non-life threatening manner. Such samples are also the choice for human studies if metabonomics ever reaches the point of becoming a benchside tool.

In a study by Nevedomskaya et al. (2010), CE-MS was used to observe differences in the urinary metabolome of wild type versus accelerated-aging TTD mice. The TTD genotype contains a mutation in the XPD gene involved in the Nucleotide Excision Repair pathway and displays a series of phenotypic changes including osteoporosis, osteosclerosis, early graying, infertility, and shortened lifespan. PCA score plots did not show any clustering of data based on genotype. The authors point out that this is a typical result when using unsupervised methods for metabolic profiling, since intrinsic variations in samples may obscure the differences of interest. PLS-DA models were able to discriminate the two genotypes for the female mice, but were poor at discriminating the males. Among the compounds observed to contribute to the difference between genotypes in the female mice were *N*-acetylspermidine, a compound involved in the processes of cell

growth and differentiation, and lysine, which is associated with calcium absorption and bone health. Also altered between the two groups was *S*-adenosyl-L-methionine, a compound which is involved in methyl group transfers and is required for cellular growth and repair.

Yan et al. (2009) have used LC-MS to look at the effects of flavones extracted from *Epimedium*, an herb used in traditional Chinese medicine for its antioxidative and antiaging effects, on the plasma metabolome of aged Sprague–Dawley rats. The authors noticed broad changes in amino acid, nucleotide, and lipid metabolism with advancing age. Specifically, they observed a change in the concentration of carnosine (a dipeptide), as well as a decrease with age in the concentrations of unsaturated fatty acids (linoleic acid, oleic acid, arachidonic acid, and palmitoleic acid). In contrast, concentrations of saturated fatty acids (myristic acid, palmitic acid, stearic acid, nonacosanoic acid) were observed to increase with increasing age. Taken together these results indicate a decline in lipid metabolism in the aged rats. Administration of the total flavones of *Epimedium* was observed to reset many of the perturbed metabolites to concentrations present in younger rats, and resulted in an increase in the concentrations of unsaturated acids and a decrease in the concentrations of saturated fatty acids (except stearic acid).

Of further interest is a study by Williams et al. (2005b), who used HPLC-TOF-MS to look at the effects of age on the profile of metabolites in the urine of male Wistar-derived rats. Samples were collected every 2 weeks from 4 to 20 weeks of age. Three-dimensional PLS-DA plots (first three principle components) were able to distinguish samples from the 4 week, 6 week, and, to a lesser extent, 8 week samples from the rest of the data when data were collected in positive-ion mode. When the 4- to 8-week data were removed from the analysis, the remaining data was clearly clustered by age (Fig. 3). While the majority of the metabolites contributing to the clustering (based on PLS loading weights) were not identified, at least one metabolite which increased across all age groups was identified as carnitine, again pointing to perturbations in lipid metabolism with increased age.

Schnackenberg et al. (2007) also investigated age-related changes in the urinary metabolic profile of rats (Sprague–Dawley). Using NMR, UPLC-MS with an ESI source operated in both positive and negative ionization modes, and PCA to analyze their data, the authors were able to distinguish 25-day, 40-day, and 80-day-old rats. The authors identified hippuric acid, a kynurenic acid salt, ferulic acid sulfate, and suberic acid as metabolites that increased with advancing age. It was also noted that the concentrations of several Krebs cycle intermediates including acetate, fumarate, oxaloacetate, pyruvate, and trans-aconitate were decreased in the aged rats, possibly indicating alterations in mitochondrial activity.

Several metabonomics studies which employed UPLC coupled to oa-TOF-MS to investigate age-related metabolite changes in urine from Zucker rats have also recently appeared (Plumb et al., 2005; Granger et al., 2007). Using PCA and PLS-DA, Plumb et al. (2005) showed that 6-week-old rats could be distinguished from 20-week-old rats in both the Zucker obese strain and a Wistar-derived control group. Additionally, it was observed that there was more variance amongst the 20-week-old Zucker obese sample group than the

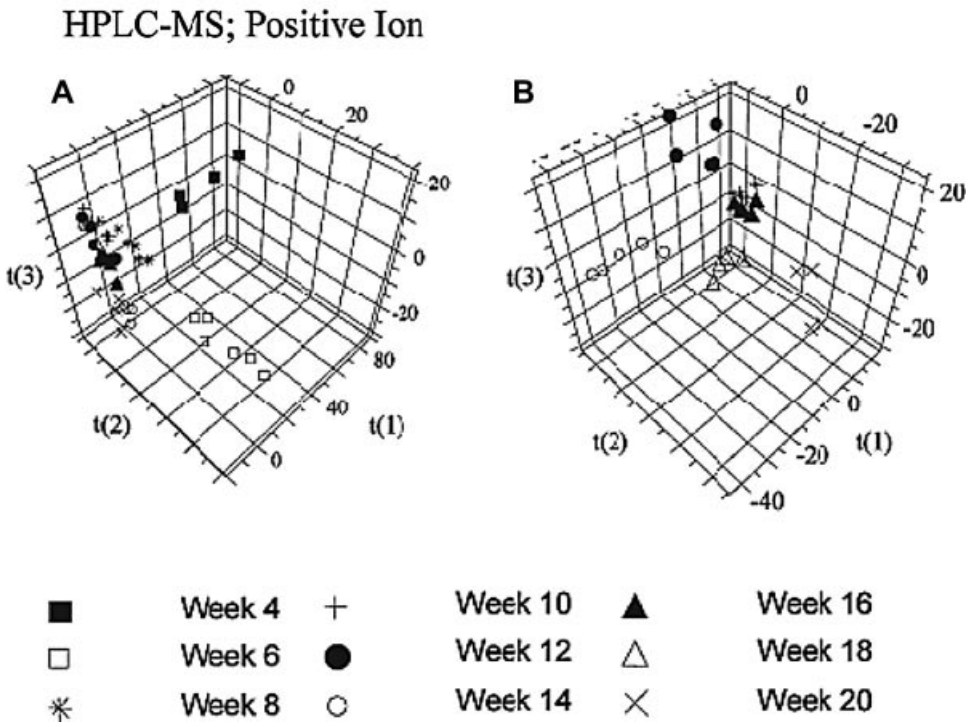

FIGURE 3. **A**: Three-dimensional PLS-DA plots obtained using HPLC-MS data from analysis of urine of male Wistar-derived rats across a 20-week period. **B**: Re-clustering of data following censoring of week 4 to week 8 data, affords greater separation in the remaining data. Reprinted from Williams et al. (2005b) with permission from The Royal Society of Chemistry.

6-week-old animals. Comparison of the ions with $m/z = 255$ and 332, which contributed strongly to the PLS-DA loading plots for the 6-week and 20-week-old animals, respectively, revealed that the concentration of the ion with $m/z = 255$ was reduced in the 20-week-old animals, whereas the concentration of the ion with $m/z = 332$ was increased; however, no attempt was made to identify these metabolites. It is interesting to note that the authors were also able to cluster data based on whether samples were collected in the morning or the evening. This diurnal variation shows that caution must be taken when acquiring samples for metabonomics analysis to ensure that as many spurious variables are eliminated as possible. A related study by Granger et al. (2007) also revealed age-related clustering in urine samples from Zucker rats. Samples were collected every 2 weeks from week 6 to week 20 and analyzed by PLS-DA. Clustering by age, and a clear age-related trajectory, was observed in the score plots for both Zucker lean and Zucker obese rats.

Age-related metabolic changes have also been observed in the blood plasma of spontaneously hypertensive rats (Lu et al., 2008b). The concentrations of several fatty acids, including linoleic acid, oleic acid, and hexadecanoic acid, were observed to increase with age (10–18 weeks) in the spontaneously hypertensive rats, and decrease in the normotensive control—again pointing to changes in lipid metabolism with age. Collectively, these studies show that MS-based metabonomics can be used to detect overall age-related changes in the metabolome of rodents. One simple hypothesis that arises from these findings is that mitochondrial dysfunction, and the subsequent inability to efficiently metabolize fats, may be the underlying etiology of lipid alteration with age in these animals.

C. Aging Studies in Humans

Naturally, the ultimate goal of much gerontology research is to find ways to delay the aging process and perhaps even increase longevity in humans. Several studies have emerged which approach aging in humans from a metabonomics standpoint; however, as with *C. elegans*, many of these studies use NMR-based methods (Slupsky et al., 2007; Psihogios et al., 2008; Gu et al., 2009; D'Adamo et al., 2010). We know from our above discussion of aging in mice and rats that MS-based technologies, while largely untapped for human gerontological studies, offer a convenient method for studying metabolic changes associated with the aging process. Since MS provides complementary information to NMR, its adoption for human metabonomics studies would allow researchers to study a larger portion of the aging metabolome. For these reasons it is expected that MS-based metabonomics will play an important role in future studies of aging in humans.

An example of the application of MS-based metabonomics to study aging in humans is given by a large-scale metabonomics study carried out by Lawton et al. (2008), who used both GC-MS and HPLC-MS to analyze the plasma of some 269 individuals. These authors found that age significantly altered the concentrations of over 100 metabolites. In contrast, only 35 metabolites were significantly altered by gender, and only 6 were statistically different between Caucasian and African-American subjects. These findings indicate that aging is clearly one of the most important factors controlling alterations in human metabolic pathways, and underscore the potential of MS-based techniques to detect a large number of age-perturbed metabolites in humans. Lawton and co-workers also noted from their data that, after separating subjects into three age groups (20–35, 56–50, 51–65 years), there was an age-dependent increase in the levels of amino acids, molecules associated with amino acid metabolism, citric acid cycle intermediates (associated with energy metabolism), oxidative stress markers (oxoproline, hippurate), and molecules associated with nucleic acid metabolism (xanthine, hypoxanthine). In addition, the authors observed that the concentrations of compounds associated with lipid metabolism (carnitine, cholesterol, fatty acids, for example, linoleic acid and arachidonic acid), as in the rodent studies, were lowest in the youngest age group.

D. Aging Studies in Other Model Organisms

There are two other metabonomics studies involving aging and alternate model organisms which we would like to briefly mention; both involve proton NMR, not MS. Wang and co-workers examined the effects of aging and caloric restriction throughout the life of Labrador dogs (Wang et al., 2007). In addition to detecting a decline in creatinine excretion in aged animals, the authors noted changes in gut microbiotal metabolism in response to both age and caloric restriction. In a second study, using the fruit fly *Drosophila melanogaster*, Coquin et al. (2008) used proton NMR to analyze the age-related decline of hypoxia tolerance in muscle tissue and found that old flies are slower to recover from hypoxic stress. Additionally, these authors observed that after hypoxic stress, the heart rates of the older flies took longer to recover, as did the levels of cellular ATP.

IV. DETECTION OF BIOMARKERS ASSOCIATED WITH THE DIAGNOSIS AND ETIOLOGY OF AGE-RELATED DISEASES

When discussing aging, one must acknowledge that many diseases manifest late in life, and their pathogenesis may affect the metabolome. As health care improves and new methods are developed to diagnose and treat diseases, the median age of our society will inevitably increase, and it is projected that the number of people worldwide aged 65 and greater will increase from 418 million in 2000 to over 1.3 billion by the year 2050 (Thun et al., 2010). The use of MS-based methods to analyze urine and blood samples has the potential to offer a minimally invasive method for the diagnosis of late-life diseases. In an interesting study, Martinez-Lozano and Fernandez de la Mora (2009) have shown that MS can even be used to detect metabolites directly from human skin vapors in a completely noninvasive manner. In this section, we survey the multitude of MS-based metabonomics studies of diseases commonly associated with age, and we highlight to the reader what we consider to be some of the most important findings. In this section we will also present several metabonomics studies which use MS to study atherosclerosis and diabetes. We recognize that with the appearance of the obesity epidemic, and consequently metabolic syndrome, both diseases are now viewed as chronic, life-long diseases rather that late-onset ones by some camps.

A. Cancer

Cancer is the third highest cause of death worldwide, with 7.6 million deaths due to cancer reported in 2007, a number which is expected to rise to 17 million per year by 2030 (Thun et al., 2010). While cancer affects people of all ages, 45% of all cancers diagnosed in 2002 were in people >65 years of age (Bray & Moller, 2006). As the prognosis for cancer patients usually depends on how early the disease is detected, having reliable biomarkers for early-stage cancer diagnosis is highly desirable. Metabolic fingerprinting allows for the identification of such biomarkers, and it can be done non-invasively using urine or plasma samples. This is in contrast to current methods for diagnosing cancer, which are either invasive, or involve exposing patients to X-ray radiation. MS-based metabonomics has been used to study esophageal (Wu et al., 2009), bladder (Pasikanti et al., 2010), breast (Frickenschmidt et al., 2008; Chen et al., 2009b; Henneges et al., 2009), gastric (Chen et al., 2010a; Wu et al., 2010), prostate (Osl et al., 2008; Sreekumar et al., 2009), kidney (Perroud et al., 2006; Kind et al., 2007), lung (Fan et al., 2009; An et al., 2010; Yang et al., 2010), ovarian (Denkert et al., 2006; Guan et al., 2009), colorectal (Denkert et al., 2008; Chan et al., 2009; Cristoni et al., 2009; Hirayama et al., 2009; Ma et al., 2009; Mal et al., 2009; Nambiar, Gupta, & Misra, 2010; Qiu et al., 2009, 2010; Wang et al., 2010), pancreatic (Boros et al., 2005), stomach (Hirayama et al., 2009), and liver cancers (Xue et al., 2008; Chen et al., 2009a; Yin et al., 2009). We will discuss a few of these reports in detail below.

1. Lung Cancer

One of the deadliest and most common forms of cancer is lung cancer. According to the Center for Disease Control, over 200,000 new incidences and over 158,000 deaths were reported in 2007 in the US alone. The World Health Organization reported 1.3 million lung cancer deaths worldwide per year in 2004, representing more than 17% of all cancer related deaths. An et al. (2010) and Yang et al. (2010) have used LC-MS to identify potential biomarkers in urine to discriminate between lung cancer patients and healthy subjects. Using a novel hyphenated HILIC-RPLC-MS technique, Yang et al. identified 11 potential biomarkers which were upregulated in lung cancer patients. These included taurine, hippuric acid, valine, betaine, and carnitine. The innovative use of HILIC-RPLC-MS allowed for the analysis of both polar and apolar components in a single injection. This was accomplished via an automated column switching procedure, thereby overcoming the loss of non-retained compounds normally experienced when using a single column (Wang et al., 2008). An and co-workers, using an integrated LC-MS approach which employed several different ionization methods including APCI and ESI, also identified 11 potential biomarkers for lung cancer. These included amino acids, nucleosides, and an indole metabolite, and imply protein degradation and an increase in amino acid and nucleoside metabolism in lung cancer patients.

In a related study, Fan et al. (2009) performed a stable isotope-resolved metabonomics analysis of lung cancer patient tissue. Patients were infused intravenously with ^{13}C labeled glucose prior to tumor resection. Tissue samples were pulverized and extracted with 10% trichloroacetic acid to recover water-soluble and polar metabolites, and then analyzed with NMR and GC-MS. Samples were matched with non-cancerous tissue samples from the same patient. The authors showed an increased incorporation of labeled carbon into lactate, alanine, citrate, glutamine, succinate, aspartate, and the ribosyl moiety of nucleotides in the cancerous tissue. These data provide evidence for enhanced glycolysis in the tumor tissues, and also suggest a more active Krebs cycle.

2. Ovarian Cancer

Ovarian cancer is the sixth most frequent cancer affecting women worldwide, and typically has a poor prognosis given that 75% of cases are diagnosed in a late stage (Tinelli et al., 2009). Therefore, it would be extremely beneficial to identify biomarkers that would enable detection of ovarian cancers earlier in the progression of the disease. To this end, Guan et al. (2009) have shown that LC-TOF-MS fingerprinting of human sera samples can be used to differentiate between ovarian cancer patients and healthy controls with an accuracy of >90%.

In contrast to invasive carcinomas, ovarian borderline tumors are non-invasive and have a significantly better prognosis. Denkert et al. (2006) used a metabonomics approach to examine differences between ovarian carcinomas and ovarian borderline tumors. The authors prepared their samples by homogenizing the biopsied tumors and then extracting them with chloroform/methanol/water. After drying and derivatizing the samples they were analyzed by GC-TOF-MS. In this manner the authors were able to identify 51 biomarkers that distinguished between the two types of tumors. The authors noted differential regulation of creatinine, lactate, glucose-L-phosphate, fumarate, malate, and a number of amino acids between the two groups. Using PCA and other supervised predictive models, they were able to discriminate almost 90% of the borderline tumors from the carcinomas.

3. Colorectal Cancer

Colorectal cancer is the second most common cause of cancer-related death in America and Europe (Hung & Chung, 2006). Ma et al. (2009) have used UPLC-TOF-MS to analyze urine samples of pre-operative and post-operative colorectal cancer patients, as well as healthy controls. The authors detected several thousand metabolite related peaks, and were able to clearly separate the three groups using PLS-DA. Two unidentified low molecular weight compounds (MW 283 and 294) were determined to be elevated in the preoperative group over the controls. The levels of both of these compounds were found to decrease following surgery, and the authors suggest that these compounds may have both prognostic and diagnostic value in the early detection of colorectal cancer. Another study which looked at metabolites excreted in urine of colorectal cancer patients was performed by Qiu et al. (2010). Using GC-MS with OPLS-DA the authors were able to discriminate colorectal cancer patients from a control group. Sixteen metabolites were identified as being altered in the colorectal cancer patients including metabolites related to the TCA cycle, tryptophan metabolism, and gut flora metabolism. In a related study, these authors also examined serum

metabolites altered by colorectal cancer using both GC- and LC-MS (Qiu et al., 2009).

Denkert et al. (2008) analyzed extracts from tissue samples of healthy and colorectal cancer patients using GC-TOF-MS coupled to an EI ionization source. With this method, the authors identified 206 unique metabolites, 82 of which were differentially expressed between the cancer patients and healthy controls. Metabolites upregulated in cancer patients included amino acids and purines, suggesting increased synthesis of DNA and higher turnover of structural proteins in cancerous cells. Downregulated metabolites included intermediates in the tricarboxylic acid cycle as well as several saturated (heptadecanoic acid, suberic acid) and unsaturated (oleic acid, arachidonic acid) fatty acids. PCA clustering analysis showed that the two groups were able to be clearly separated, validating the diagnostic value of this approach.

Another metabonomics study analyzing colon tissue extracts also successfully discriminated healthy from cancerous tissue. Mal and co-workers used GC-MS in conjunction with OPLS-DA to identify 12 metabolites that were differentially altered between the two tissue types (Mal et al., 2009). These authors observed decreased levels of monosaccharides (mannose, galactose, and glucose) in the cancerous tissue and an increase in lactate concentration. Both findings are indicative of higher levels of glucose uptake and its conversion to lactate. This is consistent with increased glycolytic energy production in colon cancer tissue, and its decreased reliance on the TCA cycle and mitochondrial oxidative phosphorylation. This phenomenon has been known to occur in cancer cells for more than half a century and is described as the Warburg Effect (Warburg, 1956). In a related study, Hirayama and co-workers used CE-TOF-MS and also found reduced glucose concentrations in colon tumor tissue relative to normal tissue (Hirayama et al., 2009). These authors also noted that the concentrations of glycolytic intermediates in the tumor tissues were equal or greater than those in normal tissues, again indicative of increased glycolysis (Fig. 4).

A study by Chan et al. (2009) also examined differences in metabolites extracted from tissue samples of healthy and colorectal cancer patients. Using a combination GC-MS and solid state NMR with OPLS-DA the authors identified 31 metabolites which differed between the two groups of patients. Similar to the study presented above by Mal et al. the authors noted consistently higher levels of lactate and decreased concentrations of glucose in the colorectal cancer patients. In addition they found glycine to be consistently elevated in tissue samples from colorectal cancer patients, which could also be related to increased glycolysis, being formed from 3-phosphoglycerate. Other metabolic perturbations noted by Chan et al. include decreased levels of saturated and unsaturated lipids and fatty acids and increased concentrations of phosphocholine and phosphoethanolamine in tissue samples from colorectal cancer patients, as evidenced by NMR.

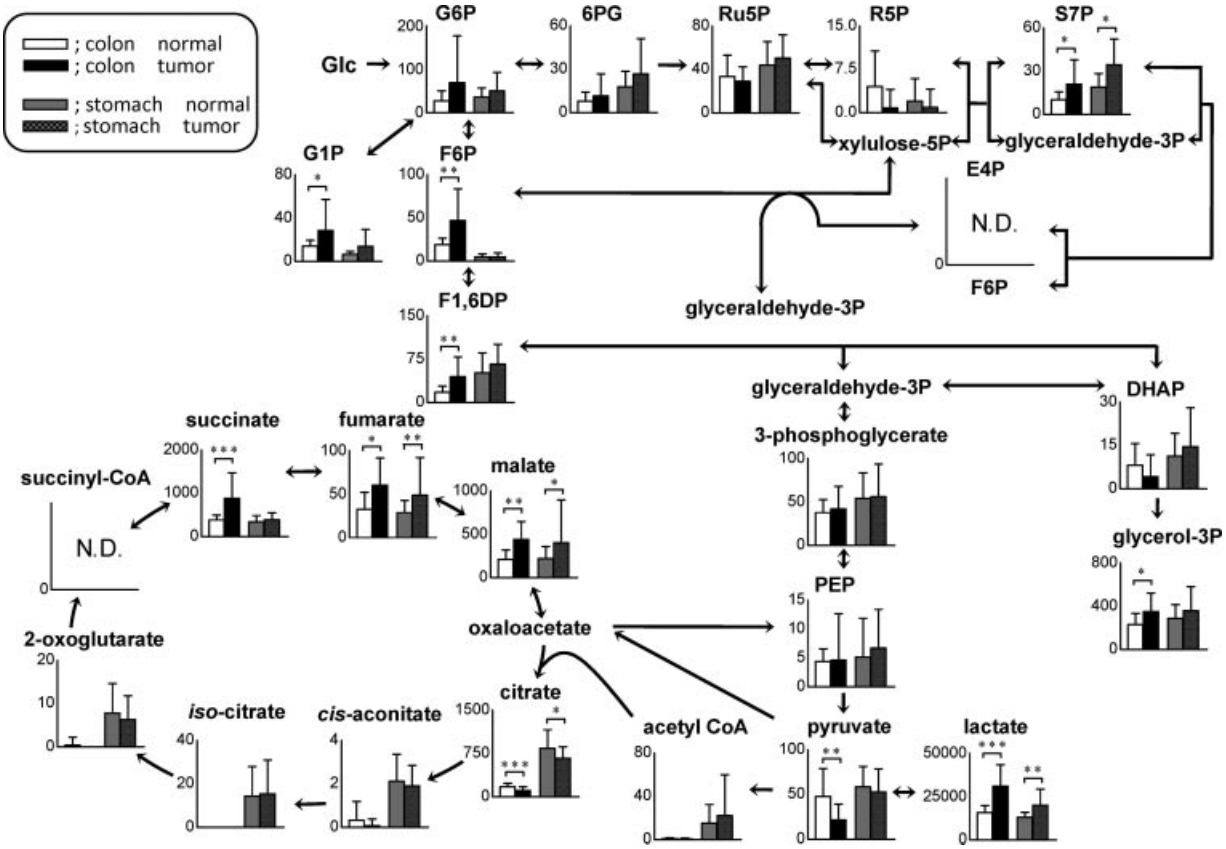

FIGURE 4. Concentrations of metabolites involved in central carbon metabolism as determined by Hirayama et al. Abcissa: average concentration of metabolite in nmol/g tissue. Reprinted from Hirayama et al. (2009) with permission from the American Association for Cancer Research.

B. Age-Related Macular Degeneration

While MS studies based on the diagnosis of cancer typically involve metabolic fingerprinting, studies of age-related macular degeneration (AMD) generally involve targeted metabolite analysis. AMD is the leading cause of blindness among the elderly in developed countries worldwide (Congdon et al., 2004; Pascolini et al., 2004; Jager, Mieler, & Miller, 2008) and is characterized by central vision loss due to damage of the retina. AMD is classified as occurring in either a wet or dry form; the dry form has been attributed to the accumulation of cellular debris, known as drusen, between the retinal pigment epithelium (RPE) and Bruch's membrane resulting in regional degeneration of the RPE and photoreceptor cell death (Murdaugh, Dillon, & Gaillard, 2009; Ni et al., 2009). Wet AMD is characterized by new, weak blood vessels growing up from the choriocapillaris through Bruch's membrane. These blood vessels can break open and leak, resulting in large amounts of bleeding which can lead to damage of the surrounding tissue and detachment of the retina (Ni et al., 2009).

A major risk factor associated with the development of AMD is the accumulation of fluorescent granules, called lipofuscin, in the RPE (Parish et al., 1998; Wang et al., 2006). Lipofuscin is a complex mixture of chromophores which is known to contain the vitamin A derivative A2E (Fig. 5) as well as several of its oxidation products (Sakai et al., 1996; Parish et al., 1998; Avalle et al., 2004; Dillon et al., 2004). A2E is able to undergo an auto-oxidation process and can also be photo-oxidized, leading to toxic oxidation products. Further, photo-oxidation of A2E by blue light has been shown to induce apoptosis in A2E-containing RPE cells (Sparrow, Nakanishi, & Parish, 2000; Sparrow & Cai, 2001). Wang et al. (2006) have analyzed the oxidation products of A2E in vitro and found that both the photo-oxidation route and auto-oxidation route produced a series of products due to single additions of oxygen across the double bonds to form epoxides, as evidenced by the presence of M + 16 ions (parental compound mass +16 Da) in the mass spectrum of the product mixture. This is consistent with a study reported by Ben-Shabat et al. (2002) where the authors describe the reaction of A2E with singlet oxygen in solution to form epoxides up to and including a nonaoxirane. In addition, Wang et al. noted a series of compounds which had masses smaller than that of A2E (592 Da), resulting from the cleavage of the double bonds of A2E along the polyene chain to form aldehydes. The presence of the aldehyde functional group was verified by reaction of the product mixture with *p*-nitrophenylhydrazine, which reacts with aldehydes to form nitrophenylhydrazones with a corresponding mass increase of 135 Da. Comparison of the mass spectrum before and after the reaction reveals that every peak with a mass of less than 592 Da completely reacts with this reagent, indicating that a series of aldehydes was formed derived from the cleavage of every double bond along the polyene chain. Further evidence for aldehyde formation comes from selected reaction monitoring of the product mixture prior to reaction with nitrophenylhydrazine which shows characteristic M-28 peaks owing to loss of the aldehyde functionality. These results are significant as both epoxides and aldehydes are highly reactive, and the formation of these species could help explain the toxicity of the degradation products of A2E.

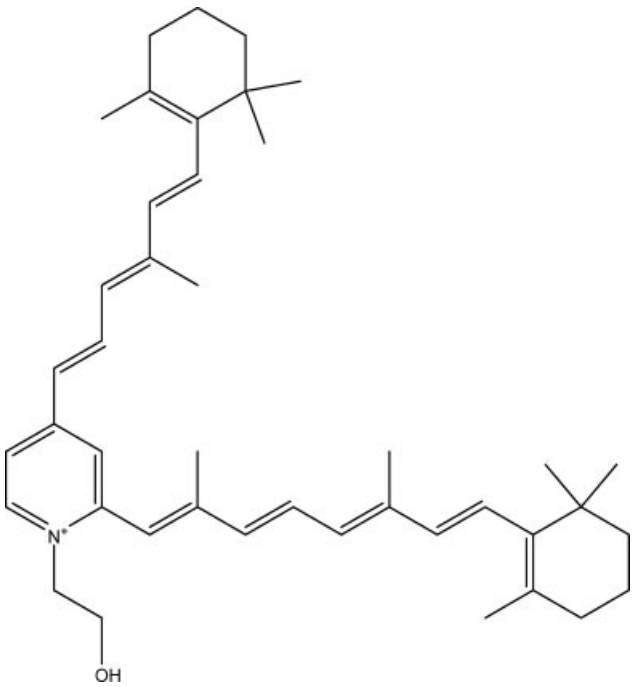

FIGURE 5. Structure of the A2E, a pyridinium bis-retinoid derived from vitamin A aldehyde.

Consumption of green leafy vegetables high in lutein, a carotenoid with antioxidative properties, is known to delay the progression of AMD by increasing the lutein concentration in the retina (Bone et al., 1993, 2003; Krinsky, Landrum, & Bone, 2003; Lakshminarayana et al., 2008). The accumulation of lutein in the retina helps to protect the eye from oxidative stress and high-energy blue light. In one study it was shown via LC-APCI-MS that intake of dietary lutein results in the accumulation of oxidative metabolites in the liver and plasma of rats (Lakshminarayana et al., 2008). Lutein oxidation products were also found in eye samples; however, the authors caution that it is unclear whether these are the result of photo-induced metabolic oxidation in the eye or they are transported via the circulatory system. Surprisingly, the metabolite 3-hydroxy-β-ε-caroten-one was observed in eye samples but not in in vitro samples, indicating possible in vivo metabolic oxidation of lutein. Several metabolites of lutein were found in the liver and plasma, but not eye samples, indicating possible involvement of lutein as an antioxidant in tissues other than the eye. Based on the fragmented ions observed in the liver and plasma samples, the authors proposed a metabolic pathway of lutein which involves oxidation to the either anhydrolutein or the diepoxide, followed by subsequent fragmentation.

C. Atherosclerosis

Atherosclerosis is a disease in which arteries become damaged due to the buildup of fatty materials such as lipids and cholesterol. The disease is characterized by chronic arterial inflammation and cell death. This often results in heart disease or stroke, and consequently atherosclerosis is the leading cause of morbidity and mortality in developed nations (Braunwald, 1997; Zhang et al., 2009a; Chen et al., 2010b). Atherosclerosis is most commonly diagnosed via angiography, an invasive

technique which also exposes the patient to radiation. Further, this technique focuses on the detection of severe narrowing of arteries as opposed to detection of the underlying disease, making a non-invasive or minimally invasive metabonomic approach an attractive alternative.

Through a metabolic fingerprinting approach, Vallejo et al. (2009) used GC-MS and PLS-DA to analyze and discriminate blood plasma derived from healthy patients and patients diagnosed with stable atherosclerosis. Teul et al. (2009) later identified at least 24 metabolites that were significantly altered in the plasma of atherosclerotic patients versus healthy controls using a combination of GC-MS and NMR. Their findings indicated elevated glucose levels and a decrease in fructose concentration in patients, which they attribute to impaired insulin response and decreased metabolism of glucose. In addition, significant variations in the concentrations of 13 amino acids were observed between the two groups.

Zhang et al. (2009a) used UFLC coupled to a hybrid IT-TOF mass spectrometer with an ESI source to analyze the plasma and urine of healthy and atherosclerotic male Wistar rats. The authors showed that PCA could be used to successfully discriminate healthy from diseased rats using either the urine or plasma samples. In addition, the authors found a decrease in butyrylcarnitine concentration in both the plasma and urine of atherosclerotic rats, a downregulation of leucine, phenylalanine, tryptophan, acetylcarnitine, propionylcarnitine, and spermine in the plasma of the diseased rats, and an upregulation of ursodeoxycholic acid, chenodeoxycholic acid, and lysophosphatidylcholine in plasma.

While all of the above studies focused on the application of metabonomics to fingerprint metabolites associated with metabolic perturbations due to atherosclerosis, they did not attempt to elucidate a specific biomarker which can be utilized for clinical diagnosis. Chen et al. (2010b), using GC-EI-MS with PCA and OPLS-DA, showed that fatty acids could be used as biomarkers for diagnosis of atherosclerosis. Specifically, the authors note that stearate and palmitate are elevated in patients with atherosclerosis, and suggest that use of palmitate as a single biomarker is sufficient to diagnose the disease. It should also be noted that, although many of the studies we have mentioned above are focused on fingerprinting diseases and using markers for disease detection, metabolomics is also a tool that can allow researchers to dive deeper into understanding a disease. Metabolite changes can signify underlying changes in metabolic pathways that may in fact be the cause of such diseases.

D. Diabetes

Diabetes is a metabolic disorder in which a person has elevated blood sugar and results either from the pancreas not producing insulin to stimulate removal of blood sugar into peripheral tissue storage sites (Type 1), or from peripheral target tissues not being able to respond to insulin that is being produced by the pancreas (Type 2). In the year 2000, the number of people diagnosed with diabetes was 171 million (worldwide). This number is expected to increase to 366 million by 2030 (Wild et al., 2004). In 2000, 10% of the world's population aged 60 years or older were affected by diabetes, with the majority of people in developed countries suffering from diabetes being >64 years of age. This number is expected to exceed 130 million by 2030 (Wild et al., 2004). In this section we will discuss studies which have taken a metabolomics approach to investigate diabetes. For brevity, we will focus our discussion on MS-based fingerprinting studies; however, metabolic profiling has also been used to study defects in metabolic pathways related to diabetes. For a broader exposure to the use of metabolomics in diabetes research we wish to direct the interested reader to a review which has recently been written on the subject by Bain et al. (2009).

Li et al. (2009) used two-dimensional GC with TOF-MS to look for biomarkers of type 2 diabetes (T2DM) in the plasma of diabetes patients. The authors analyzed the data using PLS-DA with orthogonal signal correction and identified five potential biomarkers for T2DM which included palmitic acid, 2-hydroxyisobutyric acid, and linoleic acid. In another study, Yi et al. (2008) used GC-MS and uncorrelated linear discriminant analysis (ULDA) to discriminate plasma samples from patients with T2DM from T2DM with coronary heart disease, and from healthy controls. These authors reported a correct prediction rate of 81% for discriminating between healthy and T2DM patients. Wang et al. have also used GC-MS to differentiate between plasma phospholipid samples of T2DM patients and healthy subjects (Wang et al., 2005). Furthermore, the authors used GC-MS/MS to identify several potential phospholipid biomarkers for T2DM.

Metabolic fingerprinting studies have also been performed on urine samples of T2DM patients (Yuan et al., 2007; Chen et al., 2008; Xing et al., 2010). This method has the advantage that it is completely non-invasive. Yuan et al. (2007) used GC-MS with PCA and PLS-DA to distinguish T2DM patients from controls. They also identified potential biomarkers from the PLS-DA loading plot which they determined to be predominantly organic acids, after searching libraries of known mass spectra. These included oxyl acetic acid, 4-aminobenzoic acid, 2,5-bisoxy-benzeneacetic acid, and the dimethyl ester of maleic acid.

Increased insulin resistance is a risk factor for diabetes which typically precedes T2DM. Discovery of biomarkers for this pre-diabetic state would be beneficial in the diagnosis and treatment of T2DM. Chen et al. compared urine samples from healthy individuals and insulin-resistant pre-diabetics using UPLC-Q-TOF MS with PCA followed by PLS-DA (Chen et al., 2008). They were able to completely discriminate the two groups, and identified a potential biomarker from the PLS-DA loading plot (Fig. 6). The authors isolated this compound using micropreparatory UPLC and then subjected it to analysis by negative mode ESI-FTICR mass spectrometry. This technique allows for determination of the exact mass of the component peak to within 120 ppb, and allowed the authors to deduce the elementary chemical composition of the metabolite. Using MS/MS analysis and database searching, they were then able to identify this component as 3-hydroxyhippuric acid. Similar studies aimed at identifying biomarkers in pre-diabetic individuals through a metabolic fingerprinting approach have been undertaken by Gall et al. (2010) and Zhao et al. (2010). Tsutsui et al. (2010) used a rodent model to address an analogous question. Interestingly, Gall et al. determined 2-hydroxybutyrate to be the top ranked biomarker for discriminating insulin-sensitive and insulin-resistant individuals, a biomarker which was also identified by Li et al. (2009) in patients with T2DM.

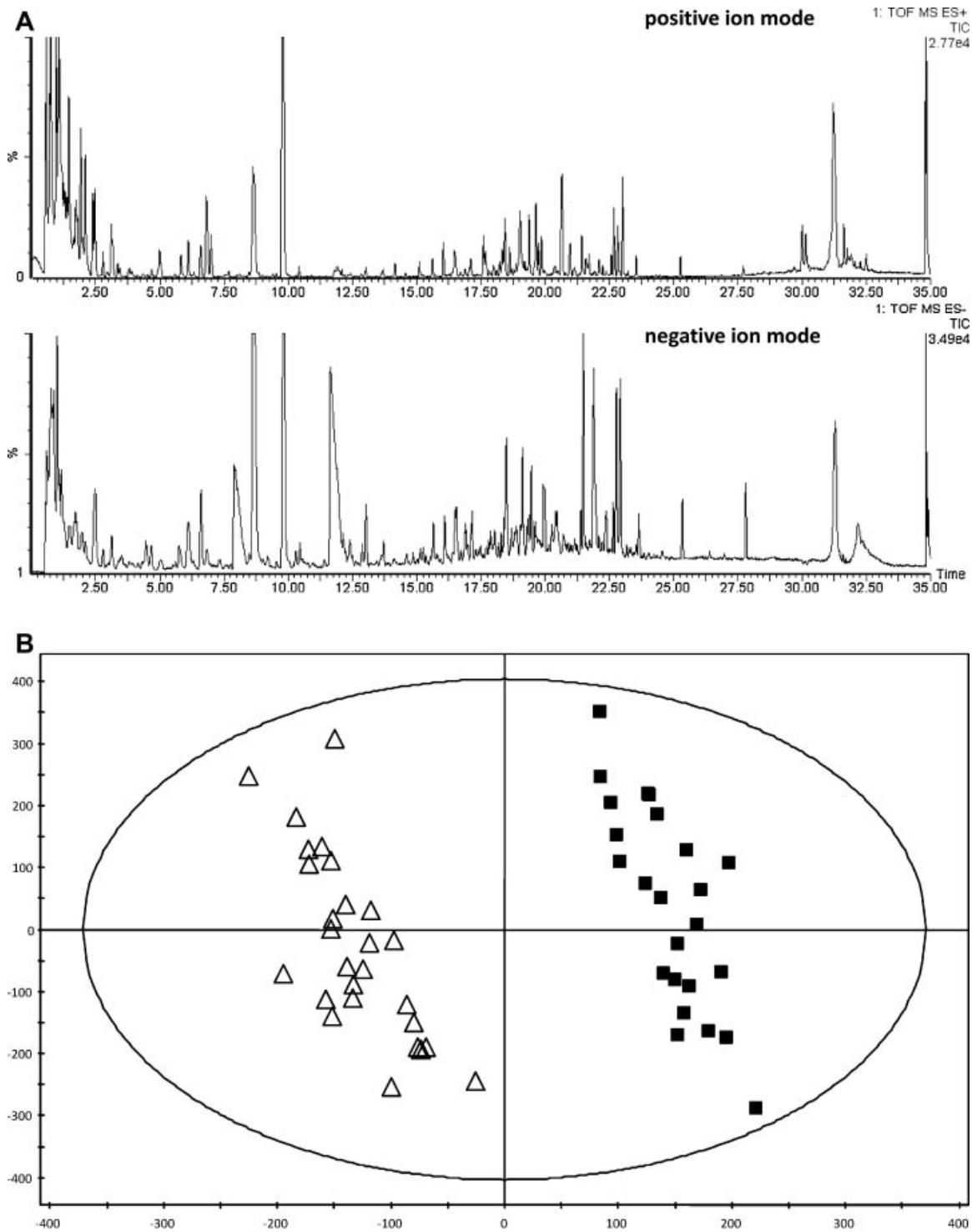

FIGURE 6. **A**: Representative UPLC-Q-TOF-MS total ion chromatogram (TIC) acquired in both positive and negative ESI mode. **B**: PLS-DA scores plot of pre-diabetic, insulin resistant subjects (Δ) and healthy controls (■). **C**: PLS-DA loading plot. **D**: The difference in peak height in the TIC of the metabolite identified by an arrow in the loading plot. Reprinted from Chen et al. (2008) with permission from the American Chemical Society.

One of the major complications of diabetes is nephropathy, a progressive kidney disease associated with a high risk of atherosclerotic disease and premature death. Twenty to 30% of patients with type 1 or type 2 diabetes develop nephropathy (Molitch et al., 2003; Zhang et al., 2009b). This has made diabetes the most common single cause of end-stage renal disease in America and Europe (Molitch et al., 2003). Zhang et al. (2009b), using a metabolic fingerprinting

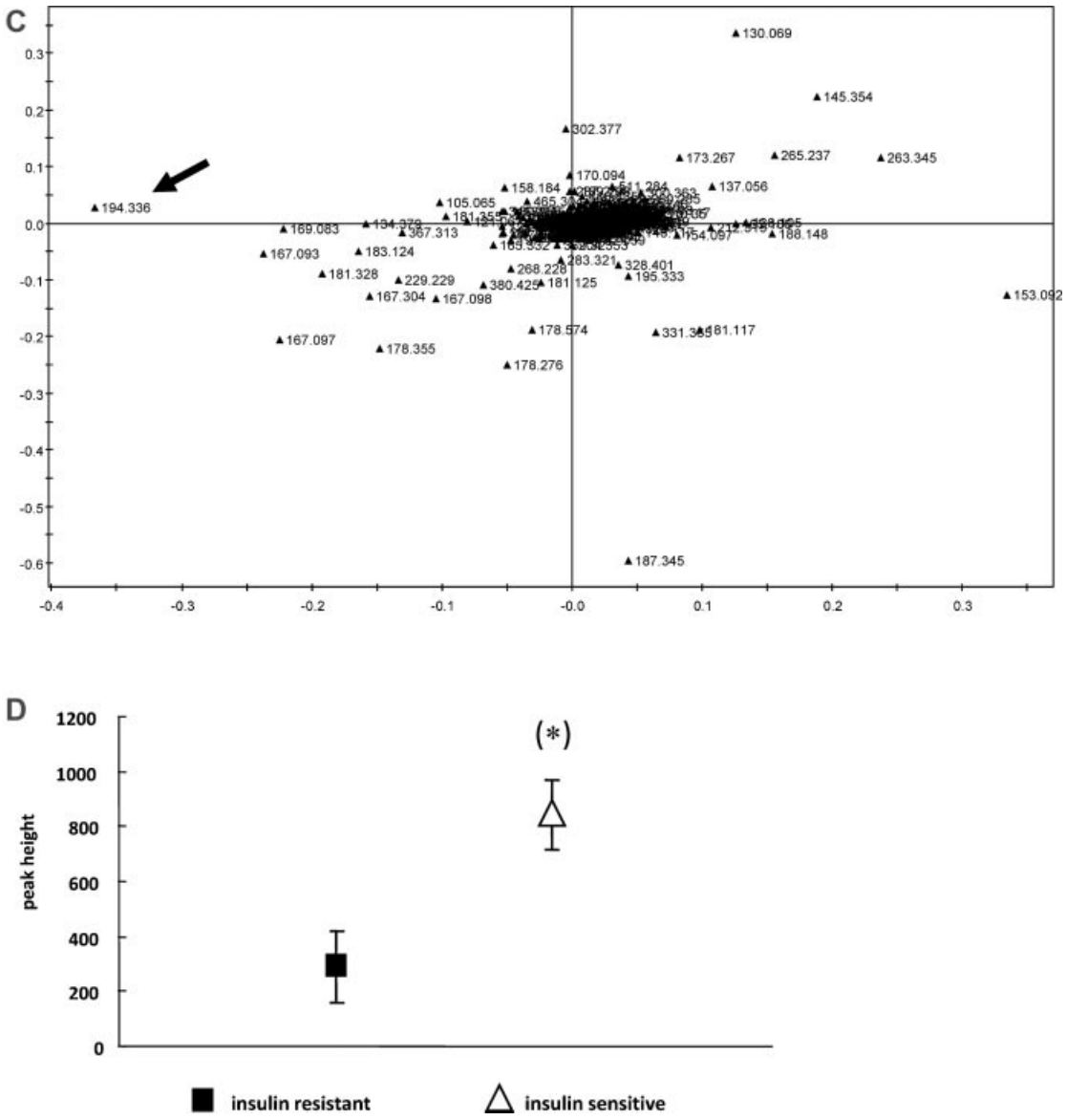

FIGURE 6. (Continued)

approach, have shown via UPLC-oa-TOF-MS and PCA that the plasma of patients with diabetic nephropathy could be distinguished from that of T2DM patients and healthy controls. In addition, they showed that plasma concentrations of leucine, dihydrosphingosine, and phytosphingosine were significantly decreased in the plasma of both T2DM and diabetic nephropathy patients versus the control group, and that the observed dihydrosphingosine content is higher in diabetic nephropathy patients than the T2DM group. Dihydrosphingosine can be coupled to a fatty acyl group to form dihydroceramide, which in turn can be converted to ceramide. Ceramide acts as a precursor for a variety of compounds, including glucosylceramide. The formation of glucosylceramide has been proposed to represent a pathway for glucose metabolism in patients with diabetic nephropathy (Shayman, 1996).

In addition to studies of T2DM in humans, multiple metabonomics studies have been carried out on rat models. The most commonly used strain for diabetes research is the Zucker rat. Zucker rats which carry the recessive trait of the leptin receptor (fa/fa) are overweight, have high levels of lipids and cholesterol in their blood, and are resistant to insulin. Metabonomic analyses of plasma and urine samples of Zucker rats have been performed using GC-MS (Major et al., 2006; Williams et al., 2006) and LC-MS (Williams et al., 2005a; Plumb et al., 2005, 2006; Williams et al., 2006; Fardet et al., 2008; Gika et al., 2008) and these are now briefly discussed.

Major et al. (2006), using capillary GC with both EI- and CI-MS, showed that PCA and OPLS could be used to discriminate the plasma metabolome of lean (*fa/fa*) obese and lean (+/*fa*) Zucker rats. They determined the metabolic profiles of the lean and lean (+/*fa*) strains to be most similar, while differing from the (*fa/fa*) obese strain. Unsurprisingly, based on library matching, the majority of the metabolites contributing to differences between lean and (*fa/fa*) obese rats were determined to belong to either fatty acids or sterols. The authors also noted higher concentrations of urea in the plasma of (*fa/fa*) obese rats, indicating kidney dysfunction. Williams and co-workers showed that GC-MS could also be used to

differentiate plasma from Zucker obese (*fa/fa*) rats and a normal Wistar-derived strain (Williams et al., 2006). It was noted by these authors that there was little overlap in metabolites identified by proton NMR, capillary GC-MS, and UPLC-MS, pointing to the complementary nature of these techniques.

In addition to GC, UPLC-oa-TOF-MS has also been used to discriminate the above three Zucker rat genotypes (Plumb et al., 2006). Again it was found through PCA and OPLS that the greatest difference in the plasma metabolomes of the three lines was between the Zucker lean and lean (+/*fa*) rats compared to the obese (*fa/fa*) animals. It has also been shown that UPLC with oa-TOF-MS can differentiate urine samples of Zucker obese and normal (Wistar-derived) rats (Plumb et al., 2005).

Metabolic fingerprinting has also been used to observe differences in the metabolic profile of Wistar rats versus Goto-Kakizaki (G-K) rats, a non-obese Wistar substrain which develops T2DM early in life (Jankevics et al., 2009). LC-MS urinary data from the two strains was easily differentiated using PCA and PLS-DA. The authors went on to use proton NMR to identify creatinine, glucose, and dimethylamine as being elevated in the urine of G-K rats, and creatine, hippurate, formate, allantoin, fumarate, citrate, acetate, and amino acids to be depleted.

E. Alzheimer's Disease

Alzheimer's disease is a degenerative form of dementia which is primarily diagnosed in people over 65 years of age. The need for identifying a reliable biomarker for early diagnosis of this disease is becoming increasingly urgent as our population ages. It is expected that as many as 1 in 85 people will be affected with Alzheimer's by 2050 (Brookmeyer et al., 2007). While there is currently no cure for Alzheimer's disease, early diagnosis could help monitor disease progression and target therapies earlier in the course of the disease. Imaging techniques such as positron emission tomography (PET) and single photon emission computed tomography (SPECT) have been used to investigate changes in oxygen and glucose metabolism in Alzheimer's disease patients, suggesting that neuroimaging biomarkers could supplement clinical data to assess progression of the disease (Jagust, 2004; Quinones & Kaddurah-Daouk, 2009). The cost and time associated with imaging, along with the need to expose the patient to radioactive probes, make MS-based metabolite analysis an attractive alternative. To this end, several metabonomics studies have been carried out on Alzheimer's patients.

Cerebral spinal fluid is the most often used biofluid for studying Alzheimer's disease as it closely resembles the composition of the brain extracellular space, but attaining this fluid is highly invasive and requires a lumbar puncture. Myint et al. (2009) have profiled polar cationic metabolites in CSF samples from Alzheimer's patients using a nano-LC/MS method they developed. To do this they employed a column with a hydrophilic inner wall coating, because traditional RPLC is inefficient at retaining hydrophilic metabolites. The authors used a mobile phase which contained a smaller percentage of organic solvent relative to what is used in HILIC, since highly polar compounds are poorly dissolved in organic-rich solvents. Using PCA on a subset of MS peaks and normalization to internal standards they were able to discriminate samples from Alzheimer's patients and age-matched controls. With this methodology, 55 putative biomarkers for the diagnosis of Alzheimer's disease were selected based on their *m/z*; however, further work is needed to elucidate the identity of these compounds.

A less invasive method is to use plasma samples, but there has been concern that these samples may not be as rich in information as CSF. Greenberg et al. (2009) evaluated human plasma samples from Alzheimer's patients using UPLC-MS with a positive ionization source. While PCA was not able to discriminate patients with Alzheimer's disease, mild cognitive impairment, and controls, this was readily accomplished via PLS-DA; however, due to inter- and intra-subject variability, this model had poor predictive power. From the PLS-DA loading plots, the authors were able to identify several high-influence metabolites, including 1-(9E-hexadecenoyl)-sn-glycero-3-phosphocholine and D-glucosaminide. The authors also noticed a trend with increases in the concentrations of the bile acid salts glycocholate, glycodeoxycholate, and glycochenodeoxycholate in patients with Alzheimer's disease and mild cognitive impairment relative to the controls. In a similar study, Li et al. (2010) also observed variations in plasma metabolites between Alzheimer's patients and healthy controls. Using UPLC-MS with an ESI source operated in the negative mode and PCA they were able to discriminate sample groups, and identified tryptophan, dihydroshingosine, phytosphingosine, hexadecasphinganine, and lysophosphatidylcholines as potential biomarkers. Further, the authors speculate that this may represent perturbation in the metabolism of amino acids, lecithin, and phospholipids in Alzheimer's patients. The successful use of PCA by Li to group subjects by disease state, but not by Greenberg, is likely to be a result of the different ionization mode used. Since MS analysis depends on the ionization efficiency of analyte molecules, the ability for a metabolite to be detected is dependent on how easily it forms either positive or negative ions. The different ionization modes therefore look at different subsets of the metabolome—species that readily form cations and species that readily form anions—again pointing to the complementary nature of analytical techniques in metabolomics and metabonomics.

A comprehensive study of metabolites perturbed in the urine, plasma, and CSF of probable Alzheimer's patients was conducted by Fonteh et al. (2007). In this study the authors used LC-ESI-MS/MS with SRM to analyze differences in free amino acid and dipeptide concentrations in probable Alzheimer's patients and gender- and age-matched controls. Among their findings was a significant increase in L-DOPA concentration and decrease in the concentrations of dopamine and carnosine in the plasma of Alzheimer's patients. They also noted a decrease in urinary glycine, and slightly lower levels of arginine and citrulline in the CSF of Alzheimer's patients. Overall, many compounds were altered with major changes in imidazole containing amino acids (histidine and methylhistidine), citrulline, ornithine, glycine, catecholamines, and antioxidant dipeptides (carnosine and anserine). Collectively, these metabolites are involved in metabolic pathways related to the urea cycle, neurotransmission, and antioxidation, and the authors propose that manipulation of these metabolic pathways could be used as a means of preventing progression of Alzheimer's disease.

A post-mortem study of metabolites in lipid extracts from the brain tissue of Alzheimer's disease patients has also been conducted (Han et al., 2002). Han et al. used negative-ion mode ESI-MS to analyze the content of sulfatides, a class of sulfated galactocerebrosides, in the brain tissue of Alzheimer's disease patients who presented with varying degrees of dementia at time of death. Altered levels of sulfatides in brain tissue matter are involved in the pathogenesis of various diseases which include metachromatic leukodystrophy and Krabbe's disease. In this study it was found that sulfatides were depleted up to 92% in all examined regions of gray matter in subjects with very mild dementia, the earliest stage of clinically identifiable Alzheimer's disease, compared to age-matched Alzheimer's disease patients with no dementia. The total content of sulfatides was also found to be depleted up to 58% in the white matter of patients with very mild dementia; however, no further decline in the concentration of sulfatides was observed going from mild dementia to severe dementia. This striking deficiency could possibly indicate that significant depletion of sulfatides occurs prior to clinical symptoms, and represents a mass loss of approximately 40 nmol/mg protein in both gray and white matter. It is interesting to note that the concentration of sulfatides in white matter was essentially constant in going from mild to severe dementia, suggesting that its concentration may be maintained at a new pathophysiological level. It was also noted that the content of ceramides, a class of potential degradation products of sulfatides, was elevated in all regions of white matter of patients with very mild dementia, with the total concentration increasing more than threefold (2.6 nmol/mg protein to 8.7 nmol/mg protein). This change was not also observed in gray matter, and may be due to degradation of sulfatides.

V. FUTURE PERSPECTIVES

While NMR has been traditionally used in metabonomics studies, use of hyphenated MS techniques is becoming increasingly common. The greater sensitivity of MS allows for detection of a larger number of metabolites as compared to NMR, which is especially useful for fingerprinting studies. However, identification of these compounds can often only be achieved through comparison to known samples or database matching. This represents the major bottleneck in use of MS in metabolomics and metabonomics when one is interested in identifying specific metabolites, such as in profiling studies. In addition there is the requirement that fragmentation conditions must be kept constant for metabolite identification and quantitation. To this end we implore metabolomics researchers interested in aging to stick to emerging conventions for sample analysis and to compile and make available their mass spectral databases for metabolite identification. In this vein, researchers are directed to free mass spectral libraries and retention time indexes for database searching provided by the Max Planck Institute of Molecular Plant Physiology (Golm Metabolome Database, http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd_msri.html) and by MassBank (http://www.massbank.jp). Compiled mass spectral libraries are also available for purchase from the National Institute of Standards and Technology (NIST) at http://www.sisweb.com/software/ms/nist.htm and from Wiley (http://www.wiley.com/WileyCDA/WileyTitle/productCd-0470606967.html).

We also wish to present to the reader some additional electronic resources which are available to researchers in metabolomics and free of charge. The Human Metabolome Database (http://www.hmdb.ca/) contains information on small molecule human metabolites. The METLIN metabolite database also contains information on metabolites, as well as tandem mass spectrometry data (http://metlin.scripps.edu/). Information on metabolic pathways and pathway maps can be found at the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/). Links to additional metabolomics resources are available on the webpage for the Metabolomics Society (http://www.metabolomicssociety.org/).

Finally, the studies which we have described above show the great power of MS-based metabolomics to uncover and explore the processes behind organismal aging. Clearly we are only at an early stage of discovery, where most of the information that is being collected is descriptive. When combined with multivariate statistical techniques, it is apparent that these new MS tools can differentiate subjects by age, and even diseased from healthy individuals within elderly populations. With time, a deeper understanding of the processes that lead to aging is sure to evolve. Use of MS-based metabolomics is not quite yet commonplace, but we predict that it will become increasingly useful as a tool in the study of aging over the coming decade, especially since MS analysis of metabolites in urine and plasma represent a cheap, minimally invasive technique that stands to expedite clinical diagnoses and ultimately improve health care of our aging citizens.

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